

# On the search for glycated lipoprotein ApoA-I in the plasma of diabetic and nephropathic patients

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The analysis of plasma samples from healthy, diabetic and nephropathic subjects was carried out by 2D gel electrophoresis. This approach shows clear differences among the three classes of subjects. In the case of diabetic and nephropathic patients intense spots appear. Their enzymatic digestion followed by matrix assisted laser desorption ionization/mass spectrometry (MALDI/MS) analysis shows that an overexpression of unglycated and glycated ApoA-I is present in both pathological states. Interestingly, this trend is also observed for the retinol-binding protein (RBP). The data obtained can be relevant to assess possible risks associated either with the glycation level of ApoA-I or with the overexpression of RBP. In fact, in the former case possibly a different functionality of the glycated protein is to be expected, reflecting a different efficiency in cholesterol transport. In the latter case, the increase of RBP level can be related to the overweight of the diabetic subjects under investigation: it is known that obesity leads to RBP overexpression. In the case of nephropathic patients, the RBP level increases in parallel with serum creatinin. Copyright © 2007 John Wiley & Sons, Ltd.

**KEYWORDS:** nonenzymatic glycation; diabetes; nephropathy; MALDI/MS; 2D gel electrophoresis

## INTRODUCTION

Data from epidemiologic studies have shown that high levels of high-density lipoproteins (HDL) in plasma offer protection from the development of atherosclerosis.<sup>1</sup> This is due to the reported actions of HDL, among which the most important is the ability to promote the efflux of cholesterol from cells in the arterial wall, a process called *reverse cholesterol transport*.<sup>2</sup> In this context, ApoA-I, which constitutes 70% of the apolipoprotein content of HDL, acts as acceptor for the transfer of phospholipids and free cholesterol from peripheral tissues and transports cholesterol in the liver and other tissues for excretion and steroidogenesis.<sup>3</sup> In particular, ApoA-I interacts with the ATP-binding cassette transporter A1 and accepts free cholesterol, and acts as a cofactor of the HDL-associated enzyme lecithin cholesterol acyl transferase (LCAT), which promotes the esterification of free cholesterol to form cholesterol esters,<sup>4</sup> and interacts with the scavenger receptor class B type I, thereby mediating the delivery of cholesterol ester to the liver.<sup>5</sup> This process is important in reducing the accumulation of foam cells in the arterial walls.<sup>6</sup>

Furthermore ApoA-I exerts antioxidant properties by removing lipid hydroperoxides from low-density lipoprotein (LDL), thereby reducing the oxidation of LDL and

consequently the atherogenicity of these lipoproteins. Therefore, ApoA-I also shows anti-inflammatory actions through the inhibition of the expression of adhesion molecules in endothelial cells and reducing the recruitment of monocytes in the arterial walls.<sup>7</sup>

Atherosclerotic vascular disease is a major complication of diabetes, and among the known risk factors for atherosclerosis, such as hyperlipoproteinemia, obesity, hypertension, hyperinsulinemia, inflammation,<sup>8</sup> low levels of HDL play an important role.<sup>9</sup> Recently, in fact, it has been suggested that in addition to the codified measurement of total cholesterol, HDL, LDL and triglycerides, the evaluation of cholesterol balance determined as the ApoB/ApoA-I ratio could be a better marker than lipids, lipoproteins and lipid ratio. The results of a series of clinical studies indicate, in fact, that the ApoB/ApoA-I ratio, because of its accuracy, can be utilized as a new risk factor for cardiovascular disease.<sup>10</sup>

Cardiovascular disease is the major cause of mortality and morbidity in patients with chronic kidney disease,<sup>11</sup> which is due, in part, to lipid abnormalities. One of the most common lipid abnormality is hypertriglyceridemia associated with an increase of ApoB-containing lipoprotein and a decrease in the antiatherogenic index, ApoB/ApoA-I ratio.<sup>12</sup>

Recently it has been shown that in patients with end-stage renal disease decreased levels of ApoA-I due to

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increased catabolism is present in comparison with the control subjects.<sup>13,14</sup>

In addition to the reduction of the levels of ApoA-I, the occurrence of post-translational modification of this apolipoprotein, among which is the nonenzymatic glycation, can contribute to its impaired action as shown by a series of studies.<sup>15–18</sup> Calvo *et al.* in a previous paper evaluated the nonenzymatic glycation of HDL in type 1 and 2 diabetic patients compared with that on control subjects. An increase of 400% of HDL glycation was found in diabetic patients with respect to control subjects, with a degree of glycation positively related with blood glucose levels. These data were confirmed by those obtained *in vitro* after incubation of native ApoA-I, either as free or as the phospholipid/ApoA-I complex, with glucose. The glycation of ApoA-I was dependent on glucose concentration, incubation time and the presence of phospholipids, suggesting that the *in vivo* glycation of HDL apolipoprotein is dependent on glucose concentration but is also influenced by the lipid environment.<sup>15</sup> In a further study, the authors purified *in vivo* glycated ApoA-I from diabetic patients and compared its lipid binding properties to those of ApoA-I from healthy subjects. Analysis of tryptophan fluorescent spectra and of fluorescence-quenching in presence of iodine pointed at evidence that glycation of ApoA-I determines a decrease in stability of the lipid–apolipoprotein interaction and in the apolipoprotein self-association. These results suggest that the nonenzymatic glycation of ApoA-I can affect the structural cohesion of HDL particles.<sup>16</sup>

