

PROGRESS REPORT SUMMARY		PROJECT NUMBER ES06000-02
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR <u>Douglass, Chester W.</u>		PERIOD COVERED BY THIS REPORT
APPLICANT ORGANIZATION <u>Harvard School of Dental Medicine</u>		FROM 9/30/92
TITLE OF PROJECT (Repeat title shown in item 1 on first page) <u>Fluoride Exposure and Osteosarcoma</u> (SEE INSTRUCTIONS)		THROUGH 9/29/93

PROGRESS REPORT SUMMARY

1. SPECIFIC AIMS

The National Toxicology Program (NTP) found "equivocal evidence" of carcinogenicity based on the occurrence of a small number of osteosarcomas in male rats fed doses of highly fluoridated water¹. A subcommittee of the Committee to Coordinate Environmental Health and Related Programs (CCEHRP) was then convened to analyze the benefits and risks of fluoride in drinking water.

An analysis of 12 "fluoridated" counties and 21 "non-fluoridated" counties within two SEER sites used as the basis for the CCEHRP Final Report that directly refuted the NTP findings². This study measured the exposure of interest, ingestion of fluoridated water, only at the county level, and no attempt was made to capture the effect of migration. To achieve the original goal of the Committee, this nation-wide hospital-based case-control study will provide a more scientifically conclusive assessment of risk for osteosarcoma from the ingestion of fluoridated water. Specific aims addressed in the on-going study include:

1. To compare the complete residential fluoride histories of osteosarcoma patients (both prevalent and incident cases) with the fluoride histories of hospital-based controls.
2. To compare total fluoride exposure from oral hygiene practices between cases and controls; including fluoride supplements, self-applied and professionally applied topical fluorides, and participation in school based fluoride programs.
3. To compare the fluoride content in bone and toenails of osteosarcoma patients with the fluoride content in bone and toenails of hospital-based controls.
4. To control for age, gender, and education in the comparison of cases and controls.

2. STUDIES AND RESULTS

To accomplish these aims, a retrospective and prospective hospital based case-control study was proposed and implemented during the first year. The retrospective aspect of the case control study collected data from prevalent cases and controls seen at 10 hospitals nation-wide. Cases are defined as patients \leq 40 years of age diagnosed with primary osteosarcoma from 11-01-89 to 12-31-92 by the participating orthopedic surgeons. Non-cancerous controls matched by age, gender, and distance from the hospital were chosen from the in- and out- patient rosters of each of the hospitals' surgery department. A 1:2 ratio of cases to controls was enrolled.

The following hospitals provided access to the patients enrolled as a case or control in the retrospective aspect of the study:

<u>Hospital</u>	<u># of Prevalent Cases/Controls</u>		Total
	<u># Cases</u>	<u>#Controls</u>	
Massachusetts General Hospital	45	90	135
Children's Hospital -Boston	18	36	54
Creighton University	9	18	27
Children's Hospital - DC	19	38	57
Memorial Sloan Kettering	27	54	81
University of Chicago	21	42	63
Rush Medical College	13	26	39
University of Florida	27	54	81
UCLA	29	58	87
Case Western/Cleveland Clinic	<u>18</u>	<u>36</u>	<u>54</u>
	226	452	678

Osteosarcoma patients and the matched controls with any prediagnosis history of the known risk factor, radiation therapy, are excluded. In addition, patients with a history of kidney dialysis are excluded as they chose to drink deionized water for medical reasons. Both genders and all races are included for study.

Protocol and Data Collection for the Retrospective Study

The Project Director, Dr. McGuire, traveled to each hospital to review the medical records and determine the eligibility of the prevalent cases. Once the age at diagnosis, gender, and distance from the hospital was transcribed from each eligible case medical record, the process to chose the corresponding controls was begun. Each hospital (except for Children's of DC) had the capability to generate a computer printout of the in- and/or out-patient rosters. A listing of the patients matching the age (± 5 years), gender, and distance from the hospital of each of the cases was requested. Once the list was received, six names were selected at random and their medical records were ordered so that abstraction of demographic and medical information could be completed. More than one return trip to each hospital was necessary to retrieve the information from the medical records of the qualifying matched controls because of the timeframe needed to generate the roster and order the medical records.

