Effects of Pre-surgical Vitamin D Supplementation and Ketogenic Diet in a Patient with Recurrent Breast Cancer

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Abstract. *Background: A woman, mother of one at the age of 19 years, was diagnosed with mammary adenocarcinoma in the right breast in 1985 at the age of 37 years. The patient underwent surgery (quadrantectomy), lymphadenectomy and radiotherapy. In 1999, an adenocarcinoma was diagnosed in the left breast, followed by adequate resection, radiotherapy and anti-oestrogen receptor treatment for 6 years. In March 2014, an infiltrating adenocarcinoma was diagnosed in the remaining part of the right breast that had been operated on and irradiated in 1985. Case Report: The pre-surgical biopsy, showed weak positivity for progesterone receptor (PgR) (<1%), high positivity for oestrogen receptor (ER) (90%), high positivity for human epidermal growth factor receptor (HER2) (>10%, score 2+), and high positivity for the nuclear protein Ki67 (30%). In the three weeks between diagnosis and operation, when no other treatment had been planned, the patient decided to self-administer high doses of oral vitamin D_3 (10,000 IU/day), and to follow a strict ketogenic diet. Results: Following right mastectomy, analysis of the surgical specimen showed no positivity for HER2 expression (negative, score 0), and significant increase in positivity of PgR (20%). Positivity for ER and Ki67 were unaltered. Conclusion: This observation indicates that a combination of high-dose vitamin D_3 and ketogenic diet leads to changes in some biological markers of breast cancer, i.e. negativization of HER2 expression and increased expression of PgR.*

Vitamin D plays a significant role in protection against breast cancer and several studies demonstrate that increased serum levels of vitamin D metabolites are associated with decreased risk of breast cancer (1-5). Since 1998, our group and others have demonstrated that in addition to serum vitamin D levels, polymorphisms of the vitamin D receptor gene are associated with the risk of breast cancer (6, 7), thus defining a complex interplay between genetic and environmental factors in the onset and progression of breast cancer.

Vitamin D_3 is generally classified as a nutritional supplement and, in the form of oral preparation, in most countries does not require a medical prescription. Therefore, patients, as well as healthy individuals, often include vitamin D_3 supplementation in their nutritional regimens, sometimes together with other vitamins and supplements. Patients with cancer are particularly sensitive to these issues since it has been thoroughly demonstrated that proper nutrition is helpful in cancer management and, consequently, there is an emerging trend in designing cancer-specific diets (8). Among the different nutritional approaches to cancer, the so-called ketogenic diet has recently acquired popular relevance (9), and patients with cancer often decide to follow such a diet as a measure to reduce cancer growth and to maximize the anticancer potential of conventional approaches.

A common denominator of ketogenic diets is the restriction of carbohydrate consumption that is balanced by an increase in the consumption of fats. In Mediterranean countries, one of the principal sources of fat-derived calories is olive oil, and it has been amply demonstrated that oleic acid consumption is beneficial in cancer management, as widely described in literature (10-14).

Regrettably, however, one of the major side-effects of a ketogenic diet is vitamin D_3 deficiency, an occurrence that was first observed in children subjected to such a diet in order to control epilepsy (15, 16). Therefore, it can be hypothesized that patients with cancer embarking on ketogenic diets may benefit from vitamin D_3 supplementation as a measure to counteract its deficiency, at the same time benefitting from the role of this vitamin in cancer management. Olive oil, frequently used in ketogenic diets to provide calories, might further contribute to the overall benefit of nutritional cancer management because of its known anticancer properties (17).

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In addition, it is worth considering that mono- and poly-unsaturated fatty acids strongly reduce the affinity of vitamin D_3 for its binding protein, thus increasing its bioavailability and, possibly, contributing to an increase in its anticancer properties (18).

Herein we report the case of a woman diagnosed with recurrent breast cancer who followed a ketogenic diet rich in olive oil and high doses of vitamin D_3, as self-administered supplementation, for three weeks prior to planned surgery. Such a nutritional strategy led to unexpected changes in her tumor markers.