Subsequently, a study performed on rats, in which glycated ApoA-I was injected, has shown, by analysis with gel chromatography of serum of rats, that glycation reduces the interaction between apolipoprotein A-I and HDL. These results suggest that the glycation of ApoA-I may contribute to the development of atherosclerosis in diabetes.<sup>17</sup> More recently, a combination of affinity chromatography and latex immunoagglutination was used to determine the glycation of plasma ApoA-I. Utilizing this assay, higher levels of glycated ApoA-I have been found in poorly controlled diabetic patients with respect to normal controls.<sup>18</sup>

The crystal structure of human ApoA-I has been recently described by Ajees *et al.*<sup>19</sup> ApoA-I is composed of an *N*-terminal four-helix bundle and two *C*-terminal helices. The *N*-terminal domain plays a relevant role in maintaining its lipid-free conformation.

Nowadays, proteomics-based approaches that examine the expressed proteins in a tissue, a cell or body fluids are increasingly being used to address biomedical application because of their ability to study simultaneously a large

number of proteins including their post-translational modification. One of the fundamental approaches in proteomics is the combination of two-dimensional electrophoresis (2-DE) for proteins separation and mass spectrometry for their identification and characterization.<sup>20</sup> The most important aim of proteomics is to identify novel disease markers by identifying differentially expressed proteins associated with a pathological state.

In the present investigation, a proteomic approach has been employed to study plasma samples from healthy, diabetic and nephropathic subjects, with the main aim addressed to identify possible post-translational modifications of ApoA-I due to nonenzymatic glycation processes. Matrix assisted laser desorption ionization (MALDI) was used to obtain the identification of the protein of interest by peptide mass fingerprinting (PMF) after enzymatic digestion.<sup>21</sup> The modified amino acids were obtained by post source decay (PSD)<sup>22</sup> experiments. The results achieved in terms of modified amino acids and the related statistical scores are shown and discussed.

## EXPERIMENTAL

### Subjects

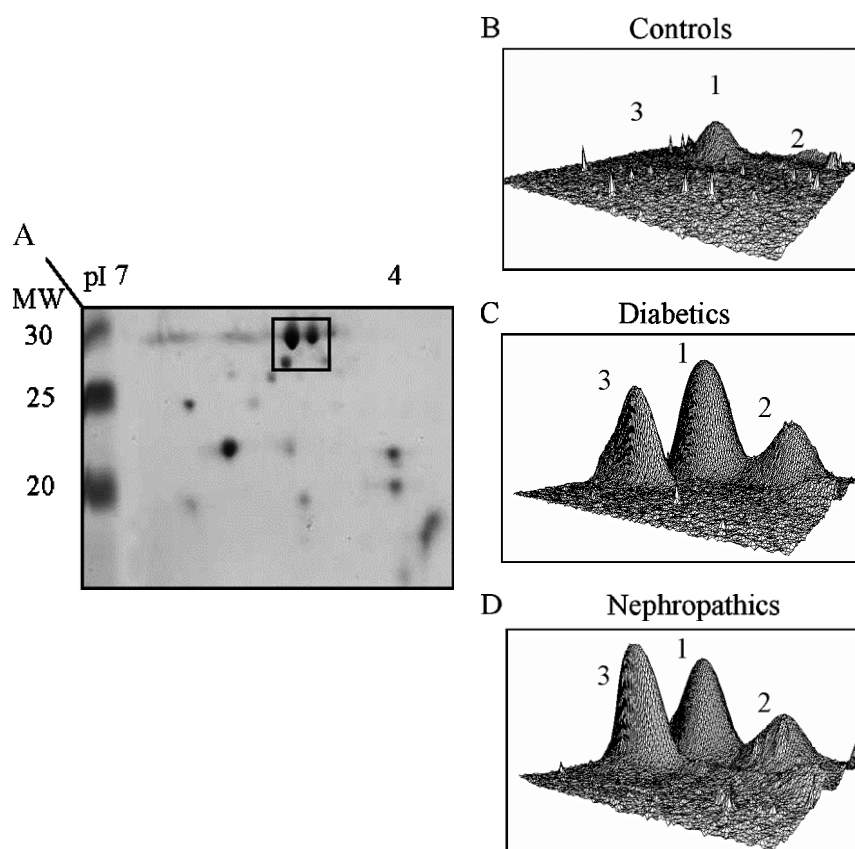
Ten type 2 diabetic patients, ten patients affected by end-stage renal disease and subjected to peritoneal dialysis and ten normal controls were evaluated. Their clinical and metabolic characteristics are reported in Table 1. All subjects gave their informed consent to the study, which was carried out following the Helsinki Declaration rules and after approval by the local Ethics Committee.

