Maternal methylmercury exposure changes the proteomic profile of the offspring’s salivary glands: Prospects on translational toxicology

Priscila Cunha Nascimento¹, Walessa Alana Bragança Aragão¹, Leonardo Oliveira Bittencourt¹, Aline Dionizio², Marilia A. R. Buzalaf³, Maria Elena Crespo-Lopez³, Rafael Rodrigues Lima¹*¹

¹ Laboratory of Functional and Structural Biology, Institute of Biological Sciences, Federal University of Para, Belém, PA, Brazil, ² Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, São Paulo, Brazil, ³ Laboratory of Molecular Pharmacology, Institute of Biological Sciences, Federal University of Para, Belém, PA, Brazil

* rafalima@ufpa.br

Abstract

Background

Methylmercury (MeHg) remains a public health issue since developing organisms are particularly vulnerable to this environmental contaminant. This study investigated the effect of maternal MeHg exposure on the modulation of proteomic profile of parotid (PA), submandibular (SM), and sublingual (SL) glands of offspring rats.

Materials and methods

Pregnant Wistar rats were daily exposed to 40 μg/kg MeHg during both gestational and lactation periods. The proteomic profiles of the major salivary glands of the offspring rats were analyzed through mass spectrometry.

Results

The offspring rats exposed to MeHg showed significant alterations in the proteomic profiles of the PA, SM, and SL glands. Altered proteins were associated with cytoskeleton components, tissue morphogenesis, and response to stimulus and stress.

Conclusion

This original study showed that maternal MeHg exposure significantly modulates the expression of proteins and induces alterations in the proteomic profiles of developing salivary glands.
Introduction

Developing organisms are particularly vulnerable to environmental toxins [1–4]. Methylmercury (MeHg) is a highly bioaccumulative toxic compound that remains a public health issue due to its natural and anthropogenic environmental distributions [4,5], such as soil erosion and biomass burning [6,7]. Primary (fossil fuel combustion, minerals, and waste) and secondary anthropogenic activities (Hg-based products and artisanal gold mining) are the main contributors to the global biogeochemical cycling of Hg and cause high levels of reemissions [1,8].

Several studies have shown that MeHg induces multiple systemic damages [9], mainly neurological impairments [4,7,10]. Our research group recently evidenced structural changes in the salivary glands of offspring rats after gestational and lactational MeHg exposure [11]. It is widely known that changes in the homeostasis of salivary glands can damage other oral tissues due to deficits in salivary production, composition, and molecular mechanisms involved in oral physiology [12–14].

It has been shown that the total Hg levels in the salivary glands of intoxicated offspring rats were associated with toxicopathologic findings evidenced by glandular morphometric changes and damages in both epithelial and myoepithelial architecture [11], which may decrease both the quality and quantity of saliva [15,16]. This fluid has been widely used in clinical and toxicological analyses of human metabolism [17,18] due to its easy and non-invasive collection and better cost efficiency for large-scale trials than the blood serum. However, the use of saliva as a diagnostic matrix for the analysis of MeHg environmental exposure is still unclear [19].

It seems relevant to explore the association between potential tissue or functional modifications and mechanisms of molecular modulation [20]; thus, this present study aimed to investigate the effect of maternal MeHg exposure on the modulation of proteins biomarkers in the parotid (PA), submandibular (SM), and sublingual (SL) glands of offspring rats.

Materials and methods

This study was previously approved by the Ethics committee on animal experimentation by of Federal University of Pará (UFPA), under protocol number 8613011217/CEUA-UFPA and is available to check at http://ceua.ufpa.br/#, by “protocol status” tool. All the experiments followed a guide for the use of laboratory animals [21] and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines [22] (S1 Table).

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Peptide Atlas, accession no: PASS01692.

Animals and experimental design

Pregnant Wistar rats (Rattus norvegicus), with 90-days-old and weighed 150 to 200g, were kept at UFPA vivarium under a 14:10h light and dark cycle (lights on 7:00 AM) and a climate-controlled room (25±2 C). The animals were kept in individual cages with food and water ad libitum in association with the experimental protocol.

A sample size calculation for the number of progenitor animals was performed, assuming a normal distribution of the variables tested. A power of 80% and a bilateral alpha level of 5% were considered with mean and standard deviation data of a previous study and detailed in Nascimento et al. [11]. The flow chart of the study design is summarized in Fig 1.

Protocol of MeHg intoxication

The pregnant female rats were equally and randomly distributed into two groups according to the experimental protocol: 1) Control group (n = 5): received vehicle only; 2) MeHg group.
(n = 5): received MeHg (40 μg/kg B.W./day) [23]. The animals were weekly weighed for dose adjustment (for review of weight data, see Nascimento et al. [11]).

The dissolution of methylmercury chloride (CH3HgCl; Sigma-Aldrich, Milwaukee, Wisconsin, USA) and the administration by cookie treats (Teddy Grahams, Nabisco, CAN) were performed as previously described [11]. Briefly, the contaminated cookies were offered once a day, only to progenitor animals and were immediately eaten, as confirmed by a visual inspection. The administration occurred during the gestational (20–21 days) and lactation periods (20–21 days).

After 24 hours of the last MeHg administration (at the end of the lactation period), the progenitor female rats were euthanized by cervical dislocation under anesthesia, with a mixture of ketamine chloride and xylazine chloride (90 mg/kg and 9 mg/kg, respectively, i. p.). Then the surgery for the collection of larger salivary glands was performed. After the assessment, the Hg levels evidence the successful intoxication protocol, as observed and described by Nascimento et al. [11].

Parallely, the offspring rats were divided by genders and maintained in collective cages (4 animals each) with food and water ad libitum, and kept in a climate-controlled room with a light/dark cycle appropriate until 40-days-old, once the salivary glands are entirely developed and thus possible to perform the analyses proposed [24].
At the 40-days-old, these pups were anaesthetized (with a mixture of ketamine and xylazine, as described above) for resection of the parotid, submandibular lingual glands for the subsequent proteomic analysis. The samples were immediately frozen in liquid nitrogen and stored at −80˚C until evaluations. The researchers were blinded regarding the analyses of the groups.

Proteomic profile

Preparation of salivary gland samples. All the steps of the proteomics approach following the protocols previously described [25,26]. Briefly, the analyses were performed by sample homogenization, protein extraction, reduction, alkylation, digestion, desalination, and purification. First, samples of larger salivary glands (parotid, submandibular and lingual glands) of the animals (n = 6 per group) were pooled 2 in 2, and the analyses were performed in biological triplicate. The sample size of 6 animals per group (3 pools from 2 animals each) has been widely used in proteomic studies involving quantitative analysis of different animal tissues [25,27–29]. By having three pools from each group, we have biological triplicates. In addition, each pool is run in the mass spectrometer three times (technical triplicates). Thus, we had nine mass spectrometer runs for each group, which is enough for proper statistical analysis using the Protein Lynx Global Server (PLGS) software embedded in the equipment.

