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Evaluation of One Step Nucleic Acid Amplification for detection of lymph node metastases compared to histopathological ultrastaging in women with endometrial cancer: a protocol for a diagnostic accuracy study

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Abstract

Background This is a multicentre, European, prospective trial evaluating the diagnostic accuracy of One Step Nucleic Acid Amplification (OSNA) compared to sentinel lymph nodes histopathological ultrastaging in endometrial cancer patients.

Methods Centres with expertise in sentinel lymph node mapping in endometrial cancer patients in Europe will be invited to participate in the study. Participating units will be trained on the correct usage of the OSNA RD-210 analyser and nucleic acid amplification reagent kit LYNOAMP CK19 E for rapid detection of metastatic nodal involvement, based on the cytokeratin 19 (CK19) mRNA detection. Endometrial cancer patients ≥ 18 years listed for surgical treatment with sentinel lymph node mapping, with no history of other types of cancer and who provide a valid written consent will be considered potentially eligible for the study. However, they will only be enrolled if a successful sentinel lymph node mapping is retrieved. Each node will be processed according to the study protocol and assessed by both OSNA and ultrastaging.

Discussion The accuracy of OSNA (index test) will be assessed against sentinel lymph node histopathological ultrastaging (reference test). This European study has the potential to be the largest study on the use of OSNA in endometrial cancer to date. OSNA could represent a modern diagnostic alternative to sentinel lymph node ultrastaging with the added benefits of standardisation and fast results.

Trial registration The study was registered in the German Clinical Trial Register – Nr. DRKS00021520, registration date 25th of May 2020, URL of the trial registry record: https://drks.de/search/en/trial/DRKS00021520.

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Keywords OSNA, Endometrial cancer, Sentinel lymph nodes, Ultrastaging

Background

Endometrial cancer (EC) is the commonest gynaecological cancer worldwide with more than 417,000 new cases diagnosed in 2020, this incidence seems to be rising [1]. Increase in the population life span and obesity, particularly in higher income countries, are definite contributing factors to this. Nonetheless, nulliparity, tamoxifen use, Lynch syndrome, early age at menarche and late menopause play an important role [2]. In an attempt to curtail this globally growing EC "epidemic", new management strategies and therapeutic algorithms are being introduced for integration into clinical practice, such as genetic testing for Lynch syndrome [3] and the EC molecular classification [4].

Therapeutic outcomes in EC are determined by tumour characteristics, its stage, grade, histological subtype, size, the presence of myometrial invasion, involvement of the lower uterine segment, lymphovascular space invasion, molecular classification and lymph nodes status. Indeed, nodal involvement is one of the key prognostic factors affecting survival. Hence, lymphadenectomy is considered an important part of surgical staging. The actual FIGO (International Federation of Gynecology and Obstetrics) staging system classifies EC into four stages. However, the vast majority of cases are diagnosed at an early stage due to their tendency to present early with abnormal vaginal bleeding [5, 6].

In patients with early-stage EC, to avoid overtreatment by undertaking systematic lymphadenectomy for nodal evaluation, a valid alternative is the sentinel lymph node biopsy (SLNB). This technique is gaining more and more acceptance also for intermediate- and high-risk patients [7–9]. The sentinel lymph node (SLN) is the first node in a lymphatic chain of nodes responsible for an organ's lymphatic drainage. For its identification in EC, a tracer is injected, preferably, into the cervix whereby it migrates to the SLN enabling its detection during surgery [10].

Surgical mapping and subsequent histological analysis of sentinel nodes, collectively known as sentinel lymph node mapping (SLNM), in EC is becoming more commonly used in oncology centres [11]. Furthermore, the integration of SLNM within a more tailored treatment approach, seems to have the potential to avoid overtreatment [12]. Despite the lack of standardisation of the ultrastaging protocol amongst pathologists, there is consensus that ultrastaging of SLNs is the gold standard for the detection of low-volume nodal involvement [13].

