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► To cite this version:

A. Mezni, Olfa Khazri, Frédérique Jarnier, Julie Hardouin, Limam Ferid, et al.. Hepatoprotective effect of grape seed and skin extract against lithium exposure examined by the window of proteomics. Dose-Response, inPress, 10.1177/15593258221141585 . hal-03855025

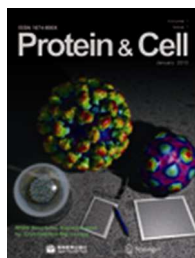
HAL Id: hal-03855025

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Submitted on 16 Nov 2022

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Journal:	<i>Protein & Cell</i>
Manuscript ID	P-C-01-2018-0026
Manuscript Type:	Original Article
Date Submitted by the Author:	10-Jan-2018
Complete List of Authors:	MEZNI, Ali; Universite de Carthage Faculte des Sciences de Bizerte, biology Khazri, Olfa; Universite de Carthage Jarnier, frederique; Universite de Rouen Hardouin, Julie; Universite de Rouen Limam, Ferid; Universite de Carthage Jouenne, Thierry; Universite de Rouen Aouani, Ezzedine; Universite de Carthage Faculte des Sciences de Bizerte Cosette, Pascal; Universite de Rouen
Keywords:	Lithium, Grape seed and skin extract, proteomics, hepatoprotection

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Hepatoprotective effect of grape seed and skin extract against lithium exposure examined by the window of proteomics

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Abstract

The aim of the present work was to study the impact of lithium exposure on liver and to evaluate the hepato-protective property of a grape extract against lithium toxicity. The experimental model consisted in rats exposed for 30 days to either various lithium concentrations, grape extract alone or lithium supplemented with grape extract. Previous liquid chromatography coupled to tandem mass spectrometry allowed to characterize the major phenolic compounds present in the grape extract. Here, proteomic analysis revealed alterations of liver protein profiles after lithium treatments that were successfully identified by mass spectrometry. These proteins are mainly involved in the respiratory electron transport chain, detoxification processes, ribosomal stress pathway, glycolysis and cytoskeleton. A dose response was observed for most of the differentially expressed proteins after lithium treatment. Interestingly, the grape extract reversed the situation and restored the level of liver proteins whose abundance was modified after lithium treatment, arguing for its protective activity. In conclusion, data demonstrated the ability of proteomic analysis to provide valuable insights into mechanisms of lithium toxicity in the liver of animal models. Based on these results, the grape extract may be envisaged as a nutritional supplement to weaken liver toxicity of lithium.

Keywords: Lithium, Grape seed and skin extract, Proteomics, Hepato-protection

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1. INTRODUCTION

Lithium is a toxic alkaline metal that could be found in the environment as industrial pollution (Kang et al, 2013). It was shown to accumulate in algae, marine animals and vegetables as tobacco leaves (Schrauzer et al 2002). On the other hand, lithium is also used as a psychiatric disease therapeutic, in particular in the case of bipolar disorders (Schou M 2001, Aral et al 2008). However, long-duration treatment with lithium has been found to cause adverse effects on various organs including the liver. There are numerous investigations reporting the toxic effects of lithium on liver structure and function (Toplan et al 2016). Inherently to its xenobiotic potential, lithium has been shown to influence the hepatic drug metabolizing enzyme system (Chadha et al 2008).

Proteomics enables the simultaneous assessment of the abundances of a large number of proteins, providing an unprecedented amount of molecular informations (Fountoulakis 2001, Gillette et al, 2013). In a biomedical context, this approach allows to explore the main metabolic pathways involved, as well as the mechanisms underlying the pathophysiological state of a central organ as the liver. Some investigators have focused on proteomic analyses of either whole protein extract or on specific fractions of the liver subjected to alcohol toxicity (Kim et al 2015). Proteomics also aims at studying posttranslational modification of proteins, but such an approach is less frequently described in the case of lithium toxicity.

Grape seed and skin extract (GSSE) is a complex mixture of polyphenolics, flavonoids, oligomeric proanthocyanidins and unsaturated fatty acids, commonly used as a nutritional supplement. GSSE exerts numerous biological activities and health-promoting properties, such as excellent scavenging of hydroxyl, superoxide and other radicals (Mokni et al, 2012), but also anti-tumor (Nandakumar et al, 2008) and anti-inflammatory properties (Kazantzis et al, 2012). GSSE is a potential health food ingredient that has been attributed the

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3 Generally Recognized As Safe (GRAS) certification from the US Food and Drug
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5 Administration (FDA, 2011).
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8 In the present study, we investigated the effect of GSSE on lithium-induced protein
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10 alteration in the liver of Wistar rats using a proteomic approach. From these observations, we
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12 suggest recommendations to clinicians on the potential protective role of GSSE.
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2. MATERIAL AND METHODS

2.1. Animals and treatments

We used forty-eight male Wistar rats (210-230 g, 2 months-old) from Pasteur Institute (Tunis) in agreement with the NIH guidelines. Rats were provided with food and water and maintained in an animal house at a fixed temperature of $22 \pm 2^{\circ}\text{C}$ under a 12-h light/dark cycle. Two series of experiment were carried out simultaneously for 30 days. The first sets of experiments aim to assess the toxic effects of lithium that was administered at 3 doses of 0, 2 and 100 mg/kg by daily intraperitoneal injection. In the second set of experiments aiming at the protective role of GSSE, 3 groups of animals (twenty four animals) received an intraperitoneal injection of GSSE (4g/kg), a binary combination of 2 mg/kg of lithium chloride + GSSE (4g/kg) or a combination of 100 mg/kg of lithium chloride + GSSE (4g/kg), respectively. Doses of lithium were chosen in the therapeutic range (2 and 5mg/kg bw) or toxic but non-lethal (100mg/kg bw) according to available literature (Milutinovic, 2016) [14].

At the end of the experiment, rats were decapitated and their liver collected, weighed and frozen in liquid nitrogen. Homogenization was conducted in lysis buffer (urea 7 M, thiourea 2 M, CHAPS 2%, C7BzO 0.5 %, DTT 20 mM, Tris-HCl 50 mM) containing a protease inhibitor cocktail (Roche Diagnostics) using an ultra-thurax homogenizator. The homogenates were then sonicated for 1 min (15% amplitude for 3 cycles of 30 s each) and all protein extraction steps were carried out on ice.

After debris elimination by centrifugation (10000g for 20 min at 4°C), supernatants were assembled and processed with a 2-D Clean-Up Kit (GE Healthcare) for protein precipitation in order to remove interfering compounds and protein level were determined using the Bradford method (Bradford, 1976).