Patients and Methods

A woman, mother of one at the age of 19 years, was diagnosed with mammary adenocarcinoma in the right breast in 1985, at the age of 37 years. The diagnosis was followed by partial resection (quadrantectomy) with lymphadenectomy and post-surgical irradiation. In 1999, at the age of 51 years, an adenocarcinoma was diagnosed in the left breast; since no testing had been done on the previous tumour, it is not known whether this was a recurrence or an independent occurrence. At that time the diagnosis was followed by adequate resection, but not full mastectomy, irradiation and anti-oestrogen receptor (ER) treatment for 6 years as a preventative measure.

In March 2014, at the age of 66 years, following routine mammography, an infiltrating adenocarcinoma was diagnosed in the remaining part of the right breast that had been operated on and irradiated in 1985. In the proximity of the areola, in the superior external quadrant, an inhomogeneous lesion of 14×9.2×12 mm (1.545 mm^3) with irregular margins, was detected and confirmed by ultrasonography. Immediately after this diagnosis, the patient underwent ultrasound-guided fine-needle (14-gauge) biopsy, and four specimens from different areas of the lesion were examined. Magnetic resonance imaging (MRI) was performed to assess local extension of the tumour. The MRI, the surgery, and the analyses on oestrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor (HER2) and nuclear protein Ki67 (both on the preoperative biopsy and on the surgical specimens) were performed at the University Hospital (Italian Public Health Service) of Firenze, Italy. The patient retains the original documents, and reports and copies are conserved in the archives of the University Hospital of Firenze, Italy.

Analyses were performed according to the European standards of quality (UNI EN ISO 9001:2008) (19), and were examined and countersigned by four different medical professionals. The patient gave her informed consent to the analyses, intervention, and treatments performed by the Italian Public Health Service, as well as written informed consent to the description of her results reported here.

The diagnostic technique routinely used to evaluate and assess tumour biomarkers status in clinical practice was immunohistochemistry (IHC) performed following the College of American Pathologists, and American Society of Clinical Oncology guidelines (www.asco.org) (20). Briefly, the pre-analytical phase was standardized for all the surgical specimens, with a cold ischemia time less than 1 h and a formalin fixation time, in 10% neutral buffered formalin, within a time frame of between 24 and 48 h. Formalin-fixed paraffin-embedded blocks were cut into 4 μm-thick sections, mounted on silane-coated glass slides, and baked overnight at 56°C. IHC was performed on four different samples using the following antibodies: anti-ER (SP1 rabbit monoclonal; Ventana Medical Systems, Tucson, AZ, USA), anti-PgR (1E2 monoclonal; Ventana Medical Systems), anti-HER2 (A 0485 rabbit polyclonal; DAKO, Glostrup, Denmark) and anti-Ki67 (MIB1 monoclonal; Ventana Medical Systems). A pathologist, specialized in molecular and cellular diagnostics, evaluated each specimen for: the percentage of stained cells (scored by evaluating the entire surgical specimen area); the intensity of the staining (weak, moderate, strong), and the pattern (complete or incomplete) of membraneous staining. Furthermore, concerning HER2 status, the score according to the Food and Drug Administration four-tier scoring system (0, 1+, 2+, 3+) was used. Evaluations and scores were performed for each of the four pre-surgical fine-needle biopsy specimens, as well as for the entire surgical specimen area.

During the three weeks between the diagnostic biopsy and the planned mastectomy, when no pharmacological treatment had been prescribed, the patient decided to follow a strict ketogenic diet and to consume high doses of oral vitamin D_3 (10,000 IU every other day). In addition, the patient self-administered a commercial preparation of oleic acid associated with glycosylated vitamin D-binding protein, branched chain amino acids, and enriched her diet with commercial olive oil and fermented milk products rich in natural glycosylated vitamin D-binding protein. It is worth noting that the patient had not received any treatment for the previous 8 years and, until the last diagnosis, had followed a typical Mediterranean diet that is relatively rich in carbohydrates.

