EVALUATING THE SUITABILITY OF THE HUMAN TOENAIL AS A BIOMONITOR FOR MANGANESE STATUS: The *One Source* Cohort

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A Thesis Presented to the Faculty of the Graduate School

at the

University of Missouri - Columbia

In Partial Fulfillment of the Requirements for the Degree

Master of Science

By

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May 2007

The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled:

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And hereby certify that in their opinion, it is worthy of acceptance.

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ACKNOWLEDGEMENTS

I owe many thanks to Dr. Robertson, who has shown an incredible amount of patience with me during this longer-than-usual process. He made a way for me "through the wilderness" and then got behind me to make sure I followed through! Thank you for not giving up on me.

To Dr. Morris, I express my deep appreciation for all of the time and thought he has put into this project from its inception through the final stages. He guided me through the bewildering world that is epidemiology and always made time to talk, even putting his own work aside to answer questions. Thank you for all those hours of instruction.

Without John Brockman's help, I wouldn't have made it. He and I worked together on planning the irradiations but he often got stuck with the worst jobs: making standards, doing graveyard shifts in the p-tube labs, interactive peak fitting, performing calculations, making plots, and the list goes on. Dr. Brockman, thank you for SO MUCH help.

A BIG thank you goes to the members (past and present) of the MURR Analytical Chemistry Group, who lent their time, bent their schedules, and sent those rabbits flying in support of this project. I list their names below, but must single out Vickie Spate for special recognition – she irradiated, transferred, and counted toenails all night, two sweltering nights straight. Thanks to her quick thinking, we were able to save the run even when a detector failed in the middle of it.

Vickie Spate
Ruth Ann Ngwenyama
Carolyn Buff
Stacy Crane
Missy Evans-Blumer
Pam Altman
Joseph Gillispie
Erin Gourley
Eric Hollabaugh

I also must thank my family for their love and support. They have put up with my inattention, substandard moods, cranky behavior, and just general "being too busy to think about anything else."

I thank the Lord for the blessings of being able to work, being able to finish, and having these wonderful people in my life.

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ABSTRACT

Numerous studies have demonstrated that the human toenail is a reliable biomonitor for the intake of Se and other elements. The objective of this study was to evaluate the hypothesis that Mn intake is reflected in toenail Mn levels. Interest in the status of Mn, a necessary trace nutrient which may also have toxic effects upon overexposure, stems from two recent developments: first, a greater awareness of the role of Mn-based enzymes in human health, and second, a renewed controversy over the use of a Mn-containing gasoline additive in Canada. In order to evaluate the hypothesis, toenail specimens from One Source™ multivitamin users and matched controls were selected. Using the NAA technique, Se was measured using established methods, and then Mn was measured via a new procedure. The Se results confirmed the accurate classification of the cohort. However, the nail did not demonstrate significant, positive response to Mn supplementation. Several explanations for this lack of response may be offered, including the confounding effect of other, unknown dietary variables; Mn blood level regulation by homeostatic mechanisms; and the masking of endogenous Mn in the toenail by persistent exogenous material.

1 INTRODUCTION

1.1 Preface to the Introduction

The purpose of the research presented herein was to determine the validity of the hypothesis that toenail manganese content is related to the amount of this mineral consumed in a dietary supplement. This introduction will begin by describing the toenail matrix and how this tissue has been employed in epidemiological studies of different types. After briefly summarizing the categories of objectives such studies seek to achieve, the elements for which the toenail has proven to be a useful biomonitor will be discussed. Toenail selenium will be highlighted as an excellent biomonitor for Se status and its known determinants will be given. The health hypotheses for Mn will be presented, along with the rationale for exploring the toenail as a Mn biomonitor. Our approach to testing the Mn toenail hypothesis is then outlined.

1.2 Biological Monitors, General Concepts

Throughout this introduction, the terms “biological monitor”, “biomonitor”, “biological marker”, and “biomarker” may be used interchangeably. These terms refer to quantifiable biological phenomena or states such as physiological responses, cellular or molecular events or alterations, or chemical concentrations in body fluids or tissues.[1, 2] Biological monitors provide information on chemical exposure, nutritional intake and status, the effects of lifestyle, or susceptibility to disease.[2] Biomonitors may be simple metrics like blood pressure, used for diagnosis of cardiovascular disease, or nicotine metabolites in urine, used by insurance companies to detect the use of tobacco. At the

other extreme, they may rely heavily on advanced bioanalytical techniques such as those employed in the quantitation of DNA adducts, yielding very specific data on xenobiotic-induced damage to macromolecules.[2] Though the most common specimens for biomonitoring include blood, urine, hair, and nails,[3] there are theoretically a large variety of possibilities that could be employed.

Many advantages accompany the use of biomonitors in epidemiological studies. They can reduce misclassification errors, for example, by identifying subjects who have provided inaccurate data with regard to diet or supplementation. In intervention studies, they can be used to assess the degree of compliance, i.e. the extent to which the test substance is being ingested. An effective biomonitor of nutritional status can identify test subjects who, though apparently healthy, begin the study with a nutritional deficiency. Often these low baseline value individuals exhibit the greatest disease risk, and their risk shows the greatest response to intervention. By the same token, in environmental studies, the biomonitor may be used to flag the subset of a population that has actually been exposed to a toxic agent.[2]

The traditional methods of determining nutritional intake have centered on dietary questionnaires and food-composition data.[1, 4] However, nutrient levels in foods may vary significantly by source and their bioavailability modified by cooking method as well as other foods consumed in the meal. The percentage of nutrient absorbed varies in complex ways from person to person and from day to day. Biomonitors show the promise of being able to cut through all of these factors and arrive at an actual, measurable, long-term nutritional status.

Biomonitors are selected based on practical considerations. They should be sensitive to the larger effect they are intended to measure, and should selectively mirror that effect only.[2] Using toenail Mn as a theoretical example, this biomonitor should ideally respond measurably to a subject's Mn intake/status, and should only respond to this stimulus, excluding environmental Mn with which the subject comes in contact but neither ingests nor absorbs. An ideal biomonitor should be easily and unobtrusively obtained from the subjects. Toenail clippings fit this description, and have the additional advantages that subjects may self-collect and mail in the specimens, which can then be stored in paper envelopes at room temperature for extended periods.

1.3 The Toenail as Biomonitor

1.3.1 Description of the Toenail Matrix

Human toenails, fingernails, and hair are body tissues that consist of keratins, which are fibrous proteins that contain disulfide[5] bridges which are thought to chelate metals present during their formation.[6] As common sense would suggest, toenails and fingernails are more similar to each other than to hair. A wide variety of elements are found in human fingernails and toenails, and many tend to exist at approximately the same order of magnitude in each type of nail.[7-13] Sulfur generally comprises approximately 2.5% (wt./wt.) of the nail. Among those elements found at the level of hundreds or thousands of ppm (μg/g) are Na, Mg, P, K, Ca, and Zn. Other elements have usually been found in the range of tenths to tens of ppm: F, Al, Cl, Ti, Cr, Mn, Fe, Ni, Cu, Se, Br, Cd, Hg, and Pb. Many others have been measured at sub-ppm levels and will

not be listed here. Many elements, with the exceptions of S and Se, exhibit a large person-to-person and sample-to-sample variance.

The high sulfur concentration in the nail provides many binding sites for metals.[7] The time frame over which metals and other substances are incorporated in the nail is a question that continues to be debated among researchers. It has been demonstrated that Se in toenail clippings represented an integrated exposure window of 6-12 months previous,[14] consistent with the idea that substances are deposited into the newly formed nail by the nail matrix at the root.[15] Other researchers, after completing an extensive review of the literature, concluded that the nail bed, which is the tissue underneath the nail, contributed little to the composition of the growing, formed nail.[6] However, the nail does increase in thickness from the root, or "lunula," to the tip, or "free margin," due to continuing contribution of layers from the nail bed.[15] Studies of patients being treated for onychomycosis show incorporation of drugs into nail clippings more rapidly than would have been expected if all nail tissue were formed in the nail matrix.[15] Reconciliation of these two schools of thought may be possible if the mechanisms of incorporation of organics differ from those of minerals. However, not all minerals are deposited in the same way. Selenium may be incorporated during the formation of keratin and be present as part of the protein itself, possibly substituting for sulfur in amino acids such as cystine or methionine.[16] Mn and other metals are likely to be chelated by sulfur groups in the proteins and could be introduced after the proteins are formed.

1.3.2 Toenails in Comparison to Other Commonly Used Biomonitors

Relative to other biomonitors, the toenail is a potentially unique tissue, especially for subjects who commonly protect it from exogenous contamination by keeping their feet in socks and close-toed shoes and/or mainly staying in clean environments. Fingernails and hair are commonly thought to be more subject to accumulation of metals due to external contamination, reflecting concentrations out of proportion to the body's actual exposure.[6, 7] For this reason, Ni and inorganic Hg are elements that are more likely to be found in fingernails versus toenails.[17] By and large, however, fingernails and toenails in one comparative study were found to be similar for most elements.[9] Though we assume that toenails are more sheltered, the possibility of external contamination cannot be excluded. For children, Cd and Pb have been found at higher levels in toenails versus hair, leading one researcher to conclude that exogenous contamination was more severe for toenails.[18] The assumption that Cd and Pb tend to have exogenous sources, however, may not be correct. Selenium, commonly an endogenous element, has also been found at higher levels in toenails versus hair.[16]

Hair, also commonly employed in biomonitor studies, differs from nails. The keratins found in hair have a different chemical composition. Specifically, they are lower in sulfur, and thus may bind metals less efficiently, though other non-fibrillar proteins may compensate.[5] In contrast to nails, metal affinities of hair may be partially a function of its melanin content, which varies greatly among persons. While nails are formed underneath the cuticle, the hair follicle is bathed in sebaceous excretions and sweat, yielding differing trace element compositions.[18] Hair, having a higher surface area, may

be more amenable to cleaning procedures than nails.[18] The flipside of this is the increased possibility of leaching endogenous metals from hair during immersion cleaning.[19, 20] The variety of hair care products and treatments, such as bleaches, dyes, and selenium- or zinc-treated shampoos, also may change the trace element composition of hair.[11, 16, 20, 21]

The metal content of blood may reflect exposure over a very short and recent period of time. On the other hand, nails and hair are thought to integrate exposure over longer periods of time and thus give a more representative picture of long-term metal body burden.[22] Plasma selenium, for example, is regarded as a biomarker of short-term selenium status and blood Se extends the range,[23] but toenail selenium monitors the intake over 6-12 months or more.[14] However, blood is a useful biomonitor for Fe and Cu, two metals whose status is not well-reflected in nails.[23, 24] Certain trace metals, such as Mn, are found at low concentrations in the blood, so low that contamination from the phlebotomist's steel needle or the collection tube may become significant.[25, 26] Aside from trace metal determinations, blood analysis reveals the body's dynamic chemistry through enzyme activity levels, protein adducts, and more.[2]

Other factors, such as cost and compliance, increase the effective utility of the toenail. No phlebotomist is needed to obtain the specimen, and it can be shipped via regular mail in a common paper envelope. Study participants more willingly comply with requests for toenail clippings versus blood or even hair samples.[16] The toenail matrix is stable for years stored at ambient conditions.[6, 27]

1.4 Two Categories of Measurement Objectives –Nutrient Status and Toxic Exposure

For some trace nutrient elements, such as Se and Cr, both deficiency and overexposure may be conditions of concern. For these and other elements, the graph of positive health effects vs. dose is generally thought to follow Bertrand's rule, graphically illustrated by an inverted U-shaped curve, (Figure 1) with the high plateau representing an intake range in which biological function is optimized.[28, 29] Most studies using the toenail as a Se biomonitor focus on a hypothesized moderate Se deficiency common among people today. Presumably, this marginal inadequacy increases risk for a wide range of diseases and/or sub-optimal health.[30] For Cr, scientists have studied a possible deficiency reflected in toenail concentrations[31] as well as potentially toxic exposure.[32]

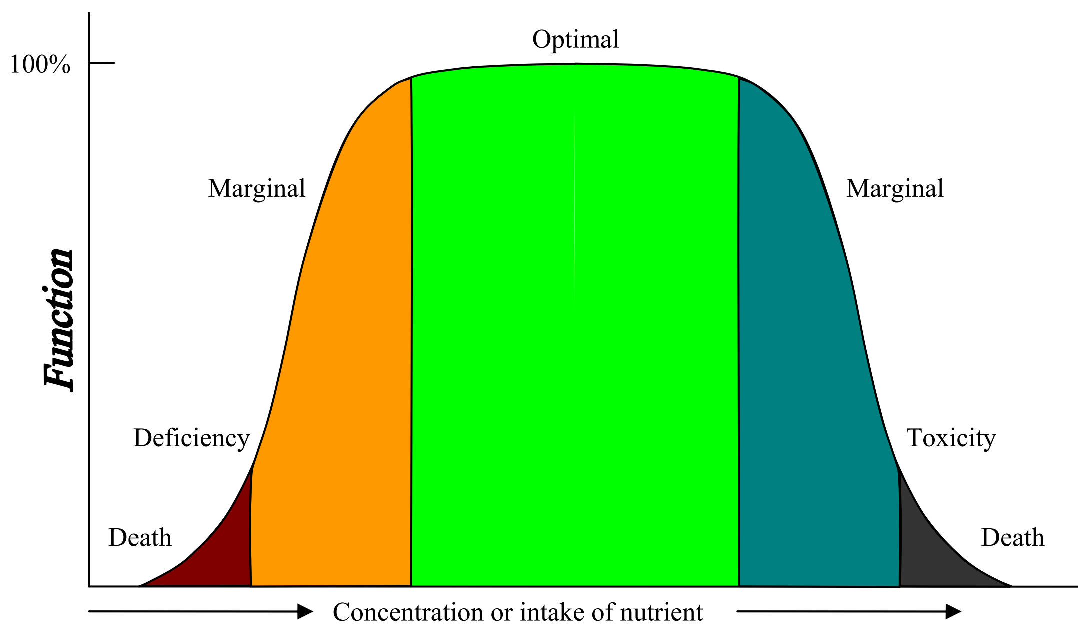


Figure 1: Dose-response curve for a trace nutrient.[29]

Other elements have no known nutritional function and are considered to be toxins at any level. (Figure 2) This group includes As, Hg, and Cd, whose concentrations in biomonitors are compared with health effects or known exposure. For

example, toenail As has been shown to be correlated with As in drinking water, even when present in water at very low levels.[33] Many associations between drinking water As and toenail As have come out of studies in West Bengal, India, and these studies show that when low-As drinking water is substituted, toenail As concentration drops.[6] Children living in U.S. towns with copper smelting operations show elevated levels of As in hair and urine.[34] Intake of Hg, primarily by consumption of fish, has been correlated with toenail and blood Hg.[35, 36] For toenail Cd concentration, on the other hand, there is scant evidence of relation to exposure[37]; it was not associated with the occurrence of ALS,[38] the risk of prostate cancer,[39] or the risk of melanoma.[40] Rather, the biomonitor of choice for Cd is urine, in which a Cd exposure is slowly eliminated over the course of decades. Increased urine Cd is significantly correlated to lung cancer and total cancer incidence.[41]

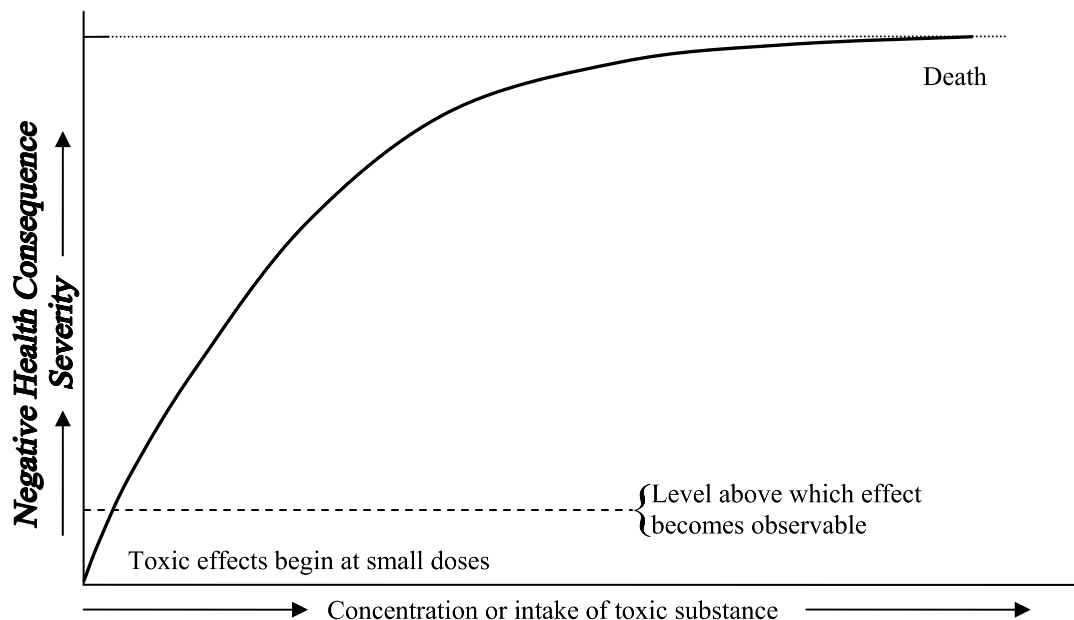


Figure 2: One model of the dose-response curve for a toxic substance.[42, 43]

1.5 Exposure Pathways

Free-living humans encounter exposures, some beneficial and some harmful, to elements in various chemical forms on a daily basis. The sources of exposure can be numerous: constituents of ingested foods, multivitamins and beverages; inhaled airborne particulates; and dermally absorbed compounds. The lure of biomonitors is their potential for integration of all absorption pathways, yielding information on what is purported to be the most important statistic: actual body burden or status of the toxin or nutrient.

1.6 Potential Applications of Biomonitors for Governmental Agencies

Biomarkers that successfully reflect toxic body burden have many potential applications in research and regulatory agencies' spheres of influence. For example, the National Institutes of Health (NIH) want to be able to understand dietary tradeoffs, such as the benefits and downsides of fish consumption[44] and to promote supplements where epidemiological data indicate dietary deficiencies. The Environmental Protection Agency (EPA) may base regulatory decisions on whether or not exposure to industrial As pollution in soil becomes a systemic hazard among children in a local area. The Occupational Safety and Health Adminstration (OSHA) is concerned with monitoring and regulating levels of airborne metals in commercial plating or metal-working facilities. The Centers for Disease Control (CDC) has released its Third National Report on Human Exposure to Environmental Chemicals, listing a number of toxic metals of

concern. The CDC aims to quantify typical exposures and provide interpretive data to the public regarding the effects of this exposure.[45]

1.7 Application of Biomonitors in Epidemiological Studies

Epidemiological, or disease endpoint, investigations fall into several different categories, including population, case-control, and intervention studies. To illustrate how the nail biomonitor has evolved, emphasis will be placed on the use of the toenail as a biomarker for Se.

1.7.1 Population Studies

In conventional population studies, disease rates are compared to dietary intakes for specific groups of people, these usually segregated geographically. When biomonitors are applied to population studies, specimens are obtained from representative subjects among the populace. From the results of specimen analysis, conclusions about the entire population are drawn. If the subject group is truly representative and its size sufficient, then analytical results coupled with intake data can yield useful epidemiological insights.[28] Nevertheless, confounding variables such as genetic variation and differences in climate and lifestyle can be the downfall of this technique. Population studies may provide correlative data but ultimately cannot be conclusive with regard to causation. While they can lay a foundation, case-control and ultimately intervention studies must follow in order to arrive at a high level of certainty for a particular hypothesis.

For Se, trace-elemental hypotheses found their beginnings with population studies in the 1970's. Some of these population studies used the blood biomonitor in order to augment their conclusions. For example, a study of cancer mortality rates versus bioavailable food Se in U.S. cities and states revealed statistically significant differences when controlling for age.[46] Areas with high Se showed a lower level of cancer mortality among residents, and the effect was more pronounced for men than women. The areas were also examined for a variety of cardiovascular, coronary, and hypertensive diseases, revealing consistent links between high-Se regions and lower rates of hypertensive heart disease.[46]

The blood biomonitor was employed in a novel way for a Se population study. Multi-state average blood Se in blood-bank samples was compared with mortality rates from cardiovascular, coronary, and hypertensive causes.[46] Again, hypertensive heart disease was inversely correlated with blood Se, which in turn was correlated to forage crop Se in the expected way.

Finnish scientists singled out two parts of their country exhibiting the extremes of mortality rates from heart disease.[46] They then analyzed for Se in water and produce consumed in these regions, and reported an inverse relationship between fatal heart disease and bioavailable Se.

Another group of researchers[46] noticed that Seneca County, New York, had the lowest incidence of colorectal cancer in the entire state. This auspicious condition was

connected to the county's high Se level in drinking water and its different soil and climate which would favor Se uptake by plants.

A later set of studies[46] utilized estimated Se intake from food consumption data along with whole blood Se from healthy donors. These variables were statistically compared with mortality rates from a wide range of cancers, and significant inverse correlations were obtained. The results were so convincing that the experimenters put forward the idea that doubling the average U.S. Se intake would drastically cut the incidence of cancer mortality in the West. The same research group also investigated estimated intakes of multiple trace elements[46], relating these data to mortality rates from specific cancers. While increasing consumption of Zn, Cd, Cu, and Cr appeared to work against Se's hypothesized anticarcinogenic effect, Mn produced an inverse correlation with rates of pancreatic cancer. This promising finding was paired, however, with the counterintuitive and likely nonsensical result that As intake seemed to lower risk for lung cancer in men. Whole blood Zn measurements from healthy donors from different parts of the country were performed and compared with cancer mortality from corresponding regions. The results suggested that blood Zn levels were positively correlated with risk of death from cancer.

More multi-elemental work was done in pursuit of factors leading to vascular and coronary disease.[46] The mortality rates from these illnesses in 25 countries were related to estimated intake of Se, Cd, Zn, Cu, Cr, As, and Mn. The study highlighted the importance of the Cd/Se intake ratio, the value of which was positively correlated with mortality from ischemic heart disease. Following up on this research, Cd/Se ratios were

measured in kidney autopsy specimens from humans who died of heart disease or other causes.[46] The kidney specimens did not exhibit differing Cd/Se ratios based on cause of death.

The incidence of lung cancer in tobacco-growing countries was compared to the composition of the tobacco produced.[46] Selenium was measured along with suspected carcinogens/toxins polonium-210, *alternaria* spore, tar, and nicotine. While the content of ^{210}Po, tar, and nicotine was relatively constant, the level of *alternaria* spore was positively correlated with lung cancer rates, while the Se concentration showed an inverse correlation. On a superficial level, it seems that tobacco Se provides a protective effect; however, it is more likely to merely be a reflection of high soil Se, which protects against cancer via dietary intake of locally-grown foods. Assumptions of this nature, as well as conclusions such as those obtained from the aforementioned multi-elemental work, must be verified through more specific case-control studies.

