COMSATS University Islamabad

Synopsis for MS ■ Ph.D. ☐

Name: Rimsha Binte Khalid	Registration No. FA23-RMG-013
Program: Master of Sciences in Molecular Genetics	Area of Specialization (if any as per approved SoS):
Department: Biosciences	Campus: Islamabad
Date of admission: Fall 2023	Date of synopsis submission: 22/10/2024
Proposed Title of the Thesis: (Use title case capitalization): Role of Bone Sialoprotein (BSP) Gene Polymorphism and Risk of Skeletal Fluorosis in Pakistani Children Population	

Supervisory Committee

Name and Designation	Role
Dr. Syed Tahir Abbas Shah (Associate Professor)	Supervisor
Dr. Syed Ali Musstjab Akber Shah (Assistant Professor)	Co-supervisor

Student's Signature _____

Summary of the Research:

Fluoride is a naturally occurring trace element found in food, plants, and water entering primarily in the human body through drinking water. Groundwater is a major source of drinking water around the globe having varying amounts of fluoride concentrations due to geological and environmental factors. It often exceeds the World Health Organization's recommended limit of 1.5 mg/L causing fluoride intoxication. Chronic exposure to excessive fluoride concentrations in drinking water may lead to a health condition called fluorosis. Dental fluorosis is an early indicator of high fluoride levels in the body causing mottling and decolorization of teeth. When exposed for a longer period, skeletal fluorosis affecting bone structure and strength transpires. Fluoride interference results in abnormal bone mineralization and increased bone fragility. Certain proteins and genes are involved in regulating bone health. Bone Sialoprotein is one of them. It is a non-collagenous protein that plays a vital role in bone mineralization. Skeletal fluorosis affects the pathway of BSP that maintains bone health and integrity. The study aims to investigate the role of BSP and BSP genes in bone health and the effect of excessive fluoride on Bone Sialoprotein pathways through the investigation of Bone Sialoprotein gene polymorphism. The research will analyze the influence of Single nucleotide polymorphism (SNPs) in BSP genes over the susceptibility to skeletal fluorosis. The end goal of this study will be to provide genetic markers for assessing fluorosis risk in the human body by examining the pathway between BSP genetic variations and fluoride-induced bone damage. It will also uncover the molecular mechanisms through which fluoride levels affect bone strength.

Student's Name: Rimsha Binte Khalid
Enrollment Number: FA23/RMG/013

Supervisor's Signature:

Introduction:

Fluorine is determined through its certain properties i.e it is a naturally occurring trace element with high electronegativity, reactive and corrosive nature. Ionic form of fluorine is fluoride (F-) which is essential for developing and maintaining bones and teeth as it promotes mineralization by interacting with calcium and phosphate. When ingested in the body, fluoride is firstly enters the gastrointestinal tract and get absorbed there then distributed through the bloodstream. 90% of absorbed fluoride accumulates in calcified tissues (bones and teeth). It poses both beneficial and adverse effects on the human body (Jhonston *et al.,* 2020). When provided in optimal concentration (0.5-1.0 ppm in water), fluoride reduces the incidence of dental caries and plays a beneficial role in bone health development. When the fluoride level exceeds this limit to 1.5 ppm it will result in a health condition called fluorosis (Srivastava *et al.,* 2020). These levels of fluorides are determined by their presence in the water, the main source of fluoride intake is through drinking water, especially groundwater. Unfortunately, clean and clear water for drinking purposes across the globe is not easily accessible due to geographical disadvantages, human interventions, and financial drawbacks. Groundwater is a major source of fresh water but various chemical compounds including elevated fluoride have contaminated it. Regions affected by fluoride contamination include areas along the East African Rift, parts of South and Southeast Asia including India, Pakistan, China, and Thailand, and certain regions of Europe and America (Shreyas J. *et al.,* 2021). High levels of fluoride (10mg/L) have been reported in parts of India, China, and Africa leading to widespread cases of dental and skeletal fluorosis. (Lacson *et al.*, 2021). These conditions cause enamel hypomineralization, bone deformities, osteoporosis, osteosclerosis, and progressive deterioration of their joints. Such conditions are responsible for reducing the life quality and overall life expectancy (A Pan *et al.,* 2021) Skeletal fluorosis is a condition caused by the long-term deposition of fluoride in bones leading to damage. Excessive fluoride is incorporated in bone tissues, altering bone structure and strength. It alters the normal hydroxyapatite with flouropatite thus disrupting bone metabolism, increasing bone density, and causing deformities, joint stiffness, and pain (Shanti lal Choubisa, 2022). If left unchecked for a long period, it leads to crippling conditions such as neurological deficits and severe skeletal deformities. The levels of fluoride in the body are widely determined through urine samples due to the renal system's critical role in fluoride excretion. Analysis of fluoride in urine is usually conducted using ion-selective electrodes method for precise quantification of fluoride concentration in the human body (Zulfiqar *et al.,* 2020). Studies reveal genetic predisposition is shown to play an important role in susceptibility to fluorosis. Certain genes involved in bone metabolism have been reported. These include matrix metalloproteinase (MMP), glutathione S-transferases (GSTs), prolactin (PRL), vitamin D receptor (VDR), and collagen type 1 alpha 2 (COLIA2)

