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Ocena przydatności klinicznej fluorescencji błękitu metylenowego w biopsji węzła wartowniczego w raku piersi

Clinical evaluation of methylene blue dye in sentinel node biopsy in breast cancer

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SPIS TREŚCI:

WYKAZ SKRÓTÓW:

MB – Methylene Blue – Błękit Metylenowy

SNB – Sentinel lymph node biopsy – Biopsja węzła wartowniczego

NIR – Near infrared – Bliskie światło podczerwone

NIRF – Near infrared fluorescence – Flurorescencja bliskiego światła podczerwonego

IGS - Image guided surgery – Chiurgia sterowana obrazem

Tc- 99m - Technetium 99m - Technet 99m

SBR - signal to background ratio – Stosunek sygnału do tła

ICG - Indigocyanine Green- Zieleń indocjaninowa

ROI - Region of Interest- Obszary zainteresowania

Słowa kluczowe:

Błękit metylenowy, chirurgia sterowana obrazem, fluorescencja bliskiego światła podczerwonego, rak piersi, biopsja węzła wartowniczego, zieleń indocjaninowa, terapia neoadjuwantowa

Key Words:

Methylene Blue, Image guided surgery, Near infrared fluorescence, fluorescence, breast cancer, Sentinel lymph node, indocyanine green, neoadjuvant treatment,

ABSTRACT

In 1876 methylene blue was discovered by German chemist Heinrich Caro who was born in Poznan (1). Initially the drug was administered for cyanide poisoning, urinary tract infection and methemoglobinemia (2). The administration in surgery is proposed as a dye in various surgical situations. Main field for methylene blue is sentinel node biopsy procedure.

Other indications for this marker are visualization of gastrointestinal, vesico-vaginal, intestino-urinary or broncho-pleural fistulas, as well during chromopertubation of the fallopian tubes during fertility diagnostics.

In the nineteenth century Virchow postulated the theory of lymph nodes being filters of the lymphatic system (3). Since 1923 the term „Glands Sentinel“ was introduced by Brathwaite, a British surgeon who described an uptake of blue dye in the lymph nodes after injection into the gastric wall. Thirty seven years later Gould used the specific term of „Sentinel Node Biopsy“ in parotid cancer (4). In 1972 Weissbach and Boedefeld proposed a surgical staging of lymph node spread as a staging method to avoid long-term damage during surgery (5). Various observations were made during 70` and 80’s without a development of lymphatic mapping.

Finally in 1977 Morton was the first physician to used colloidal gold dye, to identify lymphatic drainage for skin melanomas. The concept of a sentinel node is based on Halsted’s theory of necessary loco regional cancer treatment, where lymphatic involvement is an indicator for systemic spread of the disease (6).

Giuliano stated, in his trials, sentinel node biopsy being a feasible method as an indicator for axillary involvement. First published in 1994 with blue dyes for sentinel node biopsy in breast cancer and consequently also with radioactive nanocolloids (7).

Veronesi lastly confirmed in the 90’s sentinel node biopsy as a standard for axillary staging in breast cancer (8).

Administration of MB is proven to be a safe procedure. Subcutaneous pre- or intraoperative administration is rarely a concern for the wellbeing of patients. Side reactions are negligible, merely a drop of oxygen saturation can be measured in pulse oximetry, allergic reactions, and injection based complications i.e. bruises, discoloring, abscess, rash or ulceration are rare (9).

MB as a fluorophore is not as stable in the human body in comparison to ICG (indocyanine green) or Technetium 99m. Patient metabolization of the lipophile agent is higher which results in a higher background autofluorescence. Furthermore excitation- (668nm) and emission peaks (688nm) are very close (10).

Performing surgery with prior visualization of anatomical structures which are being removed or preserved is categorized as IGS (image guided surgery). By using near infrared fluorophores (NIRF) a surgeon is able to recognize and prioritize a distinctive specimen of interest. Administration of such fluorophores might be systemic or local to achieve different goals. In a simplified explanation, a fluorophore is activated by an infrared light beam, and, due to its properties, emits light back in a certain spectrum, visible by an imagining system, set up to catch specific wavelengths, finally displayed for the surgeon in real-time. The most common fluorophore in IGS is ICG. Digital colour labeling can help to concentrate on the main point of interest to ease the therapeutic direction. Disadvantage is a low penetration value averaging about 1 cm.

In recent years, methylene blue has shown to be a dye presenting also fluorescent properties.

Five main domains of using MB as a fluorophore were described. Identification of ureters, parathyroid glands and pancreatic tumors, intraoperatively, tumor margin visualization during breast cancer resection and first description of sentinel node biopsy in breast cancer.

Breast cancer is the most common malignant disease in the world. The number of new cases has increased in the last few years. The gold standard for detection of axillary involvement is sentinel node biopsy. The pattern of metastatic spread is via lymphatic vessels into axillary lymph nodes. Detection of prior marked sentinel nodes has replaced primary axillary dissection completely, in clinically negative axillary nodes (11).

This led to reduction of peri-and postoperative complication due to smaller likelihood for collateral damage. All the effort is done to visualize lymphatic anatomy and detect possible metastatic spread of the disease. Locoregional control is another reason for this procedure.

Aims:

Publication 1

The aim of this study is to show methylene blue fluorescent properties in sentinel lymph node detection for breast cancer patients. Moreover how different MB dilutions can enhance its fluorescence properties.

Publication 2

The aim of this article is to present a review on different selected possibilities for sentinel node biopsy mapping in breast cancer and the latest considerations where to avoid surgical axillary involvement, or consider a systemic therapeutic approach.

Material and Methods:

Publication 1

The prospective clinical study included 49 patients with a diagnosis of invasive breast cancer in the surgical department at the University Clinical Center in Gdansk, Poland. The institutional board of the ethics commits approved the study (No. NKBBN/02/2018-2020). Informed consent forms were signed by patients. Decision for patient qualification for SNB was after a multidisciplinary board review. In some cases, prior neoadjuvant treatment was given.

Sentinel node procedure was applied via the in-house standard as followed:

- 1-3 hours prior surgery a dose of 100 MBq 99m technetium nano colloid was injected periareolar in one quadrant.

- after intubation of the patient, the attending surgeon administered 1mL of MB at a standard concentration of 10mg/mL periareolar.

- massage of the injection area and breast was performed to ensure proper lymphatic drainage.

- preparation of the field of operation via scrubbing with alcoholic antiseptic and application of surgical draping.

MB (METIBLO) was purchased in 10mL vials from Laboratories STEROP NV, Scheutlaan, Brussels, Belgium, nanocolloide was obtained from the department of nuclear medicine University Clinical Center in Gdansk, Poland.

NIR fluorescence imaging hardware was a Quest Spectrum (Quest Medical Imaging, Middenmeer, The Netherlands). The optics were placed on an adjustable tripod in a range of 20-30cm from the specimen, and the visual reference monitor with color coding image was placed in clear view for the surgeon for simultaneous visualization with NIRF, allowing real time guidance.

Hands free imaging enabled even a real time visualization of the lymphatic vessels and nodes.

The quality of the image was dependent on the signal-to-background ratio (SBR) of the fluorescent light emission. To determine the SBR the Quest Research Tool was used. By using this particular software, a region of interest (ROI) from fluorescence signal to the SLN in contrast to the image from adjacent tissue, from the background. Calculating the SBR is made by dividing average pixel intensity values in the SLN ROI by averaging background pixel values. SBR of ≥ 1.1 specifies as positive NIR fluorescence

Both types of fluorescent probes, not visible for the naked eye, Cy5.5 and ICG were possible, where the Cy5.5 mode was used for MB visualization. Illumination was performed at 680 nm and visualization was approximated within 710 nm range.

After the sentinel finding, control was performed by a handheld gamma camera system, Gamma Finder II (W.O.M. World of Medicine GmbH; Berlin, Germany) as standardized in the clinical protocol. All three images - naked eye, color visualization, NIR fluorescence imagining and gamma probe were available intraoperatively.

Statistics were shown as a median with minimum and maximum values or as frequency with percentages. To test the differences between observed frequencies and frequencies that were expected under the null hypothesis, Chi-squared tests were used. Statistically significant p.value < 0.05 was considered. All statistical analyses were calculated using the SPSS version 26.0 software package for Mac (IBM Corp., Chicago, IL USA).

Publication 2

A literature review of PubMed and Medline was performed according to historical, current and future usage of dyes and markers within sentinel node biopsy in breast cancer patients. Furthermore, new approaches are being discussed for tailored treatment based on sentinel node biopsy to avoid too extensive surgical axillary involvement.

Results

Publication 1

Forty nine patients got involved in the clinical study for IGS with the double dye method, using 99m technetium and MB. The clue to this procedure was to use MB not only as visible dye, but also as fluorophore for guiding surgery.

In 23 patients (46.9%), location of SNB, or the end of the lymphatic path, was visible transcutaneously by fluorescence. In median, SBR for transcutaneous SLN location was 1.69 (range 1.66–4.35). Fluorescence in SNB was visible in 25 patients (51%). Blue-dye-colored nodes were visible to the naked eye in 40 patients (81.6%). The median SBR for SNB visualization by fluorescence was 2.54 (range 1.34–6.86). Lymphatic channels were visible under fluorescence in 14 patients (28.6%) before visualization by the naked eye, with an average SBR of 2.01 (range 1.14–5.6). In three patients (6.1%), the SLN was detected under fluorescence, but not to the naked eye. In 15 patients (30.6%), the node was visible to the naked eye, but not with fluorescence visualization.
We also prepared analyses to test the differences between observed frequencies and frequencies that were expected under the null hypothesis between transcutaneous SLN visualization, lymphatic channel fluorescence visualization, and different clinical as well as pathological factors. For transcutaneous SNL fluorescence visualization, statistically significant factors were smoking (p = 0.001) and neoadjuvant chemotherapy (p = 0.026). SLNs were visualized quicker under fluorescence during SLN preparation. Factors associated with finding included diabetes (p = 0.001), neoadjuvant chemotherapy (p = 0.003), and multifocality (p = 0.004). The only factor associated with visualization of the SLN by naked eye was neoadjuvant chemotherapy (p = 0.013).

