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# Copper isotope effect in serum of cancer patients. A pilot study

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The isotope effect describes mass-dependent variations of natural isotope abundances for a particular element. In this pilot study, we measured the $^{65}Cu/^{63}Cu$ ratios in the serums of 20 breast and 8 colorectal cancer patients, which correspond to, respectively, 90 and 49 samples taken at different times with molecular biomarker documentation. Copper isotope compositions were determined by multiple-collector inductively coupled plasma mass spectrometry (MC-ICP-MS). When compared with the literature data from a control group of 50 healthy blood donors, abundances of Cu isotopes predict mortality in the colorectal cancer group with a probability $p = 0.018$. For the breast cancer patients and the group of control women the probability goes down to $p = 0.0006$ and the AUC under the ROC curve is 0.75. Most patients considered in this preliminary study and with serum $\delta^{65}Cu$ lower than the threshold value of −0.35‰ (per mil) did not survive. As a marker, a drop in $\delta^{65}Cu$ precedes molecular biomarkers by several months. The observed decrease of $\delta^{65}Cu$ in the serum of cancer patients is assigned to the extensive oxidative chelation of copper by cytosolic lactate. The potential of Cu isotope variability as a new diagnostic tool for breast and colorectal cancer seems strong. Shifts in Cu isotope compositions fingerprint cytosolic Cu chelation by lactate mono- and bidentates. This simple scheme provides a straightforward explanation for isotopically light Cu in the serum and isotopically heavy Cu in cancer cells: $Cu^+$ escaping chelation by lactate and excreted into the blood stream is isotopically light. Low $\delta^{65}Cu$ values in serum therefore reveal the strength of lactate production by the Warburg effect.

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## A Introduction

Copper is a micronutrient and a catalytic and structural cofactor of many important enzymes involved in neoplastic tissue differentiation, such as ceruloplasmin, cytochrome oxidase, and superoxide dismutase.[1–3] Two main routes by which Cu interacts with cancer cells are angiogenesis and hypoxia. While there is little need for angiogenesis in normal tissue, the growth of cm-sized tumors is accompanied by pervasive neovascularization,[4] which secures delivery of oxygen and nutrients to tumor cells. Activation of endothelial cells,[5] which occurs in the early stage of angiogenesis, and of their subsequent proliferation[6] are both stimulated by copper contained in ceruloplasmin. In the process, copper plays a strong role in the activation of several angiogenic factors, notably VEGF, tumor necrosis factor alpha (TNF-α) and interleukin (IL1).[5]

A second role of copper in cancer is in hypoxia, a hallmark of human malignancies. HIF-1α overexpression is associated with increased tumor growth, vascularization, and metastasis.[7,8] Recent evidence suggests that the expression of certain genes involved in the epithelial–mesenchymal transition (EMT) is influenced by low oxygen levels, with hypoxia helping maintain the stem cell phenotype in cancers in certain niches.[9] Ionic serum copper is known to stabilize the hypoxia-inducible factor HIF-1α and to upregulate ceruloplasmin under hypoxic conditions.[10]

Due to its short bulk turnover time in the human body (~6 weeks[1,11]), copper is a relevant indicator of rapidly evolving cancers. Anomalously high Cu levels or Cu/Zn ratios were observed in the serum of breast cancer[12,13] and serum ceruloplasmin was found to be significantly elevated in advanced stages of solid malignant tumors.[14] Ishida *et al.*[15] demonstrated that increased levels of bioavailable copper promote tumor growth in mice. As a result, reduction of copper and ceruloplasmin using chelates such as tetrathiomolybdate and D-penicillamine[16–18] has now been approved for cancer treatment. The changes in copper concentrations, however, do not remain amenable to quantitative predictions rooted in robust biochemical processes.

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Some attributes of metals and their natural isotopes as biomarkers stand out. We use the term of biomarker for Cu isotope abundances in the human serum with the assumption that observed $^{65}Cu/^{63}Cu$ variations reflect an altered function of cancer cells relative to normal cells. Unlike complex proteins, metals do not degrade over time, regardless of how samples have been stored, which makes timing a less restrictive condition of analytical protocols. However, a significant limitation of markers merely based on metal abundances is that the direction and the intensity of enrichment or depletion of a particular metal in a particular fluid or cellular compartment cannot be quantitatively predicted. This drawback explains the so far modest success of inorganic tracers in medicine. Such a limitation does not exist, however, for isotopic tracers: isotope fractionation between coexisting molecules can usually be quantitatively predicted by *ab initio* calculations,[19] and this property can be used to assign observed isotopic variability to well-constrained biological reactions.

The structural rationale for investigating natural isotope variability is that, in most cases, heavy isotopes are expected to engage preferentially into the most stable bonds with the lowest energy.[20] The isotope effect reflects that, as required by the Heisenberg uncertainty principle, the vibrational energy of a bond is not allowed to cancel out. As vibrational frequencies, and with them the corresponding energies, decrease when the mass of vibrating atoms increases, bond energies depend on the mass of the binding partners, a property known as the isotope effect.[21] Here, we measured the variations in the abundances of the two natural copper isotopes, $^{63}Cu$ and $^{65}Cu$, in the serums of colorectal and breast cancer patients. We present results for a longitudinal study of Cu isotopes in the serums of 20 breast cancer patients (90 samples) and 8 colorectal cancer patients (49 samples) recruited into cohorts at Centre Léon Bérard in Lyon, France. The results are combined with more conventional tumor biomarkers to improve the prognostic value of Cu isotopes.

## B Materials and methods

The present set of samples does not fit the criteria expected from a cohort but is a subset of samples of frozen serum salvaged from a previous clinical investigation. As control groups, we used the Cu isotope abundances in the serum samples of 22 men and 28 women, aged 18–38, analyzed by Albarede *et al.*[22] 18/20 breast cancer patients were diagnosed with invasive ductal carcinomas and 2/20 with invasive lobular carcinomas. Analysis of estrogen or progesterone receptor (ER, PR) and human epidermal growth factor receptor 2 (Her2) amplification is reported with the rest of the data and shows that 11/20 breast cancers are ER+/PR+/Her2−, 2/20 are ER+/PR−/Her2−, 3/20 are ER+/PR+/Her2+, 2/20 are ER−/PR−/Her2−, and 2/20 are ER−/PR−/Her+. All colorectal cancer patients were diagnosed with invasive adenocarcinoma and a complete phenotype is not available. Patients for the study were selected from individuals attending a routine clinic at the Centre Léon Bérard from 1997 to 2013 at times indicated in Tables 1 and 2 as number of days relative to a common but arbitrary reference date. Informed consent was obtained from all the subjects included in the study. The study was approved by the Ethical Committee of the study hospital.

