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TITLE

Association of iron deficiency anemia with dental caries in the permanent first molars of children aged 7 to 12 years: An analytical cross-sectional study in Karachi, Sindh, Pakistan

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ABSTRACT

Introduction

Iron deficiency anemia and dental caries are prevalent diseases among Pakistani children. Limited research has been done to explore their association in permanent teeth. Given the caries susceptibility of permanent first molars and their role in the development of ideal occlusion, this study aims to estimate caries frequency in these molars and assess its association with iron deficiency anemia in 7 to 12-year-old children.

Methods and analysis

This analytical cross-sectional study will include 141 children aged 7 to 12 years visiting physicians in the pediatric outpatient department and emergency room of Dr. Ruth K.M Pfau, Civil Hospital Karachi. Using purposive sampling, children prescribed complete blood count, C-reactive protein and ferritin tests by their physicians will be screened and recruited. Data collection will involve physical examinations (including weight and height), oral examinations (including the relevant oral hygiene and caries assessments), and laboratory examinations (including the prescribed tests). Additionally, questions will be asked about socio-demographic characteristics, history of iron deficiency anemia, oral hygiene habits, smokeless tobacco use, and the frequency of cariogenic dietary consumption. Exposure variable will include the presence of iron deficiency anemia, assessed using complete blood count, C-reactive protein and ferritin tests, and treated as a dichotomous variable. Outcome variable will include dental caries in at least one permanent first molar, assessed using the DMFT index and also treated as a dichotomous variable. Analysis will include COX proportional algorithm, reporting prevalence ratios with 95% confidence intervals for the association of iron deficiency anemia and dental caries in the permanent first molars.

Frequency of children with carious permanent first molars with 95% confidence intervals will also be reported.

Ethics and dissemination

This research has been approved by ethical review committee (ERC) of Aga Khan University (AKU) (Project ID: 9692) and the institutional review board (IRB) of Dow University of Health Sciences (DUHS) (IRB Reference: IRB-3556/DUHS/Approval/2024/196) prior to participant recruitment. Results will be disseminated through seminars and peer reviewed publications.

Keywords: Iron deficiency anemia, dental caries, permanent first molar, children

ARTICLE SUMMARY

Strengths and limitations of this study

1. This study will use objective laboratory tests for the diagnosis of iron deficiency anemia and the validated DMFT index, recommended by WHO, for caries diagnosis in children.
2. It will control for the confounding associated with the mixed dentition stage, by specifically focusing on the permanent first molar which has the greatest caries risk.
3. Additionally, this study will be conducted among children belonging to the lower socioeconomic background, that are more likely to experience both the exposure and outcome; thereby controlling confounding associated with the socioeconomic status.
4. Even though this focus on permanent first molars of children belonging solely to low socioeconomic status will improve the internal validity of the study, it may compromise its external validity.

5. Moreover, since this is a cross-sectional study and the exposure and outcome are measured at one point in time, it is inherently susceptible to temporal bias.

INTRODUCTION

Dental caries is the most prevalent global disease [5], causing significant pain and impacting oral health-related quality of life [6] and nutritional intake [7] of children. In 2017, untreated dental caries affected approximately 2.3 billion people [8]. Caries prevalence in permanent teeth is highest in Africa (58.9%), followed by Asia (58.8%) and Australia (54.9%) [9]. Regionally, its prevalence in the Eastern Mediterranean Region is 61% among 12-year-olds and 70% among 15-year-olds [10].While locally, its overall prevalence in Pakistan is 56.6%, with 61.2% in mixed dentition and 57.2% in permanent dentition [11].

Anemia affects over 1.9 billion people globally [12] and had impacted 24.3% of the global population in 2021 [12]. The highest prevalence of anemia is found in Western and Central sub-Saharan Africa (47.4% and 35.7%, respectively) and South Asia (43%) [12], which overlaps with the regions heavily affected by dental caries. Dietary iron deficiency has been reported to be its leading cause among most demographic groups, including children [12].

Despite its significant impact, particularly in children, research on iron deficiency anemia (IDA) in Pakistani children aged 5-12 is limited, with varying prevalence rates reported across regions: 19% for 5-16 year-olds in rural Swat [13], 62.2% for children under 15 in Lahore [14], and 15.5% for 11-13 year-olds in Shaheed Benazirabad [15].

Studies worldwide have linked IDA with caries in children [16, 17, 18], suggesting mechanisms such as impaired saliva buffering [19], reduced enamel protection against demineralization [20], and antibacterial effects of iron [21, 22]. However, most research has focused on younger age groups, with limited representation of school-age children.

Given the prevalence of both conditions and their significant adverse effects on children's health, establishing a link between IDA and caries in Pakistani school-age children is a crucial research gap. Both conditions may strain the healthcare system and socio-economic conditions despite being preventable. This study aims to address this gap by estimating the frequency of carious permanent first molars among children aged 7 to 12 and assessing its association with iron deficiency anemia. The focus on first permanent molars is significant due to their high caries risk [2] and their role in developing ideal occlusion within this age group [23]. By providing insights into this association, the study's findings could inform future longitudinal research and enhance the integrated management of dental and hematological care for affected children.

OBJECTIVES

1. To investigate the association between iron deficiency anemia (IDA) and dental caries in the permanent first molars among children aged 7 to 12 years visiting a public tertiary care hospital in Karachi, Pakistan.
2. To estimate the frequency of children aged 7 to 12 years with carious permanent first molars visiting a public tertiary care hospital in Karachi, Pakistan.

HYPOTHESIS

The prevalence of dental caries in the permanent first molars of 7 to 12-year-old children who have iron deficiency anemia (IDA) is more than 2 times as compared to children who do not have iron deficiency anemia (IDA) after adjusting for a priori confounders and other independent variables.

METHODS AND ANALYSIS

Study design

An analytical cross-sectional study will be used. This type of design measures the burden of exposure and outcome in a specific population simultaneously and, in doing so, assesses their association in comparative terms.

To ensure that the study design and reporting are transparent and comprehensive in presenting the findings of the study, the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist will be followed.

Study site and setting

To overcome time constraints and for ethical reasons, we will conduct a hospital-based study to ensure an adequate number of children are recruited. Moreover, to obtain a homogenous sample, Dr. Ruth K. M. Pfau, Civil Hospital Karachi located in Karachi, Sindh, Pakistan will be used in this study.

Dr. Ruth K.M Pfau, Civil Hospital Karachi

Dr. Ruth K. M. Pfau, Civil Hospital Karachi is located at Mission Road in Karachi, Pakistan and is a 1900-bed public tertiary care and teaching hospital affiliated with Dow Medical College, Karachi. It is a government funded hospital that provides free treatment to patients and is staffed with highly skilled and experienced physicians. The study site will specifically include the pediatric outpatient department (OPD) and the pediatric emergency department (specifically including the less severe cases which are normally found in priority 2 (P2) and priority 3 (P3) of triage) of Dr. Ruth K. M. Pfau, Civil Hospital Karachi. These departments cater to children that are diverse in terms of age and background and mainly belong to the lower socio-economic status.

Since Dr. Ruth K.M Pfau, Civil Hospital Karachi caters to many children of lower socio-economic status, using it as a study site enhances the precision and internal validity of the study results. Additionally, the potential diversity in the age and background of these children may also contribute to the generalizability of the study findings. This study site will only be available for data collection once the Institutional Review Board (IRB) of Dow University of Health Sciences (DUHS) has given ethical approval, ensuring the preservation of ethical principles.

Data collection from this site will occur over a period of one month from the middle of September to the middle of October 2024.

Sampling technique and study population

Purposive sampling will be used to recruit study participants. These participants will include all children aged 7 to 12 years visiting physicians in the public tertiary care hospitals in Karachi, Pakistan who have been prescribed C-reactive protein (CRP), ferritin, complete blood count (CBC) tests by the physicians of Dr. Ruth K. M. Pfau, Civil Hospital Karachi in Karachi, Pakistan.

Eligibility criteria

Inclusion Criteria

Children aged 7 to 12 years, residing with an adult family member in the same household, will be included in the study. Both the children and the accompanying adults must be able to understand and communicate effectively in Urdu. Additionally, the children should be prescribed C-reactive protein (CRP), ferritin, and complete blood count (CBC) tests by the study site physicians. Participation requires the informed assent of the children and the informed consent of their accompanying adult family members.

Exclusion Criteria

However, children will be excluded from the study if they have been recently diagnosed with anemia, such as thalassemia, sickle cell anemia, or hereditary spherocytosis, by a healthcare professional; have a history of chronic diseases such as diabetes, or chronic obstructive pulmonary disease (COPD), also diagnosed by a healthcare professional; have undergone repeated blood transfusions; or are involved in long-term drug use (more than 3 months) such as antihypertensives, antihistamines, antiepileptics, and non-steroidal anti-inflammatory drugs (NSAIDs), which may adversely affect salivary flow [24, 25]. Additionally, children taking iron supplements, those with a known history of cleft lip and/or palate, those undergoing orthodontic intervention, and those with a known mental or physical disability will be excluded. Children will also be excluded if they have at least one partially erupted or unerupted permanent first molar or both permanent central incisors in either jaw, have overt fillings or crowns in the first permanent molars placed for reasons other than caries in these teeth, or have overt developmental enamel defects such as enamel hypoplasia, hypomineralization, and fluorosis.

Recruitment of participants

Potentially eligible children, along with their accompanying family members, will be respectfully approached in both the pediatric outpatient department (open during daytime hours) and the pediatric emergency department (specifically P2 and P3 of triage, operational around the clock) at Dr. Ruth K. M. Pfau, Civil Hospital Karachi, for screening purposes. Verbal consent will be obtained from both parties prior to screening. The child will then be assessed for eligibility in the study through a screening questionnaire and a brief oral examination. If deemed eligible, these children and the adult family members will be invited for participation in the study. Once informed assent and consent is obtained of both

respectively, data collection will proceed through a pre-tested questionnaire and physical, oral and laboratory examination. The adult family member will be asked questions pertaining to the socio-demographic characteristics and the medical history of the child. Subsequently, the child will be questioned with regard to their oral hygiene-related habits, other habits and cariogenic diet. After the questionnaire is filled, the child will be appropriately and respectfully approached for physical and oral examinations. Physical examination will include the assessment of their height and weight while the oral examination will include the examination of the level of oral hygiene through the simplified oral hygiene index (OHI-S) and carious status through the Decayed, Missing and Filled Teeth (DMFT/dmft) index of the permanent first molars and its adjacent teeth (permanent second premolar/primary second molar and permanent second molar). Oral examination will also include the assessment of the entire dentition for carious teeth which will be appropriately relayed to the adult family member (provided a referral slip with this information too) and the child will be referred (if needed) to the dentists in Dr. Ruth K. M. Pfau, Civil Hospital Karachi for free treatment. This will be followed by the provision of appropriate oral hygiene instructions given to the accompanying adult family member (through a flyer) for the upkeep of pediatric oral health and the appropriate laboratory tests of the child being done as prescribed. All the aforementioned data will be collected using a questionnaire via the REDCap software and all procedures will be performed by the first author of this study except for the blood samples which will be taken by trained personnel.

Study variables

The study outcome variable is dental caries in the permanent first molars which will be assessed using the DMFT index [1]. Each permanent first molar will be examined and coded as ‘D’ for decay, ‘M’ for missing, ‘F’ for filled or 0 for sound. If the DMFT index score> 0 of

at least one permanent first molar, the child will be considered carious and will be treated as such in analysis (carious/non-carious).

The primary exposure in this study is iron deficiency anemia. Anemia will be defined as hemoglobin levels less than 11.5 g/dL and 12 g/dL [26] in children aged 5 to 11 years and 12 to 14 years respectively. Iron deficiency anemia will be defined as the concurrent presence of anemia with other low parameters indicating absolute iron deficiency such as ferritin levels less than 15 microgram/L [26]. However, in the presence of an infection/inflammation (as determined by C-reactive protein (CRP) levels of greater than or equal to 10 mg/L [27]), ferritin levels less than 70 microgram/L [28] will be used instead.

Therefore, iron deficiency anemia will be diagnosed based on the concurrent presence of laboratory results in which hemoglobin and the ferritin levels (adjusted based on the CRP levels) are less than their appropriate cut-offs.

A priori confounders will include the usual monthly household expenditure, recorded as the sum of monthly household expenses related to food items, utilities (gas, water, and electricity), medical costs, clothing, social activities, and miscellaneous items. Another a priori confounder is the body mass index (BMI), calculated and categorized using the World Health Organization (WHO) growth charts for boys and girls aged 5 to 19 years [29]. Additionally, smokeless tobacco use, education level of both parents and the child, gender, and age will be considered as a priori confounders.

Other independent variables will be included as additional potential confounders, such as the number of previous diagnoses of iron deficiency anemia (since age 6), time of last dental visit and reason for the visit, tooth brushing frequency, and the use of a fluoridated toothpaste. Additional oral hygiene practices including the regular use (defined as at least once a day) of toothpowder, mouthwash, and miswak, the number of adjacent carious teeth (next to the

permanent first molar) assessed using the DMFT/dmft index [1], and a composite score of the frequency of cariogenic dietary consumption (over the last six months) will also be considered.

Moreover, the Simplified Oral Hygiene Index (OHI-S) [30] will also be included as an independent variable to indicate the level of plaque and calculus in the mouth. This will be categorized as follows: a score of 0-1.2 will be classified as 'good', a score of 1.3-3 will be classified as 'fair', and a score of 3.1-6 will be classified as 'poor' [31].

Data collection method and tools

Data collection method will include:

- Filling the screening questionnaire to assess the eligibility of the participant.
- Taking informed consent from the accompanying family member and informed assent from the child.
- Filling the structured questionnaire that asks information on the outcome variable, primary exposure variable, a priori confounders and the independent variables.

The questionnaires will be administered to the study population in Urdu.

Screening questionnaire

The screening questionnaire will be administered after obtaining appropriate verbal consent. It will include questions and oral examinations consistent with the eligibility criteria of the study. After the relevant questions are asked and the child is considered eligible based on them, oral examination will take place.

Oral cavity examination will be conducted under adequate illumination. Cheek retraction and occlusal assessment (the child will be asked to close their mouths and the relevant teeth will be examined to see if they meet the teeth of the opposing arch) will determine if the

permanent first molars and permanent central incisors have fully erupted, with unerupted/partially erupted status noted only if no prior extraction due to caries is confirmed (for the permanent first molars).

Following this procedure, the permanent first molars will be visually inspected to see if there are any overt fillings/crowns present in the permanent first molars (the accompanying adult will be asked if the filling/crown was placed because of a reason other than decay). If both permanent central incisors in either arch or at least one permanent first molar is not fully erupted and there are any fillings/crowns involving the permanent first molars that were not placed because of decay in these teeth, then that child will be considered ineligible.

The oral examination will also assess each permanent first molar for enamel developmental defects using the modified developmental defects of enamel index (mDDE) [32]. Originally introduced by the Fédération dentaire international (FDI) Commission on Oral Health in 1982 [33] and modified by Clarkson et al. in 1989 [32], this index has been widely used in various studies, including a study in Islamabad, Pakistan [34]. It will serve as a reference for detecting enamel defects predisposing the child to caries [35, 36]. Demarcated opacity of ≤1 mm will be considered sound [37], and defects will also be considered sound, if there are any uncertainties regarding them, except for demarcated brown opacities, where history will be taken.

Under proper illumination, the data collector will retract the child's cheek using a mouth mirror and utilize a dental explorer for enhanced examination sensitivity. Assessment of the buccal, lingual/palatal, and occlusal surfaces of the permanent first molar will be conducted to detect any white/cream/yellow or brown demarcated opacity. Its presence will result in exclusion. However, if there is any uncertainty regarding the brown opacities, history of frequent betel leaf and/betel nut with or without tobacco, gutkha, tea and coffee consumption will be taken and the absence of these habits will result in exclusion. Additionally, inspection

will also identify diffuse white opacity with linear/patchy/confluent (patchiness that combines leading to a chalky white area) distributions, and hypoplasia (single/multiple pits/missing enamel (including single/multiple grooves or greater area of enamel that is missing)), with any presence rendering the child ineligible for the study.