### Plasma sample preparation and 2D gel electrophoresis

The plasma samples (10 ml) obtained by mixing the samples of each group of subjects were treated with four volumes 20% (v/v) acetonitrile and applied onto a centriplus centrifugal concentrator membrane with a molecular weight cut-off (MWCO) 30 000 (Millipore). The centrifugal filter membranes were rinsed and used according to the manufacturer's instruction. The samples were centrifuged at 3000 g until >90% of the input plasma had passed through the membranes.<sup>23</sup> The filtrates were lyophilized to dryness and resuspended in water before desalting and delipidation by the trichloroacetic acid/acetone procedure. With this method we obtained a recovery of 150 µg of low molecular weight proteins from 600 mg of total plasma proteins. Desalted and delipidated proteins were resuspended in a buffer containing

**Table 1.** Clinical and metabolic parameters evaluated in the ten diabetic patients, ten nephropathic patients and ten normal controls under investigation (values are mean ±DS)

Subjects	Age (years)	Disease duration (years)	BMI (Kg/m <sup>2</sup> )	Fasting plasma glucose (mg/dl)	HbA1c (%)	HDL		Triglycerides (mg/dl)	Creatinine (mg/dl)
						Cholesterol (mg/dl)	cholesterol (mg/dl)		
Diabetics	63 ± 6.9	18 ± 9	30 ± 5	212 ± 12	9.0 ± 0.2	165 ± 31	53 ± 10	118 ± 34	1.1 ± 0.2
Nephropathic	60 ± 16	4 ± 2	26 ± 4	92 ± 5	5.8 ± 0.2	195 ± 52	47 ± 11	193 ± 90	8.2 ± 1.2
Controls	62 ± 4		25 ± 3	89 ± 4	5.5 ± 0.3	212 ± 30	53 ± 15	110 ± 45	0.87 ± 0.02



**Figure 1.** (A) 2D and 3D plots of 2-DE protein spots of (B) control, (C) diabetic and (D) nephropathic subjects.

8 mol/l urea, 2 mol/l thiourea, 4% CHAPS, 2% v/v carrier ampholytes, pH 3–10, 20 mmol/l Tris, 55 mmol/l DTT, and bromophenol blue. Two-dimensional electrophoresis was carried out on the Protean IEF cell (Biorad) as previously described.<sup>24</sup> IPG ready strips, 7 cm, pH 3–10 nonlinear gradient (Biorad), were actively rehydrated at 50 V for 24 h. Proteins were loaded at the cathode using the cup-loading tray for Protean IEF cell (Biorad) and focused for a total of 10 kVh. After focusing, the strips were first equilibrated for 15 min with a solution containing 50 mmol/l Tris-HCl, 6 mol/l urea, 30% v/v glycerol, 2% SDS and 2% DTT, and then with the same buffer containing 4.5% iodoacetamide instead of DTT. The focused proteins were then separated according to size by SDS-PAGE on 17% Tricine gel and stained with colloidal blue stain. The protein patterns were digitized with a scanner and compared with PDQUEST 6.0

