Validating the Association between Plasma Tumour Necrosis Factor Receptor 1 Levels and the Presence of Renal Injury and Functional Decline in Patients with Type 2 Diabetes.

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Aims-Elevated plasma soluble tumour necrosis factor receptor 1(TNFR1) predicts long-term progression of chronic kidney disease. We investigated the association between elevated TNFR1 and the presence of renal disease in patients with Type 2 diabetes mellitus registering a haemoglobin A1c (HbA1c) >48mmol/mol despite medical therapy.

Methods- Using sensitivity, specificity and regression analyses we interrogated the association between plasma TNFR1 and presence of chronic kidney disease as assessed by the presence of microalbuminuria and/or an estimated glomerular filtration rate of less than 60ml/min/1.73m^2 (Stages 3-5 Chronic Kidney Disease). The association of TNFR1 with C-reactive protein and leptin-adiponectin ration as plasma markers of systemic inflammation and adipose stress respectively was also investigated.

Results- Upper quartile TNFR1 is associated with elevated urinary albumin-creatinine ratios, reductions in eGFR and strongly predicts the presence of stages 3-5 chronic kidney disease in regression modelling. Elevated TNFR1 levels are associated with increased plasma C-reactive protein and augmented leptin-adiponectin ratio.

Conclusions Our study confirms plasma TNFR1 as a surrogate of renal structural and functional impairment in patients with type 2 diabetes mellitus. Association of TNFR1 with markers of systemic inflammation and adipose stress indicates that TNFR1 may be a biomarker of these processes as components of the pathogenesis of diabetic kidney disease.

1. Introduction

The UK Prospective Diabetes Study (UKPDS) indicated that at least 25% of patients develop microalbuminuria over the 10-year period following diagnosis of Type 2 diabetes mellitus (T2DM) [1]. Those patients that develop progressive diabetic kidney disease (DKD) are characterised by an accelerated decline in glomerular filtration rate (GFR) and progressive or waxing and waning albuminuria. Rapid GFR decline in patients with DKD is associated with a 10-year mortality risk of 47%, more than 6 times that of age-matched non-diabetic individuals and 4 times that of patients with T2DM but no evidence of renal microvascular complications [2,3].

As new more efficacious treatments become available, better clinical outcomes can be anticipated if clinicians can profile the risk of progressive DKD in individual patients earlier in the natural history of disease, tailoring thereafter the intensity and mode of treatment of risk factors. To this end biomarkers, particularly those that can predict long term risk of microvascular complications early in the disease course are required.

Tumour Necrosis Factor Receptor 1 (TNFR1/CD120a) is a ubiquitously expressed member of the TNF-alpha receptor superfamily[4]. Soluble plasma TNFR1 can be generated through enzymatic cleavage of the receptor by inflammatory metalloproteinases or by inclusion of the receptor in secretory exosomes [5, 6]. Circulating levels of soluble TNFR1 (TNFR1) can be accurately measured in both plasma and serum samples by enzyme linked immunosorbent assay (ELISA).

Accumulating evidence indicates that elevated levels of circulating TNFR1 can predict long-term progression of chronic kidney disease (CKD). Elevated TNFR1 is associated with risk of ESRD and death in patients with T2DM [4]. Elsewhere, circulating TNFR1 has been associated with a hazard ratio of 8.7 for the development of ESRD in T2DM [7]. Upper quartile TNFR1 associates with an adjusted hazard ratio for death of 3.0 in patients with DKD [8]. Steeper declines in GFR over 5 year follow-up have been shown in patients with DKD and high TNFR1 [9]. TNFR1 levels also correlate with hallmarks of glomerular injury including podocyte foot process effacement [10].

The potential for TNFR1 profiling to become a prognostic tool in the management of patients with DKD resulted in the test being made routinely available in our centre to help clinicians identify patients at highest risk of progressive DKD [4, 8, 10]. As part of instigating the new test we were able to generate cross-sectional data which allowed the current study to firstly aim to describe the range of circulating concentrations of TNFR1 observed in a large unselected group of patients with T2DM and HbA1c >48mmol/mol. By dividing the dataset into low and high TNFR1 categories based on quartiles we tested the degree to which elevated TNFR1 did segregate with Stage 3, 4 or 5 chronic kidney disease (CKD3, 4, or 5) in cross-sectional analysis and assessed the independent strength of the association. We also assessed whether TNFR1 status was associated with cardinal markers of systemic inflammation or adipose stress as putative drivers of accelerated renal decline in T2DM.

