# Effect of Anaesthetic Technique on Immune Cell Infiltration in Breast Cancer: A Follow-up Pilot Analysis of a Prospective, Randomised, Investigator-masked Study

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**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 intra-tumoral cancer tissue can predict prognosis and response to therapy. No study has evaluated whether the anaesthetic technique influences human breast cancer immune cell infiltration. Materials and 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: CD56 (NK cells), CD4 (T helper cells), CD8 (T suppressor cells) and CD68 (macrophages). Patients were randomised to receive either a propofol-paravertebral anaesthetic with continuing analgesia (PPA, n=12) or a balanced general anaesthesia with opioid analgesia (GA, n=16) for 24 h postoperatively. Investigators were masked to group allocation. Results: Normalised positive intensity values, (median (interquartile range (IQR)), for CD56 were lower in GA121 (116-134) versus 136 (132-142), p=0.015. CD4 was also lower in GA10.9 (5.5-27.8) versus PPA 19.7 (14.4-83.5), p=0.03 but CD8 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 the anaesthetic technique may affect perioperative immune function conducive to resisting breast cancer recurrence and metastasis.*

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While a number of retrospective studies have suggested an association between the anaesthetic technique and cancer recurrence or metastasis (1-7), almost an equal number have shown no such association (8-12). This warrants addressing in prospective, randomized clinical trials, one of which is underway in breast cancer (13), but will require many years of patient follow-up before the data can be interpreted.

The rationale underpinning a potential link between an 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 the anaesthetic technique (14-16). Therefore, translational and experimental studies are evaluating potential effects of anaesthetic techniques and agents on perioperative factors that 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 to general anaesthesia (GA), reduces cancer metastasis by attenuating routine perioperative immune impairment, particularly by preserving natural-killer (NK) cell function (17). Data from another animal model suggests that regional anaesthesia modifies T lymphocyte balance peri-operatively, in a manner conducive to resisting hepatic tumour development (18).

