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Tissue microarray validation in cervical carcinoma studies. A methodological approach

Lucília Lovane[1,3,4], Carla Carrilho[3,4] and Christina Karlsson[2]

Schools of [1]Medical and [2]Health Sciences, Faculty of Medicine and Health, Örebro University, Örebro, Sweden, [3]Maputo Central Hospital and [4]Faculty of Medicine, Eduardo Mondlane University, Maputo, Mozambique

Summary. Tissue microarrays (TMAs) are a cost-effective tool to study biomarkers in clinical research. Cervical cancer (CC) is one of the most prevalent in women worldwide, with the highest prevalence in low-middle-income countries due to a lack of organized screening. CC is associated with persistent high-risk human papillomavirus infection. Several biomarkers have been studied for diagnostic, therapeutic, and prognostic purposes. We aimed to evaluate and validate the effectiveness of TMA in CC compared to whole slide images (WSs).

We selected and anonymized twenty cases of CC. P16, cytokeratin 5 (CK5), cytokeratin 7 (CK7), programmed death-ligand 1 (PD-L1), and CD8 expression were immunohistochemically investigated. All WS were scanned and 10 representative virtual TMA cores with 0.6 mm diameter per sample were selected. Ten random combinations of 1-5 cylinders per case were assessed for each biomarker. The agreement of scoring between TMA and WS was evaluated by kappa statistics. We found that three cores of 0.6 mm on TMA can accurately represent WS in our setting. The Kappa value between TMA and WS varied from 1 for p16 to 0.61 for PD-L1. Our study presents an approach to address TMA sampling that could be generalized to TMA-based research, regardless of the tissue and biomarkers of interest.

Key words: Cervical cancer, Tissue microarray, Immunohistochemistry, Heterogeneity, Validation

Introduction

In 1986, Battifora introduced the multitumour "sausage" tissue block, in which hundreds of tissue samples were embedded in a normal-sized paraffin block (Battifora, 1986). This block allowed simultaneous immunohistochemistry testing, although in this technique the tissues had no defined orientation. In 1998, Kononen et al. evolved the concept and developed tissue microarray (TMA) technology, which uses a different sampling strategy and provides a higher level of sophistication by using tissue cores of uniform size and shape that are arrayed with a precision instrument at high density in a recipient block (Kononen et al., 1998; Nocito et al., 2001).

Thus, the TMA is a collection of tissue comprising small tissue cylinders presented on a glass slide in a grid layout, which have tens to hundreds of samples from different tissues or patient tumours, of the same or different organs. It is a form of condensed histopathology where cells or tissues are presented in a miniature multiplex platform that allows the generalisation of the findings to a large number of samples (Hewitt, 2006). There are several advantages to using this technique: TMA can accelerate *in situ* studies of tissue specimens, enables exploring the association between molecular changes for simultaneous and/or consecutive tissue specimens for molecular markers using *in situ* techniques at the DNA, RNA, or protein level (Kononen et al., 1998; Nocito et al., 2001; Hewitt, 2006) and, in such settings, with clinicopathologic information. Furthermore, the TMA technique ensures the preservation of the original tissue block for further diagnostic or research purposes. The TMA has been developed as a research tool for high-throughput molecular characterization and it is not primarily a

Corresponding Author: Lucília Trindade Lovane Matias, Department of Pathology Department, Hospital Central de Maputo, Avenida Agostinho Neto, 164, Maputo, CEP1100, Mozambique. e-mail: Lucilia.Matias@oru.se

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Abbreviations. ADCA, Adenocarcinoma; AOI, Area of interest; CC, Cervical cancer; CK 5, Cytokeratin 5; CK 7, Cytokeratin 7; CPS, Combined positive score; DNA, Desoxyribonucleic acid; EDCTP, European & Developing Countries Clinical Trials Partnership; GDPR, General data protection regulation; H&E, Haematoxylin and Eosin; HIV, Human Immunodeficiency Virus; HPV, Human papillomavirus; OUH, Orebro University Hospital; PD-L1, Programmed death ligand 1; RNA, Ribonucleic acid; SCC, Squamous cell carcinoma; SIDA, Swedish International Development Cooperation Agency; TMA, Tissue microarray; TPS, Tumour proportion score; UEM, Universidade Eduardo Mondlane; WS, Whole slide image.

technique involved in the management of individual patients (Boone et al., 2008; Karlsson et al., 2009; Simon, 2010; Franco et al., 2011). In the research setting, the method is very cost-effective (Schneider, 2006; Karlsson et al., 2009; Arafa et al., 2010; El-Mansi et al., 2006).

