**Aim:** Statins can induce pharmacologic preconditioning and thereby reduce infarct size. Cellular phosphatidylserine (PS) exposition occurs in the course of ischaemia and reperfusion and has been associated with injury. In this experiment we studied the effect of atorvastatin on PS exposition after a standardised ischaemia and reperfusion challenge.

**Methods:** In a double-blind randomised cross-over trial 30 healthy volunteers were allocated to 3 day treatment with atorvastatin (80 mg/day) and placebo (n = 24), or placebo treatment twice (n = 6). At the end of each treatment period, volunteers underwent 10 minutes of forearm ischaemic exercise. At reperfusion radiolabeled annexin A5 was administered intravenously and Gamma camera imaging of both hands was performed 1 and 4 hours after reperfusion.

**Results:** Annexin A5 targeting was not different between atorvastatin treatment (26.1 ± 9.8% and 24.0 ± 9.5% respectively at 1 and 4 hours after reperfusion) and placebo treatment (25.6 ± 11.0% and 24.5 ± 10.7%) (p = 0.99). Our time control experiment did not reveal a carry-over effect.

**Conclusions:** Our results show that treatment with atorvastatin 80 mg does not reduce forearm PS exposition after ischaemic exercise. This suggests that the role of PS exposure in the prevention of ischaemia and reperfusion injury by short term treatment with atorvastatin is limited.

**Key words:** Atorvastatin, Ischaemia-reperfusion injury, Phosphatidylserines, Annexin A5 scintigraphy, Pharmacologic preconditioning
at orvastatin prior to percutaneous coronary intervention reduced myocardial injury and improved clinical outcomes in patients with acute coronary syndromes\textsuperscript{23, 24}.

Ischaemia induced phosphatidylserine (PS) exposure on cardiomyocytes has been associated with reversible cellular damage and apoptosis\textsuperscript{23, 24}. Treatment to shield exposed PS with diannexin, a homodimer of human annexin A5 (with high affinity for exposed phosphatidylserines) significantly reduces IR-injury in the liver and kidney\textsuperscript{25, 26}. Likewise, diannexin prevents no-reflow in a rabbit cardiac ischemia-reperfusion model\textsuperscript{27}. These observations suggest a critical role for exposed PS in the development of IR-injury. We have developed an experimental model to study PS exposure after IR in healthy volunteers. Our model uses radiolabeled recombinant annexin A5 to visualise PS-exposure that occurs after 10 minutes of ischaemic exercise in healthy male volunteers, we have recently shown that annexin A5 targeting after a short pretreatment with rosuvastatin 20 mg once daily was significantly reduced (16 ± 1% and 18 ± 2% respectively at 1 and 4 hours after reperfusion) compared to placebo treatment (21 ± 3% and 25 ± 3%) (\(p<0.05\))\textsuperscript{31}. As previously reported for other statins in preclinical research with infarct size as endpoint, this effect of rosuvastatin was inhibited by caffeine, an adenosine receptor antagonist\textsuperscript{31}.

Using annexin A5 scintigraphy after voluntary ischaemic exercise in healthy males, we have recently shown that annexin A5 targeting after a short pretreatment with rosuvastatin 20 mg once daily was significantly reduced (16 ± 1% and 18 ± 2% respectively at 1 and 4 hours after reperfusion) compared to placebo treatment (21 ± 3% and 25 ± 3%) (\(p<0.05\))\textsuperscript{31}. As previously reported for other statins in preclinical research with infarct size as endpoint, this effect of rosuvastatin was inhibited by caffeine, an adenosine receptor antagonist\textsuperscript{31}.

There is ample evidence from animal and clinical research that treatment for three days with high dose atorvastatin protects against IR-injury\textsuperscript{15, 21, 32-34}. However, we do not know whether PS exposure relates to this clinical benefit of atorvastatin, as differences have been observed in the efficacy of various statins to inhibit HMG-CoA-reductase\textsuperscript{35} and to induce pleiotropic effects\textsuperscript{36}.

Aim

We aim to assess the role of PS-modulation in the beneficial effect of atorvastatin on IR-injury. We tested the hypothesis that a short pretreatment with atorvastatin 80 mg reduces annexin A5 targeting in our forearm IR-model.

