



La importancia de la cuantificación de los marcadores inmunohistoquímicos mediante la patología digital

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University of Navarra





Sumario

- **Relevancia de la cuantificación inmunohistoquímica para el desarrollo de la Patología.**
- **Condicionantes de la cuantificación en Patología.**
- **Sobre la cuantificación de biomarcadores hoy:**
 - **Diversos procedimientos de cuantificación.**
 - **Cuantificación mediante análisis de imagen.**
 - **Obtención de “immunoscores”.**
 - **Cuantificación mediante análisis multiespectral y moderna tecnología.**
- **Experiencia propia en la cuantificación mediante patología digital: Ejemplos.**
- **Conclusiones.**

Gran reto: De una Patología cualitativa a una Patología cuantitativa.

QUANTIFY
IT!

TODAY

Biomarcadores:

Actividad proliferativa: Ki67.

Predictivos de respuesta a la terapia: RE, RP, HER2...

Inmunoterapia: PD1, PD-L1, CTLA4, TILs...

Densidad vascular: CD31.

Marcadores de mutación: p53.

Otros...

Quantificación inmunohistoquímica de biomarcadores: "Immunoscores" precisos, reproducibles, rápidos y con aplicación clínica en el Cáncer.

Science

AAAS

Type, Density, and Cells Within Human Predict Clinical Outcome

Jérôme Galon,^{1*} Anne Costes,¹ Fatima Sanjaï,¹
Christine Lagorce-Pagès,³ Marie Tosolini,¹ Muriel
Franck Zinzindohoué,⁵ Patrick Bruneval,⁶ Paul
Wolf-Herman Fridman,^{1,7} Franck Pagès^{1,7}†

29 SEPTEMBER 2006 VOL 313

Journal of Pathology

J Pathol 2014; 232: 199–209

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(wileyonlinelibrary.com) DOI: 10.1002/path.4287

INVITED REVIEW

Towards the introduction of the 'Immunoscore' in the classification of malignant tumours

Jérôme Galon,^{1,2*} Bernhard Mlecnik,^{1,2,3} Gabriela Bindea,^{1,2,3} Helen K Angell,^{1,2,3} Anne Berger,⁴ Christine Lagorce,³
Alessandro Lugli,⁵ Inti Zlobec,⁶ Arndt Hartmann,⁷ Carlo Bifulco,⁸ Iris D Nagtegaal,⁹ Richard Palmqvist,¹⁰ Giuseppe
V Masucci,¹¹ Gerardo Botti,¹² Fabiana Tatangelo,¹² Paolo Delrio,¹³ Michele Maio,¹⁴ Luigi Laghi,¹⁵ Fabio Grizzi,¹⁶
Martin Asslaber,¹⁴ Corrado D'Arrigo,¹⁷ Fernando Vidal-Vanaclocha,¹⁸ Eva Zavadova,¹⁹ Lotfi Chouchane,²⁰ Pamela
S Ohashi,²¹ Sara Hafezi-Bakhtiari,²² Bradley G Wouters,²² Michael Roehri,²³ Linh Nguyen,²⁴ Yutaka Kawakami,²⁵
Shoichi Hazama,²⁶ Kiyotaka Okuno,²⁷ Shuji Ogino,²⁸ Peter Gibbs,²⁹ Paul Waring,³⁰ Noriyuki Sato,³¹ Toshihiko
Torigoe,³¹ Kyogo Itoh,³² Prabhu S Patel,³³ Shilin N Shukla,³³ Yili Wang,³⁴ Scott Kopetz,³⁵ Frank A Sinicrope,³⁶
Viorel Scripcariu,³⁷ Paolo A Ascierto,³⁸ Francesco M Marincola,³⁹ Bernard A Fox,^{40,41} and Franck Pagès^{1,2,3,42}

SCIENCE www.sciencemag.org



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Idoate M et al.

Condicionantes de la cuantificación de la ihq en Patología.

HER2 image algorithms



Algorithms for QIA

RESEARCH	CLINICAL
Many apps	Limited algorithms
Modifiable parameters	Locked down apps
Lab developed tests	Approved (FDA)
Research environment	Regulated lab (CLIA)
Continuous data	Discrete results
Variable output	Match manual scores
Researchers	Pathologist oversight
Financial benefit	Questionable ROI (CPT code)
Stand-alone system	Integrated workflow
Widespread use	Slow adoption

Table 1. The Total Test, adapted from the earlier proposal of the US Biologic Stain Commission⁴ and modified from 5

The Total Test

Pre-analytical

Specimen handling, from operating room to histology lab

Fixation: total fixation time and type of fixative

Paraffin embedding, storage and sectioning

Deparaffinization

Analytical

Antigen retrieval (exact method)

Assay (staining) method and protocol

Reagent validation

Controls (Reference Standards)

Technologist and laboratory certification

Proficiency testing and quality assurance

Post-analytical

Reading of result(s)/scoring/quantification

Report

Turn-around time

Outcomes analysis/economics/reimbursement

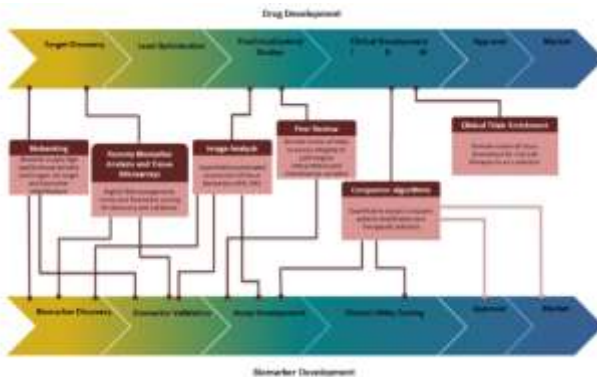


Fig. 9. The role of digital pathology in drug development and competitive biomarker discovery and validation.

Histopathology 2006, 49, 411-424. DOI: 10.1111/j.1365-2559.2006.02511.x

EXPERT OPINION

Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II

C R Taylor & R M Levenson¹

Department of Pathology, Keck School of Medicine of the University of Southern California, Los Angeles, CA and

¹Cambridge Research and Instrumentation Inc., Woburn, MA, USA

Methods (2014) 10, 71

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Digital pathology and image analysis in tissue biomarker research

Peter W. Hamilton¹, Pyter Barkhead, Yirhai Wang, Ryan Hutchinson, Declan Keenan, Darragh C. McArt, Jacqueline James, Manuel Saborido-Tellez

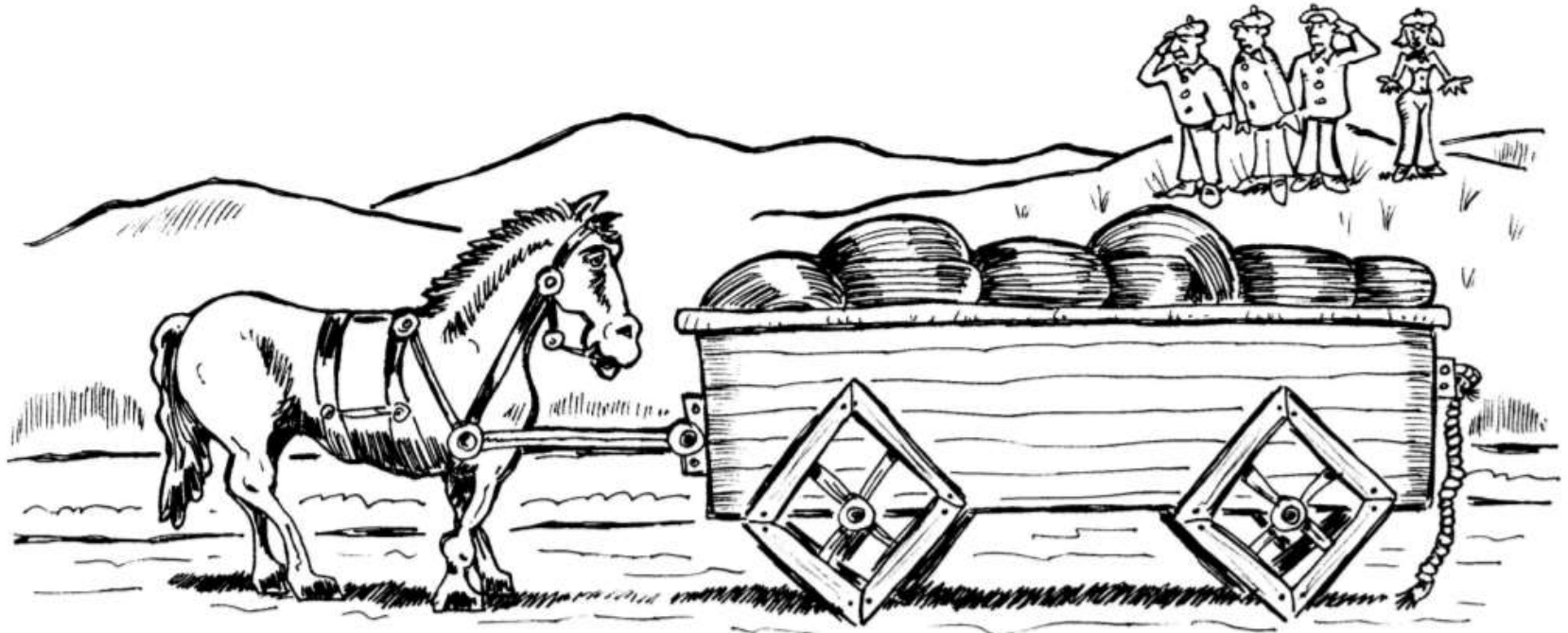
Center for Clinical Research in Cell Biology, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, United Kingdom



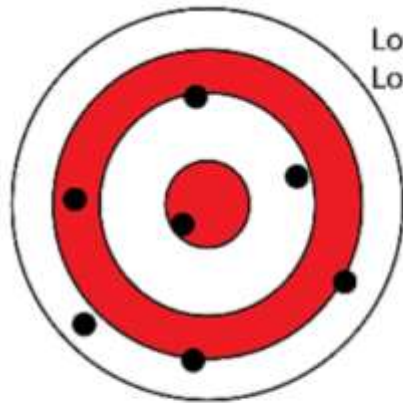
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Condicionantes: La cuantificación de la inmunohistoquímica por digitalización no avanza a suficiente ritmo



Cuantificación: Precisión. Reproducibilidad. Automatización. Eficiencia.



Low accuracy
Low precision

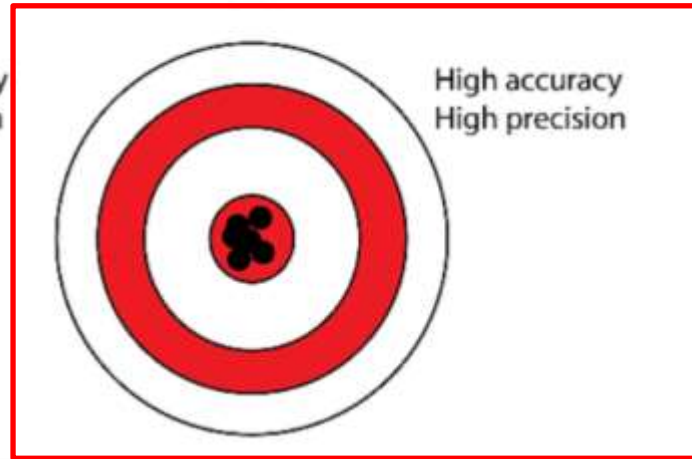


Low accuracy
High precision

Precisión y reproducibilidad



High accuracy
Low precision



High accuracy
High precision

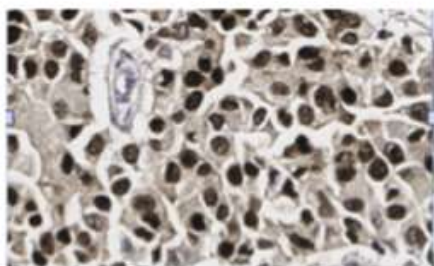
Automatización



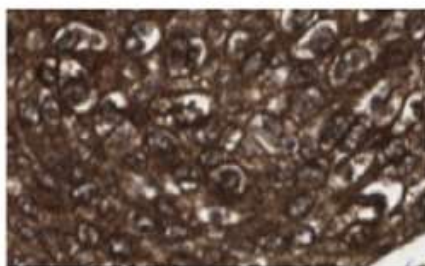


Cuantificación ihq de biomarcadores: tres tipos de inmunoexpresión

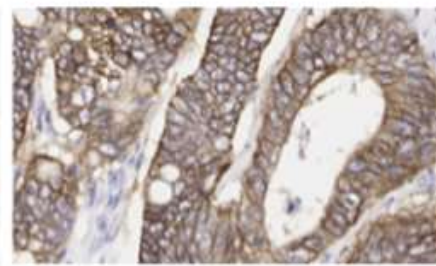
Nuclear Staining



Cytoplasmic Staining



Membrane Staining



Methods 2010, 12(1):1-14

Contents lists available at [BioRxiv](#)

Methods

Journal homepage: www.sagepub.com/journals/permissions

Antibody validation of immunohistochemistry for biomarker discovery: Recommendations of a consortium of academic and pharmaceutical based histopathology researchers

William J. Hewat^{1,2}, Arthur Lewis³, Philippa Jones⁴, Caroline Kampf⁵, Fredrik Pontén⁶, Chris M. van der Loos⁷, Neil Gray⁸, Chris Wornach⁹, Anthony Warford¹⁰

¹ Cancer Research UK Cambridge Institute, University of Cambridge, 41 Avenue Road, Cambridge CB2 0RE, UK
² Addenbrooke's Hospital, Cambridge CB2 3RQ, UK
³ ICS, Addenbrooke's Hospital, 21 Chaucer Street, London EC2A 4DF, UK
⁴ Department of Oncology, Immunology and Pathology, Maudsley Laboratories, St George's University, Upperville, Surrey
⁵ Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands
⁶ AstraZeneca Oncology Research, Macclesfield, Cheshire, UK
⁷ University of Groningen, Groningen, The Netherlands



Inmunohistoquímica cuantitativa en Patología

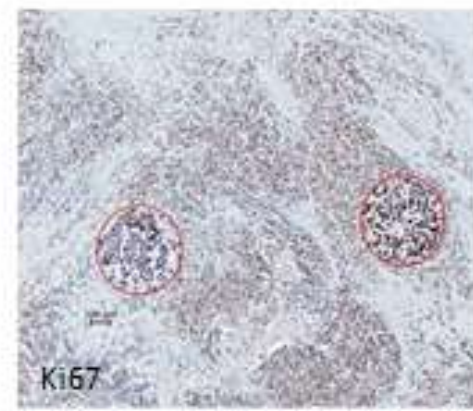
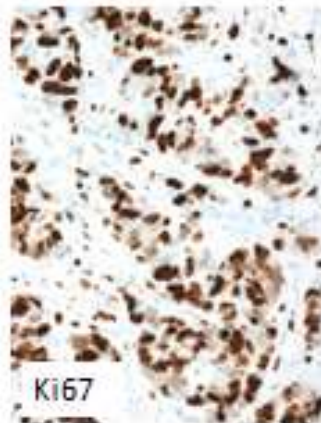
- **Proporción (%) de células inmunopositivas con/sin patrones de referencia.**
- **Cuantificación :**
 - **Mediante análisis automatizado de la imagen.**
- **Cuantificación mediante análisis de imagen multispectral.**



Inmunohistoquímica cuantitativa:

Proporción células inmunopositivas: la subjetividad en la cuantificación.

Cuantificación y heterogeneidad intratumoral en la expresión de la proteína Ki67



Assessment of Ki67 in Breast Cancer: Recommendations from the International Ki67 in Breast Cancer Working Group

Mich Ditsch, Terence D. Nislan, Roger A'Hern, John Bartlett, R. Charles Coombes, Jack Gunkel, Matthew Ellis, R. Lynn Herby, Judith C. Hugh, Tracy Leadley, Lisa McShane, Scott Pak, Frederique Penault-Llorca, Lyubmila Prudkin, Meredith Regan, Jerome Sater, Christina Schryer, Ian E. Smith, Giuseppe Viale, Jo Anne Zujewski, Daniel F. Hayes

Manuscript received March 14, 2011; revised September 1, 2011; accepted September 2, 2011.

