# Oral Sulodexide Reduces Albuminuria in Microalbuminuric and Macroalbuminuric Type 1 and Type 2 Diabetic Patients: The Di.N.A.S. Randomized Trial

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*Abstract.* Diabetic nephropathy may be effectively prevented and treated by controlling glycemia and administering angiotensin-converting enzyme (ACE) inhibitors. However, strict metabolic control can be difficult, and ACE inhibitors may be poorly tolerated and only partially effective, particularly in diabetes mellitus type 2 (DM2), warranting the search for ancillary treatment. Sulodexide is a glycosaminoglycan, a new class of drug that has demonstrated nephroprotective activity in experimental investigations. The Di.N.A.S. study was a randomized, double-blind, placebo-controlled, multicenter, dose-range finding trial to evaluate the extent and duration of the hypoalbuminuric effect of oral sulodexide in diabetic patients. A total of 223 microalbuminuric and macroalbuminuric DM1 and DM2 patients with serum creatinine $\leq$150 $\mu$mol/L and stable BP and metabolic control were recruited. They were randomly allocated to one of four groups: 50 mg/d, 100 mg/d, or 200 mg/d sulodexide daily or placebo for 4 mo (T0 to T4), with 4 mo of follow-up after drug suspension (T4 to T8). Treatment with 200 mg/d sulodexide for 4 mo significantly reduced log albumin excretion rate (logAER) from 5.25 $\pm$ 0.18 at T0 to 3.98 $\pm$ 0.11 at T4 ($P < 0.05$), which was maintained till T8 (4.11 $\pm$ 0.13; $P < 0.05$ *versus* T0). Moreover, the sulodexide-induced percent reductions in AER at T4 were significantly different from the placebo value at T4 and approximately linear to dose increments (30% [confidence limits, 4 to 49%], $P = 0.03$; 49% [30 to 63%], $P = 0.0001$; and 74% [64 to 81%], $P = 0.0001$ in the sulodexide 50, 100, and 200 mg/d groups, respectively. At T8, the sulodexide 200 mg/d group maintained a 62% (45 to 73%) AER significant reduction *versus* placebo ($P = 0.0001$). Subanalysis by type of diabetes (DM1 *versus* DM2, microalbuminuric *versus* macroalbuminuric, or on concomitant ACE inhibitors *versus* not on ACE inhibitors) demonstrated similar findings. These effects were obtained without any significant variation in metabolic control and BP or serum creatinine. Very few adverse events were reported; none were serious. In conclusion, a 4-mo course of high doses of sulodexide significantly and dose-dependently improves albuminuria in DM1 and DM2 patients and micro- or macroalbuminuric patients with or without concomitant ACE inhibition. The effect on albuminuria is long-lasting and seemingly additive to the ACE inhibitory effect.

Diabetes is the most common cause of end-stage renal disease (ESRD) in Western countries. In the United States, diabetes currently accounts for 44% of all new cases of ESRD (1). Despite advances in clinical care, the incidence of diabetes mellitus type 2 (DM2)–related cases of ESRD is rapidly increasing (2), and survival of DM-related ESRD patients on dialysis is markedly low (3,4).

The anatomic hallmarks of diabetic nephropathy (DN) include thickening of the glomerular basement membrane (GBM) and mesangial expansion with hyalinosis both in the mesangium and capillary lumen. These lesions lead to glomerular fibrosis, which progressively destroys the renal filtration unit, and eventually cause renal failure. A number of reports indicate the involvement of transforming growth factor-$\beta$ (TGF-$\beta$) in the development of DN (5). One of the first clinical markers of DN is microalbuminuria (6), commonly considered either hemodynamic in origin (7), due to endothelial dysfunction (8), or biochemical, due to alteration in GBM glycosami-

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noglycan composition, leading to an abnormal permselectivity (9).

Data from the Diabetes Control and Complications Trial (10) established that glycemic control plays a central role in the prevention and treatment of DN as shown by the effect on microalbuminuria and proteinuria. A number of studies have demonstrated that angiotensin converting enzyme inhibitors (ACEI) are also effective in reducing albuminuria and slowing the progression from DN to renal failure (11). Interestingly, both strategies can inhibit renal overexpression of TGF-$\beta$, which may constitute a pharmacologic target for the prevention and treatment of DN (12).

The search for innovative and ancillary approaches to the prevention and treatment of DN is warranted because strict metabolic control can be difficult and sometimes dangerous; even DM patients responding to ACEI therapy and metabolic control show progressive renal damage and eventually ESRD (10,11). A number of drugs are currently being investigated, glycosaminoglycans (GAGs; see Appendix) are particularly interesting because they theoretically can target the generalized endothelial dysfunction and the metabolic defect in matrix and basement membrane synthesis, which, according to the Steno hypothesis, are responsible for DN and possibly also for the high rate of cardiovascular mortality observed in DN patients (9,13).

Experiments in rats with streptozotocin-induced DM demonstrated that low molecular weight (LMW) heparin and other GAGs prevented diabetes-induced albuminuria, loss of anionic sites and thickening of the GBM, and glomerulosclerosis (14–16). Interestingly, we have demonstrated that these favorable effects accompany inhibition of renal TGF-$\beta$ (17). Furthermore, GAGs have been shown to restore anionic charges lost from the endothelial surface (18) and a number of other endothelial dysfunctions relevant to diabetic micro- and macroangiopathy (13). Recent explorative studies have also described favorable results on albuminuria in DN patients treated with a LMW heparin (19,20), danaparoid, a mixture of sulfated GAGs consisting mainly of heparan sulfate (21), and sulodexide (22–29).

Sulodexide is composed of the two GAGs that are active in preventing diabetic nephropathy in the experimental model (14). It has also been shown to inhibit TGF-$\beta$ overexpression and matrix synthesis induced by high concentrations of glucose in mesangial cells (Gambaro G and Schleicher E, manuscript in preparation) to the same extent as the single components and to rectify endothelial dysfunctions observed in DM (13).

