Impact of a High Loading Dose of Atorvastatin on Contrast-Induced Acute Kidney Injury

Running title: Quintavalle et al.; Statin and contrast-induced acute kidney injury

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Abstract:

**Background**—The role of statins in the prevention of contrast induced acute kidney injury (CIAKI) is controversial.

**Methods and Results**—We investigated 1) the *in vivo* effects of atorvastatin on CIAKI. Patients with chronic kidney disease (CKD) enrolled into the NAPLES II trial were randomly assigned to a) *Atorvastatin group* (80 mg within 24 hours before contrast media (CM) exposure; n = 202), or b) *Control group* (n = 208). All patients received high dose of N-acetylcysteine and sodium bicarbonate solution. 2) the *in vitro* effects of atorvastatin pre-treatment on CM-mediated modifications of intracellular pathways leading to apoptosis or survival in renal tubular cells. CIAKI (i.e. an increase >10% of serum cystatin C [sCyC] concentration within 24 hours after CM exposure) occurred in 9/202 patients in the *Atorvastatin group* (4.5%) and in 37/208 patients in the *Control group* (18.4%) (p = 0.005; OR = 0.22; 95% CI – 0.07-0.69). CIAKI rate was lower in the *Atorvastatin group* both in diabetics and non-diabetics and in patients with moderate CKD (estimated glomerular filtration rate [eGFR] 31 to 60 ml/min/1.73 m²). In the *in vitro* model, pre-treatment with atorvastatin 1) prevents CM-induced renal cell apoptosis by reducing stress kinases activation, and 2) restored the survival signals (mediates by Akt and Erks pathways).

**Conclusions**—A single high loading dose of atorvastatin administered within 24 hours before CM exposure is effective in reducing the rate of CIAKI. This beneficial effect is observed only in patients at low- to medium risk.

**Key words:** apoptosis; contrast media; kidney; prevention; statins
Introduction

Iodinated contrast media (CM) are used in both diagnostic and interventional cardiovascular procedures. Besides the risk for allergic reactions, the major concern for CM use is a deterioration of kidney function, named contrast-induced acute kidney injury (CIAKI). The reported incidence of CIAKI varies widely (<1% to >50%), depending on the patient population, the baseline risk factors, and definition. Hemodynamic changes of renal blood flow, which lead to hypoxia of the renal medulla, and direct toxic effects of CM on renal cells are thought to contribute to the pathogenesis of CIAKI. We have previously observed both in vitro and in vivo that CM induce apoptotic cell death via 3 important signaling pathways, a) reactive oxygen species (ROS) pathway, b) JNK/p38 pathway, and c) intrinsic apoptosis pathway, which are triggered by CM in this sequence. The causal relationship between these 3 sequential pathways supports the investigations on novel therapeutic approaches to prevent CIAKI.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) exerts several effects through their nonlipid-related mechanisms. These so-called “pleiotropic” effects encompass several mechanisms that modify inflammation responses, endothelial function, plaque stability and thrombus formation, and apoptotic pathway. The effectiveness of statins pre-treatment in reducing the incidence of CIAKI has been examined in some observational and randomized studies. Due to the controversial results, there is a general consensus that statins merit further study for the prevention of CIAKI. In the present study we investigated 1) the in vivo effects of atorvastatin pre-treatment on CIAKI; and 2) the in vitro effects of atorvastatin pre-treatment on CM-mediated modifications of intracellular pathways leading to apoptosis or survival in renal tubular cells.
Methods

Patient population

The patients included into the present study represents the subgroup with chronic kidney disease (CKD) enrolled into the NAPLES II trial (Figure 1). The design of the NAPLES II trial has been previously reported. From January 2005 to December 2008, 1348 naïve (i.e., not taking statin) patients scheduled for elective coronary angiography or percutaneous coronary intervention (PCI) in de novo lesions in native coronary arteries were considered eligible for the study. The day before the procedure all eligible patients were randomly assigned to atorvastatin treatment (Atorvastatin group) or to no atorvastatin treatment (Control group). Randomization was performed by a 1:1 ratio using a computer-generated random numbers. Patients randomized in the Atorvastatin group started atorvastatin treatment (80 mg) within 24 hours before CM exposure. The prophylaxis for CIAKI in all patients with CKD included a) N-acetylcysteine (NAC; Flumucil, Zambon Group SpA, Milan, Italy; 1200 mg PO twice daily, the day before and the day of administration of the CM), and b) hydration with sodium bicarbonate solution (154 mEq/l in dextrose and H2O): it was administered with the initial intravenous bolus of 3 ml/kg per hour for 1 hour immediately before CM injection, followed by 1 ml/kg per hour during contrast exposure and for 6 hours after the procedure. Iodixanol (Visipaque®, GE, a non-ionic, iso-osmolar (290 mOsm/kg of water) contrast agent was used in all patients. CKD was defined as an estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m². The risk score for predicting CIAKI was calculated according to the following algorithm: hypotension (integer score 5), intra-aortic balloon pump support (integer score 5), congestive heart failure (integer score 4), age >75 years (integer score 4), diabetes mellitus (integer score 3), eGFR <60 (integer score 2 to 6), pre-existing anemia (integer score 3), and CM volume (integer score 1 for each 100 cc). The global
scores ≤5, 6-10, 11-16 and ≥16 anticipate a CIAKI risk of, respectively, 7.5%, 14%, 26.1% and 57.3% 17. Serum creatinine (sCr), cystatin C (sCyC), blood urea nitrogen, sodium and potassium were measured the day before the procedure and at 24, 48 hours and 1 week after CM administration. Additional measurements were performed in all instances where there was a deterioration of baseline renal function. The primary outcome measure was the development of CIAKI, defined as an increase in sCyC concentration 10% above the baseline value at 24 hours after administration of CM 18. Secondary outcome measures were: 1) an increase of sCr concentration ≥0.5 mg/dL at 48 hours from baseline value; and 2) an increase of sCr concentration ≥25% at 48 hours from baseline value. In order to address whether a single high (80 mg) dose of atorvastatin may affect the sCr or sCyC levels, we analyzed additional 20 patients with CKD not scheduled for CM exposure and not enrolled in the original NAPLES II trial. The clinical characteristics of the 20 enrolled patients are summarized in the Supplementary Table 1S. The trial was conducted in 2 interventional cardiology centers in Italy, and was approved by our Ethic Committees.