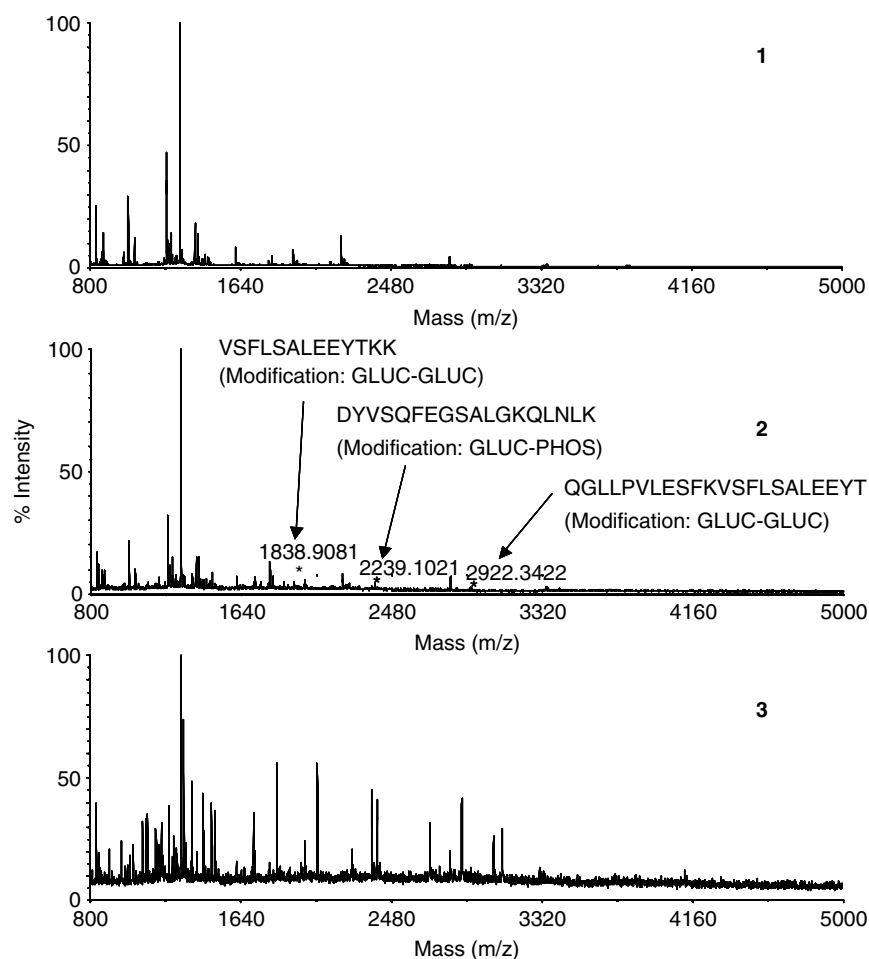
### Protein digestion

The 2D gel protein spot was washed in an Eppendorf tube two times with 200  $\mu$ l of  $\text{NH}_4\text{HCO}_3$  (50 mmol)/ $\text{CH}_3\text{CN}$  1 : 1. In each wash cycle the sample was spun for 10 min. After the wash step, the sample was left in the wash solution overnight so as to completely remove the colloidal blue stain. The Eppendorf tube containing the gel was centrifuged and the supernatant discarded. The gel pieces were treated with 1 ml of  $\text{CH}_3\text{CN}$ . The Eppendorf tube was centrifuged again and  $\text{CH}_3\text{CN}$  was discarded. The gel was dried using a Speedvac apparatus and rehydrated for 30 min at 4 °C using 20  $\mu$ l of 6 ng/ml trypsin solution. The Eppendorf tube was centrifuged and the supernatant trypsin solution was

discarded. Five microliters of  $\text{NH}_4\text{HCO}_3$  was added to the gel protein spot and the sample was incubated at 37 °C overnight. After the enzymatic digestion, the sample was centrifuged and 2  $\mu$ l of  $\text{H}_2\text{O}$  + 1% TFA solution was added to the obtained peptide mixture. The sample was sonicated in an ultrasound bath for 30 min. The Eppendorf tube was centrifuged at 13 000 g/min for 1 min and 1  $\mu$ l of the supernatant was characterized by MALDI.

### Mass spectrometry

Spectra were acquired by means of the Voyager-DE STR MALDI (Applied Biosystem, Foster City, CA, USA), a MALDI-TOF instrument capable of operating in linear, reflectron, PSD and delay extraction modes. PSD<sup>22</sup> was used to obtain the protein sequence and identify the modified aminoacids.  $\alpha$ -4-Hydroxycinnamic acid (CHCA) was used as the matrix. One microliter of the digested protein-peptide mixture was deposited on the MALDI steel plate and dried at room temperature. The matrix solution (0.5  $\mu$ l of a solution of 10 mg/ml in a  $\text{H}_2\text{O}/\text{CH}_3\text{CN}$  1/1 + 0.1% TFA) was deposited directly on the dried spot and allowed to dry at room temperature. The MALDI spectra were acquired in reflectron mode and the delay extraction time was 100 ns. A nitrogen laser ( $\lambda = 337$  nm) was used. The nitrogen laser operated at a frequency of 20 Hz and with adjustable laser energy. The laser energy was variable between 70 and 90  $\mu$ J. The accelerating voltage was 25 kV. The grid voltage was 75% of the accelerating voltage. The spectra were acquired in the  $m/z$  range 800–5000. The resolution in the  $m/z$  range was 20 000 and the mass accuracy was between 3 and 60 ppm after



**Figure 2.** MALDI mass spectra of the tryptic digested proteins corresponding to the spot 1, 2 and 3, respectively, that are differentially expressed among control, diabetic and nephropathic subjects.

calibration. The trypsin autolysis fragments at  $m/z$  842.4059 and 2211.152 were used to calibrate the mass spectra of the digested proteins.

### Data analysis

#### 2-DE image analysis

PDQuest software (Biorad) was used for spot detection, spot quantification, gel matching and statistical analysis of differences between the experimental groups. The cut-off level for a differentially expressed protein was defined as at least a two-fold increase or decrease in spot intensity. Statistically significant differences between groups for each protein were computed by ANOVA. The level of significance of difference was set at  $p < 0.05$ .

