UNIVERSITÀ DEGLI STUDI DI SALERNO

UNIVERSITÀ DEGLI STUDI DI SALERNO
Dipartimento di Farmacia

Dottorato di Ricerca
in **Scienze del Farmaco**
Ciclo XXIX — Anno accademico 2016/2017

Tesi di Dottorato in

Techniques of proteomic analysis as tools for studies in biomedical field

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Abbreviations

2-DE	Two-dimensional electrophoresis
Al	Aluminium
As	Arsenic
Ba	Barium
Be	Beryllium
BSA	Bovine serum albumin
Cd	Cadmium
Ce	Cerium
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Co	Cobalt
Cr	Chromium
Cu	Copper
DDSH	Dyssegmental dysplasia, Silverman-Handmaker type
DRC-ICP-MS	Dynamic reaction cell inductively coupled plasma mass spectrometry
DTT	Dithiotreitol
ECM	Extracellular matrix
emPAI	exponentially modified Protein Abundance Index
ESI	Electrospray
FASP	Filter-aided sample preparation
FDR	False discovery rate

Fe	Iron
GO	Gene Ontology
Hg	Mercury
HSPG2	Heparan sulfate proteoglycan 2
IAA	Iodoacetamide
IAEA	International Atomic Energy Agency
IBD	Inflammatory bowel disease
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IRSA	Idiopathic recurrent spontaneous abortion
K	Potassium
LFQ	Label-free quantification
Li	Lithium
LOD	Limit of detection
Mg	Magnesium
MGF	Mascot generic format
MMPs	Matrix metalloproteinases
Mn	Manganese
MS/MS	Tandem mass spectrometry
Ni	Nickel
PANTHER	Protein Analysis THrough Evolutionary Relationships
Pb	Lead

PBS	Phosphate-buffered saline
pI	Isoelectric point
Rb	Rubidium
RP-HPLC	Reverse-phase high-performance liquid chromatography
Sb	Antimony
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Se	Selenium
Sn	Tin
Sr	Strontium
Ti	Titanium
TIMPs	Tissue inhibitors of metalloproteinases
Tl	Thallium
TRIS21	Trisomy 21
U	Uranium
V	Vanadium
W	Tungsten
Zn	Zinc

Abstract

It is known that prenatal exposure to pollutants and particularly heavy metals can have long term damaging consequences on infants, due to their accumulation in-body. Since the 1990s, ten million tonnes of waste have been illegally dumped in the area around Caserta and Naples. Thus, direct exposure to waste and heavy metals during the last two decades was very frequent in the so-called “Lands of fires”. The number of children suffering from cancer and of malformed fetuses in Italy's "Land of Fires", an area where toxic waste has been dumped by the mafia, is reported significantly higher than elsewhere in the country. In this thesis we examined the proteome of the umbilical cords from malformed fetuses obtained by therapeutic abortions, after mothers' being exposed to the pollution on “land of fire” during early pregnancy, and analyzed the differences between umbilical cords from malformed fetuses to healthy ones. The main goals were to understand the impact of the contamination by heavy metals on the fetus development, and to identify new putative biomarkers of exposure to metal contaminants.

All umbilical cords were obtained in Campania region (Naples and Caserta, mainly in the “land of fires”). The collection of the biological samples was carried out in collaboration with the Caserta Hospital “Sant’Anna e San Sebastiano” and with the Avellino Hospital “San Giuseppe Moscati”. A proteomic approach based on Filter-Aided Sample Preparation (FASP) method was set up and performed. This bio-analytical strategy combines the advantages of in-gel and in-solution digestion for mass spectrometry–based proteomics, greatly reduces the time required for sample preparation and enables more flexibility in sample processing. Protein identification and quantification were performed by matching mass spectrometry data in on-line protein database, using the MaxQuant 1.5.2.8 software. Statistical analyses were employed to identify proteins whose levels were sensibly different in the umbilical cords from malformed fetuses. Gene Ontology (GO) classification was used in order to obtain functional information of the differentially expressed proteins and to correlate them to the embryonic development. Finally, Matrix Metalloproteinases (MMPs) have been shown to play significant roles in a number of physiological processes, including embryogenesis and angiogenesis, but they also contribute to the development of pathological processes. Thus, gelatin zymography technique

was performed to detect MMPs enzymatic activity in the umbilical cords. Our results support a significant role of MMPs in the fetus development.

Chapter I

1 Introduction

1.1 Metal contamination and fetal malformations

Despite the last decades of the XX century have been characterized from the accelerated technological innovation and the improvement of industrial implants, production activities continue to have a profound effect on the quality of the several environmental matrices (air, water and land). The risk for the population increases in function of proximity to contaminated sites and the risks associated with the contamination by heavy metals are particularly significant (Baibergenova *et al.*, 2003; Zheng *et al.*, 2012). The metal contamination has the capacity to affect the reproductive health, as observed both in industrial contests and in war zones where weapons containing toxic/carcinogenic and teratogenic metals have been used (Suarez *et al.*, 2007; Araneta *et al.*, 2003). The feature of metals, in fact, is to be "long-term teratogenic," since they remain in soil or water, accumulate over time in tissues of living organisms and the bio-accumulation increases their dangerousness, also due to possible synergism of contamination by more metals. The metal contamination has the potential to produce malformations and many of the metals which are not micro-nutrients, and some micro-nutrients at high concentrations, have shown teratogenic, mutagenic, and carcinogenic capacities (Apostoli and Catalani, 2011). Most of the so-called congenital malformations determine great alterations of the body structures and of the organs functionality, sometimes incompatible with the life but very often that allow a life with many limitations and determine a serious weight on the affected individuals and on their families. Not much is known about these malformations (which all together are in Italy between 1% and 4% of newborns), neither about the reasons nor about the modalities whereby they occur in the organism. For many malformations a single gene that determines them isn’t known, in a few cases more genes involved in the malformation have identified, in most cases it is believed that are complex events susceptible to the influence of the external environment on the mother and/or directly on the fetus (Mattison, 2010).

Epidemiological studies associated with chemical and biochemical analyses, conducted in war zones and in particular in the Gaza Strip (Figure 1.1), have shown

the existence of a correlation between heavy metal contamination and increased reproductive damage, in particular an increase of fetal malformations (Naim *et al.*, 2012). In the Gaza situation, various pieces of information useful to investigate this link were published. In recent times "metal augmented" ammunitions were developed and used in the wars and attacks in this century. Indeed, modern weapons contain within them not small amounts of powders of metals, such as Cd, Pb, Al, U, Hg, Co, V, W (Skaik *et al.*, 2010). Most metals persist in time in the environment and many of them accumulate in the bodies, suggesting that dispersion of metal powders in the environment may have both immediate and long term effects on reproductive health and cancers (Vidosavljević *et al.*, 2013; Jergovic *et al.*, 2010). By proving the fact of the presence in tissues at the site of different types of wounds of a specific metal signature different for each kind of physical damage caused by weaponry it was shown that teratogens and fetotoxicant metal contaminants (Pb, U, Al, Ti, Cu, Sr, Ba, Co, Hg, V, Cs and Sn) were delivered by weapons in the attacks in the summer of 2006 and during Operation Cast Lead in the winter of 2009 (Skaik *et al.*, 2010). Teratogens were also detected in examined large bomb craters from 2006 and 2009 bombings (Manduca *et al.*, 2014). Reconstructing the reproductive history of women delivering healthy children in 2011 at Al Shifa Hospital in Gaza, it was reported that the prevalence of birth defects has increased in Gaza since 2005, following introduction of air-delivered weaponry (Naim *et al.*, 2013). A recent study (Naim *et al.*, 2012) reported highly significant correlations between exposure of parental couples to metal delivering weaponry during Operation Cast Lead, the major recent military attack up to then, and the delivery of a baby with major structural birth defects. The progeny with birth defects was conceived 22–26 months after exposures. This information is consistent with weaponry being a source of contamination by teratogenic and toxicant metals for the population, and suggest association between reproductive damage, and exposure to attacks (Manduca *et al.*, 2014). Studies carried out by an international team in another post-war context (Fallujah area, Iraq; Figure 1.1) have shown, at a distance of 5 and 6 years respectively from war events of 2004-2005, persisting hair contamination by metals in adult and children hair, and an increase of cases of birth defects documented with a high frequency in 2011 (Alaani *et al.*, 2011; Al-Sabbak *et al.*, 2012).

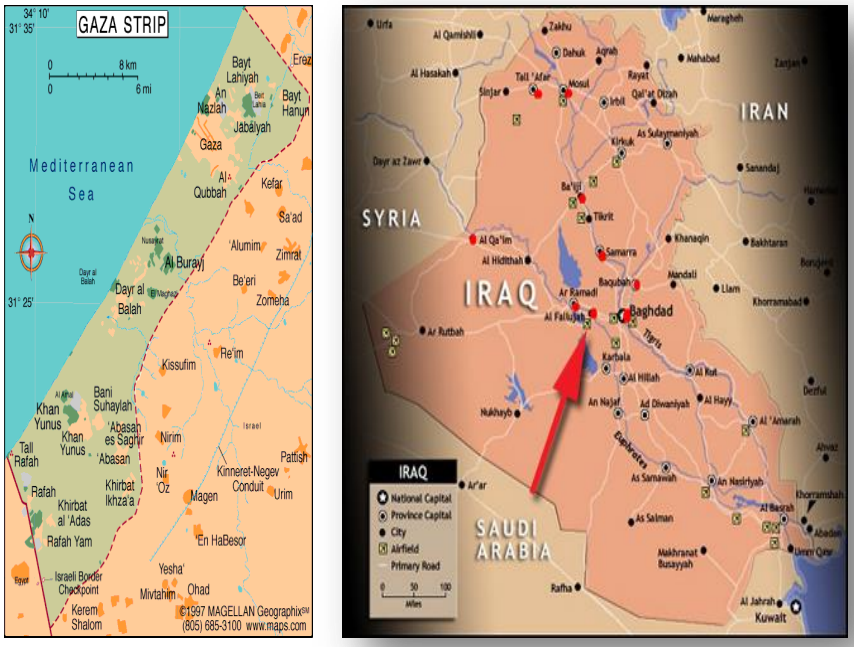

Figure 1.1. Gaza Strip and Fallujah area, Iraq. Concerns from professionals have pointed to increases in birth defects and cancers in these areas during the years following wars.

In the Campania region, where since the 80s several toxic wastes have been "deposited" legally and illegally, observations have been reported that suggest the accumulation of these materials has produced an increase of some kinds of diseases, such as allergies, cancers, adverse effects on reproductive health (infertility, malformations, low birth weight, stillbirths) (Martuzzi *et al.*, 2009). Several studies have reported an accumulation of heavy metals in vegetables and in fishes sampled in areas of Caserta, Aversa and Phlegraean Fields, which exceeded the regulatory limits. Moreover, studies performed on soil samples collected in areas with high potential of pollution have shown the presence of concentrations higher than normal levels of these metals (Cu, V, Cr, Cd, Pb) (Imperato *et al.*, 2003). Therefore the alimentary way, as well as the respiratory one, are considered to cause exposure and accumulation of metals in the exposed population. In addition, other teratogenic contaminants such as dioxins have been found in the milk of mothers in this area (Giovannini *et al.*, 2014). The metals and dioxins have the potential to affect the health of the population not only in the exposed generation, but also in their offspring. Therefore in-depth studies are needed to understand what factors

and what biochemical and physiological mechanisms are the basis of outbreaks of fetal malformations and possibly to limit their incidence.

1.2 "Omics" technologies and environmental proteomics (ecotoxicoproteomics)

Ecotoxicology is the study of the interactions between living organisms, their ecosystems and stressors (Dowling and Sheehan, 2006). The grand goal of ecotoxicology in the post-genome era is to characterize the entire set of genes and proteins that are affected when humans are exposed to environmental xenobiotics (Mussali-Galante *et al.*, 2013). Therefore, a new trend in ecotoxicology and biomedical research is the application of so-called "omics" technologies. 'Omics' technologies adopt a holistic view of the molecules that make up a cell, tissue or organism. They are aimed primarily at the universal detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a specific biological sample in a non-targeted and non-biased manner. This can also be referred to as high-dimensional biology; the integration of these techniques is called systems biology (Kell, 2007) (Figure 1.2).

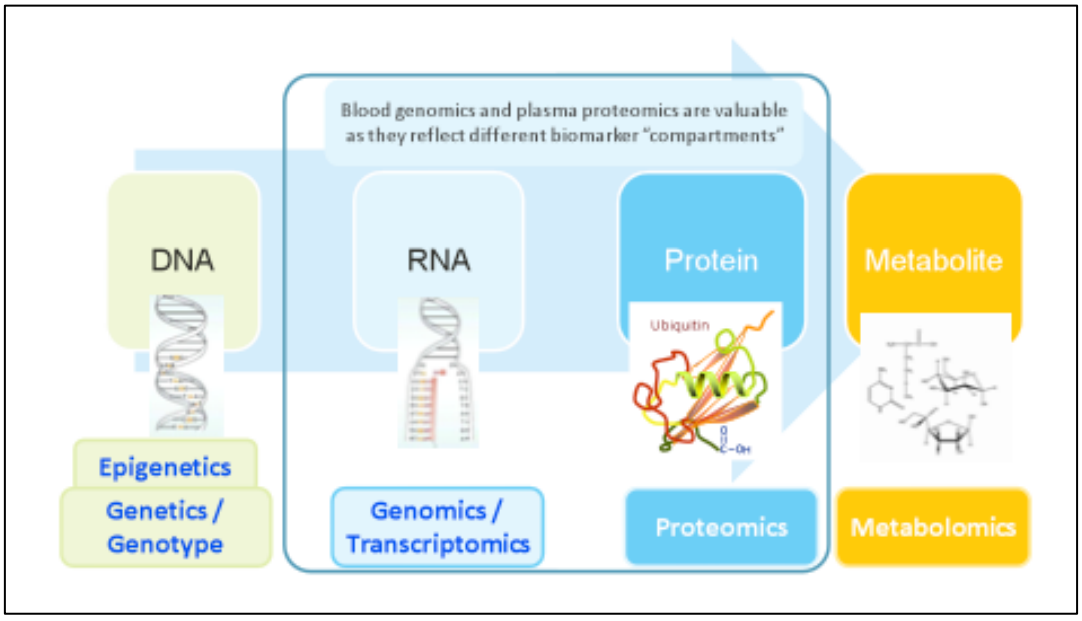

Figure 1.2. The "Omics" approach. "Omics" technologies can be used to gain a "system-wide" understanding of many important biomedical processes.

Genomics is the systematic study of an organism's genome. The genome is the total DNA of a cell or organism. The human genome contains 3.2 billion bases and an estimated 30 000–40 000 protein-coding genes (Baltimore, 2001). Traditionally, genes have been analyzed individually but microarray technology has advanced substantially in recent years. DNA microarrays measure differences in DNA sequence between individuals and the expression of thousands of genes can be analyzed simultaneously (Horgan and Kenny, 2011).

The transcriptome is the total mRNA in a cell or organism and the template for protein synthesis in a process called translation. The transcriptome reflects the genes that are actively expressed at any given moment (Horgan and Kenny, 2011).

The proteome is defined as the set of all expressed proteins in a cell, tissue or organism (Theodorescu and Mischak, 2007). Contrary to genome, the proteome is highly dynamic and changes continuously as a response to numerous intra and extracellular signaling (Barrett *et al.*, 2005). The proteome is a dynamic reflection of both genes and the environment and is thought to hold special promise for biomarker discovery because proteins are most likely to be ubiquitously affected in disease and disease response (Rifai *et al.*, 2006). Proteomics can be more broadly defined as "the effort to establish the identities, quantities, structures, and biochemical and cellular functions of all proteins in an organism, organ, or organelle, and how these properties vary in space, time, and physiological state" (Kenyon *et al.*, 2002). Proteomics is closer to physiology than genomics because post-translational regulation of proteins can reduce the correlation between mRNA abundance and protein activity (Lemos *et al.*, 2010). Proteomics aims to characterize information flow within the cell and the organism, through protein pathways and networks (Petricoin *et al.*, 2002), with the aim of understanding the functional relevance of proteins (Vlahou and Fountoulakis, 2005). It is complicated by its domain size (>100 000 proteins) and the inability to detect accurately low-abundance proteins.

Metabolomics can generally be defined as the study of global metabolite profiles in a system (cell, tissue or organism) under a given set of conditions (Goodacre *et al.*, 2004). The metabolome is the final downstream product of gene transcription and, therefore, changes in the metabolome are amplified relative to changes in the transcriptome and the proteome (Urbanczyk-Wochniak *et al.*, 2003).

Snape *et al.* (2004) proposed the term "ecotoxicogenomics" to describe the integration of genomics (transcriptomics, proteomics, and metabolomics) into ecotoxicology, and defined it as "the study of gene and protein expression in non-target organisms that is important in responses to environmental toxicant exposures."

Specifically for metal exposure assessment, very recently, "metallomics" has emerged as a new sub-discipline of toxicogenomics, which investigates the interrelationships of metal-induced proteome and metabolome changes. In this regard, searches for genes encoding metal-responsive proteins could be interesting targets for reporter genes fusions in biomarker establishing (Haferburg and Kothe, 2010).

Among "omics" technologies, **environmental proteomics** or ecotoxicoproteomics has become a powerful tool for generating hypotheses regarding how the environment affects the biology of non-target organisms. With its rapidly expanding analytical tools, it provides a means to study the changes occurring at the level of the proteome in response to both the external environment and ontogenetic events in animals, plants, and bacteria.

The approach has obvious applications to ecotoxicology since it has the potential both to identify previously unknown protein biomarkers and to gain insights into toxicity mechanisms. Since the proteome is a dynamic quantity, it holds out the promise of detecting subtle changes in sentinel species as they adapt to altered surroundings.

In the toxicological sciences, the possibility to assess the proteome is a gigantic step since the proteomic profiles embody the link between effects at both the molecular level and the whole organism level, given that proteins are the first functional stage directly or indirectly affected by toxicants (Kovacevic *et al.*, 2009). Therefore, more and more researchers are directing their efforts towards proteomic studies and have demonstrated the utility of proteomics in the ecotoxicological field. Recent studies have demonstrated that ecotoxicoproteomics allows the identification of the molecular events involved in toxicant responses and to generate hypotheses on the mode of action of stressors with a high potential for identifying novel biomarkers (Dowling and Sheehan, 2006; López-Barea and Gómez-Ariza, 2006; Monsinjon and Knigge, 2007).

1.3 Biomarkers for metal toxicity

Proteomics is one of the most powerful tools for the identification of molecules (biological markers or biomarkers) that place in evidence the exposure of organisms to a stressor. A biomarker is a measurable indicator of a specific cellular, biochemical or molecular parameter that show whether a key organism has been exposed to a stressor (van Ommen *et al.*, 2009). The use of biomarkers has been proposed as a sensitive early warning tool for biological effect measurement in environmental quality assessment (Cajarville *et al.*, 2000).

In the last 5 years, an increasing interest towards biomarkers of heavy metals have been recorded. The interest in biomarkers for heavy metals impact was defined parallels to the development of biomonitoring program, according to the test subject either had been exposed in the past or currently exposed to environmental stimuli (Sabullah *et al.*, 2015).

A variety of molecules, such as hormones (Zaccaroni *et al.*, 2009), enzymes such as cytochromes P450 (CYP), cholinesterase, catalase (CAT), glutathione S-transferase (GST) (Menezes *et al.*, 2006; Howcroft *et al.*, 2009) and other proteins such as vitellogenin, Hsp, etc. and even organelles (Ortiz-Zarragoita and Cajaraville, 2006) have been selected as biomarkers of specific chemicals or classes of chemicals (Porte *et al.*, 2006). Metabolic enzymes such as aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), superoxide dismutase (SOD), and GST had significantly increase in the activity induced by the presence of toxicant especially heavy metals (Singh *et al.*, 2012; Saliu and Bawa-Allah, 2012; Han *et al.*, 2013). Biomarkers of oxidative stress based on antioxidant enzymes such as SOD, GST, CAT, and glutathione peroxidase (GPX) have been utilized to determine the toxicity level of metal ions in aquatic organisms and have been proved to be sensitive (Radi and Matkovics, 1988; Lopes *et al.*, 2001; Farombi *et al.*, 2007).

Much of the work in the area of biomarkers has focused on metallothioneins or metallothionein-like proteins (MT). These low-molecular weight, cysteine-rich metal-binding proteins are reported to play a key role in the binding and transport of various metals (Costa *et al.*, 2008, 2009). MT are modulated by heavy metals, being an informative and specific biomarker of chronic heavy metal exposure (Mussali-Galante *et al.*, 2013).

