

see commentary on page 996

Urine haptoglobin levels predict early renal functional decline in patients with type 2 diabetes

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Diabetic nephropathy is the leading cause of end-stage renal disease. The urinary albumin to creatinine ratio is used as a predictor for the development of nephropathy but it is neither sensitive nor specific. Here we used liquid chromatography/mass spectrometry on urine of eight normoalbuminuric patients with type 2 diabetes from the VA Diabetes Trial to identify candidate markers for loss of renal function. Initial verification of seven markers (agrin, haptoglobin, mannan-binding lectin serine protease 2, LAMP-2, angiotensinogen, NGAL, and uromodulin) in the urine of an additional 30 patients showed that haptoglobin was the best predictor of early renal functional decline. We then measured this in the urine of 204 patients with type 2 diabetes who did not yet have significant kidney disease (estimated glomerular filtration rate stage 2 or better and an albumin to creatinine ratio <300 mg/g). In comparing the highest to lowest tertiles, the odds ratio for having early renal function decline was 2.70 (CI: 1.15, 6.32) using the haptoglobin to creatinine ratio compared with 2.50 (CI 1.14, 5.48) using the albumin to creatinine ratio after adjusting for treatment group and use of ACE inhibitors. Addition of the haptoglobin to creatinine ratio to a model using the albumin to creatinine ratio to predict early renal function decline resulted in improved predictive performance. Thus, the haptoglobin to creatinine ratio may be useful to predict patients with type 2 diabetes at risk of nephropathy before the development of macroalbuminuria or reduced glomerular filtration rate.

Kidney International (2013) **83**, 1136–1143; doi:10.1038/ki.2013.57; published online 27 March 2013

KEYWORDS: biological markers; chronic kidney disease; diabetes; diabetic nephropathy; type 2 diabetes; urine

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Received 4 September 2012; revised 4 December 2012; accepted 7 December 2012; published online 27 March 2013

Diabetic nephropathy is responsible for 44% of end-stage renal disease in the United States.¹ The prevalence of diabetes is estimated to be 12.9% in the US adult population.² Aggressive treatment of blood pressure, intensive diabetes therapy, and treatment with inhibitors of the renin–angiotensin–aldosterone system can slow the progression of diabetic nephropathy.^{3–5} The ability to predict which patients with diabetes will develop kidney disease would permit targeted treatment with more aggressive therapies at an earlier stage and enable new therapies to be tested.

Urine albumin to creatinine ratio (ACR) is commonly used to predict diabetic nephropathy. Microalbuminuria is associated with an increased rate of progression of diabetic renal disease in patients with both type 1⁶ and type 2 diabetes.⁷ However, multiple studies have shown that microalbuminuria (30–300 mg albumin/g creatinine) is not a specific marker for development of diabetic nephropathy in type 2 diabetes as many patients who have microalbuminuria at one point in time may not have it when measured later and it is a poor predictor of the development of macroalbuminuria.^{8–12} Diabetic glomerular lesions can be seen before the development of albuminuria.¹³ Although macroalbuminuria is generally used to define diabetic nephropathy, many patients with diabetes lose renal function before the development of macroalbuminuria.¹⁴ Between 38 and 73% of patients with type 2 diabetes who develop CKD are normoalbuminuric.^{15–20} These studies demonstrate that albuminuria is not an adequate marker to predict which patients are at risk for loss of renal function.

In order to better define loss of renal function, a definition of early renal functional decline (ERFD) has been used. The definition of ERFD was adopted based on data from the Baltimore Longitudinal Study of Aging in which a rate of decline in glomerular filtration rate (GFR) of 3.3% per year defined the 97.5 percentile.²¹ This definition has been used in a number of studies of diabetic kidney disease to define a cutoff value for ERFD in patients with diabetes.^{22–25} We used proteomic analysis to identify urinary haptoglobin as a marker for predicting which patients with type 2 diabetes will lose renal function. We performed a small verification study of the predictive ability of haptoglobin and then determined

the ability of the ratio of haptoglobin to creatinine in the urine (HCR) to predict ERF.

RESULTS

Proteomic discovery

Urine proteins from four patients who had no change in their serum creatinine over the course of the study were compared by liquid chromatography/tandem mass spectrometry to four that had a least 60% increase in serum creatinine during follow-up. Baseline serum creatinine (1.2 ± 0.09 vs. 1.2 ± 0.13 mg/dl), albuminuria (5.6 ± 4 vs. 7.4 ± 3 mg/g Cr), and length of follow-up (5.7 ± 0.09 vs. 5.6 ± 0.25 years) were not different between groups. We identified 327 proteins in at least one of the patients (Table 1 and Supplementary Table S1 online). One hundred seven proteins were expressed in at least one patient in the group that increased their serum creatinine that were not expressed in any patients in the stable group (Figure 1a). From the group of proteins that were expressed only in one group, haptoglobin was the most abundant protein and had the highest fold change. The sequence coverage of haptoglobin by mass spectrometry is shown in Supplementary Figure S1 online. Agrin was the only protein that was statistically different between the groups in the proteomic analysis ($P < 0.05$) although it would not have reached statistical significance if we had corrected for multiple comparisons. The best performing candidates were visualized with a volcano plot, which maps the P -value for the differences between groups against the fold change difference between groups (Figure 1b). Hierarchical clustering as shown in heat map (Supplementary Figure 2 online) revealed a cluster of 8 proteins including haptoglobin (apolipoprotein D, hemopexin, vitamin D-binding protein, ceruloplasmin, alpha-2-HS-glycoprotein, alpha-1B-glycoprotein, and alpha-1-antitrypsin).

