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A proteomic approach to identify metalloproteins and metal-binding proteins in liver from diabetic rats

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ABSTRACT

Proteins play crucial roles in biological systems, thus studies comparing the protein pattern present in a healthy sample with an affected sample have been widely used for disease biomarker discovery. Although proteins containing metal ions constitute only a small proportion of the proteome, they are essential in a multitude of structural and functional processes. The correct association between metal ions and proteins is essential because this binding can significantly interfere with normal protein function. Employment of a metalloproteomic study of liver samples from diabetic rats permitted determination of the differential abundance of copper-, selenium-, zinc- and magnesium-associated proteins between diabetic, diabetic treatment with insulin and non-diabetic rats. Proteins were detected by ESI–MS/MS. Seventy-five different proteins were found with alterations in the metal ions of interest. The most prominent pathways affected under the diabetic model included: amino-acid metabolism and its derivates, glycogen storage, metabolism of carbohydrates, redox systems and glucose metabolism. Overall, the current methods employed yielded a greater understanding of metal binding and how type 1 diabetes and insulin treatment can modify some metal bonds in proteins, and therefore affect their mechanism of action and function.

1. Introduction

Type 1 diabetes mellitus (DM1) is a severe metabolic disorder and a public health concern. Clinical complications of DM1 include damage and dysfunction of multiple organs with secondary complications, such as atherosclerosis, retinopathy and renal insufficiency [1].

Although metal ions comprise a small proportion of body tissues (4%), they are essential as structural components and in many life processes. The main roles of these metal ions can be described as structural and functional [2]. Metal ions bound to proteins and metalloproteins represent a large portion of the total protein. These ions are responsible for many metabolic processes, such as energy conversion in photosynthesis and respiration, gene regulation, substrate activation and other catalytic processes, transport and storage [3].

Copper (Cu) is an essential component of many metalloenzymes, including cytochrome oxidase, lysyl oxidase, superoxide dismutase, dopamine-ß-hydroxylase and tyrosinase [4]. Superoxide dismutase (SOD) is a metalloprotein that contains Cu and zinc (Zn) that catalyzes the decomposition of the superoxide anion. Therefore, it is a component of the cellular defense system protecting against oxidative damage. Cu is a vital component of electron transfer reactions of SOD, underlying its antioxidant function [5].

Experimental data shows that magnesium (Mg) is required as an important cofactor in many enzyme reactions. Importantly for DM1, Mg participates in phosphorylation reactions of glucose metabolism [6]. It is essential in almost all energy transduction systems in the glycolytic pathway and in oxidative energy metabolism, which is required for the synthesis and oxidation of fatty acids, protein synthesis, muscle contraction and ATPase activity [7]. Additionally, Mg participates in the intracellular signaling system, phosphorylation and dephosphorylation reactions that activate or inhibit a multitude of enzymes [8].

Selenium (Se) is a nonmetal, essential micronutrient for the synthesis of selenoproteins, which play an important role in the

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synthesis, metabolism and action of thyroid hormones. Furthermore, Se modifies the expression of selenoproteins, including the families of peroxidases, selenoenzymes, glutathione and thioredoxin [9]. Zn is a component of synthetases and transferases, including DNA and RNA, digestive enzymes, and associates with insulin. Zn also participates in metabolic pathways involving protein synthesis, carbohydrates, lipids and nucleic acids. In addition, zinc also plays a role in apoptotically driven programmed cell death [10].

Studies have reported alterations in metal ions by diabetes mellitus and suggested that the imbalance of specific elements may play an important role in normal glucose and insulin metabolism [11,12]. In the majority of studies, the focus is on analyzing concentrations and on the status of a single element or combinations of elements in blood, plasma or tissues; in our study, the focus is the on the integration of traditional analytical studies with inorganic and biochemical studies. For this study, we used a robust methodology to assist in understanding the variability of Cu, Mg, Se and Zn in diabetes and how treatment with insulin can alter this condition, providing valuable information about how Cu, Mg, Se and Zn are distributed with proteins, as well as the individual concentration in specific proteins, which helps to elucidate the physiological and functional aspects of biomolecules in the liver.

In this context, the present study involves the investigation Cu, Mg, Se and Zn found in liver samples from diabetic rats using flame atomic absorption spectrometry (FAAS) and graphite furnace atomic absorption spectrometry (GFAAS), by protein fractionation (two-dimensional gel electrophoresis, 2D-PAGE) and identification by electrospray ionization-tandem mass spectrometry (ESI–MS/MS).

2. Material and methods

2.1. Animals and experimental groups

A total of 24 Wistar male rats (*Rattus norvegicus*), 45 days old, were used in the experiment; these were kept in individual plastic cages with a controlled temperature and photoperiod, and they received water and a commercial diet (Purina®, Labina, Campinas-SP) *ad libitum* throughout the experimental period. The experimental design was approved by the Ethics Committee on the Use of Animals (CEUA) at the Institute of Biosciences/São Paulo State University (UNESP), Botucatu, Brazil (protocol: CEUA-436/2012). The animals were divided into three groups (n = 8): C (control group): normal rats, DM1: diabetic rats and DM1 + I: diabetic rats that received insulin. Diabetes mellitus was induced with the administration of streptozotocin (STZ; 60 mg/body weight, single dose, i.p.). Blood glucose was measured 48 h after the STZ administration, and animals with glycemic levels greater than 220 mg dL^{-1} were considered diabetic. The animals received insulin in the form of Humulin N100UI neutral protamine Hagedorn (NPH; Lilly®) with an initial dose of 3 U/animal; this dose was adjusted or maintained so that serum glucose levels were kept within the normal range. At the end of the 30 day experimental period, the animals were anesthetized (ketamine hydrochloride 10%, 0.1 mL/100 g body weight, i.p.) and sacrificed by decapitation, and the liver was collected.

2.2. Sample preparation

Approximately 1.00 g of pooled sample (liver) was weighed in triplicate and ground with 2 mL of ultrapure water, macerated, and the protein extracts were separated from the solid portion by centrifugation at 10,000g at 4 °C for 10 min in a refrigerated centrifuge. The protein extracts were used to quantify protein resuspension in a protein pellet with 0.50 mol L^{-1} NaOH. The total protein concentration of the liver samples was determined by the Biuret method using bovine serum albumin as the standard. Analytical calibration curves were constructed with concentrations of 10–100 g L^{-1} from a stock solution of albumin (100 g L^{-1}). The method used 50 mL of sample for the standard and 2.5 mL of Biuret reagent, which was mixed and placed in a water bath at 32 °C for 10 min. After 5 min at room temperature, absorbance readings were performed in a spectrophotometer at a wavelength of 545 nm.

2.3. Electrophoretic runs (2D-PAGE)

With the standardization of the gels, the electrophoretic runs were performed using liver samples for the different experimental groups. Six gels were made for each group (pooled). Aliquots of pooled liver were diluted in a urea solution containing 7 mol L^{-1}; 2 mol L^{-1} thiourea, 2% (w/v) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 0.5% (v/v) ampholytes at a pH ranging from 3 to 10, 0.002% (w/v) bromophenol blue and 2.8 mg of dithiothreitol (DTT) were added to this buffer, and the mixture was used in the electrophoretic separations.

A total of 3 μg μL^{-1} protein was added to 13 cm strips containing polyacrylamide gel with ampholytes immobilized at pH 3–10. These strips were placed onto a first dimension focusing for 12 h at room temperature to be rehydrated with the protein extract. After this period, the strips were placed into an Ettan IPGphor isoelectric focusing (IEF) unit (GE Healthcare) for the first-dimension separation, after the strips were reduced for 10 min with a solution containing 6 mol L^{-1} urea, 2% (w/v) SDS, 30% (v/v) glycerol, 50 mmol L^{-1} Tris-HCl, 0.002% (w/v) bromophenol blue and 2% (w/v) DTT and alkylated for 10 min with a similar solution, but with DTT replaced by 2.5% (w/v) iodoacetamide (IAA).

For the second dimension, the strips were placed onto a 10% polyacrylamide gel; a piece of filter paper with 10 μL of a molecular weight standard (12–225 kDa range) was placed close to the strip in the gel, and they were sealed with a hot solution of 0.5% (m/v) agarose. The second dimension of the electrophoretic run was divided into two stages: 7.5 mA/gel for 30 min and 20 mA/gel for 6 h 40 min.

After the first and second dimensions in the electrophoretic runs, the proteins in the gels were fixed in the gels using a staining solution containing 10% (v/v) acetic acid and 40% (v/v) ethanol for 1 h. In the sequence, the proteins were revealed with a colloidal Coomassie stain: 8% (m/v) ammonium sulfate, 1.6% (v/v) phosphoric acid, 0.08% (m/v) Coomassie blue G-250 and 25% (v/v) methanol for 72 h. Afterwards, the Coomassie stain was removed and the gels were washed with ultrapure water. The gels were scanned using an ImageScanner III (GE Healthcare), and the images were analyzed by ImageMaster 2D Platinum 7.0 (GeneBio, Geneva, Switzerland).

2.4. Copper, magnesium, selenium and zinc mapping by FAAS or GFAAS

The copper, selenium and zinc in the protein spots identified were determined by GFAAS, and magnesium was determined by FAAS after mineralizing the samples (spots and feed) as described by Moraes et al. [13]. The analyses used two different electrophoretic runs, and gels were obtained in duplicate for each run. The copper, magnesium, selenium and zinc determinations were performed with a Shimadzu AA-6800 atomic absorption spectrometer using wavelengths of 324.7, 285.2, 190.0 and 213.9 nm, respectively. Copper, selenium and zinc operated with a current of 400 mA and magnesium with a current of 10 mA. The analytical curves were prepared using Merck Titrisol standard solutions. The curves for copper and zinc were constructed in

concentration ranges of 5.00–20.00 μg L^{-1}; for magnesium, the range was 0.10–0.40 mg L^{-1} and for selenium the range was 10.00–60.00 μg L^{-1}. The region of the gel where no protein spots appeared was used for the analytical blank. The limits of quantification (LOQ) for copper, magnesium, selenium and zinc are 0.046, 0.94, 0.083 and 0.023 μg L^{-1}, respectively.

2.5. Characterization of protein spots by ESI–MS/MS

The protein spots were extracted from the gels using a scalpel, cut into segments of approximately 1 mm^3 and prepared for MS according to Shevchenko et al. [14] with some modification. The subsequent procedure with the segments was described by the Waters' Technical Bulletin, which can be summed up in four steps: a) dye removal (destained with 25 mM ammonium bicarbonate (Ambic)/acetonitrile (ACN) (50:50 v/v), and after destaining, the fragments were dehydrated with two ACN baths for 10 min and dried at room temperature); b) reduction and alkylation (rehydrated with 20 mM DTT in 50 mM Ambic for 40 min at 56 °C; after time, the excess reagent was removed and 55 mM IAA in 50 mM Ambic was added for 30 min at room temperature); c) tryptic digestion of proteins (incubated overnight at 37 °C with 10 ng μL^{-1} trypsin in 25 mM Ambic for 15 min; Trypsin Gold Mass Spectrometry, Promega, Madison, USA) and d) elution of peptides (extracted from gel by the addition of extraction buffer) A – 50% ACN with 1% formic acid – to each tube and incubated for 15 min at 40 °C under sonication; the supernatant was collected and transferred to a new tube, and this step was repeated with the extraction of buffer B – 60% methanol with 1% formic acid – and extraction buffer C – 100% ACN; the extracts were dried in a vacuum centrifuge and peptides were dissolved in 10 μL 3% ACN with 0.1% formic acid. Aliquots of solutions containing peptides were analyzed by obtaining the mass spectra using the nanoACQUITY UPLC-Xevo QT-MS (Waters, Manchester, UK) system. Data was acquired over 20 min, and the scan range was 50–2000 Da. ProteinLynx Global Server (PLGS) version 3.0 was used to process and search the continuum LC–MS^E data, setting carbamidomethylation of cysteines as the fixed modification and oxidation of methionines as the variable modification, allowing one missing cleavage and a maximal error tolerance of 10 ppm [15]. The identification of proteins was performed using the UniProt database (UniProtKB/Swiss-Prot – www.uniprot.org), and the search was conducted for the species *Rattus norvegicus*.

