Pre-analytics, a national survey of *Senonetwork Italia* breast centers: Much still to do ahead☆,☆☆

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ABSTRACT

Introduction: Pre-analytics involves handling and processing of microbiopsy and surgical specimen. It is critical for the preservation of morphology and the integrity of molecular markers, which are paramount as prognostic and predictive factors in breast cancer. Although pre-analytical variables in breast cancer are codified by national and international guidelines, there is currently no data on their actual endorsement in clinical practice among Breast Units (BU).

Materials and methods: An anonymous questionnaire was sent by e-mail by Senonetwork Italia, a no-profit organization representing the multidisciplinary network of BU in Italy. The questionnaire involved twenty-four questions concerning critical issues related to the average time and transport temperature of the samples, monitoring of warm and cold ischemia, average fixation time for biopsies and surgical specimens, inking of the margins, and radiography of the operating sample.

Results: Forty-nine of 113 affiliated BU (43%), involved in the management of 44% of all breast cancer treated every year in Italy, answered the questionnaire. More than 90% of the BU reported a biopsy/VABB fixation time between 6 and 24 h. Only 41% of the Centers received the fresh operative sample to be sectioned immediately, 20% used the vacuum method and the sample arrived in the laboratory within 24 −72 h. Delay in sectioning the sample was reported in as many as 40% of BU, while hot and cold ischemia time was monitored in only 4.2% and 6.2% of BU, respectively.

Conclusion: Critical issues on pre-analytics are reported by the majority of dedicated BU in Italy. This represents a major challenge regarding quality of care, and improvements are needed in order to obtain valid and reproducible results of prognostic and predictive factors.

Introduction

Pre-analytics involves methods of tissue handling (warm and cold ischemia), fixation (type of fixative and duration), processing and sectioning of microbiopsy and surgical specimens. It is a key factor to preserve morphology, antigens and nucleic acids, whose knowledge is paramount in the clinical and therapeutic decision process for patients with breast cancer.

☆ Senonetwork Italia Breast Centers Responders are present in Appendix 1.

☆☆ Senonetwork Italia Breast Centers Survey Responders are reported in Appendix 1.

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In current clinical practice, breast cancer biomarkers such as the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor gene 2 (HER2), are routinely assessed both to acquire better prognostic information and to identify subsets of patients who are appropriate candidates for specific treatments [1].

Guidelines by the American Society of Clinical Oncology (ASCO) and the College of American Pathologist (CAP) [2] have been developed to better standardize this critical process, and they have been accredited by national recommendations (the Italian Study Group on breast cancer of SIAPEC, Italian Society of Pathological Anatomy and Cytology).

Several critical parameters are involved in pre-analytics. Warm ischemic time is the time from the interruption of the blood supply to the tumor by the surgeon to the excision of the tissue specimen, whereas cold ischemic time is the time from excision to the beginning of tissue fixation [3]. Buffered Neutral Formalin (BNF) remains at the present the best fixative [3], however formaldehyde has been classified as carcinogenic [4] and has a fairly slow penetration capacity in tissues [3]. This has led to the search for temporary tissue preservation systems in order to avoid the use of formaldehyde in environments outside the Pathology laboratories, equipped with collective and individual means of chemical protection, and to allow the pathologist to have the surgical material available as soon as possible in order to be able to dissect and fix it quickly.

The optimal management of the surgical sample involves immediate transport to the laboratory, sectioning within one hour of removal, BNF fixation for 24–72 h (better 24–48 h). Alternatively, the Under Vacuum Sealing and Cooling (UVSC) can be used, with maintenance and transport of the samples at a temperature of 4 °C, sectioning within 72 h from the start of the vacuum. Sending the specimen directly in BNF to the laboratory does not allow a precise measurement of cold ischemia and it is associated with a delay in fixation (which is dependent on the penetration of BNF in the tumor tissue and correlates, therefore, with the volume of the sample). Cold ischemia should be kept to less one hour [2]. Monitoring tissue ischemia, recording the times of ischemia on the examination request, allows an implementation of the quality.

To the best of our knowledge, there are no previous studies regarding the actual implementation of such recommendations by specialized BU.

We report on a recent survey regarding this issue among Units associated with Senonetwork Italia, a no-profit organization representing the multidisciplinary network of BU in Italy.

Materials and methods

A 24-questions survey was developed by Senonetwork Italia with the support of the Study Group on Breast Pathology of the Italian Society of Pathological Anatomy (SIAPEC) and the Italian Society of Breast Surgeons (ANISC). The survey was submitted by e-mail on October 2018 to the Pathologists of the 113 Breast Units (BU) associated with Senonetwork Italia.

