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14. ABSTRACT The prevalence of breast cancer is greatest in industrialized regions and exposure to light at night has been proposed as a potential risk factor. Modulation of melatonin secretion by light has been implicated in the causal pathway linking exposure to light and breast cancer risk. Recent evidence indicates that melatonin is a natural oncostatic agent capable of functioning through a variety of anti-proliferative, anti-oxidative, and immunostimulatory mechanisms. We conduct a study to investigate the association of prediagnostic melatonin production and subsequent breast cancer risk in a prospective cohort study, the Italian ORDET study. Thus, prediagnostic melatonin production will be measured as urine levels of the 6-hydroxymelatonin sulphate (6-OHMS), its primary enzymatic metabolite, in 12-hour urine (overnight) collection. The study will be conducted as a nested case-control study. We expect 533 breast cancer cases among cohort members during the 17 year-follow-up period. Four controls will be matched to each case on age, menopausal status, recruitment center and time of recruitment for a total number of 2,132 control subjects. This study would be the first one analyzing the potential effect of melatonin on breast cancer risk. It will provide important data on risk factors that are likely key to the development of this disease at great public health impact.					
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INTRODUCTION

Melatonin (N-acetyl-5methoxytryptamine) is synthesized and released by the pineal gland in response to darkness. Thus, melatonin displays a strong variation during a 24-hour period: its serum levels are low during daylight hours and high at night. The health effect of chronic alteration of this circadian rhythm in humans has received relatively little attention. There is strong evidence to indicate that melatonin acts as a natural oncostatic substance (Blansk, 1993). Consistent experimental evidence, from both *in vitro* and *in vivo* studies, identified specific anti-carcinogenic functions of melatonin such as anti-proliferation, anti-oxidation, and immunostimulation functions (Brzezinski, 1997; Panzer and Viljoen, 1997; Reiter et al, 1997).

Environmental factors that reduce nocturnal exposure to melatonin may increase breast cancer risk by increasing levels of estrogens, by increasing exposure to oxidative stress and by reducing immune function (Cohen et al, 1978; Stevens, 1987). Nighttime plasma melatonin is reported to be lower in women affected with estrogen-receptor-positive breast cancer in comparison with women affected by other pathologies (Tamarkin et al, 1989). Melatonin was also lower in breast cancer cases than in women with benign breast disease (Bartsh et al, 1989). We are conducting a study to evaluate the relationship between melatonin and breast cancer using data from a prospective cohort study in which several sources of possible biomarker variability have been controlled by study design. We measure pre-diagnostic urine levels of the main melatonin metabolite, **6-OHMS**, in urine stored at -80° C during the 17 year follow-up period. At its completion, the study will allow us to investigate the role of prediagnostic melatonin as a potentially important factor underlying the association between environmental and life-style factors with breast cancer.

BODY OF REPORT

In accordance with the Statement of Work, during the third budget year, we completed the follow-up of the prospective cohort study and set the nested case-control study.

Background

The Lombardy Cancer Registry (LCR) conducts the follow-up of the ORDET cohort. LCR, established by the Regional County Council for Health and supervised by the Epidemiology Unit at the National Cancer Institute in Milan, has been operating since January 1, 1976. LCR registers and includes in the incidence figures all malignant tumors, according to the categories 140-208, chapter two of the International Classification of diseases, ninth revision (ICD-9). The ORDET study and LCR reside in the same institution at the Italian NCI in Milan (Istituto Nazionale per lo Studio e la Cura dei Tumori). The LCR searches for cases actively, using various information sources, primarily hospital clinical records and pathology department records. The Italian National Health Service (NHS) provides health assistance for all citizens. Most health care services are public. Private facilities are also partially supported by NHS. Among all breast cancer cases arisen among residents of Varese Province, only 1.3% is known to the registry on the basis of death certificates only, and 99% of breast cancer cases are microscopically verified. LCR incidence data are regularly published in the "Cancer Incidence in Five Continents" (International Agency for Cancer Research-World Health Organization, 1982-1997; 1998-2002) and in several international publications on population-based cancer survivals (Eurocare Study I, 1995; Eurocare Study II, 1999). In the context of these studies on cancer survival, the LCR collects clinical information of cancer cases. Among this information, LCR collects the receptor status of the breast cancers identified in the population. This variable is included in the data analysis of the present study.