The information abstracted by Dr. McGuire from the medical records of the eligible cases and qualified controls was entered directly into a software program developed by New England Research Institute (NERI). The data were then downloaded onto disks and mailed to NERI. Researchers at NERI proceeded to send a letter of explanation of the research goals of the study to the eligible osteosarcoma patients and the qualified matched controls (See Appendix A for SAMPLE CASE LETTER and SAMPLE CONTROL LETTER). Telephone contact followed within 10 days for the administration of the telephone questionnaire (See Appendix B for QUESTIONNAIRE). A parent was surveyed if the case/control was less than 18 years old. Appendix C reports the status of the dispositions for the completion of data collection from the prevalent cases and controls as of the end of the Third Quarter (6/30/93)

Approximately 25 percent of the cases and controls used well water at some point in their residential history. This rate was higher than experienced during the pilot phase. Harvard School of Dental Medicine project staff sent letters and vials for well water sample collection to the cases/controls who reported well water use. The participants are provided with a postage paid cardboard

container in which to return the well water sample. Dr. McGuire measures the level of fluoride in each sample with the use of a DR 100 Colorimeter.

Preparation for Expanded Data Collection in the Prospective Study

During Year 1, arrangements had been made to coordinate the study with an expanded data collection effort of the etiology of osteosarcoma which will be conducted by the National Cancer Institute (NCI). The funding provided for toenail analysis in Year 1 will be carried over in order to facilitate the collection of these samples so that this process can be integrated with the NCI participant interviews. We believe that the addition of the NCI background data on study participants will increase our ability to control for alternative factors in analyzing the link between fluorides and osteosarcoma.

As of the end of the Third Quarter of Year 1, final arrangements are underway to expand the protocol to adjust for the collection of an iliac crest bone sample from incident cases. The non-tumor piece of bone will provide a standardized anatomical position from which a bone sample will be taken from both the case and control. The approval for the revision from each hospital's human subject review committee has been acquired and the recruitment of research nurses at each hospital has been completed.

3. SIGNIFICANCE

When restricted to males under 20 with osteosarcoma in the SEER study in the CCEHRP Final Report, the rate of osteosarcoma increased 79% in the fluoridated counties and decreased 4% in the non-fluoridated counties from 1973-80 to 1981-87. Time-trend analyses were performed and the committee concluded that finding was not significant. However, as was stated previously, the measure of exposure, duration of ingestion of fluoridated water, was only at the county level and no attempt was made to capture the effect of the migration. Cases in the pilot study case-control study³ (primarily Iowans) lived in an average of 1.7 different towns prior to diagnosis. This finding weakens conclusions based on the assumption of no migration. The CCEHRP report says "To the extent that migration is a factor, it is likely to diminish the sensitivity of a geographic correlation study to detect possible excess risk...Failure to observe exposure-disease association can be attributed..to a community level study..This is especially true for malignancies with a very low incidence such as osteosarcoma".

Linkage of fluoride ingestion and cancer initiation could result in a large-scale defluoridation of municipal water systems under the Delaney clause. (One aspect of this EPA clause prevents the addition of carcinogenic agents to the nation's water supply).

Although the SEER study showed equivocal evidence, the pilot study results for this grant showed no linkage of the ingestion of fluoridated water to the occurrence of osteosarcoma. Therefore, an incorrect inference implicating systemic fluoride carcinogenicity and its removal from our water systems would be detrimental to the oral health of most Americans, particularly those who cannot afford to pay for increasingly expensive restorative dental care. Additionally, a greater understanding of the etiology of osteosarcoma may prevent others from experiencing it.

4. PLANS

Data Collection for Year 2

Prevalent Cases and Controls: All prevalent cases and controls were accrued in the first year. Judging from the final dispositions at the end of the 3rd Quarter, all prevalent participants will have been contacted by NERI for the completion of questionnaire by the end of Year 1. Because of higher prevalence than expected, the work to complete the gathering of all well water samples will extend into Year 2.