and bone morphogenetic proteins (BMPs) (González-Martínez F. *et al.*, 2024).

Bone Sialoprotein (BSP) is a non-collagenous protein that plays a significant role in the mineralization and structure of bones and dentin. BSP is found in the extracellular matrix of bones, essential for the nucleation of hydroxyapatite, the mineral that provides strength and rigidity to bones. Additionally, BSP is implicated in the regulation of osteoblasts and osteoclasts, the cells responsible for bone resorption and formation, respectively. BSP contains specific domains that bind to both calcium and hydroxyapatite making it important for the mineralization process (Carvalho MS *et al.*, 2021). This generates a link with fluoride exposure as it may be involved in bone metabolism and mineralization regulation. Excessive fluoride intake disrupts normal bone formation leading to osteosclerosis and osteomalacia (Kakkar *et al.,* 2020). Investigating the BSP effect due to high fluoride levels will explain the molecular mechanisms underlying the development of dental and skeletal fluorosis. Moreover, SNP within the BSP gene may influence the protein's function contributing to susceptibility to fluorosis. The study aims to explore the relationship between fluoride intoxication and BSP focusing on how genetic variations contribute to altered bone mineralization and development of fluorosis. It will also identify BSP as a potential biomarker in the early detection and management of fluorosis.

Literature Review:

The essential need of everyday life is water, and the availability of clean water is fundamental to living a quality life. Around the globe, the availability of clean drinking water is inaccessible due to geographical disadvantages, human interventions, and economic and financial drawbacks. Due to these factors, developing and especially under-developing countries are unable to meet standard drinking water conditions that pose adverse health effects (Shreyas J. *et al.*, 2021). WHO reports that 783 million people are unable to reach basic drinking water services and expected that half of the world's population will face a shortage of clean drinking water by 2025 (Lacson *et al.,* 2021) Around the world, 1.8 billion people take fecally contaminated water putting their health at extreme risk (WHO/UNICEF, 2014). Half of the world's population is consuming groundwater or hidden sea water for their drinking purpose. In India, 80% of the rural population and 50% of the urban population consume groundwater as their main source of water supply (Senthilkumar S *et al.*, 2024). This groundwater contains different minerals such as fluoride, nitrate, arsenic, sulfate, manganese, iron, selenium, and chloride. These minerals are naturally occurring, human-induced, and industrial-origin ions that degrade water quality and constitute health risks. Reports suggest that countries like India, China, Sri Lanka, Spain, Italy, and West Indies are having elevated levels of fluoride ions in groundwater. Two fluoride belts are observed along the East African Rift from Eritrea to Malawi. The second one passes through Iraq, Iran, Afghanistan, Pakistan, India, Northern Thailand, and

China (Shreyas J. *et al.*, 2021). The World Health Organization (WHO) identifies fluoride and arsenic as the most prevalent inorganic contaminants in groundwater that pose significant health risks (E. Shaji *et al.*, 2021).

