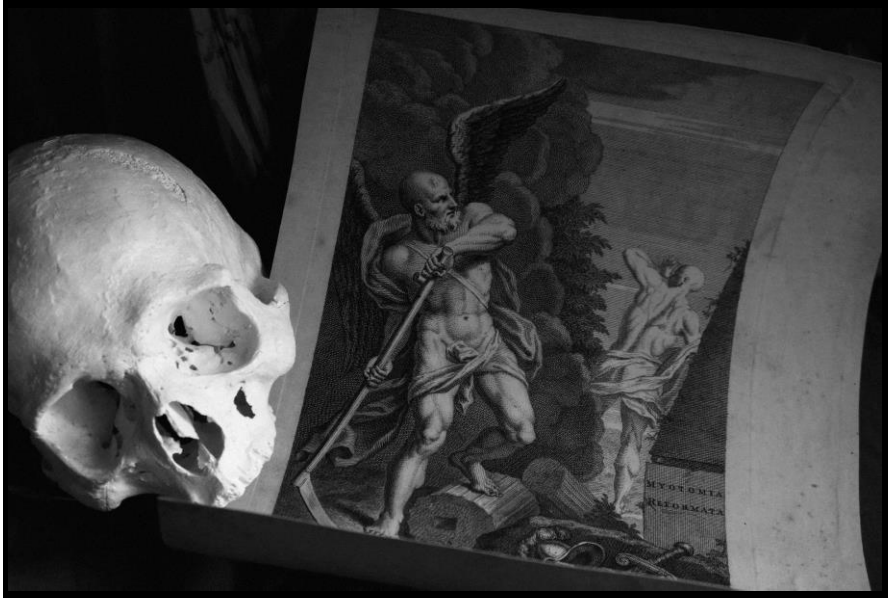


Is the right time for next generation histopathological diagnostics?

Matteo FASSAN, MD, PhD
Professor of Pathology
Department of Medicine (DIMED)
University of Padua - ITALY

1

Pathology

What my friends think I do.

What my mom thinks I do.

What society thinks I do.

What my boss thinks I do

What I think I do.

What I actually do.

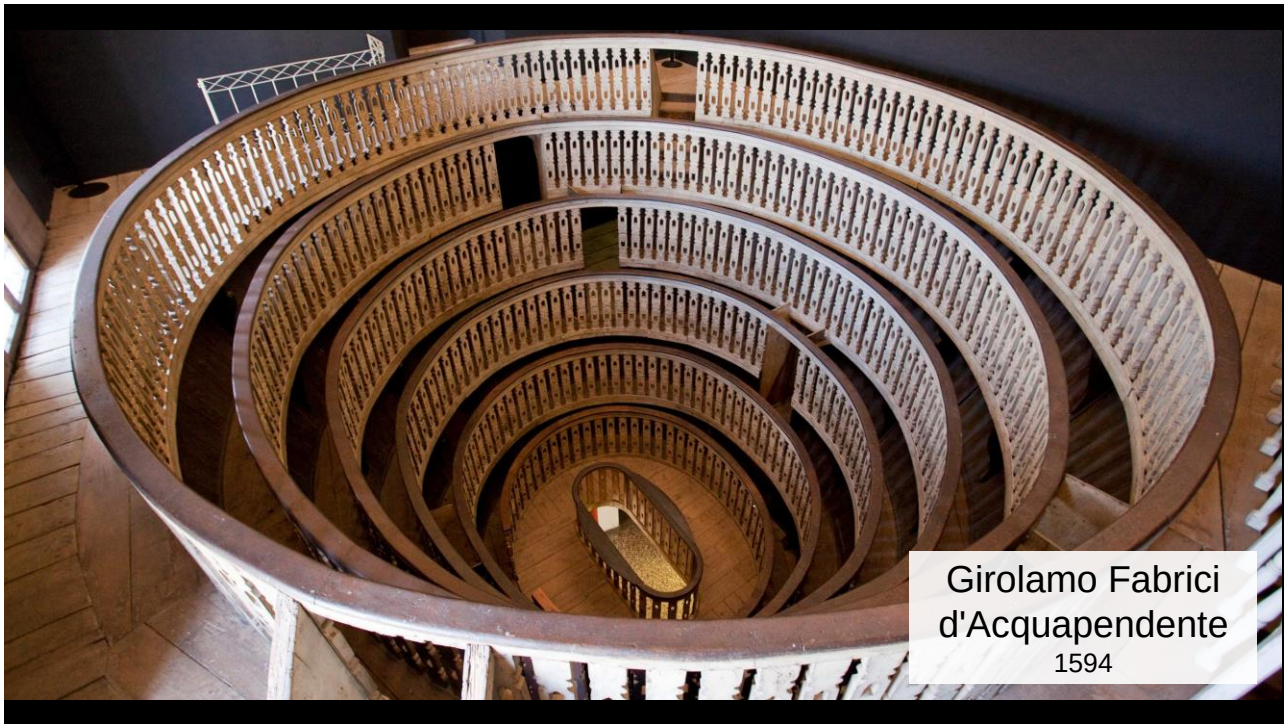
2

WIKIPEDIA
The Free Encyclopedia

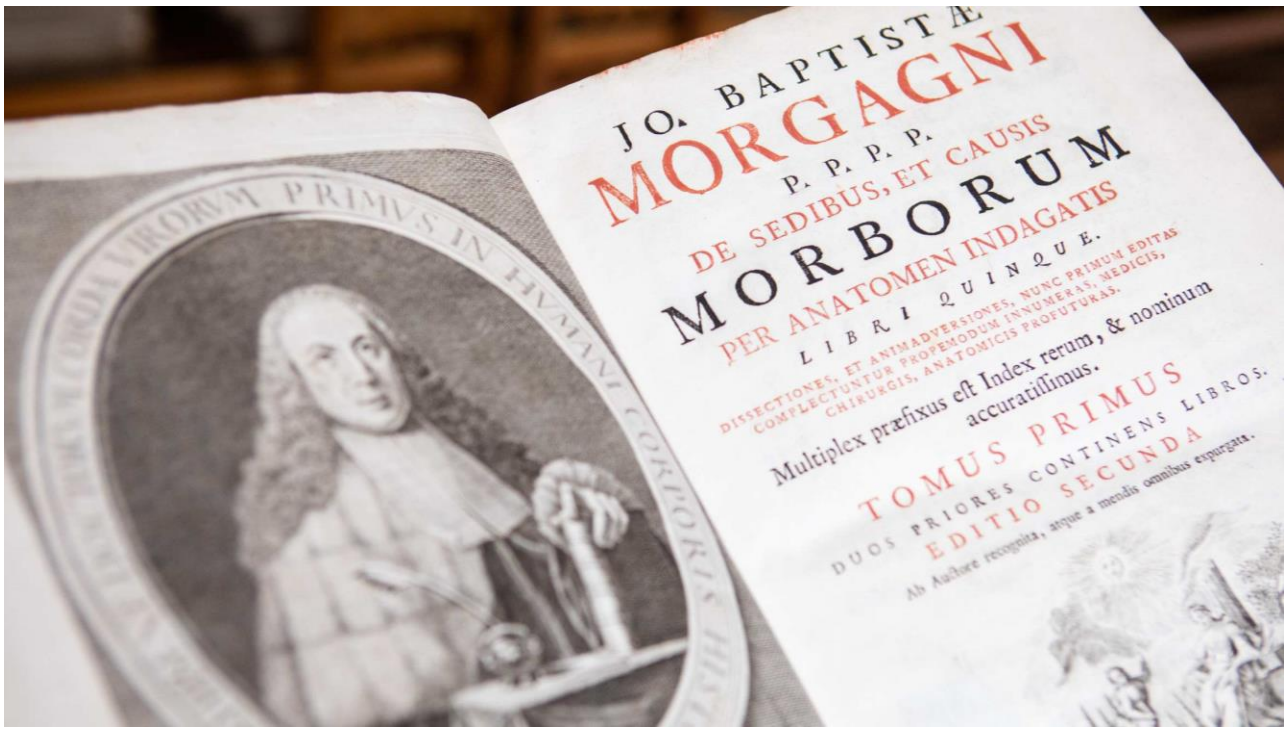
Anatomical pathology (Commonwealth) or Anatomic pathology (U.S.) is a medical specialty that is concerned with the diagnosis of disease based on the macroscopic, microscopic, biochemical, immunologic and molecular examination of organs and tissues.

3

4


Girolamo Fabrici
d'Acquapendente
1594

5


JO. BAPTISTÆ
MORGAGNI
P. P. P. P.
DE SEDIBUS, ET CAUSIS
PER ANATOMEN INDAGATIS
MORBORUM
LIBRI QUINQUE.
DISSECTIONES, ET ANIMADVERSIONES, NUNC PRIMUM EDITAS COMPLECTUNTUR PROPEMODUM INNUMERAS, MEDICIS, CHIRURGIS, ANATOMICIS PROFUTURAS.
Multiplex præfixus est Index rerum, & nominum accuratissimus.
TOMUS PRIMUS
DUOS PRIORES CONTINENS LIBROS.
EDITIO SECUNDA
Ab Auctore recognita, atque a mendis omnibus expurgata.