For TMA construction, Haematoxylin and Eosin (H&E) stained slides are assessed to select the area that represents the specimen's so-called area of interest (AOI). The sampling strategy may vary according to the needs of each specific study and depending on several factors, including tissue of origin and specific biomarkers. In cancer research, the major concern is tumour heterogeneity (Botti et al., 2016). Basically, the sampling strategy can be influenced by the diameter and number of cores included from the AOI from each case.

Sampling equipment uses punches from 0.6 to 5 mm in diameter, the larger mainly for manual TMA construction. Within a given area, this obviously affects the number of punches and, indirectly, the number of cases included in each TMA. For example, using a 0.6 mm punch strategy of sampling, the number of cases within a given area in the recipient block, increases almost three-fold compared with a 1.0 mm strategy. Studies on different types of tumours have concluded that the acquisition of multiple small tissue cores from different regions of the tumoral lesion is more adequate than increasing the punch diameter (Fons et al., 2007; Boone et al., 2008; Karlsson et al., 2009). Based on these considerations, a 0.6 mm diameter sample size is preferable, moreover having the advantage of allowing more cases within the same block, to achieve increased cost-effectiveness.

However, the number of cores that are needed to accurately represent the whole section for each biomarker may vary, therefore, before starting a TMA-based project, each biomarker needs to be validated according to the tumour type, regarding the number of cores to be representative for the specific biomarker (Nocito et al., 2001). In terms of number of cores, it is thought that a range varying from one to four cores is sufficient for analysis. This produces a representative sampling of the morphology, although this is not the consensus of all researchers (El-Mansi and Williams, 2006; Karlsson et al., 2009; Munari et al., 2017).

In an initial literature search, consisting of about 100 published articles using TMA in cervical neoplasia, the information about the performance of specific biomarkers in TMA from cervical carcinomas (CCs) compared to whole slide (WS)-based investigations was scarce (Schneider, 2006; El-Mansi and Williams., 2006; Iakovlev et al., 2007; Lesnikova et al., 2009; Choschzick et al., 2012).

In the near future, we will have a research interest in invasive CC and biomarkers of interest for the study of pathogenetic markers, with special reference to human papillomavirus (HPV)-related carcinogenesis in a Human immunodeficiency virus (HIV)-endemic milieu.

Thus, in this study, we aimed to determine the value of TMA and respective number of cores to evaluate several biomarkers in paraffin blocks of invasive CC [Cytokeratin's 5 and 7 (CKs 5 and 7), p16, programmed death-ligand 1 (PD-L1) and CD8] compared with whole slide (WS) image.

Materials and methods

Samples

Twenty cases of invasive CC were selected from the biobank at Örebro University Hospital (OUH) for the phantom TMA study. The tumours selected included 11 (55%) squamous cell carcinomas (SCC) and 9 (45%) adenocarcinomas (ADCA). New H&E sections were performed, and the cases were re-examined for morphological diagnosis by an experienced pathologist (LL).

The block that contained the largest tumour area was selected from each case.

This study protocol and consent procedure was approved by the Regional Ethical Review Board in Uppsala, which is part of the Swedish Ethical Review Authority, approval number [2008:122] with amendment 2015-06-03.

Tissue microarray phantom construction

The selection of the tumour area was performed on H&E-stained slides by a pathologist (LL). In this selection, non-representative areas, such as normal or necrotic tissue, were carefully avoided. TMA phantoms were generated within the software (0.6 mm in diameter) from the scanner supplier and scored according to established evaluation criteria. Ten different representative tumour areas were selected per sample from both the centre and the periphery of the tumour, as illustrated in Figure 1. Thus, for each biomarker, 200 virtual TMA cores, 10 per case, were scored.

Immunohistochemical staining

Immunohistochemical (IHC) staining for CD8, CK5, and CK7 was performed on DAKO OMNIS (Agilent, Santa Clara, California), with a polymer-based detection system. Positive and negative control tissues were included in each run. CINtec p16 and PD-L1 SP263 were performed on Benchmark Ultra (Roche Diagnostics, Switzerland) according to the manufacturer's instructions, which, in the case of SP263, included an isotype-specific negative control. All series included positive tissue controls. The primary antibodies, clones, and dilutions used are described in Table 1.

