Effect of Perioperative Lidocaine and Cisplatin on Metastasis in a Murine Model of Breast Cancer Surgery

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Abstract. Background/Aim: Mortality from breast cancer is usually attributable to metastasis. *In vitro* data suggest that amide local anaesthetics, e.g. lidocaine, inhibit metastasis by multiple mechanisms and recent *in vivo* data support this. Experimental data also suggest that opioids may inhibit cisplatin chemotherapy. Whether lidocaine would influence cisplatin chemotherapy has not been evaluated. Materials and Methods: 4T1 cells were injected into the mammary gland of immunocompetent female BALB/c mice, with resection of the tumour under sevoflurane anaesthesia one week later. Mice (n=45) were randomized into one of three groups: The cisplatin group received 3 mg.kg⁻¹ cisplatin; cisplatin and lidocaine group received 3 mg.kg⁻¹ cisplatin and lidocaine bolus of 1.5 mg.kg⁻¹ followed by an infusion of 2 mg.kg⁻¹.h⁻¹. The control group received sevoflurane only. All agents were given perioperatively. After 14 postoperative days, post-mortem lung, serum and liver samples were collected. Primary outcome measure was lung metastasis colony count. Results: During sevoflurane anaesthesia, the addition of lidocaine to cisplatin significantly decreased metastatic lung colony count [(mean±SD) (157±87)] compared to control [846±581, (p=0.001)], and cisplatin alone [580±383, (p=0.018)]. However, liver metastasis colony count was not reduced with the combination of cisplatin and lidocaine (9.3±13.9) when compared to control (74.7±257.3), p=0.78 or to cisplatin

alone (110±388.8), p=0.569. Serum VEGF and interleukin-6 concentrations were not significantly different. Conclusion: In a 4T1 murine model of breast cancer surgery, under sevoflurane anaesthesia, lidocaine enhanced the metastasis-inhibiting action of cisplatin. Clinical evaluation of the hypothesis that co-administration of systemic lidocaine during cisplatin chemotherapy seems warranted.

For women, the lifetime risk of developing a breast malignancy is one in eight (1) and metastatic disease accounts for the preponderance of breast cancer related deaths (2). In the treatment of breast cancer, surgery remains the linchpin of most therapeutic management pathways with 85% of patients requiring surgery in the treatment of their disease (3).

The potential influence of the perioperative period on longer-term oncological outcome has increasingly become a focus of investigation (4-6), principally the impact of anesthetic and analgesia agents on subsequent metastatic burden. This has led to calls for randomized trials to definitively examine the impact of a variety of agents used in the perioperative period (7), increased collaboration with oncological specialties (7) and the development of oncoanaesthesia as a sub-specialty (8).

Although the majority of breast cancer patients undergo surgery, adjuvant chemotherapy has contributed to increased survival and reduced mortality (9). *In vivo* models of cancer have shown that opiates, often used perioperatively to achieve postoperative analgesia, inhibit the chemotherapeutic action of common chemotherapeutic agents, (10) and subsequent work suggests that lidocaine may have chemotherapy-enhancing effects in an *in vitro* model of breast cancer (11) and an *in vivo* model of hepatocellular carcinoma (12). These findings suggest that perioperative drugs, particularly amide local anaesthetics such as lidocaine, may have previously unrecognised effects on chemotherapeutic agents.

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Systemically administered lidocaine is indicated to improve gastrointestinal and analgesic outcomes (13). Pharmacologically, it inhibits neuronal conduction through sodium channel blockade. Sodium channel activity has been implicated in enhancing the invasive potential of a number of tumour cell types. This is due to changes in the transcriptional activation of “invasion-related” genes (14, 15) and *in vitro* work has shown that inhibition of sodium channels decreases circulating tumour cells capacity for tissue invasion (16). Besides this familiar sodium blocking activity, amide local anaesthetics have also been shown to inhibit intracellular Src-family kinase signalling, reduce proliferation and increase DNA demethylation in cancer cells (17-19).

While the scientific rationale underpinning how amide local anaesthetics inhibit cancer cell metastatic potential has been described *in vitro* (14-19), whether these effects are reproducible *in vivo* when combined with standard chemotherapy agents has not been previously evaluated. This is clinically relevant because many breast and other tumour patients now have neoadjuvant chemotherapy prior to surgery. Accordingly, we tested the hypothesis that systemic perioperative lidocaine reduces post-operative metastasis when co-administered with the chemotherapeutic agent cisplatin, in a 4T1 murine model of breast cancer.