Incident Cases and Controls: Incident cases and controls will accrue throughout the entire period of Year 2. Research nurses at each hospital will perform the following duties:

1. assist the orthopedic surgeon in gaining informed consent from the incident cases,
2. locate qualified controls from departmental rosters,
3. administer the questionnaires to and collect toenail samples from the eligible cases and qualified controls,
4. collect the tumor slice, blood specimen, and iliac crest bone biopsy from the case at the time of surgery
5. collect the control autopsy bone
6. collect the control blood specimen
7. prepare all the pathology specimens for transportation (See Appendix D for Protocol)
8. send the toenail samples for analysis
9. send the questionnaire data for analysis

The Project Director and Principal Investigator will conduct the training sessions for the research nurses; travel to each hospital annually to monitor 1) the reliability of the data, 2) the process by which the controls are chosen and 3) provide guidance on the collection of specimens; and monitor the collection of data at

each hospital weekly.

ADDITION OF MINORITY RESEARCH TRAINING PROTOCOL

During year 1, a protocol was also expanded to facilitate the research training of a minority faculty member.

Description of the research experience proposed for the minority individual

Objective:

To test the hypothesis that higher socioeconomic status is positively associated with higher fluoride exposure and osteosarcoma.

Specific Aims:

1. To determine if an association exists between higher socioeconomic status and higher fluoride exposure.
2. To determine if an association exists between higher socioeconomic status and the risk for osteosarcoma and if this relationship is mediated through high fluoride exposure.

Background and Significance

The efficacy of optimal water fluoridation for the prevention of caries in the developing dentition has been well established, as has the use of fluoride supplementation in areas where fluoride levels in the water supply is sub optimal.^{1,2} Associations between indicators of socioeconomic status, such as education and income, with fluoride supplementation and fluorosis have been shown.^{3,4,5} In a study of eight hundred and fifty 11-14 year old residents of non fluoridated communities in Connecticut and Massachusetts, Pendrys and Katz⁶, found that mild to moderate enamel fluorosis was strongly associated with fluoride supplementation and median household income. The odds ratio for the increased risk of fluorosis and fluoride supplementation was 4.0. The odds ratio (OR) for the increased risk of fluorosis and median household income was 6.6. Those subjects in the middle median household income group, who had used fluoride supplements during the first six years of life, had a 28 - fold increased risk of fluorosis compared to subjects in the low median household income obtained from census tract data. According to the authors "these values must be considered conservative estimates of the true strength of association with socioeconomic status (SES), since group data to identify subjects' socioeconomic status would be expected to be

less precise than subject specific income values."⁶ Another indicator of SES, parental education, has been positively related to both prevalence of fluorosis (OR = 2.2) and use of fluoride supplements (OR = 2.7).⁷

The etiology of osteosarcoma remains unknown. Although a relatively rare tumor with less than 2100 new cases occurring per year in the United States⁸, osteosarcoma is the most commonly occurring malignant tumor of the bone⁹. Radiation^{10,11,12}, viral action^{13,14}, genetic factors¹⁵, and other exogenous factors have been implicated in the development of osteosarcomas¹⁶. Radiation induction of osteosarcoma is at present an accepted principle. A recent Seminars in Oncology review of the epidemiology of bone sarcomas however reported that conflicting study results exist for all other etiologic agents¹².

Methods

A telephone interview of 320 incident cases and 640 matched controls will be conducted. Controls will be matched by age (± 3 years), gender, and geographic residence. The telephone interview to be conducted by New England Research Institute (NERI) will be designed for the primary project. The parent grant included case - control matching on residence distance from hospital. Not matching on community of residence decreases the likelihood of similar socioeconomic status between cases and controls. This project will expand the interview to include questions regarding family socioeconomic status. Information on parental education, income and employment will be collected and uniformly coded. Cases will then be compared with controls to determine odds ratios for the association between socioeconomic status and osteosarcoma. Dr. Da Silva will develop the questionnaire in conjunction with the Principle Investigator. He will also visit each clinic site to assess the reliability of the data collection, ensure complete data collection, intervene when problems occur, and assist in ensuring study progress when personnel changes occur at the various sites.

