Simvastatin maintains steady patterns of GFR and improves AER and expression of slit diaphragm proteins in type II diabetes

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The factors determining the course of glomerular fitration rate (GFR) and albumin excretion rate (AER) and the expression of mRNA of slit diaphragm (SD) and podocyte proteins in microalbuminuric, hypertensive type II diabetic patients are not fully understood. GFR, AER, and SD protein mRNA were studied in 86 microalbuminuric, hypertensive, type II diabetics at baseline and after 4-year random double-blind treatment either with 40 mg simvastatin (Group 1) or with 30 g cholestyramine (Group 2) per day. Both groups had at baseline a GFR decay per year in the previous 2–4 years of 3 ml/min/1.73 m^2. Both Groups 1 and 2 showed a significant decrease of low-density lipoprotein cholesterol levels after simvastatin and cholestyramine treatment ($P<0.01$). No change from base line values was observed as for hs-C-reactive protein and interleukin-6. A significant decrease of 8-hydroxydeoxyguanosine urinary excretion was observed after simvastatin treatment. GFR did not change from baseline with simvstatin, whereas a decrease was observed with cholestyramine treatment (simvastatin vs cholestyramine: −0.21 vs –2.75 ml/min/1.73 m^2, $P<0.01$). AER decreased in Group 1 ($P<0.01$), but not in Group 2 patients. Real-time polymerase chain reaction measurement of mRNA SD proteins (CD2AP, FAT, Actn 4, NPHS1, and NPHS2) significantly increased in kidney biopsy specimens after simvastatin, but not cholestyramine treatment. Simvastatin, but not cholestyramine, 4-year treatment maintains steady patterns of GFR, and improves AER and expression of SD proteins in type II diabetes, despite similar hypocholesterolemic effects in circulation.

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It has been shown that the course of renal function is ominous in some type II diabetic patients, who have been called 'progressors,' whereas other patients show steady patterns of renal function for years ('non-progressors').[1–4] Similar findings have been reported also in type 1 diabetic patients.[5] Prolonged hyperglycemia causes chronic complications of diabetes.[6] Tight blood pressure control delays the progression of renal damage in diabetic patients with nephropathy.[7–10] Nevertheless, still 15–20% of the diabetic patients relentlessly develop end-stage renal disease, despite aggressive antihypertensive therapy.

Tonolo *et al.*[11,12] demonstrated that statins decrease the rate of albumin excretion rate (AER) in type II diabetics with microalbuminuria. This latter improvement of the course of renal function has been confirmed by a metanalysis of reports in literature by Fried *et al.*[13] Van Wijk *et al.*[14] reported that an increase of simvastatin treatment from 20 to 80 mg/day improved chylomicron remnant clearance in comparison with the lower 20 mg/day dose. Furthermore, the effects of statins are not merely related to their hypocholesterolemic effects.[15] Therefore, we decided to use a higher simvastatin dose in the present paper, that is 40 mg/day.

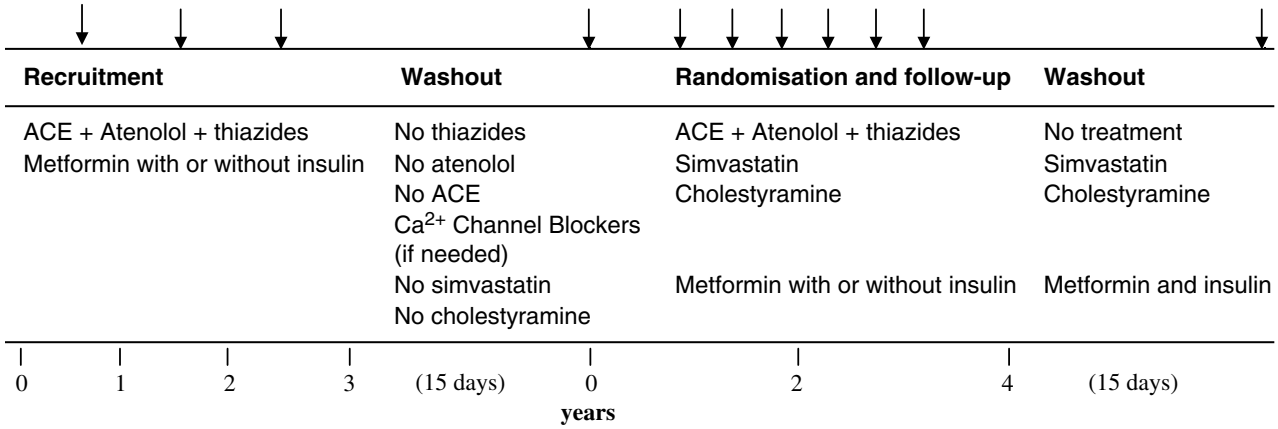
We identified a subgroup of 86 consecutive, newly recruited, type II diabetic patients, who had these clinical characteristics: a rapid deterioration of glomerular fitration rate (GFR) (progressors), hemoglobin A1c >7%, and proliferative or background diabetic retinopathy.[4,16]

Our aim was to investigate the effects of simvastatin and cholestyramine treatment on the course of renal function, in type II diabetic 'progressor' patients.

RESULTS

The two groups showed similar demographic characteristics at baseline, before treatment either with simvastatin (range

Table 1 | GFR and AER (in triplicate) evaluation



ACE, angiotensinsin-converting enzyme inhibitors; AER, albumin excretion rate; GFR, glomerular fitration rate.
The flow chart which describes the drug treatment of 86 consecutive, hypertensive type 2 microalbuminuric patients before the recruitment, during the hypoglycemic and antihypertensive, and hypocholesterolemic treatment and at the end of the follow-up period. Four patients were excluded before the first control at the 6th month. All the other patients were recruited as consecutive patients, if they had a GFR decay >3 ml/min/year/1.73 m^2 in the preceding 3 years. The statistician of the Department of Clinica Medica of the University of Sassari allocated blindly the patients to simvastatin or to cholestyramine treatment on the basis of the intention to treat principle. Each arrow on the top represents the control of GFR and AER (in triplicate as for AER).