Small-scale verification

Urine samples from 30 patients with normal renal function and an ACR < 30 mg/g at the time of enrollment were analyzed to measure a set of candidate urine markers including the two lead candidates, agrin, and haptoglobin. The stable function group included 18 patients that had no increase in serum creatinine over the next 6 years. The progressor group included 12 patients that had at least a 60% increase in serum creatinine. Seven proteins were measured simultaneously by selected reaction monitoring in the baseline sample. The proteins were chosen because they increased in the discovery proteomic analysis (haptoglobin, angiotensinogen, agrin, and mannan-binding lectin serine protease 2), decreased in the proteomic analysis (lysosome-associated membrane glycoprotein 2, LAMP-2) or were shown to increase in diabetic nephropathy in the literature (NGAL²⁶⁻²⁸ and uromodulin²⁹). Haptoglobin was able to discriminate between groups in this verification study but agrin was not (Figure 2). Angiotensinogen and NGAL appear to be promising candidate markers but were not further evaluated in this study.

Table 1 | Best performing urine proteins to predict loss of renal function

Protein	ID	Stable mean	Progressor mean	Mean change	P-value
Agrin	O00468	0	11.7	23.3	0.029
Alpha-1B-glycoprotein	P04217	1.3	190.6	141.4	0.057
Alpha-2-HS-glycoprotein	P02765	2.3	163.4	70.4	0.057
Mannan-binding lectin serine protease 2	O00187	1.3	74.2	55.0	0.057
Vesicular integral-membrane protein VIP36	Q12907	1.3	58.5	43.4	0.057
Nidogen-1	P14543	1.3	30.9	22.9	0.057
Alpha-1-antitrypsin	P01009	12.8	291.1	22.7	0.057
Secreted Ly-6/uPAR-related protein 2	Q86SR0	1.3	15.9	11.8	0.057
Lithostathine-1-alpha	P05451	8.7	92.9	10.7	0.057
Ig heavy chain V-III region TUR	P01779	0.7	19.0	28.2	0.086
Ig kappa chain V-III region B6	P01619	1.3	9.9	7.4	0.086
Hemopexin	P02790	2.3	188.6	81.3	0.114
Ig alpha-1 chain C region	P01876	2.6	127.1	48.9	0.114
Gelsolin	P06396	5.8	169.7	29.2	0.114
Kininogen-1	P01042	22.2	178.8	8.0	0.114
Beta-defensin 1	P60022	2.6	20.5	7.9	0.114
Hepatitis A virus cellular receptor 2	Q8TDQ0	2.9	22.5	7.8	0.114
Cystatin-M	Q15828	9.6	70.8	7.4	0.114
Ig kappa chain V-III region NG9 (Fragment)	P01621	5.2	31.3	6.0	0.114
Cadherin-1	P12830	36.4	206.1	5.7	0.114
Ig kappa chain V-III region SIE	P01620	26.9	143.7	5.3	0.114
Ig kappa chain V-III region VH (Fragment)	P04434	3.9	18.9	4.9	0.114
Zinc-alpha-2-glycoprotein	P25311	226.2	1034.2	4.6	0.114
Dermatopontin	Q07507	2.6	7.9	3.0	0.114
Haptoglobin	P00738	0	229.0	458	0.143
Ig alpha-2 chain C region	P01877	0	83.7	167.3	0.143
Antithrombin-III	P01008	0	63.6	127.3	0.143
Ig mu chain C region	P01871	0	59.9	119.8	0.143
Cathepsin D	P07339	0	39.6	79.1	0.143
Leucine-rich alpha-2-glycoprotein	P02750	1.3	99.3	73.7	0.143
Complement C3	P01024	0	36.8	73.5	0.143
Fibrinogen alpha chain	P02671	0.7	27.4	40.7	0.143
Beta-2-microglobulin	P61769	0.7	27.4	40.7	0.143

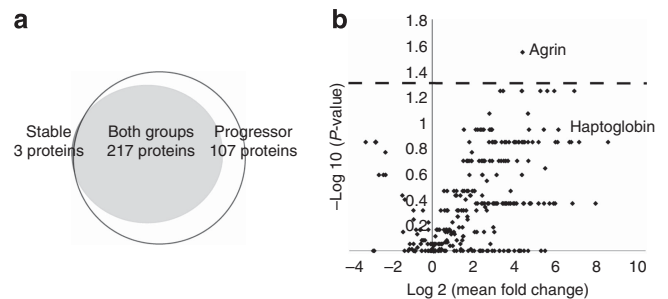


Figure 1 | Results of urine proteomic analysis. (a) Venn diagram showing the distribution of proteins between subjects who lost renal function (progressors) and those who did not (stable). **(b)** Volcano plot shows the log of change in abundance compared with the log of the P -value of differences for proteins in the urine of patients with type 2 diabetes who lost renal function compared with those who did not. We identified 327 proteins. Agrin had the smallest P -value. Haptoglobin showed the greatest fold difference between conditions. The dotted line represents a P -value of 0.05 using the Wilcoxon rank-sum test. For calculation of fold change, a value of 0.5 was assigned to proteins that were not identified in any subjects of that group.

