# Circulating TNF Receptors 1 and 2 Predict Stage 3 CKD in Type 1 Diabetes

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## ABSTRACT

Elevated plasma concentrations of TNF receptors 1 and 2 (TNFR1 and TNFR2) predict development of ESRD in patients with type 2 diabetes without proteinuria, suggesting these markers may contribute to the pathogenesis of renal decline. We investigated whether circulating markers of the TNF pathway determine GFR loss among patients with type 1 diabetes. We followed two cohorts comprising 628 patients with type 1 diabetes, normal renal function, and no proteinuria. Over 12 years, 69 patients developed estimated GFR less than 60 mL/min per 1.73 $m^2$ (16 per 1000 person-years). Concentrations of TNFR1 and TNFR2 were strongly associated with risk for early renal decline. Renal decline was associated only modestly with total TNF$\alpha$ concentration and appeared unrelated to free TNF$\alpha$. The cumulative incidence of estimated GFR less than 60 mL/min per 1.73 $m^2$ for patients in the highest TNFR2 quartile was 60% after 12 years compared with 5%–19% in the remaining quartiles. In Cox proportional hazards analysis, patients with TNFR2 values in the highest quartile were threefold more likely to experience renal decline than patients in the other quartiles (hazard ratio, 3.0; 95% confidence interval, 1.7–5.5). The risk associated with high TNFR1 values was slightly less than that associated with high TNFR2 values. TNFR levels were unrelated to baseline free TNF$\alpha$ level and remained stable over long periods within an individual. In conclusion, early GFR loss in patients with type 1 diabetes without proteinuria is strongly associated with circulating TNF receptor levels but not TNF$\alpha$ levels (free or total).

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In a companion manuscript about nephropathy in type 2 diabetes (T2D), we report that elevated plasma concentrations of TNF receptor 1 (TNFR1) and receptor 2 (TNFR2) predict the development of ESRD.[1] Particularly interesting was the ability of these values to predict ESRD not only in proteinuric patients but also in nonproteinuric patients whose ESRD onset was 6–12 years after measurement of those receptors. On the basis of this ability to anticipate far-distant events, we speculate that the concentrations of these receptors are not merely markers of the injury leading to ESRD but also are involved in the inception of renal function decline.

The 55-kD TNFR1 and 75-kD TNFR2 are cell membrane–bound receptors involved in apoptosis, survival, and key aspects of inflammation and immune response.[2,3] In addition to their presence at

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the cell surface, they are released into the extracellular space. For example, circulating TNFR1 is released by two mechanisms: the inducible cleavage of the 34-kD TNFR1 ectodomain by a disintegrin and metalloproteinase 17 (ADAM17) and constitutive release of full-length 55-kD TNFR1 within exosome-like vesicles.[4–6] Whether the same mechanisms apply to TNFR2 release, how this process is regulated and subsequent effects of the circulating forms of TNF receptors are not well known.

Some authors consider the receptors as proxies for exposure to TNF$\alpha$, but empirical support for this theory is lacking. This uncertainty is put in the spotlight by our finding in T2D that the receptors were predictors of ESRD but TNF$\alpha$ was not—despite strong correlation at baseline.[1] Tracking changes in the concentrations of TNF$\alpha$ and its receptors over time could shed light on this issue. Unlike in our companion study of patients with T2D, this was feasible in patients with type 1 diabetes (T1D) because follow-up samples were available.

In two prospective Joslin Kidney Studies of patients with T1D, we showed that progressive deterioration of renal function commences in a subset of patients with high normoalbuminuria or microalbuminuria, well before the onset of proteinuria.[7–10] Because renal function is normal at the onset of this deterioration, we designate it early GFR loss rather than late renal function loss in patients who have already developed CKD.

These two Joslin Kidney Studies in patients with T1D and high normoalbuminuria or microalbuminuria provide an opportunity to investigate whether the findings in nonproteinuric patients with T2D extend to T1D and, if so, to explore the roles of TNF markers in the development of the early GFR loss, which is manifested as progression to CKD stages 3 and higher (CKD≥3). We have reported an inverse association of concentrations of circulating TNF pathway markers with renal function in the baseline data of the second Joslin Kidney Study.[11] Although consistent with a role for TNF receptors as a determinant of renal function, that cross-sectional analysis could not distinguish a pure association parallel with GFR loss from an actual effect on the course of renal function during follow-up. By contrast, the current study traces early renal function changes for 5 to 12 years.

## RESULTS

### Characteristics of Patients According to Study Cohort

Patient characteristics are summarized in Table 1 according to cohort: 275 patients from the first Joslin Kidney Study and 353 from the second. All had high normal GFR estimated from the serum concentration of cystatin C (eGFRcystatin) at baseline. Although age at diabetes diagnosis and age at enrollment were younger in the first than in the second Joslin Kidney Study, distributions of sex, hemoglobin A1c (HbA1c), and eGFRcystatin were similar. By design, albumin excretion rate (AER) was lower in the second study because it included patients with high normoalbuminuria as well as microalbuminuria. Treatment with renin-angiotensin system inhibitors was less frequent in the earlier cohort because it was not commonly prescribed when patients were enrolled. The cohorts had similar distributions of all TNF markers (free TNF$\alpha$, total TNF$\alpha$, TNFR1, and TNFR2), even though some of the differences were significant because of different selection of the cohorts.

During follow-up, CKD≥3 determined by serial measurements of serum cystatin C developed in 38 patients in the first Joslin Kidney Study and 31 in the second, giving similar incidence rates of 15 (95% confidence interval [CI], 11–21) and 17 (95% CI, 12–24) per 1000 person-years, respectively. Given the close similarity of baseline values of TNF markers in the cohorts and their nearly identical risks of CKD≥3, the two cohorts were combined for further analysis.

### Markers of TNF Pathway and Risk for CKD≥3: Univariate Analysis

To explore how CKD≥3 risk varied with baseline concentrations of TNF pathway markers, we examined the incidence rate according to quartiles of their distributions (Table 2). A disproportionate number of cases occurred in the fourth quartile for each marker. This pattern was strongest for TNFR2: 48/1000 person-years in the fourth quartile, 9/1000 in third and second quartiles, and just 3/1000 in the first quartile ($P<0.0001$). For TNFR1, the gradient was almost identical. In contrast, it was less steep for total TNF$\alpha$ and insignificant for free TNF$\alpha$.

