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"Proteomics applications to three biological models: cell cultures, yeast and human plasma"

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Summary

"Proteome" is the entirety of proteins expressed in an organism, a cell, an organelle, but also in a tissue or a body fluid, including the modifications made to a particular set of proteins at a given time point under defined conditions. Proteome is much more dynamic than genome, and is strongly influenced by internal and external factors such as development, differentiation, temperature or stress and thus differs from cell to cell.

This work discusses our proteomics investigations conducted on three different types of biological samples obtained from three different organisms, belonging to different kingdoms and orders: NIH-3T3 cells, yeast *Saccharomyces cerevisiae* and Human Plasma.

In the first work [Magherini *et al.*, 2009] we performed a proteomic analysis of NIH-3T3 cells exposed to toxic aggregates of a protein domain not involved in any amyloid disease: the N-terminal domain of the prokaryotic HypF hydrogenase maturation factor (HypF-N). The aggregation properties and aggregate toxicity of HypF-N were previously characterized [Bucciantini *et al.*, 2004; 2005; Campioni *et al.*, 2008]. Two-dimensional gel electrophoresis followed by protein identification by MALDI-TOF MS, allowed us to find that aggregates cause to the cells changes in the expression level of proteins involved in stress response and in signal transduction. To our knowledge, this is the first proteomic study on the alterations of the protein expression profiles in cells exposed to toxic amyloid aggregates of a disease unrelated protein.

The second work [Guidi *et al.*, submitted to *B.B.A-Proteins and Proteomics*] deals with our proteomic study to understand how *Saccharomyces cerevisiae* adapts its metabolism during the exponential growth in three different glucose concentrations (2%, 0,5% and 20% glucose). From our analysis we noticed that yeast cells have a decreased growth rate during the initial phase of fermentation in high glucose and that there is a differential proteins expression depending on the environmental variations. In glucose restriction (0,5% glucose) and in high glucose

(20% glucose) we found an over-expression of a protein (Peroxiredoxin) involved in protection against oxidative stress insult. Identifying the functionally modulated proteins involved in glucose induced yeast response, will lead to a better comprehension of the mechanisms underlying the effects of different glucose concentrations; this will contribute to the complete understanding of yeast fermentation and respiration metabolism.

The last work made in our lab was a proteomic study of human plasma obtained from athletes trained to perform endurance exercise and from subjects practising various kinds of sport only for recreational purposes. Physical activity, following an increase in oxygen consumption, leads to a temporary imbalance between the production of RONS and their disposal; this phenomenon is called oxidative stress. We wanted to characterize plasma proteins that undergo carbonylation in response to different kinds of training and different physical exercises. In fact, it is widely accepted an increase in carbonylated proteins in plasma of athletes after physical exercise, but in literature there aren't works in which the targets of this oxidation are identified. This is the first work in which the carbonylated proteins in plasma are analyzed by a proteomic approach. We found not only proteins that are target of carbonylation after physical exercise, but also proteins which carbonylation is not affected by exercise and proteins which are carbonylated only in the plasma of the resting condition. A protein whose carbonylation level increases after exercise is the Haptoglobin, a glycoprotein present in plasma with important antioxidants functions: it protects Haemoglobin from oxidative damage. Then we found that endurance trained athletes showed a higher carbonylation of plasma proteins in comparison to men that practise various kind of sports with a moderate exercise. These methods allowed us to obtain an overview of the change in the oxidation of plasma proteins after physical exercise and to identify new markers of physiological stress.

The proteomic approach applied to these samples has thus provided a rich and varied set of data and we hope they will make a contribution to the appropriate individual fields of biotechnology applications.

Chapter 1

"Proteomics"

1. Proteomics

The word "*proteome*" is derived from "PROTEins expressed by a genOME" and it refers to all the proteins produced by an organism, much like the genome is the entire set of genes. Proteomics is the large-scale study of proteins, particularly their structures and functions. This term was first used by Wasinger *et al.,* in 1995 to make an analogy with genomics, and while it is often viewed as the "next step", proteomics is much more complicated than genomics.

One of the milestone achievements of biology has been the independent completion of the sequencing of the human genome by both the Human Genome Organisation (HUGO) [Lander 2001]and Celera Corporation [Venter *et al,.* 2001]. Since then, the corresponding availability of automated and high-throughput sequencers allowed the completion of several genomes of model organisms [Aparicio *et al.*, 2002; Waterston *et al.*, 2002; Gibbs *et al.*, 2004; Hillier *et al.*, 2004; Mikkelsen *et al.*, 2005]. In turn, the availability of whole-genome sequences started off large-scale searches for open reading frames (ORF's). Both *in silico* efforts and mRNA sequencing contributed greatly to this end and today we have a very good estimate of the total number of expected genes in the human genome [International Human Genome Sequencing Consortium 2004].

Although a genome provides the important basis for a better understanding of a living organism, it cannot by itself provide an explanation for the actual diversity and adaptability evident in all life. Rather, we have to look into the RNA and protein content of cells in order to get an idea of how the cell works at any given time.

One of the big surprises from the human genome sequencing was the relatively low number of about 20,000 human genes detected, only roughly three times more than present in yeast. Even though the human species appears much more complex than yeast, its difference is unlikely to be explained solely by the number of genes. Figure 1 gives an enlightening example why genetic data cannot explain many biological processes and functions of an organism.

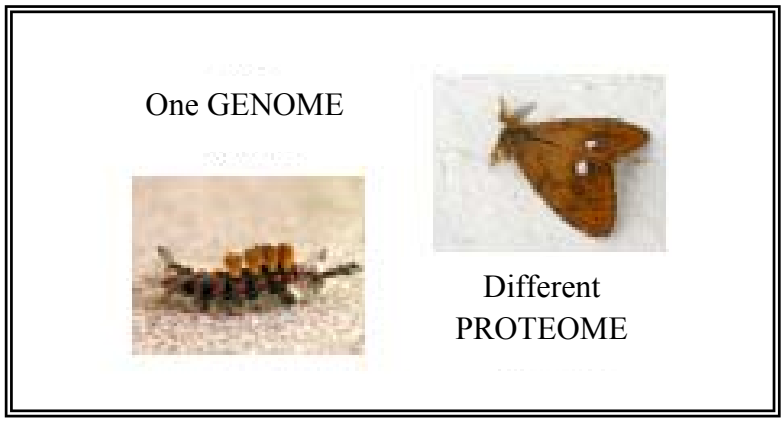

Fig. 1. Caterpillar and butterfly of Orgyia antiqua L. *Not only the genome, but in particular the proteins [the proteome] determine the appearance and state of a biological organism.*

Each somatic cell of the caterpillar and its counterpart the butterfly possess the identical genetic information, but there are two totally different phenotypes of the same insect. This fact is explained by the different expression of the individual genes into proteins, that is the different translation of the genetic information. In fact, proteins are responsible for the biological activity and function of the organism.

The proteome is the entirety of proteins expressed in an organism, a cell, an organelle, but also in a tissue or a body fluid, including the modifications made to a particular set of proteins at a given time point under defined conditions. Proteome is much more dynamic than genome, and is strongly influenced by internal and external factors such as development, differentiation, temperature or stress and thus differs from cell to cell. Furthermore, distinct genes are expressed in distinct cell types and many proteins may go through a wide variety of modifications that profoundly affect their activity. Phosphorylation, glycosylation or oxidation of certain amino acid residues can influence protein localization, stability, enzymatic activity and protein-protein interactions [Proud *et al.*, 2005; Tischer *et al.*,2003; Restle *et al.*, 2005; Spiriti *et al.*, 2008].

Understanding the functions of a living cell at a molecular level is one of the most important issues in modern research. Most of the molecular mechanisms that occur in a cell are performed by proteins [Witzmann and Li, 2002]. Given that the

whole sequence of genome of various organisms, including humans, is known, the number of proteins with unknown function has grown enormously. The *Human Genome Project* has indeed provided a wide variety of information about the sequence of individual genes. However, there isn't a simple linear relationship between the information contained in the genome and that expressed by proteins [Cahill *et al.*, 2001]. Indeed, the high post-transcriptional processing and the many post-translational variants of proteins make impossible to predict the real functional proteic product from the information contained in its *open reading frame*.

Currently, therefore, the interest is projected towards the identification of the structure, function and interactions of each protein produced by a cell, in order to understand its role both in physiological than pathological processes. The term "*proteome"* is generally attributed to Mark Wilkins, who coined the term at the "Siena Conference" in 1994 [Wasinger *et al.*, 1995]. In order to understand a living cell at the molecular level it is imperative to analyze its protein content. Analyzing the proteome presents a more daunting challenge than analyzing the genome: apart from spanning an extremely large concentration range (at least 10 orders of magnitude in plasma [States *et al.*,2006]) it is both highly dynamic in concentration as well as in modification state. Indeed, even though cells share the same genome, their proteomes can differ markedly [Collins *et al.*, 2001].

Protein sequences are not easily duplicated to large copy-numbers as is the case for nucleic acid sequences, through the application of the polymerase chain reaction (PCR). The most popular technique today for studying the proteome is by mass spectrometry, which relies on separating charged ions by their mass-to-charge ratio (m/z).

Modern proteomics studies can be divided into two main areas: expression and functional proteomics.

Expression Proteomics (also called structural): its main objective is the analysis on a large scale of proteins expressed by a cell as a function of changes in cellular conditions (different growth conditions, cellular stress, disease etc.). Expression Proteomics can be further divided into two main lines:

- Systematic proteomics : leads to the creation of reference maps of the expressed proteins and their precise identification.
- Differential proteomics: provides quantification of proteins that are differently expressed in the same biological sample, in relation to changes in physiological or pathological conditions, with the identification of proteins responsible for the expression changes.

The development of this analysis approach is the result of considerable development of analytical technologies for the separation of proteic molecules (2D electrophoresis and 2D liquid chromatography) coupled to the identification of proteins (mass spectrometry and bioinformatics). The considerable amount of new information we can get, led the proteomics studies in the biomedical field; in this field the expression proteomics aims to identify proteins involved in the development and progression of a disease, with the aim to find specific markers useful for not invasive diagnosis or prognosis of many diseases, most notably cancer [Hanashi *et al.*, 2004]. For example proteomics has been used to identify proteins that are differentially expressed between normal and tumor tissue in various cancer, such as liver, bladder, lung, prostate and others [Soldes *et al.*, 1999; Celis *et al.*, 2000; 2002; Meehan *et al.*, 2002].

Functional proteomics has the objective to identify the biological functions of proteins, whose role remains unknown. This task is also achieved through the identification of protein interactions *in vivo*. In cells, in fact, biological processes are not only controlled by the relative abundance of various proteins, but also by the transient regulation of their activity (for example by reversible covalent modification), their cellular localization and their association with other components. A large number of proteins act their functions in cells forming multi-proteic complexes; therefore, understanding biological functions of these proteins and elucidating the molecular mechanisms by which these functions are performed, is related to the identification of their molecular partners. Proteomics analysis can contribute to identify basic components of multi-proteic complexes and are an effective alternative method to the molecular biology techniques.

1.1 Two Dimensional Gel Electrophoresis (2D-GE)

Two dimensional electrophoresis allows to separate, in a single experiment, thousands of proteins expressed by a cell. In some cases more than 10,000 protein spots have been resolved in a single gel [Klose *et al.*, 1999]. The large amount of data obtained is then processed by using sophisticated bioinformatics analysis of the 2D images. The two-dimensional polyacrylamide gel electrophoresis (2D-SDS-PAGE) is a technique known since the '70s; despite the understanding of the benefits that this technique could be made in biological studies, two-dimensional electrophoresis was not actually used for many years due to some technical difficulties. In fact until the introduction of some changes in gels preparation and manipulation, to achieve clear and well reproducibly results was not possible.

Currently, two-dimensional electrophoresis is one of the methods with higher resolution for separating complex mixtures of proteins. In fact, two-dimensional electrophoresis can separate the various proteins of a highly heterogeneous protein mixture basing on their isoelectric point (isoelectric focusing), and according to their molecular weight, once masked the intrinsic charge of each protein.

However, poorly soluble proteic molecules (e.g. membrane proteins) can not be separated by two-dimensional electrophoresis, since there aren't recovered during sample preparation or during the early stages of separation processes. Small molecules (weighing less than 8 kDa) are lost during isoelectric focusing, while those weighing more than 200 kDa are not separate efficiently during the second dimension. For this reason alternative methods have been developed to integrate this method, for example two-dimensional liquid chromatography system (ion exchange / reverse phase) coupled to mass spectrometry *in tandem*. In general, these methods are called *"Multidimensional Protein Identification Technology*" [Yates *et al.*, 1997; Washburn *et al.*, 2001].

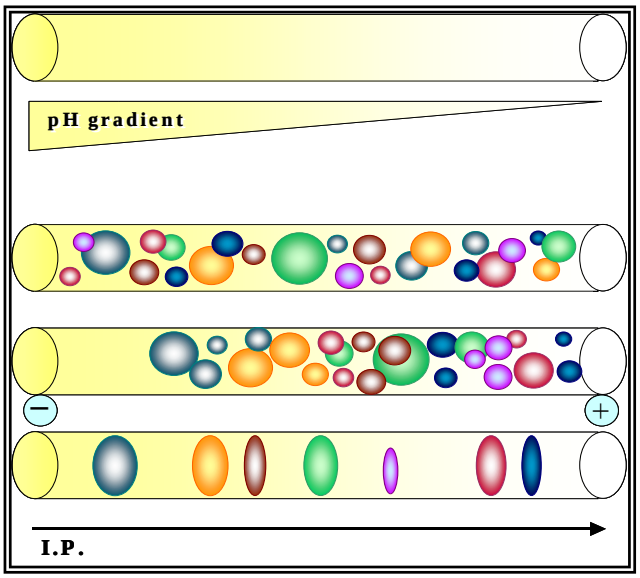

Fig. 2. Diagram of the first dimension. *At the top of the drawing there is a schematic structure of a strip with immobilized pH gradient. The bottom portion represents the phases of isoelectric-focusing. By applying a potential difference, different proteins migrate along the strip, stopping at the point where the pH corresponds to their isoelectric point.*

A common 2D-GE is achieved combining two electrophoretic techniques, which define the so-called "dimensions".

The ***first dimension*** is an isoelectric focusing (IEF), in which proteins are separated in a pH gradient until they reach an equilibrium state where their net charge is zero (isoelectric point, pI) (Fig.2). Initially, pH gradient was formed *ex novo* for each experiment, in special tubes containing the gel where sample was applied; only linear pH gradients were generated: at equal pH intervals corresponded equal parts of the gel. This methodology made the experiments less reproducible and failed to provide good resolution of heterogeneous protein mixtures. The success of two-dimensional electrophoresis came when, for the first dimension, *preformed strips* were introduced in place of the tubes containing the gel [Bjellqvist *et al.*,1982]. These strips had the polyacrylamide layer applied on. In addition pH gradient, initially generated by ampholytic carriers, was replaced by an immobilized pH gradient (IPG).

In the ***second dimension*** (Fig.3), polypeptides are separated orthogonally from the first dimension, by a polyacrylamide gel electrophoresis in the presence of sodium

dodecylsulphate (SDS, an anionic detergent). This detergent binds to proteins in a stoichiometric way, according to a 1:2 ratio with the amino acid residues.

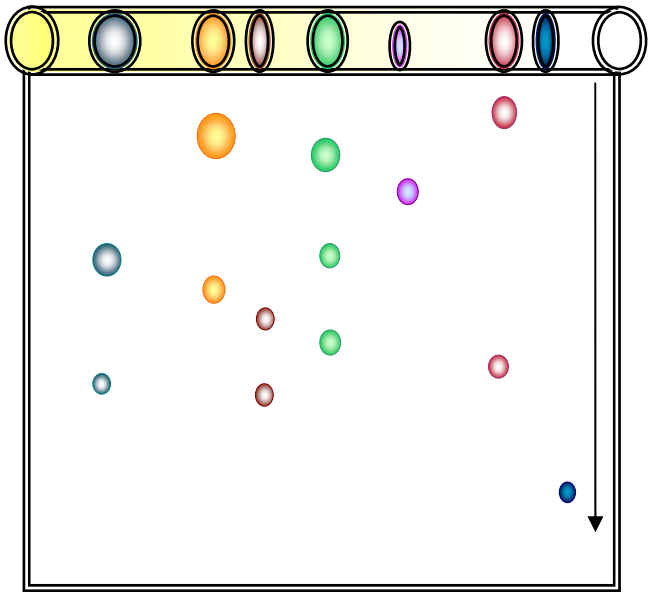

Fig. 3. Diagram of the Second dimension. *Strip containing the proteins immobilized in polyacrylamide, is placed at the top of a gel. During the run proteins migrate from the strip to the gel, maintaining the separation obtained in the first dimension and by the presence of SDS they will be further separated based on their molecular weight.*

The SDS is used in conjunction with reducing agents, in order to denature and dissociate proteins in single polypeptide chains. By using SDS the intrinsic charge of each protein is completely masked. In this way the proteins negative charge density is proportional to their molecular weight.

After the electrophoretic run, proteins are shown using appropriate specific colours and appear on the gel as a single point, called "*spot*", which can vary in size and in coloration intensity, depending on the amount of protein.

With specific software and mass spectrometry is possible quantify the differences between individual spots in different gels, in order to obtain information about changes in proteomes of samples of interest. The high resolving power of two-dimensional electrophoresis is mainly due to the fact that two distinct chemical and

physical properties of the polypeptides are exploited [Herbert *et al.*,1997]. Perhaps one of the key to the success of 2D-PAGE has been the introduction of IPG-technology (immobilized pH gradient) and in particular the generation of non-linear extended pH gradients, thereby covering the pH range 3-10. In fact the first gradients used were linear and did not allow a good resolution in the separation, due to the fact that proteins in a cell lysate do not cover uniformly all pH ranges with their isoelectric point. This problem was solved with the introduction of non-linear pH gradients. The idea of non-linear pH gradient drift from the job to Gianazza and Righetti [1980], where they evidenced that only one third of the proteins present in a generic sample focus in the region of alkaline pH scale, while more than two thirds focus in the acid region. For this reason they created a pH gradient that had a lower slope in the more acidic region and a higher slope in the alkaline one, improving the yield of the first dimension separation. For example, Giannazza *et al.*, [1985], with these modifications, achieved significant improvements in the resolution of overlapping bands in the acidic region of a *Klebsiella pneumoniae* lysate, without incurring losses in the basic region of the protein profile. A pH gradient of 3.5-10 laid the foundations for most of the non-linear gradients in commerce today.The different ranges of gradients allowed to explore a larger region of the pH scale.

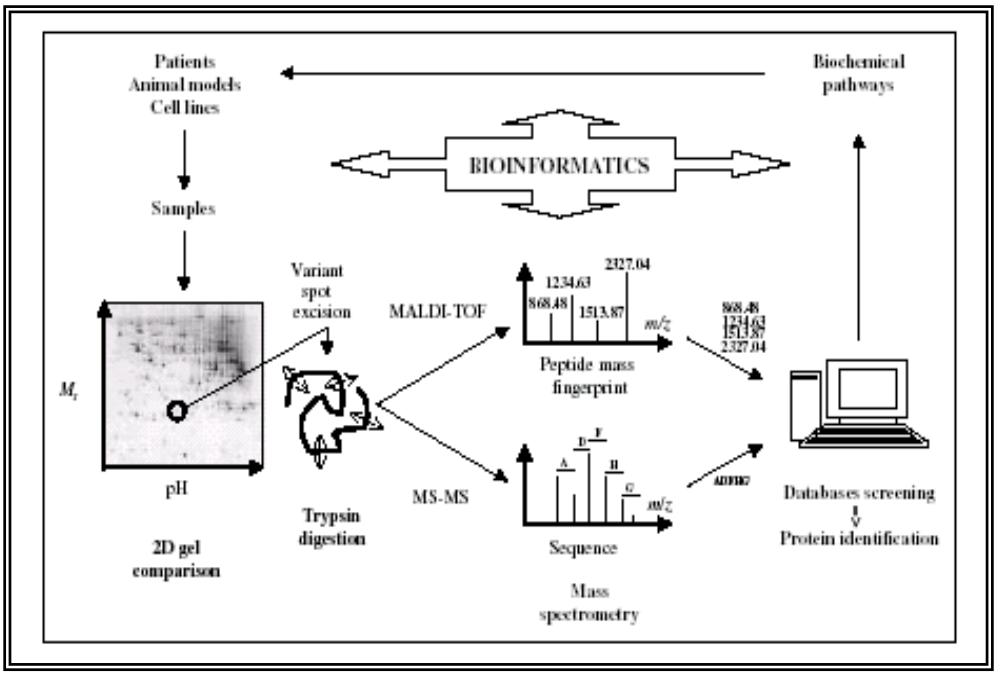

Fig. 4. *Proteomics analysis.*

Highly acidic pH ranges were described for the fractionation of acidic proteins such as pepsin [Righetti *et al.*, 1988], as well as very basic ph ranges (ph 10-12) for the analysis of protease with high and even of histones [Bossi *et al.*, 1994]. In 1990 2D-maps were described with the widest possible pH gradient (pH 2.5-11) [Sinha *et al.*, 1990]. Also sigmoidal pH gradient were optimized [Tonani and Righetti, 1991]. Despite the progress made in recent years for the development of alternative methods of protein separation, as the use of technologies based on the use of "*chips*" [Issaq *et al.*, 2002; Merchant and Weinberger, 2000] or use of "*affinity tags*" [Zhou *et al.*, 2002], two-dimensional polyacrylamide gel electrophoresis remains the most widely used in proteomics (Fig. 4).

1.2 Spots Visualization

Techniques commonly used for visualization of proteins separated on gel base on the use of dyes that bind to proteins in a non-specific way. The type of colour must be chosen according to the type of analysis (Table I). There are colour that allow viewing of spots but make impossible the analysis by mass spectrometry (for example ammoniacal silver staining). These can be used when quantitative and qualitative differences must be assessed. Other types of colouring, however, does not alter proteins structure and allow protein identification by mass spectrometry.

One such technique involves the use of colloidal Coomassie Blue staining. This dye is widely used and enables the detection of protein amounts ranging from micrograms to nanograms. A more sensitive technique, enabling detection of less abundant proteins, is the silver staining, able to detect proteins in quantities below the nanograms. Both these methods of viewing the protein, however, have drawbacks: they show a different reactivity from protein to protein, lack of linearity between the amount of protein present in the gel and the amount of dye associated and may be poorly reproducible, thereby preventing a real quantification of the protein in question. These defects were corrected with the introduction of fluorescent reagents that allow the visualization of proteins with a sensitivity of silver staining, but with fewer problems of linearity in the resolution.

Table I. *Different dyes for 2D-GE.*

Dye	*Sensibility*	*Compatibility with mass spectrometry*
radioactive	< 1 ng	yes
Coomassie Blue	30 – 100 ng	yes
Silver	1 ng	Not always
Zinc-Imidazole	10 – 20 ng	yes
SYPRO Ruby	1 ng	yes

1.3 Spots Analysis

A proteomics analysis, in spite of the sample or biological problem, requires a series of independent experiments. Generally, in order to minimize the experimental variation and to obtain the real biological information with a statistical analysis of the results, it's necessary to do the same experiments at least in triplicate. In this way, for every experiment, a lot of different gels have to be analysed, with thousands of spots to be looked in order to find qualitative and quantitative differences. In particular, to evaluate variations in spot intensities (quantitative differences) directly is impossible.

To analyze so many spots and gels in order to compare different samples and to detect both quantitative and qualitative differences, after staining, scanning the gels using a scanner or a densitometer is needed.

Thus, to analyse and quantify the differences between individual spots in different gels, in order to obtain information about changes in proteomes of samples of interest, the use of specific software is necessary. There are specific software to analyze digitized images. MELANIE (*Medical ELectrophoresys ANalysis Interactive Export system*) has bees one of the first programs and it has been updated with the program Image Master 2D (Fig.5).

The resolving power of two-dimensional electrophoresis is generally considered proportional to the total area of the gel, because it depends on the extent of both dimensions of run. On standard size gel (16cm x 18cm) between 2000 and 4000 proteins can be displayed; in order to display up to 10000 spots, gels of big dimensions have also been obtained [Klose *et al.*, 1999].

Faced with such complexity to use computerized systems of image processing is essential to obtain objectivity and reproducibility of qualitative and quantitative gels analysis. These systems will overcome the real differences between images. These differences are due to gels resolution, size and gradient, to distortion and interference. Software helps the extrapolation of information and functional expression from the huge and complex amount of data obtained by 2D-GE.

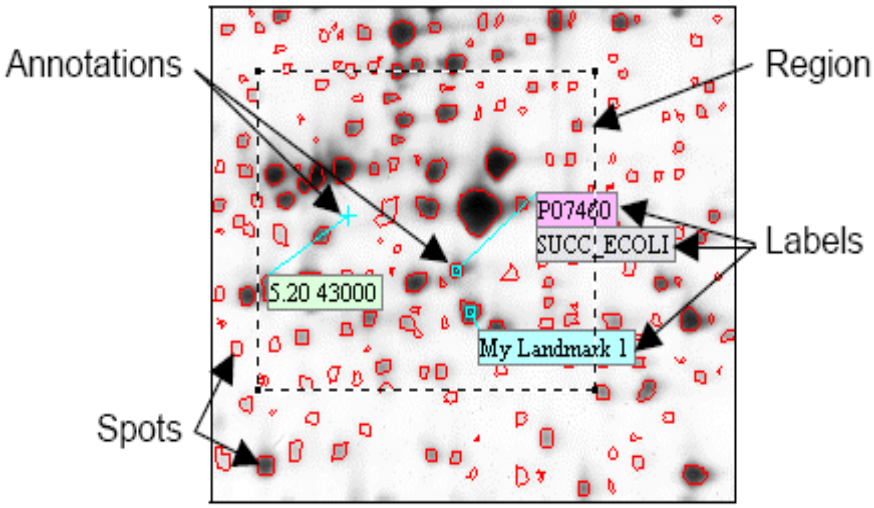

Fig. 5. *Particular of a 2D gel during the MELANIE's analysis.*

The image processing performed by the software produces an attenuation of the distortion and interference resolution, an increase in the contrast between spots and background and an images distortion allowing overlay and comparison of different gels.

Each spot recognized by the system on the gel (*spot detection*), is evaluated both qualitatively and quantitatively:

• *Qualitative analysis* is obtained in order to detect the presence or absence of spots in the gels. It's made by direct comparison overlapping different gels; the software uses specific algorithms that allow to couple different spots present in different gels as a function of their Cartesian coordinates (*gel matching*).

• *Quantitative assessment* is conducted according to the relative spot volume calculated on the total volume of all spots on the gel. The software is able to assess the volume of each spot multiplying its area to its corresponding optical intensity.

Two spots present in two different gels but characterized by the same spatial coordinates are paired and given as a *pair*. This allows a comparative analysis of the gels to understand the variations in proteins expression in relation to the different

biological conditions considered. With the numerous analysis programs a comparison of the gels obtained from samples with two-dimensional maps in database and also maps produced in different laboratories can be made directly with good results [Corbett *et al.*, 1994; Blomberg *et al.*, 1995].

Unfortunately, the procedure for computer analysis is not, as it can appear, simple and automated. The imperfection of systems analysis, both in *spot detection* that in the evaluation of "discrepancies" in images, makes the analysis an "*...exercise in frustration...*" [Witzmann *et al.*, 2002], which can take months to be completed.

1.4 Proteins identification by Mass Spectrometry

Once completed the images analysis is essential to identify and characterize the proteins that are found changed in quality or quantity [Shevchenko *et al.*, 1996]. The ability to separate high proteins loads, maintaining good resolution, gives the purification of thousands of polypeptides; these can be identified quickly and directly through specific biochemical methods. In the past, Edman degradation [Edman and Begg, 1967] was the main technique for proteins identification. Currently this method has been replaced by mass spectrometry techniques, because of problems associated with high costs and the long period of analysis [Patterson and Aebersold, 1995]. These techniques constitute the most sensitive for an accurate mass determination of various kinds of complex molecules, depending on their relationship mass/charge (*m/z*). This methodology is applicable only if the molecules can be ionized and if ions can exist in the corresponding gas phase.

