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**13. ABSTRACT** *(Maximum 200 Words)*

This four-year study is a very cost-efficient and timely longitudinal study of bone turnover markers in an ethnically diverse sample of mid-aged women as they experience the menopause transition. Building on the multisite Study of Women's Health Across the Nation (SWAN), already funded by the National Institutes of Aging and Nursing Research at the National Institutes of Health, this study proposes to analyze already collected and stored specimens of serum to measure bone formation (using an immunoradiometric assay of osteocalcin) and stored urine specimens to measure bone resorption (using urinary N-telopeptide of type I collagen). These two measures will be combined with data from SWAN on bone density (spine, hip and total body), ovarian aging (endogenous sex hormones and menstrual bleeding), medications, medical history, social and psychological assessments, and life style factors (exercise, diet, smoking, body mass) to address four research aims. For each of these aims, specific hypotheses will be investigated using data collected at up to four annual visits as well as menstrual bleeding data collected annually from monthly calendars kept by the subjects. To date, major progress on the study includes completion of bone marker assays for two of the three planned study visits, specification of priority manuscripts and conduct of preliminary analyses of the relation of bone markers and bone density to ethnicity, menopause status, and the correlation of bone density to bone turnover markers.

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Sonja M. McKinlay 11/17/99

PI - Signature Date

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## I. Introduction

### *A. Background*

In the last few years, there have been a number of studies examining the potential utility of biochemical markers of bone turnover in humans. We will review major papers published in the last 3 years, since the submission of our original contract application to the Department of Defense (DOD).

1. Use of bone turnover markers to predict changes in bone density in women not receiving anti-resorptive therapy.

A major aim of our contract with the Department of Defense was to determine if baseline measurements of bone formation, serum osteocalcin (OC), and bone resorption, urinary excretion of N-telopeptide (NTx), markers could predict subsequent changes in bone mineral density (BMD) in pre- and early perimenopausal women of multiple ethnicity who are participating the The Study of Women's Health Across the Nation (SWAN).

The ability of bone turnover markers to predict changes in bone density in women not receiving anti-resorptive therapy has been examined in several recent studies. In 226 early postmenopausal women (mean age 51 years) who were randomly assigned to receive either calcium supplementation alone (n=118) or calcium plus hormone replacement therapy (HRT) (n=108), bone loss was greatest in women with the highest urinary NTx and serum OC levels. These are the markers being assessed in the SWAN cohort (1,2). Urinary deoxypyridinoline (DPD) and serum bone-specific alkaline phosphatase (BSAP) levels did not predict changes in BMD in calcium-treated women (1,2). Similarly, in the OFELY cohort, a population based study of 305 postmenopausal women aged 50-88, baseline levels of a variety of bone formation markers [serum OC and type I collagen N-terminal propeptide, (PINP)] and bone resorption markers [(urinary N-telopeptide (NTx), urinary C-telopeptide (CTx), and serum CTx] were associated with rates of forearm bone loss (3). In contrast to the study by Rosen et al.(1), McClung et al. (4) found no relation between baseline levels of serum OC or urinary NTx and rates of bone loss from the hip or spine in 458 early postmenopausal women (age 45-59 years) (4). Similarly, Keen et al found no relation between spine or hip BMD (measured by dual-photon absorptiometry) and multiple measures of bone turnover (using older assay methods) in 141 early postmenopausal women over a 4 year period (5). In women in the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial who received placebo, baseline individual bone turnover markers explained less than 5% of the variability in the change in BMD (6). Compared to SWAN, which has over 2,200 women, these studies examined a relatively small number of women. In addition, prior studies have not examined women of varying ethnicity. Moreover, all women were postmenopausal so that these studies have not addressed the question of the predictive value of bone turnover in pre- or early postmenopausal women. Finally, the conflicting data from prior studies underscore the need for a large definitive study like ours to determine whether bone turnover markers predict changes in bone density in women. These important issues are being addressed in the SWAN cohort.

The ability of biochemical markers of bone turnover to predict bone loss from the hip in elderly white women not receiving hormone replacement therapy (HRT) has also been examined recently. In a group of 295 women, followed for an average of 3.8 years, all of the measured resorption markers (urinary NTx, DPD, pyridinoline, and CTx) predicted bone loss from the total hip (but not from the femoral neck) (7). Serum OC, but not bone-specific alkaline phosphatase (BSAP), also predicted bone loss from the

total hip. The sensitivity, specificity, and predictive values of each marker were quite low, however, so that the authors concluded that the tests have limited value in individual subjects (7).

2. Use of bone turnover markers to predict changes in bone density in women receiving anti-resorptive therapy.

Several recent studies have examined the ability of bone turnover markers to predict changes in bone density in women who are receiving anti-resorptive therapy with estrogen, raloxifene, or a bisphosphonate. In one study of early postmenopausal women, baseline urinary NTx excretion (but not DPD) and the change in NTx during HRT were related to change in spine BMD (1,2). In that study, a 30% decrease in urinary NTx after 6 months of HRT predicted a 2.2% increase in spine BMD at 1 year and women with the highest baseline levels of NTx, OC, and BSAP levels had the greatest increases in bone density during HRT (1,2). Of the various bone turnover markers, change in NTx at 6 months was the best predictor of the response to HRT (1). In contrast, a smaller study failed to find any significant relation between changes in bone turnover markers (free pyridinoline cross links, OC, urinary hydroxyproline, or alkaline phosphatase) and changes in spine BMD in women receiving transdermal estrogen, even though whole body retention of 99mTc-methylene diphosphonate did predict changes in BMD (8). Individual bone turnover markers explained very little of the variability in the change in BMD in women in the PEPI trial who received HRT (6). In early postmenopausal women treated with alendronate for 2 years, baseline serum OC and urinary NTx excretion failed to predict changes in BMD (4). Similarly, baseline serum OC, BSAP, and DPD failed to predict changes in BMD in women over age 65 treated with alendronate although baseline NTx was associated with changes in BMD in these older women (9). Changes in NTx and OC after 6 months of alendronate therapy were significantly associated with changes in spine and hip BMD in both early and late postmenopausal women (9,10). Even among those studies that report associations between bone turnover measurements and rates of subsequent bone loss, most experts feel that the associations are not strong enough to be clinically useful in individual patients.

3. Use of bone turnover markers to predict osteoporotic fracture risk in postmenopausal women.

In addition to using bone turnover markers to predict changes in BMD with or without anti-resorptive therapy, it is possible the bone turnover markers could help predict the risk of fractures independently from BMD. Cross sectional studies have reported that bone turnover (urine pyridinoline, urine CTx, and serum OC) are increased in women with histories of osteoporotic fracture (11,12). The EPIDOS study, a prospective cohort study of 7,598 healthy women over age 75, reported that increased levels of urinary CTx and free DPD (but not urinary NTx) were independent risk factors for hip fracture after adjusting for femoral neck BMD (12). In contrast, the Study of Osteoporotic Fractures (SOF), a prospective cohort study of 9,704 women over age 65, failed to find any relation between serum levels of BSAP, OC, or CTx and the risk of either hip or vertebral fracture (13). The reasons for the differences between these two studies are not known but could be related to the age of the women or the bone turnover markers that were assessed (urine markers in EPIDOS and serum markers in SOF). Changes in serum OC and BSAP and urinary CTx were directly related to the risk of vertebral fracture in women treated with raloxifene (14).

4. Ethnic and geographic variation in bone turnover.

One of the major strengths of the SWAN cohort is the ability to examine bone turnover in women of multiple ethnicity (Caucasian, African-American, Chinese, and Japanese) and in a variety of geographic regions (Boston, Ann Arbor, Pittsburgh, Los Angeles, and the San Francisco Bay area). Several recent

studies have examined bone turnover in women of differing ethnicity but none has examined a cross-section of women as diverse as in SWAN. For example, Peacock et al reported that serum OC and BSAP levels and urinary free DPD (but not NTx) excretion were lower in elderly African-American women than in Caucasian women (15). In Japanese women, serum BSAP levels were associated with changes in BMD but accounted for only about 17% of the variation in the change in BMD (16). No other ethnic groups were included in this study. One large study (n=619) investigated geographic variability in bone turnover in postmenopausal women in 10 countries on 4 continents and found that serum OC, serum BSAP, and urine CTx varied significantly by country with the lowest values in Germany and Spain and the highest values in the United States and Canada (17). In SWAN, we will be able to determine if there are differences in bone turnover in premenopausal women that are related to ethnicity or geographic regions in the US and assess whether these relationships change over time as women age and transition through the menopause.

5. Assessment of bone turnover in pre-, peri- and postmenopausal women.

Data comparing bone turnover in pre-, peri- and postmenopausal women are limited. One recent study (OFELY), however, measured bone formation markers (serum OC, BSAP and PINP) and bone resorption markers (urine NTx and CTx) in 653 randomly selected women between the aged of 35 and 89 (18). Of these women 134 were classified as premenopausal (regular menses and FSH level <16.7 IU/L ), 42 were classified as early perimenopausal (irregular menses or FSH > 16.7 IU/L, 45 were classified as Late perimenopausal (irregular menses and FSH > 16.7 IU/L), and 432 were postmenopausal (no menses for at least 12 months). Ethnicity was not specified though the women were presumably mainly Caucasian. Few significant correlations were found between bone turnover and BMD in premenopausal women though the numbers of perimenopausal women was quite small. BSAP, urine NTx, and urine CTX (but not OC or PINP) were higher in the perimenopausal than in the premenopausal women (18). These interesting findings underscore the need to perform such studies in much larger groups of women and women of multiple ethnicity as in SWAN. Bone turnover markers were recently reviewed in detail (19,20).

## *B. Aims and Hypotheses*

This four-year study is a very cost-efficient and timely longitudinal study of bone turnover markers in mid-aged women as they experience the menopause transition. Building on the multisite Study of Women Across the Nation (SWAN), already funded by the National Institutes of Aging and Nursing Research at the National Institutes of Health, this study proposes to analyze already collected and stored specimens of serum to measure bone formation (using an immunoradiometric assay of osteocalcin) and stored urine specimens to measure bone resorption (using urinary N-telopeptide of type I collagen). These two measures will be combined with data from SWAN on bone density (spine, hip and total body), ovarian aging (endogenous sex hormones and menstrual bleeding), medications, medical history, social and psychological assessments, and life style factors (exercise, diet, smoking, body mass) to address four research aims. For each of the aims, specific hypotheses will be investigated using data collected at up to four annual visits as well as menstrual bleeding data collected continually from monthly calendars kept by the subjects. The aims and hypotheses are as follows:

AIM I: To evaluate the relationships between markers of bone turnover (osteocalcin and Type I collagen N-telopeptides) and markers of ovarian aging (reproductive hormone levels, menstrual bleeding patterns, symptoms).

Hypotheses

I.1 Higher baseline levels of and/or higher rates of change in bone turnover markers will be associated

with a higher probability of transition to peri- or post-menopausal status in the 2-year study period.
I.2 Higher baseline levels of and/or higher rates of change in bone turnover markers will be associated with a higher rate of self-reported peri-menopausal symptoms.
1.3 The association of bone turnover baseline levels and/or changes over time with the probability of transition to peri- or post-menopause will vary with chronological age (for example, a 50 year old woman may have higher baseline turnover levels and lower rates of turnover change, while a 45 year old woman may have low baseline turnover levels and higher rates of turnover change associated with the same transition probabilities).
AIM II: To determine if one-time (baseline) measures of bone turnover markers or changes over time in these measures are associated with the rate of bone loss over a similar time period.
Hypotheses
II.1 Elevated levels of baseline bone turnover markers will be associated with a greater bone loss in the subsequent two-year period.
II.2 An increase in levels of bone turnover markers (measured by at least two points in a two-year period) will be more strongly associated with greater bone loss over the same period than a single, baseline measure of bone turnover.
AIM III: To assess the degree to which potential lifestyle risk factors for osteoporosis (diet, cigarette smoking, exercise, weight) modify the relationships between bone turnover and ovarian aging (Aim I above) and between bone turnover and bone density (Aim II above).
Hypotheses
III.1 Compared with non-smokers, smokers will have higher baseline levels of bone turnover markers and stronger associations between bone turnover markers and ovarian aging, and between bone turnover markers and bone loss.
III.2 Women with diets rich in phytoestrogens will have lower levels of bone turnover markers, and weaker associations between bone turnover markers and ovarian aging, and between bone turnover markers and bone loss.
III.3 Body weight will be inversely associated with bone turnover levels.
III.4 Heavier women with low levels of physical activity will have weaker associations between bone turnover markers and ovarian aging, and between bone turnover markers and bone loss.
AIM IV: To determine whether the nature or magnitude of the relationships between bone turnover and ovarian aging markers (Aim I above) and bone density (Aim II above) vary according to racial or ethnic grouping, and whether racial/ethnic differences in lifestyle factors account for any differences with respect to bone turnover markers.
Hypotheses
IV.1 Compared to Caucasians, African American women will have higher levels of bone turnover at baseline. Asian (and Mexican American) women will have baseline bone turnover levels between those for Caucasian and African American women.
IV.2 Racial/ethnic differences in lifestyle factors – such as higher smoking rates among Caucasians and African Americans, greater dietary phytoestrogens among Asians, higher weight among African Americans (and Mexican Americans) – will account for much of the hypothesized differences in baseline levels of bone turnover markers.
IV.3 There will be no detectable differences among different racial/ethnic groups with respect to change over time in bone turnover marker levels.
IV.4 The relationships between bone turnover markers and bone density, and between bone turnover markers and ovarian aging, will vary across racial/ethnic groups, but these differences will be explained in large part by racial/ethnic differences in lifestyle factors and in baseline levels of bone turnover markers.