Fluoride is highly electronegative, corrosive and reactive in nature. The body requires an optimum amount of fluoride for the normal growth and development of bones and teeth. Flouride is classified as ionisable and nonionisable as well as organic and inorganic element (Srivastava *et al.*, 2020). Organic fluorides only dissolves in water when the fluoride ion is released via any chemical reaction. Inorganic fluorides such as calcium fluoride, hydrogen fluoride, sodium fluoride etc. are abundantly present in minerals, rock phosphates and earth's crust (950 ppm) in its ionic form (Jhonston NR *et al*., 2020). Thus, can get dissolved in groundwater and make a pathway for the entrance in the human body through drinking water. Once it is ingested in the body and absorbed by the bloodstream, the compound changes its forms and spreads into the bloodstream in the form of fluoride ions. Approximately 2.6g of fluorine is present in the human body (Ferreira MKM *et al.*, 2024). When this fluoride in absorbed in the body, more than 90% of it is absorbed and distributed in bone tissues. Here, fluoride is having dual effect depending upon its concentration. It has been reported that the fluoride concentration between 1*10-5 M and 1*10-4 M prevents demineralization but has a minimum effect on mineralization. However, an excessive concentration above 1*10-4 M promotes mineralization and inhibits demineralization (Kakkar *et al.*, 2020). Naturally occurring fluoride concentration is essential for oral health, prevention of dental caries and affecting bone health and mineralization. When the fluoride limit is exceeded, it induces risk of dental and skeletal fluorosis. Fluorosis is an endemic condition that affects both hard tissues like bones and teeth and soft tissues like kidneys, liver, and cardiovascular system. Excessive accumulation of fluoride in bones causes skeletal fluorosis which is a severe health condition. In rural areas, people are exposed to prolonged intake of fluoride through drinking water which results in affecting their teeth, bones, and major joints reducing mobility (E. Shaji *et al.*, 2021). In humans, when the fluoride concentration in plasma exceeds a range of 5-8 uM, it results in skeletal fluorosis (Kakkar et *al.*, 2020). Skeletal fluorosis alters the equilibrium between formation and resorption of bones (Shanti L., Choubisa, 2024). The study reveals that more than 100 million people in South Asia, including 0.1 billion people in Pakistan are exposed to excessive levels of fluoride through drinking water that is causing mild to severe dental and skeletal fluorosis with serious health risks. (Rashid et *al.*, 2018). In Pakistan, some areas are highly contaminated and report high levels of fluoride in groundwater due to increased mineral presence, human interventions, hydrogeochemistry, and its geological context. The most affected regions include Thar (Sindh), Thal (Khushab, Mianwali, and Bhakkar), and Lahore and Kasur districts of Pakistan (Ling *et al.*, 2022; Ahmed *et al.*, 2020; Ather *et al.*, 2022). Groundwater fluoride levels in these areas often exceed the WHO recommended limit of 1.5 mg/L,

