Early Breast Cancer, Diet, and Plasma Copper Fractions

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ABSTRACT

In a study of diet and early breast cancer, blood plasma copper has been analyzed by Proton Induced X-ray Emission analysis as both total copper (P-Cu) and that ultrafiltrable from plasma (P-edu-Cu) through membranes with a cut-off at molecular mass 10,000 after equilibration with disodium ethylene diamine tetraacetic acid (EDTA) at 4°C. Ceruloplasmin (P-cer) was also measured using nephelometry of anticeruloplasmin monoclonal antibody-ceruloplasmin complexes. Dietary copper intake per day (D-Cu) was assessed over a five-day dietary record period and calculated from dietary components using a computer program. P-edu-Cu correlated significantly with both D-Cu and ceruloplasmin while P-Cu correlated only with ceruloplasmin. Further, ceruloplasmin did not significantly correlate to D-Cu. Hence, P-edu-Cu better reflects copper status than do P-cer or P-Cu as it relates to both the major copper enzyme in plasma and to daily copper intake. This may be important in drawing conclusions about the significance of copper in disease states where copper fractions other than ceruloplasmin may be most important owing, for example, to oxidative properties. Categorization as cancer or normal, by copper parameters (D-Cu, P-edu-Cu, P-Cu, P-cer), was studied in multiple correlation. In particular, the ratio P-cer/P-Cu and the ratio P-edu-Cu/D-Cu were significantly related to disease. Irrespective of age (pre- and post-menopausal), highly significant differences between normals and early stage breast cancer patients were seen with $p < 0.0001$ to $p < 0.01$. The precise role played by plasma and dietary copper fractions deserves continued attention in view of the present and earlier results in cancer studies.

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Introduction

Earlier, the role of copper was considered, measured by proton induced X-ray emission (PIXE) in early breast cancer patients as compared to pre- and postmenopausal control women on vegetarian or omnivorous diets. There was an imbalance between total plasma copper and ceruloplasmin in the cancer patients. The study is continued by analyzing for freer copper by PIXE as ultrafiltrable copper passing through membranes with a cut-off at molecular mass $10^4$, after addition of ethylene diamine tetraacetic acid (EDTA) and equilibration at 4°C overnight. Proton induced X-ray emission is sufficiently sensitive and precise for this and gives the possibility of simultaneous multi elemental analysis. The results indicate that copper could be behaving differently in cancer patients than in normal ones. Copper movements could be freer with certain adverse effects of copper, such as oxidation-reduction reactions, more prevalent. Since the patients were in an early stage of breast cancer, this could have implications for the development of cancer.

Materials and Methods

**STUDY DESIGN**

The study was performed as part of a wider study on the relationship between diet, hormones, and breast cancer in women. For both the premenopausal (pre-m) and postmenopausal (post-m) categories, there were two control groups, omnivorous (OMN) and vegetarian (VEG), and a patient group including patients with mainly stage I and a few stage II breast cancer subjects (BCA).

**SUBJECTS STUDIED**

The OMN groups included 14 premenopausal women (mean [SD] age 33 [6]) and 11 postmenopausal women (age 57 [5]). For the VEG, the pre-m group comprised 12 subjects (age 34 [7]) and the post-m 11 (age 59 [5]). The corresponding pre-m and post-m BCA included 13 (age 39 [7]) and 10 (age 66 [6]) women, so that they were, on average, about five years older than their controls. The weights of the subjects studied varied from 42 kg to 89 kg, mean 61.5 kg, and were roughly normally distributed. Only the post-m OMN had significantly higher weights, mean (±SD) 69 ± 12 kg, than on average, $p < 0.05$. The subjects’ heights, mean 163.8 cm, range 151 to 174 cm, were also fairly normally distributed, and analysis of variance showed the pre-m OMN to be slightly taller than the others (167 ± 5 cm, $p < 0.05$). The body mass index calculated as weight/(height)$^2$ ranged from 17.3 to 34.6 kg/m$^2$, mean 22.9, and was significantly higher in the post-m OMN (mean 26.3, $p < 0.01$).

**BLOOD SAMPLES**

For both patients and controls, blood samples were taken following an overnight fast on at least three consecutive days. The plasma was separated, pooled, and then frozen and stored at $-20^\circ$C until analyzed. Repeat sampling occurred after an interval of several months; typically four samplings were performed over about a year. Thus, a minimum of 12 samples were obtained for each individual (four groups of three), and each measured value represents the mean (by pooling) of three samples collected on three consecutive days.

