EFFECT OF ANAESTHETIC TECHNIQUE ON IMMUNE CELL INFILTRATION IN
BREAST CANCER: A FOLLOW-UP PILOT ANALYSIS OF A PROSPECTIVE,
RANDOMISED, INVESTIGATOR-MASKED STUDY.

F. Desmond*, J. McCormack#, N. Mulligan~, M Stokes+, DJ Buggy*^

Departments of Anaesthesia*, Pathology~ and Surgery+, Mater Misericordiae University Hospital
Dublin & Digital Core Pathology Laboratory#, University College Dublin Ireland. ^Outcomes
Research Consortium, Cleveland Clinic, USA

Corresponding author: Donal Buggy, Dept. anaesthesia, Mater Misericordiae University Hospital,
Dublin7 Ireland. donal.buggy@ucd.ie
Tel: +353-1-8032281
ABSTRACT

**Background:** Live animal studies using an inoculation model of breast cancer indicate that anaesthetic drugs and techniques differentially affect cancer metastasis, inversely related to Natural Killer (NK) cell and T lymphocyte levels. Clinical histological studies demonstrate that the distribution of these immune cells and macrophages in intratumoral cancer tissue can predict prognosis and response to therapy. No study has evaluated whether anaesthetic technique influences human breast cancer immune cell infiltration.

**Methods:** Excised breast cancer specimens from patients previously enrolled in an ongoing, prospective, randomised trial (NCT00418457) investigating the effect of anaesthetic technique on long-term breast cancer outcome, were immunohistochemically stained to enable a colour deconvolution technique to summate marked immune cell infiltration: CD 56 (NK cells), CD 4 (T helper cells), CD 8 (T suppressor cells) and CD 68 (Macrophages). Patients were randomised to receive either a propofol-paravertebral anaesthetic with continuing analgesia (PPA, n=12) or a balanced GA with opioid analgesia (GA, n=16) for 24 hr postoperatively. Investigators were masked to group allocation.

**Results:** Normalised positive intensity values, [median (IQR)], for CD 56 were lower in GA [116-134] versus 136 [132-142], P=0.015. CD 4 was also lower in GA 10.9 [5.5-27.8] versus PPA 19.7 [14.4-83.5], P=0.03, but CD 8 5.5 [4.0-9.75] versus 13.0 [5.0-14.5] respectively, P=0.24 and CD 68 infiltration 5.8[3.25-8.75] versus 8.0 [3.0-8.75], P=0.74 were not significantly different.

**Conclusion:** PPA induces increased levels of NK and T helper cell infiltration into breast cancer tissue compared with GA, but not T suppressor cells or macrophages. This is consistent with the hypothesis that anaesthetic technique may affect perioperative immune function conducive to resisting breast cancer recurrence and metastasis.
KEY WORDS:

Breast Cancer, Immunocytes, Natural Killer Cells, Anaesthetic Technique, Metastasis, Tumour Recurrance
INTRODUCTION:

While a number of retrospective studies have suggested an association between anaesthetic technique and cancer recurrence or metastasis, almost an equal number have shown no such association. This warrants addressing in prospective, randomized clinical trials, one of which is underway in breast cancer, but will require many years of patient follow-up before the data can be interpreted.

The rationale underpinning a potential link between anaesthetic technique and cancer recurrence is that a number of perioperative factors can inadvertently promote or resist tumour spread, at least partly by transient immune impairment, and that these factors may be modified by careful selection of anaesthetic technique. Therefore, translational and experimental studies are evaluating potential effects of anaesthetic techniques and agents on perioperative factors which may influence the risk of metastatic recurrence, including immune function. Experimental data from an inoculation model of breast cancer in live animal models suggest that regional anaesthesia, compared with GA, reduces cancer metastasis by attenuating routine perioperative immune impairment, particularly by preserving natural-killer (NK) cell function. Data from another animal model suggests that regional anaesthesia modifies T lymphocyte balance peri-operatively, in a manner conducive to resisting hepatic tumour development.

Immune cell infiltration into human tumour tissue, particularly into stromal tissue rather than tumour islets, can regulate anti-tumour immune resistance and be an important harbinger of prognosis in certain types of lung cancer. Moreover, in breast cancer patients, intratumoral infiltration of lymphocytes and monocytes may predict the efficacy of subsequent chemotherapy.