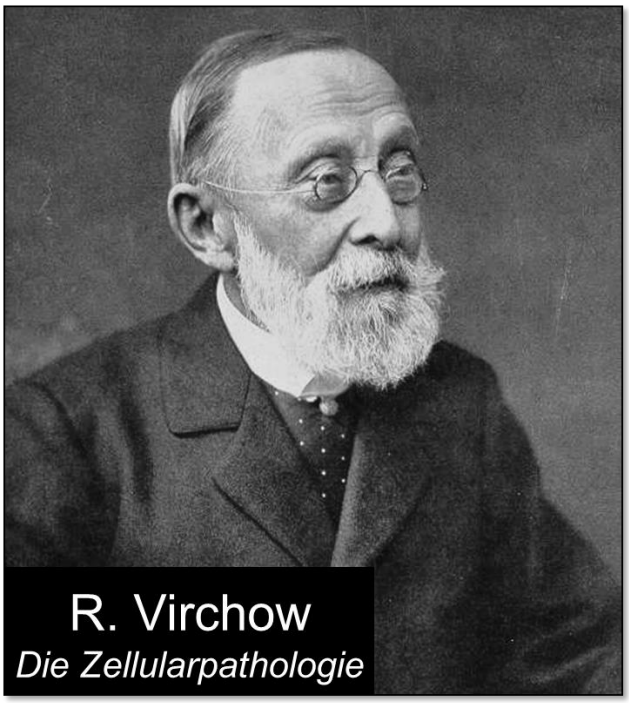
6

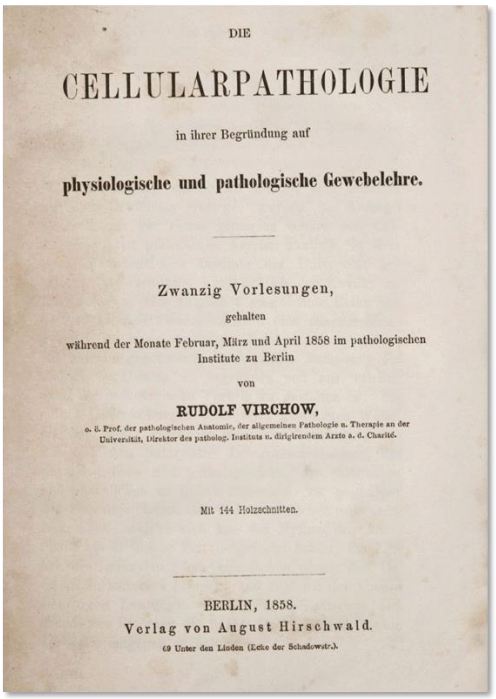
Prato della Valle: Pietro Danieletti's sculpture with the Morgagni's bust

7

8

9





DIE

CELLULARPATHOLOGIE

in ihrer Begründung auf

physiologische und pathologische Gewebelehre.

Zwanzig Vorlesungen,

gehalten

während der Monate Februar, März und April 1858 im pathologischen Institute zu Berlin

von

RUDOLF VIRCHOW,

o. ö. Prof. der pathologischen Anatomie, der allgemeinen Pathologie u. Therapie an der Universität, Direktor des patholog. Instituts u. dirigirendem Arzte a. d. Charité.

Mit 144 Holzschnitten.

BERLIN, 1858.

Verlag von August Hirschwald.

(9 Unter den Linden (Ecke der Schadowstr.).

10

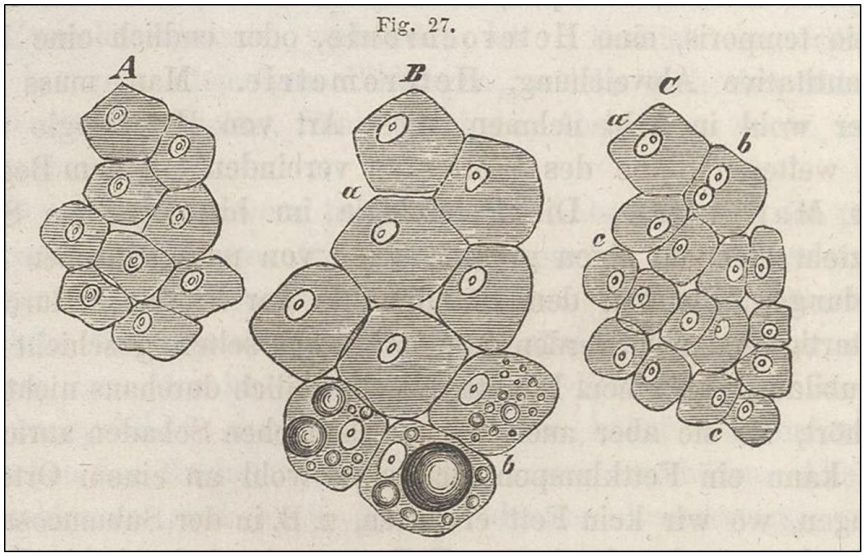
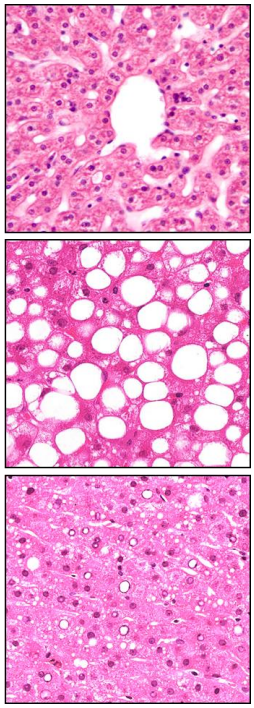


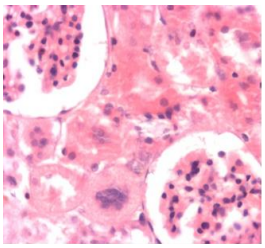
Fig. 27. Schematische Darstellungen von Leberzellen. *A* Einfache physiologische Anordnung derselben. *B* Hypertrophie, *a* einfache, *b* mit Fettaufnahme (fettige Degeneration, Fettleber) *C* Hyperplasie (numerische oder adjunctive Hypertrophie) *a* Zelle mit Kern und getheiltem Kernkörperchen. *b* getheilte Kerne. *c*, *c* getheilte Zellen.



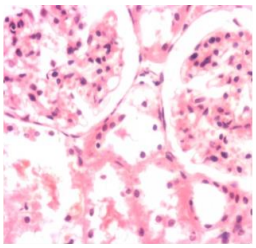
11

12

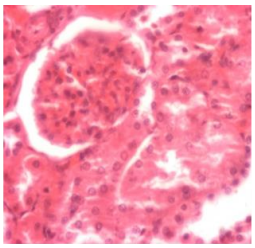
Histology after fixation: the kidney paradigm



