Simvastatin Increases AQP2 Urinary Excretion in Hypercholesterolemic Patients: A Pleiotropic Effect of Interest for Patients With Impaired AQP2 Trafficking

G Procino[1]*, P Portincasa[2]*, L Mastrofrancesco[1], L Castorani[2], L Bonfrate[2], F Addabbo[2], M Carmosino[1], A Di Ciaula[3] and M Svelto[1,4]

We previously reported that statins improve the symptoms of X-linked nephrogenic diabetes insipidus (X-NDI) in animal models. The aim of this study was to verify whether the pleiotropic effect of statins on AQP2 trafficking and kidney-concentrating ability, observed in rodents, was attainable in humans at therapeutic doses. We enrolled 24 naïve hypercholesterolemic patients and measured urine excretion of AQP2 (uAQP2) at baseline and during 12 weeks of treatment with simvastatin 20 mg/day. Simvastatin induced a rapid and significant increase of uAQP2, reduced the 24-hour diuresis, and increased urine osmolality. These effects were also maintained in patients chronically treated with statins for at least 1 year. This study strongly suggests that statins may effectively enhance the efficacy of current pharmacological treatment of patients with urine-concentrating defects caused by defective AQP2 plasma membrane trafficking, like X-NDI.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC? ☑ Statins increase plasma membrane expression of the water channel AQP2 in the mouse kidney, and increase urine concentration process. This effect is potentially beneficial for treating urine-concentrating defects due to defective AQP2 trafficking. • WHAT QUESTION DID THIS STUDY ADDRESS? ☑ The present study investigated if this additional pleiotropic effect of statins was attainable in humans at therapeutic doses. • WHAT THIS STUDY ADDS TO OUR KNOWLEDGE ☑ This study shows that simvastatin 20 mg daily given to hypercholesterolemic patients increases AQP2 urine excretion, reduces the 24-hour diuresis, and increases urine osmolality. • HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS ☑ Current treatment of X-NDI patients is not completely effective in eliminating the polyuria associated with the disease. The results of this study point to a role of statins to improve the urine-concentrating defect due to impaired AQP2 trafficking like X-linked nephrogenic diabetes insipidus (X-NDI).

Statins are the first-line recommended pharmacological therapy in patients with dyslipidemias and play a key role in both primary and secondary prevention of coronary heart disease. By decreasing plasma total and low-density lipoprotein cholesterol (LDL-C) concentrations, statins decrease the risks for atherosclerotic cardiovascular disease and associated morbidity and mortality.[1–10] Statins occupy part of the active binding site of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) and inhibit its enzymatic activity in the liver, a key step leading to the reduction of cellular sterol pool. Statins also have beneficial effects on the vascular wall by stabilizing the atherosclerotic plaques, ameliorating impaired endothelial function, and reducing vascular inflammation.

Besides the well-known metabolic and cardiovascular effects, it has been recently shown that statins increase the plasma membrane expression of the renal water channels aquaporin 2 (AQP2) (for review see, ref. 11). Water reabsorption in the kidney connecting tubule and collecting duct is regulated by the antidiuretic hormone arginine vasopressin (AVP), which promotes plasma membrane expression of AQP2, the rate-limiting step controlling reabsorption of water, thus urine concentration, in this segment of the nephron. We reported much evidence showing that statins accumulate AQP2 at the apical membrane of collecting duct cells by an AVP-independent mechanism.[12,13] The effect of statins on AQP2 is independent of classical cholesterol homeostasis, but rather depends on depletion of mevalonate-derived intermediates of the cholesterol synthetic pathways, i.e., isoprenoid intermediates, including farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP).[13]

Water balance disorders are often associated with defects of AQP2 trafficking. Nephrogenic diabetes insipidus (NDI) is

*The first two authors equally contributed to this work.

[1]Department of Biosciences, Biotechnologies & Biopharmaceutics, University of Bari, Bari, Italy; [2]Clinica "A. Murri", Department of Biomedical Sciences & Human Oncology, University of Bari Medical School, Bari, Italy; [3]Division of Internal Medicine, Hospital of Bisceglie, Bisceglie (BAT), Italy; [4]Consiglio Nazionale delle Ricerche, Bari, Italy. Correspondence: G Procino (giuseppe.procino@uniba.it)

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characterized by the inability of the kidney to respond to AVP stimulation and is caused by mutations in either the *AQP2* or vasopressin type-2 receptor (*AVPR2)* genes.[14,15] Mutations in the *AVPR2* gene lead to X-linked NDI (X-NDI) in 90% of all diagnosed congenital NDI cases.

Conventional treatment of X-NDI patients consists of a low-sodium, low-protein diet and the administration of thiazide diuretics, sometimes in combination with indomethacin or amiloride. Although these drugs cause some relief of X-NDI symptoms, they most often do not eliminate them. Due to the partial beneficial effect of the conventional treatment, much effort has been spent in the past years to uncover new and alternative methods to induce antidiuresis in X-NDI patients.[13,14,16–19]

In this regard, we recently reported that statins, in particular fluvastatin, accumulate AQP2 at the apical membrane of collecting duct cells by an AVP-independent mechanism and increase water reabsorption in both wildtype and X-NDI mice.[13,17]

The effect of statins on AQP2 trafficking in humans, however, deserves further investigation, also considering the potential efficacy of statins in patients with X-NDI.

In the present study we monitored the time-dependent effects of statin therapy on the urine excretion of AQP2, diuresis, and urine osmolality in a cohort of hypercholesterolemic subjects, with preserved renal function, initiating simvastatin or monacolin K therapy or diet for 3 months.

RESULTS

Baseline characteristics and comparison across groups

The study protocol and the assignment of patients to each experimental group is described in the Methods section and is illustrated in **Figure 1**. Naïve simvastatin (Naïve-S) were patients with hypercholesterolemia starting simvastatin treatment. On chronic statin (ONC-S) were patients who had been treated with statin for at least 1 year and continued their previous therapy during the study period. Naïve monacolin K (Naïve-MC) were patients with mild hypercholesterolemia treated with monacolin K. Naïve diet (Naïve-Diet) patients were treated with the hypolipidemic diet alone.