A general mechanism of carcinogenicity of arsenic (As), cadmium (Cd), cobalt (Co), and nickel (Ni) seems to be the inhibition of DNA repair enzymes, which include the mammalian XPA protein, the bacterial Fpg protein and the poly (ADP-ribose) polymerase. Specifically, the Fpg protein is inhibited by Cd, copper (Cu), and mercury (Hg) and Ni and Co inhibit DNA binding of XPA (Asmuss *et al*., 2000). Also, poly (ADP-ribose) polymerase is inhibited by arsenite in mammalian cells (Hartwig *et al*., 2002; Schoen *et al*., 2004). These proteins have been used as biomarkers to analyze response to toxic metals. Trace metals are reported to regulate the expression of CYP as well as heavy metals like Hg and lead (Pb) (Ki *et al*., 2009). Apolipoprotein E (Apo E) genotyping has been investigated as an indicator of susceptibility to Hg neurotoxicity (Godfrey *et al*., 2003). Gundacker *et al*. (2007) analyzed the relationship between polymorphisms in GST genes in individuals exposed to Hg. Pb inhibits several enzymes in heme formation pathway, including delta-aminolevulinic acid dehydratase (ALAD), coproporphyrinogen oxidase and ferrochelates. Because Pb effectively inhibits ALAD activity, resulting in accumulation of ALA in blood and urine, urinary ALA has also been used as a biomarker for Pb exposure or a marker of early biologic effect of Pb (Sithisarankul *et al*., 1998). The activities of pyrimidine nucleotidase and nicotinamide adenine dinucleotide synthetase in blood are decreased in Pb exposure. These can also be useful biomarkers of effect in humans (Sakai, 2000). It is suggested that Vitamin D receptor (VDR) may be playing a role in susceptibility to Pb accumulation (Onalaja and Claudio, 2000). Neurobehavioral changes due to Pb exposure have been associated with Apo E genotype (Stewart *et al*., 2002; Godfrey *et al*., 2003).

MT binds Cd strongly and is considered a good biomarker of Cd exposure (Kakkar and Jaffery, 2005). Effect biomarkers of Cd-induced renal dysfunction are β_2-microglobulin, retinol binding protein and albumin (Jin *et al*., 2002). α_1-Microglobulin is shown to be a promising marker of Cd-induced tubular dysfunction, possibly better than β_2-microglobulin (Moriguchi *et al*., 2004). Elevation in enzymes primarily of renal tubular origin, such as *N*-acetyl-β-D-glucosaminidase (NAG) and alanine aminopeptidase (AAP), has been associated with occupational Cd exposure (Lauwerys *et al*., 1994). Cd combined with As may have additive effect on renal dysfunction in workers exposed to both metals. Positive correlations and significant dose–effect have been reported by Hong *et al.*

(2003) among the concentrations of urinary Cd, As and levels of β_2-microglobulin, albumin, *N*-acetyl-β-D-glucosaminidase. Studies concerning the effects of polymorphic forms of arsenic methyl-transferase (AsMT) in regulating the toxicity of AsIII in mice (Stýblo *et al*., 2002; Aposhian *et al*., 2004; Wang *et al*., 2008) highlighted the importance of polymorphisms in the metabolic pathway in mediating formation of toxic methylated arsenical metabolites.

The use of biomarkers beyond the individual level has not always allowed for cause-effect relationships. A major limitation of biomarker use is that a variety of responses have been identified in exposed organisms, making difficult to link environmental exposure to specific chemical entities and subsequent biological effects. Variability in biomarker responses may be attributed to abiotic (temperature, salinity, dissolved oxygen, etc.) or biotic factors (genotype, phenotypic plasticity, tolerance, age, sex, body size, etc.). In this case, the use of a multi-biomarker approach, in a range of species using sentinel organisms, becomes necessary to resolve or at least have a closer insight into complex environmental problems (Mussali-Galante *et al.*, 2013).

1.4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of at least 23 endopeptidases that act as effectors of extracellular matrix remodeling in physiological and pathological conditions (Figure 1.3). All family members are secreted as inactive proenzymes (zymogens) and are thought to be activated in the tissue by cleavage of the propeptide. All MMPs contain Zn^{2+} at the catalytic site and, in addition, require Ca^{2+} for stability and activity (Birkedal-Hansen, 1993). MMPs can be subdivided into gelatinases (MMP-2 and -9), collagenases (MMP-1, -8, -13 and -18), stromelysins (MMP-3, -10 and -11) and other MMPs, according to their substrate affinity profile. Their activity is closely regulated by tissue inhibitors of metalloproteinases (TIMPs), a group of four endogenous antagonists that bind to the catalytic site of MMPs. MMPs participate in normal remodeling processes such as embryonic development, post-partum involution of the uterus, bone remodeling, ovulation and wound healing (Woessner, 1991).

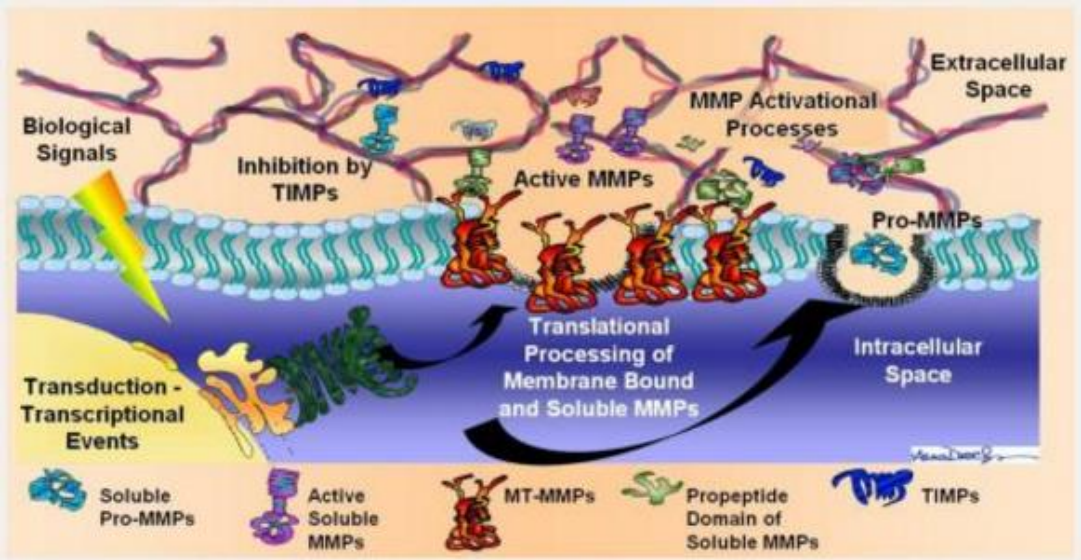

Figure 1.3. Matrix metalloproteinases (MMPs). They are Ca^{2+}-dependent Zn^{2+}-containing endopeptidases able to degrade extracellular matrix proteins in a variety of physiological and pathological processes.

MMPs hydrolyze extracellular matrix components in a variety of processes ranging from implantation and embryogenesis to inflammation, metastasis, and angiogenesis (Hernandez-Perez and Mahalingam, 2012). Metalloproteinases, whose production is enhanced by prostaglandins, play an important role in promoting uterine contractions and preterm birth by stimulating cervical ripening and membrane activation (Xu *et al.*, 2002). In particular, metalloproteinases degrade extracellular matrix proteins, such as collagens and fibronectins, leading to weakening of membranes and their subsequent rupture (Vu *et al.*, 2008). In a recent study by Sundrani *et al.* (2012), placental metalloprotein 1 and metalloprotein 9 levels were significantly increased in women with spontaneous preterm birth compared to those delivering at term, suggesting that placental metalloprotein 1/metalloprotein 9 levels may be involved in the initiation of parturition (Maymon *et al.*, 2000). Polymorphisms in metalloproteinases and TIMPs have been found to modulate the risks for preterm premature rupture of membranes (PPROM) and preterm birth (Maymon *et al.*, 2000; Cockle *et al.*, 2007).

MMPs are also thought to modulate the effects of heavy metals especially Hg. Several polymorphisms in metalloproteinases and TIMPs have been found to modulate the effects of heavy metal exposure (especially Hg) on the risk of

cardiovascular diseases (Pepper, 2001). Indeed, MMP-2 and -9 have emerged as important players in a number of cardiovascular diseases, including atherosclerosis, stroke, heart failure, ischemic heart disease and aneurysm (Kupai *et al.*, 2010). Also, the single nucleotide polymorphisms in these genes are associated with differential risk of preterm birth (Jacob-Ferreira *et al.*, 2011; Jacob-Ferreira *et al.*, 2010). Several other oxidative stress-related genes have been associated independently with heavy metals exposure and adverse pregnancy outcomes. It may be suggested that the effect of Hg on glial cell reactivity and Hg-induced oxidative stress via MMPs or paraoxonase 1 (PON1) modulating the effects of heavy metals seem also to play a role in the pathogenesis of preterm birth, perinatal hypoxia, bronchopulmonary dysplasia (BPD), preterm brain inflammation, and mortality (Türker, 2015).

Air pollutants, including heavy metals (*e.g.* Cd, Pb and Hg), can reach the respiratory system, eliciting pulmonary and/or systemic effects, which include inflammation, tissue remodeling and carcinogenesis: all phenomena where MMPs play critical roles, given their broad effects on matrix remodeling and modulation of inflammation and cell signaling (Löffek *et al.*, 2011). In an *in vitro* study, Xu *et al.* (2011) evaluated modulation of MMP-2 and -9 expression by Ni on human lung cancer cell lines A549 and H1299 in the context of the analysis of their growing capacity and invasiveness. They demonstrated that Ni could significantly enhance the invasive potential of A549 and H1299 cells in a dose-dependent manner. This was accompanied by an elevated expression of interleukin (IL)-8, transforming growth factor-β and MMP-2 and MMP-9 proteins (Dagouassat *et al.*, 2012). Fievez *et al.* (2009) examined MMP-2 and -9/TIMP-1 and -2 imbalance in rats exposed to Cd nebulisation. Such nebulisation induced a significant increase in bronchoalveolar lavage (BAL) MMP-2 and -9 and TIMP-2 expression and/or activities. In a descriptive *in vivo* study, Beaver *et al.* (2009) showed an increase in the levels of pro-MMP-9 in BAL fluid, strongly correlating with the presence of neutrophils in the airways, in mice exposed chronically to particles of hexavalent chromium (Cr(VI)), a well-known pro-inflammatory and carcinogenic agent (Fishbein, 1981). Wan *et al.* (2008) demonstrated that nanoparticles of Co, but not nanoparticles of titanium dioxide (TiO_2), induced MMP-2 and -9 activity and mRNA expression, and decreased mRNA expression of TIMP-2.

It has been demonstrated that divalent metal salts, as Zn, Cu, Hg and Sn, are capable to inhibit the activity of MMP-2 and MMP-9 at low concentration (Souza *et al.*, 2000). It has also been observed that Pb, Cd and Zn inhibit the activity of enamel MMPs in vitro (Gerlach et al., 2000; Souza and Line, 2002).
Study of the pattern of MMP expression in response to heavy metals could provide important insights into the pathogenesis of clinical syndromes associated with toxic metal exposure. Furthermore, studies of metalloproteinase expression in response to heavy metals may provide a general model for tissue injury applicable to a wide range of lesions (Evans, 2015).

1.5 Aim of the project

This research project has aimed to study the proteome of tissues of malformed fetuses exposed to heavy metals. This project is based on the proteomic analysis of umbilical cords (Figure 1.4) of malformed fetuses, obtained by therapeutic abortions, using as control umbilical cords of healthy fetuses with similar gestational age (psychiatric abortions). All umbilical cords have come from Caserta Hospital "Sant'Anna e San Sebastiano" and from Avellino Hospital "San Giuseppe Moscati", which collect cases from the whole territory of the Campania Region; the umbilical cords were obtained by therapeutic abortions of mothers resident for more than 10 years in the areas of the provinces of Naples and Caserta, unhappily known as the "land of fires". The comparison between the protein profiles observed in the umbilical cords of malformed fetuses and those of healthy fetuses with comparable gestational age may allow to individuate new possible specific biomarkers of exposure. Main aims:

- identification of down or upregulated proteins;
- study of proteomic data by complementary techniques;
- definition of putative proteins or pathways involved in the fetal malformations.

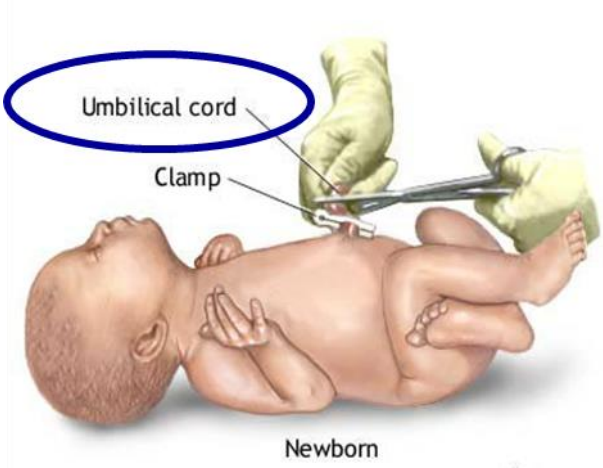

Figure 1.4. Umbilical cord. Aim of this project was to study the proteome of umbilical cords. Cord tissue contains several different cell types, including mesenchymal stem cells, epithelial cells and endothelial cells, each with different potential uses.

Chapter II

2 Materials and Methods

2.1 Umbilical cords from healthy and malformed fetuses

Caserta Hospital "Sant'Anna e San Sebastiano" and Avellino Hospital "San Giuseppe Moscati" (consent of the Ethics Committee, January 2014):

2014: GR1 (Healthy); GR2 (Healthy); GR3 (Healthy); GR4 (Healthy); GR5 (Healthy); GR6 (Healthy); GR7 (Healthy); GR8 (Healthy); GR9 (Healthy); GR10 (Healthy); CCP PSI 1 (Healthy); CC10 (Trisomy 21); G7 (Trisomy 21); CC31 (Diaphragmatic hernia); CC32 (Non-immune hydrops); CC33 (Cystic hygroma and atrioventricular canal defect); CC35 (Congenital heart disease); G6 (Trisomy 21); G9 (49,XXXXY syndrome).
2015: CCP PSI 2 (Healthy); CC3C(15) (Healthy); CCP PSI 3 (Healthy); CC(15)D (Healthy); G16 (47,XXY, Klinefelter syndrome); G5 (Trisomy 21); CC1(15) (Neural tube defect – multiple); CC5(15) (Neural tube defect); G1 (Trisomy 21 + heart disease); G2 (Dyssegmental dysplasia, Silverman-Handmaker); G3 (Trisomy 21); G4 (Trisomy 21); G11 (Neural tube defect); CC7(15) (Gastrointestinal disease); CC8(15) (47,XXY, Klinefelter syndrome); CC9(15) (Congenital heart disease, cleft lip and palate, neural tube defect).

2.2 Protein extraction and quantitative analysis

Preliminary experiments were realized to select the best method of protein extraction from umbilical cords, crucial step for the subsequent analyses. Umbilical cord proteins were extracted by cryogenic grinding with mortar and pestle in dry ice of frozen tissue (100 mg) and by suspending in 600 µl of lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM DTT, 0.5% ampholytes) added with protease inhibitor (1:100 v/v). The mixture was vortexed, centrifuged at 13,000 g for 15 min and the supernatant was collected. The protein concentration of each extract was determined according to the Bradford method (Bradford, 1976), which is a colorimetric assay based on an absorbance shift of the dye Coomassie Brilliant Blue G-250. It was constructed a calibration curve with bovine serum

albumin (BSA) (1 µg/µl, 2.5 µg/µl, 5 µg/µl, 10 µg/µl, 20 µg/µl). 1 µl of lysate was added to 1 ml of the mixture H_2O:Bradford reagent (8:2 v/v) and the assay was performed by UV-visible spectrophotometer at 595 nm.

2.3 One-dimensional gel electrophoresis and in gel digestion

In a first step umbilical cords were analyzed by one-dimensional Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) approach, followed by 'in gel' enzymatic digestion with trypsin (Wilm *et al.*, 1996). Briefly, each protein extract (70 µg) was dissolved in SDS-PAGE loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8), boiled at 100°C for 5 min, and loaded in triplicate on a 1.5-mm thick 10% polyacrylamide gel. 5 µl of protein standard marker (Protein Marker VI (10 – 245) prestained, AppliChem, Darmstadt, Germany) were loaded alongside of each gel. The protein separation was performed at constant 30 mA/gel for 2 h at room temperature. Gels were stained by colloidal Coomassie Brilliant Blue staining solution and scanned by ImageScanner III LabScan 6.0 software (GE Healthcare). Resulting electrophoretic lanes were cut in 10 slices. The gel slices were washed and destained 3 times in deionized water, cysteines were reduced with 10 mM DTT in 100 mM ammonium bicarbonate buffer for 1 h at 56°C and then alkylated with 50 mM iodoacetamide in 100 mM ammonium bicarbonate buffer for 30 min at room temperature in the dark. Digestion was performed overnight at 37°C with 13 ng/µl trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate buffer. The resulting peptides were extracted with 0.1% formic acid in acetonitrile, dried in a vacuum centrifuge, and resuspended in 30 µl 0.1% formic acid in water for mass spectrometric analysis.

2.3.1 Mass spectrometry-based protein identification

Protein identification was performed by reverse-phase high-performance liquid chromatography (RP-HPLC) coupled to electrospray ionization tandem mass

spectrometry (ESI-MS/MS). Peptide mixtures produced by tryptic digestion (5 µl) were sequentially loaded on a reversed-phase column (0.1×150 mm, packed with C18, 5 µm, 100 Å) at a flow rate of 60 µl/min with buffer A (0.1% formic acid in water). The samples were run on a 73-min gradient, which consisted of a 5%–45% buffer B (0.1% formic acid in acetonitrile/water 80/20) for 55 min, 90% buffer B for 5 min, and 5% buffer B for 13 min. The peptide analysis was performed using the LTQ ORBitrap XL (Thermo Fisher Scientific) coupled directly to the LC column via a nano-electrospray source. The MS survey scan was obtained for the m/z range 400–1800, and the MS/MS spectra were acquired from the survey scan for the 10 most intense ions as determined by the Xcalibur mass spectrometer software in real time. Dynamic mass exclusion windows of 60 s were used to limit repeated sequencing (Song *et al.*, 2009).

The thirty raw data MS/MS spectra from the three electrophoretic lanes of each sample were converted in MGF format by MM File Conversion tool for subsequent protein identification against the Swiss-Prot Protein Database (542,782 sequence entries, March 2014) through the MS search algorithm on the Mascot search engine (Perkins *et al.*, 1999). The following search parameters were set: Peptide tolerance 25 ppm; MS/MS tolerance 0.8 Da; enzyme specificity: trypsin; 2 missed cleavages permitted; fixed modification: carbamidomethylation of cysteine; variable modification: methionine oxidation; taxonomy: Homo sapiens.

The ten MGF files from each electrophoretic lane were merged into a single search in order to output a single list of identified proteins.

2.4 Two-dimensional gel electrophoresis (2-DE)

Protein lysates (500 µg for each sample) were precipitated in acetone for sample purification, rehydrated with suitable buffer (7M urea, 2M thiourea, 2% w/v CHAPS, 0.5% v/v Pharmalyte pH 3-10, 0,002% v/v Bromophenol blue stock solution 1%) with 2,8 mg/ml of dithiothreitol (DTT) and loaded on IPG strips 18 cm (pH 3-10 nonlinear). IPG strips were inserted in Ettan IPGphor3 system (GE Healthcare) for IEF in first dimension and were subsequently equilibrated with a buffer containing SDS (50mM Tris/HCl, 6M urea, 30% glycerol, 2% SDS) before

the electrophoresis in second dimension. An aliquot of equilibration buffer was added with DTT (100 mg in 10 ml of solution) for reduction of denatured proteins, a second aliquot of equilibration buffer was instead added with iodoacetamide (IAA) (250 mg in 10 ml of solution) for subsequent alkylation of the reduced sulfhydryls. Each IPG strip was then loaded on gel for the separation in second dimension. Gels were then fixed in 40% ethanol, 10% acetic acid for 3 h, stained in 0.1% Coomassie Brilliant Blue R-250 and destained in 30% ethanol, 10% acetic acid. After coloration with Coomassie blue, gels were scanned and acquired by Image Scanner III LabScan 6.0 software (GE Healthcare). Gel images were analyzed using ImageMaster 2D Platinum 6.0 software (GE Healthcare) according to manufacturer's instructions. Image analysis included spot detection, quantification and matching.