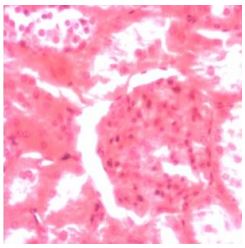
Acetic acid



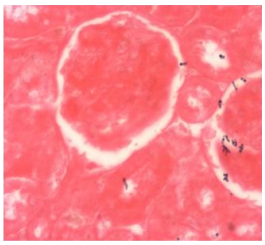
Bouin



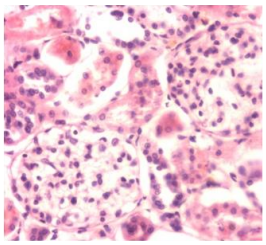
Formaldheyde



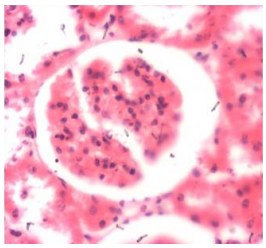
Glutaraldheyde



Mercuric chloride

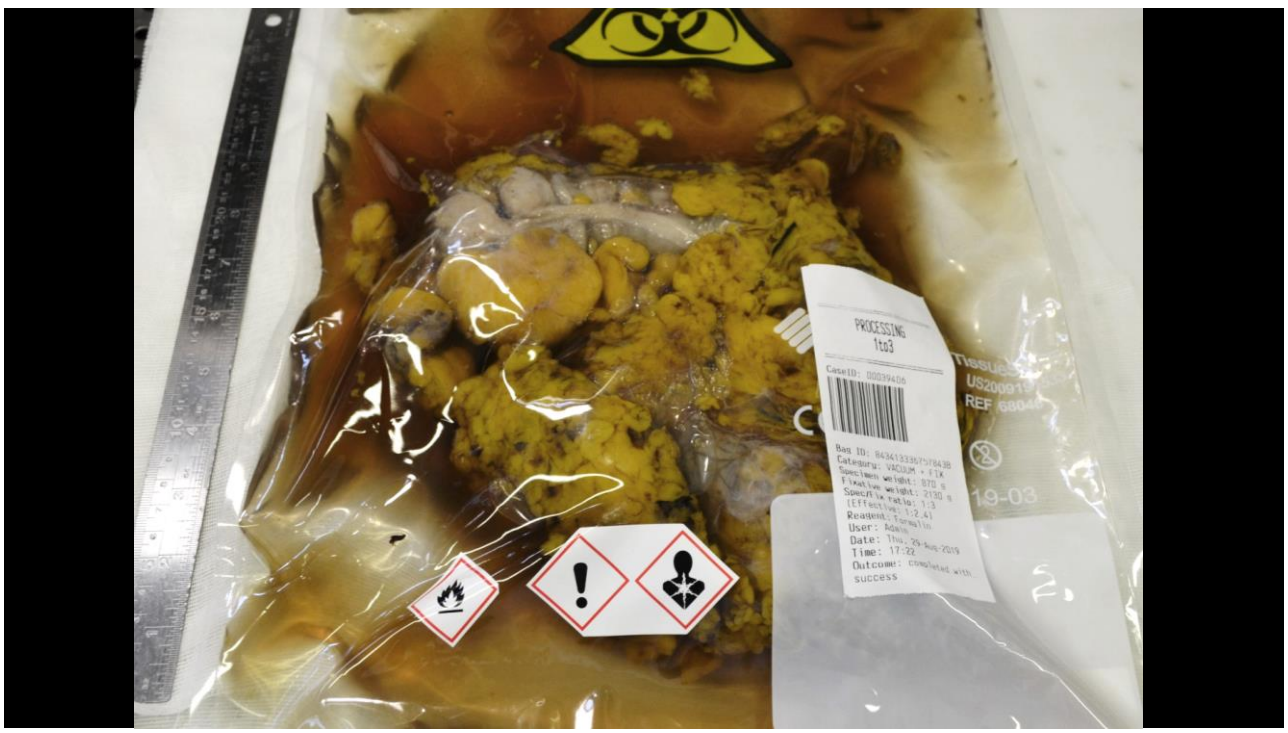


Potassium dichromate



Zenker

13



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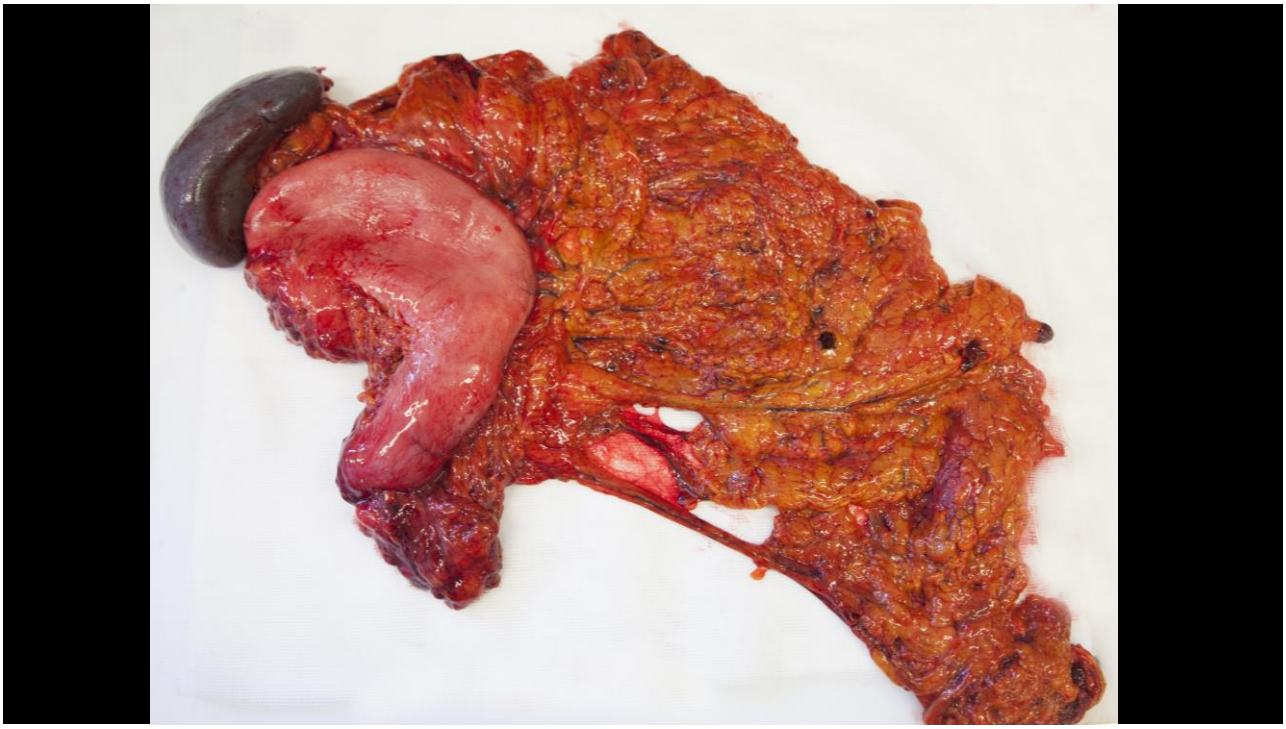

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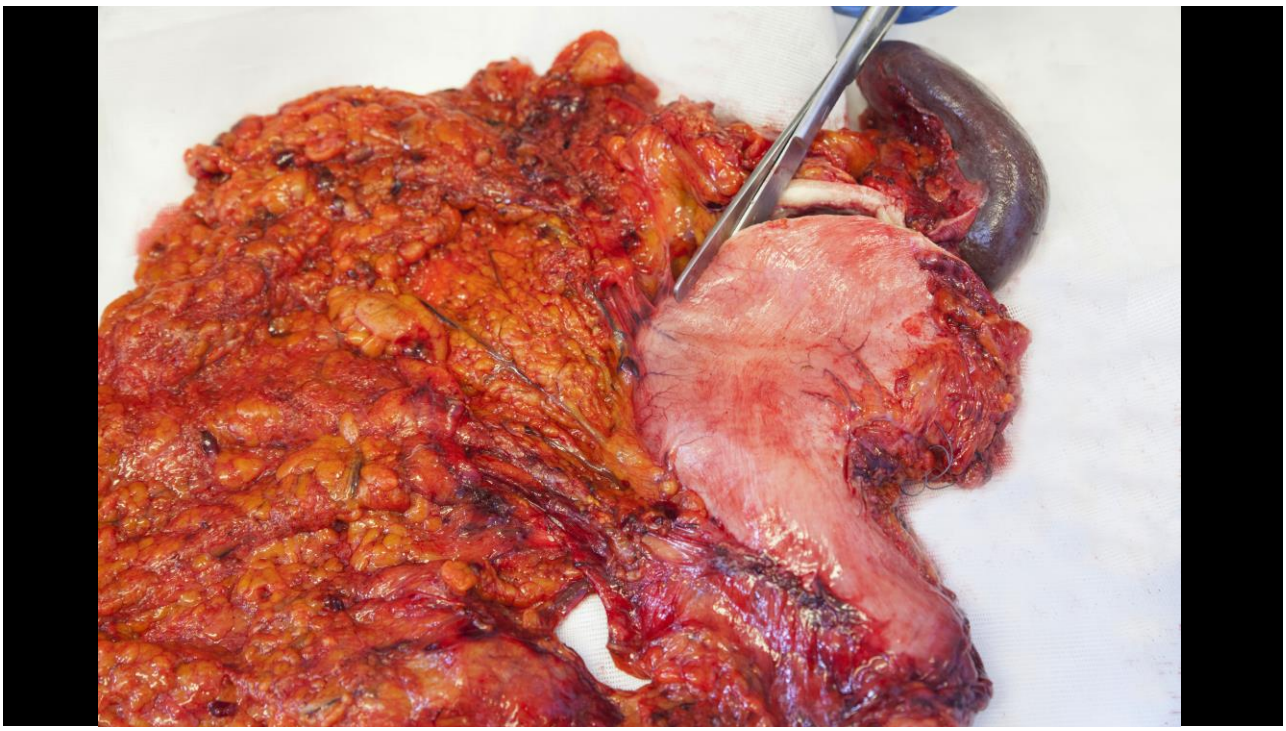

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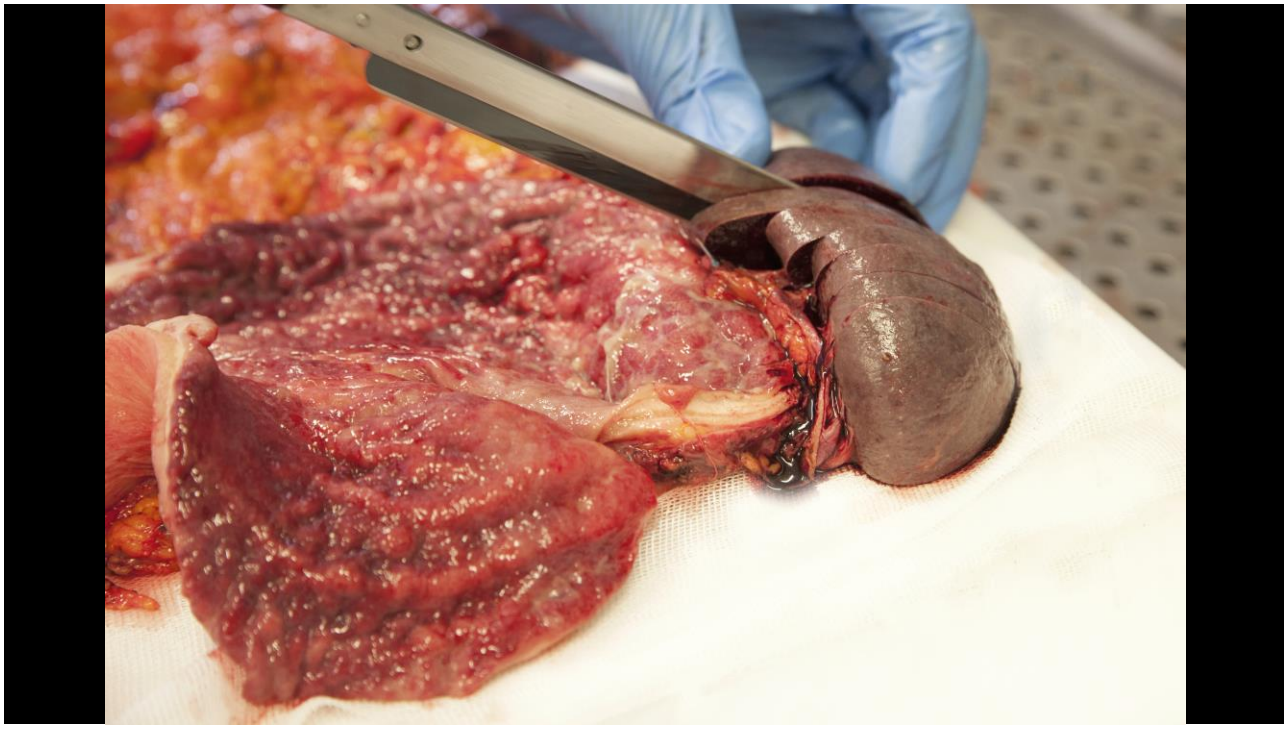

