

¹⁴C BOMB-PULSE DATING AND STABLE ISOTOPE ANALYSIS FOR GROWTH RATE AND DIETARY INFORMATION IN BREAST CANCER?

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The purpose of this study was to perform an initial investigation of the possibility to determine breast cancer growth rate with ¹⁴C bomb-pulse dating. Tissues from 11 breast cancers, diagnosed in 1983, were retrieved from a regional biobank. The estimated average age of the majority of the samples overlapped the year of collection (1983) within 3 σ . Thus, this first study of tumour tissue has not yet demonstrated that ¹⁴C bomb-pulse dating can obtain information on the growth of breast cancer. However, with further refinement, involving extraction of cell types and components, there is a possibility that fundamental knowledge of tumour biology might still be gained by the bomb-pulse technique. Additionally, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses were performed to obtain dietary and metabolic information, and to serve as a base for improvement of the age determination.

INTRODUCTION

Large amounts of the radioactive carbon isotope ¹⁴C were produced during atmospheric testing of nuclear weapons in the late 1950s and early 1960s. As a consequence, the concentration of ¹⁴C in air was almost doubled by 1963. Since the ¹⁴C produced was incorporated in atmospheric CO₂ and introduced into the global carbon cycle, all organisms living during the bomb-pulse era, including humans, have been labelled with bomb-¹⁴C^(1–3). When the Limited Test Ban Treaty from 1963 was implemented, the atmospheric ¹⁴C concentration commenced decreasing mainly due to uptake of ¹⁴CO₂ into the oceans and also into the biosphere (see Figure 1).

Already in the early 1970s, Harkness and Walton⁽⁸⁾ realised the potential of using bomb-¹⁴C as a kinetic tracer in humans. Since then, the well-known decreasing atmospheric ¹⁴C concentration has provided useful information in several fields in the medical sciences, see e.g. Falso and Buchholz⁽⁹⁾ and Spalding *et al.*⁽¹⁰⁾. For instance, this so called ¹⁴C bomb pulse has served in studies of turnover rates in human cells and tissues (e.g. in eye lenses⁽¹¹⁾, fat cells⁽¹²⁾ and Achilles tendon⁽¹³⁾). The development of various diseases has also been investigated

using the ¹⁴C bomb-pulse technique, with ¹⁴C measurements on e.g. gallstones⁽¹⁴⁾ and Alzheimer plaques⁽¹⁵⁾. A common denominator for these studies is slow turnover (years). Another example is Gonçalves *et al.*⁽¹⁶⁾, who demonstrated that human atherosclerotic plaques, known to cause e.g. heart attacks and strokes, develop slowly (mean biological ages 5–15 years). Quantification of the age of various structures in the plaque has gained a better understanding of the development of the plaque and opened for improved treatment methods.

The bomb-pulse technique has to the present authors' knowledge not yet been used to study cancer. Understanding of the time cause for the development of human cancer tissue is of utmost importance for improved prevention and treatment strategies. So far, certain assumptions have had to be made in order to estimate how long a cancer has grown. There are theoretical models to describe tumour growth rates, such as an exponential growth model or the Gompertz Model, where the latter takes into consideration a slower growth rate as the tumour size increases. These models have been applied to cancer in general⁽¹⁷⁾ and to breast cancer in particular^(18–20). These models have been shown to fit experimental and clinical data, such as observations of the doubling of tumour volume on serial mammograms. From these studies, the average

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tumour volume doubling time for human breast cancer has been estimated to be between 105 and 327 days^(21–29). Theoretically, this means that a breast cancer that is 10-mm large at detection (30 volume doublings) has been growing for 9–27 years. However, these studies are limited due to the lack of direct observations of tumour age.

The main purpose of this study was to perform an initial investigation of the possibility to determine the growth rate of breast cancer with ^{14}C bomb-pulse dating. An additional aim was to highlight the limitations of the ^{14}C bomb-pulse technique of human tissues due to dietary variations. Furthermore, the paper pays attention to how dietary information can be obtained from stable isotope ratio analysis of carbon and nitrogen, also providing means to increase the accuracy of bomb-pulse dating. Stable isotope ratio analysis may also give important information relating cancer development to diet.