Only if the child satisfied all of the aforementioned eligibility criteria, will his/her informed assent along with accompanying adult family member's informed consent be taken before proceeding to the study questionnaire.

Study questionnaire

This questionnaire will have the following seven sections (For access to Study questionnaire, refer to Supplemental Material):

Sociodemographic information

This section will include the child's age, gender and education level. It will also include questions on the education level of both parents and the usual monthly household expenditure on food items, utilities including gas, water and electricity, medical related, clothing items, social activities, and miscellaneous items.

Medical history

This section will include questions on if the child has ever been diagnosed with iron deficiency anemia from the age of 6 by a healthcare professional and how many times have they been diagnosed with iron deficiency anemia since then by a healthcare professional.

Oral hygiene related behavior

This section will include questions on when the child last visited the dentist, what was the reason for that visit, how frequently the child brushes their teeth, whether the child uses toothpaste, what toothpaste the child uses to clean their teeth and if they regularly use any of the listed oral hygiene practices at least once a day. These questions were obtained from the WHO Oral health assessment form for children [1] and were somewhat adapted to the objectives of this study.

Therefore, due to this adaptation, its content validation was done by a team of eight dental experts. These experts rated each question in terms of its clarity and relevance on a scale of 1 (not relevant/clear) to 4 (very relevant/clear) and also gave feedback on each question, which was taken into consideration for further improvement of the questions. Results indicated item-level content validity index (I-CVI) scores ranging from 0.875 to 1, a scale-level content validity index score based on the average (S-CVI/Ave) and universal agreement method (S-CVI/UA) of 0.975 and 0.8 respectively for both relevance and clarity indicating appropriate relevance and clarity of this section of the study questionnaire [38].

Other habits

This section will include one question on how often the child uses smokeless tobacco (snus, snuff, and chewable tobacco (gutkha, betel leaf and/areca nut with tobacco)). This question was also obtained from the WHO oral health assessment form for children [1] and adapted appropriately.

Dietary information

This section will include questions on how often, on average, in the last six months the child had consumed cariogenic food items. Processed starch and extrinsic sugars

enhances the cariogenicity of the diet; the food items included contain either or both of these and are commonly found in Pakistan [39].

Physical and oral examination

Physical examination

The child's height and weight will be measured using an inch tape (in centimeter (cm) which will be converted to metres for BMI calculation) and a bathroom weighing machine (in kilograms (Kg)) respectively.

Oral examination will include the simplified oral hygiene index and the DMFT/dmft index of the permanent first molars and its adjacent teeth.

The simplified oral hygiene index:

The simplified oral hygiene index was created in 1964 by Vermilion and Greene [40] and it is the addition of the simplified debris and calculus index which are measures of plaque and calculus respectively in the oral cavity [30]. It provides a quick, sensitive, quantitative measure of assessing the oral hygiene status of the individual [40].

Before beginning the oral examination, the child will be seated comfortably. Under sufficient illumination, the permanent first molars of each side and arch with the permanent central incisors will be visualized and inspected using an explorer which will improve sensitivity. His/her cheek will also be retracted, as needed, using a mouth mirror. If the right permanent central incisor of the upper arch or the left permanent central incisor of the lower arch is missing, its adjacent permanent central incisor, will be used instead.

In this study, the buccal surfaces of permanent first molars and the permanent right central incisor of the upper arch will be inspected. For the lower arch, the lingual surfaces of the permanent first molars and the buccal surface of the permanent left central incisor will be inspected.

Debris index simplified and calculus index simplified will be assessed and calculated separately for each surface. For the debris index, a code of 0 will be given, if there is no debris or extrinsic stain present. A code of 1 will be given if debris does not cover more than a third of the surface of the tooth or if there is an extrinsic stain present with no debris regardless of the tooth surface coverage. A code of 2 will be given, if the debris covers more than one third of the tooth surface but less than two thirds of the surface. A code of 3 will be given, if the debris covers more than two thirds of the tooth surface. Debris index simplified will be calculated of each child by adding all the debris index simplified scores of the buccal and lingual surfaces and dividing it by the number of surfaces visualized.

Calculus index simplified will be assessed similarly. If calculus is absent, a code of 0 will be given. If the supragingival calculus does not cover more than one-third of the tooth surface, a code of 1 will be given. If more than one-third but less than two-thirds of the tooth surface is covered in supragingival calculus, or if there are flecks of subgingival calculus in the tooth's cervical area, or both, a code of 2 will be given. If more than two thirds of the tooth surface is covered in supragingival calculus, or if there is a continuous band of subgingival calculus in the tooth's cervical area, or if both are present, the tooth surface will be given a code of 3. The calculus index simplified of the child will be calculated by adding the calculus index simplified scores of all

buccal and lingual surfaces and dividing it by the number of surfaces examined.

Simplified oral hygiene index will be calculated, subsequently, by adding the overall scores of the calculus index simplified and debris index simplified.

DMFT/dmft index of the permanent first molar and its adjacent teeth:

The DMFT index is the most used tool for caries diagnosis and is an aggregate of the total number of teeth that are decayed, filled or missing (due to caries) reflecting the teeth that have been affected by caries. It is usually used for primary/permanent dentitions but has been used for specific types of teeth such as the permanent first molars in studies as well [41]. Before beginning the oral examination, the child will be seated comfortably, and the examination of the teeth will be done using an explorer under sufficient illumination of the oral cavity. His/her cheek will also be retracted using a mouth mirror, as needed and the tooth will be dried with a gauze to improve the visual inspection. Then each permanent first molar and its adjacent permanent tooth will be examined and will be coded as 'D' for decay, 'M' for missing, 'F' for filled or 0 for sound. A code for 'D' will be given if there's an overt cavity, undermined enamel or detectably softened walls/floor are found or felt on probing with or without a permanent filling. If the tooth has a temporary filling, the tooth will still be recorded as 'D'. However, if a permanent filling is present due to previous decay (which the child or the accompanying family member will be asked about) without the aforementioned lesion, then the tooth will be recorded as filled and will be coded in the form as 'F'. If the tooth is missing and after appropriate

questioning from the child and the accompanying adult, it is found to be missing due to caries, it will be recorded as 'M' in the form. If no such case occurs, then the tooth will be considered sound and will be coded as '0'.

The DMFT score of each permanent first molar will be added (in which equal weight will be given to codes 'D', 'M' and 'F') for each child and will be recorded in the form. Similarly, the DMFT score of each adjacent permanent tooth (permanent second premolar and molar) will be added and recorded in the form. This process will also be repeated for the primary second molars, where instead of the DMFT index, the dmft index will be recorded for each tooth and added for each child [1].

Laboratory-related information

Data will be collected on ferritin, hemoglobin, and C-reactive protein for the diagnosis of iron deficiency anemia (in grams/dL, micrograms/L and milligrams/L respectively).

2 ml and 6 ml venous blood will be drawn from the patients in a ethylenediamine tetraacetic acid (EDTA) and two gel tubes respectively by experienced and trained personnel in an aseptic environment. The blood sample will be transported to nearby laboratory for analyses in ice packs (for the children presenting to the pediatric ER, the children in the pediatric OPD will have their blood drawn in the laboratory).

The laboratory will analyze the CBC using Abott Alinity hq Hematology Anlyzer (Abott), CRP using Atellica CH CRP_2 (Siemens Healthineers), and Ferritin using Attelica IM Ferritin (Siemens Healthineers).

Data management

The data will be collected through a web-based questionnaire using the REDCap software, with daily checks by the first author to ensure completeness, accuracy, and logical consistency. Inconsistent or missing information (marked as 'I don't know') will be verified by contacting the accompanying adult family member. This software's conditional logic and range checks will help maintain data accuracy, and all study questions will be set as required fields to minimize missing values.

Data cleaning and detection of missing values will be managed by the first author, who will sort each variable in ascending and descending order. Missing data will be handled using the complete-subject analysis approach. The first author, a qualified and trained dentist, will be responsible for data collection to ensure ethical and sensitive handling of information, as well as to limit bias and promote uniformity in the data collection process.

Questionnaires were translated into Urdu from English by a translator, and an expert reviewed the translation for cultural appropriateness, making necessary changes. Back translation was performed to ensure the original intent and essence of the queries were preserved. Pre-testing of the study tools will be conducted on 5% of the study sample (n=7 children) at the study site to identify any difficult questions that may require paraphrasing or re-translation.

To protect confidentiality, all documents used for data analysis and dissemination will not include participants' name and contact details, only a unique eight-character alphanumeric identification (ID) assigned to each child. The link between this ID and participant contact details will be kept in a separate file under the first author's custody. Data will be stored electronically in a password-protected database, with backup files made to prevent data loss. After seven years' post-study completion, data will be securely deleted using erasure software.

Validity

There are several potential biases that could affect the validity of this hospital-based study. Firstly, confounding bias may occur due to unequal distribution between the exposure groups of undiagnosed/unreported medical conditions, medication histories and unaccounted for cariogenic items in the child's diet that may affect caries risk. Secondly, wish bias might be introduced in the study through study questions on sociodemographic, diet, smokeless tobacco consumption and oral hygiene related behaviour. This may be mitigated by refining questions to be more neutral and less judgmental during pretesting. Moreover, a more objective assessment of oral hygiene will also be done using the simplified oral hygiene index. Thirdly, recall bias may occur during data collection of the cariogenic dietary information of the child. This will be mitigated by focusing on the child's diet over the last six months and questioning in front of the accompanying family member for verification. Lastly, non-differential misclassification may occur due to the utilization of the bathroom weighing machine. This bias will be mitigated by calibrating the bathroom weighing machine daily and checking its calibration using 1 kg weight. Calibration accuracy will be further verified using a 1kg weight. Moreover, each child will be weighed twice, and in the event of any discrepancy between the two measurements, the weighing machine will undergo recalibration and the process will be repeated.

Sample size

The sample size is calculated for both objectives as follows:

Frequency of children aged 7 to 12 with carious first permanent molars

The sample size is calculated using the World Health Organization (WHO) Sample Size Determination in Health Studies Software Version 2.0.21. A previous study conducted in Pakistan that had reported a frequency of 38.5% [42] of carious permanent first molars will

be used to calculate the sample size with an absolute precision of 9% and a confidence level of 95% (chosen to maintain the feasibility of the study), yielding a minimum sample size of 113 children aged 7 to 12 years.

Association of iron deficiency anemia (IDA) and dental caries in first permanent molars in children aged 7 to 12

The sample size is calculated using STATA software version 15.0. A power estimation of 80% and level of significance of 5% is chosen for use. The ratio of exposed to unexposed children is obtained from a previous study that was conducted in Shaheed Benazir district in Pakistan in 11 to 13-year-old children that had reported a prevalence of 15.5% [15] yielding a ratio of 1:5. Furthermore, to obtain the percent of unexposed children with the outcome, a previous study will be used that was conducted in India [43] and that had evaluated the association between iron deficiency anemia and caries, reporting 37.8% of children without iron deficiency anemia having dental caries. Using these aforementioned parameters, a minimum sample size of 76 children aged 7 to 12 years, will be required to assess the association of iron deficiency anemia and dental caries in the permanent first molars in order to detect a one-sided outcome prevalence ratio of 2.

Since the sample size of the first objective is the greater sample size calculated of the two objectives, it is selected for use in this study. After considering a 20% rate of nonresponse and ineligible participants, a final minimum sample size of approximately 141 children aged 7 to 12 is obtained.

Statistical analysis

All analysis will be done using STATA software version 15.0. The frequency of children with carious permanent first molars with 95% confidence intervals will be reported.

Descriptive statistics will also be reported of all independent variables (such as age, gender, usual monthly household expenditure, education levels of both parents and child, BMI etc.) stratified on the level of the iron deficiency anemia. All categorical variables (such as gender, child education status etc.) will be reported in the form of frequency and percentages. Normality of the quantitative variables will be assessed using boxplots, histograms and Kalmogorov-Smirnoff tests. For quantitative variables (such as age, number of adjacent carious teeth) that have distributions that are not skewed, descriptive statistics will be reported in the form of mean and standard deviation. However, if these variables have skewed distributions, median and interquartile range (IQR) will be reported instead. If the expected frequency per cell is at least 5, then chi-square test will be done to assess if the two exposure groups significantly differ in terms of their categorical predictors and their p values will be reported. However, if this frequency is less than 5, then Fischer's exact test will be used instead. To assess the same but for quantitative predictors, an independent sample t-test will be used, and their p values will be reported.

Factor analysis will be used for dimensional reduction for the cariogenic dietary construct into a single composite score. Variables with factor loadings greater than 0.4 and eigenvalues greater than 1 will be selected. This composite score will then be used to represent the frequency of cariogenic food consumption.

COX proportional hazard regression will be used to assess the association of iron deficiency anemia with dental caries in permanent first molars. Initially, univariate analysis of iron deficiency anemia and all a priori confounders and other independent variables (such as age, gender and child education level among others) will be done with caries in the permanent first molars of children and their prevalence ratios with 95% confidence intervals will be reported. Variables that are associated with caries in the permanent first molars at the univariate level (p value < 0.25) will be assessed along with iron deficiency anemia for

multicollinearity (r > 0.8). This will be followed by the assessment of confounding and multivariable analysis. Stepwise model building will be employed to construct a multivariable model. Starting with iron deficiency anemia, variables will be sequentially added to the model. With each addition to the model, likelihood ratio tests will be used to establish whether inclusion of the additional variable enhanced the fit of the model significantly (p value <0.05). Biologically plausible interaction(s) will also be assessed (p value <0.1). The parsimonious model will be determined when additional variables cannot be included and none can be removed without compromising the fit of the model. Adjusted prevalence ratios with 95% confidence intervals will be reported of caries in the permanent first molars.

Patient and public involvement statement

The patients and the public are and will not be involved in the design, conduct, reporting, or dissemination plans of this research.

ETHICS AND DISSEMINATION

All study activities will be conducted in accordance with the Declaration of Helsinki and the International Council for Harmonization (ICH) guidelines for Good Clinical Practice (GCP). Ethical approval has been obtained from the ethical review committee (ERC) of Aga Khan University (AKU) (Project ID: 9692) and the institutional review board (IRB) of Dow University of Health Sciences (DUHS) (IRB Reference: IRB-3556/DUHS/Approval/2024/196).

Informed assent and consent will be taken from the children and the accompanying adult family members respectively before study enrolment. The participants will have the right to withdraw from the study at any point without any consequences.

To ensure safety of the participants, the required blood tests will be conducted by trained personnel. Additionally, all teeth of the participant will be examined for caries and those in need will be referred to the dentists in the Dr. Ruth K. M. Pfau, Civil Hospital Karachi for free dental treatment. Moreover, to manage anxiety associated with the oral examinations, tell show do technique will be used and they will be given instructions on pediatric oral hygiene through flyers.

The study results will be disseminated through seminars and published in a peer reviewed academic journal.

LIST OF ABBREVIATIONS

AKU- Aga Khan University

BMI- Body Mass Index

CBC- Complete blood count

COPD- Chronic Obstructive Pulmonary Disease

CRP- C-reactive protein

cm- Centimetres

DMFT- Decayed, Missing, and Filled Teeth

DUHS- Dow University of Health Sciences

ER- Emergency room

ERC- Ethical Review Committee

EDTA- Ethylenediamine tetraacetic acid

FDI- Fédération Dentaire Internationale

I-CVI- Item-level Content Validity Index

ID- Identification

IDA- Iron Deficiency Anemia

IRB- Institutional Review Board

IQR- Interquartile range

Kg- Kilogram

mDDE- modified Developmental Defects of Enamel

NSAID- Non-steroidal anti-inflammatory drug

OPD- Outpatient department

P2- Priority 2

P3- Priority 3

PKR- Pakistani Rupee

S-CVI/Ave- Scale-level Content Validity Index based on the Average method

S-CVI/UA- Scale-level Content Validity Index based on the Universal Agreement method

OHI-S- Simplified Oral Hygiene Index

STROBE- Strengthening the Reporting of Observational Studies in Epidemiology

WHO- World Health Organization

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AUTHOR CONTRIBUTIONS

SS, and SST conceived and designed the study. SS will be responsible for data collection and data management. SS, SST, IA will be involved in data analysis and interpretation. SST, IA, WAJ, AFS and FRK have revised the work and have provided their intellectual input. SS wrote the first draft of the manuscript. All authors have appropriately revised, read and approved the submitted version of this protocol.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

DATA SHARING STATEMENT

Not applicable, as data collection hasn't started yet.