Digital Pathology for scanning and evaluation

After staining, all slides were scanned at 40x brightfield on a Pannoramic 250 automatic digital

scanner (3D HISTECH Ltd., Budapest, Hungary) at the Department of Clinical Research of OUH and stored within a storage system at a server facility from the same supplier, fulfilling the general data protection regulation (GDPR). The scanned material was assessed and used for evaluation through the Pannoramic Case Viewer software version 2.4.0.119028 for Microsoft Windows.

Table 1. Monoclonal antibodies, clones, dilutions, and manufacturers of the markers used in this study.

Antigen	Clone	Dilution	Manufacturer
P16	E6H4	RTU	Ventana Roche
PD-L1	SP263	RTU	Ventana Roche
CD8	C8/144B	RTU	DAKO/ Agilent
CK5	XU26	1/100	Novocastra / Leica
CK7	OV-TL	RTU	DAKO / Agilent

Scoring

Each core was coded, and IHC scoring for each marker was performed on the digitized slides by two different investigators (LL and CK). Disagreements between the investigators were resolved through discussion.

For p16, both cytoplasmatic and nuclear staining were required. Depending on the distribution of the stain in the epithelial cells, positivity was scored on a four-grade scale as strong diffuse intensity (3+), moderate intensity (2+), weak sporadic or mild intensity (1+), or no visible staining (0). Depending on the percentage of

