Instituto de Biociências

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

APLICAÇÃO DE FERRAMENTAS PROTEÔMICAS E METALOPROTEÔMICAS NA CARACTERIZAÇÃO DE BIOMARCADORES PLASMÁTICOS E HEPÁTICOS DE RATOS SUBMETIDOS AO DIABETES TIPO 1

CAMILA PEREIRA BRAGA

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biomoléculas: estrutura e função.

Prof. Dr. Pedro de Magalhães Padilha

BOTUCATU – SP
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Palavras-chave: Diabetes - Biomarcadores; Eletroforese bidimensional; Metabolismo; Metaloproteínas; Proteínas.

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ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE CAMILA PEREIRA BRAGA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA GERAL E APLICADA, DO INSTITUTO DE BIOCIÊNCIAS.

Aos 21 dias do mês de julho do ano de 2016, às 09:00 horas, no(a) Sala de Defesas da Pós-Graduação, reuniu-se a Comissão Examinadora da Defesa Pública, composta pelos seguintes membros: Prof. Dr. PEDRO DE MAGALHAES PADILHA - Orientador(a) do(a) Departamento de Química e Bioquímica / Instituto de Biociências de Botucatu - UNESP, Prof. Dr. PAULO DOS SANTOS ROLDAN do(a) Departamento de Química e Biotecnologia / Instituto de Química e Biotecnologia - Universidade Federal de Alagoas, Profa. Dra. ANA RITA DE ARAUJO NOGUEIRA do(a) Centro de Pesquisa de Pecuária do Sudeste / Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA - São Carlos, Prof. Dr. LINCOLN CARLOS SILVA DE OLIVEIRA do(a) Departamento de Química / Universidade Federal do Mato Grosso do Sul, Prof. Dr. JOSÉ MAURICIO SFORCIN do(a) Departamento de Microbiologia e Imunologia / Instituto de Biociências de Botucatu - UNESP, sob a presidência do primeiro, a fim de proceder a arguição pública da TESE DE DOUTORADO de CAMILA PEREIRA BRAGA, intitulada **APLICAÇÃO DE FERRAMENTAS PROTEÔMICAS E METALOPROTEÔMICAS NA CARACTERIZAÇÃO DE BIOMARCADORES PLASMÁTICOS E HEPÁTICOS DE RATOS SUBMETIDOS AO DIABETES TIPO 1**. Após a exposição, a discente foi arguida oralmente pelos membros da Comissão Examinadora, tendo recebido o conceito final:____ APROVADA_ . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.

Prof. Dr. PEDRO DE MAGALHAES PADILHA

Prof. Dr. PAULO DOS SANTOS ROLDAN

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SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

Introdução

α	alfa
β	beta
δ	delta
2D-DIGE	eletroforese em gel diferencial
2D-PAGE	eletroforese bidimensional em gel de poliacrilamida
µg	micrograma
µL	microlitro
1D	uma dimensão
2D	duas dimensões
AAS	espectrometria de absorção atômica
ATP	adenosina trifosfato
Ca	cálcio
CAT	catalase
Cu	cobre
DM1	diabetes mellitus tipo 1
EO	estresse oxidativo
ERO	espécies reativas de oxigênio
ESI MS MS	espectrometria de massas em sequência com ionização por eletrospray
FAAS	espectrometria de absorção atômica com atomização por chama
GSH-Px	glutationa peroxidase
Hz	hidrazida
HLA	antígeno lancocitário humano
ICP-MS	espectrometria de massas com fonte de plasma indutivamente acoplado
IDF	*internacional diabetes federation*
IEF	focalização isoelétrica
K	potássio
LC	cromatografia líquida
Mg	magnésio
mg	miligrama
mL	mililitro
Mm	massa molecular
MS	espectrometria de massas
NHS	N-hidroxi-succinimidil
pI	ponto isoelétrico
PP	polipeptídeo pancreático
SDS	dodecil-sulfato de sódio

SDS PAGE	eletroforese em gel de poliacrilamida em uma dimensão
Spot	banda 2D de proteínas
SR-XRF	fluorescência de raios-X com radiação Síncrotron
Se	selênio
SOD	superóxido dismutase
STZ	estreptozotocina
TCA	ácido tricloroacético
Zn	zinco

Capítulos

°C	degree celsius
%V	volume normalized
µg	microgram
µL	microliter
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
2D-DIGE	two-dimensional differential in-gel electrophoresis
ABC	ammonium bicarbonate
ACN	acetonitrile
AGEs	advanced glycation end-products
ANOVA	analysis of variance
B2G	blast2GO program
BCA	bichinchoninic acid assay
Ca	calcium
CEUA	ethics committee on the use of animals
CHAPS	sulphate 3-[(3-cloroaminopropil)-dimethylammonio]-1-propane
Cu	copper
Da	dalton
dL	deciliter
DM1	diabetes mellitus type 1
DMSO	dimethyl sulfoxide
DTT	1,4 Dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assays
ESI-MS/MS	electrospray ionization-tandem mass spectrometry
FA	formic acid
FDR	false discovery rate
Fe	iron
FFAs	free fatty acids
FI	functional Interaction

FAAS	furnace atomic absorption spectrometry
GFAAS	graphite furnace atomic absorption spectrometry
g	gram
h	hour
IAA	iodoacetamide
IEF	isoelectric focusing
L	liter
LC	liquid chromatography
LOQ	limit of quantification
kg	kilogram
m/m	relation mass /mass
m/v	relation mass /volume
m/z	relation mass /charge
min	minutes
Mg	magnesium
mg	milligram
mL	milliliter
mm	millimeter
mmol	milimol
Mn	manganese
MS	mass spectrometry
Ni	nickel
nL	nanoliter
nm	nanometer
NPH	neutral protamine hagedorn
pH	potential of hydrogen
pI	isoelectric point
PCA	principal component analysis
ROS	reactive oxygen species
SDC	sodium deoxycholate
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	selenium
STZ	streptozotocin
TCA	trichloroacetic acid
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TEMED	N,N,N',N-Tetramethylethylenediamine
UniProt	universal protein resource
VLDL	very low density lipoproteins

V	volt
Vh	volt-hour
Z	charge
Zn	zinc

RESUMO

APLICAÇÃO DE FERRAMENTAS PROTEÔMICAS E METALOPROTEÔMICAS NA CARACTERIZAÇÃO DE BIOMARCADORES PLASMÁTICOS E HEPÁTICOS DE RATOS SUBMETIDOS AO DIABETES TIPO 1

Diabetes mellitus tipo 1 é caracterizado pelo aumento significativo de glicose circulante no sangue, resultado da deficiência na secreção e/ou ação de insulina. O quadro hiperglicêmico, quando cronicamente instalado, leva a anormalidades no metabolismo de lipídios, proteínas e carboidratos, além de alterar o balanço entre pró-oxidantes e antioxidantes. É um importante problema de saúde pública, pois compromete a qualidade de vida e sobrevida dos indivíduos, além de envolver elevados custos no seu tratamento. Quando se consegue identificar os fatores determinantes para o desenvolvimento do diabetes, novas estratégias de prevenção e tratamento são necessárias. Nesse contexto, as proteínas e os minerais apresentam fundamental importância como componentes estruturais e funcionais dos seres vivos. Estudos proteômicos e metaloproteômicos, auxiliam na compreensão da variabilidade do diabetes, contribuindo assim na elucidação dos aspectos fisiológicos e funcionais das biomoléculas presentes em amostras biológicas, como plasma e tecido animal, além disso, a possível identificação de biomarcadores relacionados ao desenvolvimento de doenças crônicas degenerativas, como o diabetes. O objetivo geral do trabalho foi utilizar ferramentas proteômicas e metaloproteômicas na identificação de possíveis biomarcadores presentes no plasma e fígado de ratos diabéticos tipo 1 induzido experimentalmente com estreptozotocina. Foram utilizados 24 ratos *Wistar*, distribuídos em 3 grupos experimentais (n= 8): C (controle); DM1 (diabéticos tipo 1); DM1 + I (diabéticos tipo 1, tratadas com insulina). O diabetes mellitus tipo 1 foi induzido com estreptozotocina (60mg/kg, i.p., dose única) e os animais do grupo DM1 + I receberam reposição insulínica (Humulin N100^{UI} Protamina Neutra de Hagedorn - NPH, marca Lilly), com dose inicial de 3U/animal (administrada diariamente, via subcutânea, período vespertino) e de acordo com os valores obtidos de glicemia a dose inicial foi reajustada/mantida para que se atingissem os níveis séricos de euglicemia. No plasma, foi realizado estudo metaloproteômico para as proteínas que apresentaram diferença de expressão entre os grupos experimentais, utilizando-se a 2D-PAGE no processo de fracionamento de proteínas das amostras de plasma, GFAAS e FAAS na determinação quantitativa de Cu, Mg, Se e Zn nos *spots* que apresentaram diferença de expressão e ESI/MS-MS na caracterização dessas proteínas. Trinta e cinco proteínas apresentaram diferença de

expressão no plasma, indicando a alpha-1-macroglobulina e haptoglobulina como possíveis biomarcadoras do diabetes tipo 1 controlado (tratado com insulina); e as proteínas 2'-desoxinucleósido-5'-fosfato de N-hidrolase 1, proteína transmembranar 1, soro amilóide P-componente, vitamina D de ligação e biliverdina como possíveis candidatas a biomarcadoras do diabetes tipo 1 não controlado. Já a quantificação de Cu, Mg, Se e Zn nos *spots* revelaram como esses minerais poderiam estar ligados nessas proteínas e como estariam envolvidos na expressão proteica, progressão e complicações do diabetes tipo 1. No fígado, primeiramente foi realizado estudo proteômico, onde as proteínas foram caracterizadas pela técnica de *gel-free* e classificadas como dependentes ou independentes de insulina (tratamento), em relação aos dados obtidos para abundância e diferença de expressão. Esse estudo identificou 305 proteínas com diferença de expressão entre os grupos experimentais, entre elas 147 foram classificadas como dependentes de insulina e 152 como independentes de insulina. O controle dos níveis de glicose sanguínea pelo tratamento com insulina, restaurou os níveis da enzima frutose-1,6 bifosfatase indicando controle da gliconeogênese, e afetou a abundância das enzimas que funcionam na desintoxicação de EROs, restaurando o equilíbrio redox e a função mitocondrial. No fígado também foi feito o estudo das proteínas diferencialmente expressas pelo Oxi-proteoma, utilizando a 2D-DIGE com os fluoróforos Cy3 e Cy-5-hidrazida no processo de fracionamento e caracterização dos *spots* proteicos por ESI-MS/MS. A identificação das proteínas carboniladas indicou que o tratamento com insulina está associado com a diminuição do estresse oxidativo por meio da regulação positiva das enzimas antioxidantes e diminuição da produção de EROs, controle das vias metabólicas envolvidas com o metabolismo de carboidratos (glicólise e gliconeogênese) e metabolismo energético. Estudo metaloproteômico também foi realizado no fígado, utilizando a 2D-PAGE no processo de fracionamento de proteínas, GFAAS e FAAS na determinação quantitativa de Cu, Mg, Se e Zn nos *spots* obtidos no fracionamento das proteína e caracterização das proteínas por ESI/MS-MS. Os resultados obtidos nesse estudo sugeriram diferentes interações entre os minerais estudados e as proteínas entre os grupos experimentais. Essas interações podem estar associadas com alterações funcionais nessas proteínas e envolvimento na progressão e complicações do diabetes tipo 1. Assim, as informações obtidas no presente trabalho fornecem subsídios técnico-científicos na área de saúde de extrema importância, possibilitando o entendimento dos dados proteômicos e metaloproteômicos gerados relacionados com suas respectivas vias metabólicas, função molecular, processo biológico e componente celular. O caráter inédito da proposta proporcionou a elucidação de aspectos fisiológicos e funcionais das biomoléculas presentes em amostras biológicas, como plasma e tecido hepático.

ABSTRACT

PROTEOMICS AND METALLOPROTEOMICS TOOLS IN THE CHARACTERIZATION OF PLASMATIC AND HEPATIC BIOMARKERS FROM RATS SUBMITTED TO DIABETES TYPE 1

Type 1 diabetes mellitus is characterized by the significant increase in circulating glucose in the blood as a result of deficiency in the secretion and/or insulin action. The hyperglycemic state, when chronically installed, leads to abnormalities in the metabolism of lipids, proteins and carbohydrates, in addition to changing the balance between pro-oxidants and antioxidants. This is an important public health problem because it affects the quality of life and the survival of individuals, often necessitating high-cost treatment. When one can identify the determining factors for the development of diabetes, new strategies for prevention and treatment are needed. In this context, our results reflect the fundamental importance of protein and minerals such as structural and functional components of living things, proteomic and metalloproteomic studies that can assist in the understanding of diabetes variability, thus helping to elucidate the physiological and functional aspects of biomolecules in biological samples such as plasma and animal tissue. In addition, the possible identification of biomarkers related to the development of chronic degenerative diseases like diabetes. The overall objective of the study was to use proteomics and metalloproteomic in identifying potential biomarkers present in the plasma and liver of diabetic rats type 1 experimentally induced with streptozotocin. The total of 24 rats, Wistar, were divided into 3 groups (n = 8): C (control); DM1 (type 1 diabetes); DM1 + I (type 1 diabetes treated with insulin). The type 1 diabetes mellitus was induced with streptozotocin (60 mg/kg, i.p., single dose) and animals of DM1 + I group received insulin replacement (Humulin N100UI Neutral Protamine Hagedorn - NPH, brand Lilly), with an initial dose of 3U/animal (daily administered subcutaneously evening period) and in accordance with the values obtained from the initial dose of glucose was readjusted/kept so that it reached serum euglycemia. In plasma was performed metalloproteomic study for proteins that showed differences in expression between the experimental groups, 2D-PAGE was used in protein fractionation process of plasma samples, GFAAS and FAAS for quantitative determination of Cu, Mg, Se and Zn in the spots that showed differences of expression and the proteins were characterized by ESI/MS-MS. Thirty-five proteins present in the plasma had difference of expression, indicating the alpha-1-macroglobulin and haptoglobulin as potential biomarkers of type 1 diabetes controlled (insulin

treated); and 2'-deoxynucleoside 5'-phosphate N-hydrolase 1, transmembrane protein 11, serum amyloid P-component, vitamin D-binding protein and biliverdin as possible candidates for biomarkers of type 1 diabetes not controlled. The quantification of Cu, Mg, Se and Zn in spots revealed how these minerals could be linked these proteins and how they would be involved in protein expression, progression and complications of type 1 diabetes. In the liver, was first conducted the proteomic study where proteins were characterized by gel-free technique and classified as insulin dependent or independent (treatment), compared to the data obtained for abundance and difference in expression. This study identified 305 proteins with differential expression between experimental groups, among which 147 were classified as insulin-dependent and 152 as insulin-independent. The restoration of glucose levels in the blood by insulin treatment restored levels of the enzyme fructose-1,6 bisphosphatase indicating control of gluconeogenesis, and affected the abundance of enzymes that function in the detoxification of ROS, restoring redox balance and mitochondrial function. In the liver it has also been a study of the proteins differentially expressed by Oxi-proteome, using the 2D-DIGE with the fluorophores Cy3 and Cy-5-hydrazide in the fractionation process and characterization of protein spots by ESI-MS/ MS. The identification of the carbonyl proteins indicated that treatment with insulin was associated with decreased oxidative stress by upregulating the antioxidant enzymes and decreased production of ROS, controlling the metabolic pathways involved in carbohydrate metabolism (glycolysis and gluconeogenesis) and energy metabolism. The metalloproteomic study was also performed on liver, using 2D-PAGE for the protein fractionation process, GFAAS and FAAS for quantitative determination of Cu, Mg, Se and Zn in the spots obtained in the fractionation of the protein and characterization of proteins by ESI/MS-MS. The results suggested different interactions between minerals and proteins between experimental groups, and may be associated with functional alterations in these proteins and involvement in progression and complications of diabetes type 1. However, the work provides technical and scientific support in extreme health importance, enabling the understanding of proteomic and metalloproteomic data generated in relation to their respective metabolic pathways, molecular function, biological process and cellular component. The unprecedented of the proposal provided the elucidation of physiological and functional aspects of biomolecules present in biological samples, such as plasma and hepatic tissue.

Introdução

1. Introdução

1.1. Diabetes mellitus tipo 1

Dados epidemiológicos indicam que a prevalência do diabetes mellitus tipo 1 (DM1) aumenta anualmente cerca de 3% em todo mundo (1). Segundo o último Atlas publicado pela *Internacional Diabetes Federation* (IDF), 79.000 crianças são acometidas pelo DM1 por ano, sendo que os Estados Unidos, Índia e Brasil são os países com as maiores estimativas de novos casos por ano (2). A frequência crescente pode não estar relacionada apenas aos fatores genéticos, a variabilidade geográfica tem influenciado na incidência do DM1. Neste sentido, um estudo publicado em 2012, relatou que ter nascido em países com alta incidência de diabetes, aumenta o risco para o desenvolvimento do DM1 em crianças originárias de países cujo fator genético é baixo (3); apontando para a importância dos fatores ambientais sobre o desenvolvimento do DM1.

A etiologia desta doença é multifatorial, e resulta da interação entre predisposição genética e fatores ambientais (1). Os genes do sistema de histocompatibilidade humano (HLA), principalmente os da classe II (DR3, DR4 e DQ) são os responsáveis por 40% do componente genético desta doença que associados aos fatores ambientais levariam ao início do desenvolvimento do DM1 (4). Estudos epidemiológicos divulgaram alguns fatores de risco como ambiente frio, alta taxa de crescimento populacional, infecções e estresse, podem acelerar o processo patológico do desenvolvimento do DM1 (1). Enquanto outros fatores de risco que podem iniciar o processo autoimune, incluem a exposição precoce às proteínas do leite de vaca, nitrosaminas ou eventos fetais precoces, tais como infecções virais (5).

DM1 é considerado uma das principais causas de elevadas taxas de mortalidade em decorrência de seus efeitos sobre os distúrbios cardiovasculares, principalmente aterosclerose, além de outras complicações, tais como retinopatias e insuficiência renal (6). Dessa forma, o DM1 passa ser um importante problema de saúde pública, pois compromete a qualidade de vida e sobrevida dos indivíduos, além de envolver elevados custos no seu tratamento (7). No Brasil, o custo médio anual com o tratamento médico direto (consultas, tratamento ambulatorial, fornecimento de insulina, medicamentos orais, glicosímetro, bomba de insulina, procedimentos médicos, hemodiálise e hospitalizações) do DM1 por paciente é de 1.466,36; 1.252,83; 1.148,09 e 1.396,30 dólares nas regiões sudeste, sul, norte/nordeste e centro-oeste, respectivamente (7); totalizando um custo anual de aproximadamente 4 milhões de dólares (8).

Sabe-se que o pâncreas, especialmente as ilhotas de Langerhans, é o órgão diretamente implicado na causa do DM1 (9). As ilhotas pancreáticas são constituídas por cinco tipos de células: alfa (α), beta (β), delta (δ), célula que produz o polipeptídeo pancreático (PP) e células G. Em ação conjunta secretam mais de 20 hormônios, sendo o glucagon, a insulina, a somatostatina e o polipeptídeo pancreático os principais; em destaque para as células β que são responsáveis pela síntese e secreção de insulina (9).

A secreção de insulina é desencadeada pelo aumento dos níveis glicêmicos e concomitante aumento dos níveis intracelulares de ATP, que levam ao fechamento dos canais de potássio (K), dependentes de ATP, com consequente diminuição da entrada de K resultando em despolarização das células β-pancreáticas e abertura dos canais de cálcio (Ca). O aumento do Ca intracelular, por sua vez desencadeia a secreção desse hormônio (10).

Enquanto a ação da insulina é baseada em seus efeitos fisiológicos que são exercidos através da fosforilação de proteínas (mecanismo que ativa segundos mensageiros e modula as respostas biológicas), que aumenta o transporte de glicose e regulação de vias enzimáticas (11,12). A insulina é responsável pela regulação dos níveis glicêmicos, e utilização da glicose pelos tecidos periféricos e hepático, diminuindo a concentração de glicose no sangue através da inibição da produção hepática, captação e metabolização da glicose pelo músculo e tecido adiposo (13). Portanto, qualquer defeito na ação ou secreção de insulina resultará em aumento dos níveis glicêmicos e diminuição da captação da glicose pelos tecidos periféricos (13).

DM1 é caracterizado pelo aumento significativo de glicose circulante no sangue, resultado da deficiência na secreção e/ou ação de insulina (14). O quadro hiperglicêmico, quando cronicamente instalado, leva a anormalidades no metabolismo de lipídios, proteínas e carboidratos, além de alterar o balanço entre pró-oxidantes e antioxidantes (14,15).

A explicação viável para a ocorrência dessas anormalidades metabólicas é que, o papel anabólico da insulina está comprometido e, na maioria das vezes, o glucagon opõe-se ao efeito da insulina sobre o fígado, estimulando a glicogenólise e a gliconeogênese, porém com efeito reduzido sobre a utilização periférica da glicose (16). Assim, há aumento na produção hepática de glicose e baixa captação pelos tecidos periféricos, além de diminuir a conversão de glicose em glicogênio pelo fígado, e isto resulta em acentuada hiperglicemia. A cetoacidose resulta do aumento da taxa lipolítica no tecido adiposo e, consequentemente maiores concentrações de ácidos graxos livres (AGL) no plasma, que servem como substratos para síntese de corpos cetônicos (17). A hipertrigliceridemia é devido à maior produção de lipoproteínas de muito baixa densidade (VLDL), através da síntese de triacilgliceróis promovida pelos AGL que derivam do

tecido adiposo (18). Além do fato, nos indivíduos diabéticos, ocorre menor degradação de lipoproteínas pela menor atividade da enzima lipase (insulina estimula síntese e ação desta enzima) (19). Ainda, a inibição da proteólise e a maior captação de aminoácidos estimulada pela insulina estão comprometidas no diabetes, resultando no aumento das concentrações plasmáticas de dois importantes aminoácidos, alanina e glutamina, que são utilizados como substratos para a gliconeogênese hepática e renal, respectivamente (13).

Evidências demonstram que o DM1 está associado à produção aumentada de radicais livres derivados do oxigênio molecular, denominadas de espécies reativas de oxigênio (ERO) (20). A primeira linha de defesa contra o dano oxidativo são os antioxidantes endógenos enzimáticos, como as metaloproteínas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GSH-Px) (21,22). Há também os antioxidantes não enzimáticos, como os lipossolúveis (carotenos, tocoferóis, quinonas e bilirrubinas) e os hidrossolúveis (ácido ascórbico, ácido úrico e proteínas ligadas a metais) (21,22).

O estresse oxidativo (EO) é estabelecido pelo desequilíbrio entre a produção de ERO e a capacidade antioxidante endógena e o seu papel como determinante principal do início e da progressão das complicações associadas ao DM1 (23). A disfunção endotelial tem sido observada no DM1 mesmo quando a normoglicemia é alcançada, sugerindo que o estresse oxidativo tenha papel central na patogênese das complicações do DM1 (23).

Modificações oxidativas nas proteínas podem causar mudanças estruturais, perda parcial ou total de sua função e o dano oxidativo às proteínas tem um importante papel em várias doenças (24). Estudos estão sendo desenvolvidos para entender como as ERO são capazes de mediar a oxidação de proteínas em diferente doenças (25). O tipo mais comum de oxidação é a carbonilação, que resulta na formação de grupos carbonil reativos (aldeídos) que podem ser introduzidos nas proteínas em diferente sítios e mecanismos, causando danos irreversíveis e irreparáveis às proteínas (12).

Neste sentido, a fim de se compreender os mecanismos da patogênese e complicações do DM1, modelos animais têm sido extensivamente utilizados. Métodos eficientes para indução do diabetes experimental, tais como, a administração de agentes químicos β-citotóxicos como a estreptozotocina (STZ) consiste numa maneira eficaz para promover o DM1 em animais (26). A STZ é um antibiótico, de natureza glicosamina-nitrosuréia, com propriedades tóxicas, isolada de *Streptomyces achromogenes* e é captada pelas células β- pancreáticas através de transportadores de glicose GLUT-2 (27). Sugere-se que a STZ atue estimulando a produção de ERO, com elevação na peroxidação lipídica e diminuição da atividade das enzimas (27). Além disso, um

dos principais efeitos tóxicos da STZ envolve a alteração da estrutura do DNA, o qual é fragmentado pelas ERO, comprometendo, desta forma, a biossíntese e secreção de insulina (26,27).

Quando se consegue identificar os fatores determinantes para o desenvolvimento do diabetes, novas estratégias de prevenção e tratamento são necessárias. Nesse contexto, insere-se a fundamental importância das proteínas e minerais como componentes estruturais e funcionais dos seres vivos, a proteômica e metaloproteômica, áreas de estudo propostas recentemente, permitem a integração de estudos tradicionalmente analíticos com estudos inorgânicos e bioquímicos. E podem ser utilizadas como uma metodologia robusta para auxiliar na compreensão da variabilidade do diabetes, contribuindo assim na elucidação dos aspectos fisiológicos e funcionais das biomoléculas presentes em amostras biológicas, como plasma e tecido animal. Dessa forma, estudos proteômicos e metaloproteômicos permitem estudar proteínas e o envolvimento dessas biomoléculas em estados patológicos em larga escala e, além disso, a possível identificação de biomarcadores relacionados ao desenvolvimento de doenças crônicas degenerativas, como o diabetes.

1.2. Biomarcadores

A interação entre um agente causador e o organismo pode alterar diversos parâmetros biológicos. Estudos sobre biomarcadores envolvem a comparação do padrão de proteínas presentes em uma amostra saudável com o de uma amostra afetada, isto porque as proteínas desempenham papeis fundamentais nos sistemas biológicos (atividades específicas, associação com outras biomoléculas, seus níveis de expressão são essenciais para o desempenho ideal das funções celulares) (28). Uma vez que diferenças não só nos níveis de expressão, mas também na estrutura proteica geralmente indicam um quadro patológico, as proteínas são moléculas de grande interesse na busca por biomarcadores confiáveis (28).

Biomarcadores são relacionados com uma característica que é medida e avaliada como indicador de um processo biológico normal, patológico ou respostas farmacológicas a uma intervenção terapêutica (29). Contudo, estudos envolvendo biomarcadores são de extrema importância, pois auxiliam na detecção precoce de doenças, bem como a monitorá-las perante um tratamento (28).

O plasma é a amostra mais variada do proteoma presente no corpo, sendo que o perfil de proteínas pode variar com as condições fisiológicas e patológicas (6). Os objetivos da utilização do plasma para a investigação de biomarcadores é a eliminação da necessidade de biópsia,

diagnóstico de doenças específicas (utilizando proteínas como biomarcadores), compreensão da patogênese da doença, e monitoramento do progresso da intervenção terapêutica (6).

O fígado pode ser considerado como a maior glândula do organismo, cumpre importantes e complexas funções (30). Entre as funções hepáticas fundamentais estão: formação e excreção de bile, síntese de fatores de coagulação, armazenamento de vitaminas, estocagem de minerais, controle da glicemia pela glicogenólise e gliconeogênese, síntese de proteínas, formação de corpos cetônicos, participação no metabolismo de lipoproteínas e do colesterol, transformação e catabolismo de hormônios, e transformação e excreção de drogas (31). No DM1, o fígado pode desenvolver anormalidades estruturais e/ou funcionais que podem perturbar qualquer um destes processos, particularmente a regulação do metabolismo de carboidratos, lipídios e proteínas (32).

Estudos proteômicos e metaloproteômicos investigam a presença de biomarcadores relacionados com a comparação do padrão de proteínas presentes em uma amostra saudável com o de uma amostra afetada, podendo levar a compreensão e prevenção das complicações primárias e secundárias de certas patologias como no caso do DM1 e, consequentemente, diminuir a gravidade da doença.

1.3. Proteômica

Uma análise abrangente em alta-escala das proteínas é o objetivo da ciência do proteoma, a proteômica. Sua área de atuação é ampla; e engloba a identificação e quantificação de proteínas nas células, tecidos e fluidos biológicos; análise de mudanças na expressão das proteínas; caracterização de modificações pós-traducionais; e estudos de interações proteína-proteína (33).

As técnicas mais empregadas para o estudo proteômico na etapa de fracionamento/purificação das biomoléculas são: a eletroforese em gel de poliacrilamida em uma ou duas dimensões (PAGE, 2D-PAGE) e a cromatografia líquida (LC) multidimensionais.

1.3.1. Métodos para o estudo proteômico

O'Farrell introduziu em 1995 a eletroforese bidimensional em gel de poliacrilamida (2D-PAGE) para separar proteínas celulares sob condições desnaturantes e permitiu a resolução de centenas de proteínas (34). A técnica por 2D-PAGE resulta da combinação da focalização isoelétrica e da eletroforese em gel de poliacrilamida. Na primeira etapa as proteínas são separadas de acordo com seu ponto isoelétrico (pI), e na segunda etapa de acordo com suas massas moleculares(Mm) (35). Contudo a 2D-PAGE consiste em 5 etapas principais: preparo da amostra, primeira dimensão, segunda dimensão, detecção das proteínas, digitalização e tratamento das imagens (36).

O preparo da amostra é uma etapa essencial no processo de separação das proteínas, e este deve ser o mais simples possível e modificações na proteína devem ser evitadas (37). O melhor método de extração, precipitação e solubilização das proteínas varia de amostra para amostra, e cada caso particular deve ser analisado (36). Apesar disso, as proteínas da amostra precisam ser desnaturadas, desagregadas, reduzidas e solubilizadas para que atinjam um completo rompimento das interações molares e garantam que cada *spot* presente no gel represente uma proteína individual (38).

Para amostras biológicas como tecidos (como por exemplo o tecido hepático), devemos romper as células do material biológico, o rompimento celular pode ser de dois tipos: mecânico (maceração ou sonicação) ou químico (enzimático ou na presença de detergentes), esses processos são feitos a fim de se extrair as proteínas (39). O rompimento deve ser feito rapidamente e em baixas temperaturas, visto que as proteínas ficam expostas a muitos agentes que podem danificá-las de maneira irreversível (40). Além disso, alguns compostos interferentes (proteases, sais, lipídeos, ácidos nucleicos, polissacarídeos, pigmentos fenólicos e/ou proteínas muito abundantes), afetam a solubilização e o processo eletroforético, portanto devem ser removidos ou inativados durante ou após o rompimento celular, e são separados seletivamente por meio do processo de precipitação das proteínas (37). A precipitação com solução de acetato de amônio em metanol, acetona ou solução de ácido tricloroacético (TCA) em acetona, geralmente são os métodos mais utilizados para esta etapa (37).

A primeira dimensão é a focalização isoelétrica (IEF). Nela, as proteínas são separadas de acordo com o seu ponto isoelétrico (pI) em fitas contendo géis com um gradiente de pH. Uma vez submetidas a um campo elétrico, as proteínas migrarão até encontrar uma faixa de pH referente ao seu ponto isoelétrico (pI) e neste ponto ficarão com carga total neutra, interrompendo a migração no gel (35,41,42). Existem diferentes faixas de pH, as faixas amplas permitem uma visão geral das proteínas expressas, porém pode ocorrer uma comigração e sobreposição das proteínas com propriedades eletroforéticas similares, uma solução para esse problema pode ser o uso de gradientes de pH mais “estreitos” (33,43).

A segunda dimensão, é a eletroforese em gel de poliacrilamida com dodecil sulfato de sódio (SDS-PAGE), as proteínas são separadas com base na sua Mm (33,43). A mobilidade das proteínas no gel é dependente do tamanho dos poros do gel e da Mm das proteínas. O percentual de acrilamida do gel é que determina o tamanho do poro, sendo assim, deve ser escolhido levando-se em consideração a faixa de Mm das proteínas a serem identificadas, ou seja se o objetivo é identificar proteínas com alta Mm, um gel com concentração mais baixa deve ser

escolhido (por exemplo um gel de 10%), se objetivo é identificar metalotineínas deve ser utilizado um gel mais concentrado (por exemplo um gel de 15%) (44). Cada "ponto/mancha" (*spot*) visualizado no gel pode ser considerado como uma coordenada ortogonal de uma proteína que migrou especificamente em função de seu pI e sua Mm. O aparecimento ou desaparecimento de *spots* fornecem informações acerca de proteínas estágio-específicas, enquanto a intensidade dos *spots* fornece informações quantitativas a respeito da expressão diferencial de proteínas.

Geralmente o azul brilhante de Coomassie e o nitrato de prata são os mais utilizados como métodos gerais de coloração. A coloração com azul de Coomassie é menos sensível que por nitrato de prata, sendo assim, exige maior quantidade de amostra para que as proteínas sejam visualizadas. Porém o azul de Coomassie tem a vantagem de permitir melhor quantificação da abundância relativa de cada proteína (devido à reduzida gama dinâmica - gama de linearidade entre a intensidade do *spot* e concentração de proteína) em comparação com a coloração com a prata, o que garante a existência de quantidade suficiente de proteína para a sua subsequente identificação (45,46).

Após a coloração, os géis são digitalizados com o auxílio de um *scanner* específico e registra-se a intensidade de cada *spot* sob a forma de imagem. A análise das imagens dos géis por um *software* permite saber o número de *spots* presentes no gel, valores de pI e Mm, e é fundamental para a comparação das diferenças nas expressões das proteínas geradas por múltiplas corridas eletroforéticas. Proteínas podem ser quantificadas e os *spots* em múltiplos géis podem ser combinados e comparados; pode ser feita análise estatística em grupos de *spots* em conjuntos de géis, e variações, diferenças e similaridades podem ser avaliadas (42).

A identificação de diferenças estatisticamente significativas entre os géis de 2D-PAGE exige a corrida e análise de um grande número de géis dentro dos mesmos grupos experimentais, e entre os grupos experimentais que se deseja estudar, porém pode ocorrer diminuição da reprodutibilidade na comparação dos proteomas, relacionado com a variação no preparo de amostras e corridas eletroforética (47).

Neste sentido, uma forma eficaz para eliminar as possíveis variações entre os géis, é a utilização da técnica de eletroforese em gel diferencial (2D-DIGE), a qual permite a análise de até três grupos experimentais em um único gel, sendo um padrão interno comum a todos os géis, e duas amostras referentes a grupos experimentais distintos que são marcadas com diferentes fluoróforos (48). A 2D-DIGE utiliza a marcação das proteínas com corantes fluorescentes conhecidos como *CyDyes* (sendo eles: Cy2, Cy3 e Cy5) antes da IEF (49). Estes corantes possuem um grupo reativo de éster N-hidroxi-succinimidil (NHS) que se liga covalentemente aos

grupos ε-amino dos resíduos de lisina nas proteínas (49). As concentrações de corante são mantidas baixas, para que aproximadamente uma molécula de corante seja adicionado por proteína (50).

Outro método utilizado envolvendo a 2D-DIGE, para o estudo comparativo de proteínas carboniladas é o Oxi-DIGE, que está relacionado diretamente ao estresse oxidativo (51). Nesse caso, não é utilizado o éster de NHS, mas sim corantes derivados de hidrazina (Cy3- e Cy5-Hz) que marcam aldeídos de proteínas que são induzidos oxidativamente nas cadeias laterais de resíduos de lisina ou argininas das proteínas (51,52). Esse método proporciona uma melhora significativa em termos de reprodutibilidade para a análise de proteínas carboniladas, que é essencial para a identificação robusta dessa modificação, e pode ser aplicado para a identificação de proteínas carboniladas em qualquer amostra biológica (53).

No momento os esforços têm sido focados em abordagens alternativas para o estudo proteômico, como a proteômica "livre de gel" (*gel-free*), também conhecido como *shotgun* (estratégia *bottom-up*), onde frações peptídicas complexas que são geradas após a digestão da proteína são reveladas, e as vantagens da abordagem *bottom-up* são a sensibilidade e reprodutibilidade, mesmo quando trata-se de proteomas complexos como os do soro e lisados celulares (50,54).

1.4. Metaloproteoma

1.4.1. Minerais

Embora os minerais constituam uma pequena porção do tecido corporal (4%), são essenciais como componentes estruturais e funcionais em muitos processos vitais (55). No aspecto funcional, podemos destacar seu papel catalisador nos sistemas enzimáticos, por meio da ligação desses íons a substratos, orientando assim a reação e a mediação nas reações de óxido-redução devido a mudanças reversíveis no estado de oxidação do íon metálico (55).

O cobre (Cu) é necessário na respiração celular, formação óssea, função cardíaca, desenvolvimento do tecido conjuntivo, mielinização do sistema nervoso central, queratinização e pigmentação de tecidos (32,56). É componente essencial de diversas metaloenzimas incluindo a citocromo-oxidase, a lisil-oxidase, a superóxido dismutase, a dopamina-ß-hidroxilase e a tirosinase (56).

Aproximadamente 30 a 40% do zinco (Zn) presente no sistema hepático é liberado para o sangue (57). As bases bioquímicas que explicam a deficiência de Zn e a inter-relação com determinadas doenças, tornam evidentes que no caso do diabetes, as alterações metabólicas

observadas nos tipos 1 e 2 poderiam ser agravadas pela deficiência deste mineral, ou por outro lado, esta doença favoreceria os distúrbios metabólicos do Zn (58). O Zn é componente de metaloenzimas e também participa de vias metabólicas que estão envolvidas na síntese proteica, metabolismo de carboidratos, lipídeos, ácidos nucleicos, embriogênese e apoptose (59). Segundo relatos da literatura, as medidas de Zn no soro e plasma em pacientes diabéticos apresentam níveis variados, podendo estar altas, baixas ou não diferentes dos grupos controles (60,61).

O selênio (Se) ocorre nos tecidos em níveis que variam com a espécie, o órgão e a condição do animal, sendo que o fígado é o tecido com maior concentração deste elemento (62). É um micronutriente essencial para a síntese de selenoproteínas que exercem papel importante na síntese, metabolismo e ação dos hormônios tiroidianos; além disso, modifica a expressão de pelo menos 30 selenoproteínas, entre as quais, as famílias das selenoenzimas glutationa-peroxidases, tioredoxina-peroxidases e desiodases tiroidianas (62).

O magnésio (Mg) está distribuído praticamente em três compartimentos: 65% na fase mineral óssea; 34% no espaço intracelular e somente 1% no fluído extra-celular (63). Dados experimentais mostram que o Mg é um cofator importante em muitas reações enzimáticas com ação expressiva no metabolismo glicídico, especialmente aquelas envolvidas em reações de fosforilação (64). É essencial praticamente em todos os sistemas de transdução de energia, na via glicolítica, no metabolismo energético oxidativo, é requerido na biossíntese tanto de ácidos graxos como de proteínas, contração muscular e atividade da ATPase (65). Além disso, o Mg participa de sistema de sinalização intracelular, em reações de fosforilação e desfosforilação que a ativa ou inibe determinadas enzimas (66).

1.4.2. Metaloproteínas

Os íons metálicos ligados às proteínas e metaloproteínas representam uma grande porção do número total de proteínas (67). As metaloproteínas são consideradas diferentes das proteínas ligadas a metais. A primeira caracteriza-se pela alta afinidade da interação metal-proteína, enquanto que na segunda a interação metal-proteína é de baixa afinidade, com isso essa ligação é facilmente quebrada (68). Fracamente ligados às proteínas, estão os íons monovalentes como o sódio e o potássio; de intensidade moderada temos o magnésio e o cálcio; e fortemente ligados estão os metais de transição, tais como o ferro, o cobre, o zinco, o manganês, o molibdênio e o cobalto; o grau de ligação tem relação direta com suas propriedades químicas (densidade, pequeno raio atômico e forças eletrostáticas) é facilitada (69,70).

A investigação das metaloproteínas e/ou proteínas metal ligante presentes no sangue e em tecidos é de fundamental importância, tanto no aspecto estrutural como funcional. A metalômica,

nova área científica proposta recentemente, permitiu a integração de estudos tradicionalmente analíticos com estudos inorgânicos e bioquímicos. O estudo da metalômica nos organismos vivos permite obter informações sobre como o íon metálico está distribuído e coordenado às proteínas, da essencialidade e/ou toxicidade, como também da concentração individual da espécie metálica, contribuindo assim na elucidação dos aspectos fisiológicos e funcionais dessas biomoléculas (67).

Nesse contexto, várias linhas de pesquisa têm surgido na literatura com diferentes termos e abordagens. Pode-se citar, por exemplo, o metaloma – que trata da caracterização do total de espécies metálicas/metalóides presentes nos organismos; a metaloproteômica – que trata da caracterização do total de elementos presentes em um local específico dos organismos (comportamento celular, proteína, metaloproteína); a metalômica – que trata de um estudo mais aprofundado do metaloma. Nesta área buscam-se informações sobre as interações e conexões funcionais de espécies metálicas/metalóides com genes, proteínas, metabólitos e outras biomoléculas do organismo e, portanto, a elucidação do papel biológico exercido pelos íons metálicos ligados às biomoléculas (71).

Os estudos metaloproteômicos são desenvolvidos utilizando: (i) a técnica de separação das proteínas (2D- PAGE); (ii) a técnica analítica para a quantificação dos elementos (espectrometria de absorção atômica - AAS); e (iii) o detector específico para moléculas (espectrometria de massas – MS) l [51-53].

1.4.3. Espectrometria de Absorção Atômica

A AAS é utilizada para determinações quantitativas tanto de elementos metálicos e semimetálicos, quanto de alguns elementos não metálicos. Além disso, pode ser aplicada a uma grande variedade de amostras, tais como, materiais biológicos (tecidos e fluídos), ambientais (águas, solos e sedimentos), alimentares, entre outras (72). Antes das amostras serem submetidas a AAS, o procedimento comumente realizado é a digestão/mineralização úmida da porção orgânica da amostra com ácidos fortes, que visa minimizar interferências na análise de amostras biológicas (como o analito estar presente em diferentes estados de oxidação, combinado com diferentes ânions ou ligados a proteínas ou outros ligantes orgânicos) (72).

A medida da absorção da intensidade da radiação eletromagnética proveniente de uma fonte de radiação primária emitida por átomos gasosos no estado fundamental é o princípio fundamental da AAS (72). Os seis principais constituintes da espectrometria de absorção atômica são: 1) a fonte de radiação; 2) o sistema de introdução de amostras; 3) o sistema de atomização; 4) o

monocromador; 5) o sistema de detecção; e 6) a leitura. Estes componentes são conectados à sistemas computadorizados para o controle do equipamento e tratamento dos dados (73).

Os tipos de atomizadores mais usados em AAS são chama (FAAS) e forno de grafite (GFAAS) (74). A GFASS é utilizada para determinações de baixas concentrações (μg L^{-1}), é uma técnica mais sensível do que a FAAS devido a maior parte do analíto ser introduzido no tubo de grafite e atomizado no caminho óptico, além de condensar a nuvem atômica mais eficientemente por ser um sistema fechado (75,76). Já a FAAS é mais utilizada para análises elementares em níveis de mg L^{-1}, e é um sistema onde a maior parte da amostra é descartada pelo dreno do nebulizador (95%) e a parte da amostra que alcança a chama é diluída pelos gases desta, diminuindo sua sensibilidade (75,76). Outra vantagem na GFAAS quando comparada com a FAAS é a pequena quantidade de amostra utilizada, da ordem de microlitros (μL) na GFAAS, enquanto que a FAAS trabalha com amostra de alguns mililitros (mL) (74).

1.5. Caracterização das proteínas - Estudos proteômicos e metaloproteômicos

MS é um instrumento que contém uma fonte de ionização, um analisador de massas - razão massa/carga (m/z) e um detector (77). Através da técnica de MS podemos avaliar a Mm dos compostos e quantifica-los, identificar compostos desconhecidos, revelar a estrutura de moléculas e determinar as modificações pós-traducionais, ou seja, aplicada à análise de biomoléculas ela fornece uma informação precisa da Mm, identifica proteínas usando a Mm de peptídeos trípticos, sequência peptídeos e identifica modificações pós-traducionais (77,78).

As proteínas a serem analisadas nos estudos proteômicos e/ou metaloproteômicos após serem devidamente extraídas e fracionadas devem passar pelo processo de "digestão" com enzimas proteolíticas, sendo a mais utilizada a enzima tripsina para produzir peptídeos (79). No caso de estudos que utilizam o fracionamento de proteínas por 2D-PAGE, a digestão em gel é aplicada, ou seja a proteína é processada e digerida enquanto está contida em um *spot* de gel de poliacrilamida (80). A tripsina cliva as ligações peptídicas de proteínas após os grupos carboxila dos resíduos de lisina (K) e arginina (R), exceto quando estas ligações são com resíduos de prolina (P), ou seja, ligações K-P ou R-P (81). Os peptídeos obtidos na etapa de digestão tríptica podem então ser caracterizados utilizando-se a MS, entre elas a espectrometria de massas em sequência com ionização por eletrospray (ESI/MS-MS) é uma das mais utilizadas (81).

ESI/MS-MS é uma técnica de ionização branda que realiza a transferência de íons da solução para a fase gasosa, é extremamente útil para a análise de moléculas grandes, não-voláteis e que podem adquirir carga, como proteínas e ácidos nucleicos (82). A produção de íons pelo

processo eletrospray requer, essencialmente, dois passos: dispersão de gotas altamente carregadas, quase à pressão atmosférica, seguida por condições que permitam a evaporação da gota; envolve a formação de um *spray* eletrolítico, que gera pequenas gotas carregadas e destas são liberados os íons (82). Quando um potencial positivo é aplicado na solução, os íons positivos tendem a se afastar para uma região menos positiva, isto é, em direção ao contra eletrodo (82). Conforme a densidade de carga aumenta na gota, o campo elétrico formado entre o capilar e o eletrodo aumenta, provocando uma deformação na gota que está presa na ponta do capilar; a gota ganha forma de cone e permanece presa ao capilar até o momento em que a densidade de carga na superfície da gota e o aumento da repulsão entre os íons vençam a tensão superficial, ocorrendo liberação de pequenas gotas com alta densidade de carga (82). Como resultado final, os íons tornam-se completamente dessolvatados (82).

2. Justificativa

Pelo impacto socioeconômico, o DM1 é reconhecido como problema de saúde pública com reflexos sociais importantes. Além disso, o DM1 causa anormalidades metabólicas, funcionais e estresse oxidativo, determinantes principais do início e da progressão das complicações associadas ao DM1. Tanto as proteínas como minerais são componentes estruturais e funcionais que apresentam fundamental importância para os seres vivos. Nesse contexto, os estudos proteômicos e metaloproteômicos podem ser utilizados para auxiliar na compreensão e esclarecimento dos processos fisiológicos e funcionais envolvidos no DM1, além de permitir a possível identificação de biomarcadores relacionados ao desenvolvimento e progressão do DM1.

3. Hipótese e Objetivo

O DM1 desencadeia alterações metabólicas, funcionais e estresse oxidativo, que leva a modificações no proteoma e metaloproteoma.

O objetivo geral do trabalho foi identificar os possíveis biomarcadores plasmáticos e hepáticos de ratos com diabetes tipo 1 induzido experimentalmente com estreptozotocina, utilizando as ferramentas proteômicas e a metaloproteômicas.

Para atender o objetivo geral proposto o estudo foi dividido em quatro capítulos:

Capítulo I (*Metalloproteomic and differential expression in plasma in a rat model of type 1 diabetes*) – apresenta os dados obtidos do estudo metaloproteômico para as proteínas que

apresentaram diferença de expressão entre os grupos experimentais, utilizamos a 2D-PAGE no processo de fracionamento de proteínas das amostras de plasma, GFAAS e FAAS na determinação quantitativa de Cu, Mg, Se e Zn nos *spots* que apresentaram diferença de expressão, e as proteínas foram caracterizadas por ESI/MS-MS.

Capítulo II (*Insulin dependent and independent changes in liver proteome of diabetes type 1 rat model)* – apresenta os resultados obtidos para o estudo proteômico em amostras de tecido hepático. As proteínas caracterizadas pela técnica de *gel-free* foram classificadas como dependentes ou independentes de insulina (tratamento), em relação aos dados obtidos para abundância e diferença de expressão.

Capítulo III *(Alterations in oxidative damage of proteins in type 1 diabetes using a novel 2D-DIGE method: Oxi-Proteome)* – apresenta os resultados obtidos das proteínas diferencialmente expressas pelo estudo do oxi-proteoma em amostras de tecido hepático, utilizando a 2D-DIGE com os fluoróforos Cy3 e Cy-5-hidrazida no processo de fracionamento e caracterização dos *spots* proteicos por ESI-MS/MS.

Capítulo IV ***(****Using the proteomic approach to identify metalloproteins and proteins that are metal-binding with copper, magnesium, selenium and zinc in spots of liver samples from diabetic rats)* - apresenta os dados obtidos do estudo metaloproteômico em amostras de tecido hepático, 2D-PAGE foi utilizada no processo de fracionamento de proteínas das amostras de plasma, GFAAS e FAAS na determinação quantitativa de Cu, Mg, Se e Zn nos *spots* obtidos no fracionamento das proteína e caracterização das proteínas por ESI/MS-MS.

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Capítulo 1

Metalloproteomic and differential expression in plasma in a rat model of type 1 diabetes

Abstract

In the present study, we sought to develop and validate analytical methods for the application of differential proteomics to identify potential biomarkers of diabetes in rat plasma associated with copper, selenium, zinc, and magnesium fractionation in control and diabetic rats, as well as diabetic rats treated with insulin. The proteome of the plsma was separated using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The copper, selenium, and zinc were determined by graphite furnace atomic absorption spectrometry (GFAAS) and the magnesium, by flame atomic absorption spectrometry (FAAS). The protein spots that were differentially expressed between groups were characterized by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) after tryptic digestion. ESI-MS/MS analysis characterized 35 different proteins, indicating that alpha-1-macroglobulin and haptoglobulin may be potential candidates biomarkers for diabetes treated with insulin, because significant changes were detected in this group indicating a potential side effect of insulin treatment itself. Proteins as 2'-deoxynucleoside 5'-phosphate N-hydrolase 1, transmembrane protein 11, serum amyloid P-component, vitamin D-binding protein and biliverdin reductase may be potential candidates biomarkers for uncontrolled diabetes, these proteins changed in diabetic group and did not maintain at the same profile in diabetic group treated with insulin. This work provides technical and scientific support of the utmost importance for diabetes; the identification of proteins and the understanding of how bonds are altered in type 1 diabetes in plasma revealed new functional aspects that may be involved in diabetes progression.

Keywords: differential proteomic, electrospray ionization-tandem mass spectrometry, flame atomic absorption spectrometry, graphite furnace atomic absorption spectrometry, metalloproteomic, plasma, type 1 diabetes, two-dimensional electrophoresis

1. Introduction

Diabetes mellitus type 1 (DM1) is a chronic autoimmune disease of pancreatic β-cell destruction, resulting in an ongoing state of insulin deficiency (1). The rate of pancreatic β-cell destruction is variable, being rapid in children and adolescents and mainly slow in adults (2). DM1 is one of the most common chronic diseases of childhood (3), occurring between 5 and 7

years of age and also close to puberty (4). Studies suggest that most patients with longstanding DM1 have β-cell regeneration in early childhood, but not in adolescence or adulthood (5).

Plasma is the most varied sample of the proteome present in the body, the protein profiles of plasma vary with physiological and pathological conditions (6). Proteomics has been used to reflect clinically relevant molecules in the blood due to specific diseases (7). The objectives of the use of proteomics in plasma are as follows: elimination of the need for biopsy, diagnosis of specific diseases (using proteins as biomarkers), understanding the pathogenesis of disease, and monitoring the progress of therapeutic interventions. This is due to the urgent need to identify molecular targets (functional proteome) for early diagnosis and effective treatment of disease (8).