Methods

After approval of the protocol (NCT00441597) by the Institutional Review Board of the Radboud University Nijmegen Medical Centre and in accordance with the declaration of Helsinki (2008), 30 healthy male volunteers (age 18-54) signed informed consent. All participants underwent medical screening to exclude cardiovascular disease, hypercholesterolemia, hypertension, diabetes mellitus, and impaired renal function. Furthermore serum values of creatinin kinase (CK) and alanine aminotransferase (ALT) were measured to exclude participants possibly at risk for adverse events due to atorvastatin use (rhabdomyolysis and toxic hepatitis).

In this double blind randomised cross-over trial (Fig. 1) participants were allocated to treatment with either atorvastatin and placebo (\(n=24\)), or placebo treatment twice (\(n=6\)). The latter group served as a time-control. Furthermore, it allowed us to verify our assumptions on within subject variability and test-to-test correlation of our annexin A5 scans. Placebo or atorvastatin (80 mg) was administered for three days, and at least 4 weeks were allowed between the two treatment periods as a wash-out. Atorvastatin 80mg (Pfizer, Capelle a/d IJssel, The Netherlands) was capсуlated to match placebo by the department of Clinical Pharmacy (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands according to GMP standards). In both treatment periods the first tablet was administered under supervision of the investigator, after venous blood sampling to determine fasting lipid profile. The participant was instructed to take the second capsule in the morning and to abstain from caffeine during the last 24 hours prior to ischaemic exercise. On day 3 after an overnight fast the last tablet was administered under supervision, again after assessment of fasting lipid profile and serum caffeine level to verify protocol compliance.

Sixty minutes after administration of the study medication in all participants maximal voluntary contraction force of the non-dominant forearm was measured with an isometric handgrip dynamometer. Subsequently, the circulation of the non-dominant forearm was occluded for 10 minutes with an upper arm cuff inflated to 200 mmHg. Directly after occlusion volunteers were asked to perform isometric contractions of the finger flexors at 50% of their maximum contraction force. These contractions were performed rhythmically with 5 seconds of contraction followed by 5 seconds of relaxation until exhaustion. The total duration of ischaemia was 10 minutes, independent of the duration of contractions.
Immediately upon reperfusion 100 microgram recombinant human annexin A5, radiolabeled with 450 MBq Technetium-99m was administered intravenously into the dominant arm. One and four hours after this administration both hands were scanned simultaneously by use of a gamma camera (Orbiter camera equipped with low-energy high-resolution collimators; Siemens, Hoffman Estates, Ill, USA) as previously described.

Caffeine plasma concentrations were determined using reversed-phase HPLC with UV detection set at 273 nm. Serum lipid profile (total-, HDL- and LDL-cholesterol and triglycerides), creatinine kinase (CK) and alanine amino transferase (ALAT) were determined with a commercially available kit (Aerosek, Abbott, Santa Clara, Cal., USA).

**Data Analysis**

All the digitised gamma camera images were analysed offline by the same investigator (W.J.O.) using Hermes software (Nuclear Diagnostics, Stockholm, Sweden). A predefined region of interest was placed over the thenar muscle of both hands. Annexin A5 targeting was expressed as the percentage difference in counts/pixel between the experimental (non-dominant) hand and the control hand as previously described.

**Statistical Analysis**

Values are expressed as mean±standard error (SE) unless indicated otherwise.

This study is the first to use our forearm IR-model in a paired set-up in healthy volunteers. Based on previous studies, a between subject SD of 10% was predicted. Conservatively assuming a correlation coefficient of 0.5 between first and second paired observation, we calculated that with an alpha of 0.05 and a power of 90%, using 24 subjects allows us to find a difference in annexin A5 targeting of 7%. This is well in the range of our previous observation after treatment with rosuvastatin. Furthermore 6 additional participants were randomised (in a double-blind fashion) to receive placebo treatment twice to serve as time control experiment. This time-control allows us to verify our assumptions regarding this power analysis afterwards.