For the homogenization, a buffer solution was added in the samples to extract soluble proteins with lysis buffer (7 M urea, 2 M thiourea, and 40 mM DTT diluted in AMBIC; BioRad, USA) under constant stirring at 4˚ C. Then, the samples were centrifuged (at 20,817 g / 4˚ C for 30 min), and the supernatant was collected for total protein quantification by Bradford’s method [30]. After that, AMBIC (50 mM) was added in 100 μg of protein up to 50 μL. In the next stage, each sample received 10 μL of AMBIC, and 25 μL of 0,2% RapiGEST™ (Waters Co., Manchester, UK) and they were incubated (at 37˚C for 30 min). At the end of this process, 5 mM DTT was added and incubated again (37˚C for 40 min). After this, 10 mM IAA (BioRad, USA) was added and incubated for 30 min at room temperature and dark conditions.

For the digestion step, 10 μL of trypsin (Thermo Fischer, USA) was added to the samples (at 37˚C for 14 h) followed by the addition of 10 μL of 5% trifluoroacetic acid (Sigma-Aldrich, USA) and incubated (at 37˚C for 90 min). In the next step, supernatants were collected and purified using C18 spin columns (Pierce, USA). At the end of the procedure, the samples were resuspended in 12 mL of ADH (1 pmol mL1) associated with 108 mL of 3% acetonitrile (Sigma-Aldrich, USA) and 0.1% formic acid (Thermo Fischer, USA) to be submitted to Mass Spectrometry analysis.

Mass spectrometry analysis. The reading and identification of the peptides were conducted by a nanoAcquity UPLCXEvo QTof MS system (Waters, Manchester, UK), using the Protein Lynx Global Server (PLGS), as previously detailed [31]. The proteins verification was determined by downloading the Uniprot database. Then, the bioinformatics analyses were performed using Cytoscape (3.6.1 version, Java®) with the ClueGO plugin for the determination of biological process groups, based on Gene Ontology (GO) annotations [32]. Additionally, the ClusterMarker plugin was for the protein-interaction network.

Over-representation analysis (ORA). Initially, a table was built including ID code, name of the proteins and their respective Log2Ratio values. For proteins with absolute changes, -1 values were assigned for proteins detected only in control and 1 for proteins detected only in the MeHg group. For the ORA analysis, was used the R studio program [33] with the EGSEA plugin [34]. In this process, the UNIPROT database was accessed to identify proteins and the biological processes they participate in, made available by Bader Lab. After this verification, we used the Cytoscape software [35] with Enrichment Lab plugin pipeline for grouping the sets of proteins previously consulted. After that, the main biological processes were selected for
graphical analysis. In this analysis, we used the Enrichment Map Cytoscape Plugin [35], where the list of protein sets with statistically significant changes was used as input. Then, networks and clusters were built to visualize the interconnections between the protein sets. The relationship between the different protein sets was constructed based on the similarity coefficient between them (Jaccard similarity coefficient = number of proteins in common between protein sets A and B/total number of proteins in A + B). In order to identify the clusters and their respective functions, the AutoAnnotate Cytoscape App [36] was used, which names each cluster. After that, the clusters were classified into larger categories of biological processes. Then, a PPI analysis (https://www.networkanalyst.ca/) [37] was carried to results in the representative image, according to the number of interactions of the proteins with the other proteins found altered. A minimum of 10 interactions was applied. The R studio program generated the image with the GOplot plugin.

**Statistical analyses.** All identified proteins were tabulated using electronic spreadsheets (Microsoft Excel®, Windows 10 version) and representation of performing the GO analyses. For comparative analysis, the PLGS software with the Monte-Carlo algorithm was applied and to obtain the difference of protein expression between the groups, \( p < 0.05 \) for down-regulated proteins and \( p > 0.95 \) for up-regulated proteins were considered.

**Results**

**Salivary glands proteins profiling of offspring after pre- and postnatal exposure to methylmercury**

The MeHg transfer from the mothers that consumed 40 μg/kg B.W./day MeHg (an exposure of environmental meaning) modulated the proteomic profile of salivary glands of offspring. This exposure model revealed a total of 201, 603, and 228 altered proteins in Parotid, Submandibular, and Sublingual glands, respectively. In the parotid gland, 37 proteins were uniquely regulated in the control group, and 41 were uniquely in the MeHg group (Table 1). The submandibular gland proteome revealed 177 uniquely expressed in the control group and 63 uniquely in the exposed group (Table 2). Also, when analyzing the Sublingual proteome, were observed 164 proteins expressed only in the MeHg group, and there were no exclusive expressed proteins in the control group, as observed the Table 3. The proteins roster complete is described in Supplementary Tables (S1–S3 Tables).

The pre- and postnatal exposure to environmental methylmercury also changed the salivary glands of offspring rats. According to Gene Ontology, 25 categories of biological processes were affected in the Parotid Gland: the translational elongation (17.12%) and the structural constituent of cytoskeleton (9.1%) were the most affected groups (Fig 2). The proteome networks analysis revealed the interaction of proteins related mainly to the cytoskeleton, such as Glyceraldehyde-3-phosphate dehydrogenase (P04797), Actin, alpha skeletal muscle (P68136), and Profilin-1 (P62963), Tubulin beta-2A chain (P85108) (Fig 3).

In addition, the proteins related to the metabolic process (19.9%) and developmental process (14.01%) showed significant changes in the submandibular gland (Fig 4) The proteomics network revealed the interaction between proteins that are related to protein metabolism and changes in the cellular redox system, such as: Protein disulfide-isomerase A4 (P38659), Endoplasmic reticulum resident protein 29 (P52555), Endoplasmin (Q66HD0), Endoplasmic reticulum chaperone BiP (P06761), Peptidyl-prolyl cis-trans isomerase B (P24368), Hypoxia up-regulated protein 1 (Q63617), and Peroxiredoxin-4 (Q9Z0V5) (Fig 5).

While in the sublingual, the main altered biological processes were about intracellular protein transport (13.58%) and peptide metabolic process (10.49%), as detailed in Fig 6. The proteomic network showed the interaction between proteins mainly related to the
Table 1. Unique proteins in parotid gland of offspring rats after pre- and postnatal exposure to methylmercury vs control group.

