Response to reviewers:

1. Reviewer #1: The manuscript by Garcia Sevillano et al. is an interesting review that covers the integration of several omics technologies for the study and evaluation of the toxicity of environmental pollution with metals coming from the Aznalcollar pyrite mine in Doñana National Park using the rodent M. spretus as a sentinel organism.

The review adequately combines general information with the results obtained by the authors. A revision of technical issues is also provided, giving a balanced revision of the topic. Figures and graphics are adequate and informative.

On this basis, I recommend its publication in Journal of Proteomics, but before manuscript acceptance, I suggest to introduce the following minor modifications.

1.1. Please revise the English language, specially the text in the abstract.

English has been revised along the manuscript and the Abstract has been completely rewritten (marked in red in the MS).

1.2. Page 4, line 51 and Page 5, line 8. The settlement "Punta del Sebo" (PS) appears two times in the text.

The complete paragraph has been rewritten (page: 4 lines: 37-52 and page: 5 lines: 1-6)

1.3. Pages 6 and 7. Describe figures 3 and 4 in the right order. First, the description of the transcriptomic analysis and validation of GPx3 by qRT-PCR. The actual paragraph is confusing since GPx3 is describe first (page 6) and it appears again afterwards (page 7, line 13).

The complete paragraph has been rewritten according your remark (page: 6, lines: 33-53 and page: 7, lines: 1-20)

1.4. Figure 3 (C): increase in the relative transcript number corresponding to the genes analyzed. How is this calculated? Does it represent the mean of all the genes?

To prepare Fig. 3D we summed the fold changes of the ten genes represented in Fig. 3B quantified in mice captured in each of the three sampling site and this three data correlated with the sum of all the metals (Fog. 3C) quantified in the kidneys of the mice captured. We have prepared a new Fig. 3 and rephrased its legend of Fig. 3 as follows:

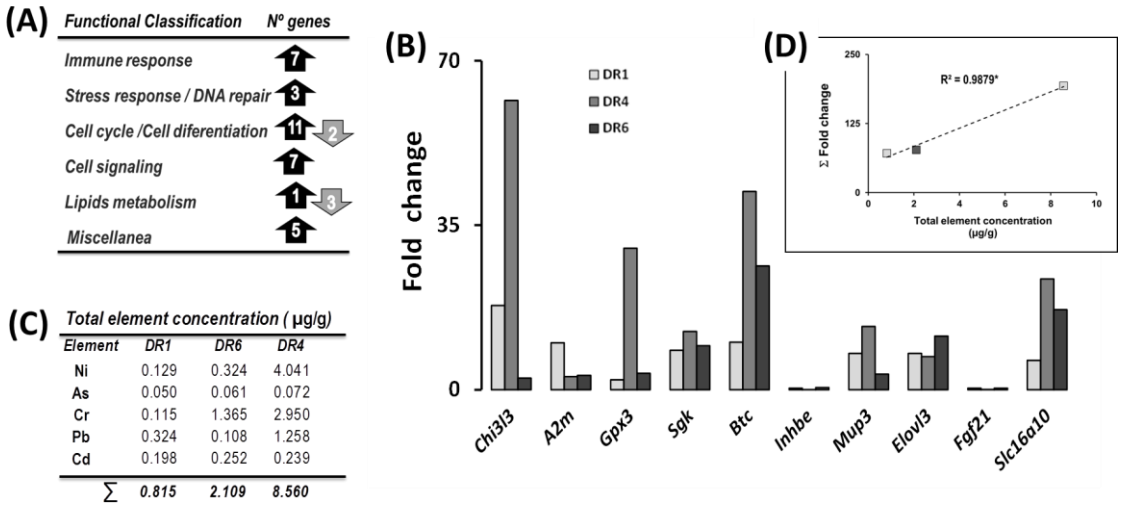

Fig 3. Results of microarray experiments with *M. spretus* (n = 9). (A) Functional classification of 39 differentially expressed genes with >10-fold-changes in at least one of the studied sites (DR1, DR4 and DR6), with indication of the number of up- or down regulated genes included in each category. (B) Comparison of the variations in the relative transcript number of a selected group of genes included in the different functional categories in animals DR1, DR4 and DR6. (C) Mice renal toxic element concentrations (ug/g). (D) Correlation between the total increase in the relative transcript number (fold.change) corresponding to the genes analyzed in (B) and the total renal toxic element concentrations given in (C). Dashed line represents the linear regression equation for the three investigated locations. Statistical significance is expressed as $*P < 0.05$.

1.5. Figure 6. Include Molecular mass and pI.

Fig.6 now includes Molecular mass and pI data

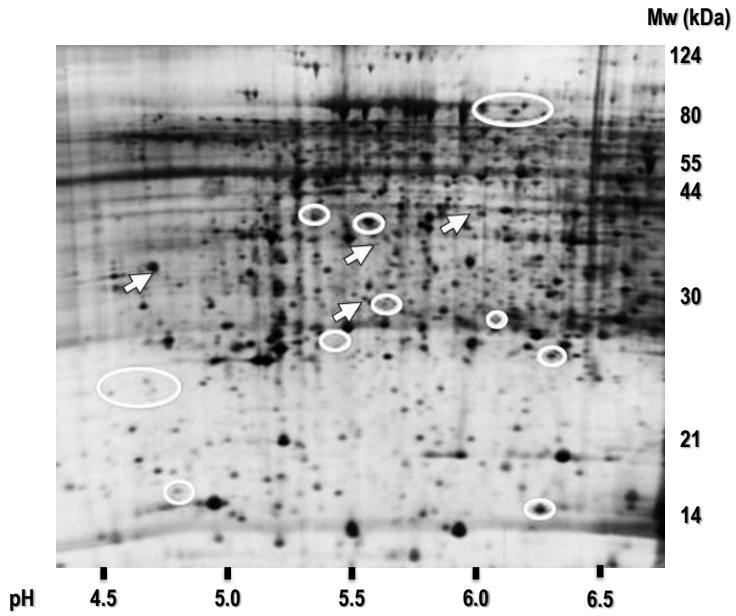

Fig. 6. 2-DE master gel of cytosolic liver proteins from *M. spretus* captured at MAT. Experiment (IEF pH 4-7, 18 cm, Amersham; 12,5% SDS-PAGE) compared the proteome of mice captured at MAT, an area heavily polluted by metals and pesticides, with that of mice collected at the reference site, RB. Arrows point to protein spots with a high intensity level in MAT samples (induced proteins); ovals frame spots with diminished intensity (repressed proteins) in MAT samples.

1.6. Page 11, line 5. Specify that this is an iTRAQ 8-plex.

The phrase now indicates that we labelled the proteins with iTRAQ® Reagent-8Plex Multiplex Kit

After being marked with iTRAQ® labels (iTRAQ® Reagent-8Plex Multiplex Kit, Applied Biosystem), proteins in different samples were mixed and analyzed by LC-MS/MS.

1.7. Page 12, line 6: add "avoid" before "mistakes".

The paragraph has been emended

(iii) reduction of disulfide bonds to promote protein denaturation and to avoid mistakes in molecular mass assignation and further identification [55], for this purpose, free thiol-containing reducing agents such as dithiothreitol (DTT), dithioerythritol (DTE), reduced glutathione (GSH) and β-mercaptoethanol are used, however, these reagents have chelating properties and are being replaced by others, such as tributylphosphine (TBP) and tris–carboxyethylphosphine (TCEP) with milder reducing properties and without chelating properties [4];

1.8. Page 14: revise since both "metabolomics" and "metabonomics" appear in the text

The Ms has been revised and the metabonomics word has been substituted by metabolomics in all the cases

2. Reviewer #2: I read with interest the manuscript of Sevillano and colleagues on "Omics technologies and their applications to evaluate metal toxicity in mice M. spretus as bioindicator. The topic of the manuscript is very important and the objective of the manuscript is well defined.

2.1. However, the results of the study are very preliminary

The manuscript has been prepared as a review compiling results previously published by the authors of this study and other authors, with the objective of providing an overview of the complementary use of omics methodologies for the study of environmental problems and the added value that methodological approach represents. Therefore, the results are not preliminary, although it is true that the complementary use of these techniques is still incipient.

2.2. and the cohorts are too small and heterogeneous to allow drawing conclusions.

Each of the different experiment has been carried out with the same mice species, and we always worked with homogeneous groups of individuals of the same sex and weight. We usually used at least 9 mice per sampling site; so, even when grouped in three pools, we could carry out an statistical analysis of data and give the statistical significance of the differences. To clarify this point, in the legends of figures has been included the *n* value for the experiment.

2.3. Unfortunately, manuscript lacks very important details concerning experimental setup, study subjects,

Due to the nature of compilation work, the experimental aspects are not developed in detail in the manuscript because they are associated with the references. However, in the legends of the figures and along the headings from 3 to 7 have been collected important experimental data to guide the reader

2.4. conclusions are not supported by the data and is misleading at many instances.

The strong point of the study is use of free-living organisms (M. spretus) as bioindicator.

The conclusions have been rewritten emphasizing results presented in the manuscript and the important role of M. spretus in comparison with *M. musculus* in environmental studies based on biological responses.

Here are few of my comments without any particular order

2.5. Title of this manuscript is misleading and should be changed with more appropriate one. This is a comparative analysis of the selected genes, proteins and metabolites in different M. spretus. Same mistake is repeated throughout the manuscript.

The purpose of the manuscript is not to perform a comparative analysis of selected genes, proteins and metabolites in M. spretus from areas with differential contamination, but an assessment of the possibilities of integrated omics methodologies for the study of the potential toxicity of metals in contaminated areas using the biological response of mouse Mus spretus used as biomarker

2.6. Authors start the discussion by claiming that this is a mechanistic paper. This is an overstatement and should be omitted.

Sorry but do not find in the text any allusion about the mechanistic character of the paper

2.7. All the studies molecules are involved in multiple pathways and this study fails to elaborate which of downstream pathways are affected.

This work aimed to review the successful applications of various omics-methodologies (heterologous microarrays, proteomics methodologies (2-DE, iTRAQ®), metallomics, ionomics or metabolomics) in environmental toxicology done by our groups. The different experiments described in this review quantified the response of M. spretus to metal exposure and pointed to common pathways affected by living in these polluted area. Nevertheless, as we stated at the end of the new abstract, the complexity of environmental monitoring of metal toxicity requires the integrated use of the multi-omics technologies to achieve a comprehensive panorama in the study of environmental issues. We do not deep insight in metabolic cycles that will be considered in future works.

Significance

This work presents new contributions in the study of environmental metal pollution in terrestrial ecosystems using *Mus spretus* mice as bioindicator in Doñana National Park (SW Spain) and surroundings. In addition, it has been demonstrated that the integration of *omics* multi-analytical approaches provides a very suitable approach for the study of the biological response and metal interactions in exposed and free-living mice (*Mus musculus* and *Mus spretus*, respectively) under metal pollution.

*Graphical Abstract (for review)

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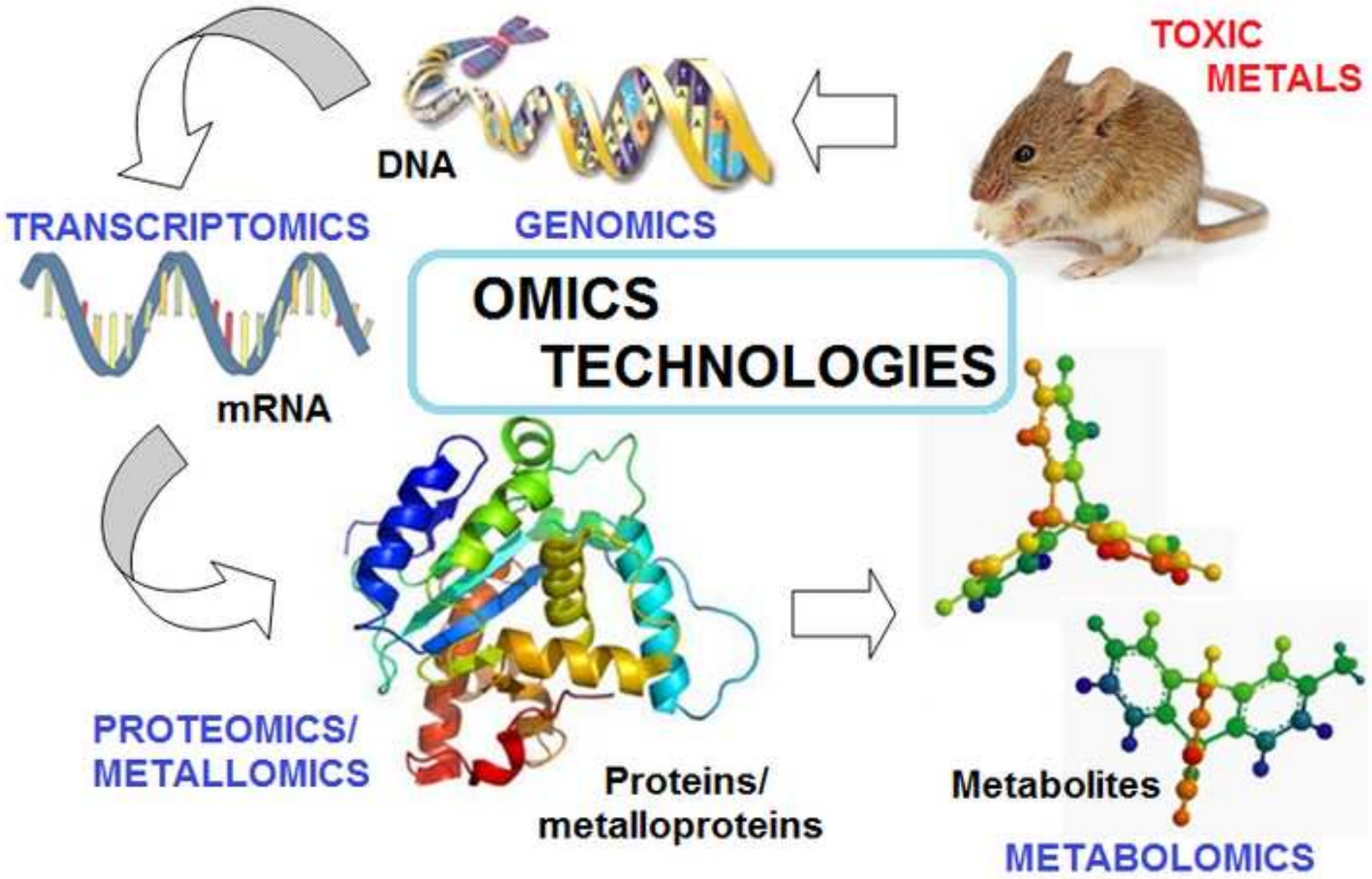

Highlights:

- Use of omics for toxicological and biochemical effects of arsenic/cadmium in mice
- Use of blood biochemicals and liver histopathology to monitor mice metal exposure
- As/Cd exposure produces changes in Se-containing proteins and metabolic pathways
- Metallomics and metabolomics provide deep information in mice exposure experiments

OMICS TECHNOLOGIES AND THEIR APPLICATIONS TO EVALUATE METAL TOXICITY IN MICE *M. spretus* AS BIOINDICATOR

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Abstract

Metals are important components of living organisms since many biological functions critically depend on their interaction with some metal in the cell. However, human activities have increased toxic metals levels in the terrestrial and aquatic ecosystems affecting living organisms. The impact of metals on cellular metabolism and global homeostasis has been traditionally assessed in free-living organisms by using conventional biomarkers; however, to obtain a global vision of metal toxicity mechanisms and the responses that metals elicit in the organisms, new analytical methodologies are needed. We review the use of omics approaches to assess the response of living organisms under metal stress illustrating the possibilities of different methodologies on the basis of our previous results. Most of this research has been based on free-living mice M. spretus, a conventional bioindicator used to monitor metal pollution in Doñana National Park (DNP) (SW Spain), which is an important European biological reserve for migrating birds affected by agricultural, mining and industrial activities. The benefits of using omic techniques such as heterologous microarrays, proteomics methodologies (2-DE, iTRAQ®), metallomics, ionomics or metabolomics has been remarked; however, the complexity of these areas requires the integration of omics to achieve a comprehensive assessment of their environmental status.

