



ORIGINAL ARTICLE

Evaluation of the chronic intoxication of fluoride on human serum metabolome using untargeted metabolomics



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Abstract Drinking water is the main source of fluoride intake for the human body and its regulated consumption helps in decreasing dental caries. However, excessive fluoride consumption over a prolonged time period causes fluorosis disease which adversely affects many tissues and organs of the body. This paper describes the evaluation of chronic intoxication of fluoride on human serum metabolome. The untargeted metabolomics approach using UPLC-QTOF-MS/MS is applied for metabolomic profiling, whereas the estimation of fluoride in serum samples was carried out using

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the ion-selective electrode (ISE). Fluoride concentration was found to be 0.16–1.25 mg/L in serum samples of 39 fluorosis patients and 0.008–0.045 mg/L in 20 healthy samples. A total of 47 metabolites were identified based on the high-resolution mass spectrometry analysis. A volcano plot was generated to discriminate features that are significantly different between the fluorosis and healthy groups at the probability of 0.05 and fold change ≥ 2 . Among all identified metabolites, intensities of ten differential identified metabolites including inosine, α -linolenic acid, guanosine, octanoyl-L-carnitine, His-Trp, phytosphingosine, lauroyl-L-carnitine, hydrocortisone, deoxyinosine and dodecanedioic acid have been found altered in disease samples compared to healthy controls. Major pathways identified based on these metabolites include energy metabolism, fatty acid oxidation, purine degradation pathway, elevated protein degradation, and increased ω -6 fatty acid linoleate signatures were observed.

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1. Introduction

Drinking water is the main source of fluoride intake for the human body (Roy & Dass, 2013). According to the World Health Organization (WHO), the permissible limit of fluoride ion concentration in water is 1.5 mg/L (Kumar & Puri, 2012). Fluoride toxicity greatly impacts human health and causes many diseases including dental and skeletal fluorosis (Strunecka & Strunecky, 2020). In skeletal fluorosis, the bones become fragile and at the severe stages, leading to a crippling disorder (Ayoob & Gupta, 2006; Ozsvath, 2009).

Around 200 million people all over the world are affected by fluorosis. It is endemic to mostly third-world countries including India, China, and Pakistan (Jacks et al., 2005). In Pakistan, fluorosis is a considerable problem in many areas, especially in the Mithi sub-district of the Thar Desert. These areas have a high concentration of fluoride in groundwater. Fluoride-containing minerals and the tropical desert climate expedites the leaching of fluoride in water (Rafique et al., 2008). Due to poor infrastructure and the unavailability of water filtration plants, people rely on groundwater for their daily use and as a consequence suffer from chronic intoxication of fluoride leading to fluorosis.

Although several perturbed mechanisms due to fluorosis are proposed in the literature yet it remains unclear, (Everett et al., 2002). Enamel development and bone metabolism could be affected by excessive consumption of fluoride which ultimately results in dental and skeletal fluorosis (Everett, 2011). It is known that mineralization is extremely sensitive to free fluoride ions. These ions greatly affect the mineralization of bones and teeth by decreasing the solubility of minerals (Aoba & Fejerskov, 2002). The minerals in bones and teeth enamel are mainly composed of hydroxyapatite, where fluoride by displacing hydroxide, forms fluorapatite. Fluoroapatite formation slows down the rebuilding of bones as it is more stable and less soluble. Therefore, enamel development and bone metabolism could be affected by excessive consumption of fluoride which ultimately results in dental and skeletal fluorosis (Everett, 2011). Chronic exposure to fluoride causes metabolic, structural, and functional damage in many tissues, e.g., gonadal, neurological, renal, and endothelial (Doull et al., 2006). Fluoride ions can attach to functional amino acid groups that are present inside and surrounding the active sites of enzymes and proteins (Edwards et al., 1984). This attachment of fluoride ions affect the production of Adenosine triphosphate (ATP) and inhibition of cellular respiration can occur (Eva Adamek 2005). Reactive oxygen species (ROS) induction, production of nitric oxide, and reduction in cellular antioxidant defenses against oxidative damage are also linked with fluoride toxicity (Barbier et al., 2010; Strunecka & Strunecky, 2020).

Metabolomics is an established field to study physiological and pathological diseases and can provide an extensive chemical fingerprint of cell metabolism (Xie et al., 2017). Metabolomics offers quick and in-depth observations of any perturbations in the phenotype of a biological system (Dunn et al., 2011). Thus it is widely being used for biomar-

ker discovery, diagnostic and prognostic purposes (Florian et al., 1995a; Florian et al., 1995b; Lokhov et al., 2010). However, metabolomics studies of fluorosis are very scarce. Metabolomic analysis was performed to study NaF-induced cytotoxicity in the HSC-2 human oral squamous cell carcinoma cell line (Sakagami et al., 2014) and, more recently, serum metabolite profiling of rats exposed to fluoride (Yue et al., 2020). To author's knowledge, metabolomics analysis of fluorosis patients has not been reported yet. Hence, the present study is focused on evaluating the chronic intoxication of fluoride on the serum metabolome of fluorosis patients in comparison with healthy controls using ultra-performance liquid chromatography-quadrupole time-of-flight tandem mass spectrometry (UPLC-QTOF-MS/MS) analysis.