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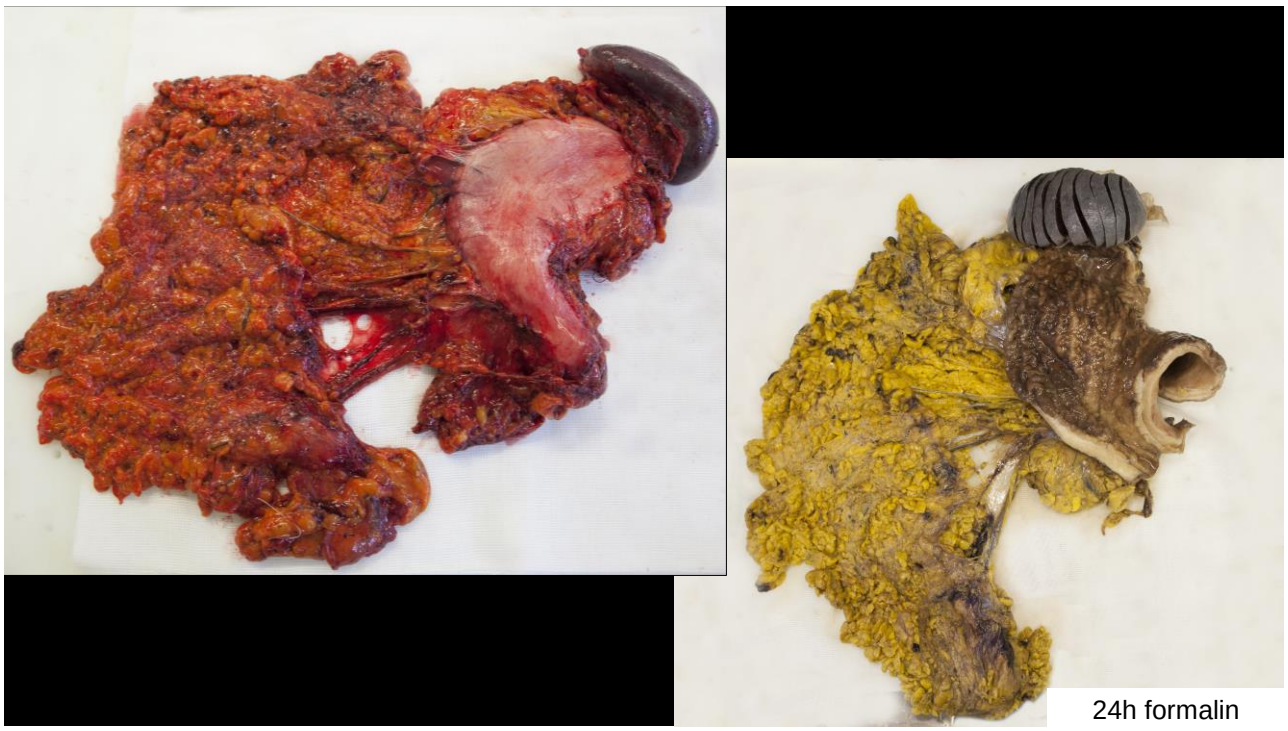
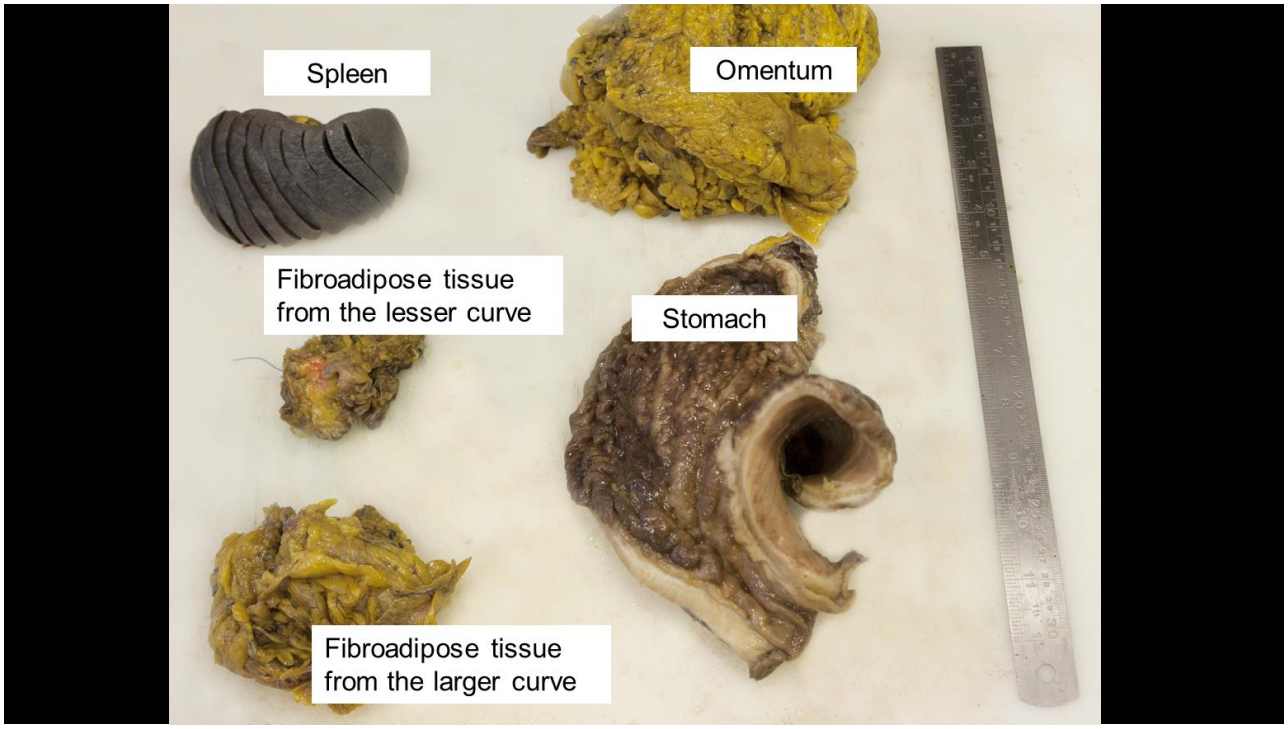
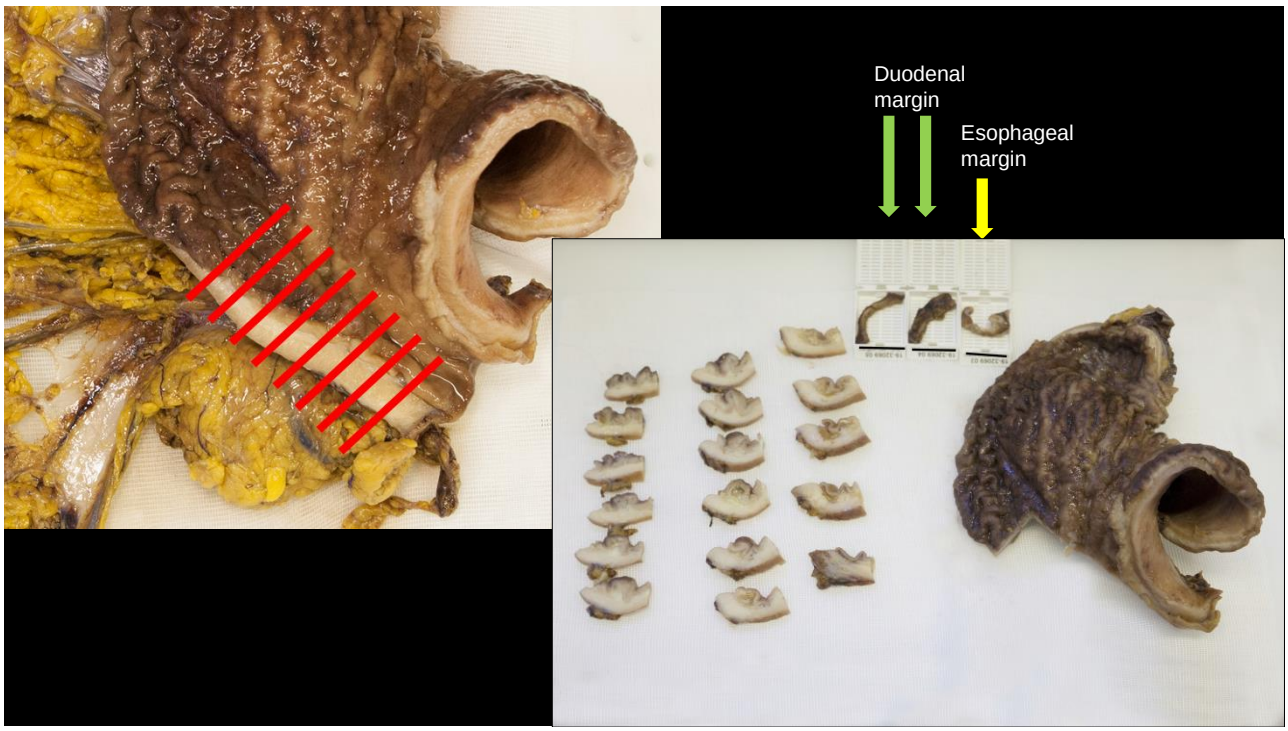


24h formalin

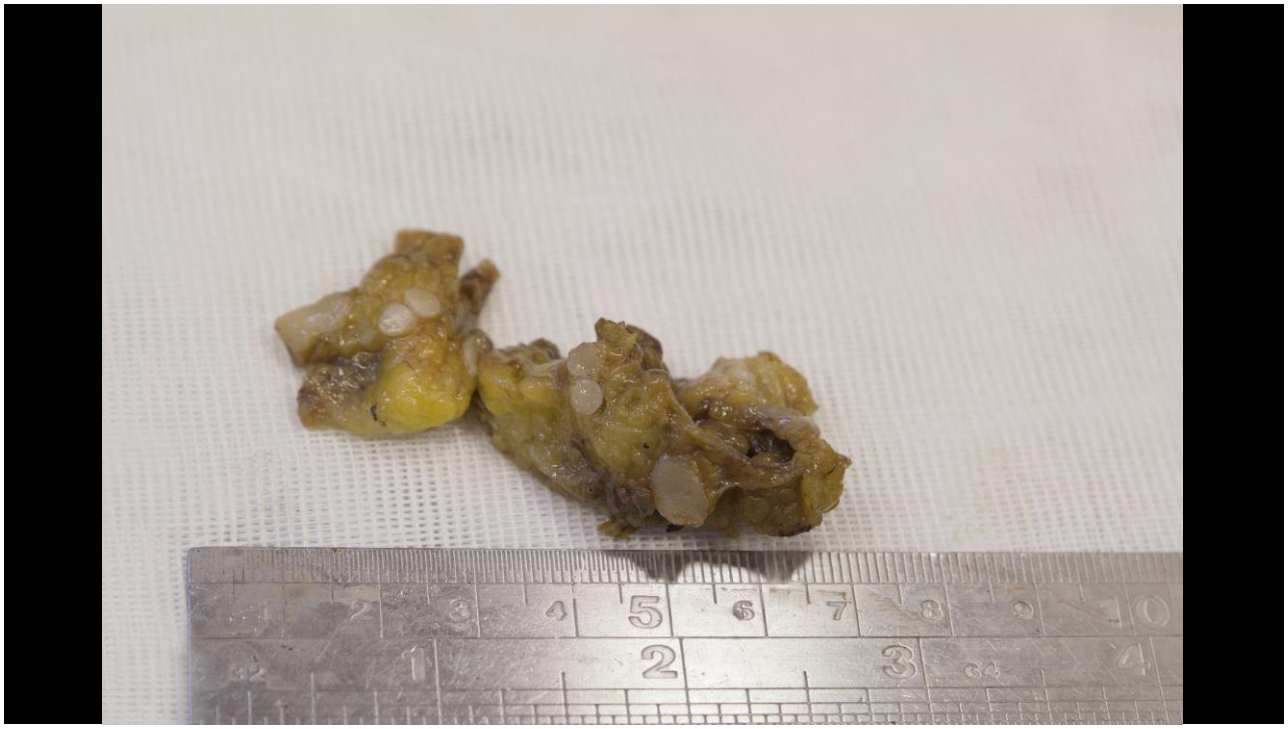
18


Spleen
Omentum
Fibroadipose tissue from the lesser curve
Stomach
Fibroadipose tissue from the larger curve

