Revista de Senología y Patología Mamaria 38 (2025) 100706

Sociedad Española de Senología y Patología Mamaria

Revista de Senología y patología mamaria
Journal of Senology and Breast Disease

www.elsevier.es/senologia

ORIGINAL

Sensitivity and specificity of MammaTyper® versus immunohistochemistry in the molecular subtyping of breast cancer: The SUBMARIN study

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Received 20 March 2025; accepted 24 April 2025
Available online 19 June 2025

KEYWORDS
Transcriptomic signature;
Molecular subtyping;
HER2-low;
Ki-67;
Prognosis;
Predictive factors

Abstract
Introduction: Immunohistochemical study for oestrogen receptor (ER/*ESR1*), progesterone receptor (PR/*PGR*), human epidermal growth factor receptor 2 (HER2/*ERBB2*), and proliferation marker Ki-67/*MKI67*PR is the standard technique for diagnosing the molecular subtype in clinical practice. However, less subjective techniques, such as the mRNA-quantifying MammaTyper® (MMT), are needed.
Materials and methods: Observational, retrospective, single-centre study carried out at the Vinalopó University Hospital, Alacant, Spain, with 109 consecutive formalin-fixed paraffin-embedded samples coming from infiltrating breast carcinoma diagnosed between 2022 and 2023.
Results: The global concordance of MMT with IHC was 76.15%. MMT results were concordant with IHC in all HER2-positive (4, 100%) or HER2-Luminal B samples (11, 100%), in 45 (95.7%) classified as Luminal B-like (HER2−) and in 11 (84.6%) Triple negative. MMT disagreed on 22 (64.7%) Luminal A-like samples, mainly because MMT detected Ki-67 expression. High values of positive agreement for ER (96.3%, 95% CI: 90.9; 98.6%), PR (89.9%, 95% CI: 82.8; 94.3%), and HER2 (87.0%, 95% CI: 67.9; 95.5%) were obtained. MMT detected more positives than IHC for Ki-67 (52.6%, 95% CI: 43.2; 62.0%) and HER2-low (74.3%, 95% CI: 66.1; 82.5%).
Conclusion: The concordance between both techniques was high for ER, PR, and HER2. Notably, MMT standardises the identification of Luminal A-B-like and HER2-low thanks to its higher sensitivity towards HER2 and Ki-67.

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https://doi.org/10.1016/j.senol.2025.100706

PALABRAS CLAVE
Firma transcriptómica;
Tipificación molecular;
HER2 baja;
Ki-67;
Pronóstico;
Factores predictivos

Sensibilidad y especificidad de MammaTyper® frente a inmunohistoquímica en la subtipificación molecular del cáncer de mama: Estudio SUBMARIN

Resumen
Introducción: En el cáncer de mama, la subtipificación molecular se realiza en la rutina clínica mediante la inmunohistoquímica (IHC) del receptor de estrógenos (ER/ESR1), el receptor de progesterona (PR/PGR), el receptor 2 del factor de crecimiento epidérmico humano (HER2/ERBB2) y el marcador de proliferación Ki-67/MKI67. Sin embargo, se necesitan técnicas menos subjetivas, como el MammaTyper® (MMT) de cuantificación de ARNm.
Materiales y métodos: Estudio observacional, retrospectivo y unicéntrico realizado en el Hospital Universitario del Vinalopó, Alicante, España, con todas las muestras consecutivas fijadas en formol e incluidas en parafina de pacientes >18 años con un carcinoma infiltrante de mama diagnosticado entre 2022 y 2023 y analizadas mediante IHC.
Resultados: Se incluyeron 109 muestras en el estudio, y la edad media de los pacientes era de 62,1 años. La concordancia global de la MMT con la IHC fue del 76,15%. Los resultados de la MMT fueron concordantes con la IHC en todas las muestras enriquecidas en HER2 (4, 100%) o HER2-Luminal B (11, 100%), en 45 (95,7%) clasificadas como Luminal B-like (HER2-) y en 11 (84,6%) Triple negativo. MMT discrepó en 22 (64,7%) muestras Luminal A-like, principalmente porque MMT detectó la expresión de Ki-67. Se obtuvieron valores elevados de concordancia positiva para RE (96,3%, IC 95%: 90,9; 98,6%), RP (89,9%, IC 95%: 82,8; 94,3%) y HER2 (87,0%, IC 95%: 67,9; 95,5%). La MMT detectó más positivos que la IHC para Ki-67 (52,6%, IC 95%: 43,2; 62,0%) y HER2-bajo (74,3%, IC 95%: 66,1; 82,5%).
Conclusión: La sensibilidad y especificidad de la MMT frente a la IHC fueron altas para RE, RP y HER2.

