

# Can Anesthetic Technique for Primary Breast Cancer Surgery Affect Recurrence or Metastasis?

Aristomenis K. Exadaktylos, M.D.,\* Donal J. Buggy, M.D., M.Sc., D.M.E., F.R.C.P.I., F.C.A.R.C.S.I., F.R.C.A.,† Denis C. Moriarty, F.C.A.R.C.S.I.,‡ Edward Mascha, Ph.D.,§ Daniel I. Sessler, M.D., Ph.D.||

**Background:** Regional anesthesia is known to prevent or attenuate the surgical stress response; therefore, inhibiting surgical stress by paravertebral anesthesia might attenuate perioperative factors that enhance tumor growth and spread. The authors hypothesized that breast cancer patients undergoing surgery with paravertebral anesthesia and analgesia combined with general anesthesia have a lower incidence of cancer recurrence or metastases than patients undergoing surgery with general anesthesia and patient-controlled morphine analgesia.

**Methods:** In this retrospective study, the authors examined the medical records of 129 consecutive patients undergoing mastectomy and axillary clearance for breast cancer between September 2001 and December 2002.

**Results:** Fifty patients had surgery with paravertebral anesthesia and analgesia combined with general anesthesia, and 79 patients had general anesthesia combined with postoperative morphine analgesia. The follow-up time was  $32 \pm 5$  months (mean  $\pm$  SD). There were no significant differences in patients or surgical details, tumor presentation, or prognostic factors. Recurrence- and metastasis-free survival was 94% (95% confidence interval, 87–100%) and 82% (74–91%) at 24 months and 94% (87–100%) and 77% (68–87%) at 36 months in the paravertebral and general anesthesia patients, respectively ( $P = 0.012$ ).

**Conclusions:** This retrospective analysis suggests that paravertebral anesthesia and analgesia for breast cancer surgery reduces the risk of recurrence or metastasis during the initial years of follow-up. Prospective trials evaluating the effects of regional analgesia and morphine sparing on cancer recurrence seem warranted.

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\* Research Fellow in Anaesthesia, Department of Anaesthesia, Mater Misericordiae University Hospital, Dublin, Ireland. † Consultant in Anaesthesia, Mater Misericordiae University Hospital and National Breast Screening Programme (Eccles Unit), and Honorary Senior Lecturer in Anaesthesia, University College, Dublin, Ireland. ‡ Professor of Anaesthesia, Mater Misericordiae University Hospital and University College, Dublin, Ireland. § Statistician, Department of Quantitative Health Sciences, Cleveland Clinic Foundation, Cleveland, Ohio. || Chair, Department of OUTCOMES RESEARCH, Cleveland Clinic Foundation Cleveland, Ohio. Director, OUTCOMES RESEARCH Institute, and Weakley Professor of Anesthesiology, University of Louisville, Louisville, Kentucky.

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Address correspondence to Dr. Buggy: Department of Anaesthesia, Mater Misericordiae University Hospital and National Breast Screening Programme Eccles Unit, Dublin 7, Ireland. donal.buggy@nbsp.ie. On the World Wide Web: www.mater.ie; www.or.org. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

CHEMOTHERAPY, endocrine therapy, and radiation therapy all have significant roles in the treatment of breast cancer, but surgical removal of the tumor offers the best prospect for a good prognosis.<sup>1</sup> However, the process of surgery inevitably induces a profound neuroendocrine, metabolic, and cytokine response.<sup>2</sup> A consequence of this stress response is transient perioperative inhibition of immune function.<sup>3</sup>

Regional anesthesia is known to prevent or attenuate the surgical stress response by blocking afferent neural transmission, which prevents noxious afferent input from reaching the central nervous system.<sup>4</sup> Paravertebral anesthesia is a regional anesthesia technique that is suitable for breast surgery when applied in the upper thoracic region.<sup>5</sup> We have previously demonstrated its efficacy in suppressing the stress response to breast surgery, although without a concomitant reduction in factors associated with tumor cell angiogenesis.<sup>6</sup> As importantly, paravertebral analgesia reduces the need for opioids, which themselves impair immune function.<sup>7,8</sup>

Experimental studies have shown that a number of perioperative factors, including regional anesthesia and optimum postoperative analgesia, independently reduce the metastatic burden in animals inoculated with a strain of breast adenocarcinoma.<sup>9-11</sup> It is plausible that inhibition of the surgical stress response by paravertebral anesthesia might attenuate perioperative factors that enhance tumor growth and spread. However, the effect of anesthetic technique on breast cancer outcome has not been evaluated in the clinical setting. In a retrospective analysis of existing medical records, we compared local recurrence and metastases in patients who had breast cancer surgery with and without paravertebral analgesia.

## Materials and Methods

After approval by the Ethics Committee of the Mater Misericordiae University Hospital, Dublin, Ireland, we reviewed the medical records of 165 patients who underwent mastectomy with axillary clearance or simple complete mastectomy between September 2001 and December 2002. Only the records from patients with a palpable breast lesion were included because the risk of recurrence is high in these patients. Furthermore, patients with smaller, screen-detected lesions tend to have less extensive operations that do not require paravertebral blocks and would have an extremely low incidence of recurrence over a longer time frame than we could review. We thus excluded patients who presented only

for wide local excision with sentinel-node axillary sampling, patients with cancers detected by the National Breast Screening Programme (because screen-detected cancers are less severe), and patients undergoing plastic or reconstructive surgery.

Paravertebral anesthesia was introduced to our department in September 2001. Local departmental policy was to offer it in combination with general anesthesia to patients undergoing extensive breast surgery involving axillary node clearance. In these patients, a catheter was inserted before induction of anesthesia into the ipsilateral paravertebral space at the level of T2 or T3 using a standard technique (paravertebral group).<sup>5</sup> Each of these patients was given a 0.2-ml/kg bolus of 0.25% levobupivacaine before induction of general anesthesia. An infusion of 0.25% plain levobupivacaine was continued for 48 h postoperatively. The same anesthetist (D.J.B.) placed all of the paravertebral catheters, the same surgeon performed all of the operations, and the same oncologist cared for all of the patients.

General anesthesia was induced with 0.5  $\mu\text{g}/\text{kg}$  fentanyl and 1.5–3.0 mg/kg propofol. After placement of a laryngeal mask airway, anesthesia was maintained with spontaneous breathing of 2–3% sevoflurane in nitrous oxide and oxygen. All patients received diclofenac, 100 mg rectally, preoperatively after induction of general anesthesia. Morphine boluses, 0.05 mg/kg, were given to patients in either group intraoperatively, at the discretion of the anesthetist.