Mass spectrometry has been made compatible with the analysis of biological polymers, mainly proteins, by the introduction of ionization methods that do not degrade the analytes: the electrospray ionization (ESI) [Fenn *et al.*, 1989] and matrix assisted desorption ionization (MALDI) [Karas and Hillenkamp, 1988]. Both techniques allow to analyze complex peptide mixtures obtained from proteolytic digestion or total lysates. The almost exclusive use of trypsin is important, since it allows to obtain peptides with known and predictable terminal residues.

Generally, a mass spectrometer consists of three main components: 1) Ion source; 2) Ion analyzer;3) Detector.

In **MALDI-TOF** (*Matrix-Assisted Laser Desorption Ionization - Time Of Flight*) mass spectrometry, peptides are incorporated into an aromatic matrix that promotes protonation if irradiated with laser. Then ions enter the chamber of the analyzer by applying a potential difference variable. The *m/z* ratio of each ion is then determined by its *time of flight* (TOF) registered by the detector. Therefore, with the same kinetic energy, ions having lower *m/z* ratio will arrive before at the end of the tube of the analyzer than heavier ions. If we want to determine the peptide sequence we proceed

to the further fragmentation of individual peptides using the TOF-TOF technique (Fig.6).

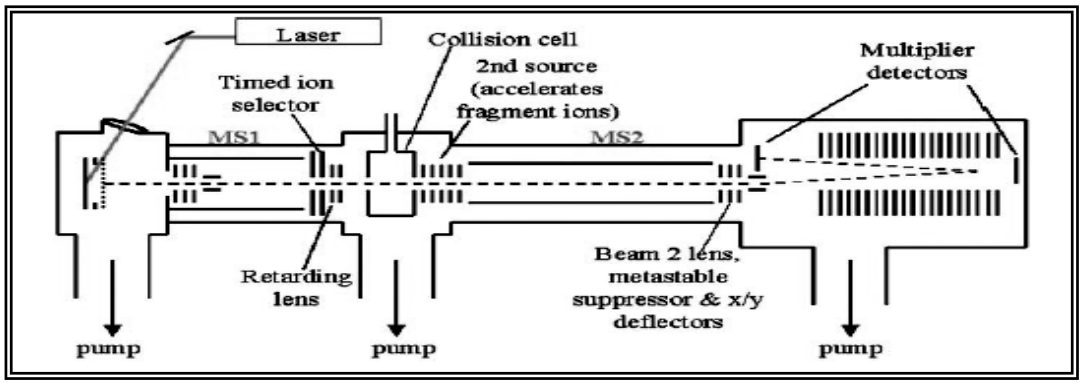

Fig. 6. *Mass Spectrometer MALDI-TOF/TOF.*

Analyzing the peptides mixture generated, we obtain a set of precise molecular weight values (*peptide mass fingerprinting*), which can be used for searching in protein sequences databases, using one of several network available software. These programs simulate the hydrolysis of all proteic sequences present in database and calculate the molecular weights values of each peptide virtually generated. Thus, for each protein, a series of virtual molecular weights is obtained; these are compared with the series of experimental values. Higher the correspondence between the measured values and theoretical ones is, greater is the probability that the protein be the one indicated in the database.

In the **ESI** mass spectrometry, analyte in liquid phase is passed through a capillary, after which the sample undergoes atomization, desolvation and ionization. In this case the analyzer is different and it is often an ion trap.

1.5 Proteomics Applications

There are countless areas of biological application of the proteomics and the amount of data, information and notional-functional integration obtained and processed is incredibly large and varied. Each type of organism, from the more complex to the most simple, is a possible object for proteomics study. It is incredible to see that even an organism generally considered "simple", like yeast, is actually an intricate functional system of proteins. Proteomics is a powerful tool that can be applied to understand a lot of biological mechanisms and processes.

For example, many applications of proteomics are used in *microbiology*. The proteome of many pathogens has been identified and placed in special databases. The proteomics approach has been useful in the development of recombinant vaccines, such as that against the *meningococcus*. Other strategies include comparative proteomic analysis of pathogens and non-pathogenic organisms, in order to identify proteins that are involved in pathogenesis. For example the proteome of the tuberculosis agent, *Mycobacterium tuberculosis*, was compared with that of relative non-pathogenic, *Mycobacterium bovis*, to identify proteins specific of the virulent strain. Proteomics has also been used to analyze all the proteins expressed by some fungi pathogenic for humans, such as *Aspergillus fumigatus* and *Candida albicans*.

Proteomics studies have also clarified that most human diseases are extremely complex, that pathogenicity involves a large number of proteins and that rarely there is a single target protein. Since proteins are the principal target of drugs, the development of proteomics techniques has provided an important aid to the identification of new active molecules. Proteomics is applied at all stages of *drug developing*: target identification, target validation, identification of *lead* compound, *lead* optimization, toxicity studies and clinical trials. Once the therapeutic target has been identified, proteomics can still be useful in the search for compounds active on that target. Techniques such as structural proteomics have been very useful in the development of protease inhibitors, drugs used to treat HIV. Additionally these techniques can be helpful in the development of broad spectrum antibiotics [Schmid

et al., 2002]. Proteomics may be useful in assessing the effectiveness of the drug and its toxicity. For example, to evaluate the proteins alterations involved in nephrotoxicity induced by gentamicin, 2D-GE was used [Charlwood *et al.*, 2002]. Proteins identified were involved in the toxicity mechanism and these studies have suggested that such markers could be used for highlighting the potential nephrotoxicity in the antibiotics screening. Correlating proteomics information with those obtained with genomic techniques, we can obtain more information on many diseases and we can clarify the action mechanisms of many drugs and many cellular processes.

1.6 Cell Culture and Proteomics

Working with cell culture is not only an established model for many diseases but also offers a quick and efficient way to analyze reactions and responses of various cells types to different effects or conditions. This includes not only looking at the cells viability, but also much deeper at the proteomics of the cells.

In cells, in fact, biological processes are not only controlled by the relative abundance of various proteins, but also by the transient regulation of their activity (for example by reversible covalent modification), their cellular localization and their association with other components. A large number of proteins act their functions in cells forming multi-proteic complexes; therefore, understanding biological functions of these proteins and elucidating the molecular mechanisms by which these functions are performed, is related to the identification of their molecular partners.

Proteomics analysis can contribute to identify basic components of multi-proteic complexes and are an effective alternative method to the molecular biology techniques. In this field the expression proteomics aims to identify proteins involved in the development and progression of a disease, with the aim to find specific markers useful for not invasive diagnosis or prognosis of many diseases, most notably cancer [Hanashi *et al.*, 2004]. Proteomics studies with cell cultures has been used to identify proteins differentially expressed between normal and tumor tissue in various cancer, such as liver, bladder, lung, prostate and others [Soldes *et al.*, 1999; Celis *et al.*, 2000; 2002; Meehan *et al.*, 2002].

In the past years, academic and industry researchers have successfully applied proteomic techniques, such as 2D-GE, liquid chromatography and mass spectrometry, to investigate protein-expression profile changes in different cell culture processes and conditions [Kim *et al.*, 2008; Shen *et al.*, 2004].

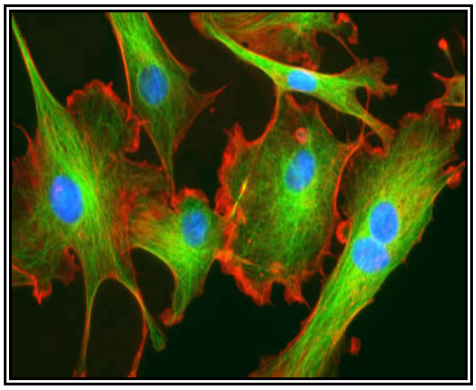

Fig.7. *Fibroblast labeled with FITC, Rhodamine, and Dapi.*

Proteomic tools have been used extensively for products characterization in cell culture industry. SDS-PAGE was used to asses purity and integrity of the product isolated from culture supernatant [Ackermann 1995]. 2D-GE has also been used to asses the consequences of cell culture conditions on protein expression [Lee 1996]. Kim et al., [2008] used a 2D-GE-based proteomics approach to the systematical analysis of the dysregulations in the cellular proteome of NIH/3T3 cells transformed by three kinds of oncogenic ras. The results obtained show that the comparative analysis of proteome from oncogenic ras expressing cells has yielded interpretable data to elucidate the differential protein expression directly and/or indirectly and contributed to evaluate the possibilities for physiological and therapeutic targets. The advantage of using cell culture is the high throughput and reproducibility throughout the different studies given through immortalized cell lines.

Therefore, cell culture proteomics is widely used to understand viral infections on cells; for example, Zhao *et al.* [2008] performed a proteomic analysis to determine the role of heat stress in production of progeny HCV in Huh7 cells harbouring intact HCV. The intact hepatitis C virus (HCV) cell culture system provided a powerful tool for studying the interaction between HCV and host cell. To study the infection mechanism of alphaherpesvirus pseudorabies virus (PrV), the etiological agent of Aujeszky's disease, Skiba *et al*. [2008] performed a quantitative proteome analysis on bovine kidney cells.

Cell culture proteomics is also used to understand drug effects on cells. For example, it's known that antidepressant drugs can have significant effects on the mood of a patient suffering from major depression or other disorders. However, many aspects of antidepressant action are not understood. McHugh *et al*. [2008] conducted a proteomic analysis in a neuronal cell culture model to identify molecules important to the operation of pathways functionally relevant to antidepressant action. Feng *et al*. [2006] performed a proteomic analysis to determine differentially expressed proteins related to the development of cancer cells resistance to Methotrexate, one of the most important and frequently used drugs in cancer therapy.

1.7 Yeast and Proteomics

Currently yeast, especially *Saccharomyces cerevisiae*, is widely used as model organism for the study of many biological mechanisms, as well as in biotechnology and pharmacology. In fact, *Saccharomyces cerevisiae* has many advantages as model system for molecular level studies. It has "technical" advantages, since the equipment and tools for its cultivation and study are fairly simple. Furthermore *Saccharomyces cerevisiae* is a unicellular organism whose genome (approximately 6000 genes) was completely sequenced and published in 1996 by Goffeau *et al.*. *The Yeast Genome Directory* and The *Saccharomyces Genome Database* (SGD) represent the definitive source of all information on *Saccharomyces cerevisiae* genome.

Through proteomic analysis has been possible to study all the proteins expressed by yeast, for example to understand the changes that occur at different stages of life of these cells, by comparing their different proteomes [Kusch *et al.*, 2008]. Given the importance of yeast in the study of the molecular mechanisms occurring in eukaryotic cells, in literature there are many works where yeast is used on with these objectives.

In recent years, with improved techniques and the importance of discoveries on yeast, studies in genomics and proteomics on these organisms have integrated. Until a few years ago, the genomic research was conducted exclusively with an approach aimed at isolating and studying a single gene at a time, to find functions and regulation pathways. This strategy has produced results of considerable interest, especially in the medical field, leading to the production of several compounds (through recombinant DNA technology) used in both therapeutic and in diagnostic field. Studies in yeast have allowed to develop both the methods that approaches for studies of structural and functional genomics using DNA microarrays [De Risi *et al.,* 1997].

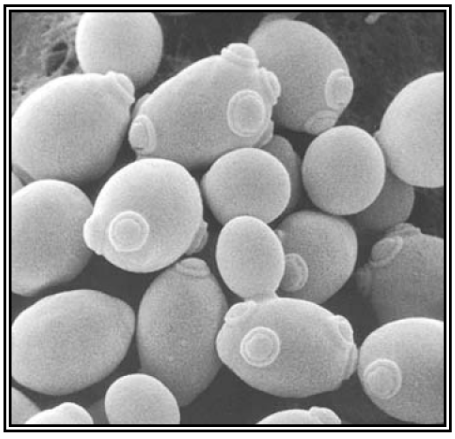

Fig.8. *Budding yeast Saccharomyces cerevisiae.*

Yeast is also the first model system in which DNA microarrays were applied to study natural populations both in laboratory conditions that in presence of natural stress [Cavalieri *et al.,* 2000, Townsend *et al.,* 2003].

Yeasts were useful to clarify the underlying mechanisms of some fundamental cellular processes and they have been used as a model for studying the mechanism of many metabolic pathways.

- In the field of *biochemistry* they contributed to understanding glycolysis: the role of some glycolytic enzymes has been clarified thanks to yeasts, in particular the role of phosphofructokinase, [Reibstein *et al.,* 1986].

- Concerning *cytology* yeasts were used to clarify organelles biogenesis [Sudarikov *et al.,* 1988] and structure and function of cytoskeleton [Takai *et al.*, 1995]. In genetics and molecular biology yeast helped to study cellular cycle: homologous of key proteins regulating cell cycle in human cells were found in yeast [Lew *et al.,* 1993]. *Saccharomyces cerevisiae* was used as model organism to understand the mechanism of oncogenes in mammalian cells, like for protooncogene RAS [Gibbs *et al.,* 1989].

- Yeast has been used also as system to produce recombinant proteins for therapeutic purposes. The first protein derived from yeast, marketed for therapeutic purposes, was the hepatitis B vaccine produced by Merck in 1986 under the name *recombivax*. Since than, many other proteins are produced by yeasts, also because, being a eukaryotic organism, is able to perform almost all post-translational modifications necessary to render the protein biologically active. Some of these recombinant proteins are hormones, like insulin [Hadfield *et al.*, 1993], interferons [Wisemann *et al.*, 1996], blood proteins like tissue plasminogen activator and factors VII, VIII and IX coagulation [Rallabhandi and Yu, 1996]. In the field of *pharmacology Saccharomyces cerevisiae* is a useful organism for the development of new drugs. For example, the application of proteomics techniques to *Saccharomyces cerevisiae* appears to be of primary importance in the early stages of drugs developing, especially in identifying the target. This includes the development of yeast strains engineered to assess the toxicity of potential drugs. Analysis based on yeast cells have been applied

successfully to identify influenza virus M2 protein inhibitors [Kurtz *et al.,* 1995].

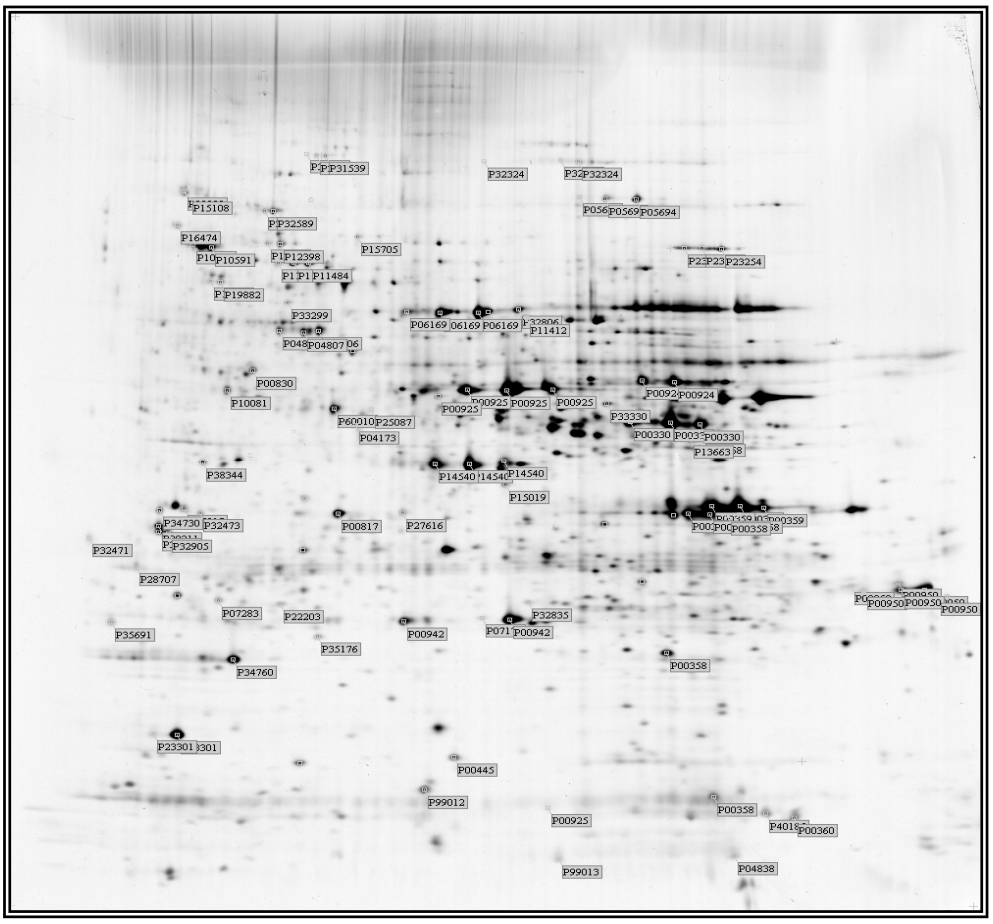

Fig. 9. *Saccharomyces cerevisiae 2D-GE Map with identified spots indicated by their accession number.*

Building yeast reference maps was possible thanks to the application of 2D–GE to *S. cerevisiae* for proteins large-scale separation and visualization [Shevchenko *et al.*, 1996; Maillet *et al.*, 1996; Norbeck *et al.*, 1997; Perrot *et al.*, 1999; Wildgruber *et al.*, 2002]. Depending on the protein staining method, more than 1000 proteins can be visualized on such gels. Also, subproteome reference maps of, for example, yeast mitochondria, have been generated [Ohlmeier *et al.*, 2004]. Moreover, 2D reference maps have been constructed for important industrial yeast strains, such as an ale-fermenting strain [Kobi *et al.*, 2004], wine and lager-brewing strain [Trabalzini *et al.*, 2003] [Joubert *et al.*, 2000;2001] (Fig. 9). These reference maps are useful tools for yeast researchers because they can be used for 2D gel comparisons.

Previews published works made in our lab used a proteomic approach and reference maps comparisons for studies on yeast. For example to do a proteome analysis in apoptotic yeast cells [Magherini *et al.*, 2007] or to study the mitochondrial proteome of yeast mutants lacking proteins Sco1p or Sco2p [Gamberi *et al.*, 2009]. Also how different carbon sources can affect protein redox state during *Saccharomyces cerevisiae* ageing was investigated [Magherini *et al.*, 2009] with a proteomic approach. In literature many quantitative 2D-gel-based studies have been reported with applications to yeast, examining, for example, yeast growth under different environmental conditions. Salusjarvi *et al.* [2003] performed a proteome analysis of recombinant xylose-fermenting yeast, comparing conditions in which glucose or xylose was the carbon source. Kolkman et al. studied yeast grown in chemostat cultures limited for glucose and ethanol [2005], which enabled the differential analysis of protein expression levels under glycolytic and gluconeogenic conditions. In other studies, 2D gel electrophoresis was used to obtain a global view of changes in the yeast proteome as a function of stimuli in the environment, such as cadmium [Vido *et al.*, 2001], lithium [Bro *et al.*, 2003], H_2O_2 [Godon *et al.*, 1998], sorbic acid [de Nobel *et al.*, 2001] and amino acid starvation [Yin *et al.*, 2004]. Proteome knowledge about industrial strains can be useful for the optimization and control of yeast proliferation during industrial fermentation.

In general, proteome analysis of yeast might contribute to the functional characterization of basic cellular mechanisms of eukaryotes [Hwang *et al.*, 2006].

1.8 Human Plasma and Proteomics

Blood plasma is an exceptional proteome in many respects. It is the most complex human-derived proteome, containing other tissue proteomes as subsets. It is collected in huge amounts (millions of liters) for preparation of protein therapeutic products. It is the most difficult protein-containing sample to characterize on account of the large proportion of albumin (55%), the wide dynamic range in abundance of other proteins and the tremendous heterogeneity of its predominant glycoproteins. And it is the most sampled proteome, with hundreds of millions of tubes withdrawn every year for medical diagnosis, making it the most important clinically. Proteins in plasma have been studied since before we knew genes existed.

Plasma is not only the primary clinical specimen but also represents the largest and deepest version of the human proteome present in any sample: in addition to the classical "plasma proteins," it contains all tissue proteins (as leakage markers) plus very numerous distinct immunoglobulin sequences, and it has an extraordinary dynamic range in more than 10 orders of magnitude of proteins concentration (Fig. 11). Although the restricted dynamic range of conventional proteomic technology (two-dimensional gels and mass spectrometry) has limited its contribution to the list of 289 proteins that have been reported in plasma to date, very recent advances in multidimensional techniques promise at least to double this number in the near future.

Abundant scientific evidence, from proteomics and other disciplines, suggests that among these are proteins whose abundances and structures change in indicative ways in many human diseases. Nevertheless, only a handful of proteins are currently used in routine clinical diagnosis, and the rate of introduction of new protein tests approved by the United States Food and Drug Administration (FDA) has paradoxically declined over the last decade to less than one new proteic diagnostic marker per year.

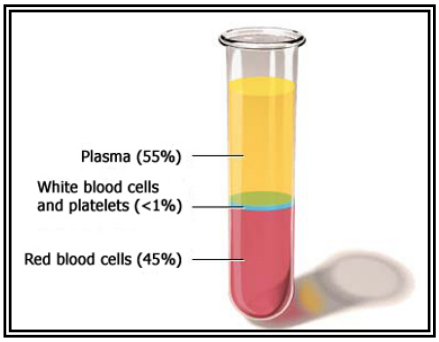

Fig.10. *Blood separated into its major components by centrifugation.*

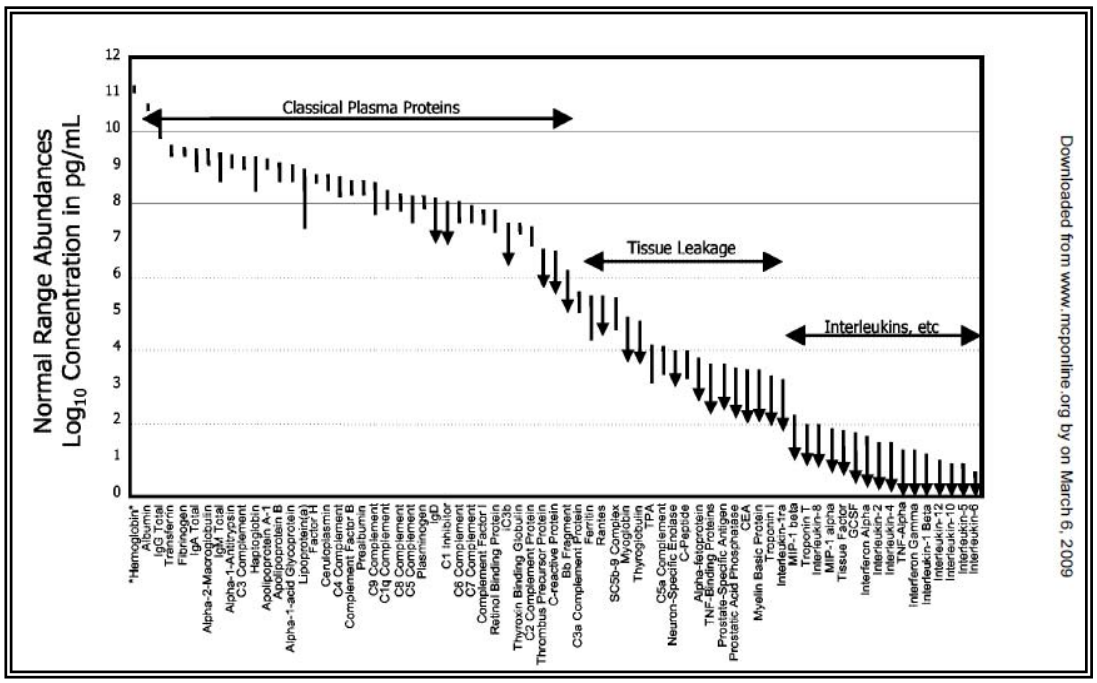

Fig.11. Reference intervals for 70 protein analytes in plasma. *Abundance is plotted on a log scale spanning 12 orders of magnitude. Where only an upper limit is quoted, the lower end of the interval line shows an arrowhead. The classical plasma proteins are clustered to the left (high abundance), the tissue leakage markers (e.g. enzymes and troponins) are clustered in the center, and cytokines are clustered to the right (low abundance).*

We use the term "plasma" to embrace all the proteins of the blood soluble phase (excluding cellular proteins) and not as a prescription for a specific sample processing technique. In his classic series entitled *The Plasma Proteins*, Putnam [1987] defined true plasma proteins as those that carry out their functions in the circulation, thus excluding proteins that, for example, serve as messengers between tissues (*e.g.* peptide hormones) or that leak into the blood as a result of tissue damage (*e.g.* cardiac myoglobin released into plasma after a heart attack). This functional definition correctly emphasized the fact that proteins may appear in plasma for a variety of different reasons, but it also hints at the fact that different methods and approaches were originally responsible for discovery of these classes.

Elaborating on Putnam's classification from a functional viewpoint, we can classify the protein content of plasma into the following design/function groups:

Proteins Secreted by Solid Tissues: classical plasma proteins are largely secreted by the liver and intestines.

***Immunoglobulins*:** they represent a unique class of proteins because of their complexity: there are thought to be on the order of 10 million different sequences of antibodies in circulation in a normal adult.

"Long Distance" Receptor Ligands: the classical peptide and protein hormones are included in this group.

"Local" Receptor Ligands: these include cytokines and other short distance mediators of cellular responses. High plasma levels may cause deleterious effects remote from the site of synthesis, *e.g.* sepsis.

Temporary Passengers: these include non-hormone proteins that traverse the plasma compartment temporarily on their way to their site of primary function, *e.g.* lysosomal proteins that are secreted and then taken up via a receptor for sequestration in the lysosomes.

Tissue Leakage Products: these are proteins that normally function within cells but can be released into plasma as a result of cell death or damage. These proteins include many of the most important diagnostic markers, *e.g.* cardiac troponins, creatine kinase, or myoglobin used in the diagnosis of myocardial infarction.

Aberrant Secretions: these proteins are released from tumors and other diseased tissues, presumably not as a result of a functional requirement of the organism. These include cancer markers, which may be normal, non-plasma-accessible proteins expressed, secreted, or released into plasma by tumor cells.

Foreign Proteins: these are proteins of infectious organisms or parasites that are released into the circulation.

A series of other body fluids including cerebrospinal fluid, synovial fluid, and urine (the ultimate destination of most of the <60-kDa protein material in plasma) share some of the protein content of plasma with specific local additions that reveal interesting clinical information. Unfortunately, these samples are more difficult to obtain in a useful state than plasma: collection of cerebrospinal fluid and synovial fluid are invasive procedures involving pain and some risk, while urine is more difficult to process to a useful sample quickly in a clinical setting (centrifugation to remove cells, prevention of microbial growth, and concentration).

Blood was first emphasized diagnostically by *Hippocrates*, who proposed that disease was due to an imbalance of four humors: blood, phlegm, yellow bile, and black bile. The importance of this idea was to propose a physical cause, and not a divine one, for human disease and it remained basic to medical practice for over a thousand years. With Wohler's synthesis of urea in 1828, the distinction between living matter and chemicals began to disappear, and with the enunciation of the cell theory by Schleiden and Schwann [1847], the question of the location of disease could be productively revisited: Virchow described the cellular (as opposed to humoral) basis of disease and finally put an end to phlebotomy as general therapy. Despite not being a humor or "vital principle," plasma remained a subject of interest throughout this period: in the 1830s Liebig and Mulder analyzed a substance called "albumin," in 1862 Schmidt coined the term "globulin" for the proteins that were insoluble in pure water, and in 1894 Gurber crystallized horse serum albumin. Enzyme activities were detectable in body fluids long before the enzyme proteins could be isolated and studied [Moss *et al.*, 1999]. Alkaline and acid phosphatase activities were related to bone disease and prostate cancer, respectively, in the decades before 1950 and in 1955 the enzyme now called aspartate aminotransferase was detected in serum following acute myocardial infarction.

The use of analytical separations to look at the plasma proteome parallels very closely the development of the separations themselves: plasma is always among the first samples to be examined. Tiselius found that serum could be fractionated into multiple components on the basis of electrophoretic mobility. His method of electrophoresis, first in liquid and then later in anticonvective media such as paper, cellulose acetate, starch, agarose, and polyacrylamide, has dominated the separative side of plasma proteome work until very recently, evolving through a series of one- and two-dimensional systems and finally to combinations with chromatography and mass spectrometry that generalize to *n*-dimensions. This evolution has resulted in an almost constant exponential increase in resolved protein species for the past 70 years.