In a more recent population study[47] of Canadians, the toenail biomonitor was employed instead of estimated intake data. Toenail specimens were collected from individuals residing in major Canadian cities distributed laterally across the continent. A significant west-to-east geographical trend was measured in the subjects' toenail Se, this monitor having reproducibly been linked to average Se status. When these analytical results were compared to cancer mortality data from each province, a highly significant inverse trend appeared for both males and females. The trend was more striking for males, who exhibited overall lower Se status.

Identification of Se deficiency as a major cause of both Keshan disease, an endemic cardiomyopathy,[46] and Kashin-Beck disease, a deforming endemic osteoarthropathy,[30] was made possible through biomonitoring population studies. Hair, blood, and urine specimens were taken from residents of affected areas. These specimens, along with food samples, were measured for Se content, yielding consistently low results. The success of preventing these disorders through Se supplementation in the population's diet left little doubt that Se is a required trace nutrient for humans.[48, 49]

1.7.2 Case-Control Studies

Typically, case-control studies entail the selection of cases from individuals who have exhibited a disease or behavior of interest. For each case, one or more control subjects from the same population are matched on the basis of equivalent characteristics. These characteristics may be age, body mass index, or other known risk factors such as cigarette smoking. Case-control studies are usually classified as either prospective or retrospective.

Prospective case-control studies often employ a cohort, a group of healthy subjects who provide dietary information and possibly pre-diagnostic specimens. The individuals of the group are followed over time and information on the diseases they develop is recorded. Disease rates are compared to diet prior to disease onset, as determined by official diagnosis or inception of symptoms, to determine any relationship. In this type of study, all participants are healthy (outcome-free) at the time of enrollment when diet questionnaires are completed and specimens are obtained, thus eliminating one

source of bias in determining dietary information. Nevertheless, the studies are large, lengthy, and costly, since they require thousands of participants in order to obtain a sufficient number of disease cases.

When discussing prospective epidemiology, it is worthwhile to mention nested studies. In these studies, researchers seek to mine conclusions from existing information and specimens, which were originally obtained as part of a large, prospective study. A follow-on study of this nature may utilize blood specimens drawn at the time of cohort formation along with current information on the disease states of the followed participants. The blood may be analyzed by new techniques in order to seek associations unanticipated by the original study designers. Here an advantage is the opportunity to apply current technology in the direction of up-to-date research trends, with little incremental cohort costs since the specimens already exist. The disadvantage is that the researchers are constrained by an older study design that may be less than optimal for their current hypotheses.

In retrospective case-control studies, cases are often identified only after diagnosis and initiation of treatment for a particular disease. The cases are then interviewed to obtain information about their pre-disease diet and the data are analyzed for correlations linking diet to disease. Specimens are taken at this time and the properties of case samples are compared with those of controls. The resolution of such studies is high since only those individuals exhibiting disease take part in the comparison. Thus, the number of subjects is low and the study management is relatively easy. However, the potential for bias in the memory of pre-disease diet information is high.

The usefulness of specimen analysis is always questioned due to the uncertainty of whether the disease state was preceded by the altered properties of the specimens (causation), or the disease itself affected these properties (reverse-causation). Nail clippings collected soon after the time of diagnosis, however, reflect body status of several months earlier, and as such can be considered a pseudo-prospective biomonitor.

Where a biological monitor shows promise in indicating body status of an element or compound, it can be employed in a larger study to link it to a disease state. Sukumar and Subramanian[13] utilized a retrospective case-control study to examine differences in elements in hair and nails of people with coronary heart disease (CHD), hypertension, and diabetes. In hypertensives, relative to controls, the researchers found significantly lower hair Cu and Mn for males and hair Cr and Zn for females. Additionally, female hypertensives exhibited lower nail Zn, and female CHD and diabetes sufferers had lower hair and nail Zn.[13] Vance et al.[50] report that certain elements (Br, Ca, Co, Hg, K, Zn) were found to be out of balance in Alzheimer's disease (AD) affected brains. These same elements in hair or nail specimens from AD patients were also found to be significantly different from controls.

The EURAMIC study is an example of a large, retrospective, case-control study. Cases (n=684) were recruited after the subjects experienced an acute myocardial infarction and entered coronary care units of hospitals in centers in eight European countries and Israel. Controls (n=724) were recruited by varying methods and were not matched to cases based on known risk factors. An analysis[51] of toenail specimens from EURAMIC indicated a significantly lower Sc concentration in cases versus controls.

Given no known physiological mechanism to enlighten the results, one might conclude that the higher Sc in cases could be due to a greater level of exogenous contamination from a healthier, more active group. A trend for Zn in the same samples was sought[52] but no statistically significant correlation was observed.

The toenail also played a role as a biomonitor in cancer research in the Nurses' Health Study, a prospective study with 68,213 female subjects providing specimens.[53] As participants subsequently were diagnosed with cancer, they entered the case group. Controls from the cohort were matched based on age and calendar month of specimen submission. The toenail samples for the two groups were pulled for Se analysis. Compiled results showed no relationship between toenail Se and risk of any type of cancer in the study.[54]

Another prospective epidemiological study involving toenails was the Health Professionals' Follow-up Study. A cohort of nearly 34,000 men submitted specimens in this study and were followed to obtain information on medical conditions as they developed. A cross-sectional study was performed on groups of men who, at the beginning of the study, reported diabetes alone or with cardiovascular disease(CVD); as well as a healthy control group. Analysis of Cr in the nail specimens revealed a significant downward trend from controls - to diabetics - to diabetics with CVD. A nested case-control analysis was performed in the same study, with the cases being men who had reported diabetes at the beginning of the study and later developed CVD. Cr analysis and comparison of the cases with healthy controls (matched on age, smoking status, and month of specimen return) showed lower toenail Cr in the cases, but with

borderline significance.[31] Se analysis of the same specimens yielded similar conclusions for toenail Se. Individuals with diabetes at the start of the study had lower toenail Se than controls. There was a significant downward trend of the odds ratio (OR) for prevalent diabetes from the lowest quartile of Se concentration to the highest.

1.7.3 Intervention Studies

Intervention trials thoroughly address the outcomes of modifying dietary intake or other behaviors. A fairly homogeneous group of individuals is formed, and then the assignment of the treatment and control sub-groups is carried out at random. Ideally, the participants, study workers, and analysts should have no knowledge of who has been assigned to what group (double-blind). All participants are typically given a pill which contains either a test substance (treatment) or placebo (control). This administration is continued for a set period of time, or until a specific outcome (i.e. stabilization of a biologic monitor) is achieved. Intervention studies theoretically will produce very specific information, since the treatment variables are limited, constrained and compared to controls in adequate numbers to observe the hypothetical effect if present. Practically, however, intervention studies are challenging because of problems with cost, long-term compliance, and ethics. This last difficulty may be due to the appearance of positive or negative health consequences in the control group, and researchers' concern for the well-being of participants. Also, interventions cannot be authorized for treatments with known deleterious health effects.[28]

In a small Finnish study, a doctor administered large amounts of Se (~1000 µg per day) to 32 patients suffering from treatment-resistant cardiac pain.[46] Significant

improvement in the group's condition without side effects was observed as a result of this intervention.

The Nutrition Intervention Trials were performed in Linxian, China, a rural, mountainous region whose residents, at the time of the study, suffered from a general state of malnutrition as well as high rates of some esophageal and gastric cancers.[55] In the General Population Trial, blood samples were drawn, at the time of study initiation ("baseline"), from a subsample (n=1,103) of the total cohort of 29,584 participants. Analysis of these blood samples was done in order to characterize the cohort in terms of nutritional status, including Se status. Results for Se and other micronutrients confirmed the participants' state of poor nutrition in comparison to U.S. standards. Sixty-nine percent of the serum specimens contained Se at a level below 1 μmol/L, the approximate concentration required in humans to saturate activity of serum selenoproteins and glutathione peroxidase. Each participant was given one of four supplement formulas or placebo. One of these supplements ("factor D") contained Se, vitamin E, and beta carotene. Over the course of the study, the factor D group displayed significantly lower rates of total mortality as well as cancer deaths, in comparison to the placebo and other three supplement groups, who were not found to have benefited.[56] Separately from supplementation effects, and considering only the baseline serum Se in the overall cohort, the highest quartile of serum Se enjoyed a significantly lower risk of esophageal and gastric cancers in comparison to the most deficient quartile.[55]

The Dysplasia Trial was a related nutritional intervention in Linxian. This trial selected only participants (n=3,318) diagnosed with esophageal dysplasia, a precursor to

esophageal or stomach cancer. The intervention was accomplished through a broad-spectrum multivitamin supplement or a placebo. Compliance, which was found to be very good, was monitored by analysis of the blood biomonitor for various non-elemental micronutrients. There was no statistically significant reduction of cancer deaths in the treatment group; however, this group did show a large and significant amelioration of risk of mortality due to cerebrovascular disease.[57]

Qidong county, China, contained townships with high incidence of primary liver cancer (PLC) and hepatitis B, a virus which is linked to liver carcinogenesis. After an intervention trial on Qidongese ducks showed that Se supplementation reduced the rate of both duck hepatitis B infection and precarcinogenic formations in liver, an analogous study was performed with humans. Se supplementation for the human trial was accomplished through distribution of selenite-containing table salt in one township, while residents of four other townships with similar rates of primary liver cancer were used as controls, receiving regular table salt. This intervention dramatically reduced the rate of PLC and hepatitis-B infection in the treatment township, with no corresponding reduction in the control areas. Another intervention was carried out among diagnosed hepatitis-B carriers only. Among those taking tablets with 200 μg of selenium in yeast form, there was zero incidence of PLC, compared with a 6% rate among the placebo group.[58]

The Nutritional Prevention of Cancer (NPC) study focused on dermatology clinic patients in the U.S. identified to be at high risk for basal cell carcinoma or squamous cell carcinoma.[59] Either a Se supplement (200 μg) or a placebo was given in treatment, the content of the doses being blinded to both patients and clinic workers. Blood serum Se

was used to monitor for compliance as well as for excessive levels of the element. These biomonitoring measurements did indeed indicate compliance, with the average serum Se among the treatment group rising by 67% after study initiation. The subjects were followed for an average of six years, during which time some developed cancers at various sites. Where possible, the cancer diagnoses were confirmed. For this study, the incidences of the initially identified diseases of interest, or "primary end points", were not significantly different between the Se and placebo groups. However, trends with high significance levels emerged for other cancers, later identified among the "secondary end points." Se supplementation among these patients significantly lowered the risks of all cancers, and specifically all carcinomas as well as prostate, colorectal, and lung cancers. Other secondary end points, such as breast cancer, bladder cancer, leukemia, and lymphoma, showed no significant differences between treatment groups. Since the subjects were not randomized for risk factors other than those of certain skin cancers, one must be cautious about applying study results too broadly.

The NPC study was extended for approximately another two years, allowing for the continued study of secondary end points. During this extension, more lung cancers appeared in the Se group versus the placebo group, eliminating the statistically significant advantage enjoyed by the treatment group for this disease. However, when the subjects were grouped by baseline Se status measured by the serum biomonitor, it was found that those in the lowest tertile of Se status benefited from supplementation with a significantly lower incidence of lung cancer.[60] Similar results were obtained for other cancers as well when the serum Se tertiles were analyzed.[61, 62] The finding suggests that individuals with

low Se status are the ones that should properly be targeted for supplementation. Interestingly, though, for the vast majority of even these low-Se subjects, serum Se was still high enough to saturate glutathione peroxidase and selenoprotein-P, suggesting that the functional mechanism of anticarcinogenesis is yet to be identified.[61]

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) study[63] is a very large, current intervention study involving the toenail biomonitor. Its cohort of 32,400 men, randomized on prostate cancer risk factors, will be used to study the effects of supplementation with Se and/or vitamin E on the risk of prostate and other age-related diseases. Prospectively collected samples of white blood cells, red blood cells, plasma, and toenails will be archived for further nested studies which will arise as disease states develop in this cohort.

1.8 Validation and Characterization of Biomonitors

The focus of much of the biomonitor literature is on employing a diverse and sometimes creative array of techniques to locate, validate, and characterize biomonitors in support of later epidemiological studies. By finding and developing meaningful, reliable biomonitoring tools, researchers have the basis of designing studies that can connect intake or exposure to a quantifiable biomarker, and in turn link this metric to disease risk, morbidity, or mortality.

1.8.1 Broad-Spectrum Approach

Experiments have been designed to test a diverse array of substances in a proposed biomonitor in order to find those which show useful response. Takagi et al.[12]

surveyed concentrations of multiple elements in nails of subjects from various countries and drew general conclusions regarding which elements tracked with known differences in dietary intakes among those populations.

One strategy is to test for cross-correlations between two biomonitors, one of which may be relatively uncharacterized. Along these lines, Altshul et al.[64] compared volunteers' hair and blood concentrations of a broad range of PCBs and pesticides, finding correlations only for a small family of PCBs. In a series of worker exposure studies, Menezes et al.[65, 66] investigated correlations of measured exposures via airborne particulates and toenail or hair concentrations of multiple elements. They found, in general, that the nails and hair did track with exposure to some degree.

1.8.2 Population-Style Approach

Analogously to purely epidemiological studies of this genre, representative specimens from differing populations are obtained and analyzed. Results are correlated to known data relating to the population, yielding information not about disease, but rather about the validity of a biomonitor. In an early study of Iranian villagers and townspeople, Reinhold et al.[67] analyzed hair specimens for Zn and Cu. The villagers, who were suspected of consuming a Zn-deficient diet, did indeed exhibit significantly lower hair Zn, though Cu was similar for both groups. Morris et al. [16] compared toenail Se of individuals from high, medium, and low geogenic Se areas and found that the toenail tracked with the varying Se intakes in these areas. Karagas et al.[33] and Schmitt et al.[68] found, in two diverse populations, a strong association between well water and toenail As. Along the same lines, Gebel et al.[69] correlated hair and blood As with soil As

in Germany. Sharma et al.[70] sought coherent trends among Indian volunteers regarding the effect of age, diet, tobacco use, and alcohol consumption on hair Zn and Cu.

Studies of this type also provide baseline and normal range data for the analytes of interest. Potential confounding variables are also considered as alternate explanations for significant correlations.

1.8.3 Two-Group Approach

Studies with case/control type matching can be used to compare two groups while minimizing confounding variables. Such is the case for the research described herein, which utilizes a two-group design with a treatment group and matched controls. The One Source Cohort (OSC) was formed by selecting individuals from the much larger Columbia Tribune Study. Cases were selected based on their self-described multivitamin supplementation. Non-supplementing controls were matched and selected from the same population. Toenail specimens, obtained along with the original dietary survey, were analyzed for Se and Mn levels, with the hypothesis that multivitamin use would affect these concentrations. Neither the OSC nor the larger health study in which it is nested is concerned with disease rates.

1.8.4 Establishment of Baseline Values

Some studies merely seek to establish baseline values in healthy populations so that abnormal values may be identified as such.[11, 12, 71] The data can also be analyzed for correlative relationships among the elemental concentrations and demographic information. Hair and fingernail concentrations of a wide variety of elements were

characterized for a non-industrial population in the late 1980's.[11] This type of investigation is important in that it gives a glimpse of expected values for these matrices, as well as their inherent variability. A comparison of hair results for this population with results published in other studies was included in the published article, along with some rationalization for compositional differences seen among populations.

1.8.5 Review of Literature Data

Periodic reviews of available data by recognized experts shed light on the validity of a biomonitor. Harkins and Susten [72] report on an Agency for Toxic Substances and Disease Registry (ATSDR) panel meeting concerned with the state of the science related to hair analysis. The panel, in broad studies and discussions, concluded that hair analysis does not reliably reflect exposure or intake of substances, with the possible exception of methylmercury. Thus, for the purpose of predicting health effects, the panel disfavored the hair matrix, at least until further studies could be performed.

1.8.6 Evaluation of Biomonitor Response

According to Slotnick and Nriagu,[6]

> "Validity of an exposure biomarker is described as the relationship between the biomarker and the actual exposure…Biomarker validation is a critical step that should be considered prior to application of the biomarker to epidemiological studies, since exposure misclassification and biomarker measurement error can result in inaccurate estimations of disease risk."

Many studies thus are designed to test the validity of a biomonitor by measuring its response to exposure.[16, 68, 73]

1.8.6.1 Characterization of Response to Toxic Exposure

Sometimes a small population of potentially exposed people can be studied in order to determine the existence and severity of the exposure. Middleport, NY is a residential site suspected of As contamination from previous pesticide manufacture in the community. In 2003, children and other residents provided samples of toenails, urine, soil from around residence, house dust, and home-grown produce. In this case, the urine and toenail samples showed little evidence that the presence of contaminated soil was increasing the subjects' As body burden.[74]

Research by Mandal et al.[75] in West Bengal, India featured an attempt to decrease the subjects' As exposure by giving them As-free water for drinking and cooking. Nail As decreased dramatically as a result but remained high relative to baseline values for a non-exposed population.[6]

1.8.6.2 Characterization of Response to Nutrient Intake

When it is well-established that a biomonitor responds to nutritional intake, it is moreover valuable to characterize the time delay for that response. Multiple studies have confirmed that toenail Se is responsive to intake at low or normal levels.[6] Toenail Se was also correlated with dietary Se at higher-than-normal intake levels.[76] This high intake was directly measured by analyzing food portions. Separately, in a longitudinal feeding study, 12 men were fed whole wheat bread on a daily basis for one year.[14] Wheat that was naturally high in Se was used to feed eight of the subjects; of these, four received medium doses and four high doses of Se in their bread. Toenail clippings, urine, and blood samples were obtained at baseline and throughout the two years following. From

the results obtained, it was possible to estimate the exposure window reflected by the toenails. For all toenails except the great toenail, three months elapsed before any change from baseline could be noted. For the great toenail, no upswing in Se concentration could be observed until after six months. This work has been regarded as useful for predicting the exposure window for other trace constituents as well where the incorporation mechanisms are assumed to be similar to that of Se.

In order to study the time between consumption of Se and the incorporation of that Se in nails, an isotopic tracer study was designed.[77] Twelve individuals ingested, for three days, 150μg/day of 96.5% enriched Se-76, which is the isotope used for toenail Se measurements by NAA but which naturally occurs at only a 9% abundance. The subjects' fingernails and toenails were sampled regularly for 15 months prior and 14 months subsequent to the supplementation. It was possible to observe a peak in the fingernail Se between 19 and 23 weeks and a wide, diffuse peak in the toenail Se between 16 and 32 weeks. This intervention demonstrated first that the baseline Se concentrations from month-to-month in the nail biomonitor are very consistent, and second, that the toenail incorporates Se over a wider exposure window compared to the fingernail.

1.8.7 Biomonitor Reproducibility

Tissue biomonitors should give consistent results in order to be considered meaningful predictors of body burden or disease risk. If two proposed biomarkers indicate different outcomes, then there should be a way to cross-correlate the results or logically explain the differences. Hambidge[78] noted that hair Cu is inconsistent, increasing in distal hair sections versus proximal sections, thus revealing that exogenous

contamination is likely responsible for the presence of this element, instead of direct deposition from the follicle. Klevay et al.[73] analyzed hair samples from one man collected over 20 years. Through a long period of stable lifestyle, diet, and residence; the hair returned rather random, variable values for multiple elements. Garland et al.[7] examined the reproducibility of the toenail biomonitor by measuring 16 trace elements in specimens obtained six years apart from the same individuals. This study found that the degree of reproducibility was widely variable from element to element, with Hg the best and Cu the worst. Wilhelm et al.[18] compared hair and toenail samples from young children for Cd, Cu, Pb, and Zn, and found only minor relationships between the two tissues. In this study, differences in growth rate, degree of exogenous contamination, and proximity to sweat or sebaceous glands were used to explain the poor correlation. When validation data are poor, they should serve as caution flags to steer researchers away from element/matrix combinations that are too erratic to be of use.

1.8.8 Characterization of Determinants

1.8.8.1 Mercury (Hg)

The Health Professionals' study was used as a platform to identify contributing factors, or determinants, relating to the concentration of Hg in toenails. Dentists, who are exposed to Hg through their occupation, comprised a majority of the cohort of participants. Subjects answered a questionnaire on fish consumption, their own dental amalgam fillings, and, if applicable, amalgam restorations performed on patients . Toenail specimens were pulled from the study archives and analyzed for Hg. The results showed that dentists have higher toenail Hg on average versus non-dental professionals.

However, for all subjects, consumption of tuna and sea fish was the strongest predictor of toenail Hg.[35]

1.8.8.2 Selenium (Se)

Many determinants of toenail Se have been identified and well characterized. These are described below.

1.8.8.2.1 Supplementation

The toenail monitor responds positively and significantly to Se supplementation, in the form of multivitamins or single supplements, both typically in pill form.[19, 21, 79] The amount of daily supplementation was the strongest predictor found for the level of toenail Se.

1.8.8.2.2 Diet

Food-frequency questionnaires have consistently failed to predict the level of toenail Se.[19, 21, 79] The reason typically given is that the levels of Se in a given food (wheat, for instance) can vary significantly depending on the level of available Se in the soil where it is grown. However, dietary Se, if it is accurately measured, is reflected in the response of the toenail biomonitor. This has been demonstrated by a feeding study involving bread made from wheat naturally high in Se, the consumption of which markedly increased toenail Se over time.[14] In another study, duplicate food portions were analyzed and found to be highly correlated with subjects' toenail Se.[76]

1.8.8.2.3 Geography

Variation in toenail Se based on place of residence is primarily due to the dietary component of locally raised food. The effect can be seen on a global level: mean Se level in the toenails of residents of seleniferous areas, such as South Dakota, was higher than specimens from medium-Se areas (Boston and Georgia) and several times higher than those from New Zealand, a region deprived of soil Se.[16] The average toenail Se level in U.S. women was found to vary based on state of residence and was correlated by the average Se concentration in forage crops in that state.[19] Even residents of low-Se areas within a state exhibited lower toenail Se than residents of adequate-Se areas in the same state.[21]

1.8.8.2.4 Age

Some studies indicate that as age increases, toenail Se significantly decreases.[19] However, there is not a consensus regarding this determinant, which has been tested in multiple studies.[6] In a study of over 3,500 specimens,[21] age was not a correlating factor.

1.8.8.2.5 Smoking Status

Studies have consistently reported lower toenail Se values for smokers versus nonsmokers.[6, 19, 21, 79] Although a dose-response relationship is sometimes suggested by the data, the reason for the smoking effect is unclear and may be due to correlating behavioral differences (i.e. unhealthy diet) instead of a direct influence.

1.8.8.2.6 Gender

Men tend to exhibit lower toenail Se levels as opposed to women, especially among non-supplementing subjects.[6, 21] However, some studies do not show this difference as significant.[79]

1.8.8.2.7 Exogenous Contamination

In particular, Se-containing anti-dandruff shampoos are a known cause of high, outlying toenail Se values found in epidemiological studies.[16, 21] However, not all specimens from users of these shampoos exhibit high Se.