II. Body

*A. Study Objectives*

This four-year project seeks to:

A. Measure osteocalcin (from serum) and Type I collagen N-telopeptides (from urine) using specimens collected annually at three time points from 2,250 women at five Field Sites across the U.S.; and

B. Combine these data with pertinent data being collected concurrently on the same women as part of the recently funded SWAN to address the Research Questions and Hypotheses as delineated in Section B above. The results of analyses will be appropriately presented and disseminated.

In order to accomplish the two technical objectives, the following key tasks and timelines were identified at the time of the application:

| | | |
|---|---|---|
| TECHNICAL OBJECTIVE A: | | Measurement and QA/QC of Osteocalcin and Type I collagen N-Telopeptides |
| Task 1: | Months 1-2: | Finalization of data acquisition protocol |
| Task 2: | Months 1-2: | Finalization of data forms/electronic file formatting |
| Task 3: | Month 3: | Finalization of Manual of Operations |
| Task 4: | Months 2-3: | Design/Testing and implementation of DMS |
| Task 5: | Months 4-39: | Monthly shipments of specimens to the Central Laboratory |
| Task 6: | Months 4-38: | Monthly transfer of data results from the Central Laboratory to the Coordinating Center |
| Task 7: | Months 5-39: | On-going monitoring of Laboratory performance, including site visits |
| Task 8: | Months 6-42: | Assessment of the stability of stored specimens using pooled samples |
| TECHNICAL OBJECTIVE B: | | Integration of bone turnovers with SWAN data, analyses and results dissemination |
| Task 9: | Months 15-40: | Integration of study and SWAN data into analytic data sets as baseline and follow-up annual data become available |
| Task 10: | Months 18-47: | Completion of all analyses. |
| Task 11: | Months 18-47: | Dissemination of results. |

*B. Study Progress*

Overall, the study has progressed well. The recruitment of participants into the study, particularly the ethnic minorities, occurred at a slower pace and at a greater expense to the parent study than was originally anticipated. Therefore, timelines for the technical objectives have been adjusted to accommodate these unforeseen changes.

1. Participant Recruitment

Recruitment into the cohort study was completed in December, 1997. Baseline data collection on those participants recruited toward the end of the recruitment period were completed by March, 1998. All recruitment goals for the study were met. Therefore, there are a total of 2,150 participants at the five sites currently participating in the bone densitometry and bone marker study. The first annual follow-up began in February 1997 and was completed in January 1999. The second annual follow-up began in March 1998 and completion is anticipated at the end of January 2000.

2. Completion of Tasks

Progress on Technical Objectives A and B are detailed below:

TECHNICAL OBJECTIVE A: Measurement and QA/QC of Osteocalcin and Type I collagen N-Telopeptides

Task 1: Months 1-2: Finalization of data acquisition protocol

Completion of this task was reported in the October 31, 1997 annual report

Task 2: Months 1-2: Finalization of data forms/electronic file formatting

Completion of this task was reported in the October 31, 1997 annual report

Task 3: Month 3: Finalization of Manual of Operations

Completion of this task was reported in the October 31, 1997 annual report

Task 4: Months 2-3: Design/Testing and implementation of DMS

Completion of this task was reported in the October 31, 1997 annual report

Task 5: Months 4-39: Monthly shipments of specimens to Central Laboratory

Shipments of specimens are occurring on a monthly basis. To date, all baseline and follow-up 01 have been shipped to the laboratory. As of September 30, 1999, 1829 serum samples and 1831 urine samples from follow-up 02 have been collected and sent to the laboratory. There are about 200 subjects who have not yet come in for their follow-up 02 visits, and about 100 who completed the interview but have

yet to complete the specimen collection protocol. The remaining follow-up 02 samples are shipped monthly as specimens are collected by the Field Sites.

Task 6: Months 4-38: Monthly transfer of data results from Central Laboratory to the Coordinating Center

The laboratory has completed analysis of 2167 baseline urine N-Teleopeptide, 2174 baseline serum osteocalcin, 1906 follow-up 01 urine N-Teleopeptide, and 1,909 follow-up 02 serum osteocalcin assays. Results were transferred by electronic data file monthly to the Coordinating Center. The Coordinating Center has received results for all baseline and follow-up 01 assays. The Central Laboratory at the request of the Coordinating Center will begin testing the follow-up 02 assays in December, 1999 and will transfer data results to the Coordinating Center monthly.

Task 7: Months 5-39: On-going monitoring of Laboratory performance, including site visits

The bone marker laboratory currently maintains quality control procedures that include internal and external quality assurances. The detailed SOP for the assays being run for this project and MRL's external certifications are included in the appendix. Overall, the laboratory has maintained a high degree of quality in their laboratory assays and are fulfilling the required QC activities.

Task 8: Months 6-42: Assessment of the stability of stored specimens using pooled samples

The SWAN study's Laboratory Oversight Committee is charged with regular review of the laboratory's Standard Operating Procedures and QC data. The LOC has reviewed all SOPs and QC data for assays associated with this study and have found them to meet or exceed all standards.

Task 9: Months 15-40: Integration of study and SWAN data into analytic data sets as baseline and follow-up annual data become available

Most of SWAN baseline data has been released for analyses by all SWAN investigators. Baseline bone densitometry data, along with the baseline bone marker data are scheduled for release in December 1999.

Task 10: Months 18-47: Completion of all analyses.

Preliminary results from bone density and bone turnover marker analyses are presented below.

## BONE DENSITY RESULTS

Table 1. Number of bone mineral density (BMD) measurements by visit and ethnicity

| Visit | Anatomic site | African American | Chinese | Japanese | Caucasian | Total |
|---|---|---|---|---|---|---|
| Baseline | Spine | 585 | 211 | 256 | 1051 | 2103 |
| | Femoral neck | 589 | 206 | 255 | 1053 | 2103 |
| | Total hip | 589 | 206 | 255 | 1053 | 2103 |
| | Whole body | 581 | 209 | 245 | 1046 | 2081 |
| Follow-up 01 | Spine | 441 | 204 | 234 | 950 | 1829 |
| | Femoral neck | 441 | 199 | 233 | 952 | 1825 |
| | Total hip | 441 | 199 | 233 | 952 | 1825 |
| | Whole body | 224 | 203 | 234 | 781 | 1442 |
| Follow-up 02* | Spine | 250 | 173 | 119 | 771 | 1313 |
| | Femoral neck | 249 | 168 | 119 | 776 | 1312 |
| | Total hip | 249 | 168 | 119 | 776 | 1312 |
| | Whole body | 149 | 169 | 112 | 636 | 1066 |

* Follow-up 02 will close on January 31, 2000.

Bone Mineral Density and Menopausal Status

Women were excluded from the following data analyses if they reported glucocorticoid use for at least 6 months, use of anticonvulsants or depo Provera for at least 1 year, or if they reported a history of hyperthyroidism, hypercalcemia, chronic liver disease, anorexia nervosa, or bulimia. A total of 2,081 women remained in the baseline analysis sample after exclusions. Mean (± SD) baseline bone mineral density (BMD) for each anatomic site for these women are shown in Table 2 by baseline menopausal status. No significant differences in BMD were observed between pre- and early perimenopausal women at any anatomic site. (Note: there were no differences in the percentage of women who were excluded from any of the ethnic groups).

Table 2. Baseline BMD measures (mean + SE) according to pre and perimenopausal status

| BMD SITE*# | All Women | Premenopausal | Early Perimenopausal |
|---|---|---|---|
| | n=2,081 | n=1,141 | n=947 |
| AP spine | 1.08 3 ± 0.003 | 1.085 ± 0.004 | 1.080 ± 0.004 |
| Femoral neck | 0.849 ± 0.003 | 0.850 ± 0.004 | 0.848 ± 0.004 |
| Total hip | 0.967 ± 0.003 | 0.966 ± 0.005 | 0.969 ± 0.005 |
| Whole body | 1.120 ± 0.002 | 1.120 ± 0.003 | 1.119 ± 0.003 |

*All values in $gm/cm^2$; #values not corrected for bone size

Table 3. Mean percent change in BMD from baseline to follow-up 01 by menopausal status

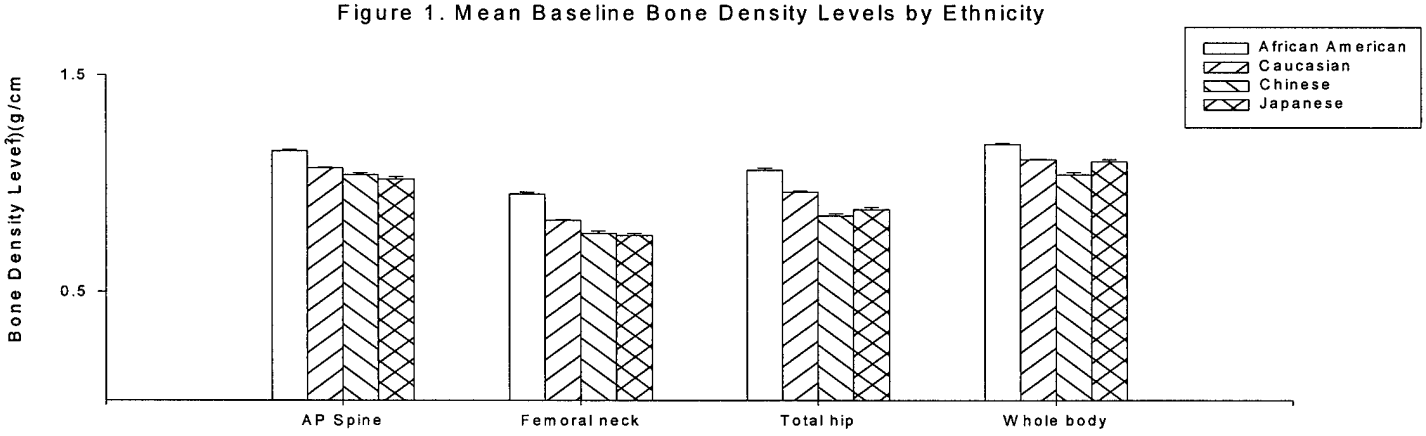
| | Percent Change | | | | | |
|---|---|---|---|---|---|---|
| | Surgical | Post | Late Peri | Early Peri | Pre | Hormone Therapy Use |
| AP Spine | -1.62 | -2.19 | -2.26 | 0.06 | 0.22 | -0.02% |
| AP Femoral Neck | -1.50 | -1.02 | -1.66 | -0.25 | -0.14 | -0.41 |
| Total Hip | -0.85 | -0.94 | -1.17 | 0.19 | 0.50 | 0.16 |
| Whole Body | -0.86 | -2.78 | -0.94 | -0.11 | 0.00 | -0.03 |

As shown in Table 3, mean bone mineral density tended to decrease from baseline to the first annual follow-up, with the highest percent change noted for women who experienced surgical menopause, or who transitioned to late perimenopause or postmenopause. For Spine BMD, the percent change was significantly ($p < 0.05$) larger for late peri and postmenopausal women than pre or early perimenopausal women, or for women who were using hormone replacement therapy (HRT) at follow-up 01. For the femoral neck and total hip sites, the percent change for premenopausal or early perimenopausal women was significantly different than for those who transitioned to late perimenopause ($p < 0.05$).

Bone Mineral Density and Ethnicity

There were large differences in baseline BMD among ethnic groups at each anatomic site ($p<0.0001$ by ANOVA at each anatomic site) (Figure 1, Table 4). Baseline BMD at each anatomic site was significantly ($p<0.0001$) higher in African-American women than in women of the other 3 ethnic groups.