Although, SLNM can be considered in the management of EC, it still attracts extensive research interest to evaluate its effectiveness as an alternative to systematic lymphadenectomy. Currently, SLNB tends to be performed either alone, in patients where systematic lymphadenectomy is not indicated, e.g., due to their FIGO stage or health condition, or to complement systematic lymphadenectomy as a way of identifying key nodes to be thoroughly analysed by ultrastaging [9, 14, 15].

The new FIGO classification for endometrial cancer published in 2023 includes several new features including a molecular classification, a new FIGO stage IIIC subdivisions for micrometastatic involvement (IIIC1i and IIIC2i) and for macrometastatic involvement (IIIC1ii and IIIC2ii) of the pelvic and paraaortic lymph nodes. This substaging is based on the better prognosis of patients with micrometastatic involvement compared with macrometastatic involvement. Furthermore, the finding of isolated tumour cells (ITC) is not considered metastatic and regarded as pN0(i+). Thus, this new subdivision confirms the importance of using ultrastaging with immunohistochemistry to detect low-volume metastatic involvement in SLNs [16].

For the analysis of SLNs the ultrastaging approach is applied, which is a more thorough pathological evaluation of the nodes. In this technique, the lymph nodes are initially examined by routine hematoxylin and eosin stain (H&E), followed by ultrastaging if the initial H&E assessment is negative. This approach is very burdensome and time-consuming. Nevertheless, it can detect micrometastases or even ITC undiagnosed by regular histology in approximately 15–20% of the cases [17]. Detection of micrometastases is very important since their presence in lymph nodes correlates with a worse prognosis compared to ITC or negative nodes [6]. A lymph node is considered positive when it has macrometastases (>2.0 mm) or micrometastases (>0.2 mm and ≤2.0 mm). In case of clusters of ITC≤0.2 mm or if no tumour cell is present, the node is considered negative [18].

Histopathological ultrastaging is not the only tool currently available to analyse LNs. An alternative innovative in vitro diagnostic test is represented by the One Step Nucleic Acid Amplification (OSNA, Sysmex Corporation, Japan). OSNA has already achieved its credibility in whole LNs analysis in breast, gastric and colorectal cancer [19–21], and in recent years is making a foray into gynaecological cancers as well [22–24]. Compared with ultrastaging, OSNA is a standardised fast molecular test capable of whole LN analysis with results readily available within 20–30 min, hence, it can be applied also intraoperatively.

To our knowledge, this is the largest prospective international multicentre diagnostic accuracy trial focusing on the comparison of precise analysis of sentinel lymph nodes in EC patients by OSNA and ultrastaging. Only

study sites with expertise in sentinel lymph node detection in EC are eligible to join this study, will be equipped with an OSNA analyser RD 210 if necessary and trained on its correct usage. For the purpose of this study, pathological ultrastaging protocol for SLN assessment will be standardised. Participating units will be able to continue with their local protocol for SLN detection, although, our preference would be the use of ICG or combination of two tracers, which is in line with current evidence [25].

It is hypothesised that OSNA has the potential to provide fast, standardised analysis of the whole lymph node with accuracy comparable to histopathological ultrastaging (reference test), thereby expediting patient's further treatment and management.

Methods

Study design

This is a prospective, multicentre, study to investigate the diagnostic accuracy of OSNA in the clinical setting. The objective of this study is to explore and evaluate the role of the OSNA in detecting lymph node metastases in women with endometrial cancer. OSNA is a CE-marked in vitro diagnostic test (CE-IVD) based on cytokeratin 19 (CK19) mRNA detection. It combines an automated gene amplification analyser RD-210 and a nucleic acid amplification reagent kit LYNOAMP. CK19, in this case, serves as a marker of the presence of tumour cells of epithelial origin (metastatic involvement). The reference test used in this study will be the ultrastaging as used for SLNs [17, 26, 27]. The study aims to analyse LNs from at least 300 eligible patients following the scheme shown in Fig. 1.