1.9 Factors that Determine Suitability of the Toenail Biomonitor

Why are not all elements equally amenable to status prediction via the toenail biomonitor? Given that the blood supply to the germinal matrix which forms the nail should contain all elements to which the body is exposed, why do we not measure a time-averaged picture of the blood composition in the nail?[80, 81] There are several reasons that explain why some elements "work" and some do not.

1.9.1 Exogenous Contamination

One factor is the extent to which exogenous contamination plays a role. Toenails, especially beyond the distal limit, may be quite porous and can harbor materials such as soil, dust, minerals from bathing water, and externally-applied creams and powders. In summary, these are substances which may have very little or no influence on the actual body burden for the element of interest. Every researcher who utilizes toenails, fingernails, or hair must be cognizant of this potential for contamination. It would be

safe to say that no cleaning procedure can be guaranteed to completely take care of all diverse exogenous materials, although most studies use a water/acetone, water/sonication, detergent, and/or a dilute acid/water wash for the specimens.[6, 17] Other researchers go to the trouble of scrupulously examining every nail and scraping away any visible dirt.

Endogenous material is defined as that matter which is added from the body into the nail during its formation. When the amount of exogenous analyte is significant in comparison to the endogenous level, random errors are introduced into the measurement, which in turn reduce the statistical power of an epidemiological study. By the same token, certain treatments might be expected to reduce the endogenous elements in the nail. Polar organics (i.e. alcohols) have been shown to easily permeate the nail, where conceivably they could dissolve and mobilize poorly bound metals.[81]

1.9.2 Affinity for Nail Components

Another basis for differences among metals is the extent to which they are bound into the proteins which constitute the nail. Keratins contain sulfur, bound in the form of the amino acid cystine. Se may be incorporated as selenocystine, the seleno-amino acid, substituting into the keratin protein due to its chemical similarity to cystine. Elements such as Hg or As[33] that form stable bonds with sulfur could be found directly associated with the keratins themselves. Other elements could diffuse into the matrix or nail bed cells from the blood and perform a nutritional or toxic function before the cells are incorporated into the nail plate. Not all elements or all species could be expected to pass the cell membranes with equal speed.

1.9.3 Homeostatic Mechanisms

Even if blood composition were accurately reflected in the nail, another confounding factor is that, for elements such as Fe or Zn, nutritional deficiencies are not reflected in the blood levels due to homeostasis, which is the tendency of the body to regulate composition of blood and tissue.[23]

1.10 Elements Amenable to Biomonitoring via the Toenail

The objective of this research is to determine whether or not the toenail can be a useful biomarker for Mn status. As we have seen, not all trace elements that may exist in the body are meaningfully represented in the toenail biomonitor. The following is a summary list of trace elements that have been studied, as reported in the literature, along with examples illustrating how the toenail has emerged as a monitor of interest for each.

1.10.1 Selenium (Se)

Toenail Se has been firmly linked to intake from diet and supplements, which makes it one of the most fortuitous, convenient, and useful biomonitors.[14, 16, 19, 76] The measurement of Se in toenails has been carried out in support of many health studies investigating a wide variety of disease. It has been utilized in studies of cancer occurring at different sites: breast,[53, 82, 83] prostate,[84-86] skin,[87, 88] bladder,[89, 90] liver,[91] colon,[92] lung,[93] and stomach.[94] In a new, large prospective prostate cancer study (SELECT) involving selenium and vitamin E supplementation, toenail specimens are being collected from all subjects.[63] Additionally, toenail Se has shown to be a predictor of incidence and mortality from all cancers.[47] Some studies of CVD and diabetes have revealed toenail Se

relationships with these diseases,[95] although in an preceding study, nail Se was not correlated with known CVD risk factors in healthy adults.[96]

1.10.2 Arsenic (As)

The first measurements of As in fingernails were for the purpose of investigating the cause of the health effect in cases where acute poisoning was suspected.[6] Continuing research has uncovered some links between toenail As and bladder cancer or melanoma.[97-99] However, no association was found between nail As and breast cancer risk.[100]

Most As studies focus on chronic, low-level environmental exposures, where the health consequences are more subtle but still potentially severe. The level of As in drinking water has, on many occasions, been shown to be a strong predictor of toenail As.[6, 33, 75, 101] Likewise, As in airborne particulates, house dust, and soil has been related to toenail As; however, external contamination could not be ruled out in some studies.[6, 101] Children's toenails failed to reflect the high soil levels of As at their residences in a U.S. study.[74] Consumption of fish was inversely related to toenail As in Pakistan, and one explanation presented was that beans, a fish replacement used by the poor, are the source of contamination.[102]

1.10.3 Mercury (Hg)

Toenails have been found to be adequate indicators of long-term body burden of Hg. As previously mentioned, toenail Hg has been related to the average amount of tuna and sea fish consumed over time.[35, 36] This dietary factor seems to be the single largest

predictor of toenail Hg in a population of individuals who are not otherwise grossly exposed to this toxic metal. Toenail Hg was also found to be more than twice the level in general dentists as compared to nondental health professionals.[35] Presumably, this is due to the dentist's work in placing and removing amalgam fillings. However, toenail Hg was not found to be significantly related to the frequency of this work as measured in a self-administered questionnaire.

In a sadly different study, the toenail biomonitor, among others, was used to gauge the Hg body burden of school-age adolescents in Ghana.[103] Many of these children are believed to be involved in crude, small-scale gold mining operations, in which they are occupationally exposed to high levels of Hg. Others may see high exposures due to their consumption of fish, which have bioaccumulated Hg released into the environment from mining operations.

1.10.4 Fluorine (F)

In addition to a historically demonstrated protective effect against dental caries, more recent studies have indicated that fluoridated drinking water reduces the risk of bone fractures. Now, evidence in three experiments presented by researchers has shown that toenail F is a reproducible parameter over time, and that it is related to intake of fluoridated drinking water.[104] This indicates that individual toenail F should be inversely associated with the risk of bone fractures. When this hypothesis was tested in a prospective case-control study, however, the risk of hip fracture was higher for the lowest quartile of toenail F, but the corresponding risk of forearm fracture was lower, overall pointing to a complex relation between toenail F and fracture risk.[105]

1.10.5 Chromium (Cr)

Cr has been studied in relation to diabetes due to the metal's role in promoting the action of insulin and thus allowing proper metabolism of glucose and fats. Indeed, lower toenail Cr was found in diabetic men versus healthy controls.[31] In order to test the hypothesis that Cr status is important for explaining similar metabolic abnormalities in HIV-positive patients, toenail Cr was measured for infected individuals, treated and untreated, as well as for healthy controls. The control group had significantly higher amounts of Cr in toenails, similar to results found for diabetes, where patients have benefited from Cr supplementation.[106] The effect of Cr status on the development of cardiovascular disease (CVD) and occurrence of myocardial infarction (MI) has been revealed by several studies utilizing the toenail monitor. The CVD study only involved adult male cases who had diabetes, and a prospective case-control toenail Cr comparison between diabetes only and diabetes/CVD groups did not yield a statistically significant trend.[31] However, as part of the same effort, a cross-sectional analysis of the specimens collected at the beginning of the study (baseline) was performed. Progressing through three groups of subjects, from healthy to diabetic to diabetic with CVD, a significant downward trend in toenail Cr was observed. As for MI studies, these generally indicate that healthy individuals with lower Cr status, as measured by toenail Cr, are at higher risk for MI.[107] Higher toenail Cr was a marginally significant predictor of increased breast cancer risk among postmenopausal women, but overall did not show a strong effect.[100]

In a different vein of research, individuals who worked with high-Cr stainless steel were found to have significantly higher levels of Cr versus controls in their nails,

other tissues, and body fluids.[32] Given the potential for high amounts of Cr in the body to cause chronic renal failure,[108] this suggests that toenail Cr could be a candidate for worker-exposure monitoring as well.

1.10.6 Iodine (I)

Dietary iodine (***I***) has many potential sources and as such is difficult to gauge from the use of diet questionnaires. For this reason, it would be useful to have a biological monitor indicative of ***I*** intake from all sources.[109] The toenail was studied as a monitor for ***I***, with the goal of ultimately employing these measurements to calculate risk for thyroid cancer. In one study, subjects classified based on a dietary questionnaire as "high" or "low" ***I*** did show the anticipated trend in toenail ***I***, although it was not statistically significant. Both of these groups' toenail ***I***, however, were well above the average for specimens collected from thyroid cancer patients. The presence of a relatively small number of individuals with unusually high toenail ***I*** in all groups was thought to have confounded the results.[110] In a study involving women with papillary thyroid cancer, toenail ***I*** was not found to be a significant predictor of disease risk.[109]

1.10.7 Cadmium (Cd)

Cd exposure has been linked to lung and prostate cancer in humans and animals.[39] Researchers have attempted to discover connections between toenail Cd and various diseases, including melanoma,[87] prostate cancer,[39] and amyotrophic lateral sclerosis.[38] Unfortunately, for these diseases, no useful associations with toenail Cd have been found.

1.10.8 Zinc (Zn)

Although it has been shown to be an element with relatively high longitudinal reproducibility in the toenail,[7] spotty results have been obtained for application of the Zn-toenail biomonitor in disease studies. The EURAMIC case-control study of myocardial infarction (MI) utilized subjects at different centers throughout Europe and Israel. In EURAMIC, no significant differences in toenail Zn were observed between MI cases and controls.[52] The same may be said for pellagra sufferers and controls, though plasma measurements did reveal significant differences.[111] Toenail Zn was also found to be uncorrelated with breast cancer risk.[100] On the other hand, a multivariate analysis of the data from a melanoma study showed, a weak, statistically poor association between toenail Zn and melanoma, in spite of the fact the raw statistics did not.[40, 87] One study in India found lower levels of Zn in nails of females with coronary heart disease and hypertension.[13] For this study, however, it was not clear if fingernails, toenails, or both were used nor were the groups matched based on other factors (such as use of Zn-containing creams or powders). In a study of neural-tube defects (NTD's),[112] counter-intuitive results were obtained. Pregnant women who later gave birth to children with NTD's such as spina bifida or anencephaly exhibited higher toenail Zn when compared to control women who bore healthy babies. Occurrence of NTD's, however, had previously been linked to Zn deficiencies, leading researchers to speculate that the abnormal NTD fetus was releasing a Zn-sequestering protein that was eventually secreted in the mother's nails and hair. Determinants of toenail Zn were sought in another study, where two groups of adults with suspected differing Zn intake or status were compared, but the toenail was not found to indicate or reflect differences.[113]

1.10.9 Scandium (Sc)

Although Sc has no known biological function, it was measured in toenails from subjects in the EURAMIC study. Higher levels of toenail Sc were significantly correlated with a lower risk of MI even after adjustments for known risk factors. There is no known mechanism by which Sc can protect against MI, and the correlation could be due to a higher level of terrestrial contamination, reflecting the fact that active people engage in a higher level of outdoor activities, and this lifestyle lowers their risk of MI.[51]

1.11 Toenail Manganese

1.11.1 Justification for Study

There would be much to be gained by establishing that the toenail is a viable monitor for other trace elements. To that end, this research has focused on manganese and the question of whether or not dietary intake is reflected in the toenail. Mn is an element of interest for several reasons. First, concerning dietary intake, is the issue of whether or not Mn imbalances, associated with health disorders, are present in the population or prevalent in subsets thereof. Second, there is a wider awareness of the role of Mn-activated enzymes in disease prevention.[114, 115] The questions arise: Is the activity of these enzymes hampered by Mn deficiency? Could their function be optimized by augmenting Mn status? Third, the use of a gasoline additive, methylcyclopendadienyl manganese tricarbonyl (MMT), has led to Mn exposure for millions of Canadians.[116] Could toenail Mn be related to disease risk in this population? A positive answer to this question could demonstrate negative health consequences from the use of MMT and be employed to shape environmental regulations.

1.11.2 Health Hypotheses

1.11.2.1 Dietary Manganese

What are the health hypotheses surrounding this element and what is its effect in the human body? Mn is a nutrient for which the health effects could be expected to follow the inverted U-shaped dose-response curve shown in Figure 1, since disorders result from deficient as well as excessive exposures. However, scientists have found it difficult to recommend the proper daily intake for Mn since the level in diets worldwide varies over one order of magnitude[117] and can be quite high for vegetarians.[118] Percentage absorption of dietary Mn, normally controlled by homeostatic mechanisms,[118, 119] is low but variable and has been shown to be influenced by co-ingestion of phytate,[120] Fe, and fat.[118] Mn absorbed by the small intestine enters the bloodstream, but is removed quickly by the liver, which secretes some of the Mn back into the gut as bile.[117] If the liver acts slowly relative to absorption or is otherwise impaired, blood Mn levels may spike beyond what would be predicted by the measured net absorption (as determined by a balance study).[117] Variability in the diets selected by free-living populations as well as the spectrum of hepatic function that individuals exhibit could conceivably give rise to situations in which deficient or, more likely, excessive Mn leads to negative health consequences.

1.11.2.2 Manganese Deficiency

1.11.2.2.1 Chronic Deficiency

Documented cases of chronic human Mn deficiency are rare due to the wide variety of food sources that contain abundant Mn.[118] However, in short-term studies,

human volunteers fed reduced-Mn diets developed such symptoms as dermatitis and hypocholesterolemia.[118, 120] Patients receiving total parenteral nutrition are susceptible to nutrient deficiencies, and one such four-year-old, unusually small for her age, was found to have low levels of Mn in her bones and blood serum. Supplemental Mn increased her growth rate significantly.[118] Osteoporosis in women has been linked to low plasma Mn and has been seen to improve with Mn supplementation.[120] Animal studies have provided much of our insight regarding Mn deficiency. In these, a low-Mn diet led to skeletal deformations and lameness, as well as other effects; among these, impaired reproductive function, diabetic-like glucose tolerance curves, and reduced HDL cholesterol.[117, 118] It is reasonable to raise the question of whether or not the prevalence of such symptoms in Western populations may have some linkage to Mn status.

1.11.2.2.2 Sub-Optimal Manganese Status

An important Mn-based enzyme in human health is manganese superoxide dismutase (MnSOD). MnSOD is locally active at the mitochondria in mammalian cells where respiration and energy production take place, incidentally producing reactive oxygen species (ROS) such as O_2^-. The ROS, if allowed to accumulate, react with biomolecules, in turn creating reactive species which can damage the DNA molecules. Such damage to DNA is the principal mechanism of carcinogenesis. MnSOD catalyzes the dismutation of O_2^- to H_2O_2, initiating the chain by which the ROS are destroyed.[121] In addition to inhibiting carcinogenesis, MnSOD is also hypothesized to suppress growth of malignant cells. In animal studies, induced overexpression of MnSOD increased resistance to carcinogenesis from chemicals and radiation.[118, 121] Studies of MnSOD

activities have lent credence to the idea that there may be value in Mn supplementation. Specifically, MnSOD activity has been observed to increase with supranutritional Mn intakes.[117]

The human MnSOD gene is the site of a common polymorphism which affects the nucleotide sequence of the enzyme. The sequence in question governs the transport of the MnSOD precursor into the mitochondria, thus the genetic polymorphism directly influences the efficiency of this transport, and the activity level of the antioxidant enzyme.[122, 123] Computer modeling and animal experiments demonstrate that the valine (V) encoded precursor protein is hindered in transport relative to the alanine (A) form.[122] This would lead one to assume that individuals with the AA genotype would be at less risk for carcinogenesis relative to VV subjects. In general, however, the opposite appears to be the case. One explanation offered[115] is that, beyond a point, high MnSOD activities lead to overproduction of H_2O_2, straining the cell's capacity to neutralize this oxidizing agent. Accumulation of H_2O_2 gives rise to other enzymatic reactions that ultimately form more ROS. Deactivation of H_2O_2 is accomplished by glutathione peroxidase, a selenium-containing enzyme whose activity is related to Se status. Thus individuals with the AA polymorphism and low Se status may be expected to experience higher risk for cancer than those with adequate Se intake.

This was indeed found to be the case. In prostate cancer studies controlled for genotype, cancer risk for subjects with AA genotype was found to be strongly and inversely correlated with antioxidant (including Se) status, but less so for VA and VV genotypes.[115] Among subjects with the AA polymorphism, the lowest quartile of Se

status (as measured by plasma Se) was five times more likely to develop aggressive prostate cancer relative to the highest quartile.

In one breast cancer study, women with the AA MnSOD genotype exhibited a higher cancer risk if they smoked or were exposed to ionizing radiation, in comparison to VA and VV subjects.[122] Again, the environmental exposures may deplete antioxidants "downstream" from MnSOD, leading to imbalances exacerbated when the MnSOD is highly active. Some data indicate, however, better breast cancer survival rates for the more active AA genotype, possibly indicating that MnSOD is involved in deactivation of chemotherapeutic agents in healthy cells. [123] It is yet to be fully investigated how Mn intake or status can affect this delicate enzymatic balance which protects cells from oxidative damage and carcinogenesis.

1.11.2.3 Toxic Exposure

Cases of toxic exposure to Mn, or manganism, while not widespread, involve specific, mainly neurological, symptoms. Mood swings, sleep disturbances, and anxiety are among the first indicators.[119] These are followed by neuromuscular disturbances in movement and muscle tone and Parkinson's disease-like symptoms with delayed onset. Animal studies have demonstrated that intravenously injected Mn concentrates in the brain and is eliminated slowly.[124] Cases of Mn exposure have been mainly via the inhalation pathway and occupationally related, such as in an alkaline-battery factory,[119] Mn mines, or metallurgical operations.[120, 125] Inhaled Mn may bypass the body's normal homeostatic mechanisms and result in a more direct pathway to the brain.[116] This is the potential route for exposure in areas where methylcyclopentadienyl manganese

tricarbonyl (MMT) is added to gasoline. Burning of MMT results in emission of an aerosol of manganese oxide particles,[126] which could concentrate to dangerous levels in urban atmospheres.

Though no symptomatic cases of excessive dietary Mn have been reported, people consuming moderate amounts of Mn in drinking water over long periods of time were observed to exhibit more neurological problems.[117] A child who drank well water with ppm-level Mn over a period of years exhibited problems with memory, listening skills, and mental focus.[127] Long-term consumption of high levels of Mn in vegetarian diets could potentially be a concern in regard to similar effects.[118]

1.11.3 Paucity of Other Biomonitors

Definitive biomonitors to assess Mn status have been sought with unsatisfactory results.[117, 120, 124] Though the metal is known to accumulate in the brain, liver, and other tissues, direct access for sampling of these is not practical for living organisms. Serum Mn has been reported to be elevated in women taking Mn supplements,[117] but serum Mn has several problems: inconsistent response to ingested or inhaled Mn,[120, 125] insensitivity to any but large changes in intake,[120] and potential for contamination during collection and storage.[26] MnSOD activity is a promising biomarker, but is nonspecific, its level also affected by diet, ethanol intake, and physical exercise, among other factors.[117] As food frequency questionnaires proved to be poor predictors of Se intake, they may also fail to predict dietary Mn.

In one case study (mentioned earlier), a child who drank high-Mn water had high levels of Mn in his hair, blood, and urine. However, an older brother who used the same water only had elevated Mn in his hair.[127] Such a circumstance, in spite of the study's obvious limitations, casts doubt on blood and urine as adequate Mn biomonitors.

1.12 One Source Cohort (OSC)

In this research, we hypothesized that toenail Mn could be related to Mn intake. The mechanism for testing this hypothesis is a treatment-control comparison of toenails from self-classified Mn supplementers. The specimens from this study were drawn from the Columbia Tribune Cohort (CTC), made up of free-living residents of central Missouri who filled out a diet, supplement, and health questionnaire included in a local newspaper insert. The participants also submitted toenail clippings with this information. A number of these subjects identified themselves as consumers of One Source multivitamin. This multivitamin, as formulated at the time of the study (1999), was unique in its content of supranutritional levels of both Se and Mn. Self-identified non-supplementing controls, also from the CTC, were matched based on sex, age, body mass index, and smoking status. Given that the correlation of toenail Se with Se supplementation is well-characterized, measurement of Se in the case and control specimens could be used to verify accuracy of the subjects' self-classification. Subsequent measurement of the same specimens for Mn content would confirm or discredit the toenail Mn hypothesis.

This mechanism was chosen for several reasons. First, the specimens were already in-house and there was no need to obtain additional permission for their use.

Second, the cost and logistics of a case-control study of exposed populations (i.e. occupationally exposed workers or urban Canadians exposed to MMT) were prohibitive. Third, since vitamin pill supplementation was the form of exposure, we could focus on ingested Mn without the confounding variable of multiple exposure pathways.

1.13 Principles of NAA

Determination of Mn in the toenail samples for this study was performed by instrumental neutron activation analysis (INAA). In general, neutron activation analysis (NAA) is, for some elements, a highly sensitive analytical technique. The basis of NAA is using neutron irradiation to induce radioactive species and measuring the gamma-ray emissions associated with their decay. If irradiation and other parameters are chosen carefully, it is often possible to determine multiple elements simultaneously, and others with a second count after the same irradiation.

No chemical separations are done in INAA, whereas for radiochemical (R)NAA, the radionuclide may be extracted from the sample and purified before detection and quantification, in order to eliminate spectral interferences and improve sensitivity. With INAA, the samples are generally weighed, irradiated, and counted without physical or chemical change. Sample handling is minimized and contact with reagents or glassware is eliminated, thus giving rise to a principal advantage of this technique, namely, low potential for contamination and mechanical or chemical loss. By the same token, since little sample manipulation is performed, INAA can also be a very rapid and convenient method for elemental analysis.

In contrast to other spectroscopic analysis techniques (e.g. atomic absorption or inductively coupled plasma - mass spectrometry) where the signal must be measured in real time as it is generated, conventional NAA utilizes planned irradiation, decay, and count times chosen based on the radionuclide(s) of interest and the sample "matrix" (other constituents of sample). The purpose of the delay is often to allow unwanted matrix radioactivity to decay, thus reducing spectral backgrounds and interferences.

In some applications, INAA is a "nondestructive" methodology, since the sample may appear to be unchanged after irradiation, and any radioactivity produced in it may decay to undetectable levels. However, for practical purposes, irradiated samples can seldom be transferred to unlicensed laboratories. Also, after long irradiations, the physical integrity of the sample may be severely degraded by radiolysis. As long as the sample maintains physical and compositional integrity, it may be re-irradiated as necessary to make other measurements or analyzed by other techniques.

INAA can often accommodate a wide range of sample sizes, limited on the low end by the smallest weight an analytical balance can accurately measure. For measurements of trace levels of platinum-group elements in geological samples, large sample sizes in the tens of grams are used.

A principal disadvantage of INAA, however, is the need for a neutron source of sufficient intensity to produce measurable amounts of the radionuclide. This source is usually a high-flux nuclear research reactor, which can generate sufficient rates of

gamma-emission and yield sensitivities in the ppm or sub-ppm ranges, competitive with other analytical techniques.