Figure 1. Mean Baseline Bone Density Levels by Ethnicity




Baseline BMD was similar in Chinese and Japanese women at each anatomic site except for whole body BMD (higher in Japanese women). Baseline BMD of the spine ($p=0.015$ vs. Chinese and $p<0.0001$ vs. Japanese), femoral neck ($p<0.0001$), and total hip ($p<0.0001$) were all significantly higher in Caucasian women than in Chinese or Japanese women. Baseline whole body BMD was significantly ($p<0.0001$) higher in Caucasian than in Chinese women. Adjustment for thickness of the vertebral bodies by taking each BMD value to the 3/2 power (BMAD) (123) did not alter any of these results. Results were similar for follow-ups 01 and 02 (Table 4).

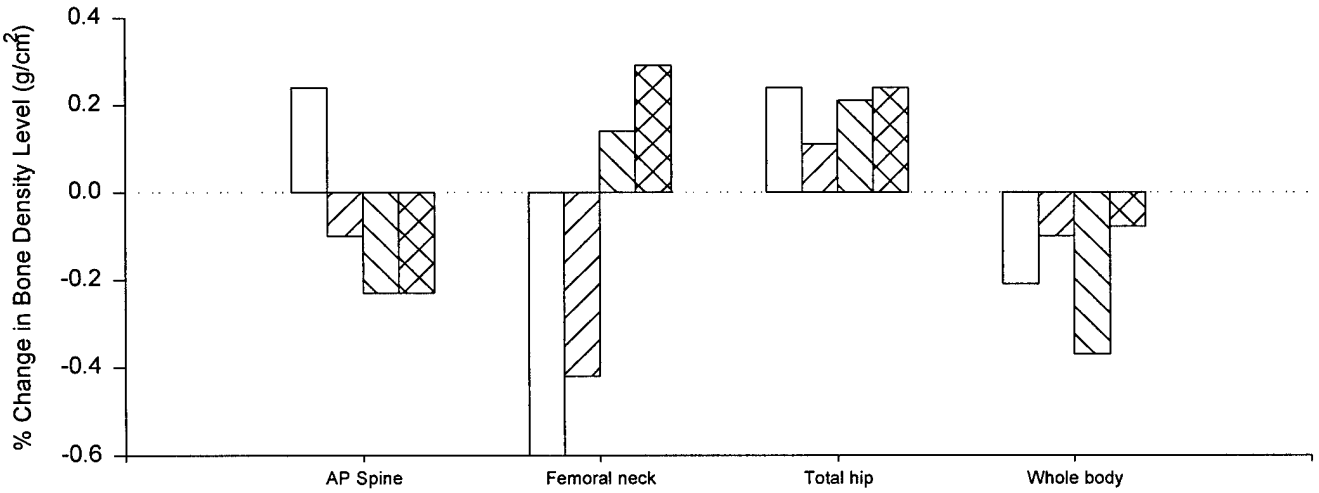
Table 4. BMD according to self-reported ethnicity in women (mean gm/cm$^2$+ SE)

| BMD SITE | African-American | Caucasian | Chinese | Japanese | p-value |
|---|---|---|---|---|---|
| **AP spine** | | | | | |
| Baseline | 1.15 ± 0.006 | 1.07 ± 0.004 | 1.04 ± 0.009 | 1.02 ± 0.007 | p < 0.0001 |
| Follow-up 1 | 1.15 ± 0.007 | 1.07 ± 0.004 | 1.03 ± 0.009 | 1.02 ± 0.008 | p < 0.0001 |
| Follow-up 2 | 1.14 ± 0.009 | 1.06 ± 0.005 | 1.02 ± 0.01 | 1.02 ± 0.01 | p < 0.0001 |
| **Femoral neck** | | | | | |
| Baseline | 0.95 ± 0.006 | 0.83 ± 0.004 | 0.77 ± 0.007 | 0.76 ± 0.006 | p < 0.0001 |
| Follow-up 1 | 0.94 ± 0.007 | 0.83 ± 0.004 | 0.77 ± 0.007 | 0.76 ± 0.006 | p < 0.0001 |
| Follow-up 2 | 0.93 ± 0.009 | 0.82 ± 0.004 | 0.76 ± 0.008 | 0.77 ± 0.01 | p < 0.0001 |
| **Total hip** | | | | | |
| Baseline | 1.06 ± 0.006 | 0.96 ± 0.004 | 0.85 ± 0.007 | 0.97 ± 0.003 | p < 0.0001 |
| Follow-up 1 | 1.06 ± 0.007 | 0.96 ± 0.004 | 0.85 ± 0.008 | 0.89 ± 0.007 | p < 0.0001 |
| Follow-up 2 | 1.05 ± 0.01 | 0.96 ± 0.004 | 0.85 ± 0.008 | 0.90 ± 0.01 | p < 0.0001 |
| **Whole body** | | | | | |
| Baseline | 1.18 ± 0.004 | 1.11 ± 0.003 | 1.04 ± 0.005 | 1.10 ± 0.007 | p < 0.0001 |
| Follow-up 1 | 1.20 ± 0.007 | 1.11 ± 0.003 | 1.03 ± 0.005 | 1.10 ± 0.006 | p < 0.0001 |
| Follow-up 2 | 1.18 ± 0.009 | 1.10 ± 0.004 | 1.03 ± 0.006 | 1.10 ± 0.003 | p < 0.0001 |

Ethnic differences in the percent change in bone mineral density from baseline to the first annual follow-up are displayed in Figure 2. African American and Caucasian women experienced a significantly lower percent change in BMD ($p < 0.05$) than did Japanese women. No significant differences in mean percent change in BMD by ethnicity were noted for any of the other anatomical bone sites.

A repeated measures linear regression model (Table 5) that included ethnicity, menopausal status (pre, early peri, late peri, post, surgical, HRT use), current smoking, number of pregnancies, age, daily

Figure 2. Mean Percent Change from Baseline to Follow-up 01 by Ethnicity



calcium intake, and body mass index confirmed that ethnicity remained a statistically significant predictor of BMD, even when controlling for these other factors. Body mass index was a major correlate of BMD at each anatomic site. Results were similar when lean body mass (by DXA) was substituted for body mass index. Adjustment for thickness of the vertebral bodies (BMAD) did not alter any the results of the multiple variable models. In this analysis, BMD for each anatomic site was lower in Chinese women and higher in African American women when compared to Caucasian women. BMD of the spine, femoral neck, and total hip was lower in Japanese than in Caucasian women.

Table 5. Repeated Measure Multivariable Regression models for BMD [β Coefficient (SE)].

| Predictor | AP Spine BMD | Femoral neck BMD | Total Hip BMD | Total Body BMD |
|---|---|---|---|---|
| Ethnicity | | | | |
| African American | 0.07 (0.01)* | 0.1 (0.01)* | 0.07 (0.01)* | 0.06 (0.01)* |
| Chinese | -0.01 (0.01)* | -0.02 (0.01)* | -0.06 (0.01)* | -0.05 (0.01)* |
| Japanese | -0.03 (0.0001)*** | -0.03 (0.0001)*** | -0.03 (0.0001)*** | 0.01 (0.0001)*** |
| Caucasian (ref) | | | | |
| Menopause status | | | | |
| Pre (ref) | | | | |
| Early peri | 0.002 (0.01)* | -0.002 (0.01)* | 0.0004 (0.01)* | -0.0001 (0.01)* |
| Late peri | -0.02 (0.01)* | -0.01 (0.01)* | -0.01 (0.01)* | -0.01 (0.01)* |
| Post | -0.02 (0.01)* | -0.01 (0.01)* | -0.02 (0.01)* | -0.03 (0.01)* |
| Surgical | -0.02 (0.0001)*** | -0.02 (0.0001)*** | -0.02 (0.0001)*** | -0.01 (0.0001)*** |
| HRT users | 0.001 (0.01)* | -0.004 (0.01)* | -0.0004 (0.01)* | -0.0004 (0.01)* |
| Current smoking | -0.003 (0.003)* | 0.001 (0.003)* | 0.004 (0.003)* | 0.01 (0.003)* |
| Pregnancies (#) | -0.002 (0.001)** | 0.001 (0.001)** | 0.0003 (0.001)** | -0.0001 (0.001)** |
| Age (yrs) | -0.001 (0.001)** | -0.003 (0.001)** | -0.0005 (0.001)** | -0.001 (0.001)** |
| $Ca^{++}$ intake (mg/d)¶ | 0.84 (0.65) | 1.89 (0.59) | 1.57 (0.60) | 1.17(0.48) |
| Body mass index ($kg/m^2$) | 0.005 (0.0004)** | 0.01 (0.0004)** | 0.01 (0.0004)** | 0.003 (0.0003)** |
| N | 3821 | 3816 | 3816 | 3434 |

*p<0.01 **p<0.001 ***p<0.0001

¶coefficient and SE have been multiplied by 100,000

## BONE TURNOVER MARKER RESULTS

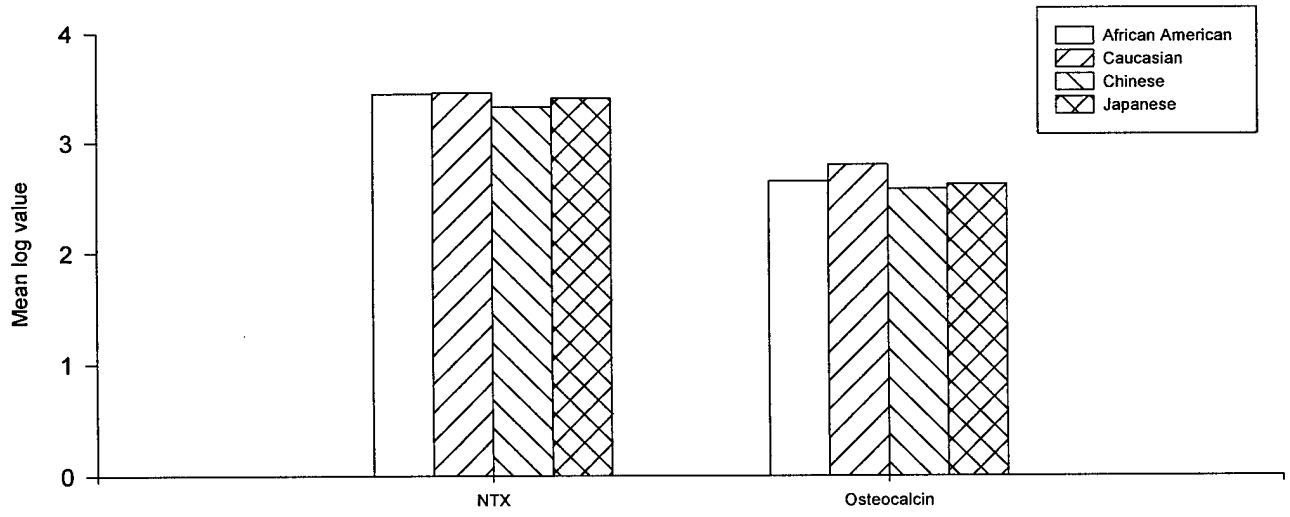
Table 6. Number of Bone Turnover Marker measurements by visit and ethnicity

| Visit | African American | Chinese | Japanese | Caucasian | Total |
|---|---|---|---|---|---|
| Baseline | | | | | |
| Urine N-Teleopeptide | 606 | 226 | 266 | 1069 | 2167 |
| Serum Osteocalcin | 601 | 228 | 267 | 1078 | 2174 |
| Follow-up 01 | | | | | |
| Urine N-Teleopeptide | 458 | 218 | 250 | 980 | 1906 |
| Serum Osteocalcin | 460 | 218 | 247 | 984 | 1909 |

Urinary N-Teleopeptide/creatinine excretion (NTx) at both baseline and follow-up 01 was significantly lower in Chinese women than in Caucasian ($p<.01$) women (Figure 3). Note that Urinary NTx and serum osteocalcin values were log transformed for analysis. Mean urinary NTx excretion was similar among African-American, Caucasian, and Japanese women. In a repeated measures linear regression model that included ethnicity, menopausal status, current smoking, number of pregnancies, age, calcium intake, and body mass index, African American, Chinese, and Japanese women had significantly lower NTx levels than Caucasian women ($p<.05$) (results not shown). Total dietary calcium and body mass index were inversely associated with NTx levels.

Mean serum osteocalcin at both baseline and follow-up 01 was significantly higher ($p<0.001$) in Caucasian women than in the other three ethnic groups (Figure 3). Mean serum osteocalcin was similar among African-American, Chinese, and Japanese women. In a repeated measures linear regression model that included ethnicity, menopausal status, current smoking, number of pregnancies, age, calcium intake, and body mass index, African American, Chinese, and Japanese women had significantly lower mean osteocalcin levels than Caucasian women ($p<.05$) (results not shown). Total dietary calcium and body mass index were inversely associated with osteocalcin levels.

