Methylene Blue Dye, an Accurate Dye for Sentinel Lymph Node Identification in Early Breast Cancer

CAROLE MATHELIN[1], SABRINA CROCE[2], DAVID BRASSE[3], BÉATRICE GAIRARD[4], MOUSLIM GHARBI[1], NOROSOA ANDRIAMISANDRATSOA[5], VIRGILE BEKAERT[3], ZIAD FRANCIS[3], JEAN-LOUIS GUYONNET[3], DANIEL HUSS[3], SAMUEL SALVADOR[3], ROLAND SCHAEFFER[6], DANIEL GRUCKER[5], CRISTI MARIN[2] and JEAN-PIERRE BELLOCQ[2]

[1]Unité de sénologie, [4]Département d'onco-hématologie,
[5]Service de Médecine Nucléaire Hôpital Civil,
[6]Département d'anesthésiologie, CHRU, 1 place de l'Hôpital, F-67091 Strasbourg Cedex;
[2]Département de pathologie, Hôpital de Hautepierre, CHRU, Avenue Molière, F-67098 Strasbourg Cedex, France;
[3]Institut Pluridisciplinaire Hubert Curien, UMR 7178 – CNRS/IN2P3
et ULP, BP 28 – 23 rue du Loess, F-67037 Strasbourg Cedex 02, France

Abstract. *Purpose: The aim of this prospective study was to analyze the safety of methylene blue dye (MBD) and compare its efficacy with that of isotopic mapping for sentinel lymph node (SLN) identification in breast cancer. Patients and Methods: The SLN procedure, involving isotopic mapping and MBD (subareolar intraparenchymal injections of 2 mL, 10 mg/mL), was performed on 100 patients with early breast cancer. Results: The procedure was safe with a success rate of 99%; SLNs were, respectively, found in 65% by MBD, in 73% by lymphoscintigraphy and in 94% by gamma-probe. Out of 40 metastatic SLNs, 37 were "hot" and 32 stained. Digital examination allowed the detection of 2 additional metastatic LNs. Conclusion: MBD is safe and combination mapping associated with digital examination is the superior method. Modification of the procedure, favouring injections of dilute MBD (4 mL, 1.25 mg/mL) increases MBD efficiency (90%) and maintains low rates of complications.*

As lymph node (LN) metastasis is one of the most important prognostic factors for survival, the assessment of regional LN is essential in the staging of breast cancer, ascertaining a prognosis, and determining optimal adjuvant treatments. The sentinel lymph node (SLN) procedure consists of recognizing and removing the first LN(s) that filter(s) lymphatic fluid from the tumour. It is now a widely accepted method of LN staging in selected early invasive breast cancer. Axillary lymph node dissection (ALND) is no longer needed if the SLN is normal (1). One main pitfall is the failure to visualize the SLN, resulting in incorrect tumour staging, leading to suboptimal treatment or axillary recurrence (2). To reduce the false negative rate of the SLN procedure, the use of a combined method (technetium and blue dye) is recommended (3-6).

Different blue dyes, isosulfan blue dye (IBD), patent blue (PB), sulfan blue, sulphane blue, patent blue violet, patent pure blue, and methylene blue dye (MBD) have been evaluated for the SLN procedure. Animal (7) and clinical (8) studies have reported that IBD has a high degree of success for SLN identification. IBD has a molecular weight of 543.7 and involves two SO_3 groups that bind to protein. The protein-dye complex has a vivid affinity for lymphatics, with a particle size small enough to travel through the lymph vessel, but sufficiently large to be trapped in the SLN. Similar to its isomer IBD, PB binds to proteins and is absorbed by the lymphatics (9). MBD is a smaller molecule (molecular weight 319.9) with no sulfonic acid groups in its structure and it does not bind to plasma proteins (8). Using a feline model, Wong *et al.* (7) demonstrated that, when injected intradermally, MBD was less satisfactory in defining lymphatic drainage patterns because of poor uptake in the lymphatics and because of tissue staining. As a result, IBD and its isomer PB were adopted for use in the SLN procedure.

