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Low TGF-β1 serum levels are a risk factor for atherosclerosis disease in ESRD patients

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Low TGF-β1 serum levels are a risk factor for atherosclerosis disease in ESRD patients.

Background. It is thought that transforming growth factor-β1 (TGF-β1) might be a key inhibitor of atherogenesis in nonuremic patients. We evaluated the intra- and post-dialytic serum levels of TGF-β1 in uremic patients to assess if TGF-β1 is an independent risk factor for cardiovascular diseases, and if any correlation exists between TGF-β1 and any yet known atherosclerotic risk factors.

Methods. We studied 155 patients who were on regular hemodialysis, with or without clinically significant atherosclerotic vascular disease. Forty-one apparently healthy people were enrolled as a control group. TGF-β1 was evaluated during the midweek dialysis session, at times 0, 30, and 120 minues, at the end of the session, and 3 hours after the session's end. All hitherto known atherosclerotic risk factors also were evaluated. The investigation was performed over a 24-month follow-up.

Results. TGF-β1 values (mean ± SD) in dialysis patients were 26.64 ± 7.0 ng/mL ($N = 155$) compared with 42.31 ± 6.0 ng/mL in the control group ($N = 41$, $P < 0.0001$). A weak inverse correlation emerged between TGF-β1 and age ($r = -0.28$), TGF-β1 and lipoprotein(a) [Lp(a); $r = -0.35$], TGF-β1 and C-reactive protein (CRP; $r = -0.27$), and TGF-β1 and plasminogen activator inhibitor-1 (PAI-1; $r = -0.41$). TGF-β1 also correlated with albumin ($r = 0.31$). In the coronary heart disease (CHD) group ($N = 32$) the TGF-β1 was 26.2 ± 4.9 ng/mL; in the cerebrovascular disease (CVD) group ($N = 8$) it was 26.7 ± 3.7 ng/mL and in the peripheral vascular disease (PVD) group ($N = 9$) it was 25.4 ± 1.7 ng/mL. In dialysis patients with no cardiovascular disease ($N = 80$) TGF-β1 was 35.1 ± 6.8 ng/mL ($P < 0.0001$ vs. CHD, CVD and PVD patients). TGF-β1 was significantly lower among those patients with triple coronary vessel disease than with the other CHD patients. The Cox analysis demonstrated that a 1 ng/mL reduction in TGF-β1 concentration was associated with a 9% increase in the relative risk of a cardiovascular event.

Conclusions. TGF-β1 was significantly reduced in hemodialysis patients, in particular in those with severe cardiovascular disease. Baseline TGF-β1, diabetes mellitus and serum albumin levels proved to be the only independent contributors to atherosclerotic risk in dialysis patients.

Key words: atherosclerosis, cardiovascular diseases, uremia, hemodialysis, ischemic heart disease, vascular complication.

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The incidence of cardiovascular disease, mainly related to atherosclerosis, increases significantly in dialysis patients, for whom it is the main cause of death [1]. The relative risk of mortality from ischemic heart disease has been estimated as 22 times higher in dialysis patients than in the normal population, a risk similar to that of survivors from a first myocardial infarction. The high incidence of vascular complications in dialysis patients also involves peripheral vascular disease and stroke, the high incidence of which likewise is thought to be related to an acceleration of atherogenesis. It is still controversial whether uremic patients per se are prone to accelerated atherogenesis, or whether the high cardiovascular morbidity and mortality are a consequence of various risk factors already present in these patients right from the predialysis phase [2, 3].

In hemodialysis patients, besides the well-known risk factors that can trigger the atherosclerotic process such as old age, smoking, hypertension, hypercholesterolemia, other factors such as hyperhomocysteinemia, hyperparathyroidism, malnutrition, and inflammation seem to accelerate the process [4–6]. However, no combination of the known risk factors has provided a convincing explanation of why accelerated atherosclerosis occurs in dialysis patients [7, 8]. Probably other mechanisms besides these are triggered or accelerated by dialysis therapy or by persisting uremia, and play an important role.

Atherosclerosis is a slowly progressing, inflammatory, proliferative disease in which various cells such as macrophages, endothelial and smooth muscle cells (SMC) are involved [9]. In the last few years, experimental evidence has suggested that transforming growth factor-β1 (TGF-β1) may have a key role in atherogenesis by inhibiting both migration and proliferation of smooth muscle cells and macrophages and by protecting endothelial function [10–12].

Previous studies in non-renal patients have demonstrated the importance of cytokines or growth factors in the atherosclerotic process and shown that atherosclerosis may be the result of an imbalance between atherogenic and proliferative growth factors such as platelet-derived growth factor (PDGF), macrophage colony-stimulating factor (MCSF) and antiatherogenic and anti-inflammatory growth factors like TGF-β1 [11–14].

In the pathological changes that characterize the atherosclerotic process and plaque formation, the cornerstone event is endothelial injury and the release of atherogenic growth factors [9]. Whatever the agent acting at the site of injury, smooth muscle cells and macrophages subsequently proliferate and migrate from the media. This proliferation is the main event in the development of atherosclerosis.

Transforming growth factor-β1 is a 25 kD homodimeric pleiotropic growth factor secreted by many kinds of cell including macrophages, lymphocytes, smooth muscle cells and platelets. It is secreted in a latent inactive form that is activated proteolytically by plasmin [15]. In turn, plasmin is produced from plasminogen by tissue plasminogen activator, the production of which is blocked by competitive inhibition from lipoprotein(a) [Lp(a)] and plasminogen activator inhibitor 1 (PAI-1). These molecules are therefore able to promote smooth muscle cell proliferation by relieving the inhibition caused by active TGF-β [16, 17]. Two kinds of TGF-β receptor (types I and II) have been identified on target cell membranes, both receptors being required for TGF-β1 signaling to occur [18].

The aims of our present study were to evaluate both the basal and the intradialytic serum levels of TGF-β1 in uremic patients and to assess whether TGF-β1 is an independent risk factor for cardiovascular disease in the hemodialysis population studied, checking for any relationship with cardiovascular disease in regular dialysis treatment (RDT) patients. We also assessed for a correlation between TGF-β1, the acute phase reactants, and any known atherosclerotic risk factors.

METHODS

The study began with a consecutive series of 155 Caucasian patients on regular hemodialysis treatment in our dialysis unit. Baseline characteristics of the 88 males and 67 females are reported in Table 1.