Figure 3. Baseline Log NTX (adjusted for creatinine) and Log Osteocalcin by Ethnicity



## RELATING BONE DENSITY TO BONE TURNOVER MARKERS

Figures 4-11 plot bone mineral density (y-axis) against log-transformed bone turnover markers (x-axis). All plots display data from baseline and follow-up 01. At both baseline and follow-up 01, the natural logarithm of NTx/creatinine was significantly negatively correlated with spine (p=0.0001), femoral neck (p=0.0001), hip (p=0.0001), and whole body (p=0.0001) BMD. Similarly, at both visits the natural logarithm of baseline osteocalcin was significantly negatively correlated with spine (p=0.0001), femoral neck (p=0.0001), hip (p=0.0001), and whole body (p=0.0001) BMD.

Figure 4. Spine BMD by Log NTX (adjusted for creatinine)

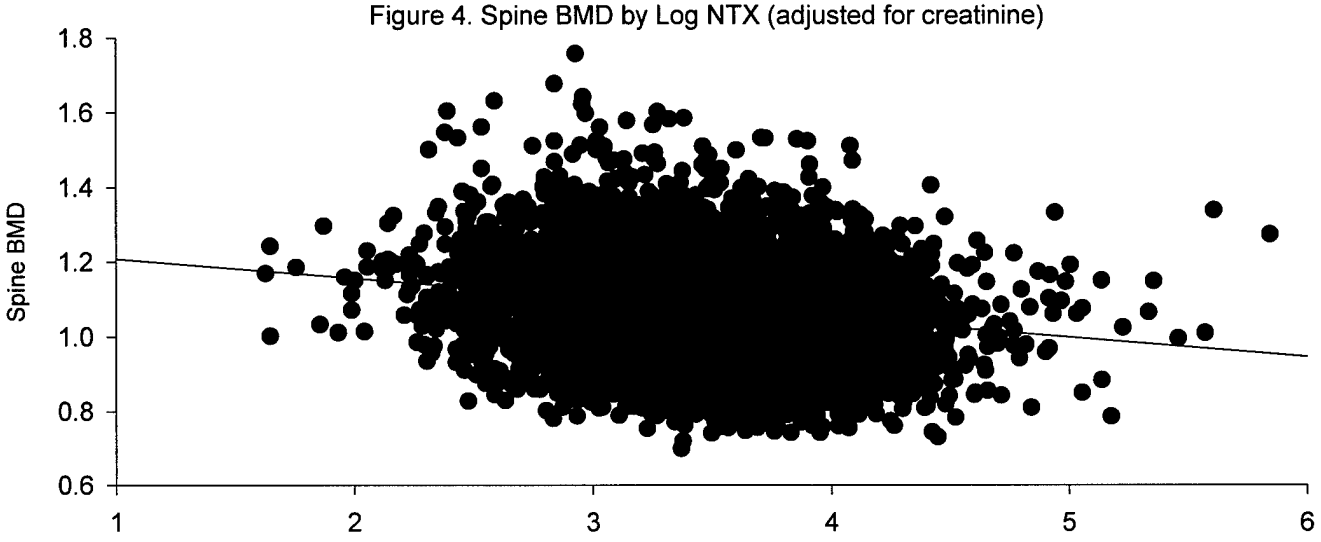



Figure 5. Hip BMD by Log NTX (adjusted for creatinine)

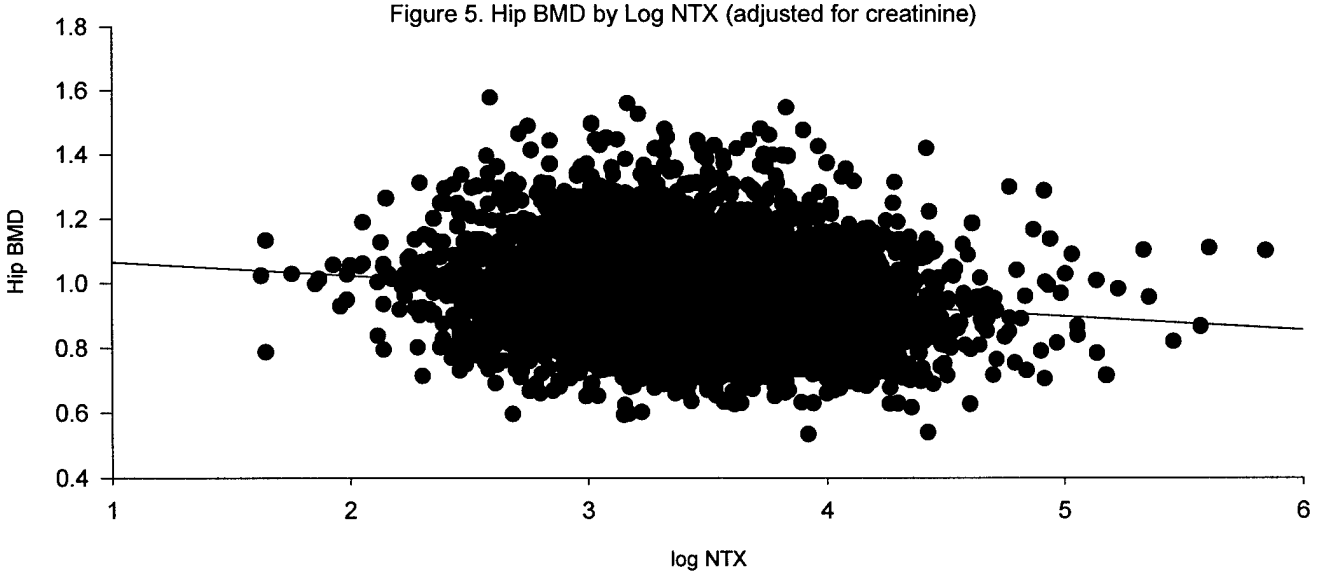


Figure 6. Femoral Neck BMD by Log NTX (adjusted for creatinine)

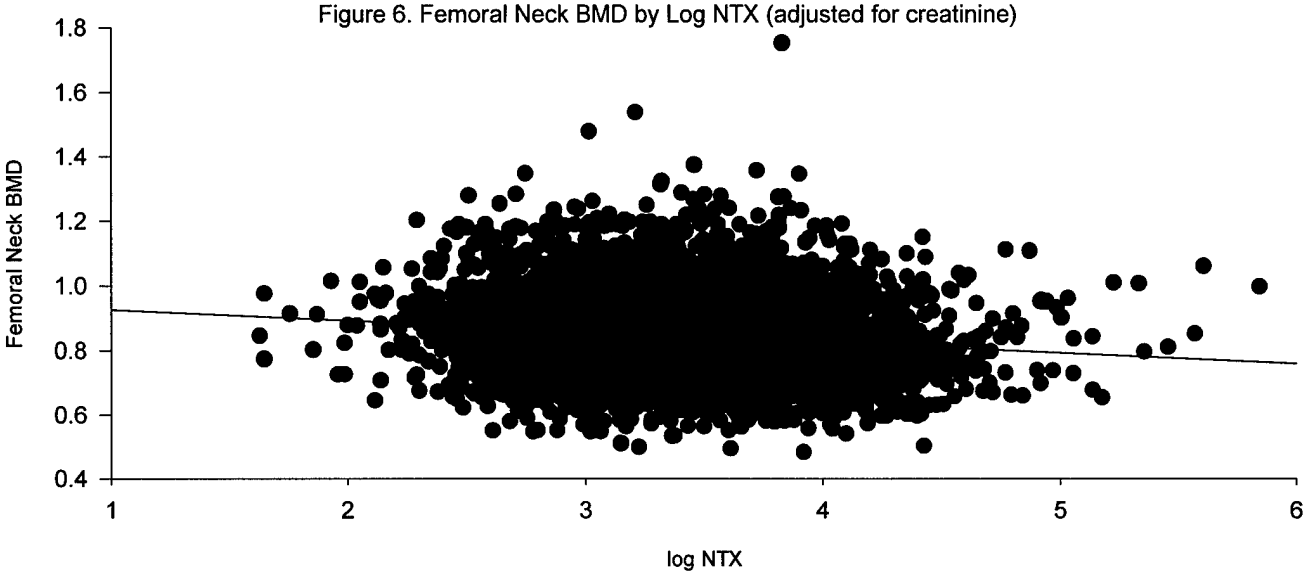



Figure 7. Whole Body BMD by Log NTX (adjusted for creatinine)