Differentializing of various dilutions of MB was presented compared to fluorescence intensity with an optimal range of 40 µM (0.0128 mg/mL) but dilutions between 20 µM (0.0064 mg/mL) to 100 µM (0.032 mg/mL) were also feasible.

Publication 2

Feasibility of sentinel nodes biopsy in breast cancer treatment is proven in one of the highest pieces of evidence among surgical procedures. Various combinations were performed, prior and post neoadjuvant therapeutic setting, involved by primary cancer in the lymphatic nodes and free in clinical evaluation. Sentinel node biopsy is safe at any age, but consequences of the axillary staging have to be put into perspective of further treatment. Since AMAROS and ACOSOG Z0011 trials lymphadenectomy has not been proven superior in comparison to radiation therapy, even in partial metastatic axillary involvement.

In the ACOSOG Z0011 trial a comparison was made between patients with one or two metastatic axillary lymph nodes after SNB who were treated either by axillary dissection, vs. radiation therapy without surgical involvement of the axilla. That trial took only pT1 and pT2 tumors in breast conserving therapy into account. In the follow up no difference in local recurrence was diagnosed, as well as disease free survival and overall survival.

AMAROS and OTOASOR trials chose a group of patients with only 1-2 involved lymph nodes with tumors larger than 3cm, lymphovascular tumor invasion, and sentinel nodes with microscopic extra capsular growth. In this population of patients an optimal adjuvant treatment plan is required for optimal overall- and disease free survival.

Sentinel node biopsy is performed by standard in a double dye method. Usually, 99m technetium and MB are considered the standard dyes. Various publications propose new dye techniques for marking sentinel nodes, i.e. ICG, or MB, fluorescein, as fluorophores, superparamagnetic iron oxide (SPIO) and contrast enhanced ultrasound (CEUS) with microbubbles have to be taken into account. Fluorophores are easy to obtain and do not require a nuclear medicine department in a hospital. ICG seems to have good properties with a high detection rate, similar to dual technique with radioactive nanocolloids and blue dye. Disadvantages are: iodine allergy risks, relatively low fluorescence brightness, discoloration of a skin. Problematic seems to be the detection of deeper located sentinel nodes, finally due to fast dispersion of the dye, while detection

times are shortened. Multispectral imaging, by using more than one fluorophore can allow further differentiation of specific structures.

Pathological findings in sentinel nodes can be classified into 3 further subgroups, except for being free of involvement. There are pN0(i+) (isolated tumor cells) or pN0(mi) (micrometastasis) and pN1 (macrometastases) grouped as involved sentinels. Various trials proved where omission of further axillary dissection does not worsen the overall survival of patients. Although radiation therapy should be proposed to lower the recurrence risk.

Neoadjuvant treatment in SNB localization was primarily handled in the SENTINA Trial. Two subgroups have been analyzed, with or without positive axillary lymph nodes prior neoadjuvant treatment. The detection rate was lower and the false negative rate was higher after neoadjuvant treatment, as if the SNB was done prior to chemotherapy.

The ACOSOG Z1071 trial discussed SNB after neoadjuvant treatment with a clinically metastatic axillary involvement. The false negative rate was up to 10% in this trial, confirming the data from SENTINA. Dual tracer method led to reduction of the false negative rates, as well as the number of sentinel nodes removed (24,3% for one node 18,5% for two nodes, and less than 10% for three or more lymph nodes). Clip marking of metastatic nodes proved to be a safe practice, when found correctly.

Finally, subgroup analysis states that omission of axillary staging after reaching a certain age, and/or with particularly favorable tumor biology is considered safe. Recommendations from the Society of Surgical Oncology, advise not to routinely use SNB in women older than 70 years with hormone positive receptor breast cancer. The SOUND trial, INSEMA Trial and BOOG are under investigation with the primary endpoint of these studies being disease free survival with secondary ones like morbidity and quality of life.

Conclusions

Publication 1

In our study an old dye was used in a new fashion with fluorescence visualization for SNB in breast cancer. Since the fluorophore properties were not sensitive enough for fluorescence visualization, application of MB in lower concentrations may raise its sensitivity. In vitro samples were tested in various solutions, indicating lower

concentrations in the SNB. To find the optimal concentration of fluorophore needed to be injected, further in vivo investigations are needed.

Publication 2

In recent years evolution and even revolution of SNB in breast cancer has become a fact. Tailored axillary approach will help in better disease staging and patient quality of life. Not only clinical but also biological factors of breast cancer have to be put into the consideration for decision making, since sometimes axillary surgery is merely an information that does not always change the concept of the treatment.

STRESZCZENIE

Abstrakt

W 1876 r. błękit metylenowy został odkryty przez niemieckiego chemika Heinricha Caro urodzonego w Poznaniu (1). Początkowo lek podawano w przypadku zatrucia cyjankami, infekcji dróg moczowych i methemoglobinemii (2). Podawanie błękitu metylenowego w chirurgii jako barwnika, jest możliwe, w różnych sytuacjach klinicznych. Głównym wskazaniem do podania błękitu metylenowego jest biopsja węzła wartowniczego.

Inne wskazania do używania tego markera to uwidocznienie przetok żołądkowo-jelitowych, pęcherzowo-pochwowych, jelitowo-moczowych, oskrzelowo-opłucnowych, a także podczas chromotubacji jajowodów w trakcie diagnostyki płodności.

W XIX wieku Virchow postulował teorię węzłów chłonnych będących filtrami układu limfatycznego (3). Od 1923 roku Brathwaite, brytyjski chirurg, wprowadził termin „Glands Sentinel", który opisał wchłanianie niebieskiego barwnika w węzłach chłonnych po wstrzyknięciu błękitu do ściany żołądka. Trzydzieści siedem lat później Gould użył specyficznego terminu „Biopsja węzła wartowniczego" w raku przyusznicy. W 1972 r (4). Weissbach i Boedefeld zaproponowali chirurgiczną ocenę badania węzłów chłonnych jako metodę określania stopnia zaawansowania choroby, aby uniknąć długotrwałego uszkodzenia po operacji (5). W latach 70. i 80. poczyniono różne obserwacje bez rozwoju mapowania limfatycznego.

Wreszcie w 1977 roku Morton był pierwszym lekarzem, który zastosował złoty barwnik koloidalny do identyfikacji drenażu limfatycznego czerniaka. Koncepcja węzła wartowniczego opiera się na teorii Halsteda o konieczności leczenia raka lokoregionalnego, gdzie zajęcie układu limfatycznego jest wskaźnikiem ogólnoustrojowego rozprzestrzeniania się choroby (6).

Giuliano stwierdził, w swoich badaniach, że biopsja węzła wartowniczego, jest metodą możliwą do wykonania, jako wskaźnik zajęcia węzłów chłonnych pachowych. Po raz pierwszy opublikowano doniesienie w 1994 roku z niebieskimi barwnikami do biopsji węzła wartowniczego w raku piersi, a w konsekwencji także z radioaktywnymi nannokoloidami (7).

Veronesi w końcu potwierdził skuteczność biopsji węzła wartowniczego w latach 90, jako standard oceny stopnia zaawansowania w raku piersi (8).

Udowodniono, że podanie MB jest bezpieczną procedurą. Podskórne podawanie przed lub śródoperacyjnie, rzadko stanowi problem dla zdrowia pacjentów. Reakcje uboczne są znikome, w pulsoksymetrii można zmierzyć jedynie spadek saturacji tlenu, reakcje alergiczne są możliwe, a powikłania po wstrzyknięciu tj. siniaki, przebarwienia, ropień, wysypka oraz owrzodzenie występują rzadko(9).

MB jako fluorofor nie jest tak stabilny w organizmie człowieka jak ICG (indocyanine green) czy Technet 99m. Metabolizacja środka lipofilowego przez pacjenta jest wyższa, co skutkuje wyższą autofluorescencją tła. Ponadto piki wzbudzenia (668 nm) i emisji (688 nm) są bardzo zbliżone(10).

Wykonywanie operacji z uprzednią wizualizacją struktur anatomicznych, które są usuwane lub zachowywane, jest klasyfikowane jako IGS (chirurgia pod kontrolą obrazu). Dzięki zastosowaniu fluoroforów w bliskiej podczerwieni (NIRF) chirurg jest w stanie rozpoznać i nadać priorytet wyróżniającej się strukturze tkankowej. Podawanie takich fluoroforów może być ogólnoustrojowe lub lokalne w celu osiągnięcia różnych celów. W uproszczeniu, fluorofor jest aktywowany przez wiązkę światła podczerwonego, a dzięki swoim właściwościom emituje światło z powrotem w określonym widmie, widocznym przez system wizyjny, nastawiony na wychwytywanie określonej długości fali, ostatecznie obraz we fluorescencji jest wyświetlany chirurgowi w czasie rzeczywistym. Najczęstszym fluoroforem w IGS jest ICG. Cyfrowe oznakowanie kolorami obrazu może pomóc skoncentrować się na głównym punkcie zainteresowania, aby ułatwić kierunek operacyjny. Niekorzystna w tym typie wizualizacji jest niska wartość penetracji, średnio około 1 cm.

W ostatnich latach błękit metylenowy okazał się barwnikiem z właściwościami fluorescencyjnymi.

Opisano pięć głównych domen wykorzystania MB jako fluoroforu. Identyfikacja moczowodów, przytarczyc i guzów trzustki, śródoperacyjna wizualizacja marginesu guza podczas resekcji raka piersi oraz pierwszy opis biopsji węzła wartowniczego w raku piersi.

Rak piersi jest najczęstszą chorobą nowotworową na świecie. Liczba nowych przypadków wzrasta w ostatnich latach. Złotym standardem wykrywania zajęcia węzłów chłonnych pachowych jest biopsja węzła wartowniczego. Wzorzec rozprzestrzeniania się przerzutów

przebiega przez naczynia limfatyczne do pachowych węzłów chłonnych. Wykrycie wcześniej zaznaczonych węzłów wartowniczych całkowicie zastąpiło pierwotną limfadenektomię pachy, w klinicznie ujemnych węzłach pachowych (11).