| Accession ID | Description                        | PLGS Score | Control | MeHg |
|--------------|------------------------------------|------------|---------|------|
| P38983       | 40S ribosomal protein SA            | 189.76     | +       | -    |
| Q63041       | Alpha-1-macroglubulin               | 34.36      | +       | 1-   |
| P24090       | Alpha-2-HS-glycoprotein             | 62.56      | -       | +    |
| Q02874       | Core histone macro-H2A.1            | 70.81      | +       | -    |
| P70623       | Fatty acid-binding protein_ adipocyte| 544.58     | -       | +    |
| P04906       | Glutathione S-transferase P         | 81.61      | +       | -    |
| G3V7G8       | Glycine—tRNA ligase                 | 30.37      | +       | -    |
| Q6IMY8       | Heterogeneous nuclear ribonucleoprotein U | 149.07     | +       | -    |
| O35274       | Neurabin-2                          | 35.6       | -       | +    |
| P13084       | Nucleophosmin                       | 201.7      | +       | -    |
| P24368       | Peptidyl-prolyl cis-trans isomerase B | 201.43     | +       | -    |
| Q6AYD3       | Proliferation-associated protein 2G4 | 66.87      | +       | -    |
| P35284       | Ras-related protein Rab-12         | 132.37     | -       | +    |
| P61107       | Ras-related protein Rab-14         | 132.37     | -       | +    |
| P51156       | Ras-related protein Rab-26         | 132.37     | -       | +    |
| Q35390       | Ras-related protein Rab-43         | 132.37     | -       | +    |
| P07340       | Sodium/potassium-transporting ATPase subunit beta-1 | 179.01     | +       | -    |
| P16086       | Spectrin alpha chain_ non-erythrocytic 1 | 32.29      | +       | -    |
| D3ZSP7       | Tetra-tripeptide repeat domain 3    | 17.24      | -       | +    |
| P63029       | Translationally-controlled tumor protein | 133.74     | +       | -    |

+58 proteins exclusive of control or MeHg group

* Accession ID according to the Uniport.org database.

Signs of + or indicate the presence or absence of the protein in one of the groups.

https://doi.org/10.1371/journal.pone.0258969.t001

Table 2. Unique proteins in Submandibular gland of offspring rats after pre- and postnatal exposure to methylmercury (MeHg) vs. control group.

| Accession ID | Description                        | PLGS Score | Control | MeHg |
|--------------|------------------------------------|------------|---------|------|
| P29314       | 40S ribosomal protein S9            | 357.01     | +       | -    |
| P62832       | 60S ribosomal protein L23           | 326.57     | +       | -    |
| B5DF11       | AN1-type zinc finger protein 5      | 121.59     | +       | -    |
| P07150       | Annexin A1                         | 45.02      | -       | +    |
| Q9WU82       | Catenin beta-1                     | 60.64      | +       | -    |
| P36375       | Glandular kallikrein-10            | 288.36     | +       | -    |
| P36373       | Glandular kallikrein-7_ submandibular/renal | 316.88     | +       | -    |
| P30713       | Glutathione S-transferase theta-2   | 431.7      | -       | +    |
| O55148       | Growth arrest-specific protein 7    | 73.71      | +       | -    |
| P25030       | Keratin_ type I cytoskeletal 20     | 87.88      | +       | -    |
| D3Z9H7       | Nuclear factor of activated T-cells_ cytoplasmic 4 | 95.45      | -       | +    |
| Q9JID1       | Programmed cell death protein 4     | 116.22     | -       | +    |
| Q9WVCO       | Septin-7                           | 97.44      | +       | -    |
| Q920J4       | Thioredoxin-like protein 1          | 162.08     | -       | +    |
| Q63355       | Unconventional myosin-Ic            | 110.53     | +       | -    |
| Q8VDA5       | Z-DNA-binding protein 1             | 72.25      | -       | +    |

+224 proteins exclusive of control or MeHg group

* Accession ID according to the Uniport.org database.

Signs of + or indicate the presence or absence of the protein in one of the groups.

https://doi.org/10.1371/journal.pone.0258969.t002
Table 3. Unique proteins in sublingual gland of offspring rats after pre- and postnatal exposure to methylmercury (MeHg) vs control group.

| Accession ID | Description                                           | PLGS Score | Control | MeHg |
|--------------|-------------------------------------------------------|------------|---------|------|
| P04764       | Alpha-enolase                                         | 671.14     | -       | +    |
| P04906       | Glutathione S-transferase P                           | 118.93     | -       | +    |
| Q63942       | GTP-binding protein Rab-3D                            | 102.33     | -       | +    |
| P0DMW0       | Heat shock 70 kDa protein 1A                          | 12.25      | -       | +    |
| P0DMW1       | Heat shock 70 kDa protein 1B                          | 12.25      | -       | +    |
| Q5XHZ0       | Heat shock protein 75 kDa_, mitochondrial             | 96.56      | -       | +    |
| P82995       | Heat shock protein HSP 90-alpha                       | 113.77     | -       | +    |
| P34058       | Heat shock protein HSP 90-beta                        | 109.19     | -       | +    |
| Q63617       | Hypoxia up-regulated protein 1                         | 31.22      | -       | +    |
| P30904       | Macrophage migration inhibitory factor                | 1074.24    | -       | +    |
| Q63716       | Peroxiredoxin-1                                       | 184.22     | -       | +    |
| P48721       | Stress-70 protein_, mitochondrial                     | 42.7       | -       | +    |

+153 proteins exclusive of the MeHg group

* Accession ID according to the Uniport.org database.

Signs of + or indicate the presence or absence of the protein in one of the groups.

https://doi.org/10.1371/journal.pone.0258969.t003

Fig 2. Functional distribution of proteins identified with differential expression proteins on the parotid gland of offspring rats after pre- and postnatal exposure to methylmercury vs. control group. The categories of proteins based on Gene Ontology annotation of biological process and the proteins access numbers were provided by UNIPROT. Terms significant (Kappa Score = 0.4) and distribution according to the percentage of the number of genes and was evaluated by ClueGO plugin of Cytoscape software 3.7.1.

https://doi.org/10.1371/journal.pone.0258969.g002
mitochondrial activity (Fig 7), such as Peroxiredoxin-5, mitochondrial (Q9R063), ATP synthase subunit alpha, mitochondrial (P15999), Sodium/potassium-tran sporting ATPase sub-unit alpha-2 (P06686), Aconitate hydratase, mitochondrial (Q9ER34), and Peptidyl-prolyl cis-trans isomerase A (P10111).

Early exposure to methylmercury modulates significantly the salivary glands proteomic profile of offspring rats

In quantitative analyses between the groups, changed expression proteins were down-regulated or up-regulated, consisting of 51 proteins with different status of regulation on parotid, 314 on submandibular, and 54 on sublingual glands (S4–S6 Tables).
analysis (ORA) resulted in 76 proteins, allowing us to observe the interactions that each protein presented in each exposed group between proteins up or down-regulated, and the proteins with exclusive regulation in each group, as shown in Fig 8.

**Discussion**

This original study showed molecular changes in the salivary glands of offspring rats caused by maternal MeHg exposure. Developing salivary glands seem a target for MeHg toxicity since significant changes in the modulation of proteins associated with cytoskeleton components, tissue morphogenesis, and stimulus/stress response was observed. This study aimed to identify alterations at the protein level and correlate them with previously reported morphological changes [11].