Culture conditions and reagents

Two cell lines were utilized: Madin Darby distal non-human tubular epithelial cells (MDCK), and human embryonic proximal tubules cells (HK2). MDCK cells were grown in a 5% CO₂ atmosphere in DMEM containing 10% heat-inactivated FBS, 2mM L-glutamine and 100U/ml penicillin-streptomycin. HK2 cell lines were grown in DMEM-F12 mixture with 10% heat-inactivated FBS, 2mM L-glutamine and 100U/ml penicillin-streptomycin. Cells were routinely passaged when 80-85% confluent. Media, sera and antibiotics for cell culture were from Sigma Aldrich (Milan, Italy). Protein electrophoresis reagents were from Bio-Rad (Richmond, VA, USA) and Western blotting and ECL reagents (GE Healthcare, Milan, Italy). The following
antibodies were used for immunoblotting: anti-beta Actin (Sigma Aldrich, Milan, Italy), anti-Phospho-JNK (P-JNK), anti-Caspase-3, anti-Phospho-Serine\(^{15}\) p53 (P-p53), anti-p53, anti-Phospho-Akt (p-Akt), anti-Akt, anti Phospho-ERK (p-ERK), anti-ERK, anti HSP70 (Cell Signaling Danvers, MA,USA) and anti-JNK (DB Bioscience, Milan Italy).

Atorvastatin was kindly donated by Pfizer (Pfizer Inc. New York, USA), while NAC was donated by Zambon (Zambon Group SpA, Milan, Italy). MDCK and HK-2 cells were pre-treated with atorvastatin, at the dose of 0.2 \(\mu\)M \(^{19}\) and or 100mMol of NAC \(^{4}\). The dose of atorvastatin was selected according to the standard doses used in cell lines. Iodixanol was used in all experiments.

**Caspase Assay**

The assay was performed using the Colorimetric CaspACETM Assay System, (Promega, Madison, Wisconsin, USA) as reported by instruction manual. Briefly, MDCK cells were pre-treated with 0.2 \(\mu\)M atorvastatin for 12 hours and then treated for 3 hours with iodixanol. Cells were harvest in caspase assay buffer and proteins were quantified by Bradford Assay. 50 \(\mu\)g of protein were used.

**Protein isolation and western blotting**

Cellular pellets were washed twice with cold PBS and resuspended in JS buffer (Hepes 50 mM, NaCl 150 nM, 1% Glicerol, 1% Triton X100, 1.5 mM MgCl\(_2\), 5 mM EGTA) containing Proteinase Inhibitor Cocktail (Roche, Basel, CH). Solubilized proteins were incubated for 1 h on ice. After centrifugation at 13,200 rpm for 10 min at 4\(^{\circ}\)C, lysates were collected as supernatants. 80 \(\mu\)g of sample extract were resolved on a 12% SDS-poliacrylamide gel using a mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA) and transferred to Hybond-C extra nitrocellulose (GE Healthcare, Milan, Italy). Membrane was blocked for 1 h with 5% non fat dry
milk in TBS containing 0.05% Tween-20 and incubated over night at 4°C with specific antibodies. For immunoblotting were used the indicated antibody. Washed filters were then incubated for 45 min with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (GE Healthcare, Milan, Italy) and visualized using chemiluminescence detection (GE Healthcare Milan Italy).

**Cell death quantification**

Cell vitality was evaluated with the CellTiter 96® AQuesus One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer’s protocol. The assay is based on reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to a colored product that is measured spectrophotometrically. Cells were plated in 96-well plates in triplicate, stimulated and incubated at 37°C in a 5% CO2 incubator. Iodixanol and atorvastatin were used in vitro at doses and time indicated. Metabolically active cells were detected by adding 20 μl of MTS to each well. After 30 min of incubation, the plates were analysed on a Multilabel Counter (Bio-Rad, Richmond, VA, USA).

Apoptosis was also analyzed via propidium iodide incorporation in permeabilized cells by flow cytometry. The cells (2 × 10⁵) were washed in PBS and resuspended in 200 μl of a solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide (Sigma Aldrich, Milan, Italy). Following incubation at 4°C for 30 min in the dark, nuclei were analyzed with a Becton Dickinson FACSScan flow cytometer. Cellular debris was excluded from analyses by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. The percentage of elements in the hypodiploid region was calculated.