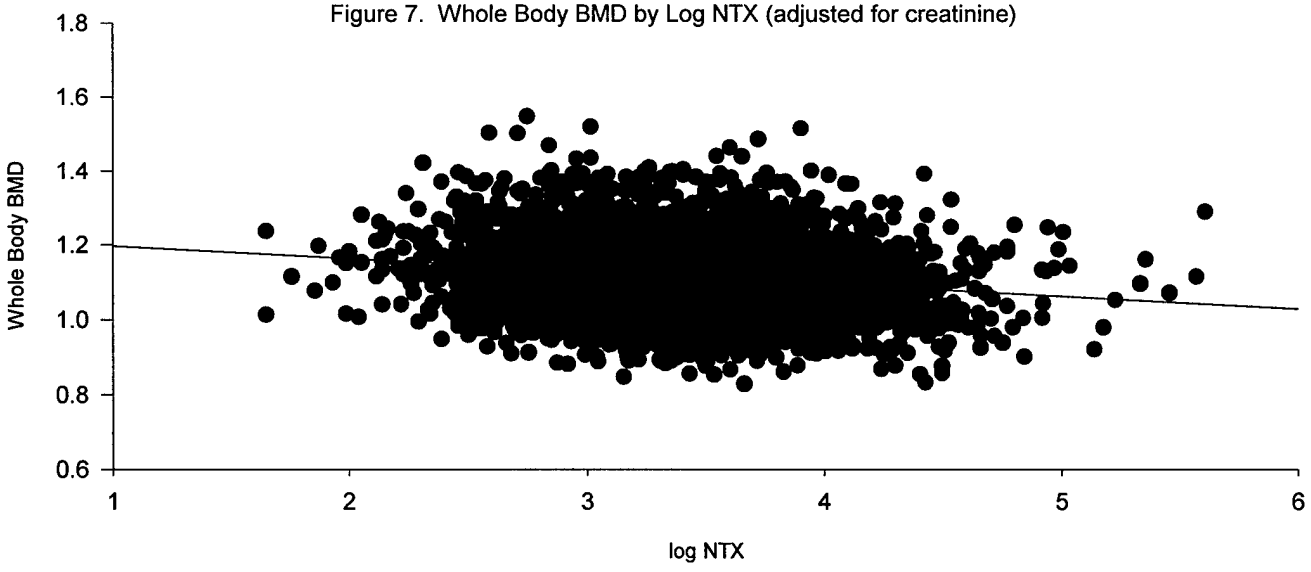


Figure 8. Spine BMD by Log Osteocalcin

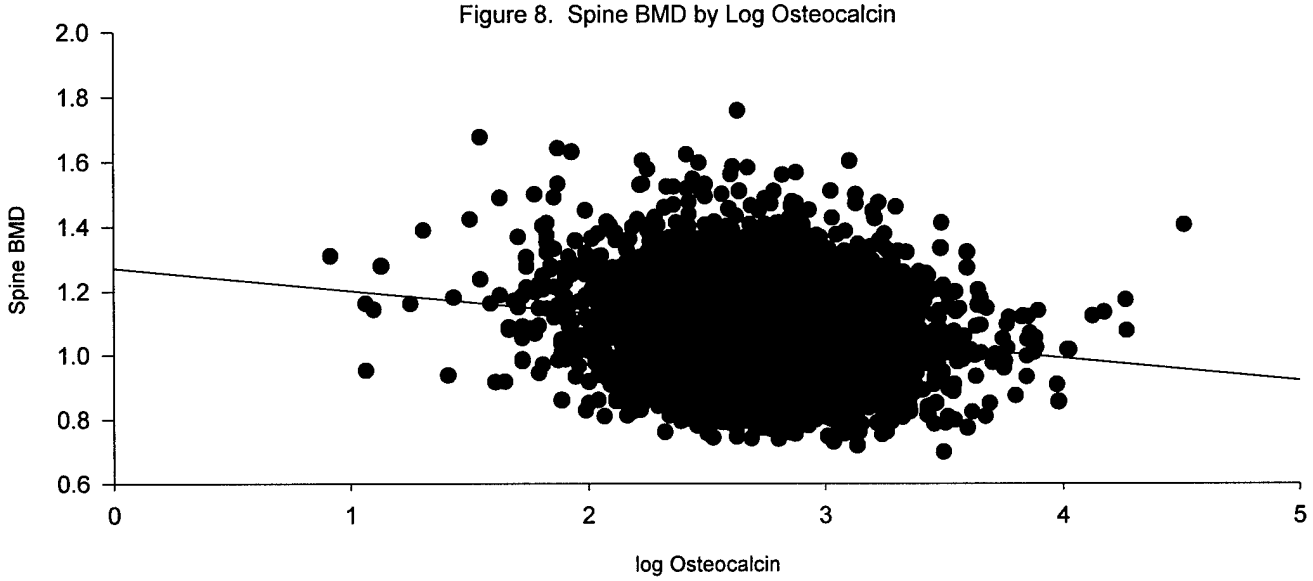



Figure 9. Hip BMD by Log Osteocalcin

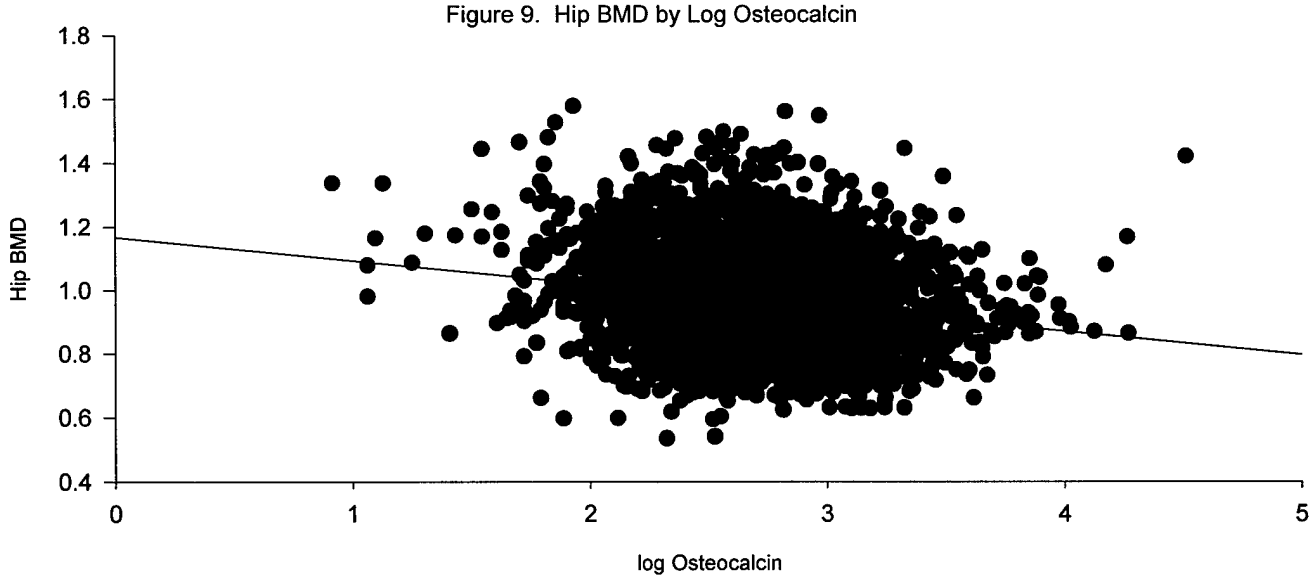


Figure 10. Femoral Neck BMD by Log Osteocalcin

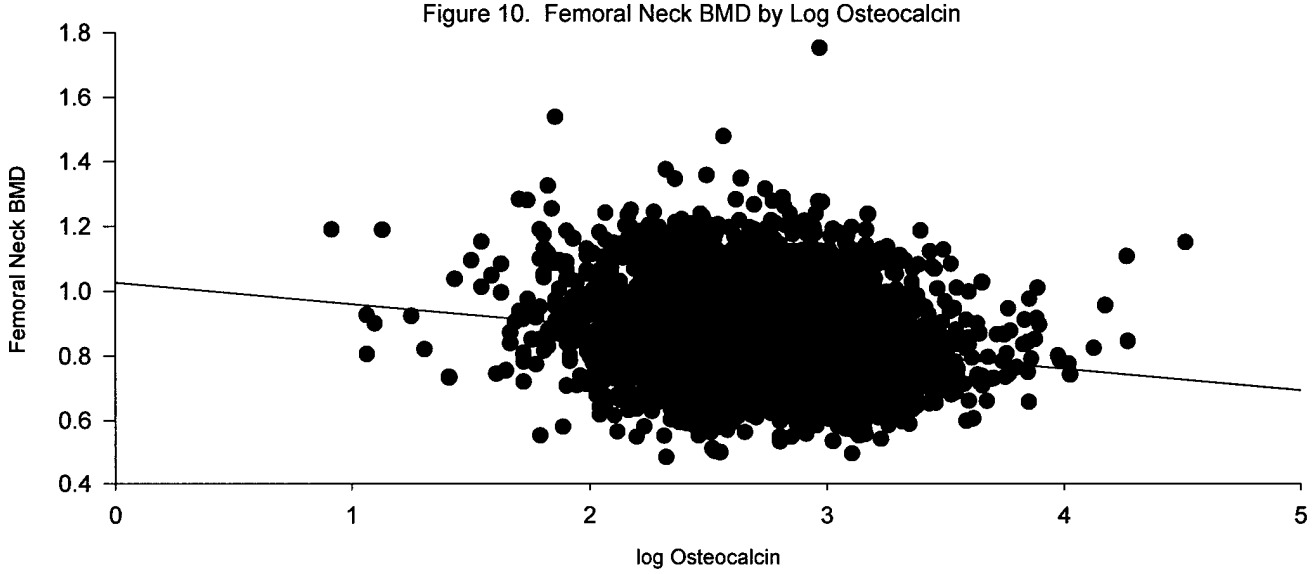
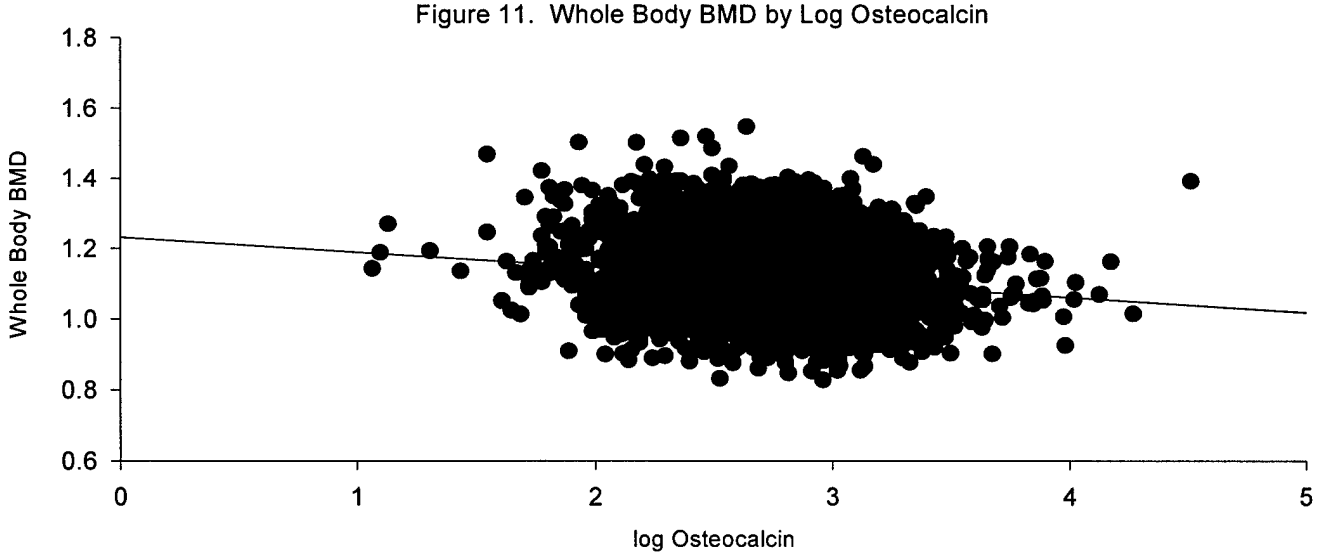



Figure 11. Whole Body BMD by Log Osteocalcin



Task 11: Months 18-47: Dissemination of results.

The following priority manuscripts have been identified that will disseminate the results of the baseline data collection. Writing groups have formed for each of these manuscripts and are now developing the analysis plans.

1. A comparison of ethnic differences in bone turnover (serum osteocalcin and urinary NTx excretion) among African American, Caucasian, Chinese and Japanese women. This paper will also include ethnic differences in bone mineral density and bone mineral apparent density.
2. Correlates of bone mineral density, bone turnover markers and menopausal status in SWAN
3. The relation of physical activity with bone mass and bone turnover in middle-aged women
4. The relation of phytoestrogen consumption with bone mineral density and bone turnover in a multi-ethnic cohort of mid-life women.
5. Correlations of bone mass with body size.

We anticipate manuscript development to continue throughout the study period and expect the first manuscripts to be submitted by the middle of 2000.

**Conclusions**

We are well on the way to completing the objectives of this project. During the next year, we will complete the remaining assays and will have longitudinal data available (from baseline, follow-up 01 and follow-up 02) to report change in bone density in relation to change in bone turnover markers. We also anticipate that during this year, the first manuscripts reporting baseline data will be submitted to professional journals and will be presented at professional meetings.

**References**

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**Appendix**

**Medical Research Laboratory SOP for Urine N-Telopeptide, Urine Creatinine, and Serum Osteocalcin and external QC certifications**

MRL
Medical Research Laboratories

Standard Operating Procedure
Procedure LAB #6000.2
Department: Laboratory
Lab Area: Miscellaneous

# TITLE: N-TELOPEPTIDE OF TYPE I COLLAGEN IN HUMAN URINE BY ELISA

Date issued: 13. August 1997
Written or Revised by: Judy Miller Manager, QA, R&D
7 Pages
Replaces Procedure: **SOP LAB #6000.1 (16. January 1996)**
Approved by: Evan Stein President

**Purpose:** To provide a standardized procedure, using NCCLS guidelines, for performing the N-telopeptide of Type I collagen assay in the laboratory.

**Contents:**





**Definitions:**

**Valid for:** All technologists trained in Special Chemistry.

**References:**

1. Osteomark™ Assay Package Insert by Ostex International, Inc., 2203 Airport Way South, Suite 301, Seattle WA 98134, 1993.

2. Jeff Morgan, Project Leader, Product Development for Ostex International, Inc., 3-5-93.

3. Daniel D. Bickford, Development Associate, Ostex International, Inc., 2-2-94.

4. Hanson DA, Weis MAE, Bollen AM, Maslan SL, Singer FR, and Eyre DR. "A Specific Immunoassay for Monitoring Human Bone Resorption: Quantitation of Type I Collagen Cross-linked N-Telopeptides in Urine" Journal of Bone and Mineral Research 7(11), 1992.

**Procedure:**

1. Principle
   1.1. Osteomark™ is an enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement o

the N-telopeptide cross-linking domain of bone collagen in human urine. The solid phase consists of microwells onto which antigen is adsorbed. Urine controls and test samples are diluted in Specimen Diluent and added to coated Microwells, along with Calibrators provided in the kit. Antibody-Horseradish Peroxidase Conjugate is then added to each well. During an initial incubation period, antigen in the sample competes with the solid-phase antigen for binding to the antibody. The wells are then washed to remove unbound material.

1.2. Buffered Substrate/Chromogen Reagent (hydrogen peroxide and 3,3',5,5' tetra-methylbenzidine) is then added to each well. During the final incubation, a blue color will develop in a reaction when bound Antibody-Horseradish Peroxidase Conjugate is present in the well. The amount of color that develops is a measure of the amount of conjugated antibody bound to the antigen adsorbed to the solid phase, and is indirectly proportional to the amount of antigen in the test sample. The reaction is stopped by addition of Stopping Reagent (1N sulfuric acid) which results in a color change from blue to yellow. The absorbance values for the Controls, Calibrators, and test samples are determined spectro-photometrically at 450 nm using 650 nm reference, by using a microplate reader.

1.3. N-telopeptide of Type I collagen in urine is used for monitoring bone resorption rates in humans. Since the cross-linked fragment of Type I collagen is excreted as a reproducible fraction of total bone-derived pyridinoline, it can act as a quantitative measure of the systemic role of bone resorption.

2. Specimen:

2.1. The determination is performed on either a 24-hour urine collection or a single "midstream" urine collection. The urine must be preservative free. Store refrigerated (2-8°C) for up to 24 hours. Store frozen (-20°C or below) for longer term storage.

2.1.1. **"Midstream" urine collection:** Patients should be instructed to collect a midstream sample, discarding approximately the first one-half of the urine voided, collecting the sample, and allowing the remainder to pass into the toilet.

2.2. Allow no more than 5 freeze/thaw cycles of the specimen.

2.3. Frozen specimens which are being assayed should be warmed to 37°C in a waterbath for 30 minutes and rotated to mix for 10 minutes.

