Study of Saliva Transcriptome and Proteome in Thai Patients with Dental Fluorosis

Miss Patcharaporn Gavila

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Geriatric Dentistry and Special Patients Care

FACULTY OF DENTISTRY

Chulalongkorn University

Academic Year 2022

การศึกษาทรานสคริปโทมและโปรตีโอมในน้ำลายของผู้ป่วยไทยที่มีฟันตกกระ

น.ส.พัชราภรณ์ กาวิละ

Thesis Title	Study of Saliva Transcriptome and Proteome in Thai Patients with Dental Fluorosis
By	Miss Patcharaporn Gavila
Field of Study	Geriatric Dentistry and Special Patients Care
Thesis Advisor	Associate Professor THANTRIRA PORNTAVEETUS, D.D.S., M.Sc., Ph.D.

Accepted by the FACULTY OF DENTISTRY, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

.... Dean of the FACULTY OF DENTISTRY

(Professor Pornchai Jansisyanont, D.D.S., M.S., Ph.D.)

THESIS COMMITTEE

.... Chairman

(Associate Professor Piyamas Sumrejkanchanakij, D.D.S., Ph.D.)

.... Thesis Advisor

(Associate Professor THANTRIRA PORNTAVEETUS, D.D.S., M.Sc., Ph.D.)

.... External Examiner

(Associate Professor PUANGWAN LAPTANASUPKUN, D.D.S., M.Sc., Ph.D.)

พัชราภรณ์ กาวิละ : การศึกษาทรานสคริปโทมและโปรตีโอมในน้ำลายของผู้ป่วยไทยที่มีฟันตกกระ. (Study of Saliva Transcriptome and Proteome in Thai Patients with Dental Fluorosis) อ.ที่ปรึกษาหลัก : รศ. ทพญ. ดร.ทัณฑริรา พรทวีทัศน์

วัตถุประสงค์: เพื่อศึกษารูปแบบการแสดงออกของอาร์เอ็นเอ และโปรตีนในน้ำลายของคนไทยที่มีภาวะฟันตกกระและเปรียบเทียบกับกลุ่มที่ไม่มีภาวะฟันตกกระ และเพื่อศึกษาความสัมพันธ์ระหว่างความรุนแรงของภาวะฟันตกกระ ระดับฟลูออไรด์ในปัสสาวะ และระดับฟลูออไรด์ในน้ำ

วิธีการ: เด็กนักเรียนในพื้นที่ที่มีระดับฟลูออไรด์สูงในจังหวัดลำพูนและราชบุรีได้รับการตรวจคัดกรองความรุนแรงของภาวะฟันตกกระ แบ่งเป็นกลุ่มตัวอย่างที่มีภาวะฟันตกกระและกลุ่มควบคุมที่ไม่มีภาวะฟันตกกระ โดยทำการเก็บตัวอย่างน้ำลาย ปัสสาวะ และน้ำบริโภค ตัวอย่างน้ำลายถูกเก็บในหลอดปลอดเชื้อและเก็บไว้ที่อุณหภูมิ -20℃ เพื่อวิเคราะห์โปรตีโอมิกส์และทรานสคริปโตมิกส์ต่อไป สำหรับตัวอย่างปัสสาวะและน้ำบริโภคจะถูกนำมาตรวจหาความเข้มข้นของฟลูออไรด์ในตัวอย่างด้วยวิธีการตรวจวัดไอออนเฉพาะเจาะจง ผลความเข้มข้นของฟลูออไรด์ในปัสสาวะและในน้ำบริโภค และการแสดงออกของอาร์เอ็นเอและโปรตีนในน้ำลายของกลุ่มที่มีฟันตกกระจะถูกนำมาเปรียบเทียบกับกลุ่มที่ไม่มีภาวะฟันตกกระ

ผลการศึกษา: กลุ่มเด็กนักเรียนอายุระหว่าง 6-16 ปี จำนวน 47 ราย พบว่า 27 รายไม่มีภาวะฟันตกกระ (Non-dental Fluorosis) 10 รายมีภาวะฟันตกกระระดับรุนแรง (Severe dental fluorosis) และ 10 รายมีภาวะฟันตกกระระดับปานกลาง (Moderate dental fluorosis) จากการศึกษาพบว่าระดับฟลูออไรด์ในปัสสาวะของกลุ่มฟันตกกระระดับรุนแรงสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (p=0.007) แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มฟันตกกระระดับปานกลาง (p=0.054) ทั้งนี้ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของระดับฟลูออไรด์ในน้ำบริโภคของทั้งสามกลุ่ม (p=0.246) ผลการวิเคราะห์การแสดงออกของอาร์เอ็นเอ พบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างกลุ่มที่มีภาวะฟันตกกระระดับรุนแรงและกลุ่มควบคุม ทั้งนี้พบว่ามีโปรตีน 12 ชนิดที่มีการแสดงออกที่แตกต่างกันอย่างมีนัยสำคัญทางสถิติระหว่างกลุ่มฟันตกกระระดับรุนแรง กลุ่มฟันตกกระระดับปานกลาง และกลุ่มที่ไม่มีภาวะฟันตกกระ และพบว่าโปรตีน S100A9 มีการแสดงออกที่เพิ่มขึ้นทั้งในกลุ่มที่มีภาวะฟันตกกระรุนแรงและปานกลางเมื่อเทียบกับกลุ่มที่ไม่มีภาวะฟันตกกระ

สรุปผลการศึกษา: ระดับฟลูออไรด์ในปัสสาวะเพิ่มขึ้นอย่างมีนัยสำคัญสอดคล้องกับภาวะฟันตกกระระดับรุนแรง พบการแสดงออกแต่งต่างกันอย่างมีนัยสำคัญของโปรตีนในน้ำลาย 12 ชนิด ระหว่างกลุ่มฟันตกกระระดับรุนแรง ฟันตกกระระดับปานกลาง และกลุ่มที่ไม่มีฟันตกกระ โดยโปรตีนเหล่านี้เกี่ยวข้องกับกระบวนการทางชีววิทยา เช่น กระบวนการอักเสบ และการตายของเซลล์

สาขาวิชา	ทันตกรรมผู้สูงอายุและการดูแลผู้ป่วยพิเศษ	ลายมือชื่อนิสิต ....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

6378015832 : MAJOR GERIATRIC DENTISTRY AND SPECIAL PATIENTS CARE

KEYWORD: Dental fluorosis Proteome RNA Sequencing Saliva Transcriptome

Patcharaporn Gavila : Study of Saliva Transcriptome and Proteome in Thai Patients with Dental Fluorosis . Advisor: Assoc. Prof. THANTRIRA PORNTAVEETUS, D.D.S., M.Sc., Ph.D.

Objectives: The purpose of this study was to examine the expression profiles of RNA and protein in Thai individuals with dental fluorosis and compare them with those without fluorosis. Additionally, the study aimed to establish a correlation between the severity of dental fluorosis, urinary fluoride levels, and water fluoride levels.

Methods: Schoolchildren in areas endemic for fluoride in Lamphun and Ratchaburi provinces of Thailand were screened for dental fluorosis severity and then enrolled in the study. Fluoride concentration in 24-hour urine and drinking water samples was measured using an ion-selective fluoride electrode. Saliva samples were collected in sterile tubes and stored at -20°C for subsequent analysis. The saliva of dental and non-dental fluorosis groups were prepared for proteomic and transcriptomic analysis. Finally, urinary fluoride levels, water fluoride levels, RNA expression (transcriptome), and protein profile (proteome) were compared between individuals with and without dental fluorosis.

Result: Among 47 schoolchildren aged between 6-16 years, 27 were non-dental fluorosis, 10 were severe dental fluorosis and 10 were moderate dental fluorosis. The urine fluoride level in the severe group was significantly higher than that in the control group (p=0.007), but there was no statistically significant difference when compared to moderate groups (p=0.054). There was no statistically significant difference in water fluoride among the three groups (p=0.246). For transcriptomic analysis, there was no statistically significant difference in RNA profiles between the severe dental fluorosis and control groups. For proteomic analysis, 12 proteins were found to be differentially expressed. S100A9 was upregulated in both the severe and moderate groups when compared to the control group.

Field of Study:	Geriatric Dentistry and Special Patients Care	Student's Signature ....
Academic Year:	2022	Advisor's Signature ....

ACKNOWLEDGEMENTS

I would like to express my deep appreciation to my supervisor, Assoc. Prof. Dr. Thantrira Porntaveetus, for her unwavering support throughout my master's degree study and research. Her patience, motivation, and vast knowledge were invaluable in guiding me during the research and thesis writing process. I could not have asked for a better advisor and mentor.

I would also like to extend my gratitude to my thesis committee members: Assoc. Prof. Dr. Piyamas Sumrejkanchanakij and Assoc. Prof. Dr. Puangwan Laptanasupkun, for their encouragement and insightful comments.

My sincere thanks go to Dr. Kanokwan Sriwatthanapong for her hard work on the laboratory processes and for motivating me during the analytical processes. I could not have completed this research without her.

Finally, I would like to express my heartfelt gratitude to the schoolchildren and teachers in fluoride-endemic areas of Ratchaburi and Lamphun provinces for their participation and assistance. I am also grateful to the staff at the Intercountry Centre for Oral Health for their help and support.

Patcharaporn Gavila

TABLE OF CONTENTS

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF TABLES

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Background and rationale

Fluoride is found in water, food, and especially dental products. It is considered a double-edged sword. Fluoride provides a protection against dental caries when consuming in adequate quantity, but a chronic and excessive fluoride intake can cause dental and skeletal fluorosis. (1)

Dental fluorosis is a developmental disturbance of dental enamel, caused by chronic and excessive ingestion of fluoride during tooth development. (2) In Thailand, the national standard of fluoride level in drinking water is ≤ 0.7 mg/liter or not exceed 1 mg/liter during high water consumption in summer. Dental fluorosis is endemic among certain areas depending on geological background such as the northern and western parts of Thailand. The incidence of dental and skeletal fluorosis has been reported in Chiangmai, Lamphun and Phayao provinces due to high fluoride concentration in natural water sources. (3) It has been estimated that fluoride level in underground water at endemic area reaches 10 mg/L(ppm). (4)

The common source of fluoride exposure is drinking/cooking water derived from underground water. Fluoride in water cannot be removed by boiling or distillation. Reverse osmosis is the successful defluoridation process in community scale but it comes with high investment and maintenance cost. (5) After ingestion within minutes, fluoride is rapidly absorbed from the stomach to plasma. The peak plasma level usually occurs within the next 30-60 minutes, and then declines due to bone uptake and urinary excretion. Urinary fluoride is a widely accepted biomarker of recent fluoride exposure and has frequently been used as an indicator of fluoride exposure from drinking water recommended by WHO. (6)

The conflicting concepts occur in debates addressing whether fluorine is an essential element for humans. The researchers of the Panel of the European Food

Safety Authority (EFSA) state that fluoride has no known essential function in human physiology and development. On the other hand, the WHO and the Centers for Disease Control and Prevention (CDC) consider fluoride to be an important dietary element for humans because of the phrase "resistance to dental caries is a physiologically important function". (7)

In the last decades, transcriptomic and proteomic studies have been increasingly used to investigate gene and protein expression in various biological contexts, pathological changes, and human diseases. (8) (9) (10) To date, there is still lack of studies about saliva transcriptome and proteome in patients affected with dental fluorosis.

To enhance the understanding of underling molecular changes by which fluoride impacts oral environment, this study will perform the transcriptomic and proteomic analyses of dental fluorosis patients' saliva. We aim to provide new knowledge about alterations in genes, protein, and signaling pathways related to dental fluorosis, leading to a better understanding of fluoride effects in humans. In addition, the identified RNA and protein changes can be the potential biological markers which are applicable to detect hidden fluoride toxicity in general population.

Research questions:

1. What is the saliva RNA profile of dental fluorosis patients?
2. What are the saliva protein characteristics of dental fluorosis patients?
3. What are the differences between saliva RNA and protein of dental fluorosis patients and non-fluorosis individuals?
4. What are the relationships between urinary fluoride level, dental fluorosis severity, and water fluoride level?

Research objectives:

1. To investigate the RNA expression profile (transcriptome) in Thai patients with dental fluorosis and to compare it with non-fluorosis individuals.

2. To characterize saliva protein (proteome) in Thai patients with dental fluorosis and to compare it with non-fluorosis individuals.

3. To demonstrate the relationship between urinary fluoride level, dental fluorosis severity, and water fluoride level.

Research hypothesis

Hypothesis A

H_0: The gene expression profile of dental fluorosis patients is similar to that in non-fluorosis individuals.

H_A: The gene expression of dental fluorosis patients is different from that in non-fluorosis individuals

Hypothesis B

H_0: The saliva protein profile of dental fluorosis patients is similar to that in non-fluorosis individuals.

H_A: The saliva protein profile of dental fluorosis patients with is different from that in non-fluorosis individuals

Hypothesis C

H_0: There is no relationship between dental fluorosis severity, urinary fluoride level, and water fluoride level.

H_A: There is a relationship between dental fluorosis severity, urinary fluoride level, and water fluoride level.

Scope of Research

This study aims to characterize transcriptomic and proteomic profiles in saliva of patients with dental fluorosis living in Lamphun and Ratchaburi provinces. A group of 10 severe dental fluorosis patients, 10 moderate dental fluorosis and 27 non-fluorosis individuals, ages 6 to 18 years old, were recruited. Dental fluorosis was diagnosed and classified based on the Dean's classification system by investigators. Written informed consents were obtained from their legal guardians. Clinical investigation, interview and collection of saliva specimens were collected. Transcriptome profiling was analyzed by next-generation sequencing (NGS). Saliva proteome will be analyzed by mass spectrometric analysis (GeLC-MS/MS). Fluoride levels in urine and water were measured.

Expected Outcomes

1. Measurement of urinary fluoride in dental fluorosis patients demonstrates the relationship between dental fluorosis severity and urinary fluoride level.

2. Characterization of the gene expression in patients with dental fluorosis provides underling molecular mechanism by which fluoride impacts human health and development.

3. Characterization of the salivary proteomic profiles in patients with dental fluorosis provides the knowledge about the difference and specification of proteins in saliva. Identification of saliva biomarker could play an important role for detecting hidden fluoride toxicity and dental fluorosis prevention.