**Biological Material**

Exfoliated cell pellets from the urine of 10 enrolled and randomly selected patients (5 in each
group) were collected by centrifugation at 1200 rpm for 25 min. A fraction of urine samples was sent to the pathologist for cytologic analysis and a fraction to the laboratory for in vitro assay. All samples were stored at -80°C for a maximum of 2 months. Urine samples were resuspended in ice-cold TRAP (Tris-HCl ph 7.5 10 mM, MgCl2 1 mM, EGTA 1mM, phenyl methylsulfonyl 0.1 mM, β-mercaptoethanol 5 mM, CHAPS 0.5% and glycerol 10%) and incubated on ice for 1 hr. The lysate was centrifuged for 20 minutes at 13,200 rpm at 4°C. The supernatant was collected. The presence of tubular cells was assessed by using morphological criteria on cytospin preparations stained by standard Papanicolaou staining method. To this end cell block preparations were employed. To ensure their adequacy cell blocks were Hematoxylin and Eosin (H&E) stained. Caspase 3 expression was detected by the rabbit polyclonal antibody (Cell Signaling 9661, Danvers MA). Signal was developed by the polyvalent LSAB-peroxidase Dako Kit (Dako, Denmark,Europe).

Statistical analysis
The sample size was selected to demonstrate a reduction in the primary end-point of CIAKI from 20% in the Control group to 10% in the Atorvastatin group. Using a two-sided Chi-square test with a significance level of 0.05, a total of at least 400 randomized patients (200 in each arm) provided the study 80% power. This is a pre-specified secondary endpoint of the NAPLES II trial.

Continuous variables are given as mean ± 1 standard deviation or median and first and third quartiles, when appropriate. The Student’s t test and the nonparametric Mann-Whitney tests were used to determine differences between values for normally and, respectively, not normally distributed variables. Categorical variables were reported as percentage and were analyzed by either Chi-squared or Fisher’s exact test, as appropriate. Multiplicity issues were addressed using
the Bonferroni adjustment. To test the impact of prophylactic regimen (as defined by the 2
groups of treatment) on changes in sCyC concentration, we used a linear mixed model taking
into account the clustered features of the data, after transforming sCyC levels into a natural
logarithm (to overcome the problem of non-normal distribution). Specifically, we considered the
treatment strategy (as defined in the Control group and Atorvastatin group), time period and
time x treatment strategy interaction as fixed effects and patients as a random effect. Probability
level <0.05 was considered significant throughout the analysis. Data were analyzed with SPSS
13.0 (Chicago, Illinois) for Windows. The authors had full access to the data and take
responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Clinical results

The clinical and biochemical characteristics were well matched between the two groups (Tables
1 and 2). sCyC increased significantly more in the Control group than in the Atorvastatin group
(p=0.005; F = 5.52 by RM-ANOVA; Fig. 2, panel A). CIAKI occurred in 9/202 patients in the
Atorvastatin group (4.5%) and in 37/208 patients in the Control group (18.4%) (p = 0.005; OR =
0.22; 95% CI = 0.07-0.69; Fig. 2, panel B). sCr increased significantly more in the Control
group than in the Atorvastatin group (p=0.018; F = 4.97 by repeated measure of variance
ANOVA model). An increase of sCr concentration ≥0.5 mg/dL at 48 hours from baseline value
occurred in 7/202 (3.5%) patients in the Atorvastatin group and in 16/208 patients (7.7%) in the
Control group (p = 0.085). An increase of sCr concentration ≥25% at 48 hours from baseline
value occurred in 6/202 (3%) patients in the Atorvastatin group and in 14/208 patients (7%) in
the Control group (p = 0.10) (Supplementary Fig. 1). We also performed a stratified analysis to
determine the benefit of atorvastatin according to the severity of CKD (eGFR ≤30 versus 31-60) and the presence of diabetes mellitus (Fig. 2, panel C). The rate of CIAKI was lower in the Atorvastatin group both in diabetics and non-diabetics and in patients with eGFR 31 to 60. On the contrary, no difference was observed in the subgroup with severe CKD (eGFR ≤30). We observed that neither sCr nor sCyC was altered by administration of a single high (80 mg) atorvastatin loading dose (Supplementary Fig. 2).

One-year outcome was available in 402/410 (98%) patients. Clinical and biochemical characteristics of the patients are reported in Supplementary Table 2S and 3S. Major adverse events (MAE, including death and dialysis) occurred in 37/402 (9%). In particular, death occurred in 29 (7%) patients, and chronic dialysis in 8 (2%) patients. MAE occurred in 9/45 (20%) patients with CIAKI (that is, a CyC ≥10% at 24 hours after contrast exposure) and in 28/357 (7.8%) patients without CIAKI (p = 0.013).