Verification

We measured urine haptoglobin concentration in 204 Veterans Affairs Diabetes Trial (VADT) participants (mean, 158 ng/ml; median 70 ng/ml). Stratifying by the haptoglobin

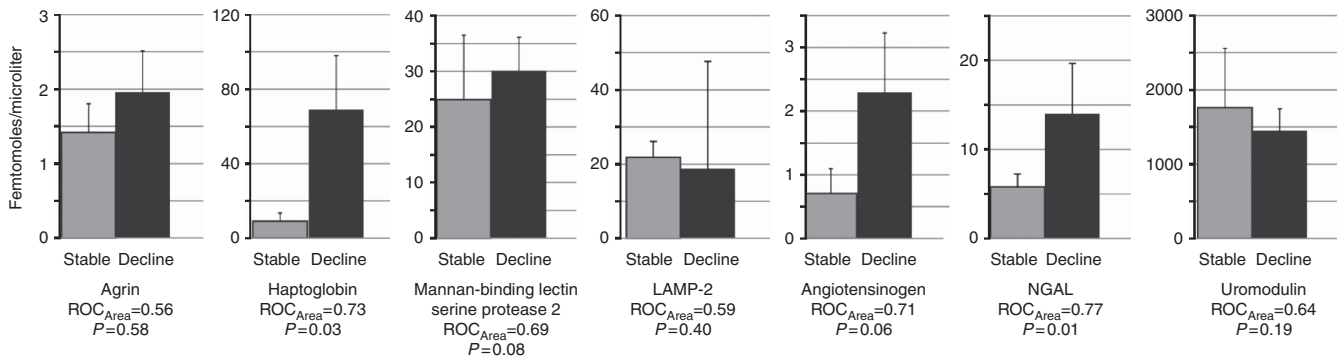


Figure 2 | Mean and s.e. values of urine protein abundance in patients with stable renal function compared with those who had a decline in renal function over the subsequent 6 years. Protein abundance was measured by multiple reaction monitoring in urine from 30 patients. The quality of the prediction was evaluated by determining the area under the receiver-operator characteristics curve. The P-value is the probability that the AUC value is different from an AUC of 0.5. AUC, area under curve; ROC, receiver-operating characteristic.

median split, subjects with high and low urinary haptoglobin had a similar mean age and diabetes duration and were equally likely to be in the intensive treatment group, current smokers, and on ACE inhibitors. In contrast, patients who had urine haptoglobin concentrations above the median had higher ACR, BMI, systolic, and diastolic blood pressures and were more likely to be male than those with haptoglobin levels below the median (Table 2).

The predetermined primary outcome measure was ERFD. Secondary outcome measures were 50% or greater increase in serum creatinine during follow-up, persistent decline in renal function and development of macroalbuminuria. Of the 204 subjects studied, 25.0% had ERFD, 18.7% had persistent decline in renal function, 15.7% had a 50% or greater increase in creatinine, 4.4% had creatinine doubling, and 3.9% developed macroalbuminuria defined as ACR ≥300 mg/g on at least two occasions. At the baseline VADT examination, stratifying by whether or not patients developed the primary endpoint, demographics, and percent in the intensive treatment group were similar across the two strata. Subjects who did not develop ERFD were more likely to smoke (Table 3). There was a strong concordance between development of the ERFD and the other renal outcomes. Although the prediction of ERFD using estimated glomerular filtration rate (eGFR) values that were >60 at baseline has limitations, the prediction of ERFD correlates with other renal outcomes. At the time of haptoglobin measurement, eGFR was similar across the two strata; however, HCR, HbA1c, and ACR levels were higher in those who later developed ERFD. After adjusting for VADT treatment group and the use of ACE inhibitors, urine haptoglobin, and HCR were higher in patients who developed EFRD than in those who did not. These data show that elevated HCR is associated with the later development of ERFD.