#### MALDI data

Data explorer software (Applied Biosystems, Foster City, CA, USA) was used to elaborate the peptide spectra and to verify the modified peptide sequence obtained by the PSD approach. The  $m/z$  ratio of the most intense 85 ions in the spectra were extracted and elaborated. Aldente<sup>25</sup> and Profound<sup>26</sup> PMFtools were used to identify the analyzed digested proteins, while the algorithm GlycoMod<sup>27</sup> was employed to identify the modified glycated peptide. Modified peptide sequences were confirmed by the PSD approach. In particular, the InSpecT software available online,<sup>28</sup> based

on the tag sequencing approach,<sup>29</sup> was employed to obtain the peptide sequence from PSD fragmentation spectra. Each modified sequence was assigned by means of a statistical score, expressed in  $p$ -value, provided by the software.

### RESULTS AND DISCUSSION

Despite its enormous potential, plasma proteomics is really complex because of the high dynamic range of plasma, in which few high abundant proteins, such as albumin and immunoglobulin, hide all the other low abundant proteins. To overcome this problem, different purification or prefractionation techniques have been applied in the past to enrich plasma samples.<sup>30</sup> Ten plasma samples for each of the groups under investigations (healthy subjects, poorly controlled patients and nephropathic patients) were mixed and each of the obtained plasma pools was analyzed by a proteomic approach. In this study we decided to focus our attention on low molecular weight proteins ( $< 30\,000$  Da), purified by centrifugal ultrafiltration, before 2-DE. This low molecular weight fraction contains ApoA-I but is made up of several classes of physiologically important proteins, such as cytokines, chemokines, peptide hormones, as well as proteolytic fragments of larger proteins.<sup>31</sup> Because albumin is known to bind and transport small molecules and peptides within the circulatory system, centrifugal ultrafiltration was

**Table 2.** Modified peptides identified by accurate mass measurement in the spots 1, 2 and 3 of control, diabetic and nephropathic subjects. The modification type is also reported. Densitometric analysis: \* $p < 0.05$  vs control

Sample (Spot - Name)	Spot density (OD*mm <sup>2</sup> )	$m/z$ (accuracy ppm)	Modification	Modified sequence	Sequence position
Control (1 - Apo A-I)	0.35 ± 0.07	–	–	–	–
Control (2- Not found)	0.10 ± 0.02	–	–	–	–
Control (3-Not Found)	0.12 ± 0.02	–	–	–	–
Diabetic (1 - Apo A-I)	2.01 ± 0.08*				
Diabetic (2 Apo A-I)	0.51 ± 0.05*	1838.908 (4)	GLUC-GLUC	VSFLSALEEYTKK	251–263
		2922.342 (58)	GLUC-GLUC	QGLLPVLESFKVSFLSALEE YTK	240–262
		2239.102 (21)	GLUC-PHOS	DYVSQFEGSALGKQLNLK	52–69
Diabetic (3- RBP)	0.62 ± 0.07*	–	–	–	–
Nephropathic (1 - Apo A-I)	1.32 ± 0.10*	–	–	–	–
Nephropathic (2 - Apo A-I)	0.43 ± 0.08*	1838.908 (4)	GLUC-GLUC	VSFLSALEEYTKK	251–263
		2922.342 (58)	GLUC-GLUC	QGLLPVLESFKVSFLSALEE YTK	240–262
		2239.102 (21)	GLUC-PHOS	DYVSQFEGSALGKQLNLK	52–69
Nephropathic (3 RBP)	0.77 ± 0.12*	–	–	–	–

conducted under solvent conditions affecting the disruption of protein–protein interactions. After this prefractionation step, the 2-DE map of plasma in the low molecular weight region resulted in different, highly enriched protein species.

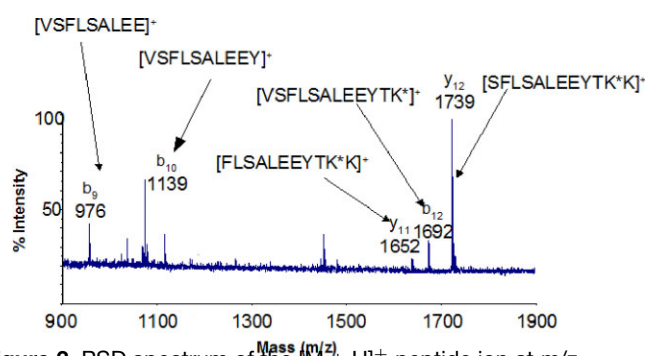
The results of the analysis of the three groups of samples by 2-DE are reported in Fig. 1. A significant difference among the three groups is clearly visualized in the 3D views of the area of interest (Fig. 1, panels B–D, Table 2). As can be easily observed, while in the case of healthy subjects practically only one peak is present in the 3D plot, in the case of diabetic and nephropathic patients three different peaks are clearly detectable in the same region.