The small number of patients investigated in the above pilot studies means a number of clinical issues have been left unsolved, particularly the optimal dosage of oral sulodexide. The Diabetic Nephropathy and Albuminuria Sulodexide (Di.N.A.S.) study was designed to answer this question.

## Materials and Methods

The Di.N.A.S study was a randomized double-blind, placebo-controlled, multicenter dose-range finding trial for sulodexide composed of 80% fast-moving heparin and 20% dermatan sulfate (Vessel 2F, Alfa Wassermann SpA, Bologna, Italy). The study was approved by the ethics committee or institutional review board of the tertiary level health institutions located in the Czech Republic (three centers), Poland (five centers), Slovak Republic (two centers), and Italy (two centers). All patients gave written informed consent in accordance with the Declaration of Helsinki.

### *Participants and Definition*

Study participants were DM1 and DM2 patients of either gender aged between 18 and 65 yr with micro- (20 to 200 $\mu$g/min) or macro- (>200 $\mu$g/min) albuminuria. They were required to have stable metabolic control: ≤10% variation from baseline in monthly HbA1C levels for over 3 mo with either insulin, oral antidiabetic agents, or diet and BP <160/90 mmHg for at least 6 mo with or without antihypertensive therapy. Exclusion criteria were as follows: neoplasms; severe liver, cardiac, or systemic disease; known hypersensitivity to any GAGs; chronic treatment with corticosteroids, corticothropine, immunosuppressants or alkylating agents; serum creatinine >150 $\mu$mol/L; urinary protein excretion rate >3 g/24 h; symptomatic urinary tract infections; hematuria; pregnancy or lactation.

### *Sample Size*

Bearing in mind the primary dose-range finding aim of the study, the sample size was estimated according to two independent hypotheses: (*1*) rejection of the hypothesis that P1 = P2 (where P is the number of patients with a 50% reduction in albuminuria after 4 mo of therapy); (*2*) rejection of the null hypothesis that $\mu 1 = \mu 2 = \mu 3$ after 4 mo of therapy, only if each independent variable accounts for at least 20% of the variation (behavior of albuminuria), $\alpha = 0.05$ and 1-$\beta$ is 90% (analyzed by one-way ANOVA with groups 1 to 3 containing the same number of subjects but treated with 3 different doses of sulodexide). A 20% withdraw and drop-out rate was also added to the calculation. Altogether, 88 patients were necessary (22 per treatment group). Therefore, the number of patients enrolled (55 per group, making a total of over 220 patients) was considered adequate. The analyses performed on the subgroups should be considered observational and not inferential.

### *Study Design*

**Randomization and Blinding.** After screening and baseline evaluation, a computer-generated block randomization list (8 per block) prepared by the Sponsor's Medical Department was used to assign all eligible patients to treatment with 25 mg, 50 mg, or 100 mg oral sulodexide twice daily or placebo. There was no stratification for clinical characteristics. Each center was expected to receive the same number of blocks. Clinical trial drug supply was managed by Univa, Bolton, UK. The study medication and placebo were packaged indistinguishably and labeled with a patient number.

**Treatment.** Each patient received four gelcaps twice daily: in the placebo group, 4 placebo gelcaps; in the 50 mg/d group, 1 sulodexide gelcap and 3 placebo gelcaps; in the 100 mg/d group, 2 sulodexide gelcaps and 2 placebo gelcaps; in the 200 mg/d group, 4 sulodexide gelcaps.

Treatment lasted for 4 mo (T0 to T4) with a subsequent 4-mo follow-up period (T4 to T8), as shown in Figure 1. Each patient was examined by a physician at randomization, every month during treatment, and every 2 mo during follow-up. At each examination, body weight (BWt) and BP were measured and complete urinalysis was performed, including albumin excretion rate (AER) on one (at T1, T2, T3, T6) and three (at T0, T4, T8) timed overnight collections. Compliance was evaluated by pill count at each monthly visit. Complete blood count, aPTT, fibrinogen, HbA1C, blood chemistry (glucose, lipids, urea, creatinine, total protein, albumin, and liver functions),

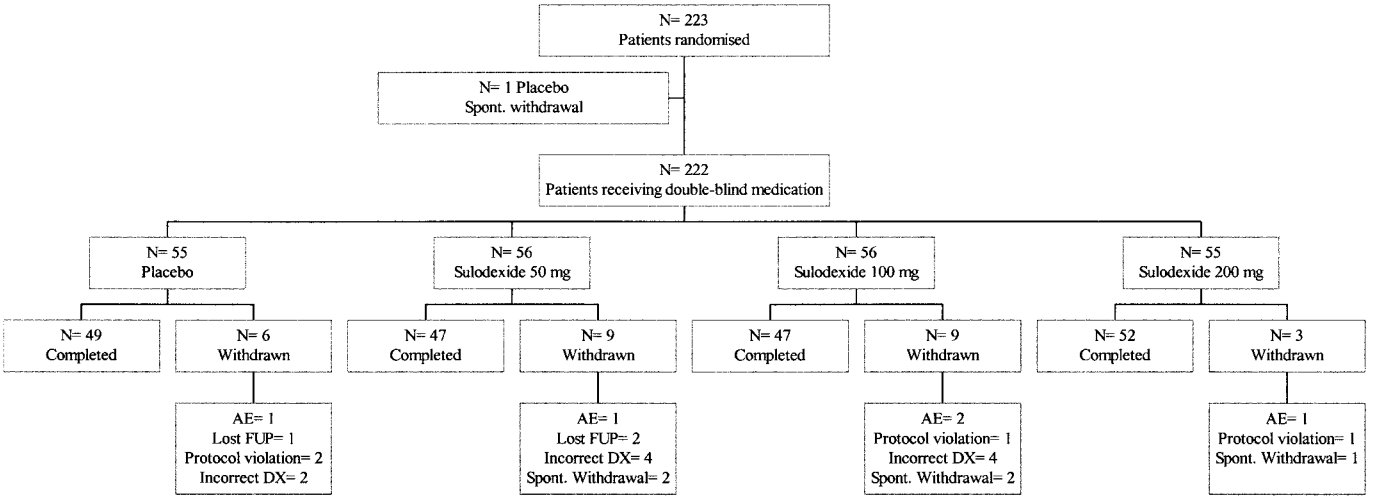


*Figure 1.* Disposition of patients. AE, adverse effect; DX, diagnosis; FUP, follow-up.

and a funduscopic examination were carried out on entry and at 4 and 8 mo.