Takie postępowanie prowadzi do zmniejszenia powikłań około- i pooperacyjnych ze względu na mniejsze prawdopodobieństwo uszkodzenia struktur limfatycznych. Dokłada się wszelkich starań, aby zwizualizować anatomię układu limfatycznego i wykryć możliwe przerzuty choroby. Innym powodem zastosowania tej procedury jest kontrola lokoregionalna.

Cele:

Publikacja 1

Celem tego badania jest wykazanie właściwości fluorescencyjnych błękitu metylenowego w wykrywaniu węzłów wartowniczych u chorych na raka piersi. Dodatkowo celem jest określenie, jak różne rozcieńczenia MB mogą poprawić jego właściwości fluorescencyjne.

Publikacja 2

Celem niniejszego artykułu jest przedstawienie przeglądu różnych wybranych możliwości mapowania biopsji węzła wartowniczego w raku piersi oraz najnowszych rozwiązań, w których można uniknąć biopsji chirurgicznej lub rozważyć systemowe podejście terapeutyczne.

Materiał i metody:

Publikacja 1

Prospektywne badanie kliniczne objęło 49 pacjentek z rozpoznaniem inwazyjnego raka piersi na oddziale chirurgicznym Uniwersyteckiego Centrum Klinicznego w Gdańsku. Badanie zostało zatwierdzone przez instytucjonalną radę etyki (nr NKBBN/02/2018-2020). Formularze świadomej zgody zostały podpisane przez pacjentów. Decyzja o kwalifikacji pacjenta do SNB została podjęta po decyzji konsylium wielodyscyplinarnego. W niektórych przypadkach zastosowano wcześniej leczenie neoadjuwantowe.

Procedura węzła wartowniczego została zastosowana zgodnie z wewnętrznym standardem w następujący sposób:

- 1-3 godzin przed zabiegiem okołootoczkowo w jednej ćwiartce wstrzyknięto dawkę 100 MBq 99m nannokoloidu technetu.

- po zaintubowaniu chorego prowadzący chirurg podał 1ml MB w standardowym stężeniu około 10 mg/ml.

- W celu zapewnienia prawidłowego drenażu limfatycznego wykonano masaż okolicy iniekcji oraz piersi.

- Przygotowanie pola operacyjnego poprzez dezynfekcję alkoholowym środkiem antyseptycznym i zastosowanie obłożenia chirurgicznego.

MB (METIBLO) zakupiono w fiolkach 10mL z Laboratories STEROP NV, Scheutlaan, Bruksela, Belgia, nannokoloid uzyskano z Zakładu Medycyny Nuklearnej Uniwersyteckiego Centrum Klinicznego w Gdańsku.

Sprzętem do obrazowania fluorescencyjnego NIR był Quest Spectrum (Quest Medical Imaging, Middenmeer, The Netherlands). Optykę umieszczono na regulowanym statywie w odległości 20-30 cm od badanej próbki, a monitor z obrazem kodowanym kolorami został umieszczony w widocznym miejscu dla chirurga w celu jednoczesnej wizualizacji z NIRF, umożliwiając prowadzenie operacji w czasie rzeczywistym.

Obrazowanie fluorescencyjne umożliwiło nawet wizualizację naczyń i węzłów chłonnych w czasie rzeczywistym.

Jakość obrazu zależała od stosunku sygnału do tła (SBR) emisji światła fluorescencyjnego. Do określenia SBR wykorzystano narzędzie Quest Research Tool. Korzystając z tego konkretnego oprogramowania, obszar zainteresowania (ROI) od sygnału fluorescencyjnego z SLN w porównaniu do obrazu z sąsiedniej tkanki- z tła. Obliczanie SBR odbywa się poprzez podzielenie średnich wartości intensywności pikseli w obszarze SLN ROI przez uśrednienie wartości pikseli tła. SBR ≥ 1,1 oznacza dodatnią fluorescencję NIR

Możliwe były do użycia obydwa typy sond fluorescencyjnych w kamerze- Cy5.5 i ICG, gdzie do wizualizacji MB wykorzystano tryb Cy5.5. Iluminację pola operacyjnego przeprowadzono przy wiązce 680 nm, a wizualizację fluorescencji w zakresie 710 nm.

Po odnalezieniu węzła wartowniczego, kontrolę prowadzono za pomocą podręcznego systemu kamery gamma, Gamma Finder II (W.O.M. World of Medicine GmbH; Berlin, Niemcy) zgodnie ze standardami protokołu klinicznego. Wszystkie trzy obrazy - wizualizacja barwna gołym okiem, obrazowanie fluorescencyjne NIR i sonda gamma były dostępne śródoperacyjnie.

Wyniki badania przedstawiono w analizie statystycznej, takiej jak mediana z wartościami minimalnymi i maksymalnymi oraz częstość wyrażona w procentach. Testy chi-kwadrat zostały użyte do sprawdzenia różnic między częstościami obserwowanymi a częstościami oczekiwanymi w ramach hipotezy zerowej. Za istotną statystycznie uznano wartość $p < 0,05$. Wszystkie analizy statystyczne przeprowadzono przy użyciu pakietu oprogramowania SPSS wersja 26.0 dla komputerów Mac (IBM Corp, Chicago, IL, USA).

Publikacja 2

Dokonano przeglądu literatury PubMed i Medline pod kątem historycznego, obecnego i przyszłego wykorzystania barwników i markerów w biopsji węzła wartowniczego u chorych na raka piersi. Co więcej, dyskutowane zostały nowe podejścia do indywidualnego leczenia opartego na biopsji węzła wartowniczego, aby uniknąć nadmiernej ingerencji chirurgicznej.

Wyniki

Publikacja 1

Czterdziestu dziewięciu pacjentów wzięło udział w badaniu klinicznym IGS metodą podwójnego barwnika z użyciem 99m technetu i MB. Kluczem do tej procedury było użycie MB nie tylko jako widocznego dla oka barwnika, ale także jako fluoroforu do śródoperacyjnej wizualizacji wybarwionych struktur.

U 23 pacjentów (46,9%) lokalizacja SNB lub końca drogi limfatycznej była widoczna przezskórnie za pomocą fluorescencji. Mediana SBR dla przezskórnej lokalizacji SLN wyniosła 1,69 (zakres 1,66–4,35). Fluorescencja w SNB była widoczna u 25 pacjentów (51%). Niebiesko wybarwione węzły były widoczne gołym okiem u 40 pacjentów (81,6%). Mediana SBR dla wizualizacji SNB metodą fluorescencji wyniosła 2,54 (zakres 1,34–6,86). Kanały limfatyczne były widoczne we fluorescencji u 14 pacjentów (28,6%) przed wizualizacją gołym okiem, ze średnim SBR 2,01 (zakres 1,14–5,6). U trzech pacjentów

(6,1%) SLN wykryto we fluorescencji, ale nie gołym okiem. U 15 pacjentów (30,6%) węzeł był widoczny gołym okiem, ale nie przy wizualizacji fluorescencji.

Przygotowaliśmy również analizy w celu sprawdzenia różnic między obserwowanymi częstościami a częstościami oczekiwanymi w hipotezie zerowej pomiędzy przezskórną wizualizacją SLN, wizualizacją fluorescencji kanału limfatycznego a różnymi czynnikami klinicznymi i patologicznymi. W przypadku przezskórnej wizualizacji fluorescencji SLN statystycznie istotnymi czynnikami były palenie tytoniu (p = 0,001) oraz chemioterapia neoadjuwantowa (p = 0,026). SLN były wizualizowane szybciej przy pomocy fluorescencji podczas preparowania SLN. Czynniki związane z ich wykryciem obejmowały cukrzycę (p = 0,001), chemioterapię neoadjuwantową (p = 0,003) oraz wieloogniskowość (p = 0,004). Jedynym czynnikiem związanym z wizualizacją SLN gołym okiem była chemioterapia neoadjuwantowa (p = 0,013).

Przedstawiono porównanie różnych rozcieńczeń MB w powiązaniu z intensywnością fluorescencji z optymalnym zakresem 40 μM (0,0128 mg/ml), ale rozcieńczenia od 20 μM (0,0064 mg/ml) do 100 μM (0,032 mg/ml) również prezentowały wysoką intensywność fluorescencji.

Publikacja 2

Wykonalność biopsji węzła wartowniczego w leczeniu raka piersi jest potwierdzona dużą liczbą dowodów naukowych. Przeprowadzono różne kombinacje, przed i po neoadjuwantowym leczeniu terapeutycznym, związane z pierwotnymi przerzutami w węzłach chłonnych jaki i wolnymi od przerzutów w ocenie klinicznej. Biopsja węzła wartowniczego jest bezpieczna w każdym wieku, ale konsekwencje zaawansowania pachowego muszą być rozpatrywane w perspektywie dalszego leczenia. Od czasu badań AMAROS i ACOSOG Z0011 nie udowodniono wyższości limfadenektomii w porównaniu z radioterapią, nawet w przypadku częściowych przerzutów do węzłów pachowych.

W badaniu ACOSOG Z0011 porównano pacjentów z jednym lub dwoma przerzutami do węzłów chłonnych pachowych po SNB, którzy byli leczeni albo wykonując limfadenektomię pachową, albo radioterapię bez leczenia chirurgicznego. W badaniu tym uwzględniono jedynie guzy pT1 i pT2 w terapii oszczędzającej pierś. W obserwacji nie stwierdzono różnic pomiędzy grupami w nawrotach miejscowych, przeżyciach wolnych od choroby i przeżyciach całkowitych.

W badaniach AMAROS i OTOASOR wybrano grupę pacjentów z tylko 1-2 zajętymi węzłami chłonnymi z guzami większymi niż 3 cm, inwazją nowotworu naczyń limfatycznych i mikroskopowym pozatorebkowym naciekiem węzłów wartowniczych. W tej populacji pacjentów dla optymalnego przeżycia całkowitego i wolnego od choroby wymagany jest optymalny plan leczenia uzupełniającego.

