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Effect of anaesthetic technique on oestrogen receptor-negative breast cancer cell function *in vitro*†

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Background. Metastatic recurrence is the main cause of breast cancer-related deaths. Tumour cell proliferation and migration are crucial steps in the metastatic process. Several perioperative factors, including general anaesthesia and opioid analgesia, adversely affect immune function, potentially increasing metastatic recurrence. Regional anaesthesia–analgesia has been consistently shown to attenuate the stress response to surgery, and also reduce opioid and general anaesthesia requirements, thereby attenuating this perioperative immunosuppression. We investigated the effect of serum from breast cancer surgery patients who received different anaesthetic techniques on breast cancer cell function *in vitro*.

Methods. Patients were randomized to receive propofol/paravertebral anaesthesia–analgesia (propofol/paravertebral, $n=11$) or sevoflurane general anaesthesia with opioid analgesia (sevoflurane/opioid, $n=11$). The ER-negative MDA-MB-231 cell line was treated with patient serum from both groups. The effects on proliferation and migration were measured.

Results. Treatment groups were well balanced for age, weight, surgical procedure, and cancer pathology. Pain scores were lower at 1 and 2 h in the propofol/paravertebral analgesia group. Compared with preoperative values, proliferation of MDA-MB-231 cells treated with postoperative patient serum at 10% concentration from the propofol/paravertebral group was significantly reduced compared with the sevoflurane/opioid group (−24% *vs* 73%, $P=0.01$). There was no significant change in MDA-MB-231 cell migration after treatment with patient serum between the two groups.

Conclusions. Serum from patients receiving propofol/paravertebral anaesthesia for breast cancer surgery inhibited proliferation, but not migration, of ER-MDA-MB-231 cells *in vitro*, to a greater extent than that from patients receiving sevoflurane/opioid anaesthesia–analgesia. This implies that anaesthetic technique alters the serum molecular milieu in ways that may affect breast cancer cell function, possibly by altering anaesthetic and opioid drug administration and resultant pain scores.

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Breast cancer is the most common malignancy in women, second only to lung cancer as cause of cancer death.[1] Metastatic recurrence is the main cause of breast cancer-related deaths. It is estimated that 30–40% of patients will die from metastatic disease, despite surgical removal of the primary tumour.[2] Tumour metastasis is a complex process that includes cellular separation from the primary tumour, invasion of and migration through surrounding tissues, invasion of the intravascular space, cellular

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transport in the bloodstream, and subsequent extravasation and proliferation in the target tissue or organ.[3]

Systemic therapy and radiation therapy are important treatment modalities for breast cancer, but surgical removal of the tumour offers the best prospect for a good prognosis.[4] However, there are a number of factors in the perioperative period that may result in immunosuppression, thereby promoting metastatic development. These include the surgery itself,[5] anaesthesia,[6] opioids,[7] pain,[8] and the stress response to surgery.[9]

Regional anaesthesia has been consistently shown to attenuate the neuroendocrine response to surgery,[10 11] and therefore perioperative immunosuppression. It also reduces the amount of general anaesthesia required, provides excellent pain relief, and reduces opioid consumption. Compared with general anaesthesia, spinal anaesthesia attenuated tumour metastasis in rats inoculated with a strain of breast adenocarcinoma.[12] Recent retrospective analyses suggested that paravertebral anaesthesia (and analgesia) for breast[13] and prostate cancer surgery[14] may reduce the risk of tumour recurrence or metastasis.

To further investigate the potential effect of perioperative anaesthetic technique on breast cancer metastasis, we conducted a prospective, randomized, controlled trial evaluating the effect of serum from primary breast cancer surgical patients who received either combined regional–general anaesthesia or general anaesthesia alone, on breast cancer cell function *in vitro*. Using the ER-negative breast adenocarcinoma cell line MDA-MB-231, we tested the hypothesis that serum from patients receiving a propofol/paravertebral anaesthetic technique would attenuate MDA-MB-231 cellular proliferation and migration to a greater extent than that from patients receiving standard general anaesthesia.