**DIETARY ASSESSMENT**

The OMN consumed all types of food. The group labelled VEG did not eat red meat, only two ate fish as well as eggs, and the rest were lactovegetarians. They had been vegetarians for a mean of 10.3 years and for at least 1.5 years. Diet was assessed over five days four times a year
by weighing dietary components on a letter balance and analyzing for nutrient composition by a computer program called Nutrica originally developed at the Finnish National Pensions Institute.

MEASUREMENT OF PLASMA COPPER LEVELS

Plasma copper levels were determined from dried plasma using proton-induced X-ray emission (PIXE) analysis where characteristic X-rays emitted from target atoms under proton bombardment are detected. The absolute concentrations of copper were determined from standard curves prepared by adding increasing volumes of Titrisol standard solutions to replicate samples of pooled normal serum, as described earlier. Plasma or serum samples are dried to constant weight under moderate vacuum and pressed into pellets. There is no chemical treatment, which minimizes contamination and which is a troublesome source of error in trace element work. Precisely 2.4 MeV protons from the 2.5 MV Van de Graaff Accelerator of the University of Helsinki emerging through a 7.5 μm thick Kapton® (polyimide (C22H10O5N2)3)* exit window impinge on the dried sample. This excites electron transitions, and, hence, produces characteristic X-ray emission which is detected by a high resolution solid state germanium detector coupled to a microcomputer used as a multichannel analyzer. The laboratory's own gravimetrically prepared standards, Seronorm®† and Seronorm protein®† were used as calibration standards and control samples.

MEASUREMENT OF PLASMA ULTRAFILTRATE COPPER CONCENTRATIONS

To 1900 μl plasma 100 μl 0.1 M Na2EDTA (Na2C10H14N2O8 · 2H2O; Titriplex III)‡ was added and allowed to stand overnight at 4°C and was shown to be very pure using the PIXE-method. The pH of the ultrafiltrate through membranes with a cut-off at molecular mass 10,000** was around 7. Because of the cut-off of the membranes the ultrafiltrate contained no ceruloplasmin total protein or subunits (Mol. mass ca. 19,000) and no larger serum proteins like albumin (Mol. mass ~68,000) or transcuprein (Mol. mass ~270,000).

The amount of ultrafiltrate was about 0.5 ml and was sufficient for PIXE anal-

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† NyCoMed, Oslo.
ysis. The ultrafiltrate was pipetted onto thin Kapton® foils and allowed to dry on an area of about 5 mm diameter. An infrared lamp was used to give a temperature of 40°C at the Kapton® membrane. Precisely 200 μl of sample were applied thus to form a dry target for PIXE. The detection of characteristic X-rays and spectral analyses were performed as for total plasma copper.

**STATISTICAL METHODS**

Results were analyzed using t-tests, the Wilcoxon U-test, analysis of variance (ANOVA), as well as by linear correlation analysis and Kendall’s rank correlation test. Unless otherwise stated, p-values quoted for the results are for non-parametric tests for clarity with regard to possible non-normality. Tabulated and summarized data are presented as arithmetic or geometrical means and ranges, including the range from −1 to +1 standard deviation of the geometric distributions.

**Results**

The plasma copper concentration ranged from 11.8 to 29.5 μmol/l (figure 1 and table I), with a variation in individuals of ±11 percent (SD). In the premenopausal BCA patients, serum copper was significantly greater than in their controls (p < 0.03); postmenopausally, there was no significant difference between groups.

Ceruloplasmin values ranged from 0.148 to 0.637 g/l (figure 2 and table I), with a variation in individuals of ±15
TABLE I

Plasma Copper, Ceruloplasmin, and Ratios of Copper-to-Ceruloplasmin*\textsuperscript{,b}