Biopsję węzła wartowniczego wykonuje się standardowo metodą podwójnego barwnika. Zwykle za standardowe uważa się technet 99m i MB. Różne publikacje proponują nowe techniki do znakowania węzłów wartowniczych – barwnikowe z wykorzystaniem fluorescencji tj. ICG lub MB, fluoresceinę; superparamagnetyczny tlenek żelaza (SPIO) i ultradźwięki wzmocnione kontrastem z mikropęcherzykami (CEUS). Fluorofory są łatwo dostępne i nie wymagają oddziału medycyny nuklearnej w szpitalu. Wydaje się, że ICG ma dobre właściwości przy wysokim współczynniku wykrywalności, podobnie jak technika podwójna z nannokoloidem i niebieskim barwnikiem. Wady ICG to: ryzyko alergii na jod, stosunkowo niska jasność fluorescencji, przebarwienia skóry. Problematyczne wydaje się wykrycie głębiej położonych węzłów wartowniczych, ostatecznie ze względu na szybkie rozproszenie barwnika czasy detekcji ulegają skróceniu. Obrazowanie wielospektralne, przy użyciu więcej niż jednego fluoroforu, może pozwolić na dalsze różnicowanie określonych struktur.

Zmiany patologiczne w węzłach wartowniczych można podzielić na 3 dalsze podgrupy. Istnieją pN0(i+) (izolowane komórki nowotworowe) lub pN0(mi) (mikroprzerzuty) i pN1 (makroprzerzuty) pogrupowane jako przerzutowe węzły wartownicze. W różnych badaniach udowodniono, że pominięcie dalszego wycięcia węzłów pachowych nie pogarsza całkowitego przeżycia pacjentów. Jednakże w takiej sytuacji należy zaproponować radioterapię, aby zmniejszyć ryzyko nawrotu.

Leczenie neoadjuwantowe w lokalizacji SNB było głównie przedmiotem badania SENTINA. Przeanalizowano dwie podgrupy, z lub bez dodatnich węzłów chłonnych pachowych przed leczeniem neoadjuwantowym. Wskaźnik wykrywalności był niższy, a odsetek wyników fałszywie ujemnych wyższy po leczeniu neoadjuwantowym, w porównaniu, gdyby SNB wykonano przed chemioterapią.

W badaniu ACOSOG Z1071 omówiono SNB po leczeniu neoadjuwantowym z klinicznym przerzutem do węzłów pachowych. Odsetek wyników fałszywie ujemnych wyniósł w tym badaniu do 10%, co potwierdza dane z badania SENTINA. Metoda podwójnego znacznika doprowadziła do zmniejszenia odsetka wyników fałszywie ujemnych, a także liczby

usuniętych węzłów wartowniczych (24,3% dla jednego węzła 18,5% dla dwóch węzłów i mniej niż 10% dla trzech lub więcej węzłów chłonnych). Oznaczanie klipsem węzłów przerzutowych okazało się bezpieczną praktyką, jeśli zostały one prawidłowo znalezione.

Wreszcie analiza podgrupy pacjentek stwierdza, że pominięcie chirurgicznego stopniowania zaawansowania pachowego po osiągnięciu pewnego wieku i/lub przy szczególnie korzystnej biologii guza jest uważane za bezpieczne. Zalecenia Towarzystwa Chirurgii Onkologicznej zalecają, aby rutynowo nie stosować SNB u kobiet w wieku powyżej 70 lat z rakiem piersi z dodatnim receptorem hormonalnym. Badania SOUND, INSEMA Trial i BOOG są obecnie badane, a pierwszorzędowym celem tych badań jest przeżycie bez choroby, z drugorzędowymi celami, takimi jak zachorowalność i jakość życia.

Wnioski

Publikacja 1

W naszym badaniu stary barwnik został użyty w nowy sposób z wizualizacją fluorescencji dla SNB w raku piersi. Ponieważ właściwości fluoroforu nie były wystarczająco czułe do wizualizacji fluorescencji w standardowym stężeniu, zastosowanie MB w niższych stężeniach może zwiększyć jego czułość. Próbki in vitro były testowane w różnych rozcieńczeniach, co wskazuje na niższe stężenia prawdopodobnie użyteczne w SNB. Aby znaleźć optymalne stężenie fluoroforu potrzebnego do wstrzyknięcia, potrzebne są dalsze badania in vivo.

Publikacja 2

W ostatnich latach ewolucja, a nawet rewolucja SNB w raku piersi staje się faktem.

Szyte na miarę podejście do układu węzłów chłonnych pachowych pomoże w lepszej ocenie zaawansowania choroby i jakości życia pacjenta.

Przy podejmowaniu decyzji należy brać pod uwagę nie tylko kliniczne, ale i biologiczne czynniki raka piersi, ponieważ czasami operacja pachowa jest jedynie informacją, która nie zawsze zmienia koncepcję leczenia.

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Publikacja 1

Methylene Blue Near-Infrared Fluorescence Imaging in Breast Cancer Sentinel Node Biopsy
Budner O, Cwalinski T, Skokowski J, Marano L, Resca L, Cwalina N, Kalinowski L, Hoveling R, Roviello F, Polom K..
Cancers (Basel). 2022 Apr 3;14(7):1817. doi: 10.3390/cancers14071817

Publikacja 2

Trends and future perspective in sentinel node biopsy in breast cancer patients.

Budner O, Polom K.

Farmacja Współczesna 2022;15:151-156

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rok	**Tytuł czasopisma**	**Char. merytor.**	**prac**	**IF**	**MEiN**
2022	Cancers	ORG	1	6,575	140
2022	Farmacja Współczesna	PPP	1	0	20
		razem	2	6,575	160

ORG – praca oryginalna

PPP – praca poglądowa/przeglądowa

cancers

MDPI

Article

Methylene Blue Near-Infrared Fluorescence Imaging in Breast Cancer Sentinel Node Biopsy

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Simple Summary: Currently the gold standard for sentinel node biopsy in breast cancer patients is radioactive nanocolloid and a blue dye. In the age of fluorescence guided surgery new fluorophores are used and methylene blue presents some fluorescent properties. This study is the first in a clinical series presenting the possible use of methylene blue as a fluorescent dye for the identification of sentinel nodes in breast cancer sentinel node biopsy. We presented a feasibility of this new method and also in additional experiments because of the quenching effect limitation, found possible dilution of methylene blue presenting improved fluorescence.

Abstract: Introduction: Fluorescence-based navigation for breast cancer sentinel node biopsy is a novel method that uses indocyanine green as a fluorophore. However, methylene blue (MB) also has some fluorescent properties. This study is the first in a clinical series presenting the possible use of MB as a fluorescent dye for the identification of sentinel nodes in breast sentinel node biopsy. Material and methods: Forty-nine patients with breast cancer who underwent sentinel node biopsy procedures were enrolled in the study. All patients underwent standard simultaneous injection of nanocolloid and MB. We visualized and assessed the sentinel nodes and the lymphatic channels transcutaneously, with and without fluorescence, and calculated the signal-to-background ratio (SBR). We also analyzed the corresponding fluorescence intensity of various dilutions of MB. Results: In twenty-three patients (46.9%), the location of the sentinel node, or the end of the lymphatic path, was visible transcutaneously. The median SBR for transcutaneous sentinel node location was 1.69 (range 1.66–4.35). Lymphatic channels were visible under fluorescence in 14 patients (28.6%) prior to visualization by the naked eye, with an average SBR of 2.01 (range 1.14–5.6). The sentinel node was visible under fluorescence in 25 patients (51%). The median SBR for sentinel node visualization with MB fluorescence was 2.54 (range 1.34–6.86). Sentinel nodes were visualized faster under fluorescence during sentinel node preparation. Factors associated with the rate of visualization included diabetes ($p = 0.001$), neoadjuvant chemotherapy ($p = 0.003$), and multifocality ($p = 0.004$). The best fluorescence was obtained using 40 μM (0.0128 mg/mL) MB, but we also observed a clinically relevant dilution range between 20 μM (0.0064 mg/mL) and 100 μM (0.032 mg/mL). Conclusions: For the first time,

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we propose the clinical usage of MB as a fluorophore for fluorescence-guided sentinel node biopsy in breast cancer patients. The quenching effect of the dye may be the reason for its poor detection rate. Our analysis of different concentrations of MB suggests a need for a detailed clinical analysis to highlight the practical usefulness of the dye.

Keywords: breast cancer; methylene blue; sentinel node biopsy; fluorescence

1. Introduction

Sentinel node biopsy (SNB) is currently the gold-standard procedure for evaluating the stage of disease in early breast cancer [1,2]. The concept of SNB has a long history, but the first description of the procedure was published by Morton and Cochrane in 1992 in melanoma patients [3]. The first application of blue dye in SNB was proposed by Giuliano et al. for breast cancer in 1994 [4]. The authors identified blue-dye-stained sentinel lymph nodes in 114 of 174 (65.5%) patients. Today's standard for SNB is a dual technique that includes blue dye and/or radioactive technetium-99m (^{99m}Tc)-labelled [5] nanocolloid. Blue dye identifies not only stained nodes but also lymphatic vessels between the injection site and axillary lymph nodes. Three different types of blue dye can be used in SNB: methylene blue (MB), isosulfan blue, and patent blue V. Recently, other dyes have been proposed for use in SNB, with indocyanine green (ICG) representing a suitable fluorophore for this application. In 2005, Kitai et al. proposed fluorescence-guided navigation of SNB in breast cancer could be performed using near-infrared light [6]. Since then, many publications have shown its practical usage and clinical potential [7–9]. Near-infrared fluorescence (NIRF) is a technique that uses light in the near-infrared range for the visualization of structures stained by a fluorophore [10]. After excitation by a specific wavelength of near-infrared (NIR) light, the fluorophore can be visualized by a special imaging system that detects the reemitted light of a different length from the excited fluorophore [11–13]. Three fluorophores have been approved for SNB NIRF by the Federal Drug Administration and the European Medicines Agency: ICG, MB, and 5-amino-levulinic acid (5-ALA). MB is not considered a pure dye that displays NIR properties [14–16].

