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Summary and General discussion
In this thesis, studies about the effect on blood platelet inhibition by several aspirin regimen adjustments and other factors of possible influence on aspirin’s efficacy to inhibit platelets, are presented. In specific, we aspired to answer the following three questions:

1. What are (pre-)analytical variables of influences within platelet research: specifically, the precision of four different subdivision methods to divide aspirin tablets, and the influence of pre-analytical variables on serum Thromboxane B₂ analysis?
2. Does changing time of intake (i.e. chronotherapy) or shorting the standard 24-hour dosing interval result in higher levels of platelet inhibition?
3. Are there other –patient related- factors, besides the circadian rhythm and aspirin’s pharmacokinetic aspects, of influence on aspirin’s efficacy to inhibit platelet (re-)activity? In specific, co-medication usage, an interplay between platelets and (pro-)thrombotic factors, and the possible influence of –psychological- stress on platelet (re-)activity.

The rationale behind these three main research questions is described in detail in the general introduction of this thesis. To visually support the factors under investigations in this thesis, we have included an overview (figure 1). In part 1 of this discussion we will deliberate on several methodological principles when it comes to platelet –activity- research. Specifically, the considerations that were raised in chapter 1 and 2 of this thesis. In part 2 of this discussion the findings of chapter 3, 4, and 5, will be discussed. To elaborate, the chapters found a higher level of platelet inhibition after aspirin evening intake in comparison with aspirin morning intake. These findings will also be taken into consideration in light of other evidence published on various other aspirin regimen adjustment to improve early morning platelet inhibition (chapter 6). In part 3 of this discussion, the studies concerning the influence of other –patient related- factors on blood platelets are debated. In specific, chapter 7, which reported on the hemostatic phenotype of intracoronary thrombi derived from patients on aspirin and subsequent statin, compared with patients without such therapy. Chapter 8, in which the difference in on-aspirin platelet activity between carriers of inheritable thrombophilia compared with controls is reported. And lastly, chapter 9, which described the influence of –psychological stress-, induced by the viewing of a horror movie, on platelet activity.

Part 1 – Methodological Considerations on Blood Platelet Research

In order to answer the question, whether, and if so, by which strategy we can best clinically relevantly influence platelet activity, one must consider how accurate platelet function testing actually is. Currently, there is no human in vivo platelet function test available. There are however several in vitro and ex vivo platelet function tests¹. Unfortunately, both in vitro and ex vivo testing coincide with transporting blood platelets out of their comfortable warm habitat (in between human vascular walls) to a cold new environment.
Blood platelets are influenced by this transport. Firstly, with the tightening of the tourniquet prior to venepuncture; the stasis of the blood is the first stimulus for blood platelets to be activated. Subsequently, blood is collected primarily into a syringe and then inserted into a vacuum tube. The diameter and the length of the puncture system however can also influence platelet function, as shear stress within the line can induce contact activation.

In chapter 2, we evaluated in healthy subjects, the subsequent influence of subjected pre-analytical time and temperature until incubation on the produced level of serum Thromboxane B₂ (TxB₂) by blood platelets. Interestingly, the study demonstrated that the level of sTxB₂ can be markedly decreased by changing pre-analytic time and temperature conditions. Moreover this decrease seemed most apparent on aspirin therapy. When viewing the results of this study one can wonder whether it is necessary to perform full incubation (i.e. 60 min. incubation at 37 °C) to measure aspirin’s inhibitory effect on blood platelets. For example, the samples without incubation off and on aspirin still differed by a mere 10-fold. Moreover, the samples incubated at room temperature differed by a 62-fold. Thus, even under suboptimal circumstances it is possible to detect a difference in certain regimens. Whereas, by enforcing the full 60 min. incubation at 37 °C rule we incorporate more pre-analytical variables. Merely incubating at room temperature could be a solution. However, those sTxB₂ levels would of course not be comparable to previous results published.
Another methodological question that emerged coincided with the hypothesis that a regimen of 40–75 mg aspirin twice daily might be superior to the standard once daily intake (chapter 4 and 5). Due to the lack of marketing authorization for aspirin tablets containing these low dosages in most countries, subdivided tablets needed to be used in research. However, inconsistencies are found in the literature concerning the best method for breaking tablets or subdividing whole aspirin tablets would result in uneven half tablets, because dose deviations or weight losses might occur. For example, conflicting results are published about the superiority of tablet splitters or breaking by hand. Subsequently, it was relevant to investigate whether subdivision of whole aspirin tablets would result in uneven half tablets, because dose deviations or weight losses might occur. Surprisingly, based on the results of our study in chapter 1, we found that breaking by hand and avoidance of cutting, with a knife or a special designed ‘tablet tool’, when subdividing aspirin tablets was best to achieve weight uniform half tablets and the least loss of mass. Hence, this was the protocol applied in chapter 4 and 5 to create twice daily half dosage aspirin regimens.