Amplification or overexpression of the oncoprotein HER2 plays an important role in the development and progression of breast cancer and has become an important biomarker and a target for therapies (21) since it is strongly associated with increased disease recurrence and poor prognosis (22). Consistent with the aggressive nature of the cancer in this patient, the preoperative biopsy on four specimens collected under ultrasound guidance showed significant positivity for HER2 (>10%) and a score of 2+ (Table I and Figure 1). The aggressive nature of this cancer is consistent with the report of the MRI performed with contrast medium, describing an area of impregnation of about 6 mm in correspondence of the *pectoralis* muscle that led to the suspicion of infiltration of the muscle itself (Figure 2). Consistent with these results, PgR expression in the preoperative biopsy was found to be low (<1%), a finding indicating poor differentiation and tumour aggressiveness (Table I and Figure 3). Study of the expression of nuclear protein Ki67 (a well-assessed cellular marker of proliferation), and ER (30% and 90% positivity, respectively) confirmed the aggressive nature of the cancer (Table I).

After 3 weeks of self-administered high doses of oral vitamin D_3 in the context of a ketogenic diet rich in olive oil, the patient underwent mastectomy; during the operation, samples of the *pectoralis* muscle were taken to assess the infiltration documented in the preoperative MRI. Histological analyses were performed on the surgical specimens.

The results reported by the Departmental Oncologic Reference Centre describe negativity for HER2 expression (Table I and Figure 4), and an increase in the positivity for PgR expression (*i.e.* 20% *vs.* <1%) (Table I and Figure 5). At the intervention, a mastectomy of the right nipple area with removal of a small specimen of the grand *pectoralis* muscle plus insertion of a tissue expander and T-loop web was performed. The report of the intervention described the presence of a ductal and lobular infiltrating carcinoma, grade 3, with a diameter of 13 mm (superior external quadrant) with minimum

Table I. *Pre-surgical and post-surgical evaluation of tumor biomarkers.*

Marker	Pre-surgical evaluation	Post-surgical evaluation
Oestrogen receptor (clone SP1)	90%	90%
Progesteron receptor (clone 1E2)	<1%	20%
Nuclear protein Ki67 (clone MIB1)	30%	30%
Human epidermal growth factor receptor (polyclonal A 0485)	>10% (score 2+)	(score 0)

Table II. *Effects on human breast cancer cells of vitamin D_3, oleic acid and ketogenic diet.*

	Vitamin D_3	Oleic acid	Ketogenic diet (9)
Effects	Induction of differentiation (26) Down-regulation of genes responsible for invasion and metastasis (27)	Down-regulation of HER2 expression (10) Cancer-specific cytotoxic effects due to perturbation of membrane structure (30)	Reduction of *de novo* synthesis of diacylglycerol (32) Reduction of protein kinase C activation (32) Reduction of HER2 expression (34)

HER2: Human epidermal growth factor receptor.

distance between the tumour and major areolar margin of 10 mm. Furthermore, the description stated the absence of vascular, haematic/lymphatic peritumoural invasion and, surprisingly, no neoplastic proliferation in the right axillary pillar (*i.e.* the *pectoralis* muscle). The status of the ER and Ki67 markers was unaltered (Table I).

Discussion

This case, although anecdotal, reports noteworthy events that are consistent with the existing literature (23). Although we have no elements to demonstrate a causal relationship between the nutritional regime followed by the patient and changes in tumor markers reported above, nevertheless, these changes are consistent with current knowledge on the effects of vitamin D_3, oleic acid and a ketogenic diet, on human breast cancer *in vitro* and *in vivo*. Thus, as presented in Table II, vitamin D_3, oleic acid and a ketogenic diet are known to influence the expression of HER2 in the context of the induction of cancer cell differentiation or apoptosis and, therefore, to play a role in the overall prognosis of breast cancer.