Table 2 | Mean $\pm$ s.e. age, duration of the disease, HbAlc, and BP

	Age (years)	Duration of the disease (years)	HbAlc %	Systolic BP (mm Hg)	Diastolic BP (mm Hg)
Microalbuminuric Type 2 diabetics Group 1 (*n*=42)	62 $\pm$ 8	13 $\pm$ 4	7.4 $\pm$ 0.7	132 $\pm$ 14	77 $\pm$ 4
Microalbuminuric Type 2 diabetics Group 2 (*n*=40)	61 $\pm$ 4	12 $\pm$ 4	7.3 $\pm$ 0.6	130 $\pm$ 13	75 $\pm$ 4

BP: blood pressure; HbA1c: hemoglobin Alc.
Mean $\pm$ s.e. clinical features of 82 (statistical comparison have been performed taking into consideration all the 86 patients) diabetic patients at baseline (age, duration) and after 4 year therapy with 40 mg per day of simvastatin or with 30 g per day of cholestyramine simvastatin (HbAlc and BP).
Group 1: simvastatin-treated patients.
Group 2: cholestyramine-treated patients.

3.7–4.2 years) or with cholestyramine (range 3.8–4.2 years) (Tables 1 and 2). The two groups of patients had similar histologic renal patterns at baseline (Figures 1 and 2, panels 1–12). Some change of the patterns of renal lesions in the kidney specimen was observed both after simvastatin and after cholestyramine treatment. For instance Figure 1 shows that an increase of arteriolar hyalinosis was observed in a patient with mild glomerular lesions at baseline after simvastatin treatment (panel 1 vs 2). On the contrary, a slight decrease of mesangial expansion was observed in a Category 2 patient after simvastatin treatment (panel 3 vs 4) (Figure 1). Figure 1 also shows a further deterioration of renal lesions in Category 3 patients after simvastatin treatment. Figure 2 shows an increase of mesangial expansion in a Category 1 patient after cholestyramine treatment (panel 7 vs 8). With regard to another patient in Category 2, the extent of mesangial expansion was similar at baseline and after cholestyramine treatment, even if also in this patient arteriolar hyalinosis appeared more severe after cholestyramine treatment. Eventually, Figure 2 shows that the patterns of interstitial lesions were more severe in a Category 3 patient after cholestyramine treatment. To accomplish a broader and more complete evaluation of the paterns of the histologic lesions at renal level, we also evaluated three numerical parameters using light microscopy (see Materials and Methods). A slightly significant improvement was observed after simvastatin, but not after cholestyramine treatment (Table 6). Glycemic control was satisfactory, having achieved an average value of 7.4 or 7.3% of hemoglobin A1c throughout the follow-up period (Table 2), as compared with previous studies from our clinical center, during which average hemoglobin A1c blood concentrations were always higher than 8.0%. Blood pressure control was also tight, showing average levels of systolic and diastolic blood pressure below 140/90 mm Hg, even if not below 130 mm Hg as for systolic levels (Table 2). Both simvastatin and cholestyramine treatment significantly decreased lipid levels to similar patterns (Table 3). On the contrary, no significant changes were observed during both treatments as regards hs-C-reactive protein levels (Table 3). Serum interleukin-6 levels were similar in Groups 1 and 2 patients at baseline (5.9 ± 0.4 vs 6.1 ± 0.5 pg/ml, NS) and no significant change from baseline was observed after both treatments. The mean urinary excretion of 8-hydroxydeoxyguanosine (8-OH-dG) (μg/g creatinine) was similar in the 43 simvastatin treated and 43 cholestyramine treated at baseline (Table 4, data regarding 42 and 40 patients, respectively, are shown). Simvastatin

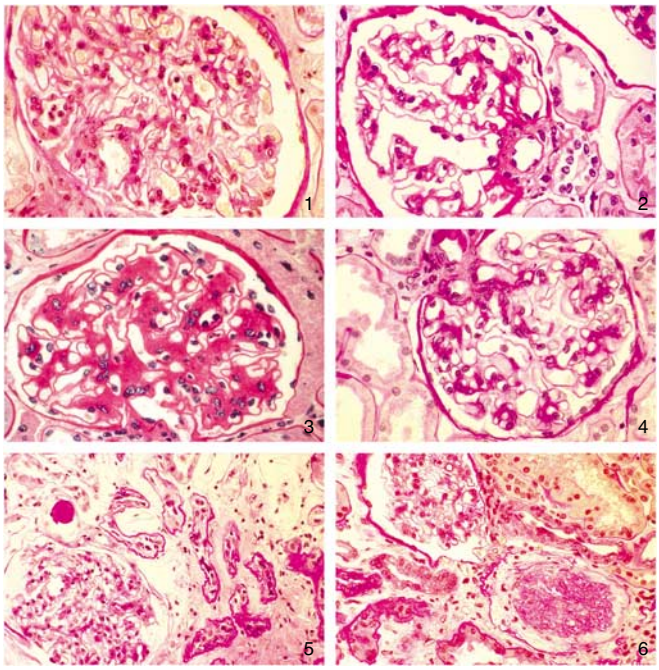


Figure 1 | Patterns of the glomerular and renal lesions in kidney specimens from Categories 1–3 patients at baseline (panels 1, 3, and 5) and after simvastatin treatment (panels 2, 4, and 6).

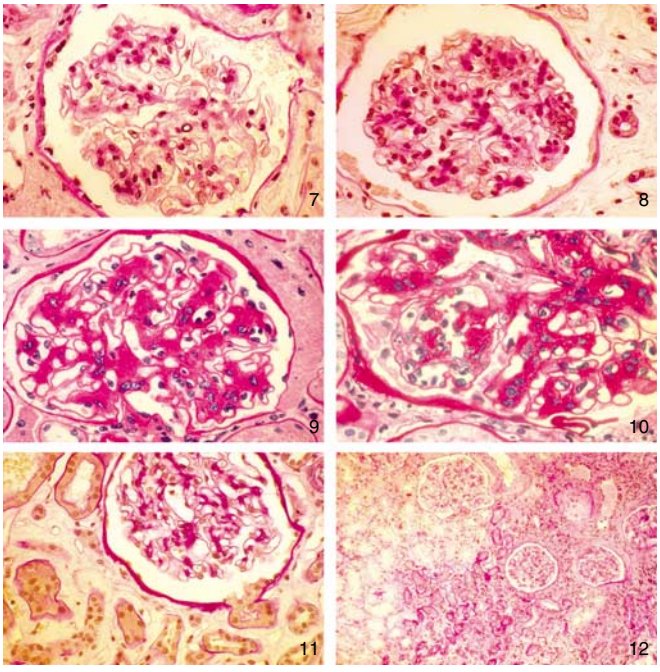


Figure 2 | Patterns of the glomerular and renal lesions in kidney specimens from Categories 1–3 patients at baseline (panels 7, 9, and 11) and after cholestyramine treatment (panels 8, 9, and 12).

tretment, but not cholestyramuine treatment, decreased the urinary excretion of 8-OH-dG (Table 4).

