# SOX10 is as specific as S100 protein in detecting metastases of melanoma in lymph nodes and is recommended for sentinel lymph node assessment

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Title page

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**Authors:** Anna Szumera-Ciećkiewicz[1,2] (ASC), Francesca Bosisio[3] (FB), Paweł Teterycz[4] (PT), Asier Antoranz[3] (AA), Francesco Delogu[5] (FD), Senada Koljenović[6] (SK), Bart A. van de Wiel[7] (BAW), Willeke Blokx[8] (WB), Léon C. van Kempen[9] (LVK), Piotr Rutkowski[4] (PR), Alexander Christopher van Akkooi[10] (ACA), Martin Cook[11] (MC), Daniela Massi[12] (DM), EORTC Melanoma Group.

**Affiliations:**

1. Department of Pathology and Laboratory Diagnostics, Maria Sklodowska-Curie National Research Institute of Oncology**,** Warsaw, Poland.
2. Department of Diagnostic Hematology, Institute of Hematology and Transfusion Medicine; Warsaw, Poland.
3. Laboratory of Translational Cell and Tissue Research and Pathology Department, KU Leuven and UZ Leuven, Leuven, Belgium.
4. Department of Soft Tissue/Bone Sarcoma and Melanoma, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland.
5. Department of Health Sciences, Clinical Pharmacology and Oncology Unit, University of Florence, Florence, Italy.
6. Department of Pathology, Erasmus MC, University Medical Centre Rotterdam, the Netherlands.
7. Department of Pathology, The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands.
8. Department of Pathology, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center, Utrecht, the Netherlands.
9. Department of Pathology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands.
10. Department of Surgical Oncology, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, the Netherlands.

11. Histopathology, Royal Surrey County Hospital, Guildford, UK.
12. Section of Pathological Anatomy, Department of Health Sciences, University of Florence, Florence, Italy.

**Corresponding author:**

Anna Szumera-Ciećkiewicz

Maria Sklodowska-Curie National Research Institute of Oncology,
Department of Pathology and Laboratory Diagnostics
5 Roentgen Str. 02-781 Warsaw, Poland

e-mail: szumann@gmail.com

ORCID: 0000-0001-5028-3422

## Abstract

**Background** Sentinel lymph node (SLN) biopsy remains crucial for melanoma staging. The European Organization for Research and Treatment of Cancer (EORTC) Melanoma Group recommends performing immunohistochemical stainings for reproducible identification of melanoma metastases. S100 protein (pS100) is a commonly used melanocytic antigen because of its high sensitivity in spite of relatively low specificity. SRY-related HMG-box 10 protein (SOX10) is a transcription factor characterizing neural crest-derived cells. It is uniformly expressed mostly in the nuclei of melanocytes, neural, and myoepithelial cells. Pathologists sometimes prefer SOX10 as a melanoma marker, but it has not yet been investigated on a large-scale to confirm that it is reliable and recommendable for routine SLN evaluation.

**Methods** Four hundred and one treatment-naïve lymph node metastatic melanomas were included in high-density tissue microarrays and were assessed for the presence of SOX10 and pS100 by immunohistochemistry. The slides were digitalized, shared, and evaluated by a panel of experienced melanoma pathologists.

**Results** The vast majority of melanomas were double-positive for pS100 and SOX10 (93.2%); A small percentage of the cases (3.9%) were double negative melanomas. Discordance between the two markers was observed: 1.9% pS100(-)/SOX10(+) and 0.75% pS100(+)/SOX10(-). SOX10 was not expressed by immune cell types in the lymph node, resulting in a less controversial interpretation of the staining.

**Conclusions** SOX10 is as equally specific as pS100 for the detection of melanoma metastases in lymph nodes. The interpretation of SOX10 staining is highly reproducible among different centers and different pathologists because of the absence of staining of immune cells.

## Introduction

Evaluation of sentinel lymph node (SLN) biopsy is considered the gold standard for the identification of early nodal metastasis and the definition of prognosis and treatment of melanoma patients (1). The updated European Organization for Research and Treatment of Cancer (EORTC) Melanoma Group protocol for the SLNs pathological assessment is based on the assessment of multiple levels of the lymph node using not only hematoxylin-eosin stained slides but also immunohistochemistry (IHC), in order to increase the efficiency in detection of small metastatic foci (2). Among the recommended antibodies traditionally used for this purpose, S100 protein (pS100) was the first marker with the highest sensitivity among all melanocytic-associated markers and practical value in melanoma diagnosis and the most frequently used for SLN evaluation (3). Although in most cases, identification of SLN metastases is straightforward, recognition of a few melanoma cells in hematoxylin-eosin sections can be challenging. In melanoma, the concept of "isolated tumor cells" does not apply, and the detection of nodal metastases of any size is pivotal for an accurate melanoma staging (4-6).

SOX10 is a member of a transcription factor family involved in the embryonal development process of the testis, neural crest, and peripheral nervous system (7-9). Synergistically with PAX3, SOX10 plays a central role in melanogenesis by direct regulation of microphthalmia transcription factor (MITF) expression (10). The role of SOX10 in the regulation of melanocyte differentiation is supported by the corollary of symptoms of SOX10 mutation disorders (i.e., Waardenburg Syndrome type 4C and 2E, central or peripheral demyelination, Hirschsprung disease) in which hypopigmentation and deafness are invariably present (11, 12). In melanoma, SOX10 is a major melanocyte enhancer, targeting many different regulatory pathways. Genetic and functional analyses revealed that SOX10 binds to a broad range of genomic sites in melanocytes, influencing distinct classes of genes by complex transcriptional mechanisms of activation and repression (13, 14).

Previous data demonstrated that SOX10 is an important melanocytic marker (15). Nevertheless, the majority of published studies comparing the different antibodies were based on statistically low-power groups and non-homogenous cases, i.e., primary vs. metastatic or desmoplastic vs. other types (16-23). Moreover, the comparability of the different studies may be hampered by the fact that different antibodies and various staining protocols were used to assess pS100 and SOX10 expression (3, 24). The summary of studies addressing SOX10 expression in the sentinel lymph node, lymph node, and metastatic melanoma were presented in Table 1.

In this study, we analyzed 401 lymph nodes (LN) with confirmed metastatic melanoma to compare the sensitivity and specificity of SOX10 and pS100. We focused not only on the detection of neoplastic cells but also explored the strengths and weaknesses of both markers for melanoma metastases detection. The paramount goal was to develop a practical recommendation that can be readily implemented in routine histopathological diagnosis and shared protocols.

