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TIME for ASPIRIN

Blood pressure and platelet reactivity

Tobias N. Bonten

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BLOOD PRESSURE AND PLATELET REACTIVITY

Tobias N. Bonten

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Time for Aspirin

BLOOD PRESSURE AND PLATELET REACTIVITY

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Voor Dick de Keijzer

CONTENTS

Chapter 1

General introduction

Despite major improvements in treatment and prevention over the last decades, cardiovascular disease is still one of the leading causes of morbidity and mortality worldwide.[1, 2] In Europe, cardiovascular disease is responsible for approximately four million deaths each year. Low-dose aspirin is a cornerstone in the prevention of cardiovascular disease, because it reduces the risk of recurrent cardiovascular events with about a quarter.[3] Historically, the natural form of aspirin (*salicin*) was extracted from willow tree bark (*salix*) which was already used in ancient Egypt and Greece to treat pain and fever.[4] Major drawbacks of this ancient drug were its side effects, especially irritation of the stomach. In 1897, a chemist named Felix Hoffman modified the compound into acetylsalicylic acid to reduce its side effects. The Bayer company registered the substance under the name "Aspirin®" as a pain killer in 1899.[5] A bleeding tendency in patients using aspirin was already reported in 1945[6], but the effect of aspirin on blood platelets was not known until 1968.[7] Even before this mechanistic discovery, a general practitioner named Lawrence Craven was one of the first to systematically investigate the possible role of aspirin to prevent myocardial infarctions in 1950 (Figure 1).[8] He recommended all his male patients between the age of 40 and 65 years to take aspirin, and observed no myocardial infarctions. Followed by large randomized placebo-controlled trials in the 80s, the role of low-dose aspirin in the prevention of cardiovascular disease was established.[9] In the current era new benefits of aspirin continue to be discovered,

A B C D

Figure 1 – **(A)** Aspirin, in the form of salicin extracted from willow bark or -leaves, was already used by the ancient Egyptians, as recorded in the Ebers Papyrus roll. The Greek medical doctors (e.g. Hippocrates) later adopted this treatment. **(B)** In 1897, a Bayer company chemist Felix Hoffman, purified acetylsalicylic acid. **(C)** Aspirin tablets in an early (pre-1921) Bayer bottle. **(D)** The general practitioner Lawrence Craven was one of the first to systematically describe the protective effect of aspirin on myocardial infarction. With permission from respectively the Leipzig University Library, Bayer Healthcare, Eric E. Johnson and the Minnesota University Library.

for example in the prevention of cancer.[10] In this thesis, we investigate a new strategy to improve the effectiveness of aspirin: the reduction of blood pressure and morning platelet reactivity by taking aspirin at bedtime instead of on awakening.

BEDTIME ASPIRIN AND BLOOD PRESSURE

High blood pressure (hypertension) is an important risk factor for cardiovascular disease. It is believed to cause 22% of all heart attacks in western Europe.[11] Even small reductions of blood pressure (3 to 5 mmHg) decrease the risk of myocardial infarction and stroke in population-based studies.[12, 13] Although there are many different antihypertensive agents, the blood pressure goal of $\leq$140/90 mmHg is reached by only half of all hypertensive patients.[14] Thus, simple and effective interventions to improve blood pressure control could have a large impact on the incidence of cardiovascular disease.

Aspirin, a non-steroidal anti-inflammatory drug (NSAID), was originally thought to increase blood pressure, because it inhibits blood pressure lowering prostaglandin synthesis. However, in contrast to other NSAIDs, aspirin was shown not to increase blood pressure in a meta-analysis.[15] In recent studies aspirin was even associated with considerable reductions of blood pressure (5 to 7 mmHg), but only when taken at bedtime instead of on awakening.[16-18] An insight into the mechanism behind this remarkable time-dependent effect of aspirin on blood pressure was given by the ASPirin In Reduction of TENSION I (ASPIRETENSION I) study.[19] In this study, aspirin intake at bedtime compared with intake on awakening reduced parameters known to influence blood pressure, such as plasma renin activity and 24 hour cortisol, dopamine and norephinephrine excretions.[19] However, these studies were conducted in healthy participants, patients with untreated hypertension or pregnant women, all without a clear clinical indication for aspirin use.[20] Use of low-dose aspirin is only indicated when benefits outweigh the harms (bleeding). Until now, this is only the case for patients who are at high risk for or have established cardiovascular disease.[9, 20, 21] Yet, the potential blood pressure lowering effect of bedtime aspirin was never assessed in patients with established cardiovascular disease, who may also use concomitant antihypertensive drugs and who have more advanced atherosclerosis than patients in previous studies. In the ASPirin In Reduction of TENSION II (ASPIRETENSION II) study, described in **chapter 3**, we assessed whether aspirin intake at bedtime compared with intake on awakening also reduces blood pressure of patients already using aspirin for prevention of cardiovascular disease.

BEDTIME ASPIRIN AND MORNING PLATELET REACTIVITY

Platelets are small anucleated cells, of which large numbers (150-450 x 10^9/L) are present in the human blood.[22] Platelets have a lifespan of 7-10 days and new platelets are constantly produced by bone marrow megakaryocytes. To maintain a stable physiological concentration, new platelets are released at a rate of 10% per day. In normal conditions platelets circulate in the bloodstream in a resting state. However, where blood vessels are damaged, platelets are exposed to stimuli and get activated, change their shape and aggregate to form a hemostatic plug.[23, 24] This is beneficial where hemostasis is needed, but harmful when a plug forms on a ruptured or eroded atherosclerotic plaque and causes myocardial infarction or stroke.

Already in the 1980s, a circadian rhythm was observed in the frequency of acute cardiovascular disease, with the highest incidence during the morning hours (6 to 12 AM).[25] In a meta-analysis the excess risk of acute cardiovascular events during morning hours was estimated to be around 40%.[26] Also in patients treated with cardiovascular drugs such as platelet inhibitors and beta-blockers, recurrent events occur in a circadian pattern.[27] After the discovery of the morning peak in cardiovascular events it was shown that platelet reactivity also follows a circadian rhythm, with a peak of platelet reactivity during the morning (6-12 AM).[28-32] Given the important role of platelets in the development of acute cardiovascular events, it is reasonable to assume that the morning peak of platelet reactivity contributes to the morning peak of acute cardiovascular events.[33] If true, reduction of morning platelet reactivity might prevent arterial thrombosis during morning hours and thereby prevent a proportion of morning cardiovascular events. This may be achieved by intake of aspirin at bedtime instead of on awakening.

After intake, aspirin is rapidly absorbed by the stomach and small intestine, reaching its maximal concentration in blood already after 20 minutes. Thereafter, aspirin is rapidly de-acetylated and cleared from the circulation.[34] Aspirin inhibits platelet reactivity by inactivating the platelet cyclo-oxygenase-1 enzyme, thereby preventing the production of thromboxane A_2, a potent amplifier of platelet aggregation. Because platelets lack the DNA to renew the enzyme, cyclo-oxygenase-1 is inhibited by aspirin for the whole lifespan of a platelet (7-10 days). Still, aspirin has to be taken each day, because new platelets are released at a rate of 10% per day.[35] These newly released platelets are uninhibited by aspirin and are capable to produce thromboxane A_2.[35] It has been suggested that the presence of 10% uninhibited platelets is enough to abolish the effect of aspirin on platelet reactivity.[7, 36] Importantly, previous studies showed that 95% of all platelets have to be inhibited by aspirin to achieve an effective reduction of platelet reactivity.[37]

With a daily platelet turnover of 10%, this implies that only 90% of all platelets are inhibited by aspirin at the end of its dosing interval (24 hours after intake), whereas 95% inhibition is needed to achieve effective inhibition of platelet reactivity.[37] This is also supported by a recent study, which showed that platelet aggregation was insufficiently inhibited 24 hours after morning aspirin intake in 25% of the patients with established cardiovascular disease.[38] The majority of patients take their aspirin on awakening, which in most cases is after the start of the morning peak of platelet reactivity at 6 AM. Consequently, after morning aspirin intake, 10% of platelets are uninhibited just before the next morning intake 24 hours later. So, with aspirin intake on awakening, 10% uninhibited platelets are present during the morning hours, when the risk of cardiovascular events is the highest (Figure 2). Because it is desirable to achieve optimal inhibition of platelet reactivity during the high risk morning hours, it might be beneficial to take aspirin at bedtime instead of on awakening. By taking aspirin at bedtime, the proportion of uninhibited platelets during morning hours would theoretically be reduced to 5%, and thereby inhibition of platelet reactivity is more effectively achieved during morning hours (Figure 2).This was already suggested by previous authors[39-41], but for the first time we evaluate this in clinical trials, described in **chapter 3** and **4**.

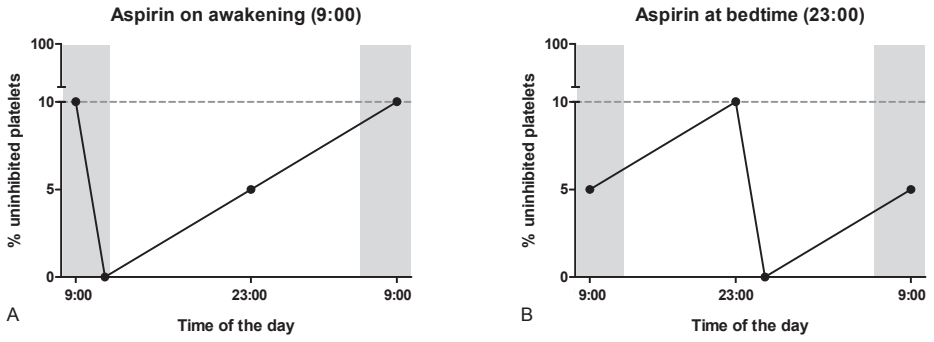

Figure 2 – Theoretical representation of the effect of aspirin intake on awakening (9:00h) or at bedtime (23:00h) on the proportion of uninhibited platelets during 24 hours. The grey shaded area represents the timing of the morning peak of cardiovascular events and platelet reactivity. Short after aspirin intake, all platelets are inhibited (0% uninhibited). Subsequently, new platelets are released at a rate of 10% per day. Due to aspirins short half-life, these newly released platelets are uninhibited. This implies that 10% uninhibited platelets are present 24 hours after aspirin intake, which is situated during the high risk morning hours when aspirin is taken on awakening (**panel A**). The proportion of uninhibited platelets during the morning hours is theoretically reduced to 5% by taking aspirin at bedtime (**panel B**). This might be beneficial, because it is known that 95% platelet inhibition is required to achieve a clinically effective reduction of platelet reactivity by aspirin.

AIMS AND OUTLINE OF THIS THESIS

The main aim of this thesis is to assess the effect aspirin intake on awakening compared with intake at bedtime on blood pressure and the morning peak of platelet reactivity. Both outcomes are particularly suitable to study with a cross-over study, which carries specific methodological advantages. **Chapter 2** gives an introduction to cross-over studies, which helps to understand the design and methodology of the studies in subsequent chapters. **Chapter 3** and **4** describe the results of two clinical trials, which were carried out to investigate the time-dependent effect of aspirin on blood pressure and platelet reactivity. To examine the effect of platelet reactivity on the risk of secondary cardiovascular events, we carried out a cohort study in men who survived a first myocardial infarction, which is described in **chapter 5**. Following international guidelines, patients with cardiovascular disease take several medications on a daily basis, of which beta-blockers are one of the most frequently prescribed. However, there is still debate about the effect of beta-blockers on platelet reactivity. By performing a systematic review and meta-analysis in **chapter 6** we synthesize all available evidence and estimate the magnitude of beta-blockers' effect on platelet reactivity. In **chapter 7** we summarize and discuss the results of the studies presented in this thesis.

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Chapter 2

Cross-over studies

Tobias N. Bonten, Bob Siegerink, Johanna G. van der Bom

Ned Tijdschr Geneesk. 2013;157:A5542

SAMENVATTING

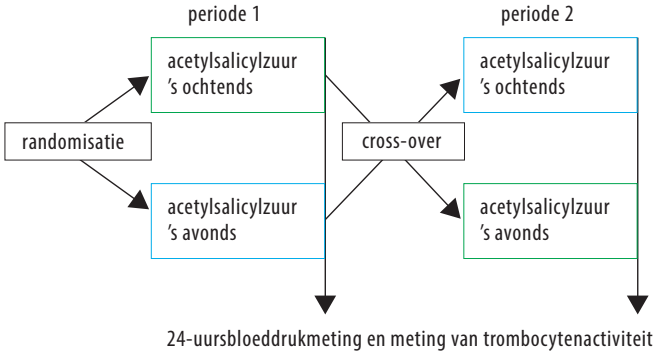
- Voor een gerandomiseerde parallelle-groep trial zijn vaak grote groepen patiënten nodig, wat veel tijd en kosten met zich meebrengt.
- In sommige gevallen kan een gerandomiseerde cross-over trial een voordeliger en efficiënter alternatief zijn.
- Cross-over studies kunnen gebruikt worden bij chronische aandoeningen, waarbij het effect van de behandeling tijdelijk is.
- Elke deelnemer krijgt in opeenvolgende perioden alle behandelingen, waarbij uitkomsten gemeten worden aan het eind van elke periode.
- Meestal is maar een kwart van de totale groepsgrootte van een parallelle-groep studie nodig.
- Resultaten kunnen worden beïnvloed door carry-over- en periode effecten, die voorkomen kunnen worden door een wash-out periode van voldoende duur in te bouwen en te randomiseren.
- Uitval van deelnemers is een groter probleem voor cross-over studies dan voor parallelle-groep studies.

INLEIDING

Effecten van behandelingen kunnen op verschillende manieren gemeten worden. In de praktijk is de meest gebruikte studie opzet een gerandomiseerde parallelle-groep trial. Deze opzet heeft echter als belangrijk nadeel dat er vaak grote groepen patiënten nodig zijn, wat veel tijd en kosten met zich meebrengt. In sommige gevallen is een cross-over studie een efficiënter en goedkoper alternatief. In dit overzichtsartikel bespreken we aan de hand van voorbeelden uit de praktijk wat een cross-over studie is, wanneer deze opzet mogelijk is en wat de voor- en nadelen zijn. Ten slotte bespreken we beknopt hoe cross-over studies geanalyseerd worden. Voor een compleet overzicht over cross-over studies verwijzen wij naar het boek van Senn.[1]

WAT IS EEN CROSS-OVER STUDIE?

In een cross-over studie krijgt elk individu in opeenvolgende perioden alle behandelingen die onderzocht worden, waarbij de uitkomsten gemeten worden aan het eind van elke periode. Hierdoor worden de onderzochte effecten vergeleken



Figuur 1 – De Aspiretension-II-studie (Aspiretension staat voor 'Asprin in reduction of tension') is een lopende studie naar het effect van inname van acetysalicylzuur 100 mg 's ochtends of 's avonds op de bloeddruk en trombocytenfunctie over de dag (www.clinicaltrials.gov; zoek op NCT01379079). De hypothese is dat inname van acetylsalicylzuur 's avonds de bloeddruk en de ochtendpiek van trombocytenreactiviteit verlaagt. In totaal worden 252 patiënten die acetylsalicylzuur gebruiken ter secundaire preventie van hart- en vaatziekten gerandomiseerd voor inname 's ochtends of 's avonds voor een periode van 3 maanden (periode 1). Na 3 maanden vindt een cross-over plaats van het tijdstip van inname, waarna deelnemers nog 3 maanden 's ochtends of 's avonds acetylsalicylzuur gebruiken (periode 2). Na 3 en 6 maanden worden een 24-uursbloeddrukmeting en een meting van de trombocytenreactiviteit in de ochtend gedaan.

binnen een individu in plaats van *tussen groepen individuen*. De meest eenvoudige opzet van een cross-over studie is een studie met twee interventies verdeeld over twee perioden. Dit wordt ook wel een 2x2-, ofwel AB/BA design genoemd. Een voorbeeld hiervan is de Aspiretension II studie, waarin de invloed van aspirine inname 's ochtends versus 's avonds op de bloeddruk en trombocytenreactiviteit wordt onderzocht (Figuur 1). Net als bij een parallelle-groep studie wordt aan het begin van een cross-over studie gerandomiseerd. Het verschil is dat niet wordt gerandomiseerd voor de behandelingen maar voor de vólgorde van de behandelingen. Het nut hiervan wordt verderop in dit artikel besproken. Idealiter worden onderzoekers en deelnemers in een cross-over studie geblindeerd voor de volgorde van behandeling en wordt gebruik gemaakt van een placebo. In een cross-over studie kunnen ook meer dan twee behandelingen vergeleken worden. Een voorbeeld hiervan is de DASH-sodium studie (Tabel 1), waarin de invloed van twee soorten dieet en drie verschillende zoutgehalten op de bloeddruk werd onderzocht.[2, 3]

Tabel 1 Voorbeeld van een cross-overstudie met meerdere behandeling

DASH-Sodium-studie
De 'Dietary approaches to stop hypertension (DASH)-Sodium'-trial is tot nu toe de grootste en meest geciteerde cross-overstudie.[2] Het bijzondere van deze studie is dat het een parallelle opzet combineert met een cross-overopzet. De deelnemers (n = 412) werden eerst gerandomiseerd voor het volgen van een satndaard Amerikaans dieet of het DASH-dieet, rijk aan groenten en fruit met weinig vet, suikers en verzadigde vetten. Vervolgens werd er binnen deze 2 groepen gerandomiseerd voor de volgorde van 3 periodes van 1 maand met een laag, gemiddeld of hoog zoutgehalte. Na elke periode van 1 maand vond cross-over plaats naar een ander zoutgehalte. Elke deelnemer gebruikte dus 1 van de 2 diëten voor 3 maanden in een opzet met parallelle groepen en elk zoutgehalte voor 1 maand in een cross-overopzet. Aan het eind van elke periode werd de bloeddruk gemeten. Bij de deelnemers met een standaard dieet zorgde het lage zoutgehalte in vergelijking met een hoog zoutgehalte voor een gemiddelde systolische bloeddrukdaling van 6,7 mmHg.

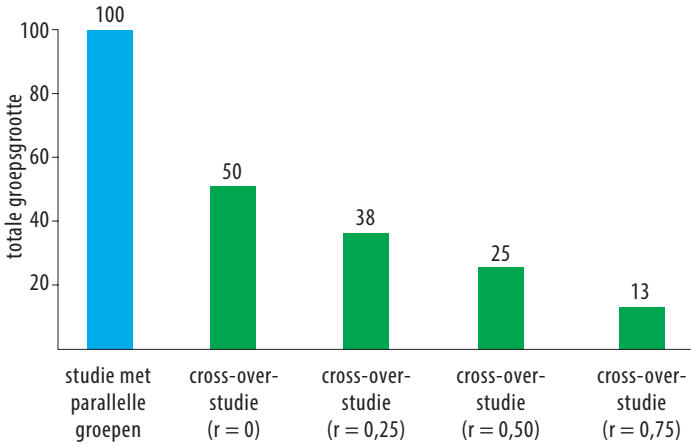
GROEPSGROOTTE

Het grootste voordeel van een cross-over studie is dat er in totaal minder patiënten nodig zijn. De rede hiervoor is dat in een cross-over studie de variatie tussen individuen geen rol meer speelt. Bij de berekening van de groepsgrootte hoeft dan alleen rekening gehouden te worden met de variatie binnen individuen, die meestal kleiner is dan de variatie tussen individuen. De variatie binnen een individu kan worden uitgedrukt als de samenhang (correlatie) tussen de herhaalde metingen binnen een individu. Hoe hoger deze samenhang, hoe lager de variatie. Als er géén samenhang is, is voor een cross-over trial maar de helft van het aantal patiënten nodig vergeleken met een parallelle-groep studie. Dit komt doordat de vergelijking plaatsvindt binnen één groep in plaats van tussen twee groepen. In

de praktijk is er echter altijd samenhang tussen herhaalde metingen binnen een individu. Dit verlaagt het aantal benodigde patiënten voor een cross-over studie verder. Als voor een parallelle-groep studie (bij gelijke power en een gelijk significantieniveau) $N_{parallel}$ patiënten nodig zijn, dan is het aantal patiënten dat nodig is voor een cross-over studie ($N_{cross\text{-}over}$) eenvoudig te berekenen met de formule:

$$N_{cross\text{-}over} = (1\text{-}r)\ N_{parallel}\ /2$$

waarbij *r* de samenhang (correlatie) is tussen de herhaalde metingen binnen een individu.[4] De groepsgrootte voor een parallelle-groep studie ($N_{parallel\ )}$ kan worden berekend volgens de gebruikelijke methoden.[5] Figuur 2 illustreert, gebaseerd op bovenstaande formule, dat de benodigde groepsgrootte voor een cross-over studie afhankelijk is van de samenhang (correlatie, *r*) tussen de herhaalde metingen binnen individuen. In de praktijk komt vaak een correlatie van rond de 0.5 voor, waardoor voor cross-over studies meestal maar een kwart van de totale groepsgrootte van een parallelle-groep studie nodig is. De precieze correlatie kan worden ontleend aan eerdere studies met herhaalde metingen of worden berekend aan de hand van gegevens uit een pilot studie.[6]



Figuur 2 – Benodigde groepsgrootte voor een cross-overstudie om met dezelfde precisie hetzelfde effect aan te kunnen tonen als een studie met parallelle groepen met 100 deelnemers. Als er geen samenhang is (r = 0) tussen de herhaalde metingen, is voor een cross-overstudie de helft van het aantal patiënten nodig. In de praktijk is de correlatie meestal rond 0,5, waardoor voor de meeste cross-overstudies maar een kwart van de totale groepsgrootte voor studie met parallelle groepen nodig is.

NADELEN VAN CROSS-OVER STUDIES

Carry-over

Als behandelingen vergeleken worden in opeenvolgende perioden kan het voorkomen dat de behandeling van periode 1 nog doorwerkt in periode 2. Het effect van de behandeling in periode 2 kan dan beïnvloed worden door het doorwerken van de behandeling in periode 1. Dit wordt het *carry-over* effect genoemd. Een manier om een carry-over effect te voorkomen is het inbouwen van een uitwasperiode (*wash-out*) tussen de periodes waarin geen behandeling wordt gegeven. Men neemt dan aan dat de metingen in de volgende periode niet meer worden beïnvloed door de behandeling in de voorafgaande periode. Een wash-out periode moet lang genoeg zijn om zoveel mogelijk te garanderen dat de interventie uitgewerkt is voordat de metingen in de volgende periode gedaan worden. In de praktijk is het van belang rekening te houden met de werkingsduur van de interventie. Aangeraden wordt om een periode van minimaal 4x de werkingsduur van het middel te nemen.[1]

Soms is een wash-out praktisch niet mogelijk of ethisch niet verantwoord. De interventie periodes kunnen dan langer gemaakt worden, zodat de uitwas van de behandeling in de voorafgaande periode zoveel mogelijk gegarandeerd wordt. In de Aspiretension II studie (Figuur 1) is het medisch en ethisch niet verantwoord om patiënten die aspirine gebruiken ter secundaire preventie van hart- en vaatziekten in een wash-out periode geen aspirine te laten gebruiken. Daarom kozen de onderzoekers voor een interventieperiode van 3 maanden, waardoor het effect van aspirine (werkingsduur 7-10 dagen) in periode 1 uitgewerkt is voordat de metingen aan het eind van periode 2 plaatsvinden. Ook in de DASH-sodium trial (Tabel 1) is gekozen om geen wash-out in te bouwen. De onderzoekers gingen ervan uit dat het effect van een hoger of lager zoutgehalte uitgewerkt was als het effect van het zoutgehalte aan het eind van een volgende periode gemeten werd.[3] Als carry-over effecten niet kunnen worden voorkomen door een wash-out of interventieperiode van voldoende duur, raden wij aan om voor een parallelle-groep studie te kiezen.

Periode effecten en het nut van randomiseren

Patiënten veranderen over de tijd. Gemiddeld nemen bloeddruk en gewicht bijvoorbeeld toe met de leeftijd.[7, 8] Deze 'vaste' verandering in de tijd wordt het *periode effect* genoemd en kan de resultaten van een cross-over studie beïnvloeden. Dit zou in de DASH trial (Tabel 1) kunnen gebeuren als alle deelnemers de verschillende zoutgehaltes in dezelfde volgorde zouden krijgen (bijvoorbeeld: hoog-, gemiddeld- en laag zoutgehalte). Een bloeddrukverlagend effect van de lagere zoutgehaltes zou dan verstoord kunnen worden door de 'vaste' bloeddruk-

verhoging in de tijd. De onderzoekers zouden dan onterecht concluderen dat er geen bloeddrukverlagend effect is van een lager zoutgehalte. Beïnvloeding door het periode effect wordt voorkomen door aan het begin van de studie te randomiseren voor de volgorde van de behandelingen.

Uitval van deelnemers

Net als bij parallelle-groep studies is het bij cross-over studies van belang dat zo min mogelijk deelnemers uitvallen tijdens de studie en dat wordt bijgehouden wat de redenen van uitval zijn. Voor een cross-over studie heeft de uitval van deelnemers echter grotere gevolgen dan voor een parallelle-groep studie. Dit komt doordat de vergelijking van interventies plaatsvindt binnen individuen. Deze vergelijking kan niet plaatsvinden als een deelnemer uitvalt voordat hij alle interventies gehad heeft. Aangezien elke deelnemer in een cross-over studie relatief meer bijdraagt aan de precisie (power) van de studie dan een deelnemer in een parallelle groep studie, verliest een cross-over studie ook sneller zijn precisie bij uitval van deelnemers dan een parallelle-groep studie. Als er een grote kans is op uitval van deelnemers, bijvoorbeeld door belastende metingen of bijwerkingen van de behandeling, kan beter gekozen worden voor een parallelle-groep studie.

