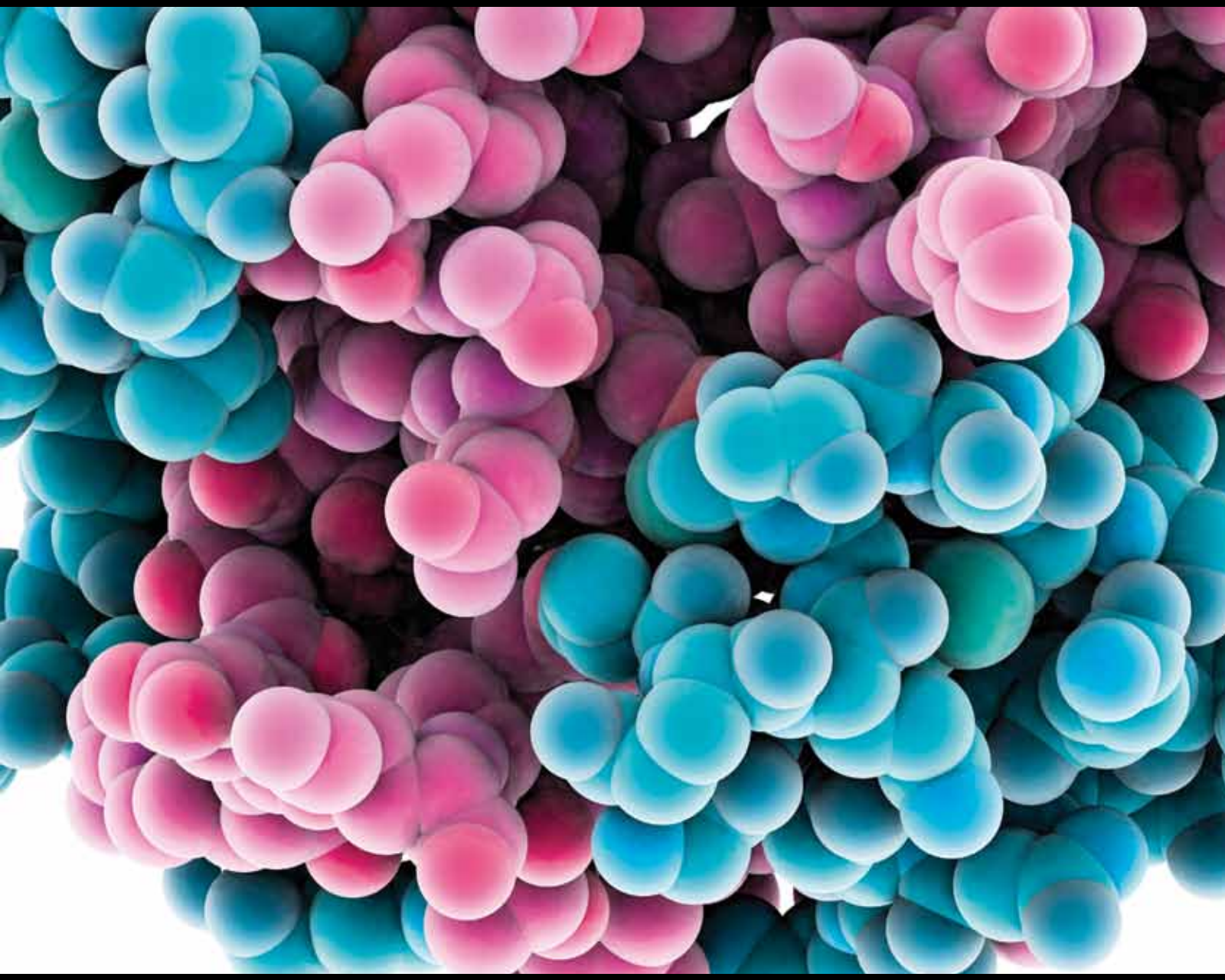
Journal of Diabetes Research

Biomarker Research in Glucose Disorders: Current Concepts and Clinical Applications

Guest Editors: D. R. Webb, K. Herbert, Melanie J. Davies, K. Khunti, N. Sattar, and C. D. A. Stehouwer



Biomarker Research in Glucose Disorders: Current Concepts and Clinical Applications

Journal of Diabetes Research

Biomarker Research in Glucose Disorders: Current Concepts and Clinical Applications

Guest Editors: D. R. Webb, K. Herbert, Melanie J. Davies, K. Khunti, N. Sattar, and C. D. A. Stehouwer

Editorial Board

Contents

Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2013, Article ID 290671, 2 pages
http://dx.doi.org/10.1155/2013/290671

Editorial

Biomarker Research in Glucose Disorders: Current Concepts and Clinical Applications

D. R. Webb,[1] K. Herbert,[2] Melanie J. Davies,[1] K. Khunti,[3] N. Sattar,[4] and C. D. A. Stehouwer[5]

[1] *Diabetes Research Unit, University of Leicester, Leicester General Hospital, Leicester LE5 4PW, UK*
[2] *Department of Cardiovascular Sciences, University of Leicester, Clinical Sciences Wing, Glenfield General Hospital, Leicester LE3 9QP, UK*
[3] *Department of Health Sciences, University of Leicester, Princess Road West, Leicester LE1 6TP, UK*
[4] *BHF Glasgow Cardiovascular Centre, Wolfson Medical School Building, University Avenue, Glasgow G12 8QQ, UK*
[5] *Maastricht University Medical Centre, P.O. BOX 5800, 6202 AZ Maastricht, The Netherlands*

Correspondence should be addressed to D. R. Webb; david.webb@uhl-tr.nhs.uk

Received 4 March 2013; Accepted 4 March 2013

The National Institute for Health working group broadly define a "biomarker" as an objectively quantifiable characteristic reflecting normal biological action, a specific pathogenic process, or pharmacologic response to therapeutic intervention.

This meaning is readily applied to an increasing array of analytes and molecules which may enhance our understanding of disease pathogenesis, aid risk stratification for diagnosis or management, or improve treatment response.

Biomarker measurements have traditionally targeted a specific pathway or mechanism proposed to be involved in a disease, for example, association of adiponectin with the development of Type 2 diabetes or glycosylated haemoglobin in development of microvascular complications. Increasingly employed are state-of-the-art metabolomic and proteomic approaches which can reflect instantaneous cellular multiparametric responses to environmental stimuli. We now have tools to explore the genome (DNA), transcriptome (mRNA), proteome (protein), and metabolome (small molecule metabolites) for such purposes. Diabetes lends itself to all of these applications as it is clear that the pathogenesis of the disease and its complications are highly complex and involves numerous biological axes. Sensitive techniques such as nuclear magnetic resonance and gas/liquid chromatography mass spectrometry now have the capacity to process large numbers of samples and in conjunction with robust bioinformatics should enable detailed characterisation of the "metabotype."

In this special issue we see examples of relevance to diabetes, including state-of-the-art tandem mass spectrometry measurement of L(+) and D(−) Lactate, proteomic profiling using isobaric tagging (iTRAQ) in patients with microalbuminuria, and two studies characterising the adipocytokine adiponectin within populations at risk of cardiovascular disease and diabetes. Also included is cross-sectional evidence assessing urine F2-isoprostane- and Intercellular Adhesion Molecule-1 (ICAM-1) as biomarkers of oxidative stress and endothelial function, respectively.

Biomarker research to date has generally disappointed by failing to provide additional predictive power over and above existing classical risk factors or only minimally advancing our understanding of diabetes. Hopefully utilising existing biomarker knowledge in a targeted fashion in addition to exploiting novel "–omics" technologies will generate more useful biomarkers which are independent of known risk factors and shed new light on disease pathogenesis.

Acknowledgment

This paper is supported by the NIHR Leicester-Loughborough Diet, Lifestyle and Physical Activity Biomedical Research Unit which is a partnership between University Hospitals of Leicester NHS Trust, Loughborough University, and the University of Leicester.

D. R. Webb
K. Herbert
Melanie J. Davies
K. Khunti
N. Sattar
C. D. A. Stehouwer

Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2013, Article ID 561016, 7 pages
http://dx.doi.org/10.1155/2013/561016

Research Article

Adipocytokine Associations with Insulin Resistance in British South Asians

D. R. Webb,[1,2] K. Khunti,[2,3] S. Chatterjee,[4] J. Jarvis,[2] and M. J. Davies[1,2]

[1] *Department of Cardiovascular Sciences, University of Leicester, Leicester LE3 9QP, UK*
[2] *Leicester Diabetes Center, Leicester General Hospital, Ward 5 (Broadleaf), Gwendolen Road, Leicester LE5 4PW, UK*
[3] *Department of Health Sciences, University of Leicester, Leicester LE1 6TP, UK*
[4] *Buckinghamshire Hospitals NHS Trust, Buckinghamshire HP7 OJD, UK*

Correspondence should be addressed to D. R. Webb; david.webb@uhl-tr.nhs.uk

Received 16 August 2012; Accepted 17 December 2012

Academic Editor: N. Sattar

Aims. Adipocytokines are implicated in the pathogenesis of type 2 diabetes and may represent identifiable precursors of metabolic disease within high-risk groups. We investigated adiponectin, leptin, and TNF-α and assessed the contribution of these molecules to insulin resistance in south Asians. *Hypothesis*. South Asians have adverse adipocytokine profiles which associate with an HOMA-derived insulin resistance phenotype. *Methods*. We measured adipocytokine concentrations in south Asians with newly diagnosed impaired glucose tolerance or Type 2 Diabetes Mellitus in a case-control study. 158 (48.5% males) volunteers aged 25–75 years with risk factors for diabetes but no known vascular or metabolic disease provided serum samples for ELISA and bioplex assays. *Results*. Total adiponectin concentration progressively decreased across the glucose spectrum in both sexes. A reciprocal trend in leptin concentration was observed only in south Asian men. Adiponectin but not leptin independently associated with HOMA-derived insulin resistance after logistic multivariate regression. *Conclusion*. Diasporic south Asian populations have an adverse adipocytokine profile which deteriorates further with glucose dysregulation. Insulin resistance is inversely associated with adiponectin independent of BMI and waist circumference in south Asians, implying that adipocytokine interplay contributes to the pathogenesis of metabolic disease in this group.

1. Introduction

The role of obesity in the pathogenesis of metabolic disease has received considerable attention since the discovery of biologically active adipose-tissue-derived circulatory proteins (adipocytokines) [1–3]. It is proposed over- or underproduction of adipocytokines in overweight individuals generates an adipose-specific inflammatory response which may be an important determinant of Type 2 Diabetes Mellitus (T2DM) [4–6]. Prospective evidence exists for this within certain populations, with low concentrations of the adipocytokine adiponectin correlating strongly with insulin resistance syndromes and or incident T2DM independent of obesity [4–15]. Adiponectin may decrease T2DM risk via a number of mechanisms including hepatic fatty acid oxidation, enhanced peripheral glucose uptake, and stimulated insulin secretion [16].

The identification of particular biomarker profiles within well-defined high-risk groups may therefore provide important pathogenic insight as well as potential clinical utility through predictive capacity [17]. Of particular interest are United Kingdom populations tracing first or second ancestry to the Indian subcontinent. Collectively termed South Asians, it appears this diaspora is particularly susceptible to the effects of Western urbanisation and manifest a disproportionate prevalence of premature T2DM [18]. There is some evidence that South Asians have lower adiponectin and higher circulating hsCRP concentrations than White Europeans even in the absence of BMI-defined obesity and glucose dysregulation. This implies hypoadiponectinemia may be a generalised phenomenon within this group and a possible mediator of premature metabolic disease [4, 19–23]. The majority of biomarker studies to date have attempted to

correlate various topographical measurements and markers of metabolic dysfunction with adiponectin [24–28]. We are unaware of any previous studies categorising adipokines across the glucose spectrum in UK South Asians and then establishing their relationship with insulin resistance independent of measures of obesity.

This study aimed to firstly characterise a range of adipokines (adiponectin, leptin, Tumour Necrosis Factor-α (TNF-α)) within age-gender-matched groups of South asians with normal glucose tolerance (NGT), impaired glucose tolerance (IGT) and Type 2 Diabetes mellitus (T2DM) and secondly determine adipocytokine interactions with known predictors of metabolic risk and insulin resistance within this group.

2. Methods

2.1. Subjects. This was a retrospective case-control study embedded within a population-based screening programme for diabetes. Seventeen general practices initially invited listed patients with at least one risk factor for diabetes to a hospital or community-based screening appointment [29]. The response rate amongst invited south Asian subjects was 12%. Nine hundred and sixty-three male and female volunteers aged 25–70 underwent a standard 75 g oral glucose tolerance test (OGTT). A diagnosis of normal glucose tolerance (NGT), impaired glucose tolerance (IGT), or Type 2 Diabetes (T2DM) was made using 1999 WHO criteria [30]. Additional consent was obtained for adipocyte biomarker analyses in south Asian individuals with no history of cardiovascular disease (Figure 1). Approval was granted by the local research and ethics committee as a substudy amendment to the original screening programme protocol. The investigation was conducted in accordance with the principles outlined in the Declaration of Helsinki.

2.2. Recruitment and Patient Flow. Of the volunteers reporting south Asian ethnicity in the parent screening study, 530 were eligible for and consented to the temporary storage of their serum for future adipocytokine biomarker analysis. Twenty-two percent of this group had a WHO-defined glucose disorder and their samples were selected within respective T2DM and IGT categories to produce equal sample sizes across the glucose spectrum. All T2DM cases and 50% (40/79) of IGT subjects were selected for biomarker analysis and an age-sex-matched normal glucose tolerant control allocated by an independent researcher blinded to biomarker data. There were no statistically significant demographic differences between those consenting for ($n = 530$) and those meeting the inclusion criteria (no cardiovascular disease) but not consenting for biomarker analysis ($n = 328$) (Figure 1).

2.3. Biomedical Measurements. Baseline demographic data captured at screening included age, sex, smoking behaviour, body mass index (BMI), waist circumference, and self-reported history of cardiovascular disease or its treatment. Ethnicity status was self-assigned using UK population census categories. Weight was measured to the nearest 0.1 kg using standard weighing scales. Height was measured to the nearest 0.1 cm using a stadiometer. Waist circumference was measured by trained staff using a nonstretching measuring tape over the tops of the iliac crests [31]. Hip circumference was measured over the greatest protrusion of the gluteal muscles. Blood pressure was measured according to a standardised operating procedure using a calibrated sphygmomanometer and brachial inflation cuff (HEM-7200 M3, Omron Healthcare, Kyoto, Japan).

2.4. Biomarker Measurements. Biomarker and insulin samples were handled separately to screening measurements. They were immediately centrifuged and stored at −80°C in 200 μL aliquots to minimise repeated defrosting cycles. These analyses were performed in a single University research laboratory with expertise in adipocytokine assays. Adiponectin, leptin, and TNF-α were all analysed using bioplex assay according to the manufacturer's instructions (Linco Research Inc., St. Charles, MO, USA). Bioplex assay sample measurement using fluorescent microbead technology allowed simultaneous quantitation of several target proteins within a single serum sample of 50–100 μL. For all the assays, the filter plate was prewetted using a specific wash buffer and then 65 μL of assay buffer followed by 10 μL of calibrator, controls or 10 μL of diluted serum samples were added to the appropriate wells. The bead bottle was thoroughly vortexed for 1 minute before adding 25 μL of the bead suspension to each well, taking care to mix intermittently to avoid settling. The filter plate was sealed, covered with aluminium foil, and incubated for 16–18 hours overnight at 2–8°C. The fluid was then gently removed by vacuum extraction and the plate was washed three times with wash buffer. Fifty microlitres of detection antibody cocktail was added to each well and then the plate was resealed, covered, and incubated with agitation for 30 minutes at room temperature. After removing contents by vacuum, the plate was washed three times, and then 50 μL of streptavidin-phycoerythrin was added to each well. After further identical incubation at 30 minutes, the contents were removed by vacuum, the plate was washed three times and 100 μL of sheath fluid was added to all wells. The beads were resuspended by shaking on a plate shaker for 5 minutes before the plate was run on the analyser. For bioplex, intraassay coefficient of variation was 1.4–7.9% and inter-assay coefficient of variation was <15%.

2.5. Statistical Analysis. A power calculation was undertaken for a biomarker (adiponectin) comparison based upon available data in south Asian subjects with type 2 diabetes [24]. A total sample size of 34 would give 80% power at the 5% level to detect a one standard deviation difference between south Asian IGT and T2DM groups. All values are given as mean ± standard deviation (SD) unless otherwise stated. Skewed distributions were log transformed. *T*-tests or non parametric comparisons of median values were used to determine statistical differences in biomedical data across glucose categories. HOMA-derived insulin resistance (HOMA-IR) was calculated as fasting insulin (μUml^{-1}) × fasting glucose (mmoll^{-1})/22.5, with a set value of more

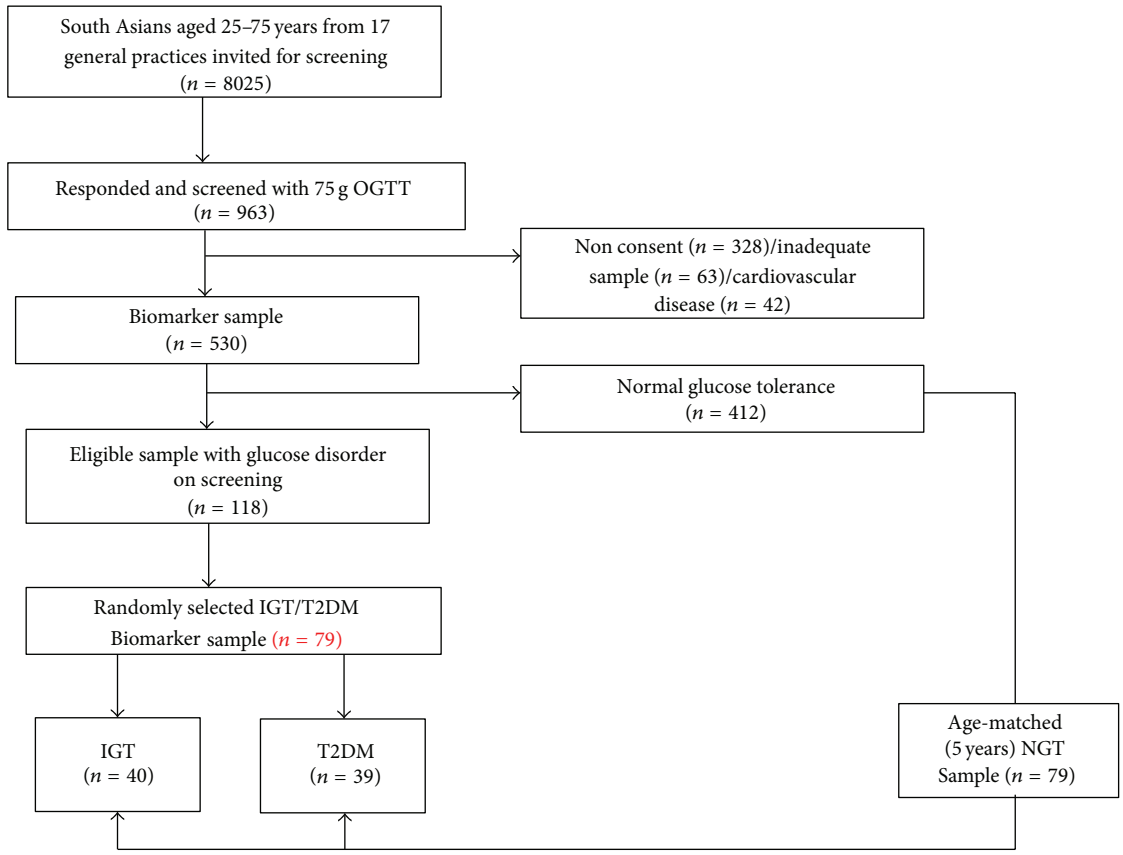


Figure 1: Recruitment and sampling procedure flow diagram.

than 3.0 indicating likely significant Insulin resistance. Conditional logistic regression analysis following logarithmic transformation of selected variables was used to determine independent biomarker relationships with insulin resistance. Only variables considered to be of importance and those significant at the level of bivariate analysis (see Supplementary Material available at http://dx.doi.org/10.1155/2013/561016) were entered simultaneously into the model. Categorical variables were coded as follows: gender: male or female, smoking: active or inactive, cardiovascular disease or lipid lowering medication: active prescription or no prescription. Summary measures of goodness of fit were performed ahead of regression analyses. Significance was assumed if $P < 0.05$. All statistical analyses were carried out using SPSS statistical software version 20.0 (SPSS, Chicago, IL, USA).

3. Results

158 Indian south Asian subjects were included, 79 with normal glucose tolerance (NGT), 40 with impaired glucose tolerance (IGT) and 39 with Type 2 Diabetes (T2DM). The mean age of the study population was 53.6 years with men and women equally represented. Biomedical characteristics categorised by glucose status are displayed in Table 1. There were statistically significant differences in Body Mass Index (BMI), waist circumference, waist hip ratio (WHR), serum triglycerides, glucose indices (fasting and two-hour plasma glucose), and derived insulin resistance (HOMA-IR) between NGT and T2DM categories. Significant differences were also observed between IGT and T2DM groups for these parameters and serum HDL-cholesterol. Fewer NGT controls were prescribed antihypertensive and or lipid lowering therapies than either of the IGT or T2DM comparator groups.

Table 2 depicts mean biomarker concentrations across NGT, IGT, and T2DM glucose categories. Adiponectin concentration decreased and leptin concentration increased across the glucose spectrum. An incremental reduction in adiponectin between NGT and T2DM groups reached statistical significance in women. Conversely a statistically significant increase in leptin concentration between NGT and T2DM was observed in men. There were no statistically significant differences in TNF-α concentration across glucose categories.

Table 3 demonstrates the independent effects of adiponectin, leptin, and TNF-α on insulin resistance (defined by HOMA-IR > 3.0) using conditional logistic regression. Omnibus test of model coefficients indicated a good fit for all models (e.g., for adiponectin chi [2] 64.4 $P < 0.001$). An independent association was observed between the defined insulin resistant state and adiponectin in models adjusting for the effects of age, gender, BMI, waist circumference, smoking, cardioprotective medications, and lipids. No independent associations of insulin resistance with either Leptin or TNF-α were observed in this selected

Table 1: Biomedical characteristics of the study population defined by normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and newly diagnosed type 2 Diabetes (T2DM).

	NGT	IGT	T2DM	*P* NGT versus T2DM	*P* IGT versus T2DM
n	79	40	39		
Age (years)	52.1 ± 9.8	55.1 ± 11.7	52.7 ± 9.2	0.08	0.18
Male sex	40 (50)	22 (54)	17 (44)	0.37	0.96
Active Smoking	8 (10)	6 (15)	6 (15)	0.71	0.85
BMI (kgm^{-2})	26.6 ± 4.2	29.1 ± 4.9	30.1 ± 5.8	0.01	0.26
Waist circumference (cm)	92.7 ± 10.6	96.9 ± 10.1	100.8 ± 10.1	<0.01	0.04
WHR	0.88 ± 0.08	0.91 ± 0.08	0.95 ± 0.08	<0.01	<0.01
SBP (mmHg)	135.3 ± 19.3	139.5 ± 20.6	144.3 ± 18.5	0.11	0.18
DBP (mmHg)	85.4 ± 10.7	86.3 ± 8.2	89.7 ± 9.1	0.52	0.07
Total cholesterol ($mmoll^{-1}$)	5.3 ± 1.0	5.2 ± 1.1	5.3 ± 1.1	0.63	0.58
HDL-C ($mmoll^{-1}$)	1.3 ± 0.3	1.2 ± 0.3	1.1 ± 0.2	0.12	0.01
Triglycerides ($mmoll^{-1}$)*	1.4 (0.8)	1.7 (1.2)	2.6 (2.1)	0.03	0.04
FPG ($mmoll^{-1}$)	5.0 ± 0.5	5.4 ± 0.6	8.1 ± 2.1	0.02	<0.01
2 h PLG ($mmoll^{-1}$)	5.4 ± 1.2	8.9 ± 1.5	14.9 ± 3.9	<0.01	<0.01
HOMA-IR (arb.)*	1.6 (1.2)	2.5 (1.8)	6.0 (4.9)	<0.01	<0.01
CVD medication					
Antihypertensive	14 (18)	15 (34)	14 (36)	0.01	0.33
Lipid lowering	16 (21)	12 (29)	11 (28)	0.03	0.89

Data are means ± standard deviation (SD) or median with inter quartile range (IQR)* and *n* (%). WC: waist circumference, WHR: waist Hip Ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HDL-C: high-density lipoprotein cholesterol subfraction, FPG: fasting plasma glucose, 2-h PLG: 2 hour post-load (75g-oral glucose tolerance test) glucose. HOMA-IR: homeostasis model insulin resistance.

Table 2: Mean biomarker concentrations within glucose groups.

	NGT	IGT	T2DM	*P*
Adiponectin (μgmL^{-1})*				
Men	10.6 (6.48)	9.3 (5.0)	6.9 (3.8)	0.07
Women	18.6 (11.8)	15.7 (10.0)	11.0 (4.9)	0.02
Total	13.6 (9.7)	12.4 (7.7)	8.5 (5.1)	0.002
Leptin ($ngmL^{-1}$)*				
Men	10.5 (6.8)	14.3 (13.0)	16.2 (13.5)	0.005
Women	34.2 (29.7)	39.9 (35.7)	43.4 (40.7)	0.07
Total	20.3 (17.6)	26.5 (29.0)	26.7 (27.8)	0.05
TNF-α ($pgmL^{-1}$)*				
Men	1.76 (1.4)	1.75 (1.2)	2.14 (2.3)	0.92
Women	1.96 (1.1)	1.65 (1.3)	1.80 (1.2)	0.67
Total	1.84 (1.3)	1.70 (1.3)	2.01 (1.6)	0.77

*Data are median + inter quartile range (IQR).
P Nonparametric samples median test.

south Asian group in the two models adjusting for these parameters.

4. Discussion

This study adds to the body of evidence implicating adipocytokine activity (or inactivity) in the pathogenesis of metabolic disease. South Asians, whether "westernised" or residing on the Indian subcontinent appear to have an adverse adipocytokine profile characterised by low total or fractionated hexameric adiponectin and increased leptin. Here we demonstrate an independent association of adiponectin with insulin resistance in a group known to develop diabetes and coronary heart disease earlier than indigenous white European populations.

Like others, we found that adiponectin is low in south Asians compared with similar published data for white Europeans and other races [23–28]. We found an independent relationship between HOMA measured insulin resistance and total adiponectin in south Asians after adjusting for the effects of age, BMI, waist circumference, cardiovascular protective medication, and lipids. This was an expected finding as plasma adiponectin has previously been shown to predict type 2 diabetes within high-risk Asian, Native American and Caucasian populations [32]. Cross-sectional studies examining glucose and adiponectin relationships have demonstrated either strong relationships [19, 25, 33] with HOMA or independent relationships confined to various glucose indices [23–25]. A recent study by Luo et al. demonstrated that total adiponectin associates with IGT and T2DM in Asian Indian women [26]. Low adiponectin levels in pregnancy have recently been shown to predict postpartum insulin resistance and beta-cell dysfunction, indicating a major role in the pathogenesis of T2DM [34]. Interestingly, weight loss (and increased insulin sensitivity) through calorie restriction does not appear to effect presumed hyperinsulinaemia-induced

Table 3: Conditional logistic regression of adipokine biomarkers with insulin resistance.

	R^2	Wald statistic	Beta coefficient (95% CI)	P
Model 1				
Adiponectin	0.29	12.41	0.91 (0.85, 0.95)	<0.01
Leptin	0.23	0.09	0.99 (0.97, 1.01)	0.78
TNF-α	0.22	0.09	1.03 (0.85, 1.23)	0.03
Model 2				
Adiponectin	0.28	13.13	0.89 (0.85, 0.95)	<0.01
Leptin	0.21	1.69	1.01 (0.99, 1.01)	0.20
TNF-α	0.20	0.07	1.00 (1.01, 1.12)	0.93

HOMA-IR > 3.0 used to define insulin resistance for categorical dependent variable.
Model 1: age, gender, BMI, smoking, Cardioprotective medication.
Model 2: age, gender, waist circumference, smoking, total cholesterol.

reduction in adiponectin in obese women, suggesting predetermined genetic causality or alternative adipocytokine biological activity [35]. We found little evidence of this on our selected population with no independent association of insulin resistance with either serum leptin or TNF-α. Adiponectin-insulin resistance interplay appears more complex than the relatively simplistic paradigm of causality currently postulated. Alternative hypotheses are emerging in which insulin sensitivity may mediate adiponectin processing and release [36]. Longitudinal work is warranted to further investigate this relationship in South Asians and to characterise primary genetic alterations that may predispose this group to hypoadiponectinemia.

Leptin is an adipocytokine with a key role in the hypothalamic satiety response regulating energy metabolism. Some studies in south Asians link this molecule to inflammation, insulin resistance and other cardiovascular risk factors [37, 38]. TNF-α is markedly upregulated in obese states and probably promotes insulin resistance by interfering with insulin receptor signalling. TNF-α concentrations are higher in urban compared with rural South Asians living in India [39]. Lack of any correlation between leptin/TNF-α and insulin resistance in our south Asian population was an unexpected finding. The absence of an association between insulin resistance and leptin independent of BMI or waist circumference in this high risk group suggests this molecule may not be a key driver to hyperinsulinemia in the advanced stages of dysmetabolism.

We recognize a number of limitations to our study design. Firstly, power to detect differences in biomarker concentrations other than adiponectin may be insufficient, especially for subgroup and gender comparisons. Secondly, the cross-sectional design of the study cannot infer causality. Longitudinal studies with many years of followup are required to determine true relationships between biomarker profiles and metabolic disease/diabetes risk. Thirdly, there is some evidence that adiponectin subfractions (high molecular weight adiponectin—12–18 multimers) rather than the total concentration measured here provides a better measure of biological activity and is a strong predictor of diabetes [40]. This could provide an explanation for the lack of independent correlation of adiponectin with insulin resistance in other south Asian groups although a recent prospective study showed similar associations of total and high molecular weight adiponectin with incident diabetes [32]. There remain relatively few studies in migrant south Asian populations who are at high risk of T2DM and strengths of this approach included the robust phenotyping methodology employed and cardiovascular disease free sample.

In summary, we have found an independent association of the biomarker Adiponectin with insulin resistance in South Asians. Further work to elucidate exactly how adipocytokine interplay contributes to metabolic risk across ethnic groups is needed.

Acknowledgments

This research was partly funded by the National Institute for Health Research (NIHR) Nutrition, Diet and Lifestyle Biomedical Research Unit, University of Leicester, United Kingdom.

References

[1] P. E. Scherer, S. Williams, M. Fogliano, G. Baldini, and H. F. Lodish, "A novel serum protein similar to C1q, produced exclusively in adipocytes," *The Journal of Biological Chemistry*, vol. 270, no. 45, pp. 26746–26749, 1995.

[2] M. Haluzik, J. Parizkova, and M. M. Haluzik, "Adiponectin and its role in the obesity-induced insulin resistance and related complications," *Physiology Research*, vol. 53, no. 2, pp. 123–129, 2004.

[3] N. Stefan and M. Stumvoll, "Adiponectin: its role in metabolism and beyond," *Hormone and Metabolic Research*, vol. 34, no. 9, pp. 469–474, 2002.

[4] S. Li, H. J. Shin, E. L. Ding, and R. M. van Dam, "Adiponectin levels and risk of type 2 diabetes. A systematic review and meta-analysis," *The Journal of the American Medical Association*, vol. 302, no. 2, pp. 179–188, 2009.

[5] R. S. Lindsay, T. Funahashi, R. L. Hanson et al., "Adiponectin and development of type 2 diabetes in the Pima Indian population," *The Lancet*, vol. 360, no. 9326, pp. 57–58, 2002.

[6] S. G. Wannamethee, G. D. O. Lowe, A. Rumley, L. Cherry, P. H. Whincup, and N. Sattar, "Adipokines and risk of type 2 diabetes in older men," *Diabetes Care*, vol. 30, no. 5, pp. 1200–1205, 2007.

[7] B. Bayés, M. L. Granada, M. C. Pastor et al., "Obesity, adiponectin and inflammation as predictors of new-onset diabetes mellitus after kidney transplantation," *American Journal of Transplantation*, vol. 7, no. 2, pp. 416–422, 2007.

[8] C. Snehalatha, B. Mukesh, M. Simon, V. Viswanathan, S. M. Haffner, and A. Ramachandran, "Plasma adiponectin is an independent predictor of type 2 diabetes in Asian Indians," *Diabetes Care*, vol. 26, no. 12, pp. 3226–3229, 2003.

[9] M. B. Snijder, R. J. Heine, J. C. Seidell et al., "Associations of adiponectin levels with incident impaired glucose metabolism and type 2 diabetes in older men and women: the Hoorn study," *Diabetes Care*, vol. 29, no. 11, pp. 2498–2503, 2006.

[10] W. Koenig, N. Khuseyinova, J. Baumert, C. Meisinger, and H. Löwel, "Serum concentrations of adiponectin and risk of type 2 diabetes mellitus and coronary heart disease in apparently healthy middle-aged men: results from the 18-year follow-up of a large cohort from Southern Germany," *Journal of the American College of Cardiology*, vol. 48, no. 7, pp. 1369–1377, 2006.

[11] J. Spranger, A. Kroke, M. Möhlig et al., "Adiponectin and protection against type 2 diabetes mellitus," *The Lancet*, vol. 361, no. 9353, pp. 226–228, 2003.

[12] H. Knobler, M. Benderly, V. Boyko et al., "Adiponectin and the development of diabetes in patients with coronary artery disease and impaired fasting glucose," *European Journal of Endocrinology*, vol. 154, no. 1, pp. 87–92, 2006.

[13] K. M. Choi, J. Lee, K. W. Lee et al., "Serum adiponectin concentrations predict the developments of type 2 diabetes and the metabolic syndrome in elderly Koreans," *Clinical Endocrinology*, vol. 61, no. 1, pp. 75–80, 2004.

[14] M. Daimon, T. Oizumi, T. Saitoh et al., "Decreased serum levels of adiponectin are a risk factor for the progression to type 2 diabetes in the Japanese population: the Funagata study," *Diabetes Care*, vol. 26, no. 7, pp. 2015–2020, 2003.

[15] R. Nakashima, N. Kamei, K. Yamane, S. Nakanishi, A. Nakashima, and N. Kohno, "Decreased total and high molecular weight adiponectin are independent risk factors for the development of type 2 diabetes in Japanese-Americans," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 10, pp. 3873–3877, 2006.

[16] K. Rabe, M. Lehrke, K. G. Parhofer, and U. C. Broedl, "Adipokines and insulin resistance," *Molecular Medicine*, vol. 14, no. 11-12, pp. 741–751, 2008.

[17] N. Sattar, S. G. Wannamethee, and N. G. Forouhi, "Novel biochemical risk factors for type 2 diabetes—pathogenic insights or prediction possibilities?" *Diabetologia*, vol. 51, no. 6, pp. 926–940, 2008.

[18] P. H. Whincup, J. A. Gilg, O. Papacosta et al., "Early evidence of ethnic differences in cardiovascular risk: cross-sectional comparison of British South Asian and white children," *British Medical Journal*, vol. 324, no. 7338, pp. 635–638, 2002.

[19] N. Abate, M. Chandalia, P. G. Snell, and S. M. Grundy, "Adipose tissue metabolites and insulin resistance in non-diabetic Asian Indian men," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 6, pp. 2750–2755, 2004.

[20] G. Valsamakis, R. Chetty, P. G. McTernan, N. M. Al-Daghri, A. H. Barnett, and S. Kumar, "Fasting serum adiponectin concentration is reduced in Indo-Asian subjects and is related to HDL cholesterol," *Diabetes, Obesity and Metabolism*, vol. 5, no. 2, pp. 131–135, 2003.

[21] M. Martin, L. P. Palaniappan, A. C. Kwan, G. M. Reaven, and P. D. Reaven, "Ethnic differences in the relationship between adiponectin and insulin sensitivity in South Asian and Caucasian women," *Diabetes Care*, vol. 31, no. 4, pp. 798–801, 2008.

[22] A. Raji, M. D. Gerhard-Herman, M. Warren et al., "Insulin resistance and vascular dysfunction in nondiabetic Asian Indians," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 8, pp. 3965–3972, 2004.

[23] W. F. Ferris, N. H. Naran, N. J. Crowther, P. Rheeder, L. van der Merwe, and N. Chetty, "The relationship between insulin sensitivity and serum adiponectin levels in three population groups," *Hormone and Metabolic Research*, vol. 37, no. 11, pp. 695–701, 2005.

[24] H. Wasim, N. M. Al-Daghri, R. Chetty, P. G. McTernan, A. H. Barnett, and S. Kumar, "Relationship of serum adiponectin and resistin to glucose intolerance and fat topography in South-Asians," *Cardiovascular Diabetology*, vol. 5, article 10, 2006.

[25] A. Mente, F. Razak, S. Blankenberg et al., "Ethnic variation in adiponectin and leptin levels and their association with adiposity and insulin resistance," *Diabetes Care*, vol. 33, no. 7, pp. 1629–1634, 2010.

[26] M. Luo, R. Oza-Frank, V. Mohan, K. M. Narayan, and K. Gokulakrishnan, "Serum total adiponectin is associated with impaired glucose tolerance in Asian Indian females but not in males," *Journal of Diabetes Science and Technology*, vol. 4, no. 3, pp. 645–651, 2010.

[27] N. Bansal, S. G. Anderson, A. Vyas et al., "Adiponectin and lipid profiles compared with insulins in relation to early growth of British South Asian and European children: the manchester children's growth and vascular health study," *Journal of Clinical Endocrinology and Metabolism*, vol. 96, no. 8, pp. 2567–2574, 2011.

[28] C. Snehalatha, A. Yamuna, and A. Ramachandran, "Plasma adiponectin does not correlate with insulin resistance and cardiometabolic variables in non-diabetic Asian Indian teenagers," *Diabetes Care*, vol. 31, no. 12, pp. 2374–2379, 2008.

[29] L. J. Gray, J. R. Tringham, M. J. Davies et al., "Screening for type 2 diabetes in a multiethnic setting using known risk factors to identify those at high risk: a cross-sectional study," *Vascular Health and Risk Management*, vol. 6, pp. 837–842, 2010.

[30] World Health Organization: Definition, Diagnosis, and Classification of Diabetes Mellitus and Its Complications: Report of a WHO Consultation. Part 1: Diagnosis and Classification of Diabetes Mellitus, World Health Organization, Geneva, Switzerland, 1999.

[31] K. Khunti, N. Taub, D. R. Webb et al., "Validity of self-assessed waist circumference in a multi-ethnic UK population," *Diabetic Medicine*, vol. 29, no. 3, pp. 404–409, 2012.

[32] N. Zhu, J. S. Pankow, C. M. Ballantyne et al., "High-molecular-weight adiponectin and the risk of type 2 diabetes in the ARIC study," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 11, pp. 5097–5104, 2010.

[33] V. Mohan, R. Deepa, R. Pradeepa et al., "Association of low adiponectin levels with the metabolic syndrome —the Chennai Urban Rural Epidemiology study (CURES-4)," *Metabolism*, vol. 54, no. 4, pp. 476–481, 2005.

[34] R. Retnakaran, Y. Qi, P. W. Connelly, M. Sermer, A. J. Hanley, and B. Zinman, "Low adiponectin concentration during pregnancy predicts postpartum insulin resistance, beta-cell dysfunction and fasting glycaemia," *Diabetologia*, vol. 53, no. 2, pp. 268–276, 2010.

[35] S. Drapeau, É. Doucet, R. Rabasa-Lhoret, M. Brochu, D. Prud'homme, and P. Imbeault, "Improvement in insulin sensitivity by weight loss does not affect hyperinsulinemia-mediated

reduction in total and high molecular weight adiponectin: a MONET study," *Applied Physiology, Nutrition and Metabolism*, vol. 36, no. 2, pp. 191–200, 2011.

[36] J. R. Cook and R. K. Semple, "Hypoadiponectinemia—cause or consequence of human "insulin resistance"?" *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 4, pp. 1544–1554, 2010.

[37] J. S. Yudkin, C. S. Yajnik, V. Mohamed-Ali, and K. Bulmer, "High levels of circulating pro-inflammatory cytokines and leptin in urban, but not rural, Indians. A potential explanation for increased risk of diabetes and coronary heart disease," *Diabetes Care*, vol. 22, no. 2, pp. 363–364, 1999.

[38] M. A. Banerji, N. Faridi, R. Atluri, R. L. Chaiken, and H. E. Lebovitz, "Body composition, visceral fat, leptin, and insulin resistance in Asian Indian men," *Journal of Clinical Endocrinology and Metabolism*, vol. 84, no. 1, pp. 137–144, 1999.

[39] G. S. Hotamisligil and B. M. Spiegelman, "Tumor necrosis factor α: a key component of the obesity-diabetes link," *Diabetes*, vol. 43, no. 11, pp. 1271–1278, 1994.

[40] Y. Wang, K. S. L. Lam, M. H. Yau, and A. Xu, "Post-translational modifications of adiponectin: mechanisms and functional implications," *Biochemical Journal*, vol. 409, no. 3, pp. 623–633, 2008.