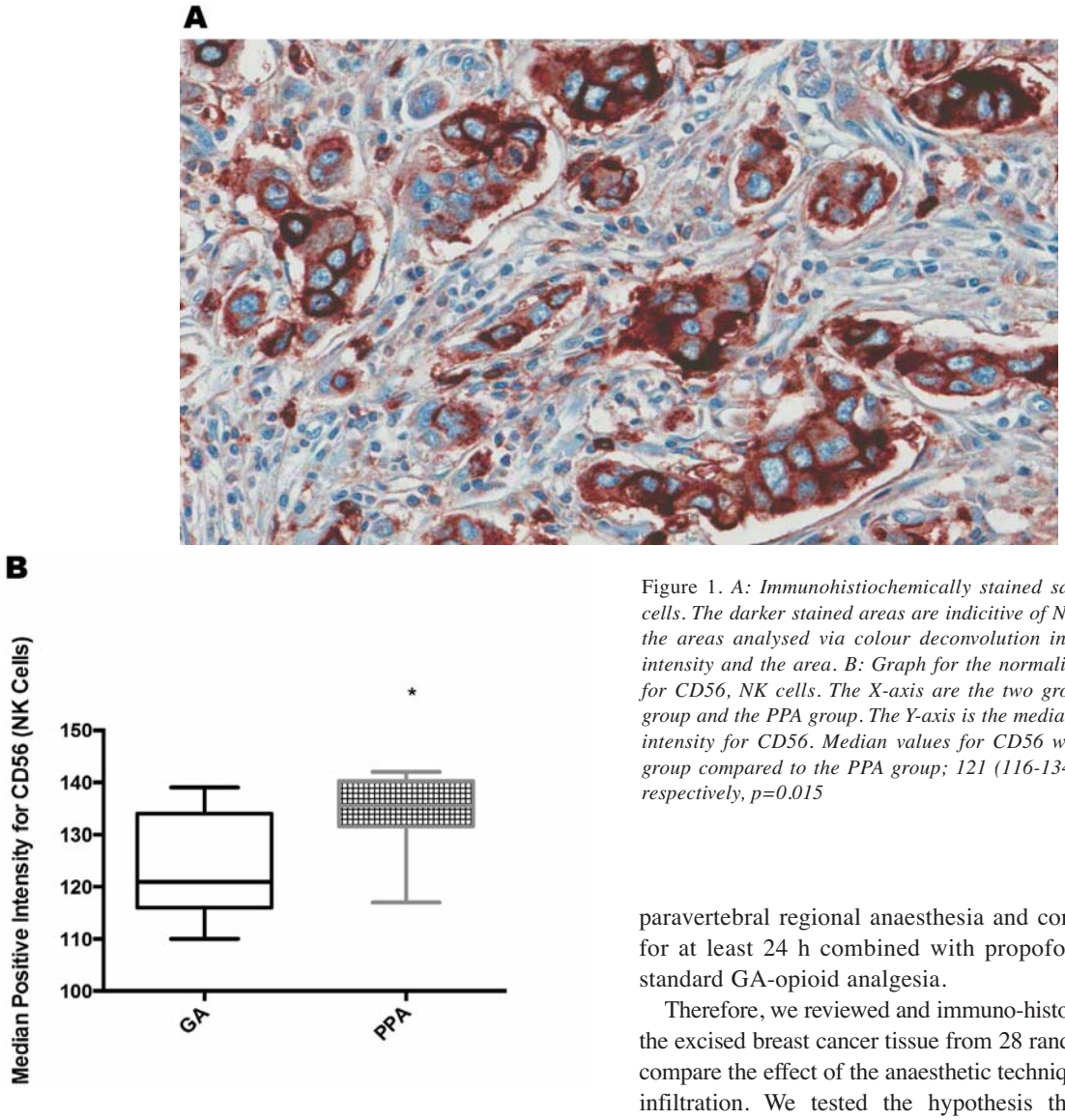



Figure 1. *A: Immunohistiochemically stained sample for CD56, NK cells. The darker stained areas are indicitive of NK cells and these are the areas analysed via colour deconvolution in order to assess the intensity and the area. B: Graph for the normalised positive intensity for CD56, NK cells. The X-axis are the two groups studied, the GA group and the PPA group. The Y-axis is the median normalised positive intensity for CD56. Median values for CD56 were lower in the GA group compared to the PPA group; 121 (116-134) vs. 136 (132-142), respectively, p=0.015*

Immune cell infiltration into human tumor tissue, particularly into stromal tissue rather than tumor islets, can regulate anti-tumour immune resistance and be an important harbinger of prognosis in certain types of lung cancer (19, 20). Moreover, in breast cancer patients, intra-tumoral infiltration of lymphocytes and monocytes may predict for efficacy of subsequent chemotherapy (21).

No study, to date, has evaluated the influence of the anaesthetic technique on immune cell infiltration in any cancer. The on-going international clinical trial (NCT 00418457) provides an opportunity to do so in breast cancer (13). In our centre, we have already enrolled >180 patients in an on-going 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 h 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 the 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.

## Materials and Methods

Research Ethics Committee approval was obtained 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 were chosen as being likely to indicate whether any significant

