Multivariate analysis approach to the serum peptide profile of morbidly obese patients

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Abstract.

BACKGROUND: Obesity is currently epidemic in many countries worldwide and is strongly related to diabetes and cardiovascular disease. Mass spectrometry, in particular matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) is currently used for detecting different pattern of expressed protein. This study investigated the differences in low molecular weight (LMW) peptide profiles between obese and normal-weight subjects in combination with multivariate statistical analysis.

MATERIALS: Serum samples of 60 obese patients and 10 healthy subjects were treated by cut-off membrane (30000 Da) to remove the most abundant proteins. The filtrates containing the LMW protein/peptides were analyzed by MALDI-TOF mass spectrometry. Dataset was elaborated to align and normalize the spectra. We performed cluster analysis and principal component analysis to detect some ionic species that could characterize and classify the subject groups.

RESULTS: We observed a down-expression of ionic species at m/z 655.94 and an over-expression of species at m/z 1518.78, 1536.77, 1537.78 and 1537.81 in obese patients. Furthermore we found some ionic species that can distinguish obese patients with diabetes from those with normal glucose level.

CONCLUSION: Serum peptide profile of LMW associate with multivariate statistical approach was revealed as a promising tool to discriminate and characterize obese patients and it was able to stratify them in relation to comorbidity that usually are associated with this disease. Further research involving a larger sample will be required to validate these findings.

Keywords: Obesity, MALDI-TOF, serum profile, principal component analysis, cluster analysis

1. Introduction

Obesity is a multifactorial disorder that is influenced by both genetic and environmental factors and may lead to various complications, including cardiovascular risks, hypertension, dyslipidemia, endothelial dysfunction, and type 2 diabetes mellitus. Obesity is becoming a worldwide epidemic in modern society, in individuals of both genders and of all ages, socio-economic strata, and ethnic groups. It is estimated that the total number of overweight adults has reached more than 1.1 billion worldwide, including 312 million obese individuals and about 10% of children that are classified as overweight or obese [1,2].

The prevalence of obesity in the United States continues to be high, exceeding 30% in most sex and age groups [3].

The European prevalence of obesity in men ranged from 4.0% to 28.3% and in women from 6.2% to

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Table 1
Casistic of individuals for the study

Subject	Tot (n°)	Sex		Age (yrs)		
		Male (n°)	Female (n°)	Median	Max	Min
Obeses	60	23	37	50	69	19
Diabetic	29	13	16	50	69	29
No diabetic	31	10	21	47	58	19
Controls	10	2	8	65	73	33

36.5%. The highest prevalence (i.e. greater than 25%) was found in regions of Italy and Spain in both sexes [4].

Obesity is formally defined as a significant increase above ideal weight and it being defined as weight which maximizes life expectancy. Life expectancy is reduced when body-mass index (BMI; defined as mass in kilograms divided by the square of the height in meters), an indicator of adiposity or fatness, is significantly increased above the ideal level [5].

Obesity is a heterogeneous group of conditions with multiple causes. Technological advances in the life time explain the increase prevalence of obesity as behavioral and environmental changes. Indeed the physiological mediators of energy intake and expenditure act to response to several interactions between genetic, environmental and psychosocial factors to determine the body weight [6].

Application of mass spectrometry (MS) to protein analysis has led to development of a variety of emerging proteomic methods applied to clinical specimens like serum/plasma and tissues. Separation, identification, and characterization of proteins and an understanding of their interactions with other proteins are the essential aims of proteomic analysis. One such technology, matrix-assisted laser desorption/ionization time of flight spectrometry (MALDI-TOF), has been used for protein-expression profiling to detect differently expressed proteins.

We applied a MS-based protein expression profiling approach to serum samples from patients with obesity to characterize obesity-related factors that can play a major role in development of the metabolic disorder.

The choice of the serum patient as the investigating sample was correlated to the rapidity and minimal invasiveness of the collecting procedure and to the best patient compliance. It is well known that the low molecular weight (LMW) proteome reflects the physiological or pathological state of cells and tissues [7]. To improve the detection of these proteins/peptides, before the MS analysis, the serum was treated by cut-off membranes in order to eliminate the high molecular

weight (HMW) proteins (e.g. albumin, immunoglobulin, transferrin and lipoproteins) that would be responsible of signal suppression effects.

In this study, a new strategy of protein profiling by MALDI/TOF combined with multivariate statistical analysis was developed to explore and characterize the disturbances of metabolic patterns.

2. Materials and methods

2.1. Patients

The study was carried out on consecutive cases of morbidly obese patients operated between October, 2001 and December, 2008 at Padova University General Hospital. The present study, which was in conformity with the principles in the Helsinki Declaration, was reviewed and approved by the local Ethics Committee (P.448); all subjects involved, submitted to bariatric surgery, gave their fully informed consent in writing.