How the experience will foster the independent research capabilities of the minority individual

This research experience will provide Dr. Da Silva with several opportunities:

1. To develop and carry out an independent research project that integrates well with an existing nationwide study. This will allow participation in the parent project while taking the main responsibility on this project.
2. Because the parent study is a multicentered project, this

project will provide Dr. Da Silva with the opportunity to interact with other established researchers at the eight different participating sites.

3. Dr. Da Silva will become an integral part of the project, working closely with the New England Research Institute and members of the Harvard School of Dental Medicine to:
 - i. Develop the telephone interview questionnaire for the incident study subjects.
- On clinical site visits he will:
- ii. Coordinate clinic site staff
 - iii. Ensure study progress as personnel turnover occurs
 - iv. Monitor the selection of appropriate controls and assess data reliability
 - v. Monitor data collection
 - vi. Monitor problems of non-response
- At Harvard and NERI:
- vii. Edit and check all data from the eight study sites in collaboration with the site clinic staff
 - viii. Develop coding schemes for data
 - ix. Prepare data files for transmission to NERI and collaborating investigators.
 - x. Produce data management and progress reports
 - xi. Check entry of and final disposition of cases and controls
 - xii. Data analysis
 - xiii. Manuscript preparation of study results

This experience, combined with Dr. Da Silva's educational background will help develop his future potential to become an independent investigator with a firsthand knowledge and understanding of the intricacies and problems associated with a large multicentered investigation. Dr. Da Silva will work with the principle investigator, project director and statistician of the parent grant to understand the complexities of data collection and analysis for a large case-control study. This analysis will also be important in the development of Dr. Da Silva's data analysis skills. He will also gain experience in the preparation and production of manuscripts of study results for publication. In sum, this opportunity would be immensely useful because it would be facilitate the minority faculty member's research experience substantially at the beginning of his academic career.

How the proposed experience relates to the specific research goals and objectives of the parent grant:

The proposed experience will allow Dr. Da Silva to become an integral part of the parent project as the developer of the telephone interview for the incident cases and their controls. In addition, he will assist in ensuring that cases have appropriately

match controls as outlined in the parent grant. He will also assist in coordinating the data collection from the various participating clinical sites, Harvard school of Dental Medicine and NERI. One of the goals of the parent grant is to determine if a relationship exists between osteosarcoma and an environmental factor - fluoride ingestion. This research supplement would allow the elucidation of an additional factor's influence - socioeconomic status. This may become an important factor in the analysis of the parent project. The issue of socioeconomic status heightens our interest as providers of health care, because it may be specifically associated with medical and dental health care provision of fluoride supplements^{17,18}. As a project whose goal it is to determine if an increased risk exists between an environmental factor (fluoride) and osteosarcoma, this supplement would allow the integration of an important factor that has been shown to be correlated with fluoride ingestion. In addition, this supplement will afford a minority investigator, with an excellent background, the opportunity to become integral to the parent project as well as embark on his own research/academic career.

(For references in this section, see Appendix F)

Determination of fluoride exposure from residential history

Prevalent and Incident Cases and Controls: The fluoride levels of the municipalities supplying water to the cases and controls are listed in the CDC Fluoridation Census in ppm. Year of fluoridation is also listed. However, we have found that many cities and towns are not listed in the Census, even though they have fluoridated water. Problem towns were identified in Year 1 and the Departments of Health in the following states were called to obtain a statewide list of the fluoride levels for all cities and towns in the state: Arizona, Arkansas, California, Colorado, Connecticut, Delaware, Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maine, Maryland, Massachusetts, New Hampshire, Ohio, Oklahoma, Pennsylvania, Rhode Island, South Carolina, Texas, Tennessee, Vermont, and Washington.

Water sample tubes will continue to be provided by mail to the participants who use well water. The participants will be requested to return a well water sample for analysis at the Harvard School of Dental Medicine. Response rate to date has been approximately 70% for well water sample collection. We expect this to improve, however 19 cases have been found in which the well in question has been shut off.