Their ages ranged from 30 to 85 years (mean age 62.3 ± 14.2 years). Hemodialysis treatment had been initiated 74.3 ± 81.2 months before the baseline TGF-β1 serum level evaluation. All patients were on regular dialysis treatment three times a week; the mean Kt/V (calculated according to Daurgirdas' second logarithmic formula) was 1.3 ± 0.3 [19]; the diuresis was <200 mL/day for all the patients. Patients were maintained on their own hemodialysis treatment throughout the study. All patients were regularly treated three times weekly with standard bicarbonate dialysis (62%) or hemodiafiltration (38%). The dialyzers used were hemophan (58%), polyacrylonitrile (PAN; 12%), and polysulfone (30%). In all procedures the same heparinization modalities were employed: in the washing phase, 2 liters of saline solution were used containing 20,000 IU of standard heparin in single pass (Sodic Heparin; Vister by Parke-Davis, Pontypool, UK), followed by 2000 ± 500 IU in a continuous intradialytic infusion.

Table 1. Baseline characteristics of the study population

No. of patients	155
Sex	
Male	88
Female	67
Age *years*	62.3 ± 14.2
Etiology of renal disease	
Chronic glomerulonephritis	30.1%
Vascular	45.4%
Diabetes	5.4%
Chronic pyelonephritis	15.7%
Adult polycystic kidney disease	2.4%
Miscellaneous	1.0%
Duration of hemodialysis *months*	74.3 ± 81.2
Diabetes	5%
Hypertension	56.6%
HDL-C *mg/dL*	26 ± 6
LDL-C *mg/dL*[a]	125 ± 30
Triglycerides *mg/dL*	189 ± 115
Smoking	10%
Lp(a) *mg/dL*	22.7 ± 20.9
Homocysteine *mmol/L*	55.4 ± 44.7
Albumin *g/L*	3.5 ± 0.3
C reactive protein *mg/dL*	1.8 ± 2.6
Fibrinogen *mg/dL*	368 ± 126

[a] Calculated by means of the Friedewald formula [55]

The distribution of TGF-β1 values in the population per hemodialysis duration quartile is shown in Figure 1.

Because there is a known genetic predisposition to be high, intermediate or low TGF-β1 producers in both the dialysis population and the control group [20], we analyzed two single-nucleotide polymorphisms in the DNA sequence encoding the leader sequence of the TGF-β1 protein, located at position +869 (codon 10, T→C, leucine→proline) and position +915 (codon 25, G→C, arginine→proline). The signal peptide sequence, where the studied polymorphisms are located, was cleaved from the TGF-β1 precursor at codon 29. The signal sequence allowed the new synthesized protein to be exported across the membranes of the endoplasmatic reticulum [21, 22]. Alteration of the signal sequence, changing one amino acid for another, can affect export efficiency.

The putative high haplotypes are T/T G/G and T/C G/G; intermediate are T/C G/C, C/C G/G and T/T G/C; low producers are C/C G/C, C/C C/C, T/T C/C and T/C C/C.

Seventy-five patients (49%) from among the 155 selected patients of our dialysis centre had a history or

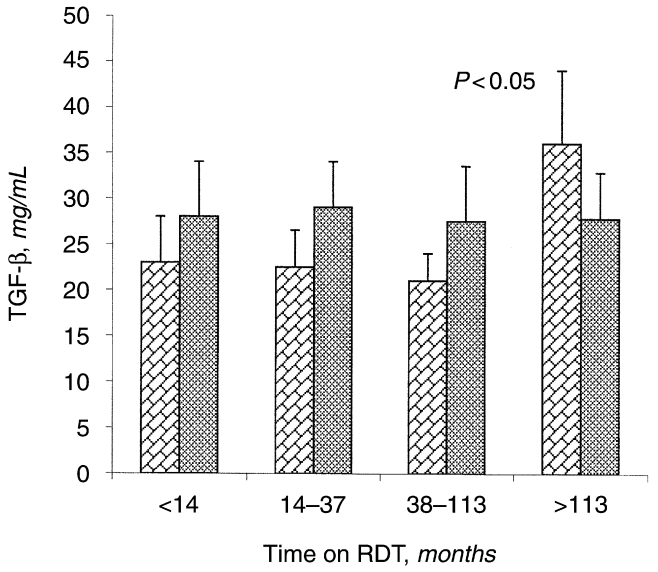


Fig. 1. Distribution of transforming growth factor-β1 (TGF-β1) values per hemodialysis duration quartile. The mean TGF-β1 values became significantly higher for males (▨) than females (▩) in the fourth quartile.

evidence of one or more "atherosclerotic related" processes on entry to our study (Table 2). Clinically significant atherosclerotic vascular disease was defined as the presence of coronary heart disease (CHD), cerebrovascular disease (CVD), or peripheral vascular disease (PVD) as described in what follows.

Coronary heart disease was diagnosed by at least one of the following criteria: (*1*) previous admission for documented myocardial infarction, not later than three months before the study started (elevated creatine kinase level and electrocardiogram changes); (*2*) a clinical history of symptoms consistent with angina confirmed by a positive exercise stress test result; or (*3*) significant positive results on coronary angiograms (stenosis >50% for 1 of the 3 major vessels) or on thallium scan (defining fixed or reversible perfusion defects). In these patients CHD was diagnosed on the basis of their previous clinical and instrumental backgrounds: these patients had undergone thallium scan (30 patients) or coronary angiograms (25 patients) at least once prior to entry in the study.

Cerebrovascular disease was defined by at least one of the following criteria: (*1*) a previous stroke (acute-onset irreversible neurological deficit) not later than three months before the study started, documented by cerebral CT or MRI or by a physician in the patient's medical records, or (*2*) evidence of stenosis >50% for carotid vessels at Doppler echography. Peripheral vascular disease was defined by the presence of claudicatio intermittens, previous peripheral vascular surgery (7 patients), including amputation for ischemic limb (3 patients), or the presence of atherosclerotic plaques at Doppler echography or at abdominal-iliac-femoral angiograms (15 patients). Peripheral vascular surgery had not been performed later than three months before the study started.