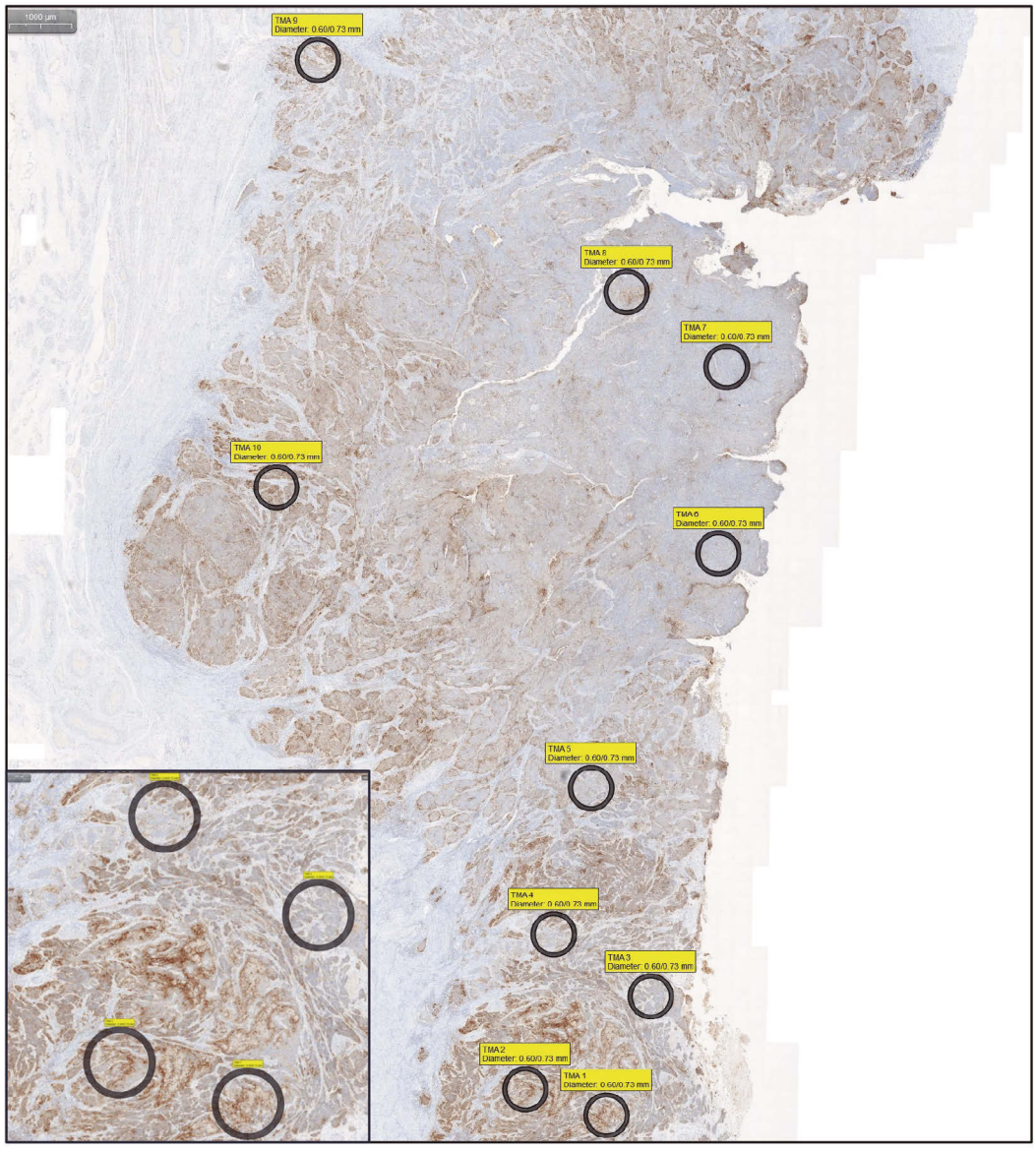

Fig. 1. Illustration of how the cores for the Phantom TMA were constructed with a close-up inserted (1.1x/5.3x magnification).

positive cells, staining was graded in a semi-quantitative manner, as Score 0: negative cells; Score 1: less than 10% of positive cells; Score 2: 10% to 50%; Score 3: > 50% (Krishnappa et al., 2014).

The expression of cytokeratins 5 and 7 was considered positive when observed as membranous staining and/or cytoplasmic. The cells were scored as negative (no staining or less than 1% of positive cells); score 1: patchy, 1% to 40% positive cells; score 2: strong diffuse staining, > 40% of positive cells (Lee et al., 2017).

PD-L1 was scored according to the Ventana algorithm for PD-L1 SP263(VENTANA, 2017). According to the interpretation guide when using the SP263 clone, epithelial cancer cells are considered positive when partial or complete circumferential staining is present on the cellular membrane. The tumour proportion score (TPS) was further categorized into two groups: high/positive when ≥ 25% of tumour cells exhibit membrane staining and low/negative when < 25% are stained (VENTANA, 2017; Zajac et al., 2019).

The combined positive score (CPS) was also assessed using a cutoff ≥ 1, as it has already been demonstrated to have almost comparable performance to the 22C3 clone (Park et al., 2020).

The scoring of CD8 was semi-quantitative; cells were considered positive when there was staining of the membrane and the cytoplasm. The cores were graded into five scores as follows: score 0: no CD8+ cell staining; score 1 (low density): 1-2 CD8+ stained cells per core; score 2 (moderate density): 3-15 CD8+ stained cells per core; Score 3 (high density): more than 15 countable CD8+ stained cells per core; or score 4 (extreme density): uncountable CD8+ stained cells per core (Enwere et al., 2017).

Statistical analysis

Ten virtually placed cores for each case and marker were evaluated. Ten unique random series were generated using Microsoft Excel (office 365). For the obtained series, we compared combinations of one, two, three, four, and five cores against the WS results.

The agreement between TMA and WS was estimated using Kappa statistics. Results are shown as value and 95% confidence intervals (95% CI).

To address the influence of a possible hot-spot pattern of staining, two approaches to statistics were used, either comparing the agreement of the mean value of TMA observations with WS or the highest value of the observations *versus* WS, the "hot-spot"-method.

The Kappa is interpreted as 0: when there is no agreement at all; 1: 0.10-0.20 slight agreement; 2: 0.21-0.40 fair agreement; 3: 0.41-0.60 moderate agreement; 4: 0.61-0.80- substantial agreement; 5: 0.81-0.99 near perfect agreement and 6: 1.0 perfect agreement.

Statistical analyses were performed using GraphPad Prism software (online version, date 2022-09-25) where kappa with a 95% confidence interval as well as the percentage of agreement were calculated.

Literature search

To find recent studies regarding the evaluation of the TMA technique in human tissue, we performed a quick PubMed search. The search comprised the following search terms: analysis, tissue array, analyses, humans, immunohistochemistry. Only free full-text journals at Örebro University Library were included in the search, the language was set as English, and the search was restricted to 2019-2020.

Results

The IHC pattern of expression of all the biomarkers used in the study is shown in Figure 2.

For p16, in TMA as well as in WS, positivity was observed in 19 cases (95%), and only one case was negative (5%). The negative case was ADCA. The concordance between TMA and WS was complete (100%) in all combinations studied.