Enrollment in Senonetwork Italia is volunteer, and BU must state they meet specific requirements regarding both an annual surgical volume of cases and a full multidisciplinary approach of the "core team" [5].

Both the Clinical Director and the Pathologist of the BU shared the responsibility to answer the questionnaire through an anonymous electronic format.

Data was processed by the secretariat of Senonetwork Italia and presented at the National AIS (Actuality in Senology) Biannual Meeting held in Florence on 6th-8th November 2019.

The survey involved

1) Questions regarding general information, geographical location of the Center and number of cases treated per year (Q1-Q5);
2) Problems inherent to the transport of biological samples from radiology or operating rooms to the Pathological Anatomy laboratories (Q6-Q7);
3) The methods of specimens submission (core biopsies/surgical samples) to the laboratories (fresh, formalin, Under-Vacuum Sealing and Cooling - UVSC) (Q8-Q9);
4) Average transport time of fresh, formalin fixed samples, UVSC, from the operating room to the laboratories (Q10-Q11-Q13);
5) Average transport temperature of the samples sent with a UVSC method (Q12);
6) Monitoring of warm and cold ischemia, and their possible indication in the report (Q14-Q15);
7) Inking the surgical sample in the operating room (Q16);
8) Radiography of the operating sample (Q17);
9) Adherence to national recommendations regarding and request for histological examination (Q18);
10) Sectioning of the surgical sample immediately at arrival in the laboratories and fixation (Q19);
11) Methods for assessing the extent of the tumor and the surgical margins (macro-sections, standard sections, radiological grids) (Q20);
12) Choice of the block (fresh, after fixation, other) for immunohistochemical and molecular determination of prognostic/predictive factors (Q21);
13) Average fixation time for biopsy specimens and surgical samples (Q22-Q23);
14) Processing protocol for biopsies and surgical samples (Q24).

Because the survey was anonymous and did not involve analysis of data regarding patients, an Institutional Review Board approval was waived.

Results

The questionnaire was answered by 49/113 BU (43%), which were involved in the management of 22,800 of the 52,000 newly diagnosed breast cancer cases every year in Italy (44%) [6].

Among the responders, BU treated 101–150 case, 151–200 cases, 201–300, or more than 300 cases per year, in 4 (8.2%), 9 (18.4%), 11 (22.4%), and 25 (51%) cases, respectively. Thirty-four BU (69.4%) were located in the North, nine (18.4%) in the Center, and six (12.2%) in southern Italy.

Analysis of data highlighted the following issues:

1) Problems regarding the transport from the operative room to the Laboratory were reported by seven BU (14.3%): over 40% of these problems were represented by the distance from the operating room or from the sampling center.
2) Transport time of the surgical specimen to the laboratory: in 20 BU (41%) the samples arrived fresh and they were immediately sectioned within 30 min in 15 cases, and in over 30 min in the other five cases. The ten BU (20.4%) using UVSC reported that the samples arrived within 48–72 h maintaining a temperature of 4 °C. In 19 BU (38.8%) the samples arrived in BNF, therefore they were not immediately sectioned and fixation was dependent upon the slow penetration of BNF through the entire sample.
3) Monitoring of warm and cold ischemia time was reported by eight Centers (17%): only three BU (6%) monitored both interval

times, three BU (4%) only the hot ischemia, and three BU (6%) the cold ischemia. In these cases the monitored data were reported in the pathologist's report.

4) Core Biopsies or Vacuum Assisted Breast Biopsy (VABB) were received by the laboratories in BNF in 47/49 Centers (96%).
5) Biopsy/VABB fixation time was reported between 6 and 24 h in 47/49 BU (96%), while for surgical samples this was reported between 12 and 72 h after sectioning in 46/49 BU (94%) (Tables 1 and 2).
6) Sampling for the determination of the prognostic/predictive factors by the pathologist was carried out on fresh tissue, after a separate fixation of 24 h, or after observing the histological sections, in 16 (33%), 25 (52%) and seven (15%) cases, respectively.
7) Inking of the margins by the surgeon in the operating room was performed in only 3 BU (6%).
8) X-ray of the operative sample was always performed in 11 BU (23%), while it was obtained only in cases of microcalcifications in 36 cases (73%). Two BU (4%) reported they never used an X-ray of surgical samples.
9) Evaluation of the resection margins and the extension of the neoplasm were carried out with standard blocks, with the aid of macro-sections, or with the radiological grid for the identification of the lesion, in 47 (96%), 10 (21%) and in six cases (13%), respectively.