19


Duodenal margin
Esophageal margin

20



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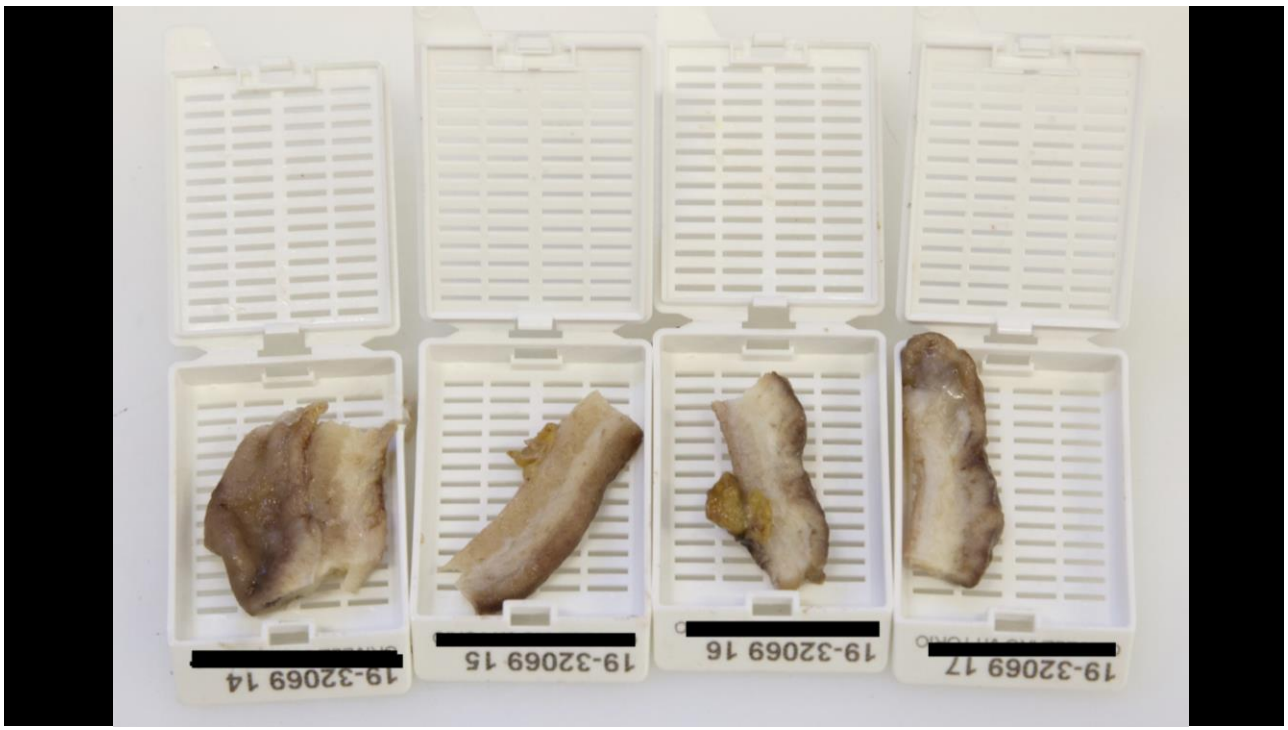

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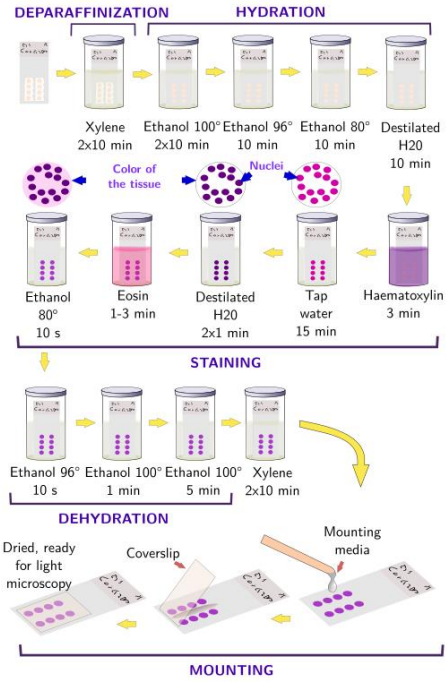
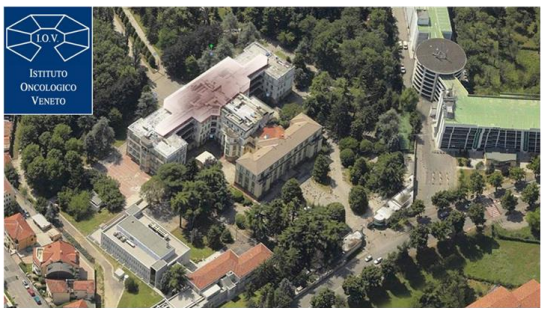


autostainer

23

SURGICAL PATHOLOGY UNIT S1



2019 - Padua

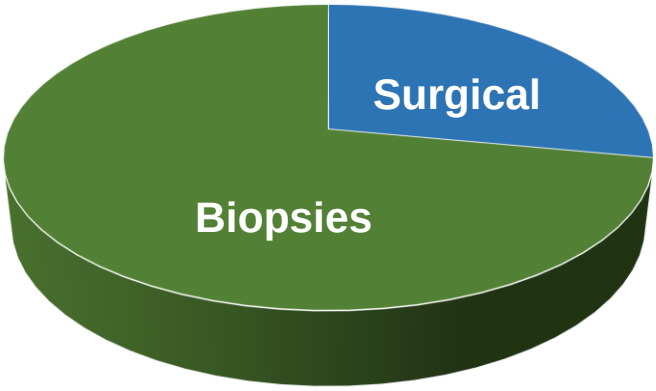
~55,000 histology reports
~30,000 cytology reports
~2,000 molecular path reports
470,500 FFPE blocks

Reference Unit for IOV (Padua)

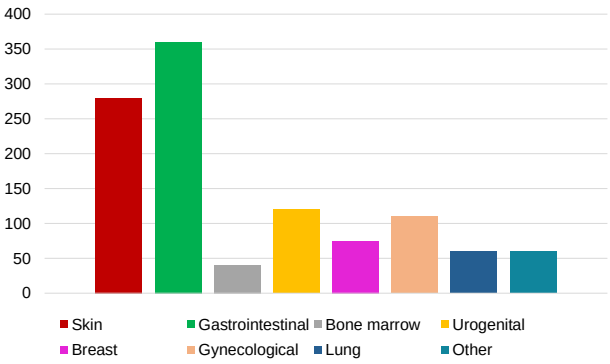
Breast surgery – Gastroenterology – Radiology - Oncology

24

Type of samples to be processed



Biopsy specimens: organ of origin



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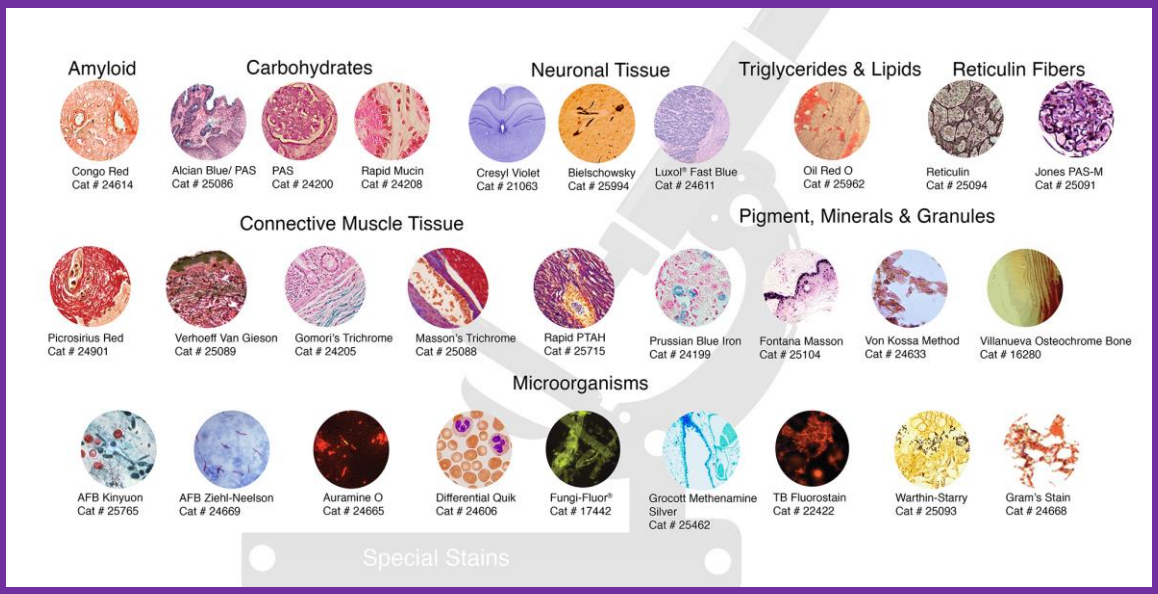