Study objectives

The focus of this study is to assess the diagnostic performance of OSNA (RD-210/LYNOAMP CK19 E) in comparison to ultrastaging for the detection of lymph node metastases in women with EC in a routine clinical setting (Fig. 1). The primary endpoint is the analysis performed at lymph node level. This will be determined by assessing the following parameters: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive and negative likelihood ratios and the receiver operating characteristic (ROC) curve. Moreover, the overall concordance rate between OSNA (index test) and ultrastaging (reference test) will be calculated, as well as the ability of both tests to stratify specimens into no metastasis, micrometastases or macrometastases.

Then the initial sensitivity evaluation will be repeated taking cluster data into account (i.e. diagnoses of lymph nodes belonging to the same patient might be correlated) using a generalised mixed model with the patient as the random factor.

We will also undertake a secondary analysis stratified by disease stage. Analysis will be undertaken on how OSNA may have influenced patient's care. Data will be collected on adjuvant treatment, disease free survival (DFS) and if recurrences occur during the study period.

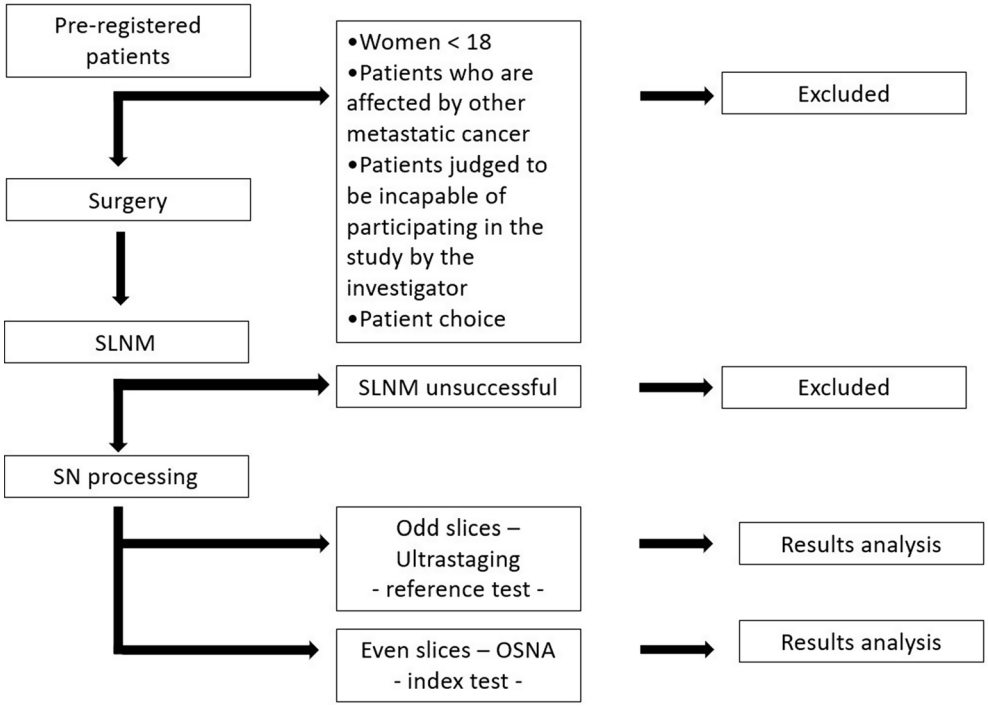

Fig. 1 Study scheme

The clinical management of the patient will rely, solely, on the reference test results (standard practice) and will not be affected by results derived from OSNA. All decisions relating to management, including adjuvant therapies, will be based on the current standard of clinical care at the respective institutions.

The sample size estimation is based on previously published data on the distribution of positive lymph nodes in patients with endometrial cancer [28, 29]. The sample size for estimation of sensitivity and specificity at the nodal level was performed using the two-sided 95% Pearson-Clopper-CI. Clustered data (i.e. multiple positive nodes in the same patient) were taken into consideration.