The UniProt protein IDs were converted to gene symbols in order to analyze them using Reactome Functional Interaction (FI) [16], a Cytoscape plugin [17]. Reactome uses a comparison with published knowledge about reactions, pathways and biological processes. Pathways with a false discovery rate (FDR) < 0.05 were considered to be significantly enriched.

3. Results and discussion

3.1. Protein separation by 2D-PAGE

The total protein concentrations in the pool of pellets in the C, DM1 and DM1 + I groups were 131.12, 92.86 and 105.79 g L^{-1} in the total extract and 26.09, 20.05 and 21.79 g L^{-1} precipitated with acetone, respectively. The pellets obtained with the acetone precipitation were used to calculate the volume of protein needed to obtain 3 μg μL^{-1} of protein to apply to the IEF strips. After standardization of the protocol to be followed, the gels obtained from different experimental groups were scanned and analyzed using ImageMaster 2D Platinum 7.0. The gels were compared in pairs because the imaging is a laborious process. After the identification of equivalent spots through the matching process, we obtained results of the correlation between the pairs of gels. The correlation between the gel replicates, encompassing the three electrophoretic runs (six gels from each group studied), were, on average in the C, DM1 and DM1 + I groups, 86%, 79% and 91%, respectively.

Correlation between the protein spots from gels was determined, considering the percentage normalized volume (%V) during the matching process. The results obtained were as follows: G1 and G2 (R > 0.75), G1 and G3 (R > 0.90) and G2 and G3 (R > 0.76). Through the correlation analysis, we can see that the proteomic profile of the C group was closer to the proteomic profile of the DM1 + I group than to the profile of the DM1 group. Most spots were distributed in the molecular weight (Mw) range of 31–76 kDa with isoelectric points (pI) ≅ 6; in general, the Mw and pI distributions were homogeneous among the different experimental groups.

3.2. Determination of Cu, Mg, Se and Zn in the spots

The concentration of Cu, Mg, Se and Zn does not provide a lot of information without characterizing the proteins in these protein spots. Thus, we converted the estimate of the protein mass in the protein spots and the Cu, Mg, Se and Zn [18,19]. Fig. 1*A* shows the protein spots containing Cu, Mg, Se and/or Zn; Fig. 1*B–D* shows the qualitative analyses that were used to identify the percentage of spots with Cu, Se, Mg and Se. In the 75 spots analyzed, the C group showed the highest percentage of Cu, the DM1 group showed the

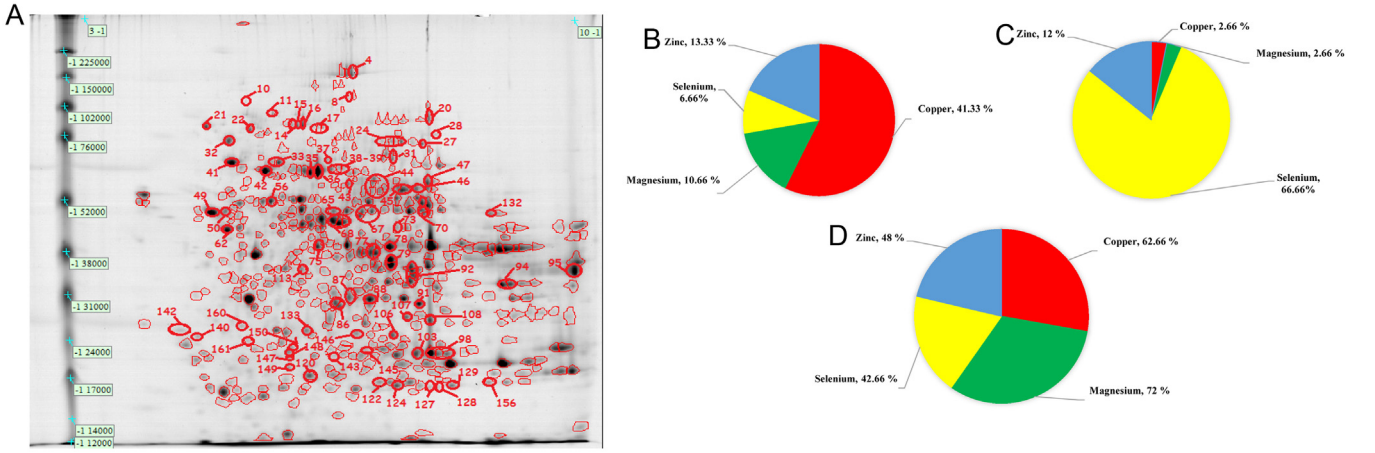

Fig. 1. *A.* Gel obtained by 2D-PAGE (pH 3–10) for liver tissue. The numbers indicate spots where Cu, Mg, Se and/or Zn as detected and the proteins characterized by ESI–MS/MS. *B.* Qualitative analysis of Cu, Mg, Se and Zn in the C group. *C.* Qualitative analysis of Cu, Mg, Se and Zn in the DM1 group. *D.* Qualitative analysis of Cu, Mg, Se and Zn in the DM1 + I group.

highest percentage of Se and the DM1 + I group showed the highest percentage of Mg.

Se exerts a beneficial influence on health, including the prevention of cancer and neurodegenerative diseases, actuating the immune system and mainly in providing antioxidant capacity through selenoproteins [20]. The literature reports that a number of metals (copper, zinc, vanadium and cadmium) have ions that are capable of eliciting insulin mimetic effects by activating the insulin signaling cascade [21]. Recent epidemiological and intervention studies related high Se levels to hyperglycemia and dyslipidemia; for example, a study conducted using the US National Health and Nutrition Examination Surveys associated a high serum selenium concentration with an increased prevalence of diabetes [22,23], and the French SUVIMAX trial population associated positive correlations between plasma Se and fasting plasma glucose [24]. The effect of Se in carbohydrate metabolism is controversial, and the adverse effect on the insulin-regulated metabolic pathway could be a fake redox paradox that facilitates insulin action by an insulin-stimulated reactive oxygen species (ROS) [25], which could explain why most protein spots had Se presence, because most Se available can bind with proteins and interfere in their function and consequently in their pathways.

The feed was analyzed for Cu, Mg, Se and Zn in order to exclude the role of feed in the results of this work. We found the following concentrations: Cu: 2.36 mg kg^{-1}, Mg: 28.90 mg kg^{-1}, Se: < LOQ and Zn: 51.40 mg kg^{-1}. Thus, the feed was observed to be a major source of Mg and Zn.

Table 1 shows the Cu, Mg, Se and Zn concentrations determined in each protein spot with their respective Mw and pI and the protein mass measured using ImageMaster 2D Platinum 7.0 software.

3.3. Protein spot analysis by ESI–MS/MS

This study analyzed 75 spots of protein and found 114 different proteins that were associated with the presence of Cu, Mg, Se and/or Zn in the C, DM1 and DM1 + I groups, shown in Table 2. Reactome

Table 1
Values for copper, selenium and zinc concentration, determination by GFAAS and magnesium by FAAS in the protein spots for liver in different experimental groups.