Keywords: Dental fluorosis, Proteome, RNA Sequencing, Saliva, Transcriptome

Type of research: Experimental research

Conceptual Framework
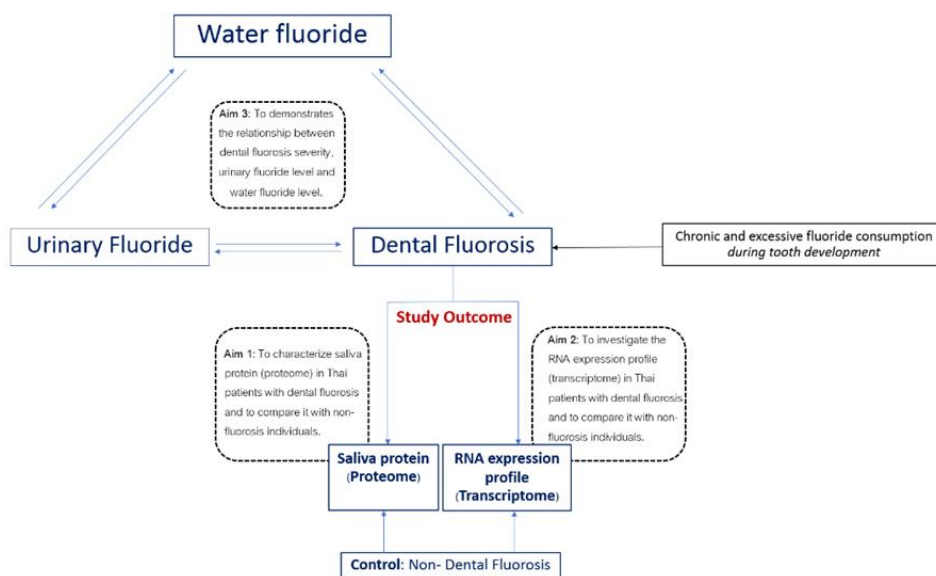
CHAPTER II

LITERATURE REVIEW

Natural Fluoride

Fluorine is a common element which accounts for about 0.3 g/kg of the Earth's crust and does not occur in the elemental state in nature because of its high reactivity. The ionic form of fluorine is fluoride which is found at significant levels in a wide variety of minerals, including fluorspar, rock phosphate, cryolite, apatite, mica, hornblende and others. (11) Fluoride is a naturally occurring mineral found in water in varying amounts and also present in some food. (12) Natural water source like lake, river, and underground water contains fluoride substance in difference levels, most of them vary under 0.5 mg/L. (13)

The distribution of fluoride in the environment is uneven and largely is derived from geogenic causes. (1) High fluoride concentrations can be found in many parts of the world, particularly in certain parts of India, China, Central Africa, and South America. The exposure to fluoride can vary markedly from one region to another. This depends on the concentration of fluoride in drinking-water and foods, drinking volume, and use of fluoridated dental preparations. In addition, fluoride exposure in some areas is considerably higher as a consequence of various factors including brick tea consumption and cooking and drying methods of food with high-fluoride coal or water. (14)

It is well known that fluoride provides protection against dental caries, both in children and in adults when consuming in adequate quantity. (11) The protective effects of fluoride increase with concentration up to about 2 mg of fluoride per liter of drinking-water. The minimum concentration of fluoride in drinking-water required to benefit the teeth is approximately 0.5 mg/l. (14) An excessive intake of fluoride at high concentration (> 1.5 mg/L) can lead to dental and skeletal fluorosis. The ingestion of fluoride during the pre-eruptive development of the teeth has a cariostatic effect due to the uptake of fluoride by enamel crystallites and formation

of fluorhydroxyapatite, which is less acid soluble than hydroxyapatite. Fluoride in the oral fluids, including saliva and dental plaque, also contributes to the cariostatic effect. This posteruptive effect is due mainly to reduced acid production by plaque bacteria and to an increased rate of enamel remineralization during an acidogenic challenge. (15)

Incidence of Fluorosis and Endemic Area

More than 500 million people live in endemic fluorosis area with excess fluoride in water source and biosphere resulting in public health problem. (7) The countries with endemic fluorosis descripted as a geological of fluoride belt covering the Syrian Arab Republic, Jordan, Egypt, Libyan Arab Jamahiriya, Algeria, Morocco, and Rift Valley. Another fluoride belt is the one stretching from Turkey through Iraq, Islamic Republic of Iran, Afghanistan, India, China, to the Northern Thailand. Similar areas can be found in America, China, and Japan. (16)

Daily exposure to fluoride depends mainly on the geographical area. In most circumstances, food seems to be the primary source of fluoride intake, with lesser contributions from drinking-water and from toothpaste. In areas with relatively high concentrations, particularly in groundwater, drinking water becomes increasingly important as a source of fluoride. Intakes in areas where high fluoride coal is used indoors may also be significant. (17)

In Thailand, dental and skeletal fluorosis situation has been reported in the Northern and Western parts of Thailand, especially in Chiangmai, Lamphun and Ratchaburi provinces due to the common practice of consuming water for drinking and cooking from underground water wells. (3)

Guideline in Drinking Water

Drinking water is typically the largest source of daily fluoride intake. (18) The World Health Organization 2006 "Guidelines for Drinking-Water Quality" set the guideline value for maximum concentration of fluoride in natural water at 1.5

mg/liter to prevent the incidence of dental fluorosis. This value is higher than the recommendation for artificial fluoridation of water supplies, which is usually 0.5–1.0 mg/liter. For individual national standards, the maximum fluoride concentration should be considered along with climatic conditions, volume of water intake and intake of fluoride from other sources. (17)

In Thailand, the national standard of fluoride level in drinking water is $\leq$ 0.7 mg/liter and not exceed the maximum level of 1 mg/liter due to warm climate region which lead to high consumption of drinking-water. (19)

Dental Fluorosis

Dental fluorosis is a developmental disturbance of dental enamel, caused by chronic and excessive ingestion of fluoride during tooth development. (2) The severity of dental fluorosis depends on the amount and timing of fluoride ingestion. (3) Dental fluorosis is characterized by hypomineralization of tooth enamel, seen as diffuse, symmetrical, discolored white opaque stains and striations. While enamel surface lesions, such as pitting, porosity, and brownish areas are occur in the more severe forms of fluorosis. (20) The mild form of dental fluorosis may not alter the general function of teeth but was considered as specific esthetic disturbance which may have psychosocial effects on many patients and impact their quality of life. While the severe form of dental fluorosis can increase the risk to attrition, erosion and dental caries due to the loss of integrity of enamel surface. (21)

Pathogenesis of Dental Fluorosis

Enamel development encompasses several stages, including the presecretory, secretory, transition, and maturation stages. In the presecretory stage, the ameloblasts differentiate and acquire their specific characteristics in preparation for the secretion of the organic matrix of enamel. During the secretory stage, the primary proteins secreted by the ameloblasts are amelogenins, with other proteins such as tuftelin, enamelin, ameloblastin, and metalloproteinases being secreted in smaller

quantities. As the ameloblasts progress into the transition stage, they undergo a shortening process and start releasing proteinases, which trigger the rapid degradation of amelogenin proteins. During the early-maturation stage, a highly porous matrix is formed, which is then overlaid with maturation-stage ameloblasts. The maturation stage is characterized by alternating cycles of ruffle-bordered and smooth-bordered ameloblasts. These ameloblasts function to remove the remaining amelogenins from the maturing enamel matrix and facilitate the final mineralization of the enamel. (22)

An elevated intake of fluoride during tooth development can lead to several changes which are related to cell/matrix/mineral interactions. In fluorosed enamel, there is a dose-related increase in amelogenin protein content during the early maturation stage. The delayed removal of amelogenins during the maturation stage of enamel development hinders the growth of enamel crystals. This hindrance in crystal growth is one of the key factors contributing to the development of fluorosis, as the impaired mineralization results in enamel with disrupted crystal structure and reduced hardness. (23)

The severity of fluorosis is influenced by factors such as the timing, duration, and exposure dose to fluoride. The transition and early-maturation stages of enamel formation are particularly vulnerable to the effects of fluoride. (24) During these stages, excessive fluoride exposure can disrupt the mineralization process and lead to the development of fluorotic lesions. Studies, such as the one conducted by Ishii & Suckling in 1991, have shown that the timing of fluoride exposure is crucial in the development of enamel fluorosis. Children exposed to high levels of fluoride during the maturation stage of tooth formation are more likely to exhibit moderate to severe fluorosis, while those primarily in the secretory stage tend to have milder or no fluorosis. (25) While the impact of fluoride on enamel maturation is crucial, its adverse effects on other stages of enamel development cannot be overlooked. These effects can be cumulative, especially when considering the severity of

fluorosis, which is often associated with long-term and continuous exposure to fluoride. (23)

In recent years, research on the pathogenesis of dental fluorosis has primarily focused on the interference of fluoride with the signaling pathway of ameloblasts. (26) Studies have shown that fluoride interferes the production and function of several molecules related to ameloblasts including glucose-regulated protein78, (27) inositol-requiring kinase 1α, and transcription factor 6 pathway in unfolded protein reactions. (28) Additionally, fluoride has been shown to induce the production of oxidative stress and reactive oxygen species, leading to apoptosis of the ameloblasts. (29, 30) These disturbances can result in the development of dental fluorosis.

There is a heterogeneity in clinical features of dental fluorosis between individuals, which can influenced by different genetic backgrounds. For instance, the occurrence of coal-burning type fluorosis has been linked to the Alu I polymorphism in the *CTR* gene. (29) In addition, investigations conducted in communities with elevated levels of fluoride in their drinking water have found an association between dental fluorosis and the polymorphism in the *COL1A2* gene. (30) However, this polymorphism has not been found to be correlated with the severity of dental fluorosis. (31)   Another study indicated that dental fluorosis is connected to polymorphisms in amelogenesis-related genes, including *AMBN, TFIP11,* and *TUFT1.* (32)

Dean Fluorosis Index

Several diagnostic scoring systems have been used to assess the severity of dental fluorosis by evaluating the tooth clinical appearance. (33) The most commonly used indices are the Dean, Thylsrup and Fejerskov Fluorosis Index (TFI) and Tooth Surface Index of Fluorosis (TSIF). (34) However, the most longest standing indices is The Dean or Dean's Fluorosis Index which was first created by Henry

Trendley Dean in 1934 and followed by its subsequent modifications. Dean's fluorosis index and diagnostic criteria are shown in table 1. (33)

Table 1 Criteria for Dean's fluorosis index for dental fluorosis

Classification	Score	Criteria
Normal	0	The enamel represents the usual translucent semivitriform (glass-like) type of structure. The surface is smooth, glossy, and usually of a pale, creamy white color.
Questionable	0.5	The enamel discloses slight aberrations from the translucency of normal enamel, ranging from a few white flakes to occasional white spots. This classification is used in those instances where a definite diagnosis of the mildest form of fluorosis is not warranted and classification of "normal" not justified.
Very mild	1	Small, opaque, paper white areas scattered irregularly over the tooth, but not involving as much as approximately 25 percent of the tooth surface. Frequently included in this classification are teeth showing no more than about 1-2 mm of white opacity at the tip of the summit of the cusps of the bicuspids or second molars.
Mild	2	The white opaque areas in the enamel of the teeth are more extensive, but do not involve as much as 50 percent of the tooth.
Moderate	3	All enamel surfaces of the teeth are affected, and surfaces subject to attrition show wear. Brown stain is frequently a disfiguring feature.
Severe	4	All enamel surfaces are affected, and hypoplasia is so marked that the general form of the tooth may be affected. The major diagnostic sign of this classification is discrete or confluent pitting. Brown stains are widespread, and teeth often present a corroded-like appearance.

Dean's fluorosis index comprises 6 categories based on tooth clinical appearance. The normal teeth are given a score of 0. The score gradually increases by severity to a score of 4 which stands for the most severe type of fluorosis. The score is based on the second most severely affected teeth, making it less reliable than the more conservative yet time consuming approach of scoring every single tooth in oral cavity. By the way this index is often used by the Word Health Organization and remains as the gold standard index in the public health armamentarium. (35)

Metabolism of Fluoride

Fluoride metabolism in humans can be divided into 3 steps: absorption, distribution, and excretion.

1. **Absorption**

After intake, 80% to 90% of ingested fluoride is absorbed in gastrointestinal tract by passive diffusion with a half-life of approximately 30 minutes. Up to 40% of ingested fluoride may be absorbed in the stomach, and the amount of absorption is inversely related to the pH of the stomach contents. (36) High concentration of dietary calcium and other cations can decrease fluoride absorption from gastrointestinal tract. (15) Unabsorbed fluoride in the stomach is then absorbed from the upper small intestine, which has a huge capacity for absorption and independent from pH factor. (36) Within minutes, fluoride can be detected in plasma, which means that fluoride is readily absorbed from the stomach. The peak plasma level usually occurs within 30-60 minutes and reverts to the original level within 3–6 hours.

(6) The absorption across the oral mucosa is limited and probably accounts for less than 1% of the daily intake. (15)

2. Distribution

Fluoride is distributed in plasma, extracellular fluid, and intracellular fluid. Intracellular fluoride concentrations are low, but they change proportionally and simultaneously with those in plasma. There are two general forms of fluoride in human plasma. The ionic form, detectable by the ion-specific electrode, is one of interest in dentistry, medicine and public health. Ionic fluoride is not bound to proteins, other compartments of plasma, or soft tissues. The concentration of ionic fluoride in soft and hard tissues is directly related to the amount of ionic fluoride intake. The levels start declining thereafter due to two main reasons: uptake in calcified tissues and excretion in urine. Plasma fluoride levels are not homeostatically regulated and vary according to the levels of intake, deposition in hard tissues, and excretion of fluoride. Many factors can modify the metabolism and effects of fluoride in the organism, such as chronic and acute acid-base disturbances, hematocrit, altitude, physical activity, circadian rhythm and hormones, nutritional status, diet, and genetic predisposition. (37)

3. Excretion

The elimination of absorbed fluoride occurs almost exclusively via the kidneys. The renal handling of fluoride is characterized by unrestricted filtration through the glomeruli followed by a variable degree of tubular reabsorption. The amount of reabsorption is inversely related to tubular fluid pH. The renal clearance of fluoride in adults is about 30 to 40 ml/minute. The rate of fluoride removal from plasma in healthy adults is approximately 75 ml/minute and is virtually equal to the sum of the renal and calcified tissues clearances.