The end of follow-up is determined by death, immigration outside Italy or last day of the follow-up: in the case of the present application the last day of the follow-up will be June 30, 2006. The latency period between cancer diagnosis and detection by the LCR is 6 months.

The present study is based on the projected incidence of cancer in the cohort by June 30th, 2006, with 533 expected breast cancer cases.

At present we are conducting the melatonin determinations for each identified case and the four related controls. Study protocols have been developed and discussed.

Methods

Breast Cancer Cases: Breast cancer cases are women with histologically confirmed invasive breast cancer diagnosed after their recruitment (date at interview) to the ORDET cohort and before the end of the last follow-up period.

Control Subjects: Eligible controls are all be women free of cancer at the time of the diagnosis of the case. For each breast cancer case, four controls are randomly chosen after matching for sources of hormone variability: a) age; b) same recruitment center to exclude differences due to transportation of samples to laboratory; c) recruitment date to control for the effect of long-term preservation of sera; d) daylight saving period to allow for possible changes in circadian rhythm.

12 Hour Urine Collection: For urine collection at baseline, each participant was asked to empty her bladder before retiring at 7:00 PM, and to collect any urine voided during the night, as well as the first morning void at 7:00 AM. Participants then delivered urine between 7:30 and 9:30 AM to the ORDET recruitment center, where it was filtered and stored at -80°C . Urine samples have not been

thawed up to now. Therefore, there will be no effects of freezing-thawing cycles and we will thaw urine for this study at the time of the proposed 6-OHMS determinations.

Analytical Methods: Melatonin production at baseline is evaluated through the urine excretion of 6-OHMS, its primary enzymatic metabolite using radioimmunoassay method (Bühlmann Laboratories AG, Switzerland). We correct concentration levels of 6-OHMS for creatinine excretion. There is evidence that total nocturnal production of melatonin is well correlated with levels of 6-OHMS in 24 hour urine samples and with morning urine samples (Markey et al, 1985 and Bojkowski et al., 1987; Cook et al., 2000). 6-OHMS shows good reliability and low intra-individual variability, at least over a short time period (Bojkowski et al., 1987), reflecting a stable rate of melatonin production in the same individual (Bojkowski et al., 1987; Arendt J, 1978). Finally, 6-OHMS is extremely stable in urine stored at -20°C and at -12°C for at least two years of cryopreservation (Bojkowski et al., 1987).

Biological specimens of all cases and matched controls are retrieved from the ORDET biological specimen bank and sent, on dry ice, to the Hormone Research Laboratory, at the Department of Preventive and Predictive Medicine of the Istituto Nazionale Tumori under the direction of Dr. Giorgio Secreto. The Laboratory is located in the same building as the ORDET specimen bank. Stored samples from cases and controls are handled identically and assayed together in the same batch. Each batch includes cases and their matched controls. Laboratory personnel are blinded to case control status of samples. In addition, we include blind control duplicates for 5% of the samples in each batch. All samples are assayed in duplicate.

At present, out of the 2132 samples (533 samples from breast cancer cases, 1599 related control subjects), 1562 have already been assayed. The analytical determinations for all the biomarkers will be completed in the next year.

KEY RESEARCH ACCOMPLISHMENTS

- Completed the follow-up of the prospective cohort study
- Set up the nested case-control study
- Began conducting the melatonin determinations for cases and controls
- Study protocols developed

REPORTABLE OUTCOMES

Publications and Presentations

We are going to publish a paper derived from the research activity developed during the evaluation of the bioassay method reliability: Barba M, Cavalleri A, Schünemann HJ, Krogh V, Micheli A, Evangelista A, Del Sette D, Fuhrman B, Berrno F, Muti P. Reliability of urinary 6-sulfatoxymelatonin as a biomarker in breast cancer. (accepted for publication, *The International Journal of Biological Markers*). See Appendix 1.