The first patient was enrolled on October 17, 1996, and last patient contact was on December 14, 1998. A policy committee reviewed all medical, ethical, and statistical issues. A quality-control evaluation committee confirmed all diagnoses.

## *Outcome Measures*

The primary outcome measure was AER at T0, T4, and T8 determined by a turbidimetric method using antibodies against human albumin. Patients were asked to collect three overnight, 8-h urine samples for each time point, indicating the beginning and end of the collection period. The adequacy of urine collection was evaluated by both interviewing the patient and determining urine creatinine excretion. If incomplete, urine collection was immediately repeated. Although sample size limits the subgroup analyses, the secondary outcome measures included AER in specific patient subgroups (all DM1 patients, all DM2 patients, all initially microalbuminuric patients, and all initially macroalbuminuric patients) at T0, T4, and T8. Additional secondary end points included the assessment of AER at T0, T4, and T8 in all patients receiving concomitant ACEI therapy.

## *Statistical Analyses*

Analyses were performed according to the intent-to-treat model on all randomized patients in their assigned groups, regardless of adherence to treatment regimen. Analysis was also performed on data from the evaluable patients. Baseline values between groups were compared using the Kruskal-Wallis test for continuous variables and the $\chi^2$ test for categorical variables. The primary endpoint was changes in AER. Three different analyses were performed:

1. To evaluate the dose-dependency of the drug effect we compared variations in AER at T4 and T8 in the three sulodexide groups with the T4 and T8 values in the placebo group. The analysis was carried out on log-transformed AER values due to the skew distribution of the AER. Analysis of covariance (ANCOVA) adjusted for baseline logAER was used. Separate analyses were performed for T4 and T8. To allow for the possible confounding effect of BP and metabolic control, the overall ANCOVA analyses of AER were also repeated with mean arterial pressure or HbA1c introduced as additional covariates.
2. Efficacy was evaluated by assessing variations from baseline AER at T4 and T8 in each group and subgroup. Log-transformed AER values were considered. ANOVA and the Bonferroni *t* test for multiple comparisons were used to evaluate percent reductions in baseline (T0) AER at T4 and T8 in each group.
3. Efficacy was also evaluated in the whole group by considering patients as responders or nonresponders. Responders were defined as showing a ≥50% reduction of basal AER at T4. Values at this time point are better defined, being the average of three determinations. Empirical values were used, rather than log-transformed data. Time of improvement was the first time in which a ≥50% reduction of the T0 AER value was observed, if confirmed by the T4 value. For this analysis, the log-rank test was used with correction for multiple comparisons. Patients who failed to maintain the sulodexide effect (>50% reduction of basal AER at T4) during the follow-up period (T4 to T8) were defined as relapsers.

For the statistical approaches in 1 and 2, the percent reduction in AER achieved by each sulodexide group relative to T0 or placebo, for each timepoint was calculated as:

$$\text{percent reduction} = 1 - \exp(\Delta)$$

where $\Delta$ is the difference in adjusted mean logAER at T4 and T8 *versus* the placebo group (analysis in 1) or the respective T0 value (analysis in 2).

For analysis purposes, the logAER (or AER in the statistical approach in 3) at a given time point was defined as the average of the logAERs (or AERs) of the three measurements. Logistic regression analysis was also performed; AER at T4 or T8 compared with T0 placebo was considered a dependent variable, and baseline AER values were considered independent variables. It was not considered appropriate to introduce center as a factor in the statistical analysis because each center entered a relatively small number of patients (mean = 17) split across four treatment groups.

The intent-to-treat data are presented; however, efficacy and dose-range finding analyses for the evaluable patients (195 subjects) at completion of treatment (T4) and completion of follow-up (T8) produced similar results (data not shown).

*Table 1.* Clinical characteristics of patient population at time of randomization[a]

| Variable | Placebo ($n = 56$) | 50 mg/d Sulodexide ($n = 56$) | 100 mg/d Sulodexide ($n = 56$) | 200 mg/d Sulodexide ($n = 55$) | *P* |
|---|---|---|---|---|---|
| Mean age (yr) | 47.0 ± 12.8 | 49.0 ± 12.4 | 47.4 ± 12.1 | 46.9 ± 12.5 | 0.745 |
| Body wt (kg) | 78.8 ± 14.1 | 77.8 ± 12.8 | 79.5 ± 13.9 | 79.9 ± 13.4 | 0.929 |
| Body mass index | 27.7 ± 5.1 | 27.4 ± 4.3 | 26.4 ± 4.2 | 27.5 ± 4.8 | 0.536 |
| Systolic BP (mmHg) | 140.9 ± 16.1 | 139.6 ± 14.2 | 136.1 ± 15.0 | 139.7 ± 15.0 | 0.316 |
| Diastolic BP (mmHg) | 80.7 ± 8.0 | 82.6 ± 6.6 | 82.1 ± 6.6 | 82.8 ± 8.0 | 0.672 |
| Patients on ACEI/not on ACEI (*n*) | 30/26 | 27/29 | 27/29 | 32/23 | 0.921 |
| DM1/DM2 patients (*n*) | 30/26 | 30/26 | 33/23 | 31/24 | 0.930 |
| Duration of diabetes | 17.2 ± 10.4 | 16.0 ± 9.6 | 15.8 ± 8.4 | 16.2 ± 10.3 | 0.923 |
| HbA1c (%) | 8.8 ± 1.5 | 8.3 ± 1.8 | 8.2 ± 1.4 | 8.4 ± 1.7 | 0.084 |
| Serum creatinine (mmol/L) | 90.4 ± 22.9 | 89.2 ± 18.5 | 94.9 ± 21.2 | 93.1 ± 21.2 | 0.460 |
| AER $\mu$g/min (log) | 5.10 ± 1.44 | 4.62 ± 1.21 | 4.58 ± 1.24 | 5.25 ± 1.34 | 0.013[b] |
| Macro/microalbuminuric patients (*n*) | 26/30 | 16/40 | 17/39 | 28/27 | 0.027[b] |