The *study group* comprised 60 morbidly obese patients (> 40 Body mass index or > 35 Body mass index with comorbidities) (23 males and 37 females), with a median age of 50 (range 19–69) years. This group comprised 29 diabetic obese subjects and 31 obese subjects with normal glucose level (Table 1). Blood samples were obtained during intraoperative treatment. The *control group* comprised 10 healthy subjects (2 males and 8 females), with a median age of 65 (range 73–33) years.

2.2. Sample preparation

For each patient, 200 μL of serum was diluted 1:4 with H_2O, and 500 μL of diluted sample was centrifuged for 20 minutes at 3000g in *Amicon Ultra-4 Centrifugal Filter Devices* at 30 kDa cut-off (Millipore, Billerica, MA, USA). Before MALDI-TOF analysis, 10 μL of filtrate was desalted and purified by ZipTip$_{C18}$ pipette tips (Millipore, Billerica, MA, USA) following the procedure described in the ZipTip user's guide.

2.3. MALDI-TOF analysis

MALDI-MS measurements were performed using an Ultraflex II MALDI-time of flight (TOF) instrument (Bruker Daltonics, Bremen, Germany), operating in reflectron positive ion mode. Ions were formed
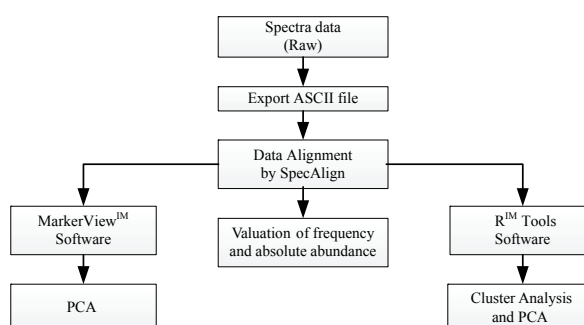
Fig. 1. Flowchart of dataset treatment after MALDI-TOF. Abbreviations: ASCII = American Standard Code for Information Interchange; PCA = Principal Component Analysis.

by a pulsed UV laser (λ = 337 nm) beam. Attempts were done to perform some post source decay (PSD) experiments on the most abundant ions but the low yield of PSD fragmentation processes did not allow us to obtain valid results. The instrumental conditions were: IS1 = 25 kV; IS2 = 21.65 kV; reflectron potential: 26.3 kV; delay time = 0 ns. The matrix was α-cyano-4-hydroxycinnamic acid (CHCA) [saturated solution in H_2O/acetonitrile (50/50 v/v) containing 0.1% trifluoracetic acid]. Five μL of purified filtrated serum sample and 5 μL of matrix solution were mixed. One μL of the resulting mixture was deposited on the stainless steel sample holder and allowed to dry before the introduction into the mass spectrometer. External mass calibration was done using the peptide calibration standard provided by Bruker Daltonics, based on the monoisotopic values of $[M+H]^+$ of Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip (1–17), ACTH clip (18–39), Somatostatin 28 at 'mass/charge' (m/z) 1046.54, 1296.69, 1347.74, 1619.82, 2093.09, 2465.20 and 3147.47, respectively.

On the basis of our previous experience [8], we focused our MS analysis on the region of LMW serum components, in the m/z range below 5000. Each MALDI spectrum was registered by the operator by summarizing 500 laser shots. The reproducibility of the MALDI data was evaluated by the calculation of discrepancy factor (D) [9].

2.4. Data treatment and statistical analysis

The overall plan of data treatment is summarized in Fig. 1.

Before conducting the statistical analysis, the MALDI-MS dataset was entered into a calculation matrix of m/z values, automatically aligned and converted to ASCII files using the SpecAlign Software version

1.22 [10]. The spectra were binned to a size of one unit of m/z, to reduce the complexity of spectra and to obviate any amplification of different species that was very closer to one another, it could be assume that they was the same protein, as described by Ragazzi et al., 2006 [11].

Moreover, for each single ionic species, we evaluated the frequency on the total of the spectra and we considered relevant only the ionic species with a frequency > 10–20%. The analysis were performed considering the absolute abundance of the peaks.

2.5. Multivariate analysis

2.5.1. DAnTE R software

DAnTE (Data Analysis Tool Extension) is a statistical tool of R Software designed to address challenges associated with quantitative bottom-up, shotgun proteomics data. This tool has also been used for microarray data and can easily be extended to other high-throughput data types [12].

2.5.2. Principal Component Analysis (PCA)

The Principal Component Analysis is a mathematical procedure that reduces the dimensionality of the data-set of variables; the data are represented in a dimensional space of n variables, which are reduced to a few *principal components* (PC) that are descriptive dimensions indicating the maximum variation within the data. These PC are linear combinations of the original variables. PCA can help to identify new meaningful underlying variables and it can detect the presence of clusters within multivariate data.

2.5.3. Cluster Analysis (CA)

To find criteria appropriate for classifying the cases according to the mass spectra pattern obtained from serum samples, a hierarchical clustering procedure (unsupervised clustering) was used without any prior knowledge of grouping.