2. Materials and methods

2.1. Ethical considerations and patient selection

A total of 39 blood samples were collected from patients with a mean age of 35.21 ± 7.85 years from different villages (Dabi, Pabe Jo Thar, Misri Memon, Arab Memon, Sakrio, Kakrario, Mau, Mithi city) of the Mithi sub-district, Tharparkar, Sindh, Pakistan. Samples were collected from those villages where the high concentration of fluoride is present in ground water (Rafique et al., 2015; Rafique et al., 2009) and people are relying to use it without any treatment process. The Mithi sub-district is approximately located between $N24^{\circ} 44' 11''$ and $E69^{\circ} 47' 50''$ in the Thar Desert. Fluorosis patients with clear dental and skeletal abnormalities (identified by a physician) were included in this study; and any type of therapy was discontinued prior to 15 days of sample collection. Additionally, 20 healthy samples with the age range of 30.75 ± 2.65 years were also collected from other villages of Sindh where people are using clean water. The approval of this study was taken by the Independent Ethics Committee, International Center for Chemical and Biological Sciences, University of Karachi. Before the collection of samples, a written informed agreement was taken from all healthy individuals and patients.

2.2. Sample collection

About 5 mL of 8 h fasting blood was taken from patients by venipuncture and immediately transferred to BD SST tube with silica clot activator, polymer gel, silicone-coated interior (BD Franklin Lakes NJ, USA, REF: 367977). The blood samples were brought to the laboratory for serum separation, cen-

trifuged at 2000 rpm for 5 min and immediately stored at -80°C .

2.3. Chemicals and reagents

Analytical grade solvents were used for all experiments unless otherwise noted. 1 and 10 ppm fluoride standard solutions were purchased from HANNA Instruments, (Woonsocket, USA). Glacial acetic acid was purchased from Lab-Scan limited (Bangkok 10330, Thailand). Sodium hydroxide was purchased from Uni-Chem Chemical Reagents. Acetonitrile and methanol were purchased from Merck KGaA, 64,271 (Darmstadt Germany). Formic acid was purchased from Daejung, chemicals and metals, Co. (Daejung, Korea). Standard N-Fmoc-L-serine (OtBu) was purchased from GL Biochem (Shanghai, China) and N-Fmoc-L-alanine was purchased from Chem-Impex International Inc and. Deionized water (Milli-Q) was used throughout the study (Millipore, Billerica, MA, USA).

2.4. Estimation of fluoride in serum

Estimation of the concentration of fluoride ion was performed according to the reported method with slight modifications (Kissa, 1987). The ion-selective electrode (ISE) (HI4110 fluoride combination, HANNA, USA) was used for the determination of fluoride in serum. Total Ionic Strength Adjustable Buffer (TISAB) was used to obtain the pH range of 5–6. By using commercially available 1 and 10 ppm fluoride standard solutions, the slope was set at -56 ± 4 for two-point calibration. After adjusting the slope, 20 mL of 0.05 ppm fluoride standard solution in TISAB was analyzed through ISE before and after the addition of 0.5 mL serum sample.

The concentration of fluoride in serum was determined using the following formula,

$$C_b = [C_s(V_o + V_b) - C_o V_o] / V_b \quad (1)$$

Where C_s = F^- ion concentration after serum addition, C_o = Concentration of standard solution V_o = Volume of standard solution, V_b = Volume of blood sample,

C_b = Concentration of F^- ion in serum sample.

2.5. Sample preparation and UPLC-QTOF-MS analysis

Serum sample having the volume of 100 μL was added to 100 μL volume of Milli-Q (containing 5 $\mu\text{g}/\text{mL}$ N-Fmoc-L-serine (OtBu) and 5 $\mu\text{g}/\text{mL}$ N-Fmoc-L-alanine as internal standards) and then added a mixture of 400 μL of acetonitrile and methanol (3:5), vortexed for 2 min and left for 10 min at room temperature. The supernatant was separated after centrifugation at 12,000 rpm for 20 min and stored at -80°C after vacuum drying until analysis. Dried samples were reconstituted in 200 μL volume of water: methanol (70:30) and transferred into an auto-sampler vial for analysis after vortexing for 2 min and centrifuged at 12,000 rpm for 20 min.