Hindawi Publishing Corporation
Experimental Diabetes Research
Volume 2012, Article ID 386041, 8 pages
doi:10.1155/2012/386041

Clinical Study

Investigating Endothelial Activation and Oxidative Stress in relation to Glycaemic Control in a Multiethnic Population

E. M. Brady,[1] D. R. Webb,[2] D. H. Morris,[2] K. Khunti,[3] D. S. C. Talbot,[4] N. Sattar,[5] and M. J. Davies[2]

[1] *Department of Diabetes Research, University Hospitals of Leicester, NHS Trust, Leicester LE5 4PW, UK*
[2] *Department of Cardiovascular Sciences, University of Leicester, Leicester LE3 9QP, UK*
[3] *Department of Health Sciences, University of Leicester, Leicester LE1 6TP, UK*
[4] *Unilever Discover, Colworth Science Park, Sharnbrook MK44 ILQ, UK*
[5] *BHF Institute for Cardiovascular and Medical Sciences, University of Glasgow, Glasgow G12 8QQ, UK*

Correspondence should be addressed to E. M. Brady, emer.brady@uhl-tr.nhs.uk

Received 12 July 2012; Revised 16 October 2012; Accepted 9 November 2012

Academic Editor: K. Herbert

Aim. An exploration of ethnic differences in measures of oxidative stress and endothelial activation in relation to known cardiovascular risk factors within South Asians (SA) and White Europeans (WE) residing in the UK. *Methods*. 202 participants within a UK multiethnic population provided biomedical and anthropometric data. Human urinary 2,3-dinor-8-iso-prostaglandin-F1α and plasma ICAM-1 were quantified as measures of oxidative stress and endothelial activation, respectively. *Results*. 2,3-Dinor-8-iso-prostaglandin-F1α levels were significantly higher in the SA group compared to WE group (10.36 (95% CI: 9.09, 11.79) versus 8.46 (7.71, 9.29), $P = 0.021$) after adjustment for age, gender, smoking status, body weight, HbA1c, and medication. Oxidative stress was positively associated with HbA1c ($\beta = 1.08$, 95% CI:1.02, 1.14, $P = 0.009$), fasting ($\beta = 1.06$, 95% CI: 1.02, 1.10, $P = 0.002$), and 2 hr glucose ($\beta = 1.02$, 95% CI: 1.00, 1.04, $P = 0.052$). In each adjusted model, SA continued to have elevated levels of oxidative stress compared to WE. ICAM-1 levels were significantly higher in the composite IGR group compared to the normoglycaemic group ($P < 0.001$). No ethnic differences in ICAM-1 were observed. *Conclusion*. These results suggest that SA are more susceptible to the detrimental effects of hyperglycaemia-induced oxidative stress at lower blood glucose thresholds than WE. Further research into the potential mechanisms involved is warranted.

1. Introduction

Recent major advances in the treatment of cardiovascular disease have so far failed to rectify recognised ethnic health inequalities in the Northern Hemisphere. For example, first and second generation UK South Asians (people tracing ancestry to India, Pakistan, Bangladesh, or Sri Lanka are termed South Asians) have disproportionately higher rates of coronary heart disease and are more likely to die earlier from cardiovascular causes than their White European counterparts [1]. Whilst an increased prevalence of diabetes within this South Asian diaspora is a well-recognised contributory factor, this and other established risk factors appear not to entirely account for continued discrepancies in rates of cardiovascular morbidity [2–5]. It is plausible that an alternative, unexplained pathophysiology drives premature arterial thromboembolic disease within this ethnic group.

Oxidative stress is probably an important determinant of conditions sharing proinflammatory aetiology, including diabetes mellitus [6, 7], hypertension [8], and atherosclerosis [9]. Failure of endogenous antioxidant defences to control deleterious reactive oxygen species (ROS) leads to protein and lipid peroxidation, the stable endproducts of which serve as increasingly reliable markers of excess ROS defining oxidative stress [10]. F_2-isoprostane is a widely researched molecule which may have utility as a biomarker in obesity [11], hypercholesterolemia [12], type 2 diabetes mellitus

[13], cardiovascular disease [14], and coronary heart disease [15, 16]. Whether South Asians are particularly susceptible to glucose-related oxidative stress or incur its detrimental consequences at lower levels of exposure is unknown.

An intact healthy endothelium is essential for normal vascular and haemodynamic function. Two established surrogates of endothelial pathology, flow-mediated dilation [17], and circulating soluble adhesion molecule (ICAM-1) [18–22] are highly linked to established cardiovascular risk factors [17, 18, 20–22] and have recently been reported to be strong predictors of T2DM [23].

There are few studies exploring the relationship between hyperglycaemia, oxidative stress, (as measured by F_2-isoprostanes), and markers of endothelial activation within high-risk ethnic minority groups. Particularly, interplay between postprandial hyperglycaemia, oxidative stress, and endothelial activation may be an important determinant of early arterial wall atherosclerosis within this population.

The purpose of this study was to explore ethnic differences in measures of oxidative stress and endothelial activation and relate these to known cardiovascular risk factors within a well-characterised population cohort. We hypothesise that oxidative stress and endothelial activation will be more prevalent in South Asians with impaired glucose tolerance compared to well-matched white Europeans and will relate to postprandial glucose within this group.

2. Methods

2.1. Subjects. Subjects were recruited from a community-based screening programme for type 2 diabetes (ADDITION-Leicester), the design of which is described elsewhere [24]. Briefly 1689 South Asians aged 25–75 yrs and 5060 white Europeans aged 40–75 yrs were screened with a standard 75 g-oral glucose tolerance test (OGTT). Additional blood was taken for determination of a standard lipid profile, HbA1c%, and liver function tests. A detailed medical history and prescription inventory were recorded and every participant underwent a standardised anthropometric assessment. After providing additional written informed consent, 202 volunteers with impaired glucose intolerance (IGT) or frank type 2 diabetes (defined by World Health Organisation (WHO) 1999 criteria) and no history of cardiovascular disease provided an additional blood and urine sample for the quantification of biomarkers of oxidative stress (2,3-dinor-8-iso-prostaglandin-F1α) and endothelial function (ICAM).

All data and samples were obtained before any antihyperglycaemic medication dietary or lifestyle modifications were initiated. Informed consent was obtained from each participating subject, and the protocol was approved by the ethical committee of Leicestershire, Northamptonshire, and Rutland, UK.

2.2. Anthropometric Data. Anthropometric measurements were collected for each participant including height and weight (Tanita TBE 611, Tanita, West Drayton, UK), to the nearest 0.5 cm, 0.1 kg, and 0.5%, resp.). Waist circumference was measured at the point of minimal abdominal circumference located halfway between the navel and the lower end of the sternum [25]. Three separate blood pressure readings were taken (sitting without crossed legs at 5-minute intervals (Omron M5-1, HEM-757-E model) to the nearest 0.5 mmHg. The mean of the last two readings was used in these analyses.

2.3. Biochemical Data. Quantification of high-density lipoprotein (HDL) cholesterol was performed using the ultra-HDL assay (UHDL) and serum cholesterol using the cholesterol enzymatic assay. Serum triglyceride was measured using the triglyceride glycerol phosphate oxidase assay (Abbott Clinical Chemistry ARCHITECH c Systems/AEROSET systems). Quantification of serum glycohemoglobin (HbA1c%) was performed using high-performance liquid chromatography (HPLC) on the automated glycohemoglobin HLC-723G analyzer (Tosoh Bioscience Ltd., UK), and plasma glucose was measured using the Hexokinase method. These assays were undertaken in the pathology laboratories within University Hospitals Leicester and repeat testing carried out if the coefficient of variance was $\geq$20%.

2.4. 2, 3-Dinor-8-iso-prostaglandin-F1α Assay. This analysis was conducted using an in-house assay on an AutoDELFIA 1235 automatic immunoassay system (PerkinElmer, Life Sciences, UK) at Unilever Colworth. All urine samples were collected and stored at -20°C until analyses took place. The quantification of human 2,3-dinor-8-iso-prostaglandin-F1α from urine was undertaken via a monoclonal antibody based competitive fluorescent immunoassay method with a 2,3-dinor-8-iso-prostaglandin-F1α (Cayman Chemical Company 15290) ovalbumin-$Eu3^{+}$-labelled-tracer (EU N1 ITC chelate, PerkinElmer, Life Sciences, UK). Procedure: 50 μL of the standard QC or urine sample were dispensed into a solid yellow low florescence anti-mouse 96-well microplate (PerkinElmer, Life Sciences, UK). The stock 2,3-dinor-8-iso-prostaglandin-F1α antibody (Unilever Discover) solution was diluted 1 : 100 in DELFIA assay buffer by the AutoDELFIA followed by the addition of 100 μL of antibody (0.03 ug/mL final concentration) to each well of the plate. The stock $Eu3^{+}$- labelled ovalbulin-8-Iso-prostaglandin-F1α tracer was automatically diluted 1/100 in assay buffer by the AutoDELFIA and then 50 μL of this solution was added to each well (final dilution 1/16,000 tracer). A 60-minute incubation period followed with shaking and the plate was then washed 6 times with DELFIA wash buffer (PerkinElmer, Life Sciences, UK). 200 μL of DELFIA enhancement solution (PerkinElmer, Life Sciences, UK) was then added to each well and the plate was shaken for 5 minutes. The fluorescence counts were then read from each well of the plate by the AutoDELFIA and the concentration of 2,3-dinor-8-iso-prostaglandin-F1α determined from the standard curve by the AutoDELFIA Multicalc data reduction programme.

Repeat testing was carried out if the coefficient of precision was $\geq$10%. This assay utilises a monoclonal rather than polyclonal antibody as it affords greater specificity, and batch consistency. To evaluate the monoclonal antibody

specificity an affinity chromatography matrix was prepared by linking 2,3-dinor-8-iso-prostaglandin-F1α monoclonal antibody to CNBr Sepharose 4B (Pharmacia). Column activity was demonstrated by the binding and elution of commercially available 2,3-dinor-8-iso-prostaglandin-F1α (Cayman Chemical Company). Good elution was obtained with 95% ethyl alcohol. Activity of the column following ethyl alcohol elution (ability to bind 2,3-dinor-8-iso-prostaglandin-F1α) was checked and found to be sufficient—indicating the column might be used for multiple purification runs. 14$*$1 mL urine samples with 2,3-dinor-8-iso-prostaglandin-F1α immunoassay concentrations > 50 ng/mL were pooled and diluted in 28.5 mLs 0.1 M phosphate pH 7.1. The column was baselined with PBS and the diluted urine sample loaded at 1 mL/minute. The column was washed with PBS to baseline, then milli-Q water and then eluted with 5 mLs 95% ethyl alcohol followed by PBS. The elution peak, evaporated to dryness, was sent for analysis by GC-MS (Dr Erin Terry & Professor Jason Morrow, Vanderbilt University School of Medicine, Nashville, USA). The GC-MS tracings reported were clean, showing one clear single peak at about 0.20 minutes and later a smaller peak about 1/8 size of the sample peak. It was concluded the main peak was 2,3-dinor-8-iso-prostaglandin-F1α and the smaller peak an unknown metabolite. This result indicated high antibody specificity and contrasted with a previous result the Vanderbilt Laboratory had obtained with a commercially available monoclonal antibody, which showed multiple peaks, indicating poor antibody specificity. The DELFIA (PerkinElmer Life Sciences, UK) immunoassay format offers advantages over traditional ELISA immunoassay technology. It utilizes the unique chemical properties of lanthanide fluorescent chelates together with time-resolved fluorescence (TRF) detection to create an assay that offers high specificity, sensitivity, wide dynamic range, and superior stability. This methodology has been previously utilized by our group [26]. Urinary concentrations of this biological marker were then corrected by urinary creatinine concentration to account for differences in renal excretory function.

2.5. ICAM Measurement. All serum samples were stood upright for 60 minutes at room temperature prior to centrifugation at 3000 rpm for 10 minutes. Each subsequent 2 mL aliquot was stored at -80°C until analyses took place. ICAM-1 was measured by ELISA (R&D systems, Abingdon, UK). The intra and inter-assay CVs were both <8%.

2.6. Statistical Analysis. All statistical analyses were conducted using Stata 10.0 (StataCorp. 2007. *Stata Statistical Software: Release 10*. College Station, TX: StataCorp LP). Differences between South Asian and White European ethnic groups in terms of baseline characteristics, cholesterol, and glycaemia measures were assessed using t-tests for continuous variables and Fisher's exact tests for categorical variables. To compare oxidative stress, as measured by 2,3-dinor-8-iso prostaglandin-F1α between these two ethnic groups, an unadjusted linear regression model and a model adjusted for age (continuous; years), gender (categorical; male, female), smoking status (categorical; nonsmoker, exsmoker, current smoker), waist circumference (continuous; centimetres), HbA1c (continuous; %) and use of medication were fitted. 2,3-dinor-8-iso-prostaglandin-F1α was found to have a skewed distribution and so was log-transformed prior to analyses; back-transformed means are reported. Also of interest was whether oxidative stress was related to measures of glycaemia and the endothelial dysfunction marker ICAM. This was investigated using two different multiple regression models. The first model was an unadjusted model. The second model was adjusted for age, gender, ethnicity, smoking, medication use (including antihypertensive, lipid-lowering, thyroid/antithyroid medication, and steroids), and waist circumference. In all regression models, continuous explanatory variables were centred around their means (HbA1c = 6%, age = 58 years, waist circumference = 98 cm, fasting glucose = 6 mmol/L, 2-hour glucose = 9 mmol/L, ICAM-1 = 19 ng/mL). P values of 0.05 or lower were considered to be significant.

3. Results

Fifty-four per cent (109) of the cohort had WHO defined impaired glucose tolerance and 46% (93) had type 2 diabetes. The baseline characteristics are shown in Table 1. Thirty-four per cent (68) were of South Asian ethnicity and 66% (134) were of White European ethnicity. There was no significant difference in the prevalence of diabetes ($P = 0.66$), gender ($P = 0.56$), or waist circumference ($P = 0.10$) between the two ethnic groups; however, the white Europeans were significantly older compared to the south Asian group (62.7 years $\pm$8.1 versus 54.9 years $\pm$10.4, $P < 0.001$) and South Asians' had significantly lower body weight (75.9 $\pm$ 2.1 versus 87.4 $\pm$ 18.5, <0.001).

Mean ($\pm$standard deviation) levels of LDL cholesterol and total cholesterol were significantly lower in the South Asian group compared to the White Europeans (LDL-C: 3.1 $\pm$ 0.8 versus 3.6 $\pm$ 1.0, $P < 0.001$, resp., and total cholesterol: 5.0 $\pm$ 1.0 versus 5.8 $\pm$ 1.2, $P < 0.001$, resp.). There were no differences between the two ethnic groups with respect to measures of glycaemia, as measured by fasting glucose ($P = 0.10$), 2-hour glucose ($P = 0.35$), and HbA1c% ($P = 0.35$).

Mean unadjusted 2,3-dinor-8-iso-prostaglandin-F1α levels were significantly higher in the South Asian group compared with the White European group (mean 11.04 nmol/mmol$_{\text{creatinine}}$ (95% CI: 9.85, 12.38) versus 9.03 nmol/mmol$_{\text{creatinine}}$ (95% CI: 8.32, 9.80), resp., $P = 0.005$). A similar difference was observed after adjustment for age, gender, smoking status, body weight, and HbA1c (Table 2).

3.1. Standard Multiple-Regression 2,3-Dinor-8-iso-prostaglandin-F1α and Variables of Interest. A 1% increase in HbA1cwas associated with a 1.09 nmol/mmol$_{\text{creatinine}}$ increase in oxidative stress (β =1.08, 95% CI: 1.02, 1.14, $P = 0.009$) after adjustment for age, gender, ethnicity, medication use, smoking status, and body weight in the

Table 1: Baseline characteristics.

Characteristic	N (%) or mean ± SD			P value for ethnicity[a]
	White Europeans	South Asians	Whole population	
Total (n)	134	68	202	
Age, years	62.7 ± 8.1	54.9 ± 10.4	60.1 ± 9.7	<0.001
Gender				
Male	63 (47.0)	35 (51.5)	98 (48.5)	0.655
Female	71 (53.0)	33 (48.5)	104 (51.5)	0.556
Smoking status (current)	51 (38.1)	6 (8.8)	57 (28.2)	<0.001
Weight, kg	87.4 ± 18.5	75.9 ± 2.1	83.5 ± 18.5	<0.001
Waist circumference, cm	101.8 ± 15.0	98.0 ± 12.4	100.5 ± 14.3	0.096
Fasting glucose, mmol/L	6.8 ± 2.2	6.4 ± 1.4	6.7 ± 2.0	0.175
2-hour glucose, mmol/L	11.4 ± 4.2	11.3 ± 3.4	11.3 ± 3.9	0.809
HbA1c, %	6.6 ± 1.4	6.7 ± 0.9	6.6 ± 1.2	0.785
Type 2 diabetes	60 (44.8)	33 (48.5)	93 (46.0)	0.656
IGT	74 (55.2)	35 (51.5)	109 (54.0)	0.752
Blood pressure, mmHg				
Systolic	147.1 ± 17.9	141.0 ± 18.1	145.1 ± 18.2	0.041
Diastolic	88.8 ± 9.9	87.5 ± 10.2	88.4 ± 10.1	0.446
Cholesterol, mmol/L				
Total	5.8 ± 1.2	5.0 ± 1.0	5.5 ± 1.2	<0.001
LDL	3.6 ± 1.0	3.1 ± 0.8	3.4 ± 1.0	0.001
HDL	1.3 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	0.010
Cardiovascular medication[b]	59 (42.4)	33 (48.5)	92 (45.5)	0.542

SD: standard deviation.
[a]P values show the difference between the two ethnic groups and were calculated using t-tests for continuous variables and Fisher's exact test for categorical variables.
[b]Defined as any of following prescription medications: angiotensin converting enzyme inhibitor, beta-blocker, calcium channel antagonist, statin, or fibrate lipid lowering treatments.

Table 2: Mean 2,3-dinor-8-iso-prostaglandin-F1α in White European and South Asian ethnic groups.

	N	Mean[a] 2,3-dinor-8-iso-prostaglandin-F1α nmol/mmol$_{creatinine}$ (95% confidence interval)		P value[b]
		White European	South Asian	
Unadjusted	202	9.03 (8.32, 9.80)	11.04 (9.85, 12.38)	0.005
Adjusted[c]	172	8.46 (7.71, 9.29)	10.36 (9.09, 11.79)	0.021

[a]Estimated using linear regression on log-transformed values, and reported as back-transformed means.
[b]The P value showing the difference between the two ethnic groups was estimated using linear regression.
[c]Adjusted for age (continuous), gender (male, female), smoking status (non-smoker, ex-smoker, current smoker), weight (continuous), HbA1c (continuous) and medication use at point of data collection.

whole cohort (Table 3). In this model levels of 2,3-dinor-8-iso-prostaglandin-F1α are 1.25 nmol/mmol$_{creatinine}$ (95% CI; 1.05, 1.49, $P = 0.011$, not in table) higher in South Asians than White Europeans independent of the potential confounding variables adjusted for.

A 1 mmol/L increase in 2 hr glucose is associated with an average increase of 1.02 nmol/mmol$_{creatinine}$ in the level of oxidative stress $\beta = 1.02$, (95% CI: 1.00, 1.04, $P = 0.042$) adjusted for age, gender, ethnicity, medication use, smoking status and body weight in the whole cohort (Table 3). In this model levels of 2,3-dinor-8-iso-prostaglandin-F1α are 1.21 nmol/mmol$_{creatinine}$ (95% CI: 1.02, 1.43, $P = 0.026$, not in table) higher in South Asians than White Europeans independent of the potential confounding variables adjusted for.

A 1 mmol/L increase in fasting blood glucose is associated with a 1.06 nmol/mmol$_{creatinine}$ increased level of oxidative stress ($\beta = 1.06$, (95% CI: 1.02, 1.10, $P = 0.001$) adjusted for age, gender, ethnicity, medication use, smoking status, and waist circumference in the whole cohort (Table 3). In this model the level of 2,3-dinor-8-iso-prostaglandin-F1α are 1.29 nmol/mmol$_{creatinine}$ (95% CI: 1.09, 1.54, $P = 0.004$,

Table 3: Multiple regression analysis showing the effect of various glycaemia and endothelial dysfunction markers on 2,3-Dinor-8-Iso-prostaglandin-F1α.

Variable	Model	R^2	β**	95% CI	P value
HbA1c, %	Unadjusted	0.027	1.07	1.01, 1.13	0.019
	Adjusted for covariates	0.124	1.08	1.02, 1.14	0.009
2 hr Glucose, mmol/L	Unadjusted	0.016	1.02	1.00, 1.03	0.074
	Adjusted for covariates	0.112	1.02	1.00, 1.04	0.052
Fasting blood glucose, mmol/L	Unadjusted	0.028	1.04	1.01, 1.08	0.018
	Adjusted for covariates	0.141	1.06	1.02, 1.10	0.002
ICAM, ng/mL	Unadjusted	0.018	1.01	0.99, 1.03	0.183
	Adjusted for covariates	0.118	1.01	0.99, 1.03	0.297

* Adjusted for age, gender, ethnicity, medication use, smoking status, and weight. Only results for variable of interest and ethnicity are shown.
** Regression coefficients were estimated using log-transformed PGF and back-transformed values are reported.

Table 4: Mean ICAM-1 ng/mL within glucose categories of normal glucose tolerance (NGT) and impaired glucose regulation (IGR)* and stratified by ethnicity.

	Combined ICAM-1 population		South Asian		White European		P[a] (SA versus WE)
	n	Mean (95% CI)	n	Mean (95% CI)	n	Mean (95% CI)	
NGT	141	17.30 (16.47, 18.14)	66	16.94 (15.70, 18.17)	75	17.62 (16.47, 18.77)	0.420
IGR*	183	19.51 (18.65, 20.38)	69	19.17 (17.87, 20.47)	114	19.72 (18.57, 20.88)	0.542
P[a] (NGT versus IGR)		<0.001		0.015		0.015	

CI: confidence interval, SA: South African, WE: White European.
IGR: Predefined composite of impaired glucose tolerance and type 2 diabetes.
[a]P values were estimated using Student's t-tests.

not in table) higher in South Asians than White Europeans independent of the potential confounding variables adjusted for.

3.2. ICAM-1. For comparative purposes ICAM-1 was matched to a normal glucose tolerant control group. Mean values of ICAM-1 within this control population were significantly lower than in a composite impaired glucose tolerant and diabetes group among all subjects ($P < 0.001$, Table 3) and when South Asian ($P = 0.015$) and white European ($P = 0.015$) subjects were analysed separately. To further illustrate this association, logistic regression analyses were performed. We report for the combined cohort a 1 ng/mL increase in ICAM is associated with an 8% increased likelihood of IGR (OR 1.08 (95% CI: 1.03, 1.13) $P < 0.001$) which was similar for South Asians' and White Europeans' alone (OR: 1.09 (1.01, 1.17), $P = 0.018$ and 1.07 (1.01, 1.13), $P = 0.015$, resp.). No ethnic differences in ICAM-1 were observed in either normal glucose tolerant ($P = 0.42$) or impaired glucose regulation ($P = 0.54$) categories (Table 4). There was no statistically significant independent association between levels of ICAM and oxidative stress in this cohort (Table 4).

4. Discussion

In this study we demonstrate evidence of enhanced oxidative stress in apparently healthy first generation UK South Asians with undiagnosed hyperglycaemia. This difference of ~17% compared with an endogenous matched White European population is independent of measured confounding effects of age, gender, smoking, use of medication, body weight and HbA1c. Causality cannot of course be inferred from cross-sectional analyses of this nature. Nevertheless, our findings suggest that oxidative stress may contribute to accelerated atherogenesis and well-documented cardiovascular disease susceptibility within South Asians migrating to northern latitudes [1, 27–29].

Oxidative stress results from an imbalance between endogenous oxidant production and the level of antioxidant activity. It is established that a hypercalorific state (caloric intake exceeds energy expenditure) induces excess production of ROS via endoplasmic reticulum stress [30]. However, in a bid to reduce the formation of excess cellular ROS, and protect against its harmful effects, insulin stimulated glucose uptake is inhibited, via the reduction of GLUT-4 translocation, further exacerbating the hyperglycaemic state [31–34]. The pancreatic β cell is more susceptible to ROS due to a relative deficiency of antioxidant enzymes. Prolonged exposure to hyperglycaemia and free fatty acids in the hypercaloric state is reported to induce β-cell dysfunction [32, 33]. This "glucolipotoxicity" has been described in a recent review [35] and oxidative stress identified as a major contributory factor to the process. Our study has demonstrated elevated levels of a marker of systemic oxidative stress in a UK South Asian cohort compared to White Europeans with comparable glycaemic status suggesting that South Asians could

be susceptible to the detrimental effects of hyperglycaemia-induced oxidative stress at lower blood glucose thresholds. This is further supported by our multiple-regression analysis. Increasing levels of HbA1c, fasting, and 2-hour glucose were all associated with increasing levels of oxidative stress (Table 3). In each model, South Asians continued to have elevated levels of oxidative stress in comparison to White Europeans following adjustment for aforementioned confounders (Table 3).

Conversely circulating cellular adhesion molecule values are not elevated in South Asians with either impaired glucose regulation or normo-glycaemia and no evidence of vascular disease compared to White Europeans (Table 4). This is perhaps not too surprising since whilst earlier studies suggested a strong link between ICAM-1 and vascular outcomes [22, 36–40], a later large prospective study and meta-analysis have contested this view [41]. Cell adhesion molecules including ICAM-1 were reported to have no significant relationship to CHD in their adjusted analysis. However, we report significantly higher levels of ICAM-1 in those with impaired glucose regulation compared to those with normo-glycaemia in the whole group and within ethnic groups. These results support recent findings from Sattar et al. [23] who reported a significant association between elevated ICAM-1 and risk of incident diabetes (HR 1.82 (95% CI: 1.27–2.63, $P = 0.0013$)). This group also reported no association between elevated ICAM-1 and incident CVD risk. This supports evidence from a smaller analysis of indigenous Indians demonstrating an upward progression of markers of lipid peroxidation and endothelial dysfunction (sVCAM-1) from healthy controls to diabetics with and without vascular disease [42]. Thus, our results support the growing body of evidence that dysregulated ICAM-1 is involved in the pathogenesis of T2DM but we suggest it is not attributable to the increased risk of vascular complications observed in South Asians residing in the UK.

Potential limitations of the present study include its cross-sectional design. It is also observational and therefore subject to residual confounding. The F_2 class of isoprostanes immerged as the "gold standard" assessment of in vivo oxidative stress and are the most widely documented of the isoprostane family [11–16, 43]. However, the F_2-isoprostanes undergo further metabolism in the kidney yielding urinary dinor derivatives [43] including 2,3-dinor-8-iso-prostaglandin-F1α. Our group decided to quantify this urinary metabolite of oxidative stress in view of its relative stability and although our findings are unique in light of this we do not view our results to be of greater impact or novelty based on this methodology but consider them to be additive to the growing body of literature for the pathogenic role of oxidative stress in atherosclerosis and cardiovascular disease alike. However, because oxidative stress is a highly complex process with numerous unstable "midproducts" it is possible this urinary metabolite may not be sensitive enough to reflect the true extent of oxidative stress in this cohort or indeed at the tissue level.

Although, only a single morning urine sample was used, we do not anticipate the alternative method of 24 hour sampling to have any influence on the results reported here. Helmersson and Basu have previously reported no significant variation between spot urine levels of F_2-isoprostanes isomers and those measured from 24-hr urine sampling and in fact suggests that a morning sample is preferable as it is representative of urine from 6–8 hours [43, 44]. Isoprostane concentrations were however expressed/mmol creatinine to adjust for urine volume fluctuations and this urinary metabolite is a stable and validated marker of oxidative stress [44, 45]. A further limitation may be the use of surrogate subclinical markers of endothelial function. Although there is utility in the identification of endothelial phenotypes using circulating serum markers that are regulated and released by the vascular endothelium, flow mediated dilation (FMD) of the brachial artery is considered to be the gold standard technique for measuring endothelial function [46]. We therefore may have lost sensitivity by not employing this technique. Further, only one marker of endothelial dysfunction was used in this cohort and only a subset of participants provided the additional fasting blood for these analyses. This may have rendered it under powered and interpretation of secondary ICAM-1-isoprostane relationships should therefore be viewed with caution. Future investigation should include E-selectin and/or von Willebrand factor and a larger sample size. Furthermore, additional measures of oxidative stress, that is, 8-hydroxy-2′-deoxyguanosine (8-OHdG) may have substantiated these results further.

Strengths of this study include the selection criteria which excluded confounders such as overt vascular disease and glucose-lowering treatments. Standard operating procedures were used throughout the study and laboratory analyses were blinded. The analysis of these biological markers was conducted using validated assay kits with good precision. Robust statistical techniques have been used which allowed us to include a larger number of potential confounders.

5. Summary

We show that ICAM-1 levels are not higher in South Asians residing in the UK despite its association with glycaemia in both groups. However, systemic oxidative stress appears to be significantly higher in glucose intolerant South Asians compared to their WE counterparts, independent of measured confounders. Our multiple-regression analysis show that HbA1c is positively associated with 2,3-dinor-8-iso-prostaglandin-F1α levels independent of confounding variables. The ethnicity variable in this model indicated that this positive association is greater in South Asians versus WE. The increase in 2,3-dinor-8-iso-prostaglandin-F1α was 1.09 $\text{nmol/mmol}_{\text{creatinine}}$ for the whole group for every 1% increase in HbA1c, for South Asians the increased levels of 2,3-dinor-8-iso-prostaglandin-F1α for every 1% increase in HbA1c was 1.25 $\text{nmol/mmol}_{\text{creatinine}}$ higher compared to WE. Subsequently, we suggest that South Asians are more susceptible to the detrimental effects of hyperglycaemia-induced oxidative stress at lower blood glucose thresholds than WE given that there is no significant difference in

HbA1c between the ethnic groups in this cohort. To substantiate this potential relationship, further research into the mechanisms involved is warranted.

Conflict of Interests

There are no direct financial relations with the commercial identities mentioned within this paper that might lead to a conflict of interests for any of the authors of this work.

Authors' Contribution

E. M. Brady and D. R. Webb shared first authorship.

Acknowledgments

This paper was funded by the Biotechnology and Biological Sciences Research Council (BBSRC), the National Institute for Health Research (NIHR) Leicester-Loughborough Diet and Lifestyle Biomedical Research Unit, and Unilever corporate research.

References

[1] N. Gholap, M. Davies, K. Patel, N. Sattar, and K. Khunti, "Type 2 diabetes and cardiovascular disease in South Asians," *Primary Care Diabetes*, vol. 5, no. 1, pp. 45–56, 2011.

[2] P. M. McKeigue, B. Shah, and M. G. Marmot, "Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians," *The Lancet*, vol. 337, no. 8738, pp. 382–386, 1991.

[3] R. Venkataraman, N. C. Nanda, G. Baweja, N. Parikh, and V. Bhatia, "Prevalence of diabetes mellitus and related conditions in Asian Indians living in the United States," *American Journal of Cardiology*, vol. 94, no. 7, pp. 977–980, 2004.

[4] K. Spronston and J. Mindell, Eds., *Health Survey For England 2004, Volume 1: The Health of Minority Ethnic Groups*, The Information Centre, Leeds, UK, 2006.

[5] A. M. Kanaya, C. L. Wassel, D. Mathur et al., "Prevalence and correlates of diabetes in South Asian Indians in the United States: findings from the metabolic syndrome and atherosclerosis in South Asians Living in America study and the multi-ethnic study of atherosclerosis," *Metabolic Syndrome and Related Disorders*, vol. 8, no. 2, pp. 157–164, 2010.

[6] A. Ceriello, "New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy," *Diabetes Care*, vol. 26, no. 5, pp. 1589–1596, 2003.

[7] J. B. Meigs, M. G. Larson, C. S. Fox, J. F. Keaney, R. S. Vasan, and E. J. Benjamin, "Association of oxidative stress, insulin resistance, and diabetes risk phenotypes: the Framingham Offspring Study," *Diabetes Care*, vol. 30, no. 10, pp. 2529–2535, 2007.

[8] R. M. Touyz, "Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance?" *Hypertension*, vol. 44, no. 3, pp. 248–252, 2004.

[9] S. Porasuphatana, S. Suddee, A. Nartnampong et al., "Glycemic and oxidative status of patients with type 2 diabetes mellitus following oral administration of alpha-lipoic acid: a randomized double-blinded placebo-controlled study," *Asia Pacific Journal of Clinical Nutrition*, vol. 21, no. 1, pp. 12–21, 2012.

[10] F. Bononmini, S. Tengattini, A. Fabiano, R. Bianchi, and R. Rezzani, "Atherosclerosis and oxidative stress," *Histology and Histopathology*, vol. 23, no. 3, pp. 381–390, 2008.

[11] J. F. Keaney Jr., M. G. Larson, R. S. Vasan et al., "Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham study," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 3, pp. 434–439, 2003.

[12] M. P. Reilly, D. Praticò, N. Delanty et al., "Increased formation of distinct F2 isoprostanes in hypercholsterolemia," *Circulation*, vol. 98, no. 25, pp. 2822–2828, 1998.

[13] G. Davì, G. Ciabattoni, A. Consoli et al., "*in vivo* formation of 8-iso-prostaglandin and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation," *Circulation*, vol. 99, no. 2, pp. 224–229, 1999.

[14] J. W. Stephens, M. P. Khanolkar, and S. C. Bain, "The biological relevance and measurement of plasma markers of oxidative stress in diabetes and cardiovascular disease," *Atherosclerosis*, vol. 202, no. 2, pp. 321–329, 2009.

[15] M. Roest, H. A. M. Voorbij, Y. T. Van der Schouw, P. H. M. Peeters, T. Teerlink, and P. G. Scheffer, "High levels of urinary F2-isoprostanes predict cardiovascular mortality in postmenopausal women," *Journal of Clinical Lipidology*, vol. 2, no. 4, pp. 298–303, 2008.

[16] Z. J. Zhang, "Systematic review on the association between F2-isoprostanes and cardiovascular disease," *Annals of Clinical Biochemistry*. In press.

[17] T. Inoue, H. Matsuoka, Y. Hiqashi et al., "Flow-mediated vasodilation as a diagnostic modality for vascular falure," *Hypertension Research*, vol. 31, no. 12, pp. 2105–2113, 2008.

[18] J. B. Meigs, F. B. Hu, N. Rifai, and J. E. Manson, "Biomarkers of endothelial dysfunction and risk of Type 2 diabetes mellitus," *Journal of the American Medical Association*, vol. 291, no. 16, pp. 1978–1986, 2004.

[19] A. Ponthieux, B. Herbeth, S. Droesch, N. Haddy, D. Lambert, and S. Visvikis, "Biological determinants of serum ICAM-1, E-selectin, P-selectin and L-selectin levels in healthy subjects: the Stanislas study," *Atherosclerosis*, vol. 172, no. 2, pp. 299–308, 2004.

[20] S. J. Hwang, C. M. Ballantyne, A. R. Sharrett et al., "Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study," *Circulation*, vol. 96, no. 12, pp. 4219–4225, 1997.

[21] R. De Caterina, G. Basta, G. Lazzerini et al., "Soluble vascular cell adhesion molecule-1 as a biohumoral correlate of atherosclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 17, no. 11, pp. 2646–2654, 1997.

[22] T. O'Malley, C. A. Ludlam, R. A. Riemermsa, and K. A. A. Fox, "Early increase in levels of soluble inter-cellular adhesion molecule-1 (sICAM-1): potential risk factor for the acute coronary syndromes," *European Heart Journal*, vol. 22, no. 14, pp. 1226–1234, 2001.

[23] N. Sattar, H. M. Murray, P. Welsh et al., "Are elevated circulating intercellular adhesion molecule 1 levels more strongly predictive of diabetes than vascular risk? Outcome of a prospective study in the elderly," *Diabetologia*, vol. 52, no. 2, pp. 235–239, 2009.

[24] D. R. Webb, K. Khunti, B. Srinivasan et al., "Rationale and design of the ADDITION-Leicester study, a systematic screening programme and Randomised Controlled Trial of multi-factorial cardiovascular risk intervention in people with Type 2 Diabetes Mellitus detected by screening," *Trials*, vol. 11, article 16, 2010.

[25] K. Khunti, N. Taub, D. Webb et al., "Validity of self-assessed waist circumference in a multi-ethnic UK population," *Diabetic Medicine*, vol. 29, no. 3, pp. 404–409, 2012.

[26] H. E. Theobald, A. H. Goodall, N. Sattar, D. C. S. Talbot, P. J. Chowienczyk, and T. A. B. Sanders, "Low-dose docosahexaenoic acid lowers diastolic blood pressure in middle-aged men and women," *Journal of Nutrition*, vol. 137, no. 4, pp. 973–978, 2007.

[27] S. Bellary, J. P. O'Hare, Raym, N. T. ond et al., "Premature cardiovascular events and mortality in South Asians with T2DM in UK Asian diabetes meliitus study—effect of ethnicity on risk," *Current Medical Research & Opinion*, vol. 26, no. 8, pp. 1873–1879, 2010.

[28] H. M. Mather, N. Chaturvedi, and J. H. Fuller, "Mortality and morbidity from diabetes in South Asians and Europeans: 11-year follow-up of the Southall Diabetes Survey, London, UK," *Diabetic Medicine*, vol. 15, no. 1, pp. 53–59, 1998.

[29] A. H. Barnett, A. N. Dixon, S. Bellary et al., "Type 2 diabetes and cardiovascular risk in the UK south Asian community," *Diabetologia*, vol. 49, no. 10, pp. 2234–2246, 2006.

[30] B. A. Maddux, W. See, J. C. Lawrence Jr., A. L. Goldfine, I. D. Goldfine, and J. L. Evans, "Protection against oxidative stress-induced insulin resistance in rat l6 muscle cells by micromolar concentrations of α-lipoic acid," *Diabetes*, vol. 50, no. 2, pp. 404–410, 2001.

[31] A. Rudich, A. Tirosh, R. Potashnik, R. Hemi, H. Kanety, and N. Bashan, "Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes," *Diabetes*, vol. 47, no. 10, pp. 1562–1569, 1998.

[32] G. Paolisso and D. Giugliano, "Oxidative stress and insulin action: Is there a relationship?" *Diabetologia*, vol. 39, no. 3, pp. 357–363, 1996.

[33] J. L. Evans, I. D. Goldfine, B. A. Maddux, and G. M. Grodsky, "Are oxidative stress-activated signaling pathways mediators of insulin resistance and β-cell dysfunction?" *Diabetes*, vol. 52, no. 1, pp. 1–8, 2003.

[34] Y. Kajimoto and H. Kaneto, "Role of oxidative stress in pancreatic β-cell dysfunction," *Annals of the New York Academy of Sciences*, vol. 1011, pp. 168–176, 2004.

[35] J. W. Kim and K. H. Yoon, "Glucolipotoxicity," *Journal of Diabetes & Metabolism*, vol. 35, no. 5, pp. 444–450, 2011.

[36] R. C. Hoogeveen, C. M. Ballantyne, H. Bang et al., "Circulating oxidised low-density lipoprotein and intercellular adhesion molecule-1 and risk of type 2 diabetes mellitus: the atherosclerosis risk in communities study," *Diabetologia*, vol. 50, no. 1, pp. 36–42, 2007.

[37] A. D. Pradhan, N. Rifai, and P. M. Ridker, "Soluble intercellular adhesion molecule-1, soluble vascular adhesion molecule-1, and the development of symptomatic peripheral arterial disease in men," *Circulation*, vol. 106, no. 7, pp. 820–825, 2002.

[38] A. S. Postadzhiyan, A. V. Tzontcheva, I. Kehayov, and B. Finkov, "Circulating soluble adhesion molecules ICAM-1 and VCAM-1 and their association with clinical outcome, troponin T and C-reactive protein in patients with acute coronary syndromes," *Clinical Biochemistry*, vol. 41, no. 3, pp. 126–133, 2008.

[39] O. Eschen, J. H. Christensen, S. P. Johnsen, C. Dethlefsen, and E. B. Schmidt, "Adhesion molecules and C-reactive protein are associated to adverse events in angina pectoris," *Scandinavian Cardiovascular Journal*, vol. 44, no. 3, pp. 153–160, 2010.

[40] Y. Higashi, K. Noma, M. Yoshizumi, and Y. Kihara, "Endothelial function and oxidative stress in cardiovascular diseases," *Circulation Journal*, vol. 73, no. 3, pp. 411–418, 2009.

[41] I. Malik, J. Danesh, P. Whincup et al., "Soluble adhesion molecules and prediction of coronary heart disease: a prospective study and meta-analysis," *The Lancet*, vol. 358, no. 9286, pp. 971–975, 2001.

[42] N. Singhania, D. Puri, S. V. Madhu, and S. B. Sharma, "Assessment of oxidative stress and endothelial dysfunction in Asian Indians with type 2 diabetes mellitus with and without macroangiopathy," *Quarterly Journal of Medicine*, vol. 101, no. 6, pp. 449–455, 2008.

[43] J. D. Morrow, "Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 2, pp. 279–286, 2005.

[44] G. L. Milne, E. S. Musiek, and J. D. Morrow, "F_2-isoprostanes as markers of oxidative stress *in vivo*: an overview," *Biomarkers*, vol. 10, pp. S10–S23, 2005.

[45] H. Helmersson and S. Basu, "F_2-isoprostane excretion rate and diurnal variation in human urine," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 61, no. 3, pp. 203–205, 1999.

[46] M. E. Widlansky, N. Gokce, J. F. Keaney, and J. A. Vita, "The clinical implications of endothelial dysfunction," *Journal of the American College of Cardiology*, vol. 42, no. 7, pp. 1149–1160, 2003.