Materials and Methods

This research was performed in accordance with the EU directive on animal studies (EU2010/63) and adherence to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (20). The study protocols were approved by the University College Dublin Animal Research Ethics Committee and authorised by the Health Products Regulatory Authority of Ireland.

Tumour model. A 4T1 BALB/c mouse model was used. As a post-surgical model of triple negative breast cancer, this syngeneic model metastasises in similar fashion to human disease, with initial spread to the lungs and liver (21). 4T1 cells have the advantage of being resistant to 6-thioguanine which aids in the detection of metastatic colonies on post-mortem specimens (22). To reduce the risk of spontaneous metastasis, tumour resection was carried out one week after inoculation (23).

Cell culture. The 4T1 cells were cultured in Roswell Park Memorial institute (RPMI) culture medium, with 10% fetal calf serum and 1% penicillin/streptomycin antibiotic added. Cells were then stored at 37°C and 5% CO₂ in sterile culture cell flasks. Prior to inoculation of study animals, the 4T1 cells were centrifuged, counted and then re-suspended in RPMI medium to obtain a final concentration of 1×10⁶ cells per millilitre (Countess Automated Cell Counter, Invitrogen, Carlsbad, CA, USA).

Test animals. Female BALB/c mice, aged eight to ten weeks were used (Charles River Laboratories, Edinburgh, Scotland). Following arrival to University College Dublin Biomedical Facility (UCD BMF), the mice underwent acclimatisation, in caged groups of five,

for five days before being enrolled into one of the study group arms. The mice were housed inside the barrier unit of UCD BMF in an air-conditioned room with the ambient temperature controlled at 21-22°C and 50% humidity under a 12-h light-dark cycle. Animals had *ad libitum* access to water and food. Following acclimatisation, animals were randomised into one of three groups

- (a) Sevoflurane (S)/Control Group: Inhaled sevoflurane 5% for induction and 3% for maintenance in 50% oxygen-air.
- (b) Cisplatin (C) Group: Inhaled sevoflurane as above and intravenous cisplatin, bolus 3 mg.kg⁻¹ given over 30-60 sec.
- (c) Cisplatin and Lidocaine (C&L) Group: Inhaled sevoflurane as above, intravenous cisplatin (bolus of 3 mg.kg⁻¹ over 30-60 sec) and intravenous lidocaine bolus of 1.5 mg.kg⁻¹ over 30-60 sec followed by infusion of 2 mg.kg⁻¹ h⁻¹ until the cessation of anaesthesia after 30 min.

Each mouse was treated as an individual unit for the purposes of randomization and a blocked design was employed to distribute the group over the duration of the study.

Establishing the tumor. After acclimatisation, animals were inoculated with 4T1 cells using a 26G hypodermic needle. The right lower inguinal mammary gland was used in all animals. Regular checks were performed to assess tumour growth for seven days. Once a palpable tumour developed, an electronic calliper was used to measure the lesion in two axes. The square root of these two measurements was taken to be the tumour diameter. Animal welfare was routinely monitored during this period by assessment of weight, behaviour, respiratory pattern and using the mouse grimace scale (MGS) (24).

Provision of anaesthesia. Seven days after inoculation, the tumour was surgically excised. This procedure took place under general anaesthesia with sevoflurane in an oxygen-air mixture. The animal was initially placed into an induction box and sevoflurane (AbbVie, Chicago, IL, USA) was delivered at a concentration of 5% with a 50% air-oxygen mixture as the carrier gas. Sevoflurane concentration was reduced to 3% once the mouse was considered to have lost pedal withdrawal response to a nociceptive stimulus and delivery of the inhalational agents was then changed from the box to a specially designed “face-mask”.

Surgical excision of the tumor. The surgical site was shaved with an electric razor and then prepared with chlorhexidine gluconate and isopropyl alcohol (Chloraprep®, CareFusion, Basingstoke, England). Analgesia was then administered and the nociceptive withdrawal response was re-checked prior to further intervention. For the administration of cisplatin, a lateral tail vein was accessed and the agent administered over 30 to 60 sec. Where lidocaine was to be administered, a 26G cannula was placed in the previously unused tail vein. The tumour was resected using an aseptic technique with closure using interrupted 7.0 polypropylene sutures (Ethicon, Somerville, New Jersey, USA). All animals were anaesthetised for 30 min as a standard.