Introduction

In Spanish women, breast cancer is the most frequently diagnosed malignancy and the main cause of cancer deaths.[1] The treatment algorithm for breast cancer patients is complex and requires a multidisciplinary team of specialists who will opt for a therapeutical strategy according to the tumour localisation/severity and its biology (including biomarkers and gene expression).[2]

Therapeutic decisions are based on classical prognostic factors (tumoral grade, tumoral diameter, axillary status, etc) and largely molecular subtyping based on the evaluation of oestrogen receptor (ER/*ESR1*), progesterone receptor (PR/*PGR*), human epidermal growth factor receptor 2 (HER2/*ERBB2*), and proliferation marker Ki-67/*MKI67*.[3] The standard protein-based semiquantitative immunohistochemistry (IHC) assay[4] is the most frequently employed technique for subtyping because of its wide availability and low cost.[5] Also, IHC is, to some extent, an observer-dependent technique. It has been reported to display up to 20% inaccurate results (false positives or negatives) for ER, PR, and HER2.[6,7] Besides, the evaluation of Ki-67 by IHC carries high intra- and inter-observer variability, and even experienced pathologists have issues reproducing their results.[8,9] Especially difficult is the diagnosis of HER2-low and ultralow expression, increasingly necessary since the results of recent or "on going" trials such as Destiny Breast 04[10] and Destiny Breast 06.[11]

Although IHC has improved over the last decades, there is a need for more reliable, accurate, and less intrinsically limited techniques to determine breast cancer biomarkers.[12]

MammaTyper® (BioNTech Diagnostics, Mainz, Germany) (MMT) is an in vitro diagnostic test quantifying the mRNA expression of the abovementioned four marker genes based on reverse transcription-quantitative real-time polymerase chain reaction.[4]

We designed this study to evaluate MMT for the molecular subtyping of breast cancer and compare it to the classification obtained by conventional IHC. Our primary outcome was to evaluate the diagnostic accuracy of MMT compared to IHC for the molecular subtyping of the four biomarkers HER2/*ERBB2*, PR/*PGR*, ER/*ESR1*, and Ki-67/*MKI67*. Secondary outcomes included evaluating qualitatively (level of agreement with IHC, sensitivity, and specificity) the four biomarkers by MMT, particularly regarding the sensitivity of MMT to detect Ki-67 and low HER2 expression.

Materials and methods

Study design and setting

This observational, retrospective, single-centre study was conducted at the Vinalopó University Hospital, Alicante, Spain. We retrieved stored formalin-fixed paraffin-embedded (FFPE) breast cancer specimens for three months,

from September 2023 to December 2023, from the Hospital's Pathological Anatomy Service. The study was approved by the Clinical Research Ethics Committee of San Carlos University Hospital, Madrid, Spain and was conducted in agreement with the Declaration of Helsinki[13] and the Spanish law on biomedical research.[14] Since data from the retrieved samples were already anonymised, the patient's informed consent was unnecessary. Data confidentiality was always ensured by identifying patients with a code only available to the study investigators. The study followed the regulations of Spanish law in terms of data protection.[14,15] We reported our results in agreement with the STARD guidelines for diagnostic accuracy studies.[16]

Participants

We included all consecutive samples from patients >18 years old with an infiltrating breast carcinoma diagnosed between 2022 and 2023 and that had been analysed by IHC. We excluded all samples from pregnant women and patients with other neoplasia.

Test methods

We analysed the molecular subtyping of stored FFPE breast cancer specimens, including Luminal A-like, Luminal B-like, HER2-Luminal B, HER2-positive (HER2+), and Triple negative according to the St Gallen classification (Table 1).[17]

MammaTyper®: MMT, the index test, required the MammaTyper® kit and its specific web software (MammaTyper Report Generator). The tissue content of the invasive tumour had to be 20% at least (tissue sections of 10 μM), and the samples should not have been stored for longer than ten years. We extracted the RNA from the samples in FFPE by using validated extraction kits (RNeasy FFPE kit, article 73,504, Qiagen) and following the manufacturer's instructions, as previously described.[18] The MMT tests were undertaken by an external laboratory (Persona Biomed Spain, S.L.) on a Roche Cobas z® 480 qPCR analyser with the LightCycler® 480 in triplicates.