## Materials and methods

### Tissue samples

A cohort of 465 LN metastatic melanoma patients was retrospectively collected from the archives of the Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland (268 cases) and the University Hospital of Leuven, Belgium (197 cases). The use of formalin-fixed paraffin-embedded (FFPE) sections of human samples left-over after diagnosis was approved by the Local Bioethical Committee. FFPE tissue sections, 2.5µm in thickness, were cut and stained with hematoxylin and eosin, reviewed to confirm the histopathological diagnosis and assessed for tissue quality control. High-density tissue microarrays (TMAs) were constructed from the archival FFPE blocks, including three representative 1.0 mm cores from each melanoma case. In each TMAs, positive and negative controls were included (tonsil, testis, liver, appendix, and normal skin).

### Immunohistochemistry

Three-µm thick tissue sections were cut from the TMAs for immunohistochemical analysis. Sections were stained using the automatic immunohistochemical stainers Ventana BenchMark XT (Ventana Medical Systems) for SOX10 (rabbit monoclonal, clone SP267 ready to use, IVD use, Ventana Medical Systems) and Dako Omnis (Dako Denmark A/S) immunostainer for pS100 (polyclonal rabbit, clone GA504, Flex ready to use, Dako Agilent). The reactions were developed with UltraMap DAB anti-Mouse Detection Kit (Ventana Medical Systems) for SOX10 and the EnVision Detection System (Agilent) for S100. All sections were counterstained with hematoxylin. Immunohistochemical scoring was performed by experienced melanoma pathologists (ASC, FB, DM, MC), blinded to sample identification data. Negative cases revealed no positive cells. The homogenous positive cases were defined as the presence of intensive staining in >75% of the neoplastic cells. The cases which presented 1-75% of positive cells with different intensity of the staining were specified as the heterogeneous positive group. The scoring system was based on previous publications (23, 25). For pS100, staining had to be present in both nucleus and cytoplasm, while for SOX10 nuclear staining was sufficient. The cases showing discrepancies in the assessment were collegially re-evaluated by the panel of pathologists in order to reach a consensus.

### Statistical analysis

Results of scoring were analyzed in (SPSS Version) with a calculation of specificity and sensitivity (true positive rate). All evaluations labeled different than “positive“ (1) or “negative” (0) in either pS100 or SOX10 were removed from further analysis. Similarly, all patients showing heterogeneous stainings across the evaluated cores were removed from further analysis. The true positive rate of four different parameters was assessed: (1) pS100 positive, (2) SOX10 positive, (3) pS100 positive and SOX10 positive, and (4) pS100 positive or

SOX10 positive. The true positive rate (TPR) for each parameter was calculated as the proportion of positive patients identified as positive.

## Results

After quality control and elimination of the samples with technical difficulties, 401 out of 465 metastatic melanoma were included in the final analysis. While pS100 was positive in 94% of the cases, SOX10 showed a slightly higher positive percentage, being detected in 95.2% of the cases [Figure 1]. Combining both markers, three different categories were observed: i) pS100(+)/SOX10(+) double-positive cases, accounting for 93.2% of the cases; ii) pS100(-)/SOX10(-) double negative cases, for a total of 3.9% of the cases; and iii) cases with isolated loss of one of the two markers, that represented the less numerous group, respectively 1.9% for pS100(-)/SOX10(+) cases and 0.75% for S100(+)/SOX10(-) cases [Figure 1 and Figure 2].
Regarding the pattern of the staining, the majority of the cases showed a homogenous staining pattern: only 6/401 cases with heterogeneous images were identified (1.49%; 6/401) [Figure 3]. SOX10 did not stain dendritic cells or histiocytes.
In addition, we explored the concordance between the dataset from different centers when stained with a homogeneous staining protocol for pS100 and SOX10. The differences in the percentage of positively evaluated cases were below 1%. The interpretation of the IHC for these two markers using adapted cut-off criteria was unquestionably easy, and reproducible.

## Discussion

Due to the wide range of cytoarchitectural features observed in melanoma, sometimes reliable IHC confirmation is needed for an accurate diagnosis. SOX10 is one of the most recently introduced diagnostic melanoma markers, while pS100 is known for 40 years (3). Strong reactions with both antibodies were identified nearly all histopathological variants of primary and metastatic melanoma (19, 26).

In our study, we found high specificity and sensitivity of SOX10 staining of lymph node melanoma metastases. The comparative study with pS100 showed excellent concordance with the true positive rate reaching 95.3%. In Table 1, we summarize the results of the largest studies investigating SOX10 expression in metastatic melanoma. In the largest study by Miettinen et al. (16), 119 cases of metastatic melanoma were investigated. In line with our results, they found a similar percentage of SOX10 positivity (95.2%); negative cases included six poorly differentiated cases with sarcomatoid morphology, a subtype known for frequently losing the expression of melanoma-specific markers, and 3 cases were also pS100 negative. From the remaining SOX10 positive group, three other cases were pS100 negative, resulting in an equal overall sensitivity for SOX10 and pS100 (16). Other studies investigated a limited number of cases. In particular, studies including SLN and LN evaluation with SOX10 showing 100% sensitivity were performed on relatively small data sets (16, 17, 22, 23, 27, 28). Moreover, the immunostainings were performed with different antibodies and in variable conditions, mostly not accepted in diagnostic practice, making the results non-comparable

and hardly applicable in clinical practice. Our study is the first large scale study on LN metastatic melanoma. The SP267 clone was chosen because it is a ready-to-use antibody and easily standardizable across different centers worldwide.

In spite of its high sensitivity, pS100 specificity is not satisfactory. Numerous pS100 positive cells with similar morphology to melanocytes (e.g., dendritic cells) can be found homing in lymph nodes (29-31). Moreover, pS100 is expressed in a broad spectrum of other tumors in the differential diagnosis of melanoma (30, 32). Some subtypes of breast and salivary gland carcinomas, rhabdomyosarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumors, and Langerhans cell histiocytosis can be diffuse or focally S100 positive (30). The low specificity of pS100 may, therefore, require to add more specific melanocytic markers such as HMB-45, Melan A, tyrosinase, MITF, or panels of melanocytic markers. In contrast to pS100, SOX10 is more specific for a neuroectodermic origin and is less expressed by other malignancies (23, 29). In particular, SOX10 can be expressed by myoepithelial/basal cell epithelial neoplasms, neurogenic tumors, and breast malignancies (16, 33, 34). A significant issue in this sense is represented by triple-negative breast carcinomas that are known to be SOX10 positive (33, 35). This pitfall needs to be kept in mind while examining axillary lymph nodes, and the diagnosis can eventually be supported in ambiguous cases by additional stainings such as cytokeratins and GATA-3 (34, 36).