Blood was drawn out in dry test tubes, centrifuged immediately, and the serum freeze-dried and stored in liquid nitrogen. 200 microliters of serum were mineralized on a hot plate in a mixture of nitric acid and hydrogen peroxide and processed on a macroporous anion-exchange resin to separate Cu. The $^{65}Cu/^{63}Cu$ ratios were determined on the Nu Instrument multiple-collector inductively-coupled plasma mass spectrometer (MC-ICP-MS) Nu Plasma HR 500 of the Ecole Normale Supérieure in Lyon. Copper contamination by the chemical and mass spectrometric procedures amounted to less than a few percent of the sample and therefore is negligible. The details of the analytical techniques used in the present study are as previously described.[22,23]

The conventional delta value $\delta^{65}Cu$ is used throughout to report the Cu isotope abundances. $\delta^{65}Cu$ is a dimensionless parameter defined as

$$\delta^{65}Cu = \frac{\left(^{65}Cu/^{63}Cu\right)_{sample} - \left(^{65}Cu/^{63}Cu\right)_{ref}}{\left(^{65}Cu/^{63}Cu\right)_{ref}} \quad (1)$$

and represents the relative deviation, usually reported in parts per 1000 (‰), of the $^{65}Cu/^{63}Cu$ ratio in the measured sample from its value in reference material NIST SRM 976. Typical external reproducibility on $\delta^{65}Cu$ at the 95 percent confidence level determined from multiple replicates of serum samples is $\sim 0.05$‰.[23] Natural variations of $\delta^{65}Cu$ in inorganic and organic material do not exceed a few per mil.

## C Results

The analytical data are given in Tables 1 and 2 together with phenotype and available biomarker information. We first plotted the two widely used indicators of breast cancer status, serum Cu($x$) and Cu/Zn($y$), against each other (Fig. 1). The control group[22] shows a strong correlation, which reflects the tight regulation of Zn concentrations in the body ($x/y$ = Zn). Women have more Cu and higher Cu/Zn than men.[22] Zinc concentrations in the serum of breast cancer patients seems to be much more variable, usually lower, relative to healthy subjects. Serum from breast cancer patients clearly shows a strong deregulation of both Cu and Zn, but although, on average, Cu/Zn increases above the value for healthy women ($p$ = 0.0006), there is still a moderate amount of overlap between the two groups.

Each $^{65}Cu/^{63}Cu$ measurement of the serum of each patient was handled as an independent measurement (Fig. 2). Copper from the serum of both men and women shows marginally distinctive $\delta^{65}Cu$ values ($p$ = 0.45[22]). The difference between $\delta^{65}Cu$ in the serum of colorectal cancer patients and of combined healthy men and women is more significant ($p$ = 0.018). Even more remarkable is the difference in $\delta^{65}Cu$ between breast cancer patients and healthy women ($p$ = 0.004). We also considered the correlation coefficient between Cu/Zn and $\delta^{65}Cu$ ($p$ = 0.06, $n$ = 86) for pooled cancer breast data and

 

Table 1 Breast cancer: phenotype, biomarkers, and analytical data on serum samples