Part 2 – Aspirin Regimen Adjustments to Induce Higher Levels of Platelet Inhibition

In light of the circadian rhythm found with (re-)occurrences of cardiovascular events (CVE), early morning hours may be essential in terms of adequate platelet inhibition. Given the high prevalence of cardiovascular disease, already a modest relative reduction of the morning peak could lead to a large absolute reduction of cardiovascular events on a population level. In part 2 of this thesis, we investigated whether various aspirin regimen adjustments might induce higher levels of platelet inhibition than the currently standard once daily low-dosage morning regimen. In specific, we investigated the chronotherapy hypothesis with aspirin therapy.

Chronotherapy involves the administration of medication in coordination with the body’s circadian rhythms to maximize therapeutic effectiveness and minimize and/or avoid adverse effects. In chapter 3 we assessed whether evening intake the OD-evening of aspirin might decrease attenuation of platelet (re-)activity, in patients with stable cardiovascular disease, in comparison with OD-morning intake of aspirin. Platelet reactivity measurements were completed for 30 patients. Interestingly, aspirin’s attenuation was most apparent in the morning regimen and slim to nonexistent in the evening regimen. However, after careful evaluation of these results, it appeared not all patients improved on the evening regimen. Within the evening regimen, 9 out of 30 patients still demonstrated suboptimal levels of platelet inhibition (i.e. a closure time of <193 seconds).

We hypothesized that an increased turnover, and the subsequent higher release of reticulated platelets might be causing trouble in these patients. Therefore, to further improve early morning platelet inhibition, we performed two randomized cross-over trials assessing the
influence of an evening regimen or a twice daily (BID) half dosage regimen in comparison with the standard once daily morning dosage. We first performed the randomized cross-over trial, comprising 12 healthy subjects (chapter 4), which demonstrated a superior level of platelet inhibition at 8.00 AM by both the OD-evening regimen and the BID half-dosage aspirin intake in comparison with the standard OD-morning regimen. However, this difference was only statistically significantly different for our primary endpoint sTxB₂ levels. Furthermore, it appeared that the BID half-dosage aspirin intake was not statistically significantly superior to the OD-evening regimen in inhibiting early morning platelet inhibition.

The possible explanation we propose for the superior level of inhibition by evening intake of aspirin 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 16. OD-evening is more likely to inhibit these peak released reticulated platelets 17; 2.) Although megakaryocytes possess the ability to produce new cyclooxygenase (COX), they can be inhibited for a maximum of 12 hours 18. Consequently, it could be that after OD-evening intake they are—partially—inhibited, and, perhaps, release fewer reticulated platelets.

We hereupon performed in chapter 5 a randomized cross-over trial, comprising 22 stable cardiovascular patients. This trial was designed to evaluate the twice daily half dosage, as well as our reticulated platelet hypothesis. The main results of the study in chapter 5 was that OD-evening intake of aspirin reduced COX-1 dependent and COX-1 independent assays, namely the sTxB₂ levels and the Platelet Function Analyzer Closure Time, during early morning hours. Interestingly, the amount of reticulated platelets was statistically significantly lower over the course of the standard 24 h dosing interval, after evening intake of aspirin in comparison with morning intake. Hence, suggesting that the turnover of thrombocytes indeed is influenced by OD-evening intake of aspirin. Moreover, reticulated platelets are more reactive than mature platelets 19, and a high level of reticulated platelets in patients with stable cardiovascular disease is associated with an increased risk of a CVE 20. Hence, although this is highly speculative, a reduction in reticulated platelet level might lead to a reduction in CVE.