Drug administration. Prior to commencing tumour excision, analgesia was administered to all animals subcutaneously; paracetamol 200 mg.kg⁻¹ and carprofen 10 mg.kg⁻¹.

Cisplatin: Where indicated, cisplatin chemotherapy 3 mg.kg⁻¹ was administered intravenously after cannulation of the tail vein and prior to tumour excision.

Lidocaine: Where indicated, lidocaine was administered intravenously as a bolus of 1.5 mg.kg^{-1} followed by an infusion of $2 \text{ mg.kg}^{-1}.\text{h}^{-1}$ for 30-40 min.

Post-operative welfare. Animals were assessed post-operatively on a regular basis and scored using a combination of the MGS (24) and appraisal of respiration, behaviour and weight. All animals received subcutaneous analgesia at 12-h intervals for two days post-operatively. Where animals scored excessively high, they were humanely euthanized to avoid further suffering.

Determining the metastatic burden of the solid organ samples. Two weeks after surgery, the study animals were culled by cervical dislocation. The liver, lungs and blood samples were then harvested. Collagenase type IV ($125 \text{ units.ml}^{-1}$) was used to treat the lungs and hyaluronidase ($500 \text{ units.ml}^{-1}$) and collagenase type I ($125 \text{ units.ml}^{-1}$) used to treat the liver. The treated solid organ tissue samples were then filtered using a cell strainer in order to better isolate cells. The cells isolated from the solid organs were then cultured at 37°C for two weeks in Iscove's Modified Dulbecco's Medium with 60 mM of 6-thioguanine (Sigma-Aldrich, MO, USA) added.

On day 35 of the study, the cultured solid organ cells were fixed to a plate using 100% methanol and then stained using 0.03% w/v methylene blue. Two researchers, blind to the experimental groups, then examined the plates for metastatic colonies (Figure 1) and the mean of the counted colonies was used for statistical analysis. A low-power magnification microscope was available to count the number of colonies per plate.

Cytokine analysis. Blood was taken immediately post-mortem on day 21. Serum was then isolated using centrifugation and, within two hours post mortem, stored at -20°C until required for analysis. A volume of $20 \mu\text{l}$ of serum was diluted to a final sample volume of $100 \mu\text{l}$ with the appropriate assay dilutant, *i.e.* a one to five dilution. Enzyme-linked immunosorbent assay (ELISA) was used to detect and quantify the presence of vascular growth endothelial factor (VEGF) (Sigma-Aldrich) and interleukin-6 (IL-6) (Abcam, Cambridge, UK).

Animals ($n=3$) with metastatic disease (as confirmed by colony count) were selected from each group for cytokine analysis. A researcher masked to the group randomisation prepared the ELISA samples, reagents and well-plates and undertook statistical analysis. Sample concentrations were interpolated from detected absorbances at 450 nm after calculation of a standard curve of known standard concentration against absorbance.

Statistical analysis. The 4T1 model has previously been successfully used to describe the impact of perioperative interventions on metastatic spread in breast cancer (23). Taking a 50% reduction in the metastatic burden to the lungs as a meaningful outcome, *e.g.* from a median of 50 metastatic colonies in control animals to 25 colonies in certain treated animals. We assumed a standard deviation of metastatic colony count expression, from our previous observations in this model, of the order of 30 colonies. Taking a Type I error of 0.05 and a Type II error of 0.1, $n=15$ animals per group were required to achieve 90% power to detect this level of difference. To allow for unexpected loss, we included ≥ 16 mice to each study group.

Results are expressed as mean $\pm$ standard deviation (SD) unless otherwise indicated. For comparison of the normally distributed murine weights and tumour sizes at the time of resection, one-way

Figure 1. Culture plate with 4T1 metastatic cells stained using methylene blue. 4T1 cells are resistant to 6-thioguanine which facilitates the detection of metastatic colonies on this example of a processed post-mortem lung tissue specimen.

ANOVA with *post-hoc* Bonferroni correction for multiple comparisons was implemented across the three groups. For non-normally distributed data, Kruskal-Wallis test was used. A p -value <0.05 was considered statistically significant.