2.2. Protein electrophoresis and in-gel digestion

Twenty-five μg of proteins from each sample were loaded and allowed for a short period of migration (1 h) in a SDS-PAGE stacking gel (7%). After Coomassie staining, the revealed protein band was excised, immersed in reductive medium (5 mM dithiothreitol), and cysteines irreversibly alkylated (25 mM iodoacetamide).

After extensive washing with water, proteins-containing band were submitted to trypsin digestion (trypsin from Promega, 0.5 μg per band). Several steps of peptide extraction were then performed in $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ mixtures (50:50) acidified with 0.1 % TFA. For each sample the peptide fragments were combined and evaporated.

2.3. Liquid nano-chromatography and mass spectrometry analysis

This analysis was achieved with a linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Elite, Thermo Scientific) equipped with a nano-ESI source and coupled to a nanoliquid chromatography (Easy-nLC II, Thermo Scientific). The separation was achieved on a reversed phase C18 column (NikkoyoTechnos, Japan) using a linear gradient of 15% to 55% B over 120 min (mobile phase A: $\text{H}_2\text{O}/0.1\%$ FA; mobile phase B: ACN/ 0.1% FA) after sample loading onto an enrichment column. Hence, the eluate was sent to the mass spectrometer with capillary voltage set at 1.5 kV. The mass spectrometer was operated in the data-dependent mode. MS scans were performed in the orbitrap analyzer (from m/z 300 to 2000 with a resolution of 30,000) and MSMS data were acquired in the linear ion trap. At last, the mass spectrometer targeted the 20 most intense peptide ions for fragmentation (top20 method) with applying dynamic exclusion for 30 s.

2.5. Statistics and bioinformatics analysis

Raw data were imported in Progenesis LC-MS software (Nonlinear Dynamics). For comparison, one sample was set as a reference and the retention times of all other samples within the experiment were aligned. After alignment and normalization, statistical analysis was performed for one-way analysis of variance (ANOVA) calculations. For quantification, peptide features presenting p value < 0.05 were kept and MS/MS spectra from selected peptides were exported for peptide identification using Mascot (Matrix Science). Total cumulative abundance of any given protein was calculated by summing the abundances of the different peptides.

Proteins were annotated with GO terms from NCBI in concordance with gene ontology (GO) standards. Additional annotations were obtained from Uniprot (<http://www.uniprot.org/>) and used to further classify proteins according to biological process, cellular component, protein class and molecular function. To integrate the observed proteomic alterations, proteins displaying significantly differential abundances between the different groups of animals were analyzed using bioinformatics research tools/databases (<http://www.pantherdb.org/> to identify molecular function and <http://stitch.embl.de/cgi/> to plot predicted protein interactions).

3. RESULTS

3.1. Evolution of protein profile after lithium treatment

Lithium induced modifications of protein expression (Figure 1) that appeared most of the time in a dose-dependent manner. First, according to their cellular localization, 42.9% of deregulated proteins belong to cell part, 14.3% are located in the extracellular region, 7.1% are involved in macromolecular protein complex, and 35.7% of proteins are found into organelles. According to their molecular function, 39 % of differentially expressed proteins are involved in catalytic activity, 30 % are associated with structural role and 29% are binding proteins.

More specifically, the most affected protein classes were respectively ribosomal proteins accounting for 15.6% (60S ribosomal protein L3, L5, L6, L8, L10 and L27 and 40S ribosomal protein S16), and transferases for another 15.6% (isovaleryl-CoA dehydrogenase, creatine kinase, acetyl-CoA C-acyltransferase, betaine-homocysteine methyltransferase, carnitine O-palmitoyltransferase and transketolase). In addition, oxido-reductases represent 13.3% (isovaleryl-CoA dehydrogenase, formyl-tetrahydrofolate dehydrogenase, betaine-homocysteine methyltransferase, transketolase, cytochrome-c oxidase, and dehydrogenase/reductase), cytoskeletal proteins 11.1% (tropomyosin beta chain, vimentin, troponin C, troponin I and myosin), transfer/carrier proteins 8.9% (sideroflexin, myoglobin, serum albumin, and glutathion-S-transferase). Less importantly represented protein classes were also present (Figure 1D), some proteins potentially belonging to multiple protein classes.

As expected, the highest modifications were detected for the highest lithium concentration (Table 1 and 2). In fact, most of the differentially expressed proteins are included in the respiratory electron transport chain (such as cytochrome c oxidase polypeptide VIc-2, +55%; cytochrome C oxidase, +48%; ATP synthase D, +133%) (Figure 2), in

metabolic processes(such as isovaleryl-CoA dehydrogenase,-71 %; creatine kinase S-type, -99%; creatine kinase M-type, -98%; betain-homocysteine S-methyltransferase, -32 %; alpha-amylase,-98 %), or related to cytoskeletal proteins (tropomyosin beta chain, -83 %; vimentin, -30 %; troponin C, -96 %; troponin I, -99%; myosin light chain, -99%), or to transfer/carrier proteins (sideroflexin, +133 %; glutathione-S-transferase kappa,+195 %).

Lithium also altered the expression of calcium-dependent proteins (such as troponin C, -96 %; myosin light chain, -99%), or oxido-reductase proteins (isovaleryl-CoA dehydrogenase; formyl-tetrahydrofolate dehydrogenase,-48 %; transketolase;betain-homocysteine S-methyltransferase; dehydrogenase/reductase;cytochrome C oxidase), or proteins involved in glycolysis pathway (such as fructose biphosphate aldolase, -88%),or also of nucleic acid binding proteins (such as 60S ribosomal protein L3, -88 %; 60S ribosomal protein L5, -64%;60S ribosomal protein L6, -75%;60S ribosomal protein L8, -81%; 60S ribosomal protein L10, -68%; 60S ribosomal protein L27, -76%; 40S ribosomal protein S16, -50 %).

3.2 Interaction network analysis

The abundance of proteins involved in the drug detoxification process in the liver was also investigated (Figure 3). These proteins displayed an increased abundance under lithium exposure (such as GST kappa, +66%; GST mu, +40%; epoxide hydrolase, +36%; cytochrome P450 2A2, +62%; cytochrome P450 2C11, +48%).