| Patient # | ID | Age | Phen | SBR | ER | PR | Her2 | Date | CA15.3 | CEA | Cu | Zn | $\delta^{65}$Cu‰ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ****990 | 58 | IDC | n.d. | + | + | − | 38 533 | 384 | 45 | 1493 | 968 | −0.39 |
| | | | | | | | | 38 575 | 277 | 18 | 2089 | 1477 | −0.50 |
| | | | | | | | | 38 624 | 111 | 10 | 1281 | 678 | −0.76 |
| | | | | | | | | 38 666 | 47 | 3 | 1873 | 5757 | −0.67 |
| | | | | | | | | 38 708 | 42 | 7 | 672 | 314 | −0.57 |
| | | | | | | | | 38 796 | 851 | 7 | 1475 | 740 | −0.50 |
| 2 | ****648 | 37 | IDC | 3 | + | + | − | 39 238 | 22 | 1 | 1965 | 1034 | −0.17 |
| | | | | | | | | 39 287 | 35 | 1 | 1165 | 839 | −0.03 |
| | | | | | | | | 39 329 | 33 | 1 | 1284 | 1147 | −0.17 |
| | | | | | | | | 39 371 | 36 | 1 | 1220 | 957 | −0.13 |
| | | | | | | | | 39 455 | 26 | 1 | 1001 | 1595 | 0.06 |
| | | | | | | | | 39 541 | 49 | 2 | 1626 | 1667 | −0.03 |
| 3 | ****474 | 45 | IDC | 3 | + | + | − | 39 009 | 57 | 10 | 1640 | 1172 | −0.57 |
| | | | | | | | | 39 051 | 26 | 6 | 1396 | 1164 | −0.52 |
| | | | | | | | | 39 077 | 27 | 3 | 1345 | 1097 | −0.65 |
| | | | | | | | | 39 100 | 34 | 3 | 1767 | 1346 | −0.32 |
| | | | | | | | | 39 146 | 18 | 2 | 1129 | 841 | −0.29 |
| | | | | | | | | 39 216 | 20 | 3 | 1647 | 1216 | −0.35 |
| | | | | | | | | 39 279 | 22 | 4 | 1422 | 1168 | −0.33 |
| | | | | | | | | 39 316 | 57 | 3 | 1880 | 1333 | −0.58 |
| 4 | ****016 | 59 | IDC | 2 | + | − | − | 39 261 | 130 | 15 | 1128 | 747 | −0.30 |
| | | | | | | | | 39 302 | 130 | 15 | 1276 | 1082 | −0.27 |
| | | | | | | | | 39 384 | 42 | 13 | 1145 | 759 | −0.40 |
| | | | | | | | | 39 482 | 38 | 12 | 1079 | 809 | −0.29 |
| | | | | | | | | 39 546 | 47 | 13 | 1105 | 713 | −0.42 |
| 5 | ****483 | 38 | IDC | 2 | − | − | + | 38 533 | 48 | 6 | 1731 | 1304 | −0.19 |
| | | | | | | | | 38 625 | 83 | 8 | 2145 | 1086 | −0.35 |
| | | | | | | | | 38 666 | 93 | 9 | 1808 | 961 | −0.53 |
| | | | | | | | | 38 709 | 126 | 10 | 2146 | 939 | −0.51 |
| 6 | ****757 | 44 | IDC | 2 | − | − | − | 39 058 | 624 | 73 | 1888 | 1225 | 0.12 |
| | | | | | | | | 39 100 | 642 | 67 | 2095 | 1164 | 0.11 |
| | | | | | | | | 39 142 | 363 | 56 | 1892 | 1081 | 0.04 |
| | | | | | | | | 39 184 | 508 | 55 | 2053 | 1097 | −0.30 |
| 7 | ****675 | 53 | IDC | 3 | − | − | − | 39 136 | 20 | 1 | 1844 | 1105 | −0.49 |
| | | | | | | | | 39 191 | 28 | 1 | 2076 | 1242 | −0.42 |
| | | | | | | | | 39 233 | 25 | 1 | 1985 | 1284 | −0.40 |
| | | | | | | | | 39 275 | 22 | 1 | 1443 | 954 | −0.46 |
| | | | | | | | | 39 318 | 23 | 1 | 2408 | 1337 | −0.53 |
| | | | | | | | | 39 356 | 21 | 1 | 2296 | 1219 | −0.40 |
| 8 | ****607 | 59 | ILC | | + | + | − | 40 066 | 1612 | 491 | 1195 | 692 | −0.60 |
| | | | | | | | | 40 094 | 580 | 202 | 1451 | 709 | −0.77 |
| | | | | | | | | 40 101 | 514 | 183 | 1786 | 829 | −0.73 |
| | | | | | | | | 40 255 | 1239 | 482 | 1665 | 1228 | −0.78 |
| 9 | ****647 | 48 | IDC | 3 | − | − | + | 39 898 | 67 | 1 | 1860 | 1355 | −0.47 |
| | | | | | | | | 39 919 | 61 | 1 | 1918 | 1279 | −0.35 |
| | | | | | | | | 39 940 | 45 | 1 | 1994 | 894 | −0.69 |
| | | | | | | | | 40 164 | 49 | 1 | 2032 | 979 | −0.67 |
| 10 | ****715 | 31 | IDC | 3 | + | + | − | 39 835 | 1402 | 340 | 2092 | 1067 | −0.94 |
| | | | | | | | | 39 863 | 89 | 154 | 1951 | 1022 | −0.59 |
| | | | | | | | | 39 892 | 214 | 31 | 1041 | 485 | −0.68 |
| | | | | | | | | 40 266 | 121 | 45 | 2070 | 1146 | −0.77 |
| 11 | ****341 | 60 | IDC | 3 | + | − | − | 39 608 | 40 | 5 | 2128 | 879 | −0.91 |
| | | | | | | | | 39 695 | 50 | 5 | 1961 | 541 | −0.77 |
| | | | | | | | | 39 723 | 63 | 5 | 2554 | 1863 | −0.69 |
| | | | | | | | | 39 751 | 45 | 5 | 2404 | 740 | −0.83 |
| 12 | ****708 | 62 | IDC | 2 | + | + | + | 39 884 | 1013 | 500 | 1680 | 732 | −0.54 |
| | | | | | | | | 39 912 | 1174 | 617 | 1649 | 754 | −0.87 |
| | | | | | | | | 39 961 | 444 | 572 | 1539 | 794 | −0.52 |
| | | | | 3 | + | + | | 40 371 | 146 | 297 | 2465 | 1671 | −0.54 |
| 13 | ****160 | 43 | IDC | | | | + | 40 702 | 19 | 1 | 1482 | 1967 | −0.41 |
| | | | | | | | | 40 759 | 20 | 2 | 1404 | 2073 | −0.44 |
| | | | | | | | | 40 822 | 29 | 1 | 2020 | 1239 | −0.72 |
| | | | | | | | | 40 941 | 19 | 1 | 1531 | 1243 | −0.54 |
| 14 | ****349 | 49 | IDC | 3 | + | + | + | 40 788 | 18 | 1 | 1098 | 2218 | −0.44 |
| | | | | | | | | 40 808 | 21 | 0 | 1733 | 3194 | −0.48 |
| | | | | | | | | 40 871 | 30 | 1 | 1279 | 1253 | −0.63 |
| | | | | | | | | 40 980 | 17 | 0 | 1345 | 1159 | −0.38 |
| 15 | ****595 | 67 | ILC | 2 | + | + | − | 38 891 | 13 | 4 | 2393 | 2911 | −0.31 |
| | | | | | | | | 38 929 | 12 | 4 | 2334 | 786 | −0.32 |
| | | | | | | | | 39 020 | 87 | 6 | 2309 | 871 | −0.57 |

Table 1 (*continued*)

| Patient # | ID | Age | Phen | SBR | ER | PR | Her2 | Date | CA15.3 | CEA | Cu | Zn | $\delta^{65}$Cu‰ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | ****145 | 42 | IDC | 3 | + | + | − | 38 495 | 16 | 19 | 2332 | 1392 | −1.05 |
| | | | | | | | | 38 582 | 21 | 3 | 1287 | 1265 | −1.10 |
| | | | | | | | | 38 624 | 16 | 3 | 1686 | 7474 | −0.73 |
| | | | | | | | | 38 673 | 14 | 9 | 1623 | 941 | −1.45 |
| 17 | ****006 | 39 | IDC | 2 | + | + | − | 39 912 | 116 | 33 | 1640 | 871 | −0.64 |
| | | | | | | | | 39 940 | 91 | 28 | 1770 | 949 | −0.63 |
| | | | | | | | | 39 968 | 61 | 23 | 1637 | 1005 | −0.40 |
| | | | | | | | | 40 163 | 39 | 28 | 1761 | 932 | −0.59 |
| 18 | ****539 | 54 | IDC | 3 | + | + | − | 39 850 | 119 | n.d. | 1961 | 1227 | −0.45 |
| | | | | | | | | 39 878 | 97 | n.d. | 1031 | 841 | −0.57 |
| | | | | | | | | 39 906 | 23 | n.d. | 1646 | 1139 | −0.41 |
| | | | | | | | | 40 095 | 46 | n.d. | 1258 | 1225 | −0.56 |
| 19 | ****228 | 49 | IDC | 3 | + | + | − | 39 163 | 5200 | 1500 | 3394 | 1019 | −0.68 |
| | | | | | | | | 39 251 | 725 | 405 | 1814 | 866 | −0.40 |
| | | | | | | | | 39 345 | 1240 | 650 | 2305 | 911 | −0.59 |
| | | | | | | | | 39 391 | 5670 | 1119 | 3616 | 972 | −0.56 |
| 20 | ****617 | 38 | IDC | 3 | + | + | − | 38 853 | 127 | n.d. | 2087 | 822 | −0.85 |
| | | | | | | | | 38 895 | 498 | 173 | 2623 | 954 | −0.65 |
| | | | | | | | | 38 965 | 571 | 187 | 2252 | 990 | −0.46 |
| | | | | | | | | 39 027 | 1430 | 514 | 1385 | 958 | −0.80 |

Age: age at diagnosis. Phen: phenotype. IDC: invasive ductal carcinoma. ILC: invasive lobular carcinoma.ER: estrogen receptor. PR: progesterone receptor. Her2: human epidermal growth factor receptor 2. CA: carbohydrate antigen. CEA: carcinoembryonic antigen. Cu and Zn in ppb (mg kg$^{-1}$).

found it non-significant. A non-parametric Kruskal–Wallis test of $\delta^{65}$Cu *vs.* Cu/Zn also indicates a poor correlation ($p$ = 0.16).