The general features of the study groups according to treatment and sex are reported in **Table 1**. Concerning age, Naïve-S and ONC-S patients were older than Naïve-MC for both sexes, while patients on the diet were of an intermediate age. Concerning the body mass index (BMI) and waist circumference, obese subjects in the ONC-S group were 6%, followed by Naïve-S and the Naïve-Diet group (50% each), and Naïve-MC group (25%). The analysis of metabolic, cardiovascular, and serum markers at baseline confirmed that Naïve patients had the greatest abnormalities, as compared with ONC-S patients (**Tables 1, 2**). Moreover, as reported in **Table 1**, **Supplementary Materials**, the study of liver steatosis and stiffness showed increased steatofibrosis in the Naïve-S group and Naïve-Diet group, as compared with Naïve-S and Naïve-MC subjects. The coefficient of liver fibrosis correlated with the steatosis score ($n = 37$; r = 0.79, $P = 0.0000$). The liver stiffness score was increased (i.e., >2.73) in Naïve-S (66.7%), Naïve-Diet (50%), ONC-S (38.5%), and Naïve-MC (24%). Intima-media thickness (IMT) was particularly increased in the ONC-S group, and was greater in Naïve-S and in Naïve-Diet, compared to Naïve-MC subjects. Both the ultrasonographic aspect and thickness of kidney cortex were comparable across groups excluding morphological alterations of the kidney.

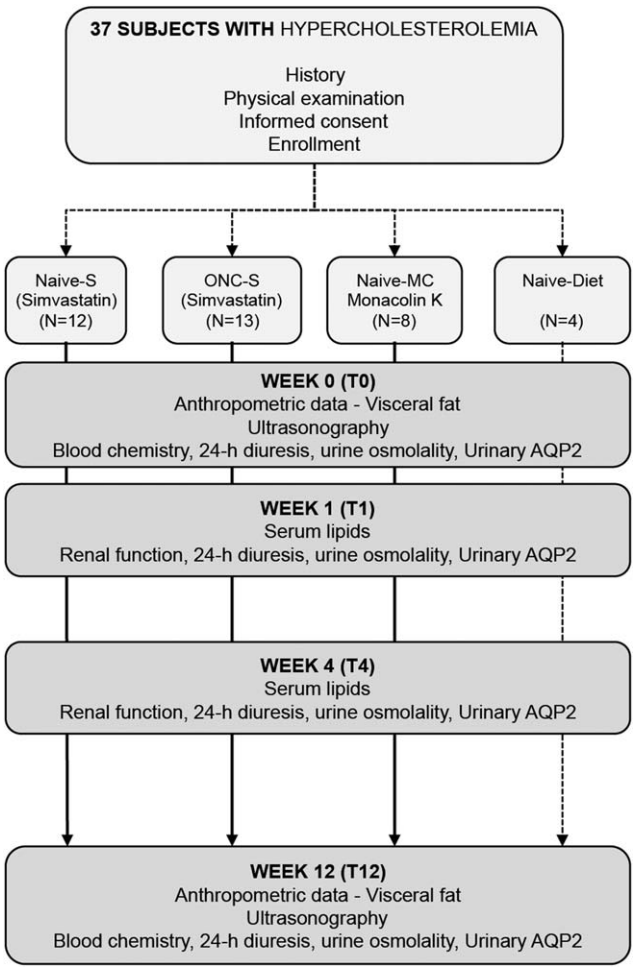

Figure 1 Flow chart design of the study. After initial screening, 37 subjects with hypercholesterolemia were enrolled in four groups. One initiated simvastatin 20 mg/day (Naïve-S), one was already on simvastatin 20 mg/day (ONC-S), one initiated monacolin K 10 mg/day (Naïve-MC), and one initiated diet (Naïve-Diet). Subjects were evaluated at baseline (week 0, T0) and at 1, 4, and 12 weeks from enrollment (T1, T4, T12). uAQP2 was assessed on week 0, 1, 4, and 12.

Patients chronically treated with statins have higher uAQP2 levels compared to naïve subjects

We first analyzed the effect of long-term statins treatment on uAQP2 levels, comparing all naïve subjects (Naïve-S, Naïve-MC, Naïve-Diet, $n = 24$) with ONC-S patients ($n = 13$), already treated with simvastatin for at least 1 year at the time of enrollment in the study. Levels of uAQP2 were significantly higher (+30%) in ONC-S, than in naïve patients (**Figure 2a**; $P < 0.05$), suggesting a significant and long-lasting effect of statin on uAQP2. Baseline (T0) total serum cholesterol and uAQP2 in the two groups are given in **Table 2** and show that total serum cholesterol was significantly lower in ONC-S than in Naïve subject

Table 1 General features of the study groups at baseline according to treatment and sex

Variable	Naïve-S	ONC-S	Naïve-MC	Naïve-Diet	Probability level (*P*) across groups
Number	12	13	8	4	—
Females:Males	6:6	7:6	5:3	2:2	—
Age (yrs)					
Females	56 ± 2[a]	64 ± 5[b]	48 ± 5[a,b]	52 ± 8	0.015
Males	54 ± 5[a,c]	67 ± 5[a, d]	49 ± 6[a]	41 ± 9[c,d]	0.020
Probability level (*P*) intra-group	NS	NS	NS	NS	—
BMI (kg/m^2)					
Females	31.0 ± 4.0	28.2 ± 0.7	31.1 ± 2.8	30.4 ± 4.8	NS (0.065)
Males	33.0 ± 2.9[a,b]	26.4 ± 0.8[a,c]	26.7 ± 1.0[a,c]	34.4 ± 3.6[c,d]	0.016
Probability level (*P*) intragroup	NS	NS	NS	NS	—
Waist circumference (cm)					
Females*	92.7 ± 5.8[a]	86.6 ± 1.1[a,b]	93.8 ± 7.5[b]	92.3 ± 4.8	0.041
Males*	96.1 ± 4.2[a]	87.8 ± 1.5[b]	88.7 ± 0.7[c]	112.5 ± 2.5[a,b,c]	0.14
Probability level (*P*) intragroup	NS	NS	NS	NS	
Overweight (%)	33%	77%	50%	33%	
Obese (%)	50%	6%	25%	50%	
Overweight+obese (%)	83%	83%	75%	83%	

Data are presented as mean ± SEM and compared by ANOVA and *post-hoc* test. Similar symbols indicate significant differences between groups; NS, not significant; BMI, Body Mass Index;
*Females <88 cm; Males <102 cm.