3. Reagents:

3.1. All reagents are supplied in the Osteomark™ Assay Kit by Ostex (Catalog #[illegible]).

3.1.1. Antigen coated 96-well plate (each plate contains 12 removable strips)

3.1.2. Antibody Conjugate Diluent

3.1.3. Antibody Conjugate Concentrate

3.2. A working conjugate solution is prepared by diluting the Antibody Conjugate Concentrate 1:101 in Antibody Conjugate Diluent. Prepare approximately 2 mL of working conjugate for each strip tested. If an entire plate is tested, dilute 240 µL of Antibody Conjugate Concentrate into 24 mL of Antibody Conjugate Diluent. Use within one hour of preparation.

3.3. Calibrators of 1, 30, 100, 300, 1000, and 3000 pMoles BCE/mL. Ready to use.

3.4. Buffered Substrate

3.5. Chromogen Reagent

3.5.1. A Chromogen/Buffered Substrate is prepared by making a 1:101 dilution of Chromogen Reagent into buffered substrate using the volumes in the table below.

| Total Number of Strips | Chromogen Reagent (μL) | Buffered Substrate (mL) |
|---|---|---|
| 2- 4 | 80 | 8 |
| 5- 8 | 160 | 16 |
| 9-12 | 240 | 24 |

3.5.2. Invert gently to mix. **DO NOT VORTEX.**

3.6. 30X Wash Concentrate

3.6.1. Dilute 1:30 in deionized water. Mix well.

3.7. Stopping Reagent

3.7.1. 1N sulfuric acid. Ready to use.

3.8. Control Levels NTX Lo, Med, High Internal/Frozen urine pools.

3.8.1. Ready to use.

3.9. Plate Sealers

3.9.1. A cellophane cover for incubation.

3.10. Reagent Storage and Stability:

3.10.1. **Storage conditions:** Store all kit reagents, except diluted wash solution, at 2-8°C. Diluted wash solution may be stored at room temperature for up to one month. Return all reagents to the storage conditions indicated.

4. Supplies

4.1. COSTAR Cluster Tubes. Catalog Number 4410.

4.1.1. 2 mL polypropylene tubes arranged in the same format as the microtitre plate.

4.2. COSTAR Pipette Tips. Catalog Number 4865.

4.2.1. Long pipette tips to facilitate pipetting out of the cluster tubes.

4.3. COSTAR Cluster Tube 8-Cap Strip. Catalog Number 4418.

4.3.1. Caps for cluster tubes after use.

4.4. 8-Channel Multipipettor, 5-50 μL range.

4.5. 8-Channel Multipipettor, 50-200 μL range.

5. Equipment

5.1. Vortex mixer

5.2. Multi-Channel Plate Washer

5.3. Behring ELISA Processor

6. Quality Control

6.1. Three levels of osteomark QC pools made from donor urine are included with each run.

6.2. Control values must have a response coefficient of variation of less than 8%.

6.3. If any level of QC is outside of established limits, the entire run is repeated. QC limits are as follows:

6.3.1. $1_{2S}$: Reject run if 1 of 3 levels of QC is greater than 3 SD from the mean.

6.3.2. $(2 \text{ of } 3)_{2S}$: Reject run if 2 of 3 levels of QC is greater than 2 SD from the mean.

6.3.3. $R_{4S}$: Reject run if 2 of 3 levels of QC exceed opposite ends of mean by 3 SD (e.g., 1=> +2 SD, 1 => -2 SD). Quality control is plotted daily on a Levy-Jennings graph.

7. Procedure:

7.1. Remove all reagents from the refrigerator. The Chromogen Reagent contains dimethylsulfoxide (DMSO) and will solidify upon refrigerated storage. ALLOW CHROMOGEN REAGENT AND BUFFERED

SUBSTRATE TO COME TO ROOM TEMPERATURE BEFORE BEGINNING STEP 2 INCUBATION. Thaw frozen specimens at room temperature. Gently vortex Calibrators, Controls, and specimens. Avoid foaming.

7.2. Aliquot standards, controls, and patients into appropriately labeled cluster tubes.

7.3. Remove appropriate number of microtitre strips from the sealed pouch. Reseal along the zipper.

7.4. Write sample identification on a worksheet.

7.5. Prepare working conjugate solution using instructions listed under REAGENTS.

7.6. Pipet 25 $\mu$L of standards, controls, and patients in duplicate into appropriate wells. This is best performed one row at a time using an 8-channel multipipettor and appropriately aligned cluster tubes. Use diagram in Figure 1.

**FIGURE 1.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 | 1 | NTX HI | NTX HI | | | | | | | | |
| B | 30 | 30 | | | | | | | | | | |
| C | 100 | 100 | | | | | | | | | | |
| D | 300 | 300 | | | | | | | | | | |
| E | 1000 | 1000 | | | | | | | | | | |
| F | 3000 | 3000 | | | | | | | | | | |
| G | NTX LO | NTX LO | | | | | | | | | | |
| H | NTX MED | NTX MED | | | | | | | | | | |

7.7. Gently mix the Working Conjugate Solution. Using an 8-channel multipipettor, pipet 200 $\mu$L of Working Conjugate Solution into each well. Pipet into all wells as quickly as possible to minimize the difference in time between the first wells and the last wells. Swirl plate gently to mix and cover with a plate sealer.

7.8. Incubate at room temperature (18-25°C) for 90 ± 15 minutes.

7.9. During incubation, prepare Wash Solution using instructions listed under REAGENTS.

7.10. Ten (10) minutes prior to the completion of the incubation, prepare the Chromogen/Buffered Substrate using instructions listed under REAGENTS.

7.11. At the end of the first incubation, carefully remove (pulling diagonally) and discard the Plate Sealer and aspirate the well contents. Wash FIVE (5) times with diluted Wash Solution as directed in the Procedural Notes. Make sure that undersides of the strips are dry. If not, wipe them gently with a tissue. Aspirate residual fluid from the wells and blot dry. Immediately add Chromogen/Buffered Substrate as described in Step 12.

7.12. Pipet 200 $\mu$L of Chromogen/Buffered Substrate into each well using an 8-channel multipipettor. Cover with a new plate sealer.

7.13. Incubate at room temperature for 13 ± 1 minute. A blue color will develop in wells containing bound Antibody-HRP Conjugate. This incubation time may alter slightly with new lots. Monitor chromogen intensity with new lots and adjust timing if necessary.

7.14. At the end of the second incubation, carefully remove and discard the Plate Sealer. Add 100 µL of Stopping Reagent to each well using an 8-channel multipipettor. The wells which have developed a blue color will turn yellow.

7.15. Read absorbance on a microplate or microstrip reader at 450 nm with a reference filter for reading at 650 nm within 30 minutes of adding Stopping Reagent.

7.16. Quantitative software with a 4-parameter curve fitting equation must be used to analyze the data.

8. **Calculations:**

8.1. Determine concentration values (picomole Bone Collagen Equivalents/mL) of specimens and controls from the calibration curve. Accurate results are best obtained using a 4-parameter curve fitting equation.

8.2. Test specimens from single urine collections may be normalized for urine dilution by urinary creatinine measurement. Final results are reported as picomoles BCE/umole creatinine.

$$\frac{pM\ BCE/mL}{creatinine\ mg/dL} \times 11.3 = pM\ BCE/uM\ creatinine$$

9. **Reporting Results:**

9.1. Results are reported in whole numbers using pM BCE/uM creatinine as units.

9.2. Patient values must have a response CV of less than 8%. If the CV is greater than 8%, the sample must be repeated.

9.3. The span of the calibrator curve (absorbance difference between 1 pM BCE/mL and 3000 pM BCE/mL calibrators) should be ≥ 1.300 absorbance value.

10. **Reference Range:**

10.1 All units are pM BCE/uM creatinine.

| STUDY GROUPS | MEAN | STANDARD DEVIATION | RANGE | N |
|---|---|---|---|---|
| Children, years 0-1 | 1639 | 915 | 102-4769 | 125 |
| 2-5 | 689 | 294 | 34-1752 | 269 |
| 6-10 | 497 | 667 | 90-1356 | 321 |
| 11-15 | 429 | 215 | 34-2158 | 209 |
| 16-20 | 192 | 147 | 34-780 | 83 |
| Premenopausal females (mean age 30 years; range 25-40 years) | 36 | 10 | 10-80 | 32 |
| Postmenopausal females (< 3 years after menopause; mean age 51 years; range 42-58 years) | 82 | 33 | 28-194 | 56 |
| Males (mean age 30 years; range 24-40 years) | 39 | 22 | 14-87 | 18 |
| Males (mean age 55 years; range 45-65 years) | 38 | 13 | 14-59 | 14 |

11. Procedural Notes:

11.1. Pipetting: Calibrators, Controls, and specimens should be pipetted into the bottom of the wells. Chan disposable pipette tip after each specimen transfer to avoid cross-contamination.

11.2. Washing procedure: It is important to wash the microwell strips five times with diluted wash solutic Too few or too many washes may give less reproducible or inaccurate results. The strips may be wash with either a strip washer or a squirt bottle. Do not allow wells to dry out between reagent addition.

11.3. Automated washer: Follow the manufacturer's instructions. First aspirate the fluid from each well. Fo each wash, the wells should be filled to 400 uL with diluted wash solution. The contents should then b aspirated. Repeat until 5 washes have been performed. Blot the microwell plate on a pad of clean pape towels to remove excess wash solution.

11.4. Squirt bottle: First aspirate the fluid from each well. For each wash, grasp the long edges of the stri holder from beneath with thumb and opposing fingers while applying slight pressure and hold horizontally over a sink. Fill the wells with diluted wash solution from a squirt bottle. In one movemen invert the strip holder and empty the wells into the sink with a flick of the wrist, holding the strip holde tightly to keep the strips in place. Repeat until 5 washes have been performed. While the plate fram is still inverted, flick it again and then blot it on a pad of clean paper towels.

11.5. Working strength conjugate should be used within 1 hour of preparation. Always use a clean disposabl container for preparation of this reagent. NEVER REUSE THE CONTAINER.

11.6. Chromogen/Buffered Substrate: This should be colorless when mixed, prior to use. A blue colo indicates that the reagent has been contaminated and should be discarded.

11.7. The Antigen Coated 96-well Plate, Calibrators and Controls of this kit contain components of humar urine and human bone tissue. These components should, therefore, be handled as potentially biohazardous material.

11.8. Handle Chromogen Reagent with care, since it contains dimethylsulfoxide (DMSO), which is readily absorbed through the skin.

11.9. The Stopping Reagent is a strong acid. Wipe up spills immediately. Flush the area of the spill with water. If Stopping Reagent contacts the skin or eyes, flush thoroughly with water and seek medical attention.

12. Limitations of Procedure

12.1. This assay is sensitive down to 10 pM BCE/mL. Any patient value less than 10 pM BCE/mL should be reported as below detectable limits.

12.1.2. Any patient yielding results of <30pM should be repeated using a 50µl sample volume and results divided by 2.

12.2. This assay is linear up to 3,000 pM BCE/mL. If the specimen exhibits a value of greater than 3,000 pM BCE/mL, dilute the specimen 1: 5 in a urine specimen or pool known to be within the range of 0 - 600 pM BCE and retest. Also test the diluent urine as a patient sample (assay value must be < 300 pM) as follows:

Example: 1040 pM BCE assay value derived from a 1 : 5 dilution of a 4000pM BCE specimen using a diluent with known osteomark® value of 300 pM BCE 1040 pM BCE - (diluent sample value) = 800pM BCE

800 pM BCE x 5 (dilution factor) = 4000 pM BCE

Note: 1 : 5 dilution represents 80% diluent (0.8) and 20% specimen (0.2) solution.

# SIGNATURES

## Procedure LAB #6000.1 NE

Originator(s): Judy Miller Date: 11/17/99

_____ Date: _____

_____ Date: _____

_____ Date: _____

**Approved By:** (must be signed by either the President, Vice President of Laboratory Operations, Vice President of Client Services, Vice President of Clinical Biostatistics and Research Data Systems, or Manager of Quality Control Research and Development).

Approval: _____ Date: 11/17/99

Title: PRESIDENT

**Yearly Review:**

Reviewed By: J Miller Date: 2/3/00

Reviewed By: J Miller Date: 2/1/99

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

**MRL**
Medical Research Laboratories

Standard Operating Procedure
Procedure LAB #4032.1
Department: Laboratory
Lab Area: Special Chemistry

# TITLE: OSTEOCALCIN IN SERUM OR PLASMA BY RIA

Date issued: 28. September 1998
Written or Revised by: Jerome C. Becker Senior Medical Technologist
7 Pages
Replaces Procedure: *Lab #4032 (15. March 1997)*
Approved by: Evan Stein President

**Purpose:** To provide a standardized procedure, using NCCLS guidelines, for performing Osteocalcin in serum by RIA.

**Contents:**


**Valid for:** Special Chemistry personnel.

**References:**
1. Directional Insert, ELSA-OSTEO, Cis Bio International, France, 1995.
2. Kent, Dr. G. Neil, Ph.D., FAACB. Markers of Bone Turnover. JIFCC 1997. Vol. 9 No. 1.