In fact, a very recent study demonstrates that vitamin D supplementation in patients with non-metastatic HER2-positive breast cancer was associated with improved disease-free survival, and this effect was attributed to the interplay between vitamin D receptor signaling and HER2 signaling through the HER2/protein kinase B (AKT)/ extracellular-signal-regulated kinases (ERK) pathway (24). These results are consistent with the observation that oral administration of a vitamin D analogue prevents mammary tumorigenesis driven by HER2 overexpression (25). As far as the molecular mechanisms underlying this effect of vitamin D_3 on HER2 expression are concerned, it can be postulated that vitamin D_3 induces differentiation of breast cancer cells or their apoptosis, with a consequent decrease of cancer cells expressing HER2. This hypothesis is consistent with the significant increase of PgR observed in this case. It appears, however, that induction of differentiation is independent of HER2 expression, since it was demonstrated that vitamin D_3 also induces differentiation of triple-negative breast cancer cells by inducing *de novo* E-cadherin expression (26). These effects are due to significant changes of expression of multiple genes induced by vitamin D_3 in breast cancer cells: it was demonstrated that at least 35 genes are regulated by vitamin D_3 in breast cancer cells and among the genes down-regulated, there are those that drive breast cancer invasion and metastasis (27). Interestingly, these effects of vitamin D_3 on gene-expression profiles also appear to occur in triple-negative breast cancer cells, thus reinforcing the idea that our observation can be extended to triple-negative breast cancers.

On the other hand, it has been demonstrated that oleic acid down-regulates HER2 expression in cancer cell lines (10), and these effects are so well assessed that the authors of one article wondered if an anti-HER2 oncogene nutraceutical was known as early as the 17th century (28). As far as the molecular mechanism is concerned, it was demonstrated that oleic acid induces the formation of inhibitory polyoma enhancer activator 3 (PEA3) transcription factor-PEA3 DNA binding site complexes at the *HER2* oncogene promoter in breast, ovarian and stomach cancer cells (29). In addition, oleic acid, when carried by proteins such as alpha-lactalbumin, exerts a selective cytotoxic effect on cancer cells (30). Since this latter effect does not appear to be dependent upon gene expression (31), it can be hypothesized that these anticancer properties may also be evident in triple-negative breast cancer.