Logistic regression was used to further examine the ability of haptoglobin, HCR, and ACR to predict ERFD and a creatinine increase of 50% or greater (Table 4). Individuals in the highest tertile of haptoglobin had a non-statistically significant two-fold increased odds of developing ERFD

Table 2 | Demographics and clinical characteristics by haptoglobin median split taken at time of haptoglobin measurement

	Haptoglobin level		P-value ^a
	Lower (n = 102)	Upper (n = 102)	
Age ^b (years)	59.5	58.7	0.3201
Diabetes duration ^b (years)	11.4	10.8	0.8916
Time to biomarker ^c (years)	1.1	1.1	0.9074
Male ^b (%)	92.2	99.0	0.0170
Non-Hispanic white ^b (%)	68.6	55.9	0.0604
Intensive treatment group ^b (%)	52.9	49.0	0.5753
Current smoker ^b (%)	16.7	17.7	0.8527
Biomarker follow-up (years)	4.8	4.6	0.4220
ACE (%)	67.7	57.8	0.1476
Hemoglobin A1c (%)	7.7	7.9	0.1702
Creatinine (mg/dl)	1.0	1.0	0.2400
eGFR (ml/min)	84	86	0.4938
ACR ^d (mg/g)	11	16	0.0106
Body mass index (kg/m ²)	31.6	33.3	0.0354
SB pressure (mm Hg)	126	131	0.0142
DB pressure (mm Hg)	72	76	0.0037

Abbreviations: ACR, albumin to creatinine ratio; DB, diastolic blood; eGFR, estimated glomerular filtration rate; ERFD, early renal functional decline; GFR, glomerular filtration rate; HCR, haptoglobin to creatinine; SB, systolic blood; VADT, Veterans Affairs Diabetes Trial.

^aχ² or Kruskal-Wallis test.

^bValues at baseline VADT.

^cTime to biomarker is the time from VADT baseline examination to collection of the samples for haptoglobin measurement.

^dGeometric mean.

relative to those in the lowest tertile of haptoglobin after controlling for treatment group and use of an ACE inhibitor. Parallel odds ratios for HCR and ACR were statistically significant with values of 2.70 (95% confidence interval (CI): 1.15, 6.32) and 2.50 (95% CI: 1.14, 5.48), respectively, (Figure 3). As creatinine based estimates of glomerular filtration rate are less accurate than iothalamate or inulin clearance estimates, we determined the ability of HCR to predict the outcomes for other measures of renal function loss. The odds ratios for these outcomes were similar (Table 4). The AUC value for the prediction of ERFD by HCR was 0.614 and by ACR was 0.621. The combined AUC was 0.664. When we used ≥50% increase in creatinine from baseline AUC value for HCR, ACR, and combine HCR with ACR were 0.654, 0.709, and 0.751, respectively.

Table 3 | Demographic and clinical characteristics stratified by decline in eGFR/year $\geq 3.3\%$ (ERFD)

VADT baseline	<3.3%/Year (n = 153)	$\geq 3.3\%$ /Year (n = 51)	P-value ^a
Age (years)	58.8	59.9	0.408
Diabetes duration (years)	10.8	11.9	0.219
Male (%)	95.4	96.1	0.844
Non-Hispanic white (%)	60.8	66.7	0.453
Intensive treatment Group (%)	49.7	47.1	0.746
Current smoker (%)	20.3	7.8	0.042
<i>At haptoglobin measurement</i>			
ACE (%)	61.4	66.7	0.504
Hemoglobin A1c (%)	7.6	8.2	0.019
Creatinine (mg/dl)	1.0	1.0	0.055
eGFR (mg/min)	83	90	0.259
Body mass index (kg/m ²)	32.6	31.8	0.282
SB pressure (mm Hg)	128	130	0.605
DB pressure (mm Hg)	74	74	0.788
<i>Biomarker concentrations</i>			
ACR ^c (mg/g)	11	21	0.004
Haptoglobin (ng/ml)	55	130	0.0512
Ln HCR (ng/g)	-10.0	-9.0	0.026
Adjusted ^b haptoglobin (ng/ml)	54	134	0.022
Adjusted ^b Ln HCR (ng/g)	-10.1	-9.0	0.008
Biomarker follow-up (years)	4.6	5	0.163
<i>Secondary outcomes</i>			
50% Creatinine increase (%)	3.9	51	<.0001
Persistent worsening of GFR (%)	4.0	62.8	<.0001
Creatinine doubling (%)	0.7	15.7	<.0001
Macroalbuminuria (%)	3.3	5.9	0.4048

Abbreviations: ACR, albumin to creatinine ratio; AUC, area under curve; DB, diastolic blood; eGFR, estimated glomerular filtration rate; ERFD, early renal functional decline; GFR, glomerular filtration rate; HCR, haptoglobin to creatinine; ROC, receiver-operating characteristic; SB, systolic blood; VADT, Veterans Affairs Diabetes Trial.

^a χ^2 Or Kruskal-Wallis test.

^bAdjusted for treatment group and use of ace inhibitors.

^cGeometric mean.