Methods

Patients and patient selection

With approval from the Ethics Committee of the Mater Misericordiae University Hospital and written informed consent, 30 women undergoing surgery for biopsy-proven primary breast cancer were approached consecutively between June 2007 and April 2008 for study inclusion. Eight (n=8) women who were invited to be randomized declined and 22 patients were enrolled into the study. Patients were aged 18–85 yr and undergoing mastectomy and axillary node clearance or wide local tumour excision without known extension beyond the breast and axillary nodes (i.e. believed to be tumour stages 1–3, nodes 0–2). Exclusion criteria were previous breast cancer surgery (except diagnostic biopsy); inflammatory breast cancer; ASA Physical Status IV or greater; any contraindication to paravertebral anaesthesia (including coagulopathy and abnormal anatomy); and any contraindication to midazolam, propofol, sevoflurane, fentanyl, or morphine.

Using a secure, web-based system that automatically records number and assignment, patients were randomly assigned to combined propofol/paravertebral anaesthesia with paravertebral analgesia (propofol/paravertebral) or general anaesthesia with postoperative opioid analgesia (sevoflurane/opioid).

Anaesthetic technique

In patients receiving propofol/paravertebral anaesthesia, a catheter was inserted into the ipsilateral paravertebral space at the second or third thoracic vertebrae using a standard technique. Before surgery, a 20 ml bolus of levobupivacaine 0.25% was injected. General anaesthesia was then induced and maintained with target-controlled infusion of propofol (Diprifusor). Patients breathed spontaneously an oxygen/air mixture through a laryngeal mask airway (LMA). Postoperative analgesia was provided by continuous paravertebral analgesia, using levobupivacaine 0.25% at an infusion rate of 8–10 ml h^{-1}. Paravertebral catheters were removed ~48 h after insertion. If required, rescue analgesia was triggered by a 10 cm visual analogue scale (VAS) pain score with arm extension of 3 cm or greater, and consisted of morphine 0.1 mg kg^{-1} i.v. bolus, followed by additional doses of 0.1 mg kg^{-1} i.m. every 3–4 h as needed.

In the sevoflurane/opioid group, general anaesthesia was induced with fentanyl 1–3 μg kg^{-1} and propofol 2–4 mg kg^{-1}. After LMA placement, anaesthesia was maintained with sevoflurane (end-tidal concentrations 1–3%) in a mixture of oxygen/air. Morphine 0.1–0.15 mg kg^{-1} was given for intraoperative analgesia. Postoperative analgesia was provided with patient-controlled morphine, with 1 mg boluses, a 6 min lockout period, and a 4 h dose limit of 30 mg. All patients received acetaminophen 1 g i.v. during surgery. White cell count (WCC) was measured before operation and 24 h after operation. VAS was measured by the anaesthetist in the postoperative anaesthesia care unit at 1 and 2 h after operation, and by nursing staff on the surgical ward 24, 48, and 72 h after operation.

Venous blood was sampled before anaesthesia and 24 h after surgery. Samples were centrifuged at 4000g and the resulting serum was stored at −20°C until further analysis.

Cell culture

The ER-negative MDA-MB-231 cell line (European Collection of Cell Cultures) was used for the analysis of cell proliferation and migration. Cells were cultured in L-Liebowitz medium supplemented with fetal bovine serum 10%, L-glutamine, and penicillin–streptomycin solution 1% at 37°C, with CO_2 5%.

Proliferation assay

Cells were cultured in a serum-free L-Liebowitz medium supplemented with L-glutamine and penicillin–streptomycin solution 1% at 37°C, with CO_2 5% for 48 h.

Cells were harvested by trypsinization, resuspended in medium, and added to 96-well plates at a density of 22 000 cells per well. The culture plates were incubated in this serum-free media for 24 h at 37°C to allow cell attachment. Serum samples were diluted in medium to produce 2%, 5%, and 10% serum concentrations, consistent with standard practice in tissue culture experiments *in vitro*. Medium alone was used as a control. Both pre- and postoperative serum samples from 11 patients in both the propofol/paravertebral and the sevoflurane/opioid groups were added in duplicate to the appropriate wells. Culture plates were incubated for a further 24 h. Cell growth was determined by the Cell Titer 96 assay (Promega, Madison, WI, USA) according to the manufacturer's protocol using a microplate reader. Cellular proliferation was measured after treatment of MDA-MB-231 cells with pre- and postoperative patient serum from both groups. Mean cell proliferation was determined for samples in duplicate and expressed as mean percentage change from pre- to postoperative values for each individual patient.