All patients studied were under the care of the same surgeon. Patients cared for by anesthetists other than D.J.B. were not offered paravertebral anesthesia because the technique had been introduced into our department only recently. These patients, and those who declined paravertebral anesthesia or in whom it was contraindicated, received balanced general anesthesia with postoperative patient-controlled morphine analgesia (general anesthesia group). Patient-controlled analgesia was administered *via* a CADD-Legacy ambulatory infusion pump (model No. 6300; Deltec Inc., St. Paul, MN) using a 1-mg morphine bolus and a lockout time of 6 min.

The main outcome measure was the incidence of metastatic spread or cancer recurrence through August 2005 (*i.e.*, follow-up interval of 2.5–4.0 yr). We obtained demographic data; tumor size, grade, type, and estrogen receptor status; the extent of axillary nodal disease; whether postoperative or preoperative adjuvant chemotherapy or radiation therapy was used; and the patients' current status as determined by documentation of their follow-up visits to the outpatient clinics or their general practitioners. In addition, we assigned a Nottingham Prognostic Index, a score for which the prognosis for breast cancer is based on the formula: 0.2 (tumor size) + histologic grade (1 = grade 1, least aggressive tumor appearance on histology; grade 2 = intermediate appearance; grade 3 = most aggressive appearance) + axillary

lymph node involvement (1 = no axillary lymph nodes involved; 2 = up to three axillary lymph nodes involved; 3 = more than three axillary lymph nodes involved).<sup>12</sup> A score below 3.4 indicates a good outcome, and a score between 3.4 and 5.4 suggests an intermediate prognosis. In addition, we recorded the tumor margins, if the tumor was estrogen positive, and if the patient received chemotherapy, radiation therapy, or endocrine therapy. The patient-scored pain rating on the 11-point Likert scale at 4 and 24 h after surgery was also retrieved from the record.

If the most recent follow-up documentation in the hospital records exceeded 3 months from the date of our assessment, we contacted the patient's general practitioner by telephone to ensure that no consultation had occurred in the interim.

We recorded the data in coded form on an Excel (Redmond, WA) spreadsheet and imported it into SAS statistical software (Cary, NC) for analysis. Normally distributed continuous variables were compared with independent sample *t* tests, and nonnormal variables were compared with Mann-Whitney U tests; categorical variables were compared with chi-square tests or Fisher exact tests, as appropriate. A Kaplan-Meier log-rank test was used for univariable analysis, and Cox proportional hazards regression was used for multivariable analysis of the time to recurrence of cancer between groups. Factors considered for the multivariable model were age, family history of cancer, tumor size, histologic grade, duration of surgery, and blood loss. All factors significant at  $P < 0.25$  were retained in the model. Associations with  $P < 0.05$  were deemed statistically significant.

## Results

We reviewed the medical records of 129 patients who underwent mastectomy and axillary clearance between September 2001 and December 2002 and who met our inclusion criteria. An additional 65 patients who had surgery during that period did not qualify. Fifty qualifying patients had surgery with paravertebral anesthesia and general analgesia, and 79 had general anesthesia with postoperative morphine analgesia. The follow-up time was  $32 \pm 5$  months (mean  $\pm$  SD). There were no significant differences in patient characteristics. The median pain score was less in the paravertebral patients than in those receiving postoperative morphine analgesia (table 1).

Paravertebral analgesia was deemed unsuccessful in two patients, one in recovery and one after 16 h. These patients were given morphine patient-controlled analgesia, but their data were analyzed as part in the paravertebral group. No other paravertebral patients required morphine. Three patients in the general anesthesia group developed infections (4%), compared with two (4%) in the paravertebral group. No patient in either

**Table 1. Anesthetic and Surgical Factors**

	Paravertebral (n = 50)	General Anesthesia (n = 79)	P Value
Age, yr	57 [51–64]	56 [50–64]	0.95
Time from surgery to recurrence or last follow-up, months	36 [24–36]	36 [24–40]	*
Duration of surgery, min	90 [69–120]	90 [60–105]	0.35
Intraoperative blood loss, ml	100 [100–200]	100 [100–100]	0.017
Pain score 4 h, Likert score	1 [0–3]	3 [2–5]	0.02
Pain score 24 h, Likert score	1 [0–2]	2 [0–4]	0.04

Likert scale: 0 = no pain; 10 = worst pain imaginable. Data are presented as median [interquartile range] and compared by Mann–Whitney test.

\* P value not meaningful; see survival analysis results.

group received a blood transfusion. Preoperatively, none of the patients were taking oral opioids, and only one patient in each group was using oral steroid therapy.

Tumor presentation and prognostic factors were similar in the two groups. Both groups had a mean Nottingham Prognostic Index score in the intermediate range. Moreover, the incidence of chemotherapy, radiation therapy, and endocrine therapy after the initial surgical treatment did not differ significantly between the groups (table 2). Recurrence or metastasis was documented in 3 of 50 patients (6%) in the paravertebral group and in 19 of 79 patients (24%) in the general anesthesia group during the follow-up period (table 3). When adjusting for the varied duration of follow-up for each patient with a Kaplan-Meier analysis, the paravertebral group had slower time to recurrence ( $P = 0.013$ , log-rank test; fig. 1). Furthermore, in a multivariable analysis adjusting for histologic grade ( $P = 0.25$ ) and axillary node involvement ( $P = 0.01$ ), the risk of recurrence was significantly less in the paravertebral group ( $P = 0.012$ ; estimated hazard ratio [95% confidence interval], 0.21 [0.06–0.71]).

Only one patient, assigned to the general anesthesia group, died during the follow-up period. Both lung and bony metastases were found in this patient.

## Discussion

Development of postoperative cancer recurrence and metastases may be analogous to development of postoperative surgical wound infection. All surgical wounds become contaminated with bacteria perioperatively. Whether contamination becomes a clinical infection depends critically on the patient's immune status. Furthermore, there is considerable evidence that the "decisive period" for establishment of clinical infections is largely restricted to the intraoperative period and subsequent few hours.<sup>13,14</sup> Perioperative factors such as maintaining normothermia<sup>15,16</sup> or providing supplemental oxygen<sup>17,18</sup> thus modulate infection risk—even though infections are not detected clinically until days later.