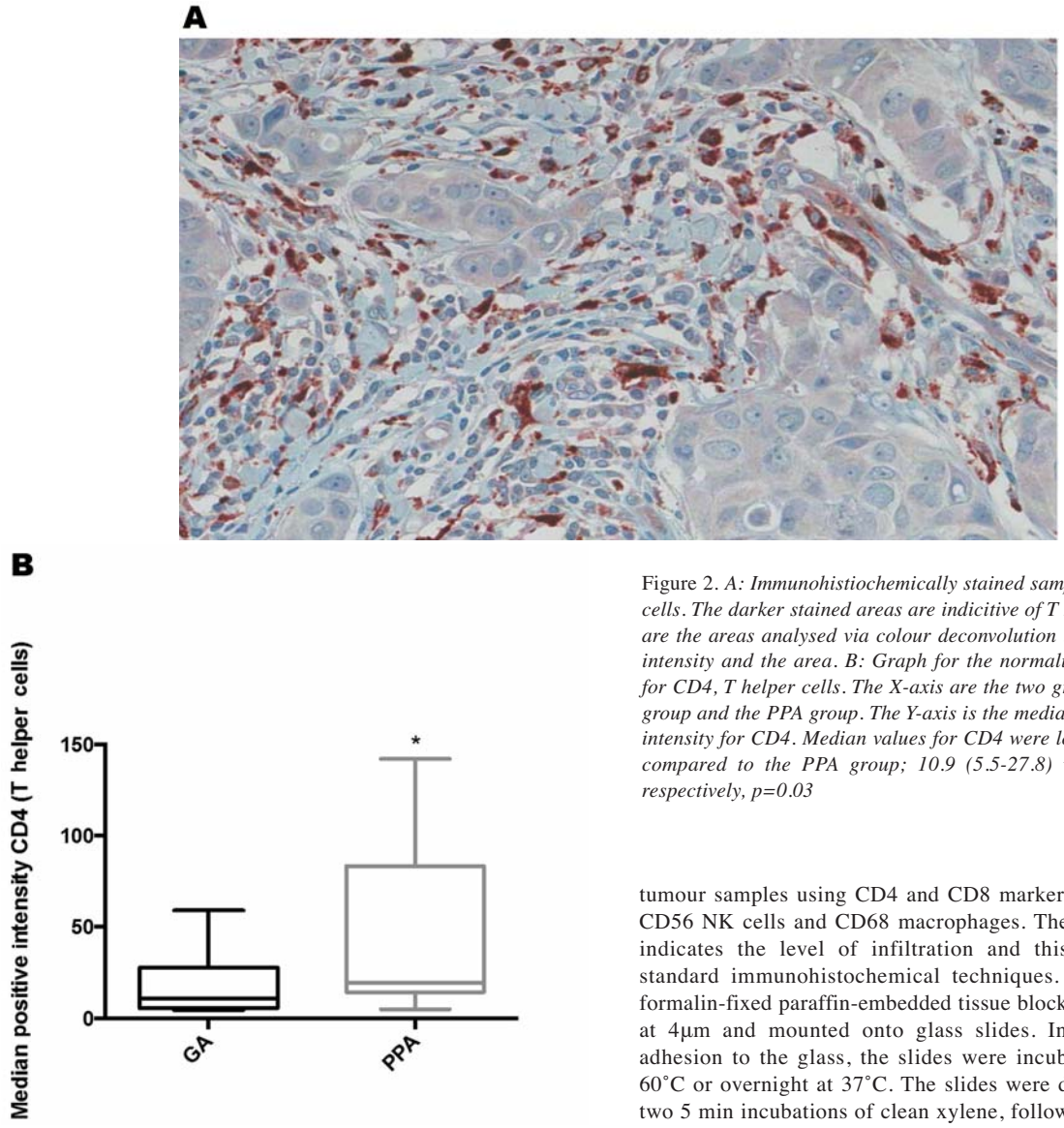



Figure 2. *A: Immunohistiochemically stained sample for CD4, T helper cells. The darker stained areas are indicitive of T helper cells and these are the areas analysed via colour deconvolution in order to assess the intensity and the area. B: Graph for the normalised positive intensity for CD4, T helper 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 CD4. Median values for CD4 were lower in the GA group compared to the PPA group; 10.9 (5.5-27.8) vs. 19.7 (14.4-83.5), respectively, p=0.03*

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. Thirty 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 CD8 markers of T lymphocytes CD56 NK cells and CD68 macrophages. The degree of staining indicates the level of infiltration and this was assessed by standard immunohistochemical 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 radio-transparent slide holder and the slide was immersed in 1 mM 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 bought to the boil in a microwave oven at max power (8 min for 800 ml) it was left boiling for 15mins at a reduced power (power 3) so that the liquid continued to simmer. It was cooled at room temperature for 30-60 min and then transferred to Tris-buffered saline. All staining was performed in a humid chamber. Double indirect alkaline phosphatase (AP) immunohistochemistry 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 (R/G/B)) corresponding to

Table I. *Demographic, morphological and breast cancer characteristics. All data shown are mean+SD or n (%).*

| Factors: | PPA group (n=12) | GA-opiate group (n=16) |
|---|---|---|
| Age (years) | 58+11 | 55+12 |
| BMI (kg/m$^2$) | 28+8 | 28+7 |
| ASA 1 | 4 (33) | 6 (37) |
| ASA 2 | 6 (50) | 8 (50) |
| ASA 3 | 2 (17) | 2 (13) |
| Type of Surgery: | | |
| Simple Mastectomy | 2 (17) | 3 (18) |
| Modified Radical | 1 (8) | 2 (12) |
| Wide Local Excision+ sentinel node | 9 (75) | 10 (63) |
| Other | 0 (0) | 1 (7) |
| Histological Grade: | | |
| Grade 1 | 2 (16) | 2 (12) |
| Grade 2 | 7 (59) | 7 (44) |
| Grade 3 | 3 (25) | 7 (44) |
| Lymph Node involvement: | | |
| Positive | 3 (25) | 6 (37.5) |
| Negative | 9 (75) | 10 (62.5) |
| Nottingham Prognostic Index | 4+3.7 | 4+3.5 |
| Hormonal Status | | |
| Oestrogen receptor-positive | 10 (83.3) | 14 (83.3) |
| Progesterone receptor- positive | 8 (66.6) | 12 (75) |
| Her2 receptor-positive | 4 (33.3) | 4 (25) |
| Define abbreviated terms | | |