Spearman rank correlation between ACR and HCR was relatively weak (0.25) and the ROC AUC values for combinations of ACR and HCR were (non-significantly) higher (Table 4). We used net reclassification to determine if HCR improved the prediction by ACR. The integrated discrimination improvement comparing the two models was statistically significant and estimated to be 0.02 (P -value = 0.045) using the probability cutpoint associated with an ACR level of 30 mg/g (Table 5). The improvement in integrated discrimination improvement demonstrates the added prognostic value that HCR provides to standard estimation using ACR. Of the 51 subjects who developed ERFD, classification improved for 7 patients using the model with HCR, but became worse for 2 patients. This resulted in a non-significant net gain in reclassification proportion for subjects who experienced an event of 0.098 (P -value = 0.095). Of the 153 subjects who did not develop ERFD, classification improved for 9 patients, but became worse for 9 other patients. The net reclassification index was estimated to be 0.098 (P -value = 0.131). Net reclassification resulted in a nonsignificant improvement in sensitivity from 37.3 to 47.1%, while specificity was unchanged at 78.4% (Figure 4).

Table 4 | Adjusted OR (and 95% CI) from logistic regression models for a one s.d. increase in the natural log of haptoglobin, creatinine corrected haptoglobin, and ACR in relation to outcomes of interest

	Outcomes of interest		
	eGFR decline $\geq 3.3\%$ /year, n = 51 (25%)	Persistent worsening of eGFR, n = 38 (18.7%)	Creatinine increase $\geq 50\%$, n = 32 (15.7%)
<i>Haptoglobin as the predictor</i>			
Lowest tertile	1.00	1.00	1.00
Second tertile	1.67 (0.73, 3.80)	1.56 (0.62, 3.93)	1.87 (0.68, 5.09)
Third tertile	2.13 (0.95, 4.79)	2.06 (0.83, 5.03)	2.10 (0.78, 5.68)
ROC AUC	0.577	0.584	0.611
<i>Haptoglobin/creatinine as the predictor</i>			
Lowest tertile	1.00	1.00	1.00
Second tertile	2.47 (1.05, 5.79)	2.46 (0.93, 6.49)	3.40 (1.15, 10.1)
Third tertile	2.70 (1.15, 6.32)	2.72 (1.04, 7.16)	3.08 (1.02, 9.25)
ROC AUC	0.614	0.602	0.654
<i>ACR as the predictor</i>			
Lowest tertile	1.00	1.00	1.00
Second tertile	1.13 (0.48, 2.65)	1.90 (0.72, 5.01)	1.34 (0.39, 4.64)
Third tertile	2.50 (1.14, 5.48)	2.91 (1.16, 7.33)	5.56 (1.94, 15.9)
ROC AUC	0.621	0.620	0.709
<i>Haptoglobin/creatinine and ACR as the predictors</i>			
ROC AUC	0.664	0.632	0.751

Abbreviations: ACR, albumin to creatinine ratio; AUC, area under curve; CI, confidence interval; eGFR, estimated glomerular filtration rate; ERFD, early renal functional decline; HCR, haptoglobin to creatinine; OR, odds ratio; ROC, receiver-operating characteristic.

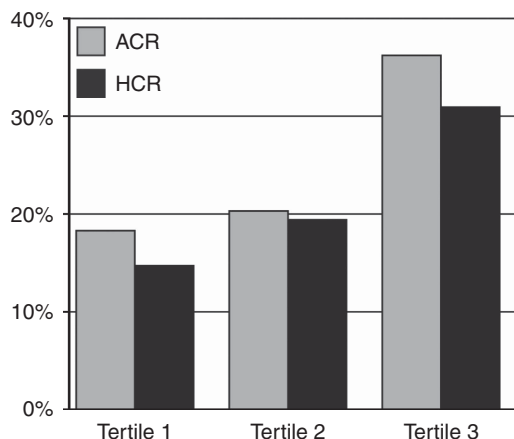


Figure 3 | Comparison of percent of patients in each tertile of ACR and HCR that met the primary outcome of early renal functional decline. ACR, albumin to creatinine ratio; HCR, haptoglobin to creatinine.

DISCUSSION

We identified urinary haptoglobin as a candidate biomarker to predict ERFD in a discovery proteomic analysis, verified the predictive ability in a set of 30 patients using selective reaction monitoring and confirmed the predictive ability of HCR in 204 patients with type 2 diabetes who had not yet manifested significant kidney disease (that is, they had eGFR >60 and normo-or microalbuminuria). In the primary analysis HCR was equivalent to urinary ACR as a predictor of ERFD. The net reclassification index showed that the use of

Table 5 | Net reclassification by adding HCR

ERFD	ACR predicted	ACR + HCR predicted	ERFD outcome	ERFD outcome	Frequency	Percent	Cumulative frequency	Cumulative percent
No	Negative	Negative	Negative	Negative	111	54.4	111	54.4
No	Negative	Positive	Negative	Positive	9	4.4	120	58.8
No	Positive	Negative	Negative	Negative	9	4.4	129	63.2
No	Positive	Positive	Negative	Positive	24	11.8	153	75
Yes	Negative	Negative	Positive	Negative	25	12.3	178	87.3
Yes	Negative	Positive	Positive	Positive	7	3.4	185	90.7
Yes	Positive	Negative	Positive	Negative	2	1.0	187	91.7
Yes	Positive	Positive	Positive	Positive	17	8.3	204	100

Abbreviations: ACR, albumin to creatinine ratio; ERFD, early renal functional decline; HCR, haptoglobin to creatinine.