2. Subjects, Materials and Methods

Study Cohorts (Table 1)

Permission was obtained from St Vincent's University Hospital Group, Dublin, Ireland for a prospective clinical audit (reference 2014/1103) evaluating reporting practice on biochemical risk factors for microvascular and macrovascular disease in patients with diabetes (fasting plasma glucose, HbA1c, lipid profile, C-reactive protein, eGFR and microalbuminuria) Contemporaneous to establishment of the audit we instigated measurement of TNFR1 as part of routine care on clinical grounds in patients with diabetes (sub-optimal glycaemic control as defined by HbA1c≥48mmol/mol). This was based on the emerging evidence base of its predictive power as a prognostic indicator of progressive renal microvascular complications of diabetes[4, 8, 10]. With the approval of The St. Vincent's University Hospital Research Ethics Committee, we matched biochemical data from the audit to cognate TNFR1 values thus permitting cross-reference of TNFR1 status with biochemical risk factor status and where available relevant clinical data. Therefore, we herein report an analysis of audit data derived from patients with HbA1c ≥48mmol/mol stratified by plasma TNFR1 level, using cut-offs for TNFR1 shown to be predictive for future renal decline elsewhere in the literature[4, 8].

Samples from patients with T2DM having routine biochemical determination of HbA1c at St. Vincent's University Hospital (SVUH), Dublin and St. Michael's Hospital (SMH), Dun Laoghaire were reflex tested for plasma TNFR1 if HbA1c analysis returned a value above 48mmol/mol (6.5%), (Data Set A, n=3444, Table 1). Complimentary clinical and biochemical data sets were obtained from hospital laboratory information systems (SVUH)

and patient paper form charts (SMH) and used to generate two refined databases of varying degrees of content (Table 1); a) HbA1c accompanied by full biochemical profile (Data Set A, n=3444) In Data Set A, 2941 of the 3444 patients had a corresponding eGFR measurement available and 1031 of the 3444 had a corresponding albumin-creatinine ratio (ACR) determination available. A second data set (Data Set B, n=763 Table 1) included patients HbA1c with eGFR and ACR for whom an accompanying full clinical profile was available.

2.1 Routine Clinical and Biochemical Measures

Biochemical measures included: HbA1c (mmol/mol), eGFR (ml/min/1.73m^2) calculated using the Modification of Diet in Renal Disease Study formula[11], creatinine (μmol/L), albumin:creatinine ratio (ACR) (mg/g), C-reactive protein (CRP) (mg/L) and TNFR1 (pg/ml). Clinical data included; age (years), sex, disease duration (years), weight (kg), body mass index (BMI-kg/m^2), systolic and diastolic blood pressure (mmHg), smoking status and presence/absence of diabetic complications namely: retinopathy, hypertension, coronary artery disease, cerebrovascular disease, peripheral vascular disease and peripheral neuropathy. CKD is defined in line with the 2013 Kidney Disease Improving Global Outcomes (KDIGO) description as evidence of renal functional impairement of at least 3 months duration with implications for health and classifiable into five stages (1-5), with 5 representing end-stage renal disease[12].

2.2 Assays

TNFR1 was measured in 10-fold diluted plasma samples using the EKF Diagnostics Human TNFR1 ELISA Assay (Cat# EIA – BIO94(EKF Diagnostics Ltd., Cardiff, UK). Assay run validity was established based on pass/fail criteria for technical duplicates for low (290pg/ml) and high (2834pg/ml) control test concentrations resulting in overall inter-assay co-efficient of variation of 6% . Leptin and Adiponectin was measured using Mesoscale Discovery (MSD-Rockville, Maryland, U.S.A.) assays K151BYC-1 (Leptin) and K151BXC-1 (Adiponectin). Inter-assay co-efficient of variation was 6% and 5% respectively for the Leptin and Adiponectin assays.

2.3 Statistical Analyses

Group values were expressed as median (inter-quartile range IQR). Group comparisons were made by Kruskall-Wallis and Mann–Whitney testing. Segregation of TNFR1 status with evidence of CKD3 or worse was examined using Receiver Operator Characteristic (ROC) curve analysis with calculation of sensitivity, specificity, negative and positive predictive values at the upper quartile cut-off for TNFR1, the value of which equated to the upper left maximum of the ROC curve. All clinically relevant variables were inputted into logistic regression models. All statistical analysis was carried out using GraphPad Prism (version 6.0)(San Diego, CA, USA) and SPSS® (version 23.0) software. All statistical analyses were two-tailed with the threshold of significance set at $p < 0.05$.