In summary, the trials in chapter 3, 4, and 5, reported promising results with respect to a higher level of platelet inhibition after OD-evening intake of aspirin. However, the participants included both healthy subjects and subjects with stable cardiovascular disease. Furthermore, three trials might be insufficient to prove superiority of the evening regimen and we therefore performed a systematic review to assess all current evidence. Consequently, in chapter 6, we performed an extensive literature search on trials reporting on various aspirin regimen adjustments on the level of platelet inhibition. In specific, 1.
A change in intake frequency; 2. A change in dosage; 3. The appliance of chronotherapy. This systematic review concluded there is no adequate evidence to determine superiority for any of the regimen adjustment in comparison with the standard once daily morning regimen.

Although the included trials are heterogeneous, when analyzing the trials reporting on sTxB₂ levels, one could conclude that an aspirin dosage between 75-350mg once daily is able to provide sufficient platelet inhibition in early morning hours²¹-²⁶. Moreover, increasing the dosage to >350mg once daily would merely provide a minor improvement in the level of platelet inhibition, whereas a higher dosage is more likely to induce side effects²⁴,²⁷,²⁸. In contrast, dosages below <75mg once daily aspirin seem to induce inadequate levels of platelet inhibition²²,²⁵,²⁸,²⁹. Furthermore, a change in intake frequency only seems beneficial if it entails an increase in intake frequency (i.e. >once daily intake). Both trials reporting on the appliance of chronotherapy demonstrated similar results: evening intake of aspirin induces a higher and more stable level of platelet inhibition in comparison with the standard once daily morning intake³⁰,³¹.

As assessed by previously published studies, the majority of patients take aspirin in the morning upon awakening³²,³³. Should we have advice the patient in the introduction of this thesis to switch to evening intake based on the studies presented in this thesis? In order to accurately answer this question, we must first determine what the clinical implications of switching to evening intake could be; both in terms of benefit and possible side-effects.

In terms of side-effects, firstly, a higher level of platelet inhibition could also induce higher rates of bleeding and gastric complications. Unfortunately, there are no large randomized clinical trials on the effect of chronotherapy with clinical endpoints (e.g. Major Cardiovascular Events- MACE or gastric complications), and the smaller randomized trials that have been published are mostly powered on surrogate endpoints (i.e. platelet function tests) without reporting side-effects. One previously published study did report that the frequency of side-effects was not statistically significantly increased by evening intake of aspirin³². However, the follow-up of this trial was merely three months and the sample size was not powered to detect a statistically significant difference in complications or side-effects³². Nevertheless, these results are in line with two other studies which report a lower gastric vulnerability to aspirin during the night than during the day³⁴,³⁵. Secondly, as is reported in chapter 4 and 5, both in healthy subjects and stable cardiovascular patients, the reported compliance is lower in the OD-evening aspirin regimens. This is in line with an earlier published trial, which compared the ingestion of a morning polypill to ingestion of an evening polypill³³, in which overall adherence assessed by pill count was highest when using the polypill in the morning.
In terms of clinical benefit, how much reduction in the incidence of cardiovascular event reoccurrences can we expect? Neither in our studies, nor in any other previously published trials is the effect of chronotherapy based on clinical endpoints (i.e. a reduction in Major Cardiovascular Events). There are however two trials currently being conducted that will hopefully answer this question. Firstly, the CARING-trial (Chronotherapy With Low-dose Aspirin for Primary Prevention) investigates the potential influence of aspirin on the primary prevention of cardiovascular, cerebrovascular and renal events in subjects with either impaired fasting glucose or previous diagnosis of type 2 diabetes mellitus, who will receive low-dose aspirin at different circadian times (i.e. upon awakening or at bedtime) (NCT00725127). This trial is an open-label randomized trial and is estimated to be completed 2026. Secondly, the TIME-ASPIRIN-trial, is not yet registered on clinicaltrial.gov, but has been awarded a research grant by ZonMw (Grant number: 848016006). This trial will be a randomized, double-blinded, controlled trial, and is estimated to be completed in 2023. Hence, in order to compose an answer prior to 2023, we will need to extrapolate from current evidence.