Chemotaxis migration assay

The effect of anaesthetic technique on breast cancer cell migration was assessed using the QCM™ Chemotaxis 96-well Cell Migration Assay. Cell migration through the membrane was quantified by the number of cells that migrate directionally through the 8 μm pore-size membrane into a lower chamber containing chemoattractant [fetal bovine serum 20% (150 μl)]. A total of 5×10^4 cells were seeded to each upper chamber of the 96-well plate in 100 μl of L-Liebowitz medium supplemented with fetal bovine serum 1%, L-glutamine, and penicillin–streptomycin 1%. Patient serum samples were diluted to a 20% concentration. One hundred microlitres of both pre- and postoperative serum samples from 11 patients in both the propofol/paravertebral and the sevoflurane/opioid groups were added in duplicate to both upper and lower chambers of the appropriate wells. Samples without cells, but containing Cell Detachment Buffer, Lysis Buffer, and CyQuant Dye were used as controls. The culture plates were incubated for 24 h at 37°C, in CO_2 5% to allow migration through the membrane. The migrated cells were recovered from the lower chamber and transferred into a 96-well flat-bottomed plate (Costar). Total cell migration was determined by measuring optical density of migrated cells using a fluorescence plate reader using 480/520 nm filter set.

Wound closure assay

A wound closure assay was used to assess the effect of treatment of MDA-MB-231 cells with patient serum on migration. MDA-MB-231 cells were grown to 90–95% confluence in 6-well plates and wounds of similar size were introduced into the monolayer by a sterile pipette tip. The monolayer was rinsed with phosphate-buffered saline (pH=7) to remove detached cells and then cultured in a medium containing either medium alone (control) or medium supplemented with 10% patient serum. The speed of wound closure was documented 6, 12, and 24 h post-wounding using the Nikon Coolpix 990 camera with the microscope at 10× objective.

Statistical analysis

Data were analysed using GraphPad Prism version 4 (GraphPad software, San Diego, CA, USA). Parametric data were compared using independent sample *t*-test for differences between the groups. The pre- to postoperative percentage change for proliferation and migration values in each individual was first calculated. Then the mean percentage change for each group was calculated. Differences in mean percentage changes were evaluated by analysis of variance (ANOVA) with *post hoc* Dunnett's test. This study was planned as a pilot study, as this is a genuinely novel area of investigation. We did not prospectively evaluate a sample size to detect a specific change in breast cancer cell proliferation and migration.

ANOVA for repeated measures was used for comparing VAS pain data between the groups, with *post hoc* Dunnett's test. χ^2 analysis or Fisher's exact tests were used for categorical data as appropriate. Results are presented as mean (SD) or median (inter-quartile range).

Results

All 22 patients completed the study according to the protocol, with 11 patients randomized to propofol/paravertebral anaesthesia–analgesia and 11 randomized to sevoflurane/opioid anaesthesia–analgesia. The same team of anaesthetists and surgeons performed all procedures; all paravertebral blocks were successful. Both the propofol/paravertebral and the sevoflurane/opioid treatment groups were well-balanced regarding age, weight, surgical procedure, and cancer pathology (Table 1). Of note, the patients enrolled in this study are a subgroup of an international multicentre trial (NCT00418457) investigating the effect of altering anaesthetic technique on outcome after primary breast cancer surgery.

Mean (SD) VAS for pain was lower in the propofol/paravertebral group compared with the sevoflurane/opioid group at 1 h (P=0.03) and 2 h (P=0.02) after surgery but were not significantly different at 24 h (Fig. 1). Morphine administration was higher in the sevoflurane/opioid group [mean (SD) 26.3 (10.7) *vs* 4.4 (2.9) mg in the propofol/paravertebral group, P=0.03]. There was no significant pre- to postoperative change in WCC in either group.

There was no significant difference in mean percentage pre- to postoperative change in cellular proliferation after treatment of cells with 2% or 5% patient serum in the sevoflurane/opioid (43% pre *vs* 55% post) group compared with the propofol/paravertebral group (4% pre *vs* 9% post).