However, it rapidly appeared that IBD and PB were associated with a significant number of allergic reactions (0.1-3%) (10-15), some of which were life-threatening (16). Moreover, an international shortage of IBD led to a search for alternative dyes for SLN mapping in breast cancer. Simmons *et al.* (17) published the first study of MBD injection for the SLN procedure in breast cancer and described localization

Correspondence to: Mathelin Carole, MD, Ph.D., Unité de sénologie, 1 place de l'Hôpital, F-67091 Strasbourg Cedex, France. Tel: +33 388116071, Fax: +33 388115391, e-mail: carole.mathelin@chru-strasbourg.fr

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rates of 90% , which was unexpectedly comparable to IBD and PB. Because MBD has been shown in numerous other studies to be equally effective in SLN identification in comparison with other blue dyes (10, 16, 18-26), it was proposed as an alternative technique for the SLN procedure. In addition, MBD, widely used in different diagnostic and therapeutic procedures (such as surgery of nipple discharge, Fallopian tube patency evaluation, chromoendoscopy), was only exceptionally associated with potentially life-threatening adverse events and caused fewer changes in oxygen saturation compared with other blue dyes (24). Furthermore, MBD offered a substantial cost reduction (17).

However, the safety of MBD during SLN procedure has remained to be addressed in randomized clinical trials (27) although, localized reactions, due to MBD injections, including necrosis of the skin and subcutaneous tissues and necrotic abscesses, have been described (26, 28, 29). More recently, pulmonary oedema has been reported with MBD during a SLN procedure in a 44-year old woman (30). The EFS (Établissement Français du Sang) proposed a new fresh frozen plasma, called PVA-BM (plasma viro-atténué par le bleu de méthylène), whose virus reduction included a MBD method (31). Eight severe allergic reactions were observed, leading in one case to the patient's death (risk of severe allergy: 1/5900 PVA-BM). The accountability of MBD is currently under evaluation.

The aim of this study was to analyze the safety of MBD and to compare the success rate of MBD, lymphoscintigraphy and gamma probe detection for SLN identification in the management of early breast cancer in a prospective series of 100 patients.

Patients and Methods

One hundred patients with invasive breast carcinomas or ductal carcinomas *in situ* (DCIS), diagnosed preoperatively by core biopsy, were enrolled in the study between the 12th of April 2006 and the 11th of April 2007 after approval and informed consent. The study (ClinicalTrials.gov Identifier: NCT00314405) was approved by the Local Research Ethics Committees and reviewed in accordance with the precepts established by the Helsinki Declaration.

None of the patients was pregnant and none of them had undergone chemotherapy or locoregional radiotherapy. Patients with suspect axillary LN were not enrolled in the clinical trial. Likewise, patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, thalassaemia or drepanocytosis were not included, because the use of MBD may aggravate methemoglobinaemia or precipitate haemolytic anaemia. After surgery, all the patients were submitted to a clinical, radiological (mammography and ultrasound) and biological (Ca 15-3) follow-up twice a year.

Methods. The SLN procedure was initiated 18 hours before surgery using a preoperative injection of colloidal rhenium sulfur and technetium (0.4 mL of Nanocis®, CIS Bio International, Gif sur Yvette, France) at the four cardinal points in the subareolar area, the total activity injected ranging from 15 MBq to 28 MBq. Scintigraphic images (anterior, lateral and oblique views) were obtained 3 hours after the rhenium sulfur injections with a conventional gamma camera (Hélix®, Elscint, Haïfa, Israel). Ten minutes before surgery, 4 intraparenchymal injections of 0.5 mL (full strength, 10 mg/mL) of MBD (Aguettant®, Lyon, France) were administered at the four cardinal points in the subareolar area (total volume: 2 mL). No massage was performed.

During surgery, the search for SLNs began with the detection and removal of all the blue-stained SLNs. The next step was to remove the "hot" nodes that were detected with a cadmium telluride (CdTe) gamma probe. The probe commercialized by Eurorad (Strasbourg, France) consisted of a semiconductor radiation detector operating at room temperature and low voltages. The 5×5×3 mm^3 CdTe detector and the charge preamplifier were mounted in an 11 mm diameter probe ensuring a detection efficiency greater than 60% for ^{99m}Tc. The collimator was an integral part of the detector head which was slightly angled to permit easier access to the axillary area.

The activity of all the removed SLNs was measured immediately after their resection by a gamma ray counter. This detector was based on a yttrium aluminum perovskite, activated by cerium (YAP:Ce) crystal cylinder (20 mm diameter, 10 mm thickness) coupled to a photomultiplier tube. The 26% geometrical efficiency of the counter was estimated by simulation (32) and validated with radioactive standard and several experimental SLN activities. The activity was measured within a 5% error. A normalization factor was applied to all the measured values to obtain the absolute SLN activity after resection.