Neutron activation analysis is based upon the production of radioactive isotopes from neutron induced nuclear reactions on stable isotopes within a sample. Many elements/isotopes may be activated, or made radioactive, through bombardment with neutrons. Frequently gamma-rays (γ-rays) accompany the radioactive decay of the isotopes. These photons are counted and their energies and intensities measured by pulse-height analysis employing a high-resolution gamma-ray spectrometer consisting of a high-purity germanium (HPGe) detector coupled through the appropriate electronics to a multi-channel analyzer. The resulting output is a γ-ray spectrum of intensity versus energy, the intensity of a given peak being proportional to the amount of analyte in the sample. The half-life (which may be measured through repeated counts) and gamma-ray energy uniquely identify a specific radionuclide.

The probability that a given isotope will capture an incident neutron is a function of the kinetic energy of that neutron. Thus the overall rate of a neutron capture reaction is dependent upon the energy spectrum of the incident neutrons. Most of the (n,γ) reactions utilized for NAA are induced by thermal or epithermal, or resonance, neutrons. Thermal neutrons, of average energy 0.0253 eV, constitute the vast majority (approximately 92%) of the total neutron flux in a light water-moderated reactor. Neutron capture probabilities, for a defined neutron energy or range of energies, are expressed as “cross-section,” which has units of area. The thermal cross-section is, in general, relatively large for most target nuclides and varies as 1/v with neutron

velocity.[128] Beyond the thermal region, epithermal neutrons with energy 0.5 eV to 100 keV make up approximately 2% of the total flux. Cross sections for (n,γ) reactions in this region are characterized by a series of sharp maxima across the neutron energy spectrum. These maxima correspond to resonances, narrow energy ranges for which the reaction probability is dramatically higher. Neutrons with energies >100 keV are called fast neutrons; these comprise approximately 6% of the total flux and give rise to (n,p), (n, α), (n, 2n), and other reactions in which massive particles are ejected from the target nuclei.

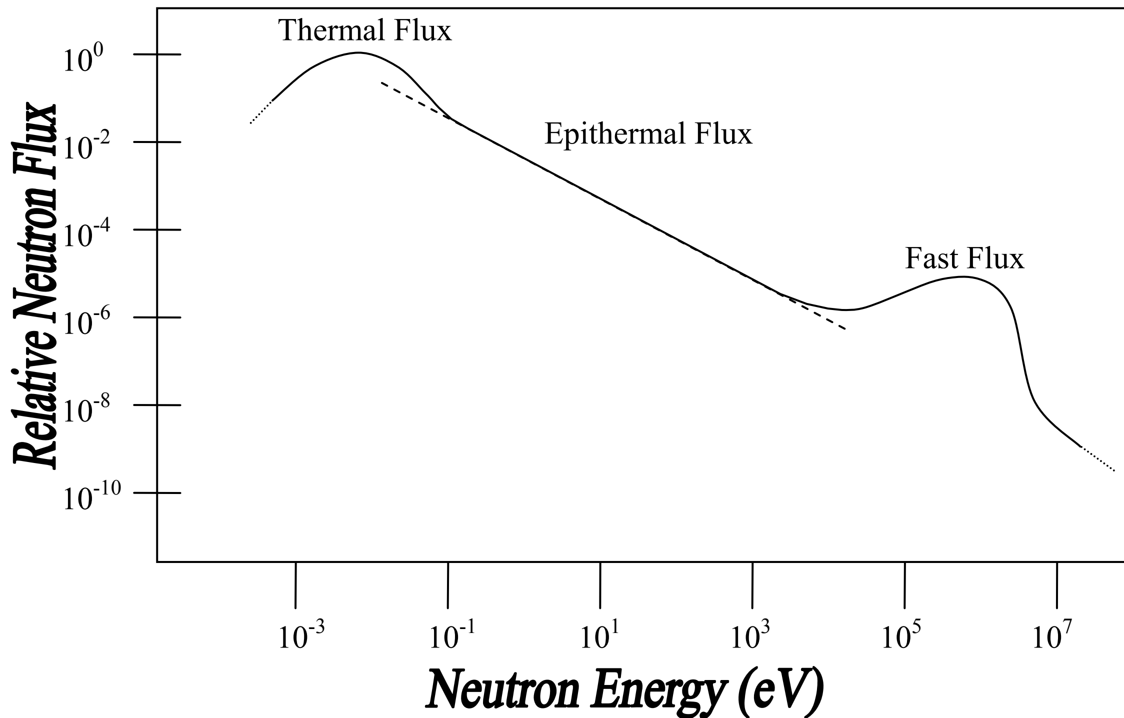


Figure 3: Typical neutron energy distribution in a nuclear fission reactor.[129]

Using shielding materials with high thermal cross-sections, such as boron or cadmium, it is possible to vastly reduce (by several orders of magnitude) the exposure of a sample to thermal neutrons. The shielding results in sample activation by epithermal

neutrons only, which can increase the signal-to-background ratio for analytes such as iodine (^{127}I (n,γ) ^{128}I) found in a sodium matrix.

For a given radionuclide produced through target nuclide neutron capture, the predicted spectral peak area for a γ-ray of a specific energy is given by the equation below.[128, 130]

$$P_{area} = N \cdot A_{\gamma} \cdot \varepsilon_{\gamma} \cdot [(\phi_{th}\,\sigma_{th}) + (\phi_{epi} \cdot RI)] \cdot (1 - e^{-\lambda t_i}) \cdot (e^{-\lambda t_d}) \cdot \{\frac{(1 - e^{-\lambda t_c})}{\lambda}\}$$

Where:
P_{area} is the spectral peak area for the γ-ray line;
N is the number of atoms of the target nuclide contained in the sample;
A_{γ} is the γ-ray abundance or average number of γ-rays of this energy emitted per decay;
ε_{γ} is the efficiency of the detector for the γ-ray energy and counting position;
ϕ_{th} is the thermal neutron flux in neutrons per second per square centimeter;
σ_{th} is the thermal reaction cross section in cm^2 (1 barn = 10^{-24} cm^2);
ϕ_{epi} is the epi-thermal (>0.5 eV) neutron flux;
RI is the resonance integral, $RI = \int_{0.5eV}^{\infty} \sigma(E) \frac{dE}{E}$
λ is the decay constant of the radionuclide formed (equal to ln 2 / $t_{1/2}$, $t_{1/2}$ being the half-life), usually in units of s^{-1};
t_i is the irradiation time in seconds; (1-$e^{-\lambda t_i}$) is called the saturation factor;
t_d is the decay time in seconds; and
t_c is the count time in seconds.

Now, N may be calculated as:

$$N = \frac{w \Theta N_A}{AW}$$

Where:
w is the weight of the element of interest in the sample, in units of g;
Θ is the fractional isotopic abundance of the activated isotope;
N_A is Avogadro’s number, 6.022×10^{23} atoms per mole; and
AW is the atomic weight of the element in grams per mole.

By carefully choosing the irradiation, decay, and count times, it is possible to increase the sensitivity and reduce interferences for the analysis.[131] Advantage may be given to short-lived isotopes, for example, through increasing the irradiation time and saturation factor, which will augment sensitivity relative to longer-lived interferences. Theoretically, if all other parameters and constants given above are measured or known, the weight of the element in the sample can be calculated. In practice, however, the calculations are simplified substantially by irradiation of standards of known composition using the same parameters as for the samples. This is known as the standard comparator method. In the standard comparator method, the detector efficiency, neutron flux, irradiation time, and sometimes decay time are all held constant. Thus the peak area of the specific γ-ray is proportional to the amount of isotope present, and this constant of proportionality is calculated from the response of the standards.

$$\frac{w_u}{w_s} = \frac{P_u}{P_s} \quad \text{or} \quad w_u = P_u \frac{w_s}{P_s}$$

Where:
w_u is the weight of element in the unknown sample;
w_s is the known weight of element in the comparator standard;
P_u is the measured γ-ray peak area for the unknown sample; and
P_s is the measured γ-ray peak area for the comparator standard, irradiated, decayed, and counted in the same way as the sample.

The pneumatic tube system at the University of Missouri Research Reactor provides a convenient way of irradiating all samples and standards equivalently but sequentially. For such irradiations, it is assumed that the neutron flux ϕ is constant throughout the experiment. Any slight variation in flux is compensated for by measuring

standards regularly during the analytical run. Due to circumstances in the laboratory, the exact decay times may be variable and so decay corrections are performed via the following equation:

$$P_u^{'} = P_u \, e^{-\lambda(t_{d,target} - t_{d,actual})}$$

Where P'_u is the decay-corrected peak area, P_u is the measured peak area, $t_{d,target}$ is the target decay time, and $t_{d,actual}$ is the actual decay time.

It is also important to note another assumption, namely, that the fractional isotopic abundance of the element in the standard is assumed to be the same for the sample as well. This assumption turns out to be very good for almost all elements. The notable exception is for Pb, where the isotopic distribution varies with the geological source. However, variability in Pb isotopic abundances is not an issue for NAA since it cannot be used to measure this element.

The HPGe detectors used for gamma-ray spectroscopy in this study feature high resolution but relatively low efficiency compared to NaI detectors. Detector efficiency and resolution are a function of energy. Typical efficiencies (relative to a NaI detector) measured at 1.33 MeV with the Co-60 γ-ray are 20-30%, and peak widths are <2 keV, full width at half-maximum (FWHM). Thus such a detector could be expected to resolve peaks whose maxima are separated by 4 keV or fewer.

A typical block diagram for a gamma-ray spectroscopy system may be found in Figure 4. The system detects incoming photons as pulses, the amplitude of a pulse event

being proportional to the energy of the photon. A critical part of the measurement consists of compensating in some way for the time lost during the count when the electronics are processing a pulse and thus unavailable or "dead" to incoming events. One method of dealing with this dead time, called Live-Time Correction (LTC), extends the real counting time as necessary to provide the requisite time for which the detection system is available. For measurements of a rapidly changing gamma count rates, such as in the case of short-lived isotopes, the LTC correction is problematic.

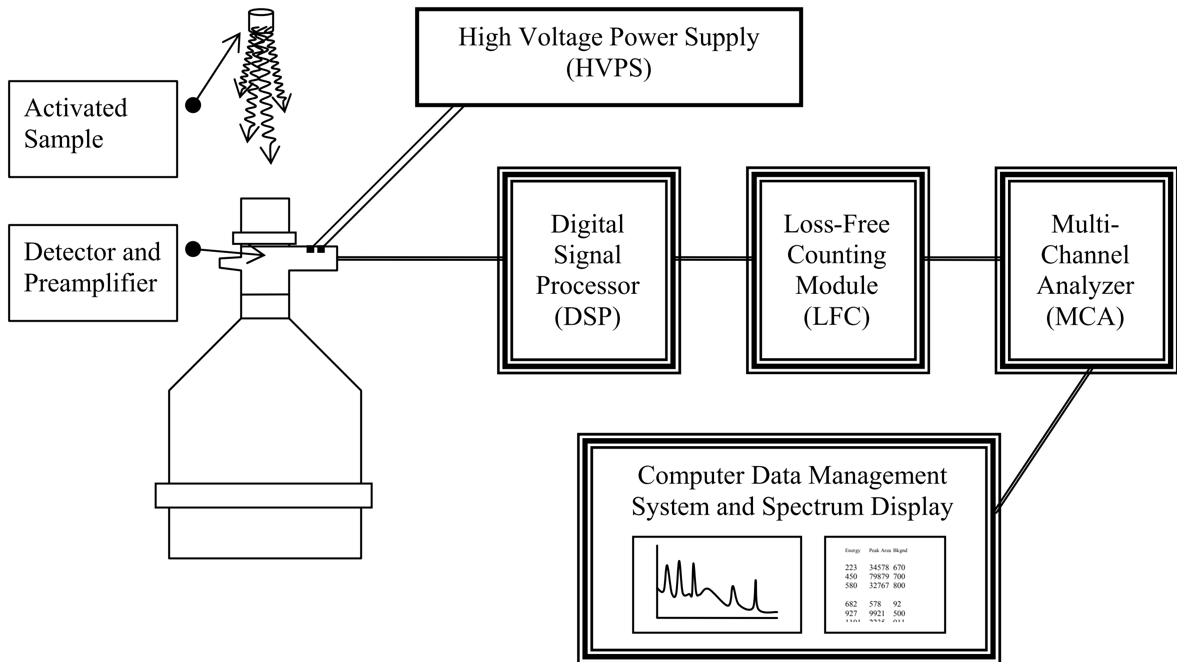
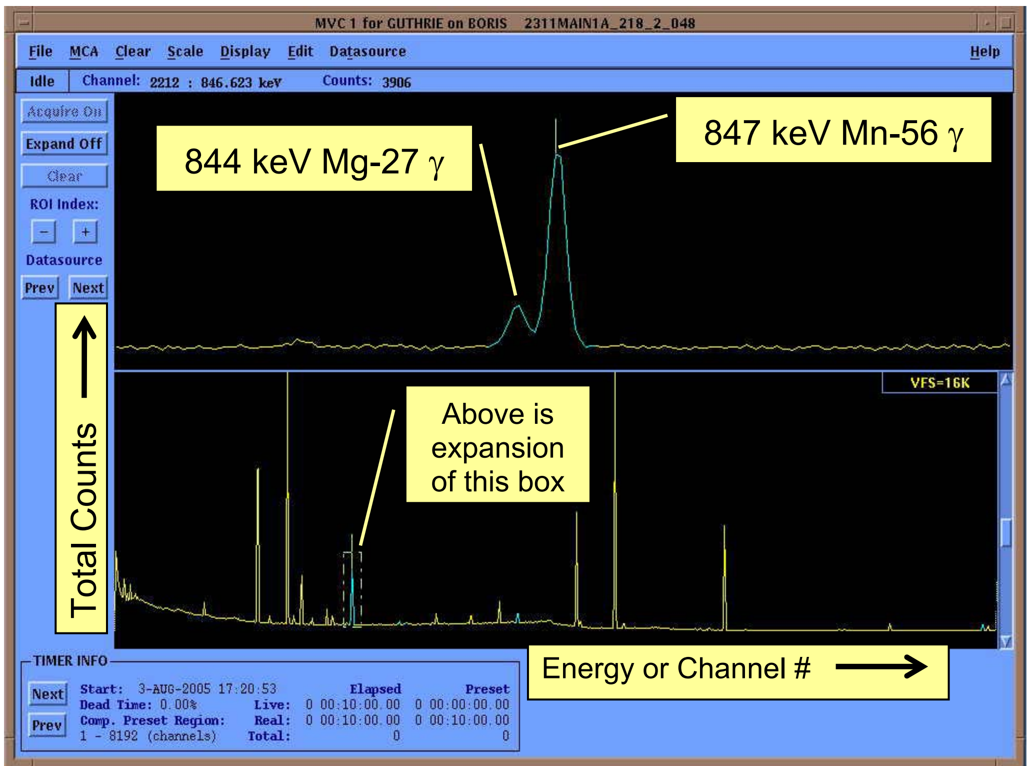


Figure 4: Block diagram of a gamma-ray spectroscopy system.

In neutron activation analysis, a Loss Free Counting (LFC) module is often utilized to compensate for dead time while maintaining a constant real counting time. Modern LFC modules, such as the Nuclear Data 599 model, make use of the Virtual Pulse Generation (VPG) technique, introduced by Westphal in 1981. In VPG, the multi-channel analyzer (MCA) "system busy" signal as well as the amplifier baseline signal are

simultaneously monitored in order to calculate the probability of a virtual pulse being accepted by the system. These calculations are performed and the resulting weighting factors applied in millisecond intervals, enabling a statistically accurate real-time correction of the gamma spectrum.[132, 133]

There are special challenges that relate to the NAA analyses for Mn and Se performed for this research. Measurement of the 847 keV ^{56}Mn γ-ray is complicated by the vicinal 843 keV γ-ray produced by decay of ^{27}Mg. Peak-fitting software can aid in separating the two, although necessary manual adjustments of the fit are tedious. One method of minimizing the size of the interfering peak is to allow a longer decay time to take advantage of the longer half-life of ^{56}Mn, 2.58 hours versus 9.46 minutes for ^{27}Mg. See Figure 5.



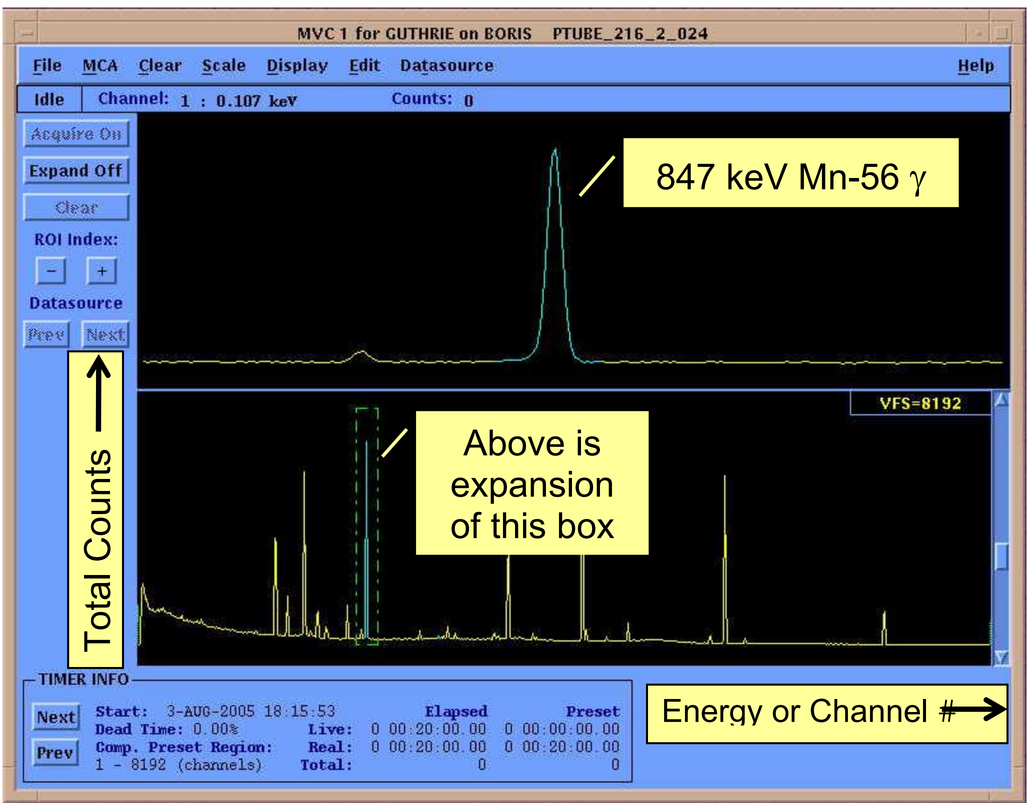


Figure 5: Gamma-ray spectra of OSC ID 39 ([Mn] = 1.62 ppm).
Top spectrum is from the first count (t_d = 5 min.)
Bottom spectrum is from the second count (t_d = 60 min.)

2 EXPERIMENTAL

2.1 Formation of the One Source Cohort

2.1.1 Columbia Tribune Study

The One Source Cohort (OSC) study is nested in the larger Columbia Tribune Study (CTS). In 1999, a newspaper advertisement was placed in the Columbia Daily Tribune in conjunction with a series of articles on Se status and disease risk. The advertisement included a questionnaire which solicited information from the volunteer subject regarding demographics, diet, supplementation, and use of medicated shampoos known to contaminate nails. Instructions and envelopes for self-collecting toenail clippings and returning these specimens were provided along with the questionnaire as well. Toenail Se results were returned to the subjects, but no follow-up contact was made in order to verify the questionnaire data. Relative to the general population, those who volunteered toenail specimens and personal information for the study tended to have a higher-than-average level of health awareness, and as such were less likely to smoke and more likely to take Se and/or multivitamin supplements.[21] Descriptive statistics for the Columbia Tribune Cohort (CTC) are included in Table 1 below.

Table 1: Demographic information for the CTC.

Demographic	Response
Total subjects, n	2711
Males, n, %	1256 (46.3%)
Females, n, %	1455 (53.7%)
Age	18 to 94 years
Males	56.7 ± 14.8
Females	54.6 ± 14.4
Race	
Caucasian	97.2%
All others	2.8%
Se supplement use	44.7%
Males	46.8%
Females	43.0%
Use of One Source™ Multivitamin	3.8%
Males	3.6%
Females	4.0%
Cigarette smoking	8.1%
Males	8.4%
Females	7.8%
Se-medicated shampoo use	4.9%
Males	7.6%
Females	2.6%

2.1.2 One Source Multivitamin

The One Source™ Multivitamin, manufactured by the Perrigo Company in Allegan, Michigan, was a popular supplement that contained super-nutritional amounts of Se and Mn. The One Source™ vitamin, as formulated in 1999, contained 375% of the Daily Value (DV) of Mn and 286% of the DV of Se[134]. Selected compositional data for this supplement is included in Table 2 below.

Table 2: Selected compositional information for the One Source™ Multivitamin.

Mineral or Trace Element	Amount per caplet	(Recommended) Daily Value (DV)[134]	% of DV per caplet
Iodine	150 µg	150 µg	100%
Magnesium	100 mg	400 mg	25%
Zinc	15 mg	15 mg	100%
Selenium	200 µg	70 µg	286%
Copper	3.5 mg	2 mg	175%
Manganese	7.5 mg	2 mg	375%
Vanadium	13 µg	Not determined	Not determined

2.1.3 Subject Selection for the One Source Cohort

A number of subjects (n=103) from the CTC identified themselves as One Source™ supplementers. Specific tests were applied to each individual in order to qualify him or her for inclusion in the OSC. The first was for the purpose of verifying the accuracy of this self-classification. Toenail Se had already been measured in the toenail specimens provided and individual results were tabulated. If the subject's toenail Se was lower than 0.9 ppm, it was judged to be unlikely that the self-reported regular use of this 200 µg/day was accurate, so the subject was excluded. Second, if the duration of supplement use was claimed to be less than six months, it was judged that the multivitamin use had been not sufficiently long-term to affect toenail concentrations. By the first test, 25 subjects were excluded as well as 15 by the second, leaving a total of 63 One Source™ supplementers (+OS) to be matched with controls.

The technique of “pair matching” was employed in order to find control subjects. Controls were selected from CTC subjects who claimed to be taking no vitamin supplements (-S). An attempt was made to find a perfect match for each subject based on the following criteria: sex, age, body mass index (BMI), and smoking status. BMI was calculated by dividing body weight (kilograms) by the square of the height (meters). Smoking status was comprised of three categories: never smoked, smoked in the past but not currently, and current smoker. When a perfect match could not be found, age and BMI were allowed to vary slightly. For example, if one imperfect match included a higher age, a lower age was sought for the next imperfect match in order to keep the mean age for both groups as equal as possible. Statistics for the resulting cohort are given below in Table 3.

Table 3: Statistics for the One Source Cohort.