[a] Values are mean ± SD or absolute numbers (*n*). ACEI, angiotensin-converting enzyme inhibitors, DM1, diabetes mellitus type 1; DM2, diabetes mellitus type 2

[b] Statistically significant value.

## Results

A total of 223 patients were allocated to the study. Table 1 summarizes the baseline clinical characteristics of the four groups. No significant difference was found between groups for age, BWt, body mass index, type of diabetes, duration of disease, frequency of hypertension and ACEI treatment, metabolic control, or serum creatinine. However, random allocation resulted in baseline logAER in patients in the 50 and 100 mg/d groups being slightly, but significantly, higher than in the placebo and 200 mg/d groups. Baseline AER values in the placebo and 200 mg/d groups were similar, as were those in the 50 and 100 mg/d groups. This trend was constant in all further subgroups: type of diabetes, micro-/macroalbuminuria range, on/not on ACEI.

### *Clinical Parameters and Adverse Effects*

Treatment and follow-up were completed by 209 of 223 patients. Fourteen patients dropped out due to adverse effects ,were lost at follow-up, or spontaneously withdrew (Figure 1). The quality-control evaluation committee recognized protocol violations or incorrect diagnosis in another 14 patients. These 28 unevaluable patients constitute 12.6% of the whole group, a figure lower than the estimated 20% withdrawl and drop-out rate added to the calculated sample size. During the whole study period, patients did not alter their usual diet, insulin, oral antidiabetic, or antihypertensive treatment. Overall patient compliance assessed by pill count at each monthly visit was excellent (approximately 96%); there was no difference in compliance between groups. Routine blood chemistry and hematologic parameters did not significantly change in any group (data not shown). Moreover, arterial BP, HbA1C (Table 2), and serum creatinine were comparable in the four groups and did not change significantly throughout the study. No serious adverse event related or unrelated to sulodexide was reported; particularly, no patient died or had an acute cardiovascular event or major variations in funduscopic examination. Adverse events leading to withdrawal from the study were as follows: rash (two cases), diarrhea (two cases), muscoloskeletal symptoms (one case). There was no substantial difference between groups for the number of unevaluable patients or prevalence and type of adverse event. The largest category of unevaluable patients was "incorrect diagnosis," ten cases (Figure 1). This was mostly accounted for by patients with microalbuminuria who resulted normoalbuminuric in ≥2 out of 3 collections at

*Table 2.* Dose-response effect of sulodexide on BP and HbA1c[a]

| Group | Systolic BP (mmHg) | | Diastolic BP (mmHg) | | HbA1C | |
|---|---|---|---|---|---|---|
| | T4 | T8 | T4 | T8 | T4/T0 | T8/T0 |
| Placebo | 139.6 ± 16.4 | 139.7 ± 18.2 | 81.6 ± 8.6 | 82.1 ± 8.4 | 0.99 ± 0.2 | 0.96 ± 0.2 |
| 50 mg/d sulodexide | 134.6 ± 15.0 | 138.0 ± 15.9 | 82.1 ± 6.4 | 82.0 ± 6.9 | 0.98 ± 0.1 | 0.97 ± 0.2 |
| 100 mg/d sulodexide | 133.9 ± 17.0 | 137.1 ± 16.3 | 82.0 ± 5.6 | 81.9 ± 6.8 | 1.01 ± 0.2 | 0.96 ± 0.2 |
| 200 mg/d sulodexide | 136.8 ± 18.9 | 137.9 ± 24.0 | 81.2 ± 13.8 | 80.4 ± 13.1 | 0.98 ± 0.2 | 0.95 ± 0.2 |

[a] Values are mean ± SD. T4, fourth and final month of treatment; T8, fourth and final month of follow-up; T0, beginning of treatment. T4/T0 and T8/T0 are the ratios between the respective values.

enrollment, a common finding as observed by Caramori *et al.* (30).

### *Dose Dependency of the Sulodexide Effect*

The primary goal of this study was to evaluate the dose-relationship of sulodexide hypoalbuminuric activity. Unfortunately, the four treatment groups differed in terms of baseline AER values, and the use of a single randomization list, rather than a list balancing enrollment between subgroups in every center, introduced a distortion between treatment groups. To obtain an insight into the dose-range finding of sulodexide, we used the ANCOVA statistical analysis, which allowed the four groups to be compared by adjusting baseline albuminuria values. Table 3 shows the adjusted mean of logAER in the four groups at the end of treatment (T4) and follow-up (T8). The overall ANCOVA F test for differences across the four groups was significant both at T4 and T8 with $P < 0.0001$ (Table 3). Figure 2 represents the percent reduction in AER (*versus* the placebo group) in each of the three sulodexide groups at T4 and T8. At T4, AER was 30%, 49%, and 74% lower than the placebo group in patients given 50, 100, and 200 mg/d sulodexide, respectively. The reduction in AER was statistically significant in each sulodexide group ($P < 0.03$, 0.0001, and 0.0001), with approximately linear dose-response (Figure 2A). At T8, the AER of the 50 mg/d sulodexide group was nearly identical to placebo, whereas the 100 and 200 mg/d sulodexide groups continued to have lower AER levels. For the 100 mg/d sulodexide group (29% reduction), the *P* value indicates a difference that is not quite statistically significant ($P = 0.07$), whereas the difference *versus* placebo (62%) in the 200 mg/d group was highly statistically significant ($P < 0.0001$). Thus the percent reduction in AER at T8 seems to persist at high levels, with approximately linear dose-response (Figure 2B). Very similar data were obtained by analyzing data in the different patient subgroups (data not shown). Results in the whole group and subgroups were furthermore confirmed after introduction of mean BP and HbA1c values as additional covariates in the ANCOVA analysis (not shown).