Keywords: *M. spretus*, Doñana National Park, environmental genomics, environmental transcriptomics, environmental proteomics, environmental metallomics, environmental metabolomics.

Introduction

Metals play an important role in the development and function of living organisms since the behavior of many genes, transcripts, proteins, metalloproteins and metabolites critically depends on their interaction with metals present into the cell, especially transition metals, such Cu, Zn, Mn, Cd or Hg. Fortunately, modern analytical technologies are providing new tools to investigate metals in depth, and generate a more comprehensive understanding of the interactions between metals and cells. The importance of monitoring the effects of heavy metals on living organisms has increased as a consequence of natural changes and anthropogenic activities that have led to an increase of toxic metals levels in terrestrial and aquatic ecosystems. In this sense, free-living organisms have been used in environmental pollution assessment since they can reflect the impact of contaminants on cellular metabolism and global homeostasis using different biomolecules related to environmental stress (biomarkers), such as genes [1], transcripts [2,3], metalloproteins [4], redox enzymes [5] and metabolites[6].

The extensive advances during the past decade in genes and genomes knowledge (genomics) have yielded several new *omics* that are very useful for the study of biological responses of organisms to toxic inputs and to understand the action mechanisms of contaminants and their toxicity. *Omics* differs from traditional hypothesis-driven research because it is a discovery-driven approach. Genomics deals with the analysis of the complete genome in order to understand the function of single genes. The majority of functional genomics is based on the analysis of gene expression (transcriptomics) and comprehensive proteins/metalloproteins analysis (proteomics/metallomics) [7]. In more recent years, metabolomics (based on the complete study of metabolites involved in different metabolic processes of organisms) has become an emerging field in analytical biochemistry and can be regarded as the end point of "*omics*" cascade (Fig. 1). Additionally, in studies based on metal exposure, the study of the ionome involves the quantitative and simultaneous measurement of the elemental composition of living organisms and the changes that undergo in response to physiological stimuli. Thus, while genomics or proteomics indicate the probability that a process may occur, metabolomics and ionomics provide information about what is really happening. Since metabolomic and ionomic profiles of gene expression involve external factors (metal exposure, diet and others) they allow understand easily the consequences of complex biological mechanisms inside the organism. In summary, monitoring environmental issues requires integrating approaches to sum up the multiple variables and factors that contribute to ecosystem behavior.

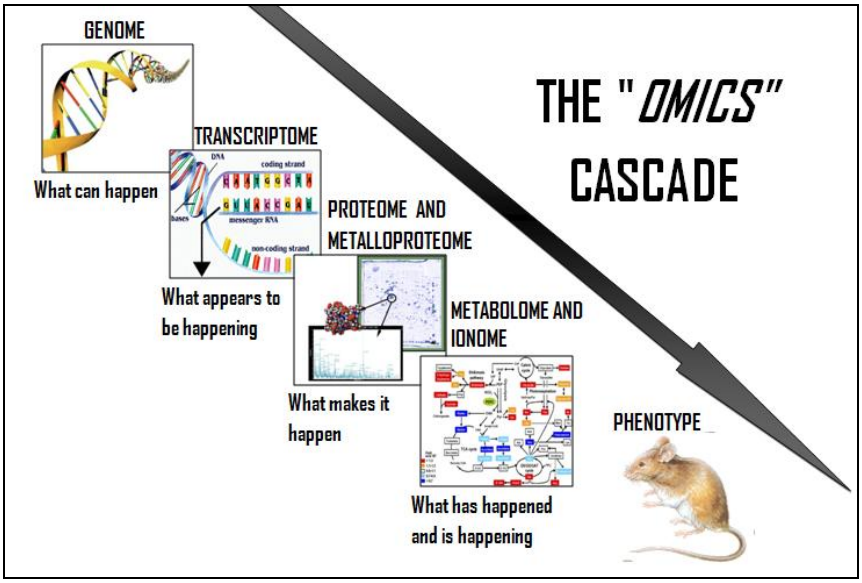

Fig. 1. "Omics" cascade to evaluate metal toxicity in free-living organisms

The influence of contamination, especially metal pollution, on biological response of free-living organisms has been used to evaluate the actual impact of xenobiotics in the environment. For this reason, some non-protected organisms, such as crayfish [8-10], fishes [11], mollusks [12], and mice [2-4, 13] have been used for environmental studies, using biological response based on genotoxic effects [1], analysis of typical biomarkers [13] and other tests [14]. More recent approaches based on *omics* techniques such as proteomics [5,10,12] and transcriptomics [2,3] have been proposed to monitor terrestrial ecosystems. Metallomics and metabolomics have recently been considered for this purpose [4,15] and now these approaches have being used to decipher the biological response to contamination [6]. For these reasons, the application of *omics* technologies, using the mouse *M. spretus* as bioindicator, has represented a good alternative, especially after the verification of genetic homology of this mouse with the conventional inbred laboratory mouse *M. musculus* that has been already sequenced [13,16]. This fact allows the use of database from *M. musculus* in the identification of transcripts, proteins/metalloproteins and metabolites that suffer changes in *M. spretus* under metal stress, avoiding the cumbersome work associated to the *de novo* sequencing.

1. Doñana National Park and the Southwest Spain region, the metal affected area

Doñana National Park (DNP) is a 50,000 ha wildlife reserve that is located northern the Guadalquivir River Estuary, in the Huelva province, SW Spain. In 1994, Doñana National Park was declared a UNESCO World Heritage Site due to its ecological importance. The park is an important bird sanctuary, each year receiving several millions of migrating birds from Africa, particularly at its core, the Doñana Biological Reserve (RB) [17]. Nevertheless, this area suffers from contamination threats caused by adjacent agricultural, mining and industrial activities responsible for the presence of metal species in the surroundings [4, 10, 18-20]. In 1998, a part of a 360 ha tailings dam of Aznalcollar pyrite mine, located 60 km north of DNP, collapsed and released to Guadiamar stream, a tributary of Guadalquivir River, four cubic hectometres of acidic water and two cubic hectometres of mud. The high toxic metals content of this mud (35% Fe, 0.8% Zn, 0.8% Pb, 0.5% As, 0.2% Cu, 0.05% Sb, 0.006% Co, 0.005% Tl, 0.005% Bi, 0.0025% Cd, 0.0025% Ag, 0.0015% Hg, 0.001% Se) threatened DNP and the Guadalquivir Estuary [17]. Since this event, several studies have provided abundant information on metal pollution in sediments and waters as well as bioaccumulation and effects on plants, invertebrates, fish, reptiles, birds, rodents, carnivorous mammals, and human populations.

In a number of works by our group, free-living mice (*M. spretus*) were collected during different periods of time since 2002 in five sampling areas from DNP and surroundings, and other areas near the Industrial Area of Huelva, in which contamination differs depending on the zone (Fig. 2). The results obtained allow the following remarks: (i) “Lucio del Palacio” (LDP- green spot) and “Reserva Biologica” (RB- black spot) located in the center of the park, can be considered as control areas with a low contamination level; (ii) “La Rocina Stream” (ROC- red spot) with strawberry, citrus fruit and grape fields in the surroundings, which is additionally affected by diffused pollution from petrochemical and chemical activities caused by the industrial belt of Huelva and acid waters and metals from north-west mining metallurgical activities in the Huelva province (Riotinto mine) can be considered a contaminated area; (iii) “El Partido” (PAR- orange spot) and (iv) “El Ajolí” (AJO- brown spot) sites, which correspond to up- and downstream from El Partido stream, respectively, which are under the influence of citrus fruit and grape fields, present a medium presence of pollutants; (v) “El Matochal” (MAT- blue spot), next to Guadiamar river, which is affected by rice growing fields and suffered the input of metals transported by the Guadiamar river during the rupture of Aznalcollar mine tailing pond in 1998 previously discussed [17], is a hot-point by the presence of contaminants; (vi) “Punta del Sebo” (PS-purple spot) is a heavily polluted industrial settlement in the Industrial Area of Huelva, where significant amounts of a very complex set of contaminants and heavy metals are daily released to the environment [21-24]; (vii) the “Domingo Rubio” stream (DR) is a contaminated marshland, part of which (DR1 - pink-spot) is under tidal influence, receiving elements of pyritic origin from the Tinto River. The medium course of the stream (DR4 - grey spot) is additionally affected by the nearby chemical and petrochemical complexes, which release, among other hazardous wastes, high amounts of atmospheric

pollutants (Fe, Cu, Cd, As, Sn, Pb, PO_4^{3-}, Zn, Sr, and Ti). An intensive agriculture activity (rice and strawberry fields) affects the upper (DR6 -yellow spot) and also medium course of DR.

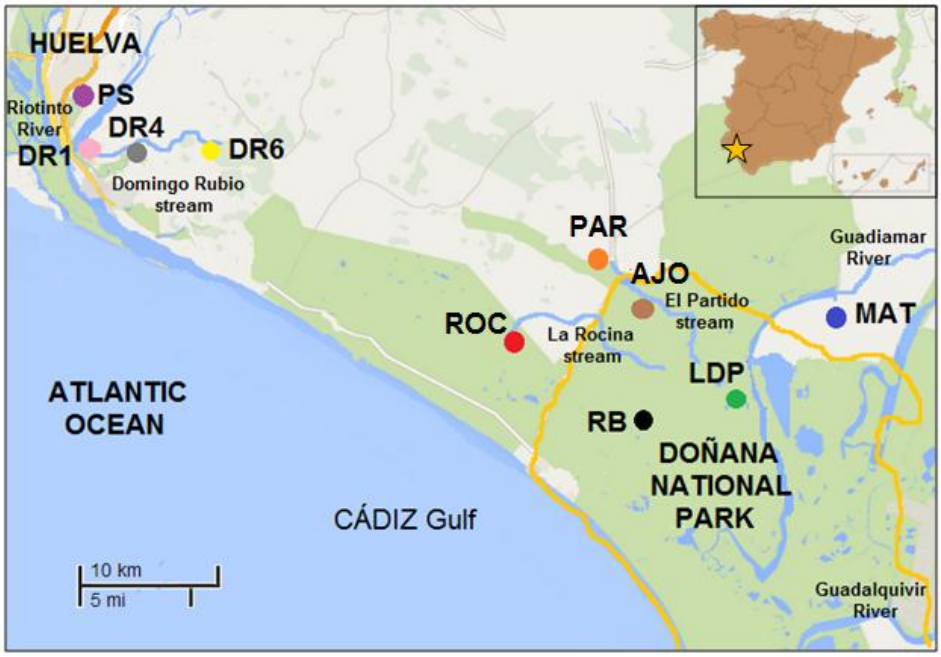

Fig. 2. Location of sampling sites for *M. spretus* mice used in the omics experiments.

2. Experimental overview of *omics* technologies for environmental studies

Environmental *omics* experiments involve a series of steps: sampling of bioindicators (e.g. mice), organs and biological fluids collection, biomolecules extraction, direct detection or chromatographic separation coupled to atomics or molecular detectors for biomolecules analysis, and finally data analysis by means of complex bioinformatics tools.

2.1. Environmental genomics

Environmental genomics seeks to characterize underlying factors, at the genetic level, that contribute to variability in organisms toxicological responses. Due to the responses diversity, environmental genomics have to integrate molecular biology, physiology, toxicology, ecology, systems biology, epidemiology and population genetics into an interdisciplinary research program. This basic information will be useful in identifying appropriated biomarkers for risk estimation, and potentially, for the development of effective strategies for the protection of the environment, global health and sustainable development. In the post-genomics era, the application of functional genomic approaches such as transcriptomics or proteomics, provides a holistic assessment of biological responses upon contamination exposure, which permit the identification of new biomolecules that may be used as biomarkers in routine environmental monitoring [2,5,7,25-31].

2.2. Environmental Transcriptomics

Genetic responses to stress conditions are often regulated at transcriptional level. Transcriptomics quantifies the levels of almost all the transcripts expressed under particular physiological

or environmental conditions. Microarray technology has evolved as a propitious platform to generate genome-wide transcriptional profiles hence providing a broad impression of how organisms respond to environmental stressors and the possibility to identify novel ecotoxicological biomarkers. This methodology has made a huge contribution to ecotoxicology [29,30,32,33], but it also has a number of pitfalls and shortcomings. One critical issue in microarray-based transcript quantification is sensitivity that imposes the subsequent use of more robust methodologies, mainly qRT-PCRT (quantitative reverse transcription-polymerase chain reaction). In fact, changes detected by microarrays have to be confirmed, at least, partially, by qRT-PCR. Quantitative RT-PCR can be carried out in a relative or absolute manner, but only absolute quantification provides the actual mRNA molecule numbers [34]. A second weakness of microarray technology has been its high cost that has limited the number of individuals, replicates, and conditions included in the studies. Methodology has now been improved and the costs reduced, and microarrays are commercially available for a range of model organisms. Finally, environmental studies generally use non-model organisms for which there are neither commercial nor custom-designed microarrays, since only limited genomic information is available at public databases. This problem may be solved by hybridizating the nucleic acids from the environmentally interesting non-model organisms onto DNA microarrays designed for phylogenetically related model species. Heterologous microarray hybridization has proven effective in studies focused mainly on identifying the responses of aquatic organisms to model pollutants in laboratory-based experiments (e.g., see [33,35,36]). The considerable gene sequence similarity between the classical *M. musculus* laboratory and *M. spretus* [2,37] has encouraged to used commercial *M. musculus* oligonucleotide microarrays for field studies in which *M. spretus* was the sentinel organism. The transcriptomes of mice collected at the low (DR1), medium (DR4), and upper (DR6) courses of the Domingo Rubio stream (Fig. 2) has been compared with animals captured in RB, the Doñana Biological Reserve, at the core of the Doñana National Park, considered a non-polluted area according to several biochemical, proteomic and transcriptional biomarkers [2,3,5,13]. Nine male *M. spretus* mice from each sampling site were selected for total RNA isolation from individual livers by the TRIzol method (Invitrogen) coupled with the GenElute Mammalian Total kit from Sigma. Equal amounts of each RNA sample were then pooled. Fluorescent complementary RNA (cRNA) generated following the manufacturer's instructions (Agilent Technologies). Hybridizations were carried out in the Whole Mouse (*M. musculus*) Genome Oligonucleotide Microarrays (4×44K, 60-mer) from Agilent Technologies, as previously described [7,38]. Almost 50% of the features in the heterologous microarray were successfully detected, indicating that the detection power of the microarray still remained considerable. The statistical analyses of microarrays identified 1872 spots differentially expressed in animals living in at least one of the contaminated DR sites as compared with RB, the negative reference. 242 out of these spots showed differential expression in mice from the three DR sites. 39 of them corresponded to transcript genes that exhibited ≥10-fold increased or decreased expression in animals from at least one DR site as compared with the control. Overall, data showed that animals collected at DR4 presented a higher number of changes in comparison with those at DR1 and DR6 (Fig 3). These data are in good agreement with our previous studies that showed a high accumulation of toxic metals (Ni, As, Cr, Cd and Pb) in mice living at DR sites, particularly in DR4 [5, 38]. By using qRT-PCR, we quantify the absolute number of transcripts of a selected group of differentially expressed genes. The qRT-PCR results verified the microarray data irrespective of their trend for up- or down-regulation, the magnitude of the genes expression change and the absolute hepatic abundance of the selected transcript [38]. Data was thereafter validated by extending the study to other different polluted sites, where absolute transcript numbers and protein amount were determined by qRT-PCR and Western-blotting, respectively. As an example, Fig. 4 shows microarray results verification of the absolute number of *Gpx3* (glutathione peroxidase 3) transcripts in livers of animals captured at the different polluted locations and compares the microarray and qRT-PCR fold-changes referred to RB animals. The proteins encoded by these differentially expressed genes were classified into six functional categories according to "Gene Ontology" (Fig. 3): Immune response (7 genes) Stress response (3 genes) Cell cycle/cell differentiation (13 genes) Lipid metabolism (4 genes). Heavy metals and pesticides have been implicated in the modulation of the immune function [39,40)] through the release of circulating pro-inflammatory and pro-oxidative mediators into the systemic circulation after particle inhalation [41-43]. Our results indicate that genes linked to the immune and cell oxidative defenses are strongly up-regulated in mice

dwelling at DR sites, and especially in DR4 animals (Fig. 3). This is the case of the Immune response category gene *Chi3l3* (60.5 fold-change in DR4 mice), coding the chitinase 3-like 3 protein, which is produced primarily by macrophages during inflammation [46]. Glutathione peroxidase 3 (GPX3; 30-fold increase in transcript levels in DR4 mice) is a selenoprotein that catalyzes the reduction of hydrogen peroxide and lipid peroxides and protects cells from damage produced by unstable reactive radicals and heavy metals [45,46]. The up-regulation of these genes was linked to oxidative stress related to pollution [47] and the inflammatory response [48]. Animals captured at a close industrial settlement, PS, were also included to validate data obtained in the microarray experiments. We found a very good correlation between fold-changes in protein and mRNA levels as shown in Fig. 4 for GPX3. The induction of MUP3, involved in the inhibition of both gluconeogenic and lipogenic genes, and the repression of the genes *Inheb* (negative regulator of cell growth) and *Fgf21*, (coding for the fibroblast growth factor 21) might be considered as a coordinated response aimed to drive energy toward inflammatory processes [38]. In conclusion, data resulting from the heterologous microarray analysis in M. spretus indicate that DR mice sustained a heavier pollutant burden than RB specimens and, therefore, suffer a chronic stress situation that elicits and maintains an immune response.