Table 2. Correlation between TGF-β1 value and other baseline risk factors for atherosclerotic disease

Variable	*r*	*P*
Age	−0.28	0.04
Duration of hemodialysis	0.20	0.05
LDL-C	0.03	0.72
Triglycerides	0.09	0.34
Lp(a)	−0.35	0.05
Homocysteine	0.07	0.41
Albumin	0.31	0.33
PAI-1	−0.41	0.05
C reactive protein	−0.27	0.05
Fibrinogen	−0.05	0.23

Eighty dialysis patients on entry to our study showed no signs or symptoms of atherosclerosis disease, in the sense that none of the selected criteria was found in their clinical history. Nonetheless, all these patients were examined by vascular Doppler echography to exclude significant and still silent atherosclerotic involvement of these arteries, or the presence of atherosclerotic plaques. Abdominal aorta, carotid arteries and iliac-femoral arteries were the vascular districts observed.

Platelet basal levels did not differ between the dialysis patients with and without atherosclerotic diseases (230,000 ± 23,000/mmc vs. 227,000 ± 27,000/mmc, P = NS) nor between 14 patients with severe CHD and 18 patients with mild CHD (223,000 ± 28,000/mm^3 vs. 228,000 ± 22,000/mmc, P = NS).

Of the 155 patients selected, 88 (56.6%) suffered from hypertension for which they were on antihypertensive treatment. Only 15 patients (9.6%) were receiving angiotensin-converting enzyme (ACE) inhibitors, 6 with ramipril 5 ± 0 mg/day, and 9 with enalapril 10 ± 5.5 mg/day.

Forty-one apparently healthy people, comparable in age (63 ± 11 years) and sex distribution (21 males and 20 females), were enrolled as a control group for TGF-β1 serum level. This latter group also underwent an electrocardiogram (ECG) and vascular Doppler echography, with the same criteria, to exclude ischemic myocardial disease and atherosclerotic disease. In these patients no pathological signs were found by the tests. All of the Doppler echography tests were performed by the same radiologist. Platelet basal levels did not differ between the control group and the dialysis patients, with and without atherosclerosis: (238,000 ± 34,000/mmc vs. 230,000 ± 23,000/mmc, P = NS, and 238,000 ± 34,000/mmc vs 227,000 ± 28,000/mmc, P = NS, respectively).

Laboratory methods

The TGF-β1 serum level was evaluated immediately before and during the midweek session; blood samples

from the arterial line were collected to check TGF-β1 at times 0 (dialysis session start), 30 minutes, 120 minutes, at the end of the session (immediately before the extracorporeal volume reinfusion) and three hours after the session's end. Activated partial thromboplastin time (aPTT) values were checked at times 0, 30 minues, 120 minutes, at the end of the session, and three hours afterwards.

During the same dialysis session a fasting blood sample was drawn to check low-density lipoprotein (LDL-C), triglycerides (TG), lipoprotein(a) [Lp(a)], homocysteine, albumin, C-reactive protein (CRP), fibrinogen, and plasminogen activator inhibitor type 1 (PAI-1) from all patients. Sampling was only performed at the dialysis start (time 0). LDL-C, TG, albumin, CRP, fibrinogen and homocysteine were evaluated by standard laboratory methods; Lp(a) was evaluated by an immunometric method (Bouty Inc., Sesto S, Giovanni, Italy), PAI-1 was evaluated by a chromatogenic method (Dade Behring Inc. Milan, Italy). We used a Genomic Prep Blood DNA Isolation Kit (Pharmacia Biotech, Inc., Uppsala, Sweden). The dosage of TGF-β1 was made as follows: first a serum separator tube was used to allow the sample to clot for one hour at room temperature. In order to obtain a complete release of TGF-β1, blood samples were maintained overnight at 2 to 8°C before centrifugation. Then the samples were centrifuged at 1000 × *g* for 10 minutes, and the serum obtained was stored at −70°C. To convert any inactive TGF-β1 to an active form, the samples were first acidified (by adding 100 μL of a 2.5 N acetic acid/10 mol/L urea solution to 100 μL of serum) and then restored to pH 7.2 to 7.6 by 100 μL of a 2.7 N NaOH/1 mol/L HEPES solution.

Concentrations were determined by means of a solid phase, TGF-β1–specific sandwich enzyme-linked immunosorbent assay (ELISA) (Quantykine; R&D Systems, Minneapolis, MN, USA). The minimum detectable level of TGF-β1 was 7 pg/mL.

The genotype polymorphism was determined as follows: blood from all individuals was collected in sterile tubes containing 0.1% of ethylenediaminetetraacetic acid (EDTA); genomic DNA was extracted using a Genomic Prep™ Blood DNA Isolation Kit (Pharmacia Biotech. Inc.). DNA samples were screened for mutations in the TGF-β1 gene by polymerase chain reaction (PCR) single strand polymosphism (SSP) methodology with the Cytokine Genotyping Tray Kit (One Lambda Inc., Los Angeles, CA, USA). Pre-optimized primers were presented, dried in different wells of a 96-well 0.2-mL thin-walled tube tray for PCR and made ready for the addition of DNA samples, recombinant Taq polymerase, and specially formulated dNTP-buffer mix (D-Mix). Each tray included a negative control reaction tube and an internal control PCR product, amplified from the human-globin gene. The amount of the primer was adjusted for optimal amplification of 100 ng of sample DNA when used in conjuction with the D-mix, and 5 U/μL of the recombinant Taq polymerase (Perkin-Elmer Amplitaq DNA Polymerase). The final reaction volume was 10 μL. The amplification program was run on a Perkin-Elmer 9700 thermalcycler: 1 cycle of 96°C for 130 seconds, 63°C for 60 seconds; 9 cycles of 96°C for 10 seconds, 63°C for 60 seconds, 20 cycles of 96°C for 10 seconds, 59°C for 50 seconds, 72°C for 30 seconds. PCR products were visualized on a 2.5% agarose gel stained with ethidium bromide.

Table 3. Intra- and post-dialytic behavior of TGF-β1 serum levels: No difference was found reflecting the membrane used, by ANOVA

Time	F	*P*
0	0.661	NS
30 minutes	0.548	NS
180 minutes	0.753	NS
240 minutes	1.215	NS
3 hours	0.134	NS

The protocol was approved by an Ethics Committee and informed consent was obtained from all the patients.

Follow-up studies

The investigation was based on a prospective study entailing 24 months of follow-up. The requirement for patients to return to our dialysis unit three times a week facilitated the determination of clinical and laboratory findings associated with baseline TGF-β1 levels during the follow up.