The effect of atorvastatin treatment on annexin A5 targeting and lipid profile was tested using ANOVA for repeated measurements. All statistical analyses were performed using SPSS 16.0 for Windows.

**Results**

Baseline characteristics of all participants are presented in Table 1. Caffeine levels were all under 0.85...
mg/L, indicating satisfactory compliance to the 24 hours caffeine abstinence. The annexin A5 targeting was not different between atorvastatin treatment (26.1 ± 2.0% and 24.0 ± 1.9% respectively at 1 and 4 hours after reperfusion) and placebo treatment (25.6 ± 2.4% and 24.5 ± 2.2%) \((p=0.999)\) (Fig.2).

Three day treatment with atorvastatin significantly reduced total cholesterol (from 4.15 ± 0.10 mmol/L to 3.44 ± 0.10 mmol/L) and LDL cholesterol (from 2.31 ± 0.10 mmol/L to 1.84 ± 0.10 mmol/L as compared to placebo treatment; \(p<0.001\) for both comparisons). HDL cholesterol and triglyceride levels were unaffected by atorvastatin (from 1.22 ± 0.04 to 1.25 ± 0.05 for HDL-C, and from 0.93 ± 0.08 to 0.80 ± 0.09 for triglycerides).

Workload (50% of the maximal strength * duration of ischaemic exercise) did not significantly differ between both study days (4260 ± 284 kg×sec and 4253 ± 219 kg×sec after atorvastatin and placebo respectively \((p=0.96)\), indicating that potential differences in ischaemic challenge did not confound the observed lack of effect on annexin A5 targeting.

In the time control group \((n=6)\) the annexin A5 targeting after the first visit was 23.5 ± 5.5% and 23.2 ± 6.5% (respectively at 1 and 4 hours after reperfusion) and 30.2 ± 6.1% and 20.3 ± 7.8% after the second visit (Fig.3). The between-subject standard deviations in annexin A5 targeting were 13.5%, 15.8%, 15.0% and 9.3% at 1 and 4 hours for visit 1 and 2 respectively. Within subject correlation was 0.93 and 0.79 at 1 and 4 hours respectively. This reproducibility should have allowed us to detect a difference in targeting between placebo and atorvastatin of at least 7%, closely resembling our initial power calculation.

### Discussion

The novel finding of this study is the lack of effect of atorvastatin on annexin A5 targeting after voluntary ischaemic exercise. This indicates that in our forearm model treatment with atorvastatin, in a dose and duration as previously used in clinical trials with patients who present with coronary syndromes \([21, 22, 32]\), does not modulate PS exposition after IR, in contrast to treatment with rosuvastatin, as reported recently \([31]\). As the protective action of atorvastatin in a setting of ischemia and reperfusion has been convincingly shown in multiple animal experiments \([33, 34]\) and even in several clinical trials \([21, 22, 38]\), we conclude that PS modulation is not likely involved in the protective mechanism of atorvastatin.

During reperfusion after an ischaemic event, multiple detrimental processes take place simultaneously including formation of radical oxygen species (ROS), cellular and mitochondrial calcium overload, and a rapid increase (restoration) in pH \([39]\). These actions result in mitochondrial damage caused by opening of the mitochondrial permeability transition pore (MPTP), activation of caspases and subsequent cell damage and possible cell death (by either necrosis or apoptosis) \([40]\).

PS exposition is recognised to be part of the signalling cascade in the course of apoptosis \([41]\). It attracts phagocytes which remove the dying cell before its remnants evoke an inflammatory response \([42]\). The importance of PS exposition in developing tissue damage after IR injury was clearly demonstrated in several experiments where IR-injury could be reduced by

### Table 1. Baseline characteristics of all participants \((n=30)\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± S.D.</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>23.3 ± 6.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.4 ± 2.4</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>126 ± 8</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>71 ± 10</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.6 ± 0.7</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.19 ± 0.94</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.33 ± 0.26</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.19 ± 0.65</td>
</tr>
</tbody>
</table>