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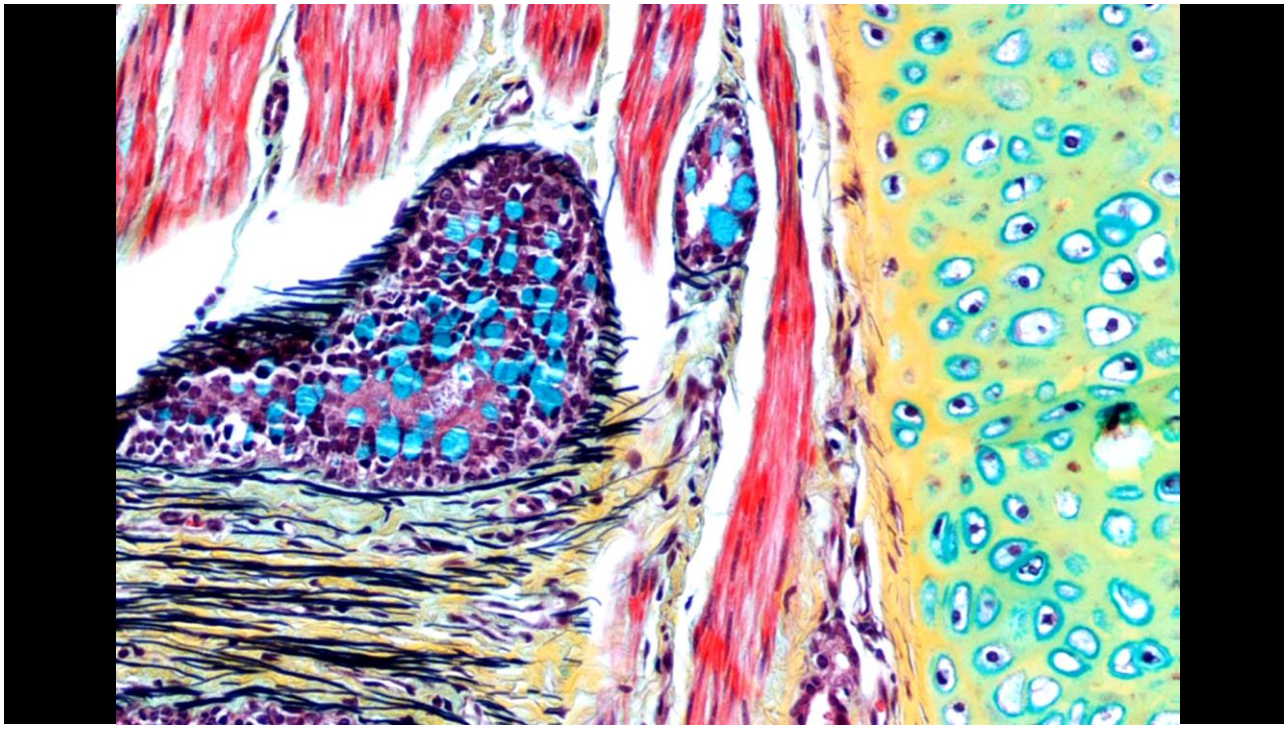

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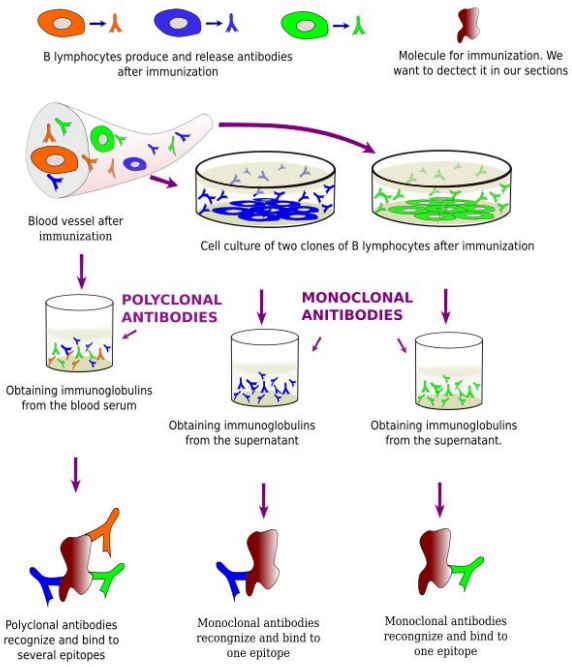
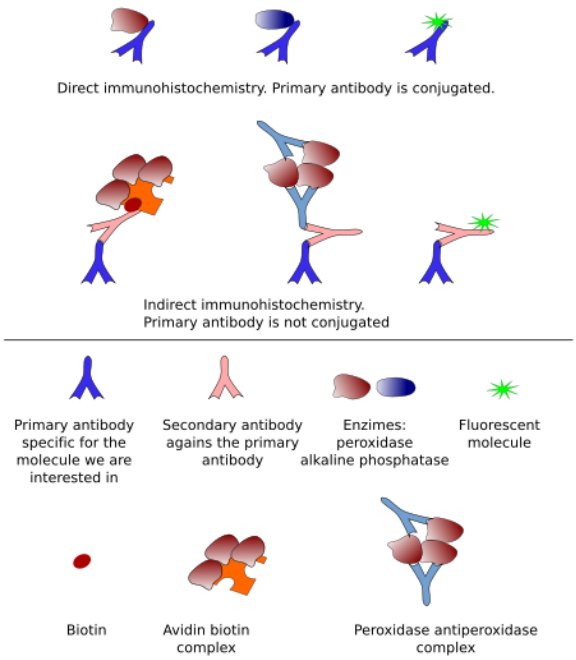
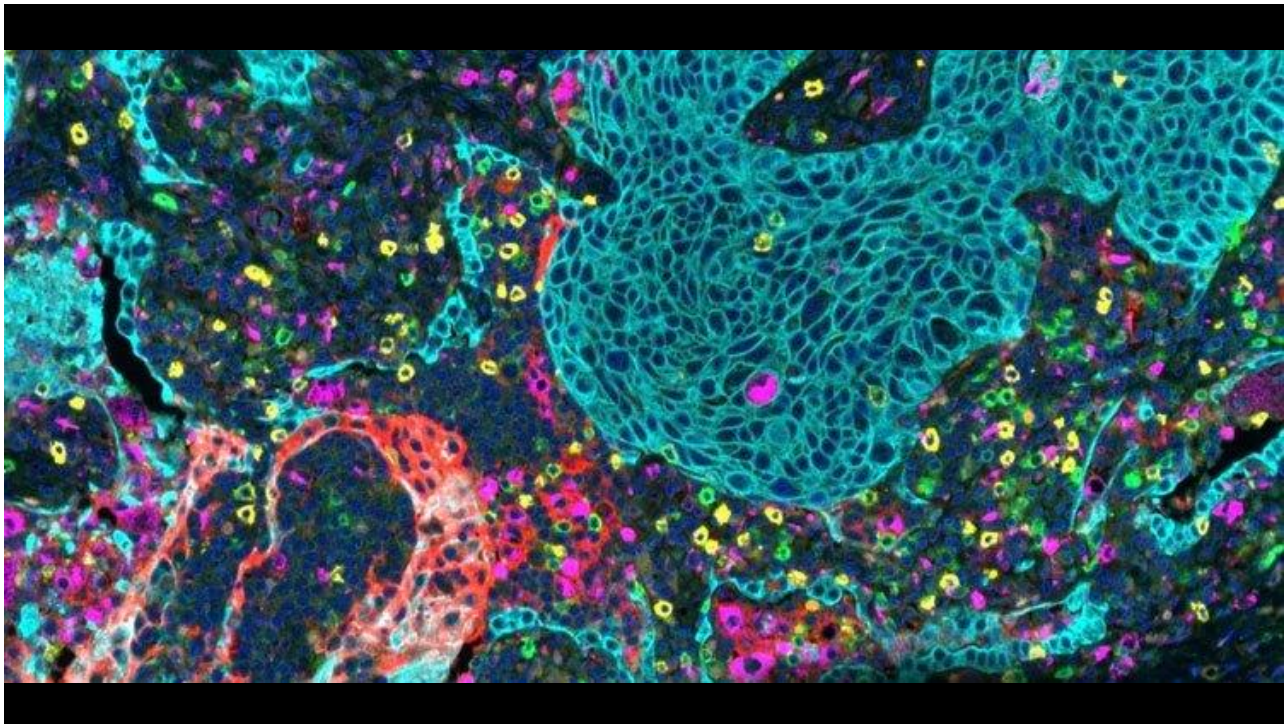

B lymphocytes produce and release antibodies after immunization
Molecule for immunization. We want to dectect it in our sections
Blood vessel after immunization
Cell culture of two clones of B lymphocytes after immunization
POLYCLONAL ANTIBODIES
MONOCLONAL ANITIBODIES
Obtaining immunoglobulins from the blood serum
Obtaining immunoglobulins from the supernatant
Obtaining immunoglobulins from the supernatant.
Polyclonal antibodies recognize and bind to several epitopes
Monoclonal antibodies recongnize and bind to one epitope
Monoclonal antibodies recongnize and bind to one epitope


Direct immunohistochemistry. Primary antibody is conjugated.
Indirect immunohistochemistry.
Primary antibody is not conjugated
Primary antibody specific for the molecule we are interested in
Secondary antibody agains the primary antibody
Enzimes: peroxidase alkaline phosphatase
Fluorescent molecule
Biotin
Avidin biotin complex
Peroxidase antiperoxidase complex

28



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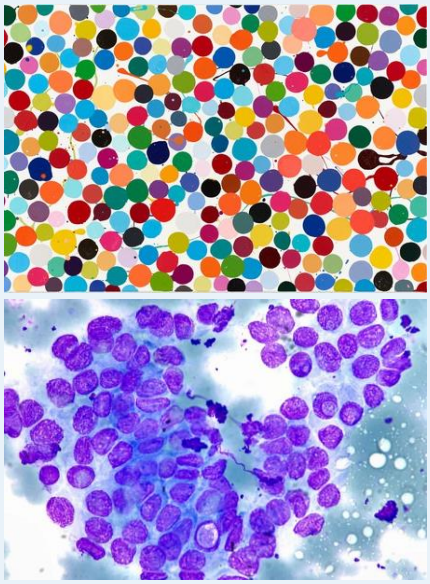
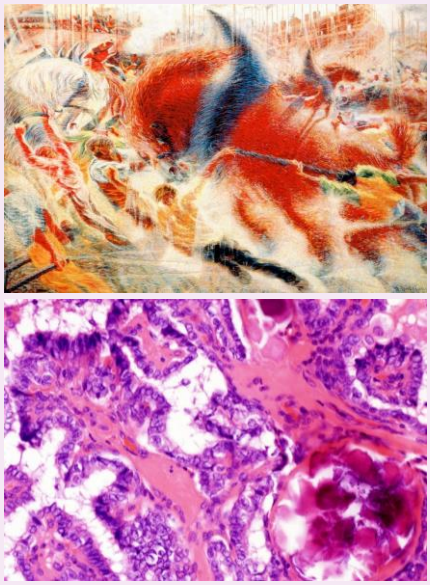