Samples were analyzed on UPLC (Thermo Scientific Dionex 3000) online linked with QTOF (HRMS) mass spectrometer (maxis II Bruker, Daltonics Bremen, Germany). An RP-C18 column (100 \times 2.0 mm, 1.8 μm , Macherey-Nagel, Düren,

Germany) was used for separation. The temperature of the column compartment was 40°C and the injection volume was 5 μL . A = 0.1% formic acid and B = methanol with 0.1 % formic acid were used as mobile phases at 0.5 mL/min of flow rate. Linear gradient starting at 1–5% B for 0–1 min, from 5 to 95% B for 1–9 min, 95% B for 9–10.5 min, 5% B over 10.7 min, 5% B over 10.7–13 min was used.

All samples were analyzed in positive ion mode with the following parameters for MS and MS/MS; drying gas temperature 270°C , drying gas flow 12.0 L/min, the capillary voltage 4500 V, 100–1200 m/z mass range, and 3.1 bars nebulizer pressure. Data was recorded using Compass HyStar 4.1 Acquisition (Bruker, Germany). Multiple collision energies of 20, 25, and 30 eV were used for MS/MS analysis.

1 N Na-formate solution was used as a mass calibrant before each sample injection. A separate pool was made by adding 10 μL from each sample, referred to as quality control (QC), given the same treatments as other samples. QC sample was subjected to analysis to provide robust quality assurance (QA) for each metabolic feature detected periodically after every 6 samples. To ensure data quality and to check instrument stability, QC samples were injected 10 times at the beginning of the batch.

2.6. Data processing and statistical analysis

After calibration of all LC-MS spectra, features were generated by Bruker's MetaboScape (version 3.0) software using the following parameters; intensity threshold 2000 counts, minimum peak length 16 spectra, minimum peak length (recursive) 13 spectra, feature signal intensity, minimum numbers of features for extraction (30% of analyses), presence of features in minimum numbers of analyses (30% of analyses), mass range 100–1500 m/z , retention-time range (0.3–12) min, primary ion $[\text{M} + \text{H}]^+$, extracted ion chromatogram (EIC) correlation 0.7. Data was normalized using internal standards mentioned in the previous section. Statistical significance was calculated using the student's t -test and features with p -value < 0.05 , after Benjamini-Hochberg corrections, were considered as significant features. Fold change in intensity of features from case to control was also calculated with the threshold of ≥ 2 . The generated features were imported to SIMCA software (version 14.1, Umetrics, Umeå, Sweden) for chemometric analysis. The distribution of distinct groups and detection of outlier by principal component analysis (PCA) was performed after mean centering and Pareto scaling to equalize weight to all variables despite their all absolute values. It was done for non-averaged samples to obtain cumulative variance which is represented by first two components on X and Y axis for measuring the differences. Prediction model was built using orthogonal partial least squares discriminant analysis (OPLS-DA). It is a supervised multivariate data analysis and was constructed using four parameters including auto scaling, N fold validation type, three numbers of fold and with ten numbers of repeats. The classes in the input data are randomly divided into three equal parts; two parts were used for training, and the remaining part was used for testing. The process was repeated ten times with a different part that is used for testing in each iteration. Thus each row is used at least once in training and testing.

Table 1 Identification of significantly differential metabolites by comparing MS/MS spectra with databases such as Mass bank of Europe, Mass bank of North America (MoNA), NIST tandem mass and annotated through exact mass measurement.

Sr. No	Name	RT	Adducts	Formula	Observed mass	Calculated mass	ppm error	P value (FDR)	Fold change
1	Inosine ^a	1.27	[M + H] +	C ₁₀ H ₁₂ N ₄ O ₅	269.0888	269.0888	< 1	0.000255	-3.734
2	α -linolenic acid ^a	10.4	[M + H] +	C ₁₈ H ₃₀ O ₂	279.2317	279.2319	< -1	0.000166	-2.667
3	Guanosine ^a	1.30	[M + H] +	C ₁₀ H ₁₃ N ₅ O ₅	284.0987	284.0989	< 1	0.000032	-5.537
4	Octanoyl-L-Carnitine ^a	5.79	[M + H] +	C ₁₅ H ₂₉ NO ₄	288.2167	288.2169	< -1	0.001222	-3.558
5	Phytosphingosine ^a	7.17	[M + H] +	C ₁₈ H ₃₉ NO ₃	318.3008	318.3002	2	0.016185	-2.049
6	His-Trp ^a	1.04	[M + H] +	C ₁₇ H ₁₉ N ₅ O ₃	342.1565	342.1561	2	0.000063	2.188
7	Lauroyl L-carnitine ^a	7.65	[M + H] +	C ₁₉ H ₃₇ NO ₄	344.2798	344.2795	< 1	0.013752	-2.657
8	Hydrocortisone ^a	7.15	[M + H] +	C ₂₁ H ₃₀ O ₅	363.2167	363.2166	-2	0.000055	-3.307
9	Deoxyinosine ^b	7.15	[M + H] +	C ₁₀ H ₁₂ N ₄ O ₄	253.0928	253.0913	6	0	6.665
10	Dodecanedioic acid ^b	9.26	[M + H] +	C ₁₂ H ₂₂ O ₄	231.1614	231.1591	10	0.007518	-6.878

a = Level 2-putative identification, b = Level 3-Tentative structure (Schymanski et al., 2014).