Atorvastatin effects on CM induced renal cell damage
In both MDCK and HK2 cells exposed to CM, pre-treatment with atorvastatin induced an increase in cell vitality and a reduction of cell death (Fig. 3). This protective effect was evident only after 6 hours and reached the peak upon 12 hours of atorvastatin pre-treatment. Interestingly, we observed that pre-treatment with atorvastatin reduced the CM-induced activation of caspase 3, Jun N-terminal Kinases (JNK) and p53 (Figs. 4-5)

We then evaluated the effects of atorvastatin pre-treatment on survival signals mediated by Akt and Erks pathways. CM induced a strong reduction of the phosphorylated (activated) forms of Akt and Erks (Fig. 5, panel C-D). Interestingly, atorvastatin almost completely restored the survival signal in kidney cells. We performed a further experiment to investigate the effect of 2 hours of NAC pre-treatment (100 mM) in the presence of atorvastatin (0.2 μM) on cell death.
after 3-hour incubation with iodixanol (200 mg I/ml). The beneficial effect of the combination of NAC and atorvastatin was higher than those obtained with the NAC or atorvastatin alone (p = 0.010; F = 10.5 by ANCOVA test; Fig. 3 panel C and D, Fig. 4 panel B). Finally, we did not observed any involvement of the JAK/STAT pathway in the atorvastatin renal cell protection. Indeed, no differences of HSP70 protein (one of the signalling molecule of this pathway) expression have been detected in cells treated with atorvastatin (Fig. 5, panel B).

In order to clarify the clinical impact of these features, we evaluated the activation of JNK, p53 and caspase 3 in epithelial tubular cells collected at 24 and 48 hours after the CM exposure from 10 patients (5 in the Atorvastatin group and 5 in the Control group). The presence of epithelial tubular cells was confirmed by immunohistochemistry by ematoxilin and cosin staining (Fig. 6). In controls, an increase of both JNK and p53 phosphorylation (activation) was observed at 24 and 48 hours after CM-exposure (Fig. 6). In contrast, in all the 5 atorvastatin-treated patients we observed a significant reduction of JNK and p53 phosphorylation (Fig. 6). Cells collected from patients were also analyzed immunoistochemically with anti-caspase 3 antibodies. Consistently with western blot data, the activation of pro-caspase 3 at 24 and 48 hours was observed only in the Control group (but not in the Atorvastatin group (Fig. 7).

Discussion

Clinical findings

The present study demonstrates that a single high (80 mg) loading dose of atorvastatin administered within 24 hours before CM exposure is effective in reducing the rate of CIAKI. This beneficial effect was observed in patients with and without diabetes mellitus as well as in those with moderate CKD (eGFR 31-60 ml/min/1.73 m2). On the contrary, no advantage was
evident in patients with severe (eGFR ≤30) CKD.

At present, the evidence of the use of statins to prevent CIAKI is conflicting and inconclusive. A number of considerations may concur to the conflicting results.

1) The sample size is often modest to detect significant difference in CIAKI rate. Often, this may be due to the enrollment of patients at low-risk for CIAKI. The lack of observed benefit may therefore represent a type II error, that is, concluding that a benefit does not exist when one really does. Toso et al., for example, did not observed any significant effect of atorvastatin loading dose on CIAKI rate. However, the 304 patients enrolled in that study were insufficient to detect the expected 50% relative decrease in CIAKI rate in the *Atorvastatin group* (from 15% in the placebo group) with 90% power at the conventional, 2-sided significance level of 5%. Indeed, more than 350 in each group would have been required to test the hypothesis. Moreover, our study is underpowered to demonstrated a impact of atorvastatin loading dose in preventing CIAKI using the current sCr cut-offs. Indeed, having an absolute sCr increase ≥0.5 mg/dl as primary endpoint, in order to demonstrate a reduction from 10% to 5%, approximately 1000 patients (450 in each arm) would be required. Also, when selecting the ≥25% sCr increase as the primary endpoint, in order to demonstrate a reduction of CIAKI from 5% to 2% approximately 1200 patients (600 in each arm) would be required. In the present study we used sCyC as marker of kidney function to detect CIAKI. This for several reasons: a) sCyC is more sensitive than sCr to rapidly detect acute changes in renal function; b) sCyC allows an early (24 h) diagnosis of CIAKI and c) sCyC predicts the occurrence of major adverse events at follow-up in patients with CKD undergoing CM exposure.

2) Primary endpoint definition. In the negative PROMISS trial, the Authors hypothesized an absolute sCr difference between baseline and 48 hours of 0.36 mg/dL in the
simvastatin group and 1.1 mg/dL in the control group. This means that, with a baseline sCr level of 1.2 mg/dL, the Authors expected a peak increase in the sCr concentration of 28% for simvastatin group and 92% percent for control group. Although such a large effect size has been observed in single center CIAKI trial, multicenter trials generally produce much smaller effect, with an absolute sCr difference between baseline and 48 hours ≤0.20 mg/dL and a peak increase in the sCr concentration of 20-30%.

Also, the absolute sCr difference is generally not a good primary outcome as it has not been validated to predict adverse outcomes (like CIAKI).

3) Type and dose of statin administered. Although results of retrospective studies (which included patients receiving a multitude of statins) may suggest the presence of a class effect, the majority of prospective randomized trials demonstrating prevention of CIAKI in patients undergoing CM exposure used short-term pretreatment with high doses of atorvastatin. Some evidence also exists on the better prophylactic effect of high versus low statin dose. Thus, when a strategy of short-term pretreatment with statins before CM exposure is adopted, it would be appropriate to use potent statins at high doses.