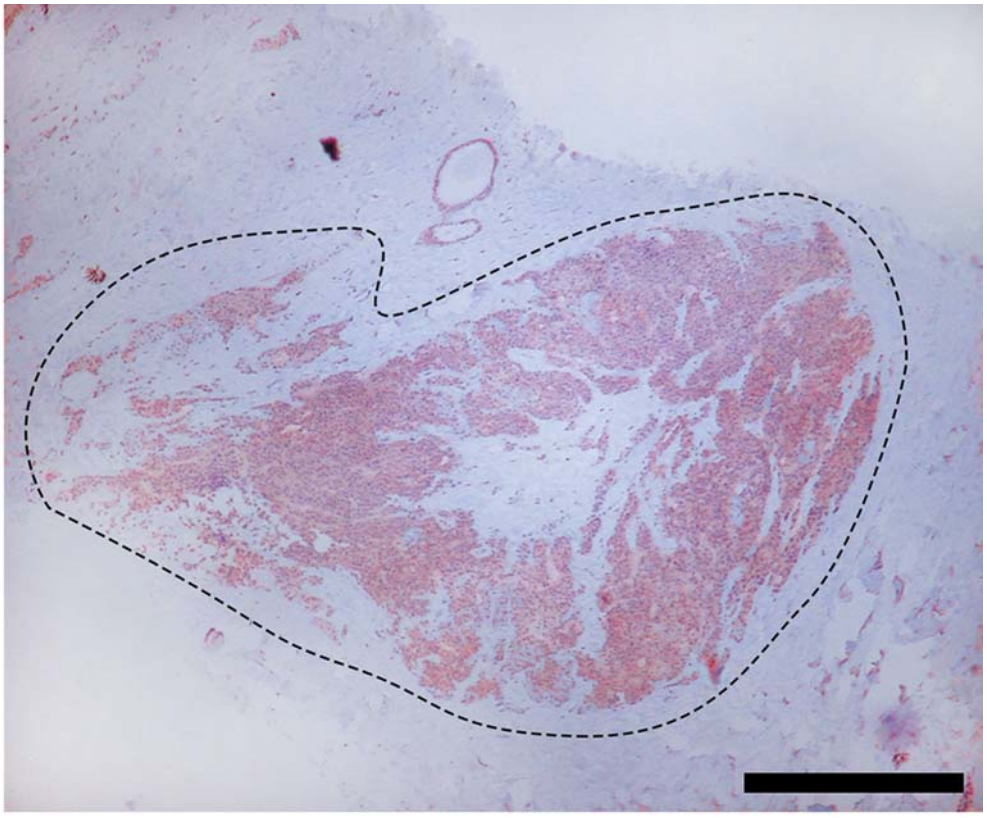

Figure 1. *Presurgical fine-needle biopsy. Original immunohistochemical specimen stained for human epidermal growth factor receptor (HER2); the image is representative of the four different biopsy specimens. Positive staining (score 2+) appears as brown areas. The dotted black line encircling the stained area was added to highlight the staining for HER2. Bar=500 μm.*

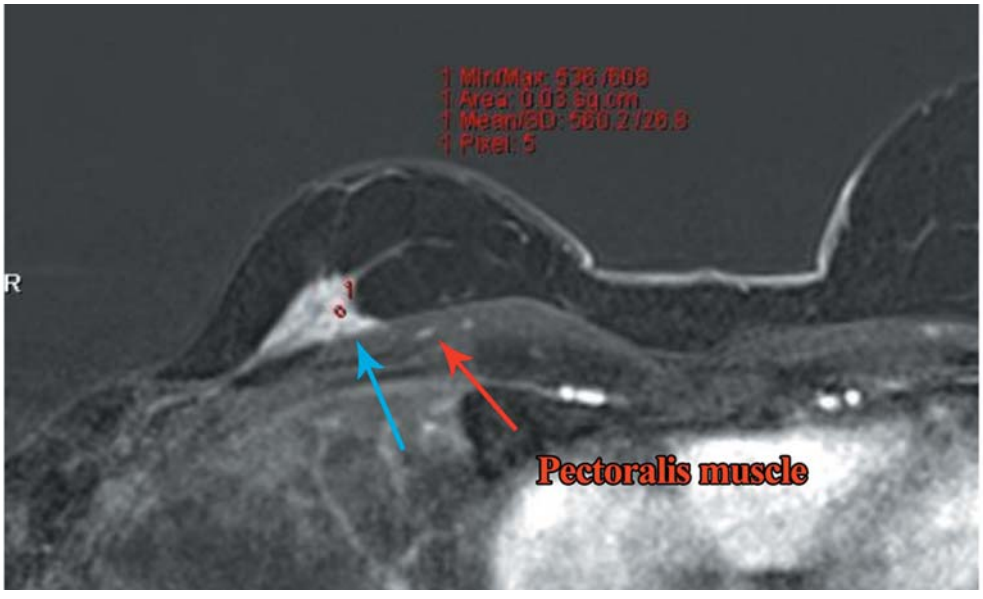

Figure 2. *Results of pre-surgical magnetic resonance imaging (MRI) with contrast medium. MRI showed an oval mass with irregular margins, inhomogeneous enhancement of 33×16×20 mm (longitudinal). The finding is at the superior external quadrant, posterior third, in strict proximity to the pectoralis muscle that shows thin impregnation of 6 mm (blue arrow) that cannot exclude the infiltration of the muscle. Images are consistent with the written report signed by the M.D. specialist in Diagnostic Radiology.*