Control (C)								Type 1 diabetes (DM1)							Type 1 diabetes + Insulin (DM1+I)						
Spot ID	pI	Mw (Da)	Prot. mass (μg)	[Cu] (mg g^{-1})	[Mg] (mg g^{-1})	[Se] (mg g^{-1})	[Zn] (mg g^{-1})	pI	Mw (Da)	Prot. mass(μg)	[Cu] (mg g^{-1})	[Mg] (mg g^{-1})	[Se] (mg g^{-1})	[Zn] (mg g^{-1})	pI	Mw (Da)	Prot. mass (μg)	[Cu] (mg g^{-1})	[Mg] (mg g^{-1})	[Se] (mg g^{-1})	[Zn] (mg g^{-1})
4				<LOQ	<LOQ	<LOQ	<LOQ	6.86	138,865	2.62	<LOQ	<LOQ	0.08 ± 0.0024	<LOQ	6.74	158,761	2.46	0.07 ± 0.0021	0.29 ± 0.0087	<LOQ	<LOQ
8				<LOQ	<LOQ	<LOQ	<LOQ	6.74	104,125	0.12	<LOQ	<LOQ	1.54 ± 0.046	8.36 ± 0.2508	6.69	126,372	0.13	2.00 ± 0.0600	1.16 ± 0.0348	<LOQ	<LOQ
10				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	5.21	107,997	0.26	1.80 ± 0.0540	<LOQ	1.20 ± 0.036	<LOQ
11				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	5.21	107,997	0.26	1.80 ± 0.0540	<LOQ	1.20 ± 0.036	<LOQ
14				<LOQ	<LOQ	<LOQ	<LOQ	5.96	80,910	0.37	<LOQ	<LOQ	1.14 ± 0.034	16.68 ± 0.5004	5.84	95,728	0.21	4.75 ± 0.1425	11.41 ± 0.3423	<LOQ	<LOQ
15				<LOQ	<LOQ	<LOQ	<LOQ	6.05	79,903	0.61	<LOQ	<LOQ	3.60 ± 0.108	0.52 ± 0.0156	5.91	94,630	0.50	0.69 ± 0.0207	4.40 ± 0.1320	<LOQ	1.87 ± 0.0561
16				<LOQ	<LOQ	<LOQ	<LOQ	6.12	80,910	0.50	<LOQ	<LOQ	1.14 ± 0.0342	<LOQ	5.98	94,630	0.70	0.44 ± 0.0132	<LOQ	<LOQ	5.55 ± 0.1665
17				<LOQ	<LOQ	<LOQ	<LOQ	6.37	80,070	0.27	<LOQ	<LOQ	1.01 ± 0.0303	<LOQ	6.26	93,275	0.26	0.43 ± 0.1329	4.80 ± 0.1440	1.02 ± 0.0306	<LOQ
20				<LOQ	<LOQ	<LOQ	<LOQ	7.86	85,065	0.97	<LOQ	<LOQ	2.11 ± 0.0633	<LOQ	7.80	100,830	0.82	0.15 ± 0.0045	1.72 ± 0.0516	<LOQ	<LOQ
21	4.75	82,042	0.89	<LOQ	1.25 ± 0.0375	<LOQ	<LOQ	4.63	81,418	0.49	<LOQ	<LOQ	2.49 ±0.0747	<LOQ	4.64	92,471	0.62	0.20 ± 0.0060	<LOQ	1.86 ± 0.0558	<LOQ
22				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	5.22	80,016	0.33	0.53 ± 0.0159	3.42 ± 0.1026	<LOQ	3.14 ± 0.0942
24				<LOQ	<LOQ	<LOQ	<LOQ	7.34	68,267	0.29	<LOQ	<LOQ	0.74 ± 0.0222	<LOQ	7.29	77,327	0.34	0.53 ± 0.0159	<LOQ	<LOQ	<LOQ
27				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	7.70	75,747	0.21	0.17 ± 0.0051	0.15 ± 0.0045	<LOQ	<LOQ
28				<LOQ	<LOQ	<LOQ	<LOQ	7.97	73,142	0.25	<LOQ	<LOQ	4.46 ± 0.1338	7.82 ± 0.2346	7.92	81,919	0.29	0.07 ± 0.0002	<LOQ	<LOQ	<LOQ
31				<LOQ	<LOQ	<LOQ	<LOQ	7.34	68,267	0.29	<LOQ	<LOQ	0.74 ± 0.0222	<LOQ	7.31	70,164	0.38	<LOQ	1.15 ± 0.0345	0.81 ± 0.0243	<LOQ
32				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	4.98	80,514	1.11	0.32 ± 0.0096	0.18 ± 0.0054	<LOQ	<LOQ
33				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	5.62	70,282	0.56	0.82 ± 0.0246	3.12 ± 0.0936	0.30 ± 0.0090	8.34 ± 0.2502
35				<LOQ	<LOQ	<LOQ	<LOQ	6.22	59,239	1.24	<LOQ	<LOQ	1.39 ± 0.0417	4.66 ± 0.1398	6.09	66,084	1.28	0.51 ± 0.153	<LOQ	0.11 ± 0.0033	<LOQ
36				<LOQ	<LOQ	<LOQ	<LOQ	6.12	80,910	0.50	<LOQ	<LOQ	1.14±0.0342	<LOQ	6.19	66,084	4.49	0.09 ± 0.0027	0.60 ± 0.0180	<LOQ	0.11 ± 0.0033
37	7.65	48,290	1.41	3.38 ± 0.1014	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ
38-39				<LOQ	<LOQ	<LOQ	<LOQ	6.71	59,815	0.34	<LOQ	<LOQ	0.88 ± 0.0264	0.72 ± 0.0216	6.54	66,823	0.23	2.29 ± 0.0687	0.65 ± 0.0195	0.97 ± 0.0291	<LOQ
41				<LOQ	<LOQ	<LOQ	<LOQ	5.04	63,227	1.58	<LOQ	<LOQ	0.48±0.0144	<LOQ	5.03	69,006	1.97	0.36 ± 0.108	0.66 ± 0.0198	<LOQ	<LOQ
42				<LOQ	<LOQ	<LOQ	<LOQ	5.56	59,695	1.73	<LOQ	<LOQ	0.73 ± 0.0219	<LOQ	5.50	66,126	0.88	0.23 ± 0.0069	<LOQ	2.40 ± 0.0720	91.65 ± 2.7495
43				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	6.67	60,807	0.27	2.05 ± 0.0615	4.13 ± 0.1239	4.36 ± 0.1308	<LOQ
44	7.17	56,576	0.30	<LOQ	0.59 ± 0.0177	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ
45				<LOQ	<LOQ	<LOQ	<LOQ	7.44	53,314	0.85	<LOQ	<LOQ	1.65 ± 0.0495	<LOQ	7.40	58,921	0.72	0.85 ± 0.0255	5.66 ± 0.1698	<LOQ	11.76 ± 0.3528
46	7.75	55,426	0.81	<LOQ	<LOQ	0.10 ± 0.0030	<LOQ	7.68	49,569	0.46	<LOQ	<LOQ	0.84 ± 0.0252	3.82 ± 0.1146	7.57	59,115	0.35	1.63 ± 0.0489	3.30 ± 0.0990	1.89 ± 0.0567	
47				<LOQ	<LOQ	<LOQ	<LOQ	6.65	43,490	2.14	<LOQ	<LOQ	1.92 ± 0.0576	<LOQ	7.82	59,365	0.56	1.54 ± 0.0462	1.66 ± 0.0498	0.01 ± 0.0003	6.39 ± 0.1917
49				<LOQ	<LOQ	<LOQ	<LOQ	4.70	47,123	2.75	<LOQ	<LOQ	0.44 ± 0.0132	<LOQ	4.76	51,827	2.45	0.16 ± 0.0048	0.82 ± 0.0246	0.47 ± 0.0141	<LOQ
50				<LOQ	<LOQ	<LOQ	<LOQ	5.43	41,025	0.35	<LOQ	6.14 ± 0.1842	2.95± 0.0885	<LOQ	3.86	53,581	0.90	0.14 ± 0.0042	0.48 ± 0.0144	<LOQ	<LOQ
56				<LOQ	<LOQ	<LOQ	<LOQ	6.06	50,138	1.24	<LOQ	<LOQ	2.63 ± 0.0789	<LOQ	5.46	55,395	0.63	0.61 ± 0.0183	<LOQ	0.53 ± 0.0159	<LOQ
60	7.83	50,912	0.75	<LOQ	<LOQ	<LOQ	5.21 ± 0.1563	7.68	49,569	0.46	<LOQ	<LOQ	0.84 ± 0.0252	3.82 ± 0.1146	7.65	54,575	0.63	9.72 ± 0.2916	1.17 ± 0.0351	0.77 ± 0.0231	24.27 ± 0.7281
62	5.05	43,140	1.65	<LOQ	<LOQ	<LOQ	2.56 ± 0.0768				<LOQ	<LOQ	<LOQ	<LOQ	4.99	46,890	2.51	<LOQ	<LOQ	0.94 ± 0.0282	<LOQ
65	6.57	48,632	0.52	<LOQ	<LOQ	<LOQ	5.33 ± 0.1599	6.55	45,951	1.07	<LOQ	<LOQ	0.58 ± 0.0174	<LOQ	6.78	54,481	0.42	1.97 ± 0.0591	6.45 ± 0.1935	1.40 ± 0.0420	<LOQ
67				<LOQ	<LOQ	<LOQ	<LOQ	6.98	46,486	0.55	11.00 ± 0.33	<LOQ	1.84 ± 0.0552	<LOQ	6.95	50,547	0.77	1.20 ± 0.0360	2.72 ± 0.0816	0.63 ± 0.0189	2.15 ± 0.0645
68				<LOQ	<LOQ	<LOQ	<LOQ	6.60	44,720	1.6	<LOQ	<LOQ	1.25 ± 0.0375	<LOQ	6.50	48,730	1.91	0.25 ± 0.0075	0.14 ± 0.0042	0.66 ±0.0198	5.83 ± 0.1749
70				<LOQ	<LOQ	<LOQ	<LOQ	7.68	49,569	0.46	<LOQ	<LOQ	0.84 ± 0.0252	3.82 ± 0.1056	7.69	51,482	1.13	0.59 ± 0.0177	6.16 ± 0.1848	<LOQ	12.83 ± 0.3849
73				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	7.05	61,417	0.79	0.34 ± 0.0102	2.43 ± 0.0729	0.18 ± 0.0054	0.65 ± 0.0195
75				<LOQ	<LOQ	<LOQ	<LOQ	6.31	38,278	0.59	<LOQ	<LOQ	0.29 ± 0.0087	<LOQ	5.92	40,622	0.33	1.83 ± 0.0549	8.55 ± 0.2565	3.13 ± 0.0939	49.52 ± 1.4856
77	7.08	38,070	0.75	<LOQ	0.63 ± 0.0189	<LOQ	<LOQ	7.05	38,060	1.13	<LOQ	<LOQ	0.31 ± 0.0093	<LOQ	7.05	40,063	1.40	0.36 ± 0.0108	1.64 ± 0.0492	0.50 ± 0.0150	8.68 ± 0.2604

Table 1 (*Continued*)

78	7.36	38,812	2.32	<LOQ	0.20 ± 0.0060	<LOQ	2.17 ± 0.0651	7.27	37,890	1.66	<LOQ	0.15 ± 0.0045	0.93 ± 0.0279	<LOQ	7.26	41,861	1.77	0.15 ± 0.0045	1.77 ± 0.0531	<LOQ	<LOQ		
79	7.38	36,070	2.09	<LOQ	<LOQ	<LOQ	1.11 ± 0.0333	7.27	35,188	1.93	<LOQ	<LOQ	0.53 ± 0.0159	<LOQ	7.27	38,265	1.64	0.25 ± 0.0075	1.44 ± 0.0432	<LOQ	21.63 ± 0.6489		
86	6.59	29,608	0.84	0.05 ± 0.0015	<LOQ	<LOQ	<LOQ	6.54	29,852	0.61	<LOQ	<LOQ	0.30 ± 0.009	<LOQ	6.46	31,747	1.26	<LOQ	3.06 ± 0.0918	<LOQ	<LOQ		
87	6.79	30,496	0.95	0.05 ± 0.0015	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	6.67	33,737	1.03	<LOQ	3.52 ± 0.1056	0.76 ± 0.0228	4.00 ± 0.1200		
88	7.08	30,197	1.84	0.10 ± 0.0030	<LOQ	<LOQ	<LOQ	7.55	38,668	0.96	<LOQ	<LOQ	0.91 ± 0.0273	<LOQ	7.19	33,206	0.56	<LOQ	5.05 ± 0.1515	<LOQ	18.18 ± 0.5454		
91				<LOQ	<LOQ	<LOQ	<LOQ	7.65	27,962	2.32	<LOQ	<LOQ	0.57 ± 0.171	<LOQ	7.63	32,597	1.93	0.02 ± 0.0006	1.1 2± 0.0336	<LOQ	10.28 ± 0.3084		
92	6.62	29,320	0.79	0.53 ± 0.0159	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	7.66	35,393	1.02	0.23 ± 0.0069	3.19 ± 0.0957	0.82 ± 0.0246	18.18 ± 0.5454		
94	9.19	32,597	0.94	0.13 ± 0.0039	<LOQ	<LOQ	<LOQ	9.02	28,940	0.21	<LOQ	<LOQ	3.77 ± 0.1131	<LOQ	9.26	37,304	1.74	<LOQ	2.20 ± 0.0660	0.03 ± 0.0009	13.35 ± 0.4005		
95	10.04	35,405	2.99	0.03 ± 0009	<LOQ	<LOQ	<LOQ	9.96	36,471	1.59	<LOQ	<LOQ	6.26 ± 0.1878	<LOQ	9.96	38,000	1.15	<LOQ	4.05 ± 0.1215	1.22 ± 0.0366	5.04 ± 0.1512		
98	8.23	19,171	4.23	0.30 ± 0.0090	<LOQ	<LOQ	<LOQ	8.05	19,525	0.97	<LOQ	<LOQ	2.15 ± 0.0645	<LOQ	8.13	18,437	0.49	0.31 ± 0.0093	11.92 ± 0.3576	<LOQ	2.72 ± 0.0816		
103	7.74	21,061	1.26	0.52 ± 0.0156	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
106	7.41	24,638	0.76	0.73 ± 0.0219	0.62 ± 0.186	6.29 ± 0.1887	<LOQ	7.43	29,162	1.49	<LOQ	<LOQ	1.29 ± 0.0387	<LOQ	5.32	24,806	0.07	<LOQ	87.04 ± 2.6112	<LOQ	110.29 ± 3.3087		
107	7.60	27,366	0.50	21.03 ± 0.6309	<LOQ	12.48 ± 0.3744	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	7.47	30,618	0.59	0.83 ± 0.0249	5.31 ± 0.1593	<LOQ	31.27 ± 0.9381		
108	7.94	26,921	1.40	0.86 ± 0.258	0.07 ± 0.0021	3.05 ± 0.915	<LOQ	7.29	23,018	0.41	<LOQ	<LOQ	2.24 ± 0.0672	<LOQ	7.81	29,869	0.76	0.79 ± 0.0237	4.84 ± 0.1452	<LOQ	26.66 ± 0.7998		
113	6.11	34,878	0.32	7.57 ± 0.2271	<LOQ	<LOQ	<LOQ	6.02	37,126	0.38	<LOQ	<LOQ	6.14 ± 0.1842	<LOQ	5.99	37,205	0.46	4.54 ± 0.1362	5.24 ± 0.1572	<LOQ	32.89 ± 0.9867		
120	6.22	17,269	0.88	0.53± 0.0159	<LOQ	<LOQ	27.97 ± 0.8391	6.45	17,000	0.47	<LOQ	<LOQ	2.80 ± 0.0840	<LOQ	6.11	19,396	1.60	1.58 ± 0.0474	2.40 ± 0.0720	0.47 ± 0.0141	8.45 ± 0.2535		
122	5.88	24,964	0.53	7.76 ± 0.2328	<LOQ	<LOQ	<LOQ	7.23	16,514	0.28	12.23 ± 0.3669	<LOQ	7.52 ± 0.2256	<LOQ	7.15	20,613	0.86	<LOQ	4.71 ± 0.1413	<LOQ	0.49 ± 0.0147		
124	7.47	16,269	0.88	1.48 ± 0.0444	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
127	7.94	16,168	0.23	4.64 ± 0.1394	<LOQ	<LOQ	<LOQ	7.77	18,195	0.77	<LOQ	0.30 ± 0.0090	2.96 ± 0.0888	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
128	8.06	16,168	0.20	2.19±0. 0657	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
129	8.26	16,269	0.50	2.5 ± 0.0774	<LOQ	1.51 ± 0.0453	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
132	8.91	47,783	0.32	0.98 ± 0.0294	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	8.83	51,654	0.51	0.87 ± 0.0261	4.43 ± 0.1329	2.00 ± 0.0600	1.51 ± 0.0453		
133	6.18	25,294	0.54	0.72 ± 0.0216	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	5.99	37,205	0.46	4.54 ± 0.1362	5.24 ± 0.1572	<LOQ	32.89 ± 0.9867		
140	4.63	24,397	0.21	7.43 ± 0.2229	1.71 ± 0.0351	<LOQ	9.53 ± 0.2859	4.62	20,101	0.44	<LOQ	<LOQ	2.25 ± 0.0675	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
142	4.37	25,294	0.43	2.06 ± 0.318	<LOQ	<LOQ	0.81 ± 0.0243	4.34	22,075	0.57	<LOQ	<LOQ	2.48 ± 0.0744	<LOQ	5.32	24,806	0.07	<LOQ	87.04 ± 2.6112	<LOQ	110.29 ± 3.3087		
143	6.71	21,061	0.21	4.20 ± 0.126	<LOQ	<LOQ	6.04 ± 0.1812	6.19	23,627	0.24	<LOQ	1.26 ± 0.0378	11.58 ± 0.3474	<LOQ	6.60	24,501	0.19	<LOQ	19.01 ± 0.5703	<LOQ	34.47 ± 1.0341		
145	7.12	23,018	0.23	4.13 ± 0.1239	<LOQ	<LOQ	<LOQ	6.86	25,761	0.29	<LOQ	<LOQ	3.83 ± 0.1149	<LOQ	7.01	24,501	0.18	<LOQ	7.53 ± 0.2259	2.16 ± 0.0648	<LOQ		
146	6.89	24,882	0.44	0.86 ± 0.0258	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
147	5.93	20,411	0.20	4.65 ± 0.1395	<LOQ	<LOQ	<LOQ	6.15	16,836	0.86	<LOQ	<LOQ	0.95 ± 0.0285	<LOQ	6.37	30,242	0.70	<LOQ	2.45 ± 0.0735	1.11 ± 0.0333	6.11 ± 0.1833		
148	5.92	21,506	0.39	2.56 ± 0.0768	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	6.46	31,747	1.26	<LOQ	3.06 ± 0.0918	<LOQ	<LOQ		
149	5.93	18,579	0.25	8.47 ± 0.2541	6.09 ± 0.1827	<LOQ	1.23 ± 0.0369				<LOQ	<LOQ	<LOQ	<LOQ	6.33	28,543	0.30	<LOQ	9.84 ± 0.2952	<LOQ	<LOQ		
150	5.96	22,307	0.23	1.68 ± 0.0504	<LOQ	<LOQ	<LOQ	6.20	19,781	0.20	<LOQ	<LOQ	15.02 ± 0.4506	<LOQ	6.67	33,737	1.03	<LOQ	3.52 ± 0.1056	0.76 ± 0.0228	4.00 ± 0.1200		
156				<LOQ	<LOQ	<LOQ	<LOQ	9.33	17,269	0.37	<LOQ	<LOQ	10.50 ± 0.315	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
160				<LOQ	<LOQ	<LOQ	<LOQ	5.25	25.989	0.79	<LOQ	1.11 ± 0.0333	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ		
161	5.34	23,751	0.16	1.09 ± 0.0327	<LOQ	<LOQ	<LOQ				<LOQ	<LOQ	<LOQ	<LOQ	5.79	18,625	0.81	<LOQ	5.08 ± 0.1524	<LOQ	21.08 ± 0.6324		