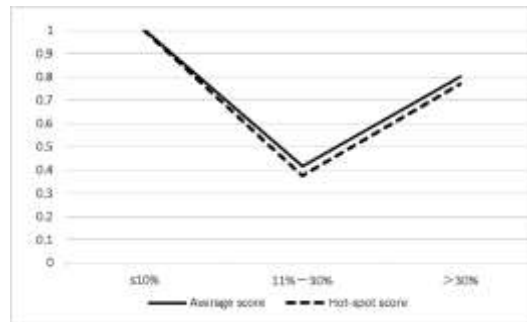
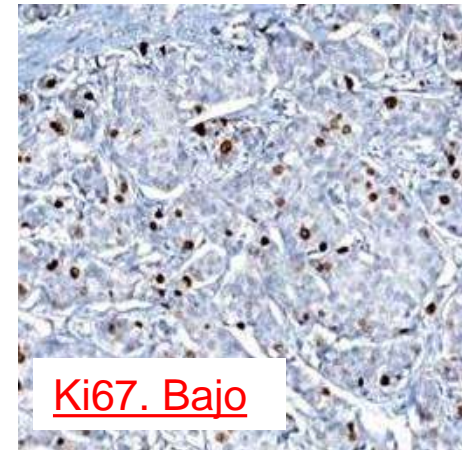
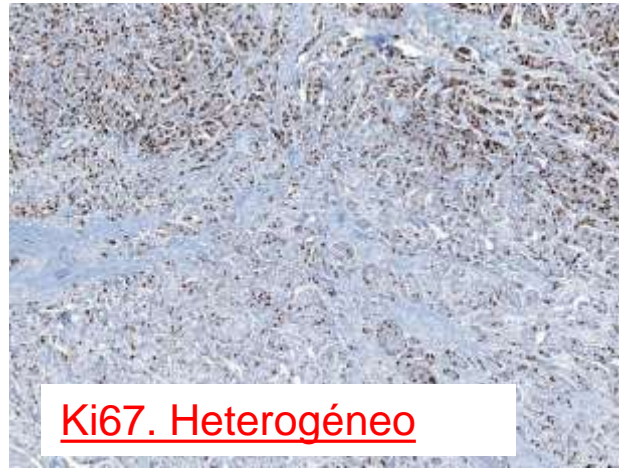
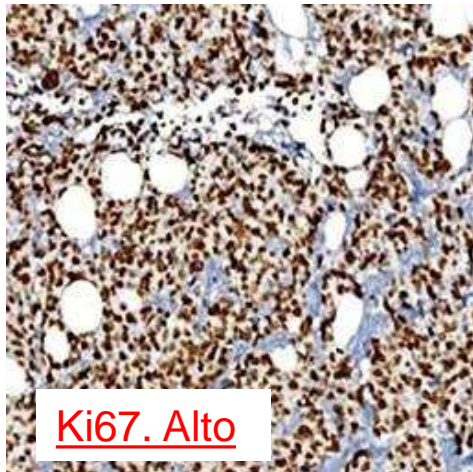


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Inmunohistoquímica cuantitativa:

Proporción células inmunopositivas.

¿Cuándo la cuantificación es compleja?



Dificultades en la evaluación en resultados en el rango 10%-30%.

Inmunohistoquímica cuantitativa:

Proporción células inmunopositivas según patrones de referencia.

Estimación respuesta patológica según sistema RCB. Web Based Calculator. MD Anderson. USA.

*Values must be entered into all fields for the calculation results to be accurate.

(1) Primary Tumor Bed

Primary Tumor Bed Area: (mm) X (mm)

→ Overall Cancer Cellularity (as percentage of area): (%)

Percentage of Cancer That Is *in situ* Disease: (%)

(2) Lymph Nodes

Number of Positive Lymph Nodes:

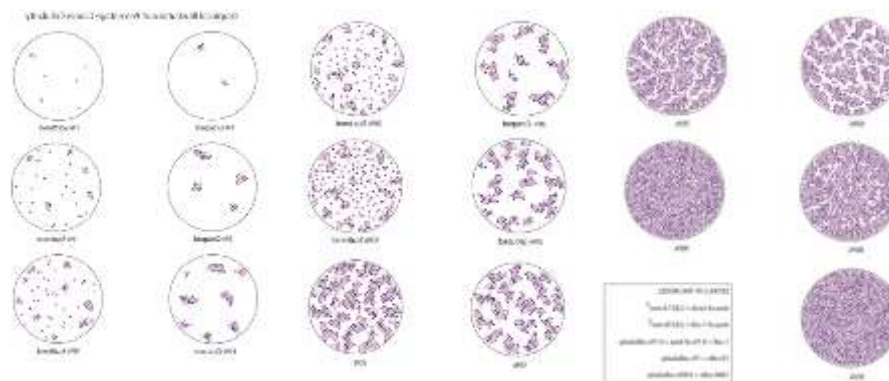
Diameter of Largest Metastasis: (mm)

Reset

Calculate

Residual Cancer Burden:

Residual Cancer Burden Class:



$$RCB = 1.4 (d_{prim} \times f_{inv})^{0.17} + [4 (d_{met} \times (1 - 0.75^{LN}))]^{0.17}$$

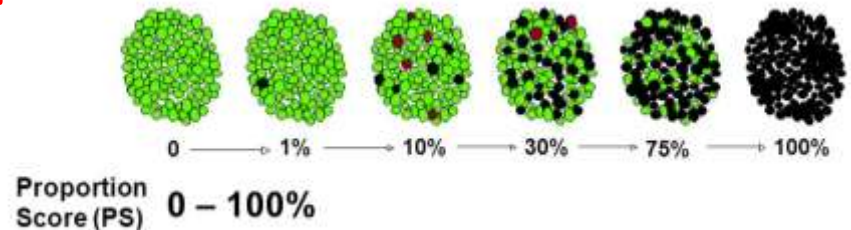
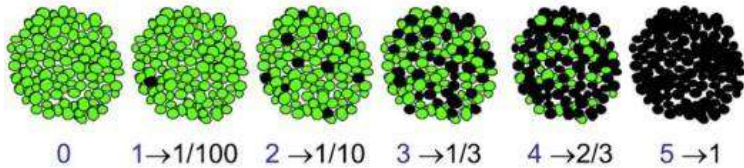
<http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3>

Inmunohistoquímica cuantitativa:

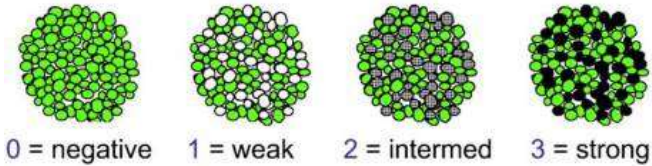
Proporción células inmunopositivas según patrones de referencia estimativos.

Allred score = Densidad + /x Intensidad

A Proportion Score (PS)



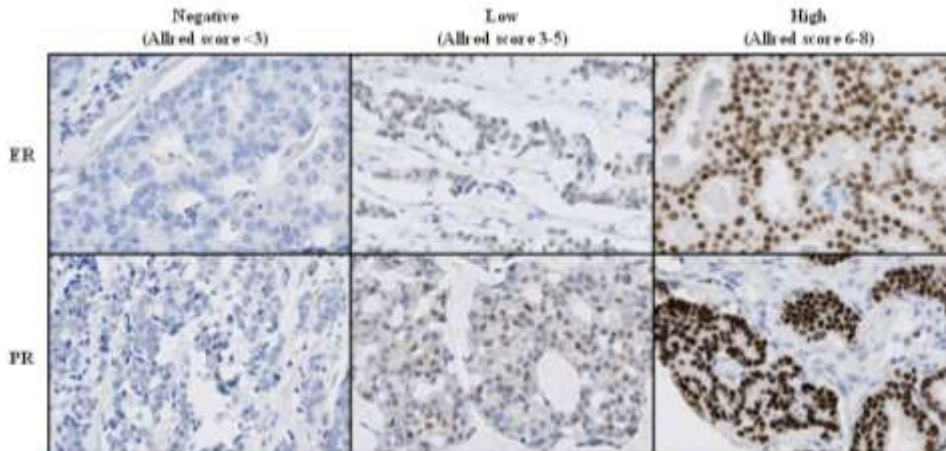
B Intensity Score (IS)



Allred Score = PS + IS (range 0-8)

Score range: 0-300

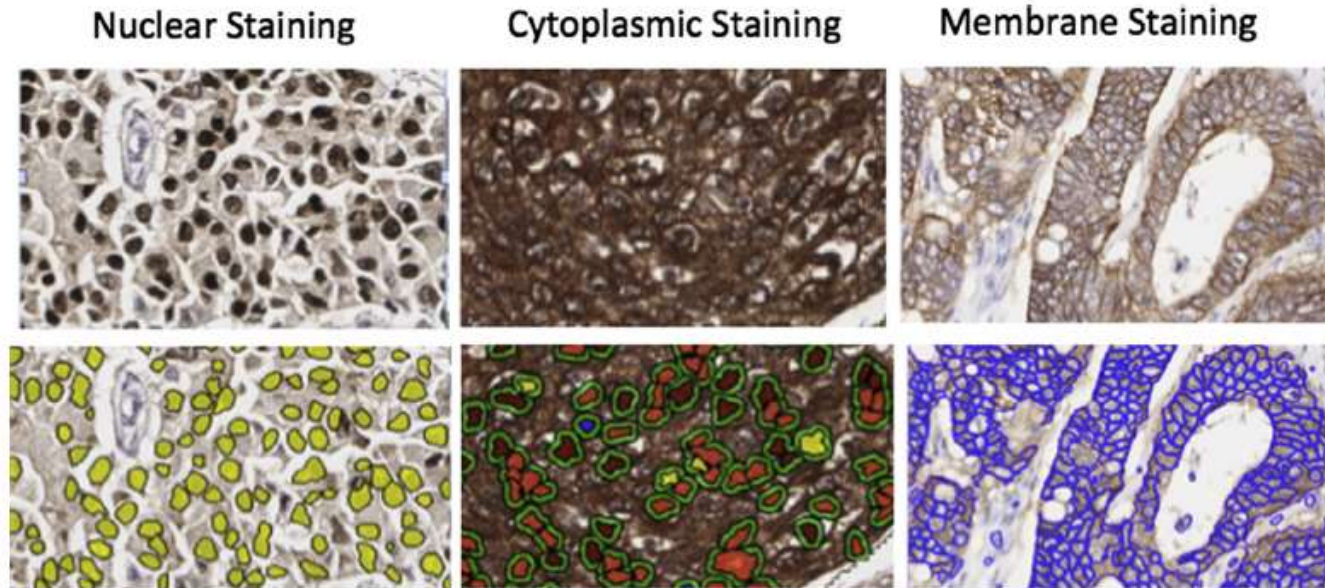
(osi) oncology







Cuantificación de biomarcadores: tres tipos de inmunoexpresión.



Methods 2010, 11(10):1-14

Contents lists available at BioRxiv.org

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Journal homepage: www.sagepub.com/journals/permissions.nav

Antibody validation of immunohistochemistry for biomarker discovery: Recommendations of a consortium of academic and pharmaceutical based histopathology researchers

William J. Horvat^{1,2}, Arthur Lewis³, Philippa Jones⁴, Caroline Kampf⁵, Fredrik Pontén⁶, Chris M. van der Loos⁷, Neil Gray⁸, Chris Wornatka⁹, Anthony Welford¹⁰

¹Proctor Research and Diagnostic Services, University of Cambridge, 41 Antrix Centre, Cambridge CB2 3RQ, UK
²Addenbrooke's Hospital, Cambridge CB2 3RQ, UK
³ICL Advanced Diagnostics, 27 Chiswick Street, London W6 7SE, UK
⁴Department of Histopathology, Immunology and Pathology, MRC Centre for Biomarker Research, 100 Brook Street, Liverpool, L69 3GB, UK
⁵Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands
⁶Amsterdam University Research Institute, Amsterdam, The Netherlands
⁷University of Groningen, Groningen, The Netherlands



Automatización del análisis cuantitativo de la imagen mediante programas específicos.

Software Type	Image Format Compatibility	Technical Knowledge Level	Customization Level	Features	Examples
Basic Science Image Analysis	Most Image Formats	Moderate	High	Variety of measurement tools Access to image processing tools Some automation	Image Pro Premier Metamorph ImageJ/FIJI Cell Profiler
Slide Scanner Based	Limited Image Formats	Low	Low-Moderate	Direct access to images Access to common algorithms US IVD for HER2/ER Pattern recognition Batch processing Designed for Digital Pathology	Roche/Ventana Leica/Aperio 3D Histech HALO PathXL TissueMark
Digital Pathology Inspired	Most Image Formats	Moderate	Moderate	Workflow based Easily adjustable parameters Batch processing Pattern recognition Access more feature data Designed for Digital Pathology	InForm, Visiopharm Definiens Tissue Studio
Algorithm Based	Most Image Formats	High	High	Fully customizable Unique algorithms Even more feature data Batch processing	MatLab Visiopharm Definiens Developer



Inmunohistoquímica cuantitativa:

Cuantificación mediante procedimientos automáticos (web-based procedures)



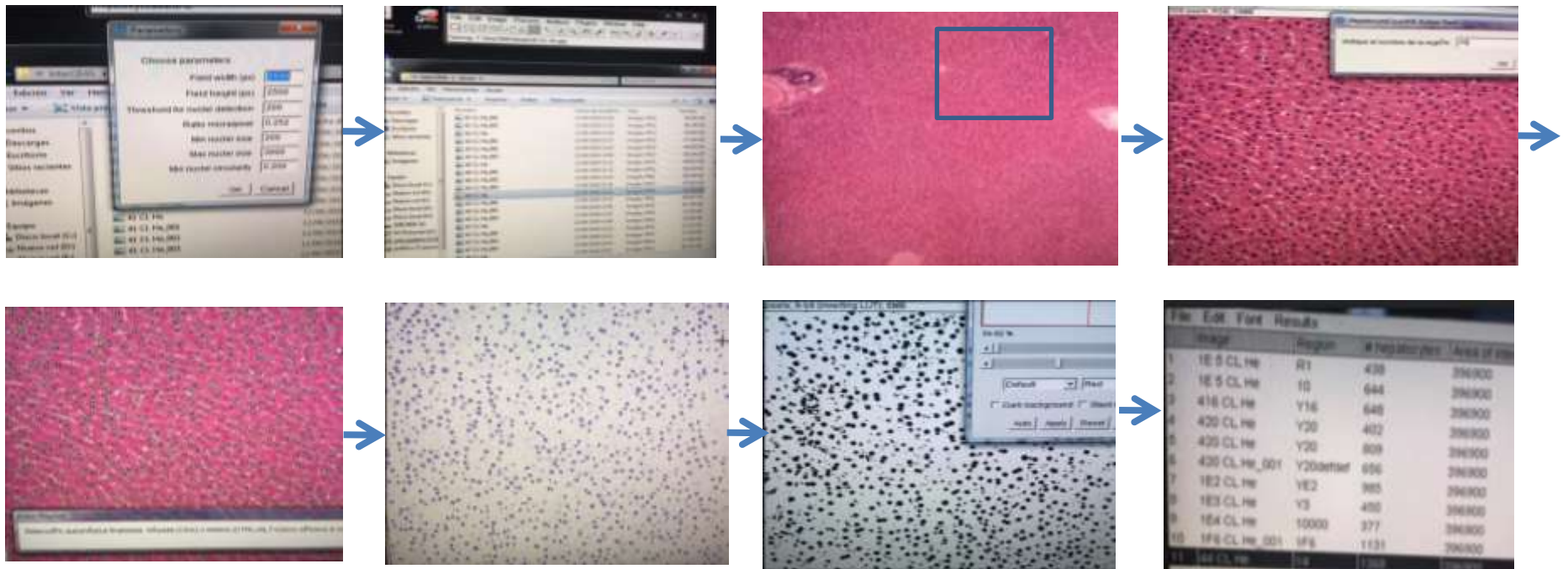
The screenshot shows the homepage of the ImageJ software project. At the top, there is a navigation bar with links for 'HOME', 'ABOUT', 'DOWNLOADS', 'LEARN', 'DEVELOP', 'NEWS', 'EVENTS', 'STATUS', and 'HELP'. Below this, a 'WELCOME' section features a microscope icon and the 'IMAGEJ' logo with the tagline 'An open platform for scientific image analysis'. Three prominent buttons are displayed: 'Download' (blue), 'Learn' (green), and 'Develop' (red). A text box below these buttons states: 'ImageJ is an open source image processing program designed for scientific multidimensional images. ImageJ is highly extensible, with thousands of plugins and scripts for performing a wide variety of tasks, and a large user community.' Further down, a 'Welcome to the Wiki' section indicates that the wiki documents all aspects of the ImageJ ecosystem, including 'ImageJ', 'Igor', 'Fiji', and 'Bio-Formats'. A 'Tools & Features' section lists key attributes: 'Open source' (source code is freely available), 'Reproducible' (tools like Script Editor and personal update sites help with workflow), and 'Interoperable' (ImageJ works with various software like KNIME, ITC, MATLAB, and scientific literature).

<https://imagej.net/Welcome>



Inmunohistoquímica cuantitativa: Cuantificación mediante procedimientos automáticos (web-based procedures)

Software original diseñado para la cuantificación de hepatocitos (núcleos) teñidos mediante H&E.



Estudio experimental de la hiperplasia hepatocitaria del lóbulo caudado tras tratamiento con partículas marcadas con Ytrio radioactivo.

Con la colaboración del Servicio de Morfometría e Imagen. Universidad de Navarra.