None of the 20 breast cancer patients survived. The evolution of $\delta^{65}$Cu with time was compared with that of biomarkers, the broadly used CEA (carcinoembryonic antigen[24]) and CA 19.9 (carbohydrate antigen 19.9[25,26]), which at the time of the study were recommended for colorectal cancer, and CA 15.3, recommended for breast cancer.[27] Plotting $\delta^{65}$Cu over time shows that all of these patients but one (breast #2) started out with either low $\delta^{65}$Cu values or experienced a strong decline in $\delta^{65}$Cu over time (Fig. 3). A $\delta^{65}$Cu alarm threshold was adopted at −0.35‰. The apparent discrepancy for case #2 seems to reflect the long time interval between the end of the $\delta^{65}$Cu record and the rise of the other biomarkers (>6 months), *i.e.*, a fast evolution of the pathology. Because of the smaller number of patients and their distribution as men and women, a similar conclusion cannot be reached for colorectal cancer. Three out of four female patients and two of the four male patients with colorectal cancer had survived at the time of writing. The $\delta^{65}$Cu of the surviving patients remained normal, *i.e.*, high with respect to non-surviving patients.

# D Discussion

### Copper isotope abundances and diagnostic

We explored whether $\delta^{65}$Cu could be relevant to cancer diagnostics by comparing the $\delta^{65}$Cu values in the serums of the 28 women in the control group and of the 20 deceased breast cancer patients. Survival is here the 'gold standard'. A weak point of the sample set is that the control group is not completely age-matched (18–38) with the cancer patient group. For the reasons discussed hereafter, this is probably not a serious drawback for the present study. The incidence of cancer tends to be higher for the elderly and Jaouen *et al.*[28] found that the total blood $\delta^{65}$Cu of woman older that 55 was lower than for younger women. Van Heghe *et al.*[29] made a similar observation between postmenopausal and menstruating women. However, 16/20 of the breast cancer patients were younger than 55 at the time of diagnostic. Whether menopause would be a primary cause for a $\delta^{65}$Cu shift in serum is unclear. It is well established that the prevalence of anemia increases with age in both men and women.[30] Erythrocytes account for one third of the total Cu in blood while their $\delta^{65}$Cu is up to 1‰ heavy relative to serum:[22] the total blood shift in $\delta^{65}$Cu observed after the age of 55[28,29] therefore cannot be simply extrapolated to serum. The present data also show short-term variations of $\delta^{65}$Cu that clearly are unrelated to any age effect. We therefore conclude that if there is an age effect on the potential diagnostic value of $\delta^{65}$Cu, it is likely to be subordinate.

Receiver Operating Characteristics (ROC) curves (*e.g.*, ref. 31) weigh the chances of a true positive *vs.* the chance of a false positive and therefore are particularly powerful tools of diagnostic medicine. ROC is the probability of a true positive decision (tp), *e.g.* that patient deceases with serum $\delta^{65}\mathrm{Cu} \leq \delta^{65}\mathrm{Cu_c}$, or that the $\delta^{65}$Cu of a young donor's serum is $\geq \delta^{65}\mathrm{Cu_c}$, *vs.* the probability of a false positive decision (fp), *e.g.*, that a patient deceases with serum $\delta^{65}\mathrm{Cu} \geq \delta^{65}\mathrm{Cu_c}$, or that the $\delta^{65}$Cu of a young donor's serum is $\leq \delta^{65}\mathrm{Cu_c}$. tp is also known as the 'sensitivity', while 1-fp is known as the 'specificity'. The ROC curve is the set of all points calculated for different cutoff values $\delta^{65}\mathrm{Cu_c}$. A widely used test is the area under the ROC curve (AUC), which varies between 0.5 (pure chance) and 1.0 (fully trustworthy test). The most reliable cutoff value can also be inferred by different techniques[31] from the elevation of the ROC curve above the first diagonal. In the present case of breast cancer diagnostic (Fig. 4), AUC is 0.76, while the optimum cutoff $\delta^{65}\mathrm{Cu_c}$ value is −0.37‰. The number of data for colorectal cancer is too small to warrant a significant test, but applying the ROC analysis to discriminate survivors from deceased patients indicates an AUC value of 0.85 and an optimum cutoff value of

 

Table 2 Colorectal cancer: phenotype, biomarkers, and analytical data on serum samples