In 2005-2006, Dr. Muti has published other papers on hormones and cancer, as listed below:

- 1) *McCann SE, Kulkarni S, Trevisan M, Vito D, Nie J, Edge S, **Muti P**, Freudenheim JL* Dietary lignan intakes and risk of breast cancer by tumor estrogen receptor status. *Breast Cancer Research and Treatment* 2006 Mar 16; [Epub ahead of print]
- 2) *Cavalieri E, Chakravarti D, Guttenplan J, **Muti P**, Jankowiak R, Rogan E, Russo J, Santen R, Sutter T* Catechol Estrogen Quinones as Initiators of Breast and Other Human Cancers. Implications for Cancer Prevention and Biomarkers of Susceptibility (in press, *BBA-Reviews on Cancer*)

- 3) *Barba M, Cavalleri A, Schünemann HJ, Krogh V, Micheli A, Evangelista A, Del Sette D, Fuhrman B, Berrino F, Muti P.* Reliability of urinary 6-sulfatoxymelatonin as a biomarker in breast cancer. (accepted for publication, *The International Journal of Biological Markers*)
- 4) *Fuhrman B, Barba M, Schünemann HJ, Hurd T, Quattrin T, Cartagena R, Carruba G, Muti P.* Basal growth hormone concentrations in blood and the risk for prostate cancer: A case-control study. *Prostate* 21:219-20, 2005.
- 5) *Berrino F, Pasanisi P, Bellati C, Venturelli E, Krogh V, Mastroianni A, Berselli G, Muti P, Secreto G.* Serum Testosterone Levels and Breast Cancer Recurrence *Int J Cancer* 113(3): 499-502, 2005.
- 6) **Platek M, Krogh V, Micheli A, Schünemann H, Sieri S, Meneghini E, Pala V, Browne R, Wilding G, Freudenheim JL, Berrino F, Muti P.* Serum fructosamine and subsequent risk of breast cancer: A Prospective Study *Cancer Epidemiol Biomark Prev* 14(1): 271-4, 2005
- 7) *Bonner MR, Han D, Nie J, Rogerson P, Vena JE, Muti P, Trevisan M, Edge SB, Freudenheim JL.* Breast cancer risk and exposure in early life to polycyclic aromatic hydrocarbons using total suspended particulates as a proxy measure *Cancer Epidemiol Biomarkers Prev.* 14:53-60, 2005
- 8) *Han D, Rogerson P, Bonner MR, Nie J, Vena JE, Muti P, Trevisan M, Freudenheim JL* Assessing Spatio-Temporal Variability of Risk Surfaces using Residential History Data in a Case Control Study of Breast Cancer *Int J Health Geographics* 4(1): 9, 2005.
- 9) **Fuhrman B, Muti P, Krogh V, Pala V, Sempos C, Trevisan M, Micheli A, Chajes V, Riboli E, Sieri S, Berrino F.* Erythrocyte membrane phospholipid composition and dietary fat composition: direct relationship and metabolic effects *Ann Nutr Metab* 50:95-102, 2006
- 10) *Ochs-Balcom HM, Grant BJ, Muti P, Sempos CT, Freudenheim JL, Browne RW, Trevisan M, Iacoviello L, Cassano PA, Schünemann HJ.* Oxidative Stress and Pulmonary Function in the General Population. *Am J Epidemiol.* 162:1137-1145; 2005
- 11) **Barba M, McCann S, Nie J, Vito D, Stranges S, Fuhrmann B, Trevisan M, Muti P, Freudenheim JL* Perinatal Exposures and Breast Cancer Risk in the Western New York Exposures and Breast cancer (WEB) Study. *Cancer Causes Control.* 17(4): 395-401, 2006
- 12) *Ochs-Balcom H, Grant BJB, Muti P, Sempos CT, Freudenheim JL, Browne R, McCann SE, Trevisan M, Iacoviello L, Schünemann HJ.* Antioxidant