As with the bacterial contamination that inevitably accompanies operations, surgery to remove primary breast cancer inadvertently disseminates tumor cells into both surrounding tissues and the systemic circulation.<sup>18</sup> And as with the progression from bacterial contamination to clinical infection, progression from local or circulating tumor cells to clinical recurrence and metastases likely depends on the host defense mechanisms.<sup>9–11,19</sup> Perioperative factors influencing immune competence may thus modulate the risk of recurrence or metastasis.<sup>3,9–11</sup>

**Table 2. Breast Cancer Details**

	Paravertebral (n = 50)	General Anesthesia (n = 79)	P Value
Tumor size at histology, cm	4.0 ± 4.9	4.6 ± 7.4	0.19†
Histologic grade			0.16†
Grade I	8 (16)	8 (10)	
Grade II	21 (42)	28 (36)	
Grade III	21 (42)	42 (54)	
Lymph node status			0.94†
Stage 1	22 (58)	35 (57)	
Stage 2	4 (11)	8 (13)	
Stage 3	12 (31)	19 (30)	
Nottingham Prognostic Index	4.5 ± 1.4	4.7 ± 1.7	0.38*
Estrogen receptor positive	38 (76)	46 (59)	0.20
Tumor margins after excision, cm	1.5 ± 0.8	1.1 ± 0.7	0.10*
Chemotherapy received	24 (51)	43 (58)	0.45
Radiation therapy received	36 (77)	60 (81)	0.55
Chemotherapy and radiation therapy received	22 (47)	39 (52)	0.48
Endocrine therapy received	27 (54)	44 (56)	0.85

All data are mean ± SD or number of patients (%). Chi-square test unless noted.

\* t test. † Mann–Whitney test.

**Table 3. Cancer Recurrence**

	Paravertebral (n = 50)	General Anesthesia (n = 79)
Crude recurrence	3 (6)	19 (24)
Percent of patients recurrence-free at 24 months (95% CI)	94 (87–100)	82 (74–91)*
Percent of patients recurrence-free at 36 months (95% CI)	94 (87–100)	77 (68–87)†
Recurrence location		
Local or axillary nodes	1	11
Liver metastasis	1	3
Bone metastasis	1	3
Lung metastasis	0	2

Data are number (%) or Kaplan-Meier survival estimate (95% confidence interval).

\*  $P = 0.038$  comparing groups on Kaplan-Meier estimates at 24 months (z test). †  $P = 0.007$  comparing groups on Kaplan-Meier estimates at 36 months (z test).

Perioperative immune competence is impaired by several factors, the most obvious being surgery *per se*, which elicits a profound neuroendocrine and cytokine flux. This stress response impairs numerous immune functions including a marked attenuation of natural killer cell function.<sup>9–11,16,19</sup> Natural killer cells are thought to play a central role in preventing tumor dissemination and establishment.<sup>3</sup> The stress response to surgery is attenuated by regional anesthesia, including paravertebral anesthesia.<sup>2,6</sup> Experimental studies in rats, for example, show that surgical stress is attenuated better by regional than by general anesthesia and that, consequently, natural killer cell function is better preserved and metastatic load to the lungs is reduced.<sup>9</sup>

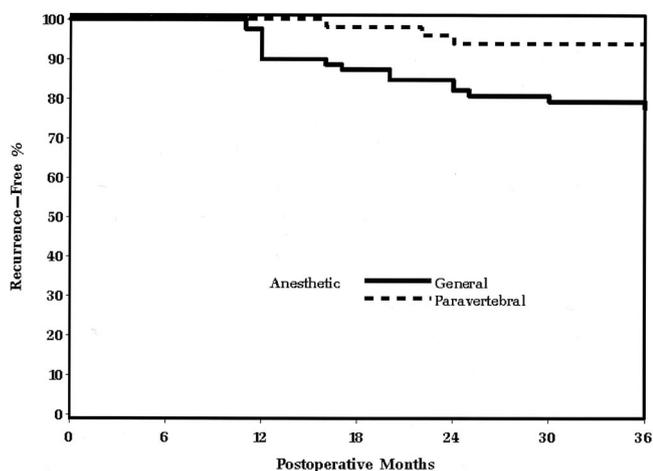
A second factor influencing perioperative immune competence is anesthesia *per se*. Anesthetic drugs impair numerous immune functions, including neutrophil and natural killer cells.<sup>7,18,20,21</sup> Patients with concomitant regional anesthesia might require less general anesthesia and thus perhaps have less anesthetic-induced immune impairment. But the major effect of regional analgesia is to spare patients postoperative opioids. This is an important effect because opioids, including the one most commonly used—morphine—suppress both cellular and hu-

moral immune function.<sup>7,8</sup> Consequently, optimum analgesia helps to preserve natural killer cell function in animals and reduces metastatic spread of cancer.<sup>10</sup> Furthermore, morphine is proangiogenic and promotes release of survival-enhancing factors that augment breast tumor growth.<sup>22</sup>

We speculate that regional anesthesia and analgesia thus might help to maintain perioperative immune function both by reducing general anesthesia requirements and by sparing postoperative opioids. To the extent that paravertebral anesthesia and analgesia help to maintain normal perioperative immune function, the technique seems likely to reduce the risk of tumor recurrence or metastasis. Our analysis is consistent with this theory: Paravertebral anesthesia and analgesia for breast cancer surgery reduced the risk of recurrence or metastasis fourfold during a 2.5- to 4-yr follow-up period.

This study has several important limitations inherent in its retrospective, observational design. Patients were not randomized and clinical care was not standardized, so that selection bias and the effects of unmeasured confounding variables cannot be excluded. For example, patients in the general anesthesia group had slightly larger tumors, smaller margins, and higher chemotherapy rates than patients in the paravertebral group, factors that could affect mortality, although these differences did not reach statistical significance. Relevant information such as the amount of morphine given and the type of chemotherapy used in each group was not available in the records. Therefore, this study should be viewed as generating a hypothesis and an estimated effect size for future large randomized controlled trials, which are being planned and which will require several years for execution and analysis. However, even a smaller effect size would be clinically important, making this hypothesis an important one to pursue, especially given its biologic plausibility according to the arguments presented above.

In summary, we observed a substantial reduction in tumor recurrence and metastases when breast cancer surgery was performed with paravertebral anesthesia and analgesia. Cancer surgery releases tumor cells into



**Fig. 1. Univariable association between paravertebral block and cancer recurrence,  $P = 0.013$  log-rank test. The association remained significant ( $P = 0.012$ ) in a multivariable model adjusting for histologic grade and number of axillary nodes.**

surrounding healthy tissue and into the systemic circulation. We speculate that whether these cells become established as recurrent cancer or metastases is likely to depend on immune competence in the immediate perioperative period. Regional anesthesia and analgesia may help to preserve immune function by attenuating the surgical stress response and diminishing the need for opioids. Although limited by its retrospective design, our study suggests that prospective trials evaluating the effects of regional analgesia and morphine sparing on cancer recurrence are warranted.