Bomb-pulse dating and stable isotope analysis

The ^{14}C bomb-pulse technique uses ^{14}C data from atmospheric clean-air CO_2 to translate the ^{14}C specific activity of the sample into a calendar date (so called CaliBomb dates, see ‘Material and Methods’ section). For humans, carbon enters the body mainly through the diet. Thus, ^{14}C data from clean-air CO_2 may not be fully representative for humans.

A previous study by Georgiadou *et al.*⁽³⁰⁾ used human blood serum samples from a biobank to estimate the accuracy of bomb-pulse dating on human material. Blood serum samples collected from residents of Malmö (Sweden) in 1978 exhibited CaliBomb dates between 3.0 ± 0.4 years before the collection date and 0.2 ± 0.5 years after the collection date (the average deviation from collection date was -1.5 ± 0.7 years). Two major effects associated with the age deviation, competing in opposite directions, were identified: (1) delay time between production and consumption of foodstuffs, which can explain CaliBomb dates obtained before the collection date and (2) excessive consumption of marine food products, which has the potential of producing CaliBomb dates after the collection date.

As discussed in the study by Georgiadou *et al.*⁽³⁰⁾, there are also other factors that have the possibility to influence the obtained CaliBomb date, however, for the majority of the population probably to a more limited extent than delay time and marine food consumption. Anthropogenic ^{14}C released from nuclear power plants or from research laboratories, industry or hospitals using ^{14}C as a tracer has the potential of producing too old CaliBomb dates (at the declining bomb-pulse curve)^(31, 32). On the other hand, fossil fuel-based products in food industry (e.g. in CO_2 used in cultivation in greenhouses) as well as food grown in heavily industrialised areas may lead to foodstuffs

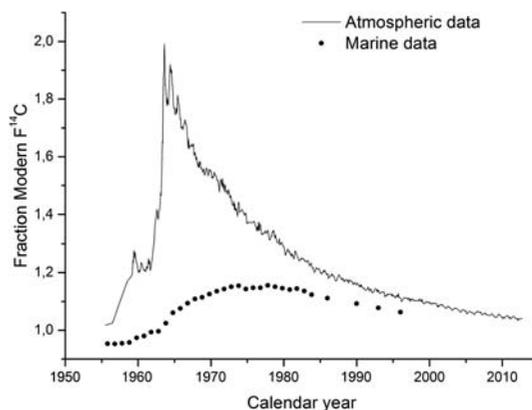


Figure 1. ^{14}C specific activity in atmospheric CO_2 and oceans representative for the northern hemisphere (expressed in units of Fraction Modern, $F^{14}\text{C}$ ⁽⁴⁾; $F^{14}\text{C}$ is ~ 1.0 before the nuclear weapons tests, corresponding to the natural $^{14}\text{C}/^{12}\text{C}$ ratio of about $10^{-10}\%$ ^(5–7)).

having lower ^{14}C specific activity than clean air, thus contributing to producing too young CaliBomb dates (at the declining bomb-pulse curve)⁽³³⁾.

^{14}C bomb-pulse dates reported in the literature usually do not take into account that there is a delay time between production and consumption of the food^(33, 34). Neither is it commonly considered that marine foodstuffs can influence the accuracy of the calibration (see the difference in marine and atmospheric calibration curve in Figure 1). However, since a large consumption of marine foodstuffs has the potential to affect the calibration curve (see Figure 1) on an individual basis, information about the diet may be important in bomb-pulse dating of human tissue samples. Additionally, the individual diet is of interest for studies of the development of various diseases, including breast cancer⁽³⁵⁾.

One technique to assess the diet of an organism is analysis of the ratios of stable isotopes of carbon and nitrogen (expressed as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in ‰, where $\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$, and R is the ratio of heavy to light isotope, i.e. $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$) (see e.g. Schoeller⁽³⁶⁾). $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ provide dietary information since the stable isotope ratios in tissues and organs of the consumer reflect those of the diet with a small shift. This discrimination of one of the stable isotopes occurs in each step of the food chain. The difference in δ -value between an organism and its diet, referred to as the discrimination factor, is generally about 1‰ for $\delta^{13}\text{C}$ and 3‰ for $\delta^{15}\text{N}$ ⁽³⁷⁾. The discrimination factor, however, also depends on various factors such as species, age, metabolic processes and environmental conditions (see e.g. Caut *et al.*⁽³⁷⁾ and O’Connell *et al.*⁽³⁸⁾). Thus, different trophic levels (positions in