FI was used to find the pathways of these 114 proteins that were considered significantly enriched (FDR < 0.05); the UniProt protein IDs were converted to gene symbols. Considering FDR < 0.05, 35 pathways were considered to be significantly enriched (Fig. 2), and the majority were related to the metabolism of amino acids and derivates (19 genes), glycogen storage diseases (13 genes), metabolism of carbohydrates (13 genes), biological oxidations (12 genes) and glucose metabolism (12 genes).

3.3.1. Amino acid metabolism

Carbamoyl phosphate synthase [ammonia] (CPS1) is ATP- and nucleotide-binding, and it is involved in the urea cycle (controls the entry of ammonia into the urea cycle). In the literature, there are no reports that this enzyme has a specific metal-binding property, but there is a report that it can be inactivated by a mixed-function oxidation binding a divalent metal and generally requiring a nucleotide [26]. However, in this work, the most important find was in the DM1 + I group, Cu and Zn were found; as these elements are divalent, it may be inferred that binding with Cu and Zn can cause the inactivation of this enzyme in the DM1 + I group associated with the control of hyperglycemia by insulin and a decrease in the production of ammonia. Ornithine carbamoyltransferase (OTC) is another enzyme involved in the urea cycle (it catalyzes the formation of L-citrulline from carbamoyl phosphate and L-ornithine). A study reported that this enzyme is regulated by Zn in two different ways: as an allosteric cofactor of the substrate-bound enzyme (site–site interactions) and by inducing inactivation by a slow, tight-binding inhibitor of the free enzyme [27]. The cysteinyl residue at position 273 of the enzyme has been identified as a metal ligand [28]. In this study, the presence of Cu was found in the C group and Cu, Mg, Se and Zn were found in the DM1 + I group. Cu, Mg and Se have not yet been reported as binding with OTC; it can be suggested that in DM1 + I inactivation of this enzyme by hyperglycemia control is induced by insulin. Arginase-1 (AGR1) was found in spot ID 79, and sorbitol dehydrogenase (SORD) was also found in this spot. AGR1 is a binuclear manganese (Mn) metalloenzyme that catalyzes the hydrolysis of arginine to ornithine and urea [29], and SORD is a Zn metalloenzyme (a tetramer containing one Zn atom/subunit) involved in the catalysis of sorbitol/fructose interconversion [30]. Some alterations in this conversion are associated in diabetes with cataract formation, neuropathy, retinopathy and nephropathy [30]. The results showed in spot 79, the presence of Zn in C, Se in DM1 and Cu, Mg and Zn in DM1 + I. No other previous reports have shown that ARG protein binds with Cu, Mg and Zn, and SORD with Cu and Mg. Beta-ureidopropionase (UPB1), 4-hydroxyphenylpyruvate dioxygenase (HPD) and fumarylacetoacetase (FAH) were identified in spot 77, where the presence of Mg was found in C, Se in DM1 and Cu, Se, Mg and Zn in DM1 + I. UPB1 is a metalloenzyme that binds two Zn^{2+} ions per subunit [31], HPD is a metalloenzyme that binds one Fe cation per subunit [32] and FAH binds with Ca^{2+} and Mg^{2+} [33]. Other proteins associated with this metabolism such as S-adenosylmethionine synthase

Table 2
Proteins identified by ESI–MS/MS in liver with the presence of copper, magnesium, selenium and/or zinc.

Spot ID	Protein entry	Protein	Score	pI (theoretical)	Mw (Da, theoretical)	Peptides	Coverage (%)	Biological process	Metabolism associated	Cu, Mg, Se and Zn presence
4	P07756	Carbamoyl-phosphate synthase [ammonia]_ mitochondrial OS = Rattus norvegicus GN=Cps1 PE = 1 SV = 1	653	6.33	164,580	13	10.95	Urea cycle	Amino-acid metabolism	DM1 = Se; DM1 + I = Cu and Mg
8	P70498	Phospholipase D2 (GN = Pld2)	131	7.44	106,037	84	1.82	Response to hydrogen peroxide, hypoxia, organic cyclic compounds and peptide hormone	Lipid metabolism	DM1 = Se and Zn; DM1 + I = Cu and Mg
10	O88600	Heat shock 70 kDa protein 4 OS = Rattus norvegicus GN=Hspa4 PE = 1 SV = 1	94	5.12	94,057	74	7.14	Stress response	Chaperone	DM1 + I = Cu and Se
11	P50475	Alanine–tRNA ligase_ cytoplasmic OS = Rattus norvegicus GN = Aars PE = 1 SV = 3	177	5.41	106,790	82	10.74	Protein biosynthesis	Nucleotide	DM1 + I = Cu and Se
14	Q63060	Glycerol kinase OS = Rattus norvegicus GN = Gk PE = 2 SV = 1	717	5.49	57,477	44	12.21	glycerol degradation via glycerol kinase pathway	Glycerol metabolism	DM1 = Se and Zn; DM1 + I = Cu and Mg
15	P28037	Cytosolic 10-formyltetrahydrofolate dehydrogenase OS = Rattus norvegicus GN = Aldh1l1 PE = 1 SV = 3	1633	6.15	101,440	79	21.51	Oxireductase	Choline and glycine metabolism	DM1 = Se and Zn; DM1 + I = Cu, Mg and Zn
16	P28037	Cytosolic 10-formyltetrahydrofolate dehydrogenase OS = Rattus norvegicus GN = Aldh1l1 PE = 1 SV = 3	1633	6.15	101,440	79	24.72	Oxireductase	Choline and glycine metabolism	DM1 = Se and Zn; DM1 + I = Cu and Zn
17	Q64380	Sarcosine dehydrogenase_ mitochondrial OS = Rattus norvegicus GN = Sardh PE = 1 SV = 2	1514	6.58	43,045	31	5.51	Kinase, transferase	Creatine metabolism	DM1 = Se; DM1 + I = Cu, Mg and Zn
20	P09034	Argininosuccinate synthase OS = Rattus norvegicus GN = Ass1 PE = 2 SV = 1	2849	7.63	46,496	33	36.65	Urea cycle	Amino-acid metabolism	DM1 = Se; DM1 + I = Cu and Mg
21	Q02253	Methylmalonate-semialdehyde dehydrogenase [acylating]_ mitochondrial OS = Rattus norvegicus GN = Aldh6a1 PE = 1 SV = 1	7429	8.47	57,808	44	35.14	Oxidoreductase	Valine and pyrimidine metabolism	C = Mg; DM1 = Se; DM1 + I = Cu and Se
22	O09171	Betaine–homocysteine S-methyltransferase 1 OS = Rattus norvegicus GN = Bhmt PE = 1 SV = 1	2238	8	39,929	29	6.06	Betaine-homocysteine S-methyltransferase activity, S-adenosylmethionine-homocysteine S-methyltransferase activity and S-methylmethionine-homocysteine S-methyltransferase activity	Mehionine metabolism	DM1 + I = Cu, Mg and Zn
24	P12346	Serotransferrin OS = Rattus norvegicus GN = Tf PE = 1 SV = 3	1104	7.14	76,395	65	14.76	ferric iron transmembrane transporter activit	Transport	DM1 = Se; DM1 + I = Cu
	Q5XHY5	Threonine–tRNA ligase_ cytoplasmic OS = Rattus norvegicus GN = Tars PE = 2 SV = 1	935	6.5	80,576	53	15.11	Protein biosynthesis	Amino-acid metabolism	
27	O35244	Peroxiredoxin-6 OS = Rattus norvegicus GN = Prdx6 PE = 1 SV = 3	6304	7.14	76,395	65	16.76	redox regulation	Oxidative stress	DM1 + I = Cu and Mg