No study to date has evaluated the influence of anaesthetic technique on immune cell infiltration in any cancer. The ongoing international clinical trial (NCT 00418457) provides an opportunity to do in breast cancer. In our centre, we have already enrolled >180 patients in an ongoing prospective, multicentre clinical trial investigating recurrence rates in women having surgery.
for primary breast cancer, who have been randomised to receive either paravertebral regional anaesthesia and continuing analgesia for at least 24 hr combined with propofol-only GA, versus standard GA-opioid analgesia.

Therefore, we reviewed and immuno-histochemically stained the excised breast cancer tissue from 28 randomised patients to compare the effect of anaesthetic technique on immune cell infiltration. We tested the hypothesis that an anaesthetic technique consisting of paravertebral regional anaesthesia with propofol-only GA increases breast cancer infiltration of NK cells, T helper cells, T suppressor cells and macrophages, compared with breast cancer tissue from patients who received standard balanced GA with opioid analgesia.
METHODS:

Research Ethics Committee approval was obtained from the Mater Misericordiae University Hospital, to re-contact women already enrolled in the ongoing clinical trial, requesting their consent to analyse their breast cancer tissue excised during primary breast cancer surgery at our hospital. Pathology specimens of n=30 women with biopsy proven breast cancer, who had been previously randomised at our centre to one of two different anaesthetic techniques in the on-going clinical trial, were randomly selected from the n=160 patients already enrolled in the clinical trial in our centre at that time. Because this is a pilot study, n=30 patient datasets was chosen as being likely to indicate whether any significant difference would be measurable. A table of random numbers was used, with the final two digits being used to indicate the patient number enrolled from our database. For example, if the final two digits from the random numbers table was ....71, then patient number 71 from our database of patients enrolled in the clinical trial was selected for inclusion in the present study. N=30 patients were enrolled for the present study in this way, n=16 from the standard GA group and n=14 from the propofol-paravertebral group (PPA).

These 30 previously enrolled patients were contacted by letter, containing a Patient Information Leaflet with follow up telephone contact by the research nurse to confirm and ascertain their consent. All 30 patients consented to the study.

Their clinical breast cancer tissue samples were reviewed and re-stained for differential expression of markers of immunocyte infiltration. Immunocyte infiltration of the breast cancer tissue samples was measured using immuno-histochemical analysis of tumour samples using CD4 and CD 8 markers of T lymphocytes CD 56 NK Cells and CD 68 macrophages. The degree of staining indicates the level of infiltration and this was assessed by standard immune-histochemical techniques. From the archival formalin-fixed paraffin-embedded tissue blocks, sections were cut at 4µm and mounted onto glass slides. In order to enhance adhesion to the glass,
the slides were incubated for 30 min at 60°C or overnight at 37°C. The slides were deparaffinised using two 5 min incubations of clean xylene, followed by three washes with absolute ethanol. The sections were placed in a radiotransparent slide holder and the slide was immersed in 1mM EDTA pH 7.5 (from a 100 mM stock) in a beaker. They were covered with a piece of Saran wrap in which holes were made. After they were brought to the boil in a microwave oven at max power (8 min for 800 ml) it was left boiling for 15 mins at a reduced power (power 3) so that the liquid continues to simmer. It was cooled at room temperature for 30-60 mins and then transferred to TBS. All staining was performed in a humid chamber. Double indirect AP immune-histochemically was conducted. The slides were scanned and the images were inspected for quality. Optimisation of the slides were undertaken where necessary due to dirt, folding, excess mounting media, air bubbles etc. The slides were analysed for the parameters indicated by both manual and automated methods.

The slides were analysed by a colour deconvolution technique. Colour deconvolution accurately separates stains, by separating the image into 3 channels (Red/Green/Blue) corresponding to the actual colours used. It allows accurate measurement of the area for each stain individually, even when the stains are superimposed at the same location. Colour deconvolution also allows a calculation of the area and the intensity for each individual stain. The mark-up image colour codes analysed pixels in the following format: negative, weak, medium, strong positive and outputs these as percentage, average intensity and area.