After SLN removal, the axillary area was thoroughly palpated by the surgeon to check for any remaining LN showing signs of extensive tumour involvement. Suspect LNs were removed and referred to as "non-SLNs", because they were neither hot nor stained.

Histological examination. The SLNs that were larger than 3 mm were bisected along their major axes and the two slice sections were macroscopically examined. Only one slice section per SLN was analyzed by frozen section to preserve the maximum integrity of the specimen and allow the best quality for definitive histological analysis. If the pathological analysis diagnosed a metastatic SLN, a complete ALND was immediately performed. All the tumour-free SLNs detected by frozen section were examined by immunohistochemistry (IHC) with cytokeratin AE1/AE3 (diluted at 1/200; Dako, Carpinteria, CA, USA) on consecutive sections, 4 μm thick, cut at 250 μm intervals, until exhaustion of the SLNs. According to the guidelines on the interpretation of TNM categories (33) issued by the European Working Group for Breast Screening Pathology (EWGBSP), macrometastases were defined as clusters of cancer cells >2 mm, micrometastases as clusters of cancer cells ≥0.2 mm and ≤2 mm and isolated tumour cells as clusters of cancer cells <0.2 mm. LN containing isolated tumour cells were considered as pN0. ALND was only performed in the case of macro- or micrometastases. The other LNs were analyzed using a standard protocol without IHC but with serial sections (3 slices 4 μm thick at 500 μm intervals).

Statistical analysis. The data are presented as median (range) or frequency (percentage). Statistical analysis was performed with Medcalc (version 10.0.2.0. MedCalc Software, Mariakerke, Belgium).

Results

Patients and breast surgery. The data are summarized in Table I. The mean age of the 100 patients was 58 years (standard deviation, SD 10.8) and their mean weight was 68 kg (SD 16). Ninety-five patients were treated for a unilateral breast cancer and 5 for a bilateral breast cancer needing a bilateral SLN procedure (representing 105 cases).

Initial breast conservative surgery and mastectomy were performed in 84 and 21 cases, respectively. In 3 cases, an immediate reconstruction with silicone-filled prosthesis placed under the pectoralis major muscle was performed. Thirty-six patients (36%) required a second operation either because of insufficient tumour margins or for an ALND. Revisional tumour site surgeries and secondary mastectomies were performed in 15 and 16 cases, respectively. Secondary ALNDs without additional breast surgery were performed in 8 cases (8%).

There were no allergic or anaphylactic reactions reported with the use of MBD. Among the adverse effects, temporary tattooing of the breast was noticed in 12 cases with complete resolution in each case. Discoloration of urine was observed, but never associated with bladder irritation. With a mean follow-up of 28 months (SD 3.7), all the patients were alive and free of recurrence and metastases, at the time of writing.

Breast tumour characteristics. There were 102 invasive breast carcinomas and 3 DCIS. The DCIS were all multifocal, whereas all the invasive tumours were unifocal with a mean size of 15.7 mm (SD 12.3) at their greatest dimension. Invasive ductal, lobular and mixed carcinomas were found in 83, 14 and 5 cases, respectively. Nineteen tumours showed lymphovascular invasion. Out of the 102 invasive breast carcinomas, oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) overexpression was found in 90, 69 and 9 cases, respectively (Table I).

Lymphoscintigraphy. In 14 cases (14/105), preoperative lymphoscintigraphy did not show any SLN. In 91 cases, lymphoscintigraphy was successful, showing at least one SLN (87%). In total, 198 SLNs were visualized preoperatively by lymphoscintigraphy.

SLN characteristics. i. Activity and staining: The identification of SLNs during surgery was successful for 104/105 cases (99%). A total of 291 LNs were removed consisting of 271 SLNs (2.6 SLNs per breast, range 1-11) and 20 non-SLNs. Among the 271 SLNs, 198 SLNs were visualized preoperatively by lymphoscintigraphy (efficiency: 73%), 256 SLNs were hot (94%), 177 were stained (65%), 162 were both hot and stained (60%), 94 were only hot (35%) and 15 were only stained (6%).