The fractional retention or balance of fluoride at any age depends on the quantitative features of absorption and excretion. For healthy, young, or middle-aged adults, approximately 50 percent of absorbed fluoride is retained by uptake in calcified tissues, and 50 percent is excreted in the urine. For young children, as much

as 80 percent can be retained owing to increased uptake by the developing skeleton and teeth. (15)

Urinary fluoride as biomarker of fluoride exposure

Total fluoride exposure of individuals or populations can be monitored by assessing fluoride concentration in biological liquids which is indicative of the level of total fluoride exposure. Urinary fluoride is one of contemporary (present/ very recent) biomarker for fluoride exposure among blood, bone surface, saliva, milk and sweat. It was widely accepted biomarker and has frequently been used as an indicator of fluoride exposure from drinking water which recommended by WHO. (6) The main advantage of using urinary fluoride excretion to estimate total fluoride exposure is that urine collections are non-invasive and more suitable than other methods for monitoring community fluoridation schemes. Most importantly, studies of urinary fluoride provide a simple and reliable means of ensuring that total fluoride intake from all sources. (38)

There are studies shown that dental fluorosis and fluoride content of enamel, plaque, saliva, urine, nails and hair are directly related to fluoride levels of drinking water and dietary fluoride intake. (39) (40) (41)

Genetic Influence the Dental Fluorosis

There are many genetic epidemiological studies shown the evidence for association between genetic polymorphisms in the susceptibility pattern of different types of fluorosis among individuals living in the same community and having the same environmental exposure. A number of studies have been explored the genes related to bone formation and development like *BGLAP* (Osteocalcin), *ESR* (Estrogen Receptor), *COL1A2* (Collagen type 1 alpha 2); genes encoding receptors that are involved in bone formation and metabolism like *VDR* (Vitamin D Receptor), *CTR* (Calcitonin Receptor); genes encoding hormones like *PTH* (Parathyroid Hormone), *PRL* (Prolactin); genes encoding detoxifying enzymes like *GSTP1* (Glutathione S-transferase

pi 1); genes encoding extracellular matrix proteins like *MMP* (Matrix Metallopeptidase); genes having roles in cognitive functions like *COMT* (Catechol-o-methyltransferase); and genes having roles in immune functions like *MPO* (Myeloperoxidase). However, only *COL1A2, CTR*, and *ESR* genes have a positive association to increase risk of dental fluorosis. (42)

RNA Sequencing

The human transcriptome comprises more than 80,000 coding transcripts and the estimated number of proteins synthesized from these transcripts is in the range of 250,000 to 1 million. These transcripts and proteins are encoded by less than 20,000 genes, suggesting extensive regulation at the transcriptional, post-transcriptional, and translational level. (43)

RNA sequencing (RNA-Seq) is a high-throughput sequencing method that provides an insight into the transcriptome of a cell/tissue/environment. This method can analyze the transcriptome by recording frequencies and alterations of transcripts in test samples and provide the understanding of basic molecular mechanisms of basic biology and pathogenesis of human diseases. Beyond quantifying gene expression, the data generated by RNA-Seq facilitates the discovery of novel transcripts, identification of alternatively spliced genes, and detection of allele-specific expression. (44)

Saliva Proteomic

Human saliva contains numerous proteins and peptides, each of them carries several significant biological functions. These proteins are not only important in maintaining the health of the oral cavity but also may can be informative for disease detection and surveillance of oral health. (45) Human saliva proteome (HSP) analysis is a comprehensive identification and quantification of the total proteins and their posttranslational modifications (PTMs) in human saliva, including their stratified origins from glandular/ductal secretions. Comprehensive analysis and identification of

the proteomic content in human whole and ductal saliva is a necessary first step toward the discovery of saliva protein markers for human disease detection. (46)

CHAPTER III

RESEARCH METHODOLOGY

Ethics Approval

The protocol for this study was approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. (Study Code HREC-DCU 2021-061, approved on 1 October 2021)

Sample size Calculation

The sample size was calculated by using the n4Studies program version 1.4.1 for 80% power and 95% confidence interval level according to the transcriptomic and proteomic study. (47) (48) (49) (50) The final estimated total sample size was 45. To compensate for error or loss of subject, a total sample size of 47 subjects were recruited.

Screening and Selection of Subjects

Participant screenings were held in fluoride endemic area of Thailand located in Lamphun and Ratchaburi provinces according to their high prevalence of dental fluorosis and low population mobility.

The inclusion criteria of case group were Thai population with severe dental fluorosis (Dean fluorosis score 4) or moderate dental fluorosis (Dean fluorosis score 3). For control group were Thai population without dental fluorosis (Dean fluorosis score 0). DFI and diagnostic criteria are shown in Table 1. A consensus score for DFI was given for each subject by four examiners (P.G., T.P., P.A and a pediatric dentist). The final score for DFI was recorded directly by the interface excel file. The exclusion criteria of subjects in this study were clinical dental caries, systemic condition, amelogenesis imperfecta, history of antibiotic, antifungal, antiviral or steroid intake or use of mouthwash in 3 months prior to sample, presence of periodontitis and mucosal lesions, smoking, drinking alcohol, and using narcotic drugs.

History taking and questionnaire.

Demographic data was collected after an informed consent form was signed. The questionnaire was completed by each participant's parent. The first section of the questionnaire collected the information about general information including age, gender, and period of staying in the area. In the second section, participants were queried about their own and their family members' medical history.

Collection and fluoride determination in water samples

The 24-hr urine samples were collected from each participant in a 2.7 liters urine container. On the morning of the collection, the subject voided the first urine (which was not collected) and recorded the time of this first (discarded) void. The subject urinated into the wide-necked collection container, and then the urine was poured into the screw-capped collection bottle using the funnel. During transportation of urine, the collection bottle was kept at 4°C. The amount of fluoride was evaluated using a fluoride electrode and TISAB III at Intercountry Centre for Oral Health, Chiangmai.

Collection and fluoride determination in urine samples

The 24-hr urine samples were collected from each participant in a 2.7 liters urine container. On the morning of the collection, the subject voided the first urine (this urine was not collected) and recorded the time of this first (discarded) void. The subject urinated into the wide-necked collection container, then the urine was poured into the screw-capped collection bottle, using the funnel. If possible, this collection bottle was kept in a refrigerator, transferred to the lab. The amount of fluoride will be evaluated by Fluoride electrode and TISAB III at Intercountry Centre for Oral Health, Chiangmai.

Saliva samples collection

Unstimulated saliva samples were obtained from eligible participants by investigator. The collection was taken between 8 AM to 10 AM, 12 hours after the last meal. The participants were instructed to rinse their mouth with 10 ml of distilled water for 30 seconds to remove debris and moisturize the mucosa. The saliva samples approximately 3 mL were collected in sterile tubes and stored at -20°C immediately.

RNA preparation and sequencing

Saliva of age- and sex- matched participants in severe fluorosis group and control group were selected for RNA sequencing. RNA extraction was performed using GeneAll RiboEx^{TM} Reagent (GeneAll Biotechnology Co., Ltd, Korea) in combination with the Rneasy mini kit (QIAGEN, Hilden, Germany). Briefly, 1 mL of saliva was centrifuged at 14,000 x g for 20 minutes at 4°C. The cell pellets were added with 1mL of RiboEx^{TM} Reagent. After incubating at room temperature for 5 minutes, 200μL of chloroform was added and the samples were centrifuged at 12,000x g for 15 minutes at 4°C to separate the upper aqueous phase. The aqueous phase was then transferred to a new sterile microcentrifuge tube and mixed with an equal volume of 70% ethanol. The resulting solution was loaded onto a Rneasy spin column and centrifuged at 12,000 x g for 30 seconds, following the manufacturer's instructions. Total RNA integrity was checked by a Bioanalyzer 2100 (Agilent, USA). RNA Integrity Number (RIN) value greater than 7 was used. RNA Sequencing was performed by Illumina NovaSeq6000 platform (SMARTer stranded RNA library (Ribo-Zero) kit) with 150 bp paired-end run mode and 60 million reads per sample (Macrogen Inc., Korea).

RNA sequencing analysis

The raw data (fastq) was aligned with the Homo sapiens reference genome (UCSC hg 19) by RNA-Seq Alignment program. Differential expressions were evaluated

via RNA-Seq Differential Expression (Illumina Inc., USA). The non-fluorosis saliva was used as a control group and the fluorosis saliva as a comparison group. Genes were considered true significance when p-value ≤ 0.05 with a false discovery rate (FDR) ≤ 0.05. The gene with true significance and log2 fold change (log2FC) ≤ -1 or ≥ 1 of fluorosis and control were selected and compared.

Quantitative real-time PCR.

To confirm RNA sequencing data, quantitative real-time PCR was performed. The RNA samples were converted to cDNA using a iScript Reverse Transcription Supermix (Bio-rad, USA). The mRNA level expression was determined using SYBR green detection system (FastStart Essential DNA Green Master; Roche Diagnostic, USA). *GAPDH* was used as an endogenous control. The expression levels were normalized to endogenous control, and subsequently calculated using the 2-ΔΔCq method.

Protein extraction and in-solution digestion.

Saliva samples were centrifuged at 14,000 x g for 20 minutes at 4°C. The proteins from 1 mL of supernatants were precipitated with 100μL of 100% trichloroacetic acid (TCA), for 30 minutes on ice, centrifuged at 20,000 x g for 20 minutes. Supernatants were discarded and the pellet washed with 500 μL of acetone, and then vortex and centrifuged at 20,000 x g for 10 minutes. The washing procedure was repeated two times. After that, samples were resuspended with 300 μL of 8M urea in 100mM triethylammonium bicarbonate (TEAB) (Thermo Fisher Scientific, USA) with Halt protease inhibitor cocktail (Thermo Fisher Scientific, USA).

Protein samples (100 μL) were added with 10 μL of dithiothreitol (DTT) and incubated at 37°C at for 30 minutes. Then the samples were alkylated with 10 μL of 100mM of iodoacetamide (IA) in a dark room for 30 min at room temperature, mixed with 40 μL of 100 mM DTT, and incubated at room temperature for at least 15

minutes. The samples were diluted with 100mM of TEAB to reduce the concentration of urea from 8M to less than 1M. The protein samples were incubated with trypsin at a ratio of 1:50 (w/w) at 37 °C for 16 hours. Before desaling, the samples were added with 100% TFA to final concentration of 0.5% TFA, incubated for 15 minutes at room temperature, then centrifuged at 15,000 x g for 10 minutes at 4℃. The quantity of tryptic peptides was measured with the Pierce Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific, USA). The peptide samples were stored at −80℃.

In-solution dimethyl labeling and fractionation.

The digested samples were reconstituted in 100 mM TEAB. The peptide samples were labeled with formaldehyde isotope including light reagents (formaldehyde and cyanoborohydride), medium reagents (formaldehyde-d2 and cyanoborohydride), and heavy reagents (deuterated and 13C-labeled formaldehyde and cyanoborodeuteride), at room temperature for an hour. Each isotope labeled sample was quenched by adding 1% (v/v) ammonia solution (25%) and formic acid. The labeled peptide samples were mixed. Then, peptides were labeled with 130 and 131 Tandem Mass Tag (TMT) reagents (Thermo Fisher Scientific, CA, USA). The pooled peptide was separated into 8 fractions using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, USA). To dry the eluted sample, the samples were evaporated using vacuum centrifugation. Dry samples were resuspended in formic acid before LC–MS/MS analysis.

LC-MS/MS and analysis

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of samples was performed on an EASY-nLC1000 system (Thermo Fisher Scientific, USA) connected to a Q-Exactive Orbitrap Plus mass spectrometer (Thermo Fisher Scientific, USA) equipped with a nanoelectrospray ion source (Thermo Fisher Scientific, San Jose, CA, United States). The raw files were processed by the Proteome Discoverer™ Software 2.1 (Thermo Fisher Scientific, USA) and searched against the human UniProt

database (August 2019). The potential contaminant was removed. The reporter ion intensity ratio of severe, moderate, and control was transformed to log2. The *p*-values of filtered protein were calculated with ANOVA based on at least three valid log2 in each group. Pair-wise comparison was used to show the significance of the differential expressed protein among 3 groups. The LSD pos-hoc test was used for the homogeneity variance protein and Game-Howell post-hoc test was used for non-homogeneity variance protein. By ratio-fold change >1.5 and *p*-value < 0.05, the relevant proteins were considered statistically different.

Bioinformatics

The selected RNA and proteins were subjected to the online resource database DAVID Bioinformatics Resources (https://david.ncifcrf.gov/) to investigate the gene ontology enriched in biological processes, cellular compartment, and molecular function. The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database was used to investigate protein-protein interactions.

Statistical Analysis

The demographic data and fluoride concentration in water and urine were summarized using descriptive statistics. The normal distribution for each variable was determined by Shapiro-Wilk test. The differences of sex, location of living, duration of living, and parent history were compared using Chi-square test. Group differences of age, water fluoride, and urine fluoride among three groups were compared using the one-way ANOVA followed by LSD post hoc test (normally distributed variables) or the Kruskal-Wallis test (not normally distribution). The data were analyzed using SPSS software version 22 (SPSS Inc. Chicago, IL, USA). The *p*-value < 0.05 was considered significant.

CHAPTER IV

RESULTS

In this study, 55 subjects were approached to participate from 3 schools located in an endemic area. However, 8 subjects either did not provide consent to participate or failed to collect samples, resulting in 47 participants being included. The oral examination and sample collection were carried out between December 2021 and January 2022.

Part 1: Fluoride Level in Urine and Drinking Water

The children were divided into three groups based on their Dean fluorosis score. The control group (n=27) had a score of 0 and included 40.74% male and 59.26% female participants. The moderate group (n=10) had a score of 3 and included 10% male and 90% female participants. The severe group (n=10) had a score of 4 included 50% male and 50% female participants. There was no statistically significant difference in sex distribution among the three groups (p=0.133). The mean age of the severe group was 10.40 ± 2.36 years, while it was 11.33 ± 1.00 in the moderate group and 10.70 ± 2.77 in the control group. There was no statistically significant difference in age among the three groups (p=0.559). Table 2 provides the general characteristics of the study population.