Example result:

(ER), prote application

ImmunoRatio regions from area over to image match

For the anal [ImmunoMe](#)



Main featu

 **Based**
Uses col
separati
(Ridler e

 **Cross-**
Supports
(Microso
and web
Mozilla F



 **Cam**
The Cam
in findi
contrast

ImmunoRatio

Language:  

[Introduction](#) [Basic mode](#) [Advanced mode](#) [Help](#) [About](#)

Example result:

Language:  

ImmunoRatio

Sample ID: example-ki67
Date: 16.11.2009 10:26
DAB / nuclear area: 14%

Original image

Pseudo-colored image showing staining components

Pseudo-colored image showing staining components

Microscope image file(s):
Seleccionar archivo: Ningún archivo seleccionado
[Add another image]

Sample identifier (optional):
[Use]

Define a region of interest (ROI) prior to analysis

Analysis settings

Blankfield correction image (optional):
Seleccionar archivo: Ningún archivo seleccionado

Image scale, pixels / μm : 0.0 (0 = auto-detection)

Brown threshold adjustment: 0 (-50 ... +50)

Blue threshold adjustment: 0 (-50 ... +50)

Result correction equation:
 $y = 6.442E x^3 + 0.0011 x^2 + 0.611 x + 0.4321$
[Reset default values]

Results table

Analyze Camera Adjustment Wizard Send feedback

Disclaimer: This image analysis software (ImmunoRatio) is a diagnostic aid for personnel trained to score immunostained samples. Analysis results should always be interpreted together with the pseudo-colored images and the original slides. The developers of ImmunoRatio expressly disclaim any and all liability for any loss or damage that may arise directly or indirectly by using the software or content available.

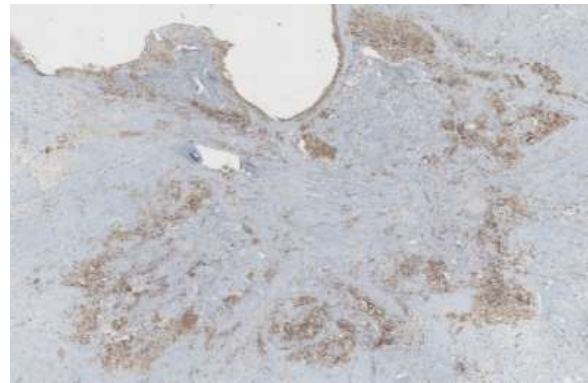
[Click here to start analyzing your images!](#)

<http://153.1.200.58:8080/immunoratio/>



Cuantificación de la expresión inmunohistoquímica de múltiples biomarcadores tisulares realizado mediante diferentes tecnologías y su aplicación clínica

Cuantificación de la expresión inmunohistoquímica mediante análisis de imagen automatizado apoyado en la tecnología.





Cuantificación de la expresión inmunohistoquímica de los biomarcadores tisulares mediante análisis automatizado de la imagen apoyado en la tecnología.

High lymphocytic infiltration in breast cancer predicts a better prognosis and a better response to neoadjuvant chemotherapy.

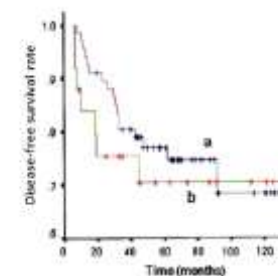
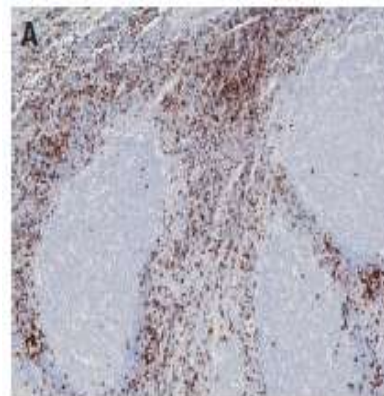
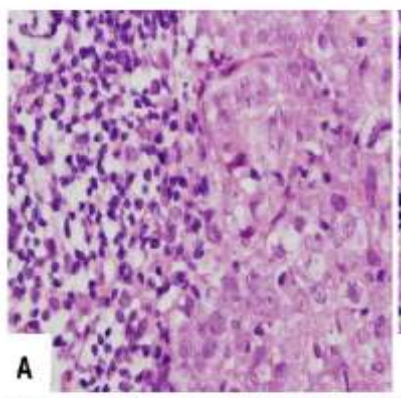
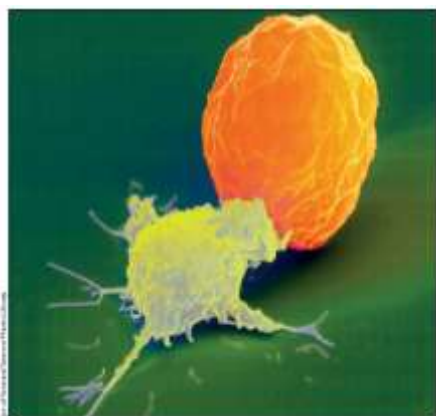


Fig. 4 Disease-free survival curves for patients with primary triple-negative breast cancer (TNBC) after neoadjuvant chemotherapy, stratified by the score of tumor-infiltrating lymphocytes (TIL). A High TIL score group ($n = 87$), B Low TIL score group ($n = 25$). Although the 5-year disease-free survival rate was slightly higher in the high TIL score group (77%) than in the low TIL score group (70%), these two curves did not differ significantly ($P = 0.58$).

Loi S et al. J Clin Oncol 2013.

Denkert G et al. J Clin Oncol 2010

Salgado R & Loi S. The lancet 2018

García Martínez E et al. Breast Cancer Research 2014.

www.lancetjournals.com/lancetonline/

Oncotarget, Vol. 7, No. 28

Research Paper

Tumor-infiltrating lymphocytes in breast cancer predict the response to chemotherapy and survival outcome: A meta-analysis

Ke Wang¹, Jianjun Xu¹, Tao Zhang¹, Dan Xue¹

¹Department of Gynecology, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, 310008, China

²School of Finance, Zhejiang University of Finance and Economics, Hangzhou, 311018, China

³Department of Plastic Surgery, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, 310009, China

Correspondence to: Dan Xue, email: xuedan@zhu.edu.cn

Keywords: breast cancer, lymphocyte infiltration, pathological complete response (PCR), prognosis, meta-analysis

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Ono et al. Breast Cancer res Treat 2012.



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Cuantificación de la expresión inmunohistoquímica de los biomarcadores tisulares mediante análisis automatizado de la imagen

Cuantificación múltipels TILs estromales para valorar el efecto de las vacunas respecto a controles pre/post tratamiento en el cáncer de mama.

160 paired pre (needle-core biopsies) and post-treatment samples (surgical specimens) of breast ductal invasive carcinomas classified as subtypes:

luminal A (24 cases),

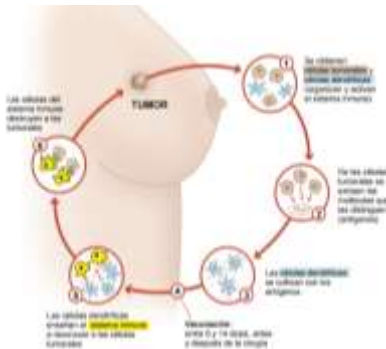
luminal B (30 cases)

triple-negative (26 cases)

localized, stages II and III, period 2011-15, corresponding to 80 patients, distributed in two groups:

Vaccine: vaccine-neoadjuvant (vaccine group), 36 patients

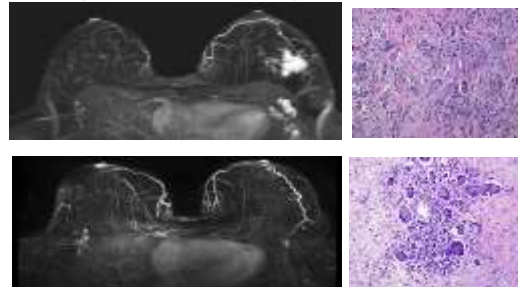
Control; only neoadjuvant treatment (control group), 44 patients



Grupo Vacunas/Grupo Control

P
r
e

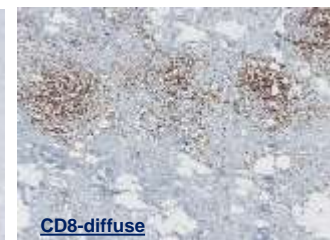
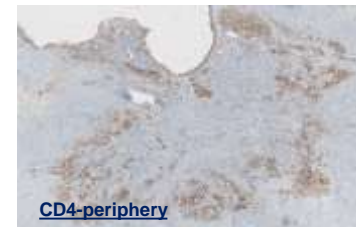
P
o
s
t



TILs estromales

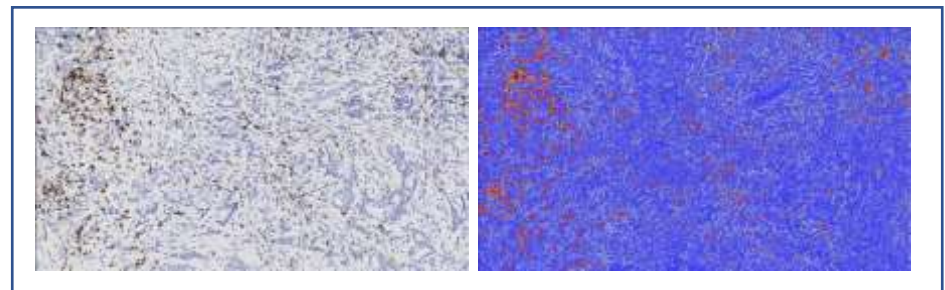
Control

Vacuna



Cuantificación de la expresión inmunohistoquímica de los biomarcadores tisulares mediante análisis automatizado de la imagen

- **Assessment of adequate staining.**
- **A previous validation of this procedure.**
- **An automatized scanning of immunostaining slides (at 20x) by ACIS III was used.**
- **Scanning of a number of fields (1-11 fields) depending of how extensive or heterogeneous the infiltration was.**

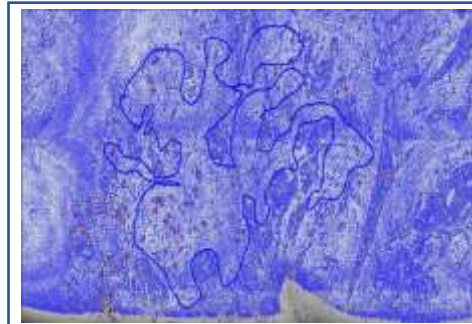


Quantificación automatizada de los Tils en preparaciones digitalizadas

Positive pixel count of whole slide image analysis.

Immune cell quantification

Immune cells identified in selected field



Stromal quantification

Stroma in the same selected field



The parameters brown and blue pixel values were carried out.

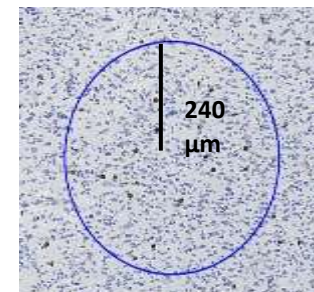
In cases of heterogeneous expression, an average of the areas with the highest and the lowest infiltration was obtained.

Brown pixel value
(immune cells,
range 268-423,468
pixels)

Blue pixel value
(stroma, range
228,712-3,08,150
pixels)

Average of ratio
brown/blue pixel
values was obtained

Results were
converted to
percentage (%)



240 μm=140,000-150,000 pixels

Quantificación automatizada de los TILs en preparaciones digitalizadas.

Quantificación de la expresión ihq de TILs (CD8 y CD4) en el cáncer de mama.

Comparación de valores de cuantificación de ihq de células TILs en cáncer de mama pre/post tratamiento según tratamiento instaurado

Control Group

Vaccine Group

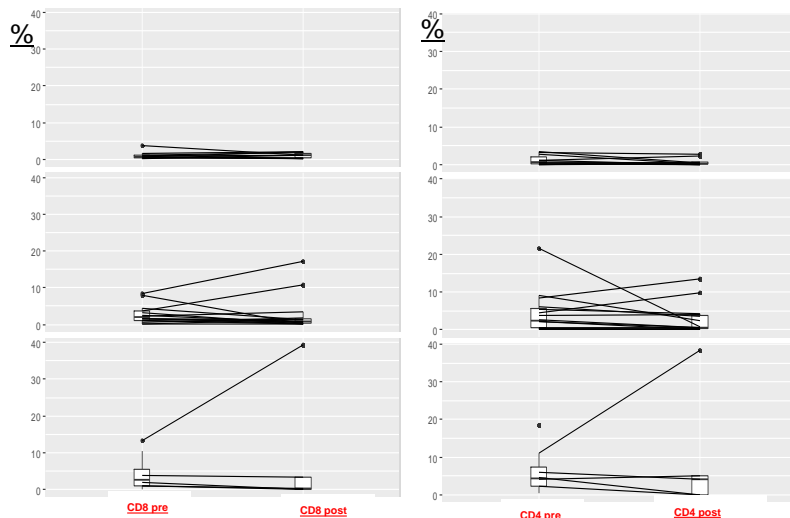
CD8 Pre/Post-treatment

CD4 Pre/Post-treatment

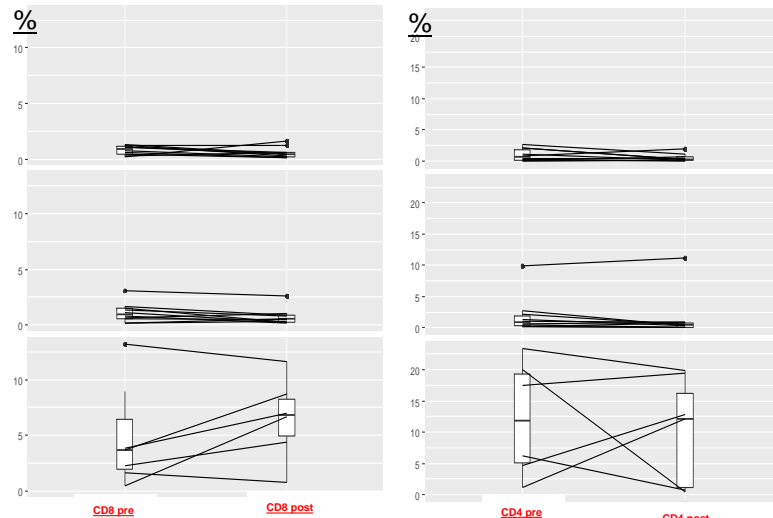
CD8 Pre/Post-treatment

CD4 Pre/Post-treatment

Lum A



Lum B



Triple-Negative

Mann-Whitney U test, $p < 0.05$

Cuantificación automatizada de los TILs en preparaciones digitalizadas.