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# Direct effect of morphine on breast cancer cell function *in vitro*: role of the NET1 gene

P. Ecimovic<sup>1</sup>, D. Murray<sup>2</sup>, P. Doran<sup>2</sup>, J. McDonald<sup>3</sup>, D. G. Lambert<sup>3</sup> and D. J. Buggy<sup>1,4\*</sup>

<sup>1</sup> Department of Anaesthesia, Mater Misericordiae University Hospital, Dublin, Ireland

<sup>2</sup> Mater-UCD Clinical Research Centre, Dublin, Ireland

<sup>3</sup> Division of Cardiovascular Sciences, Department of Anaesthesia, University of Leicester, Leicester, UK

<sup>4</sup> Outcomes Research Consortium, Cleveland Clinic, Ohio, USA

\* Corresponding author. E-mail: donal.buggy@nbsp.ie

## Editor's key points

- Morphine can influence migration and gene expression and the NET1 gene has a role in migration of cancer cells.
- Morphine increased migration of cancer cells.
- Silencing NET1 expression reversed the effect of morphine, despite the absence of the mu-opioid receptor.
- The effect of morphine on NET1 gene expression may explain the effect of morphine on breast cancer cell migration.

**Background.** Experimental data suggest that postoperative analgesia in general and opioids in particular may affect the risk of metastases after primary cancer surgery. Perioperative single-gene activation may also spark metastatic disease. The NET1 gene promotes migration in adenocarcinoma cells. We investigated opioid receptor expression in both breast cancer cell lines and the direct effect of morphine and NET-1 on breast cancer cell migration *in vitro*.

**Methods.** Proliferation and migration of oestrogen receptor-negative MDA-MB-231 and oestrogen receptor-positive MCF7 breast cancer cells were studied after incubation with morphine 10–100 ng ml<sup>-1</sup> and control. NET1 gene expression was determined by polymerase chain reaction. The effect of NET1 on cell migration was determined using gene silencing with siRNA and stimulation with lysophosphatidic acid (LPA). The effect of morphine on NET1 expression and migration of cells with silenced NET1 was investigated.

**Results.** The NET1 gene was expressed in both cell lines and stimulated by LPA (2.9-fold in MCF7 and 78-fold in MDA-MB-231). NET1 expression was decreased by 96% after gene silencing in both cell lines with corresponding changes in migration. Despite the lack of opioid receptor expression, morphine increased the expression of NET1 (by 94% in MCF7 and by 263% in MDA-MB-231 cells). Morphine also increased migration by 17–27% and 7–53% in MCF7 and MDA-MB-231, respectively. Silencing the NET1 gene reversed the effect of morphine on migration.

**Conclusions.** The NET1 gene, but not opioid receptors, is expressed in breast adenocarcinoma cells and may facilitate their migration. Morphine increased both expression of NET1 and cell migration but not when NET1 was silenced, implying that NET1 contributes to mediating the direct effect of morphine on breast cancer cell migration.

**Keywords:** analgesia; cancer; metastases; morphine

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Breast cancer is the most common cancer in women and the second leading cause of cancer death,<sup>1</sup> which is usually caused by recurrence and metastasis. Treatment is based on effective surgical removal of the primary tumour, but surgery is inevitably associated with the release of tumour cells into the lymphatic and blood streams.<sup>2–3</sup> Whether this results in clinical metastases depends on the balance between anti-metastatic immune activity and the cancer cells' ability to metastasize.<sup>4–5</sup> At least four perioperative factors affect this balance: surgery and its associated stress response,<sup>6</sup> general anaesthesia *per se*,<sup>7</sup> postoperative pain *per se*, and also opioids, given to control surgical pain and which, all, inhibit immune function and may promote angiogenesis.<sup>8–11</sup> Morphine, the archetypal opioid, depresses natural killer T-lymphocyte activity.<sup>12–13</sup> In animal models, regional

analgesia techniques preserve effective perioperative immune defences, including natural killer T-lymphocyte activity against tumour progression by attenuating the surgical stress response and by sparing postoperative opioids.<sup>14</sup>

However, morphine has been shown to have direct effects on cancer cell function essential for metastasis, specifically on their ability to proliferate, migrate, and invade, but with conflicting results from *in vitro* studies to date.<sup>15–19</sup> Mechanisms of any direct effect of morphine on cancer cells also remain to be elucidated. For example, activation of opioid receptors with morphine was shown to exert an anti-proliferative effect in one report,<sup>15</sup> but the peripheral opioid receptor antagonist methylnaltrexone has recently been demonstrated to exert anti-proliferative effects on human carcinoma cells *in vitro*.<sup>20</sup>

Opioids can influence gene expression. It has been shown that a single injection of morphine can alter the expression of major groups of genes, including those responsible for cytoskeleton-related proteins, but whether this effect is mediated by standard opioid receptors is unknown.<sup>21</sup> Recent molecular analysis has shown that activation of a single gene, affecting a process essential for metastasis, can alone be sufficient to induce metastasis *in vitro*.<sup>22–23</sup> One such gene may be the NET1 gene, which has been identified through the Serial Analysis of Gene Expression (SAGE) database as overexpressed in breast and gastric adenocarcinoma cells.<sup>24–25</sup> The NET1 gene has a key role in organization of the actin cytoskeleton and thus in the ability of cancer cells to migrate and invade.<sup>26–27</sup> It has recently been shown that NET1 expression can help identify lymph node-positive breast cancer patients at high risk of metastasis.<sup>28</sup>

Therefore, we hypothesized that morphine may increase breast cancer cell migration and that this is mediated by the NET1 gene. Accordingly, we investigated whether the NET1 gene is expressed in oestrogen receptor-positive and oestrogen receptor-negative breast adenocarcinoma cell lines and if changes in NET1 gene expression resulted in altered cellular migration. We also investigated whether these cells express opioid receptors and any effect of morphine on NET1 gene expression, migration, and proliferation in both oestrogen receptor-positive and -negative breast adenocarcinoma cells, in addition to the effect of NET1 gene silencing.

## Methods

### Cell cultures

MDA-MB-231 is an oestrogen receptor-negative human breast adenocarcinoma cell line. Cells were cultured in L-Liebowitz 15 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin–streptomycin. MCF7 is an oestrogen receptor-positive human breast adenocarcinoma cell line. These cells were grown in Eagle's Minimum Essential Medium with Earle's salts and sodium bicarbonate, also supplemented with FBS, L-glutamine, and penicillin–streptomycin. Both cell lines were incubated at 37°C, in humidified atmospheric air with 5% CO<sub>2</sub>. Cells were grown as monolayers in 75 cm<sup>2</sup> standard tissue culture dishes (Sarstedt, Ireland). The medium was changed every 3 days, and passaged weekly. For experiments, cells were harvested from 70% sub-confluent cultures.

Chinese Hamster Ovary (CHO) cells were used as positive controls for opioid receptor expression. CHO cells expressing the human nociceptin/orphanin FQ (NOP) receptor were cultured in Dulbecco's modified Eagle medium/Ham's F12 medium (50:50) supplemented with 5% FBS, penicillin (100 IU ml<sup>-1</sup>), streptomycin (100 µg ml<sup>-1</sup>), and fungizone (2.5 µg ml<sup>-1</sup>). Stock cultures were further supplemented with hygromycin B (200 µg ml<sup>-1</sup>) and geneticin (200 µg ml<sup>-1</sup>). CHO cells expressing the human kappa-opioid receptor (KOP), delta-opioid receptor (DOP), and mu-opioid receptor (MOP) were cultured in Ham's F12 medium with 10% FBS, with penicillin, streptomycin, and

fungizone. Again, geneticin was added to stock cultures. CHO cells were grown at 37°C, in humidified air with 5% CO<sub>2</sub> and were harvested when confluent.