Soon after the introduction of high resolution two-dimensional gel electrophoresis (2D-GE) in 1975 by Klose, O'Farrell and others, the technique was applied to the plasma proteins by the Anderson [1977] with the result that the number of resolved

species increased to 300 or more. The 2D-GE map of human plasma that resulted is recognizably the same as those produced later by many investigators: in contrast to cellular protein patterns, the plasma 2D-GE pattern appears the same in everyone's hands perhaps due to the very high solubility of the proteins involved and the ease with which the distinctive glycosylation trains of specific proteins can be recognized. A more comprehensive database was reported by Anderson in 1991 in which 727 spots were resolved and 376 were identified as 49 different proteins. A plasma map using an immobilized pH gradient first dimension separation was presented the following year with 40 protein identifications [Hughes *et al.*, 1992] and this has been extended [on the current Swiss 2D-Page web site: us.expasy.org/ch2d] to identify 60 proteins (613 spots identified, a majority by immunodetection) (Fig. 12).

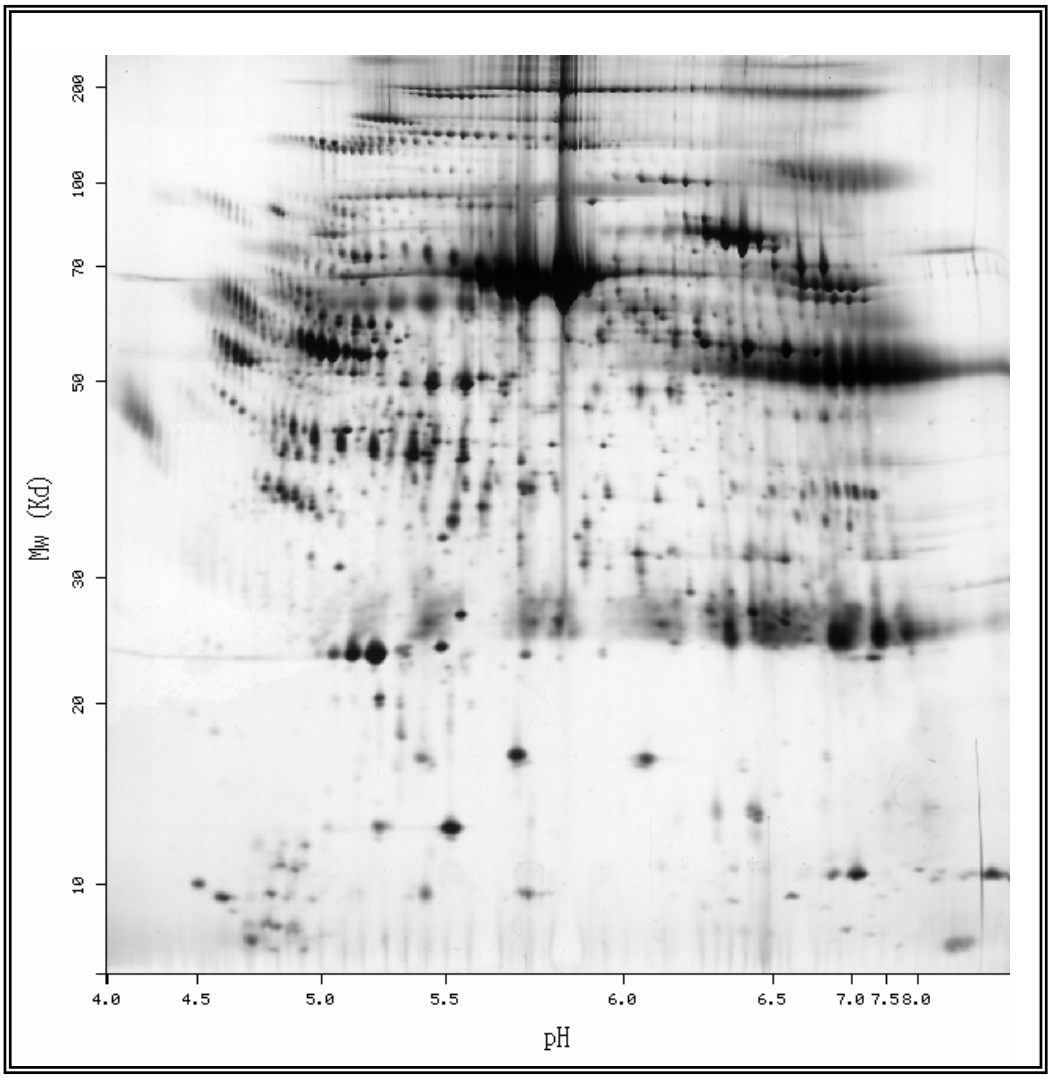

Fig. 12. *Human Plasma 2D-GE Map.*

In plasma there is a very high abundance of a few proteins (albumin, transferrin, immunoglobulins, etc.) and an extreme heterogeneity of glycoproteins and immunoglobulins. This feature may represent a limit in terms of two-dimensional gel analysis of unfractionated plasma. However, this limit can be overcome combining 2D-GE with additional separation steps. Classical chromatographic separations (such as size exclusion, ion exchange, lectin binding, and hydrophobic interaction) may be applied to further fractionate plasma.

The practical utility of 2D-GE for studies of the high abundance plasma proteome has been substantial. Because the isoelectric focusing is sensitive to molecular charge and the SDS electrophoresis is sensitive to polypeptide length, 2D-GE is very effective at revealing genetic variants (about one-third of which differ in net charge from wild type), proteolytic cleavages, and variations in sialic acid content. Several genetic variants have been discovered by 2D-GE [Tracy *et al.*, 1982; Harrison *et al.*; 1991]. Many proteins in plasma show complex combinations of post-translational modifications (particularly involving glycosylation) that can be discriminated by 2D-GE.

The immense dynamic range of plasma proteins is achieved only by technologies that presuppose the identity of the analyte: available methods for protein discovery, such as 2D-GE or LC/MS/MS, have typical dynamic ranges of only 10^2–10^4. Current approaches to the extension of this range combine independent fractionation methods (principally chromatography, immunoaffinity subtraction, preparative isoelectric focusing, or precipitation) with 2D-GE or MS to gain an additional factor of 10–10^2, which, although very productive, is still far short of the desired range.

The attraction of plasma for disease diagnosis lies in two characteristics: the ease by which it can be safely obtained and the fact that it comprehensively shows the state of the body at a particular moment. While other sample types can be obtained (saliva, tears, urine, skin, hair, etc.), each is either a small subset of plasma or else a restricted local sampling of cellular activity. Genetic markers can be sampled almost anywhere (skin, lymphocytes, and various tissues) with equivalent results, but such markers reveal only the genotype and hence do not reveal anything of the regulation of biological processes in response to lifestyle, disease or drugs that is expressed at the mRNA and protein levels. While the mRNA does reveal regulation, it is routinely

obtained for clinical purposes only from blood lymphocytes and it is not well correlated with protein expression [Anderson *et al.*, 1997] (and hence function). The use of plasma and serum for disease diagnosis is thus an obvious approach that has been undertaken with some success for many decades [Burtis *et al.*, 1999]. A series of 2D-GE studies have examined aspects of the acute phase response in which many plasma proteins increase or decrease following a range of inflammatory insults [Modesti *et al.*, 2009 Choukaife *et al.*, 1989; Saile *et al.*, 1990 Bini *et al.*, 1992;]. A work by Bini *et al.* [1996] indicates particularly interesting differences between the response of the body to bacterial and viral infection. These results indicate both the generality of the acute phase response and the power of proteomics to subdivide its features in diagnostically useful ways. Many other disease states and developmental processes have been examined using 2D-GE on plasma. Tissot *et al.* demonstrated characteristic changes in the plasma 2D-GE pattern indicative of monoclonal gammopathies, hyper-γ-globulinemia, hepatic failure, chronic renal failure, and hemolytic anemia [1991] as well as progressive changes during fetal development [Tissot *et al.*, 1993]. Other changes have been reported associated with malnutrition [Lonberg-Holm *et al.*, 1986], haptoglobin in Duchenne muscular dystrophy [John *et al.*,1989], haptoglobin in Down syndrome [Myrick *et al.*, 1990], apoA-I during parturition [Del Priore *et al.*, 1991] and apoA-I isoforms in heart disease [Burgess-Cassler *et al.*, 1992], human chorionic gonadotropin isoforms in patients with trophoblastic tumors [Hoermann *et al.*, 1993], and oxidized plasma proteins in Alzheimer's disease [Choi *et al.*, 1924].

Cancer tissue samples could be analyzed to distinguish tumor type and prognosis [Schmid *et al.*, 1995], and a panel of six cancer markers in plasma was found to be useful [Negishi *et al.*, 1987; Alaiya *et al.*, 2000]. More recently, mass spectrometry-based proteomic approaches have been used as well to discover patterns of disease-related protein features related to a specific cancer [Petricoin *et al.*, 2002].

Most of the proteins and disease markers in plasma remain to be discovered.

Chapter 2

"Proteomic analysis of cells exposed to prefibrillar aggregates of HypF-N"

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2. Abstract

Several human diseases are associated with the deposition of stable ordered protein aggregates known as amyloid fibrils. In addition, a large wealth of data shows that proteins not involved in amyloidoses, are able to form, in vitro, amyloid-like prefibrillar and fibrillar assemblies indistinguishable from those grown from proteins associated with disease. Previous studies showed that early prefibrillar aggregates of the N-terminal domain of the prokaryotic hydrogenase maturation factor HypF (HypF-N) are cytotoxic, inducing early mitochondria membrane depolarization, activation of caspase 9 and eventually cell death.

To gain knowledge on the molecular basis of HypF-N aggregate cytotoxicity, we performed a differential proteomic analysis of NIH-3T3 cells exposed to HypF-N prefibrillar aggregates in comparison with control cells. Two-dimensional gel electrophoresis followed by protein identification by MALDI-TOF MS, allowed us to identify 21 proteins differentially expressed. The changes of the expression level of proteins involved in stress response (Hsp60 and 78 kDa glucose-regulated protein) and in signal transduction (Focal adhesion kinase1) appear particularly interesting as possible determinants of the cell fate. The levels of some of the differently expressed proteins were modified also in similar studies carried out on cells exposed to Aβ or α-synuclein aggregates, supporting the existence of shared features of amyloid cytotoxicity.

2.1 Introduction

Amyloid diseases are a group of protein misfolding pathologies including either systemic forms (i.e. type II diabetes mellitus,) and neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington and prion diseases) (reviewed in [M. Stefani *et al.*, 2003;2004]). The molecular basis of these clinically different pathologies can be traced back to the presence, in the affected tissues and organs, of proteinaceous deposits of fibrillar aggregates of one out of a number of peptides or proteins, each found aggregated specifically in each disease (reviewed in [M. Stefani *et al.*, 2003; F. Chiti *et al.*, 2006]). In the last ten years it has became increasingly clear that the ability to oligomerize into amyloid assemblies is not a specific feature of the proteins and peptides found aggregated in tissues affected by amyloid diseases; in fact, since 1998, an increasing number of reports support the idea that protein misfolding following mutations, chemical modifications, presence of destabilizing surfaces or any other alteration of the chemical environment, can result a structural reorganization, favouring oligomerization/polymerization of peptides and proteins into amyloids [J.I. Guijarro *et al.*;F. Chiti *et al.*, 1999].

In addition to amyloid aggregation, aggregate toxicity has also recently resulted as a generic property of proteins and peptides. In particular, it is increasingly recognized that amyloid oligomers, preceding the appearance of mature fibrils, known as prefibrillar aggregates, are the most toxic species, among amyloid assemblies associated or not associated with disease [C.G. Glabe *et al.*, 2006;2006]. This view supports the idea that any protein can potentially become the source of toxic species impairing cell viability and that the cytotoxicity of prefibrillar aggregates results, at least in part, from shared basic structural features of the latter [M. Bucciantini *et al.*, 2004]. Moreover, a growing number of studies suggest that, in most cases, the cell membrane, can also favour protein/peptide misfolding favouring the appearance of aggregating nuclei [M. Zhu *et al.*, 2002]. Conversely, toxic prefibrillar aggregates from disease associated or disease-unrelated proteins can interact with the cell membranes modifying their structural order resulting in the early impairment of ion and redox homeostasis [M. Bucciantini *et al.*, 2005; J.I.

Kourie *et al.*, 2001]. Intense efforts are presently dedicated at unravelling the molecular basis of the appearance in tissue of protein aggregates and their cytotoxicity.

However, much must still be learnt to gain enough knowledge to allow designing therapeutic strategies aimed at counteracting the clinical symptoms of amyloid diseases. On this respect, it can be important to investigate the cytotoxic effects on cells exposed to amyloid aggregates of proteins/peptides not associated with any amyloid disease. Such study can highlight cell modifications resulting from shared structural features in amyloids excluding those associated with the specific features of any aggregated peptide/protein.

The advent of proteomics has allowed the simultaneous analysis of changes in the expression pattern of multiple proteins in complex biological systems. This appears particularly important in the case of cell dysfunctions resulting from protein aggregation. The effects protein aggregates have on cells appear highly complex and heterogeneous. Such a complex pattern of cell impairment makes proteomics one of the most useful tools to integrate these modifications into a whole systematic picture. The reports that recently appeared on the proteomic analysis of amyloid diseases such as Alzheimer's disease, Parkinson's disease and others have provided valuable data on some cell modifications allowing to explain, at least in part, cell impairment in these diseases [B. Martin *et al.*, 2008; S. Joerchel *et al.*, 2008; Y. Hu *et al.*, 2005; Z. Xun *et al.*, 2008; M.H. Chin *et al.*, 2008]. Proteomic studies provided useful information on the changes in pattern of protein expression in cells exposed to toxic aggregates of specific peptides/proteins found aggregated in the corresponding diseases. In the present study we performed a proteomic analysis of NIH-3T3 cells exposed to toxic aggregates of a protein domain not involved in any amyloid disease, the N-terminal domain of the prokaryotic HypF hydrogenase maturation factor (HypF-N) whose aggregation properties and aggregate cytotoxicity were previously characterized [M. Bucciantini *et al.*, 2004; M. Bucciantini *et al.*, 2005; S. Campioni *et al.*, 2008]. To our knowledge, this is the first proteomic study on the alterations of the protein expression profiles in cells exposed to toxic amyloid aggregates of a disease-unrelated protein. Our results highlight some generic changes in protein expression pattern elicited by the shared features of amyloids such as the basic cross-

beta structure and the exposure of hydrophobic patches. We found significant differences in the protein expression patterns in the exposed cells, with a number of up- or down-regulated proteins. Some of these proteins were also found in similar studies carried out on cells exposed to Aβ or α-synuclein aggregates in agreement with the generic nature of the cellular changes underlying amyloid cytotoxicity. Among the differentially expressed proteins, the reduced expression of Fak1 observed in the exposed cells can be related to the apoptotic process, whereas the increased expression of Hsp60 can provide protection against cell stress induced by HypF-N prefibrillar aggregates. Furthermore the treated cells showed a marked increase of the expression of both glyceraldehydes-3- phosphate dehydrogenase (Gapdh) and enolase. These two proteins are involved in energy metabolism and Gapdh has also been shown to bind the β-amyloid precursor protein [J.L. Mazzola *et al.*, 2002] and to be involved in transcriptional regulation of cell-cycle [S. Carujo *et al.*, 2006]. Finally, we observed an increase in the expression level of actin as previously shown in cell exposed to the intracellular domain of the β-amyloid precursor protein [T. Müller *et al.*, 2007].

2.2 Materials and Methods

2.2.1 HypF-N expression and purification

HypF-N was expressed and purified as previously described [S. Campioni *et al.*, 2008]. HypF-N prefibrillar aggregates were obtained by incubating the protein for 48 h at room temperature at a concentration of 0.3 mg/ml in 30% (v/v) trifluoroethanol, 50 mM sodium acetate, 2 mM dithiotreitol (DTT), pH 5.5, as previously reported [S. Campioni *et al.*, 2008]. At the end ofthe incubation the solution was centrifuged, and the resulting pellet was dried under N2 to remove the residual solvent, dissolved in DME Mat 200 μM (monomeric protein concentration) and immediately added to the cell culture medium at 2 μM final concentration.

2.2.2 Cell culture and treatment

Cell culture media and other reagents, unless otherwise stated, were from Sigma-Aldrich Fine Chemicals Co. NIH-3T3 murine fibroblasts (ATCC, Manassas, VA) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, containing 10% bovine calf serum (HyClone Lab, Perbio Company, Celbio), 3 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in a 5%CO2 humidified environment at 37 °C. Cells were used for a maximum of 10 passages. Sub-confluent NIH-3T3 cells were treated for differing lengths of time with 2 μM toxic HypF-N prefibrillar aggregates. Under these conditions it was previously shown that the aggregates are stable in the culture media [M. Bucciantini *et al.*, 2004]. Controls were performed by exposing the cells to the same amount of native, soluble HypF-N. At the end of each treatment, the cells were washed twice with phosphate-buffered saline (PBS), dried and stored at −80 °C.

2.2.3 Sample preparation and 2D-GE

Cells were scraped in RIPA buffer (50 mM Tris–HCl pH 7.0, 1% NP-40, 150 mM NaCl, 2 mM EGTA, 100 mM NaF) containing a cocktail of protease inhibitors (Sigma). The cells were sonicated (10 s) and protein extracts were clarified by centrifugation at 8000 g for 10 min. Proteins were precipitated following a

chloroform/methanol protocol [D. Wessel *et al.*, 1984] and the pellet was resuspended in 8Murea, 4% CHAPS and 20mM DTT. Three independent experiments were performed and each sample was run in triplicate in order to assess biological and analytical variation. IEF (first dimension) was carried out on non-linear wide range immobilized pH gradients (pH 3.0–10; 18 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using the Ettan™ IPGphor™ system (GE Healthcare, Uppsala, Sweden). Analytical-run IPG strips were rehydrated with 60 µg of total proteins in 350 µl of lysis buffer and 0.2% carrier ampholyte for 1 h at 0 V and for 8 h at 30 V, at20 °C. MS-preparative IPG strips were loaded with 400 µg of proteins. The strips were focused at 20 °C according to the following electrical conditions: 200 V for 1 h, from 300 V to 3500 V in 30 min, 3500 V for3 h, from 3500 V to 8000 V in 30 min, and 8000 V until a total of 80,000 V/h was reached. After focusing, analytical and preparative IPG strips were equilibrated for 12 min in 6 M urea, 30% glycerol, 2% SDS, 2% DTT in 0.05M Tris–HCl buffer, pH 6.8, and subsequently for 5 min in the same urea/SDS/Tris buffer solution where DTT was substituted with 2.5% iodoacetamide. The second dimension was carried out on 9–16% polyacrylamide linear gradient gels (18 cm×20 cm×1.5 mm) at10 °C and 40 mA/gel constant current until the dye front reached the bottom of the gel. Analytical gels were stained with ammoniacal silvernitrate as previously described [D.F. Hochstrasser *et al.*, 1998]; MS-preparative gels were stainedwith colloidal Coomassie [V. Neuhoff *et al.*,1988]

2.2.4 Western blotting analysis of proteomic candidates

For 1-DE 30 µg of protein extracts was separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore). To confirm the results obtained from 2D-GE analysis, the relative amount of Hsp60and Fak proteins were assessed by Western blot with appropriate antibodies (Santacruz). For quantification, the blots were stained with Coomassie brilliant blue R-250 and subjected to densitometric analysis performed using Quantity One Software (Bio-Rad). Statistical analysis of the data was performed by Student's t-test; p-values 0.05 were considered statistically significant. The intensity of the immunostained bands were normalized with the total protein intensities measured by Coomassie brilliant blue R-250 from the same blot.

2.2.5 Image analysis and statistics

The gel and Western blot images were acquired with an Epson expression 1680 PRO scanner. For each condition, three biological replicates were performed and only the spots present in all the replicates were taken in consideration for subsequent analysis. Computer-aided 2D image analysis was carried out using Image-Master 2-DE Platinum software version 6.0 (GE Healthcare). The relative spot volume calculated as %V (V single spot/V total spots, where V=integration of OD over the spot area) was used for quantitative analysis in order to decrease experimental errors. The normalized intensity of the spots on replicate 2D gels was averaged and standard deviation was calculated for each condition. A two-tiled non paired Student's t-test was performed using ORIGIN 6.0 (Microcal Software, Inc.) to determine whether the relative change was statistically significant.

2.2.6 In-gel trypsin digestion and MALDI-TOF mass spectrometry

The analysis was performed on the Coomassie blue-stained spots excised from the gels. The spots were washed first with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubation in 10 mM dithiothreitol (DTT) for 45 min at 56 °C. The cysteines were alkylated by incubation in 5 mM iodoacetamide for 15 min at room temperature in the dark. The gel particles were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestion was carried out with trypsin (12.5 ng/µl) in 50 mM ammonium bicarbonate buffer, pH 8.5, at 4 °C for 4 h. The buffer solution was then removed and a new aliquot of the enzyme/buffer solution was added for 18 h at 37 °C. A minimum reaction volume, enough for complete gel rehydration was used. At the end of the incubation the peptides were extracted by washing the gel particles with 20 mM ammonium bicarbonate and 0.1% TFA in 50% acetonitrile at room temperature and then lyophilised. Positive Reflectron MALDI spectra were recorded on a Voyager DE STR instrument (Applied Biosystems, Framingham, MA). The MALDI matrix was prepared by dissolving 10 mg of alpha cyano in 1 ml of acetonitrile/water (90:10 v/v). Typically, 1 µl of matrix was applied to the metallic sample plate and then 1 µl of analyte was added. Acceleration and reflector voltages were set up as follows: target voltage at 20 kV, first grid at 70% of target voltage, delayed extraction at 100 ns to obtain the best signal-to-noise ratios and the best possible isotopic resolution

with multipoint external calibration using a peptide mixture purchased from Applied Biosystems. Each spectrum represents the sum of 1500 laser pulses from randomly chosen spots per sample position. Raw data were analyzed using the computer software provided by the manufacturers and are reported as monoisotopic masses.

2.2.7 nanoLC mass spectrometry

A mixture of peptide solution was subjected to LC-MS analysis using a 4000Q-Trap (Applied Biosystems) coupled to an 1100 nano HPLC system (Agilent Technologies). The mixture was loaded on an Agilent reverse-phase pre-column cartridge (Zorbax 300 SB-C18, 5×0.3 mm, 5 μm) at 10 μl/min (A solvent 0.1% formic acid, loading time 5 min). The peptides were separated on an Agilent reverse-phase column (Zorbax 300 SB-C18,150mm×75 μm, 3.5 μm), at a flow rate of 0.3 μl/min with a 0% to 65% linear gradient in 60 min (A solvent 0.1% formic acid, 2% acetonitrile in MQwater; B solvent 0.1% formic acid, 2% MQ water in acetonitrile). Nanospray source was used at 2.5 kV with liquid coupling, with a declustering potential of 20 V, using an uncoated silica tip from New Objectives (O.D. 150 μm, I.D. 20 μm, T.D. 10 μm). The data were acquired in information-dependent acquisition (IDA) mode, in which a full scan mass spectrum was followed by MS/ MS of the 5 most abundant ions (2 s each). In particular, spectra acquisition of MS-MS analysis was based on a survey Enhanced MS Scan (EMS) from 400 m/z to 1400 m/z at 4000 amu/s. This scan mode was followed by an Enhanced Resolution experiment (ER) for the five most intense ions and then MS2 spectra (EPI) were acquired using the best collision energy calculated on the basis of m/z values and charge state (rolling collision energy) from 100 m/z to 1400 m/z at 4000 amu/s. The data were acquired and processed using the Analyst software (Applied Biosystems).

2.2.8 MASCOT analysis

The spectral data were analyzed using the Analyst software (version 1.4.1) and the MS-MS centroid peak lists were generated using the MASCOT.dll script (version 1.6b9). The MS-MS centroid peaks were threshold at 0.1% of the base peak. MS-MS spectra with less than 10 peaks were rejected. The spectra were searched against the Swiss Prot database (2006.10.17 version) using the licensed version of Mascot 2.1 (Matrix Science), after converting the acquired MS-MS spectra in MASCOT generic

file format. The MASCOT search parameters were: taxonomy *mus musculus*; allowed number of missed cleavages 2; enzyme trypsin; variable post-translational modifications, methionine oxidation, pyro-glu N-term Q; peptide tolerance 200 ppm and MS/MS tolerance 0.6 Da; peptide charge, from +2 to +3 and top 20 protein entries. Spectra with a MASCOT score b25 having low quality were rejected. The score used to evaluate the quality of matches for the MS-MS data was higher than 30. However, the spectral data were manually validated and contained sufficient information to assign peptide sequence.

2.3 Results

2.3.1 Comparative proteomic analysis between control cells and cells exposed to HypF-N prefibrillar aggregates

Previous experiments performed by *Bucciantini et al.* showed that prefibrillar HypF-N aggregates induced early Ca2+ increase and oxidative stress followed by mitochondria depolarization and caspase activation in exposed NIH-3T3 cells. After 24 h, the cells died with necrotic features possibly since the ATP levels were too low to sustain the initially triggered apoptotic program [M. Bucciantini *et al.*, 2005].

In order to investigate the changes (if any) in protein expression induced in the same cells upon exposure to HypF-N prefibrillar aggregates, we performed a 2D-GE followed by mass spectrometry. In all the experiments carried out in this study, the cells were exposed to 2 μM prefibrillar aggregates. This protein concentration was chosen to investigate finely regulated biochemical processes, such as the activation of pro-apoptotic factors, which could be hidden by a stronger cell injury [M. Bucciantini *et al.*,2005]. The cells were treated for 5 and 24 h and proteins extracts were prepared as described under Materials and methods. Then the proteins were separated by 2D-GE and the resulting silver-stained gels were analyzed using the ImageMaster 2D Platinum 6.0 software. The differences of protein expression between control and treated cells were taken into consideration if the relative volume of the spots differed reproducibly more than 1.5-fold and this difference was statistically significant. An average of about 1300 spots was detected in each silver-stained gel. Cell exposure to HypF-N prefibrillar aggregates did not affect the overall proteomic profiles both after 5 h and after 24 h (Figs. 1 and 2). However, the computer analysis highlighted 19 variations between cells treated for 5 h with 2 μM HypF-N prefibrillar aggregates (Fig. 1B) and the control cells treated for the same length of time with an equivalent amount of native HypF-N (Fig. 1A). Among these variations, only two were still present after 24 h of treatment with aggregates (Fig. 2) indicating that the alteration of the expression is a transient event, at least for this

group of proteins, except two. On the other hand, the comparison between cells treated for 24 h with 2 μM HypF-N prefibrillar aggregates and control cells showed a variation of 9 spots, whose expression was not affected after 5 h, indicating that some proteins are up- or down-regulated as a consequence of the prolonged exposure to the aggregates.