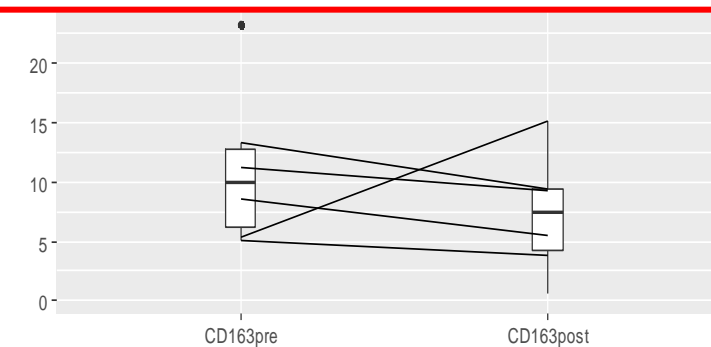
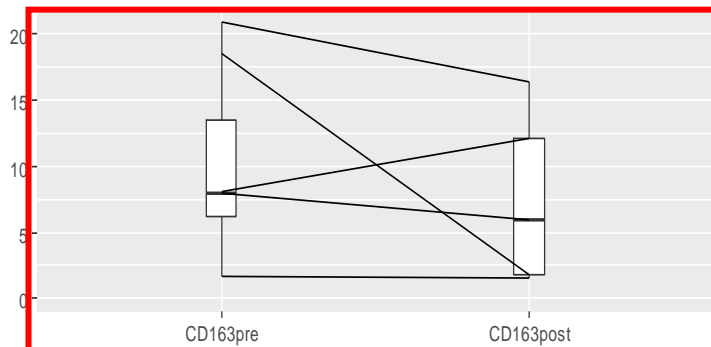
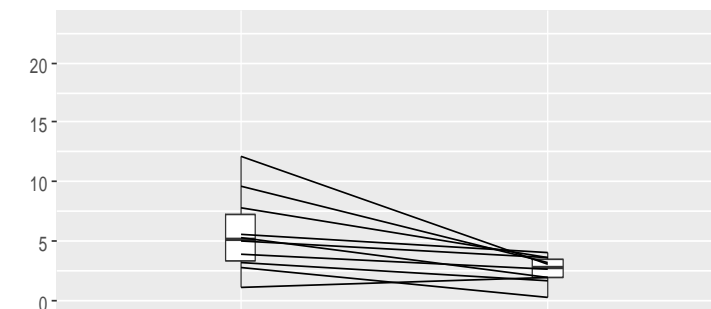
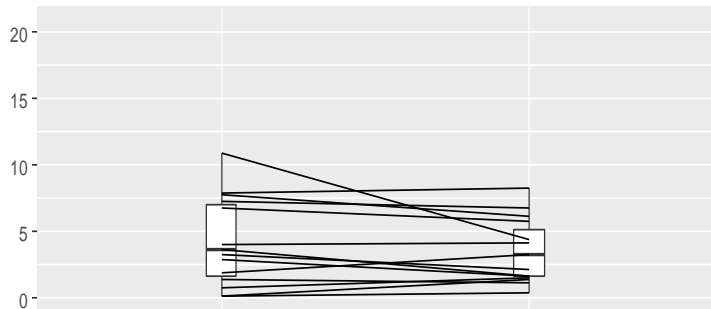
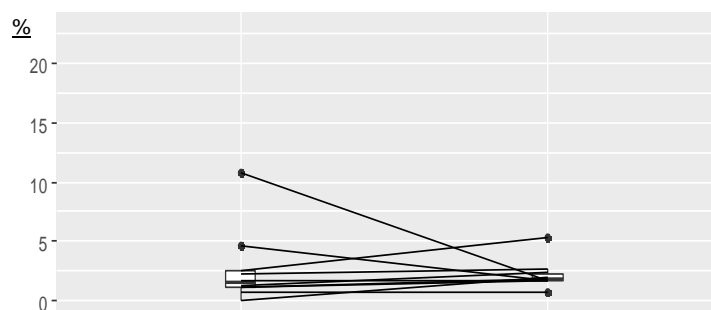
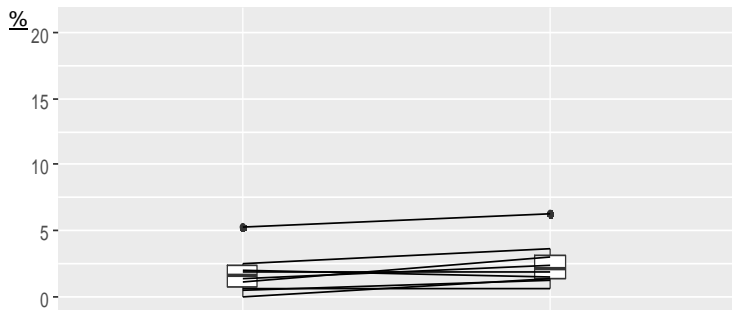
Cuantificación de la expresión ihq de CD163 (macrófagos M2) en el cáncer de mama.
La expresión fue significativamente mayor en las biopsias pretratamiento en los tumores triple negativos.

Control

Vaccine

CD163 Pre/Post-treatment

CD163 Pre/Post-treatment

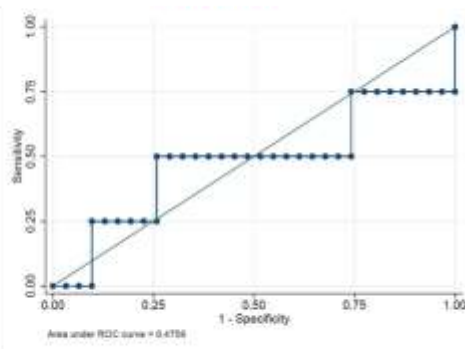


Cuantificación de la expresión inmunohistoquímica de biomarcadores tisulares realizado mediante diferentes tecnologías y su aplicación clínica

Cuantificación automatizada de los TILs en preparaciones digitalizadas.

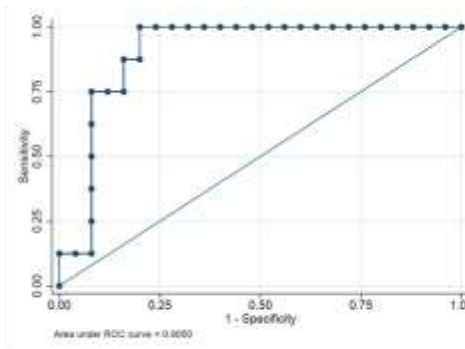
CD8

Control



AUC:0.47
Cut off 3.4%, sensitivity
50%, specificity 74%

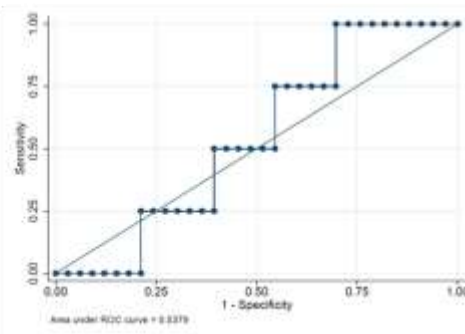
Vaccine



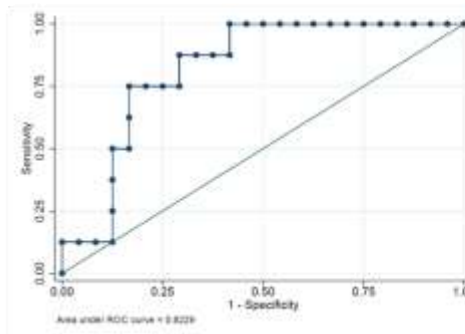
CD8

AUC:0.90
Cut off 4.3%, sensitivity
75%, specificity 92%

CD4



AUC:0.53
Cut off 3.0%, sensitivity
50%, specificity 60%



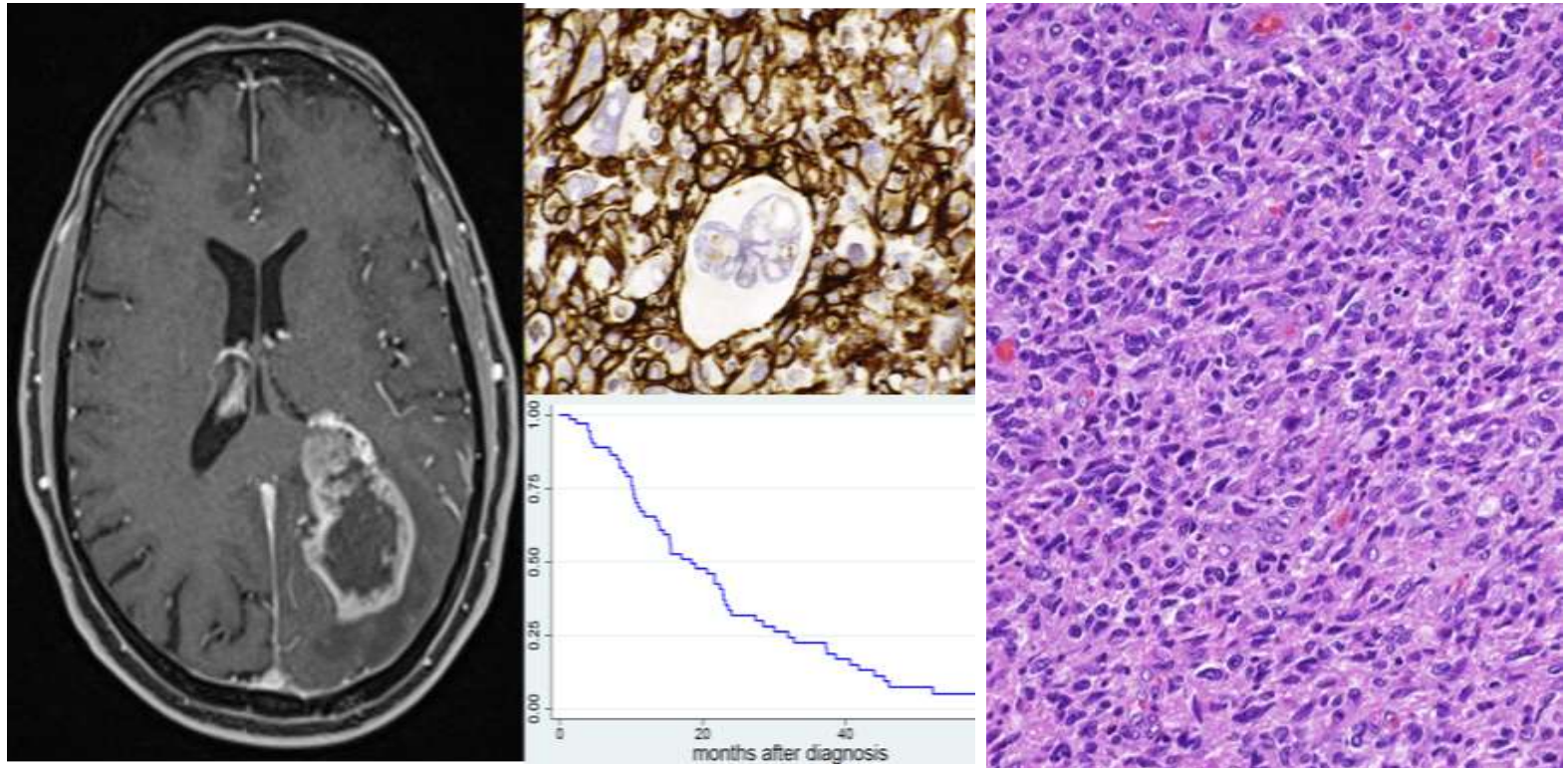
CD4

AUC: 0.82.
Cut off 7.5%, sensitivity 75%,
specificity 83%



Cuantificación de la expresión inmunohistoquímica de biomarcadores tisulares realizado mediante diferentes tecnologías y su aplicación clínica

Cuantificación expresión ihq TILs en el glioblastoma

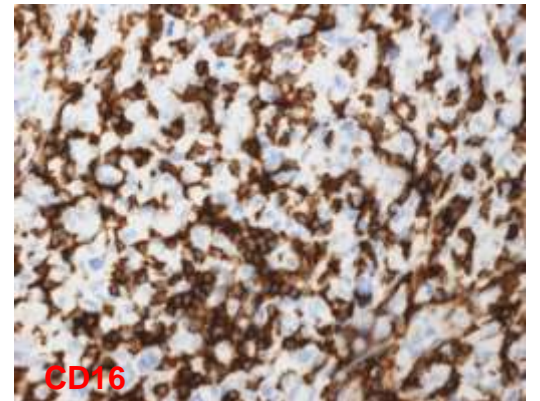
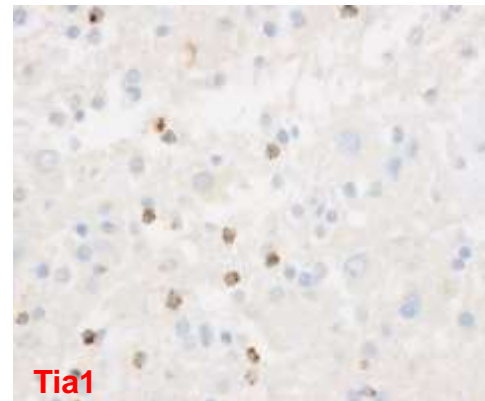
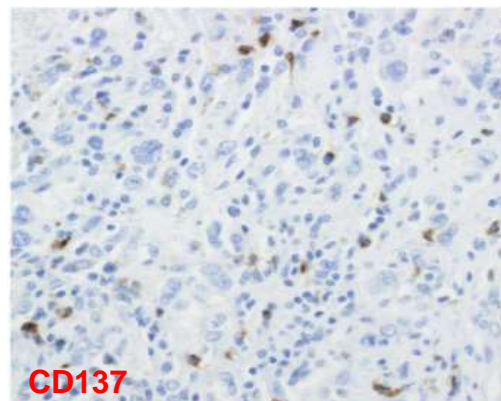
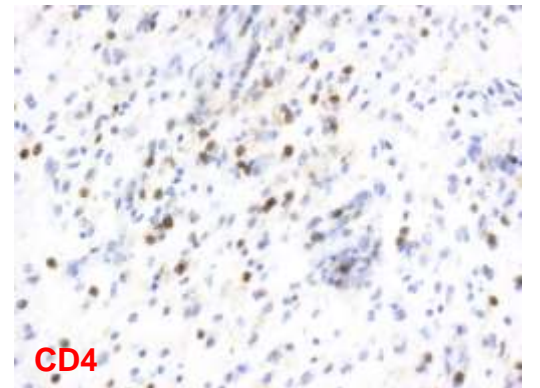
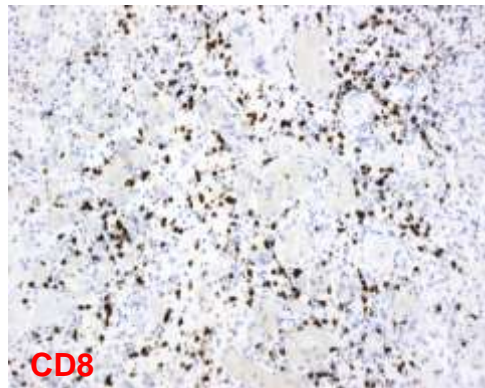
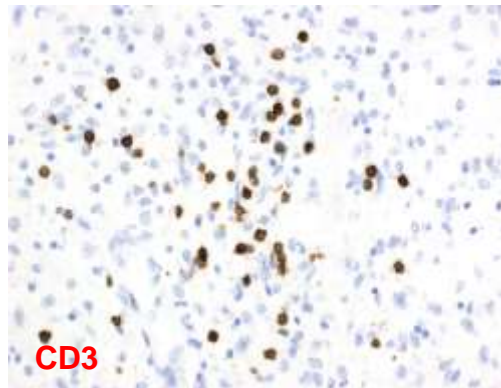
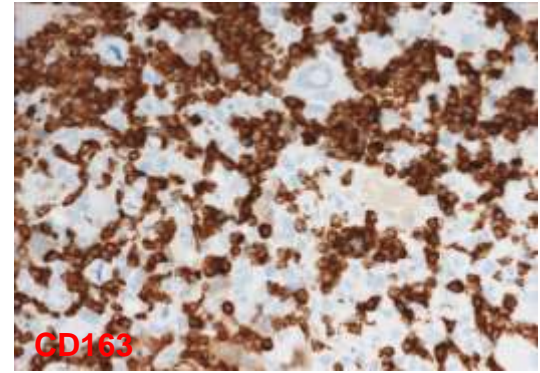
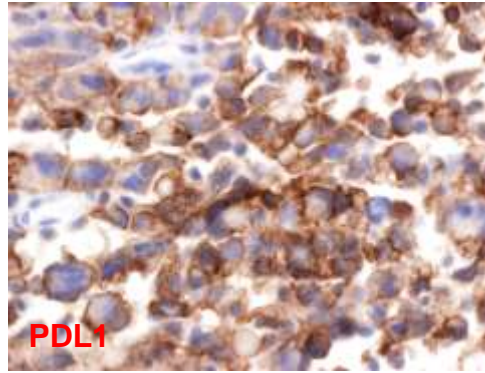
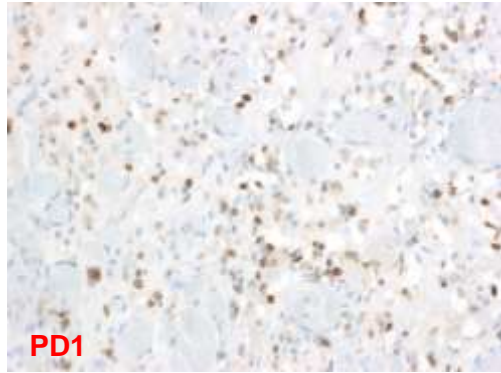


Idoate MA





Cuantificación de la expresión ihq de múltiples biomarcadores en el glioblastoma





Cuantificación de la expresión de biomarcadores ihq in GBM mediante análisis de imagen digitalizada.

A semi-quantitative assessment of the immunostained cells was performed by three observers in a blinded manner:

The **CD8 or PD1 infiltrating cell density** was evaluated according to Dahlin AM et al. (Modern Pathol. 2011) and scored as:

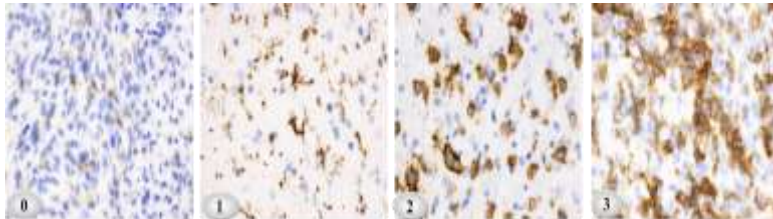
- grade 0 (no cells)
- grade 1 (sporadic cells)
- grade 2 (moderate numbers)
- grade 3 (abundant occurrence)

The extent of PD-L1 expression was scored as:

- grade 0 (0% of staining in in non-necrotic areas)
- grade 1 (isolated cells)
- grade 2 (groups, discontinuous membrane staining)
- grade 3 (groups, continuous membrane staining)

In the case of CD163, a combination of the phenotype and the density of the infiltrate was scored as:

- grades 0 and 1: density of microglia phenotype
- grades 2 and 3: density of macrophage phenotype



CD163 grading

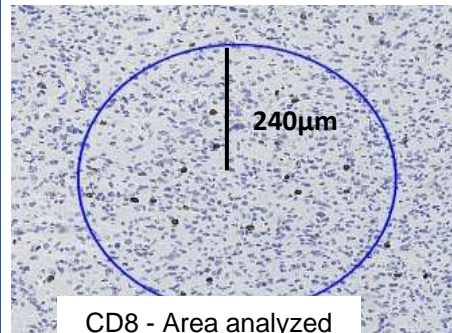
Quantification by image analysis, ACIS III (DAKO).