### Drug exposure

Morphine hydrochloride was obtained in its generic chemical form from Sigma (Sigma-Aldrich Ireland Ltd, Arklow, Ireland). It was further diluted in dimethyl sulphoxide (DMSO) to 10 µg ml<sup>-1</sup> and stored at room temperature and protected from light. To control for any effects of DMSO, control cells were also treated with DMSO. Immediately before the experiment, morphine was diluted with cell media to 100, 75, 50, 25, and 10 ng ml<sup>-1</sup>. These concentrations are similar to those obtained clinically during i.v. administration.<sup>21</sup>

Lysophosphatidic acid (LPA, Sigma) is an established driver of RhoA activation and has been shown to stimulate RhoA-mediated cytoskeletal rearrangement events.<sup>29</sup> Cells were exposed to 10 µM LPA in DMSO for 1, 2, and 4 h. After exposure, cells were harvested and used in experiments as described below.

### Cell proliferation assay

Cell proliferation was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Inc., Madison, WI, USA) according to the manufacturer's protocol. Cells were added to 96-well plates at a density of 50 000 cells per well, incubated for 24 h in a medium with FBS to allow attachment, followed by 24 h incubation in a serum-free medium. Morphine or DMSO was added to appropriate wells. The plates were incubated for 12, 24, and 36 h. Experiments were repeated three times in triplicate ( $n=3$ ). Proliferation is defined as an increase in the number of cells as a result of cell growth and division and was determined by the measuring change in absorbance at 490 nm resulting from proportion of living cells on a plate.

### Cell migration assay

Cancer cell migration across a microporous membrane was assessed using the Chemotaxis 96-well Cell Migration Assay (Chemicon International, Billerica, MA, USA) according to the manufacturer's protocol. A total of 50 000 cells were seeded to each upper chamber of the 96-well plate. Morphine and DMSO (control) diluted in media with 10% FBS were added to both upper and lower chambers of the appropriate wells. Cell migration was quantified as the number of cells that migrated directionally through an 8 µm pore-size membrane into a lower chamber containing 150 µl of media with 20% FBS as chemoattractant. Migration was measured as fluorescence at 480/520 nm and was corrected for proliferation. Independent experiments were repeated three times in triplicate ( $n=3$ ).

### RNA extraction and polymerase chain reaction

#### NET-1 expression

RNA was isolated as previously described.<sup>25–30</sup> Total RNA (2 µg) was treated with DNase I and reverse transcribed

using random hexamers and SuperScript II reverse transcriptase (Invitrogen Ltd, UK). Primers were designed using Primer3 software ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) and synthesized by Sigma-Aldrich, Ireland. The sequences of primers used for polymerase chain reaction (PCR) are available in Supplementary Table S1. PCR was carried out in a 50 µl mix containing 0.5 ml of Taq polymerase (Invitrogen) and 1 ml of cDNA. PCR products were then run on 2% agarose gel with a parallel 100 bp DNA ladder (Promega, UK). Real-time PCR was carried out according to the manufacturer's instructions using the LightCycler RNA SYBR Green 1 Amplification Kit (Roche Applied Science). All measurements were independently repeated six times ( $n=6$ ) except NET1 expression after exposure to morphine ( $n=3$ ). The maximum concentration of total RNA template used was  $0.5 \mu\text{g ml}^{-1}$ . Data are presented as cycle thresholds ( $C_t$ ), and in quantitative analysis, the  $\Delta\Delta C_t$  method was performed using the LightCycler version 4.0 software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were used to normalize NET1 expression.

### Opioid receptor expression

Total RNA was isolated from the breast cancer cell lines and CHO cells (positive control) from confluent 25 cm<sup>2</sup> flasks using either standard Tri-reagent methodology or a preparatory RNA isolation kit (*mirVana* Applied Biosystems) which uses a combination of organic- and solid-phase extraction methodologies.<sup>31</sup> Final RNA samples were resuspended in PCR grade water. RNA mass was determined using an Eppendorf Biophotometer and the purity was assessed from the 260/280 nm ratio which was  $>1.8$ . Extracted RNA was processed using a Turbo DNA-free<sup>®</sup> kit to remove any residual genomic DNA (gDNA) contamination before reverse transcription and production of copy DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). cDNA samples were probed using quantitative PCR and using commercially available TaqMan<sup>®</sup> probes from Applied Biosystems, KOP (OPRK1, Hs00175127\_m1), NOP (OPRL1-Hs00173471\_m1), MOP (OPRM1, Hs01053957\_m1), and DOP (OPRD1, Hs00538331\_m1). GAPDH was used as an endogenous control. CHO GAPDH Taqman probes were custom designed using Applied Biosystems Custom Taqman Gene Expression Assay Service (Supplementary Table S1). The thermal profile for all Q-PCR reactions in the StepOne instrument (Applied Biosystems) was 2 min at 50°C, 10 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 60°C. Non-template controls were included for all samples. Data are presented as cycle thresholds ( $C_t$ ) and in quantitative analysis,  $\Delta C_t$  is based on the differences between GAPDH and the gene of interest; MOP, DOP, KOP, and NOP. Experiments were repeated five times.

### Gene silencing of NET1 by RNA interference

Two siRNA duplexes were designed and synthesized for silencing the NET1 gene, as previously described (Qiagen Inc., CA,

USA).<sup>25</sup> Sequences of duplexes are listed in Supplementary Table S1. A chemically synthesized non-silencing siRNA duplex that had no known homology with any mammalian gene was used to control for non-specific silencing events. Cells ( $3 \times 10^5$ ) were added to each well of a 6-well plate in 3 ml growth media and incubated under the standard conditions for 24 h. After incubation, media was removed from the cells and a mix was added of 2.4 ml growth medium with 5 µg siRNA, 500 µl buffer, and 30 µl RNAifect (ratio siRNA:RNAifect=1:6) (Qiagen) dropwise. The cells were incubated for 48 h under standard conditions before assay/RNA extraction.

### Statistical analysis

Data were summarized as median and inter-quartile range. Differences between paired groups (i.e. before vs after an experimental manipulation) and between different groups were evaluated using the Wilcoxon matched pairs test and the Kruskal–Wallis tests with *post hoc* Dunn's test, respectively, as appropriate. A *P*-value of  $<0.05$  was taken to indicate the statistical significance.