2.5 Filter-Aided Sample Preparation (FASP)

In the Filter-Aided Sample Preparation (FASP) method, umbilical cord proteins were extracted by cryogenic grinding with mortar and pestle in dry ice of frozen tissue (100 mg) and by suspending in 600 µl of lysis buffer (4% SDS, 100 mM Tris/HCl pH 7.6, 0.5 mM DTT) added with protease inhibitor (1:100 v/v). The mixture was vortexed, incubated at 95°C for 3 min, centrifuged at 13,000 g for 5 min and the supernatant was collected. The protein concentration of each extract was determined according to the *DC* Protein Assay (Bio-Rad), which is a colorimetric assay for protein concentration following detergent solubilization similar to the well-documented Lowry assay (Lowry *et al.*, 1951; Peterson, 1979). The assay was performed in duplicate with a 96-well plate. It was constructed a calibration curve with BSA (1 µg/µl, 2.5 µg/µl, 5 µg/µl, 10 µg/µl, 20 µg/µl). 20 µl of reagent S (surfactant solution) were added to 1 ml of reagent A (reagent A'). 5 µl of lysate were loaded into each well. 25 µl of reagent A' and then 200 µl of reagent B were added into each well. After 15 minutes at room temperature in the dark, the absorbances were read by UV-visible spectrophotometer at 750 nm.

The FASP method uses centrifugal filters with a molecular weight cut-off, which allow to desalt and concentrate the sample. The filter unit then acts as a 'proteomic

reactor' for detergent removal, buffer exchange, chemical modification and protein digestion. The four critical steps of the FASP method are: (i) depletion of detrimental low-molecular-weight components in urea-containing buffer, (ii) carboamidomethylation of thiols, (iii) digestion of proteins and (iv) elution of peptides (Wiśniewski *et al.*, 2009). After some preliminary checks, filters with a cut-off of 10 kDa (Microcon® Centrifugal Filters, EMD Millipore^{TM}) were used. 50 µg of protein extract were loaded on each filter. Detergent removal by buffer exchange was performed in two successive washes with UA (8M urea in 100 mM Tris-HCl pH 8.5) with a 20 min spin at 13,000 g. Proteins were reduced by adding 100 mM DTT in UA. Proteins were then alkylated with 50 mM IAA in UA at room temperature for 30 min in darkness. Urea was then removed by two washes with 50 mM ammonium bicarbonate with centrifugation at 13,000 g for 15 min. Protein digestion was achieved by adding 0.5 µg of trypsin (enzyme to protein ratio 1:100) in 50 mM ammonium bicarbonate to each filter and incubating at 37°C overnight. Peptides were recovered in two washes with 40 µl of 50 mM ammonium bicarbonate with spinning at 13,000 g for 20 min each. Peptides resulting from digestion were desalted by StageTips (C18 material 200µL, Thermo Scientific^{TM}) according to manufacturer's instructions and analyzed by LC/MS/MS (LTQ-OrbiTrap XL, Thermo Scientific^{TM}).

2.5.1 Mass spectrometry-based protein identification

Peptides were separated on a EASY-nLC 1000 HPLC system (Thermo Fisher Scientific). Columns (75-µm inner diameter, 25-cm length) were in-house packed with 1.9-µm C18 particles. Peptides were loaded in buffer A (0.1% formic acid) and separated with a gradient from 7% to 60% buffer B (80% acetonitrile, 0.1% formic acid) within 3.5 h at 200 nl/min. A quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific) was directly coupled to the liquid chromatograph via a nano-electrospray source. The survey scan range was set to 400 to 1,800 *m*/*z*. Up to the 10 most abundant isotope patterns with a charge of ≥2 were subjected to Higher-energy collisional dissociation with a normalized collision energy of 25, an isolation window of 2 Th, and a resolution of 17,500 at *m*/*z* 200. To limit repeated

sequencing, dynamic exclusion of sequenced peptides was set to 60 s. Thresholds for ion injection time and ion target value were set to 20 ms and 3×10^6 for the survey scans and to 60 ms and 10^6 for the MS/MS scans (Hornburg *et al.*, 2014). Data were acquired using Xcalibur software (Thermo Fisher Scientific). LC/MS/MS analysis was performed in duplicate.

The protein identification and quantification was performed by MaxQuant 1.5.2.8 software (Max Planck Institute of Biochemistry). In MaxQuant all MS files were uploaded simultaneously, specifying the experiment name in order to get a different MaxLFQ (Label-Free Quantification) intensity for each MS run. The MS/MS spectra were searched against the UniProtKB HUMAN fasta database (70,071 forward entries; version of October 2015). MaxQuant validates scoring statistics based on hits to reversed protein entries in a target-decoy database. Therefore, it was necessary to include a reversed version for each original entry in the protein database fasta file. Reversed entries have to be indicated by a recognizable prefix to the protein ID, e.g. 'REV_' (Cox *et al.*, 2009). Enzyme specificity was set to trypsin allowing cleavage N-terminal to proline and up to 2 miss cleavages. Peptides had to have a minimum length of 5 amino acids to be considered for identification. Carbamidomethylation was set as fixed modification, acetylation (N-terminus) and methionine oxidation were set as variable modifications. A false discovery rate (FDR) cutoff of 5% was applied at the peptide and protein level. Initial precursor mass deviation of up to 4.5 ppm and fragment mass deviation up to 20 ppm were allowed. Protein identification required a minimum peptide ratio count of 1 and at least one razor peptide. Razor peptides are non-unique peptides assigned to the protein group with the most other peptides (Occam's razor principle) (Cox *et al.*, 2009). Proteins that could not be discriminated on the basis of unique peptides were grouped into protein groups. MaxLFQ intensities were used to quantify fold changes of proteins across samples, (Hornburg *et al.*, 2014).

2.5.2 Gene Ontology

Gene Ontology was performed using the PANTHER (**P**rotein **An**alysis **TH**rough **E**volutionary **R**elationships) Classification System

(http://www.pantherdb.org/), which is part of the Gene Ontology Reference Genome Project. The PANTHER matches gene function, ontology, pathways and statistical analysis, and allows to analyze data of large-scale complete genomes coming from experiments of sequencing, proteomics or gene expression. Proteins and their genes are classified according to: family and subfamily; molecular function; biological process; pathway (Mi *et al.*, 2013).

2.6 Gelatin zymography

Zymography is known as an electrophoretic technique, commonly based on SDS-PAGE, suitable for analysis of matrix metalloproteinases (MMPs) in complex biological fluids (serum, synovial fluid) and tissue extracts (heart, liver, kidney, spleen, etc.).

Samples (50 mg) were prepared in a standard, non-reducing loading buffer (62.5 mM Tris-HCl pH 6.8, 2.5% SDS, 0.002% Bromophenol Blue, 10% glycerol) for the separation by SDS-PAGE. No reducing agent (2-mercaptoetanol, DTT) or boiling are necessary since these would interfere with refolding of the enzyme. A suitable substrate (e.g. gelatin for protease detection) was embedded in the resolving gel during preparation of the acrylamide gel (10% polyacrylamide–0.1% gelatin). Protein concentration was determined by DC Protein Assay (Bio-Rad, Berkeley, CA, USA), using BSA as a standard. 150 µg of proteins, of each sample, were loaded on the gel. Following electrophoresis, the SDS was removed from the gel (or zymogram) by incubation in unbuffered Triton X-100 2,5%, followed by incubation in an appropriate digestion buffer (40mM Tris pH 7.5, 0.2M NaCl, 10mM $CaCl_2$) for an optimized length of time (~16 h) at 37 °C. Gels were stained with Coomassie blue and were then destained. Gelatinolytic activity was visualized as clear bands against a blue background. The intensity of each band present was performed with ImageJ software (http://rsbweb.nih.gov/ij/download.html). The relative mobility was determined using standard protein molecular weight markers.

2.7 Western blotting analysis

Umbilical cord whole lysates for immunoblot analysis were prepared according to the standard protocol. Protein concentration was determined by DC Protein Assay (Bio-Rad, Berkeley, CA, USA), using bovine serum albumin (BSA) as a standard. Proteins were fractionated on SDS-PAGE, transferred into nitrocellulose membranes, and immunoblotted with appropriate primary antibodies. Signals were visualized with appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences-GE Healthcare, NY, USA). Densitometry of bands was performed with ImageJ software (http://rsbweb.nih.gov/ij/download.html).

2.8 Statistical analysis

Data reported in each figure are the mean values ± SD from at least three experiments, performed in duplicate, showing similar results. Differences between treatment groups were analyzed by Student's t-test. Differences were considered significant when $p < 0.05$.

Chapter III

3 Results and Discussion

The aim of this project was to study the proteome of umbilical cords from malformed fetuses compared to healthy ones with same gestational age. All umbilical cords were obtained by therapeutic abortions of women living in Napoli or Caserta, in the so-called "land of fires" (Figure 3.1). The collection of the biological samples was carried out in collaboration with the Caserta Hospital "Sant'Anna e San Sebastiano" and with the Avellino Hospital "San Giuseppe Moscati" (The hospital ethics committee has reviewed and approved the project). The comparison between the protein profiles observed in umbilical cords of malformed and healthy fetuses may allow to identify differentially expressed proteins. The down or up-regulated proteins may indicate the biological pathways involved in the contaminants toxicity (González-Fernández *et al.*, 2008). Gene Ontology (GO) classification was used in order to obtain functional information of the differentially expressed proteins, and to correlate them to the embryonic development. Matrix Metalloproteinases (MMPs) have been shown to play significant roles in a number of physiological processes, including embryogenesis and angiogenesis, they also contribute to the development of pathological processes. Thus, gelatin zymography technique was set up and performed for detection of MMPs enzymatic activity in the umbilical cords. At delivery, mothers consented for umbilical cords of fetus and for their hair sample be taken. By the administration of a questionnaire (Appendix A) were obtained data of historical residence of the parents of the malformed fetuses (last 10 years), genetic background, reproductive history, exposures to environmental risks, to use of chemicals. This was important in order to analyze two important aspects in the study of the malformation: the possible genetic background of the family and potential risks arising from the work environment. Data of historical exposure (time of living in a place) are very important to establish correlations between a possible environmental effect and a phenotype (fetal malformation). The metal contamination was measured by Prof. Stefania Papa, Seconda Università di Napoli, and by analyzing the concentrations of the metals in mother hair during the first trimester of pregnancy. The analysis of mother hair for about 25 carcinogenic, teratogenic and toxic metals was performed by atomic spectrometry, the method

recommended by the International Atomic Energy Agency (IAEA) to verify the individual exposure to metal contamination present in the environment (Bass *et al.*, 2001). Quali-quantitative measures of the same metals were also performed on some umbilical cord samples.

Figure 3.1. "Land of fires". The umbilical cords were collected by therapeutic abortions of mothers living for more than 10 years in the towns of Acerra, Casal di Principe, Casalnuovo di Napoli and Santa Maria Capua Vetere.

3.1 Umbilical cords from healthy fetuses

In order to set up the experimental protocols, umbilical cords from healthy fetuses were collected by Dr. G. Rivezzi of the Caserta Hospital "Sant'Anna e San Sebastiano" (Table 3.1). The samples were transported in dry ice, dissected into aliquots of about 100 mg, washed with cold phosphate-buffered saline (PBS: 10 mM NaH_2PO_4; 2.7 mM KCl; 137 mM NaCl; pH 7.4) to remove contaminating blood and stored at -80°C.

Table 3.1. Umbilical cords from healthy fetuses.

	DIAGNOSIS	WEEKS OF GESTATION	DATE
GR1	Healthy	36	2014
GR2	Healthy	36	2014
GR3	Healthy	34	2014
GR4	Healthy	32	2014
GR5	Healthy	36	2014
GR6	Healthy	36	2014
GR7	Healthy	38	2014
GR8	Healthy	39	2014
GR9	Healthy	36	2014
GR10	Healthy	36	2014

3.2 Protein extraction and quantitative analysis

Umbilical cord proteins were extracted as previously described (see "materials and methods"). The protein concentration of each extract was determined according to the Bradford method (Bradford, 1976). The protein concentrations measured for the different extracts were summarized in the Table 3.2.

Table 3.2. Protein extracts.

Sample	Protein concentration (µg/µl)
GR1	3,20
GR2	14,96
GR3	7,09
GR4	12,73
GR5	3,75
GR6	9,20
GR7	17,32
GR8	5,17

3.3 One-dimensional SDS-PAGE and in-gel digestion

In a first step the umbilical cords were analyzed by one-dimensional SDS-PAGE approach, followed by 'in gel' enzymatic digestion with trypsin. Each protein extract (70 µg) was dissolved in SDS-PAGE loading buffer and loaded in triplicate on the polyacrylamide gel (Figure 3.2).

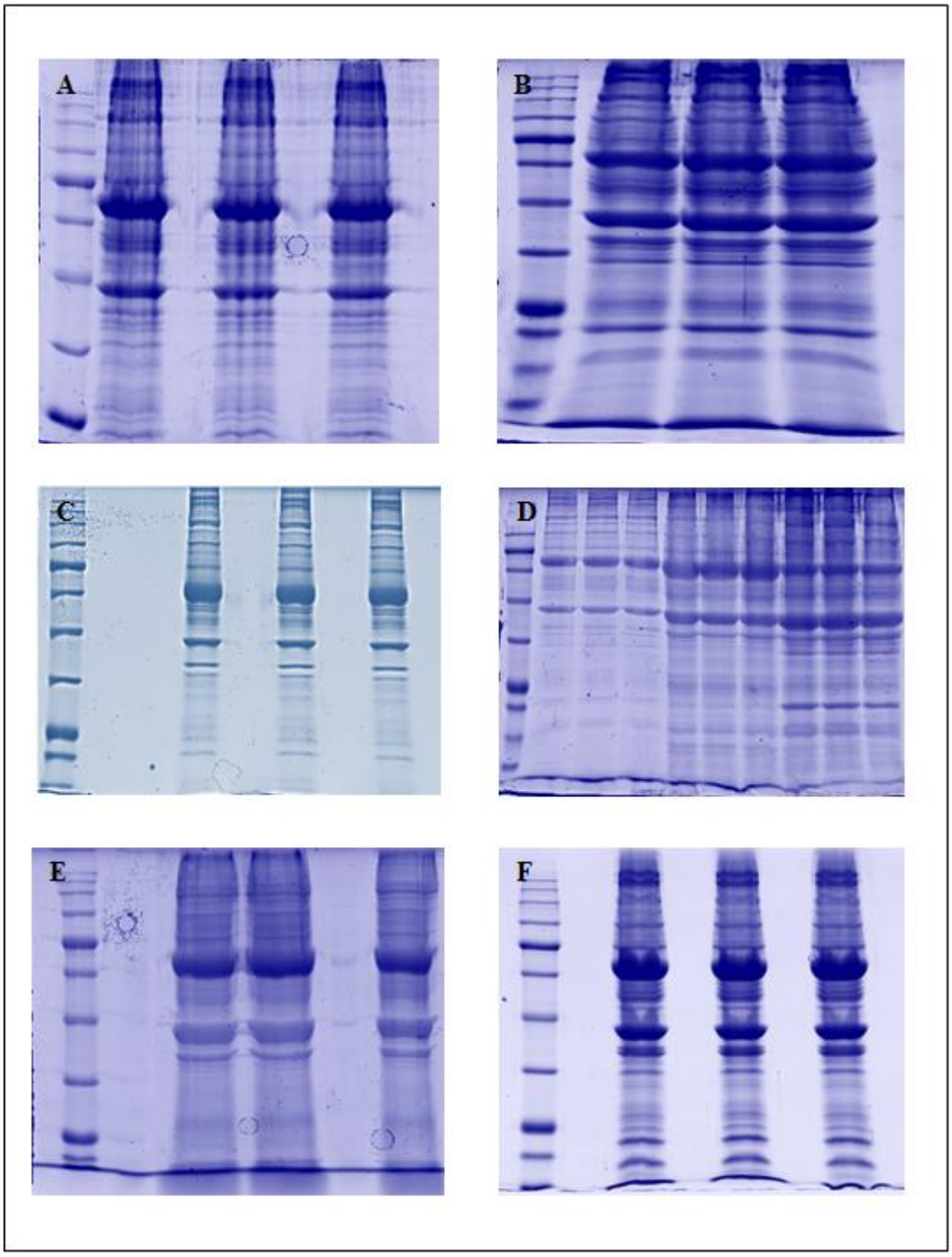

Figure 3.2. One-dimensional SDS-PAGE of protein extracts of umbilical cords from healthy fetuses: A) GR1; B) GR2; C) GR3; D) GR4, GR5, GR6; E) GR7; F) GR8. Each sample was analyzed in triplicate.

Resulting electrophoretic lanes were cut in 10 slices, that underwent in-gel tryptic digestion. Peptides were extracted, dried in a vacuum centrifuge, and resuspended for mass spectrometry-based analysis.

3.3.1 Mass spectrometry-based protein identification

The peptides resulting from the digestion were analyzed by nanoLC/MS/MS.
The protein identification was performed by Mascot search engine against the Swiss-Prot Protein Database. Mascot search engine assigns at each protein the parameter emPAI (exponentially modified Protein Abundance Index) for the absolute quantization of the protein in complex protein mixtures (Ishihama *et al.*, 2005).
Three lists of identified proteins for each sample were obtained (Table 3.3).

Table 3.3. Three lists of proteins for each sample identified by Mascot search engine.

Score	Mass	Mat-ches	Pep (sig)	Seq	Seq (sig)	emPAI	Protein names
28	21542	6	1	5	1	0,18	1,2dihydroxy3-keto-5-methylthiopentene dioxygenase
46	80879	12	1	12	1	0,05	1,4-alpha-glucan-branching enzyme
210	28179	32	8	16	7	1,74	14-3-3 protein beta/alpha
252	29326	36	9	15	6	1,07	14-3-3 protein epsilon
132	28372	29	4	16	4	0,65	14-3-3 protein eta
207	28456	29	6	11	3	0,87	14-3-3 protein gamma
62	27871	21	3	11	3	0,47	14-3-3 protein sigma
215	28032	25	6	11	2	0,66	14-3-3 protein theta
551	27899	39	17	18	9	4,25	14-3-3 protein zeta/delta
25	32038	34	1	8	1	0,12	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha
20	42387	11	2	10	1	0,09	1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon
28	122861	16	1	14	1	0,03	2'-5'-oligoadenylate synthase 3
47	37060	6	1	6	1	0,1	26S proteasome non-ATPase regulatory subunit 7
51	39872	14	1	13	1	0,09	26S proteasome non-ATPase regulatory subunit 8
30	27134	11	1	9	1	0,14	3-hydroxyacyl-CoA dehydrogenase type-2
68	32707	6	1	6	1	0,12	3-hydroxyanthranilate 3,4-dioxygenase
Score	**Mass**	**Mat-ches**	**Pep (sig)**	**Seq**	**Seq (sig)**	**emPAI**	**Protein names**
49	80879	12	2	10	2	0,09	1,4-alpha-glucan-branching enzyme
346	28179	38	7	16	3	0,46	14-3-3 protein beta/alpha
370	29326	51	11	17	5	0,84	14-3-3 protein epsilon
339	28456	38	8	15	3	0,65	14-3-3 protein gamma
399	28032	26	8	9	3	0,66	14-3-3 protein theta
644	27899	42	15	13	6	1,78	14-3-3 protein zeta/delta
44	32038	49	4	8	1	0,12	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha
18	30074	48	1	8	1	0,13	2-aminoethanethiol dioxygenase
23	26842	21	1	13	1	0,14	40S ribosomal protein S3
26	32947	14	1	12	1	0,11	40S ribosomal protein SA
15	122531	43	1	29	1	0,03	5-azacytidine-induced protein 1
39	61187	21	2	14	2	0,13	60 kDa heat shock protein, mitochondrial
34	61372	32	2	14	2	0,12	60 kDa SS-A/Ro ribonucleoprotein
34	11621	5	1	2	1	0,34	60S acidic ribosomal protein P1
158	86454	48	10	30	10	0,52	6-phosphofructokinase type C
307	72402	41	10	32	10	0,65	78 kDa glucose-regulated protein
Score	**Mass**	**Mat-ches**	**Pep (sig)**	**Seq**	**Seq (sig)**	**emPAI**	**Protein names**
128	28179	30	3	18	2	0,46	14-3-3 protein beta/alpha
88	29326	21	2	14	2	0,28	14-3-3 protein epsilon
56	28372	22	1	14	1	0,13	14-3-3 protein eta
196	28456	27	6	12	3	0,65	14-3-3 protein gamma
29	27871	20	1	12	1	0,14	14-3-3 protein sigma
61	28032	18	1	12	1	0,13	14-3-3 protein theta
468	27899	36	11	23	7	2,15	14-3-3 protein zeta/delta
38	32038	33	6	4	1	0,12	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha
21	53270	19	1	14	1	0,07	26S proteasome non-ATPase regulatory subunit 12
17	12621	15	1	4	1	0,31	28S ribosomal protein S33, mitochondrial
21	27134	10	1	8	1	0,14	3-hydroxyacyl-CoA dehydrogenase type-2
28	18886	4	1	4	1	0,2	40S ribosomal protein S10
40	13791	6	1	4	1	0,29	40S ribosomal protein S25
32	23033	8	1	4	1	0,17	40S ribosomal protein S5
63	11621	5	2	3	1	0,34	60S acidic ribosomal protein P1
33	11658	5	1	4	1	0,34	60S acidic ribosomal protein P2

3.3.2 Assessment of technical variability

The three lists were aligned manually on the basis of the quantitative mascot parameter (emPAI) in order to calculate mean and standard deviation for the measured amount of each protein. Each protein may be present in only one, two or all three lists. Where a protein wasn't detected, a zero emPAI value was added.
Finally a single list of proteins (~ 1000 identified proteins) was obtained for each sample, where a mean emPAI value and the relative standard deviation was reported for each protein (Table 3.4).
It wasn't possible to calculate mean and standard deviation for the samples GR1 and GR2, because their three lists were very different as number of identified proteins. Therefore, the samples GR1 and GR2 were discarded.