CD8, CD163 and PD1: Hot Spots stained area 240 μ m of radius.

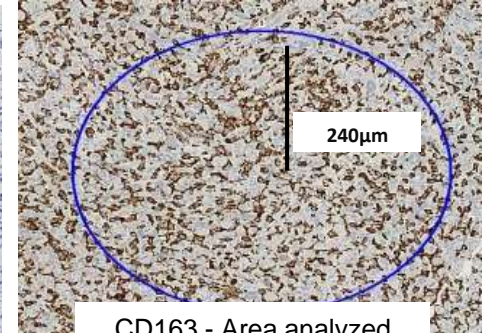
One parameter: stained area/total area (% staining).

PDL1: Hot Spots stained area 125 μ m of radius

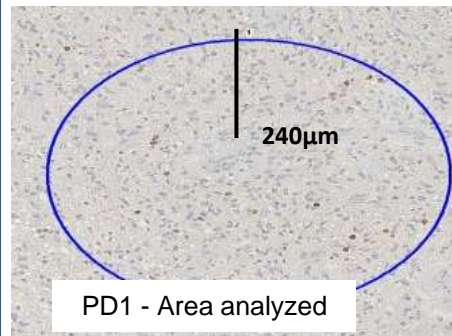
Two parameters: stained area/total area (% staining).
intensity of staining.



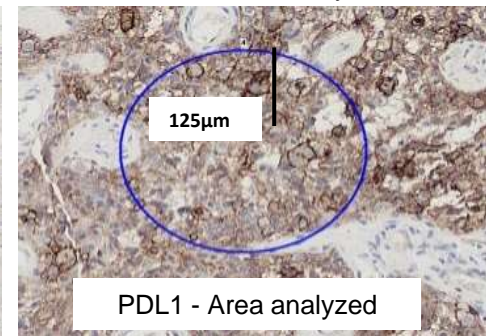
CD8 - Area analyzed



CD163 - Area analyzed



PD1 - Area analyzed

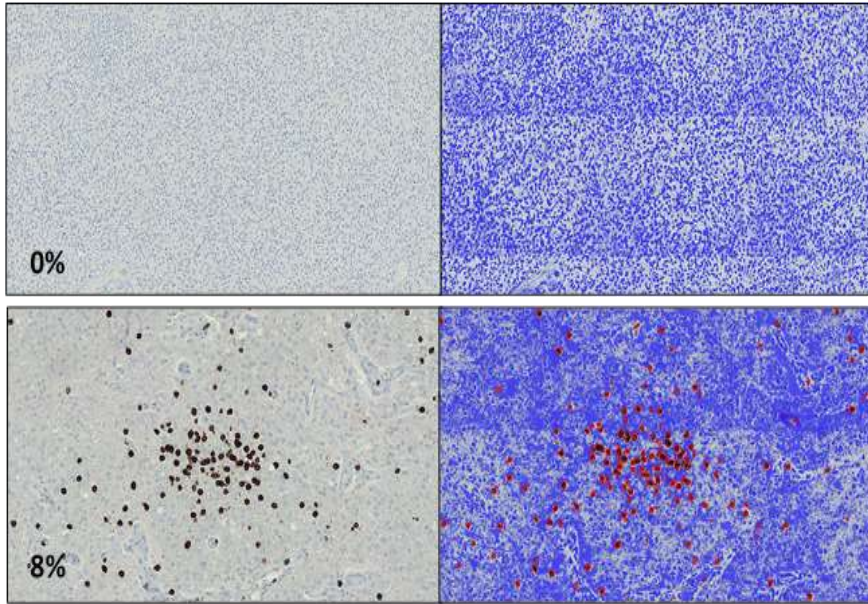


PDL1 - Area analyzed

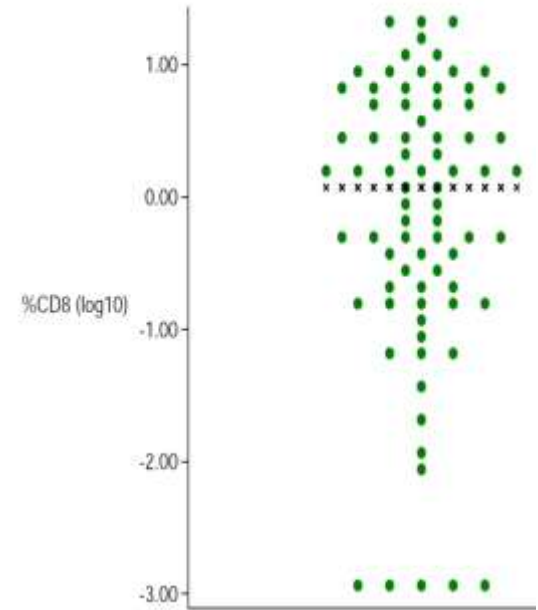


Cuantificación de expresión ihq de biomarcadores en el glioblastoma

Validation of the quantification of CD8 by digital image analysis in GBM



Digital Image Analysis

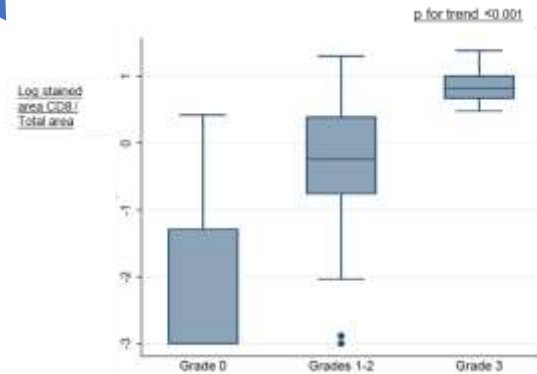


Grading

CD8	Grade 0	Grades 1-2	Grade 3	Total
Percentile 1-10	3	5	0	8
Percentile 10-75	1	45	3	49
Percentile 75-100	0	10	9	19
Total	4	60	12	76

Weights	Grade 0	Grades 1-2	Grade 3
Percentile 1-10	1	0	0
Percentile 10-75	0	1	0.2
Percentile 75-100	0	0.2	1

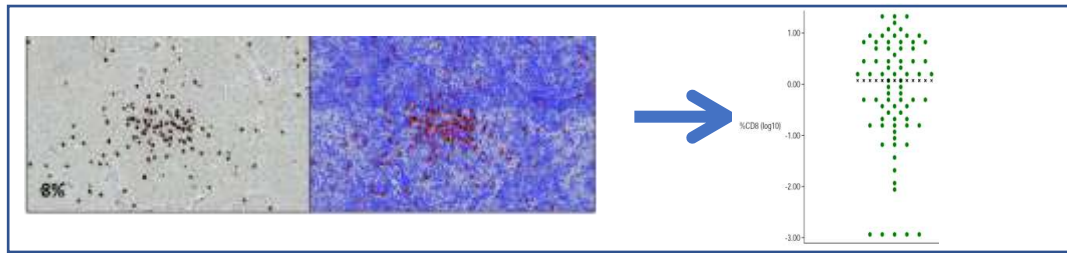
Kappa = 0.44
 Agreement = 78,4%
 Expected agreement = 61,4%



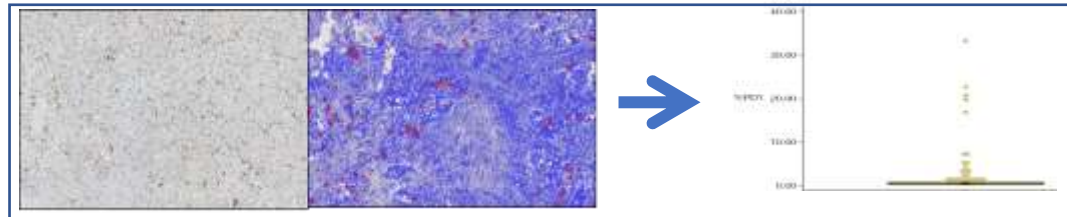


Cuantificación de expresión ihq de biomarcadores en el glioblastoma

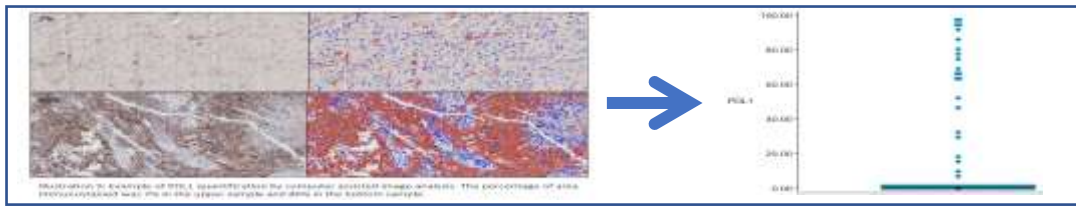
The quantified expression of CD8 correlates with significantly with survival in a series GBM



The quantified expression of PD1 does not correlate significantly with survival in a series of GBM



The quantified expression of PD-L1 does not correlate significantly with survival in a series of GBM



The quantified CD163 (M2 macrophages) expression correlates significantly with survival in a series of GBM





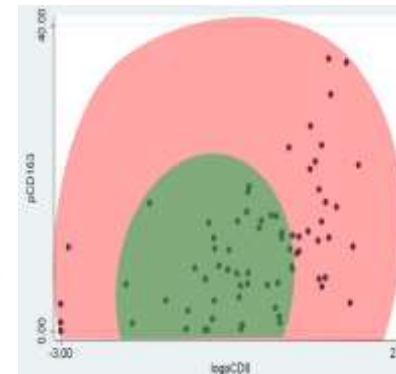
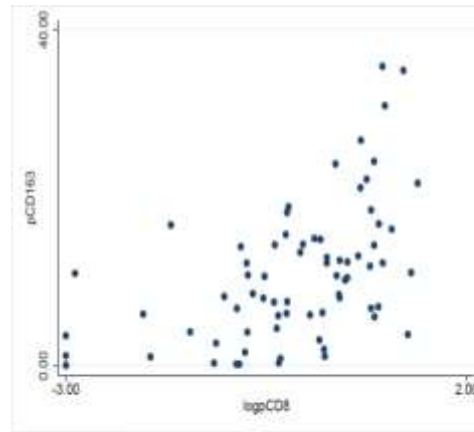
Quantificación de la expresión inmunohistoquímica de los biomarcadores tisulares realizado mediante diferentes tecnologías y su aplicación clínica.

Quantificación TILs en el glioblastoma

The higher CD8 cell infiltration is, the greater is the expression of inhibitory immune mechanisms (M2 macrophages, PD1/PDL1) in GBM.

Table 10 – Cox regression model, univariable and multivariable

Variable	Hazard Ratio	95% CI	p value	
Univariable model				
CD8 by category				
CD8 p0-10 (ref)	Reference	-	-	
CD8 p0-50	2.08	0.87	5	0.1
CD8 p75-100	2.32	1.26	4.27	<0.01
PD1 by percentage				
%PDL	1	0.89	1.01	0.44
PD1 Intensity Score				
%CD163	1	1	1	0.79
PD1 by percentage				
%PDL	1.08	0.89	1.06	0.18
CD8 by percentage				
%CD8	1.04	1.004	1.091	0.01
Multivariable model				
CD8 by category				
CD8 p0-10 (ref)	Reference	-	-	
CD8 p0-50	1.33	0.8	3.33	0.57
CD8 p75-100	2.48	1.27	4.78	<0.01
Max MGMT	0.97	0.2	0.68	<0.01
Dendritic Cell Vacc.	0.41	0.28	1.1	<0.1
Age	1.02	1	1.04	0.07
CD8 by percentage				
%CD8	1.064	1.059	1.11	0.01
Max MGMT	0.96	0.19	0.68	0.01
Dendritic Cell Vacc.	0.62	0.38	1.08	0.09
Age	1.02	1	1.04	0.04



RED ZONE= Worse prognosis
14% of patients in this zone belong to the 75th-100th percentile of Overall Survival

GREEN ZONE = Better prognosis
60% of patients in this zone belong to the 75th-100th percentile of Overall Survival

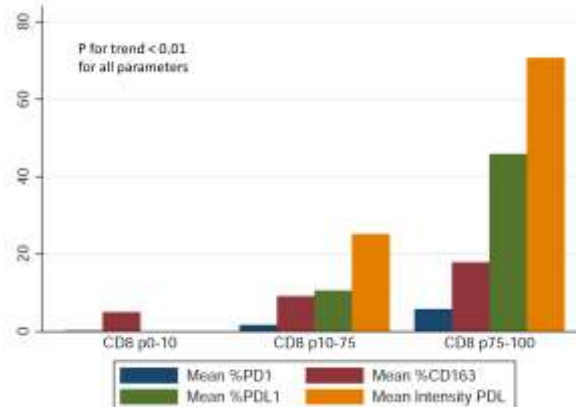
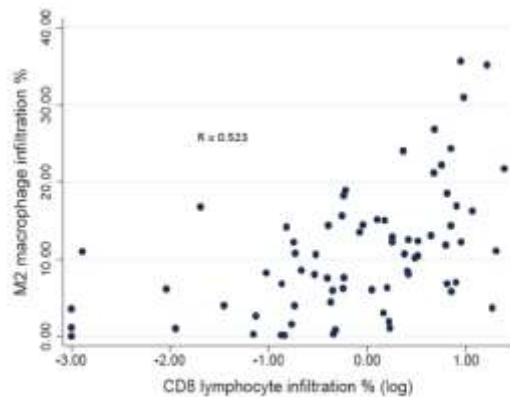


Figure 8. Scatter plot representing the variation of M2 macrophage infiltration according to CD8 lymphocyte infiltration. Each circle represents a patient. M2 macrophage infiltration becomes denser as CD8 lymphocytes does.

Cuantificación de la expresión inmunohistoquímica de biomarcadores tisulares realizado mediante diferentes tecnologías y su aplicación clínica

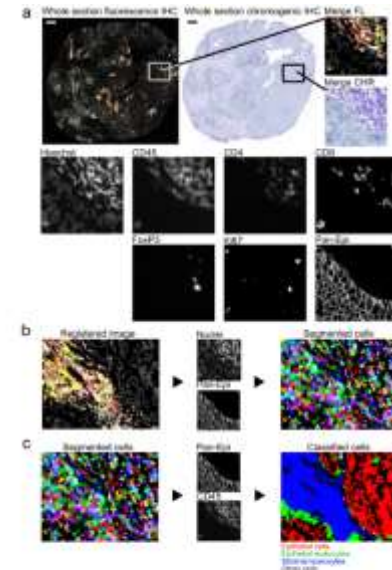
Cuantificación mediante “multiplex quantitative immunofluorescence/ immunohistochemical assay” basado en el análisis de imagen multispectral. Ejemplos y su aplicación clínica.



Inmunohistoquímica cuantitativa en cortes de bloques de parafina: “Multiplex quantitative immunohistochemistry/immunofluorescence Assay”

- Combinación de detección de múltiples anticuerpos en un único corte histológico de bloque de parafina.
- Mantiene la relación de los marcadores entre sí y con las células marcadas (relación espacial multiparamétrica).
- Análisis automático y cuantificación de los resultados de los anticuerpos comerciales en miles de células estudiadas.
- Posibilidad de obtención de “immunoscores” en cáncer.