Fig.2. Annexine A5 targeting 1 and 4 hours after reperfusion, after placebo (open bars) and atorvastatin (closed bars) treatment. Bars represent mean ± SE.
Atorvastatin and Reperfusion Injury
d
ment duration could account for the difference in annexin A5 targeting in these experiments. We have previously shown in humans that activation of ecto-5'-nucleotidase mediates prevention of PS exposure by rosuvastatin after IR. The effect of statins on ecto-5'-nucleotidase appears to occur rapidly6, 7). For example Sanada et al. found a significant increase of ecto-5'-nucleotidase activity in dogs for different statins (pravastatin, pitavastatin and cerivastatin) already 10 minutes after treatment7). Thus, our discrepant results between rosuvastatin and atorvastatin are not likely explained by differences in treatment duration but rather reflect a difference in pharmacodynamics between these two statins. There are significant differences between several statins in their efficacy to lower LDL cholesterol48). Moreover, pleiotropic effects have been shown to differ between lipophilic statins (e.g. atorvastatin) and hydrophilic statins (e.g. rosuvastatin). Lipophilic statins have been reported to render endothelial and vascular smooth muscle cells more susceptible to apoptosis than hydrophilic statins49). In humans rosuvastatin inhibits Rho/Rho kinase activity to a greater extent than atorvastatin at a dose that equally reduced LDL cholesterol36). Moreover hydrophilic statins have been suggested to be superior in reducing event rate after a primary acute coronary syndrome in normcholesterolemic patients50).

**Conclusion**

Our results show that treatment with atorvastatin 80 mg does not reduce forearm PS exposition after ischaemic exercise. This suggests that the role of PS exposure in the prevention of ischemia and reperfusion injury by short term treatment with atorvastatin is limited.

**Acknowledgements**

C.W.W. is an MD-medical research trainee funded by The Netherlands Organization for Health Research and Development (920-03-353). G.A.R. is a Clinical Established Investigator of the Netherlands Heart Foundation (2006 T035) and received an unrestricted research grant from Pfizer. For the remaining authors none were declared. Theseus Imaging Corporation, Boston, MA, USA provided GMP-graded annexin A5 for free.

**References**

1) Lewis SJ, Moye LA, Sacks FM, Johnstone DE, Timmis G, Mitchell J, Limacher M, Kell S, Glasser SP, Grant J, Davis

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**Fig. 3.** Annexine A5 targeting 1 and 4 hours after reperfusion in time-control group (placebo treatment twice), after visit 1 (open bars) and visit 2 (closed bars). Bars represent mean ± SE.

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The (cardio)protective mechanism of statins is well investigated, though its mechanism has not been fully elucidated40). Adenosine appears to play an important role in initiating the cascade (adenosine dependent phosphorylation of ERK1/2, Akt and eNOS) leading to a protective phenotype after statin therapy45). Upregulation of ecto-5'-nucleotidase has been shown in vitro and in animals in-vivo for various statins46). In humans in-vivo however, this effect has only been explored for rosuvastatin, which increases ecto-5'-nucleotidase activity with 50%47).

In our rosuvastatin trial participants were treated 7 days31), whereas in this atorvastatin trial participants were treated for 3 days. However we doubt that treatment duration could account for the difference in annexin A5 targeting in these experiments. We have previously shown in humans that activation of ecto-5'-nucleotidase mediates prevention of PS exposure by rosuvastatin after IR. The effect of statins on ecto-5'-nucleotidase appears to occur rapidly6, 7). For example Sanada et al. found a significant increase of ecto-5'-nucleotidase activity in dogs for different statins (pravastatin, pitavastatin and cerivastatin) already 10 minutes after treatment7). Thus, our discrepant results between rosuvastatin and atorvastatin are not likely explained by differences in treatment duration but rather reflect a difference in pharmacodynamics between these two statins. There are significant differences between several statins in their efficacy to lower LDL cholesterol48). Moreover, pleiotropic effects have been shown to differ between lipophilic statins (e.g. atorvastatin) and hydrophilic statins (e.g. rosuvastatin). Lipophilic statins have been reported to render endothelial and vascular smooth muscle cells more susceptible to apoptosis than hydrophilic statins49). In humans rosuvastatin inhibits Rho/Rho kinase activity to a greater extent than atorvastatin at a dose that equally reduced LDL cholesterol36). Moreover hydrophilic statins have been suggested to be superior in reducing event rate after a primary acute coronary syndrome in normcholesterolemic patients50).