Date and cause of death were recorded directly for deaths occurring in our Unit and from interviews with the physicians who verified the cause when death occurred outside the dialysis unit.

The cause of death was classified as a cardiovascular event (myocardial infarction, sudden death, and stroke) or non-cardiovascular event (infection, neoplasms, massive gastrointestinal bleeding, others including uncertain causes when no clear atherosclerotic event was found).

Statistical analysis

Statistical analysis was performed by the Statistical Package for the Social Sciences (SPSS for Windows Software Package; version 9.0.1). Data are presented as means ± standard deviation.

The Student *t* test was used for group comparison of continuous variables with normal population distribution (Kolmogorov-Smirnov test, $Z = 0.77$).

The ANOVA test was performed to evaluate the intra and post-dialytic behavior of serum TGF-β1 (Table 3).

Linear regression analysis was performed to evaluate the presence of a statistically significant relationship between two parameters, in this case between TGF-β1 and the other baseline variables commonly known as risk

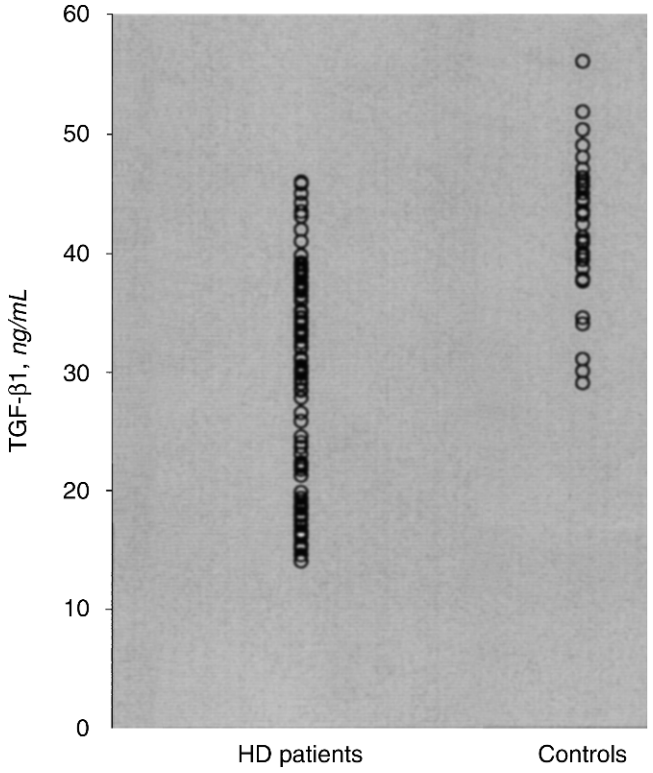


Fig. 2. Dot plot showing TGF-β1 values in hemodialysis patients (26.6 ± 7 ng/mL) versus controls (42.3 ± 6 ng/mL, $P < 0.0001$).

factors for atherosclerosis: age, sex, LDL-C, TG, Lp(a), homocysteine, albumin, CRP, fibrinogen, and PAI-1.

Multivariate analysis was performed using the Cox proportional hazard model to determine the independent association of baseline parameters with the risk of cardiovascular death during the follow-up period. Variables entered in the final analysis were: age, diabetes, systolic arterial pressure, dyastolic arterial pressure, LDL, TGF-β1 and albumin.

Kaplan-Meier event-free survival curves were analyzed separately on the two groups with TGF-β1 serum level respectively above and below the mean value 26.64 ng/mL.

RESULTS

Baseline findings

The mean TGF-β1 concentration in our hemodialysis patients was 26.6 ± 7 ng/mL compared with 42.3 ± 6 ng/mL in the control group ($P < 0.0001$; Fig. 2). No significant difference in TGF-β1 was found between males (25.2 ± 10.5 ng/mL) and females (28.1 ± 6.8 ng/mL) among the hemodialysis population.

Again in the dialysis group, no significant difference in TGF-β1 was found between the 129 (83%) high producers, 27.2 ± 8.0 ng/mL, the 18 (11%) intermediate producers, 25.2 ± 11.0 ng/mL, and the 8 (5%) low producers, 26.5 ± 9.2 ng/mL (Table 4). In the control group, the same percentage distribution of dialysis patients was found: 33 (80%) patients were high producers, 6 (14%) were intermediate and 2 (5%) low producers.

Because a certain number of our hemodialysis patients had clinical evidence of atherosclerosis on entry to the study, the TGF-β1 levels were compared among the three groups of CHD, CVD and PVD patients (Table 5). In the CHD group ($N = 32$), the TGF-β1 level was 26.2 ± 4.9 ng/mL, in the CVD group ($N = 8$) 26.7 ± 3.7 ng/mL and in the PVD ($N = 9$) 25.4 ± 1.7 ng/mL (P = NS). In hemodialysis patients with no evidence of cardiovascular disease the TGF-β1 mean value was 35.1 ± 6.8 ng/mL ($P < 0.0001$ vs. CHD, CVD and PVD patients). Interestingly, in patients with simultaneous evidence of CHD, CVD and PVD disease ($N = 20$), TGF-β1 was significantly lower (mean 16.7 ± 4.0 ng/mL; $P < 0.0001$ again versus those with CHD, CVD or PVD alone). Furthermore, the TGF-β1 serum concentration was significantly lower (20 ± 3 ng/mL) among those patients of the CHD group with triple coronary vessel disease ($N = 14$) than among patients of the same group with mild CHD ($N = 18$), whose serum concentration was 30.8 ± 5 ng/mL, $P < 0.001$ (Fig. 3).

Transforming growth factor-β1 serum levels were again nearly the same in hypertensive patients (25.2 ± 8.4 ng/mL) versus non-hypertensives (28.4 ± 9.3 ng/mL) and in diabetics (25.4 ± 8.7 ng/mL) versus non-diabetics (26.9 ± 9.0 ng/mL), and they did not correlate with the different causes of end-stage renal disease.

From the analysis of the association of TGF-β1 baseline serum levels with other potential risk factors for atherosclerotic clinical events, no significant correlation was found between TGF-β1 and LDL-C, TG, homocysteine and fibrinogen (Table 2). A weak inverse correlation emerged between TGF-β1 and age ($r = -0.28$), TGF-β1 and Lp(a) ($r = -0.35$), TGF-β1 and CRP ($r = -0.27$) and, finally, between TGF-β1 and PAI-1 ($r = -0.41$); TGF-β1 also proved to correlate with albumin ($r = 0.31$). In the population studied a positive moderate correlation also was found between CRP and albumin ($r = 0.63$) and CRP and Lp(a) ($r = 0.52$).