Group	n	Age (y) avg. ± std. dev.	BMI avg. ± std. dev.	Smoking Status (n)		
				Never	Past	Current
Females						
+OS	37	50 ± 13	24.9 ± 4.5	26	10	1
-S	37	50 ± 13	24.8 ± 4.6	25	11	1
Males						
+OS	26	58 ± 12	28.0 ± 4.0	16	9	1
-S	26	58 ± 12	27.9 ± 4.1	16	9	1
Replicates ("Splits")	62					
Reproducibility QC	20					
Nonsensical sample	1					
Total	209					

2.2 Toenail Samples for the One Source Cohort

2.2.1 Replicate Samples and Reproducibility QC's

All 126 subjects had an archived nail sample from the previous Se analysis. Of these, 62 had supplemental nail sample material archived in the original collection envelope that was used for blinded splits (duplicates) in the analysis. In addition, 20 nail samples that consisted of five replicate samples from four nail collections over 9 months from one individual were also included in the analysis. One sample originally intended to be the 63rd blind split was assigned an ID but later it was determined that there was insufficient specimen. A "nonsense" sample was substituted so that there would be a nail sample for each ID.

All above samples were randomized and the identifications were blinded to the analysts. The 209 total samples were assigned OSC ID's, placed into labeled paper envelopes, and delivered to the experimenters.

2.2.2 Preparation of Nail Samples for Analysis

The nail samples were sonicated for 10 minutes in 10 mL of 10% (v/v) nitric acid solution, decanted and then sonicated for 10 minutes in 10 mL of 18MΩ-cm water. The samples were then rinsed with 18MΩ-cm water and stored in pre-cleaned plastic liquid scintillation vials. The samples were freeze dried using an ATR Heto vacuum freeze drier and then weighed. The masses of the 208 samples ranged from 2 to 80 mg; the median sample mass was 48 mg.

2.2.3 Non-blinded Replicates

Based on the weights obtained during freeze drying, 10 of the 209 samples were larger than 80 mg and were selected to create non-blinded splits for the analysis. The samples were split while being re-weighed in preparation for Se analysis, and were identified using the original OSC ID followed by an "A" or a "B".

2.3 Determination of Selenium

The OSC samples were analyzed for Se by instrumental neutron activation analysis (INAA) in April, 2005. The samples were weighed into 0.4 mL high-density polyethylene vials and irradiated in a pneumatic tube system for 7 seconds at a flux of *ca.* 5.0 X 10^{13} $n \cdot cm^{-2} \cdot s^{-1}$. The samples were then allowed to decay for 15 seconds before counting on the face of an HPGe coaxial detector (System 1) for 30 seconds, real time,

using a loss-free pulse pile-up correction. The detector had a measured relative efficiency of 23.3% and a FWHM resolution of 1.77 keV at 1.33 MeV. All samples had a dead time of less than 5%. The peak areas were determined interactively with Genie ESP spectroscopy software package (Canberra). Comparator standards were prepared by pipetting approximately 0.5 μg of Se from a certified Se Standard (Spex) onto filter paper in a 0.4 mL high density polyethylene vial. Eleven samples of NIST SRM 1577 Bovine liver (approximately 40 mg each) were used as a quality control and co-analyzed with the samples during the Se analysis.

2.4 Toenail Simulation Experiment

A small simulation experiment was undertaken in order to optimize the irradiation and counting conditions for the Mn analysis. The inorganic chemical composition of a typical 20 mg toenail sample (Cheng 1994) was simulated by the addition of representative amounts of Mn (0.002 μg), Na (8 μg), Cl (17 μg), K (5 μg), Ca (32 μg), and Mg (14 μg) in the form of liquid standards to a ~15 mg sample of ashless paper pulp inside a 0.4 mL high density polyethylene vial. Concentrated ammonium hydroxide solution (20 μL) was added to each vial to prevent loss of Cl as HCl. Samples were irradiated for 1 min or 2 min and then counted for 10 to 30 minutes over a two-hour period.

2.5 Pilot Study

In order to practice the mechanics of the main analysis, a pilot study was undertaken in May, 2005. The objectives of the pilot study were to investigate detector counting positions, evaluate the proposed decay and counting times, check for

consistency in standard preparation, evaluate Standard Reference Materials (SRM's) for use, test sensitivity for other elements, and to explore and understand the flow of the analysis as well as how the tasks will be divided among the analysts. Nine of the "B" non-blinded split samples were irradiated, along with mixed-element standards, iodine standards, one quality control (QC) sample each of SRM 1577 Bovine Liver (24.0 mg) and SRM 1566b Oyster Tissue (29.6 mg), and blank polyvials with filter paper. Samples were irradiated for 1 minute at a thermal neutron flux of *ca.* 5.0×10^{13} $n \cdot cm^{-2} \cdot s^{-1}$ and allowed to decay for 5 minutes. During this time nail samples were removed from the vials in which they were irradiated and were weighed into new 0.4 mL polyethylene vials. An expanded polyethylene plug was placed on top in order to hold the nail sample onto the bottom of the vial. All samples were counted on a small rotating holder at a source-to-detector distance of *ca.* 1 cm on a HPGe coaxial detector (System 1) for 600 seconds. After this count, samples were allowed to decay for a total of 60 minutes after the end of irradiation, and then were re-counted for 15 minutes on the face of one of two HPGe detectors (Systems 2 and 3).

2.6 Determination of Manganese

2.6.1 Comparator Standards

Comparator standards were prepared using a special mix standard from High Purity Standards (Charleston, SC). The special mix standard contained 2000 µg/mL of Ca and Zn, 100 µg/mL of Cu, 500 µg/mL of Mg, 10 µg/mL of Mn, and 2 µg/mL of V in 4% HNO_3. Each standard was prepared by pipetting 25 µL of the special mix onto ~20 mg of ashless paper pulp in a 0.4 mL polyvial. The exact weight of standard added to

each polyvial was recorded in order to calculate the amount of each element added. Twenty-three standards were prepared in this way. Four blanks were prepared with paper pulp in a polyvial, as well as two more blanks with an empty polyvial.

2.6.2 Bovine Liver Quality Control Samples

Thirteen QC samples of National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1577 Bovine Liver were prepared for co-irradiation with the toenail samples. Aliquots of SRM (~20 mg) were weighed into polyvials. A filter paper circle was placed on top of the material, then an expanded polyethylene foam plug was inserted into the vial to hold the contents in the bottom of the vial for reproducible counting geometry.

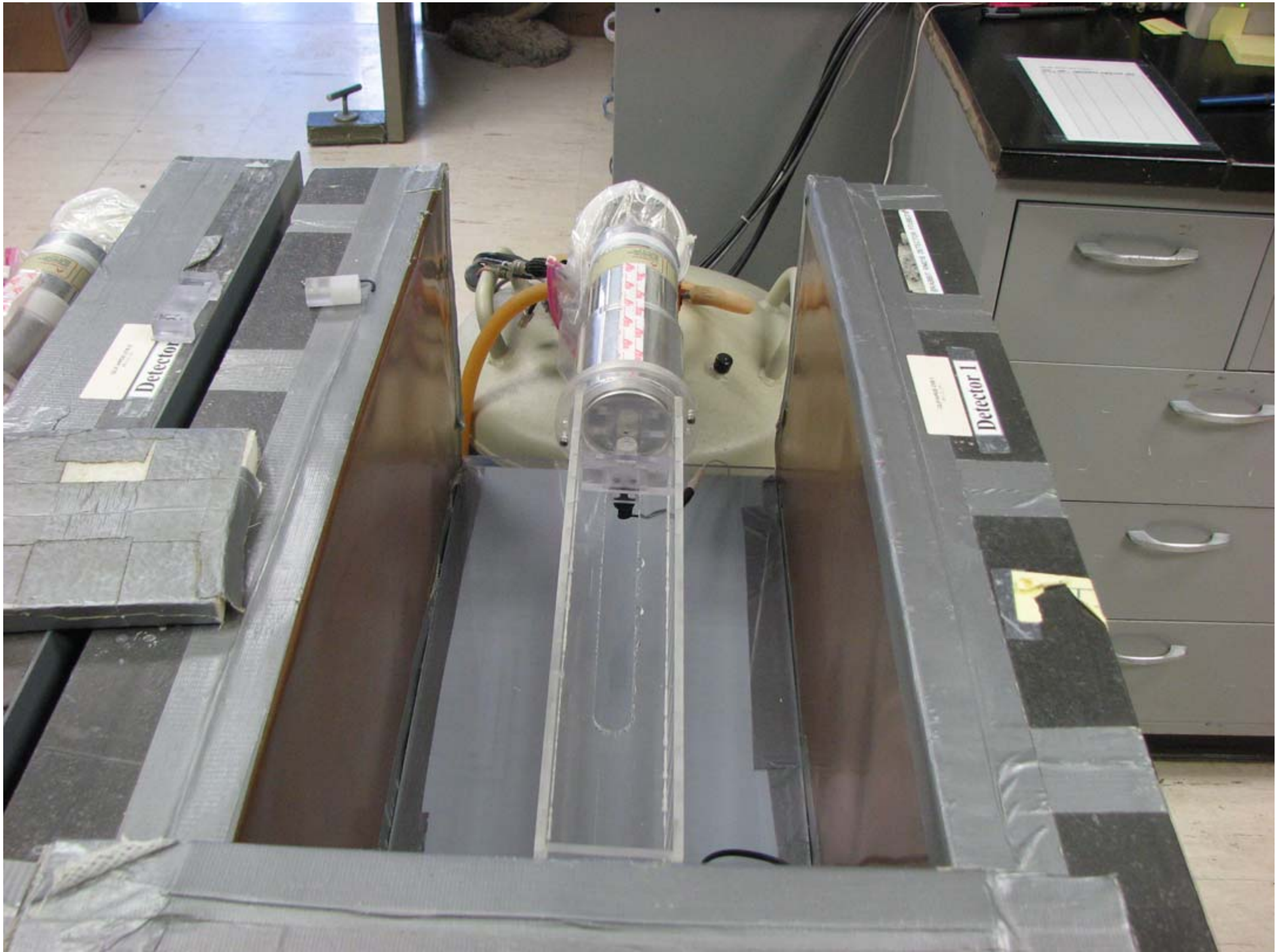
2.6.3 Neutron Activation Analysis for Manganese

The OSC samples were analyzed for Mn by INAA in August, 2005. The samples used in the earlier Se analysis were re-irradiated in a pneumatic tube system for 90 seconds at a flux of *ca.* 5.0×10^{13} $n \cdot cm^{-2} \cdot s^{-1}$. The samples were then allowed to decay for 300 seconds during which time the samples were transferred from the irradiation vials into clean 0.4 mL polyethylene vials, re-weighed, and, in order to obtain a uniform counting geometry, held against the bottom of the vial using expanded polyethylene plugs. The samples were then counted in a small rotating holder at a source-to-detector distance of *ca.* 1 cm on a HPGe coaxial detector (System 1) for 600 seconds in order to observe the signal from the shorter lived elements in this "mid" irradiation. Elements observed in this initial count include Cu, Mg, Ti, V, Sc and Al. The detector had a measured relative efficiency of 23.3% and a FWHM resolution of 1.77 keV at 1.33 MeV.

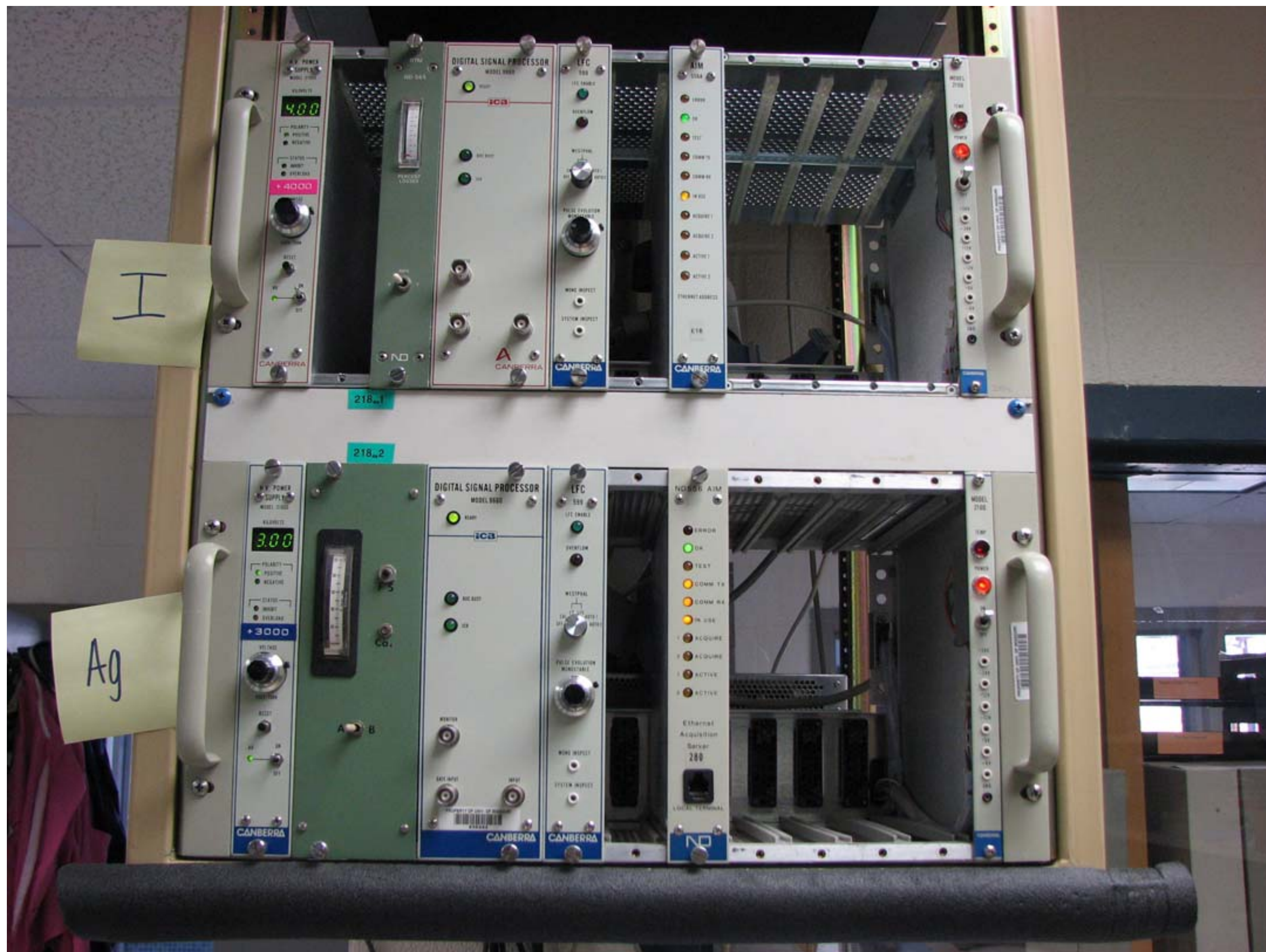
Following this initial count, the transferred sample was allowed to decay for one hour from the end of irradiation and then counted for twenty minutes on the face of one of three HPGe detectors for the Mn analysis. (Only two detectors were in use at any given time.) The three detectors (Systems 2, 3, and 4) had measured relative efficiencies of 27.3%, 30.9%, and 19.7% and a FWHM resolution of 1.68, 1.69, and 1.73 keV at 1.33 MeV, respectively. See Table 4 and Photographs 1-5 for a complete description of the gamma-ray detection systems used. The peak areas were determined interactively with Genie ESP spectroscopy software package (Canberra). Transfer of the sample from the irradiation vial was necessary because the Mn signal observed in blank irradiation vials was, on average, ten times greater than the lowest Mn signal observed in the nail samples. Of the thirteen comparator standards prepared, eleven were used in this analysis. Eleven of the Bovine Liver QC samples were irradiated during the analytical run.

Photograph 1: Detector systems in MURR Lab 218. All photos taken Feb. 26, 2007.

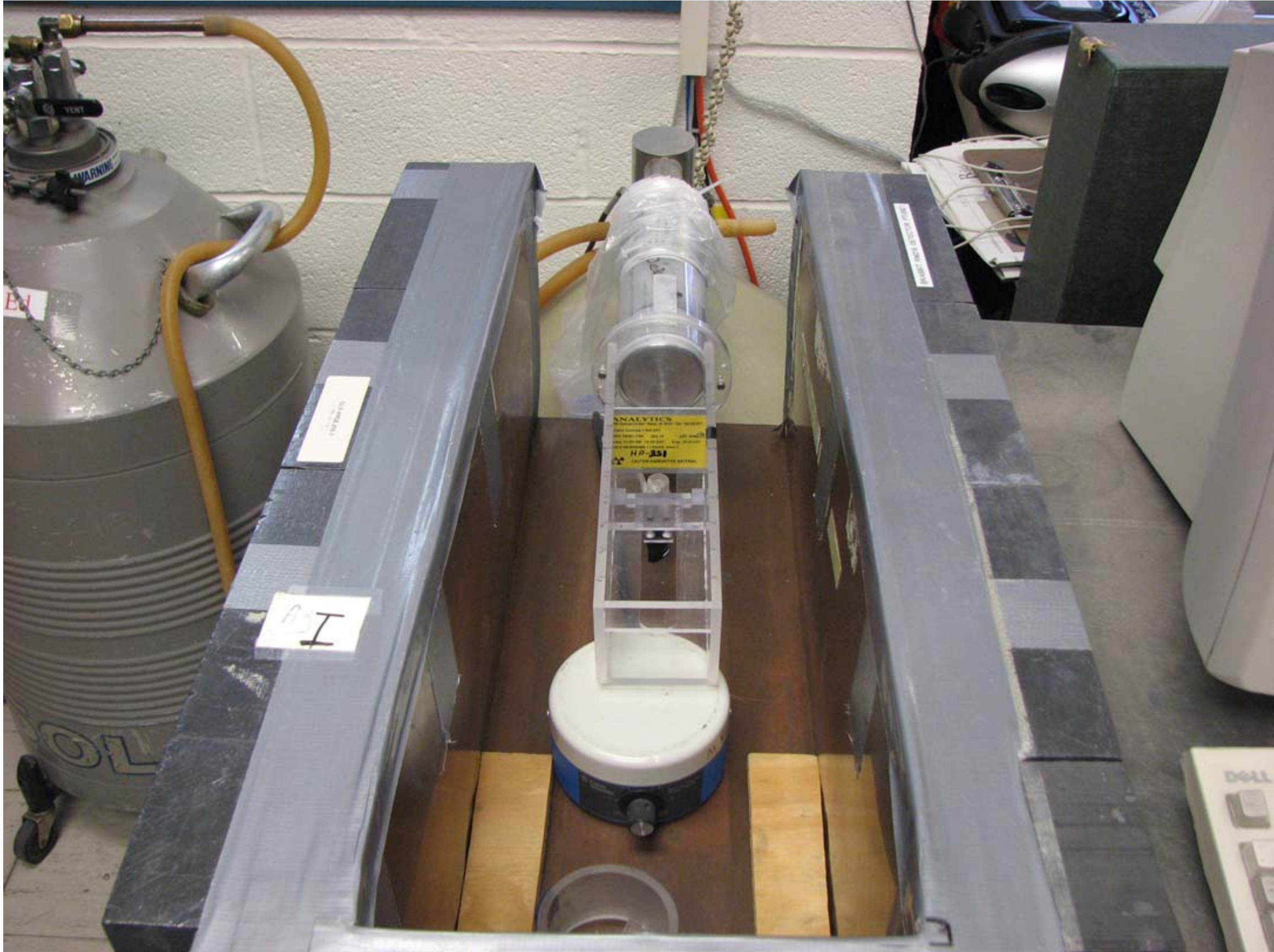
Photograph 2: Position 1 spin on Lab 218 detector 1.



Photograph 3: Signal processing electronics for detectors 1 and 2, Lab 218.



Photograph 4: Detector 1 in Lab 216.



Photograph 5: Signal processing electronics for detectors 1 and 2, Lab 216.

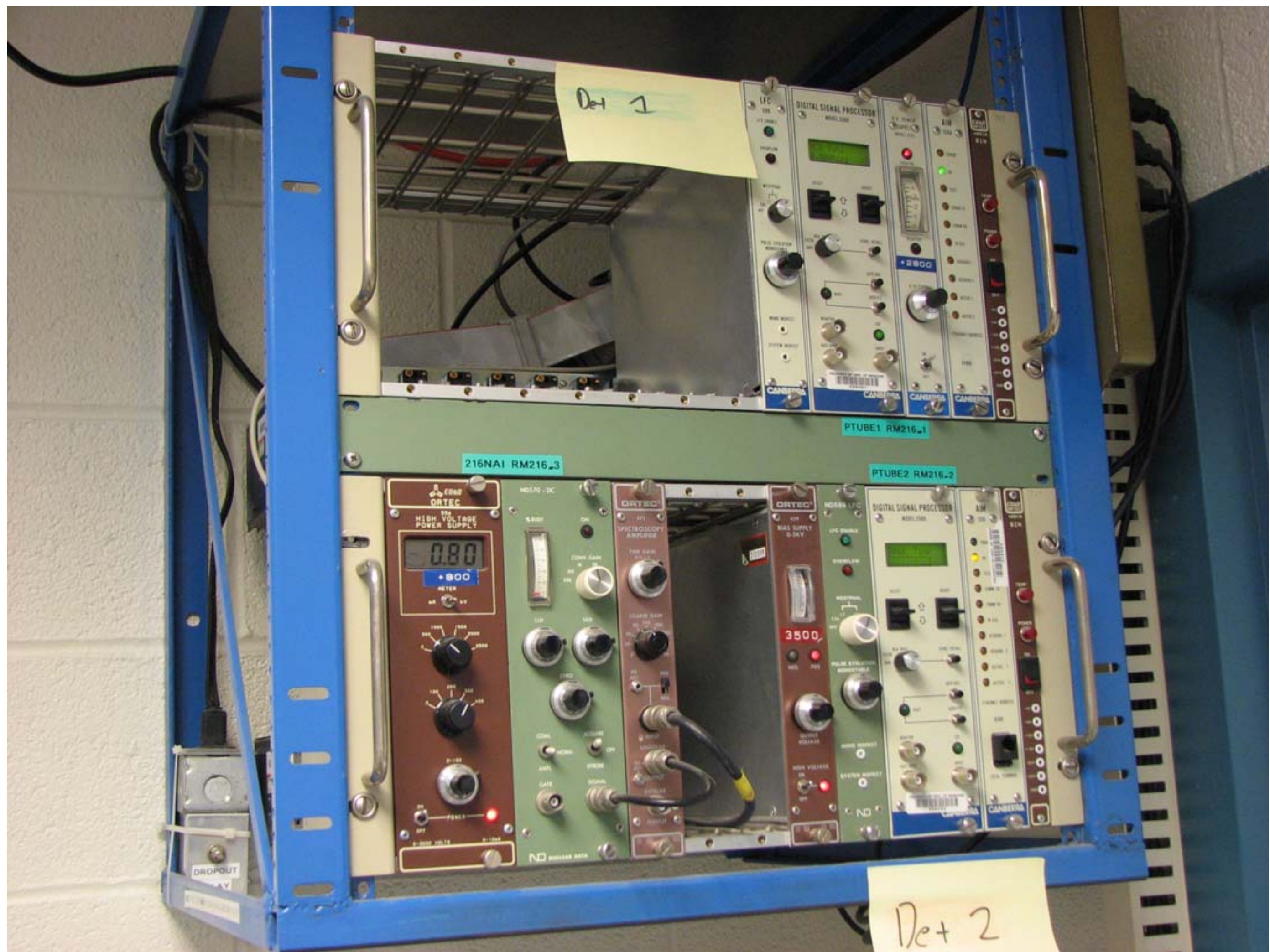


Table 4: Detection systems used in this work.