	P12346	Serotransferrin OS = Rattus norvegicus GN = Tf PE = 1 SV = 3	709	7.14	76,395	55	14.49	ferric iron transmembrane transporter activit	Transport	
28	Q9ER34	Aconitate hydratase_ mitochondrial OS = Rattus norvegicus GN = Aco2 PE = 1 SV = 2	245	5.97	73,858	69	19	Response to toxic substance	Chaperone	DM1 = Se and Zn; DM1 + I = Cu
31	P34058	Heat shock protein HSP 90-beta OS = Rattus norvegicus GN=Hsp90ab1 PE = 1 SV = 4	3813	4.96	83,281	67	30.66	chaperone, cellular response to interleukin-4, negative regulation of neuron apoptotic process	Chaperone	DM1 = Se; DM1 + I = Mg and Se
	P82995	Heat shock protein HSP 90-alpha OS = Rattus norvegicus GN=Hsp90aa1 PE = 1 SV = 3	2081	4.93	84,815	91	18.14	Stress response	Chaperone	
32	P20059	Hemopexin OS = Rattus norvegicus GN=Hpx PE = 1 SV = 3	182	7.58	51,351	36	15.65	cellular iron ion homeostasis	Transport	DM1 + I = Cu and Mg
33	P48721	Stress-70 protein_ mitochondrial OS = Rattus norvegicus GN=Hspa9 PE = 1 SV = 3	3792	5.37	70,871	50	17.03	mRNA processing, mRNA splicing, stress response, transcription, transcription regulation	Chaperone	DM1 + I = Cu, Mg, Se and Zn
	P63018	Heat shock cognate 71 kDa protein OS = Rattus norvegicus GN=Hspa8 PE = 1 SV = 1	93	5.50	69,642	57	17.68	Stress response	Chaperone	
35	P02793	Ferritin light chain 1 OS = Rattus norvegicus GN = Ftl1 PE = 1 SV = 3	220	5.98	20,749	16	27.87	Iron storage	Other	DM1 = Se and Zn; DM1 + I = Cu and Se
36	P02770	Serum albumin OS = Rattus norvegicus GN = Alb PE = 1 SV = 2	3023	6.09	68,731	50	20.07	Transport	Other	DM1 = Se; DM1 + I = Cu, Mg and Zn
37	P14882	Propionyl-CoA carboxylase alpha chain_ mitochondrial OS = Rattus norvegicus GN = *Pcca* PE = 1 SV = 3	531	7.6	81,623	60	16.01	cellular amino acid and fatty acid catabolic process	Amino-acid metabolism	C = Cu
38–39	P07379	Phosphoenolpyruvate carboxykinase_ cytosolic [GTP] OS = Rattus norvegicus GN = Pck1 PE = 1 SV = 1	2569	6.75	71,615	47	10.52	Tricarboxylic acid cycle	Carbohydrate metabolism	DM1 = Se and Zn; DM1 + I = Cu, Mg and Se
	Q920L2	Succinate dehydrogenase [ubiquinone] flavoprotein subunit_ mitochondrial OS = Rattus norvegicus GN = Sdha PE = 1 SV = 1	633	6.45	62,200	46	17.94	Glycolysis	Carbohydrate metabolism	
	P12928	Pyruvate kinase PKLR OS = Rattus norvegicus GN = Pklr PE = 2 SV = 2	107	6.23	60,647	49	9.54	Toxin transport	Chaperone	
	Q6P502	T-complex protein 1 subunit gamma OS = Rattus norvegicus GN=Cct3 PE = 1 SV = 1	98	5.07	72,347	52	26.91	Stress response	Chaperone	
41	P06761	78 kDa glucose-regulated protein OS = Rattus norvegicus GN=Hspa5 PE = 1 SV = 1	8726	5.91	70,549	51	16.54	Stress response	Chaperone	DM1 = Se; DM1 + I = Cu and Mg
	P55063	Heat shock 70 kDa protein 1-like OS = Rattus norvegicus GN=Hspa1 L PE = 2 SV = 2	932	5.91	70,550	48	9.83	Stress response	Chaperone	
	Q07439	Heat shock 70 kDa protein 1A/1 B OS = Rattus norvegicus GN=Hspa1a PE = 2 SV = 2	897	5.37	70,871	50	6.19	Stress response	Chaperone	
	P63018	Heat shock cognate 71 kDa protein OS = Rattus norvegicus GN=Hspa8 PE = 1 SV = 1	894	5.50	69,642	50	6.32	Stress response	Chaperone	
	P14659	Heat shock-related 70 kDa protein 2 OS = Rattus norvegicus GN=Hspa2 PE = 1 SV = 2	894	5.37	70,871	50	40.09	Stress response	Chaperone	

Table 2 (*Continued*)

Spot ID	Protein entry	Protein	Score	pI (theoretical)	Mw (Da, theoretical)	Peptides	Coverage (%)	Biological process	Metabolism associated	Cu, Mg, Se and Zn presence
42	P63018	Heat shock cognate 71 kDa protein OS = Rattus norvegicus GN=Hspa8 PE = 1 SV = 1	7256	5.50	69,642	50	12.64	Stress response	Chaperone	DM1 = Se; DM1 + I = Cu, Se and Zn
	Q07439	Heat shock 70 kDa protein 1A/1 B OS = Rattus norvegicus GN=Hspa1a PE = 2 SV = 2	3042	5.91	70,550	49	19.66	Stress response	Chaperone	
	P14659	Heat shock-related 70 kDa protein 2 OS = Rattus norvegicus GN=Hspa2 PE = 1 SV = 2	2938	5.91	70,549	51	12.64	Stress response	Chaperone	
	P55063	Heat shock 70 kDa protein 1-like OS = Rattus norvegicus GN=Hspa1 L PE = 2 SV = 2	2128	5.07	72,347	52	5.5	Stress response	Chaperone	
43	P38652	Phosphoglucomutase-1 OS = Rattus norvegicus GN = Pgm1 PE = 1 SV = 2	4463	6.3	61,403	44	37.01	Glycolysis/gluconeogenesis	Carbohydrate metabolism	DM1 + I = Cu, Mg and Se
44	P04762	Catalase OS = Rattus norvegicus GN=Cat PE = 1 SV = 3	657	7.07	59,757	42	21.14	Response to reactive oxygen species	Oxidative stress	C = Mg
	P0C2 × 9	Delta-1-pyrroline-5-carboxylate dehydrogenase_ mitochondrial OS = Rattus norvegicus GN = Aldh4a1 PE = 1 SV = 1	627	7.13	61,869	33	30.10	Oxidoreductase	Proline metabolism	
	P38652	Phosphoglucomutase-1 OS = Rattus norvegicus GN = Pgm1 PE = 1 SV = 2	590	6.3	61,403	44	17.79	glycolysis/gluconeogenesis pathway	Carbohydrate metabolism	
	P32232	Cystathionine beta-synthase OS = Rattus norvegicus GN=Cbs PE = 1 SV = 3	453	6.29	62,308	54	19.07	Carboxylic ester hydrolase activity	Lipid metabolism	
	Q64573	Liver carboxylesterase 4 OS = Rattus norvegicus PE = 2 SV = 2	426	6.25	62,495	57	11.23	Carboxylic ester hydrolase activity	Lipid metabolism	
	Q63010	Liver carboxylesterase B-1 OS = Rattus norvegicus PE = 1 SV = 1	372	6.10	62,147	36	11.68	Carboxylic ester hydrolase activity	Lipid metabolism	
	P16303	Carboxylesterase 1D OS = Rattus norvegicus GN=Ces1d PE = 1 SV = 2	345	5.64	61,715	39	5.35	Carboxylic ester hydrolase activity	Lipid metabolism	
	Q63108	Carboxylesterase 1E OS = Rattus norvegicus GN=Ces1e PE = 2 SV = 1	305	6.23	60,647	49	11.01	Toxin transport	Chaperone	
	Q6P502	T-complex protein 1 subunit gamma OS = Rattus norvegicus GN=Cct3 PE = 1 SV = 1	199	6.45	62,200	46	14.98	Glycolysis	Carbohydrate metabolism	
45	P04762	Catalase OS = Rattus norvegicus GN=Cat PE = 1 SV = 3	8595	7.07	59.757	42	21.49	Response to reactive oxygen species	Oxidative stress	DM1 = Se; DM1 + I = Cu, Mg and Zn
	P0C2 × 9	Delta-1-pyrroline-5-carboxylate dehydrogenase_ mitochondrial OS = Rattus norvegicus GN = Aldh4a1 PE = 1 SV = 1	2270	7.13	61.849	46	33.78	Oxidoreductase	Proline metabolism	
46	P04762	Catalase OS = Rattus norvegicus GN=Cat PE = 1 SV = 3	4785	7	66,093	42	20.43	Glutamate and proline biosynthetic process	Proline metabolism	C = Se, DM1 = Se and Zn; DM1 + I = Cu, Mg and Se
	P0C2 × 9	Delta-1-pyrroline-5-carboxylate dehydrogenase_ mitochondrial OS = Rattus norvegicus GN = Aldh4a1 PE = 1 SV = 1	1043	7.13	61.849	20	24.57	Oxidoreductase	Proline metabolism	
47	P13255	Glycine N-methyltransferase OS = Rattus norvegicus GN = Gnmt PE = 1 SV = 2	12867	5.79	58,914	46	36.97	Lyase, Transferase	Histidine metabolism	DM1 = Se; DM1 + I = Cu, Mg, Se and Zn
49	P00564	Creatine kinase M-type OS = Rattus norvegicus GN=Ckm PE = 1 SV = 2	63	6.58	43,045	31	5.51	phosphocreatine biosynthetic process, response to heat	Other	DM1 = Se; DM1 + I = Cu, Mg and Se