Colour deconvolution is used for stain colour calibration. Colour calibration defines the stain colour vector (R/G/B) so stained cells will be correctly identified by the analysis tool. Default colour vectors are colour 1- haematoxylin, colour 2- eosin, and colour 3- diaminobenzidine (DAB). Colour vector numbers can be changed if different stains are used. The colour for each stain is calibrated separately for each stain that differs from the default. Separate control slides for each stain are used. After calibration is complete, the modified parameter settings are saved as a macro. By using a control slide with one colour, the Average Optical Density for the stain’s RGB (Red/Green/Blue)
colour components can be measured. These values became the Colour Channel inputs for that stain when running colour deconvolution.

Raw image data are in RGB format. Intensity is the average of RGB channels in the raw data. Large intensity is bright and corresponds to very light staining. Low intensity is very dark and corresponds to dark staining.

Total stained area is the cumulative total area of positive and negative pixels. Total analysis area is the total area of analysis including any clear glass areas of the digital slide. Results are colour coded to match the mark up area.

Data analysis

Average positive intensity data was divided by the total analysis area to give normalised positive intensity data for each patient’s staining for each marker in turn. Because this data was not normally distributed, we compared differences between the median (interquartile range) for the anaesthetic techniques using the Mann-Whitney test. For patient characteristics, differences between normally distributed continuous data was tested using the unpaired t-test and for categorical data using Fischer’s exact test.
RESULTS:

While n=30 patients were randomly selected from the patients previously randomised to ongoing trial NCT00418457, n=16 GA and n=14 PPA, the specimens from two patients in the PPA group were not suitable for immunohistochemical analysis for technical difficulties with the quality of the staining. Therefore, n=16 patients from the GA group and n=12 from the PPA group remained for analysis.

Table 1 shows the patient demographic, morphometric and breast cancer characteristics. There were no significant differences between the groups for any of the parameters measured, including the grade and clinical stage of the breast cancers diagnosed. Table 2 shows the anaesthetic and analgesic data for drugs administered intraoperatively in addition to visual analogue scale pain data on sitting forward (moving) in the post anaesthesia care unit. Predictably, there was significantly higher opioid use (both fentanyl and morphine) and sevoflurane in the GA-opioid group and higher propofol use in the propofol-paravertebral group, as would be expected from the study protocol.

Figure 1 shows the CD 56 (Natural Killer cell) data. Figure 1A shows an indicative stain of CD 56. Figure 1B shows the normalised positive intensity for the two groups of CD 56 data. Median values for CD 56 were lower in the GA group compared with the PPA group 121 [116-134] vs 136 [132-142] respectively, P=0.015.

Figure 2 shows CD4 (T helper cell) data. Figure 2A shows an indicative stain of CD 4. Figure 2B shows the normalised positive intensity for the two groups of CD 4 data. Median values for CD 4 were again lower in the GA group compared with the PPA group 10.9 [5.5-27.8] vs 19.7 [14.4-83.5] respectively, P=0.03.

Figure 3 shows CD 8 (T suppressor cell) data. Figure 3A shows an indicative stain of CD 8. Figure 3B shows the normalised positive intensity for the two groups of CD 8 data. Median values for CD 8 were not significantly different in the GA group compared with the PPA group, median [interquartile range] 5.5 [4.0-9.75] vs 13.0 [5.0-14.5] respectively, P=0.24.
Figure 4 shows CD 68 (macrophage) data. Figure 4A shows an indicative stain of CD 68. Figure 4B shows the normalised positive intensity for the two groups of CD 68 data. Median values for CD 68 were not significantly different in the GA group compared with the PPA group, median [interquartile range] likewise 5.8 [3.25-8.75] vs 8.0 [3.0-8.75] respectively, P=0.74.
DISCUSSION:

In this pilot study to evaluate the effect of anaesthetic technique, on immune cell infiltration in breast cancer tissue, in women with primary breast cancer, we have found that there is indeed differential expression of natural killer and T helper cells, but not T suppressor cells or macrophages. Anaesthetic technique consisting of paravertebral regional anaesthesia with propofol only GA increases NK cell and T helper cell infiltration into breast cancer tissue but not T suppressor cells or macrophages. Because these patients were randomized into a clinical trial of women with primary breast cancer, the differences are convincingly attributable to an effect of anaesthetic technique on perioperative immune cell behaviour.