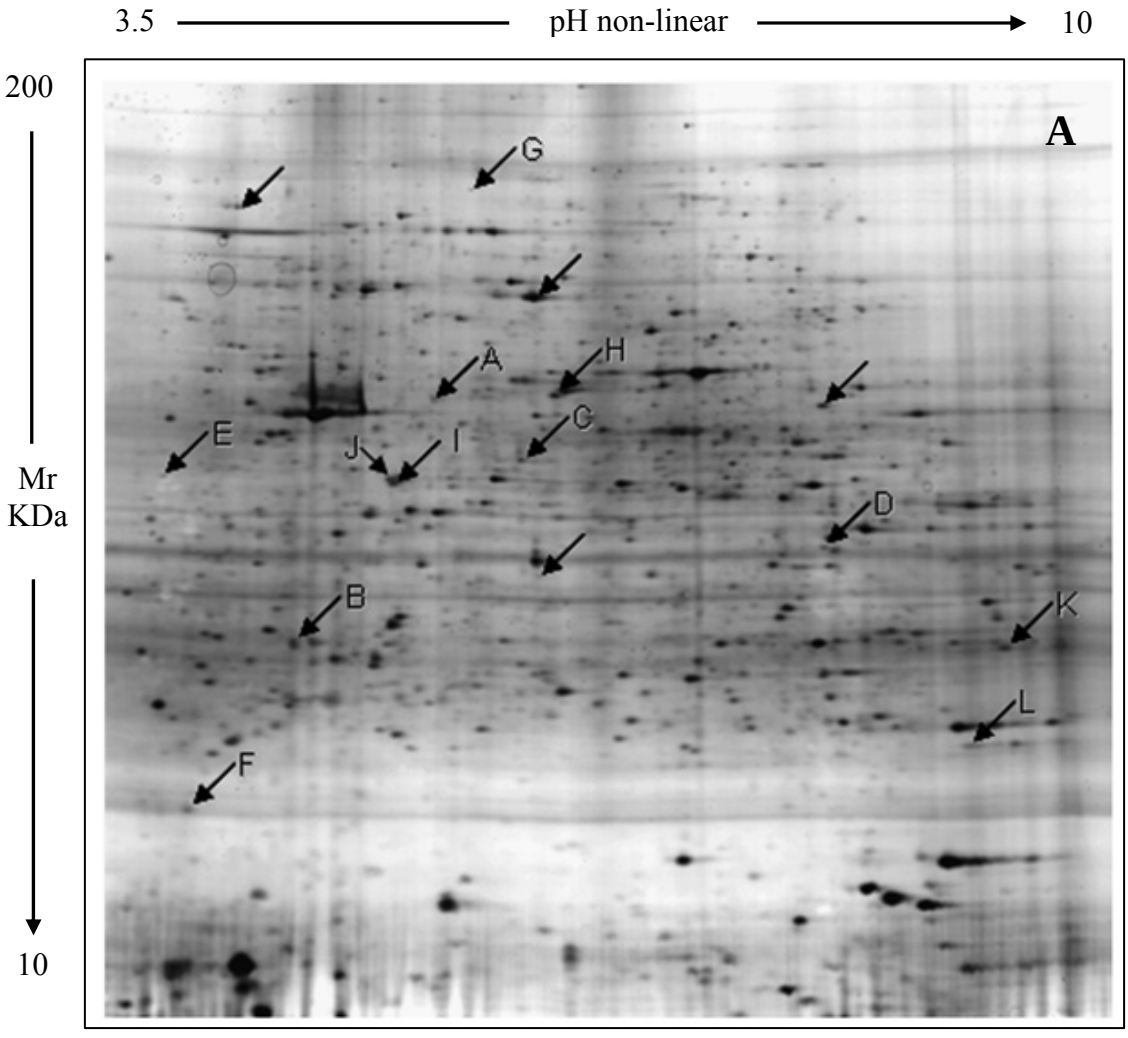

Fig.1A. *2D images of silver stained gels of total proteins extracted from control and HypF-N prefibrillar aggregate treated cells for 5 h. Arrows indicate variations between control (panel A) and treated cells (panel B, next page). Letters indicate the identified proteins. Controls were performed by exposing the cells to native, soluble HypF-*

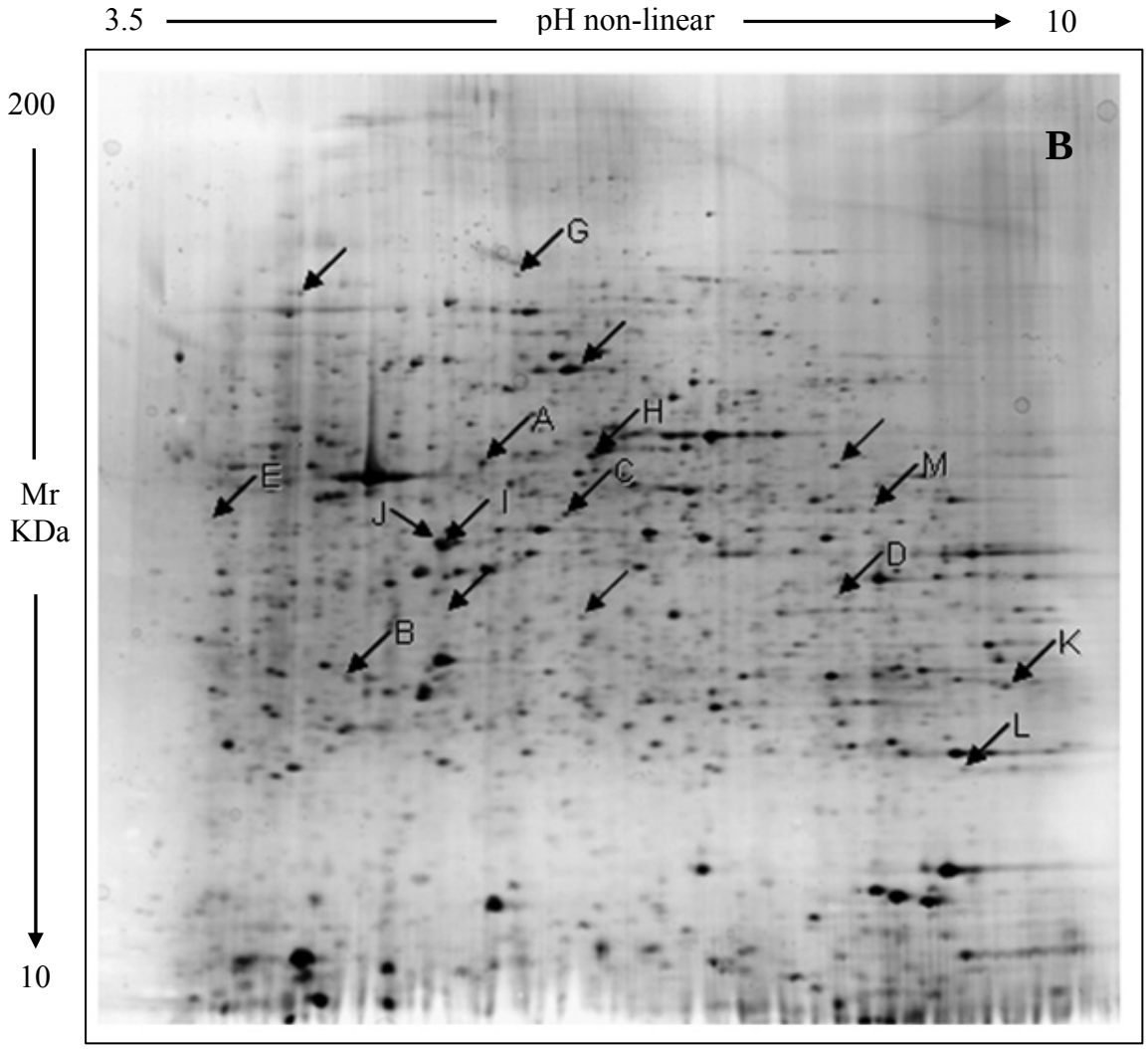

Fig. 1B. *See preview page.*

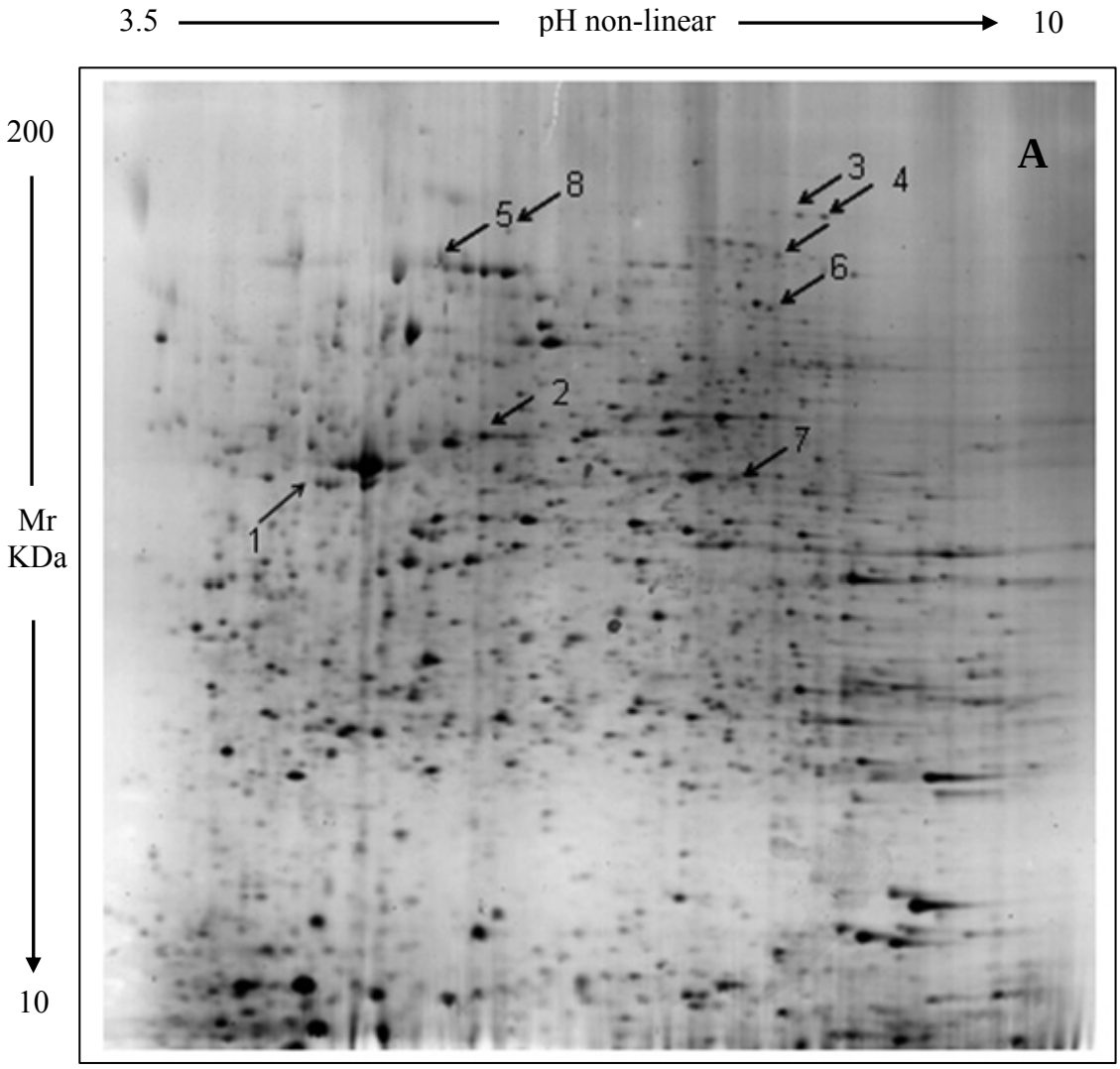

Fig. 2A. *2D images of silver stained gels of total proteins extracted from control and HypF-N prefibrillar aggregate treated cells for 24 h. Arrows indicate variations between control (panel A) and treated cells (panel B, next page). Numbers indicate the identified proteins. Controls were performed by exposing the cells to native, soluble HypF-N.*

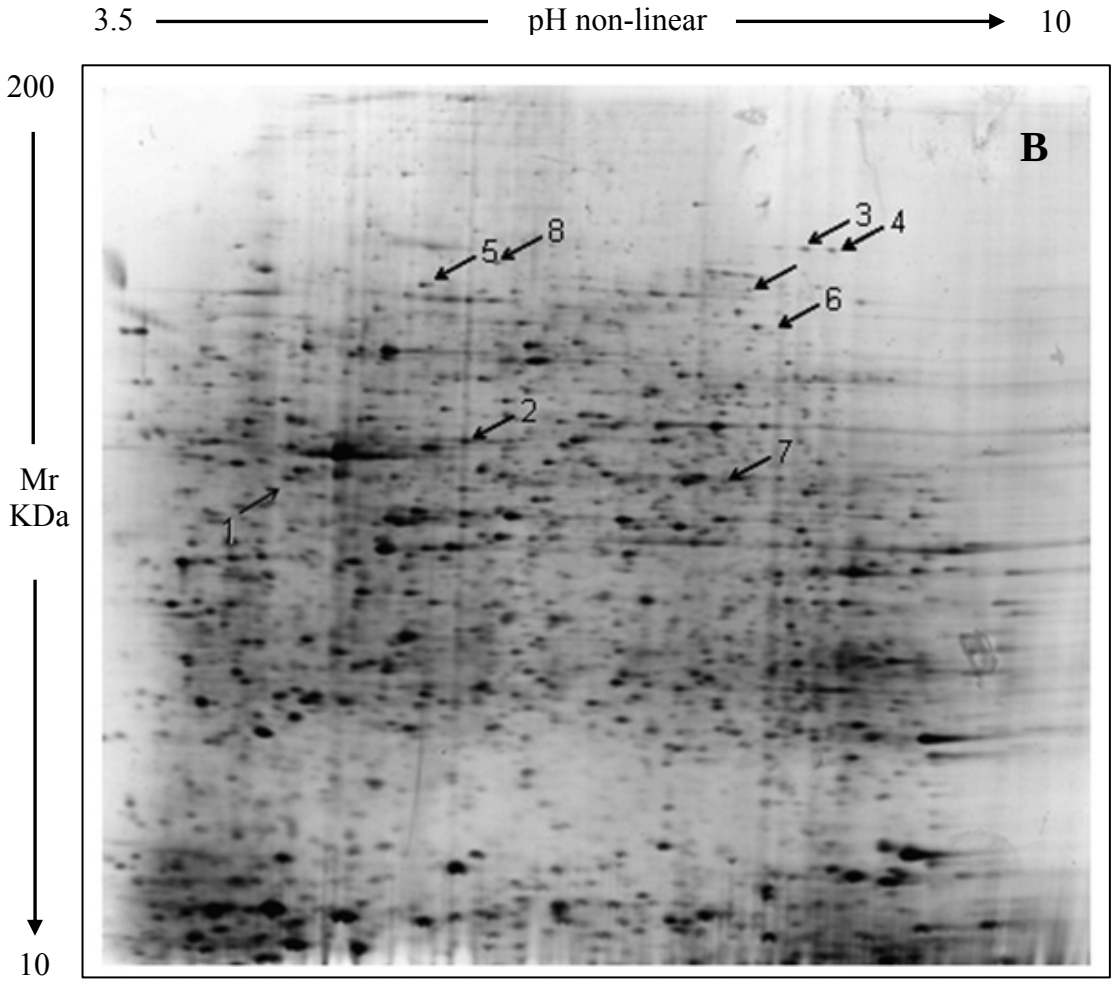

Fig. 2B. *See preview page.*

2.3.2 Identification of differentially expressed proteins

In order to identify the proteins of interest, 400 μg of protein lysates was loaded on preparative gels and stained with colloidal Coomassie. The spots indicated by arrows in representative gels shown in Figs. 1 and 2 were selected for mass spectral identification after merging the images of preparative and analytical gels. The proteins excised from the gels were reduced, alkylated and in situ digested with trypsin. The resulting peptide mixtures were directly analyzed by MALDI/MS according to the peptide mass fingerprinting procedure.

The peaks detected in the MALDI spectra were used to search for a non redundant sequence database using the in house MASCOT software, taking advantage of the specificity of trypsin and the taxonomic category of the samples. The number of measured masses that matched within the given mass accuracy of 200 ppm was recorded and the proteins that received the highest number of peptide matches were examined. Some spots could not be identified unambiguously either due to the low protein content of the spot or to the presence of more than one protein per spot.

Among the 19 spots differentially expressed after 5 h of cell exposure to the aggregates, 13 spots were successfully identified and are indicated by arrows and letters in Fig. 1. Among the 9 spots differentially expressed in cells treated for 24 h with 2 μM HypF-N prefibrillar aggregates, 8 spots were identified and are indicated by arrows and numbers in Fig. 2. Some spots gave no confident identification by the peptide mass fingerprinting procedure. Additional data were then provided by nano LC/MS/MS experiments. The peptide mixtures were fractionated by nano HPLC and sequenced by tandem mass spectrometry leading to the unambiguous identification of the protein candidate.

The lists of proteins identified by these approaches are reported in Table 1 (5 h treatment) and in Table 2 (24 h treatment). The identified proteins included cytoskeleton elements (actin, tubulin alpha 1C chain, microtubule-actin cross-linking factor 1), enzymes involved in energy metabolism and transcriptional regulation

(Gapdh, enolase), proteins involved in stress response (Hsp60 and 78 kDa glucose-regulated protein) and the focal adhesion kinase, Fak1. Among these proteins only Fak1 and Hsp60 showed an expression variation persisting over time, since it was observed both after 5 and after 24 h of cell exposure to the aggregates.

Table 1. *Relative change in protein expression in cells treated for 5 h with HypF-N prefibrillar aggregates versus control cells.*

Spot	Protein name	AC	Mascot score	No. matched peptides	Sequence coverage	Fold change	*p*-value	Functional categorization
Up-regulated by HypF-N								
A	Hsp60	P63038	162	5	12%	+1,58	0,0005	Protein folding and stress response
B	Actin	P60710	79	5	22%	Only in treated cells.	N.D.	Cytoskeletal organization
C	Actin	P60710	241	7	18%	+9.8	0.007	Cytoskeletal organization
D	Glyceraldehyde-3-phosphate dehydrogenase	P16858	120	7	33%	+9,7	0,0081	Energy related (glycolysis)
E	78 kDa glucose-regulated protein	P20029	78	2	5%	+4.8	0.1	Protein folding and stress response (response to unfolded proteins)
F	Nucleophosmin	Q61937	165	5	15%	Only in treated cells.	N.D.	Associated with nucleolar ribonucleoprotein structures and bind single-stranded nucleic acids
Down-regulated by HypF-N								
G	Focal Adhesion kinase-1	P34152	77	17	22%	1,6	0,01	Non-receptor protein-tyrosine kinase involved in cell motility, proliferation and apoptosis.
H	Enolasi-1	P17182	118	12	32%	-2,4	0,09	Energy related (glycolysis)
I	Tubulin alpha-1C chain	P68373	80	9	31%	-8,3	0,015	major constituent of microtubules
J	Annexin-A3	O35639	111	13	43%	-2	0,019	Inhibitor of phospholipase A2
K	Hsp60	P63038	85	2	5%	-1,6	0,032	Protein folding and stress response
L	Transgelin-2	Q9WVA4	171	7	41%	Only in control cells	N.D.	Belongs to the calponin family
M	Heparan sulfate glucosamine 3-O-sulfotransferase 2	Q673U1	47	2	4%	Only in control cells	N.D.	Catalyzes the O-sulfation of glucosamine

Table 2. *Relative change in protein expression in cells treated for 24 h with HypF-N prefibrillar aggregates versus control cells.*

Spot	Protein name	Accession number	Mascot score	No. matched peptides	Sequence coverage	Fold change	*p*-value	Functional categorization
Up-regulated by HypF-N								
1	Ovostatin homolog	Q3UU35	48	4	15%	+1,4	0,28	Proteases inhibitor
2	Hsp60	P63038	162	5	12%	+1,58	0,0005	Protein folding and stress response
Down-regulated by HypF-N								
3	Microtubule-actin cross-linking factor 1	Q9QXZ0	82	14	10%	-1.66	0.035	F-actin-binding protein which may play a role in cross-linking actin to other cytoskeletal proteins.
4	Microtubule-actin cross-linking factor	Q9QXZ0	88	10	8%	-2,24	0,023	
5	Pol protein	Q7M6W3	73	10	17%	-1,67	0,1	Unknown
6	Pdia2 protein	Q14AV9	43	2	5%	-3,21	0,004	Protein disulfide isomerase
7	Poly(rC)-binding protein 2	Q61990	71	2	6%	-3,78	0,014	Single-stranded nucleic acid binding protein
8	Fak1 Focal Adhesion kinase	P34152	77	17	22%	-1,53	0,015	Non-receptor protein-tyrosine kinase involved in cell motility, proliferation and apoptosis.

2.3.3 Validation of proteomics results

In order to validate the proteomic results, the amounts of Fak1 and Hsp60 were evaluated by Western blot analysis with specific antibodies as shown in Fig. 3, panels A and B, respectively. Thirty μg of proteins was loaded on 12% SDS-PAGE and transferred onto a PVDF membrane. For quantification, the intensities of the immunostained bands were normalized to the total protein intensities in the same blot, as measured by Coomassie brilliant blue. In Fig. 3, panel C the histograms representing the variation of the expression of Fak1 and Hsp60 are also reported. Such analysis confirmed the decrease of Fak1 expression and the increase of Hsp60 expression both after 5.0 and 24 h.

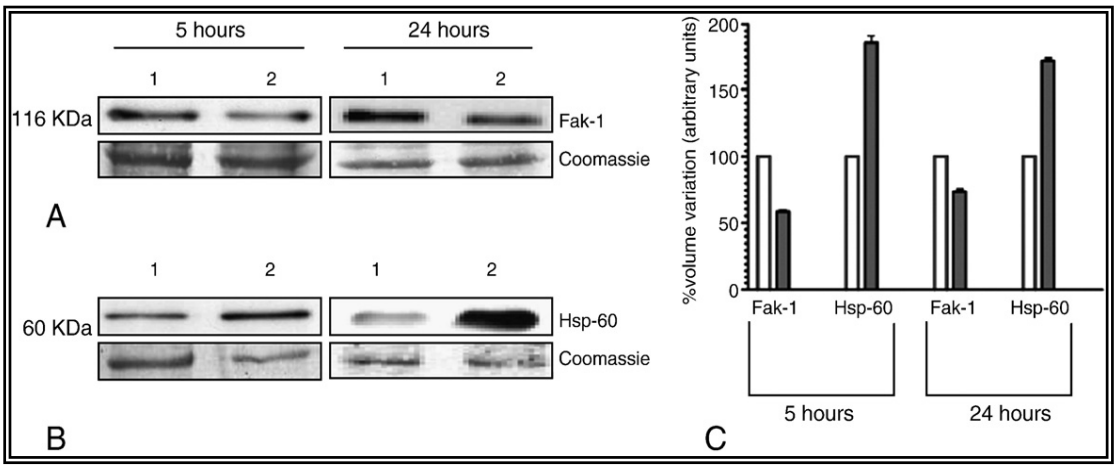

Fig. 3. *Validation of proteomic results by western blot analysis. Western blot were probed with antibodies against Fak1 and Hsp60 proteins identified by proteomic screening. The intensity of immunostained bands was normalized with the total protein intensities measured from the same blot stained with Coomassie brilliant blue (in panel A and panel B a representative band of the lane is reported). (A) Aggregate-induced reduced expression of Fak1 after 5 and 24 h of treatment: lane 1, cells exposed to native HypF-N; lane 2, cells exposed to HypF-N prefibrillar aggregates. (B) Aggregate-induced increased expression of Hsp60 after 5 and 24 h of treatment: lane 1, cells exposed to native HypF-N; lane 2, cells exposed to HypF-N prefibrillar aggregates. (C) Histograms representing Fak1 and Hsp60 protein expression variation. The two-tailed non paired Student's t-test was performed using ORIGIN 6.0. (pb0.05).*

2.3.4 Protein expression changes in cells exposed for 5 h to HypF-N prefibrillar aggregates

In cells exposed to HypF-N prefibrillar aggregates for 5 h, 6 spots (A to F in Table 1) appeared up-regulated, whereas 7 spots (G to M in Table 1) appeared down-regulated. Among the up-regulated proteins, we found the heat shock protein Hsp60 (spot A). Hsp60 belongs to a family of highly homologous chaperone proteins that are induced in response to environmental, physical and chemical stresses, including accumulations of misfolded proteins and reactive oxygen species [H.R. Saibil *et al.*,2008; R. Arya *et al.*, 2007]. The increase of Hsp60 expression limits the consequences of damage facilitating cell recovery. Hsp60 was also identified in the spot K, which was down-regulated upon cell treatment with the aggregates. However, the position in the gel and the peptide coverage, indicates that this spot is probably a fragment arising from a proteolytic cleavage of Hsp60.

A further indication of a stress condition induced in cells exposed to the HypF-N aggregates is the marked increase in the expression of the key glycolytic enzyme Gapdh (spot D). This protein plays a central role in glycolysis, catalyzing the reversible conversion of glyceraldehyde- 3-phosphate to 1,3-bisphosphoglycerate. More recent studies have highlighted unexpected non-glycolytic functions of Gapdh in physiological and pathological processes, including transcriptional regulation of cell-cycle [S. Carujo *et al.*, 2006]. In addition two spots corresponding to actin were up-regulated.

Among the proteins whose expression appeared decreased upon exposure to HypF-N prefibrillar aggregates, the focal adhesion kinase (Fak1; spot G) is particularly interesting. Fak1 is a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of proliferation and migration of normal and tumour cells [M.D. Schaller *et al.*, 1992; 2001]. Interestingly, Fak1 and Hsp60 are the only two proteins, among those differentially expressed after 5 h of cell exposure, that do not recover a normal expression level after 24 h.

2.3.5 Protein expression changes in cells exposed for 24 h to HypF-N prefibrillar aggregates

It was previously shown that NIH-3T3 cells treated for 24 h with 10 μM HypF-N prefibrillar aggregates die with necrotic features, including cytoplasmic vacuolization and nuclear swelling after an initial apoptotic activation [M. Bucciantini *et al.*, 2005]. The amount of HypF-N aggregates (2 μM, soluble protein concentration) used in this work is not so high to induce cell death, thus allowing the cells to overcome damage. After 24 h of cell exposure to HypF-N aggregates, few other proteins, besides Fak1 and Hsp60, displayed altered expression.

In particular, we identified 6 new spots (1 up-regulated and 5 down-regulated). Among the proteins down-regulated, the microtubule-actin crosslinking factor 1 (MACF1, spot 3) belongs to the Plakin family, that includes proteins involved in the linkage of cytoskeletal elements and the junctional complex. MACF1 was found to regulate microtubule remodelling in response to the activation of signal transduction pathways, although its function has not yet been fully explored [I. Karakesisoglou *et al.*, 2000; H.J. Chen *et al.*, 2006].

2.4 Discussion

To our knowledge, this study is the first proteomic investigation focused on highlighting the alterations of the protein expression profiles in a cultured cell model exposed to toxic amyloid aggregates of a protein not involved in any amyloid disease. Our analysis was performed using a non lethal dose of HypF-N prefibrillar aggregates, allowing the detection of fine variations more directly implicated in a response to the cell injury given by the aggregates, instead of the complex pattern of changes arising during the process of cell death.

Our approach led us to identify a subset of cell proteins whose levels were significantly altered upon cell exposure to the aggregates for 5 h or 24 h. Some of the proteins detected in our investigation, including Hsp60, actin, enolase-1 and Gapdh had previously been identified in other proteomic studies carried out on cells exposed to Aβ42 or α-synuclein. In Table 3 a comparison between the protein identified in our study and the proteins identified in other studies is shown, indicating that there is a general response of cells to toxic aggregates that is not sequence specific [B. Martin *et al.*, 2008; S. Joerchel *et al.*, 2008; M.A. Lovell *et al.*, 2005; S.J. Shin *et al.*, 2004; D.C. David *et al.*, 2006]

Protein	Amyloid aggregates	Cell type or animal model	
GAPDH	HypF-N	NIH3T3 fibroblast cells	This study
	Amyloid β-peptide	Mitochondria from primary neuron	[30]
		Tg2576 transgenic mice	[31]
		3xTgAD Alzheimer's mice (hippocampal protein)	[12]
	β-amyloid (1–42)	P301L tau overexpressing SH-SY5Y	[32]
Tubulin α-chain	HypF-N	NIH3T3 fibroblast cells	This study
	β-amyloid (1–42)	SN56.B5.C4 cholinergic cells	[13]
		3xTgAD Alzheimer's mice (hippocampal protein)	[12]
	β-amyloid (1–42)	Amygdalae from P301L tau transgenic mice	[32]
78 kDa glucose-regulated protein	HypF-N	NIH3T3 fibroblast cells	This study
		3xTgAD Alzheimer's mice (hippocampal protein)	[12]
	β-amyloid (1–42)	P301L tau overexpressing SH-SY5Y	[32]
Enolase	HypF-N	NIH3T3 fibroblast cells	This study
		Tg2576 transgenic mice	[31]
		3xTgAD Alzheimer's mice (hippocampal protein)	[12]
Hsp60		3xTgAD Alzheimer's mice	[12]
Actin	HypF-N	NIH3T3 fibroblast cells	This study
	β-amyloid (1–42)	Amygdalae from P301L tau transgenic mice	[32]

Table 3.*Protein expression changes observed in this study in comparison to previous protein reported in other studies.*

Actually, changes in the expression levels of Gapdh, actin, tubulin and heat shock proteins have frequently been reported in amyloid-linked proteomic studies possibly because they are related to a generic response to stress conditions [J. Petrak *et al.*, 2008] such as that associated with the growth of amyloid aggregates. In our experiments, cell exposure to toxic amyloid aggregates induced an increase of the expression levels of several proteins such as Hsp60. In addition to its chaperone activity, Hsp60 has been suggested to perform complex functions, producing both anti- and pro-apoptotic effects. In fact, cytosolic Hsp60, can promote either cell survival or caspase mediated cell death by preventing the translocation of the pro-apoptotic protein Bax into the mitochondria or by favouring the maturation of procaspase-3, respectively [J.C. Ghosh *et al.*, 2008; S.R. Kirchhoff *et al.* 2002; S. Xanthoudakis *et al.*, 1999 ; A. Samali *et al.*, 1999; D. Chandra *et al.*, 2007]. In a recent study, Hsp60, Hsp70, and Hsp90 were shown to provide differential protection against intracellular stress caused by β-amyloid by maintaining the efficiency of the mitochondrial oxidative phosphorylation and the tricarboxylic acid cycle enzymes. In particular, Hsp60was shown to prevent the inhibition of complex IV activity by β-amyloid, thus preventing apoptosis [V. Veereshwarayya *et al.*, 2006].