Hindawi Publishing Corporation
Experimental Diabetes Research
Volume 2012, Article ID 350852, 6 pages
doi:10.1155/2012/350852

Clinical Study

Plasma Plasminogen Activator Inhibitor-1 Is Associated with End-Stage Proliferative Diabetic Retinopathy in the Northern Chinese Han Population

Ze-Long Zhong[1, 2] and Song Chen[1, 2]

[1] *Clinical College of Ophthalmology, Tianjin Medical University, Tianjin 300020, China*
[2] *Center of Vitreoretinopathy, Tianjin Eye Hospital, Tianjin 300020, China*

Correspondence should be addressed to Ze-Long Zhong, zzl8150@yahoo.com.cn

Received 26 June 2012; Revised 2 September 2012; Accepted 9 October 2012

Academic Editor: D. R. Webb

Objective. To identify predictors of end-stage proliferative diabetic retinopathy (PDR) in a cohort of individuals with type 2 diabetes mellitus (T2DM) from the Northern Chinese Han population. *Methods*. We investigated characteristics of 153 consecutive diabetic patients with end-stage PDR (62 males, 91 females), 123 consecutive PDR patients without end-stage PDR (48 males, 75 females), and 151 normal subjects (63 males, 88 females). Only one eye of each patient or healthy subject was included in this study. Univariate logistic regression models and multivariate logistic regression models were constructed to evaluate the predictors of end-stage PDR. *Results*. In univariate analysis, systolic blood pressure, diastolic blood pressure, duration of diabetes, family history of T2DM, and plasminogen activator inhibitor-1 (PAI-1) were significently associated with end-stage PDR. After multivariate analysis, family history of T2DM, plasma PAI-1 levels, smoking, and duration of diabetes were four positive predictors associated with end-stage PDR. *Conclusions*. Higher plasma levels of PAI-1 were associated with end-stage PDR in the Northern Chinese Han population with T2DM.

1. Introduction

There has been much discussion recently about the risk factors for proliferative diabetic retinopathy (PDR). It remains uncertain what independent risk factors and predictors associated with end-stage PDR in the Northern Chinese Han population with type 2 diabetes mellitus (T2DM) exist. End-stage PDR is the most serious phrase of PDR. Diet, lifestyle, and depression [1] have been associated with a rapid increase in the incidence of DM in many countries throughout the world, particularly in developing countries such as China [2]. T2DM and its complications have been studied by many researchers in the fields of endocrinology [3, 4] and ophthalmology [5] since it is thought that identification and reduction of risk factors of DR may lead to a decrease of vision loss that is associated with this condition. Many previous studies have demonstrated that hereditary [6, 7] and environmental factors [8, 9] were both likely to be risk factors for PDR. Furthermore, it has been inferred that some cytokines in the peripheral blood might be predictors of end-stage PDR.

Systemic risk factors, including age, duration of diabetes, glycemic control, hypertension, renal impairment, the use of insulin, smoking, abdominal obesity, and genetic factors, have been reported to be associated with the progression of DR [10–15]. Additionally hyperopic refractive error [16] and axial length [17] are reported to be related to the development and progression of DR. However, whether there are circulating predictors of end-stage PDR in peripheral blood is unknown. The gene encoding human plasminogen activator inhibitor (PAI-1) was cloned from the lambda EMBL3 genomic library and was found to span approximately 12 kb and contain eight introns [18]. SERPINE1 (PAI-1) and other inflammatory mediators, such as ICAM1, IL-1, coagulation factor III, and VEGF, can cause retinal ischemia-reperfusion injury (RIRI) and retinal neovascularization [19]. The interaction between PAI-1 and TGF-β may ultimately induce the neovascularization seen in retinopathy of

T2DM via the action of VEGF [20]. Therefore, it is plausible therefore that this circulating protein may predict the onset of end-stage PDR. Thus, targeting PAI-1 in addition to or instead of the traditional aforementioned risk factors may prevent progression to end-stage PDR. The aim of this study was to identify risk factors and predictors of end-stage PDR in a Northern Chinese Han population.

2. Methods

2.1. Study Design. From Jan 2009 to Jan. 2012, 153 consecutive patients with T2DM (62 males, 91 females) that had a clinical diagnosis of end-stage PDR were enrolled. 123 consecutive diabetic patients without end-stage PDR (48 males, 75 females) and 151 normal subjects (63 males, 88 females) also participated in the study. One eye, with the higher degree of damage in each patient, was included in this study when the severity of diabetic retinopathy in each of two eyes of T2DM was different. If two eyes were eligible, one eye was randomly included in this study using a randomization envelope. Control subjects were defined as the patients with PDR but without end-stage proliferative retinopathy. The eligibility criteria in the patients included known or newly diagnosed T2DM and age of 40 yr or older. Patients were excluded if they had acute complications of DM, type 1 or other types of DM, gestational diabetes, serious cardiovascular disease, hepatic, nephritic, or other complications, or other serious primary diseases or mental illness [9]. Individuals screened for the normal control group were excluded if they had any diseases of any system, as identified from their history and physical examinations [9].

2.2. Procedures. All examinations followed a similar protocol that was approved by the institutional human subjects committee of Tianjin Medical University and Tianjin Eye Hospital. The study was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all subjects enrolled in this study.

The ocular and laboratory examinations included blood pressure, eye examination after dilating the pupils, stereoscopic color fundus photographs, family history of diabetes (yes or no), age, gender, duration of diabetes, duration of hypertension, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting blood glucose (FBG), postprandial blood glucose (PBG), blood urea nitrogen (BUN), creatinine (CREA), triglycerides (TGs), total cholesterol (TC), urine albumin, white blood cells (WBCs), red blood cells (RBCs), mean platelet volume (MPV), PAI-1 levels, and history of smoking.

2.3. Definitions. T2DM was diagnosed according to the World Health Organization Expert Consultation Report [21] and consisted of one of the following: fasting blood glucose (FBG) $\geq$7.0 mmol/L, blood glucose $\geq$11.1 mmol/L 2 hours after an oral glucose tolerance test (OGTT), or random blood glucose $\geq$11.1 mmol/L. DR was clinically graded in accordance with the International Clinical Diabetic Retinopathy guidelines [22]. Early treatment diabetic retinopathy study (ETDRS) levels were as follows: 60–71, proliferative retinopathy (new vessels, vitreous haemorrhages, fibrovascular proliferation, and tractional detachment of retina) or scars of photocoagulation; 85, end-stage proliferative retinopathy (macula obscured by haemorrhage, retinal detachment at centre of macula, phthisis bulbi, or enucleation secondary to complications of diabetic retinopathy) [23]. The duration of diabetes was the period between the age at diagnosis and the age at the baseline examination [23]. Blood pressure was recorded twice with a random zero sphygmomanometer (Hawksley, Lancing, UK). Hypertension was defined as a systolic blood pressure $\geq$140 mmHg or diastolic blood pressure $\geq$90 mmHg and/or the use of blood pressure-lowering drugs [24]. A person was classified as never smoked if he/she had smoked fewer than 100 cigarettes in his/her lifetime and as a current smoker if he/she had not stopped smoking. Passive smoking was defined as in a previous study [25]. Controls were matched to end-stage PDR subjects based on age ($\leq$5 years), gender, place of residency, and economical condition.

2.4. Laboratory Measurements. The plasma levels of PAI-1 were measured using ELISA arrays (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) according to the manufacturer's instructions. The measurement of plasma levels of PAI-1, BUN, CREA, plasma glucose, and triglycerides was done in accordance with the studies in [26–30].

2.5. Statistical Analysis. Continuous variables are presented as the mean with the standard deviation and were compared between end-stage PDR and the controls with a Student's t-test. Dichotomous variables were presented as percentages and were compared utilizing an χ^2 test. After one-way analysis of variance (ANOVA), the LSD test was used for the comparison among the mean values of three groups for different ages or plasma levels of PAI-1.

In the first part of the analysis, all potential risk factors including age, gender, type of diabetes, duration of diabetes, family history of diabetes, age, sex, duration of diabetes, SBP, DBP, FBG, PBG, BUN, CREA, TG, TC, urine albumin, WBC, RBC, MPV, PAI-1 level, and history of smoking (passive smoking was definited; active smokers with passive smoking were defined) were analyzed by univariate logistic regression analysis. The univariate relationships among the factors associated with PDR and the severity of PDR were analyzed with linear regression models.

In the second part of the analysis, PAI-1 levels and other variables with $P < 0.1$ in the univariate analysis were included as independent variables in multivariate logistic regression models. Because the plasma PAI-1 levels were not normally distributed, they had been log transformed before the courses of multivariate logistic regression. We used the SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) as the software for the statistical analyses. $P < 0.05$ was considered to indicate statistical significance. All P values were two sided.

3. Results and Discussion

In the present study, comparisons of baseline data between the patients with PDR and the normal subjects are shown

Table 1: Baseline data of patient characteristics in three groups.

	End-stage PDR		Normals ($n = 151$)
	Yes ($n = 153$)	No ($n = 123$)	
Male (n[%])	62 (40.523)*	48 (39.024)*	63 (41.722)
Age, years#	52.719 ± 6.661*§	57.626 ± 8.006*	53.136 ± 7.342
PAI-1, pmol/dL#	7.394 ± 2.084*§	6.216 ± 0.989*	1.952 ± 0.276
95%CI	7.062–7.727	6.040–6.393	1.851–2.053
Family history of DM (n[%])	13 (8.497)§	36 (29.268)	—
History of hypertension (n[%])	89 (58.170)§	75 (60.976)	—
Smoking (n[%])			
Active	61 (39.869)	46 (37.398)	—
Passive	153 (100.000)	123 (100.000)	—
Duration of DM#	12.496 ± 6.173§	9.954 ± 4.441	—
FBG, mmol/L#	7.125 ± 1.987§	6.512 ± 2.157	—
PBG, mmol/L#	9.919 ± 3.401	9.226 ± 3.786	—
SBP, mmHg#	136.272 ± 11.114§	131.952 ± 14.719	—
DBP, mmHg#	84.771 ± 6.985§	82.602 ± 9.373	—
TC, mmol/L#	5.161 ± 0.784	5.222 ± 1.101	—
TG, mmol/L#	2.115 ± 1.509	1.854 ± 1.628	—
CREA, umol/L#	82.971 ± 52.230§	70.085 ± 33.491	—
BUN, mmol/L#	6.890 ± 2.630	6.345 ± 2.242	—
PAI-1, pmol/dL#	7.394 ± 2.084§	6.216 ± 0.989	—
WBC, 10^9/L#	6.709 ± 1.606	6.513 ± 1.953	—
RBC, 10^{12}/L#	4.350 ± 0.396	4.347 ± 0.563	—
HGB, g/L#	54.831 ± 65.599	130.452 ± 18.130	—
Lymphocyte counts, 10^9/L#	7.643 ± 2.164§	1.784 ± 0.555	—
MPV, #	9.871 ± 0.792§	10.192 ± 0.971	—

*$P < 0.01$ versus normals; §$P < 0.05$ versus no end-stage PDR; *$P > 0.05$ versus normal.
#Continuous variables are presented as the mean with the standard deviation.

in Table 1. The results from univariate logistic regression models demonstrated that SBP, DBP, duration of diabetes, family history of DM, PAI-1, FBG, MPV, CREA, and urine albumin are associated with the end-stage DR (Table 2). The results of multivariate logistic regression models demonstrated that patients who had a family history of T2DM, history of active smoking and passive smoking, and longer duration of diabetes were more likely to have end-stage DR (Table 3).

A striking finding from this study was that plasma levels of PAI-1 were an independent predictor of end-stage PDR in Northern Chinese Han patients with T2DM (Table 3). In our results, plasma levels of PAI-1 in patients with end-stage PDR were significantly higher than in patients without end-stage PDR or normals (Table 1). This is consistent with the fact that PAI-1 which is a risk factor for DR may serve as a useful marker of increased DR susceptibility [31]. Yan et al. reported that PAI-1 and other inflammatory mediators can lead to retinal ischemia-reperfusion injury (RIRI) [19] which is associated with oxidative stress. We therefore inferred that there might be a link between the activity of PAI-1 and oxidative stress in ocular tissues under hyperglycemic conditions. Consequently, elevated PAI-1 levels, a marker for underlying endothelial dysfunction, may be linked to an increased risk for diabetes [32] and might be a marker of diabetic microvascularization [31]. On the contrary, higher plasma levels of PAI-1 are reported to be independently associated with a lower risk of retinopathy [33]. Besides, Tarnow et al. reported that PAI-1 does not contribute to genetic susceptibility to DR [34]. The conflicting results between our studies and theirs might be related to differences in nationalities and the methodologies. Whether elevated PAI-1 levels are a significant biomarker of end-stage PDR must be testified by larger studies with subjects from different populations in the future.

The results of this study also suggested that family history of diabetes, smoking, and the duration of DM were independent risk factors of end-stage PDR in Northern Chinese Han patients with T2DM (Table 3). These are consistent with the fact that the previous studies reported [9, 35–40]. Therefore, genetic factors which were not measured easily might be an important risk factor of end-stage PDR. We also infer that smoking cessation and a reduction in exposure to smoke in people with T2DM may reduce the development of PDR and the onset of end-stage PDR. Besides, we consider that the length of duration of diabetes might predict the severity of PDR.

Table 2: Univariate analysis of the progress of PDR.

	OR	P value	95% CI	
SBP	1.027	0.007	1.007	1.047
DBP	1.034	0.031	1.003	1.065
Duration of diabetes	1.914	$P < 0.001$	1.872	1.957
Family history of DM	7.446	$P < 0.001$	3.701	14.982
PAI-1	1.607	$P < 0.001$	1.329	1.942
FBG	0.869	0.017	0.775	0.975
PBG	0.948	0.115	0.888	1.013
TC	0 .932	0.588	0.723	1.202
TG	1.118	0.172	0.953	1.312
CREA	0.993	0.019	0.987	0.999
BUN	1.079	0.129	0.978	1.191
Urine albumin	0.734	0.004	0.594	0.907
WBC	1.068	0.406	0.914	1.249
REC	1.014	0.961	0.569	1.808
MPV	0.650	0.008	0.473	0.894
History of hypertension	0.933	0.802	0.545	1.600
Smoking	1.298	0.073	1.291	1.305

Table 3: Multivariate analysis of the progress of PDR.

	OR	P	95% CI	
Family history of DM	28.346	$P < 0.001$	5.554	144.678
Log value of PAI-1	3.113	0.001	3.066	3.169
Smoking	2.246	0.023	2.125	2.373
Duration of diabetes	1.748	$P < 0.001$	1.626	1.871
Urine albumin	0.141	0.103	0.013	1.484
CREA	0.937	0.001	0.902	0.973
FBG	0.573	0.001	0.690	1.320
MPV	1.393	0.520	0.409	0.803
SBP	1.024	0.383	0.971	1.080
DBP	1.135	0.051	1.029	1.251

The first limitation of this study is that all patients were consecutively but not randomly selected. Our findings need further confirmation from larger numbers of randomly sampled cases. Second, the medications and treatment of the patients were not recorded in this study because medication and treatment conditions were not clear for some of the patients. In addition, the difference in nationalities and/or methods might have biased the results of this study to some extent relative to other studies.

In conclusion, our data suggested that PAI-1 levels are an independent risk factor of end-stage PDR. Because PAI-1 is associated with RIRI, which is associated with oxidative stress, we infer that targeting PAI-1 might prevent excessive oxidative stress in ocular tissues. Furthermore, assessing T2DM based on these identified independent risk factors, including PAI-1 levels, family history of T2DM, smoking, and duration of diabetes, might be a promising strategy to adopt to prevent the progression of PDR. Our results might provide novel approaches to the issue of preserving vision and preventing blindness.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by a Grant from the Tianjin Science and Technology Project, China (no. 08ZCGYSF01700). The authors thank all the editors and anonymous reviewers in relation to this paper in the course of its preparation.

References

[1] N. Sieu, W. Katon, E. H. B. Lin, J. Russo, E. Ludman, and P. Ciechanowski, "Depression and incident diabetic retinopathy: a prospective cohort study," *General Hospital Psychiatry*, vol. 33, no. 5, pp. 429–435, 2011.

[2] J. Stevens, K. P. Truesdale, E. G. Katz, and J. Cai, "Impact of body mass index on incident hypertension and diabetes in Chinese Asians, American Whites, and American Blacks:

The People's Republic of China study and the atherosclerosis risk in communities study," *American Journal of Epidemiology*, vol. 167, no. 11, pp. 1365–1374, 2008.

[3] T. Y. H. Wong, P. Poon, C. C. Szeto, J. C. N. Chan, and P. K. T. Li, "Association of plasminogen activator inhibitor-1 4G/4G genotype and type 2 diabetic nephropathy in Chinese patients," *Kidney International*, vol. 57, no. 2, pp. 632–638, 2000.

[4] B. Li, S. Liu, L. Miao, and L. Cai, "Prevention of diabetic complications by activation of Nrf2: diabetic cardiomyopathy and nephropathy," *Experimental Diabetes Research*, vol. 2012, Article ID 216512, 7 pages, 2012.

[5] A. Gumieniczek, B. Owczarek, and B. Pawlikowska, "Oxidative/ nitrosative stress and protein damages in aqueous humor of hyperglycemic rabbits: effects of two oral antidiabetics, pioglitazone and repaglinide," *Experimental Diabetes Research*, vol. 2012, Article ID 653678, 2012.

[6] M. Y. Chun, H. S. Hwang, H. Y. Cho et al., "Association of vascular endothelial growth factor polymorphisms with nonproliferative and proliferative diabetic retinopathy," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 7, pp. 3547–3551, 2010.

[7] D. Galetović, D. Karlica, K. Bućan, and L. Znaor, "The role of clinical and metabolic factors in the genesis and development of diabetic retinopathy," *Acta Medica Croatica*, vol. 62, no. 1, pp. 23–27, 2008.

[8] J. Esteves, A. F. Laranjeira, M. F. Roggia et al., "Diabetic retinopathy risk factors," *Arquivos Brasileiros de Endocrinologia e Metabologia*, vol. 52, no. 3, pp. 431–441, 2008.

[9] Z. L. Zhong, M. Han, and S. Chen, "Risk factors associated with retinal neovascularization of diabetic retinopathy in type 2 diabetes mellitus," *International Journal of Ophthalmology*, vol. 4, no. 2, pp. 182–185, 2011.

[10] H. A. Van Leiden, J. M. Dekker, A. C. Moll et al., "Risk factors for incident retinopathy in a diabetic and nondiabetic population: The Hoorn study," *Archives of Ophthalmology*, vol. 121, no. 2, pp. 245–251, 2003.

[11] T. C. Rodrigues, M. Pecis, M. J. Azevedo, and J. L. Gross, "Blood pressure homeostasis and microvascular complications in diabetic patients," *Arquivos Brasileiros de Endocrinologia e Metabologia*, vol. 49, no. 6, pp. 882–890, 2005.

[12] X. L. Cai, F. Wang, and L. N. Ji, "Risk factors of diabetic retinopathy in type 2 diabetic patients," *Chinese Medical Journal*, vol. 119, no. 10, pp. 822–826, 2006.

[13] C. B. Leitão, L. H. Canani, S. P. Silveiro, and J. L. Gross, "Ambulatory blood pressure monitoring and type 2 diabetes mellitus.," *Arquivos Brasileiros de Cardiologia*, vol. 89, no. 5, pp. 315–354, 2007.

[14] X. W. Xie, L. Xu, Y. X. Wang, and J. B. Jonas, "Prevalence and associated factors of diabetic retinopathy. The Beijing Eye Study 2006," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 246, no. 11, pp. 1519–1526, 2008.

[15] J. Li and Y. H. Hu, "Susceptibility genes for diabetic retinopathy," *International Ophthalmology*, vol. 2, no. 1, pp. 1–6, 2009.

[16] X. W. Xie, L. Xu, Y. X. Wang, and J. B. Jonas, "Prevalence and associated factors of diabetic retinopathy. The Beijing Eye Study 2006," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 246, no. 11, pp. 1519–1526, 2008.

[17] K. J. Yang, C. C. Sun, W. C. Ku et al., "Axial length and proliferative diabetic retinopathy," *Optometry & Vision Science*, vol. 89, pp. 465–470, 2012.

[18] M. Follo and D. Ginsburg, "Structure and expression of the human gene encoding plasminogen activator inhibitor, PAI-1," *Gene*, vol. 84, no. 2, pp. 447–453, 1989.

[19] S. F. Yan, D. J. Pinsky, N. Mackman, and D. M. Stern, "Egr-1: is it always immediate and early?" *Journal of Clinical Investigation*, vol. 105, no. 5, pp. 553–554, 2000.

[20] H. Liao, M. C. Hyman, D. A. Lawrence, and D. J. Pinsky, "Molecular regulation of the PAI-1 gene by hypoxia: contributions of Egr-1, HIF-1α, and C/EBPα," *FASEB Journal*, vol. 21, no. 3, pp. 935–949, 2007.

[21] R. Kahn, "Report of the expert committee on the diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 20, no. 7, pp. 1183–1197, 1997.

[22] R. Klein, M. D. Knudtson, K. E. Lee, R. Gangnon, and B. E. K. Klein, "The Wisconsin Epidemiologic Study of Diabetic Retinopathy XXII. The twenty-five-year progression of retinopathy in persons with type 1 diabetes," *Ophthalmology*, vol. 115, no. 11, pp. 1859–1868, 2008.

[23] M. D. Davis, M. R. Fisher, R. E. Gangnon et al., "Risk factors for high-risk proliferative diabetic retinopathy and severe visual loss: early treatment diabetic retinopathy study report 18," *Investigative Ophthalmology and Visual Science*, vol. 39, no. 2, pp. 233–252, 1998.

[24] V. A. Koivisto, L. K. Stevens, M. Mattock et al., "Cardiovascular disease and its risk factors in IDDM in Europe," *Diabetes Care*, vol. 19, no. 7, pp. 689–697, 1996.

[25] L. Zhang, G. C. Curhan, F. B. Hu, E. B. Rimm, and J. P. Forman, "Association between passive and active smoking and incident type 2 diabetes in women," *Diabetes Care*, vol. 34, no. 4, pp. 892–897, 2011.

[26] Y. T. Kruszynska, J. G. Yu, J. M. Olefsky, and B. E. Sobel, "Effects of troglitazone on blood concentrations of plasminogen activator inhibitor 1 in patients with type 2 diabetes and in lean and obese normal subjects," *Diabetes*, vol. 49, no. 4, pp. 633–639, 2000.

[27] M. Ugajin, K. Yamaki, N. Iwamura, T. Yagi, and T. Asano, "Blood urea nitrogen to serum albumin ratio independently predicts mortality and severity of community- acquired pneumonia," *International Journal of General Medicine*, vol. 5, pp. 583–589, 2012.

[28] J. Závada, E. Hoste, R. Cartin-Ceba et al., "A comparison of three methods to estimate baseline creatinine for RIFLE classification," *Nephrology Dialysis Transplantation*, vol. 25, no. 12, pp. 3911–3918, 2010.

[29] G. T. C. Ko, H. P. S. Wai, and J. S. F. Tang, "Effects of age on plasma glucose levels in non-diabetic Hong Kong Chinese," *Croatian Medical Journal*, vol. 47, no. 5, pp. 709–713, 2006.

[30] S. Wang, L. Xu, J. B. Jonas, Y. X. Wang, Q. S. You, and H. Yang, "Dyslipidemia and eye diseases in the adult Chinese population: The Beijing Eye Study," *PLoS One*, vol. 7, no. 3, article e26871, 2012.

[31] I. Ezzidi, N. Mtiraoui, M. Chaieb, M. Kacem, T. Mahjoub, and W. Y. Almawi, "Diabetic retinopathy, PAI-1 4G/5G and -844G/A polymorphisms, and changes in circulating PAI-1 levels in Tunisian type 2 diabetes patients," *Diabetes and Metabolism*, vol. 35, no. 3, pp. 214–219, 2009.

[32] J. B. Meigs, J. Dupuis, C. Liu et al., "PAI-1 gene 4G/5G polymorphism and risk of type 2 diabetes in a population-based sample," *Obesity*, vol. 14, no. 5, pp. 753–758, 2006.

[33] L. Brazionis, K. Rowley, A. Jenkins, C. Itsiopoulos, and K. O'Dea, "Plasminogen activator inhibitor-1 activity in type 2

diabetes: a different relationship with coronary heart disease and diabetic retinopathy," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 4, pp. 786–791, 2008.

[34] L. Tarnow, C. D. A. Stehouwer, J. J. Emeis et al., "Plasminogen activator inhibitor-1 and apolipoprotein E gene polymorphisms and diabetic angiopathy," *Nephrology Dialysis Transplantation*, vol. 15, no. 5, pp. 625–630, 2000.

[35] D. M. Hallman, J. C. Huber, V. H. Gonzalez, B. E. K. Klein, R. Klein, and C. L. Hanis, "Familial aggregation of severity of diabetic retinopathy in Mexican Americans from Starr County, Texas," *Diabetes Care*, vol. 28, no. 5, pp. 1163–1168, 2005.

[36] Diabetes Control and Complications Trial Research Group, "Clustering of long-term complications in families with diabetes in the Diabetes Control and Complications Trial," *Diabetes*, vol. 46, pp. 1829–1839, 1997.

[37] L. Zhang, G. C. Curhan, F. B. Hu, E. B. Rimm, and J. P. Forman, "Association between passive and active smoking and incident type 2 diabetes in women," *Diabetes Care*, vol. 34, no. 4, pp. 892–897, 2011.

[38] M. Bamashmus, A. Gunaid, and R. Khandekar, "Diabetic retinopathy, visual impairment and ocular status among patients with diabetes mellitus in Yemen: a hospital-based study," *Indian Journal of Ophthalmology*, vol. 57, no. 4, pp. 293–298, 2009.

[39] P. K. Rani, R. Raman, A. Chandrakantan, S. S. Pal, G. M. Perumal, and T. Sharma, "Risk factors for diabetic retinopathy in self-reported rural population with diabetes," *Journal of Postgraduate Medicine*, vol. 55, no. 2, pp. 92–96, 2009.

[40] I. P. Chatziralli, T. N. Sergentanis, P. Keryttopoulos, N. Vatkalis, A. Agorastos, and L. Papazisis, "Risk factors associated with diabetic retinopathy in patients with diabetes mellitus type 2," *BMC Research Notes*, vol. 3, pp. 153–156, 2010.

Hindawi Publishing Corporation
Experimental Diabetes Research
Volume 2012, Article ID 168602, 31 pages
doi:10.1155/2012/168602

Research Article

Differential Proteome Profiling Using iTRAQ in Microalbuminuric and Normoalbuminuric Type 2 Diabetic Patients

Jonghwa Jin,[1] Yun Hyi Ku,[2] Yikwon Kim,[1] Yeonjung Kim,[1] Kyunggon Kim,[1] Ji Yoon Lee,[3] Young Min Cho,[2] Hong Kyu Lee,[2] Kyong Soo Park,[2,4] and Youngsoo Kim[1]

[1] *Department of Biomedical Sciences, Seoul National University College of Medicine, 28 Yongon-Dong, Seoul 110-799, Republic of Korea*
[2] *Department of Internal Medicine, Seoul National University College of Medicine, 28 Yongon-Dong, Seoul 110-799, Republic of Korea*
[3] *National Instrumentation Center for Environmental Management, Seoul National University, Seoul 151-921, Republic of Korea*
[4] *Genome Research Center for Diabetes and Endocrine Disease, Seoul National University College of Medicine, 28 Yongon-Dong, Seoul 110-799, Republic of Korea*

Correspondence should be addressed to Kyong Soo Park, kspark@snu.ac.kr and Youngsoo Kim, biolab@snu.ac.kr

Received 2 July 2011; Revised 27 October 2011; Accepted 22 November 2011

Academic Editor: K. Herbert

Diabetic nephropathy (DN) is a long-term complication of diabetes mellitus that leads to end-stage renal disease. Microalbuminuria is used for the early detection of diabetic renal damage, but such levels do not reflect the state of incipient DN precisely in type 2 diabetic patients because microalbuminuria develops in other diseases, necessitating more accurate biomarkers that detect incipient DN. Isobaric tags for relative and absolute quantification (iTRAQ) were used to identify urinary proteins that were differentially excreted in normoalbuminuric and microalbuminuric patients with type 2 diabetes where 710 and 196 proteins were identified and quantified, respectively. Some candidates were confirmed by 2-DE analysis, or validated by Western blot and multiple reaction monitoring (MRM). Specifically, some differentially expressed proteins were verified by MRM in urine from normoalbuminuric and microalbuminuric patients with type 2 diabetes, wherein alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, and prostate stem cell antigen had excellent AUC values (0.849, 0.873, and 0.825, resp.). Moreover, we performed a multiplex assay using these biomarker candidates, resulting in a merged AUC value of 0.921. Although the differentially expressed proteins in this iTRAQ study require further validation in larger and categorized sample groups, they constitute baseline data on preliminary biomarker candidates that can be used to discover novel biomarkers for incipient DN.

1. Introduction

Diabetes mellitus is a chronic disease with potentially devastating complications. For example, diabetes mellitus is associated with macrovascular complications, such as cardiovascular and cerebrovascular diseases, and microvascular complications, including diabetic nephropathy (DN) and retinopathy [1]. DN is a long-term complication of diabetes that is caused by specific renal structural alterations, such as mesangium expansion due to the progressive accumulation of extracellular matrix (ECM), and by functional losses, such as elevated glomerular basement membrane (GBM) permeability [2].

DN occurs in 15% to 25% of type 1 diabetic patients and 30% to 40% of type 2 diabetic patients [3] and accounts for approximately one-half of all new cases of end-stage renal disease (ESRD). Furthermore, ESRD has a 5-year survival rate of only 21% [4]. Because the progression of ESRD in DN is irreversible, the early diagnosis of DN is necessary to prevent or delay progression to ESRD [5]. Microalbuminuria represents a potentially reversible incipient stage of nephropathy and is used as a noninvasive index for the detection of diabetic renal disease. Microalbuminuria is defined as a state in which abnormal amounts of albumin are excreted in urine (30–300 mg/24 h versus <30 mg/24 h in normoalbuminuria) [5, 6].

The use of microalbuminuria to predict incipient DN, particularly in type 2 diabetic patients, is limited for several reasons [7]: the microalbuminuric state also predicts cardiovascular disease in diabetic and nondiabetic individuals [8, 9], and it is associated with inflammation and hypertension [5]. Consequently, the likelihood of detecting nondiabetic renal disease or normal glomerular structure is observed with microalbuminuria patients [10]. Thus, more accurate biomarkers for incipient DN in type 2 diabetic patients are required that can differentiate incipient DN from other conditions in microalbuminuria patients, including cardiovascular disease, inflammation, and hypertension.

Recently, to compare DN patients with non-DN patients, proteomic technologies have been developed to identify urinary marker candidates that are associated with the development of DN. Various proteomic approaches have been used for this purpose, including 2-DE, 2-DE DIGE, and SELDI-TOF [5, 11, 12]. However, because many studies have focused on restricted sets of targeted proteins, alterations in comprehensive urinary protein profiles in type 2 diabetes have not been monitored. In particular, SELDI-TOF has been shown to be a valuable technology for urinary proteomic analysis, but the absolute identification of differentially excreted proteins remains challenging [13].

To scan a comprehensive differential proteome for preliminary DN candidate biomarkers, we used a 4-plex isobaric tag for relative and absolute quantification (iTRAQ, 4-plex), allowing us to identify and quantify proteins in up to 4 samples [14]. The advantages of iTRAQ include whole labeling of representative or pooled samples, comparatively high throughput, and retention of posttranslational modification (PTM) data; one of its shortcomings is that it cannot be applied easily to a large collection of individual clinical samples due to reagent cost and the required mass spectrometry effort [15]. To date, iTRAQ has been applied to a variety of sample sets, including *E. coli*, mammalian cells, yeast, plant cells, and human biological fluids [16–22].

Therefore, in this study, we used iTRAQ to identify and quantify differentially excreted urinary proteins in microalbuminuric versus normoalbuminuric type 2 diabetic patients and investigate the associations that would reflect the progress of DN. Afterward, those differentially excreted urinary proteins have been confirmed by 2-DE, followed by MALDI-TOF/TOF, or validated by Western blot and MRM.

2. Materials and Methods

2.1. Urine Sample Preparation. Type 2 diabetic subjects (age ≥ 40 years) with or without microalbuminuria who were patients at the Diabetes Center of Seoul National University Hospital, Seoul, Republic of Korea, were enrolled in 2006. Microalbuminuria patients were randomly selected out of these outpatients, whereas normoalbuminuric patients were selected to be matched to age, sex, body mass index (BMI), and DM duration with microalbuminuric patients.

Forty-three subjects with diabetic retinopathy and persistent microalbuminuria formed the microalbuminuria group (MA). Persistent microalbuminuria was defined as an albumin : creatinine ratio (ACR) between 30 and 300 mg/g in 2 urine samples that were taken over 3 months. The normoalbuminuria group (NA) comprised subjects who had no diabetic retinopathy, did not use angiotensin inhibitors or angiotensin receptor blockers that lowered albuminuria, and showed no microalbuminuria in their urine in the past year (urinary albumin < 30 mg/g creatinine). Forty-three subjects formed the NA group.

There were no significant differences in age, sex, body mass index, or diabetes mellitus duration between the 2 study groups. Subjects with hematuria, uncontrolled hypertension (blood pressure ≥ 140/90 mm Hg), uncontrolled hyperglycemia (glycated hemoglobin A1c ≥ 8.5%), urinary tract infection, acute febrile illness, congestive heart failure, or malignancy were excluded. Individuals who were receiving peroxisome proliferator-activated receptor gamma agonists were also excluded. Midstream urine of spot urine samples were collected in sterile 50-mL tubes that contained 50 μL 0.1 mM PMSF (serine protease inhibitor) and 500 μL 1 mM sodium azide from 86 patients and were stored at −80°C until use. Informed consent was obtained from all subjects after obtaining approval for the study from the Institutional Review Board at Seoul National University Hospital.

Urine albumin and creatinine were measured in spot urine samples by immunoturbidimetric method using the TIA Micro Alb Kit (Nittobo, Tokyo, Japan) and enzymatic creatinine assay (Roche, Mannheim, Germany), respectively, on a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan).

For the iTRAQ and 2-DE experiments, pooled urine samples, based on average albumin-to-creatinine ratios, were used; the clinical characteristics of the study subjects are summarized in Table 1. Because the protein concentration of each urine sample varied widely, depending on the urine volume in the morning, equal amounts of total protein from each patient were pooled to prepare the urine samples (NA1–NA4 and MA1–MA4).

To prepare the protein samples, approximately 50 mL aliquots of normoalbuminuric and microalbuminuric urine were centrifuged at 3000 g for 30 min at 4°C. Supernatants were filtered through a 0.22 μm MILLEX GP membrane (Millipore, Carrigtwohill, Cork, Ireland) and concentrated to 3 mL in an Amicon ultrafiltration cell (YM2, 3 kDa MW cut-off, Millipore). The concentrated urine samples were then desalted by dialysis twice using a Slide-A-Lyzer dialysis cassette kit (3.5 kDa, Pierce, Rockford, ILUSA) against 1000 volumes of distilled water, containing 0.1 mM PMSF (serine protease inhibitor) and 1 mM β-ME, at 4°C. Proteins in the dialyzed urine were precipitated with 5 volumes of acetone for 4 hrs at −20°C, and the resulting pellets were washed 3 times with cold acetone; the supernatants were discarded.

2.2. Labeling with iTRAQ Reagents. Aliquots of 100 μg of protein were reduced, alkylated, digested, and labeled according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Briefly, 1 μL of denaturant (2% SDS) and 1 μL of reducing reagent (50 mM tris-[2-carboxyethyl] phosphine) were added to each sample and incubated for 1 hr at 60°C. Each sample was allowed to cool at room temperature, and 1 μL of cysteine blocking reagent (200 mM methyl methanethiosulfonate (MMTS) in isopropanol) was

Table 1: Clinical characteristics of normoalbuminuric (NA) and microalbuminuric (MA) type 2 diabetic patients.

Characteristics	NA 1[a] ($n = 9$)	MA 1[a] ($n = 9$)	NA 2[b] ($n = 9$)	MA 2[b] ($n = 9$)	NA 3[c] ($n = 9$)	MA 3[c] ($n = 9$)	NA 4[d] ($n = 16$)	MA 4[d] ($n = 16$)
Gender (M/F)	4/5	5/4	5/4	4/5	5/4	4/5	9/7	9/7
Mean age (years)	62.4 ± 8.0 (49–72)	66.4 ± 7.8 (55–82)	60.7 ± 4.7 (54–67)	61.9 ± 2.7 (56–65)	64.9 ± 5.3 (56–73)	62.3 ± 4.4 (41–72)	63.7 ± 7.5 (49–72)	63.2 ± 9.8 (44–82)
Duration of diabetes (years)	9.1 ± 4.4	10.4 ± 7.0	8.7 ± 5.0	7.3 ± 4.7	13.0 ± 9.0	10.6 ± 7.7	9.9 ± 4.8	11.6 ± 7.3
BMI (kg/m^2)	25.4 ± 3.0	24.9 ± 3.3	23.8 ± 2.1	24.9 ± 3.6	23.1 ± 2.7	22.6 ± 2.6	24.4 ± 2.9	25.1 ± 2.9
Fasting plasma glucose (mg/dL)	130.8 ± 21.1	133.8 ± 36.4	131.3 ± 31.9	135.8 ± 41.3	117.8 ± 21.7	117.6 ± 26.6	132.4 ± 19.2	144.8 ± 35.7
HbA1C (%)	6.8 ± 0.7	6.8 ± 0.9	6.9 ± 0.7	7.4 ± 0.8	7.2 ± 0.7	7.2 ± 0.7	6.8 ± 0.6	7.0 ± 0.8
Blood urea nitrogen (mg/dL)	15.1 ± 4.8	17.1 ± 4.9	13.8 ± 2.4	15.7 ± 3.9	16.8 ± 3.4	17.7 ± 4.4	15.1 ± 3.9	16.4 ± 4.1
Serum creatinine (mg/dL)	1.0 ± 0.1	1.1 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	0.98 ± 0.13	1.0 ± 0.15
Serum total cholesterol (mg/dL)	184.4 ± 34.5	180.6 ± 29.1	164.2 ± 25.5	165.6 ± 24.5	164.9 ± 22.2	175.4 ± 50.7	181.5 ± 29.7	182.1 ± 25.5
Serum HDL cholesterol (mg/dL)	48.1 ± 12.2	46.1 ± 8.8	52.3 ± 9.5	48.9 ± 8.3	55.0 ± 7.8	41.5 ± 4.7	46.4 ± 10.8	46.8 ± 7.7
Serum LDL cholesterol (mg/dL)	99.8 ± 26.6	102.6 ± 22.3	91.0 ± 21.6	92.0 ± 27.6	85.9 ± 20.3	98.8 ± 30.0	102.0 ± 23.4	104.1 ± 19.7
Serum triglycerides (mg/dL)	119.9 ± 37.0	158.6 ± 55.9	132.9 ± 66.1	177 ± 134.7	128.3 ± 82.8	186.3 ± 66.8	126.5 ± 56.5	145.4 ± 51.6
Albumin : creatinine ratio (mg/g)	12.2 ± 7.1	120.5 ± 70.7[e]	10.0 ± 3.6	82.6 ± 41.9[f]	8.8 ± 1.6	86.1 ± 47.1[g]	9.8 ± 6.9	107.4 ± 69.4[h]
Pooled urine concentration (mg/mL)	3.7 ± 1.6	6.9 ± 3.4	4.1 ± 1.2	8.1 ± 4.2	4.7 ± 1.2	7.6 ± 3.8	4.3 ± 1.8	7.1 ± 4.5

[a–c] Sample sets for the three iTRAQ experiments, [d] sample sets for 2-DE, [e] $P < 0.001$ for NA1 versus MA1, [f] $P < 0.001$ for NA2 versus MA2, [g] $P < 0.05$ for NA3 versus MA3, and [h] $P < 0.001$ for NA4 versus MA4. Data are expressed as the mean ± SD.

added and incubated for 20 min at room temperature. The tubes were digested with trypsin (Promega, Madison, WI, USA) at a protein-to-enzyme ratio of 10 : 1 at 37°C overnight, and the contents of one vial of iTRAQ reagent, dissolved in 70 μL of ethanol, were added to each peptide mixture and incubated for 1 hr at room temperature.

In this study, 3 iTRAQ experiments were performed. The detailed iTRAQ labeling strategy is summarized for the specified NA/MA urine samples in Figure 1 and Table 1; iTRAQ Experiments 1, 2, and 3 were performed for labeling (a) and (b), (c) and (d), and (e), respectively. Each normoalbuminuric peptide was labeled with iTRAQ reagents 114, 115, and 116, and the microalbuminuric peptide was labeled with iTRAQ reagents 115 and 117 (Figure 1). The 2 sample sets (microalbuminuric and normoalbuminuric) were combined and dried. To analyze the proteome quantitatively using iTRAQ labeling, we determined the labeling efficiency, as described [23]; the number of possible labeling sites (the N-termini of all peptides and lysine side chains) in 21,610 peptides were compared manually with that of completely labeled sites, represented by the Pro GroupTM Algorithm in ProteinPilot.

2.3. Strong Cation Exchange Chromatographic Fractionation. iTRAQ-labeled samples were subjected to LC-MS/MS at the National Instrumentation Center for Environmental Management, Seoul National University, and fractionated using strong cation exchange (SCX) chromatography, as follows. Dried samples were reconstituted in 500 μL of buffer A (25% v/v acetonitrile (ACN) and 5 mM ammonium formate, adjusted to pH 2.7 with formic acid) and loaded onto a PolySULFOETHYL A column (4.6 mm id $\times$ 100 mm, 5 μm, 200 Å; PolyLC, Columbia, MD, USA) in a HP1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA). The column was equilibrated for 5 min in buffer A, and the peptides were eluted using a gradient of 0% to 30% buffer B (25% v/v ACN and 1 M ammonium formate [pH 3] with formic acid) over 80 min and 30% to 90% buffer B for 40 min at a flow rate of 0.7 mL/min. Absorbance was monitored at 280 nm, and the fractions were collected every 2 min after injection.