Vitamins and Pulmonary Function in individuals diagnosed with asthma or COPD (*in press, Eur J Clin Nutr*)

- 13) Colombo C, **Muti P**, Pala V, Cavalleri A, Venturelli E, Locardi M, Berrino F, Secreto G. Plant-based diet, serum fatty acid profile, and free radicals in postmenopausal women: the diet and androgens (DIANA) randomized trial. *Int J Biol Markers*. 20(3):169-76, 2005

She has also presented new results from other studies at the Annual Meeting of the American Association for Cancer Research (2005):

- 1) Platek M, Freudenheim JL, Quick S, Nie J, Muti P, McCann S, Trevisan M, Shields P, Edge S *Methylenetetrahydrofolate reductase (MTHFR) and risk of breast cancer: The Western New York Exposures and Breast Cancer Study (WEB Study) Annual Meeting American Association for Cancer Research, Anaheim, California, April 2005*
- 2) Teter B*, Cavalleri A, Fuhrman B, Krogh V, Barba M, Schünemann HJ, Evangelista A, Del Sette D, Micheli A, Meneghini E, Secreto G, Berrino F, Muti P *Urinary Nocturnal Excretion of 6-sulfatoxymelatonin and Breast Cancer Risk Meeting of the American Association for Cancer Research "Frontiers in Cancer Prevention Research", Baltimore, October 2005*
- 3) Barba M*, Cavalleri A, Schünemann HJ, Krogh V, Evangelista A, Secreto G, Micheli A, Qi Zhou, Fuhrman B, Reter B, Berrino F, Muti P *Effects of different storage temperatures on 6-Sulfatoxymelatonin concentration in urine Meeting of the American Association for Cancer Research "Frontiers in Cancer Prevention Research", Baltimore, October 2005*
- 4) Crespo CJ, Fuhrman B, Smit E, Freudenheim JL, Garcia Palmieri M, Lee IM, Muti P, Marrero FR. *Anthropometric Measures and Fasting Glucose as Predictors of Fatal Prostate Cancer among Puerto Rican Men: The Puerto Rico Heart Health Study Cohort Meeting of the American Association for Cancer Research "Frontiers in Cancer Prevention Research", Baltimore, October 2005*
- 5) Barba Maddalena, Terrenato Irene, Fuhrman Barbara, Teter Barbara, Schunemann Holger, Muti P. *Secondary sexual characteristics and body size at different ages in relation to risk of prostate cancer: results from a case-control study Annual Meeting American Association for Cancer Research, Washington, April 2006*
- 6) McCann SE, Muti P, Vito D, Edge SB, Trevisan M, Freudenheim JL.

Dietary lignan intakes and risk of breast cancer by tumor estrogen receptor status Annual Meeting American Association for Cancer Research, Washington, April 2006

Two of these studies have been submitted for publication. In addition, Dr. Muti has several other manuscripts submitted for publication on hormone and related factors and cancer.

CONCLUSIONS

We are continuing the hormone determinations phase for this grant. Therefore, there are no conclusions to report at this time.

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APPENDIX

Appendix 1:

Reliability of urinary 6-sulfatoxymelatonin as a biomarker in breast cancer

Running head: urinary 6-sulfatoxymelatonin in breast cancer

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Abstract

The objective of this study is to evaluate the effect of cryopreservation at different storage temperatures on urinary 6-sulfatoxymelatonin concentration. Overnight urine from 28 postmenopausal women participating in the ORDET cohort study was filtered and separated into 6 mL aliquots. Urine samples were stored at -80°C and at -30°C for an average of 14 years. Urinary 6-sulfatoxymelatonin concentration was assessed using a competitive immunoassay. Mean values of aMT6s stored at -30°C were systematically lower than those stored at -80°C (10.7 ng/ml versus 15.8 ng/ml, $p < 0.001$, respectively). Bland Altman plots showed disagreement between determinations at different storage temperatures at the highest levels of the metabolite concentration. The degree of agreement evaluated in terms of ICC was 0.68 (95% CI 0.41-0.84 $p < 0.0001$). Pearson correlation coefficient between aMT6-s values of the two differently stored samples was 0.93 ($p < 0.001$), while the Kendal tau coefficient for rank distribution was 0.73 ($p < 0.001$). Our data suggest that storage temperatures might affect degradation of aMT6-s during storage time. However, effects on

individual characterization by melatonin levels seem not to be affected by cryopreservation conditions.