Immunohistochemistry: IHC was chosen as the reference standard since it remains the most extensively used technique in routine clinical practice for the molecular subtyping of breast cancer samples.[3] IHC had been performed before this study according to the local standard procedures for ER/*ESR1* (clone EP11, Dako-Agilent), PR/*PGR* (Clone PgR 1294, Dako-Agilent), HER2/*ERBB2* (Hercep-Test, Dako-Agilent), and Ki-67/*MKI67* (Clone MIB-1, Dako-Agilent).

Table 1 Molecular subtypes of breast cancer according to the St Gallen classification.[17]

	ER	PR	HER2	Ki-67
Luminal A-like	Pos	Pos	Neg	Low
Luminal B (HER2-neg)	Pos	Neg/Low	Neg	High
HER2-Luminal B	Pos	Pos/Neg	Pos	Any
HER2-E (HER2-enriched)	Neg	Neg	Pos	Any

ER: Oestrogen receptor; Neg: Negative; Pos: Positive; PR: Progesterone receptor.

We defined those samples with >1% stained nuclei as ER and PR positive (we considered them 'low' if the value was between 1% and 10%) and as Ki-67 'high' those with >20% stained nuclei (or 'low' otherwise). We considered HER2-positive the HER2 (3+) and HER2 (2+) amplified by fluorescence in situ hybridisation (FISH) samples, HER2-low those HER2 (2+) not amplified by FISH and HER2 (1+), and HER2-negative those HER2 (0). IHC results from eligible samples were retrieved from the patient's clinical history. After MMT analysis, a second reading of the IHC biomarkers studied (all of them) was performed in those cases where the results were discordant.

Statistical analysis and sample size

We described quantitative variables with measures of central tendency and dispersion and qualitative variables with absolute and relative frequencies, with 95% confidence intervals (CIs) for both variable types. We did not impute missing or lost values from available data. We measured global agreement or diagnostic accuracy in molecular subtyping between MMT and IHC by the 95% CI of the percentage of concurring results calculated with the exact method of Clopper-Pearson. We calculated the correlation in the five molecular subtypes between IHC and MMT with the Spearman correlation coefficient with its 95% CI. We set the statistical significance level at $P < 0.05$ and did not undertake multiplicity adjustments to control for type I errors. We carried out all analyses using R version 4.3.2 (2023-10-31 ucrt).

We expected the percentage of concurring results not to be under 90%. Therefore, we needed to include 108 samples for the inferior limit of the 95% CI of unilateral concurring results not to be under 90%, assuming a CI maximum extent of 5%. We calculated the CI using the exact method of Clopper-Pearson.

Results

Clinicopathological characteristics

A total of 109 samples were included in the study. The mean age of the patients when the sample was obtained was 62.1 years old (standard deviation: 14.1). The cases included are from either core biopsy, Assisted Vacuum Biopsy or surgical specimen. Most were taken by core needle biopsy or surgical specimen (64, 58.7%) and were either invasive no special type (49, 45.0%) or invasive ductal carcinoma (34, 31.2%) (Table 2).

Diagnostic accuracy

The global concordance of MMT with IHC was **76.15%.** MMT results were concordant with IHC in all samples identified as HER2-positive (4, 100%) or -Luminal B (11, 100%), in 45 (95.7%) samples classified as Luminal B-like and in 11 (84.6%) Triple-negative samples. However, MMT disagreed on 22 samples (64.7%) classified by IHC as Luminal A-like. Most of the disagreement was caused by the detection of Ki-67 expression by MMT, while IHC detected no expression on ten

Table 2 Clinicopathological parameters of the analysed breast cancer samples. Figures are absolute numbers (and %) unless otherwise stated.