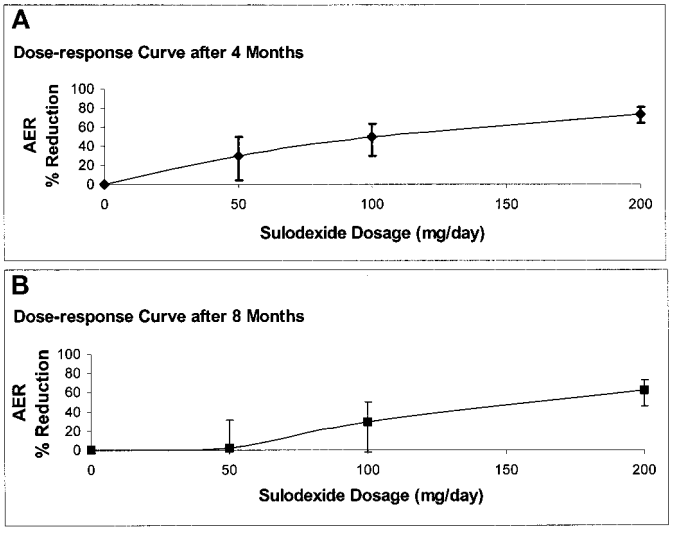


*Figure 2.* Sulodexide's dose-response curves.

### *Efficacy Evaluation of Sulodexide Treatment*

**Effect of Sulodexide on AER in the Whole Study Group.** The highest dosage of sulodexide (200 mg/d) reduced baseline albuminuria by 43%, which was statistically significant ($P < 0.05$; Table 3). Interestingly, the T8 AER value was 30% lower than the T0 basal value ($P < 0.05$; Table 3). The hypoalbuminuric effect of sulodexide is highlighted if the rate of responders in the four treatment groups is considered. There were no responders in the placebo group, and the number of responders increased with increasing sulodexide dosage, reaching statistical significance with 200 mg/d ($P < 0.0001$; Figure 3A). In this group, there was no difference in the number of DM1 and DM2 responders (data not shown), and 60% of micro- and 40% of macroalbuminuric patients were responders. These data analyses also confirm the long-lasting hypoalbuminuric effect. At T8, more than 60% of responders in the 200 mg/d group (16 of 25) maintained AER 50% lower than baseline values (Figure 3B).

No correlation emerged between the hypoalbuminuric effect

*Table 3.* Effect of sulodexide on albumin excretion in the whole study group[a]

| Group | Log AER | | | AER% Reduction (Efficacy Analysis) | | AER% Reduction (Dose-Range Finding Analysis) | |
|---|---|---|---|---|---|---|---|
| | T0 | T4 | T8 | T4 *versus* T0 | T8 *versus* T0 | T4 *versus* T4 Placebo | T8 *versus* T8 Placebo |
| Placebo | 5.10 ± 0.19 | 5.31 ± 0.11 | 5.07 ± 0.13 | | | | |
| 50 mg/d sulodexide | 4.62 ± 0.16 | 4.95 ± 0.12 | 5.05 ± 0.13 | | | 30[b] (4 to 49) | |
| 100 mg/d sulodexide | 4.58 ± 0.17 | 4.63 ± 0.12 | 4.73 ± 0.13 | | | 49[c] (30 to 63) | |
| 200 mg/d sulodexide | 5.25 ± 0.18 | 3.98 ± 0.11 | 4.11 ± 0.13 | 43[d] (31 to 54) | 30[d] (8 to 51) | 74[c] (64 to 81) | 62[c] (45 to 73) |

[a] Values are log mean ± SEM or mean and confidence limits. AER, albumin excretion rate. Only statistical significant reductions are reported.
[b] $P < 0.03$.
[c] $P < 0.0001$ *versus* placebo.
[d] $P < 0.05$ *versus* T0.