Fig. 3. The principal component analysis provides useful information that helps in the understanding of the differences within and between the groups. OPLS-DA is a discriminant analysis that uses a PLS algorithm to establish the predictive ability of the data. Moreover, R^2X and R^2Y are the prediction parameters in which R^2Y and R^2X show the interpretation rate of the established model to Y and X matrix respectively and Q2 shows the prediction ability of the model.

After removing outliers, PCA was generated and the score plot showed fine separation as shown in Fig. 4. OPLS-DA was constructed for establishing a model for the discrimination between healthy and disease groups as shown in Fig. 5. VIP values of ten significantly different metabolites were found to be greater than 1. The OPLS-DA model showed fine separation between disease and healthy groups with sensitivity (95%), specificity (100%) and 98.64% classification rate. For validation, permutation tests (500) on OPLS-DA model were performed. The intercept (Q2 and R2 when the correlation coefficient is zero) for HS and FU shows that model is satisfactory as shown in Fig. 6.

4. Discussion

A wide range of normal concentrations (0.008–0.045 mg/L) of fluoride in plasma has been reported in the literature. In comparison, our study shows that the concentration of fluoride was found to be 0.16–1.25 mg/L in 39 disease samples and 0.03–0.007 mg/L in 20 healthy samples. Fluoride ion concentrations were found to agree with the reported data from healthy (Şener et al., 2007) and disease (Rafique et al., 2015) samples. The toxic levels of fluoride are the root cause of many pathological problems as they cause the inhibition of metalloproteinase within the organelles or different physiological pathways (Agalakova & Gusev, 2012). The sporadic nature of the disease roots in the lack of complete understanding of

the metabolomics of fluorosis. Long-term exposure to fluorine and its associated metabolites results in a wide array of toxicity traits including oxidative stress, DNA damage, cell cycle arrest, apoptosis, skeletal and soft tissue degradation (Johnston & Strobel, 2020). Therefore, this study mainly focuses on the investigation of alteration of key metabolites responsible for the progression of the disease and the identification of possible therapeutic approaches for better disease management.

In this study, a comparison of fluorosis and healthy samples showed metabolites perturbation of purine degradation pathway (e.g., guanosine and inosine), decreased levels of corticosteroids, elevated protein degradation indicated by the increased level of dipeptides (His-Trp), increased ω -6 fatty acid linoleate signatures, and decreased mitochondrial activity.

As mentioned in table 1, two of the significantly identified metabolites are L-carnitine with different fatty acid chains. L-carnitine is an important factor involved in the normal function of mitochondria. L-carnitine (D-isomer is toxic and not present in humans) is actively involved in energy metabolism, beta-oxidation regulation, and maintenance of the endogenous antioxidant defense system (Reuter & Evans, 2012). In a recent study, it was reported that circulating mitochondrial DNA content is negatively correlated with low-to-moderate fluoride exposure to dental fluorosis in Chinese school-age children (Zhou et al., 2019). Hence reduced levels of L-carnitine in this study would be due to reduced mitochondrial DNA content which might be a causal intermediate in biological mechanisms linking fluoride exposure to dental fluorosis. Another effect of chronic fluoride intoxication on disturbed energy metabolism is due to the loss of mitochondrial transmembrane potential, as fluoride has a high charge density (Gutowska et al., 2010). This perturbed the mitochondrial membrane function of ATP synthesis, and this is well known that low ATP synthesis is linked to increased ROS production (Strunecka & Strunecky, 2020).

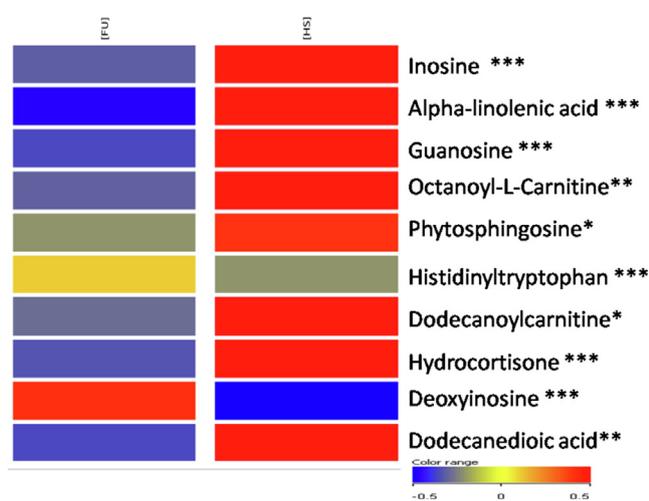


Fig. 2 Heatmap visualization in the serum of ten identified and significant metabolites based on their normalized intensities. The red indicates the significantly up-regulated metabolites and blue represents the significantly down-regulated metabolites. p-values * = < 0.05; ** = ≤ 0.01 ; *** = ≤ 0.001 .