Serum concentrations of TNFR1 and TNFR2 were highly correlated (Spearman $r=0.78$; $P<0.0001$) (Figure 1). In Figure 1, red squares represent patients who developed CKD≥3, and gray circles represent those whose renal function remained stable and within the normal range over time. Vertical and horizontal lines are quartile boundaries. Almost all cases of CKD≥3 in the fourth quartile of one receptor were also in the fourth quartile of the other. This finding illustrates the redundancy of information in the two receptors.

To see the effects of marker concentration on the temporal pattern of occurrence of CKD, we plotted the cumulative risk for CKD≥3 according to follow-up time and marker quartile. For patients in the highest quartile of TNFR2, the cumulative risk for CKD≥3 increased steeply at a constant rate from the start of observation (Figure 2). For patients in the lower quartiles of TNFR2, cases were few and occurred late. The 12-year cumulative risk for CKD≥3 for the highest TNFR2 quartile was 60% and ranged from 5% to 19% for the lower quartiles. Results were similar for quartiles of TNFR1 (data not shown).

### Markers of the TNF Pathway and Risk for CKD≥3: Multivariate Analysis

Table 3 shows results of univariate and multivariate Cox proportional analysis. Whereas many baseline clinical covariates showed an association with risk for CKD≥3 in univariate analysis, almost all of them became insignificant when analyzed together. Only HbA1c, AER, and eGFRcystatin remained significant (see Concise Methods) in multivariate analysis.

**Table 1.** Baseline characteristics of study groups with T1D and incidence of CKD≥3 during follow-up according to cohort

| Variable | First Joslin Kidney Study (*n*=275) | Second Joslin Kidney Study (*n*=353) | *P* Value[a] |
|---|---|---|---|
| Study characteristics | | | |
| albuminuria category | MA | High NA or MA | |
| calendar years of recruitment | 1991–1992 | 2003–2005 | |
| follow-up interval (yr) | 10-12 | 5-7 | <0.0001 |
| Patient characteristics | | | |
| men (%) | 51 | 53 | 0.61 |
| age (yr) | 31±8 | 39±12 | <0.0001 |
| body mass index (kg/m$^2$) | 25±4 | 27±5 | <0.0001 |
| systolic BP (mmHg) | 122±14 | 121±13 | 0.45 |
| diastolic BP (mmHg) | 76±8 | 73±8 | <0.0001 |
| serum cholesterol (mg/dl) | 200±42 | 187±30 | <0.0001 |
| ever smoking (%) | 51 | 44 | 0.10 |
| age at diabetes diagnosis (yr) | 14±8 | 18±11 | <0.0001 |
| diabetes duration (yr) | 18±9 | 21±9 | <0.0001 |
| HbA1c (%) | 8.9±1.5 | 8.4±1.3 | <0.0001 |
| AER ($\mu$g/min) | 56 (37, 101) | 41 (24, 79) | 0.07 |
| eGFRcystatin (ml/min per 1.73 m$^2$) | 133±30 | 129±30 | 0.06 |
| treated with RASi/AHTN (%) | 34 | 58 | <0.0001 |
| Markers at baseline (pg/mL) | | | |
| free TNF$\alpha$ | 4.2 (3.2, 5.4) | 5.0 (3.4, 6.7) | 0.30 |
| total TNF$\alpha$ | 8.7 (5.7, 12.2) | 8.9 (4.9, 13.5) | 0.08 |
| TNFR1 | 1345 (1156, 1598) | 1382 (1180,1709) | 0.17 |
| TNFR2 | 2161 (1732, 2673) | 2230 (1869, 2695) | 0.12 |
| Incidence of CKD≥3 | | | |
| rate per 1000 person-years (95% CI) | 15 (11–21) | 17 (12–24) | |
| cases (*n*) | 38 | 31 | 0.05 |

Data expressed with a plus/minus sign are the mean ± SD. Markers at baseline are expressed as the median (25th, 75th percentiles). MA, microalbuminuria; NA, normoalbuminuria; NS, nonsignificant; RASi/AHTN, renin-angiotensin system inhibitors and/or other antihypertensive agent.
[a]Bonferroni correction was applied.

**Table 2.** Incidence of CKD≥3 in patients with T1D during 5- to 12-year follow-up according to quartiles of distributions of baseline circulating TNF pathway marker concentrations

| Quartile | Patients (*n*) | Incidence per 1000 Person-Years (No. of Events) | | | |
|---|---|---|---|---|---|
| | | Free TNF$\alpha$ | Total TNF$\alpha$ | TNFR1 | TNFR2 |
| Q1 | 157 | 10 (11) | 9 (9) | 5 (6) | 3 (4) |
| Q2 | 157 | 11 (12) | 8 (9) | 9 (10) | 9 (10) |
| Q3 | 157 | 14 (13) | 9 (10) | 10 (11) | 9 (10) |
| Q4 | 157 | 24 (21) | 33 (29) | 45 (42) | 48 (44) |
| *P* for trend[a] | | 0.048 | <0.0001 | <0.0001 | <0.0001 |

Q1–Q4, quartiles 1–4. Quartile boundaries for 25th, 50th, and 75th percentiles, respectively, are as follows (pg/ml for all values). Free TNF$\alpha$: 3.3, 4.6, 6.1; total TNF$\alpha$: 5.5, 8.8, 12.7; TNFR1: 1170, 1371, 1670; TNFR2: 1810, 2197, 2690.
[a]Bonferroni correction was applied.