Humans are mainly exposed to MeHg through the consumption of contaminated fish and seafood [4,38,39]. Even if mothers are not affected, MeHg can be transferred to fetuses during pregnancy and lactation and induce long-term adverse effects on developing organs. Alterations in neonates and children induced by maternal MeHg exposure have been observed in chronically exposed populations such as Amazonian rivers [10]. Therefore, this exposure model aimed to mimic the daily intake of MeHg contaminated food by pregnant women. The determination of a safe level of MeHg exposure is complex, especially for vulnerable groups such as fetuses and infants. Although the World Health Organization (WHO) indicates 1.6 μg/kg/BW MeHg as the tolerable weekly intake (PTWI) [40], chronically exposed populations are usually found with higher MeHg levels [14–16]. Recent biomonitoring has revealed hair Hg
levels as high as 75 ppm [41–43], which may represent a MeHg weekly intake of 52.5 μg/kg as suggested by Crespo-Lopez [1]. Thus, the MeHg dose used in this study (40 μg/kg/BW) resembles the exposure detected in chronically exposed humans and is widely used in validated research with rats [11,23,44–46].

Organic Hg is mainly absorbed by the gastrointestinal tract, enters into the blood circulation, and is rapidly distributed to other tissues [6,39]. Due to its high lipophilicity, MeHg can immediately cross biological barriers; thus, MeHg consumed by pregnant women easily crosses the placenta [6,47]. The central nervous system is traditionally reported as the main target for MeHg and its neurotoxin mechanisms have been investigated by several studies [1]; however, MeHg can also damage other systems, such as immunological, renal, and cardiovascular [1,10]. Recent findings of our research group indicated that MeHg induced alterations in oral cell lines [48], alveolar bone [49], and salivary glands [11,44–46]. Since developing salivary glands significantly accumulate Hg [11], saliva can be potentially used as a matrix for biomonitoring environmental human exposure to MeHg [13]. This study results determined the proteins involved in the modulation of the salivary glands of offspring rats after pre-and postnatal MeHg exposure and thus contribute to validate the saliva as an additional diagnostic tool.

Increasing evidence has shown that significant environmental MeHg levels can change biological functions in humans and animals; however, adverse molecular implications are not yet fully understood [20]. Therefore, this data described the association between Hg accumulation in offspring rats and the modulation of the salivary gland proteomic profiles. A total of 1032 proteins were found altered in the three salivary gland pairs (S1–S6 Tables). When compared

**Fig 5. Subnetwork created in the ClusterMark app to identify protein-protein interactions (PPI) in submandibular glands of the off-spring exposed to MeHg.** The proteins are identified according to the accession ID of the UNIPROT database. The colors of the nodes represent proteins with differences in expression (pink: downregulated) and proteins exclusive to the control group (red). The gray nodes indicate proteins that were not identified in the samples but interacted through the database analysis. Identification of the proteins found in the samples according to the accession ID: Q63617 (Hypoxia up-regulated protein 1), P52555 (Endoplasmic reticulum resident protein 29), P06761 (Endoplasmic reticulum chaperone BiP), P38659 (Protein disulfide-isomerase A4), Q66HD0 (Endoplasm), Q92IV5 (Peroxiredoxin-4), P24368 (Peptidyl-prolyl cis-trans isomerase B).
to a study with adult animals [45], the offspring rats evaluated in this study interestingly presented a greater number of altered proteins, which suggests that early exposure to MeHg is more harmful. Moreover, the abovementioned intoxication model may have strengthened the findings of this study.

Our research group has previously, observed that the PA glands were more susceptible to Hg accumulation than SM and SL glands when evaluated under the same MeHg exposure protocol [11]; however, PA glands were the second most affected in terms of proteins number, which, suggests that the cells present in these glands play a role on defense mechanisms against MeHg. This study indicated that Hg levels mainly induced alterations in the biological process associated with translational elongation (Fig 1), such as the 40S ribosomal protein (P38983) not expressed in the MeHg group that may lead to tissue morphogenesis [50]. The structural constituent of the cytoskeleton was also changed and can be explained by previous immuno-histochemistry findings [11]. In addition, the proteomics network analysis showed some protein-protein interactions that participate in cytoskeleton cellular processes, such as the up-regulation of glyceraldehyde-3-phosphate dehydrogenase (P04797) and actin alpha skeletal

Fig 6. Functional distribution of proteins identified with differential expression proteins on Sublingual gland of offspring rats after pre- and postnatal exposure to methylmercury vs. control group. The categories of proteins based on Gene Ontology annotation of biological process and the proteins access number were provided by UNIPROT. Terms significant (Kappa Score = 0.4) and distribution according to the percentage of number of genes and was evaluated by ClueGo plugin of Cytoscape software 3.7.1.
muscle (P68136), which indicates the modulation of PA cytoskeleton as a response to MeHg stimuli. By contrast, the downregulation of proteins associated with actin filaments and microtubules formation [51,52] such as profilin-1 (P62963) and tubulin beta-2A chain (P85108) indicates an impairment in the maintenance of cytoskeleton components that jeopardizes the cell morphology.

Regarding the proteomic changes of SM glands, a total of 603 altered proteins among 27 biological processes were observed. Both metabolic (20%) and developmental (14%) processes were the most affected by MeHg, while kallikreins (glandular kallikrein-10, P36375; glandular kallikrein-7_submandibular/renal, P36373) and annexin (P07150) were the most altered protein groups. The kallikreins directly maintain oral health by acting as vasodilatation mediators in damaged oral mucosa, which favor both defense and cicatricial processes [53]; in addition, they indirectly protect and repair dental enamel against dental caries due to proteolytic activation of proteins, such as the proline-rich protein [54]. The association of kallikreins with
survival and proliferation cells has also been suggested since these proteins bind to glandular receptors that may activate critical survival pathways such as PI3K-Akt [55]. Moreover, kallikreins are predominantly found in the striated ducts and ductal cells of mammals’ SM glands [56,57]. Therefore, the lack of kallikreins expression in the MeHg group of this study may compromise the regeneration capacity of the SM glands and also explain previous MeHg-induced toxicopathologic findings in the ductal area [11].

The proteomic analysis showed the expression of annexins only in the SM glands of offspring rats exposed to MeHg (Table 2). These calcium-binding proteins can be involved in intracellular transport [58] and some family members such as annexin A1 (ANXA1, P07150) are associated with the inflammatory process [59]. Moreover, ANXA1 has an important functional attribute in the phagocytic clearance of apoptotic cells [59]. It is well known that Hg

![Circos plot of protein-protein interaction (PPI). The analysis of the Parotid (PA), Sublingual (SL), and Submandibular (SM) glands of offspring rats after pre- and postnatal exposure to methylmercury (MeHg) vs. control group in categories apoptosis (green), cell cycle (blue), development (red) cytoskeleton (lime green), response to stimulus (orange), stress response (pink) and mitochondrial activity (grey). Blue-scale proteins are down-regulated and red up-regulated in the MeHg group when compared to the control group.](https://doi.org/10.1371/journal.pone.0258969.g008)
exposure leads to inflammation [60]; however, this study specifically describes the molecular pathways of damage associated with MeHg exposure in the salivary glands of offspring rats. The oxidative stress was also a pathway of damage associated with MeHg exposure. The proteomic network analysis revealed protein-protein interactions related to protein metabolism. The disulfide isomerase A4 protein (P38659), which controls disulfide bonds between two thiol groups, was exclusively observed in the group control. The down-regulation of endoplasmic reticulum resident protein 29 (P52555), endoplasmic (Q66HD0), and endoplasmic reticulum chaperone BiP (P06761) associated with secretory proteins processing in the endoplasmic reticulum (ER) can compromise protein folding and cell survival [61]. Conversely, the down-regulation of peptidyl-prolyl cis-trans isomerase B (P24368), hypoxia up-regulated protein 1 (Q63617), and peroxiredoxin-4 (Q9Z0V5) proteins indicate changes in redox homeostasis control. It must be highlighted that peroxiredoxin-4 is responsible for cellular protection against oxidative stress by reducing hydrogen peroxide in water and alcohol [5]; thus, its downregulation favors cellular oxidative reactions.