Previous trials in aspirin treated patients have shown that poor clinical outcomes are associated with both high values of COX-1-dependent and COX-1-independent assays\textsuperscript{36-38}. Firstly, it has been shown that the risk of recurrent cardiovascular events is increased in patients with higher Platelet Function Analyzer aspirin platelet reactivity values. Stable
cardiovascular disease patients with a PFA Closure time of <193 seconds have an odds ratio of 2.1 (95% CI: 1.4–3.4, P< 0.001) for developing a composite cardiovascular endpoint in comparison with stable cardiovascular disease patients with a PFA Closure Time of >193 seconds\(^5\). This meta-analyses suggests that already a modest reduction platelet reactivity, as found in our studies in chapter 3, 4, and 5, could result in clinical benefit. This possible clinical benefit could be calculated with known cardiovascular disease statistics (See Box 1. Thought experiment).

In conclusion, given the high prevalence of cardiovascular disease, as mentioned above, a slight reduction of the morning peak platelet activity and release, might lead to a large absolute reduction of cardiovascular events on a population level. Studies in this thesis suggest that, for patients who already take aspirin on a daily basis for cardiovascular disease prevention, switching to aspirin intake in the evening seems prudent, safe, and potentially beneficial. However, future clinical trials are needed to evaluate with which magnitude this reduction in platelet reactivity affects clinical outcomes.

Part 3 – Other Factors of Influence on Blood Platelets

The thought experiment, as reported in box 1, on the possible reduction in the prevalence of recurrent cardiovascular events is painting a pretty picture. After a first cardiovascular event, classical risk factors (e.g. blood pressure, cholesterol) are aggressively treated. Still, 20% to 40% of all patients develop a recurrent cardiovascular event\(^40,42\). So, other risk factors than basal platelet activity are likely to play a role in the development of a recurrent event\(^43,44\).

Over the years, there have been countless attempts to predict which patients will endure a CVE despite the use of aspirin. In general, as a result of possible pigeonholing, the majority of research has focused on characterizing platelet phenotype or aspirin pharmacodynamics. Subsequently, interplay between platelets, inflammation and other pro-thrombotic factors have been under-investigated. In view of this, in part 3 of this thesis we took into consideration other factors which might cross-talk with platelets: co-medication, pro-thrombotic factors, and the possible influence of –psychological- stress.

Firstly, in chapter 7, we characterized haemostatic phenotype of intracoronary thrombi derived from patients with a ST-elevation Myocardial Infarction (STEMI), both with and without aspirin and subsequent statin therapy. Interestingly, recent data in patients with myocardial infarction suggested additional preventative mechanisms: both aspirin and statin exhibit anti-inflammatory effects within intracoronary thrombi\(^45,46\). Moreover, statin therapy also inhibits platelets\(^46,47\). As inflammation, aggregation, and thrombosis are closely intertwined\(^45,48\), we hypothesized that thrombi derived from patients on aspirin and subsequent statin might demonstrate a different haemostatic content than patients without
such therapy. Although the data in chapter 7 suggests that haemostatic parameters within intracoronary thrombi are different in coronary thrombi derived from patients on secondary cardiovascular prevention therapy, these findings are somewhat limited.

In order to establish the precise role of aspirin and statin therapy separately, and their possible complementary and/or synergistic anti-haemostatic effects in thrombus formation in patients with a ST-elevation myocardial infarction, future studies should be undertaken.

Secondly, in chapter 8, we assessed whether on-aspirin platelet activity might differ in carriers of pro-thrombotic factors (i.e. inheritable thrombophilia) in comparison with controls. The two most common mutations are the factor V Leiden mutation and the prothrombin G20210A mutation. Interestingly, both factor V and thrombin are known to influence platelet (re-)activity. The data demonstrated a possible increased on-aspirin platelet (re-)activity in carriers of the prothrombin G20210A mutation, as measured by the PFA-200 and the VerifyNow. Surprisingly, the increased on-aspirin platelet (re-)activity in the prothrombin G20210A mutation carriers was present in spite of sTxB₂ levels demonstrating sufficient COX suppression. In specific, the PFA-CT was borderline statistically significantly lower in comparison with the control group and the VerifyNow ARU was borderline statistically significantly higher in comparison with the controls. This increased on-aspirin platelet (re-)activity however was not present in carriers of the factor V Leiden mutation.