Table 2 General Characteristics of Study Population

Characteristics	Severe fluorosis (n=10)	Moderate fluorosis (n=10)	Control (n=27)	*p*-value
Age (years)				0.559[a]
○ Mean ± SD	10.40 ± 2.36	11.33 ± 1.00	10.70 ± 2.77	
○ Median	11.5	11	11	
○ Range	6-13	11-14	7-16	
Sex, n (%)				0.133[b]
○ Male	5 (50%)	1 (10%)	11 (40.7%)	
○ Female	5 (50%)	9 (90%)	16 (59.3%)	

Characteristics	Severe fluorosi (n=10)	Moderate fluorosis (n=10)	Control (n=27)	*p*-value
Urine Fluoride (ppm)				**0.024**[a]
○ Mean ± SD	5.44 ± 3.39^A	2.79 ± 1.01^{A,B}	2.49 ± 1.80^B	
○ Median	4.6	3.19	1.86	
○ Minimum	0.7	1.24	0.50	
○ Maximum	11.4	4.01	6.78	
Water Fluoride (ppm)				0.246[a]
○ Mean ± SD	1.93 ± 2.40	0.16 ± 0.10	0.97 ± 1.82	
○ Median	0.20	0.20	0.20	
○ Minimum	0.17	<0.10	<0.10	
○ Maximum	5.9	0.3	6.1	
Location, n (%)				**0.021**[b]
○ Lamphun	2 (20%)	5 (50%)	19 (70.4%)	
○ Ratchaburi	8 (80%)	5 (50%)	8 (29.6%)	
Parent's Duration of Living, n (%)				0.584[b]
○ 1-5 years	2 (20%)	2 (20%)	5 (19%)	
○ 5-10 years	1 (20%)	4 (40%)	10 (33.3%)	
○ 10-20 years	2 (20%)	0	4 (14.3%)	
○ More than 20 years	5 (50%)	4 (40%)	8(33.3%)	
Parent History, n (%)				**<0.001**[b]
○ None	3 (30%)	6 (60%)	22 (81.5%)	Reference
○ Bone Fracture	0	0	0	-
○ Joint Pain	0	0	5 (18.5%)	0.380[c]
○ Tooth Discolor	7 (70%)	4 (40%)	0	<0.001[c]

[a] Difference among groups, analyzed by Kruskal-Wallis test. Different superscript capital letter indicated difference between groups, analyzed by Dunn's post-hoc test with Bonferroni multiple testing correction.

[b] Difference among groups, analyzed by Pearson's Chi-square test.

[c] Difference between groups comparing with the reference group, analyzed by Pearson's Chi-square test.

A significant difference at *p*-value < 0.05 indicated in bold.

Abbreviation: SD, standard deviation.

The urine fluoride levels were measured in the severe, moderate, and control groups, with mean values of 5.54 ± 3.39 ppm (range 0.7 to 11.4 ppm), 2.79 ± 1.01 ppm (range 1.24 to 4.01 ppm), and 2.49 ± 1.80 ppm (range 0.50 to 6.78 ppm), respectively. Kruskal-Wallis test analysis revealed a statistically significant difference among the three groups (p=0.024). Pairwise comparison showed that the urine fluoride level in the severe group was significantly higher than that in the control group (p=0.007), but there was no statistically significant difference between the severe and moderate groups (p=0.054). There was also no statistically significant difference between the moderate and control groups (p=0.700) (Figure 1). The detailed results are shown in Table 3.
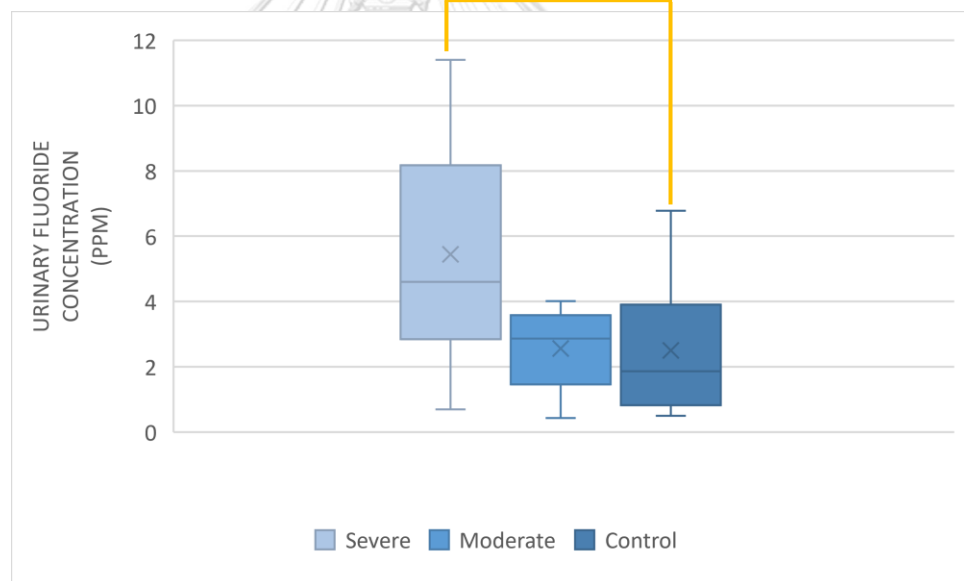
*Statistically significant difference ($p<0.05$), analyzed by Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni multiple testing correction.

Figure 1 Urinary fluoride concentration (ppm) in the severe, moderate and control groups

The mean water fluoride levels in the severe group, moderate group, and control group were 1.93 ± 2.40 ppm (range: 0.17 to 5.9 ppm), 0.16 ± 0.10 ppm (range: <0.1 to 0.3 ppm), and 0.97 ± 1.82 ppm (range: 0.1 to 6.1 ppm), respectively. However, the Kruskal-Wallis test indicated that there was no statistically significant difference among the three groups (p=0.246) (Figure 2).
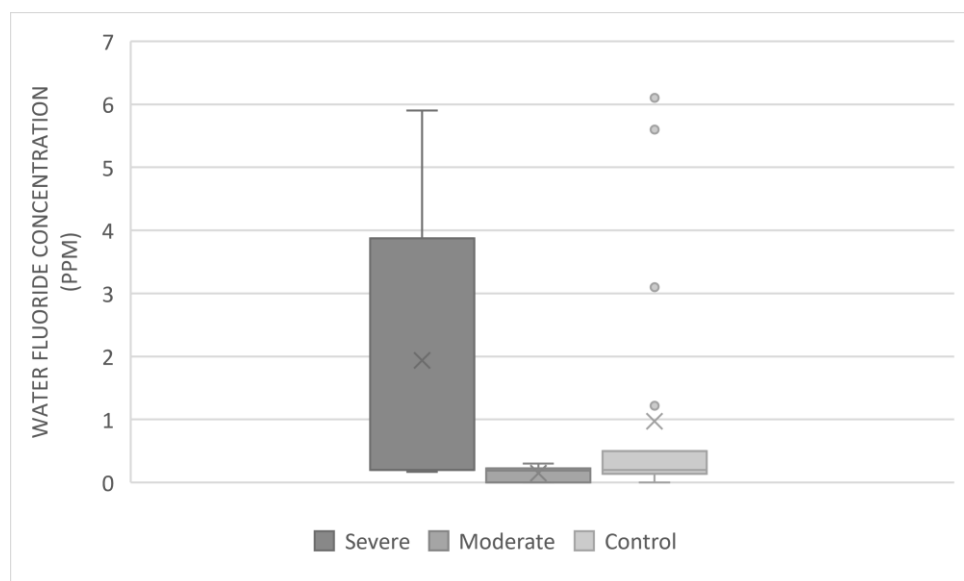
Figure 2 Water fluoride concentration (ppm) in severe, moderate and control groups

The location of residence was found to have a statistically significant difference in proportion among the three groups (p=0.021). The majority of participants in the control group (70.4%) were from Lamphun province, while most of those in the severe group (80%) were from Ratchaburi province. Participants in the moderate group were equally represented from both provinces.

The proportion of parent's duration of living in the endemic area among the three groups revealed no statistically significant difference (p=0.584). However, there was a statistically significant difference in the proportion of parent history among the three groups (p<0.001). Parents of participants in the severe (70%) and moderate

(30%) groups reported tooth discoloration significantly more frequently than those in the control group (0%) (*p*<0.001). The proportion of parent history with joint pain did not differ significantly among the groups (*p*=0.380). Additionally, there were no reports of parent history with bone fracture in these three groups.

Part 2: Saliva Transcriptomic Profile

Age- and sex-matched participants were recruited for RNA preparation and sequencing, divided into two groups: severe (n = 7) and control (n = 7). A total of 27,914 annotated genes were counted, with 7,532 genes assessed for differential expression between the two groups. The sample correlation matrix and the principal component analysis (PCA) plot between the severe dental fluorosis group and control group did not show a segregation between two groups (Figure 3 and Figure 4).
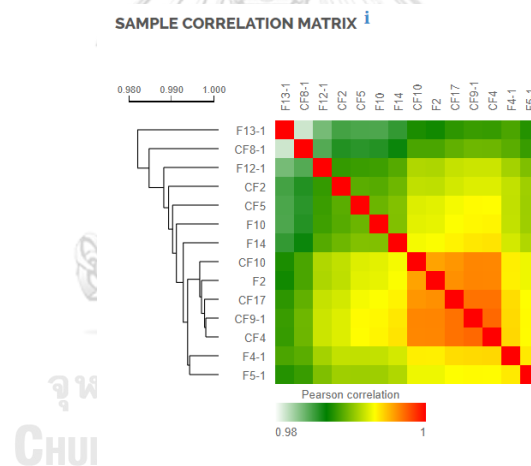
Figure 3 Sample correlation matrix between severe dental fluorosis and control group
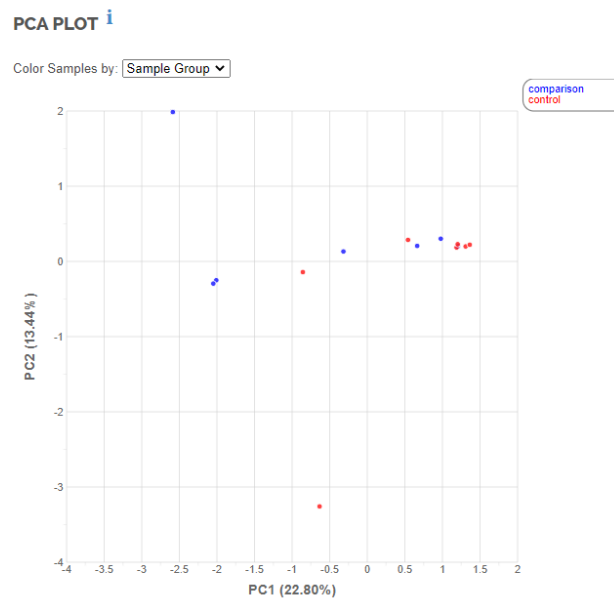
Figure 4 The PCA plot between severe dental fluorosis and control groups

Part 3: Saliva Proteomic Profile

A total of 166 proteins were identified and quantified, with an average of 6 peptides per protein and an FDR < 5% for all proteins. The coefficient of variance (CV) was less than 0.3 across all samples and replicates. Potential contaminants were filtered out, resulting in 161 proteins (Table S1) that were grouped into severe, moderate, and control groups. These 161 proteins were further filtered to include only those with at least three valid values in each group, resulting in the identification of 95 proteins. The significance of the expressed proteins was determined using a one-way ANOVA test among the three groups, followed by pairwise comparison testing. Using a ratio-fold change > 1.5 and p-value < 0.05, 11 proteins were found to be differentially expressed by Dunn's post-hoc test with Bonferroni multiple testing correction (Figure 5).
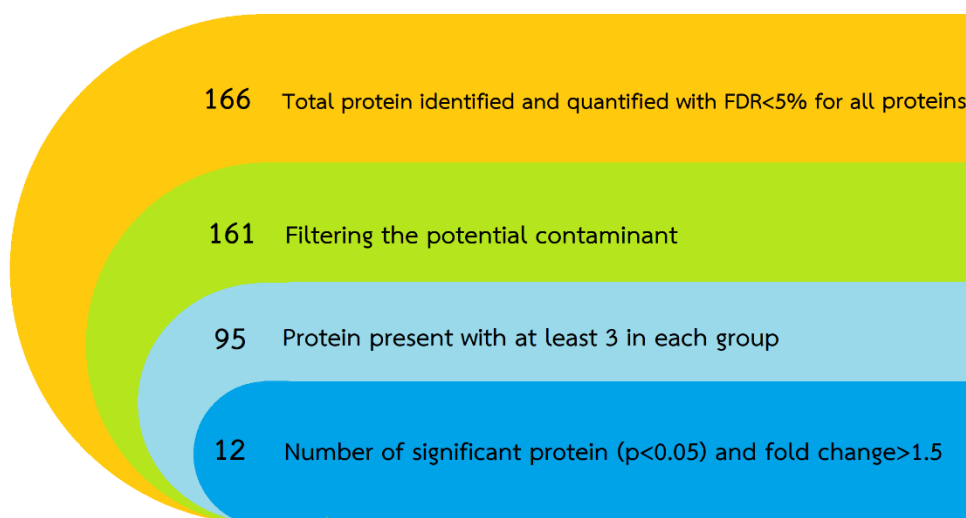
Figure 5 Diagram showing the filtration step applied to the total number of protein

Among the 12 proteins, the expression of 4 out of 12 proteins including 1) Isoform 2 of Transketolase (TKT), 2) Protein S100-A9 (S100A9), 3) Plastin-2 (LCP1), and 4) Histone H4 (HIST1H4A) was significantly increased in the severe group compared to the control group. Comparing the moderate group to the control group, 1 out of 12 proteins, S100-A9 (S100A9), was significantly increased while 4 out of 12 including, 1) Desmocollin-2 (DSC2), 2) Thymidine phosphorylase (TYMP), 3) Neutrophil gelatinase-associated lipocalin (LCN2), and 4) Superoxide dismutase (SOD1) were significantly decreased.

Comparing the severe group to the moderate fluorosis group, 10 out of 12 proteins were significantly different. The severe fluorosis group showed a significant increase in 7 proteins, including, 1) Desmocollin-2 (DSC2), 2) Glyceraldehyde-3-phosphate dehydrogenase (GADPH), 3) Thymidine phosphorylase (TYMP), 4) Plastin-2 (LCP1), 5) Neutrophil gelatinase-associated lipocalin (LCN2), 6) Superoxide dismutase (SOD1) and 7) Histone H4 (HIST1H4A) and a significant decrease in 3 proteins including, 1) Immunoglobulin kappa constant (IGKC), 2) Protein LEG1 homolog (LEG) and 3) Isoform 2 of Fructose-bisphosphate aldolase A (ALDOA). (Table 3 and Figure 6)

Table 3 List of 12 proteins that are differentially expressed among 3 groups.