Body mass index was 27 ± 0.4 and $28 \pm 0.6\,kg/m^2$ at baseline in Groups 1 and 2, respectively, and no significant change from baseline was observed after both treatments. GFR did not change from baseline at the end of the 4-year treatment with simvastatin (Table 5). On the contrary, a significant decrease of GFR was observed in the cohort of patients treated with cholestyramine (cholestyramine vs simvastatin: $-2.75\,ml/min/1.73\,m^2$ per year vs $-0.21\,ml/min/1.73\,m^2$ per year, $P<0.01$). However, also cholestyramine treatment slightly blunted the decay of GFR, as the average GFR decrease per year in the overall group of patients before entering the present study was $-3.9\,ml/min/1.73\,m^2$/year. AER significantly decreased in the patients treated with simvastatin, but not in those treated with cholestyramine (Table 5). The percentage of patients who had steady normoalbuminuria, during the 4th year of follow-up instead of microalbuminuria, was three-fold higher during simvastatin than during cholestyramine treatment (29 vs 8%, $P<0.01$). Overt proteinuria did develop in 15% of cholestyramine-treated patients and in 4% of simvastatin-treated patients ($P<0.01$).

Glomeruli from type II diabetic patients were always positive for ox-low-density lipoprotein (ox-LDL), with a variable intensity and pattern of deposition (Figures 4–5). No deposition of ox-LDL was ever observed in the glomeruli of the five non-diabetic, control hypertensive subjects (Figure 6). We described such latter observation in two different ways: (1) in a qualitative and quantitative fashion (Figures 4, 5, and 7, Table 6). More particularly as regards the quantitative fashion the deposition of ox-LDL was expressed as % of fluorescence staining for each glomerular area. These data (ranging from 10–20% to 60–80%) were represented in relationship with NPHS1 mRNA normalized for the amount of extracted RNA (Figure 7). At baseline both (15 simvastatin- and 15 cholestyramine-treated patients) had a deposition of ox-LDL broadly dispersed in a wide range, always associated with markedly low NPHS1 patterns (Figure 7). Only simvastatin treatment resulted in a significant decrease of the deposition of ox-LDL and in a significant increase of NPHS1 patterns (Figure 7). The deposition of ox-LDL was similar in patients in Categories 1–3 according to the histologic classification we have previously described. Table 7 shows the means $\pm$ s.d. of some slit diaphragm (SD) mRNA expression in specimens from kidney biopsy of Groups 1 and 2 patients at baseline and after simvastatin and cholestyramine treatment. CD2AP, FAT, Actn4, NPHS1, and NPHS2 mRNA expressions reported as percentage of the internal standard mRNA 18S were all significantly higher in the simvastatin-treated patients, in comparison with both baseline values in the simvastatin Group 1, and baseline and 4-year values after follow-up in the cholestyramine cohort. No change was observed after cholestyramine treatment.

DISCUSSION

The results of the present study demonstrate that both simvastatin and cholestyramine reduce circulating LDL levels in microalbuminuric, type II diabetic, hypertensive patients, but that only simvastatin abates progression of renal damage. Moreover, our results show that simvastatin markedly

Table 3 | Mean ± s.e. and geometric mean with range serum and plasma levels of CH, TG, LDL cholesterol, HDL cholesterol, and highly sensitive C-protein-reactive protein (hsCRP) at baseline (B) and after 4 years of simvastatin or cholestyramine treatment

	CH (mg/dl)	TG (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	HsCRP (mg/l)
Simvastatin					
B	231 ± 12	191 ± 45	150 ± 13	43 ± 5	2.25 (0.7-2.99)
4 years	170 ± 13*	148 ± 39*	99 ± 15*	42 ± 6	2.19 (0.8-2.81)
Cholestyramine					
B	227 ± 16	188 ± 50	148 ± 16	44 ± 7	2.11 (0.7-2.70)
4 years	166 ± 18*	146 ± 48*	97 ± 18*	45 ± 8	2.13 (0.6-2.90)

CH: total cholesterol; HDL: high-density lipoprotein; hsCRP: highly sensitive C-protein-reactive protein; LDL: low-density lipoprotein; TG: triglycerides.
Mean ± s.e. lipid patterns and hsPCR values of 82 diabetic patients at baseline and after 4-year therapy with 40 mg per day of simvastatin or with 30 g per day of cholestyramine simvastatin (statistical comparison have been performed taking into consideration all the 86 patients).
Group 1: simvastatin-treated patients.
Group 2: cholestyramine-treated patients.
*$P < 0.01$, baseline vs 4th year.

Table 4 | Mean ± s.e. urinary 8-OH-dG levels (μg/g creatinine) in type 2 hypertensive microalbuminuric diabetic patients (82) at baseline, and in 42 after 4-year simvastatin and in 40 after 4-year cholestyramine treatment

	Baseline	4th year
Simvastatin	4.45 ± 0.15	3.20 ± 0.20**
Cholestyramine	4.49 ± 0.11	3.91 ± 0.14^{NS}

8-OH-dG: 8-hydroxydeoxyguanosine.
Group 1: Simvastatin treated patients.
Group 2: Cholestyramine treated patients.
**$P < 0.01$ baseline vs 4th year.
NS=not significant baseline vs 4th year.

Table 5 | Mean ± s.e. and median with ranges of GFR and AER at baseline and after 4 years treatment with simvastatin and cholestyramine

	GFR (ml/min/1.73m^2)	AER (μg/mg)	Normo	Prot.
Simvastatin				
B	91 ± 8	77 (31–259)	—	—
4 years	90 ± 7	40* (10–319)	29%	4%
Cholestyramine				
B	90 ± 7	88 (34–261)	—	—
4 years	79 ± 8*,**	81 (17–399)	8%**	15%**

AER: albumin excretion rate; GFR: glomerular fitration rate.
Group 1: Simvastatin treated patients.
Group 2: cholestyramine-treated patients.
*$P < 0.05$ baseline vs 4th year. **$P < 0.01$ baseline vs 4th year.
NS=not significant baseline vs 4th year.
Mean ± s.e. and Median with ranges of GFR and AER at baseline and after 4 year treatment with simvasatatin and cholestyrramine. Precentage of patients showing regression to normoalbuminuria (normo) and progression to proteinuria (prot).