Mechanisms of prevention of CM-induced renal cells damage by atorvastatin

The cornerstone of the prophylaxis of CIAKI is hydration; however, strategies preventing CM-induced renal cell apoptosis seem to have a clinical role. Previous studies have demonstrated that CM induce an increase in ROS production. This leads to eventual activation of the stress kinases JNK1/2 and p38. For this reason, clinical trials have been performed using various antioxidant compounds with the aim of lowering the occurrence of CIAKI by scavenging ROS. The present study shows the additive protective effect of atorvastatin over the combined administration of sodium bicarbonate solution and NAC: this supports the hypothesis that the combination of different antioxidant compounds seems to be more effective than a single agent.
in preventing CIAKI. The Acetylcysteine for Prevention of Renal Outcomes in Patients Undergoing Coronary and Peripheral Vascular Angiography (ACT) trial showed no advantages in routine NAC use. However several aspects need to be addressed before reaching the strong conclusion that NAC should be abandoned, including: baseline chronic kidney disease severity, consistency of hydration protocol and impact of CM selection. A recent meta-analysis of 30 trials, indeed, showed a renoprotective benefit with NAC. NAC pre-treatment inhibits CM-induced ROS production and therefore inhibits JNK and p38 activation as well as apoptosis, suggesting the existence of a specific target for NAC upstream to the apoptosis-executing stress kinases in the CM-activated signaling pathway. Atorvastatin may work at different level in preventing activation of the intrinsic apoptotic pathway. Statins reduces the intracellular ROS levels in many cellular systems by acting on the inhibition of ROS-producing enzymes. In our in vitro model, pre-treatment with high dose of atorvastatin reduced contrast-induced JNK activation, which therefore leads to intrinsic apoptosis pathway activation. On the contrary, activation of JAK2/STAT5 pathway does not seems to have a role in atorvastatin protective effect on contrast-induced renal cell damage: indeed, unlike to asialoeritropoietin, atorvastatin does not induce an increase in the HSP70 cellular levels. Atorvastatin induces an increase in the survival signals and a reduction of the death signals mediated by CM treatment of kidney cells. This effect was time dependent, reaching a maximum effect upon 12 hours of statins incubation. We also, for the first time, confirmed in vivo these mechanisms. In vitro studies addressing the pathophysiology of CM-induced apoptosis have been usually criticized due to several limitations, including 1) the assessment of only one potential mechanism of the CM-induced renal cell damage, in the absence of confounding variables which can be found in vivo (eg. hypoxia due to hemodynamic changes or other systemic mechanisms), 2) the exposure to a
constant concentration of CM to all cells line, whereas in vivo the more distal epithelial tubular
cells are exposed to much higher concentration, 3) the potentially high dose of CM, and 4) differences in the tested drug/compound metabolism and transport across cell membrane.

Cultured cells are attached with their basolateral membrane to the culture dish. This may preclude the access of atorvastatin to the cells through the active mechanism, since the organic amino-transporting polypeptide is mainly present on the basolateral side of the epithelial renal cells. On note, however, the cytochrome 3A4, which metabolizes atorvastatin into active metabolites, is consistently expressed in proximal tubular epithelial cells. Moreover, several studies indicate that the parent drug is equipotent to the active metabolites in vitro.

**Study Limitations**

The present study is a pre-specified secondary endpoint of the NAPLES II trial, which was designed to assess whether a single, high (80 mg), loading (within 24 hours) dose of atorvastatin is effective in preventing elevation of biomarkers of myocardial infarction following elective coronary stent implantation. The lack of randomization of the patients with CKD may represent a limitation. However, the most important characteristics of the 2 groups were well balanced without significant differences. The present study was powered having sCyC as marker of kidney damage. The current gold standard for kidney function is still sCr. Having the sCr cutoffs as primary end-points would have requested a much larger (>1000 patients) sample size to detect the beneficial prophylactic effect of atorvastatin. Moreover, serum CyC is a reliable marker for both an early (24 h) diagnosis of CIAKI, and for predicting the occurrence of major adverse events (MAE) at follow-up in patients with CKD undergoing CM exposure. Finally, the 4% loss to follow-up rate should be taken into account when interpreting our results. Indeed, although the 17 patients lost at follow-up were largely similar to those analyzed (Supplementary
data), every patient lost to follow-up can be considered as a potential threat to robust and precise inference.

Conclusions

A single high loading dose of atorvastatin administered within 24 hours before CM exposure is effective in reducing the rate of CIAKI, by preventing CM-induced epithelial tubular renal cells apoptosis and increasing the survival signaling pathways. The advantage of adding atorvastatin loading dose to sodium bicarbonate solution and NAC seems to be effective only in patients at low-to-medium risk, but not in those at high risk.

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Conflict of Interest Disclosures: None.

References:


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Table 1. Clinical characteristics of the patients enrolled into the 2 groups