key words: 6-sulfatoxymelatonin, cryopreservation, storage temperature, biomarker, breast cancer

Introduction

Melatonin is the chief secretory product of the pineal gland. Over the last few years, the body of evidence regarding the properties of melatonin in combating cancer has grown stronger. Melatonin may play a role in the initiation and progression of cancer as well as in neoplastic cell growth regulation. In vitro and in vivo studies consistently demonstrated specific anti-carcinogenic functions of melatonin, such as the anti-proliferative, anti-oxidant and immunostimulating effects (1-4).

Serum melatonin has a very short half-life and is rapidly metabolized, mainly in the liver. 6-sulfatoxymelatonin (aMT6-s), which is excreted in urine, represents the principal metabolite and biomarker of melatonin. In view of the increasing scientific interest in melatonin as a determinant of cancer risk and as anti-cancer agent, the accurate measurement of its chief metabolite in biological specimens is of great interest.

A number of studies have shown a high correlation between measurements of aMT6-s in urine and plasma (5-10). A single morning urinary melatonin measurement is a reliable marker for long-term melatonin levels (11). However, there is a lack of evidence regarding potential storage effects on levels of

melatonin. For example, differences in assay methodologies and sample handling, including the method of storage, could influence aMT6-s reported levels. The aim of our study was to evaluate the effect of cryopreservation on aMT6-s levels in urine samples stored in two different cryopreservation systems at -30° C and -80°C, using a competitive immunoassay.

Materials and Methods

Subjects

Urine samples were collected from postmenopausal women participating in a prospective study of hormones, diet and breast cancer risk, the Hormones and Diet in the Etiology of Breast Cancer Risk (ORDET) study, carried out between June 1987 and June 1992. During the study period, 10,786 healthy women were enrolled, aged 35 to 69, in northern Italy. All members of the cohort were volunteers recruited from the general population of Varese province. The total number of women recruited in the cohort represented ~7% of the general population of women. A major focus of the ORDET study was to investigate endogenous hormones and their relationship to breast cancer risk. Therefore, several sources of hormone variability were controlled for by excluding those women who were pregnant or breast-feeding, those on oral contraceptives or hormone replacement therapy, those affected by metabolic diseases, as well as those women with a previous history of cancer (12).

The study is based on 28 women initially recruited in the ORDET study and subsequently excluded because recognized by the Cancer Registry as breast

cancer cases at the time of recruitment. Subjects were postmenopausal women, reporting their last menses more than one year prior to recruitment in the ORDET study and urine collection.

Participants donated a sample of overnight urine according to a collection protocol calling for discarding the last void at 7 pm and collecting urine during the night up to 7 am. All participants reported accurate overnight urine collection. Overnight urine was kept at room temperature during collection. No preservatives were added, either at collection or during storage. Urine samples were delivered to the ORDET recruitment center the day after overnight collection, between 7:30 and 9:00 am.

At the center, samples were processed as soon as delivered. Urine was filtered and separated so that 6 mL aliquots were stored at -80°C and 6 mL at -30°C . All samples were taken out of the freezer simultaneously and sent to the laboratory in the same parcel on dry ice. The samples were stored for an average of 14 years.