2.5.4. MarkerView$^{\text{TM}}$ Software

MarkerView Software uses sophisticated processing algorithms that accurately find peaks in complex data sets. The data alignment compensates for minor variations in mass values, ensuring that identical compounds in different samples are accurately compared to one another. It allows to rapidly review data acquired on mass spectrometers to determine up- and down-regulation of endogenous species in complex samples. With statistic capabilities including principal compo-
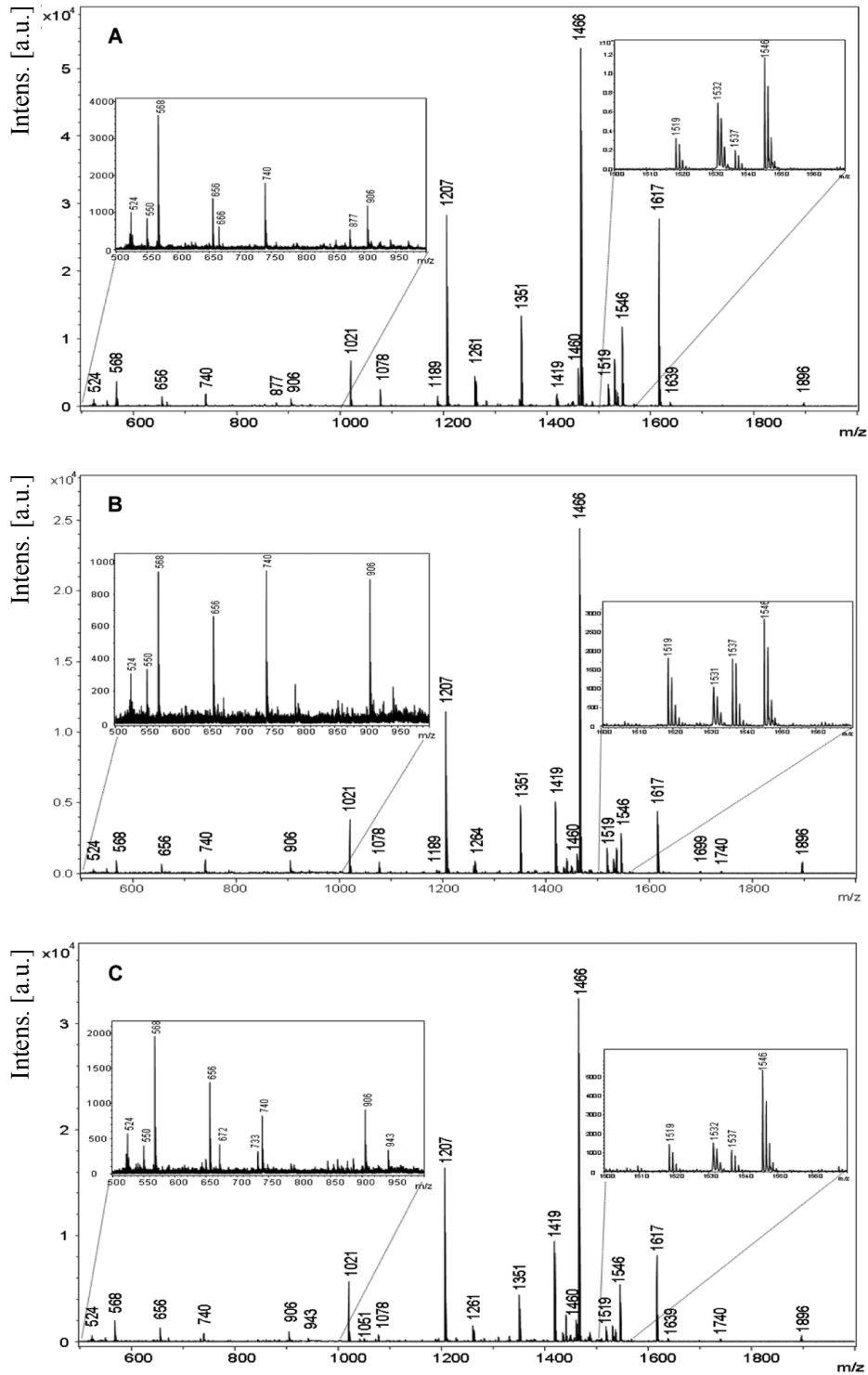