Results

In total, 50 mice were inoculated. Three animals were euthanized post-operatively due to scoring excessively high on welfare checks (Figure 2). One animal was found to have an excessively burdensome intraperitoneal tumour load perioperatively and was culled under anaesthesia. One animal failed to develop a tumour after inoculation and was culled. Therefore, a total of $n=45$ animals were available for data analysis.

Group characteristics. There were no significant differences in tumour diameter between the sevoflurane control and cisplatin group, or the cisplatin group and cisplatin & lidocaine (C&L) group. However, there was a significant difference between the C&L group and the control group, with the C&L group having a larger primary tumour diameter at the time of resection, $p=0.027$. The tumour diameters for the control, cisplatin group and C&L group were $1.84 \text{ mm} \pm 0.22$, 2.39 ± 0.19 and 2.54 ± 0.14 respectively (Figure 3A).

Figure 2. Overview of study and animal use. A total of 50 animals were initially inoculated. Seventeen animals were randomised to the control group, 17 to the cisplatin group and 16 to the cisplatin and lidocaine group. One animal was determined not have developed a primary tumour. One animal was found to have a massive intraperitoneal tumour at surgical resection and was culled under anaesthesia. Three animals scored excessively high on post-operative welfare checks and were culled. Two of these animals were in the cisplatin group and one in the cisplatin and lidocaine group. A total of 45 mice were left available for analysis with n=15 in each group.

There were no significant differences in weight between the treatment groups at the time of primary tumour resection. The weights of the animals in the sevoflurane control group, cisplatin group and the cisplatin and lidocaine group were 19.24 ± 0.24 , 19.01 ± 0.29 and 18.91 ± 0.27 , respectively (Figure 3B).

Metastatic colony counts. In the presence of sevoflurane anaesthesia, lidocaine reduced the metastatic burden to the lung tissue when administered perioperatively with cisplatin. Animals receiving peri-operative cisplatin (580 ± 383) had similar colony count to the control animals (846 ± 581) ($p=0.182$). With the addition of lidocaine, a >75% decrease in pulmonary metastatic colonies (157 ± 87) was found when compared to the control and cisplatin groups, ($p=0.001$ and $p=0.018$ respectively) (Figure 4). This is despite an increased primary tumour diameter measurement at the time of surgery in the C&L group when compared to control

Hepatic metastases. The hepatic colony count was not lowered in the cisplatin group (9.3 ± 13.9) when compared to the control group (74.7 ± 257.3 , $p=0.78$). The addition of lidocaine to cisplatin (110 ± 388.8) did not significantly decrease the colony count when compared to cisplatin alone (9.3 ± 13.9), $p=0.569$.

Cytokine analysis. Serum vascular endothelial growth factor and interleukin-6 concentrations were not significantly altered between the different groups by the introduction of perioperative cisplatin or a combination of cisplatin and lidocaine. The respective mean (SD) VEGF concentrations (pg.ml^{-1}) detected in the sevoflurane control (S), Cisplatin (C) group and C&L groups were 2068 (3007), 866.7 (1190) and 2120 (1660), $p=0.897$ (Figure 5). The respective means (SD) IL-6 concentrations (pg.ml^{-1}) detected in the sevoflurane control, cisplatin group and cisplatin and lidocaine groups were 17.46 (20.07), 46.27 (47.31) and 19.03 (32.96), $p=0.563$ (Figure 6).

Figure 3. Tumour diameter and animal weight at the time of surgery. A) Tumour diameter at time of surgery. Median, interquartile range and full range of tumour diameters in each group at the time of surgery. The tumour diameter in the cisplatin and lidocaine group (S&L) was significantly larger than the control group. B) Animal weight at time of surgery. Mouse weight (in grams) at the time of surgery on day 7. Median, interquartile range and full range of each experimental group included. There were no statistically significant differences between the groups. S: Sevoflurane control group; C: cisplatin group. C&L: cisplatin and lidocaine group. * $p=0.027$ when compared to the sevoflurane control group.