Similarly, the results of SL glands analysis also emphasized the role of oxidative stress mechanisms in MeHg intoxication of developing salivary glands. Interestingly, a lack of protein expression was observed in the control group (Table 3), while the majority of proteins expressed in the MeHg group was related to stress response such as the heat shock proteins (HSP) 70 kDa 1A (P0DMW0), HSP 70 kDa 1B (P0DMW1), HSP 75 kDa mitochondrial (Q5XHZ0), HSP 90-alpha (P82995), and HSP 90-beta (P34058)). The HSP is produced as a response to stress conditions such as exposure to toxins [62]. The findings of this study with offspring rats are similar to those found in a study with adult animals exposed to MeHg and corroborate with the oxidative imbalance evidenced by Bittencourt et al. [45].

Furthermore, proteins associate with stimulus-response were only expressed in the SL glands exposed to MeHg. The alpha-enolase (P04764) presents different functions and can be associated with HSP and cytoskeletal structures [64]. This protein was recently indicated as an antigentic target in primary Sjogren’s syndrome [65], which is an autoimmune disease mainly associated with xerostomia; thus, this study findings regarding salivary glands exposed to MeHg may be directly related to oral dryness [66]. The proteomic network analysis of the MeHg group revealed protein-protein interactions such as peroxiredoxin-5 mitochondrial (Q9R063) and aconitate hydratase mitochondrial (Q9ER34) in the mitochondrial activity, which suggests a response to oxidative reactions induced by reactive oxygen species (ROS). Meanwhile, the ATP synthase subunit alpha mitochondrial (P15999), sodium/potassium-transporting ATPase subunit alpha-2 (P06686), and peptidyl-prolyl cis-trans isomerase A (P10111) proteins were up-regulated, which indicates increased mitochondrial activity as a cellular response to MeHg toxicity.

Overall, the main protein categories affected by MeHg in the salivary glands are related to apoptosis, cell cycle, development, cytoskeleton, stimulus-response, stress, and mitochondrial activity (Fig 4). Considering the previously reported MeHg-induced structural changes in salivary glands, a down-regulation of the proteins associated with glandular architecture was expected; however, the variations observed in the over-representation analysis need a more detailed investigation. A subexpression of the actin gamma-enteric smooth muscle (P63269), actin alpha cardiac muscle 1 (P68035), and actin alpha skeletal muscle (P68136) proteins was observed in all salivary glands. The three main actin isoforms (alpha, beta, and gamma) have been identified in vertebrate animals. The alpha actins are found in muscle tissues and are significant constituents of the contractile apparatus, while both beta and gamma acts coexist as cytoskeleton components in most cell types and are mediators of internal cell motility [63].

The modulation of proteins associated with the ubiquitin-proteasome system, which is vital for proteostasis, was observed in all salivary glands. The abovementioned HSP are associated
with maturation, re-folding, and degradation of proteins [64,65]. The HSP 70-binding 1 (Q6IMX7) was only expressed in PA glands exposed to MeHg, while the HSP 10 kDa mitochondrial (P26772) was only expressed in the SM glands of the control group and HSP with different molecular weights such as 10 kDa (P26772; exclusive in control), 70 kDa, and 90 kDa (P0DMW0 and P82995, respectively) were only observed in the SL glands exposed to MeHg. Moreover, the E3 ubiquitin-protein ligase TRIM23 (P36407) was only observed in the SM glands exposed to MeHg, the polyubiquitin-B (P0CG51) was only expressed in the SL glands exposed to MeHg, and the ubiquitin-fold modifier 1 (Q5BJP3), which act as a quality control [66,67], was only observed in the control PA glands. Therefore, the potential proteostasis impairment can cause protein aggregation or dysfunctionality, and compromise glandular activity.

Overall, this study suggests that salivary glands are targets for MeHg-induced molecular changes during early life, especially at an environmental exposure dose. This experimental–environmental model demonstrated the main proteomic changes in PA, SM, and SL glands associated with significant modulation of proteins involved in cytoskeleton components, tissue morphogenesis, and response to stimulus and stress. Therefore, further studies are needed to investigate whether such changes can also alter saliva biomarkers in vulnerable populations such as neonates and infants.

Supporting information

S1 Table. Unique proteins in parotid gland of offspring rats of the MeHg group vs. control group.

S2 Table. Unique proteins in submandibular gland of offspring rats of the MeHg group vs. control group.

S3 Table. Unique proteins in sublingual gland of offspring rats of the MeHg group vs. control group.

S4 Table. Identified proteins with significantly different expression altered in Parotid Gland of offspring rats of the MeHg group vs. control group.

S5 Table. Identified proteins with significantly different expression altered in submandibular gland of offspring rats MeHg group vs. control group.

S6 Table. Identified proteins with significantly different expression altered in sublingual gland of offspring rats of the MeHg group vs. control group.

S7 Table. The ARRIVE guidelines checklist.

Author Contributions

Conceptualization: Priscila Cunha Nascimento, Maria Elena Crespo-Lopez, Rafael Rodrigues Lima.
Data curation: Maria Elena Crespo-Lopez, Rafael Rodrigues Lima.

Formal analysis: Priscila Cunha Nascimento, Maria Elena Crespo-Lopez, Rafael Rodrigues Lima.

Funding acquisition: Rafael Rodrigues Lima.

Investigation: Priscila Cunha Nascimento, Walessa Alana Bragança Aragão, Leonardo Oliveira Bittencourt, Aline Dionizio.

Methodology: Priscila Cunha Nascimento, Walessa Alana Bragança Aragão, Leonardo Oliveira Bittencourt, Aline Dionizio.

Project administration: Rafael Rodrigues Lima.

Software: Priscila Cunha Nascimento, Walessa Alana Bragança Aragão, Leonardo Oliveira Bittencourt, Aline Dionizio, Marilia A. R. Buzalaf.

Supervision: Rafael Rodrigues Lima.

Validation: Priscila Cunha Nascimento, Aline Dionizio, Marilia A. R. Buzalaf, Maria Elena Crespo-Lopez, Rafael Rodrigues Lima.