When used as a fluorophore, MB is excited by light at a wavelength of 668 nm, with an emission of 688 nm, which is detected within the visible light spectrum of 400–700 nm [14]. Few initial reports about the use of MB's NIRF properties have been published [17–19]. MB has been used to visualize the parathyroid glands during surgery, to localize the ureters for the prevention of accidental damage to them during operations, to localize different pancreatic tumors, and in breast cancer margin detection during breast conserving therapy [17,19–23]. A recent publication by Zhang et al. presented the first videos of an SNB using MB as a fluorophore [7].

The aim of the current study was to present, for the first time in a clinical series of patients, the possible use of MB as a fluorescent dye for SNB performed during surgery for breast cancer. We also explored the detection rate of different dilutions of MB and their corresponding fluorescence.

2. Methods

In this prospective series of 49 patients, only patients with a diagnosis of invasive breast cancer were included in the study. All patients received care at the Surgical Oncology Department at the University Clinical Center in Gdansk, Poland. An institutional review board approved the study (No. NKBBN/92/2018-2020). The patients signed an informed consent form. Each patient qualified for SNB after a multidisciplinary board review. Certain patients (9) received pre-operative treatment in the form of chemotherapy (6/49; 12.2%) or hormonal therapy (3/49; 6.1%) depending on their qualifications according to current breast cancer treatment guidelines.

All patients underwent the standard sentinel node procedure. MB (METIBLO) in 10 mg/mL vials was purchased from Laboratoires STEROP NV, Scheutlaan, Brussels, Belgium. On the day of surgery, approximately 1–3 h prior to the operation, a dose of 100 MBq 99m technetium nanocolloid was administered at an injection site close to the areola. Directly before the operation, the attending surgeon injected 1 mL of MB, at a standard concentration of 10 mg/mL, at the injection site close to the areola. After the injection of MB, the area was massaged to aid in the assessment of proper lymphatic drainage. After surgical scrubbing and preparation of the operative field with sterile draping, NIR fluorescence imaging was performed using a Quest Spectrum (Quest Medical Imaging, Middenmeer, The Netherlands) fluorescence imaging device after dimming the operative lights. The Quest Spectrum (Quest Medical Imaging, Middenmeer, The Netherlands) is designed and developed for open and minimally invasive image-guided surgery using NIRF imaging. The fluorescence imaging system is designed to visualize two types of fluorescent probes that are not visible to the naked eye: Cy5.5 and ICG. It can also be used with any other probe that has similar fluorescence properties. For this study, the Cy5.5 mode was used to visualize MB. Tissues were illuminated with a wavelength of 680 nm and visualized at approximately 710 nm. During this process, a color image of the surgical field can be visualized simultaneously with NIRF, allowing surgical guidance.

The camera was installed onto a flexible arm. Fluorescence imaging was performed at a distance of 20–30 cm from the surgical field. As darkness is necessary for better visualization of MB's fluorescence, the surgical field itself was illuminated using the white light source of the camera system. Afterward, a standard inspection by a handheld gamma camera system, Gamma Finder II (W.O.M. World of Medicine GMBH; Berlin, Germany), was performed using the standard SNB protocol. Each surgeon had access to all three images—gamma probe, naked eye for color visualization, and NIR fluorescence imaging—to aid in the detection of the sentinel node (SLN).

The possibility of the camera system visualizing fluorescence with "hands free" imaging enabled continuous visualization of the lymphatic vessels and nodes during the surgery (Figures 1 and 2). The visibility of the fluorescence signal of the SLN depends on the signal-to-background ratio (SBR). To determine SBR, the Quest Research Tool (Quest Medical Imaging, Middenmeer, The Netherlands) was used. The software allows the selection of a region of interest (ROI) from the fluorescence signal of the SLN in the fluorescence image and from adjacent tissue, the background. The SBR is calculated by dividing the average intensity of the pixel values in the SLN ROI by the average intensity of the pixel values in the background. An SBR $\geq$ 1.1 is considered positive by NIR fluorescence. Sentinel nodes visible under normal light (Figure 1) and under fluorescence (Figure 2) were observed.

Dilution Range to Support Findings

As our clinical research results showed, the standard concentration for injection of methylene blue (10 mg/mL) revealed a promising, but not ideal, result. We hypothesized that quenching at this high concentration might be the effect causing the suboptimal detection of fluorescence in the sentinel nodes. To support this hypothesis, ex vivo studies with the aim of finding an optimal dilution were performed, resulting in the finding that lower concentrations of methylene blue could potentially result in better visualization of the fluorescence of the dye. In future studies, the optimal dose will be one of the topics to focus on.

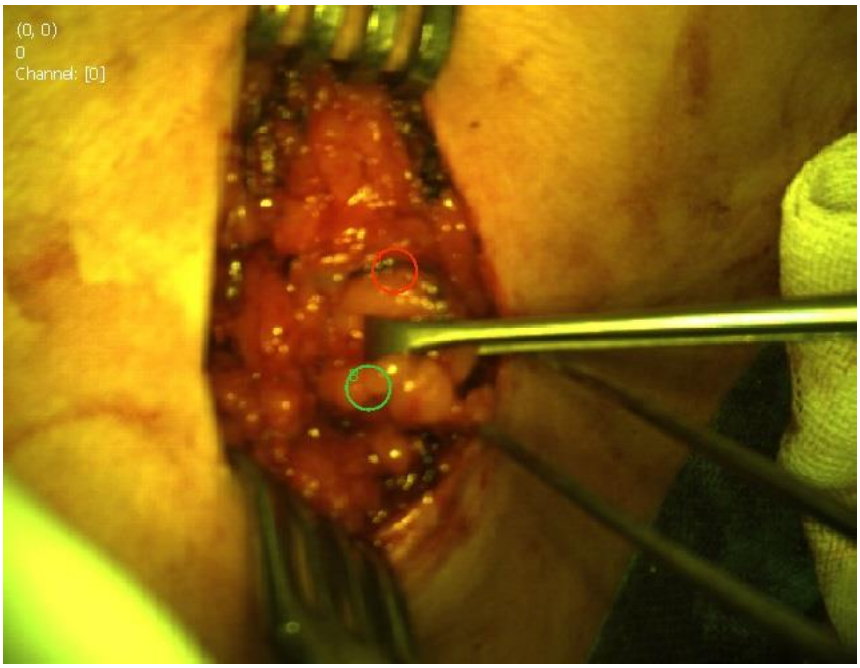

Figure 1. Sentinel node biopsy visible under normal light.

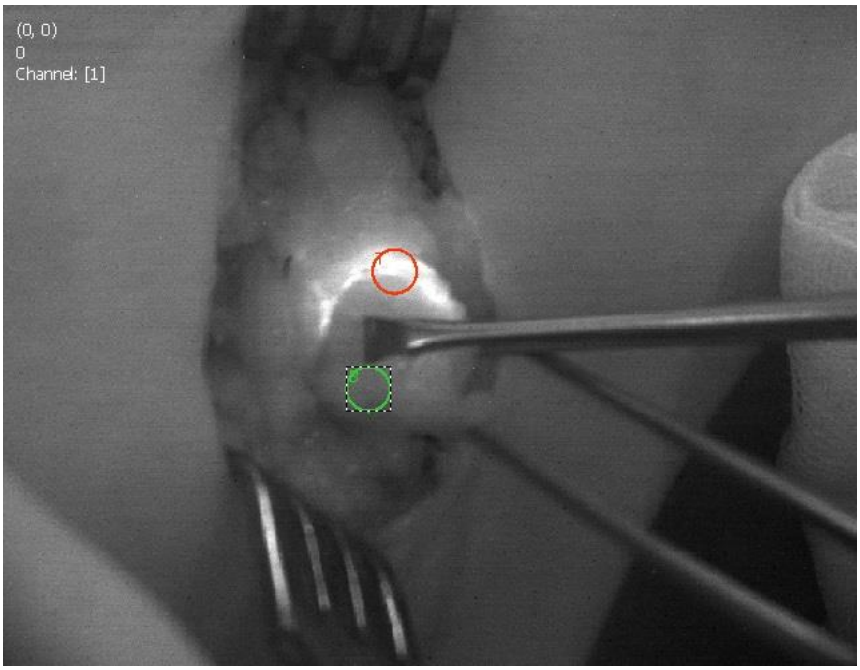

Figure 2. Sentinel node biopsy visible under fluorescence. The ROI are marked.

We conducted additional experiments to support the clinical findings. Two dilution series ranging from 0.125 to 1000 µM were created using MB stock solutions. Eppendorf vials were filled with different dilutions and the vials were imaged using a Quest Spectrum (Quest Medical Imaging, Middenmeer, The Netherlands) fluorescence imaging device, maintaining fixed camera settings, distance to the sample, and sample location (Figure 3). Analysis of the fluorescence signal in the vials was performed using the Quest Research Tool. In the fluorescence image, an ROI was selected in the sample region, and another at a fixed point in the background. Fluorescence intensities were calculated by subtracting the background intensity from the fluorescence intensity in the sample. We used MB at a basic concentration of 10 mg/mL.

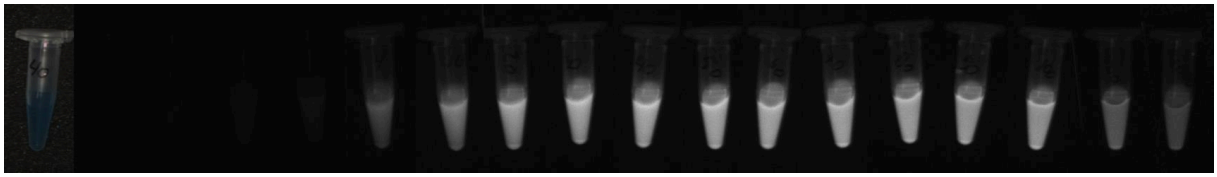

Figure 3. Dilution series of methylene blue visualized by fluorescence imagining device.

3. Statistical Analysis

Descriptive statistics were reported either as a median with minimum and maximum values or as a frequency with percentages. Chi-squared tests were used to test for differences between observed frequencies and frequencies that were expected under the null hypothesis. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS version 26.0 software package for Mac (IBM Corp., Chicago, IL, USA).