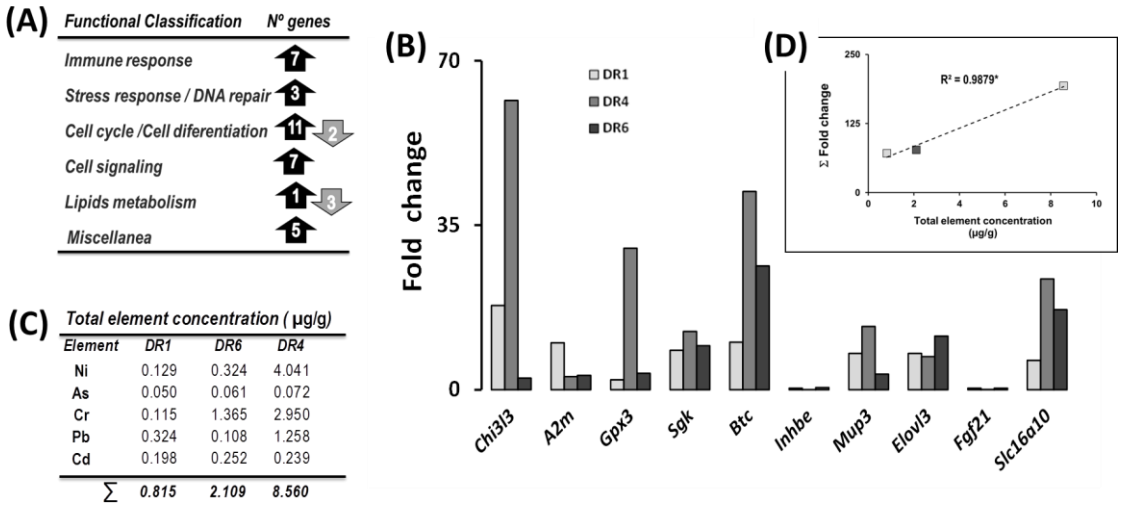

Fig 3. Results of microarray experiments with *M. spretus* (A) Functional classification of 39 differentially expressed genes with >10-fold-changes in at least one of the studied sites (DR1, DR4 and DR6), with indication of the number of up- or down regulated genes included in each category. (B) Comparison of the variations in the relative transcript number of a selected group of genes included in the different functional categories in animals DR1, DR4 and DR6. (C) Mice renal toxic element concentrations (ug/g). (D) Correlation between the total increase in the relative transcript number (fold.change) corresponding to the genes analyzed in (B) and the total renal toxic element concentrations given in (C). Dashed line represents the linear regression equation for the three investigated locations. Statistical significance is expressed as $*P < 0.05$. $n = 9$ mice per sampling site.

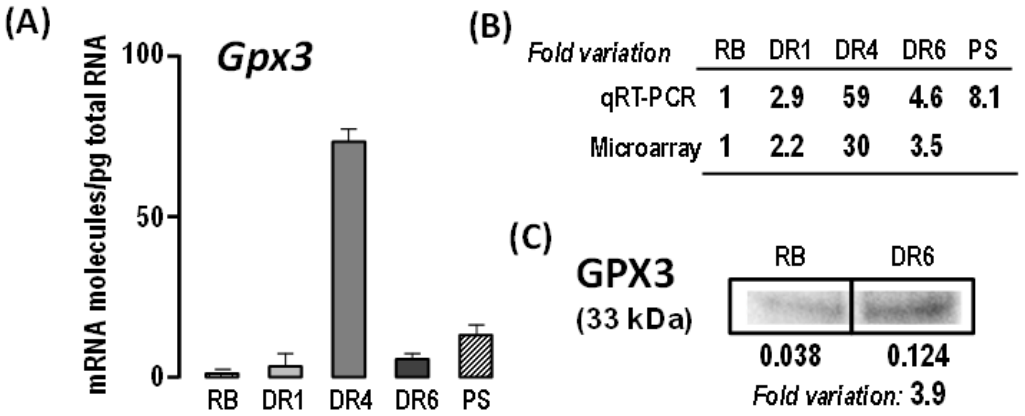

Fig 4. Verification and validation of the microarray experiments with *M. spretus*. (A) Absolute quantification by real time qRT-PCR of the *Gpx3* transcripts in the same sample pools used in the microarray experiments, for data verification. A similar sample corresponding to animals captured in PS was included for data validation. Data are mean ± SEM of four technical replicates. (B) Comparison of microarray and qRT-PCR fold variations between the reference and each problem site. (C) Western blotting of GPX3 proteins. Numbers give the intensities of immunoblotting signals normalized with total protein contents.

2.3. Environmental Proteomics

Protein expression levels are the balance among transcription, translation and degradation of proteins in cells, including the different steps of transcripts and proteins maturation and modification. Proteomics is the global analysis of these proteins and provide additional and complementary information to the transcriptomics approach. As an omics methodology, environmental proteomics provides a wide comprehensive assessment of toxic and defensive mechanisms triggered by pollutants without requiring any previous knowledge of their toxicity mechanism. Proteomic approaches permit to identify proteins that are significantly altered in an organism after pollution exposure, what can to help to reveal the toxicity mechanism. Additionally, these proteins, once identified, can be used as new biomarkers in ecosystems biomonitoring. The lack of data relative to protein sequences of non-model organisms with environmental interest is a drawback that we solved using model sequenced organisms genetically homologous to free-living bioindicators, that is the case of the couple of mice M. musculus/*M. spretus*, previously mentioned, used as bioindicator. The Fig 5 compares the hepatic proteomes from the laboratory mouse *M. musculus* (BALB/c), and the aboriginal species, *M. spretus* (SPRET/EiJ), both commercial inbred strains (Jackson Laboratory, USA). Though quantitative and qualitative differences were visible (*e.g.*, the spot of pI 6.5 and 6 kDa or the set of pI ~4.5 and ~16 kDa), both proteomes were quite similar. The nature of the proteomic differences between the two mouse species has not been further studied, but we assume that at least some of them most probably arise from differences in the cellular machinery responsible for post-translational modifications [49], as suggested by the clear differences shown by a "spot train" of pI 4.0–4.5 and ~40 kDa and from the similitude of both species genomic sequences [16].

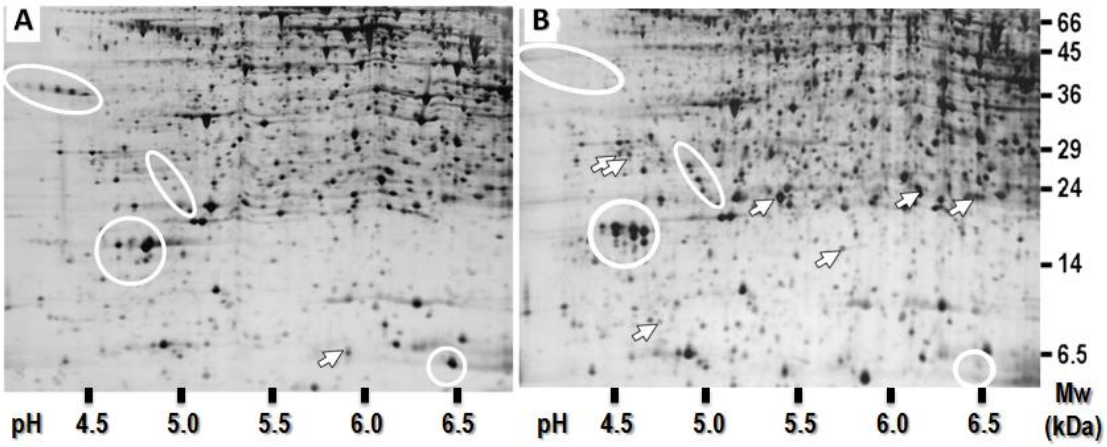

Fig. 5. 2-DE master gel of cytosolic liver proteins from *(*A) *M. musculus* (BALB/c, n=6) and (B) *M. spretus (*SPRET/EiJ, n=6). Comparison was focused on hepatic soluble proteins of Mr 5–100 kDa and pI 4.0–7.0.Oval frames and arrows point to protein spots with different intensity levels between both inbred strains (Jackson Laboratory, USA).

The authors of this work have applied proteomic approaches to evaluate the environmental quality status of DNP and its surroundings, using soluble hepatic proteins from *M. spretus* for 2-DE analysis. The proteomes of mice from areas heavily polluted by metals and pesticides (MAT), and from the reference area (LDP) were compared. Over 2900 proteins were resolved by 2-DE in 18-cm wide gels with a 4–7 pH range. Twenty protein spots were significantly different between both areas: 16 predominated at the reference area (repressed proteins in polluted mice, framed by ovals in Fig 6), and 4 spots were found at the pesticide-polluted area (induced proteins; Fig 6, arrows).The diminution in these protein amounts detected in MAT polluted mice might be the cause of the decreased antioxidative enzymes – G6PDH, 6PGDH, Cat – paralleled to the increase in oxidative damaged molecules –MDA, GSSG –, previously reported in *M. spretus* mice. [7]

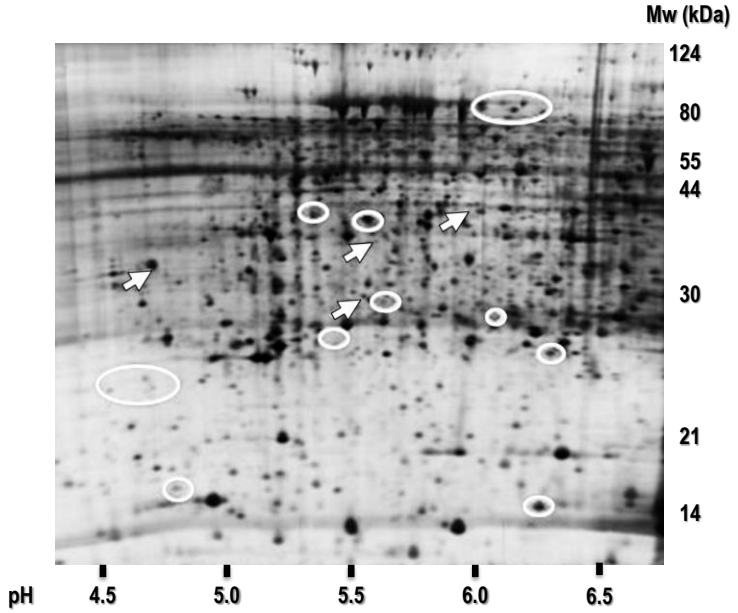

Fig. 6. 2-DE master gel of cytosolic liver proteins from *M. spretus* captured at MAT. Experiment (IEF pH 4-7, 18 cm, Amersham; 12,5% SDS-PAGE) compared the proteome of mice captured at MAT, an area heavily polluted by metals and pesticides, with that of mice collected at the reference site, RB. Arrows point to protein spots with a high intensity level in MAT samples (induced proteins); ovals frame spots with diminished intensity (repressed proteins) in MAT samples. *n* = 9 mice per sampling site.

The proteomic methodology has also been used to complement the transcriptomic studies in animals captured in DR1, DR4 and DR6. Equal amounts of livers from four male mice/site were pooled and homogenized and 2-DE electrophoresis was applied as described [5,12], using 24 cm (pH 4–7) Amersham Immobiline Dry-Strips®. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done in 12.5 % gels using the BioRad Protean® Plus Dodeca cell (20 °C) at 2.5 W/gel, 10 min, and 10 W/gel until separation was finished. Gels were silver-stained following a protocol compatible with MS analysis [5]. Gel images of three replicates per sample were obtained with a BioRad GS-800 densitometer. Spot volumes were quantitated using the PDQuest software (v7.1, BioRad). Around 2500 protein spots were resolved in the pH and Mw ranges indicated in Fig 7. One-way analysis of variance followed by the Student–Newman–Keuls post-test was then used for a definitive selection of the spots showing altered expression patterns (over/underexpression ratio of at least 3-fold) between the different animal groups and the reference. Thirty six differentially expressed spots were manually excised, reduced (10 mM DTT), alkylated (55 mM iodoacetamide), digested overnight at 30 °C with trypsin (Promega) and the peptides extracted with ACN/TFA (acetonitrile/trifluoroacetic acid) as previously described [5]. Aliquots of 0.5 µL were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) peptide mass fingerprint (PMF) in a Voyager DE-PRO instrument (Applied Biosystems) in reflectron mode. PMF data were contrasted against mammalian sequences included at Swiss-Prot (EBI) and non-redundant NCBI (Bethesda, USA) databases using Protein Prospector (California University, USA) and MASCOT (Matrix Science, UK) software [5]. Sixteen spots were finally identified, as summarized in Fig 7, which are involved in different cellular functions: axonal transport and cell division, proteolysis, central pathways of glucid, fatty acid, amino acid, methyl, and urea metabolism, biotransformation, and adaptation to oxidative stress [5]. Some changes in protein expression probably protect mice from the toxic effects of pollutants. This would be the case for the increase of glutathione *S*-transferase O1 (GSTO1), that protected from As toxicity, or the increase of glycine N-methyltransferase (GNMT), protective against polycyclic aromatic hydrocarbon (PAH)-derived hepatotoxicity. Proteomic data were consistent with metal biomonitoring and conventional biomarker responses, and with transcriptomic data above mentioned, indicating that DRS (and PS) mice sustained a heavier pollutant burden than RB or LDP specimens and suffered a chronic oxidative stress. In fact, 10 of the 16 identified proteins, protected polluted mice from oxidative stress or were targets of oxidative damages, directly or due to increased proteolytic susceptibility [5,7].

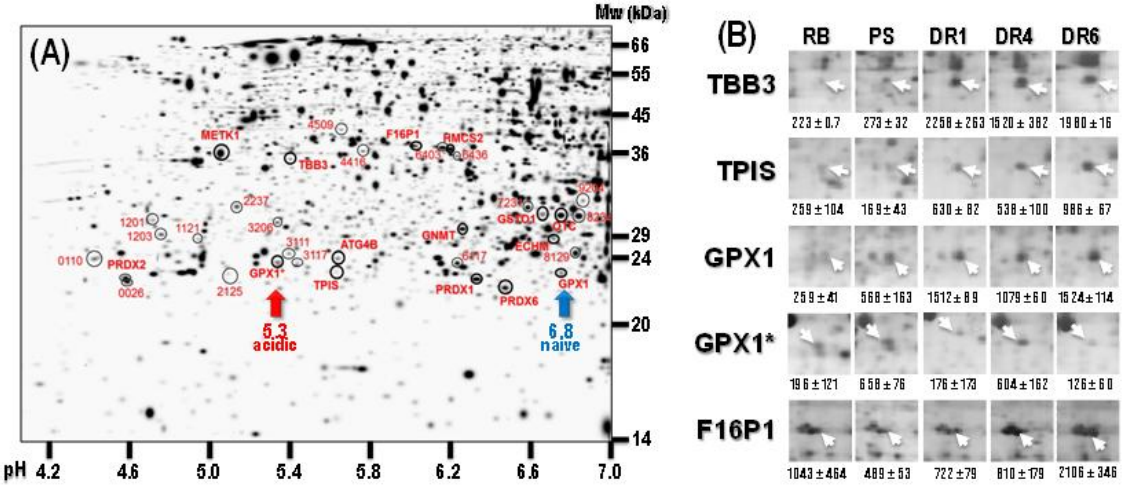

Fig. 7. (A) 2-DE master gel of cytosolic liver proteins from *M. spretus*. The 36 spots excised for MALDI-TOF-PMF analysis are encircled. Identified proteins are indicated by their Swiss-Prot entry name. Arrows point the two GPX1 isoforms identified by their different pI. (B) proteins that were up-regulated in the animals collected in at least one of the contaminated sites. n = 9 mice per sampling site.