Fig. 2. Example of MALDI/MS spectrum of serum sample from a healthy not obese subject (A), an obese subject with normal glucose level (B) and a diabetic obese subject (C). The first zoom area is in the range of 500–1000 m/z and the second one is between 1500–1570 m/z.
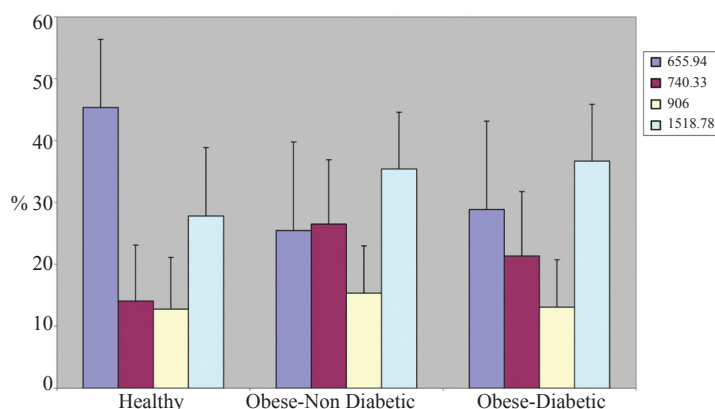
Fig. 3. Histograms of the abundances of ions at m/z 655.94, 740.33, 906 and 1518.78 as found in serum samples of healthy subjects, obese subjects with normal glucose level and diabetic obese subjects. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130971)
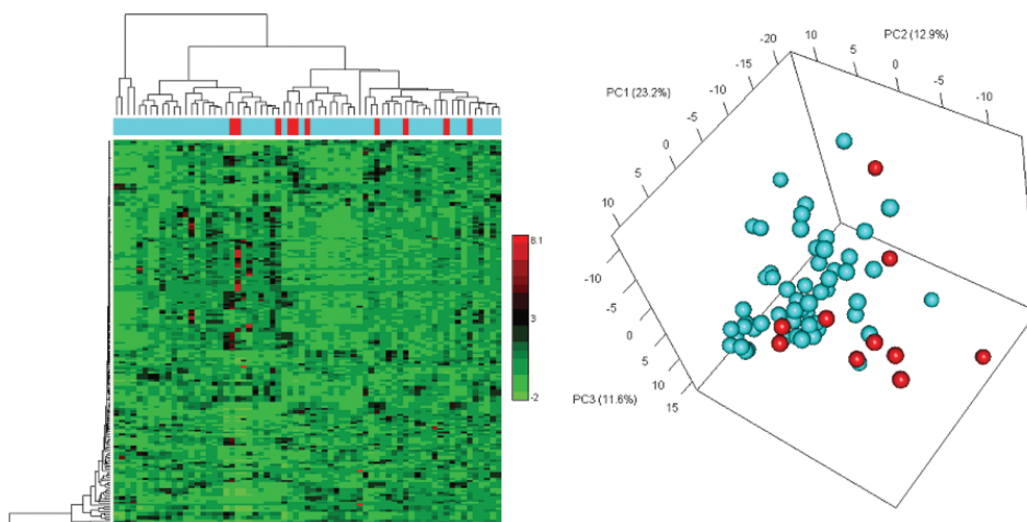