Multiplexed immunohistochemistry TILs in prostatic cancer



SCIENTIFIC REPORTS

OPEN Systems pathology by multiplexed immunohistochemistry and whole-slide digital image analysis

100 24 January 2017

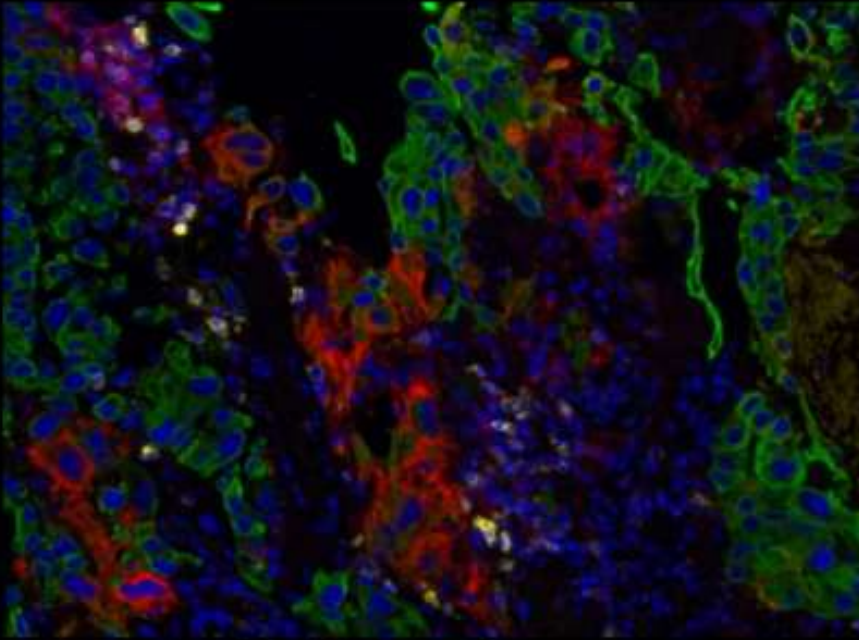
vol. 7 | no. 1 | 1-12 | DOI: 10.1038/s41598-017-00000-0

Full article: 10.1038/s41598-017-00000-0

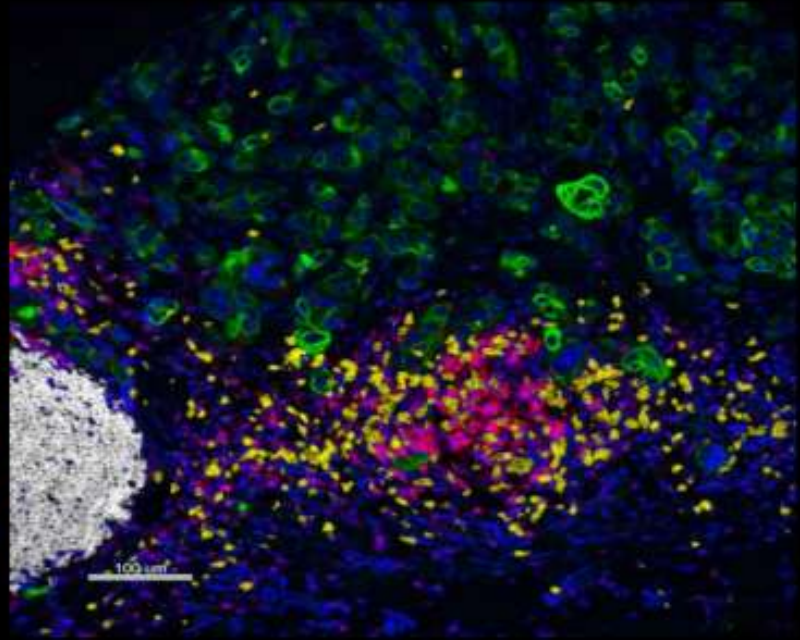
Sami Blom¹, Lassi Paavola¹, Dmitri Bychkov², Riku Turkki¹, Petra Mäki-Terä¹, Annabritha Hemmes¹, Katja Väikmäki¹, Johan Lundin², Olli Kallioniemi^{1,3} & Teijo Pellinen¹

Multiplex QIF testing in Pathology CLIA Labs

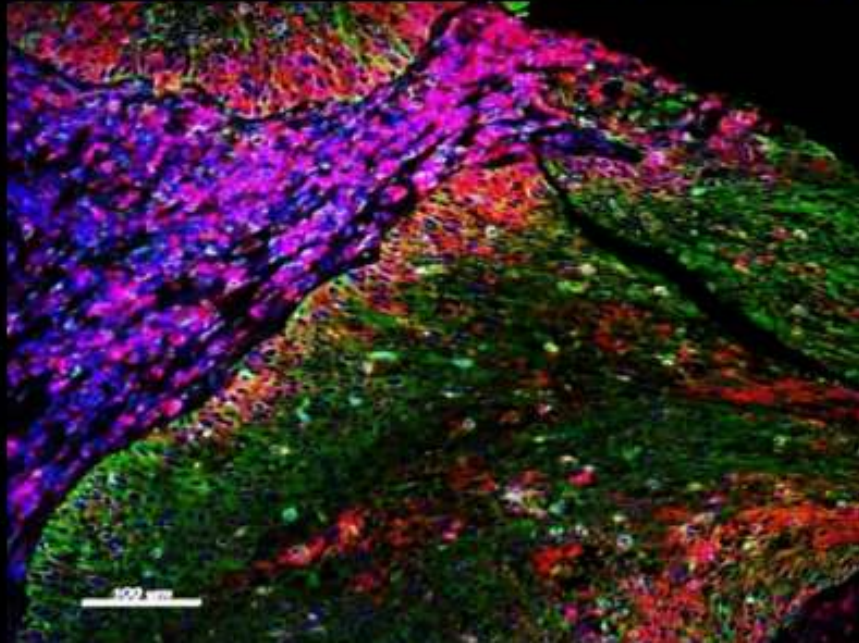
DAPI/CK/PD-1/PD-L1/PD-L2



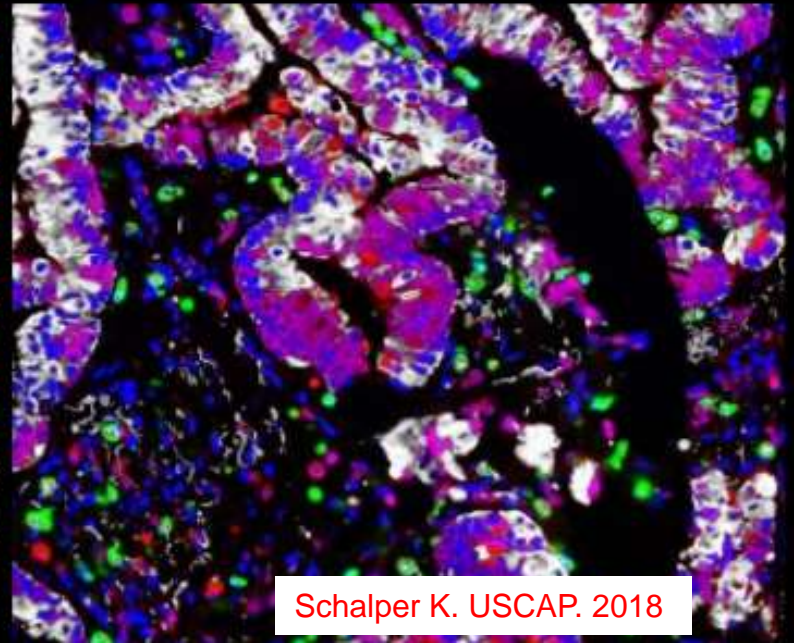
DAPI/CK/CD4/CD8/CD20



DAPI/CK/B2M/Class I/Class II



DAPI/CK/HLA-DR/CD68/CD11b





Cox Multivariate analysis including Immunoscore

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Histopathologic-Based Prognostic Factors of Colorectal Cancers Are Associated With the State of the Local Immune Reaction

Bernhard Mlecnik, Marie Tosolini, Amos Kirilovsky, Anne Berger, Gabriela Bindea, Tchao Meatchi, Patrick Bruneval, Zlatko Trajanoski, Wolf-Herman Fridman, Franck Pages, and Jérôme Galon

COX analysis for DFS	HR	Log Rank P-Values
Tumor (T) stage	1.24	0.29
N Stage	1.31	0.17
Gender	1.47	0.18
Number of total Lymph nodes	1.13	0.68
Histological grade	0.69	0.29
Mucinous Colloids	1.29	0.47
Occlusion	1.03	0.94
Perforation	4.03	0.0084
Immunoscore	0.65	0.0003

Cox Analysis	DFS		OS		DSS	
	HR	P-value	HR	P-value	HR	P-value
AJCC/UICC-TNM	1.38	0.09 ns	1.18	0.29 ns	1.43	0.10 ns
Immunoscore	0.64	<0.0001	0.71	<0.0001	0.63	<0.0001

JOURNAL OF CLINICAL ONCOLOGY

EDITORIALS

TNM Staging in Colorectal Cancer: T Is for T Cell and M Is for Memory

Elizabeth K. Broussard and Mary L. Disis, Tumor Vaccine Group, Center for Translational Medicine in Women's Health, University of Washington, Seattle, WA

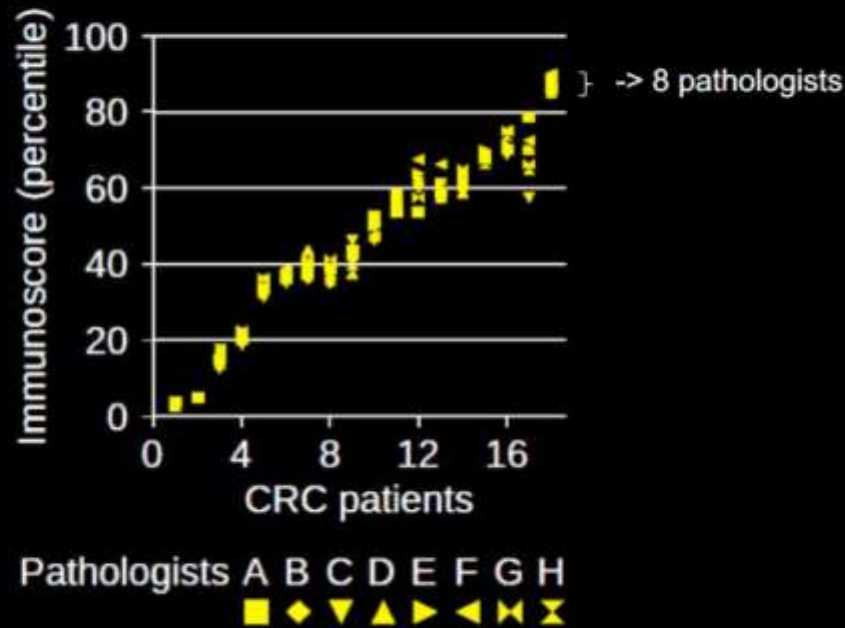
- ✓ Tumor progression, invasion and recurrence are dependent on the immune contexture and Immunoscore
- ✓ Pre-existing immunity is determining the fate and survival of the patient

Immunoscore (I) using whole slide FFPE

Consensus Immunoscore

Immunoscore quality controls

Immunoscore quantification with digital pathology performed by 8 independent pathologists

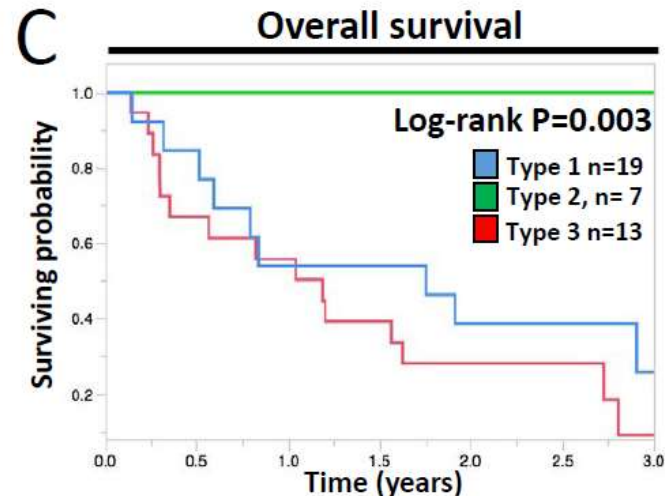
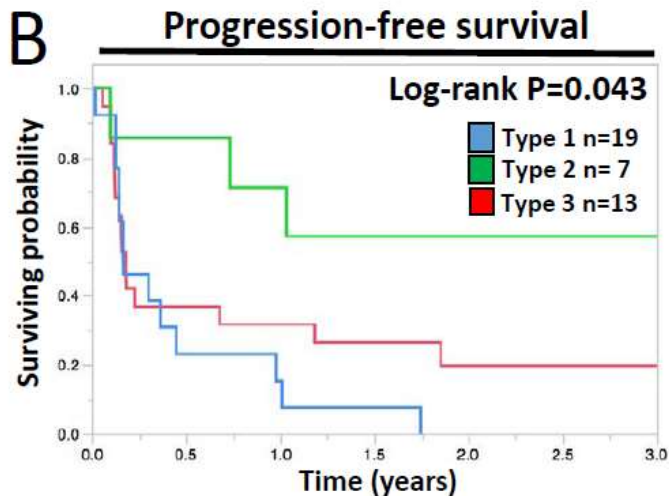
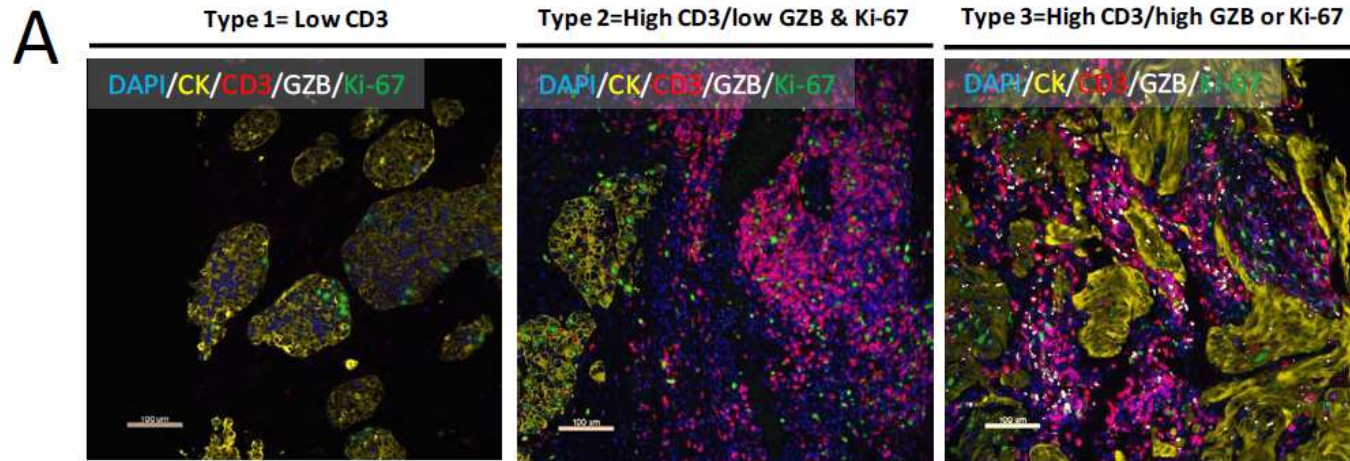


- ✓ Very good concordance between independent digital quantification of Immunoscore (Immunoscore software) by 8 pathologists
- ✓ Immunosome : mean percentile of the 4 parameters
- ✓ Similar quantification were performed for CD3_{CT}, CD3_{IM}, CD8_{CT}, CD8_{IM}
- ✓ Immunoscore is **Standardized, Objective, Quantitative**

(death)

Cuantificación inmunohistoquímica de biomarcadores: Immunoscores precisos, reproducibles y con aplicación clínica.

Análisis multiespectral de TILs estromales en el carcinoma no microcítico de pulmón



Cuantificación mediante “multiplex quantitative immunofluorescence/ immunohistochemical assay” basado en análisis multispectral.

- Se puede aplicar sobre tejido fijado en formol e incluido en parafina.
- Capacidad de los fluoróforos de ser excitados por una longitud de onda y emitir a una longitud de onda específica más larga.
- Hay múltiples fluoróforos comerciales disponibles.
- Se puede aplicar sobre anticuerpos comerciales.

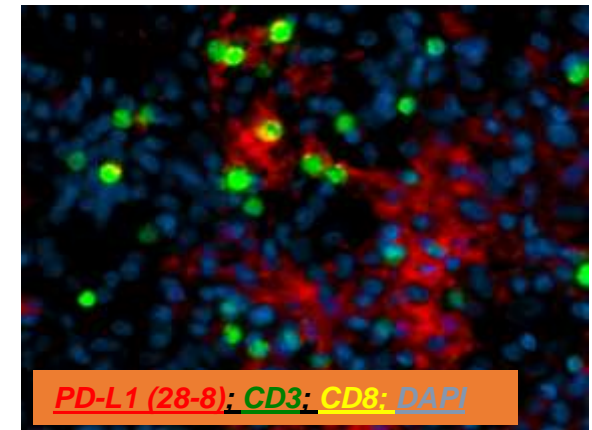
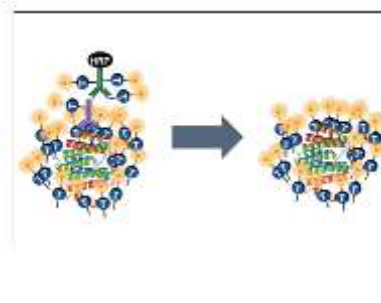
Table 2
Sample fluorescent dyes for multiplexed IHC assays.

Fluorescent dye	Excitation λ (nm)	Emission λ (nm)
Coumarin	402	443
→ Fluorescein	494	517
TMR	550	570
Cyanine 3	550	570
Cyanine 3.5	581	596
Cyanine 5	648	667
Cyanine 680	669	688



Cuantificación de la expresión inmunohistoquímica de biomarcadores tisulares realizado mediante diferentes tecnologías y su aplicación clínica

Cuantificación mediante “multiplex quantitative immunofluorescence/ immunohistochemical assay” basado en análisis multispectral.