CK7 was positive in TMA and WS in 14 cases (70%) and negative in three cases (15%). There were, however, three cases with heterogeneous patterns of expression, which had positive cells in focal areas with less than 10% or focal single cells in the TMA and the overall scores in WS were classified as negative, focal, and positive, respectively. As shown in Figure 3, kappa values indicated substantial to almost perfect agreement with ≥ 90% actual agreement *versus* WS for all numbers of cores studied.

CK5 was generally positive in both TMA and WS in nine cases (45%) and negative in five cases (25%). The remaining six cases showed heterogeneous patterns of expression in TMA; in WS, three were classified as positive and three had focal staining patterns (score = 1). In the case of CK5, the "hot-spot" method yielded slightly better kappa values and agreement, however, with overlapping confidence intervals. Data for the hot-spot method is shown in Figure 4.

For PD-L1, both TPS and CPS were applied. Applying TPS, eight cases (40%) were completely negative or had one core with less than 1% of stained cells, and two cases were positive. In 10 cases (50%), the pattern of expression was heterogeneous, with different percentages of positive tumour cells or negative TMAs. Some cases were considered positive with TMA, however, the overall WS score was negative because the percentage of positive tumoral cells was < 25%.

The agreement as well as the Kappa statistics between TMA and WS for PD-L1 using the "mean method" is shown in Figure 5, i.e., when assessing three cores, the kappa value was 0.61 (95% CI: 0.48-0.74) with an agreement of 87%; using the "hot-spot" method, almost the same values were found (data not shown).

Concerning CPS with WS, 13 out of the 20 cases scored CPS ≥ 1. TMA scoring on 2 to 5 cores with mean