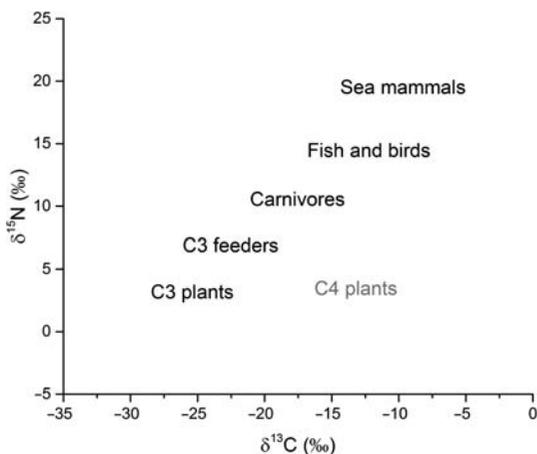


Figure 2. Generalised isotopic trophic level diagram for terrestrial and marine food webs (values from Georgiadou *et al.*⁽³⁰⁾). The $\delta^{13}\text{C}$ values of C3 plants (e.g. wheat and potatoes) differ from those of C4 plants (e.g. maize and sugar cane) due to photosynthetic pathways. For the Swedish diet, of relevance for this study, C3 plants dominate over C4 plants.

the food chain) are characterised by different ranges of δ values (see Figure 2).

Despite the complexity and variation of discrimination factors, several studies have shown that $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ are valuable biomarkers of dietary intake (see e.g. Georgiadou *et al.*⁽³⁰⁾ and Patel *et al.*⁽³⁹⁾ and references therein). The approach can additionally be used as a tool to identify samples where extreme dietary conditions (high intake of marine products) might influence the ^{14}C bomb-pulse date (decreasing the applicability of the atmospheric calibration curve).

The serum study⁽³⁰⁾ showed that the age deviation (difference between CaliBomb date and actual sampling date) correlated strongly with $\delta^{13}\text{C}$, which was interpreted as influence from marine dietary components. $\delta^{15}\text{N}$, however, did not show any correlation with age deviation in that study. $\delta^{13}\text{C}$ data from the serum study have also been used to demonstrate the possibility to develop general methods increasing accuracy as well as precision of the age determination using the ^{14}C bomb-pulse technique⁽⁴⁰⁾. However, more data are needed to produce a general correction model.

MATERIAL AND METHODS

Frozen breast cancer tissues from 11 women collected in 1983 were retrieved from the South Swedish Breast Cancer Group Biobank. In three cases, the samples were large and sub-samples from each tumour could be taken (maximum of three sub-samples per cancer).

Prior to ^{14}C analysis, parts of each tissue sample were graphitised according to the procedures described by Andersson Georgiadou *et al.*⁽⁴¹⁾ and Genberg *et al.*⁽⁴²⁾. Total carbon in the graphite samples ranged between 32 and 124 μg . The $^{14}\text{C}/\text{C}$ ratio, expressed as F^{14}C ⁽⁴⁾, was analysed with accelerator mass spectrometry (AMS) at the Lund Single Stage AMS facility at Lund University^(43, 44). The analytical precision of the F^{14}C measurements for human tissue samples is usually 0.5–1% for this instrument⁽⁴⁰⁾. The obtained F^{14}C values were translated into calendar years using the CaliBomb software (<http://calib.qub.ac.uk/CALIBomb/>), choosing the calibration dataset ‘Levin’ (representative for European clean-air CO_2) and ‘smoothing’ (averaging the dataset) of 0.5 years. A given analytical uncertainty in F^{14}C results in various uncertainties in the CaliBomb date depending e.g. on the steepness of the declining bomb-pulse curve and on seasonal variations (see Figure 1).

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were analysed using isotope ratio mass spectrometry (IRMS) at the Department of Biology, Lund University, using between 0.28 and 0.40 mg of dried tumour material for each sample. The analytical precision obtained for standards at this instrument is usually <0.4‰ for carbon and <0.12‰ for nitrogen (1σ). Further information about the IRMS analysis can be found in Andersson Georgiadou⁽⁴⁰⁾.

RESULTS AND DISCUSSION

The results of the ^{14}C measurements and the IRMS analysis are shown in Table 1. A few samples lack IRMS data due to insufficient amount of sample material. The analytical uncertainties of the ^{14}C measurements were higher than normally because of the difficulty of accurately weighing the samples and due to smaller and more varying sample sizes than usually.