Aandachtspunten voor deelnemers

Voor deelnemers aan een cross-over studie kan het een nadeel zijn dat de tijd die zij doorbrengen in het onderzoek tweemaal zo lang is als in een parallelle-groep studie. Met een wash-out periode is dit nog langer. Terwijl in een parallelle-groep studie patiënten meestal maar 50% kans hebben om gerandomiseerd te worden voor de nieuwe behandeling, krijgt elke deelnemer in een cross-over studie gegarandeerd de experimentele behandeling. Dit kan een nadeel zijn omdat deelnemers in een cross-over studie alle interventies en metingen moeten ondergaan. Aan de andere kant kan het juist prettig zijn voor deelnemers om te weten dat ze in ieder geval een periode de experimentele behandeling krijgen. Mogelijk is de bereidheid tot deelname hierdoor groter bij een cross-over studie dan bij een studie met parallelle groepen. De voor- en nadelen voor de deelnemers hangen dus af van de bestudeerde interventie en de cross-overopzet.

WANNEER KAN EEN CROSS-OVER STUDIE GEBRUIKT WORDEN?

In een cross-overstudie moet de interventie een tijdelijk effect hebben, waarbij na het staken van de interventie de patiënt weer terugkeert in de staat van vóór het begin van de interventie. Hierbij valt te denken aan aandoeningen die een

chronisch beloop hebben en de behandeling geen genezing brengt maar gericht is op symptoombestrijding of op vertraging van het ziekteproces. Voorbeelden zijn hypertensie, astma, reuma, migraine, epilepsie en diabetes mellitus.

In een cross-over studie moet de uitkomst van de bestudeerde interventie op relatief korte termijn te meten zijn, bijvoorbeeld bloeddruk, aanvalsfrequentie bij epilepsie, longfunctie of pijn scores. Cross-over studies zijn niet geschikt voor het bestuderen van interventies waarbij een lange follow-up nodig is om de uitkomst te meten. Ook zijn cross-over studies niet geschikt om interventies te bestuderen met een blijvend effect, zoals genezing of overlijden. De patiënt keert dan immers niet in zijn oorspronkelijke staat terug. Alle voor- en nadelen van cross-over studies zijn samengevat in tabel 2. Kortom, een cross-overstudie vormt een alternatief voor een studie met parallelle groepen als: (a) een stabiele chronische aandoening bestudeerd wordt; (b) de bestudeerde interventie een tijdelijk effect heeft (geen genezing of overlijden); (c) het carry-over-effect kan worden vermeden door een uitwasperiode of interventieperiodes van voldoende duur; (d) een laag percentage uitval van deelnemers verwacht wordt; (e) voor deelnemers een langere studieduur acceptabel is.

Tabel 2 Voor- en nadelen van cross-overstudies

voordelen
• meestal maar een kwart van het aantal deelnemers nodig in vergelijking met studies met parallelle groepen
• vergelijking bij 1 individu in plaats van tussen individuen; elk individu is zijn of haar eigen controle
• mogelijk bij chronische stabiele ziekten zoals hypertensie, reuma, migraine, diabetes
• efficiënter en goedkoper dan studie met parallelle groepen
nadelen
• niet mogelijk bij interventies met een onomkeerbaar effect op de uitkomst (genezing of overlijden)
• mogelijke verstoring van vergelijking tussen interventies door 'carry over'- en periode-effecten
• angere onderzoekstijd voor deelnemers
• uitval van deelnemers groter probleem dan in studies met parallelle groepen
• niet mogelijk als een lange follow-upduur nodig is om het effect van de interventie te meten

ANALYSE

In een cross-overstudie is er sprake van herhaalde (gepaarde) metingen bij 1 individu. De meest gebruikte methode om data uit een cross-overstudie weer te geven is een tabel waarin de uitkomst in kolommen gesorteerd is op de behandeling,

uitgesplitst naar de volgorde waarvoor deelnemers gerandomiseerd zijn.[1] Met een gepaarde t-test kan het verschil in bloeddruk tussen de 2 interventies (inname 's ochtends vs. 's avonds) statistisch getest worden. In de praktijk is echter vaak sprake van ontbrekende waarden en worden meerdere metingen verricht in 2 of meer periodes. Een gepaarde t-toets of variantieanalyse (ANOVA) is dan niet geschikt, omdat deze testen individuen met ontbrekende waarden niet meenemen in de analyse. Voor analyse van cross-overstudies wordt daarom meestal gebruik gemaakt van analysemethoden voor herhaalde metingen ('mixed models').[9] Met deze methoden worden individuen van wie waarden ontbreken, wél meegenomen in de analyse en kunnen ook periode-effecten geanalyseerd worden.

CONCLUSIE

In sommige gevallen zijn cross-over studies een goedkoop en efficiënt alternatief voor parallelle-groep studies. De belangrijkste voordelen zijn dat maar een kwart tot de helft van het aantal patiënten nodig is en dat de onderzochte behandelingen vergeleken worden bij de individuele patiënt. Een cross-over studie vormt een alternatief voor een parallelle-groep studie als een stabiele chronische aandoening bestudeerd wordt en de bestudeerde interventie een tijdelijk effect heeft (geen genezing, geen overlijden). Het is van belang dat het carry-over effect zoveel mogelijk wordt vermeden door een wash-out of interventieperiodes van voldoende duur. Voor deelnemers moet de langere studieduur van een cross-over studie acceptabel zijn. De kans op uitval van deelnemers moet laag zijn omdat uitval voor cross-over studies grotere negatieve gevolgen heeft dan voor parallelle-groep studies. Als aan deze voorwaarden wordt voldaan is een cross-over studie voordeliger en efficiënter dan een parallelle-groep studie.

DANKNOOT

Prof. Theo Stijnen, statisticus, afdeling Medische Statistiek LUMC, leverde commentaar op een deel van dit artikel.

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Chapter 3

Aspirin intake at bedtime: does it lower blood pressure and morning platelet reactivity in patients with stable cardiovascular disease? A randomized cross-over trial

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ABSTRACT

Aims

To evaluate whether intake of low-dose aspirin at bedtime, compared with intake on awakening, reduces 24-hour ambulatory blood pressure and morning platelet reactivity.

Methods and Results

In this randomized open-label cross-over trial, 290 patients were randomized to take 100mg aspirin on awakening or at bedtime during two periods of 3 months. At the end of each period 24-hour blood pressure and morning platelet reactivity was measured. The primary analysis population comprised 263 (blood pressure) and 133 (platelet reactivity) patients. Aspirin intake at bedtime did not reduce blood pressure compared with intake on awakening (difference systolic/diastolic: -0.1 [95% CI: -1.0; 0.9] / -0.6 [95% CI: -1.2; 0.0] mmHg). Platelet reactivity during morning hours was reduced with bedtime aspirin intake (difference: -22 ARU [95% CI -35; -9]).

Conclusions

This study showed that the intake of aspirin at bedtime compared with intake on awakening does not reduce blood pressure of patients taking aspirin for cardiovascular disease prevention. However, bedtime aspirin reduced morning platelet reactivity. Future studies are needed to assess the impact of this promising simple intervention on the excess of cardiovascular events during the high risk morning hours.

INTRODUCTION

Cardiovascular disease (CVD) is still a leading cause of mortality and morbidity worldwide.[1, 2] One of the most important modifiable risk factors for CVD is blood pressure. Even small reductions of blood pressure significantly decrease the risk of myocardial infarction and stroke.[3] However, almost half of the patients with hypertension remain uncontrolled despite blood pressure lowering medication.[4] Thus, simple interventions to improve blood pressure control are needed.

Aspirin traditionally was assumed to have no effect on blood pressure[5], but in recent studies aspirin intake at bedtime compared with intake on awakening considerably reduced blood pressure.[6-11] Additionally, we previously found that aspirin intake at bedtime compared with on awakening reduced plasma renin activity and cortisol, dopamine and norepinephrine excretions over 24 hours.[12] However, all previous studies included healthy subjects, pregnant women or patients with mild hypertension.[6-11, 13] If the effect of bedtime aspirin intake on blood pressure also holds for patients who already use aspirin for CVD prevention, simply changing the time of intake from awakening to bedtime could substantially reduce their risk for recurrent cardiovascular events.

Furthermore, platelet aggregation peaks during morning hours, which is thought to contribute to the observed peak of CVD from 6 to 12 AM.[14, 15] Due to its short half-life, aspirin only inhibits the platelets that are present at the time of intake, while new platelets are released at a rate of 10% per day in healthy subjects.[16, 17] Thus, just before each aspirin intake, these newly released platelets are uninhibited and can induce platelet aggregation. However, it is desirable to achieve optimal platelet aggregation inhibition particularly during those high risk morning hours. As already suggested by previous authors, intake of aspirin at bedtime might attenuate the morning peak of platelet reactivity, but this was never evaluated in a clinical trial.[18, 19]

To assess whether aspirin intake at bedtime compared with intake on awakening reduces blood pressure and morning platelet reactivity we conducted a randomized cross-over trial in patients using low-dose aspirin for prevention of CVD.

METHODS

Study population

Patients between 18-75 years of age using aspirin for secondary prevention of CVD were recruited from general practitioner (GP) practices around Leiden, the Netherlands. Subjects with a baseline blood pressure (BP) of <120/70 or >160/100

mmHg were excluded. Additionally, subjects were excluded if they used other anti-platelet drugs than aspirin or had changed any antihypertensive medication in the 3 months before baseline to guarantee stability of CVD and blood pressure before entry into the study. Other exclusion criteria were regular use of non-steroidal anti-inflammatory drugs (NSAIDs), employment as shift worker, evidence of secondary arterial hypertension (e.g. pheochromocytoma) and pregnancy.

Study design

This prospective, randomized, open-label, blinded endpoint (PROBE), 2-period crossover study was conducted at a single center in the Netherlands and registered at www.clinicaltrials.gov/ct2/show/NCT01379079. Benefits of the PROBE design and its validity for studies measuring ambulatory blood pressure have been previously documented.[20] The study was conducted in accordance with the Declaration of Helsinki, approved by the Leiden University Medical Center (LUMC) Ethics Committee and all subjects gave written informed consent. Randomization was performed with a computer-generated randomization code by an independent person at the Department of Clinical Epidemiology of the LUMC and was inaccessible to the investigators. Eligible subjects were randomized (1:1 ratio) to take aspirin on awakening followed by aspirin at bedtime or the opposite order during two intervention periods of 3 months. The two intervention periods were not separated by a wash-out period, because withholding aspirin to the included patients was considered unethical. The duration of each intervention period was analogous to previous studies.[9-11] All subjects received 100 mg effervescent aspirin (Carbasalate Calcium, Vemeda Manufacturing, the Netherlands). At the end of each intervention period, subjects visited the research site for two consecutive days. At day 1, 24-hour ambulatory blood pressure measurement (ABPM) was started between 8-12 AM and subjects took aspirin at the same time as in the preceding 3 months. At day 2, subjects refrained from taking aspirin in the morning, ABPM was ended and blood was drawn. The time of ABPM start at day 1 and blood draw at day 2 was similar for each participant at each visit.

Measurements

Blood pressure

At baseline, BP was measured by an automatic device (Mobil-O-Graph NG device; IEM GmbH, Germany) every 2 minutes in seated position after 10 minutes of rest. The average of 6 readings was used to determine baseline blood pressure. As the primary endpoint, ABPM was performed during participants normal daily routine with a validated and calibrated Mobil-O-Graph NG device (IEM GmbH, Germany).

Measurements started between 8-12AM and the same device was used at each visit. The BP cuff was adjusted to the arm circumference and worn on the non-dominant arm. Systolic and diastolic BP were automatically measured every 20 minutes during the day and every 30 minutes during the night for 24 consecutive hours, with the screen turned off to blind subjects for BP readings. Activities, bed- and awakening times were recorded in a diary. ABPM was considered valid if at least 70% of the measurements were valid, sleep time during ABPM was between 6 and 12 hours and data were not missing for an interval of more than 2 hours.

Platelet reactivity

As a secondary endpoint, platelet reactivity was measured during morning hours (between 8 and 12 AM). At the morning of blood sampling subjects refrained from taking aspirin. Blood was sampled without stasis from the antecubital vein and platelet reactivity was measured with the VerifyNow® Aspirin Assay (Accumetrics, San Diego, USA).[21] According to manufacturer's instructions, platelet reactivity was measured between 30 minutes and 4 hours after the blood draw.

Questionnaires, compliance and patient preference

Subjects completed a questionnaire to assess eligibility criteria, medical history, medication use and chronobiological rhythm at baseline. Missing information was completed with the use of general practitioner or pharmacy records. At each follow-up visit, side effects and change in other medication were registered by questionnaires. Subjects were instructed to take aspirin within 1 hour after awakening or 1 hour before bedtime. Compliance was assessed and optimized with electronic pill boxes (Evalan, Amsterdam, the Netherlands), which registered the time of intake and sent an SMS text message if subjects were non-compliant. Additionally, a pill count was performed at each visit. Subjects were instructed not to change timing of co-medication, which was checked with questionnaires at each follow-up visit. Pre-randomization timing of aspirin use and patient preference after study completion was assessed by questionnaires.

Statistical analyses

To detect an inter-individual difference of 3 mmHg in blood pressure with 80% power at a 5% significance level, we calculated a required sample size of 250 patients. For this calculation we assumed an intra-individual standard deviation of 12.9 mmHg, as derived from a previous study.[12] Estimating a drop-out of 10% and invalid ABPM of 5%, we randomized 290 subjects. As planned on beforehand, platelet reactivity was measured in the first 160 patients, yielding a power of 90% to detect a difference of 17 ARU at a 5% significance level. For this calculation

we used an intra-individual standard deviation of 46.85 ARU, as derived from a previous study.[22] Continuous characteristics are described as mean ± standard deviation(SD) if normally distributed or as median (interquartile range [IQR]) if not normally distributed. Categorical variables are expressed as numbers (percentages). For analysis, we edited ABPM values according to conventional criteria to remove measurement errors and outliers. Because the sampling frequency was denser during the day (3x/hour) than during the night (2x/hour), we calculated a weighted overall mean BP, as suggested previously[23]:

$$\frac{\text{sum day or night measurements}}{\text{nr day or night measurements}}$$

$$\frac{(\text{mean day BP} * \text{nr day measurements}) + (\text{mean night BP} * \text{nr night measurements})}{\text{nr day measurements} + \text{nr night measurements}}$$

Mean day- and night BP was calculated as:

For these calculations, the start of day- and night times was obtained from the ABPM diaries.

The primary endpoint was assessed in a primary- and secondary analysis population. The primary analysis population included all subjects who were randomized and completed measurements of the endpoints. The secondary analysis population excluded subjects with one or more invalid ABPM, change of antihypertensive medication, or compliance <90%. Paired t-tests were performed to analyze day-, night- and overall mean BP after intake of aspirin on awakening and at bedtime. Additionally, we used linear mixed models to assess the treatment effect and possible period or carry-over effects. Subgroup analyses were pre-specified for users of beta-blockers, inhibitors of the renin-angiotensin system (users versus non-users), users of no- versus 1 or more blood pressure lowering drugs and subjects with baseline systolic BP of >140 versus ≤140 mmHg.

The secondary endpoint platelet reactivity was analysed with a paired t-test and linear mixed models. Subjects who forgot to take aspirin on the day before platelet reactivity measurements (n=3) were excluded from analysis. Subgroup analyses were pre-specified for diabetic subjects, current smokers (yes versus no) and mean platelet volume (MPV) values (divided into quartiles). Although not pre-specified, an additional subgroup analysis for body mass index (BMI) was performed, because obesity, as a marker for metabolic syndrome, may be associated with platelet reactivity.[24]

Side effects and patients' preferences were analysed descriptively and using McNemar's test. All analyses were performed in SPSS 20.0 (IBM corp., USA) and were 2-sided, with a level of significance of 0.05.

RESULTS

Study population and compliance

Between June 2011 and December 2012, 3479 subjects were screened at 30 GP practices, of whom 1704 did not meet inclusion criteria, primarily because of age >75 years (n=1080) and use of other platelet inhibiting drugs (n=386). A further 1182 subjects did not respond or declined to participate and 303 were excluded for other reasons (Figure 1). A total of 290 subjects were randomized, and baseline characteristics were similar between groups (Table 1). The study follow-up was discontinued by 26 subjects, primarily because study participation was too aggravating (18/26; 70%). The primary- and secondary analysis populations comprised 263 and 150 subjects, respectively, for assessment of the primary endpoint. Measurements for the secondary endpoint platelet reactivity were complete for 136

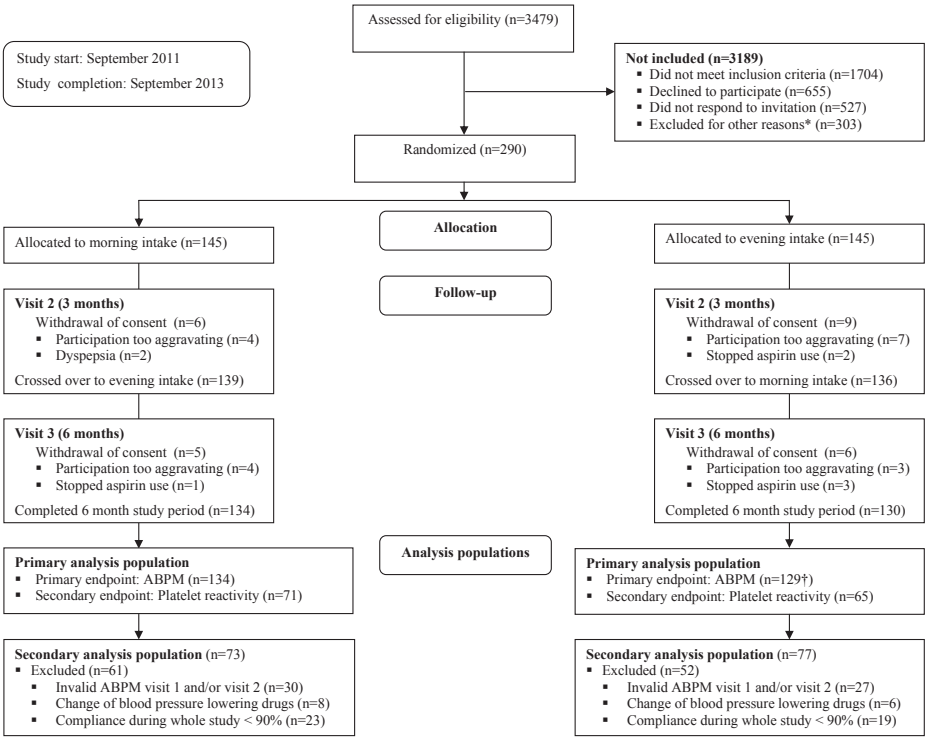

Figure 1 – Patient flow. ABPM: ambulatory blood pressure measurement. * Other reasons: stopped aspirin use before inclusion, not able to participate in clinical trial as judged by general practitioner, changed address, not speaking Dutch language. † 1 subject refused ABPM at the last follow-up visit.

subjects. Compliance as measured by electronic pill boxes and pill count was high, and similar with aspirin intake on awakening (99% [97 to 100%] and 100% [100 to 100%], respectively) and intake at bedtime (98% [94 to 100%] and 100% [100 to 100%]).

Table 1. Baseline clinical characteristics of randomized study participants (n=290)*

	Awakening – Bedtime group (n=145)	Bedtime – Awakening group (n=145)
Sex (M/F)	106/39	106/39
Age (yr)	64±7	64±7
Current smokers	21 (15)	28 (19)
Body mass index (kg/m^2)	28.4±4.7	28·1±4.6
Systolic blood pressure (mmHg)	137±10	137±10
Diastolic blood pressure (mmHg)	88±8	88±8
Diabetics	17 (12)	14 (10)
Cardiovascular history		
Myocardial infarction	53 (37)	59 (41)
Stable angina pectoris	59 (41)	61 (42)
Stroke/Transient ischemic attack	28 (19)	23 (16)
Atrial fibrillation	14 (10)	13 (9)
Peripheral artery disease	12 (8)	9 (6)
Other†	3 (2)	1 (1)
Aspirin use at baseline		
On awakening	106 (73)	100 (69)
Duration (years)	6 (3-11)	6 (4-14)
Medication use		
Number of blood pressure lowering drugs§	2 (1-2.5)	2 (1-3)
β-blockers	74 (51)	80 (55)
Ace-inhibitors	60 (41)	55 (38)
Angiotensin II inhibitors	37 (26)	33 (23)
Calcium antagonists	29 (20)	27 (19)
Diuretics	37 (26)	46 (32)
Lipid lowering drugs	116 (80)	123 (85)

* Continuous values are presented as means ± standard deviation (SD) or medians + interquartile range if not normally distributed. Categorical values are presented as number (%).

† Other cardiovascular disease: heart valve disease (n=3), myelodysplastic syndrome (n=1).

§ Blood pressure lowering drugs: β-blockers, α-blockers, ace-inhibitors, angiotensin-II inhibitors, calcium antagonists, thiazide and loop diuretics, nitrates (daily use).

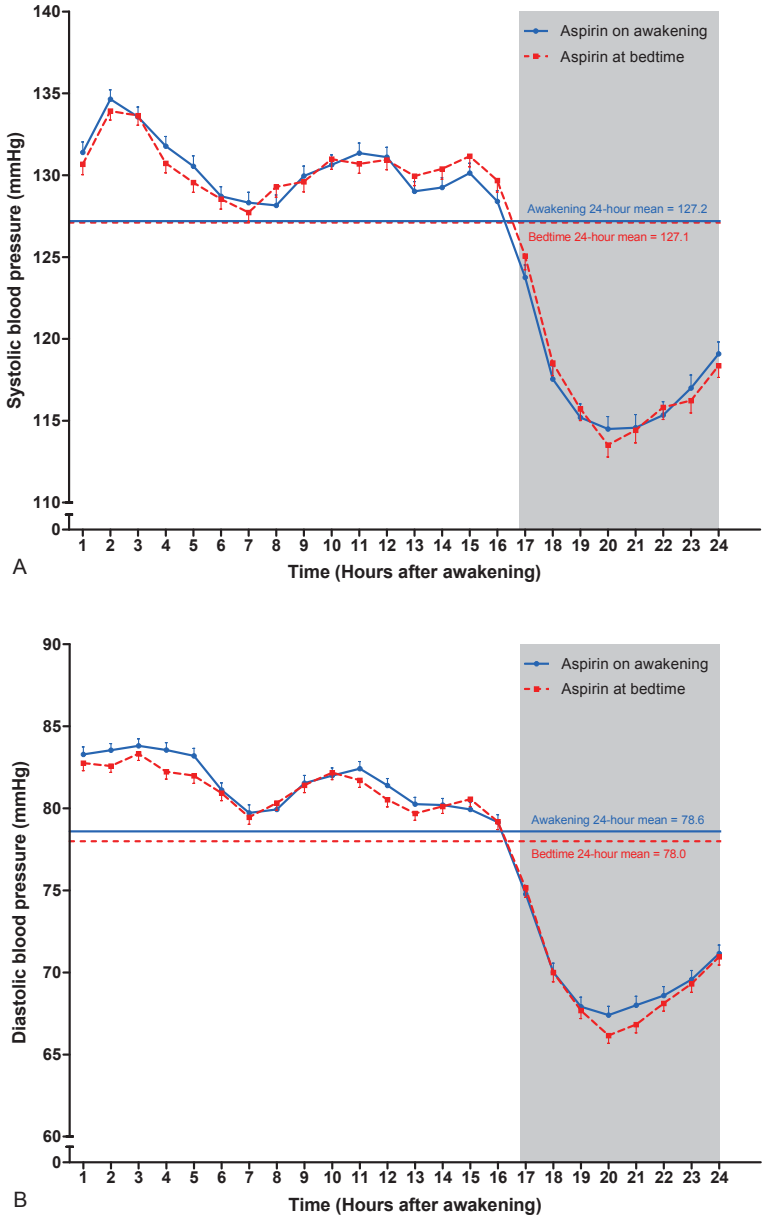

Figure 2 – Effect of low-dose aspirin intake at bedtime compared with intake on awakening on 24-hour ambulatory blood pressure profile in the primary analysis population (n=263). **A.** Systolic blood pressure. **B.** Diastolic blood pressure. Each graph shows hourly means and standard errors of blood pressure measured after 3 months of low-dose aspirin intake on awakening (continuous blue line) and 3 months of low-dose aspirin intake at bedtime (dashed red line). Hours on the x-axis refer to hours after awakening from nocturnal sleep, as recorded in diaries during ABPM. The shaded area represents the average nocturnal period for all subjects.