Transforming growth factor-β1 serum levels were evaluated before the start of the midweek dialysis session, separating patients dialyzed with hemophan (24.8 ± 7.4 ng/mL) from those on PAN (mean value 26.5 ± 5.4 ng/mL) and those on polysulfonee (27.7 ± 9.3 ng/mL). No statistically significant difference was found either among the three groups of membranes or between the two techniques (standard bicarbonate dialysis 25.8 ± 5.4 ng/mL vs. hemodiafiltration 26.9 ± 4.7 ng/mL). Checking the intradialytic behavior of TGF-β1 serum levels, only at 30 minutes was there a significant reduction (by about 30%, $P < 0.001$), without any difference reflecting the

Table 4. Genotypes and TGF-β1 levels in hemodialysis patients

Producer	High		Intermediate		Low	
Codon 10	T/C	T/T	C/C	T/C	C/C	C/C
Codon 25	G/G	G/G	G/G	G/C	C/C	G/C
TGF-β1 *ng/mL*	26.5 ± 8.2	27.9 ± 9.5	19.8 ± 10	30.5 ± 11.5	28.4 ± 8.7	24.5 ± 9.5
Frequency %	28	55	6	5	3	2

Table 5. Transforming growth factor-β1 values in patients with and without atherosclerotic disease

Clinical event	*N* patients	TGF-β1 *ng/mL*	Significance[a]
Atherosclerotic disease	75	26.1 ± 2.3	<0.0001
CHD only	32	26.2 ± 4.9	<0.0001
CVD only	8	26.7 ± 3.7	<0.0001
PVD only	9	25.4 ± 1.7	<0.0001
CHD + PVD + CVD	26	16.7 ± 4.0	<0.0001
No atherosclerotic disease	80	35.1 ± 6.8	—

[a]Student *t* test versus patients with no atherosclerotic disease.

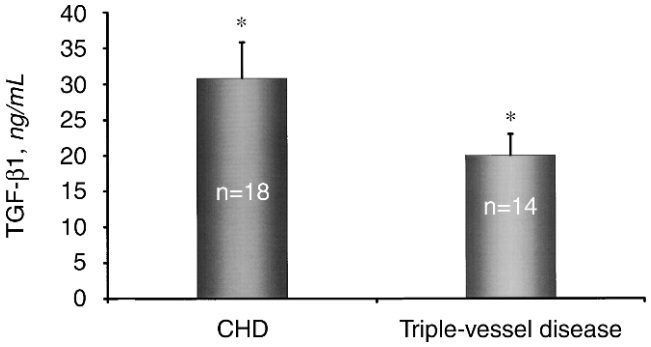


Fig. 3. TGF-β1 in the coronary heart disease (CHD) group showing patients with mild CHD versus patients with triple coronary vessel disease. *$P < 0.001$.

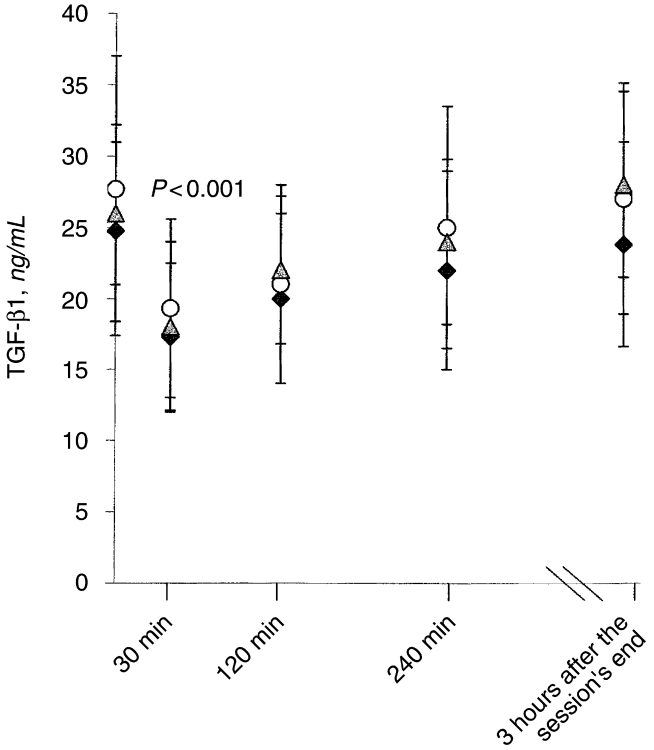


Fig. 4. Intradialytic behavior of TGF-β1 serum levels. No difference was found reflecting the membrane or technique used using the Student *t* test. At 30 minutes a significant ($P < 0.001$) reduction in TGF-β1 was observed over T0 values. Symbols are: (◆) hemophan; (○) polysulfone; (△) PAN.

membrane or the technique used (Fig. 4 and Table 3). By three hours after the session's end, levels had reverted to basal values (Fig. 4).

Looking at the distribution of TGF-β1 values per hemodialysis duration quartile (Fig. 1), interestingly the mean values were nearly the same until 113 months of dialysis (P = NS), becoming significantly higher, but only for the male gender, in the fourth quartile (>113 months; $P < 0.05$).

Follow-up

During the 24 months of follow-up 18 deaths occurred (17 cardiovascular and 1 non-cardiovascular; Table 6). Three patients discontinued dialysis treatment after renal transplantation.

Age, LDL, systolic arterial pressure, diastolic arterial pressure, male sex, diabetes, albumin and TGF-β1 levels were entered into the stepwise logistic regression analysis as variables to determine their contribution to the risk of cardiovascular death. Baseline TGF-β1, serum albumin and the presence of diabetes mellitus levels proved the only independent contributors to risk in the regression model (Table 7).