Fig. 4. Elaboration of all MALDI-TOF dataset of Control (red) and Study (blue) group with unsupervised hierarchical Cluster Analysis and unsupervised Principal Component Analysis visualizing in 3D. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130971)

nent analysis and t-tests, it is easy to mine data resulting from experiments where the sample groupings are known ahead of time (supervised), from experiments where there is no knowledge of the inherent sample groupings prior to analysis (unsupervised), or from a combination of the two. After data analysis, MarkerView Software provides report generation capabilities that can help to record the putative biomarkers.

3. Results

3.1. MALDI/TOF analysis

Serum of 60 obese subjects and 10 healthy subjects

were analyzed in order to observe the presence of some differences in the low molecular weight serum protein profile (Fig. 2). We acquired data from 500 to 5000 m/z and in particular we observed more ionic species at low m/z until 2000. In this range we found the ionic species at 1466 m/z as the higher peak and some difference of peak's intensity (as 655.94, 1518.78 and 1536.77 m/z).

The reproducibility of MALDI-TOF data was evaluated by the analysis of the spectra obtained by the same sample (serum + CHCA matrix) deposited on the MALDI plate for five times (D values in the range 0.13–0.21) and the spectra obtained by the same serum
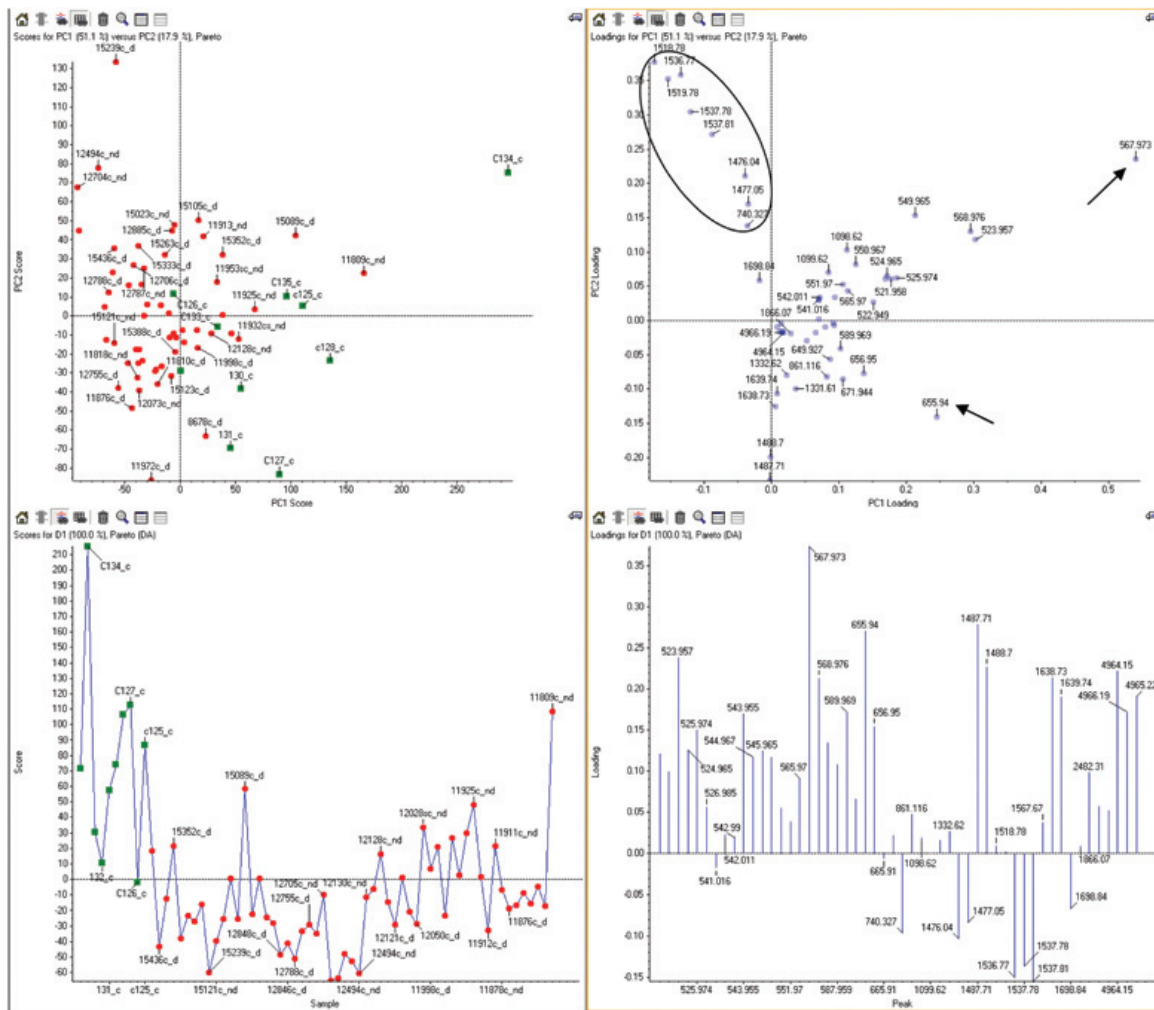
Fig. 5. Score and Loading Graphics of Control (■, green) versus Study (●, red) group with unsupervised (top) and supervised (down) PCA using ionic species with *t*-test > 95%. In evidence on Loading Graphics, we found the ionic species more characteristics of each group. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130971)

sample prepared in five different days (range was 0.18–0.25). The values are indicative for a good reproducibility, taking into account the complexity of a MALDI experiment. Just to give an evaluation of the significance of the above D range values, it must be considered that comparing the spectrum of a healthy subject with that of an obese patient, the D value increase to 1.21.

3.2. Evaluation of the frequency and the abundance of ionic species

We observed some differences among the *study* and *control groups*: the major differences can be obtained considering the frequency and the absolute abundance

of the species at m/z 655.94, 740.33, 906 and 1518.78. We calculated the ratio abundance (i.e. the abundance A_s of each species S was calculated keeping $\sum_i A_{s_i} = 100$) of the 4 most interesting ions (Fig. 3). In particular the species at m/z 655.94 significantly underexpressed in obese subjects.