Blood pressure

The circadian 24h ABPM profile after 3 months aspirin intake on awakening and 3 months intake at bedtime is depicted in Figure 2. The mean (SD) 24-hour systolic and diastolic blood pressures were 127 (12) and 79 (9) mmHg with aspirin intake on awakening, whereas this was 127 (12) and 78 (8) with aspirin at bedtime. This resulted in differences of -0.1 mmHg (95% confidence interval (CI) -1.0 to 0.9) and -0.6 mmHg (CI -1.2 to 0.0). Furthermore, systolic and diastolic blood pressures during day- and nighttime did not differ by the timing of aspirin intake (Table 2). Mixed model analysis showed the same results and no evidence for carry-over- or period effects (data not shown). Additionally, the findings among subgroups of subjects using or not using β-blockers, angiotensin inhibitors, blood pressure lowering drugs in general or subjects with baseline office BP >140 or ≤140 mmHg were similar to the overall results (Table 3). Finally, in the secondary analysis, comprising only patients with valid ABPM at both visits who did not change their antihypertensive medication between visit 2 and 3 and were ≥90% compliant as registered with electronic pill boxes, aspirin intake at bedtime was not associated with a reduction of mean 24-hour blood pressure or day- and night time blood pressure (Appendix table 1).

Table 2. Mean 24-hour, day- and night ambulatory blood pressure values (mmHg) according to time of aspirin administration in the primary analysis population (n=263)

	Aspirin on awakening	Aspirin at bedtime	Mean difference (bedtime – awakening) [95% CI]*
24-hour SBP	127 ± 12	127 ± 12	-0.1 [-1.0 to 0.9]
24-hour DBP	79 ± 9	78 ± 8	-0.6 [-1.2 to 0.0]
Day SBP	131 ± 12	131 ± 12	0.0 [-1.0 to 1.0]
Day DBP	82 ± 9	81 ± 9	-0.6 [-1.2 to 0.1]
Night SBP	117 ± 15	117 ± 14	-0.1 [-1.4 to 1.1]
Night DBP	69 ± 10	69 ± 9	-0.4 [-1.2 to 0.3]

*Mean difference and 95% CI obtained with paired t-tests. Values are mean ± standard deviation. SBP: systolic blood pressure; DBP: diastolic blood pressure; CI: confidence interval

Platelet reactivity

Three subjects forgot to take aspirin on the day before platelet reactivity measurements, and were excluded from this analysis. In the remaining 133 subjects, aspirin intake at bedtime reduced morning platelet reactivity (mean difference -22 ARU [95% CI -35 to -9]; p=0.001; Figure 3). Subgroup analysis showed that, besides in subjects with diabetes, aspirin intake at bedtime reduced platelet reactivity in all subgroups (Table 4).

Table 3. Subgroup analysis of the effect of Aspirin intake on awakening or at bedtime on mean 24-hour blood pressure

	n	Difference (Bedtime – Awakening) [95% CI]*	P-value†
β-blocker use			
No	121	-0.3[-1.7 to 1.2] / -0.8[-1.7 to 0.2]	
Yes	142	0.1[-1.1 to 1.3] / -0.3[-1.2 to 0.4]	0.80
Angiotensin inhibitor use‡			
No	106	-0.2[-1.5 to 1.0] / -1.0[-1.9 to -0.2]	
Yes	157	0.0[-1.3 to 1.4] / -0.2[-1.0 to 0.6]	0.68
Blood pressure lowering drugs use§			
No	51	0.3[-1.5 to 2.1] / -1.3[-2.7 to 0.0]	
Yes	212	-0.2[-1.2 to 0.9] / -0.4[-1.0 to 0.3]	0.78
Baseline systolic office blood pressure			
>140 mmHg	92	0.9[-0.8 to 2.5] / 0.0[-1.1 to 1.2]	
≤140 mmHg	171	-0.6[-1.7 to 0.6] / -0.9[-1.6 to -0.2]	0.14

All blood pressure differences are depicted as systolic[95% CI] / diastolic[95% CI] blood pressure, in mmHg; *Mean difference and 95% confidence interval (CI) obtained from paired t-test within each subgroup; †P-value for interaction obtained from linear mixed model analysis; ‡Angiotensin receptor inhibitors: use of ace-inhibitors and/or angiotensin-2-inhibitors. § Blood pressure lowering drugs: β-blockers, α-blockers, ace-inhibitors, angiotensin-II inhibitors, calcium antagonists, thiazide and loop diuretics, nitrates (daily use).

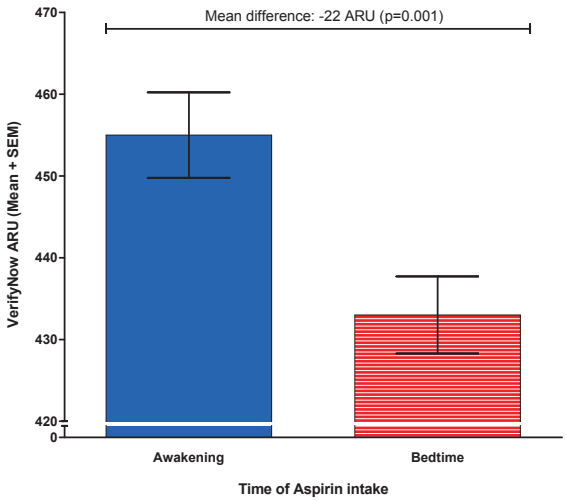

Figure 3 – Effect of low-dose aspirin intake at bedtime versus on awakening on morning platelet reactivity. Filled bars represent VerifyNow platelet reactivity values after aspirin intake on awakening. Dashed bars represent values after aspirin intake at bedtime.

Table 4. Subgroup analysis of the effect of low-dose aspirin intake on awakening or at bedtime on morning platelet reactivity (n=133)

	n	Aspirin on awakening	Aspirin at bedtime	Difference (Bedtime-Awakening) [95% CI]*	P-value†
Diabetes					
No	115	455 ± 61	429 ± 50	-26 [-40 to -13]	
Yes	18	455 ± 55	463 ± 71	8 [-34 to 50]	0.03
BMI (kg/m²)‡					
18.5 – 25	37	452 ± 52	425 ± 52	-27 [-47 to -8]	
25 – 30	60	454 ± 66	439 ± 58	-15 [-36 to 7]	0.41§
≥ 30	36	459 ± 58	432 ± 51	-27 [-53 to -1]	0.94§
Smoking					
No	110	453 ± 60	433 ± 57	-20 [-35 to -5]	
Yes	23	463 ± 61	433 ± 43	-29 [-58 to -1]	0.77
Mean platelet volume (fl), quartile (range)					
1 (9.1 – 10.1)		441 ± 57	424 ± 57	-16 [-93 to 9]	
2 (10.2 – 10.6)		457 ± 60	441 ± 55	-10 [-46 to 25]	0.77§
3 (10.7 – 11.3)		471 ± 68	443 ± 55	-25 [-53 to 4]	0.49§
4 (11.4 – 12.6)		445 ± 48	427 ± 59	-18 [-45 to 9]	0.98§

All platelet reactivity values are depicted in Aspirin Reaction Units (ARU)±standard deviation. Higher ARU represents higher platelet reactivity.

* Mean difference and 95% CI obtained from paired t-test within each subgroup

† P-value for interaction obtained from linear mixed model analysis

‡ BMI classified according to World Health Organizations' classification of obesity

§ P-value for interaction with the first group as reference group

Side effects and patient preference

Three subjects did not complete the study due to side effects (Supplemental table 2). Of these subjects, 2 developed dyspepsia after the switch from aspirin intake in the evening (before study entry) to intake on awakening in the first study period, and 1 developed headache after the switch from intake on awakening to intake at bedtime during study follow-up. The frequency of well-known aspirin side effects (dyspepsia, nausea, heartburn) was similar between aspirin intake on awakening and at bedtime (Appendix table 3).

At baseline 206/290 (71%) of the subjects took aspirin on awakening. After completion of the study, 53/264 (20%) preferred to switch to another time of aspirin intake than prior to study entry. A total of 32/264 (12%) switched from intake on awakening to intake at bedtime and 21/264 (8%) from at bedtime to on awakening. So, no clear patient preference was present for time of intake.

DISCUSSION

In this large cross-over trial among patients using low-dose aspirin for CVD prevention, 24-hour blood pressure did not differ between aspirin intake at bedtime and intake on awakening. However, aspirin intake at bedtime was associated with lower morning platelet reactivity.

Comparison with previous studies

The finding that aspirin intake at bedtime compared with intake on awakening does not reduce blood pressure is in contrast with previous studies, mostly from a single source in this field.[6-11, 13, 25] This may be explained by differences in study populations. First, previous studies included subjects who did not use blood pressure lowering drugs such as β-blockers or inhibitors of the renin-angiotensin-aldosterone system- (RAAS). This is an important difference, because the mechanism behind the time-dependent effect of aspirin on blood pressure was previously related to a reduction in renin-angiotensin-aldosterone system- (RAAS) and catecholamine activity over 24 hours.[12] However, in our subgroup analyses, we did not find an effect in both users and non-users of β-blockers or RAAS-inhibitors. Even in the subgroup who did not use any blood pressure lowering drugs, there was no effect. Our findings corroborate those of an earlier study which also did not find a blood pressure lowering effect of bedtime aspirin intake among treated hypertensive patients.[26] Second, patients in all previous studies did not use aspirin before study entry. In contrast, all patients in our study had a medical indication for aspirin use and had used aspirin for median 6 years. It is possible that the time-dependent effect of aspirin on blood pressure weakens over time because of increased arterial stiffening.[27] However, a potential blood pressure lowering effect of bedtime aspirin intake would only be clinically relevant in patients already using aspirin for CVD prevention, and we are the first in this field to include this clinically relevant patient group.

The circadian rhythm of platelet reactivity and its relation with the morning peak of cardiovascular events has been thoroughly studied.[15, 28] Previous authors suggested that platelet inhibition during these high risk morning hours could be optimized by aspirin intake at bedtime.[18, 19] However, to the best of our knowledge, this has never been evaluated in a clinical trial. Our study suggests that morning platelet reactivity can be reduced by taking aspirin at bedtime instead of on awakening. This effect was homogeneously present in all subgroups, except in diabetic subjects. However, the size of this subgroup was too small (n=18) to rule out any effect in diabetic patients. The reduction of platelet reactivity during the vulnerable morning hours might be beneficial for patients with CVD, who have

higher platelet turnover and of which in 25% platelet reactivity is inadequately inhibited 24 hours after aspirin intake.[29, 30] Previous authors suggested that twice daily dosing of aspirin is yields more effective platelet inhibition over the whole day in selected patient populations with high platelet turnover (e.g. diabetic patients).[31-33] Although this seems valuable for selected patient populations, we would not recommend twice daily dosing for all CVD patients, because twice daily dosing could result in more side effects and less compliance.[34, 35]

Clinical interpretation

It has been shown that the risk for recurrent cardiovascular events is increased in patients with higher VerifyNow-aspirin platelet reactivity values.[36, 37] Stable CVD patients with platelet reactivity >550 ARU had an absolute risk of 15.6% for developing the composite cardiovascular endpoint, whereas this was only 5.3% in patients with ARU values <550.[37] In another study, the absolute risk for the primary endpoint (all-cause death and recurrent cardiovascular events) was 13.3% in patients >454 ARU and 5.9% in patients <454 ARU.[36] These observational studies suggest that already a modest reduction in platelet reactivity could result in clinical benefit for patients with CVD.

Because the CVD morning peak is a multifactorial process, we do not expect that bedtime aspirin would abolish the CVD morning peak completely.[38] Still, given the high prevalence of CVD, already a modest reduction of the morning peak would lead to a large absolute benefit. For example, 280.000 recurrent cardiovascular events occur in the United States (US) every year, and thus on average 767 each day.[1] With the known excess of 40% during the morning hours (6-12 AM)[39], this would mean that 241 events occur from 6 to 12 AM, and 526 during the rest of the day. If aspirin intake at bedtime would reduce this morning peak by 20%, it would lead to an absolute reduction of 4853 recurrent events each year in the US alone. In summary, switching to bedtime aspirin intake is a simple and possible effective intervention, but future clinical trials should evaluate whether this intervention indeed translates in a reduction of recurrent cardiovascular events.

Strengths and limitations

The major strength of our study is that we used a cross-over design, which yields high statistical power and enables comparison of treatment effects within each patient. Furthermore, this is the first study in this field which registered the actual time of aspirin intake by electronic pill boxes, which is of major importance to study time-dependent effects.

The main limitation of our study is that only 150/263 (57%) patients complied perfectly with the study protocol. This was mainly due to invalid ABPM (n=57) or

compliance of less than 90% within the prespecified time of intake (n=42). However, sensitivity analysis among patients with complete follow-up and compliance revealed exactly the same results. Importantly, the statistical power of this secondary analysis was still adequate, as reflected by the narrow confidence intervals in our secondary analysis (Supplemental table 1). Another limitation could be the open-label design of this study. However, benefits and validity of the open-label PROBE design have been previously documented and we do not think that the design influenced the objective ABPM and platelet reactivity measurements as endpoints.[20]

Regarding platelet reactivity measurements, it is a limitation that we measured platelet reactivity at only one time point during the morning, although comparability within subjects was optimized by drawing blood at the same time at each visit. Yet, it is possible that the peak of platelet reactivity is shifted to another time of the day, which is currently being investigated by our group (www.clinicaltrials.gov/ct2/show/NCT01900639). However, a shift of the platelet reactivity peak away from the morning hours to another time of the day would still be beneficial, because other triggers of acute vascular events present during the high risk morning hours (e.g. morning blood pressure surge, rise of cortisol and catecholamines) are absent during the rest of the day.[38]

CONCLUSIONS

Previous studies suggested that low-dose aspirin taken at bedtime reduces blood pressure. However, this surprising phenomenon was never studied in patients using aspirin for CVD prevention. This study showed that the intake of aspirin at bedtime compared with intake on awakening does not reduce blood pressure in patients with stable CVD using low-dose aspirin on a daily basis. Yet, bedtime aspirin intake did reduce platelet reactivity during morning hours. Future studies are needed to assess the impact of this simple intervention on the excess of cardiovascular events during morning hours.

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Chapter 4

Effect of aspirin intake on awakening or at bedtime on circadian rhythm of platelet reactivity in healthy subjects: a randomized cross-over trial

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ABSTRACT

Background

The risk of acute cardiovascular events is highest during morning hours, and platelet activity peaks during morning hours. The effect of the timing of aspirin intake on circadian rhythm and morning peak of platelet reactivity is not known.

Methods and Results

A randomized open-label cross-over trial in healthy subjects (n=14) was conducted. Participants used acetylsalicylic acid (80mg) for two periods of two weeks on awakening and at bedtime, or the opposite order. At the end of both periods, participants were admitted and blood was drawn every 3 hours to measure cyclo-oxygenase-1 (COX-1)-dependent (VerifyNow-Aspirin; Serum Thromboxane B_2 [$STxB_2$]) and COX-1-independent (flow cytometry surface CD62p expression; microaggregation) platelet activity.

Mean VerifyNow platelet reactivity over the whole day was similar with intake on awakening and at bedtime (mean difference: -9 [95% confidence interval (CI) -21 to 4]). However, the morning increase in COX-1-dependent platelet activity was more efficiently reduced by intake of aspirin at bedtime compared with on awakening (mean difference VerifyNow: -23 Aspirin Reaction Units [CI -50 to 4]; $STxB_2$: -1.7 ng/ml [CI -2.7 to -0.8]). COX-1-independent assays were not affected by aspirin intake or its timing.

Conclusions

Low-dose aspirin taken at bedtime compared with intake on awakening reduces COX-1-dependent platelet reactivity during morning hours in healthy subjects. Future clinical trials in larger patient groups are required to investigate whether simply switching to aspirin intake at bedtime reduces the risk of cardiovascular events during the high risk morning hours and offers an overall benefit.

INTRODUCTION

Low-dose aspirin is one of the most used drugs worldwide, and a cornerstone in the prevention of cardiovascular disease (CVD).[1] It reduces the risk of recurrent CVD with about 25%.[2] Although not supported by evidence, aspirin is usually taken on awakening. However, it may be more beneficial to take aspirin at bedtime. Aspirin's preventive action is based on the inhibition of platelet aggregation and the formation of arterial thrombi, which plays an important role in the pathogenesis of acute CVD.[3, 4] Platelet aggregation and activation surface markers follow a circadian rhythm, with a peak between 6 AM to noon.[5, 6] This might play a role in the observed peak of acute CVD during morning hours, which is present in patients with- and without previous CVD.[7-10] Moreover, patients with a myocardial infarction during morning hours have larger infarct size than those with events during the rest of the day, which has a worse prognosis.[11, 12] Therefore, it could be useful to develop interventions specifically aimed at reducing the morning peak of CVD.

Due to its short half-life, aspirin only inhibits platelets which are present at the time of intake, while new platelets are released at a rate of 10%/day in healthy subjects.[13, 14] This turnover rate is even higher in patients with atherosclerotic disease.[15, 16] Newly released platelets are more reactive and are uninhibited just before the next aspirin intake.[17] This is supported by a recent study, which showed that platelet aggregation was insufficiently inhibited 24 hours after morning aspirin intake in 25% of the patients with established CVD.[18] Because it is desirable to achieve optimal inhibition of platelet aggregation during the high risk morning hours, it might be beneficial to take aspirin at bedtime.[19, 20] However, the effect of the timing of aspirin intake on the morning peak and circadian rhythm of platelet reactivity during 24 hours has not been investigated. Therefore, the aim of this study was to assess whether aspirin intake at bedtime compared with intake on awakening alters the circadian rhythm and reduces the morning peak of platelet reactivity in healthy subjects.

METHODS

Subjects

Eligible participants were all healthy adults aged 18 or older, who had the capacity to give informed consent (IC). Exclusion criteria were: active chronic disease, use of any other medication, allergy to salicylates, platelet count < $150*10^9$/L, Verifynow Aspirin Reaction Units (ARU) < 550, pregnancy, current smoking shift work <2 months, history of major bleeding events, known bleeding diathesis, cardiovas-

cular disease, malignancy and extreme awakening- or bedtime hours, defined as regular (>2 days a week) bedtime <10 PM or >midnight and/or awakening <6 AM or >9 AM. Participants were recruited through local advertisements in the Leiden University Medical Center (LUMC, Leiden, the Netherlands).

Design

This study was designed as a prospective randomized open-label blinded-end-point (PROBE) two-period crossover trial.[21] A computer-generated randomization code was prepared by an independent person at the department of Clinical Epidemiology of the LUMC, which was inaccessible to the investigators to guarantee treatment allocation concealment. The study was designed and reported in accordance with the CONSORT guidelines for randomized, controlled trials and registered at www.clinicaltrials.gov/ct2/show/NCT01900639. The study was approved by the LUMC Ethics Committee and all participants gave written informed consent.

After a screening visit, participants were randomized into two groups. One group took aspirin on awakening during two weeks and subsequently at bedtime during two weeks, whereas the other group received the same interventions in opposite order. The intervention periods were separated by a washout period of 4 weeks. Subjects were instructed to take 80 mg of effervescent acetylsalicylic acid (TEVA Pharmaceuticals, Amsterdam, the Netherlands) in the morning between 7 and 10 AM or in the evening between 10 PM and midnight, respectively. Participants visited the research site at the beginning of each 2-week intervention period for instructions and new study medication for the subsequent period. At the end of both intervention periods, subjects were admitted for 24 hours to the research department after an overnight fast (Table 1). Thus, each subject was admitted twice for 24 hours, both after intervention with aspirin on awakening and after intervention with aspirin at bedtime.

All study medication was prepared by the pharmacy of the LUMC. Medication compliance was registered and optimized with electronic pill boxes (Evalan, Amsterdam, the Netherlands), which sends a text message by phone when subjects do not open the box within the pre-specified time window. Additionally, a pill count was performed after both intervention periods. Noncompliance was defined as an adherence of <90% as registered by the electronic pill box, as a remaining pill count of $\geq$3, or the subject's acknowledgement of noncompliance. At each study visit, we registered possible adverse events and side effects of aspirin use by structured questionnaires.

Table 1. Day scheme of admission at visit 2 and 4

Time		Action*
Day 1	**8:30**	Insertion intravenous catheter
	9:00	Blood sampling
	9:05	Intake of aspirin when on awakening
	9:30	Breakfast
	12:00	Blood sampling
	12:05	Lunch
	15:00	Blood sampling
	18:00	Blood sampling
	18:05	Diner
	21:00	Blood sampling
	23:00	Blood sampling (+$STxB_2$)
	23:05	Intake of aspirin when at bedtime
Day 2	**00:00**	Blood sampling – Lights off
	03:00	Blood sampling
	6:00	Blood sampling (+$STxB_2$ + Flow cytometry)
	8:00	Lights on - awakening
	8:15	15 minutes ambulating
	9:00	Blood sampling (+$STxB_2$ + Flow cytometry)
	9:05	Intake of aspirin when taking it at awakening
	9:30	Breakfast
	12:00	Blood sampling (+$STxB_2$ + Flow cytometry)

* At every blood sampling moment a VerifyNow tube was drawn.
$STxB_2$: serum thromboxane B_2. The grey area represents the morning peak of platelet reactivity at day 2.

Twenty-four-hour Admission Periods

After each intervention period, subjects were admitted from 8 AM to noon the next day. An overview of the schedule during admission is given in table 1. Because the absorption of aspirin after intake could be affected by food intake, the timing of breakfast, lunch, and dinner, these variables were fixed for all subjects.[22] Subjects were allowed to sleep from midnight to 8 AM, during which the lights were turned off. After awakening, subjects were asked to ambulate for 15 minutes to resemble normal morning activity. During the rest of the day, subjects behaved according to their personal preference, but were not allowed to perform physical exercise.

Using an 18 gauge antecubital venous catheter (VC) (Becton Dickinson Inc., Franklin Lakes, NJ, USA), blood was sampled every 3 hours. This sampling rate is analogous to a previous study on the circadian rhythm of platelet aggregation.[23] Additional samples were drawn at 9 AM and 11 PM, just before each supervised

aspirin intake. Each first 5 mL was discarded, after which blood for platelet function testing was drawn, followed by infusion of saline to keep the VC patent. Additional tubes for determination of serum thromboxane B_2 ($STxB_2$) were drawn at 11 PM on day 1, and 6 AM, 9 AM and 12 AM on day 2.

Laboratory measurements

At baseline and at the beginning of each 24-hour admission period, hematologic variables were determined according to standard procedures. Platelet activity was evaluated with COX-1-dependent and COX-1-independent assays.

COX-1-dependent assays

Platelet reactivity was measured with the VerifyNow Aspirin Assay (Accumetrics, San Diego, CA, USA) as described previously.[24] The VerifyNow Aspirin System converts measured platelet aggregation into Aspirin Reaction Units (ARU). Higher ARU values correspond with higher platelet reactivity. Although manufacturer's instructions allow measurement of platelet reactivity between 30 min – 4 hours after blood draw, we measured all samples 3 hours after each blood draw, to prevent time-dependent changes of platelet reactivity within the determination window.[25] $STxB_2$ is a stable metabolite of thromboxane A_2 (TxA_2), which directly reflects platelet COX-1 enzyme capacity, and is pharmacologically the most specific assay to evaluate the effect of aspirin on platelets.[14, 26] Whole blood was allowed to clot at 37°C in non-anticoagulated tubes for 1 hour, after which serum was separated by centrifugation and stored at -80°C until analysis. $STxB_2$ was measured with an enzyme immuno-assay according to manufacturers' instructions (Thromboxane B_2 Express, Cayman Chemicals, Ann Arbor, MI, USA).

COX-1-independent assays

For COX-1-independent assays, blood was drawn at 6 AM, 9 AM and 12 AM of day 2. Four weeks after the last aspirin intake blood was drawn for controls. Platelet reactivity was determined by agonist induced platelet surface CD62P (P-selectin) expression and platelet microaggregation. As agonists, thrombin receptor–activating peptide (TRAP; activates the thrombin receptor proteinase-activated receptors–1)[27], adenosine diphosphate (ADP; activates P2Y1, P2Y12, and P2X1 receptors)[28], phorbol 12-myristate 13-acetate (PMA; activates protein kinase C which in turn indirectly activates αIIbβ3 integrin)[29] and ristocetin (binds von Willebrand factor, changes its conformation and subsequently activates platelets through glycoprotein Ib binding) were used.[30] Induced platelet surface CD62p expression was determined as described previously.[31] Concentration series ranged from 625 µmol/L – 38 nmol/L (TRAP; H-2936, Bachem, Weil am Rhein, Germany), 125 µmol/L – 8

nmol/L (ADP; 01897, Sigma-Aldrich, Zwijndrecht, the Netherlands) and 8 µmol/L – 0.5 nmol/L (PMA; P8139, Sigma-Aldrich), Platelet microaggregation was performed as described previously.[30] In short, citrated blood was labelled with either FITC Mouse Anti-Human CD31 (1:100; 555445; BD Pharmingen™, San Diego, CA, USA) or Alexa Fluor® 647 Mouse anti-Human CD31 (1:100; 558094, BD Pharmingen™). The differently labeled platelets were mixed and subsequently activated with TRAP (250 µmol/L), ADP (50 µmol/L), PMA (160 nmol/L) or ristocetin (700 µmol/L; R7752, Sigma-Aldrich). Samples were taken at 0s, 30s, 60s, 120s, 180s, 300s and 480s, measured by flow cytometry (Coulter FC-500-MPL, Beckman Coulter, Fullerton, CA, USA) and analyzed with Kaluza Analysis Software (Beckman Coulter). CD62P expression was determined in the CD61 positive population. The CD62P-positive gate was established using unlabelled cells and isotype controls. The mean percentage of CD62P-positive in the eight concentrations was calculated. Microaggregation was quantified using a quadrant in the dot plot of non-stimulated samples. The percentage double positive samples were calculated relative to the total amount of positive events. Platelet microaggregation increased over time and after 30 s for TRAP, ADP and ristocetin and after 180 s for PMA. The largest discrimination was observed and these values are reported in this manuscript.