It was calculated that 53 positive nodes (corresponding to 38 patients) would be required to achieve 90% sensitivity at 95% Pearson-Clopper-CI width of 17%. The same estimation was made for specificity. To achieve a specificity of 98% with a 95% Pearson-Clopper-CI width of 7%, 96 negative lymph nodes would be needed (corresponding to 32 patients).

Based on the above mentioned assumptions, the number of patients needed to obtain the sufficient number of positive lymph nodes is estimated to be 300 patients, of whom 38 patients with at least 1 positive lymph node. Therefore, a total of 60 positive nodes can be expected (Fig. 2). PASS (PASS 15 Power Analysis and Sample Size Software (2017), NCSS, LLC. Kaysville, Utah, USA, nccs.com/software/pass) was used for statistical analysis.

Research ethics approval and data management

The study protocol was approved by the institutional review board and ethical committee at the leading study site (University Hospital in Pilsen, Charles University Prague, Czech Republic) and subsequently approved by all participating sites. The study was registered in the German Clinical Trial Register – Nr. DRKS00021520. The study is being conducted across multiple sites. All patient recruitment and trial data collection is being undertaken in accordance with Good Clinical Practice (GCP). The design, analysis, interpretation of data, drafting, and revisions conform the Helsinki Declaration, the Committee on Publication Ethics (COPE) guidelines (https://publicationethics.org/), the STARD checklist, and SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) statements, available through the EQUATOR (enhancing the quality and transparency of health research) network (www.equator-network.org).

Study population and selection criteria

Inclusion Criteria for the study were set as follows: EC patients ≥ 18 years old listed for surgical treatment with SLNM, with no history of other types of cancer and who provide a valid written consent will be considered potentially eligible for the study. However, they will only be enrolled upon a successful SLNM and SLN retrieval.

SLN detection and processing

The samples included are SLNs from women with EC. The SLNs will be analysed using ultrastaging and OSNA. The detection of sentinel nodes in endometrial cancer patients is still not a fully standardised procedure [6]. For this purpose, any kind of tracer (e.g. radiocolloid, magnetic tracers, indocyanine green - ICG, blue dye or other) can be applied, as routinely performed at the study site according to the institutional guidelines. All commonly used surgical approaches are acceptable – open surgery or minimal invasive techniques including robotic surgery.

SLNs will be carefully dissected from the surrounding adipose tissue and serially sectioned at 2 mm thickness perpendicular to the longest axis of the node. The odd slices will be sent for ultrastaging. The even slices will be shock frozen in liquid nitrogen and stored at – 80°C for further OSNA analysis. SLNs that are 4 mm or less in their shorter axis will be cut into halves with one half attributed to each method (Fig. 3). The laboratory personnel performing the OSNA method will be blinded to the results of the reference test and vice versa.

	endometrial cancer		300	assumption					
stage	proportion	N+ (% within stage*)	number of patients within stage	N+ **patients** within stage	Pat. with 1 pos. node (%)	Pat. with 2 pos. node (%)	Pat. with 3 pos. node (%)	check 100%	pos. **Lymph-nodes**
I	72,0%	3,0%	216	6,5	80%	20%	0%	100%	7,776
II	12,0%	10,0%	36	3,6	70%	30%	0%	100%	4,68
III	13,0%	68,0%	39	26,5	50%	30%	20%	100%	45,084
IV	3,0%	21,0%	9	1,9	40%	40%	20%	100%	3,402
N+				38					60,942
N-				262				rounded value (to the lower value)	60

Fig. 2 The excel sheet used for calculating number of patients necessary to obtain 60 positive lymph nodes