Table 6. Cardiovascular events and causes of death in the study cohort during follow-up

Cardiovascular events (fatal and non-fatal)	*N*	Causes of death	*N*
Angina pectoris	41	Cardiovascular	17
Heart failure	18	Myocardial infarction	7
Arrhythmia	10	Stroke	4
Peripheral artery disease	10	Heart failure	3
Transient ischemic attack	9	Sudden death	2
Stroke	7	Arrhythmia	1
Sudden death	2	Non-cardiovascular	
Pulmonary embolism	1	Neoplasia	1

Table 7. Multiple logistic regression analysis of risk factors for cardiovascular death

Variable	β	SEM	Odds ratio	95% Confidence interval	*P*
LDL-C	−0.092	0.0115	0.99	(0.96, 1.01)	0.42
PAS	−0.069	0.0317	0.99	(0.93, 1.05)	0.82
PAD	0.041	0.0506	1.04	(0.94, 1.15)	0.40
Diabetes	1.2194	0.615	3.38	(1.01, 11.3)	0.04
Albumin	−0.785	0.0052	1.1	(0.93, 1.01)	0.02
Age at event	0.044	0.0037	1.0	(0.99, 1.00)	0.98
TGF-β1	−0.898	0.0451	0.91	(0.83, 0.99)	0.04

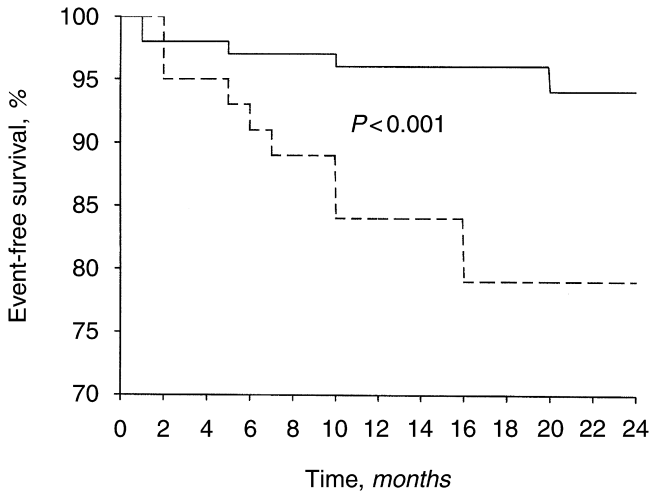


Fig. 5. Kaplan-Meier survival curves. Comparison between the TGF-β1 high values group (>26.64 ng/mL; solid line) and the TGF-β1 low values group (<26.64 ng/mL; dashed line) was $P < 0.001$ by the log rank test.

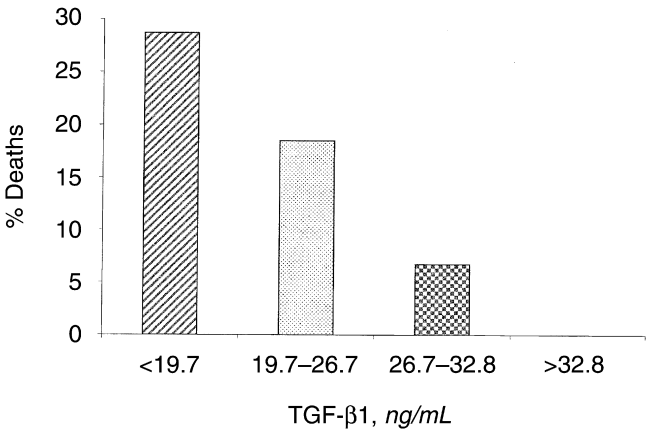


Fig. 6. Mortality histograms for quartiles of TGF-β1 levels. The higher the TGF-β1 value, the lower the percentage of deaths. Each histogram shows the percentage of deaths in the group with TGF-β1 levels indicated. In the first quartile there were 9 deaths (28.6%), in the second quartile 7 (18.5%), in the third there was 1 death (6.7%), in the fourth quartile with the highest TGF-β1 levels no death occurred.

The Cox analysis demonstrated that a 1 ng/mL reduction in TGF-β1 concentration was associated with a 9% increase in the relative risk of undergoing a cardiovascular event ($P = 0.04$). The presence of diabetes ($P = 0.04$) and hypoalbuminemia ($P = 0.02$) were the only parameters that contributed to the regression model.

Finally, using the mean TGF-β1 concentration value of 26.64 ng/mL as an arbitrary cut off value to define patients with higher and lower TGF-β1 values, the Kaplan-Meier survival curves were calculated separately.

The Kaplan-Meier survival curves showed significant differences between the two groups: $P < 0.001$ by the log rank test (Fig. 5).

To confirm the Kaplan-Meier survival curves, mortality histograms for quartiles of TGF-β1 levels were provided (Fig. 6). Cut-off values for quartiles were TGF-β1 concentrations of 19.7 ng/mL, 26.7 ng/mL and 32.8 ng/mL. The data demonstrated that the higher the TGF-β1 value, the lower the percentage of deaths: in the first quartile there were 9 deaths (28.6%), in the second quartile 7 (18.5%), in the third there was one death (6.7%), and in the fourth quartile with the highest TGF-β1 levels no death occurred.

DISCUSSION

The importance of cardiovascular disease in RDT patients emerges from both clinical and statistical evaluations that show the precocity and the impact of atherosclerotic disease on dialysis patients in terms of morbidity and mortality.

No combination of Framingham risk factors has provided a convincing explanation of why dialysis patients have accelerated atherosclerosis.

Recently several experimental studies in non-renal patients have focused on the role cytokines may play in generating atherosclerosis. Artherosclerotic changes could be partly an expression of an imbalance between antiatherogenic (TGF-β1) and atherogenic (MCSF and IL-6) cytokines and growth factors [11–14].

Transforming growth factor-β1 is a pleiotropic protein. Some of its effects are the result of its neutralizing the main inflammatory cytokines by inhibiting their synthesis [as with tumor necrosis factor-alpha (TNF-α)] or by downscaling their effects (as with IL-1β, interferon-γ and TNF-α) [23, 24].

Evidence also exists that TGF-β1 down-regulates (*1*) the inflammatory cytokine-induced expression of VCAM-1,

(*2*) the leukocyte adhesion to the endothelium, and (*3*) decreases macrophage activity by releasing IL-1 Ra [25–27].

Transforming growth factor-β1 has four actions to (*1*) inhibit both migration and proliferation of smooth muscle cells and macrophages [15, 28]; (*2*) induce apoptosis in numerous cells involved in the vascular lesions [29, 30]; (*3*) reduce adhesiveness of the endothelium for inflammatory cells [15, 31–33]; and (*4*) contribute to vascular protection by inhibiting human metalloelastase identified in vulnerable areas of plaque and responsible for its instability [34].