posing serious health risks to local populations (Zulfiqar *et al.,* 2019). Fluorosis of various organs and tissues including the brain, kidneys, liver, endometrium, bone marrow, leukemic cells, and erythrocytes has been reported. Fluoride can pass through the blood-brain barrier and testis barriers resulting in different types of fluorosis (Guth *et al.,* 2020). The stomach absorbs the fluoride and it is released by the urine thus making it an important biomarker for the identification of fluoride levels in the body (Saeed *et al.,* 2021). It is also observed that children in areas with endemic fluorosis tend to have lower IQs as compared to healthy children (AL. Choi *et al.,* 2015; Sebastian and Sunitha, 2015). Genetic predispositions play a crucial in determining the individual's susceptibility to fluoride toxicity. Several genes are related to fluoride metabolism and its effects on bone and tooth development. These genes are involved in the formation of extracellular matrix, collagen formation, and bone remodeling. Among these Bone Sialoprotein (BSP) is of major concern in the context of fluoride-induced skeletal and dental fluorosis. Bone Sialoprotein (BSP) is a non-collagenous protein in mineralized connective tissues like bones, teeth, and cartilage. It plays a vital role in the regulation of hydroxyapatite crystal formation in bones and teeth (Carvalho MS *et al.,* 2021). Hydroxyapatite is a calcium phosphate mineral that is an important component of bone and dental enamel. The interaction of BSP with hydroxyapatite is vital for healthy mineralization. The protein's polyglumatic acid and RGD (arginine-glycine-aspartate) allow it to bind to hydroxyapatite and interact with cell surface integrins, facilitating bone and dental tissue formation (Ao M *et al.,* 2017). However, this precise mechanism becomes disrupted in fluorosis, where excessive fluoride impairs BSP's function in bone formation. The normal interaction between the BSP and hydroxyapatite is altered due to high fluoride levels in the fluoride-exposed individuals. Brittle hydroxyapatite which affects bone strength and structure. The role of BSP in the nucleation of hydroxyapatite makes it a mediator of mineralization defects seen in fluorosis (Kakkar *et al.,* 2020). The excessive fluoride affects the nucleation activity of BSP leading to the formation of defective mineral deposits. It is reported that the genetic expression of BSP can influence individual susceptibility to fluoride toxicity (Everett et al., 2011). Single nucleotide polymorphism (SNPs) in BSP-related genes may alter the bone response to fluoride exposure making certain individuals more prone to developing fluorosis. Fluoride's ability to induce oxidative stress in bone cells makes the role of BSP more complex in fluorosis. When fluoride is present in excessive amounts it generates reactive oxygen species (ROS) that disrupt BSP's normal activity and damage cellular components. Studies show that fluoride may affect BSP's capacity to regulate mineralization through oxidative pathways (Wei *et al.,* 2022). BSP plays a valuable biomarker for early diagnosis of fluorosis due to its central role in bone and teeth mineralization. Thus, BSP involvement in hydroxyapatite nucleation, bone remodeling, and mineralization makes it a vital protein in understanding the pathogenesis of fluorosis (Bouet G *et al.,* 2015). BSP's regulatory mechanisms and genetic variations including SNP analysis will

be crucial in identifying biomarkers for early detection and intervention of fluorosis (Malaval L *et al.*, 2008). Understanding the complex pathway between fluoride exposure, oxidative stress, and genetic variations will provide insights for addressing health challenges posed by fluoride toxicity.

Problem Statement:

Fluoride exposure in drinking groundwater in excessive amounts is posing a major health concern in different parts of Pakistan leading to a condition called fluorosis including dental and skeletal fluorosis. In Pakistan, some areas are highly contaminated and report high levels of fluoride in groundwater due to increased mineral presence, human interventions, hydrogeochemistry, and its geological context. The most affected regions include Thar (Sindh), Thal (Khushab, Mianwali, and Bhakkar), and Lahore and Kasur districts of Pakistan. Fluoride has a pathological effect on bone health while the molecular mechanism by which fluoride disrupts bone mineralization is not fully understood.

Bone Sialoprotein (BSP) is a non-collagenous protein involved in hydroxyapatite crystal formation. It is involved in bone and tooth mineralization. When fluoride is present in excessive amounts it generates reactive oxygen species (ROS) that disrupt BSP's normal activity and damage cellular components. Studies show that fluoride may affect BSP's capacity to regulate mineralization through oxidative pathways. BSP plays a valuable biomarker for early diagnosis of fluorosis due to its central role in bone and teeth mineralization. Thus, BSP involvement in hydroxyapatite nucleation, bone remodeling, and mineralization makes it a vital protein in understanding the pathogenesis of fluorosis. The contribution of BSP to fluoride-induced dysregulation of bone health is not fully explored. Investigating the BSP effect due to high fluoride levels will explain the molecular mechanisms underlying the development of skeletal fluorosis. Moreover, SNP within the BSP gene may influence the protein's function contributing to susceptibility to fluorosis. This research aims to investigate the molecular and genetic pathways involving BSP in fluoride toxicity. It focuses on how alterations in BSP gene function are linked to the development of fluorosis. The study aims to provide insight into the role of BSP in fluorosis through Single nucleotide Polymorphism (SNP) analysis in fluoride-affected populations, potentially identifying biomarkers for early diagnosis and therapeutic targets.