Table 3.4. Mean and standard deviation of proteins for each sample.

Protein names	**Mean**	**Standard deviation**
14-3-3 protein beta/alpha	1,10	0,91
14-3-3 protein epsilon	0,68	0,56
14-3-3 protein eta	0,39	0,37
14-3-3 protein gamma	0,76	0,16
14-3-3 protein sigma	0,31	0,23
14-3-3 protein theta	0,40	0,37
14-3-3 protein zeta/delta	3,20	1,48
1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	0,12	0,00
3-hydroxyacyl-CoA dehydrogenase type-2	0,14	0,00
40S ribosomal protein S10	0,20	0,00
60S acidic ribosomal protein P1	0,34	0,00
6-phosphofructokinase type C	0,29	0,16
Actin, aortic smooth muscle	46,50	8,49
Actin, cytoplasmic 1	13,83	2,67
Actin-related protein 2	0,08	0,00
Actin-related protein 2/3 complex subunit 4	0,19	0,00
Adapter molecule crk	0,11	0,00
Adenosylhomocysteinase	0,12	0,06
Adenylate kinase isoenzyme 1	0,63	0,00
Adenylyl cyclase-associated protein 1	0,28	0,06
Adenylyl cyclase-associated protein 2	0,07	0,00
Adipocyte enhancer-binding protein 1	0,06	0,04
ADP-ribosylation factor 1	0,82	0,22
Afamin	0,05	0,00
Aldehyde dehydrogenase X, mitochondrial	0,17	0,06
Alpha-1-antichymotrypsin	0,26	0,13
Alpha-1-antitrypsin	3,14	0,23
Alpha-2-HS-glycoprotein	0,20	0,16

Subsequently, the intra-sample variability was calculated with the following mode (Auger *et al.*, 2000):

$$\textbf{Intra-sample variability} = \frac{\text{Mean of the standard deviations}}{\text{Mean of the means}} \times 100$$

The intra-sample variabilities were reported in the Table 3.5.

Table 3.5. Intra-sample variabilities.

Sample	Intra-sample variability
GR3	67,23%
GR4	24,28%
GR5	37,81%
GR6	66,53%
GR7	101,22%
GR8	39,33%

In order to minimize these variabilities, the proteins present in only one of the three lists were omitted, while the proteins present in at least two lists on three were considered. The intra-sample variabilities thus were recalculated and reported in Table 3.6.

Unfortunately, they were still too high and actually not satisfactory.

Table 3.6. Intra-sample variabilities recalculated.

Sample	Intra-sample variability
GR3	65,35%
GR4	21,86%
GR5	29,71%
GR6	63,71%
GR7	100,22%
GR8	34,20%

3.4 Two-dimensional gel electrophoresis (2-DE) and image analysis

In order to overcome the high technical variabilities showed by one-dimensional SDS-PAGE analysis, it was then set up a proteomic approach based on two-dimensional gel electrophoresis (2-DE). All 2-DE experiments were performed on test samples of umbilical cords from healthy term fetuses. The umbilical cord proteins were separated by IEF using 18 cm IPG strips (pH 3-10NL; GE Healthcare) on the basis of their charge (separation in first dimension) and by SDS-PAGE on the basis of their molecular weight (separation in second dimension). The result is an array of protein spots, characterized by precise coordinates x and y: isoelectric point (pI) and apparent molecular weight (Mw), respectively. Each spot potentially corresponds to a single protein in the sample (Figure 3.3). The gel image analysis was performed by ImageMaster 2D Platinum 6.0 software (GE Healthcare) according to manufacturer's instructions; the most significant spots, were subjected to 'in gel' enzymatic digestion and LC/MS/MS analysis for protein identification. The image analysis included spot detection, gel-to-gel matching and spot quantification (Raman *et al.*, 2002). Manual editing (e.g. deletion of false protein spots and correction of spot shape) during the spot detection is nearly impossible to avoid due to complexity of spots on a gel as well as manual matching because of dissimilarity of spot positions. However, an excessive manual interventions should be avoided to increase the reliability of the image control and the reproducibility of the procedure (Magdeldin *et al.*, 2014). Ambiguous regions, such as crowded areas or areas containing high-molecular-weight proteins that were not well defined, were discarded. Some of the spots detected at the boundaries of the gels and not satisfactorily resolved were also excluded from further analysis. Therefore, the number of the spots detected was always below that expected from the resolution capability of the technique; this result is related to our choose to use a conservative approach for the analysis of the gels, counting only clearly defined spots and not considering outermost and confusing areas.

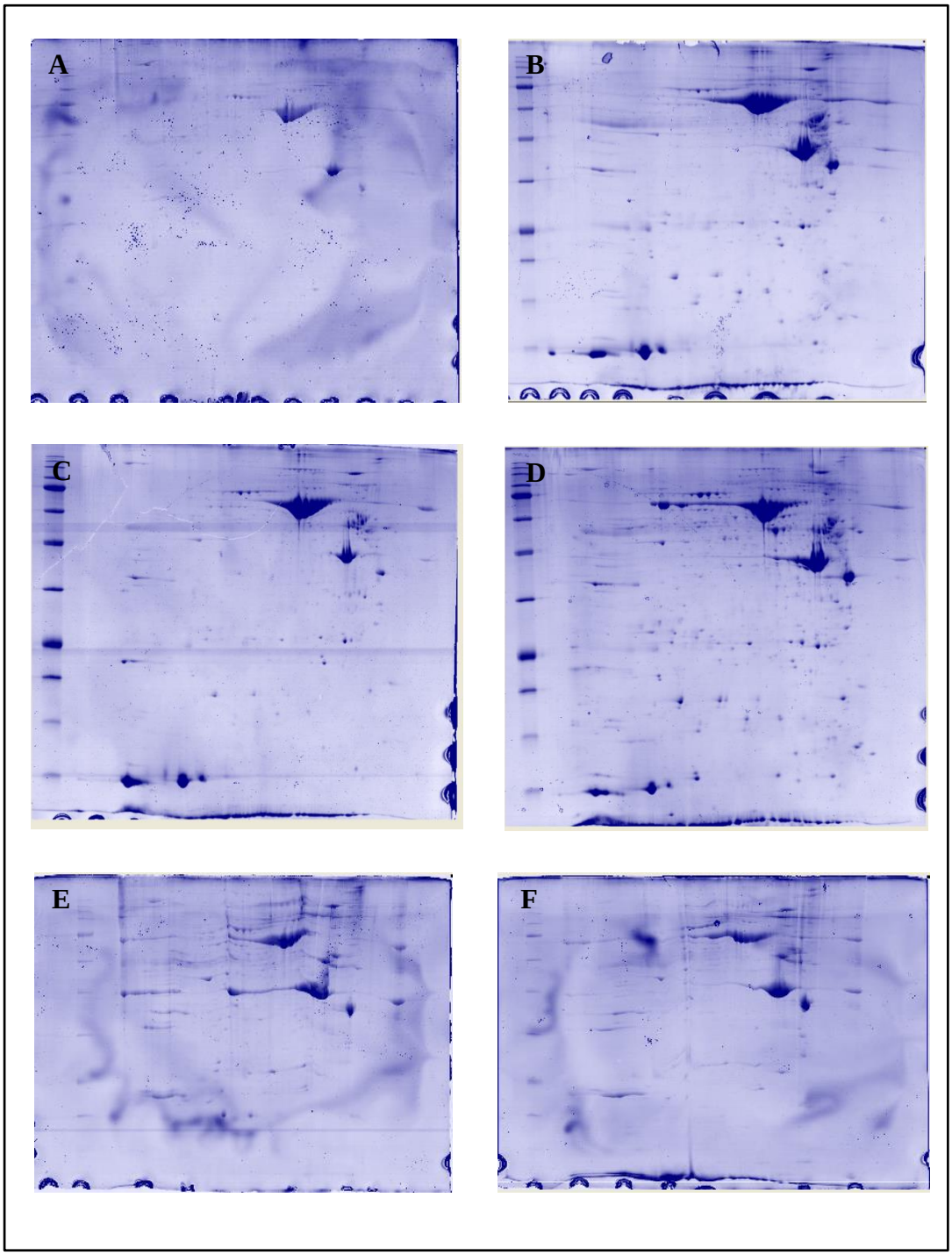

Figure 3.3. 2-DE of protein extracts of umbilical cords from healthy fetuses: A) GR1; B) GR2; C) GR3; D) GR4; E) GR6; F) GR7.

3.4.1 Limitations of 2-DE

Several limitations intrinsically related to the use of a 2-DE electrophoresis-based approach emerged. Firstly, this project involved the screening of a large number of clinical samples; since 2-DE is labor-intensive and has a relative low throughput, that represented a serious obstacle for a project like this.

Secondarily, there could also be a progressive loss of basic proteins during long running of electro-focusing under electric field, leading to low reproducibility and requiring careful monitoring of electric field (Magdeldin *et al.*, 2012).

Different types of proteins can always be missing, due to the difficulty in separating membrane-bound (hydrophobic) and extreme proteins (highly acidic or basic) (Wilkins *et al.*, 1998).

Low dynamic range of proteins is one of the challenging problems encountered during 2-DE. Low-abundance proteins are rarely seen on traditional 2D maps because large quantities of abundant soluble proteins obscure their detection. Moreover, the visualization of faint protein spots (low abundant) separated on 2-DE gels is also governed by staining sensitivity (Chevalier, 2010).

Spot detection software sometimes lead to missing some data because of the mismatching error resulted due to shifted spots between gels or spots overlapping. Furthermore, false positive spots are commonly detected due to staining artifacts. The relationship between the original protein quantity in the sample and the measured spot intensity is affected by various intervening factors (e.g., sample loss occurring during the IEF or while transferring to the second dimension) (Magdeldin *et al.*, 2014).

Finally, the umbilical cord is a heterogeneous tissue containing several different cell types, including mesenchymal stem cells, epithelial cells and endothelial cells. Several umbilical cords had also a significant contamination by blood that represented a serious obstacle for the success of the technique.

Therefore, all these reasons led us to introduce a different gel-free proteomic approach based on “Filter-Aided Sample Preparation (FASP)” technique.

3.5 Umbilical cords from malformed fetuses

The umbilical cords obtained by therapeutic abortions were collected in collaboration with Dr. G. Rivezzi of the Caserta Hospital "Sant'Anna e San Sebastiano" and with Dr. C. Ciccone of the Avellino Hospital "San Giuseppe Moscati" (consent of the Ethics Committee, January 2014). Twentyfive umbilical cords obtained by therapeutic abortions were collected; 20 samples were from fetuses affected by different malformations and 5 were control samples from healthy fetuses (psychiatric abortions) with similar gestational age (Table 3.7). The umbilical cords collected at the Caserta Hospital were indicated with the letter G, while the umbilical cords collected at the Avellino Hospital were indicated with the letters CC.

Table 3.7. Umbilical cords obtained by therapeutic abortions.

	DIAGNOSIS (WEEKS OF GESTATION ~19-21)	**DATE**
CONTROLS		
CCP PSI 1	Healthy	2014
CCP PSI 2	Healthy	2015
CC3C(15)	Healthy	2015
CCP PSI 3	Healthy	2015
CC(15)D	Healthy	2015
PATHOLOGICALS		
CC10	Trisomy 21	2014
G7	Trisomy 21	2014
CC31	Diaphragmatic hernia	2014
CC32	Non-immune hydrops	2014
CC33	Cystic hygroma and atrioventricular canal defect	2014
CC35	Congenital heart disease	2014
G6	Trisomy 21	2014
G9	49,XXXXY syndrome	2014
G16	47,XXY (Klinefelter syndrome)	2015
G5	Trisomy 21	2015
CC1(15)	Neural tube defect – multiple	2015
CC5(15)	Neural tube defect	2015
G1	Trisomy 21 + heart disease	2015
G2	Dyssegmental dysplasia, Silverman-Handmaker	2015
G3	Trisomy 21	2015
G4	Trisomy 21	2015
G11	Neural tube defect	2015
CC7(15)	Gastrointestinal disease	2015
CC8(15)	47,XXY (Klinefelter syndrome)	2015
CC9(15)	Congenital heart disease, cleft lip and palate, neural tube defect	2015

The samples were transported in dry ice, dissected into aliquots of about 100 mg, washed with cold PBS to remove contaminating blood and stored at -80°C for further analysis.

There were several problems for the collection of umbilical cords, such as:

– authorization by the Ethics Committee of the Caserta Hospital;

– consent denied by some mothers;
– impossibility by some gynecologists to collect and properly treat the material collected (-80°C in cryogenic liquid), in particular when the therapeutic abortion was performed during the night shift;
– detachment of the liquid nitrogen dewar at the Avellino Hospital and loss of all umbilical cords collected in the end of 2015 and all 2016 (Figure 3.4).


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CORRIERE

Giornale dell'Irpinia fondato da Gianni Festa

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UNA SOLA SOSTA E COMPRI TUTTO
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Ricerca in fumo

Ospedale Moscati di Avellino, due anni di ricerca sui feti malformati in fumo. Si tratta di sabotaggio interno o di negligenza? A breve potrebbe essere avviata un'indagine interna per chiarire la vicenda. E non è detto che non si investa anche la Procura della Repubblica, visto che gli studi erano stati garantiti tramite un finanziamento di 27 mila euro della Provincia di Avellino, presidente Alberta De Simone. In questi anni si stava procedendo con la raccolta di campioni embrionali, tessuti del cordone ombelicale principalmente, per capire se vi fosse una connessione tra la malformazione e l'ambiente circostante. I contenitori per la raccolta del materiale organico avrebbero dovuto garantire una temperatura di meno ottanta gradi per la conservazione dei tessuti. Ma dallo scorso ventisei gennaio non è stato prodotto l'azoto liquido e la temperatura è vorticosamente scesa a meno venti gradi. In tal modo il lavoro di due anni dei ricercatori è andato letteralmente in fumo e il materiale raccolto non è più utilizzabile per gli scopi prefissati.

Massimo Gardini pag. 8-9

Tessuti dei cordoni inutilizzabili, la temperatura passa da -80 a -20 gradi, perchè non è stato prodotto l'azoto liquido

Editoriale

A ciascuno la sua....

di GIANNI FESTA

L'assenza di una classe dirigente che sia riferimento della politica del fare si evince da

RUBRICHE

DIARIO
Domenico Gallo Pag.4

CARTA STRACCIA
Gianni Marino Pag. 4

IRPINIAITALIA
Angelo Flammia Pag. 4

Figure 3.4. Avellino Hospital "San Giuseppe Moscati": temperature of the dewar passed from -80°C to -20°C for missed production of liquid nitrogen and loss of all samples collected in the end of 2015 and all 2016.

3.6 Protein extraction and quantitative analysis

Umbilical cord proteins were extracted as previously described. The protein concentration of each extract was determined according to the *DC* Protein Assay (Bio-Rad), and the protein concentrations measured for the different extracts were summarized in the Table 3.8.

Table 3.8. Protein extracts.

Sample	Protein concentration (µg/µl)
CCP PSI 1	1,78
CCP PSI 2	1,44
CCP PSI 3	2,01
CC3C(15)	3,13
CC(15)D	2,13
CC10	6,10
CC31	2,85
CC32	2,81
CC33	0,50
CC35	3,95
CC1(15)	3,23
CC5(15)	2,93
CC7(15)	2,26
CC8(15)	2,51
CC9(15)	4,11
G1	3,47
G2	3,58
G3	2,46
G4	3,31
G5	2,34
G6	1,98
G7	3,33
G9	2,39
G11	2,62
G16	1,98

3.7 Filter-Aided Sample Preparation (FASP)

The Filter-Aided Sample Preparation (FASP) method is actually considered a crucial technology in the sample preparation for the analysis of complex proteomes. It combines the advantages of in-gel and in-solution digestion for mass spectrometry–based proteomics. Notably, it combines a strong detergent (SDS) for universal solubilization with a chaotropic agent (8 M urea) to efficiently ‘clean up’ the proteome before digestion and to obtain purified peptides after digestion,

avoiding the disadvantages of the gel format. SDS is the reagent of choice for total solubilization of cells and tissues, used as denaturant and as an efficient inactivator of proteases (Hernandez-Valladares *et al.*, 2016). Unfortunately, it can preclude enzymatic digestion and dominate mass spectra. Therefore, depletion of SDS is a prerequisite for efficient mass-spectrometric analysis in proteomics (Wiśniewski *et al.*, 2009). The FASP method greatly reduces the time required for sample preparation and minimizes sample loss (Manza *et al.*, 2005). It enables more flexibility in sample processing, such as successive cleavage, and sample fractionation, which increases depth of proteomics analysis and facilitates identification of low abundant proteins. An ultrafiltration device was used to retain and concentrate the sample, for detergent removal, for depletion of detrimental low-molecular-weight components, for protein digestion and peptide elution. Proteins in the filter unit were alkylated in the urea buffer and were then exchanged into ammonium bicarbonate (Ambic) buffer for enzymatic digestion. Advantage of the ultrafiltration approach is the purity of the digests, which is a prerequisite for effective peptide fractionation and mass spectrometry (Wiśniewski, 2016). Filters with relative molecular mass (Mr) cut-offs of 3k and 10k were tested. The number of identified peptides with a molecular weight above about 1,5 kDa was much reduced for the 3k filter compared to the 10k filter while peptides with masses over 2,5 kDa were almost completely retained by the 3k filter. As the 10k filter efficiently retained small proteins (5–10 kDa) and efficiently released peptides up to 5 kDa, it was used as the standard in the subsequent experiments. However, it is well known that typically 40–60% of the sample can not be recovered in the filtrate because of filter clogging probably produced from poorly digested proteins, large peptides, nucleic acids and lipids. Moreover, the sample of interest can be lost in case of occasional filter failure (Hernandez-Valladares *et al.*, 2016). All umbilical cords obtained by therapeutic abortions were analyzed in duplicate. 50 µg of protein extract were loaded on each filter. The method has demonstrated to give excellent performance for samples between 25 and 100 µg of total protein. For higher loads, a continuous decrease of peptide yield was observed (Wiśniewski, 2016). 0.5 µg of trypsin (enzyme to protein ratio 1:100) were added to each filter. Peptides resulting from digestion were desalted by StageTips (C18 material 200µL) prior to LC/MS/MS analysis (Figure 3.5).

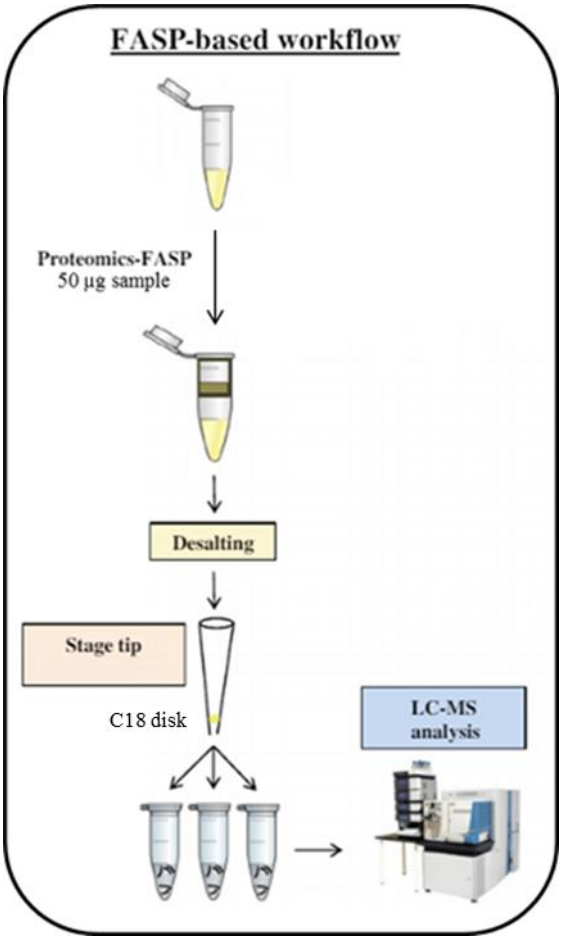

Figure 3.5. Proteomic workflow. FASP method is followed by StageTip desalting to prepare samples for the proteome data set.