The interaction between a causative agent and the organism may modify various biological parameters. Several studies have involved comparing the biomarker protein pattern present in a healthy sample with that of an affected sample because proteins play crucial roles in biological systems (specific activities associated with other biomolecules, where their expression levels are essential for optimal performance of cellular functions) (9). The diagnosis of primary and secondary complications of DM1 using biomarkers will help to prevent secondary complications of diabetes and thus reduce the severity of the disease.

On the other hand, one-third of all proteins possess a bound metal (10). Metalloproteins involve diverse classes of proteins, with intrinsic metal atoms providing different functions (catalytic, regulatory, and structural) (11). Metalloproteomes are investigated by a combination of approaches: annotation of a protein and identification of the intrinsic metal and bioinformatics analysis (11). The Protein Data Bank shows that the most abundant are magnesium (Mg) and zinc (Zn), and frequently observed are calcium (Ca), manganese (Mn), iron (Fe), and nickel (Ni) (10).

DM1 causes metabolic and functional alterations can alter the proteome and metalloproteome, the comparative metalloproteome was not reported in the literature involving the DM1. In this context the present study involves the study of differential protein expression in diabetic rat plasma protein following fractionation by two-dimensional electrophoresis (2D PAGE) and identification by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) followed by Cu, Se, and Zn investigation of these spots by graphite furnace atomic absorption spectrometry (GFAAS) and of Mg by flame atomic absorption spectrometry (FAAS).

2. Material and Methods

2.1. Experimental animals

The experimental procedures were approved by the Ethics Committee on the Use of Animals (CEUA) of the Institute of Biosciences/São Paulo State University (UNESP) - Botucatu, which were in accordance with the ethical principles in animal research provided by the Brazilian College of Animal Experimentation (Protocol: CEUA-436/2012).

The animals used were male Wistar rats (n=24, *Rattus norvegicus*) with an average initial weight of approximately 250 g and age 45 days; these were kept in individual plastic cages under controlled temperature (25 ± 2 ºC) and photoperiod (12:12 h light/dark cycle) conditions. The animals received water and commercial diet (Purina Labina, Campinas-SP) *ad libitum* throughout the experimental period. Water and food intake was controlled daily and weighing of the animals was performed weekly.

The animals were randomly assigned to the following experimental groups (n = 8 per group): C (control) - normal rats that received water and food, DM1 (diabetic rats) - diabetic rats that received water and food and DM1 + I (treated diabetic rats with insulin) - diabetic rats treated with insulin replacement, which received water and food. Experimental DM1 was induced by the intraperitoneal administration of streptozotocin (STZ, single dose, 60 mg kg^{-1} body weight). The STZ was diluted with sodium citrate buffer (0.1 M, pH 4.5) and the animals received 1 mL of the prepared solution. Those animals with glucose concentrations above 220 mg dL^{-1} were considered diabetic and used in the experiment. The DM1 + I group received insulin replacement at an initial dose of 3U/animal (subcutaneously) of Humulin N100UI Neutral Protamine Hagedorn (NPH), Lilly brand. The values of blood glucose were checked daily and the initial insulin dose was adjusted or maintained to obtain normal glucose levels (110 mg dL^{-1}).

At the end of the 30-day experimental period, the animals were anaesthetized (ketamine hydrochloride 10%, 0.1 mL/100 g body weight, i.p.) and sacrificed by decapitation. The blood was collected in the presence of heparin, and the plasma was separated by centrifugation for 15 min at 6,000 xg.

2.2. Biochemical determinations

Blood glucose was determined using the method of Moura (1982) (12), the staining intensity of which is proportional to the glucose concentration in the sample; this was performed prior to the pooling of plasma samples in order to verify that the animals in the DM1 group were

presenting hyperglycemia and that the DM1 animals were able to achieve normoglycemia with insulin replacement, thus ensuring that the animals used in the experiment were reproducing the pathological findings typical of DMI and responding appropriately to insulin therapy. Having made this determination from the individual glucose concentrations of these animals, n= 8 animals were selected for each experimental group in order to pool plasma samples for each group.

The total protein concentrations in rat plasma samples was determined by the Biuret method using bovine serum albumin as standard. Analytical calibration curves were constructed with concentrations of 10 to 100 g L^{-1} from a stock solution of albumin (100 g L^{-1}). The method used 50 mL of sample for standards and 2.5 mL of Biuret reagent, which were mixed and placed in a water bath at 37 °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 separation of protein fractions

Six gels were made for each group (pooled plasma), resulting in a total of 18 gels. To retain the solubilized proteins, aliquots of pooled plasma were diluted in urea solution containing 7 mol L^{-1}; thiourea 2 mol L^{-1}; CHAPS (sulphate 3-[(3-cloroaminopropil)-dimethylammonio]-1-propane) 2% (w/v); ampholytes 0.5% (v/v) at pH ranging from 4 to 7; and 0.002% bromophenol blue (w/v); 2.8 mg of DTT (1,4-Dithiothreitol) was then added to this buffer. Dilution of the plasma pool was made in such a way that the resulting concentration of total proteins in the rehydration solution was the same for all samples: 1.5 mg μL^{-1}.

Prior to the electrophoretic separation process, a mass of approximately 375 µg of rat plasma proteins was applied to strips of 13 cm for isoelectric focusing; these strips contained a precast gel with immobilized ampholytes at pH 4 to 7. These strips were placed in the apparatus and allowed to stand for 12 h at room temperature to allow rehydration of the protein extract. After this period, the rehydrated strip was brought to the isoelectric focusing system (Ettan™ IPGphor™, GE Healthcare Life Sciences) for isoelectric focusing in the first dimension, which was performed for a total of 18,000 Vh.

After separation in the first dimension, the strip proteins, which had been separated according to pI, were equilibrated in two steps. First, we used 10 mL of solution containing urea 6 mol L^{-1}, SDS (Sodium dodecyl sulfate) 2% (w/v), 30% glycerol (v/v), Tris-HCl 50 mmol L^{-1} (pH 8.8), 0.002% bromophenol blue (w/v), and 2% (w/v) DTT, in order to keep the proteins in their reduced forms. The second stage used a solution of similar composition, substituting DTT

for iodoacetamide (IAA) 2.5% (w/v) to obtain alkylation of the protein thiol groups and thus prevent possible reoxidation. Each step lasted for 15 min and was performed under low agitation on the table. In the second step of the electrophoretic (SDS-PAGE) process, the strips were applied to polyacrylamide gels at 10% (w/v), previously prepared between 180 x 160 x 1.5 mm plates. We applied the molecular mass standards (12 to 225 kDa) to the side of the strip, and both were sealed with an agarose solution (0.5% w/v); the run was done in two steps: 7.5 mA/gel at 30 min and 15 mA/gel at 6 h 10 min.

After the running period, the proteins in the gel were fixed for 1 h using a solution containing 10% acetic acid (v/v) and 40% (v/v) ethanol. Proteins were visualized by employing the colloidal Coomassie stain, and the gel was scanned and the image analyzed using image processing ImageMaster Platinum, version 7.0 to obtain the following program parameters: number of spots, percentage of matching between gels, pI, and molecular mass of spots.

2.4. Analysis of differential expression

For the analysis of expression of spots, to see whether they were being over- or understated in plasma samples from normal, diabetic, and insulin-treated diabetic rats, the Platinum ImageMaster software was used. We calculated the mean and the corresponding parameter volume normalized (%V) standard deviation of each spot for this study; a value of 95% was considered relevant for the change in expression.

2.5. Protein spot characterization by ESI-MS/MS

The protein spots were extracted from the gels and prepared for MS according Shevchenko et al. (2006) (13) with some modifications, using the following steps: stain removal, reduction and alkylation of proteins, trypsin digestion, and elution of peptides. Protein identification was obtained with the embedded ion accounting algorithm of the software and searching in *Rattus norvegicus* database (UniProtKB/Swiss-prot at www.uniprot.org). Some parameters are commonly used in ESI-MS/MS table (13), such as the protein access is the identification used in database (UniProtKB/Swiss-prot at www.uniprot.org); the protein score is derived from the ions scores, for a search that contains a small number of queries, the protein score is the sum of the highest ions score for each distinct sequence; the protein pI and Mw experimental are obtained in the Platinum ImageMaster software for each gel run; the protein pI and Mw theoretical are obtained in database for each protein using the protein access (UniProtKB/Swiss-prot at www.uniprot.org); and coverage are used to determine the percent of

the residues in each protein sequence that have been identified. FASTA sequences of the detected proteins were obtained and imported to the Blast2GO program (B2G), which enabled separation into two levels (cellular component and molecular function) (14).

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

The protein spots identified with differential expression were analyzed by GFAAS, and FAAS after mineralizing the samples (spots, feed, and total plasma) as described by Moraes et al. (2013) (15). We used two different electrophoretic runs, and Cu, Mg, Se, and Zn determinations were performed with a Shimadzu AA-6800 atomic absorption spectrometer using wavelengths of 324.7 nm, 285.2 nm, 190.0 nm, and 213.9 nm, respectively. The current used for Cu, Se and Zn determinations was 400 mA and 10 mA for Mg, using curves with concentration ranges 5.00-20.00 µg L^{-1} for Cu and Zn; 0.10-0.40 mg L^{-1} for Mg, and 10.00-60.00 µg L^{-1} for Se. The region of the gel where no protein spots appeared was used for the analytical blank. The limit of quantification (LOQ) to Cu, Mg, Se and Zinc were 0.046 µg L^{-1}, 0.94 µg L^{-1}, 0.083 µg L^{-1} and 0.023 µg L^{-1}, respectively.

2.7. Statistical analyses

For biochemical and nutritional variables, a completely randomized design was used according to analysis of variance (ANOVA). The level of significance for statistical analyses was $\alpha = 0.05$ and F statistics were significant at $p < 0.05$. Tukey's test was used to compare means between groups (16). To determine the statistical significance between the spots, ANOVA was performed using the ImageMaster Platinum, version 7.0 software. The significance level adopted was also $\alpha = 0.05$.

3. Results and Discussion

3.1. Analysis of experimental groups

Animals were chosen to compose the results set on the basis of glycemic analyses of experimental groups, associated with weight loss/gain, and symptoms observed, such as polyuria and polyphagia over the 30 experimental days (Table 1).

After the induction of experimental diabetes, it was noted that the DM1 animals had lower ($p < 0.05$) final body weight compared with the other experimental groups (C and DM1 + I), which did not differ from each other. This indicates a greater weight loss in diabetic untreated

animals, as compared with groups C and DM1 + I. Weight loss, which is commonly observed in DM1, is one of the most common manifestations and may result either from excessive protein degradation or high lipolysis (17). The concentrations of final glucose in untreated diabetic rats (DM1) showed a significant increase when compared with the other groups (C and DM1 + I). The hyperglycemia observed in DM1 may have been due to low peripheral glucose utilization or abnormalities in glucose metabolism, in particular, activation of gluconeogenesis and glycogenolysis (18).

DM1 animals showed symptoms of polyuria and polyphagia. An increase in both feed and water intake was noted for DM1 animals compared with C and DM1 + I animals, which did not differ from each other. The increase in food consumption in DM1 could have been related to the availability of intracellular glucose molecules and also to high glycosuria (19), whereby water intake is high due to brisk diuresis because the glucose concentration in the glomerular filtrate is increased due to hyperglycemia. With insulin replacement in DM1 + I animals, there was an improvement in the above-mentioned parameters, such as increased weight gain and reduced food, water, and glucose consumption; this may be attributed to improved glycemic control.

Table 1. Body weight (g, initial and final), glucose (mg dL^{-1}; initial and final) feed intake (g/day), and water consumption (mL/day) for the different experimental groups.

	C	**DM1**	**DM1+I**
Initial weight (g)	298.56±10.59a	295.47±15.34a	288.52±18.06a
Final weight (g)	371.66±14.68a	234.91±34.86b	341.82±17.93a
Initial glucose (mg dL^{-1})	108.88±10.88a	101.29±19.38a	104.17±4.17a
Final glucose (mg dL^{-1})	110.29±23.73a	427.97±40.34b	126.02±15.03a
Feed intake (g/dia)	25.93±2.01a	44.44±8.55b	27.68±7.12a
Water cosnumption (mL/dia)	33.66±3.62a	161.34±20.88b	51.96±14.51a

All values are expressed as means ± SD. ^{a,b,c}. Means followed by different letters indicate significant differences between the groups ($p < 0.05$).

The results for the concentration of protein by Biuret method, in g L^{-1} were as follows: C= 61.70, DM1 = 55.60, and DM1 + I = 58.50. Based on the results of the total protein present in rat plasma samples, the amount to be added in the hydration steps prior to isoelectric focusing (separation of proteins by isoelectric point) was calculated.

3.2. Electrophoretic runs

The images were compared between pairs of gels by means of the matching process using the ImageMaster Platinum program v.7.0, which identifies the spots that are equivalent between pairs. The result of the image processing procedure yielded a correlation between the pairs of gels presented. Image processing of the electrophoretic runs disclosed, for example, average correlations between gels of 78%, 85%, and 85% for C, DM1, and DM1 + I groups, respectively; this simplified mode means that the protein spots were present in two replications of these gels. This indicates good repeatability and reproducibility of repetitions performed on the same gels.

In the study of gel images, correlations between protein spots of different experimental groups were estimated and compared with the %V, which enabled us to evaluate a possible correlation between protein expression and the set of protein spots analyzed. Since we seek to understand the influence of protein expression in DM1, this strategy can provide information that allows us to discuss and understand them. The correlation between protein spots on gels C and DM1 ($r > 0.78$); C and DM1 + I ($r > 0.87$); and gels DM1 and DM1+I ($r > 0.67$) considering the %V. Correlation analysis allows us to observe that the proteomic profiles of groups C and DM1 + I were closer to each other than those of groups C and DM1. On the basis of this information, it can be inferred that the expression of plasma proteins in group C is most similar to that of group DM1 + I.

It is generally observed that gels have a homogeneous distribution of protein spots according to their respective pIs and molecular masses in the different experimental groups. We also observed that most of the protein spots that make up the sample proteomic map in rat plasma were found to be distributed mainly in the molecular mass range of 31 to 76 kDa, more frequently in the range 5 to 6 pIs.

3.3. Differences in protein expression and quantitative analysis of Cu, Mg, Se, and Zn

A comparison between C and DM1 showed 149 coincident spots and 14 spots with differential expression, while C and DM1 + I showed 175 coincident spots and 11 spots with differential expression, and DM1 and DM1 + I showed 143 coincident spots and 20 spots with differential expression, according to the analysis performed by ImageMaster Platinum v.7.0.

In Figure 1 and Table 2, data regarding the characterization obtained in the analysis of proteins identified by ESI-MS/MS are shown. Of the 45 spots analyzed by ESI-MS/MS, three were not identified as proteins, which may be related to a fault in the tryptic - or even the low-

protein concentration - digestion step not generating peptides that were possible to sequence; 35 different proteins were identified among the 45 protein spots analyzed.

Of these 35 proteins, their associated sequences were compared with those of cellular component and molecular function-related sequences described in Figures 2, *A* and *B*. These sequences were most associated with the cellular components cell (103) and extracellular regions (19) and organelles (16), and with the molecular functions of binding (25) and catalytic activity (8).

The following concentrations in feed analysis: Se: < limit of quantification (LOQ), Cu: 2.36 mg kg^{-1}, Mg: 28.90 mg kg^{-1}, and Zn: 51.40 mg kg^{-1}, with major sources of Mg and Zn. In total plasma, the concentrations of Cu: 84.06 mg kg^{-1}, Mg: 2,141.10 mg kg^{-1}, Se: 157.33 mg kg^{-1}, and Zn: 2,340.73 mg kg^{-1} in C; Cu: 59.40 mg kg^{-1}, Mg: 2,122.20 mg kg^{-1}, Se: 250.48 mg kg^{-1}, and Zn: 2,377.02 mg kg^{-1} in DM1; and Cu: 82.74 mg kg^{-1}, Mg: 2,115.90 mg kg^{-1}, Se: 146.98 mg kg^{-1}, and Zn: 4,129.65 mg kg^{-1} in DM1 + I. High plasma Se was found in our study, as observed in other studies that associated high serum Se concentration with an increased prevalence of diabetes (20,21). In Table 3 is the quantitative determination of Cu, Mg, Se and Zn in the protein spots with difference expression between the experimental groups.

Figure 1. Proteins differentially expressed ($p < 0.05$) between the different experimental groups, considering the %V. It was selected an image to illustrate the gel. Spots 1-14: proteins differentially expressed between C and DM1. Spots 15-25: proteins differentially expressed between C and DM1+I. Spots 25-45: proteins differentially expressed between DM1 and DM1+I.

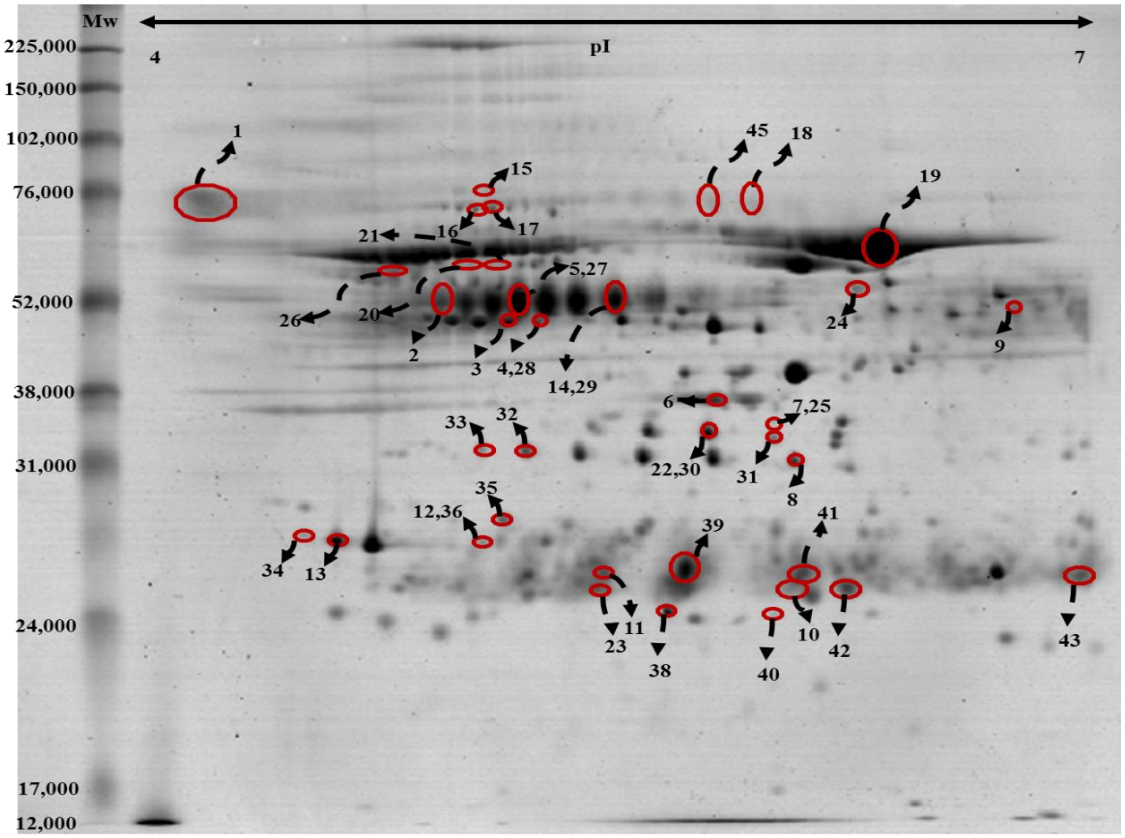


Table 2. Characterisation of protein spots that showed differential expression ($p < 0.05$) by ESI-MS/MS. Positive and negative values indicate up or down regulation of proteins in relation to the spot analysed.

Spot ID	Protein	Access	Score	pI/Mw experimental	pI/Mw theoretical	Coverage (%)	p<0.05	Variation in expression relative to C
1 (-C/ +DM1)	Histidine-rich glycoprotein	HRG_RAT	507	5.08/ 63,445	7.76/ 59,049	8		
	Plasma protease C1 inhibitor	IC1_RAT	460		5.53/ 55,611	14.68	0.035	-70.691
2 (+C/ –DM1)	Small kinetochore-associated protein	SKAP_RAT	65	4.84/ 48,794	5.33/ 34,885	6.09	0.035	+146.644
3 (+C/ – DM1)	Alpha-1-antiproteinase	A1AT_RAT	1882	5.11/ 48,093	5.70/ 46,136	7.79	0.038	+104.754
4 (+C/ – DM1)	Alpha-1-antiproteinase	A1AT_RAT	1078	5.20/ 48,100	5.70/ 46,136	12.41	0.007	+166.346
5 (-C/ + DM1)	Transmembrane protein 11, mitochondrial	TMM11_RAT	124	5.21/ 52,609	6.98/ 21,342	3.68	0.014	+266.918
	2'-deoxynucleoside 5'-phosphate N-hydrolase 1	DNPH1_RAT	102		4.95/ 17,781	9.82		
6 (-C/ + DM1)	Serum albumin	ALBU_RAT	740	5.89/ 35,808	6.09/ 68,731	8.72	0.016	+226.866
7 (+C/ – DM1)	Alpha-1-antiproteinase	A1AT_RAT	94	5.97/ 32,346	5.70/ 46,136	3.67	0.029	+77.333
8 (-C/ + DM1)	Haptoglobin	HPT_RAT	474	6.03/ 30,236	6.10/ 38,563	8.07	0.019	-67.743
9 (-C/ + DM1)	Fibrinogen beta chain	FIBB_RAT	751	6.72/ 48,687	7.89/ 54,235	16.28	0.013	-67.510

	Ig gamma-1 chain C region	IGHG1_RAT	184		6.43/ 35,946	4.91		
	Taste receptor type 2 member 114	TR114_RAT	54		9.27/ 35.604	3.24		
10 (-C/ + DM1)	Galanin peptides	GALA_RAT	112		5.76/ 13,328	18.55		
	Protein phosphatase 1 regulatory subunit 11	PP1RB_RAT	84	6.05/ 20,170	6.21/ 13,936	24.41	0.040	-97.987
11 (-C/ + DM1)	Ig kappa chain C region, A allele	KACA_RAT	257	5.38/ 21,296	4.99/ 11,732	28.3	0.048	-97.199
12 (+C/ – DM1)	Serum amyloid P-component	SAMP_RAT	593	4.92/ 23,098	5.50/ 26,176	16.23	0.047	+77.574
13 (-C/ + DM1)	Neutrophil cytosol factor 2	NCF2_RAT	13	4.56/ 23,453	5.67/ 59,573	2.66	0.035	-75.479
14 (-C/ + DM1)	Vitamin D-binding protein	VTDB_RAT	535	5.48/ 51.,551	5.65/ 53,544	2.94	0.049	-273.580
	Biliverdin reductase A	BIEA_RAT	135		33,566	13.56		

Spot ID	**Protein**	**Acess**	**Score**	**pI/Mw experimental**	**pI/Mw theoretical**	**Coverage (%)**	**p<0.05**	**Variation in expression relative to C**
15 (+C/ – DM1+I)	Afamin	AFAM_RAT	145	5.11/ 72,357	5.87/ 69,335	12.5	0.026	+68.139
16 (-C/ + DM1+I)	Serotransferrin	TRFE_RAT	154	4.99/ 68,771	7.14/ 76,395	2.58	0.011	-74.965
17 (-C/ + DM1+I)	Serotransferrin	TRFE_RAT	378	5.05/ 68,468	7.14/ 76,395	10.89	0.015	-80.478

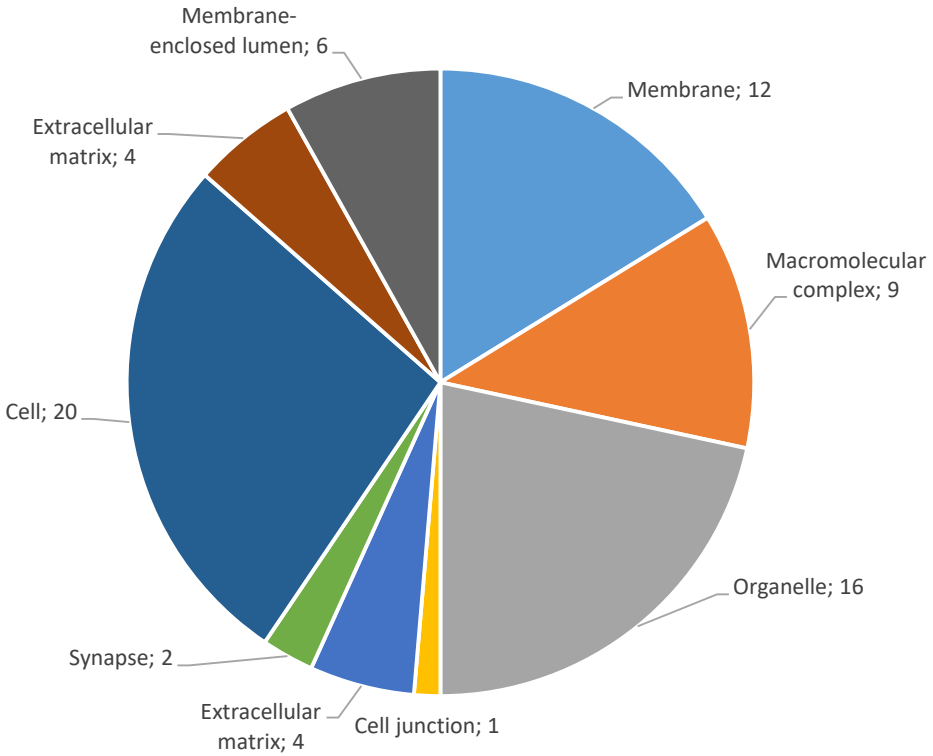
18 (+C/ -DM1+I)	Hemopexin	HEMO_RAT	1078	5.20/ 48,100	7.58/ 51,351	14.78	0.001	+96.869
19 (+C/ -DI)	Serum albumin	ALBU_RAT	245	6.30/ 59,343	6.09/ 68,731	8.72	0.005	+148.522
20 (-C/ + DM1+I)	Serine protease inhibitor A3L	SPA3L_RAT	252	4.95/56,047	5.48/ 46,277	7.26	0.018	-83.390
	Serine protease inhibitor 2.1	SPI21_RAT	251		6.83/ 24,218	11.21		
21 (-C/ + DM1+I)	Alpha-1-macroglobulin	A1M_RAT	574	5.05/ 56,000	6.46/ 167,125	5.8	0.024	-107.114
22 (-C/ + DM1+I)	Etoposide-induced protein 2.4 homolog	EI24_RAT	94	5.21/ 20,121	9.75/ 38,893	4.71	0.016	-66.531
	F-box/SPRY domain-containing protein 1	FBSP1_RAT	74		8.41/ 25,759	10.43		
23 (-C/ + DM1+I)	Fuctinin-2	GALA_RAT	143	5.38/ 17,721	4.30/ 2,489	54.55	0.014	-81.532
24 (+C/ -DM1+I)	Serum albumin	ALBU_RAT	124	6.24/ 51,484	6.09/ 68,731	4.44	0.003	+98.274
25 (-C/ + DM1+I)	Alpha-1-antiproteinase	A1AT_RAT	94	5.98/ 31,395	5.70/ 46,136	3.67	0.016	-67.629
Spot ID	**Protein**	**Access**	**Score**	**pI/Mw experimental**	**pI/Mw theoretical**	**Coverage (%)**	**p<0.05**	**Variation in expression relative to DM1**
26 (-DM1/ + DM1+I)	Serine protease inhibitor A3L	SPA3L_RAT	523	4.72/ 56,371	5.48/ 46,277	5.81	0.013	-518.280

27 (+DM1/ – DM1+I)	Transmembrane protein 11, mitochondrial	TMM11_RAT	124	5.21/ 52,609	6.98/ 21,342	3.68	0.010	+1681.505
	2'-deoxynucleoside 5'-phosphate N-hydrolase 1	DNPH1_RAT	102		4.95/ 17,781	9.82		
28 (+DM1/ – DM1+I)	Probable N-acetyltransferase CML5	CMLO5_RAT	108	5.22/ 48.304	8.99/ 25.167	15.11	0.016	-179.709
29 (+DM1/ – DM1+I)	Vitamin D-binding protein	VTDB_RAT	535	5.49/ 51.247	5.65/ 53,544	2.94	0.046	+328.497
	Biliverdin reductase A	BIEA_RAT	135		33,566	13.56		
30 (-DM1/ +DM1+I)	Etoposide-induced protein 2.4 homolog	EI24_RAT	94	6.24/ 51.484	9.75/ 38,893	4.71	0.011	-88.058
	F-box/SPRY domain-containing protein 1	FBSP1_RAT	74		8.41/ 25,759	10.43		
31 (-DM1/ + DM1+I)	Alpha-1-macroglobulin	A1M_RAT	81	5.99/ 32,532	6.46/ 167,125	0.6	0.003	-117.212
32 (-DM1/ + DM1+I)	Haptoglobin	HPT_RAT	281	5.18/ 31,546	6.10/ 38,563	13.26	0.001	-82.892
33 (-DM1/ + DM1+I)	Haptoglobin	HPT_RAT	67	5.18/ 31,546	6.10/ 38,563	3.46	0.015	-80.308
34 (-DM1/ + DM1+I)	C-reactive protein	CRP_RAT	76	4.41/ 26,661	4.89 /25,468	5.22	0.038	-123.931
35 (-DM1/ + DM1+I)	Not identified			5.11/ 24,969			0.024	-64.181

36 (-DM1/ + DM1+I)	Serum amyloid P-component	SAMP_RAT	124	4.92/ 23,098	5.50/ 26,176	16.23	0.047	-107.984	
37 (-DM1/ + DM1+I)	Not identified			5.41/ 18,385			0.013	-74.797	
38 (-DM1/ + DM1+I)	Src kinase-associated phosphoprotein 1	SKAP1_RAT	39	5.64/ 16,826	4.35/ 40,903	4.8	0.045	-141.844	
	Synaptogyrin-2	SNG2_RAT	28		4.61/ 24,711	8.04			
39 (+DM1/ - DM1+I)	Ig lambda-2 chain C region	LAC2_RAT	759	5.70/ 20,029	5.76/ 11,318	14.42	0.041	+79.695	
40 (-DM1/ + DM1+I)	Apolipoprotein A-I	APOA1_RAT	64	5.99/ 16,766	5.52 /30,062	3.47	0.049	-88.126	
41 (+DM1/ - DM1+I)	Ig kappa chain C region, A allele	KACA_RAT	363	6.07/ 19,496	4.99/ 11,732	28.3	0.014	+118.837	
42 (+DM1/ - DM1+I)	cAMP-regulated phosphoprotein 19	ARP19_RAT	88	6.22/ 18,449	9.07/ 12,293	38.39	0.011	+82.475	
	Proline-rich nuclear receptor coactivator 2	PNRC2_RAT	87		10.19/ 14.881	6.72			
43 (+DM1/ - DM1+I)	Not identified			6.99/ 19.839			0.014	+146.888	
44 (-DM1/ + DM1+I)	Gamma-glutamylaminecyclotransferase	GGACT_RAT	48	5.78/ 73,265	6.03/ 16,925	16.78	0.013	-518.280	
45 (+DM1/ - DM1+I)	Alpha-1-antiproteinase	A1AT_RAT	2143	5.78/ 49,014	5.70/ 46,136	14.11	0.001	+171.363	

Figure 2. Classification of sequences of proteins differentially expressed ($p < 0.05$). A. Cellular component. B. Molecular function

A.



B.

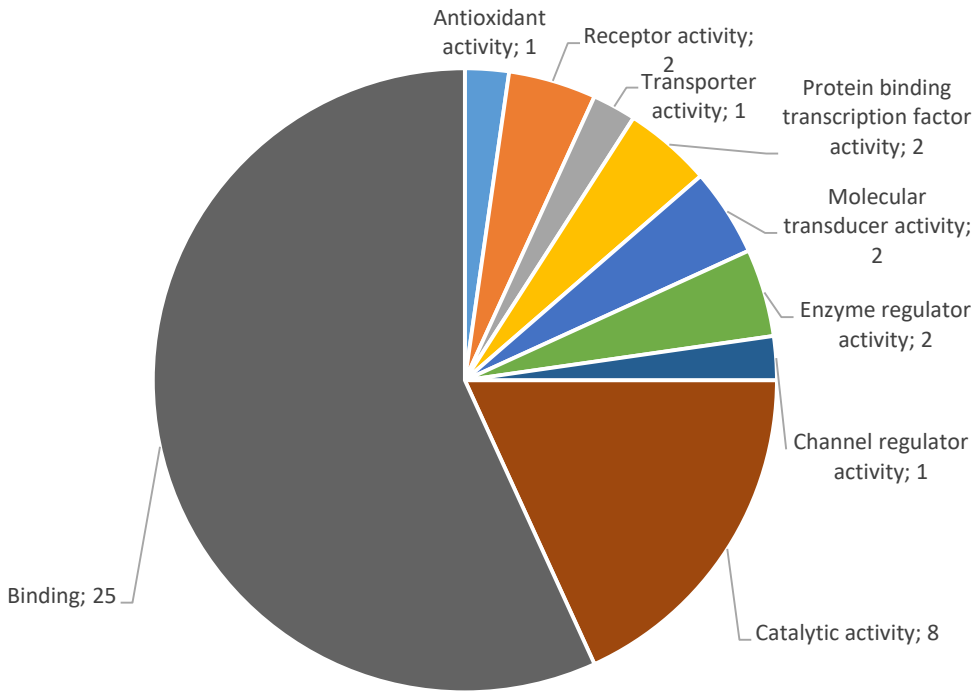


Table 3. Determination of Cu, Mg, Se and Zn in the different experimental groups.

C	DM1	DM1+I

Spot Id	Cu	Mg	Se	Zn	Spot Id	Cu	Mg	Se	Zn	Spot Id	Cu	Mg	Se	Zn
	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}		µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}		µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}
1	<LOQ	<LOQ	<LOQ	<LOQ	1	<LOQ	<LOQ	<LOQ	<LOQ	1	<LOQ	97	41	<LOQ
2	23	<LOQ	0.42	94	2	<LOQ	<LOQ	<LOQ	<LOQ	2	<LOQ	152	0.67	<LOQ
3	17	<LOQ	23	12	3	<LOQ	<LOQ	<LOQ	<LOQ	3	<LOQ	260	11	<LOQ
4	22	<LOQ	0.96	<LOQ	4	<LOQ	<LOQ	<LOQ	<LOQ	4	<LOQ	237	17	<LOQ
5	0.74	<LOQ	13	<LOQ	6	<LOQ	<LOQ	<LOQ	<LOQ	5	<LOQ	82	0.56	<LOQ
6	<LOQ	<LOQ	<LOQ	<LOQ	5	<LOQ	<LOQ	<LOQ	<LOQ	6	<LOQ	127	41	569
7	<LOQ	<LOQ	0.65	<LOQ	7	63	<LOQ	<LOQ	<LOQ	7	<LOQ	245	15	524
8	<LOQ	<LOQ	20	<LOQ	8	46	<LOQ	56	<LOQ	8	<LOQ	225	0.79	578
9	<LOQ	<LOQ	16	<LOQ	9	<LOQ	<LOQ	<LOQ	<LOQ	9	<LOQ	<LOQ	<LOQ	<LOQ
10	<LOQ	<LOQ	<LOQ	<LOQ	10	31	<LOQ	10	<LOQ	10	<LOQ	<LOQ	<LOQ	14
11	0.97	<LOQ	17	<LOQ	11	<LOQ	<LOQ	<LOQ	<LOQ	11	<LOQ	67	<LOQ	<LOQ
12	<LOQ	<LOQ	<LOQ	<LOQ	12	<LOQ	<LOQ	<LOQ	<LOQ	12	<LOQ	<LOQ	<LOQ	<LOQ
13	32	<LOQ	22	<LOQ	13	0.67	<LOQ	54	<LOQ	13	<LOQ	212	11	674
14	<LOQ	<LOQ	<LOQ	<LOQ	14	<LOQ	<LOQ	<LOQ	<LOQ	14	<LOQ	<LOQ	<LOQ	<LOQ
15	0.87	<LOQ	0.90	<LOQ	15	<LOQ	<LOQ	0.65	105	15	<LOQ	220	0.90	<LOQ
16	0.37	<LOQ	12	<LOQ	16	27	<LOQ	0.65	<LOQ	16	<LOQ	102	36	<LOQ
17	0.95	<LOQ	0.87	<LOQ	17	<LOQ	<LOQ	<LOQ	<LOQ	17	<LOQ	<LOQ	<LOQ	<LOQ
18	<LOQ	<LOQ	0.20	<LOQ	18	<LOQ	<LOQ	<LOQ	95	18	<LOQ	0.5	33	723
19	<LOQ	<LOQ	<LOQ	<LOQ	19	<LOQ	<LOQ	<LOQ	<LOQ	19	<LOQ	<LOQ	<LOQ	<LOQ
20	0.46	<LOQ	0.96	<LOQ	20	<LOQ	<LOQ	<LOQ	<LOQ	20	<LOQ	<LOQ	<LOQ	<LOQ
21	<LOQ	<LOQ	<LOQ	<LOQ	21	<LOQ	<LOQ	<LOQ	<LOQ	21	<LOQ	<LOQ	<LOQ	<LOQ
22	<LOQ	<LOQ	<LOQ	<LOQ	22	41	185	<LOQ	<LOQ	22	<LOQ	397	0.11	0.07
23	<LOQ	<LOQ	<LOQ	<LOQ	23	<LOQ	<LOQ	<LOQ	<LOQ	23	<LOQ	<LOQ	<LOQ	<LOQ
24	<LOQ	<LOQ	13	<LOQ	24	<LOQ	<LOQ	<LOQ	<LOQ	24	<LOQ	412	11	<LOQ
25	<LOQ	<LOQ	<LOQ	<LOQ	25			1	107	25	<LOQ	<LOQ	<LOQ	<LOQ

26	<LOQ	<LOQ	<LOQ	<LOQ	26	<LOQ	<LOQ	<LOQ	<LOQ	26	<LOQ	182	34	<LOQ
27	0.20	<LOQ	0.99	<LOQ	27	34	70	<LOQ	<LOQ	27	<LOQ	<LOQ	<LOQ	<LOQ
28	<LOQ	<LOQ	<LOQ	<LOQ	28	<LOQ	32	<LOQ	<LOQ	28	<LOQ	<LOQ	<LOQ	<LOQ
29	0.73	<LOQ	32	<LOQ	29	<LOQ	<LOQ	<LOQ	<LOQ	29	<LOQ	<LOQ	<LOQ	<LOQ
30	<LOQ	<LOQ	20	23	30	125	<LOQ	65	<LOQ	30	<LOQ	<LOQ	<LOQ	<LOQ
31	<LOQ	<LOQ	<LOQ	<LOQ	31	<LOQ	<LOQ	<LOQ	<LOQ	31	<LOQ	<LOQ	<LOQ	<LOQ
32	37	<LOQ	15	<LOQ	32	0.58	<LOQ	42	<LOQ	32	<LOQ	112	28	58
33	<LOQ	<LOQ	29	<LOQ	33	0.85	<LOQ	46	<LOQ	33	<LOQ	275	34	546
34	<LOQ	<LOQ	<LOQ	<LOQ	34	<LOQ	<LOQ	<LOQ	<LOQ	34	<LOQ	<LOQ	<LOQ	<LOQ
35	<LOQ	<LOQ	<LOQ	<LOQ	35	0.087	<LOQ	59	<LOQ	35	<LOQ	<LOQ	<LOQ	<LOQ
36	<LOQ	<LOQ	<LOQ	<LOQ	36	<LOQ	<LOQ	<LOQ	<LOQ	36	<LOQ	<LOQ	<LOQ	<LOQ
37	37	<LOQ	10	<LOQ	37	<LOQ	<LOQ	<LOQ	<LOQ	37	<LOQ	<LOQ	<LOQ	<LOQ
38	<LOQ	<LOQ	0.87	<LOQ	38	<LOQ	<LOQ	11	<LOQ	38	<LOQ	<LOQ	11	<LOQ
39	<LOQ	<LOQ	<LOQ	<LOQ	39	24	<LOQ	<LOQ	<LOQ	39	<LOQ	<LOQ	0.28	<LOQ
40	<LOQ	<LOQ	0.60	<LOQ	40	20	112	<LOQ	<LOQ	40	<LOQ	<LOQ	0.78	
41	<LOQ	<LOQ	22	<LOQ	41	<LOQ	<LOQ	<LOQ	<LOQ	41	<LOQ	<LOQ	<LOQ	<LOQ
42	12	1	16	<LOQ	42	26	<LOQ	<LOQ	<LOQ	42	<LOQ	<LOQ	<LOQ	<LOQ
43	<LOQ	<LOQ	<LOQ	<LOQ	43	<LOQ	<LOQ	<LOQ	<LOQ	43	<LOQ	<LOQ	<LOQ	<LOQ
44	<LOQ	<LOQ	27	<LOQ	44	0.20	<LOQ	<LOQ	<LOQ	44	<LOQ	<LOQ	<LOQ	<LOQ
45	<LOQ	<LOQ	<LOQ	<LOQ	45	<LOQ	<LOQ	1	<LOQ	45	<LOQ	<LOQ	<LOQ	<LOQ

In spot 1 (-C/ +DM1), two proteins: histidine-rich glycoprotein and plasma protease C1 inhibitor. Histidine-rich glycoprotein is involved in the transport of glucose and negatively regulates fibrinogens and signaling of vascular endothelial growth factor (22). Plasma protease C1 inhibitor is involved in the innate immune response, the regulation of proteolysis, and the negative regulation of complement activation. With regard to lectin and endopeptidase activity, these were upregulated in DM1. In this study, Mg and Se in the plasma of DM1 animals. Histidine-rich glycoproteins bind with divalent metals (23) and the literature reports binding with Cu, Zn, Ni, and mercury (Hg), for example; this can explain why found Mg and Se.

Small kinetochore-associated protein (+C/ -DM1) is related to cell division as an essential component of the mitotic spindle that is required for chromosome segregation and anaphase progression; this promotes the metaphase to anaphase transition and is necessary for the alignment of chromosomes and the normal segregation of sister chromatids (24). There are no reports in the literature that this protein has a specific metal-binding property, but in our work, was found Cu, Se, and Zn in C and Mg and Se in DM1. The presence of a cysteine in the amino-acid composition can explain the binding of these metals.

Alpha-1-antiproteinase was found in spots 3, 4, 7 (+C/ –DM1), 25 (-C/ +DM1 + I), and 45 (+DM1/ –DM1 + I). Alpha-1-antiproteinase is an inhibitor of serine proteases; its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin, exerts responses to hypoxia, triglycerides, and lipopolysaccharide, stimulates estradiol, cytosine, and peptide hormone, downregulates endopeptidase activity, and is involved in the regulation of proteolysis (25). Alpha-1-antiproteinase protects the connective tissue from inflammatory enzymes and helps to prevent blood clotting, which can be related to why this protein was more highly expressed in group C than in group DM1. Levels of the serum acute-phase protein groups were shown to correlate positively with blood pressure in humans (26); this is one of the most abundant proteins in the urine of patients with proteinuria, and has been suggested as a marker of glomerulopathy. The somewhat increased expression of this protein in DM1 compared with DM1 + I may be due to some secondary complication of the disease. In C, it was shown to bind to Cu, Se, and Zn, while in DM1 + I, it bound to Mg and Se. We did not find any reports in the literature, which may suggest that this is a newly discovered interaction.

In spots 5 (-C/ +DM1) and 27 (+DM1/ -DM1+I), was identified protein 2'-deoxynucleoside 5'-phosphate N-hydrolase 1 and transmembrane protein 11. The protein 2'-deoxynucleoside 5'-phosphate N-hydrolase 1 is involved in the metabolic processes for positive regulation of nucleotide synthesis, cell proliferation, and cell growth; this enzyme catalyzes the

cleavage of N-glycosidic deoxyribonucleoside-5'-monophosphate to produce deoxyribose 5-phosphate and a purine or pyrimidine base can be upregulated in response to c-myc and by partial hepatectomy (27). This protein presents no reports related to diabetes, but it is known to be mainly upregulated in response to c-myc; this gene encodes proteins that regulate gene transcription and thus may influence the cell response (28). *In vitro* studies of mesangial cells by Wolf et al. (1992) (29) showed that c-myc is expressed when cells are exposed to high glucose concentrations, which may explain to some extent why this protein is upregulated in diabetic animals. Mitochondrial transmembrane protein 11 is related to negative regulation of cell growth by gluconeogenesis and glycolysis (30), which explains its upregulation in animals from group DM1. There are no reports in the literature on metal binding to this protein; in our study, was binding of Cu and Se in C, and Cu, Mg, and Zn in DM1 + I.

Haptoglobin (spot 8: -C/ +DM1; spot 32 and 33: -DM1/ +DM1 + I) captures and combines with free plasma hemoglobin to allow hepatic recycling of heme iron to prevent kidney damage, acts as an antioxidant, and plays a modulatory role in various aspects of the acute-phase response (31). This protein was shown to bind with Cu (DM1 and C), Se (C, DM1 and DM1 + I), Mg (DM1), and Zn (DM1 + I).

Fibrinogen beta chain (-C/ +DM1) acts on translational signaling protein polymerization, tissue regeneration, and cellular responses to the stimulation of leptin and interleukin-1, because they are associated with the inflammatory response and the risk of secondary complications, such as the risk of coronary heart disease observed in DM1 (32). Also in spot 9 (-C/ +DM1) was identified Ig gamma-1 chain C region, taste receptor type 2 member 114, and the presence of Se in C.

Galanin peptides (-C/ +DM1) are involved in the regulation of inflammatory immune responses, which are related to carbohydrate metabolism and the regulation of glucocorticoid metabolism and inhibit glucose-induced insulin release (33). Protein phosphatase 1 regulatory subunit 11 (C/DM1) negatively regulates catalytic activity (34). In spot 10, Cu and Se was found in DM1, and Zn in DM1 + I. There are no reports about these proteins binding to Cu, Se, and Zn.

Serum amyloid P-component (spot 12: +C/ -DM1; spot 36: -DM1/ +DM1 + I) binds 2 calcium ions per subunit. A precursor of amyloid component P, which is found in the basal and associated with amyloid deposits in the membrane, it also acts as a complex-forming protein (35), and in this case was downregulated in animals from DM1. In our study, there was no presence of Cu, Mg, Se, or Zn associated with this protein in spots 12 and 36.

Neutrophil cytosol factor 2 (-C/ +DM1) was upregulated in DM1; this may be because its function is related to response to glucose stimulus, catabolic processes involved in the NADP-dependent generation of superoxide anions and cellular defense responses (36). Neutrophil cytosol factor 2 showed the presence of Cu and Se in C and DM1, and Mg, Se, and Zn in DM1 + I.

Vitamin D-binding protein (Spot 14: -C/ +DM1; spot 29: +DM1/ - DM1 + I) in plasma carries the vitamin D sterols and prevents polymerization of actin monomers, connecting their present response to tumor necrosis factor, vitamin D metabolic processes, and cellular death factor (37). This is related to cardiovascular diseases arising from diabetes, and this mechanism was found to be upregulated in DM1. Biliverdin reductase A (Spot 14: -C/ +DM1; spot 29: +DM1/ - DM1 + I) is involved in metabolic processes of small molecules, heme, and catabolic processes in redox processes (with concomitant oxidation of the NADH or NADPH cofactor) (38). A previous study has shown that biliverdin reductase suppresses insulin signaling and acts as a negative regulator of glucose uptake (38), in a way explaining its upregulation in DM1 animals; it also binds to the p85 regulatory subunit of PI3K/Akt (39), a mechanism that may also lead to an improvement in insulin sensitivity (40). However, these animals may have increased the expression of this protein, trying to supply insulin or reset the mechanism responsible for insulin deficiency in the pathological state. In addition, there was no association with the presence of Cu, Mg, Se, or Zn in this spot.

Afamin (+C/ -DM1) is a protein linked to vitamin E that can be carried in body fluids under conditions where the system does not contain sufficient lipoproteins. Afamin has already been reported to be an ovarian cancer marker (41), but its relationship to diabetes has not been previously reported. In this study, it was shown to be downregulated, which may have resulted in increased oxidative stress in diabetes due to the overproduction of reactive oxygen species (ROS) and decreased efficiency of antioxidant defenses. Moreover, the carrying capacity of vitamin E, as a non-enzymatic antioxidant system in diabetes, may be reduced due to a decreased ability to remove ROS, thus explaining the downregulation of afamin in DM1. The presence of Cu and Se in C, Se and Zn in DM1, and Mg and Se in DM1 + I were observed.

Alpha-1-macroglobulin (spot 21**:** -C/ +DM1 + I; spot 31**:** -DM1/ +DM1 + I) is able to inhibit all four classes of proteases by a single mechanism. This protein has a length of peptide that contains specific cleavage sites for different proteases: proteinase cleaves a certain region, and a conformational change in the protein induces proteinase to retain the encapsulated enzyme, which remains active against low-molecular-weight substrates (activity against substrates of high

molecular weight is very low) (42). Then, one thioester bond in the cleavage region is hydrolyzed and mediates binding of the protein to a covalent proteinase, causing further downregulation of endopeptidase activity (42). The DM1 + I group showed increased expression of this protein, which may be explained by insulin treatment in these animals having reduced the effects of endotoxin-induced shock, but also being able to modulate the immunological response in plasma, somewhat controlling the secondary complications of DM1. In addition, our study suggests that α1 macroglobulin could play a local anti-inflammatory role (43). The presence of Cu, Mg, Se, and Zn was not observed in these two spots (21 and 31).

C-reactive protein (-DM1/ +DM1 + I) is a metalloprotein (binds 2 calcium ions per subunit) that has several functions associated with host defense: it promotes agglutination, phagocytosis and complement fixation through calcium-dependent binding to phosphorylcholine, can interact with DNA and histones and clean nuclear material released from damaged circulating cells, and presents negative regulation for the storage of lipids (44). C-reactive protein is a marker of inflammation; in patients with diabetes, low-grade inflammation is evidenced by increased plasma levels (44). However, in our study, animals in the DM1 + I group showed upregulation of this protein, which can also be associated with a decreased risk of cardiovascular disease in diabetes. The presence of Cu, Mg, Se, and Zn was not observed in this protein.

Apolipoprotein A-I (-DM/ +DM1 + I) is part of the reverse transport of cholesterol from the tissues to the liver for excretion, by promoting cholesterol efflux from tissues and acting as a cofactor for lecithin cholesterol acyltransferase. Some studies have reported lower levels of apolipoprotein A-I in the presence of coronary complications (45). The hyperglycemia observed in type 1 diabetes is closely related to lipidemic disorders, such as increased LDL cholesterol and triglycerides and reduced HDL cholesterol, which increase the risk of ischemic heart disease. Thus, the reduction of hyperglycemia that was observed in the animals of group DM1 + I has been found to be beneficial as a determining factor for decreased oxidative stress and reduced diabetic atherogenesis; in turn, this protein was upregulated in these animals (45). In this protein, was found the presence of Cu in C and DM1 + I, and Cu and Mg in DM1.