3.3. DAnTE R software analysis

All the ionic species of the spectrum of *control* and *study groups* were analyzed using *DAnTE R* unsupervised hierarchical clustering analysis and unsupervised PCA (Fig. 4). No significant cluster of the sample groups was found by clustering analysis, while a
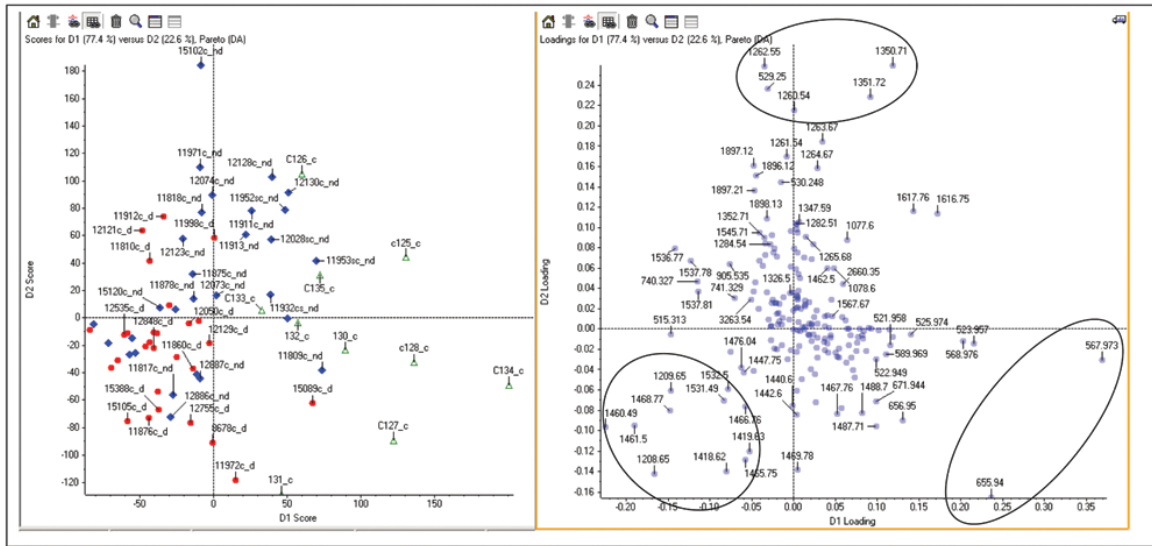
Fig. 6. Supervised PCA: Score and Loading Graphic of the three groups of subjects (controls: △, green; obese-diabetics: ●, red, and obese-non diabetics: ◇, blue). In the circle we found the ionic species more characteristics of each group. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130971)
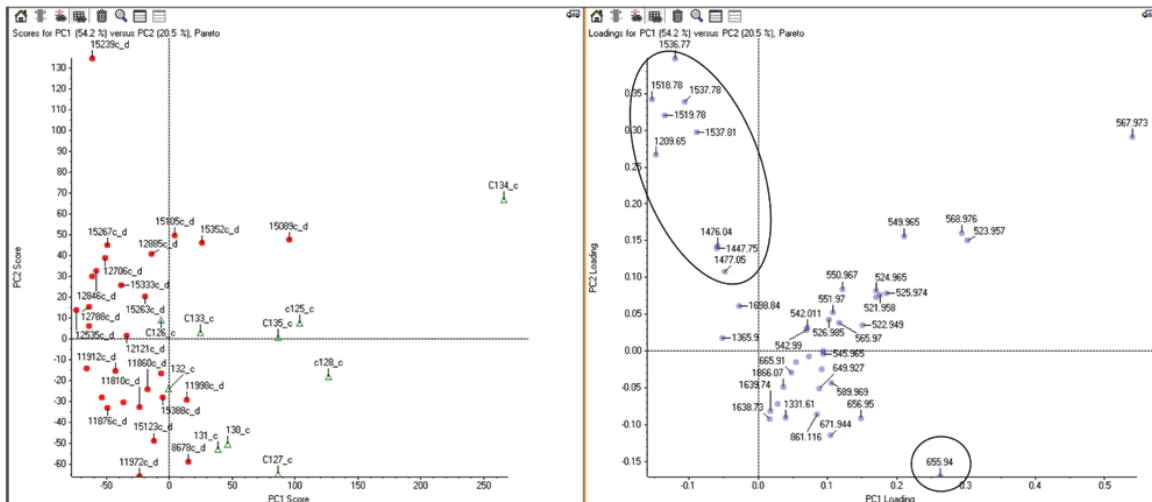

Fig. 7. Unsupervised PCA: Score and Loading Graphic of ionic species of control (△, green) and obese-diabetics (●, red) group. In the circles we found the ionic species more characteristics of each group. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130971)

grouping of control samples (in red) was showed along the PC2.

This software allows to visualize PCA analysis in 3D, but it does not perform a graphical visualization of the ionic species score calculated on PC1, PC2 and PC3.

Furthermore, we analyzed all ionic species of the *control group* with the ones of *obese-diabetic samples* and *obese-non diabetic samples* by cluster analysis. No

significant clusters of the sample groups were found.