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<th>Control group (n= 208)</th>
<th>Atorvastatin group (n= 202)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>70 ± 8</td>
<td>70 ± 6</td>
</tr>
<tr>
<td>Male</td>
<td>120 (58%)</td>
<td>103 (51%)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75±15</td>
<td>76±13</td>
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<tr>
<td>Height (m)</td>
<td>1.67±0.5</td>
<td>1.65±0.5</td>
</tr>
<tr>
<td>Body-mass index (kg/m²)</td>
<td>28±5</td>
<td>28±4</td>
</tr>
<tr>
<td>Blood Pressure (mm Hg)</td>
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<td></td>
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<tr>
<td>Systolic</td>
<td>150±22</td>
<td>151±23</td>
</tr>
<tr>
<td>Diastolic</td>
<td>76±10</td>
<td>77±13</td>
</tr>
<tr>
<td>Mean</td>
<td>101±13</td>
<td>102±15</td>
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<tr>
<td>LV ejection fraction (%)</td>
<td>50±8</td>
<td>50±9</td>
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<tr>
<td>Systemic Hypertension</td>
<td>182 (87.5%)</td>
<td>172 (85.5%)</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>80 (38.5)</td>
<td>89 (44%)</td>
</tr>
<tr>
<td>Drugs:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>83 (40%)</td>
<td>76 (38%)</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>64 (31%)</td>
<td>64 (32%)</td>
</tr>
<tr>
<td>Angiotensin II receptor inhibitor</td>
<td>64 (31%)</td>
<td>66 (33%)</td>
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<tr>
<td>Diuretics</td>
<td>100 (48%)</td>
<td>111 (55%)</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>137(66%)</td>
<td>131 (65%)</td>
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<tr>
<td>Procedure performed</td>
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<tr>
<td>Coronary angiography</td>
<td>68 (23%)</td>
<td>78 (27%)</td>
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<tr>
<td>PCI</td>
<td>140 (77%)</td>
<td>124 (73%)</td>
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<tr>
<td>Volume of contrast media (ml)</td>
<td>184±78</td>
<td>177±74</td>
</tr>
<tr>
<td>Contrast ratio &gt;1</td>
<td>55 (26%)</td>
<td>51 (25%)</td>
</tr>
</tbody>
</table>

LV = left ventricular; ACE = angiotensin converting enzyme; PCI = percutaneous coronary intervention. Continuous values are expressed as mean ± standard deviation; categorical values are expressed as a total number and as a percentage of the global population (in parenthesis).
**Table 2.** Clinical characteristics of the patients enrolled in the 2 groups

<table>
<thead>
<tr>
<th></th>
<th>Control group (n= 208)</th>
<th>Atorvastatin group (n= 202)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum creatinine, median (range), mg/dl</strong></td>
<td>1.29 (0.88-1.61)</td>
<td>1.32 (0.96-1.62)</td>
</tr>
<tr>
<td><strong>Serum cystatin C, median (range), mg/dl</strong></td>
<td>1.25 (1.0-1.59)</td>
<td>1.23 (1.06-1.62)</td>
</tr>
<tr>
<td><strong>eGFR (ml/min/1.73 m²)</strong></td>
<td>43 ± 14</td>
<td>42 ± 13</td>
</tr>
<tr>
<td>≤30</td>
<td>38 (18.5%)</td>
<td>37 (18.5%)</td>
</tr>
<tr>
<td><strong>Contrast nephropathy risk score</strong>*</td>
<td>7.5 ± 2.7</td>
<td>8.1 ± 2.8</td>
</tr>
<tr>
<td><strong>Serum Urea Nitrogen (mg/dl)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>78 ± 31</td>
<td>80 ± 35</td>
</tr>
<tr>
<td>After 48 hours</td>
<td>70 ± 30</td>
<td>76 ± 35</td>
</tr>
<tr>
<td><strong>Serum Sodium (mEq/l)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>140 ± 5</td>
<td>140 ± 3</td>
</tr>
<tr>
<td>After 48 hours</td>
<td>140 ± 5</td>
<td>141 ± 4</td>
</tr>
<tr>
<td><strong>Serum Potassium (mEq/l)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.7 ± 0.7</td>
<td>4.6 ± 0.7</td>
</tr>
<tr>
<td>After 24 hours</td>
<td>4.5 ± 0.7</td>
<td>4.6 ± 0.7</td>
</tr>
</tbody>
</table>

*GFR = estimated glomerular filtration rate. *According to Mehran et al. *7. Continuous values are expressed as median and first and third quartiles (serum creatinine and cystatin C) or mean ± standard deviation; categorical values are expressed as a total number and as a percentage of the global population (in parenthesis).

**Figure Legends:**

*Figure 1.* Diagram showing the flow of participants through each stage of the trial according to the CONSORT guidelines.

*Figure 2. Panel A:* serum cystatin C concentration at baseline, 24 and 48 hours after contrast media administration in the **Control group** (*open symbol, continuous line*) and in the **Atorvastatin group** (*closed symbol, dashed line*); p=0.005; F = 5.32 by linear mixed model. **Panel B:** incidence of contrast-induced-acute kidney injury (CIAKI) in the **Control group** and in the **Atorvastatin group.** **Panel C:** benefit of atorvastatin according to the severity of CKD (eGFR ≤30 versus 31-60) and the presence of diabetes mellitus.
Figure 3. Effects of atorvastatin (Ato; 0.2 μM) pre-treatment for different times (2, 4, 6, 12, 24, 36 hours) on CM-induced MDCK and HK2 cells damage, assessed as viability (Cell titer proliferation assay) (Panel A and B), percent of apoptotic cells evaluated by FACS analysis (Panel C and D). Pre-treatment with both N-acetylcysteine (NAC; 100mMol) and atorvastatin (0.2 μM) was more effective than each single compound alone in the prevention of CM-induced apoptosis; * p<0.001 versus column [NAC - and atorvastatin -]; ‡ p = 0.03 versus columns [NAC - and atorvastatin +] and [NAC+ and atorvastatin -]. All cells were incubated for 3 hours with iodixanol (200mg iodine/mL). Each experiment was repeated three times. In Panel A and B a mixed linear model for repeated measures was used, with Bonferroni adjustment. In Panel C and D the Student’s t test was used.