Study Title: Association of iron deficiency anemia with dental caries in the permanent first molars of children aged 7 to 12 years: An analytical cross-sectional study in Karachi, Sindh, Pakistan

Instructions:

1. Separate form should be filled for each subject.
2. This form should only be filled after the subject has been screened successfully and has been considered eligible for the study.

Child ID: _____

Version number and Date: __ (_ _/_ _/_ _ _ _)

Section A: Socio-demographic information

(asked from the legal guardian)

S. No	Question	Code	Skip	Response
1.	Gender of child?	1. Female 2. Male		
2.	What is the age of child?	Write exact age in years		__ __ Years
3 a.	Has the child ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q4a.	
3 b.	What is the education status of your child?	1. Illiterate 2. Literate but no formal schooling 3. Schooling 3.1 if schooling= please specify Grade _____ 9. I don't know		
4 a.	Has the mother ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q5 a	
4 b.	What is the mother's education status?	1. Illiterate 2. Literate but no formal schooling 3. Primary education		

		4. Secondary education 5. Higher secondary education 6. Above higher secondary education 9. I don't know		
5 a.	Has the father ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q6.	
5 b.	What is the father's education status?	1. Illiterate 2. Literate but no formal schooling 3. Primary education 4. Secondary education 5. Higher secondary education 6. Above higher secondary education 9. I don't know		
6.	What is the usual monthly household expenditure on food items?	Write the exact amount in PKR '98' is I don't remember		
7.	What is the usual monthly household expenditure on utilities such as gas, water and electricity?	Write the exact amount in PKR '98' is I don't remember		
8.	What is the usual monthly household medical expenditure?	Write the exact amount in PKR '98' is I don't remember		
9.	What is the usual monthly household expenditure on clothing items?	Write the exact amount in PKR '98' is I don't remember		
10.	What is the usual monthly household expenditure on social activities such as gifts, parties etc.?	Write the exact amount in PKR '98' is I don't remember		
11.	What is the usual monthly household expenditure on miscellaneous items?	Write the exact amount in PKR '98' is I don't remember		

Section B: Medical history

(taken from the legal guardian)

S. No	Question	Code	Skip	Response
12 a.	Has the child ever been diagnosed with iron deficiency anemia from the age of 6 by a healthcare professional?	1. Yes 2. No 9. I don't know	If 'No' or 'I don't know', skip to S. No 13 a.	
12 b.	How many times have your child been diagnosed with iron deficiency anemia from the age of 6 by a healthcare professional?	Write the exact number of times '98' is I don't remember		

Section C: Oral hygiene-related behavior

(taken from the child)

S.No	Question	Code	Skip	Response
13 a.	When was the last time you went to the dentist?	1. Never 2. >1 year ago 3. Between 6 months and a year ago 4. <6 months ago 9. I don't know	If 'Never' skip to S. No 14	
13 b.	What was the reason for your visit to the dentist?	1. Pain or trouble with teeth, gums or mouth 2. Treatment/Follow-up treatment 3. Routine check-up of teeth 4. Other reasons Please specify: _____ 9. I don't know		
14.	How frequently do you brush your teeth?	1. Never 2. 2-3 times a month 3. 2-6 times a week 4. Once a day 5. ≥ 2 times a day 9. I don't know	If 'Never' or 'I don't know' skip to S. No 16.	

15 a.	Do you use a toothpaste?	1. Yes 2. No 9. I don't know	If 'No' or 'I don't know', skip to S. No 16	
15 b.	What toothpaste do you use to clean your teeth?	Write the exact response. '98' is I don't remember		
16.	Do you regularly use any of these other oral hygiene practices atleast once a day?	1. Toothpowder 2. Mouthwash 3. Miswak 4. All of the above 5. None of the above 6. Toothpowder and mouthwash 7. Toothpowder and miswak 8. Mouthwash and miswak 9. I don't know		

Section D: Other habits

(taken from the child)

S. No	Question	Code	Skip	Response
17.	How often do you use smokeless tobacco including snus, snuff, chewable tobacco (gutkha, betel leaf and/areca nut with tobacco)?	1. Never 2. 2-3 times a month 3. Once a week 4. 2-6 times a week 5. Every day 9. I don't know		

Section E: Dietary information

(taken from the child)

S. No 18. How often have you consumed, on average, the following food items in the last six months?

	Items	Never/ < once a month	1-3 times a month	Once a week	2 to 4 times a week	5 to 6 times a week	Once a day	2 to 3 times a day	4 to 5 times a day	6 to 7 times a day
	Bread and Cereals									
1	Porridge with sugar									
2	Frozen paratha/ Street food paratha									
3	Homemade paratha (Refined Flour)									
4	White bread/ Bun									
5	Rusk/ Cake Rusk									
6	Two-minute noodles/ Macaroni/ Spaghetti									
7	Biscuit / Cookie									
8	Sandwich biscuit									
9	Donut									
10	Cupcake/Muffin/Plain Cake									
11	Pizza									
	Desserts									
12	Kheer/ Custard/Pudding									
13	Cake/Pastry									
14	Meethai/halwa									
15	Sweetened yoghurt									
16	Ice cream									
17	Plain Jelly									
18	Fruit chaat (that has added sugar)									
	Beverages									
19	Tea with sugar									
20	Sweet lassi (flavored or unflavored)									
21	Soft drink									
22	Sharbat / Instant Drink									
23	Fruit juice									
24	Flavored milk									
	Miscellaneous									
25	French fries									
26	Chocolate									
27	Candy									
28	Cotton candy									
29	Chewing gum (not the sugar-free one)									
30	Lollipop									
31	Jam (for example on bread)									

32	Honey (for example on bread)									
33	Chocolate spread (for example on bread)									
34	Chips									

Section F: Physical and oral examination

(taken of the child)

S. No	Measurement	Finding
19.	Height (in cm)	
20.	Weight (in kg)	

S. No 21 **Oral hygiene status assessment**

Debris Index Simplified:

	Right permanent first molar		Permanent central incisor		Left permanent first molar		Total	
	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual
Upper	_____		_____ (right)		_____		_____	
Lower		_____	_____ (left)			_____	_____	_____

Debris Index Simplified= (The buccal scores) + (The lingual scores)/ (Total number of examined buccal and lingual surfaces)

=_____

Calculus Index Simplified:

	Right permanent first molar		Permanent central incisor		Left permanent first molar		Total	
	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual
Upper	_____		_____ (right)		_____		_____	
Lower		_____	_____ (left)			_____	_____	_____

Calculus Index Simplified = (The buccal scores) + (The lingual scores)/ (Total number of examined buccal and lingual surfaces)

=_____

Simplified Oral Hygiene Index= Debris Index Simplified + Calculus Index Simplified=

_____

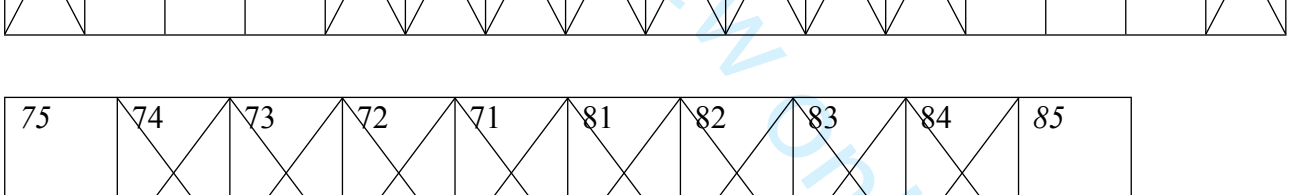
S. No 22 **Carious assessment of the permanent first molars and adjacent teeth**

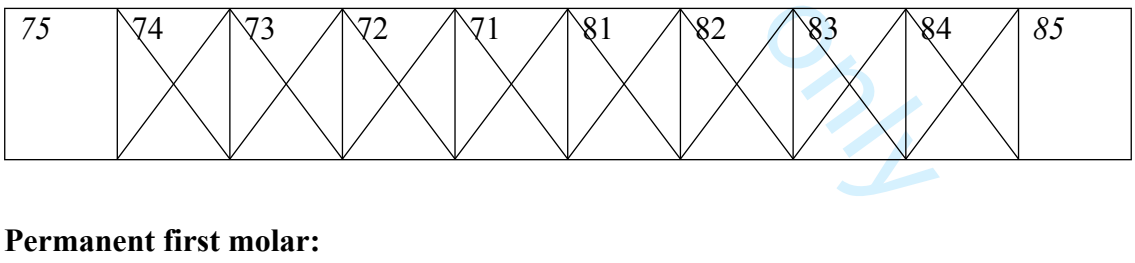
DMFT/dmft index:

Permanent teeth	Primary teeth
D= Decayed	d= decayed
M= Missing	m= missing
F= Filled	f= filling

Maxilla

Mandible





Permanent first molar:

Total Decayed=_____ Total Missing=_____ Total Filled=_____

Total DMFT index score=_____

Adjacent teeth:

Total Decayed=_____ Total Missing=_____ Total Filled=_____

Total DMFT index score=_____

Total decayed=_____ Total missing=_____ Total filled=_____

Total dmft index score=_____

Section F: Laboratory-related information

S. No	Blood parameter	Code	Finding
23.	Hemoglobin (g/dL)		
24.	Ferritin (microgram/L)		
25.	C-reactive protein (milligram/L)		

Form filled by: _____ Date: _ _/_ _/_ _ _ _ *(Day/Month/Year)*

Form checked and edited by: _____ Date: _ _/_ _/_ _ _ _ *(Day/Month/Year)*

BMJ Open

Protocol for an Analytical Cross-Sectional Study on the Association of Iron Deficiency Anemia with Dental Caries in the Permanent First Molars of Children Aged 7 to 12 Years in Karachi, Pakistan.

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2024-092312.R1
Article Type:	Protocol
Date Submitted by the Author:	11-Nov-2024
Complete List of Authors:	Syed, Sadaf; The Aga Khan University, Community Health Sciences Azam, Iqbal; Aga Khan University, Community Health Sciences Jamalvi , Waseem ; Dr Ruth KM Pfau Civil Hospital Karachi, Paediatrics; Dow University of Health Sciences, Paediatrics Khan, Farhan; Aga Khan University, Department of Surgery, Section of Dental Surgery Saleem, Ali; Aga Khan University, Paediatrics and Child Health Tikmani, Shiyam; The Aga Khan University, Community Health Sciences
Primary Subject Heading:	Dentistry and oral medicine
Secondary Subject Heading:	Epidemiology, Nutrition and metabolism, Paediatrics, Public health, Global health
Keywords:	Anaemia < HAEMATOLOGY, Child, Cross-Sectional Studies

WORD COUNT: Abstract: 319 words, Text (from Introduction to Ethics and Dissemination): 5419 words

TITLE

Protocol for an analytical cross-sectional study on the association of iron deficiency anemia with dental caries in the permanent first molars of children aged 7 to 12 years in Karachi, Sindh, Pakistan

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ABSTRACT

Introduction

Iron deficiency anemia and dental caries are prevalent diseases among Pakistani children. Limited research has been done to explore their association in permanent teeth. Given the caries susceptibility of permanent first molars and their role in the development of ideal occlusion, this study aims to estimate caries frequency in these molars and assess its association with iron deficiency anemia in 7 to 12-year-old children.

Methods and analysis

This analytical cross-sectional study will include 141 children aged 7 to 12 years visiting physicians in the pediatric outpatient department of Dr. Ruth K.M Pfau, Civil Hospital Karachi. Using consecutive sampling, children who met initial screening criteria were further evaluated to determine eligibility for the study. Data collection will involve physical examinations (including weight and height), oral examinations (including the relevant oral hygiene and caries assessments), and laboratory examinations (including the prescribed tests). Additionally, questions will be asked about socio-demographic characteristics, history of iron deficiency anemia, oral hygiene habits, smokeless tobacco use, and the frequency of cariogenic dietary consumption. Exposure variable will include the presence of iron deficiency anemia, assessed using complete blood count, C-reactive protein and ferritin tests, and treated as a dichotomous variable. Outcome variable will include dental caries in at least one permanent first molar, assessed using the DMFT index and also treated as a dichotomous variable. Analysis will include COX proportional algorithm, reporting prevalence ratios with 95% confidence intervals for the association of iron deficiency anemia and dental caries in the permanent first molars. Frequency of children with carious permanent first molars with 95% confidence intervals will also be reported.

Ethics and dissemination

This research has been approved by Ethical Review Committee of Aga Khan University (Reference number: 2024-9692-30593) and the Institutional Review Board of Dow University of Health Sciences (IRB Reference: IRB-3556/DUHS/Approval/2024/196) prior to participant recruitment. Results will be disseminated through seminars and peer reviewed publications.

Keywords: Anemia, Iron deficiency; Child; Dental Caries; Dentition, Permanent; Molar

ARTICLE SUMMARY

Strengths and limitations of this study

1. This study will use objective laboratory tests for the diagnosis of iron deficiency anemia and the validated DMFT index, recommended by WHO, for caries diagnosis in children.
2. It will control for the confounding associated with the mixed dentition stage, by specifically focusing on the permanent first molar which has the greatest caries risk.
3. Additionally, this study will be conducted among children belonging to the lower socioeconomic background, that are more likely to experience both the exposure and outcome; thereby controlling confounding associated with the socioeconomic status.
4. Even though this focus on permanent first molars of children belonging solely to low socioeconomic status will improve the internal validity of the study, it may compromise its external validity.
5. Moreover, since this is a cross-sectional study and the exposure and outcome are measured at one point in time, it is inherently susceptible to temporal bias.

INTRODUCTION

Dental caries is the most prevalent global disease [1], causing significant pain and impacting oral health-related quality of life [2] and nutritional intake [3] of children. In 2017, untreated dental caries affected approximately 2.3 billion people [4]. Caries prevalence in permanent teeth is highest in Africa (58.9%), followed by Asia (58.8%) and Australia (54.9%) [5]. Regionally, its prevalence in the Eastern Mediterranean Region is 61% among 12-year-olds and 70% among 15-year-olds [6].While locally, its overall prevalence in Pakistan is 56.6%, with 61.2% in mixed dentition and 57.2% in permanent dentition [7].

Anemia affects over 1.9 billion people globally [8] and had impacted 24.3% of the global population in 2021 [8]. The highest prevalence of anemia is found in Western and Central sub-Saharan Africa (47.4% and 35.7%, respectively) and South Asia (43%) [8], which overlaps with the regions heavily affected by dental caries. Dietary iron deficiency has been reported to be its leading cause among most demographic groups, including children [8].

Despite its significant impact, particularly in children, research on iron deficiency anemia (IDA) in Pakistani children aged 5-12 is limited, with varying prevalence rates reported across regions: 19% for 5-16 year-olds in rural Swat [9], 62.2% for children under 15 in Lahore [10], and 15.5% for 11-13 year-olds in Shaheed Benazirabad [11].

Studies worldwide have found a significant association of IDA with caries in children [12, 13, 14], though, most have focused on younger age groups, with limited representation of school-age children. Some studies have proposed mechanisms supporting this association such as impaired saliva buffering [15], reduced enamel protection against demineralization [16], and antibacterial effects of iron [17, 18].