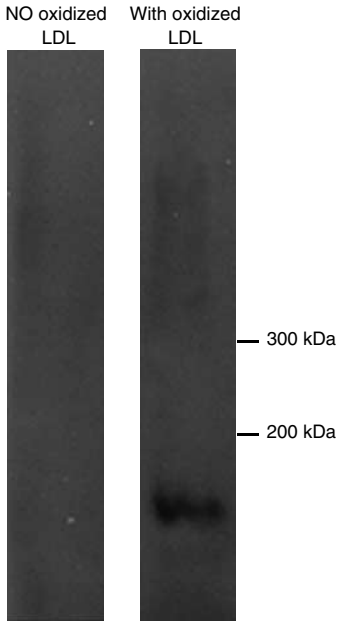


Figure 3 | LDLs were purchased from Sigma. Before oxidation, LDLs were dialyzed against sterile PBS at 4°C for 2 h. The oxidation was performed by incubating LDL solution with 10 μmol/l $CuSO_4$ at 37°C for 24 h. Western blot analysis demonstrates the specificity of the antibody against oxidized LDL.

improves mRNA expression of SD proteins. It does not appear from our data that either simvastatin or cholestyramine treatment modifies hs-C-reactive protein- and interleukin-6-circulating levels. Eventually the amelioration of AER and expression of mRNA of SD proteins, as well as the stabilization of GFR patterns, were associated with decrease of 8-OH-dG urinary excretion.

Our findings suggest that statins increase the expression of nephrin, podocin, CD2AP, and FAT, alpha-actin proteins. These proteins are tightly associated and are embedded into lipid rafts.[17–21] The response of SD proteins to exogenous signals is orchestrated by a number of molecules that cluster in cholesterol-rich areas of the cell, the lipid rafts. Several evidences suggest that the inhibition of cholesterol synthesis by statins modifies these lipid rafts.[17–21] With regard to our findings on reactive oxygen species, it has to be pointed out that reactive oxygen species lead to oxidative damage of DNA, aging, and degenerating disease. 8-OH-dG was first reported as causing oxidative DNA damage.[22–25]

The mechanisms underlying the relationship between the decrease of 8-OH-dG and the amelioration of renal function could be due to a decreased overproduction of reactive oxygen species.[26] This latter hypothesis is suggested by the observation from other research groups[17-19,27,28] that SD structure is rescued by oxygen radical scavengers. The

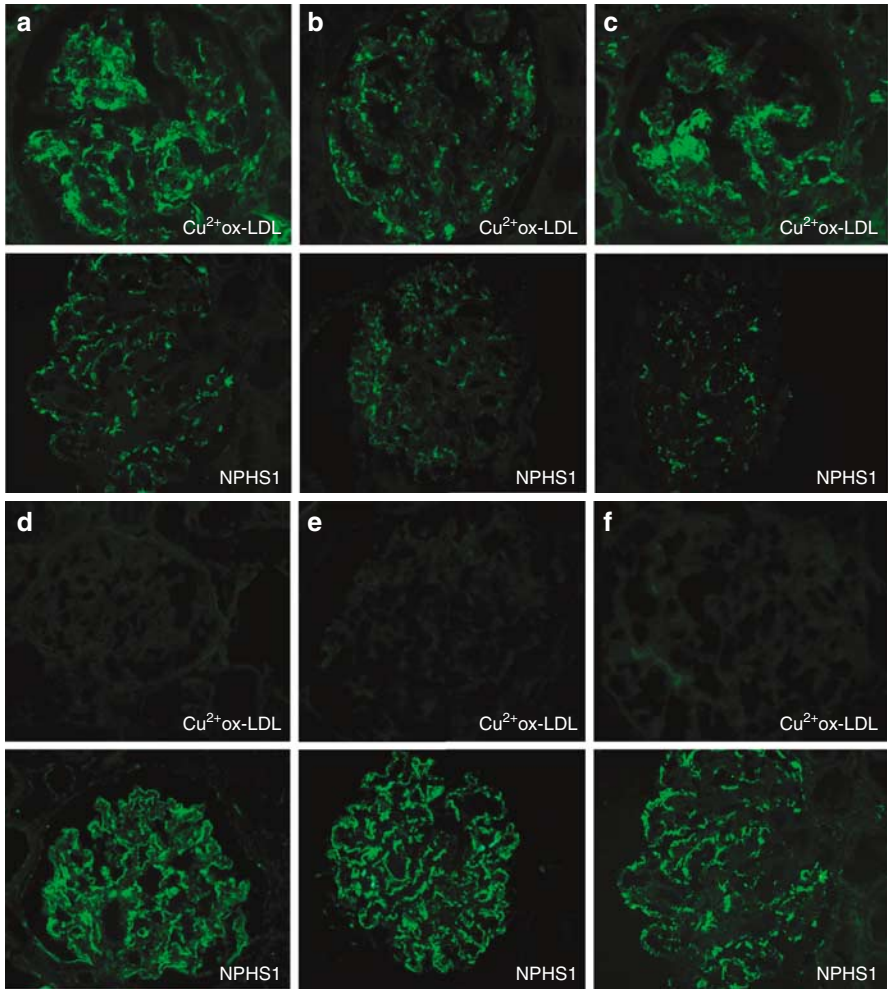


Figure 4 | Qualitative expression of the fluorescence patterns of ox-LDL and NPHS1 at glomerular level. From three out of 15 type II diabetic, hypertensive, microalbuminuric patients, at (**a–c**) baseline and (**d–f**) after 4-year therapy with 40 mg per day of simvastatin. These images are typical examples of three patients ((**a**) 1st patient, (**b**) 2nd patient, (**c**) 3rd patient at baseline, and (**d–f**) after treatment) out of 15 similar analyses carried out in 15 patients before and after treatment with simvastatin treatment.

decrease of ox-LDL and the pleiotrophic effects secondary to simvastatin administration, at glomerular level, might play a role from this point of view.[21] Eventually, the protective effect of statins at glomerular level is not associated with significant changes of the circulating levels of hs-C-reactive protein and interleukin-6. These latter finding do not support the tenet that a modulation of the systemic immunologic action is the most important mechanism accounting for the benefits of simvastatin at renal level.

Whatever the mechanism, which explains the improvement or the maintaining at steady levels of several parameters of renal function after simvastatin, we believe that the present study may be useful to prevent end-stage renal disease in type II diabetes.