## Results

### NET1 expression in breast cancer cell lines

We performed these experiments to determine if the NET1 gene was expressed in the breast cancer cell lines and if expression was changed with LPA or siRNA, which would imply that it can also be changed with exposure to other effectors such as morphine. NET1 expression was detected in both MCF7 and MDA-MB-231 cells. The effect of cell exposure to LPA is shown in Table 1. Treatment of cells with 10 µM LPA significantly increased expression of the NET1 gene in MCF7 by 2.9-fold [median cycle threshold ( $C_t$ ) 26.05] and in MDA-MB-231 cell line by 78-fold (median  $C_t$  23.06) after 2 h of incubation. Treatment with 10 µM LPA also caused a corresponding increase in migration by 234%

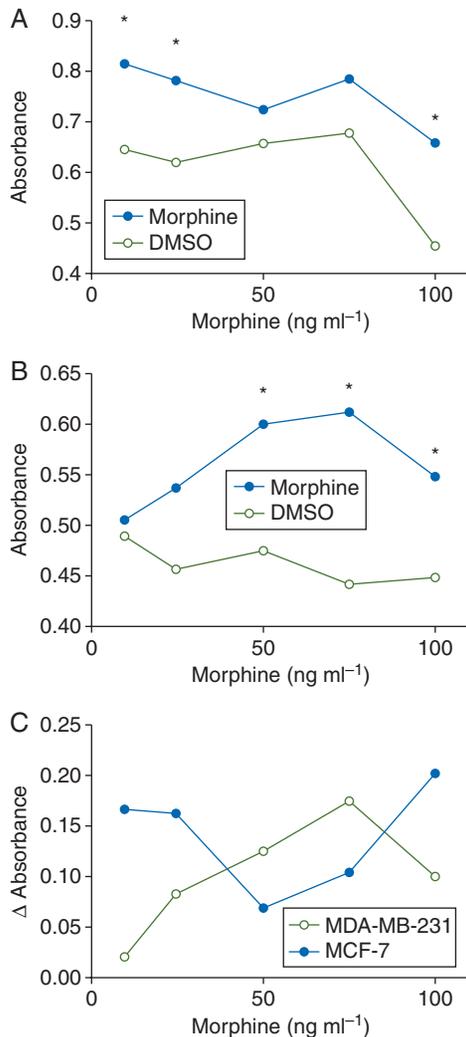
**Table 1** NET1 gene expression and migration in MCF7 and MDA-MB-231 cell lines after stimulation with 10 µM LPA for 1, 2, and 4 h. Individual data points are given ( $n=3$ )

	MCF-7	MDA-MB-231
T=1 h		
GAPDH $C_t$	15.29, 18.80, 25.78	16.02, 18.72, 25.33
NET1 $C_t$	23.50, 26.26, 33.33	22.48, 23.86, 26.27
$\Delta C_t$	7.55	5.14
T=2 h		
GAPDH $C_t$	17.20, 20.39, 24.39	14.42, 20.45, 33.30
NET1 $C_t$	26.32, 27.05, 27.71	22.63, 23.06, 23.66
$\Delta C_t$	6.66	2.61
T=4 h		
GAPDH $C_t$	18.08, 19.89, 23.46	15.1, 17.44, 18.21
NET1 $C_t$	24.17, 28.49, 36.08	22.00, 24.35, 27.85
$\Delta C_t$	8.60	6.92

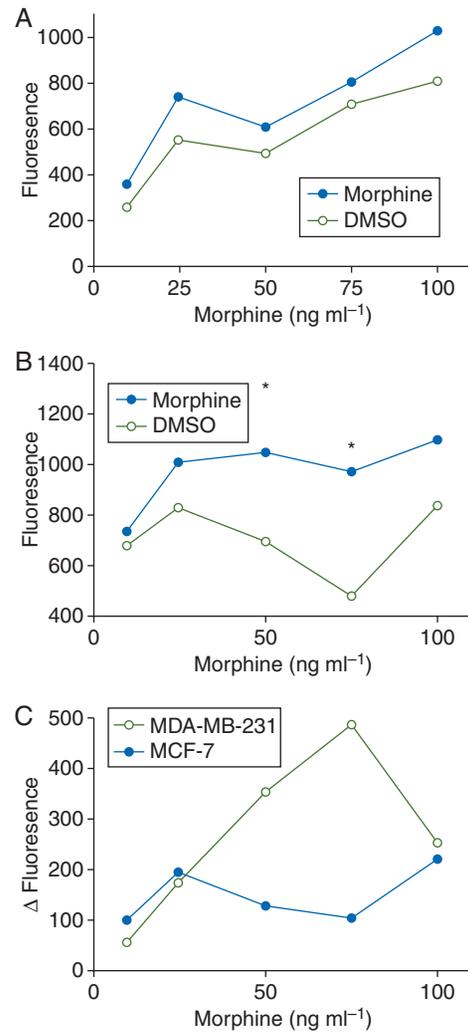
in MCF7 cells ( $P=0.007$ ) and by 299% in MDA-MB-231 ( $P=0.004$ ) compared with control. The effect of cell exposure to siRNA is shown in Table 2. Targeted siRNA reduced NET1 gene expression by 96% in both cell lines (median  $C_t$  23.03

**Table 2** NET1 gene expression and migration in MCF7 and MDA-MB-231 cell lines after gene silencing with siRNA. Individual data points are given ( $n=3$ )

	MCF-7	MDA-MB-231
GAPDH $C_t$	15.38, 16.94, 19.47	17.98, 29.58, 24.87
NET1 $C_t$	21.34, 26.13, 32.94	27.31, 29.23, 31.35
$\Delta C_t$	9.19	8.65



**Fig 1** Proliferation of breast cancer cells after incubation with morphine or DMSO control. Proliferation of (A) MCF7 cells and (B) MDA-MB-231 cells. Median value ( $n=3$ ) is shown. (C) Difference between morphine and control.  $*P<0.05$  compared with control.