Therefore, while S100 interpretation in SLN requires careful evaluation by experienced pathologists due to possible misdiagnosis and frequent overestimation of the presence of metastatic disease, SOX10 evaluation is easier due to unambiguous metastases evaluation. The pS100 positive dendritic cells, in particular, in the lymph node, tend to be present isolated or in small aggregates and are not stained by SOX10. Therefore, SOX10 is certainly more helpful than pS100 in the detection of micrometastases in SLN.
However, it should be underlined that some concerns may arise with the use of SOX10 instead of pS100. SOX10, being exclusively located in the nucleus in most cases (with only moderate intense cytoplasmic staining in some cases), may be less visible than pS100, expressed both in the cytoplasm and the nucleus while scanning at low power the SLN. Nevertheless, the panel of pathologists participating in the present study did not have serious difficulties in the SOX10 staining evaluation. Another concern may be the preservation of cell morphology. In fact, nuclear pleomorphism is the most important clue leading to the diagnosis of melanoma metastasis in the lymph node and helping the pathologist in the differential diagnosis with intranodal benign nevi. Nuclear morphology can be masked if evaluated on the immunohistochemistry slide, but this problem may be avoided if the EORTC Melanoma Group recommendations for the evaluation of the SLN are followed, and the SOX10 staining is correlated with the cytomorphological features of the suspicious metastatic focus on the HE of the immediately adjacent tissue section (2).
In the literature, the cut-off to define SOX10 positivity is not precisely defined. The majority of studies consider a melanoma positive when at least 1% of the neoplastic cells are positive irrespective of staining intensity (17, 23, 27). By contrast, some studies do not take

heterogeneity into consideration (16). In our analysis, most of our cases were characterized by strong homogenous staining, with over 75% of immunopositive cells. Cases with SOX10-positive melanoma cells below 75% were considered positive, but we labeled them as “heterogeneous”. Vrotsos et al. showed that even if 86% of melanoma metastases are strongly positive for SOX10 in more than 75% of melanoma cells, in 14% of cases positivity can be in less 75% of the cells, even reaching a very subtle 1% (23). On the contrary, Willis et al. found a more generally widespread SOX10 expression by most of the melanoma cells, with high mean intensity and mean percentage cell staining reaching 99.6% (17). In our study, we observed a very low percentage of heterogeneous cases (1.49%). This heterogeneous category was most likely due to the presence of different melanoma cell clones in the same metastatic deposits. Dedifferentiated or transdifferentiated melanomas are good examples of this phenomenon (37, 38). However, in our data set, we observed this was a very infrequent problem (6 cases per 401 lymph node metastatic melanomas).

In conclusion, we demonstrate that SOX10 is a highly specific marker for melanoma metastasis in lymph nodes, comparable to pS100 regarding true positive rates but easier to interpret and strongly reproducible if a ready-to-use antibody is used. According to the presented results, SOX10 has the same level of recommendation as pS100 for the detection of melanoma metastasis in SLN protocols.

Table 1. Summary of studies addressing SOX10 expression in a sentinel lymph node, lymph node, and metastatic melanoma.

| *Type of material* | *No. of all cases/No. of cases with MM* | *No./% SOX10(+) cases* | *SOX10 cut-off* | *Antibody* | *Year* | *Ref.* |
|---|---|---|---|---|---|---|
| SLN | 77/58 | 58/100% | >1% | Goat anti-rodent, polyclonal, 1:100, Santa-Cruz, Biotechnology Inc, Santa Cruz, CA | 2015 | (17) |
| SLN/LN | 93/40 | 43*/100% | NA | Polyclonal, Santa Cruz Biotechnology, Inc, Santa Cruz, CA | 2011 | (27) |
| SLN | 121/33 | 33/100% | NA | NA | 2009 | (22) |
| LN | 50/50 | 50/100 | >1% | Rabbit, polyclonal, prediluted; Cell Marque, Rocklin, CA | 2016 | (23) |
| MM | 125/125 | 119/95.2% | NA | EP268; Epitomics Inc, AC-0237, Burlingame, CA; 1:250 | 2015 | (16) |
| MM | 87/87 | 73/83.9% | >1% | BC34, 1:100 | 2015 | (28) |
| SLN/LN | 401/401 | 382/95.3% | >75% | SP267; Rabbit monoclonal, RTU, IVD; Ventana Medical Systems | 2020 | presented study |

*additional three sentinel lymph nodes, which were not initially diagnosed as positive for metastasis, were identified with Sox-10, S100, Melan-A, and HMB-45 immunostains.

No. number; MM metastatic melanoma % percentage; Ref. reference; SLN sentinel lymph node; LN lymph node; NA not available data; RTU ready to use; IVD in vitro diagnostics.

## Figure Captions

*Figure 1. Distribution of the positive stainings for pS100 and SOX10 in the data set of the Maria Sklodowska-Curie National Research Institute of Oncology (red) and of the University Hospital of Leuven (grey). The true positive rate (TPR) is shown for pS100 and SOX10 separately (two groups of histograms on the right) and in combination (two groups of histograms on the left). The TPR for the single institutes can be compared to the cumulative percentage (Total, in blue), and they are overall comparable.*

*Figure 2. Representative photomicrographs of hematoxylin and eosin (H&E), SOX10 and pS100 staining in melanoma metastasis of three different categories: A, case #1, double positive pS100(+)/SOX10(+) (number of cases=374 accounting for 93.2% of the total cases); the residual of the lymph node (yellow arrow) with a strong reaction in dendritic cells (red arrow); B, case #2 and C, case #3, isolated loss of one of the two marker, pS100(-)/SOX10(+) (number of cases=3, accounting for 0.75% of the total cases) and pS100(+)/SOX10(-) (number of cases=8, accounting for 1.99% of the total cases), pS100 strong reaction in dendritic cells (red arrow); D, case #4, double negative pS100(-)/SOX10(-)((number of cases=16, accounting for 3.98% of the total cases); pS100 strong reaction in dendritic cells (red arrow) and, S100 moderate to strong reaction in histiocytes (yellow arrow).*

*Figure 3. Representative photomicrographs of hematoxylin and eosin (H&E), SOX10 and pS100 staining in melanoma metastasis of heterogeneous cases (number of cases=6 accounting for 1.49% of the total cases): A and C, case #5 and case #7, SOX10 positive reaction in nearly 100% of cells, pS100 positive only partially (<75% of cells); B, case #6, SOX10 isolated positive cells, pS100 strong positive reaction.*

Conflict of interest statement: None declared.