Statistical Analysis

To achieve a power of 90% with a 95% confidence level to find a reduction of 65 ARU at the morning peak with aspirin intake at bedtime, 12 subjects were needed in this cross-over study[32]. For this calculation, we used an intra-individual standard deviation of 46.85 ARU, as extracted from a previous study[33]. 65 ARU corresponds with 1.5 times the intra-individual standard deviation, which we regarded as a possibly clinically relevant difference. Anticipating drop-out of approximately 10%, 14 subjects were randomized. The primary analysis was guided by the intention to treat principle. Second, a per-protocol analysis was performed in which participants were excluded who started smoking, started using other medication, or who did not take aspirin in the pre-specified time windows during the study. Third, a sensitivity analysis was performed, in which only measurements without the use of a tourniquet were analysed. Tourniquet use during blood draws results in higher intravascular pressure and can induce a turbulent blood flow which subsequently activates platelets and thereby influence the results.[34] Linear mixed models were used to compare platelet reactivity over the whole 24 hour (VerifyNow) and during morning hours on day 2 (6 AM – noon; all platelet activity measurements). Additionally, the area under the curve (AUC) during morning hours on day 2 was calculated for each participant using the linear trapezoidal method, which uses the following equation: $\sum(x_n - x_{n-1})(y_n + y_{n-1})/2$. Paired t-tests were used to test differences in AUCs.

SPSS statistics 20 (IBM Corp., USA) and GraphPad Prism 6 (GraphPad Software Inc., USA) were used for statistical calculations and graphical presentation of results.

RESULTS

Recruitment and study population

From June 2013 to October 2013, a total of 44 subjects were screened for eligibility, of whom 14 subjects refused to participate and 8 subjects did not meet the inclusion criteria. A total of 3 subjects were excluded because of chronic medication use

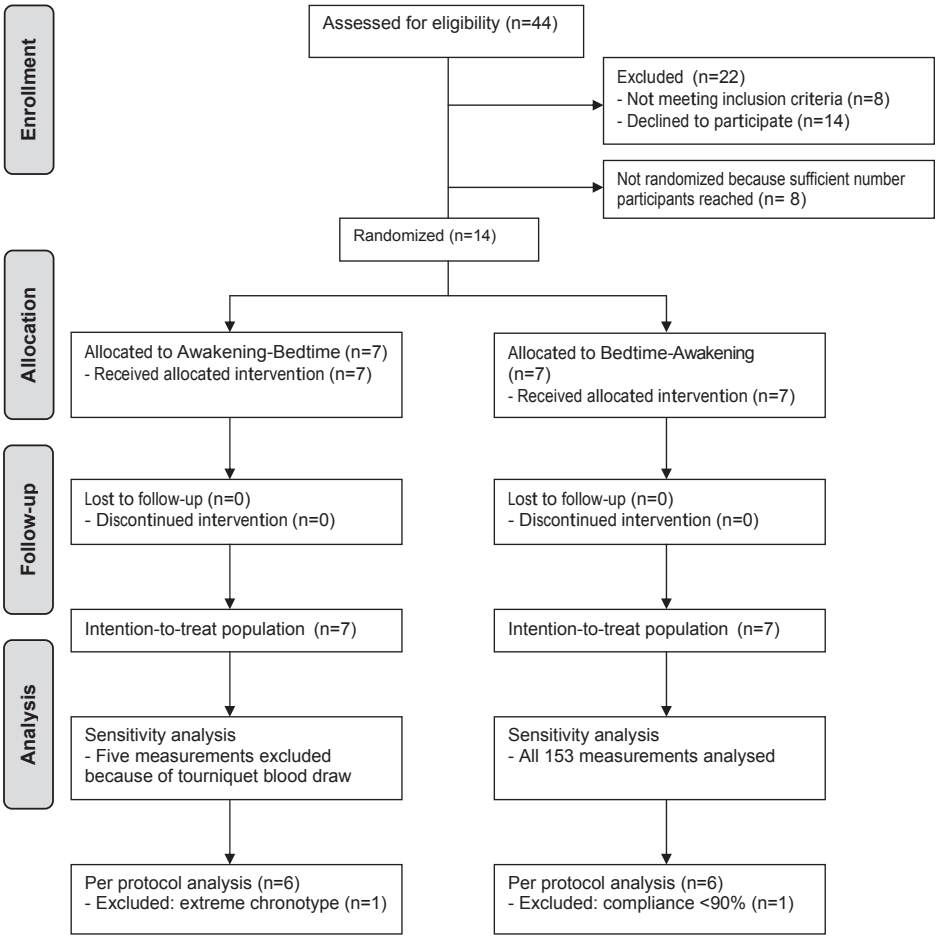


Figure 1 – Flowchart of study patients.

and 2 because of a known bleeding diathesis. Another 3 subjects were excluded because of extreme awakening- or bedtime hours, shift work in the preceding two months or current smoking. In addition, 8 subjects were meeting the inclusion criteria but were not randomized because sufficient number of participants had already been included. Finally, 14 subjects were randomized (Figure 1). Baseline characteristics of the randomized subjects are listed in table 2. Compliance, validated by electronic pill boxes, was high during the whole study, and similar with intake on awakening and at bedtime (96.5% and 97.2%, respectively).

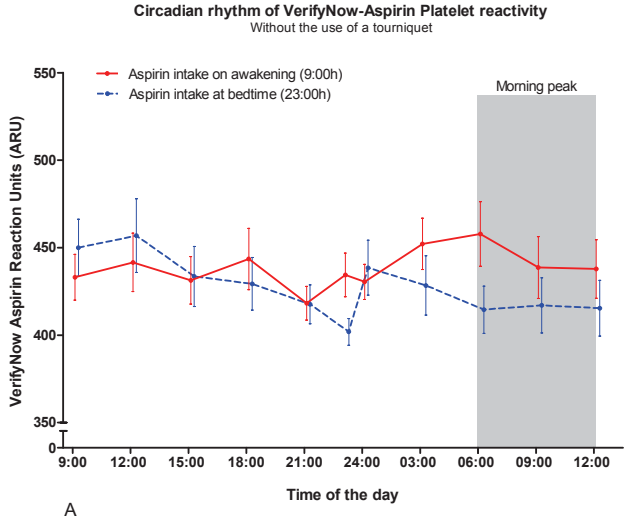
Table 2. Baseline characteristics of 14 randomized subjects

Characteristic		Healthy Volunteers (n=14)
Age (years)		22.2 ± 1.8
Sex (female)		9 (64.3)
Body Mass Index (kg/m^2)		22.8 ± 2.7
Hemoglobin (mmol/L)	males	9.3 ± 0.7
	females	8.2 ± 0.7
Platelet count (x10^9/L)		236 ± 33
Mean platelet volume (fl)		10.8 ± 1.1
Platelet distribution width (µm)		13.2 ± 2.4
VerifyNow (Aspirin Reaction Units [ARU])		642 ± 20

Data are number (%) or mean ± SD

COX-1-dependent platelet reactivity

The effect of intake of low-dose aspirin on awakening or at bedtime on the circadian rhythm and morning peak of COX-1-dependent platelet assays are shown in figure 2. VerifyNow platelet reactivity reached its maximum at 6 AM with aspirin intake on awakening and shifted to noon with intake at bedtime. Mean VerifyNow platelet reactivity over the whole day was 438 (SD 54) ARU with intake on awakening and 430 (SD 59) ARU with intake at bedtime (mean difference: -9 [95% CI -21 to 4]; p=0.190). Mean VerifyNow platelet reactivity during the morning peak was 445 (SD 63) with intake on awakening, whereas this was 421 (SD 61) with aspirin intake at bedtime (mean difference -23 ARU [95% CI -50 to 4; p=0.09]). The mean VerifyNow AUC was higher during the morning peak with intake of aspirin on awakening (882 ± SD 107) than with intake at bedtime (851 ± SD 105), although this was not statistically significant (mean difference -31 [95% CI: -88 to 26] P=0.256). Aspirin intake at bedtime reduced $STxB_2$ mean and AUC during morning hours. Mean levels of $STxB_2$ during the morning peak with aspirin intake on awakening was 4.3 (SD 2.8) ng/ml with intake on awakening, whereas this was 2.6 (SD 1.3) ng/ml with intake at bedtime (mean difference -1.7 [95% CI -2.7 to -0.8]; p=0.001).



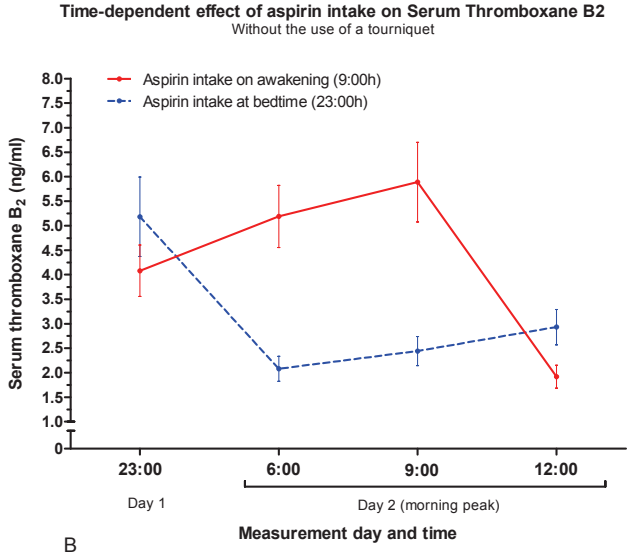


Figure 2 – Effect of aspirin intake on awakening or at bedtime on circadian rhythm and morning hour COX-1-dependent platelet activity. Panel **A**) VerifyNow-Aspirin platelet reactivity. Grey shaded area depicts the morning peak of platelet reactivity and cardiovascular events. Panel **B**) Serum Thromboxane B_2. Figures are the results of the sensitivity analysis, in which measurements after tourniquet blood draw (n=5 (1.6%)) were excluded. Values are depicted as means ± standard error.

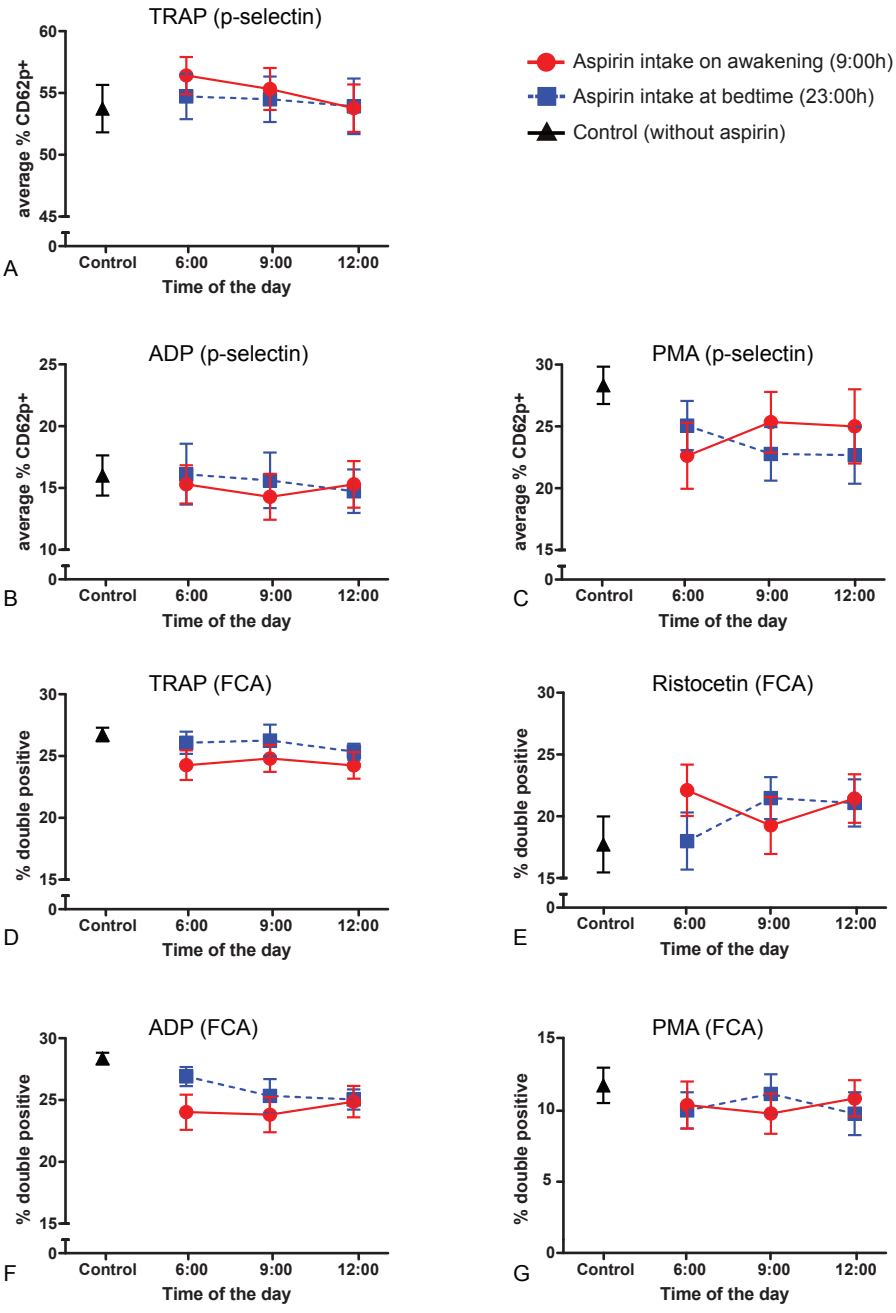


Figure 3 – Effect of aspirin intake on awakening or at bedtime on COX-1-independent platelet assays during morning hours. Panel **A** to **C**) Flow cytometry CD62 (p-selectin) surface expression after activation with respectively Thrombin receptor agonist peptide (TRAP), adenosine diphosphate (ADP) and phorbol 12-myristate 13-acetate (PMA). Panel **D** to **F**) Flow cytometry based aggregation (FCA) after activation with respectively TRAP, Ristocetin, ADP and PMA.

The mean AUC of $STxB_2$ levels during the morning peak was also lower with aspirin intake at bedtime (mean difference -4.7 [95% CI -6.9 to -2.5]).

Two subjects were excluded in the per-protocol analysis, because of compliance <90% and change of chronotype during the study. The results of the per protocol analysis for VerifyNow platelet reactivity and $STxB_2$ were similar to the intention to treat analysis (Appendix table). In our sensitivity analysis, which excluded tourniquet blood draws (5/304; 1.6%), aspirin intake at bedtime reduced morning VerifyNow platelet reactivity with 30 ARU (95% CI -56 to -3; p=0.03) compared with on awakening. Additionally, the mean difference in AUC during the morning peak was larger (-52 [95% CI: -105 to 2]; p=0.057) in this sensitivity analysis.

COX-1-independent platelet reactivity

The mean percentage platelet surface p-selectin expression during morning hours after activation with respectively TRAP, ADP and PMA was 55.5 (SD 6.0), 15.2 (SD 6.3) and 22.5 (SD 4.9) after aspirin intake on awakening and 56.0 (SD 6.6), 16.0 (SD 8.1) and 22.1 (SD 7.7) after aspirin intake at bedtime (Figure 3; Appendix table). In the per-protocol- and sensitivity analysis results were similar as the intention-to-treat analysis (Appendix table). So, the mean CD62p expression after stimulation with TRAP, ADP and PMA was not affected by the time of aspirin intake. Spontaneous and maximum platelet microaggregation did not differ between aspirin intake upon awakening or at bedtime (*data not shown)*. The mean percentage of double positive events during morning hours after stimulation with TRAP, ADP, PMA and ristocetin was 25.1 (SD 3.6), 23.5 (SD 4.7), 9.6 (SD 5.0) and 22.5 (SD 4.9) after aspirin intake on awakening and 26.6 (SD 3.9), 25.4 (SD 3.8), 10.4 (SD 4.7) and 22.7 (SD 4.1) after aspirin intake at bedtime, respectively. Similar results were obtained in the per protocol and sensitivity analysis (Appendix table). Notably, both the platelet responsiveness- and microaggregation assay were not affected by aspirin intake at all, irrespective of time of intake (Figure 3).

DISCUSSION

The main finding of this study is that aspirin intake at bedtime compared with intake on awakening reduces COX-1-dependent platelet reactivity during the morning hours. Furthermore, aspirin intake at bedtime appeared to shift the peak of platelet reactivity away from the morning hours.

Comparison with previous studies

Previous authors suggested that the morning peak of platelet reactivity could be reduced by taking aspirin at bedtime instead of on awakening.[19, 20, 35] However, to our knowledge, this has never been evaluated in a clinical trial. The Physicians Health Study showed that the protective effect of aspirin was most pronounced for morning myocardial infarctions, although the time of aspirin intake was not reported.[36] In the only study which specifically assessed the effect of aspirin intake on morning platelet reactivity, aspirin was taken at 9 PM.[37] In this study, aspirin intake at 9 PM compared with placebo abolished the morning peak of platelet aggregation, which is in line with our study. However, only one dose of 325 mg enteric coated aspirin was administered and no direct comparison with morning intake was made in that study. In our study, 80 mg of plain effervescent aspirin was taken for 14 consecutive days and the effect of intake on awakening and bedtime was directly compared. It is known that stable platelet inhibition by low-dose aspirin (80-100 mg) can take several days.[14] Furthermore, recent clinical guidelines recommend a daily dose of 50 to 160 mg for CVD prevention.[38] Thus, our study most closely represents current clinical practice with respect to chronic daily use of low-dose aspirin for CVD prevention.

We found time-dependent differences with COX-1-dependent-, but not with COX-1-independent platelet reactivity measurements. This can be explained by aspirin not affecting these COX-1-independent pathways, as indicated by our results in which COX-1-independent platelet activity was not affected by aspirin in general.

Strengths and limitations

The major strength of this study is that we used a cross-over design, which eliminated between-person variability and determinants of platelet reactivity.[39] We selected individuals with a pre-specified non-extreme chronotype, which optimized homogeneity of circadian biological rhythms between study participants. Furthermore, compliance during the intervention periods was optimally controlled with the combined use of electronic pill boxes and pill counts. Only 2 subjects were less than 90% compliant or changed their chronotype during the study, which did not materially affect our results as confirmed by the results of a per protocol analysis.

Platelet reactivity during morning hours is affected by physical activity.[40] Therefore, a limitation of our study is that participants were not admitted on the day before the start of our measurements to standardize behaviour and physical activity in the first measurement hours. This limitation is reflected by the difference in VerifyNow platelet reactivity during the morning hours on measurement day 1 and 2 (Figure 2). This could be explained by differences in physical activity necessary to

reach the study site that might have activated platelets. Behaviour and physical activity during admission were standardized, and therefore more reliable to assess treatment effects during the morning peak on day 2. Another limitation is that we only used the VerifyNow-Aspirin device to measure aspirin-related platelet reactivity, while several other devices are available. Although light transmission aggregometry (LTA) is the historical golden standard, LTA is more time consuming than the point-of-care VerifyNow, requires specialized laboratory technicians and additional blood manipulation. Of all alternatives, VerifyNow shows the strongest correlation with LTA.[41] In addition, we measured serum levels of TxB_2, which is pharmacologically the most specific test for aspirins' effect on platelets.[26]

Finally, our study was conducted in healthy individuals and not in CVD patients. Yet, patients with CVD have higher platelet turnover, which would result in a higher proportion of uninhibited platelets 24 hours after aspirin intake. Therefore, we expect that the observed effects will be even larger in CVD patients.[15] This is supported by a recent study, in which higher platelet turnover was associated with insufficient platelet inhibition 24 hours after morning aspirin intake in 25% of CVD patients.[18]

Clinical implications

The morning peak of platelet reactivity is likely to contribute to the morning peak of acute cardiovascular events.[5] However, despite antiplatelet therapy, a morning peak is still present in patients with recurrent events.[7] Aspirin intake at bedtime instead of on awakening might attenuate this morning peak by optimizing platelet inhibition during these high risk morning hours. Because acute arterial thrombosis is a multifactorial process and COX-1 only affects a part of total platelet reactivity , we do not expect that more efficient COX-1-dependent platelet inhibition abolishes the morning peak of cardiovascular events completely.[42] For example, in a previous study in aspirin treated patients, poor clinical outcomes correlated with both high values of COX-1-dependent ($STxB_2$) and COX-1-independent assays.[43] The expected benefit of specifically reducing COX-1-dependent platelet reactivity could also be derived from previous studies, in which the risk of recurrent cardiovascular events was increased in patients with higher VerifyNow-Aspirin platelet reactivity.[44, 45] Stable CVD patients with platelet reactivity >550 ARU had an absolute risk of 15.6% for developing the composite cardiovascular endpoint, whereas this was only 5.3% in patients with ARU values <550.[45] In another study, the absolute risk for the primary endpoint (all-cause death and recurrent cardiovascular events) was 13.3% in patients >454 ARU and 5.9% in patients <454 ARU.[44] Although these observational studies suggest that already a modest reduction in COX-1- depen-

dent platelet reactivity could result in clinical benefit, future clinical trials should evaluate whether this indeed leads to a reduction of cardiovascular events.

CONCLUSION

This study suggests that low-dose aspirin taken at bedtime compared with intake on awakening reduces COX-1-dependent platelet reactivity during morning hours in healthy subjects. Future clinical trials in larger patient groups are required to investigate whether simply switching to aspirin intake at bedtime reduces the risk of cardiovascular events during the morning hours.

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Chapter 5

Platelet reactivity is not associated with recurrent cardiovascular events in men with a history of myocardial infarction: a cohort study

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Letter to the editor. J Thromb Haemost. 2012;10:2616-8

Cardiovascular disease is one of the leading causes of morbidity and mortality worldwide. In spite of improvements in secondary prevention over the last decades 20-40% of patients develop a recurrent event.[1] Platelets are key players in the development of arterial thrombosis. Consequently, a cornerstone of secondary prevention comprises the inhibition of platelets.[2, 3] Following international guidelines, classical risk factors are aggressively treated after a first cardiovascular event. Yet, other biological mechanisms may play a role in developing a recurrent event. Platelet characteristics, such as increased basal reactivity, has been proposed to be one of those mechanisms. At the occurrence of arterial thrombosis, platelets are activated and release granule contents into the vasculature, including chemokines which interact with other blood cells and the endothelium.[4, 5] Three of these chemokines are NAP-2 (neutrophil-activating peptide-2), which is an activation product of CXCL7 (CXC chemokine ligand 7, also known as precursor of beta-thromboglobulin), CXCL4 (CXC chemokine ligand 4 ,also known as platelet factor 4) and RANTES (regulated on activation, normal T cell expressed and secreted), which modulates monocytes involved in the progression of atherosclerotic plaques.[6] We measured NAP-2, CXCL-4 and RANTES because these markers were studied previously in relation to cardiovascular disease and serve as a marker of basal platelet activity.[6-17] We hypothesized that increased basal activity of these platelet markers increase the risk of a recurrent cardiovascular event. In the present study we studied the association between plasma concentrations of NAP-2, CXCL4 and RANTES and the incidence of recurrent cardiovascular events in men with a history of myocardial infarction.

A cohort study was performed among men who experienced a first myocardial infarction (MI) between 1990 and 1996. Details of the study have been reported previously.[18] In short, patients were followed until september 1st 2004 to asses the occurrence of recurrent major arterial cardiovascular events (rMACE). Follow-up information was collected from hospital files, general practioners and patient questionnaires. The study was approved by the local ethics committee and all patients gave written informed consent. A blood sample was collected from each patient after the first event to determine levels of platelet markers (NAP-2, CXCL4 and RANTES) using semi-automated ELISA as described previously.[12, 19] High levels of platelet reactivity were defined as levels above the 90th percentile. Continuous data are summarized as medians with interquartile range (IQR) and categorical data are presented as counts and percentages. Cox proportional hazard models were used to estimate hazard ratios for the occurrence of rMACE according to different concentrations of the platelet markers. Hazard ratios were adjusted for putative confounding factors in

the relation between the platelet reactivity markers and rMACE. Sensitivity analyses using continuous variables and different cut-off points were performed.

A total of 542 men were included in the cohort who experienced a first myocardial infarction at a median age of 57 years (IQR 49-64), and for whom median follow-up time was 9.2 years (IQR 2.7 – 11.4). During follow-up, 254 (47%) men developed at least one rMACE. Median time between first MI and blood sampling was 2.6yrs (range 0.2 – 6yrs), which did not differ between the patients with rMACE and no rMACE. Platelet reactivity markers were missing in 41 patients, of whom clinical characteristics did not differ from the non-missing patients. For all platelet reactivity markers, patients with a level above the 90th percentile did not have an increased risk of developing an rMACE compared with patients with platelet reactivity markers below the 90th percentile. Adjustment for potential confounders did not change (CXCL4 and RANTES) or attenuated (NAP-2) the risk estimates even further (table). Changing the cut-off points or using platelet reactivity as continuous variable did not affect the results.