Interactions of these proteins with other protein partners were obtained by searching the STRING online database (Figure 4). The presented protein functional association networks are the result of the entry proteins “Epoxide hydrolase” (Ephx) and “Cytochrome P 450 2A2” (Cyp2a2). Interestingly, Ephx binds directly to proteins such as glutathione-S-transferase (Gstm1,Gsto1,Gsta4,Gstt1, Gstt2 and Mgst1) and cytochrome P450 (Cyp1a1,

Cyp1a2 and Cyp 2e1). Cytochrome P 450 2A2 binds directly to proteins such as cytochrome P450 (Cyp 4a2, Cyp 4a1, Cyp 4a8, Cyp 2c7 and Cyp 4f1), 3 beta hydroxysteroid dehydrogenase (hsd3b and hsd3b5), epoxide hydrolase 2 (Ephx 2), glutathion-S-transferase theta 2 (Gstt2) and UDP-glucuronosyltransferase 2B15 (Ugt2b15).

In addition, a more global network was represented (Figure 5) which enables the visualization of important regulations at multiple levels (protein-protein interactions and protein-chemical interactions). This pointed out proteins previously described and also proteins involved in transition metal ion binding (Gstm1, Alb, Cp, and Cpa2), in lipid oxidation (Cpt2, Ivd, Hadha and Hadhb), in muscle system processes (Myl1, Tnni2, Tnnc2, Acta 1, Tmp2, and Aldolase). Most interestingly, one can notice that all proteins have a direct or indirect relationship with ROS, mainly with oxygen and hydroxyl radicals, by different relations such as proteins directly involved in oxidative stress mechanism and associated antioxidant properties.

3.3. Hepatoprotective effect of GSSE

In an amazing manner, GSSE modulates the expression of proteins affected by lithium treatment (Table 1 and 2). The effect of GSSE was clearly noticed concerning the abundance of proteins involved in the respiratory electron transport chain (such as cytochrome c oxidase polypeptide VIc-2 and cytochrome C oxidase, ATP synthase D) that were up-regulated with lithium exposure and down-regulated with GSSE co-treatment. On contrast, lithium treatment down-regulated the abundance of ribosomal proteins (such as 60S RPL4, RPS16, RPL18a, RPL3, RPL10, RPL8, RPL6 and RPL27), a situation that was reversed with GSSE co-administration. The treatment with lithium also down-regulated proteins involved in oxidative stress and detoxification system (such as GST1, Gstk, and up-regulated Ephx, Cyp2a2 and Cyp2c23), while their level were re-established under GSSE exposure. The same also apply

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for cytoskeletal proteins (such as tropomyosin chain beta, vimentin, troponin C, troponin I, and myosin light chain) whose level was restored after co-treatment with GSSE. In addition, lithium treatment affects the abundance of many oxido-reductase proteins (isovaleryl-CoA dehydrogenase, formyltetrahydrofolate dehydrogenase,transketolase, betain homocysteine S-methyltransferase, dehydrogenase/reductase, and cytochrome C oxidase) and GSSE re-established the abundance of these proteins.

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4. DISCUSSION

Lithium is used as a mood stabilizer and was the first drug approved by the US FDA (FDA 2011) in the treatment of bipolar disorder (Schou M, 1999). However, many side effects remain an obstacle to a widespread clinical use (McKnight et al, 2012). The present study highlighted the effects of a lithium exposure on liver protein profile but also the protective effect afforded by co-administration of a grape extract.

The treatment of healthy rats with various doses of lithium altered the expression of proteins involved in the respiratory electron transport chain. More specifically, lithium induced the increase in abundance of the terminal oxidase of the mitochondrial respiratory chain (Rak et al, 2016), regulating oxidative phosphorylation (Begum et al, 2013). In addition, lithium treatment also up-regulated ATP synthase D. Almost all functions of mitochondria are either directly or indirectly linked to the oxidative phosphorylation machinery and energy coupling. The major part of this machinery, located in the inner mitochondrial membrane, comprises the four electron transfer chain complexes (complexes I, II, III, and IV) and ATP synthase (complex V) (Srinivasan et al, 2012). The goal of oxidative phosphorylation is to generate a proton gradient within the inner mitochondrial membrane, the potential energy of this gradient being used to synthesize ATP. Since this process is not completely efficient, electron transfer to O₂ may occur at complex I or III, resulting in generation of reactive oxygen species (ROS) (Fukuda et al, 2007). In this regard, many GST protein abundances were decreased after lithium treatment. These proteins constitute the main regulators of the reduced/oxidized glutathion ratio in cells and are commonly used as a measure of cellular redox potential and stress (Martyniuk et al, 2012). Low levels of GSTs may render animals less protected against oxidative stress, the lower levels potentially due to either lower production or higher consumption of GST in detoxification processes (Miller et al, 2016).

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3 Interestingly, lithium also up-regulated epoxide hydrolase and decreased the
4 abundance of P450 cytochromes which constitute the major enzyme family capable of
5 catalyzing the oxidative biotransformation of most drugs and xenobiotics and are therefore of
6 particular relevance in the context of clinical pharmacology (Nelson, 2006; Guengerich, 2008;
7 Zanger et al 2008). Epoxide hydrolase is an enzyme that metabolizes xenobiotics by
8 generating reactive epoxides into water-soluble compounds for excretion (Morisseau et al,
9 2005). These proteins are described to complement each other in detoxifying a wide array of
10 mutagenic, toxic, and carcinogenic xenobiotics (Kitteringham et al, 1996) and are called drug
11 metabolizing enzymes (DMEs), involved in biotransformation of associated substrates (Liu et
12 al 2016; Zhou et al 2013).

13
14 In addition, numerous ribosomal proteins (RP) were affected by lithium treatment and
15 there was a general trend for a decrease in ribosome protein abundance associated with
16 exposure. In contrast, the level of RPL5, a cytosolic ribosomal protein increased with lithium
17 exposure. Interestingly, it has been proposed that a defect in a RP causes increased levels of
18 free RPL5, that has been already described in other contexts to activate p53. The deficiency of
19 a ribosomal component triggers a pathological condition, known as ribosomal stress,
20 characterized by activation of p53 leading to apoptosis (Ellis 2011). Ribosomal proteins are
21 involved in DNA repair, cell cycle arrest, apoptosis and proliferation, and moderately
22 expressed in various cancers (Warner et al, 2009).

23
24 Another important point was the modification of the expression level of several
25 proteins associated with glycolysis. Lithium treatment has been reported to disrupt glycolysis
26 and gluconeogenesis by altering relevant enzyme activity (Lu et al 2010). Among these
27 proteins, fructose-bisphosphate aldolase A was down-regulated and enolase up-regulated after
28 lithium exposure. Fructose bisphosphate aldolase is involved in the fourth step of glycolysis,
29 catalyzing the cleavage of fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate

(Macomber et al, 2011). Enolase is a key glycolytic enzyme that catalyzes the dehydration of 2-phosphoglycerate to phosphoenol pyruvate in the last step of the catabolic glycolytic pathway (Chen et al, 2016). Because the glycolytic pathway is the major energy source for many cells, the effect of lithium on these proteins suggests that it might both disturb energy metabolism. Moreover, lithium induced an increase of abundance of transketolase, reported to be involved in the reductive CBB cycle (Calvin-Benson-Bassham cycle) and in the non-oxidative part of the pentose phosphate pathway, thus playing a critical role in connecting the pentose phosphate pathway to glycolytic intermediates(Hu et al, 2011).