No.	Protein			Severe versus Control			Moderate versus Control			Severe versus Moderate		
	Description	Symbol	Entry	Change	p-value	Log2 fold-change	Change	p-value	Log2 fold-change	Change	p-value	Log2 fold-change
1	Immunoglobulin kappa constant	IGKC	P01834	DOWN	0.047	-0.23920102084	UP	0.000191	0.46497221825	DOWN*	0.000008	-0.70417323909
2	Protein LEG1 homolog	LEG1	Q6PSS2	DOWN	0.1	-0.24451453735	UP	0.000077	0.5218088738	DOWN*	0.00001	-0.7969542473
3	Isoform 2 of Transketolase	TKT	P29401-2	UP*	0.005	0.77323815035	DOWN	0.146	-0.0433190376	UP	0.08	0.81655718030
4	S100-A9	S100A9	P06702	UP*	0.04	0.63095510322	UP*	0.023	0.69918438423	DOWN	0.774	-0.0682928101
5	Dsmocollin-2	DSC2	Q02487	UP	0.258	0.1668062886	DOWN*	0.04	-0.6810454136	UP*	0.01	0.84785170471
6	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	P04406	UP	0.508	0.12035126028	DOWN	0.027	-0.5365704665	UP*	0.023	0.65692172686 7
7	Thymidine phosphorylase	TYMP	P19971	UP	0.907	0.025820389	DOWN*	0.000022	-1.046004911	UP*	0.000075	1.0718253
8	Plastin-2	LCP1	P13796	UP*	0.018	0.592307865	DOWN	0.104	-0.595004611	UP*	0.001	1.187312476
9	Isoform 2 of Fructose-bisphosphate aldolase A	ALDOA	P04075-2	DOWN	0.101	-0.606982326	UP	0.039	0.453176474	DOWN*	0.003	060158801
10	Neutrophil gelatinase-associated lipocalin	LCN2	P80188	UP	0.4	0.169526584	DOWN*	0.045	-0.91751969	UP*	0.001	1.087046274
11	Superoxide dismutase	SOD1	P00441	UP	0.77	0.031181834	DOWN*	0.000226	-1.239313469	UP*	0.002	1.270495303
12	Histone H4	HIST1H4A	P62805	UP*	0.003	1.387331831	UP	0.637	-4.30044926	UP*	0.015	5.68778

* One-way ANOVA test followed by Dunn's post-hoc test with LSD or Game-Howell multiple testing correction: Statistically significant ($p<0.05$)

and Log2 fold change > 0.58 or < - 0.58. Colored table cells indicate significant difference.
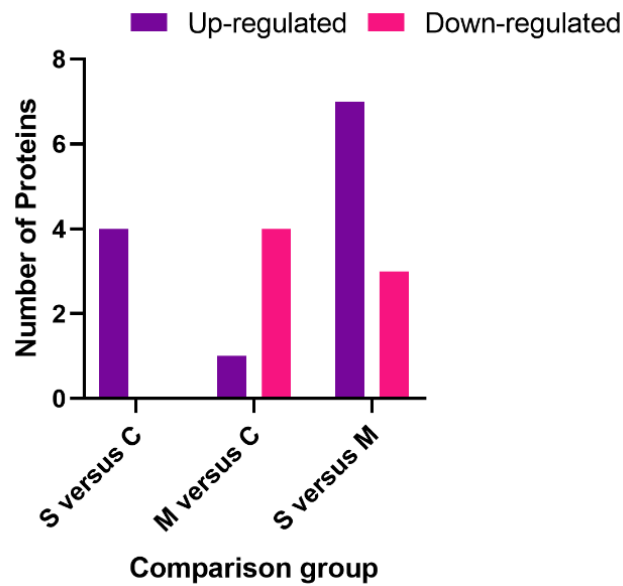
S = Severe dental fluorosis group, M = Moderate dental fluorosis group, C = Control dental fluorosis group

Figure 6 Number of proteins showing upregulate and down regulation in each pair-comparison

Among the 12 proteins that were differentially expressed, LCP1 and HIST1H4A were significantly increase in the severe group compared to the moderate and the control groups, suggesting that these proteins are related to the severe form of fluorosis (Figure 7A, B). Moreover, TKT was significantly increased in the severe fluorosis group compared to the control (Figure 7C).
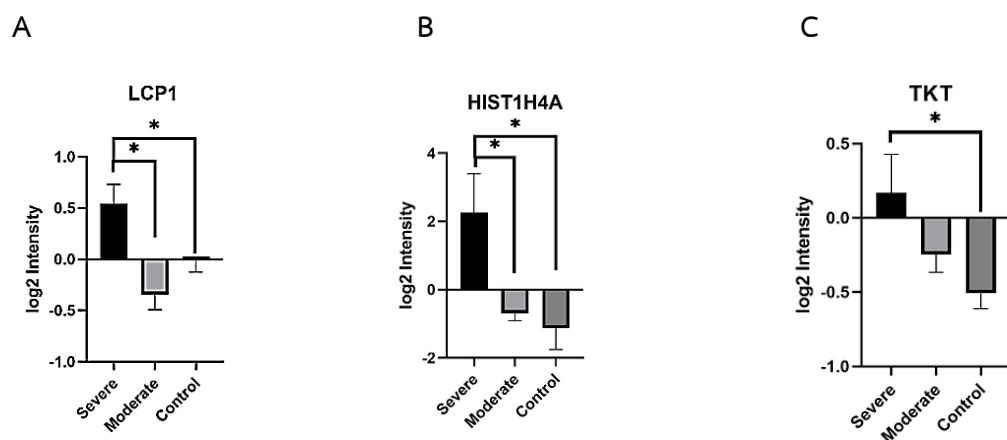
Figure 7 Bar graphs showing upregulated proteins in the severe group compared to moderate group and/or control group.

Four proteins including DSC2, TYMP, LCN2, and SOD1 were downregulated in the moderate fluorosis group compared to the severe fluorosis and control groups (Figure 8A – D).
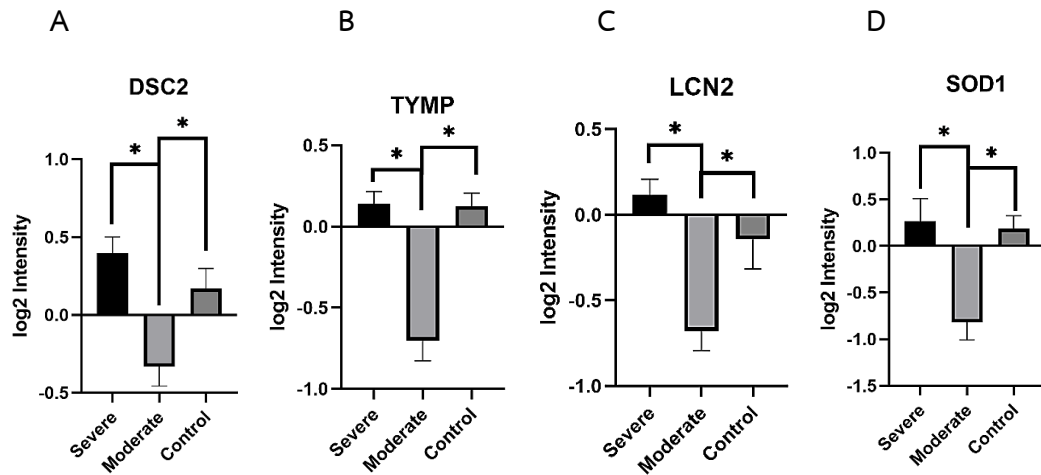
Figure 8 Bar graphs showing downregulated proteins in the moderate fluorosis group compared to the severe fluorosis and the control groups.

Additionally, three proteins (IGKC, ALDOA, and LEG1) were increased and one protein (GAPDH) was decreased in the moderate fluorosis group compared to the severe fluorosis group (Figure 9A – D), implying that these four proteins may differentiate the severe fluorosis group from the moderate fluorosis group.
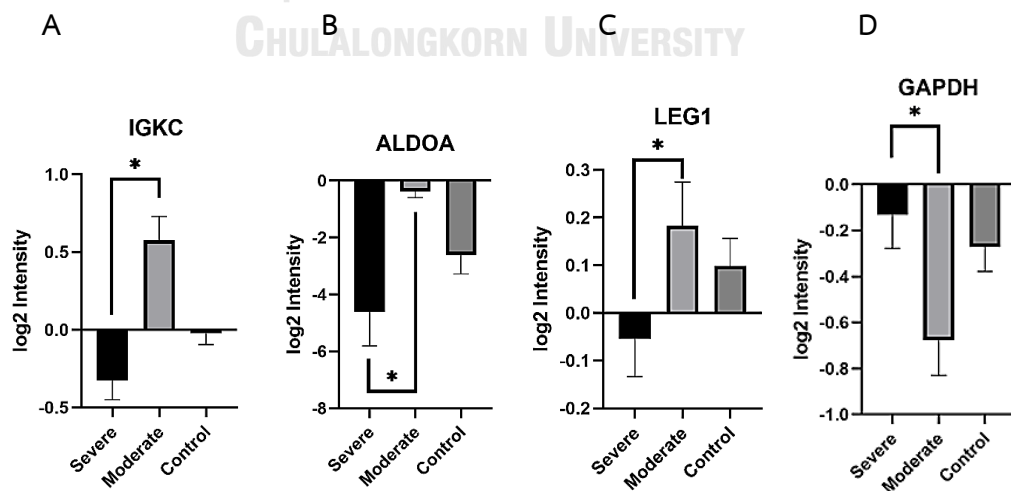
Figure 9 Bar graphs showing downregulated and upregulated proteins in the moderate fluorosis group compared to the severe fluorosis group.

Protein S100A9 showed a significant decrease in the control group compared to both the severe and moderate fluorosis groups. This suggests that S100A9 may have the potential to differentiate fluorosis patients from the control group (Figure 10).

Figure 10 Bar graphs showing downregulated protein S100A9 in control group compared to the severe and moderate fluorosis groups.

GO analysis conducted on the 12 differentially expressed proteins revealed that these proteins were involved in several biological processes, cellular compartment, and molecular function as follows. (Figure 11 and Table 4)
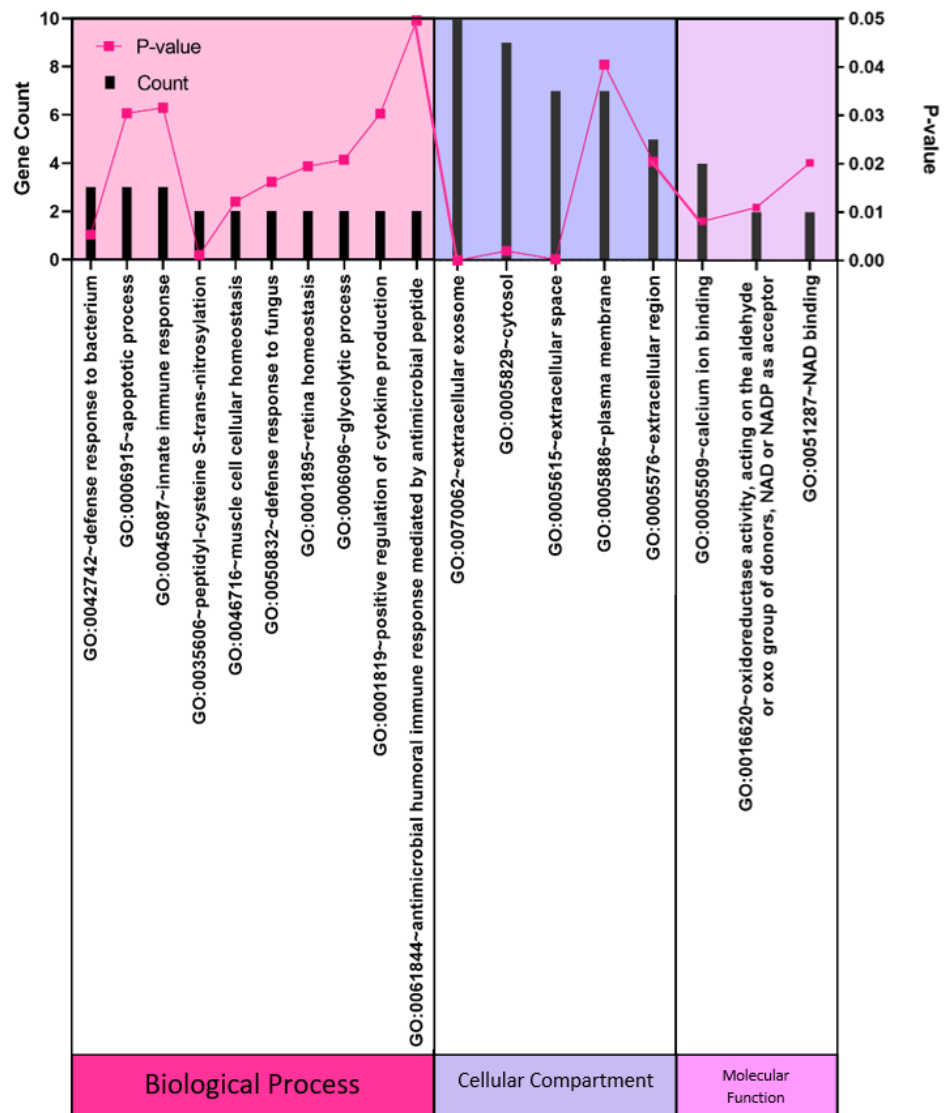
Figure 11 Functional classification of 12 proteins analyzed using GO analysis

Table 4 Functional classification and gene list analyzed using GO analysis.