These experimental backgrounds have been confirmed by several clinical studies. In particular, Grainger et al found that serum levels of active TGF-β1 were more severely depressed in non-renal patients with advanced atherosclerosis than in persons without coronary atherosclerosis [11]. McDonald et al found that increased serum TGF-β1 may exert a cardioprotective effect [35]. Subsequently Erren et al confirmed that TGF-β1 serum levels are low in patients with severe ischemic heart disease [13].

In RDT patients, Suthanthiran et al found higher levels of serum TGF-β1 in black than in white patients, and suggested that this may be associated with the reduced incidence among the former of ischemic heart disease and death from myocardial infarction, and that this may explain the observation that blacks survive better than whites on hemodialysis [36]. Grainger et al also found that (active + latent) TGF-β1, and the proportions of TGF-β1 that were active, were similar in serum and platelet poor plasma prepared from the same blood samples [37]. Tashiro found significantly lower TGF-β1 plasma levels in patients with ischemic heart disease than in controls [14].

Research into the role of TGF-β1 in atherosclerotic and restenotic processes has given conflicting results. Several articles indicate that TGF-β1 tends to be highly expressed at sites of vascular injury, particularly in human restenotic lesions [38, 39]. Conversely other authors maintain that TGF-β1 is a potent antiproliferative agent for vascular cells [40, 41] and that decreased activation of TGF-β1 is associated with the progression of atherosclerosis [11].

The differing pattern of expression of TGF-β1 receptors may hold a key to understanding the role of TGF-β1 in the atherosclerotic process [30].

The absence of a TGF-β1 inhibitory effect [42, 43] in the end leads to increased chemotaxis, increased extracellular matrix formation, cell proliferation and a decrease in apoptosis. Thus, the resistance to apoptosis may lead to the slow proliferation of cell subsets resistant to TGF-β1, thereby contributing to the progression of atherosclerotic or restenotic lesions [30, 31, 44].

TGF-β and cardiovascular disease

Using a stepwise multiple regression analysis, only low TGF-β1 levels, apart from diabetes and serum albumin, prove to be predictive of cardiovascular mortality. Some protective role is surely posited if we reflect that a reduction by 1 ng/mL of TGF-β1 is associated with a 9% increase in the risk of mortality.

The main result that emerges from our study is that our RDT patients had a significantly lower concentration of active TGF-β1 than the control group of healthy subjects. The low TGF-β1 serum levels in our RDT patients exclude the possibility that the two investigated genetic patterns of production may ultimately affect TGF-β1 serum levels. Indeed, the low-producer prevalence in our populations is only 5%.

The most significantly lowered levels occurred in patients with simultaneous evidence of coronary, cerebral and peripheral vascular disease. Among the patients with ischemic heart disease, the lowest levels were found in those with significant stenoses of three major coronary vessels. These results are in agreement with previous reports regarding non-renal patients with so-called triple vessel disease [11, 14].

Such a correlation between reduced TGF-β1 levels and ischemic heart disease is related both to the antiproliferative effect of this cytokine on SMC and to its specific cardioprotective effect. TGF-β1 is able to counteract the release of TNF-α into the blood after myocardial ischemia via a transcription and translation mechanism [45]. TGF-β1 also reduces superoxide production in the ischemic coronary circulation by its endothelium protecting effect, which it also achieves by suppressing leukocyte adhesion to endothelial cells even in the presence of inflammatory cytokines.

The cardiovascular protecting effect of TGF-β1 is all the more important considering that the cells targeted by TGF-β1 come under the effect of other growth factors such as PDGF-AB and MCSF, which interact with specific receptors promoting proliferative activity and thus counteract the effects of TGF-β1. To further complicate this broad scenario, our previous papers reported the significant intradialytic platelet release of PDGF-AB that might predispose RDT patients to atherosclerosis [46, 47].

Unfortunately, the patients we studied had no ascertained TGF-β1 levels for the pre-dialytic period, so we were unable to clarify if and how its concentration varied as hemodialysis treatment got underway.

TGF-β and traditional atherosclerotic risk factors

Among the traditional atherosclerotic risk factors, age seems to affect basal TGF-β1 values only partially. In our study there was only a weak inverse correlation of TGF-β1 levels with age occurring both for hemodialyis patients (with or without cardiovascular diseases) and for the control group. There are no conclusive data in the literature on any correlation between age and TGF-β1 in non-renal patients [11]. This may be due to the shortage of studies on the subject.

No statistically significant difference in TGF-β1 levels emerges from our study between males and females among our hemodialysis patients. By contrast in non-renal patients the TGF-β1 serum level is thought to be correlated with gender. Grainger in particular speculates that women's TGF-β1 levels may be under hormonal regulation, especially considering the ascertained secretion of TGF-β1 from fetal human fibroblasts by antiestrogens [11]. Clearly, more extensive studies are needed to define the role of gender and age on TGF-β1 levels.

The incidence of diabetes mellitus in our population was around 5%. Among the evaluated cardiovascular risk factors, diabetes proved to have a higher role in terms of mortality (odds ratio 3.38) in our study population. However, no statistical difference emerged between diabetics and non-diabetics where TGF-β1 levels were concerned.

It is well-known that high levels of LDL and reduced levels of HDL are associated with an increased risk of cardiovascular mortality. Grainger finds an inverse correlation between serum LDL cholesterol and the proportion of active TGF-β1, and proposes that the sequestration of TGF-β1 to lipoprotein represents a possible mechanism for depression of TGF-β1 activity in advanced atherosclerosis [48].

If we look at our own patients, no correlation between LDL and TGF-β1 values was found; indeed, patients with the highest survival on dialysis show the highest levels of LDL. Again, the multivariable regression model did not show any statistically significant correlation between total serum cholesterol or LDL concentration and cardiovascular disease. As with total serum cholesterol, we did not find any significant association between systolic blood pressure and cardiovascular disease. The fact that multivariable analysis showed no association between the serum total cholesterol level or systolic blood pressure and the presence of atherosclerotic cardiovascular disease is at first sight somewhat surprising, but in line with the results of other cohort studies [7, 8]. These observations once again raise questions as to whether the traditional cardiovascular risk factors are applicable to the chronic hemodialysis population.

TGF-β, albuminemia, and acute phase reaction

The finding that hypoalbuminemia predicts death in dialysis patients has been attributed to inflammation, malnutrition and underdialysis [49]. Some authors have recently found a link between both CRP and hypoalbuminemia and cardiovascular disease in both the general population and in hemodialysis patients (abstract; Bergström et al, *J Am Soc Nephrol* 6:573, 1995) [50–52].