2.4. LC-MS/MS Analysis. Fractions were reconstituted in solvent A and injected into an LC-ESI-MS/MS system. LC-MS/MS was performed using an integrated system, which consisted of an autosampler switching pump and a micropump (Tempo Nano LC system; Applied Biosystems) with a hybrid quadrupole-TOF LC-MS/MS spectrometer (QStar Elite; Applied Biosystems) that was equipped with a nanoelectrospray ionization source (Applied Biosystems) and fitted with a 10 μm fused silica emitter tip (New Objective, Woburn, MA, USA).

Peptides were first trapped on a Zorbax 300SB-C18 trap column (300 μm id $\times$ 5 mm, 5 μm, 100 Å; Agilent Technologies), washed for 10 min with 98% solvent A (water/ACN [98 : 2 v/v] and 0.1% formic acid) and 2% solvent B (water/ACN [2 : 98 v/v] and 0.1% formic acid) at a flow rate of 10 μL/min, and separated on a Zorbax 300SB-C18 capillary column (75 μm id $\times$ 150 mm, 3.5 μm, 100 Å) at a flow rate of 300 nL/min. The LC gradient was run at 2% to 35% solvent B over 120 min and from 35% to 90% over 10 min, followed by 90% solvent B for 15 min, and finally 5% solvent B for 35 min. The resulting peptides were electrosprayed through a coated silica tip (New Objective) at an ion spray voltage of 2300 eV.

For data acquisition, the mass spectrometer was set in the positive ion mode at a selected mass range of 400–1600 *m/z* for a 1 sec TOF-MS survey scan to detect precursor ions. The 5 most abundant peptides (count >20) with charge states of +2 to +4 were selected to perform the information-dependent acquisition (IDA) of MS/MS data. Once selected, the precursor ions were dynamically excluded for 60 sec at a mass tolerance of 100 ppm.

2.5. Data Analysis. Data file processing, protein identification, and relative abundance quantification were performed using ProteinPilot v.2.0.1 (Applied Biosystems; MDS-Sciex, Concord, Canada) and the Paragon algorithm [24]. Database searches were performed against the Celera human database (human KBMS 5.0, 2005-03-02; a total of 187,748 entries provided by Applied Biosystems). The search parameters used were: a peptide and fragment ion mass tolerance of 0.2 Da; 1 missed trypsin cleavage; fixed cysteine modification by MMTS; variable oxidation of methionine; and iTRAQ labeling of the N-termini of peptides and lysine side chain residues.

The confidence threshold for protein identification was an unused ProtScore >1.3 (95% confidence interval). ProteinPilot computes a percentage confidence that reflects the probability that a hit is a false positive; thus, at the 95% confidence level, the false positive identification rate is approximately 5% [24, 25]. Although this program automatically accepts all peptides that have a confidence level >1%, only proteins with at least 1 peptide that had a confidence level >95% were initially recorded. At these low confidence levels, peptides do not identify a single protein by themselves but, rather, support protein identification in the presence of other peptides [24, 25]. Quantification results were reported only when the error factor (EF) was <2, which indicates a standard deviation of quantification <20%.

2.6. GO Ontology Analysis. The "biological process" and "molecular function" classifications were analyzed using PANTHER ID numbers (http://www.pantherdb.org/), provided in the ProteinPilot output when a Celera human database is used. To construct a graphical representation of differentially excreted proteins, MultiExperiment Viewer (Version 4.3) was used, allowing us to generate a "heatmap" of differentially excreted proteomes (http://www.tm4.org/mev/).

2.7. 2-DE Urinary Proteome and PMF Analysis. Urine samples were pooled from 16 type 2 diabetic patients with normoalbuminuria and 16 type 2 diabetic patients with microalbuminuria. The characteristics of the pooled urine samples for 2-DE are shown in Figure 1 and Table 1. For the PMF analysis, a MALDI-TOF/TOF mass spectrometer (ABI 4700 Proteomics Analyzer, Applied Biosystems) was used as described in our previous papers [26, 27].

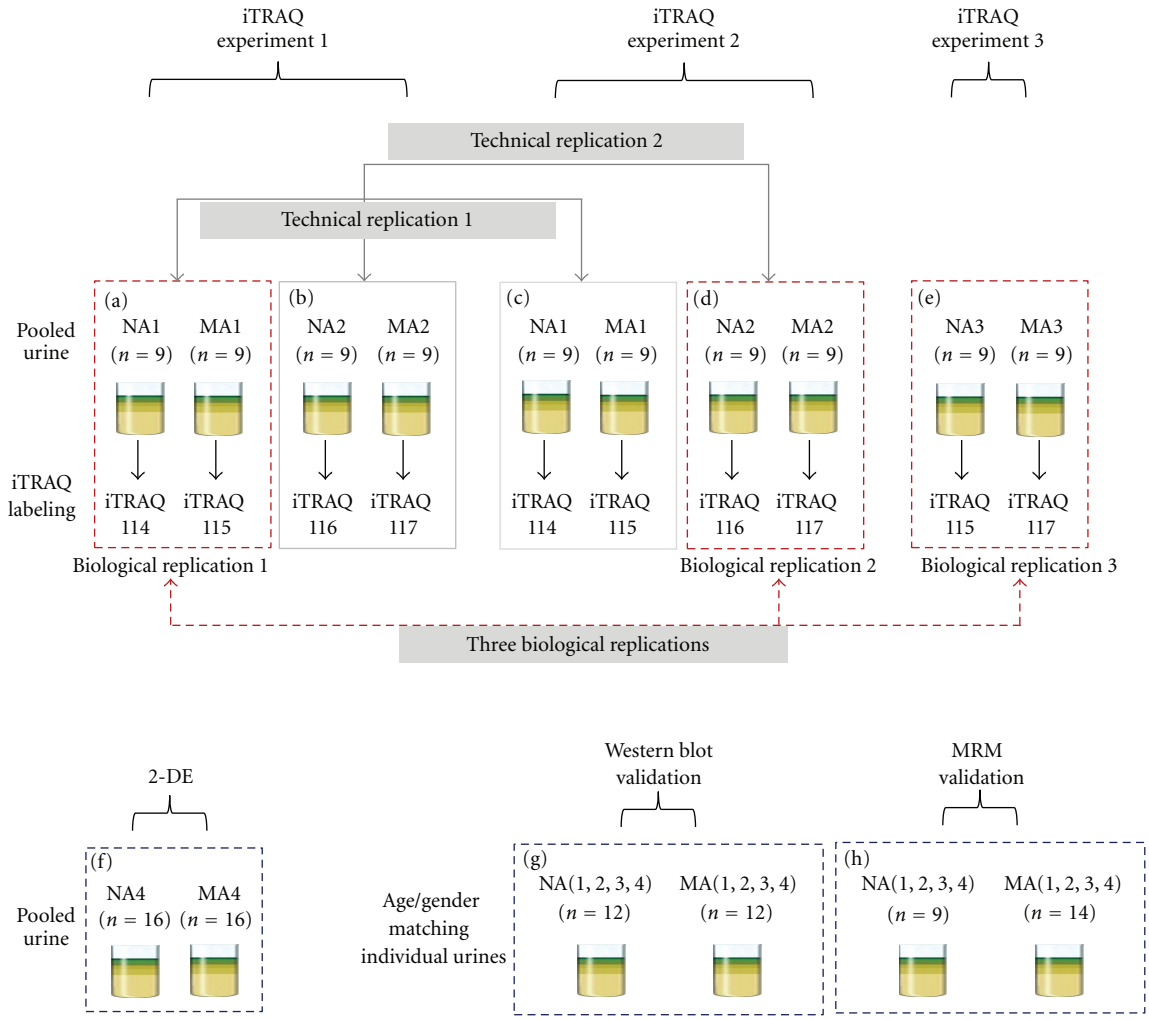


Figure 1: Workflow of iTRAQ, 2-DE, Western blot, and MRM of the urinary proteome. For analysis of the urinary proteome, 3 iTRAQ experiments were performed, 2-DE, Western blot, and MRM were conducted to confirm and validate the iTRAQ results. iTRAQ experiments 1, 2, and 3 were performed, labeled (a) and (b), (c) and (d), and (e), respectively, wherein 3 biological replicates (labeled (a), (d), and (e), resp.), technical replicate 1 (labeled (a) and (c)), and technical replicate 2 (labeled (b) and (d)) were performed in microalbuminuric versus normoalbuminuric urine. 2-DE, Western blot, and MRM analysis of the urinary proteome were conducted using labeled (f), (g), and (h), respectively.

2.8. Western Blot Analysis. Twenty-four urine samples that were matched for gender and age (NA: 6 females and 6 males, and MA: 6 females; 6 males) were selected from the urine sample groups (NA1–NA4 and MA1–MA4, resp.) and subjected to Western blot validation of the 6 representative candidates from the iTRAQ experiments (Figures 1 and 6, and Table 1). The primary antibodies were directed against transferrin (1 : 500, AbFrontier, Seoul, Korea), ceruloplasmin (1 : 1000, AbFrontier), α1-antitrypsin (1 : 1000, AbFrontier), vitamin D-binding protein (1 : 1000, AbFrontier), α1-acid glycoprotein (1 : 2000, AbFrontier), and haptoglobin (1 : 1000, AbFrontier).

2.9. Candidate Validation Using Multiple Reaction Monitoring. In addition to Western blot, multiple reaction monitoring (MRM) was performed to verify the candidate biomarkers using 9 NA and 14 MA urine samples from the urine sample groups (NA1–NA4 and MA1–MA4, resp.) (Figure 1 and Table 1). In our MRM experiment [28], triple quadrupole linear ion trap MS (4000 Qtrap, coupled with a nano Tempo MDLC, Applied Biosystems) was performed; the detailed procedure is previously described [28]. Data were processed using the MultiQuant program (Applied Biosystems, version 1.0), and each peak area of the transitions was normalized to an input internal standard (Q1/Q3 transitions at 542.3/636.3 *m/z* for beta-galactosidase peptide) [28]. In the statistical analysis, receiver operating characteristic (ROC) curves and interactive plots were generated using Medicalc (MedCalc Software, Mariakerke, Belgium, version 10.0.1.0).

3. Results

3.1. Identification of Urinary Proteomes from Normoalbuminuric and Microalbuminuric Patients. For the iTRAQ experiments, 3 biological replicates (biological replicate 1 was

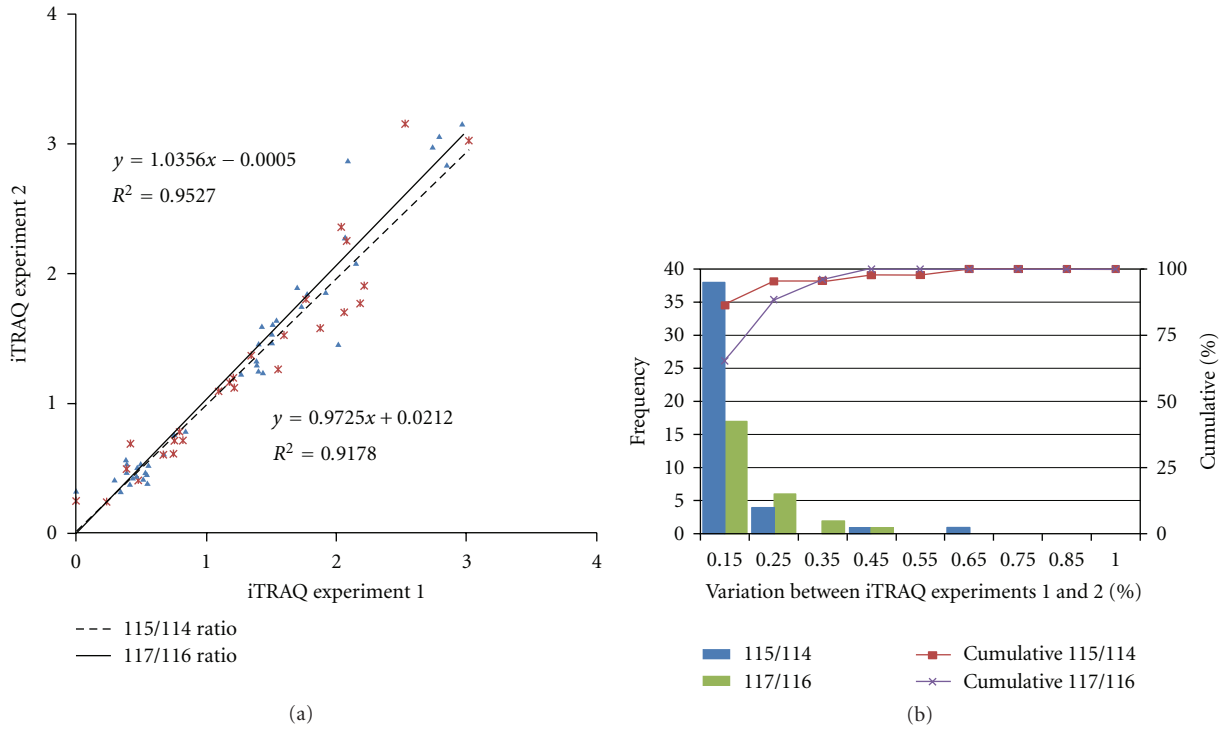


Figure 2: Correlation between the 2 technical replicates and determination of the cutoff value for significant fold changes. (a) Plots of iTRAQ ratios for two technical replicates. Forty-four proteins were commonly observed from technical replicate 1 (labeled 115/114), and 26 proteins were commonly observed from technical replicate 2 (labeled 117/116). These differentially excreted proteins (P value < 0.05, more than two unique peptides: >95%) were plotted in the linear dynamic range. The technical variations yielded a correlation coefficient of r^2 = 0.9527 and r^2 = 0.9178 between iTRAQ experiments 1 and 2, respectively. (b) The % variations for the common proteins from the two technical replicates. The 44 and 26 common proteins from the 2 technical replicates were used as inputs to calculate % variations. The vertical axis represents the number of proteins, and the horizontal axis denotes % variation. Ninety percent of the proteins fell within 25% of the respective experimental variation. Thus, we considered a fold-change of >1.25 or <0.80, a meaningful cutoff that represented actual differences in the iTRAQ experiments.

labeled (a), replicate 2 was labeled (d), and replicate 3 was labeled (e)); 2 technical replicates (technical replicate 1 was labeled (a) and (c), replicate 2 was labeled (b) and (d)) were generated from normoalbuminuric and microalbuminuric urine (Figure 1). The 3 biological replicates were used to profile and quantitate the urinary proteome; the 2 technical replicates were used solely to determine the cutoff for significant fold-changes.

Seven hundred ten proteins were identified from 21,610 peptides of the 3 combined biological replicates at a minimum confidence level of 95% (unused ProtScore > 1.3). Of the proteins that were identified by iTRAQ, 27% comprised 1-peptide proteins; 14% was 2-peptide proteins; 8% was 3-peptide proteins; 5% was 4-peptide proteins; 46% comprised proteins that had 5 or more peptides. In our iTRAQ experiment, 83 proteins (unused ProtScore > 1.3) were common to all 3 biological replicates at a minimum confidence level of 95%, using 3 different pooled urine samples.

3.2. Determination of Cutoff for Significant Fold-Change in iTRAQ Experiments. To generate the quantitative proteome using iTRAQ labeling, we first determined the labeling efficiency, which exceeded 98% (data not shown). Next, the cutoff for significant fold-change was determined based on the 2 technical replicates ((a) and (c) of iTRAQ experiment 1, (b); (d) of iTRAQ experiment 2) (Figure 1). In the 2 replicate experiments, the number of commonly identified proteins was 173 (2 [115/114] ratios from technical replicate 1) and 107 (2 [117/116] ratios from technical replicate 2), and the number of selected proteins was 44 (2 [115/114] ratios from technical replicate 1) and 26 (2 [117/116] ratios from technical replicate 2), which were chosen based on the following criteria: it contained more than 2 unique peptides (>95%), and P value <0.05 for the 115/114 and 117/116 reporter ions. The 70 proteins were used to monitor technical variations and confirm the threshold for meaningful differences.

The technical variations for the 115/114 and 117/116 reporter ions, calculated using the ratios of the 44 and 26 commonly observed proteins between the 2 technical replicates, were r^2 = 0.9527 and r^2 = 0.9178, respectively (Figure 2(a)). Accordingly, 90% of the commonly observed in the technical replicates fell within 25% of the respective experimental variation (Figure 2(b)). Therefore, we set fold-change thresholds of >1.25 or <0.80 to identify true differences between the expression of 115/114 and 116/117 reporter ions, as described in [23].

3.3. Differential Proteomes between Microalbuminuria and Normoalbuminuria. In our iTRAQ study, we obtained diverse biomarker candidates from 3 pooled biological NA/MA

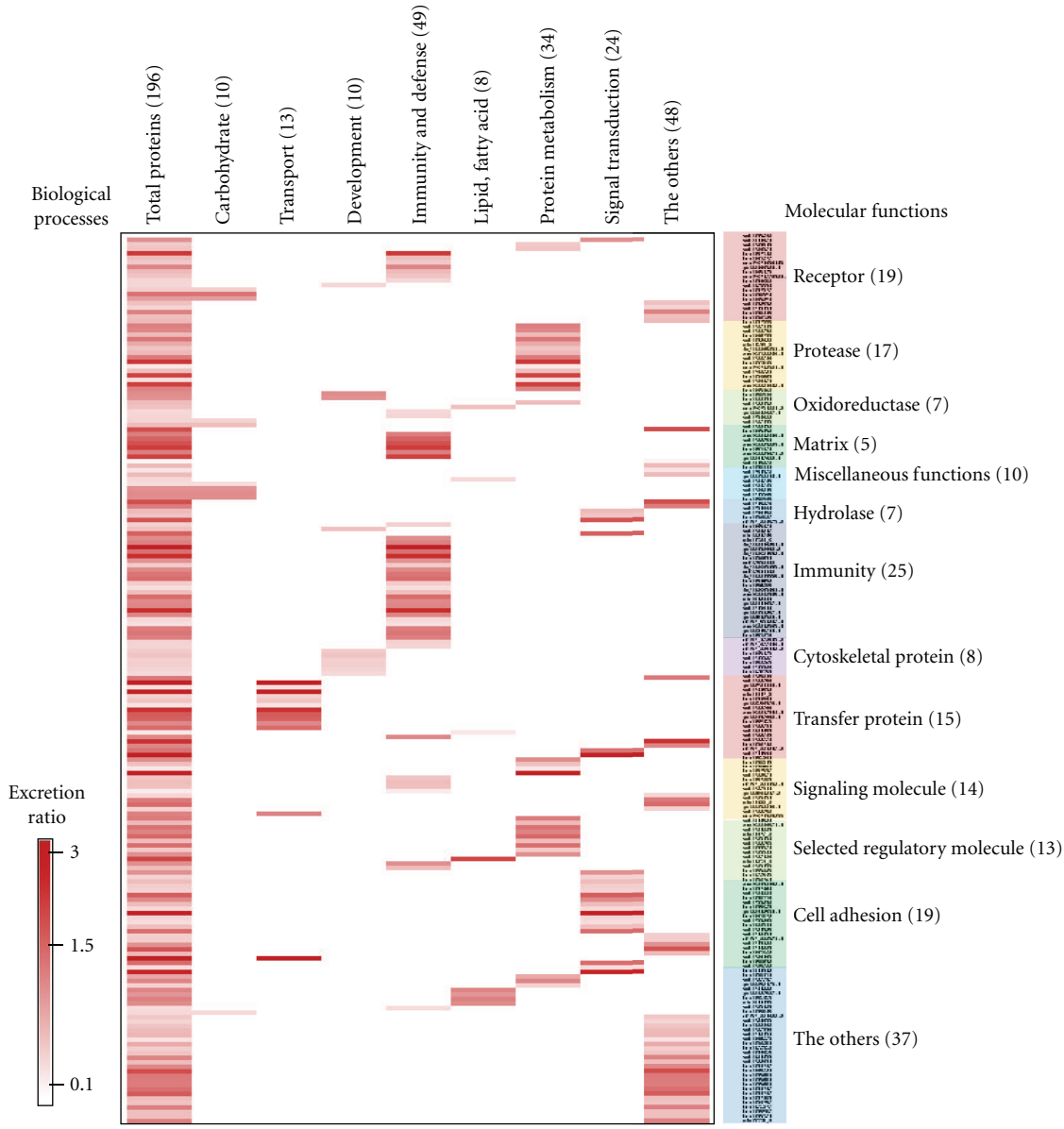


Figure 3: Comprehensive functional annotation of the differentially excreted proteome. The 196 quantitated urinary proteins were annotated for the "biological process" (x-axis) and "molecular function" subcategories (y-axis) in a heatmap. The "biological process" and "molecular function" categories comprised 8 and 13 subcategories, respectively. The 196 quantitated urinary proteins are individually assigned into the "biological process" and "molecular function" subcategories.

urine samples (each pooled NA or MA urine sample consisted of 9 individual urine specimens; thus, the 3 pooled NA, and 3 pooled MA urine samples comprised 54 different individual urine samples). Further, biomarker candidates were confirmed and validated by 2-DE, Western blot, and MRM.

To analyze urinary proteomes in normoalbuminuria and microalbuminuria subjects, 3 biological replicates were generated, wherein 196 proteins met the following criteria: P value < 0.05, EF < 2, more than 2 unique peptides with >95% confidence level, and protein expression >1.25 or <0.80 for all reporter ions; 99 and 97 proteins were upregulated and downregulated, respectively (Appendix A).

These proteins were further analyzed by differential proteomic expression. All quantified proteins were classified into "biological process" and "molecular function" subcategories using the PANTHER classification program, allowing us to analyze phenotypic features and molecular functions between microalbuminuria and normoalbuminuria (Figure 3). Moreover, to visualize the comprehensive functional annotations graphically, such as in a heatmap, the 196 proteins

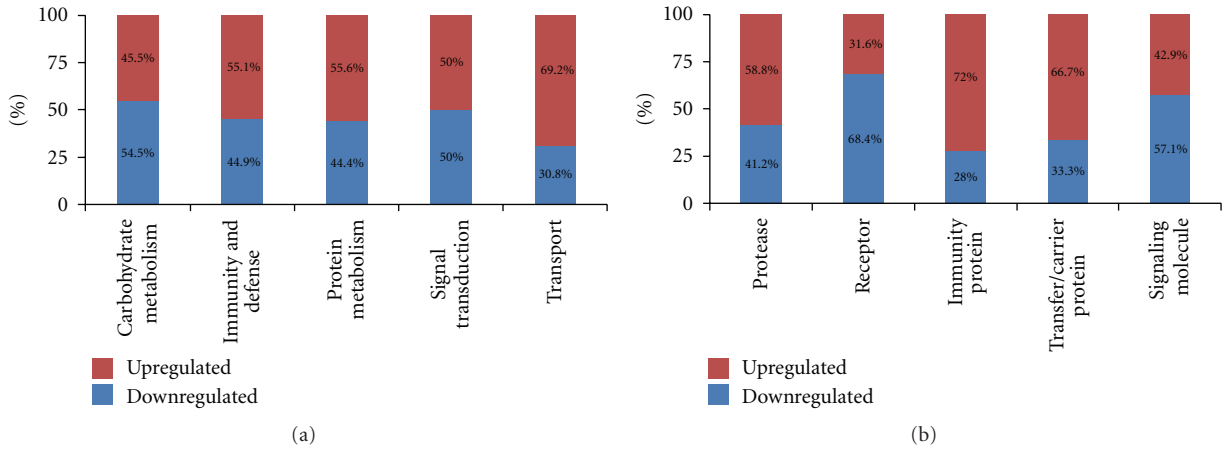


Figure 4: Functional distribution of differentially excreted proteins in microalbuminuria versus normoalbuminuria. Functional classification of differentially excreted proteins into (a) "biological process" and (b) "molecular function." Only 5 major subcategories for "biological process" and "molecular function" are shown; each subcategory is presented as the percentage of up- and down-regulated proteins.

were first categorized by "biological process," and a second dimension was added to coordinate the "molecular function" subcategories (Figure 3). The 196 proteins constituted a preliminary list of biomarkers; Figure 3 shows the expression ratios of the iTRAQ dataset and differentially excreted proteins in microalbuminuria versus normoalbuminuria urine.

3.4. Classification of Urinary Proteomes in Microalbuminuria versus Normoalbuminuria. The 196 proteins from the 3 biological replicates were categorized by PANTHER ID number into "biological process" and "molecular function" groups; certain subcategories are summarized in Figures 4(a) and 4(b).

The "biological process" subcategories accounted for 196 differentially excreted proteins, wherein "immunity and defense" and "protein metabolism" represented 49 and 34 of the quantitated proteins, respectively—the 2 largest components (Figure 3). Moreover, in the "biological process" subcategories, "carbohydrate metabolism" (54.5%) and "signal transduction" (50%) were downregulated in microalbuminuric versus normoalbuminuric urine (Figure 4(a)). In contrast, 69.2%, 55.6%, and 55.1% of the 196 proteins were upregulated in the "transport," "protein metabolism," and "immunity and defense" subcategories, respectively (Figure 4(a)).

The "molecular function" subcategories accounted for 196 differentially excreted proteins, in which "immunity" and "receptor" represented 25 and 19 of the quantitated proteins, respectively—the 2 largest components (Figure 3). In the "molecular function" subcategories, "receptor" (68.4%) and "signaling molecule" (57.1%) proteins were down-regulated in microalbuminuric versus normoalbuminuric urine (Figure 4(b)). In contrast, 72.0%, 58.8%, and 66.7% of the 196 proteins were up-regulated in the "immunity protein," "protease," and "transfer/carrier protein" subcategories, respectively (Figure 4(b)).

3.5. Differentially Excreted Urinary Proteins Are Associated with Pathogenic Status. One hundred ninety-six tentative biomarker candidates were differentially expressed, based on the iTRAQ data, and they were characterized biologically according to "biological process" and "molecular function." Moreover, in a detailed association study of diabetic nephropathy and differentially excreted proteins using references and databases, we prioritized 10 candidates, of the 196 differentially excreted proteins (Figure 3), that were associated with pathogenic status, such as glomerular and tubular dysfunction and other types of diseases. Accordingly, these proteins were classified into categories of pathogenesis in Table 2.

In the 3 biological replicates, transferrin (*TF*), ceruloplasmin precursor (*CP*), mannose-binding lectin-associated serine protease-2 precursor (*MASP2*), alpha-1-antitrypsin (*A1AT*), haptoglobin (*HP*), and basement membrane-specific heparin sulfate proteoglycan core protein (*HSPG*) were associated with glomerular dysfunction; except for *MASP2* and *HSPG*, all were upregulated in microalbuminuric versus normoalbuminuric urinary proteomes (Table 2).

Moreover, several differentially excreted proteins that were related to tubular dysfunction, such as vitamin D-binding protein (*VDBP*) and alpha-1-acid glycoprotein 1 precursor (*AGP1*) were selected for further validation. *VDBP* and *AGP1* were upregulated in microalbuminuria versus normoalbuminuria (Table 2).

In addition, *FABP* (fatty acid-binding protein) and *PSCA* (prostate stem cell antigen) correlate with other types of disease, and *PSCA* was selected for further validation. In this iTRAQ experiment, *FABP* was downregulated, whereas *PSCA* expression increased in the microalbuminuric versus normoalbuminuric urinary proteome (Table 2).

3.6. Identification of Differentially Excreted Proteins Using 2-D Gel Electrophoresis. Differential protein expression between microalbuminuric and normoalbuminuric urine was also measured using the 2-D gel electrophoresis in pooled NA4 and MA4 urine (Figure 1 and Table 1). In triplicate 2-DE analysis (Figures 5(a) and 5(b)), two proteins (regulator of telomere elongation helicase 1: *RTEL1* and serum albumin:

Table 2: Selected 10 differentially excreted proteins related to pathogenic status in microalbumiuric versus normoalbuminuric urines.

Pathogenic status	*N*	Number of unique peptides[a]	Accession number[b]	Gene name[c]	MA : NA expression		
					iTRAQ[d]	2-DE[e]	WB[f]
Glomerular dysfunction	1	15	spt|P02787	*TF*	1.86	—	4.66 ± 1.41
	2	47	Spt|P00450	*CP*	2.09	—	11.16 ± 0.38
	3	209	spt|P01009	*A1AT*	1.42	—	3.36 ± 0.03
	4	49	spt|P00738	*HP*	2.35	—	7.28 ± 5.52
	5	10	trm|Q9UMV3	*MASP2*	0.29	0.10 ± 0.005	—
	6	235	spt|P98160	*HSPG*	0.68	0.28 ± 0.041	—
Tubular dysfunction	7	9	spt|P02774	*GC*, *VDBP*	2.44	—	2.88 ± 0.11
	8	414	spt|P02763	*ORM1*, *AGP1*	2.04	—	1.82 ± 0.08
Other types of diseases	9	9	spt|Q01469	*FABP*	0.29	0.27 ± 0.037	—
	10	5	trm|O43653	*PSCA*	1.70	—	—

[a]The numbers of unique peptides and MS/MS spectrum observed by ProteinPilot software were determined only for those peptides with ≥95% confidence. [b]Accession numbers represent entries in the Human CDS database (human KBMS 5.0, 2005-03-02; a total of 187,748 entries provided by Applied Biosystems). [c]Gene name from the Expasy database correspond to protein accession number [b]from the Human CDS database (human KBMS 5.0, 2005-03-02; a total of 187,748 entries provided by Applied Biosystems). [d–f]Ratio of differentially excreted protein for iTRAQ, 2-DE and WB in microalbumiuric versus normoalbuminuric urines, respectively.

ALB) were upregulated, and 5 proteins (basement membrane-specific heparan sulfate proteoglycan core protein: *HSPG*, fatty acid-binding protein: *FABP*, mannose binding lectin-associated serine protease-2: *MASP2*, AMBP protein: *AMBP*, and Fibulin-5: *FBLN5*) were downregulated in microalbuminuric urine (Figures 5(a) and 5(b)).

Of the 7 proteins that were identified by 2-DE, 4 had the same pattern of differential excretion as in the iTRAQ experiment. Specifically, the spots that corresponded to serum albumin were upregulated by 2-DE (Figure 3(b), spots 1: 50.8 ± 15.3, 2: 17.5 ± 4.0, and 3: 14.2 ± 2.5) and iTRAQ (iTRAQ: 3.09). In contrast, *HSPG* (spot 4: 0.28 ± 0.041 and iTRAQ: 0.68), *FABP* (spot 5: 0.27 ± 0.037 and iTRAQ: 0.29), and *MASP2* (spot 6: 0.10 ± 0.005 and iTRAQ: 0.29) were significantly downregulated in both techniques (Table 3). *AMBP* (spot 7) was downregulated by 2-DE (0.20 ± 0.002) but upregulated in the iTRAQ experiment (1.44). Two proteins were identified by 2-DE alone—regulator of telomere elongation helicase 1 (spot 8: 3.0 ± 0.5) was upregulated and fibulin-5 precursor (spot 9: 0.12 ± 0.007) was downregulated (Table 3).

3.7. Validation of Differentially Expressed Proteins from iTRAQ by Western Blot. To validate the differentially excreted proteins from the iTRAQ results, 6 proteins (*TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, and *HP*) that were associated with pathogenic status were subjected to Western blot. The Western blot results were consistent with the iTRAQ findings (Figure 6 and Table 2): *TF* (4.66 ± 1.41 and $P < 0.0005$), *CP* (11.16 ± 0.38 and $P < 0.01$), *A1AT* (3.36 ± 0.03 and $P < 0.005$), *VDBP* (2.88 ± 0.11 and $P < 0.05$), *AGP1* (1.82 ± 0.08 and $P < 0.05$), and *HP* (7.28±5.52 and $P < 0.05$) were upregulated in microalbumiuric versus normoalbuminuric urine.

3.8. MRM Validation for 7 Selected Biomarker Candidates. To verify the 7 biomarker candidates (*TF*, *CP*, *A1AT*, *HP*, *VDBP*, *AGP1*, and *PSCA*), MRM was performed using 9 individual normoalbuminuric and 14 microalbuminuric samples (Figure 1 and Table 1). The peak area for each Q1/Q3 transition (Table 4) for the candidates was first normalized to the peak area of beta-galactosidase that was spiked with 50 fmol as the internal standard and compared between microalbuminuric versus normoalbuminuric samples.

MRM validation was assessed by interactive plots and ROC curves, represented by the peak area of each Q1/Q3 transition. Figure 7 shows the interactive plots and ROC curves for *TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, *HP*, and *PSCA* with regard to sensitivity, specificity, and relative concentrations versus beta-galactosidase. In the ROC curves, *TF*, *A1AT*, *AGP1*, *HP*, and *PSCA* had excellent area under the curve (AUC) values (0.762, 0.849, 0.873, 0.754, and 0.825, resp.), as did *CP* and *VDBP*, to a lesser extent (0.683 and 0.675, resp.) (Figure 7). Particularly, the merged ROC curve combining 3 biomarker candidates (alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, and prostate stem cell antigen) resulted in the improved AUC value of 0.921, which is greater than those of the individual proteins (0.849, 0.873, and 0.825 for alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, and prostate stem cell antigen, resp.) (Figure 8).

In the interactive plots, *TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, and *PSCA* were upregulated in microalbuminuric versus normoalbuminuric urine, whereas *HP* was down-regulated. *TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, and *PSCA* had the same excretion patterns by iTRAQ and western blot; conversely, *HP* had the opposite excretion pattern.

4. Discussion

4.1. Differentially Excreted Proteomes between Microalbuminuria and Normoalbuminuria. To identify and quantify proteins that were associated with diabetic nephropathy in microalbuminuric and normoalbuminuric urine, we used relative quantitative proteomic techniques, such as iTRAQ, 2-DE, Western blot, and MRM. In our iTRAQ experiment, 710 urinary proteins were identified at a >95% confidence level, of which 196 were differentially excreted by >1.25

Table 3: Differentially expressed proteins by 2-DE in microalbuminuria versus normoalbuminuria.

Gene name[a]	Accession number[b]	Up-/down-regulated	Mol. Mass, Da (pI)[c]	Peptides matched	Total ion C.I.%[d]	Ma/Na (2-DE)[f]	Ma/Na (iTRAQ)[g]
ALB	P02768	Up	71317.2 (5.92)	2	100.00	50.8 ± 15.3	3.09
ALB	P02768	Up	71317.2 (5.92)	2	99.84	17.5 ± 4.0	3.09
ALB	P02768	Up	71317.2 (5.92)	2	100.00	14.2 ± 2.5	3.09
HSPG	P98160	Down	468527.5 (6.06)	2	100.00	0.28 ± 0.041	0.68
FABP	Q01469	Down	15496.7 (6.6)	1	97.76	0.27 ± 0.037	0.29
MASP2	Q9UMV3	Down	75684.6 (5.47)	2	100.00	0.10 ± 0.005	0.29
AMBP	P02760	Down	38974 (5.95)	2	99.91	0.20 ± 0.002	1.44
RTEL1	Q9NZ71	Up	152278.2 (8.68)	1	96.77	3.0 ± 0.5	—
FBLN5	Q9UBX5	Down	50146.7 (4.58)	1	99.36	0.12 ± 0.007	—

[a–b]Gene name from the Expasy database correspond to protein accession number. [b]Accession numbers represent entries in the Human CDS database. [c]Molecular mass (mol. mass) is presented by Da, while isoelectric point stands for pI. [d]Total ion score and total ion CI % for MALDI-TOF/TOF were calculated using GPS v3.5 in the MASCOT search program (v2.0). [f–g]Ratio of differentially excreted protein for 2-DE and iTRAQ in microalbumiuric versus normoalbuminuric urines, respectively. Data are expressed as the mean ± SD.

Table 4: Parameters of MRM Experiment for seven candidate proteins.

Protein name	Q1[a]	Q3[b]	Sequence[c]	Fragment[d]	Charge[e]	CE[f]
Transferrin	482.8	682.4	APNHAVVTR	y6	2+	26
	315.2	558.3	AVGNLR	y5	2+	19
	315.2	459.3	AVGNLR	y4	2+	19
Ceruloplasmin	686.4	1080.0	GAYPLSIEPIGVR	y10	2+	35
	686.39	870.5	GAYPLSIEPIGVR	y8	2+	35
Alpha-1-antitrypsin	555.81	997.5	LSITGTYDLK	y9	2+	29
	555.81	797.4	LSITGTYDLK	y7	2+	29
	393.2	587.3	VVNPTQK	y5	2+	22
	393.2	473.3	VVNPTQK	y5	2+	22
Haptoglobin precursor	729.8	1084.5	NLFLNHSENATAK	y10	2+	37
	352.2	517.3	VSVNER	y4	2+	20
Vitamin D-binding protein	400.2	700.4	VLEPTLK	y6	2+	26
	400.2	587.3	VLEPTLK	y5	2+	26
Alpha-1-acid glycoprotein 1	556.8	811.4	SDVVYTDWK	y6	2+	29
	580.8	974.5	WFYIASAFR	y8	2+	31
	580.8	827.4	WFYIASAFR	y7	2+	31
Prostate stem cell antigen	501.0	830.5	AVGLLTVISK	y8	2+	30
	501.0	660.4	AVGLLTVISK	y6	2+	30

[a–b]Q1 and Q3 (m/z) represent the Q1 and Q3 transitions for proteotypic peptide, respectively. [c]Sequence represents the sequence of proteotypic peptide for target protein. [d]Fragment type indicates the ion type of the Q3 transition. [e]Charge represents the charge state of precursor ion. [f]CE represents collision energy.

or <0.80—99 and 97 proteins were up- and down-regulated, respectively (Appendix A).

Recently, the Urinary Protein Biomarker (UPB) database was constructed and published, in which 205 publications were curated manually [29]. Using this database, we can easily determine whether a biomarker candidate has been identified by another group for the same disease and evaluate its disease specificity. Thirty-six of the 196 quantified proteins from our iTRAQ experiment were registered in the UPB database; the remaining 160 proteins were not listed.

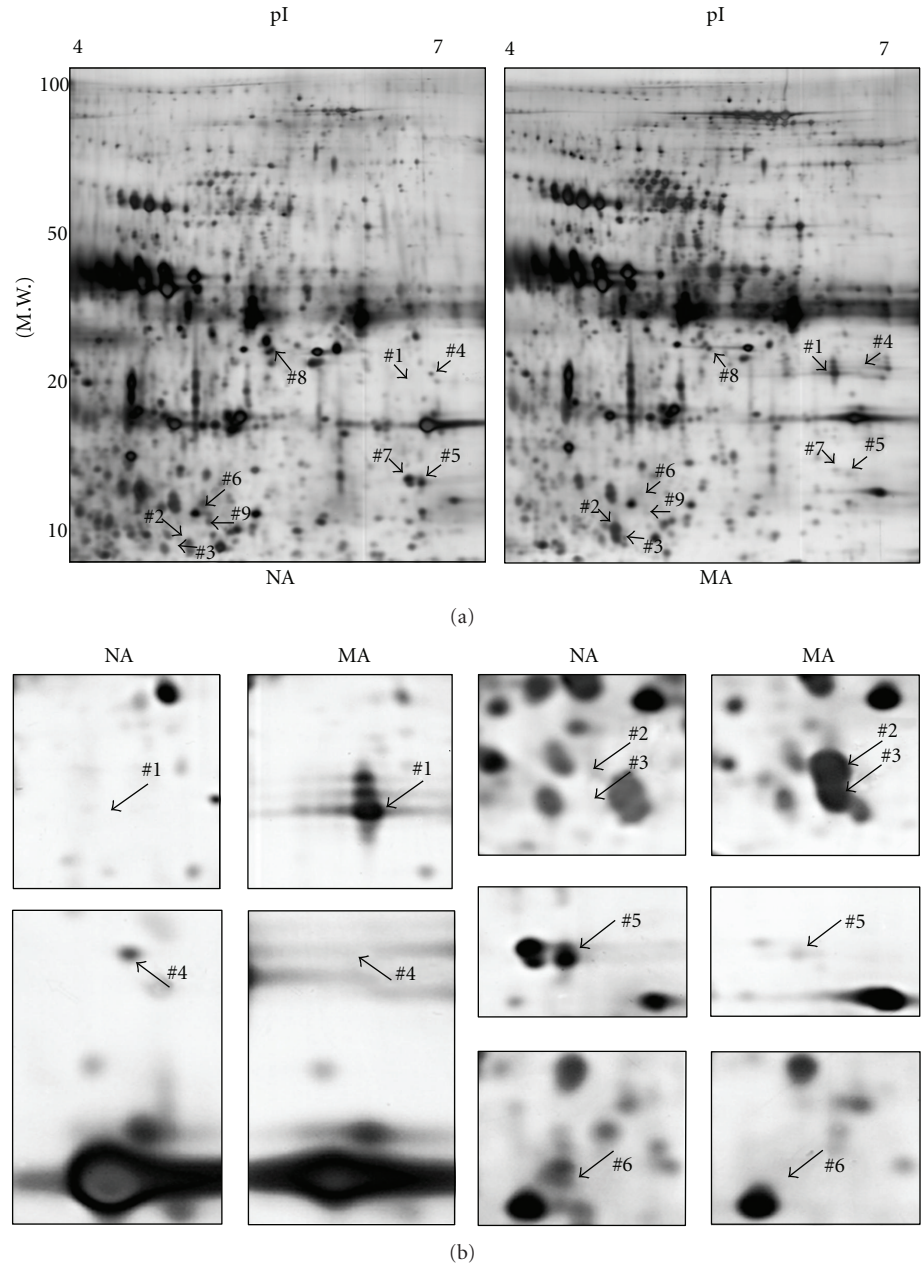


Figure 5: Differentially excreted proteins by 2-DE in microalbuminuria versus normoalbuminuria. (a) Representative whole 2-DE images of normoalbuminuric (NA) and microalbuminuric (MA) urine. Total protein (100 μg) samples were loaded onto IPG strips (pH 4–7, nonlinear) for IEF and separated in the second dimension on a 12% polyacrylamide gel. The horizontal and vertical axes represent p*I* and molecular weight, respectively. The arrowed numbers denote for differentially excreted proteins and correspond to the proteins in Table 3. (b) Magnified sections of differentially excreted proteins in the 2-DE gel. Some arrowed proteins in the 2-DE gel (Figure 5(a)) were magnified side by side to compare their relative expression.