Furthermore, lithium treatment also induced a modified abundance of proteins involved in lipid oxidation. Here, it was found that CPT, Slc27 and Ivd levels were decreased upon lithium treatment. This led to an accumulation of intracellular lipids and insulin resistance in insulin-targeting cells (Guo et al 2013). Noteworthy that restricted production of Slc27 leads to liver steatosis (Kazantzis et al 2012). Moreover, fatty acid oxidation has been considered as a major contributor for the development of hepatic steatosis and the progression of non-alcoholic fatty liver disease (Rector et al 2010). In addition, lithium altered abundance of pancreatic lipase (Pnlip), a protein secreted by the acinar pancreas, essential for the hydrolysis and absorption of long-chain triglyceride fatty acids (Kiba, 2015).

Another groups of proteins regulated by lithium treatment participates to cytoskeleton organization and dynamics. Indeed, in our proteomic study, the levels of the cytoskeletal proteins dramatically decreased in the treated group. These changes might be associated to the disruption of liver metabolism and function. The cytoskeleton is a basic structural element of all cell types, playing a pivotal role in the maintenance of cell architecture, adhesion, migration, differentiation, division and organelle transport. Accordingly, the alteration of cytoskeletal genes expression has a high probability to be associated with cytoskeleton structural disruption, potentially leading to cell death (Tammimies et al, 2013).

From another point of view, cytoskeletal organization is crucial for normal mitochondrial morphology, motility and function. Cytoskeleton is involved in the metabolic regulation of mitochondrial respiration and energy fluxes (Appaix et al, 2003). The altered abundances of cytoskeletal proteins and mitochondria dysfunction likely result from the altered mitochondrial oxidative phosphorylation (OXPHOS) system and ensuing ROS formation, which plays a crucial role in the disruption of cytoskeleton organization (Ding et al 2001).

The most relevant result drawn from the current study is the hepato-protection afforded by GSSE against lithium toxicity. GSSE treatment abrogated almost all alterations such as oxidative stress, disruption of cytoskeleton, mitochondrial dysfunction and metabolism. Our study paralleled the data of Muzik and coworkers (Musik et al 2015) who used selenium as protective agent against lithium hepato-toxicity. To our knowledge, our data are the first to demonstrate a potent protection of a natural extract as GSSE against lithium hepato-toxicity. In our case, high dosage GSSE reaching 4g/kg, were highly safe when used in various experimental settings as nephro-protection against doxorubicin (Mokni et al 2016), neuroprotection against lithium exposure (Mezni et al 2016), cardio-protection against arsenic (Sfaxi et al 2015), protect lung subjected to bleomycin (Khazri et al 2016) or pancreas submitted to fat treatment (Aloui et al 2016).

In conclusion, GSSE exerts a potent protection against lithium-induced liver toxicity in healthy rat even at high concentration. We propose that GSSE, through the regulation of the damages induced by lithium, can be considered as a potential hepato-protector, able to minimize the negative impact of lithium in humans. Consequently, GSSE may be used as an adjuvant to therapeutic doses of lithium to avoid its harmful effects.

Conflict of interest

The authors have no conflict of interest to disclose

Acknowledgments

This work was supported by grants from the Ministry of Higher Education of Tunisia.

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Legends of figures

Figure 1: Lithium-induced alterations of the liver proteome. Significantly regulated proteins are classified in GO categories; the results are presented as percentage of molecular function (a), biological process (b), cellular component (c), protein class (d), and pathway (e).

Figure 2: Quantitative analysis of proteins involved in the respiratory electron transport chain affected by lithium and GSSE. Cytochrome-c oxidase chain IV (A), Cytochrome c oxidase polypeptide VIc-2 (B) and ATP synthase D chain (C).

Figure 3: Quantitative analysis of proteins involved in detoxification. GST kappa (A), GST mu (B), cytochrome P450 2C11 (C), cytochrome P450 2A2 (D) and epoxide hydrolase (E).

Figure 4: Protein-protein interaction network (STRING) of proteins involved in complex of detoxification. Gstm1 (A) and Ephx (B).

Figure 5: Interaction network with STITCH (protein-protein interactions, protein-chemical interactions and interactions with hydroxyl and oxygen radicals).

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Table 1: List of identified proteins that displayed differential abundances between exposure to lithium alone or supplemented to GSSE.

Protein ID	Gene name	Anova p-value	Description	[Lithium]			[Lithium] +GSSE		
				0	2	100	0	2	100
Q6I7R1	Dhrs7l1	9.23E-05	Dehydrogenase/reductase (SDR family) member 7	+	+	++	+	+	+
P02680	Fgg	2.08E-03	Fibrinogen, gamma polypeptide	+++	++	+	+++	++	++
P31399	Atp5h	4.82E-04	ATP synthase D chain, mitochondrial (EC 3.6.3.14)	+	++	+++	+	+	+
P50878	Rpl4	2.01E-04	60S ribosomal protein L4 (L1)	++	+	+	++	++	+++
Q63965	Sfxn1	1.56E-03	Sideroflexin 1	+	++	+++	++	++	+
P62250	Rps16	2.83E-04	Ribosomal protein S16	++	+	+	++	++	+++
P24473	Gstk1	4.60E-04	Glutathione S-transferase kappa 1 (EC 2.5.1.18)	++	+	+	++	++	+++
P58775	Tpm2	3.15E-05	Tropomyosin beta chain, skeletal muscle	+++	++	+	++	++	+
P50137	Tkt	6.81E-04	Transketolase (EC 2.2.1.1) (TK).	+	+	+++	+	+	+
P05065	Aldoa	4.96E-04	Fructose-bisphosphatealdolase (EC 4.1.2.13) A	+++	++	+	++	++	++
P12007	Ivd	2.76E-03	Isovaleryl-CoAdehydrogenase (EC 1.3.99.10)	+++	+	+	++	++	++
Q9QZ76	Mb	4.73E-06	Myoglobin	+++	+	-	+++	++	+
Q304F3	Tnnc2	2.48E-06	Troponin C, fast skeletal muscle	++	-	-	+	+	+
P27768	Tnni2	7.09E-06	Troponin I	++	-	-	+	+	+
P09605	Ckmt2	4.18E-06	Creatine kinase (EC 2.7.3.2)	++	-	-	+	+	+
P31000	Vim	6.16E-05	Vimentin	++	+	+	++	++	+
P04937	Fn1	1.98E-04	Fibronectinprecursor	++	+	+	++	++	+
P62718	Rpl18a	8.35E-04	Ribosomal protein L18a	++	+	+	++	++	+++
P11951	Cox6c2	2.84E-05	Cytochrome c oxidase polypeptide VIc-2 (EC 1.9.3.1)	+	++	++	++	+	+
P05503	cox1	2.06E-04	Cytochrome-c oxidase (EC 1.9.3.1) chain IV	+	++	++	+	+	+
P07338	Ctrb1	1.75E-04	Chymotrypsinogen B (EC 3.4.21.1) B	++	++	+	++	+	+