| Patient # | Gender | ID | Age | Phen | Surv stat | Date | CA19.9 | CEA | Cu | Zn | $\delta^{65}$Cu‰ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | ***960 | 66 | LCA | Alive | 39 498 | 3.8 | 7.1 | 1181 | 1047 | −0.11 |
| | | | | | | 40 367 | 4.2 | 8.9 | 1121 | 1331 | −0.16 |
| | | | | | | 40 609 | 7.39 | 11.7 | 1139 | 1500 | −0.43 |
| | | | | | | 40 623 | 1.74 | 7.6 | 1281 | 1481 | −0.33 |
| | | | | | | 40 693 | 1.4 | 3 | 1526 | 1344 | −0.18 |
| | | | | | | 40 751 | 1.2 | 2.4 | 963 | 1161 | −0.23 |
| 2 | F | ***326 | 55 | LCA | Alive | 39 608 | 53 | n.d. | 1673 | 1350 | −0.21 |
| | | | | | | 39 728 | 14.8 | 1.92 | 1820 | 1610 | −0.17 |
| | | | | | | 39 792 | 11.23 | 1.32 | 1940 | 1221 | −0.28 |
| | | | | | | 40 469 | 2.4 | n.d. | 1882 | 1554 | −0.28 |
| | | | | | | 40 485 | 13 | 8 | 1946 | 1398 | −0.21 |
| | | | | | | 40 555 | 2.4 | 7.1 | 1784 | 1220 | −0.17 |
| | | | | | | 40 625 | 3 | 7.4 | 2117 | 1587 | −0.15 |
| | | | | | | 40 840 | 429.1 | 8.8 | 1556 | 1460 | −0.26 |
| | | | | | | 40 861 | 199 | 10.6 | 1073 | 2060 | −0.36 |
| | | | | | | 40 903 | 11.4 | 76.2 | 1863 | 1466 | −0.34 |
| 3 | F | ****793 | 39 | LCA | Deceased | 40 375 | 67.1 | 94.6 | 1449 | 1063 | −0.53 |
| | | | | | | 40 392 | n.d. | 28.1 | 1452 | 2452 | −0.50 |
| | | | | | | 40 422 | 25.3 | 7.4 | 1239 | 1821 | −0.33 |
| | | | | | | 40 492 | 18.8 | 0.94 | 1193 | 1276 | −0.35 |
| | | | | | | 40 555 | 27 | n.d. | 1909 | 1197 | −0.49 |
| | | | | | | 40 588 | 18.1 | 0.36 | 2363 | 1618 | −0.45 |
| | | | | | | 40 630 | 18.7 | 0.5 | 2210 | 1471 | −0.33 |
| | | | | | | 40 686 | 17.7 | 0.2 | 2057 | 1430 | −0.39 |
| 4 | M | ***391 | 63 | ACA | Deceased | 40 198 | 7 | 0.1 | 1478 | 993 | −0.13 |
| | | | | | | 40 576 | 206 | 3.1 | 1234 | 1266 | −0.14 |
| | | | | | | 40 590 | 125 | 2.6 | 1187 | 1182 | −0.38 |
| | | | | | | 40 653 | 138 | 3.2 | 1721 | 1054 | −0.30 |
| | | | | | | 40 750 | 163 | 2.5 | 1378 | 1332 | −0.25 |
| | | | | | | 40 779 | 303 | 4 | 1345 | 1383 | −0.39 |
| 5 | F | ****733 | 55 | LCA | Alive | 40 617 | 871 | 4294 | 1451 | 1356 | −0.28 |
| | | | | | | 40 631 | 586 | 3587 | 1685 | 1571 | −0.20 |
| | | | | | | 40 715 | 36 | 139 | 1897 | 1466 | −0.25 |
| | | | | | | 40 757 | 15.1 | 12.4 | 1749 | 1302 | −0.29 |
| | | | | | | 40 855 | 7.4 | 1.3 | 1597 | 1318 | −0.17 |
| | | | | | | 40 893 | 8.3 | 1.2 | 2003 | 1228 | −0.39 |
| 6 | F | ****673 | 62 | ACA | Alive | 40 616 | 39 | 2.7 | 1792 | 1613 | 0.04 |
| | | | | | | 40 644 | 15 | 2 | 1909 | 1391 | −0.03 |
| | | | | | | 40 723 | 8 | 3.2 | 1530 | 1419 | −0.27 |
| | | | | | | 40 763 | 11 | 3.7 | 1478 | 1765 | −0.21 |
| | | | | | | 40 828 | 8 | 3.4 | 2318 | 1734 | −0.09 |
| 7 | M | ****781 | 63 | ACA | Alive | 40 653 | 292 | 416.3 | 1838 | 1423 | −0.18 |
| | | | | | | 40 674 | 292 | 416 | 1135 | 1137 | −0.13 |
| | | | | | | 40 737 | 35 | 51 | 1568 | 1680 | −0.15 |
| | | | | | | 40 779 | 26 | 34.46 | 1697 | 1493 | −0.43 |
| | | | | | | 40 954 | 98 | 104 | 968 | 1291 | −0.34 |
| 8 | M | ****290 | 81 | LCA | Deceased | 40 262 | 137 | 26 | 1218 | 1228 | −0.30 |
| | | | | | | 40 793 | 156 | 61 | 1428 | 1386 | −0.53 |
| | | | | | | 40 807 | 1720 | 100 | 1214 | 981 | −0.55 |
| | | | | | | 40 849 | 86 | 14 | 1423 | 1413 | −0.61 |
| | | | | | | 40 919 | 8176 | 136 | 1325 | 1244 | −0.65 |

LCA: lieberkuhnian adenocarcinoma. ACA: adenocarcinoma on adenoma. Surv stat = survival status. Other: see Table 1.

$\delta^{65}Cu_c = -0.30$. In the following discussion, we will use a single cutoff value for all patients of $\delta^{65}Cu_c = -0.35$.

The data further show a number of salient features:

(1) The serums of all patients who did not survive breast or colorectal cancer, but one, was characterized by protracted periods with serum $\delta^{65}Cu < -0.35$ (Fig. 3)

(2) Low levels of biomarkers, typically less than 30 U ml$^{-1}$, which are taken as a 'negative', are commonly observed even for patients with $\delta^{65}Cu < -0.35$ (colorectal #3, breast # 3, 4, 9, 13–18, 20).

(3) A decrease of $\delta^{65}$Cu typically precedes an increase of the other biomarkers by about 3–6 months (Fig. 5). Rapidly decreasing $\delta^{65}$Cu should also be considered a negative indication.

The present study is still at the pilot stage implying that a claim at this time that $\delta^{65}$Cu is a reliable biomarker is premature, especially because the age-match is insufficient. Nevertheless, the potential of $\delta^{65}Cu_c$ as a diagnostic tool for both breast and colorectal cancer appears real. Low $\delta^{65}$Cu may help direct attention to an oncologic condition at an early stage, even when molecular biomarkers remain within their normal range. The technique is non-invasive and only involves drawing blood following routine laboratory techniques. However small, the leading time of $\delta^{65}$Cu with respect to molecular biomarkers may also help the decision process in adapting therapies to a particular patient. Contrary to molecular biomarkers, the integrity of Cu levels and

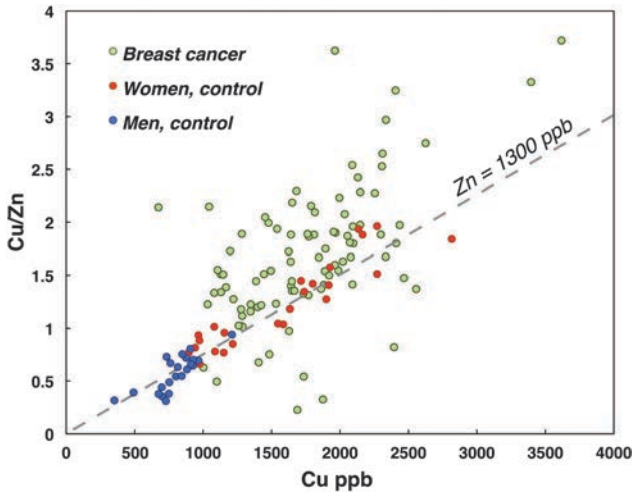


Fig. 1 Cu and Cu/Zn as indicators of breast cancer status (ppm is μg $kg^{-1}$). The control group[22] shows a strong correlation, reflecting the tight regulation of Zn concentrations in the body (*x*/*y* = Zn). Zinc in the serums of breast cancer patients seems to be more variable, usually lower, relative to healthy subjects.