Discussion

Several problems associated with technical organization and logistics do emerge from our survey, and likely may have an impact on the overall results of pathology laboratories.

To the best of our knowledge this is the first national study regarding pre-analytics of microbiopsies and surgical specimen among qualified BU. Although national guidelines regarding this issue have been developed and implemented by many scientific societies, there is no knowledge on how they are actually implemented in the clinical setting.

All the steps in this phase are critical in order to obtain good histological and immunohistochemical preparations and reliable biomolecular and genomic profiles [7]. Nowadays, such sophisticated tumor analysis at a molecular level identifies the presence or absence of certain cancer gene signatures or biomarkers. It represents the basis for building both personalized therapy, as such information is often linked to specific therapies, and to monitor the risk of disease recurrence, as this might be variably relevant. NCCN guidelines [1], AJCC TNM staging 8th Ed., as long as many other national protocols now incorporate genomic testing in many particular clinical and biological conditions, and this requires optimal preservation of DNA and RNA in the tumor sample if results must be accounted for.

Because the pathologist is increasingly asked for high quality and standardization of these results, pre-analytics is gaining more relevance in this process.

Bass et al. [8] performed a review of the literature on how these parameters can influence the analysis of the nucleic acids (DNA and RNA), proteins and morphology in different ways. Neumeister et al. [9] documented the progressive loss of activity of labile molecules after the surgical interruption of blood flow, leading to tissue ischemia, acidosis and enzymatic degradation. Most studies concluded that delay of formalin fixation had negative effect on ER and PR and on HER2 immunohistochemistry, as well as on other markers used. Pekmezci et al. [10] evaluated the expression of the ER and PR according to ischemia time of less or more than one hour, reporting that five and nine out of 190 samples showed a negative effect on ER and PR measurements, respectively. Hicks et al. [11] showed that ER, PR and other markers are thermolabile proteins whose levels of expression are altered by prolonged cold ischemic time. Khoury et al. [12] reported that a cold ischemia time greater than two hours led to significant morphological changes in three out of 10 cases, and significant immunohistochemical changes in seven out of 10 cases, especially on membrane markers (such as E-Caderina, Epidermal Growth Factor Receptor and HER2). The same group reported that HER2 fluorescence in situ hybridization intensity begins to show compromised interpretation after only one hour [13]. However, another study reported that a cold ischemia time of up to three hours had no deleterious effect on in situ hybridization or IHC for HER2 [14].

Although not addressed by our survey, it has also been reported that an extensive use of electrocautery during breast conservation surgery may cause tissue damage due to denaturation of proteins and problems with interpretation of free margin of excision [15].

Both type of fixative and the duration of tissue fixation influence profoundly the quality of the process and subsequently the results. Formalin penetration and cross-linking process is slow and both processes are temperature dependent [16,17]. In general, the average size of DNA extracted from tissues fixed in buffered formalin decreases with increasing fixation time [17]. Not only cross-linking of nucleic acids with histones does occur with formalin fixation, but formalin reacts directly with nucleotides, causing molecular degradation and alteration sequences [18–20].

Fifteen percent of the BU had problems transporting the samples to the Pathology laboratory, often because they were not located in the same building, with an increase in the time of cold ischemia in the case fresh materials was sent. The ideal situation is when both the operating room and the laboratory are located in the same building. In many BU it was reported that the surgical specimens were immersed in formalin unsectioned. This is associated with two critical problem. First, the use in the operating room of a substance, such as formaldehyde, classified as a Class 1 carcinogenic agent by the International Agency for Research of Cancer (IARC) [4]. Second, it is well known that fixation begins from the periphery of the specimen, and fixation of the cancerous lesion, usually at the center of the surgical resection, can take many hours, depending on the penetration rate of the fixative [21]. Immersion in formalin of a large specimen is not equivalent to fixation. Sectioning 24–48 h after the immersion in formalin of the entire specimen means a delay of fixation: formalin penetration has been reported to be 1 mm per hour, and the mechanism of action of formalin, an aqueous solution of formaldehyde, involves an

Table 1
Fixation time for biopsies/VABB.

Time (hours)	Biopsies/VABB
<6	4%
6–12	29%
12–18	37%
18–24	50%
>24	4%

Table 2
Fixation time for surgical sample.