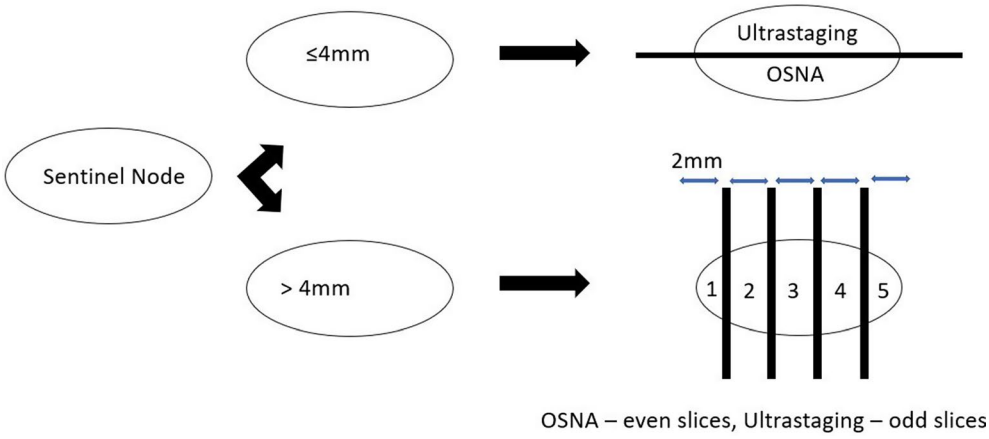

Fig. 3 Sentinel lymph node processing

Specimen collection and storage

Excised lymph nodes will be immediately refrigerated (2 to 8 °C) or stored on ice (0 to 4 °C) without drying to avoid RNA degradation. Sterile biopsy sample containers will be used, tightly fastened to avoid tissue dehydration. Recommended transport time between surgery and laboratory is within 15 min. If OSNA cannot be performed within eight hours after resection, lymph nodes will be stored at -80 °C.

Odd slices undergoing ultrastaging

The odd slices will be fixed in 10% buffered formalin, embedded in paraffin. From each paraffin block, levels at 150 µm interval will be sliced. At each level, three adjacent sections will be cut, and the first section of each level will be stained with H&E. If no tumour can be detected, then the second section will be stained with IHC using the anti-cytokeratin AE1/AE3 antibody. The third is kept as a spare section and can be used to repeat staining if necessary. A lymph node is considered positive when it presents macrometastases (>2.0 mm) or micrometastases (>0.2 mm and ≤2.0 mm). The presence of ITCs (clusters of tumour cells no greater than 0.2 mm) will be documented in the results table, but the node will be considered negative [18].

Even slices undergoing the OSNA analysis

OSNA enables the detection and quantification of CK19 mRNA in surgically removed lymph node(s) lysate, using the automated Gene Amplification Detector RD-210. CK19 serves here as a tumour marker for the detection and quantification of lymph node metastasis (micro/macrometastases) depending on the CK19 mRNA level. Lymph nodes will be homogenised in 4 mL of LYNORHAG lysis buffer using the automated sample pre-treatment system RP-10 (Sysmex Corporation, Kobe, Japan). Primer solution, enzyme solution and sample will be automatically pipetted into the respective detection cell in the reaction block of the RD-210 analyser (Fig. 4). The analysis is performed based on the manufacturer´s protocol. The reaction solution will be mixed, and the reaction block subsequently heated up to the defined reaction temperature (64 °C). The first reverse transcription reaction allows the amplification reaction of target genes (CK19 mRNA), if any, in the sample.

Lymph nodes are defined as 'negative' or 'positive' according to established cut-off values: negative for CK19 mRNA cCP/µL if less than 250, positive for micrometastases (+) if mRNA CK19 levels are 250-4,999 cCP/µL and macrometastases (++) if 5,000 mRNA cCP/µl or more are detected.

Analysis of the primary tumour

The primary tumour will be tested for the expression of CK19 by using IHC (anti-cytokeratin 19 antibody).