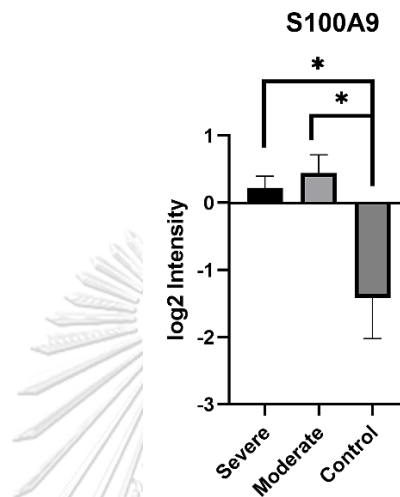| Functional analysis by GO term	Protein count	% protein count of all proteins	p-value	Gene List
Biological process				
Defense response to bacterium	3	25	0.005161	IGKC, LCN2, S100A9
Apoptotic process	3	25	0.030348	LCN2, S100A9, SOD1
Innate immune response	3	25	0.031474	IGKC, LCN2, S100A9
Peptidyl-cysteine S-trans-nitrosylation	2	16.67	9.30E-04	GAPDH, S100A9
Muscle cell cellular homeostasis	2	16.67	0.012026	ALDOA, SOD1
Defense response to fungus	2	16.67	0.016159	GAPDH, S100A9
Retina homeostasis	2	16.67	0.019363	IGKC, SOD1
Glycolytic process	2	16.67	0.020733	ALDOA, GAPDH
Positive regulation of cytokine production	2	16.67	0.030277	GAPDH, SOD1
Antimicrobial humoral immune response mediated by antimicrobial peptide	2	16.67	0.049562	GAPDH, S100A9
Cellular Compartment				
Extracellular exosome	10	83.33	8.69E-08	LEG1, IGKC, LCN2, LCP1, ALDOA, TKT, GAPDH, S100A9, DSC2, SOD1
Cytosol	9	75	0.001952	LCP1, ALDOA, TKT, GAPDH, S100A9, DSC2, TYMP, SOD1
Extracellular space	7	58.33	2.15E-04	LEG1, IGKC, LCN2, LCP1, ALDOA, S100A9, SOD1
Plasma membrane	7	58.33	0.040489	IGKC, LCP1, GAPDH, S100A9, DSC2, SOD1
Extracellular region	5	41.67	0.020412	IGKC, LCN2, ALDOA, S100A9, SOD1
Molecular Function				
Calcium ion binding	4	33.33	0.008101	LCP1, TKT, S100A9, DSC2
Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	1	8.33	0.010998	GAPDH
NAD binding	1	8.33	0.020174	GAPDH

CHAPTER V

DISCUSSION

According to the "Guidelines for Drinking-Water Quality" published by the World Health Organization (WHO) in 2006, the suggested guideline value for preventing the incidence of dental fluorosis in natural water is a maximum concentration of 1.5 parts per million (ppm) of fluoride. On the other hand, the recommendation for artificial fluoridation of water supply is at 0.5-1.0 ppm. (51)

The WHO recommends an optimal fluoride concentration of 0.7-1.0 ppm for regions with hot climate where the temperatures range from 38-40 degrees Celsius. (18) In Thailand, the Bureau of Dental Health has established a national standard for fluoride concentration in drinking water at 0.7 ppm. (19) Our study revealed that the mean water fluoride values in endemic areas were 1.93 (range 0.17 – 5.9) in the severe fluorosis group, 0.16 (0.1 – 0.3) in the moderate fluorosis group, and 0.97 (0.2 – 6.1) in the control group. Overall, the mean water fluoride values in the severe and control groups exceeded the national concentration.

The fluoride concentrations in urine and blood are proposed as the most reliable indicators of exposure to fluoride. (52) The acceptable concentration of urine fluoride is 1 ppm. (53) The mean urinary fluoride was 5.44 ppm (0.7 – 11.4) in the severe group, 2.79 ppm (1.24 – 4.01) in the moderate fluorosis group, and 2.49 ppm (0.5 – 6.78) the in control group, which were all higher than the accepted level. Studies in other endemic areas showed the urinary fluoride levels were 0.17 – 47.50, 0.15 – 1.99, 0.9 – 3.25, 1.07 – 4.0, and 0.05 – 2.8 ppm in Youssoufia city of Morocco (54), Barcelona (55), Gurgaon (56), Nellore (53), and Jhajjar (57) respectively.

The effect of fluoride is cumulative and mainly determined by the duration and quantity of exposure. (58) Epidemiological research has shown that individuals living in fluoride-endemic or artificially fluoridated areas are more susceptible to dental fluorosis compared to those residing in non-endemic or non-fluoridated

regions. However, it is important to note that the susceptibility and severity of fluorosis are influenced by a multitude of factors. These factors include the total fluoride consumption, the mode of fluoride intake (such as drinking water, food, or dental products), renal function (which affects fluoride excretion), the rate of bone metabolism, metabolic activity, high altitude of residence (which can alter the acid-base balance in the body), nutritional status, composition of the diet, and even genetic factors. (59) In our study, we did not find any significant differences in the duration of living in endemic areas among the three groups. However, we did observe that the parents of individuals with severe and moderate dental fluorosis had significantly higher incidences of dental fluorosis compared to the control group, where no reports of parental fluorosis were observed. These findings indicate that factors such as genetics and household water consumption behavior may play a role in the incidence and severity of dental fluorosis in individuals, even when they reside in the same fluorosis-endemic area.

Urinary fluoride has been proposed as a marker of fluoride exposure, and our study confirms this evidence by showing that higher levels of urinary fluoride are associated with severe dental fluorosis. This suggests that urinary fluoride can be used as a tool to assess current fluoride availability. However, it is important for future studies to also investigate other biomarkers of fluoride exposure, such as hair and nail samples. By exploring multiple biomarkers, a more comprehensive understanding of fluoride exposure and its impact on dental fluorosis can be obtained.

Saliva transcriptome

Our findings found that there was no significant difference in the RNA profiles between severe dental fluorosis and non-dental fluorosis, as evidenced by the absence of delta-gene count. However, it is important to consider certain limitations of our study. Firstly, the RNA sequencing raw data exhibited high variability, which

may have influenced our ability to detect differential expressions accurately. : Secondly, the limited number of transcriptomic samples, resulting from the low expression of human RNA in the saliva samples, combined with the high cost of the library preparation kit required for saliva transcriptome analysis, can have a substantial impact on the statistical analysis. Therefore, future studies should aim to address the limitations by recruiting a larger sample size. This will help mitigate the potential impact of high variant data and enhance the statistical robustness of the analysis.

The lack of differential expression at the RNA level does not necessarily imply the absence of differential expression at the protein level. One possible explanation for this discrepancy could be the differences in the methods employed for RNA extraction and protein preparation. In our study, a whole cell method was used for RNA extraction, whereas a cell-free method was used for protein preparation. The differences of laboratory process may impact to the disparity between the RNA and protein expression profiles. To gain a more comprehensive understanding, future investigations should aim to harmonize the methodologies employed for RNA extraction and protein preparation.

Saliva Proteome

Through proteomic analysis, we successfully identified 12 significant proteins that exhibited differentiation among the severe, moderate, and control groups. Notably, these proteins are involved in critical biological processes such as apoptosis and the defense response to bacteria, as supported by gene ontology analysis.

S100A9 is a calcium- and zinc- binding protein that plays a prominent role in the regulation of inflammatory processes and early stages of cancer development. (60) Additionally, it can induce cell death via autophagy and apoptosis, and serve as a powerful amplifier of inflammation in autoimmunity. (61) Recent research has suggested that salivary S100A9 could be a potential biomarker for periodontitis which

is a chronic inflammatory condition. (62) A discovery from a study conducted in the fluoride-endemic region of Eldoret, Kenya, revealed a positive correlation between esophageal squamous cell carcinoma (ESCC) and dental fluorosis (63). Furthermore, a comparative proteomic analysis demonstrated an upregulation of S100A9 in ESCC (64). Interestingly, our study also identified an upregulation of S100A9 in cases of severe and moderate dental fluorosis. These suggest that S100A9, a potential biomarker of inflammation, could be involved in fluorosis in terms of chronic inflammation and immune dysregulation in affected tissues. However, more research is needed to establish a direct link between S100A9 and fluorosis.

LCP1 and HIST1H4A exhibit significant increases in the severe fluorosis group compared to both moderate fluorosis group and control group, indicating a correlation between these proteins and severe dental fluorosis. Previous studies have shown that *LCP1* gene expression is increased in chronic periodontitis and generalized aggressive periodontitis. (65) Furthermore, it has been observed in osteoclast that the inflammation was induced by phosphorylated LCP1 through the TNFR1 signaling cascade leads to bone resorption, ultimately resulting in bone loss. (66) This suggests that LCP1 may play a role in the bone remodeling process and the development of mineralized-related disorders such as dental and skeletal fluorosis and periodontitis. HIST1H4A is a protein involved in chromatin structure and gene regulation through its role in packaging DNA. Histone modifications, such as acetylation, methylation, and phosphorylation involve in various biological processes, play a crucial role in gene expression regulation for development and disease. (67) Dysregulation of histone modifications can impact gene expression patterns and contribute to disease development and progression. In the context of fluorosis, fluoride exposure may affect histone modifications and potentially alter gene expression patterns during tooth development as well as epigenetic mechanisms. (68) However, the specific mechanisms and epigenetic alterations associated with fluorosis are still not well understood and require further investigation.

Furthermore, in severe cases of dental fluorosis, there was a significant increase in TKT, compared to the moderate group and the control group. TKT has been associated with the ability to increase cell proliferation and migration while inhibiting cell apoptosis. (69, 70) Interestingly, recent research has shed light on the concept of anastasis, which refers to the natural recovery of cells from apoptotic death. Anastasis involves the re-entry of cells into the cell cycle, subsequent proliferation, and cytoskeletal rearrangement, leading to increased migratory ability. (71) For prolonged exposure to fluoride, which can lead to severe dental fluorosis, cells may undergo apoptosis and required a recovery process. In this context, the increased expression of TKT protein observed in severe dental fluorosis may potentially stimulate cell proliferation and migration, aiding in the restoration of cells to a normal state. This suggests that TKT could play a role in the recovery and repair mechanisms of cells affected by fluoride-induced damage.

The severe fluorosis group demonstrated lower levels of ALDOA, IGKC, and LEG1 when comparing to moderate fluorosis group. ALDOA is an enzyme involved in glycolysis, which is the metabolic pathway responsible for breaking down glucose to produce energy. It catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. To date, there is limited research specifically focusing on the relationship between ALDOA protein and fluorosis. However, glycolytic enzymes, including aldolase, have been involves in various cellular processes, such as cell growth, proliferation, and differentiation. (72) It has also been shown that ALDOA can inhibit DNA repair and induce cell cycle arrest via DNA damage in pancreatic cancer cells (73), suggesting that an alteration in the ALDOA level might lead to the disturbance of DNA repair and cell cycle. Excessive fluoride exposure may disrupt glycolysis process and can impact cellular metabolism which contribute to the development and progression of certain diseases. Apart from ALDOA, IGKC is a protein that is part of the immunoglobulin molecule, specifically the constant region of the kappa light chain. Immunoglobulins

are antibodies produced by plasma cells which play an important role in the immune response which helping to recognize and neutralize pathogens. (74) It is possible that fluoride can also affect immune function and the production of antibodies in the human body.

Four proteins including DSC2, TYMP, LCN2, and SOD1 were particularly decreased in the moderate fluorosis group compared to the severe fluorosis group and the control group, and the GAPDH level was decreased in the moderate group compared to the severe group. DSC2 is a protein that belongs to the desmocollin family and is involved in cell adhesion, specifically in the formation of desmosomes that provide structural integrity and stability to tissues, including epithelial tissues such as the skin and oral mucosa, (64) implying that fluoride might affect cell adhesion and the integrity of epithelial tissues. LCN2 has been shown to inhibit cell apoptosis through various mechanisms. It can increase the expression of superoxide dismutases (SOD1 and SOD2), which are antioxidant enzymes that help reduce oxidative stress. LCN2 also decreases the expression of the proapoptotic protein Bax and reduces the formation of reactive oxygen species (ROS) in CHO cells. (75) TYMP has been demonstrated to suppress the activation of caspases 3 and 9, which are key enzymes involved in the apoptotic pathway. It also inhibits the release of mitochondrial cytochrome c, another event associated with cell apoptosis. (76) GAPDH plays a role in both energy metabolism and apoptosis regulation. It has been shown to have pro-survival functions and can regulate apoptosis. Reduced levels of GAPDH can trigger an increase in cellular apoptosis by decreasing ATP levels through glycolysis and inhibiting the autophagy-mediated clearance of permeabilized mitochondria. (77) In animal studies, exposure to fluoride (NaF) has been shown to increase osteoblast apoptosis, and the rate of apoptosis tends to increase with higher doses of fluoride. Fluoride can also affect the expression of collagen type I, an important component of the extracellular matrix. (78) At the protein and molecular levels, fluoride-induced ameloblast apoptosis has been observed through the

activation of the FasL/Fas signaling pathway. Additionally, fluoride exposure can promote the release of cytochrome c from mitochondria, which is associated with apoptosis. Taken together, the findings from our study and previous research suggest that a decrease in these proteins such as LCN2, TYMP, and GAPDH could potentially trigger cell apoptosis. The modulation of apoptosis-related pathways and proteins may contribute to the pathogenesis of fluorosis and the harmful effects of excessive fluoride exposure on various cell types and tissues. However, the mechanisms underlying their regulation in moderate cases remain unclear. Factors such as the duration and intensity of fluoride exposure, individual variations in genetic susceptibility, and the complex interaction of cellular signaling pathways could all contribute to the differential expression of these proteins in moderate cases. Additional research is needed to investigate the precise molecular mechanisms that lead to the downregulation of these proteins specifically in moderate fluorosis. Investigating the role of other regulatory factors, such as microRNAs or transcription factors, in modulating the expression of LCN2, TYMP, and GAPDH in the context of moderate fluorosis could provide further insights. By understanding the factors that influence the downregulation of these proteins in moderate fluorosis cases, we can gain a better understanding of the molecular mechanisms underlying the progression and severity of fluorosis and potentially identify new targets for therapeutic interventions or preventive strategies.

These findings highlight the potential of saliva-based proteomics as a promising tool for assessing the impact of dental fluorosis on an individual's health. By clarify the underlying molecular mechanisms associated with this condition, we can deepen our understanding of its pathogenesis and potentially develop targeted interventions for prevention and management.

Limitations of the study

One limitation of this study is the limited geographic scope of the participants. The study only included individuals from two specific locations in a fluoride-endemic area of Thailand. Therefore, the findings may not be representative of dental fluorosis patients from other regions or populations. Additionally, the proportion of case and control participants may not have been evenly distributed, which could introduce bias and affect the generalizability of the results. Another limitation is the relatively small sample size for the transcriptomic profiling analysis. With a small sample size, the statistical power to detect differences between the dental fluorosis group and the control group may be limited. This could impact the reliability and validity of the findings. A larger sample size would provide more statistical power and strengthen the study's conclusions. Future studies with larger and more diverse sample sizes are needed to validate and expand upon the results obtained in this study.
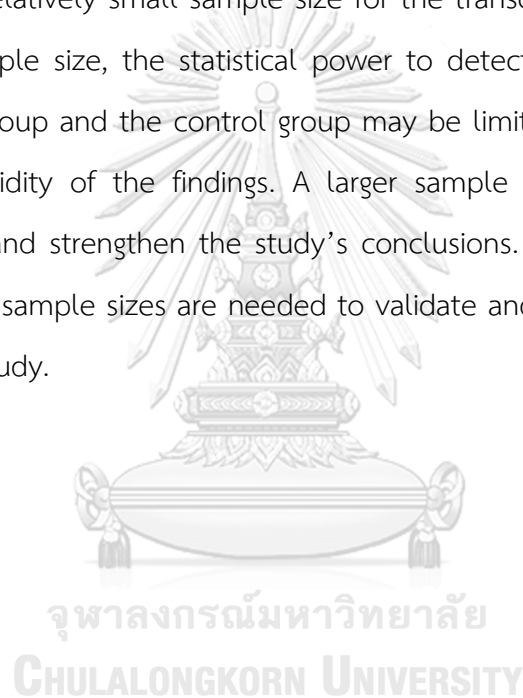
CHAPTER VI

CONCLUSION

The findings from analyzing urinary fluoride levels, water fluoride levels, transcriptomic profiles, and proteomic profiles in individuals with dental fluorosis compared to those without dental fluorosis provide valuable insights into several important aspects.