We assessed the independent effect of each TNF marker on the risk for CKD≥3 by adding it to a Cox proportional hazards model of the influential clinical characteristics: HbA1c, AER, and eGFRcystatin. Each marker was represented in this analysis by an indicator variable for the contrast of the fourth quartile with the lower quartiles, a representation that captured the dose-response relationship more completely than a second-order polynomial (See Table 2 and Statistical Analyses in Concise Methods). The results of this set of models are summarized in Figure 3 with the hazard ratio and 95% CI for each TNF pathway marker after adjustment for clinical covariates. The effect of TNFR2 was strongest and that of TNFR1 a close second. The effect of total TNF$\alpha$ and free TNF$\alpha$ did not remain significant in the multivariate analysis. When markers were added pairwise, TNFR2 emerged as the dominant influence. With TNFR2 and total TNF$\alpha$ in the model, the effect of TNFR2 was only slightly reduced (broken lines in Figure 2). With TNFR1 and total TNF$\alpha$ in the model, the result was the same (data not shown). Finally, with TNFR1 and TNFR2 in the model, the effect of TNFR2 was again only slightly reduced (hazard ratio, 2.5; 95% CI, 1.3–4.8) whereas that for TNFR1 was not significant (hazard ratio, 1.6; 95% CI, 0.8–3.3).

### Temporal Variation in Concentrations of TNF Pathway Markers

From the ability of a single assay of TNF pathway markers to predict the future onset of CKD≥3, we inferred that baseline concentrations either persisted unchanged or changed, but

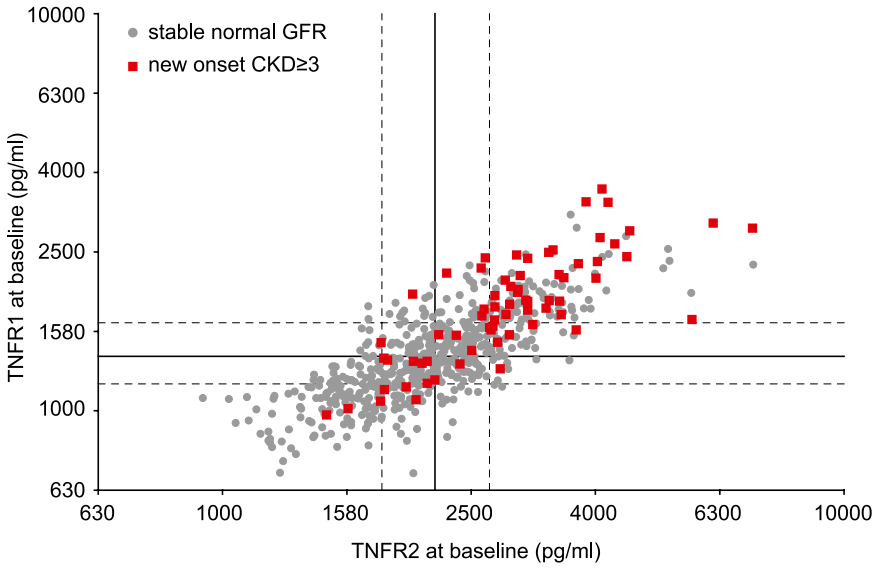


**Figure 1.** Scatter plot of circulating concentrations of TNFR1 and TNFR2 in baseline samples from study participants with T1D and normal renal function (Spearman correlation between TNFR1 and TNFR2: $r$=0.78; $P$<0.0001). Gray circles represent patients whose renal function remained normal at the end of follow-up. Red squares represent patients whose renal function declined to CKD≥3. Vertical and horizontal lines are the 25th, 50th, and 75th percentiles of the respective markers.

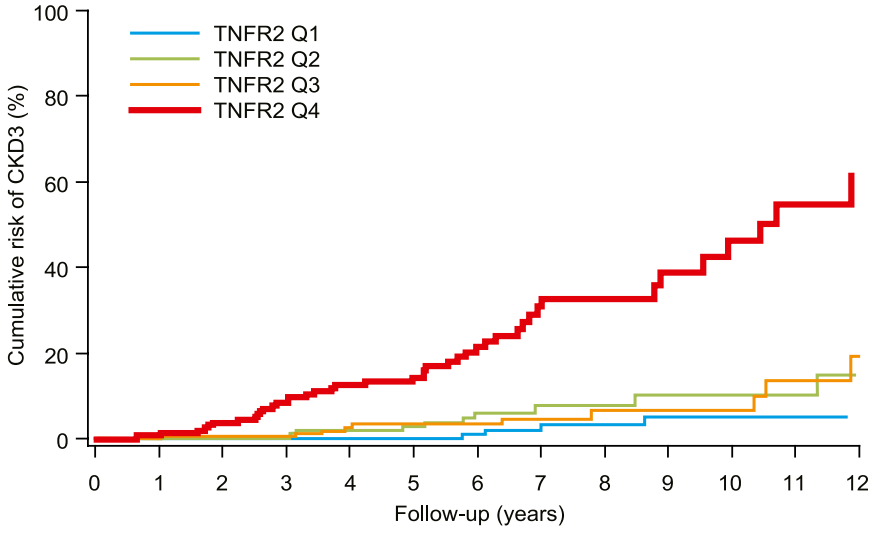


**Figure 2.** Cumulative risk for CKD≥3 in patients with T1D during 12 years of follow-up according to quartile (Q1–Q4) of circulating TNFR2 at baseline.

with rank order preserved. To test this, we measured the concentrations of TNF markers in 77 patients from the first Joslin Kidney Study in samples obtained at baseline and follow-up samples obtained 2–3 years later (Table 4).

Rank correlations between the two measurements were high (0.72–0.81) except for the moderate correlation for total TNFα (0.56). Moreover, changes in the means were tiny. Together these findings indicate that concentrations were stable over time within the individual. Furthermore, the belief that in circulation free TNFα drives TNFRs levels was not supported by the negative and mostly nonsignificant correlations between baseline free TNFα concentration and subsequent changes in the concentrations of the receptors.

### TNF Pathway Protein Expression on Leukocytes and Soluble Receptors

In an ancillary study we measured the expression of proteins of the TNF pathway on fresh leukocytes in patients with high or low serum total concentrations of TNF receptors. We recruited 34 nonproteinuric patients with T1D or T2D and eGFRcystatin greater than 60 ml/min per 1.73 $m^2$ in the Joslin Clinic and measured circulating TNFR1 and TNFR2. We divided the group at the median serum TNFR2 concentrations and compared the expression of membrane-bound TNFα, TNFR1, and TNFR2 and ADAM17 on their leukocytes. The results are summarized in Table 5. Expression of those proteins on neutrophils, monocytes, or lymphocytes did not differ between the two subgroups.