Given the prevalence of both conditions and their significant adverse effects on children's health, establishing a link between IDA and caries in Pakistani school-age children is a crucial research gap. Both conditions may strain the healthcare system and socio-economic conditions despite being preventable. This study aims to address this gap by estimating the frequency of carious permanent first molars among children aged 7 to 12 and assessing its association with iron deficiency anemia. The focus on first permanent molars is significant due to their high caries risk [19] and their role in developing ideal occlusion within this age group [20]. By providing insights into this association, the study's findings could inform future longitudinal research and enhance the integrated management of dental and hematological care for affected children.

OBJECTIVES

1. To investigate the association between IDA and dental caries in the permanent first molars among children aged 7 to 12 years visiting a public tertiary care hospital in Karachi, Pakistan.
2. To estimate the frequency of children aged 7 to 12 years with carious permanent first molars visiting a public tertiary care hospital in Karachi, Pakistan.

HYPOTHESIS

The prevalence of dental caries in the permanent first molars of 7 to 12-year-old children who have IDA is more than 2 times as compared to children who do not have IDA after adjusting for a priori confounders and other independent variables.

METHODS AND ANALYSIS

Study design

An analytical cross-sectional study will be used. This type of design measures the burden of exposure and outcome in a specific population simultaneously and, in doing so, assesses their association in comparative terms.

To ensure that the study design and reporting are transparent and comprehensive in presenting the findings of the study, the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist will be followed.

Study site and setting

To overcome time constraints and for ethical reasons, we will conduct a hospital-based study to ensure an adequate number of children are recruited. Moreover, to obtain a homogenous sample, Dr. Ruth K. M. Pfau, Civil Hospital Karachi located in Karachi, Sindh, Pakistan will be used in this study.

Civil Hospital Karachi (CHK)

CHK is a 1900-bed public tertiary care and teaching hospital located on Mission Road in Karachi, Pakistan. Affiliated with Dow Medical College, it is government-funded and provides free treatment to all patients, mainly including patients from diverse backgrounds, predominantly from lower socio-economic groups. The pediatric outpatient department (OPD) at CHK, operational Monday through Saturday during daytime hours, sees approximately 130 children aged 7 to 12 years daily. It has been selected as the study site due to its diversity and practicality in recruitment, offering a balanced sample of healthier children, including those who may not attend school. This selection helps enhance the precision and internal validity of the study while acknowledging potential limitations in external validity.

Data collection will commence after receiving ethical approval from the Institutional Review Board (IRB) and Ethical Review Committee (ERC) of Dow University of Health Sciences

(DUHS) and Aga Khan University (AKU) respectively. A pre-testing phase will take place from December 2nd to 7th, 2024, followed by recruitment and data collection from 9th December 2024 to mid-February 2025. The recruitment process aims to enroll between 1 to 4 participants per day. Despite the limited operational hours and detailed selection criteria, the high patient volume ensures a feasible and steady recruitment pace. Data cleaning and analysis will take approximately one month, and manuscript preparation is expected to be completed by mid-April 2025.

Sampling technique and study population

In this study, the target population includes children aged 7 to 12 years visiting physicians at CHK, who have been prescribed C-reactive protein (CRP), ferritin, and complete blood count (CBC) tests. Consecutive sampling technique will be used as participants will be recruited in the order they present at the pediatric OPD. This approach will also allow for efficient identification of eligible participants through an initial screening process based on key criteria that can be quickly assessed. The initial screening will determine whether the child is accompanied by a family member from the same household, whether both individuals can understand Urdu, whether the child has been prescribed the relevant blood tests, and if the child has at least one fully erupted permanent central incisor in both arches, along with all fully erupted permanent first molars.

After this preliminary screening, a more thorough evaluation will be conducted to ensure that participants meet the full set of inclusion and exclusion criteria. This sampling approach not only facilitates the rapid identification of potential participants but also ensures that all eligibility requirements are rigorously verified, allowing for a targeted yet efficient selection process for the study.

Eligibility criteria

Inclusion Criteria

The study will include children aged 7 to 12 years who reside with an adult family member in the same household and are able to communicate effectively in Urdu. Both must understand Urdu, as the first author, the sole data collector, is proficient only in this language, and hiring translators or additional data collectors is not feasible due to financial constraints. This requirement ensures clear communication and improves the accuracy and reliability of the data collected.

A key criterion for inclusion is that the child must be prescribed CRP, ferritin, and CBC tests by a physician at the study site. This requirement ensures that the blood tests are medically justified, reassuring parents that these tests serve both clinical and research purposes, which helps increase their comfort with participation.

Additionally, the child must be accompanied by an adult family member who resides in the same household. This is crucial for obtaining accurate socio-demographic information, details about the child's health behaviors, and relevant household data, such as monthly expenditure. Children visiting the pediatric OPD are sometimes accompanied by relatives or non-family members who may not have sufficient knowledge of the child's health and household circumstances. Therefore, the inclusion of a household-residing family member helps ensure the accuracy of the information collected.

Furthermore, informed assent from the child and informed consent from the accompanying adult family member are mandatory for participation, in line with ethical guidelines to protect participant autonomy.

Exclusion Criteria

However, children will be excluded from the study if they have been recently diagnosed with anemia, such as thalassemia, sickle cell anemia, or hereditary spherocytosis, by a healthcare

professional; have a history of chronic diseases such as diabetes, or chronic obstructive pulmonary disease (COPD), also diagnosed by a healthcare professional; have undergone repeated blood transfusions; or are involved in long-term drug use (more than 3 months) such as antihypertensives, antihistamines, antiepileptics, and non-steroidal anti-inflammatory drugs (NSAIDs), which may adversely affect salivary flow [21, 22]. Additionally, children taking iron supplements, those with a known history of cleft lip and/or palate, those undergoing orthodontic intervention, and those with a known mental or physical disability will be excluded. Children will also be excluded if they have at least one partially erupted or unerupted permanent first molar or both permanent central incisors in either jaw, have overt fillings or crowns in the first permanent molars placed for reasons other than caries in these teeth, or have overt developmental enamel defects such as enamel hypoplasia, hypomineralization, and fluorosis in the same teeth.

Recruitment of participants

Potentially eligible children, along with their accompanying family members, will be respectfully approached in the pediatric OPD of CHK, for screening purposes. The child will be assessed for eligibility in the study through a screening questionnaire and a brief oral examination. If deemed eligible, these children and the adult family members will be invited for participation in the study.

The data collection process will include a pre-tested questionnaire, as well as physical, oral, and laboratory examinations. The adult family member will provide information on the child's socio-demographic background and medical history, while the child will answer questions about oral hygiene habits, other relevant behaviours, and their cariogenic diet. Once the questionnaire is completed, the child will undergo a physical examination to assess height and weight, followed by an oral examination to evaluate oral hygiene using the Simplified

Oral Hygiene Index (OHI-S) and carious status through the Decayed, Missing, and Filled Teeth (DMFT/dmft) index. This examination will focus on the permanent first molars and adjacent teeth but will also assess the entire dentition for carious teeth. Any findings from the oral examination will be communicated to the family member, and a referral slip for free treatment at the hospital's dental OPD will be provided if needed. Additionally, oral hygiene instructions will be offered to the family through an educational flyer.

All data will be collected via the REDCap software, with the first author responsible for administering the questionnaires, conducting the physical and oral examinations, and overseeing the study procedures. Blood samples, however, will be collected by trained personnel, and the costs for laboratory tests, including CBC, CRP, and ferritin, will be covered by the first author. These test results, typically available within 12 to 48 hours, will be accessible directly to both the first author and the participants. All study procedures will be completed within a single visit, ensuring convenience for participants while maintaining ethical standards through pre-obtained informed consent. Follow-up visits will not be required.

Study variables

The study outcome variable is dental caries in the permanent first molars which will be assessed using the DMFT index [23]. Each permanent first molar will be examined and coded as 'D' for decay, 'M' for missing, 'F' for filled or 0 for sound. If the DMFT index score> 0 of at least one permanent first molar, the child will be considered carious and will be treated as such in analysis (carious/non-carious).

The primary exposure in this study is IDA, defined by anemia with hemoglobin below 11.5 g/dL [24] and absolute iron deficiency, indicated by ferritin below 15 µg/L [25] when CRP is

under 10 mg/L or ferritin below 70 µg/L when CRP is 10 mg/L or higher to account for the effect of infection or inflammation on ferritin [26, 27].

A priori confounders will include the usual monthly household expenditure, recorded as the total monthly household spending on food, utilities (gas, water, and electricity), medical costs, clothing, social activities, and miscellaneous items. Body mass index (BMI) will be calculated and categorized using the World Health Organization (WHO) growth charts for boys and girls aged 5 to 19 years [28]. Smokeless tobacco use will also be included as an a priori confounder, given its prevalence in Pakistan, where around 7.7% of adults use smokeless tobacco [29], suggesting its use in children particularly among the lower socioeconomic groups [30]. Other a priori confounders include parental and child education levels, the child's gender, and age. Additional independent variables that will be assessed as potential confounders, including previous diagnoses of iron deficiency anemia (since age 6), time of last dental visit, reasons for the visit, tooth brushing frequency, and use of fluoridated toothpaste. Oral hygiene practices such as regular use (defined as at least once a day) of toothpowder, mouthwash, miswak, and dandasa along with caries in the number of adjacent teeth (to the permanent first molar) assessed using the DMFT/dmft index [23], will also be considered. A composite score of the frequency of cariogenic dietary consumption over the past six months will be another independent variable.

Lastly, OHI-S [31] will be included as an independent variable to reflect plaque and calculus levels in the mouth. It will be categorized as follows: scores between 0-1.2 will be classified as 'good,' scores between 1.3-3 will be classified as 'fair,' and scores between 3.1-6 will be classified as 'poor' [32].

Data collection method and tools

Data collection method will include:

- Filling the screening questionnaire to assess the eligibility of the participant.
- After obtaining appropriate consent, filling the structured questionnaire that asks information on the outcome variable, primary exposure variable, a priori confounders and the independent variables.

The questionnaires will be administered to the study population in Urdu and are expected to take around 35 to 50 minutes to complete.

Screening questionnaire

The screening questionnaire will be administered after obtaining appropriate verbal consent. It will include questions and oral examinations consistent with the eligibility criteria of the study. After the relevant questions are asked and the child is considered eligible based on them, oral examination will take place.

The oral cavity examination will be conducted under adequate illumination. Cheek retraction and occlusal assessment (the child will be asked to close their mouths and the relevant teeth will be examined to see if they meet the teeth of the opposing arch) will verify if the permanent first molars and permanent central incisors have fully erupted, with unerupted/partially erupted status noted only if no prior extraction due to caries is confirmed (for the permanent first molars).

Following this procedure, the permanent first molars will be visually inspected for overt fillings or crowns (the accompanying adult will be asked whether the filling or crown was placed for reasons other than decay). If both permanent central incisors in either arch or at least one permanent first molar is not fully erupted and there are any fillings/crowns involving the permanent first molars that were not placed because of decay in these teeth, then that child will be considered ineligible.

The oral examination will also assess each permanent first molar for enamel developmental defects using the modified developmental defects of enamel index (mDDE) [33]. Originally introduced by the Fédération dentaire international (FDI) Commission on Oral Health in 1982 [34] and modified by Clarkson et al. in 1989 [33], this index has been widely used in various studies, including a study in Islamabad, Pakistan [35]. It will serve as a reference for detecting enamel defects predisposing the child to caries [36, 37]. Demarcated opacity of ≤1 mm will be considered sound [38], and defects will also be considered sound, if there are any uncertainties regarding them, except for demarcated brown opacities, where history will be taken.

Under proper illumination, the data collector will retract the child's cheek with a mouth mirror and use a dental explorer to enhance examination sensitivity. Assessment of the buccal, lingual/palatal, and occlusal surfaces of the permanent first molar will be conducted to detect any white/cream/yellow or brown demarcated opacity. Its presence will result in exclusion. However, if there is any uncertainty regarding the brown opacities, history of frequent betel leaf and/betel nut with or without tobacco, gutkha, tea and coffee consumption will be taken and the absence of these habits will result in exclusion. Additionally, inspection will identify diffuse white opacity with linear, patchy, or confluent (combining into a chalky white area) distributions, as well as hypoplasia (single/multiple pits, missing enamel, (grooves, or larger areas of missing enamel)). The presence of these conditions will render the child ineligible for the study.

Study questionnaire

This questionnaire will have the following seven sections (For access to Study questionnaire, refer to Supplemental Material):

Sociodemographic information

This section will include the child's age, gender and education level. It will also include questions on the education level of both parents and the usual monthly household expenditure on food items, utilities including gas, water and electricity, medical related, clothing items, social activities, and miscellaneous items.

Medical history

This section will include questions on if the child has ever been diagnosed with iron deficiency anemia from the age of 6 by a healthcare professional and how many times have they been diagnosed with iron deficiency anemia since then by a healthcare professional.

Oral hygiene related behavior

This section will include questions on when the child last visited the dentist, what was the reason for that visit, how frequently the child brushes their teeth, whether the child uses toothpaste, what toothpaste the child uses to clean their teeth and if they regularly use any of the listed oral hygiene practices at least once a day. These questions were obtained from the WHO Oral health assessment form for children [23] and were somewhat adapted to the objectives of this study.

Therefore, due to this adaptation, its content validation was done by a team of eight dental experts. These experts rated each question in terms of its clarity and relevance on a scale of 1 (not relevant/clear) to 4 (very relevant/clear), and provided feedback, which was considered for further improvement of the questions. Results indicated item-level content validity index (I-CVI) scores ranging from 0.875 to 1, a scale-level content validity index score based on the average (S-CVI/Ave) and universal agreement method (S-CVI/UA) of 0.975 and 0.8 respectively for both relevance and

clarity indicating appropriate relevance and clarity of this section of the study questionnaire [39].

Other habits

This section will include one question on how often the child uses smokeless tobacco (snus, snuff, and chewable tobacco (gutkha, betel leaf and/areca nut with tobacco)). This question was also obtained from the WHO oral health assessment form for children [23] and adapted appropriately.

Dietary information

This section will include questions on how often, on average, in the last six months the child had consumed cariogenic food items. Processed starch and extrinsic sugars enhances the cariogenicity of the diet; the food items included contain either or both of these and are commonly found in Pakistan [40].

Physical and oral examination

Physical examination

The child's height will be measured using an inch tape, recorded in centimeters (cm) and later converted to meters (m) for calculating body mass index (BMI). Weight will be measured in kilograms (kg) using the Tanita HA621 Manual Weight Scale, manufactured by Tanita Corporation, Japan.

Oral examination will include the simplified oral hygiene index and the DMFT/dmft index of the permanent first molars and its adjacent teeth.

The Simplified Oral Hygiene Index:

The OHI-S, developed by Greene and Vermilion in 1964 [41], provides a quick, quantitative assessment of an individual's oral hygiene [41] by combining the simplified debris and calculus indices, which measure the presence of plaque and calculus, respectively [31].

Before the oral examination begins, the child will be comfortably seated, and the permanent first molars and central incisors will be visually inspected under sufficient lighting, using an explorer to enhance detection. A mouth mirror will be used to retract the cheek as needed. If the upper right or lower left permanent central incisor is missing, the adjacent permanent central incisor will be inspected instead. For the OHI-S, the buccal surfaces of the upper permanent first molars, upper right and lower left permanent central incisors, and the lingual surfaces of the lower permanent first molars will be inspected.

Debris Index Simplified (DI-S)

DI-S will assess the amount of debris present on each tooth surface. Scores will range from 0 to 3, with 0 indicating no debris and extrinsic stain, 1 indicating debris not covering more than one-third of the tooth surface or the presence of extrinsic stain, 2 indicating debris covering between one-third and two-thirds, and 3 indicating debris covering more than two-thirds of the tooth surface. The overall debris index for each child will be calculated by summing the scores of all surfaces examined and dividing by the number of surfaces assessed.

Calculus Index Simplified (CI-S)

CI-S will similarly assess the presence of supragingival or subgingival calculus. A score of 0 indicates no calculus, 1 indicates supragingival calculus

covering not more than one-third of the surface, 2 indicates coverage between one-third and two-thirds, or subgingival calculus flecks, and 3 indicates supragingival calculus covering more than two-thirds, or/and a continuous band of subgingival calculus. The overall calculus index will be calculated similarly to the debris index, by summing the scores of all surfaces and dividing by the number of surfaces examined.