Firstly, the analysis of urinary fluoride levels gives us an indication of the bioavailability of fluoride in the body. By measuring fluoride levels in urine, we can understand how fluoride is being absorbed and excreted, which helps us assess the extent of fluoride exposure and its potential health effects.

Secondly, the assessment of water fluoride levels is crucial in understanding the impact of fluoride concentration in drinking water on the development of dental fluorosis. The results highlight the importance of access to safe water, particularly in fluoride-endemic areas, to prevent excessive fluoride intake and reduce the risk of dental fluorosis.

Furthermore, the study of transcriptomic and proteomic profiles in saliva provides valuable information about the molecular changes associated with dental fluorosis using non-invasive and cost-effective saliva-based methods. The observed differences in gene expression and protein levels indicate the potential impact of chronic fluoride exposure during tooth development. Specifically, the identified biological processes such as apoptosis and inflammation suggest the involvement of these processes in the development and progression of dental fluorosis.

These findings collectively contribute to our understanding of the mechanisms and effects of dental fluorosis at the molecular level. They emphasize the importance of monitoring and controlling fluoride exposure, particularly during critical periods of tooth development, and provide insights that can inform future preventive and therapeutic approaches for dental fluorosis.

To gain a comprehensive understanding of fluoride exposure and its impact on human health, it is recommended to conduct further investigations focusing on the molecular profile of urine which can provide valuable insights into the current level of fluoride exposure and the extent of fluoride accumulation in the body, leading to identifying specific biomarkers related to fluoride exposure and its potential health effects. With this knowledge, we can enhance our understanding of the health implications associated with fluoride and make informed decisions regarding public health policies, guidelines, and preventive measures.

APPENDIX

Table 5 All proteins that were discovered in the study after filtering the potential contaminant.

Protein description	Entry Name	Entry
Mucin-5B	Q9HC84	MUC5B
Cystatin-SN	P01037	CST1
Cystatin-S	P01036	CST4
Serum albumin	P02768	ALB
Immunoglobulin heavy constant alpha 1	P01876	IGHA1
Alpha-amylase 1	P04745	AMY1A
Cystatin-SA	P09228	CST2
Deleted in malignant brain tumors 1 protein	Q9UGM3	DMBT1
Polymeric immunoglobulin receptor	P01833	PIGR
Immunoglobulin heavy constant alpha 2	P01877	IGHA2
Immunoglobulin alpha-2 heavy chain	P0DOX2	IGA2
Zinc-alpha-2-glycoprotein	P25311	AZGP1
Lactotransferrin	P02788	LTF
Immunoglobulin kappa constant	P01834	IGKC
Cystatin-C	P01034	CST3
Immunoglobulin J chain	P01591	JCHAIN
Serotransferrin	P02787	TF
Immunoglobulin lambda constant 2	P0DOY2	IGLC2
Actin, cytoplasmic 1	P60709	ACTB
Lipocalin-1	P31025	LCN1
Histatin-1	P15515	HTN1
Immunoglobulin lambda constant 3	P0DOY3	IGLC3
Lysozyme C	P61626	LYZ
Isoform H7 of Myeloperoxidase	P05164-3	MPO
Alpha-enolase	P06733	ENO1
Hemoglobin subunit beta	P68871	HBB
Immunoglobulin kappa light chain	P0DOX7	IGK
Lactoperoxidase	P22079	LPO
Protein LEG1 homolog	Q6P5S2	LEG1
Mucin-7	Q8TAX7	MUC7
Immunoglobulin lambda constant 6	P0CF74	IGLC6
BPI fold-containing family B member 2	Q8N4F0	BPIFB2
Statherin	P02808	STATH
Submaxillary gland androgen-regulated protein 3B	P02814	SMR3B
Immunoglobulin heavy constant mu	P01871	IGHM

Uteroglobin	P11684	SCGB1A1
BPI fold-containing family A member 2	Q96DR5	BPIFA2
Immunoglobulin gamma-1 heavy chain	P0DOX5	IGG1
Haptoglobin	P00738	HP
Prolactin-inducible protein	P12273	PIP
Neutrophil defensin 3	P59666	DEFA3
Peroxiredoxin-5, mitochondrial	P30044	PRDX5
Isoform 2 of Transketolase	P29401-2	TKT
Immunoglobulin heavy constant gamma 3	P01860	IGHG3
Protein S100-A9	P06702	S100A9
Kallikrein-1	P06870	KLK1
Alpha-1B-glycoprotein	P04217	A1BG
Desmocollin-2	Q02487	DSC2
Immunoglobulin kappa variable 3-11	P04433	IGKV3-11
Isoform 2 of Histone H2B type 2-F	Q5QNW6-2	HIST2H2BF
Glyceraldehyde-3-phosphate dehydrogenase	P04406	GAPDH
Hemoglobin subunit delta	P02042	HBD
Galectin-3-binding protein	Q08380	LGALS3BP
Desmoglein-3	P32926	DSG3
Thymidine phosphorylase	P19971	TYMP
Isoform 2 of Carbonic anhydrase 6	P23280-2	CA6
Protein S100-A8	P05109	S100A8
Immunoglobulin heavy constant gamma 2	P01859	IGHG2
Phosphoglycerate mutase 1	P18669	PGAM1
Cathepsin G	P08311	CTSG
Plastin-2	P13796	LCP1
Leukocyte elastase inhibitor	P30740	SERPINB1
Calmodulin-like protein 3	P27482	CALML3
Cystatin-D	P28325	CST5
Folate receptor alpha	P15328	FOLR1
Macrophage migration inhibitory factor	P14174	MIF
Immunoglobulin kappa variable 3-15	P01624	IGKV3-15
Immunoglobulin kappa variable 3D-15	A0A087WSY6	IGKV3D-15
Histone H3.1	P68431	HIST1H3A
Immunoglobulin kappa variable 3-20	P01619	IGKV3-20
Isoform 3 of Interleukin-1 receptor antagonist protein	P18510-3	IL1RN
Alpha-actinin-4	O43707	ACTN4
Immunoglobulin heavy variable 3-15	A0A0B4J1V0	IGHV3-15
Trefoil factor 3	Q07654	TFF3

Metalloproteinase inhibitor 1	P01033	TIMP1
Complement C4-A	P0C0L4	C4A
Fatty acid-binding protein 5	Q01469	FABP5
Alpha-2-macroglobulin	P01023	A2M
Kallikrein-11	Q9UBX7	KLK11
Complement factor B	P00751	CFB
Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	ITIH4
Isoform 4 of Extracellular matrix protein 1	Q16610-4	ECM1
Folate receptor gamma	P41439	FOLR3
Galectin-7	P47929	LGALS7
Isoform 3 of Cysteine-rich secretory protein 3	P54108-3	CRISP3
Immunoglobulin heavy variable 3-9	P01782	IGHV3-9
Isoform long of Serine protease inhibitor Kazal-type 5	Q9NQ38-3	SPINK5
Vitronectin	P04004	VTN
Cytidine deaminase	P32320	CDA
Immunoglobulin kappa variable 2-40	A0A087WW87	IGKV2-40
Isoform 2 of Fructose-bisphosphate aldolase A	P04075-2	ALDOA
Coronin-1A	P31146	CORO1A
Neutrophil gelatinase-associated lipocalin	P80188	LCN2
Heat shock 70 kDa protein 6	P17066	HSPA6
Keratin, type II cytoskeletal 1	P04264	KRT1
Endoplasmic reticulum chaperone BiP	P11021	HSPA5
Isoform 3 of L-lactate dehydrogenase A chain	P00338-3	LDHA
Keratin, type I cytoskeletal 9	P35527	KRT9
Immunoglobulin heavy variable 3-74	A0A0B4J1X5	IGHV3-74
Complement C3	P01024	C3
Transcobalamin-1	P20061	TCN1
Antileukoproteinase	P03973	SLPI
Rab GDP dissociation inhibitor beta	P50395	GDI2
14-3-3 protein sigma	P31947	SFN
Immunoglobulin heavy variable 4-39	P01824	IGHV4-39
Heat shock protein beta-1	P04792	HSPB1
Isoform 2 of Histone H2A.J	Q9BTM1-2	H2AFJ
Alpha-2-HS-glycoprotein	P02765	AHSG
Histidine-rich glycoprotein	P04196	HRG
Profilin-1	P07737	PFN1
Pyruvate kinase PKM	P14618	PKM
Low affinity immunoglobulin gamma Fc region receptor III-A	P08637	FCGR3A
Immunoglobulin lambda variable 2-18	A0A075B6J9	IGLV2-18

Immunoglobulin lambda variable 7-46	A0A075B6I9	IGLV7-46
Alpha-2-macroglobulin-like protein 1	A8K2U0	A2ML1
Immunoglobulin lambda variable 1-51	P01701	IGLV1-51
Superoxide dismutase [Cu-Zn]	P00441	SOD1
Apolipoprotein A-I	P02647	APOA1
Immunoglobulin kappa variable 4-1	P06312	IGKV4-1
Adenylyl cyclase-associated protein 1	Q01518	CAP1
Isoform 2 of Tropomyosin alpha-3 chain	P06753-2	TPM3
Isoform 3 of Vitamin D-binding protein	P02774-3	GC
SPARC-like protein 1	Q14515	SPARCL1
Phosphoglycerate kinase 1	P00558	PGK1
Isoform 3 of Actin-related protein 2/3 complex subunit 4	P59998-3	ARPC4
Protein-glutamine gamma-glutamyltransferase E	Q08188	TGM3
Thioredoxin	P10599	TXN
Antithrombin-III	P01008	SERPINC1
Isoform 2 of Neutral alpha-glucosidase AB	Q14697-2	GANAB
14-3-3 protein zeta/delta	P63104	YWHAZ
Isoform 2 of Adenylosuccinate synthetase isozyme 1	Q8N142-2	ADSSL1
Immunoglobulin lambda variable 2-14	P01704	IGLV2-14
Heat shock cognate 71 kDa protein	P11142	HSPA8
Cystatin-B	P04080	CSTB
Fibromodulin	Q06828	FMOD
Isoform 2 of Mucin-21	Q5SSG8-2	MUC21
Isoform 4 of Alpha-actinin-1	P12814-4	ACTN1
Proline-rich protein 4	Q16378	PRR4
D-dopachrome decarboxylase	P30046	DDT
Immunoglobulin heavy variable 6-1	A0A0B4J1U7	IGHV6-1
Puromycin-sensitive aminopeptidase	P55786	NPEPPS
Desmoglein-1	Q02413	DSG1
Moesin	P26038	MSN
Histone H4	P62805	HIST1H4A
Immunoglobulin lambda variable 1-44	P01699	IGLV1-44
Immunoglobulin kappa variable 1D-33 OS	P01593	IGKV1D-33
Ezrin	P15311	EZR
Nucleolar protein 8	Q76FK4	NOL8
Ubiquitin-like modifier-activating enzyme 1	P22314	UBA1
Alpha-1-antitrypsin	P01009	SERPINA1
Isoform 3 of Zinc finger protein 74	Q16587-3	ZNF74
Immunoglobulin kappa variable 2D-29	A0A075B6S2	IGKV2D-29

Myeloblastin	P24158	PRTN3
Serpin B3	P29508	SERPINB3
Serpin B5	P36952	SERPINB5
Protein YIF1B	Q5BJH7	YIF1B
Immunoglobulin lambda-like polypeptide 1	P15814	IGLL1
Transaldolase	P37837	TALDO1
Leukocyte receptor cluster member 9	Q96B70	LENG9
Retinoic acid receptor responder protein 1	P49788	RARRES1
Fibrinogen gamma chain	P02679	FGG

REFERENCES

1.   Jha SK, Mishra VK, Sharma DK, Damodaran T. Fluoride in the environment and its metabolism in humans. Reviews of Environmental Contamination and Toxicology Volume 211. 2011:121-42.

2.   Elsherbini MS, Alsughier Z, Elmoazen RA, Habibullah MA. Prevalence and severity of dental fluorosis among primary school children in AlRass, Saudi Arabia. Int J Med Health Res. 2018;4(3).

3.   McGrady MG, Ellwood RP, Srisilapanan P, Korwanich N, Worthington HV, Pretty IA. Dental fluorosis in populations from Chiang Mai, Thailand with different fluoride exposures–Paper 1: assessing fluorosis risk, predictors of fluorosis and the potential role of food preparation. BMC Oral Health. 2012;12:1-12.

4.   Theerawasttanasiri N, Taneepanichskul S, Pingchai W, Nimchareon Y, Sriwichai S. Implementing a geographical information system to assess endemic fluoride areas in Lamphun, Thailand. Risk Management and Healthcare Policy. 2018:15-24.

5.   Sawangjang B, Hashimoto T, Wongrueng A, Wattanachira S, Takizawa S. Assessment of fluoride intake from groundwater and intake reduction from delivering bottled water in Chiang Mai Province, Thailand. Heliyon. 2019;5(9):e02391.

6.   Baez RJ, Petersen PE, Marthaler T, Organization WH. Basic methods for assessment of renal fluoride excretion in community prevention programmes for oral health: World Health Organization; 2014.

7.   Strunecka A, Strunecky O. Mechanisms of fluoride toxicity: from enzymes to underlying integrative networks. Applied Sciences. 2020;10(20):7100.

8.   Casamassimi A, Federico A, Rienzo M, Esposito S, Ciccodicola A. Transcriptome profiling in human diseases: new advances and perspectives. International journal of molecular sciences. 2017;18(8):1652.

9.   Ran L-Y, Xiang J, Zeng X-X, Tang J-l, Dong Y-T, Zhang F, et al. Integrated transcriptomic and proteomic analysis indicated that neurotoxicity of rats with chronic fluorosis may be in mechanism involved in the changed cholinergic pathway and oxidative stress. Journal of Trace Elements in Medicine and Biology. 2021;64:126688.

10.   Esteves CV, de Campos WG, Amorim dos Santos J, Kobayashi Velasco S, Guerra ENS, Siqueira WL, et al. Proteomic profile of saliva collected directly from ducts: a systematic review. Clinical Oral Investigations. 2020;24:559-68.