## DISCUSSION

Elevated serum concentrations of TNFR1 and TNFR2 are strongly associated with early renal function loss (progression to CKD3 or higher) that starts in patients with T1D who have normal renal function. The association is independent of circulating TNFα (free or total) and relevant clinical covariates: age, HbA1c, urinary AER, baseline eGFRcystatin, BP, and treatment with renin-angiotensin system inhibitors. These data in T1D are consistent with and further expand our findings in T2D detailed in our companion manuscript.[1] In T2D, high concentrations of TNF receptors are strong predictors of renal function loss (progression to ESRD) in nonproteinuric as well as proteinuric patients. Together, these associations point to the involvement of TNF receptors and their independent role in the cause of early and late renal function loss in both types of diabetes.

Our study demonstrates the strongest association with TNFR2, with TNFR1 being a close second. TNFR1 is abundant on all nucleated cells, but TNFR2 expression is restricted mainly to endothelial cells and leukocytes,[12] although this varies between normal and diseased tissues.[13–15] Each receptor

**Table 3.** Cox proportional hazard analysis of risk for CKD≥3 in patients with T1D according to clinical characteristics and circulating markers of TNF pathway in univariate and multivariate models

| Baseline Characteristic | Univariate Model: Hazard Ratio (95% CI) | Multivariate Model: Hazard Ratio (95% CI) | |
|---|---|---|---|
| | | Clinical Predictors Only | Clinical Predictors and Markers[a] |
| Clinical predictors[b] | | | |
| sex (male) | 0.55 (0.33–0.90) | | |
| age | 1.44 (1.15–1.81) | — | — |
| body mass index | 1.11 (0.93–1.32) | — | — |
| systolic BP | 1.12 (0.95–1.32) | — | — |
| diastolic BP | 1.03 (0.83–1.28) | — | — |
| serum cholesterol | 1.39 (1.11–1.75) | — | — |
| ever smoking | 1.18 (0.73–1.91) | — | — |
| age at diabetes diagnosis | 1.37 (1.10–1.71) | — | — |
| Diabetes duration | 1.08 (0.91–1.30) | — | — |
| HbA1c | 1.52 (1.28–1.79) | 1.61 (1.36–1.90) | 1.59 (1.33–1.90) |
| AER | 1.59 (1.33–1.91) | 1.20 (0.99–1.47) | 1.16 (0.95–1.42) |
| eGFRcystatin | 2.00 (1.71–2.32) | 1.88 (1.61–2.19) | 1.64 (1.37–1.95) |
| treated with RASi/AHTN | 2.17 (1.30–3.61) | — | — |
| cohort (JKS2 versus JKS1) | 2.03 (1.13–3.66) | — | — |
| Circulating markers: Q4 versus Q1–Q3[c] | | | |
| free TNF$\alpha$ | 2.14 (1.23–3.74) | — | 1.44 (0.80–2.59)[d] |
| total TNF$\alpha$ | 4.89 (2.81–8.51) | — | 1.87 (1.00–3.48)[d] |
| TNFR1 | 6.42 (3.90–10.6) | — | 2.53 (1.36–4.67) |
| TNFR2 | 7.16 (4.31–11.9) | — | 3.04 (1.67–5.52) |

RASi/AHTN, renin-angiotensin system inhibitors and/or other antihypertensive agent; JKS, Joslin Kidney Study; Q1–Q4, quartiles 1 to 4.
[a]The effect of each circulating marker was examined separately while controlling for clinical predictors. Hazard ratios for clinical predictors are from the multivariate model with TNFR2.
[b]Hazard ratios for each clinical covariate are for a one-quartile increase in their respective distribution except for categorical variables and for eGFRcystatin, for which it is a one-quartile decrease.
[c]Clinical characteristics of patients with fourth quartile versus first through third quartiles are shown in Supplemental Table 1.
[d]Effects of free and total TNF$\alpha$ were not significant in the multivariate model.

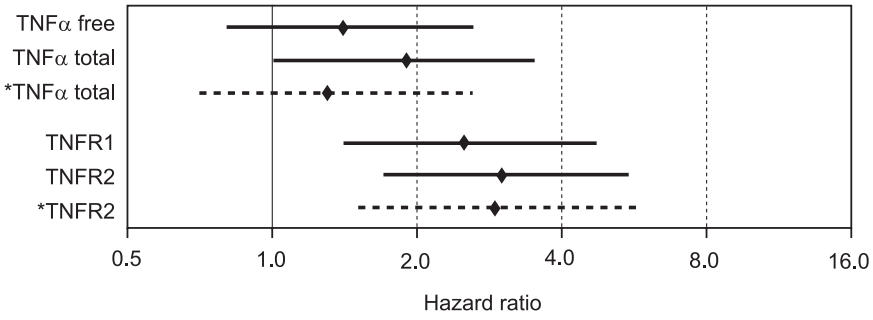


**Figure 3.** Effect of each TNF pathway marker at baseline on the risk for CKD≥3 in T1D patients followed up to 12 years. Hazard ratios are estimates from a Cox proportional hazards model for a fourth-quartile effect versus first through third quartiles and adjusted for HbA1c, AER, and eGFRcystatin. Diamonds and solid lines represent the point estimates and 95% CIs for individual markers adjusted by clinical covariates. Diamonds and broken lines marked with an asterisk represent point estimates and 95% CIs adjusted by clinical covariates and with both TNF$\alpha$ total and TNFR2 in the model.

may play distinct roles in inflammation and apoptosis,[13,14,16] but they also cooperate to regulate many of their downstream effects.[17–19] TNFRs are released from the cell surface by the action of ADAM17.[4,5] In our study, however, variation in circulating TNFRs was not accompanied by differences in their surface expression or that of ADAM17 on peripheral blood mononuclear cells. This finding indicates that leukocytes might not be the most relevant source of circulating receptors or implies a more complex regulation of circulating TNFR levels.[3,6,20] TNFR1 has been recently reported to be released in the intact form through exocytosis and to circulate afterward in exosome-like vesicles.[6] Indeed, this mechanism would not directly affect levels of TNFR1 expression on the cell surface.