A novel, MS-based approach has been used for the relative quantification of proteins, relying on the derivatization of primary amino groups in intact proteins using isobaric tag for relative and absolute

quantitation (iTRAQ®). The method allows address biomarkers identification in environmental studies. iTRAQ® is a technique that utilizes a multiplexed isobaric chemical tagging reagent which allows multiplexing of up to eight protein samples and produces identical MS/MS sequencing ions for all eight versions of the same derivatized tryptic peptide. This greatly facilitates peptide identification due to the resulting higher intensities of the parent and fragment ions. Quantitation is achieved by comparison of the peak areas and resultant peak ratios for either eight MS/MS reporter ions, which range from 113-119 and 121 Da. Cytosolic hepatic proteins from *M. spretus* mice captured at LDP, PAR, MAT and ROC areas (nine male mice per site, extracted after pooling equal amounts of cryohomogenized tissue), were reduced (10 mM DTT), alkylated (55 mM iodoacetamide), digested overnight at 30 °C with trypsin (Promega) and the peptides extracted with ACN/TFA (acetonitrile/trifluoroacetic acid) as described [5]. After being marked with iTRAQ® labels (iTRAQ® Reagent-8Plex Multiplex Kit, Applied Biosystem), proteins in different samples were mixed and analyzed by LC-MS/MS. Identical peptides derived from different samples have the same mass and were separated in a single peak. However, in the second tandem mass analyser, the reported groups (m/z: 113, 114, 115, 116, 117, 118, 119, and 121) are separated and their signal intensity ratios indicated the ratios of the peptide quantities to determine the relative quantities of the peptides of each sample relative to the reference sample. A total of 97,384 peptides were identified and analyzed with the adequate software (Proteome Discoverer, Thermo) and a total of 225 proteins, with *Xcorr* ≥ 2 and represented by more than two peptides, were selected. In a preliminary analysis, proteins were classified (Ingenuity Pathways Analysis) into five functional categories related with the mice defense against xenobiotic-caused stress (Fig 8). Most of them are involved in the aryl hydrocarbon receptor (AhR) signaling pathway. Many xenobiotics exert direct biological effects by binding to cytosolic AhR, which results hence activated. AhR is induced by PAHs and its primary role is the control of xenobiotic metabolism through cytochrome P450 [50]. Activation of AhR has a variety of downstream effects which influence on tumor genesis, inflammation, formation of DNA adducts, cell proliferation, and loss of cell-cell adhesion. Other important groups of proteins were linked to protection against oxidative stress and phase II detoxification reactions and the immune response. All these three groups were similarly present in the three studied site samples, which indicate a global response to contamination. In contrast, fatty acid metabolism is differentially represented in the three samples, what probably indicates an association with specific pollutants present in each zone. We are currently performing experiments for verification of this data and trying to associate the different responses to particular pollutants.

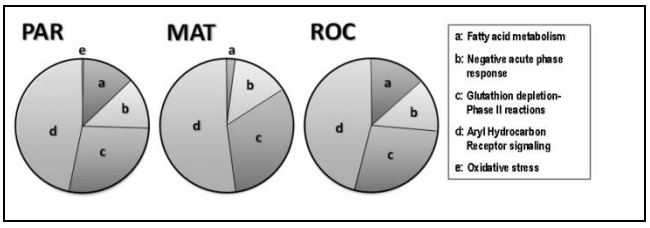

Fig. 8. A) Functional classification of proteins differentially expressed in *M. spretus* mice captured at three polluted sites (PAR; MAT, ROC) in relation to reference mice (RB) as identified by iTRAQ® methodology. *n* = 9 mice per sampling site.

2.4. Environmental metallomics to evaluate terrestrial ecosystems affected by metal pollution

Most metabolic pathways require enzymes with a transition metal acting as an essential cofactor. In the systems biology of transition metal metabolism, the metallome is the whole of cellular metal content, considering its speciation, and localization in each cell and organism [51]. A systematic view of transition metal metabolism requires the understanding of how organisms sense, adapt and use these metals, as well as their traffic and interactions in cells and tissues, considering transport by biological fluids and cross through biological barriers [52]. Metal ions manifest their importance in far more than the ~50% of proteins discovered so far, and metalloproteins represent to 30% of proteins in known genomes.

Metallomics is one of the most recent *-omics* [51] which uses metals or metalloids, as heteroatomic markers or tags to track these molecules in complex biological matrices [2,5,7,25,28,31,53,54]. Metallomics provides a good alternative to deep insight into the fate of elements in exposed organisms, and gives information about metals trafficking, interactions and homeostasis.

A critical step in metallomics of tissues and biological fluids is sample preparation. This step could be understood as any manipulation that modifies the sample matrix in order to make it more suitable for analysis. Sample preparation for metallomic from tissues or biological fluid requires several steps: (i) cell disruption or a partial degradation of sample matrix for later extraction of the analyte using mechanical treatments in presence of liquid nitrogen [4], such as cryhomogenization step; (ii) analyte extraction and solubilization assisted by different buffers, mainly with ion strength suitable to the type of protein present in the sample [55], e.g. Tris-buffer and ammonium acetate, this later more suitable for sample analysis with ESI-MS because its greater volatility [4]; (iii) reduction of disulfide bonds to promote protein denaturation and to avoid mistakes in molecular mass assignation and further identification [55]. For this purpose, free thiol-containing reducing agents such as dithiothreitol (DTT), dithioerythritol (DTE), reduced glutathione (GSH) and β-mercaptoethanol are used. However, these reagents have metal chelating properties and are being replaced by others, such as tributylphosphine (TBP) and tris–carboxyethylphosphine (TCEP) with milder reducing properties and without chelating properties [4]; (iv) inhibition of proteases by means of a proteases inhibitor without chelating properties, such as phenylmethylsulfonyl fluoride (PMSF) [4]. Optionally, when element speciation is required addition of detergents is necessary, such as SDS or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), since the element is incorporated or strongly complexed by the biomolecules and detergents disrupt the cell membranes, breaking lipid–protein interaction and, consequently, solubilizing the metalbinding proteins and preventing hydrophobic interactions [56].

In metallomic approaches is fundamental the use of high sensitivity and multielemental atomic detectors mainly ICP-MS [57], generally coupled to a chromatographic module (in single or multidimensional arrangements), and mass spectrometry for parallel biomolecules identification in an integrated workflow [4,57,58]. The use of ICP-MS is critical in speciation and metallomics since it allows: (i) multielemental capabilities (including non-metals such as S, P, Se), (ii) elevated sensitivity, (iii) high tolerance to matrix and, (iv) great linearity range. Several analyzers can be used in ICP-MS detection such as quadrupole (Q), time of flight (TOF), sector field (SF) or multicollector (MC). Sample introduction can be performed by nebulization of HPLC outflow, capillary electrokinetic chromatography (CEC), or CE, capillary zone electrophoresis (CZE). Alternatively, laser ablation (LA) from gel electrophoresis (GE) spots of living organisms organs can be used for this purpose [59]. Several modes of chromatographic separations can be used for species discrimination at preparative, capillary or nano scale, and usually two or three orthogonal systems can be off-line combined to increase metal-biomolecules resolution, such as size exclusion chromatography (SEC) [18], reverse phase (RP) [60], ion exchange chromatography (IEC) [4], hidrophilic interaction liquid chromatography (HILIC) [61] and affinity chromatography (AF) [62]. However, as commented before, the combination with organic mass spectrometry is mandatory for unknown species in biochemical issues, especially electrospray ionization (ESI) or matrix assisted laser desorption (MALDI). The use of ESI-MS is more suitable than MALDI-MS for tandem mass spectrometry and on-line couplings with separation techniques (HPLC, CE). However, MALDI-TOF-MS is recommended for matrices with low complexity. Moreover, ESI-MS is a technique sensitive to concentration and both covalent and non-covalent bonds are preserved, while MALDI-MS is mass sensitive and only covalent bonds are preserved. Several mass analyzers can be used to obtain structural information of species such as triple quadrupole (QqQ), triple quadrupole time of flight (QqQTOF), ion trap (IT), quadrupole trap (QTrap) or Fourier transform ion cyclotron resonance (FT-IRC) [58].

The biological response to metal contamination accounted in mice organs and fluids of free-living mice *M. spretus* (male mice weight about 12 g) from contaminated (MAT, AJO, PAR, ROC) and non-contaminated (LDP) sites of Doñana National Park and surroundings was studied (Fig. 2) by our research group [4,6]. The up and down-regulation of metal-biomolecules caused by pollutants was traced by SEC-ICP-MS. The most interesting results were obtained by the response of Cu-, Zn- and Cd-biomolecules, and the chemical species from the toxic element As [4]. The distribution, accumulation and

transference of zinc, copper and cadmium in living organisms have been considered in detail in the literature. Several facts such as the modulation of zinc concentration by homeostatic mechanisms [63] and the importance of transport mechanisms on copper distribution [64] are relevant issues to explain the relative presence of metal-binding molecules in the different organs of exposed organisms. In relation to this, the induction of Cd and Zn-metallothioneins in mice (*M. musculus*) exposed to industrial dust rich in metals has been reported [65], and these experimental data confirm the antagonistic interactions among Cd, Zn, Cu and Pb, as well as the differential rate of excretion of these elements from kidney/liver under increasing exposure [65]. The intensity of Cu-peak from the contaminated area MAT is clearly higher than that from other sites also affected by contamination, such as ROC and AJO (that represent a 40 % reduction of intensity respect to MAT) and LDP and PAR (60-70% reduction of intensity respect to MAT). A similar peak located at 7 kDa was observed for Zn although in this case LDP exhibits the highest intensity followed by MAT, which fact can be related with the correlative concentration of this element in the cytosolic extract. The equivalent peak traced by Cd (7 kDa) shows lower sensitivity, with remarkable intensity of peaks corresponding to MAT and LDP [4]. Another peak traced by Cu at 32 kDa shows higher intensity for samples from LDP, MAT, ROC and PAR, but the response is lower in the area with low pollution (AJO). To confirm that Cu,Zn-peak of molecular mass of 32 kDa is superoxide dismutase, it was collected from liver extract using a preparative size exclusion column coupled to ICP-MS (Fig. 9A). The fraction was desalted, lyophilized, and analyzed by AEC-ICP-MS (Fig. 9B). In this later chromatogram it is possible to observe a predominant peak traced by Cu and Zn which can be collected with a preparative AEC column, lyophilized, desalted and submitted to tryptic digestion for identification by nESI-QqQ-TOF (Fig. 5C). The mass spectrum of peptides resulted after tryptic digestion was used for protein identification, using doubly charged peptide ions of m/z 584.31, 684.39 and 756.84 for MS/MS analysis and protein identification in MASCOT database (Fig. 9D), following an integrated metallomic workflow (Fig. 9). The results confirm the presence of Cu,Zn-superoxide dismutase associated to the peak of 32 kDa. In all cases, the identified peptides were assigned to *M. musculus* mice, a sequenced model organism genetically homologous to *M. spretus*.

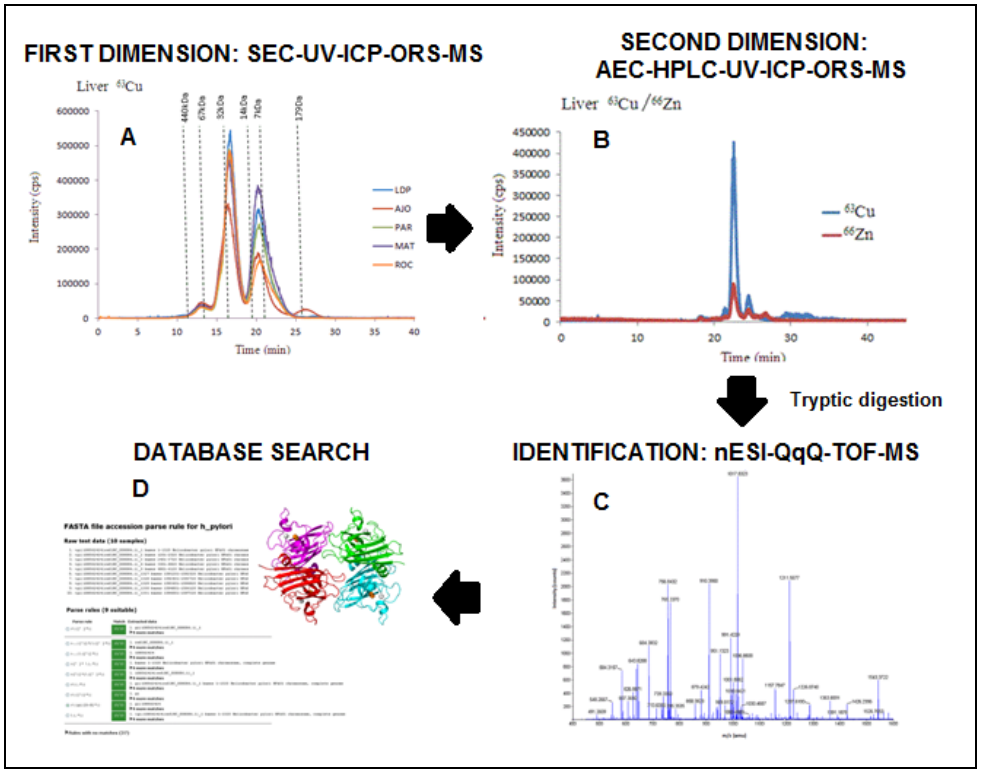

Fig. 9. Workflow of metallomic approach for mice exposed to metals

Additionally, the high presence of arsenic in the surrounding of DNP has been checked [19], and speciation of this element in plasma from free-living mice shows the presence of iAs(III) but especially DMA in contaminated areas, such as MAT and ROC (Fig. 2). These results confirm the suggestion of Suzuki *et al.* [66] about the potential use of arsenic in blood and plasma as biomarker of exposure to this element.

2.5. Environmental metabolomics in mice subjected to metal pollution

Environmental metabolomics is an emerging field referred to the application of metabolomics to characterize the interactions of living organisms with their environment [67]. This information is of great value in several issues such as risk assessment of chemicals in the environment, study of mode of action (MOA) of toxicants and discovery of indicators for the health of animals. In particular, the use of metabolomics to study metal toxicity is gaining importance in recent years [68]. In metabolomics we consider molecules with molecular mass less than 1000 Da, which are usually intermediate metabolites and end products of cellular functions, and their levels can be considered as the response of biological systems to environmental or genetic manipulation. The metabolome is very diverse including lipid soluble usually associated to cell membranes, polar metabolites from the cell, and acid and basic ions.

Recently, the correct choice of sample treatment procedure for metabolites extraction from tissues and biological fluids is starting to gain importance, because it affects both the observed metabolite content and biological interpretation of data [70]. For comprehensive and reproducible metabolomic analysis, these procedures must be non-selective, simple and fast [71]. Thus, analysis of biofluids is normally carried out after protein precipitation [72], while extraction of tissues requires a previous lysis of cells, involving usually a cryhomogenization step, as in metallomics approaches, in combination with a simple extraction protocol based on the use of polar: lipophilic solvents mixtures [73]. Up to date, there is not a universal method to extract and analyse all the metabolites.