Detection System	Lab 218 detector 2	Lab 216 detector 1	Lab 216 detector 2	Lab 218 detector 1
System #	1	2	3	4
Use in this work	Count in Se run, first (short) count in Mn run	Second (long) count in Mn run, failed during analysis	Second (long) count in Mn run	Second (long) count in Mn run, substituted for system #2 after failure
HPGe Detector				
Mfr. and model #	Canberra model # GC2018S	Ortec model # GEM-25185-P	Ortec model # GEM-30185-P	Ortec model # GEM-20180-P
Resolution: FWHM at 1.33 MeV	1.77 keV	1.68 keV	1.69 keV	1.73 keV
Relative efficiency at 1.33 MeV	23.3%	27.3%	30.9%	19.7%
Signal Processing Electronics				
High voltage power supply	Canberra model # 3106D	Canberra model # 3105	Ortec model # 459	Canberra model # 3106D
Digital signal processor	Canberra model # 3660	Canberra model # 2060	Canberra model # 2060	Canberra model # 3660
Loss-free counting module	Canberra model # 599	Canberra model # 599	Nuclear Data model # 599	Canberra model # 599

3 RESULTS

Note: Many values given in this section are expressed as (mean ± standard deviation).

3.1 Toenail Simulation Experiment

The simulated toenail experiment showed that the maximum signal to noise ratio for the ^{56}Mn 846 keV gamma ray was achieved with a 1 minute irradiation and a 60 minute decay time (see Table 5 below). The actual irradiation time used for the Mn measurement was 90 seconds in order to increase sensitivity for other elements measured with the same irradiation. In addition to achieving a high signal to noise ratio, the 60 minute decay reduced or eliminated the interference from the 843 keV γ-ray emitted by ^{27}Mg ($t_{½}$ = 9.46 m). Subsequently to this experiment, it was discovered that the paper pulp and polyvials used contained significant amounts of Mn contamination. Although this fact did not substantially distort this optimization, it gave rise to an initial overoptimistic estimate of the Mn sensitivity of the method. As previously stated, the issue of polyvial contamination was addressed in the actual analysis by post-irradiation sample transfer into a cold polyvial. Where transfer was not feasible (standards and SRM's), blank subtraction was performed. For all these cases where blank correction was used, the counts subtracted were less than 1% of the total.

Table 5: Data from toenail simulation experiment.

1 minute irradiation of TS1 - count on Detector 2 Room 218							
		846.77 keV peak			1810.77 keV peak		
Decay Time (min)	Count time (min)	Peak area	Background	Peak/ Bkgrnd Ratio	Peak area	Background	Peak/ Bkgrnd Ratio
5	10	*No data collected*					
20	30	12493	10885	1.15	1199	7401	0.16
60	30	10465	6522	1.60	1509	4771	0.32
120	30	7942	5116	1.55	1042	2776	0.38
2 minute irradiation of TS2 - count on Detector 1 Room 218							
		846.77 keV peak			1810.77 keV peak		
Decay Time (min)	Count time (min)	Peak area	Background	Peak/ Bkgrnd Ratio	Peak area	Background	Peak/ Bkgrnd Ratio
5	10	3943	8075	0.49	263	4288	0.06
26	30	10488	11370	0.92	381	4572	0.08
60	30	9427	7162	1.32	1090	5237	0.21
120	30	7310	6406	1.14	578	2520	0.23

3.2 Pilot Study

No results per se were calculated from the pilot study. Dead time monitoring during this pilot study assisted in determining the closest gamma-ray detector counting position that could be used. In the position utilized for the first count, the nail sample dead times ranged from 7% to 18%. Bovine Liver or Oyster Tissue QC samples produced dead times of 23% and 43%, respectively. Based on this excessive dead time from the Oyster Tissue SRM, it was decided that this QC would not be used for the main experiment. As previously mentioned, the 60 second irradiation time used for the pilot study was increased to 90 seconds for the main experiment.

3.3 Comparator Standards

The Se and Mn concentrations of the nail samples were determined using standard comparator INAA. The Se analysis was performed utilizing the $^{76}Se(n,\gamma)^{77m}Se$ reaction and measuring the 161.9 keV gamma ray ($t_{½}$ = 17.4 s). The average (n=12) response function for the 0.5 μg Se comparator standards was 1.16 ± 0.01 x 10^5 counts/μg Se. The average (n=10) observed Se value in the NIST SRM 1577 Bovine Liver samples was 1.06 ± 0.03 μg/g; the certified Se value in this SRM is 1.1 ± 0.1 μg/g.

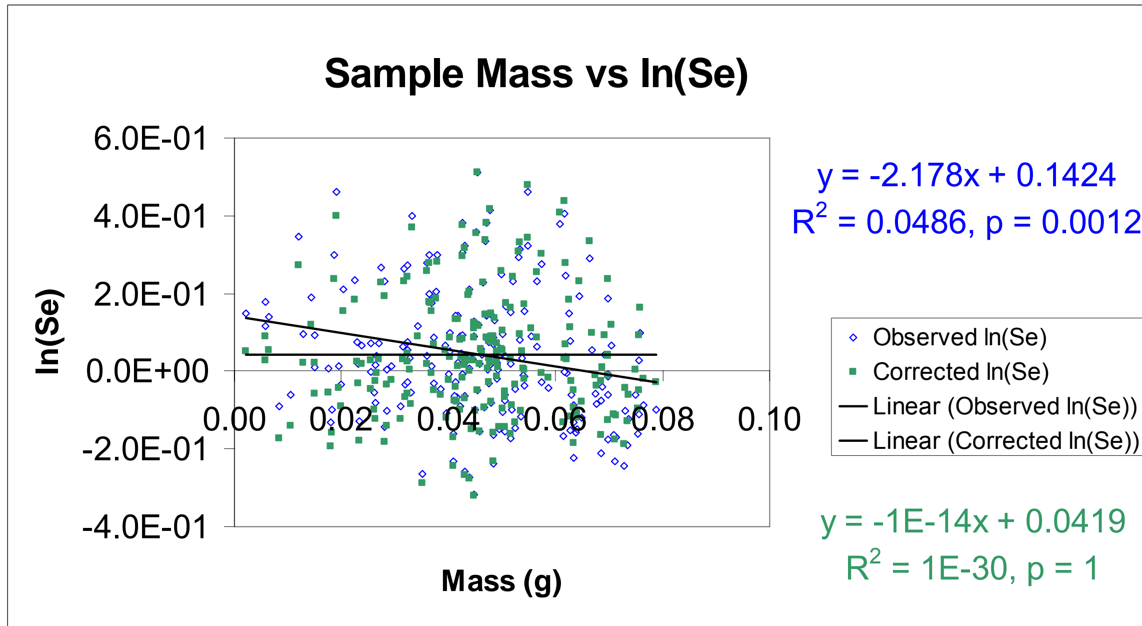
3.4 Mass Bias Correction in Selenium Results

As in previous studies,[7, 84] a mild correlation was observed between the measured Se concentration and the nail sample mass. The correlation was corrected as described in Garland, Morris et al.[7] Briefly, the steps involved in this mass-bias correction are as follows:

1. The natural logarithm of each sample result in ppm ["ln(Se)"] is calculated.
2. The log values (y axis) are regressed against the sample mass in grams (x axis). A line with a negative slope is obtained.
3. The predicted ln(Se) for each sample mass and the median sample mass is calculated based on the linear regression equation.
4. The residuals are calculated by subtracting each predicted ln(Se) from the actual ln(Se) values.
5. Each residual value is added to the predicted ln(Se) for the median sample mass.

6. The resulting values are exponentiated (e^Y) to yield the corrected Se results in ppm.

Five data points, all representing toenail Se values greater than 1.7 ppm, were deemed to be outliers and were not used in the correction process. Figure 6 displays a graphic comparison of the observed and corrected Se concentration values.



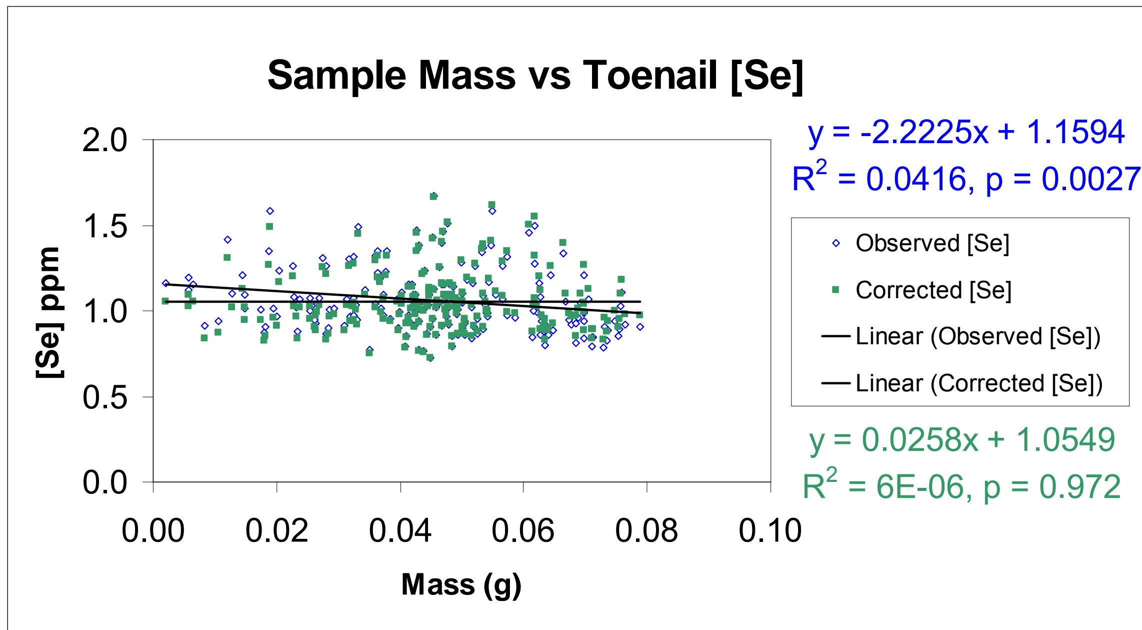


Figure 6: Graphical illustration of the Se mass bias correction.

3.5 Manganese Results

The Mn analysis was performed using the $^{55}Mn(n,\gamma)^{56}Mn$ reaction and measuring the 846.8 keV gamma ray ($t_{½}$ = 2.58 h). Three detector systems (Systems 2, 3, and 4) were used for this second and longer count; at any given time two were in use simultaneously, in order to increase sample throughput during the experiment. The average response function for the 0.25 µg Mn comparator standards on System 2 (n=6) was 1.14 ± 0.02 x 10^6 counts/µg Mn and on System 3 (n=10) was 1.28 ± 0.02 x 10^6 counts/ µg Mn. System 2 developed technical problems during the course of the experiment, necessitating a switch to another detector (System 4). The average response of the System 4 (n=4) was 9.45 ± 0.10 x 10^5 counts/µg Mn. The average observed Mn value in the NIST SRM 1577 Bovine Liver samples on System 2 (n=3) was 10.7 ± 0.1 µg/g, on System 3 (n=6) was 10.4 ± 0.8 µg/g, and on System 4 (n=2) was 10.5 ± 0.1 µg/g; the certified value for Mn in this SRM is 10.3 ± 1.0 µg/g.

3.6 Calculation of Minimum Detectable Amount

The average 95% confidence level minimum detectable amount (MDA) was calculated by the following procedure. Spectra for the 20 unblinded split samples ("A" and "B" samples) were taken to be representative of the entire experiment. For Mn, the spectra from the second (long) count were used. The background at 162 keV (Se) or 847 keV (Mn) was taken from the PEAKOUT printout corresponding to each spectrum. The theoretical minimum detectable peak area at this energy was calculated as three times the square root of the background. The resulting value was divided by the mean Se or Mn standard response (in counts/ng) for the corresponding detector, yielding an MDA

estimate in units of nanograms. The twenty MDA estimates were averaged to calculate the experiment MDA of Se and Mn in the nail samples as 0.8 ng and 0.4 ng, respectively. These values correspond to a limit of detection of 17 ng/g for Se and 8 ng/g for Mn when using the median sample mass of 48 mg. For the 62 individuals for whom a blinded split sample was available, the split sample results were averaged. The average relative standard deviation between the two analyses for these 62 individuals was 4% for Se and 24% for Mn. A similar disparity in variance was also observed in the 20 control nail samples collected from a single individual over the course of a year - the mean Se concentration was 0.87 ± 0.04 µg/g while the mean Mn concentration was 0.11 ± 0.06 µg/g.

3.7 Sample Results

The concentration of Se in the nail samples ranged from 0.7 to 3.0 µg/g and the concentration of Mn in the nails ranged from 0.02 to 1.6 µg/g. The mean Se concentration values obtained for the female (n=35) and male (n=26) supplementers (+OS) were 1.15 ± 0.13 µg/g and 1.17 ± 0.19 µg/g, respectively, and the mean Se concentrations obtained for the female (n=37) and male (n=25) non-supplementers (–S) were 1.01 ± 0.13 µg/g and 0.99 ± 0.17 µg/g, respectively. Females and males in the +OS group were found to have mean toenail Mn concentrations of 0.12 ± 0.14 µg/g and 0.081 ± 0.040 µg/g, respectively, while females and males in the –S group had mean toenail Mn concentrations of 0.16 ± 0.24 µg/g and 0.20 ± 0.34 µg/g, respectively.

3.8 Statistical Analyses

In order to account for the non-normal distribution of the nail concentrations, the statistical analyses were performed on the natural log transformed data. The boxplots for the natural log transformed toenail Se and Mn concentration in the +OS and –S groups are shown in Figure 7. The statistical analysis of the log transformed Se and Mn results was performed by matching each treatment sample (+OS) with its matched control (–S). This procedure assumes that there is less variability between matched pairs than between unmatched pairs. A mixed statistical model was employed which accounts for random effects, such as element concentration, and fixed variables such as +OS or –S status. Because duplicate measures of one individual are correlated, the correlation must be accounted for in the statistical analysis. A compound symmetry model was used in the Mixed procedure in SAS (SAS Institute Inc., Cary, NC) to account for the 62 duplicate samples. The Mixed model analysis shows, for a two-sided alternative hypothesis, a highly significant difference between the means of the ln(Se concentration) between the +OS and –S group; +OS mean (0.16 ± 0.18) is higher than the –S mean (-0.0114 ± 0.14) with a p value < 0.0001. The same analysis indicates a borderline significant result between the means of the ln(Mn concentration) between the +OS and control group ($p = 0.045$). The marginal difference is, however, in the unanticipated direction as the control group has a higher mean value (-2.37 ± 0.81) than the supplement group (-2.56 ± 0.70).

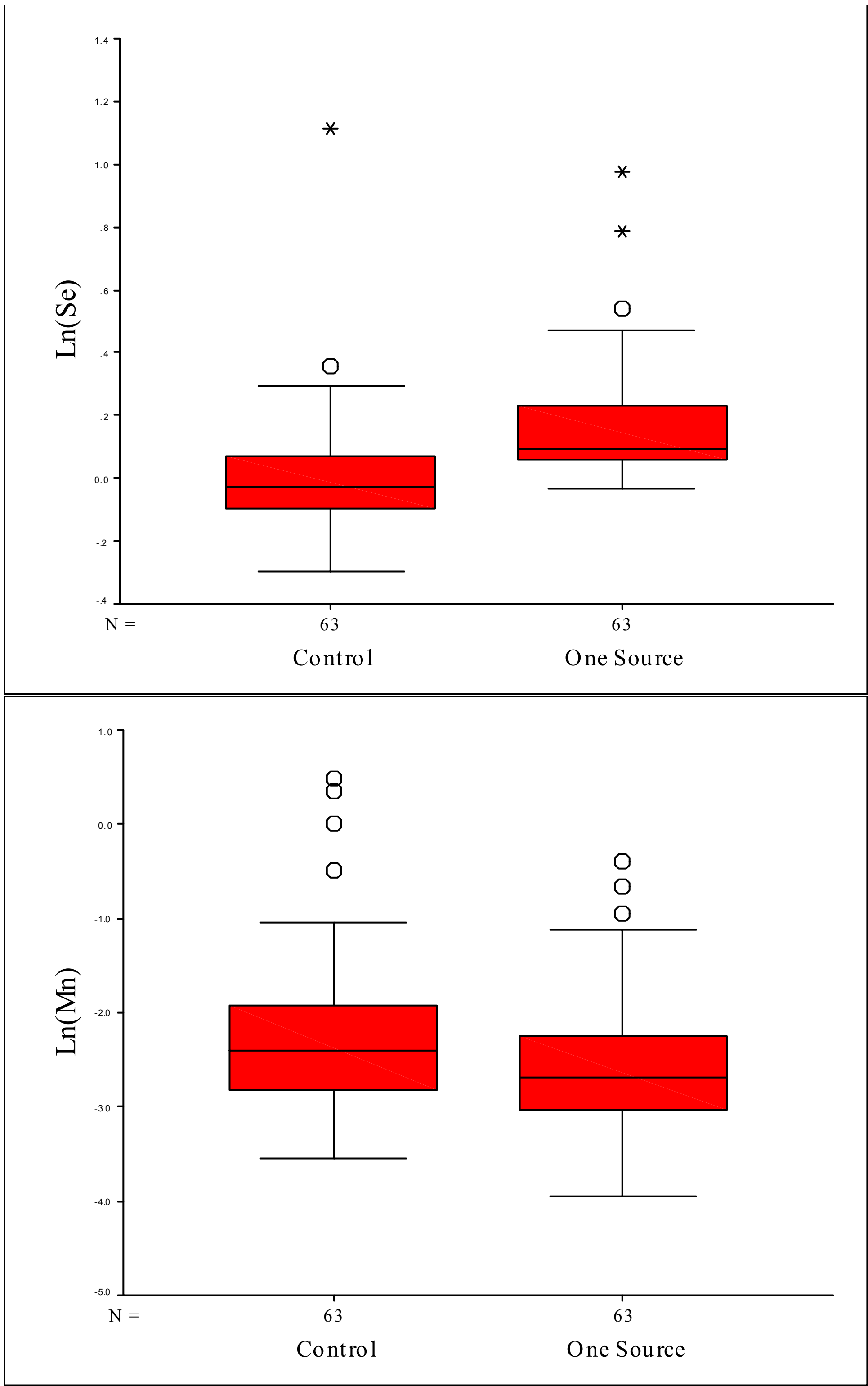


Figure 7: Boxplots showing the One Source and Control group distributions of log-transformed toenail Se and Mn concentrations.[135]

4 DISCUSSION

4.1 Proper Classification of Subjects

Measurement of Se in the nail provided an important internal control for this experiment. In the original CTS study, Se supplementation was observed to yield a significant increase in toenail Se concentration relative to non-supplementers. Because the same result was obtained in this subset, we conclude that any misclassification of +OS or –S status by the participants was minimal.

4.2 Intra-Subject Variability

In contrast to Se, the nail did not show a significant, positive response to Mn supplementation. A major difference between the Se and Mn results was, however, the larger variability in the Mn values in multiple samples from a single individual. As noted earlier, the percent relative standard deviations (%RSD) were calculated for the Se and Mn results for each of the 62 pairs of split samples; the mean %RSD for Se was 4% whereas the mean %RSD for Mn was 24%. A similar difference in the variance between Se and Mn was also observed in the 20 control nail samples taken from a single individual over the course of a year; 4% RSD for Se and a 58% RSD for Mn. (Figure 8)

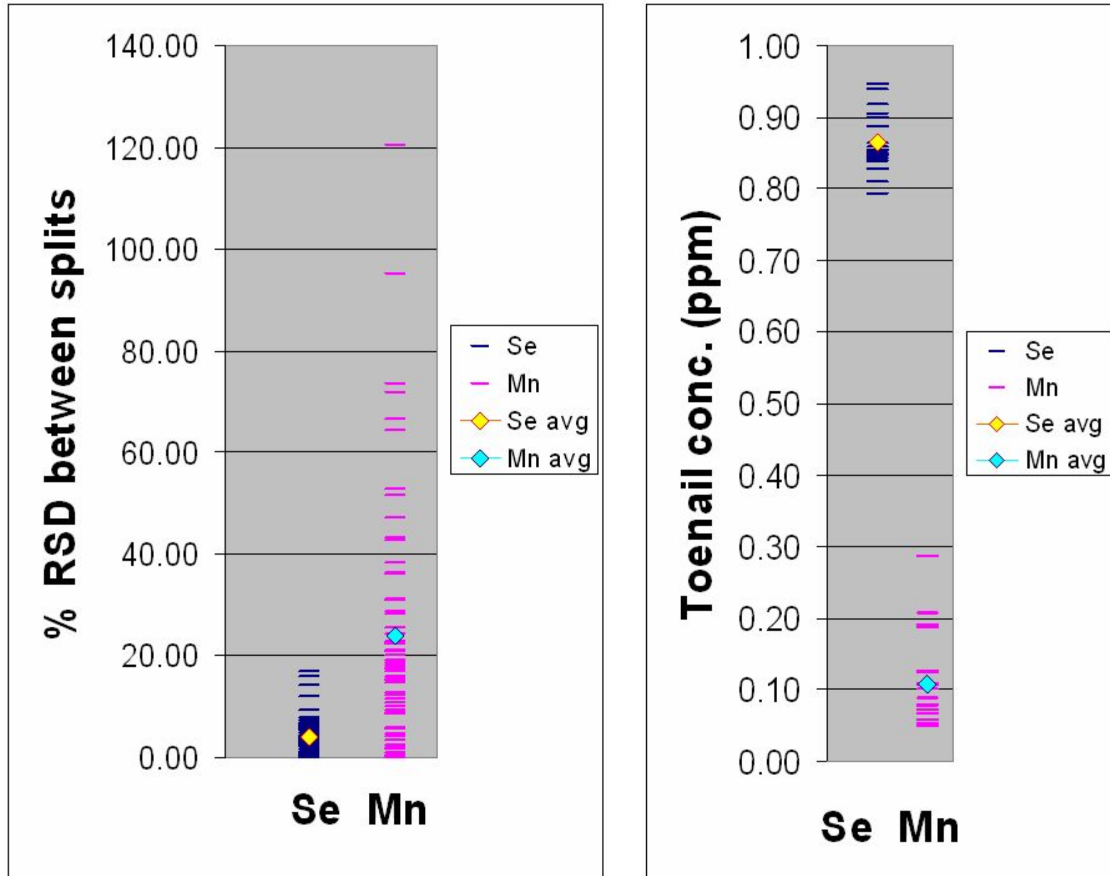


Figure 8: Variability of Mn and Se results shown in distribution graphs – on left, %RSD's between replicate ("split") pairs, and on right, the concentrations measured in 20 control samples from one individual.[135]