P21531	Rpl3	2.29E-04	60S ribosomal protein L3 (L4).	++	+	-	+	+	++
Q6PDV7	Rpl10	2.38E-04	60S ribosomal protein L10	++	+	+	+	++	+++
P07687	Ephx1	9.59E-05	Epoxide hydrolase (EC 3.3.2.3)	+	++	+++	++	+	+
Q66HN6	Slc27a2	2.29E-04	Solute carrier family 27 (Fatty acid transporter)	++	+	+	+	+	++
O09171	Bhmt	8.50E-04	Betaine-homocysteinemethyltransferase	++	+	+	+	+	++
P04905	Gstm1	1.26E-03	Mu class glutathione s-transferase (EC 2.5.1.18)	++	+	-	+	++	+++
P68134	Acta1	6.57E-06	Actin alpha, skeletal muscle	++	+	-	+	++	+++
P62919	Rpl8	3.07E-04	Ribosomal protein L8, cytosolic	++	+	-	+	++	+++
P21533	Rpl6	1.62E-04	Rpl6 protein.	+	-	-	+	++	+++
P15149	Cyp2a2	2.23E-04	Cytochrome P450 2A2 (EC 1.14.14.-1)	-	+	+++	-	+	+
P61354	Rpl27	4.99E-05	Ribosomal protein L27, cytosolic	++	+	+	+	++	+++
Q9QUN4	Pnlip	1.20E-04	Pancreatic lipase	+	++	+++	-	-	++
Q9JL97	Cp	1.45E-04	GPI-anchored ceruloplasmin	+++	-	-	++	++	+
P19222	Cpa2	1.11E-04	Carboxypeptidase A2 (EC 3.4.17.15)	++	+	-	+	+	++
P18886	Cpt2	1.03E-04	Carnitine O-palmitoyltransferase (EC 2.3.1.21)	++	+	-	+	+	++
Q5EBC8	Ugt2b	5.24E-04	UDP glycosyltransferase 2 family, polypeptide B	++	++	+	++	+	+
P15429	Eno3	1.27E-04	Enolase 3, beta.	+	++	+++	++	+	-
Q68G40	Cyp2c23	2.47E-06	Cytochrome P450 2C11	+	+	++	+	+	+
P09895	Rpl5	3.77E-05	Ribosomal protein L5, cytosolic	+	++	++	+	++	+++
P02770	Alb	3.92E-05	Serumalbuminprecursor	+++	+	+	+	+	++
Q60587	Hadhb	2.18E-04	Acetyl-CoA C-acyltransferase (EC 2.3.1.16)	+	++	+++	+	+	+
P00564	Ckm	1.23E-06	Creatine kinase (EC 2.7.3.2) chain M	++	+	-	+	+	+
P28037	Aldh1l1	8.25E-06	Formyltetrahydrofolate dehydrogenase	++	+	+	+	+	++
P02600	Myl1	6.38E-09	Myosin light chain 1	++	+	-	+	+	+
P00689	Amy2	1.41E-04	Alpha-amylase (EC 3.2.1.1) precursor	+++	+	-	+	++	++

Table 2: Annotation of proteins that displayed modified abundances.

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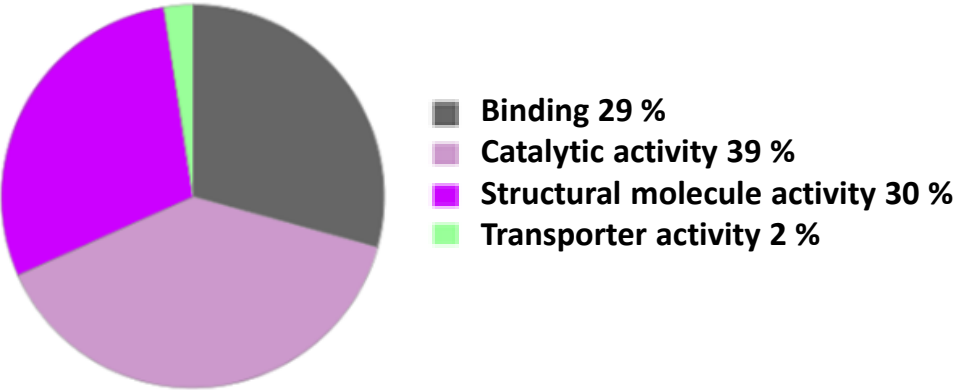
Accession	Gene Name	Localisation	Fonction
Q6I7R1	Dhrs7l1	Unknown	Oxidoreductase activity
P02680	Fgg	Extracellular region	Metal ion binding
P31399	Atp5h	Mitochondrion	Mitochondrial inner membrane, mitochondrial proton-transporting ATP synthase complex, membrane, proton-transporting ATP synthase complex, coupling factor F(o), hydrogen ion transmembrane transporter activity
P50878	Rpl4	Ribosome	Large ribosomal subunit, intracellular ribonucleoprotein complex, protein binding
Q63965	Sfxn1	Mitochondrion	Membrane, integral component of membrane, mitochondrial membrane, ion transmembrane transporter activity
P62250	Rps16	Ribosome	Small ribosomal subunit, intracellular ribonucleoprotein complex, structural constituent of ribosome
P24473	Gstk1	Mitochondrion	Mitochondrial matrix, glutathione transferase activity, transferase activity
P58775	Tpm2	Cytoplasm	Cytoskeleton, actin binding
P50137	Tkt	Peroxisome	Endoplasmic reticulum membrane,intracellular membrane-bounded organelle, transketolase activity, transferase activity, metal ion binding
P05065	Aldoa	Cytoplasm	Fructose-bisphosphate aldolase activity, lyase activity
P12007	Ivd	Mitochondrion	Mitochondrial matrix, oxidoreductase activity
Q9QZ76	Mb	Extracellular exosome	Oxygen transporter activity, metal ion binding
Q304F3	Tnnc2	Troponin complex	Calcium ion binding
P27768	Tnni2	Troponin complex	Actin binding
P09605	Ckmt2	Mitochondrion	Mitochondrial inner membrane, membrane, nucleotide binding, creatine kinase activity, ATP binding, kinase activity, transferase activity
P31000	Vim	Nucleus	Cytoplasm, intermediate filament, nuclear matrix, structural constituent of cytoskeleton, protein