Metabolomics requires analytical techniques with high sensitivity and ability for molecules identification that in addition be accessible for most laboratories, as mass spectrometry that together to nuclear magnetic resonance (NMR) spectroscopy are major analytical tools in this *omic* [74]. NMR is only a detection technique which does not rely on separation of analytes, but the interpretation of spectra is difficult for complex samples. MS is usually coupled to high performance liquid chromatography (HPLC) [75], gas chromatography (GC) [76], or capillary electrophoresis (CE) [77], in order to reduce the complexity of mass spectra, avoiding isobaric interferences and making possible metabolite identification and quantification. Gas chromatography, especially when interfaced with mass spectrometry (GC-MS), is one of the approaches most widely used in metabolomics, since it offers very high chromatographic resolution, but requires chemical derivatization for many metabolites. Nevertheless, high mass and polar metabolites cannot be analysed with this technique [78]. On the other hand, HPLC has lower chromatographic resolution, but the number of metabolites able to be analyzed is greater [79]. In addition, CE is most appropriate for charged analytes [80]. Finally, considering the advantages and drawbacks of separations techniques coupled to mass spectrometry, a number of studies propose the use of sample direct infusion to mass spectrometer without any previous separation [81], this approach makes analysis simpler and faster and the traditional isobaric interferences affecting direct infusion are overcome with the use of high resolution mass analyzers, such as hybrid systems triple quadrupole-time-of-flight (QqQ-TOF) [6,81] or Orbitrap [82]. These technological advances require the improvement of bioinformatics and chemometrics methods, mainly related to chromatographic data alignment and discrimination of components in large datasets.

A number of studies based on metabolomics in plasma and kidneys from *M. spretus* mice captured in DNP and surroundings (LDP, PAR, MAT), show perturbations in metabolic pathways related with oxidative stress, which maybe provoked by metal presence in these areas [6]. Metabolites from plasma and kidney of free-living mice *M. spretus* were extracted and analyzed in positive (ESI+) and negative (ESI-) ion mode (Fig. 10A), resulting different spectral profiles in a wide spectral range (m/z 50-1100) by DI-ESI-QqQ-TOF-MS. After the discriminant analysis of the results (Fig. 10B) and the

identification of metabolites associated to this discrimination (Fig. 10C), the metabolomic analysis was complemented with the analysis of plasma samples by GC-MS (Fig. 10D). Thereafter, a biological interpretation is mandatory (Fig. 10E) [6].

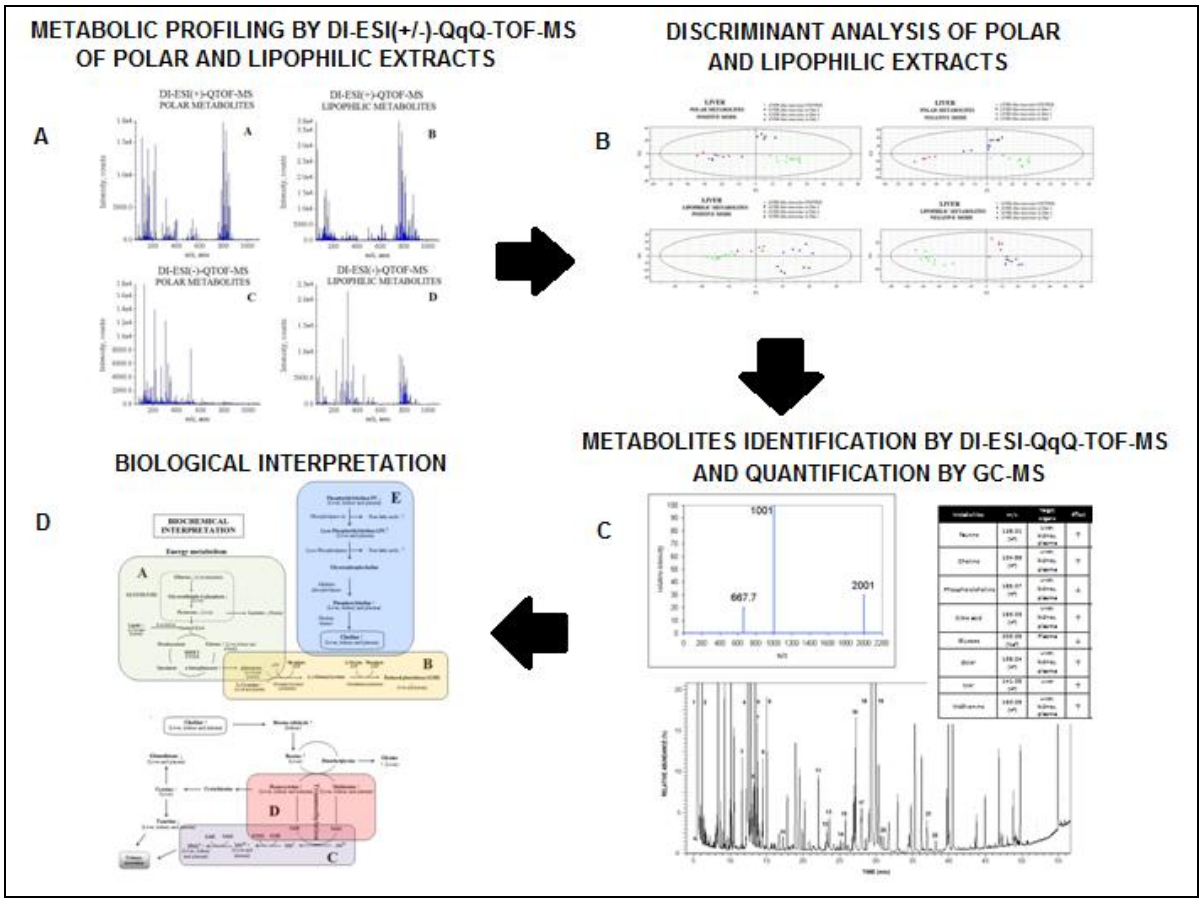

Fig. 10. Workflow of metabolomic approach for mice exposed to metals

Metabolomics of plasma and kidneys from mice captured in DNP and surroundings (LDP, PAR and MAT, Fig. 2) showed perturbations in metabolic pathways due to the presence of metals in the ecosystem, such as breakdown of the membrane phospholipids (phosphatidylcholines (PCs), Lyso-phosphatidylcholines (LPCs), free fatty acids (FFAs) and triglycerides (TGs)), energy metabolism (carnitine, citric acid, glutamic acid, glucose) and some metabolites related with oxidative stress (taurine, reduced glutathione) (Table 1) [4].

Table 1. Metabolites with altered abundance profiles in kidney tissue and plasma of mice (*M. spretus*) affected by metal pollution [4].

ALTERED METABOLITES	m/z	Effect	Target organs	Acquisition MODE	Associated Pathology
Creatinine	114.1	↑ PAR ↑↑ MAT	Kidney and plasma	ESI+	Renal damage
Glutathione	308.1	↓ PAR ↓ MAT	Kidney and plasma	ESI+	
Taurine	124.0	↑ PAR ↑ MAT	Kidney	ESI-	Oxidative stress
Glutamic acid	146.0	↑ PAR ↑↑ MAT	Kidney and plasma	ESI-	
Choline	103.0	↑ PAR ↑ MAT	Kidney and plasma	ESI-	
	105.0	↑ PAR ↑ MAT	Kidney and plasma	ESI+	
Lyso-Phosphatidylcholines	450-550	↑ PAR ↑↑ MAT	Kidney and plasma	ESI+	Degradation of membrane phospholipids (cell apoptosis)
Phosphatidylcholines	700-850	↓ PAR ↓↓ MAT	Kidney and plasma	ESI+	
Pipecolic acid	130.1		Kidney	ESI+	
Arachidonic acid	303.2	↑ PAR ↑↑ MAT	Kidney	ESI-	
Oleic acid	281.3		Kidney	ESI+	
L-Carnitine	162.1	↓ PAR ↓ MAT	Kidney	ESI+	Responsible for transporting fatty acids from the cytosol to the mitochondria for energy production by β oxidation

*Variations compared to control mice (LDP): ↑, increasing signal intensity, ↓, decreasing signal intensity.

2.6. Ionomics

The ionome is defined as the mineral nutrient and trace element composition of an organism and represents the inorganic components of cellular, tissue or biological fluids. Ionomics involves the quantitative and simultaneous measurement of the elemental composition of living organisms and the changes in this composition as response to physiological stimuli, developmental state, and genetic modifications [82]. In this field, ICP-MS technology plays an important role as a consequence of multielement capabilities, sensitivity and small amount of sample needed to perform the analysis. These advantages make it essential in studies involving micro-mammals. After multielemental determination in different tissues and biological fluids (Fig. 11A), results obtained are subjected to statistical studies that allow us to discriminate between the groups under study, normally discriminant analysis, such as PLS-DA (Fig. 11B).

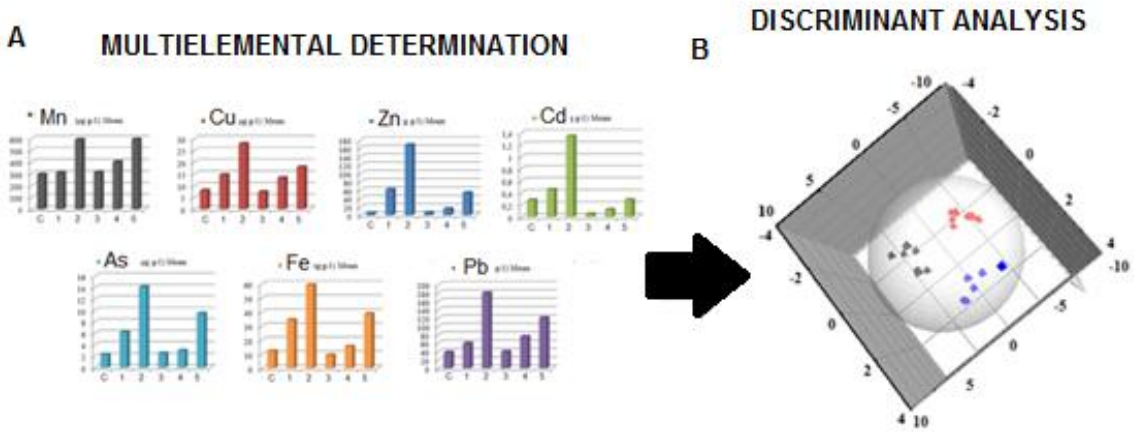

Fig. 11. Workflow of ionomic approach for mice exposed to metals

The application of ionomics to kidney from free-living mice *M. spretus*, captured in DNP and surroundings, allows us to discriminate between mice occurring in different areas on the basis of multi-element concentration in this organ. We have measured metal concentrations in kidney from *M. spretus* mice captured in two polluted sites, PAR and MAT, and compared their levels with those of the reference LDP animals. It is remarkable the high levels of Cu, Zn, Mn and Fe in kidney extracts from free-living mice in all sampled animals, independently of their capture site [4]. In contrast, toxic elements, such as Cd, As, Ni, Cr and Pb showed very low concentrations in these samples, but with significantly higher levels in mice captured in PAR and particularly in MAT. Chemometrics analysis revealed that samples from PAR and MAT can be discriminated from LDP samples because of their higher concentration of Mn, Fe, Zn, Cu, As, Cd and Pb in kidney.

3. Metals interactions under environmental metal stress

Since living organisms are usually exposed to a complex environment in which different elements and their species are present together and interact, it is particularly difficult the interpretation of triggered biological mechanisms. Hence, analytical methods for multi species analysis, considering their biological, synergistic and inhibitory effects, are claimed. The antagonistic interactions more studied during the last years are those corresponding to the couples Se/As and Se/Hg. A good example is the antagonistic effect of Se on Hg toxicity that was reported in 1967 based on experiments with rats treated with mercury chloride and selenite [84].

Interactions between elements depend on the chemical species and organism type [85]. In rodents, Se prevents As-induced cytotoxicity when arsenite and selenite are simultaneously administered to mice and hamster [86-88]. In rats, co-administration of selenobetaine (SeBet) and arsenite enhances the tumour-suppressive effect of selenium [89].

Taking into account the literature, Se and Zn are the most interesting elements in relation with their antagonistic and synergistic actions with other toxic elements, such as Cd, As and Hg. Zn counteracts the toxic effect of Cd in rats [90]; Zn consumption may be beneficial against Cd hepatotoxicity [91]; Zn reduces the Cd-induced metallothionein synthesis [92] and Zn prevents Cd-induced alterations in lipid metabolism [93]. Finally, several studies in rats confirm that Se presents numerous antagonistic interactions with Cd, such as prevention of Cd-induced oxidative stress [90], protection against Cd-induced nephrotoxicity and hepatotoxicity [94], antagonism of Cd-induced inhibition of hepatic drug metabolism [95] and Cd-induced testicular damage [96]. On the other hand, antagonistic effects of Se on Hg toxicity have been object of numerous studies along the time. In humans,

Se prevents Hg-induced cardiovascular diseases [97], inhibits Hg-induced neurotoxicity [98], prevents Hg-induced apoptosis [99], represents a defence against methylmercury ($MeHg^{+}$) toxicity [100] and causes Hg detoxification [101], between others. The study of species interactions of elements in biological organisms requires experts from various fields such as biology, chemistry, medicine, biochemistry, molecular biology and genetics [85]. In addition, in real environments, animals are exposed to multiple elements that interact among them, so that the assessment of biological response has to be oriented to the toxicological effects caused by the combination of metal/metalloids and their species.

4. The role of isotopic dilution analysis in environmental metallomics

In recent years numerous applications of isotope dilution (IDA) methodologies have been proposed for novel fields, such as metallomics and speciation, with the purpose of determinate individual chemical species in in different tissues or biological fluid [102,103]. This requires a previous separation of the species to be determined before the mass spectrometric measurement by ICP-MS.

During the application of IDA in elemental speciation, new quantitation forms have been described. For example, post-column isotope dilution (species unspecific mode), when standards for the species are not available, allows the accurate determination of elemental species even if the structure of the compounds is unknown, while multiple isotopically enriched species can be applied for the evaluation and correction of species interconversion reactions [62]. However, this mode of spiking can only be applied when the ionisation efficiency of the element is independent of the chemical form in which the elements reach the ion source. On the other hand, when standards are available, the species-specific spiking mode requires the use of a spike solution containing the species to be analysed in an isotopically labeled form, similarly to classical organic isotope dilution technique [104].

Model organisms exposure experiments using enriched stable isotopes are a good alternative to evaluate the metabolic response to these elements under established conditions. In order to understand the toxicology and metabolic pathways of toxic and essential elements, analytical strategies have been developed to obtain qualitative and quantitative information concerning the elements, element species, their interactions, transformations and functions in biological systems. In this sense, selenium stable isotopes have been extensively used to study selenium metabolism in rats and humans and its bioavailability from food to feces or urine [105]. In most recent years, isotope pattern deconvolution (IPD) quantitative data of endogenous and exogenous essential (or toxic) elements has been used to discriminate the fate of endogenous and supplemented selenium and their catabolic species in rat urine, by using ICP-MS equipped with a collision/reaction cell coupled to HPLC. In this case, two different enriched isotopes are necessary, a metabolic tracer (^{77}Se) and a quantitation tracer (^{74}Se) [106]. In this sense, have been reported different studies based on the application of enriched stable isotope tracers in terrestrial ecosystems to investigate the uptake and distribution of the classical pollutants Cd, Pb, and Hg in connection with environmental studies [107-109]. In summary, IDA and IPD analysis play an important role in the quantification of metalloproteins to compare the biological response in organs and biological fluids from mice captured in areas affected by metal pollution.

5. Laboratory exposure experiments of *M. musculus* to arsenic and cadmium.

A number of toxic elements, such as As and Cd, increases cancer incidence and toxicological disorders in living organisms. However, the molecular mechanisms underlying cell damage remain unknown. For this reason controlled exposure experiments with model organisms (*M. musculus*) can provide suitable information about the biological response of model organisms against these contaminants, as well as metal homeostasis, metals interactions and their traffic between organs, which can be correlated with similar responses in environmental areas [6]. In controlled exposure experiments the concentration and bioavailability of xenobiotics, exposure conditions and duration of experience are important parameters, which can be selected on the basis of the experiment purpose. These experiences are a good starting point for further correlations to complex environmental issues.