Visualization: Priscila Cunha Nascimento, Aline Dionizio, Marilia A. R. Buzalaf, Maria Elena Crespo-Lopez, Rafael Rodrigues Lima.

Writing – original draft: Priscila Cunha Nascimento.

Writing – review & editing: Aline Dionizio, Marilia A. R. Buzalaf, Maria Elena Crespo-Lopez, Rafael Rodrigues Lima.

References

1. Crespo-Lopez ME, Augusto-Oliveira M, Lopes-Araújo A, Santos-Sacramento L, Yuki Takeda P, Macchi BM, et al. Mercury: What can we learn from the Amazon? Environ Int. 2021; 146:106223. Epub 2020/10/30. https://doi.org/10.1016/j.envint.2020.106223 PMID: 33120229

2. Hughes C, Waters M, Allen D, Obasanjo I. Translational toxicology: a developmental focus for integrated research strategies. BMC Pharmacology and Toxicology. 2013; 14(1):51. https://doi.org/10.1186/2050-6511-14-51 PMID: 24079609

3. van de Bor M. Fetal toxicity. Handbook of clinical neurology. 2019; 162:31–55. Epub 2019/07/22. https://doi.org/10.1016/B978-0-444-64029-1.00002-3 PMID: 31324317

4. WHO. Mercury and health World Health Organization 2017 [Accessed 21th January 2021]. Available from: https://www.who.int/news-room/fact-sheets/detail/mercury-and-health.

5. Yang J, Wang Z, Liu X, Lu P. Modulation of vascular integrity and neuroinflammation by peroxiredoxin 4 following cerebral ischemia-reperfusion injury. Microvascular Research. 2021; 135:104144. https://doi.org/10.1016/j.mvr.2021.104144 PMID: 33515567

6. Bjerklund G, Dadar M, Mutter J, Aaseth J. The toxicology of mercury: Current research and emerging trends. Environmental research. 2017; 159:545–54. Epub 2017/09/11. https://doi.org/10.1016/j.envres.2017.08.051 PMID: 28889024

7. UNEP. Global Mercury Assessment 2018. In: Program UNE, editor. United Nations Environment Program 2019.

8. Esdaile LJ, Chalker JM. The Mercury Problem in Artisanal and Small-Scale Gold Mining. Chemistry-A European Journal. 2018; 24(27):6905–16. https://doi.org/10.1002/chem.201704840 PMID: 29314284

9. Ursinyova M, Masanová V, Uhnaková I, Murinova LP, Patayova H, Rausova K, et al. Prenatal and Early Postnatal Exposure to Total Mercury and Methylmercury from Low Maternal Fish Consumption. Biological trace element research. 2019; 191(1):16–26. Epub 2018/12/01. https://doi.org/10.1007/s12111-018-1585-6 PMID: 30499063.

10. Santos-Sacramento L, Arrifano GP, Lopes-Araújo A, Augusto-Oliveira M, Albuquerque-Santos R, Takeda PY, et al. Human neurotoxicity of mercury in the Amazon: A scoping review with insights and critical considerations. Ecotoxicology and environmental safety. 2021; 208:111686. Epub 2020/01/06. https://doi.org/10.1016/j.ecoenv.2020.111686 PMID: 33396018.
11. Nascimento PC, Ferreira MKM, Balbinot KM, Alves-Júnior SM, Viana Pinheiro JdJ, Silveira FM, et al. Methylmercury-Induced Toxicopathologic Findings in Salivary Glands of Offspring Rats After Gestational and Lactational Exposure. Biological trace element research. 2020. https://doi.org/10.1007/s12011-020-02409-z PMID: 33009984

12. Nanci A. Ten Cate Histologia Oral: Elsevier Editora Ltda.; 2019.

13. Michalke B, Rossbach B, Göten T, Schäferhenrich A, Scherer G. Saliva as a matrix for human biomonitoring in occupational and environmental medicine. Int Arch Occup Environ Health. 2015; 88(1):1–44. Epub 2014/03/13. https://doi.org/10.1007/s00420-014-0938-5 PMID: 24619390.

14. Porcheri C, Mitsiadis TA. Physiology, Pathology and Regeneration of Salivary Glands. Cells. 2019; 8(9). Epub 2019/08/29. https://doi.org/10.3390/cells8090076 PMID: 3155013; PubMed Central PMCID: PMC6769486.

15. Hand AR, Frank ME. Fundamentals of Oral Histology and Physiology: Wiley; 2015.

16. Marti-Álamo S, Mancheño-Franch A, Marzal-Gamarra C, Carlos-Fabuel L. Saliva as a diagnostic fluid. Literature review. J Clin Exp Dent. 2012; 4(4):e237–e43. https://doi.org/10.4317/jced.50865 PMID: 24558562.

17. Greabu M, Battino M, Mohora M, Totan A, Didilescu A, Spinu T, et al. Saliva—a diagnostic window to the body, both in health and in disease. Journal of medicine and life. 2009; 2(2):124–32. Epub 2010/01/30. PMID: 20108531; PubMed Central PMCID: PMC3018981.

18. Michalke B, Rossbach B, Göten T, Schäferhenrich A, Scherer G. Saliva as a matrix for human biomonitoring in occupational and environmental medicine. International Archives of Occupational and Environmental Health. 2015; 88(1):1–44. https://doi.org/10.1007/s00420-014-0938-5 PMID: 24619390.

19. Pesch A, Wilhelm M, Rostek U, Schmitz N, Weishoff-Houben M, Ranft U, et al. Mercury concentrations in urine, scalp hair, and saliva in children from Germany. Journal of exposure analysis and environmental epidemiology. 2002; 12(4):252–8. Epub 2002/06/28. https://doi.org/10.1038/sj.jeea.7500228 PMID: 12087431.

20. Yang L, Zhang Y, Wang F, Luo Z, Guo S, Strähle U. Toxicity of mercury: Molecular evidence. Chemosphere. 2020; 245:125586. Epub 2019/12/28. https://doi.org/10.1016/j.chemosphere.2019.125586 PMID: 31881386.

21. Council NR, Studies DEL, Research ILA, Animals CUGCUL. Guide for the Care and Use of Laboratory Animals: Eighth Edition: National Academies Press; 2011. https://doi.org/10.1258/la.2010.010031 PMID: 21123303

22. Pericie du Sert N, Ahiwuwalia A, Alam S, Avey MT, Baker M, Browne WJ, et al. Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. PLoS biology. 2020; 18(7): e3000411. Epub 2020/07/15. https://doi.org/10.1371/journal.pbio.3000411 PMID: 32663221; PubMed Central PMCID: PMC7660025.

23. Kong HK, Wong MH, Chan HM, Lo SC. Chronic exposure of adult rats to low doses of methylmercury induced a state of metabolic deficit in the somatosensory cortex. Journal of proteome research. 2013; 12(11):5233–45. Epub 2013/08/30. https://doi.org/10.1021/pr400356v PMID: 23984759.