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Vegetarians</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Premenopause</strong></td>
<td></td>
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</tr>
<tr>
<td>Copper (µmol/l)</td>
<td>16.5 ± 0.30 (51)</td>
<td>16.7 ± 0.43 (48)</td>
<td>18.7 ± 0.62\textsuperscript{c} (48)</td>
</tr>
<tr>
<td>Ceruloplasmin (g/l)</td>
<td>0.327 ± 0.007 (52)</td>
<td>0.322 ± 0.011 (48)</td>
<td>0.335 ± 0.009 (48)</td>
</tr>
<tr>
<td>Ratio (µg/g)</td>
<td>3.20 ± 0.07 (51)</td>
<td>3.40 ± 0.086 (48)</td>
<td>3.44 ± 0.061\textsuperscript{c,d} (48)</td>
</tr>
<tr>
<td><strong>Postmenopause</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper (µmol/l)</td>
<td>18.4 ± 0.40\textsuperscript{a} (45)</td>
<td>18.5 ± 0.47\textsuperscript{a} (38)</td>
<td>18.2 ± 0.31 (32)</td>
</tr>
<tr>
<td>Ceruloplasmin (g/l)</td>
<td>0.387 ± 0.013\textsuperscript{f} (45)</td>
<td>0.355 ± 0.011\textsuperscript{f} (40)</td>
<td>0.309 ± 0.011\textsuperscript{g} (33)</td>
</tr>
<tr>
<td>Ratio (µg/g)</td>
<td>3.02 ± 0.074 (45)</td>
<td>3.22 ± 0.083 (38)</td>
<td>3.94 ± 0.096\textsuperscript{h,i} (32)</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of number of determinations in parentheses.

b Statistical significance (by U test) is as follows: significant difference between patients and controls, (\textsuperscript{c} p < 0.05; \textsuperscript{d} p < 0.01; \textsuperscript{h} p < 0.001); significant difference between premenopausal and postmenopausal controls (\textsuperscript{e} p < 0.05; \textsuperscript{f} p < 0.01); significant difference between premenopausal and postmenopausal patients (\textsuperscript{g} p < 0.001); \textsuperscript{d} versus premenopausal omnivores (p < 0.01).

percent (SD). The levels in the pooled premenopausal controls were significantly lower than those in the pooled postmenopausal controls (p < 0.001), with no significant effect of diet. The postmenopausal BCA patients had significantly lower ceruloplasmin levels than the pooled postmenopausal controls (p < 0.001).

The copper-to-ceruloplasmin ratios (figure 3 and table I), ranging from 2.0 to 5.85 µg/g were significantly higher in the pooled BCA patients than in the pooled controls (p < 0.001). Although the pattern in the pre- and postmenopausal groups was similar, the most striking difference was in the latter (p < 0.001 for BCA vs. omnivores; p < 0.01 for BCA vs. vegetarians). Premenopaually, the patients also had a higher ratio than the controls; however, only for the omnivores was the ratio significantly higher in patients than in controls (p < 0.04).

The EDTA-chelatable ultrafiltrable copper (P-edu-Cu) levels in plasma of patients and controls with an omnivorous or vegetarian diet are shown in figure 4. As expected for chelatable ultrafiltrable copper, there is a wide range from 75.2 µg/l to 516 µg/l geometric mean 227 (mean +1 SD 324, mean −1 SD 160). Even after accounting for the logarithmic distribution of the results, there were no significant differences between the groups. Unlike total plasma copper and ceruloplasmin, the P-edu-Cu correlated significantly with the dietary copper intake (figure 5 correlation coefficient 0.2, p < 0.005). The correlation, however, weakened for higher dietary cop-
per intakes (figure 6); after the mid-range of diet, copper P-edu-Cu levels tended to plateau.

**Dietary Copper and Total Fibre**

Dietary copper concentrations calculated from the weighed five-day dietary components by computer program are shown in table II. The values are given for the different pre- and post-m groups as crude data and also normalized per square meter indexed as the square of height. Also shown are the fiber intakes in the different groups. Pre-menopausally, the copper intakes were lower in the patients than in the controls (p < 0.05); post-m, the vegetarians had significantly higher copper and fiber intake than in both other groups.

**DISCRIMINATION BETWEEN BREAST CANCER AND NORMAL CONTROLS**

In this study, because dietary copper, dietary fiber, total P-Cu and EDTA-chelatable ultrafiltrable P-Cu (both expressible as per plasma dry mass or wet volume; the PIXE method involves drying of samples) as well as P-ceruloplasmin were all available, it was possible to use all these variables together in analyses. The results showed better discrimination between dietary normals and breast cancer patients than by using plasma variables alone. For the ratio of the plasma variables P-Cu/P-cer (figure 3), the significances of the higher ratios in cancer patients over controls were as follows: for all controls against all patients, ie, overall, p < 0.001; pre-m
EARLY BREAST CANCER, DIET, AND PLASMA COPPER FRACTIONS

FIGURE 3. The ratio of plasma copper to ceruloplasmin (µg/g) in pre- and postmenopausal omnivorous (OMN) and vegetarian (VEG) controls and breast cancer patients (BCA).