The response to toxic effects of arsenic in the free-living mice *M. spretus* has been evaluated in a laboratory experiment with *M. musculus* [81]. Mice *M. musculus* (inbred BALB/c strain) were exposed to arsenic during 12 days, using animals of 7 weeks of age fed ad libitum with feed metals deficient pellets. The animals were allowed to acclimate for 5 days with free access to food and water under controlled condition (temperature (25-30ºC) and a 12 h light-dark cycle) prior to start exposure experiment. For this experiment exposure, 24 animals of *M. musculus* mice were divided into three groups, one used as control, other exposed to As(III) (in the form As_2O_3) and a third group exposed to Cd (II) (in the form $CdCl_2$), using oral administration of 100 µL of a solution of 3 mg As per kg of body weight and subcutaneous injection of 100 µL containing 0.1 mg of Cd per kg of body weight and per day during a total period of exposure of 12 days. The control mice were subjected to oral administration of 100 µL of 0.9% NaCl in ultrapure water per day during 12 days.

Under As exposure, the results show that the ability of inorganic arsenic methylation in the liver may be overcome under chronic exposure to the element, and as a consequence arsenite is not methylated to MA^V and DMA^V, respectively. MA^V, DMA^V and low concentrations of the arsenic can be found in plasma and liver [81]. This observation has also been reported by others authors in relation to chronic arsenic exposure [110]. In addition, this fact agrees with previous reports, establishing the biotransformation of inorganic arsenic to methylated species (MA^V and DMA^V) in the liver of mammals. [111,112], which are finally excreted in the urine as pentavalent methylated arsenic forms [113]. On the other hand, SEC-ICP-MS coupling provided information about metal containing-proteins and metabolites related to arsenic exposure (metallomics), which has been correlated with the changes in the global metabolism (metabolomics), considering their consequences on in the redox status of protein and protein expression. Our study shows that arsenic causes biochemical pathways alterations, such as energy metabolism (*e.g.*, glycolysis, Kreb´s cycle), amino acid metabolism, choline metabolism and degradation of membrane phospholipids (apoptosis) (Table 2) [81]. Similar effects were obtained by Lai et al. when realgar (As_4S_4) was administered to rat using nuclear magnetic resonance as a metabolomic methodology [114].

On the other hand, when Cd is administered during 14 days to *M. musculus* the coupling SEC-ICP-MS provides the Cd, Cu and Zn-traced peaks from plasma which allows check the fate and potential interactions of metal-biomolecules in plasma and organs. Other study based on *M. musculus* showed antagonistic interactions among Cd, Zn, Cu, as well as differential rate of excretion of these elements from kidney/liver under increasing exposure to cadmium [65]. Cd-containing biomolecules in plasma present two remarkable peaks one at retention time matching with proteins of about 55 kDa, and other eluting with the MTs fraction (7 kDa). The accumulation of Cd in plasma along the exposure in the fraction of 55kDa can be related with the high affinity of this element by selenoprotein P [115]. The increasing intensity of Cd-MT peak reveals the higher concentration of metallothionein in plasma for detoxifying purposes. In this sense, it is well-known that Cd exposure causes changes in the distribution of endogenous Zn and Cu in plasma and organs, playing both metals a protective role against Cd toxicity due to their contribution in MTs induction [116]. Cd intake causes increasing intensity of Cu and Zn-traced peaks in plasma at about 67 kDa. This fact can be related with two important copper and zinc transport proteins in bloodstream, such as BSA and transferrin (Tf) with 67kDa and 79kDa molecular mass, respectively. Metabolomic results show (Table 2) perturbations in triglycerides (TGs) and diglycerides (DGs) levels and membrane phospholipids composition (PCs and LPCs), as well as the increasing presence of choline and polyunsaturated fatty acids (PUFAs), and decreasing of glucose, taurine, glutamic acid, and citric acid. These metabolic changes observed during Cd exposure can be related to perturbations in different metabolic cycles, such as degradation of membrane phospholipids, energy metabolism and metabolites related with oxidative stress.

Table 2. Metabolites with altered abundance profiles in plasma of mice (*M. musculus)* subjected to As and Cd controlled exposure.

Altered metabolites	m/z	*M. musculus* exposed to As	*M. musculus* exposed to Cd
Methionine	150.05 (H^+)	↑	
Choline	104.09 (H^+)	↑	↑
Phosphorylcholine	185.07 (H^+)	↑	
MA^V	141.05 (H^+)	↑	
DMA^V	139.04 (H^+)	↑	
Citric acid	193.03 (H^+)	↑	↓
Glutathione	308.04 (H^+)	↓	
Glucose	203.05 (Na^+)	↓	↓
Taurine	124.01 ($-H^+$)	↑	↓
Arginine	175.11 (H^+)	↓	
Glutamic acid	148.05 (H^+)	↑	
Creatine	132.07 (H^+)	↑	
Homocysteine	136.03 (H^+)	↓	
Free fatty acids (FFAs)	200-400	↑	↑
Phosphatidylcholines (PC)	700-850	↓	↓
Lyso-phosphatidylcholines (Lyso-PC)	450-600	↑	↑
Diglycerides (DGs)	600-700	↑	↑
Triglycerides (TGs)	750-950	↑	↑

Variations compared to control mice: ↑, increasing signal intensity, ↓, decreasing signal intensity

6. Correlations between laboratory metal exposure experiments of mice *M. musculus* and free-living mice *M. spretus* from Doñana Natural Park and surrounding affected by metal pollution

The homology between *M. musculus* and *M. spretus*, previously commented, allows interesting comparisons between the response of *M. musculus* under exposure experiments to toxic elements and *M. spretus* under environmental stress.

Response of *M. spretus* to As presence in the contaminated area MAT (Fig. 2) reveals the presence of low molecular species of this element, which is in agreement with the exposure experiments of *M. musculus* to arsenic, previously mentioned. In the Cu-chromatogram traced by SEC-ICP-ORS-MS a peak of high intensity at about 67 kDa was obtained, which can be related with transport proteins such as Cu/Zn-BSA and Cu-Tf of 67 kDa and 79kDa, respectively. Similar results were obtained for Zn that confirms this hypothesis. Higher intensities of both metals were detected in plasma from mice captured in MAT, which agrees with the increased presence of these peaks when Cd is administered to the laboratory mice *M. musculus*. The profile traced by Cd exhibits differences between mice captured in MAT and those from LDP. In MAT several peaks were obtained due to the high affinity of this element to thiol groups of proteins, as stated by other authors [66]. Similar pattern is traced by plasma from *M. musculus*

mice under cadmium exposure during 12 days. In addition, the difference in intensities among the peaks traced by Cu and Zn respect to Cd is also similar between laboratory exposed animals and free-living ones.

The Table 1 shows an increase in the levels of creatinine, LPCs, choline and glutamic acid in plasma from mice captured in MAT in comparison with control area (LDP). In addition, a decrease in the levels of GSH, PCs, arginine and glucose were observed. These results present high similarity with the metabolites altered in plasma of *M. musculus* mice under As and Cd exposure (Table 2).

In summary, the biological response of free-living mice *M. spretus* captured in MAT, affected by As, Cd pollution could be related with the high concentrations of these elements in MAT after the breakdown of the rafts from Aznalcollar pyrite mine in 1998, which presents high similarity with the response of *M. musculus* mice under As and Cd exposure, evaluated by metallomics and metabolomics approaches.

7. Concluding remarks

Due to the complexity of environmental systems, a comprehensive assessment of the effect of contaminants on living organisms requires the use of multi-disciplinary tools to sum up the multiple variables and factors contributing to ecosystem status. For this reason the use of massive information analytical approaches, the –omics, represents a good alternative to conventional biomarkers since this latter approach is limited by the requirement of deep knowledge of toxicity mechanisms from the contaminants under study and its target analysis character. However, the omics are non-target methodologies and therefore open to unexpected responses under particular environmental conditions. Several points can be remarked in relation to the results collected in the present appraisal: (a) the interest of use in environmental assessments bioindicators (such as *M. spretus*) that are genetically homologous to sequenced animals (*M. musculus* in this case), which allows interesting relationships between genetic response, protein expression, metabolites production enhancement or inhibition, metalloproteins and metallometabolites profile changes, and metal species transformations, considering the interplay among them. (b) The response to toxic elements, such as As, of free-living mice *M. spretus* can be evaluated using exposure experiments in the laboratory with the mice *M. musculus*, showing arsenic methylation in liver and alterations in energy metabolism and cell membrane degradation; with Cd exposure interactions between Cd, Cu and Zn in liver are confirmed and the accumulation of Cd in plasma associated to selenoprotein P and metallothionein, which is related to detoxification processes. In addition, metabolic changes related to degradation of membrane phospholipids, energy metabolism and oxidative stress were observed. (c) Conversely, results of biological response obtained from mice living in contaminated and non-contaminated areas from Doñana National Park can be correlated with laboratory experiments with *Mus musculus*.

In conclusion, the complexity of environmental monitoring of metal toxicity requires the integrated use of multi –*omics* technologies to achieve a comprehensive panorama in the study of environmental issues and the diagnosis of contamination threats.

8. LIST OF ABBREVIATIONS

DNP: Doñana National Park, qRT-PCR: quantitative reverse transcription-polymerase chain reaction, GPx3: glutathione peroxidase 3, LC-MS/MS: liquid chromatography-tandem mass spectrometry, SDS-PAGE: sodium dodecylsulphate - polyacrylamide gel electrophoresis, PMF: peptide mass fingerprint MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight, GSTO1: glutathione *S*-transferase O1, GNMT: glycine N-methyltransferase, PAH: polycyclic aromatic hydrocarbon, iTRAQ: isobaric tag for relative and absolute quantitation, AhR: aryl hydrocarbon receptor, DTT: dithiothreitol,

DTE: dithioerythritol, GSH: reduced glutathione, TBP: tributylphosphine, CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Q: quadrupole, SF: sector field, MC: multicollector, CEC: capillary electrokinetic chromatography, CZE: capillary zone electrophoresis, LA: laser ablation, GE: gel electrophoresis, HILIC: hidrophilic interaction liquid chromatography, IEC: ionic exchange chromatography, AF: affinity chromatography, IT: ion trap, QTrap: quadrupole trap, FT-IRC: Fourier transform ion cyclotron resonance, NMR: nuclear magnetic resonance, SeBet: selenobetaine, $MeHg^{+}$: methylmercury, IPD: isotope pattern deconvolution, MTs: metallothioneins, BSA: Bovine serum albumin, Tf: transferrin, PUFAs: polyunsaturated fatty acids, SEC: size exclusion chromatography, ICP-MS: Inductively coupling plasma-mass spectrometry, AF: affinity chromatography, IDA: isotopic dilution analysis, DI-ESI-QTOF-MS: direct infusion mass spectrometry, PC: phosphatidylcholine, LPC: lysophosphatidylcholine, FFA: free fatty acid, GC-MS: gas chromatography-mass spectrometry, CE: capillary electrophoresis, NMR: nuclear magnetic resonance, LDL: low density lipoproteins, HDL: high density lipoproteins, PMSF: Phenylmethanesulfonyl fluoride, TCEP: tris(2-carboxyethyl)phosphine hydrochloride, MTSFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide, TMCS, trimethylchlorosilane, RBCs: red blood cells, PTFE: polytetrafluoroethylene, ORS: octopole reaction systems, IDA: isotopic dilution analysis, MTs: metallothioneins, CA: carbonic anhydrase, SeCys: selenocysteine, Cys: cystine, SeP: selenoprotein P, SeAlb: selenoalbumin, eGPx: extracellular glutathione peroxidase, TCA: tricarboxylic acid cycle, GSH: reduced glutathione, QTOF-MS: quadrupole-time-of-flight mass spectrometry, ESI: electrospray ionization, ICP-MS: inductively coupled plasma mass spectrometry, PLS-DA: partial least squares discriminant analysis, VIP: variable importance in the projection, TGs: triglycerides, DGs: diglycerides, iAs^{III}: arsenite, iAs^{V}: arsenate, MA^{V}: monomethylarsonic acid, DMA^{V}: dimethylarsinic acid.

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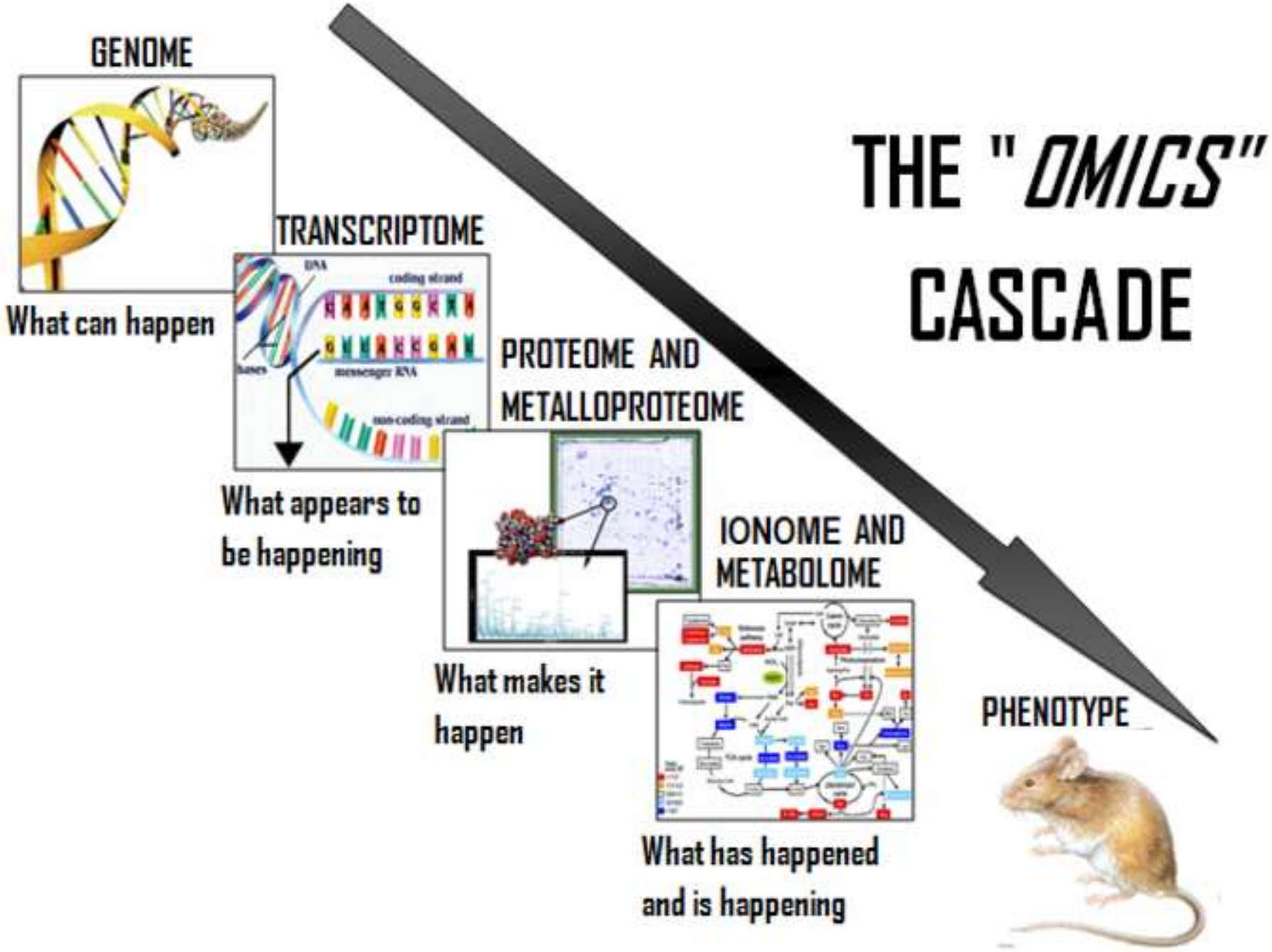