Our findings in a cohort of men who survived a first myocardial infarction do not confirm the notion that increased basal platelet reactivity increases the risk of recurrent cardiovascular events. This important negative finding contradicts previous studies which suggested that these inflammatory markers play a role in the development of first and recurrent cardiovascular events.[12-17] Regarding the beta-thromboglobulin NAP-2, our findings are in line with the recent study of Berent et al., in which no difference in recurrence of cardiovascular events was found between patients with low and high beta-thromboglobulin levels.[20] A possible explanation for the absence of an association between platelet reactivity markers and recurrent cardiovascular events in our study is that our study population represented a group of stable patients with chronic cardiovascular disease, whereas patients in previous studies were studied in an acute setting.[13-17] However, platelet inflammatory markers might play a more dominant role in the development of cardiovascular events in stable patients with a low prevalence of classical risk factors.[12] A major strength of our study is the large sample size and long follow-up, since platelet inflammatory markers may affect the vascular endothelium over a long period of time before a recurrent cardiovascular event takes place. Additionally, our results can be generalized to other men with a first myocardial infarction, because our cohort was representative of a typical population of men who survived a first myocardial infarction. A limitation of our study is that renal function, which affects the level of beta-thromboglobulin[21], was not measured and could not be controlled for as a confounding factor. However, because renal function and beta-thromboglobulin

are inversely correlated, additionally controlling for the effect of renal function would only attenuate the hazard ratios. Unfortunately, due to practical reasons, we did not measure soluble glycoprotein Ib (sGPIb) in the current study, while a previous study in premenopausal women found an association between high levels of sGPIb and myocardial infarction.[12]

In conclusion, we found that high platelet reactivity, as measured by CXCL4, NAP-2 and RANTES, does not increase the risk of recurrent cardiovascular events in men who survived a first myocardial infarction.

Table Hazard ratios for recurrent MACE (rMACE) in men with a first myocardial infarction according to platelet reactivity markers

	No rMACE (n=263)	rMACE (n=238)	Hazard ratio (95% CI)	
			Unadjusted	Fully Adjusted*
NAP-2				
< 90th percentile	240	211	1 [ref]	1 [ref]
≥ 90th percentile	23	27	1.3 (0.9-1.9)	1.1 (0.8-1.7)
CXCL4				
< 90th percentile	239	212	1 [ref]	1 [ref]
≥ 90th percentile	24	26	1.2 (0.8-1.8)	1.2 (0.8-1.8)
RANTES				
< 90th percentile	239	212	1 [ref]	1 [ref]
≥ 90th percentile	24	26	1.2 (0.8-1.8)	1.2 (0.8-1.8)

Nap-2: neutrophil activating peptide 2; CXCL4: CXC chemokine ligand 4; RANTES: regulated on activation, normal T cell expressed and secreted.

* Adjusted for (at time of blood drawing): age, total cholesterol, systolic blood pressure, current smoking, C reactive protein and use of aspirin, oral anticoagulants, glucose lowering drugs, cholesterol lowering drugs.

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Chapter 6

Effect of beta-blockers on platelet aggregation: a systematic review and meta-analysis

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ABSTRACT

Background

Platelets play an important role in cardiovascular disease (CVD) and beta-blockers are often prescribed for CVD prevention. Beta-blockers may directly affect platelet aggregation, because beta-adrenergic receptors are present on platelets. There is uncertainty about the existence and magnitude of an effect of beta-blockers on platelet aggregation.

Methods and Results

We performed a systematic review and meta-analysis on the effect of beta-blockers on platelet aggregation. We retrieved 31 studies (28 clinical trials, 3 observational studies). Beta-blockers decreased platelet aggregation: standardized mean difference -0.54 (95% CI -0.85 to -0.24, $p<0.0001$). This corresponds to a reduction of 13% (95% CI 8 to 17%). Non-selective lipophilic beta-blockers decreased platelet aggregation more than selective non-lipophilic beta-blockers.

Conclusion

Clinically used beta-blockers significantly reduce platelet aggregation. Non-selective lipophilic beta-blockers seem to reduce platelet aggregation more effectively than selective non-lipophilic beta-blockers. These findings may help to explain why some beta-blockers are more effective than others in preventing CVD.

INTRODUCTION

Cardiovascular disease (CVD) is a major cause of mortality and morbidity worldwide.[1,2] Anti-platelet and beta-blocking drugs are cornerstones in the secondary prevention of CVD.[3-6] The aim of anti-platelet therapy is to inhibit prothrombotic and anti-inflammatory platelet properties, which contribute to the pathogenesis of CVD[7]. Beta-blockers are recommended for secondary prevention of CVD because of their beneficial effects on heart rate, blood pressure and myocardial oxygen demand.[5,6,8] Yet, beta-blocking agents may also affect platelet aggregation, because beta-adrenergic receptors are present on platelets and catecholamine levels are known to potentiate platelet reactivity.[9] The beta-adrenergic receptor on human platelets is mainly from the beta-2-subtype, which is only inhibited by non-selective beta-blockers.[10] This suggest that non-selective beta-blockers would have a more pronounced effect on platelet aggregation than selective beta-blockers.[11] Besides, beta-blockers have a membrane stabilizing effect which, depending on the lipophilicity of the compounds, could also affect platelet aggregation.[10,12]

Although beta-blockers are one of the most frequently prescribed drugs for CVD prevention and notwithstanding the mechanistic plausibility, there is still uncertainty about the existence and magnitude of an effect of beta-blockers on platelet aggregation.[13] Our aim was to synthesize the currently available evidence on the effect of beta-blockers on platelet aggregation and to examine whether this effect is modulated by the selectivity and lipophilicity of the beta-blockers.

METHODS

Search strategy

MEDLINE and EMBASE were searched until June 2013. The search terms used were "Platelet Aggregation" and "Beta blockers" or "Adrenergic beta-antagonists" (an overview of the complete search string is shown in Appendix methods 1). The search was extended by review of bibliographies from articles included in the final selection.

Eligibility criteria

Eligible articles had to report on the effect of beta-blockers on platelet aggregation measured by Light Transmission Aggregometry (LTA). In vitro studies, defined as studies in which beta-blockers were added after blood drawing, were not eligible. Non-English articles, animal studies and platelet aggregation studies performed under conditions affecting platelet aggregation, i.e. physical or psychological

stress, acute cardiovascular disease or pregnancy, were not included. Unpublished trials and data presented in short reports, conference abstracts or letters to the editor were also not eligible. Only studies on beta-blockers WHO-registered for clinical use were considered (http://www.whocc.no/atc_ddd_index). Studies were excluded if platelet aggregation was induced by an infrequently studied agonist, defined as an agonist that was used in one eligible study only.

Risk of bias assessment

We assessed the risk of bias of the included studies according to the Cochrane Collaboration's tool for assessing risk of bias.[36] For the assessment of cross-over studies we added the characteristics 'Reported on carry-over effects' and 'Presence of carry-over effects' to the domain 'other bias'. For observational studies, we added the characteristics 'Reporting in- and exclusion criteria' and 'Adequate control for confounding factors' to the domain 'other bias'. In accordance with the tool's instruction, all dimensions were scored as 'low risk of bias' 'high risk of bias' or 'unclear risk of bias' by two independent reviewers (TNB and CEIP). Disagreement occurred in 5/264 (2%) scorings and was resolved by consensus.

To estimate the impact of studies with a high risk of bias, we performed a sensitivity analysis by restricting the analysis to randomized studies, assuming a lower risk of bias for randomized studies. We regarded the domains on blinding as less important in the risk of bias assessment, because platelet aggregation is measured quantitatively. Publication bias was examined using a funnel plot. To estimate the impact of possible publication bias, we performed a cumulative meta-analysis based on study precision (standard error).

Data Extraction

Two reviewers (TNB and CEIP) independently extracted data using standardized coding forms. For one-group (single treatment arm, no placebo) and cross-over (multiple treatment arms, interventions and placebo in each arm) trials we extracted data on platelet aggregation before and after intervention. For two-group trials (multiple treatment arms, intervention or placebo in each arm) we extracted data before and after intervention in the intervention and placebo groups. One two-group trial reported only data after intervention for the two trial arms, in which case we used that data for meta-analysis.[37] Of cross-sectional studies, we compared data on platelet aggregation between exposed and non-exposed groups. If results were presented in figures only, we extracted outcome measures from the appropriate figures where possible.[38] When multiple measurements of platelet aggregation were performed in time, the last measurement was selected for analysis.

Effect size calculation and statistical procedures

Because the measurement scale of platelet aggregation varied across studies, we calculated standardized mean differences (SMDs) for each study. As four types of study designs were included (cross-sectional, one-group-, two-group- and cross-over trials), we calculated SMDs using the appropriate formulas for each design (Appendix methods 2).[36,39-41] In brief, an SMD was calculated by dividing the mean difference by the pre-test standard deviation (SD) or SD of the non-exposed group for cross-sectional studies. SMD values were negative when the intervention (beta-blocker) reduced the outcome (platelet aggregation), with effect sizes of -0.20, -0.50 and -0.80 representing small, medium and large reductions respectively.[42] To facilitate interpretation, we additionally recalculated the overall effect size back to percentage platelet aggregation, a commonly used measurement scale in platelet aggregation studies, by multiplying the overall SMD with the mean SD (and 95% CI) of the studies which used percentage platelet aggregation as measurement scale. Most studies used multiple agonists to measure platelet aggregation. First, we analysed the effect of all agonists separately. Second, we calculated the mean of the effect sizes for each study, so that each study contributed with only one effect size to the analysis.

A random-effects meta-analysis was performed by default, because of expected between-study heterogeneity.[43] Subgroup analyses were performed for studies with non-selective versus selective beta-blockers, non-lipophilic versus lipophilic beta-blockers, short- (intake < 1 week) versus long-term (intake > 1 week) exposure and healthy versus diseased study population. If exposure duration was not reported, we assumed long-term exposure.[44-46] Univariate meta-regression analysis was performed for each subgroup. All analyses were performed with STATA 12 (StataCorp LP, USA).

RESULTS

Literature search and study characteristics

A total of 1065 articles were identified through the literature search, of which 70 were reviewed in detail. No additional publications were identified by review of bibliographies. A total of 31 studies, reported in 25 articles, were included (Figure 1). We included 13 cross-over trials, 8 one-group trials, 7 two-group trials and 3 cross-sectional studies in which a total of 454 subjects (range 4-43) were studied (Details of included studies are shown in Appendix table 1). The majority of the studies (n=25) included patients with cardiovascular disease or cardiovascular disease risk factors (hypertension, coronary artery disease, diabetes or previous

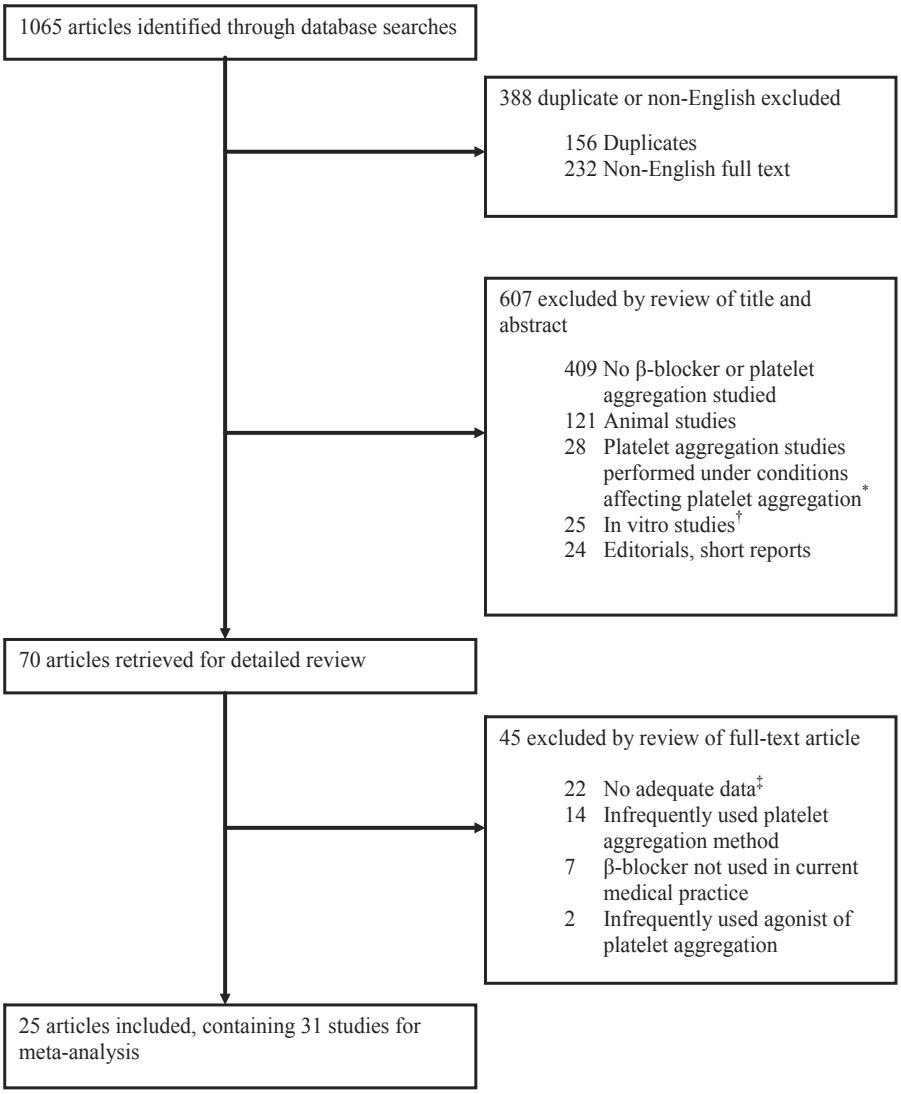

Figure 1 – Flow chart of study selection
* Conditions affecting platelet aggregation: physical or psychological stress, acute cardiovascular disease or pregnancy.
† In vitro was defined as studies in which beta-blockers were added after blood drawing.
‡ No outcome measures and/or no measure of variability (SE or SD), t-statistic or exact p-value reported.

myocardial infarction). Mean age ranged from 33 to 74.5 years. Most studies (n=26) reported on long-term exposure (>1 week) and non-selective beta-blockers such as carvedilol, propranolol, labetalol or timolol (n=23).

Meta-analysis of effect of beta-blockers on platelet aggregation

The 31 included studies contained a total of 81 observations on platelet aggregation. The most used agonist to induce platelet aggregation was adenosine diphosphate (ADP) (52%), followed by Collagen (20%) and Epinephrine (20%), whereas Arachidonic acid (n=5) and Thrombin (n=2) were used in the minority of experiments. The effect of beta-blockers on platelet aggregation was maximal with the use of epinephrine as an antagonist (Figure 2).

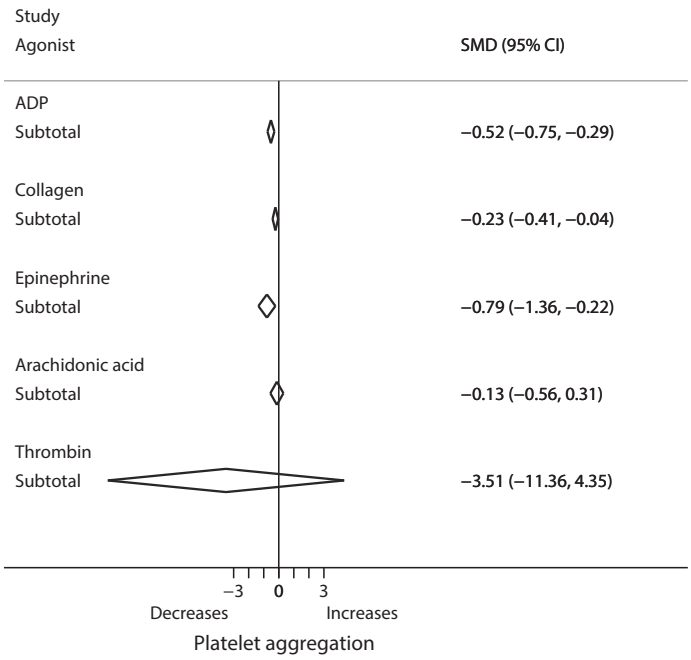

Figure 2 – Effect of beta-blockers on platelet aggregation by agonists
Forest plot of standardized mean differences (SMD) of the effects of beta-blockers on platelet aggregation by different agonists. ADP: adenosine diphosphate.

When study observations and antagonists were pooled, 13% (n=4) of the studies showed an increase in platelet aggregation, whereas the majority (84%; n=26) showed a decrease in platelet aggregation due to beta-blockers. Random effects meta-analysis showed a decrease in platelet aggregation with exposure to beta-blockers: SMD -0.54 (95% CI -0.85 to -0.24, p<0.0001; Figure 3). This corresponds to a reduction of 13% (95% CI 8-17%) in platelet aggregation. Subgroup analysis showed that non-selective and lipophilic beta-blockers (SMD -0.78 [95% CI -1.21 to -0.35] and -0.66 [95% CI -1.04 to

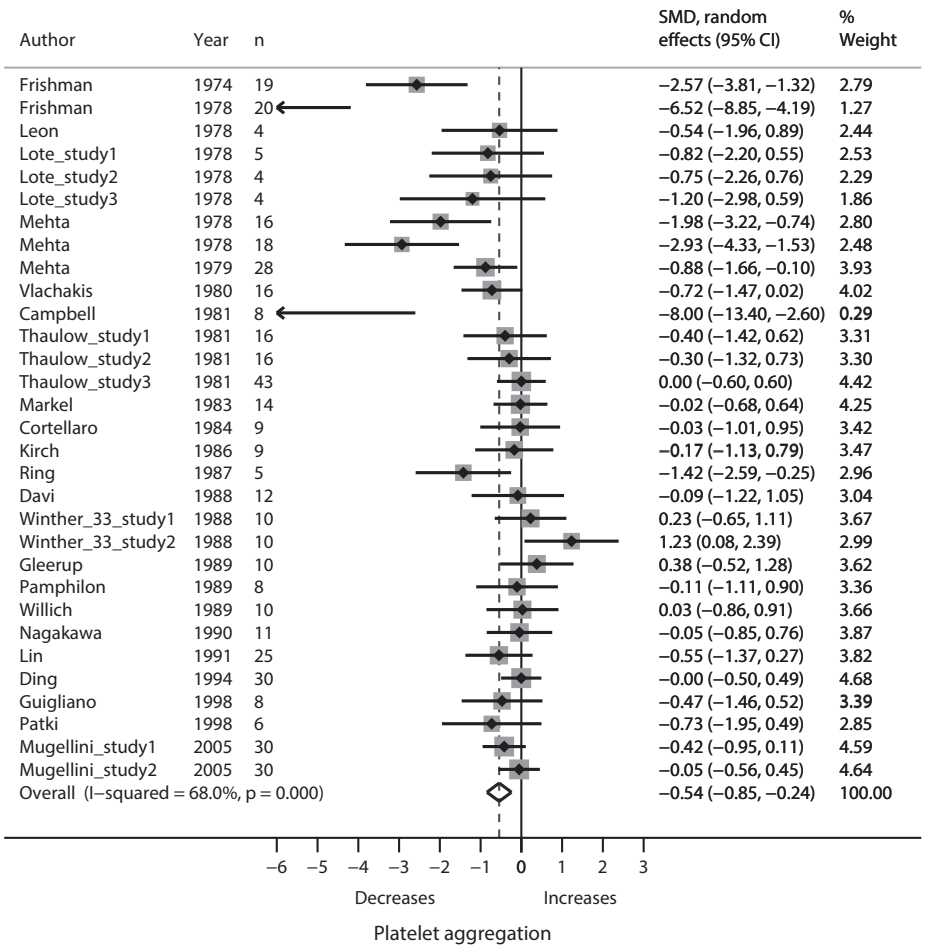

Figure 3 – Overall effect over beta-blockers on platelet aggregation
Forest plot of standardized mean differences (SMD) of the effects of beta-blockers on platelet aggregation. The black diamonds represent the effect estimate (SMD), where a negative SMD represents a decrease of platelet aggregation. The size of the grey squares around the effect estimates corresponds to the weight of the study in the meta-analysis. Horizontal lines represent corresponding 95% confidence intervals (CI). The estimate and CI of the pooled effect is indicated by the diamond.

-0.27]) decreased platelet aggregation more than selective and non-lipophilic beta-blockers (SMD -0.14 [95% CI -0.40 to 0.12] and -0.21 [95% CI -0.51 to 0.08]; Figure 4). These differences were not statistically significant with meta-regression analysis (non-selective vs. selective: p=0.18; non-lipophilic vs. lipophilic: p=0.47). The effect was not different for studies with short- or long-term exposure and in healthy or non-healthy populations (p=0.94 and p=0.77; Figure 4).

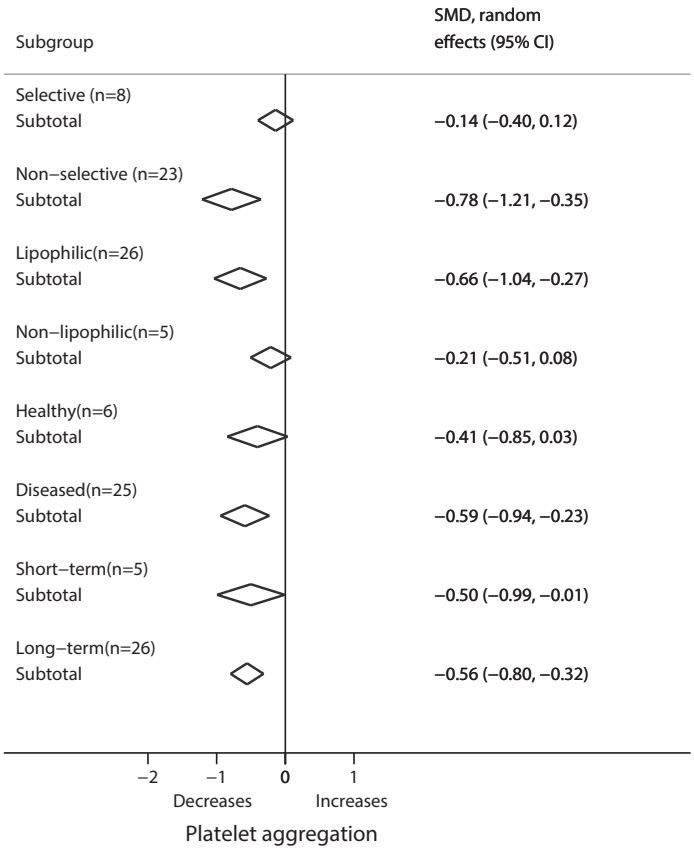

Figure 4 – Subgroup analyses
Forest plot of standardized mean differences (SMD) of the effects of subgroups beta-blockers on platelet aggregation. The effect estimates and CI of each subgroup are indicated by the diamonds. *Subgroups: Selective (metoprolol, atenolol), Non-selective (propranolol, labetalol, timolol, carvedilol), Lipophilic (propranolol, labetalol, timolol, metoprolol, carvedilol), Non-lipophilic (atenolol), Healthy subjects, Diseased (hypertension, coronary artery disease, diabetes or previous myocardial infarction), Short-term treatment (<1 week), Long-term treatment (>1 week).

Risk of bias assessment and publication bias

A summary of the risk of bias assessment for clinical trials and observational studies is shown in Appendix figure 1. Blinding was performed in 21/28 (75%) trials, but only 17/28 (61%) were double- or triple blinded. Of cross-over trials, only 6/13 (46%) reported on carry-over effects. Only 20/28 (71%) of all trials were randomized and only one trial described the randomisation procedure. In a sensitivity analysis with restriction to randomized trials only (n=20), the pooled effect estimate was SMD -0.42 (95% CI -0.80 to -0.04).

The funnel plot showed a relative lack of small studies with negative results (Figure 5). Two studies showed extreme effects, although their contributions to the overall effect were marginal (weights of 1.2% and 0.3%).[14,15] Exclusion of these two studies from our analysis did not materially affect the pooled effect estimate (SMD after exclusion: -0.41 [95% CI -0.65 to -0.17]). To estimate the impact of possible publication bias, we conducted a cumulative meta-analysis based on study precision (Appendix figure 2). This indicated that the overall effect estimate was influenced by small studies. However the effect estimate of the largest studies with the smallest standard errors was robust around SMD -0.25 to -0.30, again indicating a decrease in platelet aggregation with exposure to beta-blockers.