3. Results

3.1 Demographics

Table 1 details each patient cohort analysed. Twenty-four percent of patients in Dataset A and 23% of patients in Dataset B had an eGFR measurement equal to or lower than 60ml/min/1.73m^2 indicating CKD 3, 4 or 5.

Elevations in Plasma TNFR1 are Associated with Renal Injury and Reduced eGFR in Patients with T2DM and Off-target Glycaemic Control

Figure 1A shows the distribution of plasma TNFR1 concentrations in 3444 patients. The data was divided into quartiles. A marked rightward skew in variance was notable with Q4 ranging from 2061pg/ml to 90000pg/ml with a median and inter-quartile range of 2787 (1410) pg/mL. Proportionately the spread of data within the first 3 quartiles (Q1-Q3) was much lesser and accordingly as elsewhere in the literature, the proportion of the population within Q1-Q3 was collapsed into a single class defined as TNFR1 low (≤2061pg/mL in this study) and the proportion of the population above the Q4 cut-off defined as TNFR1 high.

Urinary Albumin-Creatinine-Ratio (ACR) data was available for 1031 patients in Dataset A and served as an index of renal injury. When the patient cohort in Dataset A was split according to TNFR1 status into Q1-Q3 and Q4, median ACR in Q1-Q3 was 1.10 (2.3)mg/mmol versus 3.15 (20.2) mg/mmol in patients in Q4 ($p<0.01$) (Figure 1B).

We were able to calculate eGFR for 2941 patients in dataset A. In Q1-Q3 of TNFR1 the median serum creatinine was 76 (22) µmol/L versus 105 (59) µmol/L in Q4 ($p<0.01$) and resultant Q1-Q3 median eGFR was 86.3 (27.3) mL/min/1.73m^2 versus 54.9 (33.9) mL/min/1.73m^2 in Q4 ($p<0.01$, Figure 1C)

To validate the univariate association of elevated TNFR1 with renal functional impairment, sensitivity and specificity and regression analyses were undertaken using information from the 2941 eGFR profiled patients in dataset A using age, gender, glycaemia and cholesterol status as relevant clinical and biochemical factors. The Q4 cut-off of 2061pg/ml had a sensitivity of 68%; specificity of 86%; positive predictive value of 58%, and negative predictive value of 90% with a likelihood ratio of 4.8 for the presence of CKD 3, 4 or 5 (Table 2). In 763 patients from Dataset B we furthermore examined the sensitivity and specifity of the Q4 TNFR1 cut-off as a means of identifying patients with a combination of albuminuria (3mg/mmol) and eGFR<60 ml/min/1.73 m2. A total of 72 patients (9.5%) met with this definition and the Q4 cut-off for TNFR1 had respectively a sensitivity of 69%, a specificity of 79% and likelihood ratio of 3.3.

The relative ability of the upper quartile cut-off for TNFR1 to discriminate between patients with and without an eGFR of 60ml/min/1.73m^2 (at least CKD3) was examined in multivariable logistic regression analysis of Dataset B including the following parameters; age, diabetes duration, smoking status, body, body mass index, systolic blood pressure, diastolic blood pressure, haemoglobin A1c in addition to the presence or absence of

retinopathy, coronary artery disease, cerebrovascular disease, peripheral vascular disease and peripheral neuropathy.

The model was statistically significant ($p<0.001$), explained 36.8% (Nagelkerke R^2) of the variance in CKD classification and correctly classified 83% of cases. Patients with upper quartile TNFR1 were 6.5 times more likely to have an eGFR diagnostic of CKD3 or worse than patients in the lower 3 quartiles for TNFR1 (OR-6.51, 95% CI 4.25-9.99, $p<0.001$). Whilst the age of patients as a continuous variable only had a small significant predictive power this is accounted for the by generally narrow skewed distribution of ages around the mean of 63. The presence of retinopathy did however have a good predictive power with regard to identifying those patients with CKD3 or worse (OR-2.06, 95% CI 1.28- 3.32, p<0;01).

3.2 Elevated Plasma TNFR1 is associated with Systemic Inflammation and Adipose Tissue Stress.

We performed univariate analysis of the relationship between TNFR1 status and levels of CRP and Leptin Adiponectin Ratio (LAR) in 757 samples from Dataset A. Elevated CRP and LAR reflect systemic inflammation and adipose stress respectively and are as such cardinal measures of each of these processes that have been reported to be linked to accelerated renal decline in diabetes [13, 14]. Median CRP at the Q1-3 interval of TNFR1 was 3.0 (4.7) mg/L while median for Q4 was 5.6 (8.9)mg/L) ($p <0.001$, Figure 2A). Median Leptin Adiponectin Ratio at TNFR interval Q1-Q3 was 0.003 (0.007) while median for Q4 was 0.009 (0.029) ($p <0.001$, Figure 2B).