Table 1 only includes CaliBomb dates after the peak in 1963 (all calibrations also return a result between the mid-1950s and beginning of the 1960s). It is however not likely that the results from the rising part of the bomb-pulse curve are relevant in this study. A result from the rise of the bomb pulse would imply that the carbon in the analysed tissue was about 20 years old at the time of collection for all the subjects (not likely, the subjects were between about 30 and 60 years of age in the late 1950s). Another alternative would be that the turnover of cells is very slow resulting in an average F^{14}C value corresponding to the rise of the bomb pulse. Neither this explanation appears likely. The most plausible explanation seems to be that the carbon in the breast tumour samples mainly originates from recently consumed food.

Table 1. Results from the ¹⁴C and IRMS analyses of the breast tumour samples.

| Sample | Year of birth | Lab code | ¹⁴ C analysis | | | IRMS analysis | | |
|--------|---------------|----------|--------------------------|------------------------|--------------------|---------------|-----------------------|-----------------------|
| | | | m C (µg) | F ¹⁴ C ± 1σ | CaliBomb date ± 1σ | m (mg) | δ ¹³ C (‰) | δ ¹⁵ N (‰) |
| T1 | 1924 | GEO_1511 | 45 | 1.211 ± 0.014 | 1984.8 ± 1.1 | 0.30 | -22.3 | 11.0 |
| T2 | 1901 | GEO_1512 | 56 | 1.193 ± 0.013 | 1986.2 ± 1.9 | 0.30 | -22.0 | 11.7 |
| T3A | 1928 | GEO_1513 | 130 | 1.309 ± 0.015 | 1979.3 ± 0.6 | 0.35 | -22.5 | 13.3 |
| T3B | | GEO_1514 | 63 | 1.241 ± 0.014 | 1982.8 ± 0.9 | 0.28 | -22.7 | 12.8 |
| T3C | | GEO_1515 | 88 | 1.240 ± 0.014 | 1982.9 ± 0.9 | | | |
| T4 | | GEO_1516 | 54 | 1.239 ± 0.014 | 1982.9 ± 0.9 | | | |
| T5 | 1919 | GEO_1517 | 53 | 1.222 ± 0.014 | 1984.1 ± 0.8 | 0.39 | -21.2 | 13.2 |
| T6 | 1901 | GEO_1518 | 124 | 1.244 ± 0.014 | 1982.7 ± 0.9 | 0.37 | -21.0 | 13.0 |
| T6:2 | | GEO_1519 | 103 | 1.232 ± 0.014 | 1983.1 ± 0.8 | | | |
| T7 | 1898 | GEO_1520 | 83 | 1.274 ± 0.014 | 1980.9 ± 1.0 | 0.32 | -27.3 | 9.3 |
| T8 | 1932 | GEO_1521 | 101 | 1.247 ± 0.014 | 1982.3 ± 1.3 | 0.40 | -23.3 | 11.0 |
| T9A | 1902 | GEO_1522 | 52 | 1.184 ± 0.013 | 1987.5 ± 1.7 | 0.35 | -22.8 | 9.4 |
| T9B | | GEO_1523 | 37 | 1.190 ± 0.013 | 1986.7 ± 1.7 | 0.28 | -24.7 | 7.8 |
| T9C | | GEO_1524 | 32 | 1.157 ± 0.013 | 1990.2 ± 1.7 | | | |
| T10 | 1915 | GEO_1525 | 98 | 1.137 ± 0.013 | 1992.8 ± 3.3 | 0.37 | -20.6 | 13.7 |
| T11 | 1911 | GEO_1526 | 79 | 1.223 ± 0.014 | 1984.1 ± 0.7 | 0.32 | -21.9 | 10.3 |

All samples were collected in 1983. Only results from the time after the bomb-pulse peak in 1963 are shown. A few samples lack IRMS data due to insufficient amount of sample material (m means mass of carbon (m C (µg)) and sample (m (mg)), respectively).

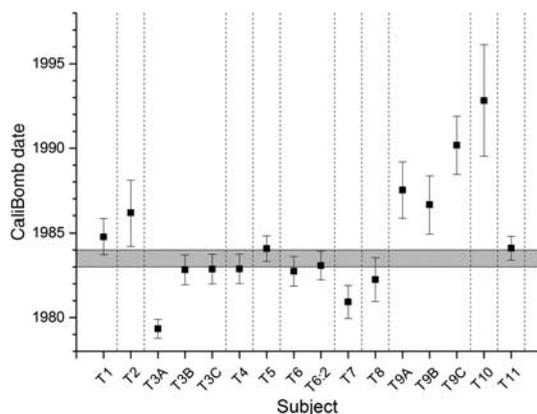


Figure 3. Results from the ¹⁴C measurements (±1σ) for each subject (the year of collection, 1983, is indicated in grey).