	Overall (*N* = 109)
Sex	
Female	109 (100)
Age (years), *mean* (SD), *N* = 107	62.1 (14.1)
Sample type, *N* = 106	
Core needle biopsy	71 (66.9)
Vacuum-assisted biopsy	18 (17.1)
Surgical Specimens	17 (16.0)
Histology, *N* = 104	
Invasive No Special Type	49 (48.8)
Invasive ductal carcinoma	40 (38.4)
Invasive lobular carcinoma	4 (3.8)
Others	11 (10.6)
Tumoral stage, *N* = 92	
G1	43 (46.7)
G2	42 (45.7)
G3	7 (7.6)

SD: Standard deviation; TX: Primary tumour could not be assessed.

samples (45.5%). In addition, eight (36.4%) of the 22 discordant samples were classified as HER2-Luminal B (one [4.5%] because low levels of PR were detected and seven [31.8%] because HER2 expression was found) (Table 3).

Biomarker detection

The qualitative evaluation was undertaken based on the 2x2 contingency tables for each biomarker (Supplementary Table S1). For ER, MMT showed high positive agreement (96.3%, 95% CI: 90.9; 98.6%) with IHC, sensitivity (98.9%, 95% CI: 94.2; 99.8%), and specificity (81.2%, 95% CI: 57.0; 93.4%). The same was true for PR, with high positive agreement (89.9%, 95% CI: 82.8; 94.3%) with IHC, sensitivity (94.7%, 95% CI: 87.2; 97.9%), and specificity (78.8%, 95% CI: 62.2; 89.3%) of MMT. The best results were achieved in HER2 detection (positive agreement: 87.0%, 95% CI: 67.9; 95.5%, sensitivity: 100%, 95% CI: 74.1; 100%, and specificity: 75.0%, 95% CI: 46.8; 91.1%). For Ki-67, the sensitivity was high (91.5%, 95% CI: 82.8; 96.1%), but the specificity was low (47.4%, 95% CI: 32.5; 62.7%). However, MMT was able to detect 52.6% (95% CI: 43.2; 62.0%) more positives than IHC. Similarly, for HER2-low, MMT's sensitivity was high (90.5%, 95% CI: 81.7; 95.3%) but specificity was low (25.7%, 95% CI: 14.2; 42.1%), although, again, MMT detected 74.3% (95% CI: 66.1; 82.5%) more positives than IHC (Table 4).

Discussion

To the best of our knowledge, this was the first Spanish study to explore the level of agreement between MMT and IHC focused on Ki-67 and HER2-low. MMT was demonstrated to identify HER2-positive, HER2-Luminal B, Luminal B-like, and Triple-negative with high accuracy. However, MMT disagreed with IHC on two-thirds of Luminal A-like samples, mainly because of differences in Ki67 evaluation since MMT could better detect its expression. Likewise, the second important disagreement was in the evaluation of low HER2 levels where, again, MMT could be superior to IHC in identifying HER2-low cases. Overall, the advantages of MMT over IHC included its higher reproducibility (since different centres may use different IHC antibodies and pre-analytical conditions are variable) and objectivity, its quantitative nature, the reliability of source material (RNA from FFPE specimens, whereas IHC is based upon the analysis of much more labile proteins), and the shorter time to obtain the results (around eight hours), potentially enabling to inform the multidisciplinary team for therapeutical decisions on the same day of the biopsy diagnosis.

In our study, we used the MMT results to classify breast cancer patients into the five molecular subtypes determined by the St. Gallen classification,[17] which had not been extensively explored heretofore. MMT showed an excellent agreement with IHC with respect to HER2 and was even more sensitive than the reference method to detect HER2 expression, which may have therapeutical implications since more patients could benefit from anti-HER2 therapy if classified with MMT. However, disparities between MMT and IHC became apparent for Luminal A-like cases. Most of the discordance originated from the detection of Ki-67 expression by MMT in those samples, also illustrating a higher sensitivity of MMT than IHC towards this biomarker.

Table 3 Diagnostic concordance between MammaTyper® and IHC, *N* (%).