Figure 4. Mean and standard deviation of colonies counted in the prepared lung samples of each experimental group. The combined cisplatin and lidocaine group had a statistically significant smaller number of metastatic colonies in the lungs when compared to both the sevoflurane control group ($p=0.001$) and the cisplatin group ($p=0.018$). S: sevoflurane control group; C: cisplatin group; C&L: cisplatin and lidocaine group. * $p=0.018$ when compared to cisplatin group.

Figure 5. Post-mortem serum vascular endothelial growth factor (VEGF) concentration detected by ELISA in picograms per millilitre. Three animals, known to have metastases to the lungs, were used from each group. Sample concentrations were derived from an interpolated curve of known standard concentration against measured absorbance. The mean and standard deviation of the three experimental groups are displayed. There was no statistically significant difference between the groups. S: sevoflurane control group; C: cisplatin group; C&L: cisplatin and lidocaine group.

Discussion

In this murine model of breast cancer surgery, using sevoflurane general anaesthesia, administering perioperative lidocaine with intravenous cisplatin decreased lung metastatic colony counts significantly when compared to perioperative intravenous cisplatin administration alone. These results are consistent with the hypothesis that perioperative lidocaine attenuates metastatic activity after

cancer surgery. Our data also raises the prospect that co-administration of systemic lidocaine could enhance standard cisplatin chemotherapy distinct from perioperative use, *i.e.* in oncologic practice.

Previous *in vivo* and *in vitro* work has examined the impact of lidocaine on standard chemotherapeutic agents when used to treat hepatocellular and breast cancer, but, these efforts were either cell culture-based projects or examined the impact of lidocaine alongside chemotherapy

Figure 6. Post-mortem serum interleukin-6 (IL-6) concentration detected by ELISA in picograms per millilitre. Three animals, known to have metastases to the lungs, were used from each group. Sample concentrations were derived from an interpolated curve of known standard concentration against measured absorbance. The mean and standard deviation of the three experimental groups displayed. There was no statistically significant difference between the groups. S: Sevoflurane control group; C: cisplatin group; C&L: cisplatin and lidocaine group.

on primary tumor growth and not metastatic disease (11, 12). What we present here is the first examination of the impact of perioperative lidocaine on the effectiveness of cisplatin and metastatic burden after primary resection in a breast cancer surgery model. The majority of breast cancer-related deaths are linked to metastatic disease (2) and thus examining the impact of intervention on metastasis offers a greater translation to clinical practice than using cell-based results, biochemical markers or tumour size as a sole outcome measure.

Cisplatin was the first platinum compound to receive FDA approval for use in the treatment of cancers and has been used to treat a variety of cancers (25). Cisplatin exerts its anti-tumour effect on breast cancer cells through the promotion of apoptosis (26), and it is known that this effect can be modulated by interaction at the molecular level (27).

We accept that in current oncological practice, cisplatin has a limited role in the treatment of breast cancer and it may be difficult to justify using platinum-containing chemotherapeutic compounds in treating breast cancer that is not metastatic triple negative disease (mTNBC) given that alternative agents with more advantageous side-effect profiles are available (28). However, cisplatin does not appear to have any lack of cytotoxic activity against breast cancer cells *per se*, and its use in clinical practice is avoided due to the availability of less toxic, more easily administered agents (29). Conversely, a recent review recognised that platinum-containing agents may confer survival benefit in mTNBC (28). The 4T1 murine

model can be considered an analogue of a post-surgical Stage IV triple negative disease burden due to lack of receptor expression.

Also, it is accepted that in clinical practice, besides exceptions such as hyperthermic intraperitoneal chemotherapy (or HIPEC), chemotherapeutics are not routinely given perioperatively. Where indicated, chemotherapy is usually administered after surgical excision over four to eight cycles taking 12 to 24 weeks. Occasionally, when large or locally advanced tumours are present, pre-operative (neo-adjuvant) chemotherapy is implemented to reduce the surgical resection required (30).

In our model, cisplatin was administered in the perioperative phase, immediately prior to surgery. Cisplatin alone, when administered this way, did not lead to a statistically significant reduction in pulmonary colony count when compared to the control group, $p=0.182$. Pulmonary metastatic colony count decreased significantly with the addition of lidocaine to cisplatin, when compared to the sevoflurane control and cisplatin groups (Figure 4). This fold reduction in the magnitude of pulmonary metastatic disease when compared to control occurred in spite of a larger primary tumour size (Figure 3A).