Immune cell markers have recently been the focus of translational research to predict efficacy of chemotherapy treatment of breast cancer, following a study that suggested that intratumoral lymphocytic infiltrates could be associated with better prognosis after chemotherapy in breast cancer patients. Moreover, in a mouse model of breast cancer, blocking vascular endothelial growth factor (VEGF), essential in angiogenesis of metastatic breast tumours, affected tumour infiltration with cancer-resisting immune cells including NK cells, T helper cells and reduces tumour-associated macrophage infiltration in an orthotopic mouse model breast cancer xenografts. This alteration in immune cell infiltration by inhibition of VEGF correlated with serum cytokine levels of IL-1B.22,23 Previously, our group demonstrated that serum from patients randomised to the GA-opioid arm of the ongoing clinical trial outlined had higher levels of VEGF, IL-1B and certain matrix metalloproteinases compared with patients receiving the putative cancer-resisting propofol-paravertebral anaesthetic technique24,25 Our study showed no difference in overall macrophage infiltration with anaesthetic technique, although unfortunately we missed the opportunity to look for specific TAM subsets of macrophage infiltration. Recent work has also shown that adaptive immune responses by B and T lymphocytes and their role in the inflammatory response can
specifically regulate multiple pro-tumour properties of myeloid cells that in turn can control cancer
development\textsuperscript{26}.

The mechanism by which anaesthetic technique might cause such an early alteration in immune cell
expression into breast cancer (which would have been excised within 30-90 min after induction of
anaesthesia) is unclear. Perhaps the fact that the PPA patients received local anaesthetics, whereas
the GA patients did not, might be contributory. Recent laboratory evidence has shown that amide-
local anaesthetics (which were used in the paravertebral arm of this study) may provide anti-
metastatic and anti-inflammatory effects. Tissue from patients with lung adenocarcinoma was
exposed to these different classes of local anaesthetics in vitro and analysed for cell migration, Src-
activation and intercellular adhesion molecule 1-phosphorylation\textsuperscript{27}. Amide local anaesthetics (LA)
attenuated cancer cell activation and migration. Furthermore, local anaesthetics have also been
observed to have direct cytotoxic effects on T-lymphoma cells \textit{in vitro}, causing apoptosis at lower
concentrations and necrosis at higher concentrations, and their cytotoxic effects appeared to
correlate with their lipophilicity and potency\textsuperscript{28}.

Previous studies have shown that NK and T helper cell activity is better preserved with epidural
anaesthesia compared with general anaesthesia in humans\textsuperscript{29, 30}.

The use of local anaesthetics perioperatively in regional anaesthesia techniques attenuates the
surgical stress response and decreases consumption of opiates. Opiates have been shown in multiple
studies to have an immune-suppressant effect and therefore could reduce the tissue immunocyte
migration\textsuperscript{16, 29-32}. All participants in the GA arm had fentanyl and morphine within 30-90 min of the
tissue sample excision. Perhaps the contemporaneous opiate administration could also have had a
direct effect on breast cancer cells which, like many immune cells, express opioid receptors and
which in turn might affect immune cell infiltration. A translational study of > 2,000 women with
breast cancer indicated that a single gene polymorphism of the MOR gene (A118G) is associated
with increased survival after 10 yrs. Whether this observation is attributable to quality analgesia
or the use of specific opioids is unclear.

It is also plausible that sevoflurane use in the GA group contributed to a reduction in the NK cell
expression, because volatile agents have been shown to protect cancer cells in vitro against tumour
necrosis factor induced apoptosis, which may in turn stimulate immune cell infiltration.

Moreover, it is known that the surgical stress response transiently impairs perioperative immune-
competence. Suppression of NK cell activity occurs within hours of surgery, lasts for days, and is
proportional to the invasiveness of the surgery. Reducing further exposure to opiates and
sevoflurane, may preserve perioperative immune function, including NK cells. Inhibition of the
stress response to surgery by paravertebral anaesthesia was associated with reduce risk of metastasis
during the initial years of retrospective follow-up of patients undergoing breast cancer surgery.