4. Discussion

The sample population distribution for TNFR1 in the present study aligned with other studies in the literature in which the upper quartile cut-off oscillates around 2000pg/ml. Stratification at the upper quartile cut-off independently associates with increased albuminuria and lower eGFR on cross-sectional analyses. Upper quartile plasma TNFR1 was also associated with elevated CRP levels and LAR ratios suggesting that TNFR1 may serves as a sentinel of chronic systemic inflammation and adipose stress.

Progression of chronic renal disease to renal insufficiency and ultimately renal failure is a relatively slow process given that the kidney is an organ with a large degree of functional redundancy and adapative capacity. Thus, the nadir of renal function below which insufficiency and progression towards end-organ failure proceeds (60ml/min1.73m^2) usually requires 10-20 years of disease progression. Our cross-sectional study showed that many patients with established markers of renal damage such as raised urine ACRs as well as many patients with established renal impairments (CKD 3 or worse) have elevated TNFR1 and thus may be at risk for more rapid progression towards end stage disease. However, we also identified that many of this cross-sectional cohort have raised sTNFR1 but do not at least yet manifest evidence of renal damage or functional impairments. Given the raised likelihood ratio of 4.8 for finding in elevated TNFR1 in association with renal functional decline, the implication is that many from this subgroup of patients are at a high risk of subsequently developing renal damage and functional impairment and thus may be appropriate to consider for prophylactic treatment intensification.

Interestingly, although impaired glycaemic control was present in all patients studied this did not translate into an increase in the relative prevalence of CKD 3 or worse versus the generally reported penetrance of CKD in patients with diabetes [15]. That rates of CKD in the study remained comparable to generally accepted overall prevalence rates highlighting the emerging consensus that factors superimposed upon the presence of hyperglycaemia determine susceptibility to renal injury and progressive functional decline in DKD. In line with this, TNFR1 was not a surrogate of hyperglycaemia as a wide range of TNFR1 levels can be observed across hyperglycaemia.

However, the identified association between elevated TNFR1, albuminuria and CKD 3, 4, or 5, point to this biomarker potentially being of clinical prognostic utility especially when combined with established risk factors as well as other prognostic biomarkers such as TNF-R2 and Kidney Injury Molecule-1[16]. The ability to stratify patients according to risk may help determine the intensity of pharmacotherapy and frequency of follow-up. This would allow for resource to be focussed on the most at-risk cohort whilst simultaneously limiting side effects of intensive treatments in patients at lower risk of microvascular complications. The last point is particularly relevant given the high specificity of TNFR1 (90%) as a rule-out tool for the exclusion of CKD on cross-sectional analysis.

Pavkov et al reported that high levels of TNFR1 associate with global renal sclerosis scores, increased mesangial volumes, reductions in effective filtration surface and widening of podocyte foot processes (Pavkov et al. 2015) in patients with T2DM. Such linkage of

TNFR1 to renal structural and functional impairment raises questions on the nature of the injury process that elevated TNFR1 levels reflect. We showed that upper quartile levels of sTNFR1 were associated with higher levels of CRP and Leptin Adiponectin Ratios, suggesting that high levels of TNFR1 may be a proxy for ongoing systemic inflammation and adipose stress. Elevated levels of both CRP and Leptin Adiponectin Ratio have been associated with renal decline [13, 14]. Elevated levels of CRP also associate with mesangial expansion and glomerular basement membrane thickening in patients with T2DM [17].

Limitations of our study include its cross-sectional nature and lack of granularity on specific medication usage which may have informed to some degree the multivariable analyses. Moreover, we have focussed on DKD in patients with poorer glycaemic control to the exclusion of patients with progressive renal injury despite well controlled glycaemia.

These things considered, our data suggest further refined follow-up studies should be pursued in these cohorts of patients. Repeat trajectory analysis should be carried out spanning 5 to 10 years to allow time-qualified interpretations of the risk associated with elevated TNFR1 especially in those patients with raised TNFR1 at baseline without evidence of coincident renal damage or renal functional impairment. Information on disease management strategies including lifestyle intervention, pharmacotherapy and surgical interventions and renal outcome in these patients would add to the value of associations tested in multivariable analysis. Specific linkage of TNFR1 to therapy - modifiable disease pathways can be obtained from prospective study of these patients,

allowing determination of whether any of the novel anti-diabetic treatments reduces TNFR1.