MATERIALS AND METHODS

Subjects and methods

Table 1 describes the procedures we followed to recruit type II diabetics with hypertension ($>130/85$ mm Hg), and microalbuminuria, treated by angiotensinsin-converting enzyme inhibitors (5 mg ramipril or 20 mg lisinopril/day), 12.5 mg/day thiazides, and 100 mg/day atenolol in the last 3 years, with a glycemic control accomplished by 1500 mg/day metformin with either three insulin analogs before meals or once daily long-acting insulin injection. The definition of microalbuminuria was a median between 30 and 300 μg/mg of albumin/creatinine, in three consecutive urine specimens (Tables 1 and 2).[29] The main criterion for the inclusion into the study was that a decrease of GFR >1 ml/min/1.73m^2/year had to be observed during the 3 years before the recruitment. The rationale is that a decrease of 1 ml/year is usually observed in all Caucasian subjects older than 50 years.[30] We evaluated GFR from the clearance rate of ^{51}Cr-ethylenediamine tetraacetic acid technique.[16,31] GFR course was evaluated using a permutation approach to a Hotelling's T(2) statistic.[32] A list of 86 consecutive patients who had a GFR decline during the last 3 years of follow-up (see Table 5) >-3 ml/min/1.73 m^2/year were randomly allotted, either to 40 mg/day simvastatin or to 30 g/day cholestyramine treatment. The analysis of the results was carried out on the basis of the intention to treat principle. All the 86 patients were included in the statistical analysis on the basis of the intention to treat principle. In the Result and

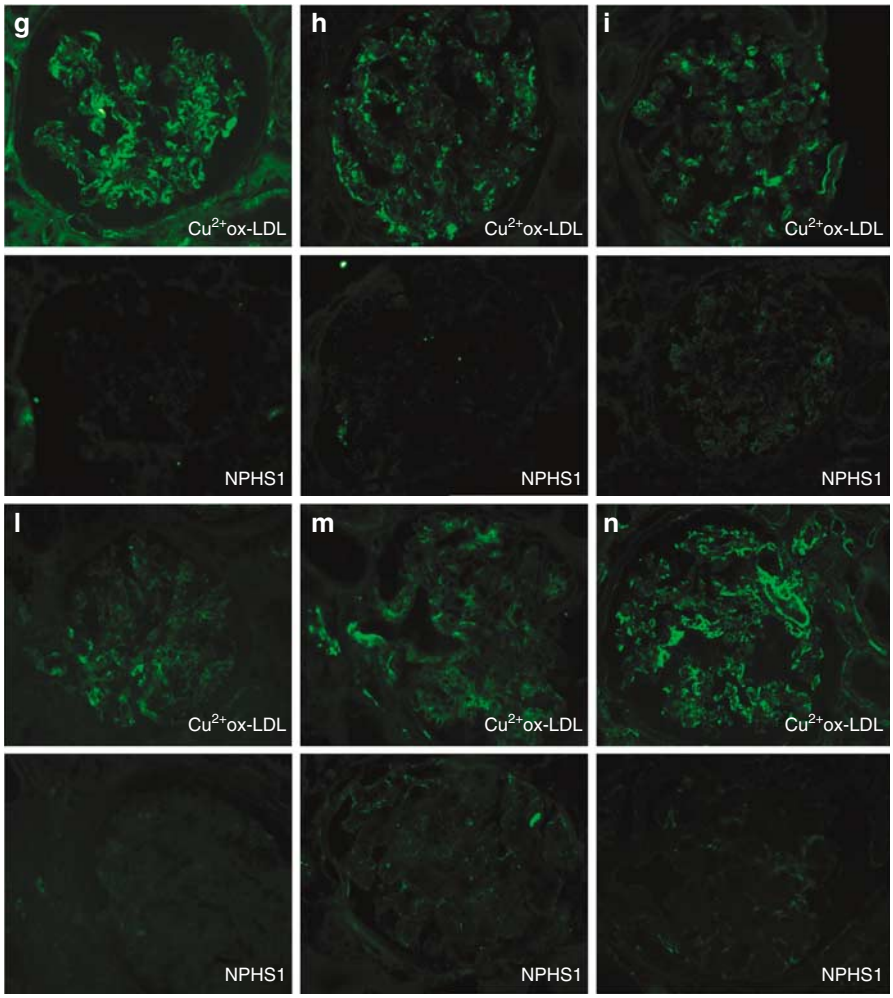


Figure 5 | Qualitative expression of the fluorescence patterns of ox-LDL and NPHS1 at glomerular level from three out of 15 type II diabetic patients, at (g–i) baseline and (l–n) after 4-year therapy with 30 g per day of cholestyramine. These images are typical examples of three patients ((**g**): 1st patient, (**h**) 2nd patient, (**i**) 3rd patient at baseline, and (**l–n**) after treatment) out of 15 similar analyses carried out in 15 patients before and after treatment 30 g per day of cholestyramine.

Table sections we showed the data of 82 patients at baseline. Two patients developed renal cancer. One patient developed myocardial infarction. One patient showed a 3- to 4-fold increase of transaminases above baseline levels during the first 5 months after recruitment. Forty-two patients on simvastatin and 40 on cholestyramine ended the study without clinical side effects (Table 1). Thirty patients out of the above-mentioned two groups of patients were studied as for ox-LDL after random selection (first one yes, the second one no, the third one yes, etc.) after casual mixing of the entire series of patients till the number of 15 was reached in each of the two groups of simvastatin- and cholestyramine-treated patients. Plasma triglycerides were measured as glycerol by colorimetric method after enzymatic hydrolysis. High-density lipoprotein cholesterol levels were measured enzymatically after precipitation of very low-density lipoprotein, intermediate-density-lipoprotein, and LDL from plasma. LDL cholesterol was measured using the formula LDL cholesterol = total cholesterol − high-density lipoprotein cholesterol − triglycerides/5.[33] Measurement of highly specific hs-C-reactive protein levels was obtained by nephelometry with a highly sensitive immunoassay that used a monoclonal antibody coated to polystrene particles (hs-C-reactive protein). The interassay coefficient of variation for the CRP measurement was 4%.[34] Fasted serum levels of interleukin-6 were determined by enzyme-linked immunosorbent assay (Quantitative IL-6; R&D Systems, Oxford, UK).[35] The sensitivity of the assay was 0.70 pg/ml.