Our data has a number of potential limitations. We measured indirectly only the quantitative
number of immunocytes expressed, but this may not reflect immunocyte cytotoxicity function,
which would require a dynamic study in living cancer tissue, rather than preserved, excised breast
cancer tissue. Our measurement technique of immune cell presence was indirect, based on intensity
of staining rather than on actual cell counts. An alternative measure of immune cell infiltration
would have been aggregating the total number of actual immune cells visualised. A difficulty with
this direct counting method is choosing a consistent area of the mounted specimen in which to
conduct the count. Inevitably, some areas of a given mounted specimen have greater density of
immune cell infiltration than others. Flow cytometry would also have been technically difficult in
these specimens because they are histological samples mounted on slides, rather than blood
samples. Therefore, we elected to use the staining intensity method as described above, because it
quantifies immune cell presence throughout the specimen, which was then normalised to take
account of the area of sample evaluated.

Furthermore, our CD4 data should also be interpreted with caution, because this marker also stains
for CD4+FOXP3+ cells, which are in fact immunosuppressive, the opposite effect from T helper
cells. Unfortunately we did not stain for the CD4+FOXP3+ cells in our study. Similarly, our CD8
marker includes both T suppressor and T effector cells, but our staining mechanism does not
distinguish between these subsets.

In addition, the time from the induction of anaesthesia to time of breast cancer tissue excision was
in the order of 30-90 min. This is a limited time for anaesthetic technique or any other perioperative
factor to influence immune cell migration into and expression within breast cancer tissue, yet we
identified the differences described. It is possible that the observed differences were present
preoperatively, but the fact that there were no significant differences in breast tumour type and
grade between the groups makes this explanation unlikely.

CONCLUSION:

Our findings in this pilot study, together with available data from breast and lung cancer patients
that immune cell infiltration is an important marker not only of prognosis but also of response to
treatment, suggest that a prospective, randomised study of the effect of anaesthetic technique on
immune cell infiltration in breast and other forms of cancer is warranted. Such studies should
include a comparison of the excised breast cancer tissue samples with preoperative needle biopsy
and more detailed T cell subset analysis, including immunosuppressant CD4+FOXP3+ T cells and
the ratio of effector to regulatory T cells within the tumour. Further useful data would be obtained
from evaluating matrix metalloproteinase and mu-opioid receptor (MOR) status within the tumour.
Confirmation of these findings would be consistent with the inoculation live animal model of breast
cancer, and provide a mechanistic rationale for a potential clinical effect of anaesthetic technique on breast cancer outcome. Definitive data from the ongoing, randomised, clinical follow up trial in breast cancer patients is awaited.

AUTHOR CONTRIBUTIONS:

Fiona Desmond contributed to the study design and protocol development. She arranged the pathological staining of specimens and interpreted the colour deconvolution technique. She assisted with data analysis and critically revised the manuscript for intellectual content. She approved the final manuscript.

Janet McCormack implemented the colour deconvolution technique as applied to our pathological data in the digital core technology laboratory. She was involved in the design of the study. She assisted with the data analysis and interpretation of the results. She approved the final manuscript.

Niall Mulligan provided immunohistological expertise for the study, arranged the initial and antibody specific staining of the specimens, assisted with data analysis and interpretation, approved final manuscript.

Maurice Stokes provided surgical oncological advice for the study, assisted drafting the protocol and breast cancer sample provision, assisted with data analysis and interpretation, and approved final manuscript.

Donal Buggy designed and instigated the study, having previously established the multicentre clinical trial. He obtained funding, developed the protocol, built the necessary collaboration, analysed and interpreted the data and drafted the manuscript.
ABBREVIATIONS:

-NK: Natural Killer

-PPA: Paravertebral, Propofol Anaesthesia

-GA: General Anaesthesia

-IQR: Interquartile Range

-TBS: Tris Buffered Saline

-AP: Alkaline Phosphatase

-DAB: Diaminobenzidine

-RGB: Red/Green/Blue

-VEGF: Vascular Endothelial Growth Factor

-TAM: Tumour Associated Macrophages

-LA: Local Anaesthetics

-MOR: Mu-Opioid Receptor

COMPETING INTERESTS:

The authors declare that they have no competing interests.