This high variability in Mn results is not due exclusively to measurement error. The mean amount of Mn found in the 209 nail samples was 5.6 ng, which is 14 times the MDA of 0.4 ng. Percent RSD's for the 62 pairs of split samples were plotted against the average concentration for the pair. A negative correlation of variability (%RSD) with sample concentration would provide evidence that measurement error was a large factor in the results. A linear fit of the data shows that, if anything, the opposite is the case. (Figure 9)

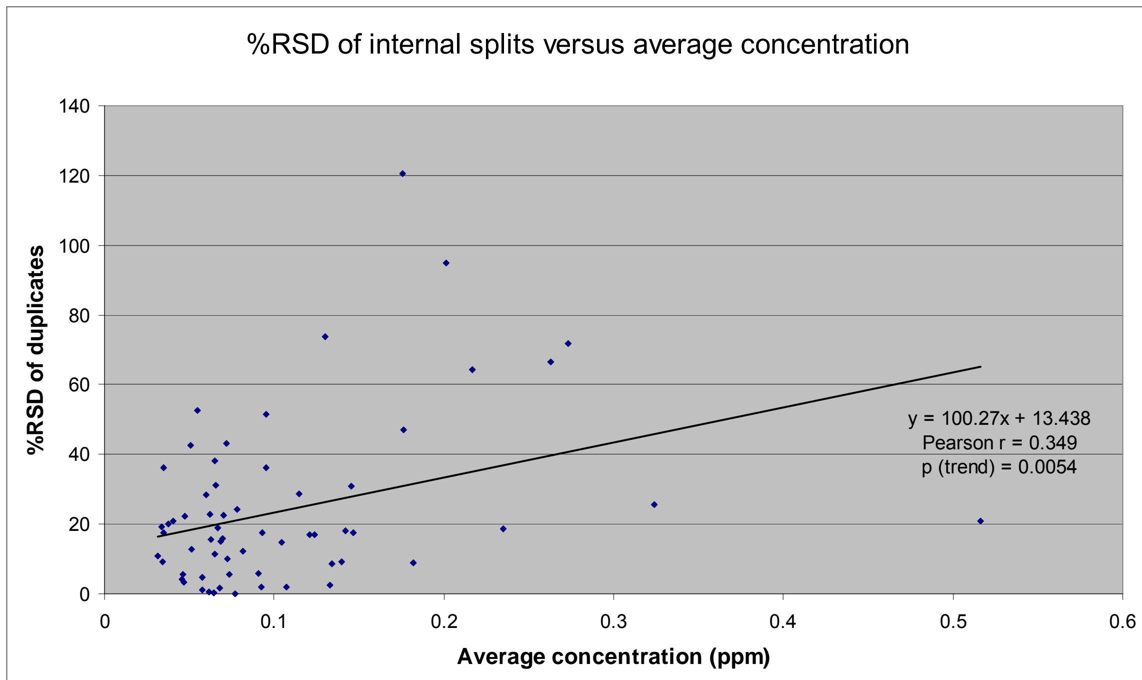
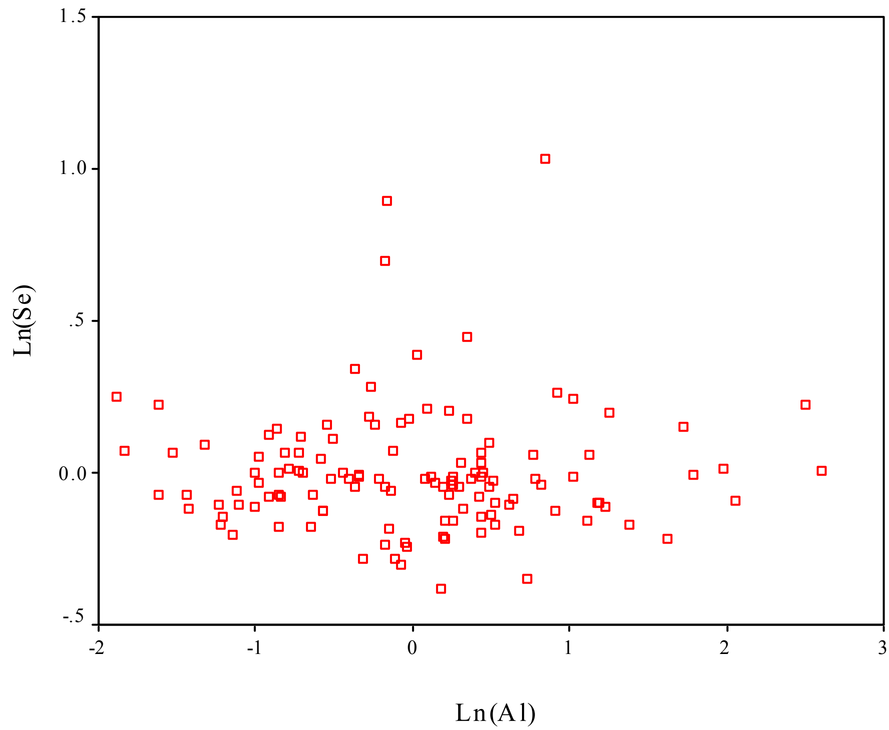


Figure 9: Percent RSD of 62 duplicate samples versus average concentration (ppm).

4.3 Exogenous Material

The large variance in the Mn concentration in the sample splits and the control nail samples suggests that Mn is not distributed homogenously throughout the sample. This observation led us to hypothesize that a portion of the Mn in the nail originates from an external source. That is, while the nail samples were cleaned and visually inspected in an attempt to remove exogenous material, the procedure is not removing all contamination. To test this idea, the relationship between the Al signal (counts/μg) observed in the short count of the "mid" irradiation and the nail Mn and Se concentration was examined. Aluminum and silicon, which contributes to the Al-28 activity, are widely distributed in the terrasphere and their presence in the nail suggests exogenous contamination. As can be seen in the plots of the log transformed Mn and Se

concentrations versus the log transformed Al signal presented in Figure 10, Mn ($p < 0.0001$) is highly correlated with the Al in the nail whereas Se ($p = 0.67$) is not. The strong correlation between Mn and Al may indicate that a significant portion of the Mn measured in the nail originates as terrestrial contamination.



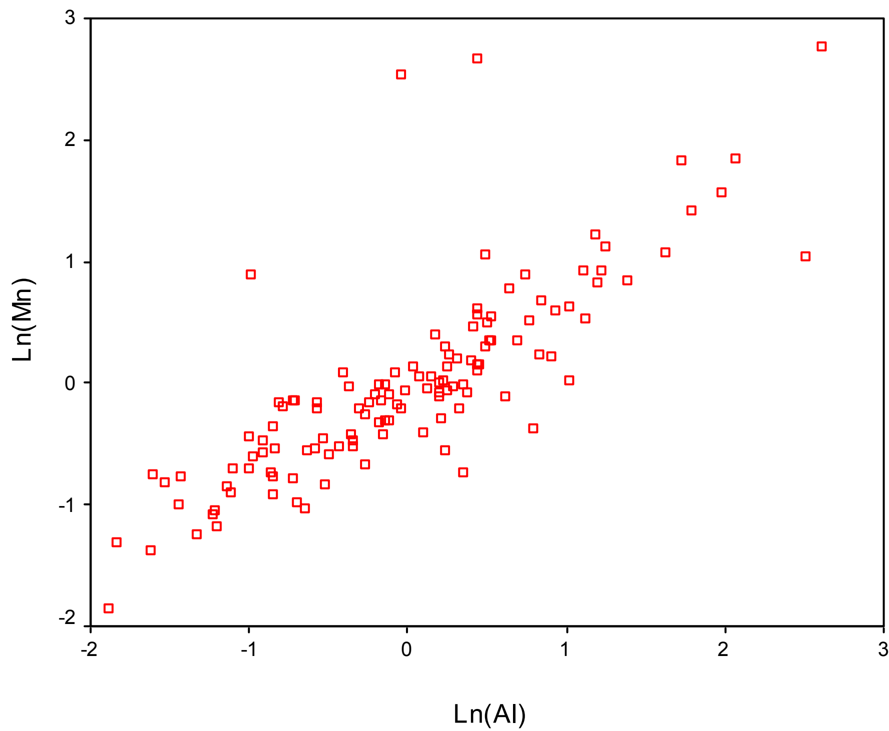


Figure 10: Scatter plots for Se vs. Al (no correlation, Pearson coefficient -0.032) and Mn vs. Al (positive correlation, Pearson coefficient 0.811)[135]

4.4 Covariant Model

The Mn and Se data were reanalyzed using a covariant model that normalized the Al concentration between the +OS and –S groups. Again, a marginal difference was observed ($p = 0.055$) in the mean ln(Mn concentration) between the two groups with a higher value observed for the –S group.

4.5 Explanatory Hypotheses

There are several factors that could explain the toenail's apparent lack of measurable response to Mn intake. First, the bioavailable fraction of the Mn consumed is highly variable, being determined by a number of factors, including other dietary components. Second, blood Mn is regulated via homeostatic mechanisms and may not be substantially modified by supranutritional intake. Third, this element may show little chemical affinity for the keratinaceous proteins that compose the nail. Fourth, the quantity of metabolically deposited Mn may be small relative to that present due to exogenous contamination.

4.5.1 Absorbable Fraction and Bioavailability of Dietary Mn

The absorbable fraction for a nutrient is one factor in its overall bioavailability, which takes into account absorption and utilization as well. For dietary Mn, the fraction available for absorption in the GI tract is low and variable.[136] As is the case with many fortified foods and multivitamins, One Source contains Mn in the sulfate form, $MnSO_4$ being a highly soluble compound[137] which is thought to be more bioavailable than the oxide.[138] However, even in fortified or liquid foods, the absorbable fraction of Mn, as measured by a simulated *in vitro* gastric and intestinal digestion, is small, averaging 5%

in a survey of dairy products.[136] The true absorbable fraction for Mn in humans and animals may be influenced by the concurrent consumption of foods containing solids to which the Mn may bind, such as dietary fiber or protein, or chemicals that complex and precipitate Mn, such as soy phytate.[139] Outside of *in vitro* experiments, however, any modifications to the absorbable fraction are not independently measurable, but rather only the effects on total nutrient absorption or bioavailability can be quantified.

Bioavailable Mn is taken up into the bloodstream via the GI tract. The absorption appears to take place by active transport mechanisms which are regulated based on the current body status of Mn.[120] Past intake of Mn, therefore, presumably influences current retention.[140] Other dietary components, though they may have no effect on the absorbable fraction of Mn, can nevertheless affect its bioavailability. Ascorbic acid, tea polyphenols, and lactoferrin (in human breast milk) are known to bind to Mn and modify the efficiency with which it is transported into the blood.[139-142] Other minerals present, such as Ca, may block Mn uptake;[141] conversely, in Fe-deficient diets, Mn may be absorbed in excess.[138]

4.5.2 Homeostatic Mechanisms

Once Mn enters the blood, it is subject to homeostatic mechanisms which regulate its levels. Circulating Mn concentrates in the liver, which rapidly excretes Mn into the bile and releases this back into the gut.[117, 139] Other tissues, such as the brain, kidneys, and skeletal muscle, also absorb Mn and release it at varying rates.[124] Retention of Mn is low, and has been reported in the range of 0.6-9.2%, in contrast to Se retention of approximately 80%.[120] Balance studies have demonstrated that Mn retention is not

substantially related to intake.[117] It is conceivable that the regular consumption of Mn and other minerals via multivitamin supplementation actually decreases the body's propensity for Mn absorption.

Testimony to the effectiveness of the body's regulation of Mn absorption comes from the scarcity of reports of toxicity resulting from consumption of Mn via dietary intake.[120, 139] The multiplicity of factors relating to absorption of Mn from diet and supplements confound the differences between treatment and control groups in this study.

4.5.3 Binding of Mn to Keratin

Though limited data exist, Mn appears to bind poorly to keratin proteins. Other metals, such as Cr, Fe, Ni, Cu, Zn, Cd, Pb, and Hg have been found to bind to avian feather keratin in wastewater treatment applications.[143] Mn is conspicuously absent from this element set, which overlaps reasonably well with the set of elements for which the toenail biomonitor has indicated a positive response. The sulfides of these metals are also relatively insoluble compared to MnS,[137] indicating that Mn forms relatively weak bonds to sulfur, a major component and metal binding site in keratin. Separately, human hair keratin has been investigated for its ability to bind Mn, Cu, Zn, and As cations,[144] and the sorption of Mn was found to be inherently minimal, and even lower when other metals were present to compete for binding sites. Taken as a whole, the literature contains little specific information regarding Mn and keratin, logically leading one to conclude that the probable attempts to adsorb Mn onto keratin have been fruitless. Extrapolating from this, it is reasonable to suggest that cationic Mn circulating in the nail matrix blood supply may not be absorbed into the newly produced nail keratin.

4.5.4 Exogenous Contamination

Crustal abundances are 0.1% for Mn and 50-200 ppb for Se.[120] Typical Mn levels in soil and sediment SRM's range in the hundreds of ppm while Se levels in the same material are much lower, generally in the single-digit ppm range.[145] With the assumption that these values are representative of many soil types, it is clear that the potential for soil contamination is much higher with Mn. Similarly, other elements applicable to the toenail biomonitor, such as As, Hg, and Cd, are far less ubiquitous than is Mn. The aforementioned high degree of correlation between toenail Mn and Al also highlights the likelihood that toenail Mn is largely determined by exogenous sources.

4.6 Study Strengths and Weaknesses

Overall, the study accomplished its goal of testing the Mn toenail hypothesis. As with every scientific study, though, this one had strengths and weaknesses.

4.6.1 Strengths

Several fortuitous circumstances contributed to the strength of the study. Because of prior specimen collection and questionnaire administration in the Missouri Study, we had a ready supply of properly stored, analyzable specimens with relevant, accompanying information about the subjects, most of whom were long-time residents at their current address. Our facility is uniquely equipped with the means to measure Mn and Se in the specimens with sufficient sensitivity and selectivity, and our NAA method sidesteps large analytical errors due to laboratory contamination. The samples were not destroyed during the measurement and are still available for future re-analysis. A single standard reference material (SRM 1577 Bovine Liver) was adequate for demonstrating the

accuracy of the method for both Mn and Se. Split samples and 20 single-individual replicates could be analyzed in order to characterize intra-person variability of toenail Mn.

Perhaps the greatest advantage our study enjoyed was that Se measurement of the same specimens produced an internal check on several critical aspects of the experiment. Because the One Source™ multivitamin contained relatively large amounts of both Se and Mn, the proper classification of the subjects in terms of supplement use was demonstrated by the Se measurement. The expected low intra-individual variability for toenail Se was confirmed for the samples and provided a point of reference for comparing the corresponding variability of Mn results. The acceptable Se results obtained from these quality controls indicate that samples were not mislabeled or interposed during the analysis.

4.6.2 Weaknesses

Weak points of the current study include the lack of a thorough cleaning study for optimizing the method of removal of exogenous Mn. Our acid cleaning method, while it can be expected to selectively solubilize only external Mn, was not extensively tested prior to being applied to the specimens. It was not possible to control for exogenous Mn or know what portion of the Mn signal was due to contamination present on the nail before it was submitted.

The early method development work did not take into account the later finding that the polyvials contained a substantial amount of Mn. While this ultimately had little

effect on the method and the analysis did not suffer from the problem, it initially gave an exaggerated estimate of Mn sensitivity. The issue of polyvial contamination was solved by transferring the sample to a cold vial post-irradiation.

The use or non-use of the One Source™ multivitamin could not be confirmed by follow-up with the participants. As described earlier, these participants were drawn from a larger cohort of individuals who responded to newspaper advertisement following a series of articles on selenium and human health. As a result, the health-conscious segment of the population was over-represented in this group, which in turn yielded specimens with higher-than-average Se compared to the general population. Based on the OSC Se analysis, there was a lesser difference between control and treatment groups than would be expected if representative population samples were used instead. It may be expected, then, that this same phenomenon mitigated any differences for Mn as well.

The final disadvantage of the study is its low power due to a small number of subjects. However, the information gained does not provide any impetus for a larger study of similar nature.

5 CONCLUSIONS

In agreement with previous work, this study demonstrates that the toenail is an excellent biomonitor for Se intake. In contrast, the nail does not appear to be an appropriate biomonitor for supplemental intake of Mn in the diet, since no increase in toenail Mn was observed with supranutritional consumption of this trace nutrient.

As discussed previously, the absorption of Mn is a complex process, highly influenced by individual body chemistry and other dietary components. In contrast to Se, there are no clear-cut data to indicate that Mn supplementation actually increases blood Mn concentrations, a factor which is regulated by mineral stores in tissues. Mn has not been shown to exhibit a chemical affinity to the nail's keratinaceous material, thus it may not be deposited into the nail via the blood supply. Beyond these unknown factors, we hypothesize that any increase in metabolically deposited Mn resulting from supplementation is masked by a relatively large amount of persistent exogenous contamination.

The present data do not encourage the formation of a larger treatment-control study involving Mn supplementation and toenails. However, a purely chemical investigation of Mn binding using nail keratin could help elucidate the potential mechanism for endogenous Mn deposition into the nail. If the results showed an unanticipated affinity of Mn to keratin, building credence for the blood-deposition mechanism, then it could be worthwhile to continue work. The next step would be to optimize toenail cleaning methods to reduce or eliminate exogenous Mn while retaining the endogenous fraction. Potentially, the improved cleaning procedure could be applied to the existing samples, facilitating an improved method for testing the Mn/toenail hypothesis.

APPENDIX: Data and Results Table

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
2220	125	1		F	30	24	never	no	0.00	0	0.00		0.07563	0.08206	1.10E+00	9.44E-02
1862	145	2		F	48	21	current	no	0.00	0	0.00		0.07291	0.0768	8.30E-01	8.28E-02
956	104	3		F	48	33	never	no	0.00	0	0.00		0.02333	0.0243	8.36E-01	9.12E-02
1861	71	4		F	48	32	never	yes	200.00	1	0.50	One Source	0.06884	0.07251	1.01E+00	4.44E-02
CLK951001-4	196	5											0.07124	0.07544	8.92E-01	1.91E-01
2413	122	6		F	33	18	never	no	0.00	0	0.00		0.02842	0.02874	8.68E-01	1.11E-01
1933	150	7		F	55	34	past	no	0.00	0	0.00		0.04156	0.04335	9.26E-01	5.58E-02
1936	29	8		F	48	21	past	yes	200.00	4	2.00	One Source	0.03603	0.03792	1.02E+00	5.93E-01
CLK960103-5	114	9											0.03971	0.04148	8.86E-01	8.00E-02
1744	51	10		F	41	19	never	yes	200.00	7	1.00	One Source	0.04895	0.05152	1.08E+00	8.34E-02
CLK960103-1	113	11											0.05188	0.05426	9.16E-01	1.06E-01

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
CLK950701-3	67	12											0.07098	0.07432	8.95E-01	1.25E-01
1235	34	13		M	54	28	never	yes	200.00	1	1.20	One Source	0.0324	0.03519	1.05E+00	7.97E-02
176	5	14		M	35	31	never	yes	200.00	0	5.00	One Source	0.0618	0.06516	1.07E+00	8.87E-02
1841	42	15		F	65	24	never	yes	200.00	0	6.00	One Source	0.03113	0.03258	2.22E+00	9.37E-02
590	19	16		F	42	22	never	yes	200.00	1	1.50	One Source	0.01204	0.01266	1.31E+00	6.68E-02
2525	180	17		F	70	21	past	no	0.00	0	0.00		0.03223	0.03358	1.02E+00	1.20E-01
919	201	18		M	70	28	past	no	0.00	0	0.00		0.0485	0.05088	7.93E-01	7.19E-02
2344	158	19		F	35	21	never	no	0.00	0	0.00		0.02932	0.03106	9.77E-01	2.66E-01
1307	48	20		F	68	22	past	yes	200.00	0	5.00	One Source	0.03240	0.03355	1.27E+00	4.30E-02
1614	126	21		F	44	20	past	no	0.00	0	0.00		0.03602	0.03784	1.29E+00	3.04E-01
JSM041005fn	207	22											0.04521	0.04795	1.43E+00	1.72E-01
CLK950401-2	85	23											0.07113	0.07517	8.38E-01	4.78E-02
1933	105	24		F	55	34	past	no	0.00	0	0.00		0.06848	0.0723	9.74E-01	5.95E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
2292	163	25		M	50	25	never	no	0.00	0	0.00		0.05045	0.05308	8.65E-01	1.01E+00
1733	137	26		M	83	25	never	no	0.00	0	0.00		0.03844	0.03991	9.40E-01	1.18E-01
2447	130	27		M	35	31	never	no	0.00	0	0.00		0.02612	0.02738	9.05E-01	2.88E-02
419	26	28		M	70	31	past	yes	200.00	1	2.00	One Source	0.03645	0.03835	1.32E+00	5.16E-02
92	111	29		F	49	29	never	no	0.00	0	0.00		0.02631	0.02744	8.84E-01	1.27E-01
1648	28	30		M	66	28	never	yes	200.00	0	1.00	One Source	0.04764	0.04919	1.03E+00	5.65E-02
1841	32	31		F	65	24	never	yes	200.00	0	6.00	One Source	0.04298	0.04554	2.24E+00	4.99E-02
1856	87	32		M	60	30	past	yes	200.00	0	1.50	One Source	0.02288	0.02416	1.03E+00	6.60E-02
2344	200	33		F	35	21	never	no	0.00	0	0.00		0.04529	0.04736	9.51E-01	2.04E-01
CLK950401-3	86	34											0.06272	0.066	8.89E-01	4.86E-02
2666	187	35		F	44	21	never	no	0.00	0	0.00		0.03996	0.04164	9.78E-01	6.13E-01
1557	168	36		F	74	24	past	no	0.00	0	0.00		0.06980	0.07369	9.91E-01	4.56E-02
CLK951001-1	173	37											0.06976	0.07339	8.83E-01	1.01E-01