DMFT/dmft index of the permanent first molar and its adjacent teeth:

The DMFT index, widely used for caries diagnosis, is an aggregate of the total number of decayed, missing (due to caries), and filled teeth, reflecting the carious status of the dentition. Although it is typically used for both primary and permanent dentitions, it can also be applied specifically to certain teeth, such as the permanent first molars [42], as is the case in this study.

Oral examination will be conducted under adequate illumination. The cheek will be retracted with a mouth mirror as necessary, and the teeth will be dried with gauze to enhance the visual inspection. Moreover, an explorer will also be used to aid caries detection. Each permanent first molar and its adjacent permanent tooth will be assessed. The teeth will be classified as ‘D’ (decayed), ‘M’ (missing due to caries), ‘F’ (filled), or ‘0’ (sound). A tooth will be coded as ‘D’ if an overt cavity, undermined enamel, or softened walls/floor are detected upon probing, with or without a permanent filling. If a temporary filling is present, the tooth will still be recorded as decayed (‘D’). If a permanent filling is present due to previous decay (as confirmed by questioning the child and the accompanying adult), and no signs of active decay are observed, the tooth will be coded as ‘F’. In cases where a tooth is

missing and confirmed to be missing due to caries (through questioning the child and the accompanying adult), it will be coded as 'M'. If no such condition is present, the tooth will be considered sound and coded as '0'.

The DMFT score for each child will be calculated separately for the permanent first molars and their adjacent permanent teeth by adding their respective scores, with equal weight given to the 'D,' 'M,' and 'F' codes. This process will also be applied to primary second molars, using the dmft index, with results recorded accordingly [23].

Laboratory-related information

Data will be collected on ferritin, hemoglobin, and CRP to diagnose iron deficiency anemia, measured in micrograms/L, grams/dL, and milligrams/L, respectively. All blood tests will be conducted at the Dow Diagnostic Research and Reference Laboratory (DDRRL), located adjacent to Civil Hospital Karachi. Established in 2007, DDRRL adheres to international standards, providing high-quality diagnostic services, emphasizing on accuracy and reliability [43]. The laboratory maintains internal quality assurance through daily controls [43], and its medical directorate is trained in compliance with ISO 15189:2022 standards for medical laboratories [44].

For each participant, 2 ml of venous blood will be drawn into an ethylenediamine tetraacetic acid (EDTA) tube for hemoglobin analysis, as EDTA tubes prevent coagulation and preserve cellular components [45]. Additionally, 6 ml of blood will be collected in two 3 ml gel tubes for CRP and ferritin analyses. Gel tubes are necessary for serum separation, which is required for these tests [45]. The collected samples will be transported to DDRRL on the same day, using ice packs to maintain appropriate temperatures during transit. The ice packs will be monitored and replaced as needed to preserve sample integrity.

Upon arrival at the laboratory, the samples will be promptly analyzed. CBC will be analyzed using the Abott Alinity hq Hematology Analyzer, CRP using the Atellica CH CRP_2 by Siemens Healthineers, and ferritin using the Atellica IM Ferritin system, also by Siemens Healthineers.

Data management

Data will be collected using a web-based questionnaire through REDCap software, with daily checks by the first author to ensure completeness, accuracy, and logical consistency. Any inconsistent or missing information (marked as "I don't know") will be verified by contacting the accompanying adult family member. REDCap's conditional logic and range checks will aid in maintaining data accuracy, with all study questions set as required fields to minimize missing values.

Data cleaning and detection of missing values will be managed by the first author, who will sort each variable in ascending and descending order. As a qualified and trained dentist, the first author will also conduct data collection to ensure ethical and sensitive handling of information, reduce bias, and promote uniformity in the data collection process.

The questionnaires were translated from English to Urdu by a translator, and an expert reviewed the translation for cultural appropriateness, making necessary adjustments. A back translation was performed to ensure that the original intent and meaning of the questions were preserved. Pre-testing of the study tools will be conducted on 5% of the study sample (n=7 children) at the study site to identify any questions that may require paraphrasing or re-translation.

Validity

Several potential biases could affect the validity of this hospital-based study. First, confounding bias may arise due to unequal distribution of undiagnosed or unreported medical

conditions, medication histories, and unaccounted-for cariogenic items in the child's diet, which could influence caries risk.

Second, wish bias might be introduced through study questions on sociodemographic factors, diet, smokeless tobacco consumption, and oral hygiene behaviours. This can be mitigated by refining the questions to be more neutral and less judgmental during pre-testing. Additionally, a more objective assessment of oral hygiene will be performed using the OHI-S to complement self-reported data.

Third, recall bias could occur when collecting information on the child's cariogenic diet. This will be mitigated by focusing on the child's diet over the past six months and verifying responses with the accompanying family member.

Lastly, non-differential misclassification could result from using a manual weight scale. This will be minimized by calibrating the machine daily and verifying its accuracy with a 1 kg weight. Each child will be weighed twice; if discrepancies arise between the measurements, the scale will be recalibrated and the weight reassessed.

Sample size

The sample size is calculated for both objectives as follows:

Frequency of children aged 7 to 12 with carious first permanent molars

The sample size is calculated using the WHO Sample Size Determination in Health Studies Software Version 2.0.21. A previous study conducted in Pakistan reported a frequency of 38.5% [46] for carious permanent first molars. Using this figure, with an absolute precision of 9% and a 95% confidence level (chosen to maintain study feasibility), a minimum sample size of 113 children aged 7 to 12 years is required.

Association of IDA and dental caries in first permanent molars in children aged 7 to 12

The sample size for this objective is calculated using STATA software version 15.0, with a power of 80% and a significance level of 5%. The ratio of exposed to unexposed children is derived from a study conducted in the Shaheed Benazir district of Pakistan among 11 to 13-year-olds, which reported an IDA prevalence of 15.5% [11], yielding a ratio of 1:5. The proportion of unexposed children with the outcome (dental caries) is obtained from a study conducted in India [47], which reported 37.8% of children without IDA having dental caries. Using these parameters, a minimum sample size of 76 children aged 7 to 12 years is required to assess the association between IDA and dental caries in first permanent molars, in order to detect a one-sided prevalence ratio of 2.

Since the sample size calculated for the first objective is larger, it will be used for this study. After accounting for a 20% non-response and ineligibility rate, a final minimum sample size of approximately 141 children aged 7 to 12 years is obtained.

Statistical analysis

All analyses will be conducted using STATA software version 15.0. The frequency of children with carious permanent first molars will be reported along with 95% confidence intervals.

Descriptive statistics will be presented for all independent variables (such as age, gender, monthly household expenditure, parental and child education levels, and BMI etc.) stratified by IDA status. Categorical variables (e.g., gender, child's education status) will be reported as frequencies and percentages. The distribution of quantitative variables will be assessed for normality using box plots, histograms, and the Kolmogorov-Smirnov test. For normally distributed variables (e.g., age, number of adjacent carious teeth), means and standard deviations will be provided; for skewed distributions, medians and interquartile ranges (IQRs) will be reported.

Differences between exposure groups for categorical variables will be assessed using the chi-square test, provided the expected frequency per cell is at least 5; otherwise, Fisher's exact test will be used. For quantitative predictors, independent sample t-tests will be conducted, and p-values will be reported.

To analyze the cariogenic dietary construct, Principal Component Analysis (PCA) will reduce this variable into a single composite score, categorizing the frequency of cariogenic food consumption into low, medium, and high levels.

To assess the association between IDA and dental caries in permanent first molars, COX proportional hazards regression will be used. Initially, univariate analyses will assess IDA, along with a priori confounders and other independent variables (e.g., age, gender, education level, tooth brushing frequency etc.), for their association with caries in permanent first molars. Crude prevalence ratios with 95% confidence intervals will be reported. Variables with a p-value < 0.25 at the univariate level will be checked for multicollinearity along with IDA ($r > 0.8$).

Subsequently, confounding and multivariable analyses will be conducted. A stepwise model-building approach will be used to construct the multivariable model, starting with IDA and sequentially adding variables. Likelihood ratio tests will be used to determine whether each added variable significantly improves the model fit (p-value < 0.05). Biologically plausible interactions will also be assessed (p-value < 0.1). The final, parsimonious model will be determined when no additional variables can be included, and none can be removed without affecting the model's fit. Adjusted prevalence ratios with 95% confidence intervals for caries in permanent first molars will be reported.

Patient and public involvement statement

The patients and the public are and will not be involved in the design, conduct, reporting, or dissemination plans of this research.

ETHICS AND DISSEMINATION

All study activities will be conducted in accordance with the Declaration of Helsinki and the International Council for Harmonization (ICH) guidelines for Good Clinical Practice (GCP). Ethical approval has been obtained from the ERC of AKU (Reference number: 2024-9692-30593) and the IRB of DUHS (IRB Reference: IRB-3556/DUHS/Approval/2024/196).

Prior to enrollment, informed assent and consent will be obtained from the participating children and their accompanying adult family members, respectively. Data collection will take place in a private space to protect their confidentiality. Sensitive questions, including those related to tobacco use, will be posed in a neutral and non-judgmental manner. This approach will foster a comfortable environment for participants, thereby minimizing any potential discomfort associated with discussing sensitive topics and upholding the highest ethical standards in our research.

To ensure participant safety, required blood tests will be performed by trained personnel, and any child needing dental treatment will be referred to dentists at CHK for free dental care. The "tell-show-do" technique will be used to ease anxiety during the oral examination, and instructions on pediatric oral hygiene will be provided via flyers.

Confidentiality will be maintained throughout the study. Data will be anonymized by assigning each participant a unique eight-character alphanumeric ID, with no direct link to their personal information in documents used for analysis or dissemination. The link between participant contact details and ID will be kept in a separate file under the custody of the first

author. Data will be stored in a password-protected database, with backup files, and will be securely deleted seven years after study completion using erasure software.

The study results will be disseminated through seminars and published in a peer reviewed academic journal.

LIST OF ABBREVIATIONS

AKU- Aga Khan University

BMI- Body Mass Index

CBC- Complete blood count

CI-S - Calculus Index Simplified

CHK- Civil Hospital Karachi

COPD- Chronic Obstructive Pulmonary Disease

CRP- C-reactive protein

cm- Centimetres

DI-S- Debris Index Simplied

DMFT- Decayed, Missing, and Filled Teeth

DUHS- Dow University of Health Sciences

ERC- Ethical Review Committee

EDTA- Ethylenediamine tetraacetic acid

FDI- Fédération Dentaire Internationale

I-CVI- Item-level Content Validity Index

ID- Identification

IDA- Iron Deficiency Anemia

IRB- Institutional Review Board

IQR- Interquartile range

Kg- Kilogram

mDDE- modified Developmental Defects of Enamel

NSAID- Non-steroidal anti-inflammatory drug

OPD- Outpatient department

PCA- Principal Component Analysis

PKR- Pakistani Rupee

S-CVI/Ave- Scale-level Content Validity Index based on the Average method

S-CVI/UA- Scale-level Content Validity Index based on the Universal Agreement method

OHI-S- Simplified Oral Hygiene Index

STROBE- Strengthening the Reporting of Observational Studies in Epidemiology

WHO- World Health Organization

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AUTHOR CONTRIBUTIONS

SS, and SST conceived and designed the study. SS will be responsible for data collection and data management. SS, SST, IA will be involved in data analysis and interpretation. SST, IA, WAJ, AFS and FRK have revised the work and have provided their intellectual input. SS wrote the first draft of the manuscript. All authors have appropriately revised, read and approved the submitted version of this protocol. SS is the guarantor.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

DATA SHARING STATEMENT

Not applicable, as data collection hasn't started yet.

Study Title: Protocol for an Analytical Cross-Sectional Study on the Association of Iron Deficiency Anemia with Dental Caries in the Permanent First Molars of Children Aged 7 to 12 Years in Karachi, Pakistan

Instructions:

1. Separate form should be filled for each subject.
2. This form should only be filled after the subject has been screened successfully and has been considered eligible for the study.

Child ID: _____

Version number and Date: __ (_ _/_ _/_ _ _ _)

Section A: Socio-demographic information

(asked from the legal guardian)

S. No	Question	Code	Skip	Response
1.	Gender of child?	1. Female 2. Male		
2.	What is the age of child?	Write exact age in years		__ __ Years
3 a.	Has the child ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q4a.	
3 b.	What is the education status of your child?	1. No formal schooling 2. Schooling 2.1 if schooling= please specify Grade _____ 9. I don't know		
4 a.	Has the mother ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q5 a	
4 b.	What is the mother's education status?	1. No formal schooling 2. Primary school education 3. Middle school education		

		4. Secondary school education 5. Higher secondary school education 6. Above higher secondary school education 9. I don't know		
5 a.	Has the father ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q6.	
5 b.	What is the father's education status?	1. No formal schooling 2. Primary school education 3. Middle school education 4. Secondary school education 5. Higher secondary school education 6. Above higher secondary school education 9. I don't know		
6.	What is the usual monthly household expenditure on food items?	Write the exact amount in PKR '98' is I don't remember		
7.	What is the usual monthly household expenditure on utilities such as gas, water and electricity?	Write the exact amount in PKR '98' is I don't remember		
8.	What is the usual monthly household medical expenditure?	Write the exact amount in PKR '98' is I don't remember		
9.	What is the usual monthly household expenditure on clothing items?	Write the exact amount in PKR '98' is I don't remember		
10.	What is the usual monthly household expenditure on social activities such as gifts, parties etc.?	Write the exact amount in PKR '98' is I don't remember		
11.	What is the usual monthly household expenditure on miscellaneous items?	Write the exact amount in PKR '98' is I don't remember		

Section B: Medical history

(taken from the legal guardian)

S. No	Question	Code	Skip	Response
12 a.	Has the child ever been diagnosed with iron deficiency anemia from the age of 6 by a healthcare professional?	1. Yes 2. No 9. I don't know	If 'No' or 'I don't know', skip to S. No 13 a.	
12 b.	How many times have your child been diagnosed with iron deficiency anemia from the age of 6 by a healthcare professional?	Write the exact number of times '98' is I don't remember		

Section C: Oral hygiene-related behavior

(taken from the child)

S.No	Question	Code	Skip	Response
13 a.	When was the last time you went to the dentist?	1. Never 2. >1 year ago 3. Between 6 months and a year ago 4. <6 months ago 9. I don't know	If 'Never' skip to S. No 14	
13 b.	What was the reason for your visit to the dentist?	1. Pain or trouble with teeth, gums or mouth 2. Treatment/Follow-up treatment 3. Routine check-up of teeth 4. Other reasons Please specify: _____ 9. I don't know		
14.	How frequently do you brush your teeth?	1. Never 2. 2-3 times a month 3. Once a week	If 'Never' or 'I don't	

		4. 2-6 times a week 5. Once a day 6. ≥ 2 times a day 9. I don't know	know' skip to S. No 16.	
15 a.	Do you use a toothpaste?	1. Yes 2. No 9. I don't know	If 'No' or 'I don't know', skip to S. No 16	
15 b.	What toothpaste do you use to clean your teeth?	Write the exact response. '98' is I don't remember		
16.	Do you regularly use any of these other oral hygiene practices atleast once a day?	1. Toothpowder 2. Mouthwash 3. Miswak 4. Dandaasa 5. All of the above 6. None of the above 7. Toothpowder and mouthwash 8. Toothpowder and miswak 9. Toothpowder and dandaasa 10. Mouthwash and miswak 11. Mouthwash and dandaasa 12. Miswak and dandasa 13. Toothpowder, mouthwash and miswak 14. Toothpowder, mouthwash and dandasa 15. Toothpowder, dandasa and miswak 16. Mouthwash, dandasa and miswak		

Section D: Other habits

(taken from the child)