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**References**

1) Lewis SJ, Moye LA, Sacks FM, Johnstone DE, Timmis G, Mitchell J, Limacher M, Kell S, Glasser SP, Grant J, Davis


13) Bell RM, Yellon DM: Atorvastatin, administered at the onset of reperfusion, and independent of lipid lowering, protects the myocardium by up-regulating a pro-survival pathway. J Am Coll Cardiol, 2003; 41: 508-515


mice. Gastroenterology, 2007; 133: 632-646
26) Weyer KE, Wagener FADTG, Friedlink C, Boerman OC, Scheffer GJ, Allison A, Masereeuw R, Rongen GA: Dia-


pone.0024276
27) Hale SL, Allison AC, Kloner AR: Diannexin reduces no-

reflow after reperfusion in rabbits with large ischemic myocardial risk zones. Cardiovasc Ther, 2011; 29: e42-

52. doi: 10.1111/j.1755-5922.2010
28) Riksen NP, Zhou Z, Oyen WJ, Ramakers BP, Rongen GA: Caffeine prevents protection in two human

models of ischemic preconditioning. J Am Coll Cardiol, 2006; 48: 700-707

30) Pasceri V, Patti G, Nusca A, Pristipino C, Richichi G, Di SG: Randomized trial of atorvastatin for reduction of myocardial damage during coronary intervention: results from the ARMYDA (Atorvastatin for Reduction of Myocar-

33) Naoumova RP, Dunn S, Rallidis L, bu-Muhana O, Neuwirth C, Rendell NB, Taylor GW, Thompson GR: Pro-

longed inhibition of cholesterol synthesis explains the effi-
cacy of atorvastatin. J Lipid Res, 1997; 38: 1496-1500

vastatin (10 mg) versus atorvastatin (40 mg) on rho kinase activity in caucasian men with a previous atherosclerotic event. Am J Cardiol, 2009; 103: 437-441
41) Shen XD, Ke B, Zhai Y, Tsuchihashi SI, Gao F, Duarte S, Coito A, Busuttil RW, Allison AC, Kupiec-Weglinski JW: Dia-
nexitin, a novel annexin α homodimer, protects rat liver transplants against cold ischemia-reperfusion injury. Am J Transplant, 2007; 7: 2463-2471
42) Ludman A, Venugopal V, Yellon DM, Hausenloy DJ: Statins and cardioprotection--more than just lipid lower-
ing? Pharmacol Ther, 2009; 122: 30-43
44) Osman L, Amrani M, Ilsley C, Yacoub MH, Smolenski RT: Atorvastatin accelerates extracellular nucleotide de-
45) Meijer P, Wouters CW, van den Broek PH, de RM, Scheffer GJ, Smits P, Rongen GA: Uptregulation of ecto-5'-nu-

pone.0024276
46) Zitoimolas K, Chatzizisis Y, Elisaf M, Hatziotis AI: Effect of HMG-CoA reductase inhibitors on vas-

cular cell apoptosis: beneficial or detrimental? Atheroscle-
rrosis, 2010; 56: 722-727
47) Jones PH, Davidson MH, Stein EA, McKenney JM, Miller E, Cain VA, Blasetto JW: Comparison of the efficacy and safety of rosuvastatin versus atorvastatin, sim-
vastatin, and pravastatin across doses (STELLAR® Trial). Am J Cardiol, 2003; 92: 152-160
48) Katsiki N, Tziomalos K, Chatzizisis Y, Elisaf M, Hatziotis AI: Effect of HMG-CoA reductase inhibitors on vascu-

lar cell apoptosis: beneficial or detrimental? Atheroscler-
orosis, 2010; 211: 9-14
49) Sakamoto T, Kojima S, Ogawa H, Shimomura H, Kimura K, Ogata Y, Sakaino N, Kitagawa A: Usefulness of hydro-
philic vs lipophilic statins after acute myocardial infarc-