Table 2 Serum and urine analyses at baseline in the study groups according to treatment

Variable	Naïve-S	ONC-S	Naïve-MC	Naïve-Diet	Probability level (*P*) across groups
Number	12	13	8	4	
Serum cholesterol (mg/dl)					
Total	236.9 ± 6.3[a,b]	178.2 ± 6.[b,c]	218.1 ± 7.2[a,c]	230.5 ± 12.8[a,c]	0.0000
HDL	47.4 ± 2.7	54.8 ± 4.1	48.4 ± 5.0	51.0 ± 2.4	NS
LDL	153.6 ± 4.4[a,b]	112.5 ± 8.7[b,c]	147.1 ± 9.4[a,c]	152.2 ± 7.8[a,c]	0.0002
Serum triglycerides (mg/dl)	139.5 ± 29.7[a]	78.9 ± 8.6[a,b]	90.3 ± 10.3	149.5 ± 26.3[b]	0.062
Serum glucose (mg/dl)	90.8 ± 3.7	75.3 ± 4.7[a,b]	93.8 ± 4.6[a]	94.7 ± 5.6[b]	0.04
Serum creatinine (mg/dl)	0.87 ± 0.06[a]	1.0 ± 0.04[a,b]	0.83 ± 0.05[b]	0.90 ± 0.07	0.03
Creatinine Clearance (g/L)	106.9 ± 5.8	87.3 ± 5.5	105.1 ± 6.4	97.6 ± 6.0	NS
Urinary AQP2 (pmol/24h)	2992 ± 223	3719 ± 341	2833 ± 470	2904 ± 287	NS
Urinary creatinine (mg/dl)	0.74 ± 0.03	0.85 ± 0.05	0.63 ± 0.10	0.78 ± 0.05	NS
Urinary Creatinine (mg/24h)	11.2 ± 1.4[a]	14.0 ± 1.3[a]	9.7 ± 1.4[a]	15.4 ± 1.9[a]	0.000
Diuresis (ml)	1657 ± 125	1638 ± 112	1650 ± 163	1958 ± 166	NS
AST (UL)	34.5 ± 9.6[a]	24.9 ± 2.9[a]	45.9 ± 13.3[a]	83.2 ± 12.7[a]	0.0006
ALT (UL)	30.4 ± 8.4[a]	22.6 ± 2.7[a]	41.4 ± 12.6	66.2 ± 15.8[a]	0.018
γGT (UL)	30.9 ± 6.1	28.0 ± 4.0	33.6 ± 8.7	53.3 ± 9.5	NS

Data are present as mean ± SEM and compared by ANOVA and post-hoc test. Similar superscript letters indicate significant differences between groups; NS, not significant.

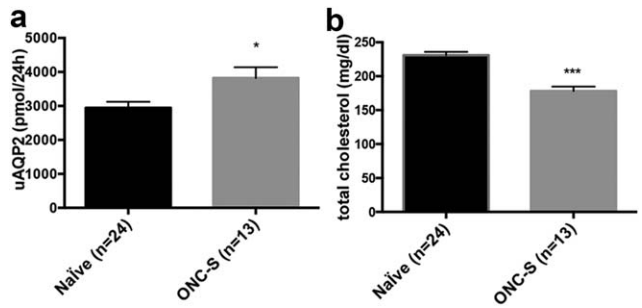

Figure 2 Basal excretion of uAQP2 in the study groups according to treatment. (**a**) uAQP2 levels were significantly higher in hypercholesterolemic patients on statin therapy (ONC-S) compared to the whole group of Naïve patients (i.e., Naïve statin, Naïve-MC, Naïve-Diet). (**b**) Total blood cholesterol was significantly lower in ONC-S compared to Naïve patients. Data were analyzed by Student's *t*-test for unpaired data ($*P < 0.05$, $***P < 0.0001$).

(178.2 ± 6.6, $n = 13$ vs. 230.9 ± 5.1, $n = 24$) (**Figure 2b**; $P < 0.0001$). Importantly, urine creatinine excretion (uCre) was not significantly modified by statin treatment throughout the entire duration of the study (**Table 2**).

Simvastatin treatment affects both uAQP2 and urine parameters

We next evaluated uAQP2, diuresis, urine osmolality, and serum cholesterol in the group of Naïve-S patients, before initiation of simvastatin treatment (T0), and at weeks 1, 4, and 12 after starting a treatment regimen with simvastatin 20 mg/day (T1, T4, and T12, respectively). The results are reported in **Figure 3**. Quantitation of uAQP2 levels by enzyme-linked immunosorbent assay (ELISA) test (**Figure 3a**) showed an early and dramatic increase at T1 (+53%) and remained constantly high at T4 (+63%) and T12 (+65%) compared with T0. In the same patients, measurement of the 24-hour urine collection volume showed a progressive, slight contraction of diuresis (**Figure 3b**) that became statistically significant at T12 (–15%) compared with T0. Urine osmolality (**Figure 3c**) rapidly increased at T1 (+36%) and stood high at T4 (+38%) and T12 (+54%). Compared with baseline (T0), total cholesterol (**Figure 3c**) significantly decreased at T4 (–16%) and T12 (–23%), HDL-cholesterol (**Figure 3e**) did not show significant changes, and LDL-cholesterol (**Figure 3f**) decreased only at T12 (–32%).