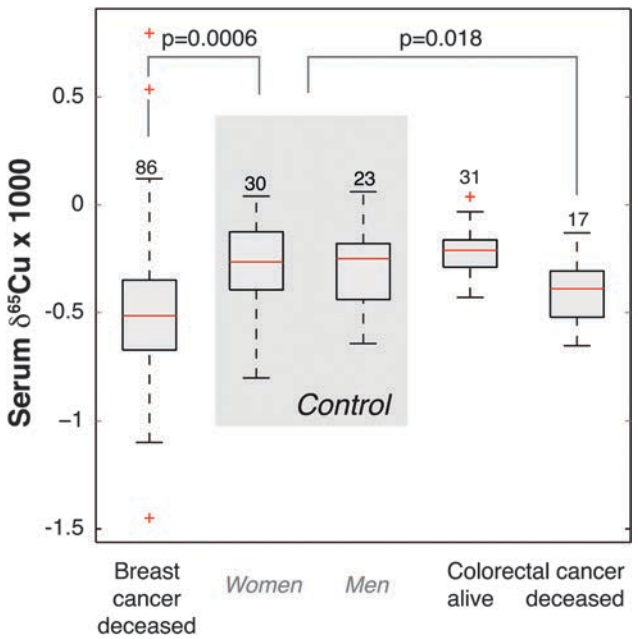


Fig. 2 Whisker plots of serum $\delta^{65}$Cu values for healthy men and women compared to breast cancer and colorectal cancer patients. Boxes represent the 75 percent middle quantiles and the whiskers 95 percent quantiles. Red lines: median; red crosses: outliers. Separation between breast cancer patients and healthy women is strong and seems to depend on mortality.

isotope abundances does not spontaneously change in serum samples. As an additional benefit, material sampled at earlier stages of the disease may be retrieved and analyzed and legacy biospecimens from biobanks can be entered into broad studies.

### Interpreting Cu isotope abundance shifts

An interpretation is needed to explain why cytosolic $Cu^{+}$ shuttled to ATP7A for excretion into the blood stream is isotopically lighter in cancer patients than in the control group.

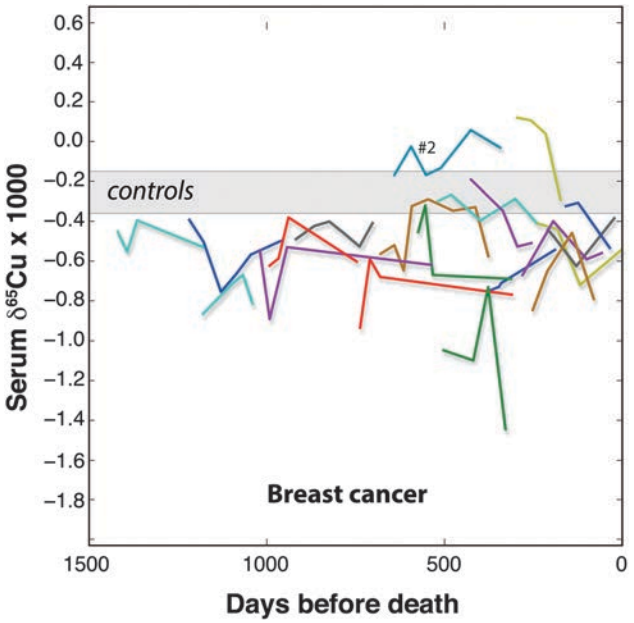


Fig. 3 Evolution of serum $\delta^{65}$Cu for the 20 breast cancer cases up to patient death. Each line represents a different patient with color used for differentiation purposes. The limits of the grey-shaded band (controls) were taken from Fig. 2.

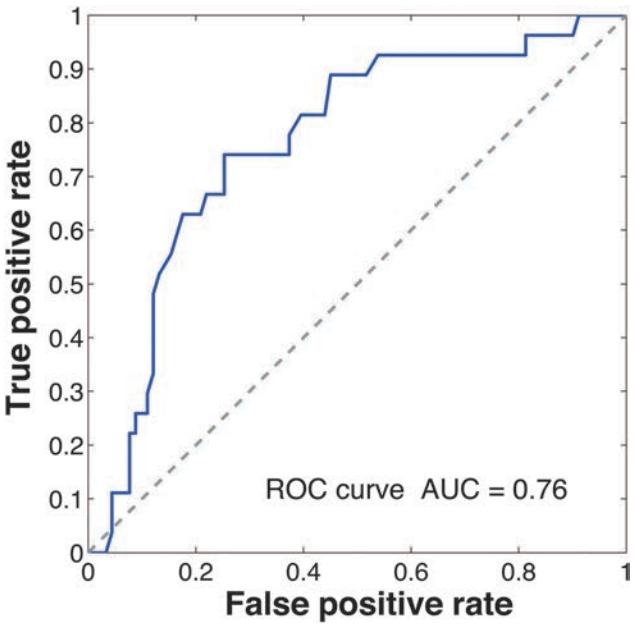


Fig. 4 Receiver Operating Characteristics (ROC) curve (*e.g.*, ref. 31) for $\delta^{65}$Cu in the serums of cancer patients. The ordinate is the probability of a True Positive decision for a particular cutoff value $\delta^{65}Cu_c$, and the abscissa the probability of False Positive decision. The ROC curve plots the points for all possible values of the cutoff $\delta^{65}Cu_c$. The optimum cutoff value for breast cancer is $\delta^{65}Cu_c$ = −0.37‰. The area under the ROC curve (AUC) may vary between 0.5 (pure chance) and 1.0 (fully trustworthy test): the value of 0.76 obtained for the present data set supports the worth of $\delta^{65}$Cu as a diagnostic tool.