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**Affiliations:**

1. Department of Pathology and Laboratory Diagnostics, Maria Sklodowska-Curie National Research Institute of Oncology**,** Warsaw, Poland.
2. Department of Diagnostic Hematology, Institute of Hematology and Transfusion Medicine; Warsaw, Poland.
3. Laboratory of Translational Cell and Tissue Research and Pathology Department, KU Leuven and UZ Leuven, Leuven, Belgium.
4. Department of Soft Tissue/Bone Sarcoma and Melanoma, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland.
5. Department of Health Sciences, Clinical Pharmacology and Oncology Unit, University of Florence, Florence, Italy.
6. Department of Pathology, Erasmus MC, University Medical Centre Rotterdam, the Netherlands.
7. Department of Pathology, The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands.
8. Department of Pathology, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center, Utrecht, the Netherlands.
9. Department of Pathology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands.
10. Department of Surgical Oncology, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, the Netherlands.

11. Histopathology, Royal Surrey County Hospital, Guildford, UK.
12. Section of Pathological Anatomy, Department of Health Sciences, University of Florence, Florence, Italy.

**Corresponding author:**

Anna Szumera-Ciećkiewicz

Maria Sklodowska-Curie National Research Institute of Oncology,
Department of Pathology and Laboratory Diagnostics
5 Roentgen Str. 02-781 Warsaw, Poland

e-mail: szumann@gmail.com

ORCID: 0000-0001-5028-3422

## Abstract

**Background** Sentinel lymph node (SLN) biopsy remains crucial for melanoma staging. The European Organization for Research and Treatment of Cancer (EORTC) Melanoma Group recommends performing immunohistochemical stainings for reproducible identification of melanoma metastases. S100 protein (pS100) is a commonly used melanocytic antigen because of its high sensitivity in spite of relatively low specificity. SRY-related HMG-box 10 protein (SOX10) is a transcription factor characterizing neural crest-derived cells. It is uniformly expressed mostly in the nuclei of melanocytes, neural, and myoepithelial cells. Pathologists sometimes prefer SOX10 as a melanoma marker, but it has not yet been investigated on a large-scale to confirm that it is reliable and recommendable for routine SLN evaluation.

**Methods** Four hundred and one treatment-naïve lymph node metastatic melanomas were included in high-density tissue microarrays and were assessed for the presence of SOX10 and pS100 by immunohistochemistry. The slides were digitalized, shared, and evaluated by a panel of experienced melanoma pathologists.

**Results** The vast majority of melanomas were double-positive for pS100 and SOX10 (93.2%); A small percentage of the cases (3.9%) were double negative melanomas. Discordance between the two markers was observed: 1.9% pS100(-)/SOX10(+) and 0.75% pS100(+)/SOX10(-). SOX10 was not expressed by immune cell types in the lymph node, resulting in a less controversial interpretation of the staining.

**Conclusions** SOX10 is as equally specific as pS100 for the detection of melanoma metastases in lymph nodes. The interpretation of SOX10 staining is highly reproducible among different centers and different pathologists because of the absence of staining of immune cells.

## Introduction

Evaluation of sentinel lymph node (SLN) biopsy is considered the gold standard for the identification of early nodal metastasis and the definition of prognosis and treatment of melanoma patients (1). The updated European Organization for Research and Treatment of Cancer (EORTC) Melanoma Group protocol for the SLNs pathological assessment is based on the assessment of multiple levels of the lymph node using not only hematoxylin-eosin stained slides but also immunohistochemistry (IHC), in order to increase the efficiency in detection of small metastatic foci (2). Among the recommended antibodies traditionally used for this purpose, S100 protein (pS100) was the first marker with the highest sensitivity among all melanocytic-associated markers and practical value in melanoma diagnosis and the most frequently used for SLN evaluation (3). Although in most cases, identification of SLN metastases is straightforward, recognition of a few melanoma cells in hematoxylin-eosin sections can be challenging. In melanoma, the concept of "isolated tumor cells" does not apply, and the detection of nodal metastases of any size is pivotal for an accurate melanoma staging (4-6).

SOX10 is a member of a transcription factor family involved in the embryonal development process of the testis, neural crest, and peripheral nervous system (7-9). Synergistically with PAX3, SOX10 plays a central role in melanogenesis by direct regulation of microphthalmia transcription factor (MITF) expression (10). The role of SOX10 in the regulation of melanocyte differentiation is supported by the corollary of symptoms of SOX10 mutation disorders (i.e., Waardenburg Syndrome type 4C and 2E, central or peripheral demyelination, Hirschsprung disease) in which hypopigmentation and deafness are invariably present (11, 12). In melanoma, SOX10 is a major melanocyte enhancer, targeting many different regulatory pathways. Genetic and functional analyses revealed that SOX10 binds to a broad range of genomic sites in melanocytes, influencing distinct classes of genes by complex transcriptional mechanisms of activation and repression (13, 14).

Previous data demonstrated that SOX10 is an important melanocytic marker (15). Nevertheless, the majority of published studies comparing the different antibodies were based on statistically low-power groups and non-homogenous cases, i.e., primary vs. metastatic or desmoplastic vs. other types (16-23). Moreover, the comparability of the different studies may be hampered by the fact that different antibodies and various staining protocols were used to assess pS100 and SOX10 expression (3, 24). The summary of studies addressing SOX10 expression in the sentinel lymph node, lymph node, and metastatic melanoma were presented in Table 1.

In this study, we analyzed 401 lymph nodes (LN) with confirmed metastatic melanoma to compare the sensitivity and specificity of SOX10 and pS100. We focused not only on the detection of neoplastic cells but also explored the strengths and weaknesses of both markers for melanoma metastases detection. The paramount goal was to develop a practical recommendation that can be readily implemented in routine histopathological diagnosis and shared protocols.

## Materials and methods

### Tissue samples

A cohort of 465 LN metastatic melanoma patients was retrospectively collected from the archives of the Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland (268 cases) and the University Hospital of Leuven, Belgium (197 cases). The use of formalin-fixed paraffin-embedded (FFPE) sections of human samples left-over after diagnosis was approved by the Local Bioethical Committee. FFPE tissue sections, 2.5µm in thickness, were cut and stained with hematoxylin and eosin, reviewed to confirm the histopathological diagnosis and assessed for tissue quality control. High-density tissue microarrays (TMAs) were constructed from the archival FFPE blocks, including three representative 1.0 mm cores from each melanoma case. In each TMAs, positive and negative controls were included (tonsil, testis, liver, appendix, and normal skin).