One plausible nonleukocyte cellular source of both the cleaved and the intact circulating TNFRs is the activated endothelium.[18,21] Urinary concentrations of TNFRs have not correlated strongly with the circulating receptors and have not confounded the effect of the latter ones on risk for CKD3 (data shown in Supplemental Tables 2 and 3). It points out that a systemic rather than a local kidney source of TNF receptors is contributing to increased risk for early renal function loss.

How may circulating concentrations of TNFR1 and TNFR2 be associated with early GFR loss? One possibility is that their elevation reflects the presence of a disease process in kidneys that leads to early GFR loss. However, this interpretation is undermined by our findings that high circulating concentrations

**Table 4.** Concentrations of circulating TNF markers in 77 patients with T1D at baseline and early follow-up__

| Markers | Time of Examination | | Change from Baseline versus Follow-Up[a] | Spearman Correlations | |
|---|---|---|---|---|---|
| | Baseline (pg/ml) | Early Follow-Up (pg/ml)[b] | | Baseline versus Follow-Up | Baseline Free TNF$\alpha$ versus Change during Follow-Up |
| free TNF$\alpha$ | 5.3±2.0 | 5.3±2.2 | 0.04±1.6 | 0.72[c] | NA |
| total TNF$\alpha$ | 9.2±5.5 | 8.6±4.8 | −0.6±5.5 | 0.56[c] | −0.07 |
| TNFR1 | 1473±446 | 1478±606 | 4±409 | 0.77[c] | −0.11 |
| TNFR2 | 2519±725 | 2522±1049 | 3±726 | 0.81[c] | −0.23[d] |

Data expressed with a plus/minus sign are the mean ± SD. NA, not applicable.
[a]No change was statistically significant.
[b]Median (25th, 75th percentiles) = 2.5 (2, 3) years after the baseline examination.
[c]$P<0.001$.
[d]$P<0.05$.

**Table 5.** Serum concentrations of soluble TNF receptors and flow cytometry analysis of surface expression of TNF$\alpha$-pathway proteins on peripheral blood cells stratified by group-specific median serum concentration of TNFR2

| Characteristic | Group Stratification (TNFR2 Median)[a] | | *P* Value[b] |
|---|---|---|---|
| | Low (*n*=17) | High (*n*=17) | |
| Serum sTNFR2 (pg/ml) | 2442±260 | 4079±1098 | By design |
| Serum sTNFR1 (pg/ml) | 1310±276 | 2291±773 | <0.001 |
| Leukocyte counts ($\times 10^9$/L) | 6.6±1.7 | 6.4±1.9 | 0.63 |
| neutrophils | 4.4±2.0 | 4.2±1.3 | 0.81 |
| lymphocytes | 2.8±4.5 | 1.5±0.6 | 0.25 |
| monocytes | 0.4±0.1 | 0.5±0.2 | 0.07 |
| Surface expression of TNF$\alpha$-pathway proteins[c] | | | |
| TNF$\alpha$ protein on | | | |
| neutrophils | 24±21 | 17±25 | 0.24 |
| T lymphocytes, CD3$^+$ | 1.4±1.8 | 1.5±1.8 | 0.87 |
| monocytes | 24±17 | 25±31 | 0.76 |
| TNFR1 protein on | | | |
| neutrophils | 105±58 | 87±82 | 0.24 |
| T lymphocytes, CD3$^+$ | 2.0±1.7 | 1.5±2.8 | 0.50 |
| monocytes | 135±71 | 121±104 | 0.37 |
| TNFR2 protein on | | | |
| neutrophils | 27±10 | 24±7 | 0.34 |
| T lymphocytes, CD3$^+$ | 9±6 | 9±4 | 0.97 |
| monocytes | 122±32 | 115±31 | 0.48 |
| ADAM17 protein on | | | |
| neutrophils | 77±6.2 | 74±6.6 | 0.15 |
| T lymphocytes, CD3$^+$ | 3.1±1.1 | 3.3±1.3 | 0.62 |
| monocytes | 141±13 | 133±17 | 0.12 |

Data are the mean ± SD.
[a]Low and high denote strata below and above median TNFR2 in each group (2769 pg/ml), accordingly.
[b]*P* value for *t* test on log-transformed values.
[c]Data are mean fluorescence intensities, the differences between the intensities for the surface marker and the isotype control.

of TNF receptors persist in patients, are stable within the individual, and precede the development of CKD by 5–12 years. An alternative is that their elevation contributes directly to the renal injury underlying early GFR loss, or that it is a risk factor for the injury. We also discussed this issue in our earlier cross-sectional report.[11]

How could circulating TNFRs affect kidney function? Exposure of kidney organ culture to either receptor increases apoptosis, mainly in tubules.[13] Individual knockout mice models of TNFR1 or TNFR2 have a delay in the fibrotic response in a mouse model of tubulointerstitial fibrosis,[22] whereas TNFR2 deficiency alone protects from development of GN.[14] Unfortunately, little research has been done on this in the diabetic kidney. Laboratory studies have focused mainly on the effects of TNF$\alpha$ on the kidney, as recently reviewed,[15,23] and a particular role of TNFRs in diabetic nephropathy has not

been investigated. TNF$\alpha$ increases albumin permeability,[24] and its inhibition in a diabetic animal model decreases albuminuria.[25] However, improved albuminuria is not necessarily paralleled by improvement in GFR.[26,27] Interestingly, as we examined in the patients participating in the second Joslin Kidney Study, circulating TNFRs did not affect the course of albuminuria during follow-up (Supplemental Table 4). The effect of TNF$\alpha$ pathway inhibition on a GFR-oriented outcome has not been studied so far. The current studies prompt a question about whether therapeutic interventions in diabetic nephropathy should focus on modulation of TNFRs specifically.