We also found significantly increased levels of Gapdh and actin. As far as Gapdh is concerned, several recent studies have shown that, in addition to glycolysis, it is involved in several glycolysis-unrelated activities; these include a role in vesicle fusion and transport [E.J. Tisdale *et al.*, 2001], microtubule bundling [P. Huitorel *et al.*, 1985], nuclear RNA transport [R. Singh *et al.*, 1993], and transcription [G. Morgenegg *et al.*, 1986]. Furthermore, increased expression and nuclear translocation of Gapdh have recently been reported to participate to the apoptotic pathway in different cell types [M.R. Hara *et al.*, 2005; D.M. Chuang *et al.*, 2005; Z. Dastoor *et al.*, 2001; F. Magherini *et al.*, 2007]. Finally, Gapdh has also been reported to bind to a variety of proteins involved in neuronal diseases, including the amyloid precursor protein and huntingtin [J.L. Mazzola *et al.*, 2002]. The increased levels of actin expression in cells exposed to prefibrillar HypF-N aggregates are similar to the effects previously reported by Muller *et al.*; these authors found up-regulation of the actin gene expression in cells harbouring the cytoplasmic domain of the amyloid precursor protein [T. Müller *et al.*, 2007].

Finally, we found a significant decrease of Fak1 in cells exposed both 5 h and 24 h to the HypF-N aggregates. The repression of Fak1 synthesis in exposed cells, confirmed by Western blot analysis, is one of the major results of this proteomic analysis. In vivo animal studies have shown that Fak1 expression is increased in a number of human cancers, thus contributing to tumour development and malignancy [M.D. Basson *et al.*, 2008]. Moreover, Fak1 has recently been shown to be a critical protein in survival signalling, since it blocks apoptosis induced by several stimuli [D.H. Crouch *et al.*, 1996]. Fak1 expression decrease following proteolytic cleavage in various cell types has been associated with various cell dysfunctions including c-Myc-induced apoptosis of chicken embryo fibroblasts (CEF) [D.H. Crouch *et al.*, 1996], growth factor deprivation-induced apoptosis of human umbilical vein endothelial cells [B. Levkau *et al.*, 1998], and detachment-induced cell death (anoikis) of intestinal epithelial cells [J. Grossmann *et al.*, 2001]. In a recent study, different epithelial cell lines treated with thimerosal displayed increased levels of hydrogen peroxide resulting in caspase activation, Fak1 cleavage and apoptosis [M.F. Mian *et al.*, 2008]. All these observations suggest that the decreased Fak1 expression in NIH-3T3 cells treated with HypF-N aggregates could be related to the apoptotic process: in particular its decreased intracellular levels could be the consequence of a proteolytic cleavage making the cells more vulnerable to death.

The reported changes in protein expression profiles in exposed cells suggest some alterations in specific signalling pathways involved in the control of gene transcription and translation and/or in protein degradation pathways. These alterations could be triggered, at least in part, by modifications of signalling pathways following the reported interaction of amyloid aggregates with the cell membrane [M. Bucciantini *et al.*, 2005; J.I. Kourie *et al.*, 2001; L. Zhang *et al.*, 2001]. Overall, our results can provide useful information on the crucial events underlying cytotoxicity induced by amyloid aggregates of different peptides or proteins both related and unrelated to disease.

Acknowledgements

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Chapter 3

"Effect of different glucose concentrations on proteome of Saccharomyces cerevisiae"

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Submitted

3. Abstract

We performed a proteomic study to understand how *Saccharomyces cerevisiae* adapts its metabolism during the exponential growth on three different concentrations of glucose; this information will be necessary to understand yeast carbon metabolism in different environments. We induced a natural diauxic shift by growing yeast cells in glucose restriction thus having a fast and complete glucose exhaustion. We noticed differential expressions of groups of proteins. Cells in high glucose have a decreased growth rate during the initial phase of fermentation; in glucose restriction and in high glucose we found an over-expression of a protein (Peroxiredoxin) involved in protection against oxidative stress insult. The information obtained in our study validates the application of a proteomic approach for the identification of the molecular bases of environmental variations such as fermentation in high glucose and during a naturally-induced diauxic shift.

Keywords: Yeast fermentation, proteomics, diauxic shift, glucose metabolism

3.1 Introduction.

Yeast is a facultative anaerobe able to live on various fermentable and non-fermentable carbon sources. *Saccharomyces cerevisiae* uses glucose in different ways depending on the availability of oxygen and quantity of carbon source. When yeast is grown on fermentable substrates such as glucose, the metabolic energy essentially originates from glycolysis. The Pasteur effect relates oxygen with sugar catabolism, hence glycolysis in resting cells proceeds more rapidly under anaerobic conditions than under aerobic conditions [Lagunas *et al.*, 1983]. The situation is very different in growing cultures where, irrespective of oxygen availability, fermentation is the predominant route of sugar metabolism. If the glucose concentration is high, the Pasteur effect is no longer operable and is replaced by the Crabtree effect, by which cells continue to ferment due to the repression/inactivation of respiratory enzymes or to the inherent limited respiratory capacity of cells [Walker *et al.*, 1999].

The Crabtree effect can be either a short-term or a long-term effect [Petrik *et al.*, 1983]. The short-term effect is characterized by its capability of triggering alcoholic fermentation upon a sudden glucose excess condition whereas the long-term effect is characterized by the respiratory fermentative metabolism observed in batch cultivation or in continuous culturing. During fermentation in grapes, for example, sugar concentrations can vary between 1 M and 10^{-5} M [Kruckeberg *et al.*, 1996]. To survive changes in its nutritional environment, yeast is able to detect the availability of nutrients and rapidly adapt its metabolism. In *S. cerevisiae*, growth in rich media with low glucose concentration increases both replicative and chronological longevity [Jiang *et al.*, 2000; Lin *et al.*, 2000]. The beneficial effects of glucose restriction in yeast are related to the increase in respiratory rates that occurs when glucose levels in the media are low [Lin *et al.*, 2002; Barros *et al.*, 2004]. These enhanced respiratory rates increase intracellular NAD^+ levels which may be involved in the regulation of replicative lifespan. This modulates the activity of Sir2 family proteins [Lin *et al.*, 2003] and reduces the releasing of mitochondrial reactive oxygen species (ROS) [Tahara *et al.*, 2007]. ROS are normal by-products of cell metabolism; however an increase in their production can cause oxidative modifications in cell

macromolecules such as proteins, DNA and lipids. This can induce alterations of cell structure that, in many cases, lead to a loss of function. A great number of recent reports seems to confirm the Harman "free radical theory of ageing" [Harman *et al.*, 1992] proposed some fifty years ago notwithstanding the many still remaining unknown details.

In our previous study, a proteomic approach was used to evaluate the redox state of yeast protein cysteines during chronological ageing [Magherini *et al.*, 2009]. These data demonstrated that glucose restriction and growth on glycerol supplemented media extend *S. cerevisiae* lifespan and that oxidative damage in cells grown on high glucose content, mostly affects glycolytic enzymes. Studying how yeast adapts to changes in its environment is important, not only because it might be relevant for the optimization of its industrial applications but also because knowledge gained during such experiments could help understand some important eukaryotic cells biological processes. Comprehensive transcriptome analyses have been performed to study the effects of different nutrient conditions on yeast [Boer *et al.*, 2003; Wu *et al.*, 2004; Saldanha *et al.*, 2004; Tai *et al.*, 2005]. These genome-wide expression profiles reflect the physiological status of cells and show how they respond to different nutritional environments at transcriptional level. Unlike transcriptome studies, proteome studies allow the analysis of all proteins that are present in a certain condition. However, increasing evidence shows that mRNA abundance is not always correlated with protein expression levels [Anderson *et al.*, 1997; Gygi *et al.*, 1999; Le Naour *et al.*, 2001; Ideker *et al.*, 2001; Griffin *et al.*, 2002]. Therefore, it is essential to study yeast adaptation and other biological processes at a proteomic level.

Two-dimensional reference maps have been constructed for important industrial yeast strains [Kobi *et al.*, 2004; Trabalzini *et al.*, 2003; Joubert *et al.*, 2000; Joubert *et al.*, 2001]. Many quantitative 2D-gel-based studies with applications to yeast have been reported, examining, for example, yeast growth under different environmental conditions [Salusjarvi *et al.*, 2003; Brejning *et al.*, 2005; Trabalzini *et al.*, 2003]. Global analyses of protein profiles have become a powerful tool to understand how cells respond to changes in environment. Ethanol fermentation is a clear one example of a process during which yeast cells have to adapt to significant variations. We

performed a proteomic study to understand how *S. cerevisiae* adapts its metabolism during the exponential growth in medium supplemented with three different concentrations of glucose: 2%, 0.5% and 20%. We selected a 2% and 20% glucose concentration for yeast growth because both induce a fermentative metabolism. Furthermore, media with 20% glucose concentration is very similar to natural must. Throughout alcoholic fermentation, *S. cerevisiae* cells have to cope with several stress conditions that could affect their growth and viability. Glucose *per se* is a powerful signalling molecule in yeast. When glucose concentration falls below 0.2%, cells stop dividing for a few hours. After this lag phase, cells start to consume ethanol (obtained from the former glucose catabolism) by respiration (natural diauxic shift) [Westerbeek-Marres *et al.*, 1988]. A yeast model of glucose restriction (0.5% glucose) was chosen in order to study the effect of glucose limitation on its proteome.

The aim of this study was to analyze the proteome changes during growth under both high glucose concentration and glucose restriction in yeast. As a result, we notice differential expressions of groups of proteins. Yeast grown in these conditions shows different growth rates and fermentative behaviours. The different expression patterns could be related to the stress caused by high glucose concentration and to the previously demonstrated beneficial effect of glucose restriction.

3.2 Materials and Methods

3.2.1 Strains.

The *S.cerevisiae* strain used in this study is wild-type strain W303-1A, MATa, ura3-52, trp1Δ2, leu2-3_112, his3-11, ade2-1, can1-100 (accession no. 20000A; EUROSCARF, Frankfurt a.M., Germany).

3.2.2 Growth conditions.

Yeast cells were grown at 30°C in synthetic complete (SC) medium containing 0.67% w/v of Yeast Nitrogen Base without Amino Acids (USbiological) supplemented with complete Amino Acid dropout solution (USbiological). 0.5% glucose, 2% glucose and 20% glucose were used as carbon sources. Cells were picked from fresh colonies and grown overnight in SC medium with 0.5%, 2% and 20% glucose. Cells were then diluted in their respective fresh media to an optical density at 600 nm of 0.2 O.D./ml using flasks with volume/medium ratio of 3:1. Growth was monitored by measuring the turbidity of the culture at 600 nm (OD600) on a spectrophotometer until they reached the cellular density of 0.8 O.D./ml.

3.2.3 Ethanol and glucose measurement.

For ethanol and glucose determination, cells were grown in SC medium containing a fermentable carbon source (0.5% glucose, 2% glucose and 20% glucose) to an optical density (600 nm) of 0.8 O.D. /ml. One millilitre of the growth was harvested at exponential phase and then centrifuged. The cleared supernatant was collected to estimate the ethanol production and the residual glucose. Ethanol was determined using the alcohol-dehydrogenase/aldehyde-dehydrogenase method (the assay was performed according to the "K-etoh" kit from Megazymes (Ireland)). Glucose was determined according to the Accu-Chek® Active Glucose (Roche Diagnostics) protocol.

3.2.4 Intracellular ROS evaluation.

To evaluate the ROS production, cells were grown in SC medium supplemented with 0.5%, 2% and 20% glucose and were harvested when they reached the cellular density of 0.8 O.D./ml. One O.D. of cells was washed twice in 10 mM HEPES buffer, then resuspended in the same buffer and incubated at 30° C in the dark for 2 hours with dihydrorhodamine 123 (Molecular Probes) in order to highlight ROS production on a Leica TCS SP5 confocal microscope.

3.2.5 Sample preparation and 2D-GE.

For two dimensional experiments, cells were harvested during the exponential phase at a cellular density of 0.8 O.D./ml. Cells were broken in RIPA buffer (50 mM Tris-HCl pH 7, 1% NP-40, 150 mM NaCl, 2mM EGTA, 100mM NaF) plus a cocktail of yeast protease inhibitors (Sigma) with glass beads in a Fastprep instrument (Savant). Protein extracts were clarified by centrifugation at 8000g for 10 minutes.
Proteins were precipitated following the chloroform/methanol protocol [Wessel *et al.*, 1984] and the pellet was resuspended in 8M urea, 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 20 mM dithiothreitol (DTT). For each experimental condition at least three samples were run in order to assess biological and analytical variation. Isoelectrofucusing (IEF) was carried out on nonlinear wide-range immobilized pH gradients (pH 3-10; 18 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using the EttanTM IPGphorTM system (GE Healthcare, Uppsala, Sweden). MS-Preparative-run IPG-strips were rehydrated at 16°C with 350 µg of proteins in 350 µl of lysis buffer and 0.2% carrier ampholyte for 1h at 0 V and for 8h at 30 V. The strips were then focused at 16°C according to the following electrical conditions: 200 V for 1h, from 300 V to 3500 V in 30 min, 3500 V for 3h, from 3500 V to 8000 V in 30 min, 8000 V until a total of 80000 Vh was reached. After focusing MS-preparative IPG strips were equilibrated for 12 min in 6 M urea, 30% glycerol, 2% Sodium Dodecyl Sulfate (SDS), 0.05 M Tris-HCl, pH 6.8, 2% DTT, and subsequently for 5 min in the same urea/SDS/Tris buffer solution but substituting the 2% DTT with 2.5% iodoacetamide. The second dimension was carried out on 9-16% polyacrylamide linear gradient gels (18 cm x 20 cm x 1.5 mm) at 40 mA/gel constant current and 10°C until the dye front reached the

bottom of the gel. The MS-preparative gels were stained with colloidal Coomassie [Vilain *et al.*, 2001].

3.2.6 Image analysis and statistics.

Gels were acquired with an Epson expression 1680 PRO scanner. For each strain condition, 2D gels were run in triplicate and only spots present in all the replicates were taken into consideration for subsequent analysis. Computer-aided 2D image analysis was carried out using ImageMaster 2-D Platinum software version 6.0 (GE Healthcare). Relative spot volume (%V were V=integration of OD over the spot area and were %V = V single spot/V total spots) was used for quantitative analysis in order to reduce experimental errors. The normalized intensity of spots on three replicates 2-D gels was averaged and standard deviation was calculated for each condition. A two-tailed non-paired Student's t-test was performed using ORIGIN 7.5 (Microcal Software, Inc.) to determine if the variations in relative spot volume was statistically significant

3.2.7 In-gel trypsin digestion and MALDI-TOF Mass spectrometry.

Protein spots were manually excised from the gel, washed with high-purity water and with 50% acetonitrile/water and dehydrated with 100% acetonitrile. The gel slices were swollen at room temperature in 20 µl of 40 mM NH_4HCO_3/10% acetonitrile containing 25 ng/µl trypsin (Trypsin Gold, mass spectrometry grade, Promega). After 1 h, 50 µl of 40 mM NH_4HCO_3/10% acetonitrile were added and digestion proceeded overnight at 37 °C. The generated peptides were than extracted with 50% acetonitrile/5% trifluoroacetic acid (TFA, 2 steps, 20 min each at room temperature), dried by vacuum centrifugation, suspended in 0.1% TFA, passed through micro ZipTip C18 pipette tips (Milllipore, Bedford, MA, U.S.A.) and directly eluted with the MS matrix solution (10 mg/ml $\tilde{\alpha}$ cyano-4-hydroxycinnamic acid in 50% acetonitrile/1% TFA). Mass spectra of the tryptic peptides were obtained using a Voyager-DE MALDI-ToF mass spectrometer (Applied Biosystems). Peptide mass fingerprinting database searching was performed using the MASCOT search engine (http://www.matrixscience.com) in NCBInr/Swiss-Prot databases. Parameters were set to allow one missed cleavage / peptide, a mass tolerance of 0.5 Da and

considering carbamido-methylation of cysteines as a fixed modification and oxidation of methionines as a variable modification.

3.3 Results and Discussion.

3.3.1 Growth and fermentation profile during aerobic cultivation.

The W303-1A yeast strain was aerobically cultivated in SC medium supplemented with 2%, 0.5% and 20% glucose in order to characterize the molecular basis of the yeast's respiratory and fermentative metabolism during exponential growth in the presence of different amounts of glucose. The strain is Crabtree positive and to analyze its behaviour, we first evaluated cell growth rate. The three different growth behaviours are shown in Figure 1 panel a. In 0.5% and 2% glucose the yeast enters the exponential phase more rapidly than in 20% glucose. We choose the standard condition of 2% glucose concentration as control.

We found that cells grown in glucose restriction show a higher growth rate compared to cells grown in high glucose. After cellular acclimation the exponential growth begins. When the cultures reach a cellular density of 0.8 O.D./ml (i.e. $8x10^6$ cells/ml) we evaluate cellular ethanol production and glucose consumption. We choose this particular cellular density because in yeast grown in glucose restriction, this point is coincides with the onset of the natural diauxic shift [Maris, 2001] (glucose concentration in the medium reaches 0.2%, see Table 1A). In this particular moment cells undergo a metabolic adaptation accompanied by a global reprogramming of gene expression (including mitochondrial biosynthesis) which precedes complete glucose exhaustion. The ethanol production in the three different amounts of glucose is shown in Figure 1 panel b. The ethanol concentration at 0.8 O.D./ml is: 2.6 mg/O.D. for 2% glucose; 2.4 mg/O.D for 0.5% glucose and 4.2 mg/O.D. for 20% glucose. Under high glucose concentration (20%) ethanol production is not as high as expected. Pham T. K. *et al.* [2006, 2008] had previously reported that to minimize the osmotic stress caused by a high glucose concentration, *S. cerevisiae* accelerates its production of acidic compounds and glycerol rather than synthesize ethanol. The differences related to sugar consumption among the cultures are made evident in Figure 1 panel c: at 0.8 O.D./ml a low percentage of glucose

consumption (9.9%) is observed in yeast grown in 20% glucose; at that cellular density these cells leave approximately 90% residual glucose. On the contrary, glucose consumption percentually increases in cells grown in glucose restriction: cells leave about 32% residual glucose in the medium. In Table 1 the differences in fermentative abilities are shown using two parameters: glucose consumption (A) and ethanol production (B). As is evident in Table 1A and Figure 1c, the cells in glucose restriction are the ones to use the most glucose. In high glucose concentration ethanol is the major product but as shown in Table 1B and Figure 1c, the percentage of glucose used for fermentation, in comparison to the total glucose consumption (9.9%), is only 37.6%.

Table 1.A Total, residual and used glucose in SC medium supplemented with 2%, 0.5% and 20% glucose, measured at the cellular density of 0.8 O.D./ml. Glucose is expressed in mg/O.D. and in percentage (relative and absolute (in brackets)).

Glucose	2%		0.5%		20%	
	mg/O.D.	%	mg/O.D.	%	mg/O.D.	%
total	22.7	100 (2.0)	5.3	100 (0.5)	217	100 (20.0)
residual	14	65.4 (1.3)	1.8	32 (0.17)	195	90.1(18.0)
consumption	8	34.6 (0.7)	3.6	68 (0.33)	21.8	9.9 (2.0)

Table 1.B Correlation between ethanol production, expressed in mg/O.D. and in mol/O.D., and glucose consumption. The percentage represents the rate of glucose used for fermentation in comparison to total glucose consumption.

	Ethanol		Glucose	
	mg/O.D.	mol/O.D.	consumption (mol/O.D.)	% used for ethanol production
2%	2.6	$5.6*10^{-5}$	$4.4*10^{-5}$	63.0
0.5%	2.4	$5.2*10^{-5}$	$2.0*10^{-5}$	100
20%	4.2	$9.1*10^{-5}$	$12.1*10^{-5}$	37.6

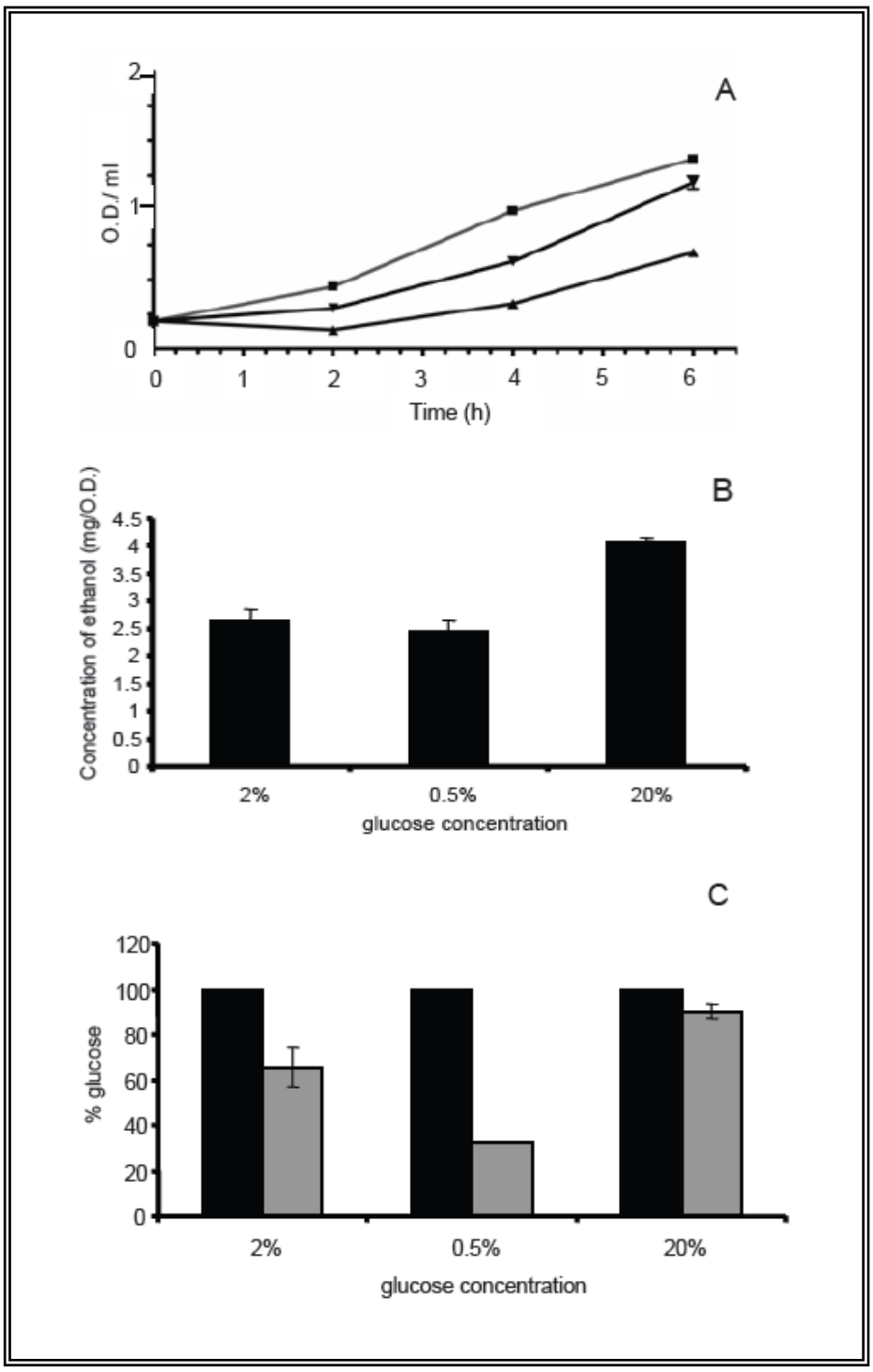


Fig. 1. a) *Aerobic exponential growth of W303 1A in SC medium supplemented with.: ■, 2% glucose; ▲, 20% glucose; ▼, 0.5% glucose. Growth rate of cells was determined by O.D. measurements at 600 nm for the indicated times.* ***b)*** *Ethanol concentration (mg/O.D.) was evaluated at the cellular density of 0.8 O.D./ml on SC plus 2%,0.5% and 20% glucose.* ***c)*** *Percentage of residual glucose evaluated at 0.8 O.D./ml on SC medium containing an initial glucose concentration of: 2% glucose; 0.5% glucose and 20% glucose. Black bars indicate the amount of initial glucose in each medium which is to be considered 100%. Grey bars represent the % of residual glucose in each medium. Data represent the mean and standard deviation obtained from three independent experiments.*

3.3.2 Evaluation of intracellular generation of free radicals.

ROS production in the three different glucose concentrations (2%, 0.5% and 20%) was measured with dihydrorhodamine (DHR123) when the cells reach a cellular density of 0.8 O.D./ml. DHR123 is a non-fluorescent dye that is able to enter cells [Unterluggauer, 2003; Madeo, 1999]. Yeast cells grown in 0.5% and 2% glucose concentration show a relatively low and comparable ROS production. Oxidation of DHR123 results considerably higher in yeast grown in 20% glucose. Figure 2 shows that approximately 50% of cells grown in 20% glucose are stained with fluorescent Rodamine at 0.8 O.D./ml. thus indicating a high level of ROS production.

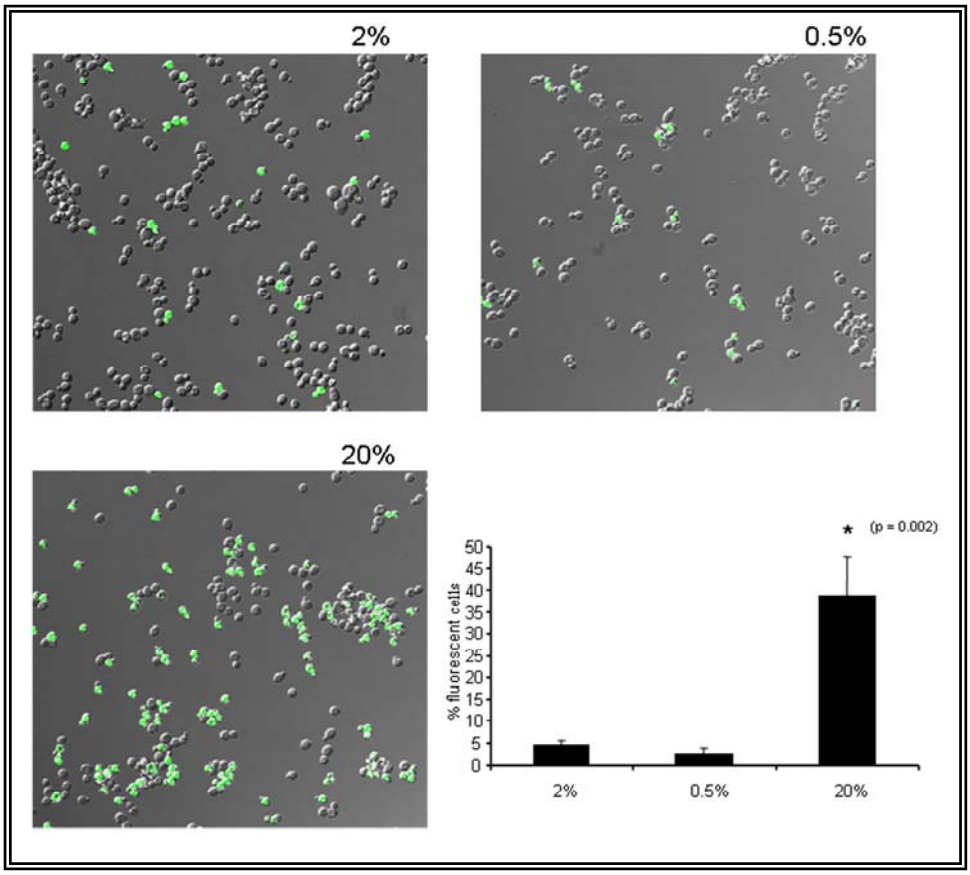


Fig. 2. *ROS production, evaluated using the DHR123 fluorescent probe in cells grown exponentially (0.8 O.D./ml) on different glucose concentrations: figures represent a merge between DIC and fluorescent images obtained with a 40X objective. In the histogram, the percentage of fluorescent cells stained with DHR123 at the cellular density of 0.8 O.D./ml is shown. A two-tailed non-paired Student's t-test was performed using ORIGIN 6.0 (Microcal Software, Inc.) to determine if the relative change in the conditions of 0.5% and 20% glucose was statistically significant in comparison to the control cells. Differences were considered statistically significant when p < 0.01 (*).*

On the contrary, the percentage of fluorescent cells at the same growth point is less than 10% in 0.5% and 2% glucose. Considering that intracellular levels of ROS depend on the balance between their production and scavenging, the high level of ROS production in cells grown in 20% glucose indicates an alteration of this equilibrium.