### Immunohistochemistry

Three-µm thick tissue sections were cut from the TMAs for immunohistochemical analysis. Sections were stained using the automatic immunohistochemical stainers Ventana BenchMark XT (Ventana Medical Systems) for SOX10 (rabbit monoclonal, clone SP267 ready to use, IVD use, Ventana Medical Systems) and Dako Omnis (Dako Denmark A/S) immunostainer for pS100 (polyclonal rabbit, clone GA504, Flex ready to use, Dako Agilent). The reactions were developed with UltraMap DAB anti-Mouse Detection Kit (Ventana Medical Systems) for SOX10 and the EnVision Detection System (Agilent) for S100. All sections were counterstained with hematoxylin. Immunohistochemical scoring was performed by experienced melanoma pathologists (ASC, FB, DM, MC), blinded to sample identification data. Negative cases revealed no positive cells. The homogenous positive cases were defined as the presence of intensive staining in >75% of the neoplastic cells. The cases which presented 1-75% of positive cells with different intensity of the staining were specified as the heterogeneous positive group. The scoring system was based on previous publications (23, 25). For pS100, staining had to be present in both nucleus and cytoplasm, while for SOX10 nuclear staining was sufficient. The cases showing discrepancies in the assessment were collegially re-evaluated by the panel of pathologists in order to reach a consensus.

### Statistical analysis

Results of scoring were analyzed in (SPSS Version) with a calculation of specificity and sensitivity (true positive rate). All evaluations labeled different than “positive“ (1) or “negative” (0) in either pS100 or SOX10 were removed from further analysis. Similarly, all patients showing heterogeneous stainings across the evaluated cores were removed from further analysis. The true positive rate of four different parameters was assessed: (1) pS100 positive, (2) SOX10 positive, (3) pS100 positive and SOX10 positive, and (4) pS100 positive or

SOX10 positive. The true positive rate (TPR) for each parameter was calculated as the proportion of positive patients identified as positive.

## Results

After quality control and elimination of the samples with technical difficulties, 401 out of 465 metastatic melanoma were included in the final analysis. While pS100 was positive in 94% of the cases, SOX10 showed a slightly higher positive percentage, being detected in 95.2% of the cases [Figure 1]. Combining both markers, three different categories were observed: i) pS100(+)/SOX10(+) double-positive cases, accounting for 93.2% of the cases; ii) pS100(-)/SOX10(-) double negative cases, for a total of 3.9% of the cases; and iii) cases with isolated loss of one of the two markers, that represented the less numerous group, respectively 1.9% for pS100(-)/SOX10(+) cases and 0.75% for S100(+)/SOX10(-) cases [Figure 1 and Figure 2].
Regarding the pattern of the staining, the majority of the cases showed a homogenous staining pattern: only 6/401 cases with heterogeneous images were identified (1.49%; 6/401) [Figure 3]. SOX10 did not stain dendritic cells or histiocytes.
In addition, we explored the concordance between the dataset from different centers when stained with a homogeneous staining protocol for pS100 and SOX10. The differences in the percentage of positively evaluated cases were below 1%. The interpretation of the IHC for these two markers using adapted cut-off criteria was unquestionably easy, and reproducible.

## Discussion

Due to the wide range of cytoarchitectural features observed in melanoma, sometimes reliable IHC confirmation is needed for an accurate diagnosis. SOX10 is one of the most recently introduced diagnostic melanoma markers, while pS100 is known for 40 years (3). Strong reactions with both antibodies were identified nearly all histopathological variants of primary and metastatic melanoma (19, 26).

In our study, we found high specificity and sensitivity of SOX10 staining of lymph node melanoma metastases. The comparative study with pS100 showed excellent concordance with the true positive rate reaching 95.3%. In Table 1, we summarize the results of the largest studies investigating SOX10 expression in metastatic melanoma. In the largest study by Miettinen et al. (16), 119 cases of metastatic melanoma were investigated. In line with our results, they found a similar percentage of SOX10 positivity (95.2%); negative cases included six poorly differentiated cases with sarcomatoid morphology, a subtype known for frequently losing the expression of melanoma-specific markers, and 3 cases were also pS100 negative. From the remaining SOX10 positive group, three other cases were pS100 negative, resulting in an equal overall sensitivity for SOX10 and pS100 (16). Other studies investigated a limited number of cases. In particular, studies including SLN and LN evaluation with SOX10 showing 100% sensitivity were performed on relatively small data sets (16, 17, 22, 23, 27, 28). Moreover, the immunostainings were performed with different antibodies and in variable conditions, mostly not accepted in diagnostic practice, making the results non-comparable

and hardly applicable in clinical practice. Our study is the first large scale study on LN metastatic melanoma. The SP267 clone was chosen because it is a ready-to-use antibody and easily standardizable across different centers worldwide.

In spite of its high sensitivity, pS100 specificity is not satisfactory. Numerous pS100 positive cells with similar morphology to melanocytes (e.g., dendritic cells) can be found homing in lymph nodes (29-31). Moreover, pS100 is expressed in a broad spectrum of other tumors in the differential diagnosis of melanoma (30, 32). Some subtypes of breast and salivary gland carcinomas, rhabdomyosarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumors, and Langerhans cell histiocytosis can be diffuse or focally S100 positive (30). The low specificity of pS100 may, therefore, require to add more specific melanocytic markers such as HMB-45, Melan A, tyrosinase, MITF, or panels of melanocytic markers. In contrast to pS100, SOX10 is more specific for a neuroectodermic origin and is less expressed by other malignancies (23, 29). In particular, SOX10 can be expressed by myoepithelial/basal cell epithelial neoplasms, neurogenic tumors, and breast malignancies (16, 33, 34). A significant issue in this sense is represented by triple-negative breast carcinomas that are known to be SOX10 positive (33, 35). This pitfall needs to be kept in mind while examining axillary lymph nodes, and the diagnosis can eventually be supported in ambiguous cases by additional stainings such as cytokeratins and GATA-3 (34, 36).