Collection of toenail sample and determination of fluoride concentration

Incident Cases and Controls: The research nurses will collect set of toenail clippings from all 10 toes from all incident participants. The clippings will be cleaned in deionized water at each hospital. This procedure is highly effective at removing superficial contamination. The clippings will then be sent to the laboratory of Dr. J Steven Morris at the University of Missouri Research Reactor. The case control pairs will be analyzed in the same assay run by neutron activation analysis; the specimens will be labeled by ID number only, and the position of the case and control specimen will vary randomly. After exposure to thermal neutrons, gamma emissions from the ^{20}F isotope (half-life 11.03 seconds) are counted and, with the use of a certified standard, converted to weight of fluoride. The coefficient of variation for these measurements on separate nail specimens from the same person (which includes biological variation and technical error) is 10-15%.

Determination of fluoride content in bone

About one cm^{-3} of resected bone from the incident osteosarcoma tumor surgical site of patients will be analyzed for fluoride content NMR imaging and spectroscopy. Collecting the resected specimen will not in any way alter the type of surgical procedure that given patient will receive. Additionally, while the incident case is under general anesthesia for the treatment of the sarcoma lesion, an iliac crest bone biopsy will be obtained. At a point 2 cm distal and 2 cm posterior to the anterior-superior iliac spine, a 4 cm incision will be made. the soft tissues will be retracted to expose the outer cortex of the ilium. The trochar is then positioned so that the needle will acquire a transiliac bicortical core biopsy with a diameter of approximately 1 cm. Hemostasis will be achieved by electrocoagulation or minor bleeders. The soft tissues are allowed to fall back into place. The wound is closed in a routine fashion. The bone biopsy from the incident osteosarcoma patient will be analyzed for fluoride content. The non-tumor piece of bone will provide a standardized anatomical position from which a bone sample will be taken from both the case and the control. Cadaver iliac crest bone samples (the control bone) matched by age and gender will also undergo fluorine analysis. Specimens will be stored at -60°C and then transported to MGH in dry ice until ready for use.

The specimen will be cleaned of adhering soft tissue and periosteum following brief thawing, placed under liquid nitrogen, and ground to a fine powder with a large ceramic mortar and pestle. The resulting powder will be lyophilized overnight at room temperature, and then extracted with chloroform-methanol. This treatment will remove most of the water and lipids, producing a dry powder which is easy to spin at high speed. The mildness of the

treatment insures that no chemical alteration of the mineral will occur. In our experience, ground but otherwise untreated specimens often cannot be spun above about 1.5 kHz, which would be sufficient for fluorapatite, but insufficient for calcium fluoride-type constituents. Lyophilizing yields a pasty material that can usually be coaxed above 2.5 kHz. Removal of the majority of the lipid is required to produce a dry, free flowing powder which can dynamically balance itself as the spinner approaches speeds above 6 kHz. Specimens will be equilibrated to laboratory temperature and humidity for 24 hr prior to NMR analysis.

Quantitative ^{19}F MAS NMR spectra of an accurately weighed sample of each powdered specimen will be obtained on a Bruker MSL-400 NMR spectrometer operating at a field strength of 9.4 T and a ^{19}F frequency of 376 MHz. Spinning speeds will be precisely 7.00 kHz so that the distribution of signal intensities among the sidebands will be fixed for all specimens. Weights will be taken both before and after NMR analysis to check for possible water absorption. Quantitation will be performed by comparison of the total integral of the baseline corrected spectrum with that of a reagent grade CaF_2 standard. CaF_2 is chosen as a primary standard because it may be accurately determined by gravimetry alone. Data processing will be performed offline from the spectrometer using the NMRI software package from New Methods Research (Syracuse, NY). Replicate analyses will be performed for each specimen on two different days.

The figure of 7 kHz represents the approximate maximum spinning speed achievable by our equipment. As shown by Kreinbrink, et al³¹ this is more than adequate to fully narrow apatitic fluoride, and sufficient to narrow CaF_2 -type resonances enough for reasonable quantitation. Although combined MAS/multiple pulse methods⁴ provide the sharpest, potentially most useful, resonances, there is often a loss in total signal/noise ratio, and so we will avoid these techniques because of their sometimes questionable absolute quantitative accuracy.