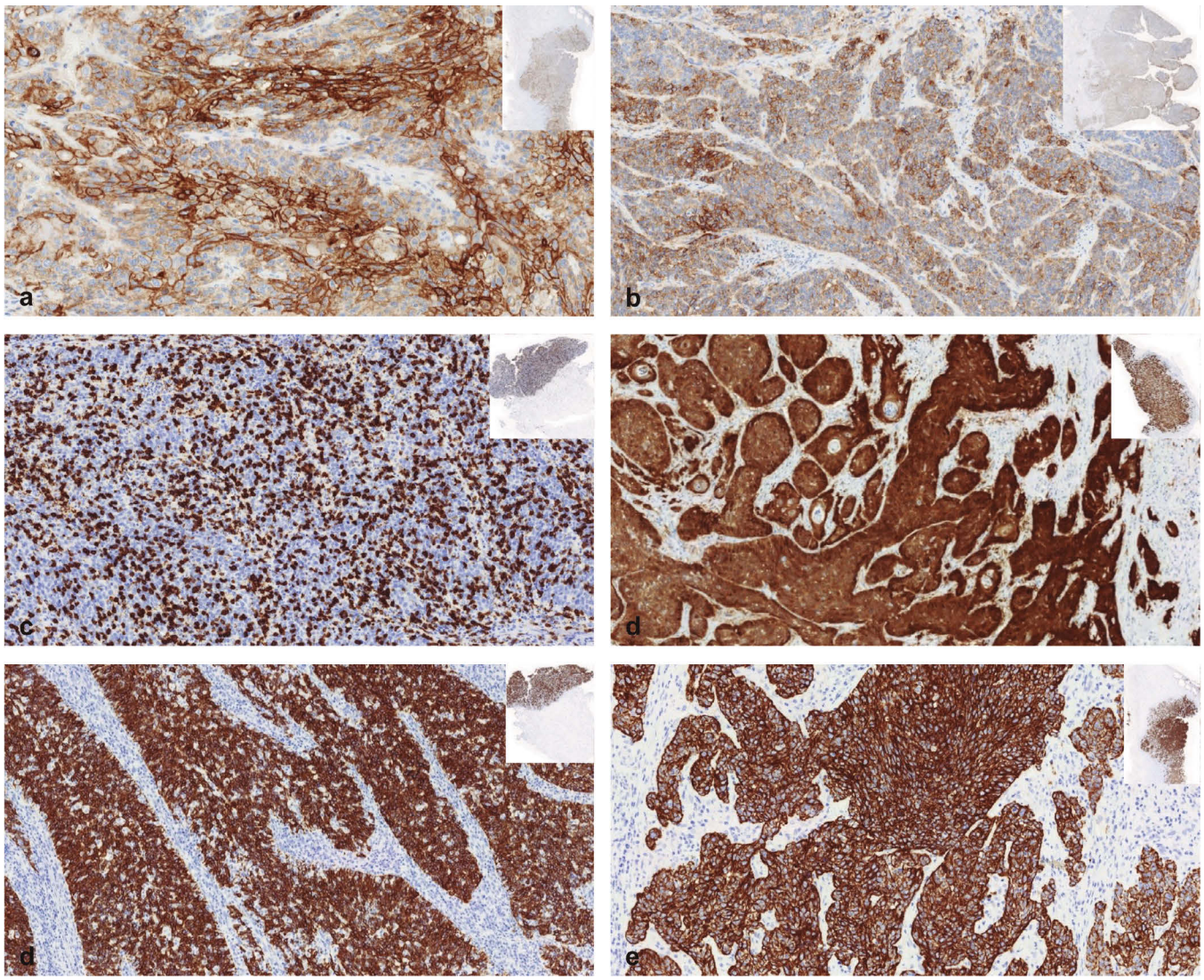

Fig. 2. Patterns of expression of PD-L1, CD8, p16 and cytokeratins 5 and 7 in squamous cell carcinomas. Membrane expression of PD-L1, high positive in >25% of tumour cells (**a**; 47.8x and **b**; 35.9x); cytoplasmatic and membrane positivity of CD8 lymphocytes 31.9x **(c)**; cytoplasmatic and nuclear strong positivity for P16 in tumour cells 22.2x **(d)**; cytoplasmatic and membrane positivity for CK5 in tumour cells 26.2x **(e)**; cytoplasmatic and membrane positivity for CK7 in tumour cells 44.2x **(f)**. The whole slide is inserted for each marker.

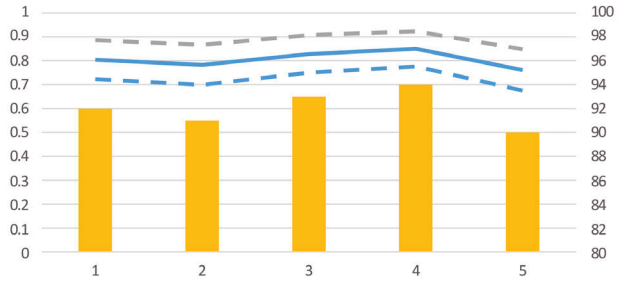

Fig. 3. CK7 data concerning the number of cores (x-axis) evaluated with the "mean method", on the left y-axis; kappa value and the right y-axis; agreement between whole-section scoring and TMA evaluation with the %. Line and dotted lines; kappa value with 95% confidence interval, bars; agreement.

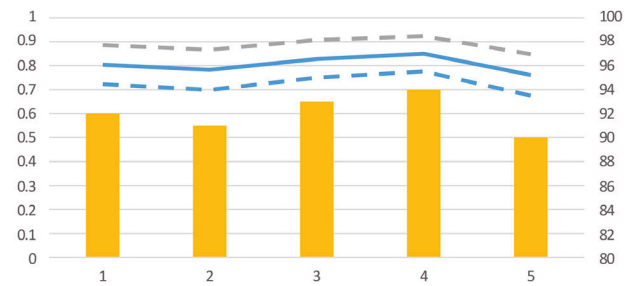

Fig. 4. CK5 data concerning the number of cores (x-axis) evaluated with the "hot-spot method", on the left y-axis; kappa value and the right y-axis; agreement between whole-section and TMA evaluation with the %. Line and dotted lines; kappa value with 95% confidence interval, bars; agreement.

and hot-spot methods showed an agreement ranging from 82.0-86.5% to 83.5-86.5%, respectively. Kappa values ranged from 0.634 (95% CI 0.521-0.747) to 0.717 (95% CI 0.619-0.815) applying the mean method, and 0.634 (95% CI: 0.521-0.747) to 0.727(95% CI 0.626-0.829) applying the "hot-spot" method. The best kappa value was achieved using the "hot-spot" method on three cores.

Data for CD8 is presented in the same manner in Figure 6. For CD8, a good agreement was achieved with three or more cores, even though the kappa values indicated only a moderate agreement.

The literature search generated 127 articles, as seen in Figure 7; of these, five did not use TMAs in their studies. Of the 122 remaining papers, 67 stated the number of cores used and 64 the diameter. Only four articles had references about evaluation in the same tissue as in the present study.

Discussion

In our present study, we present a simple and robust approach to address the question of representativity for a selection of biomarkers using a CC study as a model.