Figure 4. Effect of atorvastatin (Ato; 0.2 μM) pre-treatment on CM-induced MDCK (Panel A) and HK2 (Panel B) cells damage, assessed by caspase 3 assay or by Western blot of the procaspase 3. *p <0.001 versus control; ‡ p <0.001 versus column [iodixanol + and atorvastatin -]. Pre-treatment with both N-acetylcysteine (NAC; 100mMol) and atorvastatin (0.2 μM) was more effective than each single compound alone in the prevention of CM-induced caspase-3 activation. * p<0.001 versus column [NAC - and atorvastatin -]; ‡ p = 0.03 versus columns [NAC - and atorvastatin +] and [NAC+ and atorvastatin -]. Each experiment was repeated three times. The Student’s t test was used.

Figure 5. Western blot analysis showing the effect of atorvastatin (Ato; 0.2 μM) pre-treatment on CM-activation of JNK and p53 in MDCK (Panel A) and HK-2 (Panel B) renal cells. The activation of JNK and p53 was evaluated by detection of the phosphorylated (activated) form of
the proteins (p-JNK and p-ser15-p53). Pre-treatment with both N-acetylcysteine (NAC; 100mMol) and atorvastatin (0.2 μM) was more effective than each single compound alone. No differences of HSP70 protein (one of the signalling molecule of JAK/STAT pathway) expression have been detected in cells treated with atorvastatin (Panel B) Western blot analysis showing that atorvastatin (Ato; 0.2 μM) pre-treatment induced an increase of the phosphorylated (activated) levels of both AKT and ERKs (p-AKT and p-ERK) in presence of iodixanol in MDCK (Panel C) and HK-2 (Panel D) cells.

**Figure 6.** Immunoistochemestry of kidney tubular cells. Urine cytological cell block was prepared as described in methods section. This specimen type was used to perform specific tubular cell marker immunostaining preparation (H&E staining 400x) and to asses the activation of active caspase 3 from Control group (Panel A) and Atorvastatin group (Panel B) patients at different times.

**Figure 7.** In vivo effects of contrast media on epithelial tubular renal cells. Western blot analysis assessing JNK and p53 phosphorylation (activation) levels in epithelial tubular cells from 2 patients in Control group (Panel A) and 2 patients in Atorvastatin group (Panel B). The analysis revealed that JNK and p53 activation was higher in the Control group than in the Atorvastatin group.
Patients assessed for eligibility (n= 1385)

Excluded (n= 37):
✓ 9 refused to participate
✓ 28 with history of intolerance to statins

1348 patients randomized

✓ 676 allocated to Atorvastatin group
✓ 676 received the allocated treatment

212 patients with eGFR < 60 ml/min/1.73 m²: 10 were lost at follow-up

202 patients included

✓ 672 allocated to Control group
✓ 672 received the allocated treatment

215 patients with eGFR < 60 ml/min/1.73 m²: 7 were lost at follow-up

208 patients included
A

Serum Cystatin C (median)

- Atorvastatin group
- Control group

\[ p = 0.005; \text{F} = 5.32 \]

Time (hours)

Baseline 24 48

B

CI-AKI (%)

- Control group
- Atorvastatin group

\[ \text{OR} = 0.22, 95\% \text{CI} = 0.07-0.69 \]
\[ p = 0.005 \]

37/208

4.5%

9/202

C

Subgroups

GFR ≤30 ml/min/1.73 m²
GFR 31-60 ml/min/1.73 m²
Diabetes mellitus yes
Diabetes mellitus no

Odds Ratio

(95% CI)

0.73 (0.10-4.93)
0.13 (0.03-0.59)
0.56 (0.37-0.86)
0.62 (0.44-0.88)

0.75
<0.001
0.006
0.002
Impact of a High Loading Dose of Atorvastatin on Contrast-Induced Acute Kidney Injury
Cristina Quintavalle, Danilo Fiore, Francesca De Micco, Gabriella Visconti, Amelia Focaccio, Bruno Golia, Bruno Ricciardelli, Elvira Donnarumma, Antonio Bianco, Maria Assunta Zabatta, Giancarlo Troncone, Antonio Colombo, Carlo Briguori and Gerolama Condorelli

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**SUPPLEMENTAL MATERIAL**

Effect of high loading dose of atorvastatin on serum creatinine concentrations after contrast media exposure. sCr increased significantly more in the *Control group* than in the *Atorvastatin group* (p=0.018; F = 4.97 by repeated measure of variance). An increase of sCr concentration ≥0.5 mg/dL at 48 hours from baseline value occurred in 7/202 (3.5%) patients in the *Atorvastatin group* and in 16/208 patients (7.7%) in the *Control group* (p = 0.085). An increase of sCr concentration ≥25% at 48 hours from baseline value occurred in 6/202 (3%) patients in the *Atorvastatin group* and in 14/208 patients (7%) in the *Control group* (p = 0.10) (Figure 1S).