Copper is known to occupy a number of sites in various protein families, but by far the most common Cu–protein bond is with histidine (Cu–N), cysteine and methionine (Cu–S).[32,33] Cu–O

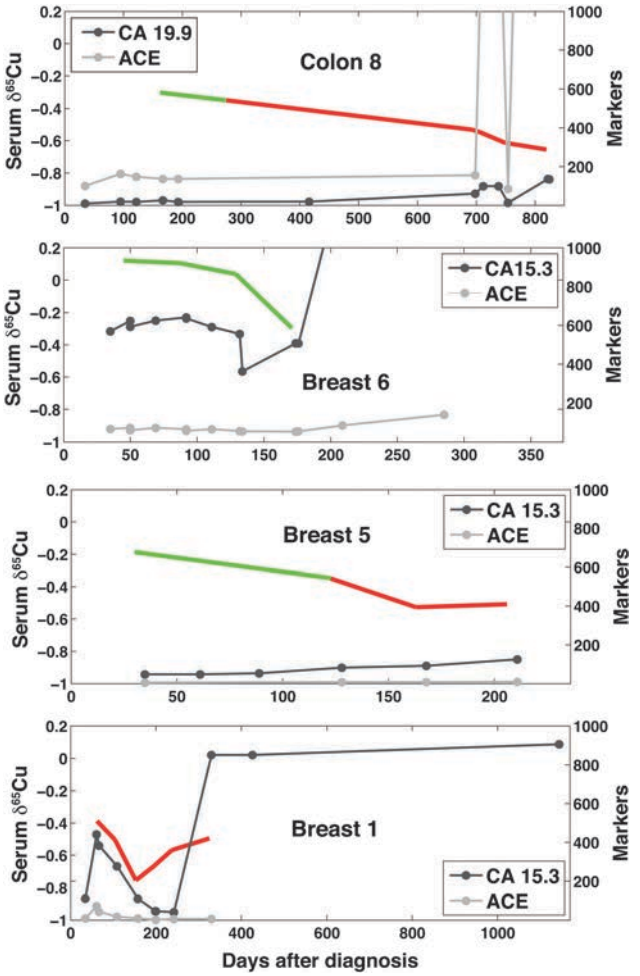


Fig. 5 Early alarm by $\delta^{65}$Cu. The colors (left axis) highlight the position of the serum Cu isotope composition relative to the $\delta^{65}$Cu alarm threshold of −0.35‰ (per mil). Molecular biomarkers (right axis): CEA (carcino-embryonic antigen) in dark grey and CA 19.9 and CA 15.3 (carbohydrate antigens) in light grey. These four patients show that a $\delta^{65}$Cu drop may lead the increase of biomarkers by several months.

bonds are less frequent and usually involve sulfate and hydroxide. It is expected that the strength of the bond will increase with ionization energy or electronegativity from S to N and O and therefore that Cu binding with histidine, such as in superoxide dismutase, will be isotopically heavy (high $\delta^{65}$Cu), whereas Cu binding with cysteine, such as in ceruloplasmin, will be isotopically light (low $\delta^{65}$Cu).[22] *Ab initio* calculations of isotope effects for a variety of Cu bonds[19] corroborated this prediction, hence opening up new possibilities for assessing which ligands are involved in the distribution of copper within cells of in-body fluids such as serum.

Kinetics potentially interferes with the *rate* at which the endpoint of a reaction will be attained. Isotope fractionation down concentration gradients, as invoked for Ca isotope effects,[34,35] is, however, an intrinsically transient process and should disappear at steady state. Fluxes and masses must be conserved, for both elements and their constituent isotopes, meaning that elements and isotopes cannot indefinitely accumulate in any particular cell or tissue. Inputs and outputs must eventually match each other. An isotope effect can be observed between multiple competing outputs, but not between a single input and a single output.

The present data cannot be explained by a simple hematocrit effect, whether shifted by the hypoxic conditions of cancer cells or not. Enhanced erythropoiesis would upregulate superoxide dismutase production (SOD). It would deplete the residual Cu stores normally allocated to ceruloplasmin production and make them isotopically lighter. However, considering the 0.7 to 1‰ difference in $\delta^{65}$Cu between erythrocytes and serum,[22] the shift observed for $\delta^{65}$Cu in patient serum is inconsistent with any viable change in hematocrits.

Having excluded diffusion and hematocrit effects, we will now explore how Cu isotope reflect the tight connection between lactate production, copper redox reactions, and the strong Cu chelation by lactate. Glucose burning (glycolysis) is the primary source of ATP, which is achieved by the attachment of inorganic phosphate $P_i$ to adenosine diphosphate (ADP). In normal *aerobic glycolysis* ATP is produced by a set of reactions summarized as follows:

$$\text{Glucose} + 2\text{ADP} + 2\text{NAD}^+ + 2\text{P}_i \Leftrightarrow 2\text{ATP} + 2\text{pyruvate}^- + 2\text{NADH} + 2\text{H}_2\text{O} + 4\text{H}^+ \quad (2)$$

where nicotinamide adenine dinucleotide ($NAD^+$) is an ubiquitous electron acceptor. NADH is further re-oxidized at the surface of mitochondrion with consumption of $H^+$. In cancer cells, in contrast, pyruvate is used as electron acceptor and aerobic glycolysis is replaced by *anaerobic glycolysis*:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \Leftrightarrow 2\text{ATP} + 2\text{lactate}^- + 2\text{H}_2\text{O} + 2\text{H}^+ \quad (3)$$

a reaction comparable to fermentation and known as the Warburg effect. Excess protons produced by the latter reaction are pumped out of the cell into the blood stream, which decreases its pH and greatly favors metastasis.

Lactate is painfully known to long-distance runners for accumulating in the muscles during prolonged strenuous anaerobic activity, but is normally metabolized back to glycogen by the liver after 24 hours. Lactate levels are also observed to be elevated in critically ill patients and correlate well with disease severity.[36,37] It has been observed that reduction or inhibition of lactate dehydrogenase, which catalyzes the forward and backward conversion of pyruvate to lactate, increases oxidative stress in cancer cells and promotes cellular death.[38] L-lactate concentrations of about 10 mM or even higher have been observed in the cytosol of cells taken from biopsies of metastatic tumors.[39] Unfortunately, excretion of lactate from the cytosol into the blood stream being regulated in a complex way by a family of proton-linked membrane transport proteins known as monocarboxylate transporters (MCTs),[40] data on serum lactate cannot easily be used to infer the status of lactate in cancer cells.

L-lactate (lact) binds to Cu(II) as Cu-lact$^+$ and Cu-lact$_2$, and the chelation constants reaches 330 and 19 500,[41,42] respectively.