3.7.1 Mass spectrometry-based protein identification

The desalted peptides resulting from the digestion were separated and analyzed by nanoLC/MS/MS (Figure 3.6). The protein identification and quantification was performed by the MaxQuant 1.5.2.8 software. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification (Cox and Mann, 2008). Its algorithm was developed to measure a new intensity determination and normalization procedure called MaxLFQ, that is fully compatible with any peptide or protein separation prior to LC-MS analysis (Cox *et al.*, 2014). An output of 531 identified proteins was thus obtained.

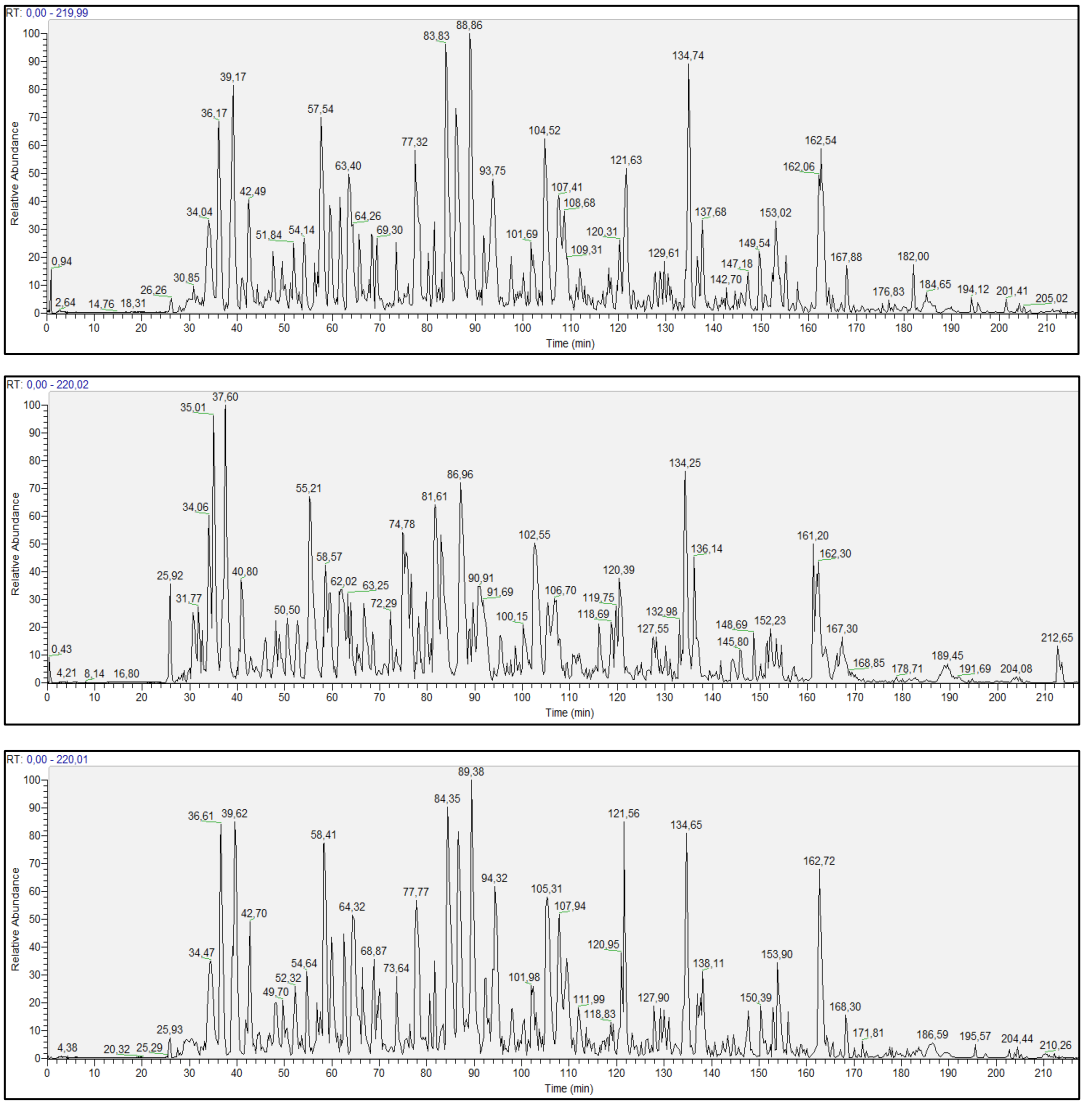

Figure 3.6. Three chromatographic profiles of umbilical cord peptides by nanoHPLC-nanoESI-MS/MS.

3.7.2 Statistical analysis

For the statistical analysis Perseus 1.5.1.6 software was employed, which is part of MaxQuant. Since the most of the samples are affected by different malformations, they were treated individually and compared with the quadruplicates of all 5 control samples pooled as a single big control sample (CTR). So in Perseus the MaxLFQ intensities of CTR and the MaxLFQ intensities of a pathological sample in quadruplicate were uploaded. Mean and standard deviation were calculated inside

the 2 groups. To identify proteins differentially expressed between the 2 groups the *t*-student test was applied, using a p value of 5% for the significances (Roxas and Li, 2008). Subsequently a ratio between pathological mean/control mean was calculated on the differentially expressed proteins. In the resulting (Table 3.9) table the proteins upregulated compared to the control group were indicated in light blue and the downregulated ones were in yellow.

Table 3.9. Upregulated and downregulated proteins obtained by pathological/control ratio.

Protein names	**Ratio CC7(15)/CTR**
Laminin subunit beta-1	N
Hemopexin	N
Heat shock protein HSP 90-beta	N
Annexin A6;Annexin	N
GTP-binding nuclear protein Ran	N
Malate dehydrogenase, cytoplasmic;Malate dehydrogenase	N
Fermitin family homolog 1;Fermitin family homolog 2	N
Angiotensinogen;Angiotensin-1;Angiotensin-2;Angiotensin-3;Angiotensin-4; Angiotensin 1-9;Angiotensin 1-7;Angiotensin 1-5;Angiotensin 1-4	N
Alpha-1-acid glycoprotein 1	N
Tropomyosin alpha-1 chain	N
Laminin subunit gamma-1	N
Nidogen-1	N
Protein-glutamine gamma-glutamyltransferase 2	N
Moesin	N
BTB/POZ domain-containing protein KCTD12	N
Mitogen-activated protein kinase kinase kinase kinase 4	N
Myb/SANT-like DNA-binding domain-containing protein 4	N
Peroxiredoxin-6	9,41
Myosin-9	6,22
78 kDa glucose-regulated protein	4,67
Myosin light polypeptide 6	0,00
Mimecan	0,00
Keratin, type II cytoskeletal 8	0,00

N = not divisible by 0, not identified in the control group

All the umbilical cords coming from fetuses affected by trisomy 21 were pooled as a single sample (TRIS21) and compared with CTR.

Fourteen different pairwise comparisons were performed. In order to identify and correlate interesting proteins to the embryonic development, common proteins in at least three on fourteen different pairwise comparisons were selected (Tables 3.10A-3.10B).

Table 3.10A. Six of fourteen different pairwise comparisons (pathological samples of 2014).

Protein names	CC31	CC32	CC33	CC35	G9	TRIS21
Tropomyosin beta chain	2,34	2,71		2,05	2,10	1,60
Myosin-11	2,70	3,89		3,35	2,73	1,81
Periostin		1,87	2,71	0,15		
Heat shock protein HSP 90-beta	N	N		N	N	N
Keratin, type II cytoskeletal 8		2,21				
Endoplasmin	3,15	2,99		3,33		6,28
Annexin A1	2,32	2,30				2,55
Annexin A2;Annexin;Putative annexin A2	1,58	1,82	0,43			1,52
Calponin-1	6,95	7,05		5,08	3,55	
Myosin light polypeptide 6	1,86	1,94				
Myosin regulatory light polypeptide 9	3,16	3,70		3,38	3,18	2,13
Heat shock-related 70 kDa protein 2	N	N		N	N	N
Collagen alpha-1(XIV) chain	34,70	71,75		22,42	39,79	41,49
Cysteine and glycine-rich protein 1	8,73	12,83		7,65	5,48	4,53
Myosin-9	6,49	8,94		8,34	6,01	7,35
Annexin A6;Annexin		N		N	N	N
Histone H1.2	15,05					42,87
Desmin		2,23				
Filamin-A	2,36	2,44				
Glutathione S-transferase P	11,26	28,52		16,56		14,12
Collagen alpha-1(XII) chain	5,94	3,12		4,00		2,69
Tubulin beta chain	5,10	8,14		5,91		
Creatine kinase B-type		N		N	N	N
Procollagen C-endopeptidase enhancer 1	N	N		N	N	N
Mimecan	2,95	2,74				1,89
Septin-7;Septin-14	N	N		N		
Protein disulfide-isomerase A3		7,71			5,76	11,34
Triosephosphate isomerase		16,08		7,51	7,06	5,33
Gelsolin		8,13		7,71	8,39	4,59
Thioredoxin domain-containing protein 5		5,61		5,01		3,58
78 kDa glucose-regulated protein		4,95				5,96
Heat shock cognate 71 kDa protein		3,17				2,12
Protein-glutamine γ-glutamyltransferase 2		N			N	
Cofilin-1			6,26		4,69	4,77
Keratin, type II cytoskeletal 5	N	N		N		
Fibulin-1	4,62	3,66		3,86		
14-3-3 protein zeta/delta	4,15	5,69				4,07
Collagen alpha-3(VI) chain	4,10	5,29		3,05		2,36
Neuroblast diff.-associated protein AHNAK	4,10					
Cytoskeleton-associated protein 4	3,34					3,37
Lumican	2,70	1,88				1,56
Decorin	2,08	1,79				2,13
Peroxiredoxin-6		9,03				
Protein S100-A11		N		N	N	
Prolargin	N	N		N	N	
L-lactate dehydrogenase B chain		N		N	N	
Heat shock 70 kDa protein 1A/1B	N	N			N	
14-3-3 protein epsilon	N		N			
Moesin		N				
Myosin regulatory light chain 12A; 12B	N	N				
Peptidyl-prolyl cis-trans isomerase A		2,81				
Transgelin-2		N		N		N
Tropomyosin alpha-3 chain	N					N
Vimentin	1,54					
Calmodulin		5,59				
Pyruvate kinase PKM;Pyruvate kinase		6,00				3,69
Tubulin beta-4B chain;Tubulin beta-4A chain			3,29			
Pigment epithelium-derived factor				4,90		
Alpha-actinin-4		3,55				
Phosphoglycerate kinase 1		6,01				3,88
Collagen alpha-1(V) chain		N			N	N
Collagen alpha-1(VI) chain	3,19	3,15				2,67
Collagen alpha-2(VI) chain	3,49	4,50				2,08
PDZ and LIM domain protein 7		N		N		
Myosin light chain kinase, smooth muscle		N		N		
Caldesmon					5,35	0,11

Table 3.10B. Eight of fourteen different pairwise comparisons (pathological samples of 2015).

Protein names	CC1 (15)	CC5 (15)	CC7 (15)	CC8 (15)	CC9 (15)	G2	G11	G16
Tropomyosin beta chain		2,65		0,00	2,85	0,00	0,00	1,62
Myosin-11		3,27		0,00	3,53	0,00	0,00	
Periostin	5,35	2,20		0,00	4,95	0,00	0,00	
Heat shock protein HSP 90-beta	N	N	N		N	N		
Keratin, type II cytoskeletal 8		2,92	0,00	0,00	2,87	12,64	0,00	
Endoplasmin	6,39	7,28			5,04	3,91		
Annexin A1		2,07		8,58	3,10	2,55	0,00	
Annexin A2;Annexin;Putative annexin A2		2,05			2,23	2,39	0,00	
Calponin-1		3,17			7,01			3,64
Myosin light polypeptide 6			0,00	0,00	1,63	0,00	0,00	
Myosin regulatory light polypeptide 9		3,82			3,80			2,40
Heat shock-related 70 kDa protein 2	N	N			N			
Collagen alpha-1(XIV) chain	13,66	71,42			13,23			
Cysteine and glycine-rich protein 1	4,02	8,48			15,93			
Myosin-9		11,12	6,22		8,84			
Annexin A6;Annexin	N	N	N		N			
Histone H1.2	67,20	44,48			29,03	35,70		20,30
Desmin		2,52		0,00	3,34	0,00	0,00	
Filamin-A		1,85			3,76	0,00	0,00	
Glutathione S-transferase P	12,94	18,68			24,22			
Collagen alpha-1(XII) chain		4,08		7,06	4,33			
Tubulin beta chain	10,40	10,96			16,79			
Creatine kinase B-type		N			N			
Procollagen C-endopeptidase enhancer 1		N						
Mimecan		1,92	0,00				0,00	
Septin-7;Septin-14		N			N			
Protein disulfide-isomerase A3	8,40	13,29			12,82			
Triosephosphate isomerase		7,40			10,99			
Gelsolin		11,66			10,81			
Thioredoxin domain-containing protein 5		7,83			7,43			
78 kDa glucose-regulated protein		7,17	4,67		7,20			
Heat shock cognate 71 kDa protein	3,75	3,45			2,55			
Protein-glutamine γ-glutamyltransferase 2	N		N					
Cofilin-1	4,80	6,59						
Keratin, type II cytoskeletal 5					N			
Fibulin-1		4,68						
14-3-3 protein zeta/delta		5,28			4,67			
Collagen alpha-3(VI) chain		3,56						
Neuroblast diff.-associated protein AHNAK	5,28	5,04			4,04			
Cytoskeleton-associated protein 4	3,77	6,85			3,97			
Lumican				0,00			0,00	
Decorin				0,00			0,00	
Peroxiredoxin-6		11,09	9,41		11,54			
Protein S100-A11					N			
14-3-3 protein epsilon	N							
Moesin	N		N					
Myosin regulatory light chain 12A; 12B					N			
Peptidyl-prolyl cis-trans isomerase A		2,11			3,15			
Transgelin				0,00		0,00	0,00	
Transgelin-2					N			
Tropomyosin alpha-3 chain					N	N		
Vimentin				0,00			0,01	
Calmodulin				8,43	4,81			
Pyruvate kinase PKM;Pyruvate kinase	5,07				4,08			
Tubulin beta-4B chain;Tubulin beta-4A chain		2,55			3,20			
Pigment epithelium-derived factor		4,23			6,41			
Alpha-actinin-4		4,12			5,61			
Phosphoglycerate kinase 1		6,26			5,57			
Collagen alpha-1(V) chain					N			
Collagen alpha-1(VI) chain		2,41						
Collagen alpha-2(VI) chain		3,16						
PDZ and LIM domain protein 7					N			
Myosin light chain kinase, smooth muscle					N			
Caldesmon		5,99			9,00			

3.7.3 Gene Ontology (GO)

The obtained differentially expressed proteins were subjected to GO classification via the PANTHER Classification System database (http://www.pantherdb.org/). PANTHER is a comprehensive system that combines gene function, ontology, pathways and statistical analysis tools, enabling to analyze large-scale, genome-wide data from sequencing, proteomics or gene expression experiments. It is widely used by bench scientists, bioinformaticians, computer scientists and systems biologists (Mi *et al.*, 2013). The proteins identified in this study were classified according to the 3 GO terms (molecular function, cellular component and biological process) and to PANTHER pathways. According to molecular function, most of the differently expressed proteins showed structural molecule activity (38.8%), followed by binding (31.0%) and catalytic activity (27.6%) (Figure 3.7).

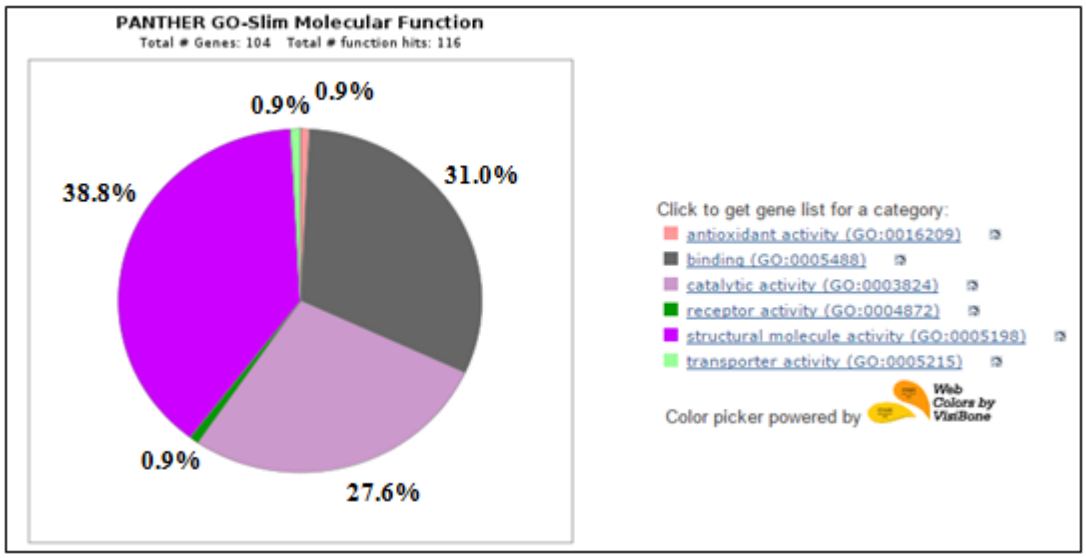

Figure 3.7. Classification of proteins according to molecular function (www.pantherdb.org/).

These proteins were then classified according to their cellular localization. Cellular components were mainly distributed in cell (40.5%) and organelle (31.9%) structures, but they displayed scattering into cell junction (6.0%), membrane (6.0%), macromolecular complex (5.2%), extracellular region (5.2%) and extracellular matrix (5.2%) (Figure 3.8).

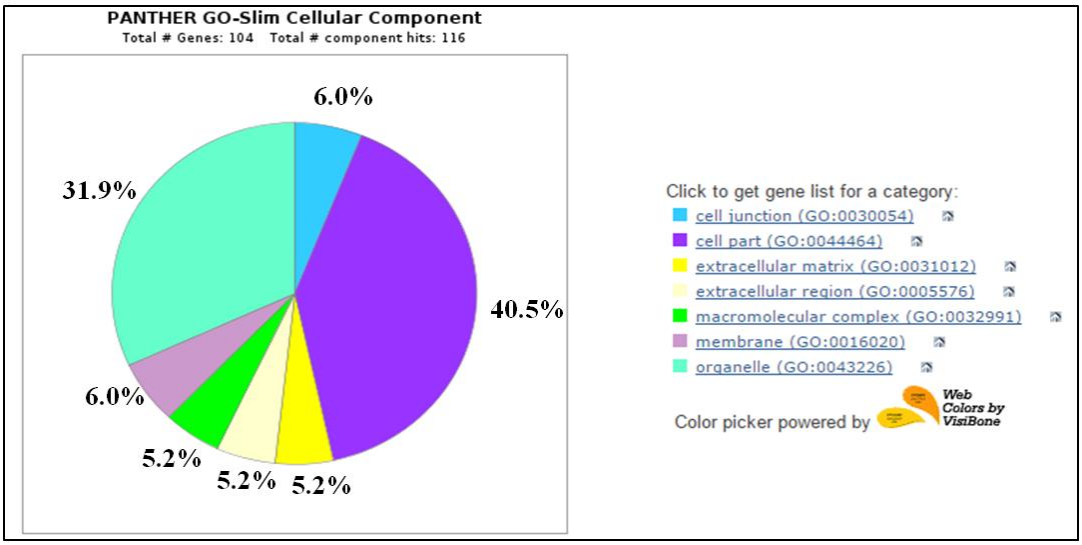

Figure 3.8. Classification of proteins according to cellular localization (www.pantherdb.org/).

In addition, the proteins were also analyzed according to PANTHER pathway database. Pathway analysis revealed that many of the proteins detected participate in: cytoskeletal regulation (15.8%), inflammation mediated by chemokine and cytokine signaling pathway (13.7%), integrin signaling pathway (9.5%), nicotinic acetylcholine receptor signaling pathway (7.4%), Parkinson (6.3%) and Huntington (6.3%) diseases (Figure 3.9).

