PROGRESS REPORT SUMMARY	ANT NUMBER ES06000-03	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR Chester W. Douglass	PERIOD COVERED BY THIS REPORT FROM	THROUGH
APPLICANT ORGANIZATION Harvard School of Dental Medicine	9/30/93	9/29/94

TITLE OF PROJECT *(Repeat title shown in item 1 on first page)*
Fluoride Exposure and Osteosarcoma
(SEE INSTRUCTIONS)

PROGRESS REPORT SUMMARY

1. Specific Aims

The National Toxicology Program (NTP) found "equivocal evidence" of carcinogenicity based on the occurrence of a small number osteosarcomas in male rats fed doses of highly fluoridated water[1]. A subcommittee of the Committee to Coordinate Environmental Health and Related Programs (CCEHRP) was then convened to analyze 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 a basis for the CCEHRP Final Report that directly refuted the NTP findings[2]. 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 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 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 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:

Hospital	# of Prevalent Cases/Controls		
	#Cases	#Controls	Totals
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	18	36	54
	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 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 listing 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 the 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. Telephone contact followed within 10 days for the administration of the telephone questionnaire (See Appendix A for the retrospective questionnaire). A parent was surveyed if the case/control was less than 18 years old. Appendix B 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 (6/30/94). Dispositions are final for all but three percent (22/678) of the participants. We have yet to obtain well water samples for these remaining 22 participants. The last reminder telephone call was made in June to elicit the remaining samples.

Approximately 25 percent of the cases and controls used well water at some point in their residential history. This rate was higher than expected 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.

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 2 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.

The Project Director and Principal Investigator conducted the training sessions for the research nurses in Washington DC at the end of January. With the assistance of NCI staff, the following subjects were covered during the four day session:

* Study Objectives and Training Objectives
* Data Collection Components
* Site Coordinator Duties
* Interview Requirements for Certification
* Interviewing Video
* Questionnaire and Q by Q's
* Role Plays: Community Dyads
* Subject Data Form
* Assigning ID's
* Case and Control Enrollment and Forms
* Record of Contacts and Form
* Co-respondents Screen Worksheet
* Informed Consent
* Specimen Collection
* Practice Specimen Handling and Labelling
* Site Plan
* Practice for Certification
* Quality Control and Editing
* Reporting to and Communicating with Project Staff
* Overall Review

As of the end of the Third Quarter of Year 2, the protocol has been expanded to adjust for the collection of an iliac crest bone sample and a blood sample from incident cases and a blood sample from the incident controls. See Appendix C for the updated protocol. 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. The approval for the revision from each hospital's human subject review committee has been acquired, the recruitment of research nurses at each hospital has been completed, and data collection from incident cases and controls has begun.

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 study[3] (primarily Iowans) lived in an average of 1.7 different towns prior to the diagnosis. This finding weakens conclusions based on the assumption on 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 defluoiridation 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 systematic 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 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 3

Prevalent Cases and Controls: All prevalent cases and controls were accrued in the first year. Because of higher prevalence than expected, the work to complete the gathering all well water samples was extended into year 2. By the end of the 4th Quarter of Year 2, final dispositions will have been issued, the analysis will have been completed and a manuscript submitted for publication.

Incident cases and controls: Incident cases and controls will accrue throughout the entire period of year 3. Research nurses at each hospital are preforming the following duties:

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

The Project Director and Principal Investigator will work with NCI staff as they travel to each hospital 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.

MINORITY RESEARCH TRAINING PROTOCOL

During the past year, Dr. Da Silva has become an integral part of the project. He has worked closely with the Harvard personnel, NERI and NCI. Dr. Da Silva worked directly with Harvard personnel and NCI to incorporate his research protocol into the prospective portion of the project. He assisted in the development of the interview questionnaire to ensure that the specific aims of his portion of the project would be addressed:

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.

Dr. Da Silva has also been involved in follow up with NERI to identify short falls in data acquisition. He made a site visit, with Dr. McGuire, to Washington D.C.'s Children's Hospital. During the site visit he learned the on site protocol for identifying matched controls and assisted in obtaining the remaining necessary controls.

Dr. Da Silva participated, along with other Harvard personnel, in the training session held at NCI for the Site Coordinators who

will be involved in the prospective portion of the study. He participated in all aspects of the training including the consent requirements, data collection components, role playing and certification as an interviewer. Dr. Da Silva will also serve as an additional contact person for the individuals at each of the sites and will assist in the resolution of any problems that arise.

In response to a suboptimal acquisition of participating controls from UCLA Medical Center, Dr. Da Silva made a site visit to update data on previously identified controls, contact them and request their consent to participate in the telephone interview conducted by NERI.

Dr. Da Silva has also been trained in the protocol for the measurement of fluoride in well water samples at Harvard. He has assisted in the follow-up on missing well water samples. Thus reducing the number of missing data points. He has also been involved in data transmission of water sample fluoride levels to NERI and in data error checking.

Determination of fluoride exposure from residential history

Prevalent and Incidental 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 Department 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 samples tubes will continue to be provided by mail to the participants in the prospective study 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 in over 30 incidences the well in question has been shut off.

Collection of toenail samples and determination of fluoride concentration

Incident cases and controls: The research nurses will collect

a 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 2^0F 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 a 4 cm incision will be made. The soft tissue 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 fluoride analysis. Specimens will be stored at -60^0 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 pestal. 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 lipid, 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 1.5 kHz, which would be sufficient for

fluorapatite, but insufficient for calcium fluoride- type constituents. Lyophilizing yields a pasty material that can be 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 both before and after NMR analysis to check for possible water absorption. Quantitation will be performed by comparison of the 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 al[31] this is more than adequate to fully narrow apatitic fluoride, and sufficient to narrow CaF2-type resonances enough for reasonable quantitation. Although combined MAS/multiple pulse methods[4] 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 characterized[5] 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 the 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. By the end of the 4th Quarter of Year 2, a the analysis of the relationship between exposure to fluoride and osteosarcoma will be complete and a manuscript submitted for publication. One publication is complete and has been accepted for publication. "The Genetic Risk Factors for Bone and Soft Tissue Tumors" has been accepted for publication in the Surgical Oncology Clinics of North America. The definitive data however, will accrue from the findings of the prospective study.