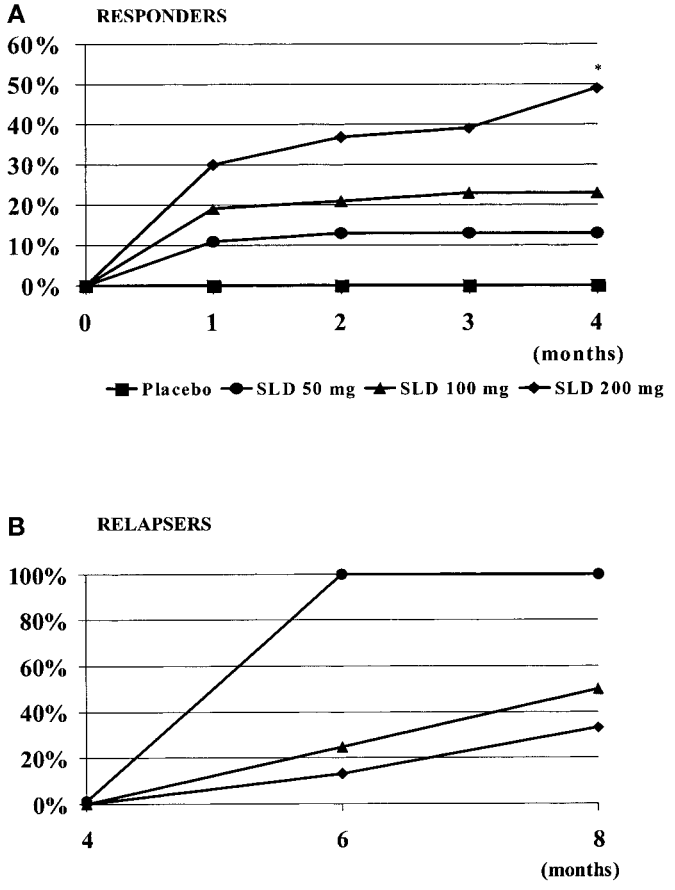


*Figure 3.* Responders (A) and relapsers (B) in the whole study group. The analysis was performed on the whole group of patients. A responder is a patient disclosing an AER reduction ≥50% of his/her basal (T0) value during the sulodexide treatment; a relapser is the responder at T4 who loses the AER reduction of ≥50% of his/her basal (T0) value effect in the follow-up period. * statistically signific-sant variation.

of sulodexide and baseline values of AER, duration of diabetes, basal and T4/T0 ratios of HbA1c, BP, cholesterol, triglycerides, blood-urea nitrogen, creatinine, creatinine clearance, aPTT, or fibrinogen.

**Effect of Sulodexide on AER in DM1 and DM2 Patients.** Sulodexide was shown to be effective in both DM1 and DM2 patients (Table 4). In DM1 patients, a 17% reduction in baseline AER was observed in the 100 mg/d sulodexide group, and 44% in the 200 mg/d group (both $P < 0.05$). Interestingly, AER was still 23% lower than baseline in the 200 mg/d group at the end of follow-up ($P < 0.05$).

Similarly, a 40% reduction in AER was observed in DM2 patients in the 200 mg/d group ($P < 0.05$). Again, the AER was significantly (38%) lower than baseline at T8 ($P < 0.05$).

**Effect of Sulodexide on AER in Micro- and Macroalbuminuric Diabetic Patients.** Both micro- and macroalbuminuric patients were sensitive to sulodexide (Table 5). Administration of 100 and 200 mg/d sulodexide reduced baseline AER in microalbuminuric patients by 23% and 40%, respectively (both $P < 0.05$). In the 200 mg/d group, reduction in AER was

*Table 4.* Effect of sulodexide on albumin excretion in DM1 and DM2 (efficacy evaluation)[a]

| Group | DM1 Log AER | | | DM1 AER% Reduction *versus* T0 | | DM2 Log AER | | | DM2 AER% Reduction *versus* T0 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T0 | T4 | T8 | T4 | T8 | T0 | T4 | T8 | T4 | T8 |
| Placebo | 4.80 ± 0.26 | 5.16 ± 0.17 | 5.07 ± 0.19 | | | 5.47 ± 0.29 | 5.51 ± 0.15 | 5.12 ± 0.17 | | |
| 50 mg/d sulodexide | 4.67 ± 0.23 | 4.99 ± 0.16 | 5.07 ± 0.19 | | | 4.57 ± 0.23 | 4.87 ± 0.16 | 4.99 ± 0.18 | | |
| 100 mg/d sulodexide | 4.61 ± 0.22 | 4.51 ± 0.17 | 4.59 ± 0.19 | 17[b] (−3 to 36) | | 4.55 ± 0.25 | 4.77 ± 0.16 | 4.92 ± 0.17 | | |
| 200 mg/d sulodexide | 5.26 ± 0.24 | 3.94 ± 0.16 | 4.09 ± 0.18 | 44[b] (31 to 58) | 23[b] (−14 to 60) | 5.24 ± 0.28 | 4.02 ± 0.16 | 4.12 ± 0.17 | 40[b] (20 to 61) | 38[b] (20 to 56) |

[a] Values are log mean ± SEM or mean and confidence limits. Only statistically significant reductions are indicated.
[b] $P < 0.05$ *versus* T0.

*Table 5.* Dose-response effect of sulodexide on albumin excretion in micro- and macroalbuminuric diabetic patients (efficacy evaluation)[a]

| Group | Microalbuminuria Log AER | | | Microalbuminuria AER% Reduction *versus* T0 | | Macroalbuminuria Log AER | | | Macroalbuminuria AER% Reduction *versus* T0 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T0 | T4 | T8 | T4 | T8 | T0 | T4 | T8 | T4 | T8 |
| Placebo | 3.94 ± 0.14 | 4.33 ± 0.18 | 4.03 ± 0.20 | | | 6.41 ± 0.14 | 7.02 ± 0.10 | 6.81 ± 0.15 | | |
| 50 mg/d sulodexide | 4.02 ± 0.12 | 3.98 ± 0.15 | 4.03 ± 0.17 | | | 6.12 ± 0.16 | 6.59 ± 0.14 | 6.59 ± 0.20 | | |
| 100 mg/d sulodexide | 3.93 ± 0.12 | 3.63 ± 0.16 | 3.74 ± 0.17 | 23[b] (8 to 39) | | 6.10 ± 0.16 | 6.34 ± 0.13 | 6.27 ± 0.19 | | |
| 200 mg/d sulodexide | 4.17 ± 0.14 | 2.97 ± 0.19 | 3.00 ± 0.20 | 40[b] (18 to 63) | 31[b] (−14 to 75) | 6.29 ± 0.17 | 5.68 ± 0.09 | 5.84 ± 0.13 | 45[b] (35 to 54) | 29[b] (14 to 44) |

[a] Values are log mean ± SEM or mean and confidence limits. Only statistical significant reduction are reported.
[b] $P < 0.05$ *versus* T0.