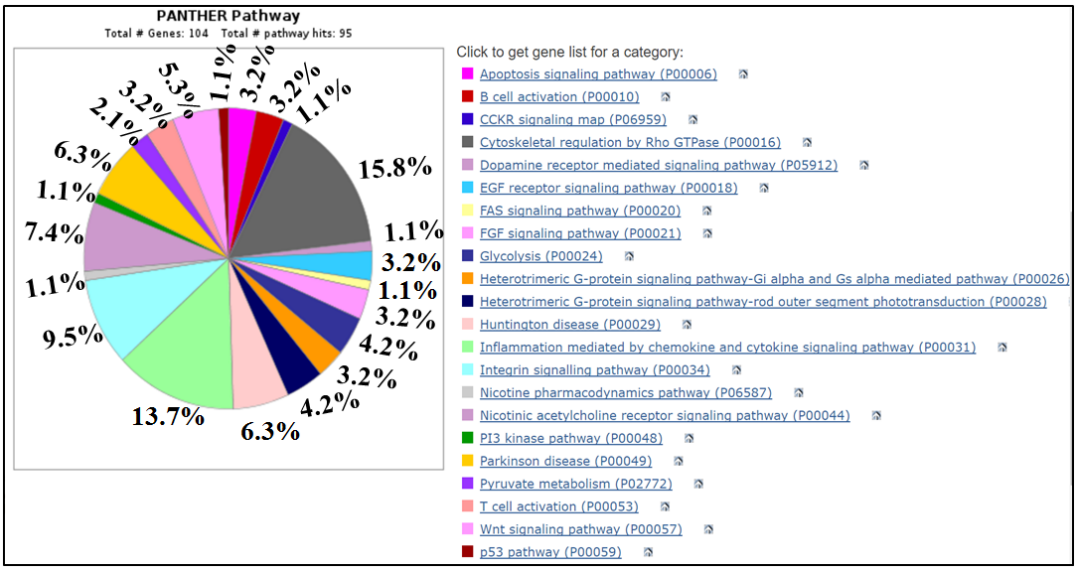

Figure 3.9. Classification of proteins according to PANTHER pathway database (www.pantherdb.org/).

Biological process classification revealed that most of the differentially expressed proteins were involved in cellular processes (30.0%), cellular component organization or biogenesis (19.5%), metabolic processes (13.6%), multicellular organismal processes (10.0%) and developmental processes (8.6%) (Figure 3.10).

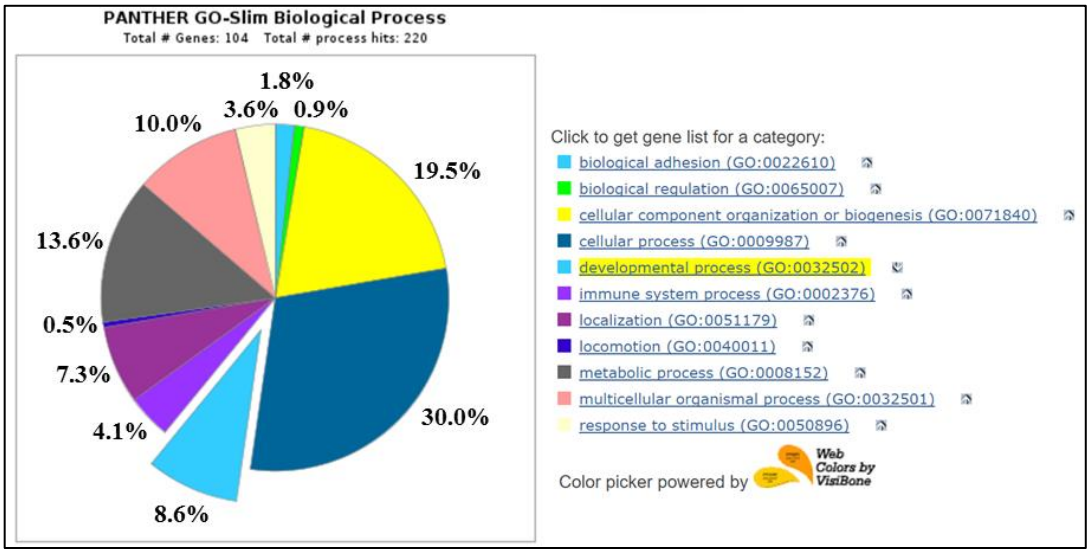

Figure 3.10. Classification of proteins according to biological process (www.pantherdb.org/).

Moreover, the proteins involved in developmental processes were identified and reported in Table 3.11.

Table 3.11. Main proteins involved in developmental processes.

Protein name	Gene
Mimecan	OGN
Myosin regulatory light chain 12A	MYL12A
Myosin-3	MYH3
Myosin-11	MYH11
Collagen alpha-1(V) chain	COL5A1
PDZ and LIM domain protein 7	PDLIM7
Tropomyosin beta chain	TPM2
Procollagen C-endopeptidase enhancer 1	PCOLCE
Fibulin-1	FBLN1
Myosin-7	MYH7
Cysteine and glycine-rich protein 1	CSRP1
Tropomyosin alpha-3 chain	TPM3
Myosin-13	MYH13
Lumican	LUM
Myosin-7B	MYH7B
Prolargin	PRELP
Myosin-6	MYH6
Myosin-9	MYH9
Collagen alpha-1(VI) chain	COL6A1

Developmental process (GO:0032502) was further investigated and sub-classified (Figure 3.11). The two most represented classes of proteins were system (34.3%) and mesoderm (34.3%) development proteins. Also proteins involved in the ectoderm development were present (17.1%). Proteins involved in the cell differentiation (8.6%) and in the anatomical structure morphogenesis (5.7%) were also observed.

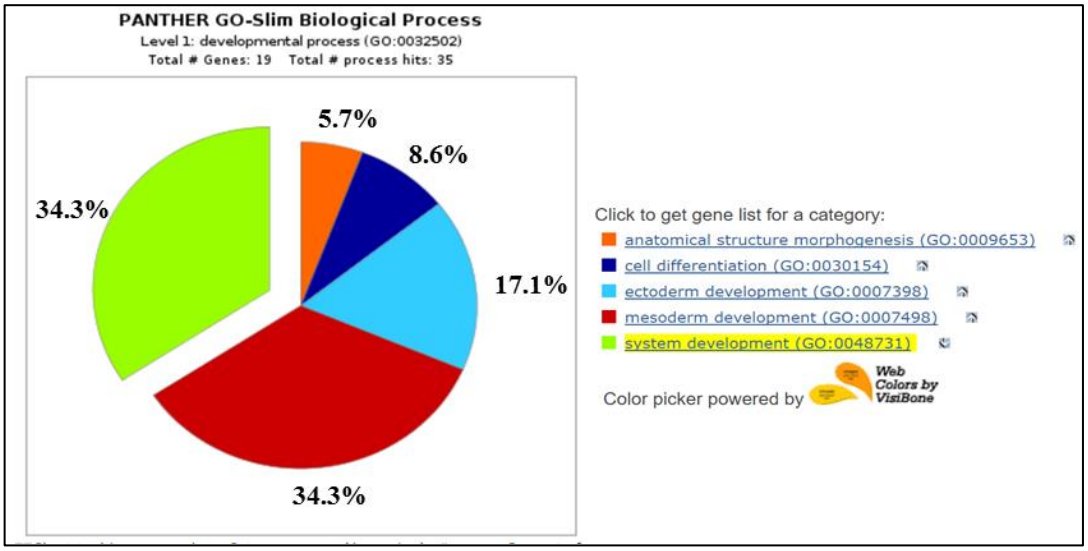

Figure 3.11. Classification of proteins according to developmental process (www.pantherdb.org/).

Finally, the proteins were annotated according to system development (GO:0048731) (Figure 3.12). In this category, most of the proteins were those involved in the muscle organ development (44.4%). The next biggest group contains proteins involved in the nervous system development (27.8%). There were also some proteins which participate in the development of the heart (11.1%), of the skeletal system (11.1%), and in the angiogenic process (5.6%).

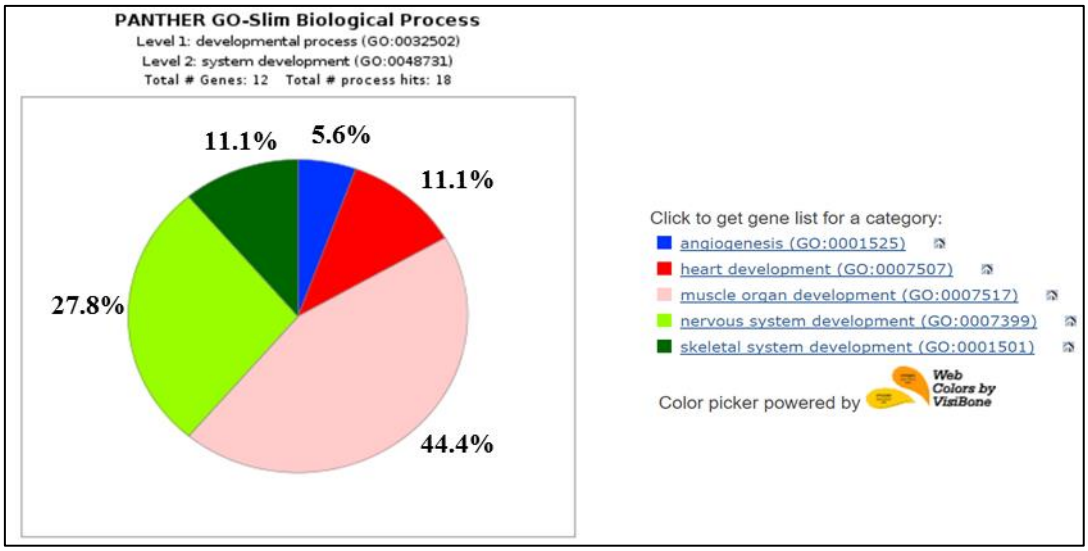

Figure 3.12. Classification of proteins according to system development (www.pantherdb.org/).

The proteins annotated as involved in system development were reported in Table 3.12.

Table 3.12. Main proteins involved in system development.

Protein name	Gene	System development
Myosin-11	MYH11	muscle organ
PDZ and LIM domain protein 7	PDLIM7	muscle organ, heart, skeletal system
Myosin-7	MYH7	muscle organ
Cysteine and glycine-rich protein 1	CSRP1	muscle organ
Tropomyosin alpha-3 chain	TPM3	muscle organ, nervous system
Myosin-7B	MYH7B	muscle organ
Myosin-6	MYH6	muscle organ
Myosin-9	MYH9	muscle organ
Mimecan	OGN	nervous system
Procollagen C-endopeptidase enhancer 1	PCOLCE	nervous system, heart, skeletal system, angiogenesis
Lumican	LUM	nervous system
Prolargin	PRELP	nervous system

We investigated the proteins involved in system development in the umbilical cords affected by malformations of the nervous system: CC1(15) (Neural tube defect – multiple), CC5(15) (Neural tube defect), CC9(15) (Neural tube defect, congenital heart disease, cleft lip and palate), G11 (Neural tube defect) (Table 3.13). Unfortunately, the low number of samples collected did not allow us to achieve accurate evaluations.

Table 3.13. Differentially expressed proteins in the samples affected by malformations of the nervous system.

Protein name	System development	CC1 (15)	CC5 (15)	CC9 (15)	G11
Myosin-11	muscle organ		3,27	3,53	0,00
Cysteine and glycine-rich protein 1	muscle organ	4,02	8,48	15,93	
Myosin-9	muscle organ		11,12	8,84	
Procollagen C-endopeptidase enhancer 1	nervous system, heart, skeletal system, angiogenesis		N		
Mimecan	nervous system		1,92		0,00
Lumican	nervous system				0,00
Tropomyosin alpha-3 chain	muscle organ, nervous system			N	
PDZ and LIM domain protein 7	muscle organ, heart, skeletal system			N	

The differentially expressed proteins identified in the umbilical cords affected by trisomy 21 (TRIS21) were classified according to the 3 GO terms (molecular function, cellular component and biological process). According to molecular function, the majority of the proteins showed binding activity (42.3%), followed by catalytic activity (33.3%) and structural molecule activity (19.2%) (Figure 3.13).

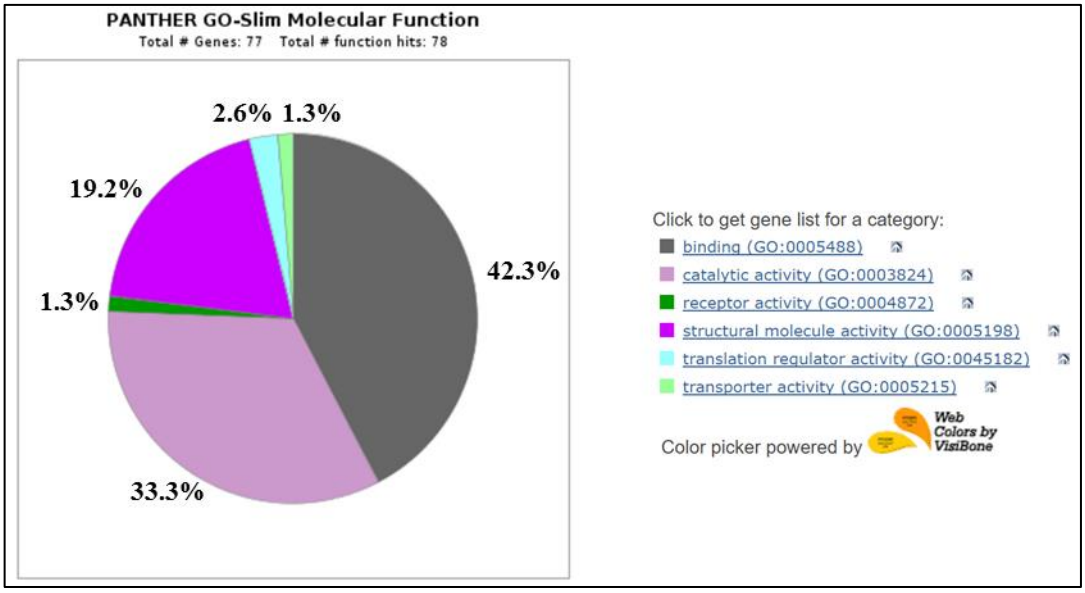

Figure 3.13. Classification of proteins identified in TRIS21 according to molecular function (www.pantherdb.org/).

The differentially expressed proteins identified in TRIS21 were then classified according to their cellular localization. Cellular components were mainly distributed in cell (37.7%) and organelle (20.8%) structures, but they displayed scattering into cell junction (13.2%), membrane (13.2%), extracellular region (7.5%) and extracellular matrix (7.5%) (Figure 3.14).

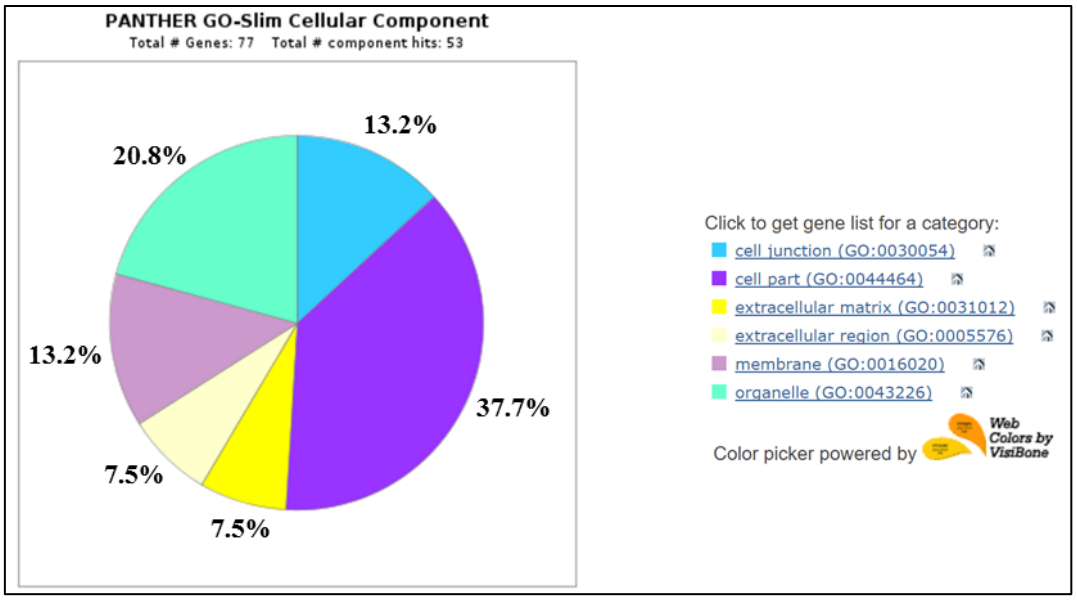

Figure 3.14. Classification of proteins identified in TRIS21 according to cellular localization (www.pantherdb.org/).

Biological process classification of the differentially expressed proteins identified in TRIS21 revealed that the most proteins were involved in cellular processes (23.2%), metabolic processes (22.1%), cellular component organization or biogenesis (17.7%), multicellular organismal processes (9.4%) and developmental processes (8.3%) (Figure 3.15).

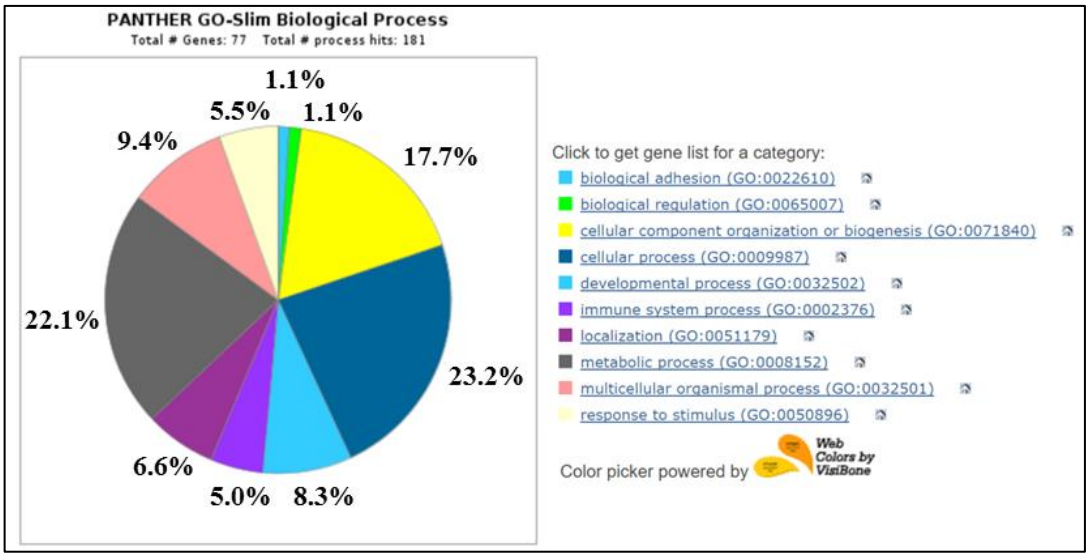

Figure 3.15. Classification of proteins identified in TRIS21 according to biological process (www.pantherdb.org/).

The differentially expressed proteins identified in the umbilical cord CC35 (Congenital heart disease) were classified according to the 3 GO terms.

According to molecular function, the most proteins showed structural molecule activity (36.6%), followed by binding activity (32.4%) and catalytic activity (25.4%) (Figure 3.16).

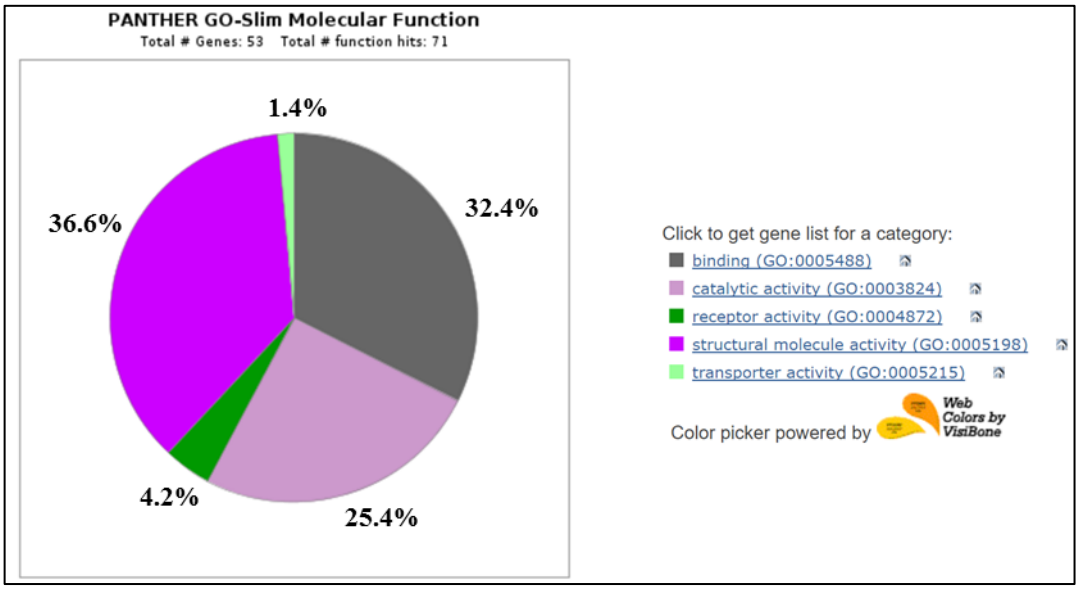

Figure 3.16. Classification of proteins identified in CC35 according to molecular function (www.pantherdb.org/).