3.4. MarkerView^TM Software analysis

3.4.1. Control group versus study group

A *t*-test for each ionic species detected in the *control* and *study group* was performed and we selected the ionic species with *t*-test probability > 95%. A supervised PCA was performed and we found that the
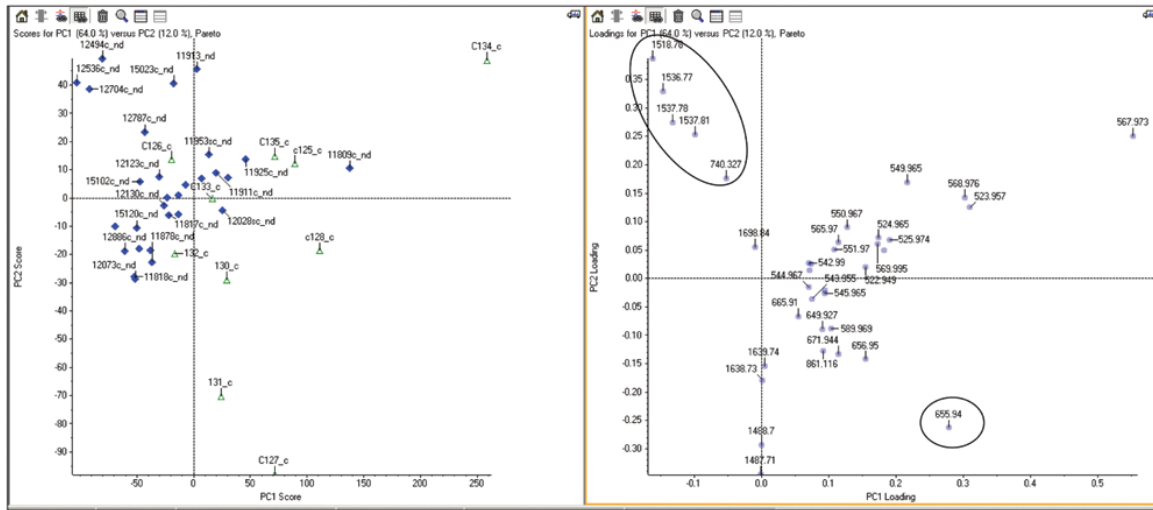
Fig. 8. Unsupervised PCA: Score and Loading Graphic of samples of control (Δ, green) and obese-non diabetics ($\lozenge$, blue) group. In the circles we found the ionic species more characteristics for each group. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130971)

52 ionic species selected with t-tests did not distinguish the two groups of interest. Then an unsupervised PCA was carried out. Figure 5 shows a clear cluster of the *control* samples along the PC1 with value > 0. However, looking at the Loading Graph that shows the weight of the all ionic species in the mathematical construction of PC, some m/z intensities can be related to one specific group; in particular ions at m/z 567.97 and 655.94 were showed over-expressed in the *control group* and the ions at m/z 740.33, 1476.04, 1477.05, 1518.78, 1519.78, 1536.77, 1537.78 and 1537.81 were showed over-expressed in the *study group*.

Then we compared the *control group* with the *obese-diabetics* and the *obese-non diabetics* subjects using a supervised PCA. As showed on the Score Graph, we found a mild grouping of control samples on the PC1 with value > 30. Most of the *obese-diabetics* and *obese-non diabetics* subjects were grouped over and under value 0 on PC2, respectively. We identified 2 ions (at m/z 567.97 and 655.94) as characteristic for *control group*, 5 ions (at m/z 529.25, 1260.54, 1262.55, 1350.71 and 1351.72) as characteristics for *obese-non diabetics group,* and 11 ions (at m/z 1208.65, 1209.65, 1418.62, 1419.63, 1460.49, 1461.50, 1465.75, 1466.76, 1468.77, 1531.49 and 1531.50) as characteristics for *obese-diabetics group* (Fig. 6).

3.4.2. Control group versus obese-diabetics group

We analyzed the ionic species with t-test probability $> 95\%$ of the *control group* versus the *obese-diabetics*

group. We performed the unsupervised PCA and we found that there was a slight separation between the 2 groups along PC1 (Fig. 7): the ion at m/z 655.94 was maintained more expressed in *control* samples, while the ions at m/z 1447.75, 1476.04, 1477.05, 1519.78, 1536.77, 1537.78 and 1537.81 were found expressed in *obese-diabetics group*. Moreover we identified another ionic species at m/z 1209.65, which also was characteristic of *obese-diabetics group* using supervised PCA (Fig. 6).

3.4.3. Control group versus obese-non diabetics group

We analyzed the ionic species related to the *control group* and *obese-non diabetic group* with t-test $> 95\%$ using an unsupervised PCA. In Fig. 8, a slight separation between the 2 groups along PC1 is showed: m/z 655.94 was more expressed in *control* samples, while m/z 740.33, 1518.78, 1536.77, 1537.78 and 1537.81 were over-expressed in *obese-non diabetics group*. The last 4 ionic species were also characteristics of *obese-diabetics group*.

3.4.4. Obese-diabetics group versus obese-non diabetics group

Although the PCA analysis of *diabetics* versus *non diabetics* subgroups highlighted no significant differences in the LMW serum peptide profile, through the previous independent comparison of each subgroup with *control group*, we identified 4 common ions at m/z 1518.78, 1536.77, 1537.78 and 1537.81 in both

subgroup and the ion at m/z 740.33 as characteristic species of *obese-non diabetic group* and 5 ions at m/z 1209.65, 1447.75, 1519.78, 1476.04 and 1477.05 as characteristic species of *obese-diabetics group*.