FIGURE 4. Ultrafiltrable EDTA-plasma copper levels in pre- and postmenopausal omnivorous (OMN) and vegetarian (VEG) controls and breast cancer patients (BCA).
BCA vs. pre-m OMN, p < 0.001; pre-m BCA vs. pre-m VEG, p < 0.01; post-m BCA vs. post-m OMN, p < 0.04.

Statistically more significant discrimination between breast cancer and controls was seen using the compound ratio of edu-Cu/dt-Cu to P-Cu/P-cer (Figure 7), where edu-Cu is expressed per unit of plasma dry weight and dt-Cu is normalized per kg body weight. In breast cancer, the ratio is higher than in dietary controls both pre- and postmenopausally. For all controls against all patients, i.e., overall, p < 0.0005; pre-m BCA vs. pre-m OMN, p < 0.001; pre-m BCA vs pre-m VEG, p < 0.05; post-m BCA vs post-m OMN, p < 0.03; post-m BCA vs post-m VEG, p < 0.0005.

When the copper tightly incorporated into plasma proteins (ceruloplasmin) was calculated as total P-Cu minus P-edu-Cu (abbreviated b-Cu), a highly significant
### TABLE II

Daily Dietary Copper and Total Fiber Intake as Geometric Means with Single Standardized Ranges

<table>
<thead>
<tr>
<th></th>
<th>Omnivores</th>
<th>Vegetarians</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet copper (mg/day)</strong></td>
<td>Premenopause⁺⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.71 (1.40 - 2.09)</td>
<td>1.56 (1.28 - 1.89)</td>
<td>1.37 (1.10 - 1.70)</td>
</tr>
<tr>
<td><strong>Diet copper (mg/m²/day)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.61 (0.50 - 0.75)</td>
<td>0.56 (0.47 - 0.68)</td>
<td>0.51 (0.40 - 0.64)</td>
</tr>
<tr>
<td><strong>Diet fiber (g/day)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.6 (13.9 - 24.9)</td>
<td>23.5 (18.4 - 30.0)</td>
<td>13.4 (9.4 - 18.9)</td>
</tr>
<tr>
<td><strong>Diet copper (mg/day)</strong></td>
<td>Postmenopause⁻⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.30 (0.99 - 1.72)</td>
<td>1.61 (1.34 - 1.94)</td>
<td>1.24 (1.05 - 1.47)</td>
</tr>
<tr>
<td><strong>Diet copper (mg/m²/day)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50 (0.40 - 0.63)</td>
<td>0.61 (0.50 - 0.75)</td>
<td>0.47 (0.41 - 0.53)</td>
</tr>
<tr>
<td><strong>Diet fiber (g/day)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.6 (12.7 - 21.7)</td>
<td>20.2 (13.8 - 29.7)</td>
<td>15.5 (12.5 - 19.3)</td>
</tr>
</tbody>
</table>

⁺⁺ Premenopausally, the patients' copper and fiber intakes were significantly less than in the normals (p < 0.05).
⁻⁻ Postmenopausally, the vegetarians' copper and fiber intake were greater than the other groups' (p < 0.05).

A significant difference between both pre- and postmenopausal breast cancer patients and both vegetarian and omnivorous controls was seen (figure 8). The function used is: $(b$-$Cu/P$-$cer$) $\times$ $(edu$-$Cu/dt$-$Cu$). Here, $b$-$Cu$ = plasma tightly incorporated copper.
Figure 8. Discrimination between breast cancer and controls using the plasma ultrafiltrable copper/dietary copper per square-meter and the plasma bound copper/ceruloplasmin ratios, taking into account colloid content of plasma and dietary fibre. Discriminator: \((b\text{-Cu/cer})(edu\text{-Cu} / dt\text{-Cu})\), where: \(b\text{-Cu}\) = plasma bound copper; \(ie\), total Cu–EDTA-ultrafiltered Cu; \(cer\) = plasma ceruloplasmin; \(P\text{-edu-Cu} = EDTA\text{-ultrafiltered Cu/plasma dry weight}\); and \(dt\text{-Cu}\) = dietary copper \(\times\) diet fibre/height squared. The levels in breast cancer patients are significantly elevated \((p < 0.0001)\).