24. Taga R, Sesso A. Ultrastructural studies on developing parotid gland of the rat at early postnatal periods. Archivum histologicum Japonicum = Nihon soshikigaku kiroku. 1979; 42(4):427–44. Epub 1979/10/01. https://doi.org/10.1679/aohc1950.42.427 PMID: 575618.

25. Dionizio AS, Melo CGS, Sabino-Arias IT, Ventura TMS, Leite AL, Souza SRG, et al. Chronic treatment with fluoride affects the jejunum: insights from proteomics and enteric innervation analysis. 2018; 8(1):1–12. https://doi.org/10.1038/s41598-018-21533-4 PMID: 29453425.

26. Ferreira MKM, Araúgo WAB, Bittencourt LO, Puty B, Dionizio A, Souza MPC, et al. Fluoride exposure during pregnancy and lactation triggers oxidative stress and molecular changes in hippocampus of offspring rats. Ecotoxicology and environmental safety. 2021; 208:111437. Epub 2020/10/24. https://doi.org/10.1016/j.ecoenv.2020.111437 PMID: 33096359.

27. Dionizio A, Melo CGS, Sabino-Arias IT, Araújo TT, Ventura TMO, Leite AL, et al. Effects of acute fluoride exposure on the jejunum and ileum of rats: Insights from proteomic and enteric innervation analysis. Science of The Total Environment. 2020; 741:140419. https://doi.org/10.1016/j.scitotenv.2020.140419 PMID: 32866984

28. Melo CGS, Perles J, Zanoni JN, Souza SRG, Santos EX, Leite AL, et al. Enteric innervation combined with proteomics for the evaluation of the effects of chronic fluoride exposure on the duodenum of rats. Scientific reports. 2017; 7(1):1070. Epub 2017/04/23. https://doi.org/10.1038/s41598-017-01090-y PMID: 28432311; PubMed Central PMCID: PMC5430799.

29. Charone S, De Lima Leite A, Peres-Buzalaf C, Silva Fernandes M, Ferreira de Almeida L, Zardin Graeff MS, et al. Proteomics of Secretary-Stage and Maturation-Stage Enamel of Genetically Distinct Mice. Caries Res. 2016; 50(1):24–31. Epub 2016/01/29. https://doi.org/10.1159/000442301 PMID: 26820156.
30. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976; 72(1):248–54. https://doi.org/10.1006/abio.1976.9999 PMID: 942051

31. Leite AL, Lobo JGVM, da Silva Pereira HAB, Fernandes MS, Martini T, Zucki F, et al. Proteomic analysis of gastrocnemius muscle in rats with streptozotocin-induced diabetes and chronically exposed to fluoride. 2014; 9(9):e106646. https://doi.org/10.1371/journal.pone.0106646 PMID: 25180703

32. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics. 2009; 25(8):1091–3. https://doi.org/10.1093/bioinformatics/btp101 Bioinformatics. PMID: 19237447

33. Team RC. R: A language and environment for statistical computing. Vienna, Austria; 2013.

34. Alhamdoosh M, Ng M, Wilson NJ, Sheridan JM, Huynh H, Wilson MJ, et al. Combining multiple tools outperforms individual methods in gene set enrichment analyses. Bioinformatics. 2017; 33(3):414–24. Epub 2016/10/04. https://doi.org/10.1093/bioinformatics/btw623 PMID: 27694195; PubMed Central PMCID: PMC5408797.

35. Merico D, Isserlin R, Stueker O, Emili A, Bader GD. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. PLoS one. 2010; 5(11):e13984. Epub 2010/11/19. https://doi.org/10.1371/journal.pone.0013984 PMID: 21085593; PubMed Central PMCID: PMC2981572.

36. Kucera M, Isserlin R, Arkhangorsky A, Bader GD. AutoAnnotate: A Cytoscape app for summarizing networks with semantic annotations. F1000Research. 2016; 5:1717. Epub 2016/11/11. https://doi.org/10.12688/f1000research.9090.1 PMID: 27830058; PubMed Central PMCID: PMC5082607.

37. Xia J, Benner MJ, Hancock RE. NetworkAnalyzer—integrative approaches for protein-protein interaction network analysis and visual exploration. Nucleic acids research. 2014; 42(Web Server issue):W167–74. Epub 2014/05/28. https://doi.org/10.1093/nar/gku443 PMID: 24861621; PubMed Central PMCID: PMC4086107.

38. Gad SC. Methylmercury. In: Wexler P, editor. Encyclopedia of Toxicology ( Third Edition). Oxford: Academic Press; 2014. p. 318–20.

39. Clarkson TW, Magos L. The toxicology of mercury and its chemical compounds. Critical reviews in toxicology. 2006; 36(8):609–62. Epub 2006/09/16. https://doi.org/10.1080/10408440600845619 PMID: 16973445.

40. WHO. Guidance for Identifying Populations at Risk from Mercury Exposure In: Organization WH, editor. 2008.

41. de Paula Fonseca Arrifano G, Del Carmen Rodriguez Martin-Doimeadios R, Jimenez-Moreno M, Augusto-Oliveira M, Rogerio Souza-Monteiro J, Paraense R, et al. Assessing mercury intoxication in isolated/remote populations: Increased S100B mRNA in blood in exposed riverine inhabitants of the Amazon. Neurotoxicology. 2018; 68:151–8. Epub 2018/08/05. https://doi.org/10.1016/j.neuro.2018.07.018 PMID: 30076990.

42. Arrifano GPF, Martin-Doimeadios RCR, Jiménez-Moreno M, Ramírez-Mateos V, da Silva NFS, Souza-Monteiro JR, et al. Large-scale projects in the amazon and human exposure to mercury: The case-study of the Tucurui Dam. Ecotoxicology and environmental safety. 2018; 147:299–305. Epub 2017/09/01. https://doi.org/10.1016/j.ecoenv.2017.08.048 PMID: 28858702.

43. Arrifano GPF, Martin-Doimeadios RCR, Jiménez-Moreno M, Fernández-Trujillo S, Augusto-Oliveira M, Souza-Monteiro JR, et al. Genetic Susceptibility to Neurodegeneration in Amazon: Apolipoprotein E Genotyping in Vulnerable Populations Exposed to Mercury. Frontiers in genetics. 2018; 9:285. Epub 2018/08/14. https://doi.org/10.3389/fgene.2018.00285 PMID: 30100920; PubMed Central PMCID: PMC6073741.

44. Farias-Junior PMA, Teixeira FB, Fagundes NCF, Miranda GHN, Oliveira Bittencourt L, de Oliveira Paraense RS, et al. Chronic intoxication by methylmercury leads to oxidative damage and cell death in salivary glands of rats. Metallomics: integrated biometal science. 2017; 9(12):1778–85. Epub 2017/10/31. https://doi.org/10.1039/c7mt00168a PMID: 29082389.

45. Bittencourt LO, Puty B, Charone S, Aragão WAB, Farias-Junior PM, Silva MCF, et al. Oxidative Biochemistry Disbalance and Changes on Proteomic Profile in Salivary Glands of Rats Induced by Chronic Exposure to Methylmercury. Oxidative medicine and cellular longevity. 2017; 2017:5653291. Epub 2017/08/16. https://doi.org/10.1155/2017/5653291 PMID: 28811865; PubMed Central PMCID: PMC5546058.