In parallel, we followed the same parameters in the ONC-S group, already under statin treatment at the time of enrollment in this study, and continuing simvastatin 20 mg/day during the 12 weeks of this study (**Figure 4**). In the ONC-S group of patients, uAQP2 levels (**Figure 4a**) were already elevated at T0 and remained statistically unchanged at T1, T4, and T12, suggesting that uAQP2 reached a plateau of excretion, induced by chronic simvastatin treatment. In particular, at each timepoint uAQP2 levels in the ONC-S group were not statistically different from those measured in Naïve-S at T12 (one-way analysis of variance (ANOVA), multiple comparison). In the ONC-S group, in line with the lack of changes in uAQP2, also diuresis and urine

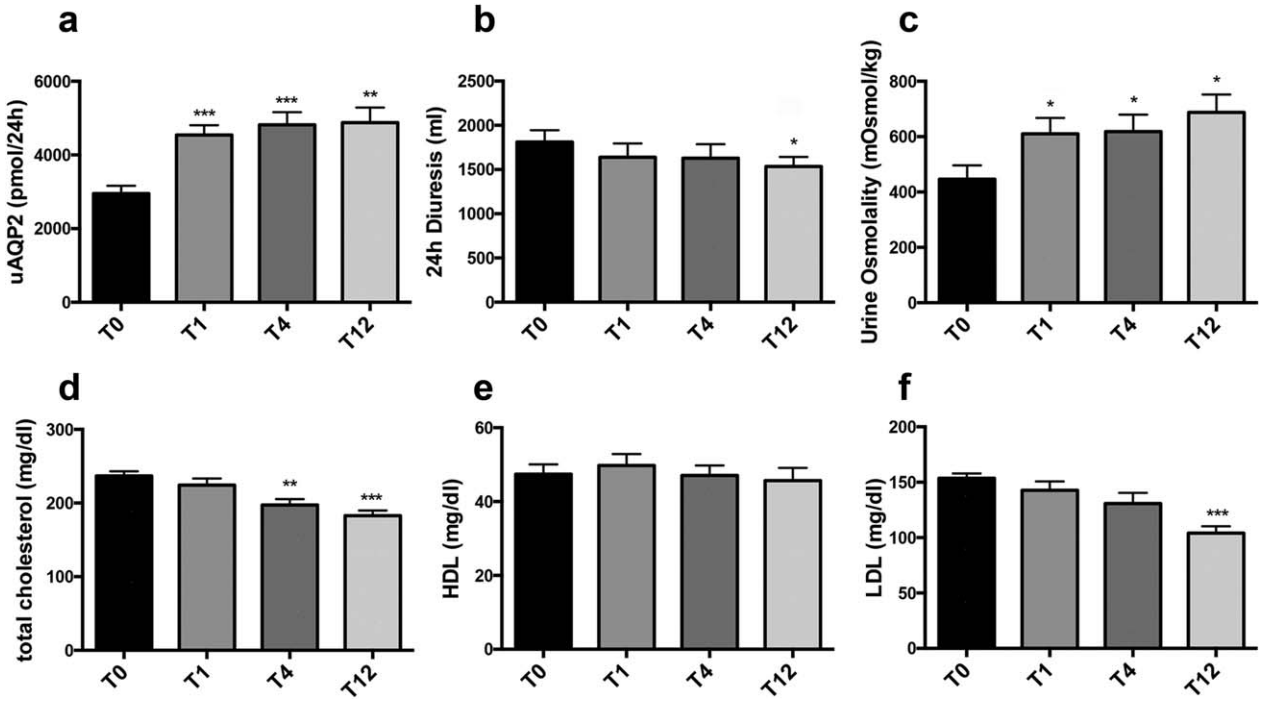

Figure 3 Effect of simvastatin on Naïve-S group of patients. Effect of simvastatin therapy (20 mg/die) on uAQP2 excretion, diuresis, urine osmolality, and serum lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol) in hypercholesterolemic patients undergoing simvastatin treatment for 12 weeks (Naïve-S group). Analyses were performed at weeks 0, 1, 4, 12 (T0, T1, T4, T12). uAQP2 levels (**a**) time-dependently increased during simvastatin treatment. Diuresis (**b**) was significantly lowered by T12 while urine osmolality (**c**) rapidly and significantly increased by T1. Simvastatin significantly lowered total cholesterol (**d**) and LDL-cholesterol (**f**) but not HDL (**e**). The values obtained were compared by one-way ANOVA multiple comparison test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