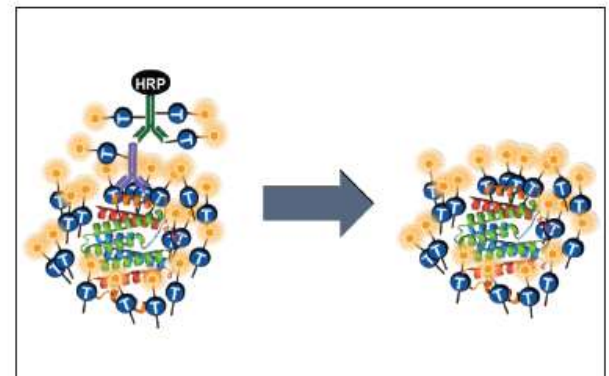


Se trata de una solución integral:

- Método de inmunotinción avanzada (técnica Opal).
- Análisis multispectral.
- Sistema de análisis de imagen

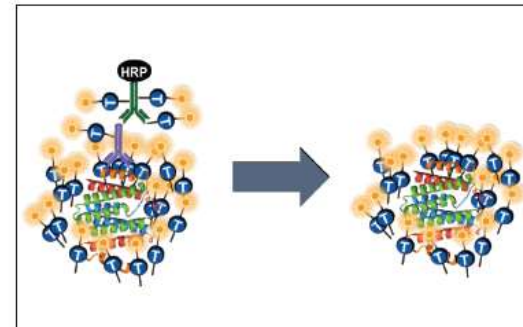
Cuantificación mediante “multiplex quantitative immunofluorescence/ immunohistochemical assay” basado en multiespectral análisis.

- Los casos se escanearon mediante Vectra (PerkinElmer).
- Vectra capturó los espectros fluorescentes a intervalos de longitud de onda de 20 nm y combinó estas capturas para crear una imagen única.
- Para el análisis de los espectros de los fluoróforos se utilizó el software de análisis de imágenes InForm (PerkinElmer).
- El sistema generó un registro de intensidad para todos los marcadores estudiados de cada célula tumoral analizada.
- Para cada marcador se determinó la intensidad fluorescente media basada en la intensidad de píxeles para cada célula.



Cuantificación mediante “multiplex quantitative immunofluorescence/ immunohistochemical assay” basado en multispectral análisis Procolo “Opal”.

- Desparafinización de los cortes histológicos.
- Recuperación antigénica.
- Aplicación secuencial de anticuerpos primarios, seguido de sistema de detección (SuperPicture Polymer Detection HRP kit) y visualización mediante fluoróforo. Eliminación del anticuerpo primario entre cada aplicación. Visualización de los núcleos mediante DAPI (1:2000) y montaje.
- Escaneo de las preparaciones mediante el “Vectra slide scanner (PerkinElmer)” en el entorno de evaluación de fluorescencia (captura de espectro de fluorescencia a intervalos de 20 nm de longitud de onda entre 420 nm y 720 nm) y obtención de una única “stack image” o “firma espectral” para cada marcador analizado.
- Análisis de la imagen espectral de fluorescencia para cada célula analizada mediante el programa “InForm image analysis “ de (PerkinElmer) y la obtención de la intensidad media de fluorescencia basado en los pixeles de fluorescencia.
- Análisis de los datos de la intensidad media de fluorescencia para cada caso de acuerdo al umbral establecido.





Cuantificación inmunohistoquímica de biomarcadores:

Immunescores precisos, reproducibles y con aplicación clínica.

Efecto del tratamiento con Nivolumab (anti PD1 en el glioblastoma). Estudio pre/post en casos y controles.

Estudio realizado por la Universidad de Navarra en colaboración con la Universidad de Yale.

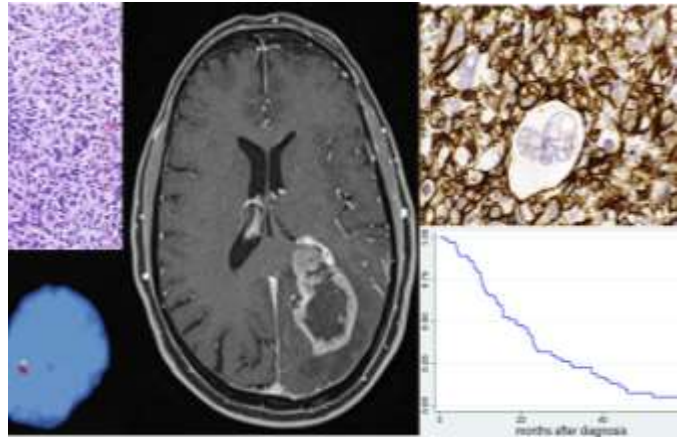
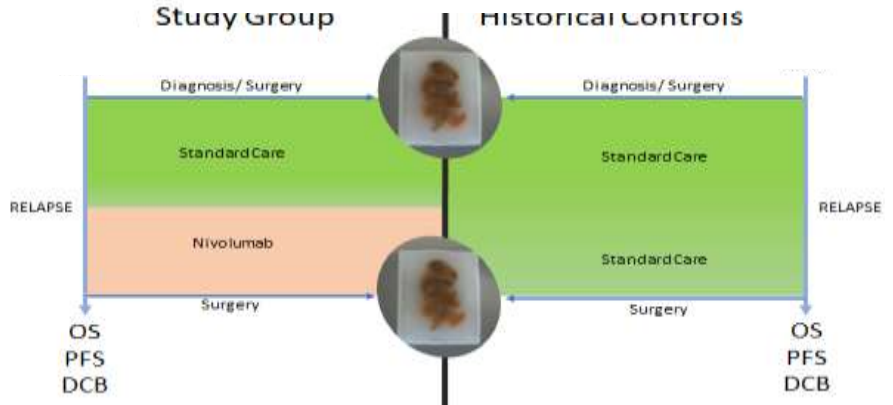


Table S2

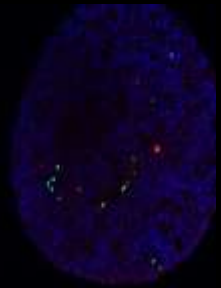
Sequence	Subject	Sex	Age	No. of prior therapies	Most recent prior therapy	Time (hr)	Description	PD-L1 expression	CD8 expression	CD8+ expression	CD8+ expression	CD8+ expression	CD8+ expression	CD8+ expression	CD8+ expression	CD8+ expression	CD8+ expression	CD8+ expression	CD8+ expression	CD8+ expression
1	1000001	M	45	1	Surgery	720	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
2	1000002	M	48	1	Surgery	750	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
3	1000003	F	55	1	Surgery	800	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
4	1000004	M	58	1	Surgery	850	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
5	1000005	M	62	1	Surgery	900	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
6	1000006	M	65	1	Surgery	950	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
7	1000007	M	70	1	Surgery	1000	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
8	1000008	M	75	1	Surgery	1050	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
9	1000009	M	80	1	Surgery	1100	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
10	1000010	M	85	1	Surgery	1150	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
11	1000011	M	90	1	Surgery	1200	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
12	1000012	M	95	1	Surgery	1250	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
13	1000013	M	100	1	Surgery	1300	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
14	1000014	M	105	1	Surgery	1350	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
15	1000015	M	110	1	Surgery	1400	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
16	1000016	M	115	1	Surgery	1450	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
17	1000017	M	120	1	Surgery	1500	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
18	1000018	M	125	1	Surgery	1550	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
19	1000019	M	130	1	Surgery	1600	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
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25	1000025	M	160	1	Surgery	1900	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
26	1000026	M	165	1	Surgery	1950	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
27	1000027	M	170	1	Surgery	2000	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
28	1000028	M	175	1	Surgery	2050	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
29	1000029	M	180	1	Surgery	2100	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
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32	1000032	M	195	1	Surgery	2250	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
33	1000033	M	200	1	Surgery	2300	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
34	1000034	M	205	1	Surgery	2350	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
35	1000035	M	210	1	Surgery	2400	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
36	1000036	M	215	1	Surgery	2450	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
37	1000037	M	220	1	Surgery	2500	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
38	1000038	M	225	1	Surgery	2550	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
39	1000039	M	230	1	Surgery	2600	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
40	1000040	M	235	1	Surgery	2650	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
41	1000041	M	240	1	Surgery	2700	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
42	1000042	M	245	1	Surgery	2750	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
43	1000043	M	250	1	Surgery	2800	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
44	1000044	M	255	1	Surgery	2850	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
45	1000045	M	260	1	Surgery	2900	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
46	1000046	M	265	1	Surgery	2950	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
47	1000047	M	270	1	Surgery	3000	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
48	1000048	M	275	1	Surgery	3050	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
49	1000049	M	280	1	Surgery	3100	Control	100	1	100	100	100	100	100	100	100	100	100	100	100
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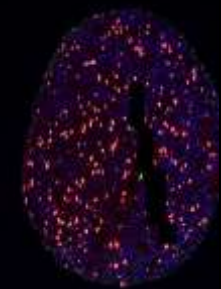
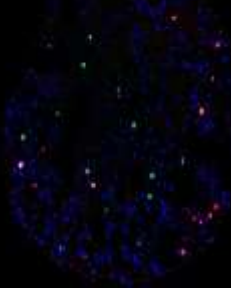
CLUSTER 1

PATIENT 11

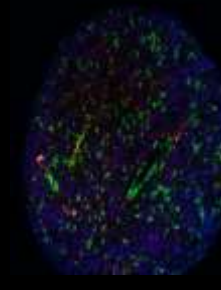
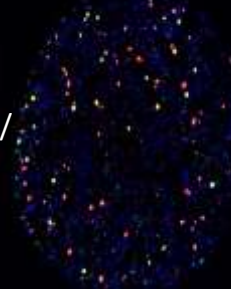
PATIENT 20



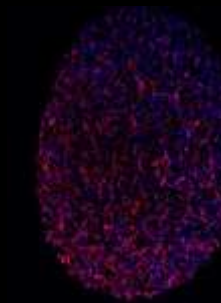
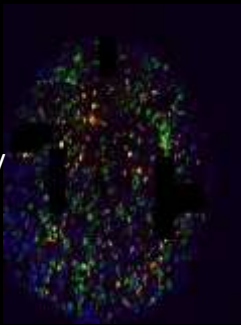
DAPI/CD3/CD8



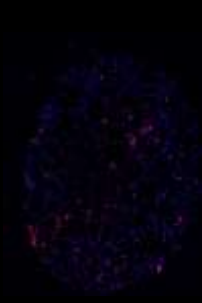
DAPI/CD3/Granzb/
Ki67



DAPI/CD11b/CD68/
HLA-DR



DAPI/PDL1



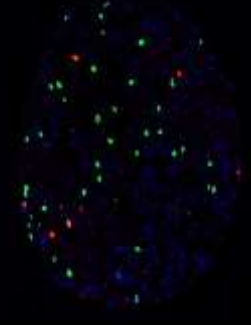
CLUSTER 2

PATIENT 3

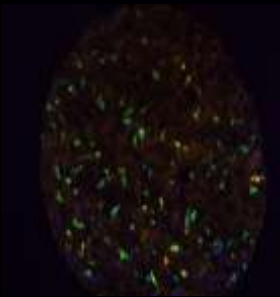
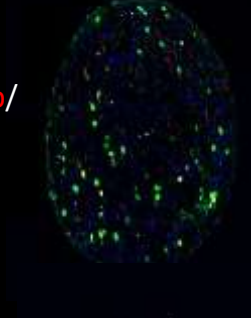
PATIENT 12