Human biopsies

Light microscopy. Kidney biopsies were performed under ultrasound guidance. After kidney biopsy, tissue was immediately examined under a dissecting microscope to ensure adequate numbers of glomeruli. Most of the core was placed in Zenker' fixative, embedded in paraffin, and processed for light microscopy. Light microscopy sections (2 μm thick) were stained with hematoxylin and eosin and periodic acid Shiff. The following classification was adopted: Category 1, normal or near normal renal structure. These patients had biopsies that were normal or showed very mild mesangial expansion, tubulointerstitial changes, or arteriolar hyalinosis in any combination (Figures 1 and 2, panels 1 and 2 and panels 7 and 8). Category 2, typical diabetic nephropathy—these patients had biopsies that were balanced severity of glomerular,

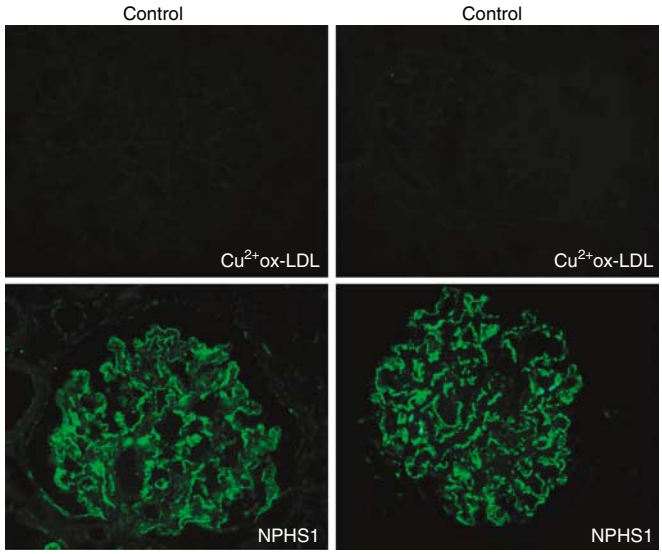


Figure 6 | Qualitative expression of the fluorescence patterns of ox-LDL and NPHS1 in a glomerulus from one control subject out of the five controls, (details in the text), with hypertension at baseline.

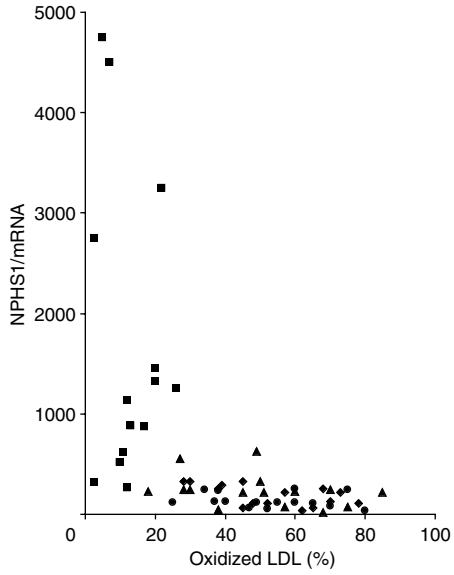


Figure 7 | Quantitative reverse transcriptase-PCR analysis of NPHS1 normalized for mRNA content in relationship with the quantitative evaluation of fluorescence staning of ox-LDL in the 30 diabetic patients before and after simvastatin (15 patients) and cholestyramine (15 patients) treatment (see details in the text for the recruitment and blind evaluation of this cohort of patients from the overall 86 patients). This approach was not applied in Table 7, which reported quantitative reverse transcriptase-PCR analysis of the other SD proteins calculated in the same fashion used for NPHS1 in Figure 7, after correction for the internal standard without further correction for RNA content, which was considered interesting, even if not needed, for the comparison with the glomerular content of ox-LDL. Closed circles represent patients at baseline before simvastatin. Squares represent patients after simvastatin. Rhombs represent patients at baseline before cholestyramine. Triangles represent patients after cholestyramine.

Table 6 | Mean ± s.e. morphologic findings in 82 type 2 diabetic patients treated either with simvastatin or with cholestyramine at baseline and after 4 year follow-up

	Index of mesangial expansion	Index of interstitial thickness	Index of arteriolar hyalinosis
Simvastatin			
B	0.61 ± 0.13	0.38 ± 0.09	0.65 ± 0.17
4 years	0.47 ± 0.09*	0.30 ± 0.17^{NS}	0.44 ± 0.11*
Cholestyramine			
B	0.60 ± 0.15	0.40 ± 0.10	0.67 ± 0.15
4 years	0.59 ± 0.13^{NS}	0.38 ± 0.13^{NS}	0.58 ± 0.16^{NS}

Group 1: simvastatin-treated patients.
Group 2: Cholestyramine-treated patients.
*$P < 0.05$ baseline vs 4th year.
NS=not significant baseline vs 4th year.
Means ± s.e. of the numerical parameters using light microscopy to calculate the percent global glomerular sclerosis, the index of mesangial expansion and that of interstitial thickness in specimens from kidney biopsies in Groups 1 and 2 82 diabetic patients at baseline and after 4-year therapy with 40 mg per day of simvastatin or with 30 g per day of cholestyramine simvastatin.

severe not mild mesangial expansion, tubulointerstitial changes, or arteriolar hyalinosis changes. This picture is typical of that seen in Type I diabetes with peculiar diabetic nephropathy changes (Figures 1 and 2, panels 3 and 4 and panels 9 and 10). More details have been provided further on in the text. Category 3, atypical patterns of renal injury—these patients had absent or only mild glomerular diabetic changes with severe renal structural lesions, including the following: (a) tubular atrophy, tubular basement membrane thickening and reduplication and interstitial fibrosis; (b) advanced glomerular arteriolar hyalinosis commonly associated with atherosclerosis of large vessels; (c) global glomerular sclerosis ($>25\%$) in the presence or absence of mild mesangial expansion, (see later on in the details for this classification (Figure 1 and 2, panels 5 and 6; panels 11 and 12). Other details have been described elsewhere.[4,16,36,37] The index of mesangial expansion (IME) was determined by a semiquantitative estimate of the width of mesangial zones in each glomerulus. 0 was used as normal, 1.0 as twice normal thickness, 2.0 as three times normal thickness, etc. The mean of the grades for each glomerulus for IME was determined for each patient and represents the IME score reported in the current study (Table 6). The mean difference between two readings was 10.5%, with mean differences in IME index of 0.10. The index of interstitial fibrosis was determined as a semiquantitative estimate of the space occupied by fibrous tissue separating cortical tubules; further details are given in.[36] The index of arteriolar hyalinosis was determined as a semiquantitative estimate of replacement of arteriolar smooth muscle by periodic acid Shiff-positive materials.[36] Patients in Category 1 were four in number. Patients in Category 2 were 56 patients with typical diabetic nephropathology (30 in the simvastatin group and 26 in cholestyramine group). Patients in Category 3 were 22 patients who had atypical patterns of renal injury as described before (10 in the simvastatin group and 12 in the cholestyramine group).[4,16,37]