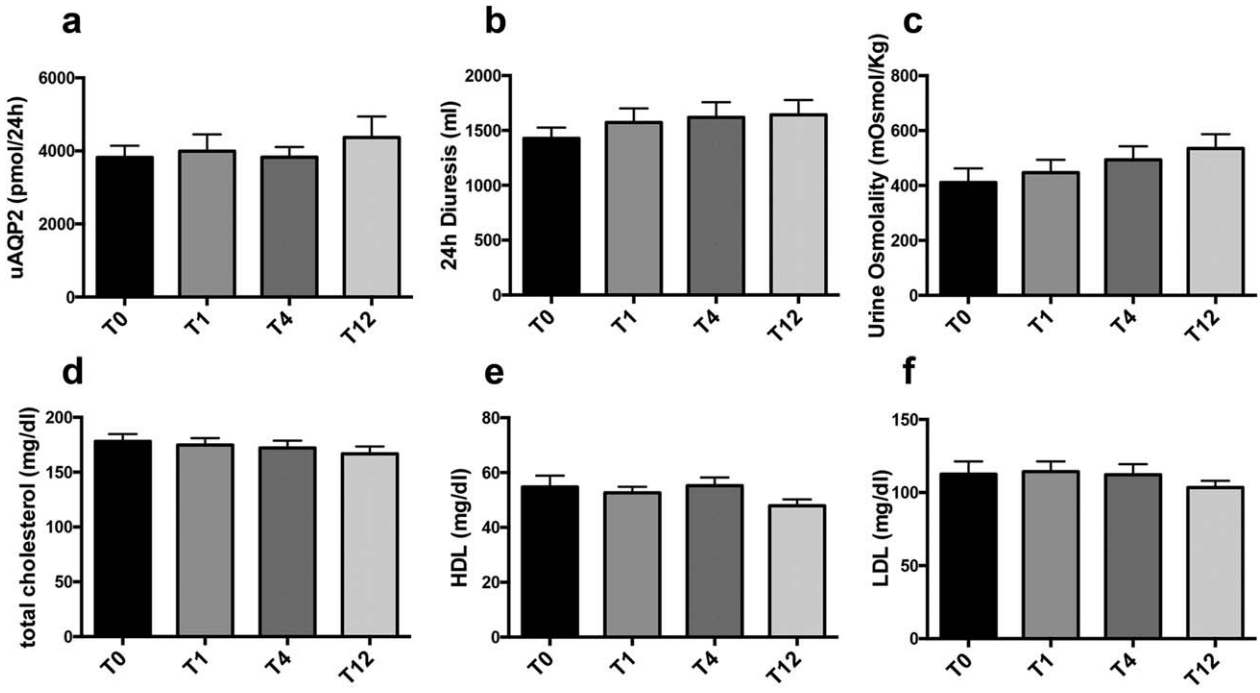

Figure 4 Effect of simvastatin on ONC-S group of patients. Effect of 12 weeks of statin therapy on uAQP2 excretion, 24-hour diuresis, urine osmolality, and serum lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol), in patients already on statin treatment (ONC-S group). uAQP2 levels (**a**) were already elevated at baseline, with stable values throughout the study period. No changes in diuresis (**b**) and urine osmolality (**c**) were measured. No significant change in serum lipids (**d–f**) was recorded. Data were analyzed by one-way ANOVA multiple comparison test.

osmolality (**Figure 4b,c**) remained unchanged at each timepoint during the study period.

In the same patients both total cholesterol and LDL levels (**Figure 4d,f**) at T0 were already comparable to those observed in Naïve-S subjects after 12 weeks (T12) of simvastatin treatment and did not further decrease at any of the observed timepoints.

A comparison of the results obtained in Naïve-S and ONC-S groups, with respect to uAQP2, diuresis, urine osmolality, and total serum cholesterol, is reported in **Figure 5**.

In particular, we show that 12 weeks of simvastatin treatment increased uAQP2 excretion in the Naïve-S group (**Figure 5a**; $P < 0.01$ compared to baseline) to values not statistically different from those measured in the ONC-S group ($P = 0.48$). We also show that the reduction of diuresis (**Figure 5b**; $P < 0.05$) and increase in urine osmolality (**Figure 5c**; $P < 0.05$) observed in the Naïve-S group after 12 weeks of simvastatin treatment were maintained in the ONC-S group over time. Twelve weeks of simvastatin treatment significantly reduced total cholesterol in the Naïve-S group ($P < 0.0001$ compared to baseline) to values that were not statistically different from those measured in the ONC-S group (**Figure 5d**; $P = 0.11$).

Neither dietary supplement of monacolin K nor hypolipidic diet alone affects uAQP2 and urine parameters

In the Naïve-MC group, uAQP2 levels tended to progressively increase throughout the study period, although the differences did not reach statistical significance compared to T0 (**Figure 1a**, **Supplementary Materials**). Diuresis and urine osmolality were also unaffected by monacolin K supplement (**Figure 1b,c**, **Supplementary Materials**). A mild, although statistically significant, decrease of total serum cholesterol was observed at T12 (–15%) compared with T0 (**Figure 1d**, **Supplementary Materials**). Monacolin K dietary supplement affected neither HDL nor LDL cholesterol (**Figure 1e,f**, **Supplementary Materials**; ANOVA test for multiple comparison).

A similar effect was observed in the group of patients on the hypolipidemic diet (Naïve-Diet group): compared to baseline (T0), we only observed a small, statistically significant, reduction of total cholesterol at T12 (**Figure 2d**, **Supplementary Materials**), which was not accompanied by a significant modification of any of the other parameters considered in this study (see **Figure 2**, **Supplementary Materials**).

Changes of metabolic parameters during the observation time

By week 12, the analysis across the four study groups showed that in the Naïve-S group BMI, liver steatosis score, and L/F index had decreased significantly ($0.007 < P < 0.038$) (see **Table 1**, **Supplementary Materials**). Females in this group had the greatest and significant decrease and the change was associated with a significant decrease of waist circumference (from 92.7 ± 5.8 cm at T0 to 88.3 ± 4.4 cm at T12, equal to –4.4 ± 1.3%, $P = 0.035$) as compared with males (from 96.1 ± 4.2 cm at T0 to 95.2 ± 3.8 cm at T12, equal to –0.9 ± 1.0%, P = NS).