The prothrombin G20210A mutation is a gain of function mutation, hence carriers exhibit elevated plasma levels of prothrombin and subsequent elevated thrombin levels. Thrombin, a primary player in the secondary hemostasis, also is a potent regulator of platelet adhesion and activation. It does so by proteolysis of the protease activated receptors (PARs). Furthermore, thrombin binds glycoprotein Ib (alpha), which amplifies platelet activation by accelerating PAR-1 activation. In mouse models, inhibition of thrombin activity or genetic deletion of the platelet thrombin receptor results in significantly impaired platelet accumulation and p-selectin expression. This rational is in accordance with studies demonstrating that thrombin inhibitors (i.e. factor II) also inhibit platelets. It can thus be hypothesized that elevated secondary hemostasis parameters can induce high on-aspirin platelet (re-)activity. These preliminary findings raise intriguing questions regarding the choice of arterial thromboprophylaxis in carriers of a prothrombin G20210A mutation, but also in the general population. The COMPASS trial demonstrated that patients treated with rivaroxaban 2.5 mg twice daily in combination with aspirin experienced fewer cardiovascular events but more bleeding complications than those who received aspirin monotherapy. Rivaroxaban is a selective direct factor Xa inhibitor and hereby also an indirect thrombin inhibitor (i.e. factor Xa converts prothrombin to thrombin). The results of the COMPASS trial suggest that in the general population the number needed to harm is
too high for monotherapy of rivaroxaban to be beneficial. However, perhaps in a population with elevated secondary hemostasis parameters, like in prothrombin G20210A carriers, monotherapy with a direct and/or indirect thrombin inhibitor might be more beneficial than in the general population.

And lastly, in chapter 9, we applied an innovative method to investigate the influence of -psychological- stress on platelet activity. Abundant epidemiological data have demonstrated that – psychological - stress increases risk of morbidity and mortality from atherothrombotic cardiovascular disease61-65. An illustrative example is the more than doubling of CVE risk among German men during home team soccer matches of the 2006 FIFA World Cup66. Although unproven, a plausible hypothesis is that events may be triggered by increases in adrenergic activity, heart rate, systemic blood pressure, and blood coagulability67,68. The viewing of a scary movie has been proven to induce a physiological stress response69. A major player inducing increased platelet reactivity during stress is the sympathetic nervous system (SNS). The SNS exerts physiological effects at times of acute stress, for example an increase in heartrate, blood pressure, and bronchodialation. Furthermore, the SNS induces adrenomedullary release of catecholamines, particularly epinephrine70. Catecholamines, and specifically epinephrine, are potent platelet aggregation agents. Moreover, catecholamines are also known to potentiate platelet activation71. Interestingly, a recent study demonstrated an increase in factor VIII after watching a horror movie, suggesting an effect of acute fear on the coagulation system72. However, primary haemostasis is thought to play a more prominent role in the etiology of CVE than secondary hemostasis73. The data in chapter 9 demonstrated that a ‘blood curdling’ horror movie increases platelet reactivity, particularly in men. There are two likely causes for this ambiguous sex difference. Firstly, men have a higher sympathoadrenal responsiveness than women74,75. Secondly, although women typically report higher levels of negative affect than men in response to psychological stressors, greater acute hypothalamic–pituitary–adrenal and autonomic responses have been found in adult men as compared to adult women76-78. If we would perform a thought experiment, these results are also in line with the historical gender-role differences between men and women. Whereas men had to explore the area, with an imminent risk of getting injured and therefore have a ‘fight or flight’ stress response system, women, being physically inferior to most men, have a ‘tend-and-befriend’ stress response system79.
CONCLUDING REMARKS