*Table 6.* Dose-response effect of sulodexide on albumin excretion in patients with or without ACE inhibitors (efficacy evaluation)[a]

| Group | ACEI Log AER | | | ACEI AER% Reduction *versus* T0 | | No ACEI Log AER | | | No ACEI AER% Reduction *versus* T0 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T0 | T4 | T8 | T4 | T8 | T0 | T4 | T8 | T4 | T8 |
| Placebo | 5.45 ± 0.28 | 5.77 ± 0.13 | 5.67 ± 0.15 | | | 4.73 ± 0.25 | 4.79 ± 0.20 | 4.39 ± 0.21 | | |
| 50 mg/d sulodexide | 4.85 ± 0.26 | 5.34 ± 0.14 | 5.55 ± 0.16 | | | 4.41 ± 0.19 | 4.47 ± 0.19 | 4.44 ± 0.20 | / | |
| 100 mg/d sulodexide | 4.88 ± 0.22 | 5.05 ± 0.14 | 5.34 ± 0.15 | | | 4.31 ± 0.24 | 4.12 ± 0.20 | 3.95 ± 0.21 | 17[b] (−5 to 39) | 35[b] (18 to 53) |
| 200 mg/d sulodexide | 5.62 ± 0.20 | 4.41 ± 0.12 | 4.76 ± 0.14 | 40[b] (25 to 55) | 16[b] (−16 to 49) | 4.73 ± 0.30 | 3.45 ± 0.21 | 3.28 ± 0.22 | 46[b] (27 to 65) | 50[b] (26 to 74) |

[a] Values are log mean ± SEM or mean and confidence limits. Only statistical significant results are reported.
[b] $P < 0.05$ *versus* T0.

still statistically significant (31%) at the end of follow-up. In macroalbuminuric patients, a 45% statistically significant reduction in AER was observed in the 200 mg/d sulodexide group ($P < 0.05$), and AER was still significantly lower (29%) than baseline at T8 ($P < 0.05$).

**Effect of Sulodexide on AER in Patients with or without Concomitant ACEI Therapy.** Table 6 reports changes in AER in diabetic patients in the subgroups receiving and not receiving concomitant ACEI treatment. A 40% statistically significant reduction in AER was observed in the 200 mg/d sulodexide group at T4 ($P < 0.05$), even among diabetic patients already receiving ACEI. The reduction was still significant at T8 (16%). In diabetic patients not receiving concomitant ACEI treatment, a 17% reduction in AER was observed in the 100 mg/d sulodexide group at T4, and 46% in the 200 mg/d group, both statistically significant ($P < 0.05$). At the end of follow-up AER, was 35% and 50% lower than baseline in the 100 and 200 mg/d groups, respectively ($P < 0.05$).

## Discussion

The Di.N.A.S. study shows that 4 mo of treatment with 200 mg/d oral sulodexide remarkably reduces AER in albuminuric diabetic patients and that sulodexide not only slows progression but can actually improve proteinuria in diabetic patients. Furthermore, there appears to be a dose-response relationship with no evidence of a plateau effect. Although the small sample size limited the subgroup analyses, these effects seem largely independent of the type of diabetes, extent of basal albuminuria or concomitant ACEI treatment, and do not significantly effect HbA1C, BP, or serum creatinine. Our findings are therefore congruent with those previously reported from small explorative investigations using oral and parenteral sulodexide (22–29), parenteral LMW heparins (19,20), and other parenteral GAGs (21).

The hypoalbuminuric effect of 200 mg/d oral sulodexide was particularly evident in microalbuminuric patients, and 60% of them can be considered responders.

Microalbuminuria in diabetic patients has been considered to be of hemodynamic origin (7), due to endothelial dysfunction (8) or abnormal charge permselectivity (9). Our data do not support an effect of sulodexide on renal hemodynamics. Indeed, the hypoalbuminuric effect was achieved in our study without any detectable variation in renal hemodynamics, reflected by serum creatinine and creatinine clearance. Moreover, we did not observe any change in BP control during treatment or follow-up, suggesting that the hypoalbuminuric effect of GAGs is probably not mediated through mechanisms related to renal hemodynamics (14). This concept is also supported by the unprecedented findings that the hypoalbuminuric effect of sulodexide was noted in the subgroup of patients already benefiting from ongoing ACEI therapy (roughly to the same extent as that observed in patients not on ACEI), in which the hemodynamic-dependent component of albuminuria was conceivably already offset by ACE inhibition.

The concept of abnormal charge permeability as a cause of albuminuria has been recently challenged, at least in severe proteinuric conditions (31). Furthermore, the significance of alterations in differential clearance of neutral and anionic-dextrans as a marker of glomerular charge permselectivity has also been disputed (32). Finally, mechanisms underlying albuminuria in diabetic patients have been also linked to the tubular degradation of albumin during renal passage (33). All three observations question the concept that abnormal charge permselectivity is the initial GBM disorder responsible for microalbuminuria. Therefore, the most plausible explanation of the improvement in microalbuminuria is sulodexide's well-known favorable effect on the endothelium (13).

However, macroalbuminuric patients also responded to 200 mg/d sulodexide. Thus, even at the more advanced stage of overt DN, a high dose of sulodexide significantly reduces AER. Macroalbuminuria is associated with clear, pronounced glomerular abnormalities, which alter glomerular permeability due to the development of large pores (34), implying sulodexide probably effects the glomerular and GBM structure and not only endothelial permeability dysfunction, as in microalbuminuria.

It has already been proposed that GAGs may reduce albuminuria in DN by favorably interfering with mechanisms responsible for the altered GBM and composition and function (permselectivity) of the mesangial extracellular matrix (13,35). Several observations suggest that sulodexide activity in the kidney is complex, possibly modulating the renal expression of genes involved in renal remodeling: first, the persistence of the hypoalbuminuric effect up to 4 mo after cessation of therapy with the higher doses of sulodexide; second, the number of responders increases over the 4 mo of treatment, suggesting the hypoalbuminuric effect of sulodexide increases over time; and third, the similar extent of the hypoalbuminuric effect in patients with/without concomitant ACEI-therapy and the sharp difference between the persistent, posttreatment urinary albumin lowering effect of sulodexide and the rapid rise in AER seen shortly after discontinuation of ACEI therapy (36).