Assay

In order to quantify aMT6s concentration in urine, we used an ELISA kit EK-M6S (Buhlmann Laboratories AG, Switzerland). The procedure consists of a competitive immunoassay, using a capture antibody technique. In brief: pre-diluted (1:200) urine samples, ready to use Calibrators and Controls and biotinylated aMT6s are pipetted into the appropriate wells. Biotinylated aMT6s competes with the aMT6s present in the sample for the binding sites of an anti-aMT6s-antibody to form a complex which is captured by a second antibody

coated on the wells. The addition of the Enzyme Label (streptavidin conjugated to horseradish peroxidase) and, subsequently, of the tetramethylbenzidine (TM) substrate generates a blue-coloured product the amount of which is in inverse proportion to the quantity of aMT6s originally present in the sample. Adding acidic Stop Solution turns the colour from blue to yellow. The colour intensity is measured at a wavelength of 450 nmol in a microtiter plate reader in order to plot a Standard Curve and to calculate the aMT6-s concentration.

We measured the aMT6-s concentration in samples from the same subject, stored at -30°C and -80°C, in double wells using the same kit. We analyzed all urine samples using two kits of the same lot. The intra and inter-assay variability of the aMT6-s were 14.5% and 14.4% at low concentrations and 3.7% and 6.24% at high concentrations. The inter-lot variability was 16.3% at low concentrations and 2.19% at high concentrations.

Statistical Analysis

We compared the mean and standard deviation for aMT6-s cryopreservation at -30°C and -80°C with the *t-test* for paired data.

We examined agreement between levels measured at the two different storage temperatures in two ways; that is we examined the extent to which two different storage temperatures might possibly affect aMT6s concentration in urine. First, we constructed a Bland-Altman plot that allows the investigation of the extent of agreement between the variables of interest (13). We plotted the mean of the two storage methods (X-axis) against the difference between the two methods.

Second, we calculated the intra-class correlation coefficient (ICC), to assess the relationship between two variables that have the same metric and variance. We used a 2-ways mixed model of the ICC, treating the mean of the concentrations from the two storage temperatures as a fixed variable and subjects as a random variable.

We then computed Pearson correlation coefficient (r) to assess the degree of correlation between aMT6-s concentration in urine stored at -30°C and -80°C . Furthermore, in order to evaluate the effect of cryopreservation on individual categorization, we used Kendall's tau coefficient for rank distribution.

Results

Table 1 shows aMT6s concentration at both -30°C and -80°C . Mean values of aMT6s stored at -30°C were systematically lower than those stored at -80°C (10.7 ng/mL versus 15.8 ng/mL in urine stored at -30°C versus -80°C , $p < 0.001$). The table suggests presence of an outlier (number 20), possibly deriving from a technical bias.

On the basis of the Bland Altman plots (Figure 1), the disagreement between determinations at different storage temperatures is particularly evident at the highest levels of aMT6s concentration. Levels at -80°C were higher and, on average, the differences between -30°C and -80°C correlated with the level of melatonin concentration. The degree of agreement evaluated in terms of ICC was 0.68 (95% CI 0.41-0.84 $p < 0.0001$).

Pearson correlation coefficient between aMT6-s values of the two differently stored samples was 0.93 ($p < 0.001$), while the Kendal tau coefficient for rank distribution was 0.73 ($p < 0.001$).

Conclusions

We compared the effect of different storage temperatures on aMT6-s levels and observed a significant difference between the absolute urine levels of aMT6-s in specimens stored at -30°C and -80°C . While the individual characterization of exposure to aMT6s remained evident for both storage conditions (high levels remained high and low levels remained low), storage at lower temperatures led to a decline in absolute levels. Moreover, the degree of agreement between aMT6 levels at different storage temperatures was lower at higher metabolite's concentration.

Our data support the hypothesis that storage temperatures might affect degradation of aMT6-s during storage time. Based on our results, a potential, even though not severe, misclassification bias could arise from the use of urine stored at -30° . As a consequence, differences in storage methodologies should be carefully taken into account in the interpretation and discussion of results obtained from clinical and epidemiological studies based on urinary aMT6-s concentration.