Subsequently, 196 differentially excreted proteins yielded 10 preliminary biomarker candidates for further validation.

The "molecular function" subcategories accounted for 196 differentially excreted proteins, of which "immunity" represented 25 of the quantitated proteins—the largest component (Figure 3). This proportion reflects the increased inflammatory reactions and higher vascular lesion counts in kidneys during the development of diabetic nephropathy. Through detailed association searches between diabetic nephropathy and the 196 differentially excreted proteins using relevant databases and references, we identified and classified several biomarker candidates that were associated with pathogenic status, such as glomerular and tubular dysfunction and other types of disease (Table 2).

Consequently, 10 proteins were selected for preliminary validation studies such as 2-DE, Western blot, and MRM.

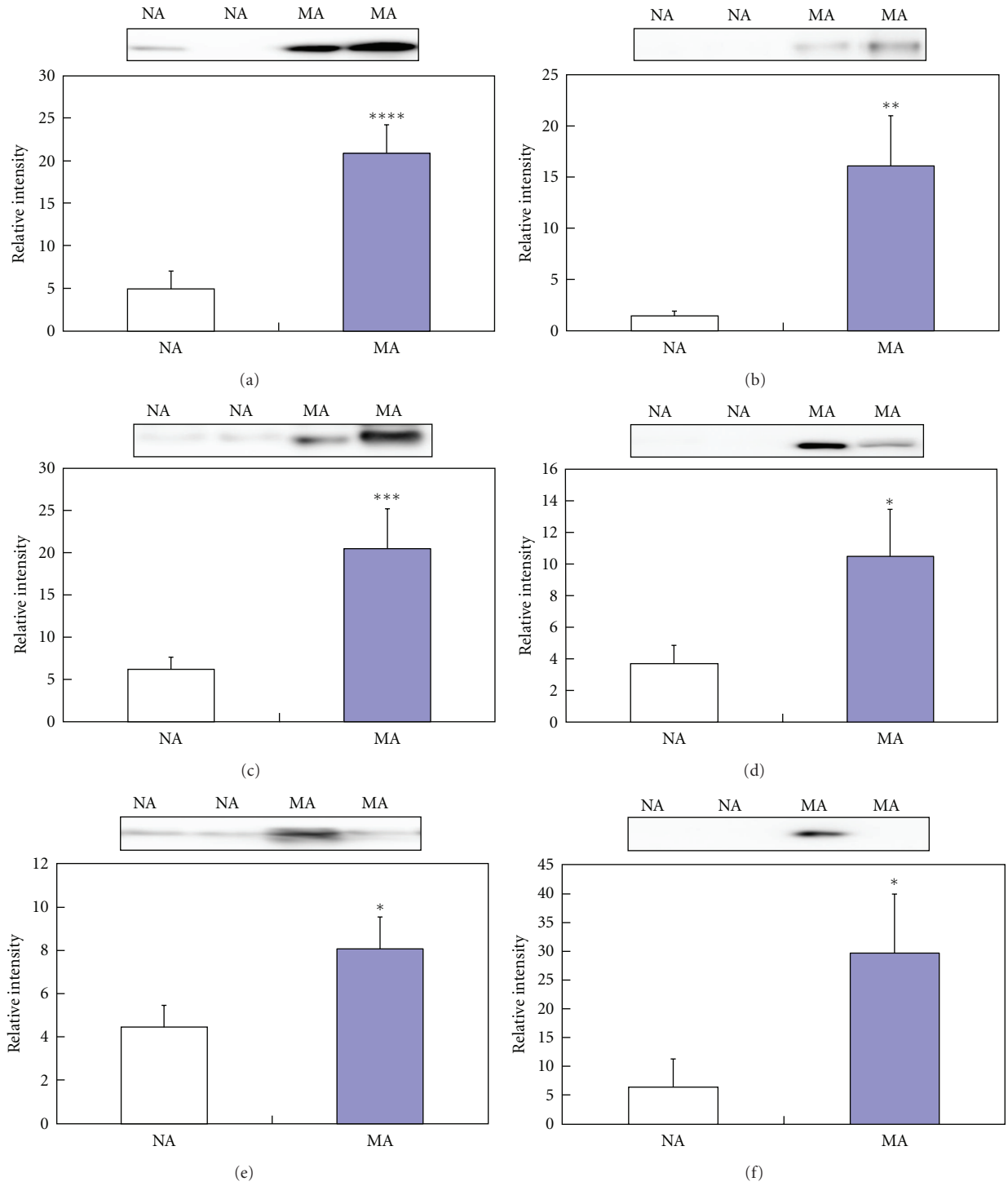


Figure 6: Validation using Western blot for six representative differentially excreted proteins. The concentrations of transferrin (a), ceruloplasmin (b), α1-antitrypsin (c), vitamin D-binding protein (d), α1-acid glycoprotein (e), and haptoglobin (f) are significantly higher in microalbuminuric patients versus normoalbuminuric urine. The relative intensities on the vertical axis indicate normalized values versus the representative control. Each bar represents the mean ± SEM, based on the relative intensities of the gel bands. Statistical significance for the differences ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.0005$) were determined by paired Student's t-test. Two representative NA and MA blots are shown at the top of the bar graphs.

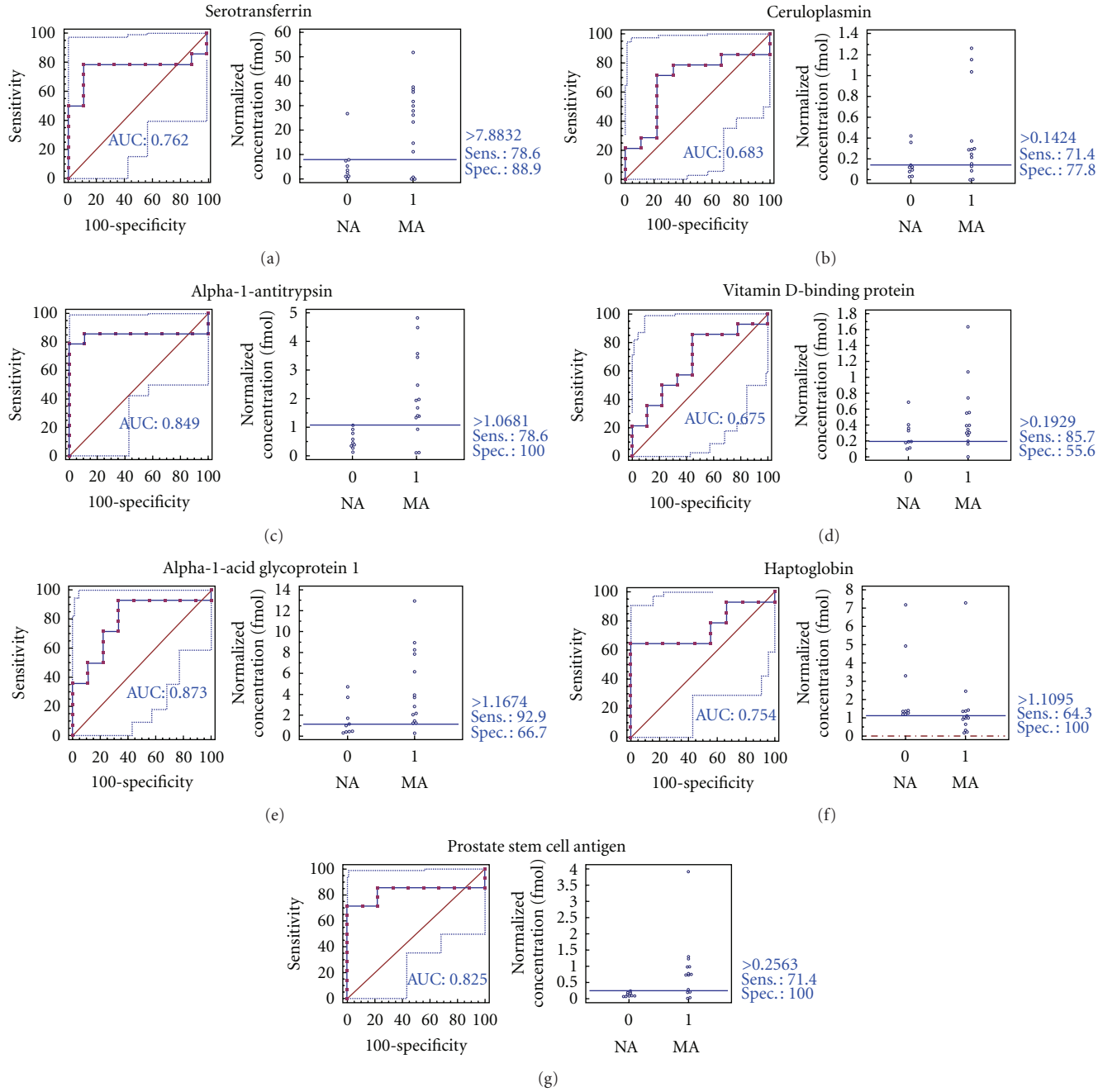


Figure 7: ROC curves and interactive plots for MRM validation in normoalbuminuric versus microalbuminuric urine. Seven biomarker candidates (*TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, *HP*, and *PSCA*) were validated by MRM, in which 9 normoalbuminuric and 14 microalbuminuric urine samples were used. (a)–(g) Interactive plots and ROC curves for *TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, *HP*, and *PSCA*. In the ROC curves, the solid lines represent the corresponding score in sensitivity (*x*-axis) and 100-specificity (*y*-axis). In the interactive plots, the *y*-axis indicates the normalized concentration of the target protein against the spiked internal standard (50 fmol of beta-galactosidase peptide). Sens. and Spec. represent the sensitivity and specificity for the target proteins, respectively. The AUC values are shown inside the ROC curves.

For example, 3 proteins (*HSPG*, *FABP*, and *MASP2*) that were identified by 2-DE had the same pattern of differential excretion in the 2-DE and iTRAQ experiments (Table 2). Six differentially excreted proteins (*TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, and *HP*) were validated by Western blot, for which the patterns of excretion were consistent with the iTRAQ results (Table 2).

Recently, an optimized quantitative proteomic strategy in urinary proteomic analysis was proposed for urine biomarker discovery using a small set of samples [21]. According to this report, the initial amount of proteins that is analyzed and the precipitation method (methanol precipitation) of the urine proteins are critical. Notably, our preparation methods for urinary proteomes approximated this optimized

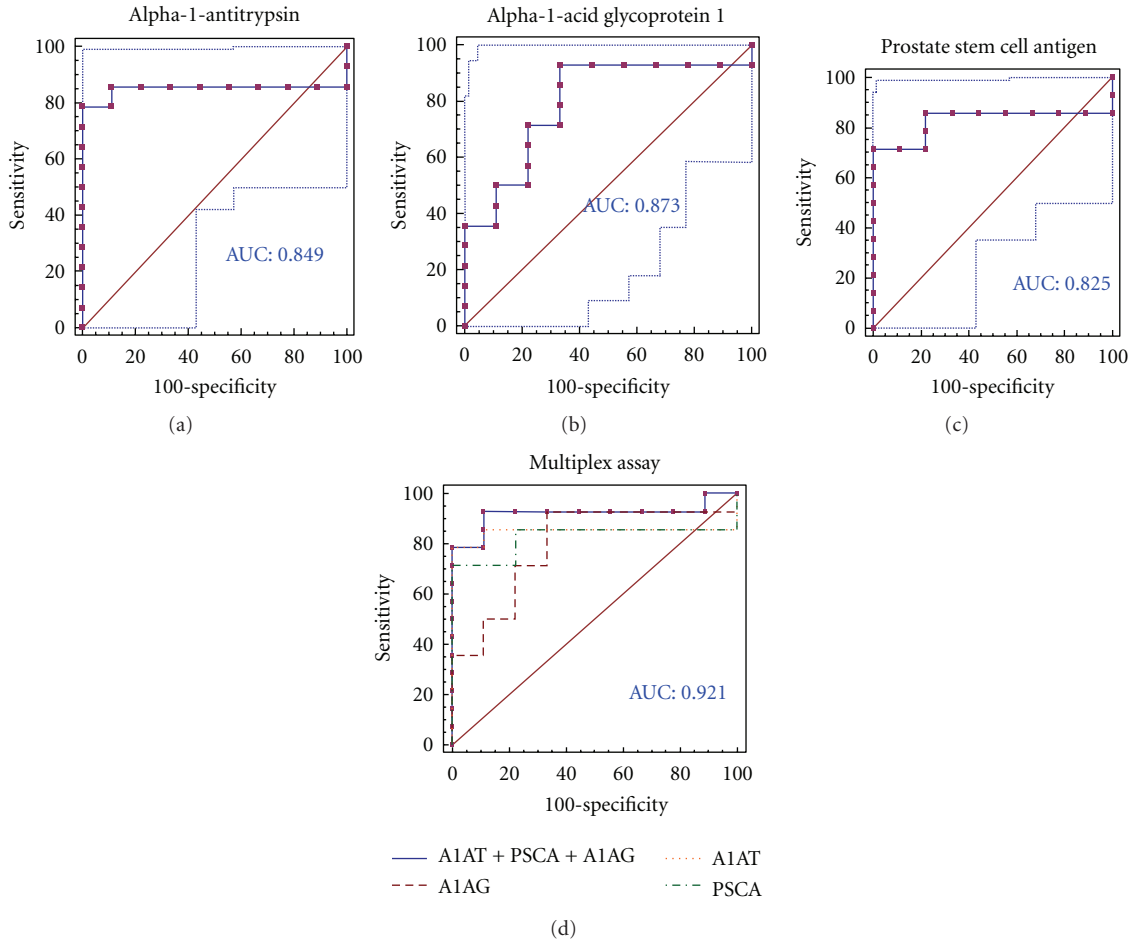


Figure 8: ROC curves for three candidate biomarkers and the 3-marker panel. MRM validation was performed for (a) alpha-1-antitrypsin, (b) alpha-1-acid glycoprotein 1, (c) prostate stem cell antigen, and (d) their combination, generating AUC values of 0.849, 0.873, and 0.825, respectively, whereas the combination resulted in a merged AUC of 0.921.

protocol, although we used acetone precipitation instead of methanol precipitation.

Nevertheless, an advantage of our study was that we used a large collection of urine samples from 86 diabetic patients to perform 3 iTRAQ experiments, including 3 biological replicates and 2 technical replicates, resulting in more reliable statistical significance.

4.2. Differentially Excreted Proteome and Glomerular Dysfunction. Glomerular dysfunction is caused by GBM thickening and mesangial expansion due to ECM accumulation [2], and several proteins, such as *TF*, *CP*, *MASP2*, *A1AT*, *HP*, and *HSPG*, were associated with glomerular dysfunction.

Transferrin-to-creatinine and ceruloplasmin-to-creatinine ratios are known to reflect changes in renal hemodynamics, and these ratios are significantly higher microalbuminuric patient than normoalbuminuric patients [30]. This result is caused by elevated intraglomerular hydraulic pressure, which leads to the development of diabetic glomerulosclerosis [30–32]. Moreover, *TF* and *CP* were listed in the UPB database, showing upregulation. *TF* is associated with diabetic nephropathy, normoalbuminuric type 2 diabetes, kidney calculi, and ureteropelvic junction obstruction, whereas *CP* is linked to diabetic nephropathy and normoalbuminuric type 2 diabetes. In our iTRAQ and 2-DE, *TF* and *CP* urinary proteins were commonly upregulated in the comparison of microalbuminuric and normoalbuminuric urinary proteome (Table 2).

MASP2 is a serum protease that activates the complement cascade, which regulates the maintenance of glomerular permeability and the pathogenesis of focal segmental glomerulosclerosis [33]. *MASP2* was not listed in the UPB database, and in this iTRAQ and 2-DE, this protein was commonly downregulated in comparison of microalbuminuric and normoalbuminuric urinary proteome (Table 2).

A1AT is a serine protease inhibitor, which prevents neutrophil elastase by degrading ECM proteins, which maintains vascular elasticity and glomerular integrity [13, 34]. In a previous study, *A1AT* excretion was elevated in microalbuminuria, which can cause matrix molecules to accumulate [35]. *A1AT* was also listed in the UPB database, showing upregulation; it is associated with diabetic nephropathy, severe acute pancreatitis, kidney calculi, nephrotic syndrome, and ureteropelvic junction obstruction. In our iTRAQ and

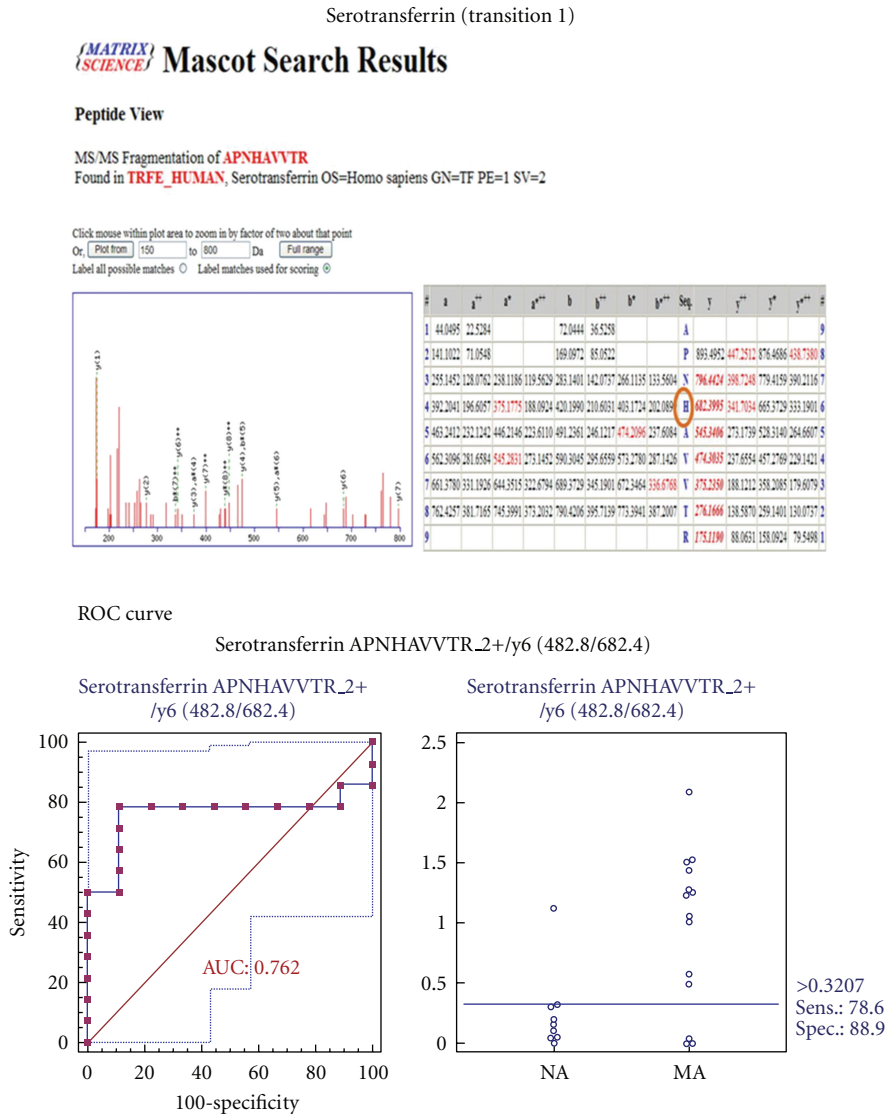


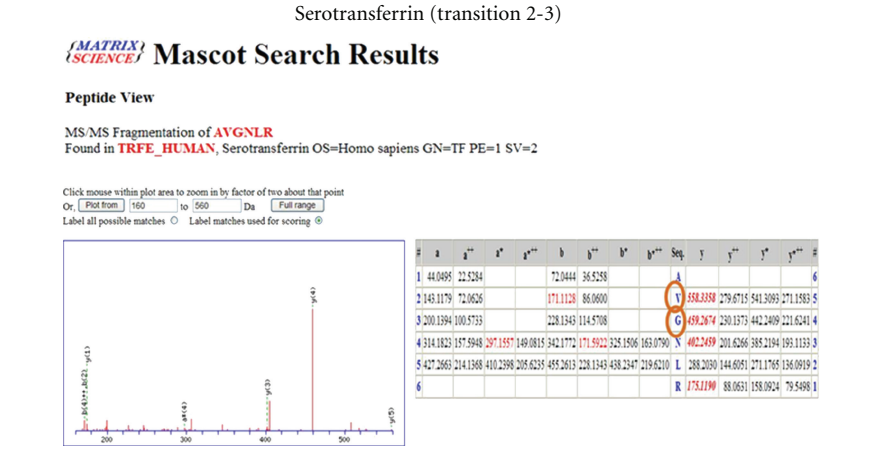
Figure 9

Western blot, *A1AT* was also upregulated in the comparison of microalbuminuric and normoalbuminuric urinary proteome (Table 2).

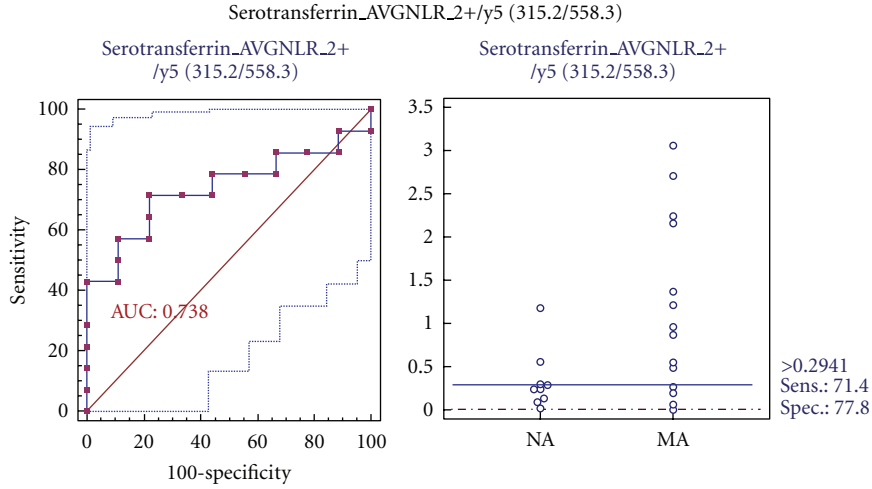
Haptoglobin (*HP*) is an acute phase protein that binds to free hemoglobin (Hb) with the highest affinity. Formation of the Hb-HP complex prevents the loss of renal iron and oxidative damage that are driven by free Hb. Specifically, *HP* mediates complement-dependent podocyte damage [36]. Thus, complement activation results in the release of proteases, oxidants, and growth factors, damaging the functional integrity of the GBM. *HP*, which was downregulated and is associated with diabetic nephropathy and type 2 diabetes mellitus, appeared in the UPB database. However, in our iTRAQ and Western blot, *HP* was commonly upregulated in the comparison of microalbuminuric and normoalbuminuric urinary proteome (Table 2).

HSPG is present in the basement membrane of every vascularized organ, including the GBM. The highly negatively charged side chains of *HSPG* are important determinants for the charge-selective permeability of the GBM [37]. Under hyperglycemic conditions, the loss of *HSPG* from the GBM alters the charge-selective properties of the glomerular capillary, causing increased filtration of negatively charged albumin [37, 38]. *HSPG* is not registered in the UPB database, and in our iTRAQ and 2-DE, *HSPG* was commonly downregulated in the comparison of microalbuminuric and normoalbuminuric urinary proteome (Table 2).

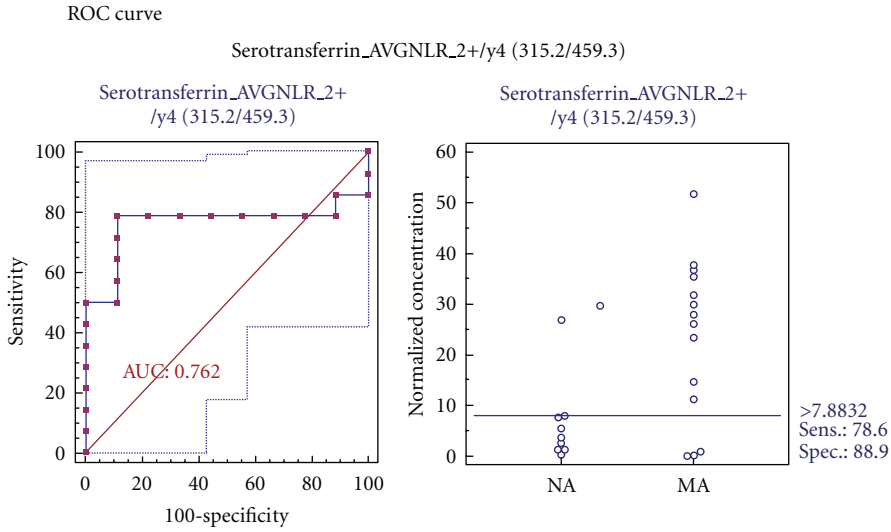
4.3. Differentially Excreted Proteome and Tubular Dysfunction and Other Types of Diseases. Low-molecular-weight proteins, such as *VDBP* and *AGP1*, are associated with kidney tubular dysfunction and were commonly upregulated in the iTRAQ and Western blot (Table 2). Unlike high-molecular-weight proteins, they are filtered in the glomerulus on the basis of charge selectivity and pore size of the GBM and are reabsorbed into proximal renal tubules under normal conditions



(a)



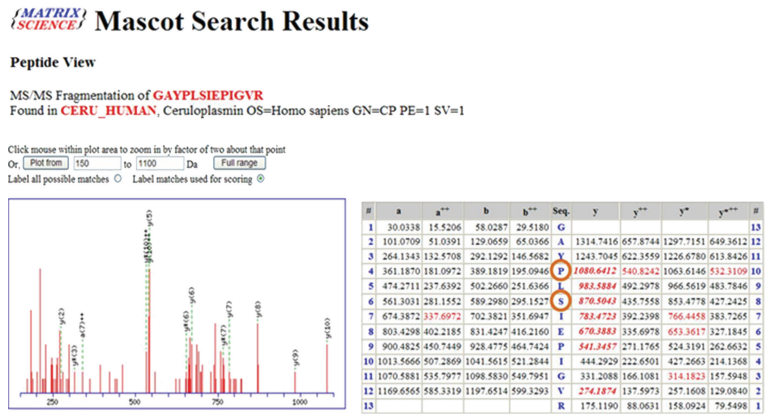
(b)



(c)

Figure 10

Ceruloplasmin (transition 1-2)

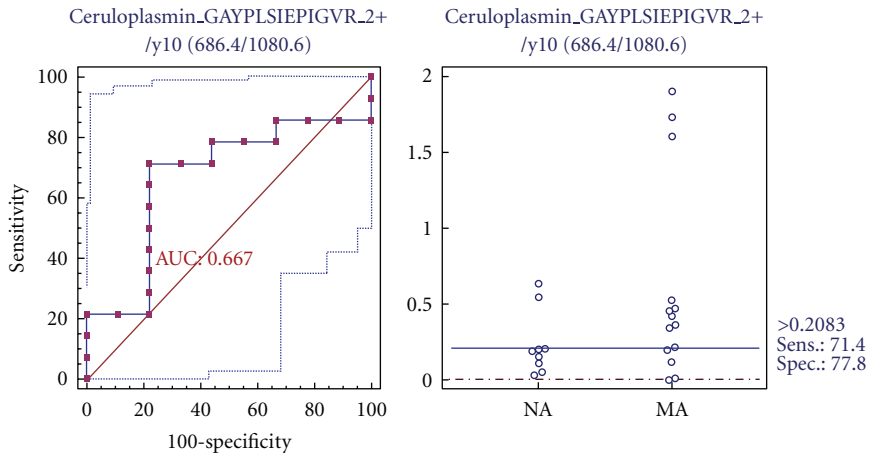


#	a	a++	b	b++	Seq.	y	y++	y*	y*++	#
1	30.0338	15.5206	58.0287	29.5180	G					13
2	101.0709	51.0391	129.0659	65.0366	A	1314.7416	657.8744	1297.7151	649.3612	12
3	264.1343	132.5708	292.1292	146.5682	Y	1243.7045	622.3559	1226.6780	613.8426	11
4	361.1870	181.0972	389.1819	195.0946	P	*1080.6412*	540.8242	1063.6146	532.3109	10
5	474.2711	237.6392	502.2660	251.6366	L	*983.5884*	492.2978	966.5619	483.7846	9
6	561.3031	281.1552	589.2980	295.1527	S	*870.5043*	435.7558	853.4778	427.2425	8
7	674.3872	337.6972	702.3821	351.6947	I	*783.4723*	392.2398	766.4458	383.7265	7
8	803.4298	402.2185	831.4247	416.2160	E	*670.3883*	335.6978	653.3617	327.1845	6
9	900.4825	450.7449	928.4775	464.7424	P	*541.3457*	271.1765	524.3191	262.6632	5
10	1013.5666	507.2869	1041.5615	521.2844	I	444.2929	222.6501	427.2663	214.1368	4
11	1070.5881	535.7977	1098.5830	549.7951	G	331.2088	166.1081	314.1823	157.5948	3
12	1169.6565	585.3319	1197.6514	599.3293	V	*274.1874*	137.5973	257.1608	129.0840	2
13					R	175.1190	88.0631	158.0924	79.5498	1

(a)

ROC curve

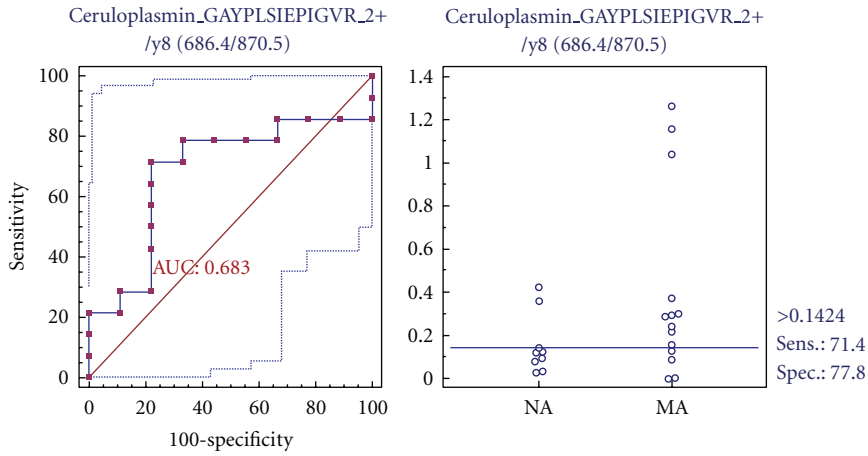
Ceruloplasmin_GAYPLSIEPIGVR_2+/y10 (686.4/1080.6)



(b)

ROC curve

Ceruloplasmin_GAYPLSIEPIGVR_2+/y8 (686.4/870.5)



(c)

Figure 11

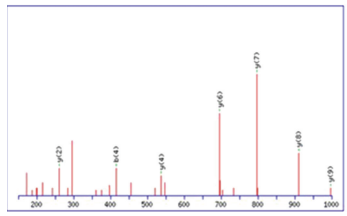
Alpha-1-antitrypsin (transition 1-2)

MATRIX SCIENCE **Mascot Search Results**

Peptide View

MS/MS Fragmentation of **LSITGTYDLK**
Found in **A1AT_HUMAN**, Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3

Click mouse within plot area to zoom in by factor of two about that point
Or, Plot from 150 to 1000 Da Full range
Label all possible matches ○ Label matches used for scoring ◉

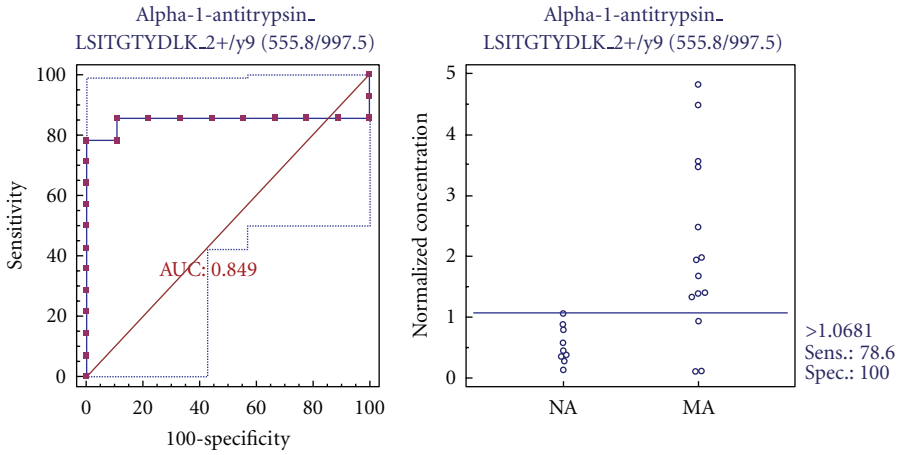


#	a	a^{++}	b	b^{++}	Seq.	y	y^{++}	y^*	y^{*++}	#
1	86.0964	43.5519	114.0913	57.5493	L					10
2	173.1285	87.0679	201.1234	101.0653	S	997.5201	499.2637	980.4935	490.7504	9
3	286.2125	143.6099	314.2074	157.6074	I	910.4880	455.7477	893.4615	447.2344	8
4	387.2602	194.1337	415.2551	208.1312	T	797.4040	399.2056	780.3774	390.6923	7
5	444.2817	222.6445	472.2766	236.6419	G	696.3563	348.6818	679.3297	340.1685	6
6	545.3293	273.1683	573.3243	287.1658	T	639.3348	320.1710	622.3083	311.6578	5
7	708.3927	354.7000	736.3876	368.6974	Y	538.2871	269.6472	521.2606	261.1339	4
8	823.4196	412.2134	851.4145	426.2109	D	375.2238	188.1155	358.1973	179.6023	3
9	936.5037	468.7555	964.4986	482.7529	L	260.1969	130.6021	243.1703	122.0888	2
10					K	147.1128	74.0600	130.0863	65.5468	1

(a)

ROC curve

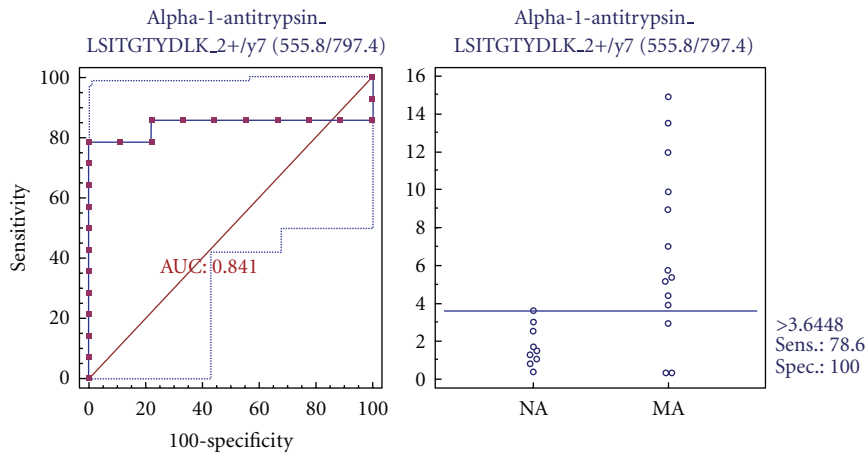
Alpha-1-antitrypsin_LSITGTYDLK_2+/y9 (555.8/997.5)



(b)

ROC curve

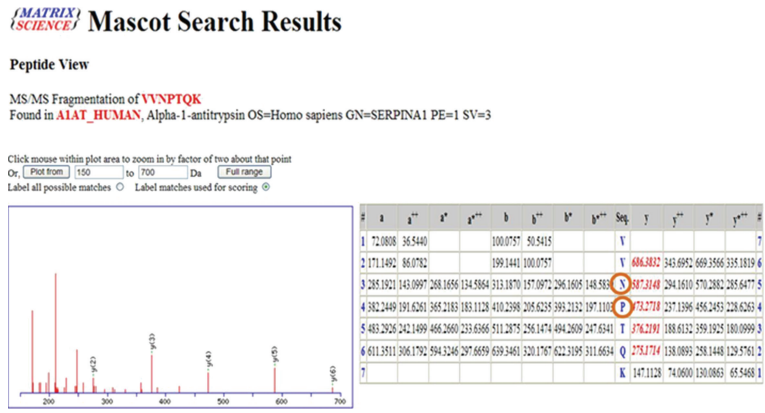
Alpha-1-antitrypsin_LSITGTYDLK_2+/y7 (555.8/797.4)



(c)

Figure 12

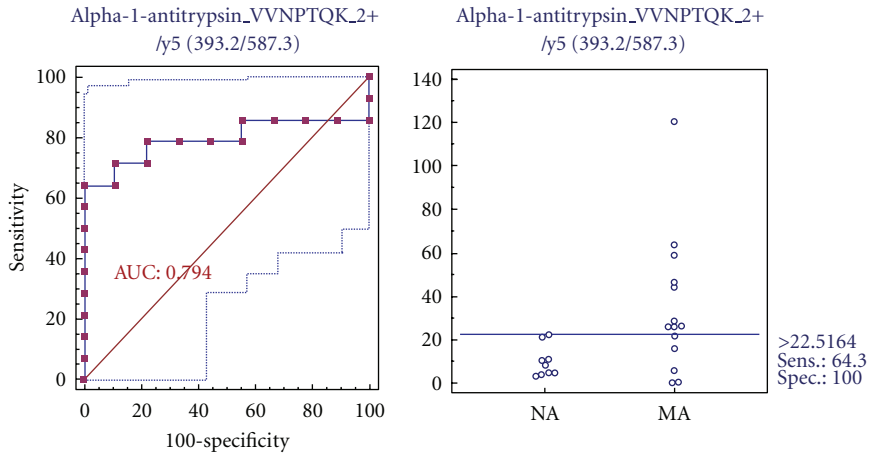
Alpha-1-antitrypsin (transition 3-4)



(a)

ROC curve

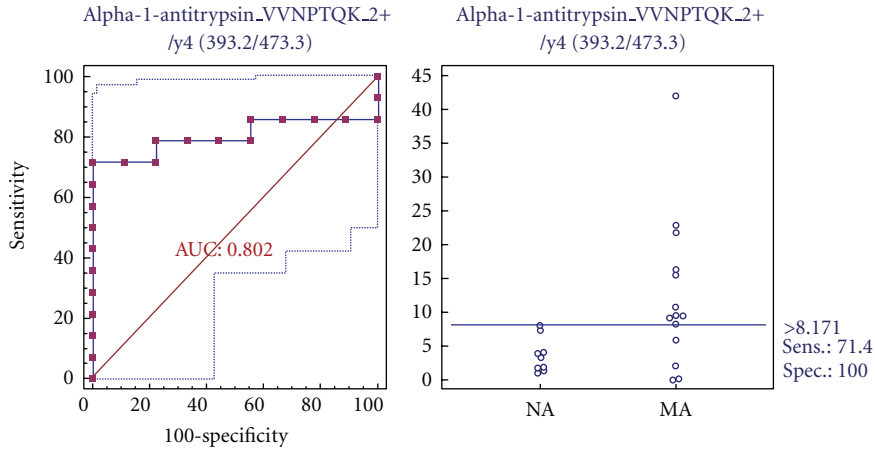
Alpha-1-antitrypsin_VVNPTQK_2+/y5 (393.2/587.3)



(b)

ROC curve

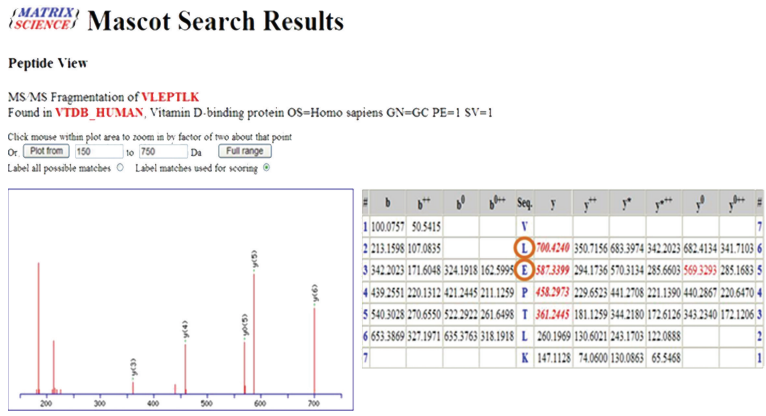
Alpha-1-antitrypsin_VVNPTQK_2+/y4 (393.2/473.3)



(c)

Figure 13

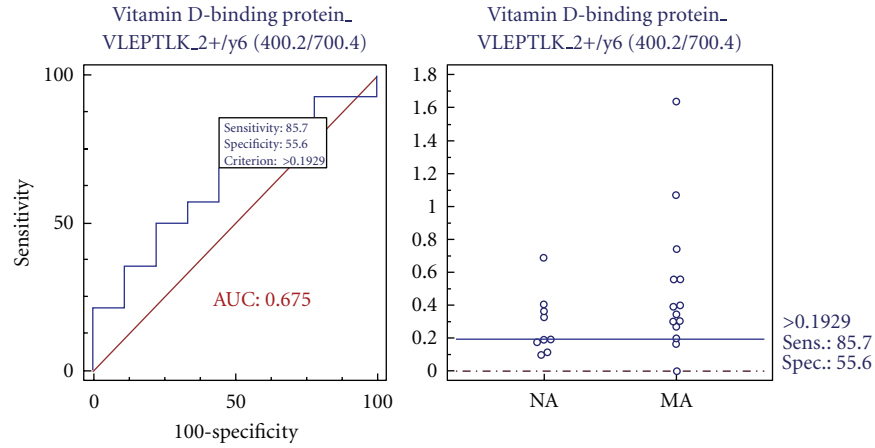
Vitamin D-binding protein (transition 1-2)



(a)

ROC curve

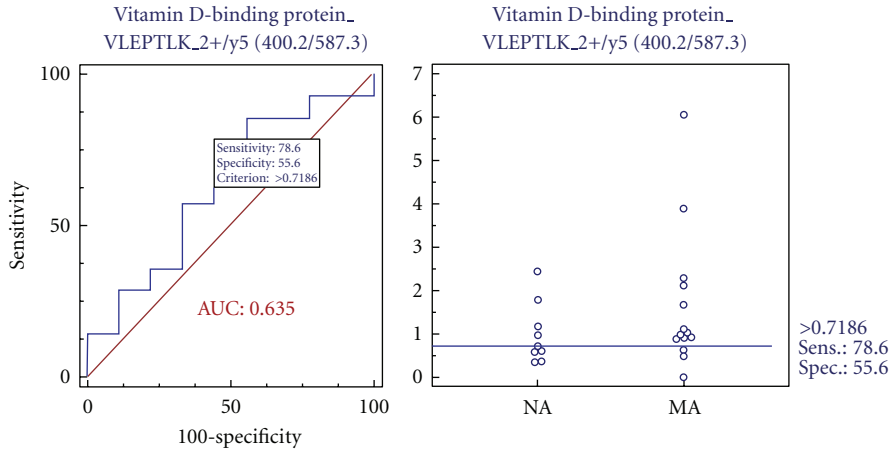
Vitamin D-binding protein_VLEPTLK_2+/y6 (400.2/700.4)



(b)

ROC curve

Vitamin D-binding protein_VLEPTLK_2+/y5 (400.2/587.3)



(c)

Figure 14

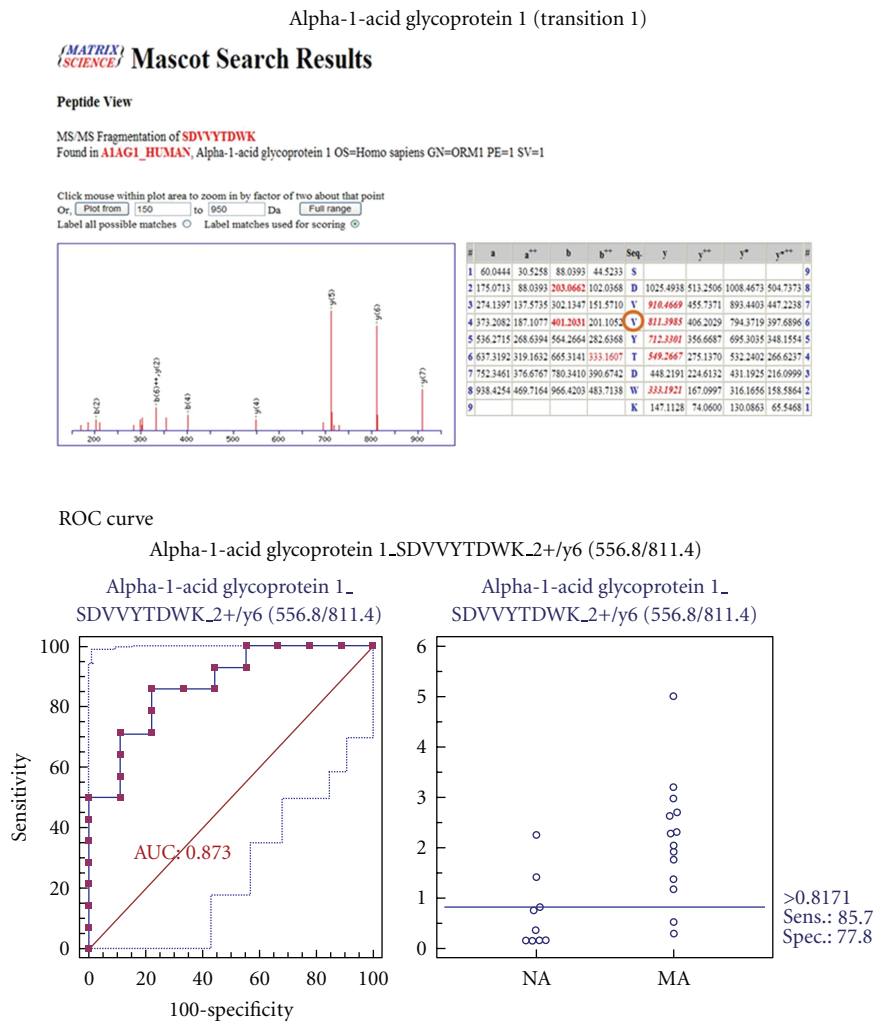


Figure 15

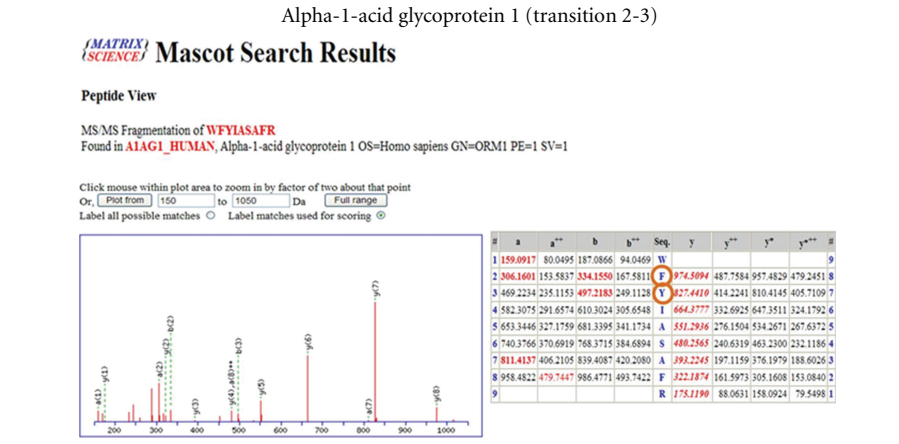
[38]. If reabsorption is impaired, however, these proteins can be overexcreted into urine.