Figure 3 shows the results from the ¹⁴C measurements (±1σ, results only for the declining part of the bomb-pulse curve) for each subject (the year of collection was 1983). The CaliBomb date of all samples except T3A and T9C overlaps with the year of collection 1983 within 3σ.

Figure 4 shows the δ¹³C and δ¹⁵N values obtained for the tumour samples. Sample T7 seems to lie apart from rest of the samples. In contrast to the other samples, T7 contained mainly fat tissue, alternatively necrosis, which might explain the separate location of the samples in the diagram. In stable isotope analysis

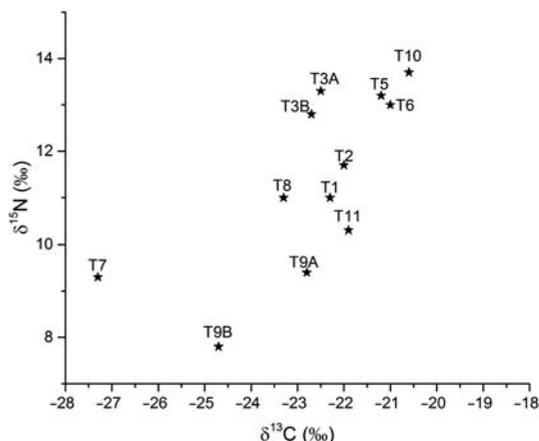


Figure 4. δ¹³C and δ¹⁵N obtained for the tumour samples (compare with Figure 2). A few tumour samples lack IRMS data due to insufficient amount of sample material.

of human tissues, it is also known that different tissues do not have identical δ¹³C and δ¹⁵N values (see e.g. Caut *et al.*⁽³⁷⁾ and Tieszen *et al.*⁽⁴⁵⁾). Fatty acids, e.g., are known to be depleted in δ¹³C (having a negative discrimination factor) compared to other tissues as well as diet. Schoeller *et al.*⁽⁴⁶⁾ found that δ¹³C (plasma lipid) < δ¹³C(plasma protein) in 10 American subjects. Tieszen *et al.*⁽⁴⁵⁾ report that δ¹³C values in gerbil were ranked as fat < liver < muscle < brain < hair (the discrimination factor between diet and fat being about -3‰). The present results for sample T7 follow

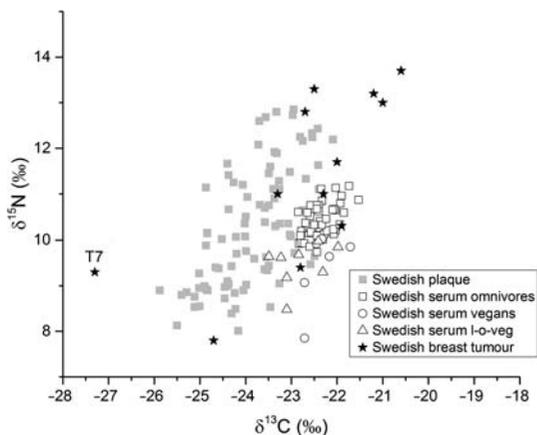


Figure 5. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for the tumour samples and previous measurements on Swedish plaque⁽⁴¹⁾ and serum⁽³⁰⁾. Note that serum for the omnivores, in general, has higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ than vegans and lacto-ovo vegetarians (l-o-veg), in concordance with the generalised isotopic trophic diagram in Figure 2.

the same trend as these observations and indicate that $\delta^{13}\text{C}(\text{fat cells/fat tissue}) < \delta^{13}\text{C}(\text{cancer cells})$.

Figure 5 shows the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values obtained for the tumour samples including previous measurements of serum and atherosclerotic plaques from Swedish patients (see Gonçalves *et al.*⁽⁴⁷⁾). It should be noted that the subjects are not the same, neither is the year of collection (serum from 1978 and plaque from 2007–2009). However, in this diagram sample T7 still lies apart from the rest of all sample types. Figure 5 also indicates linear correlations between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for the various sample types. This can be expected considering the trophic level diagram in Figure 2.