3. Conclusion

Thirty-five proteins showed differential expression in plasma, indicating that alpha-1-macroglobulin and haptoglobulin may be potential candidates biomarkers for diabetes controlled

(treated with insulin), because significant changes were detected in this group indicating a potential side effect of insulin treatment itself. Proteins as 2'-deoxynucleoside 5'-phosphate N-hydrolase 1, transmembrane protein 11, serum amyloid P-component, vitamin D-binding protein and biliverdin reductase may be potential candidates biomarkers for uncontrolled diabetes, these proteins changed in diabetic group and did not maintain at the same profile in diabetic group treated with insulin. The proteins showed different interactions with Cu, Mg, Se and Zn, suggesting how bonds are altered in type 1 diabetes in plasma revealing new functional aspects that may be involved in diabetes progression and protein expression.

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Capítulo 11

Insulin dependent and independent changes in liver proteome of diabetes type 1 rat model

Abstract

Type 1 diabetes is a major public health problem that continues to burden the healthcare system, costing more and more as the epidemic grows. New strategies and actions that aid in earlier diagnosis of type 1 diabetes to prevent secondary complications are essential in alleviating this burden. Identification of differentially expressed hepatic proteins, with and without insulin treatment in type 1 diabetes rat model generated potential biomarkers of disease progression and response to insulin treatment. Proteomic data were processed to assess protein alterations specific to each condition, 305 proteins were identified that had significantly altered abundance (p <0.05) between control, type 1 diabetes, and type 1 diabetes treated with insulin. Out of these proteins, 147 were classified as insulin dependent and 152 as insulin independent. Affected proteins were mapped using Cytoscape with FDR $\leq$ 0.05 to reveal protein interactions. Bioinformatics analysis of their metabolic function revealed 9 pathways alterations present in insulin dependent group and 30 in insulin independent group. Analysis of control, diabetic and insulin treated diabetic groups in rat model revealed the abundance changes of various enzymes that function in key metabolic pathways and stress responses. Gluconeogenesis appeared to be idled to the normal levels in diabetic group by insulin treatment with the restoration of control group levels of the Fbp1 enzyme. However, Pc participating in the initial steps of gluconeogenesis was insulin independent, may be related to oxalate that enters and maintains intermediates of the TCA cycle. The majority of the enzymes of the TCA cycle displayed insulin dependency, this may be explained as an anapleurotic response to maintain intermediates of the TCA cycle in response to insulin. The abundance of proteins in the electron transport chain, specifically complex I, II, and V were insulin independent. Restoration of the blood glucose levels by insulin treatment effects the abundance of enzymes that function in the detoxification of ROS, restoring the redox balance and mitochondrial function.

Keywords: diabetes, insulin, liver, metabolism, pathway, proteomic

1. Introduction

Diabetes mellitus type 1 (DM1) is a chronic autoimmune disease causing pancreatic β-cell destruction and insulin deficiency (1). Chronic hyperglycemia present in DM1 leads to abnormalities in the metabolism of lipids, proteins and carbohydrates (2). Additionally, the

imbalance between pro-oxidants and antioxidants induces oxidative stress via several mechanisms resulting in glucose autoxidation, formation of advanced glycation end-products (AGEs), and activation of the polyol pathway (3,4). Complications associated with the diabetic state include: alteration of energy and glucose metabolism, coronary artery disease, peripheral arterial disease, stroke, nephropathy, neuropathy, and retinopathy (5).

The occurrence of metabolic abnormalities in DM1 results from the imbalance of insulin and glucagon, a peptide hormone produced by pancreatic α-cells that opposes the effects of insulin stimulating hepatic glycogenolysis and gluconeogenesis, and reduces peripheral glucose utilization. Thus, hepatic glucose production is increased, glycogen production is decreased, and peripheral uptake of glucose is decreased resulting in severe hyperglycemia. In addition, lipolysis increases in adipose tissue with a subsequent increase in plasma free fatty acids (FFAs) that serve as substrates for the synthesis of ketone bodies causing ketoacidosis. The increase in plasma FFAs promotes the synthesis of triglycerides causing an increase in very low density lipoproteins (VLDL) and hypertriglyceridemia. Furthermore, insulin deficiency impairs proteolysis and peripheral amino acid uptake resulting in an increase in plasma alanine and glutamine. Alanine and glutamine are used as substrates for hepatic and renal gluconeogenesis, respectively.

The liver is an essential organ in the human body responsible for a multitude of functions. Some of these functions include: bile formation and excretion, vitamin and mineral storage, control of plasma glucose levels, protein synthesis, formation of ketone bodies, participation in lipoprotein and cholesterol metabolism, hormone processing, and xenobiotic breakdown and excretion (6). In DM1, the liver can develop structural and/ or functional abnormalities that may disrupt any of these processes, particularly regulation of carbohydrate and lipid metabolism (7).

The use of biomarkers in biological and medical fields is a growing approach in the diagnosis of disease. Protein expression is tightly regulated to ensure optimal cellular function and the changes in their levels have direct impact on both metabolic and physiological pathways. On the other hand, these changes may reflect various disease states or functional abnormalities. Utilizing proteins or peptides as biomarkers has been applied in the diagnosis of many specific diseases, understanding of disease pathogenesis, and monitoring the progress of therapeutic interventions (8). Many studies have been successfully conducted to identify potential biomarkers by comparing protein patterns present in healthy samples to affected or diseased samples (9). Therefore the discovery of new DM1 biomarkers may help in early and reliable diagnosis before secondary complications arise and reduce the severity of the disease outcome.

Many proteomics studies of DM1 have been reported in the literature including examination of various tissues (10,11) and plasma (12,13) from drug induced (14,15) and/or genetic models (16,17). The majority of these studies focus on proteomics in the diabetic state without treatment. The major findings were in plasma of DM1 patients, hemopexin is upregulated and can be modulated by glucose through a ROS-dependent mechanism (12), in heart of DM1 rat model the mitochondrial heat shock protein 70 was downregulated contributing with protein import dysfunction and this process implicating in the pathogenesis of the diabetic heart (10) and in investigations of proteome in DM1 akita mice showed tissue-specific remodeling causing the mitochondrial dysfunction in heart and preservation of mitochondrial function in kidney, brain and liver (16). In the present study focusing on the hepatic proteome, streptozotocin (STZ) was used to induce DM1 in a rat model. This is a common model used to induce DM1 and hyperglycemia that results in metabolic disbalance including dysregulation of energy and glucose metabolism, oxidative stress and cell death (18). Although STZ induced proteomics studies have been published, subset of the proteins dysregulated due to STZ treatment rather than DM1 conditions has not been well defined. Therefore the liver proteome of STZ induced DM1 rats with and without insulin treatment was analyzed and compared, generating sets of proteins and pathways that were either dependent or independent upon insulin treatment.

2. Materials and Methods

2.1. Experimental animals and procedures

The experimental procedures were approved by the Ethics Committee on the Use of Animals (Protocol: CEUA-436/2012) of the Institute of Biosciences/Sao Paulo State University (UNESP) - Botucatu, which were in accordance with the ethical principles in animal research provided by the Brazilian College of Animal Experimentation.

Fifteen Wistar male rats (*Rattus norvegicus*) (45 days old) with an average initial weight of approximately 250 g were used. Animals were kept in individual plastic cages at a controlled temperature (25 ± 2ºC) and photoperiod (12:12 h cycles light/dark) receiving water and commercial diet (Purina® Labina, Campinas-SP) *ad libitum* throughout the experimental period.

The animals were randomly assigned into the three experimental groups including control group (C), diabetic rats (DM1) and diabetic rats treated with insulin (DM1+I). Control group (n = 5) received water and food without any additional treatment. In diabetic group, the DM1 was induced by the intraperitoneal administration of streptozotocin (STZ) in a single dose. The STZ

was diluted with sodium citrate buffer (100 mM, pH 4.5) and the animals received 1 mL (60 mg kg^{-1} body weight) of the prepared solution. After 48 hours, blood samples were collected from the punctured tail and blood glucose was determined by glucometer. The animals with glucose concentrations above 220 mg dL^{-1} were considered diabetic and used in the experiment (n = 5). For diabetic, insulin treated group, diabetes was induced by STZ as described above and the animals with blood glucose concentrations above 220 mg dL^{-1} (n=5) were used for additional insulin treatment. The insulin (Humulin N100UI Neutral Protamine Hagedorn- NPH, Lilly®) was administrated subcutaneously at an initial dose of 3 U/animal. Following insulin daily doses depended on the blood glucose levels measured just before insulin administration and were adjusted to maintain glucose levels within the normal range (110 mg dL^{-1}).

At the end of the 30 day experimental period, the animals in all three groups were anaesthetised (10% ketamine hydrochloride at 0.1 mL/100 g of body weight, i.p.), sacrificed by decapitation and the liver was collected, flash frozen and lyophilized.

2.2. Protein extraction

The samples were reconstituted in Lysis buffer **(**25 mM Tris buffer pH 8.0, 8 M Urea) and homogenized in a Bullet Blender 24 (Next Advance). For every 100 mg of liver tissue, 200 µL of Lysis buffer was added along with ~ 50 µL of ZrO grinding balls (Next Advance). The samples were vortexed until completely homogenized (~3 x 2 minutes, placed on ice for 30 seconds between each vortex cycle). Beads were then allowed to settle on ice for 2 minutes, crude extracts placed into new tubes and centrifuged for 5 minutes at 15,000 xg. Protein concentrations in supernatant was determined by Bichinchoninic acid assay (BCA) using samples diluted 1:10 and 1:100 in water.

2.3. Protein delipidation

For each sample, volume corresponding to 100 µg of proteins was transferred to the new tube, mixed with cold acetone (3 times the volume of sample) and incubated at -20˚C for 30 minutes. Samples were centrifuged for 2 minutes at 15,000 xg to pellet the precipitated proteins and supernatants were discarded. Pellets were washed twice with cold acetone, dried in speed vac (approximately 15 minutes) and stored at -80˚C.

2.4. Protein digestion

Delipidated pellets (100 µg proteins) were fully solubilized in 20 µL Denaturation Buffer (25 mM ammonium bicarbonate, pH8.0; 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP); 5% SDC (sodium deoxycholate) and incubated for 10 min at 60˚C. Thiol alkylation was achieved by adding 5 µL Alkylation Buffer (100 mM Iodoacetamide in water, freshly prepared) and incubating the samples for 60 min at room temperature in dark. Following alkylation, samples were diluted with 175 µL Dilution buffer (25 mM ammonium bicarbonate, pH8.0) and 2 µL Trypsin solution (1µg/µL) was added. Samples were incubated overnight at 37˚C. To stop reaction and remove SDC, 10 µL of 10% Trifluoroacetic acid (TFA) was added, mixture incubated for 30 min at room temperature and centrifuged at 15,000 xg (SDC precipitates at low pH). Supernatants were transferred into the new tubes and directly used for LC-MS analysis.

2.5. LC-MS^E Analysis

All analyses were carried out using a Waters nanoAcquity UPLC System coupled to a Waters Synapt G2 TOF mass spectrometer. The mobile phases were composed of Solvent A (0.1% formic acid in H_2O) and Solvent B (0.1% formic acid in acetonitrile- ACN). Injection volume was 2 µL. Following injection, the peptides were concentrated on Trap C-18 enrichment column (0.3 x 1 mm, Waters) and washed at 10 µL/min with Solvent A for 3 min. The enrichment column was then switched into the nanoflow path (500 nL/min) and further separated on C-18 reversed phase nanocolumn (0.075 x 250 mm; Waters) coupled with the nanoelectrospray ionization (nESI) source of Synapt G2 mass spectrometer. Separation of peptides was achieved at the following gradient: T=0 min: 5% B; T=95 min: 50% B; T=96 min: 85% B; T=97 min: 85% B; T=98 min: 5% B; T=99 min: 5% B; T=100 min: 85%; B T=101 min: 85% B T=102 min: 5% B and T=120 min: 5% B (column re-equilibration). MS data was collected in positive, Data Independent Acquisition (MS^E) mode under following conditions: a capillary voltage of 2,900 V; the source temperature was set at 70°C; cone gas flow was maintained 6 L/min; acquisition range was 50 – 2,000 m/z. MS^E data was collected with alternating low (4 eV) and elevated (ramp from 17 to 42 eV) energy over a 100–1500 **m/z** range. Spectra and statistical analysis was carried out using Progenesis software (Waters) and searched against NCBI database.

2.6. LC-MS^E Data processing and statistical analysis

LC-MS^E raw data was processed and analyzed by Progenesis QI software package for protein identification and quantitation. All runs were aligned to the most suitable reference as determined by Progenesis QI. For peak picking a chromatographic peak width of 0.2 minutes and the most sensitive setting in the automatic sensitivity method was used. Data normalization was done using total ion intensities referring to the automatically selected least different run. Ata quality was assessed by Q metrices including sample preparation, instrument and experiment matrix. Data was searched against the NCBI database with peptide tolerance and fragment tolerance set to auto. Search parameters also included up to 2 missed cleavages, fixed modification of carbamiodomethyl, and variable modifications of methionine oxidation. For ion matching the requirements were set at least 2 fragments per peptide with 5 fragments per protein and one peptide per protein. Progenesis QI software was then used to generate normalized protein abundance counts. Statistical analysis (a repeated measures ANOVA) was performed using Progenesis QI. All conflicts identified data processing, quantification and protein ID were resolved by manual inspection.

2.7. Pathway enrichment analysis

The list of proteins identified as a significantly changed (p <0.05) was submitted to the batch retrieval service ("Retrieve/ID mapping") at The Universal Protein Resource (UniProt) and the Uniprot protein IDs were converted to gene symbols that were further analyzed by Reactome Functional Interaction (FI) (19) using Cytoscape plugin (20). The Reactome allows analysis of proteomic dataset by the comparison of the experimentally obtained data with published knowledge database identifying related reactions, pathways and biological processes. Pathways with a false discovery rate (FDR) <0.05 were considered to be significantly enriched and implied a potential impact of identified proteins on the metabolic pathways and cellular function.

3. Results and Discussion

3.1. Comparative analysis

Proteomics data was processed and analysed using Progenesis QI software package. Principal Component Analysis (PCA) revealed clear separation of all three groups and a repeated measures ANOVA identified 305 proteins significantly different in abundance (p <0.05) between the control (C), diabetic (DM1) and insulin treated diabetic (DM1+I) groups (Table 1 summarizes

the significantly changed proteins with fold change > 1.5; full list of the proteins is shown in Supplementary Table 1). Significantly changed proteins were further categorized as insulin dependent (> 20% comparing the abundances, proteins changed in diabetic group and their levels were completely or partially restored by insulin treatment) and insulin independent (< 20% comparing the abundances, proteins changed in diabetic group and maintained at the same levels or trends in diabetic group treated with insulin). Out of these 305 significantly changed proteins, 147 were classified as insulin dependent and 152 were classified as insulin independent. Interestingly, 6 identified proteins did not show difference between control and diabetic group, however significant changes were detected in insulin treated group indicating a potential side effect of insulin treatment itself.

Table 1. Proteins differentially expressed in diabetes (Fold Change > 1.5).

Access	Peptides	Proteins	Score	Anova (p)*	Fold Change - groups	DM1 regulation	Insulin condition	Mw (Da)	pI	Function	Average Normalised Abundances		
											C	DM1	DM1+I
Amino acid metabolism													
P07756	193 (154)	Carbamoyl-phosphate synthase [ammonia], mitochondrial OS=Rattus norvegicus GN=Cps1 PE=1 SV=1	1770.95	4.86E-05	1.54	up	dependent	164,580	6.33	urea cycle	51.23	78.96	55.57
P09034	38 (29)	Argininosuccinate synthase OS=Rattus norvegicus GN=Ass1 PE=2 SV=1	423.56	2.15E-04	2.00	up	dependent	46,496	7.63	urea cycle	21.03	42.12	25.94
P20673	6 (3)	Argininosuccinate lyase OS=Rattus norvegicus GN=Asl PE=2 SV=1	48.16	4.84E-06	3.43	up	dependent	51,550	5.99	urea cycle	1.17	4.02	1.6
P18757	19 (10)	Cystathionine gamma-lyase OS=Rattus norvegicus GN=Cth PE=1 SV=2	161.7	8.75E-03	1.50	up	independent	43,605	8.2	amino-acid biosynthesis, Cysteine biosynthesis	14.09	21.19	20.89
P09606	13 (10)	Glutamine synthetase OS=Rattus norvegicus GN=Glul PE=1 SV=3	98.15	9.86E-04	1.72	down	dependent	42,268	6.64	glutamate metabolic process	3.22	2.6	4.48
P10868	8 (6)	Guanidinoacetate N-methyltransferase OS=Rattus norvegicus GN=Gamt PE=1 SV=2	65.16	1.13E-04	1.66	down	dependent	26,407	5.69	amine and polyamine biosynthesis; creatine biosynthesis	1.88	1.22	2.03
Q5I0C3	7 (4)	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial OS=Rattus norvegicus GN=Mccc1 PE=1 SV=1	40.62	1.95E-03	1.72	up	dependent	79,330	6.66	leucine catabolic proces	0.75	1.18	0.69

P25409	26 (18)	Alanine aminotransferase 1 OS=Rattus norvegicus GN=Gpt PE=1 SV=2	175	2.41E-04	1.55	up	independent	55,110	6.08	amino-acid degradation, biosynthetic process, L-alanine catabolic process	2.63	4.08	3.78
P50554	12 (6)	4-aminobutyrate aminotransferase, mitochondrial OS=Rattus norvegicus GN=Abat PE=1 SV=3	69.77	3.17E-03	1.70	up	dependent	56,456	8.15	neurotransmitter degradation	1.73	2.93	2.12
Energy Metabolism													
P19112	26 (20)	Fructose-1,6-bisphosphatase 1 OS=Rattus norvegicus GN=Fbp1 PE=1 SV=2	175.82	0.01	1.53	up	dependent	39,609	5.54	gluconeogenesis	7.27	11.16	8.75
P07379	32 (23)	Phosphoenolpyruvate carboxykinase, cytosolic [GTP] OS=Rattus norvegicus GN=Pck1 PE=1 SV=1	257.86	1.05E-08	1.82	up	dependent	69,416	6.09	gluconeogenesis	2.46	4.49	3.04
O35077	16 (12)	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic OS=Rattus norvegicus GN=Gpd1 PE=1 SV=4	98.3	4.11E-05	1.66	down	dependent	37,453	6.16	glycolysis/gluconeogenesis pathway	3.02	1.93	3.21
P38652	4 (2)	Phosphoglucomutase-1 OS=Rattus norvegicus GN=Pgm1 PE=1 SV=2	25.76	3.06E-05	1.74	down	dependent	61,403	6.3	glycolysis/gluconeogenesis pathway	0.95	0.54	0.9
Q9ER34	17 (9)	Aconitate hydratase, mitochondrial OS=Rattus norvegicus GN=Aco2 PE=1 SV=2	136.97	2.27E-05	1.75	up	dependent	85,433	7.87	Tricarboxylic acid cycle	1.38	2.41	1.77
P14408	24 (18)	Fumarate hydratase, mitochondrial OS=Rattus norvegicus GN=Fh PE=1 SV=1	189.87	8.60E-05	1.51	down	dependent	54,464	9.06	Tricarboxylic acid cycle	3.08	4.66	3.65
P10719	46 (37)	ATP synthase subunit beta, mitochondrial OS=Rattus	460.48	5.72E-05	1.54	up	independent	56,354	5.18	ATP synthesis, Hydrogen ion transport, Ion transport, Transport	34.83	45.68	53.57

		norvegicus GN=Atp5b PE=1 SV=2											
Q68FY0	20 (14)	Cytochrome b-c1 complex subunit 1, mitochondrial OS=Rattus norvegicus GN=Uqcrc1 PE=1 SV=1	164.12	9.02E-04	1.82	up	dependent	52,849	5.57	electron transport, respiratory chain, transport	2.63	4.77	3.49
P12075	13 (11)	Cytochrome c oxidase subunit 5B, mitochondrial OS=Rattus norvegicus GN=Cox5b PE=1 SV=2	97.71	3.31E-04	1.83	up	dependent	13,915	7.68	hydrogen ion transmembrane transport	4.37	7.98	6.17
P10888	10 (5)	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial OS=Rattus norvegicus GN=Cox4i1 PE=1 SV=1	81.2	1.08E-05	1.54	up	dependent	10,515	9.45	response to nutrient	3.95	6.1	4.77
P10818	7 (6)	Cytochrome c oxidase subunit 6A1, mitochondrial OS=Rattus norvegicus GN=Cox6a1 PE=1 SV=2	68.41	3.57E-03	1.86	up	dependent	12,301	9.3	mitochondrial respiratory chain complex IV	6.98	12.95	8.43
P20788	8 (5)	Cytochrome b-c1 complex subunit Rieske, mitochondrial OS=Rattus norvegicus GN=Uqcrfs1 PE=1 SV=2	57.26	4.77E-03	1.50	up	independent	29,446	9.04	electron transport, respiratory chain, transport	2.2	3.3	3.28
Q5M9I5	3 (2)	Cytochrome b-c1 complex subunit 6, mitochondrial OS=Rattus norvegicus GN=Uqcrh PE=3 SV=1	30.39	6.29E-03	1.68	up	independent	10,424	4.9	electron transport, respiratory chain, transport	3.17	5.31	4.59
Q7TQ16	3 (2)	Cytochrome b-c1 complex subunit 8 OS=Rattus norvegicus GN=Uqcrq PE=3 SV=1	25.29	5.40E-03	1.54	up	dependent	9,849	10.52	electron transport, respiratory chain, transport	0.64	0.98	0.73
P10715	4 (3)	Cytochrome c, testis-specific OS=Rattus norvegicus GN=Cyct PE=2 SV=2	22.38	7.96E-05	1.80	up	dependent	11,743	9.34	apoptosis, electron transport, Respiratory chain, Transport	0.53	0.95	0.73

P00406	5 (4)	Cytochrome c oxidase subunit 2 OS=Rattus norvegicus GN=Mtco2 PE=2 SV=3	18.55	2.38E-05	2.33	up	dependent	25,928	4.6	electron transport, respiratory chain, transport	0.97	2.25	1.51
P35171	1 (1)	Cytochrome c oxidase subunit 7A2, mitochondrial OS=Rattus norvegicus GN=Cox7a2 PE=1 SV=1	6.5	2.92E-04	1.51	up	dependent	9,353	10.28	mitochondrial respiratory chain	2.08	3.13	2.45
P00173	14 (11)	Cytochrome b5 OS=Rattus norvegicus GN=Cyb5a PE=1 SV=2	144.57	5.75E-04	1.69	up	dependent	15,355	4.87	electron transport, transport	23.24	39.19	24.67
P19234	8 (5)	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial OS=Rattus norvegicus GN=Ndufv2 PE=1 SV=2	46.35	8.64E-03	1.59	up	dependent	27,378	6.23	electron transport, respiratory chain, transport	1.14	1.82	1.33
P13803	24 (19)	Electron transfer flavoprotein subunit alpha, mitochondrial OS=Rattus norvegicus GN=Etfa PE=1 SV=4	254	8.57E-05	1.55	up	independent	34,951	8.62	electron transport,transport	14.72	19.41	22.77
P23965	15 (11)	Enoyl-CoA delta isomerase 1, mitochondrial OS=Rattus norvegicus GN=Eci1 PE=1 SV=1	125.11	0.01	1.67	up	independent	32,254	9.55	fatty acid metabolism, lipid metabolism	5.94	9.91	8.25
Q64591	9 (3)	2,4-dienoyl-CoA reductase, mitochondrial OS=Rattus norvegicus GN=Decr1 PE=1 SV=2	70.49	5.87E-03	1.94	up	dependent	36,133	9.08	fatty acid beta-oxidation	1.07	2.08	1.23
Q62651	5 (3)	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial OS=Rattus norvegicus GN=Ech1 PE=1 SV=2	39.63	8.44E-06	2.67	up	dependent	36,172	8.14	lipid metabolism; fatty acid beta-oxidation.	0.56	1.49	0.73
Q5FVR5	2 (1)	Acyl-coenzyme A amino acid N-acyltransferase 2	9.16	2.95E-04	1.57	up	independent	46,011	8.64	fatty acid metabolism, lipid metabolism	1.2	1.64	1.88

		OS=Rattus norvegicus GN=Acnat2 PE=2 SV=1											
Q811X6	3 (3)	Lambda-crystallin homolog OS=Rattus norvegicus GN=Cryl1 PE=2 SV=3	21.07	3.24E-04	3.25	down	dependent	35,341	5.94	fatty acid metabolism, lipid metabolism	2.71	0.92	3
Lipid Metabolism													
P22791	36 (20)	Hydroxymethylglutaryl-CoA synthase, mitochondrial OS=Rattus norvegicus GN=Hmgcs2 PE=2 SV=1	340.17	6.86E-03	1.69	up	independent	56,912	8.86	cholesterol biosynthesis, cholesterol metabolism, lipid biosynthesis and metabolism, steroid biosynthesis and metabolism	9.44	15.95	13.26
P04639	15 (11)	Apolipoprotein A-I OS=Rattus norvegicus GN=Apoa1 PE=1 SV=2	118.08	5.62E-04	1.72	up	dependent	30,062	5.52	cholesterol metabolism, lipid metabolism and transport, steroid metabolism	1.74	3	2.31
Q63010	9 (3)	Acyl-coenzyme A thioesterase 2 OS=Rattus norvegicus PE=1 SV=1	56.85	0.02	1.51	up	independent	62,495	6.25	detoxification of xenobiotics	0.19	0.29	0.28
P02692	18 (14)	Fatty acid-binding protein, liver OS=Rattus norvegicus GN=Fabp1 PE=1 SV=1	185.11	6.45E-04	1.82	down	dependent	14,273	7.79	lipid transport, transport	28.35	15.56	27.98
Q64573	17 (7)	Liver carboxylesterase 4 OS=Rattus norvegicus PE=2 SV=2	119.2	6.77E-03	1.55	down	dependent	62,308	6.29	metabolic process	7.38	5.27	8.15
Q5BK32	3 (3)	FAS-associated factor 2 OS=Rattus norvegicus GN=Faf2 PE=2 SV=1	23.18	2.37E-03	1.61	up	independent	41,080	5.7	lipid particle organization	1.47	2.23	2.37
Nucleotide Metabolism													
P61980	13 (10)	Heterogeneous nuclear ribonucleoprotein K OS=Rattus norvegicus GN=Hnrnpk PE=1 SV=1	108.05	2.37E-05	1.59	down	dependent	50,976	5.39	mRNA processing, mRNA splicing, Transcription, Transcription regulation	7.98	12.67	9.45

Q5U2T8	1 (1)	Corepressor interacting with RBPJ 1 OS=Rattus norvegicus GN=Cir1 PE=2 SV=1	11.8	0.02	13.12	up	dependent	51,416	9.88	mRNA processing, mRNA splicing, Transcription, Transcription regulation	0.15	2.03	0.18
O70351	17 (15)	3-hydroxyacyl-CoA dehydrogenase type-2 OS=Rattus norvegicus GN=Hsd17b10 PE=1 SV=3	99.7	9.46E-04	1.50	up	independent	27,246	8.91	tRNA processing	6.86	10.26	8.94
Q4QR75	1 (1)	Exosome complex component RRP45 OS=Rattus norvegicus GN=Exosc9 PE=2 SV=1	5.84	0.01	1.64	up	independent	48,882	5.16	rRNA processing	1.17	1.91	1.6
P29410	17 (12)	Adenylate kinase 2, mitochondrial OS=Rattus norvegicus GN=Ak2 PE=2 SV=2	192.86	3.48E-05	1.55	up	independent	26,379	6.33	ATP metabolic process,Kinase, Transferase, oxidative phosphorylation	6.73	10.43	10.25
P62083	6 (5)	40S ribosomal protein S7 OS=Rattus norvegicus GN=Rps7 PE=1 SV=1	40.34	6.73E-04	1.51	up	independent	22,127	10.9	required for rRNA maturation	1.4	1.81	2.11
P63324	2 (2)	40S ribosomal protein S12 OS=Rattus norvegicus GN=Rps12 PE=1 SV=2	11.12	1.04E-04	1.50	down	dependent	14,525	6.82	translation	0.52	0.42	0.63
P52759	30 (25)	Ribonuclease UK114 OS=Rattus norvegicus GN=Hrsp12 PE=1 SV=3	207.5	1.93E-06	1.59	down	dependent	14,303	7.8	RNA phosphodiester bond hydrolysis, endonucleolytic, negative regulation of translation	33.52	23.61	37.46
P37805	1 (1)	Transgelin-3 OS=Rattus norvegicus GN=Tagln3 PE=1 SV=2	5.72	0.02	1.59	up	independent	22,501	6.84	negative regulation of transcription from RNA polymerase II promoter	0.73	1.17	1.15
Q4V7D6	1 (1)	Putative GTP cyclohydrolase 1 type 2 Nif3l1 OS=Rattus norvegicus GN=Nif3l1 PE=2 SV=1	5.1	0.04	4.85	down	dependent	41,519	6.57	negative regulation of nucleic acid-templated transcription, positive regulation of transcription, DNA-templated	0.1	0.02	0.03
Q63803	4 (3)	Guanine nucleotide-binding protein G(s) subunit alpha isoforms	26.19	1.75E-03	1.80	up	dependent	122,887	4.73	modulators or transducers in various transmembrane signaling systems	0.56	1	0.78

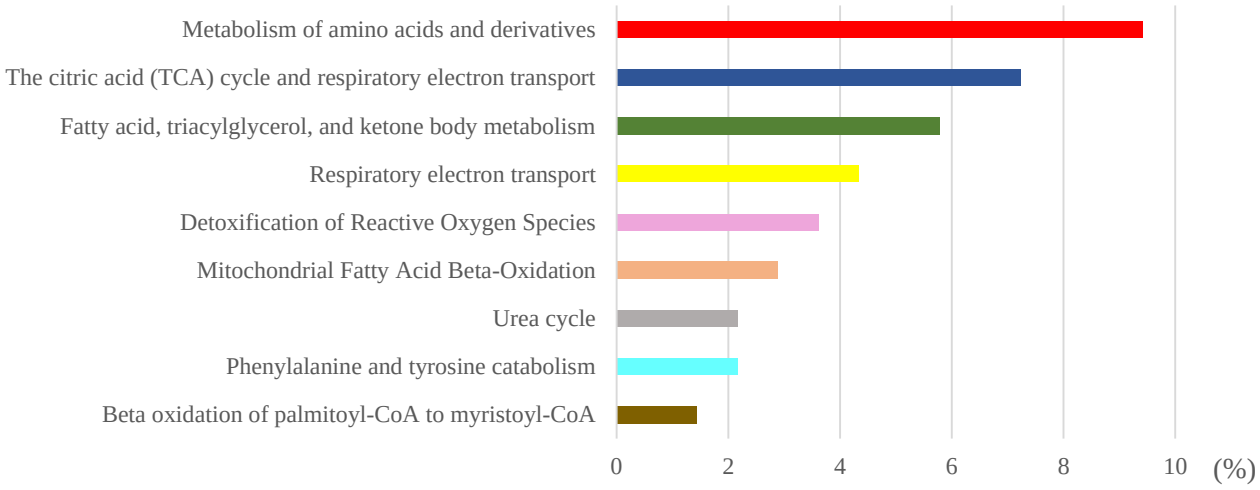
		XLas OS=Rattus norvegicus GN=Gnas PE=1 SV=3											
P20595	3 (3)	Guanylate cyclase soluble subunit beta-1 OS=Rattus norvegicus GN=Gucy1b3 PE=1 SV=2	16.17	2.86E-03	1.66	up	independent	70,456	5.23	nitric oxide mediated signal transduction, cGMP biosynthetic process	1.97	3.27	3.18
Q9WTT6	10 (6)	Guanine deaminase OS=Rattus norvegicus GN=Gda PE=1 SV=1	55.85	9.08E-04	3.18	up	dependent	51,016	5.56	guanine catabolic proces	1.77	5.63	2.08
Q62655	2 (1)	Transcription factor 4 OS=Rattus norvegicus GN=Tcf4 PE=2 SV=2	9.1	0.02	1.62	down	other	63,053	6.95	transcription, transcription regulation	0.09	0.08	0.13
P85834	7 (6)	Elongation factor Tu, mitochondrial OS=Rattus norvegicus GN=Tufm PE=1 SV=1	59.71	5.95E-03	1.91	up	dependent	49,522	7.23	protein biosynthesis	6.51	12.42	7.39
Chaperones and oxidative stress													
P14659	28 (12)	Heat shock-related 70 kDa protein 2 OS=Rattus norvegicus GN=Hspa2 PE=1 SV=2	208.44	0.04	1.53	up	independent	69,642	5.5	chaperone	6.44	9.87	9.19
P35565	5 (4)	Calnexin OS=Rattus norvegicus GN=Canx PE=1 SV=1	32.42	8.96E-03	1.50	up	dependent	67,255	4.48	chaperone	2.92	4.39	3.35
P97576	2 (2)	GrpE protein homolog 1, mitochondrial OS=Rattus norvegicus GN=Grpel1 PE=1 SV=2	17.78	7.56E-03	1.59	up	dependent	24,297	8.57	chaperone	0.39	0.62	0.39
Q66H94	1 (1)	Peptidyl-prolyl cis-trans isomerase FKBP9 OS=Rattus norvegicus GN=Fkbp9 PE=2 SV=1	11.59	2.62E-04	1.87	up	independent	63,127	4.93	chaperone-mediated protein folding	6.44	11.63	12.05
Q9Z0V5	9 (6)	Peroxiredoxin-4 OS=Rattus norvegicus GN=Prdx4 PE=2 SV=1	85.3	1.04E-04	1.72	down	dependent	31,007	6.18	response to reactive oxygen species	0.85	0.49	0.77

P35704	4 (4)	Peroxiredoxin-2 OS=Rattus norvegicus GN=Prdx2 PE=1 SV=3	34.26	3.43E-04	1.39	up	independent	21,784	5.34	removal of superoxide radicals	1.31	1.82	1.59
P07632	26 (19)	Superoxide dismutase [Cu-Zn] OS=Rattus norvegicus GN=Sod1 PE=1 SV=2	207.88	8.38E-03	1.54	down	dependent	15,912	5.88	antioxidant, oxidoreductase	65.94	49.48	75.97
P04762	54 (43)	Catalase OS=Rattus norvegicus GN=Cat PE=1 SV=3	546.24	3.05E-05	1.81	down	dependent	59,757	7.07	hydrogen peroxide	25.66	14.41	26.02
P46418	29 (6)	Glutathione S-transferase alpha-5 OS=Rattus norvegicus GN=Gsta5 PE=1 SV=2	192.64	1.15E-04	2.01	up	dependent	25,347	8.42	xenobiotic catabolic process, response to drug, response to nutrient levels	1.26	2.53	1.56
P24473	3 (2)	Glutathione S-transferase kappa 1 OS=Rattus norvegicus GN=Gstk1 PE=1 SV=3	26.74	2.96E-03	1.66	up	dependent	25,493	9.13	glutathione metabolic process	1.11	1.83	1.41
P04906	1 (1)	Glutathione S-transferase P OS=Rattus norvegicus GN=Gstp1 PE=1 SV=2	6.2	5.48E-03	1.53	up	dependent	23,439	6.89	cellular response to insulin stimulus, Glutathione conjugation	0.63	0.97	0.76
Q9Z0V6	5 (4)	Thioredoxin-dependent peroxide reductase, mitochondrial OS=Rattus norvegicus GN=Prdx3 PE=1 SV=2	32.31	4.50E-05	1.61	up	dependent	28,295	7.14	negative regulation of neuron apoptotic process	0.57	0.92	0.63
P28492	5 (4)	Glutaminase liver isoform, mitochondrial OS=Rattus norvegicus GN=Gls2 PE=2 SV=3	37.92	1.55E-03	1.89	up	dependent	66,248	7.07	glutamine metabolic process, reactive oxygen species metabolic process	0.72	1.36	0.95
P14141	31 (25)	Carbonic anhydrase 3 OS=Rattus norvegicus GN=Ca3 PE=1 SV=3	342.99	9.17E-09	14.22	down	dependent	29,431	6.89	response to oxidative stress	60.41	4.25	48.54
Q9Z0U5	8 (4)	Aldehyde oxidase 1 OS=Rattus norvegicus GN=Aox1 PE=1 SV=1	52.15	0.02	1.68	down	dependent	146,921	6.55	prominent source of superoxide generation	1.12	0.67	1.09
Transport and Binding													

P01946	55 (45)	Hemoglobin subunit alpha-1/2 OS=Rattus norvegicus GN=Hba1 PE=1 SV=3	271.39	6.74E-03	1.50	up	independent	15,329	7.81	oxygen transport, transport,	180.38	270.65	237.64
Q498T9	9 (3)	Volume-regulated anion channel subunit LRRC8C OS=Rattus norvegicus GN=Lrrc8c PE=2 SV=1	49.37	9.99E-04	1.54	up	dependent	90,162	8.15	differentiation, ion transport, transport	0.31	0.48	0.38
Q498T9	9 (3)	Volume-regulated anion channel subunit LRRC8C OS=Rattus norvegicus GN=Lrrc8c PE=2 SV=1	49.37	9.99E-04	1.54	up	dependent	92,463	7.97	ion transport, transport	0.31	0.48	0.38
P36201	3 (3)	Cysteine-rich protein 2 OS=Rattus norvegicus GN=Crip2 PE=2 SV=1	36.39	0.01	1.59	down	dependent	22,696	8.94	binding protein	1.29	1.31	2.06
Q6AYA5	2 (1)	Transmembrane protein 106B OS=Rattus norvegicus GN=Tmem106b PE=1 SV=1	16.07	0.03	2.44	up	dependent	31,152	6.89	transport	0.05	0.1	0.04
Q9JHW5	2 (2)	Vesicle-associated membrane protein 7 OS=Rattus norvegicus GN=Vamp7 PE=1 SV=1	10.71	3.88E-04	1.99	up	dependent	24,776	8.71	exocytosis, protein transport, transport	0.37	0.74	0.41
Q5U206	2 (2)	Calmodulin-like protein 3 OS=Rattus norvegicus GN=Calml3 PE=2 SV=1	15.6	2.18E-04	1.51	down	dependent	16,803	4.18	calcium, metal-binding	0.41	0.35	0.52

Out of the 147 proteins classified as insulin dependent, 138 were converted to gene symbols and analysed using Reactome FI. Analysis revealed 9 significantly enriched (FDR<0.05) pathways (Figure 1). Most of these pathways are functionally related to metabolism and include: metabolism of amino acids and related pathways (urea cycle and phenylalanine and tyrosine catabolism), the tricarboxylic acid (TCA) cycle and respiratory electron transport chain, and metabolism of lipids (mitochondrial fatty acid beta-oxidation; fatty acid, triacylglycerol, and ketone body metabolism; beta oxidation of palmitoyl-CoA to myristoyl-CoA). In addition, the detoxification of reactive oxygen species pathway was identified as significantly affected.

Figure 2. Insulin dependent pathways. Uniprot protein IDs were converted to gene symbols that were further analyzed by Reactome FI (considering FDR < 0.05) using Cytoscape plugin.



Considering the 152 insulin independent proteins, 149 were successfully converted to gene symbols. Reactome FI analysis revealed 30 pathways (FDR<0.05) that were significantly enriched (Figure 2). The pathways identified for the insulin independent proteins are primarily related to energy metabolism, redox balance amino acid metabolism and lipid metabolism including glucose metabolism (gluconeogenesis, glycolysis), formation of ATP by chemiosmotic coupling, degradation of cysteine and homocysteine, synthesis of ketone bodies, pyruvate metabolism and TCA cycle, metabolism of nucleotides, sulfur amino acid metabolism, branched-chain amino acid catabolism and phase II conjugation.

All identified pathways with corresponding genes for both insulin dependent and independent changes are summarized in Table 2.

Figure 3. Insulin independent pathways. Uniprot protein IDs were converted to gene symbols that were further analyzed by Reactome FI (considering FDR < 0.05) using Cytoscape plugin.

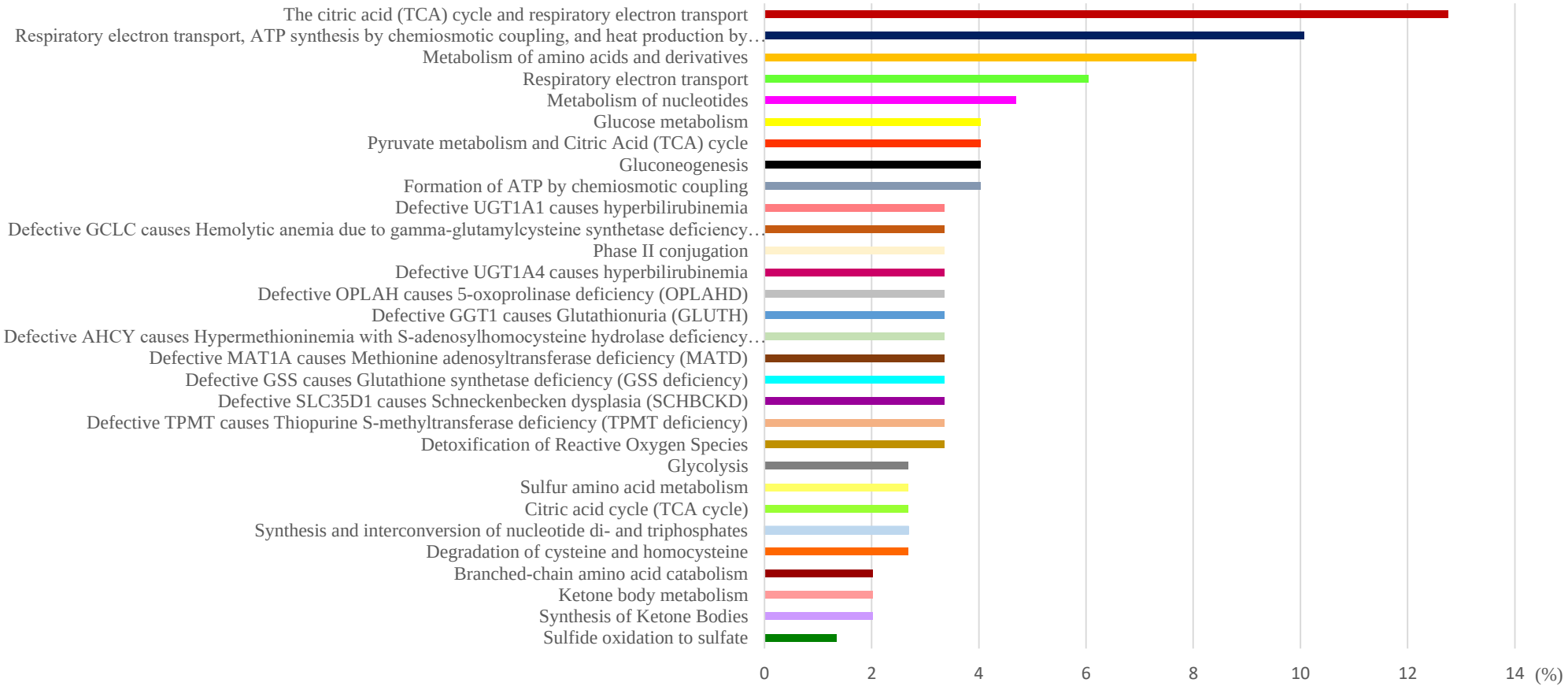


Table 2. Insulin dependent and insulin independent pathways and their associated genes/ proteins. Genes in red correlate to a decrease in protein abundance; while, genes in green correlate to an increase in protein abundance. Green: upregulated in DM1, Red: downregulated in DM1.

ReactomePathway	P-value	FDR	Genes
Insulin Dependent - genes associated			
Metabolism of amino acids and derivatives	0	<5.000e-04	Mat1a,Pcbd1,Tat,Psma1,Glul,Ass1,Asl,Cps1,Mccc1,Hpd,Gls2,Haao,Gamt
The citric acid (TCA) cycle and respiratory electron transport	0	1.67E-03	Ldha,Cox5b,Idh2,Fh,Ndufv2,Cox6a1,Uqcrc1,Uqcrq,Aco2,Cox4i1
Detoxification of Reactive Oxygen Species	0	1.25E-03	Prdx3,Prdx1,Cat,Gstp1,Sod1
Mitochondrial Fatty Acid Beta-Oxidation	0	2.80E-03	Pccb,Hadhb,Decr1,Acadvl
Phenylalanine and tyrosine catabolism	0.0001	3.83E-03	Pcbd1,Tat,Hpd
Urea cycle	0.0001	9.14E-03	Ass1,Asl,Cps1
Beta oxidation of palmitoyl-CoA to myristoyl-CoA	0.0007	3.53E-02	Hadhb,Acadvl
Respiratory electron transport	0.0007	3.34E-02	Cox5b,Ndufv2,Cox6a1,Uqcrc1,Uqcrq,Cox4i1
Fatty acid, triacylglycerol, and ketone body metabolism	0.001	4.92E-02	Apoa1,Pccb,Hadhb,Gpd1,Fabp1,Decr1,Acadvl,Acsl1
Insulin independent - genes associated			
The citric acid (TCA) cycle and respiratory electron transport	0	<5.000e-04	Cox5a,Pdhb,Suclg1,Adhfe1,Atp5b,Uqcrfs1,Atp5l,Atp5o,Atp5i,Atp5j,Cycs,Ndufa10,Uqcrh,Etfb,Etfa,Dlst,Sdha,Sdhb,Atp5a1
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins	0	<3.333e-04	Cox5a,Atp5b,Uqcrfs1,Atp5l,Atp5o,Atp5i,Atp5j,Cycs,Ndufa10,Uqcrh,Etfb,Etfa,Sdha,Sdhb,Atp5a1
Formation of ATP by chemiosmotic coupling	0	<2.500e-04	Atp5b,Atp5l,Atp5o,Atp5i,Atp5j,Atp5a1

Metabolism of amino acids and derivatives	0	<2.000e-04	Hsd17b10,Glud1,Arg1,Csad,Aldh4a1,Tst,Suox,Otc,Cth,Bckdha,Dlst,Bckdhb
Respiratory electron transport	0	<1.667e-04	Cox5a,Uqcrfs1,Cycs,Ndufa10,Uqcrh,Etfb,Etfa,Sdha,Sdhb
Gluconeogenesis	0	<1.429e-04	Mdh1,Pc,Eno3,Gapdh,Eno1,Gapdhs
Degradation of cysteine and homocysteine	0	<1.250e-04	Csad,Tst,Suox,Cth
Synthesis of Ketone Bodies	0	6.67E-04	Hmgcl,Hmgcs2,Bdh1
Detoxification of Reactive Oxygen Species	0	9.00E-04	Txn2,Prdx5,Prdx2,Cycs,Txn
Pyruvate metabolism and Citric Acid (TCA) cycle	0	1.27E-03	Pdhb,Suclg1,Adhfe1,Dlst,Sdha,Sdhb
Metabolism of nucleotides	0.0001	1.50E-03	Nme2,Nme1,Ak2,Adk,Txn,Dpys,Aprt
Ketone body metabolism	0.0001	1.92E-03	Hmgcl,Hmgcs2,Bdh1
Synthesis and interconversion of nucleotide di- and triphosphates	0.0001	3.71E-03	Nme2,Nme1,Ak2,Txn
Citric acid cycle (TCA cycle)	0.0002	5.27E-03	Suclg1,Dlst,Sdha,Sdhb
Sulfur amino acid metabolism	0.0004	8.94E-03	Csad,Tst,Suox,Cth
Glycolysis	0.0004	1.11E-02	Eno3,Gapdh,Eno1,Gapdhs
Glucose metabolism	0.0005	1.15E-02	Mdh1,Pc,Eno3,Gapdh,Eno1,Gapdhs
Branched-chain amino acid catabolism	0.0013	3.60E-02	Hsd17b10,Bckdha,Bckdhb
Defective TPMT causes Thiopurine S-methyltransferase deficiency (TPMT deficiency)	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Defective SLC35D1 causes Schneckenbecken dysplasia (SCHBCKD)	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Defective GSS causes Glutathione synthetase deficiency (GSS deficiency)	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat

Defective MAT1A causes Methionine adenosyltransferase deficiency (MATD)	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Defective AHCY causes Hypermethioninemia with S-adenosylhomocysteine hydrolase deficiency (HMAHCHD)	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Defective GGT1 causes Glutathionuria (GLUTH)	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Defective OPLAH causes 5-oxoprolinase deficiency (OPLAHD)	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Defective UGT1A4 causes hyperbilirubinemia	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Phase II conjugation	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Defective GCLC causes Hemolytic anemia due to gamma-glutamylcysteine synthetase deficiency (HAGGSD)	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Defective UGT1A1 causes hyperbilirubinemia	0.0014	2.55E-02	Cyp1a2,Ugt1a1,Gsta4,Mat2a,Glyat
Sulfide oxidation to sulfate	0.0027	4.73E-02	Tst,Suox

3.2. Amino acid metabolism

Insulin dependent changes in protein abundance - During fasting, glucogenic amino acids from the breakdown of skeletal muscle are converted by the liver to glucose via gluconeogenesis. During this process ammonia is generated. The excess ammonia is detoxified by the urea cycle and excreted by the kidneys (21). The data shows an increased abundance of carbamoyl phosphate synthetase I (Cps1, 1.54-fold increase), argininosuccinate synthetase (Ass1, 1.54-fold increase), argininosuccinate lyase (As1, 3.43-fold increase) in DM1 group. Upon insulin treatment, these enzymes were restored to control levels demonstrating that their abundance is insulin dependent. In the diabetic condition the catabolism of skeletal muscle to release glucogenic amino acids and subsequent hepatic gluconeogenesis increases ammonia. Results indicate that the increased levels of these enzymes play important role in detoxification of ammonia excess generated by these processes. Insulin treatment prevents the breakdown of skeletal muscle and release of ammonia to generate glucose and therefore restores enzymes necessary for ammonia detoxification to control levels.

Insulin independent changes in protein abundance - Similarly, the protein abundance of Arginase-1 (Arg1, 1.42-fold increase) was increased in the DM1 group; a result which could potentially be explained by reduced insulin action and increased protein catabolic processes. However, Arg1 protein levels were not restored to control levels in the insulin treated group. Reduced insulin activity in the diabetic state may cause impaired endothelial function and remodeling resulting from the Arg1 reaction which ultimately decreases NO production from eNOS pathway to the arginase pathway (22). The abundance of ornithine transcarbamylase (Otc, 1.16-fold decrease) was also decreased in the DM1 group and not restored to control levels in the DM+I group. This potentially suggests that activation by Mn^{2+} supports increase/decrease flux without altering Otc protein levels (23). Two branched-chain amino acids (BCAA) catabolic process had increased abundance in DM1 group: 2-oxoisovalerate dehydrogenase subunit alpha and beta (Bckdha, 1.30-fold increase and Bckdhb, 1.27-fold increase). Previous studies have displayed that these enzymes are particularly responsive to the inhibitory action of insulin where disturbances in BCAA protein abundances have been associated with diabetic complications (24). However, the present study did not show significant differences in BCAA protein abundances between the DM1 group and the DM1+I group. This discrepancy could potentially be due to the diabetic state induced excessive damage in the model systems examined. Delta-aminolevulinic acid dehydratase (Alad) is a metalloenzyme containing sulfhydryl groups and zinc. Its cysteinyl residues are highly sensitive to pro-oxidant conditions as hyperglycemia (25).

Aminolevulinic acid (ALA) is produced by the condensation of glycine and succinyl coenzyme A. Alad and its substrate ALA function in the porphyrin and heme biosynthetic pathway (26) Alad demonstrated a decrease in abundance in both the DM1 and DM1+I groups. Previous studies have also shown a decrease in Alad in DM1 (25,27,28). Also, decreased abundance of Alad is associated with high glucose levels and decreased antioxidants defense (29).