VDBP is a multifunctional serum glycoprotein and is an important mediator in immunopathogenesis because it is associated with immunoglobulin receptors on the surfaces of B- and T-lymphocytes [12]. Moreover, *VDBP* binds to circulating vitamin D metabolites with high affinity [39], and *VDBP*-bound 25-hydroxyvitamin D3 crosses the GBM and is reabsorbed by proximal tubular cells in a megalin-dependent manner, suggesting that it controls renal uptake and the activation of metabolites [4, 40]. *VDBP*, also listed in the UPB database, was upregulated and is associated with diabetic nephropathy and Dents disease. In our iTRAQ and Western blot, *VDBP* was commonly upregulated in the comparison of microalbuminuric and normoalbuminuric urinary proteome (Table 2).

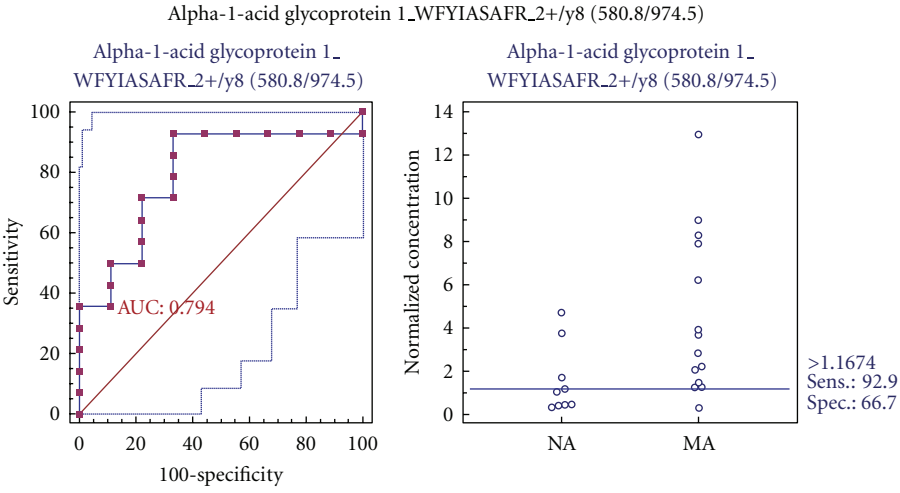
AGP1 is synthesized in response to systemic tissue injury, inflammation, and infection, like most other acute phase proteins [7, 41]. Further, increased levels of *AGP1* reflect elevated levels of cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α), which are associated with type 2 diabetes [7]. A study has demonstrated that *AGP1* is an indicator of tubular disorder in multiple myeloma [42], and another has shown that serum and urinary *AGP1* levels are elevated in type 2 diabetic patients with kidney disorders [5, 7]. *AGP1* was listed in the UPB database, showing upregulation, and is associated with diabetic nephropathy, diabetic kidney disorder, preeclampsia, and acute appendicitis. In our iTRAQ and Western blot, *AGP1* was also commonly upregulated in the comparison of microalbuminuric and normoalbuminuric urinary proteome (Table 2).

FABP (fatty acid-binding protein: *FABP*) correlated with other types of diseases [43] and was also chosen for further validation. *FABP* was not listed in the UPB database, and in this iTRAQ and 2-DE, *FABP* was commonly downregulated in the comparison of microalbuminuric and normoalbuminuric urinary proteome (Table 2).

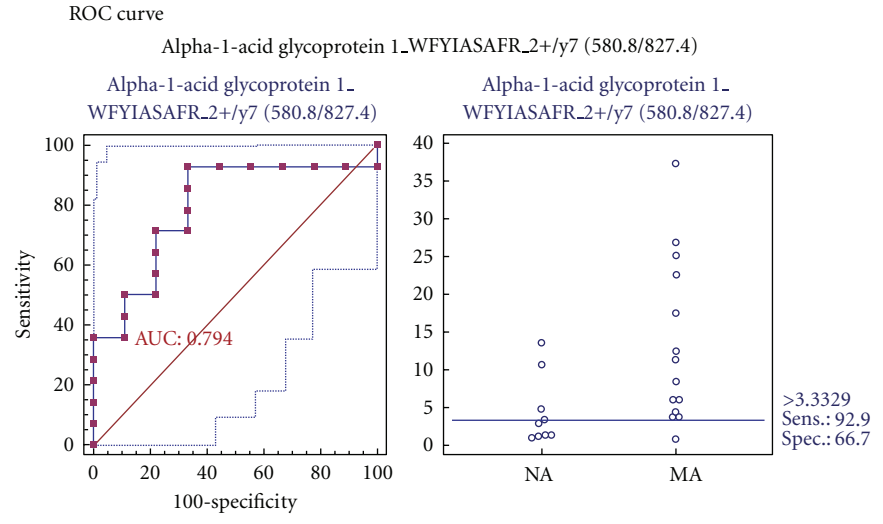
PSCA is highly expressed in the prostate and, to a lesser extent, in the bladder, placenta, colon, kidney, and stomach. Moreover, it is upregulated in prostate cancer and is detected in cancers of the bladder and pancreas [44]. *PSCA* was not listed in the UPB database but was upregulated in the microalbuminuric versus normoalbuminuric urinary proteome in



(a)



(b)



(c)

Figure 16

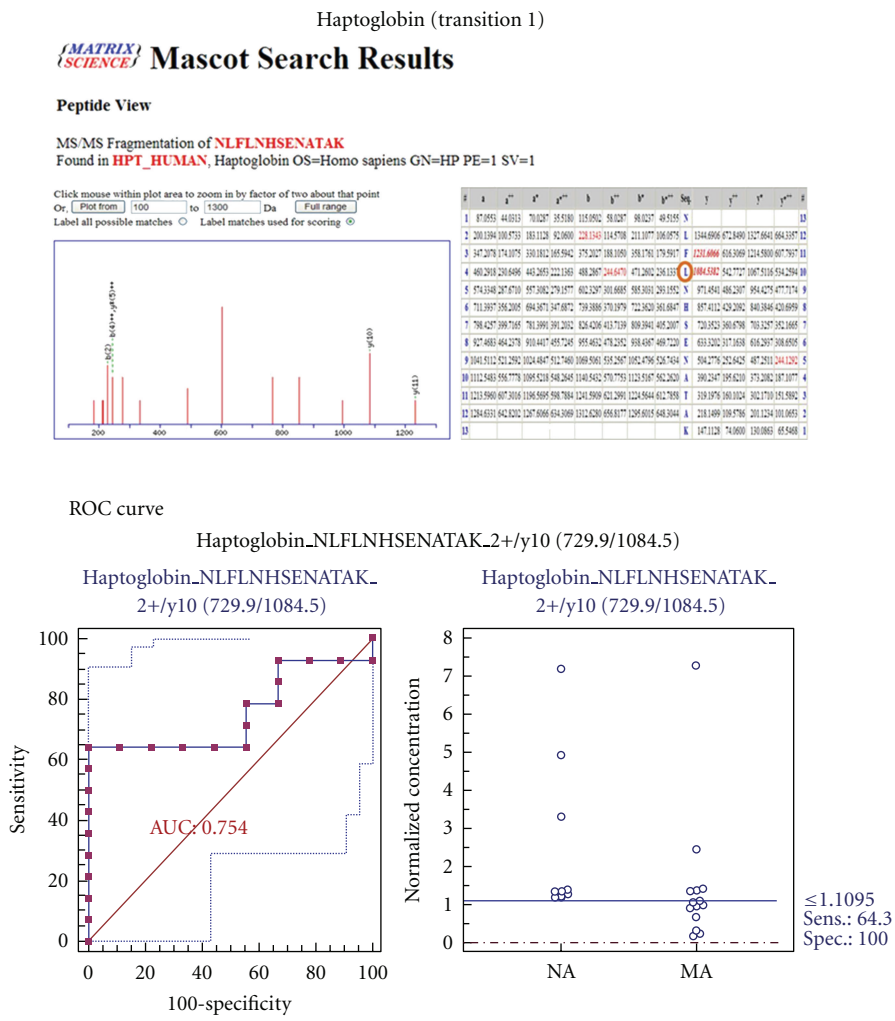


Figure 17

our iTRAQ experiment (Table 2). No relationship between prostate stem cell antigen and diabetic nephropathy has been reported.

4.4. Validation of Differentially Excreted Proteins Using MRM. For the MRM experiments, we used a bacterial beta galactosidase peptide as the internal standard for relative quatitation [28]. Seven preliminary biomarker candidates (*TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, *HP*, and *PSCA*) were confirmed in 9 normoalbuminuric and 14 microalbuminuric urine samples by MRM (Figure 7).

In the interactive plots, *TF*, *CP*, *A1AT*, *VDBP*, *AGP1*, and *PSCA* were preferentially excreted in microalbuminuria versus normoalbuminuria, whereas *HP* was downregulated. *TF*, *CP*, *A1AT*, *VDBP*, and *AGP1* had the same pattern of excretion in the iTRAQ and western analysis, and *HP* had the opposite pattern between the MRM and Western analysis. *HP* consists of α-chain (amino acid sequence: 19–160) and β-chain (aminoacid sequence: 162–406), which are connected by disulfide bridges. In the MRM experiment, the transition (amino acid sequence: 203–215) in the β-chains was used for the relative quantitation of *HP*. In contrast, portions of both the α-chain and β-chain were used in the iTRAQ quantitation (sequence coverage: 31.0%), and an antibody that targeted a sequence in the α-chain was used for the Western blot analysis. It is conceivable that these disparate targets resulted in contradictory patterns between iTRAQ, Western blot, and MRM. Regardless of the methods or targets, reproducible patterns must be obtained with each method.

Furthermore, we performed a multiplex assay to improve AUC values with 3 biomarker candidates (alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, and prostate stem cell antigen), obtaining a merged AUC value of 0.921, which is greater than those of the individual proteins (0.849, 0.873, and 0.825 for alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, and prostate stem cell antigen, resp.) (Figure 8).

Although our results require further validation in a larger collection of urine samples that contains various control samples, it appears that *A1AT*, *AGP1*, and *PSCA* are excellent biomarker candidates, with AUC values > 0.8; combining the candidates improved the AUC value of 0.921. Accordingly, the other differentially expressed proteins from our iTRAQ experiment in Appendix A are potential candidates for further validation in obtaining DN biomarkers.

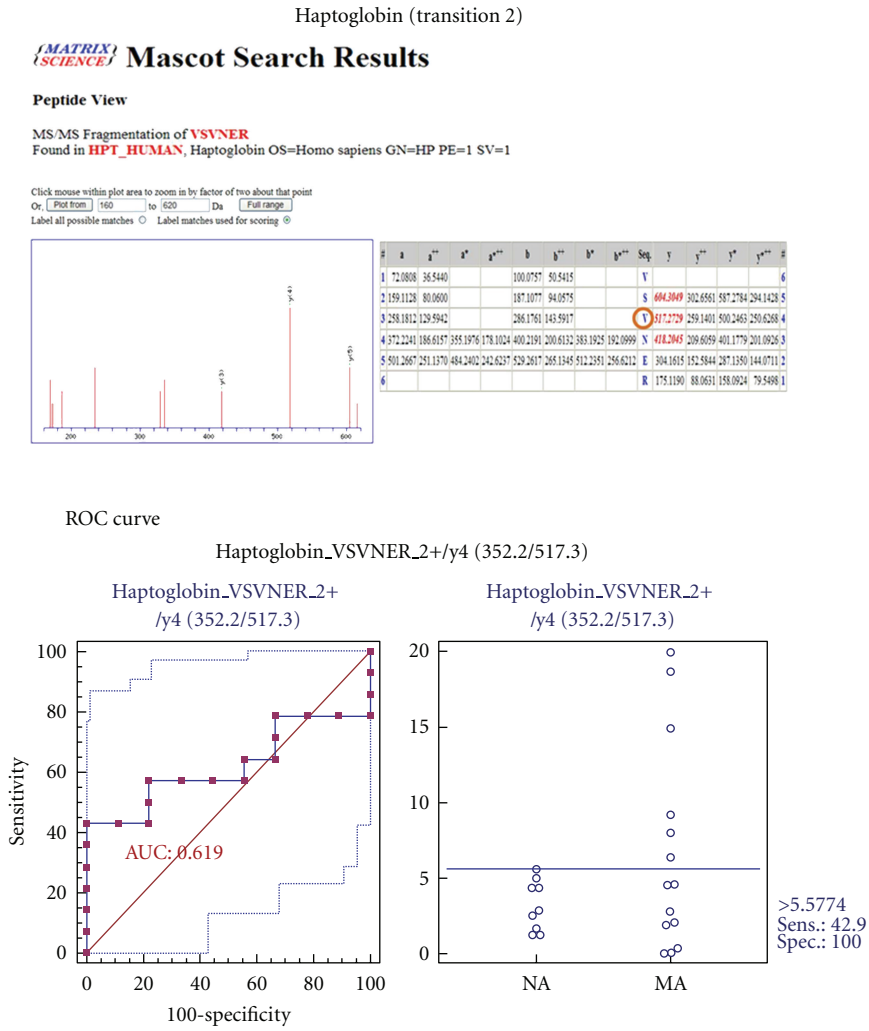


Figure 18

5. Conclusions

Microalbuminuria is used as a noninvasive index for the detection of diabetic renal disease. Yet, more specific and accurate biomarkers for DN are required, particularly in type 2 diabetic patients, due to several reasons, including nonspecific detection in nondiabetic renal disease, cardiovascular disease, inflammation, and hypertension. In our iTRAQ experiments, 710 urinary proteins were identified at a >95% confidence level, of which 196 were differentially excreted by >1.25 or <0.80—99 and 97 proteins were up- and down-regulated, respectively (Appendix A).

We prioritized 196 proteins to select preliminary biomarker candidates by characterizing them with regard to "biological process" and "molecular function" and associating them with pathogenesis. Consequently, 10 proteins were selected. To confirm and validate these candidates, 2-DE, Western blot, and MRM were performed. Based on the MRM results, *A1AT*, *AGP1*, and *PSCA*, which had AUC values > 0.8, are good biomarker candidates, and we improved the AUC value to 0.921 on combining the 3 proteins.

Further validation studies on other differentially excreted proteins might contribute to a greater understanding of the mechanism of renal dysfunction and its association with the pathogenesis of DN, facilitating the development of better biomarkers for DN.

Appendices

A. Differentially Excreted Urinary Proteome in Microalbumiuric versus Normoalbuminuric Urine

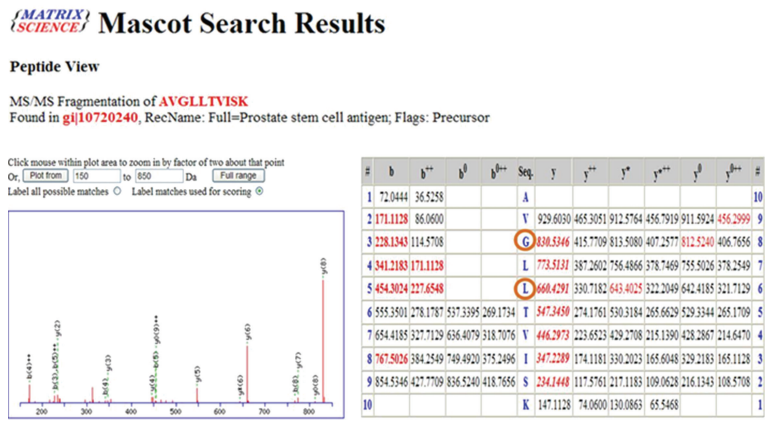
For more details, see Table 5.

B. Mascot Search and ROC Curves for Each Transition of MRM

To verify the MRM validation, we analyzed minimum of 2 transitions for each biomarker candidate protein.

Results from Mascot search and ROC curves for each transition are summarized in Figures 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19.

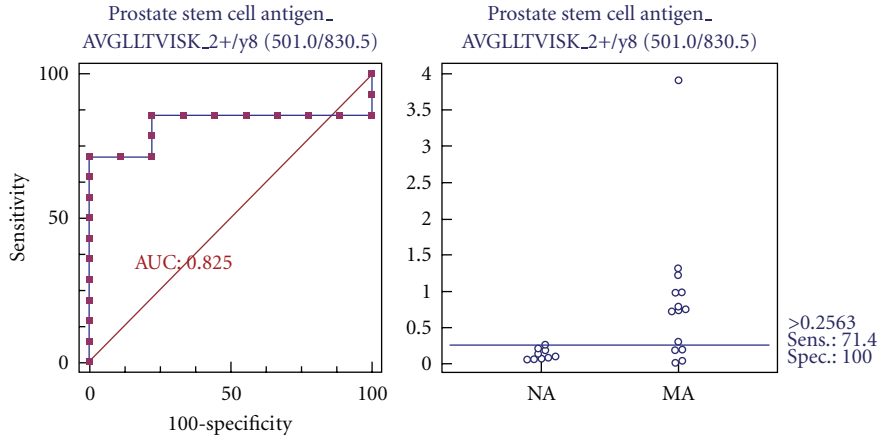
Prostate stem cell antigen (transition 1-2)



(a)

ROC curve

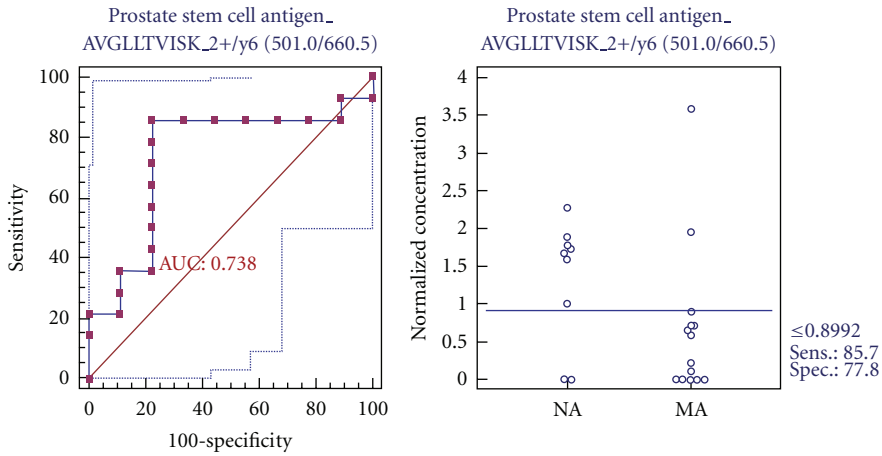
Prostate stem cell antigen_AVGLLTVISK_2+/y8 (501.0/830.5)



(b)

ROC curve

Prostate stem cell antigen_AVGLLTVISK_2+/y6 (501.0/660.4)



(c)

Figure 19

Table 5: Differentially excreted urinary proteome in microalbumiuric versus normoalbuminuric urine.

N	Unique peptides[a]	Accession number[b]	Protein name	Ratio[c] MA : NA	Pval[d] MA : NA	EF[e] MA : NA
1	651	spt|P02768	Serum albumin	3.09	0.00	1.04
2	337	gb|AAF01333.1	Serum albumin	0.36	0.00	1.08
3	669	trm|Q8N4N0	Alpha-2-glycoprotein 1	1.48	0.00	1.02
4	639	rf|NP_003352.1	uromodulin	0.72	0.00	1.04
5	269	emb|CAA42438.1	Zn-alpha2-glycoprotein	1.80	0.00	1.06
6	235	spt|P98160	HSPG	0.68	0.00	1.04
7	209	spt|P01009	Alpha-1-antitrypsin	1.42	0.00	1.04
8	414	spt|P02763	Alpha-1-acid glycoprotein 1	2.04	0.00	1.03
9	265	spt|P02788	Serotransferrin	2.46	0.00	1.10
10	46	spt|P02760	AMBP protein	1.44	0.00	1.12
11	300	spt|P07911	Uromodulin	0.24	0.00	1.08
12	106	prf|765044A	Ig G1 H Nie	0.61	0.00	1.15
13	223	dbj|BAC85395.1	Unnamed protein product	1.37	0.00	1.10
14	217	emb|CAA29229.1	Alpha-1-acid glycoprotein 1	2.29	0.00	1.11
15	107	trm|Q5VU27	Heparan sulfate proteoglycan 2	2.00	0.00	1.18
16	306	spt|P07998	Ribonuclease pancreatic	0.80	0.00	1.08
17	62	spt|P41222	Prostaglandin-H2 D-isomerase	1.40	0.00	1.13
18	47	spt|P00450	Ceruloplasmin	2.09	0.00	1.12
19	117	prf|763134A	Ig A1 Bur	1.60	0.02	1.39
20	46	cra|hCP1909255	Serine proteinase inhibitor	1.52	0.00	1.07
21	90	spt|P10451	Osteopontin	0.57	0.00	1.43
22	9	spt|P04746	Pancreatic alpha-amylase	0.41	0.00	1.33
23	39	spt|P02749	Beta-2-glycoprotein I	1.37	0.00	1.07
24	50	trm|Q9UII8	E-cadherin	1.36	0.00	1.12
25	34	rf|NP_006112.2	Keratin 1	0.60	0.00	1.16
26	88	spt|Q14624	ITIH4	0.78	0.00	1.08
27	21	trm|Q6N025	FN	1.27	0.01	1.20
28	21	trm|Q8N175	Keratin 10	0.67	0.00	1.27
29	34	emb|CAA48671.1	Alpha1-antichymotrypsin	1.64	0.00	1.17
30	50	spt|P00738	Haptoglobin	2.36	0.01	1.24
31	22	gb|AAA52014.1	Cholesterol esterase	0.46	0.00	1.10
32	63	spt|P05451	Lithostathine 1 alpha	1.54	0.00	1.05
33	31	trm|Q6PAU9	Kininogen 1	0.75	0.00	1.18
34	20	spt|P04217	Alpha-1B-glycoprotein	1.86	0.00	1.31
35	34	spt|P05155	Plasma protease C1 inhibitor	0.75	0.00	1.12
36	14	trm|Q8N473	Alpha 1 type I collagen	0.72	0.00	1.14
37	139	trm|Q6IB74	ORM2 protein	1.48	0.00	1.23
38	22	spt|Q8WZ75	Roundabout homolog 4	0.34	0.00	1.23
39	29	spt|P02791	Hemopexin	1.73	0.01	1.33
40	33	trm|Q6LBL5	GM2 activator protein	1.45	0.00	1.06
41	19	pdb|1HP7_A	A Chain A, uncleaved alpha-1-antitrypsin	1.76	0.00	1.18
42	23	spt|P55290	Cadherin-13	0.76	0.00	1.13
43	21	trm|Q8IZY7	Poly-Ig receptor	0.63	0.00	1.36
44	25	spt|P05154	Plasma serine protease inhibitor	0.76	0.00	1.12
45	10	trm|Q96CZ9	Cadherin 11, type 2, isoform 1 preproprotein	0.44	0.00	1.50
46	16	gb|AAR84237.2	Truncated epidermal growth factor	0.48	0.00	1.25
47	35	spt|P24855	Deoxyribonuclease I	0.50	0.00	1.12
48	17	trm|Q7Z645	Collagen, type VI, alpha 1	0.51	0.00	1.17
49	15	dbj|BAA19556.1	Immunoglobulin light chain V-J region	1.69	0.01	1.38
50	33	emb|CAA23842.1	Unnamed protein product	1.43	0.00	1.05

Table 5: Continued.

N	Unique peptides[a]	Accession number[b]	Protein name	Ratio[c] MA : NA	Pval[d] MA : NA	EF[e] MA : NA
51	32	spt|P08571	CD14	2.36	0.00	1.62
52	26	trm|Q6GMX2	Hypothetical protein	0.59	0.01	1.24
53	111	emb|CAA29873.2	Alpha-1-acid glycoprotein 2	2.25	0.00	1.18
54	7	trm|Q8WY99	Cathepsin C	1.58	0.00	1.17
55	11	spt|Q92820	Gamma-glutamyl hydrolase	0.54	0.01	1.33
56	13	spt|P15586	N-acetylglucosamine-6-sulfatase	1.30	0.02	1.23
57	10	gb|AAQ88523.1	AQGV3103	0.79	0.05	1.26
58	8	trm|Q8N2F4	Hypothetical protein PSEC0200	0.71	0.01	1.29
59	20	trm|Q6MZU6	Hypothetical protein DKFZp686C15213	0.39	0.00	1.28
60	27	trm|Q6LDS3	APS protein	1.34	0.00	1.08
61	9	pdb|1L9X_D	Structure Of Gamma-Glutamyl Hydrolase	0.69	0.00	1.12
62	15	spt|P07339	Cathepsin D	1.38	0.01	1.26
63	10	spt|P51884	Lumican	0.78	0.01	1.20
64	130	dbj|BAC85483.1	Unnamed protein product	0.73	0.00	1.14
65	9	pdb|1ATH_B	B Chain B, Antithrombin Iii	1.29	0.00	1.17
66	15	rf|NP_001822.2	Clusterin isoform 1	0.63	0.00	1.25
67	23	trm|Q5VW91	Decay accelerating factor for complement	1.26	0.00	1.09
68	7	spt|P54802	Alpha-N-acetylglucosaminidase	0.50	0.00	1.25
69	12	spt|Q16270	IGFBP-7	0.79	0.00	1.14
70	8	trm|Q5VZE3	Golgi phosphoprotein 2	0.38	0.02	1.46
71	10	spt|P05543	Thyroxine-binding globulin	1.27	0.00	1.11
72	9	spt|P02774	Vitamin D-binding protein	2.44	0.00	1.15
73	63	rf|NP_000573.1	Secreted phosphoprotein 1	0.58	0.00	1.24
74	56	spt|P02671	Fibrinogen alpha/alpha-E chain	0.67	0.00	1.11
75	10	trm|Q9UBG3	Tumor-related protein	0.32	0.00	1.67
76	15	trm|O00391	Quiescin Q6	0.77	0.03	1.25
77	36	trm|Q5VY30	Retinol binding protein 4, plasma	1.38	0.00	1.04
78	8	rf|NP_004675.2	SPARC-like 1	0.55	0.03	1.70
79	9	spt|Q92692	Herpesvirus entry mediator B	0.50	0.00	1.31
80	39	trm|Q96FE7	HGFL protein	0.73	0.00	1.15
81	6	spt|P43652	Afamin	4.67	0.00	1.33
82	24	pdb|1QDD_A	Lithostathine	1.73	0.00	1.20
83	24	spt|P10153	Nonsecretory ribonuclease	0.83	0.01	1.13
84	8	spt|P16278	Beta-galactosidase	1.50	0.00	1.20
85	7	trm|Q5VYK1	Collagen, type XII, alpha 1	0.75	0.00	1.15
86	9	emb|CAA37914.1	Precursor (AA-19 to 692)	1.91	0.01	1.62
87	15	trm|Q7Z5L0	Unnamed secretory protein	0.56	0.00	1.31
88	11	spt|P08236	Beta-glucuronidase	1.33	0.00	1.12
89	13	cra|hCP51001.2	superoxide dismutase 3	0.45	0.00	1.38
90	17	pir|S13195	Ganglioside M2 activator protein	1.33	0.00	1.18
91	12	cra|hCP1858145	Protein C receptor, endothelial	1.42	0.00	1.22
92	12	trm|Q6IAT8	B2M protein	1.48	0.00	1.08
93	7	trm|Q9Y5X6	Glutamate carboxypeptidase	1.47	0.00	1.04
94	13	spt|P06702	Calgranulin B	0.41	0.00	1.36
95	11	emb|CAB90482.1	Human type XVIII collagen	0.56	0.01	1.48
96	7	trm|O00533	Neural cell adhesion molecule	0.73	0.02	1.29
97	72	spt|P04745	Salivary alpha-amylase	1.25	0.00	1.12
98	40	spt|O75594	Peptidoglycan recognition protein	0.56	0.00	1.08
99	127	emb|CAA40946.1	Immunoglobulin lambda light chain	1.71	0.01	1.47
100	15	trm|Q9UJ36	Transmembrane glycoprotein	0.74	0.01	1.25
101	4	gb|AAH17802.1	SPRR3 protein	0.14	0.00	1.25
102	9	spt|Q01469	Fatty acid-binding protein	0.29	0.00	1.34

Table 5: Continued.

N	Unique peptides[a]	Accession number[b]	Protein name	Ratio[c] MA : NA	Pval[d] MA : NA	EF[e] MA : NA
103	4	trm|Q6FGL5	LCN2 protein	1.36	0.01	1.33
104	10	trm|Q9UMV3	MBL-associated serine protease 2	0.29	0.00	1.34
105	10	gb|AAH30653.1	Cadherin 13, preproprotein	2.91	0.00	1.76
106	6	spt|Q9H8L6	Multimerin 2	0.69	0.02	1.34
107	33	trm|Q9UD19	Intron-containing kallikrein	0.73	0.02	1.30
108	4	spt|P07195	L-lactate dehydrogenase B chain	0.75	0.00	1.16
109	9	spt|P08185	Corticosteroid-binding globulin	3.22	0.00	1.79
110	14	trm|Q5UGI3	Ubiquitin C splice variant	1.37	0.01	1.24
111	6	pdb|1O1P_D	D Chain D, Deoxy Hemoglobin	0.55	0.00	1.32
112	7	spt|P80723	Brain acid soluble protein 1	2.24	0.00	1.46
113	7	spt|P19320	Vascular cell adhesion protein 1	1.25	0.03	1.23
114	6	spt|P27797	Calreticulin	1.39	0.00	1.13
115	10	spt|Q01459	Di-N-acetylchitobiase	1.43	0.01	1.26
116	6	trm|Q6PN97	Alpha 2 macroglobulin	3.04	0.00	1.84
117	5	gb|AAV40827.1	superoxide dismutase 3	0.48	0.00	1.15
118	3	spt|P08473	Neprilysin	2.26	0.00	1.51
119	61	trm|Q9Y5Y7	LYVE-1	1.63	0.00	1.03
120	2	spt|P26038	Moesin	1.56	0.00	1.18
121	9	trm|Q6PIJ0	FCGR3A protein	0.77	0.00	1.06
122	7	spt|P14209	T cell surface glycoprotein E2	2.02	0.02	1.61
123	6	spt|P02765	Alpha-2-HS-glycoprotein	1.69	0.00	1.20
124	215	pir|A23746	Ig kappa chain V-III	1.31	0.00	1.14
125	3	trm|Q9NT71	Hypothetical protein DKFZp761A051	0.74	0.03	1.31
126	6	trm|Q9Y4W4	Type XV collagen	0.52	0.00	1.25
127	5	cra|hCP42501.1	Complement component 1	0.74	0.00	1.20
128	11	spt|P09564	T-cell antigen CD7	0.67	0.01	1.26
129	6	dbj|BAA86053.1	Carboxypeptidase E	0.79	0.02	1.20
130	6	spt|P15151	Poliovirus receptor	1.46	0.01	1.51
131	13	trm|Q8IUP2	Protocadherin 1, isoform 1	0.49	0.02	1.75
132	3	trm|Q8NBK0	Hypothetical protein	1.31	0.01	1.19
133	5	spt|P22891	Vitamin K-dependent protein Z	0.76	0.01	1.21
134	6	trm|Q9UNF4	Hyaluronic acid receptor	1.50	0.01	1.29
135	11	trm|Q9HCU0	Tumor endothelial marker 1	0.46	0.00	1.36
136	8	spt|P35527	Keratin, type I cytoskeletal 9	0.55	0.00	1.29
137	3	spt|P55285	Cadherin-6	0.41	0.00	1.39
138	3	trm|Q9BYH7	Scavenger receptor with C-type lectin type I	1.39	0.01	1.23
139	2	trm|Q13942	Calmodulin	2.64	0.00	1.39
140	8	spt|P04004	Vitronectin	1.87	0.00	1.42
141	11	trm|Q86Z23	Hypothetical protein	2.11	0.01	1.49
142	140	trm|Q9NWE3	Hypothetical protein FLJ10084	1.55	0.00	1.04
143	99	trm|Q9NWE3	Hypothetical protein FLJ10084	1.53	0.00	1.06
144	11	spt|P05109	Calgranulin A	0.42	0.00	1.34
145	58	trm|Q9NWE3	Hypothetical protein FLJ10084	1.50	0.00	1.08
146	3	trm|Q9BYH7	Scavenger receptor with C-type lectin type I	1.65	0.00	1.24
147	4	gb|AAA52018.1	Chromogranin A	0.52	0.00	1.26
148	4	emb|CAI20248.1	PPGB	1.51	0.00	1.24
149	92	pir|S12443	Ig lambda chain (Ke+O−)−human	1.35	0.03	1.24
150	7	trm|Q5TEQ5	OTTHUMP00000044363	0.29	0.00	1.58

Table 5: Continued.

N	Unique peptides[a]	Accession number[b]	Protein name	Ratio[c] MA : NA	Pval[d] MA : NA	EF[e] MA : NA
151	18	trm|Q6UX86	GPPS559	2.08	0.01	1.60
152	2	trm|Q8TCZ2	MIC2L1	0.80	0.01	1.16
153	5	spt|P61970	Nuclear transport factor 2	0.78	0.01	1.18
154	284	pdb|1T04_C	Anti-ifn-gamma fab in C2 space group	1.45	0.02	1.28
155	8	spt|P00790	Pepsin A	0.80	0.00	1.15
156	2	cra|hCP1778903.1	CD7 antigen	0.48	0.00	1.03
157	5	trm|O00480	Butyrophilin, subfamily 2	0.68	0.00	1.12
158	5	trm|O43653	Prostate stem cell A	1.70	0.00	1.25
159	4	trm|Q9BX83	Hemoglobin alpha 1 globin chain	0.67	0.05	1.47
160	9	spt|P11684	Uteroglobin	3.16	0.00	1.86
161	7	trm|Q7LDY7	Alpha-KG-E2	1.47	0.00	1.17
162	3	trm|Q9BYH7	Scavenger receptor	1.92	0.00	1.25
163	4	trm|Q6AZK5	KRT13 protein	0.56	0.02	1.58
164	5	spt|P09619	PDGF-R-beta	0.66	0.01	1.29
165	5	pdb|5TTR_H	Leu 55 Pro Transthyretin	1.46	0.02	1.33
166	3	rf|NP_877418.1	Mucin 1, transmembrane	0.49	0.02	1.78
167	2	trm|Q5SWW9	OTTHUMP00000060590	0.39	0.01	1.76
168	2	rf|NP_003217.2	Trefoil factor 3	1.63	0.00	1.22
169	3	spt|Q99574	Neuroserpin	0.63	0.01	1.36
170	21	trm|Q9Y3U9	Hypothetical protein DKFZp566C243	0.75	0.00	1.06
171	23	gb|AAB27607.1	Prostaglandin D synthase	1.20	0.00	1.10
172	4	gb|AAO11857.1	Immunoglobulin	1.38	0.00	1.14
173	2	trm|Q5VTA6	Cubilin	0.79	0.03	1.23
174	3	trm|Q5SY67	OTTHUMP00000059857	0.69	0.02	1.33
175	54	spt|P15814	Immunoglobulin lambda-like polypeptide 1	2.50	0.01	1.37
176	2	spt|P22352	Plasma glutathione peroxidase	0.70	0.00	1.13
177	7	gb|AAL68978.1	Mutant beta globin	0.32	0.00	1.77
178	6	spt|P35908	KCytokeratin 2e	0.50	0.03	1.74
179	284	dbj|BAB18261.1	Anti-HBs antibody light chain	3.19	0.00	1.53
180	3	rf|XP_370615.2	Hypothetical protein	0.53	0.03	1.72
181	164	gb|AAB50880.2	Anitubulin IgG1 kappa VL chain	1.64	0.00	1.30
182	170	dbj|BAC01692.1	Immunoglobulin kappa light chain	2.49	0.01	1.79
183	2	trm|Q7RTN9	Type II keratin K6h	0.43	0.01	1.52
184	3	spt|P21926	Motility-related protein	1.70	0.03	1.45
185	2	spt|Q13873	Bone morphogenetic protein receptor	0.61	0.01	1.08
186	4	gb|AAA62175.1	Heat shock protein 27	0.53	0.00	1.38
187	15	spt|P02787	transferrin	1.86	0.00	1.25
188	2	spt|P07108	Acyl-CoA-binding protein	2.20	0.02	1.47
189	2	spt|Q9NZH0	G protein-coupled receptor family C	1.27	0.04	1.25
190	2	trm|Q96E46	Fructose-1,6-bisphosphatase 1	0.44	0.00	1.01
191	44	gb|AAB53267.1	Immunoglobulin V-region light chain	1.40	0.03	1.30
192	8	gb|AAR32503.1	Immunoglobulin heavy chain	0.46	0.00	1.19
193	7	rf|NP_653247.1	Immunoglobulin J chain	0.37	0.01	1.88
194	29	emb|CAA12585.1	Ig heavy chain variable region	1.50	0.00	1.13
195	4	gb|AAD16731.1	Immunoglobulin lambda light chain	1.58	0.00	1.11
196	33	trm|Q6UXB8	HGSC289 (OTTHUMP00000039678)	1.27	0.04	1.22

[a]The numbers of unique peptides and MS/MS spectrum observed by ProteinPilot software were determined only for those peptides with ≥95% confidence.
[b]Accession numbers represent entries in the Human CDS database (human KBMS 5.0, 2005-03-02; a total of 187,748 entries provided by Applied Biosystems).
[c–e]The iTRAQ ratio, *P* value, and EF value in microalbumiuric versus normoalbuminuric urine, respectively.