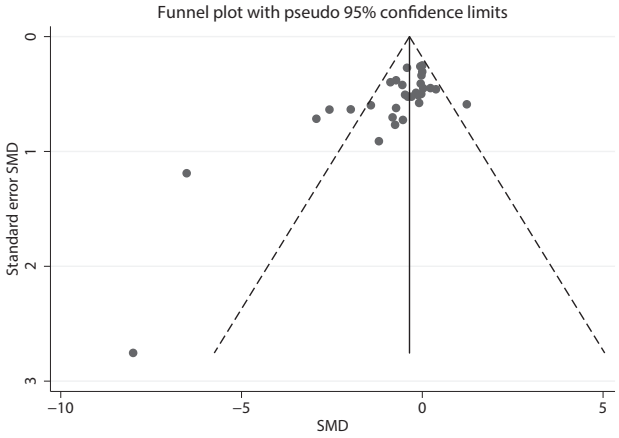

Figure 5 – Funnel plot
Funnel plot of all included studies. Two studies with extreme effects are the studies of Campbell (1981; SMD -8) and Frishman (1978; SMD -6.5)

DISCUSSION

Beta-blockers are one of the most frequently used drugs in prevention of CVD and platelets play an important role in the pathogenesis of CVD. Until now it was unclear whether beta-blockers affected platelet aggregation. This meta-analysis demonstrates that clinically used doses of beta-blockers significantly reduce platelet aggregation. Additionally, it indicates that non-selective lipophilic beta-blockers reduce platelet aggregation more effectively than selective and non-lipophilic beta-blockers.

Previous studies

Recent meta-analyses found that beta-blockers, as a class, effectively reduce cardiac- and all-cause mortality in patients with systolic heart failure and acute myocardial infarction.[16,17] It is known that this is the result of beneficial effects on heart rate, blood pressure and myocardial oxygen demand.[5,6,8] Besides these established beneficial effects, the present meta-analysis suggests that part of the protective effect of beta-blockers could be the result of platelet aggregation inhibition. Interestingly, recent meta-analyses found that the non-selective beta-blocker carvedilol was superior to selective beta-blockers, which is in line with the results and subgroup analysis of the COMET trial.[18,19] In these studies it was suggested that the non-selective lipophilic beta-blocker carvedilol exerts an additional beneficial effect through improvement of endothelial function, stimulating β-arresting signalling and anti-oxidant properties.[20-22] Our findings suggest that lipophilic non-selective beta-blockers decrease platelet aggregation most effectively, which could in part explain the superiority of non-selective lipophilic beta-blockers.

Mechanisms of platelet aggregation reduction by beta-blockers

The inhibition of platelet aggregation by beta-blockers can be explained by multiple mechanisms. First, beta-blockers may exert their antiplatelet effect by a chemical interaction with the platelet cell membrane. The strength of this interaction depends on the lipophilicity of the compound, and results in stabilisation of the platelet cell membrane, making it less sensitive to agonists.[12] Second, beta-2 receptors on platelets may be blocked by non-selective beta-blockers. This would affect intra-platelet adenosine 3,5-cyclic monophosphate (cAMP) levels, which decreases calcium availability and subsequent activation of platelets.[23] However, compared with the number of alpha-receptors per platelet, the number of beta-2 receptors is small.[24] Therefore it is more logical to assume that the beta-blockers exert part of their antiplatelet effect not directly, by binding platelet receptors, but indirectly, by decreasing plasma catecholamine levels. This is also supported by the fact that platelet aggregation was maximally inhibited by beta-blockers in experiments were epinephrine was used as an agonist (Figure 2). It is known that catecholamine levels reached in vivo potentiate platelet aggregation, thereby even overcoming inhibition of platelet aggregation by aspirin.[23] Interestingly, non-selective beta-blockers reduce catecholamine levels more effectively than selective beta-blockers, which supports our finding that platelet aggregation is more effectively inhibited by non-selective beta-blockers.[25,26] Third, high blood pressure activates platelets through multiple pathways, and a decrease of blood pressure itself could therefore decrease platelet aggregation.[13]

Strengths and limitations

A major strength of this meta-analysis is that we excluded in vitro studies from our analysis.[27-33] In these studies, beta-blockers were added in concentrations that exceeded those currently used in medical practice, making it difficult to generalize the effects to the clinical situation. Another strength is that we did not exclude any type of study design. Calculating effects sizes specific for each study design facilitated meta-analysis of the studies.

Because different agonists induce different platelet activation pathways, this could influence the observed results. Therefore, before pooling all agonists, we first analysed the effect of beta-blockers on platelet aggregation separately by all agonists (Figure 2). This analysis showed that platelet aggregation was inhibited with the use of all agonists, and more pronounced with epinephrine. This could be expected because platelet sensitivity is modified by catecholamine levels.[23] We think that pooling of all agonists to estimate the overall effect of beta-blockers on platelet aggregation is valid because all agonists showed a clear reduction in platelet aggregation. Moreover, ADP was the most frequently used agonist and did not show an extreme effect estimate which could have overestimated our overall pooled effect estimate.

A limitation of this meta-analysis is that the risk of bias of the included studies is quite high. We think this can be partly explained by the fact that most studies were performed in years when reporting according to international standards for clinical trials and observational studies was not yet established. However, we do not think that for example non-blinding would have affected our results, because platelet aggregation is not affected by placebo effects and it has been shown that open unblinded studies with objective endpoints yield the same results as double-blind placebo-controlled studies.[34] Fifty percent of the included trials were designed as cross-over experiments, in which carry-over effects could influence the results.[35] However, the treatment periods in all included cross-over studies were long enough to ensure adequate wash-out. This is supported by our finding that the pooled effect did not differ between short-term (<1 week) or long-term (>1 week) administration of beta-blockers. Finally, only 65% of the trials were randomized and only one trial described the randomisation procedure. However, in a sensitivity analysis we showed that the overall pooled effect size was robust with restriction to randomized studies only. The funnel plot showed evidence for small study effect, which might be explained by publication bias. Nevertheless, cumulative meta-analysis still indicated a clear and statistically significant effect of beta-blockers on platelet aggregation. Finally, it is known that platelet aggregation is influenced by numerous characteristics, which were not all registered in the included studies. However, the majority of included studies were clinical trials, in

which randomization ensured that known and unknown variables affecting platelet aggregation were equally distributed over treatment groups and were kept constant during the trial. Therefore, confounding of our results by other platelet affecting variables is highly unlikely.

CONCLUSION

In conclusion, this meta-analysis suggests that clinically used doses of beta-blockers reduce platelet aggregation. Non-selective, lipophilic beta-blockers appear to reduce platelet aggregation most effectively. These findings may help to explain why some beta-blockers are more effective than others in preventing CVD.

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Chapter 7

General discussion and summary

The main aim of this thesis was to assess the effect aspirin intake on awakening compared with intake at bedtime on blood pressure and the morning peak of platelet reactivity. The rationale behind these two research questions is described in detail in **chapter 1**. The design of our studies in **chapter 3** and **4** was based on the specific methodological considerations of cross-over studies, which is reviewed in **chapter 2**. In this discussion we first apply these considerations on the design of our cross-over studies in this thesis. Secondly, we will summarize the results of **chapter 3** and **4**, and discuss the implications of our findings. Finally, our studies on the influence of beta-blockers on platelet reactivity (**chapter 6**) and the role of platelet inflammatory markers in the development of recurrent cardiovascular events (**chapter 5**) are discussed.

CROSS-OVER STUDIES

A cross-over design is an efficient alternative for a parallel-group design to study chronic disorders in which treatments have temporary effects. In general, only a quarter of the total group size is needed for cross-over studies compared with parallel group studies, saving costs and time. Treatments of blood pressure and platelet reactivity have temporary effects, and are therefore particularly suitable to study with a cross-over design. With a cross-over design, two major concerns have to be acknowledged: period-effects and carry-over effects. First, period-effects can arise when the measured outcome changes over time. In **chapter 3**, for example, we measured blood pressure, which is known to increase with age. If all participants in our study would have received the same treatment order (e.g. 3 months aspirin on awakening followed by 3 months aspirin at bedtime), this fixed change of blood pressure over time could distort our results. A solution to this problem is to randomize participants for the sequence of interventions. The fixed changes over time are then equally distributed over the interventions. So, randomisation is equally important in cross-over studies as in parallel-group studies, but for a different reason: in parallel-group studies randomization is used to achieve comparability between intervention groups, whereas in cross-over studies randomization is needed to achieve comparability between periods.

Second, carry-over needs to be considered when using a cross-over design. A carry-over effect arises when an intervention in the previous period affects the results in the subsequent period. Usually, a wash-out period is incorporated in cross-over studies to restore participants' characteristics to their baseline state before the next intervention is started. However, in our study in **chapter 3** a wash-out period was unnecessary. To achieve adequate wash-out of any blood pressure

effect of the first intervention period before measurement in the second period, we assumed that aspirins' effect on blood-pressure would not exceed the duration of the intervention periods (3 months). Furthermore, incorporation of a washout period would have been unethical, because patients used aspirin for prevention of cardiovascular disease. In our study in **chapter 4** we did incorporate a wash-out period of 2 to 4 weeks. This was enough to ensure restoration of normal platelet reactivity, which takes 7-10 days, after the first intervention period with aspirin.

To summarize, we carefully considered the specific methodological issues of cross-over studies for the design of our studies in **chapter 3** and **4**, which enhanced the validity of our results.

ASPIRIN ON AWAKENING OR AT BEDTIME?

Bedtime aspirin to reduce blood pressure

In **chapter 3** we studied whether intake of low-dose aspirin at bedtime compared with intake on awakening reduced blood pressure of patients with stable cardiovascular disease. We randomized 290 patients, who already used aspirin for prevention of cardiovascular disease for a median duration of 6 years, to 3 months aspirin intake at bedtime followed by 3 months intake on awakening, or the opposite order. At the end of both intervention periods, 24-hour blood pressure was measured, which was complete for 263 patients. We found that aspirin intake at bedtime did not reduce 24-hour blood pressure compared with intake on awakening (difference systolic/diastolic: -0.1 [CI: -1.0; 0.9] / -0.6 [CI: -1.2; 0.0] mmHg).

Why did we find no effect on blood pressure?

Previous studies suggested that aspirin reduces blood pressure when taken at bedtime instead of on awakening.[1-4] Additionally, a biological plausible mechanism was found: compared with intake on awakening, aspirin intake at bedtime reduced plasma renin activity, cortisol, dopamine and norepinephrine excretions over 24 hours.[5] Differences in patient characteristics and co-medication may explain why we did not find a blood pressure lowering effect of bedtime aspirin intake. All previous studies were performed in healthy subjects or patients with mild- or untreated hypertension or pregnancy. In contrast, patients in our study all had cardiovascular disease, already used aspirin before study entry and used concomitant antihypertensive drugs. Yet, in our pre-specified subgroup analyses, based on the use of specific types of antihypertensive drugs, we also did not find a blood pressure lowering effect. Importantly, even in the subgroup that did not use any blood pressure lowering drugs, there was no difference between intake

on awakening and at bedtime. This suggests that in patients already using aspirin for cardiovascular disease prevention, blood pressure is not affected by the time of aspirin intake, regardless of the concomitant use of antihypertensive drugs. These findings corroborate those of an earlier study, which also did not find a blood pressure lowering effect of bedtime aspirin intake among treated hypertensive patients.[6] Another possible explanation for our findings is that the effect of aspirin intake at bedtime on blood pressure weakens over time because of increased arterial stiffening and progressive atherosclerosis.[7] Finally, non-compliance with the time of aspirin intake cannot explain our negative findings. A major strength of our study is that we registered the actual time of aspirin intake by electronic pill boxes. Exclusion of participants who were less than 90% compliant did not affect our results. In summary, the contrasting results between previous studies and the present study might be explained by differences in study population. However, the patient populations of previous studies had low cardiovascular risk and therefore had no medical indication for aspirin.[8] In low risk hypertensive patients, other antihypertensive medications, with less side effects than aspirin, are preferable. Therefore, a potential blood pressure lowering effect of bedtime aspirin intake is only clinically relevant in patients already using aspirin for cardiovascular disease prevention. Given the large size of our cross-over study and the absence of a blood pressure lowering effect of bedtime aspirin in any subgroup, no further studies are needed to assess the blood pressure lowering effect of bedtime aspirin.

Bedtime aspirin to reduce morning platelet reactivity

In our study in **chapter 3** we assessed the effect of taking aspirin at bedtime compared with intake on awakening on platelet reactivity during morning hours in patients with established cardiovascular disease. Platelet reactivity measurements were complete for 133 patients, a pre-specified subgroup of the total 290 randomized patients. Aspirin intake at bedtime reduced morning platelet reactivity with a mean of -22 VerifyNow-Aspirin reaction units (ARU) (CI -35 to -9). In **chapter 3** we measured platelet reactivity at only one time-point during the morning hours. To further assess the effect of bedtime aspirin intake on the circadian rhythm of platelet reactivity, we performed a 24-hour rhythm study in **chapter 4**. The main result of the study in **chapter 4** was that bedtime aspirin intake reduced two COX-1-dependent assays, namely VerifyNow-Aspirin (-23 ARU [CI -50 to 4]) and $STxB_2$ (-1.8 ng/ml [CI -2.8 to -0.8]), during morning hours. The magnitude of this reduction is similar to the results in **chapter 3**. In contrast, aspirin intake at bedtime versus on awakening did not affect COX-1-independent assays.

A possible explanation is that platelet activation by COX-1-independent pathways is not affected by aspirin in general. However, this also raises questions about the

relative clinical importance of COX-1-dependent and COX-1-independent platelet reactivity assays, which will be discussed below. Finally, the studies in **chapter 3** and **4** included patients with chronic cardiovascular disease and healthy participants respectively, whereas numerous patients take aspirin in combination with other antiplatelet drugs in the acute phases of cardiovascular disease. So, the results of our studies cannot be generalized to patients in the acute phase of cardiovascular disease. However, a previous study found that, compared with morning intake, the morning surge of platelet aggregation was also decreased by the combined intake of aspirin and clopidogrel at bedtime.[9]

In summary, these studies suggest that platelet reactivity during the high risk morning hours can be reduced by taking aspirin at bedtime instead of on awakening. Next, we will discuss the clinical implications of these results.

Should we advise patients to take aspirin at bedtime?

Safety of bedtime aspirin

The majority (70%) of the patients in our study in **chapter 3** took aspirin on awakening before study entry. Should we advise these patients to switch to bedtime intake, based on the studies presented in this thesis? The medical principle 'first do no harm' applies to this suggested intervention. In our study we compared the number of side effects after aspirin intake on awakening with intake at bedtime. We observed that drop-out of patients (n=26) was not related to the timing of aspirin intake, and that the frequency of side-effects was not increased by aspirin intake at bedtime. In line with this observation, previous studies suggest that gastric vulnerability to aspirin is lower during night than during the day.[10, 11] In summary, with respect to harms, it seems safe to advise patients to take aspirin at bedtime.

Clinical effect of bedtime aspirin

When doing no harm, is it beneficial for patients to take aspirin at bedtime instead of on awakening? What are the clinical implications of switching to bedtime aspirin? No previous study evaluated the effect of this intervention. In our studies we did not follow patients for the occurrence of clinical end points. In **chapter 4** we observed that bedtime aspirin intake only affects circadian rhythm of COX-1-dependent assays, while in a previous study in aspirin treated patients, poor clinical outcomes were associated with both high values of COX-1-dependent ($STxB_2$) and COX-1-independent assays.[12] Moreover, acute arterial thrombosis is a multifactorial process and COX-1 only affects a part of platelet reactivity in vivo. Similarly, the morning peak of cardiovascular events is also caused by multiple factors (Figure 1).

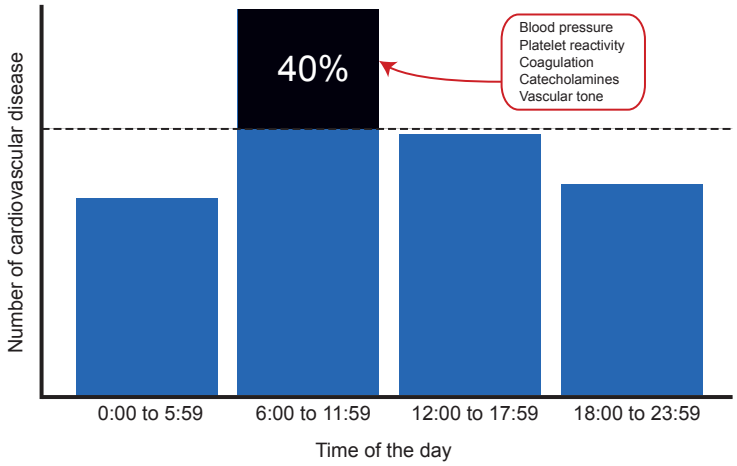

Figure 1 – Distribution of acute cardiovascular events over the day in 6 hour time windows. The horizontal line corresponds to the number expected cardiovascular events if events occurred evenly throughout the day. However, a clear peak of cardiovascular events during the morning hours (6-12 AM) is present. Multiple mechanisms, including the morning peak of platelet reactivity, probably contribute to this peak. (Adapted from: Elliot WJ, Am J Hypertens 2001;14:291-95S).

We therefore do not expect that more efficient COX-1-dependent platelet inhibition abolishes the morning peak of cardiovascular events completely.[13] Still, the specific expected benefit of reducing COX-1-dependent platelet reactivity might be derived in two ways: 1) from previous observational studies and 2) by calculating the possible benefit derived from known cardiovascular disease statistics. First, it has been shown that the risk of recurrent cardiovascular events is increased in patients with higher VerifyNow-aspirin platelet reactivity values.[14, 15] Stable cardiovascular disease patients with VerifyNow-Aspirin platelet reactivity >550 ARU had an absolute risk of 15.6% for developing the composite cardiovascular endpoint, whereas this was only 5.3% in patients with ARU values <550.[14] In another study, the absolute risk for the primary endpoint (all-cause death and recurrent cardiovascular events) was 13.3% in patients >454 ARU and 5.9% in patients <454 ARU.[15] These observational studies suggest that already a modest reduction in COX-1-dependent platelet reactivity, as found in our studies in **chapter 3** and **4**, could result in clinical benefit.

Second, the clinical benefit can be calculated with known cardiovascular disease statistics.[16] These statistics, from the United States, show that each year 280 000 patients develop a recurrent cardiovascular event. On average, this equals 767 events each day. With the known excess of 40% during the morning hours (6-12 AM), this would mean that 241 events occur from 6 to 12 AM, and 526 during the

rest of the day. Compared with the other three 6-hour time periods, the absolute excess of recurrent cardiovascular events due to the morning peak equals 66 events per day. If aspirin intake at bedtime reduces platelet reactivity during the morning hours, and thereby would reduce the morning peak of cardiovascular events by 10%, this would lead to a reduction of 6.6 events per day and 2427 events per year in the United States. With 50% reduction of the morning peak, this equals 12 133 events per year. The total number of recurrent cardiovascular events would then be reduced from 280 000 to 267 867 (Figure 2).

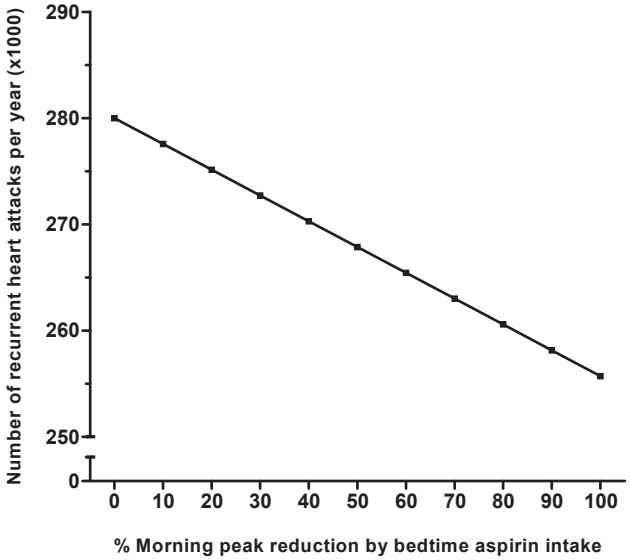

Figure 2 – Estimation of the effect of bedtime aspirin intake on the incidence of recurrent heart attacks in the United States.

Given the high incidence of cardiovascular disease, already a modest relative reduction of the morning peak would lead to a large absolute reduction of cardiovascular events on a population level. However, although switching to bedtime aspirin intake is a simple intervention, future clinical trials need to be performed to validly quantify the reduction of cardiovascular events.

To summarize, studies in this thesis suggest that, for patients who already take aspirin on a daily basis for cardiovascular disease prevention, switching to aspirin intake at bedtime is safe and potentially beneficial because platelet reactivity during the high risk morning hours is reduced by taking aspirin at bedtime instead of on awakening. However, future studies are needed to evaluate with which magnitude this reduction in platelet reactivity affects clinical outcomes.

BASAL PLATELET ACTIVITY AND RECURRENT CARDIOVASCULAR EVENTS

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.[17] So, other risk factors are likely to play a role in the development of a recurrent event. Basal platelet activity has been proposed as one of those mechanisms.[18, 19] In **chapter 5** we studied whether increased basal platelet activity, as measured by the chemokines NAP-2, CXCL4 and RANTES, is associated with the incidence of recurrent cardiovascular events. These platelet chemokines interact with inflammatory cells in the progression of atherosclerosis and leukocyte recruitment in acute arterial thrombi.[20, 21] The Study of Myocardial Infarctions Leiden (SMILE) follow-up study was used, which is a cohort study of men (n=542) who experienced a myocardial infarction (MI) between 1990 and 1996. Basal platelet activity markers were measured in blood drawn after (median 2.6 years) the first MI, and patients were followed for a median of 9.2 years, registering recurrent major adverse cardiovascular events (rMACE). During follow-up 254 (47%) patients developed at least one rMACE. The main finding was that patients with an increased basal platelet activity, defined as >90th percentile, were not at increased risk of rMACE (adjusted hazard ratios: NAP-2: 1.1 [95% CI 0.8-1.7]; CXCL4: 1.2 [0.8-1.8]; RANTES 1.2 [0.8-1.8]).

These findings contradict previous studies, in which basal levels of these platelet inflammatory markers appeared to play a role in the development of first and recurrent cardiovascular events.[18, 19, 22-25] We suggest two explanations for this finding. First, our study population represented a group of stable cardiovascular disease patients who survived a first MI, whereas patients in previous studies were studied in an acute setting.[18, 19, 23-25] These studies showed that platelet chemokine markers increase when an acute cardiovascular event occurs, and decrease after the event. It is possible that platelet derived chemokines are merely a marker of acute platelet activation, and as such do not contribute to acute arterial thrombus formation. Second, basal platelet activity might play a more dominant role in the development of cardiovascular events in patient populations with a low prevalence of classical risk factors. When classical risk factors are absent, it is likely that other factors, such as inflammatory platelet markers, contribute to acute thrombus formation. This theory is supported by the previous finding that basal levels of inflammatory platelet markers increased the risk of myocardial infarction in young women.[22]

We conclude that basal platelet inflammatory markers are not associated with recurrent cardiovascular events in men who survived a myocardial infarction. Levels of platelet inflammatory markers might still affect the risk of cardiovascular

events, but this probably depends on the prevalence of classical cardiovascular risk factors in the studied population.

BETA-BLOCKERS AND PLATELET REACTIVITY

Following international guidelines, beta-blockers are frequently prescribed to patients with cardiovascular disease.[26] For example, in our clinical trial in **chapter 3**, 53% of the patients used a beta-blocker. Platelet reactivity is affected by numerous characteristics.[27, 28] Because beta-adrenergic receptors are present on human platelets, an effect of beta-blockers on platelet reactivity may be expected.[29] However, the existence and magnitude of this effect still was unclear. In a systematic review and meta-analysis, described in **chapter 6**, we synthesized all currently available evidence of the effect of beta-blockers on platelet reactivity. We included 31 studies (28 trials, 3 observational studies) in which a total of 454 participants were studied. The main finding was that beta-blocker use reduced platelet aggregation with 13% (95% CI 8-17%). Interestingly, we found that this effect was most pronounced with epinephrine-induced platelet aggregation, which suggests a role of platelet beta-adrenergic receptors or catecholamine levels as underlying mechanisms. Furthermore, non-selective and lipophilic beta-blockers decreased platelet aggregation more than selective and non-lipophilic beta-blockers. A limitation was that most studies were of moderate to low methodological quality. For example, only 20/28 (71%) of all trials were randomized and only one trial described the randomisation procedure. However, in a sensitivity analysis of randomized studies only, the effect size remained similar. Additionally, we suspected publication bias, because the funnel plot revealed the absence of small studies with negative results. To estimate the magnitude of this bias, we performed a cumulative meta-analysis based on study precision. Although the overall effect was attenuated, this analysis still indicated that beta-blockers reduce platelet aggregation. Another limitation is that the included studies solely used light transmission aggregometry (LTA) to assess platelet aggregation, whereas multiple other assays are available nowadays. However, LTA is the historical golden standard to test platelet aggregation, and correlates with the measurements we used in **chapters 3** and **4** (VerifyNow and $STxB_2$).[30] Additionally, a reduction of platelet aggregation was observed with the use of different antagonists, representing the most important platelet activation pathways.[31] A major strength of this meta-analysis is that we excluded *in vitro* studies from our analysis. In these studies, beta-blockers were added in concentrations that exceeded those currently used in medical practice, making it difficult to

generalize the effects to the clinical situation. Another strength is that we did not exclude any type of study design.