4. Discussion

Morbid obesity is a chronic, stigmatized and costly disease that is rarely curable and is increasing in prevalence in most of the world. To date available treatments, including drugs, are palliative and are effective in the long term. However, the delay in treatment reduces life expectancy *per se* and increases the risk of future development of comorbidities such as diabetes, being the most threatened for its related cardiac implications [13]. Broad evidences suggest that bariatric surgery may increase longevity, particularly through reducing cardiovascular cause of death [14].

Moreover bariatric surgery continues to be the most sustainable form of weight loss available to morbidly obese patients. It is unsurprising that results have improved and better data are emerging regarding improvement of obesity-related comorbid conditions.

Serum proteomic is an important discipline that focuses on the evaluation of circulating proteins/peptides, which can be consequences of genetic and/or metabolic changes. However, it is necessary to reduce the sample complexity and in particular to remove the high abundant proteins, to focus on the low molecular weight peptide serum profile. In this way MALDI-MS serum profile, can be used as an initial screening to identify ionic species pattern associated to pathological conditions and comorbidity.

In our preliminary study, we analyzed the LMW protein/peptide profile of a group of obese adult subjects by multivariate approaches. We performed data analysis with several and different software at the same time to improve the quality of outputs. The DAnTE R Software analysis by PCA using the complete native MALDI dataset showed only a cluster between control and study group. Possibly the collinearity of many data considered could have masked most informative information present in samples. Therefore, to cluster the various groups, MS data analysis has required an additional selection of ionic species based on the presence of a statistically significant difference. Indeed we performed a t-test analysis by MarkerView Software to reduce and select the ionic species and to obtain a more informative dataset.

In this preliminary study, we observed contrasting results regarding the analysis of the groups that we compared. We performed supervised PCA, where we directed the performance of analysis importing information associated with each sample, and we identified in total 18 ions that characterized controls group (2 ions), obese-non diabetics group (5 ions) and obese-non diabetics group (11 ions). In the following step, we performed a unsupervised PCA, not based on *a priori* assumptions. In particular, we observed no significant difference between obese-diabetics and obese-non diabetics in a direct comparison that could suggest an insufficient sample size and/or an influence of other unknown variables such as those related to drug treatment or linked to the presence of a metabolic syndrome. Thus, we performed independent and separate unsupervised analysis of the obese subgroups with control group and we compared the ionic species to identify in total 10 ions, referred as 4 common ions for both subgroups, 1 for obese-non diabetics group and 5 for obese-diabetics group. In particular, according to the evaluation of frequency and ratio abundance we observed the ionic species at m/z 655.94, 740.33, 906 and 1518.78 while by PCA we observed ions at m/z 567.97, 655.94 and the ions at m/z 740.33, 1476.04, 1477.05, 1518.78, 1519.78, 1536.77, 1537.78 and 1537.81. Therefore we compared the detected ionic species and we identified some common ions between the different analysis: the under-expression of ion at m/z 655.94 and the over-expression of 4 ions at m/z 1518.78, 1536.77, 1537.78 and 1537.81 as characteristic of obese subjects.

Furthermore, we identified the ions at m/z 740.33, and 1209.65, 1447.75, 1519.78, 1476.04 and 1477.05 as characteristic species of obese-non diabetics and obese-diabetics subjects, respectively.

In the obese-diabetics group, we found a higher number of ionic species than in obese-non diabetics group. This observation allows us to hypothesize that these species could be originating from protein glycation [15], and from inflammatory response [16] as previously reported associated with diabetics.

Our findings indicated that LMW serum protein/peptide profile obtained by MALDI-MS is a promising tool for discriminating obese and healthy subjects. However, further studies on larger group of patients are needed to validate the results and for identifying the ionic species that we detected by LMW peptide profiles and multivariate statistical analysis.

5. Conclusions

Although standardized bariatric procedures have been performed for several decades, it remains unclear

which technique should be considered the treatment of choice on a patient-specific basis, as the various surgical treatments provide different metabolic follow-up results, thus hampering comparison. Excess weight loss is generally accepted as the key parameter for determining success rates after bariatric surgery. However, in morbidly obese patients, the effect on body weight is not always paralleled by a comparable decrease in comorbid conditions. For this reason, it is becoming clear that there is a need for new endpoints and sophisticated research tools that would provide an increased understanding of the underlying biofunctional mechanisms. The study of peptidoma has the remarkable potential to enhance our understanding and practice of weight regulation. It is increasingly becoming apparent that proteomics, more than gene analysis, is the research tool of choice in understanding the complexities of patho-physiologic mechanisms.

Although in this study significant evidence was not obtained in obese-diabetic and obese-non diabetic patients, we believe that the time is ripe for this kind of approach. One of the areas in which proteomics may have its greatest potential is in the discovery of new diagnostic and prognostic markers of diseases such as morbid obesity and in understanding the response to surgery.

In conclusion, it is increasingly evident that proteomics, aside to gene expression analysis, could be the research tool of choice that helps to understand the complexities of patho-physiologic mechanisms. We show that one of the areas where proteomics might be validly and effectively applied is the characterization, through a protein/peptide profile, of morbid obesity.

Acknowledgments

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