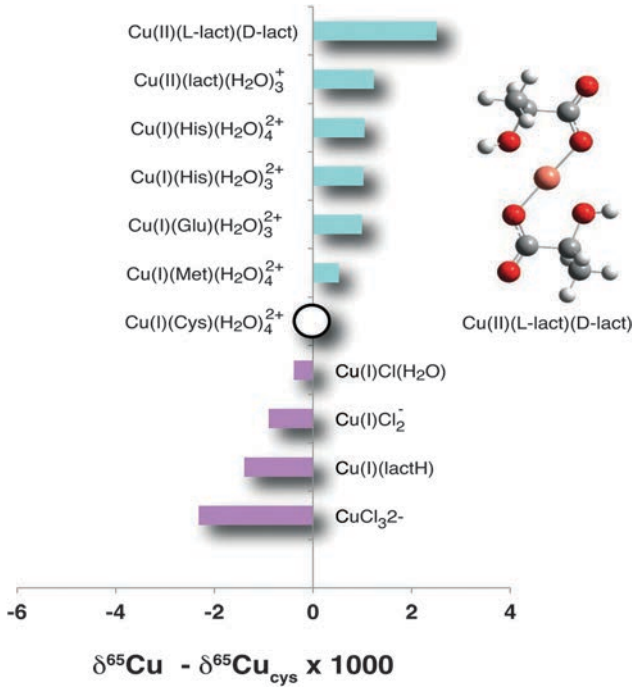


Fig. 6 Relative shift of $\delta^{65}$Cu for various Cu(I) and Cu(II) complexes relative to Cu(I) bound to cysteine as predicted by *ab initio* calculations.[19] As a rule of thumb, Cu(I) compounds are depleted in $^{65}$Cu relative to Cu(II) compounds, while Cu compounds with oxygen and nitrogen are enriched relative to compounds with sulfur. Right: the particularly stable [Cu(II)(L-lact)(D-lact)] complex.

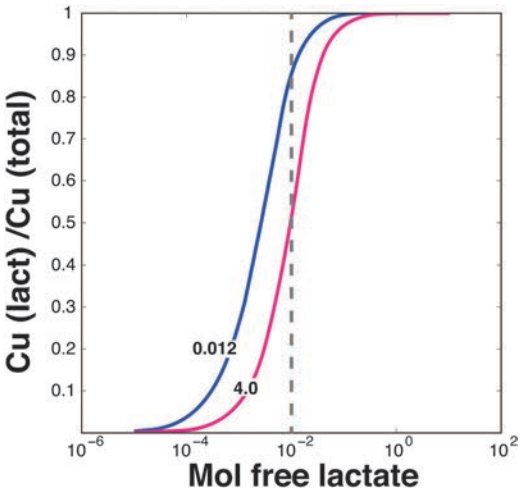


Fig. 7 Proportion of total copper chelated by lactates *vs.* free lactate concentration in the cytosol. The numbers on the curves represent the $Cu^+/Cu^{2+}$ ratio for a redox potential of 0.153 V (copper ions)[50] in blue and for a body potential of 0.27 V[51] in red. The vertical dashed line corresponds to a lactate concentration of 10 mmol typical of tumor cells.[39]

Chelation is relevant to the present study because *ab initio* calculations[19] indicated that the side hydroxyl renders Cu bound to lactate isotopically heavy, even more so than Cu bound to histidine (Fig. 6). The extent of $^{65}$Cu preference over $^{63}$Cu in Cu-lact$^+$ with respect to Cu engaged into a cysteine bond is more than 1‰, which is very large with respect to most common compounds.

The effect of Cu chelation on Cu isotopes in serum is better understood by first observing that $Cu^+$ oxidation is a potential source or electrons for pyruvate (pyr) reduction to lactate:

$$\text{L-pyr}^- + 2\text{Cu}^+ + 2\text{H}^+ \Leftrightarrow \text{L-lact}^- + 2\text{Cu}^{2+} \qquad (4)$$

Excess $H^+$, acidosis, caused by release of respiratory $CO_2$, and removal of lactate and $Cu^{2+}$ would, however, drive reaction (4) to the right. This is where Cu chelation becomes important: copper forms unusually stable mono-and bidentate lactate complexes following the reactions:

$$\text{Cu}^{2+} + \text{L-lact}^- \Leftrightarrow \text{Cu(L-lact}^-)$$

$$\text{Cu}^{2+} + 2\text{L-lact}^- \Leftrightarrow \text{Cu(L-lact}^-)_2 \qquad (5)$$

If lactate is produced faster than it is eliminated in the blood stream, the amount of free $Cu^{2+}$ is strongly reduced by chelation and reaction (4) proceeds to the right, thereby producing even more lactate. Although pyruvate does not spontaneously reduce to lactate, the lactate $Cu^{2+}$ complexes are therefore strong enough to secure massive cytoplasmic oxidation of $Cu^+$ to $Cu^{2+}$ and promote the correlative reduction of pyruvate to lactate. To a large extent, oxidative copper chelation by lactic acid mimics fermentation even in a relatively oxic environment. *Oxidative copper chelation* starts being significant at lactate concentrations of 0.1 mmol and is essentially complete at lactate concentrations of 100 mmol. At a lactate concentration of 10 mmol typical of tumor cells,[39] and under redox conditions typical of the human body, 50–80 percent of total cellular Cu is chelated by lactate (Fig. 7).

Expected shifts of measured $\delta^{65}$Cu due to Cu chelation are quite significant, notably low $\delta^{65}$Cu in serum due to free $Cu^+$ escaping chelation in lactate-rich cancer cells and shuttled to ATP7A for export, and the symmetrically elevated $\delta^{65}$Cu values in the tissue of hepatocarcinomas relative to healthy liver tissue (Balter *et al.*, PNAS, *in revision*).

Depending on lactic acid chirality, two enantiomers, Cu(L-lact)$_2$ and Cu(D-lact)$_2$, and the diastereomer Cu(L-lact)(D-lact) are present. Increased glyoxalase 1 expression by malignant transformation has recently been emphasized.[43–46] D-lactate is produced during glycolysis by the detoxification of cytoplasmic methylglyoxal,[47] a cytotoxic α-oxoaldehyde, which is normally disposed of by glutathione in a process regulated by glyoxalases. The DL diastereomer configuration being particularly stable, Cu isotopes therefore have some potential to clarify the processes involved in the glyoxalase pathway.

### Expected Cu isotope signature of therapeutic *vs.* endogenous Cu chelation

Copper chelation has been suggested as a therapeutic strategy to act on the pyruvate-lactate system and Cu isotopes may be used to test the mechanisms behind the Warburg effect. Tetrathiomolybdate (TTM and ATN-224, ref. 16) preferentially chelates $Cu^+$, which tends to starve cellular Cu uptake and shift cytosolic reaction (3) to the left. TTM counteracts lactate

 

production but meanwhile should also increase $H^+$. TTM should therefore help liberate isotopically heavy $Cu^{2+}$ into the blood stream and reverse the $\delta^{65}$Cu cancer signal. In contrast, the effect of methotrexate, which has been found to inhibit glyoxylase in acute lymphoid leukemia,[48] and D-penicillamine,[18] a strong chelate of divalent metals,[48,49] should be opposite to the effect of TTM and enhance the $\delta^{65}$Cu cancer signature. The potential of Cu isotopes as an indicator of how patients react to treatments targeting the Warburg effect in all its forms is worthy of further clinical and theoretical work.

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