ACR and HCR in combination provides better ability to predict ERFD than either biomarker alone. This is the first discovery of a candidate marker that can predict ERFD as well as ACR. The prediction was made in patients who had not yet developed macroalbuminuria. It may result in better clinical prediction of risk of rapid loss of renal function. The predictive ability of HCR will need to be determined in larger sets of patients, the threshold values for prediction will need to be refined and the confounding effects on HCR of other medical conditions and medications determined. The results of our study are promising as HCR was predictive in patients with normal renal function and before the development of macroalbuminuria.

Haptoglobin is an alpha-2 sialoglycoprotein with the ability to bind hemoglobin to prevent loss of iron through the kidneys as well as to prevent oxidative damage to the nephron.^{30,31} Haptoglobin is heavily glycosylated resulting in a net negative charge.^{32,33} Because of its large size and negative charge, the haptoglobin-hemoglobin complex is not filtered at the glomerulus. Haptoglobin is translated as a single protein, which is cleaved into alpha and beta subunits. The alpha chain of haptoglobin has two major allelic forms alpha-1 and alpha-2. The alpha subunits of haptoglobin can exist as combinations of 1-1, 1-2, or 2-2.³³ The mature haptoglobin exists as multimers. Patients with the 1-2 or 2-2 genotype have larger multimers (200-400 kDa) than patients with 1-1 genotype who have haptoglobin tetramers with an apparent molecular weight of about 100 kDa.³⁰ Haptoglobin 1-1 genotype has been shown to be protective relative to the haptoglobin 2-2 genotype.³⁴ The reason for this protective effect of the haptoglobin 1-1 form may be due to its higher antioxidant capacity and hemoglobin binding capacity. Recombinant 1-1 haptoglobin produces a larger inhibition of oxidation by hemoglobin *in vitro* than other genotypes.³⁵ Patients with the haptoglobin 1-1 genotype are more likely to be hypertensive and salt sensitive.³⁶ In our analysis, patients with haptoglobin concentrations higher than the median had higher blood pressures but it is not clear how this correlates with genotype in our study.

Urinary concentrations of haptoglobin are correlated with the loss of GFR before the development of macroalbuminuria. As patients with a 2-2 genotype have larger circulating

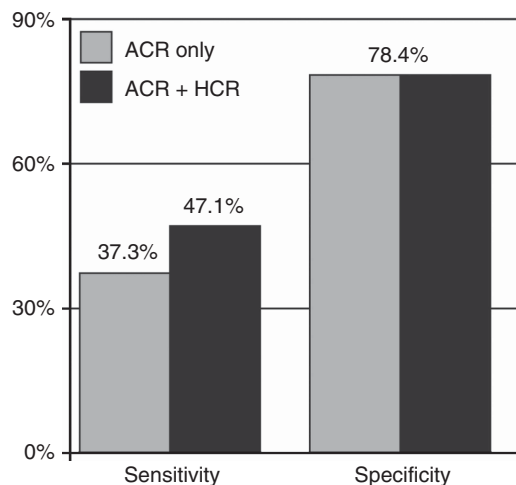


Figure 4 | Improvement in sensitivity and specificity of classification using ACR and HCR in combination. The sensitivity and specificity of ACR for prediction of early renal functional decline were 37.3% and 78.4%, respectively. Net reclassification resulted in a non-significant improvement in sensitivity to 47.1% without a change in the specificity. ACR, albumin to creatinine ratio; HCR, haptoglobin to creatinine.

haptoglobin complexes, these patients should have haptoglobin, which is less likely to be filtered because of the larger size. One possible interpretation of these findings is that glomerular permeability increases sufficiently to allow haptoglobin in the serum to leak into the urinary space before the development of overt nephropathy. In this scenario, haptoglobin is a marker of increased glomerular permeability similar to albumin. A second hypothesis is that tubular injury causes increased expression of haptoglobin as a protective mechanism against oxidative injury. Haptoglobin is primarily produced in the liver but other tissues including kidney have been shown to express it. In mice, haptoglobin is expressed in the kidney after induction by LPS.³⁷ Haptoglobin may be expressed in the renal tubules either as a response to injury or to increased oxidative stress. A third potential mechanism is that tubular injury permits haptoglobin to leak into the tubules from the peritubular capillaries. Increased measured urinary haptoglobin could also be a surrogate marker of patients that have the 2-2 haptoglobin genotype as patients with a 2-2 genotype are at increased risk of progression. DNA was not collected as part of the VADT study so this hypothesis could not be tested in the current study.