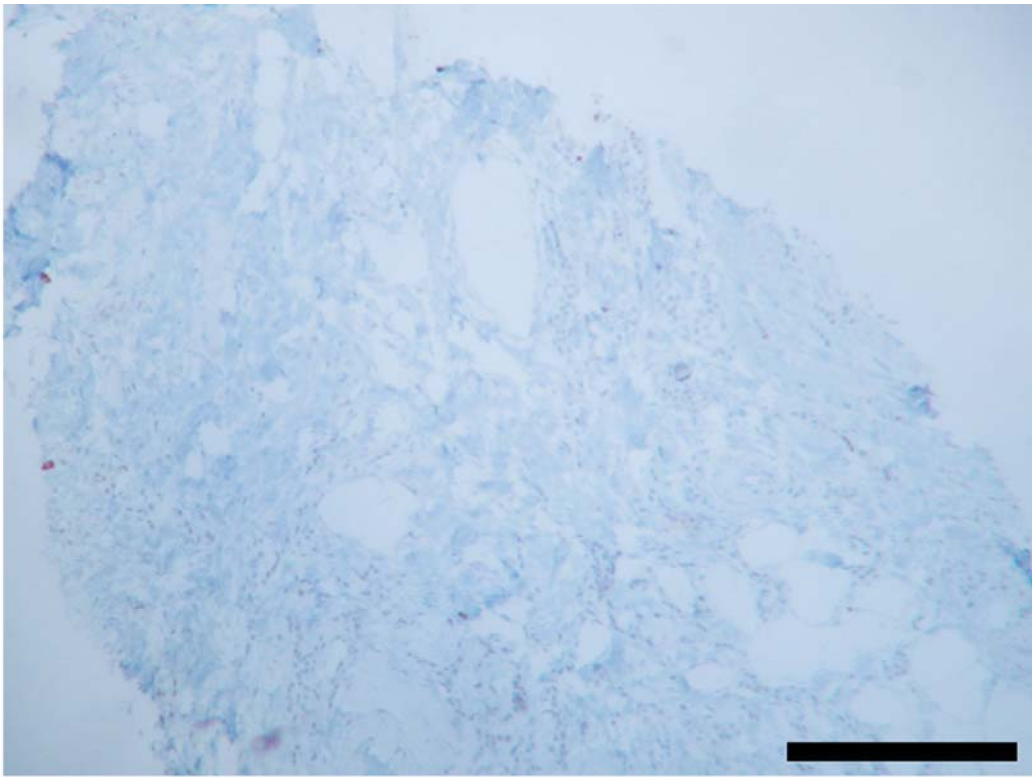

Figure 3. *Pre-surgical fine-needle biopsy. Original immunohistochemical specimen stained for progesterone receptor (PgR). No significant staining was detected; only clear blue non-specific background can be seen. The image is representative of the four different biopsy specimens. Bar=250 μm.*

An association between the ketogenic (low carbohydrate) diet undertaken by the patient and the changes in HER2 expression may be hypothesized on the basis of down-regulation of HER2 expression *via* the diacylglycerol-protein kinase C pathway. Many years ago, we demonstrated that diacyglycerol synthesised *de novo* from glucose, activated and down-regulated protein kinase C in transformed cells (32), and subsequently, we observed that *ERB*-transformed cells exhibited elevated steady-state levels of diacyglycerol (33). More recently, it was demonstrated that protein kinase C is responsible for the preferential recycling of the HER2 protein in breast cancer cells and it has been hypothesized that this protein kinase C-dependent endosomal signaling may be responsible for the oncogenic potential of HER2 (34). Therefore, a nutrition-associated reduction of glucose available for *de novo* synthesis of diacylglycerol and, therefore, reduced protein kinase C activation, may explain the negativity of HER2 expression observed for this patient. According to this hypothesis, reduced synthesis of diacylglycerol through the glycolytic pathway would lead to low protein kinase C activation and, consequently, most HER2 protein would be driven by endocytic traffic into lysosomes instead of recycling, with the final result of limiting the overexpression of HER2 protein.

It is worth noting that diacylglycerol-activated isoforms of protein kinase C are overexpressed in several types of breast cancer, including triple-negative cases (35). Therefore, it can be hypothesized that a nutritional approach aimed at reducing *de novo* synthesis of diacylglycerol through the glycolytic pathway might also be beneficial in triple-negative breast cancer. These considerations about the role of *de novo* synthesis of diacylglcyerol and protein kinase C activation may also suggest the utility of assessing the status of protein kinase C activation in biopsies as a further step in the molecular characterization of breast cancer.

In conclusion, an approach based on nutrition that includes vitamin D_3 and oleic acid in the context of a ketogenic diet may be useful in the complementary therapy of breast cancer and is consistent with century-old observations that may now be interpreted at the molecular level (28). Although the effects that we observed were pertinent to a case of HER2-/ER-positive breast cancer, it can be hypothesized that such a nutritional approach may also be beneficial in triple-negative breast cancer.