The most of the enzymes that function in the urea cycle were restored to control levels upon insulin treatment. Interestingly, Arg1 displayed increased abundance in both DM1 and DM1+I groups suggesting that the remodeling occurring under the initial diabetic state in insulin treated group cannot be completely reversed by insulin treatment.

3.3. Energy metabolism

Insulin dependent changes in protein abundance - In DM1 group, glycogen metabolism regulates blood glucose by stimulating gluconeogenesis and inhibiting glycolysis, and in effect producing glucose and urea from lactate, glutamine and alanine (30). Two enzymes that function in gluconeogenesis had increased abundance in DM1 group not treated with insulin: fructose 1,6-bisphosphatase (Fbp1, 1.53-increase) and cytosolic phosphoenolpyruvate carboxykinase (Pck1, 1.82-increase). Both of these enzymes function solely in gluconeogenesis. Fbp1 converts fructose-1,6-bisphosphate to fructose-6-phosphate in gluconeogenesis and Pck1 is involved in the irreversible conversion of oxaloacetate to phosphoenolpyruvate in the first step of gluconeogenesis. Insulin treatment in the insulin treated group restored both enzymes to control levels. Previous studies have reported increased hepatic and renal Fbp1 protein abundance in diabetic rats (31–33) and a correlation between Pck1 activity and glycemic control (34). Additional studies have also reported increased Fbp1 and Pck1 protein abundance in models of DM that were restored to steady state levels upon insulin treatment (35–37).

Phosphoglycerate kinase 1 (Pgk1, 1.25-fold decrease), glycerol-3-phosphate dehydrogenase [NAD[+]] (Gpd1, 1.66-fold decrease) and phosphoglucomutase-1 (Pgm1, 1.74-fold decrease) were found decreased in the DM1 group. Interestingly, these enzymes are involved in the second part of glycolysis generating both ATP on the substrate level and providing intermediates to TCA cycle. Lower levels suggest potential mitochondrial damage and increased demand for NADPH generation by pentose phosphate pathway indicating high levels of oxidative stress. In addition, increased abundance of lactoylglutathione lyase (Glo1) is associated with oxidative stress and production advanced glycation end products (AGEs). The abundance of Glo1 was found decreased in the DM1 group (1.31-fold decrease) and restored to

control levels in the insulin treated group. Previous studies have shown similar levels of Glo1 associated with high glucose levels in the formation of AGEs and AGEs precursors such as, methylglyoxal and glyoxal in DM1 that were reversed by insulin treatment (38).

The tricarboxylic acid cycle (TCA) is considered the hub of cellular fuel metabolism located within the mitochondrial matrix. The TCA cycle links amino acid catabolism to gluconeogenesis, is the primary source of electrons for respiration, and the site of terminal oxidation. In DM1 group, TCA cycle induction has been correlated to the development of mitochondrial respiratory dysfunction, hepatic oxidative stress, and inflammation. TCA cycle consist of eight reactions that oxidize the acetyl group of acetyl-CoA, derived from pyruvate, fatty acids and amino acid degradation resulting in the release of two molecules of CO_2 while conserving the liberated energy in the reduced NADH and $FADH_2$ molecules. Three enzymes that function in the TCA cycle: mitochondrial aconitate hydratase (Aco2, 1.75-fold increase), mitochondrial fumarate hydratase (Fh), and cytoplasmic isocitrate dehydrogenase [NADP] (Idh1) were shown to have altered abundances in the DM1 group that were restored to control levels in the DM+I group. In contrast to the results presented here, previous examination of diabetic models has not reported altered abundances of the above mentioned enzymes (16,17). However, insulin dependency of these enzymes can be explained by the fact that they provide mitochondrial derived substrates for elevated glycolysis and decrease electron deposition related to oxygen species production.

Two isoforms of NADH dehydrogenase (complex II of electron transport chain) had increased abundance in DM1 group that were restored to control levels in the insulin treated group including flavoprotein 2 (Ndufv2, 1.59-fold increase) and 1 alpha subcomplex subunit 10 (Ndufa10, 1.47-fold increase). This suggests that insulin plays a role in the activation of mitochondrial oxidative phosphorylation in this experimental model of DM1. Also related to the electron transport chain, nine isoforms of cytochromes were identified to have altered abundances in the DM1 group that were restored to control levels upon in the insulin treatment. Four of these cytochrome oxidases are present in complex IV of the electron transport chain. To our knowledge none of the alterations in electron transport chain enzyme abundances have been previously reported. These cytochromes are the primary system for chemical defense in animals (39); therefore, insulin treatment may be associated with decreasing ROS generation and oxidative stress.

Insulin independent changes in protein abundance - Some enzymes participating in energy metabolism displayed changes in abundance in both DM1 and insulin treated groups

compared to control group. Pyruvate carboxylase which regulates hepatic glucose production by catalyzing the first step in gluconeogenesis (40) displayed increased abundance (Pc 1.19-fold increase) in both DM1 and insulin treated DM1+I group (Figure 5). Contrary to results presented here, previous studies have not reported an increase in Pc abundance upon insulin treatment. Our data suggests that Pc may be involved in anaplerotic reactions to maintain TCA cycle intermediates such as producing oxaloacetate from pyruvate.

Additionally, three enzymes involved in glycolysis and gluconeogenesis including (Eno1, 1.22-fold increase), beta-enolase (Eno3, 1.38-fold increase), glyceraldehyde-3-phosphate dehydrogenase (Gapdh, 1.34-fold increase) were identified as the insulin independent (Figure 5). These enzymes however participate in reversible reactions and their increased abundance in the DM1 group may reflect increased gluconeogenesis; while, in the insulin treated group may be reflective of glycolytic activation and higher metabolic flux by constant supply of insulin.

In this study, L-lactate dehydrogenase A chain that is responsible for conversion of lactate to pyruvate was also found to be decreased in abundance (Ldha, 1.26-fold decrease) in the DM1 group (Figure 5). This can be attributed to the glucose utilization through the pentose phosphate pathway, promoting the peripheral glucose utilization by anaerobic glycolysis and interfering with the mitochondrial respiratory chain (41,42). Ldha displayed insulin independence, as the DM1+I group contained similar levels as the DM1 group. In DM1-I group the increased abundance of Ldha compared to control could be an overall increased efficiency of carbon flux through glycolysis with subsequent pyruvate accumulation (43).

Most of the enzymes involved in the TCA cycle were found to have increased abundance in both DM1 and DM1+I groups compared to control. These enzymes include: mitochondrial succinate dehydrogenase [ubiquinone] flavoprotein subunit and iron-sulfur, (Sdha, 1.32-fold increase; Sdhb, 1.25-fold increase), succinyl-CoA ligase [ADP/GDP-forming] subunit alpha (Suclg1, 1.22-fold increase), cytoplasmic malate dehydrogenase (Mdh1, 1.28-fold increase), cytoplasmic aconitate hydratase (Aco1, 1.36-fold increase), mitochondrial pyruvate dehydrogenase E1 component subunit beta (Pdhb, 1.28-fold increase), hydroxyacid-oxoacid transhydrogenase (Adhfe1, 1.48-fold increase), mitochondrial isocitrate dehydrogenase (Idh2, 1.35-fold increase), and dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (Dlst, 1.19-fold increase). The insulin independency of TCA cycle enzymes may be related to that of Pc insulin independency: to restore the intermediates of TCA cycle by promoting the oxaloacetate generation from pyruvate upon a constant insulin treatment.

Interestingly, L-xylulose reductase (Dcxr, 1.38-fold increase) was also found to have increased abundance in DM1 and DM+I groups. It may be involved in glucuronate pathway of animal systems, an alternative route of glucose-6-phosphate oxidation. Current literature does not report Dcxr protein alterations associated with diabetes; however, this enzyme has been reported to have increased abundance in prostate cancer and is associated with melanoma progression (44).

ATP serves as a signal molecule in many cells (45). Recent studies have shown that in STZ-induced type 1 diabetic mice hepatic ATP is significantly reduced (46). On the other hand, studies have also shown that cellular oxygen consumption along with hepatic ATP production is increased under diabetic conditions (16,47). Six proteins associated with ATP synthase also displayed increased abundance in both DM1 and DM1+I group. These proteins include: ATP synthase subunit beta (Atp5b, 1.54 fold-increase), ATP synthase subunit alpha (Atp5a1, 1.30 fold-increase), ATP synthase subunit O (Atp5o, 1.30 fold-increase), ATP synthase-coupling factor 6 (Atp5j, 1.40 fold-increase), ATP synthase subunit e (Atp5i, 1.39 fold-increase), and ATP synthase subunit g (Atp5l, 1.14 fold-increase). This suggests that independent of insulin, increased ATP synthase abundance could potentially result in increased ATP generation and suppression of gluconeogenesis, while activating glycolysis to ameliorate hyperglycemia present in DM1.

Electron transfer flavoprotein subunit alpha and beta displayed an increased abundance in DM1 and DM1+I groups (Etfa, 1.55-fold increase and Etfb, 1.26-fold increase). They are an obligatory electron acceptor of reactions involved in fatty acid oxidation (48). Network analysis indicated that increases in ATP independent of insulin generated a reaction sequence involving increased protein levels of enzymes of the respiratory electron chain.

Hydroxymethylglutaryl-CoA lyase was also revealed to have increased abundance in DM1 and insulin treated groups (Hmgcl, 1.47 fold-increase). Hmgcl is cytoplasmic enzyme and higher levels suggest that acetoacetate synthesis can occur in the cytoplasm creating a surplus of acetyl-CoA when lipogenesis is inhibited.

Increased abundance of adenosine kinase (Adk, 1.24-fold increase) were found in both DM1 and DM1+I groups suggesting an association of Adk with glucose levels. However, previous study showed that 10 days after the STZ injection there was 2-fold increase in hepatic Adk content and administration of insulin decrease Adk levels under normal conditions (49).

3.4. Lipid metabolism

Insulin dependent changes in protein abundance - In DM1 both hepatic gluconeogenesis and ketogenesis are excessive (50). Currently, a major area of interest is focused on the elevated free fatty acids (FFAs) and activation/regulation of enzymes participating in fatty acid β-oxidation (FAO) and gluconeogenesis (51). Hepatic FAO is increased in DM1 for ATP generation. The enzymes upregulated in FAO can promote undesired ketogenesis with excessive production of acetyl-coA by increased FAO. This fact is supported by upregulation of four enzymes including mitochondrial trifunctional enzyme subunit beta (Hadhb, 1.24-fold increase), mitochondrial enoyl-CoA hydratase domain-containing protein 3 (Echdc3, 1.67-fold increase), mitochondrial 2,4-dienoyl-CoA reductase (Decr1, 1.94-fold increase), and mitochondrial delta[3,5]-delta[2,4]-dienoyl-CoA isomerase (Ech1, 2.67-fold increase) that promotes FAO in mitochondria supplying TCA cycle with Acetyl-CoA and can be restored to control levels by insulin treatment.

Sulfotransferase 1 family member D1 (Sult1d1, 1.31-fold increase) abundance was increased in DM1 group and restored to control levels in insulin treated group. Previous study of the diabetic state reported similar alterations in sulfotransferases and indicated that these changes occurred concomitantly with shifts in glucose metabolism, ketone homeostasis, protein Kinase C isoforms, and P450 metabolism (52).

DM1 is also associated with accumulation of the intermediates of the fatty acids metabolism and their accumulation in liver (53). Apolipoprotein A-I (Apoa1) participates in the transport of cholesterol from peripheral tissues to the liver. Previous studies have shown negative correlation between plasma glucose and Apoa1 protein abundance (54,55). Increased abundance of Apoa1 (1.72-fold increase) was found in the DM1 group and restored to control levels by insulin treatment. On the other hand, non-specific lipid-transfer protein (Scp2, 1.26-fold decrease) was downregulated in DM1 group. These changes in both ApoA1 and Scp2 suggest that they may be responsible for aberrant transfer of all common diacylglycerophospholipids, cholesterol as well as glycosphingolipids and gangliosides between membranes (56) and promotes the development of steatosis of liver. In addition, several downregulated enzymes involved in lipid degradation including isoamyl acetate-hydrolyzing esterase 1 (Iah1; 1.45-fold decrease), long-chain-fatty-acid-CoA ligase 1 (Acsl1, 1.34-fold decrease), very long-chain specific acyl-CoA dehydrogenase (Acadvl, 1.45 fold decrease), mitochondrial acyl-CoA synthetase family member 2 (Acsf2, 1.46-fold decrease), acyl-protein thioesterase 1 (Lypla1, 1.44-fold decrease) were identified indicating that they may play an important role in the steatosis

process and accumulation of intermediates of fatty acid metabolism. This is further supported by fact that fatty acid-binding protein (Fabp1) was downregulated (1.82-fold decrease) in DM1. Fabp1 is intracellular lipid chaperone, involved in lipid homeostasis and regulation of inflammatory pathways (57). While reduced levels of Fabp1 are linked to disorders in lipid metabolism, increased concentrations may alleviate some secondary complications common in DM1 (58). For all these enzymes, their control levels were restored by insulin treatment.

Insulin independent changes in protein abundance – Eight enzymes directly functioning in FAO displayed increased abundance in DM1 and DM+I groups. These enzymes include mitochondrial trifunctional enzyme subunit alpha (Hadha, 1.48-fold increase), peroxisomal 3-ketoacyl-CoA thiolase A (Acaa1a, 1.35-fold increase), mitochondrial enoyl-CoA delta isomerase 1 (Eci1, 1.37-fold increase), bile acyl-CoA synthetase (Slc27a5, 1.28-fold increase), acyl-coenzyme A synthetase (Acsm5, 1.28-fold increase), enoyl-CoA delta isomerase 2 (Eci2, 1.36-fold increase), mitochondrial 2,4-dienoyl-CoA reductase (Decr1, 1.94-fold increase), and acyl-coA amino acid N-acyltransferase 2 (Acnat2, 1.57-fold increase). Previous studies have not shown similar patterns of FAO enzymes; however, the lipotoxicity generated in the diabetic state may be influenced by other factors of insulin treatment. Increased abundance of mitochondrial hydroxymethylglutaryl-CoA synthase (Hmgcs2, 1.47 increase) was found in both DM1 and DM1+I groups. Hmgcs2 is involved in the synthesis of ketone bodies. The insulin independency suggests that Hmgcs2 may be involved in supplying ketone bodies through FAO as an alternative carbon source to glucose (59).

In addition, two carboxylesterases (CES) were found to have decreased abundance in both DM1 and insulin treated groups. These enzymes are hepatic carboxylesterase 4 (1.55-fold decrease) and carboxylesterase 1D (Ces1d, 1.42-fold decrease). The downregulation of these enzymes may be associated with hepatic hydrolytic biotransformation as recently published study reported that different concentrations of glucose influence CES abundance (60). However, this particular study was not examining the diabetic state.

3.5. Chaperone and oxidative stress

Insulin dependent changes in protein abundance - Uncontrolled diabetes generates uncontrolled oxidative stress; other important conditions in diabetes are dyslipidemia, modification of proteins and lipids, and perturbations in the tissue antioxidant defense (61). Calnexin (Canx, 1.50-fold increase) was upregulated in DM1 (Canx) with insulin dependency, we did not find some relation with insulin or diabetes with this protein, but this protein is used to

describe folding/quality control in ER and essential for the folding of glycoproteins can be related with decrease of mitochondria dysfunction and increase of quality control in ER associated with insulin treatment. The calreticulin (Calr, 1.42-fold decrease) was downregulated in DM1 and insulin dependent, this is a calcium-binding protein of the endoplasmic reticulum, recent studies have reported important role in tissue remodeling how this protein was found insulin dependent can suggest the tissue remodeling with decrease in mitochondria dysfunction in treated animals. The 78 kDa glucose-regulated (Hspa5,1.29-fold decrease) was found with insulin dependency can have some control of glucose metabolism in these animals. The 90-beta (Hsp90ab1, 1.34-fold decrease) was downregulated in DM1 and insulin dependent, important in maintenance of protein kinase activity, was reported in previous studies be involved in the regulation of glucose transport and glycogen synthesis by insulin mediated (62) confirming ours results. Thioredoxin-dependent peroxide reductase, mitochondrial (Prdx3, 1.61-fold increase) were upregulated in DM1 and insulin dependent, can be related with a compensatory mechanism of diabetes induced oxidative stress. Enzymes involved in ROS scavenging and protect against oxidative stress such as peroxiredoxin-1 and 4 (Prdx1, 1.30-fold decrease and Prdx4, 1.72-fold decrease), superoxide dismutase (Cu-Zn-Sod1, 1.54-fold decrease), catalase (Cat, 1.81-fold decrease) and glutathione S-transferase theta-2 (Gstt2, 1.32-fold decrease) were downregulated DM1 with insulin dependency, suggesting ROS metabolism dysregulation with production increased of ROS and increase protein oxidation associated with DM1 and regulation with insulin treatment (63). Carbonic anhydrase 3 (Ca3, 14.22-fold decrease) might function to protect cells from oxidative damage (64,65), as our study, previous study related decreased in concentration of this enzyme in liver of diabetics rats and restored to control value by administration of insulin (66). Aldehyde oxidase 1 (Aox1, 1.62-fold decrease) was downregulated and insulin dependent, can be associated with ROS generation and oxidative liver injury (67), in the literature we did not found the same results, a study related after induction of diabetes in rats Aox1 is increased significant different results was observed in our study (67). Glutaminase liver isoform, mitochondrial (Gls2) was found upregulated in DM1 and insulin got to control this regulation in DM1-I. Plasma glutamine carrier energy between organs that is used in hepatic urea synthesis and gluconeogenesis in liver, and glutamine can exert its effects by redox homeostasis (68). Glutamine is used in hepatocytes from STZ diabetic rats more rapidly than normal or insulin diabetic rats, stimulated by glucagon, the upregulation of Gls2 increased glutamine uptake in diabetes (68). Ribonuclease inhibitor (Rnh1, 1.35-fold decrease) contributes to intracellular redox

homeostasis (69) in DM1 was downregulated and insulin dependent, can be associated with increased of oxidative stress in liver in DM1.

Insulin dependent changes in protein abundance - Heat shock proteins (Hsps), also known as stress proteins function to protect proteins, lipids, and nucleic acids from damage and denaturation (70). The crucial role of Hsps in diabetes is their ability to counteract denaturation of tissue proteins and defense mechanisms, their expression may contribute to diabetes complications (51). Five Hsps had increased abundance and one had decreased abundance in both the DM1 and DM1+I groups. The proteins with increased abundance include: 60 kDa (Hspd1, 1.26-fold increase), 70 kDa (Hspa2, 1.53-fold increase), mitochondrial stress-70 protein (Hspa9, 1.22-fold increase), 10 kDa (Hspe1, 1.33-fold increase), and 70 kDa protein 1-like (Hspa1l, 1.24-fold increase); indicating a stress response to damage and oxidative stress observed in DM1 and DM1+I. Hspa2, Hspa9 and Hspa1l have capacities to inhibit apoptosis by release of initiator effector caspases (71). Previous examination of DM1 have shown an elevation of these proteins (72). Hspe1 is a small Hsp that is regulated by differential serine (73). The single Hsp with decreased abundance was Hsp 71 kDa (Hspa8, 1.13-fold decrease). Endoplasmin (Hsp90b1) is known to be upregulated by glucose starvation and stress conditions (74). It contains several endoplasmic reticulum stress elements (ERSE) and under stress conditions are responsible for the enhanced expression (75). Hsp90b1 displayed increased abundance in both DM1 and DM1+I groups.

Disturbance of antioxidant defense mechanisms present in uncontrolled DM1 create an imbalance in cellular redox state and oxidative stress (57). Peroxiredoxins (Prxs) are thiol-dependent enzymes responsible for the degradation of peroxides (76). In the present study, Prx2 and Prx5 were shown to have increased abundance (1.22 and 1.39-fold increase). Prx2 and Prx5 protect against oxidative stress induced apoptosis in DM1 (77). Interestingly, insulin treatment in the DM1+I group did not restore the Prx proteins to control levels. In contrast to the results obtained in the current study, Bast et al. (2002) reported upregulation of Prx1 and Prx2, but not Prx5, in cultured insulinoma cells exposed to diabetic inducing xenobiotics (STZ and aloxan) (78).

Glutathione S-transferases (Gst) consist of a family of isozymes that function in cellular detoxification through the catalytic fusion of the reduced form of glutathione (GSH) to potentially toxic molecules (79). Gsts are identified according to structure, sequence, and behavior as alpha, kappa, mu, theta, pi, omega and sigma. Glutathione S-transferase alpha-5 (Gsta5), glutathione S-transferase A6 (Gsta6), glutathione S-transferase alpha-4 (Gsta4), glutathione S-transferase

kappa 1 (Gstk1) and glutathione S-transferase P (Gstp1) all displayed increased abundances in DM1. The alpha subfamily has glutathione peroxidase like activity that functions mostly in detoxification of lipid peroxidation products (80). The single pi and kappa family member (Gstp1 and Gstk1) function in xenobiotic metabolism and prevention of ER stress, respectively (81,82).

While, some studies reported that after the injection of STZ the activity of Gst was reduced in DM1 (83–85); other studies have shown GST to be upregulated in DM1 and may be associated with in short-term diabetes (86).

The thioredoxin (Trx) antioxidant system utilizes NADPH as a cofactor to protect cells from oxidative stress (87). Others studies have reported that high glucose levels induce an increase in Trx expression (87). Similarly, Trx displayed an increased abundance in DM1 and DM1+I groups. This suggests that insulin can control high glucose but cannot restore Trx protein alteration.

Rho GDP-dissociation inhibitor 1 (Arhgdia) plays a negative modulatory role in glucose-stimulated insulin secretion (64); it displayed increased abundance in the DM1 and DM1+I groups. In addition to its role in hormonal regulation, Arhgdia also functions in the activation of NADPH oxidase with subsequent superoxide formation (88).

4. Conclusion

Gluconeogenesis appeared to be idled to the normal levels in diabetic group by insulin treatment with the restoration of control group levels of the Fbp1 enzyme. However, Pc participating in the initial steps of gluconeogenesis was insulin independent, this response may be related to oxalate that enters and maintains intermediates of the TCA cycle. Interestingly, the abundance of several enzymes that function in both gluconeogenesis and glycolysis displayed insulin independence, this may be explained by the concept that insulin independent enzymes play more of a role in the activation of gluconeogenesis in the absence of insulin treatment and stimulation of glycolytic flux upon insulin administration once a day. Similarly, the insulin dependent enzymes are associated with activation of glycolysis under insulin treatment. The majority of the enzymes of the TCA cycle displayed insulin dependency, this may be explained as an anapleurotic response to maintain intermediates of the TCA cycle in response to insulin. The abundance of proteins in the electron transport chain, specifically complex I, II, and V were insulin independent, consequently result in mitochondrial ROS generation and an oxidative environment within the cell. Restoration of the blood glucose levels by insulin treatment effects

the abundance of enzymes that function in the detoxification of ROS, restoring the redox balance and mitochondrial function.

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Supplementary Table 1. Complete table of proteins differentially expressed.

Access	Peptides	Names	Score	Anova (p)*	Fold Change - groups	DM1 regulation	Insulin condition	Mw (Da)	pI	Function	Average Normalised Abundances		
											C	DM1	DM1+I
Amino acid metabolism													
P07756	193 (154)	Carbamoyl-phosphate synthase [ammonia], mitochondrial OS=Rattus norvegicus GN=Cps1 PE=1 SV=1	1770.95	4.86E-05	1.54	up	dependent	164,580	6.33	urea cycle	51.23	78.96	55.57
P09034	38 (29)	Argininosuccinate synthase OS=Rattus norvegicus GN=Ass1 PE=2 SV=1	423.56	2.15E-04	2.00	up	dependent	46,496	7.63	urea cycle	21.03	42.12	25.94
P07824	34 (28)	Arginase-1 OS=Rattus norvegicus GN=Arg1 PE=1 SV=2	358.47	7.10E-04	1.42	up	independent	34,973	6.76	urea cycle	15.1	21.49	18.96
P20673	6 (3)	Argininosuccinate lyase OS=Rattus norvegicus GN=Asl PE=2 SV=1	48.16	4.84E-06	3.43	up	dependent	51,550	5.99	urea cycle	1.17	4.02	1.6
P00481	24 (21)	Ornithine carbamoyltransferase, mitochondrial OS=Rattus norvegicus GN=Otc PE=1 SV=1	195.93	0.02	1.16	down	independent	39,886	9.12	urea cycle	14.93	15.16	17.38
Q03248	21 (13)	Beta-ureidopropionase OS=Rattus norvegicus GN=Upb1 PE=1 SV=1	167.62	0.02	1.22	down	other	44,042	6.47	amino-acid biosynthesis; beta-alanine biosynthesis.	10.46	10.58	12.72
P18757	19 (10)	Cystathionine gamma-lyase OS=Rattus norvegicus GN=Cth PE=1 SV=2	161.7	8.75E-03	1.50	up	independent	43,605	8.2	amino-acid biosynthesis, Cysteine biosynthesis	14.09	21.19	20.89
Q63342	36 (28)	Dimethylglycine dehydrogenase, mitochondrial OS=Rattus	279.63	2.63E-06	1.35	up	independent	96,048	6.91	amino-acid betaine catabolic process	11.07	14.47	14.9

		norvegicus GN=Dmgdh PE=1 SV=1											
P12007	16 (12)	Isovaleryl-CoA dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Ivd PE=1 SV=2	105.67	3.79E-04	1.34	down	independent	46,435	8.03	amino-acid degradation; L-leucine degradation	2.59	2.38	3.18
P09606	13 (10)	Glutamine synthetase OS=Rattus norvegicus GN=Glul PE=1 SV=3	98.15	9.86E-04	1.72	down	dependent	42,268	6.64	glutamate metabolic process	3.22	2.6	4.48
P11960	14 (8)	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial (Fragment) OS=Rattus norvegicus GN=Bckdha PE=1 SV=1	94.86	0.01	1.30	up	independent	50,164	7.68	branched-chain amino acid catabolic process	3.95	5.12	4.65
P35738	13 (8)	2-oxoisovalerate dehydrogenase subunit beta, mitochondrial OS=Rattus norvegicus GN=Bckdhb PE=1 SV=3	88.35	6.72E-04	1.27	up	independent	42,823	6.4	branched-chain amino acid catabolic process	1.74	2.21	2.17
P10868	8 (6)	Guanidinoacetate N-methyltransferase OS=Rattus norvegicus GN=Gamt PE=1 SV=2	65.16	1.13E-04	1.66	down	dependent	26,407	5.69	amine and polyamine biosynthesis; creatine biosynthesis	1.88	1.22	2.03
P62260	11 (3)	14-3-3 protein epsilon OS=Rattus norvegicus GN=Ywhae PE=1 SV=1	62.48	4.50E-05	1.18	down	other	29,174	4.63	protein target	1.09	1.09	1.28
P35213	3 (1)	14-3-3 protein beta/alpha OS=Rattus norvegicus GN=Ywhab PE=1 SV=3	25.75	0.01	1.35	up	independent	28,054	4.81	protein target	2.19	2.96	2.61
P32755	9 (6)	4-hydroxyphenylpyruvate dioxygenase OS=Rattus norvegicus GN=Hpd PE=1 SV=3	61.61	8.68E-04	1.36	up	dependent	45,112	6.29	L-phenylalanine catabolic proces, tyrosine catabolic process	1.71	2.33	1.86
Q5I0C3	7 (4)	Methylcrotonoyl-CoA carboxylase subunit alpha,	40.62	1.95E-03	1.72	up	dependent	79,330	6.66	leucine catabolic proces	0.75	1.18	0.69

		mitochondrial OS=Rattus norvegicus GN=Mccc1 PE=1 SV=1											
P07633	5 (3)	Propionyl-CoA carboxylase beta chain, mitochondrial OS=Rattus norvegicus GN=Pccb PE=2 SV=1	33.44	8.85E-03	1.38	up	dependent	58,626	7.19	cellular amino acid catabolic process, fatty acid catabolic process	1.89	2.6	2.09
P07153	5 (4)	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Rattus norvegicus GN=Rpn1 PE=2 SV=1	33.1	1.58E-04	1.19	down	independent	68,304	6.05	protein glycosylation, peptide transport	1.32	1.11	1.25
P25409	26 (18)	Alanine aminotransferase 1 OS=Rattus norvegicus GN=Gpt PE=1 SV=2	175	2.41E-04	1.55	up	independent	55,110	6.08	amino-acid degradation, biosynthetic process, L-alanine catabolic process	2.63	4.08	3.78
P50554	12 (6)	4-aminobutyrate aminotransferase, mitochondrial OS=Rattus norvegicus GN=Abat PE=1 SV=3	69.77	3.17E-03	1.70	up	dependent	56,456	8.15	neurotransmitter degradation	1.73	2.93	2.12
Energy Metabolism													
P19112	26 (20)	Fructose-1,6-bisphosphatase 1 OS=Rattus norvegicus GN=Fbp1 PE=1 SV=2	175.82	0.01	1.53	up	dependent	39,609	5.54	gluconeogenesis	7.27	11.16	8.75
P07379	32 (23)	Phosphoenolpyruvate carboxykinase, cytosolic [GTP] OS=Rattus norvegicus GN=Pck1 PE=1 SV=1	257.86	1.05E-08	1.82	up	dependent	69,416	6.09	gluconeogenesis	2.46	4.49	3.04
P52873	48 (31)	Pyruvate carboxylase, mitochondrial OS=Rattus norvegicus GN=Pc PE=1 SV=2	357.83	7.54E-04	1.19	up	independent	129,777	6.34	gluconeogenesis, lipid biosynthesis, lipid metabolism	5.2	5.71	6.2
P04694	15 (9)	Tyrosine aminotransferase OS=Rattus norvegicus GN=Tat PE=1 SV=1	84.88	5.82E-04	1.34	down	dependent	50,635	5.27	2-oxoglutarate metabolic process	4.72	3.52	4.49

P04764	31 (17)	Alpha-enolase OS=Rattus norvegicus GN=Eno1 PE=1 SV=4	276.65	6.37E-03	1.22	up	independent	47,128	6.16	glycolysis/gluconeogenesis pathway	6.92	8.46	8.26
P15429	16 (3)	Beta-enolase OS=Rattus norvegicus GN=Eno3 PE=1 SV=3	108.34	3.42E-03	1.38	up	independent	47,014	7.08	glycolysis/gluconeogenesis pathway	2.62	3.61	2.98
P04797	29 (18)	Glyceraldehyde-3-phosphate dehydrogenase OS=Rattus norvegicus GN=Gapdh PE=1 SV=3	244.97	2.52E-04	1.34	up	independent	35,828	8.14	glycolysis/gluconeogenesis pathway	14.67	19.67	19.01
Q9ESV6	23 (9)	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific OS=Rattus norvegicus GN=Gapdhs PE=1 SV=1	146.54	6.78E-05	1.39	up	independent	46,708	8.17	glycolysis/gluconeogenesis pathway	1.67	2.33	2.29
P16617	35 (24)	Phosphoglycerate kinase 1 OS=Rattus norvegicus GN=Pgk1 PE=1 SV=2	306.6	5.16E-04	1.25	down	dependent	44,538	8.02	glycolysis/gluconeogenesis pathway	4.79	4.26	5.33
O35077	16 (12)	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic OS=Rattus norvegicus GN=Gpd1 PE=1 SV=4	98.3	4.11E-05	1.66	down	dependent	37,453	6.16	glycolysis/gluconeogenesis pathway	3.02	1.93	3.21
P38652	4 (2)	Phosphoglucomutase-1 OS=Rattus norvegicus GN=Pgm1 PE=1 SV=2	25.76	3.06E-05	1.74	down	dependent	61,403	6.3	glycolysis/gluconeogenesis pathway	0.95	0.54	0.9
P04642	12 (10)	L-lactate dehydrogenase A chain OS=Rattus norvegicus GN=Ldha PE=1 SV=1	94.14	4.47E-03	1.27	down	dependent	36,451	8.45	lactate metabolic process	2.48	2.08	2.63
Q6P7Q4	6 (6)	Lactoylglutathione lyase OS=Rattus norvegicus GN=Glo1 PE=1 SV=3	42.23	6.97E-03	1.32	down	dependent	20,820	5.12	pyruvate metabolic pathway	1.67	1.47	1.94
Q9ER34	17 (9)	Aconitate hydratase, mitochondrial OS=Rattus norvegicus GN=Aco2 PE=1 SV=2	136.97	2.27E-05	1.75	up	dependent	85,433	7.87	Tricarboxylic acid cycle	1.38	2.41	1.77

Q920L2	21 (14)	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial OS=Rattus norvegicus GN=Sdha PE=1 SV=1	122.47	0.01	1.32	up	independent	71,615	6.75	Tricarboxylic acid cycle	2.33	3.09	2.71
P14408	24 (18)	Fumarate hydratase, mitochondrial OS=Rattus norvegicus GN=Fh PE=1 SV=1	189.87	8.60E-05	1.51	down	dependent	54,464	9.06	Tricarboxylic acid cycle	3.08	4.66	3.65
P13086	15 (9)	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial OS=Rattus norvegicus GN=Suclg1 PE=2 SV=2	117.85	0.02	1.22	up	independent	36,148	9.54	Tricarboxylic acid cycle	3.4	3.74	4.16
P21913	12 (8)	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial OS=Rattus norvegicus GN=Sdhb PE=2 SV=2	92.27	6.23E-03	1.25	up	independent	31,830	8.96	Tricarboxylic acid cycle	1.88	2.35	2.18
O88989	14 (8)	Malate dehydrogenase, cytoplasmic OS=Rattus norvegicus GN=Mdh1 PE=1 SV=3	92.16	0.01	1.28	up	independent	36,483	6.16	Tricarboxylic acid cycle	5.06	6.46	5.75
Q63270	8 (6)	Cytoplasmic aconitate hydratase OS=Rattus norvegicus GN=Aco1 PE=1 SV=1	59.9	1.12E-04	1.36	up	independent	98,128	6.72	Tricarboxylic acid cycle	5.49	7.49	7.11
P49432	3 (2)	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial OS=Rattus norvegicus GN=Pdhb PE=1 SV=2	17	1.24E-03	1.28	up	independent	38,982	6.2	Tricarboxylic acid cycle	3.02	3.48	3.87
Q4QQW3	18 (13)	Hydroxyacid-oxoacid transhydrogenase, mitochondrial OS=Rattus norvegicus GN=Adhfe1 PE=1 SV=1	122.38	2.52E-03	1.48	up	independent	50,226	7.25	Tricarboxylic acid cycle	2	2.97	2.56

P41562	17 (16)	Isocitrate dehydrogenase [NADP] cytoplasmic OS=Rattus norvegicus GN=Idh1 PE=1 SV=1	125.43	3.35E-04	1.41	down	dependent	46,734	6.53	Tricarboxylic acid cycle	2.98	2.12	2.86
Q01205	21 (15)	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial OS=Rattus norvegicus GN=Dlst PE=1 SV=2	132.47	3.64E-03	1.19	up	independent	48,925	8.89	Tricarboxylic acid cycle	4.13	4.93	4.59
P56574	2 (2)	Isocitrate dehydrogenase [NADP], mitochondrial OS=Rattus norvegicus GN=Idh2 PE=1 SV=2	11.13	3.04E-03	1.35	up	dependent	50,967	8.88	intermediary metabolism and energy production, Glyoxylate bypass, Tricarboxylic acid cycle	0.95	1.27	0.96
Q920P0	9 (7)	L-xylulose reductase OS=Rattus norvegicus GN=Dcxr PE=1 SV=1	65.35	3.84E-03	1.38	up	independent	25,720	6.83	Xylose metabolism	3.2	4.41	3.95
Q6AYQ8	7 (6)	Acylpyruvase FAHD1, mitochondrial OS=Rattus norvegicus GN=Fahd1 PE=2 SV=1	44.21	3.36E-03	1.32	down	dependent	24,480	7.21	Oxaloacetate decarboxylase activity	2.27	2.12	2.81
Q02974	8 (6)	Ketohexokinase OS=Rattus norvegicus GN=Khk PE=1 SV=1	38.39	3.39E-03	1.49	down	dependent	32,749	6.24	Fructose metabolism	1.14	0.76	1.03
P10719	46 (37)	ATP synthase subunit beta, mitochondrial OS=Rattus norvegicus GN=Atp5b PE=1 SV=2	460.48	5.72E-05	1.54	up	independent	56,354	5.18	ATP synthesis, Hydrogen ion transport, Ion transport, Transport	34.83	45.68	53.57
P15999	50 (39)	ATP synthase subunit alpha, mitochondrial OS=Rattus norvegicus GN=Atp5a1 PE=1 SV=2	412.06	4.68E-05	1.30	up	independent	59,754	9.22	ATP synthesis, Hydrogen ion transport, Ion transport, Transport	19.09	22.1	24.83
Q06647	19 (14)	ATP synthase subunit O, mitochondrial OS=Rattus norvegicus GN=Atp5o PE=1 SV=1	152.74	1.17E-05	1.30	up	independent	23,398	10.03	ATP synthesis, Hydrogen ion transport, Ion transport, Transport	4.74	5.53	6.14

P31399	12 (10)	ATP synthase subunit d, mitochondrial OS=Rattus norvegicus GN=Atp5h PE=1 SV=3	119.61	3.14E-03	1.25	down	other	18,763	6.16	ATP metabolic process	9.63	9.91	12.02
P21571	9 (9)	ATP synthase-coupling factor 6, mitochondrial OS=Rattus norvegicus GN=Atp5j PE=1 SV=1	59.89	9.10E-03	1.40	up	independent	12,494	9.43	hydrogen ion transport, Ion transport, transport	4.41	5.58	6.18
P29419	6 (3)	ATP synthase subunit e, mitochondrial OS=Rattus norvegicus GN=Atp5i PE=1 SV=3	47.97	0.01	1.39	up	independent	8,225	9.34	ATP synthesis, Hydrogen ion transport, Ion transport, transport	3.09	3.93	4.29
Q6PDU7	2 (2)	ATP synthase subunit g, mitochondrial OS=Rattus norvegicus GN=Atp5l PE=1 SV=2	17.52	0.03	1.14	up	independent	11,433	9.57	ATP synthesis, Hydrogen ion transport, Ion transport, transport	2.21	2.38	2.51
Q68FY0	20 (14)	Cytochrome b-c1 complex subunit 1, mitochondrial OS=Rattus norvegicus GN=Uqcrc1 PE=1 SV=1	164.12	9.02E-04	1.82	up	dependent	52,849	5.57	electron transport, respiratory chain, transport	2.63	4.77	3.49
P11240	15 (10)	Cytochrome c oxidase subunit 5A, mitochondrial OS=Rattus norvegicus GN=Cox5a PE=1 SV=1	122.43	0.01	1.49	up	independent	16,130	6.08	Hydrogen ion transmembrane transport	13.89	20.76	17.13
P12075	13 (11)	Cytochrome c oxidase subunit 5B, mitochondrial OS=Rattus norvegicus GN=Cox5b PE=1 SV=2	97.71	3.31E-04	1.83	up	dependent	13,915	7.68	hydrogen ion transmembrane transport	4.37	7.98	6.17
P62898	9 (7)	Cytochrome c, somatic OS=Rattus norvegicus GN=Cycs PE=1 SV=2	83.51	0.03	1.49	up	independent	11,605	9.61	apoptosis, electron transport, respiratory chain, transport	4.26	5.5	6.34
P10888	10 (5)	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial OS=Rattus norvegicus GN=Cox4i1 PE=1 SV=1	81.2	1.08E-05	1.54	up	dependent	10,515	9.45	response to nutrient	3.95	6.1	4.77

P10818	7 (6)	Cytochrome c oxidase subunit 6A1, mitochondrial OS=Rattus norvegicus GN=Cox6a1 PE=1 SV=2	68.41	3.57E-03	1.86	up	dependent	12,301	9.3	mitochondrial respiratory chain complex IV	6.98	12.95	8.43
P20788	8 (5)	Cytochrome b-c1 complex subunit Rieske, mitochondrial OS=Rattus norvegicus GN=Uqcrfs1 PE=1 SV=2	57.26	4.77E-03	1.50	up	independent	29,446	9.04	electron transport, respiratory chain, transport	2.2	3.3	3.28
Q5M9I5	3 (2)	Cytochrome b-c1 complex subunit 6, mitochondrial OS=Rattus norvegicus GN=Uqcrh PE=3 SV=1	30.39	6.29E-03	1.68	up	independent	10,424	4.9	electron transport, respiratory chain, transport	3.17	5.31	4.59
Q7TQ16	3 (2)	Cytochrome b-c1 complex subunit 8 OS=Rattus norvegicus GN=Uqcrq PE=3 SV=1	25.29	5.40E-03	1.54	up	dependent	9,849	10.52	electron transport, respiratory chain, transport	0.64	0.98	0.73
P10715	4 (3)	Cytochrome c, testis-specific OS=Rattus norvegicus GN=Cyct PE=2 SV=2	22.38	7.96E-05	1.80	up	dependent	11,743	9.34	apoptosis, electron transport, Respiratory chain, Transport	0.53	0.95	0.73
P00406	5 (4)	Cytochrome c oxidase subunit 2 OS=Rattus norvegicus GN=Mtco2 PE=2 SV=3	18.55	2.38E-05	2.33	up	dependent	25,928	4.6	electron transport, respiratory chain, transport	0.97	2.25	1.51
P35171	1 (1)	Cytochrome c oxidase subunit 7A2, mitochondrial OS=Rattus norvegicus GN=Cox7a2 PE=1 SV=1	6.5	2.92E-04	1.51	up	dependent	9,353	10.28	mitochondrial respiratory chain	2.08	3.13	2.45
P00173	14 (11)	Cytochrome b5 OS=Rattus norvegicus GN=Cyb5a PE=1 SV=2	144.57	5.75E-04	1.69	up	dependent	15,355	4.87	electron transport, transport	23.24	39.19	24.67
P04799	3 (1)	Cytochrome P450 1A2 OS=Rattus norvegicus GN=Cyp1a2 PE=1 SV=2	16.01	1.36E-03	1.32	up	independent	58,259	8.95	NADPH-dependent electron transport pathway	3.78	4.99	4.46
P19234	8 (5)	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial OS=Rattus	46.35	8.64E-03	1.59	up	dependent	27,378	6.23	electron transport, respiratory chain, transport	1.14	1.82	1.33

		norvegicus GN=Ndufv2 PE=1 SV=2											
Q561S0	4 (2)	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial OS=Rattus norvegicus GN=Ndufa10 PE=1 SV=1	22.91	0.02	1.47	up	independent	40,493	7.64	electron transport, respiratory chain, transport	0.82	1.2	1.03
Q07116	20 (12)	Sulfite oxidase, mitochondrial OS=Rattus norvegicus GN=Suox PE=1 SV=2	146.06	0.02	1.22	up	independent	60,806	6.41	energy metabolism; sulfur metabolism.	2.1	2.42	2.57
P97519	14 (8)	Hydroxymethylglutaryl-CoA lyase, mitochondrial OS=Rattus norvegicus GN=Hmgcl PE=2 SV=1	102.62	7.75E-04	1.47	up	independent	34,192	8.69	metabolic intermediate metabolism, acyl-CoA metabolic process,	1.36	2	1.87
Q64640	7 (5)	Adenosine kinase OS=Rattus norvegicus GN=Adk PE=1 SV=3	65.13	1.27E-03	1.24	up	independent	40,134	5.72	purine salvage	2.64	3.13	3.28
P13803	24 (19)	Electron transfer flavoprotein subunit alpha, mitochondrial OS=Rattus norvegicus GN=Etfa PE=1 SV=4	254	8.57E-05	1.55	up	independent	34,951	8.62	electron transport,transport	14.72	19.41	22.77
Q68FU3	14 (10)	Electron transfer flavoprotein subunit beta OS=Rattus norvegicus GN=Etfb PE=2 SV=3	135.97	2.13E-03	1.26	up	independent	27,687	7.61	electron transport, transport	7.87	9.89	9.41
Q64428	45 (32)	Trifunctional enzyme subunit alpha, mitochondrial OS=Rattus norvegicus GN=Hadha PE=1 SV=2	324.17	1.30E-05	1.48	up	independent	82,665	9.16	fatty acid metabolism, lipid metabolism	3.93	5.82	5.44
P18163	30 (16)	Long-chain-fatty-acid--CoA ligase 1 OS=Rattus norvegicus GN=Acsl1 PE=1 SV=1	198.67	0.03	1.34	down	dependent	78,179	6.6	fatty acid metabolism, lipid metabolism	6.25	4.88	6.54

Q60587	25 (18)	Trifunctional enzyme subunit beta, mitochondrial OS=Rattus norvegicus GN=Hadhb PE=1 SV=1	182.01	4.16E-04	1.24	up	dependent	51,414	9.5	fatty acid metabolism, lipid metabolism	4.18	5.2	4.19
P21775	17 (14)	3-ketoacyl-CoA thiolase A, peroxisomal OS=Rattus norvegicus GN=Acaa1a PE=1 SV=2	132.26	0.01	1.35	up	independent	43,833	8.53	fatty acid metabolism, lipid metabolism	2.78	3.75	3.16
P23965	15 (11)	Enoyl-CoA delta isomerase 1, mitochondrial OS=Rattus norvegicus GN=Eci1 PE=1 SV=1	125.11	0.01	1.67	up	independent	32,254	9.55	fatty acid metabolism, lipid metabolism	5.94	9.91	8.25
Q9ES38	18 (11)	Bile acyl-CoA synthetase OS=Rattus norvegicus GN=Slc27a5 PE=1 SV=1	116.36	1.24E-03	1.28	up	independent	76,265	8.54	fatty acid metabolism, lipid metabolism	1.31	1.65	1.68
Q3MIE0	11 (4)	Enoyl-CoA hydratase domain-containing protein 3, mitochondrial OS=Rattus norvegicus GN=Echdc3 PE=2 SV=1	79.56	2.58E-03	1.30	up	dependent	32,385	8.83	catalytic activity	0.49	0.64	0.51
Q5XIC0	12 (10)	Enoyl-CoA delta isomerase 2, mitochondrial OS=Rattus norvegicus GN=Eci2 PE=1 SV=1	107.44	5.15E-04	1.36	up	independent	43,021	9.21	fatty acid beta-oxidation	2.12	2.87	2.52
Q64591	9 (3)	2,4-dienoyl-CoA reductase, mitochondrial OS=Rattus norvegicus GN=Decr1 PE=1 SV=2	70.49	5.87E-03	1.94	up	dependent	36,133	9.08	fatty acid beta-oxidation	1.07	2.08	1.23
Q6AYT9	7 (3)	Acyl-coenzyme A synthetase, mitochondrial OS=Rattus norvegicus GN=Acsm5 PE=2 SV=1	52.41	5.57E-04	1.28	up	independent	64,623	8.12	fatty acid metabolism, lipid metabolism	0.71	0.85	0.9
Q62651	5 (3)	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial OS=Rattus norvegicus GN=Ech1 PE=1 SV=2	39.63	8.44E-06	2.67	up	dependent	36,172	8.14	lipid metabolism; fatty acid beta-oxidation.	0.56	1.49	0.73

Q5FVR5	2 (1)	Acyl-coenzyme A amino acid N-acyltransferase 2 OS=Rattus norvegicus GN=Acnat2 PE=2 SV=1	9.16	2.95E-04	1.57	up	independent	46,011	8.64	fatty acid metabolism, lipid metabolism	1.2	1.64	1.88
P45953	6 (5)	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Acadvl PE=1 SV=1	36.64	9.97E-05	1.45	down	dependent	70,750	9.01	fatty acid metabolism, lipid metabolism	0.94	1.01	1.37
O70490	5 (4)	Acyl-CoA synthetase family member 2, mitochondrial OS=Rattus norvegicus GN=Acsf2 PE=2 SV=1	30.79	0.02	1.46	down	dependent	67,886	8.39	fatty acid metabolism, lipid metabolism	0.31	0.21	0.26
P70470	4 (2)	Acyl-protein thioesterase 1 OS=Rattus norvegicus GN=Lypla1 PE=1 SV=1	21.92	5.44E-04	1.44	down	dependent	24,709	6.04	Fatty acid metabolism, Lipid metabolism	0.89	0.77	1.11
Q811X6	3 (3)	Lambda-crystallin homolog OS=Rattus norvegicus GN=Cryl1 PE=2 SV=3	21.07	3.24E-04	3.25	down	dependent	35,341	5.94	fatty acid metabolism, lipid metabolism	2.71	0.92	3
P70473	23 (18)	Alpha-methylacyl-CoA racemase OS=Rattus norvegicus GN=Amacr PE=1 SV=3	204.7	0.03	1.49	up	independent	41,828	6.38	Lipid metabolism; fatty acid metabolism.	6.54	9.74	8.49
Lipid Metabolism													
P22791	36 (20)	Hydroxymethylglutaryl-CoA synthase, mitochondrial OS=Rattus norvegicus GN=Hmgcs2 PE=2 SV=1	340.17	6.86E-03	1.69	up	independent	56,912	8.86	cholesterol biosynthesis, cholesterol metabolism, lipid biosynthesis and metabolism, steroid biosynthesis and metabolism	9.44	15.95	13.26
P52847	18 (13)	Sulfotransferase family cytosolic 1B member 1 OS=Rattus norvegicus GN=Sult1b1 PE=1 SV=2	126.84	6.00E-03	1.19	up	independent	34,835	8.16	lipid metabolism, steroid metabolism	4.86	5.35	5.78

G3V9R3	7 (5)	Sulfotransferase 1 family member D1 OS=Rattus norvegicus GN=Sult1d1 PE=2 SV=1	52.86	9.41E-04	1.31	up	dependent	35,004	5.58	catecholamine metabolism, lipid metabolism	4.82	6.29	4.95
P04639	15 (11)	Apolipoprotein A-I OS=Rattus norvegicus GN=Apoa1 PE=1 SV=2	118.08	5.62E-04	1.72	up	dependent	30,062	5.52	cholesterol metabolism, lipid metabolism and transport, steroid metabolism	1.74	3	2.31
O55171	14 (8)	Acyl-coenzyme A thioesterase 2, mitochondrial OS=Rattus norvegicus GN=Acot2 PE=1 SV=1	91.9	3.76E-03	1.26	up	independent	49,701	6.79	acyl-CoA metabolic process	1.96	2.39	2.46
Q63010	9 (3)	Acyl-coenzyme A thioesterase 2 OS=Rattus norvegicus PE=1 SV=1	56.85	0.02	1.51	up	independent	62,495	6.25	detoxification of xenobiotics	0.19	0.29	0.28
Q711G3	7 (3)	Isoamyl acetate-hydrolyzing esterase 1 homolog OS=Rattus norvegicus GN=Iah1 PE=2 SV=2	45.14	2.85E-03	1.45	down	dependent	28,004	5.63	lipid degradation, lipid metabolism	1.07	0.79	1.14
P02692	18 (14)	Fatty acid-binding protein, liver OS=Rattus norvegicus GN=Fabp1 PE=1 SV=1	185.11	6.45E-04	1.82	down	dependent	14,273	7.79	lipid transport, transport	28.35	15.56	27.98
P11915	29 (22)	Non-specific lipid-transfer protein OS=Rattus norvegicus GN=Scp2 PE=1 SV=3	218.36	0.01	1.26	up	dependent	58,813	6.62	lipid transport, transport	8.95	8.41	10.61
Q9JLJ3	31 (23)	4-trimethylaminobutyraldehyde dehydrogenase OS=Rattus norvegicus GN=Aldh9a1 PE=1 SV=1	251.98	5.51E-04	1.23	down	independent	53,653	6.57	amine and polyamine biosynthesis; carnitine biosynthesis	5.54	6.24	6.82
Q64573	17 (7)	Liver carboxylesterase 4 OS=Rattus norvegicus PE=2 SV=2	119.2	6.77E-03	1.55	down	dependent	62,308	6.29	metabolic process	7.38	5.27	8.15
P16303	25 (23)	Carboxylesterase 1D OS=Rattus norvegicus GN=Ces1d PE=1 SV=2	200.04	0.01	1.42	down	independent	62,147	6.1	lipid degradation, lipid metabolism	7.66	5.41	6.31