Therefore, while S100 interpretation in SLN requires careful evaluation by experienced pathologists due to possible misdiagnosis and frequent overestimation of the presence of metastatic disease, SOX10 evaluation is easier due to unambiguous metastases evaluation. The pS100 positive dendritic cells, in particular, in the lymph node, tend to be present isolated or in small aggregates and are not stained by SOX10. Therefore, SOX10 is certainly more helpful than pS100 in the detection of micrometastases in SLN.
However, it should be underlined that some concerns may arise with the use of SOX10 instead of pS100. SOX10, being exclusively located in the nucleus in most cases (with only moderate intense cytoplasmic staining in some cases), may be less visible than pS100, expressed both in the cytoplasm and the nucleus while scanning at low power the SLN. Nevertheless, the panel of pathologists participating in the present study did not have serious difficulties in the SOX10 staining evaluation. Another concern may be the preservation of cell morphology. In fact, nuclear pleomorphism is the most important clue leading to the diagnosis of melanoma metastasis in the lymph node and helping the pathologist in the differential diagnosis with intranodal benign nevi. Nuclear morphology can be masked if evaluated on the immunohistochemistry slide, but this problem may be avoided if the EORTC Melanoma Group recommendations for the evaluation of the SLN are followed, and the SOX10 staining is correlated with the cytomorphological features of the suspicious metastatic focus on the HE of the immediately adjacent tissue section (2).
In the literature, the cut-off to define SOX10 positivity is not precisely defined. The majority of studies consider a melanoma positive when at least 1% of the neoplastic cells are positive irrespective of staining intensity (17, 23, 27). By contrast, some studies do not take

heterogeneity into consideration (16). In our analysis, most of our cases were characterized by strong homogenous staining, with over 75% of immunopositive cells. Cases with SOX10-positive melanoma cells below 75% were considered positive, but we labeled them as “heterogeneous”. Vrotsos et al. showed that even if 86% of melanoma metastases are strongly positive for SOX10 in more than 75% of melanoma cells, in 14% of cases positivity can be in less 75% of the cells, even reaching a very subtle 1% (23). On the contrary, Willis et al. found a more generally widespread SOX10 expression by most of the melanoma cells, with high mean intensity and mean percentage cell staining reaching 99.6% (17). In our study, we observed a very low percentage of heterogeneous cases (1.49%). This heterogeneous category was most likely due to the presence of different melanoma cell clones in the same metastatic deposits. Dedifferentiated or transdifferentiated melanomas are good examples of this phenomenon (37, 38). However, in our data set, we observed this was a very infrequent problem (6 cases per 401 lymph node metastatic melanomas).

In conclusion, we demonstrate that SOX10 is a highly specific marker for melanoma metastasis in lymph nodes, comparable to pS100 regarding true positive rates but easier to interpret and strongly reproducible if a ready-to-use antibody is used. According to the presented results, SOX10 has the same level of recommendation as pS100 for the detection of melanoma metastasis in SLN protocols.

Table 1. Summary of studies addressing SOX10 expression in a sentinel lymph node, lymph node, and metastatic melanoma.

| *Type of material* | *No. of all cases/No. of cases with MM* | *No./% SOX10(+) cases* | *SOX10 cut-off* | *Antibody* | *Year* | *Ref.* |
|---|---|---|---|---|---|---|
| SLN | 77/58 | 58/100% | >1% | Goat anti-rodent, polyclonal, 1:100, Santa-Cruz, Biotechnology Inc, Santa Cruz, CA | 2015 | (17) |
| SLN/LN | 93/40 | 43*/100% | NA | Polyclonal, Santa Cruz Biotechnology, Inc, Santa Cruz, CA | 2011 | (27) |
| SLN | 121/33 | 33/100% | NA | NA | 2009 | (22) |
| LN | 50/50 | 50/100 | >1% | Rabbit, polyclonal, prediluted; Cell Marque, Rocklin, CA | 2016 | (23) |
| MM | 125/125 | 119/95.2% | NA | EP268; Epitomics Inc, AC-0237, Burlingame, CA; 1:250 | 2015 | (16) |
| MM | 87/87 | 73/83.9% | >1% | BC34, 1:100 | 2015 | (28) |
| SLN/LN | 401/401 | 382/95.3% | >75% | SP267; Rabbit monoclonal, RTU, IVD; Ventana Medical Systems | 2020 | presented study |

*additional three sentinel lymph nodes, which were not initially diagnosed as positive for metastasis, were identified with Sox-10, S100, Melan-A, and HMB-45 immunostains.

No. number; MM metastatic melanoma % percentage; Ref. reference; SLN sentinel lymph node; LN lymph node; NA not available data; RTU ready to use; IVD in vitro diagnostics.

## Figure Captions

*Figure 1. Distribution of the positive stainings for pS100 and SOX10 in the data set of the Maria Sklodowska-Curie National Research Institute of Oncology (red) and of the University Hospital of Leuven (grey). The true positive rate (TPR) is shown for pS100 and SOX10 separately (two groups of histograms on the right) and in combination (two groups of histograms on the left). The TPR for the single institutes can be compared to the cumulative percentage (Total, in blue), and they are overall comparable.*

*Figure 2. Representative photomicrographs of hematoxylin and eosin (H&E), SOX10 and pS100 staining in melanoma metastasis of three different categories: A, case #1, double positive pS100(+)/SOX10(+) (number of cases=374 accounting for 93.2% of the total cases); the residual of the lymph node (yellow arrow) with a strong reaction in dendritic cells (red arrow); B, case #2 and C, case #3, isolated loss of one of the two marker, pS100(-)/SOX10(+) (number of cases=3, accounting for 0.75% of the total cases) and pS100(+)/SOX10(-) (number of cases=8, accounting for 1.99% of the total cases), pS100 strong reaction in dendritic cells (red arrow); D, case #4, double negative pS100(-)/SOX10(-)((number of cases=16, accounting for 3.98% of the total cases); pS100 strong reaction in dendritic cells (red arrow) and, S100 moderate to strong reaction in histiocytes (yellow arrow).*

*Figure 3. Representative photomicrographs of hematoxylin and eosin (H&E), SOX10 and pS100 staining in melanoma metastasis of heterogeneous cases (number of cases=6 accounting for 1.49% of the total cases): A and C, case #5 and case #7, SOX10 positive reaction in nearly 100% of cells, pS100 positive only partially (<75% of cells); B, case #6, SOX10 isolated positive cells, pS100 strong positive reaction.*