Cytology

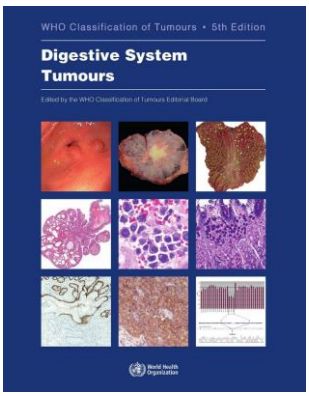


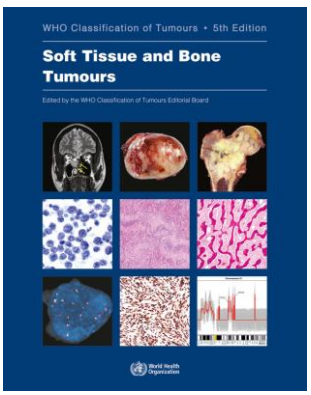
Histology

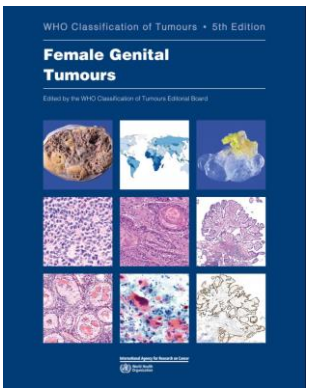
30


Normal
Inflammation
Cancer
Autoimmune disease

31


WHO Classification of Tumours • 5th Edition
Digestive System Tumours


WHO Classification of Tumours • 5th Edition
Soft Tissue and Bone Tumours


WHO Classification of Tumours • 5th Edition
Female Genital Tumours

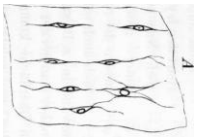
32

The multistep model of scientific paradigms

1761

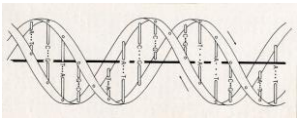
De sedibus et causis morborum per anatomen indagatis

1858



Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebenlehre

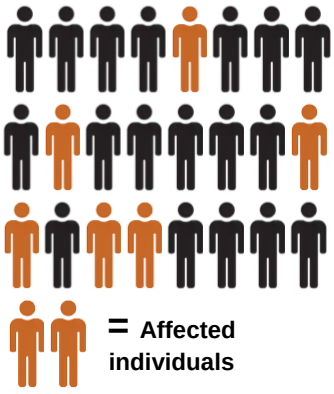
1953

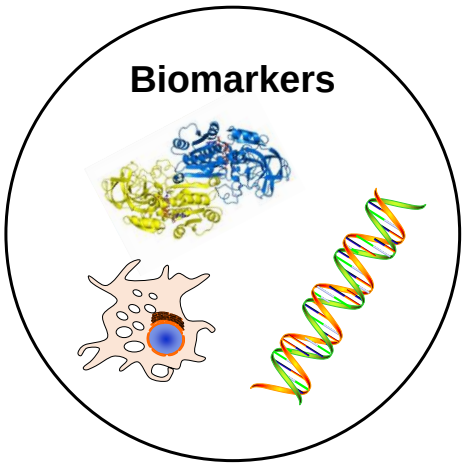


Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid

33

Diagnostic





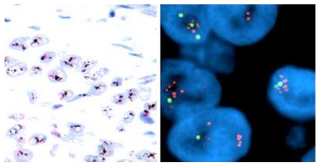
Prognostic

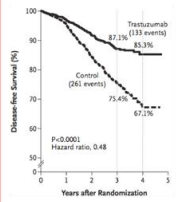
Predictive

34

From the molecular alteration to the targeted therapy

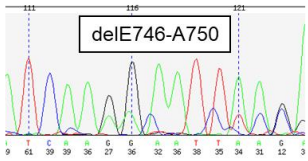
HER2 amplification
20-30% Breast ductal k

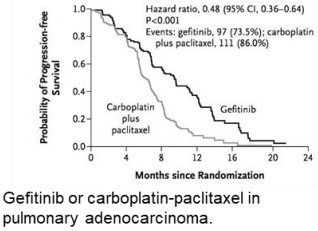




Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer.
Romond et al. - _NEJM_ 2005

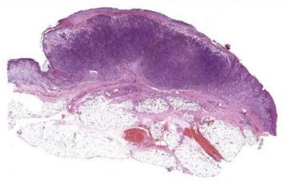
EGFR mutation
15-20% Lung adenocarcinomas

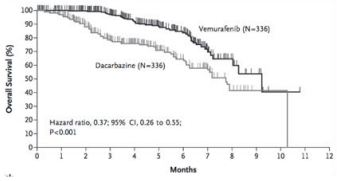




Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma.
Mok et al. - _NEJM_ 2009

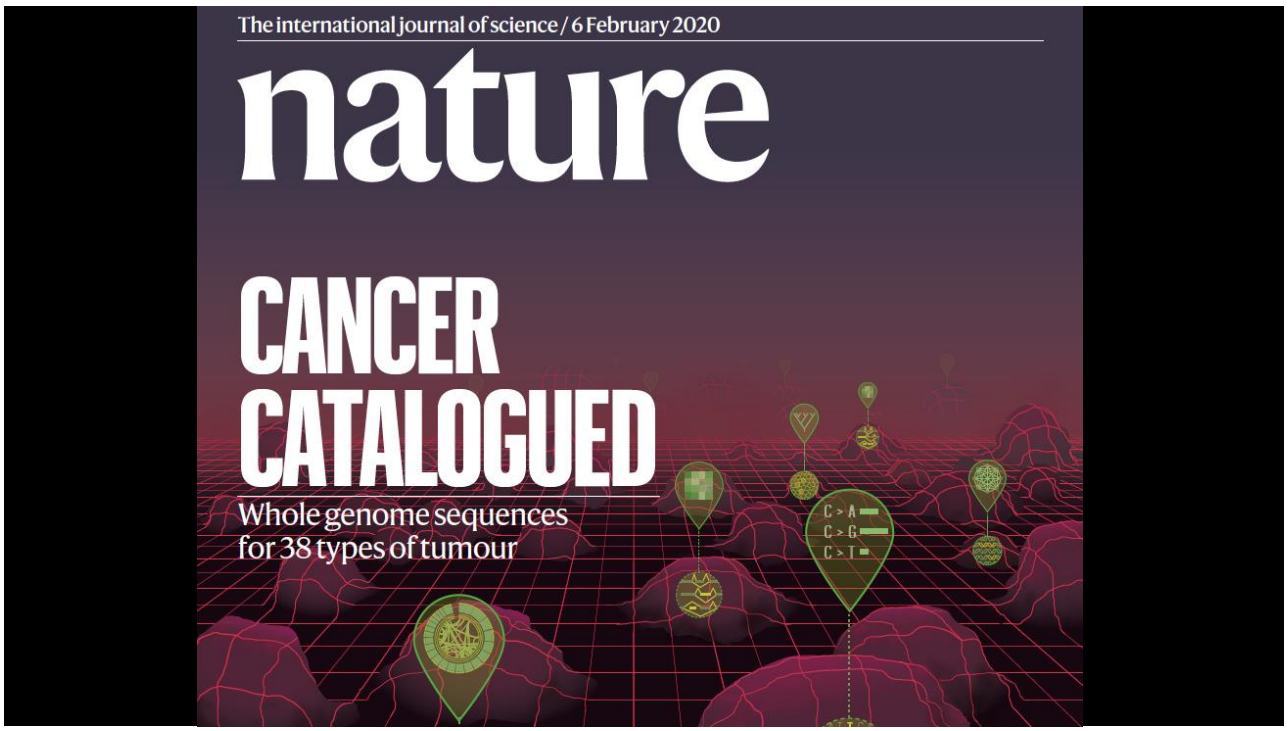
BRAF V600E mutation
50-60% Melanoma





Improved survival with vemurafenib in melanoma with _BRAF_ V600E mutation.
Chapman et al. - _NEJM_ 2011

35



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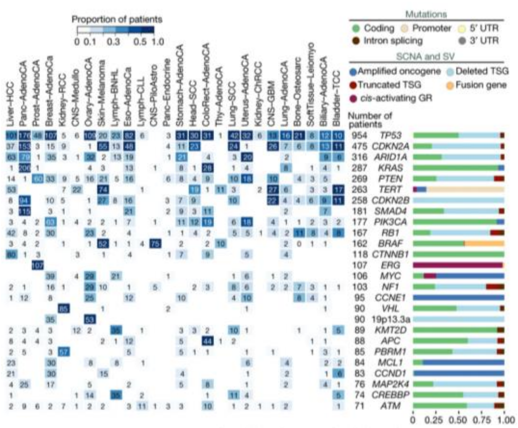
nature

Article | Open Access | Published: 05 February 2020

Pan-cancer analysis of whole genomes

The ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium

Nature 578, 82–93(2020) | Cite this article



PCAWG PanCancer Analysis of WHOLE GENOMES

"Cancer is driven by genetic change, and **the advent of massively parallel sequencing** has enabled systematic documentation of this variation at the whole-genome scale."

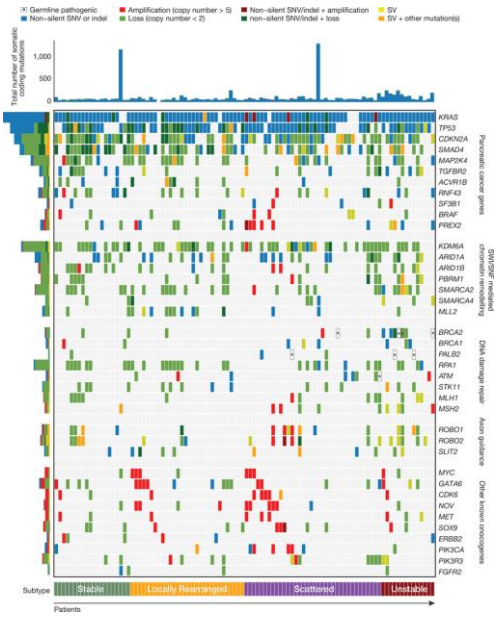
"On average, cancer genomes contained **4-5 driver mutations** when combining coding and non-coding genomic elements; however, in around 5% of cases no drivers were identified, suggesting that cancer driver discovery is not yet complete."