			binding
P04937	Fn1	Extracellular region	Proteinaceous extracellular matrix, heparin binding
P62718	Rpl18a	Ribosome	Intracellular ribonucleoprotein complex, structural constituent of ribosome
P11951	Cox6c2	Mitochondrion	Mitochondrial inner membrane, membrane, integral component of membrane, cytochrome-c oxidase activity
P05503	Mtco1	Mitochondrion	Mitochondrial inner membrane, membrane, integral component of membrane, respiratory chain complex IV, respiratory chain, cytochrome-c oxidase activity, oxidoreductase activity, metal ion binding
P07338	Ctrb1	Extracellular region	Extracellular space, peptidase activity, serine-type peptidase activity, hydrolase activity
P21531	Rpl3	Nucleus	Nucleolus, cytoplasm, ribosome, intracellular ribonucleoprotein complex, structural constituent of ribosome
Q6PDV7	Rpl10	Ribosome	Intracellular ribonucleoprotein complex, structural constituent of ribosome
P07687	Ephx1	Endoplasmic reticulum	Endoplasmic reticulum membrane, membrane, integral component of membrane, organelle membrane, intracellular membrane-bounded organelle, hydrolase activity, cis-stilbene-oxide hydrolase activity
Q66HN6	Slc27a2	Membrane	Integral component of membrane, catalytic activity, long-chain fatty acid-CoA ligase activity, very long-chain fatty acid-CoA ligase activity
O09171	Bhmt	Cytoplasm	Methyltransferase activity, transferase activity, metal ion binding, betaine-homocysteine S-methyltransferase activity
P04905	Gstm1	Cytoplasm	Glutathione transferase activity, transferase activity
P68134	Acta1	Stress fiber	Cytoplasm, cytoskeleton, striated muscle thin filament, actin filament, nucleotide binding, ATP binding
P62919	Rpl8	Cytoplasm	Ribosome, intracellular ribonucleoprotein complex, RNA binding, rRNA binding
P21533	Rpl6	Ribosome	Intracellular ribonucleoprotein complex, structural constituent of ribosome
P15149	Cyp2a2	Endoplasmic reticulum	Endoplasmic reticulum membrane, membrane, organelle membrane, intracellular membrane-bounded organelle, monooxygenase activity, oxidoreductase activity, metal ion binding, aromatase activity
P61354	Rpl27	Ribosome	Intracellular ribonucleoprotein complex, structural constituent of ribosome

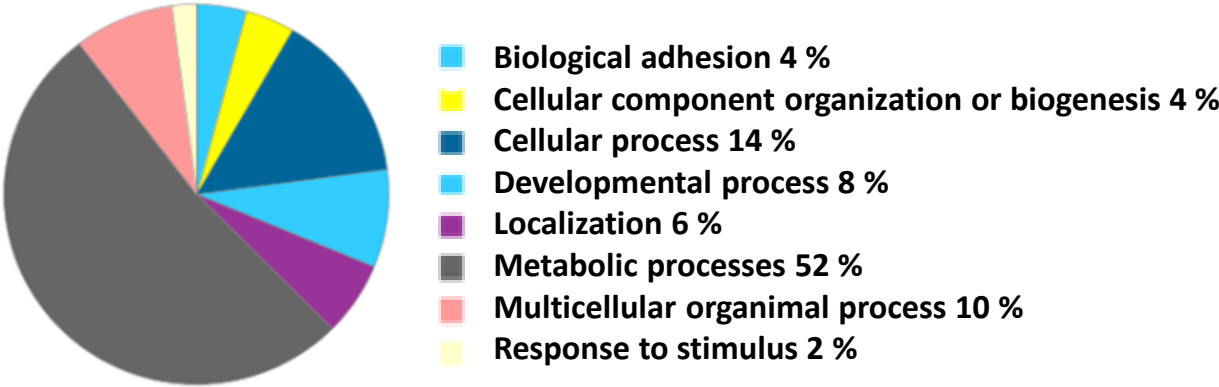
Q9QUN4	Pnlip	Extracellular region	Triglyceride lipase activity, carboxylic ester hydrolase activity
Q9JL97	Cp	Cell	Oxidoreductase activity, metal ion binding
P19222	Cpa2	Extracellular region	Carboxypeptidase activity, peptidase activity, metallopeptidase activity, hydrolase activity, metal ion binding
P18886	Cpt2	Mitochondrion	Mitochondrial inner membrane, membrane, carnitine O-palmitoyltransferase activity, transferase activity, transferring acyl groups
Q5EBC8	Ugt2b	Membrane	Integral component of membrane, glucuronosyltransferase activity, transferase activity, transferring glycosyl groups
P15429	Eno3	Cytoplasm	Phosphopyruvate hydratase activity, lyase activity, metal ion binding
Q68G40	Cyp2c23	Unknown	Monooxygenase activity, oxidoreductase activity, metal ion binding
P09895	Rpl5	Nucleus	Nucleolus, cytoplasm, ribosome, intracellular ribonucleoprotein complex, RNA binding, rRNA binding
P02770	Alb	Extracellular region	Protein complex, DNA binding, fatty acid binding, drug binding, lipid binding, toxic substance binding, pyridoxal phosphate binding, metal ion binding, oxygen binding
Q60587	Hadhb	Mitochondrion	Mitochondrial outer membrane, mitochondrial inner membrane, endoplasmic reticulum, membrane, acetyl-CoA C-acyltransferase activity, transferase activity, transferring acyl groups
P00564	Ckm	Cytoplasm	Nucleotide binding, creatine kinase activity, ATP binding, kinase activity, transferase activity
P28037	Aldh1l1	Cytoplasm	Formyltetrahydrofolate dehydrogenase activity, oxidoreductase activity, aldehyde dehydrogenase (NADP+) activity
P02600	Myl1	Myosin complex	Calcium ion binding
P00689	Amy2	Extracellular region	Extracellular space, alpha-amylase activity, calcium ion binding, hydrolase activity, acting on glycosyl bonds, metal ion binding