Due to the lesser levels of L-carnitine in fluorosis, it would also suggest that the patient might suffer from decreased carnitine palmitoyltransferase 1A (CPT1A) activity (van der Leij et al., 2000). CPT1A deficiency is characterized by hypoketotic dicarboxylic aciduria with high urinary levels of dodecanedioic acid (Scaglia et al., 1998). Moreover, it was observed in a study that dodecanedioic acid is an indicator of CPT1A deficiency (Korman et al., 2005). However, the contradicting results from these two studies were observed in our case, where dodecanedioic acid was found to be decreased in

fluorosis patients when compared to healthy individuals. It can be hypothesized for this anomaly that higher excretion of dodecanedioic acid results in lower serum levels. A validation study is needed for dodecanedioic acid to affirm the observations of this study.

Another identified metabolite was α -linolenic acid (ALA), a poly-unsaturated fatty acid, an exogenous metabolite that belongs to the omega-3 fatty acid class. ALA works as the precursor metabolite for long-chain unsaturated fatty acids by a series of alternating desaturation and elongation reactions. ALA is observed more than 2.5 times decreased in patients as compared to healthy individuals. The lower levels of ALA ultimately cause a decrease in the concentration of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are the common products of ALA metabolism and help to reduce inflammation and may help in lowering the risk of chronic diseases (Chen & Nilsson, 1993). It was also reported for different mammals that higher fluorine content decreases the fatty acid composition in biological samples (Wang et al., 2012). Therefore, deficient ALA intake and higher fluorine content collectively make the situation worse for normal regulation of the fatty acid metabolic pathway.

Phytosphingosine (PHS) is one of the most widely distributed natural sphingoid bases. It metabolizes to odd-numbered fatty acids and is incorporated into glycerophospholipids (Kondo et al., 2014). Higher fluoride levels elevate the pH (Sharma et al., 2010) which ultimately causes dental erosion and dental fluorosis. PHS and other sphingoid bases with their anti-erosive properties form layers on the surface of enamel and act as diffusion barriers against H^+ ions (Valentijn-Benz et al., 2015). Decreased levels of PHS indicate the increased susceptibility to enamel erosion.

In our study, significant changes are found in functionally diverse metabolites of the purine degradation pathway which

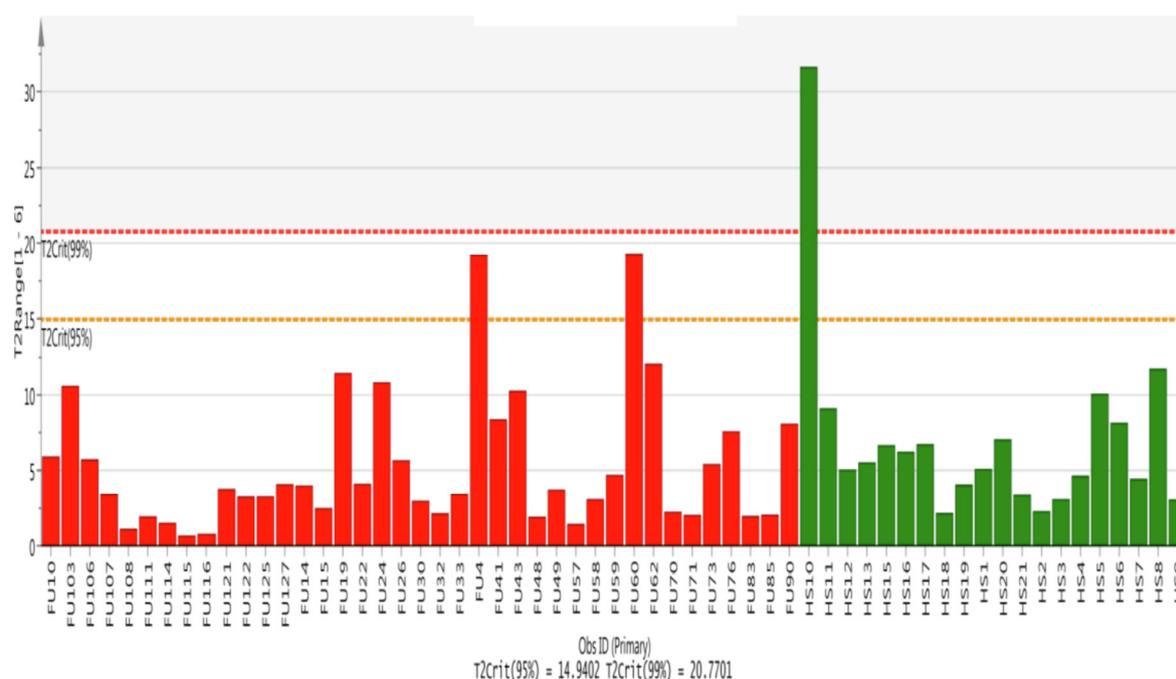


Fig. 3 Hotelling's T^2 plot with confidence limits 99% and 95% Hotelling's showing three outliers [healthy (green) and fluorosis (red)].