Research Objectives:

1. To evaluate skeletal fluorosis in the pediatric population of Punjab and identify contributing factors based on environment, fluoride exposure, and demographics.

2. To elucidate the role of SNP rs17013181 (A/G) in the BSP gene, which is linked to skeletal fluorosis

in affected individuals.

3. To undergo *In silico* analysis using bioinformatics tools and software.

Research Methodology:

Ethical Approval:

The COMSATS University Islamabad Ethical Review Board will grant approval for the planned study.

Design of Study:

There will be a retrospective case-control research.

Study Settings and Sample Collection:

The fluoride concentration in drinking water is mapped across different regions of Punjab, Pakistan based on previous studies (Ling *et al.,* 2022). Analyzing the two parameters i.e water quality data and fluoride exposure levels in four different groups. Two to three villages will be selected from each region representing different exposure levels to fluoride. A total of 775 children aged between 7 and 15 years (male and female participants) will be randomly selected from the mentioned sub-regions. Urine and blood samples will be collected from all participants to analyze the fluoride exposure and association with fluorosis. This will provide a context for correlating clinical manifestations of fluorosis with fluoride exposure levels in each region.

Samples of urine and blood will be taken and analyzed. Spot urine samples (first-morning void) will be taken from 775 children and stored in pre-labeled, dry, clean sampling bottles. Paired household drinking water samples (N=550) will be collected from the same participants. Both samples will be carried to the analytical laboratory with extreme care, Urine samples will stored at a low temperature in an icebox at -20°C for preservation before analysis. From 550 children, 5mL of fasting peripheral blood samples will be taken using sterile syringes and transferred to EDTA tubes for subsequent testing. Control data will be collected from nearby villages/areas with low or negligible fluoride contamination ensuring participants have similar lifestyles and dietary habits. The ion selective electrode (ISE) method will be used for urine fluoride level testing. Additionally, urinary creatinine levels will be determined in all samples through colorimetric analysis.

In silico Analysis:

Bioinformatics tools and software will be used for *in silico* analysis. These tools will help to explore the molecular basis of fluoride-induced health issues important to understand and identify the potential genetic markers and various variations.

DNA Extraction:

First, DNA will be isolated from both affected and healthy individuals. Manual extraction of DNA will be performed using Phenol/chloroform/isopropanol (PCI) protocol and the quality of DNA will be evaluated on 2% agarose gel electrophoresis. A Nanodrop spectrophotometer will be used for the quantification of DNA and then the DNA will be stored at -20°C till further use (Zekri *et al.*, 2012).

PCR:

Polymerase Chain Reaction (PCR) will be performed to reveal the BSP gene polymorphism genotypes of cases and controls. A housekeeping gene such as GPDH, B-action, or HPRT-1 will serve as the internal control.

Statistical Analysis:

SPSS (Version 22) will conduct multivariate and univariate statistical analyses to assess the data. Microsoft Excel will be used for data visualization. Logistic regression analysis will be employed to adjust for potential confounding factors such as age, gender, and demographic variables. OriginPro 2016 (Origin Lab, Northampton, MA) with the target gene CT values normalized to those of control genes.

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Tentative Schedule

Phase I (1-18 months)

- Literature Review
- *In silico* analysis
- Identification and sample collection
- DNA Extraction
- Amplification of DNA Using PCR

Phase II (28-36 months)

- Statistical analysis for verification of Data
- Thesis writing

Months	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**
Literatrure review & sampling											
DNA extraction & primer designing											
Experimentation											
Analysis of results											
Thesis writing											

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Details of Completed Coursework

	Course Code and Title	Credit Hours	Grade Points	Semester
1.	BIO726 CLINICAL GENETICS	3	A	1st
2.	BIO710 RESEARCH TECHNIQUES	3	A	1st
3.	BIO707 RECOMBINANT DNA TECHNOLOGY	3	B	1st
4.	BIO601 ADVANCES IN MOLECULAR BIO	3	C	2nd
5.	BIO603 MOLECULAR GENETICS	3	B	2nd
6.	BIO730 HISTOLOGY	3	C	2nd

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