Figure 1

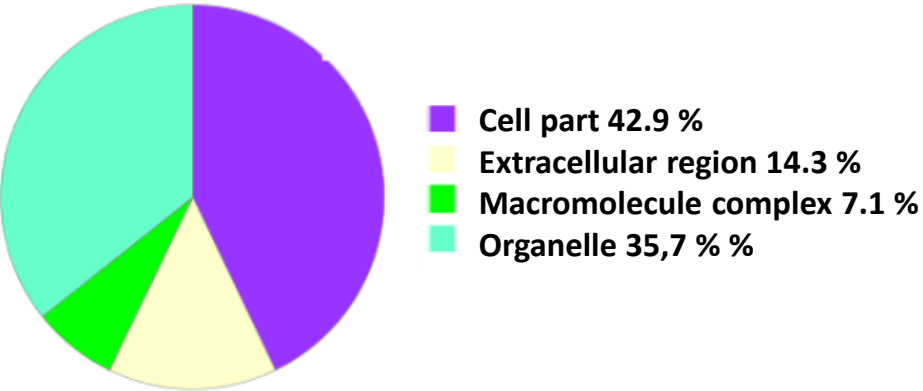
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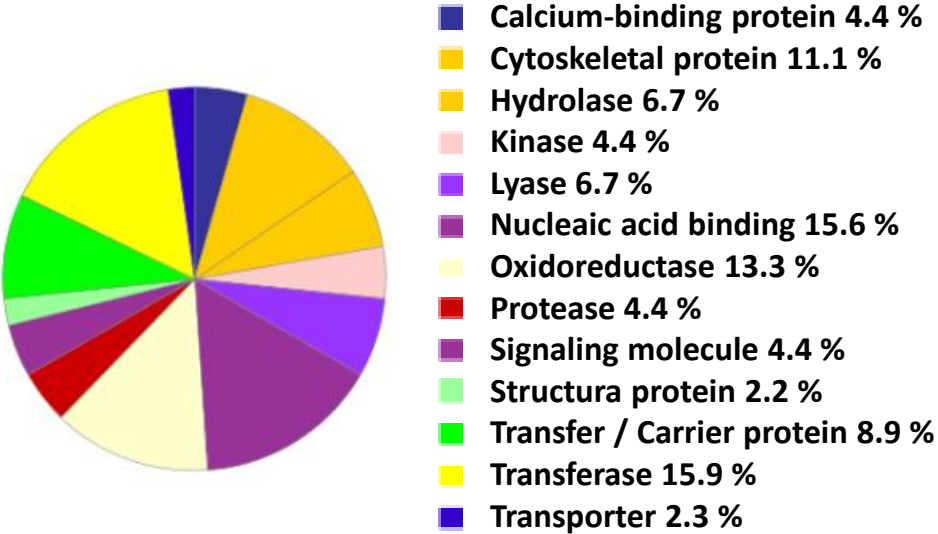
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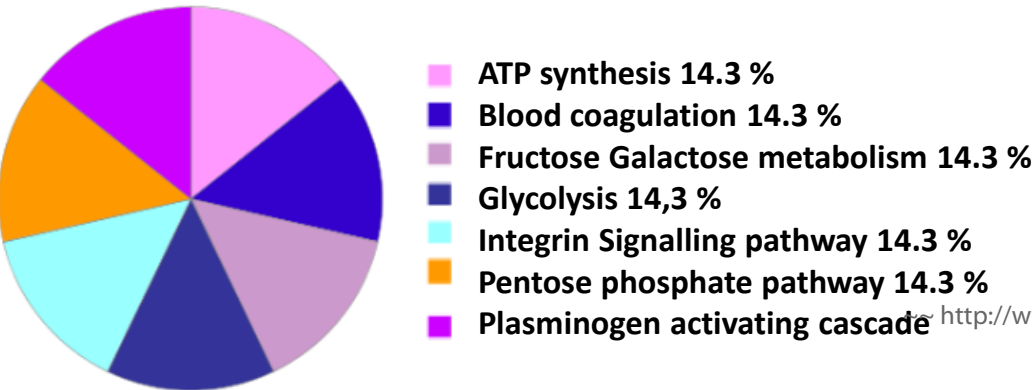
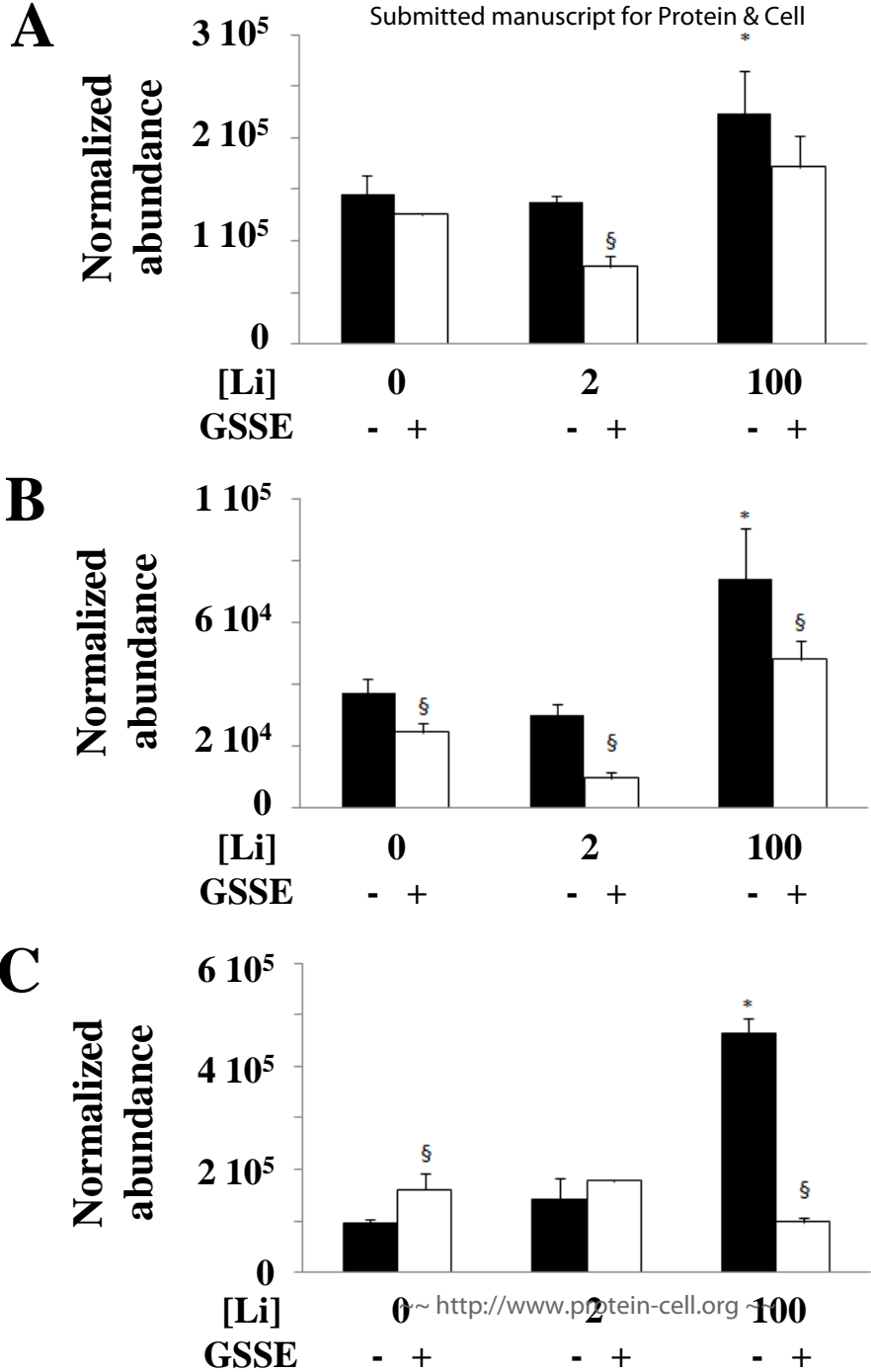