A broad range of TMA-based studies thus contribute to state-of-the-art medical practices in many settings, mainly regarding cancer biomarkers. However, an observation is that, for various studies using TMA techniques, limited or no evaluation regarding sample size or core diameter and/or core numbers was performed before the studies, i.e., we only found four articles in our literature search (Schneider, 2006; El-Mansi and Williams, 2006; Iakovlev et al., 2007; Choschzick et al., 2012) that addressed the TMA sampling procedure in the context of cervical neoplasia and representativity for the studied biomarker. Thus, a model that can be generalised for different biomarkers, as well as different tissue materials, is urgently needed to ensure the quality of the very cost-effective TMA technique for biomarker studies, especially in the context of potential therapeutic implications.

Since the construction and determination of sample size in TMA is crucial in many aspects, general representativity, hot-spot dependent biomarkers, tumour heterogeneity, and established biomarker cut-off limits must be considered. The need for cost-effectiveness in large cohort studies, especially considering research in resource-limited settings, must also be taken into account when deciding upon the preliminary sampling protocol. Safeguarding the putative future needs of the individual patient contributing to the TMA cohort is also of the utmost importance.

As shown in previous studies (Karlsson et al., 2009; Sauter, 2010; Jones and Prasad, 2012; Oshiro et al., 2016) performed in several different tissues, our data supports that the use of 0.6 mm punches is reliable in

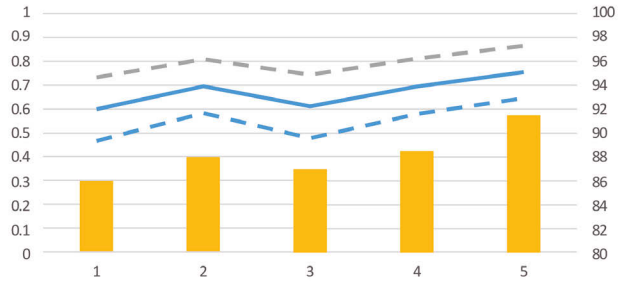

Fig. 5. PD-L1 data concerning the number of cores (x-axis) evaluated with the "mean method", on the left y-axis; kappa value and the right y-axis; agreement between whole-section scoring and TMA evaluation with the %. Line and dotted lines; kappa value with 95% confidence interval, bars; agreement.

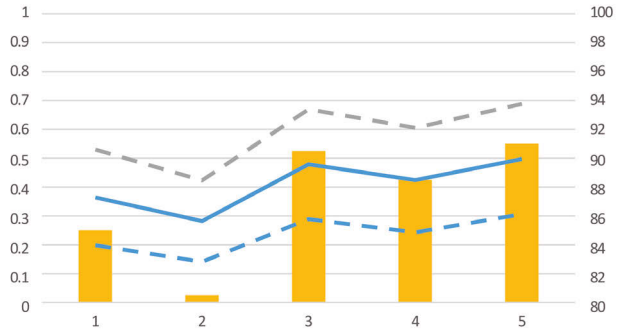

Fig. 6. CD8 data concerning the number of cores (x-axis) evaluated with the "mean method", on the left y-axis; kappa value and the right y-axis; agreement between whole-section scoring and TMA evaluation with the %. Line and dotted lines; kappa value with 95% confidence interval, bars; agreement.

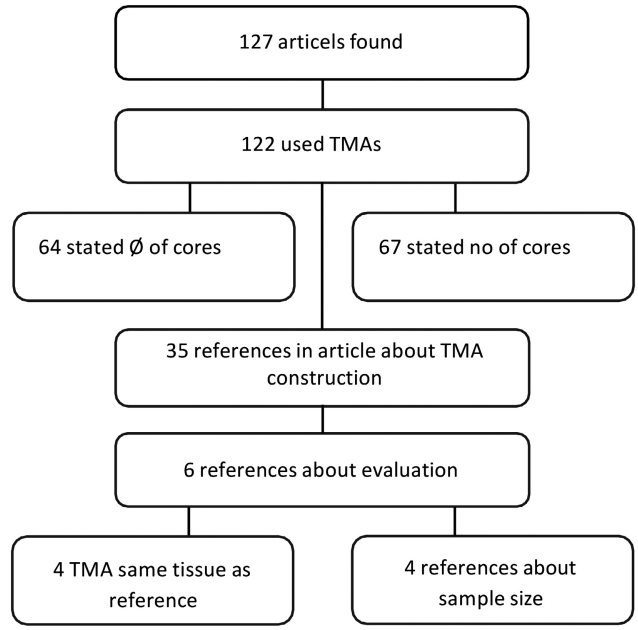

Fig. 7. Flow chart describing the results of the literature search.

evaluating IHC tests. Increasing the diameter of each single punch does not seem to contribute to the representativity of the "microenvironment" within the tissue. From these findings, the present study concentrated on the number of cores, in our context of invasive CC, striving to develop and imply a statistical approach that could be generalised.