Evaluation of NEPSH1 and other podocyte and SD proteins

Sequences specific for intracellular (amino acid (aa) 101–1126: TEAGSEEDRV RNEYEESQT GERDQ) and extracellular (aa 1039–1056: HQ PSGEPDQLPTEPPSG) oligopeptides were selected over

Table 7 | Mean ± s.d. values of mRNA expression of slit-diaphragm proteins in specimens from kidney biopsy from hypertensive, microalbuminuric type 2 diabetics expressed as % of each protein (CD2AP, FAT, Actn 4, NPHS1, and NPHS2) of the 18S mRNA expression (internal control)

	CD2AP	FAT	Actn 4	NPHS1	NPHS2
Simvastatin					
B	0.049 ± 0.039	0.024 ± 0.010	0.019 ± 0.012	0.012 ± 0.010	0.022 ± 0.017
4 years	1.011 ± 0.042*	0.88 ± 0.15*	0.97 ± 0.16*	0.85 ± 0.22*	0.75 ± 0.19*
Cholestyramine					
B	0.055 ± 0.041	0.026 ± 0.013	0.023 ± 0.014	0.014 ± 0.011	0.025 ± 0.018
4 years	0.060 ± 0.040	0.030 ± 0.011	0.026 ± 0.013	0.019 ± 0.014	0.030 ± 0.022

SD: slit diaphragm.
Means ± s.d. of some SD mRNA expression in specimens from kidney biopsy in Groups 1 and 2 82 diabetic patients at baseline and after 4-year therapy (statistical comparison have been performed taking into consideration all the 86 patients). Expressed as percent of each protein of the 18S mRNA expression (internal control) (statistical comparison have been performed taking into consideration all the 86 patients).
Group 1: simvastatin-treated patients.
Group 2: cholestyramine-treated patients.
*$P < 0.01$ baseline vs 4th year.

the human nephrin sequence (Gene Bank, AF035835) using the PredictProtein program via internet at the European Molecular biology Laboratory network available in the University of Sassari. A similar approach was followed as regards CDA2, Actn4, FAT, and NEPHS2 proteins. These peptides showed no homology to other known protein sequences and were synthesized and purified. The proteins were run through reducing 10% polyacrilamide gel in the Protean Mini-gel electrophoresis system (BioRad Laboratories) and then transferred to nitrocellulose filters (Amersham, Biosciences, Milan, Italy). After blocking for 2 h at room temperature with 3% non-fat-dried milk (Valio, Helsinki, Finland) in phosphate-buffered saline (PBS), the filters were incubated with antibodies Aff 338 (1:5) and Aff 380 (1:5) in PBS containing 1% non-fat-dried milk and 0.002% sodium azide for 1 h at room temperature. The filters were then washed several times in PBS containing 0.2% Tween 20, further incubating with peroxidase-conjugated, affinity-purified goat anti-rabbit immunoglobulin G antibody (Jackson Immunoresearch Lab, West Grove, PA, USA, dilution 1:40) for 1 h at room temperature and washed as above. The bound antibodies were detected with Super Signal enhanced chemiluminiscence substrate (Pierce Rockford, IL, USA). The Aff 338 and Aff 380 rabbit polyclonal antibodies were originally raised against the major splicing variant designated as nephrin alpha, using a recombinant fusion protein alpha 435 as the antigen as described before in the text. Raising polyclonal antibodies against CDA2, Actn4, FAT, and NEPHS2 were accomplished using the extracellular segments of several human podocyte proteins analyzed by hydrophobicity plots using Kyte–Doolittle curves and the scale website. The most hydrophilic portions specific to the protein were selected for peptide synthesis, also using the PredictProtein program via internet. Peptide sequences that were chosen were homologous (maximum of one aa difference) with the corresponding human sequences. Before immunization, 15 ml of preimmune serum was obtained from each rabbit. Two peptides (of 15 aa each) for each protein were used to immunize two rabbits each to raise polyclonal antibodies. The initial immunization was carried out with complete Freund's adjuvant, whereas boosters at days 20, 40, and 60 were given with incomplete Freund's adjuvant. Enzyme-linked immunosorbent assay titers for antigen-specific reactivity were checked after the first bleed, at day 70, using peptide-coated wells. The following antibodies were raised: anti-human antibodies CD2AP, anti-FAT, anti-Actn4, and anti-NPPHS2 (dilution 1:20). Secondary antibodies for immunofluorescence were purchased from Sigma-Aldrich (St Louis, MO, USA).

Immunofluorescence microscopy

Immunofluorescence staininig was performed on 4 μm frozen sections and on semithin frozen sections from kidney specimens. After rinsing with PBS, uspecified binding sites were blocked with 2% fetal calf serum, 2% bovine serum albumin and 0.2% fish gelatin in PBS for at least 30 min. Primary antibodies (prediluited in blocking solution) were applied for 60 min at room temperature. Antigen–antibody complexes were visualized using fluorochrome (Cy2 or Cy3)-conjugated secondary antibodies (Biotrand, Cologne, Germany) prediluited in blocking solutions. Sections were washed with PBS, rinsed with H_2O, and mounted in 15% mowiol (Calbiochem, Bad Sodem, Germany) and 50% glycerol PBS. After overnight drying, specimens were analyzed and documented with Polyvar 2 photomicroscope (Leica, Bensheim, Germany). Simultaneous confocal double fluorescence microscopy was carried out with Zeiss 410 ultraviolet laser scanning microscope (Carl Zeiss, Obertochen, Germany) equipped with appropiate filters. Micrographs were taken using an imaging recorder (Focus Graphics, Foster City, CA, USA) and digitally processed using the Adobe, Phosop 4.0 program. These procedures were basically based on the study of Mundel *et al.*[38] with the necessary modifications.