Table I. *Main pathological characteristics of the breast tumours and surgical procedures.*

	Number
Patients	100
Bilateral carcinomas	5
SLN procedures	105
DCIS	3
ICs	102
Tumour size for ICs in mm (SD)	15.7 (SD 12.3)
SBR grade of ICs	
Low grade	38 (37%)
Intermediate grade	42 (41%)
High grade	22 (22%)
Histological type of ICs	
Ductal	83 (81%)
Lobular	14 (14%)
Mixed	5 (5%)
Angio-invasions	19 (19%)
Predictive factors for ICs	
ER^+	90 (88%)
PR^+	69 (68%)
HER-2^+	9 (9%)
Surgery	
Initial breast conservative surgery	84 (80%)
Initial mastectomy	21 (20%)
Initial ALND	18 (17%)
Second surgery	36 (36%)
Tumour site surgery	15 (14%)
Secondary mastectomy	16 (15%)
Secondary ALND (total)	12 (11%)
Secondary ALND (without breast surgery)	8 (8%)

SLN: Sentinel lymph node; DCIS: ductal carcinoma *in situ*; ICs: invasive carcinoma; SD: standard deviation; SBR: Scarf-Bloom-Richardson; ER: oestrogen receptor; PR: progesterone receptor; HER-2: human epidermal growth factor receptor-2; ALND: axillary lymph node dissection.

ii. Pathological analysis: Among the 271 SLNs analyzed with frozen sections, 245 removed from 82 patients were found to be free of metastases while 26 removed from 18 patients were invaded by cancer cells (1 micrometastasis and 25 macrometastases). For these 18 patients, ALND was performed during the same surgical operation and contained metastatic LNs in 3 cases. Serial sectioning and IHC showed cancer cells in 14 false-negative SLNs removed from 14 patients (10 micrometastases and 4 macrometastases). Twelve ALNDs were performed during a second operation and contained metastatic LN(s) in 1 case. In 2 cases, ALND was proposed but the patients refused it due to lymphoedema risk. The data relating to the patients with metastatic SLNs and the pathological findings are summarized in Tables II and III. Among the 40 metastatic SLNs, 37 were hot and 32 were stained; 8 metastatic SLNs were not stained and 3 were not hot.

Table II. *Sentinel lymph node procedures.*

	Patients with tumour-free SLN on FS, SS and IHC	Patients with metastatic SLN(s) on FS	Patients with tumour-free SLN on FS but metastatic on SS and/or IHC	Total
Patients	68	18	14	100
Procedures	72	18	15	105
SLNs	180	49	42	271
Non-SLNs	15	5	0	20
Hot SLNs	171	45	40	256
Blue SLNs	116	32	29	177
Metastatic SLNs	0	26	14	40
ALNDs	0	18	12	30
Metastatic ALNDs	0	3	1	4

FS: Frozen section; SS: serial section; IHC: immunohistochemistry; SLN: sentinel lymph node; ALND: axillary lymph node dissection.

Non-SLN characteristics. Careful intraoperative digital examination allowed the detection of 2 additional metastatic LNs, among the 20 non-SLNs that were removed. Histological examination showed that these LNs were massively metastasized.

Follow-up. Neither local or regional recurrences, nor systemic metastases were diagnosed after a mean follow-up of 28 months.

Discussion

In this study, intraparenchymal subareolar injection of MBD was safe, no major adverse events being noticed and no axillary recurrences being diagnosed after a mean follow-up of 28 months. In particular, no allergic or anaphylactic reactions were observed during the SLN procedure, in accordance with most published series, except the work of Teknos *et al*. (30) reporting a pulmonary oedema during a SLN procedure using MBD. In the present clinical trial, no necrotic skin lesions were observed, in contrast to published data. Stradling *et al*. (28) reported 5 necrotic skin lesions (21%) after the injection of 3 to 5 mL of MBD (full-strength 1%, 10 mg/mL) in a series of 24 patients. MBD was injected into both the parenchyma and the skin. None of the patients required surgical debridement. A severe skin and fat necrosis was reported by Salhab *et al*. (29) with a peritumoural MBD injection, but the concentration of the MBD was not mentioned in the case report. In the recent prospective study of Saha *et al*. (34) comparing IBD and MBD, 7% of skin necrosis was observed after intraparenchymal and intradermal area injection of 3-5 mL of 1% MBD. Zakaria *et al*. (26) analyzed 381 patients receiving MBD and described 5 cases of skin necroses. One of these patients developed full-thickness skin necrosis that required excision. In the remaining 4 cases, the areas of skin necrosis healed without intervention. The absence of skin necrosis in the present study could probably be explained by the use of deep intraparenchymal injections with a lower volume of MBD (2 mL of MBD, 10 mg/mL).