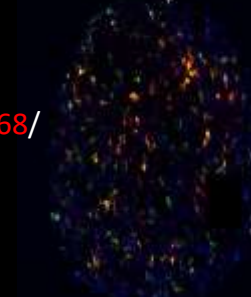
DAPI/CD3/CD8



DAPI/CD3/Granzb/
Ki67



DAPI/CD11b/CD68/
HLA-DR



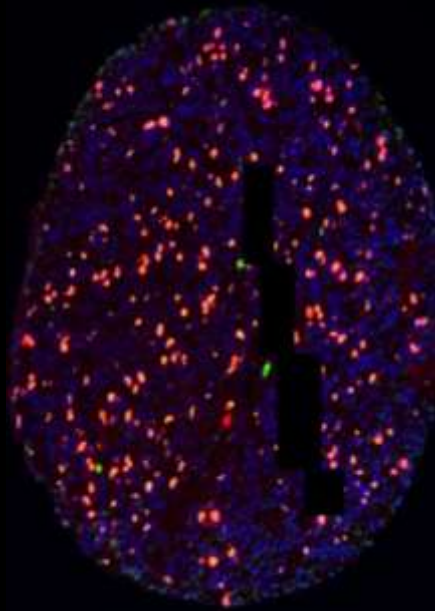
DAPI/PDL1



CLUSTER 1

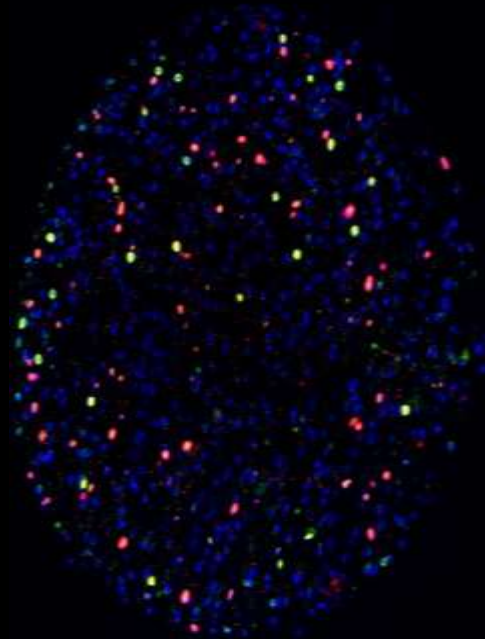
DAPI/CD3/Granzb/Ki67

PATIENT 11



OS = 41 days

PATIENT 20

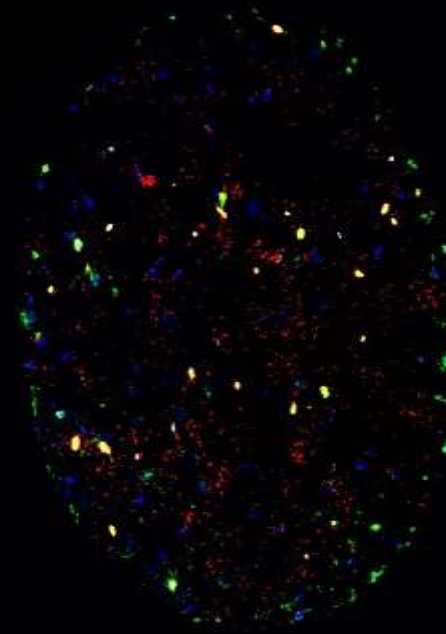


OS = 191
days

CLUSTER 2

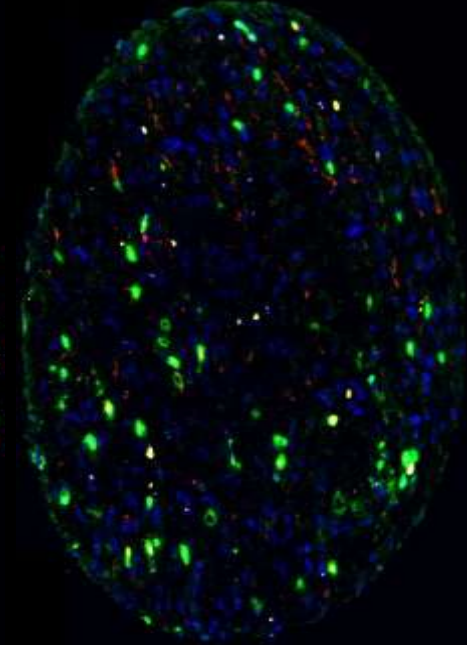
DAPI/CD3/Granzb/Ki67

PATIENT 3



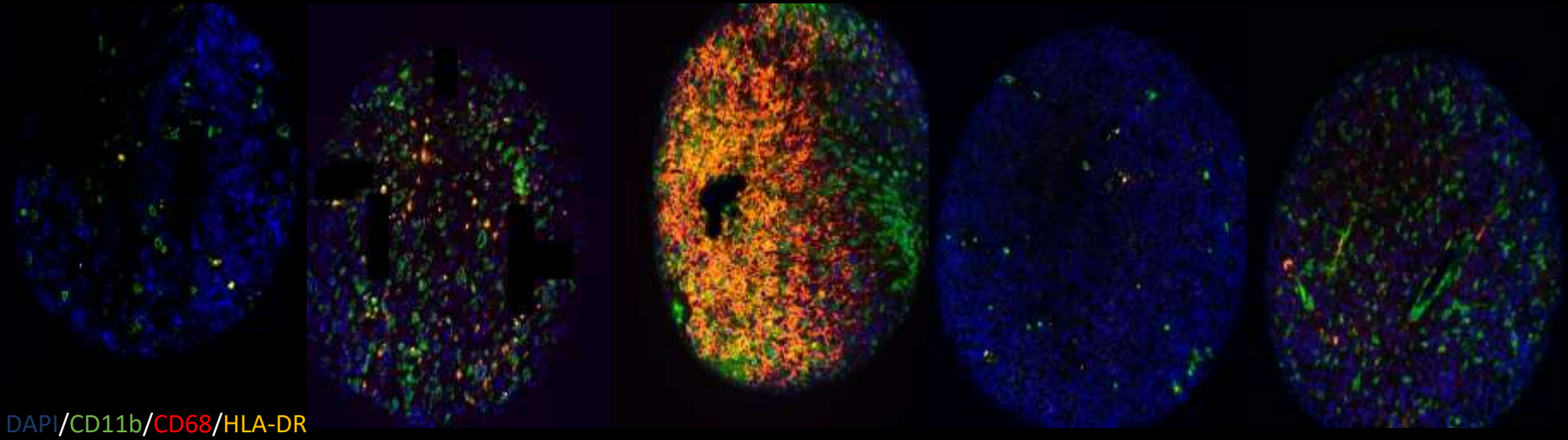
OS = 764
days
Still alive

PATIENT 12



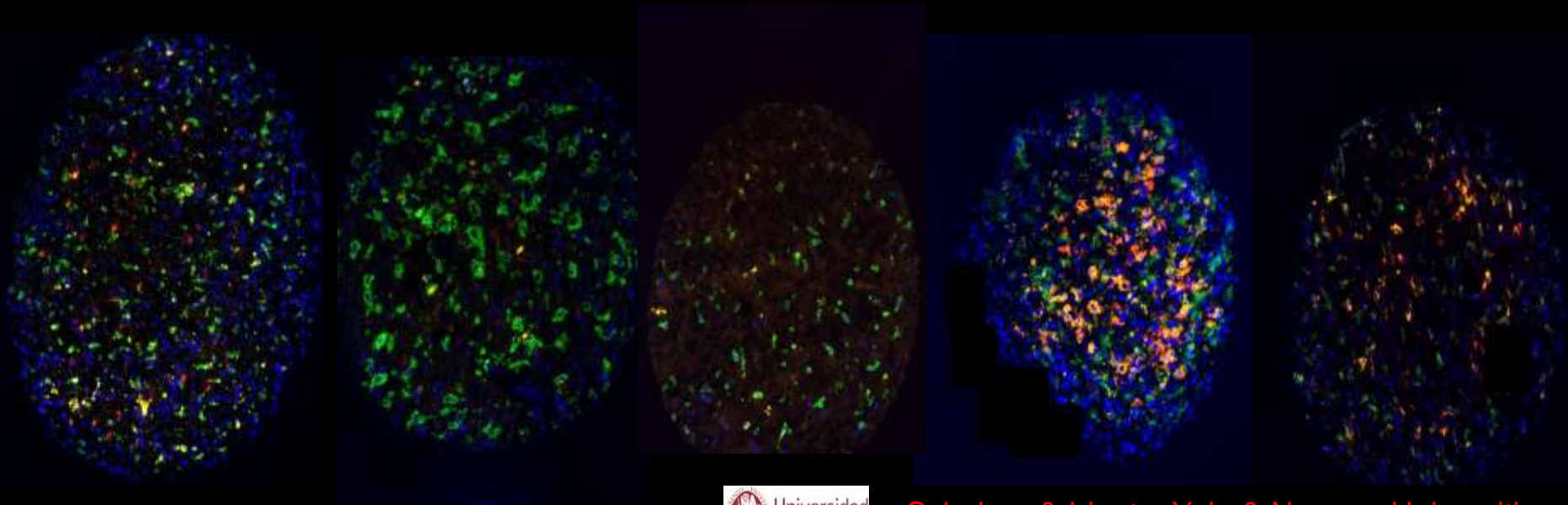
OS = 313
days

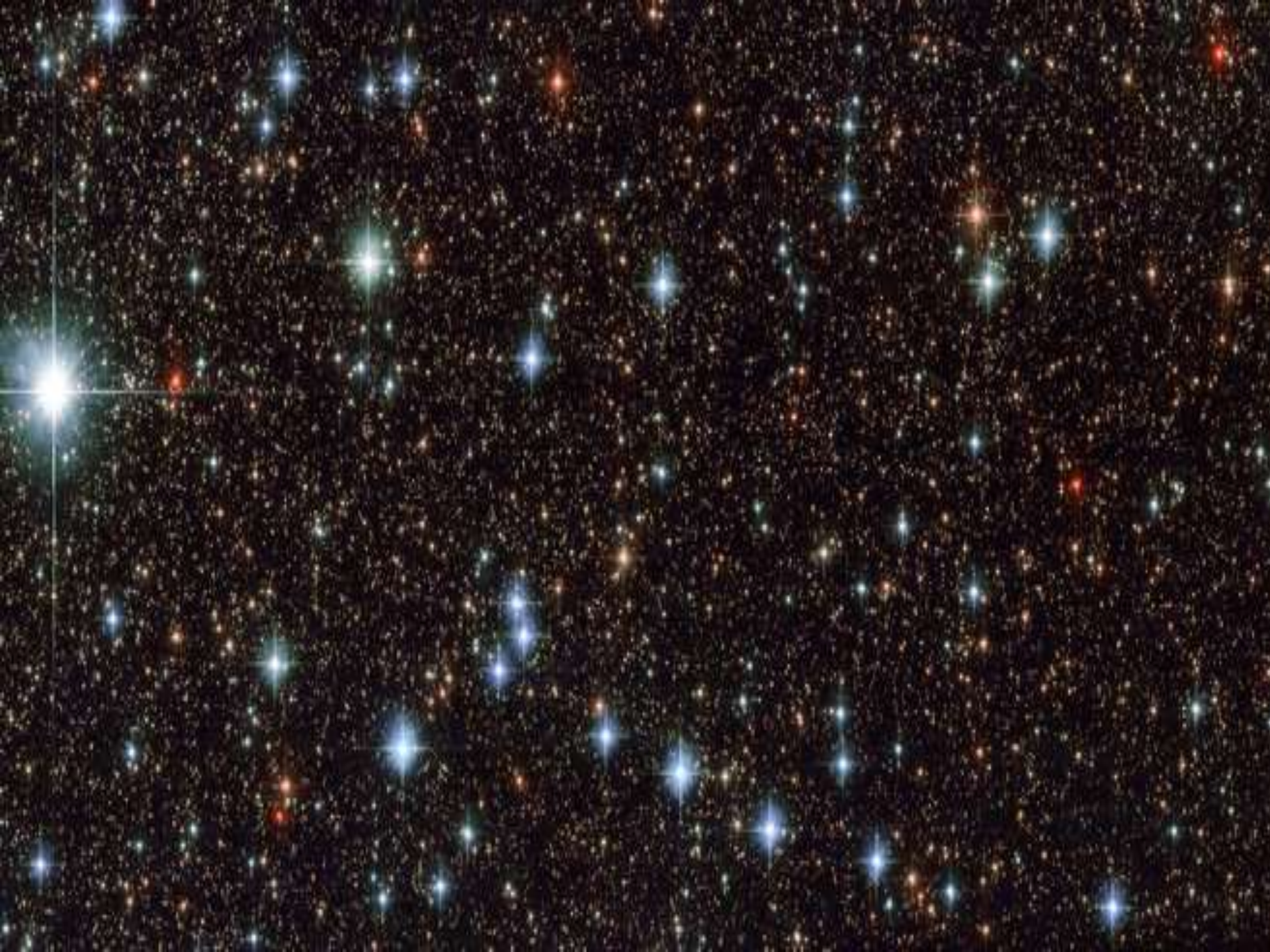
Cluster 1



DAPI/CD11b/CD68/HLA-DR

Cluster 2





Quantificación expresión ihq mediante multiplex quantitative immunofluorescence

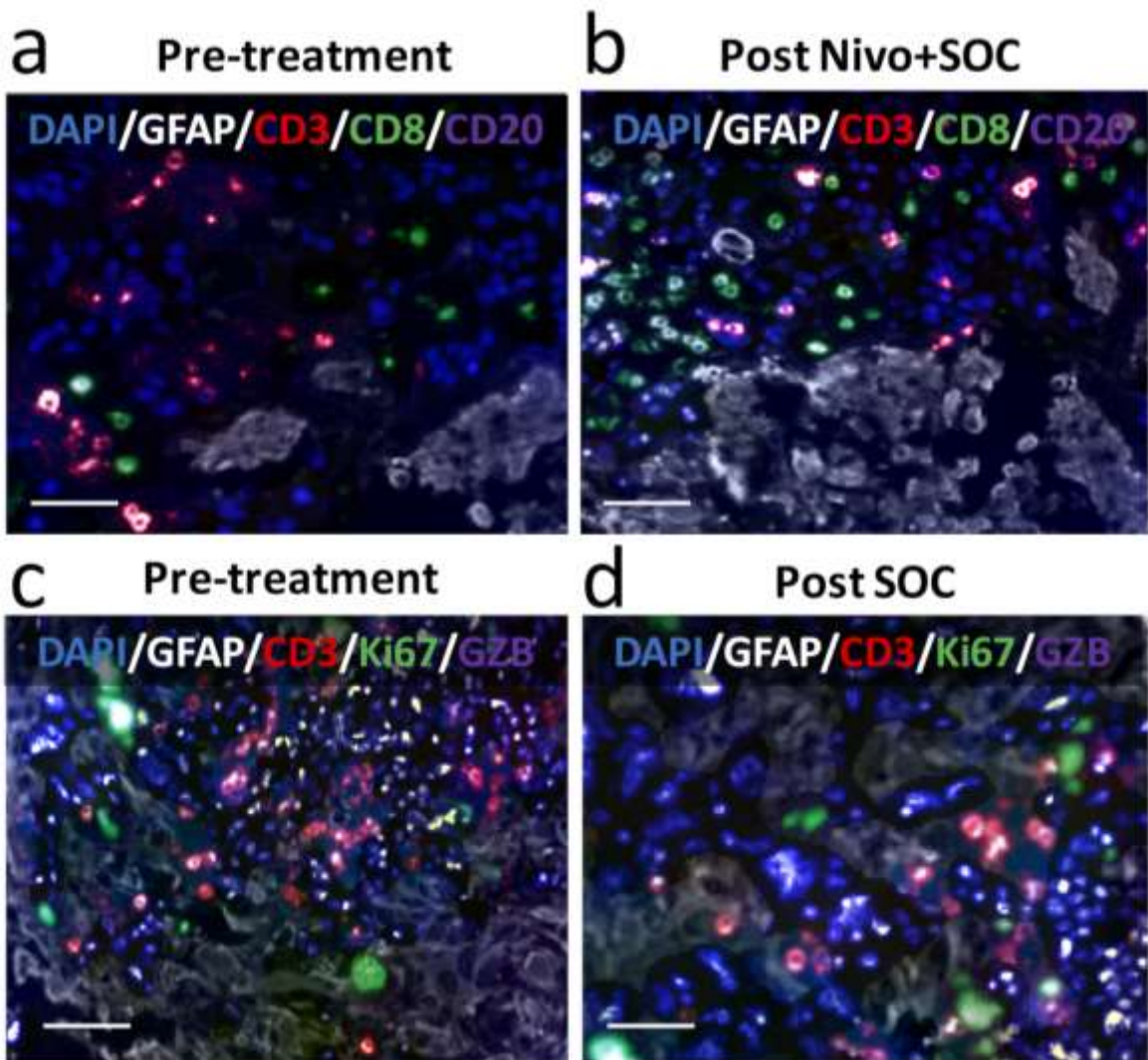
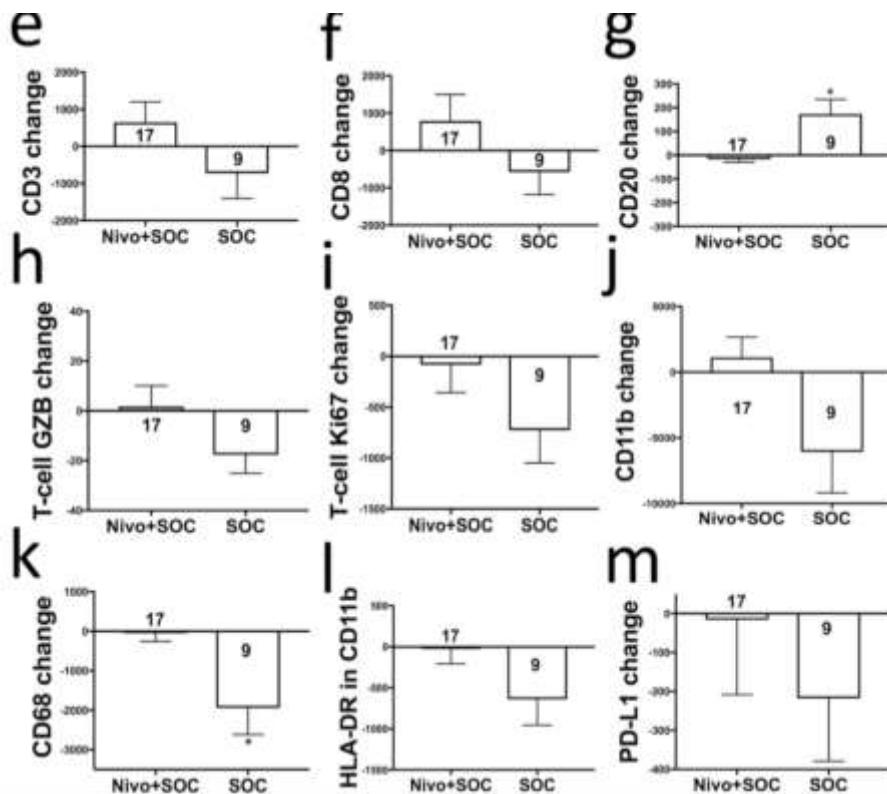


Figure 4: Multiplex immunofluorescence assessment of immune cells in the tumor microenvironment before and after treatment. (a-d) Representative fluorescence captions of pre/post treatment glioblastoma samples simultaneously stained with a 5-color multiplex panel containing the markers DAPI (blue), GFAP (white), CD3 (red), CD8 (green) and CD20 (magenta) (a-b); or with another panel including DAPI (blue), GFAP (white), CD3 (red), Ki-67 (green) and GZB (magenta) (c-d). Bar=200 μ m. (e-m) Charts showing the pre/post level change in each treatment.

Schalper & Idoate. Yale & Navarra Universities

Cuantificación de la expresión inmunohistoquímica de biomarcadores tisulares realizado mediante diferentes tecnologías y su aplicación clínica

Cuantificación expresión ihq múltiples biomarcadores mediante "multiplex quantitative immunofluorescence" apoyado en el análisis multiespectral y facilitado por la moderna tecnología.



(e) Sp=200 (µm). (e-m) Charts showing the pooled level change in each treatment group for different immune markers measured using multiplex quantitative analysis including CD3 (e), CD8 (f), CD20 (g), T-cell GZB (h), T-cell Ki67 (i), CD11b (j), CD68 (k), myeloid cell HLA-DR (l) and PD-L1 protein. The number of cases in each group is indicated within each bar. *P<0.05 using Wilcoxon-rank test. All cases were measured together in the same experimental runs using linear histograms.

Treatment with nivolumab was associated with a mild (non-significant) increase in tumor T-cell infiltration.

Schalper K &, Idoate MA. Yale & Navarra Universities.



A LOS LUCHADORES POR
LA DIGITALIZACION
INMUNOHISTOQUIMICA

LA
PATOLOGIA
DIGITAL

INMUNOHISTOQUIMICA
LA PATOLOGIA DIGITAL

INMUNOHISTOQUIMICA
LA PATOLOGIA DIGITAL



Conclusiones

- La Patología y la Industria deben ir de la mano para un rápido avance en el paso de la Patología Cualitativa a una Patología Cuantitativa que se apoya en la evaluación de la inmunohistoquímica.
- Es necesaria la concienciación por parte del patólogo y otros especialistas, además de las instituciones implicadas, especialmente en el área de la Oncología, en la necesidad de *la evaluación precisa, reproducible y rápida de múltiples marcadores inmunohistoquímicos mediante la moderna tecnología de análisis automatizada de la imagen.*
- La nueva Patología Diagnóstica Cuantitativa permitirá disponer de valiosos datos de biomarcadores relevantes predictivos de respuesta a la terapia y de significado pronóstico para el bien de nuestros pacientes.



Dpto. de Anatomía Patológica. Sede CUN Pamplona y sede CUN Madrid.





Paisaje Otoñal Pireinaico Navarro.