Q5BK32	3 (3)	FAS-associated factor 2 OS=Rattus norvegicus GN=Faf2 PE=2 SV=1	23.18	2.37E-03	1.61	up	independent	41,080	5.7	lipid particle organization	1.47	2.23	2.37
Nucleotide Metabolism													
P61980	13 (10)	Heterogeneous nuclear ribonucleoprotein K OS=Rattus norvegicus GN=Hnrnpk PE=1 SV=1	108.05	2.37E-05	1.59	down	dependent	50,976	5.39	mRNA processing, mRNA splicing, Transcription, Transcription regulation	7.98	12.67	9.45
P62961	4 (3)	Nuclease-sensitive element-binding protein 1 OS=Rattus norvegicus GN=Ybx1 PE=2 SV=3	21.78	7.59E-06	1.38	down	dependent	35,730	9.87	mRNA processing, mRNA splicing, Transcription, Transcription regulation	0.75	0.54	0.74
Q5U2T8	1 (1)	Corepressor interacting with RBPJ 1 OS=Rattus norvegicus GN=Cir1 PE=2 SV=1	11.8	0.02	13.12	up	dependent	51,416	9.88	mRNA processing, mRNA splicing, Transcription, Transcription regulation	0.15	2.03	0.18
O70351	17 (15)	3-hydroxyacyl-CoA dehydrogenase type-2 OS=Rattus norvegicus GN=Hsd17b10 PE=1 SV=3	99.7	9.46E-04	1.50	up	independent	27,246	8.91	tRNA processing	6.86	10.26	8.94
Q4QR75	1 (1)	Exosome complex component RRP45 OS=Rattus norvegicus GN=Exosc9 PE=2 SV=1	5.84	0.01	1.64	up	independent	48,882	5.16	rRNA processing	1.17	1.91	1.6
P67779	19 (13)	Prohibitin OS=Rattus norvegicus GN=Phb PE=1 SV=1	158.77	1.44E-03	1.21	up	independent	29,820	5.57	DNA synthesis	4.08	4.89	4.94
Q0VGK3	26 (17)	Glycerate kinase OS=Rattus norvegicus GN=Glyctk PE=2 SV=1	180.26	0.02	1.20	up	independent	55,187	5.84	protein phosphorylation, kinase, transferase	12.25	14.09	14.69
P29410	17 (12)	Adenylate kinase 2, mitochondrial OS=Rattus norvegicus GN=Ak2 PE=2 SV=2	192.86	3.48E-05	1.55	up	independent	26,379	6.33	ATP metabolic process,Kinase, Transferase, oxidative phosphorylation	6.73	10.43	10.25
Q9WUS0	19 (11)	Adenylate kinase 4, mitochondrial OS=Rattus	147.79	6.98E-03	1.22	down	dependent	25,203	7.8	ATP metabolic process, nucleoside diphosphate phosphorylation,	7.47	7.37	8.97

		norvegicus GN=Ak4 PE=2 SV=1								nucleoside triphosphate biosynthetic process, response to drug			
P29411	7 (3)	GTP:AMP phosphotransferase AK3, mitochondrial OS=Rattus norvegicus GN=Ak3 PE=2 SV=2	41.17	6.11E-03	1.30	up	independent	25,438	8.89	homeostasis of cellular nucleotides	0.96	1.19	1.25
P38983	13 (10)	40S ribosomal protein SA OS=Rattus norvegicus GN=Rpsa PE=1 SV=3	84.88	4.56E-03	1.19	down	dependent	32,824	4.8	endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	4.91	4.37	5.18
P62083	6 (5)	40S ribosomal protein S7 OS=Rattus norvegicus GN=Rps7 PE=1 SV=1	40.34	6.73E-04	1.51	up	independent	22,127	10.9	required for rRNA maturation	1.4	1.81	2.11
P05765	4 (2)	40S ribosomal protein S21 OS=Rattus norvegicus GN=Rps21 PE=1 SV=1	23.9	3.34E-04	1.29	down	independent	9,127	8.71	translational elongation	1.32	1.03	1.2
P63324	2 (2)	40S ribosomal protein S12 OS=Rattus norvegicus GN=Rps12 PE=1 SV=2	11.12	1.04E-04	1.50	down	dependent	14,525	6.82	translation	0.52	0.42	0.63
P09895	2 (2)	60S ribosomal protein L5 OS=Rattus norvegicus GN=Rpl5 PE=1 SV=3	19.09	2.94E-03	1.15	down	independent	34,459	9.75	translation	2.61	2.49	2.87
P62832	2 (2)	60S ribosomal protein L23 OS=Rattus norvegicus GN=Rpl23 PE=2 SV=1	11.39	2.71E-03	1.29	down	other	14,865	10.51	translation	1.51	1.57	1.95
P19944	2 (2)	60S acidic ribosomal protein P1 OS=Rattus norvegicus GN=Rplp1 PE=3 SV=1	25.66	5.75E-03	1.37	down	dependent	11,498	4.23	translational elongation	7.83	6.77	9.26
P02401	6 (5)	60S acidic ribosomal protein P2 OS=Rattus norvegicus GN=Rplp2 PE=1 SV=2	56.07	0.01	1.23	down	dependent	11,692	4.4	translational elongation	4.21	4.04	4.97

P19804	14 (8)	Nucleoside diphosphate kinase B OS=Rattus norvegicus GN=Nme2 PE=1 SV=1	127.7	1.10E-03	1.17	up	independent	17,283	6.91	Nucleotide metabolism	7.03	8.26	8.25
Q05982	6 (1)	Nucleoside diphosphate kinase A OS=Rattus norvegicus GN=Nme1 PE=1 SV=1	40.24	6.33E-04	1.47	up	independent	17,193	5.94	differentiation, endocytosis, neurogenesis, nucleotide metabolism	0.24	0.34	0.36
P52759	30 (25)	Ribonuclease UK114 OS=Rattus norvegicus GN=Hrsp12 PE=1 SV=3	207.5	1.93E-06	1.59	down	dependent	14,303	7.8	RNA phosphodiester bond hydrolysis, endonucleolytic, negative regulation of translation	33.52	23.61	37.46
P37805	1 (1)	Transgelin-3 OS=Rattus norvegicus GN=Tagln3 PE=1 SV=2	5.72	0.02	1.59	up	independent	22,501	6.84	negative regulation of transcription from RNA polymerase II promoter	0.73	1.17	1.15
Q5UEM7	1 (1)	Cyclic AMP-responsive element-binding protein 3-like protein 4 OS=Rattus norvegicus GN=Creb3l4 PE=2 SV=1	9.99	4.11E-03	1.46	up	independent	40,281	6.04	transcription, transcription regulation, unfolded protein response	4.46	5.51	6.52
P61459	8 (2)	Pterin-4-alpha-carbinolamine dehydratase OS=Rattus norvegicus GN=Pcbd1 PE=1 SV=2	79.66	2.05E-03	1.42	down	dependent	12,000	6.28	tetrahydrobiopterin biosynthesis, transcription, transcription regulation	0.26	0.18	0.22
Q4V7D6	1 (1)	Putative GTP cyclohydrolase 1 type 2 Nif3l1 OS=Rattus norvegicus GN=Nif3l1 PE=2 SV=1	5.1	0.04	4.85	down	dependent	41,519	6.57	negative regulation of nucleic acid-templated transcription, positive regulation of transcription, DNA-templated	0.1	0.02	0.03
Q63803	4 (3)	Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas OS=Rattus norvegicus GN=Gnas PE=1 SV=3	26.19	1.75E-03	1.80	up	dependent	122,887	4.73	modulators or transducers in various transmembrane signaling systems	0.56	1	0.78

P20595	3 (3)	Guanylate cyclase soluble subunit beta-1 OS=Rattus norvegicus GN=Gucy1b3 PE=1 SV=2	16.17	2.86E-03	1.66	up	independent	70,456	5.23	nitric oxide mediated signal transduction, cGMP biosynthetic process	1.97	3.27	3.18
Q9WTT6	10 (6)	Guanine deaminase OS=Rattus norvegicus GN=Gda PE=1 SV=1	55.85	9.08E-04	3.18	up	dependent	51,016	5.56	guanine catabolic proces	1.77	5.63	2.08
P24329	19 (15)	Thiosulfate sulfurtransferase OS=Rattus norvegicus GN=Tst PE=1 SV=3	191.07	4.48E-03	1.29	up	independent	33,407	7.71	epithelial cell differentiation synthesis	19.76	24.41	25.48
P36972	7 (5)	Adenine phosphoribosyltransferase OS=Rattus norvegicus GN=Aprt PE=1 SV=1	45.58	2.22E-04	1.49	up	independent	19,546	6.15	purine metabolism	1.18	1.75	1.45
P50475	7 (5)	Alanine--tRNA ligase, cytoplasmic OS=Rattus norvegicus GN=Aars PE=1 SV=3	46.75	0.01	1.15	down	dependent	106,790	5.41	catalyzes the attachment of alanine to tRNA(Ala)	1.71	1.51	1.75
Q62655	2 (1)	Transcription factor 4 OS=Rattus norvegicus GN=Tcf4 PE=2 SV=2	9.1	0.02	1.62	down	other	63,053	6.95	transcription, transcription regulation	0.09	0.08	0.13
Q5XI72	5 (3)	Eukaryotic translation initiation factor 4H OS=Rattus norvegicus GN=Eif4h PE=1 SV=1	40.02	2.65E-03	1.40	down	dependent	27,324	6.66	protein biosynthesis	3.65	4.25	5.1
P05197	37 (27)	Elongation factor 2 OS=Rattus norvegicus GN=Eef2 PE=1 SV=4	253.83	0.03	1.15	up	independent	95,284	6.41	protein biosynthesis	4.21	4.85	4.65
P85834	7 (6)	Elongation factor Tu, mitochondrial OS=Rattus norvegicus GN=Tufm PE=1 SV=1	59.71	5.95E-03	1.91	up	dependent	49,522	7.23	protein biosynthesis	6.51	12.42	7.39
Chaperones and oxidative stress													
P63039	53 (42)	60 kDa heat shock protein, mitochondrial OS=Rattus	540.64	1.77E-03	1.26	up	independent	60,955	5.91	chaperone	12.5	15.76	15.24

		norvegicus GN=Hspd1 PE=1 SV=1											
P48721	50 (29)	Stress-70 protein, mitochondrial OS=Rattus norvegicus GN=Hspa9 PE=1 SV=3	371.5	0.01	1.22	up	independent	73,858	5.97	chaperone	6.4	7.84	7.32
Q66HD0	33 (24)	Endoplasmin OS=Rattus norvegicus GN=Hsp90b1 PE=1 SV=2	245.16	8.45E-03	1.35	up	independent	92,771	4.72	chaperone	6.61	8.94	8.9
P14659	28 (12)	Heat shock-related 70 kDa protein 2 OS=Rattus norvegicus GN=Hspa2 PE=1 SV=2	208.44	0.04	1.53	up	independent	69,642	5.5	chaperone	6.44	9.87	9.19
P26772	25 (19)	10 kDa heat shock protein, mitochondrial OS=Rattus norvegicus GN=Hspe1 PE=1 SV=3	171.89	0.01	1.33	up	independent	19,902	8.89	chaperone	12.95	16.18	17.19
P55063	19 (9)	Heat shock 70 kDa protein 1-like OS=Rattus norvegicus GN=Hspa1l PE=2 SV=2	143.06	3.23E-03	1.24	up	independent	70,549	5.91	chaperone	2.84	3.53	3.47
P13084	5 (3)	Nucleophosmin OS=Rattus norvegicus GN=Npm1 PE=1 SV=1	41.46	0.02	1.18	up	independent	32,560	4.62	chaperone	1.22	1.44	1.33
P35565	5 (4)	Calnexin OS=Rattus norvegicus GN=Canx PE=1 SV=1	32.42	8.96E-03	1.50	up	dependent	67,255	4.48	chaperone	2.92	4.39	3.35
P62076	3 (2)	Mitochondrial import inner membrane translocase subunit Tim13 OS=Rattus norvegicus GN=Timm13 PE=3 SV=1	24.51	6.07E-03	1.21	up	independent	10,458	8.42	chaperone, preotin transporter	2.57	3.1	2.97
P97576	2 (2)	GrpE protein homolog 1, mitochondrial OS=Rattus norvegicus GN=Grpel1 PE=1 SV=2	17.78	7.56E-03	1.59	up	dependent	24,297	8.57	chaperone	0.39	0.62	0.39

Q9WV97	3 (2)	Mitochondrial import inner membrane translocase subunit Tim9 OS=Rattus norvegicus GN=Timm9 PE=1 SV=3	16.24	1.85E-04	1.32	up	independent	10,376	6.71	chaperone	0.48	0.64	0.52
Q66H94	1 (1)	Peptidyl-prolyl cis-trans isomerase FKBP9 OS=Rattus norvegicus GN=Fkbp9 PE=2 SV=1	11.59	2.62E-04	1.87	up	independent	63,127	4.93	chaperone-mediated protein folding	6.44	11.63	12.05
P06761	42 (31)	78 kDa glucose-regulated protein OS=Rattus norvegicus GN=Hspa5 PE=1 SV=1	359.35	1.82E-03	1.29	down	dependent	72,347	5.07	chaperone	8.38	6.82	8.78
P63018	40 (19)	Heat shock cognate 71 kDa protein OS=Rattus norvegicus GN=Hspa8 PE=1 SV=1	331.82	0.04	1.13	down	independent	70,871	5.37	chaperone, repressor	6.51	6.75	7.38
P18418	26 (24)	Calreticulin OS=Rattus norvegicus GN=Calr PE=1 SV=1	217.37	4.64E-03	1.42	down	dependent	47,995	4.33	chaperone	8.09	5.68	7.78
P34058	22 (11)	Heat shock protein HSP 90-beta OS=Rattus norvegicus GN=Hsp90ab1 PE=1 SV=4	139.55	1.19E-03	1.34	down	dependent	83,281	4.96	chaperone, cellular response to interleukin-4, negative regulation of neuron apoptotic process	6.26	4.97	6.67
Q63716	27 (24)	Peroxiredoxin-1 OS=Rattus norvegicus GN=Prdx1 PE=1 SV=1	302.98	0.02	1.30	down	dependent	22,109	8.27	redox regulation	18.21	16.07	20.95
Q9R063	16 (9)	Peroxiredoxin-5, mitochondrial OS=Rattus norvegicus GN=Prdx5 PE=1 SV=1	119.8	0.01	1.22	up	independent	21,179	8.94	cellular response to reactive oxygen species	3.04	3.73	3.71
Q9Z0V5	9 (6)	Peroxiredoxin-4 OS=Rattus norvegicus GN=Prdx4 PE=2 SV=1	85.3	1.04E-04	1.72	down	dependent	31,007	6.18	response to reactive oxygen species	0.85	0.49	0.77
P35704	4 (4)	Peroxiredoxin-2 OS=Rattus norvegicus GN=Prdx2 PE=1 SV=3	34.26	3.43E-04	1.39	up	independent	21,784	5.34	removal of superoxide radicals	1.31	1.82	1.59

P07632	26 (19)	Superoxide dismutase [Cu-Zn] OS=Rattus norvegicus GN=Sod1 PE=1 SV=2	207.88	8.38E-03	1.54	down	dependent	15,912	5.88	antioxidant, oxidoreductase	65.94	49.48	75.97
P04762	54 (43)	Catalase OS=Rattus norvegicus GN=Cat PE=1 SV=3	546.24	3.05E-05	1.81	down	dependent	59,757	7.07	hydrogen peroxide	25.66	14.41	26.02
P30713	3 (2)	Glutathione S-transferase theta-2 OS=Rattus norvegicus GN=Gstt2 PE=1 SV=3	19.51	1.91E-03	1.32	down	dependent	27,439	7.75	glutathione metabolic process	1.38	1.14	1.51
P46418	29 (6)	Glutathione S-transferase alpha-5 OS=Rattus norvegicus GN=Gsta5 PE=1 SV=2	192.64	1.15E-04	2.01	up	dependent	25,347	8.42	xenobiotic catabolic process, response to drug, response to nutrient levels	1.26	2.53	1.56
Q6AXY0	19 (6)	Glutathione S-transferase A6 OS=Rattus norvegicus GN=Gsta6 PE=1 SV=1	123.35	8.36E-05	1.45	up	independent	25,808	5.9	glutathione metabolic process	1.07	1.56	1.3
P14942	18 (8)	Glutathione S-transferase alpha-4 OS=Rattus norvegicus GN=Gsta4 PE=1 SV=2	101.76	1.14E-03	1.22	up	independent	25,510	6.77	xenobiotic metabolic process	1.96	2.35	1.92
P24473	3 (2)	Glutathione S-transferase kappa 1 OS=Rattus norvegicus GN=Gstk1 PE=1 SV=3	26.74	2.96E-03	1.66	up	dependent	25,493	9.13	glutathione metabolic process	1.11	1.83	1.41
P04906	1 (1)	Glutathione S-transferase P OS=Rattus norvegicus GN=Gstp1 PE=1 SV=2	6.2	5.48E-03	1.53	up	dependent	23,439	6.89	cellular response to insulin stimulus, Glutathione conjugation	0.63	0.97	0.76
P11232	8 (7)	Thioredoxin OS=Rattus norvegicus GN=Txn PE=1 SV=2	55.24	0.04	1.20	up	independent	11,673	4.8	Electron transport, Transcription, Transcription regulation, Transport	4.97	5.49	5.99
Q9Z0V6	5 (4)	Thioredoxin-dependent peroxide reductase, mitochondrial OS=Rattus norvegicus GN=Prdx3 PE=1 SV=2	32.31	4.50E-05	1.61	up	dependent	28,295	7.14	negative regulation of neuron apoptotic process	0.57	0.92	0.63

P97615	2 (2)	Thioredoxin, mitochondrial OS=Rattus norvegicus GN=Txn2 PE=2 SV=1	12.7	0.01	1.38	up	independent	18,232	7.74	cell redox homeostasis, Electron transport, Transport	1.35	1.86	1.82
P28492	5 (4)	Glutaminase liver isoform, mitochondrial OS=Rattus norvegicus GN=Gls2 PE=2 SV=3	37.92	1.55E-03	1.89	up	dependent	66,248	7.07	glutamine metabolic process, reactive oxygen species metabolic process	0.72	1.36	0.95
Q5XI73	4 (3)	Rho GDP-dissociation inhibitor 1 OS=Rattus norvegicus GN=Arhgdia PE=1 SV=1	37.71	0.01	1.22	up	independent	23,407	5.1	cellular response to redox state	1.45	1.77	1.58
P14141	31 (25)	Carbonic anhydrase 3 OS=Rattus norvegicus GN=Ca3 PE=1 SV=3	342.99	9.17E-09	14.22	down	dependent	29,431	6.89	response to oxidative stress	60.41	4.25	48.54
P06214	14 (8)	Delta-aminolevulinic acid dehydratase OS=Rattus norvegicus GN=Alad PE=1 SV=1	96.67	5.27E-03	1.25	down	independent	36,032	6.03	heme biosynthesis, Porphyrin biosynthesis	1.76	1.41	1.63
Q9Z0U5	8 (4)	Aldehyde oxidase 1 OS=Rattus norvegicus GN=Aox1 PE=1 SV=1	52.15	0.02	1.68	down	dependent	146,921	6.55	prominent source of superoxide generation	1.12	0.67	1.09
P29315	6 (2)	Ribonuclease inhibitor OS=Rattus norvegicus GN=Rnh1 PE=1 SV=2	47.27	0.02	1.35	down	dependent	49,974	4.67	may play a role in redox homeostasis	0.7	0.52	0.64
Q68FT9	4 (3)	Selenocysteine lyase OS=Rattus norvegicus GN=Scly PE=1 SV=1	31.23	0.04	1.45	down	other	47,256	6.16	negative regulation of cellular response to oxidative stress, response to insulin	0.38	0.37	0.54
Transport and Binding													
P02091	50 (7)	Hemoglobin subunit beta-1 OS=Rattus norvegicus GN=Hbb PE=1 SV=3	347.37	4.85E-03	1.45	up	independent	15,979	7.87	oxygen transport, transport,	150.28	217.73	210.2
P11517	48 (5)	Hemoglobin subunit beta-2 OS=Rattus norvegicus PE=1 SV=2	335.68	5.03E-03	1.40	up	independent	15,982	8.91	oxygen transport, transport,	17.5	24.5	23.87

P01946	55 (45)	Hemoglobin subunit alpha-1/2 OS=Rattus norvegicus GN=Hba1 PE=1 SV=3	271.39	6.74E-03	1.50	up	independent	15,329	7.81	oxygen transport, transport,	180.38	270.65	237.64
Q8VIF7	26 (18)	Selenium-binding protein 1 OS=Rattus norvegicus GN=Selenbp1 PE=1 SV=1	213.32	0.04	1.17	up	independent	52,532	6.1	protein transport	5.29	5.93	6.2
Q9QXQ0	25 (19)	Alpha-actinin-4 OS=Rattus norvegicus GN=Actn4 PE=1 SV=2	171.22	0.04	1.15	down	independent	104,915	5.27	protein transport, transport	12.16	10.81	12.45
Q498T9	9 (3)	Volume-regulated anion channel subunit LRRC8C OS=Rattus norvegicus GN=Lrrc8c PE=2 SV=1	49.37	9.99E-04	1.54	up	dependent	90,162	8.15	differentiation, ion transport, transport	0.31	0.48	0.38
Q5U308	7 (3)	Volume-regulated anion channel subunit LRRC8D OS=Rattus norvegicus GN=Lrrc8d PE=2 SV=1	39.06	1.41E-05	1.31	up	independent	97,940	7.14	ion transport, transport	0.85	1.1	1.12
Q498T9	9 (3)	Volume-regulated anion channel subunit LRRC8C OS=Rattus norvegicus GN=Lrrc8c PE=2 SV=1	49.37	9.99E-04	1.54	up	dependent	92,463	7.97	ion transport, transport	0.31	0.48	0.38
P02761	6 (6)	Major urinary protein OS=Rattus norvegicus PE=1 SV=1	42.37	6.54E-05	1.45	down	dependent	20,737	5.85	behavior, transport	3.04	2.1	2.74
P02696	6 (3)	Retinol-binding protein 1 OS=Rattus norvegicus GN=Rbp1 PE=1 SV=2	41.79	0.01	1.42	up	independent	15,834	5.1	retinol metabolic process	0.87	1.23	1.2
P06768	1 (1)	Retinol-binding protein 2 OS=Rattus norvegicus GN=Rbp2 PE=1 SV=3	5.08	9.90E-04	1.20	down	independent	15,585	5.9	retinol-binding, vitamin A, transport	1.29	1.31	1.55
P04276	6 (5)	Vitamin D-binding protein OS=Rattus norvegicus GN=Gc PE=1 SV=3	39.08	4.85E-05	1.46	down	dependent	53,544	5.65	transport	1.28	0.9	1.32
P63029	5 (4)	Translationally-controlled tumor protein OS=Rattus norvegicus GN=Tpt1 PE=1 SV=1	36.89	0.03	1.27	down	dependent	19,462	4.76	involved in calcium binding	1.38	1.31	1.66

P36201	3 (3)	Cysteine-rich protein 2 OS=Rattus norvegicus GN=Crip2 PE=2 SV=1	36.39	0.01	1.59	down	dependent	22,696	8.94	binding protein	1.29	1.31	2.06
P02764	3 (1)	Alpha-1-acid glycoprotein OS=Rattus norvegicus GN=Orm1 PE=2 SV=1	21.06	1.39E-04	1.24	up	independent	23,575	5.64	acute phase, transport	2.54	3.13	2.93
Q6AYA5	2 (1)	Transmembrane protein 106B OS=Rattus norvegicus GN=Tmem106b PE=1 SV=1	16.07	0.03	2.44	up	dependent	31,152	6.89	transport	0.05	0.1	0.04
Q9Z2L0	10 (8)	Voltage-dependent anion-selective channel protein 1 OS=Rattus norvegicus GN=Vdac1 PE=1 SV=4	70.85	5.90E-05	1.37	up	independent	30,756	8.62	apoptosis, ion transport, transport	1.14	1.56	1.47
Q9JHW5	2 (2)	Vesicle-associated membrane protein 7 OS=Rattus norvegicus GN=Vamp7 PE=1 SV=1	10.71	3.88E-04	1.99	up	dependent	24,776	8.71	exocytosis, protein transport, transport	0.37	0.74	0.41
Q5U206	2 (2)	Calmodulin-like protein 3 OS=Rattus norvegicus GN=Calml3 PE=2 SV=1	15.6	2.18E-04	1.51	down	dependent	16,803	4.18	calcium, metal-binding	0.41	0.35	0.52
Other functions													
Q10758	44 (31)	Keratin, type II cytoskeletal 8 OS=Rattus norvegicus GN=Krt8 PE=1 SV=3	375.21	4.90E-03	1.65	up	dependent	54,019	5.83	contractile apparatus	5.49	9.04	6.39
Q6IFU7	6 (3)	Keratin, type I cytoskeletal 42 OS=Rattus norvegicus GN=Krt42 PE=3 SV=1	38.01	0.01	1.57	down	dependent	50,213	5.09	intermediate filamen	1.08	0.98	1.55
P45592	15 (10)	Cofilin-1 OS=Rattus norvegicus GN=Cfl1 PE=1 SV=3	108.41	2.10E-04	1.28	up	independent	18,533	8.22	actin cytoskeleton dynamics	2.13	2.73	2.61
P80254	19 (13)	D-dopachrome decarboxylase OS=Rattus norvegicus GN=Ddt PE=1 SV=3	192.35	0.03	1.33	down	dependent	13,133	6.09	melanin biosynthesis, inflammatory response	41	32.73	43.44

P04905	34 (18)	Glutathione S-transferase Mu 1 OS=Rattus norvegicus GN=Gstm1 PE=1 SV=2	314.46	5.57E-04	2.09	down	dependent	25,914	8.27	olfaction, Sensory transduction	18.54	8.89	16.89
Q03336	31 (24)	Regucalcin OS=Rattus norvegicus GN=Rgn PE=1 SV=3	305.18	3.28E-03	1.45	down	dependent	33,390	5.27	ascorbate biosynthesis	13.95	9.64	13.07
P51635	26 (19)	Alcohol dehydrogenase [NADP(+)] OS=Rattus norvegicus GN=Akr1a1 PE=1 SV=2	197.4	3.26E-04	1.28	down	independent	36,506	6.84	aldehyde catabolic process	3.58	3.87	4.57
P22734	18 (14)	Catechol O-methyltransferase OS=Rattus norvegicus GN=Comt PE=1 SV=2	189.76	1.83E-06	1.63	down	dependent	29,597	5.41	catecholamine metabolism, neurotransmitter degradation	11.11	7.5	12.22
Q68G31	18 (10)	Phenazine biosynthesis-like domain-containing protein OS=Rattus norvegicus GN=Pbld PE=2 SV=1	152.91	5.31E-04	1.18	up	independent	31,687	5.9	biosynthetic process, negative regulation of epithelial cell migration	3.57	4.22	4.08
P50237	20 (17)	Sulfotransferase 1C1 OS=Rattus norvegicus GN=Sult1c1 PE=1 SV=1	148.14	1.49E-04	1.76	down	dependent	35,763	6.1	metabolic process	4.87	2.76	4.62
P14046	25 (6)	Alpha-1-inhibitor 3 OS=Rattus norvegicus GN=A1i3 PE=1 SV=1	136.21	8.14E-04	1.34	down	dependent	163,773	5.7	inflammatory response	1.9	1.41	1.72
Q00729	15 (2)	Histone H2B type 1-A OS=Rattus norvegicus GN=Hist1h2ba PE=1 SV=2	82.82	2.41E-03	1.35	up	independent	14,225	10.29	inflammatory response	1.9	2.57	2.54
Q00715	18 (4)	Histone H2B type 1 OS=Rattus norvegicus PE=1 SV=2	114.34	1.11E-03	1.30	up	independent	13,990	10.37	defense response to bacterium	10.58	13.7	13.09
Q5U2Q3	16 (10)	Ester hydrolase C11orf54 homolog OS=Rattus norvegicus PE=1 SV=1	134.36	1.14E-03	1.58	down	dependent	34,993	6.16	hydrolase activity, acting on ester bonds	2.67	1.75	2.76
P13255	14 (8)	Glycine N-methyltransferase OS=Rattus norvegicus GN=Gnmt PE=1 SV=2	129.29	5.35E-05	2.07	up	independent	32,549	7.1	methyltransferase, transferase	3.33	6.9	5.72

P29147	17 (11)	D-beta-hydroxybutyrate dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Bdh1 PE=1 SV=2	125.67	2.65E-04	1.35	up	independent	38,202	9.01	adipose tissue development, response to drug and insulin	6.57	7.8	8.89
P46953	16 (10)	3-hydroxyanthranilate 3,4-dioxygenase OS=Rattus norvegicus GN=Haao PE=1 SV=2	119.06	1.11E-03	1.30	down	dependent	32,582	5.57	cofactor biosynthesis; NAD(+) biosynthesis	3.09	2.54	3.31
P20760	16 (11)	Ig gamma-2A chain C region OS=Rattus norvegicus GN=Igg-2a PE=1 SV=1	117.94	4.42E-03	1.74	up	dependent	35,186	7.72	regulation of actin dynamics for phagocytic cup formation, FCGR activation	3.31	5.09	2.93
P70580	11 (7)	Membrane-associated progesterone receptor component 1 OS=Rattus norvegicus GN=Pgrmc1 PE=1 SV=3	94.48	4.68E-03	1.40	up	dependent	21,598	4.43	axon guidance	5.61	4.34	6.06
P0CC09	9 (7)	Histone H2A type 2-A OS=Rattus norvegicus GN=Hist2h2aa3 PE=1 SV=1	82	6.91E-03	1.29	down	independent	14,095	10.9	component of nucleosome	19.84	22.87	25.5
P05545	10 (5)	Serine protease inhibitor A3K OS=Rattus norvegicus GN=Serpina3k PE=1 SV=3	77.67	3.64E-07	2.63	up	dependent	46,562	5.31	negative regulation of angiogenesis	1.95	0.74	1.81
P62804	11 (8)	Histone H4 OS=Rattus norvegicus GN=Hist1h4b PE=1 SV=2	70.01	1.06E-04	1.31	down	independent	11,367	11.36	component of nucleosome	6.09	6.97	7.98
P56571	8 (7)	ES1 protein homolog, mitochondrial OS=Rattus norvegicus PE=1 SV=2	67.73	7.30E-04	1.39	up	dependent	28,172	9.11	mitochondrial component	3.16	4.37	3.49
P51652	10 (3)	Aldo-keto reductase family 1 member C18 OS=Rattus norvegicus GN=Akr1c18 PE=1 SV=1	66.47	2.75E-03	1.37	up	independent	37,300	5.9	progesterone metabolic process	4.9	6.45	6.72
P62959	8 (5)	Histidine triad nucleotide-binding protein 1	64.82	0.02	1.38	up	independent	13,777	6.36	apoptosis, transcription, transcription regulation	7.03	9.69	8.72

		OS=Rattus norvegicus GN=Hint1 PE=1 SV=5											
P02767	6 (3)	Transthyretin OS=Rattus norvegicus GN=Ttr PE=1 SV=1	64.22	9.39E-04	1.76	down	dependent	15,720	5.77	hormone, thyroid hormone	6.63	3.76	6.18
P28841	10 (6)	Neuroendocrine convertase 2 OS=Rattus norvegicus GN=Pcsk2 PE=1 SV=1	63.8	5.74E-03	1.25	down	independent	70,753	5.91	hormone	2.15	1.89	2.35
P38918	10 (7)	Aflatoxin B1 aldehyde reductase member 3 OS=Rattus norvegicus GN=Akr7a3 PE=1 SV=2	63.17	8.57E-03	1.46	down	dependent	36,747	6.8	aflatoxin catabolic process	2.01	1.52	2.22
Q5XI32	8 (3)	F-actin-capping protein subunit beta OS=Rattus norvegicus GN=Capzb PE=1 SV=1	59.73	0.02	1.33	up	independent	30,629	5.69	actin cytoskeleton organization	1.81	2.41	2
O09175	8 (4)	Aminopeptidase B OS=Rattus norvegicus GN=Rnpep PE=1 SV=2	58.19	0.02	1.82	up	dependent	72,720	5.47	aminopeptidase, hydrolase, metalloprotease, protease	0.99	1.51	0.83
P49889	7 (1)	Estrogen sulfotransferase, isoform 3 OS=Rattus norvegicus GN=Ste PE=1 SV=1	56.81	3.84E-06	17.62	down	dependent	35,416	5.57	estrogen metabolic process	5	0.28	3.83
O88794	9 (8)	Pyridoxine-5'-phosphate oxidase OS=Rattus norvegicus GN=Pnpo PE=1 SV=1	55.63	2.18E-03	1.37	up	independent	51,016	5.16	Cofactor biosynthesis	1.15	1.58	1.35
Q64634	9 (1)	UDP-glucuronosyltransferase 1-8 OS=Rattus norvegicus GN=Ugt1a8 PE=2 SV=1	54.39	0.03	1.49	down	dependent	60,060	8.92	cellular response to hormone stimulus, flavonoid biosynthetic process	0.54	0.45	0.36
Q64550	9 (2)	UDP-glucuronosyltransferase 1-1 OS=Rattus norvegicus GN=Ugt1a1 PE=1 SV=1	52.83	0.02	1.19	up	independent	59,663	8.77	acute-phase response	0.95	1.05	1.14

P20767	6 (5)	Ig lambda-2 chain C region OS=Rattus norvegicus PE=4 SV=1	49.98	1.52E-04	2.06	up	dependent	11,318	5.76	antigen binding	0.76	1.29	0.62
O08557	8 (5)	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 OS=Rattus norvegicus GN=Ddah1 PE=1 SV=3	49.52	5.59E-03	1.30	up	independent	31,426	5.75	inhibited by zinc ion	3.86	4.62	5.01
P17475	7 (3)	Alpha-1-antiproteinase OS=Rattus norvegicus GN=Serpina1 PE=1 SV=2	48.94	0.04	1.29	up	independent	46,136	5.7	Acute phase	6.64	8.57	7.13
Q9ESN0	9 (6)	Protein Niban OS=Rattus norvegicus GN=Fam129a PE=2 SV=2	48.52	1.09E-05	1.96	up	dependent	103,465	4.64	stress response, translation regulation	1.54	3.01	1.7
Q6IE52	9 (1)	Murinoglobulin-2 OS=Rattus norvegicus GN=Mug2 PE=1 SV=1	48.08	0.02	1.26	down	dependent	161,589	6.51	protease inhibitor, serine protease inhibitor	0.93	0.81	1.02
Q63617	8 (5)	Hypoxia up-regulated protein 1 OS=Rattus norvegicus GN=Hyou1 PE=1 SV=1	47.46	9.62E-03	1.93	down	dependent	11,289	5.11	negative regulation of hypoxia-induced intrinsic apoptotic signaling pathway	0.57	0.29	0.53
P05182	5 (4)	Cytochrome P450 2E1 OS=Rattus norvegicus GN=Cyp2e1 PE=1 SV=4	45.25	1.85E-03	4.71	up	dependent	56,627	8.6	metabolizes several precarcinogens, drugs	1.21	5.7	1.21
Q5PQT3	6 (2)	Glycine N-acyltransferase OS=Rattus norvegicus GN=Glyat PE=2 SV=1	45.18	4.25E-06	1.39	up	independent	33,899	8.82	detoxification	1.13	1.57	1.32
P06399	9 (3)	Fibrinogen alpha chain OS=Rattus norvegicus GN=Fga PE=1 SV=3	44.17	3.85E-04	1.82	down	dependent	86,686	5.51	adaptive immunity, blood coagulation, hemostasis, immunity,innate immunity	3.55	2.04	3.72
P63102	7 (2)	14-3-3 protein zeta/delta OS=Rattus norvegicus GN=Ywhaz PE=1 SV=1	43.92	0.03	1.10	down	independent	27,771	4.73	establishment of Golgi localization, regulation of cell death	1.13	1.05	1.02
Q64595	7 (4)	cGMP-dependent protein kinase 2 OS=Rattus	43.74	2.53E-03	1.28	down	dependent	87,182	8.6	circadian regulation of gene expression	13.33	10.49	13.45

		norvegicus GN=Prkg2 PE=1 SV=1											
Q5RKJ1	6 (2)	Macrophage erythroblast attacher OS=Rattus norvegicus GN=Maea PE=2 SV=2	41.5	0.02	1.69	up	dependent	45,336	8.95	cell cycle, cell division, erythrocyte maturation	2.11	2.96	3.57
P04466	5 (3)	Myosin regulatory light chain 2, skeletal muscle isoform OS=Rattus norvegicus GN=Mylpf PE=2 SV=2	41.31	3.11E-03	1.34	up	independent	18,969	4.79	immune response	0.46	0.61	0.52
P01835	4 (2)	Ig kappa chain C region, B allele OS=Rattus norvegicus PE=1 SV=1	40.42	7.73E-03	3.37	up	dependent	11,601	4.97	antigen binding	2.53	7.81	2.32
Q69CM7	8 (5)	MYCBP-associated protein OS=Rattus norvegicus GN=Mycbpap PE=2 SV=1	40.16	3.13E-03	1.25	up	independent	106,345	7.88	differentiation, spermatogenesis	0.72	0.91	0.85
P07943	6 (2)	Aldose reductase OS=Rattus norvegicus GN=Akr1b1 PE=1 SV=3	39.37	0.04	1.14	down	independent	35,797	6.26	cellular response to hydrogen peroxide	0.75	0.73	0.83
Q78EJ9	6 (5)	Calpain-8 OS=Rattus norvegicus GN=Capn8 PE=2 SV=1	35.83	0.03	1.34	up	independent	79,555	6	calcium-regulated	1.63	2.19	2.14
Q5RJR8	5 (5)	Leucine-rich repeat-containing protein 59 OS=Rattus norvegicus GN=Lrrc59 PE=1 SV=1	34.16	1.27E-03	1.26	down	dependent	34,869	10	required for nuclear import of FGF1	2.17	2.13	2.67
P31000	4 (2)	Vimentin OS=Rattus norvegicus GN=Vim PE=1 SV=2	33.96	8.68E-06	1.40	up	independent	53,733	5.05	stabilization of type I collagen mRNAs	0.67	0.94	0.8
Q6AYR8	7 (4)	Secernin-2 OS=Rattus norvegicus GN=Scrn2 PE=2 SV=1	37.17	1.08E-04	2.08	down	dependent	46,502	5.25	dipeptidase activity	1.49	0.78	1.61
P80067	5 (3)	Dipeptidyl peptidase 1 OS=Rattus norvegicus GN=Ctsc PE=1 SV=3	30.01	9.83E-05	2.48	down	independent	52,235	6.41	activation of cysteine-type endopeptidase activity involved in apoptotic process	0.64	0.26	0.46

P04550	2 (1)	Parathymosin OS=Rattus norvegicus GN=Ptms PE=1 SV=2	28.52	9.48E-03	1.37	up	independent	11,559	4.15	immune response	0.54	0.74	0.73
P20761	4 (3)	Ig gamma-2B chain C region OS=Rattus norvegicus GN=Igh-1a PE=1 SV=1	28.16	0.03	1.66	up	dependent	36,497	7.69	antigen binding	0.77	0.96	0.57
P13832	4 (2)	Myosin regulatory light chain RLC-A OS=Rattus norvegicus GN=Rlc-a PE=2 SV=2	27.16	0.02	2.29	down	dependent	19,895	4.65	protein targeting to plasma membran	0.46	0.2	0.41
P04355	3 (3)	Metallothionein-2 OS=Rattus norvegicus GN=Mt2 PE=1 SV=1	23.86	0.01	6.41	up	independent	6,145	8.23	bind various heavy metals	0.16	1.03	0.84
Q66HL2	3 (1)	Src substrate cortactin OS=Rattus norvegicus GN=Cttn PE=1 SV=1	21.77	0.04	1.16	down	independent	56,942	5.11	endocytosis	0.56	0.55	0.64
Q68FP2	4 (2)	Serum paraoxonase/lactonase 3 OS=Rattus norvegicus GN=Pon3 PE=2 SV=1	21.66	9.13E-03	1.93	up	dependent	39,458	5.49	activity towards the organophosphate paraxon and aromatic carboxylic acid esters	0.38	0.74	0.41
P97888	3 (1)	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B gamma isoform OS=Rattus norvegicus GN=Ppp2r2c PE=2 SV=1	21.49	6.15E-08	2.17	down	dependent	51,474	5.92	signal transduction	3.8	2.02	4.38
Q9EQV9	4 (2)	Carboxypeptidase B2 OS=Rattus norvegicus GN=Cpb2 PE=2 SV=1	21.23	1.49E-03	1.37	up	dependent	48,827	8.6	blood coagulation, fibrinolysis, hemostasis	4.13	5.64	4.39
P0C0S7	4 (1)	Histone H2A.Z OS=Rattus norvegicus GN=H2afz PE=1 SV=2	20.23	0.02	1.21	up	dependent	13,553	10.58	cellular response to insulin stimulus	0.06	0.08	0.06
Q8R5M5	4 (2)	2-amino-3-carboxymuconate-6-semialdehyde decarboxylase OS=Rattus	19.76	1.87E-03	1.49	up	dependent	38,091	6.02	secondary metabolite metabolism; quinolate metabolism.	0.51	0.77	0.56

		norvegicus GN=Acmsd PE=1 SV=1											
Q9JM53	3 (1)	Apoptosis-inducing factor 1, mitochondrial OS=Rattus norvegicus GN=Aifm1 PE=1 SV=1	19.74	6.64E-03	1.23	up	independent	66,723	9.06	apoptosis	1.91	2.14	2.34
Q6TMG5	4 (1)	NF-kappa-B essential modulator OS=Rattus norvegicus GN=Ikbkg PE=2 SV=1	19.7	0.01	1.58	up	dependent	48,066	5.59	transcription, transcription regulation, B cell homeostasis	1.62	2.57	1.78
P08661	3 (2)	Mannose-binding protein C OS=Rattus norvegicus GN=Mbl2 PE=1 SV=2	19.61	1.54E-03	1.40	down	independent	26,014	5.24	complement activation lectin pathway, complement pathway, immunity, innate immunity	0.39	0.28	0.3
Q00981	2 (1)	Ubiquitin carboxyl-terminal hydrolase isozyme L1 OS=Rattus norvegicus GN=Uchl1 PE=1 SV=2	17.13	7.38E-03	1.75	up	independent	24,838	5.14	Ubl conjugation pathway	0.24	0.42	0.41
P01581	3 (2)	Interferon gamma OS=Rattus norvegicus GN=Ifng PE=2 SV=1	16.25	0.04	1.22	down	independent	17,918	9.41	antiviral defense, growth regulation	3.42	3.62	4.17
P18420	3 (1)	Proteasome subunit alpha type-1 OS=Rattus norvegicus GN=Psma1 PE=1 SV=2	16.16	7.12E-04	1.47	down	dependent	29,518	6.14	immunity	1.44	0.98	1.41
B2RYU8	2 (1)	LYR motif-containing protein 1 OS=Rattus norvegicus GN=Lyrm1 PE=2 SV=1	16.15	0.02	1.97	up	dependent	14,259	9.73	inhibition of apoptosis of preadipocytes.	0.63	1.24	0.84
Q9JHX0	3 (3)	Regulator of G-protein signaling 2 OS=Rattus norvegicus GN=Rgs2 PE=2 SV=1	16.13	0.02	1.15	down	independent	24,323	8.94	cell cycle, translation regulation	1.91	1.67	1.8
Q4V888	2 (2)	Type 2 phosphatidylinositol 4,5-bisphosphate 4-phosphatase OS=Rattus	15.91	0.03	1.32	up	independent	28,024	9.07	hydrolase activity	1.52	2.01	1.9

		norvegicus GN=Tmem55a PE=2 SV=1											
Q9ERW3	3 (1)	Fibroblast growth factor 13 OS=Rattus norvegicus GN=Fgf13 PE=1 SV=2	15.68	0.01	246.27	down	dependent	27,588	9.92	neurogenesis	0.23	9.24E-04	0.11
P08733	3 (2)	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform OS=Rattus norvegicus GN=Myl2 PE=1 SV=2	15.51	1.88E-03	1.51	down	dependent	18,880	4.83	cell growth involved in cardiac muscle cell development , cardiac muscle contraction	1.72	1.57	2.38
A4GG66	3 (1)	Gap junction gamma-1 protein OS=Rattus norvegicus GN=Gjc1 PE=2 SV=1	15.08	0.03	3.18	down	dependent	45,666	6.97	cardiac muscle tissue development, cell-cell signaling	0.8	0.25	0.68
P62839	2 (2)	Ubiquitin-conjugating enzyme E2 D2 OS=Rattus norvegicus GN=Ube2d2 PE=1 SV=1	10.99	0.02	1.17	down	independent	16,735	7.69	Ubl conjugation pathway	0.68	0.59	0.7
Q5U2Y0	2 (1)	WD repeat domain phosphoinositide-interacting protein 4 OS=Rattus norvegicus GN=Wdr45 PE=2 SV=1	10.86	0.04	1.12	down	independent	34,587	6.5	autophagy	0.81	0.83	0.91
Q5XIQ2	2 (1)	ELMO domain-containing protein 3 OS=Rattus norvegicus GN=Elmod3 PE=1 SV=1	10.65	6.10E-04	11.74	down	dependent	40,014	6.73	phagocytosis	0.19	0.02	0.09
P35234	2 (2)	Tyrosine-protein phosphatase non-receptor type 5 OS=Rattus norvegicus GN=Ptpn5 PE=2 SV=2	10.21	0.01	1.31	down	dependent	42,366	4.98	response to immobilization stress , positive regulation of protein phosphorylation	3.11	2.46	3.23
Q6AXU6	2 (1)	Hematological and neurological expressed 1 protein OS=Rattus norvegicus GN=Hn1 PE=2 SV=3	10.01	3.29E-03	1.29	up	independent	15,575	5.03	developmental process	0.38	0.49	0.41

P08426	1 (1)	Cationic trypsin-3 OS=Rattus norvegicus GN=Try3 PE=2 SV=1	9.89	3.30E-04	1.30	up	independent	26,269	7.46	digestion	3.67	4.4	4.76
P02803	1 (1)	Metallothionein-1 OS=Rattus norvegicus GN=Mt1 PE=1 SV=1	8.44	4.57E-03	5.96	up	dependent	6,006	8.38	cellular response to zinc ion, negative regulation of growth	0.3	1.78	1.15
P16409	1 (1)	Myosin light chain 3 OS=Rattus norvegicus GN=Myl3 PE=2 SV=2	5.92	1.20E-05	3.18	up	dependent	22,156	5.03	muscle contraction , regulation of the force of heart contraction	1.17	3.59	1.13
P02706	1 (1)	Asialoglycoprotein receptor 1 OS=Rattus norvegicus GN=Asgr1 PE=1 SV=2	5.52	3.88E-05	2.50	down	dependent	32,849	5.77	endocytosis	0.51	0.2	0.4
O35476	1 (1)	Medium-wave-sensitive opsin 1 OS=Rattus norvegicus GN=Opn1mw PE=1 SV=2	5.34	0.01	1.34	down	independent	40,200	8.56	sensory transduction, vision	0.34	0.25	0.26
O70249	3 (1)	N-glycosylase/DNA lyase OS=Rattus norvegicus GN=Ogg1 PE=2 SV=1	13.97	3.11E-04	1.66	down	dependent	38,711	8.91	DNA damage, DNA repair	0.47	0.34	0.56
P63012	1 (1)	Ras-related protein Rab-3A OS=Rattus norvegicus GN=Rab3a PE=1 SV=1	4.95	0.02	1.35	down	dependent	24,970	4.85	exocytosis, protein transport, transport	0.62	0.52	0.7
P50137	35 (28)	Transketolase OS=Rattus norvegicus GN=Tkt PE=1 SV=1	280.55	0.02	1.37	up	independent	67,644	7,22	ribose phosphate biosynthetic process, pentose-phosphate shunt, non-oxidative branch	10.94	15.03	14.71
O09171	53 (29)	Betaine--homocysteine S-methyltransferase 1 OS=Rattus norvegicus GN=Bhmt PE=1 SV=1	552.47	5.38E-04	1.54	up	independent	44,976	8.02	methyltransferase, transferase	24.67	32.62	38.03
Q6UPE0	25 (20)	Choline dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Chdh PE=1 SV=1	221.62	2.77E-04	1.48	up	independent	66,389	8.75	amine and polyamine biosynthesis; betaine biosynthesis	6.27	8.52	8.52
P18298	13 (6)	S-adenosylmethionine synthase isoform type-2 OS=Rattus norvegicus GN=Mat2a PE=1 SV=1	107.28	5.86E-04	1.41	up	independent	43,716	5.93	amino-acid biosynthesis; S-adenosyl-L-methionine biosynthesis	3.14	4.43	4.42

P13444	17 (9)	S-adenosylmethionine synthase isoform type-1 OS=Rattus norvegicus GN=Mat1a PE=1 SV=2	142.48	4.84E-07	1.85	up	dependent	43,698	5.61	amino-acid biosynthesis; S-adenosyl-L-methionine biosynthesis; S-adenosyl-L-methionine from L-methionine	6.13	11.33	6.94
Q68FT5	24 (8)	S-methylmethionine--homocysteine S-methyltransferase BHMT2 OS=Rattus norvegicus GN=Bhmt2 PE=2 SV=1	177.85	0.02	1.32	down	dependent	39,929	6.18	amino-acid biosynthesis, methionine biosynthetic process, S-methylmethionine cycle	1.16	1.22	1.53
P10860	54 (42)	Glutamate dehydrogenase 1, mitochondrial OS=Rattus norvegicus GN=Glud1 PE=1 SV=2	487.5	0.03	1.27	up	independent	61,416	8.05	oxidoreductase, allosteric regulation	13.04	15.79	16.59
P57113	25 (21)	Maleylacetoacetate isomerase OS=Rattus norvegicus GN=Gstz1 PE=1 SV=2	182.33	7.52E-04	1.26	up	independent	23,961	7.63	phenylalanine catabolism, tyrosine catabolism	5.89	7.39	6.83
Q63150	18 (12)	Dihydropyrimidinase OS=Rattus norvegicus GN=Dpys PE=1 SV=2	111.99	0.01	1.15	down	independent	56,815	6.77	beta-alanine metabolic process, thymine catabolic process, uracil metabolic and catabolic process	3.53	3.46	3.97
Q03626	19 (4)	Murinoglobulin-1 OS=Rattus norvegicus GN=Mug1 PE=2 SV=1	108.95	5.37E-04	1.28	up	independent	165,326	5.68	acute phase	0.29	0.38	0.32
Q0D2L3	14 (10)	Agmatinase, mitochondrial OS=Rattus norvegicus GN=Agmat PE=2 SV=1	108.57	7.80E-04	1.59	up	independent	37,987	6.7	putrescine biosynthesis, spermidine biosynthesis	2.66	4.22	4.13
Q64611	12 (6)	Cysteine sulfinic acid decarboxylase OS=Rattus norvegicus GN=Csad PE=1 SV=1	79.51	1.22E-03	1.37	down	independent	55,249	6.84	carboxylic acid metabolic process,	2.56	1.87	2.08
Q5U300	9 (6)	Ubiquitin-like modifier-activating enzyme 1 OS=Rattus norvegicus GN=Uba1 PE=1 SV=1	46.15	9.39E-05	1.43	down	dependent	117,788	5.36	Ubl conjugation pathway	1.44	1.24	1.77

P69060	1 (1)	N-acylneuraminate cytidylyltransferase OS=Rattus norvegicus GN=Cmas PE=2 SV=1	6.01	4.72E-04	4.02	up	dependent	48,129	8.51	N-acetylneuraminate metabolic process	0.25	1	0.55
P0C2X9	34 (27)	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Aldh4a1 PE=1 SV=1	301.43	2.70E-03	1.19	up	independent	61,869	7.13	proline metabolism	10.48	12.24	12.42
Uncharacterized–associated proteins													
Q68FQ4	2 (1)	Uncharacterized protein C11orf70 homolog OS=Rattus norvegicus PE=2 SV=1	10.67	3.13E-03	1.31	up	dependent	31,058	6.4		0.91	1.2	0.94
Q5RJN9	2 (2)	Uncharacterized protein C14orf79 homolog OS=Rattus norvegicus PE=2 SV=1	9.97	1.38E-04	1.42	up	dependent	34,736	5.31		0.59	0.84	0.61

Capítulo III

Alterations in oxidative damage of proteins in type 1 diabetes using a novel 2D-DIGE method – Oxi-proteome

Abstract

Type 1 diabetes is associated with oxidative damage due to the increased production of reactive oxygen species, which is caused by either the overproduction of reactive oxygen species or the decreased efficiency of antioxidant defenses. This process implicates oxidative stress in the progression of certain diseases, including diabetes. One common measure of oxidative damage is protein oxidation via carbonylation, which can lead to irreversible and irreparable damage to the protein. The carbonylation of proteins is of great interest due to the modification of protein function after carbonylation; therefore, the identification and accurate quantification of altered proteins may lead to the discovery of novel diagnostic and predictive biomarkers. A new method that combines two-dimensional electrophoresis with hydrazide fluorophore derivatization was used to identify carbonylated proteins, in order to identify differentially oxidatively damaged proteins in type 1 diabetes and to annotate their function into different classes of metabolism. Proteomic data were processed; the pathways involved in molecular function, biological processing, and cellular components were identified through bioinformatics. One hundred and forty-four proteins were found to have differential carbonylation ($p<0.05$) in type 1 diabetes, and the pathway most affected in insulin-dependent and -independent diabetes was the metabolism of amino acids and derivatives. These results suggest that oxidative damage alters protein function in the livers of animal without insulin treatment, contributing to disease progression as well as complications in DM1 associated with hyperglycemia.