To our knowledge, this is the first study to address the issue of potential storage effects on levels of melatonin metabolites in biologic samples. Strength of this study include the use of highly standardized procedures for urine collection,

processing and cryopreservation. Furthermore, our multiple statistical approaches produced not only descriptive but also reliability and correlation data, allowing the consideration of multiple aspects of the investigated relationships.

This study has some limitations. The presence of a potential outlier in one of the samples (individual number 20) could have partly attenuated the relationship between the investigated variables.

Conclusively, in investigating melatonin properties as an anti-cancer agent, the compelling need of further scientific evidence stating the implications of different storage methodologies clearly emerges, in order to overcome some of the potential methodological limitations currently existing in study design and results interpretation.

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Table 1. aMT6 concentrations at -30° and -80°. ORDET STUDY, 1997-1992		
Sample	aMT6s (ng/mL) -30°C	aMT6s (ng/mL) -80°C
1	12.23	30.46
2	14.88	17.00
3	12.89	20.42
4	5.24	9.81
5	6.51	7.00
6	4.21	6.52
7	37.61	46.43
8	6.29	16.62
9	12.90	23.34
10	8.16	11.15
11	14.76	19.22
12	6.71	13.44
13	18.72	26.86

14	8.91	12.35
15	1.83	3.33
16	3.05	5.04
17	10.96	15.17
18	9.57	11.85
19	11.07	10.31
20	32.76	3.83
21	12.87	16.21
22	2.03	2.44
23	13.69	19.15
24	26.4	35.83
25	2.40	4.26
26	7.56	9.78
27	6.59	14.96
28	9.91	16.53

**Table 1. aMT6s concentration at -30° C and -80° C
ORDET STUDY, 1987-1992**

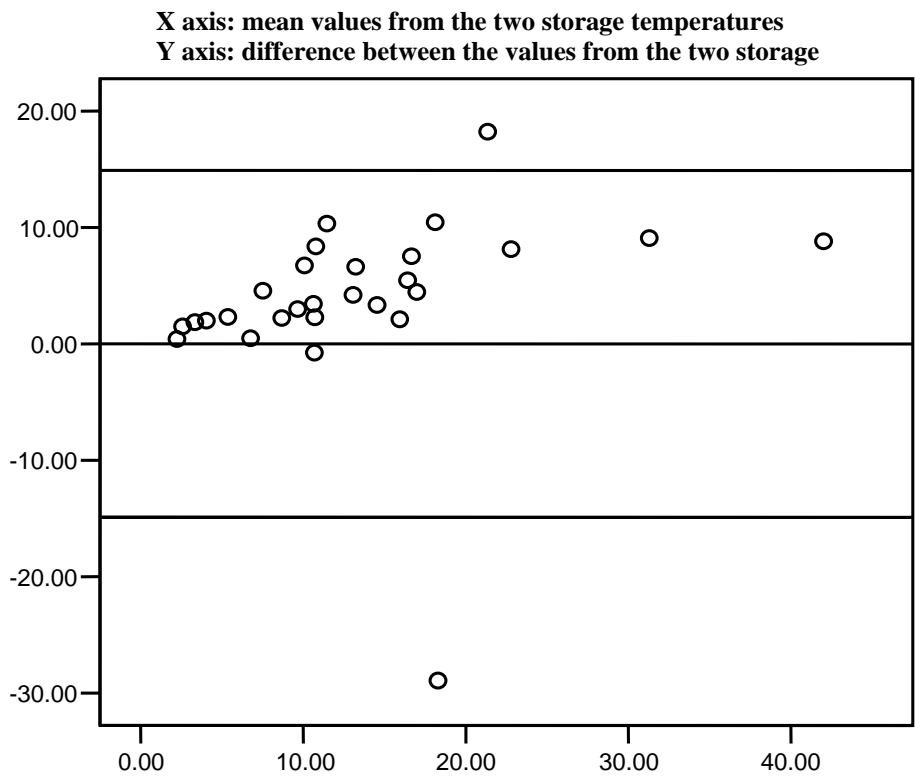


Figure 1. Bland-Altman graph comparing aMT6-s levels in urine samples stored at -30° C versus -80°C