The inhibition of platelet aggregation by beta-blockers can be explained by multiple mechanisms. First, beta-blockers may chemically interact with the lipophilic platelet cell membrane, which could result in stabilisation of the cell membrane and thereby make platelets less sensitive to stimuli.[32] This is supported by our finding that lipophilic beta-blockers reduce platelet aggregation more than non-lipophilic beta-blockers. Second, beta-blockers may affect platelets directly, by binding platelet receptors, or indirectly, by decreasing platelet potentiating plasma catecholamine levels.[29] However, the number of beta-2 receptors on platelets is small.[33] Therefore it is more logical to assume that the beta-blockers exert part of their antiplatelet effect by decreasing plasma catecholamine levels. This is also supported by our findings that platelet aggregation was maximally inhibited by beta-blockers in experiments were epinephrine was used as an antagonist. Third, high blood pressure activates platelets through multiple pathways, and a decrease of blood pressure itself could therefore decrease platelet aggregation.[34]

In conclusion, our meta-analysis in **chapter 6** suggests that besides beneficial effects on heart rate, blood pressure and myocardial oxygen demand, part of the protective effect of beta-blockers could be the result of platelet aggregation inhibition.

CONCLUSIONS

In summary, aspirin intake at bedtime did not reduce blood pressure of patients with stable cardiovascular disease. However, the studies in this thesis suggest that platelet reactivity during the high risk morning hours can be reduced by aspirin intake at bedtime. Future studies should evaluate whether this simple intervention indeed leads to a reduction of cardiovascular events. The study on basal levels of platelet inflammatory markers does not support previous evidence that these markers contribute to the development of recurrent cardiovascular events in a high risk cardiovascular disease population. However, platelet reactivity, as measured in our studies on the effect of aspirin intake at bedtime, was associated with cardiovascular events in previous observational studies. In a meta-analysis, we suggest that beta-blockers, which are frequently prescribed to patients with cardiovascular disease, reduce platelet reactivity, which in part could explain their beneficial clinical effects.

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Appendices

CHAPTER 3, APPENDIX TABLE 1

Appendix table 1. Mean 24-hour, day- and night ambulatory blood pressure values (mmHg) according to time of aspirin administration in the secondary analysis population (n=150)

	Aspirin on awakening	Aspirin at bedtime	Mean difference (bedtime – awakening) [95% CI]*
24-hour SBP	125 ± 10	125 ± 9	0.0 [-1.1 to 1.1]
24-hour DBP	78 ± 8	77 ± 8	-0.4 [-1.2 to 0.3]
Day SBP	129 ± 10	129 ± 10	0.1 [-1.2 to 1.3]
Day DBP	81 ± 9	80 ± 8	-0.5 [-1.3 to 0.2]
Night SBP	115 ± 12	115 ± 12	0.0 [-1.5 to 1.5]
Night DBP	68 ± 10	68 ± 9	-0.2 [-1.2 to 0.7]

*Mean difference and 95% CI obtained with paired t-tests. Values are mean ± standard deviation.
SBP: systolic blood pressure; DBP: diastolic blood pressure; CI: confidence interval

CHAPTER 3, APPENDIX TABLE 2

Appendix table 2. Side effects and relation with timing of aspirin intake of subjects that did not complete study follow-up (n=26)

Patient code	Period of drop-out	Timing of Aspirin intake at drop-out	Reason drop-out	Related to side effect of aspirin
105	1	At bedtime	Stopped aspirin use after advise cardiologist	No
113	1	On awakening	Stomach pain after switch from evening intake to intake on awakening	Yes
132	1	At bedtime	Study participation too aggravating	No
151	1	On awakening	Withdrawal of consent to participate in other clinical trial	No
173	1	At bedtime	Study participation too aggravating	No
180	1	At bedtime	Study participation too aggravating	No
250	1	On awakening	Stomach pain after switch from evening intake to intake on awakening	Yes
251	1	At bedtime	Study participation too aggravating	No
327	1	On awakening	Study participation too aggravating	No
329	1	At bedtime	Stopped aspirin use after head trauma; advise of first aid physician	No
365	1	At bedtime	Study participation too aggravating	No
436	1	On awakening	Study participation too aggravating	No
447	1	On awakening	Study participation too aggravating	No
455	1	At bedtime	Study participation too aggravating	No
462	1	At bedtime	Study participation too aggravating	No
203	2	At bedtime	Study participation too aggravating	No
271	2	At bedtime	Study participation too aggravating	No
334	2	On awakening	Stopped aspirin use after advise cardiologist	No
344	2	On awakening	Study participation too aggravating	No
366	2	On awakening	Switch to vitamin k antagonist instead of aspirin after advise cardiologist	No
368	2	On awakening	Switch to vitamin k antagonist instead of aspirin after advise cardiologist	No
387	2	At bedtime	Switch to vitamin k antagonist instead of aspirin after advise cardiologist	No
405	2	At bedtime	Did not want to take aspirin at bedtime due to practical reasons	No
417	2	On awakening	Study participation too aggravating	No
465	2	At bedtime	Headache after switch from morning intake to intake at bedtime	Possible
437	2	On awakening	Study participation too aggravating	No

CHAPTER 3, APPENDIX TABLE 3

Appendix table 3. Self-reported side effects of randomized study subjects at baseline and subjects who completed study follow-up

	At Baseline (n=290), n (%)	**During study follow-up (n=264), n (%)**		
		Aspirin on awakening	Aspirin at bedtime	p-value*
Dyspepsia	15 (5.2)	12 (4.5)	12 (4.5)	1.00
Nausea	8 (2.8)	4 (1.5)	9 (3.4)	0.18
Heartburn	27 (9.3)	16 (6.1)	20 (7.6)	0.50
Nose bleeding	13 (4.5)	9 (3.4)	6 (2.3)	0.55
Bruises	45 (15.5)	32 (12.1)	35 (13.3)	0.75
Bloody stool	4 (1.4)	5 (1.9)	5 (1.9)	1.00

*P-value obtained by McNemar's test

Supplemental Table 1. Effect of aspirin intake on awakening or at bedtime on Cox-1 dependent and non-Cox-1 dependent platelet reactivity.

		Cox-1-dependent assays								
		Awakening			Bedtime			Difference, Bedtime – Awakening* (95% CI)		
		Intention to treat	Per protocol	Sensitivity	Intention to treat	Per protocol	Sensitivity	Intention to treat	Per protocol	Sensitivity
VerifyNow (ARU)	Whole day, AUC (SD)	4379.9 (385.3)	4412.6 (398.9)	4394.7 (436.4)	4300.4 (454.2)	4343.2 (479.4)	4289.8 (526.9)	-79.4 (-229.4;70.4)	-69.4 (-237.4;98.6)	-104.9 (-320.8;110.9)
	Whole day, Mean (SD)	438.1 (54.1)	441.4 (56.1)	438.2 (54.3)	429.6 (58.8)	433.9 (62.5)	428.2 (56.8)	-8.5 (-21.3;4.3)	-7.5 (-22.0;7.0)	-10.0 (-22.6;2.6)
	Morning peak, AUC (SD)	882.4 (107.3)	887.9 (113.4)	892.1 (34.5)	851.1 (105.0)	858.2 (112.5)	840.3 (31.9)	-31.3 (-88.2;25.6)	-26.7 (-96.4;37.0)	-51.8 (-105.4;1.8)
	Morning peak, Mean (SD)	445.1 (63.3)	448.6 (66.6)	445.1 (63.3)	421.7 (60.9)	424.8 (65.4)	415 (51.9)	-23.4 (-50.7;3.9)	-23.8 (-55.3;7.6)	-29.5 (-55.7;-3.2)
$STxB_2$ (ng/ml)	Morning peak, AUC (SD)	9.9 (4.6)	9.6 (5.1)	10.9 (4.9)	5.2 (2.0)	5.2 (2.3)	5.4 (2.1)	-4.7 (-6.9;-2.5)	-4.5 (-7.2;-1.7)	-5.6 (-8.2;-2.9)
	Morning peak, Mean (SD)	4.4 (2.8)	4.3 (3.0)	4.4 (2.8)	2.6 (1.3)	2.6 (1.4)	2.5 (1.1)	-1.8 (-2.8;-0.8)	-1.7 (-2.8;-0.6)	-1.9 (-2.9;-0.9)
		Cox-1-independent assays – Surface CD62 (P-selectin) expression								
		Awakening			Bedtime			Difference, Bedtime – Awakening* (95% CI)		
		Intention to treat	Per protocol	Sensitivity	Intention to treat	Per protocol	Sensitivity	Intention to treat	Per protocol	Sensitivity
ADP	Morning peak, AUC (SD)	30.5 (12.8)	33.3 (11.3)	32.3 (13.2)	31.6 (15.1)	33.5 (15.3)	33.4 (15.6)	1.1 (-3.2;5.3)	0.2 (-4.7;5.1)	1.0 (-4.4;6.4)
	Morning peak, Mean (SD)	15.2 (6.3)	16.6 (5.5)	16.1 (6.5)	16.0 (8.1)	16.9 (8.3)	16.9 (8.4)	-0.8 (-1.8;3.3)	0.3 (-2.7;3.2)	0.8 (-2.5;4.0)
TRAP	Morning peak, AUC (SD)	110.2 (12.0)	111.5 (11.5)	109.5 (11.5)	112.1 (13.0)	112.9 (13.1)	113.2 (13.7)	1.9 (-2.5;6.4)	1.4 (-3.8;6.6)	3.7 (-0.9;8.2)
	Morning peak, Mean (SD)	55.5 (6.0)	56.2 (5.8)	55.3 (5.6)	56.0 (6.6)	56.4 (6.6)	56.7 (6.9)	0.5 (-1.9;2.9)	0.1 (-2.7;2.9)	1.4 (-1.1;3.9)
PMA	Morning peak, AUC (SD)	50.5 (16.6)	53.1 (15.3)	48.5 (18.3)	44.6 (15.0)	46.9 (15.1)	42.7 (14.7)	-5.9 (-13.8;2.0)	-6.2 (-14.8;2.4)	-5.7 (-14.0;2.6)
	Morning peak, Mean (SD)	25.1 (8.3)	26.5 (7.6)	24.0 (9.0)	22.1 (7.7)	23.3 (7.7)	21.4 (7.5)	-3.0 (-7.3;1.2)	-3.2 (-7.8;1.4)	-2.6 (-7.2;2.0)

Cox-1-independent assays – Flow cytometry based aggregation (FCA)										
		Awakening			Bedtime			Difference, Bedtime – Awakening* (95% CI)		
		Intention to treat	Per protocol	Sensitivity	Intention to treat	Per protocol	Sensitivity	Intention to treat	Per protocol	Sensitivity
Ristocetin	Morning peak, AUC (SD)	42.4 (12.1)	43.6 (12.5)	40.8 (13.0)	42.5 (10.4)	43.1 (9.3)	41.5 (10.3)	0.1 (-8.6;8.8)	-0.5 (-9.2;8.1)	0.7 (-10.1;11.5)
	Morning peak, Mean (SD)	22.5 (4.9)	22.8 (5.2)	22.5 (4.8)	22.7 (4.1)	22.4 (3.2)	22.3 (4.4)	0.1 (-3.4;3.7)	-0.4 (-3.9;3.1)	-0.2 (-4.2;3.7)
TRAP	Morning peak, AUC (SD)	49.9 (6.4)	49.6 (6.8)	51.5 (5.3)	52.7 (6.7)	51.7 (5.1)	52.5 (7.2)	2.8 (-2.5;8.1)	2.1 (-3.1;7.4)	1.0 (-4.7;6.7)
	Morning peak, Mean (SD)	25.1 (3.6)	25.0 (3.9)	26.0 (2.7)	26.6 (3.9)	25.9 (2.9)	26.5 (4.2)	1.5 (-1.6;4.7)	0.9 (-2.2;4.0)	0.5 (-2.7;3.7)
ADP	Morning peak, AUC (SD)	47.4 (7.9)	46.8 (8.1)	49.2 (7.0)	50.4 (6.7)	50.3 (6.6)	49.8 (7.4)	2.9 (-3.3;9.2)	3.5 (-3.2;10.1)	0.6 (-6.0;7.2)
	Morning peak, Mean (SD)	23.5 (4.7)	23.2 (4.9)	24.7 (3.7)	25.4 (3.8)	25.2 (3.9)	25.0 (4.2)	1.9 (-1.8;5.6)	2.0 (-2.0;6.0)	0.4 (-3.2;3.9)
PMA	Morning peak, AUC (SD)	19.6 (9.2)	20.1 (9.7)	21.6 (8.5)	20.2 (8.7)	19.8 (9.3)	20.4 (9.6)	0.6 (-5.3;6.6)	-0.3 (-7.0;6.3)	-1.3 (-8.5;5.9)
	Morning peak, Mean (SD)	9.6 (5.0)	9.9 (5.3)	10.8 (4.4)	10.4 (4.7)	9.8 (4.8)	10.6 (5.1)	0.8 (-2.7;4.3)	-0.1 (-3.8;3.5)	-0.2 (-4.5;4.2)

*Difference (Bedtime – Awakening): for AUC estimated by paired t-tests; Mean differences estimated by linear mixed model analysis. ARU: aspirin reaction units; AUC: area under the curve; $STxB_2$: serum thromboxane B_2; ADP: adenosine diphosphate; TRAP: Thrombin receptor agonist peptide; PMA: phorbol 12-myristate 13-acetate; FCA: flow cytometry based aggregation.

CHAPTER 6, APPENDIX METHODS 1

Overview of the complete search strategy per database. Results for search on June 1st 2013:

Pubmed search strategy (331 hits)

("Platelet function Tests"[Mesh] OR "Platelet function Tests"[ti] OR "VerifyNow"[tiab] OR "PFA-100"[tiab] OR "multiple electrode aggregometry"[tiab] OR "light transmission aggregometry"[tiab] OR "serum thromboxane"[tiab] OR "Platelet Aggregation"[Majr] OR "Platelet Aggregation"[ti] OR "Platelet Aggregation Inhibitors"[Majr] OR "antiplatelet"[ti] OR "Platelet Adhesiveness"[Majr] OR "Platelet Adhesiveness"[ti] OR "Platelet Antiaggregant"[ti] OR "Platelet Antiaggregants"[ti]) AND ("adrenergic beta-antagonists"[Majr] OR "adrenergic beta-antagonists"[ti] OR "beta blockers"[ti] OR "beta-blockers"[ti] OR "betablockers"[ti] OR "beta blocker"[ti] OR "beta-blocker"[ti] OR "betablocker"[ti] OR "adrenergic beta-antagonists"[Pharmacological Action] OR "β blocker"[ti] OR "β-blocker"[ti] OR "β blockers"[ti] OR "β-blockers"[ti] OR "beta-Adrenergic Blocking"[ti])

Embase search strategy (734 hits)

("platelet function test*".ti,ab. OR "platelet aggregation" .ti,ab. OR exp thrombocyte aggregation/ OR "VerifyNow".ti,ab. OR "PFA-100".ti,ab. OR "multiple electrode aggregometry".ti,ab. OR "light transmission aggregometry".ti,ab. OR "serum thromboxane".ti,ab. OR "platelet adhesion".ti,ab. OR exp thrombocyte adhesion/ OR antiplatelet*.ti,ab. OR "platelet adhesiveness".ti,ab. OR "platelet antiaggregant*".ti,ab.)AND (exp *beta adrenergic receptor blocking agent/ OR "adrenergic beta-antagonist*".ti. OR "beta blocker*".ti. OR "beta-blocker*".ti. OR "betablocker*".ti. OR "beta-Adrenergic Blocking".ti.)

CHAPTER 6, APPENDIX METHODS 2

Calculation of effect size estimates (SMDs) and standard errors (SEs). Formulas used to calculate SMDs and SEs for each study design:

Study Design	Effect size estimate (SMD)	Standard error (SE) of SMD
Unpaired		
Two-group trial	$\frac{M_{D,i} - M_{D,p}}{SD_{pooled}}$	$\sqrt{\frac{N_{tot}}{N_i * N_p} + \frac{SMD^2}{2(N_{tot}-2)}}$
Cross-sectional study	$\frac{M_e - M_{ne}}{SD_{ne}}$	$\sqrt{\frac{N_{tot}}{N_e * N_{ne}} + \frac{SMD^2}{2(N_{tot}-2)}}$
Paired		
One-group trial	$\frac{M_{post} - M_{pre}}{SD_{pre}}$	$\sqrt{\frac{1}{0.5 * N_{tot}} + \frac{SMD^2}{N_{tot}}} \times \sqrt{2(1-\rho)}$
Cross-over trial	$\frac{M_{post} - M_{pre}}{SD_{pre}}$	$\sqrt{\frac{1}{0.5 * N_{tot}} + \frac{SMD^2}{N_{tot}}} \times \sqrt{2(1-\rho)}$

SMD = standardized mean difference; $M_{D,i}$ = mean difference post – pre-test in intervention group; $M_{D,p}$ = mean difference post – pre-test in placebo group; M_e = mean in exposed group; M_{ne} = mean in non-exposed group; M_{post} = mean post-test; M_{pre} = mean pre-test.

SD_{pooled} = pooled standard deviation at baseline, calculated as $\sqrt{\frac{(Ni-1)SDi^2 + (Np-1)SDc^2}{Ntot}}$

SD_{ne} = standard deviation in non-exposed; SD_{pre}= standard deviation pre-test.

N_{tot} = total number of subjects ; N_i = number of subjects in intervention group; N_p= number of subjects in placebo group; N_e=number of subjects in the exposed group; N_{ne}= number of subjects in non-exposed group.

ρ = correlation, calculated as $\frac{SD_{pre}{}^2 + SD_{post}{}^2 - SD_{diff}{}^2}{2 * SD_{pre} * SD_{post}}$ with SD_{diff} = standard deviation of the differences.

If not reported, we calculated SD_{pre} assuming equal variances over treatment periods and using the mean correlation of the other included paired studies (mean correlation 0.54): $SD_{pre} = SD_{pre} = \sqrt{\frac{SD_{diff}{}^2}{2(1-\rho)}}$

Appendix Table 1 Characteristics of all included studies

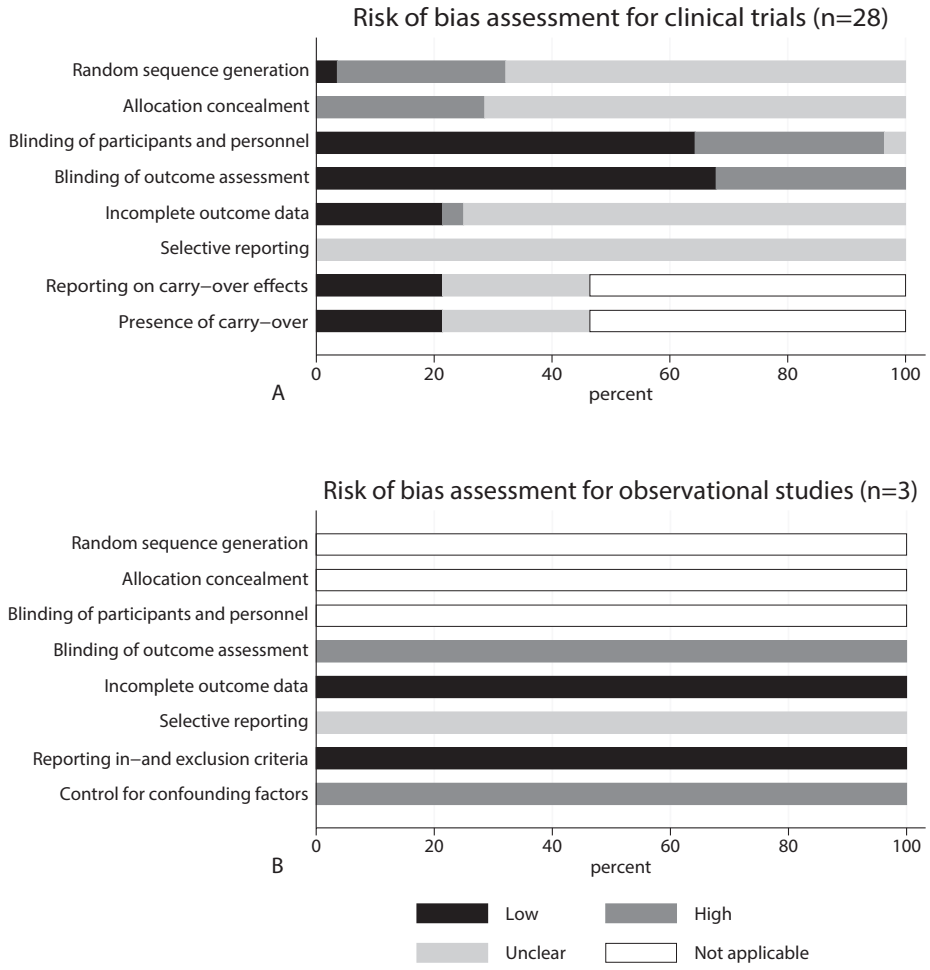
Ref	Author	Year	Design	Population	N (% male)	Age mean or range (yrs)	β-blocker	Dose (mg)*	Duration	Aggregation agonist (LTA)	Outcome Measure
1	Vlachakis	1980	1-group trial	Hypertension	16 (75)	54	Propranolol	60	8 wks	ADP	Threshold concentration
2	Frishman	1974	2-group trial	CAD	19 (70)	54	Propranolol	40	4 wks	ADP	Threshold concentration
3	Mehta	1978	cross-sectional	CAD	18 (ns)	57	Propranolol	80	ns	ADP, Epi	% platelet aggregation
4	Mehta	1978	cross-sectional	CAD	16 (ns)	46-64	Propranolol	80	ns	ADP, Epi	% platelet aggregation
5	Frishman	1978	2-group trial	CAD	20 (ns)	52	Propranolol	40	50 wks	ADP	Threshold concentration
6	Lote study1	1978	cross-over trial	Hypertension	5 (80)	30-45	Labetalol	200	2 wks	Coll, ADP, Epi	% platelet aggregation
6	Lote study2	1978	cross-over trial	Hypertension	4 (100)	30-45	Labetalol	400	2 wks	Coll, ADP, Epi	% platelet aggregation
6	Lote study3	1978	cross-over trial	Hypertension	4 (100)	30-45	Propranolol	80	2 wks	Coll, ADP, Epi	% platelet aggregation
7	Leon	1978	1-group trial	Healthy	4 (ns)	34	Propranolol	160	2 d	Coll, ADP, Epi	% light transmittance
8	Mehta	1979	cross-sectional	CAD	28 (ns)	41-64	Propranolol	20	ns	ADP, Epi	% platelet aggregation
9	Thaulow study1	1981	2-group trial	Healthy	16 (63)	23-37	Timolol	5	once	ADP	Threshold concentration
9	Thaulow study2	1981	2-group trial	Healthy	16 (63)	23-37	Propranolol	40	once	ADP	Threshold concentration
9	Thaulow study3	1981	2-group trial	Previous MI	43 (91)	41-76	Timolol	10	> 1 yr	ADP	Threshold concentration
10	Campbell	1981	1-group trial	Hypertension	8 (ns)	21-63	Propranolol	320	4 wks	Thromb	Threshold concentration
11	Markel	1983	cross-over trial	Hypertension	14 (100)	42-58	Atenolol	100	4 wks	Coll, ADP, Epi	AUC, mg

12	Cortellaro	1984	cross-over trial	Healthy	9 (100)	33	Metoprolol	100	3 wks	ADP, Thromb, AA, Epi	Threshold concentration
13	Kirch	1986	1-group trial	Hypertension	9 (ns)	51	Atenolol	100	12 wks	ADP, Coll	AUC, cm²/min
14	Ring	1987	cross-over trial	Healthy	5 (100)	28-32	Propranolol	40	once	ADP, Epi	% platelet aggregation
15	Winther study1	1988	cross-over trial	CAD	10 (100)	60	Metoprolol	100	2 wks	ADP	Threshold concentration
15	Winther study2	1988	cross-over trial	CAD	10 (100)	60	Timolol	10	2 wks	ADP	Threshold concentration
16	Davi	1988	2-group trial	Hypertension	12 (ns)	>60	Propranolol	80	1 wks	ADP, Coll, AA	Threshold concentration
17	Willich	1989	cross-over trial	CAD	10 (80)	52-77	Metoprolol	200	9 d	ADP, Epi	Threshold concentration
18	Gleerup	1989	cross-over trial	Hypertension	10 (80)	54	Timolol	5	2 wks	ADP	Threshold concentration
19	Pamphilon	1989	1-group trial	Healthy	8 (ns)	18-37	Propranolol	160	once	Coll, ADP, AA, Epi	Angle Slope
20	Nagakawa	1990	1-group trial	Hypertension	11 (27)	69	Carvedilol	20	8 wks	AA	% platelet aggregation
21	Lin	1991	2-group trial	Hypertension	25 (80)	44	Labetalol	600	4 wks	Coll, ADP, Epi	% light transmittance
22	Ding	1994	cross-over trial	Hypertension	30 (57)	59	Propranolol	75	10 wks	Coll, ADP, AA, Epi	max. amplitude
23	Giugliano	1998	2-group trial	DM	8 (75)	53	Carvedilol	25	12 wks	ADP	% platelet aggregation
24	Patki	1998	1-group trial	Hypertension	6 (83)	47	Atenolol	50	1 wks	ADP	% platelet inhibition
25	Mugellini study1	2005	cross-over trial	DM	30 (43)	75	Atenolol	50	6 wks	ADP, Coll	Delta T-max%
25	Mugellini study2	2005	cross-over trial	Hypertension	30 (47)	74	Atenolol	50	6 wks	ADP, Coll	Delta T-max%

CAD: coronary artery disease; DM: diabetes mellitus; MI: myocardial infarction; N: total number of patients; yrs: years; mg: milligrams; wks: weeks; d: days; yr: years; LTA: light transmission aggregometry; Coll: collagen; ADP: adenosine diphosphate; Epi: epinephrine; AA: arachidonic acid; Thromb: thrombine; ns: not specified;

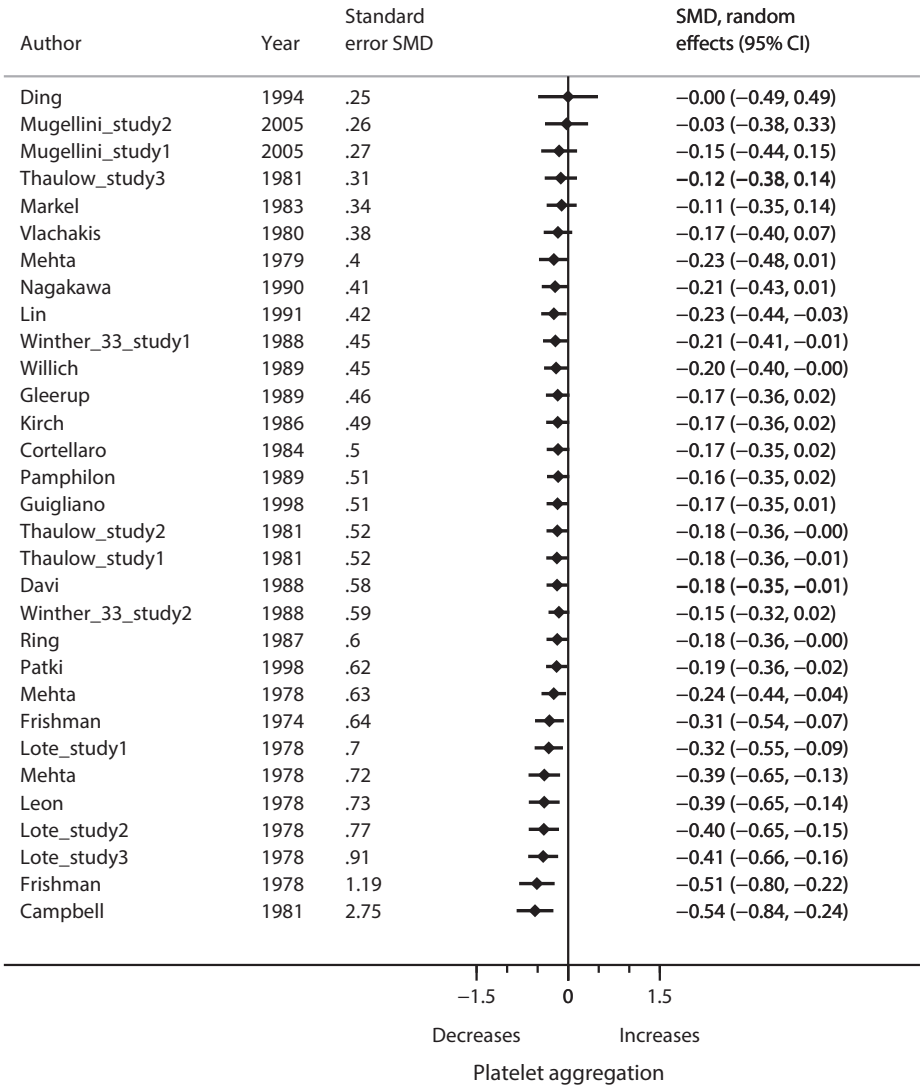
* When studies used a run-in period for beta-blocker dosage titration, the final reached dose of the beta-blocker or the mean dose was used.