In terms of practice, while measurement of plasma TNFR1 levels in patients with T2DM does not add any benefit as regards identifying patients who have renal damage or renal impairments, its clinical utility may lie in its value as a means of adding a tentative estimate of the risk of progressive renal complications. Elevated levels of TNFR1 may indicate that a patient is more likely to show rapid decline in renal function. Levels of TNFR1 in the lower 3 quartiles may be of more reliable use as a means of identifying patients at a lower risk of progression, even if some degree of albuminuria is present. Thus, the biomarker may provide an additional layer of value in guiding both the intensity of risk factor control and the frequency of clinical visits and review of renal function.

Finally, the results of our study indicate that there is a sufficiently large cohort of patients with type 2 diabetes, raised TNFR1 with only limited renal impairment which could be recruited into prospective randomized controlled trial to test new strategies to reduce the incidence and progression of chronic kidney disease.

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6. References

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7. Figure and Table Title and Captions

Figure 1. Univariate Association of TNFR1 Status with ACR, Serum Creatinine and eGFR in Patients with T2DM and Off-target Glycaemic Control.

A) Distribution of TNFR1 levels in the whole cohort from Dataset A broken into quartiles. B) Overall ACR levels (All) and levels above and below the upper quartile cut-off for TNFR1 (2061pg/ml). C) Overall eGFR (All) and levels above and below the upper quartile cut-off for TNFR1 (2061pg/ml). *p<0.05 between groups above and below the upper quartile cut-off for TNFR1. *eGFR-estimated Glomerular Filtration Rats ACR-Albumin-Creatinine Ratio*

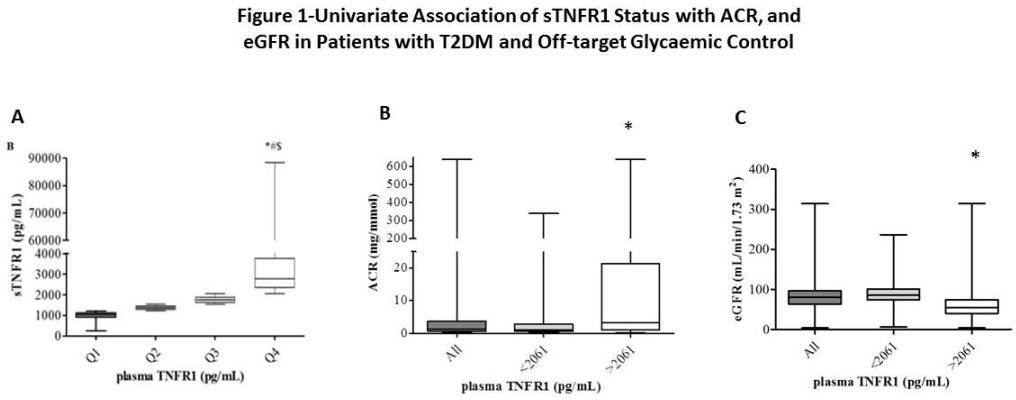

Figure 2. C-Reactive Protein and Leptin -Adiponectin Ratios According to TNFR1 Status.

Leptin and Adiponectin were measured in plasma samples from Dataset B for which complimentary time-matched measures of TNFR1 and CRP were available (n=757). Levels of CRP (A) and the derived Leptin-Adiponectin ratio (LAR, B) at two intervals of plasma TNFR1 level, below (Q1-3) and above (Q4) the upper quartile cut off for plasma TNFR1 (2061pg/ml) were compared. *p<0.05 Q1-3 versus Q4.

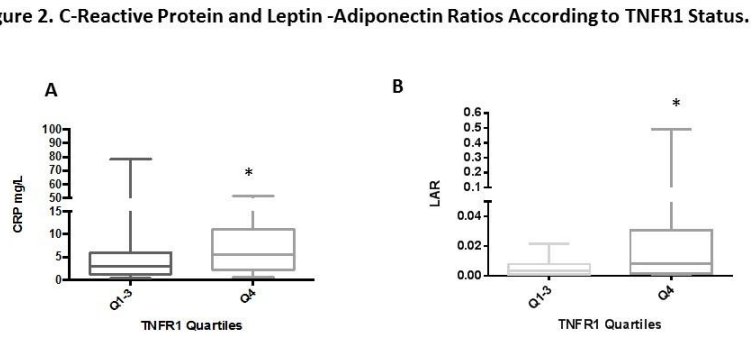

Table 1. Characteristics of Study Cohorts

*HbA1c-Haemoglobin A1c, eGFR-estimated Glomerular Filtration Rats ACR-Albumin-Creatinine Ratio, CRP-C-reactive Protein, TNFR1-Tumour Necrosis Factor Receptor 1, *1031 of 3444 patients had ACR values. Continuous data presented as mean (SD) except in case of ACR (median (interquartile range-25th to 75th percentile)*