Keywords: 2D-DIGE, hydrazide, diabetes, insulin, liver, proteomic

1. Introduction

Annually, some 79,000 children worldwide are estimated to develop DM1, and countries such as the United States, India and Brazil have the highest estimated number of new cases per year (1). The development of DM1 is associated strongly with a genetic predisposition, but in some cases environmental factors can cause the autoimmune destruction of β cells with dependence on insulin treatment (1).

Evidence has shown that DM1 is associated with the increased production of free radicals derived from molecular oxygen referred to as reactive oxygen species (ROS) (2). ROS are

believed to act on the majority of cellular structures, causing damage to proteins, cell membranes and nucleic acids, which results in cell death and dysfunction with tissue injury, thus contributing to several pathological conditions (3). Oxidative stress (OS) is described as the imbalance between the overproduction of ROS and decreased efficiency of the antioxidant defenses, a process that takes place early in disease and progresses gradually (4). OS is implicated in many changes that occur not just in aging but also during the progression of certain diseases such as diabetes, neurodegenerative diseases, inflammatory diseases and cancer (5).

Oxidative modification of protein leads to structural changes that may cause partial or total loss of their function; thus, protein oxidative damage plays an important role in many diseases (6). Proteins are known to be oxidized in up to 35 different ways, and studies are being developed to understand the effects of ROS-mediated protein oxidation in disease (7). The most common type of protein oxidation is carbonylation, which results in the introduction of carbonyl groups (ketones and aldehydes) into proteins at different sites by different mechanisms and lead to irreparable damage to the protein (8).

Various analytical methods have been used to detect protein carbonyls using specific chemical derivatizations of the carbonyl groups, including avidin, streptavidin, biotin hydrazide, hydrazines, hydrazides and hydroxylamines. The conventional methods used include calorimetric methods, enzyme-linked immunosorbent assays (ELISA), western blotting, chromatography, mass spectrometry, and fluorescence (9). However, a new technique, 2D-DIGE (two-dimensional differential in-gel electrophoresis), has been reported which uses derivatization with hydrazide fluorophores. With this method, different protein samples are labeled with up to three spectrally distinct fluorescent dyes known as CyDye Dige fluors (Cy2, Cy3 and Cy5), (10), and labeled samples are combined and separated by 2D gel electrophoresis, then visualized separately by their specific excitation wavelengths. The advantages of 2D-DIGE are the ability to run multiple samples on the same gel along with other experimental designs unique to this technique (11). This method has been widely used despite the fact that it is relatively new; however, a search of the literature does not show that this method has been used to detect oxidative damage in the livers of diabetic rats.

The identification of carbonylated proteins is of great interest as their identification and quantification can lead to the discovery of new diagnostic and predictive biomarkers. In this context, the liver proteome of type 1 diabetic rats was analyzed to measure the carbonylation of proteins related to insulin-independent or -dependent DM1.

2. Material and Methods

2.1. Sample collection and preparation

Twenty-four Wistar rats at 45 days of age were used in this study and maintained in an environmentally controlled room (25±2ºC and 12:12 h cycles light/dark) and fed with a standard rat pellet diet and water *ad libitum.* The experimental procedures were approved by the Ethics Committee on Animal Experimentation of the Institute of Biosciences/São Paulo State University (UNESP, Protocol: CEUA-436/2012). The animals were divided in three experimental groups: C, normal rats that received water and food; DM1, diabetic rats that received water and food; DM1+I, diabetic rats treated with insulin replacement, which received water and food. Diabetes mellitus type 1 was induced by the intraperitoneal administration of streptozotocin (STZ, single dose, 60 mg kg^{-1} body weight). After 48 hours, animals with glucose concentrations above 220 mg dL^{-1} were considered diabetic and used in the experiment. DM1+I group received an initial dose of 3U/animal of insulin, Humulin N100UI Neutral Protamine Hagedorn (NPH), Lilly brand, and according to the values obtained for blood glucose, the initial insulin dose was adjusted to maintain serum glucose levels 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 then sacrificed by decapitation. Livers were collected, and 1 g of liver was lyophilized.

The lyophilized samples (~20 mg) were reconstituted in lysis buffer **(**25 mM Tris buffer pH 8, 8 M Urea). The protein concentrations of rat liver samples were determined using the bicinchoninic acid (BCA) method with bovine serum albumin as the standard (Pierce™ BCA Protein Assay Kit). With the standardization of the gels, the electrophoretic runs with liver samples for the different experimental groups were performed. Eight gels were made for each group (one per sample), resulting in a total of 24 gels.

2.2. Derivatization procedure

Two cyanine dyes coupled with hydrazides, Cy3-Hz and Cy5-Hz, were used in the derivatization of carbonylated proteins. Cyanine dyes diluted to 50 mM in dimethyl sulfoxide (DMSO) were added to the derivatization buffer (1:10, 0.1 M sodium acetate pH 5, 1 mM EDTA and 1% SDS). The derivatization solution (cyanine dyes and derivatization buffer) was added to the protein extracts at a concentration of 1 mg μL^{-1} and incubated with light vortexing for 3 hours in the dark. After incubation, Tris (2 M) and $NaCNBH_4$ (200 mM) were added to each tube

containing protein extracts. Samples were then left at room temperature in the dark for 15 min to stop the reaction and stabilize the hydrazone formed.

2.3. Electrophoretic runs

The derived proteins were precipitated with acetone (100%, 1:5 v/v, cold acetone, -20°C, for 2 hours) and centrifuged at 15,000 *g* for 15 min after which the pellets were washed twice with cold acetone. To retain the solubilised proteins, pellets were resuspended in 150 uL of IEF sample buffer with low agitation. The buffer used contained 8 M urea, 2 M thiourea, 2% (w/v) CHAPS (sulfate 3-[(3-cloroaminopropil)-dimethylammonio]-1-propane), 0.5% (v/v) ampholytes at pH ranging from 3 to 10 and 0.002% bromophenol blue (w/v). Then 50 mM DTT was added to this buffer, and the mixture was used for the electrophoretic separations. Individual samples labeled with Cy5-Hz were combined with a Cy3-Hz labeled DM1 pool to remove the variation among animals. Combined samples labeled with fluorophores were added to a 17-cm IEF strip (BioRad) with passive rehydration for 12 h. Strips were placed gel-side down onto the sample and overlaid with mineral oil. After this period, the rehydrated strip was isoelectrically focused to resolve the first dimension (IEF, Step 1: 250 V for 15 min, Step 2: 10,000 V for 3 hours, Step 3: 10,000 V until 55,000 Vh, Step 4: 500 V).

After separation in the first dimension, the strip was equilibrated in two steps. First, strips were incubated in 10 mL of a solution containing 6 M urea, 2% (w/v) SDS, 30% glycerol (v/v), 50 mM Tris-HCl (pH 8.8), 0.002% bromophenol blue (w/v) and 2% (w/v) DTT to reduce the proteins. Second, the strips were incubated using the same solution with 2.5% (w/v) iodoacetamide (IAA) in place of the DTT to alkylate free thiols. Each step lasted for 15 min and was performed under low agitation at room temperature. To resolve the second dimension, the strips were applied to 10% polyacrylamide gels. The strips were sealed to the gels with an agarose solution (0.5% w/v); and the run was done in two steps: 125 V for 20 min and 250 V for 5h 25 min. After running the proteins, gels were fixed for 3 h using 10% acetic acid (v/v) and 40% (v/v) ethanol. Labeled proteins were visualized by fluorescence using a Typhoon FLA-9500 imager. Voltages were optimized using oxidized and derived BSA standards for Cy3 (filter: LPG, excitation: 532 nm, emission: ≥ 575 nm) and Cy5 (Filter: LPR, excitation: 635 nm, emission: ≥ 665 nm). The images were aligned and analyzed using Progenesis SameSpots. To determine the statistical significance between the groups, ANOVA was performed using the Progenesis SameSpots software. The significance level was set to 5%. Following fluorescent detection, the

gels were stained with colloidal Coomassie stain and identified spots were excised for protein identification.

2.4. Protein identification

The gel fragments were treated with cycles of chemical washing and incubation with acetonitrile (ACN) and 50 mM ammonium bicarbonate (ABC) solution (40:60% v/v) at room temperature for 15 min. This process was repeated until all of the stain was removed and the gel fragments are opaque. The gel slice was shrunk in 100% ACN. After ACN removal, the gel slice was placed in a 50°C heat bath to evaporate residual ACN. Trypsin working solution, 1ng/µL in 50 mM ABC buffer, was added to the gel fragment and incubated overnight at 37°C in the dark to digest proteins. The supernatant was transferred to new 1.5-mL Eppendorf tubes, and the tryptic peptides were further extracted from the gel by sonication after the addition of 60% ACN/5% TFA, using a volume sufficient to cover the gel fragment. The supernatant was combined with the previous supernatant and the process was repeated twice more. The pooled supernatants were dried in a SpeedVac, and the tryptic peptides were resuspended in 25 µL of 0.1% formic acid (FA). The resupended peptides (5 µL) were injected for analysis onto a LTQ Velos Pro mass spectrometer coupled to a dual pump Dionex 3000 RSLCnano LC. Samples were loaded onto a C18 PepMap100 µ-precolumn (300 µm i.d. x 5 mm) at a flow rate of 40 µL/min for 4 minutes with Solvent A (0.1% formic acid). Peptides were then eluted onto a 15 cm New Objective PicoFrit Reprosil-PUR analytical column at a flow rate of 400nL/min with the gradient of 3 minutes hold at 3% Solvent B (0.1% formic acid in ACN), ramped to 15% B over 7 minutes, ramped to 28% B over 20 minutes, ramped to 99% B over 6 minutes and held for one minute. The gradient was returned to 3% B in one minute and held for 22 minutes to re-equilibrate the column. Spectra were acquired in the data dependent mode from m/z 400 to 2000 with the top 3 ions selected for CID fragmentation at 35% normalized collision energy and a dynamic exclusion time of 30 s. The MS was set to a nano spray voltage of 1.9 kV and heated capillary temperature of 250°C. Raw data files were converted to mascot generic format using MSConvert and searched against an in-house Mascot database (SwissProt 51.6). The Mascot search allowed for 1 missed cleavage, variable modification of methionine oxidation, and a peptide and fragment ion mass tolerance of 0.6 Da. The protein data were evaluated considering the highest score and/or coverage, and the molecular weight and pI obtained in the 2D-DIGE experiment were compared with data from the UniProt database. After identification, FASTA sequences of the detected proteins were obtained and imported to the Blast2GO program (B2G), which enabled separation

into two levels (molecular function and cellular component) for both insulin-dependent and -independent (12).

2.5. Pathway enrichment analysis

The oxidatively damaged proteins found were separated into insulin-dependent and -independent DM1 groups. Their Unihprot protein IDs were converted to gene symbols for analysis by Reactome Functional Interaction (FI) (13), a Cytoscape plugin (14). Reactome FI allows analysis of proteomic data sets through the comparison with published knowledge about interactions, pathways and biological processes. Pathways with a false discovery rate (FDR) <0.05 were considered to be significantly enriched.

3. Results and discussion

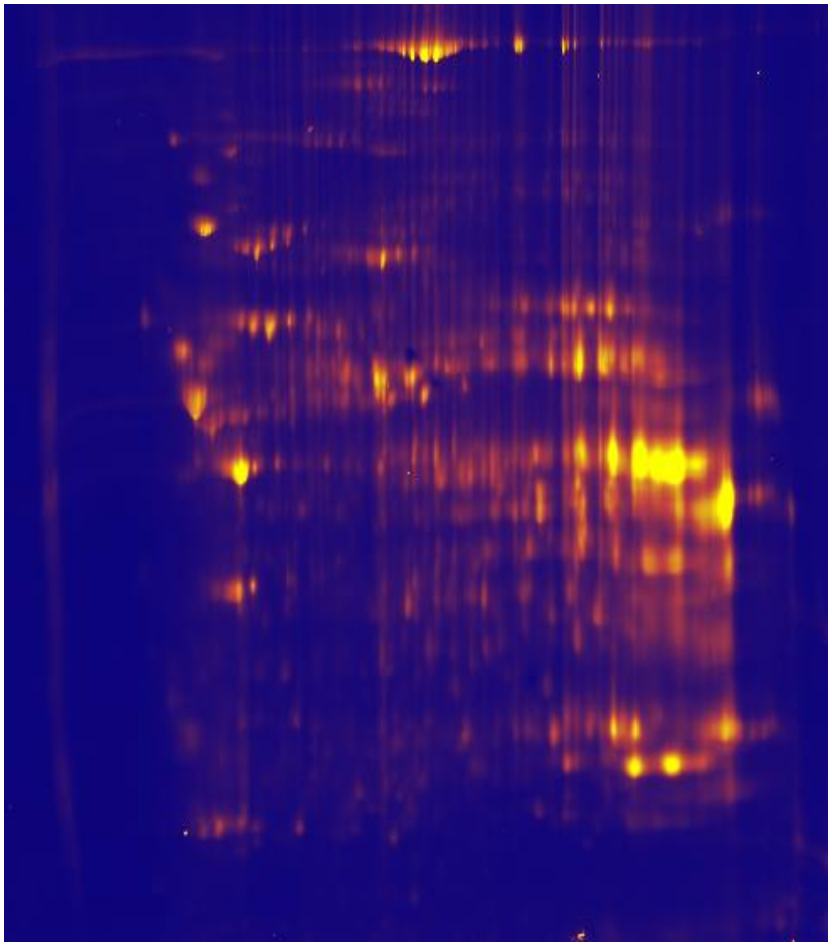
Protein carbonylation is directly associated with protein damage under oxidative stress conditions. This manuscript uses the detection of protein carbonyls by Cy3 and Cy5 hydrazide labeling after combining samples in the same gel to identify modified proteins in insulin-dependent and -independent models. This replaces other strategies such as western blotting, ELISA, and immunodectection as described by Baraibar et al. (2013). (15). In this study, DM1 is associated with oxidative stress, which induces protein oxidative damage as reflected by the level of protein carbonylation. Further, this study showed what proteins were modified and determined the effect of insulin treatment in DM1. Protein targets were mapped to pathways identified in cellular components, molecular processes and molecular pathways.

3.1. Comparative analysis: insulin dependent or independent

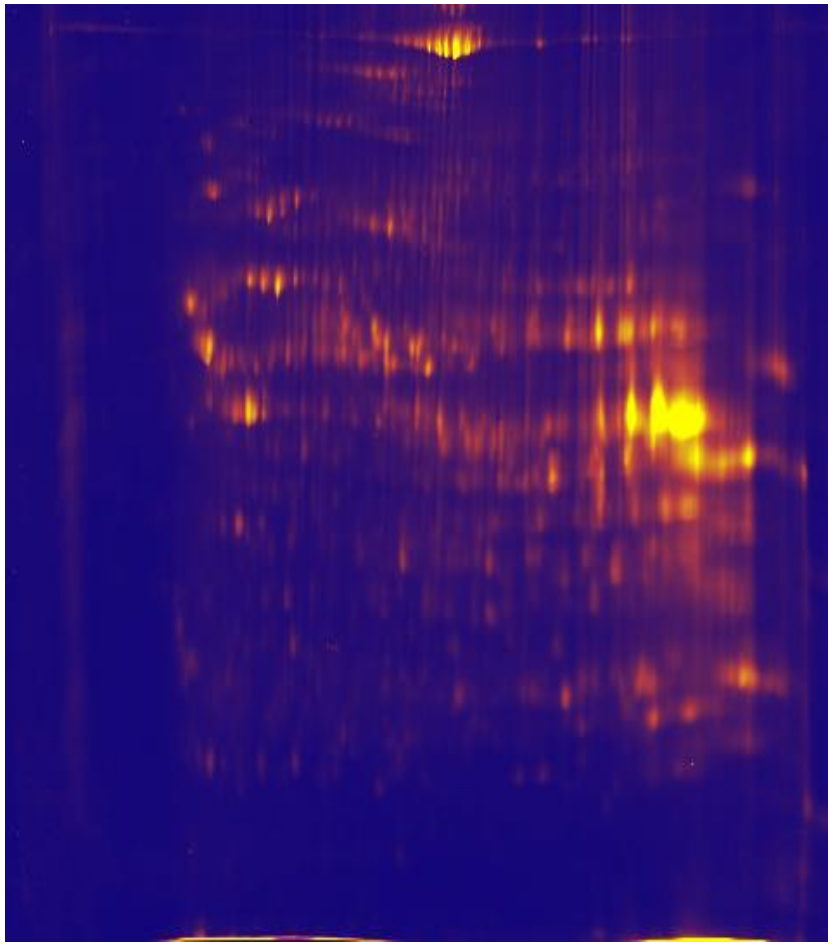
The presence of protein carbonyls has been used as marker of oxidative stress in different research models such as aging and diseases. This study identified 144 differentially oxidatively modified proteins with a $p<0.05$ between C, DM1, DM1+I and DM1 pool (Figure 1). The spot identification (ID), protein name, accession, fold change, mascot score, sequence coverage and peptides, molecular mass and theoretical pI for each protein are shown in Table 1.

Figure 1. *A.* Overlay of control vs. type 1 diabetes pool. *B.* Overlay of type 1 diabetes vs. type 1 diabetes pool. **C.** Overlay of type 1 diabetes pool treated with insulin vs. type 1 diabetes pool. D. Oxidavely damaged proteins with difference expression using 2D-DIGE of diabetic liver.

A. Overlay of control vs. type 1 diabetes pool

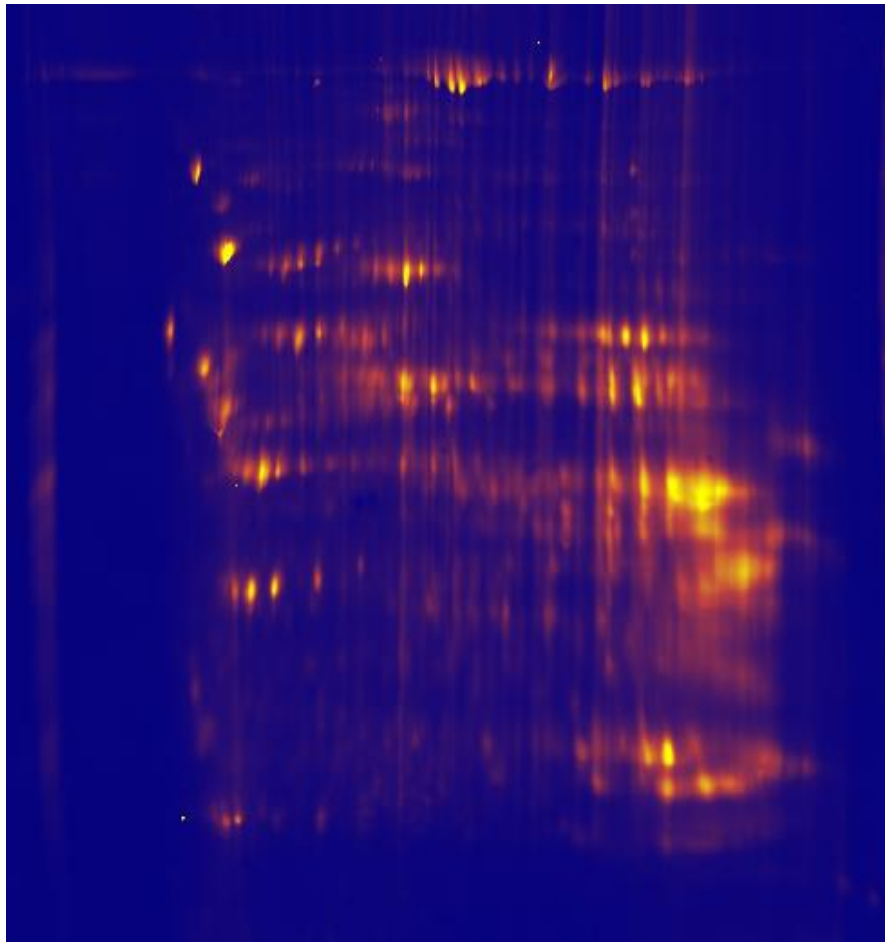


B. Overlay of type 1 diabetes vs. type 1 diabetes pool



C. Overlay of type 1 diabetes pool treated with insulin vs. type 1 diabetes pool

D. Oxidavely damaged proteins with difference expression using 2- DIGE



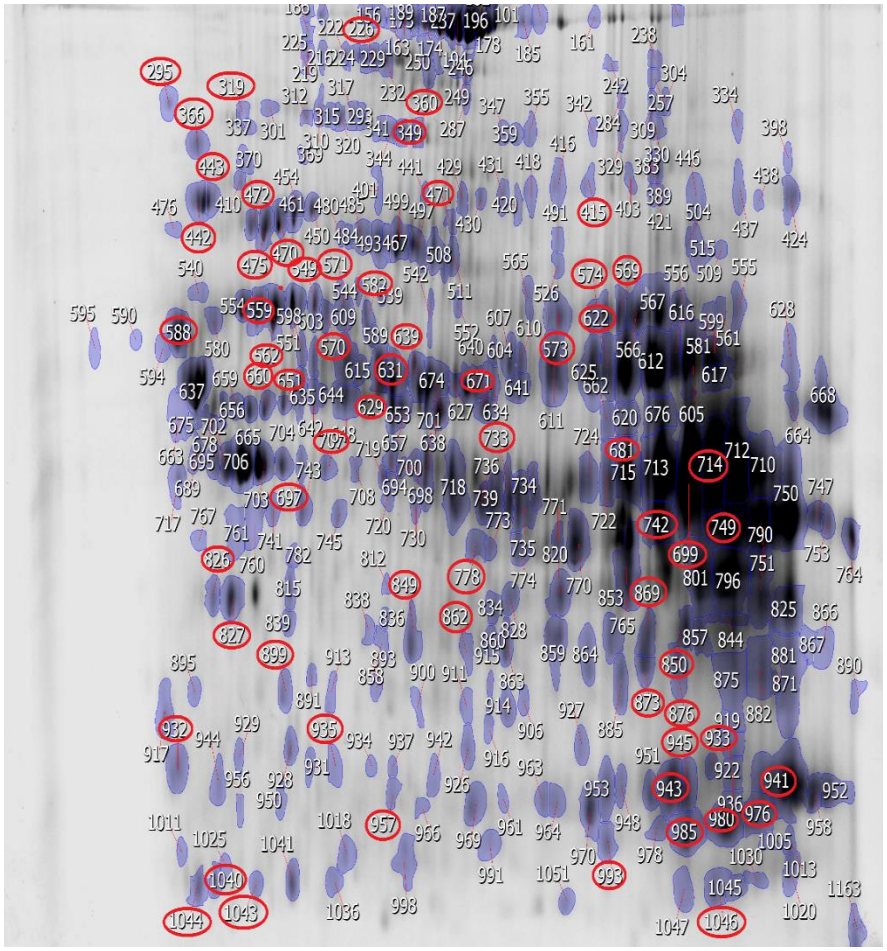
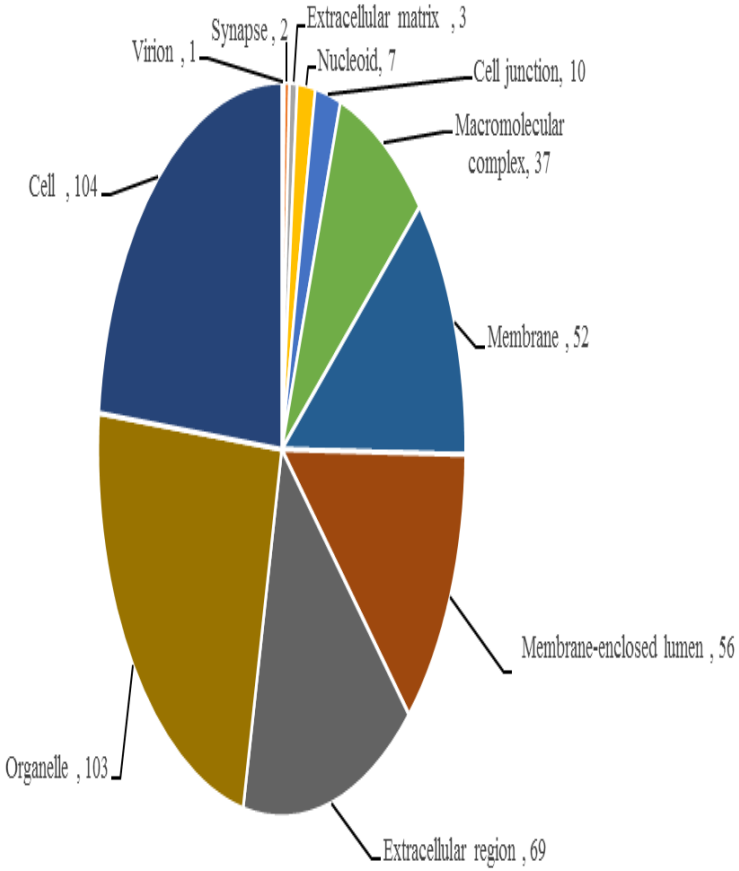
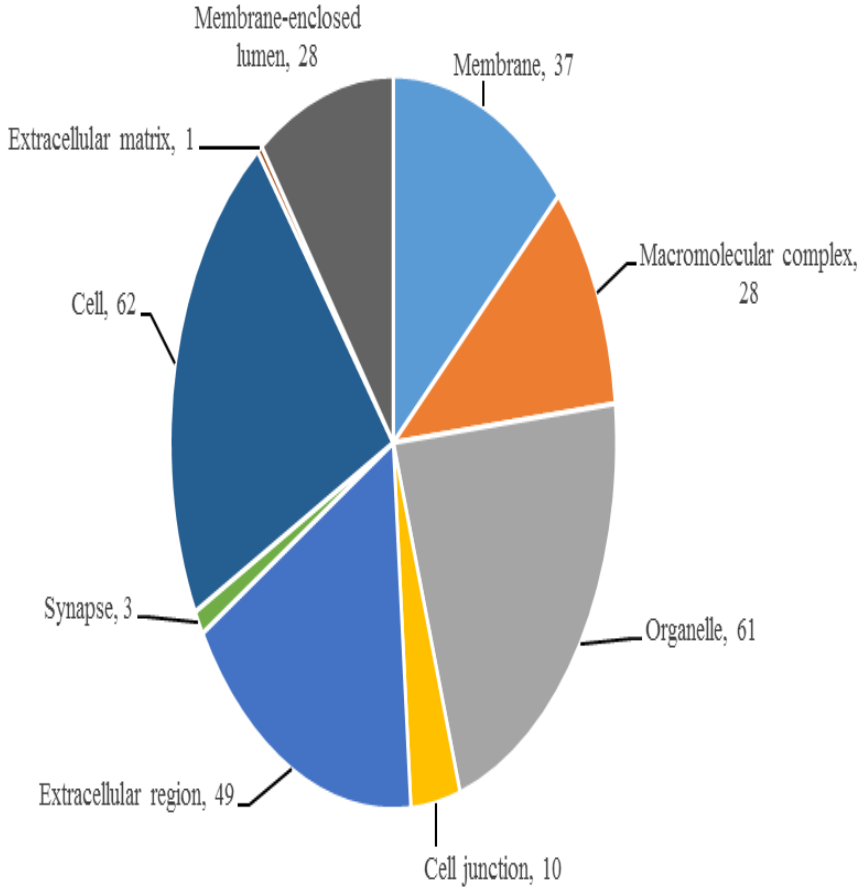


Figure 2. Analysis by the B2G of liver tissue with the sequences of proteins identified as insulin dependent and independent. *A1*. Cellular component- insulin dependent. *A2*. Cellular component- insulin independent. *B1*. Molecular function- insulin dependent. *B2*. Molecular function- insulin independent.

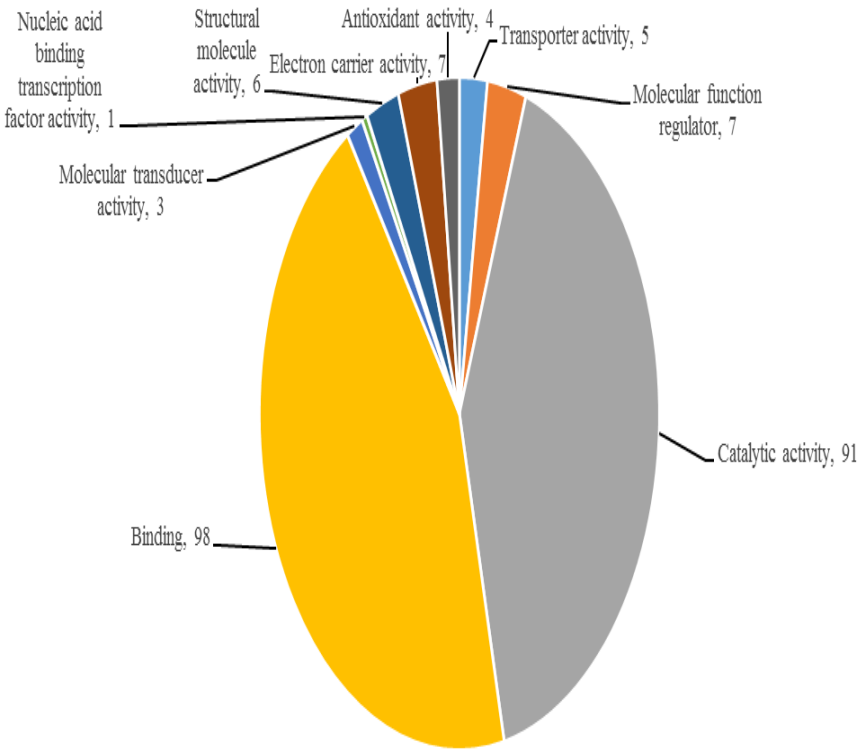
A1. Cellular component - insulin dependent



A2. Cellular component - insulin independent



B1. Molecular function - insulin dependent



B2. Molecular function- insulin independent

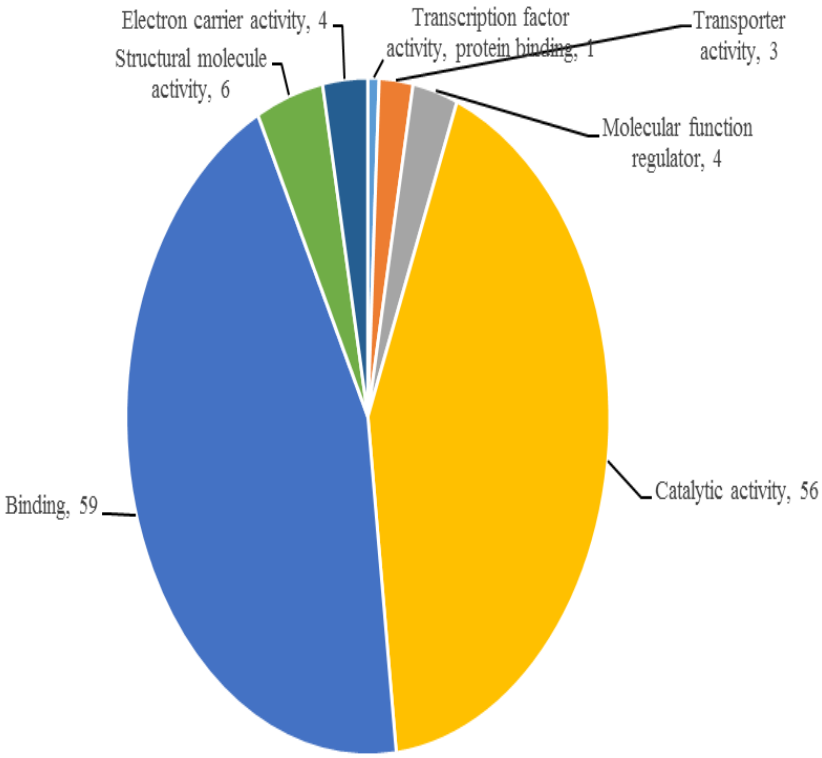


Table 1. Oxidatively damage proteins related as insulin dependent or independent, and DM1 regulation.

Spot ID	Acession	Protein	Score	Mw	Anova (p)	Fold	C	DM1	DM1 +I	DM1 pool	DM1 pool regulation	Insulin	Function	Metabolism
Insulin dependent														
443	HS90B_RAT	Heat shock protein HSP 90-beta OS=Rattus norvegicus GN=Hsp90ab1 PE=1 SV=4	359	83,229	4.31E-10	4.3	5.09E+06	1.17E+06	3.47E+06	2.00E+06	down	dependent	Stress response	Chaperone
	HS90A_RAT	Heat shock protein HSP 90-alpha OS=Rattus norvegicus GN=Hsp90aa1 PE=1 SV=3	211	84,762									Stress response	Chaperone
862	3HIDH_RAT	3-hydroxyisobutyrate dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Hibadh PE=1 SV=3	1406	35,280	2.82E-09	1.4	1.24E+06	9.67E+05	1.13E+06	8.65E+05	up	dependent	Branched-chain amino acid catabolism	Amino acid
	PNPH_RAT	Purine nucleoside phosphorylase OS=Rattus norvegicus GN=Pnp PE=1 SV=1	240	32,281									Purine nucleoside salvage	Purin
	ETFA_RAT	Electron transfer	123	34,929									Electron transport, Transport	Energy

		flavoprotein subunit alpha, mitochondrial OS=Rattus norvegicus GN=Etfa PE=1 SV=4												
985	GSTM2_RAT	Glutathione S-transferase Mu 2 OS=Rattus norvegicus GN=Gstm2 PE=1 SV=2	1257	25,686	8.94E-09	2.6	1.99E+07	7.52E+06	1.73E+07	9.49E+06	up	dependent	Olfaction, Sensory transduction	Glutathione
	GSTM1_RAT	Glutathione S-transferase Mu 1 OS=Rattus norvegicus GN=Gstm1 PE=1 SV=2	664	25,897									Olfaction, Sensory transduction	Glutathione
	GSTM4_RAT	Glutathione S-transferase Yb-3 OS=Rattus norvegicus GN=Gstm3 PE=1 SV=2	221	25,664									Transferase	Glutathione
943	DHPR_RAT	Dihydropteridine reductase OS=Rattus norvegicus GN=Qdpr PE=1 SV=1	483	25,536	1.37E-07	2.9	1.19E+07	5.38E+06	1.56E+07	8.08E+06	up	dependent	Tetrahydrobiopterin biosynthesis	Other
	ETFB_RAT	Electron transfer flavoprotein subunit beta OS=Rattus norvegicus GN=Etfb PE=2 SV=3	389	27,670									Electron transport, Transport	Energy

	AGT2_RAT	Glutathione S-transferase Mu 2 OS=Rattus norvegicus GN=Gstm2 PE=1 SV=2	273	25,686								Olfaction, Sensory transduction	Glutathione
	CAH3_RAT	Carbonic anhydrase 3 OS=Rattus norvegicus GN=Ca3 PE=1 SV=3	197	29,413								Response to oxidative stress	Oxidative stress
	KAD2_RAT	Adenylate kinase 2, mitochondrial OS=Rattus norvegicus GN=Ak2 PE=2 SV=2	180	26,363								ATP metabolic process,Kinase, Transferase, oxidative phosphorylation	Nucleotide
442	GRP78_RAT	78 kDa glucose-regulated protein OS=Rattus norvegicus GN=Hspa5 PE=1 SV=1	1655	72,302	1.76E-07	2.5	8.51E+06	3.44E+06	6.81E+06	4.20E+06	down	dependent	Cellular response to glucose starvation, response to endoplasmic reticulum stress	Chaperone
827	RGN_RAT	Regucalcin OS=Rattus norvegicus GN=Rgn PE=1 SV=3	769	33,368	2.01E-06	3.4	8.47E+06	2.49E+06	7.37E+06	3.61E+06	down	dependent	Ascorbate biosynthesis	Other
850	GNMT_RAT	Glycine N-methyltransferase OS=Rattus norvegicus GN=Gnmt PE=1 SV=2	225	32,528	8.33E-06	2	1.81E+06	2.93E+06	2.60E+06	3.62E+06	up	dependent	methyltransferase, transferase	Methionine

	ANXA2_RAT	Annexin A2 OS=Rattus norvegicus GN=Anxa2 PE=1 SV=2	140	38,654									angiogenesis	Other
	G3P_RAT	Glyceraldehyde-3-phosphate dehydrogenase OS=Rattus norvegicus GN=Gapdh PE=1 SV=3	120	35,805									glycolysis/gluconeogenesis	Carbohydrate
	LDHA_RAT	L-lactate dehydrogenase A chain OS=Rattus norvegicus GN=Ldha PE=1 SV=1	109	36,427									lactate metabolic process	Carbohydrate
573	LMNA_RAT	Prelamin-A/C OS=Rattus norvegicus GN=Lmna PE=1 SV=1	236	74,279	8.89E-06	1.7	6.90E+06	4.06E+06	5.31E+06	4.51E+06	down	dependent	negative regulation of adipose tissue development	Other
	AL4A1_RAT	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Aldh4a1 PE=1 SV=1	189	61,830									proline catabolic process to glutamate, proline and glutamate biosynthetic process	proline metabolism
	PGM1_RAT	Phosphoglucomutase-1 OS=Rattus norvegicus GN=Pgm1 PE=1 SV=2	167	61,365									glycolysis/gluconeogenesis	Carbohydrate

1040	PSB6_RAT	Proteasome subunit beta type-6 OS=Rattus norvegicus GN=Psmb6 PE=1 SV=3	78	25,273	1.29E-05	6.5	2.21E+06	3.38E+05	1.40E+06	8.99E+05	down	dependent	proteasome-mediated ubiquitin-dependent protein catabolic process, proteasomal ubiquitin-independent protein catabolic process	Other
	COMT_RAT	Catechol O-methyltransferase OS=Rattus norvegicus GN=Comt PE=1 SV=2	65	29,578									neurotransmitter degradation	catecholamine
	ECHM_RAT	Enoyl-CoA hydratase, mitochondrial OS=Rattus norvegicus GN=Echs1 PE=1 SV=1	62	31,496									fatty acid beta-oxidation	Lipid
980	SODM_RAT	Superoxide dismutase [Mn], mitochondrial OS=Rattus norvegicus GN=Sod2 PE=1 SV=2	845	24,659									response to oxidative stress	Oxidative stress
873	ETFA_RAT	Electron transfer flavoprotein subunit alpha, mitochondrial OS=Rattus norvegicus GN=Etfa PE=1 SV=4	319	34,929	4.16E-05	2.5	1.32E+06	3.35E+06	2.36E+06	2.96E+06	up	dependent	Electron transport, Transport	Energy
	GBLP_RAT	Guanine nucleotide-	229	35,055									Apoptosis, Biological rhythms, Cell	Other

		binding protein subunit beta-2-like 1 OS=Rattus norvegicus GN=Gnb2l1 PE=1 SV=3										cycle, Gastrulation, Growth regulation, Translation regulation	
	GNMT_RAT	Glycine N-methyltransferase OS=Rattus norvegicus GN=Gnmt PE=1 SV=2	155	32,528								methyltransferase, transferase	Methionine
	ECH1_RAT	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial OS=Rattus norvegicus GN=Ech1 PE=1 SV=2	103	36,148								lipid metabolism; fatty acid beta-oxidation	Lipid
	CAH1_RAT	Carbonic anhydrase 1 OS=Rattus norvegicus GN=Ca1 PE=1 SV=1	99	28,282								one-carbon metabolic process	Oxidative stress
	CAH2_RAT	Carbonic anhydrase 2 OS=Rattus norvegicus GN=Ca2 PE=1 SV=2	69	29,096								one-carbon metabolic process	Oxidative stress
707	PON3_RAT	Serum paraoxonase/lactonase 3 OS=Rattus norvegicus	144	39,433	5.85E-05	2	1.51E+06	7.61E+05	1.27E+06	7.56E+05	down	dependent	activity towards the organophosphate paraxon and aromatic carboxylic acid esters	Other

		GN=Pon3 PE=2 SV=1											
	IVD_RAT	Isovaleryl-CoA dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Ivd PE=1 SV=2	120	46,406								amino-acid degradation; L-leucine degradation	Amino acid
	PH4H_RAT	Phenylalanine-4-hydroxylase OS=Rattus norvegicus GN=Pah PE=1 SV=3	101	51,789								Phenylalanine catabolism	Other
	AMPL_RAT	Cytosol aminopeptidase OS=Rattus norvegicus GN=Lap3 PE=1 SV=1	90	56,115								aminopeptidase and metalloexopeptidase activity	Other
574	AL4A1_RAT	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Aldh4a1 PE=1 SV=1	699	61,830	1.28E-04	1.9	7.98E+06	4.12E+06	6.68E+06	5.26E+06	down	dependent	proline catabolic process to glutamate, proline and glutamate biosynthetic process	proline metabolism
	LMNA_RAT	Prelamin-A/C OS=Rattus norvegicus GN=Lmna PE=1 SV=1	298	74,279								negative regulation of adipose tissue development	Other

DHE3_RAT	Glutamate dehydrogenase 1, mitochondrial OS=Rattus norvegicus GN=Glud1 PE=1 SV=2	185	61,377	oxidoreductase, allosteric regulation	Other
CATA_RAT	Catalase OS=Rattus norvegicus GN=Cat PE=1 SV=3	176	59,719	hydrogen peroxide	Oxidative stress
ACSM3_RAT	Acyl-coenzyme A synthetase ACSM3, mitochondrial OS=Rattus norvegicus GN=Acsm3 PE=2 SV=1	112	65,671	fatty acid biosynthetic process	Lipid
PUR9_RAT	Bifunctional purine biosynthesis protein PURH OS=Rattus norvegicus GN=Atic PE=1 SV=2	65	64,168	Purine biosynthesis	Purine
ACSF2_RAT	Acyl-CoA synthetase family member 2, mitochondrial OS=Rattus norvegicus GN=Acsf2 PE=2 SV=1	62	67,843	fatty acid metabolism, lipid metabolism	Lipid

	EHD1_RAT	EH domain-containing protein 1 OS=Rattus norvegicus GN=Ehd1 PE=1 SV=1	60	60,565									Cilium biogenesis/degradation	Other
	DHAK_RAT	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing) OS=Rattus norvegicus GN=Dak PE=1 SV=1	60	59,406									carbohydrate phosphorylation, glycerol metabolic process, regulation of innate immune response	Carbohydrate
935	GSTM4_RAT	Glutathione S-transferase Yb-3 OS=Rattus norvegicus GN=Gstm3 PE=1 SV=2	154	160,464	3.97E-04	2.1	7.02E+05	4.22E+05	8.80E+05	5.98E+05	down	dependent	glutathione metabolic process	Oxidative stress
	GSTM2_RAT	Glutathione S-transferase Mu 2 OS=Rattus norvegicus GN=Gstm2 PE=1 SV=2	81	25,664									Olfaction, Sensory transduction	Other
571	PDIA3_RAT	Protein disulfide-isomerase A3 OS=Rattus norvegicus GN=Pdia3 PE=1 SV=2	2655	56,588	4.05E-04	1.7	4.41E+06	2.64E+06	4.23E+06	3.42E+06	down	dependent	oxidation-reduction process, response to endoplasmic reticulum stress	Other

	SUOX_RAT	Sulfite oxidase, mitochondrial OS=Rattus norvegicus GN=Suox PE=1 SV=2	105	60,768								energy metabolism; sulfur metabolism.	Energy
549	PDIA3_RAT	Protein disulfide-isomerase A3 OS=Rattus norvegicus GN=Pdia3 PE=1 SV=2	724	56,588	4.95E-04	1.7	3.48E+06	2.09E+06	2.98E+06	2.45E+06	down	dependent	oxidation-reduction process, response to endoplasmic reticulum stress	Other
	TCPE_RAT	T-complex protein 1 subunit epsilon OS=Rattus norvegicus GN=Cct5 PE=1 SV=1	343	59,499									response to virus, toxin transport	chaperone
	FTCD_RAT	Formimidoyltransferase-cyclodeaminase OS=Rattus norvegicus GN=Ftcd PE=1 SV=4	98	58,877									histidine catabolic process to glutamate and formamide, histidine catabolic process to glutamate and formate	Histidine
472	HSP7C_RAT	Heat shock cognate 71 kDa protein OS=Rattus norvegicus GN=Hspa8 PE=1 SV=1	471	70,827	5.66E-04	1.4	2.14E+06	1.57E+06	2.11E+06	1.49E+06	down	dependent	mRNA processing, mRNA splicing, Stress response, Transcription, Transcription regulation	chaperone, repressor
	GRP78_RAT	78 kDa glucose-regulated protein	64	72,302									cellular response to glucose starvation	chaperone

		OS=Rattus norvegicus GN=Hspa5 PE=1 SV=1												
	ANXA6_RAT	Annexin A6 OS=Rattus norvegicus GN=Anxa6 PE=1 SV=2	152	75,706									Calcium/phospholipid-binding	Other
849	MDHC_RAT	Malate dehydrogenase, cytoplasmic OS=Rattus norvegicus GN=Mdh1 PE=1 SV=3	728	36,460	6.99E-04	2.3	3.07E+06	2.01E+06	4.65E+06	2.33E+06	down	dependent	Tricarboxylic acid cycle	Carbohydrate
	BIEA_RAT	Biliverdin reductase A OS=Rattus norvegicus GN=Blvra PE=1 SV=1	94	33,545									oxidation-reduction process, heme catabolic process	Porphyrin-containing compound
957	PSB3_RAT	Proteasome subunit beta type-3 OS=Rattus norvegicus GN=Psmb3 PE=1 SV=1	184	22,949	7.38E-04	1.6	6.68E+05	4.26E+05	6.89E+05	4.62E+05	down	dependent	proteasome-mediated ubiquitin-dependent protein catabolic process, proteasomal ubiquitin-independent protein catabolic process	Other
976	HCD2_RAT	3-hydroxyacyl-CoA dehydrogenase type-2 OS=Rattus norvegicus GN=Hsd17b10 PE=1 SV=3	775	27,229	9.94E-04	2.3	1.34E+07	5.77E+06	1.15E+07	8.51E+06	down	dependent	tRNA processing	Nucleotide

	GSTM1_RAT	Glutathione S-transferase Mu 1 OS=Rattus norvegicus GN=Gstm1 PE=1 SV=2	370	25,897								Olfaction, Sensory transduction	Glutathione
	GSTM2_RAT	Glutathione S-transferase Mu 2 OS=Rattus norvegicus GN=Gstm2 PE=1 SV=2	76	25,686								Olfaction, Sensory transduction	Glutathione
697	CGL_RAT	Cystathionine gamma-lyase OS=Rattus norvegicus GN=Cth PE=1 SV=2	588	43,577	0.001	2	1.27E+06	6.31E+05	1.08E+06	7.48E+05	up	dependent	amino-acid biosynthesis, Cysteine biosynthesis	Amino acid
	BHMT1_RAT	Betaine--homocysteine S-methyltransferase 1 OS=Rattus norvegicus GN=Bhmt PE=1 SV=1	416	44,948									methyltransferase, transferase	Homocysteine
	THIM_RAT	3-ketoacyl-CoA thiolase, mitochondrial OS=Rattus norvegicus GN=Acaa2 PE=2 SV=1	163	41,844									fatty acid beta-oxidation	Lipid
	NFS1_RAT	Cysteine desulfurase, mitochondrial OS=Rattus norvegicus	108	49,981									Molybdenum cofactor biosynthesis	Other

	GN=Nfs1 PE=2 SV=1				
AADAT_RAT	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial OS=Rattus norvegicus GN=Aadat PE=1 SV=1	98	47,754	2-oxoglutarate metabolic process, glutamate metabolic process, kynurenine metabolic process, L-kynurenine metabolic process	Other
ACADM_RAT	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Acadm PE=1 SV=1	89	46,526	Fatty acid metabolism	Lipid
BAAT_RAT	Bile acid-CoA:amino acid N-acyltransferase OS=Rattus norvegicus GN=Baat PE=1 SV=2	86	46,435	Fatty acid metabolism	Lipid
THIL_RAT	Acetyl-CoA acetyltransferase, mitochondrial OS=Rattus norvegicus GN=Acat1 PE=1 SV=1	74	44,666	response to organic cyclic compound, response to starvation, response to hormone	Other
PGK1_RAT	Phosphoglycerate kinase 1	69	44,510	glycolysis/gluconeogenesis	Carbohydrate