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
CLK951001-2	147	38											0.06150	0.06432	8.76E-01	8.77E-02
580	103	39		M	76	27	past	no	0.00	0	0.00		0.01491	0.01499	1.02E+00	1.62E+00
621	109	40		M	51	27	never	no	0.00	0	0.00		0.05402	0.0568	1.01E+00	1.71E-01
1461	84	41	A	F	74	23	past	yes	200.00	0	1.00	One Source	0.06983	0.0729	1.04E+00	6.70E-02
1461	84	41	B	F	74	23	past	yes	200.00	0	1.00	One Source	0.06740	0.07001	9.89E-01	5.61E-02
1645	73	42		F	46	22	past	yes	200.00	3	2.00	One Source	0.03692	0.03859	9.94E-01	3.82E-01
1757	116	43		M	48	24	never	no	0.00	0	0.00		0.03315	0.03422	1.45E+00	1.14E-01
983	65	44		M	56	32	past	yes	200.00	1	1.00	One Source	0.06269	0.06496	1.12E+00	3.47E-02
1554	78	45		F	43	39	never	yes	200.00	1	0.50	One Source	0.05342	0.05584	1.39E+00	1.39E-01
2585	90	46	A	F	58	27	never	yes	200.00	1	10.00	One Source	0.04632	0.04865	1.05E+00	1.16E-01
2585	90	46	B	F	58	27	never	yes	200.00	1	10.00	One Source	0.04992	0.05234	1.06E+00	1.01E-01
1426	20	47		F	44	20	past	yes	20.00	3	0.50	One Source	0.04480	0.04423	1.12E+00	3.43E-02
1310	127	48		M	66	26	past	no	0.00	0	0.00		0.06672	0.07007	1.10E+00	3.65E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
2136	70	49		F	49	30	never	yes	200.00	3	0.50	One Source	0.04681	0.04954	1.40E+00	6.59E-02
43	148	50		M	66	29	never	no	0.00	0	0.00		0.01054	0.01107	8.70E-01	8.17E-02
180	93	51		M	81	27	past	yes	200.00	0	2.50	One Source	0.02854	0.02979	9.68E-01	4.83E-02
1392	112	52		F	55	26	past	no	0.00	0	0.00		0.06333	0.06704	9.10E-01	8.96E-02
1492	108	53		F	43	38	never	no	0.00	0	0.00		0.05077	0.05378	1.19E+00	1.34E-01
CLK950701-5	74	54											0.05161	0.05437	8.51E-01	6.68E-02
1024	143	55		M	64	27	never	no	0.00	0	0.00		0.02635	0.02768	9.74E-01	1.60E-01
991	75	56		M	64	24	never	yes	200.00	0	1.00	One Source	0.04751	0.05	1.08E+00	5.18E-02
1461	36	57		F	74	23	past	yes	200.00	0	1.00	One Source	0.04930	0.05205	1.01E+00	6.21E-02
636	96	58		F	28	24	never	yes	200.00	2	4.00	One Source	0.02368	0.02501	1.02E+00	4.19E-02
424	55	59		M	66	25	past	yes	200.00	0	3.00	One Source	0.03681	0.03849	1.17E+00	3.88E-02
1326	161	60		F	48	21	past	no	0.00	0	0.00		0.01792	0.01884	8.24E-01	4.45E-02
2195	136	61		M	47	24	never	no	0.00	0	0.00		0.03502	0.03609	7.50E-01	2.16E-01

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
1389	206	62		F	28	21	never	no	0.00	0	0.00		0.02800	0.02954	8.34E-01	3.52E-01
319	13	63		F	41	25	never	yes	200.00	3	1.00	One Source	0.04845	0.0512	1.08E+00	8.32E-02
1910	141	64		F	67	32	never	no	0.00	0	0.00		0.05415	0.05701	1.00E+00	6.60E-02
1842	27	65		M	64	33	never	yes	200.00	0	6.00	One Source	0.04702	0.04881	1.47E+00	4.67E-02
1701	189	66		M	43	24	current	no	0.00	0	0.00		0.04222	0.04473	9.06E-01	2.87E-02
858	167	67		F	68	22	past		0.00	0	0.00		0.05656	0.05938	1.29E+00	5.51E-02
424	44	68		M	66	25	past	yes	200.00	0	3.00	One Source	0.04853	0.05118	1.15E+00	3.02E-02
689	7	69		M	60	31	past	yes	200.00	2	2.00	One Source	0.04737	0.04731	1.01E+00	7.65E-02
2210	154	70		F	63	23	never	no	0.00	0	0.00		0.02319	0.0223	9.68E-01	3.64E-02
CLK950701-1	17	71											0.04108	0.04259	9.28E-01	1.08E-01
CLK951001-3	177	72											0.07530	0.07977	9.08E-01	5.83E-02
1876	160	73		M	38	24	never	no	0.00	0	0.00		0.04484	0.04751	7.25E-01	1.42E-01
1693	121	74		F	29	27	never	no	0.00	0	0.00		0.01444	0.0154	1.13E+00	5.57E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
1807	198	75		M	60	34	past	no	0.00	0	0.00		0.05054	0.05206	9.50E-01	1.18E-01
557	119	76		F	66	24	never	no	0.00	0	0.00		0.03284	0.02918	1.00E+00	4.29E-02
1615	140	77		M	68	27	never	no	0.00	0	0.00		0.04382	0.04435	1.00E+00	6.16E-02
917	24	78		F	33	16	never	yes	200.00	1	2.00	One Source	0.04798	0.05046	1.09E+00	3.86E-02
400	1	79		M	64	22	never	yes	200.00	0	1.00	One Source	0.04255	0.05046	9.83E-01	1.28E-01
1445	58	80		M	48	23	never	yes	200.00	0	1.50	One Source	0.05210	0.05493	1.28E+00	5.21E-02
547	15	81		M	43	24	current	yes	200.00	2	2.00	One Source	0.04027	0.04186	1.04E+00	1.35E-01
652	30	82		M	47	24	never	yes	200.00	7	1.50	One Source	0.04475	0.0471	1.09E+00	4.09E-02
443	152	83		F	46	24	past	no	0.00	0	0.00		0.04490	0.04715	9.04E-01	1.07E-01
2255	131	84		M	64	23	never	no	0.00	0	0.00		0.03780	0.03937	1.32E+00	7.46E-02
2669	178	85		F	35	23	past	no	0.00	0	0.00		0.05414	0.05752	9.82E-01	6.71E-02
CLK950701-2	100	86											0.06371	0.06708	8.88E-01	7.79E-02
1910	133	87		F	67	32	never	no	0.00	0	0.00		0.03720	0.03952	9.51E-01	3.54E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
858	146	88		F	68	22	past		0.00	0	0.00		0.04367	0.04603	1.21E+00	4.01E-02
2422	170	89		F	65	22	never	no	0.00	0	0.00		0.04639	0.04793	1.26E+00	8.65E-02
1326	174	90		F	48	21	past	no	0.00	0	0.00		0.05345	0.05663	9.04E-01	4.81E-02
2087	209	91		F	49	21	never	no	0.00	0	0.00		0.05740	0.06003	9.95E-01	2.27E-01
14140	199	92		M	57	36	past	no	0.00	0	0.00		0.06780	0.07778	2.84E+00	1.91E-01
1757	155	93	A	M	48	24	never	no	0.00	0	0.00		0.05321	0.05514	1.36E+00	1.77E-01
1757	155	93	B	M	48	24	never	no	0.00	0	0.00		0.05473	0.05376	1.41E+00	1.77E-01
2185	208	94		M	60	29	past	no	0.00	0	0.00		0.03243	0.0343	9.44E-01	1.31E-01
1121	184	95		F	58	22	never	no	0.00	0	0.00		0.02002	0.0208	9.14E-01	5.10E-02
480	62	96		F	74	27	never	yes	200.00	0	2.00	One Source	0.01751	0.0176	9.46E-01	5.89E-02
1915	31	97		M	38	27	never	yes	200.00	4	0.50	One Source	0.07591	0.08027	1.18E+00	9.38E-02
1850	193	98		F	58	28	never	no	0.00	0	0.00		0.07484	0.06198	1.00E+00	5.87E-02
418	97	99		F	66	24	never	yes	200.00	1	2.00	One Source	0.02805	0.02963	1.21E+00	3.64E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
1649	45	100		F	63	23	never	yes	200.00	0	1.00	One Source	0.04258	0.04455	1.35E+00	2.35E-02
557	186	101		F	66	24	never	no	0.00	0	0.00		0.04298	0.04521	1.02E+00	3.23E-02
CLK951001-5	138	102											0.06348	0.06621	9.52E-01	2.87E-01
2185	191	103		M	60	29	past	no	0.00	0	0.00		0.06777	0.07075	9.64E-01	1.49E-01
1145	153	104		F	43	24	never		0.00	0	0.00		0.03178	0.03357	1.26E+00	9.12E-02
1414	56	105	A	M	57	36	past	yes	200.00	0	0.75	One Source	0.05484	0.05715	1.61E+00	1.39E-01
1414	56	105	B	M	57	36	past	yes	200.00	0	0.75	One Source	0.06173	0.06326	1.55E+00	1.21E-01
1915	102	106		M	38	27	never	yes	200.00	4	0.50	One Source	0.06978	0.07392	1.27E+00	9.11E-02
2544	99	107		M	50	38	never	yes	200.00	0	1.00	One Source	0.06180	0.06539	1.32E+00	1.15E-01
43	169	108		M	66	29	never	no	0.00	0	0.00		0.04093	0.04139	8.43E-01	1.05E-01
1615	203	109		M	68	27	never	no	0.00	0	0.00		0.04784	0.05034	1.02E+00	7.73E-02
147	59	110		M	58	27	past	yes	200.00	1	3.00	One Source	0.04140	0.04298	1.14E+00	4.13E-02
1726	194	111		F	47	31	never	no	0.00	0	0.00		0.07311	0.07729	9.58E-01	1.29E-01

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
1733	134	112	A	M	83	25	never	no	0.00	0	0.00		0.07553	0.07902	9.53E-01	3.64E-01
1733	134	112	B	M	83	25	never	no	0.00	0	0.00		0.07883	0.08208	9.73E-01	2.67E-01
1696	82	113		F	55	31	past	yes	200.00	0	2.00	One Source	0.04263	0.04346	1.46E+00	6.47E-02
1932	142	114		M	42	28	past	no	0.00	0	0.00		0.04582	0.0486	8.56E-01	5.78E-02
2294	12	115		M	42	27	past	yes	200.00	1	1.00	One Source	0.04681	0.04936	1.16E+00	1.35E-01
1807	123	116	A	M	60	34	past	no	0.00	0	0.00		0.07370	0.07816	9.40E-01	2.50E-01
1807	123	116	B	M	60	34	past	no	0.00	0	0.00		0.07651	0.08121	9.80E-01	2.20E-01
634	132	117		M	66	29	never	no	0.00	0	0.00		0.04944	0.05179	8.67E-01	5.98E-02
2406	92	118		M	68	30	never	yes	200.00	0	3.00	One Source	0.04388	0.04605	1.23E+00	1.40E-01
621	188	119		M	51	27	never	no	0.00	0	0.00		0.05153	0.05468	1.03E+00	1.94E-01
1024	162	120		M	64	27	never	no	0.00	0	0.00		0.04603	0.04869	1.06E+00	1.24E-01
2544	94	121		M	50	38	never	yes	200.00	0	1.00	One Source	0.04995	0.05239	1.29E+00	9.35E-02
680	49	122		F	43	23	never	yes	200.00	1	8.00	One Source	0.04373	0.0463	2.65E+00	5.74E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sampling (y)	Calculated Body Mass Index (kg/m^2)	Smoking	Selenium Supplementation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
2526	91	123		M	83	25	never	yes	200.00	0	10.00	One Source	0.04771	0.05011	1.52E+00	6.72E-02
1230	149	124		F	57	21	past	no	0.00	0	0.00		0.03199	0.03385	9.37E-01	9.96E-02
547	88	125		M	43	24	current	yes	200.00	2	2.00	One Source	0.03948	0.04146	1.05E+00	1.06E-01
534	106	126		F	48	27	never	no	0.00	0	0.00		0.01947	0.02071	9.57E-01	3.54E-02
CLK960103-3	156	127											0.03298	0.03474	9.19E-01	1.24E-01
1139	164	128		F	42	23	never	no	0.00	0	0.00		0.03097	0.03266	8.85E-01	3.83E-02
CLK950401-5	8	129											0.06483	0.06836	9.23E-01	1.87E-01
2690	176	130		M	69	36	never	yes	10.00	0			0.06441	0.06814	1.26E+00	2.60E-02
1554	83	131		F	43	39	never	yes	200.00	1	0.50	One Source	0.01865	0.01936	1.27E+00	3.86E-01
2585	43	132		F	58	27	never	yes	200.00	1	10.00	One Source	0.04553	0.04738	1.12E+00	1.06E-01
CLK960103-2	120	133											0.04840	0.05116	8.54E-01	5.26E-02
1068	11	134		F	30	24	never	yes	200.00	3	0.50	One Source	0.04210	0.04429	1.08E+00	5.73E-02
1607	144	135		M	58	26	past	no	0.00	0	0.00		0.04667	0.04868	9.12E-01	6.23E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
CLK950401-1	57	136											0.06844	0.07176	8.49E-01	7.14E-02
590	52	137		F	42	22	never	yes	200.00	1	1.50	One Source	0.04800	0.05071	1.16E+00	6.85E-02
1068	81	138		F	30	24	never	yes	200.00	3	0.50	One Source	0.02650	0.02802	9.96E-01	5.82E-02
CLK960103-4	118	139											0.04434	0.04695	8.60E-01	7.76E-02
1862	182	140		F	48	21	current	no	0.00	0	0.00		0.05250	0.05544	8.76E-01	4.76E-02
1557	135	141		F	74	24	past	no	0.00	0	0.00		0.04896	0.05351	1.03E+00	4.78E-02
689	4	142		M	60	31	past	yes	200.00	2	2.00	One Source	0.00576	0.00613	1.09E+00	7.07E-02
1310	107	143		M	66	26	past	no	0.00	0	0.00		0.04512	0.04699	1.13E+00	3.20E-02
1743	14	144		M	51	27	never	yes	200.00	7	2.00	One Source	0.04714	0.04891	1.09E+00	4.75E-02
1936	60	145		F	48	21	past	yes	200.00	4	2.00	One Source	0.04714	0.04942	1.16E+00	4.40E-01
1492	183	146		F	43	38	never	no	0.00	0	0.00		0.03633	0.03848	1.19E+00	4.12E-01
1932	115	147		M	42	28	past	no	0.00	0	0.00		0.04090	0.04343	7.84E-01	7.58E-02
1445	40	148		M	48	23	never	yes	200.00	0	1.50	One Source	0.02744	0.02916	1.25E+00	7.21E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
1306	37	149	A	M	66	30	never	yes	200.00	1	2.00	One Source	0.06159	0.06501	1.03E+00	1.43E-01
1306	37	149	B	M	66	30	never	yes	200.00	1	2.00	One Source	0.06210	0.06453	1.03E+00	1.32E-01
2294	47	150		M	42	27	past	yes	200.00	1	1.00	One Source	0.06247	0.06625	1.20E+00	1.31E-01
983	66	151	A	M	56	32	past	yes	200.00	1	1.00	One Source	0.06297	0.06488	9.83E-01	5.82E-02
983	66	151	B	M	56	32	past	yes	200.00	1	1.00	One Source	0.05867	0.0613	9.76E-01	3.53E-02
1726	172	152		F	47	31	never	no	0.00	0	0.00		0.05047	0.0534	9.36E-01	1.65E-01
688	89	153		M	76	22	never	no	0.00	0	0.00		0.02034	0.02131	1.17E+00	6.53E-02
2525	185	154		F	70	21	past	no	0.00	0	0.00		0.04459	0.04597	1.03E+00	7.08E-02
511	41	155		F	29	29	never	yes	200.00	2	1.00	One Source	0.03433	0.03636	1.09E+00	1.18E-01
1426	33	156		F	44	20	past	yes	20.00	3	0.50	One Source	0.00205	0.00213 (very low)	1.05E+00	7.51E-02 (<LOD)
321	128	157		M	56	30	past	no	0.00	0	0.00		0.04894	0.05116	1.05E+00	1.17E-01
CLK950401-4	21	158											0.07346	0.07783	8.78E-01	7.11E-02
1414	63	159		M	57	36	past	yes	200.00	0	0.75	One Source	0.04538	0.04757	1.67E+00	6.05E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
71	9	160		M	76	24	never	yes	200.00	0	1.50	One Source	0.05004	0.05237	1.11E+00	6.47E-02
229	165	161		F	41	20	past	no	0.00	0	0.00		0.02544	0.02625	1.03E+00	1.06E-01
1842	35	162		M	64	33	never	yes	200.00	0	6.00	One Source	0.06078	0.06351	1.51E+00	5.60E-02
2447	124	163		M	35	31	never	no	0.00	0	0.00		0.04304	0.04538	9.32E-01	3.35E-02
1850	197	164		F	58	28	never	no	0.00	0	0.00		0.04910	0.05211	9.86E-01	8.11E-02
688	39	165		F	58	22	never	yes	200.00	2	1.50	One Source	0.03771	0.03993	1.20E+00	4.06E-02
2275	159	166		F	41	26	never	no	0.00	0	0.00		0.04577	0.04833	1.02E+00	1.09E-01
1542	10	167		F	44	21	never	yes	200.00	0	2.00	One Source	0.02696	0.02813	1.03E+00	1.67E-01
1306	16	168		M	66	30	never	yes	200.00	1	2.00	One Source	0.04441	0.04668	1.03E+00	9.12E-02
1645	22	169		F	46	22	past	yes	200.00	3	2.00	One Source	0.05242	0.0567	9.68E-01	2.66E-01
2131	38	170		F	55	31	past	yes	200.00	3	3.00	One Source	0.03730	0.03891	1.07E+00	5.54E-02
1856	72	171		M	60	30	past	yes	200.00	0	1.50	One Source	0.05374	0.05668	1.05E+00	3.36E-01
71	18	172		M	76	24	never	yes	200.00	0	1.50	One Source	0.02547	0.02599	9.55E-01	9.16E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
886	166	173		M	50	38	never	no	0.00	0	0.00		0.04130	0.04302	9.61E-01	3.88E-02
2615	25	174		F	47	29	never	yes	200.00	1	4.00	One Source	0.05663	0.05957	1.09E+00	9.16E-02
2097	129	175		F	53	25	never	no	0.00	0	0.00		0.04849	0.05125	1.10E+00	1.10E-01
1876	202	176		M	38	24	never	no	0.00	0	0.00		0.04385	0.04439	7.58E-01	1.26E-01
1572	64	177		F	49	22	never	yes	200.00	0	2.00	One Source	0.04185	0.04414	1.14E+00	2.60E-02
177	6	178		F	34	28	never	yes	200.00	0	5.00	One Source	0.05169	0.055	1.18E+00	2.94E-02
2275	117	179		F	41	26	never	no	0.00	0	0.00		0.01493	0.01589	9.45E-01	1.39E-01
1531	46	180		F	35	23	past	yes	200.00	6	2.00	One Source	0.04300	0.04556	1.03E+00	8.66E-02
1693	110	181		F	29	27	never	no	0.00	0	0.00		0.04476	0.04688	1.13E+00	6.94E-02
2112	77	182		M	50	25	never	yes	200.00	4	1.00	One Source	0.05007	0.05325	1.06E+00	7.00E-02
2690	157	183		M	69	36	never	yes	10.00	0			0.04028	0.04285	1.09E+00	3.26E-01
1450	53	184		F	35	21	never	yes	200.00	0	2.00	One Source	0.04239	0.0437	1.04E+00	1.20E-01
1861	69	185		F	48	32	never	yes	200.00	1	0.50	One Source	0.04747	0.05001	9.71E-01	4.71E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
302	171	186		F	69	28	never	no	0.00	0	0.00		0.00568	0.00602	1.03E+00	1.42E+00
924	2	187		F	69	27	never	yes	200.00	0	1.00	One Source	0.07051	0.07471	1.13E+00	7.67E-02
682	139	188		F	34	27	never	no	0.00	0	0.00		0.01823	0.01929	8.53E-01	4.31E-02
2696	175	189		M	54	28	never	no	0.00	0	0.00		0.03144	0.03241	1.03E+00	4.00E-02
1087	23	190		F	70	22	past	yes	200.00	4	1.00	One Source	0.00653	0.00681	1.06E+00	1.71E-01
1109	181	191		F	74	27	never	no	0.00	0	0.00		0.00833	0.00878	8.42E-01	7.56E-02
919	179	192	A	M	70	28	past	no	0.00	0	0.00		0.06407	0.06213	8.96E-01	4.23E-02
919	179	192	B	M	70	28	past	no	0.00	0	0.00		0.06345	0.06539	8.31E-01	5.35E-02
1696	76	193		F	55	31	past	yes	200.00	0	2.00	One Source	0.01896	0.02013	1.49E+00	6.44E-02
2686	3	194		F	48	27	never	yes	200.00	1	3.00	One Source	0.05342	0.05654	1.10E+00	3.86E-01
1236	54	195		F	53	28	never	yes	200.00	0	3.00	One Source	0.05427	0.05743	1.19E+00	3.95E-02
2526	68	196		M	83	25	never	yes	200.00	0	10.00	One Source	0.03135	0.03247	1.98E+00	7.75E-02
1139	192	197		F	42	23	never	no	0.00	0	0.00		0.04790	0.05062	9.24E-01	2.91E-02

ID Information				Cohort Data									Analysis Data and Results			
Proj 1892 or other ID	Blind 1 ID	OSC ID	Split (A/B)	Sex (M/F)	Age at sam-pling (y)	Calcu-lated Body Mass Index (kg/m^2)	Smoking	Selenium Supple-mentation	µg Se / day	Days per week forget to take supp.	Years taking supp.	Supp. Brand	Se Analysis Mass (g)	Mn Analysis Mass (Transfer) (g)	Mass-bias corrected Se (ppm)	Mn (ppm)
340	98	198		F	57	18	past	yes	0.00	0	30.00	One Source	0.04303	0.0457	1.37E+00	1.92E-02
2112	80	199		M	50	25	never	yes	200.00	4	1.00	One Source	0.06912	0.0721	1.10E+00	5.95E-02
1121	204	200		F	58	22	never	no	0.00	0	0.00		0.04300	0.04404	7.66E-01	7.98E-02
1572	101	201		F	49	22	never	yes	200.00	0	2.00	One Source	0.03651	0.03851	1.19E+00	4.39E-02
2255	195	202	A	M	64	23	never	no	0.00	0	0.00		0.06634	0.07034	1.40E+00	1.12E-01
2255	195	202	B	M	64	23	never	no	0.00	0	0.00		0.05729	0.05915	1.35E+00	6.53E-02
2407	61	203		F	67	30	never	yes	200.00	1	3.00	One Source	0.02259	0.02375	1.20E+00	6.76E-01
CLK950701-4	95	204											0.05013	0.05273	9.08E-01	2.06E-01
1109	151	205		F	74	27	never	no	0.00	0	0.00		0.04174	0.04351	1.01E+00	6.10E-02
924	79	206		F	69	27	never	yes	200.00	0	1.00	One Source	0.05544	0.0589	1.12E+00	7.68E-02
1607	190	207		M	58	26	past	no	0.00	0	0.00		0.06985	0.07434	1.04E+00	1.98E-01
2220	205	208		F	30	24	never	no	0.00	0	0.00		0.04251	0.04512	1.09E+00	8.70E-02
97	50	209		F	48	20	current	yes	200.00	0	1.00	One Source	0.01281	0.01349	1.02E+00	7.80E-02

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