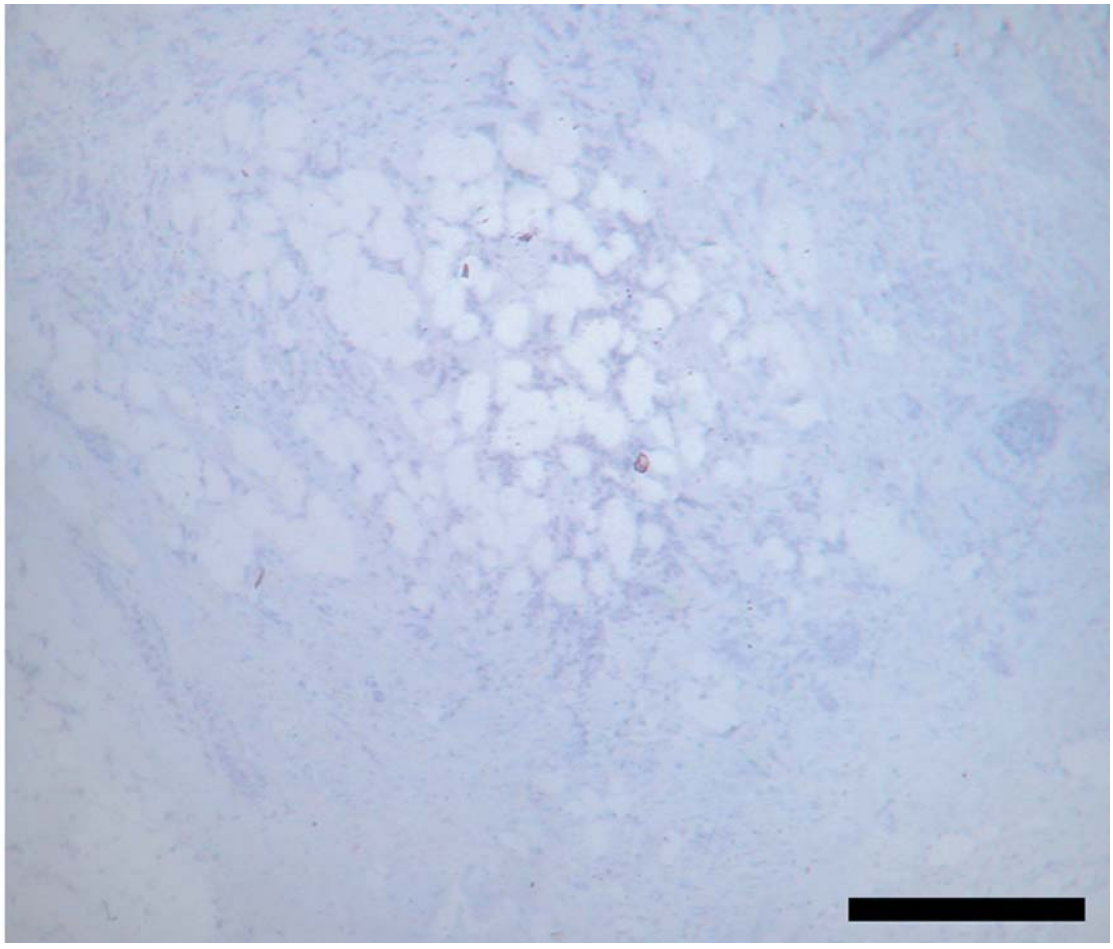

Figure 4. *Post-surgical specimen. Original immunohistochemical staining for human epidermal growth factor receptor (HER2). The absence of staining is clearly evident and consistent with the operator's evaluation (score 0). Only clear non-specific background staining can be seen. The score derives from the evaluation of the entire surgical specimen area. Bar=500 μm.*