Figure 2



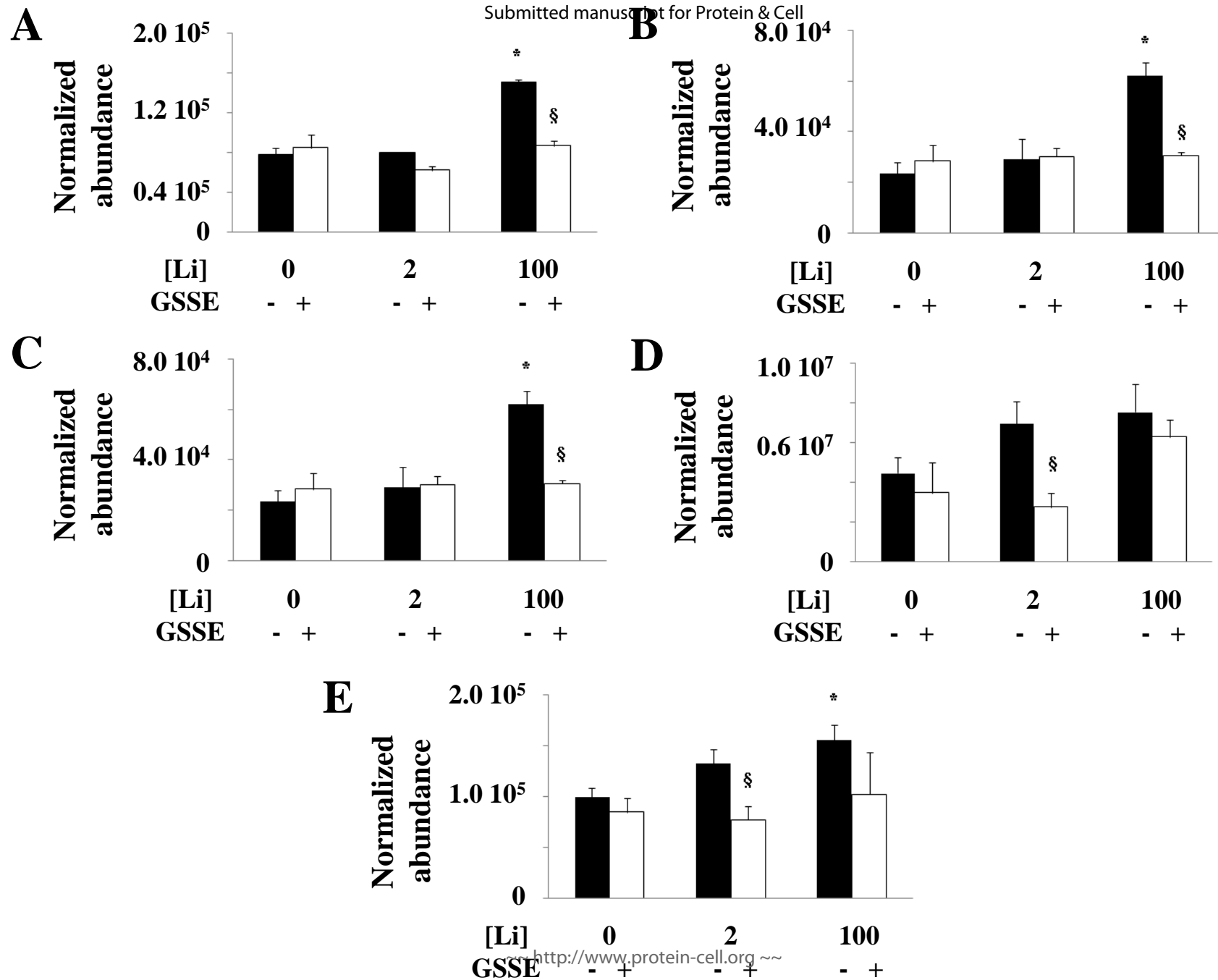
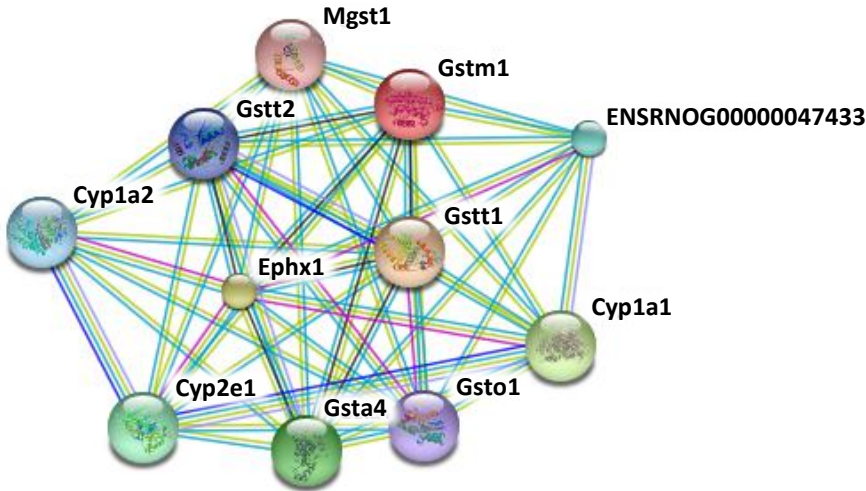


Figure 4

A



B