4. Results

This study prospectively enrolled 49 female patients with invasive breast cancer who then underwent an SLN biopsy under both radioactive and MB guidance. The fluorescence properties of MB were also used for guidance during the SLN biopsy. The median age of the patients was 62.49 years (range 42–84 years). All other clinicopathological characteristics of the study patients are listed in Table 1. Analysis of different factors associated with sentinel node biopsy are listed in Table 2. Uptake of ^{99m}Tc was present in all patients. In 23 patients (46.9%), the location of the SLN, or the end of the lymphatic path, was visible transcutaneously using fluorescence. The median SBR for transcutaneous SLN location was 1.69 (range 1.66–4.35). Fluorescence in the SLN was visible in 25 patients (51%). Blue-dye-stained nodes were visible to the naked eye in 40 patients (81.6%). The median SBR for SLN visualization by fluorescence was 2.54 (range 1.34–6.86). Lymphatic channels were visible under fluorescence in 14 patients (28.6%) prior to visualization by the naked eye, with an average SBR of 2.01 (range 1.14–5.6). In three patients (6.1%), the SLN was visible under fluorescence, but not to the naked eye. In 15 patients (30.6%), the node was visible to the naked eye, but not with fluorescence visualization. We also performed analyses to test the differences between observed frequencies and frequencies that were expected under the null hypothesis between transcutaneous SLN visualization, lymphatic channel fluorescence visualization, and different clinical as well as pathological factors (Table 3). For transcutaneous SNL fluorescence visualization, statistically significant factors were smoking (p = 0.001) and neoadjuvant chemotherapy (p = 0.026). SLNs were visualized faster under fluorescence during SLN preparation. Factors associated with this included diabetes (p = 0.001), neoadjuvant chemotherapy (p = 0.003), and multifocality (p = 0.004). The only factor associated with visualization of the SLN by naked eye was neoadjuvant chemotherapy (p = 0.013).

Table 1. Clinicopathological characteristic of 49 enrolled patients.

Variable	Category	N. Cases (%)
All patients		49 (100)
Age, years [median (min-max)]		62 (42–84)
BMI [median (min-max)]		28 (17–52)
Diabetes	No	47 (95.9)
	Yes	2 (4.1)
Smoking	No	34 (69.4)
	Yes	15 (30.6)
Tumor localization	Upper outer	29 (59.2)
	Upper medial	8 (16.3)
	Lower outer	8 (16.3)
	Lower medial	4 (8.2)

Table 1. *Cont.*

Variable	Category	N. Cases (%)
Multifocality	No	41 (83.7)
	Yes	8 (16.3)
Neoadjuvant chemotherapy	No	43 (87.8)
	Yes	6 (12.2)
Neoadjuvant hormone therapy	No	46 (93.9)
	Yes	3 (6.1)
Histological grade	I	18 (36.7)
	II	19 (38.8)
	III	11 (22.4)
	Missing	1 (2.0)
Molecular type	Luminal A	21 (41.9)
	Luminal B	10 (20.4)
	Luminal HER2+	7 (14.3)
	Non-luminal HER2+	2 (4.1)
	Triple negative	8 (16.3)
	Missing	1 (2.0)
Estrogen	No	10 (20.4)
	Yes	38 (77.6)
	Missing	1 (2.0)
Progesterone	No	14 (28.6)
	Yes	34 (69.4)
	Missing	1 (2.0)
HER2	No	36 (73.5)
	Yes	12 (24.5)
	Missing	1 (2.0)
Ki-67 [median (min-max)]		10 (1–85)

Table 2. Analysis of sentinel lymph node data.

Variable	Category	Number of Cases (%)
Number of sentinel nodes	1	26 (53.1)
	2	14 (28.6)
	3	4 (8.2)
	4	3 (6.2)
	5	1 (2.0)
	Missing	1 (2.0)
Type of metastatic nodes	Missing	1 (2.0)
	No metastasis	30 (61.2)
	Micrometastasis	1 (2.0)
	Macrometastasis	14 (28.6)
	Isolated cancer cells	2 (4.1)
	Missing	1 (2.0)
Radiocolloid nodes stained	Negative	0
	Positive	49
NIRF visibility through skin	Negative	26 (53.1)
	Positive	23 (46.9)
NIRF visibility of nodes	Negative	24 (49)
	Positive	25 (51)
Blue nodes stained (eye)	Negative	9 (18.4)
	Positive	40 (81.6)
NIRF visibility of lymphatic vessels	Negative	35 (71.4)
	Positive	14 (28.6)

NIRF, near-infrared fluorescence.

Table 3. Critical values of the Chi-Square test for factors associated with near-infrared fluorescence visibility through skin and of sentinel lymph nodes in comparison to naked eye visibility of blue-stained nodes.

Variable	Category	NIRF Visibility through Skin		(*p* Value)	NIRF Visibility of Nodes		(*p* Value)	Blue Nodes Stained (Eye)		(*p* Value)
		0	**1**		**0**	**1**		**0**	**1**	
Diabetes	No	24	23	1.84	23	24	0.001	8	39	1.39
	Yes	2	0		1	1		1	1	
Smoking	No	18	16	0.001	17	17	0.046	7	27	0.365
	Yes	8	7		7	8		2	13	
Neoadjuvant chemotherapy	No	23	20	0.026	21	22	0.003	8	35	0.013
	Yes	3	3		3	3		1	5	
Neoadjuvant hormone therapy *	No	24	21	0.201	22	23	0.356	7	38	4.82
	Yes	2	1		2	1		2	1	
Multifocality	No	24	17	3.02	20	21	0.004	7	34	0.281
	Yes	2	6		4	4		2	6	
Molecular type *	Luminal A	11	10	0.762	10	11	3.73	4	17	3.11
	Luminal B	6	4		6	4		3	7	
	Luminal HER2+	3	4		2	5		0	7	
	Non-luminal HER2+	1	1		2	0		0	2	
	Triple negative	5	3		4	4		2	6	
Histological grade *	I	12	6	2.25	9	9	1.29	3	15	1.44
	II	8	11		11	8		5	14	
	III	6	5		4	7		1	10	
NIRF_lymphatic channel	0	19	16	0.074	10	25	20.4	8	27	1.65
	1	7	7		14	0		1	13	

NIRF, near-infrared fluorescence. * Values are given according to number of patients per group after exclusion of patients with potential missing data.

As presented in Table 4, the optimal dilution for the highest fluorescence intensity for MB was 40 µM (0.0128 mg/mL). However, there may be potential clinical relevance for MB dilutions in the range 20 µM (0.0064 mg/mL) to 100 µM (0.032 mg/mL). We also visualized the performance of the different concentrations of MB and their corresponding fluorescence intensity (Figure 3).

Table 4. Fluorescence intensity in different Methylene Blue dilutions.

uM	mg/mL	Average Intensity	Standard Deviation
0.125	0.00004	8.8	1.7
0.25	0.00008	9.8	1.8
0.5	0.00016	13	2
1	0.00032	17.2	2.3
4	0.00128	60	4.8
10	0.0032	106.6	8.2
20	0.0064	140.3	8.4
30	0.0096	159.1	8.6
40	0.0128	164.4	7.4
50	0.016	162.4	7.2
60	0.0192	157.3	7
70	0.0224	157.7	6.2
80	0.0256	156.6	6.9
90	0.0288	146.1	5.8
100	0.032	141.8	5.5
500	0.16	79.3	4.1
1000	0.32	59.5	3.7

5. Discussion

The primary endpoint of our research was the measurement of fluorescence imaging regarding the effectiveness of MB for SLN mapping in breast cancer patients. Our results revealed that in 51% of patients, we were able to visualize the fluorescence signal in the lymph nodes using the fluorescence imaging device. Based on our prior experience using ICG fluorescence during SLN biopsies for breast cancer, it appears that the performance of NIR fluorescence is comparable to that of radioactive dyes. This may lead to changes in our daily practice in the future. However, we need to further investigate why we were able to visualize fluorescence of the SLNs in only half of our study's patients.

MB presents with different characteristics in comparison to ICG, when used as a fluorophore. By analyzing the technical differences between ICG and MB, we may be able to draw more conclusions about their functions. The main difference between the two fluorophores is the peak of visible light excitation, which presents around 700 nm for MB and 800 nm for ICG [11,14,15]. This causes MB to have a lower tissue penetration capacity, so background tissue can show more autofluorescence [11]. In the literature, the depth of penetration for ICG was determined to be between 1–1.5 cm, and for MB, up to 1 cm [24]. Additional studies are needed to examine the penetration depth of MB when employing its fluorescent properties. Both ICG and MB are currently used to identify lymph nodes, in part due to their small size. ICG has a diameter of 1.2 nm, 776 Da, and MB has a diameter of 1.43 nm, 320 Da [13]. However, their small size, especially their very small diameter, can cause these dyes to pass quickly through the lymph nodes, eventually losing the dye beyond the SLN [25,26].

The next challenge regarding the use of MB is the necessity of dimming the operative lights during surgical procedures. When ICG was first used as a fluorophore during surgery, the operative lights were dimmed during the procedure; however, advancements in technology have allowed for the use of new cameras under the normal lights used in the operating theater. This is less disturbing to the operator during surgery [13,27]. For now, dimming the operative lights is necessary when using MB, as the range of excitation and detection of MB is within the daylight spectrum. Thus, in using MB as a fluorophore, surgeons must work using only the light emitted from the camera.

5.1. Doses

Another problem that presents with the use of MB as a fluorophore is the determination of an optimal dose of blue dyes for an SLN biopsy. In our study, each patient received a standard dose of radiocolloid, 1 mCi in 1 mL, which was injected intradermally around the areola at a single injection point prior to the operation. The standard dose of blue dyes varies from 1–5 mL, depending on the meta-analysis. Li et al. [28] showed that dosages can vary from 0.1 mL to 10 mL. The dose of 1 mL of MB was determined as the current standard in our department.