50	Q6P9T8	Tubulin beta–4 B chain OS = Rattus norvegicus GN = Tubb4b PE = 1 SV = 1	5873	4.79	49,801	30	43.37	microtubule-based process	Other	DM1 = Mg and Se; DM1 + I = Cu and Mg
	P69897	Tubulin beta-5 chain OS = Rattus norvegicus GN = Tubb5 PE = 1 SV = 1	4701	4.78	49,671	30	24.32	microtubule-based process	Other	
56	O88618	Formimidoyltransferase-cyclodeaminase OS = Rattus norvegicus GN = Ftcd PE = 1 SV = 4	5832	5.88	56,623	53	26.53	Cell redox homeostasis	Oxidative stress	DM1 = Se; DM1 + I = Cu and Se
	P11598	Protein disulfide-isomerase A3 OS = Rattus norvegicus GN = Pdia3 PE = 1 SV = 2	5409	6.41	60,806	37	41.39	sulfur metabolism	Energy metabolism	
	Q07116	Sulfite oxidase_ mitochondrial OS = Rattus norvegicus GN = Suox PE = 1 SV = 2	1751	6.41	60,806	39	18.88	energy metabolism; sulfur metabolism	Energy metabolism	
60	Q63150	Dihydropyrimidinase OS = Rattus norvegicus GN = Dpys PE = 1 SV = 2	2776	6.77	56,815	44	12.9	beta-alanine metabolic process, thymine catabolic process, uracil metabolic and catabolic process	Other	C = Zn; DM1 = Se and Zn; DM1 + I = Cu, Mg, Se and Zn
	P70619	Glutathione reductase (Fragment) OS = Rattus norvegicus GN = Gsr PE = 2 SV = 2	120	8.06	46,301	40	33.47	cell redox homeostasis	Oxidative stress	
62	P10719	ATP synthase subunit beta_ mitochondrial OS = Rattus norvegicus GN = Atp5b PE = 1 SV = 2	10854	5.18	56,354	36	68.62	ATP synthesis, Hydrogen ion transport, Ion transport, Transport	Energy metabolism	C = Zn; DM1 + I = Se
	Q63081	Protein disulfide-isomerase A6 OS = Rattus norvegicus GN = Pdia6 PE = 1 SV = 2	188	5	48,173	34	14.09	cell redox homeostasis	Chaperone	
65	Q8VIF7	Selenium-binding protein 1 OS = Rattus norvegicus GN = Selenbp1 PE = 1 SV = 1	1690	5.1	52,532	31	5.77	protein transporter	Transport	C = Zn; DM1 = Se; DM1 + I = Cu, Mg and Se
67	P04764	Alpha-enolase OS = Rattus norvegicus GN = Eno1 PE = 1 SV = 4	5530	6.16	47,128	31	38.71	gglycolysis/gluconeogenesis	Carbohydrate metabolism	DM1 = Cu and Se; DM1 + I = Cu, Mg, Se and Zn
	P15429	Beta-enolase OS = Rattus norvegicus GN = Eno3 PE = 1 SV = 3	2236	7.8	47,014	29	19.59	gglycolysis/gluconeogenesis	Carbohydrate metabolism	
	P07323	Gamma-enolase OS = Rattus norvegicus GN = Eno2 PE = 1 SV = 2	2108	5.03	47,141	29	26.96	gglycolysis/gluconeogenesis	Carbohydrate metabolism	
	Q9JLJ3	4-trimethylaminobutyraldehyde dehydrogenase OS = Rattus norvegicus GN = Aldh9a1 PE = 1 SV = 1	417	6.57	53,653	40	18.42	amine and polyamine biosynthesis; carnitine biosynthesis	Lipid metabolism	
	P25409	Alanine aminotransferase 1 OS = Rattus norvegicus GN = Gpt PE = 1 SV = 2	305	6.08	55,110	36	17.74	amino-acid degradation, biosynthetic process, L-alanine catabolic process	Amino-acid metabolism	
68	P13444	S-adenosylmethionine synthase isoform type-1 OS = Rattus norvegicus GN = Mat1a PE = 1 SV = 2	4457	5.61	43,698	31	21.16	amino-acid biosynthesis; S-adenosyl-L-methionine biosynthesis; S-adenosyl-L-methionine from L-methionine	Amino-acid metabolism	DM1 = Se; DM1 + I = Cu, Mg, Se and Zn
	P18298	S-adenosylmethionine synthase isoform type-2 OS = Rattus norvegicus GN = Mat2a PE = 1 SV = 1	1377	5.93	43,716	32	6.58	amino-acid biosynthesis; S-adenosyl-L-methionine biosynthesis	Amino-acid metabolism	
70	P10860	Glutamate dehydrogenase 1_ mitochondrial OS = Rattus norvegicus GN = Glud1 PE = 1 SV = 2	3974	8.05	61,416	44	19.81	oxidoreductase, allosteric regulation	Other	DM1 = Se and Zn; DM1 + I = Cu, Mg and Zn

Table 2 (*Continued*)

Spot ID	Protein entry	Protein	Score	pI (theoretical)	Mw (Da, theoretical)	Peptides	Coverage (%)	Biological process	Metabolism associated	Cu, Mg, Se and Zn presence
73	P85968	6-phosphogluconate dehydrogenase_ decarboxylating OS = Rattus norvegicus GN = Pgd PE = 1 SV = 1	110	6.57	53,236	36	3.52	Gluconate utilization, Pentose shunt	Carbohydrate metabolism	DM1 + I = Cu, Mg, Se and Zn
75	Q64640	Adenosine kinase OS = Rattus norvegicus GN = Adk PE = 1 SV = 3	2104	5.72	40,134	36	26.59	purine salvage	Lipid metabolism	DM1 = Se; DM1 + I = Cu, Mg, Se and Zn
77	Q03248	Beta-ureidopropionase OS = Rattus norvegicus GN = Upb1 PE = 1 SV = 1	2812	6.74	44,042	33	25.45	amino-acid biosynthesis; beta-alanine biosynthesis	Amino-acid metabolism	C = Mg, DM1 = Se and DM1 + I = Cu, Mg, Se and Zn
	P32755	4-hydroxyphenylpyruvate dioxygenase OS = Rattus norvegicus GN=Hpd PE = 1 SV = 3	2625	6.29	45,112	29	15.04	L-phenylalanine catabolic proces, tyrosine catabolic process	Amino-acid metabolism	
	P25093	Fumarylacetoacetase OS = Rattus norvegicus GN = Fah PE = 1 SV = 1	1804	6.67	45,976	31	20.47	Phenylalanine catabolism, Tyrosine catabolism	Amino-acid metabolism	
78	Q6P756	Adaptin ear-binding coat-associated protein 2 OS = Rattus norvegicus GN=Necap2 PE = 1 SV = 2	139	7.72	28,405	18	10.27	endocytosis	Transport	C = Mg and Zn; DM1 = Mg and Se; DM1 + I = Cu and Mg
79	P07824	Arginase-1 OS = Rattus norvegicus GN = Arg1 PE = 1 SV = 2	8906	6.76	34,973	31	41.8	Urea cycle	Amino-acid metabolism	C = Zn; DM1 = Se; DM1 + I = Cu, Mg and Zn
	P27867	Sorbitol dehydrogenase OS = Rattus norvegicus GN = Sord PE = 2 SV = 4	176	7.14	38,235	30	7.84	Fructose biosynthesis	Carbohydrate metabolism	
86	P46844	Biliverdin reductase A OS = Rattus norvegicus GN = Blvra PE = 1 SV = 1	807	5.82	33,566	28	13.9	biliverdin reductase activity	Other	C = Cu; DM1 = Se, DM1 + I = Mg
87	D3ZHP7	Serine/threonine-protein kinase ULK3 OS = Rattus norvegicus GN = Ulk3 PE = 3 SV = 1	181	6.34	53,419	20	51.85	Autophagy	Nucleotide	C = Cu; DM1 + I = Mg, Se and Zn
88	Q5I0J9	Putative L-aspartate dehydrogenase OS = Rattus norvegicus GN = Aspdh PE = 2 SV = 1	5716	5.52	31,260	23	29.21	aspartate dehydrogenase activity	Other	C = Cu; DM1 = Se, DM1 + I = Mg and Zn
	P17988	Sulfotransferase 1A1 OS = Rattus norvegicus GN = Sult1a1 PE = 1 SV = 1	2500	6.37	33,906	20	26.62	Transferase	Lipid metabolism	
91	P13255	Glycine N-methyltransferase OS = Rattus norvegicus GN = Gnmt PE = 1 SV = 2	12867	7.1	32,549	20	24.57	methyltransferase, transferase	Other	DM1 = Se; DM1 + I = Cu, Mg and Zn
92	P00481	Ornithine carbamoyltransferase_ mitochondrial OS = Rattus norvegicus GN=Otc PE = 1 SV = 1	2516	9.12	39,886	27	44.63	Urea cycle	Amino-acid metabolism	C = Cu, DM1 + I = Cu, Mg, Se and Zn
94	P04797	Glyceraldehyde-3-phosphate dehydrogenase OS = Rattus norvegicus GN = Gapdh PE = 1 SV = 3	3136	8.14	35,828	29	19.88	glycolysis/gluconeogenesis	Carbohydrate metabolism	C = Cu; DM1 = Se; DM1 + I = Mg, Se and Zn
	P04642	L-lactate dehydrogenase A chain OS = Rattus norvegicus GN = Ldha PE = 1 SV = 1	812	8.45	36,451	24	2.71	lactate	Carbohydrate metabolism	
	P19629	L-lactate dehydrogenase C chain OS = Rattus norvegicus GN = Ldhc PE = 1 SV = 3	180	7.56	35,687	30	24.26	lactate	Carbohydrate metabolism	
	P04636	Malate dehydrogenase_ mitochondrial OS = Rattus norvegicus GN = Mdh2 PE = 1 SV = 2	170	8.93	35,684	19	4.26	tricarboxylic acid cycle	Carbohydrate metabolism	
95	P00884	Fructose-bisphosphate aldolase B OS = Rattus norvegicus GN = Aldob PE = 1 SV = 2	4190	8.66	39,618	19	25.34	Glycolysis	Carbohydrate metabolism	C = Cu; DM1 = Se; DM1 + I = Mg, Se and Zn
	P00507	Aspartate aminotransferase_ mitochondrial OS = Rattus norvegicus GN = Got2 PE = 1 SV = 2	1130	6.73	46,429	21	9.01	Amino-acid biosynthesis	Amino-acid metabolism	

	P04903	Glutathione S-transferase alpha-2 OS = Rattus norvegicus GN = Gsta2 PE = 2 SV = 2	1111	8.89	25,559	20	21.62	glutathione transferase activity	Oxidative stress	
	P00502	Glutathione S-transferase alpha-1 OS = Rattus norvegicus GN = Gsta1 PE = 1 SV = 3	1111	8.87	25,607	21	10.11	glutathione transferase activity	Oxidative stress	
98	P08010	Glutathione S-transferase Mu 2 OS = Rattus norvegicus GN = Gstm2 PE = 1 SV = 2	8573	6.91	25,703	21	16.51	glutathione transferase activity	Oxidative stress	C = Cu; DM1 = Se; DM1 + I = Cu, Mg and Zn
	P08009	Glutathione S-transferase Yb-3 OS = Rattus norvegicus GN = Gstm3 PE = 1 SV = 2	2575	6.84	25,681	22	30.28	glutathione transferase activity	Oxidative stress	
	P04905	Glutathione S-transferase Mu 1 OS = Rattus norvegicus GN = Gstm1 PE = 1 SV = 2	1602	8.27	25,914	15	7.11	glutathione transferase activity	Oxidative stress	
103	P14141	Carbonic anhydrase 3 OS = Rattus norvegicus GN=Ca3 PE = 1 SV = 3	1436	6.89	29,431	23	9.23	Response to oxidative stress	Oxidative stress	C = Cu
	B0BNN3	Carbonic anhydrase 1 OS = Rattus norvegicus GN=Ca1 PE = 1 SV = 1	322	6.86	28,300	18	8.81	Response to oxidative stress	Oxidative stress	
106	Q497B0	Omega-amidase NIT2 OS = Rattus norvegicus GN=Nit2 PE = 1 SV = 1	4320	6.9	30,701	21	16.55	omega-amidase activity	Other	C = Cu, Mg and Se; DM1 = Se; DM1 + I = Mg and Zn
	Q6AXX6	Redox-regulatory protein FAM213A OS = Rattus norvegicus GN = Fam213a PE = 1 SV = 1	117	9.19	25,763	24	31.83	Antioxidant	Oxidative stress	
107	P13803	Electron transfer flavoprotein subunit alpha_ mitochondrial OS = Rattus norvegicus GN = Etfa PE = 1 SV = 4	1862	8.62	34,951	12	6.94	electron transport	Energy metabolism	C = Cu and Se; DM1 + I = Cu, Mg and Zn
108	P52847	Sulfotransferase family cytosolic 1 B member 1 OS = Rattus norvegicus GN = Sult1b1 PE = 1 SV = 2	935	8.16	34,835	23	8.46	lipid metabolism, steroid metabolism	Lipid metabolism	C = Cu, Mg and Se; DM1 = Se; DM1 + I = Cu, Mg and Zn
113	P19112	Fructose-1_6-bisphosphatase 1 OS = Rattus norvegicus GN = Fbp1 PE = 1 SV = 2	5045	5.54	39,609	33	34.16	gluconeogenesis	Carbohydrate metabolism	C = Cu; DM1 = Se; DM1 + I = Cu, Mg and Zn
	Q9Z1N1	Fructose-1_6-bisphosphatase isozyme 2 OS = Rattus norvegicus GN = Fbp2 PE = 2 SV = 1	424	6.76	36,887	26	5.31	gluconeogenesis	Carbohydrate metabolism	
120	O35244	Peroxiredoxin-6 OS = Rattus norvegicus GN = Prdx6 PE = 1 SV = 3	8422	7.14	76,395	17	6.84	redox regulation	Oxidative stress	C = Cu and Zn; DM1 = Se; DM1 + I = Cu, Mg, Se and Zn
	P34067	Proteasome subunit beta type-4 OS = Rattus norvegicus GN = Psmb4 PE = 1 SV = 2	117	6.44	29,197	20	9.73	Hydrolase, Protease, Threonine protease	Other	
	Q9Z0V6	Thioredoxin-dependent peroxide reductase_ mitochondrial OS = Rattus norvegicus GN = Prdx3 PE = 1 SV = 2	107	7.14	28,295	18	18.59	negative regulation of neuron apoptotic process	Oxidative stress	
122	P04041	Glutathione peroxidase 1 OS = Rattus norvegicus GN = Gpx1 PE = 1 SV = 4	2858	7.7	22,305	15	38.81	Oxidoreductase, Peroxidase	Oxidative stress	C = Cu; DM1 = Cu and Se; DM1 + I = Mg and Zn
124	Q63716	Peroxiredoxin-1 OS = Rattus norvegicus GN = Prdx1 PE = 1 SV = 1	2729	8.27	22,109	19	33.33	redox regulation	Oxidative stress	C = Cu
	P40307	Proteasome subunit beta type-2 OS = Rattus norvegicus GN = Psmb2 PE = 1 SV = 1	2650	6.96	22,912	17	6.31	Hydrolase, Protease, Threonine protease	Other	