The Naïve-MC group showed a trend to decreased BMI mainly in females (–8.3 ± 4.2%, $P = 0.043$). Further comparisons in the other study groups (i.e., ONC-S and Naïve-Diet) did

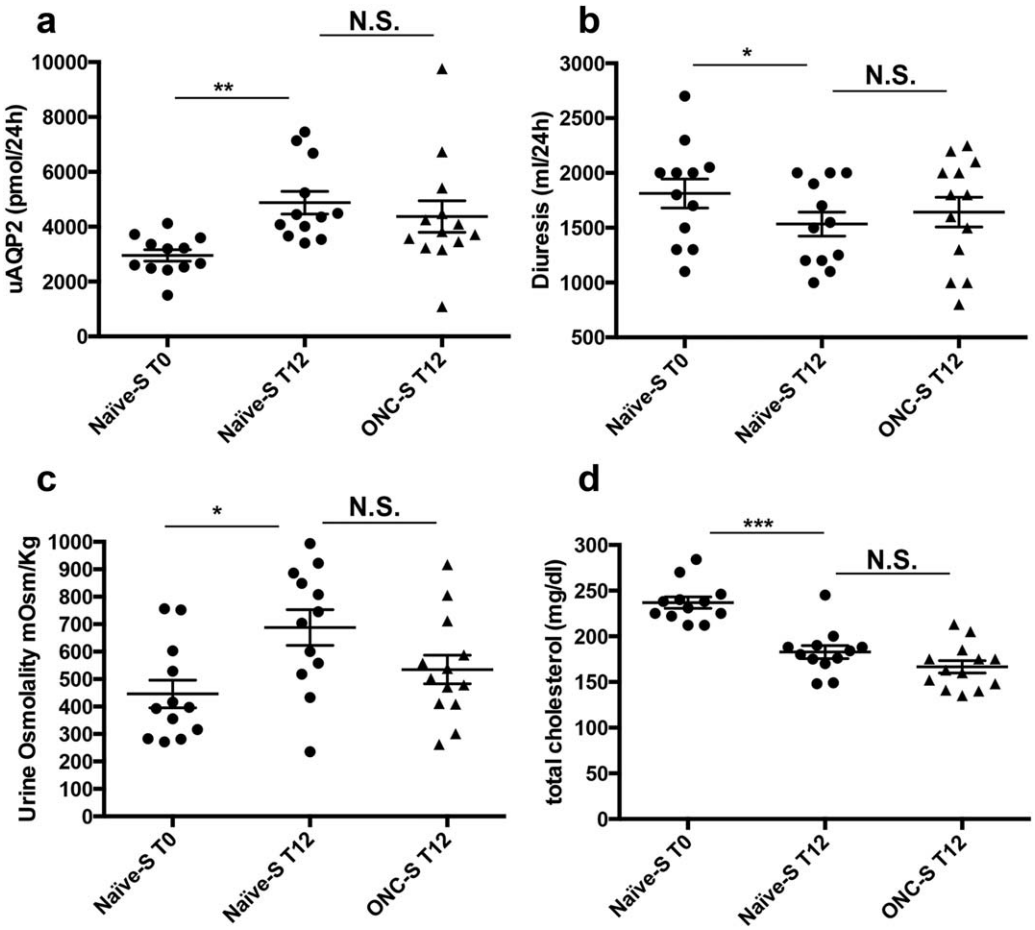

Figure 5 Effects of simvastatin treatment over time. Scatterplot showing single values and mean with SEM of uAQP2 excretion (**a**), 24-hour diuresis (**b**), urine osmolality (**c**), and serum total cholesterol (**d**) at baseline (T0) and after 12 weeks of simvastatin treatment (T12) in Naïve-S patients, and at T12 in patients already on statin (ONC-S). All changes induced by simvastatin treatment in Naïve-S at T12 were maintained in ONC-S at T12. Data were analyzed by Student's *t*-test for paired data (Naïve-S, T0 vs. T12) and unpaired data (Naïve-S T12 vs. ONC-S T12). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$, N.S. = not significant).

not show significant changes of BMI, liver steatosis score, or L/F index at the end of the study (see **Table 1**, **Supplementary Materials**).

No significant change in IMT, kidney cortex thickness, occurred in either sexes across all study groups.

DISCUSSION

Here we show, for the first time, that simvastatin treatment is associated with increased uAQP2 excretion, contraction of the diuresis, and increased urine osmolality in human subjects. These effects appear to be time-dependent and reached a plateau by week 12 of statin treatment.

Statins competitively inhibit hepatic HMG CoA reductase, the rate-limiting step in cholesterol biosynthesis[20]; this step is crucial to reduce plasma total and LDL cholesterol levels. Additionally, statins exert a series of "pleiotropic" effects that are either established on atherosclerotic diseases or potential in nonatherosclerotic diseases.[11] The effect of statins on uAQP2, however, goes beyond the well-known lipid-lowering effect.

In humans, uAQP2[21] is assumed as a noninvasive urinary biomarker reflecting the abundance of AQP2 at the apical membrane of collecting duct cells and used to evaluate a number of physiopathological conditions.[20,22–25]

In recent years, starting from evidence in cellular and animal models, attention has focused on the statin-dependent increase of AQP2 levels at the apical membrane of the kidney collecting duct principal cells.[26] Statins, by increasing AQP2 expression at this site, would also increase water reabsorption in the kidney, thus reducing water excretion in urine, as shown in mice[12,13] and rats. This pleiotropic effect of statins (for review see ref. 11) might be exploited to improve the efficacy of the current treatment of pathological conditions like lithium-induced nephrotoxicity,[27] urinary tract obstructions,[28] chronic hypokalemia,[29] hypercalciuria,[30,31] and X-linked nephrogenic diabetes insipidus,[14] caused by reduced expression of AQP2 and characterized by a decrease in urinary concentrating ability.

Interestingly, the effect on uAQP2 is not restricted to one particular statin. In renal MCD4 cells both lovastatin and fluvastatin

promoted accumulation of AQP2 at the apical plasma membrane. In LL-CPK1 cells simvastatin induced the same effect on AQP2. *In vivo*, fluvastatin increased plasma membrane expression of AQP2 in wildtype mice and in the mouse model of X-linked NDI in a vasopressin-independent fashion[13,17] and simvastatin showed the same effect in collecting duct principal cells of vasopressin-deficient Brattleboro rats.