	Immunohistochemistry					**MMT Total**
	HER2-E (*N* = 4)	Lum. A (*N* = 34)	Lum. B (HER2−) (*N* = 47)	HER2-Lum. B (*N* = 11)	TN (*N* = 13)	
MammaTyper®						
HER2-E	4 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4
Lum. A	0 (0.0)	12 (35.3)	1 (2.1)	0 (0.0)	0 (0.0)	13
Lum. B (HER2−)	0 (0.0)	14 (41.2)	45 (95.7)	0 (0.0)	2 (15.3)	61
HER2-Lum. B	0 (0.0)	8 (23.5)	1 (2.7)	11 (100)	0 (0.0)	20
TN	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	11 (84.6)	11
Agreement	4 (100)	12 (35.3)	45 (95.7)	11 (100)	11 (84.6)	–
Disagreement	0 (0.0)	22 (64.7)	2 (4.3)	0 (0.0)	2 (15.4)	–

IHC: Immunohistochemistry; HER2-E: HER2-enriched; Lum. A: Luminal A-like; Lum. B: Luminal B; MMT: MammaTyper®; TN: Triple-negative.

Table 4 Qualitative assessment of Mammatyper® vs. immunohistochemistry. Figures are % (and 95% CI) unless otherwise stated.

	HER2-low [a]	HER2	Ki-67	PR	ER
Positive agreement	69.7 (60.5; 77.6)	87.0 (67.9; 95.5)	76.1 (67.3; 83.2)	89.9 (82.8; 94.3)	96.3 (90.9; 98.6)
Disagreement	30.3 (22.4; 39.5)	13.0 (4.5; 32.1)	23.9 (16.8; 32.7)	10.1 (5.7; 17.2)	3.7 (1.4; 9.1)
Sensitivity	90.5 (81.7; 95.3)	100 (74.1; 100)	91.5 (82.8; 96.1)	94.7 (87.2; 97.9)	98.9 (94.2; 99.8)
Specificity	25.7 (14.2; 42.1)	75.0 (46.8; 91.1)	47.4 (32.5; 62.7)	78.8 (62.2; 89.3)	81.2 (57.0; 93.4)
False negative	9.5 (4.0; 15.0)	0.0 (0.0; 0.0)	8.5 (3.3; 13.7)	5.3 (1.1; 9.5)	1.1 (−0.9; 3.1)
False positive	74.3 (66.1; 82.5)	25.0 (7.3; 42.7)	52.6 (43.2; 62.0)	21.2 (13.5; 28.9)	18.8 (11.5; 26.1)
PPV	72.0 (62.2; 80.1)	78.6 (52.4; 92.4)	76.5 (66.4; 84.2)	91.1 (82.8; 95.6)	96.8 (91.1; 98.9)
NPV	56.2 (33.2; 76.9)	100 (70.1; 100)	75.0 (55.1; 88.0)	86.7 (70.3; 94.7)	92.9 (68.5; 98.7)
Kappa coefficient	0.189 (0.116; 0.263)	0.742 (0.564; 0.921)	0.424 (0.332; 0.517)	0.754 (0.674; 0.835)	0.844 (0.776; 0.912)

CI: Confidence interval; ER: Oestrogen receptor; NPV: Negative predictive value; PPV: Positive predictive value; PR: Progesterone receptor.

[a] Defined as HER2 (1+) and HER2 (2+) amplified or not by fluorescence in situ hybridisation.

Our results suggest that IHC may underestimate proliferation, potentially leading to underclassification of Luminal B-like biology. This has prognostic and therapeutic implications, as Ki-67 $\geq$ 20% correlates with higher recurrence risk. Our results were partially in agreement with those previously published, where not only Luminal A-like, but especially Luminal B-like samples, showed the most discrepancies between these two molecular subtyping techniques.[5] In any case, our results seem to resolve inconsistencies in the immunohistochemical evaluation of Ki67, either by over- or under-staining of tissue.

When analysing each biomarker separately, the level of positive agreement for ER (96.3%) was one of the highest reported, with high sensitivity and specificity. An investigation from Stefanovic et al. on biomarker changes between primary and metastatic breast cancer tumours described an 81.0% ER/*ESR1* concordance between MMT and IHC.[19] Likewise, Wallwiener et al. found that the technical limitations of IHC were apparent when analysing primary sites and metastases, and that MMT was more sensitive and consistent in both sites, especially for ER determination.[20] In addition, two posters by Teng et al.[21] and Shaaban et al.[22] showed high overall percentages of agreement between MMT and IHC for ER (95.4%[21] and 95.5%[22]), even when using low cut-off values calibrated against $\geq$1% IHC staining.[21]