S. No	Question	Code	Skip	Response
17.	How often do you use smokeless tobacco including snus, snuff, chewable tobacco (gutkha, betel leaf and/areca nut with tobacco)?	1. Never 2. 2-3 times a month 3. Once a week 4. 2-6 times a week 5. Every day 9. I don't know		

Section E: Dietary information

(taken from the child)

S. No 18. How often have you consumed, on average, the following food items in the last six months?

	Items	Never/ < once a month	1-3 times a month	Once a week	2 to 4 times a week	5 to 6 times a week	Once a day	2 to 3 times a day	4 to 5 times a day	6 to 7 times a day
	Bread and Cereals									
1	Porridge with sugar									
2	Frozen paratha/ Street food paratha									
3	Homemade paratha (Refined Flour)									
4	White bread/ Bun									
5	Rusk/ Cake Rusk									
6	Two-minute noodles/ Macaroni/ Spaghetti									
7	Biscuit / Cookie									
8	Sandwich biscuit									
9	Donut									
10	Cupcake/Muffin/Plain Cake									
11	Pizza									
	Desserts									
12	Kheer/ Custard/Pudding									
13	Cake/Pastry									

14	Meethai/halwa									
15	Sweetened yoghurt									
16	Ice cream									
17	Plain Jelly									
18	Fruit chaat (that has added sugar)									
	Beverages									
19	Tea with sugar									
20	Sweet lassi (flavored or unflavored)									
21	Soft drink									
22	Sharbat / Instant Drink									
23	Fruit juice									
24	Flavored milk									
	Miscellaneous									
25	French fries									
26	Chocolate									
27	Candy									
28	Cotton candy									
29	Chewing gum (not the sugar-free one)									
30	Lollipop									
31	Jam (for example on bread)									
32	Honey (for example on bread)									
33	Chocolate spread (for example on bread)									
34	Chips									

Section F: Physical and oral examination

(taken of the child)

S. No	Measurement	Finding
19.	Height (in cm)	
20.	Weight (in kg)	

S. No 21 **Oral hygiene status assessment**

Debris Index Simplified:

	Right permanent first molar		Permanent central incisor		Left permanent first molar		Total	
	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual
Upper	_____		_____ (right)		_____		_____	
Lower		_____	_____ (left)			_____	_____	_____

Debris Index Simplified= (The buccal scores) + (The lingual scores)/ (Total number of examined buccal and lingual surfaces)
=_____

Calculus Index Simplified:

	Right permanent first molar		Permanent central incisor		Left permanent first molar		Total	
	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual
Upper	_____		_____ (right)		_____		_____	
Lower		_____	_____ (left)			_____	_____	_____

Calculus Index Simplified = (The buccal scores) + (The lingual scores)/ (Total number of examined buccal and lingual surfaces)
=_____

Simplified Oral Hygiene Index= Debris Index Simplified + Calculus Index Simplified=
_____

S. No 22 **Carious assessment of the permanent first molars and adjacent teeth**

DMFT/dmft index:

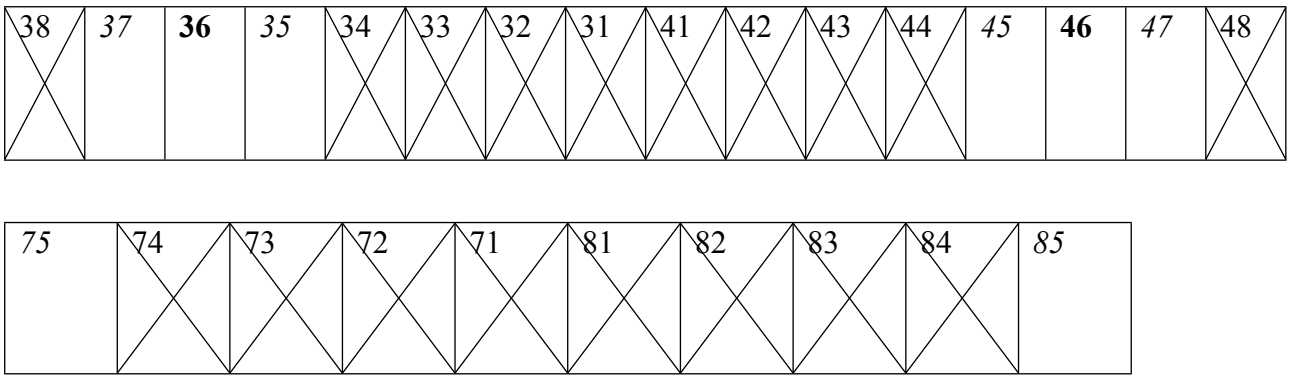
Permanent teeth	Primary teeth
D= Decayed	d= decayed
M= Missing	m= missing
F= Filled	f= filling

Maxilla

28	*27*	**26**	*25*	24	23	22	21	11	12	13	14	*15*	**16**	*17*	18

65	64	63	62	61	51	52	53	54	*55*

Mandible



Permanent first molar:

Total Decayed=_____ Total Missing=_____ Total Filled=_____

Total DMFT index score=_____

Adjacent teeth:

Total Decayed=_____ Total Missing=_____ Total Filled=_____

Total DMFT index score=_____

Total decayed=_____ Total missing=_____ Total filled=_____

Total dmft index score=_____

Section F: Laboratory-related information

S. No	Blood parameter	Code	Finding
23.	Hemoglobin (g/dL)		
24.	Ferritin (microgram/L)		
25.	C-reactive protein (milligram/L)		

Form filled by: _____ Date: _ _/_ _/_ _ _ _ *(Day/Month/Year)*

Form checked and edited by: _____ Date: _ _/_ _/_ _ _ _ *(Day/Month/Year)*

BMJ Open

Protocol for an Analytical Cross-Sectional Study on the Association of Iron Deficiency Anemia with Dental Caries in the Permanent First Molars of Children Aged 7 to 12 Years in Karachi, Pakistan.

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TITLE

Protocol for an analytical cross-sectional study on the association of iron deficiency anemia with dental caries in the permanent first molars of children aged 7 to 12 years in Karachi, Sindh, Pakistan

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ABSTRACT

Introduction

Iron deficiency anemia and dental caries are prevalent diseases among Pakistani children. Limited research has been done to explore their association in permanent teeth. Given the caries susceptibility of permanent first molars and their role in the development of ideal occlusion, this study aims to estimate caries frequency in these molars and assess its association with iron deficiency anemia in 7 to 12-year-old children.

Methods and analysis

This analytical cross-sectional study will include 141 children aged 7 to 12 years visiting physicians in the pediatric outpatient department of Dr. Ruth K.M Pfau, Civil Hospital Karachi. Using consecutive sampling, children who met initial screening criteria were further evaluated to determine eligibility for the study. Data collection will involve physical examinations (including weight and height), oral examinations (including the relevant oral hygiene and caries assessments), and laboratory examinations (including the prescribed tests). Additionally, questions will be asked about socio-demographic characteristics, history of iron deficiency anemia, oral hygiene habits, smokeless tobacco use, and the frequency of cariogenic dietary consumption. Exposure variable will include the presence of iron deficiency anemia, assessed using complete blood count, C-reactive protein and ferritin tests, and treated as a dichotomous variable. Outcome variable will include dental caries in at least one permanent first molar, assessed using the DMFT index and also treated as a dichotomous variable. Analysis will include Poisson regression with robust variance, reporting prevalence ratios with 95% confidence intervals for the association of iron deficiency anemia and dental caries in the permanent first molars. Frequency of children with carious permanent first molars with 95% confidence intervals will also be reported.

Ethics and dissemination

This research has been approved by Ethical Review Committee of Aga Khan University (Reference number: 2024-9692-30593) and the Institutional Review Board of Dow University of Health Sciences (IRB Reference: IRB-3556/DUHS/Approval/2024/196) prior to participant recruitment. Results will be disseminated through seminars and peer reviewed publications.

Keywords: Anemia, Iron deficiency; Child; Dental Caries; Dentition, Permanent; Molar

ARTICLE SUMMARY

Strengths and limitations of this study

1. This study will use objective laboratory tests for the diagnosis of iron deficiency anemia and the validated DMFT index, recommended by WHO, for caries diagnosis in children.
2. It will control for the confounding associated with the mixed dentition stage, by specifically focusing on the permanent first molar which has the greatest caries risk.
3. Additionally, this study will be conducted among children belonging to the lower socioeconomic background, that are more likely to experience both the exposure and outcome; thereby controlling confounding associated with the socioeconomic status.
4. Even though this focus on permanent first molars of children belonging solely to low socioeconomic status will improve the internal validity of the study, it may compromise its external validity.
5. Moreover, since this is a cross-sectional study and the exposure and outcome are measured at one point in time, it is inherently susceptible to temporal bias.

INTRODUCTION

Dental caries is the most prevalent global disease [1], causing significant pain and impacting oral health-related quality of life [2] and nutritional intake [3] of children. In 2017, untreated dental caries affected approximately 2.3 billion people [4]. Caries prevalence in permanent teeth is highest in Africa (58.9%), followed by Asia (58.8%) and Australia (54.9%) [5]. Regionally, its prevalence in the Eastern Mediterranean Region is 61% among 12-year-olds and 70% among 15-year-olds [6].While locally, its overall prevalence in Pakistan is 56.6%, with 61.2% in mixed dentition and 57.2% in permanent dentition [7].

Anemia affects over 1.9 billion people globally [8] and had impacted 24.3% of the global population in 2021 [8]. The highest prevalence of anemia is found in Western and Central sub-Saharan Africa (47.4% and 35.7%, respectively) and South Asia (43%) [8], which overlaps with the regions heavily affected by dental caries. Dietary iron deficiency has been reported to be its leading cause among most demographic groups, including children [8].

Despite its significant impact, particularly in children, research on iron deficiency anemia (IDA) in Pakistani children aged 5-12 is limited, with varying prevalence rates reported across regions: 19% for 5-16 year-olds in rural Swat [9], 62.2% for children under 15 in Lahore [10], and 15.5% for 11-13 year-olds in Shaheed Benazirabad [11].

Studies worldwide have found a significant association of IDA with caries in children [12, 13, 14], though, most have focused on younger age groups, with limited representation of school-age children. Some studies have proposed mechanisms supporting this association such as impaired saliva buffering [15], reduced enamel protection against demineralization [16], and antibacterial effects of iron [17, 18].

Given the prevalence of both conditions and their significant adverse effects on children's health, establishing a link between IDA and caries in Pakistani school-age children is a crucial research gap. Both conditions may strain the healthcare system and socio-economic conditions despite being preventable. This study aims to address this gap by estimating the frequency of carious permanent first molars among children aged 7 to 12 and assessing its association with IDA. The focus on first permanent molars is significant due to their high caries risk [19] and their role in developing ideal occlusion within this age group [20]. By providing insights into this association, the study's findings could inform future longitudinal research and enhance the integrated management of dental and hematological care for affected children.

OBJECTIVES

1. To investigate the association between IDA and dental caries in the permanent first molars among children aged 7 to 12 years visiting a public tertiary care hospital in Karachi, Pakistan.
2. To estimate the frequency of children aged 7 to 12 years with carious permanent first molars visiting a public tertiary care hospital in Karachi, Pakistan.

HYPOTHESIS

The prevalence of dental caries in the permanent first molars of 7 to 12-year-old children who have IDA is more than 2 times as compared to children who do not have IDA after adjusting for a priori confounders and other independent variables.

METHODS AND ANALYSIS

Study design

An analytical cross-sectional study will be used. This type of design measures the burden of exposure and outcome in a specific population simultaneously and, in doing so, assesses their association in comparative terms.

To ensure that the study design and reporting are transparent and comprehensive in presenting the findings of the study, the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist will be followed.

Study site and setting

To overcome time constraints and for ethical reasons, we will conduct a hospital-based study to ensure an adequate number of children are recruited. Moreover, to obtain a homogenous sample, Dr. Ruth K. M. Pfau, Civil Hospital Karachi located in Karachi, Sindh, Pakistan will be used in this study.

Civil Hospital Karachi (CHK)

CHK is a 1900-bed public tertiary care and teaching hospital located on Mission Road in Karachi, Pakistan. Affiliated with Dow Medical College, it is government-funded and provides free treatment to all patients, mainly including patients from diverse backgrounds, predominantly from lower socio-economic groups. The pediatric outpatient department (OPD) at CHK, operational Monday through Saturday during daytime hours, sees approximately 130 children aged 7 to 12 years daily. It has been selected as the study site due to its diversity and practicality in recruitment. This setting, offers a balanced sample of healthier children, including those who may not attend school. This selection helps enhance the precision and internal validity of the study while acknowledging potential limitations in external validity.

Data collection will commence after receiving ethical approval from the Institutional Review Board (IRB) and Ethical Review Committee (ERC) of Dow University of Health Sciences

(DUHS) and Aga Khan University (AKU) respectively. A pre-testing phase will take place from December 2nd to 7th, 2024, followed by recruitment and data collection from 9th December 2024 to mid-February 2025. The recruitment process aims to enroll between 1 to 4 participants per day. Despite the limited operational hours and detailed selection criteria, the recruitment process remains feasible. The high patient volume ensures a steady pace of enrollment. Data cleaning and analysis will take approximately one month, and manuscript preparation is expected to be completed by mid-April 2025.

Sampling technique and study population

In this study, the target population includes children aged 7 to 12 years visiting physicians at CHK, who have been prescribed C-reactive protein (CRP), ferritin, and complete blood count (CBC) tests. Consecutive sampling technique will be used as participants will be recruited in the order they present at the pediatric OPD. This approach will also allow for efficient identification of eligible participants through an initial screening process based on key criteria that can be quickly assessed. The initial screening will determine whether the child is accompanied by a family member from the same household, whether both individuals can understand Urdu, whether the child has been prescribed the relevant blood tests, and if the child has at least one fully erupted permanent central incisor in both arches, along with all fully erupted permanent first molars.

After this preliminary screening, a more thorough evaluation will be conducted to ensure that participants meet the full set of inclusion and exclusion criteria. This sampling approach facilitates the rapid identification of potential participants while ensuring rigorous verification of eligibility. It allows for a targeted and efficient selection process for the study.

Eligibility criteria

Inclusion Criteria

The study will include children aged 7 to 12 years who reside with an adult family member in the same household and are able to communicate effectively in Urdu. Both the child and the family member must understand Urdu. This requirement is necessary because the first author, who is the sole data collector, is proficient only in this language. Financial constraints make hiring translators or additional data collectors infeasible. This requirement ensures clear communication and improves the accuracy and reliability of the data collected.

A key criterion for inclusion is that the child must be prescribed CRP, ferritin, and CBC tests by a physician at the study site. This requirement ensures that the blood tests are medically justified. It also reassures the parents that the tests serve both clinical and research purposes, increasing their comfort with participation.

Additionally, the child must be accompanied by an adult family member who resides in the same household. This is crucial for obtaining accurate socio-demographic information, details about the child's health behaviors, and relevant household data, such as monthly expenditure. Children visiting the pediatric OPD are sometimes accompanied by relatives or non-family members who may not have sufficient knowledge of the child's health and household circumstances. Therefore, the inclusion of a household-residing family member helps ensure the accuracy of the information collected.

Furthermore, informed assent from the child and informed consent from the accompanying adult family member are mandatory for participation, in line with ethical guidelines to protect participant autonomy.

Exclusion Criteria

However, children will be excluded from the study if they have been recently diagnosed with anemia (e.g. thalassemia, sickle cell anemia, or hereditary spherocytosis) by a healthcare professional. Other exclusion criteria include a history of chronic diseases such as diabetes,

or chronic obstructive pulmonary disease (COPD) as diagnosed by a healthcare professional., repeated blood transfusions; or long-term drug use (more than 3 months). Drugs such as antihypertensives, antihistamines, antiepileptics, and non-steroidal anti-inflammatory drugs (NSAIDs), are included, as they may adversely affect salivary flow [21, 22]. Children will also be excluded if they are taking iron supplements, have a known history of cleft lip and/or palate, are undergoing orthodontic intervention, or have a known mental or physical disability. Exclusion criteria also includes partially erupted or unerupted permanent first molar or both permanent central incisors in either jaw. Additionally, children with overt fillings or crowns in the first permanent molars (placed for reasons other than caries in these teeth) or with overt developmental enamel defects such as enamel hypoplasia, hypomineralization, and fluorosis in these teeth will be excluded.