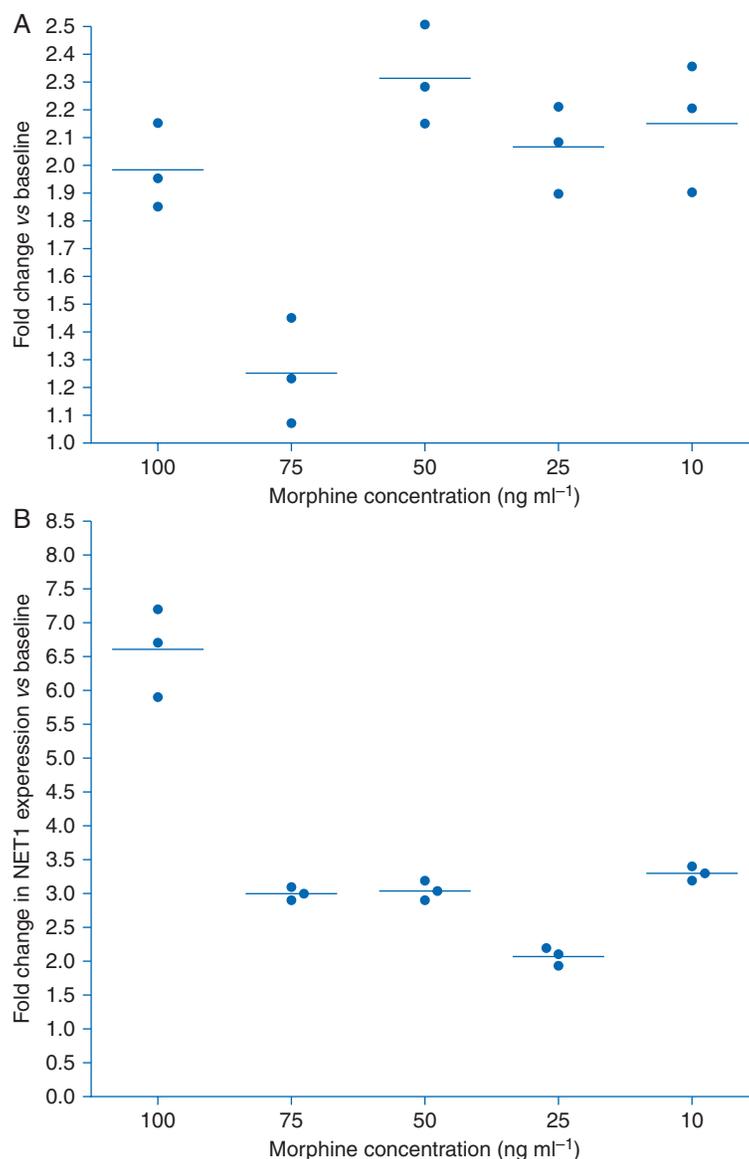


**Fig 2** Migration of breast cancer cells after incubation with morphine or DMSO control. Migration of (A) MCF7 cells and (B) MDA-MB-231 cells. Median value ( $n=3$ ) is shown. (C) Difference between morphine and control.  $*P<0.05$  compared with control.

in MCF7 cells and 29.23 in MDA-MB-231 cells). Migration of cells incubated with siRNA was not decreased compared with controls (data not shown).

### Effect of morphine on proliferation and migration

Proliferation experiments were carried out to determine that the chosen concentrations of morphine were not lethal to cells, and to enable migration results to be controlled for any change in proliferation. Morphine increased proliferation in both cell lines, with a 10–31% increase in MCF7 and a 2–16% increase in MDA-MB-231 cells (Fig. 1A–C). Morphine increased migration in both breast cancer cell lines. Migration was increased dose dependently by 17–27% in MCF7 and by 7–53% in MDA-MB-231 cells (Fig. 2).



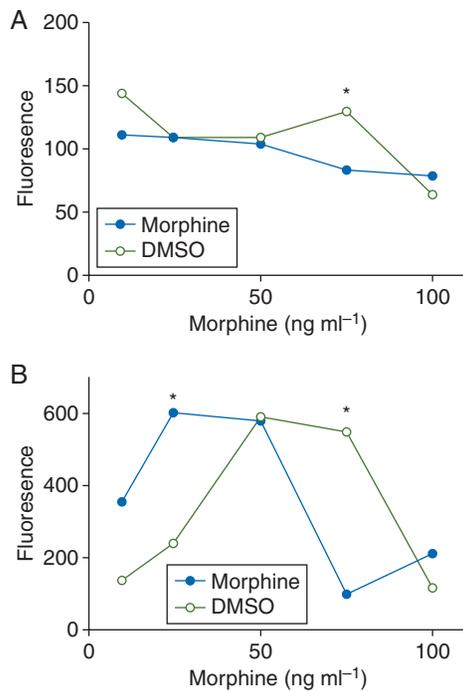
**Fig 3** Fold change of NET1 expression after incubation of cells with morphine. NET1 expression in (A) MCF7 cells and (B) MDA-MB-231 cells. All values are statistically significant ( $P < 0.05$ ). Individual raw data points are shown.

### Effect of morphine on NET1 expression

Treatment of breast cancer cells with morphine resulted in increased expression of the NET1 gene. A 1.2–4.3-fold increase in expression was observed in MCF7 cells ( $P = 0.04$ ) (mean  $C_t$  26.53–31.34), and a 2.1–6.7-fold increase in expression was observed in MDA-MB-231 cells ( $P = 0.003$ ) (mean  $C_t$  22.91–31.05) (Fig. 3A and B). This corresponded with increased migration after incubation with morphine. However, when NET1 expression was silenced, morphine did not increase migration of cells in either of the cell lines (Fig. 4A and B). NET1 silencing on average decreased morphine-induced migration of MCF7 cells by 13%, the decrease being largest at 36% at 75 ng ml<sup>-1</sup> ( $P = 0.019$ ).

### Expression of opioid receptors

In order to determine if the effects of morphine were due to activation of opioid receptor, we assessed the opioid receptor complement by PCR in both cell types. High  $\Delta C_t$  values (approaching 20) were measured for DOP in MDA-MB-231 cells and for MOP and KOP in both cell types (Table 3). This suggests that translation into active protein is unlikely. MCF-7 cells did express DOP with  $\Delta C_t$  values of  $\sim 13$ . Interestingly, both MCF-7 and MDA-MB-231 expressed mRNA encoding the NOP receptor with MDA-MB-231 expressing  $\sim 6.5$ -fold higher levels ( $P < 0.05$ ). High levels of expression of all targeted was seen in the CHO cells (positive controls) as expected.



**Fig 4** Migration of breast cancer cells with silenced NET1 gene after incubation with morphine or DMSO control. Migration of (A) MCF7 cells and (B) MDA-MB-231 cells. Median value ( $n=3$ ) is shown. \* $P<0.05$  compared with control.