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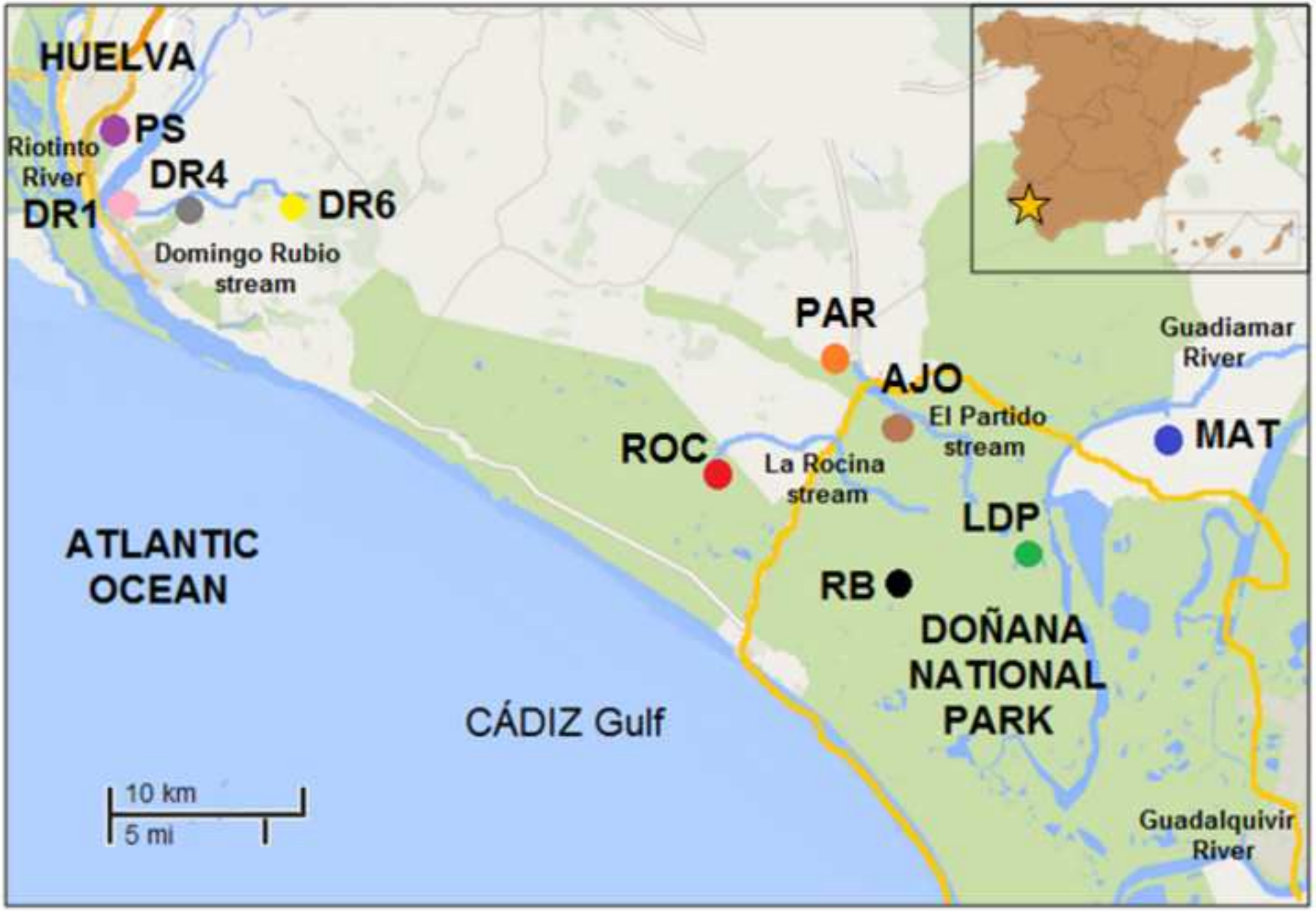

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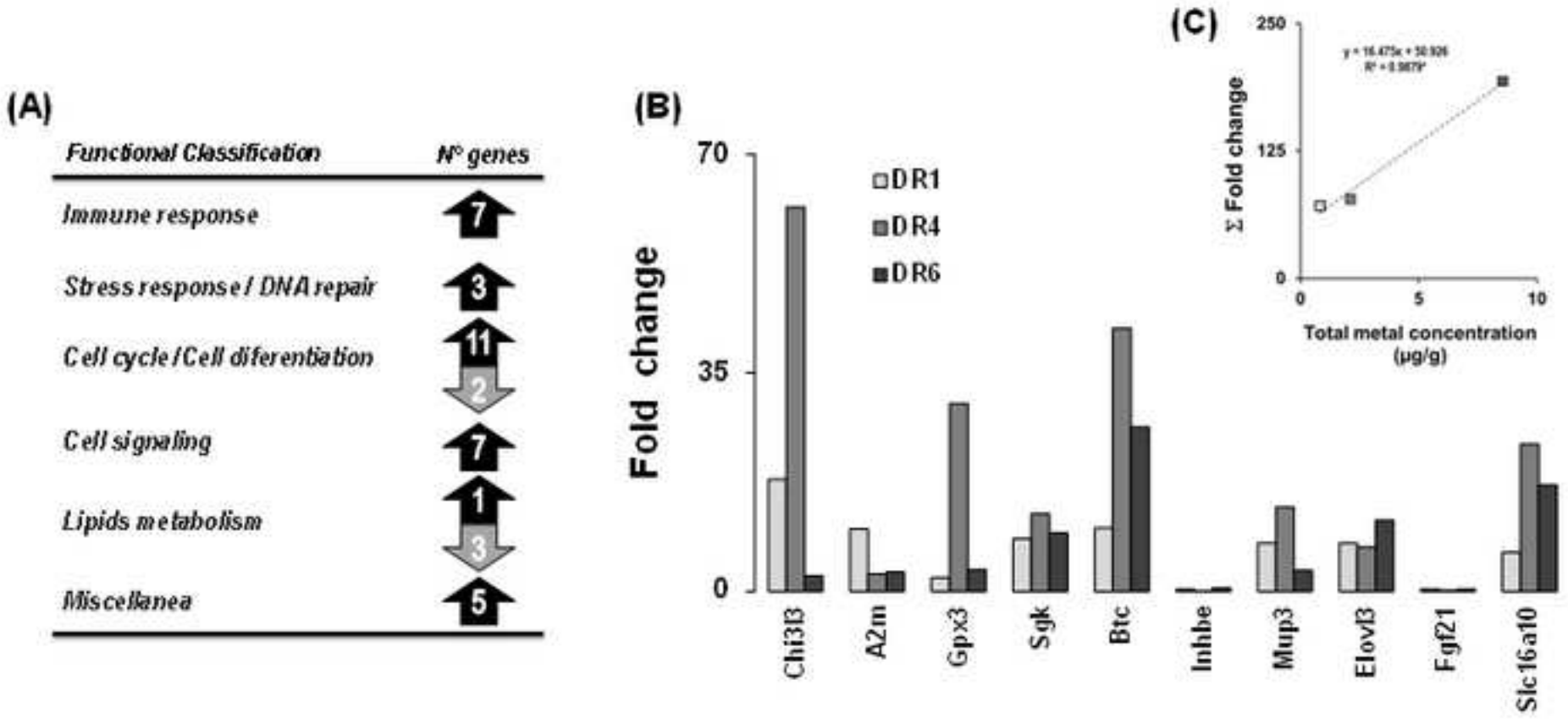

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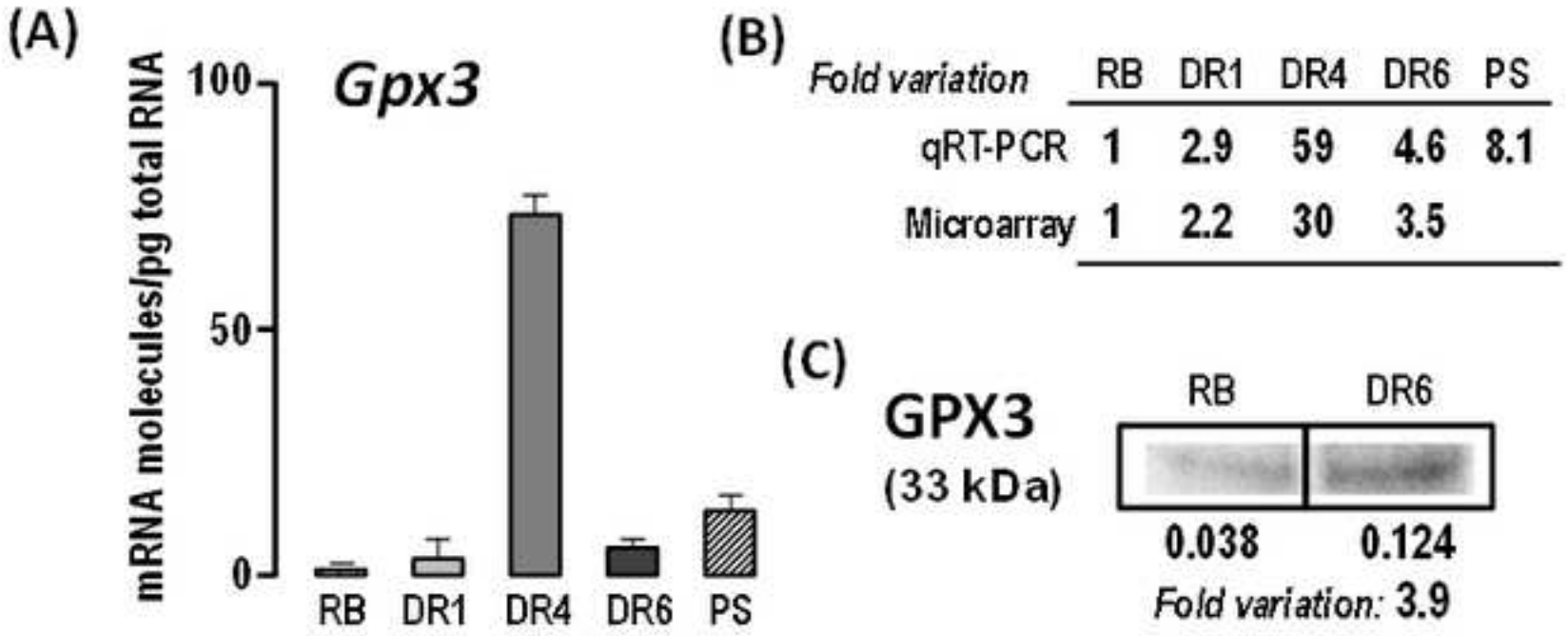

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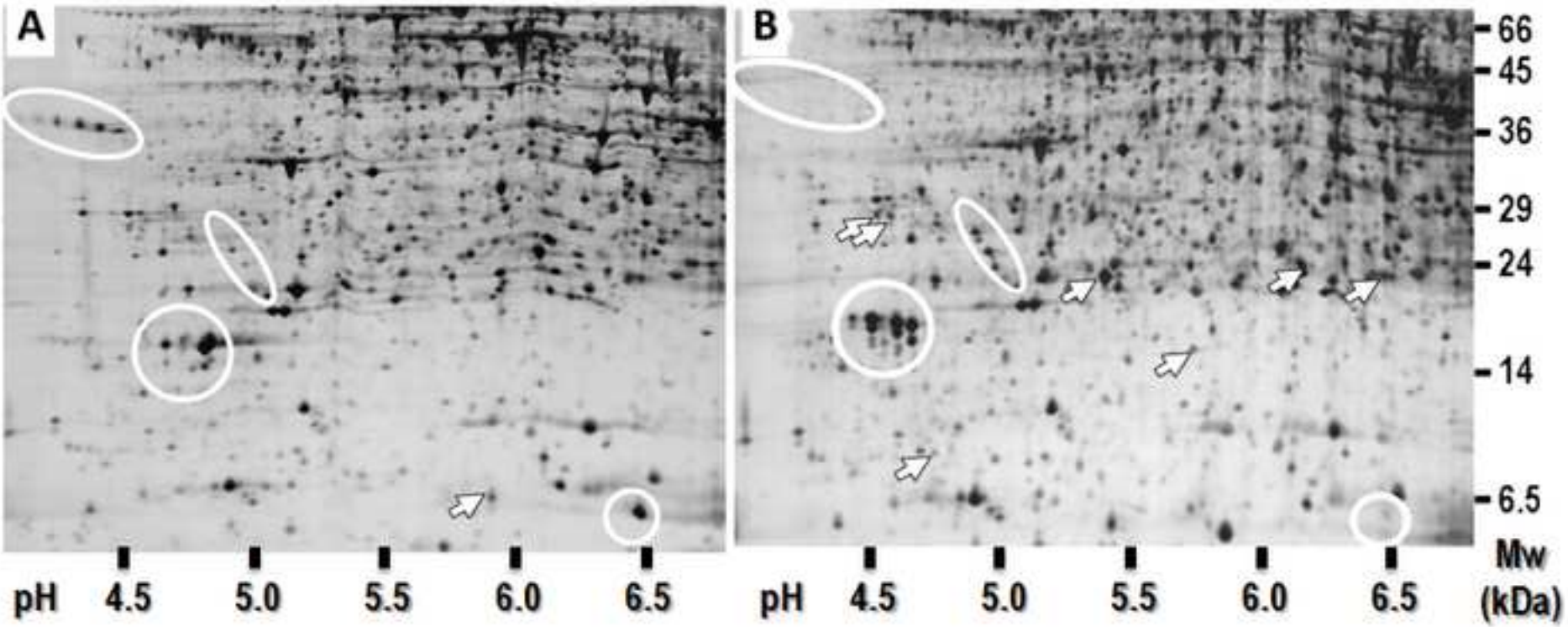

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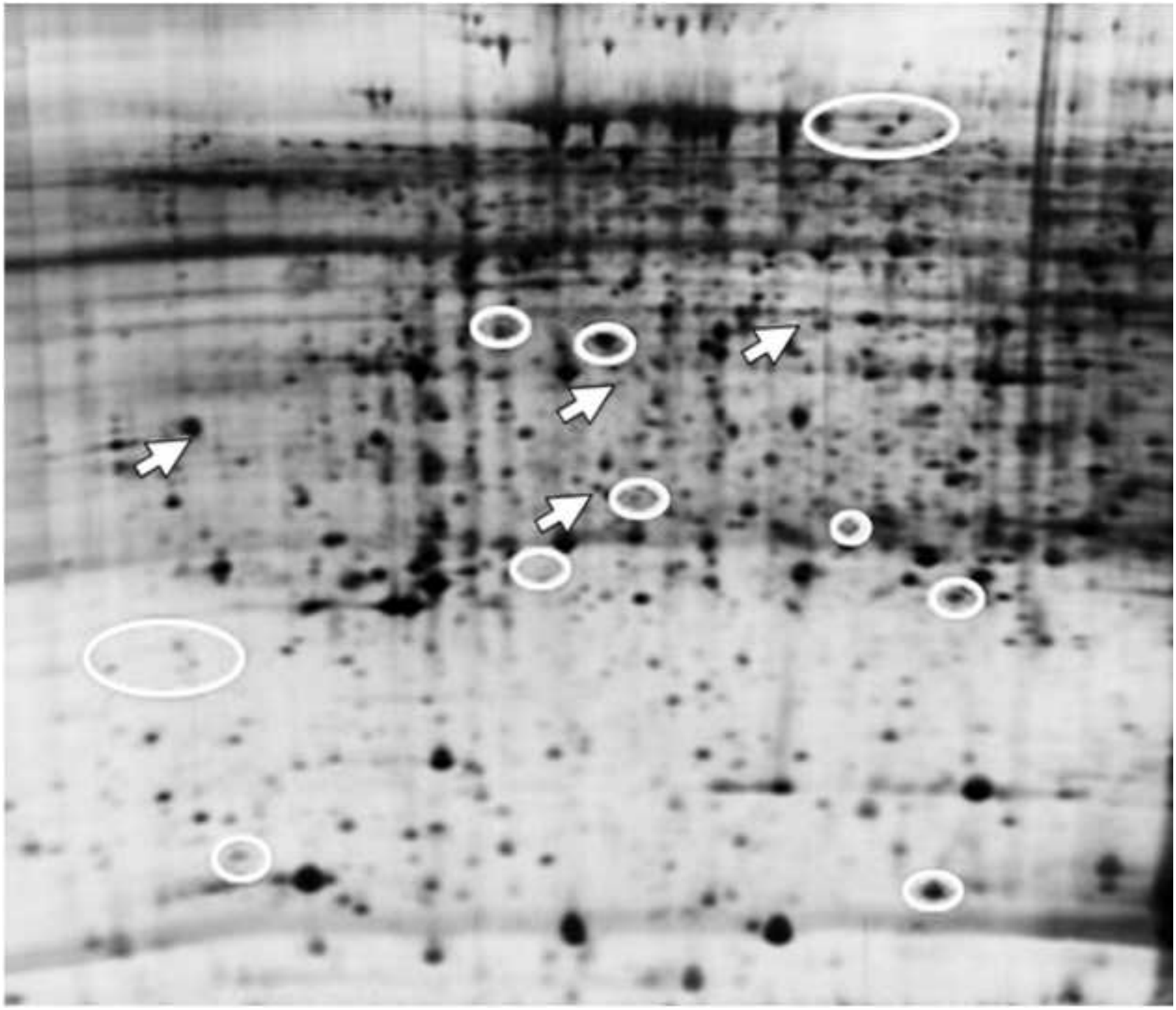