Prior to the start of this aspect of the study, we will characterize the quantitative accuracy of our methods by blind analysis of a series of weighed mixtures of hydroxyapatite, previously characterized⁵ fluorapatite, and reagent grade CaF_2 . A standard set of conditions and instrument settings will be developed. Samples of these mixtures and occasional bone samples will be sent to commercial laboratories for fluoride determination by dissolution and potentiometric analysis in order to establish a comparison between NMR and conventional methods. Occasional bone NMR and potentiometric analyses will be checked by the method of standard additions using reagent grade CaF_2 .

Report Writing

After the return of all obtainable well water samples from the prevalent cases and controls, data collection for the retrospective aspect of the study will be complete. Analysis will commence immediately. Thus, in year 2, we will have a preliminary report on the findings from the 226 retrospective cases and 452 controls. Additionally, an article is in progress reviewing the current literature on the genetic risk factors for osteosarcoma and other bone and soft tissue tumors. The definitive data however, will accrue from the findings of the prospective study.

PROGRESS REPORT (Personnel and Study Subjects)

NT NUMBER
ESO6000-02

All Personnel for the Current Budget Period
and Any Planned Changes in Personnel for the Next Budget Period

Use two sections. In the first section list All Current Personnel. In the second section list Planned Personnel Changes.

Name	Degree(s)	SSN	Role on Project (e.g., PI, Res. Assoc.)	Date of Birth (MM/DD/YY)	Annual % Effort
<u>All Current Personnel</u>					
Douglass, Chester	DDS, PhD		Principal Investigator		
Mankin, Henry	MD		Investigator		
McGuire, Sheila	DDS, DMSc		Proj. Direct.		
Ackerman, Jerome	PhD		Investigator		
Hunter, David	PhD		Investigator		
Pfleiderer, Bettina	PhD		Res. Assoc.		
Ferraro, Ilona			Administrat.		
Duffy, Mary		Secretary			
<u>Planned Personnel Changes</u>					
McGuire, Sheila	BDS. MPH		Project Dir.		
Joshi, Anil			Programmer		
To Be Named			Statistician		

Provide the number of subjects enrolled in the study to date according to the following categories. (See Page 8 for definitions.)

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic Origin	Hispanic	White, not of Hispanic Origin	Other or Unknown	TOTAL
Female		1	19		205	13	238
Male		4	34		301	17	356
Unknown							
TOTAL		5	53	0	506	30	594

5. HUMAN SUBJECTS

Protocol Changes

In addition to the data and pathology samples that we originally proposed, an iliac crest bone biopsy will be performed at the time of surgery to gather a non-tumor piece of bone at a standardized anatomical position. A similar bone sample will be taken from an autopsy control. The approval for the revision from each hospital's human subject review committee has been acquired. See Appendix E for a sample set of Informed Consents submitted for successful institutional approval.

Inclusion of Women and Minorities

All women and minorities meeting the eligibility criteria will be enrolled in the proposed study. The gender ratio found by previous studies for the occurrence of osteosarcomas is 3:2 male:female. Osteosarcoma, a rare bone cancer, has a low prevalence rate in people of color.

6. VERTEBRATE ANIMALS

Not Applicable

7. PUBLICATIONS

Not Applicable

8. INVENTIONS AND PATENTS

Not Applicable

Literature Cited

1. National Toxicology Program. Toxicology and carcinogenesis of sodium fluoride in F344/N rats and B6C3F1 mice. Technical Report Series 393, NIH Publication No. 90-2848, 1990.
2. Review of Fluoride: Benefits and Risks. Report of the Ad Hoc Subcommittee on Fluoride of the Committee to coordinate Environmental Health and Related Programs, Public Health Service, Department of Health and Human Services. 1991.

3. McGuire SM, Vanable ED, McGuire MH, Buckwalter JA, and Douglass CW. Is there a link between fluoridated water and osteosarcoma? JADA 122:39-45, 1991.
4. Smith KA, Burum DP. Applications of fluorine-19 CRAMPS to the analysis of calcium fluoride/fluorapatite mixtures. J Magn Reson 84:85-94, 1989.
5. Shimoda S., AobaR, Moreno EC, Miake Y. Effect of solution composition on morphological and structural features of carbonated calcium apatites. J Dent Res. 69:1731-1740, 1990.