Table 1-Characteristics of Study Cohorts

	Database A n=3444	Dataset B n=763
Age (yrs)	62.7(15.5)	63.8(16.07)
Sex (m/f)	M(59%) F(41%)	M(60%) F(40%)
HbA1c (mmol/mol)	63.1(19.6)	61.9(14.2)
Cholesterol (mmol/l)	4.35(2.2)	na
Trig (mmol/l)	1.82(3.38)	na
HDL (mmol/l)	1.38(2.29)	na
LDL (mmol/l)	2.18(2.25)	na
eGFR (ml/min/1.73m2)	79.9(27.5)	73.5(19.6)
ACR (mg/mmol)	1.3(2.9)*	1(3.2)
Creatinine (umol/l)	94.4(65.1)	90.9(45)
CRP (mg/l)	16.9(37.2)	na
TNFR1 (pg/ml)	2124(3942)	1902(1406)
Disease duration (yrs)	na	16.6(11.4)
Smoker (y/n)	na	y(24%) n(76%)
Weight (kg)	na	84.7(19.7)
BMI (kg/m2)	na	29.9(8)
SBP (mmhg)	na	134(18.9)
DBP (mmhg)	na	75(11.5)
CLINICAL		
Nephropathy	Y24% N26%	Y23% N77%
Retinopathy	na	Y23% N77%
Hypertension	na	Y4% N96%
CAD	na	Y13% N87%
Cerebrovascular disease	na	Y3% N97%
PVD	na	Y6% N94%
Peripheral Neuropathy	na	Y14% N86%

Table 2. Sensitivity and Specificity of TNFR1 Status as a Correlate of the Presence/Absence of CKD3 or Worse

Table 2- Sensitivity and Specificity of TNFR1 Status as Correlate of Presence/Absence of CKD3 or Worse

		CKD3 or worse				
		YES	NO	TOTAL		
TNFR1 INTERVAL	Q4	450	329	779		
	Q1-Q3	213	1949	2162		
	TOTAL	663	2278	2941		
Overall CKD3 or Worse Prevalence(%)					663/2941*100	22.543
Sensitivity(%)					450/663*100	67.873
Specificity (%)					1949/2278*100	85.558
Positive Predictive Value (PPV-%)					450/779*100	57.766
Negative Predictive Value (NPV-%)					1949/2162*100	90.148
Likliehood Ratio (LR)					68/(100-86)	4.8571

Table 3. Multivariable Clinical and Biochemical Logistic Regression Analysis Predictors of Risk of CKD3 or Worse

Table 3-Multivariable Clinical and Biochemical Logistic Regression Analysis of Predictors of Risk of CKD3 or Worse

			95% C.I.for OR	
	(p-value)	OR	Lower	Upper
Sex (M:F)	0.78	1.1	0.7	1.7
Age (years)	**<0.01**	**1.1**	**1.0**	**1.1**
Disease Duration (years)	0.36	1.0	1.0	1.0
Smoking Status	0.59	0.8	0.4	1.6
Body weight (kg)	0.86	1.0	1.0	1.0
Body Mass index (kg/m2)	0.20	1.0	1.0	1.1
Systolic Blood Pressure (mmHg)	0.66	1.0	1.0	1.0
Diastolic Blood Pressure (mmHg)	0.62	1.0	1.0	1.0
Haemogloblin A1c (mmol/mol)	0.91	1.0	1.0	1.0
Retinopathy	**<0.01**	**2.1**	**1.3**	**3.3**
Hypertension	0.86	1.0	0.7	1.6
Coronary Artery Disease	0.87	1.0	0.6	1.8
Cerebrovascular Disease	0.68	1.3	0.4	3.7
Peripheral Vascuar Disease	0.28	0.7	0.3	1.4
Peripheral Neuropathy	0.13	1.5	0.9	2.5
Plasma TNFR1 category (Q1-3 or Q4)	**<0.01**	**6.5**	**4.2**	**10.0**