		OS=Rattus norvegicus GN=Pgk1 PE=1 SV=2												
	AATC_RAT	Aspartate aminotransferase, cytoplasmic OS=Rattus norvegicus GN=Got1 PE=1 SV=3	65	46,400									Amino-acid biosynthesis	Amino acid
569	AATM_RAT	Aspartate aminotransferase, mitochondrial OS=Rattus norvegicus GN=Got2 PE=1 SV=2	1762	47,284	0.002	2.2	4.41E+06	2.57E+06	5.68E+06	3.74E+06	down	dependent	Amino-acid biosynthesis	Amino acid
	ECHB_RAT	Trifunctional enzyme subunit beta, mitochondrial OS=Rattus norvegicus GN=Hadhb PE=1 SV=1	100	51,382									fatty acid metabolism, lipid metabolism	Lipid
1044	GBLP_RAT	Guanine nucleotide-binding protein subunit beta-2-like 1 OS=Rattus norvegicus GN=Gnb2l1 PE=1 SV=3	97	35,055	0.002	3.1	3.25E+06	1.09E+06	3.38E+06	1.56E+06	down	dependent	Apoptosis, Biological rhythms, Cell cycle, Gastrulation, Growth regulation, Translation regulation	Other
	URIC_RAT	Uricase OS=Rattus	88	34,912									purine nucleobase catabolic process	Purine

		norvegicus GN=Uox PE=1 SV=3											
	HMGCL_RAT	Hydroxymethylglutaryl-CoA lyase, mitochondrial OS=Rattus norvegicus GN=Hmgcl PE=2 SV=1	81	34,170								metabolic intermediate metabolism, acyl-CoA metabolic process,	Energy
	HCDH_RAT	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Hadh PE=2 SV=1	72	34,426								fatty acid beta-oxidation, negative regulation of insulin secretion, response to insulin	Lipid
876	OAT_RAT	Ornithine aminotransferase, mitochondrial OS=Rattus norvegicus GN=Oat PE=1 SV=1	731	48,302	0.002	1.5	2.15E+06	1.39E+06	1.69E+06	1.48E+06	up	dependent	L-proline biosynthetic process, ornithine metabolic process and urea cycle	Amino acid
	SAHH_RAT	Adenosylhomocysteinase OS=Rattus norvegicus GN=Ahcy PE=1 SV=3	546	47,507									One-carbon metabolism	
	ODBA_RAT	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial (Fragment)	174	50,133									branched-chain amino acid catabolic process, branched-chain amino acid catabolic process,	Amino acid

	OS=Rattus norvegicus GN=Bckdha PE=1 SV=1			response to glucocorticoid	
ADK_RAT	Adenosine kinase OS=Rattus norvegicus GN=Adk PE=1 SV=3	137	40,108	purine salvage	Nucleotide
NAKD2_RAT	NAD kinase 2, mitochondrial OS=Rattus norvegicus GN=Nadk2 PE=2 SV=1	95	48,087		
IDHC_RAT	Isocitrate dehydrogenase [NADP] cytoplasmic OS=Rattus norvegicus GN=Idh1 PE=1 SV=1	94	46,705	Tricarboxylic acid cycle	Carbohydrate
HPPD_RAT	4-hydroxyphenyl pyruvate dioxygenase OS=Rattus norvegicus GN=Hpd PE=1 SV=3	90	45,084	L-phenylalanine catabolic proces, tyrosine catabolic process	Amino acid
CH60_RAT	60 kDa heat shock protein, mitochondrial OS=Rattus norvegicus GN=Hspd1 PE=1 SV=1	88	60,917	B cell cytokine production and proliferation, response to hydrogen peroxide	chaperone

NDUS2_RAT	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial OS=Rattus norvegicus GN=Ndufs2 PE=1 SV=1	83	52,528	Electron transport, Respiratory chain, Transport	Energy
METK1_RAT	S-adenosylmethionine synthase isoform type-1 OS=Rattus norvegicus GN=Mat1a PE=1 SV=2	71	43,670	amino-acid biosynthesis; S-adenosyl-L-methionine biosynthesis; S-adenosyl-L-methionine from L-methionine	Methionine
ASSY_RAT	Argininosuccinate synthase OS=Rattus norvegicus GN=Ass1 PE=2 SV=1	66	46,467	Urea cycle	Amino acid
KAT3_RAT	Kynurenine--oxoglutarate transaminase 3 OS=Rattus norvegicus GN=Ccbl2 PE=2 SV=1	66	51,011	biosynthetic process, cellular amino acid metabolic process, kynurenine and L-kynurenine metabolic process	Amino acid
NLTP_RAT	Non-specific lipid-transfer protein OS=Rattus norvegicus GN=Scp2 PE=1 SV=3	60	58,775	lipid transport, transport	Lipid

	PRS7_RAT	26S protease regulatory subunit 7 OS=Rattus norvegicus GN=Psmc2 PE=1 SV=3	60	48,544								ER-associated ubiquitin-dependent protein catabolic process, positive regulation of proteasomal protein catabolic process	Nucleotide
471	M2GD_RAT	Dimethylglycine dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Dmgdh PE=1 SV=1	230	95,987	0.003	1.8	6.45E+05	8.17E+05	4.51E+05	7.19E+05	up	dependent	amino-acid betaine catabolic process	Amino acid
	LONM_RAT	Lon protease homolog, mitochondrial OS=Rattus norvegicus GN=Lonp1 PE=2 SV=1	229	105,726									chaperone-mediated protein complex assembly, oxidation-dependent protein catabolic process, regulation of mitochondrial DNA replication, response to hormone an hypoxia	Chaperone
319	TERA_RAT	Transitional endoplasmic reticulum ATPase OS=Rattus norvegicus GN=Vcp PE=1 SV=3	460	89,293	0.003	1.9	6.62E+05	4.73E+05	8.75E+05	5.83E+05	down	dependent	DNA damage, DNA repair, Transport	Transport
	MAGI3_RAT	Membrane-associated guanylate kinase, WW and PDZ domain-	140	160,464									regulation of JNK cascade, signal transduction	Other

		containing protein 3 OS=Rattus norvegicus GN=Magi3 PE=1 SV=2												
933	DHPR_RAT	Dihydropteridine reductase OS=Rattus norvegicus GN=Qdpr PE=1 SV=1	153	25,536	0.003	1.6	1.08E+06	6.57E+05	1.03E+06	7.74E+05	down	dependent	Tetrahydrobiopterin biosynthesis	Other
	ECHM_RAT	Enoyl-CoA hydratase, mitochondrial OS=Rattus norvegicus GN=Echs1 PE=1 SV=1	65	31,496									fatty acid beta-oxidation	Lipid
869	G3P_RAT	Glyceraldehyde-3-phosphate dehydrogenase OS=Rattus norvegicus GN=Gapdh PE=1 SV=3	777	35,805	0.005	1.6	2.92E+06	4.12E+06	3.12E+06	4.57E+06	up	dependent	glycolysis/gluconeogenesis	Carbohydrate
	G3PT_RAT	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific OS=Rattus norvegicus GN=Gapdhs PE=1 SV=1	24	46,678									glycolysis/gluconeogenesis pathway	Carbohydrate
	ROA2_RAT	Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Rattus	66	37,455									mRNA processing, mRNA splicing	Nucleotide

		norvegicus GN=Hnrnpa2b1 PE=1 SV=1												
226	CPSM_RAT	Carbamoyl-phosphate synthase [ammonia], mitochondrial OS=Rattus norvegicus GN=Cps1 PE=1 SV=1	781	164,476	0.005	1.5	1.28E+06	1.49E+06	1.17E+06	1.75E+06	up	dependent	urea cycle	Amino acid
	MAGI3_RAT	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 3 OS=Rattus norvegicus GN=Magi3 PE=1 SV=2	125	160,464									regulation of JNK cascade, signal transduction	Other
360	AASS_RAT	Alpha-aminoadipic semialdehyde synthase, mitochondrial OS=Rattus norvegicus GN=Aass PE=2 SV=1	174	102,842	0.005	1.7	8.85E+05	1.09E+06	8.91E+05	1.49E+06	up	dependent	L-lysine catabolic process to acetyl-CoA via saccharopine	Other
681	AGT2_RAT	Alanine--glyoxylate aminotransferase 2, mitochondrial OS=Rattus	209	57,164	0.007	3.1	1.79E+06	5.59E+06	2.31E+06	3.93E+06	up	dependent	glycine biosynthetic process, by transamination of glyoxylate, glyoxylate catabolic process, positive	Amino acid

		norvegicus GN=Agxt2 PE=1 SV=2											regulation of nitric oxide biosynthetic process	
	HMCS2_RAT	Hydroxymethylglutaryl-CoA synthase, mitochondrial OS=Rattus norvegicus GN=Hmgcs2 PE=2 SV=1	172	56,876									cholesterol biosynthesis, cholesterol metabolism, lipid biosynthesis and metabolism, steroid biosynthesis and metabolism	Lipid
566	CATA_RAT	Catalase OS=Rattus norvegicus GN=Cat PE=1 SV=3	387	59,719	0.01	2	9.25E+06	4.52E+06	9.03E+06	6.75E+06	down	dependent	hydrogen peroxide	Oxidative stress
	AL4A1_RAT	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Aldh4a1 PE=1 SV=1	204	61,830									proline catabolic process to glutamate, proline and glutamate biosynthetic process	proline metabolism
1043	PRDX2_RAT	Peroxiredoxin-2 OS=Rattus norvegicus GN=Prdx2 PE=1 SV=3	132	21,770	0.017	1.7	7.20E+05	5.24E+05	7.80E+05	4.55E+05	down	dependent	removal of superoxide radicals	Oxidative stress
622	DHE3_RAT	Glutamate dehydrogenase 1, mitochondrial OS=Rattus	532	61,377	0.018	1.6	7.19E+06	5.06E+06	7.96E+06	6.83E+06	down	dependent	oxidoreductase, allosteric regulation	Other

		norvegicus GN=Glud1 PE=1 SV=2											
	ATPA_RAT	ATP synthase subunit alpha, mitochondrial OS=Rattus norvegicus GN=Atp5a1 PE=1 SV=2	494	59,717								ATP synthesis, Hydrogen ion transport, Ion transport, Transport	Energy
	MMSA_RAT	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial OS=Rattus norvegicus GN=Aldh6a1 PE=1 SV=1	112	57,771								thymine catabolic and metaolic process, valine catabolic and metabolic process	Nucleotide
	AL7A1_RAT	Alpha-aminoadipic semialdehyde dehydrogenase OS=Rattus norvegicus GN=Aldh7a1 PE=1 SV=2	65	58,711								glycine betaine biosynthetic process from choline	Other
631	K2C8_RAT	Keratin, type II cytoskeletal 8 OS=Rattus norvegicus GN=Krt8 PE=1 SV=3	1525	53,985	0.02	1.3	8.32E+06	6.38E+06	8.58E+06	7.73E+06	down	dependent	contractile apparatus	Other
	K2C75_RAT	Keratin, type II cytoskeletal 75 OS=Rattus norvegicus	74	58,991								contractile apparatus	Other

		GN=Krt75 PE=3 SV=2											
	K2C1_RAT	Keratin, type II cytoskeletal 1 OS=Rattus norvegicus GN=Krt1 PE=2 SV=1	72	64,791								May regulate the activity of kinases such as PKC and SRC	Other
	ODO2_RAT	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial OS=Rattus norvegicus GN=Dlst PE=1 SV=2	267	48,894								Tricarboxylic acid cycle	Carbohydrate
	ALDH2_RAT	Aldehyde dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Aldh2 PE=1 SV=1	160	56,453								cellular response to fatty acid, negative regulation of apoptotic process, ethanol catabolic process	Alcohol
941	GSTA3_RAT	Glutathione S-transferase alpha-3 OS=Rattus norvegicus GN=Gsta3 PE=1 SV=3	1317	25,303	0.029	1.7	3.79E+07	2.19E+07	3.80E+07	2.98E+07	down	dependent	xenobiotic catabolic process	Oxidative stress
	ECI1_RAT	Enoyl-CoA delta isomerase 1, mitochondrial	338	32,234								fatty acid beta-oxidation	Lipid

		OS=Rattus norvegicus GN=Eci1 PE=1 SV=1											
	HCD2_RAT	3-hydroxyacyl-CoA dehydrogenase type-2 OS=Rattus norvegicus GN=Hsd17b10 PE=1 SV=3	86	27,229								tRNA processing	Nucleotide
	KAD3_RAT	GTP:AMP phosphotransferase AK3, mitochondrial OS=Rattus norvegicus GN=Ak3 PE=2 SV=2	83	25,422								homeostasis of cellular nucleotides	Nucleotide
	SDHB_RAT	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial OS=Rattus norvegicus GN=Sdhb PE=2 SV=2	80	31,809								tricarboxylic acid cycle, respiratory electron transport chain	Carbohydrate
475	HSP7C_RAT	Heat shock cognate 71 kDa protein OS=Rattus norvegicus GN=Hspa8 PE=1 SV=1	1819	70,827	0.032	1.3	4.35E+06	4.08E+06	4.56E+06	3.43E+06	down	dependent	mRNA processing, mRNA splicing, Stress response, Transcription, Transcription regulation	chaperone, repressor

	ANXA6_RAT	Annexin A6 OS=Rattus norvegicus GN=Anxa6 PE=1 SV=2	115	75,706									Calcium/phospholipid-binding	Other
	GRP75_RAT	Stress-70 protein, mitochondrial OS=Rattus norvegicus GN=Hspa9 PE=1 SV=3	97	73,812									cellular response to interleukin-1, response to toxic substance	chaperone
470	HSP7C_RAT	Heat shock cognate 71 kDa protein OS=Rattus norvegicus GN=Hspa8 PE=1 SV=1	1915	70,827	0.047	1.6	3.18E+06	2.28E+06	3.58E+06	2.87E+06	down	dependent	mRNA processing, mRNA splicing, Stress response, Transcription, Transcription regulation	chaperone, repressor
	ANXA6_RAT	Annexin A6 OS=Rattus norvegicus GN=Anxa6 PE=1 SV=2	263	75,706									Calcium/phospholipid-binding	Other
	GRP75_RAT	Stress-70 protein, mitochondrial OS=Rattus norvegicus GN=Hspa9 PE=1 SV=3	104	73,812									cellular response to interleukin-1, response to toxic substance	chaperone
1046	PRDX1_RAT	Peroxiredoxin-1 OS=Rattus norvegicus GN=Prdx1 PE=1 SV=1	248	22,095	0.047	2.2	2.03E+06	4.43E+06	2.27E+06	3.13E+06	up	dependent	redox regulation	Oxidative stress
Insulin independent														

714	BHMT1_RAT	Betaine--homocysteine S-methyltransferase 1 OS=Rattus norvegicus GN=Bhmt PE=1 SV=1	1179	44,948	3.05E-06	2.1	2.85E+07	4.06E+07	5.87E+07	5.80E+07	up	independent	methyltransferase, transferase	Homocysteine
	ASSY_RAT	Argininosuccinate synthase OS=Rattus norvegicus GN=Ass1 PE=2 SV=1	791	46,467									Urea cycle	Amino acid
	THIM_RAT	3-ketoacyl-CoA thiolase, mitochondrial OS=Rattus norvegicus GN=Acaa2 PE=2 SV=1	317	41,844									fatty acid beta-oxidation	Lipid
	CGL_RAT	Cystathionine gamma-lyase OS=Rattus norvegicus GN=Cth PE=1 SV=2	125	43,577									amino-acid biosynthesis, Cysteine biosynthesis	Amino acid
	PGK1_RAT	Phosphoglycerate kinase 1 OS=Rattus norvegicus GN=Pgk1 PE=1 SV=2	119	44,510									glycolysis/gluconeogenesis pathway	Carbohydrate
349	SARDH_RAT	Sarcosine dehydrogenase, mitochondrial OS=Rattus norvegicus	529	101,376	3.83E-06	1.7	9.53E+05	1.02E+06	1.13E+06	1.60E+06	up	independent	choline catabolic process	Other

		GN=Sardh PE=1 SV=2												
582	SUOX_RAT	Sulfite oxidase, mitochondrial OS=Rattus norvegicus GN=Suox PE=1 SV=2	430	60,768	1.97E-04	2.9	1.25E+06	4.30E+05	8.25E+05	9.43E+05	down	independent	energy metabolism; sulfur metabolism.	Energy
	PDIA3_RAT	Protein disulfide-isomerase A3 OS=Rattus norvegicus GN=Pdia3 PE=1 SV=2	231	56,588									oxidation-reduction process, response to endoplasmic reticulum stress	Other
	PCCB_RAT	Propionyl-CoA carboxylase beta chain, mitochondrial OS=Rattus norvegicus GN=Pccb PE=2 SV=1	129	58,589									cellular amino acid catabolic process, fatty acid catabolic process	Amino acid
	AL9A1_RAT	4-trimethylaminobutyraldehyde dehydrogenase OS=Rattus norvegicus GN=Aldh9a1 PE=1 SV=1	108	53,618									amine and polyamine biosynthesis; carnitine biosynthesis	Lipid
	FTCD_RAT	Formimidoyltransferase-cyclodeaminase OS=Rattus norvegicus	103	58,877									histidine catabolic process to glutamate and formamide, histidine catabolic	Histidine

		GN=Ftcd PE=1 SV=4											process to glutamate and formate	
	DHAK_RAT	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing) OS=Rattus norvegicus GN=Dak PE=1 SV=1	86	59,406									carbohydrate phosphorylation, glycerol metabolic process, regulation of innate immune response	Carbohydrate
	AMPL_RAT	Cytosol aminopeptidase OS=Rattus norvegicus GN=Lap3 PE=1 SV=1	77	56,115									Magnesium, Manganese, Metal-binding, Zinc	Metal-binding
993	GSTM2_RAT	Glutathione S-transferase Mu 2 OS=Rattus norvegicus GN=Gstm2 PE=1 SV=2	1335	25,686	2.79E-04	2.2	4.73E+06	2.12E+06	2.81E+06	2.23E+06	down	independent	Olfaction, Sensory transduction	Other
	GSTM4_RAT	Glutathione S-transferase Yb-3 OS=Rattus norvegicus GN=Gstm3 PE=1 SV=2	254	25,664									glutathione metabolic process	Oxidative stress
	GSTM1_RAT	Glutathione S-transferase Mu 1 OS=Rattus norvegicus GN=Gstm1 PE=1 SV=2	234	25,897									olfaction, Sensory transduction	Other

	TPIS_RAT	Triosephosphate isomerase OS=Rattus norvegicus GN=Tpi1 PE=1 SV=2	113	26,832									Gluconeogenesis, Glycolysis, Pentose shunt	Carbohydrate
639	ALDH2_RAT	Aldehyde dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Aldh2 PE=1 SV=1	374	56,453	8.16E-04	1.5	8.72E+06	5.92E+06	6.04E+06	6.74E+06	down	independent	cellular response to fatty acid, negative regulation of apoptotic process, ethanol catabolic process	Alcohol
	ODO2_RAT	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial OS=Rattus norvegicus GN=Dlst PE=1 SV=2	319	48,894										
	SBP1_RAT	Selenium-binding protein 1 OS=Rattus norvegicus GN=Selenbp1 PE=1 SV=1	101	52,498									protein transport	Transport
	AL9A1_RAT	4-trimethylaminobutyraldehyde dehydrogenase OS=Rattus norvegicus	98	53,618									amine and polyamine biosynthesis; carnitine biosynthesis	Lipid

		GN=Aldh9a1 PE=1 SV=1												
	ATPA_RAT	ATP synthase subunit alpha, mitochondrial OS=Rattus norvegicus GN=Atp5a1 PE=1 SV=2	79	59,717									ATP synthesis, Hydrogen ion transport, Ion transport, Transport	Energy
	TCPB_RAT	T-complex protein 1 subunit beta OS=Rattus norvegicus GN=Cct2 PE=1 SV=3	77	57,422									chaperone-mediated protein complex assembly, protein folding, toxin transport	Chaperone
570	CH60_RAT	60 kDa heat shock protein, mitochondrial OS=Rattus norvegicus GN=Hspd1 PE=1 SV=1	1777	60,917	0.001	1.5	1.58E+06	1.23E+06	1.23E+06	1.02E+06	down	independent		
	TCPE_RAT	T-complex protein 1 subunit epsilon OS=Rattus norvegicus GN=Cct5 PE=1 SV=1	140	59,499									response to virus, toxin transport	chaperone
	PDIA3_RAT	Protein disulfide-isomerase A3 OS=Rattus norvegicus GN=Pdia3 PE=1 SV=2	127	56,588									oxidation-reduction process, response to endoplasmic reticulum stress	Other

742	DLDH_RAT	Dihydrolipoyl dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Dld PE=1 SV=1	379	54,004	0.001	1.4	1.18E+07	1.40E+07	1.44E+07	1.69E+07	up	independent	cell redox homeostasis, acetyl-CoA biosynthetic process from pyruvate, mitochondrial electron transport, NADH to ubiquinone	Pyruvate and lysine metabolism,
	DHE3_RAT	Glutamate dehydrogenase 1, mitochondrial OS=Rattus norvegicus GN=Glud1 PE=1 SV=2	200	61,377									oxidoreductase, allosteric regulation	Other
	ASSY_RAT	Argininosuccinate synthase OS=Rattus norvegicus GN=Ass1 PE=2 SV=1	129	46,467									Urea cycle	Amino acid
	NLTP_RAT	Non-specific lipid-transfer protein OS=Rattus norvegicus GN=Scp2 PE=1 SV=3	125	58,775									lipid transport, transport	Lipid
	CGL_RAT	Cystathionine gamma-lyase OS=Rattus norvegicus GN=Cth PE=1 SV=2	115	43,577									amino-acid biosynthesis, Cysteine biosynthesis	Amino acid
	DHAK_RAT	Bifunctional ATP-dependent dihydroxyacet	68	59,406									carbohydrate phosphorylation, glycerol metabolic process, regulation of	Carbohydrate

		one kinase/FAD-AMP lyase (cyclizing) OS=Rattus norvegicus GN=Dak PE=1 SV=1											innate immune response	
366	HS90B_RAT	Heat shock protein HSP 90-beta OS=Rattus norvegicus GN=Hsp90ab1 PE=1 SV=4	831	83,229	0.002	1.7	2.41E+06	3.28E+06	3.20E+06	4.13E+06	down	independent	cellular response to interleukin-4, negative regulation of neuron apoptotic process	Chaperone
	HS90A_RAT	Heat shock protein HSP 90-alpha OS=Rattus norvegicus GN=Hsp90aa1 PE=1 SV=3	495	84,762									Stress response	Chaperone
660	QCR1_RAT	Cytochrome b-c1 complex subunit 1, mitochondrial OS=Rattus norvegicus GN=Uqcrc1 PE=1 SV=1	203	52,815	0.002	1.5	1.03E+06	1.23E+06	1.17E+06	1.51E+06	up	independent	electron transport, respiratory chain, transport	Energy
	MAGI3_RAT	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 3 OS=Rattus	169	160,464									regulation of JNK cascade, signal transduction	Other

		norvegicus GN=Magi3 PE=1 SV=2											
	FTCD_RAT	Formimidoyltransferase-cyclodeaminase OS=Rattus norvegicus GN=Ftcd PE=1 SV=4	100	58,877								histidine catabolic process to glutamate and formamide, histidine catabolic process to glutamate and formate	Histidine
	DCTN2_RAT	Dynactin subunit 2 OS=Rattus norvegicus GN=Dctn2 PE=1 SV=1	88	44,121								mitotic nuclear division, mitotic spindle organization	Other
	ACTB_RAT	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1	69	41,710								cellular response to electrical stimulus, retina development in camera-type eye, axonogenesis	Other
	HMCS2_RAT	Hydroxymethylglutaryl-CoA synthase, mitochondrial OS=Rattus norvegicus GN=Hmgcs2 PE=2 SV=1	69	56,876								cholesterol biosynthesis, cholesterol metabolism, lipid biosynthesis and metabolism, steroid biosynthesis and metabolism	Lipid
671	AL7A1_RAT	Alpha-aminoadipic semialdehyde dehydrogenase OS=Rattus norvegicus GN=Aldh7a1 PE=1 SV=2	774	58,711	0.002	1.5	2.26E+06	2.40E+06	1.88E+06	2.83E+06	up	independent	glycine betaine biosynthetic process from choline	Other

DHE3_RAT	Glutamate dehydrogenase 1, mitochondrial OS=Rattus norvegicus GN=Glud1 PE=1 SV=2	128	61,377	oxidoreductase, allosteric regulation	Other
SSDH_RAT	Succinate-semialdehyde dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Aldh5a1 PE=1 SV=2	108	56,096	central nervous system development, gamma-aminobutyric acid catabolic process, oxidation-reduction process, succinate metabolic process	Amino acid
CH60_RAT	60 kDa heat shock protein, mitochondrial OS=Rattus norvegicus GN=Hspd1 PE=1 SV=1	86	60,917	B cell cytokine production and proliferation, response to hydrogen peroxide	chaperone
SBP1_RAT	Selenium-binding protein 1 OS=Rattus norvegicus GN=Selenbp1 PE=1 SV=1	83	52,498	protein transport	Transport
QCR1_RAT	Cytochrome b-c1 complex subunit 1, mitochondrial OS=Rattus norvegicus GN=Uqcrc1 PE=1 SV=1	74	52,815	electron transport, respiratory chain, transport	Energy
ENOA_RAT	Alpha-enolase OS=Rattus	73	47,098	glycolysis/gluconeogenesis pathway	Carbohydrate

		norvegicus GN=Eno1 PE=1 SV=4												
749	ADH1_RAT	Alcohol dehydrogenase 1 OS=Rattus norvegicus GN=Adh1 PE=1 SV=3	815	39,620	0.004	2.2	1.15E+07	2.44E+07	2.49E+07	2.17E+07	up	independent	acetaldehyde biosynthetic process, ethanol oxidation, response to progesterone	Alcohol
	THIL_RAT	Acetyl-CoA acetyltransferase, mitochondrial OS=Rattus norvegicus GN=Acat1 PE=1 SV=1	605	44,666									response to organic cyclic compound, response to starvation, response to hormone	Other
	ALDOB_RAT	Fructose-bisphosphate aldolase B OS=Rattus norvegicus GN=Aldob PE=1 SV=2	254	39,593									Glycolysis	Carbohydrate
	ALDOA_RAT	Fructose-bisphosphate aldolase A OS=Rattus norvegicus GN=Aldoa PE=1 SV=2	125	39,327									Glycolysis	Carbohydrate
	THIM_RAT	3-ketoacyl-CoA thiolase, mitochondrial OS=Rattus norvegicus GN=Acaa2 PE=2 SV=1	131	41,844									fatty acid beta-oxidation	Lipid

	PGK1_RAT	Phosphoglycerate kinase 1 OS=Rattus norvegicus GN=Pgk1 PE=1 SV=2	126	44,510									glycolysis/gluconeogenesis pathway	Carbohydrate
	CGL_RAT	Cystathionine gamma-lyase OS=Rattus norvegicus GN=Cth PE=1 SV=2	93	43,577									amino-acid biosynthesis, Cysteine biosynthesis	Amino acid
	IDHP_RAT	Isocitrate dehydrogenase [NADP], mitochondrial OS=Rattus norvegicus GN=Idh2 PE=1 SV=2	76	50,935									Tricarboxylic acid cycle	Carbohydrate
	BHMT1_RAT	Betaine--homocysteine S-methyltransferase 1 OS=Rattus norvegicus GN=Bhmt PE=1 SV=1	74	44,948									methyltransferase, transferase	Homocysteine
651	K2C8_RAT	Keratin, type II cytoskeletal 8 OS=Rattus norvegicus GN=Krt8 PE=1 SV=3	163	53,985	0.005	1.5	5.89E+05	9.00E+05	7.89E+05	7.46E+05	up	independent	contractile apparatus	Other
	IVD_RAT	Isovaleryl-CoA dehydrogenase, mitochondrial	74	46,406									amino-acid degradation; L-leucine degradation	Amino acid

		OS=Rattus norvegicus GN=Ivd PE=1 SV=2												
932	1433G_RAT	14-3-3 protein gamma OS=Rattus norvegicus GN=Ywhag PE=1 SV=2	350	28,285	0.005	2.2	3.52E+06	1.57E+06	2.07E+06	2.01E+06	down	independent	cellular response to insulin stimulus, regulation of neuron differentiation	Amino acid
	1433Z_RAT	14-3-3 protein zeta/delta OS=Rattus norvegicus GN=Ywhaz PE=1 SV=1	218	27,754									histamine secretion by mast cell, protein targeting to mitochondrion, response to drug	Amino acid
	1433B_RAT	14-3-3 protein beta/alpha OS=Rattus norvegicus GN=Ywhab PE=1 SV=3	147	28,037									negative regulation of protein dephosphorylation, negative regulation of transcription, DNA-templated, positive regulation of catalytic activity, cytoplasmic sequestering of protein	Amino acid
	1433F_RAT	14-3-3 protein eta OS=Rattus norvegicus GN=Ywhah PE=1 SV=2	131	28,194									membrane depolarization during action potential, regulation of sodium ion transmembrane transporter activity	Amino acid
	1433T_RAT	14-3-3 protein theta OS=Rattus norvegicus GN=Ywhaq PE=1 SV=1	49	27,761									negative regulation of ion transmembrane transport, negative regulation of ion	Amino acid

													transmembrane transport	
699	ASSY_RAT	Argininosuccinate synthase OS=Rattus norvegicus GN=Ass1 PE=2 SV=1	2261	46,467	0.006	1.8	4.07E+07	5.41E+07	6.60E+07	7.19E+07	up	independent	Urea cycle	Amino acid
	BHMT1_RAT	Betaine--homocysteine S-methyltransferase 1 OS=Rattus norvegicus GN=Bhmt PE=1 SV=1	667	44,948									methyltransferase, transferase	Homocysteine
	CGL_RAT	Cystathionine gamma-lyase OS=Rattus norvegicus GN=Cth PE=1 SV=2	332	43,577									amino-acid biosynthesis, Cysteine biosynthesis	Amino acid
	THIM_RAT	3-ketoacyl-CoA thiolase, mitochondrial OS=Rattus norvegicus GN=Acaa2 PE=2 SV=1	234	41,844									fatty acid beta-oxidation	Lipid
	ACADM_RAT	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Acadm PE=1 SV=1	136	46,526									Fatty acid metabolism	Lipid

THTR_RAT	Thiosulfate sulfurtransferase OS=Rattus norvegicus GN=Tst PE=1 SV=3	113	33,386	epithelial cell differentiation, rRNA import into mitochondrion, rRNA transport	Nucleotide
QCR2_RAT	Cytochrome b-c1 complex subunit 2, mitochondrial OS=Rattus norvegicus GN=Uqcrc2 PE=1 SV=2	97	48,366	Electron transport, Respiratory chain, Transport	Energy
FUMH_RAT	Fumarate hydratase, mitochondrial OS=Rattus norvegicus GN=Fh PE=1 SV=1	76	54,429	Tricarboxylic acid cycle	Carbohydrate
AADAT_RAT	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial OS=Rattus norvegicus GN=Aadat PE=1 SV=1	75	47,754	2-oxoglutarate metabolic process, glutamate metabolic process, kynurenine metabolic process, L-kynurenine metabolic process	Other
HMCS2_RAT	Hydroxymethylglutaryl-CoA synthase, mitochondrial OS=Rattus norvegicus GN=Hmgcs2 PE=2 SV=1	63	56,876	cholesterol biosynthesis, cholesterol metabolism, lipid biosynthesis and metabolism, steroid biosynthesis and metabolism	Lipid

899	ODPB_RAT	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial OS=Rattus norvegicus GN=Pdhb PE=1 SV=2	461	38,957	0.012	2	8.22E+05	1.63E+06	1.17E+06	1.04E+06	down	independent	tricarboxylic acid cycle	Carbohydrate
588	TBB2A_RAT	Tubulin beta-2A chain OS=Rattus norvegicus GN=Tubb2a PE=1 SV=1	145	49,875	0.016	1.9	1.18E+07	6.07E+06	7.92E+06	7.85E+06	up	independent	protein polymerization, microtubule-based process	Nucleotide
	TBB5_RAT	Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1	142	49,639									protein polymerization, spindle assembly	Nucleotide
	TBB4B_RAT	Tubulin beta-4B chain OS=Rattus norvegicus GN=Tubb4b PE=1 SV=1	121	49,769									protein polymerization, spindle assembly	Nucleotide
733	HPPD_RAT	4-hydroxyphenyl pyruvate dioxygenase OS=Rattus norvegicus GN=Hpd PE=1 SV=3	964	45,084	0.018	1.3	3.28E+06	4.00E+06	3.83E+06	4.13E+06	up	independent	L-phenylalanine catabolic proces, tyrosine catabolic process	Amino acid
	ACADL_RAT	Long-chain specific acyl-CoA dehydrogenase	279	47,842									fatty acid beta-oxidation using acyl-CoA dehydrogenase, lipid homeostasis,	Lipid

		, mitochondrial OS=Rattus norvegicus GN=Acadl PE=1 SV=1										long-chain fatty acid catabolic process, protein homotetramerization	
	IDHC_RAT	Isocitrate dehydrogenase [NADP] cytoplasmic OS=Rattus norvegicus GN=Idh1 PE=1 SV=1	138	46,705								Tricarboxylic acid cycle	Carbohydrate
	SAHH_RAT	Adenosylhomocysteinase OS=Rattus norvegicus GN=Ahcy PE=1 SV=3	101	47,507								One-carbon metabolism	
Other													
945	ETFB_RAT	Electron transfer flavoprotein subunit beta OS=Rattus norvegicus GN=Etfb PE=2 SV=3	1068	27,670	1.48E-07	3.4	8.24E+06	3.12E+06	1.05E+07	4.24E+06	up	other	Electron transport, Transport	Energy
	GSTM2_RAT	Glutathione S-transferase Mu 2 OS=Rattus norvegicus GN=Gstm2 PE=1 SV=2	401	25,686									Olfaction, Sensory transduction	Glutathione
	KAD2_RAT	Adenylate kinase 2, mitochondrial OS=Rattus	153	26,363									ATP metabolic process,Kinase, Transferase,	Nucleotide

		norvegicus GN=Ak2 PE=2 SV=2											oxidative phosphorylation	
295	ENPL_RAT	Endoplasmin OS=Rattus norvegicus GN=Hsp90b1 PE=1 SV=2	588	92,713	1.33E-05	7.5	4.48E+06	6.00E+05	2.11E+06	1.45E+06	down	other	cellular response to manganese ion, ER-associated ubiquitin-dependent protein catabolic process, protein folding	chaperone
826	RGN_RAT	Regucalcin OS=Rattus norvegicus GN=Rgn PE=1 SV=3	187	33,368	1.55E-05	2.8	2.63E+06	9.47E+05	2.40E+06	1.19E+06	down	other	ascorbate biosynthesis	Other
	ATPB_RAT	ATP synthase subunit beta, mitochondrial OS=Rattus norvegicus GN=Atp5b PE=1 SV=2	94	56,318									ATP synthesis, Hydrogen ion transport, Ion transport, Transport	Energy
	EF1D_RAT	Elongation factor 1-delta OS=Rattus norvegicus GN=Eef1d PE=1 SV=2	82	31,311									Protein biosynthesis, Transcription, Transcription regulation	Nucleotide
559	CH60_RAT	60 kDa heat shock protein, mitochondrial OS=Rattus norvegicus GN=Hspd1 PE=1 SV=1	2258	60,917	5.41E-04	1.3	5.09E+06	5.58E+06	6.07E+06	4.54E+06	down	other	chaperone	Chaperone
562	CH60_RAT	60 kDa heat shock protein, mitochondrial OS=Rattus norvegicus	274	60,917	0.002	1.4	7.67E+06	7.62E+06	9.63E+06	7.05E+06	down	other	B cell cytokine production and proliferation, response to hydrogen peroxide	chaperone

		GN=Hspd1 PE=1 SV=1											
629	FTCD_RAT	Formimidoyltransferase-cyclodeaminase OS=Rattus norvegicus GN=Ftcd PE=1 SV=4	1659	58,877	0.002	1.7	6.78E+06	6.77E+06	1.17E+07	7.64E+06	down	other	histidine catabolic process to glutamate and formamide, histidine catabolic process to glutamate and formate	Histidine
778	AMACR_RAT	Alpha-methylacyl-CoA racemase OS=Rattus norvegicus GN=Amacr PE=1 SV=3	378	41,801	0.007	2.5	1.83E+06	2.05E+06	4.60E+06	2.53E+06	down	other	Lipid metabolism; fatty acid metabolism.	Lipid
	ARGI1_RAT	Arginase-1 OS=Rattus norvegicus GN=Arg1 PE=1 SV=2	362	34,951									urea cycle	Amino acid
	ACDSB_RAT	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Acadsb PE=1 SV=1	152	47,793									fatty acid beta-oxidation using acyl-CoA dehydrogenase, fatty acid beta-oxidation, lipid homeostasis	Lipid
	EIF3H_RAT	Eukaryotic translation initiation factor 3 subunit H OS=Rattus norvegicus	143	39,880									formation of translation preinitiation complex	Amino acid

	GN=Eif3h PE=2 SV=1				
ACADS_RAT	Short-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Acads PE=1 SV=2	109	44,737	Fatty acid metabolism,	Lipid
ADHX_RAT	Alcohol dehydrogenase class-3 OS=Rattus norvegicus GN=Adh5 PE=1 SV=2	90	39,550	ethanol oxidation, ethanol catabolic process, aging	Alcohol
BHMT1_RAT	Betaine--homocysteine S-methyltransferase 1 OS=Rattus norvegicus GN=Bhmt PE=1 SV=1	88	44,948	methyltransferase, transferase	Homocysteine
ASSY_RAT	Argininosuccinate synthase OS=Rattus norvegicus GN=Ass1 PE=2 SV=1	87	46,467	Urea cycle	Amino acid
GABT_RAT	4-aminobutyrate aminotransferase, mitochondrial OS=Rattus	62	56,419	neurotransmitter degradation	Other

	norvegicus GN=Abat PE=1 SV=3	

Out of these proteins, 105 were classified as insulin dependent (>20%, proteins changed in DM1 and insulin treatment restored), and 62 proteins as insulin independent (<20%, proteins changed in DM1 and insulin treatment did not restore) and 20 proteins were related in other conditions (significant changes in insulin treated group indicating a potential side effect of insulin treatment itself). The carbonylated proteins in liver tissue were mapped using the B2G program, and the groups identified in insulin dependent and independent DM1 to be most affected by oxidative stress when classified to cellular component were organelle (103 insulin dependent and 61 insulin independent) and cell (104 insulin dependent and 62 insulin independent) while classifying proteins into molecular function identified the major groups as catalytic (91 insulin dependent and 56 insulin independent) and binding (98 insulin dependent and 59 insulin independent) (Figure 2).

The genes identified in insulin dependent and independent DM1 in each pathway are demonstrated in Table 2 (color description based in DM1 regulation; red – downregulation and green - upregulation). Of the insulin dependent proteins (104 converted to gene symbol), 55 pathways were found to be significantly enriched (FDR<0.05), and the most enriched metabolic pathways included amino acid and derivatives, cellular responses to stress, fatty acid, triacylglycerol, ketone body metabolism, and biological oxidations. For the insulin independent proteins (61 converted to gene symbol), 30 pathways were found to be significantly enriched (FDR<0.05), and the most enriched metabolic pathways included metabolism of amino acids and derivatives, the citric acid cycle (TCA) and respiratory electron transport. Twelve proteins were associated with other conditions but did not have a FDR<0.05.

Table 2. Representative genes in pathway showed be insulin dependent and independent with oxidatively damage and regulation (up or downregulation) in DM1. Green: upregulated in DM1, Red: downregulated in DM1.

ReactomePathway	P-value	FDR	HitGenes
Insulin Dependent - genes associated			
Beta oxidation of butanoyl-CoA to acetyl-CoA	0.0006	7.00E-03	Hadh,Echs1
Lysine catabolism	0.0024	2.68E-02	Aadat,Dlst
Neurotransmitter Clearance In The Synaptic Cleft	0.0024	2.68E-02	Aldh2,Comt
Purine catabolism	0.0024	2.68E-02	Pnp,Cat
Uptake and function of diphtheria toxin	0.0024	2.68E-02	Hsp90ab1,Hsp90aa1
Reversible hydration of carbon dioxide	0.0024	2.68E-02	Ca2,Ca1
O2/CO2 exchange in erythrocytes	0.0042	3.89E-02	Ca2,Ca1
Degradation of cysteine and homocysteine	0.0042	3.89E-02	Suox,Cth
Urea cycle	0.0042	3.89E-02	Ass1, Cps1
Erythrocytes take up oxygen and release carbon dioxide	0.0042	3.89E-02	Ca2,Ca1
Erythrocytes take up carbon dioxide and release oxygen	0.0042	3.89E-02	Ca2,Ca1
Purine salvage	0.0042	3.89E-02	Pnp,Adk
Synthesis of Ketone Bodies	0	1.82E-04	Hmgcl,Acat1,Hmgcs2
Beta oxidation of hexanoyl-CoA to butanoyl-CoA	0	3.85E-04	Hadh,Hadhb,Echs1
Beta oxidation of lauroyl-CoA to decanoyl-CoA-CoA	0	3.85E-04	Hadh,Hadhb,Echs1
Ketone body metabolism	0	3.85E-04	Hmgcl,Acat1,Hmgcs2
Phenylalanine and tyrosine catabolism	0.0001	6.43E-04	Qdpr/ Qdpr,Pah,Hpd

Mitochondrial fatty acid beta-oxidation of unsaturated fatty acids	0.0001	6.43E-04	Hadhb,Acadm,Eci1
Methylation	0.0002	2.07E-03	Mat1a,Ahcy,Comt
Attenuation phase	0.0003	3.40E-03	Hsp90ab1,Hsp90aa1,Hspa8
HSF1 activation	0.0004	4.69E-03	Vcp, Hsp90ab1,Hsp90aa1
HSF1-dependent transactivation	0.0018	1.99E-02	Hsp90ab1,Hsp90aa1,Hspa8
Glycolysis	0.0027	2.88E-02	Gapdh,Gapdhs,Pgk1
Beta oxidation of octanoyl-CoA to hexanoyl-CoA	0	<2.500e-04	Hadh,Hadhb,Acadm,Echs1
Beta oxidation of decanoyl-CoA to octanoyl-CoA-CoA	0	<2.500e-04	Hadh,Hadhb,Acadm,Echs1
mitochondrial fatty acid beta-oxidation of saturated fatty acids	0	1.43E-04	Hadh,Hadhb,Acadm,Echs1
Amino acid synthesis and interconversion (transamination)	0	1.91E-04	Got2,Got1,Glud1,Oat
Branched-chain amino acid catabolism	0	4.35E-04	Hibadh,Hsd17b10/ Hsd17b10,Acat1,Bckdha
Purine metabolism	0.0002	2.25E-03	Pnp,Cat,Adk,Atic
Detoxification of Reactive Oxygen Species	0.0002	2.49E-03	Prdx2, Prdx1, Cat,Sod2
Glutathione conjugation	0.0003	3.09E-03	Gstm1,Gstm2,Gstm3,Gsta3
Cellular response to heat stress	0.0009	1.01E-02	Vcp,Hsp90ab1,Hsp90aa1,Hspa8
AUF1 (hnRNP D0) destabilizes mRNA	0.004	4.15E-02	Psmb6,Psmb3,Psmc2,Hspa8
Hh ligand biogenesis disease	0.0045	4.09E-02	Psmb6,Psmb3,Vcp,Psmc2
Mitochondrial Fatty Acid Beta-Oxidation	0	2.00E-04	Hadh,Hadhb,Acadm,Echs1,Eci1
Sulfur amino acid metabolism	0	1.58E-04	Got1,Mat1a,Ahcy,Suox,Cth
Gluconeogenesis	0	1.67E-04	Got2,Got1,Mdh1,Gapdh,Gapdhs,Pgk1
Metabolism of nucleotides	0.0001	1.07E-03	Pnp,Ak2,Agxt2,Cat,Adk,Atic
Defective TPMT causes Thiopurine S-methyltransferase deficiency (TPMT deficiency)	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3

Defective SLC35D1 causes Schneckenbecken dysplasia (SCHBCKD)	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Defective GSS causes Glutathione synthetase deficiency (GSS deficiency)	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Defective MAT1A causes Methionine adenosyltransferase deficiency (MATD)	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Defective AHCY causes Hypermethioninemia with S-adenosylhomocysteine hydrolase deficiency (HMAHCHD)	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Defective GGT1 causes Glutathionuria (GLUTH)	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Defective OPLAH causes 5-oxoprolinase deficiency (OPLAHD)	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Defective UGT1A4 causes hyperbilirubinemia	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Phase II conjugation	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Defective GCLC causes Hemolytic anemia due to gamma-glutamylcysteine synthetase deficiency (HAGGSD)	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Defective UGT1A1 causes hyperbilirubinemia	0	5.56E-05	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3
Glucose metabolism	0	1.50E-04	Got2,Got1,Pgm1,Mdh1,Gapdh,Gapdhs,Pgk1
The citric acid (TCA) cycle and respiratory electron transport	0.0016	1.82E-02	Ldha,Ndufs2,Etfb/ Etfb, Etfa,Dlst,Sdhb,Atp5a1
Biological oxidations	0.0001	8.97E-04	Gstm1,Gstm2,Gstm3,Mat1a,Ahcy,Comt,Gsta3, Aldh2
Fatty acid, triacylglycerol, and ketone body metabolism	0.0008	8.53E-03	Hadh,Hmgcl,Acat1,Hadhb,Hmgcs2,Acadm,Echs1,Eci1
Cellular responses to stress	0.0022	2.72E-02	Vcp,Hsp90ab1,Prdx2,Prdx1,Cat,Hsp90aa1,Hspa8,Sod2
Metabolism of amino acids and derivatives	0	<5.000e-04	Hibadh,Got2,Got1,Mat1a,Aadat,Qdpr/Qdpr,Hsd17b10/ Hsd17b10,Ahcy,Ass1,Glud1,Pah,Acat1,Psmb6,Psmb3,Aldh4a1,Cps1,Psmc2,Agxt2,Hpd,Suox,Cth,Oat,Bckdha, Dlst,Ftcd

Insulin Independent - Genes Associated			
Synthesis of Ketone Bodies	0.0005	7.75E-03	Acat1,Hmgcs2
Sulfide oxidation to sulfate	0.0008	1.09E-02	Suox,Tst
Ketone body metabolism	0.0008	1.09E-02	Acat1,Hmgcs2
Uptake and function of diphtheria toxin	0.0011	1.12E-02	Hsp90ab1,Hsp90aa1
mitochondrial fatty acid beta-oxidation of unsaturated fatty acids	0.0011	1.12E-02	Acadm,Acadl
mitochondrial fatty acid beta-oxidation of saturated fatty acids	0.0019	1.92E-02	Acadm,Acadl
Association of TriC/CCT with target proteins during biosynthesis	0.0025	2.24E-02	Cct2,Cct5
Attenuation phase	0.0036	3.05E-02	Hsp90ab1,Hsp90aa1
HSF1 activation	0.0043	3.50E-02	Hsp90ab1,Hsp90aa1
Branched-chain amino acid catabolism	0.0058	4.17E-02	Dld,Acat1
Regulation of pyruvate dehydrogenase (PDH) complex	0.0058	4.17E-02	Pdhb,Dld
Lysine catabolism	0	3.57E-04	Aadat,Dld,Dlst
Degradation of cysteine and homocysteine	0	3.68E-04	Suox,Cth,Tst
Folding of actin by CCT/TriC	0.0001	3.50E-04	Cct2,Actb,Cct5
Amino acid synthesis and interconversion (transamination)	0.0001	1.14E-03	Got2,Got1,Glud1
Mitochondrial Fatty Acid Beta-Oxidation	0.0002	2.35E-03	Acadm,Acadl,Pccb
Glutathione conjugation	0.0012	1.24E-02	Gstm1,Gstm2,Gstm3
Citric acid cycle (TCA cycle)	0	3.33E-04	Dld,Idh2,Fh,Dlst
Formation of tubulin folding intermediates by CCT/TriC	0	3.53E-04	Tubb2a,Cct2,Tubb4b,Cct5
Defective TPMT causes Thiopurine S-methyltransferase deficiency (TPMT deficiency)	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy

Defective SLC35D1 causes Schneckenbecken dysplasia (SCHBCKD)	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Defective GSS causes Glutathione synthetase deficiency (GSS deficiency)	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Defective MAT1A causes Methionine adenosyltransferase deficiency (MATD)	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Defective AHCY causes Hypermethioninemia with S-adenosylhomocysteine hydrolase deficiency (HMAHCHD)	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Defective GGT1 causes Glutathionuria (GLUTH)	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Defective OPLAH causes 5-oxoprolinase deficiency (OPLAHD)	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Defective UGT1A4 causes hyperbilirubinemia	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Phase II conjugation	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Defective GCLC causes Hemolytic anemia due to gamma-glutamylcysteine synthetase deficiency (HAGGSD)	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Defective UGT1A1 causes hyperbilirubinemia	0.001	9.70E-03	Gstm1,Gstm2,Gstm3,Ahcy
Loss of Nlp from mitotic centrosomes	0.0016	1.55E-02	Hsp90aa1,Dctn2,Tubb4b,Ywhag
Loss of proteins required for interphase microtubule organizationÂ from the centrosome	0.0016	1.55E-02	Hsp90aa1,Dctn2,Tubb4b,Ywhag
Centrosome maturation	0.0025	2.15E-02	Hsp90aa1,Dctn2,Tubb4b,Ywhag
Recruitment of mitotic centrosome proteins and complexes	0.0025	2.15E-02	Hsp90aa1,Dctn2,Tubb4b,Ywhag
Regulation of PLK1 Activity at G2/M Transition	0.004	3.30E-02	Hsp90aa1,Dctn2,Tubb4b,Ywhag
Anchoring of the basal body to the plasma membrane	0.0057	4.31E-02	Hsp90aa1,Dctn2,Tubb4b,Ywhag

Activation of BAD and translocation to mitochondria	0	<2.500e-04	Ywhaz,Ywhab,Ywhag,Ywhah,Ywhaq
Sulfur amino acid metabolism	0	<1.667e-04	Got1,Suox,Cth,Ahcy,Tst
Glycolysis	0	<1.250e-04	Aldoa,Aldob,Tpi1,Eno1,Pgk1
Activation of BH3-only proteins	0	<1.250e-04	Ywhaz,Ywhab,Ywhag,Ywhah,Ywhaq
Prefoldin mediated transfer of substrate to CCT/TriC	0	<1.111e-04	Tubb2a,Cct2,Tubb4b,Actb,Cct5
Cooperation of Prefoldin and TriC/CCT in actin and tubulin folding	0	<1.000e-04	Tubb2a,Cct2,Tubb4b,Actb,Cct5
Chaperonin-mediated protein folding	0	<9.091e-05	Tubb2a,Cct2,Tubb4b,Actb,Cct5
Protein folding	0	8.33E-05	Tubb2a,Cct2,Tubb4b,Actb,Cct5
Intrinsic Pathway for Apoptosis	0	1.54E-04	Ywhaz,Ywhab,Ywhag,Ywhah,Ywhaq
Pyruvate metabolism and Citric Acid (TCA) cycle	0	3.89E-04	Pdhb,Dld,Idh2,Fh,Dlst
Translocation of GLUT4 to the plasma membrane	0.0001	3.81E-04	Ywhaz,Ywhab,Ywhag,Ywhah,Ywhaq
Biological oxidations	0.0028	2.33E-02	Gstm1,Gstm2,Gstm3,Ahcy,Aldh2
Membrane Trafficking	0.0047	3.73E-02	Ywhaz,Ywhab,Ywhag,Ywhah,Ywhaq
Apoptosis	0.0052	3.97E-02	Ywhaz,Ywhab,Ywhag,Ywhah,Ywhaq
Programmed Cell Death	0.0069	4.99E-02	Ywhaz,Ywhab,Ywhag,Ywhah,Ywhaq
Assembly of the primary cilium	0.0025	2.28E-02	Cct2,Hsp90aa1,Dctn2,Tubb4b,Ywhag,Cct5
Gluconeogenesis	0	<3.333e-04	Aldoa,Aldob,Tpi1,Eno1,Pgk1
Glucose metabolism	0	<2.000e-04	Aldoa,Aldob,Tpi1,Eno1,Pgk1
Metabolism of carbohydrates	0.002	1.82E-02	Aldoa,Aldob,Tpi1,Eno1,Pgk1
Glycogen storage diseases	0.002	1.82E-02	Aldoa,Aldob,Tpi1,Eno1,Pgk1
Myoclonic epilepsy of Lafora	0.002	1.82E-02	Aldoa,Aldob,Tpi1,Eno1,Pgk1
The citric acid (TCA) cycle and respiratory electron transport	0	3.13E-04	Uqcrc2,Uqcrc1,Pdhb,Dld,Idh2,Fh,Dlst,Atp5a1

Metabolism of amino acids and derivatives	0	<5.000e-04	Hpd,Aadat,Suox,Cth,Dld,Ahcy,Ass1,Glud1,Acat1,Dlst,Ftcd,Tst

3.2. Oxidatively damaged proteins associated with insulin dependent

Insulin dependent. Forty-eight spots with oxidative damage corresponding to 44 proteins were found to be significantly upregulated in DM1, whereas 87 spots corresponding to 69 proteins were downregulated.