Table 2 (*Continued*)

Spot ID	Protein entry	Protein	Score	pI (theoretical)	Mw (Da, theoretical)	Peptides	Coverage (%)	Biological process	Metabolism associated	Cu, Mg, Se and Zn presence
127	P07895	Superoxide dismutase [Mn]_ mitochondrial OS = Rattus norvegicus GN = Sod2 PE = 1 SV = 2	3761	8.96	24,674	18	13.07	response to oxidative stress	Oxidative stress	C = Cu; DM1 = Mg and Se
128	Q63716	Peroxiredoxin-1 OS = Rattus norvegicus GN = Prdx1 PE = 1 SV = 1	2872	8.27	22,109	19	31.56	redox regulation	Oxidative stress	C = Cu
129	P30713	Glutathione S-transferase theta-2 OS = Rattus norvegicus GN = Gstt2 PE = 1 SV = 3	4035	7.75	27,439	22	33.03	glutathione metabolic process	Oxidative stress	C = Cu and Se
	P04905	Glutathione S-transferase Mu 1 OS = Rattus norvegicus GN = Gstm1 PE = 1 SV = 2	3785	8.27	25,914	18	18.59	olfaction, Sensory transduction	Other	
	P08010	Glutathione S-transferase Mu 2 OS = Rattus norvegicus GN = Gstm2 PE = 1 SV = 2	119	6.91	25,703	19	16.29	olfaction, Sensory transduction	Other	
	P04904	Glutathione S-transferase alpha-3 OS = Rattus norvegicus GN = Gsta3 PE = 1 SV = 3	110	8.78	25,319	20	13.96	Transferase	Oxidative stress	
	Q6AXY0	Glutathione S-transferase A6 OS = Rattus norvegicus GN = Gsta6 PE = 1 SV = 1	87	5.9	25,808	19	3.62	glutathione metabolic process	Oxidative stress	
	P46418	Glutathione S-transferase alpha-5 OS = Rattus norvegicus GN = Gsta5 PE = 1 SV = 2	79	8.42	25,347	20	3.6	xenobiotic catabolic process, response to drug, response to nutrient levels	Oxidative stress	
	P14942	Glutathione S-transferase alpha-4 OS = Rattus norvegicus GN = Gsta4 PE = 1 SV = 2	79	6.77	25,510	21	3.6	glutathione metabolic process	Oxidative stress	
	P04903	Glutathione S-transferase alpha-2 OS = Rattus norvegicus GN = Gsta2 PE = 2 SV = 2	79	8.89	25,559	20	3.6	glutathione metabolic process	Oxidative stress	
	P00502	Glutathione S-transferase alpha-1 OS = Rattus norvegicus GN = Gsta1 PE = 1 SV = 3	79	8.87	25,607	46	31.46	glutathione metabolic process	Oxidative stress	
132	P15999	ATP synthase subunit alpha_ mitochondrial OS = Rattus norvegicus GN = Atp5a1 PE = 1 SV = 2	1435	9.22	59,754	22	7.34	ATP synthesis, Hydrogen ion transport, Ion transport, Transport	Energy metabolism	C = Cu; DM1 + I = Cu, Mg, Se and Zn
133	P52844	Estrogen sulfotransferase_ isoform 1 OS = Rattus norvegicus GN = Sult1e1 PE = 2 SV = 1	1811	5.78	35,509	29	21.36	Transferase	Other	C = Cu; DM1 + I = Cu, Mg and Zn
	P49889	Estrogen sulfotransferase_ isoform 3 OS = Rattus norvegicus GN = Ste PE = 1 SV = 1	1500	5.57	35,416	29	16.61	Transferase	Other	
	P52845	Estrogen sulfotransferase_ isoform 2 OS = Rattus norvegicus GN = Ste2 PE = 2 SV = 1	1500	5.57	35,365	28	16.61	Transferase	Other	
140	P08010	Glutathione S-transferase Mu 2 OS = Rattus norvegicus GN = Gstm2 PE = 1 SV = 2	550	6.91	25,703	30	25	olfaction, Sensory transduction	Other	C = Cu, Mg and Zn; DM1 = Se
142	P09634	Homeobox protein Hox-A7 (Fragment) OS = Rattus norvegicus GN=Hoxa7 PE = 3 SV = 1	62	4.86	12,552	9	25.71	Developmental protein	Other	C = Cu and Zn; DM1 = Se; DM1 + I = Mg and Zn

143	O35077	Glycerol-3-phosphate dehydrogenase [NAD(+)]_ cytoplasmic OS = Rattus norvegicus GN = Gpd1 PE = 1 SV = 4	782	6.16	37,453	28	18.62	glycolysis/gluconeogenesis	Carbohydrate metabolism	C = Cu and Zn; DM1 = Mg and Se; DM1 + I = Mg and Zn
	Q8CG45	Aflatoxin B1 aldehyde reductase member 2 OS = Rattus norvegicus GN = Akr7a2 PE = 1 SV = 2	245	8.35	40,675	21	28.34	Oxidoreductase	Other	
145	P31210	3-oxo-5-beta-steroid 4-dehydrogenase OS = Rattus norvegicus GN = Akr1d1 PE = 1 SV = 1	2741	6.18	37,378	101	26.38	bile acid catabolic process	Lipid metabolism	C = Cu; DM1 = Se; DM1 + I = Mg and Se
146	P24329	Thiosulfate sulfurtransferase OS = Rattus norvegicus GN = Tst PE = 1 SV = 3	1131	7.77	33,407	20	10.44	epithelial cell differentiation synthesis	Other	C = Cu
147	Q63797	Proteasome activator complex subunit 1 OS = Rattus norvegicus GN = Psme1 PE = 2 SV = 1	1544	5.77	28,577	25	20.08	innate immune response	Other	C = Cu; DM1 = Se; DM1 + I = Mg, Se and Zn
	P13803	Electron transfer flavoprotein subunit alpha_ mitochondrial OS = Rattus norvegicus GN = Etfa PE = 1 SV = 4	182	8.62	34,951	24	5.11	electron transport	Energy metabolism	
	P23680	Serum amyloid P-component OS = Rattus norvegicus GN = Apcs PE = 2 SV = 2	106	5.5	26,176	17	5.26	innate immune response	Other	
148	Q5XI73	Rho GDP-dissociation inhibitor 1 OS = Rattus norvegicus GN = Arhgdia PE = 1 SV = 1	3089	5.1	23,407	44	22.06	cellular response to redox state	Oxidative stress	C = Cu; DM1 + I = Mg
	P46953	3-hydroxyanthranilate 3_4-dioxygenase OS = Rattus norvegicus GN=Haao PE = 1 SV = 2	2132	5.57	32,582	100	27.62	cofactor biosynthesis; NAD(+) biosynthesis	Other	
149	Q63797	Proteasome activator complex subunit 1 OS = Rattus norvegicus GN = Psme1 PE = 2 SV = 1	733	5.77	28,577	25	21.69	innate immune response	Other	C = Cu, Mg and Zn; DM1 + I = Mg
150	P85973	Purine nucleoside phosphorylase OS = Rattus norvegicus GN = Pnp PE = 1 SV = 1	5621	6.46	32,302	21	38.41	immune response	Nucleotide	C = Cu; DM1 = Se; DM1 + I = Mg, Se and Zn
156	Q63716	Peroxiredoxin-1 OS = Rattus norvegicus GN = Prdx1 PE = 1 SV = 1	2026	8.27	22,109	18	21.11	redox regulation	Oxidative stress	DM1 = Se
160	Q68FU3	Electron transfer flavoprotein subunit beta OS = Rattus norvegicus GN = Etfb PE = 2 SV = 3	7067	7.61	27,687	17	26.27	electron transport, transport	Energy metabolism	DM1 = Mg
	P08010	Glutathione S-transferase Mu 2 OS = Rattus norvegicus GN = Gstm2 PE = 1 SV = 2	2130	6.91	25,703	22	33.49	glutathione transferase activity	Oxidative stress	
161	P04905	Glutathione S-transferase Mu 1 OS = Rattus norvegicus GN = Gstm1 PE = 1 SV = 2	10826	8.27	25,914	22	36.24	glutathione transferase activity	Oxidative stress	C = Cu; DM1 + I = Mg and Zn
	P29411	GTP:AMP phosphotransferase AK3_ mitochondrial OS = Rattus norvegicus GN = Ak3 PE = 2 SV = 2	278	8.89	25,438	20	14.98	homeostasis of cellular nucleotides	Nucleotide	