Amide local anaesthetics have previously shown metastatic attenuating effects on an assortment of tumour cell types, probably due to mechanisms beyond their well-recognized nerve conduction and membrane stabilizing effects (11, 14, 31). As the archetypal amide local anaesthetic, with a long history of use, proven safety profile with infusion and potential benefits when administered in the perioperative phase (13), lidocaine was used in our study.

We used lidocaine doses which are reflective of clinical practice and, ideally, we would have continued lidocaine infusions into the post-operative recovery period. Unfortunately, this was neither feasible or practical due to logistical and staffing concerns. Infusions were discontinued at cessation of anaesthesia. Regardless of the duration of surgery, anaesthesia was continued for 30 min and then terminated and this is the point at which any lidocaine infusion was stopped. The mean duration of lidocaine infusion was 21.8 min (SD 1.7 min, range=19-23 min).

The 4T1 model of mammary cell carcinoma was used as the analogue for human breast cancer. 4T1 cells metastasise primarily to the lungs, liver and bone in a pattern comparable to that of human disease (21, 32) and the model might be considered a post-surgical model of “triple negative” disease with a Stage IV metastatic burden. In a syngeneic, murine counterpart to human disease, inoculation can be performed on immunocompetent animals without risk of rejection and the role of immunity does not require specific attention (33). 4T1 cells are resistant to 6-thioguanine and this facilitates quantifying metastatic colonies (22). Finally, we have experience in using this model of breast cancer and our

previous work helped determine the optimal time for resection and the conduct of anaesthesia (23).

Lidocaine, in the absence of traditional tumoricidal agents, has been found to have an attenuating effect on cancerous tissues through both directly and indirectly acting mechanisms (17, 27, 31). The actual mechanism of cytotoxicity remains elusive, yet there is evidence that lidocaine may alter the balance between pro- and anti-apoptotic protein expression (12) and alter tumour suppressor gene expression (34) leading to the promotion of apoptosis in tumour cells. Lidocaine has also been found, *in vitro*, to promote DNA demethylation in breast cell cancer lines (11, 19) which may have a protective effect through regulation of tumour suppressor gene expression (34).

Apoptosis is ultimately the mechanism through which cisplatin exerts an anti-tumour effect (35) and a number of different signalling pathways can influence tumour cell sensitivity to cisplatin (36). It is plausible that lidocaine acts synergistically with cisplatin to more heavily tip the balance, at both the molecular and epigenetic level, towards a pro-apoptotic state through manipulation of apoptosis-controlling protein and gene expression (12).

Our group previously found reduced IL-6 expression associated with perioperative lidocaine administration using a semi-quantitative technique in a similar model of breast cancer surgery (23). Interleukin-6 is a pleiotropic cytokine, but elevated serum IL-6 levels appear to have a negative prognostication in breast cancer patients and blockade of IL-6 signalling receptors confers benefit *in vitro* (37). Similarly, elevated levels of VEGF are correlated with poorer clinical outcomes in breast cancer patients (38) and VEGF family member overexpression results in enhanced metastasis to lung tissue (39).

We used ELISA in order to better quantify the effects of peri-operative lidocaine on specific cytokines, and chose pro-inflammatory IL-6 and pro-angiogenic VEGF for evaluation. Using the serum from three animals known to have metastases from each group, it was determined that VEGF and IL-6 concentrations were not altered in a statistically significant way by the introduction of perioperative cisplatin or a combination of cisplatin and lidocaine when compared to control. Caution should be exercised when interpreting the findings of our cytokine analysis. Immunity is a bewilderingly complex interplay between a multitude of cytokines and it should not be assumed that lidocaine does not have any anti-inflammatory or anti-angiogenic action based on this analysis of a secondary outcome.

In conclusion, in this model of breast cancer, a potential metastasis-inhibiting effect of systemic lidocaine was found when given perioperatively in combination with cisplatin. This supports the hypothesis that lidocaine during cancer surgery may influence the risk of metastasis, and a prospective randomised clinical trial seems justified. Further clinical evaluation of a potential role of lidocaine in enhancing the

effectiveness of chemotherapeutic agents, independent of perioperative administration, also seem warranted.

Conflicts of Interest

D.M and D.J.B are Editorial Board members of the British Journal of Anaesthesia. All other Authors have no competing interests to declare.

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