3.3.3 Proteomic analysis.

To investigate *S. cerevisiae* protein expression during exponential growth under different glucose concentrations we compared the proteomes of yeast grown in 0.5% and 20% to that grown in 2% glucose (as control). Yeast cells were harvested during exponential phase (0.8 O.D./ml) and proteins were extracted and resolved by two-dimensional SDS-PAGE (2D-GE). Figure 3 shows typical 2D-GE images of soluble proteins from yeast cells grown in 2% (control cells, panel a), 0.5% (panel b), and 20 % (panel c) glucose concentration. The pattern of protein distribution and relative abundance displays corresponds well with yeast 2D-GE protein profiles produced previously by our laboratory [Magherini *et al.*, 2007] and to a 2D-GE *S. cerevisiae* reference gel available on the internet (www.expasy.org).

An average of about 1000 spots is detected in each 2D-GE gel stained with Coomassie. The computer analysis points out a total of 156 protein spots changing significantly. Of these protein spots, 82 are differentially expressed (quantitative difference) whereas 74 are not detected in all the analyzed strains (qualitative difference). The general pattern of migration is maintained between replicate gels, thus indicating the high degree of reproducibility of sample preparation and of 2D-GE procedures.

MALDI-TOF mass spectrometry was used to identify proteins of interest. It is important to remember that proteins are affected by post-translational regulation and that, although we characterized only the spots with different abundance levels, we found several isoforms of proteins. The results reported in this study indicate that, during exponential phase, there are significant alterations in protein expression profile of cells grown on different glucose concentrations.

Identification of differentially expressed proteins (quantitative difference).

A total of 21 differentially expressed proteins was successfully identified by MS analysis. The locations of these spots are marked with circles and capital letters in the representative gels in Figure 3 (panels a, b and c). Fourteen spots in cells grown in 0.5% glucose (spots A, C, E, H, I, J, M, O, P, Q, R, S, T and U) and 12 in cells grown in 20% glucose (spots C, E, F, I, J, K, L, M, N, P, Q, and R) are down-regulated in comparison to cells grown in 2% glucose. Of these: 8 spots (C, E, I, J, M, P, Q and R) show a down-regulation in both 0.5% and 20% glucose *versus* control cells. Nine spots are up-regulated (spots A, B, D, O, G, H, S, T and U) in 20% glucose grown cells in comparison to control cells. Spot G, corresponding to Peroxiredoxin TSA1, is up-regulated in both 0.5% and 20% glucose in comparison to the control. This protein is involved in protection against oxidative stress and its expression depends on nutrient availability or on stress conditions. Peroxiredoxin belongs to a set of stress defence proteins known as "environmental stress response", thus suggesting the importance a redox control complex has in stress conditions [Wong *et al.*, 2003].

In 0.5% glucose grown cells 6 spots (spots B, D, F, K, N and L) show no variation in comparison to the control cells. This suggests that an increase in the expression level of a number of specific proteins is a dominant phenomenon in cells grown in high glucose concentration. The identities of the found proteins are summarized in Table 2. Some differentially expressed proteins are present in multiple forms, thus suggests post-translational modification and/or the proteolysis of the corresponding native proteins. We identified 3 spots (spots A, B and C) corresponding to Pyruvate decarboxylase 1 (Pdc-1p); 2 spots corresponding to Enolase 2 (spots O and N); 6 spots (S, T, U and P, Q, R) corresponding to Glyceraldehyde-3-phosphate dehydrogenase 3; 2 spots that correspond to Alcohol dehydrogenase 1 (Adh-1p) (spots K and L) and finally 2 spots (E and F) that correspond to S-adenosylmethionine synthetase-2. Proteins with significantly decreased molecular masses in our 2D gels are a sign of protein fragmentation.

Identification of qualitative differences

A computer analysis pointed out a total of 40 qualitative spot variations between control cells and cells grown in 0.5% and 20% glucose. These spots were all identified by MS analysis. The data are summarized in Table 3. The location of the spots is marked with circles and numbers in the representative gels shown in Figure 3 (panels a, b, and c). Seven spots (spots 1 to 7; Figure 3a) are detected exclusively in the control cells and 7 spots (spots 8 to 14; Figures 3a and 3b) are detected exclusively in the 20% glucose yeast cells. Seven spots (spots 15 to 21; Figure 3c) are detected both in the control cells and in the 0.5% glucose yeast cells whilst 19 spots (spots 22 to 40; Figures 3a and 3c) are detected both in the control cells and in the 20% glucose yeast cells.

- **Proteins detected exclusively in 2% glucose.**

Seven proteins are detected exclusively in cells grown in 2% glucose. Of these, a majority is involved in cell's glucose metabolism and protein synthesis (Figure 3a and Table 3). Among these we identified the Enolase 2 enzyme (spot 1). This enzyme catalyses the first common step of glycolysis and gluconeogenesis; its expression is glucose induced. We also identify Pyruvate kinase1 (Pyk-1, spot 3), catalyst in the final step of glycolysis. At this glucose concentration we identified 2 more spots (spots 6 and 7) as Bmh1 and Bmh2 which are the yeast members of the highly conserved 14-3-3 protein family present in all eukaryotic organisms.

These two proteins are involved in cells growth control, in post-transcriptional regulation and in the regulation of many processes including exocytosis, vesicle transport, Ras/MAPK signalling (Kakiuchi *et al.*, 2007).

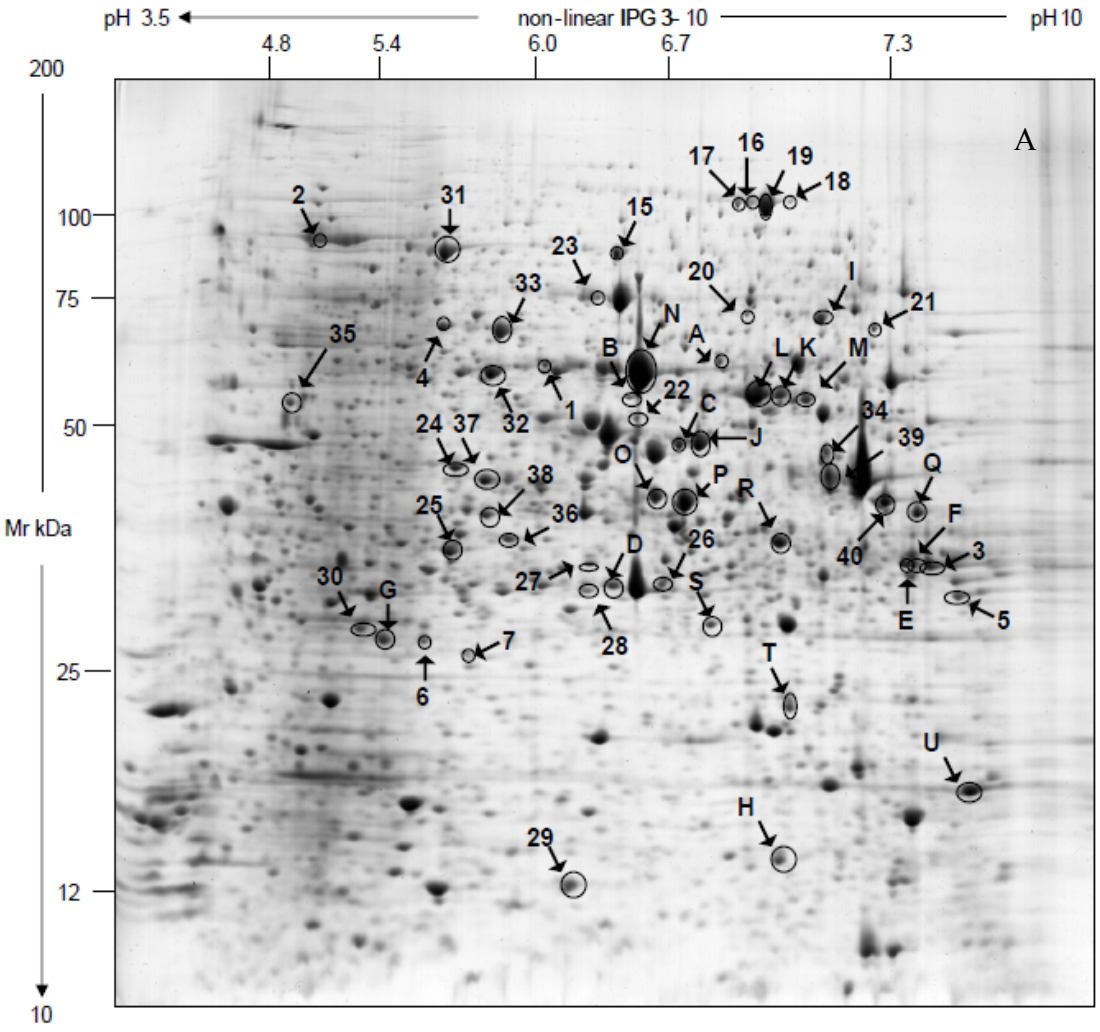
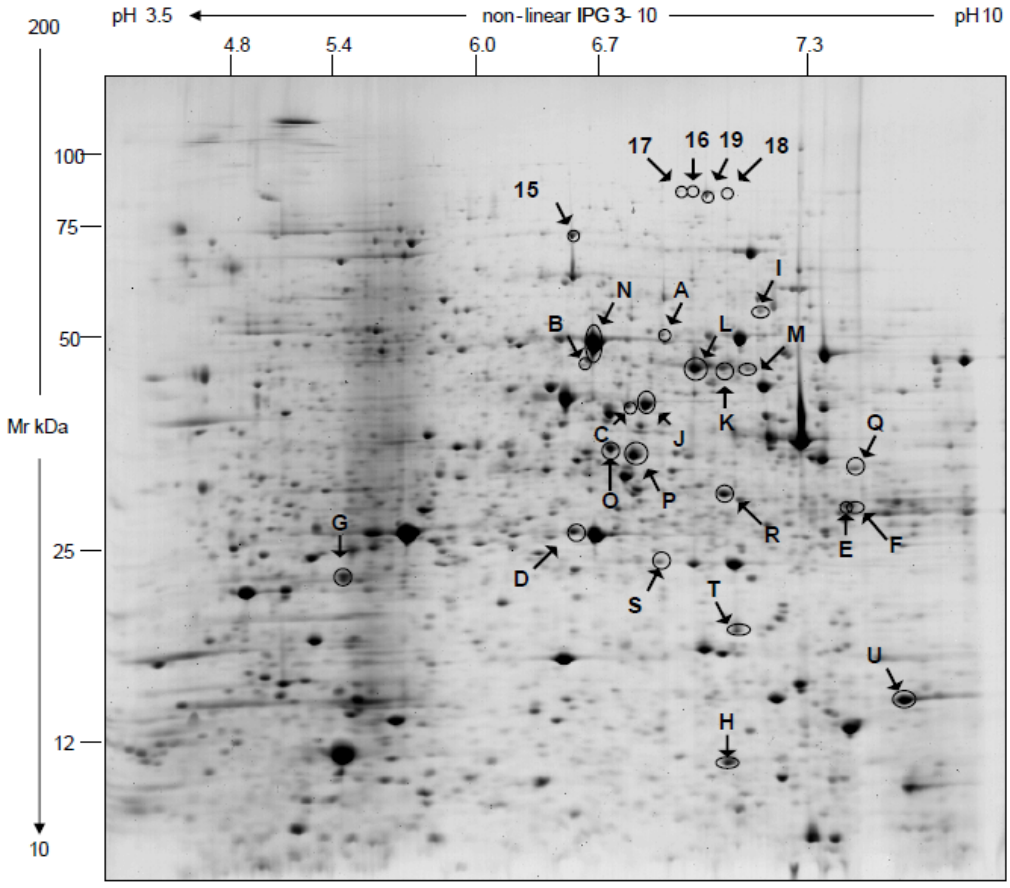


Fig. 3. *Representative Blue Coomassie 2D gels of total protein extract from cells grown exponentially in 2% glucose (panel A), 0.5% glucose (panel B) and 20% glucose (panel C). Quantitative and qualitative variations are displayed with circles. Quantitative variations between cells grown in 0.5% glucose (panel B, next page) and 20% glucose (panel C, next parge) vs 2% glucose (panel A) are indicated by capital letters. They are listed in Table 2. Qualitative variations identified are displayed with numbers and represent conditions described in Sections 3.5. They are listed in Table 3.*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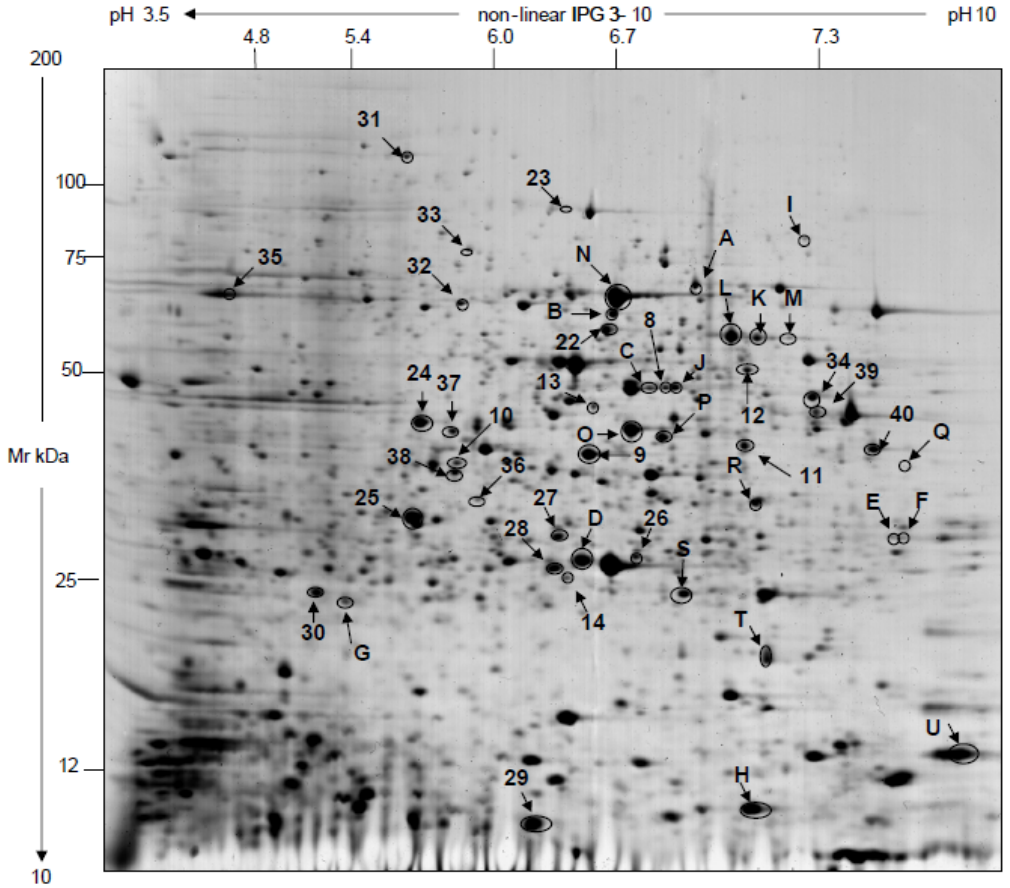
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- **Proteins detected exclusively in 20% glucose.**

The 7 spots detected exclusively in yeast cells during exponential growth in 20% glucose are listed in Table 3. The locations of the identified proteins are marked with circles and numbers in the representative gel shown in Figure 3c. Some of these proteins are present in multiple forms. We found the enzyme Phosphoglycerate kinase (spot 8) expressed in 20% glucose; this is a glycolitic/gluconeogenic enzyme. When cells produce a high amount of ethanol, they use this metabolite as a carbon source and they convert it into glucose through gluconeogenic pathway. We also identified 2 spots of the Pyruvate decarboxylase 1 enzyme (spots 10 and 11). Pyruvate decarboxylase 1 is the first enzyme of the fermentation pathway and its expression in cells grown in 20% glucose agrees with the high rate fermentation that we evaluated in these cells. Furthermore we saw that the translation protein Elongation factor 2 (spot 13) and the ATP-dependent RNA helicase (spot 14) which is involved in mitochondrial splicing and also required for efficient mitochondrial translation, are expressed exclusively in 20% glucose.

- **Proteins exclusively detected both in 2%glucose and 0.5% glucose.**

Among the 7 protein spots, whose expression during the exponential growth resulted detectable exclusively both in 2% and in 0.5% glucose (Figures 3a and 3b), is the 5-methyltetrahydropteroyl-triglutamate-homocysteine-methyltransferase which is present in multiple forms. We identified, in fact, 4 spots (spots 16, 17, 18 and 19) that correspond to this enzyme which is involved in amino acids metabolism. We also identified the Poly-(A)-binding protein (spot 15), which is part of the 3'-end RNA-processing complex and interacts with translation factor eIF-4G, and the Alpha subunit of mitochondrial F1F0 ATP synthase (spot 21), which is a large, evolutionarily conserved enzyme complex required for ATP synthesis.

- **Proteins exclusively detected both in 2% glucose and 20%glucose.**

Of the 18 proteins (Figures 3a and 3c) whose expression results inhibited in 0.5% glucose we identified several proteins related to glycolysis and alcoholic fermentation. Two spots (spots 22 and 23) correspond to the enzyme Pyruvate decarboxylase 1. One spot correspondsto Phosphoglycerate kinase (spot 34), a key enzyme in glycolysis. Two spots (spots 24 and 25) correspond to Fructose 1,6-bisphosphate aldolase, which is required for glycolysis and 2 spots correspond to Enolase 2 (spots 27 and 28) whose expression is glucose-induced. We also identified 2 spots corresponding to Glyceraldehyde-3-phosphate dehydrogenase 3 (spots 39 and 40). Among the proteins detected in 0.5% glucose we identified a protein involved in the synthesis of amino acids: 5-methyltetrahydropteroyltriglutamate-homocysteine-methyltransferase. Moreover, we found the cytoplasmatic and nuclear Poly(A)-binding protein involved in the regulation of peptide formation on ribosomes. In our study the naturally-induced diauxic shift resulted in an induction of the global protein synthesis. This, in turn, allows the *de novo* biosynthesis of functional mitochondria. Glucose restriction also induces the synthesis of a few proteins necessary for respiratory growth such as the mitochondrial ATP synthase subunit alpha that produces mitochondrial ATP This confirmed the induction of respiration during the diauxic shift.

In the current study we detected that, during the first hours of fermentation, in yeast cells is increased the expression of proteins involved in response to oxidative stress. In particular, we found two members of the heat shock protein 70 family (HSP70) which are stress-induced and were not detected 0.5% glucose: Ssa2p and Ssb1p. These stress response proteins can indicate a better adaptation during fermentation and be important for maintaining the viability of cells during fermentative conditions. In our study we see that, in high glucose, the percentage of glucose used for fermentation is low compared to total glucose consumption and that the ethanol production is inhibited. We deduced that glucose was also used to produce other molecules, such as glycerol. Further evidence was observed, since Adh1p, an enzyme related to ethanol production, was down-regulated. Moreover, we found an increase in the expression of many proteins involved in glycerol biosynthesis. We determined that DL-glycerol-3-phosphatase1 (involved in glycerol

biosynthesis) was expressed exclusively in this condition. This is induced in response to osmotic stress. These results confirmed the theory according to whom *S. cerevisiae* accelerates its production of glycerol and acidic compounds rather than synthesizing ethanol to minimize the effects of the high osmotic conditions generated by a high glucose concentration and ethanol production [33 Pham, 2006]. We can conclude that yeast cells in high glucose concentration have a decreased growth rate during the initial phase of fermentation, because part of their metabolism is occupied in the synthesis of compounds to resist osmotic stress.

Table2: Relative protein expression changes of 2% glucose *versus* 0.5% glucose and 20% glucose.

Spot N°	Protein name	AC [a]	Score [d]	N° of matching peptide [e]	Sequence coverage [f] (%)	%V(x10^{-4}) mean (±SD)[g]			Fold change (i)	
						Glucose2%	Glucose0.5%	Glucose20%	2% / 0.5%	2% / 20%
A	Pyruvate decarboxylase isozyme 1	P06169	102	8	22	998 ± 74	521 ± 37[c]	1891 ± 463	1.9	0.52
B	Pyruvate decarboxylase isozyme 1	P06169	100	6	25	554 ± 141	733 ± 96	1916 ± 6[b]	0.75	0.29
C	Pyruvate decarboxylase isozyme 1	P06169	102	8	22	2845 ± 90	1037 ± 142	1527 ± 170[b]	2.7	1.9
D	Adenylate kinase cytosolic	P07170	59	6	31	3681 ± 1870	3560 ± 70[b]	7763 ± 472	1	0.47
E	S-adenosylmethionine synthetase 2	P19358	86	11	33	2493 ± 280	1270 ± 220	331 ± 20[b]	1.9	7.5
F	S-adenosylmethionine synthetase 2	P19358	89	12	35	1373 ± 408	1027 ± 113	603 ± 50	1.3	2.2
G	Peroxiredoxin TSA1	P34760	104	8	51	436 ± 244	1820 ± 80[c]	1063 ± 85	0.23	0.41
H	Peptidyl-prolyl cis-trans isomerase	P14832	83	9	43	2042 ± 274	920 ± 32[b]	9392 ± 1100	2.2	0.21
I	Cystathionine beta-synthase	P32582	93	15	34	2908 ± 107	1280 ± 120	25 ± 12[b]	2.3	11.8
J	Phosphoglycerate kinase	P00560	111	13	42	9666 ± 3460	3515 ± 25	2074 ± 43	2.7	4.7
K	Alcohol dehydrogenase 1	P00330	104	12	47	3058 ± 682	3605 ± 1025	1629 ± 169	0.84	1.9
L	Alcohol dehydrogenase 1	P00330	111	13	50	16641 ± 117	17850 ± 350	5548 ± 348[b]	0.93	2.9
M	Heat shock protein homolog SSE1	P32589	66	10	19	1940 ± 1	788 ± 98	331 ± 15[b]	2.4	6
N	Enolase 2	P00925	150	20	58	46594 ± 8776	52400 ± 10400	24660 ± 5927	0.88	1.9
O	Enolase 2	P00925	148	17	51	4884 ± 401	1375 ± 115	8955 ± 379[b]	3.5	0.54
P	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	69	9	39	11174 ± 813	3125 ± 315	2601 ± 145[b]	3.5	4.3
Q	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	68	9	40	2268 ± 588	540 ± 15	102 ± 82[c]	4.2	22.2
R	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	70	10	43	5383 ± 598	1690 ± 80[c]	1576 ± 79	3.2	3.4
S	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	65	7	42	1331 ± 40	341 ± 97	2504 ± 134	3.9	0.53
T	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	68	9	40	2195 ± 193	840 ± 115	3638 ± 268[c]	2.6	0.6
U	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	70	11	37	6042 ± 1453	1725 ± 125[b]	16573 ± 1	3.5	0.36

a) Swiss-Prot/TrEMBL accession number.
b) p-Value 6 <0.01.
c) p-Value 6 <0.05.
d) MASCOTscore (Matrix Science, London, UK; http://www.matrixscience.com).
e) Number of peptide masses matching the top hit from Ms-Fit PMF.
f) Percentage of amino acid sequence coverage of matched peptides in the identified proteins.
g) Each value represents the mean 6 SD of individually computed %*V* (*V* = integration of OD over the spot area;%*V* = *V* single spot/*V* total
spots) in three different gels of control, 0.5% and 20%.
i) Fold change (control *vs* 0.5% and control *vs* 20% glucose) was calculated dividing %*V* from control by the %*V* from 0.5% glucose and from 20% glucose.

Table 3. MS identification of qualitative differences.

Spot Nº	Protein name	AC [a]	Score [b]	Nº of matching peptide [c]	Sequence coverage [d] (%)
Proteins detected exclusively in Glucose 2%					
1	Enolase 2	P00925	148	17	51
2	HSP82	P10591	47	7	14
3	Pyruvate kinase 1	P00549	67	6	19
4	Eukaryotic translation initiation factor 4B	P34167	98	10	21
5	Ketol-acid reductoisomerase, mitochondrial precursor	P06168	63	8	31
6	Protein BMH1	P29311	63	6	31
7	Protein BMH2	P34730	72	5	27
Proteins detected exclusively in Glucose 20%					
8	Phosphoglycerate kinase	P00560	148	17	51
9	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	68	9	40
10	Pyruvate decarboxylase isozyme 1	P06169	102	8	22
11					
12	YNN4	P53912	56	6	25
13	Elongation factor 2	P32324	62	10	12
14	ATP-dependent RNA helicase MSS116, mitochondrial precursor	P15424	41	5	10
Proteins detected exclusively in Glucose 2% and Glucose 0.5%					
15	Polyadenylate-binding protein, cytoplasmic and nuclear (Poly(A) binding protein) (PABP)	P04147	64	7	18
16					
17	5methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	P05694	70	9	14
18					
19					
20	GMP synthase [glutamine-hydrolyzing]	P38625	55	7	14
21	ATP synthase subunit alpha, mitochondrial precursor	P07251	91	14	26
Proteins detected exclusively in Glucose 2% and Glucose 20%					
22	Pyruvate decarboxylase isozyme 1	P06169	102	8	22
23	Pyruvate decarboxylase isozyme 1	P06169	100	6	20
24	Fructose-bisphosphate aldolase	P14540	94	8	50
25	Fructose-bisphosphate aldolase	P14540	56	5	23
26	Adenylate kinase cytosolic	P07170	59	6	31
27	Enolase 2	P00925	150	20	58
28	Enolase 2	P00925	150	20	58
29	Enolase 2 fragment				
30	Heat shock protein SSA2	P10592	74	12	23
31	Heat shock protein SSB1	P11484	93	17	35
32	Heat shock protein SSB1	P11484	80	15	30
33	NADP-specific glutamate dehydrogenase 2	P39708	68	9	27
34	Phosphoglycerate kinase	P00560	117	13	42
35	Small glutamine-rich tetratricopeptide repeat-containing protein 2	Q12118	65	7	29
36	(DL)-glycerol-3-phosphatase 1	P41277	78	7	35
37	Inorganic pyrophosphatase	P00817	94	14	57
38	NADPH dehydrogenase2	Q03558	88	10	34
39	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	117	12	53
40	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	68	9	40

a) Swiss-Prot/TrEMBL accession number.
b) MASCOTscore (Matrix Science, London, UK; http://www.matrixscience.com).
c) Number of peptide masses matching the top hit from Ms-Fit PMF.
d) Percentage of amino acid sequence coverage of matched peptides in the identified proteins.