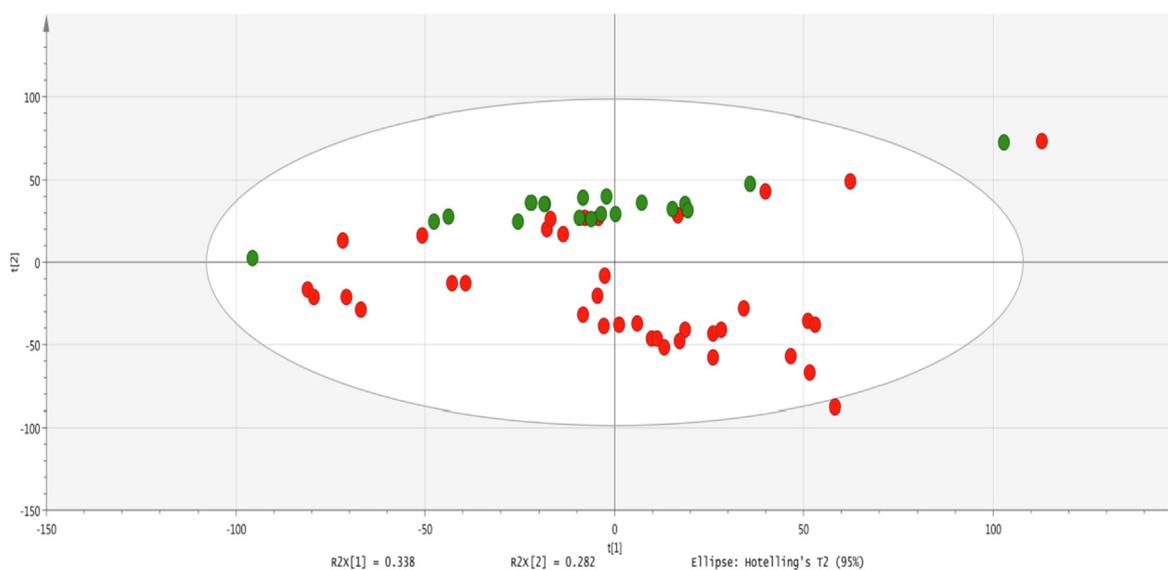


Fig. 4 PCA scores scatter plot of 39 diseases and 20 healthy samples using a normalized intensity of all features extracted from mass spectra. Boundary showed the 95% of confidence limit of Hotelling's T2 test. Each dot represents a sample and each color represents the type of the sample. Red = fluorosis samples; green = healthy.

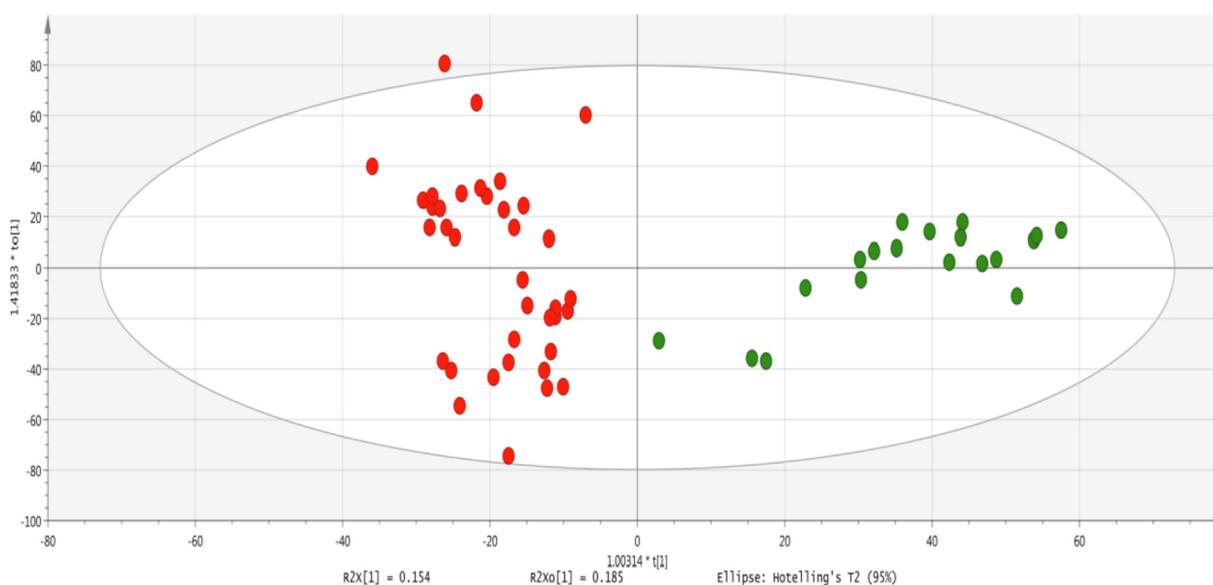


Fig. 5 Two-dimensional OPLSDA score plot of a total of 59 samples from disease and healthy individuals showing the separation of a study group on the x-axis and variations within the group on the y-axis. Each dot represents a sample and each color represents the study group. Red = fluorosis samples; green = healthy.

