# Correlation between Increased Serum sFas Levels and Microalbuminuria in Type 1 Diabetic Patients

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**Abstract**

*Objective:* The aim of this study was to elucidate if apoptosis dysregulation is present in type 1 diabetic patients with microalbuminuria. *Subjects and Methods:* The following variables were determined in 29 type 1 diabetic patients: the duration of diabetes, soluble Fas (sFas), Bcl-2, hemoglobin $A_{1c}$ levels, glomerular filtration rate (GFR) and microalbuminuria, using the urine albumin to urine creatinine ratio (ACR). Age and gender were assessed and patients were categorized into two groups, according to their ACR: the microalbuminuric (MA) group with an ACR $\geq 30$ mg/g, and the normoalbuminuric (NA) group with an ACR $< 30$ mg/g. *Results:* The differences between the two groups regarding sFas, Bcl-2 and GFR were not statistically significant. However, in the MA group, a significant positive relationship between sFas and ACR was observed (r = 0.736, p = 0.015). Dividing patients into two subgroups – mild versus severe (ACR $\geq 150$ mg/g) microalbuminuric patients – significant differences in sFas (60.4 vs. 87.2 pg/ml; p = 0.047) and GFR (113 vs. 69.5 ml min$^{-1}$ 1.73 m$^{-2}$; p = 0.021) were observed, whereas in Bcl-2, the difference was not significant (77.96 vs. 71.13 ng/ml).

*Conclusions:* At the early stages of diabetic nephropathy in type 1 diabetic patients, there seems to be a dysregulation of apoptosis, as expressed by enhanced sFas levels, leading to the speculation that the prevalence of antiapoptotic mechanisms (sFas) may promote mesangial proliferation.

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## Introduction

Diabetic nephropathy (DN) is one of the most common complications of diabetes that lead to renal replacement therapy. Notably, the incidence of chronic renal failure is considered to be higher in type 1 than in type 2 diabetes mellitus [1]. Three main histopathologic changes occur with DN: mesangial expansion, thickening of the glomerular basement membrane which leads to glomerular hyperfiltration, and glomerulosclerosis (in late stages). The early phase of DN is characterized by microalbuminuria and an increased glomerular filtration rate (GFR), and the presence of macroalbuminuria is accompanied by reduced GFR. Although the histopathologic changes have been described clearly, the exact pathogenetic mechanism of DN still remains unclear. It should be stressed that mesangial expansion (which implies a disturbance in renal cell homeostasis) seems to be the

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leading cause of renal function decline [2, 3]. Apoptosis, namely programmed cell death, has a central role in this homeostatic mechanism and is regulated by certain molecules and genes [4–6]. Remarkably, disturbances of this homeostatic mechanism have been implicated in the pathophysiology of several renal diseases [6].

Fas is a transmembrane receptor, and its activation via Fas ligand leads to apoptosis induction (death receptor or exogenous pathway). A soluble form of Fas is sFas, where Fas has lost its transmembrane domain through alternative splicing [5]. This form (sFas) suppresses apoptosis by inhibiting the binding competition between Fas and Fas ligand. On the other hand, Bcl-2 is one of the most important antiapoptotic molecules, governing the endogenous pathway of apoptosis [5]. Currently, the role of apoptosis in DN is of great interest and is being investigated extensively.

To date, the role of apoptosis has been investigated only in type 2 diabetic patients with macroalbuminuria. The aim of the present study was to examine whether or not apoptosis dysregulation is present in type 1 diabetic patients with microalbuminuria. Moreover, to our knowledge, this is the first study which evaluates Bcl-2 serum levels in patients with DN.

## Materials and Methods

A total of 29 patients with type 1 diabetes mellitus were included in the study. All patients gave informed consent, and the Hospital Ethics Committee approved the study. Patients with an underlying disease in which apoptosis might play a considerable pathogenetic role or patients with any diabetic complication (except DN) were excluded from the study. Specifically, those with a history of autoimmune disease, malignancy, pulmonary emphysema and thyroid disease were not eligible to participate. All patients underwent direct ophthalmoscopy in order to rule out diabetic proliferative retinopathy. Peripheral neuropathy was excluded by means of a biothesiometer, whereas the presence of coronary heart disease was excluded by a negative history and a negative treadmill exercise test according to the Bruce protocol. The presence of peripheral artery occlusive disease was checked by measuring the ankle-brachial index in each patient. None of the participants was receiving antihypertensive drugs. In addition, the following variables were also determined: age, diabetes duration, serum creatinine level, sFas, Bcl-2, hemoglobin $A_{1c}$ ($HbA_{1c}$) and GFR.