**Procedure:**

1. **Principle:**
   1.1. Osteocalcin or Bone GLA Protein (BGP) is a low molecular weight protein. Synthesized by osteoblasts, it is specific to bone tissue and represents approximately 20% of non-collagenous proteins.
   1.2. ELSA-OSTEO is a solid-phase immunoradiometric assay. Two monoclonal antibodies were prepared against sterically remote sites. The first is coated on the ELSA solid phase, the second is radiolabeled with iodine 125.
   1.3. Osteocalcin molecules present in the standards or samples are sandwiched between the two antibodies. Excess unbound tracer is removed during a wash procedure, and the ELSA retains only the absorbed antibody/antigen/tracer antibody combination. The amount of radioactivity bound to the ELSA is proportional to the amount of osteocalcin present at the beginning of the assay.

2.. **Specimen:**
   2.1. The assay is performed on serum or plasma. Heparin or EDTA plasma samples may be used. Citrate plasma

samples should not be used.

2.2. Hemolyzed or hyperlipidemic samples should not be used.

2.3. Samples must be frozen at -20°C if not analyzed immediately.

3. **Reagents:**

3.1. The following are supplied in a ELSO-STEO kit.

3.1.1. External control pool from donor serum is also included on each run. This pool is kept stored at -70°C.

3.2. ELSA Tubes

3.2.1. 48 tubes contain monoclonal anti-human osteocalcin antibody coated on the ELSA. The ELSA is fixed to the bottom of the tubes.

3.2.2. Tubes are stored at 2-8°C until the expiration date. Tubes removed from packages must be stored in the zip lock bag provided in the kit.

3.3 ANTI-OSTEIO $I^{125}$

3.3.1. The 16 mL vial contains ≤ 8 $\mu$Ci of $I^{125}$ monoclonal antihuman osteocalcin antibody, buffer, animal proteins, sodium azide, red dye, non-immunized mice immunoglobulins.

3.3.2. Store at 2-8°C until expiration date.

3.4. Standard 0

3.4.1. Lyophilized buffer, and animal proteins.

3.4.2. Reconstitute the vial's contents by volumetrically adding 4 mL of deionized water.

3.4.3. Store at 2-8°C until expiration date. After reconstitution store at -20°C for one month.

3.5. Standards 1 - 4

3.5.1. Lyophilized buffer, animal proteins, human osteocalcin.

3.5.2. Reconstitute the vial's contents by volumetrically adding 0.5 mL of deionized water.

3.5.3. Store at 2-8°C until expiration date. After reconstitution store at -20°C for one month.

3.6. Control

3.6.1. Lyophilized buffer, animal proteins, human osteocalcin.

3.6.2. Reconstitute the vials contents by volumetrically adding 0.5 mL of deionized water.

3.6.3. Store at 2-8°C until expiration date. After reconstitution store at -20°C for one month.

3.7. TWEEN 20:

3.7.1. Dilute 3 mL of TWEEN 20 in one liter of deionized water.

3.7.2. Store at 2-8°C until expiration date. After dilution store in a capped container for a maximum of 15 days.

4. **Equipment and Supplies:**

4.1. MLA pipet or equivalent pipet capable of accurately dispensing 50$\mu$l.

4.2. Eppendorf repeating pipet.

4.3. Eppendorf Combitip. Two sizes needed.

4.3.1. A tip which can accurately dispense 300 $\mu$l.

4.3.2. A tip which can accurately dispense 3 ml.

4.4. Vortex

4.5. Rotating shaker capable of rotating at 175 rotations/min.

4.6. Foam decanter

4.7. Decanting blotters.

4.8. Gamma Counter with Iso-data software.

5. **Calibration:**
   5.1. Five calibrating standards are included in the kit.
   5.2. Each standard must be volumetrically reconstituted with deionized $H_20$. Let reconstituted standards sit for 15 minutes before use.
   5.3. Reconstituted standards are stable for one month at -20°C.
   5.4. Avoid repeated freeze thaw.
   5.5. The standards are set up in triplicate.
   5.6. Any triplicate that has a CV over 10% must be reviewed by a supervisor to evaluate the effect on the curve.
   5.7. The actual dose result and the calculated dose result must not differ by more than 15% for each standard.

6. **Quality Control:**
   6.1. Two quality control samples are included on each run.
   6.2. The kit control is reconstituted with 0.5 mL of deionized water. Let reconstituted control sit for 15 minutes before use.
   6.3. Reconstituted control is stable for one month at -20°C. The control pool from donor serum is thawed at room temperature prior to use.
   6.4. Avoid repeated freeze thaw.
   6.5. Control is set up in duplicate at the beginning and end of each run. If a run is longer than 20 patients, 1 level of QC should be included in the middle of run and one at the end.
   6.6. If the duplicate control tubes have a CV of over 10%, the run must be repeated.
   6.7. All QC must fall within established guidelines or the entire run is repeated..

7. **Assay Procedure:**
   7.1. All reagents must be brought to room temperature (18-25°C) at least thirty minutes before their use. Standards and control must be reconstituted 15 minutes before use.
   7.2. Label appropriate ELSA tubes in triplicate for the standards and in duplicate for the controls and samples.
   7.3. Use a MLA pipet to dispense 50$\mu$l of standards, controls, or samples into the correcsponding labeled ELSA tube.
   7.4. Add 300 $\mu$l of $I^{125}$ anti-human osteocalcin to each ELSA tube with an Eppendorf pipet.
   7.5. Vortex each tube.
   7.6. Parafilm and Incubate for 2 hours ± 5 minutes at room temperature (18-25°C) on a rotating shaker at a 175 rotations/minute. Decant prior to washing.
   7.7. Then use Eppendorf to dispense3 ml of diluted Tween 20 wash solution into each ELSA tube.
   7.8. Place tubes into a foam decanter and decant liquid.
   7.9. Repeat wash process twice for a total of 3 washes.
   7.10. Blot tubes by inverting and tapping tubes on a blotting pad.
   7.11. Measure the remaining radioactivity bound to the ELSA on a gamma counter for one minute per tube.

8. **Calculations:**
   8.1. Use Iso data reduction software to do the following:
      8.1.1. Calculate B/B0 for each tube using the 0 standard as B0.
      8.1.2. Calculate the mean of the triplicate results for each standard.
      8.1.3. Use a Linear v. Linear graph to plot dose response v. mean B/B0.
      8.1.4. Draw a weighted linear line through the mean of each standard.
      8.1.5. Calculate the dose response of each duplicate control and sample from the curve drawn.

9. **Reporting Results:**

9.1. Results are reported to one decimal point in ng/mL.
9.2. Duplicate sample tubes with CV > 10% must be repeated.
9.3. Results are reported from 0.4 ng/mL to value of high standard.
9.4. If result is greater than the highest standard, dilute with zero standard and reassay.
9.5. Reference Range:

The chart below shows the distribution of values obtained in presumably normal adult subjects.

| | Age (years) | Number of cases | Mean ng/ml | Median ng/ml | Extreme values ng/ml |
|---|---|---|---|---|---|
| Males | 20 - 30 | 48 | 23.8 | 22.0 | 11.3 - 37.0 |
| | 31 - 40 | 51 | 21.5 | 19.4 | 10.7 - 34.1 |
| | 41 - 50 | 49 | 20.3 | 19.9 | 5.2 - 34.5 |
| | 51 - 60 | 91 | 18.7 | 18.6 | 6.3 - 30.7 |
| | 61 - 70 | 60 | 19.1 | 19.2 | 8.8 - 29.7 |
| Females | 20 - 30 | 70 | 21.8 | 21.1 | 8.8 - 39.4 |
| | 31 - 40 | 87 | 17.1 | 16.2 | 7.7 - 31.9 |
| | 41 - 50 | 74 | 15.7 | 15.0 | 8.0 - 36.0 |
| | 51 - 60 | 85 | 24.4 | 22.6 | 8.0 - 50.5 |
| | 61 - 70 | 32 | 24.4 | 24.3 | 12.9 - 55.9 |

10. **Appendix**
A. Linearity
B. Interference Studies

Appendix A:

## ELSA-OSTEOCALCIN
## LINEARITY

| POOL # | POOL 1 ML | POOL 0 ML | OSTEO (NG/ML) ACTUAL | OSTEO (NG/ML) THEO | % RECOV |
|---|---|---|---|---|---|
| 1 | 0.100 | 0.000 | 222.60 | | |
| 2 | 0.100 | 0.200 | 73.38 | 74.20 | 98.9 |
| 3 | 0.050 | 0.250 | 37.32 | 37.10 | 100.6 |
| 4 | 0.050 | 0.400 | 26.14 | 24.73 | 105.7 |
| 5 | 0.050 | 0.500 | 21.44 | 20.24 | 105.9 |
| 6 | 0.025 | 0.500 | 10.69 | 10.60 | 100.8 |

POOLS 1-6 *ACTUAL OSTEO* are mean of duplicate readings.

| POOL # | POOL 1 ML | POOL 0 ML | OSTEO (NG/ML) ACTUAL | OSTEO (NG/ML) THEO | % RECOV |
|---|---|---|---|---|---|
| 7 | 0.100 | 0.000 | 18.22 | | |
| 8 | 0.200 | 0.100 | 11.95 | 12.15 | 98.4 |
| 9 | 0.100 | 0.200 | 5.84 | 6.07 | 96.2 |
| 10 | 0.050 | 0.250 | 2.57 | 3.04 | 84.5 |
| 11 | 0.025 | 0.275 | 1.41 | 1.52 | 92.8 |

POOLS 7-11 *ACTUAL OSTEO* are mean of duplicate readings.

Appendix B:

## ELSA-OSTEOCALCIN
## LIPEMIA INTERFERENCE

| POOL # | POOL 1 ML | TRIG SPIKE ML | TRIG MG/DL | OSTEO (NG/ML) ACTUAL | OSTEO (NG/ML) THEO | % RECOV |
|---|---|---|---|---|---|---|
| 1 | 0.100 | 0.000 | 99 | 16.82 | | |
| 2 | 0.275 | 0.025 | 340 | 15.68 | 16.42 | 95.5 |
| 3 | 0.250 | 0.050 | 581 | 15.34 | 16.03 | 95.7 |
| 4 | 0.200 | 0.100 | 1063 | 14.86 | 15.23 | 97.6 |
| 5 | 0.150 | 0.150 | 1545 | 14.20 | 14.44 | 98.3 |
| 6 | 0.100 | 0.200 | 2026 | 13.20 | 13.65 | 96.7 |
| 7 | 0.050 | 0.250 | 2508 | 12.14 | 12.85 | 94.5 |
| 8 | 0.025 | 0.275 | 2749 | 12.22 | 12.46 | 98.1 |
| TRIG SPIKE | 0.000 | 0.100 | 2990 | 13.26 | 12.06 | 110.0 |

*TRIG SPIKE Theo Osteo result* based on duplicate reading of airfuged sample.

POOLS 1-9 *ACTUAL OSTEO* are mean of duplicate readings.

| POOL # | POOL 1 ML | HB SPIKE ML | HB MG/DL | OSTEO (NG/ML) ACTUAL | OSTEO (NG/ML) THEO | % RECOV |
|---|---|---|---|---|---|---|
| 1 | 0.100 | 0.000 | 0.0 | 18.06 | | |
| 2 | 0.275 | 0.025 | 150.0 | 15.99 | 16.56 | 96.6 |
| 3 | 0.250 | 0.050 | 300.0 | 14.23 | 15.05 | 94.6 |
| 4 | 0.200 | 0.100 | 600.0 | 11.41 | 12.04 | 94.7 |
| 5 | 0.150 | 0.150 | 900.0 | 8.76 | 9.03 | 97.0 |
| 6 | 0.100 | 0.200 | 1200.0 | 5.72 | 6.02 | 94.9 |
| 7 | 0.050 | 0.250 | 1500.0 | 3.41 | 3.01 | 113.3 |
| 8 | 0.000 | 0.100 | 1800.0 | 0.00 | | |

POOLS 1-8 *ACTUAL OSTEO* are mean of duplicate readings.

# SIGNATURES

## Procedure LAB #4032.1

Originator(s): _____ Date: _____

_____ Date: _____

_____ Date: _____

_____ Date: _____

**Approved By:** (must be signed by either the President, Vice President of Laboratory Operations, Vice President of Client Services, Vice President of Clinical Biostatistics/Resarch Data Systems, or Manager of Quality Control Research and Development.)