Recruitment of participants

Potentially eligible children, along with their accompanying family members, will be respectfully approached in the pediatric OPD of CHK, for screening purposes. The child will be assessed for eligibility in the study through a screening questionnaire and a brief oral examination. If deemed eligible, these children and the adult family members will be invited for participation in the study.

The data collection process will include a pre-tested questionnaire, as well as physical, oral, and laboratory examinations. The adult family member will provide information on the child's socio-demographic background and medical history. The child will answer questions about oral hygiene habits, other relevant behaviours, and their cariogenic diet. Once the questionnaire is completed, the child will undergo a physical examination to assess height and weight. This will be followed by an oral examination, to evaluate oral hygiene using the Simplified Oral Hygiene Index (OHI-S) and carious status through the Decayed, Missing, and

Filled Teeth (DMFT/dmft) index. This examination will focus on the permanent first molars and adjacent teeth but will also assess the entire dentition for carious teeth. Any findings from the oral examination will be communicated to the family member, and a referral slip for free treatment at the hospital's dental OPD will be provided if needed. Additionally, oral hygiene instructions will be offered to the family through an educational flyer.

All data will be collected via the REDCap software. The first author will be responsible for administering the questionnaires, conducting the physical and oral examinations, and overseeing the study procedures. Blood samples, however, will be collected by trained personnel. The costs for laboratory tests, including CBC, CRP, and ferritin, will be covered by the first author. These test results, typically available within 12 to 48 hours, will be accessible directly to both the first author and the participants. All study procedures will be completed within a single visit, ensuring convenience for participants while maintaining ethical standards through pre-obtained informed consent. Follow-up visits will not be required.

Study variables

The study outcome variable is dental caries in the permanent first molars, assessed using the DMFT index [23]. Each permanent first molar will be examined and coded as 'D' for decay, 'M' for missing, 'F' for filled or 0 for sound. If the DMFT index score> 0 of at least one permanent first molar, the child will be considered carious and will be treated as such in analysis (carious/non-carious).

The primary exposure in this study is IDA, defined by anemia with hemoglobin level below 11.5 g/dL [24] and absolute iron deficiency. Iron deficiency will be indicated by ferritin levels below 15 µg/L [25], when CRP is under 10 mg/L or by ferritin level below 70 µg/L

when CRP is 10 mg/L or higher. These thresholds will account for the effect of infection or inflammation on ferritin [26, 27].

A priori confounders will include the usual monthly household expenditure, recorded as the total monthly household spending on food, utilities (gas, water, and electricity), medical costs, clothing, social activities, and miscellaneous items. Body mass index (BMI) will be calculated and categorized using the World Health Organization (WHO) growth charts for boys and girls aged 5 to 19 years [28]. Smokeless tobacco use will also be included as an a priori confounder, given its prevalence in Pakistan, where around 7.7% of adults use smokeless tobacco [29], suggesting its use in children particularly among the lower socioeconomic groups [30]. Other a priori confounders include parental and child education levels, the child's gender, and age.

Additional independent variables that will be assessed as potential confounders, including previous diagnoses of IDA (since age 6), time of last dental visit, reasons for the visit, tooth brushing frequency, and use of fluoridated toothpaste. Oral hygiene practices such as regular use (defined as at least once a day) of toothpowder, mouthwash, miswak, and dandasa, will also be considered. The number of carious adjacent teeth (to the permanent first molar) will be assessed using the DMFT/dmft index [23]. A composite score of the frequency of cariogenic dietary consumption over the past six months will also be included as another independent variable. Lastly, OHI-S [31] will reflect plaque and calculus levels in the mouth. Scores will be categorized as follows: 0-1.2 as 'good,' 1.3-3 as 'fair,' and 3.1-6 as 'poor' [32].

Data collection method and tools

Data collection method will include:

- Filling the screening questionnaire to assess the eligibility of the participant.

- After obtaining appropriate consent, filling the structured questionnaire that asks information on the outcome variable, primary exposure variable, a priori confounders and the independent variables.

The questionnaires will be administered to the study population in Urdu. They are expected to take around 35 to 50 minutes to complete.

Screening questionnaire

The screening questionnaire will be administered after obtaining appropriate verbal consent. It will include questions and oral examinations consistent with the eligibility criteria of the study. After the relevant questions are asked and the child is considered eligible based on them, oral examination will take place.

The oral cavity examination will be conducted under adequate illumination. Occlusal assessment will involve retracting the cheek and asking the child to close their mouths to determine whether the relevant teeth of the opposing arches meet. This will verify if the permanent first molars and permanent central incisors have fully erupted. Unerupted or partially erupted status will only be noted for the molars if no prior extraction due to caries is confirmed.

Following this procedure, the permanent first molars will be visually inspected for overt fillings or crowns. The accompanying adult will be asked whether these restorations were placed for reasons other than decay. If both permanent central incisors in either arch or at least one permanent first molar is not fully erupted, and there are any fillings or crowns not related to decay in the permanent first molars, the child will be considered ineligible.
The oral examination will also assess each permanent first molar for enamel developmental defects using the modified developmental defects of enamel index (mDDE) [33]. This index was originally introduced by the Fédération dentaire international (FDI) Commission on Oral

Health in 1982 [34] and modified by Clarkson et al. in 1989 [33]. It has been widely used in various studies, including one in Islamabad, Pakistan [35]. It will serve as a reference for detecting enamel defects predisposing the child to caries [36, 37]. Demarcated opacity of ≤1 mm will be considered sound [38]. Defects will also be considered sound, if uncertainties arise, except for demarcated brown opacities, in which case a history will be taken.

Under proper illumination, the data collector will retract the child's cheek with a mouth mirror and use a dental explorer to enhance examination sensitivity. Assessment of the buccal, lingual/palatal, and occlusal surfaces of the permanent first molar will be conducted to detect any white/cream/yellow or brown demarcated opacity. Its presence will result in exclusion. However, if there is any uncertainty regarding the brown opacities, history of frequent betel leaf and/betel nut (with or without tobacco), gutkha, tea, or coffee consumption will be taken. The absence of these habits will result in exclusion. Additionally, inspection will identify diffuse white opacity with linear, patchy, or confluent (combining into a chalky white area) distributions. It will also assess for hypoplasia including single or multiple pits and missing enamel (grooves, or larger areas of missing enamel). The presence of any of these conditions will render the child ineligible for the study.

Study questionnaire

This questionnaire will have the following seven sections (For access to Study questionnaire, refer to Supplemental Material):

Sociodemographic information

This section will include the child's age, gender and education level. It will also include questions on the education level of both parents and the usual monthly household expenditure on food items, utilities including gas, water and electricity, medical related, clothing items, social activities, and miscellaneous items.

Medical history

This section will include questions on if the child has ever been diagnosed with IDA from the age of 6 by a healthcare professional, and how many times have they been diagnosed with it since then.

Oral hygiene related behavior

This section will include questions on when the child last visited the dentist, the reason for that visit, how frequently the child brushes their teeth, whether the child uses toothpaste, what toothpaste they use, and whether they regularly use any of the listed oral hygiene practices at least once a day. These questions were obtained from the WHO Oral Health Assessment Form for Children [23] and were somewhat adapted to the objectives of this study.

Therefore, due to this adaptation, content validation was done by a team of eight dental experts. These experts rated each question for clarity and relevance on a scale of 1 (not relevant/clear) to 4 (very relevant/clear) and provided feedback for further improvement. Results indicated item-level content validity index (I-CVI) scores ranging from 0.875 to 1. The scale-level content validity index score, based on the average (S-CVI/Ave) and universal agreement method (S-CVI/UA), were 0.975 and 0.8, respectively, for both relevance and clarity. These scores indicate that this section of the study questionnaire is appropriately relevant and clear [39].

Other habits

This section will include one question on how often the child uses smokeless tobacco (such as snus, snuff, and chewable tobacco like gutkha, betel leaf and/areca nut with tobacco). This question was also obtained from the WHO Oral Health Assessment Form for Children [23] and adapted appropriately.

Dietary information

This section will include questions on how often, on average, in the last six months the child had consumed cariogenic food items. Recognizing that processed starch and extrinsic sugars enhance the dietary cariogenicity, the questionnaire focuses on food items that contain either or both and are commonly found in Pakistan [40].

Physical and oral examination

Physical examination

The child's height will be measured using an inch tape, recorded in centimeters (cm). It will be later converted to meters (m) for calculating body mass index (BMI). Weight will be measured in kilograms (kg) using the Tanita HA621 Manual Weight Scale, manufactured by Tanita Corporation, Japan.

Oral examination will include the simplified oral hygiene index and the DMFT/dmft index of the permanent first molars and its adjacent teeth.

The Simplified Oral Hygiene Index:

The OHI-S, developed by Greene and Vermilion in 1964 [41], provides a quick, quantitative assessment of an individual's oral hygiene [41] by combining the simplified debris and calculus indices, which measure the presence of plaque and calculus, respectively [31].

Before the oral examination begins, the child will be comfortably seated. The permanent first molars and central incisors will be visually inspected under sufficient lighting, using an explorer to enhance detection. A mouth mirror will be used to retract the cheek as needed. If the upper right or lower left permanent central incisor is missing, the adjacent permanent central incisor

will be inspected instead. For the OHI-S, the buccal surfaces of the upper permanent first molars, upper right and lower left permanent central incisors, and the lingual surfaces of the lower permanent first molars will be inspected.

Debris Index Simplified (DI-S)

DI-S will assess the amount of debris present on each tooth surface. Scores will range from 0 to 3: 0 indicates no debris or extrinsic stain, 1 indicates debris covering no more than one-third of the tooth surface or the presence of extrinsic stain, 2 indicates debris covering between one-third and two-thirds, and 3 indicates debris covering more than two-thirds of the tooth surface. The overall debris index for each child will be calculated by summing the scores of all surfaces examined and dividing by the number of surfaces assessed.

Calculus Index Simplified (CI-S)

CI-S will similarly assess the presence of supragingival or subgingival calculus. A score of 0 indicates no calculus. A score of 1 indicates supragingival calculus covering no more than one-third of the surface. A score of 2 indicates coverage between one-third and two-thirds, or the presence of subgingival calculus flecks. A score of 3 indicates supragingival calculus covering more than two-thirds, or/and a continuous band of subgingival calculus. The overall calculus index will be calculated similarly to the debris index, by summing the scores of all surfaces and dividing by the number of surfaces examined.

DMFT/dmft index of the permanent first molar and its adjacent teeth:

The DMFT index, widely used for caries diagnosis, is an aggregate of the total number of decayed, missing (due to caries), and filled teeth, reflecting the carious status of the dentition. Although it is typically used for both primary and permanent dentitions, it can also be applied specifically to certain teeth, such as the permanent first molars [42], as is the case in this study.

Oral examination will be conducted under adequate illumination. The cheek will be retracted with a mouth mirror as necessary, and the teeth will be dried with gauze to enhance the visual inspection. Moreover, an explorer will also be used to aid caries detection. Each permanent first molar and its adjacent permanent tooth will be assessed. The teeth will be classified as 'D' (decayed), 'M' (missing due to caries), 'F' (filled), or '0' (sound). A tooth will be coded as 'D' if an overt cavity, undermined enamel, or softened walls/floor are detected upon probing, with or without a permanent filling. If a temporary filling is present, the tooth will still be recorded as decayed ('D'). If a permanent filling is present due to previous decay (as confirmed by questioning the child and the accompanying adult), and no signs of active decay are observed, the tooth will be coded as 'F'. In cases where a tooth is missing and confirmed to be missing due to caries (through questioning the child and the accompanying adult), it will be coded as 'M'. If no such condition is present, the tooth will be considered sound and coded as '0'.

The DMFT score for each child will be calculated separately for the permanent first molars and their adjacent permanent teeth by adding their respective scores, with equal weight given to the 'D,' 'M,' and 'F' codes. This process will also be applied to primary second molars, using the dmft index, with results recorded accordingly [23].

Laboratory-related information

Data will be collected on ferritin, hemoglobin, and CRP to diagnose IDA. Ferritin will be measured in micrograms/L, hemoglobin in grams/dL, and CRP in milligrams/L. All blood tests will be conducted at the collection center of Dow Diagnostic Research and Reference Laboratory (DDRRL), located adjacent to CHK. Established in 2007, DDRRL adheres to international standards, providing high-quality diagnostic services, emphasizing on accuracy and reliability [43]. The laboratory maintains internal quality assurance through daily controls [43]. Its medical directorate is also trained in compliance with ISO 15189:2022 standards for medical laboratories [44].

For each participant, 2 ml of venous blood will be drawn into an ethylenediamine tetraacetic acid (EDTA) tube for hemoglobin analysis. EDTA tubes prevent coagulation and preserve cellular components [45]. Additionally, 6 ml of blood will be collected in two 3 ml gel tubes for CRP and ferritin analyses. Gel tubes are necessary for serum separation, which is required for these tests [45]. The collected samples will be transported to DDRRL on the same day, using ice packs to maintain appropriate temperatures during transit. The ice packs will be monitored and replaced as needed to preserve sample integrity.

Upon arrival at the laboratory, the samples will be promptly analyzed. CBC will be analyzed using the Abott Alinity hq Hematology Analyzer, CRP using the Atellica CH CRP_2 by Siemens Healthineers, and ferritin using the Atellica IM Ferritin system, also by Siemens Healthineers.

Data management

Data will be collected using a web-based questionnaire through REDCap software. The first author will perform daily checks to ensure completeness, accuracy, and logical consistency. Any inconsistent or missing information (marked as "I don’t know") will be verified by

contacting the accompanying adult family member. REDCap's conditional logic and range checks will help maintain data accuracy. All study questions will be set as required fields to minimize missing values.

Data cleaning and detection of missing values will be managed by the first author by sorting each variable in ascending and descending order. To further ensure data quality, the first author, a qualified and trained dentist, will be actively involved in conduct data collection. This involvement will ensure ethical and sensitive handling of information, minimize bias, and promote uniformity throughout the process.

To ensure cultural appropriateness, the questionnaires were translated from English to Urdu by a translator, and reviewed by an expert. Necessary adjustments were made during the review, and a back translation was performed to preserve the original intent and meaning of the questions. Pre-testing of the study tools will also be conducted on 5% of the study sample (n=7 children) at the study site to identify any questions requiring paraphrasing or re-translation.

Validity

Several potential biases could affect the validity of this hospital-based study. First, confounding bias may arise due to unequal distribution of undiagnosed or unreported medical conditions, medication histories, and unaccounted-for cariogenic items in the child's diet, which could influence caries risk.

Second, wish bias might be introduced through study questions on sociodemographic factors, diet, smokeless tobacco consumption, and oral hygiene behaviours. This can be mitigated by refining the questions to be more neutral and less judgmental during pre-testing. Additionally, a more objective assessment of oral hygiene will be performed using the OHI-S to complement self-reported data.

Third, recall bias could occur when collecting information on the child's cariogenic diet. This will be mitigated by focusing on the child's diet over the past six months and verifying responses with the accompanying family member.

Lastly, non-differential misclassification could result from using a manual weight scale. This will be minimized by calibrating the machine daily and verifying its accuracy with a 1 kg weight. Each child will be weighed twice; if discrepancies arise between the measurements, the scale will be recalibrated and the weight reassessed.

Sample size

The sample size is calculated for both objectives as follows:

Frequency of children aged 7 to 12 with carious first permanent molars

The sample size is calculated using the WHO Sample Size Determination in Health Studies Software Version 2.0.21. A previous study conducted in Pakistan reported a frequency of 38.5% [46] for carious permanent first molars. Using this figure, with an absolute precision of 9% and a 95% confidence level (chosen to maintain study feasibility), a minimum sample size of 113 children aged 7 to 12 years is calculated.