Amino acid metabolism. Carbamoyl-phosphate synthase (Cps1) and ornithine aminotransferase (Oat) are enzymes involved in urea cycle and associated with the increase of gluconeogenesis via amino acid catabolism and showed an increase oxidative damage (16). DM1 is characterized by inflammation and oxidative stress, and Cps1 plays a pivotal role in diabetic nephropathy. Theses enzymes were found upregulated in DM1 and insulin treatment was found to abrogate carbonylation. Aspartate aminotransferase cytoplasmic and mitochondrial (Got1 and Got2) showed a downregulation in DM1. The metabolism of amino acids is initiated by aminotransferases which control the production of glutamate, and the transamination reaction mediates the synthesis of aspartate, glutamine, and glutamate from ammonia and intermediates of the glycolysis (17). Got1 and Got2 activity is also involved in hepatic glucose synthesis. In DM1 glucose synthesis by glycolysis decreased and oxidative stress increased indicating that the oxidative modification of these enzymes in DM1 is directly associated with oxidative stress and liver damage, while treatment with insulin restored these enzymes indicating a decrease in oxidative stress. Branched-chain amino acids (BCAA) catabolic process 3-hydroxyisobutyrate dehydrogenase (Hibadh) which is an oxidoreductase and 2-oxoisovalerate dehydrogenase subunit alpha and beta (Bckdha) were found to have increased oxidative modification in DM1. The metabolism of BCAAs is altered in association with insulin deficiency. BCAAs are particularly responsive to the inhibitory action of insulin (18), and are associated with liver dysfunction in DM1. The upregulation of Hibadh and Bckdha suggests an increase in energy requirement. Other proteins which showed upregulation which were abrogated by insulin treatment included Kynurenine--oxoglutarate transaminase 3 (Ccbl2), kynurenine--oxoglutarate transaminase (Ccbl2), dimethylglycine dehydrogenase (Dmgdh) and alanine--glyoxylate aminotransferase 2 (Agxt2).

Carbohydrate and energy metabolism. In DM1 gluconeogenesis is increased and glycolysis decreased. Two isoforms of glyceraldehyde-3-phosphate dehydrogenases (Gapdh and Gapdhs) were found with upregulation (increased oxidative damage) in DM1. In glucose metabolism these proteins are involved in both gluconeogenesis and glycolysis, and carbonylation may be related to the activation of gluconeogenesis and the inactivation of glycolysis in DM1. Treatment with insulin decreased modification of these enzymes. Gapdh also

has non-glycolytic activities such as binding to DNA and RNA, transcription regulation, interactions with small molecules and oxidative modification which interferes with its activity and structure (19). Phosphoglucomutase-1 (Pmg-1) is involved in gluconeogenesis and glycolysis and had upregulation in DM1 which may be associated with activation of gluconeogenesis. In DM1 oxidative stress and damage are common, and the activation of gluconeogenesis in DM1 is associated with uncontrolled diabetes which may result in diabetic complications. L-xylulose reductase (Dcxr) participates in an alternative route of glucose-6-phosphate oxidation (20) and was found to have upregulation in DM1. Other enzymes involved in energy metabolism, such as electron transfer flavoprotein subunit alpha and beta (Etfa, Etfb) and NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (Ndufs2) were found to have an upregulation in DM1 are involved in reactions of fatty acid oxidation (21); and ATP synthase subunit alpha (Atp5a1) showed a downregulation in DM1. These data indicate an increase in ATP production via fatty acid oxidation.

Lipid metabolism. In metabolic conditions observed in diabetes fatty acids are β-oxidized to acetyl CoA, which are oxidized in the citric acid cycle by the reduced forms of NADH and $FADH_2$, which are reoxidized by electron transport chain resulting in higher electron flow through the respiratory chain (22). This can provoke the leakage of electrons from complexes I and III, which reduces the levels of molecular oxygen. This favors mitochondrial ROS generation and consequently oxidative stress leading to protein carbonylation (23). Excessive fatty acid oxidation and lipotoxicity are associated with ROS generation in diabetes (24). Enzymes associated with fatty acid metabolism (FAO) and oxidative damage were found to have downregulation in DM1 including enoyl-CoA hydratase (Echs1), enoyl-CoA delta isomerase 1 (Eci1), acyl-coenzyme A synthetase ACSM3 (Acsm3), acyl-CoA synthetase family member 2 (Acsf2), 3-ketoacyl-CoA thiolase (Acaa2), hydroxyacyl-coenzyme A dehydrogenase (Hadh) as well as upregulation of delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase (Ech1) and medium-chain specific acyl-CoA dehydrogenase (Acadm).

Nucleotide metabolism. Adenylate kinase (Ak2) and GTP:AMP phosphotransferase (Ak3) had up or downregulation, respectively, in DM1. These enzymes maintain cellular ATP levels by increasing AMP. A protein involved in RNA process identified as having decreased oxidative damage in DM1, 3-hydroxyacyl-CoA dehydrogenase type-2 (Hsd17b10), suggests involvement in defects in the regulation of fat oxidation and of β cell function (25). A protein involved in mRNA processing and splicing, heterogeneous nuclear ribonucleoprotein K (Hnrnpk), was found in association with increased oxidative damage, and is associated with

insulin activated mitochondria gene expression in DM1 (26). Hnrnpk regulates mRNA transcription, gene stability and translation involved in the control of metabolism, oxidative stress and apoptosis (27). Treatment with insulin restored Hnrnpk carbonylation to normal levels in DM1+I. Ribosomal protein 26S protease regulatory subunit 7 was found to have upregulation and its function catalyzes protein translation and extraribosomal functions. The oxidative modification of this protein may alter the regulation of apoptosis, development and cellular transformation (28) and is implicated in complications and the progression of DM1.

Chaperone and oxidative stress. Protein oxidation, lipid peroxidation and DNA damage has been observed following the induction of oxidative stress (19), and as may be expected the antioxidant enzymes superoxide dismutase [Mn] (Sod2) and catalase (Cat) showed downregulation which was restored with insulin treatment. Other antioxidant enzymes with altered oxidative damage found in this study included peroxiredoxin 1 and 2 (Prx1 and Prx2). These enzymes are thiol-dependent in the degradation of H_2O_2 and alkyl peroxides (29), but interestingly it was found that Prx1 had increased carbonylation while Prx2 was decreased. The upregulation of Prx1 may be associated with the increase of cytokines and immunological dysregulation (30) in DM1, and insulin treatment restored Prx1 carbonylation levels. Three isoforms of carbonic anhydrase (Ca1, Ca2, Ca3) were found to exhibit upregulation, and a previous study showed that the inhibition of these enzymes is associated with the prevention of hyperglycemia and induces endothelial cell death (31). Hyperglycemia can be a strong inducer of Ca that can result in liver damage and treatment with insulin controls hyperglycemia. Stress-70 protein (Hspa9), catechol (Comt) and 78 kDa glucose-regulated protein (Hspa5) were found downregulated, indicating a decrease in the stress response to damage and oxidative stress observed in DM1 (32).

3.3. Oxidatively damaged proteins associates with insulin independent

Insulin independent. Seven spots with oxidative damage corresponding to 40 different proteins were found to have significantly increased carbonylation in DM1, whereas 10 spots corresponding to 26 different proteins were decreased.

Amino acid metabolism. Proteins targets found downregulated in DM1 include 14-3-3 proteins (Ywhaz, Ywhae, Ywhah, Ywhag). These proteins perform regulatory steps in insulin-regulated glucose homeostasis and are involved in the intrinsic pathway for apoptosis and translocation of GLUT4 to the plasma membrane (33). Treatment with insulin did not restore these enzymes to their normal oxidative state.

Carbohydrate and energy metabolism. DM1 is associated with glucose disturbance and Triosephosphate isomerase (Tpi1), bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (Dak), alpha-enolase (Eno1) and fructose-bisphosphate aldolase B (Aldob, Aldob) all showed alterations of protein carbonylation which can be associated with an imbalance between glycolysis and gluconeogenesis. Cytochrome b-c1 complex subunit 1 (Uqcrc1) and cytochrome b-c1 complex subunit 2 (Uqcrc1) showed downregulation. Cytochromes are the primary system for chemical defense in animals and is involved in some complications associated with DM1 (34).

Lipid metabolism. The enzyme 4-trimethylaminobutyraldehyde dehydrogenase (Aldh9a1) showed downregulation in DM1 and may be involved in alterations of lipid metabolism.

3.4. Oxidatively damaged proteins associates with insulin dependent and independent

Alcohol metabolism. Aldehyde dehydrogenase 2 (Aldh2) is involved in ethanol metabolism and is responsible for the detoxification and oxidation of aromatic and aliphatic aldehydes. The accumulation of aldehydes can increase liver damage. Aldh2 is an antioxidant enzyme which is inactivated by free radicals (35), and previous studies have shown it to be a target of oxidative modification during hepatic ischemia-reperfusion (36) and glyceryl trinitrate tolerance (37). Adlh2 was found downregulation in both insulin dependent and independent DM1.

Amino acid metabolism. The enzyme argininosuccinate synthetase (Ass1) was found upregulated in both insulin dependent and independent DM1. Isovaleryl-CoA dehydrogenase (Ivd) was found to have both up and downregulation in insulin dependent and independent DM1 respectively. This enzyme has a catalytic oxidoreductase activity. Cystathionine gamma-lyase (Cth) was found upregulated in both insulin dependent and independent spots. This enzyme creates H_2S and may generate a redox imbalance (38) which can lead to liver injury. 4-hydroxyphenylpyruvate dioxygenase (Hpd) was found upregulated, associated with insulin dependent and independent DM1 and is responsible to catalyze the formation of homogentisate from 4-hydroxyphenylpyruvate (4-HPP) and molecular oxygen (39).

Carbohydrate and energy metabolism. Phosphoglycerate kinase 1 (Pgk1) was found upregulated in both insulin dependent and independent DM1, but as this enzyme is involved in gluconeogenesis and glycolysis, its modification may be related to inactivation of glycolysis in

DM1 and activation of gluconeogenesis in DM1+I. Isocitrate dehydrogenase [NADP] (Idh1) and dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (Dlst) are enzymes involved in the TCA which were found up and downregulated, respectively. Sulfite oxidase (Suox) is involved in the accumulation of sulfide (40) and downregulated in insulin dependent and independent.

Chaperone and oxidative stress. Different isoforms of glutathione S-transferase (Gst) were identified as having up and downregulation oxidative damage. This data is in accord with previous studies of the oxidation of Gst in DM1 which are contradictory (41–44). Heat shock proteins HSP 90-alpha and HSP 90-beta (Hsp90aa1 and Hsp90ab1) both show downregulation in insulin dependent and independent DM1 and are associated with defects in glycogen synthesis mediated by insulin.

4. Conclusion

The Oxi-proteome presented here constitutes potential proteins that may be associated with cellular dysfunctions described during the progression of DM1. The carbonylated proteins identified represent potential pre-symptomatic biomarkers for DM1. Treatment with insulin is shown to be associated with a decrease of oxidative stress through the upregulation of antioxidant enzymes and a decrease of ROS generation, control of glucose carbohydrate metabolic pathways (glycolysis and gluconeogenesis) and energy metabolism.

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Capítulo IV

Using the proteomic approach to identify metalloproteins and proteins that are metal-binding with copper, magnesium, selenium and zinc in spots of liver samples from diabetic rats

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 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 electrospray ionization mass spectrometry. 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 new bound and how type 1 diabetes and insulin treatment can modify some metal bonds in proteins, and therefore affect their mechanism of action and function. Thereby, identifying metalloproteins that may be used as biomarkers in diabetic diagnosis and diabetic progression upon insulin treatment.

Keywords: electrospray ionization-tandem mass spectrometry, flame atomic absorption spectrometry, graphite furnace atomic absorption spectrometry, metalloproteomic, type 1 diabetes, two-dimensional electrophoresis

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). The 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 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 copper and zinc that catalyzes the decomposition of the superoxide anion. Therefore, it is a component of the cellular defense system protecting against oxidative damage. Copper is a vital component of electron transfer reactions of SOD, underlying its antioxidant function (5).

Experimental data shows that magnesium is required as an important cofactor in many enzyme reactions. Importantly for DM1, magnesium 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, magnesium participates in the intracellular signaling system, phosphorylation and dephosphorylation reactions that activate or inhibit a multitude of enzymes (8).

Selenium is an essential micronutrient for the synthesis of selenoproteins, which play an important role in the synthesis, metabolism and action of thyroid hormones. Furthermore, selenium modifies the expression of selenoproteins, including the families of peroxidases, selenoenzymes, glutathione and thioredoxin (9). Zinc is a component of synthetases and transferases, including DNA and RNA, digestive enzymes, and associates with insulin. Zinc also participates in metabolic pathways involving protein synthesis, carbohydrates, lipids, and nucleic acids. In addition, zinc also plays a role in apoptoically 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 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 metal ions in diabetes and how treatment with insulin can alter this condition providing valuable information about how a metal ion is distributed to and coordinated 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 of copper (Cu), magnesium (Mg), selenium (Se) and zinc (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 years 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: diabetics 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 hours after the STZ administration, and the animals with glycemic levels greater that 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 3U/animal; this dose was adjusted or maintained so that it reached serum glucose levels within the normal range. At the end of the 30 day experimental period, the animals were anaesthetised (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, macerate, and the protein extracts were separated from the solid portion by centrifugation at 10,000 g, 4°C, 10 min in a refrigerated centrifuge. The protein extracts were used to quantify protein resuspending in 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 to 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 minutes at room temperature, absorbance readings were performed in a spectrophotometer at a wavelength of 545 nm.

2.3. Eletrophoretic runs (2D-PAGE)

With the standardisztion 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}; thiourea 2 mol L^{-1}; CHAPS (Sulphate 3-[(3-cloroaminopropil)-dimethylammonio]-1-propane) 2% (w/v); ampholytes 0.5% (v/v) at a pH ranging from 3 to 10; 0.002% bromophenol blue (w/v) 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 (250 µL of the solution described) was added to 13-cm strips containing polyacrylamide gel with ampholytes immobilized at a pH 3 to 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 the isoelectric focusing Ettan IPGphor (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 DTT was replaced by 2.5% (w/v) iodoacetamide.

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 ImageScanner III (GE Healthcare), and the images were analyzed by the ImageMaster 2D Platinum 7.0 (GeneBio, Geneva, Switzerland).

2.4. Characterization of protein spots by ESI-MS/MS

The protein spots were extracted from the gels using a scalpel and cut into segments of approximately 1 mm^3, prepared for MS according Shevchenko et al. (2006) (13) 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 bath 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 ^oC; after time, the excess reagent was removed and 55 mM IAA in Ambic 50 mM was added for 30 min at room temperature); c) tryptic digestion of proteins (incubated overnight at 37 ^oC 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 ^oC under sonication; the supernatant was collected and transferred to 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 acquisition was obtained during 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 (14). 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 the Reactome Functional Interaction (FI) (15), a Cytoscape plugin (16). The 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.

2.5. 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. (2013) (17). 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 nm; 285.2 nm; 190.0 nm 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.

3. Results and Discussion

3.1. Protein separation by 2D-PAGE

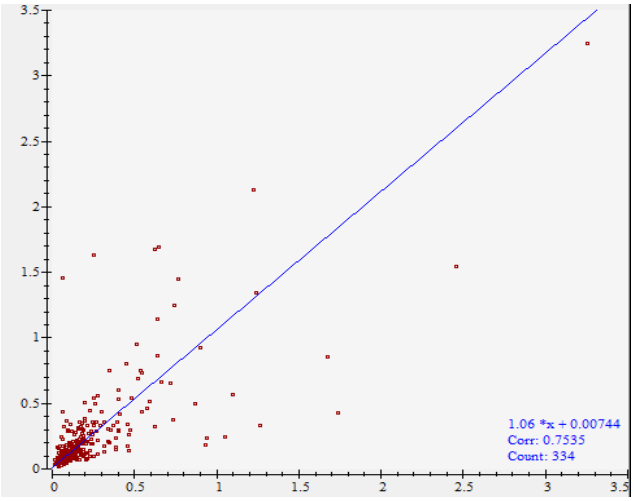
The total protein concentrations in the pool of pellets in the C, DM1 and DM1+I groups were 131.12, 92.86 and 105.79 g L^{-1} in the total extract and 26.09, 20.05 and 21.79 g L^{-1} precipitating with acetone, respectively. The pellets obtained with the acetone precipitation were used to calculate the 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 the ImageMaster 2D Platinum v.7. 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 the results of the correlation between the pairs of gels. The correlation between the gel replicates, encompassing the three electrophoretic runs (six gels of each group studied), showed that, on average in the C, DM1 and DM1+I groups, there were 86, 79 and 91%, respectively.

Figure 1 shows the correlation between the protein spots from gels, considering the % 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 ($R > 0.76$). The nearer the point is to one, the lower will be the %V difference between the equivalent spots. It is inferred that there are both equivalent protein spots and different protein spots in the respective gels analyzed. Through the correlation analysis, we can see that the proteomic profile of the C group is closer to the proteomic profile of the DM1+I group than to the profile of the DM1 group. The most spots were distributed in the molecular weight (Mw) range of 31 to 76 kDa with isoelectric points

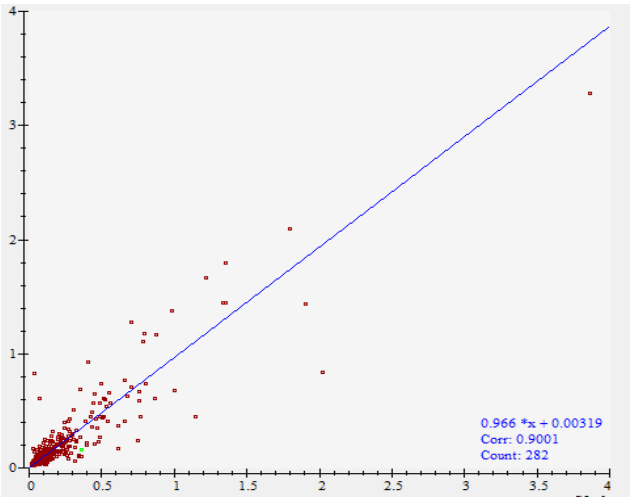
(pI) ≅ 6; in general, the Mw and pIs distribution were homogeneous among the different experimental groups.

Figure 1. Correlation between C and DM1 (A), C and DM1+I (B), DM1 and DM1+I (C). The nearer the point is to one, the lower will be the %V difference between the equivalent spots. It is inferred that there are both equivalent protein spots and different protein spots in the respective gels analyzed.

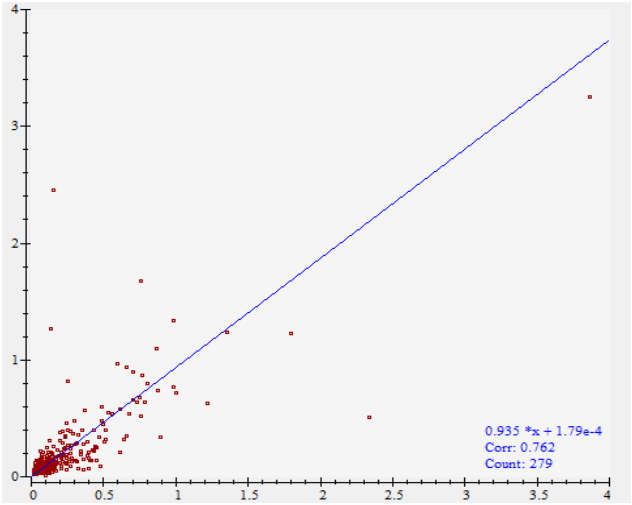
A.



B.



C.



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

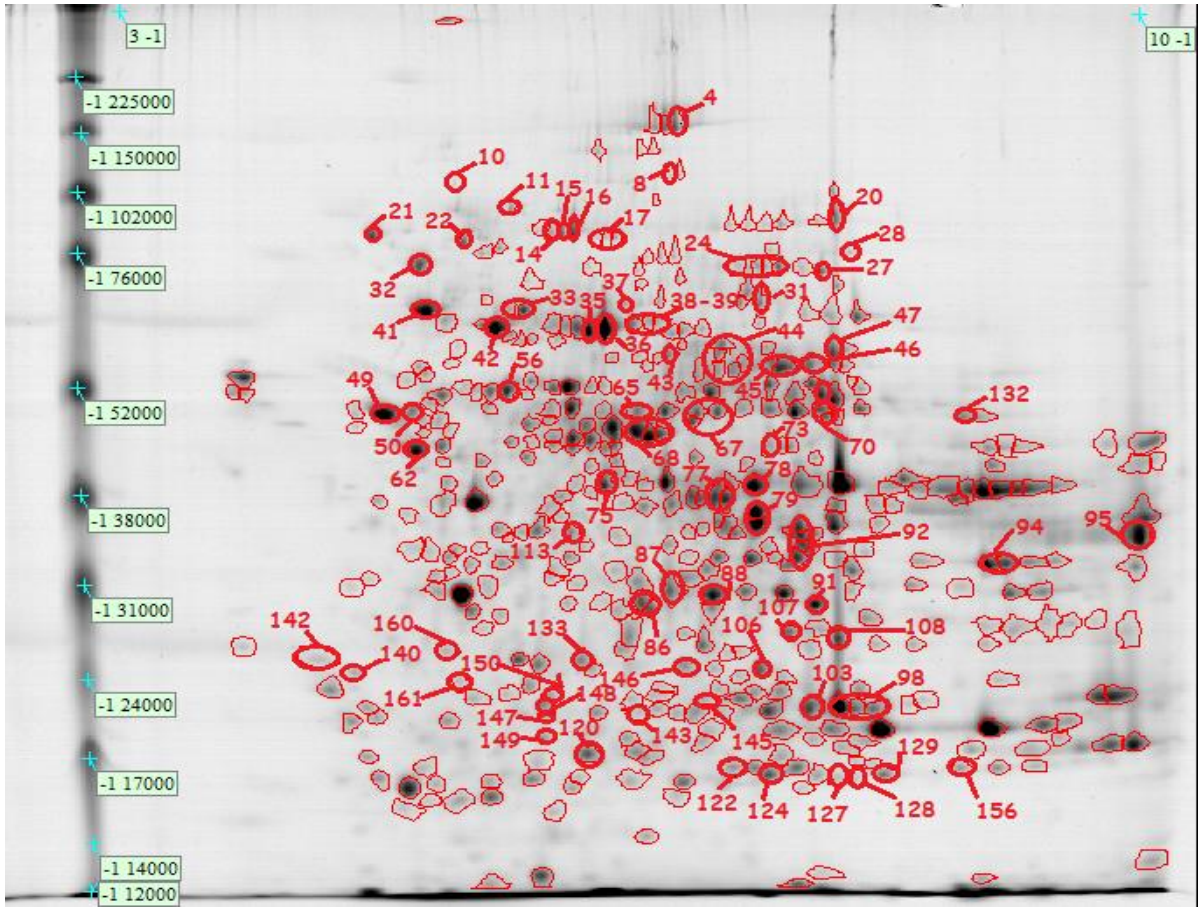
mass in the protein spots and the Cu, Mg, Se and Zn masses to the number of protein molecule spots and the number of atoms of Cu Mg, Se and Zn. These calculations made it possible to estimate how many atoms of Cu Mg, Se and Zn would be present per molecule of protein spots (18,19). Figure 2*A* shows the protein spots containing the metals of interest, and nonmetals are also indicated; Figures 2*B–D* show 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 highest percentage of Se and the DM1+I group showed the highest percentage of Mg.

Selenium 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 selenium 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 selenium and fasting plasma glucose (24), The effect of selenium 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 the most protein spots with Se presence, because the most Se available that can bind with protein and interfere in theirs function and consequently in their pathways.

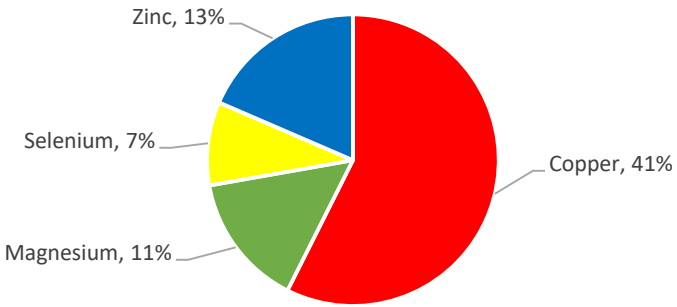
The feed was analyzed for Cu, Mg, Se and Zn in order to exclude the role of feed in the results of this work. The following concentrations: Se: <limit of quantification (LOQ), Cu: 2.36 mg kg^{1}, Mg: 28.90 mg kg^{1}, and Zn: 51.40 mg kg^{1}. Thus, the feed was observed to be a major source of Mg and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

Figure 2. *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 control group. *C.* Qualitative analysis of Cu, Mg, Se and Zn in type 1 diabetes group. *D.* Qualitative analysis of Cu, Mg, Se and Zn in type 1 diabetes treated with insulin group.

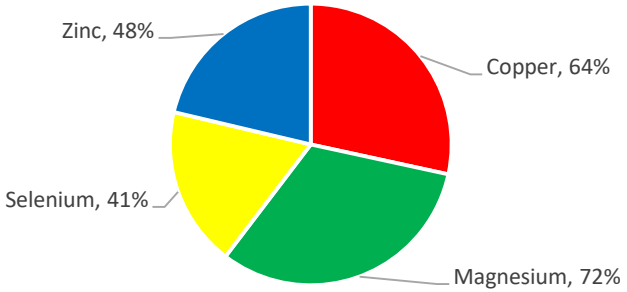
A. Gel obtained by 2D-PAGE (pH 3–10) for liver tissue



B. Qualitative analysis of Cu, Mg, Se and Zn in control group



C. Qualitative analysis of Cu, Mg, Se and Zn in type 1 diabetes group



D. Qualitative analysis of Cu, Mg, Se and Zn in type 1 diabetes treated with insulin group

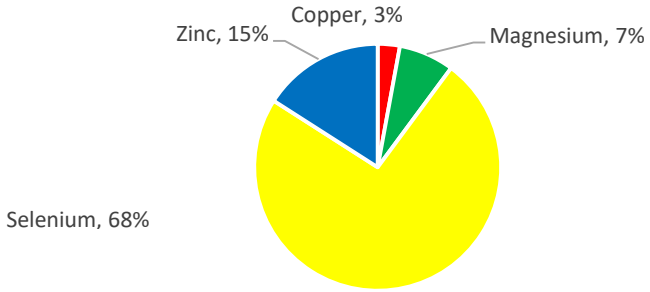


Table 1 shows the Cu Mg, Se and Zn concentrations determined in each protein spot with their respective Mw and pIs and the protein mass measured using the ImageMaster 2D Platinum 7.0 software.

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)	PTN mass (µg)	[Cu] (mg g^{-1})	[Mg] (mg g^{-1})	[Se] (mg g^{-1})	[Zn] (mg g^{-1})	pI	Mw (Da)	PTN mass (µg)	[Cu] (mg g^{-1})	[Mg] (mg g^{-1})	[Se] (mg g^{-1})	[Zn] (mg g^{-1})	pI	Mw (Da)	PTN (µg)	[Cu] (mg g^{-1})	[Mg] (mg g^{-1})	[Se] (mg g^{-1})	[Zn] (mg g^{-1})
4								6.86	138,865	2.62			0.08		6.74	158,761	2.46	0.07	0.29		
8								6.74	104,125	0.12			1.54	8.36	6.69	126,372	0.13	2.00	1.16		
10															5.21	107,997	0.26	1.80		1.20	
11															5.21	107,997	0.26	1.80		1.20	
14								5.96	80,910	0.37			1.14	16.68	5.84	95,728	0.21	4.75	11.41		
15								6.05	79,903	0.61			3.60	0.52	5.91	94,630	0.50	0.69	4.40		1.87
16								6.12	80,910	0.50			1.14		5.98	94,630	0.70	0.44	0.00		5.55
17								6.37	80,070	0.27			1.01		6.26	93,275	0.26	0.43	4.80	1.02	
20								7.86	85,065	0.97			2.11		7.80	100,830	0.82	0.15	1.72		
21	4.75	82,042	0.89		1.25			4.63	81,418	0.49			2.49		4.64	92,471	0.62	0.20	0.00	1.86	
22															5.22	80,016	0.33	0.53	3.42		3.14
24								7.34	68,267	0.29			0.74		7.29	77,327	0.34	0.53			
27															7.70	75,747	0.21	0.17	0.15		
28								7.97	73,142	0.25			4.46	7.82	7.92	81,919	0.29	0.07			

31								7.34	68,267	0.29			0.74		7.31	70,164	0.38		1.15	0.81	
32															4.98	80,514	1.11	0.32	0.18		
33															5.62	70,282	0.56	0.82	3.12	0.30	8.34
35								6.22	59,239	1.24			1.39	4.66	6.09	66,084	1.28	0.51		0.11	
36								6.12	80,910	0.50			1.14		6.19	66,084	4.49	0.09	0.60		0.11
37	7.65	48,290	1.41	3.38																	
38-39								6.71	59,815	0.34			0.88	0.72	6.54	66,823	0.23	2.29	0.65	0.97	
41								5.04	63,227	1.58			0.48		5.03	69,006	1.97	0.36	0.66		
42								5.56	59,695	1.73			0.73		5.50	66,126	0.88	0.23		2.40	91.65
43															6.67	60,807	0.27	2.05	4.13	4.36	
44	7.17	56,576	0.30		0.59																
45								7.44	53,314	0.85			1.65		7.40	58,921	0.72	0.85	5.66		11.76
46	7.75	55,426	0.81			0.10		7.68	49,569	0.46			0.84	3.82	7.57	59,115	0.35	1.63	3.30	1.89	
47								6.65	43,490	2.14			1.92		7.82	59,365	0.56	1.54	1.66	0.01	6.39
49								4.70	47,123	2.75			0.44		4.76	51,827	2.45	0.16	0.82	0.47	
50								5.43	41,025	0.35		6.14	2.95		3.86	53,581	0.90	0.14	0.48		
56								6.06	50,138	1.24			2.63		5.46	55,395	0.63	0.61		0.53	
60	7.83	50,912	0.75				5.21	7.68	49,569	0.46			0.84	3.82	7.65	54,575	0.63	9.72	1.17	0.77	24.27
62	5.05	43,140	1.65				2.56								4.99	46,890	2.51			0.94	

65	6.57	48,632	0.52				5.33	6.55	45,951	1.07			0.58		6.78	54,481	0.42	1.97	6.45	1.40	
67								6.98	46,486	0.55	11.00		1.84		6.95	50,547	0.77	1.20	2.72	0.63	2.15
68								6.60	44,720	1.6			1.25		6.50	48,730	1.91	0.25	0.14	0.66	5.83
70								7.68	49,569	0.46			0.84	3.82	7.69	51,482	1.13	0.59	6.16		12.83
73															7.05	61,417	0.79	0.34	2.43	0.18	0.65
75								6.31	38,278	0.59			0.29		5.92	40,622	0.33	1.83	8.55	3.13	49.52
77	7.08	38,070	0.75		0.63			7.05	38,060	1.13			0.31		7.05	40,063	1.40	0.36	1.64	0.50	8.68
78	7.36	38,812	2.32		0.20		2.17	7.27	37,890	1.66		0.15	0.93		7.26	41,861	1.77	0.15	1.77		
79	7.38	36,070	2.09				1.11	7.27	35,188	1.93			0.53		7.27	38,265	1.64	0.25	1.44		21.63
86	6.59	29,608	0.84	0.05				6.54	29,852	0.61			0.30		6.46	31,747	1.26		3.06		
87	6.79	30,496	0.95	0.05											6.67	33,737	1.03		3.52	0.76	4.00
88	7.08	30,197	1.84	0.10				7.55	38,668	0.96			0.91		7.19	33,206	0.56		5.05		18.18
91								7.65	27,962	2.32			0.57		7.63	32,597	1.93	0.02	1.12		10.28
92	6.62	29,320	0.79	0.53											7.66	35,393	1.02	0.23	3.19	0.82	18.18
94	9.19	32,597	0.94	0.13				9.02	28,940	0.21			3.77		9.26	37,304	1.74		2.20	0.03	13.35
95	10.04	35,405	2.99	0.03				9.96	36,471	1.59			6.26		9.96	38,000	1.15		4.05	1.22	5.04
98	8.23	19,171	4.23	0.30				8.05	19,525	0.97			2.15		8.13	18,437	0.49	0.31	11.92		2.72
103	7.74	21,061	1.26	0.52																	
106	7.41	24,638	0.76	0.73	0.62	6.29		7.43	29,162	1.49			1.29		5.32	24,806	0.07		87.04		110.29

107	7.60	27,366	0.50	21.03		12.48										7.47	30,618	0.59	0.83	5.31		31.27
108	7.94	26,921	1.40	0.86	0.07	3.05		7.29	23,018	0.41			2.24			7.81	29,869	0.76	0.79	4.84		26.66
113	6.11	34,878	0.32	7.57				6.02	37,126	0.38			6.14			5.99	37,205	0.46	4.54	5.24		32.89
120	6.22	17,269	0.88	0.53			27.97	6.45	17,000	0.47			2.80			6.11	19,396	1.60	1.58	2.40	0.47	8.45
122	5.88	24,964	0.53	7.76				7.23	16,514	0.28	12.23		7.52			7.15	20,613	0.86		4.71		0.49
124	7.47	16,269	0.88	1.48																		
127	7.94	16,168	0.23	4.64				7.77	18,195	0.77		0.30	2.96									
128	8.06	16,168	0.20	2.19																		
129	8.26	16,269	0.50	2.58		1.51																
132	8.91	47,783	0.32	0.98												8.83	51,654	0.51	0.87	4.43	2.00	1.51
133	6.18	25,294	0.54	0.72												5.99	37,205	0.46	4.54	5.24		32.89
140	4.63	24,397	0.21	7.43	1.71		9.53	4.62	20,101	0.44			2.25									
142	4.37	25,294	0.43	2.06			0.81	4.34	22,075	0.57			2.48			5.32	24,806	0.07		87.04		110.29
143	6.71	21,061	0.21	4.20			6.04	6.19	23,627	0.24		1.26	11.58			6.60	24,501	0.19		19.01		34.47
145	7.12	23,018	0.23	4.13				6.86	25,761	0.29			3.83			7.01	24,501	0.18		7.53	2.16	
146	6.89	24,882	0.44	0.86																		
147	5.93	20,411	0.20	4.65				6.15	16,836	0.86			0.95			6.37	30,242	0.70		2.45	1.11	6.11
148	5.92	21,506	0.39	2.56												6.46	31,747	1.26		3.06		
149	5.93	18,579	0.25	8.47	6.09		1.23									6.33	28,543	0.30		9.84		

150	5.96	22,307	0.23	1.68			6.20	19,781	0.20	15.02			6.67	33,737	1.03	3.52	0.76	4.00
156							9.33	17,269	0.37	10.50								
160																		
161	5.34	23,751	0.16	1.09									5.79	18,625	0.81	5.08		21.08

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. We used the Reactome FI to find the pathways of these 114 proteins that were considered significantly enriched (FDR <0.05, Figure 3); were used the Uniprot protein IDs were converted to gene symbols. Considering FDR <0.05, 35 pathways were considered to be significantly enriched, 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).

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+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+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+I= Cu and Se
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 and Mg
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 and Zn; DM1+I= Cu 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 Se
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	

	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	
	P55063	Heat shock 70 kDa protein 1-like OS=Rattus norvegicus GN=Hspa1l PE=2 SV=2	932	5.91	70,550	48	9.83	Stress response	Chaperone	
41	Q07439	Heat shock 70 kDa protein 1A/1B OS=Rattus norvegicus GN=Hspa1a PE=2 SV=2	897	5.37	70,871	50	6.19	Stress response	Chaperone	DM1= Se; DM1+I= Cu and Mg
	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	
	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	
	Q07439	Heat shock 70 kDa protein 1A/1B OS=Rattus norvegicus GN=Hspa1a PE=2 SV=2	3042	5.91	70,550	49	19.66	Stress response	Chaperone	DM1= Se; DM1+I= Cu, Se and Zn
42	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=Hspa1l 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
	P0C2X9	Delta-1-pyrroline-5-carboxylate dehydrogenase_ mitochondrial OS=Rattus	627	7.13	61,869	33	30.10	Oxidoreductase	Proline metabolism	

		norvegicus GN=Aldh4a1 PE=1 SV=1								
	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
	P0C2X9	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
	P0C2X9	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 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+I= Cu and Mg
50	Q6P9T8	Tubulin beta-4B 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
	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
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 and Zn ; DM1+I= Cu, Mg 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= 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+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	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	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	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+I= 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	DM1= Se; DM1+I= Mg 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	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	DM1= Se; DM1+I= 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 1B 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 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;

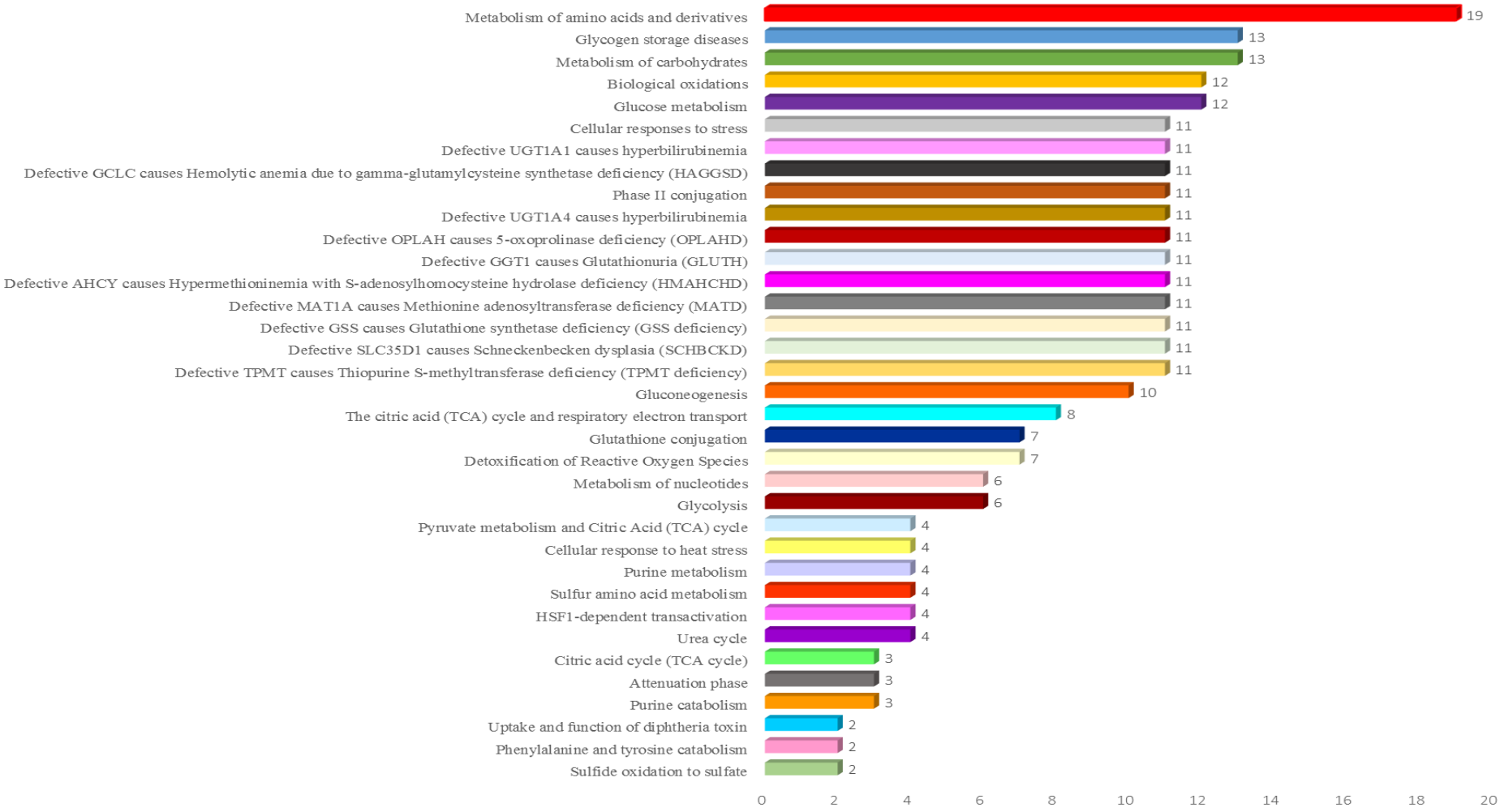
	P34067	Proteasome subunit beta type-4 OS=Rattus norvegicus GN=Psmb4 PE=1 SV=2	117	6.44	29,197	20	9.73	Hydrolase, Protease, Threonine protease	Other	DM1+I= Cu, Mg and Zn
	Q9Z0V6	Thioredoxin-dependent peroxide reductase_ mitochondrial OS=Rattus norvegicus GN=Prdx3 PE=1 SV=2	107	7.14	28,295	18	18.59	negative 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	
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
	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	
129	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	C= Cu and Mg
	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
	P52844	Estrogen sulfotransferase_ isoform 1 OS=Rattus norvegicus GN=Sult1e1 PE=2 SV=1	1811	5.78	35,509	29	21.36	Transferase	Other	
133	P49889	Estrogen sulfotransferase_ isoform 3 OS=Rattus norvegicus GN=Ste PE=1 SV=1	1500	5.57	35,416	29	16.61	Transferase	Other	C= Cu; DM1+I=Cu, Mg and Zn
	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	M1= Mg; DM1+I=
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= Mg
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	
	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	
161	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	C= Cu; DM1+I=Cu, Mg and Zn

Figure 3. 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.

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 this enzyme can be inactivated by a mixed-function oxidation bind a divalent metal and generally require a nucleotide (26). However, in this work, in the DM1+I group, was found Cu and Zn, as these elements are divalent, may be inferred these bind 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 (catalyzes the formation of L-citrulline from carbamoyl phosphate and L-ornithine). A study reported that this enzyme is regulated by zinc 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, was found the presence of Cu in the C group; and Cu, Mg, Se and Zn in the DM1+I group. The Cu, Mg and Se were not report yet bind with Otc, can be suggest in DM1+I the inactivation of this enzyme by hyperglycemia control induces 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 zinc metalloenzyme (a tetramer containing one Zn atom/subunit), Sord is involved in the catalysis of sorbitol/fructose interconversion. Some alterations in this conversion are associated in diabetes with cataract formation, neuropathy, retinopathy and nephropathy (30). The results showed the presence in Sord of Zn in C; Se in DM1; and Cu, Mg, and Zn in DM1+I**.** No other previous report have shown that this protein binds with Cu, Mg and Zn. Argininosuccinate synthase (Ass1) showed Se, Cu and Mg; this related enzyme, which is involved in the urea cycle, is not reported as metal binding in the literature. In spot 77, were identified beta-ureidopropionase (Upb1), 4-hydroxyphenylpyruvate dioxygenase (Hpd) and fumarylacetoacetase (Fah) where was found in C the presence of Mg, Se in DM1, and Cu, Se, Mg and Zn in DM1+I. Upb1 is a metalloenzyme that binds 2 Zn^{2+} ions per subunit (31), Hpd is a metalloenzyme that binds 1 Fe cation per subunit (32) and Fah binds with Ca^{2+} and $Mg.^{2+}$ (33). Other proteins associated with this metabolism as S-adenosylmethionine synthase isoform type-1 and 2 (Mat1a and Mat2a) are reported in literature as a metal ion binding protein, this protein has magnesium-finger metal binding domains (34). Mat1a and Mat2a catalyze the formation of S-adenosylmethionine from methionine and ATP. In the DM1, was found Cu, and

Cu, Mg, Se and Zn in DM1+I. The most significant was the protein bound in DM1+I with Zn, as Zn and Mg have the same proprieties that can interfere with some modifications in this protein. The propionyl-CoA carboxylase alpha chain (Pcca) was detect 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 bond because Pcca is composed of 737 amino acids where 2.4 are histidine, 1.5% cysteine and 2.8% methionine.

Chaperone and Oxidative stress metabolism. Heat shock proteins, which 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 Zn in DM1+I. Spot 33, Stress-70 protein (Hspa9) and heat shock cognate 71 kDa (Hspa8) showed the presence of Cu, Mg, Se and Zn . Spot 41, 78 kDa glucose-regulated protein (Hspa5), heat shock 70 kDa protein 1-like and 1A/1B (Hspa1l and Hspa1a), heat shock cognate 71 kDa protein (Hspa8) and heat shock-related 70 kDa protein 2 (Hspa2) showed the presence of Se in DM1; and Cu and Mg in DM1+I. Spot 42, heat shock cognate 71 kDa (Hspa8), heat shock 70 kDa protein 1A/1B (Hspa1a), heat shock-related 70 kDa protein 2 (Hspa2) and Heat shock 70 kDa protein 1-like (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 increases dynamics at the dimer interface and modulates anti-aggregation (37). The literature does not have any other report of metal binding or association with metals in heat shock proteins, our study can suggest some alterations to the treatment with insulin in that the ions can bind with these proteins to protect them from oxidative stress generated in group DM1. Peroxiredoxins are responsible for the degradation of peroxides and enzymes thiol-dependent, was found peroxiredoxin-1 (Prx1) in three spots (124, 128 and 156), and was detected the presence of Cu in C; and Se in DM1. Other 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 and Zn in DM1+I. The literature does not report any binding or association of these enzymes with metal ions, but can interfere that in group DM1 the Prx-1 and 6 had a presence of Se that can change the function of these proteins in the degradation of peroxides while groups C and DM1+I had the presence of Cu and Zn. Different isoforms of Glutathione S-transferase were found in this study. This enzyme plays a key role in enzymatic detoxification (38). The results showed the association with Cu in C, Se in DM1, and Mg and Zn in DM1+I; the literature does not show these enzymes associaiton with metals. Carbonic anhydrase 1 and 3 (Ca1 and Ca3) are Zn metalloenzymes. In this study, was found these enzymes with the presence of Cu in C. As

Zn has many characteristics similar to Cu, Cu can bind to the Ca1 and Ca3 domains, as occurs with other divalent ions.

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 1 Mg^{2+} ion per subunit. In this study, was found Cu, Mg and Se presence in Pgm1. The presence of cysteine (weak base) in the peptide sequence may promote the association of Cu and Se (weak acid) in this enzyme. In spot 67, was found alpha, beta and gamma enolase (Eno1, Eno3 and Eno2) that are involved in gluconeogenesis and glycolysis (40). Eno1 is a magnesium 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, was found the presence of Cu and Se in DM1, and Cu, Mg, Se and Zn in DM1+I. Thus, it may infer some alteration in this enzyme in DM1 that can interfere in carbohydrate metabolism and glycaemia control. Spot 113 showed two Mg metalloenzymes: fructose-1_6-bisphosphatase 1 (Fbp1) and fructose-1_6-bisphosphatase isozyme 2 (Fbp2) binds 3 Mg^{2+} ions per subunit, is involved in production of fructose 1,6-biphosphate (42) 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 association of Cu and Se observed in this enzyme. In spot 34, was found glyceraldehyde-3-phosphate dehydrogenase (Gapdh), L-lactate dehydrogenase A and C chain (Ldha and Ldhc), and malate dehydrogenase (Mdh2) 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 a cysteines 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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Conclusões

Conclusões

- 35 proteínas apresentaram diferença de expressão no plasma, indicando a alpha-1-macroglobulina e haptoglobulina como possíveis biomarcadoras do DM1 controlado (tratado com insulina); e as proteínas 2'-desoxinucleósido-5'-fosfato de N-hidrolase 1, proteína transmembranar 1, soro amilóide P-componente, vitamina D de ligação e biliverdina como possíveis candidatas a biomarcadoras do diabetes tipo 1 não controlado.
- O estudo metaloproteômico no plasma revelou diferentes interações entre as proteínas com o cobre, magnésio, selênio e zinco nos grupos experimentais; sugerindo como esses minerais poderiam estar ligados nas proteínas que apresentaram diferença de expressão e estarem envolvidos na expressão proteica, progressão e complicações do diabetes tipo 1.
- O tratamento com insulina em animais diabéticos foi capaz de restaurar os níveis da enzima frutose-1,6 bifosfatase indicando controle na produção de glicose hepática pela possível inibição da gliconeogênese nesses animais. Enquanto a piruvato carboxilase foi classificada como independente de insulina, como essa enzima está envolvida nas etapas iniciais da gliconeogênese, esta resposta pode ter relação com a geração de oxalatoacetato, mantendo, desta froma a concentração dos intermediários do ciclo do ácido cítrico.
- A maioria das enzimas do ciclo do ácido cítrico foram classificadas como dependentes de insulina, apresentando uma resposta anapleurótica para manter os intermediários do ciclo ácido cítrico à resposta insulínica.
- A restauração da glicemia, pelo tratamento com insulina afetou a abundância das enzimas que funcionam na desintoxicação de EROs, restabelecendo o equilíbrio redox e a função mitocondrial.
- A carbonilação de proteínas está diretamente associada a danos proteicos sob condições de estresse oxidativo. A análise do Oxi-proteoma identificou 105 proteínas com diferença de expressão que foram classificadas como dependentes de insulina e 62 como independentes de insulina; essas proteínas carboniladas podem estar relacionadas às disfunções celulares descritas na progressão do DM1.
- A identificação das proteínas carboniladas indicou potenciais biomarcadores pré-sintomáticos para o DM1. O tratamento com insulina atenuou o estresse oxidativo por favorecer as enzimas antioxidantes e diminuir a produção de EROs, controlou o metabolismo de carboidratos e metabolismo energético.

- O estudo metaloproteômico no fígado sugeriu diferentes interações entre os minerais (cobre, magnésio, selênio e zinco) e as proteínas entre os grupos experimentais, podendo associar com as alterações funcionais dessas proteínas e envolvimento na progressão e complicações do DM1.

Anexo

Anexo 1. Aprovação da Comissão de ética no uso de animais (CEUA)

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Botucatu

35 Anos 1977 - 2012 Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o Protocolo nº **436-CEUA**, sobre "Desenvolvimento de métodos analíticos para aplicação da metalômica na identificação de possíveis biomarcadores em plasma e fígado de ratos diabéticos", sob a responsabilidade de **Pedro de Magalhães Padilha**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Botucatu, 24 de agosto de 2012.

Profª Drª Patrícia Fernanda Felipe Pinheiro
Presidenta da CEUA

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