Some authors consider elevated circulating concentrations of TNFRs as a slow-release reservoir of TNF$\alpha$,[2] but the role of circulating TNFRs must be more complex. The concentration of circulating TNF$\alpha$ at baseline was correlated with serum concentrations of TNFRs to some extent; at the same time, in both our studies of T1D and T2D, none of the circulating TNF$\alpha$ forms (free or total) was associated with renal function loss if the effect of one TNF receptor was accounted for. Moreover, TNF$\alpha$ concentration at baseline was not associated with subsequent changes in circulating TNFRs. These findings call into question speculation on whether circulating forms of TNFRs function as decoys for TNF$\alpha$ or that their concentrations reflect long-term exposure to this pro-inflammatory cytokine.

In patients from the second Joslin Kidney Study, we also investigated the association between early renal function loss and circulating concentrations of other inflammatory markers (IL-8, monocyte chemotactic protein-1, interferon-$\gamma$–inducible protein-10, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, Fas ligand, and C-reactive protein), receptors of other cytokines (Fas, IL-1R, IL-2R, IL-4R, and IL-6R), and receptor for advanced glycation end products. None were significantly associated with early GFR loss (data not shown). These findings agree with our results reported in the companion manuscript. In that study we showed that in patients with T2D, high risk for ESRD was predicted specifically by circulating levels of TNFR1 and TNFR2, but not by any of the other markers of systemic inflammation or markers of endothelial damage.[1] Therefore, our findings in T1D and T2D point to the specific involvement of TNFR-mediated pathway rather than a general inflammation in the development of renal function decline and progression to ESRD. We acknowledge that chronic low-grade inflammation has been postulated in the literature[23,28–30] as being involved in the development of diabetic nephropathy. However, those studies were inconclusive regarding the specific mechanisms or biomarkers involved in the disease process underlying renal function decline. We hope that our findings will reinvigorate of this area of research.

Among the strengths of our study are its large size, prospective design, consistency with the data in T2D, and further expansion of these data. Some may consider the lack of direct measurements of GFR a weakness. However, multiple determinations of serum cystatin C over time reliably indicate the course of renal function change.[7,31] It is unlikely that the associations between elevated serum cystatin C as an indicator of CKD3 and high serum levels of TNFRs are spurious as result of the inflammatory properties of both.[32] For example, in our recent study we demonstrated that in the patients with diabetes, CKD$\geq$3 determined by serum cystatin C was a much stronger predictor of ESRD than was CKD$\geq$3 determined by serum creatinine (Krolewski *et al.*, submitted). In addition, in the companion manuscript we demonstrated that high serum levels of TNFRs were very strong predictors of ESRD in patients with T2D.[1]

Finally, there are some differences between the findings of this study in T1D and our findings in T2D. However, the differences are more apparent than real. First, the hazard ratios for the development of ESRD according to circulating levels of TNF receptors in the T2D study were much higher than those for the development of CKD3 in the T1D study. However, the CIs for the hazard ratios overlap. Second, in the univariate analyses for both T1D and T2D, the major effect on risk for early and late renal function loss was clearly confined to the highest concentrations of TNF receptors. Because of the lack of cases of ESRD in the lowest quartile of the plasma TNFR concentrations, we were not able to examine a nonlinear relationship in T2D as we did in this study examining risk for CKD3 in T1D.

## CONCISE METHODS

### Study Cohorts

The study group comprised two cohorts of patients with T1D who were recruited while attending the Joslin Clinic (Table 1). The protocols and consent procedures for both studies were approved by the Joslin Diabetes Center Institutional Review Board. The first Joslin Kidney Study cohort was recruited in 1991–1992 and was followed for 8–12 years. For the subset of 302 patients with an AER in the microalbuminuria range (30–300 $\mu$g/min), results regarding the natural history of microalbuminuria and the occurrence of early GFR loss have been published.[7,33] From that study, we included the 275 patients whose follow-up was complete and for whom a baseline blood sample was still available for analysis. The second Joslin Kidney Study cohort was recruited in 2003–2005, and 353 patients have had 5–7 years of follow-up.[10] Eligible for the current study were those with high-normal AER (15–29 $\mu$g/min) ($n$=146) or microalbuminuria (30–300 $\mu$g/min) ($n$=207) at enrollment. The 314 patients with low-normal AER less than 15 $\mu$g/min were ineligible because they had not been followed as a result of their very low risk for early GFR loss.[7,10] In both studies 94% of patients identified themselves as white. Recruitment and examination protocols and definitions of clinical characteristics have been described elsewhere.[7,10,11,33]

### Measurement of Markers of TNF Pathway

Protocols for measuring concentrations of TNF$\alpha$ (free and total) and its receptors (TNFR1 and TNFR2) have been described elsewhere.[1,11] The first two were measured in plasma. We discovered that measurements obtained from serum underestimated total TNF$\alpha$ in particular (data not shown). Assay results for the receptors are identical in serum and plasma (data not shown) and were measured in serum. Protocols to measure additional markers in ancillary studies are described at the end of the Concise Methods.

### Measurement of Renal Function

The eGFRcystatin values were calculated according to the following formula:[34]

$$eGFRcystatin\ in\ ml/min\ per\ 1.73m^2 = 127.7 \times cystatin\ C\ in\ mg/L^{-1.17} \times age^{-0.13} (\times 0.91\ when\ female)$$

The eGFRcystatin correlates closely with measured GFR and, unlike GFR estimates based on serum creatinine, tracks changes accurately even in the normal or elevated range.[7,31] Moreover, in patients with T1D and T2D, CKD stage 3 according to eGFRcystatin predicts the risk for ESRD more accurately than do estimates based on serum creatinine (Krolewski *et al.*, submitted).[35]

In the first Joslin Kidney Study, cystatin C was assayed in all available serum samples (median, six) in the Joslin Research Laboratory between 2004 and 2006 using the BN ProSpec System nephelometer (Dade Behring Diagnostics, Newark, DE [currently Siemens Healthcare Diagnostics Inc]).[7] Our interassay coefficient of variation was less than 3.9%. In the second Joslin Kidney Study, all available serum samples (median, five) were assayed in 2009 and 2010 at the Collaborative Studies Clinical Laboratory at the University of Minnesota using BN ProSpec and calibrated to internal standards supplied by the manufacturer. Their interassay coefficient of variation was approximately 4.7%. To develop a formula (based on duplicate assays) to calibrate the 2003–2006 serum cystatin C determinations to the 2009–2010 determinations, a random sample of 60 serum specimens from the first Joslin Kidney Study was included with the 2009–2010 set. The serum cystatin C values from the first study were then adjusted to be comparable to those from the second study.