Authors' Contribution

J. Jin and Y. H. Ku contributed equally to this study.

Acknowledgments

This paper was supported by the 21C Frontier Functional Proteomics Project of the Korean Ministry of Science and Technology (Grant no. FPR 08-A2-110) and a Grant (no. 10035353) from the Seoul R&BD Program.

References

[1] R. Klein, B. E. K. Klein, S. E. Moss, and K. J. Cruickshanks, "The wisconsin epidemiologic study of diabetic retinopathy: XVII. The 14- year incidence and progression of diabetic retinopathy and associated risk factors in type 1 diabetes," *Ophthalmology*, vol. 105, no. 10, pp. 1801–1815, 1998.

[2] F. P. Schena and L. Gesualdo, "Pathogenetic mechanisms of diabetic nephropathy," *Journal of the American Society of Nephrology*, vol. 16, supplement 1, pp. S30–S33, 2005.

[3] B. F. Schrijvers, A. S. De Vriese, and A. Flyvbjerg, "From hyperglycemia to diabetic kidney disease: the role of metabolic, hemodynamic, intracellular factors and growth factors/cytokines," *Endocrine Reviews*, vol. 25, no. 6, pp. 971–1010, 2004.

[4] Y. Wang, J. Zhou, A. W. Minto et al., "Altered vitamin D metabolism in type II diabetic mouse glomeruli may provide protection from diabetic nephropathy," *Kidney International*, vol. 70, no. 5, pp. 882–891, 2006.

[5] S. Jain, A. Rajput, Y. Kumar, N. Uppuluri, A. S. Arvind, and U. Tatu, "Proteomic analysis of urinary protein markers for accurate prediction of diabetic kidney disorder," *Journal of Association of Physicians of India*, vol. 53, pp. 513–520, 2005.

[6] M. Berger, D. Mönks, C. Wanner, and T. H. Lindner, "Diabetic nephropathy: an inherited disease or just a diabetic complication?" *Kidney and Blood Pressure Research*, vol. 26, no. 3, pp. 143–154, 2003.

[7] M. B. Gomes and V. G. Nogueira, "Acute-phase proteins and microalbuminuria among patients with type 2 diabetes," *Diabetes Research and Clinical Practice*, vol. 66, no. 1, pp. 31–39, 2004.

[8] H. L. Hillege, V. Fidler, G. F. H. Diercks et al., "Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population," *Circulation*, vol. 106, no. 14, pp. 1777–1782, 2002.

[9] H. C. Gerstein, J. F. E. Mann, Q. Yi et al., "Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals," *Journal of the American Medical Association*, vol. 286, no. 4, pp. 421–426, 2001.

[10] Y. H. Yang, S. Zhang, J. F. Cui et al., "Diagnostic potential of serum protein pattern in type 2 diabetic nephropathy," *Diabetic Medicine*, vol. 24, no. 12, pp. 1386–1392, 2007.

[11] H. H. Otu, H. Can, D. Spentzos et al., "Prediction of diabetic nephropathy using urine proteomic profiling 10 years prior to development of nephropathy," *Diabetes Care*, vol. 30, no. 3, pp. 638–643, 2007.

[12] P. V. Rao, X. Lu, M. Standley et al., "Proteomic identification of urinary biomarkers of diabetic nephropathy," *Diabetes Care*, vol. 30, no. 3, pp. 629–637, 2007.

[13] K. Sharma, S. Lee, S. Han et al., "Two-dimensional fluorescence difference gel electrophoresis analysis of the urine proteome in human diabetic nephropathy," *Proteomics*, vol. 5, no. 10, pp. 2648–2655, 2005.

[14] L. R. Zieske, "A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies," *Journal of Experimental Botany*, vol. 57, no. 7, pp. 1501–1508, 2006.

[15] K. Aggarwal, L. H. Choe, and K. H. Lee, "Shotgun proteomics using the iTRAQ isobaric tags," *Briefings in Functional Genomics and Proteomics*, vol. 5, no. 2, pp. 112–120, 2006.

[16] K. Aggarwal, L. H. Choe, and K. H. Lee, "Quantitative analysis of protein expression using amine-specific isobaric tags in *Escherichia coli* cells expressing *rhsA* elements," *Proteomics*, vol. 5, no. 9, pp. 2297–2308, 2005.

[17] A. Glen, C. S. Gan, F. C. Hamdy et al., "ITRAQ-facilitated proteomic analysis of human prostate cancer cells identifies proteins associated with progression," *Journal of Proteome Research*, vol. 7, no. 3, pp. 897–907, 2008.

[18] Y. Ogata, M. C. Charlesworth, L. Higgins, B. M. Keegan, S. Vernino, and D. C. Muddiman, "Differential protein expression in male and female human lumbar cerebrospinal fluid using iTRAQ reagents after abundant protein depletion," *Proteomics*, vol. 7, no. 20, pp. 3726–3734, 2007.

[19] P. L. Ross, Y. N. Huang, J. N. Marchese et al., "Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents," *Molecular and Cellular Proteomics*, vol. 3, no. 12, pp. 1154–1169, 2004.

[20] A. Matheson, M. D. P. Willcox, J. Flanagan, and B. J. Walsh, "Urinary biomarkers involved in type 2 diabetes: a review," *Diabetes/Metabolism Research and Reviews*, vol. 26, no. 3, pp. 150–171, 2010.

[21] M. Afkarian, M. Bhasin, S. T. Dillon et al., "Optimizing a proteomics platform for urine biomarker discovery," *Molecular and Cellular Proteomics*, vol. 9, no. 10, pp. 2195–2204, 2010.

[22] J. Luo, T. Ning, Y. Sun et al., "Proteomic analysis of rice endosperm cells in response to expression of HGM-CSF," *Journal of Proteome Research*, vol. 8, no. 2, pp. 829–837, 2009.

[23] J. Jin, J. Park, K. Kim et al., "Detection of differential proteomes of human β-cells during islet-like differentiation using iTRAQ labeling," *Journal of Proteome Research*, vol. 8, no. 3, pp. 1393–1403, 2009.

[24] I. V. Shilov, S. L. Seymourt, A. A. Patel et al., "The paragon algorithm, a next generation search engine that uses sequence temperature values sequence temperature values and feature probabilities to identify peptides from tandem mass spectra," *Molecular and Cellular Proteomics*, vol. 6, no. 9, pp. 1638–1655, 2007.

[25] A. Pierce, R. D. Unwin, C. A. Evans et al., "Eight-channel iTRAQ enables comparison of the activity of six leukemogenic tyrosine kinases," *Molecular and Cellular Proteomics*, vol. 7, no. 5, pp. 853–863, 2008.

[26] J. Park, S. Kim, J. K. Oh et al., "Identification of differentially expressed proteins in imatinib mesylate-resistant chronic myelogenous cells," *Journal of Biochemistry and Molecular Biology*, vol. 38, no. 6, pp. 725–738, 2005.

[27] J. Park, H. Kwon, Y. Kang, and Y. Kim, "Proteomic analysis of O-GlcNAc modifications derived from streptozotocin and glucosamine induced β-cell apoptosis," *Journal of Biochemistry and Molecular Biology*, vol. 40, no. 6, pp. 1058–1068, 2007.

[28] K. Kim, S. J. Kim, H. G. Yu et al., "Verification of biomarkers for diabetic retinopathy by multiple reaction monitoring," *Journal of Proteome Research*, vol. 9, no. 2, pp. 689–699, 2010.

[29] C. Shao, M. Li, X. Li et al., "A tool for biomarker discovery in the urinary proteome: a manually curated human and animal urine protein biomarker database," *Molecular and Cellular Proteomics*, vol. 10, no. 11, 2011.

[30] T. Narita, M. Hosoba, M. Kakei, and S. Ito, "Increased urinary excretions of immunoglobulin G, ceruloplasmin, and transferrin predict development of microalbuminuria in patients with type 2 diabetes," *Diabetes Care*, vol. 29, no. 1, pp. 142–144, 2006.

[31] S. Anderson and B. M. Brenner, "Pathogenesis of diabetic glomerulopathy: hemodynamic considerations," *Diabetes/Metabolism Reviews*, vol. 4, no. 2, pp. 163–177, 1988.

[32] R. Zatz, T. W. Meyer, H. G. Rennke, and B. M. Brenner, "Predominance of hemodynamic rather than metabolic factors in the pathogenesis of diabetic glomerulopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 17, pp. 5963–5967, 1985.

[33] L. Musante, G. Candiano, M. Bruschi et al., "Characterization of plasma factors that alter the permeability to albumin within isolated glomeruli," *Proteomics*, vol. 2, no. 2, pp. 197–205, 2002.

[34] V. Thongboonkerd and P. Malasit, "Renal and urinary proteomics: current applications and challenges," *Proteomics*, vol. 5, no. 4, pp. 1033–1042, 2005.

[35] V. Thongboonkerd, M. T. Barati, K. R. McLeish et al., "Alterations in the renal elastin-elastase system in type 1 diabetic nephropathy identified by proteomic analysis," *Journal of the American Society of Nephrology*, vol. 15, no. 3, pp. 650–662, 2004.

[36] H. H. Y. Ngai, W. H. Sit, P. P. Jiang, V. Thongboonkerd, and J. M. F. Wan, "Markedly increased urinary preprohaptoglobin and haptoglobin in passive heymann nephritis: a differential proteomics approach," *Journal of Proteome Research*, vol. 6, no. 8, pp. 3313–3320, 2007.

[37] J. T. Tamsma, J. Van Den Born, J. A. Bruijn et al., "Expression of glomerular extracellular matrix components in human diabetic nephropathy: decrease of heparan sulphate in the glomerular basement membrane," *Diabetologia*, vol. 37, no. 3, pp. 313–320, 1994.

[38] C. Y. Hong and K. S. Chia, "Markers of diabetic nephropathy," *Journal of Diabetes and its Complications*, vol. 12, no. 1, pp. 43–60, 1998.

[39] W. Z. Ye, D. Dubois-Laforgue, C. Bellanné-Chantelot, J. Timsit, and G. Velho, "Variations in the vitamin D-binding protein (Gc locus) and risk of type 2 diabetes mellitus in French Caucasians," *Metabolism: Clinical and Experimental*, vol. 50, no. 3, pp. 366–369, 2001.

[40] A. Nykjaer, D. Dragun, D. Walther et al., "An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D_3," *Cell*, vol. 96, no. 4, pp. 507–515, 1999.

[41] T. Fournier, N. Medjoubi-N, and D. Porquet, "Alpha-1-acid glycoprotein," *Biochimica et Biophysica Acta*, vol. 1482, no. 1-2, pp. 157–171, 2000.

[42] A. Corso, G. Serricchio, P. Zappasodi et al., "Assessment of renal function in patients with multiple myeloma: the role of urinary proteins," *Annals of Hematology*, vol. 78, no. 8, pp. 371–375, 1999.

[43] E. A. Reece, I. Ji, Y. K. Wu, and Z. Zhao, "Characterization of differential gene expression profiles in diabetic embryopathy using DNA microarray analysis," *American Journal of Obstetrics and Gynecology*, vol. 195, no. 4, pp. 1075–1080, 2006.

[44] Z. Gu, G. Thomas, J. Yamashiro et al., "Prostate stem cell antigen (PSCA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer," *Oncogene*, vol. 19, no. 10, pp. 1288–1296, 2000.

Hindawi Publishing Corporation
Experimental Diabetes Research
Volume 2012, Article ID 234812, 10 pages
doi:10.1155/2012/234812

Research Article

L(+) and D(−) Lactate Are Increased in Plasma and Urine Samples of Type 2 Diabetes as Measured by a Simultaneous Quantification of L(+) and D(−) Lactate by Reversed-Phase Liquid Chromatography Tandem Mass Spectrometry

Jean L. J. M. Scheijen,[1] Nordin M. J. Hanssen,[1,2] Marjo P. H. van de Waarenburg,[1] Daisy M. A. E. Jonkers,[3] Coen D. A. Stehouwer,[1,2] and Casper G. Schalkwijk[1,2]

[1] *Laboratory for Metabolism and Vascular Medicine, Department of Internal Medicine, Maastricht University, P. Debeyelaan 25, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands*
[2] *Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands*
[3] *Division of Gastroenterology and Hepatology, Department of Internal Medicine, Maastricht University, Maastricht, The Netherlands*

Correspondence should be addressed to Jean L. J. M. Scheijen, j.scheijen@maastrichtuniversity.nl

Received 7 July 2011; Revised 8 November 2011; Accepted 9 November 2011

Academic Editor: N. Sattar

Background. Plasma and urinary levels of D-lactate have been linked to the presence of diabetes. Previously developed techniques have shown several limitations to further evaluate D-lactate as a biomarker for this condition. *Methods*. D- and L-lactate were quantified using ultraperformance liquid chromatography tandem mass spectrometry with labelled internal standard. Samples were derivatized with diacetyl-L-tartaric anhydride and separated on a C_{18}-reversed phase column. D- and L-lactate were analysed in plasma and urine of controls, patients with inflammatory bowel disease (IBD), and patients with type 2 diabetes (T2DM). *Results*. Quantitative analysis of D- and L-lactate was achieved successfully. Calibration curves were linear ($r^2 > 0.99$) over the physiological and pathophysiological ranges. Recoveries for urine and plasma were between 96% and 113%. Inter- and intra-assay variations were between 2% and 9%. The limits of detection of D-lactate and L-lactate in plasma were 0.7 μmol/L and 0.2 μmol/L, respectively. The limits of detection of D-lactate and L-lactate in urine were 8.1 nmol/mmol creatinine and 4.4 nmol/mmol creatinine, respectively. Plasma and urinary levels of D- and L-lactate were increased in patients with IBD and T2DM as compared with controls. *Conclusion*. The presented method proved to be suitable for the quantification of D- and L-lactate and opens the possibility to explore the use of D-lactate as a biomarker.

1. Introduction

There are several conditions in which D-lactate can become increased in blood and urine in humans [1]. Recent studies demonstrated increased levels of D-lactate in diabetes and in infection, ischemia, and trauma, suggesting the use of D-lactate as a biomarker. However, to further explore the use of D-lactate as such a biomarker, there is a need of an improved method for analysing D-lactate.

Lactate has two optical isomers, L-lactate and D-lactate (Figure 1(b)). L-lactate is the most abundant enantiomer of lactate. It is formed mainly during anaerobic glycolysis by conversion of pyruvate to L-lactate by lactate dehydrogenase [2]. D-lactate is often considered as the nonphysiological counterpart of L-lactate [1]. Under physiologic conditions, the concentration of D-lactate is a 100-fold lower when compared to L-lactate [3]. The origin of D-lactate in human metabolism is thought to be derived from two major sources, namely, degradation of methylglyoxal into D-lactate by the glyoxalase pathway and production by intestinal bacteria. Indeed, disturbances in these metabolic pathways are associated with increased levels of D-lactate [3–7]. Although

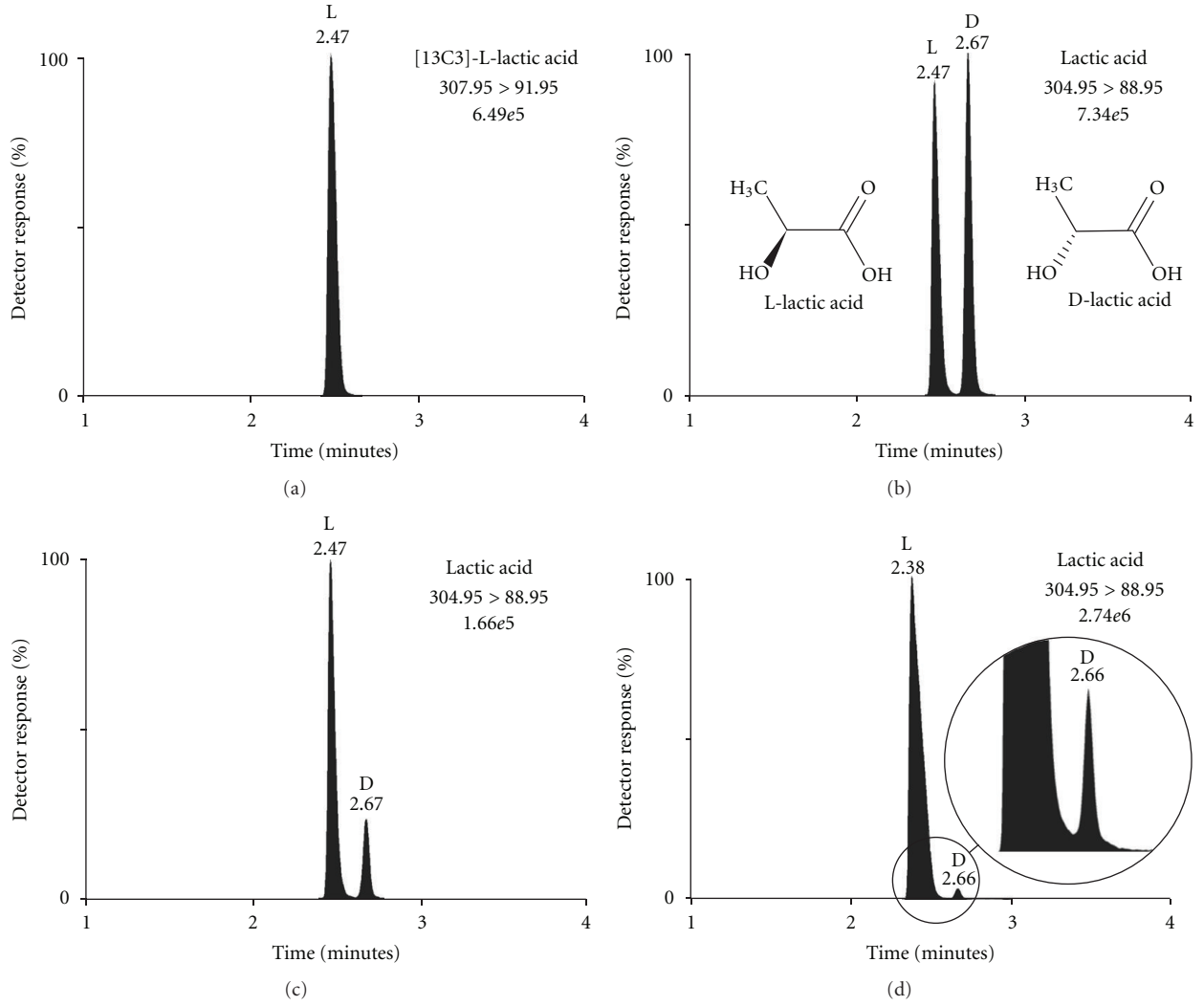


Figure 1: Representative chromatograms of D- and L-lactic acid derivatives[(a)]. (a) Internal standard [$^{13}C_3$]-L-lactate chromatogram (435 μmol/L, 72 pmol). (b) Representative chromatogram of a standard solution of D- and L-Lactate (351 μmol/L, 58.2 pmol, 501 μmol/L, and 83.0 pmol, resp.) and molecular structures of optical isomers L- and D-lactates. (c) Representative chromatogram of a urine sample (D- and L-Lactate; 14.1 μmol/L, 2.3 pmol, 74.6 μmol/L, and 12.4 pmol, resp.). (d) Representative chromatogram of a plasma sample (D- and L-Lactate; 11.2 μmol/L, 1.9 pmol, 1375.0 μmol/L, and 227.7 pmol, resp.). [(a)] Injection volume: 2 μL.

some enzymes capable of metabolizing D-lactate have been described [8], its metabolism is very inefficient, and D-lactate is mainly excreted in urine [1].

Methylglyoxal is a highly reactive compound formed in the process of glycolysis and lipid peroxidation. Methylglyoxal is increased in diabetes and is a major precursor in the formation of advanced glycation endproducts [9]. Methylglyoxal is degraded by the glyoxalase system resulting in D-Lactate. D-lactate in plasma and urine has been shown to be increased in patients with diabetes [3, 7]. D-lactate can be used as a reflection of methylglyoxal and is much easier to measure than the very reactive methylglyoxal.

In the colon, many commensal bacteria produce D-lactate as a result of anaerobic glycolysis. Under physiological circumstances, this D-lactate is further metabolized by the commensal bacteria to acetate. Therefore, D-lactate produced in the intestinal tract does not significantly contribute to levels of D-lactate in the systemic circulation under physiological circumstances [10]. However, under pathologic conditions, systemic D-lactate levels may rise due to intestinal production by bacteria. In patients with ulcerative colitis, gut ischemia, and appendicitis, increased levels of D-lactate have indeed been demonstrated [4–6]. The most extreme example of impaired gut permeability and bacterial overgrowth is short bowel syndrome, which is associated with D-lactate acidosis [10].

So far, D- and L-Lactate have been analysed by several different techniques ranging between chiral stationary phase liquid chromatography using UV or fluorescence detection [3, 11–15], enzymatic assays [7, 16–21], gas chromatography mass spectrometry (GC/MS) methods [22, 23], liquid chromatography mass spectrometry (LC/MS) methods [24, 25], and reversed phase liquid chromatography using fluorescence detection [26]. However, these techniques have

Table 1: MRM settings.

Component	Parent ion (m/z)	Daughter ion (m/z)	Collision energy (eV)	Dwell (secs)
[$^{13}C_3$]-L-lactate	307.95	91.95	8.0	0.1
D-lactate	304.95	88.95	8.0	0.1
L-lactate	304.95	88.95	8.0	0.1

several shortcomings such as low sensitivity [11, 24, 27], large sample volume [19, 21, 22], complex chromatographic systems [3, 12, 13], and long run times [3, 11, 26, 27]. To further explore the use of D-lactate as a biomarker, there is a need of an improved method for analysing D-lactate.

In this paper, we describe a highly sensitive, specific and fast ultraperformance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) method for the analysis of D- and L-Lactate in plasma and urine without the need of a chiral stationary phase. We achieved a significant improvement over the methods described in the literature and obtained a strong tool for the analysis of D- and L-Lactate in large studies. With this method, we measured plasma and urine concentrations of D- and L-Lactate in controls, patients with inflammatory bowel disease (IBD), and in patients with type 2 diabetes (T2DM).

2. Materials and Methods

2.1. Chemicals and Reagents. L(+)-lactate (98%) and dichloromethane (≥99.9%) were obtained from Sigma-Aldrich. Ammonia solution (25%) and acetic acid anhydrous (100%) were obtained from Merck. Formic acid (p.a.), (+)-O,O′-diacetyl-L-tartaric anhydride (≥97%) (DATAN) and lithium D-lactate (≥99%) were obtained from Fluka. Water and acetonitrile (ULC-MS grade) were obtained from Biosolve. [$^{13}C_3$]-Sodium L-lactate (20%, w/w in water) was obtained from Cambridge Isotope Laboratories.

2.2. Chromatographic Conditions. Samples were analysed by reversed phase LC-tandem MS using an Acquity UPLC BEH C_{18} analytical column (100 × 2.1 mm, 1.7 μm, Waters). Detection was carried out using a Xevo TQ tandem mass spectrometer (Waters), which was operated in negative multiple-reaction-monitoring (MRM) mode. UPLC analysis was performed using a binary gradient at a flow of 0.5 mL/min using an Acquity UPLC (Waters). Solvent A was 1.5 mM ammonium formate (pH = 3.6), and solvent B was acetonitrile. A linear gradient was started at 99.5% solvent A, which was changed within 3 minutes to 97% solvent A. After cleaning the column with 40% solvent B during 2 minutes, the column was equilibrated for 1 minute at the initial composition. Injection volume was 2 μL, and column temperature was set at 31°C. Samples were kept at 6°C. Chromatograms were acquired and processed with Masslynx V4.1 SCN 644 (Waters).

2.3. Mass Spectrometry Conditions. MRM transitions were optimised using direct infusion of D-lactate (500 μmol/L), [$^{13}C_3$]-L-Lactate (400 μmol/L), and L-lactate (1000 μmol/L) standard solution into the tandem MS at a flow of 20 μL/min. Optimal conditions for all parents were found at a capillary voltage of 1.5 kV and a cone voltage of 10 V. The source and desolvation temperature were 150 and 450°C, respectively. The cone gas flow and desolvation gas flow were 0 and 800 l/hour, respectively. To establish the most sensitive daughter ions, the collision energy was set at 8 eV with a collision gas flow of 0.15 mL/min. Table 1 shows the optimised MRM settings.

2.4. Plasma and Urine Samples. Three groups were selected for D- and L-Lactate measurements. Diabetic individuals and nondiabetic controls were sex- and age-matched subsets recruited from the cohort study of diabetes and atherosclerosis in Maastricht (CODAM). The characteristics of these subjects have been described in detail elsewhere [28]. In short, the control group ($n = 52$) was 55.8 ± 0.7 years old, 46% female, and had an HbA1C of 5.6 ± 0.1% and a fasting plasma glucose of 5.2 ± 0.1 mmol/L. Group 2, the patients with T2DM ($n = 52$), were 56.3 ± 0.6 years old, 39% female, and had an HbA1C of 6.9 ± 0.2% and fasting plasma glucose of 8.0 ± 0.2 mmol/L. Group 3 consisted of patients with IBD in remission: 32 plasma samples (52.3 ± 8.6 years, 44% female) and 34 urine samples (54.6 ± 14.1 years, 59% female). These samples were recruited from the outpatient-clinic of the Maastricht University Medical Center.

For comparison of the proposed UPLC tandem MS method with the enzymatic method, we analysed plasma and urinary D-lactate, with both methods, in rat samples. These animals were described in details elsewhere [29].

2.5. Plasma Sample Preparation. To 25 μL of internal standard solution (containing 434.75 μmol/L [$^{13}C_3$]-L-lactate) 25 μL of plasma was added. Samples were mixed thoroughly and subsequently deproteinized with 600 μL of a mixture of methanol : acetonitrile (1 : 1, by volume) and centrifuged at room temperature during 10 minutes at 14000 rpm. The supernatant was pipetted into a reaction vial and evaporated to dryness under a gentle stream of nitrogen at a temperature of 50°C. Fifty microliters of freshly made DATAN (50 mg/mL dichloromethane : acetic acid (4 : 1, by volume)) was added. The vial was capped, vortexed, and heated at 75°C for 30 minutes. After 30 minutes, the vial was allowed to cool down to room temperature, and the mixture was evaporated to dryness with a gentle stream of nitrogen. The derivatized residue was reconstituted with 150 μL acetonitrile : water (1 : 2, by volume).

2.6. Urine Sample Preparation. Twenty five microliters of internal standard solution (containing 434.75 μmol/L [$^{13}C_3$]-L-lactate), 25 μL urine, and 300 μL of methanol were pipetted

Table 2: Linearity tested in different matrices.

Matrix	Slope[(a)]	Y-intercept	r^2	Concentration range (μmol/L)
D-lactate				
Water	1.2927	−4.5	0.9971	0–105
Plasma A	1.4307	21.2	0.9987	0–105
Plasma B	1.3820	51.3	0.9991	0–105
Plasma C	1.5037	31.9	0.9990	0–105
Mean	1.4023			
CV(%)	6.3			
L-lactate				
Water	0.7768	−4.9	0.9996	0–3008
Plasma A	0.7566	1032	0.9998	0–6016
Plasma B	0.7164	996	0.9997	0–6016
Plasma C	0.7534	1346	0.9997	0–3008
Mean	0.7502			
CV(%)	3.2			
D-lactate				
Water	1.1265	−15.4	0.9999	0–702
Urine A	1.1092	105.24	0.9984	0–351
Urine B	1.1114	99.7	0.9999	0–702
Urine C	1.0287	69.8	0.9992	0–351
Mean	1.094			
CV(%)	4.04			
L-lactate				
Water	0.7967	0.2	0.9998	0–1002
Urine A	0.8039	60.4	0.9995	0–501
Urine B	0.7918	48.6	0.9995	0–1002
Urine C	0.7749	135.0	0.9993	0–501
Mean	0.7967			
CV(%)	1.56			

[(a)] Slope: {concentration (μmol/L)} versus {response = (peak area component/peak area internal standard). ∗Internal standard concentration (μmol/L)}.

into a reaction vial. Samples were mixed thoroughly and evaporated to dryness under a gentle stream of nitrogen at a temperature of 50°C. Fifty microliters of freshly made DATAN (50 mg/mL dichloromethane : acetic acid (4 : 1, by volume)) was added. The vial was capped, vortexed, and heated at 75°C for 30 minutes. After 30 minutes, the vial was allowed to cool down to room temperature, and the mixture was evaporated to dryness with a gentle stream of nitrogen. The derivatized residue was reconstituted with 300 μL acetonitrile : water (1 : 2, by volume).

2.7. Method Validation. Linearity of the detection of D- and L-Lactate was tested in water and matrix by adding D- and L-Lactate standard to water and during preparation of plasma or urine samples (Table 2). Calibration curves were obtained by linear regression of a plot of the analyte concentration (x) versus the peak-area ratio of the analyte/internal standard area (y). For both the analytes, [$^{13}C_3$]-L-lactate was used as internal standard.

The lower limit of detection was determined by calculating the concentration at a signal-to-noise ratio of six (s/N: 6, injection volume: 2 μL).

For recovery experiments, standard solutions of D- and L-Lactate were added to urine or plasma and subsequently prepared as described in the sample preparation section.

The intra-assay variation of the method was determined in two different plasma and urine samples ($n = 10$) analysed in one batch during one day. The interassay variation of the method was determined in two different plasma and urine samples divided into batches and analysed during 10 different days.

Freeze-thaw stability was tested in two different plasma and urine samples by snap-freezing these samples in liquid nitrogen and thawing them for 5 subsequent cycles.

To investigate the stability of plasma and urine samples, stored at 6°C in the autosampler, replicate injections of two different plasma and urine samples were done every hour during 24 hours.

2.8. Determination of Fasting Plasma Glucose, Hba1C, and Urinary Creatinine. After an overnight fast, plasma glucose concentrations (mmol/L) were measured with a hexokinase glucose-6 phosphate dehydrogenase method (ABX Diagnostics, Montpellier, France). Hba1C (%) was determined

by ion-exchange high-performance liquid chromatography (HPLC) (Bio-Rad, Veenendaal, the Netherlands). Fasting plasma glucose concentrations and Hba1C were determined in the CODAM participants only.

Urinary D- and L-Lactate concentrations were expressed as μmol/mmol creatinine. Creatinine concentration in urine was analysed using a Beckman LX20 analyser (Beckman Coulter) based on the Jaffé reaction method [30].

2.9. D-Lactate Enzymatic Assay. For method comparison, an enzymatic-spectrophotometric method, based on the oxidation of D-lactate to pyruvate by NAD^+ in the presence of bacterial D-lactate hydrogenase, was used [21].

2.10. Statistical Analysis. The method validation data were expressed as mean and standard deviation (SD). To investigate agreement between the enzymatic and UPLC tandem MS method, we used linear regression and a Bland-Altman plot after log normalisation of the rat urine samples. Limits of agreement were defined as 2 times the SD. The patient study data were expressed as mean and standard error of the mean (SEM). To detect group differences, we applied analysis of variance (ANOVA) with post hoc Bonferroni correction. P value <0.05 was considered statistically significant.

3. Results

3.1. Reversed Phase Chromatography. D- and L-Lactate DATAN derivatives yielded a baseline separation on a reversed phase UPLC column with a retention time of 2.7 minutes for D-lactate and 2.5 minutes for L-lactate. Representative chromatograms are shown in Figure 1.

3.2. Stability of D- and L-Lactate. After 5 freeze-thaw cycles no change of D- and L-Lactate levels was observed, as tested in two different plasma and urine samples (data not shown).

To make large number of measurements within one run possible, we tested the stability of D- and L-Lactate when samples were stored in the autoinjector at 6°C. D- and L-Lactate were at least stable for 24 hours, and no degradation was observed after replicate injections of two different plasma and urine samples (data not shown).

3.3. Linearity, Lower Limit of Detection, Recovery, and Precision. Linearity of the detection of D- and L-Lactate was tested in matrix and water. For plasma, the slope, tested in three different plasma samples and in water measured on different days, was 1.4 ± 6.3% (mean ± CV%) for D-lactate and 0.75 ± 3.2% for L-lactate (Table 2). For urine, the slope, tested in three different urine samples and in water measured on different days, was 1.09 ± 4.0% for D-lactate and 0.80 ± 1.6% for L-lactate (Table 2). The limits of detection of D-lactate and L-lactate in plasma were 0.65 μmol/L (108 fmol) and 0.2 μmol/L (33 fmol), respectively. The limits of detection of D-lactate and L-lactate in urine were 8.1 nmol/mmol creatinine (40 fmol); and 4.4 nmol/mmol creatinine (22 fmol) respectively.

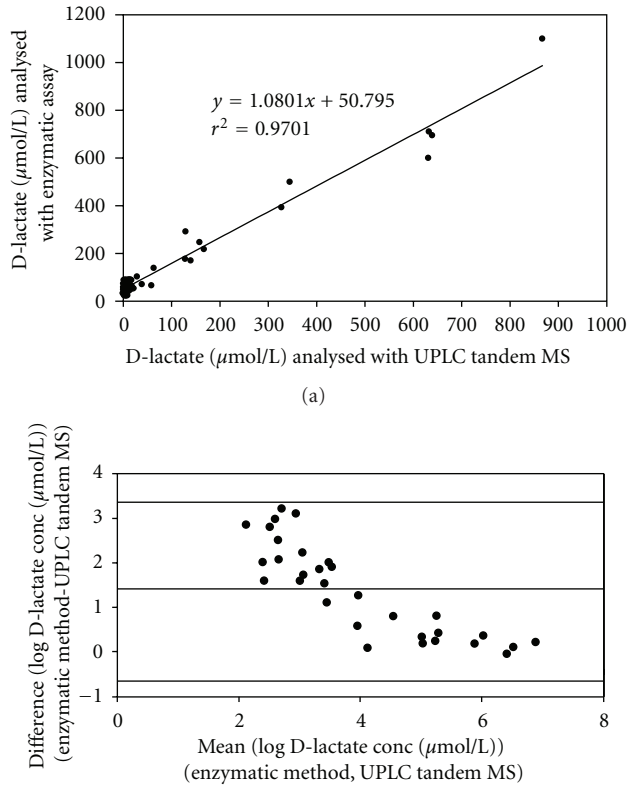


Figure 2: Comparison of urinary D-lactate in rat urine as measured by UPLC tandem MS and enzymatic method. (a) Correlation between D-lactate levels measured by UPLC tandem MS and enzymatic method. (b) Bland-Altman plot of log-transformed D-lactate levels as measured by UPLC tandem MS and enzymatic method.

We found recoveries, for urine and plasma, between 96% and 113% (Table 3). The validation data demonstrated inter- and intra-assay variations between 2% and 9% (Table 4).

3.4. Comparison of the UPLC Tandem MS Method versus the Enzymatic Assay. We compared the proposed UPLC-tandem MS method with the enzymatic assay, by analysing plasma and D-lactate levels in rat urine with both methods. However, due to low D-lactate levels in plasma, it was not possible to analyse these samples with the enzymatic assay (data not shown). For urine, linear regression of the data resulted in the equation $y = 1.08x + 50.795$, with excellent correlation ($r = 0.985$) between both techniques (Figure 2(a)). However, the Bland Altman plot showed that although in the higher range both techniques are in excellent agreement, in the lower range considerately higher values were measured with the enzymatic technique as compared with the UPLC tandem MS method (Figure 2(b)).

3.5. Comparison of Urinary and Plasma D- and L-Lactate Concentration between Controls, Individuals with T2DM, and Individuals with IBD. We next investigated D- and L-Lactate plasma and urine concentration of nondiabetic controls,

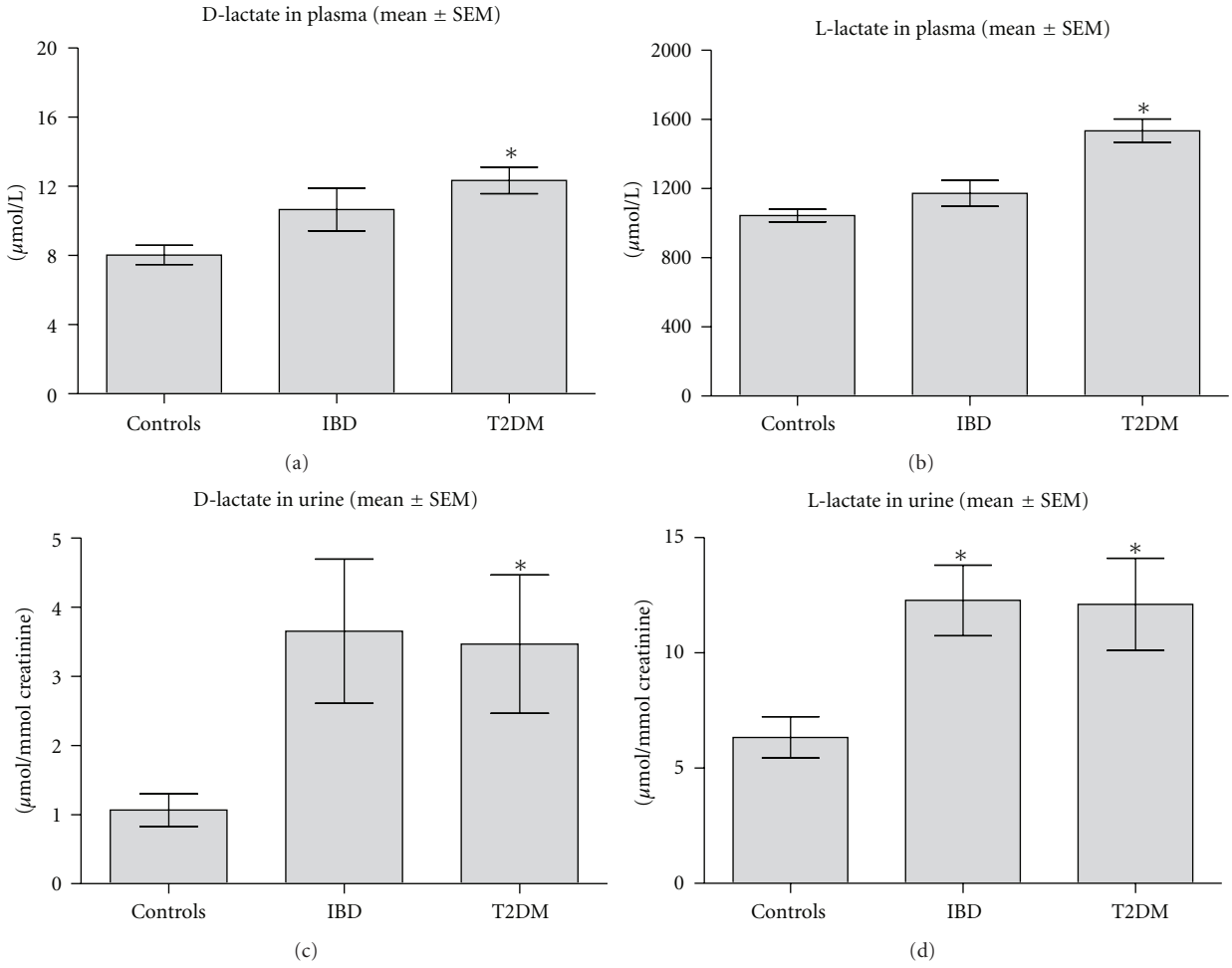


Figure 3: Urinary and plasma D- and L-Lactate concentrations of controls, patients with inflammatory bowel disease (IBD), and patients with type 2 diabetic patients (T2DM). Data are presented as Mean ± SEM: ($^*P < 0.05$).

patients with IBD, and T2DM patients (Figure 3). In healthy controls, D- and L-Lactate concentrations were 8.0 ± 0.6 and 1044.8 ± 36.7 μmol/L in plasma, respectively, and 1.1 ± 0.2 and 6.3 ± 0.9 μmol/mmol creatinine in urine, respectively (mean ± SEM). In IBD patients, levels of D- and L-Lactate were higher, when compared to the nondiabetic control group in both plasma (10.7 ± 1.2 and 1172.4 ± 74.6 μmol/L, resp.) and urine (3.1 ± 0.8 and 11.8 ± 1.4 μmol/mmol creatinine, resp.), which was significant for urinary L-lactate.

In T2DM patients, the concentrations of D- and L-Lactate were significantly higher in plasma (12.3 ± 0.8 and 1534.7 ± 67.5 μmol/L, resp.) and urine (3.4 ± 1.0 and 12.1 ± 2.0 μmol/mmol creatinine, resp.). Both plasma and urinary D-lactate levels, as determined in T2DM and controls, correlated with HbA1C ($r = 0.392$, $P < 0.001$ and $= 0.421$, $P < 0.001$, resp.) and fasting plasma glucose ($r = 0.360$, $P < 0.001$ and $r = 0.416$, $P < 0.001$, resp.).