Concerning the number of cores, previous results have been achieved in different tissue and/or biomarker contexts, mainly associated with proliferation and therapeutic biomarkers. In breast cancer, studies favour that three or four cores of 0.6 mm can accurately represent the biomarker pattern of WS (Camp et al., 2000; Hoos and Cordon-Cardo, 2001; Torhorst et al., 2001; Fons et al., 2007; Boone et al., 2008; Karlsson et al., 2009), as well as in lung and endometrial carcinomas (Karlsson et al., 2009), while other studies on breast and bladder cancer state that replicate cores are adequate (Kyndi et al., 2008; Eskaros et al., 2017).

The PD-L1 staining pattern can be focal or heterogeneous, which can lead to an underrepresentation in the TMA (Botti et al., 2016). Different evaluation models, TPS and CPS using cut-off levels of 1-50% in different tumour settings make it troublesome to evaluate by TMA (Botti et al., 2016; Park et al., 2020; de Ruiter et al., 2021).

In non-small cell lung carcinoma, PD-L1 was scored for triplicate TMA cores as positive when at least one of the three cores was positive (Gagné et al., 2018), whilst Munari et al. concluded that the heterogeneity of PD-L1 expression can be overcome by using four cores of 0.6 mm to accurately classify PD-L1. Furthermore, they observed a discordance rate of 20% and 7.9% when using > 1% and > 50% cut-offs for PD-L1 scoring algorithms (Munari et al., 2017).

In colorectal tumours, using multiple TMA cores can accurately address the heterogeneous expression of PD-L1 (Lee et al., 2016). Ye et al. in a study of PD-L1 heterogeneity in gastric cancer, showed that five cores were accurately representative of WS when applying a 1% cut-off level, with an almost perfect Kappa agreement of 0.8. They also showed that an increase in the number of cores reduces the specificity from 95% with less than four cores to 89% with six cores (Ye et al., 2020), highlighting an interesting aspect of overinterpretation of hot-spot staining patterns in TMA with extensive sampling. A similar observation could be made in our dataset, where the kappa value of the hot-spot method declined with more than three cores. Thus, the evaluation model specific for biomarkers with known heterogeneity/hot-spot patterns is complex. Concerning our findings, the proposed model of a statistical approach to TMA could easily be adopted in both the hot-spot and mean expression models of evaluation since likely different biomarkers need different evaluation models, even within the same study protocol.

Regarding the limitations of the present study, it only addresses a limited number of biomarkers within the two major carcinoma types at a specific anatomical location. In our opinion, there is no general answer for all tumour or tissue types or all biomarkers. The goal of the present study was, however, to present an approach that can be generalised. In particular, the use of digital sampling techniques makes it cost and tissue-effective to apply for any biomarker and tissue within a study-specific setting.

Histopathology has been proven to be a most powerful prognostic instrument in medicine since the 19th century (Masic, 2019), we must still be humble about the fact when discussing the different core sizes and numbers needed to determine new biomarkers within a tumour of only e.g., 1 cm in size, we are still only looking at <0.05% of the tumour volume, regardless of the approach (Sauter, 2010). However, we present an approach to describe the accuracy of the TMA sampling procedure, at least compared with WS evaluation, which has been the gold standard of histopathology since the early days of Virchow.

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Conflict of Interest Statement. The authors declare no competing interests concerning the research, authorship, and publication of this article.

Author Contributions. LL conceptualized and designed the study, performed data acquisition, revised the morphological diagnosis, interpreted whole slides and TMA phantom immunohistochemistry, and drafted the manuscript. CC conceptualized and designed the study, revised the morphological diagnosis, interpreted TMA phantom immunohistochemistry, and critically revised the manuscript. CK conceptualized and designed the study, selected cases for the study, performed data acquisition and analysis, interpreted whole slides and TMA phantom immunohistochemistry, and critically revised the manuscript. All authors have read and approved the final manuscript.

Data Availability Statement. All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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