Table 1 Patient characteristics, reported as mean (range), mean (SD) or number

	Sevoflurane/ opioid (n=11)	Propofol/ paravertebral (n=11)
Age (yr)	55.8 (34–64)	59.1 (48–78)
Weight specimen excised (g)	439.8 (443.9)	252.2 (381.1)
Mastectomy and axillary node clearance [% (n)]	5	3
Wide local clearance and sentinal node biopsy [% (n)]	6	8
Oestrogen receptor positive [% (n)]	10	11
Progesterone receptor positive [% (n)]	9	8
HER2 positive [% (n)]	1	2
Node positive [% (n)]	1	2
Mean percentage pre- to postoperative change in white cell count	34 (23)	30 (45)

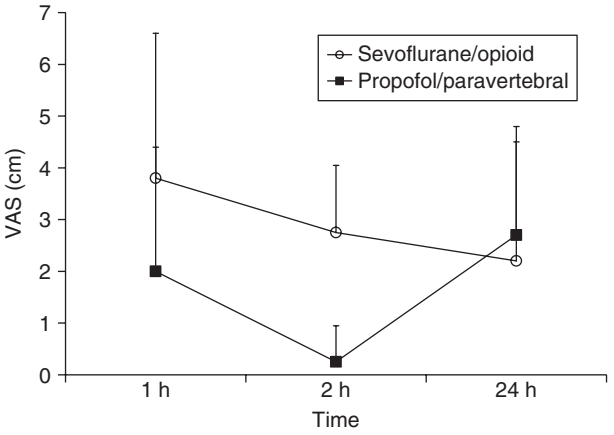

Fig 1 Mean (SD) VAS pain scores in patients after breast cancer surgery with sevoflurane anaesthesia and opioid analgesia (sevoflurane/opioid) or combined propofol/paravertebral anaesthesia and paravertebral analgesia (propofol/paravertebral). Pain scores were significantly lower at 1 (P=0.03) and 2 h (P=0.02) in the propofol/paravertebral group.

However, when cells were treated with 10% patient serum in the propofol/paravertebral group, there was a significant attenuation in mean percentage change in cell proliferation (−24%) compared with the increased proliferation observed in the sevoflurane/opioid group (+74%) (P=0.01) (Fig. 2). Conversely, there was no significant difference in migration of MDA-MB-231 cells through the 8 μm membrane of the QCM™ Chemotaxis 96-well Cell Migration Assay when MDA-MB-231 cells were treated with pre- and postoperative serum samples from either the sevoflurane/opioid group or the propofol/paravertebral group (5% *vs* 6%, P=0.92) (Fig. 3). In addition, there was no observed difference in cell migration using the wound scratch assay after treatment of MDA-MB-231 cells with pre- and postoperative patient serum in either group (Fig. 4).

Discussion

We assessed the effects of anaesthetic technique on breast cancer cell proliferation and migration by treating

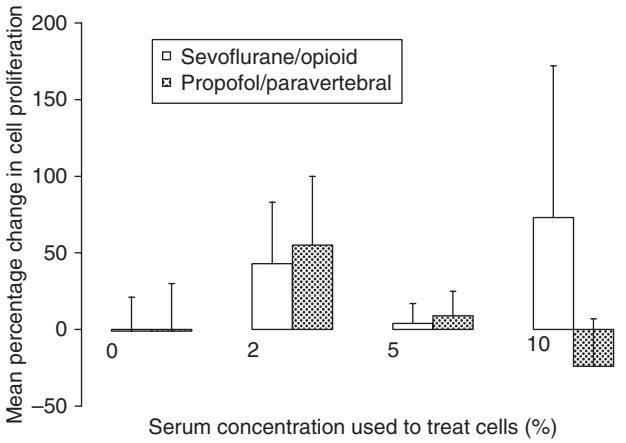

Fig 2 Mean percentage change in MDA-MB-231 cell proliferation from pre- to postoperative values in the sevoflurane/opioid group compared with the propofol/paravertebral group after treatment with patient serum at 0%, 2%, 5%, and 10% concentrations. Compared with values in the sevoflurane/opioid group, proliferation was reduced significantly at 10% patient serum concentration in the propofol/paravertebral group (P=0.01).