A potential concern in these studies is the determination of ERFD based on creatinine measurements. Estimated GFR values calculated using the MDRD equation are not as accurate as measurements using iothalamate or inulin clearance. We used these estimates of GFR because they have previously been used in this population to report renal outcomes from the VADT trial.³⁸ However, we used other outcome measures ($\geq 50\%$ increase in serum creatinine and persistent decline in renal function), which should not be affected by the calculations. In all, 15.7% of the population

had an increase in serum creatinine $\geq 50\%$. The analysis of both these secondary endpoints showed a more robust predictive ability for HCR than the analysis of prediction of ERFD (third tertile odds ratio of 3.08 and 2.72 compared with 2.70). A second study limitation is our limited sample size, which prevented us from determining whether the predictive ability of HCR differed depending on baseline albuminuria levels.

In summary, we identified HCR as a biomarker for the diagnosis of ERFD in patients with type 2 diabetes. The predictive ability of HCR appears equivalent to ACR in the current study. Larger studies will be necessary to determine its true predictive ability. Combining HCR with ACR increases the predictive ability. If future studies confirm the prognostic capabilities of this marker, it could enhance the ability for early intervention to delay or prevent the development of diabetic nephropathy. Although adding an additional test (HCR) could potentially increase diagnostic costs, if diagnostic results improve, the addition of HCR to potentially improve outcomes and permit better personalization of care.

MATERIALS AND METHODS

Study sample

The study design of the VADT study has been previously reported.^{39,40} Biomarker samples were collected as part of the Markers and Mechanisms of Vascular Disease in Diabetes sub-study. Urine samples were collected at the time of the VADT visit and stored without protease inhibitors at -80°C . Baseline biomarker samples were collected on 1007 VADT subjects an average of 1.9 years after a participant's enrollment in the VADT examination. Participants were subsequently followed an average of 3.8 years. Urine samples were obtained from the VADT trial after completion of the study so we were not able to exclude other causes of chronic kidney disease.

Proteomic discovery phase

Control subjects for the discovery proteomic analysis had serum creatinine values that were identical at the beginning and end of the study period. Patients with progression had at least 60% increase in serum creatinine over the study period. Four subjects from each group were chosen as cases and controls. Case-control subjects were matched on baseline serum creatinine and quantity of urinary albumin.

Initial verification phase

We selected 30 patients with normal renal function and an ACR $< 30\text{ mg/g}$ at baseline in which to measure a set of candidate urine markers. The group had 12 subjects with at least a 60% increase in serum creatinine and 18 with no change in creatinine.

Verification phase

The first 280 sequentially numbered urine samples were selected for measurement of urine haptoglobin concentration. Sample size was selected based on power analysis using alpha 0.05, response distribution 25%, confidence level 90%. Before statistical analyses, 76 subjects were excluded because they had either GFR $\leq 60\text{ ml/min per }1.73\text{ m}^2$ or ACR $\geq 300\text{ mg/g}$ (macroalbuminuria) at

the time of the biomarker sample collection. The remaining 204 subjects were used to determine the ability of urine haptoglobin to creatinine ratio (HCR) to predict verification phase outcomes.

Proteomic discovery analysis

To account for technical variability, 200 ng of the recombinant HIV protein gp160 was spiked into each urine sample (100 μl). Proteins were denatured, alkylated, and digested with trypsin. Each sample was pre-fractionated using offline reversed phase solid phase extraction. Solid phase extraction fractions were analyzed by LC-MS/MS as described in the on-line methods. Tandem mass spectrometry was performed using an AB SCIEX (Framingham, MA) Triple-TOF 5600 mass spectrometer.

Protein identification and quantification

Acquired spectra were searched against the 2011_6 release of the Human UniProtKB/Swiss-Prot database (20,127 entries) using the Mascot search engine. The error tolerances were 10 p.p.m. and 0.5 Da for peptides and MS/MS fragments, respectively. Mascot search results were loaded into Scaffold. Protein probabilities were assigned by the Protein Prophet algorithm.⁴¹ The Scaffold unweighted spectral counts of identified proteins were normalized to the internal standard recombinant HIV protein present in each biological sample, and the relative abundance of each protein is reported in normalized spectral counts. Three hundred twenty seven proteins were identified in at least one sample with a false discovery rate of 0.9%.

Selection of candidates

The fold change between conditions was calculated as the ratio of normalized spectral counts in the progressor group to the counts in the stable group. A mean spectral count value of 0.5 was used for calculation of fold change if the protein was not observed in any subjects in the group. A volcano plot was created to illustrate the magnitude of the difference in the abundance between the groups compared with the P -value calculated from the Wilcoxon rank-sum test. We used the plot to select candidate biomarkers that had a larger magnitude fold change (> 20 fold change) and lower P -values < 0.4 . As we had only one protein with statistical significant P -value, other proteins with non-significant P -values, but a very high confidence of protein prediction (≥ 7 high confidence peptides seen) and higher fold changes, were selected. We selected agrin (23-fold increase, $P=0.03$, 4 peptides), haptoglobin (458-fold increase, $P=0.14$, 18 peptides), mannan-binding lectin serine protease 2 (55-fold increase, $P=0.057$, 7 peptides), and angiotensinogen (69-fold increase, $P=0.43$, 8 peptides) as proteins, which increased in our discovery proteomic analysis and LAMP-2 (0.4-fold change, $P=0.40$, 3 peptides) as a protein which decreased. We selected NGAL and uromodulin because of published evidence that they increase in patients with diabetic nephropathy. In the discovery proteomic analysis, NGAL was 5.6-fold higher ($P=0.43$) and uromodulin was 1.2-fold higher in patients that progressed ($P=0.69$). Unsupervised hierarchical clustering identified a set of proteins, which clustered with haptoglobin that was similar to the one identified using the volcano plot (Supplementary Figure S2 online).