Enzymatic digestion of the differentially expressed spots followed by MALDI analysis (Fig. 2) showed with high statistical confidence ( $p$ -value from  $3.6 \times 10^{-24}$  to  $1 \times 10^{-7}$ ) that spots 1 and 2 correspond to ApoA-I and spot 3 to RBP (Table 3), indicating a significant overexpression of these proteins in the examined pathological cases. In particular, the enzymatic digestion of spot 2 followed by MALDI analysis and data evaluation (Tables 2 and 3) shows that this protein corresponds to glycosylated ApoA-I, present also in the case of normal subjects but in a much smaller extent. The glycosylation sites have been individually identified by the analysis of digestion fragments (Table 2). In the case of the sample 2, new mono- and di-glycosylated peptides are evidenced (mass accuracy between 4 and 58 ppm), proving the occurrence of *in vivo* glycosylation processes.

However, the peptide mass even if obtained with high mass accuracy, is not enough to correctly assign the modified amino acid. Therefore, the modified peptide sequences were confirmed by PSD approach followed by the peptide sequence tags (PSTs) approach to filter the database.<sup>21,28,29</sup> In particular, InSpecT PSTs software<sup>28</sup> has been used. This algorithm identifies modified peptides by calculating tag sequence obtained by the peptide fragments reported in the mass spectrometry (MS/MS) spectrum. The modified peptide sequences are reported in Table 4, together with their probability score expressed as the  $p$ -value. As can be

seen (Table 4), the  $p$ -value was highly significant thanks to the high-quality PSD spectra (between  $6 \times 10^{-7}$  and  $5 \times 10^{-5}$ ). As an example, the PSD spectrum of the identified di-glycosylated VSFLSALEEYTK\*K peptide is reported in Fig. 3. In this case, the modification involves only the Lys262 and not the Lys263. This is confirmed by the fragment ions at  $m/z$  1692, 1739 and 1652 corresponding to  $b_{12}$  (VSFLSALEEYTK\*),  $y_{12}$  (SFLSALEEYTK\*K) and  $y_{11}$  (FLSALEEYTK\*K) fragments, respectively. The presence of ions at  $m/z$  1129 and 976 corresponding to  $b_{10}$  (VSFLSALEEY) and  $b_9$  (VSFLSALEE), in which the K\* loss is observed, fully confirms the previous hypothesis. Moreover, the fact that trypsin does not induce cleavage at the Lys 262 sites of the modified peptide but at Lys263 fully confirms the PSD spectrum data. In fact, if Lys263 were modified, this enzymatic cleavage site would not be recognized and cleaved by the enzyme as it happens in the case of Lys262-modified residues.

These data imply that in plasma samples of both diabetic and nephropathic subjects glycosylated ApoA-I is present in an abundance comparable to that of unglycosylated protein. Furthermore, considering equal amounts of plasma sample, both unglycosylated and glycosylated proteins are overexpressed in these groups in comparison with control subjects.

**Figure 3.** PSD spectrum of the  $[M+H]^+$  peptide ion at  $m/z$  1838.9 (peptide sequence: VSFLSALEEYTK\*K). \* Glu-Glu modified amino acid.

**Table 3.** Identification of the differentially expressed proteins named 1, 2 and 3 obtained by peptide mass fingerprint (PMF) approach

Name	Accession code	Mol. mass (kDa)	Isoelectric point (pI)	Score (p-value)	Sequences	Band no.
Apolipoprotein A-I(1-242).	P02647	28	5.3	6.2*10 <sup>-24</sup>	DLATVYVDVLK DSGRDYVSQFEFSALGK DYVSQFEFSALGK LLDNWDSVTSTFSK LREQLGPVTQEFWDNLEK EQLGPVTQEFWDNLEK VQPYLDDDFQK WQEEMELYR QKVEPLR AELQEGAR LSPLGEEMR THLAPYSDELRL LAEYHAK AKPALEDLR QGLLPVLESFK VSFLSALEEYTK	1
Apolipoprotein A-I(1-242).	P02647	28	5.3	3.6*10 <sup>-24</sup>	DEPPQSPWDR DYVSQFEFSALGK LLDNWDSVTSTFSK LREQLGPVTQEFWDNLEK EQLGPVTQEFWDNLEK VQPYLDDDFQK WQEEMELYR QKVEPLR AELQEGAR LSPLGEEMR THLAPYSDELRL AKPALEDLR QGLLPVLESFK VSFLSALEEYTK	2
RETBP_HUMAN	P02753	21	5.2	1*10 <sup>-7</sup>	FSGTWYAMAK FSGTWYAMAKK GNDDHWIVDTDYDYAVQY SCR LLNLDGTCADSYSFVFSR QRQEELCLAR QEELCLAR LIVHNGYCDGR	3