The trials in this thesis demonstrate the challenges and limitations of platelet–activity-research. There are numerous platelet tests available, however, there is currently no consensus in the scientific community on their clinical relevance. All platelet function tests are – to a various extent- capable of marking an increased risk for enduring a CVE in aspirin users\textsuperscript{15,80}. Despite their capability of marking this increased risk, it remains to be proven that tailoring of antiplatelet therapy based on the current platelet tests reduces cardiovascular event reoccurrences. For example, the ANTARCTIC-trial, a large multicentre, open-label, blinded-endpoint, randomized controlled superiority trial, reported that platelet function monitoring with treatment adjustment did not improve the clinical outcome of elderly patients treated with coronary stenting for an acute coronary syndrome\textsuperscript{81}. So if these platelet function tests supposedly measure ‘hyper-active’ platelets, but intensifying antiplatelet therapy does not further reduce cardiovascular event reoccurrence, perhaps these hyper-active platelets are not the problem but rather an epiphenomenon of an underlying problem. Although we like to think of blood platelets as monogamous soldiers in the hemostatic army, they originate from the same progenitor cells as immune cells, and subsequently also –at least partially- dictate the inflammation and immune process\textsuperscript{82}. There is some evidence that the marking by platelet function tests coincides with increased levels of cardiovascular inflammation parameters. Hence, it is perhaps not the antiplatelet therapy that needs tailoring, but rather the anti-inflammatory therapy\textsuperscript{83,84}. On this note, it would be interesting to retrospectively analyze ANTARCTIC-trial data to investigate whether the participants who were marked as ‘non-responsive’ to antiplatelet therapy have elevated cardiovascular inflammatory markers.

In line with this inflammatory hypothesis, the CANTOS-trial (a randomized, double-blind trial of canakinumab vs. placebo on nonfatal myocardial infarction, nonfatal stroke, or cardiovascular death) reported that anti-inflammatory therapy with canakinumab led to a statistically significantly lower rate of recurrent cardiovascular events than placebo, independent of lipid-level lowering\textsuperscript{85}. Furthermore, it was demonstrated that the magnitude of risk reduction for major cardiovascular events, cardiovascular death, and death from any cause with canakinumab was greatest among patients with the largest reductions in levels of interleukin-6 and high-sensitivity C-reactive protein, which suggests that the benefit was related to the targeting of the interleukin-1β- interleukin-6-C-reactive protein pathway of immunity. Interestingly, the CIRT-trial (a randomized, double-blind trial of low-dose methotrexate vs. matching placebo in patients with previous myocardial infarction or multisvessel coronary disease who additionally had either type 2 diabetes or the metabolic syndrome) did not demonstrate a reduction in levels of interleukin-1β, interleukin-6, or C-reactive protein, nor did it result in fewer cardiovascular events than placebo\textsuperscript{87}. It can thus
be hypothesized, that it is through the inhibition of interleukin-1β-interleukin-6 process, that a reduction in cardiovascular events can be established.

Despite the scientific and clinical excitement about the results of the CANTOS-trial, canakinumab is priced at approximately $200,000 per year\(^8\). Hence, such pricing is not suitable for a common disease such as coronary artery disease. Two possible solutions could be: 1.) Further investigation to find lower-cost agents that target the interleukin-1β–interleukin-6 signaling; 2.) Tailor made anti-inflammatory therapy: perhaps the patients who are marked as non-responsive to antiplatelet therapy are the ones most in need for such exceptional therapy. This would markedly reduce the size of the targeted population.

As there are currently so many other antiplatelet agents available, a much heard commentary regarding the studies presented in this thesis is that soon aspirin will be declared obsolete. In line with this statement, in 2015, there were 0.81 million people in the Netherlands who were prescribed daily usage of acetylsalicylic acid 80mg, whereas by 2017 this number had decreased -6.6% to 0.76 million\(^8\). In my opinion, however, the rationale as to why chronotherapy of aspirin therapy might be beneficial in reducing cardiovascular event reoccurrences, applies to the other ‘main’ antiplatelet agents as well\(^89\).\(^90\). Hence, the subtitle of this thesis is called: lessons learned from aspirin.

In conclusion, firstly, randomized clinical trials powered on clinically relevant endpoints (i.e. major cardiovascular events) are pending and will hopefully confirm whether chronotherapy of aspirin can indeed further reduce cardiovascular event reoccurrences. Secondly, platelet function testing had its limitations, however, it might be of benefit in the future investigations of tailoring anti-inflammatory therapy. And lastly, it might be of relevance to further investigate the chronotherapy hypothesis on other anti-platelet agents.