**Table 3** Opioid receptor expression in breast cancer cells.  $C_t$  values for GAPDH and NOP, DOP, KOP, and MOP from five samples of MCF-7 and MDA-MB-231 breast cancer cells. Individual data points are given. \* $P<0.05$  compared with MCF-7. Control samples are recombinant CHO<sub>hNOP/MOP/DOP/KOP</sub>. <sup>†</sup>DOP only NTC for MCF-7, MDA-231, and control samples showed amplification at  $\sim 37$  cycles and greater. This is a background fluorescence that can occur with some of the Taqman assays. MCF7: KOP—one sample showed no amplification, two samples  $\Delta C_t$  was determined from the single positive assay well. MOP—two samples undetermined, two samples  $\Delta C_t$  was determined from the single positive assay well

	MCF-7	MDA-MB-231	Control
GAPDH $C_t$	16.88, 17.52, 17.89	16.53, 16.84, 17.18	18.56
NOP $C_t$	30.04, 30.53, 30.76	26.85, 27.13, 27.39	19.22
$\Delta C_t$	12.86, 13.01, 13.17	10.21, 10.31, 10.35*	0.65
GAPDH $C_t$	16.68, 16.90, 17.68	16.80, 16.98, 17.69	17.78
DOP $C_t$	26.98, 30.03, 30.82	34.47, 35.18, 35.52	17.73
$\Delta C_t$	10.30, 13.13, 13.22	17.60, 17.78, 18.38	-0.05
GAPDH $C_t$	16.90, 17.08, 17.66	17.72, 17.97, 18.15	18.42
KOP $C_t$	36.76, 36.86, 37.09	36.71, 36.78, 36.93	18.28
$\Delta C_t$	19.72, 19.83, 20.00	18.66, 18.81, 19.23	0.14
GAPDH $C_t$	16.91, 17.04, 17.82	16.30, 16.57, 16.74	17.78
MOP $C_t$	36.82, 37.12, 37.14	36.02, 36.40, 37.99	15.66
$\Delta C_t$	19.32, 19.45, 20.19	19.72, 19.81, 21.25	-2.12

## Discussion

This is the first study to our knowledge to investigate direct effects of morphine on breast adenocarcinoma cell function *in vitro* using two different cells lines of the same histological type but different metastatic potential (oestrogen receptor positive and negative). It is also the first experimental study to evaluate the role of the metastases-promoting NET1 gene in migration and proliferation of breast adenocarcinoma cells.

We have shown that morphine caused an overall increase in proliferation and migration of cell lines. This increase was more apparent in oestrogen receptor-negative MDA-MB-231 cells. The consistency of results in both cell lines convincingly suggests that morphine directly stimulates migration and proliferation of breast adenocarcinoma cells. However, there was no clear dose-response relationship. A previous study using MCF7 cell suggests that morphine stimulates angiogenesis and thus indirectly promotes tumour growth,<sup>13</sup> but this study did not investigate direct effects of morphine on cell proliferation or migration. In another study using MCF7 cells, an inhibitory effect of morphine on cell growth was observed to be dependent on stimulation of the cancer cells by  $17\beta$ -estradiol.<sup>17</sup> When  $17\beta$ -estradiol was removed, the effect of morphine on proliferation of cancer cells became positive. These, and our results, suggest interplay between opioids and oestrogen receptors in the regulation of breast cancer cell function, and would be consistent with the more pronounced effect of morphine on oestrogen receptor-negative cells in our study. It remains to be seen if the stimulatory effect of morphine on cell migration *in vitro*, observed in our present study, is translated clinically in terms of metastatic ability *in vivo* and hence into changes in the incidence of metastasis in breast cancer patients receiving non-opioid analgesia.

On the other hand, it is well known that the opioid system is one of the main inhibitory systems in the body. Morphine has been shown to induce cancer cell apoptosis *in vitro* in leukaemia cell lines, lung adenocarcinoma cell lines, and also in MCF7 breast adenocarcinoma cell lines<sup>15</sup> and cause inhibition of *in vitro* proliferation, migration, and invasion of some colon, pancreatic, and head and neck cancer lines.<sup>15</sup> Our data contradict these findings. Therefore, we determined the opioid receptor status of breast adenocarcinoma cells and found that the NOP receptor was expressed to a greater extent in oestrogen receptor-positive cells and DOP was expressed to a greater extent in oestrogen receptor-negative cells. We believe that the target for morphine, MOP, was absent. This suggests a complex interplay of morphine on breast cancer cells that does not involve MOP activation and as such does not contradict the above findings. Interpretation of our findings is limited, since we do not know what may be happening at the protein level. However, the relative absence of mRNA is highly suggestive of the absence of protein. Morphine and other opioids may cross cell membranes.<sup>21</sup> We therefore investigated genetic control of the function of the cells in terms of migration.

The NET1 gene has been identified as overexpressed in breast and gastric adenocarcinoma cells.<sup>23 24</sup> Furthermore, its expression can identify node-positive breast cancer patients at high risk for distant metastasis.<sup>28</sup> It has been shown that it has a key role in organization of the actin cytoskeleton and thus in the ability of cancer cells to migrate.<sup>26 27 30</sup> In our study, when NET1 gene expression was stimulated with LPA, an established driver of RhoA activation, this was associated with enhanced migration in both breast cell lines, but to a much greater extent in oestrogen receptor-negative cells. On the other hand, when NET1 expression was silenced by incubating cells with siRNA, the expected decrease in cell migration was minimal, suggesting that the absence of NET1 does not affect baseline cell migration. Our results concur with the present knowledge on NET1 function in gastric adenocarcinoma cells.<sup>30</sup> Also, oestrogen receptor-negative breast cancer is known to have greater metastatic potential, which could explain the difference in the NET1 gene expression following LPA exposure in our two cell lines. It has been suggested that certain gene expressions are dependent on oestrogen receptor status,<sup>24</sup> which suggests that further studies are needed to determine a possible involvement of NET1 in a metastatic potential of oestrogen receptor-negative breast adenocarcinoma cells.

We also found that morphine affects NET1 gene expression in breast adenocarcinoma cells and thus changes the cells' ability to migrate. Little is known so far on the effect of morphine on gene expression and the role of changes in gene expression in direct effects of drugs on cells. Pentobarbital, isoflurane, and propofol have been shown to affect gene expression *in vivo*.<sup>32</sup> It has also been shown that a single dose of morphine can alter expression of two major groups of genes: for proteins involved in mitochondrial respiration and for cytoskeleton-related proteins,<sup>29</sup> but it has not been determined if this change in gene expression causes a change in cell function.

In conclusion, our results have shown that morphine significantly increases expression of the NET1 gene in both cell lines. This corresponded to an increase in migration of cells incubated with morphine compared with controls. To investigate the link between the effect of morphine on NET1 gene expression and effect of morphine on cell migration further, we exposed cells with silenced NET1 gene to morphine. In cells with silenced NET1, the stimulating effect of morphine on migration was lost. These results indicate that NET1 is required for morphine to fully exert its direct effect on breast cancer migration. Further studies are needed to further elucidate the mechanistic pathway of direct morphine effect on cell migration, perhaps involving protein end-products of the NET1 gene.

## Supplementary material

Supplementary material is available at *British Journal of Anaesthesia* online.

## Conflict of interest

D.J.B. and D.G.L. are members of the BJA Board. D.G.L. and J.M. have collaborative links with *University of Ferrara Peptides* (UFPeptides) that is involved in the development of opioid ligands. D.G.L. holds a consultancy with Grunenthal GmbH.

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