After overnight fasting, peripheral venous blood samples were obtained slowly from an antecubital vein and transferred to tubes containing $Na_2$ EDTA (1.5 mg/ml). Then, the plasma was separated by centrifugation (1,500 g for 20 min) at 4°C and stored at –80°C until laboratory analysis.

Plasma levels of sFas and Bcl-2 were measured using commercially available enzyme-linked immunosorbent assay kits (Bender MedSystems, Vienna, Austria). Aliquots of early morning urine
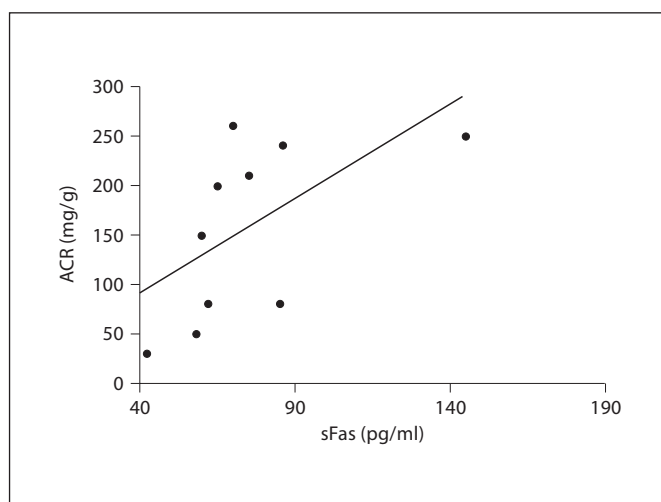


**Fig. 1.** Two-way scatter plot of ACR and sFas in the MA group.

samples were tested for albuminuria by the DCA 2000+ (Bayer Healthcare LLC, Elkhart, Ind., USA), a microalbumin/creatinine assay system that provides a calculated albumin/creatinine ratio (ACR) result [7]. The patients were categorized into two groups according to the ACR: the microalbuminuric (MA) group with an ACR ≥30 mg/g, and the normoalbuminuric (NA) group with an ACR <30 mg/g [8]. GFR was calculated by the abbreviated Modification of Diet in Renal Disease study equation (based on serum creatinine, patient age, race and sex) [9].

Statistical analysis was performed using the nonparametric test of Mann-Whitney and Spearman's rank correlation coefficient (without making any assumption about the normal distribution of the variables as the sample size is small) [10]. Multiple regression analysis was performed in order to determine the effect of age, diabetes duration, sFas, Bcl-2 and ACR on GFR. A p value <0.05 was used to reject the null hypothesis in either of the tests applied.

## Results

Of the 29 participants who were included in the study, 16 (55%) were females and 13 (45%) were males. Their mean age was 34.48 years. Ten of the patients (34.49%) were included in the MA group and 19 (65.51%) in the NA group. None of the participants had macroalbuminuria. The NA group had a significantly higher age compared with the MA group. The differences between the two groups regarding diabetes duration, the levels of $HbA_{1c}$, sFas, Bcl-2 and GFR were not statistically significant (table 1). However, in the MA group, a significant positive relationship between sFas and ACR was observed (r = 0.736, p = 0.015) (fig. 1).

**Table 1.** Characteristics of diabetic patients categorized according to the presence of albuminuria

|  | 30 ≤ ACR ≤ 300 mg/g (MA group) | ACR <30 mg/g (NA group) | p value |
|---|---|---|---|
| Patients | 10 | 19 |  |
| Diabetes duration, years | 14.60 ± 2.45 | 17.41 ± 2.50 | NS |
| Age, years | 28.30 ± 5.20 | 38.0 ± 11.53 | 0.021 |
| HbA$_{1c}$, % | 7.70 ± 0.64 | 8.13 ± 0.38 | NS |
| sFas, pg/ml | 73.8 ± 8.71 | 70.71 ± 5.33 | NS |
| Bcl-2, ng/ml | 74.55 ± 21.37 | 65.16 ± 16.26 | NS |
| GFR, ml/min/1.73 m$^2$ | 73.43 ± 9.02 | 88.85 ± 3.73 | NS |

NS = Not significant.