All these reports show that the effect of statins on AQP2 membrane expression is associated *in vivo* with reduced urine output and increased urine osmolality. It should be highlighted that the rapid effect of statins on AQP2 suggests that it is not due to cholesterol depletion at the plasma membrane, but depends on the statin-induced depletion of intermediates on the mevalonate pathway, regulating the actin cytoskeleton organization dynamics controlling AQP2 intracellular localization.[13]

We designed this study to verify whether the effect of statins on AQP2 demonstrated in rodents is attainable in humans at pharmacological doses of these drugs. In particular, we followed the time-dependent effect of simvastatin 20 mg/day on uAQP2 in naïve hypercholesterolemic patients (Naïve-S) and in patients already on simvastatin treatment (ONC-S). Statin therapy effectively decreased serum lipids, confirming a good compliance with therapy. We found that the simvastatin-dependent increase of uAQP2 was evident in patients with preserved (normal) renal function and morphology.

In naïve patients starting statin therapy, uAQP2 progressively increased by the first week of treatment, reaching a plateau at week 12. We next extended our observation to a group of patients already on chronic simvastatin treatment for at least 1 year and found that uAQP2 levels in these patients were comparable to those reached by naïve patients after 12 weeks of simvastatin treatment.

The effect of simvastatin on uAQP2 in Naïve-S patients was also accompanied by a slight but significant reduction of the 24-hour urine output and an early increase of urine osmolality that was observed at each timepoint after initiation of simvastatin treatment. Any possible confusing effect of diuretic therapy was excluded in this study, since none of the patients were taking diuretics. Interestingly, the effect of simvastatin on both uAQP2 and urinary parameters was stable over time. In fact, as summarized in **Figure 5**, at the end of the observational study (T12) we observed that uAQP2 in the Naïve-S group significantly increased to a level comparable to that already displayed by the ONC-S group, suggesting that the effect of simvastatin on uAQP2 does not reverse over time. The same trend was observed for the reduction of diuresis and increase of urine osmolality, likely induced by simvastatin treatment. This observation is fundamental, as it indicates that statins would provide a long-lasting improving effect on the kidney concentrating ability of patients affected by X-NDI.

Due to wide interindividual variation, the effect of statins on uAQP2 levels were mildly related to total serum cholesterol levels (data not shown), pointing to a rather pleiotropic effect of statins. In this respect is important to highlight that, in this study, a mild reduction of total blood cholesterol, obtained through administration of monacolin K or hypolipidic dietary regimen, neither affected uAQP2 nor urine parameters (see **Figures 1 and 2**, **Supplementary Materials**).

Naturally occurring monacolins in red yeast rice, such as monacolin K, have HMG CoA reductase inhibitor activity,[32] and monacolin K has the same chemical formula of lovastatin, a natural statin still used in clinical practice. We showed here that in a group of eight patients a dietary supplement of monacolin 10 mg/day reduced total blood cholesterol of about 15% at T12 compared to the 23% reduction induced by simvastatin 20 mg/day at the same timepoint. uAQP2 showed a tendency to increase during the 12 weeks of observation, although these changes were not statistically relevant. We hypothesize that longer observation times (i.e., more than 12 weeks), a larger group of patients, or higher doses of monacolin might be necessary to demonstrate an affect of monacolin K on uAQP2 and urinary parameters.

On the other hand, in agreement with a pleiotropic effect of statins on AQP2, we observed that in a small group of patients on a hypolipidic dietary regimen total blood cholesterol was reduced but none of the renal parameters investigated in this study were modified (see **Figure 2**, **Supplementary Materials**). This evidence supports our hypothesis that, rather that cholesterol levels, depletion of intermediates of the cholesterol biosynthetic pathway is responsible for the effect on AQP2.

The effect of statins on uAQP2 was also independent of variations in BMI, abdominal circumference, liver index, liver steatosis score, or IMT, as little variation of these parameters were noticed between the start and the end of the observation period. Likely an initial, albeit minor, improvement in metabolic aspects depended on the intensive follow-up of the patients and better compliance to healthier lifestyles, especially in females.

Also, the Naïve-S group displayed a significant decrease of both BMI and liver steatosis. We speculate that patients in this group have a greater perception of "disease" (hypercholesterolemia) due to the initiation of statin therapy. Thus, Naïve-S patients show better dietary compliance to improve drug efficacy and achievement of the therapeutic goal. The significant decrease of liver steatosis in the Naïve-S group appears to follow this outcome.

In conclusion, simvastatin administration rapidly increases uAQP2 in hypercholesterolemic patients. This effect lasted for the 12-week duration of the study and was still evident in patients on chronic statin treatment. The increase of uAQP2, correlated with increased AQP2 expression in the renal collecting duct, likely promotes water reabsorption in the kidney tubule, as we observed a small contraction of diuresis and a significant increase of urine osmolality in these patients. Quite unexpectedly, the increase of water reabsorption in the statin-treated patients did not activate a compensatory mechanism associated with a return of urine AQP2 and osmolality towards baseline levels. Future studies, including measurements of water intake, plasma sodium/osmolality, and levels of circulating AVP will help to clarify this aspect.

Importantly, in this work we extended in humans previous results obtained in renal cell lines and animal models. To our best knowledge this is the first report demonstrating that statins promote AQP2 membrane accumulation in the kidney and increase urine-concentrating abilities in patients. Along with

much evidence showing the improving effect of statins on symptoms of X-linked NDI in animal models, our data strongly suggest that statins may effectively improve the efficacy of the current pharmacological treatment of those urine-concentrating defects caused by impaired AQP2 plasma membrane trafficking, such X-NDI.