46. Lima LAO, Bittencourt LO, Puty B, Fernandes RM, Nascimento PC, Silva MCF, et al. Methylmercury Intoxication Promotes Metallothionein Response and Cell Damage in Salivary Glands of Rats. Biological trace element research. 2018; 185(1):135–42. Epub 2018/01/15. https://doi.org/10.1007/s12011-017-1230-9 PMID: 29332268.
47. Björnberg KA, Vahter M, Berglund B, Niklasson B, Blennow M, Sandborn-Englund G. Transport of methylmercury and inorganic mercury to the fetus and breast-fed infant. Environ Health Perspect. 2005; 113(10):1381–5. https://doi.org/10.1289/ehp.7856 PMID: 16203251.

48. Nogueira LS, Vasconcelos CP, Mitre GP, da Silva Kataoka MS, Lima MO, de Oliveira EHC, et al. Oxidative Damage in Human Periodontal Ligament Fibroblast (hPLF) after Methylmercury Exposure. Oxidative medicine and cellular longevity. 2019; 2019:8470857. https://doi.org/10.1155/2019/8470857 PMID: 3185822

49. de Oliveira Lopes G, Aragão WAB, Bittencourt LO, Puty B, Lopes AP, dos Santos SM, et al. Imaging Microstructural Damage and Alveolar Bone Loss in Rats Systemically Exposed to Methylmercury: First Experimental Evidence. Biological trace element research. 2021. https://doi.org/10.1007/s12011-020-02492-2 PMID: 33409908

50. Thapa M, Bommakantes A, Shamsuzzaman M, Gregory B, Samsel L, Zengel JM, et al. Repressed synthesis of ribosomal proteins generates protein-specific cell cycle and morphological phenotypes. Mol Biol Cell. 2013; 24(23):3620–33. Epub 2013/10/09. https://doi.org/10.1091/mbc.E13-02-0097 PMID: 24108599.

51. Ree R, Kind L, Kaziales A, Varland S, Daa M, Richter K, et al. PFN2 and NAA80 cooperate to efficiently acetylate the N-terminus of actin. Journal of Biological Chemistry. 2020; 295(49):16713–31. https://doi.org/10.1074/jbc.RA120.015468 PMID: 32978259

52. Leandro-García LJ, Leskelä S, Landa I, Montero-Conde C, López-Jiménez E, Letón R, et al. Tumoral and tissue-specific expression of the major human β-tubulin isoforms. 2010; 67(4):214–23. https://doi.org/10.1002/cm.20436 PMID: 20191564

53. Fábian TK, Fejédery P, Csermely P. Saliva in Health and Disease, Chemical Biology of. Wiley Encyclopedia of Chemical Biology2008. p. 1–9.

54. Wong RS, Madapallimattam G, Bennick A. The role of glandular kallikrein in the formation of a salivary proline-rich protein A by cleavage of a single bond in salivary protein C. The Biochemical journal. 1983; 211(1):35–44. Epub 1983/04/01. https://doi.org/10.1042/bj2110035 PubMed Central PMCID: PMC1154326. PMID: 6553499

55. Levine M. Susceptibility to dental caries and the salivary proline-rich proteins. International journal of dentistry. 2011; 2011:953412. Epub 2011/12/23. https://doi.org/10.1155/2011/953412 PMID: 22190937; PubMed Central PMCID: PMC3235478.

56. Yahiro J, Miyoshi S. Immunohistochemical localization of kallikrein in salivary glands of the Japanese monkey, Macaca fuscata. Archives of oral biology. 1996; 41(2):225–8. Epub 1996/02/01. https://doi.org/10.1016/0003-9969(95)00116-6 PMID: 8712979

57. Garrett JR, Smith RE, Kidd A, Kyniacou K, Grabske RJ. Kallikrein-like activity in salivary glands using a new tripeptide substrate, including preliminary secretory studies and observations on mast cells. The Histochemical journal. 1982; 14(6):967–79. Epub 1982/11/01. https://doi.org/10.1016/S0003-9969(05)80249-2 PMID: 6924654

58. Gerke V, Creutz CE, Moss SE. Annexins: linking Ca2+ signalling to membrane dynamics. Nature reviews Molecular cell biology. 2005; 6(6):449–61. Epub 2005/06/02. https://doi.org/10.1038/nrm1661 PMID: 15928709.

59. Lim LH, Pervaiz S. Annexin 1: the new face of an old molecule. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2007; 21(4):968–75. Epub 2007/01/12. https://doi.org/10.1096/fj.06-7464rev PMID: 17215481.

60. Pollard KM, Cauvi DM, Toomey CB, Hultman P, Kono DH. Mercury-induced inflammation and autoimmunity. Biochimica et biophysica acta General subjects. 2019; 1863(12):129299–. Epub 2019/02/10. https://doi.org/10.1016/j.bbagen.2019.02.001 PMID: 30742953.

61. Olevik PA, Brattás M, Lie KK, Goksøyr A. Transcriptional responses in juvenile Atlantic cod (Gadus morhua) after exposure to mercury-contaminated sediments obtained near the wreck of the German WW2 submarin e U-864, and from Bergen Harbor, Western Norway. Chemosphere. 2011; 83(4):552–63. https://doi.org/10.1016/j.chemosphere.2010.12.019 PMID: 21195448

62. Ghosh S, Sarkar P, Basak P, Mahalanobish S, Sil PC. Role of Heat Shock Proteins in Oxidative Stress and Stress Tolerance. In: Asea AAA, Kaur P, editors. Heat Shock Proteins and Stress. Cham: Springer International Publishing; 2018. p. 109–26.

63. Consortium TU. UniProt: the universal protein knowledgebase in 2021. Nucleic acids research. 2020; 49(D1):D480–D9. https://doi.org/10.1093/nar/gkaa1100%J Nucleic Acids Research.

64. Miller DJ, Fort PE. Heat Shock Proteins Regulatory Role in Neurodevelopment. 2018; 12(821). https://doi.org/10.3389/fnins.2018.00821 PMID: 30483047

65. Reichmann D, Voth W, Jakob U. Maintaining a Healthy Proteome during Oxidative Stress. Molecular cell. 2018; 69(2):203–13. https://doi.org/10.1016/j.molcel.2017.12.021 PMID: 29351842
66. Löw P. The role of ubiquitin–proteasome system in ageing. General and Comparative Endocrinology. 2011; 172(1):39–43. https://doi.org/10.1016/j.ygcen.2011.02.005 PMID: 21324320

67. Kirkin V, Dikic I. Role of ubiquitin- and Ubl-binding proteins in cell signaling. Current opinion in cell biology. 2007; 19(2):199–205. Epub 2007/02/17. https://doi.org/10.1016/j.ceb.2007.02.002 PMID: 17303403.