### Definition of Early GFR Loss

We monitored renal function during follow-up to determine which patients developed impaired function (CKD≥3), defined as an eGFRcystatin less than 60 ml/min per 1.73 $m^2$. The date of the first sample with a qualifying eGFRcystatin was taken as the date of onset.

### Statistical Analyses

Analyses were performed using SAS software, version 9.2 (SAS Institute, Cary, NC). We explored the dose-response relationship between TNF pathway markers and risk for CKD≥3 by analyzing each marker in quartiles. The pattern appeared nonlinear, with a disproportionate risk in the fourth quartile. To accommodate this pattern, markers were examined both as a continuous second-order polynomial and as quartiles in Cox proportional hazards models. With both representations in the model, an indicator variable for the fourth quartile fully captured the shape of the dose-response relationships between markers and risk for CKD≥3, leaving an insignificant effect for the second-order polynomial (likelihood ratio test). Therefore, the markers were modeled in subsequent analyses as indicator variables for the fourth quartile.

The baseline clinical covariates were examined in a Cox proportional hazards model (Table 3). The model was subsequently reduced by minimizing the Schwartz (Bayesian) information criterion, and the independent effect of each TNF marker on the risk for CKD≥3 was assessed in the presence of the relevant clinical covariates that contained HbA1c, AER, and eGFRcystatin and was controlled for the cohort. There were no missing data in the variables retained in the final model except for HbA1c, for which 8% of observations were missing. The other clinical covariates (age, systolic BP, renoprotective treatment, and smoking) did not remain significant and did not confound the effects of TNF$\alpha$ or TNFRs, so they were not retained in the final model.

### Methods for Studies of Peripheral Blood Cells

Thirty-four patients attending the Joslin Clinic with T1D ($n$=19) and T2D ($n$=15), an AER below the proteinuria range, and an eGFRcystatin greater than 60 ml/min per 1.73 $m^2$ were recruited to the study in September–October 2010. Fifteen were male (44%) and twenty-three (63%) had microalbuminuria. Medical questionnaire data, blood, and urine were collected, and circulating marker concentrations were measured as described in earlier in the Concise Methods.

Blood was obtained in EDTA-anticoagulant for a three-color fluorescence flow cytometric analysis of TNF$\alpha$-pathway–related proteins (TNF$\alpha$, TNFR1, TNFR2, and ADAM17) on peripheral blood cell subsets. Commercial sources of phycoerythrin (PE), fluorescein-isothiocyanate (FITC), allophycocyanin (APC)-conjugated mouse (m) antihuman monoclonal antibodies, and IgG isotype controls are as follows: anti-membrane TNF$\alpha$/PE (mIgG1), anti-TNFR1/PE (mIgG1), anti-TNFR2/FITC (mIgG2a), and anti-ADAM17/FITC (mIgG1) from R&D Systems (Minneapolis, MN); mIgG1/FITC, mIgG1/PE, mIgG1/APC, mIgG2a/FITC, and mIgG2a/APC from Biolegend (San Diego, CA); APC conjugated anti-CD3 for T cells, anti-CD11b for neutrophils (gating on CD11b high cells), and anti-CD14 for monocytes from Biolegend (San Diego, CA).

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## DISCLOSURES

None.

## REFERENCES

1. Niewczas MA, Gohda T, Skupien J, Smiles AM, Walker WH, Rosetti F, Cullere X, Eckfeldt JH, Doria A, Mayadas TN, Warram JH, Krolewski AS: Circulating TNF receptors 1 and 2 predict ESRD in type 2 diabetes. *J Am Soc Nephrol* 23: 507–515, 2012