METHODS

Patients

From October 2013 to July 2015, we enrolled 37 subjects for the study of urinary AQP2 (uAQP2). Patients had hypercholesterolemia requiring either statin treatment ($n = 33$) or initiating a hypolipidic diet ($n = 4$), which served as control. The following four groups entered the study:

1. Naïve simvastatin (Naïve-S; $n = 12$): six females and six males with hypercholesterolemia starting statin treatment for primary or secondary prevention of cardiovascular diseases. Treatment consisted of simvastatin 20 mg once daily (evening).
2. On chronic statin (ONC-S; $n = 13$): seven females and six males with hypercholesterolemia who had been treated with statin for at least 1 year and continued their previous therapy during the study period. Treatment was simvastatin 20 mg once daily (evening).
3. Naïve monacolin K (Naïve-MC; $n = 8$): five females and three males patients with mild hypercholesterolemia either refusing initial statin treatment or intolerant to standard statin treatment and starting with monacolin K (Liposcudil Plus, http://www.piamfarmaceutici.com), 10 mg once daily (evening). According to the manufacturer, each pill of monacolin K is equivalent to lovastatin 10 mg.
4. Naïve diet (Naïve-Diet; $n = 4$): two females and two males refusing initial pharmacologic treatments and choosing the hypolipidic diet.

Inclusion criteria were patients on primary prevention requiring moderate reduction of LDL-cholesterol according to the ACC/AHA guidelines to reduce the risk of atherosclerotic cardiovascular disease. Patients aged 40 to 75 years with an estimated 10-year ASCVD risk $\geq$7.5% and requiring moderate intensity therapy.[33] Exclusion criteria included concomitant type 1 or type 2 diabetes (to avoid any possible interference with renal function), blood hypertension, concomitant use of diuretics or other drugs interfering with simvastatin (i.e., amiodarone, amlodipine, ranolazine, diltiazem, verapamil). Exclusion was based on the results of a global clinical, instrumental, and laboratory work-up in all subjects, prior to enrollment into the study.

All subjects maintained a typical isocaloric "Mediterranean diet" (rich in fruits, vegetables, whole grains, beans, nuts, and seeds). Olive oil was the important source of monosaturated fat. The diet also included low to moderate amounts of fish, poultry, and dairy products, and little red meat.

In the group of hypercholesterolemic patients choosing to undergo the initial diet, an isocaloric hypolipidic diet was suggested.

Ethics statement

The design of the study was observational, since no additional intervention was planned apart from routine medical care. Samples were collected after the patients gave their written informed consent and the study was reviewed and approved by the Institutional Review Board of the Polyclinic Hospital, University of Bari Medical School (study no. 4200 STAT-NDI-01, prot. 765/CE/24-09-13), Bari, Italy, and carried out in accordance with the Helsinki Declaration of 1975 (as revised in 1983). No individual patient data are reported in this article. Registration of the trial was obtained at ClinicalTrials.gov with Identifier NCT02523001.

Study design

As shown in **Figure 1**, subjects were evaluated at baseline (week 0, T0) and at 1, 4, and 12 weeks from enrollment (T1, T4, T12). Subjects were also enquired by phone interview every week to check for overall compliance.

After initial evaluation, including history and a general physical examination, subjects signed the informed consent form and were enrolled in one of the four study groups.

At baseline (T0), all subjects underwent measurement of anthropometric data, ultrasonography studies, and blood chemistry. All hypercholesterolemic patients underwent specific measurements of renal function and uAQP2 repeated after 1, 4, and 12 weeks from enrollment. Anthropometric data (i.e., BMI, waist circumference),[34–36] ultrasonography, and blood chemistry were reassessed in all subjects at the end of the study (week 12).

Assessment of liver steatosis/stiffness and carotid ITM

Ultrasonography was performed with the Hitachi Noblus-E ecocolordoppler (Hitachi Medical, Tokyo, Japan) to evaluate kidney cortex thickness, echogenicity of the liver parenchyma, as a sensitive marker of liver steatosis[37–39] (ranging from grade 0 to grade 3), hepatic elasticity expressed as L/F liver index (<2.73 for a normal liver parenchyma, while from 2.73 to 3.93 for increased fibrosis),[41,42] the carotid IMT.[43]

Serum and urine analyses

Blood analyses were planned at baseline and at the end of the study (T0, T12). All participants were asked to fast for at least 12 hours before the examination and to provide blood samples to measure plasma levels of total cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL),[44] triglycerides (TG) plasma aminotransferase (AST and ALT), gamma-glutamyl-transpeptidase (GGT) levels, total/direct bilirubin, glucose, and creatinine (Instrumentation Laboratories, Werfen, Milan, Italy).

Twenty-four hours diuresis was collected at each timepoint (i.e., 0, 1, 4, and 12 weeks). Urine samples were stored at −80°C and analyzed at the end of the study period. Urine creatinine was measured enzymatically (IL Test TM Creatinine, Instrumentation Laboratories, Werfen) and results used to calculate creatinine clearance and excretion (24 hours) in all patients.

Quantitation of urinary AQP2 was performed using a standard ELISA protocol originally established by Umenishi et al.,[45] with some modifications.[46] uAQP2 values were normalized to the 24-hour diuresis and expressed as picomoles of AQP2/24h (pmol/24h). Urine osmolality was measured using a vapor pressure osmometer (Vapror 5520; www.wescor.com).

Statistical analysis

Results are given as means ± standard error of the mean (SEM). Comparison of continuous variables within the same group was performed by nonparametric tests including repeated-measures one-way ANOVA, with the Greenhouse-Geisser correction, Bonferroni's multiple comparison test in case of significant probability levels. Comparison of variables was performed by Students' *t*-test: for unpaired data when comparing different groups and for paired data for comparisons before (T0) and after (T12) treatment in the same group. Statistical analyses were performed by PRISM 6.0 software (GraphPad Software, San Diego, CA). A *P*-value of less than 0.05 was considered statistically significant.

Additional supporting information can be found in the online version of this article

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

G.P., P.P., L.B., M.C., and M.S. wrote the article; G.P., P.P., L.B., A.D.C., and M.S. designed the research; G.P., L.M., L.C., and F.A. performed the research; G.P., P.P., L.M., L.C., M.C., and A.D.C. analyzed the data; G.P. and P.P. contributed new reagents/analytical tools.

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