11.   Supplies RCoPoLCotFoW. Fluoride, Teeth and Health: A Report and Summary on Fluoride and Its Effect on Teeth and Health: Pitman Medical; 1976.

12.   Guissouma W, Hakami O, Al-Rajab AJ, Tarhouni J. Risk assessment of fluoride exposure in drinking water of Tunisia. Chemosphere. 2017;177:102-8.

13.   Edmunds W, Smedley P. Essentials of medical geology. Essentials of Medical Geology Springer. 2013:311-36.

14.   Cotruvo JA. 2017 WHO guidelines for drinking water quality: first addendum to the fourth edition. Journal-American Water Works Association. 2017;109(7):44-51.

15.   Intakes IoMSCotSEoDR. Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. 1997.

16.   Organization WH. Fluorides and oral health: report of a WHO Expert Committee on Oral Health Status and Fluoride Use [meeting held in Geneva from 22 to 28 November 1993]: World Health Organization; 1994.

17.   Water S, Organization WH. Guidelines for drinking-water quality [electronic resource]: incorporating first addendum. Vol. 1, Recommendations. 2006.

18.   Murray JJ, Organization WH. Appropriate use of fluorides for human health: World Health Organization; 1986.

19.   Bureau of Dental Health DoH, Ministry of Public Health Guidelines to manage high fluoride in drinking water to prevent dental fluorosis. 2005.

20.   Fejerskov O, Manji F, Baelum V. The nature and mechanisms of dental fluorosis in man. Journal of dental research. 1990;69(2_suppl):692-700.

21.   Di Giovanni T, Eliades T, Papageorgiou SN. Interventions for dental fluorosis: A systematic review. Journal of Esthetic and Restorative Dentistry. 2018;30(6):502-8.

22.   DenBesten P. Biological mechanisms of dental fluorosis relevant to the use of fluoride supplements. Community dentistry and oral epidemiology. 1999;27(1):41-7.

23.   DenBesten P, Li W. Chronic fluoride toxicity: dental fluorosis. Fluoride and the oral environment. 2011;22:81-96.

24.   Evans RW, Darvell BW. Refining the estimate of the critical period for susceptibility to enamel fluorosis in human maxillary central incisors. Journal of Public Health Dentistry. 1995;55(4):238-49.

25.   Ish T, Suckling G. The severity of dental fluorosis in children exposed to water with a high fluoride content for various periods of time. Journal of Dental Research. 1991;70(6):952-6.

26.   Wei W, Pang S, Sun D. The pathogenesis of endemic fluorosis: Research progress in the last 5 years. Journal of cellular and molecular medicine. 2019;23(4):2333-42.

27.   Zhang Y, Zhang K, Ma L, Gu H, Li J, Lei S. Fluoride induced endoplasmic reticulum stress and calcium overload in ameloblasts. Archives of Oral Biology. 2016;69:95-101.

28.   Wei W, Gao Y, Wang C, Zhao L, Sun D. Excessive fluoride induces endoplasmic reticulum stress and interferes enamel proteinases secretion. Environmental toxicology. 2013;28(6):332-41.

29.   Jiang M, Mu L, Wang Y, Yan W, Jiao Y. The relationship between Alu I polymorphisms in the calcitonin receptor gene and fluorosis endemic to Chongqing, China. Medical Principles and Practice. 2015;24(1):80-3.

30.   Huang H, Ba Y, Cui L, Cheng X, Zhu J, Zhang Y, et al. COL1A2 gene polymorphisms (Pvu II and Rsa I), serum calciotropic hormone levels, and dental fluorosis. Community dentistry and oral epidemiology. 2008;36(6):517-22.

31.   Escobar-García D, Mejía-Saavedra J, Jarquín-Yáñez L, Molina-Frechero N, Pozos-Guillén A. Collagenase 1A2 (COL 1A2) gene A/C polymorphism in relation to severity of dental fluorosis. Community dentistry and oral epidemiology. 2016;44(2):162-8.

32.   Küchler EC, Dea Bruzamolin C, Ayumi Omori M, Costa MC, Antunes LS, Pecharki GD, et al. Polymorphisms in nonamelogenin enamel matrix genes are associated with dental fluorosis. Caries research. 2018;52(1-2):1-6.

33.   Medina-Solis CE, Pontigo-Loyola AP, Maupome G, Lamadrid-Figueroa H, Loyola-Rodríguez JP, Hernández-Romano J, et al. Dental fluorosis prevalence and severity using Dean's index based on six teeth and on 28 teeth. Clinical oral investigations. 2008;12:197-202.

34.   Mohamed AR, Thomson WM, Mackay TD. An epidemiological comparison of Dean's index and the Developmental Defects of Enamel (DDE) index. Journal of public health dentistry. 2010;70(4):344-7.

35.   Organization WH. Oral health surveys: basic methods: World Health Organization; 2013.

36.   Zohoori FV, Duckworth RM. Fluoride: intake and metabolism, therapeutic and toxicological consequences. Molecular, genetic, and nutritional aspects of major and trace minerals: Elsevier; 2017. p. 539-50.

37.   Buzalaf MAR. Fluoride and the oral environment: Karger Medical and Scientific Publishers; 2011.

38.   Marthaler T. Monitoring of renal fluoride excretion in community preventive programmes on oral health: World Health Organization; 1999.

39.   Yadav J, Lata S. Urinary fluoride levels and prevalence of dental fluorosis in children of Jhajjar District, Haryana. Indian journal of medical sciences. 2003;57(9):394-9.

40.   Grimaldo M, Borjaaburto VH, Ramirez AL, Ponce M, Rosas M, Diazbarriga F. Endemic fluorosis in San-Luis-Potosi, Mexico. 1. Identification of risk-factors associated with human exposure to fluoride. Environmental Research. 1995;68(1):25-30.

41.   Das K, Mondal NK. Dental fluorosis and urinary fluoride concentration as a reflection of fluoride exposure and its impact on IQ level and BMI of children of Laxmisagar, Simlapal Block of Bankura District, WB, India. Environmental monitoring and assessment. 2016;188:1-14.

42.   Pramanik S, Saha D. The genetic influence in fluorosis. Environmental toxicology and pharmacology. 2017;56:157-62.

43.   de Klerk E, AC't Hoen P. Alternative mRNA transcription, processing, and translation: insights from RNA sequencing. Trends in Genetics. 2015;31(3):128-39.

44.   Kukurba KR, Montgomery SB. RNA sequencing and analysis. Cold Spring Harbor Protocols. 2015;2015(11):pdb. top084970.

45.   Jasim H, Olausson P, Hedenberg-Magnusson B, Ernberg M, Ghafouri B. The proteomic profile of whole and glandular saliva in healthy pain-free subjects. Scientific reports. 2016;6(1):39073.

46.   Hu S, Loo JA, Wong DT. Human saliva proteome analysis. Annals of the New York Academy of Sciences. 2007;1098(1):323-9.

47.   Hartenbach FARR, Velasquez É, Nogueira FC, Domont GB, Ferreira E, Colombo APV. Proteomic analysis of whole saliva in chronic periodontitis. Journal of proteomics. 2020;213:103602.

48.   Oyelakin A, Horeth E, Song E-AC, Min S, Che M, Marzullo B, et al. Transcriptomic and network analysis of minor salivary glands of patients with primary Sjögren's syndrome. Frontiers in immunology. 2021;11:606268.

49.   Belstrøm D, Constancias F, Liu Y, Yang L, Drautz-Moses DI, Schuster SC, et al. Metagenomic and metatranscriptomic analysis of saliva reveals disease-associated microbiota in patients with periodontitis and dental caries. NPJ biofilms and microbiomes. 2017;3(1):23.

50.   Jeon Y-S, Cha J-K, Choi S-H, Lee J-H, Lee J-S. Transcriptomic profiles and their correlations in saliva and gingival tissue biopsy samples from periodontitis and healthy patients. Journal of Periodontal & Implant Science. 2020;50(5):313.

51.   WHO G. Guidelines for drinking-water quality. World health organization. 2011;216:303-4.

52.   Kokot Z, Drzewiecki D. Fluoride levels in hair of exposed and unexposed populations in Poland. Fluoride. 2000;33(4):196-204.

53.   Jagmohan P, Narayana Rao S, Sambasiva Rao K. Prevalence of high fluoride concentration in drinking water in Nellore district, AP, India: a biochemical study to develop the relation to renal failures. World J Med Sci. 2010;5(2):45-8.

54.   Abdennebi E, Fandi R, Lamnaouer D. Human fluorosis in Morocco: analytical and clinical investigations. Veterinary and human toxicology. 1995;37(5):465-8.

55.   Torra M, Rodamilans M, Corbella J. Serum and urine ionic fluoride: normal range in a nonexposed population. Biological trace element research. 1998;63:67-71.

56.   Singh B, Gaur S, Garg V. Fluoride in drinking water and human urine in Southern Haryana, India. Journal of hazardous materials. 2007;144(1-2):147-51.

57.   Kumar S, Lata S, Yadav J, Yadav J. Relationship between water, urine and serum fluoride and fluorosis in school children of Jhajjar District, Haryana, India. Applied Water Science. 2017;7:3377-84.

58.   Jarquín-Yañez L, Mejía-Saavedra JdJ, Molina-Frechero N, Gaona E, Rocha-Amador DO, López-Guzmán OD, et al. Association between urine fluoride and dental fluorosis as a toxicity factor in a rural community in the state of San Luis Potosi. The Scientific World Journal. 2015;2015.

59.   Martínez-Mier EA. Fluoride: its metabolism, toxicity, and role in dental health. Journal of Evidence-Based Complementary & Alternative Medicine. 2012;17(1):28-32.

60.   Zhang XL, Wu ZZ, Xu Y, Wang JG, Wang YQ, Cao MQ, et al. Saliva proteomic analysis reveals possible biomarkers of renal cell carcinoma. Open Chemistry. 2020;18(1):918-26.

61.   Yi W, Zhu R, Hou X, Wu F, Feng R. Integrated Analysis Reveals S100a8/a9 Regulates Autophagy and Apoptosis through the MAPK and PI3K-AKT Signaling Pathway in the Early Stage of Myocardial Infarction. Cells. 2022;11(12):1911.

62.   Shin M-S, Kim Y-G, Shin YJ, Ko BJ, Kim S, Kim H-D. Deep sequencing salivary proteins for periodontitis using proteomics. Clinical oral investigations. 2019;23:3571-80.

63.   Menya D, Maina SK, Kibosia C, Kigen N, Oduor M, Some F, et al. Dental fluorosis and oral health in the African Esophageal Cancer Corridor: Findings from the Kenya ESCCAPE case–control study and a pan‑African perspective. International journal of cancer. 2019;145(1):99-109.

64.   Fan N-J, Gao C-F, Wang C-S, Zhao G, Lv J-J, Wang X-L, et al. Identification of the up-regulation of TP-alpha, collagen alpha-1 (VI) chain, and S100A9 in esophageal squamous cell carcinoma by a proteomic method. Journal of proteomics. 2012;75(13):3977-86.

65.   Öztürk V-Ö, Emingil G, Osterwalder V, Bostanci N. The actin-bundling protein L-plastin: A novel local inflammatory marker associated with periodontitis. Journal of Periodontal Research. 2014;50.

66.   Chellaiah MA. L-Plastin Phosphorylation: Possible Regulation by a TNFR1 Signaling Cascade in Osteoclasts. Cells. 2021;10(9).

67.   Holt MV, Wang T, Young NL. High-throughput quantitative top-down proteomics: histone H4. Journal of the American Society for Mass Spectrometry. 2019;30(12):2548-60.

68.   Bhowmik AD, Podder S, Mondal P, Shaw P, Bandyopadhyay A, Das A, et al. Chronic exposure to environmentally relevant concentration of fluoride alters Ogg1 and Rad51 expressions in mice: Involvement of epigenetic regulation. Ecotoxicology and Environmental Safety. 2020;202:110962.

69. Qin Z, Xiang C, Zhong F, Liu Y, Dong Q, Li K, et al. Transketolase (TKT) activity and nuclear localization promote hepatocellular carcinoma in a metabolic and a non-metabolic manner. Journal of Experimental & Clinical Cancer Research. 2019;38(1):154.

70. Zhang L, Huang Z, Cai Q, Zhao C, Xiao Y, Quan X, et al. Inhibition of Transketolase Improves the Prognosis of Colorectal Cancer. Frontiers in Medicine. 2022;9.

71. Sun G, Guzman E, Balasanyan V, Conner CM, Wong K, Zhou HR, et al. A molecular signature for anastasis, recovery from the brink of apoptotic cell death. Journal of Cell Biology. 2017;216(10):3355-68.

72. Chang Y-C, Yang Y-C, Tien C-P, Yang C-J, Hsiao M. Roles of aldolase family genes in human cancers and diseases. Trends in Endocrinology & Metabolism. 2018;29(8):549-59.

73. Xu W. ALDOA inhibits cell cycle arrest induced by DNA damage via the ATM-PLK1 pathway in pancreatic cancer cells. 2021.

74. Lefranc M-P, Lefranc G. Immunoglobulins or antibodies: IMGT® bridging genes, structures and functions. Biomedicines. 2020;8(9):319.

75. Wang G, Liu X, Meng L, Liu S, Wang L, Li J, et al. Up-regulated lipocalin-2 in pulmonary hypertension involving in pulmonary artery SMC resistance to apoptosis. International Journal of Biological Sciences. 2014;10(7):798.

76. Ikeda R, Furukawa T, Mitsuo R, Noguchi T, Kitazono M, Okumura H, et al. Thymidine phosphorylase inhibits apoptosis induced by cisplatin. Biochemical and biophysical research communications. 2003;301(2):358-63.

77. Colell A, Green D, Ricci J-E. Novel roles for GAPDH in cell death and carcinogenesis. Cell death and differentiation. 2009;16:1573-81.

78. Yan X, Yan X, Morrison A, Han T, Chen Q, Li J, et al. Fluoride induces apoptosis and alters collagen I expression in rat osteoblasts. Toxicology letters. 2011;200(3):133-8.

VITA

NAME   PATCHARAPORN GAVILA

DATE OF BIRTH   20 DEC 1990

PLACE OF BIRTH   CHIANGMAI, THAILAND

INSTITUTIONS ATTENDED   FACULTY OF DENTISTRY, CHIANGMAI UNIVERSITY

HOME ADDRESS   31/1 RATUTIT ROAD SOI 7 WATGATE MUANG, CHIANGMAI