The differentially expressed proteins identified in CC35 were then classified according to their cellular localization. Cellular components were mainly distributed in cell (34.6%) and organelle (29.5%) structures, but they displayed also cell junction (9.0%), membrane (9.0%), extracellular region (7.7%), macromolecular complex (5.1%) and extracellular matrix (5.1%) (Figure 3.17).

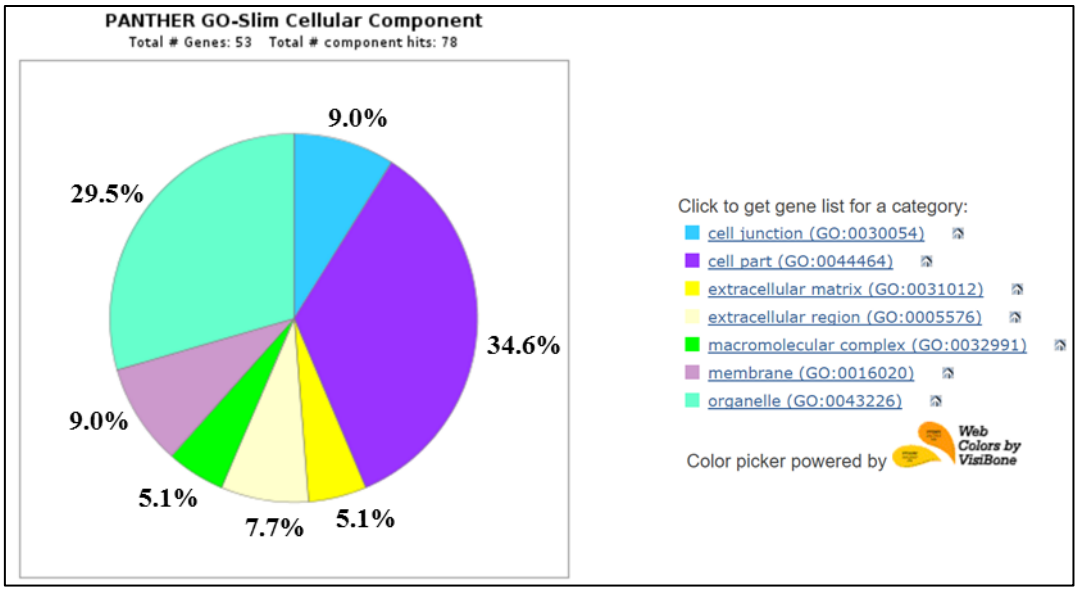

Figure 3.17. Classification of proteins identified in CC35 according to cellular localization (www.pantherdb.org/).

The proteins identified in CC35 were then classified according to their biological process. Biological process classification revealed that the most proteins were involved in cellular processes (24.6%), cellular component organization or biogenesis (16.4%), multicellular organismal processes (11.9%), localization (11.9%), developmental (10.4%) and metabolic processes (8.2%) (Figure 3.18).

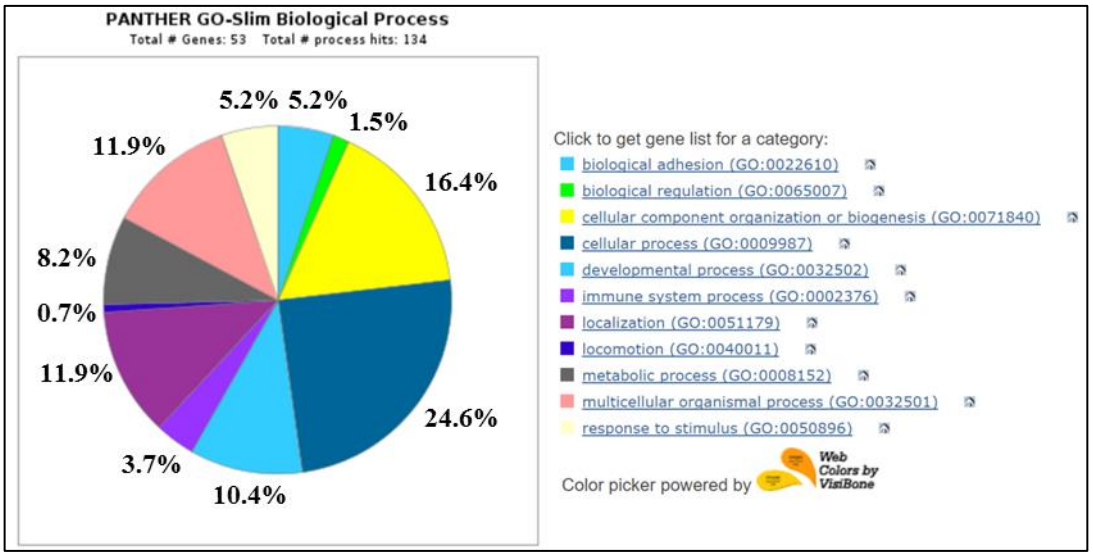

Figure 3.18. Classification of proteins identified in CC35 according to biological process (www.pantherdb.org/).

Finally, the differentially expressed proteins identified in the umbilical cord CC7(15) (malformation of the gastrointestinal system) were classified according to the 3 GO terms.

According to molecular function, the most proteins showed catalytic activity (35.0%), followed by structural molecule activity (30.0%), binding activity (20.0%), receptor (10.0%) and antioxidant activity (5.0%) (Figure 3.19).

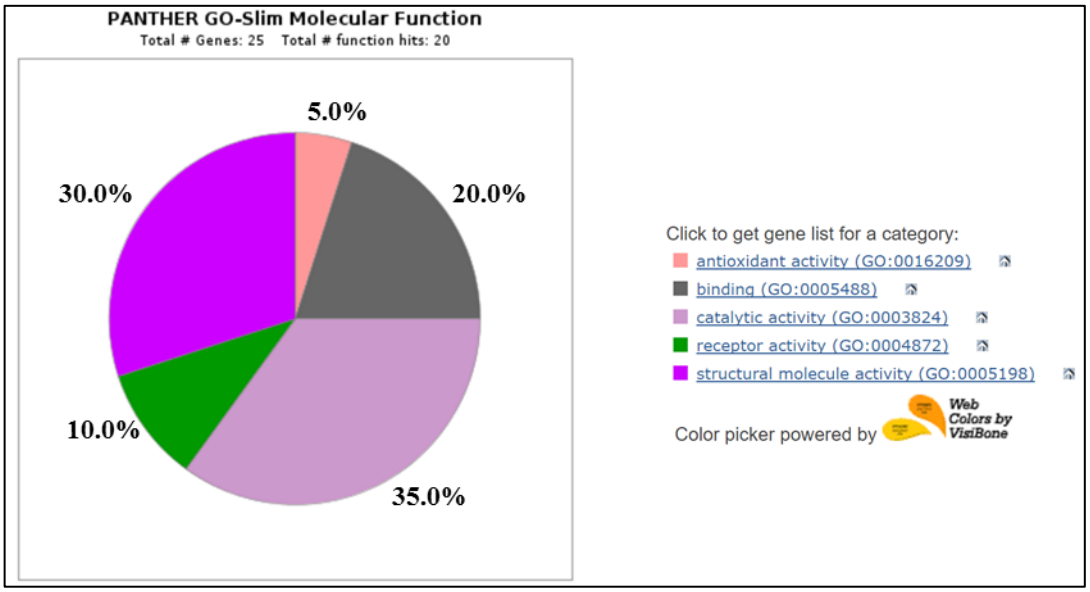

Figure 3.19. Classification of proteins identified in CC7(15) according to molecular function (www.pantherdb.org/).

The differentially expressed proteins identified in CC7(15) were then classified according to their cellular localization. Cellular components were mainly distributed in cell (31.8%), organelle structures (22.7%), extracellular matrix (18.2%), extracellular region (18.2%), but they displayed also cell junction (4.5%), and membrane (4.5%) (Figure 3.20).

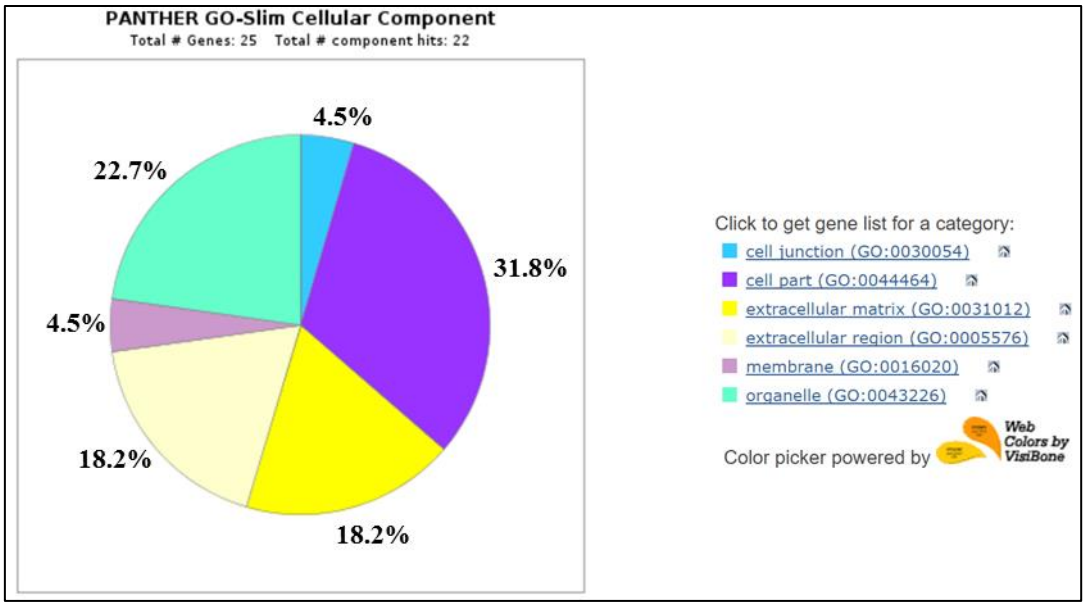

Figure 3.20. Classification of proteins identified in CC7(15) according to cellular localization (www.pantherdb.org/).

Biological process classification of the differentially expressed proteins identified in CC7(15) revealed that the most proteins were involved in cellular processes (19.0%), metabolic processes (16.7%), multicellular organismal processes (16.7%), cellular component organization or biogenesis (14.3%), developmental (11.9%) and localization processes (9.5%) (Figure 3.21).

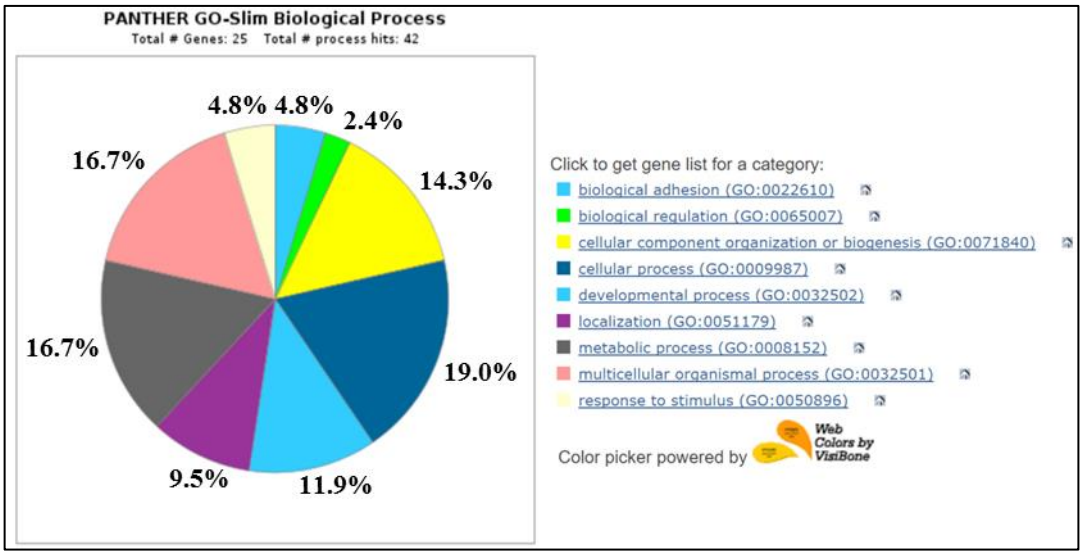

Figure 3.21. Classification of proteins identified in CC7(15) according to biological process (www.pantherdb.org/).

3.8 Gelatin zymography

MMPs have been shown to play significant roles in different physiological processes, including embryogenesis and angiogenesis, but also contribute to pathological processes such as tumor metastasis, inflammation and arthritis (Kupai *et al.*, 2010). Of this diverse family of enzymes, the gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been extensively studied owing to their consistent association with tumor invasion and metastasis (Toth and Fridman, 2001). It has been reported that MMP-2 and MMP-9 functional gene polymorphisms might be associated with an increased risk of idiopathic recurrent spontaneous abortion (IRSA) in women (Pereza *et al.*, 2012). Gelatin zymography was performed for the detection and analysis of the level and type of the gelatinases MMP-2 and MMP-9 expressed in umbilical cords. Thus extracts of umbilical cords from malformed fetuses and from a healthy one with similar gestational age as control showed two major bands in zymograms: MMP-2 (72 kDa) and MMP-9 (92 kDa) (Figure 3.22).

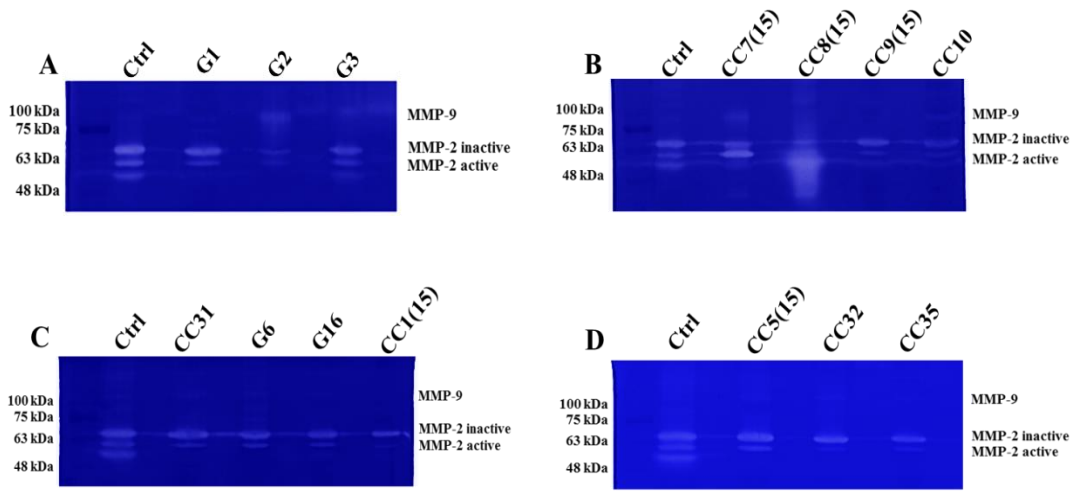

Figure 3.22. Representative gelatin zymograms showing MMP-9 and MMP-2 activities in extracts of umbilical cords from malformed fetuses.

Our results showed a significantly increased activity of MMP-2 in CC8(15) (Klinefelter syndrome) and in CC7(15) (malformation of the gastrointestinal system) compared to control. The activity of MMP-2 was significantly lower in G2 [Dyssegmental dysplasia, Silverman-Handmaker type (DDSH)], CC9(15) (Congenital heart disease, cleft lip and palate, neural tube defect), CC10 (Trisomy 21), G16 (Klinefelter syndrome), CC1(15) (Neural tube defect – multiple), CC32 (Non-immune hydrops), CC35 (Congenital heart disease) (**$p < 0.001$) (Figure 3.23). However, no significant differences in MMP-2 levels were observed in the other malformed samples [G1, G3, CC31, G6, CC5(15)].

In order to verify the observed gelatinolytic activity of MMP-2, we performed a Western blotting analysis, but no significant variation of MMP-2 levels was observed. Based on zymography results, we performed a more MS analysis of our proteomic data aimed to investigate a possible difference of MMP-2 expression, but no significant difference was highlighted in CC8(15).

Indeed, zymography measures the proteolytic activity, not expression levels, of MMPs (Keles *et al.*, 2014). MMP protein levels do not directly reflect MMP activity as only the inactive form of the protein may be present, or an excess of an inhibitor may block activity (Lowrey *et al.*, 2008).

So it's not surprising that we have not found an up or downregulation of MMPs in proteomic data. Although a significant difference of MMP-2 was not observed in

proteomic data, the analysis of the differentially expressed proteins in CC8(15) showed some correlations with MMP-2.

MMP-2 is constitutively expressed on the amnion during gestation in several animal species, including humans. The activity of MMP-2 is enhanced during labour (Vadillo-Ortega and Estrada-Gutiérrez, 2005). An appropriate level of MMP-2 is needed for normal pregnancy (Zhang *et al.*, 2003). It is involved in cell invasion and in embryonic development and organogenesis. A growing number of reports suggest that MMP-2 is involved in renal development, renal tubule physiology and glomerular pathophysiology (Lelongt *et al.*, 2001). MMP-2 is expressed in neurons and glia, supporting a primary role in embryonic neuronal development (Miller *et al.*, 2008). MMP-2 appears to have a crucial role in embryonic heart development including angiogenesis, valve development, and heart tube formation. Inhibition of MMP-2 causes severe heart tube defects, cardia bifida, and a disruption in the looping direction, which suggest a key role of MMP-2 in cell migration and remodeling required for normal heart development (Youssef and Schulz, 2011). MMP-2 plays also a direct role in early skeletal development and bone cell growth and proliferation. Its loss results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth (Mosig *et al.*, 2007).

By studying the proteins differentially expressed in CC8(15), we could highlight that their up or downregulation may have been influenced by the activity of MMP-2. Decorin, that was absent in CC8(15), is a ubiquitous proteoglycan comprised of a core protein attached to a single dermatan/chondroitin sulphate glycosaminoglycan chain. It may play a role in regulation of collagen fibrillogenesis and function as a reservoir of transforming growth factor beta (TGF-beta) in the extracellular milieu. It was demonstrated that decorin is susceptible to many MMPs and is degraded by MMP-2 (Imai *et al.*, 1997).

Desmin [absent in CC8(15)], is a muscle-specific, type III intermediate filament that integrates the sarcolemma, Z disk, and nuclear membrane in sarcomeres and regulates sarcomere architecture (Sequeira *et al.*, 2014). It was found that desmin is susceptible to degradation by MMP-2 *in vitro* (Kandasamy *et al.*, 2010).

Calmodulin, that was upregulated in CC8(15), is a small Ca^{2+}-binding protein and acts in signal transduction in cell growth, differentiation, proliferation, survival, and motility through association with calmodulin-binding proteins (Agell *et al.*, 1998).

Binding of calmodulin to K-Ras4B-GTP activates the PI3K/Akt (also known as protein kinase B, PKB) pathway and enhances cell migration through inducing the expression of MMP-2 which breaks down type IV collagen, a major structural component of basement membranes (Nussinov *et al.*, 2015).