5.2. Quenching

Researchers observed that a reduction in fluorescence emission occurs as the concentration of fluorophore increases [29]. From this point of view, using a high concentration of fluorophore for SLN mapping while using NIR fluorescence may prove disruptive [30]. It is probable that the dilution of NIR fluorophores may decrease the effect of quenching and affect the NIR signal of the dye. Another concern is that the high concentration of MB in the sentinel lymph node may be responsible for the difficulties in fluorescence visualization of these structures using NIR light. This phenomenon may play a role in the observations in our study's group of patients. We hypothesized that lower doses of MB may improve our visualization. In our preclinical study, we found that, with lower concentration, the fluorescence of MB increased, reaching its peak at 40 μM (0.0128 mg/mL). In our analysis of different dilutions for the highest fluorescence intensity, it seemed that dilution might be key to improving the intensity of fluorescence; thus, a future study should focus on this idea to show the optimal dilution in a clinical setting, which may be different from the laboratory value presented in our study.

5.3. Safety Profile and Side Effects

MB has been used in the field of surgery for decades, typically presenting with a good safety profile [3,19,31–34]. Skin necrosis has been reported, as well as fat and parenchymal necrosis [35–37]. Other skin reactions were also reported by Kaklamanos et al. [38]. In some patients, skin discoloration may be seen after blue dye injection, which can persist as a tattoo for up to one year after surgery [39]. Persistent skin discoloration is also observed after the use of other fluorophores such as ICG for SLN biopsy in breast cancer [40]. In our study, no adverse reactions or events were noted by our patients.

5.4. Identification Rate

The identification rate of SNB when using only blue dye is about 91%. We can improve the detection rate by performing SNB using a second dye with radioactive properties, especially nanocolloids. With this double dye technique, detection rates improved to 96% [5]. When ICG was used, sentinel lymph nodes could be identified in breast cancer patients by the naked eye 50% of the time. When using ICG's fluorescent properties, SLN could be visualized 94% of the time [6]. In our study, SLNs were stained blue during surgery in 81.6% of patients (40/49). Using a near-infrared fluorescence imaging camera system, we were able to visualize fluorescence in the SLNs with MB in 51% of cases (25/49). Interestingly, in three patients (7%), SLNs were seen only with MB fluorescence. We must

underline the fact that in 17 patients (37%), the SLNs were seen only with the naked eye, but were not visible under fluorescence.

It appears that the visualization of MB under fluorescence is the reverse of ICG, where identification of SLN using the naked eye is much lower than when using its fluorescent properties. According to Motomura et al., in a group of 172 patients, SLNs stained with ICG were visualized by the naked eye in 73.8% of the patients [41].

The low rate of fluorescent lymph nodes when using MB is likely due to the quenching effect phenomenon. Similar problems were identified after initial studies using ICG for SNB were published. In a study by Mieog et al., researchers found that, with an increase in concentration, the quenching effect disturbed the visualization of ICG-stained structures. They recommended a dose of 0.62 mg of ICG as an optimal dose for fluorescence navigation [30]. Other papers reported the use of doses of ICG between 0.625 mg and 15 mg in a volume of between 1 and 5 mL respectively, achieving similar detection rates [42]. We presented an in vitro analysis of different dilutions of MB, which showed that its fluorescence intensity reached its peak with a dilution of 40 μM (0.0128 mg/mL), beyond which the fluorescence intensity became much smaller. Thus, further research is needed to determine the optimal dose of MB in a clinical setting when using its fluorescent properties.

The most important advantage of MB is its long-term clinical use and well-documented experience by many surgeons. We must point out that a low-detection rate of SLN under fluorescence guidance is a critical point of our study. Future technological advancements, the use of new cameras, or the possible use of other blue dyes as fluorophores may all be solutions to this dilemma.

6. Conclusions

Our study presents the possibility of performing SNB using an old dye in a new fashion with fluorescence visualization. The standard dose of MB did not seem to be sensitive enough in fluorescence visualization of SLNs in breast cancer. However, we suppose that MB as a fluorophore may prove to be a useful tool in the future, especially at lower concentrations. As the in vitro dilution test represents samples that have only been diluted and not injected into the patient, it should be considered that the concentration in the SLN may be lower than the concentration injected. Therefore, further in vivo investigation is needed to indicate the optimal injection concentration for the best fluorescence visualization of SLNs using MB.

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Trends and future perspective in Sentinel Node Biopsy in Breast Cancer Patients

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Abstract

The current approach to lymph node staging in early breast cancer is sentinel lymph node biopsy (SLNB), which is essential for prognosis and regional control of the disease. No imagining method is capable to detect lymph node metastasis and only SLNB is considered a gold standard to identify even the smallest metastatic foci in regional lymph nodes. Nevertheless, despite years of experience in this procedure, important clinical aspects and some variabilities are still under investigation. Also, for this SLNB procedure, new techniques and indications are explored if a more precise and individualised approach is possible for the tailored treatment of our patients. This review aimed to discuss selected questions about modern and possible future directions in SLNB for breast cancer. *(Farm Współ 2022; 15: 151-156) doi: 10.53139/FW.20221520*

Keywords: Breast Cancer, Sentinel Node Biopsy, Axillary Lymph Node Dissection, Indocyanine Green, Blue Dye, Technetium

Introduction

Breast cancer is the most commonly diagnosed malignant disease worldwide, with an estimated number of around 2.3 million new cases in 2020 [1]. In Poland, in 2020, the detection rate of breast cancer, according to world health organisation data, is 24,644 cases [2]. Sentinel lymph node biopsy (SLNB) for many decades showed its clinical usefulness, improving staging diagnosis in many cancer patients [3]. Using this method, we can visualise the lymphatic anatomy and get information about the cancer metastasis to the lymphatic system. Not only the stage of the disease but also regional control of cancer might be observed. Historical term, „sentinel", was described for lymphatic nodes in 1923 by Braithwaite, later by Gould et al. in 1960, and by Sayegh in 1966 [4-6]. The term „Sentinel Lymph Node" (SLN), for the first time was proposed by Cabanas et al. [7]. The details of the physiological concept of "sentinel lymph node biopsy "was published in melanoma patients in 1992 by Morton and Cochran [8].

The concept of SLN is based on hypothetical lymph node drainage from the primary tumour. Currently, no imaging system can detect lymph node metastasis in the early stage of breast cancer. However, by performing SLNB, we can stage regional lymph nodes and diagnose not only macro- but also micrometastasis of the lymphatic system. By drainage of the lymphatic area, the cancer cells flow towards the sentinel lymph node(s) and subsequently are transported to higher tiers of lymphatic system [8].

Today SLNB is a standard alternative technique to axillary lymph node dissection (ALND) for breast cancer staging. This surgical approach reduced the postoperative complications associated with ALND-like lymphedema, pain, nerve damage, postoperative serum collection, thromboembolic events, and infections.

The beginning of SLNB was based on the usage of methylene blue as a visible blue dye during the SLNB procedure [8]. The current gold standard is based on a dual method procedure using radioactive nanocolloide with an addition of a blue dye [9].

The detection rate of the double dye technique is about 96%, and using only blue dye is around 91% [10].

This paper aims to describe the current methods and trends for selected subjects in SLNB for breast cancer.

One or two metastatic sentinel nodes

ACOSOG Z0011

This trial was proposed to prove whether SLNB impacts survival compared to ALND in breast-conserving therapy (BCT) [11]. For early-stage breast cancer, presenting one or two metastatic lymph nodes after

SLNB, the patients were randomised to ALND vs whole breast irradiation therapy, with standardised adjuvant treatment either way.

The first results revealed no benefit in ALND vs SLNB and radiation therapy in case of locoregional recurrence in patients with BCT.

This trial's long-term follow-up showed no significant difference in local recurrence free survival and overall survival. Ten-year cumulative locoregional recurrence was similar for both groups (6.2% for ALND and 5.3% for sentinel node biopsy p=0.036)

As a result of this trial, a new standard was set for T1/T2 breast cancer with SLNB, where we can omit ALND when radiotherapy is given afterwards.

Still, three and more metastatic lymph nodes, or macroscopically suspicious lymph nodes, remain an indication for ALND [12].

AMAROS Trial

In another clinical trial (AMAROS), after surgical staging of the axilla, it was proven that postoperative radiotherapy compared to ALND showed similar oncological outcomes, with significantly lower morbidity and reduced risk of postoperative lymphedema [13].

Based on the results of these trials, still the group of selected patients with only 1-2 positive lymph nodes after SLNB procedure, with tumours greater than 3 cm, lymphovascular tumour invasion, and sentinel nodes microscopic extra capsular extensions, are a group of patients where an optimal adjuvant treatment plan is required [14].

Similar results were presented in another clinical trial (OTOASOR trial) [15].

NEW DYES

However, the standardised dual tracer technique in SLNB in breast cancer staging presents excellent results in terms of detection and false negative ratio. New dyes are being developed to improve some aspects of this procedure.

Fluorescent Dyes

From 2005, indocyanine green (ICG) has proved to be a valuable dye for breast cancer SLNB [16].

Using the fluorescent properties of this fluorophore, we can visualise the lymphatic vessels between the injection site and the sentinel lymph node in real time. A relatively cheap method is a good alternative for hospitals without access to nuclear medicine department. It is especially important because the detection rate is higher than nanocolloid and blue dye alone, similarly to the dual technique [17].

The advantages of this tracer include the visualization of lymphatic vessels through the skin, long-term proven safety profile, high-resolution real-time tracking and simple administration protocol with a short learning curve.

The main disadvantages are possible iodine allergy risks, relatively low fluorescence brightness, and skin discolouration. The detection of deeper sentinel nodes also seems problematic, ultimately due to the quick dispersion and then shortening of the detection time [18].

Not only ICG but also other fluorophores showed the feasibility of lymph node biopsy using fluorescent properties of these dyes like methylene blue or fluorescein [19,20].

Using different fluorophores during the same operation - a multispectral imagining - is possible to differentiate specific structures during operation [21].

Superparamagnetic iron oxide (SPIO)

With this tracer, non-invasive magnetic properties are used for handheld magnometer detection. Preoperative magnetic resonance imaging can visualise sentinel lymph nodes preoperatively and handheld magnometers intraoperatively. A non-inferiority of this technique in comparison to the dual tracer technique was proved in a meta-analysis by Zada et al. [22]. Like ICG, this procedure stains more lymph nodes [23]. We must remember that if using this approach, the surgical field must not be covered with any tools with magnetic properties.