Statistical analysis

The diagnostic accuracy of OSNA (index test) will be determined by assessment of the following parameters: sensitivity, specificity, PPV, NPV, positive and negative likelihood ratios, ROC curve. Moreover, the overall concordance rate between the OSNA method and the reference test ultrastaging will be calculated, as well as the ability of both tests to differentiate between no metastasis, micro/macrometastases.

Data and results recording and quality assurance

All submitted patient data will be anonymised. The results of OSNA assay and ultrastaging will be reported on electronic study report form (eSRF). Upon completion of both assays, the recorded data will be reviewed for legibility, completeness, and accuracy. The OSNA results are for clinical performance study only and will not be used for diagnostic or management purposes.

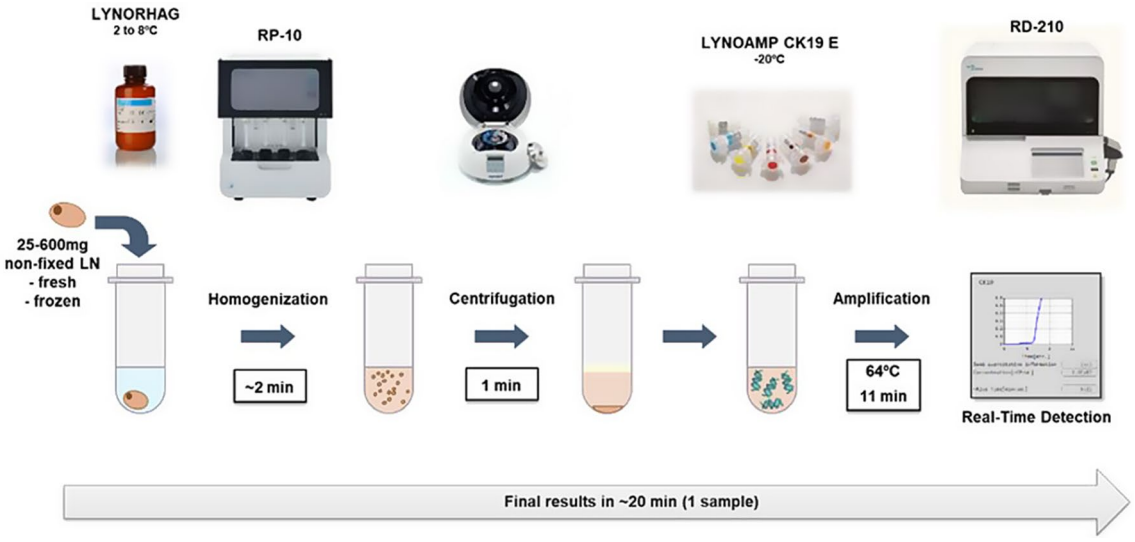

Fig. 4 Sample processing with OSNA

Testing of the samples must be done by a trained person only. Monitoring activities will be performed to verify compliance with the study protocol.

For quality assurance purposes, submission of all specimens (preferably in digital form) and full pathology report (preferably in digital form, original language, and English translation), will be required at least from two patients per centre (randomly selected by the Study Coordinator or Principal Investigator) and reviewed centrally at Sikl's Department of Pathology, University Hospital Pilsen, Charles University, Prague, Czech Republic. Additional cases will be reviewed in case a major discrepancy is found from the protocol.

Analysis

The primary analysis will be performed at the lymph node level. A sensitivity evaluation will be repeated taking the clustered data structure based on the assumption that the diagnosis of lymph nodes belonging to the same patient might be correlated, using a generalised mixed model with patient as the random factor. A second sensitivity analysis for negative nodes will be performed based on whether the patient had other positive nodes or not.