4. Discussion

We describe here a rapid, sensitive, and highly specific method for the simultaneous determination of D- and L-Lactate in plasma and urine by UPLC MS/MS. The derivatisation of D- and L-Lactate with DATAN makes it possible to separate both enantiomers on a reversed-phase-based analytical column. This results in a robust chromatographic system without the need of a column-switching or solid phase extraction (SPE) presample cleanup. We found that urinary and plasma levels of D- and L-Lactate were significantly increased in T2DM as compared with nondiabetic controls. In addition, we observed higher L- and D-Lactate levels in patients with IBD, but only significant for urinary L-lactate.

Many other techniques have been used to quantify D- and L-Lactate, with several disadvantages, such as long run times [3, 11, 26, 27], large sample volume [19, 21, 22, 24, 27], a column-switching preseparation technique [3, 12, 13], or low sensitivity [11, 24, 27]. Moreover, a disadvantage of the enzymatic method is that it is not possible to measure D- and L-Lactate in a single run.

Chiral stationary phase liquid chromatography has been applied for the enantiomeric separation of D- and L-Lactate [11, 14, 25]. SPE or prereversed phase liquid chromatographic separation is obligatory for good chiral chromatographic performance [3, 12, 13, 31]. Furthermore,

Table 3: Recovery data for plasma and urine.

Plasma	Mean (SD) μmol/L	CV, %	Recovery, %
D-lactate added[a] μmol/L ($n = 5$)			
0	8.1 (0.2)	2.9	—
52.7	67.3 (1.2)	1.8	112.4
105.4	127.7 (3.7)	2.9	113.5
L-lactate added[a] μmol/L ($n = 5$)			
0	1365 (44.6)	3.3	—
1504	2931 (26.2)	0.9	104.2
3008	4443.2 (125.1)	2.8	102.3
Urine[b]	Mean (SD) μmol/mmol creatinine	CV, %	Recovery, %
D-lactate added[a] μmol/L ($n = 5$)			
0	0.91 (0.03)	3.4	—
98.8	8.03 (0.10)	1.3	110.9
197.6	14.34 (0.29)	2.0	104.7
L-lactate added[a] μmol/L ($n = 5$)			
0	4.84 (0.13)	2.6	—
94	10.99 (0.38)	3.5	100.6
188	16.56 (0.14)	0.8	96.0

[a] Addition of 25 μL standard solution to 25 μL plasma or urine.
[b] Creatinine concentration: 15.4 mmol/L.

Table 4: Precision data for D- and L-Lactate in plasma and urine.

Matrix	D-lactate mean (SD), μmol/L	CV,%	L-lactate mean (SD), μmol/L	CV,%
Intra-assay, $n = 10$				
Plasma A	13.0 (0.7)	5.1	1265.3 (36.2)	2.9
Plasma B	85.7 (2.5)	2.9	6605.8 (190.4)	2.9
Interassay, $n = 10$				
Plasma A	12.4 (0.6)	5.2	1338.7 (48.8)	3.6
Plasma B	85.4 (3.8)	4.4	6452.6 (245.8)	3.8
	D-lactate mean (SD), μmol/mmol creatinine		L-lactate mean (SD), μmol/mmol creatinine	CV, %
Intra-assay, $n = 10$				
Urine A[1]	0.857 (0.03)	3.8	4.40 (0.27)	6.0
Urine B[2]	16.26 (0.48)	2.9	10.58 (0.60)	5.7
Interassay, $n = 10$				
Urine A[1]	0.718 (0.04)	5.6	3.75 (0.33)	8.8
Urine B[2]	14.90 (1.05)	7.0	8.68 (0.81)	9.3

[1] Urine A, creatinine 15.4 mmol/L.
[2] Urine B, creatinine 5.9 mmol/L.

the shorter lifetime, higher cost, and difficult selection of a suitable chiral column [32, 33] have made an alternative method for enantiomeric separation desirable. More recently, Cevasco et al. [26] used a reversed phase liquid chromatography method for separation of D- and L-lactic acid. However, a run-to-run time of 35 minutes and an obligatory SPE sample preparation step make this method less feasible for large cohort studies. Moreover, the used derivatisation reagent was not commercially available and had to be synthesised before use.

Anhydrides of tartaric acid were used successfully as chiral derivatisation reagents of hydroxy acids and other enantiomeric compounds [34–36]. In this paper, we describe the derivatisation of the enantiomeric D- and L-Lactate

with DATAN. This derivatisation step results in a highly sensitive and specific D- and L-Lactate derivative which is baseline separated on a UPLC reversed phase column and detected with tandem MS. The advantage of this technique, as compared to the described methods in the literature, is that there is no need for sample cleanup or preseparation of the sample matrix, and only 25 μL of sample is necessary. Also the highly efficient and specific fragments of these DATAN derivatives generated in the collision cell is an improvement against the nonderivatized analysis of D- and L-Lactate with LC/MS [25]. With a run-to-run time of 6 minutes we established a fast and reliable method suitable for measuring D- and L-Lactate in large cohort studies.

The D- and L-Lactate concentrations we measured in plasma and urine from healthy controls are in reasonable agreement with data obtained by other techniques [3, 18, 19, 25]. Indeed, we found an acceptable correlation of the new UPLC tandem MS method with the enzymatic assay in urine samples. However, the enzymatic assay is not adequately sensitive for lower levels of D-lactate, as reflected in the Bland-Altman plot. The enzymatic method measures higher levels of D-lactate than the UPLC tandem MS in the lower range.

D-lactate was not significantly increased in patients with IBD compared with nondiabetic controls in both plasma and urine. Another study, however, found a significant increase of D-lactate in hospitalised patients with active IBD [6]. This difference may be explained by the fact that the patients we have included were in remission. In addition, due to the relatively small sample size, the power to detect statistically differences was low.

We found a statistically significant increase of urine and plasma levels of D- and L-Lactate in T2DM as compared with nondiabetic controls. The fact that both D-lactate and L-lactate are increased in patients with T2DM suggests that the hyperglycaemic state is an important source of D-lactate elevations in diabetes. L-lactate is mainly formed during glycolysis by conversion of pyruvate to L-lactate by lactate dehydrogenase. D-lactate is an endproduct of the metabolism of methylglyoxal, formed during hyperglycaemia, by the glyoxalase pathway [37]. In line with this, we demonstrate that D-lactate correlates significantly with HbA1C, a marker for prolonged hyperglycaemia. However, based on our small study, we cannot definitely conclude whether D-lactate is merely a reflection of methylglyoxal, gut-flora, or both, as several possible residual confounding factors such as BMI and gut permeability may explain the differences we observed between our patient groups.

Methylglyoxal is produced in small amount from carbohydrates, fat, and protein metabolism. It is has been demonstrated that methylglyoxal is the most important precursor in the formation of advanced glycation endproducts. Methylglyoxal and methylglyoxal-derived advanced glycation endproducts are believed to be implicated in the development of diabetic vascular complications. Because D-lactate is elevated in diabetes and may be used as an indicator of methylglyoxal, the measurement of D-lactate needs to be evaluated in cohort studies with D-lactate as a possible predictor of diabetic complications. In addition, mechanistic studies are needed to elucidate the relative contribution of several metabolic pathways to the total urinary and plasma D-lactate pool, in both healthy and diabetic individuals.

In conclusion, this specific measurement of D- and L-Lactate shows promise in the investigation of diabetes and metabolic diseases.

Abbreviations

CTMM: The Center for Translational Molecular Medicine
IBD: Inflammatory bowel disease
T2DM: Type 2 diabetes
GC/MS: Gas chromatography mass spectrometry
LC/MS: Liquid chromatography mass spectrometry
UPLC: Ultraperformance liquid chromatography
MS/MS: Tandem mass spectrometry
DATAN: (+)-O,O'-diacetyl-L-tartaric anhydride
MRM Multiple-reaction-monitoring
SEM Standard error of the mean
ANOVA Analysis of variance
SPE Solid phase extraction.

Acknowledgments

This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (http://www.ctmm.nl/), project PREDICCt (Grant 01C-104), and supported by the Netherlands Heart Foundation, Dutch Diabetes Research Foundation, and Dutch Kidney Foundation.

References

[1] J. B. Ewaschuk, J. M. Naylor, and G. A. Zello, "D-lactate in human and ruminant metabolism," *Journal of Nutrition*, vol. 135, no. 7, pp. 1619–1625, 2005.

[2] B. Stallknecht, J. Vissing, and H. Galbo, "Lactate production and clearance in exercise. Effects of training. A mini-review," *Scandinavian Journal of Medicine and Science in Sports*, vol. 8, no. 3, pp. 127–131, 1998.

[3] H. Hasegawa, T. Fukushima, J. A. Lee et al., "Determination of serum D-lactic and L-lactic acids in normal subjects and diabetic patients by column-switching HPLC with pre-column fluorescence derivatization," *Analytical and Bioanalytical Chemistry*, vol. 377, no. 5, pp. 886–891, 2003.

[4] A. P. Duzgun, G. Bugdayci, B. Sayin, M. M. Ozmen, M. V. Ozer, and F. Coskun, "Serum D-lactate: a useful diagnostic marker for acute appendicitis," *Hepato-Gastroenterology*, vol. 54, no. 77, pp. 1483–1486, 2007.

[5] M. J. Murray, M. D. Gonze, L. R. Nowak, and C. F. Cobb, "Serum D(−)-lactate levels as an aid to diagnosing acute intestinal ischemia," *American Journal of Surgery*, vol. 167, no. 6, pp. 575–578, 1994.

[6] W. B. Song, Y. H. Lv, Z. S. Zhang et al., "Soluble intercellular adhesion molecule-1, D-lactate and diamine oxidase in patients with inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 15, no. 31, pp. 3916–3919, 2009.

[7] J. P. Talasniemi, S. Pennanen, H. Savolainen, L. Niskanen, and J. Liesivuori, "Analytical investigation: assay of d-lactate in diabetic plasma and urine," *Clinical Biochemistry*, vol. 41, no. 13, pp. 1099–1103, 2008.

[8] P. K. Tubbs, "The metabolism of D-alpha-hydroxy acids in animal tissues," *Annals of the New York Academy of Sciences*, vol. 119, no. 3, pp. 920–926, 1965.

[9] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[10] M. L. Halperin and K. S. Kamel, "D-lactic acidosis: turning sugar into acids in the gastrointestinal tract," *Kidney International*, vol. 49, no. 1, pp. 1–8, 1996.

[11] J. B. Ewaschuk, J. M. Naylor, W. A. Barabash, and G. A. Zello, "High-performance liquid chromatographic assay of lactic, pyruvic and acetic acids and lactic acid stereoisomers in calf feces, rumen fluid and urine," *Journal of Chromatography B*, vol. 805, no. 2, pp. 347–351, 2004.

[12] T. Fukushima, J. A. Lee, T. Korenaga, H. Ichihara, M. Kato, and K. Imai, "Simultaneous determination of D-lactic acid and 3-hyroxybutyric acid in rat plasma using a column-switching HPLC with fluorescent derivatization with 4-nitro-7-piperazino-2, 1,3-benzoxadiazole (NBD-PZ)," *Biomedical Chromatography*, vol. 15, no. 3, pp. 189–195, 2001.

[13] H. Ichihara, T. Fukushima, and K. Imai, "Enantiomeric determination of D- and L-lactate in rat serum using high-performance liquid chromatography with a cellulose-type chiral stationary phase and fluorescence detection," *Analytical Biochemistry*, vol. 269, no. 2, pp. 379–385, 1999.

[14] S. Okubo, F. Mashige, M. Omori et al., "Enantiomeric determination of L- and D-lactic acid in human cerebrospinal fluid by chiral ligand exchange high-performance liquid chromatography," *Biomedical Chromatography*, vol. 14, no. 7, pp. 474–477, 2000.

[15] O. O. Omole, D. R. Brocks, G. Nappert, J. M. Naylor, and G. A. Zello, "High-performance liquid chromatographic assay of (+/−)-lactic acid and its enantiomers in calf serum," *Journal of Chromatography B*, vol. 727, no. 1-2, pp. 23–29, 1999.

[16] M. Demircan, S. Cetin, S. Uguralp, N. Sezgin, A. Karaman, and E. M. Gozukara, "Plasma D-lactic acid level: a useful marker to distinguish perforated from acute simple appendicitis," *Asian Journal of Surgery*, vol. 27, no. 4, pp. 303–305, 2004.

[17] S. R. Goodall and F. M. Byers, "Automated micro method for enzymatic L(+) and D(−) lactic acid determinations in biological fluids containing cellular extracts," *Analytical Biochemistry*, vol. 89, no. 1, pp. 80–86, 1978.

[18] E. Haschke-Becher, M. Baumgartner, and C. Bachmann, "Assay of D-lactate in urine of infants and children with reference values taking into account data below detection limit," *Clinica Chimica Acta*, vol. 298, no. 1-2, pp. 99–109, 2000.

[19] R. B. Brandt, S. A. Siegel, M. G. Waters, and M. H. Bloch,- "Spectrophotometric assay for D(−)-lactate in plasma," *Analytical Biochemistry*, vol. 102, no. 1, pp. 39–46, 1980.

[20] C. W. Ludvigsen, J. R. Thurn, G. L. Pierpont, and J. H. Eckfeldt, "Kinetic enzymic assay for D(−)-lactate, with use of a centrifugal analyzer," *Clinical Chemistry*, vol. 29, no. 10, pp. 1823–1825, 1983.

[21] R. Martí, E. Varela, R. M. Segura, J. Alegre, J. M. Suriñach, and C. Pascual, "Determination of D-lactate by enzymatic methods in biological fluids: study of interferences," *Clinical Chemistry*, vol. 43, no. 6, pp. 1010–1015, 1997.

[22] Y. Inoue, T. Shinka, M. Ohse, H. Ikawa, and T. Kuhara, "Application of optical isomer analysis by diastereomer derivatization GC/MS to determine the condition of patients with short bowel syndrome," *Journal of Chromatography B*, vol. 838, no. 1, pp. 37–42, 2006.

[23] Y. Inoue, T. Shinka, M. Ohse et al., "Changes in urinary level and configuration ratio of d-lactic acid in patients with short bowel syndrome," *Journal of Chromatography B*, vol. 855, no. 1, pp. 109–114, 2007.

[24] E. J. Franco, H. Hofstetter, and O. Hofstetter, "Determination of lactic acid enantiomers in human urine by high-performance immunoaffinity LC-MS," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 49, no. 4, pp. 1088–1091, 2009.

[25] D. Norton, B. Crow, M. Bishop, K. Kovalcik, J. George, and J. A. Bralley, "High performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) assay for chiral separation of lactic acid enantiomers in urine using a teicoplanin based stationary phase," *Journal of Chromatography B*, vol. 850, no. 1-2, pp. 190–198, 2007.

[26] G. Cevasco, A. M. Piatek, C. Scapolla, and S. Thea, "A simple, sensitive and efficient assay for the determination of d- and l-lactic acid enantiomers in human plasma by high-performance liquid chromatography," *Journal of Chromatography A*, vol. 1218, pp. 787–792, 2011.

[27] L. Tan, Y. Wang, X. Liu, H. Ju, and J. Li, "Simultaneous determination of L- and D-lactic acid in plasma by capillary electrophoresis," *Journal of Chromatography B*, vol. 814, no. 2, pp. 393–398, 2005.

[28] M. Jacobs, M. M. J. Van Greevenbroek, C. J. H. Van Der Kallen et al., "Low-grade inflammation can partly explain the association between the metabolic syndrome and either coronary artery disease or severity of peripheral arterial disease: the CODAM study," *European Journal of Clinical Investigation*, vol. 39, no. 6, pp. 437–444, 2009.

[29] O. Brouwers, P. M. Niessen, I. Ferreira et al., "Overexpression of glyoxalase-I reduces hyperglycemiainduced levels of advanced glycation end products and oxidative stress in diabetic rats," *Journal of Biological Chemistry*, vol. 286, no. 2, pp. 1374–1380, 2011.

[30] M. Jaffé, "Ueber die Niederschlag, welchen Pikrinsaure in normalem Ham erzeugt und uber eine neue Reaction des Kreatinins," *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*, vol. 10, p. 391, 1886.

[31] A. J. Buglass and S. H. Lee, "Sequential analysis of malic acid and both enantiomers of lactic acid in wine using a high-performance liquid chromatographic column-switching procedure," *Journal of Chromatographic Science*, vol. 39, no. 11, pp. 453–458, 2001.

[32] A. M. Krstulovic, "Chiral stationary phases for the liquid chromatographic separation of pharmaceuticals," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 6, no. 6–8, pp. 641–656, 1988.

[33] B. A. Persson and S. Andersson, "Unusual effects of separation conditions on chiral separations," *Journal of Chromatography A*, vol. 906, no. 1-2, pp. 195–203, 2001.

[34] W. Lindner and I. Hirschböck, "Tartaric acid derivatives as chiral sources for enantioseparation in liquid chromatography," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 2, no. 2, pp. 183–189, 1984.

[35] E. A. Struys, E. E. W. Jansen, N. M. Verhoeven, and C. Jakobs, "Measurement of urinary D- and L-2-hydroxyglutarate enantiomers by stable-isotope-dilution liquid chromatography-tandem mass spectrometry after derivatization with diacetyl-L-tartaric anhydride," *Clinical Chemistry*, vol. 50, no. 8, pp. 1391–1395, 2004.

[36] X. X. Sun, L. Z. Sun, and H. Y. Aboul-Enein, "Chiral derivatization reagents for drug enantioseparation by high-performance liquid chromatography based upon pre-column

derivatization and formation of diastereomers: enantioselectivity and related structure," *Biomedical Chromatography*, vol. 15, no. 2, pp. 116–132, 2001.

[37] P. J. Thornalley, "The glyoxalase system in health and disease," *Molecular Aspects of Medicine*, vol. 14, no. 4, pp. 287–371, 1993.

Hindawi Publishing Corporation
Experimental Diabetes Research
Volume 2012, Article ID 250621, 5 pages
doi:10.1155/2012/250621

Research Article

Relation of Adiponectin to Glucose Tolerance Status, Adiposity, and Cardiovascular Risk Factor Load

N. Wolfson,[1] D. Gavish,[1] Z. Matas,[2] M. Boaz,[3] and M. Shargorodsky[4]

[1] *Department of Medicine, Edith Wolfson Medical Center and Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*
[2] *Department of Biochemistry, Edith Wolfson Medical Center, Holon 58100, Israel*
[3] *Epidemiology and Research Unit, Edith Wolfson Medical Center and Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*
[4] *Department of Endocrinology, Edith Wolfson Medical Center and Sackler School of Medicine, Tel Aviv University, P. O. Box 5, Holon 58100, Israel*

Correspondence should be addressed to M. Shargorodsky, marinas@wolfson.health.gov.il

Received 17 July 2011; Accepted 10 November 2011

Academic Editor: K. Khunti

Objective. Adiponectin has anti-atherogenic and anti-inflammatory properties. We investigated the influence of adiponectin on glucose tolerance status, adiposity and cardiovascular risk factors (CVRFs). *Design and Patients*. Study consisted of 107 subjects: 55 with normal glucose tolerance (NGT) and 52 with impaired glucose regulation (IGR) who were divided into two groups: 24 subjects with impaired fasting glucose (IFG Group) and 28 patients with type 2 diabetes mellitus (DM Group). In additional analysis, study participants were divided into two groups, according to CVRFs: low and high risk. Measurements: Patients were evaluated for glucose, HbA1C, insulin, lipids, CRP, HOMA-IR and adiponectin. *Measurements*. Patients were evaluated for glucose, HbA1C, insulin, lipids, CRP, HOMA-IR and adiponectin. *Results*. Adiponectin was significantly higher in NGT group than in IFG ($P = 0.003$) and DM ($P = 0.01$) groups. Adiponectin was significantly, positively associated with HDL and inversely associated with glucose, HbA1c, ALT, AST, TG, HOMA-IR. Patients with higher CVRFs load have lesser adiponectin compared to patients with low cardiovascular risk $P < 0.0001$). Adiponectin was inversely associated with the number of risk factors ($r = -0.430$, $P = 0.0001$). *Conclusions*. Circulating adiponectin was significantly lower in subjects with different degree of IGR compared to subjects with normal glucose homeostasis. Adiponectin was significantly lower in high risk group than low risk group and decreased concurrently with increased number of CVRFs.

1. Introduction

Adiponectin, a collagen-like protein specifically and highly expressed in human adipose cells, plays an important role in insulin sensitivity, inflammation, lipid metabolism, and atherogenesis [1–3]. Low plasma adiponectin levels are significantly correlated with endothelial dysfunction, increased intima media thickness, and progression of coronary artery calcification independently of other cardiovascular risk factors [4–6]. Plasma adiponectin levels are reduced not only among obese patients but also in disease states frequently associated with obesity, such as type 2 diabetes, hypertension, metabolic syndrome, and coronary artery disease [7–9]. Altogether, these data suggest that adiponectin may mediate its effects via obesity-independent mechanisms, but whether obesity per se or other pathways play a regulatory role is unclear and deserves further evaluation. The present study was designed to assess the relation between plasma adiponectin levels to glucose tolerance status, adiposity, and cardiovascular risk factors (CVRFs) load.

2. Methods

The study group consisted of 107 Caucasian subjects, (67 females, mean age 56.4 ± 10.0 years) who were recruited from the outpatient metabolic clinic and evaluated for the study. The study participants were divided into three groups according to glucose tolerance status: 55 with normal glucose

Table 1: Clinical characteristics of study patients by glucose tolerance status.

Variables	NGT group $n = 55$	IFG group $n = 24$	DM group $n = 28$
Female/male	39/16	7/17	17/11
Age (y)	55.7 ± 9.5	58.8 ± 9.6	55.9 ± 11.3
BMI (kg/m^2)	33.3 ± 6.5	32.9 ± 6.8	31.6 ± 6.2
Current smokers, *n* (%)	7 (13%)	3 (8%)	3 (11%)
Hypertension, *n* (%)	28 (52%)	10 (42%)	15 (54%)
Dyslipidemia, *n* (%)	24 (44%)	12 (50%)	17 (61%)
Systolic blood pressure (mmHg)	131.5 ± 18.4	134.7 ± 18.7	134.9 ± 19.6
Diastolic blood pressure (mmHg)	73.4 ± 14.1	76.5 ± 12.1	76.0 ± 9.6
Fasting glucose (mg/dL)	89.5 ± 7.3	109.4 ± 7.6*	156.0 ± 53.5*
HBAIC (%)	5.7 ± 0.4	5.9 ± 0.5*	8.0 ± 2.1*
HDL-cholesterol (mg/dL)	56.6 ± 21.3	47.6 ± 13.4*	45.9 ± 18.3*
Triglycerides (mg/dL)	119.9 ± 59.4	150.6 ± 62.0*	195.3 ± 158.4*
Hs-CRP (mg/dL)	0.9 ± 0.4	0.7 ± 1.2	0.9 ± 1.1
ALT (mg/dL)	26.2 ± 12.8	30.9 ± 16.2	33.6 ± 29.1*
AST (mg/dL)	22.9 ± 7.8	27.5 ± 8.6	28.0 ± 18.0
Fasting insulin (IU/mL)	12.6 ± 7.2	26.0 ± 20.1*	18.8 ± 16.0*
HOMA-IR	2.8 ± 1.7	7.2 ± 5.7*	7.2 ± 7.6*
Adiponectin (ng/mL)	12597.2 ± 7237.6	7567.5 ± 4193.5*	7484.2 ± 4563.9*

* *P* value versus NGT group (significant at the 0.05 level).

tolerance (NGT Group), 24 subjects with impaired fasting glucose (IFG Group), and 28 patients with type 2 diabetes mellitus (DM Group).

The cutoff for IFG was defined as FBG = 100 − 125 mg/dL, according to current ADA criteria [10].

In additional analysis study, participants were divided into two groups, according to CVRFs: low- and high-risk subjects. Cardiovascular risk factors were defined using the ATP III definition and included the following disorders: hypertriglyceridemia (≥150 mg/dL and/or pharmacological treatment), low HDL cholesterol level (<40 mg/dL in men and <50 mg/dL in women), high blood pressure (systolic blood pressure ≥130 mmHg and/or diastolic BP ≥ 85 mmHg and/or pharmacological treatment), and elevated plasma glucose ≥100 mg/dL and/or pharmacological treatment). Obesity was defined using the World Health Organization criteria (BMI ≥ 30 kg/m^2). According to the above-mentioned CVRFs, the high-risk group included patients with three or more CVRFs ($n = 55$), whereas low risk had two or less CVRFs ($n = 52$).

2.1. Biochemical Parameters. Blood sampling for full chemistry and metabolic parameters, including fasting glucose, HbA1C, c-peptide, insulin, lipids profile, fibrinogen, hs-CRP, and plasma adiponectin, will be performed. Adiponectin will be determined by a commercial sandwich enzyme immunoassay technique, R&D Systems, Minneapolis, MN, USA (catalog number DRP300) with 2.8% intra-assay and 6.5% interassay variability.

Insulin resistance and β-cell function was estimated using homeostasis model assessment. Homeostasis model assessment-insulin resistance (HOMA-IR) will be calculated by the following formula: fasting plasma insulin (mU/mL) × fasting plasma glucose (mg/dL)/405 [11], HOMA for β-cell function (HOMA-β) as follows: 20 × fasting plasma insulin (mU/mL)/fasting plasma glucose (mmol/L)−3.5.

2.2. Statistical Analysis. Analysis of data was carried out using SPSS 9.0 statistical analysis software (SPSS Inc., Chicago, IL, USA, 1999). For continuous variables, such as hemodynamic and biochemistry measures, descriptive statistics were calculated and reported as mean ± standard deviation. Distributions of continuous variables were assessed for normality using the Kolmogorov-Smirnov test (cutoff at $P = 0.01$). Associations between continuous variables with approximately normal distributions including anthropometric, metabolic, and hemodynamic parameters were described using Pearson's correlation coefficients. Associations between continuous variables with distributions significantly deviating from normal were described using Spearman's rho coefficients. All tests are twosided and considered significant at $P < 0.05$.

3. Results

Demographic and clinical characteristics of the study groups according to glucose tolerance status are presented in Table 1. As can be seen, all groups were similar in terms of age, BMI, and presence of concomitant cardiovascular risk factors such as hypertension, dyslipidemia, and smoking. Fasting glucose differed significantly between each group and every other group, lowest in NGT subjects and highest in patients with DM, as expected. HOMA-IR as well as fasting insulin was significantly lower in NGT group than in IFG and DM

Table 2: Correlations.

		Circulating adiponectin
Age	r-value	0.295(**)
	P value	0.002
BMI	r-value	−0.008
	P value	0.931
AST	r-value	−0.279(**)
	P value	0.004
ALT	r-value	−0.348(**)
	P value	0.0001
HDL-cholesterol	r-value	0.418(**)
	P value	0.0001
LDL-cholesterol	r-value	0.128
	P value	0.206
Triglycerides	r-value	−0.453(**)
	P value	0.0001
Hs-CRP	r-value	−0.169
	P value	0.085
Fasting glucose	r-value	−0.375(**)
	P value	0.0001
HbA1C	r-value	−0.328(**)
	P value	0.001
Fasting insulin	r-value	−0.537(**)
	P value	0.0001
HOMA-IR	r-value	−0.623(**)
	P value	0.0001

**Correlation is significant at the 0.01 level (2-tailed).

groups, but not different between IFG and DM groups. Additionally, significant intergroup differences were detected for HDL-cholesterol and triglycerides.

Adiponectin was significantly higher in NGT group than in IFG ($P = 0.003$) and DM ($P = 0.01$) groups. A difference in plasma adiponectin levels between IFG and DM groups was not detected.

As shown in Table 2, adiponectin was significantly, positively associated with HDL, significantly inversely associated with glucose, HbA1c, ALT, AST, triglycerides, insulin, HOMA-IR, and marginally inversely associated with CRP. No association between adiponectin and BMI was observed ($r = -0.008$, $P = 0.931$). Multiple regression models which were arrived at using a backward, stepwise approach were performed to identify variables independently associated with plasma adiponectin levels. In this model, adiponectin was significantly inversely associated with HbA1C (standardized beta $= -0.385$, $P = 0.009$) and ALT (standardized beta $= -0.453$, $P = 0.040$). The overall model is significant ($P < 0.0001$) and explains 38.2% of variability in plasma adiponectin levels.

In additional statistical analysis, the study participants were divided into two groups according to the presence of CVRFs: low-risk and high-risk. As can be seen in Table 3, age and BMI were not different between low-risk and high-risk patients. High risk-subjects had a median of 3 CVRFs (range 3–4), while low-risk subjects had a median of 2 CVRFs (range 0–2). Systolic and diastolic blood pressure as well as fasting glucose, HbA1C, fasting insulin, HOMA-IR, triglycerides, AST, and LDL-cholesterol were significantly higher and HDL-cholesterol significantly lower in high versus low-risk patients. Plasma adiponectin levels were significantly lower in high-risk group compared to low-risk subjects (6755.6 ± 3492.2 versus 13701.1 ± 7051.5 ng/mL, $P < 0.0001$).

Adiponectin was significantly inversely associated with the number of risk factors ($r = -0.430$, $P = 0.0001$) (Table 4).

4. Discussion

In the present study, circulating adiponectin was significantly lower in subjects with different degree of IGR compared to subjects with normal glucose homeostasis. Furthermore we confirm previously observed positive correlations of adiponectin with HDL and negative correlations with glucose, HbA1c, triglycerides, insulin, and HOMA-IR and report an inverse correlation with ALT. In multiple regression models HbA1C as well as ALT was independent determinant of adiponectin levels and explained 38.2% of variability in plasma adiponectin levels. Additionally, adiponectin was significantly lower in high-risk group than low-risk group and decreased concurrently with increased number of CVRFs. Thus, hypoadiponectinemia is more intensively related to glucose intolerance and CVRFs load than to adiposity.

The site and mechanism of adiponectin actions on glucose metabolism remain unknown. Recent genome-wide scans have mapped a diabetes susceptibility locus to chromosome 3q27, where the adiponectin gene (apM1) is located [12]. Evidence of an association between type 2 diabetes and single nucleotide polymorphisms at positions 45 and 276 and in the proximal promoter and exon 3 of the adiponectin gene has been also reported [11]. Furthermore, it has been shown that circulating adiponectin levels are significantly lower in healthy first-degree relatives of type 2 diabetic patients [13].

In our study, plasma adiponectin levels were significantly lower in both IFG and DM groups compared to subjects with normal glucose tolerance. Moreover, HbA1C levels were independent determinant of circulating adiponectin. Therefore, there is a scientific rationale that plasma adiponectin is a target for future research in the treatment and prevention of diabetes. We suggest that increase in adiponectin levels by combination of lifestyle modifications and medications may have a beneficial effect in patients with impaired glucose regulation.

Our study confirmed previously observed strong inverse associations between low adiponectin levels and some of the well-known risk factors for atherosclerosis, such as low HDL-cholesterol levels, hypertriglyceridemia, and insulin resistance [14–16]. Additionally, we found significant inverse correlation between adiponectin and ALT, a surrogate marker for nonalcoholic fatty liver disease, which has recently been considered as a predictor for development of type 2 diabetes and metabolic syndrome [17–19]. These findings clearly support the point of view that adiponectin may have an active role in the pathogenesis of cardiovascular diseases.

Table 3: Clinical characteristics of study patients by CVRFs load.

Variables	Low-risk subjects $n = 52$	High-risk subjects $n = 55$	P value
Age	57.7 ± 9.1	55.2 ± 10.8	0.200
Sex (M/F)	13 (25%)	27 (49%)	0.01
BMI	31.8 ± 6.7	33.7 ± 6.2	0.135
CVRFs median (range)	2 (range 0–2)	3 (range 3–4)	0.002
Systolic blood pressure (mmHg)	128.1 ± 18.0	137.9 ± 18.2	0.006
Diastolic blood pressure (mmHg)	72.7 ± 12.5	77.7 ± 11.1	0.032
Mean arterial pressure (mmHg)	94.7 ± 14.2	99.0 ± 14.4	0.126
Fasting blood glucose (mg/dL)	93.7 ± 19.0	128.2 ± 45.8	0.0001
HBAIC (%)	5.7 ± 0.6	6.9 ± 1.8	0.0001
HDL-cholesterol (mg/dL)	56.5 ± 16.1	45.0 ± 15.4	0.0001
Triglycerides (mg/dL)	104.0 ± 47.5	186.0 ± 119.4	0.0001
Hs-CRP (mg/dL)	0.9 ± 0.4	0.9 ± 1.1	0.408
Fasting insulin (IU/mL)	11.9 ± 7.6	22.6 ± 17.4	0.0001
HOMA-IR	2.7 ± 1.8	7.0 ± 4.5	0.0001
AST (mg/dL)	22.4 ± 7.0	28.0 ± 14.3	0.011
ALT (mg/dL)	25.6 ± 12.1	32.6 ± 23.6	0.204
Adiponectin (ng/mL)	13701.1 ± 7051.5	6755.6 ± 3492.2	0.0001

Table 4: Correlations between adiponectin levels and number of CVRFs.

Correlations			CVRF's	Adiponectin
Spearman's rho	CVRF's	Correlation coefficient	1.000	−0.430(**)
		Sig. (2-tailed)		0.0001
		N	107	107
	Adiponectin	Correlation coefficient	−0.430(**)	1.000
		Sig. (2-tailed)	0.0001	
		N	107	107

**Correlation is significant at the 0.01 level (2-tailed).

In the present study, no associations of adiponectin with BMI have been found; however, other studies have been unable to detect associations between adiponectin and whole-body fat mass and subcutaneous fat volume [20]. Nevertheless, an association between BMI and circulating adiponectin levels has been reported by other authors [21, 22]. Although we did not find the associations of adiponectin with BMI as well as differences in adiponectin levels between obese (BMI $\geq 30\,kg/m^2$) and nonobese study participants, the possibility that the relatively small sample size in the present study limited exploration of these potential correlations cannot be excluded.

The present study observed strong associations between adiponectin levels and glucose tolerance status as well as number of cardiovascular risk factors. Thus, despite the fact that adiponectin is highly specific to adipose tissue, hypoadiponectinemia is more intensively related to impaired glucose regulation and CVRFs load than to adiposity. The findings of our study are in accordance with published data that circulating adiponectin level is a strong risk marker for metabolic syndrome independent of measures of adiposity [15]. Moreover, decreased plasma adiponectin levels are independently associated with the presence of coronary artery disease in men even after adjustment for BMI [23]. Among healthy men in the Health Professionals Follow-up Study, a doubling of adiponectin levels was associated with a 30% decreased risk for myocardial infarction after adjustment for BMI, alcohol consumption, physical activity, diabetes, and hypertension [24]. Similarly, among patients with type 1 and type 2 diabetes mellitus, increased circulating adiponectin levels were associated with a lower risk of coronary artery disease after adjusting for standard risk factors [25, 26]. These combined findings indicate that adiponectin may have a direct antiatherogenic role or mediate its effects via obesity-independent mechanisms.

Thus, plasma adiponectin integrates with the cardiovascular risk factors and is a potential target for research in reducing morbidity and mortality of atherosclerotic disease. Future research should focus on clinical endpoints such as prevention of diabetes and reduction of cardiovascular morbidity and mortality following adiponectin increase, as well as the ability of adiponectin values to predict these outcomes.

References

[1] N. Stefan, B. Vozarova, T. Funahashi et al., "Plasma adiponectin concentration is associated with skeletal muscle insulin receptor tyrosine phosphorylation, and low plasma concentration precedes a decrease in whole-body insulin sensitivity in humans," *Diabetes*, vol. 51, no. 6, pp. 1884–1888, 2002.

[2] T. Yokota, K. Oritani, I. Takahashi et al., "Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages," *Blood*, vol. 96, no. 5, pp. 1723–1732, 2000.

[3] N. Ouchi, S. Kihara, Y. Arita et al., "Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages," *Circulation*, vol. 103, no. 8, pp. 1057–1063, 2001.

[4] K. C. B. Tan, A. Xu, W. S. Chow et al., "Hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 2, pp. 765–769, 2004.

[5] M. Shargorodsky, M. Boaz, Y. Goldberg et al., "Adiponectin and vascular properties in obese patients: is it a novel biomarker of early atherosclerosis," *International Journal of Obesity*, vol. 33, no. 5, pp. 553–558, 2009.

[6] S. Störk, M. L. Bots, P. Angerer et al., "Low levels of adiponectin predict worsening of arterial morphology and function," *Atherosclerosis*, vol. 194, no. 2, pp. e147–e153, 2007.

[7] W. Koenig, N. Khuseyinova, J. Baumert, C. Meisinger, and H. Löwel, "Serum concentrations of adiponectin and risk of type 2 diabetes mellitus and coronary heart disease in apparently healthy middle-aged men. Results from the 18-year follow-up of a large cohort from Southern Germany," *Journal of the American College of Cardiology*, vol. 48, no. 7, pp. 1369–1377, 2006.

[8] Y. Iwashima, T. Katsuya, K. Ishikawa et al., "Hypoadiponectinemia is an independent risk factor for hypertension," *Hypertension*, vol. 43, no. 6, pp. 1318–1323, 2004.

[9] K. Hotta, T. Funahashi, Y. Arita et al., "Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 6, pp. 1595–1599, 2000.

[10] S. Genuth, K. G. Alberti, P. Bennett et al., "The expert committee on the diagnosis and classification of diabetes mellitus follow-up report on the diagnosis of diabetes mellitus," *Diabetes Care*, vol. 26, pp. 3160–3167, 2003.

[11] L. L. Li, X. L. Kang, X. J. Ran et al., "Associations between 45T/G polymorphism of the adiponectin gene and plasma adiponectin levels with type 2 diabetes," *Clinical and Experimental Pharmacology and Physiology*, vol. 34, no. 12, pp. 1287–1290, 2007.

[12] M. Stumvoll, O. Tschritter, A. Fritsche et al., "Association of the T-G polymorphism in adiponectin (Exon 2) with obesity and insulin sensitivity: interaction with family history of type 2 diabetes," *Diabetes*, vol. 51, no. 1, pp. 37–41, 2002.

[13] F. Pellmé, U. Smith, T. Funahashi et al., "Circulating adiponectin levels are reduced in nonobese but insulin-resistant first-degree relatives of type 2 diabetic patients," *Diabetes*, vol. 2, no. 5, pp. 1182–1186, 2003.

[14] Y. Yamamoto, H. Hirose, I. Saito et al., "Correlation of the adipocyte-derived protein adiponectin with insulin resistance index and serum high-density lipoprotein-cholesterol, independent of body mass index, in the Japanese population," *Clinical Science*, vol. 103, no. 2, pp. 137–142, 2002.

[15] J. Hung, B. M. McQuillan, P. L. Thompson, and J. P. Beilby, "Circulating adiponectin levels associate with inflammatory markers, insulin resistance and metabolic syndrome independent of obesity," *International Journal of Obesity*, vol. 32, no. 5, pp. 772–779, 2008.

[16] A. J. Krentz, D. V. Mühlen, and E. Barrett-Connor, "Adipocytokines, sex hormones, and cardiovascular risk factors in postmenopausal women: factor analysis of the Rancho Bernardo study," *Hormone and Metabolic Research*, vol. 41, no. 10, pp. 773–777, 2009.

[17] S. G. Kim, H. Y. Kim, J. A. Seo et al., "Relationship between serum adiponectin concentration, pulse wave velocity and nonalcoholic fatty liver disease," *European Journal of Endocrinology*, vol. 152, no. 2, pp. 225–231, 2005.

[18] B. Vozarova, N. Stefan, R. S. Lindsay et al., "High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes," *Diabetes*, vol. 51, no. 6, pp. 1889–1895, 2002.

[19] K. M. Choi, J. Lee, K. W. Lee et al., "Serum adiponectin concentrations predict the developments of type 2 diabetes and the metabolic syndrome in elderly Koreans," *Clinical Endocrinology*, vol. 61, no. 1, pp. 75–80, 2004.

[20] L. Lenchik, T. C. Register, F. C. Hsu et al., "Adiponectin as a novel determinant of bone mineral density and visceral fat," *Bone*, vol. 33, no. 4, pp. 646–651, 2003.

[21] Y. Arita, S. Kihara, N. Ouchi et al., "Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity," *Biochemical and Biophysical Research Communications*, vol. 257, no. 1, pp. 79–83, 1999.

[22] M. Matsubara, S. Maruoka, and S. Katayose, "Inverse relationship between plasma adiponectin and leptin concentrations in normal-weight and obese women," *European Journal of Endocrinology*, vol. 147, no. 2, pp. 173–180, 2002.

[23] M. Kumada, S. Kihara, S. Sumitsuji et al., "Coronary artery disease association of hypoadiponectinemia with coronary artery disease in men," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, pp. 85–89, 2003.

[24] T. Pischon, C. J. Girman, G. S. Hotamisligil, N. Rifai, F. B. Hu, and E. B. Rimm, "Plasma adiponectin levels and risk of myocardial infarction in men," *Journal of the American Medical Association*, vol. 291, no. 14, pp. 1730–1737, 2004.

[25] M. B. Schulze, I. Shai, E. B. Rimm, T. Li, N. Rifai, and F. B. Hu, "Adiponectin and future coronary heart disease events among men with type 2 diabetes," *Diabetes*, vol. 54, no. 2, pp. 534–539, 2005.

[26] T. Costacou, J. C. Zgibor, R. W. Evans et al., "The prospective association between adiponectin and coronary artery disease among individuals with type 1 diabetes. The Pittsburgh epidemiology of diabetes complications study," *Diabetologia*, vol. 48, no. 1, pp. 41–48, 2005.