ACKNOWLEDGEMENT:

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REFERENCES:


22. Roland CL, Dineen SP, Lynn KD et al. Inhibition of vascular endothelial growth factor reduces angiogenesis and modulates immune cell infiltration of orthotopic breast cancer xenografts. Mol Cancer Ther 2009;8: 1761-71


34. Kawaraguchi Y, Horikawa YT, Murphy AN et al. Volatile anesthetics protect cancer cells against tumour necrosis factor related apoptosis-inducing ligand-induced apoptosis via caveolins. *Anesthesiology* 2011; 115:499-508
Table 1: Demographic, Morphological and Breast cancer characteristics. All data shown are mean±SD or n(%)

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<th>GA-Opiate Group (N = 16)</th>
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Table 2: Intraoperative anaesthetic and analgesic and VAS pain on moving in post anaesthesia care unit. All data shown are median [minimum, maximum].

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<td>[4, 15]</td>
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Figure Legends:

Figure 1A: This is an immunohistiochemically stained sample for CD 56, NK cells. The darker stained areas are indicative of NK cells and these are the areas analysed via colour deconvolution in order to assess the intensity and the area.

Figure 1B: This is a graph for the normalised positive intensity for CD 56, NK cells. The X-axis are the two groups studied, the GA group and the PPA group. The Y-axis is the median normalised positive intensity for CD 56. Median values for CD 56 were lower in the GA group compared with the PPA group; 121 [116-134] vs 136 [132-142] respectively. P = 0.015

Figure 2A: This is an immunohistiochemically stained sample for CD 4, T helper cells. The darker stained areas are indicative of T helper cells and these are the areas analysed via colour deconvolution in order to assess the intensity and the area.

Figure 2B: This is a graph for the normalised positive intensity for CD 4, T helper cells. The X-axis are the two groups studied, the GA group and the PPA group. The Y-axis is the median normalised positive intensity for CD 4. Median values for CD 4 were lower in the GA group compared with the PPA group; 10.9 [5.5-27.8] vs 19.7 [14.4-83.5] respectively. P = 0.03

Figure 3A: This is an immunohistiochemically stained sample for CD 8, T suppressor cells. The darker stained areas are indicative of T suppressor cells and these are the areas analysed via colour deconvolution in order to assess the intensity and the area.

Figure 3B: This is a graph for the normalised positive intensity for CD 8, T suppressor cells. The X-axis are the two groups studied, the GA group and the PPA group. The Y-axis is the median normalised positive intensity for CD 8. Median values for CD 8 were not
significantly different in the GA group compared with the PPA group; 5.5 [4.0-9.75] vs 13.0 [5-14.5] respectively, P = 0.24

Figure 4A: This is an immunohistiochemically stained sample for CD 68, tumour associated macrophage cells. The darker stained areas are indicative of tumour associated macrophages and these are the areas analysed via colour deconvolution in order to assess the intensity and area.

Figure 4B: This is a graph for the normalised positive intensity for CD 68, tumour associated macrophages. The X-axis are the two groups studied, the GA group and the PPA group. The Y-axis is the median normalised positive intensity for CD 68. Median values for CD 68 were not significantly different in the GA group compared with the PPA group; 5.8 [3.25-8.75] vs 8.0 [3.0-8.75] respectively, P = 0.74
Figure 1B

Median Positive Intensity for CD56 (NK Cells)

Median [25-75%], 121 [116-134] vs. 136 [132-142], GA PPA respectively, P=0.015
Figure 2A
Figure 2B

Median Positive Intensity for CD4 (T Cells)

Median [25-75%], 10.9 [5.5-27.8] vs. 19.7 [14.4-83.5], GA PPA respectively, P=0.03
Figure 3A
Median Positive Intensity CD8 (T Suppressor Cells)

Median [25-75%], 5.5 [4.0-9.75] vs. 12.0 [5.0-14.5], GA PPA respectively, P=0.24
Figure 4A
Median [25-75%], 5.8 [3.25-8.75] vs. 8.0 [3.00-8.75], GA PPA respectively, P=0.74