are concomitant with oxidative stress and compromised antioxidant capacity. Particularly, the reduction of an oxygen molecule by xanthine oxidase and the production of the superoxides (O_2^-) results in decreased intrinsic radical scavengers (Rzeuski, 1998), with an increased vulnerability to the generation of free oxygen radicals (Quinlan et al., 1997). This is reflected in this study as guanosine and inosine were found to be down-regulated while deoxyinosine levels were found to be up-regulated. The perturbation in these metabolites is similar to the previous study (Li et al., 2020). The lower levels of guanosine and inosine, with increased levels of deoxyinosine

in the fluorosis sample, indicate that interconversion of inosine and adenosine occurs to replenish cellular energy levels in stress conditions by increasing the metabolic flux to regenerate ATP through purine nucleotide cycle (PNC) (Takahashi & Sato, 2014) or hypoxanthine-IMP-AMP pathway (Shafy et al., 2012). These pathways utilize the intermediate of purine degradation metabolites to regenerate AMP from IMP via AMP deaminase, adenylosuccinate synthetase (ADSS), and adenylosuccinate lyase (ADSL) and phosphoribosyl pyrophosphate (PRPP) enzymes, respectively (Simoni et al., 2007). However, further investigation of these interconnected path-

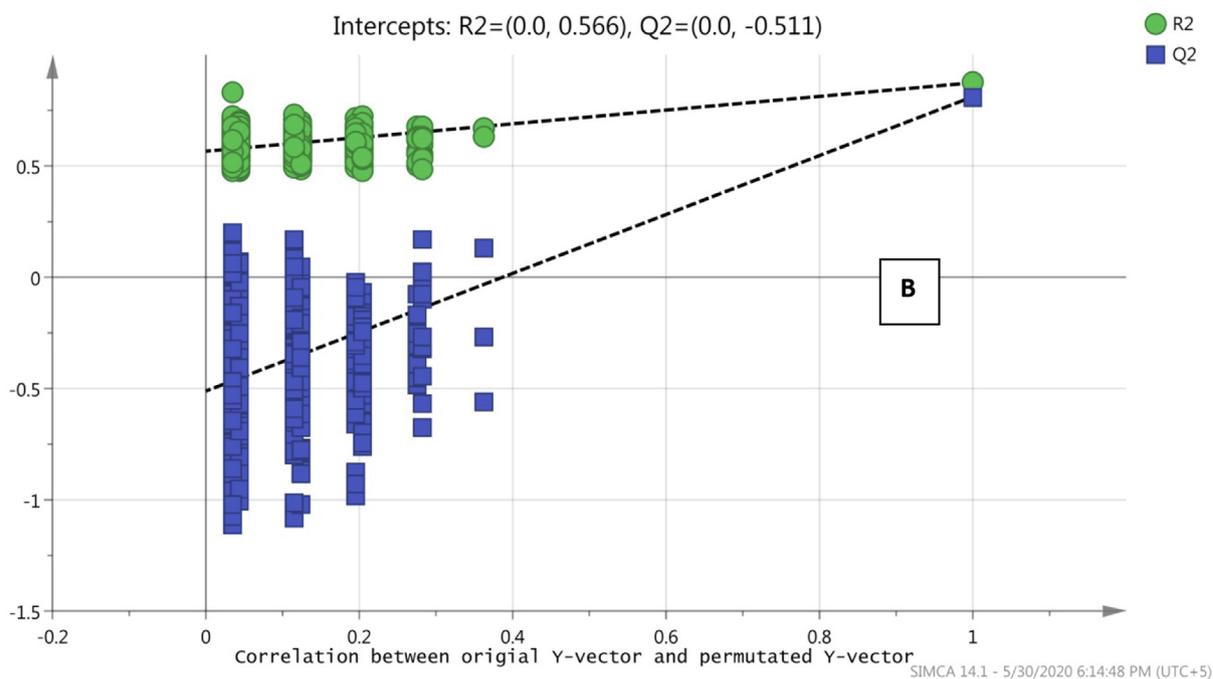
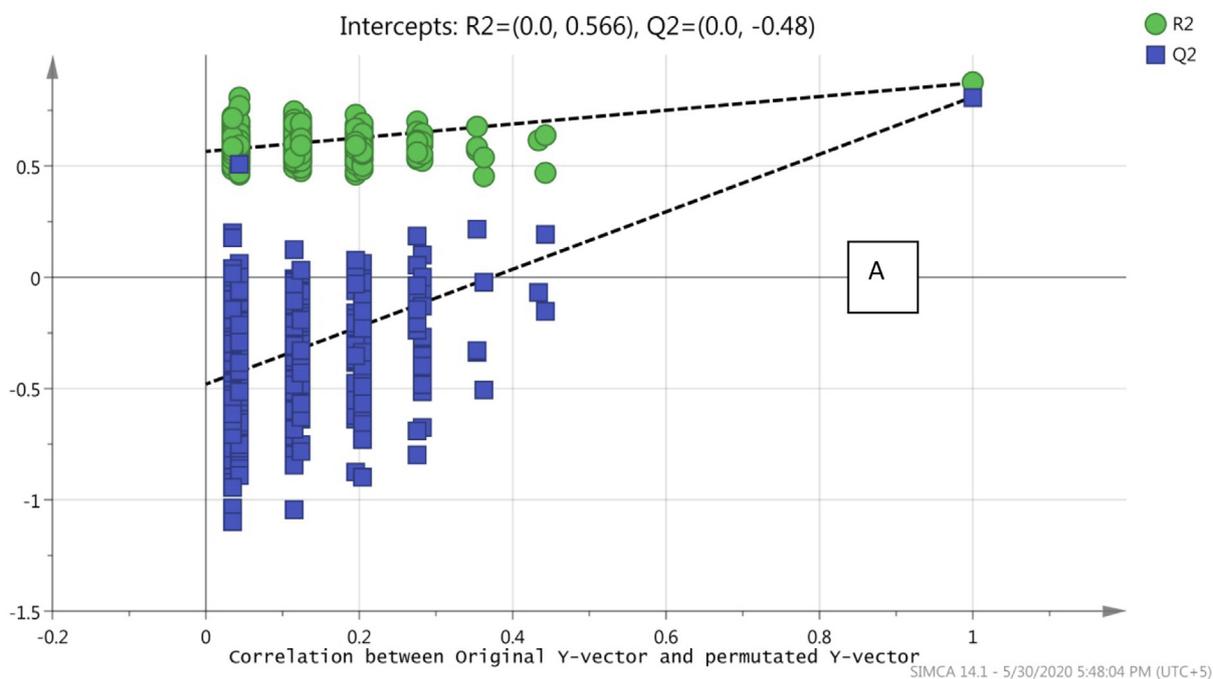