These findings are partially in agreement with already published work on ApoA-I levels: in fact, the overexpression of ApoA-I in the case of diabetic subjects is perfectly in agreement with that found by Calvo *et al.*,<sup>15-17</sup> while the same behavior observed here for nephropathic subjects is in contrast with the data published by Batista *et al.*<sup>13</sup> and Okubo *et al.*<sup>14</sup> based on stable isotope studies. This discrepancy can be due to an increased catabolism of triglycerides occurring in patients with end-stage renal disease. The glycation of ApoA-1 in the case of diabetic patients is perfectly in line with what has already been observed by us for other circulating proteins such as human serum albumin, hemoglobin and IgG. The present investigation evidences an undescribed

behavior: in the case of nephropathic subjects a glycation level analogous to that present for diabetics is observed. This can be due to (1) carbonyl stress or (2) glucose from peritoneal dialysis.

The spot 3, corresponding to RBP, was completely undetectable in the case of healthy subjects, while it was abundant for both the pathological states. Its function has been described as deliverer of retinol from the liver stores to the peripheral tissues. In plasma, the RBP-retinol complex interacts with transthyretine: this prevents its loss by filtration through kidney glomeruli. The levels of RBP secreted by liver and adipocytes have been recently evaluated from lean healthy, obese and obese type 2 diabetic

**Table 4.** Modified peptides confirmed by PSD approach followed by PSTs sequence elaboration. The \* is placed on the right of the modified amino acid

Sample	PSD sequence	Score ( <i>p</i> -value)
Diabetic (2-Apolipoprotein A-I)	VSFSALEEYTK*K	$8 \times 10^{-7}$
	QGLLPVLESFK*VSFSALEEYTK	$4 \times 10^{-6}$
	DYVSQFEFSALGK*QLNLK	$3 \times 10^{-5}$
Nephropathic (2-Apolipoprotein A-I)	VSFSALEEYTK*K	$7 \times 10^{-7}$
	QGLLPVLESFK*VSFSALEEYTK	$4 \times 10^{-6}$
	DYVSQFEFSALGK*QLNLK	$5 \times 10^{-5}$

subjects. It has been shown that the levels of this protein increase at least twice in the case of obese diabetic patients. Also in the case of obese, nondiabetic subjects an increase has been observed.<sup>32</sup> This behavior has been explained as related to the insulin-resistant state, already present in obese subjects, that precedes the type 2 diabetes development. Furthermore, elevated RBP levels have been associated with a reduced expression of glucose transporters 4 (GLUT4) in adipocytes, which is considered an early pathological feature of insulin resistance. Pertaining to the high RBP levels in nephropathic subjects, the concentration of free RBP and the percentage of free RBP in serum were on logarithmic scales inversely correlated with the endogenous creatinine clearance and increase in parallel with the serum creatinine.<sup>33</sup> As shown in Table 1, the nephropathic patients under study exhibited high creatinine levels and the results obtained on RBP are perfectly in line with what has already been published.

The data obtained indicate that the evaluation of ApoA-I, glycosylated ApoA-I and RBP can be considered a valid diagnostic tool to assess the metabolic state of diabetic and/or nephropathic patients. In fact, while glycosylated ApoA-I levels can be related with the glyco-oxidation stress experienced by the patient during the half-life of the protein, the change in functionality of the protein due to glycation necessarily reflects a different cholesterol transport efficiency. This aspect could give a rationale for some of the long-term diabetic complications. It is to be emphasized that the same trend is also observed for end-stage renal disease patients, but originating by a different mechanism related to the efficiency of glycosylated ApoA-I clearance. This result explains the occurrence of macrovascular disease in both types of patients well.

The increase of RBP levels in the case of patients must be related to two different mechanisms, typical of the kind of disease. Then, in the case of diabetic subjects the RBP overexpression can be justified by the obesity of the patients, while in the case of nephropathic subjects it can be related to the impaired excretion due to tubular damage.