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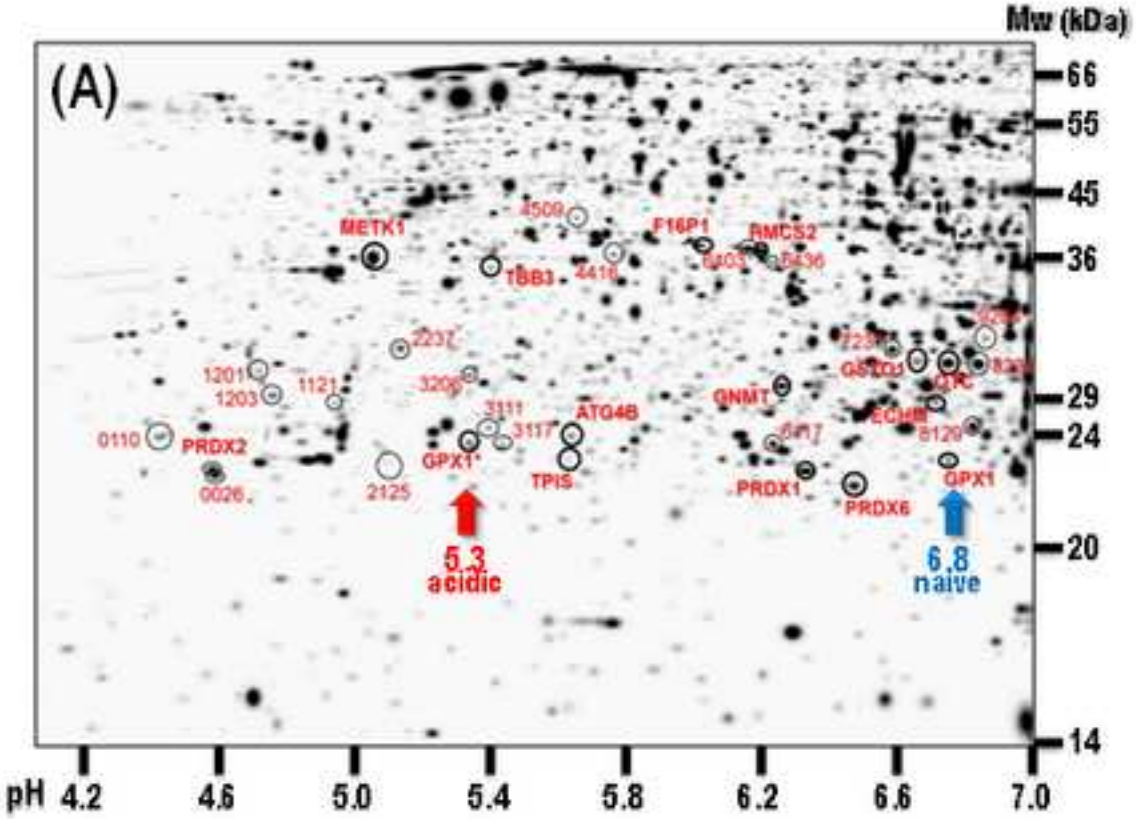

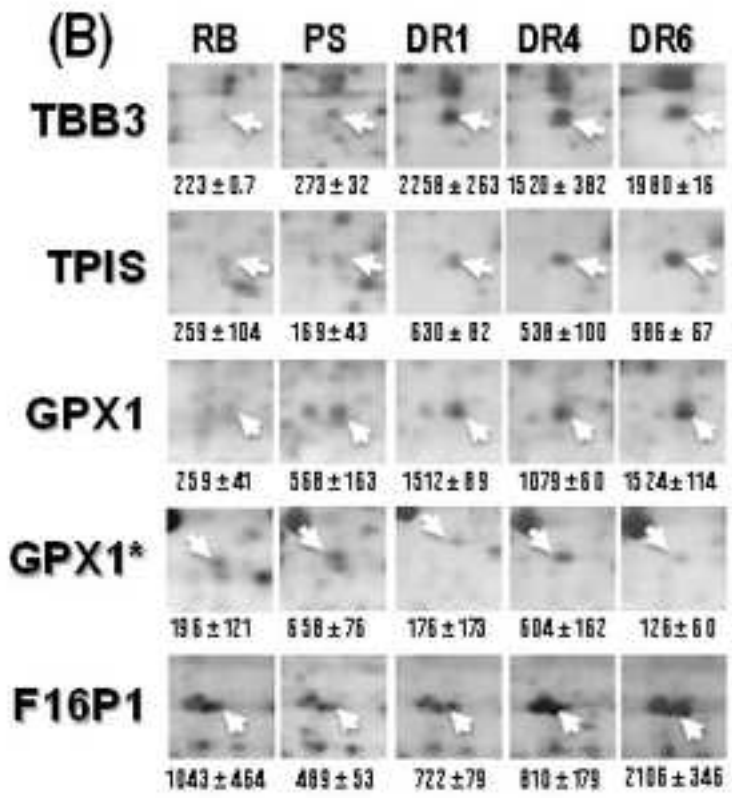

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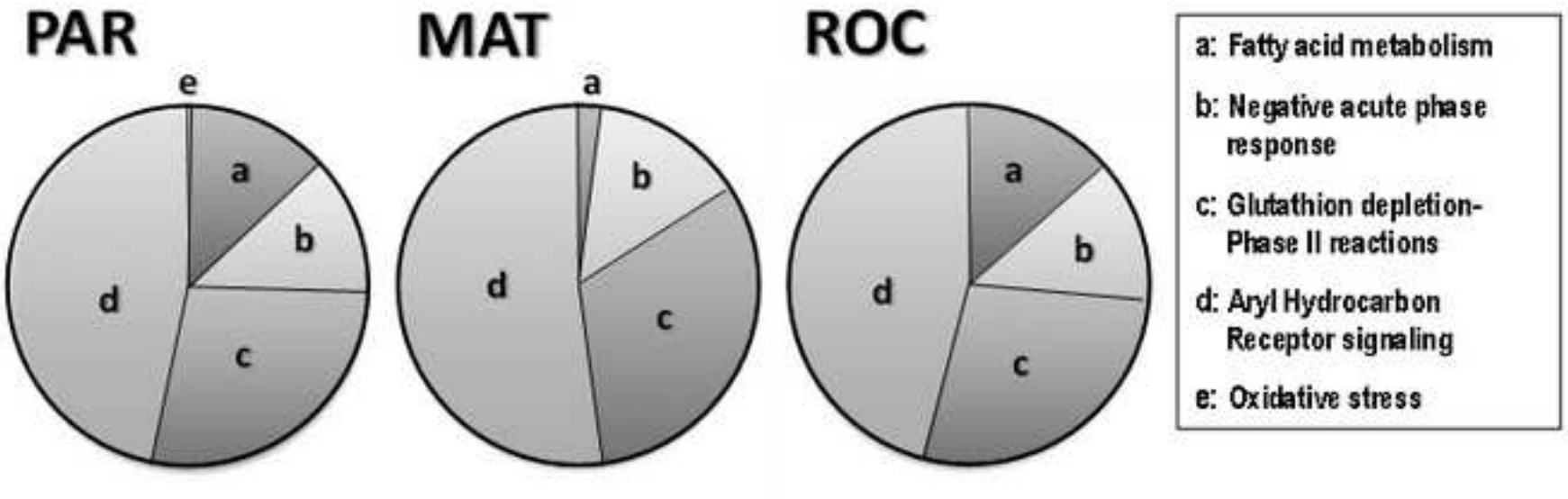

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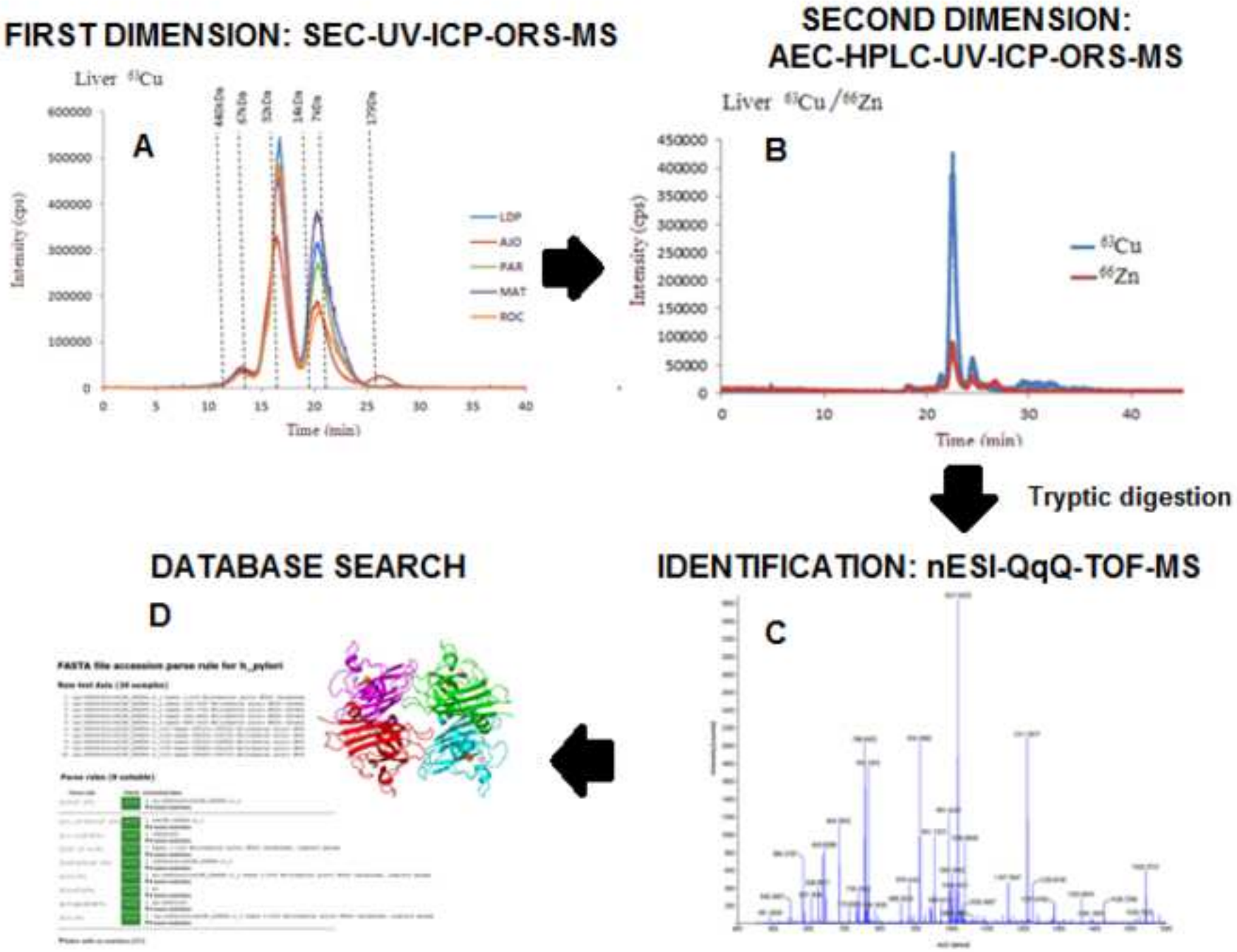

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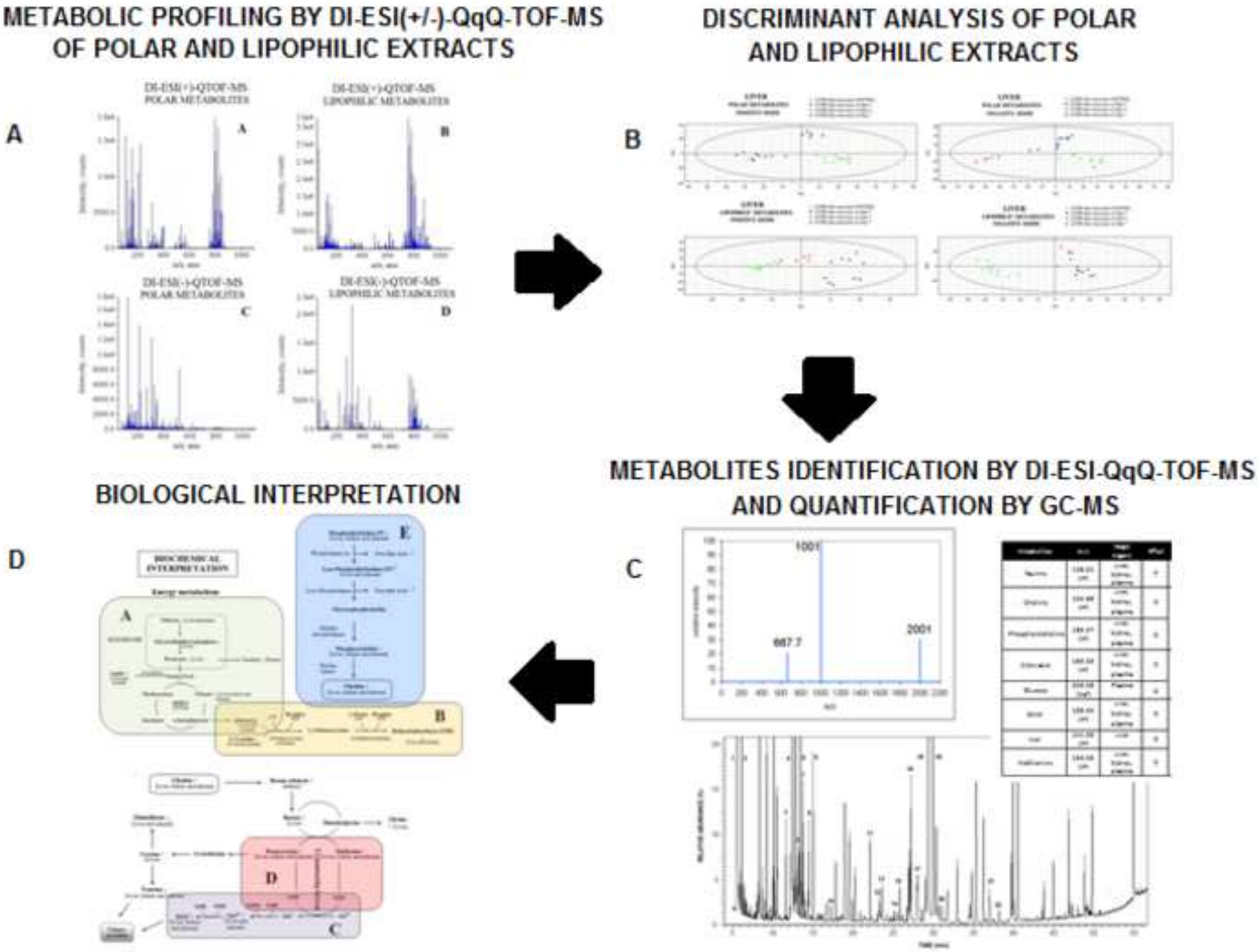

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