Ox-LDL preparation and evaluation

Before oxidation, LDL solution at the concentration of 1 mg/ml was dialyzed against sterile PBS at 4°C for 2 h. The oxidation was performed by incubating LDL solution with 10 μmol/l $CuSO_4$ at 37°C for 24 h, according to Jialal *et al.*[39] (Figure 3). In order to check the specificity of the antibody against ox-LDL used in this study, native LDL and Cu^{2+} ox-LDL (10 μg) were run on a 5% sodium dodecyl sulfate polyacrylamide electrophoresis gel and transferred to a nitrocellulose membrane by electroblocking. Membranes were then incubated with primary rabbit anti-Cu^{2+} LDL antibody[39] followed by secondary antibody. Positive reaction products were identified by enhanced chemiluminiscence (ECL, Amersham) and autoradiography. Negative controls were performed concurrently by loading buffer instead of proteins on sodium dodecyl sulfate polyacrylamide electrophoresis gel or by substituting buffer (Figure 3).

mRNA renal expression of podocyte and SD proteins in kidney specimens

mRNA has been extracted from kidney biopsies using the guanidinium thiocyanate phenol–chloroform method with RNA STAT-60. RNA quality and quantity was controlled by microfluid

electrophoresis using the RNA 6000 LabChip on a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) yielding 0.07–2.5 μg of total RNA. From the extracted RNA, single-strand cDNA was obtained using a reverse transcriptase (Superscript II) and random hexamer primers. We designed polymerase chain reaction amplification primers and probes, for each gene, which have been analyzed in an Applied Biosystem analyzer (ABI 7900, Roche, Milano, Italy) in the presence of a standard, to calibrate the reactions. For nephrin, primers were forward 5′-CAGGAGCACGGCATCACA, reverse 5′-TGTTCTCAGTCTGGGATGCAT, and probe was 6FAM-TGCAGGT CACCTTTCCCCCTAGTGC-MGBNFQ. For podocin primers were forward 5′- AATTATATTCCGACTGGGACATCTG, reverse 5′-GCA GGGCAAAAAAAAGAAAAGA, and probe was 6FAM-TTCCTGGA AGAGCCAAAGGCCCTG-MGBNFQ. For the analysis of the remaining genes (CD2AP, Actn4, and FAT), the following kits from ABI (USA) were used: Hs00183713_m1-CD2AP, Hs00245168_m1-ACTN4, Hs00170627_m1-FAT. The reverse transcriptase-PCR reaction contained buffer, 200 μM deoxyribonucleotide triphosphate, 5.5 mM $MgCl_2$, 100 nM forward and reverse primers, 50 nM FAM probe, 0.05 U/μl AmpliTaqGold, and 5 μl cDNA. The thermocycler protocol consisted of 40 cycles (15′ at 95° and 1′ at 60° each). Then, 18S RNA was choosen as internal standard. The internal standard (18S) and the selected genes were analyzed for any single sample in the same batch. All PCR products were confirmed to be the expected cDNA by subcloning the PCR product using the PGEM-T vector system (Promega, Milano, Italy) and then sequencing the cloned fragments. For the selected genes (CD2AP, FAT, Actn4, NPHS1, and NPHS2) we calculated their relative expression in comparison with the internal standard (18S RNA) in each sample. After correction for the 18S, the amount of mRNA was corrected in each single sample, also in comparison with the expression of the same gene measured in a pool of RNA obtained from five non-diabetic subjects (control) undergoing surgical nephrectomy for renal cancer (biopsy obtained from the opposite kidney pole during surgery). From the computer-generated plot of amplification during the reverse transcriptase-PCR reaction, a threshold line was arbitrarily drawn in a position where in all samples both internal standard (18S) and the genes under evaluation were in the exponential phase. This threshold line identifies in the exponential part of the plot a cycle, which is a direct function of the original amount of mRNA in the sample, called cycle threshold (C_t). Correction for the internal standard and control sample is carried out using the following formula: C_t gene$-C_t$ 18S $=\Delta C_t$ then ΔC_t sample $-\ \Delta C_t$ control $=\Delta\Delta C_t$. The relative expression of the sample under analysis for each gene is finally calculated as $-2^{\Delta\Delta C_t}$. Data on this methodology has been previously presented.[16] NPHS1 was also expressed after normalization for extracted RNA to evaluate its relationship with oxidized LDL in 15 renal human biopsies in the group on simvastatin and in 15 renal human biopsies in the group on cholestyramine and in five normal controls (patients with hypertension who underwent surgical removal of one kidney for cancer).[16]

Analysis of 8-OH-dG in human urine

Urinary 8-OH-dG levels were measured using the automated coupled-column HPLC-ECD system according to the method of Kasai.[22,23] The 8-OH-dG used for standards was obtained from Sigma (Milano, Italy). The reversed phase column (Capcell Pak C18, 5 μM, 4.6 × 250 mm^2) used in HPLC-2 for analysis of the 8-OH-dG fraction was purchased from Shiseido (Japan). HPLC grades of methanol and acetonitrile were purchased from Wako Pure Chemical Industries and the Kanto Chemical Co. (Milano, Italy).

Primary end point

We aimed to evaluate the number of patients with a decrease of GFR > 3 ml/min/1.73m^2/year, during a follow-up period of 4 years (range 3.7–4.2) in two groups of patients treated either by simvastatin (43 patients) or by cholestyramine (43 patients). The study was approved by the Ethical Committee of the University of Sassari.

Calculation of the size of the subjects to be studied in each group

The calculation of the size of subjects was based on the following formula:. Bilateral significativity 0.05 with a power of 80%, if we assume a difference between the two groups of 0.251.[24] If $z_{2\text{alpha}} = 1.96$, $z_{2\text{beta}} = 1.64$, sigma = 3.5 (see[24] as for the coefficient of error for repeated GFR measurement), and delta = 4.25 we have

$$\text{Number} = \frac{2\,\text{Sigma}_T^2(\ z\,\text{alpha}/2 + \ z\,\text{beta})^2}{\text{delta}^2}$$

Thus, in each group we needed at least 35 subjects. However, we studied 43 patients in each group, because we were not sure that all the patients would have accepted the entire protocol.

Statistics

Data are reported as mean ± s.e. or s.d. when normally distributed, and as medians with ranges when the data were not normally distributed. The unpaired and paired Student's *t*-test for parametric parameters, the Wilcoxon test for non-parametric parameters, and the quadratic chi test for the comparison of the data expressed as percentages were calculated using the software SPSS analysis approach.[24]

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