The main purpose of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ measurements of the breast tumour samples was to evaluate if the diet of any of the subjects contained a large proportion of fish products, which could influence the ^{14}C value (according to Figure 1). High values of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (upper right corner of Figure 2) correspond to more fish products in the diet than low values (lower left corner of Figure 2). According to Figures 4 and 5, tumour samples with high $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (upper right corner) could indicate that these subjects consume more marine foodstuffs than the others (e.g. the diet of subjects 5, 6 and 10 contains more fish products than others). The excess marine influence could subsequently affect the bomb-pulse dating (see Figure 1), and possibly give dates after the collection date (above the grey area in Figure 3). This is not observed (see Figure 3). In view of the limited amount of samples, the magnitude of the uncertainty of the measurements, the

variability of discrimination factors and probably variability in growth rate of breast tumours, it is difficult to draw any general conclusions from the findings.

It is evident that further development of the application of ^{14}C bomb-pulse dating technique for breast cancer research is needed before it can be of use. A main drawback of the technique is that the ^{14}C content in the tumour tissue originating from the time when the tumour was in its initial stage is small relative to the ^{14}C content from the later created part of the tumour. Analysis of extracted tumour DNA may be a way to obtain more relevant growth rate information, since 50% of carbon in DNA originates from the latest cell division⁽⁹⁾. A better approach to estimate the onset of the tumour growth would be to isolate and analyse cancer stem cell DNA. The main challenge of this approach will be to obtain samples large enough for the ^{14}C analysis (at least $10\ \mu\text{g}$ of carbon is required for the system used). An associated difficulty is that the slope of the bomb pulse nowadays is approaching zero. This means that the resulting uncertainty in the age determination increases to inapplicability when approaching present days. Thus bomb-pulse dating of fresh material is only possible for cells or tissues with a slow turnover (several years). For materials with shorter turnover, relevant age information can only be obtained from the steeper part of the bomb-pulse curve, and biological material from biobanks must be used. The available amounts of sample material in the biobanks are however often very limited.

If the estimate of the ^{14}C turnover rate can be more exact, the information gained can be of value, primarily in the understanding of tumour biology: which is the tumour growth rate? Also, the clinical implications of the dating of breast cancer could be the revision or validation of existing tumour growth models. Furthermore, in breast cancer screening one major drawback is the overdiagnosis of slow-growing indolent tumours. Overdiagnosis occurs when women without symptoms are diagnosed with a disease that will not cause them to experience symptoms or lead to early death⁽⁴⁸⁾. It has been estimated that the overdiagnosis in breast cancer screening is 10–20%^(49, 50). It is not possible to incorporate ^{14}C bomb-pulse dating in clinical practice since the method is both laborious and expensive and most importantly the atmospheric levels of ^{14}C are now down at a low level. That is why the ^{14}C bomb-pulse dating technique applied on breast cancer combined with an analysis of micro- and macro-structural changes in the tumour, in order to find a signature of a slow growing cancer, could be of interest. For example, in parallel to the efforts of obtaining relevant tumour growth rate information, samples of the same breast tumour could be investigated with a high-resolution imaging technique, i.e. synchrotron

radiation-based small angle X-ray scattering to detect changes in the collagen structure of the tumour tissue⁽⁵¹⁾. If structural changes in a breast tumour can be correlated to growth rate, this could provide means to find new biomarkers or a morphological signature that can be used to further refine breast cancer diagnosis and ultimately optimise the treatment, e.g. less aggressive treatment of indolent tumours.

CONCLUSIONS

This is a first pilot study assessing the potential of carbon and nitrogen isotope analysis for obtaining growth rate (¹⁴C/¹²C) and dietary (¹³C/¹²C and ¹⁵N/¹⁴N) information for breast cancer research. Stable carbon and nitrogen isotope analysis may give information about the relation between diet and breast cancer development and progression.

The measurements in this limited, initial study could not demonstrate the possibility to obtain age information on the onset of the breast cancer. However, analysis of total tumour material may primarily reflect the current turnover of the carbon in the various components of the metabolically active tumour tissue. Analysis of extracted tumour DNA, or the isolation and analysis of cancer stem cells, may be a way to obtain more relevant growth rate and age information. Furthermore, ¹³C/¹²C and ⁵N/¹⁴N analysis may provide important dietary information of value for studies of possible correlations between diet and tumour development.

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