**Table 2.** Characteristics of diabetic patients with microalbuminuria

|  | 30 ≤ ACR < 150 mg/g (subgroup a) | ACR ≥150 mg/g (subgroup b) | p value |
|---|---|---|---|
| Patients | 5 | 5 |  |
| Diabetes duration, years | 12.0 ± 3.20 | 17.20 ± 3.66 | NS |
| Age, years | 26.0 ± 7.60 | 30.60 ± 3.41 | NS |
| HbA$_{1c}$, % | 8.90 ± 0.85 | 6.50 ± 0.50 | NS |
| sFas, pg/ml | 60.4 ± 6.75 | 87.2 ± 14.36 | 0.047 |
| Bcl-2, ng/ml | 77.96 ± 34.41 | 71.13 ± 29.40 | NS |
| GFR, ml/min/1.73 m$^2$ | 113.0 ± 6.88 | 69.5 ± 9.35 | 0.021 |

NS = Not significant.

The MA patients were further classified according to the median value of ACR (150 mg/g): subgroup a with an ACR ≥30 and <150 mg/g, and subgroup b with an ACR ≥150 and <300 mg/g, as in Altman [10]. Significant differences between the two subgroups in sFas (60.4 vs. 87.2 pg/ml; p = 0.047) and GFR (113 vs. 69.5 ml min$^{-1}$ 1.73 m$^{-2}$; p = 0.021) were observed, but for Bcl-2, the difference was not significant (77.96 vs. 71.13 ng/ml) (table 2).

After performing multiple regression analysis in all our patients, GFR was found to be negatively correlated with age (b = –0.70, p < 0.001) and ACR (b = –0.48, p < 0.01), but in the MA subgroup, GFR was negatively correlated with age (b = –0.8, p < 0.01) and sFas (b = –0.45, p < 0.03).

## Discussion

Initial studies, which investigated the pathogenesis of DN, have found increased apoptotic and decreased antiapoptotic activity [11–13]. In the present study, sFas was shown to have a significant correlation with ACR, suggesting that there is a dysregulation of apoptosis in MA type 1 diabetic patients. According to Shimizu et al. [14], dysregulation of apoptosis (imbalance between preapoptotic and antiapoptotic mechanisms) leads to mesangial expansion in proliferative glomerulonephritis, provided that apoptosis is the principal counteracting mechanism of mesangial cell proliferation. It could be hypothesized that in type 1 DN, there is a similar apoptosis dysregulation mechanism: the increased antiapoptotic activity (assumed by increased sFas levels) could be responsible for mesangial expansion and glomerular capillary impingement, initially resulting in hyperfiltration, and finally, in GFR reduction. Furthermore, our results indicate that GFR falls as microalbuminuria worsens in MA patients (table 2). Notably, in our multivariate analysis, this GFR decline occurs in parallel with an sFas level increase. These results are in agreement with those of Perianayagam et al. [15].

Onozaki et al. [16] proposed that plasma sFas might be a predictive factor for the prognosis of DN, since they demonstrated a positive correlation between plasma sFas and urine albumin levels in type 2 diabetic patients with various stages of DN. Moreover, the plasma sFas levels

were significantly higher in the advanced stages (3B and 4) than in the early stages of DN. However, among patients with mild albuminuria (20–420 mg/day), no significant difference in sFas was observed. In contrast, we found a significant difference in sFas among MA patients – mild versus severe (ACR ≥150 mg/g) microalbuminuric patients. It should be noted that there is no conclusive evidence that the elevation of plasma sFas in DN is protective against apoptosis. It is possible that increased production of an antiapoptotic molecule like sFas could represent an enhanced inhibitory response to ongoing renal apoptosis.

Regarding Bcl-2 levels, no statistically significant differences were observed between the two groups. However, it is noteworthy that the mean serum levels of Bcl-2 differ remarkably in the MA and NA groups (table 1). Therefore, it could be speculated that if our sample was larger, a significant association might be found. Nevertheless, recent experimental studies have shown that Bcl-2 contributes to mesangial expansion. Specifically, mesangial cell proliferation in DN is governed by vascular endothelial growth factor [16], a growth factor which acts through Bcl-2 [17, 18]. To date, (possibly) this is the first study that evaluates Bcl-2 plasma levels in patients with DN.