Table II. *Intraoperative anaesthetic and analgesic and VAS pain on moving in post-anaesthesia care unit. All data shown are median (minimum, maximum).*

| Outcome | Propofol-paravertebral n=12 | General-opioid n=16 |
|---|---|---|
| Propofol (mg) | 800 (400, 1295) | 200 (150, 230) |
| Sevoflurane (MAC-hours) | 0.0 (0) | 1.2 (0.9, 1.8) |
| Intraoperative fentanyl (μg) | 50 (0, 125) | 200 (100, 300) |
| Morphine (mg) | 0 (0, 0) | 8 (4, 15) |
| VAS Pain (cm) | 1 (0, 3) | 3 (1, 4) |

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 (R/G/B) 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 these data was not normally distributed, we compared differences between the median (interquartile range (IQR)) 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 the Fischer's exact test.

## Results

While n=30 patients were randomly selected from 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 I shows the patients' 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 II 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 CD56 (natural killer cell) data. Figure 1A shows an indicative stain of CD56. Figure 1B shows the normalised positive intensity for the two groups of CD56 data. Median values for CD56 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 CD4. Figure 2B shows the normalised positive intensity for the two groups of CD4 data. Median values for CD4 were again lower in the GA group compared to the PPA group 10.9 (5.5-27.8) *vs*. 19.7 (14.4-83.5), respectively, $p=0.03$.

Figure 3 shows CD8 (T suppressor cell) data. Figure 3A shows an indicative stain of CD8. Figure 3B shows the normalised positive intensity for the two groups of CD8 data. Median values for CD8 were not significantly different in the GA group compared with the PPA group, median (IQR) 5.5 (4.0-9.75) *vs*. 13.0 (5.0-14.5), respectively, $p=0.24$.

Figure 4 shows CD68 (macrophage) data. Figure 4A shows an indicative stain of CD68. Figure 4B shows the normalised positive intensity for the two groups of CD 68 data. Median values for CD68 were not significantly different in the GA group compared with the PPA group, median (IQR) 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 found that there is indeed differential expression of natural killer and T helper cells but not T suppressor cells or macrophages. The 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 the anaesthetic technique on perioperative immune cell behaviour.

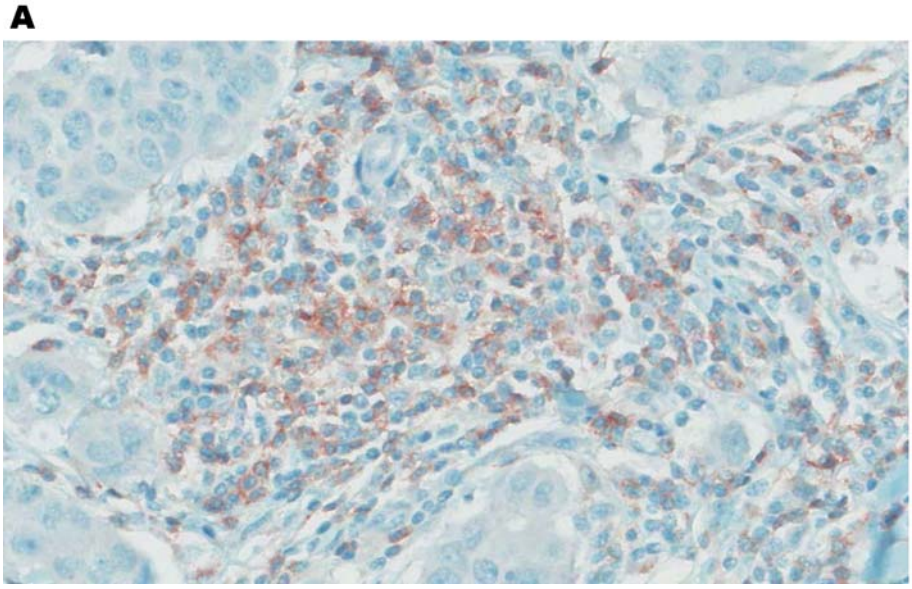
Immune cell markers have recently been the focus of translational research to predict efficacy of chemotherapy treatment of breast cancer, following a study that suggested that intratumoral lymphocytic infiltrates could be associated with better prognosis after chemotherapy in breast cancer patients. Moreover, in a mouse model of breast cancer, blocking vascular endothelial growth factor (VEGF), essential in angiogenesis of metastatic breast tumours, affected tumor infiltration with cancer-resisting immune cells, including NK cells, T helper cells, and reduced 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 interleukin (IL)-1β (22, 23). Previously, our group demonstrated that serum from patients randomised to the GA-opioid arm of the ongoing clinical trial had higher levels of VEGF, IL-1β and certain matrix metalloproteinases compared with patients receiving the putative cancer-resisting propofol-paravertebral anaesthetic technique (24, 25) Our study showed no difference in overall macrophage infiltration with anaesthetic technique, although, unfortunately, we missed the opportunity to look for specific tumor-associated macrophage 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 (26).