## Conflict of interest statement: None declared.

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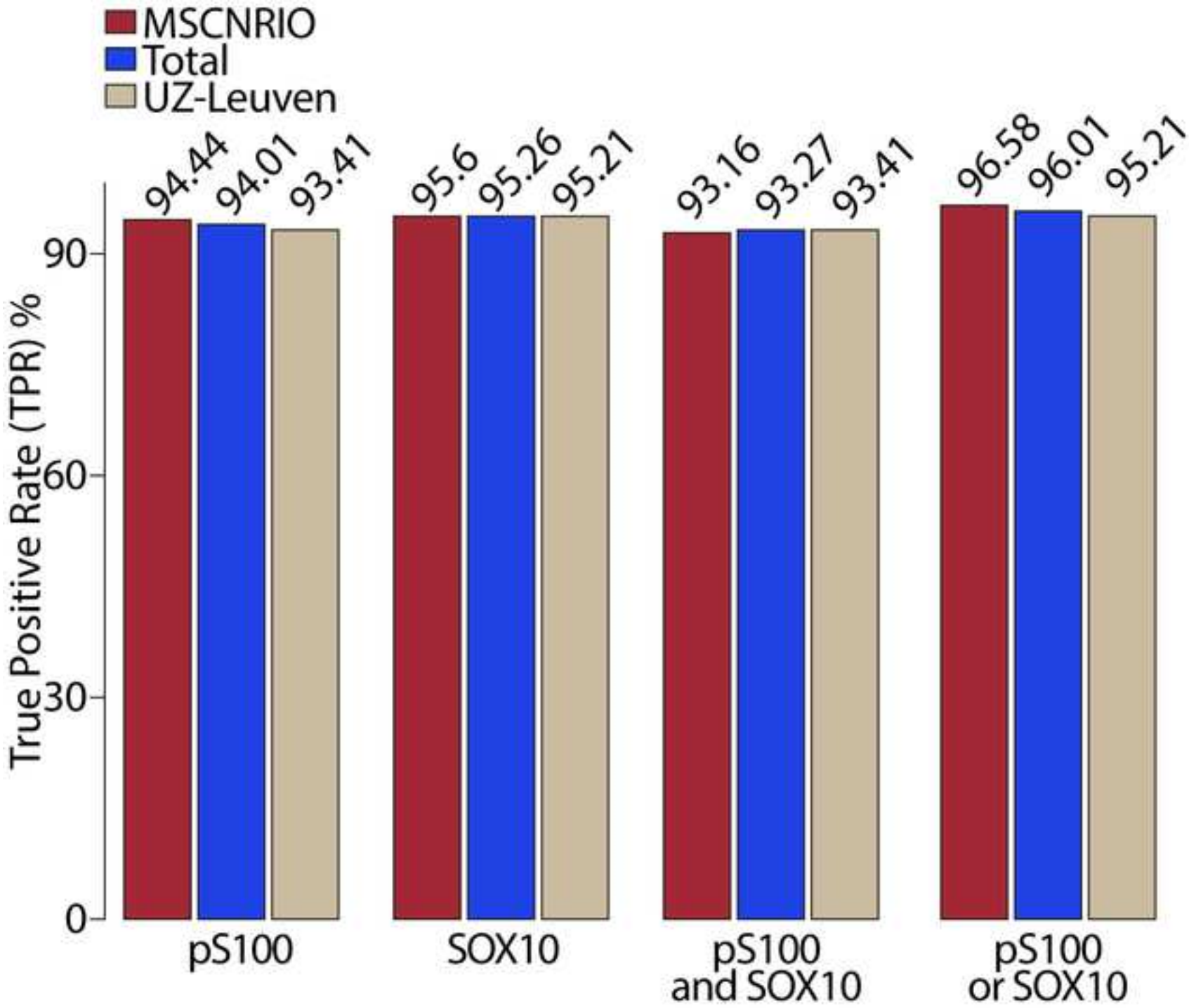