Conflicts of Interest

At the time of these observations, *i.e.* in the first half of 2014, Marco Ruggiero was Full Professor of Molecular Biology at the Department of Experimental and Clinical Biomedical Sciences of the University of Firenze (retired in December 2014) and consulting Scientific Director of Immuno Biotech Ltd. (employment terminated in February 2015), a company producing supplements. However, he did not participate in any step of the diagnostic and therapeutic procedures involving this patient, nor did he advise the patient or influence them in any way. In particular, he was not involved in nor had any prior knowledge of the pre- and post-operative analyses of the specimens. As explained in the text, such analyses were carried-out by the University Hospital of Firenze (Italy), part of the Italian Public Health Service. All the analyses, interventions and treatments for this patient were performed according to the European standards of quality (UNI EN ISO 9001:2008). The results were examined, validated and countersigned by four different medical professionals. In addition, since all the supplements consumed by the patient were commercially available, no reference to any specific brand is made anywhere here.

Preliminary reports of this case have been presented in the form of an abstract and poster presentation at the Ninth International Conference of Anticancer Research, October 6-10, 2014, Sithonia, Greece, and at the Controlling Cancer Summit, May 12-14, 2015, London, U.K.

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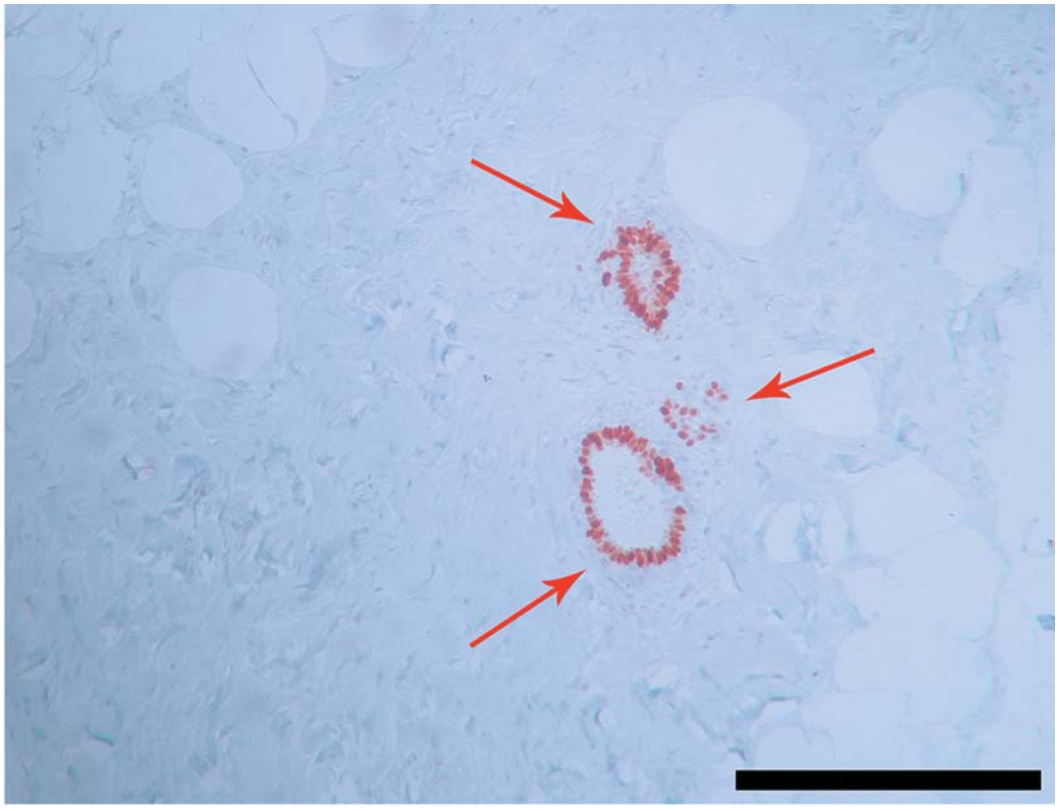

Figure 5. *Postsurgical specimen. Original immunohistochemical staining for progesterone receptor (PgR). The brown staining is clearly evident and consistent with the operator's evaluation (positive, 20%). Arrows indicate the areas positive for PgR expression. The percentage derives from the evaluation of the entire surgical specimen area. Bar=250 μm.*

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