The mechanism by which an 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 (27). Amide local anaesthetics (LA) attenuated cancer cell activation and migration. Furthermore, LAs have also been observed to have direct cytotoxic effects on T-lymphoma cells *in vitro*, causing apoptosis at lower concentrations and necrosis at higher concentrations, and their cytotoxic effects appeared to correlate with their lipophilicity and potency (28).

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

The use of LAs 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 immunosuppressive effect and, therefore, could reduce the tissue immunocyte migration (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 that, 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 years (33). Whether this observation is attributable to quality of 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 as 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 (34).



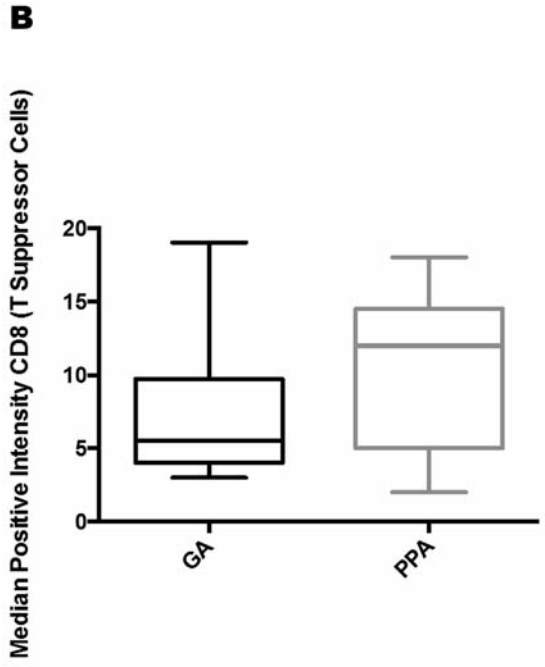




Figure 3. *A: Immunohistiochemically stained sample for CD8, T suppressor cells. The darker stained areas are indicitive of T suppressor cells and these are the areas analysed via colour deconvolution in order to assess the intensity and the area. B: Graph for the normalised positive intensity for CD8, 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 CD8. Median values for CD8 were not significantly different in the GA group compared to the PPA group; 5.5 (4.0-9.75) vs. 13.0 (5-14.5), respectively, p=0.24*

Moreover, it is known that the surgical stress response transiently impairs perioperative immunocompetence. Suppression of NK cell activity occurs within hours of surgery, lasts for days and is proportional to the invasiveness of the surgery (15, 16, 29-31). 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 reduced risk of metastasis during the initial years of retrospective follow-up of patients undergoing breast cancer surgery (1).

Our data suffer a number of potential limitations. We measured indirectly only the quantitative number of immunocytes expressed, an approach that 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 the 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 visualized. 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 the sample evaluated.