Table III. *Pathological findings concerning metastatic SLNs.*

	Micrometastases	Macrometastases	Total
Number	11	29	40
Diagnosis with FS	1	25	26
Diagnosis with SS and/or IHC	10	4	14
Capsular invasion	0	9	9
Hot SLNs	10	27	37
Blue SLNs	8	24	32
Positive ALNDs	1	3	4

FS: Frozen section; SS: serial section; IHC: immunohistochemistry; ALND: axillary lymph node dissection.

In the present series, the only minor adverse effect was temporary tattooing of the breast which was noticed in 12 cases (11%), with complete resolution in each case. This frequency was higher than expected. In Zakaria *et al*.'s study (26), blue staining was only observed in 5 cases (1.25%) and resolved within 3 weeks in 4 cases, further follow-up not being available for the other patient. Zakaria *et al*. (26) analyzed the influence of different dilutions of MBD (first group: 43 patients with full strength (10 mg/mL) MBD and peritumoural injection, second group: 236 patients with intermediate dilution and subareolar injection, third group: 102 patients with a dilution of 1.25 mg/mL and subareolar injection) and noticed a reduction in the frequency of local inflammatory reactions in the third group using the lowest dose of MBD. The higher frequency of temporary blue staining in the present study could probably be explained by the use of full strength MBD.

Table IV. *Main MBD injection protocols published in the English literature.*

Authors (reference), year	Patient number	Injection site	Injection depth	Volume (mL)	Concentration (mg/mL)	Massage (minutes)	MBD alone success rate (%)	Combined technique success rate (%)
Blessing *et al*. (18), 2002	112	PT	-	3 to 5*	-	5	-	99.0
Simmons *et al*. (19), 2003	112	PT	IP	5	10	5	92.0	95.5
Eldrageely *et al*. (20), 2004	81	PT	IP	5	10	5	93.0	98.0
Nour (21), and Nakhlis 2004	54	SA	-	5	-	5	83.0	-
Golshan (22), *et al*. 2006	141	SA	-	5	10	5	96.5	NA
Varghese (25), *et al*. 2008	329	SA	SD	1	10	1 to 2	96.5	98.7
Zakaria (26), *et al*. 2008	43	PT	SD		10	-	74.0	
	236	SA	SD	Less than 3 to 4	Intermediate	-	88.0	99.7
	102	SA	SD		1.25	-	92.0	
Saha (34), 2008	72	SA	IP and SD	3 to 5	10	-	100	NA
Present study, 2009	100	SA	IP	2	10	No	65.0	99.0
	75	SA	IP	4	1.25	No	90.0	

SA: Subareolar area; PT: peritumoural; IP: intraparenchymal; SD: subdermal; NA: non-applicable; - : not reported. *The decision as to the volume of dye was based on the distance from the primary tumour to the axilla. When the tumour was located in the upper outer quadrant of a small-sized breast, a 3 ml injection was used. In all other cases, a greater volume (up to 5 ml) of dye was injected.

In the present study population, 3 patients underwent immediate breast reconstruction without adverse events. Singh-Ranger *et al*. (35) reported a case of capsular contraction necessitating revisional surgery, occurring in a patient who underwent immediate breast reconstruction during surgery for breast cancer, where a subdermal periareolar injection of 1.5 mL of 1% MBD was used to locate the SLN and was found to have heavily discoloured the prosthesis at subsequent revisional surgery performed nine months later. The authors concluded that capsular contraction may have been caused by a localised tissue reaction initiated by the dye. The position of the prosthesis, under the pectoralis major muscle, is probably safer, avoiding contact of the prosthesis with MBD, as reported in this study.

Olliver *et al*. (36) reported DNA damage caused by MBD in chromoendoscopy for Barrett's oesophagus. However, the evidence was only from 15 patients and larger series are needed to confirm these findings. Furthermore the DNA damage observed with MBD in chromoendoscopy was dependent on the presence of endoscopic white light. Further studies as regards potential biological effects such as DNA damage are required.