Similarly, the concordance of PR/*PGR* (89.9%) found in our study was on the high end of those previously published. Saracchini et al. found moderate levels of agreement between MMT and IHC for PR (76.3%), but their sample size was modest (*N* = 76).[23] Fasching et al. have also reported moderate percentages of concordance for PR (82.4%),[24] which tended to be higher in other studies, such as those from Teng et al. (91.1%)[21] or from Sinn et al. (92.9%).[25] Although these percentages could always be considered high, the discrepancies could be explained by sampling issues (small amounts of invasive carcinoma or small tumour content), weak ER and PR expression, and the inclusion of normal mammary tissue.[26] The sensitivity and specificity of MMT found in our study were also strong.

The positive agreement of HER2/*ERBB2* observed in our study was good (87.0%), taking into account that some studies have reported higher values, up to 100%.[19,20] The sensitivity was excellent but at the expense of a moderate-high specificity. Interestingly, Stefanovic et al. found a lower percentage of agreement in metastatic than in primary tumours for HER2 and correlated the higher rate of HER2 expression in metastatic tumours (initially Triple negative primary tumours) with brain and bone metastases, implying a benefit of real-time HER2 monitoring techniques, such as MMT, over standard IHC.[19]

HER2-low is not considered a separate biological subtype, and HER2 (1+) and (2+) not FISH-amplified samples were considered HER2-negative in clinical practice until recently.[27] However, the prolonged progression-free and overall survival observed in HER2-low patients treated with trastuzumab deruxtecan support studying HER2-low as a separate clinical entity.[26] Unfortunately, the discrimination between HER2 (0)—negative—and HER2 (1+)—HER2-low—with IHC is challenging and poorly reproducible.[28] Our results showed that MMT reclassifies as HER2-low 74.3% of samples identified as HER2-negative by IHC, which is higher than previously reported by Badr et al. (55.6%)[26] and by Teng et al. (45.2%).[29] This may have therapeutic implications since HER2-low patients have been described to have a better prognosis than HER2-negative in the medium-term.[30]

Regarding Ki-67/*MKI67*, current guidelines do not consider it a reliable factor for implementation in clinical practice due to its lack of reproducibility, and experts advise to pay careful attention to preanalytical issues and to calibrate standardised visual scoring in IHC.[31] In our study, the modest agreement found between MMT and IHC for Ki-67 (76.1%) was in line with values previously reported by Wirtz et al. (75.0%),[5] highlighting the robustness of the MMT method.

Our study should be interpreted in light of its limitations, mainly originating from its single-centre and retrospective design; if other antibodies had been used for IHC, the concordance rates would have been different. Regarding the MMT test procedure, the need to run eight samples to leverage its price and the requirement of well-fixed RNA in FFPE samples (otherwise prone to false negatives) are its main disadvantages compared to standard IHC.

Conclusion

MMT was a quantitative and simple procedure for molecular subtyping of breast cancer patients. The sensitivity and specificity of MMT versus IHC were high for ER, PR, and HER2.

Remarkably, MMT could improve the definition and identification of Luminal A- and B-like and HER2-low tumours compared to IHC, thanks to its higher sensitivity towards HER2 and Ki-67. Finally, we consider that our results and previous findings support a future prospective study in our setting comparing the molecular subtyping of breast cancer patients between MMT and IHC and a prospective, well-designed trial to the MMT value as a predictive test.

Funding

Sysmex España, S.L. funded the medical writing, statistics and the MammaTyper Kits free of charge for this study.

CRediT authorship contribution statement

Laia Bernet-Vegué: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Supervision. **Stella Peláez Malagón:** Investigation, Resources, Writing – review & editing. **Marina Berna Rubio:** Patholigist Assistant.

Conflict of interest

Laia Bernet-Vegué is an Associate Editor of Revista de Senología y Patología Mamaria and the rest of the authors declare there are no conflicts of interest.

Acknowledgements

The authors would like to thank Matías Rey-Carrizo, PhD, and Sara Cervantes, PhD, for their medical writing support on behalf of i2e3 ProComms.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.senol.2025.100706.

Data availability statement

The data supporting the findings of this study are available from the corresponding author (L. B.-V.) upon reasonable request and with permission of Vinalopó University Hospital, Alicante, Spain.

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