Contrast-enhanced ultrasound (CEUS) with microbubbles

The idea behind this technique is based on dispersion with sulphur hexafluoride gas, stabilised by phospholipids [24].

The SLNB together with lymphatic vessels, are visualised by contrast-enhanced ultrasound (CEUS). The live visualisation using live ultrasound imagining can help in marking by guide wire of SLN(s) before operation. Identification rates ranged from 92.8%, with no statistical difference between CEUS and blue dyes [17]. We must keep in mind that this technique is highly dependent on ultrasound skills and requires a long

learning curve. The main advantage of this method is its cost effectiveness.

Micrometastasis and isolated tumour cells

According to sentinel node histopathological classification, we can distinguish three types of metastases based on the size of metastatic foci: macrometastasis, micrometastasis and isolated tumour cells.

The first clinical question in case of pN0(i+) or pN0(mi) is an additional non-sentinel involvement in other lymph nodes. For pN0(i+) the involvement varies from 4.9-16% [25,26], and for pN0(mi) involvement varies from 0-21% [27].

The second clinical question is the prognosis for pN0(i+) or pN0(mi) after SLNB. In the MIRROR study, patients with pN0(i+) or pN0(mi), did or did not receive systemic adjuvant therapy, and 10% decrease in 5-year disease-free survival was found in these patients in comparison to the pN0 group [28]. Nevertheless, with additional systemic therapy, 10% 5-year disease-free survival improvement was observed for pN0(i+) or pN0(mi).

In another research additional pathological section of histological negative sentinel lymph nodes resulted in finding occult metastasis in 15.9% of patients, including 11.1% of ITC, 4.4% of micrometastasis and 0.4% macrometastasis [29].

Statistically significant less disease-free and overall survival was observed. However, the presence of occult metastasis was found not to be a predictor for cancer recurrence.

In the IBCSG 23-01 trial micrometastatic sentinel lymph nodes with or without lymphadenectomy showed similar disease-free survival rates [30]. Similar results were presented in AATRM 048/13/2000 trial [31]. The omission of axillary lymphadenectomy should be proposed for patients with or without radiotherapy afterwards.

Neoadjuvant Treatment

Due to new advancements in neoadjuvant treatment and an increasing number of patients qualified for this procedure, new challenges occur. Neoadjuvant therapy can change the axillary stage status and helps classify more patients eligible for SNB. Primary systemic therapy is responsible for up to 30-40% of complete pathological remission in the axillary lymph nodes [32].

We need to analyse two different subgroups of patients for SLNB, with or without positive axillary lymph nodes in preoperative settings.

Sentinel node biopsy after neoadjuvant treatment in node-negative patients (cN0)

The SENTINA trial showed that after neoadjuvant treatment, SLNB is feasible and showed a lower detection rate and higher false negative rate, as if the SLNB was done before the neoadjuvant treatment [33]. The false negative rate of SLNB in cN0 patients, who underwent neoadjuvant chemotherapy, was 5.9% vs 4.1% with the surgery-first approach [34].

A meta-analysis based on 23 studies proved an excellent identification rate and false negative rate [34].

Sentinel node biopsy after neoadjuvant treatment in node-positive patients (cN1)

In an ACOSOG Z1071 trial, the false negative rate of axillary involvement was less than 10% after prior treatment of cN1 status patients.

The false negative rate was 20.3%, and for the dual technique, 10.8% [35]. With the removal of at least three or more lymph nodes, the false negative rate dropped to 9.1% compared to 21.1% if two nodes were removed.

The GANEA 2 Trial showed independent predictor factors associated with a higher false negative rate after neoadjuvant chemotherapy for SLNB if the residual tumour size was ≥ 5 mm and lymphovascular invasion was present [36].

In the SENTINA trial, similar to ACOSOG Z1071, the reduction in false negative rate from 16-8.6% was found if the dual method was used instead of one tracer [37].

Moreover, the false negative rate decreased when more sentinel lymph nodes were removed (24.3% for one node, 18.5% for two nodes, and less than 10% for three or more lymph nodes). Another factor involves pathological immunohistochemical evaluation of the lymph node. Using this specific method, the false negative rate dropped to 8.7%. Interestingly, 6.8% of false negative ratio could be achieved if lymph nodes were marked by a clip, via ultrasound, prior neoadjuvant treatment [36].

The other useful tool for localising pre-neoadjuvant treatment positive lymph nodes is pretreatment labelling by clip [38].

The feasibility and placement of a clip into the most suspicious biopsy-proven lymphatic nodes were

investigated with a postoperative identification rate of 87.8% in the specimen.

The false negative rate for these patients was 4.2 % when the marked node was found as a sentinel node; however, if this clip node was not a sentinel node, the false negative rate was 16.7% [39]. In some cases, the clip-marked lymph node can be found preoperatively by ultrasound. If not, there is the possibility of marking via a guide wire in the department of radiology. The lymph node can be found during surgery. After performing SLNB, the confirmation of the clip in the lymph node can be confirmed by X-Ray mammography.

Many of the questions may be resolved by the results of ongoing clinical trials [40-42].

Omission of SLNB in Elderly patients

Older patients with estrogen receptor positive tumours, treated with hormonal therapy, showed that omission of SLNB in tumours with a good prognosis is possible [43,44].

In an Italian study, patients aged 65-80 years, who underwent BCT were randomised to ALND vs. no axillary intervention at all. All patients received five years of treatment with tamoxifen. No difference was found in case of cancer-specific mortality, overall mortality or crude cumulative incidence of breast events.

In another retrospective study in cN0, patients aged 70 years and older who underwent ALND vs no--ALND showed no difference in breast cancer mortality in 15 years of follow-up [45].

In the IBCSG Trial 10-93 in patients aged 60 years and older, randomised with or without ALND followed by five years of tamoxifen, similar results in terms of disease-free survival and overall survival was achieved, with significantly better quality of life, in the subgroup of patients without axillary surgery [43].

According to the Recommendation of Society of Surgical Oncology, SLNB should not be routinely used in women older than 70 years with hormone positive receptor breast cancer.

Omission of SLNB/Avoidance in Axillary staging

Following low recurrence rates for SLNB negative and selective SLNB positive cases, the rate of lymphedema after SLNB may impact the patients' outcome. Currently, ongoing trials are investigating the feasibility of omitting SLNB in patients with cN0 and biologically not aggressive tumours after BCT. The SOUND trial, INSEMA Trial and BOOG 2013/08 are under investigation [40-42].

The primary endpoint of these studies is a disease--free survival with secondary ones including morbidity and quality of life.

Conclusions

Recently, an evident trend toward deescalation of axillary surgical treatment in breast cancer patients is observed. Additionally, new technologies are being helpful for pre- and intraoperative sentinel node visualisation. ACOSOG Z0011 trial changed the dogma in some cases of positive sentinel nodes, where one or two metastatic sentinel nodes are no longer an indication for axillary lymph node dissection in patients qualified for breast-conserving therapy.

Indocyanine green and other fluorescent dyes, like methylene blue or fluorescein, might be useful tools for intraoperative visualisation of sentinel nodes and lymphatic vessels. Also, other new techniques for SLNB, like SPIO and CEUS found their place in clinical research.

The presence of ITC or micrometastasis in the lymph nodes is a risk factor for non-sentinel lymph node involvement. Moreover, the presence of ITC or micrometastatic sentinel lymph nodes is responsible for a 10% decrease in 5-year disease-free survival; however, this difference disappears with additional systemic therapy.

Occult sentinel node metastases are not predictive factors for cancer recurrence. Following IBCSG 23-01 trial, ALND seems to be an overtreatment in case of patients with micrometastatic sentinel lymph nodes.

Also, after neoadjuvant therapies, a sentinel node biopsy is offered for all cN0 patients and in case of clinical downstaging to ycN0 cases. Still positive and palpable lymph nodes remain an indication for ALND.

Older patients with estrogen receptor positive cancer, together with anti-hormonal therapy, are good candidates for the omission of SLNB. Ongoing clinical trials will probably find a subgroup where omitting axillary staging in younger subgroups also will be possible.

In the last years, the revolution and evolution of SLNB in breast cancer has become a fact.

A tailored axillary approach will help improve disease staging and patient quality of life.

Conflict of interest
None

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OŚWIADCZENIE

Jako współautor pracy pt. "Methylene Blue Near-Infrared Fluorescence Imaging in Breast Cancer Sentinel Node Biopsy" oświadczam, iż mój własny wkład merytoryczny w przygotowaniu, przeprowadzenie oraz opracowanie badań oraz przedstawienie pracy w formie publikacji to: współpraca z autorem przy koncepcji pracy, zbieraniu materiału, poprawkach merytorycznych publikacji.

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Oświadczam, iż samodzielnie i możliwa do wyodrębnienia cześć ww. pracy wykazuje indywidualny wkład lek. Olivera Budnera przy opracowaniu, koncepcji, wykonywaniu części eksperymentalnej opracowaniu i interpretacji wyników tej pracy.

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(podpis współautora)

Dr n. med. Jarosław Skokowski

(tytuł zawodowy, imię i nazwisko)

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(podpis współautora)

Natalia Cwalina MD
(title, name and surname)

Detroit 15.09.2022

STATEMENT

As a co-author of the work entitled "Methylene Blue Near-Infrared Fluorescence Imaging in Breast Cancer Sentinel Node Biopsy", I declare that my own substantive contribution in the preparation, conduct and development of research and presentation of the work in the form of a publication is: collaboration with the author on the concept of work, material collection, substantive corrections of the publication.

At the same time, I consent to the submission of the above-mentioned work by Oliver Budner as part of a doctoral dissertation in the form of a thematically coherent set of articles published in scientific journals.

I declare that independently and separately identifiable part of the above-mentioned work shows the individual contribution of Oliver Budner in the development, concept, and performance of the experimental part, preparation and interpretation of the results of this work.

Natalia Cwalina MD

(co-author signature)

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