MDA-MB-231 cells with serum from patients who had received either sevoflurane general anaesthesia with opioid analgesia or combined general anaesthesia and regional anaesthesia for primary breast cancer surgery. The principal finding of this study was that cellular proliferation of the human breast carcinoma cell line MDA-MB-231 was significantly reduced when cells were treated with post- *vs* preoperative patient serum from the propofol/paravertebral group at 10% serum concentration. However, there was no significant change in breast cancer cell migration between the two groups.

The ability of cancer cells to metastasize to different sites of the body is the major cause of morbidity and mortality of breast cancer. The metastatic process involves several steps, all of which must be successfully completed for a tumour to establish and grow as a secondary deposit. These include growth of the tumour at the primary site, angiogenesis, invasion of this new vasculature by cancer cells, and extravasation at distant sites. There are a number of factors in the perioperative period that may result in immunosuppression, thereby promoting metastasis. While the surgery removes the main bulk of the tumour, manipulation of the primary tumour during surgery is associated with release of tumour cells into the circulation. Removal of the tumour may also cause a reduction in anti-angiogenic factors and release of growth factors, both of which have tumour-promoting effects.[5] In addition, surgery induces a profound stress response, including neuroendocrine, cytokine, and metabolic responses.[9] This, in turn, causes suppression of natural killer (NK) cell activity. This compromises host immune function, which has been shown to enhance tumour development.[15]

Anaesthesia itself contributes to perioperative immunosuppression. Anaesthetic drugs impair a number of immune functions, including neutrophil, macrophage, T cell, and NK

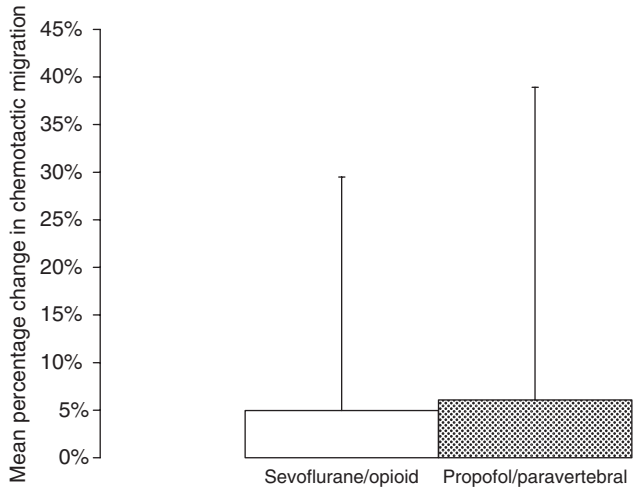

Fig 3 Effect of exposure of MDA-MB-231 cells to pre- and postoperative patient serum on cell migration. There was no significant change in mean percentage change of pre- to postoperative cellular migration after treatment of MDA-MB-231 cells with serum from either group.

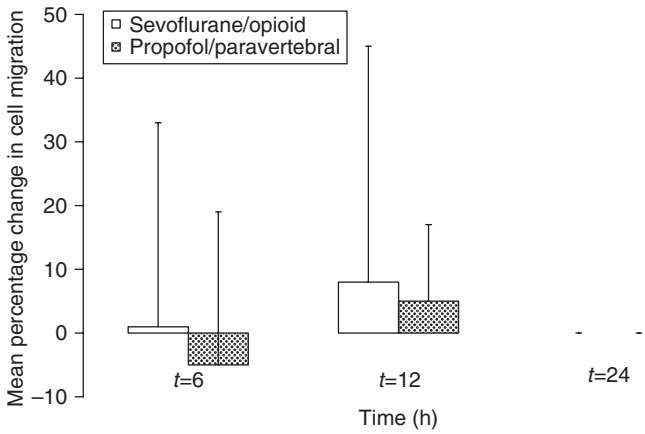

Fig 4 Mean percentage change of wound closure between pre- and postoperative serum treatment at t=6, 12, and 24 h. There was no significant change in speed of wound closure from pre- to postoperative values after treatment of MDA-MB-231 cells with patient serum between the groups at t=6 or 12 h. At 24 h post-wounding, all wounds were completely closed.

cell function.[6] Opioids, including morphine and fentanyl, are commonly used analgesics during the perioperative period in breast cancer surgery. Opioids have been shown to inhibit both cellular and humoral immune functions in humans,[7] and animal studies have demonstrated a dose–response effect with increasing doses of morphine associated with greater immunosuppression.[16] In addition, morphine has been shown to be pro-angiogenic, promoting breast tumour growth in mice.[17] And finally, pain suppresses cell-mediated immunity, and has been shown to enhance the tumour promoting effects of surgery,[8] which highlights the need for optimum perioperative analgesia.