In our study the serum albumin levels are significantly lower in hemodialysis patients with cardiovascular disease, and this correlates with CRP levels: albumin emerges as an independent risk factor for cardiovascular death (odds ratio 1.1). Again, among hemodialysis patients with cardiovascular disease, albumin shows a significant, though moderate, direct correlation with serum TGF-β1 levels. In our view, this result lends itself to at least to two interpretations that need not be mutually exclusive: (*1*) both parameters reflect the severity of vascular impairment and/or the degree of vascular inflammation; (*2*) as with hypoalbuminemia, low TGF-β1 values might express a state of malnutrition.

Among the acute phase proteins, Lp(a) is thought to correlate in part with an inhibition of TGF-β1 activation [16, 53]. In accordance with previous reports our mean values of Lp(a) prove higher in patients with known cardiovascular disease than other RDT patients or the control group [54–56]. Our results show a slight inverse correlation between Lp(a) and TGF-β1. This does not seem sufficient in itself to account for the reduced basal serum levels of TGF-β1.

It is possible, however, that the inflammatory process inside the arterial wall enhances the pathophysiological effects of Lp(a), thus further reducing the activation of TGF-β1 [57]. In this connection we found a slight correlation between Lp(a) and PCR.

Plasminogen activator inhibitor-1 is a 50 kD protein synthesized and released by endothelial cells. High PAI-1 levels have been shown to predict the risk of recurrent myocardial infarction in younger non-uremic men and the occurrence of ischemic accidents in atherosclerotic patients [58]. PAI-1 production can be stimulated by oxidized LDL and cytokines such as TNF, both of which are elevated in hemodialysis patients [17]. The pro-atherogenic role of PAI-1 also is probably linked to its property of blocking the activation of latent TGF-β1 by competitively inhibiting tPA. PAI-1 and Lp(a) promote SMC proliferation in culture by reducing the autocrine inhibition caused by active TGF-β1 [59, 60].

In agreement with previous observations [61, 62], PAI-1 values in our hemodialysis patients proved higher than among controls. The increase was more marked in cardiovascular patients. These results could be related to the high plasma cytokine levels found in hemodialysis patients and the degree of endothelium dysfunction. In our study PAI-1 levels showed a moderate inverse correlation with TGF-β1 levels. These results seem to confirm the experience of other authors with non-renal patients, that is to say, a moderately inhibiting effect by PAI-1 on TGF-β1 exists. This effect might combine with the similar inhibition by Lp(a) and partly explain the low TGF-β1 values.

TGF-β and the hemodialysis procedure

Neither the type of membrane [cellulose modified (HE) or synthetic (PAN, PS)] nor the technique used seemed to affect the basal serum TGF levels, there being no significant differences among the membranes or the

Table 8. aPTT intra- and post-dialytic values

Time	aPTT *seconds*
30 minutes	107 ± 24
120 minutes	90 ± 22
240 minutes	78 ± 16
3 hours	43 ± 8

techniques used. These findings would appear to discount the possibility of TGF-β1 basal values and the activation pathway being somehow related to the biological reaction directly caused by the blood-membrane contact, or this result being considered an expression of higher or lower biocompatibility of the artificial materials. Our results are broadly in line with Suthanthiran's in vivo study that found no significant difference between "biocompatible" and "bioincompatible" membranes [36]. On the other hand, they diverge from the Mege et al's in vitro experience [63].

The intradialytic behavior of TGF-β1 serum levels showed a marked reduction at 30 minutes (about 30%) with a progressive return to basal values three hours after the session's end and without any difference related to the membrane and/or technique used. Though we have no proper explanation for this result, we do think that removal across the dialyzer membrane may be ruled out in view of the high molecular weight of this growth factor. However, the biology and the activation pathway of TGF-β1 might well hold the key to interpreting the result. In the circulation TGF-β1 exists in two distinct, biologically inactive forms: the original latent complex and a second complex with the protease inhibitor α_2-macroglobulin, which can be dissociated by heparin [64, 65]. The drop in total TGF-β1 occurring at 30 minutes thus might reflect the activating effect of the heparin (washing dose), and this would be borne out by the concomitant peak in aPTT values observed at 30 minutes (Table 8). We hypothesize that this heparin action is probably followed by activation of TGF-β1 and the link with specific cell receptors, leading to a fall in circulating values. These findings also suggest the hypothesis that the lower TGF-β1 basal serum levels found in our hemodialysis patients could partly stem from the heparin effect, as this growth factor is cyclically activated and may be exhausted in the process.

What is particularly interesting to analyze is the correlation between TGF-β1 and the time on RDT (Fig. 1). Interestingly, the highest TGF-β1 levels among our patients were found in the males of the subgroup with longest survival on dialysis (>113 months). In the light of the multivariate analysis results, such a finding among these patients may contribute to the theory that TGF-β1 plays a protective role against progression of the atherosclerotic process.

Conclusion

Our study shows that TGF-β1 serum levels are significantly reduced in hemodialysis patients, particularly in those with severe cardiovascular disease. The lower TGF-β1 concentration may predispose the hemodialysis patients to a worsening of atherosclerosis, in agreement with what has been argued in non-renal patients. Hemodialysis patients with severe cardiovascular disease showed a negative moderate correlation of TGF-β1 with raised levels of Lp(a) and PAI-1 and a moderate direct correlation with serum albumin levels. From the multivariable analysis it emerged that TGF-β1 is an independent cardiovascular risk factor along with diabetes and hypoalbuminemia. Nonetheless, if we consider that reduced TGF-β1 levels also have been found in hemodialysis patients without cardiovascular disease, we can argue that the observed correlations are not the only possible explanation of such a change in hemodialysis patients.

Therefore, the low TGF-β1 levels also could be hypothetically related to (*1*) subclinical endothelium damage, or (*2*) heparin-mediated activation of TGF-β1, which might lead to a reduction in TGF-β1 serum values (exhaustion) as a consequence of the subsequent link between TGF-β1 and the TGF-β1 receptor. Nevertheless, reduced TGF-β1 levels still only can be considered a necessary, and not a sufficient, condition. The role of TGF-β1 in atherosclerosis needs further evaluation and correlation with other known risk factors by means of longer prospective controlled studies.

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