3.4 Conclusions

The information obtained in our study validates the application of a proteomic approach for the identification of the molecular bases of environmental variations such as fermentation in high glucose and during a naturally-induced diauxic shift. Identifying the functionally modulated proteins involved in glucose induced yeast response, will lead to a better comprehension of the mechanisms underlying the effects of different glucose concentrations and will contribute to the complete understanding of yeast fermentation and respiration metabolism.

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Chapter 4

"Plasma proteins carbonylation and physical exercise"

4. Abstract

Physical activity, following an increase in oxygen consumption, leads to a temporary imbalance between the production of RONS and their disposal; this phenomenon is called oxidative stress. Proteins are one of the most important targets of oxidation during physical exercise and carbonylation is one of the more common oxidative protein modifications. In cells a regulated level of oxidized proteins exists and this doesn't interference with cell function; however, an increase in oxidized protein levels may cause a series of cellular malfunctions that could lead to a disease state. For this reason a quantification of protein oxidation is of major importance to distinguish healthy and disease states.

The aim of our research was to characterize plasma proteins carbonylated in response to physical exercise in men trained to perform endurance exercise and in men practising sport for recreational purposes. It is widely accepted the increase of carbonylated proteins in the plasma of athletes after exercise, but in literature there aren't works in which the targets of this oxidation are identified. We analyzed the plasma taken at resting condition and after two different kinds of PE by a proteomic approach using 2D-GE followed by western blot with specific antibodies against marked carbonylated proteins. We noticed that "endurance hard trained" athletes showed a higher carbonylation of plasma proteins in comparison to men practising various kind of sports with a moderate training. From the 2D analysis we found proteins target of carbonylation after physical exercise, but also proteins which carbonylation is not affected by exercise and proteins carbonylated only at resting condition.

These methods have allowed to obtain an overview of the change in the oxidation of plasma proteins after physical exercise and to identify new markers of physiological stress.

4.1 Introduction

Physical activity is defined as any body movement induced by skeletal muscle which leads to an excess energy expenditure compared to the condition of rest. There is a great interest in investigating the effects of a regular physical activity on the human health and physiology.

Medicine recognizes that physical activity plays an essential role in ensuring good health. This depends on the fact that a physically active life induces organic changes and modifications that are positive for the proper functioning of organs and apparatuses. We know that a steady exercise causes an adaptive response that allows the improvement of sports performance [Davies *et al.*, 1981]. These adjustments include an increase in the size and number of mitochondria in skeletal muscle and an altered expression of several genes. These changes are relatively stable over time and are effective only when sport is practiced on a regular basis. In fact, proper practice of sport leads to cardiovascular protection, reduction of the risk of obesity and of diabetes, reinforcement of the joints, hormonal control, delay of aging.

On the contrary physical inactivity, which has general implications for the entire body, is a risk factor for health. The physical inactivity has an important part in the development of obesity, dyslipidemia and hypertension, and it is also one of the determining factors in the genesis of coronary diseases.

However physical activity, following an increase in oxygen consumption, leads to a temporary imbalance between the production of toxic substances for the body (reactive oxygen/nitrogen species (RONS)) and their disposal [Davies *et al.*, 1982; Ashton *et al.*, 1998; 1999; Bailey *et al.*, 2004; 2007; Groussard *et al.*, 2003; Ogonovszky *et al.*, 2005]. The generation of RONS occurs regularly as part of normal cellular metabolism and is increased under conditions of physical stress. RONS generate as a result of electron leakage from the mitochondrial respiratory chain or other physiological and non-physiological processes. The body's antioxidant defence system serves to protect the cells from the excess in RONS production. This system comprises both endogenous (bilirubin, uric acid, superoxide dismutases,

catalase, glutathione peroxidase, etc.) and exogenous (carotenoids, tocopherols, ascorbate, bioflavonoids, etc.) compounds [Urso *et al.*, 2003]. The exogenous compounds are consumed in the diet and come primarily from ingestion of fruits and vegetables [Watson *et al.*, 2005].

Physical activity leads to a temporary imbalance between the production of free radicals and their disposal, and this phenomenon is called *oxidative stress* [Finaud *et al.*, 2006]. There is a great deal of controversy over whether or not *oxidative stress* and subsequent damage are truly associated with exercise. In the past, exercise studies have varied in the intensity, duration, and mode of activity chosen for the study model. Additionally, variations in the fitness levels of subjects and assays used to assess oxidative damage have contributed to the inconsistent findings. Together these factors have contributed to the lack of consensus regarding exercise-induced oxidative stress. Since the initial finding of an increase in lipid peroxidation following acute aerobic exercise in 1978 [Dillard *et al.*, 1978], the research about of oxidative stress and exercise has expanded substantially. This increased interest is fuelled by several factors, including the enhanced awareness of the role of RONS in human disease, as well as the widespread development and availability of various antioxidant agents (of which efficacy is often tested using exercise as a stimulus of RONS). Although much of the early work has viewed exercise-induced RONS production as a potential detriment to physiological function (i.e., decreased performance and immune function, and increased fatigue), more recent work is investigating an alternative role for RONS production in regards to favourable exercise-induced adaptations.

An excessive accumulation of free RONS causes oxidation of proteins, lipids and nucleic acids; the oxidation modifies these macromolecules and can damage them [Alessio *et al.*, 1988; Davies *et al.*, 1982; Gomez-Cabrera *et al.*, 2006; Ikeda *et al.*, 2006; Ji *et al.*, 2006; Mahoney *et al.*, 2005; Paroo *et al.*, 2002; Poulsen *et al.*, 1998; Russell *et al.*, 2005; Halliwell B, *et al.*, 1994].

Proteins are one of the most important targets of oxidation, based on their high concentration in cells. Among the many possible oxidative modification of proteins,

irreversible introduction of carbonyl groups is one of the more common [Dalle-Donne *et al.*, 2006].

Proteins carbonylation is a post-translational modification which can be obtained through various reactions and consists in the addition of carbonyl groups to protein structure, especially the aminoacidical residues of proline, arginine and lysine. Protein carbonyls can be generated directly as a result of amino acids side chain oxidation and protein backbone cleavage or indirectly, by Michael addition of 4-hydroxy-2-nonenal to protein lysine, histidine or cysteine residues or reaction of protein ammonium substituent with lipid peroxidation products.

In cells a regulated level of oxidized proteins exists and this doesn't interference with cell function even if reactive oxygen species are continuously generated both intracellular and exogenously. However, an increase in oxidized protein levels may cause a series of cellular malfunctions that could lead to a disease state. For this reason a quantification of protein oxidation is of major importance to distinguish healthy and disease states. As one of the most common features of oxidative damage, protein carbonyls have been used as a marker for identification and quantification of protein damage [Lin *et al.*, 2009; Lamprecht *et al.*, 2008; Bloomer *et al.*, 2007]. For identification and quantification of oxidized proteins carbonylated peptides have been a major target. In fact, protein carbonyl groups have been quantified in several ways. One is by derivatization with 2,4-dinitrophenyhydrazine or tritiated borohydride followed by quantification with UV spectroscopy or radiography [Mirzaei *et al.*, 2006].

The sport activity in our body leads to an increase in endogenous defences against this type of stress, therefore reducing the damage. Several manufacturers of supplements focus on sales of antioxidants to reduce the free radicals production, thereby facilitating the recovery between workouts or competitions. However, there are currently significant limitations of the knowledge of the relationship between performance, supplements and oxidative stress [Bloomer *et al.*, 2006; Margaritis *et al.*, 2008; Khassaf *et al.*, 2003].

In this study we focused on carbonylation of plasma proteins and its modification during the physical exercise (PE in this work). Plasma is the biological sample for excellence; it contains the highest concentration of different proteins than any other type of sample.

Unlike other tissues, plasma proteome is extremely complex, because it contains specific proteins of the blood and also proteins released from other tissues as a result of leakage, damage or other factors [Anderson *et al.*, 2002]. In medicine plasma proteins have been widely used to obtain useful information on physiopathology, to define the various clinical cases in a uniform way and to reach an early diagnosis for many diseases. In particular, plasma proteins have been widely used as a useful alarm bell, so that an altered expression of some of them may be considered the key to early diagnosis of certain diseases.

It is known that endurance training can cause damage to the active muscle, as indicated by increased release of muscular enzymes into the plasma, the ultrastructural disruption of the sarcomere [Maxwell *et al.,* 1993] and a substantial impairment in maximal torque production [Millet *et al.,* 2003]. The practical implications of this damage have been reviewed [Warren *et al.,* 2001] and include decreased joint range of motion, increased fatigability, decreased shortening velocity, and prolonged strength loss. Evidence that oxidative damage by reactive oxygen species (ROS) mediates skeletal muscle damage is accumulating [Maxwell *et al.,* 1993].

The aim of our research was to characterize plasma proteins that undergo carbonylation in response to physical exercise in men trained to perform endurance exercise. It is widely accepted the increase of carbonylated proteins in the plasma of athletes after exercise [Bloomer *et al.,*2007, Nikolaidis *et al.*, 2007], but in literature there aren't works in which the targets of this oxidation are identified. To study the carbonylation target proteins we analyzed the plasma of athletes taken at resting condition and after two different kinds of PE by a proteomic approach using two-dimensional electrophoresis followed by western blot with specific antibodies against marked carbonylated proteins. We then analyzed the two-dimensional images identifying several proteins that are targets of carbonylation.

By proteomic analysis we found not only proteins that are target of carbonylation after physical exercise, but also proteins which carbonylation is not affected by exercise and also proteins that are carbonylated only in the plasma of the resting condition. These methods have allowed to obtain an overview of the change in the oxidation of plasma proteins after physical exercise and to identify new markers of physiological stress.

4.2 Materials and methods

4.2.1 Subjects

To our research 19 (8 endurance athletes and 11 recreational athletes) volunteers subjects cooperated. These men volunteered to participate following explanation of all experimental procedures. A medical history and physical activity questionnaire were completed by all subjects in order to determine eligibility. No subject used antioxidant supplements. Plasma samples of all subjects were taken at rest condition and after an hour of run. Eight endurance trained healthy volunteers were recruited among athletes practising endurance activity in Siena. Mean age was 48 ± 10 years; weight was 70 ± 12 kg. They averaged 60 ± 12 Km training/week (see Tab I). Eleven recreational athletes volunteers were recruited among athletes practising various kinds of physical activity in Florence. Mean age was 45 ± 5 years; weight was 73 ± 8 kg. Subjects were selected on the basis of non-smoking status, age, stable body weight, and maintenance of regular exercise patterns. Subjects were excluded if they were antioxidant supplement users (vitamin C, vitamin E, selenium, or carotenoids). Subjects with previous personal history of cardiovascular disease, diabetes mellitus, dyslipemia, physical disability, or chronic respiratory disease, as well as those with a body mass index (BMI) over 30 kg/m^2, alcohol consumption greater than 40 g per day, or long-term medication use, including mineral or vitamin supplements, were excluded. The local research ethics committee approved the protocol and all participants provided informed consent.

4.2.2 Physical fitness assessment

The endurance trained participants followed an individualized and supervised training program for 2 weeks before the running tests. Subjects recorded on a daily basis the type, duration, and intensity of exercise performed each day. The training program counted total of 112 Km of run, 48 Km in the first week and 64 Km during the second week. After this training period the athletes performed two different running test: an "aerobic resistance test", in which they made a run of 12 Km at a constant speed but 5% -10% under their aerobic threshold and an "aerobic power

test", in which they made five runs of 1000 meters with speed 2%-5% above their aerobic threshold. The recreational athletes followed their normal physical activity during the two weeks before the test without a common training program or a specific diet.

4.2.3 Diet

The endurance trained subjects consumed a specified diet for 15 d before the two running tests. The daily diet provided ~2500 Kcal and consisted of approximately 20% protein, 60% carbohydrate, and 20% fat. Nutrient composition of the specified diets was determined using an Excel Program.

4.2.4 Handling of plasma samples (Blood analysis)

A 5 mL blood sample from each athlete was taken via vacutainer from an antecubital vein by a medical staff at resting condition and after each test (aerobic and anaerobic) following a 10 min quiet rest. Blood samples were analyzed for whole blood lactate using an Accutrend portable lactate analyzer (Roche Diagnostics, Mannheim, Germany). The remainder of whole blood was immediately separated to plasma and stored in several aliquots in liquid nitrogen to be used for the measurement of oxidative stress biomarkers. Protein carbonyls were analyzed using an ELISA according to the procedures recommended by the manufacturer (kit Protein Carbonyl Assay, Cayman). Assays were performed in duplicate on first thaw.

4.2.5 Albumin and IgG depletion from plasma samples

The deep frozen crude plasma samples were returned to 4 ◦C. Using the commercially available ion exchange based Blue Albumin and IgG Depletion kit (Sigma), 50 μL of crude plasma was diluted with 150 μL of equilibration buffer (kit reagent) before application to the column. The protocol was executed as described in the Blue Albumin and IgG Depletion Kit user guide. The flow-through fraction contained the albumin depleted plasma.

4.2.6 Two-dimensional polyacrylamide gel electrophoresis

The frozen albumin and IgG-depleted plasma samples were solubilised in a lysis buffer containing 8M urea, 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM dithiothreitol (DTT) and 2.0% 4–7 non-linear immobilized pH gradient (IPG) buffer (Amersham Bioscience, Uppsala, Sweden). For each experimental condition at least three samples were run in order to assess biological and analytical variation. Isoelectrofocusing (IEF) was carried out on nonlinear wide-range immobilized pH gradients (pH 4-7; 7 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using the EttanTM IPGphorTM system (GE Healthcare, Uppsala, Sweden). MS-Preparative-run IPG-strips were rehydrated at 16°C with 150 μg of proteins in 125 μl of lysis buffer and 0.2% carrier ampholyte and focused at 16°C according to the following electrical conditions: for 12h at 30V and for 0.30h at 500V, from 500 V to 1000 V in 30 min, from 1000 V to 5000 V in 1,30h, 5000 V until a total of 18000 Vh was reached. After focusing MS-preparative IPG strips were equilibrated for 12 min in 6 M urea, 30% glycerol, 2% Sodium Dodecyl Sulfate (SDS), 0.05 M Tris-HCl, pH 6.8, 2% DTT, and subsequently for 5 min in the same urea/SDS/Tris buffer solution but substituting the 2% DTT with 2.5% iodoacetamide. The second dimension was carried out on 9-16% polyacrylamide linear gradient gels (18 cm x 20 cm x 1.5 mm) at 40 mA/gel constant current and 10°C until the dye front reached the bottom of the gel. The MS-preparative gels were stained with colloidal Coomassie [Vilain *et al.*, 2001].

4.2.7 Derivatization of protein carbonyls and DNP immunostaining

Following plasma sample rehydration and IEF, the IPG strips used for carbonylation analysis were placed in 10mL tubes and incubated in 2N HCl with 10mM DNPH (2,4-dinitrophenylhydrazine, Sigma, St. Louis, MO) at 25 ◦C for 20 min. Following the incubation enabling protein-bound carbonyls derivatization, the marked IPG strips were washed with 6M Urea, 20% Glycerol, 1% SDS, 150mM Tris-HCl pH 6.8. The marked IPG strips were then prepared for the second dimension, followed by protein blotting to a PVDF membrane as described previously [Korolainen *et al.,* 2007; Reinheckel *et al.,* 2000]. Next, the PVDF membranes were incubated overnight at 4 ◦C for immunostaining with the primary antibody solution consisting of a 1:10.000 dilution of the anti-DNP IgG antibody (Sigma) in the Phosphate-

buffered saline (PBS) containing 3.0% non-fat dry milk. Next, the blots were washed with PBS, 20% Tween and incubated with the goat anti-rabbit IgG/HRP conjugate (1:3.000 dilution in PBS/Milk) for 1 h at room temperature. An enhanced chemiluminescence kit (Immobilon Western Chemiluminescent AP substrate, Millipore) was used for detection. The protein spots were quantified using ImageMaster 2-D Platinum software version 6.0 (GE Healthcare).

4.2.8 Image analysis

Gels and Oxyblots images were acquired with an Epson expression 1680 PRO scanner. Computer-aided 2D image analysis was carried out using ImageMaster 2-D Platinum software version 6.0 (GE Healthcare). Relative spot volume (%V were V=integration of OD over the spot area and were %V = V single spot/V total spots) was used for analysis in order to reduce experimental errors. The intensity of carbonylated spots in the oxyblots was normalized on Coomassie gels.

4.2.9 In-gel enzymatic digestion and MALDI-TOF mass spectrometry

Protein spots were manually excised from the gel, washed with high-purity water and with 50% acetonitrile/water and dehydrated with 100% acetonitrile. The gel slices were swollen at room temperature in 20 µl of 40 mM NH_4HCO_3/10% acetonitrile containing 25 ng/µl trypsin (Trypsin Gold, mass spectrometry grade, Promega). After 1 h, 50 µl of 40 mM NH_4HCO_3/10% acetonitrile were added and digestion proceeded overnight at 37 °C. The generated peptides were than extracted with 50% acetonitrile/5% trifluoroacetic acid (TFA, 2 steps, 20 min each at room temperature), dried by vacuum centrifugation, suspended in 0.1% TFA, passed through micro ZipTip C18 pipette tips (Milllipore, Bedford, MA, U.S.A.) and directly eluted with the MS matrix solution (10 mg/ml $\tilde{\alpha}$ cyano-4-hydroxycinnamic acid in 50% acetonitrile/1% TFA). Mass spectra of the tryptic peptides were obtained using a Voyager-DE MALDI-ToF mass spectrometer (Applied Biosystems). Peptide mass fingerprinting database searching was performed using the MASCOT search engine (http://www.matrixscience.com) in NCBInr/Swiss-Prot databases. Parameters were set to allow one missed cleavage per peptide, a mass tolerance of 0.5 Da and considering carbamido-methylation of cysteines as a fixed modification and oxidation of methionines as a variable modification. The criteria used to accept

identifications included the extent of sequence coverage, the number of matching peptides and the probabilistic score, as detailed in Table II.

4.3 Results and Discussion

4.3.1 Subjects.

The aim of our research was to characterize plasma proteins that undergo carbonylation in response to physical exercise in 8 volunteers subjects trained to perform endurance exercise. These men (called "hard trained" in this work) are used to perform endurance activity for agonistic purposes. They train at least 5 times at week according to a specific training program with a personal trainer who constantly monitors their performance. Furthermore they periodically perform races and marathons. These subjects never use supplements during their training and races. These subjects could be processed to a very controlled performance. Tab I summarizes subjects features. For this work they followed for two weeks before the tests a common training program, a specific diet and the abstention from smoke and drugs. This common treatment let us to have the athletes in the same physical condition for the tests.

The athletes performed two different running tests; an "aerobic resistance test", in which they made a run of 12 Km at a constant speed but 5% -10% under their aerobic threshold. Doing so they had a constant and sustained aerobic effort without lactate accumulation. The second test was an "aerobic power test", in which they made five runs of 1000 meters with speed 2%-5% above their aerobic threshold. Doing so they had a sub maximal and repeated anaerobic efforts with lactate accumulation. We made a blood collection (5 ml) before and after these exercises.

	Weight (kg)	Age (years)	Training (km/week)	Anaerobic treshold (Km/h)	Energy intakes (Kcal/day)
All subjects (n=8)	70 ± 12	48 ± 10	60 ± 12	13 ± 0.5	2549.43

[a]Mean±SE.

Tab. I. *Subjects characteristics*.

4.3.2 Protein carbonylation in the post exercise condition of "hard trained" subject.

In order to have a preliminary determination of total protein carbonyls, we used a kit supplied by Cayman company, which is based on the ability of 2,4-dinitrophenylhydrazine (DNPH) to react with proteins carbonyls, producing the corresponding 2,4-dinitrofenilidrazone, which will be then identified with spectrophotometric methods. We performed the assay on the plasma obtained from all the subjects in order to evaluate a difference in the proteic carbonylation level between their resting condition and their post-exercise condition. The assay demonstrated that the carbonyls levels are subjective for each sample, and no significant differences were found (data not shown). Furthermore, due to the low sensitivity of the method it is not possible to find little differences involving small groups of proteins in the whole plasma assayed. For this reason we used a proteomic approach to study protein carbonylation targets.

We analyzed the plasma before and after exercise in all the subjects. We marked carbonylated proteins with DNPH which is detected by a specific antibody as described in methods. Then we performed the experiments using 2D-GE followed by western blot. Comparison and normalization of plasma protein oxidation levels was obtained by matching the 2D-oxyblots and subsequent Coomassie-stained 2D-gel images from the same sample. It's known that albumin and IgG constitute approximately 85–90% of the total protein mass of crude human plasma [Dayarathna *et al.*, 2008; Chromy *et al.*, 2004; Issaq *et al.*, 2007]. So we depleted plasma samples from Albumin and IgG using a specific kit (see methods). In fact, an increase in the number of lower abundant protein spots may be considered as much more powerful diagnostic biomarkers [Jacobs *et al.*, 2004] and this approach has be implemented after removal of highly abundant proteins. Thus, after the depletion, a relatively large amount of albumin was still contained in the albumin-depleted plasma samples, as is visible in the 2-DE gels given in Fig. 1. However, a perfect and complete removal of albumin from plasma samples would also cause highly distorted proteomic results such as removing the broad range of other low mass and low-abundant

physiologically important regulatory and/or transient proteins which were bound to albumin as the main carrier/transport protein in the blood.

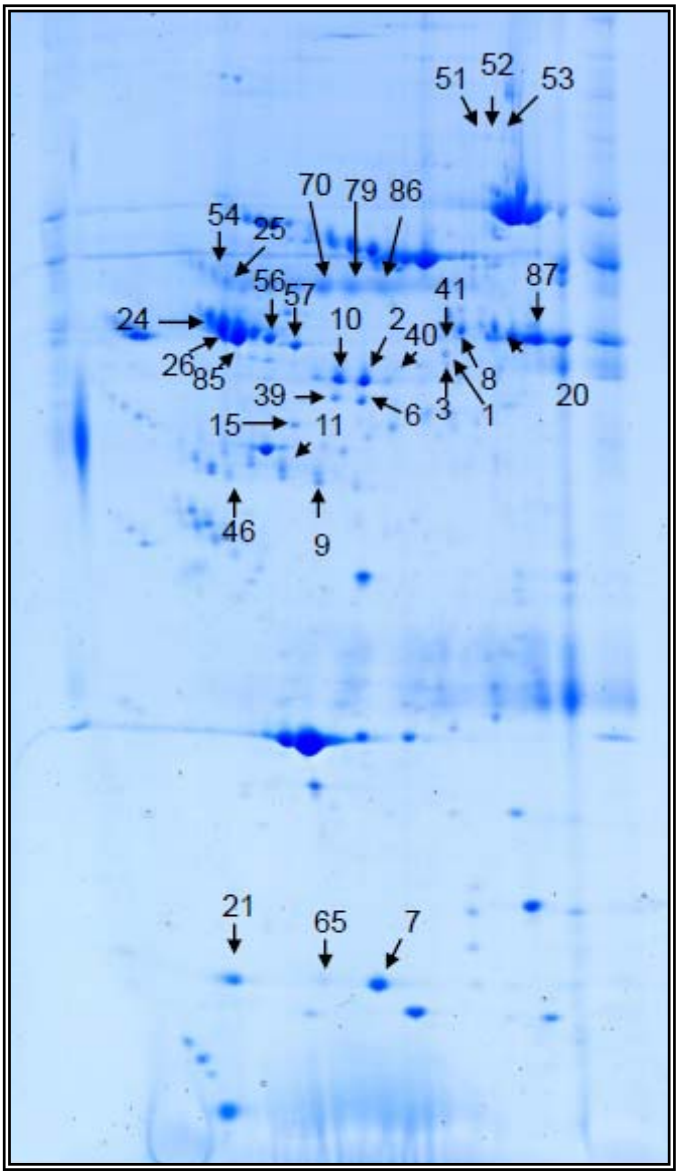


Fig. 1*: Representative Coomassie gel of total plasma proteins. Arrows and numbers indicate the proteins spots target of carbonylation, identified by MS and by matching with plasma proteome map in database.*

To reduce the experimental variability we decided to use 7 cm length strips, collocating on the same gel two strips corresponding to resting-condition plasma and to post-exercise plasma of each subject. Figure 2 shows some representative images of the gels and oxyblots obtained. In panel A and B there are two images

representative of the oxyblots. In each picture the number 1 refers to the resting-condition plasma, while the number 2 relates to the post-exercise plasma. Panel A shows a carbonylation profile of a subject presenting a low level of protein carbonylation at resting condition and an increase after the exercise. Panel B shows a protein carbonylation profile of a subject presenting a high protein carbonylation at resting condition and an increase after the exercise. Panels a and b show the corresponding Coomassie stained gels. We used the same criteria for each subject, analysing all the almost 100 images obtained. In order to verify the experimental reproducibility we also performed several replicates of random samples. Reproducible gel patterns were observed between the replicates.

A total of about 300 protein spots appeared in the Coomassie staining 2-DE gel of albumin/IgG-depleted plasma samples as reported in the representative gel in Fig 1. In order to evaluate the effect of PE on spots oxidation, we compared the intensity values of the spots of the oxyblots signals in the resting condition with those obtained post the PE. From this analysis, we found 31 protein spots, identified by overlapping with the map in databases and confirmed by mass spectrometry, differentially carbonylated in all the samples and in both conditions. These proteins are reported in Tab II and indicated by arrows and numbers in Fig 1.

From our analysis we observed a high individual variability in protein carbonylation, maybe depending on the habits of the single subject and we didn't find any proteins spots changing significantly following the PE in all the subjects. In the figure 3 is shown an example of this individual variability: a particular of the 2D-oxyblots of each sample underlines the different carbonylated states of the spot 2, 10, 6 and 39 (corresponding to various isoforms of beta fibrinogen chain) at the resting condition and after the two different physical exercises. As shown in the figure more spots corresponded to isoforms of the same protein. We noticed that not all the isoforms are carbonylated following the PE in all the subjects. This fact probably means that some isoforms are more susceptible to carbonylation than others; this is maybe due to their structural specific features.