2. Aderka D: The potential biological and clinical significance of the soluble tumor necrosis factor receptors. *Cytokine Growth Factor Rev* 7: 231–240, 1996
3. Levine SJ: Molecular mechanisms of soluble cytokine receptor generation. *J Biol Chem* 283: 14177–14181, 2008
4. Bell JH, Herrera AH, Li Y, Walcheck B: Role of ADAM17 in the ectodomain shedding of TNF-alpha and its receptors by neutrophils and macrophages. *J Leukoc Biol* 82: 173–176, 2007
5. Edwards DR, Handsley MM, Pennington CJ: The ADAM metalloproteinases. *Mol Aspects Med* 29: 258–289, 2008
6. Hawari FI, Rouhani FN, Cui X, Yu ZX, Buckley C, Kaler M, Levine SJ: Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: A mechanism for generation of soluble cytokine receptors. *Proc Natl Acad Sci U S A* 101: 1297–1302, 2004
7. Perkins BA, Ficociello LH, Ostrander BE, Silva KH, Weinberg J, Warram JH, Krolewski AS: Microalbuminuria and the risk for early progressive renal function decline in type 1 diabetes. *J Am Soc Nephrol* 18: 1353–1361, 2007
8. Rosolowsky ET, Niewczas MA, Ficociello LH, Perkins BA, Warram JH, Krolewski AS: Between hyperfiltration and impairment: Demystifying early renal functional changes in diabetic nephropathy. *Diabetes Res Clin Pract* 82(Suppl 1): S46–S53, 2008
9. Perkins BA, Ficociello LH, Roshan B, Warram JH, Krolewski AS: In patients with type 1 diabetes and new-onset microalbuminuria the development of advanced chronic kidney disease may not require progression to proteinuria. *Kidney Int* 77: 57–64, 2010
10. Ficociello LH, Rosolowsky ET, Niewczas MA, Maselli NJ, Weinberg JM, Aschengrau A, Eckfeldt JH, Stanton RC, Galecki AT, Doria A, Warram JH, Krolewski AS: High-normal serum uric acid increases risk of early progressive renal function loss in type 1 diabetes: Results of a 6-year follow-up. *Diabetes Care* 33: 1337–1343, 2010
11. Niewczas MA, Ficociello LH, Johnson AC, Walker W, Rosolowsky ET, Roshan B, Warram JH, Krolewski AS: Serum concentrations of markers of TNFalpha and Fas-mediated pathways and renal function in nonproteinuric patients with type 1 diabetes. *Clin J Am Soc Nephrol* 4: 62–70, 2009
12. Bradley JR: TNF-mediated inflammatory disease. *J Pathol* 214: 149–160, 2008
13. Al-Lamki RS, Wang J, Vandenabeele P, Bradley JA, Thiru S, Luo D, Min W, Pober JS, Bradley JR: TNFR1- and TNFR2-mediated signaling pathways in human kidney are cell type-specific and differentially contribute to renal injury. *FASEB J* 19: 1637–1645, 2005
14. Vielhauer V, Stavrakis G, Mayadas TN: Renal cell-expressed TNF receptor 2, not receptor 1, is essential for the development of glomerulonephritis. *J Clin Invest* 115: 1199–1209, 2005
15. Ernandez T, Mayadas TN: Immunoregulatory role of TNFalpha in inflammatory kidney diseases. *Kidney Int* 76: 262–276, 2009
16. MacEwan DJ: TNF receptor subtype signalling: differences and cellular consequences. *Cell Signal* 14: 477–492, 2002
17. Pinckard JK, Sheehan KC, Schreiber RD: Ligand-induced formation of p55 and p75 tumor necrosis factor receptor heterocomplexes on intact cells. *J Biol Chem* 272: 10784–10789, 1997
18. Slowik MR, De Luca LG, Fiers W, Pober JS: Tumor necrosis factor activates human endothelial cells through the p55 tumor necrosis factor receptor but the p75 receptor contributes to activation at low tumor necrosis factor concentration. *Am J Pathol* 143: 1724–1730, 1993
19. Tartaglia LA, Pennica D, Goeddel DV: Ligand passing: The 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J Biol Chem* 268: 18542–18548, 1993
20. Doedens JR, Black RA: Stimulation-induced down-regulation of tumor necrosis factor-alpha converting enzyme. *J Biol Chem* 275: 14598–14607, 2000
21. Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, Wong GHW, Gatanaga T, Granger GA, Lentz R, Raab H, Kohr WJ, Goeddel DV: Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell* 61: 361–370, 1990
22. Guo G, Morrissey J, McCracken R, Tolley T, Klahr S: Role of TNFR1 and TNFR2 receptors in tubulointerstitial fibrosis of obstructive nephropathy. *Am J Physiol* 277: F766–F772, 1999
23. Navarro JF, Mora-Fernández C: The role of TNF-alpha in diabetic nephropathy: pathogenic and therapeutic implications. *Cytokine Growth Factor Rev* 17: 441–450, 2006
24. McCarthy ET, Sharma R, Sharma M, Li JZ, Ge XL, Dileepan KN, Savin VJ: TNF-alpha increases albumin permeability of isolated rat glomeruli through the generation of superoxide. *J Am Soc Nephrol* 9: 433–438, 1998
25. Moriwaki Y, Inokuchi T, Yamamoto A, Ka T, Tsutsumi Z, Takahashi S, Yamamoto T: Effect of TNF-alpha inhibition on urinary albumin excretion in experimental diabetic rats. *Acta Diabetol* 44: 215–218, 2007
26. Mauer M, Zinman B, Gardiner R, Suissa S, Sinaiko A, Strand T, Drummond K, Donnelly S, Goodyer P, Gubler MC, Klein R: Renal and retinal effects of enalapril and losartan in type 1 diabetes. *N Engl J Med* 361: 40–51, 2009
27. Pavkov ME, Mason CC, Bennett PH, Curtis JM, Knowler WC, Nelson RG: Change in the distribution of albuminuria according to estimated glomerular filtration rate in Pima Indians with type 2 diabetes. *Diabetes Care* 32: 1845–1850, 2009
28. Hasegawa G, Nakano K, Sawada M, Uno K, Shibayama Y, Ienaga K, Kondo M: Possible role of tumor necrosis factor and interleukin-1 in the development of diabetic nephropathy. *Kidney Int* 40: 1007–1012, 1991
29. Bohle A, Wehrmann M, Bogenschütz O, Batz C, Müller CA, Müller GA: The pathogenesis of chronic renal failure in diabetic nephropathy. Investigation of 488 cases of diabetic glomerulosclerosis. *Pathol Res Pract* 187: 251–259, 1991
30. Rodríguez-Iturbe B, Pons H, Herrera-Acosta J, Johnson RJ: Role of immunocompetent cells in nonimmune renal diseases. *Kidney Int* 59: 1626–1640, 2001
31. Perkins BA, Nelson RG, Ostrander BE, Blouch KL, Krolewski AS, Myers BD, Warram JH: Detection of renal function decline in patients with diabetes and normal or elevated GFR by serial measurements of serum cystatin C concentration: Results of a 4-year follow-up study. *J Am Soc Nephrol* 16: 1404–1412, 2005
32. Stevens LA, Schmid CH, Greene T, Li L, Beck GJ, Joffe MM, Froissart M, Kusek JW, Zhang YL, Coresh J, Levey AS: Factors other than glomerular filtration rate affect serum cystatin C levels. *Kidney Int* 75: 652–660, 2009
33. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS: Regression of microalbuminuria in type 1 diabetes. *N Engl J Med* 348: 2285–2293, 2003
34. Stevens LA, Coresh J, Schmid CH, Feldman HI, Froissart M, Kusek J, Rossert J, Van Lente F, Bruce RD 3rd, Zhang YL, Greene T, Levey AS: Estimating GFR using serum cystatin C alone and in combination with serum creatinine: A pooled analysis of 3,418 individuals with CKD. *Am J Kidney Dis* 51: 395–406, 2008
35. Peralta CA, Katz R, Sarnak MJ, Ix J, Fried LF, De Boer I, Palmas W, Siscovick D, Levey AS, Shlipak MG: Cystatin C identifies chronic kidney disease patients at higher risk for complications. *J Am Soc Nephrol* 22: 147–155, 2011


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