Fig. 6 Permutation (500 times) tests for OPLS-DA model showing R2 (goodness of fit) and Q2 (predictive ability) values, A; Healthy, B; Fluorosis.

ways is essential for evaluating the role of purine degradation metabolites in fluorosis in meeting the energy requirement of the body.

The significant change in hydrocortisone (cortisol) level in fluorosis patients defines one of the suggestive physiological links between corticosteroids and calcium complexes, as exces-

sive fluoride causes calcium overload, which in turn modulates physiochemical properties of corticosteroids (Monder et al., 1988; Reid et al., 1986). A previous study has shown that excessive fluoride exposure can cause calcium overload by exerting endoplasmic reticulum stress, which disrupts mitochondrial oxidative phosphorylation, decreases ATP level,

and causes an increase in intracellular Ca⁺ thus resulting in calcium overload (Pandya et al., 2013; Zhang et al., 2016). But this demands further exploration to critically describe potential relationships between calcium-related disease conditions in fluorosis and the calcium chelation capabilities of the corticosteroids.

5. Conclusion

Evaluation of the chronic intoxication of fluoride on human serum metabolome has shown that the concentration of identified metabolites either increased or decreased in patients as compared to healthy. All metabolites identified in this study have clear connection to main physiological reactions of human body. The possible mechanisms involved different key metabolites and molecular pathways associated with the pathogenesis of fluorosis have been proposed, as like purine degradation pathway, decreased levels of corticosteroids, elevated protein degradation, increased ω -6 fatty acid linoleate signatures, and decreased mitochondrial activity in fluorosis individuals. The identified metabolites could be helpful for a better understanding of the disease progression and diagnosis. These identified metabolites could be useful biomarkers for fluorosis however, an extensive study is needed for the validation of these biomarkers in population of different origins.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

S.G.M. proposed the subject, designed the study and actively participated in manuscript writing; M.U. performed the experiments, interpreted the data and actively involved in the write-up of the manuscript; A.A. actively participated in results, discussion and manuscript checking; T.R. and S.S.H. involved in the estimation of fluoride; S.K. assisted in sample preparation, statistical analysis and reviewing the manuscript; R.J. actively participated in sample collection; A.J.S. and F.I. involved in statistical analysis and interpretation of results. J.U. actively participated in the manuscript writing and revision. All authors reviewed and approved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.arabjc.2022.103928>.

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