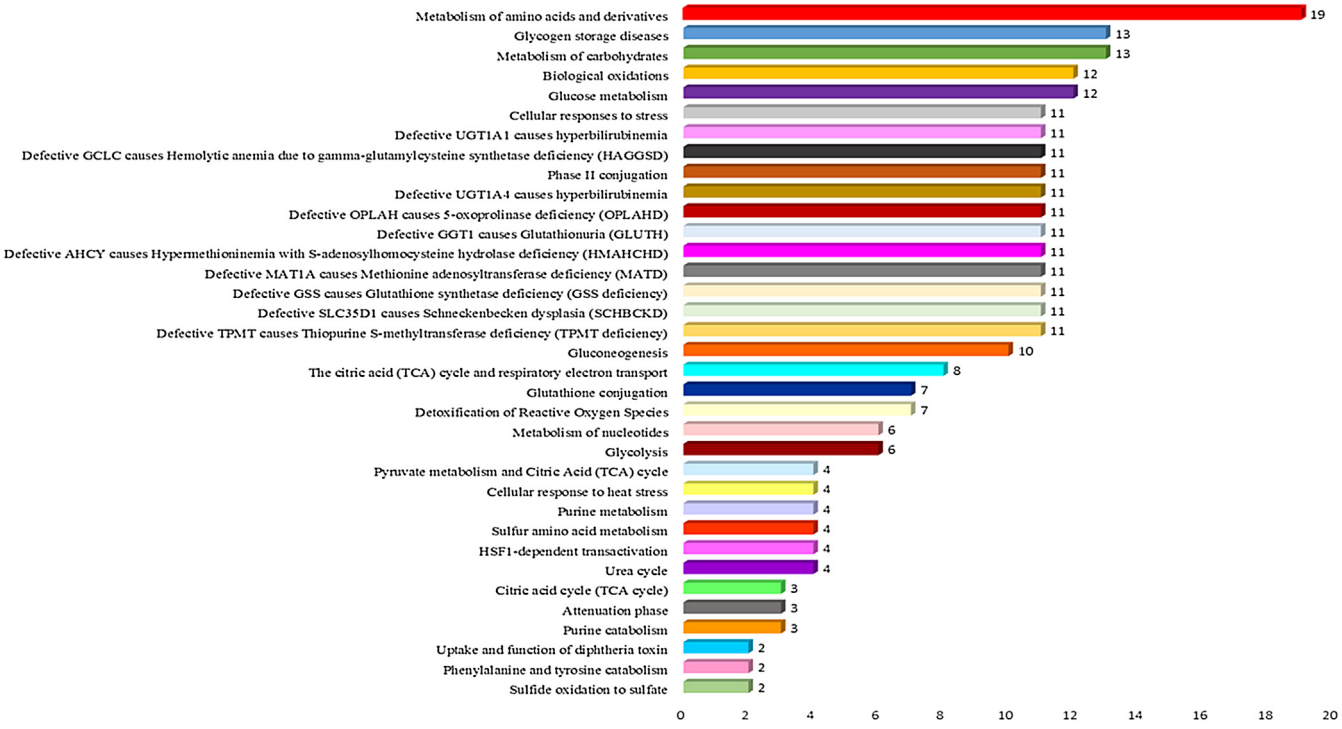

Fig. 2. Pathways with FDR < 0.05 using the Reactome FI.
Genes related in each pathway. Sulfide oxidation to sulfate: *Tst,Suox;* Phenylalanine and tyrosine catabolism: *Fah,Hpd;* Uptake and function of diphtheria toxin: *Hsp90ab1,Hsp90aa1;* Purine catabolism: *Pnp,Gpx1,Cat;* Attenuation phase: *Hsp90ab1,Hsp90aa1,Hspa8;* Citric acid cycle (TCA cycle): *Mdh2,Aco2,Sdha;* Urea cycle: *Ass1,Arg1,Cps1,Otc;* HSF1-dependent transactivation: *Hspa1l,Hsp90ab1,Hsp90aa1,Hspa8;* Sulfur amino acid metabolism: *Mat1a,Tst,Suox,Cbs;* Purine metabolism: *PNP,GPX1,CAT,ADK;* Cellular response to heat stress: *Pnp,Gpx1,Cat,Adk;* Pyruvate metabolism and Citric Acid (TCA) cycle: *Ldha,Mdh2,Aco2,Sdha;* Glycolysis: *Aldob,Pklr,Eno2,Eno3,Gapdh,Eno1;* Metabolism of nucleotides: *Pnp,Gpx1,Gsr,Cat,Adk,Dpys;* Detoxification of Reactive Oxygen Species: *Prdx3,Prdx1,Gpx1,Gsr,Cat,Prdx6,Sod2;* Glutathione conjugation: *Gstm1,Gstm2,Gstm3,Gsta1, Gsta2,Gsta3,Gsta4;* The citric acid (TCA) cycle and respiratory electron transport: *Ldha,Mdh2,Atp5b,Aco2,Etfb,Etfa,Sdha,Atp5a1;* Gluconeogenesis: *Got2, Fbp1, Fbp2, Mdh2, Aldob, Eno2, Eno3, Gapdh, Eno1, Pck1;* Defective TPMT causes Thiopurine S-methyltransferase deficiency (TPMT deficiency), Defective SLC35D1 causes Schneckenbecken dysplasia (SCHBCKD), Defective GSS causes Glutathione synthetase deficiency (GSS deficiency), Defective MAT1A causes Methionine adenosyltransferase deficiency (MATD), Defective AHCY causes Hypermethioninemia with S-adenosylhomocysteine hydrolase deficiency (HMAHCHD), Defective GGT1 causes Glutathionuria (GLUTH), Defective OPLAH causes 5-oxoprolinase deficiency (OPLAHD), Defective UGT1A4 causes hyperbilirubinemia, Phase II conjugation, Defective GCLC causes Hemolytic anemia due to gamma-glutamylcysteine synthetase deficiency (HAGGSD) and Defective UGT1A1 causes hyperbilirubinemia: *Gstm1,Gstm2,Gstm3,Mat1a,Sult1a1,Sult1e1,Gsta1,Gsta2,Gsta3,Gsta4,Mat2a;* Cellular responses to stress*: Hspa1l, Hsp90ab1, Prdx3, Prdx1, Gpx1, Gsr, Cat, Hsp90aa1, Prdx6, Hspa8, Sod2;* Glucose metabolism: *Got2, Fbp1, Fbp2, Pgm1, Mdh2, Aldob, Pklr, Eno2, Eno3, Gapdh, Eno1, Pck1;* Biologicaloxidations: *Gstm1,Gstm2,Gstm3,Mat1a,Sult1a1,Akr7a2,Sult1e1,Gsta1,Gsta2,Gsta3,Gsta4,Mat2a;* Glycogen storage diseases and Metabolism of carbohydrates: *Pgd, Got2, Fbp1, Fbp2, Pgm1, Mdh2, Aldob, Pklr, Eno2, Eno3, Gapdh, Eno1, Pck1;* Metabolism of amino acids and derivatives: *Fah, Got2, Mat1a, Ass1, Glud1, Arg1, Psmb4, Psmb2, Aldh4a1, Cps1, Tst, Hpd, Suox, Otc, Ckm, Psme1, Haao, Ftcd, Cbs.*

isoform type 1 and 2 (MAT1A and MAT2A) are reported in the literature as metal ion-binding proteins; this protein has Mg-finger metal-binding domains [34]. MAT1A and MAT2A catalyze the formation of S-adenosylmethionine from methionine and ATP [34]. Most significant was the protein bound in DM1 and DM1 + I with Zn, as Zn and Mg have the same properties that can interfere with some modifications in these proteins. Propionyl-CoA carboxylase alpha chain (PCCA) was detected with Cu in C. Cu is expected to bind by amino acids containing side chains with soft or borderline ligands such as histidine, cysteine and methionine [35], which could explain this presence because PCCA is composed of 737 amino acids where 2.4% are histidine, 1.5% cysteine and 2.8% methionine (UniProtKB/Swiss-Prot – www.uniprot.org).

3.3.2. Chaperone and oxidative stress metabolism

Heat shock proteins protect other proteins from stress, promote protein folding and prevent protein aggregation [36]. In spot 10, heat shock 70 kDa protein 4 (HSPA4) showed the presence of Cu and Se in DM1 + I. In spot 33, stress-70 protein (HSPA9) and heat shock cognate 71 kDa (HSPA8) showed the presence of Cu, Mg, Se and Zn. In spot 41, 78 kDa glucose-regulated protein (HSPA5), heat shock 70 kDa protein 1-like and 1A/1 B (HSPA1L and HSPA1A), HSPA8 and heat shock-related 70 kDa protein 2 (HSPA2) showed the presence of Se in DM1 and Cu and Mg in DM1 + I. In spot 42, HSPA8, HSPA1A, HSPA2 and HSPA1L showed the presence of Se in DM1 and Cu, Mg and Se in DM1 + I. The literature reported metal binding just in the small heat shock protein α-crystallin that binds with Cu in the core domain, inducing increased dynamics at the dimer interface and modulating anti-aggregation [37]. The literature does not have any other report of metal binding or association with metals in heat shock proteins; our study could suggest some alterations to treatment with insulin in that the ions can bind with these proteins to protect them from oxidative stress generated in the diabetic condictions. Peroxiredoxins are responsible for the degradation of peroxides and thiol-dependent enzymes; peroxiredoxin-1 (PRX1) was found in three spots (124, 128 and 156) with the presence of Cu detected in C and Se in DM1. Another isoform of PRX (peroxiredoxin-6, PRX6) was found in two spots: spot 27 with Cu and Mg in DM1, and spot 120 with Cu and Zn in C, Se in DM1 and Cu, Mg, Se and Zn in DM1 + I. The literature does not report any binding or association of these enzymes with Cu, Mg, Se and Zn; in DM1 the PRX-1 and 6 had a presence of Se that could change the function of these proteins in the degradation of peroxides. Different isoforms of glutathione S-transferase were found in this study. This enzyme plays a key role in enzymatic detoxification [38]. The results showed association with Cu in C, Se in DM1 and

Mg and Zn in DM1 + I; can be associated with some modification of GST thiol groups that can interfere in their function. Carbonic anhydrase 1 and 3 (CA1 and CA3) are Zn metalloenzymes. In this study, these enzymes were found with the presence of Cu in C. As Zn has many characteristics similar to Cu, Cu can bind to the CA1 and CA3 domains.

3.3.3. Carbohydrate and energy metabolism

Phosphoglucomutase-1 (PGM1) plays a key role in carbohydrate metabolism by reversibly catalyzing the interconversion of glucose-1-phosphate to glucose-6-phosphate by the transfer of a phosphate between the C6 and C1 hydroxyl groups of glucose [39]. PGM1 is a ubiquitous metalloenzyme that binds one Mg^{2+} ion per subunit. In this study, Cu, Mg and Se presence was found in PGM1. The presence of cysteine (weak base) in the peptide sequence may promote the association of Cu and Se in this enzyme. Alpha, beta and gamma enolase (ENO1, ENO3 and ENO2) that are involved in gluconeogenesis and glycolysis were found in spot 67 [40]. ENO1 is a Mg metalloenzyme that binds two Mg^{2+} per subunit, and ENO2 and 3 require Mg^{2+} for catalysis and for stabilizing the dimer [41]. In our study, the presence of Cu and Se was found in DM1 and Cu, Mg, Se and Zn in DM1 + I. Thus, it may imply some alteration in this enzyme in DM1 and DM1 + I that can interfere in carbohydrate metabolism and glycemia control. Spot 113 showed two Mg metalloenzymes: fructose 1,6-bisphosphatase 1 (FBP1) and fructose 1,6-bisphosphatase isozyme 2 (FBP2) which bind three Mg^{2+} ions per subunit and are involved in the production of fructose 1,6-bisphosphate [42]. They showed the presence of Cu in C, Se in DM1 and Cu, Mg and Se in DM1 + I. The cysteine present in the peptide sequence may promote the association of Cu and Se observed in this enzyme. In spot 34, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), L-lactate dehydrogenase A and C chain (LDHA and LDHC) and malate dehydrogenase (MDH2) were found with the presence of Se in DM1 and Se and Zn in DM1 + I. The literature does not report any metal-binding association with these enzymes. Since these enzymes have cysteine in their sequences, we can infer this association in groups DM1 and DM1 + I.

4. Conclusion

The determination of copper, magnesium, selenium and zinc bound to different proteins related to different metabolic processes can provide important information about the activity and function of the entire proteome. The literature has poor information about these associations, particularly related to diabetes and insulin treatment. However, the results shown in this manuscript could represent a mechanism for understanding the interactions and changes in DM1 pathology.

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