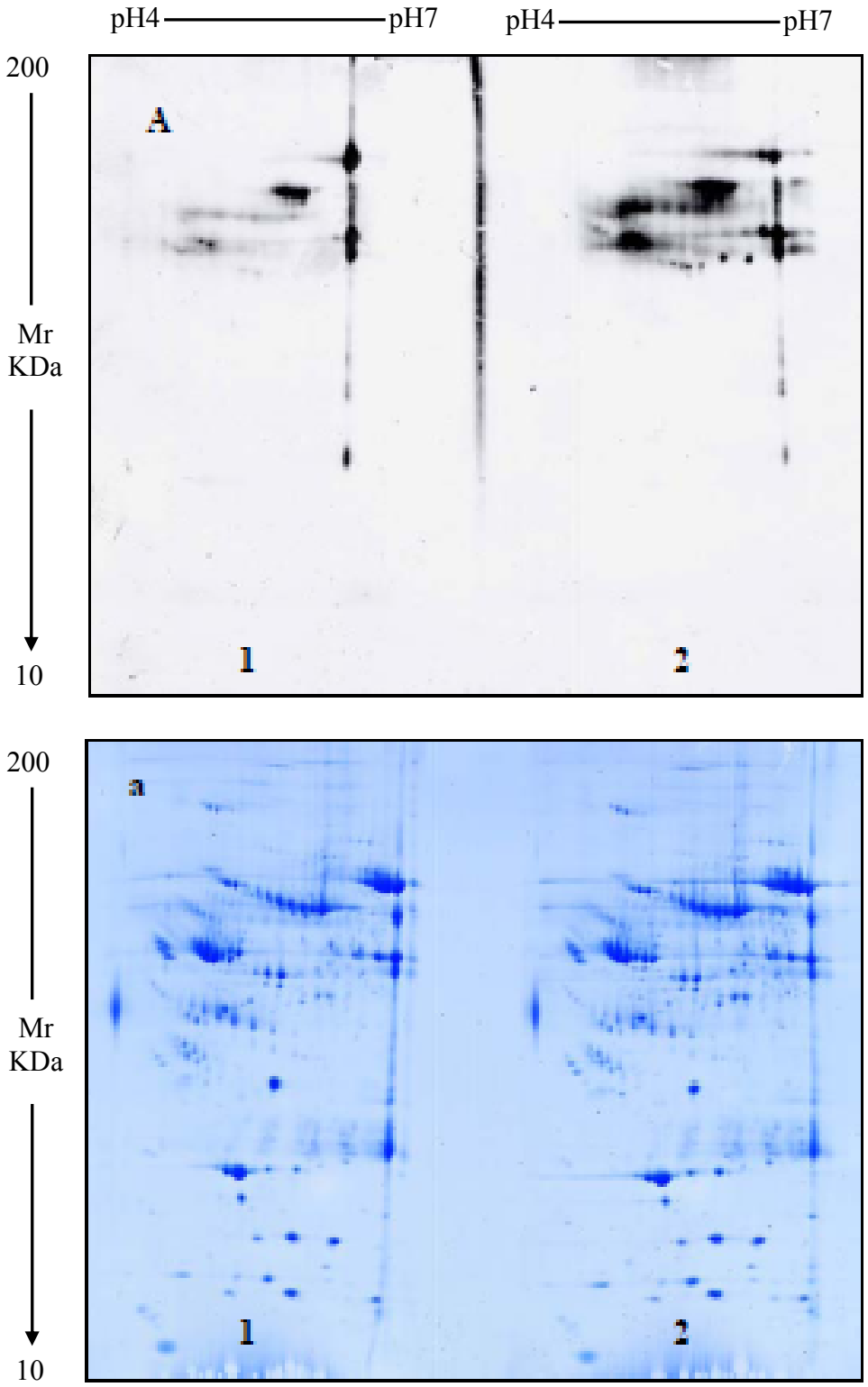


Fig. 2. *Representative images of gels and oxyblots obtained. In panel A and B (next page) there are two images representative of the oxyblots. Number 1 refers to the resting-condition plasma, number 2 relates to the post-exercise plasma. Panel A shows a carbonylation profile of a subject presenting a low protein carbonylation at resting condition and an increase after the exercise. Panel B shows a protein carbonylation profile of a subject presenting a high protein carbonylation at resting condition and an increase after the exercise. Panels a and b show the corresponding Coomassie stained gels.*

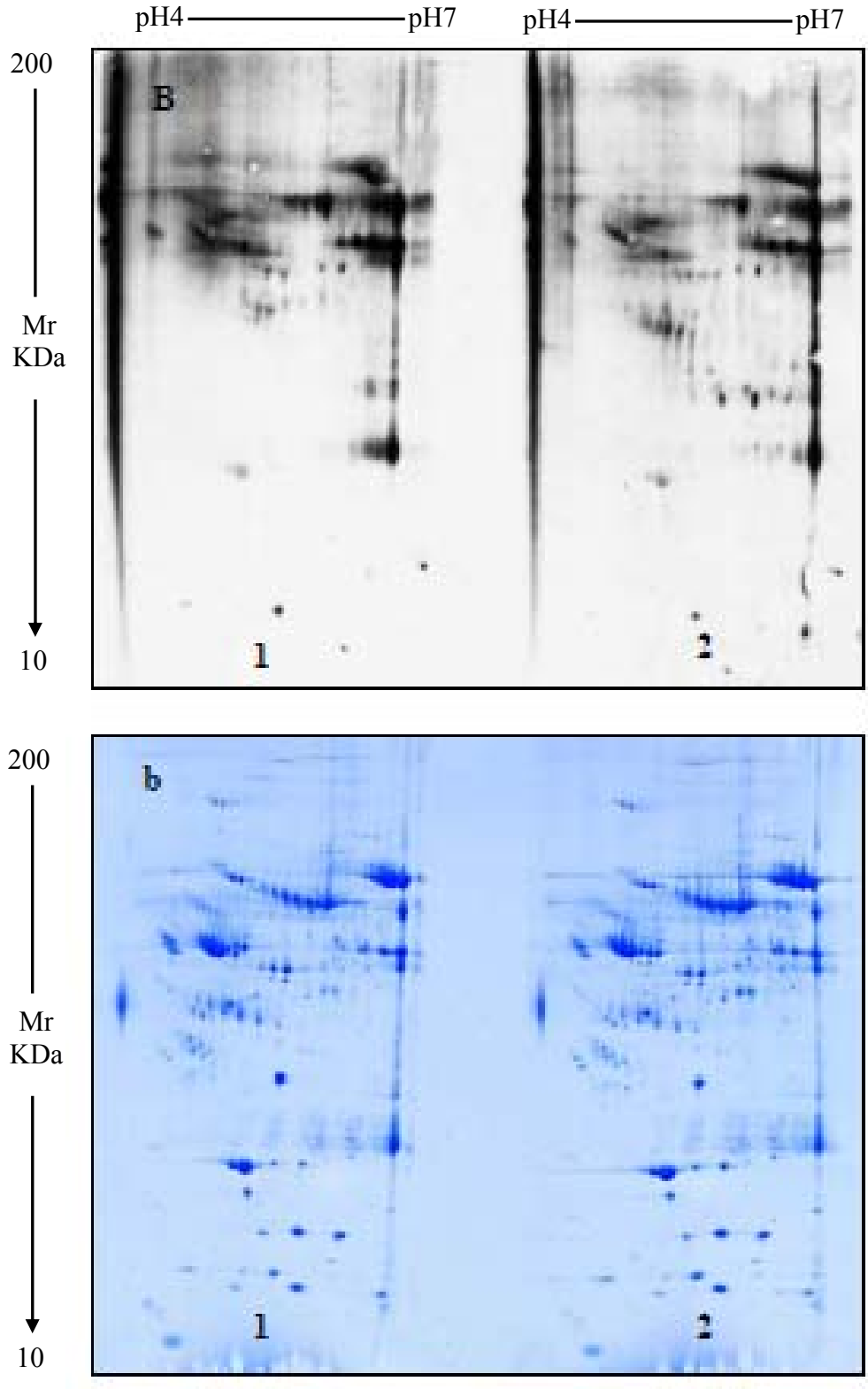


Fig. 2. *See previews page.*

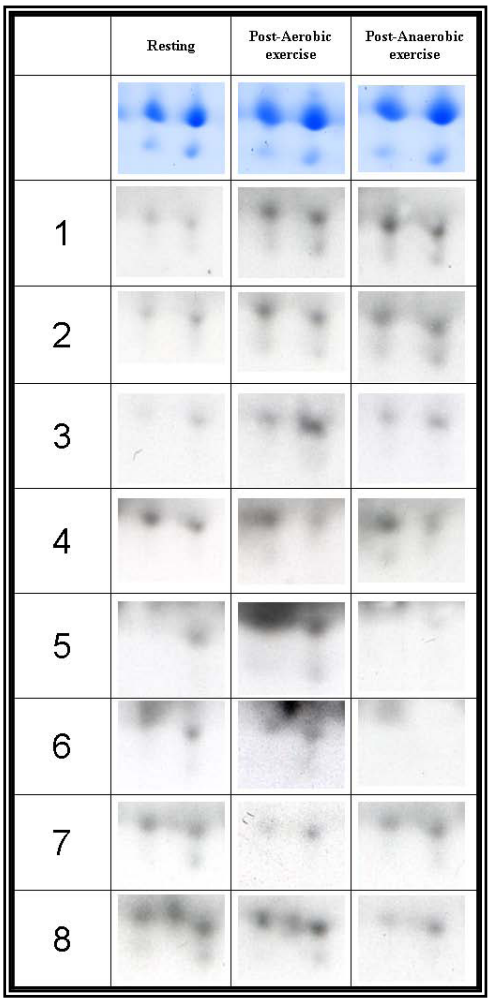


Fig.3. *Example of individual variability in spot carbonylation. Particular of the 2D-oxyblots of each sample underlining the different carbonylated states of the spot 2, 10, 6 and 39 (corresponding to various isoforms of beta fibrinogen chain) at the resting condition and after the two different physical exercises*

4.3.3 Protein carbonylation trend following the aerobic and anaerobic exercise.

In order to better analyze our results and to compare the effects of the aerobic and the anaerobic exercise in endurance trained men, we indicated the carbonylation trend of each protein spot after the exercise in a coloured table. We considered as modified in carbonylation those spots whose spot intensity in post-exercise oxyblots was at least 2 times higher or lower than in the resting condition. The table IIIA shows the spots carbonylation trend after the aerobic exercise, while the table IIIB

shows the spots carbonylation trend after the anaerobic exercise. In these tables are included all subjects (horizontally) and all carbonylated spots (vertically), using three different colours to indicate the carbonylation trend of each spot following the exercise. Green boxes represent major spot carbonylation before the PE, red boxes represent an increase in carbonylation following the PE and orange boxes indicate that the spot carbonylation remains constant in spite of PE. Asterisks represent spots carbonylated only in resting condition (asterisk and green box) or only after the exercise (asterisk and red box).

This kind of table gives also an idea of the carbonylation profile of the single subject. In fact there are subjects showing high proteins carbonylation following both the exercises (for example subject number 1, 4 and 5), while there are subjects who have a low carbonylation profile in both the PE (for example subjects 3 and 7). In particular the subject number 2 presents spots carbonylated exclusively after the activity. The lines in the tables represent the carbonylation trend of each protein spot after the physical exercise. Some spots are always carbonylated in all the subjects and keep a constant carbonylation (orange boxes) level after PE. These spots are the numbers 24, 26, 85, 56, 57, 70, 79 and 86 and correspond to the proteins a-1-antitrypsin, Vitamin D binding protein and Ig alpha chain C region. These proteins, through their abundance, may function as scavengers of free radicals. a-1-antitrypsin is a glycoprotein synthesized by the liver which plays a regulative role for the trypsin and other proteolytic enzymes activity. We also found that albumin and other immunoglobulin are carbonylated in most subjects irrespective of the PE (data not shown).

A protein whose carbonylation level increases after exercise is the Haptoglobin (spot 9, 11, 46), a glycoprotein present in plasma with important antioxidants functions: it protects Haemoglobin from oxidative damage. The spots corresponding to Haptoglobin are carbonylated after the physical activity (boxes marked with asterisks) and in more subjects following the anaerobic exercise. There are also protein spots which carbonylation decreases with the PE (green boxes) or that are carbonylated only before the activity (green boxes marked with asterisks). These spots are present in all the subject and in both conditions. Given that carbonylation is an irreversible proteins modification, the fact that we find a group of spot more carbonylated before the exercise maybe is due to the plasma proteins turnover.

In Fig. 4 we summarised all the results obtained representing the percentage of carbonylated spots after the two types of exercise. We can see that the values and the spots distributions are practically the same between the two types of exercises and we could see that only the 21-23% of the analyzed spots remains not carbonylated after the PE.

All these results indicate that the PE induces a strong variation on plasma protein carbonylation related to the individual conditions. We identify that the only protein which carbonylation level increases after the PE is the Haptoglobin. This protein could be indicated as a potential marker of oxidative stress due to an hard endurance training. Moreover from our results this protein shows a mainly increase in carbonylation following an anaerobic effort.

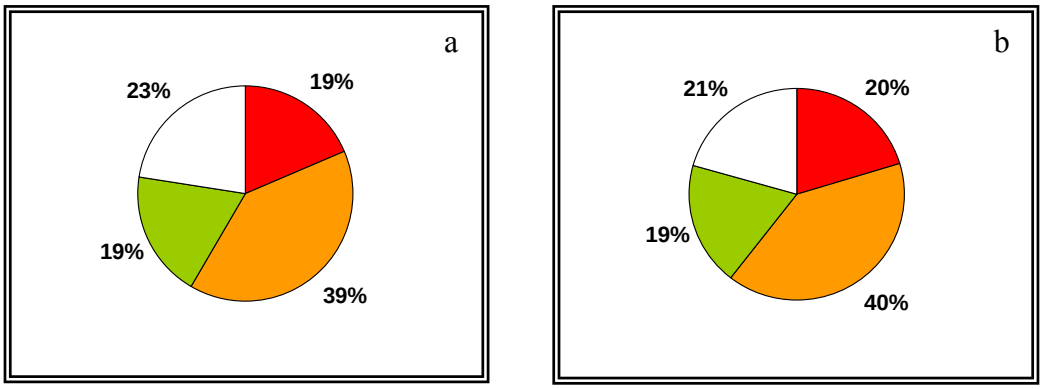
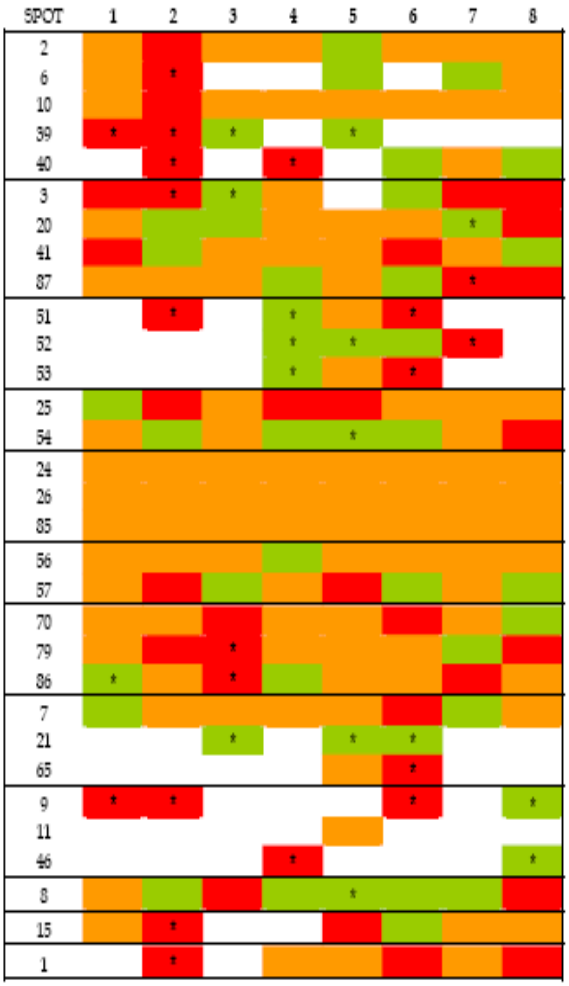
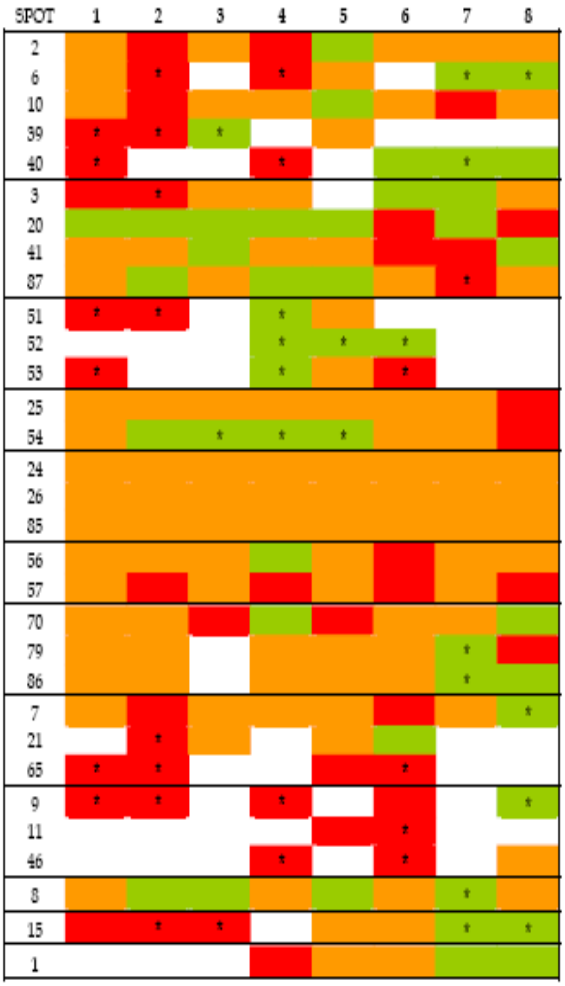


Fig. 4. *Percentage of carbonylated spots after the two types of exercise in endurance trained subjects. a) relates to the aerobic exercise. b) relates to the anaerobic exercise. Green: major spot carbonylation before PE. Red: increase in carbonylation after PE. Orange: constant carbonylation in spite of PE. White: no carbonylation.*

A: Aerobe PE



B: Anaerobe PE



Tab. III: *Spots carbonylation trend after PE in endurance trained men. Panel A relates to the aerobic exercise, while panel B relates to the anaerobic exercise. Horizontal numbers indicate subjects and vertical numbers indicate all carbonylated spots. Colours indicate the carbonylation trend of each spot following the exercise. Green: major spot carbonylation before PE. Red: increase in carbonylation after PE. Orange: constant carbonylation in spite of PE. White: no carbonylation. Asterisks represent spots carbonylated only in resting condition (asterisk and green box) or only after the exercise (asterisk and red box).*

Spot	(e)	AC [(a)]	Score[(b)]	N° of matching peptide [(c)]	Sequence coverage[(d)] (%)	Protein name
2	MS	P02679	250	16	51	Fibrinogen gamma chain
6	MS	P02679	116	9	20	Fibrinogen gamma chain
10	MS	P02679	144	10	30	Fibrinogen gamma chain
39	MS	P02679	132	9	20	Fibrinogen gamma chain
40	MS	P02679	116	9	20	Fibrinogen gamma chain
3	MS	P02675	90	6	12	Fibrinogen beta chain
20	MS	P02675	107	10	25	Fibrinogen beta chain
41	map	P02675				Fibrinogen beta chain
87	map	P02675				Fibrinogen beta chain
51	map	P00751				Complement factor B
52	MS	P00751	94	7	13	Complement factor B
53	map	P00751				Complement factor B
25	MS	P01042	109	9	21	Kininogen 1
54	MS	P01042	89	9	13	Kininogen 1
24	MS	P01009	181	14	40	Alpha-1 antitrypsin
26	MS	P01009	161	13	36	Alpha-1 antitrypsin
85	MS	P01009	128	11	29	Alpha-1 antitrypsin
56	MS	P02774	98	8	20	Vitamin D binding protein
57	MS	P02774	117	9	22	Vitamin D binding protein
70	MS	P01876	98	7	20	Ig alpha-1 chain C region
79	MS	P01876	85	7	22	Ig alpha-1 chain C region
86	map	P01876				Ig alpha-1 chain C region
7	MS	P02766	178	9	58	Transthyretin
21	MS	P02766	66	3	25	Transthyretin
65	MS	P02766	66	3	25	Transthyretin
9	MS	P00738	93	7	16	Haptoglobin
11	MS	P00738	112	10	24	Haptoglobin
46	MS	P00738	78	6	15	Haptoglobin
8	MS	P02787	135	12	16	Serotransferrin
15	MS	O43866	88	7	18	CD5 antigen-like
1	map	P99006				IgG heavy chain

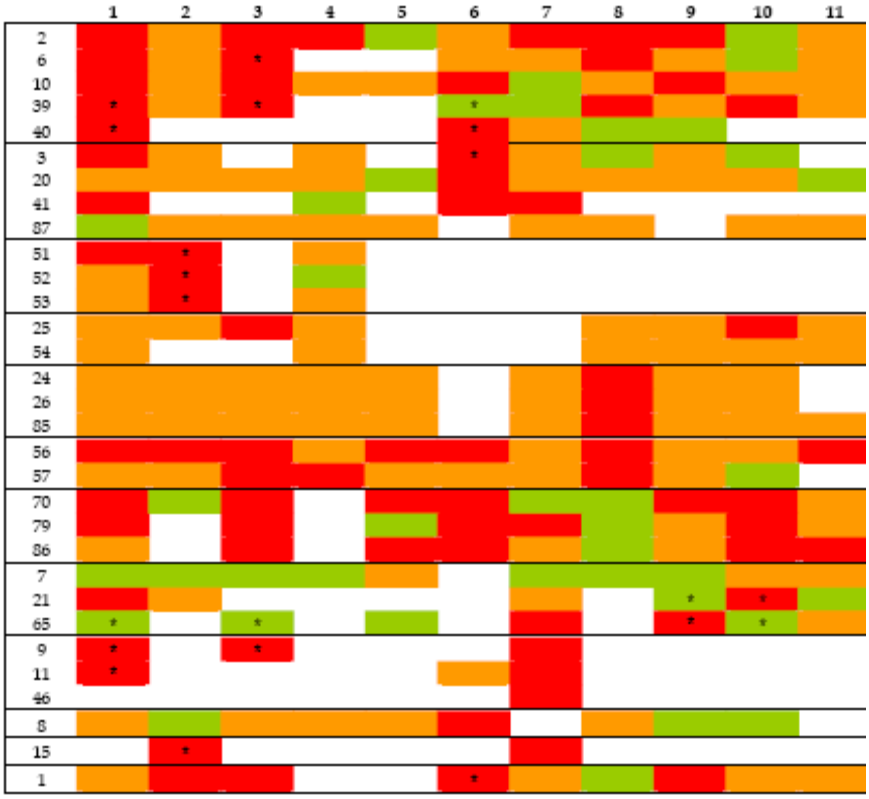
a) Swiss-Prot/TrEMBL accession number.
b) MASCOTscore (Matrix Science, London, UK; http://www.matrixscience.com).
c) Number of peptide masses matching the top hit from Ms-Fit PMF.
d) Percentage of amino acid sequence coverage of matched peptides in the identified proteins.
e) Identification method

***Tab. II**: proteins identified as target of carbonylation.*

4.3.4 Protein carbonylation in the post exercise condition of "less trained" subjects

In order to evaluate the influence on protein carbonylation in subject not trained for endurance activity, we analyzed the same trend on plasma proteins of subject who practise various kind of sport for recreational purposes. This group was formed by 11 recreational athletes. They practise regular physical activity less than 3 times at week according to their physical possibility and without a personal trainer. To process these athletes to the same effort intensity for our study they performed 1 hour of run according to their physical possibility; a sample of blood collection (5 ml) was taken before and after this exercise. Furthermore, they quantify the effort during the hour of run by using the Borg Scale. This is a simple method of rating perceived exertion (RPE) and can be used to gauge an athlete's level of intensity in training and competition. In fact, researchers found that there is a correlation between an athlete's rate of perceived exertion (RPE) and their heart rate, lactate levels, $\%VO_2$ max and breathing rate [Chen *et al.,* 2002]. Their perceived exertion was between 13 and 15 RPE, corresponding to a moderate exercise, considered like an aerobic exercise.

Also for these subjects we analyzed the plasma protein carbonylation by marking the carbonylated proteins with DNPH and using 2D-GE followed by western blot. In order to analyze our results we indicated, as previously, the carbonylation trend of each protein spot in the table IV. Analyzing the same proteins we identified for the hard trained subjects we saw a different carbonylation trend. First of all is interesting to notice that the global protein carbonylation level, "carbonylome", following the PE is lower in comparison to the hard trained subjects (33% *vs* 23%, fig. 5-4). There are some proteins spot (70, 79 and 86) susceptible to carbonylation following the exercise corresponding to the Ig alpha chain C region. Also for the recreational athletes the Haptoglobin resulted carbonylated after the exercise. As previously visualized for the hard trained subjects there are individual variations in the carbonylation profile of the recreational athletes. In fact we can see from the table that some subjects present high carbonylation level following the exercise (subjects 1and 7), while some subjects have a low and constant level of carbonylated spots (subjects 2, 4 and 11).



Tab. IV: *Spots carbonylation trend after PE in recreational athletes. Horizontal numbers indicate subjects and vertical numbers indicate all carbonylated spots. Colours indicate the carbonylation trend of each spot following the exercise. Green: major spot carbonylation before PE. Red: increase in carbonylation after PE. Orange: constant carbonylation in spite of PE. White: no carbonylation. Asterisks represent spots carbonylated only in resting condition (asterisk and green box) or only after the exercise (asterisk and red box).*

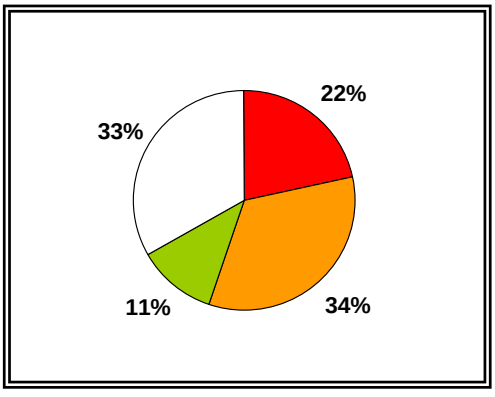


Fig. 5. *Percentage of carbonylated spots after PE in recreational athletes. Green: major spot carbonylation before PE. Red: increase in carbonylation after PE. Orange: constant carbonylation in spite of PE. White: no carbonylation.*

4.3.5 Protein carbonylation in resting condition

To understand if a constant and strenuous training may have effect on proteins oxidation in spite of the physical exercise, we decided to study the plasma carbonylome at the resting condition (basal level) for both the group of subjects. To do so, we compared the proteins carbonylation at resting condition in the hard athletes and in the recreational athletes. We considered the presence or the absence of the carbonylated spot in the western blots of each subject. The table V shows the results of this comparison between the two groups. Spots marked with a "X" are carbonylated in the corresponding subject. Even analysing the basal carbonylation profile of each subject it's possible to notice the individual variability, previews found with the proteomic analysis.

	Recreational athletes											Endurance trained men							
	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8
2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
6	x	x			x	x	x	x	x	x	x	x				x		x	x
10	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
39		x				x	x	x	x	x	x			x		x			
40							x	x	x								x	x	x
3	x	x		x			x	x	x	x		x		x	x		x	x	x
20	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
41	x			x			x					x	x	x	x	x	x	x	x
87	x	x	x	x	x		x	x		x	x	x	x	x	x	x	x		x
51	x			x											x	x			
52	x			x											x	x	x		
53	x			x											x	x			
25	x	x	x					x	x	x	x	x	x	x	x	x	x	x	x
54	x	x						x	x	x	x	x	x	x	x	x	x	x	x
24		x		x	x		x	x	x			x	x	x	x	x	x	x	x
26		x		x	x		x		x			x	x	x	x	x	x	x	x
85		x		x	x		x	x	x			x	x	x	x	x	x	x	x
56	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
57	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x
70	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
79	x		x		x	x	x	x	x	x	x	x	x		x	x	x	x	x
86	x		x		x	x	x	x	x		x	x	x		x	x	x	x	x
7	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x
21	x	x					x		x		x			x		x	x		
65	x	x	x		x		x				x					x			
9								x									x		x
11							x									x			
46							x												x
8	x	x	x	x	x	x		x	x	x		x	x	x	x	x	x	x	x
15							x					x				x	x	x	x
1	x	x	x				x	x	x	x	x				x	x	x	x	x

Tab. V. *Spots Carbonylation at resting condition in recreational athletes and in Endurance trained men. "X" indicate the spot carbonylation in each subject.*

As we can see from the table there are spots, corresponding to the same protein, always carbonylated in the resting condition in both the groups. For example the spots 2 and 10 corresponding to isoforms of fibrinogen gamma chain, spot 20 corresponding to fibrinogen beta chain and 56, corresponding to Vitamin D binding protein. Maybe some isoforms of these proteins are more susceptible to carbonylation than others. This fact may be due to conformational features of the various isoforms. The others spots corresponding to these proteins are carbonylated only in some subjects and we found that there is a majority of endurance trained subjects showing this characteristic. In fact, our analysis indicates that recreational athletes present at resting conditions a 62% of carbonylation of the total spots analyzed, in comparison to the high trained athletes presenting a 70%.

The higher oxidation level at resting condition in the more trained men maybe means that a constant and strenuous training causes an oxidative stress higher than a moderate physical exercise.

4.4 Conclusions

The aim of our research was to characterize plasma proteins that undergo carbonylation in response to physical exercise in men trained to perform endurance exercise and in men practising sport for recreational purposes. By a proteomic analysis we found not only proteins that are target of carbonylation after physical exercise, but also proteins which carbonylation is not affected by exercise and proteins that are carbonylated only in the plasma of the resting condition. A protein whose carbonylation level increases after exercise is the Haptoglobin, a glycoprotein present in plasma with important antioxidants functions: it protects Haemoglobin from oxidative damage. Then we noticed that "endurance hard trained" athletes showed a higher carbonylation of plasma proteins in comparison to men that practise various kind of sports with a moderate exercise.

These methods have allowed to obtain an overview of the changes in the oxidation of plasma proteins with physical exercise and to identify new markers of physiological stress.

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