Furthermore, our CD4 data should also be interpreted with caution because this marker also stains for $CD4^{+}FOXP3^{+}$ cells,

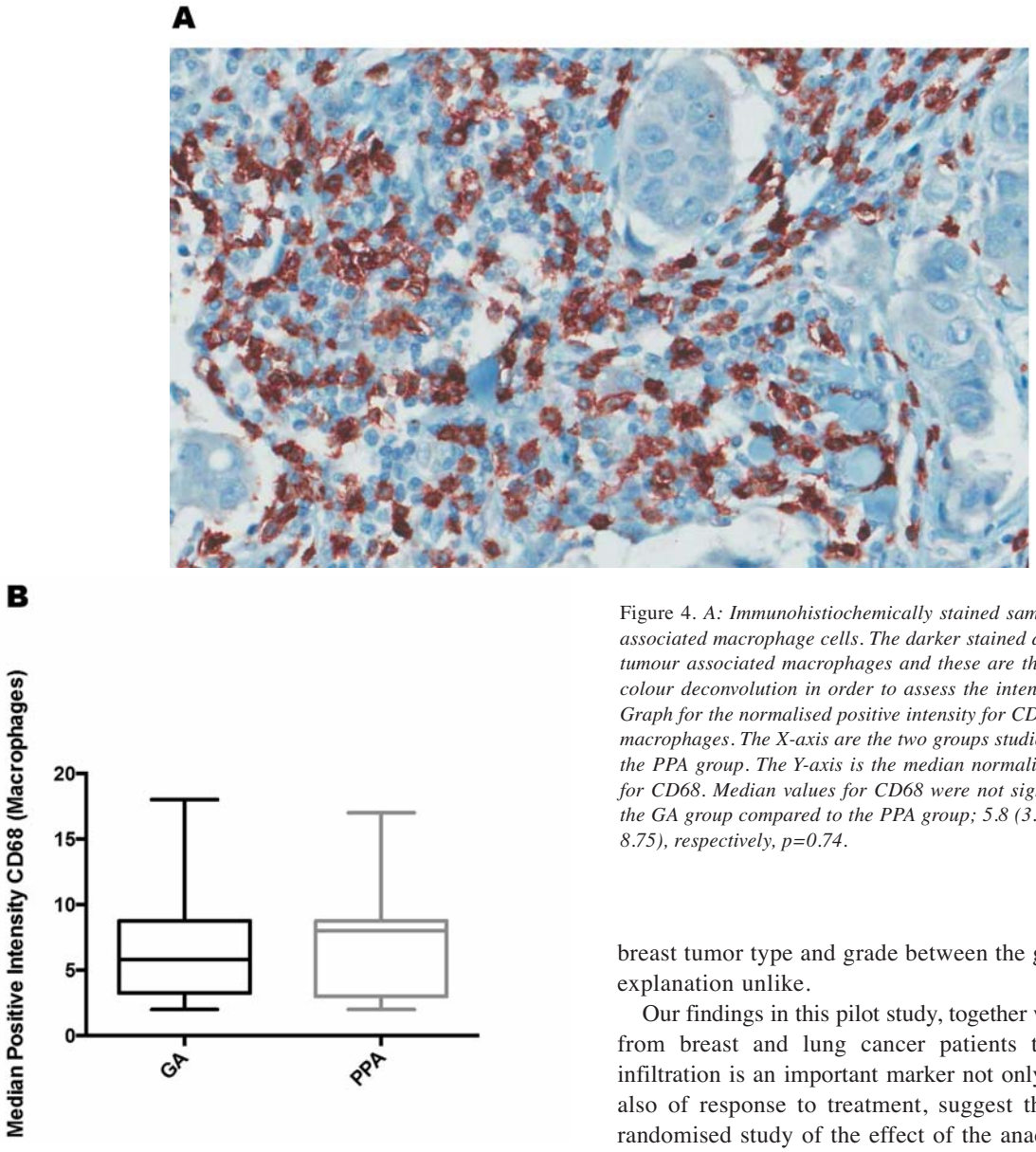



Figure 4. *A: Immunohistiochemically stained sample for CD68, tumor associated macrophage cells. The darker stained areas are indicitive of tumour associated macrophages and these are the areas analysed via colour deconvolution in order to assess the intensity and the area. B: Graph for the normalised positive intensity for CD68, tumor-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 CD68. Median values for CD68 were not significantly different in the GA group compared to the PPA group; 5.8 (3.25-8.75) vs. 8.0 (3.0-8.75), respectively, p=0.74.*

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 the 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 tumor type and grade between the groups makes this explanation unlike.

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 the 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-opoioid receptor (MOR) status within the tumour. Confirmation of these findings would be consistent with the inoculation of breast cancer cells in an animal model and provide a mechanistic rationale for a potential clinical effect of the anaesthetic technique on breast cancer outcome. Definitive data from the ongoing, randomised, clinical follow-up trial in breast cancer patients is awaited.

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