CHAPTER 6, APPENDIX FIGURE 1



Appendix figure 1 – Risk of bias assessment. Methodological quality graphs: review authors' judgements about each methodological quality item presented across included trials (**panel a**) and observational studies (**panel b**).

CHAPTER 6, APPENDIX FIGURE 2



Appendix figure 2 – Cumulative meta-analysis based on study precision.

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Nederlandse samenvatting

Het doel van dit proefschrift was om het effect van aspirine inname 's ochtends of 's avonds op de bloeddruk en ochtendpiek van bloedplaatjesactiviteit te onderzoeken. De achtergrond hiervan is uitgebreid beschreven in **hoofdstuk 1** en zal hieronder kort worden samengevat.

Onze studies in **hoofdstuk 3** en **hoofdstuk 4** waren 'cross-over' studies. Specifieke methodologische kenmerken hiervan zijn beschreven in **hoofdstuk 2**. In deze samenvatting worden eerst de specifieke kenmerken van cross-over studies toegepast op de studies in dit proefschrift. Daarna worden de resultaten van **hoofdstuk 3** en **hoofdstuk 4** samengevat en bespreken wij conclusies en aanbevelingen. Tot slot vatten wij de bevindingen over de invloed van bloedplaatjes markers op het recidief risico hart- en vaatziekten (**hoofdstuk 5**) en de invloed van bèta-blokkers op bloedplaatjesactiviteit (**hoofdstuk 6**) samen.

CROSS-OVER STUDIES

Cross-overstudies zijn veelal goedkoper en efficiënter dan studies met parallelle groepen. In een cross-overstudie krijgt elk individu in opeenvolgende perioden alle behandelingen die onderzocht worden, waarbij de uitkomsten gemeten worden aan het eind van elke periode. De effecten worden dus vergeleken per individu in plaats van tussen groepen individuen. Er zijn echter wel specifieke voorwaarden voor het uitvoeren van een cross-overstudie. Cross-overstudies kunnen gebruikt worden bij het bestuderen van chronische aandoeningen waarbij het effect van de behandeling tijdelijk is. Behandeling van bloeddruk en bloedplaatjesactiviteit, onderzocht in **hoofdstuk 3** en **hoofdstuk 4**, zijn hiervan goede voorbeelden. De resultaten van een cross-over studie kunnen worden beïnvloed door periode-effecten en 'carry over'-effecten. Periode-effecten kunnen ontstaan als een uitkomst verandert over de tijd. In **hoofdstuk 3** bijvoorbeeld werd bloeddruk gemeten, en die stijgt met de leeftijd. Als alle deelnemers in onze studie alle behandelingen in dezelfde volgorde zouden hebben gekregen (bijvoorbeeld: 3 maanden aspirine 's ochtends, gevolgd door 3 maanden aspirine 's avonds), zou de vaste stijging van bloeddruk over de tijd onze resultaten kunnen beïnvloeden. Een oplossing hiervoor is om deelnemers te randomiseren voor de volgorde van de interventie. De vaste stijging van bloeddruk over de tijd wordt dan gelijk verdeeld over de interventies. Kortom, randomisatie is even belangrijk in cross-over studies als in parallelle-groep studies, maar vanwege een andere reden: randomisatie in parallelle-groep studies wordt gebruikt om vergelijkbaarheid tussen de interventiegroepen te bereiken, terwijl randomisatie bij cross-over studies nodig is om vergelijkbaarheid tussen de interventieperioden te bereiken.

Verder moet er bij de opzet van een cross-over studie gedacht worden aan 'carry-over' effecten. Als behandelingen vergeleken worden in opeenvolgende perioden kan het voorkomen dat de behandeling van periode 1 nog doorwerkt in periode 2. Dit wordt het 'carry-over'-effect genoemd. Een manier om dit effect te voorkomen is door tussen de behandelperioden een uitwasperiode ('wash-out') in te bouwen waarin geen behandeling wordt gegeven. Het idee erachter is dat de metingen in de volgende periode niet meer worden beïnvloed door de behandeling in de voorafgaande periode omdat het effect daarvan uitgewerkt is tijdens de uitwasperiode. Echter, in de studie in **hoofdstuk 3** was een uitwasperiode niet nodig. Om een goede wash-out te bewerkstelligen van het effect van aspirine op de bloeddruk in de eerste periode werd aangenomen dat het effect van aspirine op de bloeddruk niet langer zou duren dan de duur van de interventieperiode (3 maanden). Ook zou een uitwasperiode in deze studie onethisch zijn geweest, omdat patiënten aspirine gebruikten ter preventie van hart- en vaatziekten. In onze studie in **hoofdstuk 4** werd wel een uitwasperiode ingebouwd. De duur hiervan, 2 tot 4 weken, was voldoende om de normale bloedplaatjesactiviteit te herstellen (7 tot 10 dagen) na aspirine inname tijdens de eerste interventieperiode.

Samenvattend hebben wij rekening gehouden met de specifieke methodologische kenmerken van cross-over studies bij de opzet van de studies in **hoofdstuk 3** en **hoofdstuk 4**, wat de validiteit van deze studies ondersteunde.

ASPIRINE 'S OCHTENDS OF 'S AVONDS?

Een lage dosis aspirine (carbasalaatcalcium of acetylsalicylzuur) is de hoeksteen in de behandeling van hart- en vaatziekten: het verlaagt het risico op het opnieuw optreden van hart- en vaatziekten met 25%. Daarmee is het een van de meest effectieve geneesmiddelen die we kennen. De werking van aspirine berust op het remmende effect op de bloedplaatjes. Als bloedplaatjes samenklonteren en een stolsel vormen in een slagader kan dit een hart- of herseninfarct tot gevolg hebben. Aspirine remt de klonterneiging van bloedplaatjes en voorkomt daarmee een gedeelte van de hart- en herseninfarcten.

In eerdere studies werd echter nog een effect van aspirine gevonden: aspirine kan de bloeddruk verlagen als het 's avonds wordt ingenomen in plaats van 's ochtends. Dit verassende effect werd gevonden in studies met gezonde proefpersonen, zwangeren of patiënten met licht verhoogde bloeddruk. Aangezien aspirine ook bijwerkingen kent, dient het alleen te worden voorgeschreven als het voordelige effect ervan opweegt tegen het risico op bijwerkingen. Alleen bij patiënten met hart- en vaatziekten is dit het geval. Voor deze patiënten is een

bloeddruk verlagend effect van aspirine waarschijnlijk ook voordelig, aangezien bloeddruk een van de belangrijkste risicofactoren is voor het optreden van hart- en vaatziekten.

Avond-aspirine om bloeddruk te verlagen

In **hoofdstuk 3** is onderzocht of aspirine-inname 's avonds in plaats van 's ochtends de bloeddruk verlaagt van patiënten die dagelijks aspirine gebruiken ter preventie van hart- en vaatziekten. Hiervoor werden uit huisartsenpraktijken in de omgeving Leiden 290 patiënten willekeurig ingedeeld in twee groepen. De eerste groep gebruikte 3 maanden aspirine 's ochtends, gevolgd door 3 maanden aspirine 's avonds. De tweede groep gebruikte 3 maanden aspirine 's avonds, gevolgd door 3 maanden aspirine 's ochtends. Aan het eind van elke 3 maanden werd gedurende 24 uur de bloeddruk gemeten en werden bijwerkingen van aspirine geregistreerd. De bijwerkingen met aspirine-inname 's avonds kwamen gemiddeld niet vaker voor dan met inname 's ochtends. Voor 263 patiënten waren de bloeddrukmetingen compleet. We constateerden dat de gemiddelde bloeddruk over 24 uur niet lager was met avond-inname van aspirine dan met ochtend-inname (gemiddeld verschil systolisch / diastolisch: -0.1 [CI: -1.0; 0.9] / -0.6 [CI: -1.2; 0.0] mmHg). Ook was er geen verschil in bloeddruk over de dag- en nacht, en bij subgroepen patiënten die wel of geen antihypertensiva gebruikten. Een mogelijke verklaring voor onze negatieve bevinding wat betreft bloeddruk is dat het bloeddrukverlagend effect van avond-aspirine afneemt naarmate patiënten langer aspirine gebruiken door toename van stijfheid van de bloedvaten. Geconcludeerd kan worden dat inname van aspirine 's avonds in plaats van 's ochtends de bloeddruk niet verlaagt van patiënten die aspirine gebruiken ter preventie van hart- en vaatziekten. Gezien de hoge validiteit van ons onderzoek en de afwezigheid van enig bloeddrukverlagend effect van avond-aspirine is er geen toekomstig onderzoek meer nodig naar het bloeddrukverlagende effect van avond-aspirine in patiënten die al aspirine gebruiken.

Avond-aspirine om de ochtendpiek van bloedplaatjesactiviteit te verlagen

Eerst zal kort de achtergrond worden samengevat, waarna de resultaten van de studies in **hoofdstuk 3** en **hoofdstuk 4** worden besproken.

De werking van aspirine berust op het remmende effect op de bloedplaatjes. Deze remming is onomkeerbaar voor de gehele levensduur van elk bloedplaatje (7-10 dagen). Toch moet aspirine elke dag worden ingenomen, omdat dagelijks ongeveer 10% van de bloedplaatjes worden vernieuwd. Ook is bekend dat de bloedplaatjesactiviteit een 24-uurs ritme volgt, met een piek in de ochtenduren. Deze piek valt samen met de piek in het optreden van hart- en vaatziekten van

6-12 uur ’s ochtends. De verhoogde activiteit van bloedplaatjes in de ochtenduren draagt dus waarschijnlijk bij aan die van hart- en vaatziekten in de ochtend. Na inname remt aspirine bloedplaatjes die op dat moment aanwezig zijn in het bloed, maar wordt binnen 30 minuten afgebroken en uitgescheiden uit het lichaam. Alle bloedplaatjes die daarna worden aangemaakt zijn dus ongeremd door aspirine. De meeste mensen nemen aspirine ’s ochtends in. Eerder onderzoek toonde aan dat, bij aspirine inname in de ochtend, de bloedplaatjesactiviteit de volgende ochtend (vlak voor inname van de volgende aspirine) onvoldoende geremd werd in 25% van de patiënten die dagelijks aspirine slikten. Dit terwijl juist in de ochtenduren een optimale remming gewenst is vanwege de verhoogde kans op hart- en vaatziekten in de ochtend. Een manier om de remming van bloedplaatjes in de ochtend te verbeteren is wellicht door aspirine ’s avonds in te nemen in plaats van ’s ochtends. Dit zou kunnen zorgen voor een verlaging van de ochtendpiek in bloedplaatjesactiviteit en daarmee mogelijk ook voor een verlaging van het optreden van hart- en vaatziekten in de ochtend. Of de ochtendpiek van bloedplaatjesactiviteit verlaagd kan worden door aspirine ’s avonds in te nemen in plaats van ’s ochtends is nog niet eerder onderzocht.

In **hoofdstuk 3** werd bij een gedeelte van de patiënten (n=133) bloedplaatjesactiviteit in de ochtenduren gemeten. De bloedplaatjesactiviteit werd gemeten met behulp van een apparaat waarmee de klonterneiging van bloedplaatjes uitgedrukt werd in een getal (aspirine reactie units; ARU). Een hogere waarde betekent een hogere bloedplaatjesactiviteit. Wij constateerden dat de bloedplaatjesactiviteit in de ochtenduren lager was als aspirine ’s avonds werd ingenomen (gemiddeld verschil: 22 ARU [95% betrouwbaarheidsinterval (BI): -35 tot -9]). Een beperking van dit onderzoek was dat er maar één meting tijdens de ochtenduren kon worden verricht, terwijl bekend is dat bloedplaatjesactiviteit een 24-uurs ritme volgt. Daarom werd een 24-uurs ritme studie uitgevoerd, die beschreven is in **hoofdstuk 4**. Veertien gezonde vrijwilligers namen 2 weken aspirine ’s ochtends in, gevolgd door 2 weken aspirine ’s avonds, of de omgekeerde volgorde. Aan het eind van beide perioden van 2 weken werd de bloedplaatjesactiviteit elke 3 uur gemeten. Het belangrijkste resultaat was dat aspirine-inname ’s avonds de bloedplaatjesactiviteit tijdens de ochtenduren (6-12 uur) verlaagde met gemiddeld 23 ARU (95% BI: -50 tot 4). Ook werd het serum tromboxane B_2, een directe maat voor het effect van aspirine op bloedplaatjes, verlaagd tijdens de ochtenduren door aspirine ’s avonds in te nemen in plaats van ’s ochtends (gemiddeld verschil: -1.8 ng/ml [95% BI: -2.8 tot -0.8])

Samengevat kunnen we uit de bevindingen in **hoofdstuk 3** en **hoofdstuk 4** concluderen dat bloedplaatjesactiviteit tijdens de ochtenduren beter wordt geremd als aspirine ’s avonds wordt ingenomen in plaats van ’s ochtends.

Advies aan patiënten die aspirine gebruiken

Onze studie in **hoofdstuk 3** suggereert dat bijwerkingen gemiddeld niet vaker voorkomen met aspirine-inname 's avonds dan met inname 's ochtends. Het lijkt dus veilig om aspirine 's avonds in te nemen.

Aspirine-inname 's avonds zorgde in dit onderzoek niet voor een verlaging van de bloeddruk. Wel was duidelijk dat de activiteit van bloedplaatjes in de ochtenduren beter geremd werd door aspirine 's avonds in te nemen. Dit zou voordelig kunnen zijn voor het voorkómen van hart- en vaatziekten in de ochtenduren, omdat de kans op het ontwikkelen van een bloedstolsel in een slagader (met een hart- of herseninfarct tot gevolg) groter is als de bloedplaatjesactiviteit hoger is. Aspirine remt de klonterneiging van bloedplaatjes, en een maximale werking van aspirine tijdens de ochtenduren zou een deel van het optreden van een bloedstolsel in de ochtenduren kunnen voorkomen. Wel moet worden opgemerkt dat in onze studies patiënten niet werden gevolgd om te onderzoeken of het optreden van hart- en vaatziekten in de ochtenduren inderdaad wordt verlaagd door aspirine 's avonds in te nemen. Dit zal toekomstig onderzoek moeten uitwijzen.

BASALE BLOEDPLAATJESACTIVITEIT EN RISICO OP RECIDIEF HART- EN VAATZIEKTEN

In **hoofdstuk 5** werd onderzocht of hoge waarden van basale activiteit van bloedplaatjes, gemeten met ontstekingswaarden in bloed (NAP-2, CXCL4 en RANTES), het risico op een recidief hartinfarct verhogen. De gedachte hierachter is dat deze bloedplaatjes-specifieke ontstekingswaarden bijdragen aan het ontstaan van aderverkalking (atherosclerose) en ontstekingscellen aantrekken in bloedstolsels. In totaal 542 mannen, die een hartinfarct hadden overleefd tussen 1990 en 1996, werden gemiddeld 9.2 jaar gevolgd. Tijdens de follow-up kregen 254 (47%) mannen een vorm van recidief hartinfarct. Het belangrijkste resultaat was dat hoge waarden van de gemeten ontstekingswaarden het risico op een recidief niet verhoogden (gecorrigeerde hazard ratio's: NAP-2: 1.1 [95% BI 0.8-1.7]; CXCL4: 1.2 [0.8-1.8]; RANTES 1.2 [0.8-1.8]). Deze resultaten spreken eerder onderzoek tegen. Een eerste mogelijke verklaring hiervoor is dat onze studiepopulatie stabiele patiënten vertegenwoordigde, terwijl eerdere studies juist patiënten met acute vormen van hart- en vaatziekte bestudeerden. Ten tweede is het mogelijk dat de verhoogde waarden in ons onderzoek niet bijdroegen aan het risico omdat bij onze patiënten (oudere mannen die een hartinfarct overleefden) andere klassieke risicofactoren veel meer aanwezig waren dan bij patiënten in een eerder onderzoek (jonge vrouwen). Als klassieke risicofactoren afwezig zijn, zoals bij jonge vrouwen, en zij

toch een hartinfarct krijgen, is het waarschijnlijk dat andere risicofactoren een veel grotere rol spelen.

Uit deze studie kan geconcludeerd kan worden dat de basale activiteit van bloedplaatjes niet gerelateerd is aan het risico op recidief hart- en vaatziekten bij mannen die een eerste hartinfarct overleefden. Dit sluit niet uit dat de basale activiteit van bloedplaatjes, gemeten met ontstekingswaarden, van invloed kan zijn op het hart- en vaatziekten risico. Waarschijnlijk hangt dit af van de prevalentie van klassieke risicofactoren in de onderzochte populatie.

BÈTA-BLOKKERS EN BLOEDPLAATJESREACTIVITEIT

In navolging van internationale richtlijnen worden bèta-blokkers vaak voorgeschreven aan patiënten met hart- en vaatziekten. Bloedplaatjes spelen een grote rol bij het ontstaan van hart- en vaatziekten, maar het effect van bèta-blokkers op bloedplaatjesreactiviteit was nog onduidelijk. Daartoe hebben werd in **hoofdstuk 6** alle beschikbare literatuur hierover samengevat en het effect van bèta-blokkers op de bloedplaatjesreactiviteit samengevoegd in een meta-analyse. Uiteindelijk werden 31 studies geïncludeerd (28 experimentele-, 3 observationele onderzoeken), waarin in totaal 454 patiënten bestudeerd werden. Het belangrijkste resultaat was dat het gebruik van bèta-blokkers de bloedplaatjesreactiviteit verlaagde met gemiddeld 13% (95% BI: 8-17%). Verder vonden we dat dit effect het meest aanwezig was als adrenaline gebruikt werd om de bloedplaatjesreactiviteit te testen. Ook verlaagden niet-selectieve lipofiele bèta-blokkers (zoals carvedilol) de bloedplaatjesreactiviteit meer dan selectieve niet-lipofiele bèta-blokkers (zoals metoprolol). Samenvattend suggereert deze studie dat het beschermende effect van bèta-blokkers, naast een verlaging van het hartritme, bloeddruk en zuurstofbehoefte van het hart, mogelijk mede berust op een verlaging van de bloedplaatjesreactiviteit.

CONCLUSIES

De studies in dit proefschrift laten zien dat aspirine-inname 's avonds in plaats van 's ochtends niet de bloeddruk, maar wel de ochtendpiek van bloedplaatjesactiviteit verlaagt van patiënten die aspirine gebruiken ter preventie van hart- en vaatziekten. Dit werd bevestigd in een 24-uurs ritme studie bij gezonde vrijwilligers. Toekomstig onderzoek moet uitwijzen of deze simpele interventie inderdaad leidt tot een verlaging van het risico op hart- en vaatziekten.

Uit onderzoek in dit proefschrift blijkt dat hoge waarden van basale bloedplaatjesreactiviteit, gemeten met bloedplaatjes-specifieke ontstekingswaarden, waarschijnlijk niet bijdragen aan het risico op een recidief hartinfarct in mannen die eerste hartinfarct overleefd hebben. In eerder onderzoek was verhoogde bloedplaatjesreactiviteit geassocieerd met het risico op hart- en vaatziekten. Uit de meta-analyse in dit proefschrift blijkt dat bèta-blokkers, die vaak worden voorgeschreven aan patiënten met hart- en vaatziekten, bloedplaatjesreactiviteit verlagen.

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Curriculum vitae

Tobias Nicolaas Bonten werd op 1 augustus 1984 geboren in Heerlen. In 2002 behaalde hij zijn gymnasium diploma aan het Bernardinus College te Heerlen, waarna hij 5 maanden rondreisde in Australië. In 2003 begon hij zijn studie Geneeskunde aan de Universiteit Leiden. Tijdens de doctoraal fase nam hij deel aan een uitwisseling met het Karolinska Instituut te Stockholm. In het vierde jaar van zijn studie was hij als student assistent op de afdelingen Verloskunde en Neurologie van het Leids Universitair Medisch Centrum (LUMC) betrokken bij de praktische uitvoering van wetenschappelijk onderzoek. Ook gaf hij op de snijzaal van het LUMC anatomie les aan studenten geneeskunde. Aan het eind van zijn studie volgde een wetenschappelijke stage op de afdeling Infectieziekten van het LUMC naar diagnostiek en predictie van gecompliceerde urineweginfecties. Tijdens deze stage volgde hij een cursus epidemiologie onder begeleiding van dr. J.D. Snoep. Het arts-examen werd in april 2010 cum laude afgelegd, waarna hij werkte als arts-assistent interne geneeskunde in het Diaconessenhuis Leiden.

Van december 2010 tot februari 2014 deed hij promotieonderzoek op de afdeling Klinische Epidemiologie van het LUMC onder begeleiding van prof. F.R. Rosendaal, prof. J.G. van der Bom en dr. J.D. Snoep. Gedurende deze jaren bleef hij als arts-assistent avond- en nachtdienst doen in het Diaconessenhuis Leiden en volgde hij de opleiding tot Epidemioloog B onder begeleiding van prof. F.R. Rosendaal en prof J.G. van der Bom. De resultaten van het onderzoek naar het tijdsafhankelijke effect van aspirine op bloeddruk en bloedplaatjes activiteit zijn in dit proefschrift beschreven. Ook werden de resultaten gepresenteerd op internationale congressen, wat niet onopgemerkt bleef door nationale en internationale media. In 2013 kreeg hij samen met prof. J.G. van der Bom subsidie van het Leids Universitair Fonds om onderzoek te doen naar de invloed van het innametijdstip van aspirine op het 24-uurs ritme van bloedplaatjes activiteit.

Sinds 1 maart 2014 volgt hij de opleiding tot huisarts in de regio Leiden.