Discussion

The potential of OSNA for the analysis of lymph nodes in endometrial cancer patients was shown in several studies [23, 30–34]. OSNA showed a sensitivity up to 100%, a specificity up to 98.4% and concordance up to 97.1% compared to histological examination. Fanfani et al. compared standard ultrastaging and OSNA in their ability to detect metastatic lymph node involvement and declared a higher detection rate of micrometastases with OSNA [22]. Recently several groups have reported on OSNA use in endometrial cancer management. La Fera et al. analysed 668 nodes by OSNA and ultrastaging. They declared specificity of 98.4%, diagnostic accuracy of 96.7% and so far the lowest published sensitivity of 50% [35]. Togami et al. published a comparison of ultrastaging with OSNA analysis in 133 patients with cervical and endometrial cancers. A total of 437 sentinel nodes were examined with a resulting diagnostic accuracy of 97.9%, sensitivity of 91.8% and specificity of 98.9% [36].

Kostun et al. [23] compared OSNA to ultra-staging and demonstrated that OSNA resulted in more frequent detection of micrometastases and upstaging of more than 20% of patients. However, this study analysed data of 58 patients from a single institution using the automated Gene Amplification Detector RD-100i. For the introduction of OSNA into routine therapeutic strategy of EC a multicentre study with sufficiently large cohort of patients is crucial. For the purpose of this study, it was calculated that in a cohort of 300 patients, 60 positive LNs would be expected. This setting should provide sufficient statistical power for our primary endpoint. To our knowledge this study is the largest cohort of EC patients managed in multiple centres to be examined by OSNA for the detection of metastatic involvement of SLNs.

The reference method used in this study, ultrastaging, is a widely accepted method for examining sentinel nodes to detect low volume disease. Nevertheless, the methodology for performing the technique is yet to be standardised. Indeed, there is currently no widely accepted standardised ultrastaging protocol in place and the approach may vary among different institutions and sites [26]. Fanfani et al. [30] reported the institutional ultrastaging protocol consisting of two adjacent 5 µm sections taken at 200 µm distances from a paraffin block lacking metastatic carcinoma on routine H&E staining. One of the sections is then stained with H&E, while the

other with IHC, using the anti-cytokeratin AE1:AE3 antibody (Ventana Medical Systems, Inc., Tucson, AZ), for the detection of low volume metastatic disease by light microscopy. López-Ruiz et al. [31] described an ultrastaging protocol which differs from the protocol described in Fanfani et al. [27] in section's thickness, where 4 µm instead of 5 µm is used. Furthermore, one section was examined with H&E staining and the other section was processed using IHC for cytokeratin 19 (mouse monoclonal, clone RCK 108, prediluted, Dako, Glostrup, Denmark). Cibula et al. [26] suggested cutting 4 sections at 200 µm intervals through the paraffin block, not specifying their thickness. They proposed that one section would be stained with H&E and if no tumour was detected, the second would be stained using pan-cytokeratin antibody. Two extra unstained sections would be available in case there is a problem with stain or further evaluation of detected tumour is needed.

OSNA could, if successfully introduced into clinical practice, contribute to the standardisation and availability of detailed sentinel node examination in endometrial cancer. This method is already used for lymph node analysis in breast, colorectal and gastric cancer patients [18–20]. In endometrial cancer, OSNA is still considered an experimental method and, to our knowledge, no studies have yet reported on OSNA analysis for whole nodes. However, due to the nature of this method, it is not possible to process the same tissue sample simultaneously using both methods – OSNA and ultrastaging. It is understood that the correct use of the terms 'sensitivity', 'specificity' and 'accuracy' would require the analysis of the identical lymph node with both methods. Sample preparation for OSNA precludes its histopathological examination and vice versa. Since different slices of the same lymph node are analysed using two different approaches, it could theoretically be that metastases are present in one slice but not in the other, giving possible rise to tissue allocation bias. In order to minimize this bias, the SLNs are carefully stripped of surrounding adipose tissue before processing and then cut into 2 mm slices according to the study protocol (nodes up to and including 4 mm are only halved). However, this is still controversial because individual slices, and not the whole node, are always examined by only one of the methods, thus compromising the overall accuracy of the two methods being compared. Therefore, depending on the results of our study, a sufficiently robust multicentre study analysing whole lymph nodes in endometrial cancer by OSNA is the next logical step in the future.