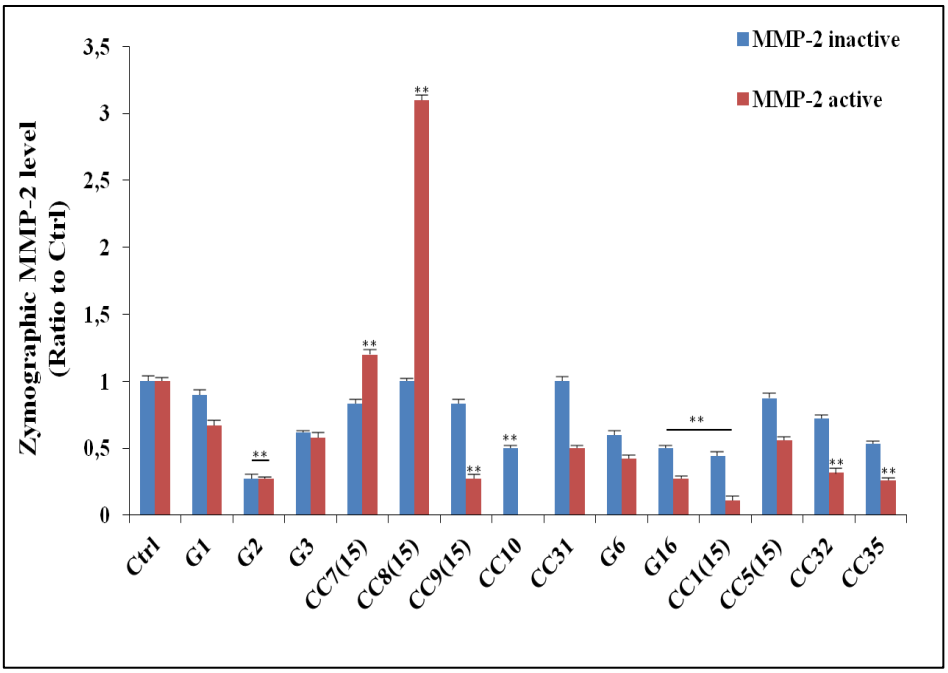

Figure 3.23. Zymographic MMP-2 levels in umbilical cords. Densitometric ratio in Ctrl was arbitrarily indicated as equal to the unit and represented as mean values ± SD of three separate experiments (**p < 0.001).

Zymography showed significantly higher levels of MMP-9 in G2 (DDSH) and in CC7(15) (malformation of the gastrointestinal system) than in control (**p < 0.001) (Figure 3.24). However, there were no significant differences in MMP-9 levels for the other samples of malformed fetuses (data not shown).

DDSH is a lethal autosomal recessive form of dwarfism with characteristic anisospondylic micromelia and is caused by functional null mutations of the perlecan gene (HSPG2) (Arikawa-Hirasawa *et al.*, 2001). Perlecan is a heparan sulfate proteoglycan expressed in basement membranes and cartilage. It in cartilage plays a critical role in endochondral bone formation by promoting angiogenesis essential for cartilage matrix remodeling and subsequent endochondral bone formation. In the absence of perlecan, chondrocytes were able to differentiate into

mature hypertrophic chondrocytes. MMP-9 is expressed in the hypertrophic chondrocytes of wild-type mice and Hspg2$^{-/-}$ mice and is involved in degradation of the hypertrophic matrix (Ishijima *et al.*, 2012).

The umbilical cord CC7(15) is affected by a malformation of the gastrointestinal system. Recent studies have implicated a pathogenic role for MMP-9 in inflammatory bowel disease (IBD) (Nighot *et al.*, 2015). It has been shown that epithelial-derived MMP-9 is crucial in intestinal damage (Castaneda *et al.*, 2005). Indeed, epithelial-derived MMP-9 is absent in normal colonic tissue but is upregulated during IBD and could be responsible for degradation of extracellular matrix (ECM) components with subsequent loss of mucosal integrity and increase of epithelial permeability (Liu *et al.*, 2013; Medina and Radomski, 2006).

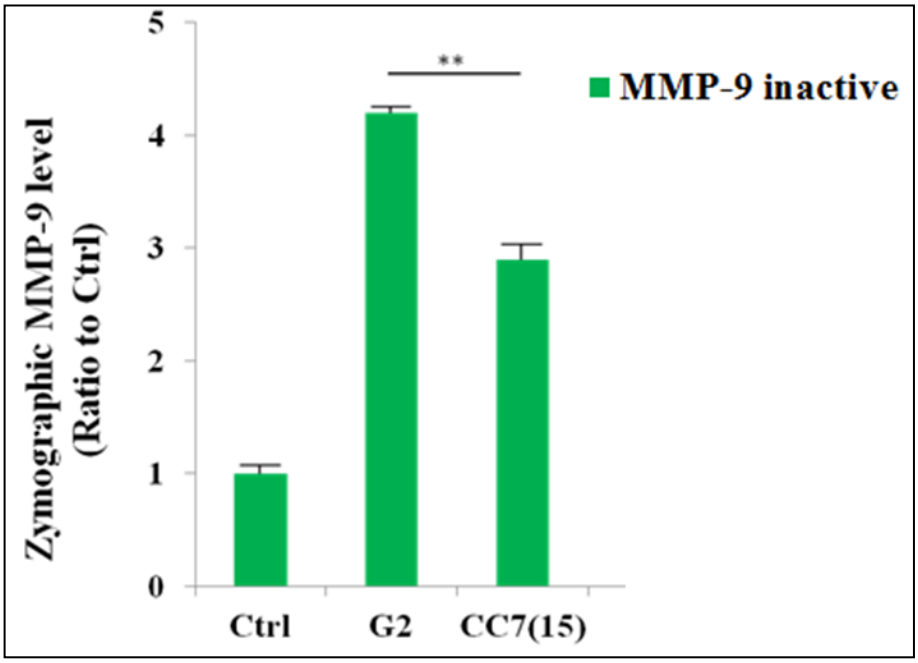

Figure 3.24. Zymographic MMP-9 levels in umbilical cords. Densitometric ratio in Ctrl was arbitrarily indicated as equal to the unit and represented as mean values ± SD of three separate experiments (**$p < 0.001$).

All these assessments will require further confirmation based on the analysis of a greater number of biological samples.

3.9 Western blotting analysis

In order to verify the observed gelatinolytic activity on zymography of MMP-2 and MMP-9, we analyzed the protein levels through Western blotting. No significant variation of MMP-2 levels was observed. Instead, both zymography and Western blotting analysis showed that MMP-9 was significantly increased in G2 and in CC7(15) (Figure 3.25).

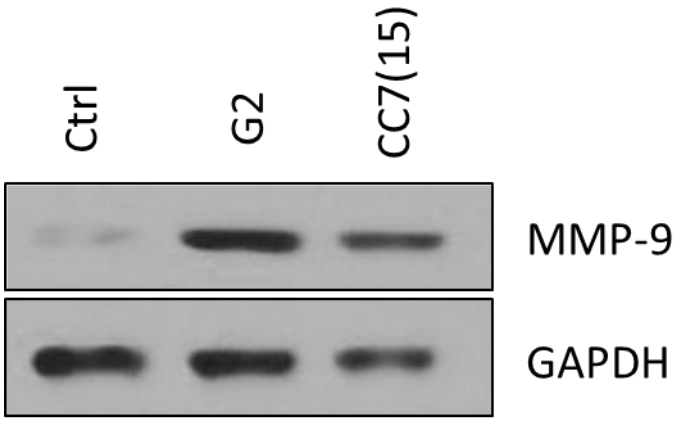

Figure 3.25. MMP-9 levels in umbilical cords. GAPDH was used as loading control. Blots shown are from one experiment representative of at least two with similar results.

3.10 Metal analyses in mother's hair and umbilical cords

The analyses were performed, with a standard protocol, at the Laboratories of the Maugeri Foundation (Pavia, Italy), and Prof. Stefania Papa , Seconda Università di Napoli and included additional seven samples of hair with already known metal load, as technical controls. Unfortunately, it is currently not available a international standard for the heavy metals in umbilical cords, based on samples with already known metal load. Briefly, hair and umbilical cord samples were weighed and mineralized in a microwave oven in 65% HNO_3 (3.5 mL) + 30% H_2O_2 (0.5 mL, Suprapur-Merck), diluted 1:20 in bidistilled H_2O and analyzed in DRC-ICP-MS with 20 sweeps/reading. The DRC-ICP-MS instrument was an ELAN DRC II (Perkin Elmer, SCIEX Instruments, Toronto, ON, Canada) with a

quadrupolar analyzer, mass interval 5-270 amu, source ICP, radiofrequency 40 MHz, max. power 1,600 W. Analysis of metal load by DRC-ICP/MS were run for Li, Be, Co, Zn, Hg, Cd, Ba, V, Mn, Sn, Sb, Ti, Ce, Rb, Pb, W, Tl, As, Ni, Se, V, Cr, Cu, U, Mg, K. Limits of detection (LOD) varied from 0.01 for Se, to 0.0004 for U. Our results showed significantly increased levels of Sn, Ti, and W in mother's hair. The umbilical cords seem to show significantly increased levels of Ti (Appendix B). It was suggested that metal-based particles containing Ti penetrate into the amniotic fluid and may affect human fetuses (Barošová *et al.*, 2015).

Because of the small number of samples analyzed and the absence of an international standard, it is actually not possible to make a critical assessment of these results.

4 Conclusions

It is known by studies conducted in the Gaza Strip and in Iraq that prenatal exposure to pollutants and particularly heavy metals can have long term damaging consequences on infants, due to their accumulation in-body. Thus, direct exposure to waste and heavy metals during the last two decades was very frequent in the so-called “Land of fires”. The number of children suffering from cancer and of malformed fetuses in Italy's "Land of Fires", an area where toxic waste has been dumped by the mafia, is reported significantly higher than elsewhere in the country. In this thesis we examined the proteome of the umbilical cords from malformed fetuses obtained by therapeutic abortions, after mothers' being exposed to the pollution on “land of fires” during early pregnancy, and analyzed the differences between umbilical cords from malformed fetuses to healthy ones. The main goals were to understand the impact of the contamination by heavy metals on the fetus development, and to identify new putative biomarkers of exposure to metal contaminants.

The proteomic study of heterogeneous tissue samples as the umbilical cord was performed by the FASP technique. This bio-analytical strategy combines the advantages of in-gel and in-solution digestion for mass spectrometry–based proteomics, greatly reduced the time required for sample preparation and enabled more flexibility in sample processing.

Many differentially expressed proteins were identified. By a Gene Ontology (GO) study it was possible to obtain functional informations of these proteins and to correlate them to the embryonic development. Most of the differently expressed proteins showed structural molecule activity.

Gelatin zymography was set up and performed for the detection and analysis of the levels of the gelatinases MMP-2 and MMP-9 expressed in the umbilical cords. A different activity of these MMPs in the malformed umbilical cords was assessed. In particular, the increased activity observed for MMP-2 may be related to some proteins, such as decorin, desmin, and calmodulin, that were differentially expressed in samples where MMP-2 showed increased activity. Our results could support a significant role of the MMPs in the fetus development.

This was a pilot project still in progress. However, our preliminary results support the hypothesis that heavy metals alter proteome expression and can induce significant changes in proteomic profiles of the exposed organisms. This work demonstrates and confirms the importance of proteomics to assess the biochemical changes and to elucidate the underlying mechanisms of toxicity induced by heavy metals in malformed fetuses. Additional studies, including collection of umbilical cords from malformed fetuses and from healthy ones as controls, genomic and transcriptomic analyses, are in progress to identify new protein pathways involved in heavy metal toxicity and to discover novel biomarkers.

Although all obstacles expected and not expected that sometimes made us think of a boycott, we hope that our project will continue and that the situation in the "land of fires" will become an important objective for scientific community, politics, and society.

"Nella Terra dei Fuochi i bambini continuano a morire. Negli ultimi venti giorni sono otto i bambini morti di tumore. Questi bambini non riposeranno mai in pace. Per loro non c'è giustizia". Avevano tutti tra i sette mesi e gli undici anni. È il grido di denuncia delle mamme aderenti al comitato "Vittime della terra dei fuochi" del 6 Febbraio scorso.

"In the Land of Fires children continue to die. In the last twenty days eight children died of cancer. These children never rest in peace. For them there is no justice". They all had between seven months and eleven years. It is the cry of complaint of the mothers participating in the committee "Victims of the land of fires" of last 6 February. (http://www.terredifrontiera.info/tumori-terra-fuochi/)

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Appendix A

Nome del medico in servizio

Nome di infermiera in servizio

Numero file x RICERCA:

Numero tessera sanitaria madre:

Campioni prelevati da, iniziali operatore
Capelli madre []
Cordone []
Placenta []
Altro [] specifica:

Il questionario contiene le informazioni sul bambino (B), il padre (P) e la madre (M):

Data di intervento
Data del colloquio:
Ospedale:

Sezione I

Luogo di residenza della famiglia :
Strada
Riferimento Telefonico
Relazione tra i genitori: 1 - cugini [] 2 - dalla famiglia [] 3 - nessuna parentela []
Sesso del bambino 1 - Maschio [] 2 - Femmina []
Questo bambino: 1 - Singolo[] 2 - Gemelli []
Ordine di nascita del neonato

Dati clinici
Le informazioni qui di seguito devono essere raccolte dal personale medico responsabile
Settimane di gestazione (numero)
Peso alla nascita in grammi
Tipo di malformazione:
1 - Difetto cardiaco congenito, CHD [] Q20-Q28, specifica

2 - Labbro / palatoschisi, CL / P []Q35-Q37, specifica

3- Difetti agli arti []Q65-Q79 , specifica

4 - Difetto sistema nervoso [],Q00-Q07 specifica

5- Difetto sistema digerente []Q38-Q45, specifica

6- Difetto muscolo scheletrico []Q65-Q79, specifica

7- Difetto sistema respiratorio [] Q30-Q34, specifica

8-Difetto sistema urinario []Q60-Q64, specifica

9-Difetto sistema genitale [Q50-Q56, specifica

10 -Malformazioni multiple [] specifica

11-Difetti occhio, orecchio, collo e viso [] Q10-Q18, specifica

12 – Altre [] Q80-Q89, specifica

13- trisomie ed altre anomalie cromosomiche [] Q90-Q99, specifica

Diagnosi :

Ecografica

1

Genetica

Anatomo-patologica

Sezione II
Inizia l'intervista dicendo al genitore che stiamo iniziando un programma di studio sulle malformazioni congenite e chiediamo la collaborazione per poter investigare i motivi e le alterazioni biologiche coinvolte.
Perciò useremo in modo anonimo le informazioni che ci daranno e i materiali che raccogliamo: cordone ombelicale, placenta e un ciuffetto di capelli della madre.

Tutti i dati forniti saranno trattati con la massima riservatezza e soltanto da personale sanitario e di ricerca per scopi scientifici e clinici. I nomi delle persone che collaborano **non potranno mai** essere resi noti nè i loro indirizzi e telefoni.

Lei è d'accordo di rispondere?
Firma per consenso di Madre: _____
e (facoltativa)
Firma per consenso del Padre: _____

1.
-MADRE
-Nome: _____
-Età: _____
-Lavoro attuale: _____ Passato: _____ attività lavorative di rischio*_____
-PADRE
-Nome: _____
-Età: _____
2. -Lavoro attuale: _____ Passato: _____ attività lavorative di rischio*_____

Per la madre:
1. Prima gravidanza ? []

Quante gravidanze/ figli ha avuto?

Data di nascita-mese anno							
Sesso							
Sano							
Con malformazione + tipo							
Interruzione gravidanza +causa							
Nato morto							
Figli con tumori +tipo							
Figlio con malattia cronica +tipo							
Aborti spontanei + settimana di gestazione + aveva malformazioni?							

Durante la gravidanza, ha preso FARMACI [] se si quali e in che dosi _____

Durante la gravidanza, ha preso VITAMINE [] se si quali e in che dosi _____

Durante la gravidanza, ha preso acido folico [] se si quali e in che dosi _____

Durante la gravidanza o prima ha usato Trattamenti estetici [] e quali (tinture, creme con acido retinoico..ecc)
_____

DIETA : Nessuna particolarità [] vegetariana [] senza-molto poco latte [] senza-molto poco latticini [] senza-molto poco pesce [] senza-molto poco verdure [].

Per la madre ed il padre
Questa gravidanza è con fecondazione in vitro?

Siete fumatori? _____ quante sigarette al giorno? _____
Siete esposti a sostanze tossiche sul luogo di lavoro? Se si, quali?
Usate /avete usato negli ultimi 10 anni o usano i vostri vicini:
pesticidi / insetticidi / fertilizzanti e altri prodotti chimici per la coltivazione della terra?
Se si, quali?

Nelle adiacenze della casa attuale si trova:
Discarica di immondizia Sì [] o No [], distanza……..., data di apertura……

Inceneritore Sì [] o No [],distanza……..., data di apertura……

Luoghi dove si brucia immondizia all'aperto? Sì [] o No [],distanza……..., data di apertura……

Fabbrica, Sì [] o No [], specifica tipo di produzione……, distanza……..., data di apertura……

Strada di alto traffico Sì [] o No [],distanza……..., data di apertura……

Quando è stata costruita la casa attuale?
Da quanto tempo vive nella attuale residenza?
Dove abitava prima, negli ultimi 10 anni?
Quando era stata costruita la precedente residenza?

Che acqua usa per bere/cucinare?

Avete lavorato/lavorate nel settore di scavi e /o dei materiali da costruzione?
Sì [] o No [],
Se sì, quando e per quanto a lungo e con che ruolo

Dal momento che avete avuto un bambino con difetti alla nascita, vorremmo esaminare le possibili cause e se ci sono altri casi nella famiglia estesa. Apprezziamo la vostra risposta alle seguenti domande.

	Madre	Padre
Soffre di malattie? quali		
Un genitore ha malformazioni?		
Uno zio/zia ha malformazioni?		
Cugini o cugine con malformazioni?		
Quanti fratelli ha?		
Quante sorelle ha?		
Sono tutti in salute i fratelli?		
Se qualcuno ha una malformazione, di che tipo?		
Sono tutti in salute le sorelle?		
Se qualcuna ha una malformazione, di che tipo?		
Quanti figli hanno i fratelli?		
Sono tutti in salute i figli dei fratelli?		
Se qualcuno ha una malformazione, di che tipo?		
Quanti figli hanno le sorelle?		
Sono tutti in salute i figli delle sorelle?		
Se qualcuno ha una malformazione, di che tipo?		
Qualcuno dei fratelli o sorelle abita vicino a voi?		

Se possibile, allegare UN'IMMAGINE (o un riferimento a un file di immagini) delle pertinenti caratteristiche morfologiche.

Modificato 25 Settembre, 2013 dal Prof. Paola Manduca, Università di Genova, Italia

Appendix B

MOTHER'S HAIR	DIAGNOSIS											
Analyte Symbol			Al	Fe	Mg	Cr	Hg	Ni	Pb	Sn	Sr	Ti	W
Unit Symbol			µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
Detection Limit			0,4	0,4	0,7	0,02	0,0004	0,15	0,04	0,03	0,01	0,04	0,03
Analysis Method			ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS
Standard hair Germany			<8	4,8-17	20-130	0,02-0,21	<0,6	<2	<3	<0,7	0,65-6,9	<0,15	<0,002
	CC3C(15)	Healthy	2,61	5,45	94,8	0,03	2,93	0,34	0,29	0,54	2,77	0,08	0,2
	CC5(15)	Neural tube	1,1	7,91	26	0,07	0,345	<0,15	0,37	0,22	0,74	<0,04	<0,03
	CC7(15)	Gastrointestinal	4,43	12,89	34,5	0,06	1,23	0,23	1,01	7	0,74	0,27	0,06
	CC8(15)	47,XXY	1,46	9,29	337	0,08	2,13	<0,15	0,23	0,86	9,62	0,17	<0,03
	G3	Trisomy 21	3,27	9,19	525	0,05	0,346	0,39	0,45	2,56	6,4	0,14	0,07
	G11	Neural tube	2,52	8,91	181	0,05	0,0974	<0,15	0,29	0,54	7,21	0,21	0,18

UMBILICAL CORDS	DIAGNOSIS											
Analyte Symbol			Al	Fe	Mg	Cr	Hg	Ni	Pb	Sn	Sr	Ti	W
Unit Symbol			µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
Detection Limit			0,4	0,4	0,7	0,02	0,0004	0,15	0,04	0,03	0,01	0,04	0,03
Analysis Method			ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS	ICP-MS
	CC3C(15)	Healthy	<2,05	31,67	743	<0,1	0,0191	<0,77	0,99	<0,2	0,64	<0,20	<0,2
	CC5(15)	Neural tube	<1,73	26,71	768	0,09	0,0103	<0,65	0,25	<0,1	1,08	0,27	<0,1
	CC7(15)	Gastrointestinal	3,4	136,04	696	0,15	0,125	<0,61	6,8	0,41	1,47	0,39	<0,1
	CC8(15)	47,XXY	<1,91	12,66	689	<0,10	0,0135	<0,72	<0,2	<0,1	0,48	<0,19	<0,1
	G3	Trisomy 21	4,54	33,15	262	0,21	0,0192	<0,58	<0,2	<0,1	0,19	0,36	<0,1
	G11	Neural tube	<3,64	40,96	222	<0,2	0,0137	<1,36	<0,4	<0,3	0,15	14,68	<0,3