Discussion

Breast cancer causes about 45,000 deaths per year in the USA, and, like other cancers, arises owing to gene defects causing changes in control of cell metabolism. In particular, the control of the cell cycle is defective leading to unregulated growth of the defective, cancerous cells. The diverse defects\(^4\) include emergence of oncogenes such as \(c\text{-myc, int-2 and c-erbB2}\), with amplification of DNA in their regions, and loss of heterozygosity on various chromosomes – \(1p, 1q, 3p, 7, 11p, 13q, 17p, 17q\) and \(18q\).\(^5\)

Suppressor genes like \(p53\) often have defective products. In familial forms of breast cancer, different genetic changes may be at work, as with the \(BRCA1\) and \(BRCA2\) genes,\(^7\) but these syndromes also have a relationship to sporadic breast cancer,\(^8\) eg, where a second mutation involving the good allele to an inherited defective suppressor gene leads to cancer development. The frequency of genetic changes may be correlated with histological tumour grade.\(^6\)

Hence, any factor which can damage the cell’s DNA (also that of repair genes)\(^9\) is potentially important for the development of cancer and its progression. One category of substances which can induce mutations is metal ions, like \(Cu^{II}/II\) and \(Fe^{II}/III\). These can take part in and catalyse oxidation-reduction reactions of the Fenton or Haber-Weiss types yielding hydroxyl radical (\(\cdot\mathrm{OH}\)) from hydrogen peroxide or superoxide.\(^10,11\) The hydroxyl radical is highly reactive and destructive to macromolecules and may lead to local damage to the molecules of
life, if its production is uncontrolled. Oxidation may also be due to other active oxygen species, like superoxide radicals and peroxide$^{12}$ in the absence of sufficient superoxide dismutase$^{13}$ or catalase activities, respectively. Hence, DNA bases can be hydroxylated to yield 8-hydroxyguanine and cytosine or thymine glycols. This particularly causes cytosine-to-thymine mismatching during copying, with mutations occurring clustertwise.$^{14}$ Copper ions bind to bases and not to the sugar-phosphate moiety (as, for example, Mg does) and Cu-2+ then tends to destabilize DNA. Copper's capacity to cause oxidative damage in the presence of H$_2$O$_2$ has been reported as 50-fold that for iron.$^{15}$

Although for some time it had been believed that about 95 percent of plasma copper was associated with ceruloplasmin,$^{16}$ recent studies have shown that as little as 65 percent may be nearer the truth with about 8 percent in a ca. 270,000 molecular mass protein called transcuprein, 14 percent attached to albumin, and about 5 percent attached to small molecules like aminoacids.$^{17}$ Plasma and extracellular copper pass into the cell at least partly by mediation of ceruloplasmin receptors$^{18}$ on the cell surface which are not internalized themselves in the process. Some have reported transcuprein copper in malignancies and elevated total as well as ceruloplasmin copper;$^{19}$ the elevation in Wilson's disease and Indian childhood cirrhosis is due to an increase in non-ceruloplasmin copper probably released from the copper laden liver.$^{19}$ The elevated serum copper after a myocardial infarction would be a similar phenomenon.

Ultrafiltrable copper has been studied in gynecological malignancies with the conclusion that because levels are similar in controls and patients, copper is not the etiological element in these cancers.$^{20}$ In that work, no chelating agent was used so that freer copper was limited to a very small fraction of the freer copper of our study. Also, the ultrafiltration membranes had a cut-off of 30,000 so that smaller fragments (eg, the 19,000 fragments) of ceruloplasmin could have passed through.

The damage inflicted by copper on macromolecules is not necessarily all due to freer copper. Although it has been thought that ceruloplasmin is primarily antioxidant in nature, it can also cause oxidative damage to macromolecules such as low density lipoprotein.$^{21}$ Again, simple oxidative mechanisms may not account for all the damaging potential of copper in the case of DNA.$^{15}$ Some seem to have shown in certain systems using spin traps that iron, rather than copper, may be responsible for DNA damage via hydroxyl radicals.$^{11}$ Even if copper does not produce these in the vicinity of DNA, it could act detrimentally in another way.$^{14}$

Copper/zinc ratios, which tend to be more statistically significant in advanced cancer, have earlier been used to discriminate cancer patients from normals when copper levels or zinc levels alone have not shown this capability. Distinguishing between cancer and normals in early cancer is more demanding, and provision of efficient prognostic indices yet again more difficult. The patients in this study had early stage breast cancer with, however, statistically significant alterations in the P-Cu/P-cer ratio. The inclusion, in discrimination between normals and cancer patients, of other copper parameters like dietary copper and dietary fiber, which can bind metal ions and, hence, influence bioavailability, gave better statistical significances than those for the simple P-Cu/P-cer ratio. Further, the patterns of differences between normals and cancer patients were similar pre- and post-menopaually. Further research involving this more comprehensive approach could help uncover possible important differ-
ences in copper metabolism between normals on various diets and early cancer patients.

Acknowledgments

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References