Though this was never proved in previous studies, OSNA results could theoretically be influenced by the presence of benign epithelial inclusions or endosalpingiosis in the nodule sample due to their expression of CK19. However, their frequency in sentinel node samples is reported in the literature to be fairly low (1–2%) and in the case of taking non-sentinel nodes even lower (0.1–0.2%). Their clinical impact can thus be considered negligible [23, 31].

OSNA is building its reputation on intraoperative whole node analysis aiming for precision at ultrastaging level. Moreover, application of OSNA could lead to significant cost saving to health authorities [37, 38]. Recruitment of the study began in autumn 2020 and is still open. We would like to use this work not only to raise awareness on OSNA as a valid alternative diagnostic test, but also to encourage other centres to join the study.

Abbreviations

AE1/AE3	Anti-cytokeratin antibody
CK19	Cytokeratin 19
CE-IVD	The Conformité Européenne - marked in vitro diagnostic test
DFS	Disease free survival
EC	Endometrial cancer
eSRF	Electronic study report form
FIGO	International Federation of Gynecology and Obstetrics
H&E	Hematoxylin and eosin stain
ICG	Indocyanine green
IHC	Immunohistochemistry
ITC	Isolated tumour cells
LYNOAMP	Nucleic acid amplification reagent kit
NPV	Negative predictive value
OSNA	One Step Nucleic Acid Amplification
PPV	Positive predictive value
ROC	Receiver operating characteristic curve
RD 210	OSNA analyser
SLN	Sentinel lymph node
SLNM	Sentinel lymph node mapping
SLNB	Sentinel lymph node biopsy

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Author contributions

Dr Jan Kostun is the major contributor in writing the manuscript. All the other authors have contributed with their expertise and writing corrections.

Funding

This study is supported by a grant from Sysmex Corporation (Kobe, Japan). The Sysmex company had a role in the design of the study.

Data availability

All submitted patient data will be anonymised. The results of OSNA assay and ultrastaging will be reported on electronic study report form (eSRF) in the REDCap database (Research Electronic Data Capture, Vanderbilt University, Tennessee, USA) and available upon approval (contact person: Jan Kostun, Department of Gynaecology and Obstetrics, University Hospital Pilsen, Charles University, Czech Republic, email: kostunj@fnplzen.cz).

Declarations

Ethical approval and consent to participate

The study protocol was approved by the ethical committee at the leading study site: The Local Ethical Committee of the University Hospital in Pilsen

and the Medical Faculty in Pilsen, Charles University in Prague, Czech Republic and subsequently approved by all participating sites. The study was registered in the German Clinical Trial Register – Nr. DRKS00021520. The study is being conducted across multiple sites. All patient recruitment and trial data collection is being undertaken in accordance with Good Clinical Practice (GCP). The design, analysis, interpretation of data, drafting, and revisions conform the Helsinki Declaration, the Committee on Publication Ethics (COPE) guidelines (https://publicationethics.org/), the STARD checklist, available through the EQUATOR (enhancing the quality and transparency of health research) network (www.equator-network.org). Only endometrial cancer patients ≥ 18 years listed for surgical treatment with sentinel lymph node mapping, with no history of other types of cancer and who provide a valid written informed consent (obtained from participants and/or their legal guardians) will be considered potentially eligible for the study.

Consent for publication

Not applicable- as the manuscript does not contain any identifiable information or images of study participants.

Competing interests

The co-author and study coordinator of this project, Alessandra Mescalchin, is an employee of Life Science, Sysmex Europe SE, Germany, the EMEA regional headquarter of the funder, Sysmex Corporation (Kobe, Japan) that had a role in the design of the study. Therefore she has a competing interest.All other authors declare no competing interests.

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