Initial verification by selected reaction monitoring

We used selected reaction monitoring to verify the ability of seven candidate markers to predict loss of renal function in 30 patients from the VADT trial with normal renal function and an

ACR <30 mg/g. The group had 12 subjects with a decline in renal function of at least 60% and 18 with stable serum creatinine during follow-up. We measured agrin (STVPVNTNR), haptoglobin (VTSIQDWVQK), mannan-binding lectine serine protease 2 (WTLTAPPGYR), LAMP-2 (IPLNDLFR), angiotensinogen (ALQDQLVLAAG), NGAL (VPLQQNFQDNQFQGK) and uromodulin (VGGTGMFTVR) using selected reaction monitoring. The isotopically labeled peptide was synthesized for each protein in which the labeled peptide was 8 or 10 Da heavier than the unlabeled peptide. A mixture of the labeled peptides was made and standard concentration curves were constructed for each peptide based on one product ion (MS/MS product). A volume of urine normalized for the creatinine concentration was added to a solution of Rapigest SF surfactant. Each sample was spiked with the cocktail of isotopically labeled peptides. The samples were reduced, alkylated, digested with trypsin, and loaded onto a Strata-X polymeric reversed phase solid phase extraction column and eluted with 40% acetonitrile. The eluted fraction was separated on a C18 column and injected into an AB SCIEX 5600 triple-ToF mass spectrometer for analysis using parameters that had been optimized for each peptide. Protein abundance was determined by comparing the summed intensity of the appropriate product ion of the endogenous peptide to the summed intensity of the peptide containing the stable isotope using the multiquant software package (ABSciex).

Measurement of urine haptoglobin by ELISA in the verification phase

Haptoglobin measurement was done using the Human Cardiovascular Disease Single Plex Haptoglobin Assay kit (Millipore, Billerica, MA) according to manufactures instruction. Aliquots of two quality control samples were included in each assay for estimating the inter assay coefficient of variation which was always $\leq 14\%$. The team measuring the urine concentrations of haptoglobin and creatinine was blinded to the outcome of the individual patients. All samples were run in duplicate and the mean value was used.

Verification phase study endpoints

The average follow-up time after haptoglobin measurement was 3.7 years. Outcomes of interest utilize the serial values of eGFR, ACR, and creatinine, which were collected yearly throughout the VADT study. Our primary outcome (ERFD) was defined as having $\geq 3.3\%$ decline in eGFR per year.²³ Serum creatinine was measured as part of the VADT trial. The MDRD equation was used to estimate GFR. Decline in eGFR was defined by fitting a regression model with random intercept and slope using Proc Mixed in SAS 9.3 to obtain yearly decline in eGFR at the level of the individual. Other outcomes of interest were a 50% increase in creatinine level or a persistent worsening of GFR defined as having the two final GFR values lower than the baseline value. The definition of persistent worsening of GFR was used in the original report of renal outcomes from the VADT.³⁸

Statistical analyses

Unadjusted proportions and means were determined for baseline characteristics stratified by haptoglobin median split as well as our primary outcome, ERFD. Baseline differences across the haptoglobin median split as well as our primary outcome were tested using either χ^2 or the Kruskal-Wallis test. For regression analysis, the association between biomarker tertile and each outcome was assessed separately for each biomarker using logistic regression after controlling for

treatment arm and use of an ACE inhibitor. In addition, the comparative discriminatory power of various multivariate logistic regression models was assessed using the concordance statistic (c-statistic; an approximation to the area under the receiver-operating-characteristic (ROC) curve (AUC)), the net reclassification index and the integrated discrimination index. All analyses were performed using SAS v. 9.3 (SAS Institute, Cary, NC).

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This study was supported by a merit review award (JMA) and a career development 2 award (MGJ) from the Biomedical Laboratory Research and Development Program of the Department of Veterans Affairs and NIH grants R01 DK080234 and UL1 RR029882. The VADT study was sponsored by the Cooperative Studies Program of the Department of Veterans Affairs Office of Research and Development. The contents do not necessarily represent the views of the Department of Veterans Affairs or the United States Government. Portions of the data were presented in abstract form at the 2012 American Society of Nephrology meeting.

Sources of support that require acknowledgment:

VA Merit Review Award
NIH R01DK080234
UL1 RR029882

SUPPLEMENTARY MATERIAL

Figure S1. Sequence of haptoglobin.

Figure S2. Unsupervised hierarchal clustering analysis of all 340 proteins identified between eight patients was performed using Matlab (R2012b; Mathworks, Natick, MA).

Table S1. All identified proteins.

Comprehensive methods.

Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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