Association of IDA and dental caries in first permanent molars in children aged 7 to 12

The sample size for this objective is calculated using STATA software version 15.0, with a power of 80% and a significance level of 5%. The ratio of exposed to unexposed children is derived from a study conducted in the Shaheed Benazir district of Pakistan among 11 to 13-year-olds, which reported an IDA prevalence of 15.5% [11]. This yields a ratio of 1:5. The proportion of unexposed children with the outcome (dental caries) is obtained from a study conducted in India [47], which reported that 37.8% of children without IDA have dental caries. Using these parameters, a minimum sample size of 76 children aged 7 to 12 years is

calculated to assess the association between IDA and dental caries in first permanent molars. This sample size will detect a one-sided prevalence ratio of 2.

Since the sample size calculated for the first objective is larger, it will be used for this study. After accounting for a 20% non-response and ineligibility rate, a final minimum sample size of approximately 141 children aged 7 to 12 years is obtained.

Statistical analysis

All analyses will be conducted using STATA software version 15.0. The frequency of children with carious permanent first molars will be reported along with 95% confidence intervals.

Descriptive statistics will be presented for all independent variables (such as age, gender, monthly household expenditure, parental and child education levels, and BMI etc.) stratified by IDA status. Categorical variables (e.g., gender, child's education status) will be reported as frequencies and percentages. The distribution of quantitative variables will be assessed for normality using box plots, histograms, and the Kolmogorov-Smirnov test. For normally distributed variables (e.g., age, number of adjacent carious teeth), means and standard deviations will be provided. For skewed distributions, medians and interquartile ranges (IQRs) will be reported.

Differences between exposure groups for categorical variables will be assessed using the chi-square test, provided the expected frequency per cell is at least 5; otherwise, Fisher's exact test will be used. For quantitative predictors, independent sample t-tests will be conducted, and p-values will be reported.

To analyze the cariogenic dietary construct, Principal Component Analysis (PCA) will reduce this variable into a single composite score, categorizing the frequency of cariogenic food consumption into low, medium, and high levels.

To assess the association between IDA and dental caries in permanent first molars, Poisson regression with robust variance will be used. Initially, univariate analyses will assess IDA, along with a priori confounders and other independent variables (e.g., age, gender, education level, tooth brushing frequency etc.), for their association with caries in permanent first molars. Crude prevalence ratios with 95% confidence intervals will be reported. Variables with a p-value < 0.25 at the univariate level will be checked for multicollinearity along with IDA (r > 0.8).

Subsequently, confounding and multivariable analyses will be conducted. A stepwise model-building approach will be used to construct the multivariable model, starting with IDA and sequentially adding variables. Likelihood ratio tests will be used to determine whether each added variable significantly improves the model fit (p-value < 0.05). Biologically plausible interactions will also be assessed (p-value < 0.1). The final, parsimonious model will be determined when no additional variables can be included, and none can be removed without affecting the model's fit. Adjusted prevalence ratios with 95% confidence intervals for caries in permanent first molars will be reported.

Patient and public involvement statement

The patients and the public are and will not be involved in the design, conduct, reporting, or dissemination plans of this research.

ETHICS AND DISSEMINATION

All study activities will be conducted in accordance with the Declaration of Helsinki and the International Council for Harmonization (ICH) guidelines for Good Clinical Practice (GCP). Ethical approval has been obtained from the ERC of AKU (Reference number: 2024-9692-30593) and the IRB of DUHS (IRB Reference: IRB-3556/DUHS/Approval/2024/196).

Prior to enrollment, informed assent and consent will be obtained from the participating children and their accompanying adult family members, respectively. Data collection will take place in a private space to protect their confidentiality. Sensitive questions, including those related to tobacco use, will be posed in a neutral and non-judgmental manner. This approach will foster a comfortable environment for participants, thereby minimizing any potential discomfort associated with discussing sensitive topics and upholding the highest ethical standards in our research.

To ensure participant safety, required blood tests will be performed by trained personnel. Any child needing dental treatment will be referred to dentists at CHK for free dental care. The "tell-show-do" technique will be used to help ease anxiety during the oral examination, and instructions on pediatric oral hygiene will be provided via flyers.

Confidentiality will be maintained throughout the study. Data will be anonymized by assigning each participant a unique eight-character alphanumeric identification (ID), with no direct link to their personal information in documents used for analysis or dissemination. The link between participant contact details and ID will be kept in a separate file under the custody of the first author. Data will be stored in a password-protected database, with backup files, and will be securely deleted seven years after study completion using erasure software.

The study results will be disseminated through seminars and published in a peer reviewed academic journal.

LIST OF ABBREVIATIONS

AKU- Aga Khan University

BMI- Body Mass Index

CBC- Complete blood count

CI-S - Calculus Index Simplified

CHK- Civil Hospital Karachi

COPD- Chronic Obstructive Pulmonary Disease

CRP- C-reactive protein

cm- Centimetres

DI-S- Debris Index Simplied

DMFT- Decayed, Missing, and Filled Teeth

DUHS- Dow University of Health Sciences

ERC- Ethical Review Committee

EDTA- Ethylenediamine tetraacetic acid

FDI- Fédération Dentaire Internationale

I-CVI- Item-level Content Validity Index

ID- Identification

IDA- Iron Deficiency Anemia

IRB- Institutional Review Board

IQR- Interquartile range

Kg- Kilogram

mDDE- modified Developmental Defects of Enamel

NSAID- Non-steroidal anti-inflammatory drug

OPD- Outpatient department

PCA- Principal Component Analysis

PKR- Pakistani Rupee

S-CVI/Ave- Scale-level Content Validity Index based on the Average method

S-CVI/UA- Scale-level Content Validity Index based on the Universal Agreement method

OHI-S- Simplified Oral Hygiene Index

STROBE- Strengthening the Reporting of Observational Studies in Epidemiology

WHO- World Health Organization

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AUTHOR CONTRIBUTIONS

SS, and SST conceived and designed the study. SS will be responsible for data collection and data management. SS, SST, IA will be involved in data analysis and interpretation. SST, IA, WAJ, AFS and FRK have revised the work and have provided their intellectual input. SS wrote the first draft of the manuscript. All authors have appropriately revised, read and approved the submitted version of this protocol. SS is the guarantor.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

DATA SHARING STATEMENT

Not applicable.

Study Title: Protocol for an Analytical Cross-Sectional Study on the Association of Iron Deficiency Anemia with Dental Caries in the Permanent First Molars of Children Aged 7 to 12 Years in Karachi, Pakistan

Instructions:

1. Separate form should be filled for each subject.
2. This form should only be filled after the subject has been screened successfully and has been considered eligible for the study.

Child ID: _____

Version number and Date: __ (_ _/_ _/_ _ _ _)

Section A: Socio-demographic information

(asked from the legal guardian)

S. No	Question	Code	Skip	Response
1.	Gender of child?	1. Female 2. Male		
2.	What is the age of child?	Write exact age in years		__ __ Years
3 a.	Has the child ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q4a.	
3 b.	What is the education status of your child?	1. No formal schooling 2. Schooling 2.1 if schooling= please specify Grade _____ 9. I don't know		
4 a.	Has the mother ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q5 a	
4 b.	What is the mother's education status?	1. No formal schooling 2. Primary school education 3. Middle school education		

		4. Secondary school education 5. Higher secondary school education 6. Above higher secondary school education 9. I don't know		
5 a.	Has the father ever attended school?	1. Yes 2. No 9. I don't know	If "No" or "I don't know" then go to Q6.	
5 b.	What is the father's education status?	1. No formal schooling 2. Primary school education 3. Middle school education 4. Secondary school education 5. Higher secondary school education 6. Above higher secondary school education 9. I don't know		
6.	What is the usual monthly household expenditure on food items?	Write the exact amount in PKR '98' is I don't remember		
7.	What is the usual monthly household expenditure on utilities such as gas, water and electricity?	Write the exact amount in PKR '98' is I don't remember		
8.	What is the usual monthly household medical expenditure?	Write the exact amount in PKR '98' is I don't remember		
9.	What is the usual monthly household expenditure on clothing items?	Write the exact amount in PKR '98' is I don't remember		
10.	What is the usual monthly household expenditure on social activities such as gifts, parties etc.?	Write the exact amount in PKR '98' is I don't remember		
11.	What is the usual monthly household expenditure on miscellaneous items?	Write the exact amount in PKR '98' is I don't remember		

Section B: Medical history

(taken from the legal guardian)

S. No	Question	Code	Skip	Response
12 a.	Has the child ever been diagnosed with iron deficiency anemia from the age of 6 by a healthcare professional?	1. Yes 2. No 9. I don't know	If 'No' or 'I don't know', skip to S. No 13 a.	
12 b.	How many times have your child been diagnosed with iron deficiency anemia from the age of 6 by a healthcare professional?	Write the exact number of times '98' is I don't remember		

Section C: Oral hygiene-related behavior

(taken from the child)

S.No	Question	Code	Skip	Response
13 a.	When was the last time you went to the dentist?	1. Never 2. >1 year ago 3. Between 6 months and a year ago 4. <6 months ago 9. I don't know	If 'Never' skip to S. No 14	
13 b.	What was the reason for your visit to the dentist?	1. Pain or trouble with teeth, gums or mouth 2. Treatment/Follow-up treatment 3. Routine check-up of teeth 4. Other reasons Please specify: _____ 9. I don't know		
14.	How frequently do you brush your teeth?	1. Never 2. 2-3 times a month 3. Once a week	If 'Never' or 'I don't	

		4. 2-6 times a week 5. Once a day 6. ≥ 2 times a day 9. I don't know	know' skip to S. No 16.	
15 a.	Do you use a toothpaste?	1. Yes 2. No 9. I don't know	If 'No' or 'I don't know', skip to S. No 16	
15 b.	What toothpaste do you use to clean your teeth?	Write the exact response. '98' is I don't remember		
16.	Do you regularly use any of these other oral hygiene practices atleast once a day?	1. Toothpowder 2. Mouthwash 3. Miswak 4. Dandaasa 5. All of the above 6. None of the above 7. Toothpowder and mouthwash 8. Toothpowder and miswak 9. Toothpowder and dandaasa 10. Mouthwash and miswak 11. Mouthwash and dandaasa 12. Miswak and dandasa 13. Toothpowder, mouthwash and miswak 14. Toothpowder, mouthwash and dandasa 15. Toothpowder, dandasa and miswak 16. Mouthwash, dandasa and miswak		

Section D: Other habits

(taken from the child)

S. No	Question	Code	Skip	Response
17.	How often do you use smokeless tobacco including snus, snuff, chewable tobacco (gutkha, betel leaf and/areca nut with tobacco)?	1. Never 2. 2-3 times a month 3. Once a week 4. 2-6 times a week 5. Every day 9. I don't know		

Section E: Dietary information

(taken from the child)

S. No 18. How often have you consumed, on average, the following food items in the last six months?

	Items	Never/ < once a month	1-3 times a month	Once a week	2 to 4 times a week	5 to 6 times a week	Once a day	2 to 3 times a day	4 to 5 times a day	6 to 7 times a day
	Bread and Cereals									
1	Porridge with sugar									
2	Frozen paratha/ Street food paratha									
3	Homemade paratha (Refined Flour)									
4	White bread/ Bun									
5	Rusk/ Cake Rusk									
6	Two-minute noodles/ Macaroni/ Spaghetti									
7	Biscuit / Cookie									
8	Sandwich biscuit									
9	Donut									
10	Cupcake/Muffin/Plain Cake									
11	Pizza									
	Desserts									
12	Kheer/ Custard/Pudding									
13	Cake/Pastry									

14	Meethai/halwa									
15	Sweetened yoghurt									
16	Ice cream									
17	Plain Jelly									
18	Fruit chaat (that has added sugar)									
	Beverages									
19	Tea with sugar									
20	Sweet lassi (flavored or unflavored)									
21	Soft drink									
22	Sharbat / Instant Drink									
23	Fruit juice									
24	Flavored milk									
	Miscellaneous									
25	French fries									
26	Chocolate									
27	Candy									
28	Cotton candy									
29	Chewing gum (not the sugar-free one)									
30	Lollipop									
31	Jam (for example on bread)									
32	Honey (for example on bread)									
33	Chocolate spread (for example on bread)									
34	Chips									

Section F: Physical and oral examination

(taken of the child)

S. No	Measurement	Finding
19.	Height (in cm)	
20.	Weight (in kg)	

S. No 21 **Oral hygiene status assessment**

Debris Index Simplified:

	Right permanent first molar		Permanent central incisor		Left permanent first molar		Total	
	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual
Upper	_____		_____ (right)		_____		_____	
Lower		_____	_____ (left)			_____	_____	_____

Debris Index Simplified= (The buccal scores) + (The lingual scores)/ (Total number of examined buccal and lingual surfaces)

=_____

Calculus Index Simplified:

	Right permanent first molar		Permanent central incisor		Left permanent first molar		Total	
	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual	Buccal	Lingual
Upper	_____		_____ (right)		_____		_____	
Lower		_____	_____ (left)			_____	_____	_____

Calculus Index Simplified = (The buccal scores) + (The lingual scores)/ (Total number of examined buccal and lingual surfaces)

=_____

Simplified Oral Hygiene Index= Debris Index Simplified + Calculus Index Simplified=

_____

S. No 22 **Carious assessment of the permanent first molars and adjacent teeth**

DMFT/dmft index:

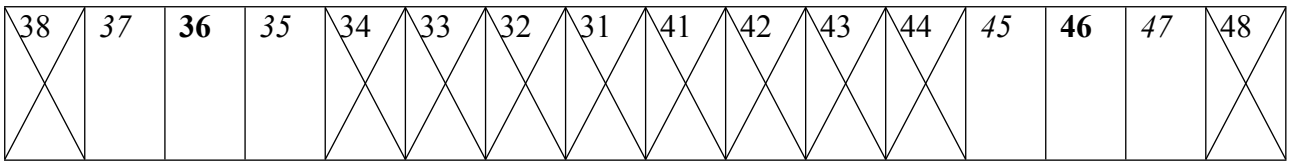
Permanent teeth	Primary teeth
D= Decayed	d= decayed
M= Missing	m= missing
F= Filled	f= filling

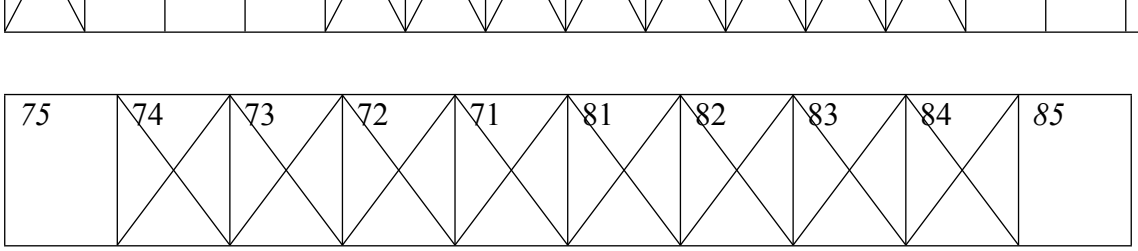
Maxilla

28	*27*	**26**	*25*	24	23	22	21	11	12	13	14	*15*	**16**	*17*	18

65	64	63	62	61	51	52	53	54	*55*

Mandible





Permanent first molar:

Total Decayed=_____ Total Missing=_____ Total Filled=_____

Total DMFT index score=_____

Adjacent teeth:

Total Decayed=_____ Total Missing=_____ Total Filled=_____

Total DMFT index score=_____

Total decayed=_____ Total missing=_____ Total filled=_____

Total dmft index score=_____

Section F: Laboratory-related information

S. No	Blood parameter	Code	Finding
23.	Hemoglobin (g/dL)		
24.	Ferritin (microgram/L)		
25.	C-reactive protein (milligram/L)		

Form filled by: _____ Date: _ _/_ _/_ _ _ _ *(Day/Month/Year)*

Form checked and edited by: _____ Date: _ _/_ _/_ _ _ _ *(Day/Month/Year)*