**Figure 1S**

A

![Graph showing serum creatinine concentrations over time for Atorvastatin and Control groups](image)

B

![Bar chart showing CIAKI percentages for Atorvastatin and Control groups](image)

C

![Bar chart showing CIAKI percentages for Atorvastatin and Control groups](image)
Effect of high loading dose of atorvastatin on serum creatinine and cystatin C concentrations. We analyzed 20 patients with chronic kidney disease and assessed the effects of a single high (80 mg) atorvastatin loading dose on renal function in the absence of contrast media exposure, using two surrogate markers of GFR, serum creatinine and cystatin C. These markers were measured simultaneously before and 24 h and 48 h after atorvastatin administration. These patients were not part of the original NAPLES II trial. This amendment of the NAPLES II trial was approved by our Ethic Committee, and all the 20 patients signed the informed consent. The clinical characteristics of the 20 enrolled patients are summarized in the Table 1S. We observed that neither serum creatinine nor cystatin C was altered by administration of a single high (80 mg) atorvastatin loading dose (Figure 2S).

Table 1S. Clinical characteristics of the 20 patients.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>75±11</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>10 (50%)</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>28±6</td>
</tr>
<tr>
<td><strong>sCr, mg/dL</strong></td>
<td>1.45 (1.06-1.67)</td>
</tr>
<tr>
<td><strong>eGFR, ml/min/1.73 m²</strong></td>
<td>43±9</td>
</tr>
<tr>
<td><strong>sCyC, mg/dL</strong></td>
<td>1.48 (1.30-1.80)</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td>9 (45%)</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>15 (75%)</td>
</tr>
<tr>
<td><strong>LV ejection fraction, %</strong></td>
<td>55±7</td>
</tr>
</tbody>
</table>

**Drugs:**
- ACE inhibitor: 8 (40%)
- Calcium channel blocker: 6 (30%)
- Angiotensin II receptor inhibitor: 6 (30%)
- Diuretics: 10 (50%)
- Beta blockers: 14 (70%)

BMI = body mass index. sCr = serum creatinine; eGFR = estimated glomerular filtration rate; sCyC = serum cystatin C; LV = left ventricular. sCr and sCyC are expressed as median and Q1-Q3.
Figure 2S

A

B

* \( p > 0.05 \) by paired \( t \) test versus baseline.
Patients lost at follow-up.

Table 2S. Clinical characteristics of the patients lost at follow-up

<table>
<thead>
<tr>
<th></th>
<th>Patients included (n= 410)</th>
<th>Patients lost at follow-up (n= 17)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>70 ± 9</td>
<td>68 ± 6</td>
<td>0.53</td>
</tr>
<tr>
<td>Male</td>
<td>223 (54%)</td>
<td>10 (59%)</td>
<td>0.80</td>
</tr>
<tr>
<td>Body-mass index (kg/m²)</td>
<td>28±5</td>
<td>27±2</td>
<td>0.37</td>
</tr>
<tr>
<td>LV ejection fraction (%)</td>
<td>50±9</td>
<td>51±7</td>
<td>0.58</td>
</tr>
<tr>
<td>Systemic Hypertension</td>
<td>354 (86%)</td>
<td>15 (88%)</td>
<td>0.95</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>169 (41%)</td>
<td>7 (41%)</td>
<td>0.80</td>
</tr>
<tr>
<td>Volume of contrast media (ml)</td>
<td>180±76</td>
<td>171±49</td>
<td>0.51</td>
</tr>
<tr>
<td>Contrast ratio &gt;1</td>
<td>106 (26%)</td>
<td>4 (23%)</td>
<td>0.96</td>
</tr>
</tbody>
</table>

LV = left ventricular; Continuous values are expressed as mean ± standard deviation; categorical values are expressed as a total number and as a percentage of the global population (in parenthesis).

Table 3S. Clinical characteristics of the patients lost at follow-up

<table>
<thead>
<tr>
<th>Landmark</th>
<th>Patients included (n= 410)</th>
<th>Patients lost at follow-up (n= 17)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine, median (range), mg/dl</td>
<td>1.30 (0.88-1.62)</td>
<td>1.30 (1.20-1.45)</td>
<td>0.56</td>
</tr>
<tr>
<td>Serum cystatin C, median (range), mg/dl</td>
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<td>1.22 (1.02-1.55)</td>
<td>0.56</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>43 ± 14</td>
<td>45 ± 8</td>
<td>0.54</td>
</tr>
<tr>
<td>Contrast nephropathy risk score*</td>
<td>7.8 ± 2.7</td>
<td>8.1 ± 2.8</td>
<td>0.65</td>
</tr>
</tbody>
</table>

eGFR = estimated glomerular filtration rate. *According to Mehran et al. 7. Continuous values are expressed as median and first and third quartiles (serum creatinine and cystatin C) or mean ± standard deviation; categorical values are expressed as a total number and as a percentage of the global population (in parenthesis).
FIGURE LEGENDS Supplement

Figure 1S. Panel A: serum creatinine concentration at baseline, 24 and 48 hours after contrast media administration in the Control group (open symbol, continuous line) and in the Atorvastatin group (closed symbol, dashed line); Panel B: incidence of contrast-induced-acute kidney injury (CIAKI; defined as a serum creatinine increase ≥0.5 mg/dl at 48 hours) in the Control group and in the Atorvastatin group. Panel C: incidence of contrast-induced-acute kidney injury (CIAKI; defined as a serum creatinine increase ≥25% at 48 hours) in the Control group and in the Atorvastatin group.

Figure 2S. Serum creatinine (panel A) and cystatin C (panel B) concentrations at baseline and at 24 and 48 hours after a single high (80 mg) loading dose of atorvastatin in patients with chronic kidney disease. *p >0.05 by paired t test versus baseline.