Time (hours)	Surgical resections
<12	4%
12–24	52%
24–36	31%
36–48	18%
48–72	8%
>72	2%

additional fixation time for chemically crosslinks of proteins and nucleic acids [22].

The use of UVSC can avoid the problem of fixation delay due to the slow penetration of BNF within the specimen, as long as a the cold chain at 4 °C of UVSC is maintained. In any case, the surgical samples should arrive in the Pathology Department within 48–72 h [23–26] to be sectioned and fixed. Of course, UVSC does not guarantee the preservation of tissues [26], but it prevents dehydration and favors cooling by removing air. Cold is the main preserving factor because it inhibits enzymatic autolysis, and the specimen can be thereafter sectioned and fixed. In our survey, UVSC was used in one fifth of the BU.

It is critical that even the formalin samples should arrive within the shortest possible time to the laboratory, and the time of immersion in formalin should be indicated in the request form to check the total fixation time. Sampling of the formalin-fixed material by the Pathology department should be carried out as soon as possible, in order to guarantee that the surgical sample is fixed, thus preventing autolytic changes. Fifteen percent of BU stated they did not fulfill these indications, and this problem seems particularly evident of the surgical specimen rather than for the biopsy/VABB.

The fixation, hot and cold ischemia times should be monitored and reported in the pathologist's report. This would allow to improve the quality of histological preparations, but above all to obtain a greater reproducibility of the results, especially those of immunohistochemistry and molecular biology. Currently, Italian BU only rarely monitor hot and cold ischemia. Greater attention is given to checking the fixation time, and in almost all cases the fresh surgical sample is sectioned as soon as it arrives in the laboratory, to allow better fixation [27].

Assessment of the surgical margins after breast conservation surgery (BCS) was usually carried out after orientation of the specimen by means of orientation landmarks, and in only 6% of cases by inking in the operating room by the surgeon, which allows the pathologist to have more precise limits between the various margins of excision. Particularly during oncoplastic procedure this might be relevant, as it allows to limit the anterior (subcutaneous) from the other margins, and we believe that this simple method might be of benefit for a more precise assessment by the pathologist.

An X-ray in two projections allows you to have the security of having removed the lesion, especially in small, non-palpable lesions, or in case of microcalcifications, also having an assessment on the margins of exeresis.

For the identification and evaluation of the size of neoplastic lesions, the use of histological macro-sections and radiological grids can help the pathologist. Macro-sections in breast pathology are useful to a proper definition of stage disease, tumor size, multifocality and resection margins. The presence and extension of in situ carcinoma is more completely observed, overcoming the difficulties of gross inspection at naked eye. In addition, they play an important role in treated breast (post neo-adjuvant chemotherapy), as they are useful to better evaluate the residual cancer burden and the degree of tumor regression [28].

There are several limitations of our study. First, this is a retrospective survey based on answers given by the pathologists of Italian BU, and not an investigation of analytic data obtained and confirmed by chart examination or institutional review. Second, only a minority of BU in Italy participated to the survey, and we do not know if the data presented here are indeed representative of all BU actually present in Italy.

Conclusions

Although national and international guidelines state specific requirements for handling, and processing of breast cancer specimens, our study indicates that several specific flaws are still present in the clinical setting. This may have a profound impact on the subsequent pathology evaluation regarding resection margins, prognostic and predictive factors, including genomic evaluation for assessment of oncological therapies.

Knowledge of these problems underlies the importance of improving our organization in this regard.

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CRediT authorship contribution statement

Leopoldo Costarelli: Data curation, Conceptualization, Formal analysis, Study concepts, Study design, Data analysis and interpretation, Manuscript editing. **Antonio Rizzo:** Formal analysis, Statistical analysis. **Bortul Marina:** Manuscript review. **Francesca Pietribiasi:** Data curation, Data acquisition. **Mario Taffurelli:** Manuscript review. **Corrado Tinterri:** Data curation, Formal analysis, Funding acquisition, Data acquisition, Statistical analysis. **Burlizzi Stefano:** Manuscript review. **Lavinia Bargiacchi:** Data curation, Formal analysis, Funding acquisition, Data acquisition, Quality control of data and algorithms, Data analysis and interpretation. **Lucio Fortunato:** Data curation, Conceptualization, Formal analysis, Study concepts, Study design, Data analysis and interpretation, Manuscript preparation.

Declaration of competing interest

The authors have no conflicts of interest in regard to the content of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejso.2020.08.029.

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