## REFERENCES

- Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham study. *The Journal of the American Medical Association* 1986; **256**: 2835.
- Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circulation Research* 2005; **1221**: 1232.
- Rader D. Regulation of reverse cholesterol transport and clinical implications. *The American Journal of Cardiology* 2003; **92**: 42J.
- Jonas A. Lecithin cholesterol acyltransferase. *Biochimica et Biophysica Acta* 2000; **1529**: 245.
- Krieger M. Charting the fate of the "Good Cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. *Annual Review of Biochemistry* 1999; **68**: 523.
- Vainio S, Ikonen E. Macrophage cholesterol transport. A critical player in foam cell formation. *Annals of Medicine* 2003; **35**: 146.
- Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, Fogelman AM. Antiinflammatory properties of HDL. *Circulation Research* 2004; **95**: 764.
- Roher I, Hersberger M, von Eckardstein A. High density lipoproteins in the intersection of diabetes mellitus, inflammation and cardiovascular disease. *Current Opinion in Lipidology* 2004; **15**: 269.
- Quintao EC, Medina WL, Passarelli M. Reverse cholesterol transport in diabetes mellitus. *Diabetes/Metabolism Research and Reviews* 2000; **16**: 237.
- Walldius G, Jungner I. The apo/apoAI ratio: a strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy- a review of the evidence. *Journal of Internal Medicine* 2006; **259**: 493.
- US Renal Data System. *Death Rates by Primary Cause of Death, 2000 Annual Data Report, Reference Tables, Table H 18*. NIH, NIDDK: Bethesda, 2000; 659.
- Attman PO, Samuelsson O, Alaupovic P. Lipoprotein metabolism in renal failure. *American Journal of Kidney Diseases* 1993; **21**: 573.
- Batista MC, Welty FK, Diffenderfer MR, Sarnak MJ, Schaefer EJ, Lamou-Fava S, Asztalos BF, Dolnikowski GG, Brousseau ME, Marsh JB. Apolipoprotein A-I, B-100 and B-48 metabolism in subjects with chronic kidney disease, obesity and the metabolic syndrome. *Metabolism* 2004; **53**: 1255.
- Okubo K, Ikewaki K, Sakai S, Tada N, Kawaguchi Y, Mochizuki S. Abnormal HDL apolipoprotein A-I and A-II kinetics in hemodialysis patients: a stable isotope study. *Journal of the American Society of Nephrology* 2004; **15**: 1008.
- Calvo C, Ponsin G, Berthezene F. Characterization of the non enzymatic glycation of high density lipoprotein in diabetic patients. *Diabetes and Metabolism* 1988; **14**: 264.
- Calvo C, Talussot C, Ponsin G, Berthezene F. Nonenzymatic glycation of apolipoprotein A-I. Effects on its self-association and lipid binding properties. *Biochemical and Biophysical Research Communications* 1988; **153**: 1060.
- Calvo C, Verdugo C. Association in vivo of glycosylated apolipoprotein A-I with high density lipoproteins. *European Journal of Clinical Chemistry and Clinical Biochemistry* 1992; **30**: 3.
- Shishino K, Murase M, Makino H, Sadeki S. Glycosylated apolipoprotein A-I assay by combination of affinity chromatography and latex immunoagglutination. *Annals of Clinical Biochemistry* 2000; **37**: 498.
- Ajees AA, Anantharamaiah GM, Mishra VK, Hussain MM, Murthy HMK. Crystal structure of human apolipoprotein A-I: insights into its protective effect against cardiovascular diseases. *Proceedings of the National Academy of Sciences* 2006; **103**: 2126.

20. Patterson SD, Aebersold RH. Proteomics: the first decade and beyond. *Nature Genetics* 2003; **33**: 311.
21. Cristoni S, Bernardi LR. Bioinformatics in mass spectrometry data analysis for proteomics studies. *Expert Review of Proteomics* 2004; **1**: 469.
22. Moneti G, Francese F, Mastrobuoni G, Pieraccini G, Seraglia R, Valitutti G, Traldi P. Do collisions inside the collision cell play a relevant role in CID-LIFT experiments? *Journal of Mass Spectrometry* 2007; **42**: 117.
23. Harper RG, Workman SR, Schuetzner S, Timperman AT, Sutton JM. Low-molecular-weight human serum proteome using ultrafiltration, isoelectric focusing and mass spectrometry. *Electrophoresis* 2004; **25**: 1299.
24. Banfi C, Brioschi M, Wait R, Begum S, Gianazza E, Fratto P, Polvani G, Vitali E, Parolari A, Mussoni L, Tremoli E. Proteomic analysis of membrane microdomains derived from both failing and non-failing human hearts. *Proteomics* 2006; **6**: 1976.
25. <http://www.expasy.org/tools/aldente/> [accessed 2007].
26. <http://prowl.rockefeller.edu/> [accessed 2007].
27. <http://www.expasy.ch/tools/glycomod/> [accessed 2007].
28. <http://peptide.ucsd.edu/> [accessed 2007].
29. Tanner S, Shu H, Frank A, Wang LC, Zandi E, Mumby M, Pevzner PA, Bafna V. InSpecT: fast and accurate identification of post-translationally modified peptides from tandem mass spectra. *Analytical Chemistry* 2005; **77**: 4626.
30. Anderson NL, Anderson NG. The human plasma proteome: history, character and diagnostic prospects. *Molecular and Cellular Proteomics* 2002; **1**: 845.
31. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. *Molecular and Cellular Proteomics* 2003; **2**: 1096.
32. Graham TE, Yang Q, Blucher M, Hammerstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn B. Retinol-binding protein 4 and insulin resistance in lean, obese and diabetic subjects. *The New England Journal of Medicine* 2006; **354**: 2552.
33. Kirsztajn MG, Nishida SK, Ajzen H, Pereira AB. Urinary retinol-binding protein as prognostic marker in the treatment of nephrotic syndrome. *Nephron* 2000; **86**: 424.