In the present study, the overall identification rate of SLNs was 99% , but among 271 SLNs, only 177 were stained (65%). Moreover, among 40 metastatic SLNs, 8 were not stained (20%). Some authors have hypothesized that the small molecular weight of MBD might lead to the identification of an increased number of axillary SLNs (18). This was not apparent from the present results. The SLN identification rate was lower with MBD than with dye-alone techniques (83% to 93%), in other reports (17, 18, 20, 21, 23, 26) and Table IV. These discrepancies could be explained by four main hypotheses. First, the use of intraparenchymal injection, which probably reduces the risk of superficial tissue necrosis, seems to minimize the technical success of lymphatic mapping (which appears to be best when tracers are injected superficially) (26, 37). Secondly, the dilution of MBD (1.25 mg/mL) instead of full-strength (10 mg/mL) dosage increases the success of MBD. In the study of Zakaria *et al*. (26), based on the analysis of 3 groups of patients receiving different dilutions of MBD, a blue node was identified in 74% of the first group (10 mg/mL), 88% of the second group (intermediate dilution), and 92% of the third group (1.25 mg/mL). A statistically significant difference in mapping success with MBD was observed between patients in the first group compared to the third group (p=0.004). Third, the volume used in the present series (2 mL) was perhaps insufficient, the majority of authors (17-19, 21, 22, 26), except Varghese *et al*. (23, 25), used 3 to 5 mL (Table IV). Finally, massage at the MBD injection site can increase the drainage of MBD (38). However, the safety of breast massage being controversial (39-41), it was avoided in this study to minimize the risk of passive transport of normal or malignant epithelial cells (42).

The present MBD injection protocol did however decrease the false-negative rate of the procedure. Indeed, MBD allowed the identification of 3 metastatic SLNs among 40 (7.5%), which were neither hot nor suspicious at palpation. Moreover, MBD use facilitated the visual detection of SLNs.

Preoperative lymphoscintigraphy also had low efficiency (73%) as only 198 SLNs among 271 SLN were visualized preoperatively. The difference between the results obtained with lymphoscintigraphy and those obtained with the gamma probe could mostly be explained by lymphatic drainage of the colloid during the night before surgery and the intrinsic performance of the two systems. Nevertheless, the precise

localization of the SLN given by lymphoscintigraphy also facilitated surgery. In a retrospective series, Degnim *et al*. (43) demonstrated that omission of the blue dye tracer would have increased the false-negative rate by approximately 2.5% in patients with positive lymphoscintigraphy. In our series, 91 cases underwent successful lymphoscintigraphy and in 3 of those, the metastatic SLN was blue, but not hot, hence the omission of MBD in the case of successful lymphoscintigraphy would have increased the false-negative rate by 3.3% .

All these data prompted us to continue using MBD in the SLN procedure whatever the result of the lymphoscintigraphy, but to modify the mapping technique in order to increase technical success and maintain low rates of complications. Currently, dilute MBD (injection of 4 mL with a concentration of 1.25 mg/mL) is used with a subareolar intraparenchymal injection without massage. In the first 75 cases, the identification rate reached 90% , without adverse events. Further study will check if these modifications increase mapping success in a series involving more patients.

Hirsch *et al*. (44) reported that the injection of MBD into a breast to localize breast tumours altered the results of the ER ligand-binding assay, due to a reduction in specific binding capacity. This effect was not seen with IBD (45). To our knowledge, there are no other studies published on these findings. In the present trial, no alteration of the ER or PR immunodetection in the tumoural tissue was observed, since ER or PR determination in the preoperative core biopsies and the surgical specimens did not differ. The discrepancies between Hirsch *et al*.'s data (44) and the present results may be explained by the histological technique (formol, alcohol and xylene) used in this study which dissolved the MBD; the injection of MBD at the four cardinal points in the subareolar area and not near the tumour or by the differing hormone receptor analyses (ligand binding assay *versus* IHC).

Conclusion

MBD is safe for SLN identification in early breast cancer and a mapping technique of subareolar intraparenchymal injections of dilute MBD without massage increases technical success and maintains low rates of complications. The superiority of the combined method including not only MBD and isotope mapping, but also careful intraoperative digital examination is highlighted.

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The authors declare that they have no competing interests.

Authors' Contributions

CM carried out the clinical trial, performed surgical treatments and drafted the manuscript. MG helped to perform surgical treatment. RS performed anesthesia. SC, CrM and JPB performed the pathological analysis and helped to draft the manuscript. NA and DG performed the lymphoscintigraphies and were medical investigators of the trial. JLG, SS, DB, VB, ZF and DH were scientific investigators of the trial. BG performed the statistical analysis. All authors read and approved the final manuscript.

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