Approval: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

**MRL**
Medical Research Laboratories

Standard Operating Procedure
Procedure LAB #4224.1
Department: Laboratory
Lab Area: Chemistry

TITLE: **CREATININE IN SERUM, PLASMA OR URINE - Hitachi 747-200**

| | | |
|---|---|---|
| Date issued: | 9. March 1999 | |
| Written or Revised by: | Kim Bryant | Medical Technologist |
| 6 Pages | | |
| Replaces Procedure: | *Lab #4224/TT-KD/22.Mar95* | |
| Approved by: | Evan Stein | President |

**Purpose**: To provide a standardized procedure, using NCCLS guidelines, for performing the creatinine assay on the Hitachi 747-200.

**Contents**:


**Definitions**: **NCCLS**
National Committee for Clinical Laboratory Standards

**Valid for**: All MRL technologist.

**References**:

1. Jaffe M: Ztschr Physiol Chem, 10:391, 1886.
2. Henry RJ: Clinical Chemistry Principles and Technics, Harper & Row, New York, 1969, p. 287.
3. Teger-Nilsson AC: Scand J Clin Lab Invest, 13:326, 1961.
4. Creatinine Application Sheet, Boehringer Mannheim Diagnostics, Indianapolis, IN, 1990.
5. Kaplan LA, Pesce AJ: Methods in Clinical Chemistry, C.V. Mosby Co., pp. 16, 1297-8, 1987.
6. Jacobs, David S. Laboratory Test Handbook. Lexi-Comp/Mosby, 1988, p. 101.

**Procedure:**

1. **Principle:**

1.1. In alkaline medium, creatinine forms a yellow-orange-colored complex with picric acid.

$$\text{creatinine} + \text{picric acid} \xrightarrow{\text{alkaline}} \text{creatinine-picric acid complex}$$

1.2. The color intensity is proportional to the concentration of creatinine present and is measured photometrically at 505 nm.

1.3. Creatinine measurements are used as a renal function test, providing an index of glomerular filtration.

## 2. Specimen:

2.1. Use serum (SST or plain tubes) or plasma, free of icteria. Heparin or EDTA in the usual concentrations will not interfere. Use of other common anticoagulants will interfere with the test.

2.1.1. No special patient instructions are required.

2.1.2. Creatinine is stable in serum or plasma for 24 hours at 2-8°C. For longer storage, freeze specimens at -20°C.

2.2. For a random urine, patients should be instructed to collect a midstream sample, discarding approximately the first one-half of the urine voided, collecting the sample, and allowing the remainder to pass into the toilet.

2.3. For creatinine clearance determination, one needs a precisely timed urine collection and a blood sample taken during the collection period. A 24-hour collection yields the best results.

2.3.1. To initiate the test, the patient empties his/her bladder at the beginning of the timed period. Urine is collected throughout the period, and the bladder is again emptied at the end of the timed period.

2.3.2. No special preservative is necessary for the urine collection, though the urine should be refrigerated at 4-8°C.

2.3.3. The 24-hour urine volume is measured and a 10 ml aliquot is centrifuged at 2400 g for 10 minutes. Dilute urine x 20 with 0.9% NaCl prior to analysis.

## 3. Reagents:

3.1. Reagents are obtained from Boehringer Mannheim Corporation.

3.2. **NaOH** (Catalog #1127632) (5 x 600 mL)

3.2.1. Reactive Ingredient:
0.2 mol/L Sodium hydroxide

3.3. **Picric Acid** (Catalog #1127659) (5 x 300 mL)

3.3.1. Reactive Ingredient:
25 mmol/L Picric Acid

3.4. **Precautions and Warnings:**

3.4.1. For in vitro diagnostic use.

3.4.2. Never pipette by mouth. Exercise the normal precautions required for the handling of all

laboratory reagents.

3.4.3. **WARNING. CORROSIVE.** Bottle 1 contains sodium hydroxide. In case of contact, flush affected areas with copious amounts of water. Get immediate medical attention for eyes or if ingested.

3.4.4. **DANGER. TOXIC.** Bottle 2 contains picric acid. In case of contact, flush affected areas with copious amounts of water. Get immediate medical attention for eyes or if ingested.

3.5. Reagent Preparation:

3.5.1. Preparation of Working Solutions:

3.5.1.1. For R1 Working Solution, use contents of one Bottle 1 (NaOH) as supplied. No preparation is required.

3.5.1.2. For R2 Working Solution, use contents of one Bottle 2 (Picric Acid) as supplied. No preparation is required.

3.5.2. Storage and Stability:

3.5.2.1. Store system reagents at 20-25°C. For stability of the unopened components, refer to the box or bottle labels for the expiration dates.

3.5.2.2. The opened R1 Working Solution is stable at 2-12°C for three weeks.

3.5.2.3. The opened R2 Working Solution is stable at 2-12°C until the expiration date on the bottle.

## 4. Calibration:

4.1. See Standard Operating Procedure LAB #4202.2 for instructions regarding calibration.

## 5. Quality Control:

5.1. Unassayed normal and abnormal controlled pools are run. See Standard Operating Procedure LAB #4502 for guidelines to evaluate unassayed control pools. See Standard Operating Procedure LAB #4202.2 for instructions for preparing and running quality control pools as well as for acceptance of data.

5.2. Instrumentation Laboratories Urichem Control for urine (Catalog #2934-80). Reconstitute vial volumetrically with 25 ml $XXH_2O$. Aliquot and freeze at -70°C. Stable one year at -70°C. Dilute x 20 with 0.9% NaCl prior to analysis.

5.3. Elevated urine pool. Aliquot and freeze at -70°C. Stable one year at -70°C. Dilute X20 with 0.9% NaCl prior to analysis. Run once daily.

## 6. Instrument Settings:

6.1. **4.2. Chemistry Parameters:**

| | |
|---|---|
| Temperature | : 37°C |
| TEST NAME | :[CREA] |
| ASSAY CODE | :[RATE-A] [30]-[35] |
| WAVELENGTH (SUB) | :[570] nm |
| WAVELENGTH (MAIN) | :[505] nm |

| | SERUM | URINE |
|---|---|---|
| SAMPLE VOLUME | :[10] [10] | [10] [10] |
| EXPECTED VALUE | :[0.7]-[1.4] | [ 0]-[150] |
| PANIC VALUE | :[0.4]-[1.9] | [ 0]-[150] |
| ABS LIMIT | :[4500] [INCREASE] | [4500] [INCREASE] |
| PROZONE LIMIT | :[ 0] [LOWER] | [ 0] [LOWER] |

| | |
|---|---|
| R1 VOLUME | :[250] uL |
| R2 VOLUME | :[ 50] uL |
| R1 DUMMY INTERVAL | :[ 0] |
| R2 DUMMY INTERVAL | :[ 0] |
| DILUTION VOLUME | :[ 0] |
| CALIBRATION METHOD | :[LINEAR] |
| POINTS | :[ 0] |
| SD LIMIT | :[ 0.1] |
| DUPLICATE LIMIT | :[ 10] |
| SENSITIVITY LIMIT | :[ 0] |
| STD1 ABS LEVEL | :[ 0]-[4000] |
| INSTRUMENT FACTOR | :[ 1.0] |

| STD | CONC | RACK | POS |
|---|---|---|---|
| 1(SALINE) | 0 | 1 | 1 |
| 2(PRECICAL) | * | 1 | 2 |
| 3 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 |

*ASSIGNED VALUE

7. **Calculation:**

7.1. The analyzer computer uses absorbance measurements to calculate creatinine concentration as follows:

$$Cx = K(Ax - Ab) + Cb$$

Where:

Cx = Concentration of Sample
K = Factor for determining creatinine concentration, determined by the use of the calibrator
Ax = Change in absorbance per minute of Sample + R1 + R2 from positions 30 through 35
Ab = Change in absorbance per minute of Blank + R1 + R2 from positions 30 through 35
Cb = Concentration of Reagent Blank

7.2. To calculate clearance, use the following formula:

$$\text{Clearance} = \frac{UV}{P} \times \frac{1.73}{S} = \frac{\text{urine creatinine(mg/dl)}}{\text{serum creatinine(mg/dl)}} \times \frac{\text{volume(ml)}}{\text{time(min)}} \times \frac{1.73}{S}$$

Where:

S = Calculated surface area of the patient
1.73 = surface area ($m^2$) of a standard 70 kg person

See attached nomogram for correcting body weight and height to surface area.

## 8. Reporting Results:

8.1. Results are reported to one decimal point in mg/dl.
8.2. Reference Range:
8.2.1. Serum: 0.7 - 1.4 mg/dl
8.2.2. 24-hour Urine: 1.0 - 1.8 gm/TV
8.2.3. Creatinine Clearance:
8.2.3.1. Male: 75 - 135 ml/min
8.2.3.2. Female: 65 - 125 ml/min

## 9. Procedural Notes:

9.1. The "Compensated Test" display of the PARAMETER job is used to multiply the urine result by 20. On the "Compensated Test Entry": CREAT. TEST # (URINE) = CREAT X 20.

## 10. Limitations of Procedure:

10.1. No significant interference from hemoglobin (686 mg/dl) or lipemia (1,120 mg/dl triglycerides) is observed.
10.2. Significant negative interference from bilirubin at levels > 10 mg/dl is noted.
10.3. For ketones, acetone to 50 mg/dl, acetoacetate to 20 mmol/L, and β-hydroxybutyrate to 25 mmol/L do not significantly interfere with the assay.
10.4. The assay is linear up to 15.0 mg/dl.
10.5 The assay is sensitive to 0.1 mg/dl.

# SIGNATURES

## Procedure LAB #4224.1

Originator(s): _____ Date: _____

_____ Date: _____

_____ Date: _____

_____ Date: _____

**Approved By:** (must be signed by either the President, Vice President of Lab Operations, Vice President of Client Services, Vice President of CBRDS or Laboratory Manager .)

Approval: _____ Date: _____

Title: _____

**Yearly Review:**

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Reviewed By: _____ Date: _____

Revision Reason(s):

College of American Pathologists

Accredited Laboratory

# The College of American Pathologists

*certifies that the laboratory named below*

## *Medical Research Laboratories*
## *Evan A. Stein, MD, PhD*

Laboratory Number: 31719-01

*has met all applicable standards for accreditation and is hereby fully accredited by the College of American Pathologists Laboratory Accreditation Program. Reinspection should occur within 30 days of February 15, 2001 to maintain accreditation.*

Accreditation does not automatically survive a change in director, ownership, or location and assumes that all interim requirements are met.

William B. Stanley M.D.
Chair, Commission on Laboratory Accreditation

Thomas R. Wood M.D.
President, College of American Pathologists

# CENTERS FOR DISEASE CONTROL AND PREVENTION
# NATIONAL HEART, LUNG, and BLOOD INSTITUTE
# LIPID STANDARDIZATION PROGRAM

*This certifies that*

## Medical Research Laboratories

*Highland Heights, Kentucky*

*under the direction of* Evan A. Stein, M.D., Ph.D.

*has successfully met the criteria of precision and accuracy for the measurement of*

**Hitachi-747**

| | | | | |
|---|---|---|---|---|
| *Total Cholesterol* | QTR I | QTR II | QTR III | |
| *HDL - Cholesterol* | QTR I | QTR II | QTR III | |
| *Triglyceride* | QTR I | QTR II | QTR III | |

*as Specified for Standardization by the Centers for Disease Control-National Heart, Lung and Blood Institute Lipid Standardization Program*

**1999**

Medical Director

Scientific Director

DEPARTMENT OF HEALTH & HUMAN SERVICES

# Health Care Financing Administration

**Laboratory:** MEDICAL RESEARCH LABORATORIES

**Mailing Address:** 2 TESSENEER DR
HIGHLAND HEIGHTS KY 41076-9753

**Laboratory Director:** EVAN A STEIN

**Physical Location:** 2 TESSENEER DR
HIGHLAND HEIGHTS KY 41076-9753

**CLIA ID#: 36D0346528**

**Effective Date:** February 09, 1999

**Expiration Date:** February 08, 2001

## CLIA LABORATORY CERTIFICATE OF ACCREDITATION

Pursuant to Section 353 of the Public Health Service Act (42 U.S.C. 263a) as revised by the Clinical Laboratory Improvement Amendments (CLIA), Public Law 100-578, the above named laboratory located at the address shown hereon (and other locations registered under this certificate) is hereby authorized to accept human specimens for the purposes of performing laboratory examinations.

This certificate shall be valid until the expiration date above, but is subject to revocation, suspension, limitation, or other sanctions for violation of the Act or the regulations promulgated thereunder.

David S. Cade, Director
Family and Children's Health Programs Group
Center for Medicaid and State Operations