Regional anaesthesia and analgesia provides excellent pain relief in the perioperative period. It also reduces the amount of general anaesthesia and opioid analgesia required. Regional anaesthesia has been consistently shown to attenuate the neuroendocrine response to surgery,[10 11] thereby attenuating perioperative immunosuppression. The addition of neuraxial block to general anaesthesia markedly attenuated tumour metastasis in rats inoculated with a strain of breast adenocarcinoma when compared with general anaesthesia alone.[12] A recent retrospective analysis comparing anaesthetic technique for primary breast cancer surgery found that the use of paravertebral anaesthesia and analgesia combined with general anaesthesia compared with general anaesthesia and opioid analgesia was associated with a 79% decrease in the incidence of recurrence or metastasis 3–4 yr later.[13] We therefore tested the hypothesis that the use of a regional anaesthetic technique in combination with general anaesthesia compared with general anaesthesia alone for primary breast cancer surgery would reduce the metastatic behaviour of breast cancer cells *in vitro*.

The ER-negative MDA-MB-231 cell line was chosen, as this cell type is highly aggressive both *in vitro* and *in vivo*, and associated clinically with a worse overall prognosis compared with ER-positive breast cancer. Therefore, the ability to attenuate the proliferation or migration of this highly aggressive cell line by safely and easily altering the way in which anaesthesia is administered for primary cancer surgery could have strong clinical implications.

Many patients harbour micrometastases and scattered tumour cells at the time of surgery.[18] However, a smaller number develop clinically significant metastases,[19] which may be due to the immune system's ability to eradicate minimal residual disease. Prognosis from breast cancer does not just depend on the presence of distant metastases, but on whether they can proliferate or not.[2] In general, markers of increased proliferation rate correlate with a worse prognosis in untreated patients, and may predict benefit from chemotherapy.[20] Our study demonstrated that cellular proliferation of the human breast carcinoma cell line MDA-MB-231 was significantly reduced when cells were treated with post- *vs* preoperative patient serum from the propofol/paravertebral group at 10% serum concentration. A possible explanation for this finding is that the molecular profile of the serum of these patients may be altered as a result of anaesthetic technique. Our group has previously shown that the use of combined propofol/paravertebral anaesthesia with paravertebral analgesia compared with general anaesthesia alone for primary breast cancer surgery has resulted in a reduction in serum concentrations of pro-tumorigenic cytokines and MMPs (IL-1β, MMP-3, and MMP-9) and an increase in the serum concentration of the anti-tumorigenic cytokine IL-10. There was no significant difference in cellular proliferation after treatment of cells with 2% or 5% patient serum between the two groups. This is not an unexpected finding, as these concentrations are below standard serum

concentrations (10%) used for *in vitro* cell culture and therefore any biologically active constituents may be too dilute to exert any significant effect.

Cell migration is a critical component of many physiological processes, including embryonic development, lymphocyte trafficking, haemostasis, and wound healing.[21] Increased cell migration is also one of the critical steps in the development of metastases.[22] In addition, it plays a central role in the progression of tumours from a non-invasive to an invasive and metastatic phenotype. We did not detect any significant difference in breast cancer cell migration in cells treated with patient serum from either the propofol/paravertebral or the sevoflurane/opioid group.

A potential limitation of this study is the small sample size. We did not prospectively evaluate a sample size to detect a specific change in breast cancer cell proliferation and migration as this study was planned as a pilot study. However, the sample size was large enough to detect a statistically significant difference in breast cancer cell proliferation between the two groups.

In summary, in this *in vitro* MDA-MB-231 model of breast carcinoma cells, 10% serum from patients receiving a propofol/paravertebral anaesthetic technique reduced cancer cell proliferation but not migration, compared with that from patients receiving sevoflurane/opioid anaesthesia. This suggests that relatively minor alterations in anaesthetic technique may alter the serum molecular profile of breast cancer patients in a way that may influence breast cancer cell proliferation. Further investigations into the mechanisms behind the effect of anaesthetic technique on breast cancer cell behaviour seem warranted.

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