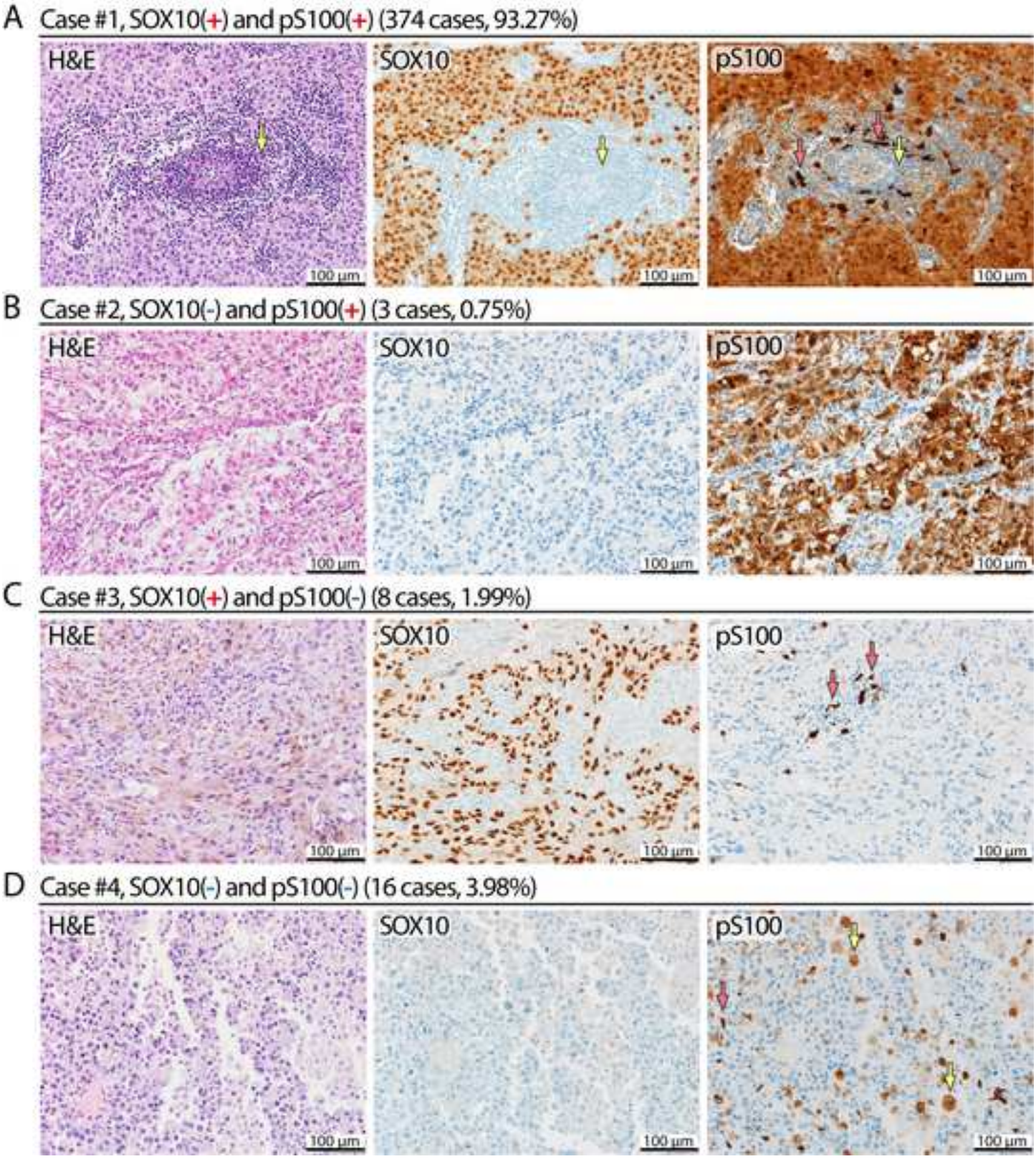

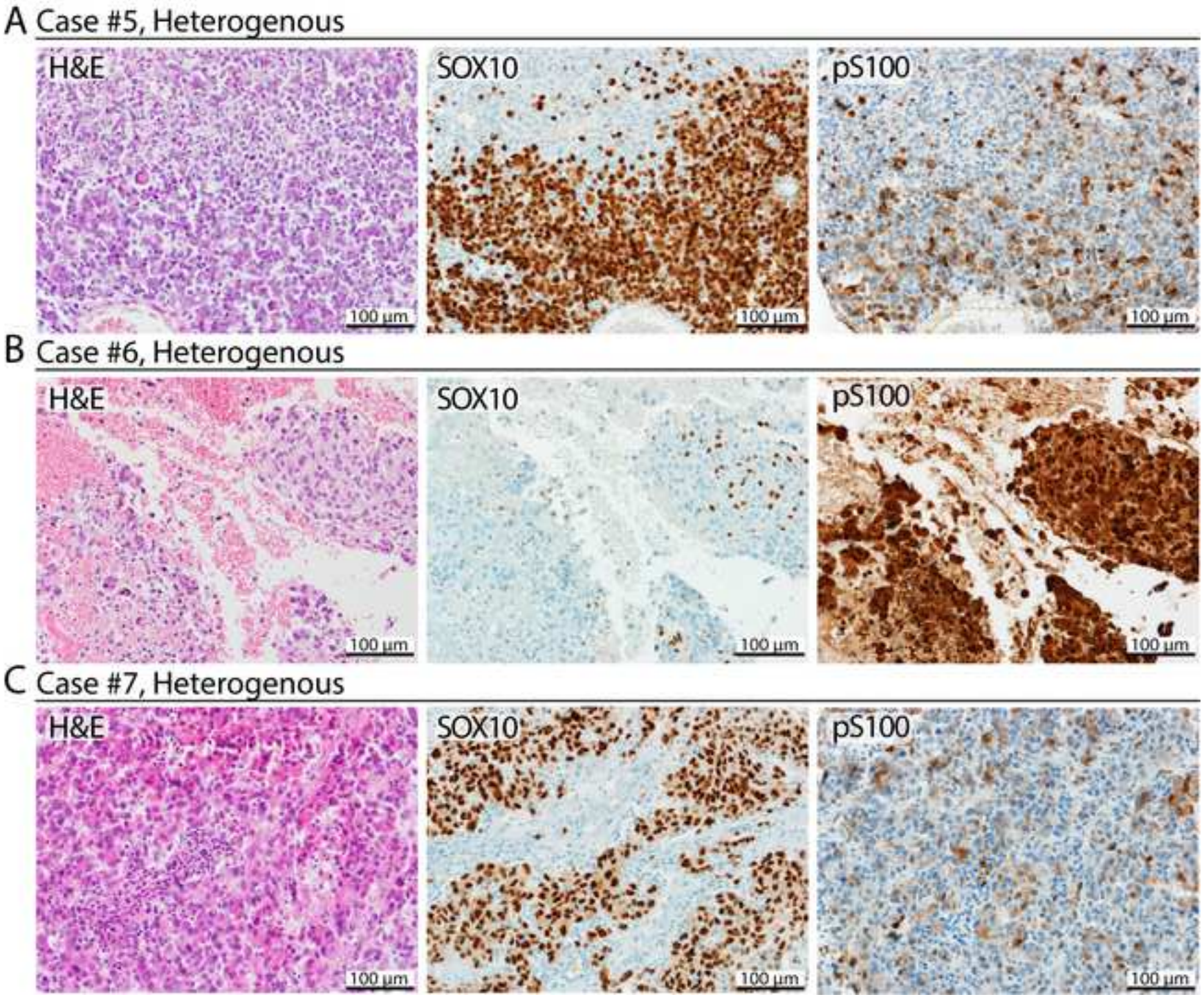

**Authors with affiliations listed below and contributing the paper:** SOX10 is as specific as S100 protein in detecting metastases of melanoma in lymph nodes and is recommended for sentinel lymph node assessment **declare no conflicts of interests:**

**Authors:** Anna Szumera-Ciećkiewicz[1,2] (ASC), Francesca Bosisio[3] (FB), Paweł Teterycz[4] (PT), Asier Antoranz[3] (AA), Francesco Delogu[5] (FD), Senada Koljenović[6] (SK), Bart A. van de Wiel[7] (BAW), Willeke Blokx[8] (WB), Léon C. van Kempen[9] (LVK), Piotr Rutkowski[4] (PR), Alexander Christopher van Akkooi[10] (ACA), Martin Cook[11] (MC), Daniela Massi[12] (DM), EORTC Melanoma Group.

**Affiliations:**

1. Pathology Laboratory, Department of Pathology and Laboratory Diagnostics, Maria Sklodowska-Curie **National Research Institute of Oncology,** Warsaw, Poland.
2. Department of Diagnostic Hematology, Institute of Hematology and Transfusion Medicine; Warsaw, Poland.
3. Laboratory of Translational Cell and Tissue Research and Pathology Department, KU Leuven and UZ Leuven, Leuven, Belgium.
4. Department of Soft Tissue/Bone Sarcoma and Melanoma, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland.
5. Department of Health Sciences, Clinical Pharmacology and Oncology Unit, University of Florence, Florence, Italy.
6. Department of Pathology, Erasmus MC, University Medical Centre Rotterdam, the Netherlands.
7. Department of Pathology, The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands.
8. Department of Pathology, Division of Laboratories, Pharmacy and Biomedical Genetics, University Medical Center, Utrecht, the Netherlands.
9. Department of Pathology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands.
10. Department of Surgical Oncology, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, the Netherlands.
11. Histopathology, Royal Surrey County Hospital, Guildford, UK.
12. Section of Pathological Anatomy, Department of Health Sciences, University of Florence, Florence, Italy.