Treatment with GAGs prevents and rectifies the abnormal metabolism of glycosaminoglycans seen in the GBM and mesangium of diabetic animals (15). In addition, heparin has been shown to induce heparan-sulfate proteoglycan synthesis and enhance its sulfation in endothelial cells (37). Furthermore, in diabetic animals, GAGs very similar in structure to sulodexide suppress the TGF-$\beta$1–mediated enhanced expression of mesangial matrix and collagens (15–17). Finally, sulodexide suppresses high-glucose–induced overexpression of TGF-$\beta$1 and fibronectin in cultured mesangial cells (Gambaro G and Schleicher E, manuscript in preparation).

Previous studies in DM1 patients advanced the possibility that the hypoalbuminuric effect of sulodexide is long-lasting (24,26). The present investigation confirms this, and shows that it also occurs in DM2, a much more heterogeneous condition.

Despite the limitation in the subgroup analysis due to the small sample size, the additive antiproteinuric effect of sulodexide in patients with well-controlled BP and already receiving ACEI therapy is noteworthy because it promises a favorable effect on the dismal evolution of DN. The actions of ACEI and sulodexide overlap in some ways, but there are differences

that may explain sulodexide's additive effects to those of ACE inhibition alone. For example, although sulodexide diminishes the hyperglycemia-induced overexpression of TGF-$\beta$1, the mechanism of TGF-$\beta$1 modulation by ACEI is most likely different (35,38).

The effect of sulodexide on albuminuria probably reflects a favorable effect on glomerular remodeling. In the experimental model, the reduction of AER obtained with similar GAGs accompanies the prevention of glomerulosclerosis (15–17). It is interesting to observe that like strict glycemic control and ACEI therapy, the two well-known strategies capable of improving DN, sulodexide also improves both AER and TGF-$\beta$ overexpression, suggesting that it too may have a favorable effect on DN evolution. The reduction of AER by sulodexide *per se* could have a positive effect on the kidney by reducing the albuminuria-induced nephrotoxicity. Albumin (in the macroalbuminuric range) (39), glycated albumin (40), and lipid-rich albumin (41) (possibly also in the microalbuminuric range) in the glomerular filtrate are toxic to tubular epithelial cells and cause cytokine and endothelin-mediated tubulointerstitial inflammation and scarring.

The efficacy of oral GAGs on overt (macroalbuminuric) DN in DM2 patients represents an interesting new finding. In fact, the effect of oral sulodexide in DM2 has only been investigated in two smaller, randomized studies, which included only microalbuminuric patients (27,28). Both demonstrated that sulodexide was effective as a hypoalbuminuric agent. The effect of GAGs on macroalbuminuria in DM2 patients has been explored in two studies; however, tinzaparin (42) and danaparoid (21) were administered parenterally, making direct comparison difficult. The negative results obtained from both studies have raised the doubt that DM2 patients are less sensitive to GAGs than DM1 patients. Different GAG preparations and different routes of administration were used in the above cited studies; therefore, we cannot make any direct comparison.

During the Di.N.A.S. study, no severe adverse effect was observed. This is consistent with findings from numerous human trials with sulodexide for a variety of vascular indications, including one large study in which 2016 postmyocardial infarction patients, 25% of which were diabetic, received sulodexide for 1 yr (43). Interestingly, there were no clinically significant alterations in coagulation parameters, platelet count, or ophthalmologic findings, confirming the conclusions from previous studies that modest, clinically significant changes in blood coagulation tests are observed only at very high doses of oral heparins and GAGs. Although the use of anticoagulant doses of heparins may increase the risk of vitreal and retinal hemorrhage in diabetic retinopathy, reports have shown that the long-term use of a parenteral GAG, such as danaparoid, does not worsen diabetic retinopathy in DM2 patients (44). In DM1 patients, the long-term use of a parenteral GAG (45) can even reduce retinal hard exudates, lesions pathophysiologically resembling albuminuria.

In conclusion, this study has demonstrated that a 4-mo course of oral sulodexide can significantly improve albuminuria in both DM1 and DM2 patients with either micro- or macroalbuminuria, with approximately linear dose-response. Most importantly, the effect of 200 mg/d sulodexide on albuminuria is a sustained one (rather than simple stabilization). This strongly suggests that a chemical/anatomical change was induced in renal tissues by sulodexide. The albuminuria-lowering effect was additive to and independent of the effects achieved with ACEI therapy. Major adverse effects were not seen. Although this was a short-term study and longer trials with different end points (either clinical or GFR) must be performed to confirm safety and efficacy on the evolution of DN, the results suggest some important possible uses for sulodexide: (*1*) as a valid complementary treatment for patients who respond incompletely to ACEI therapy; (*2*) to treat patients unable to tolerate ACEI due to concomitant renal vascular disease, advanced renal insufficiency, hyperkalemia, or cough; and (*3*) to provide cardiovascular protection as well as nephroprotection. Sulodexide possesses antithrombotic activity (13) and has been shown to significantly reduce cardiovascular mortality in nondiabetic patients (43), a potentially very useful effect in a population at high risk of cardiovascular morbidity and mortality as albuminuric diabetic patients certainly are (9).

## Appendix

The term GAGs is generally used in the text indicating a broad category of molecules including heparin(s), LMW heparin(s), heparan sulfate, dermatan-sulfate, and mixed formulation of glycosaminoglycans, such as sulodexide and danaparoid. This use has its rationale in the fact that they share common biologic and namely nephroprotective activities. Specific GAG molecules have been defined whenever necessary.

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