This study has its limitation in a small number of subjects; however, the strict inclusion and exclusion criteria offer an acceptable level of reliability. The small number of patients investigated also accounts for the fact that this was a pilot study. Additionally, no data regarding the origin of Bcl-2 and sFas is available. The low serum levels of sFas in our study could have the following three explanations. In our study, all the participants were type 1 diabetic patients, whereas in other studies, type 2 diabetics were studied. The average age of our patients was lower compared with that of other studies. Our patients were suffering from very early stages of DN in contrast with those in other studies who had end-stage renal disease.

## Conclusion

There seems to be a dysregulation of apoptosis, as expressed by enhanced sFas levels during early stages of DN, in type 1 diabetic patients. It could also be assumed that at these stages of DN, the antiapoptotic mechanisms (sFas) prevail, thus promoting mesangial proliferation. We believe that clarification of the pathogenetic role of apoptosis in DN could provide new therapeutic tools against this serious complication.

## References

1 Anderson AR, Christiansen JS, Andersen JK, Kralner S, Dockert K: Diabetic nephropathy in type I (insulin-dependent) diabetes: an epidemiologic study. Diabetologia 1985; 25:496–501.

2 Steffes MW, Osterby R, Chavers B, Mauer SM: Mesangial expansion as a central mechanism for loss of kidney function in diabetic patients. Diabetes 1989;38:1077–1081.

3 Ortiz A, Lorz C, Justo P, Catalán MP, Egido J: Contribution of apoptotic cell death to renal injury. J Cell Mol Med 2001;5:18–32.

4 Zeiss CJ: The apoptosis-necrosis continuum: insights from genetically altered mice. Vet Pathol 2003;40:481–495.

5 Reed JC: Mechanisms of apoptosis. Am J Pathol 2000;157:1415–1430.

6 Baker AJ, Mooney A, Hughes J, Lombardi D, Johnson RJ, Savill J: Mesangial cell apoptosis: the major mechanism for resolution of glomerular hypercellularity in experimental proliferative nephritis. J Clin Invest 1994;94: 2105–2116.

7 Poulsen PL, Mogensen CE: Clinical evaluation of a test for immediate and quantitative determination of urinary albumin to creatinine ratio. Diabetes Care 1998;21:97–98.

8 Molitch ME, DeFronzo RA, Franz MJ, Keane WF, Mogenesen CE, Parving HH, Steffes MW: Nephropathy in diabetes. Diabetes Care 2004;27(suppl 1):S79–S83.

9 National Kidney Foundation: National Kidney Foundation Kidney Disease Outcomes Quality Initiative. 2006. http://www.kidney.org/professionals/kdoqi/gfr_page.cfm.

10 Altman DG: Categorizing continuous variables; in Armitage P, Colton T (eds): Encyclopedia of Biostatistics. Chichester, Wiley, 1998, pp 563–567.

11 Ortiz A, Lorz C, Justo P, Catalan M, Egido J: Role and regulation of apoptotic cell death in the kidney. Front Biosci 2000;5:D735–D749.

12 Kikuchi Y, Nonoguchi H, Machida K, Wakamatsu S, Koga H, Tomita K: Regulation of the apoptosis-related genes, Bax and Bcl-2, in the early stage of diabetes mellitus. Nephrology 2002;7:294–302.

13 Haas CS, Schocklmann HO, Lang S, Kralewski M, Sterzel RB: Regulatory mechanism in glomerular mesangial cell proliferation. J Nephrol 1999;12:405–415.

14 Shimizu A, Kitamura H, Masuda Y, Ishizaki M, Sugisaki Y, Yamanaka N: Apoptosis in the repair process of experimental proliferative glomerulonephritis. Kidney Int 1995;47: 114–121.

15 Perianayagam MC, Murray SL, Balakrishnan VS, Guo D, King AJ, Pereira BJ, Jaber BL: Serum soluble Fas (CD95) and Fas ligand profiles in chronic kidney failure. J Lab Clin Med 2000;136:320–327.

16 Onozaki A, Midorikawa S, Sanada H, Hayashi Y, Baba T, Katoh T, Watanabe T: Rapid change of glucose concentration promotes mesangial cell proliferation via VEGF: inhibitory effects of thiazolidinedione. Biochem Biophys Res Commun 2004;317:24–29.

17 Gerber HP, Dixit V, Ferrara N: Vascular endothelial growth factor induces expression of antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 1998; 273:1313–1316.

18 Nor JE, Christensen J, Mooney DJ, Polverini PJ: Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol 1999;154:375–384.