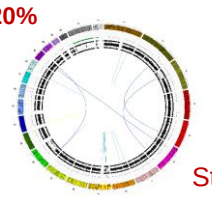
37

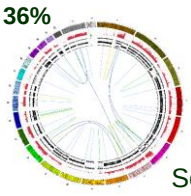
The mutational landscape of pancreatic cancer

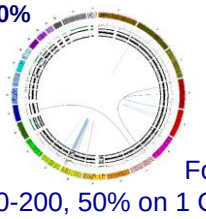
38

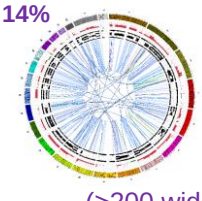
Whole genomes redefine the mutational landscape of PDAC











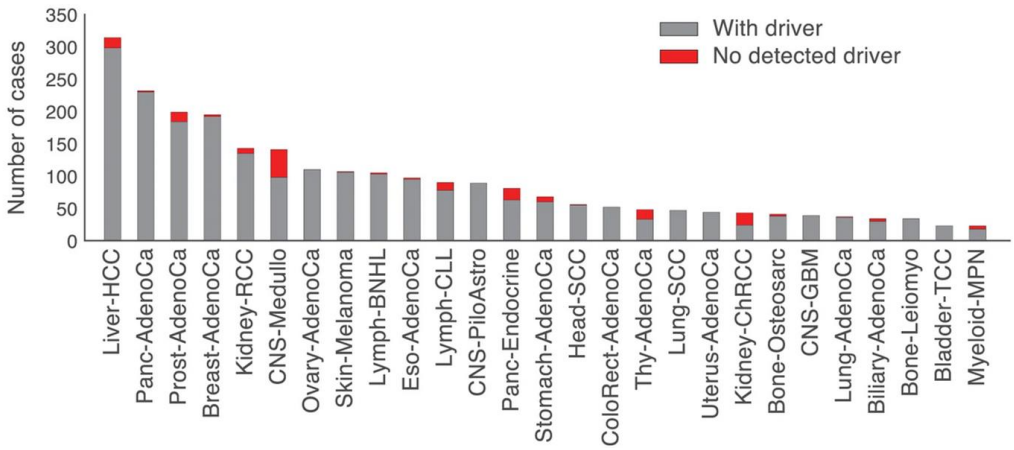
Waddell N, *et al.* – Nature 2015

39

Pan-cancer analysis of whole genomes

The ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium

Nature **578**, 82–93(2020) | Cite this article



91% of tumors had at least one identified driver mutation

40

Tissue and molecular diagnostics

- Choice of the right diagnostic approach for the available tissue sample
- Tumor is a tissue and the patologist's evaluation matter!
- Next generation sequencing in old generation laboratories

41

Tissue and molecular diagnostics

- Choice of the right diagnostic approach for the available tissue sample
- Tumor is a tissue and the patologist's evaluation matter!
- Next generation sequencing in old generation laboratories

42

The molecular diagnostics' recipe

The ingredients
(i.e. the samples)

The kitchen accessories
(i.e. the molecular methods)

43

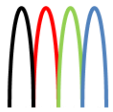
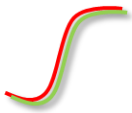
The molecular diagnostics' recipe

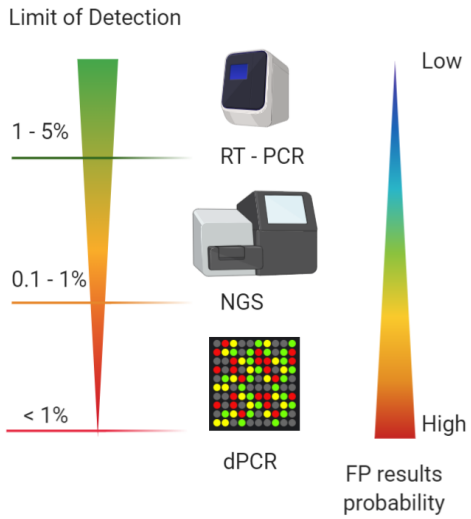
The ingredients
(i.e. the samples)

The kitchen accessories
(i.e. the molecular methods)

44

The heterogeneous landscape of diagnostic kits for targeted mutational assessment

	Sanger Sequencing	Real Time PCR	Digital PCR	Next Generation Sequencing
				
Limit of detection	10 – 20%	1 – 5%	0.1 – 1%	0.01 – 5%
Reference Range	All the mutations present in the analyzed gene regions	Only «hot spot» Mutations (probe based)	Only «hot spot» Mutations (probe based)	All the mutations present in the analyzed gene regions



Passiglia F, *et al.* – J Thorac Oncol 2019

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The heterogeneous landscape of diagnostic kits for targeted mutational assessment

IHC, FISH, RT-PCR, Pyrosequencing, Sanger, Real Time-PCR, ddPCR, NGS **<u>are methods, not tests</u>**!

Passiglia F, *et al.* – J Thorac Oncol 2019

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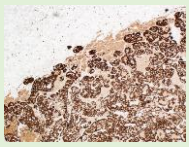
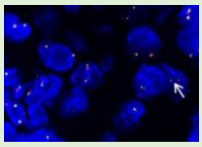
We can apply different filters, but she still is Marilyn Monroe!

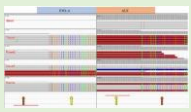
We can apply different methods to perform a test (and get an adequate result; *ALK* fusion)



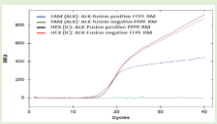
IHC



FISH



NGS

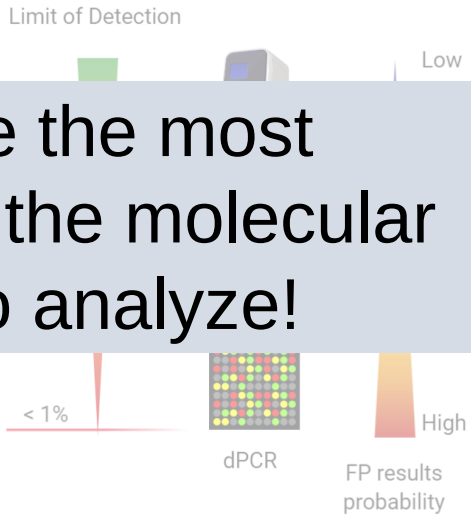


Real time

47

The heterogeneous landscape of diagnostic kits for targeted mutational assessment

	Sanger Sequencing	Real Time PCR	Digital PCR	Next Generation Sequencing
Reference Range	All the mutations present in the analyzed gene regions	Only «hot spot» Mutations (probe based)	Only «hot spot» Mutations (probe based)	All the mutations present in the analyzed gene regions



Passiglia F, *et al.* – J Thorac Oncol 2019

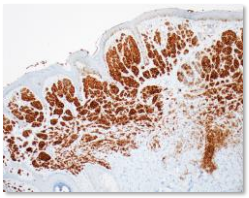
We have to chose the most adequate method for the molecular lesion we have to analyze!

48

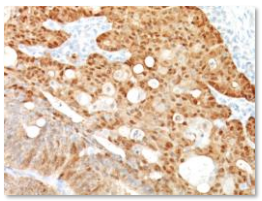
Situation 1

We have to test 1 gene with a known alteration (mutation/translocation/amplification/deletion)

- The best option is a «hot spot» single gene method such as Real time, FISH, IHC depending on the alteration we are looking for.
- NGS is not the best option in this case.



***BRAF* V600E**



B-catenin

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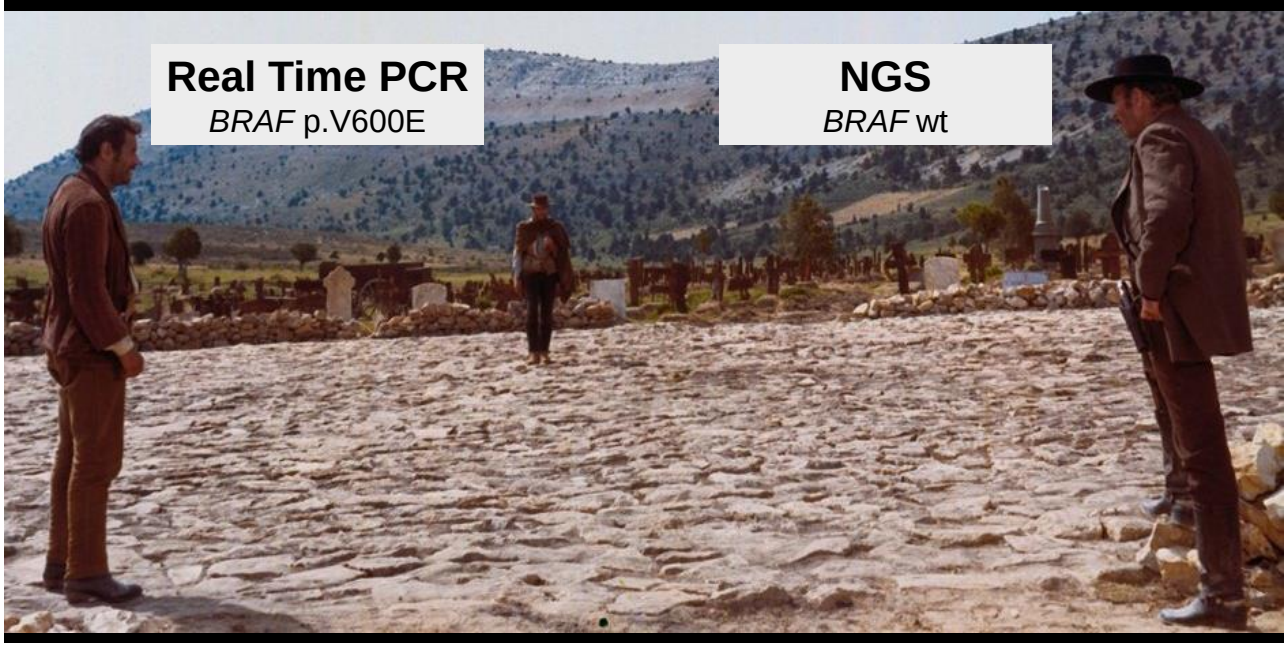
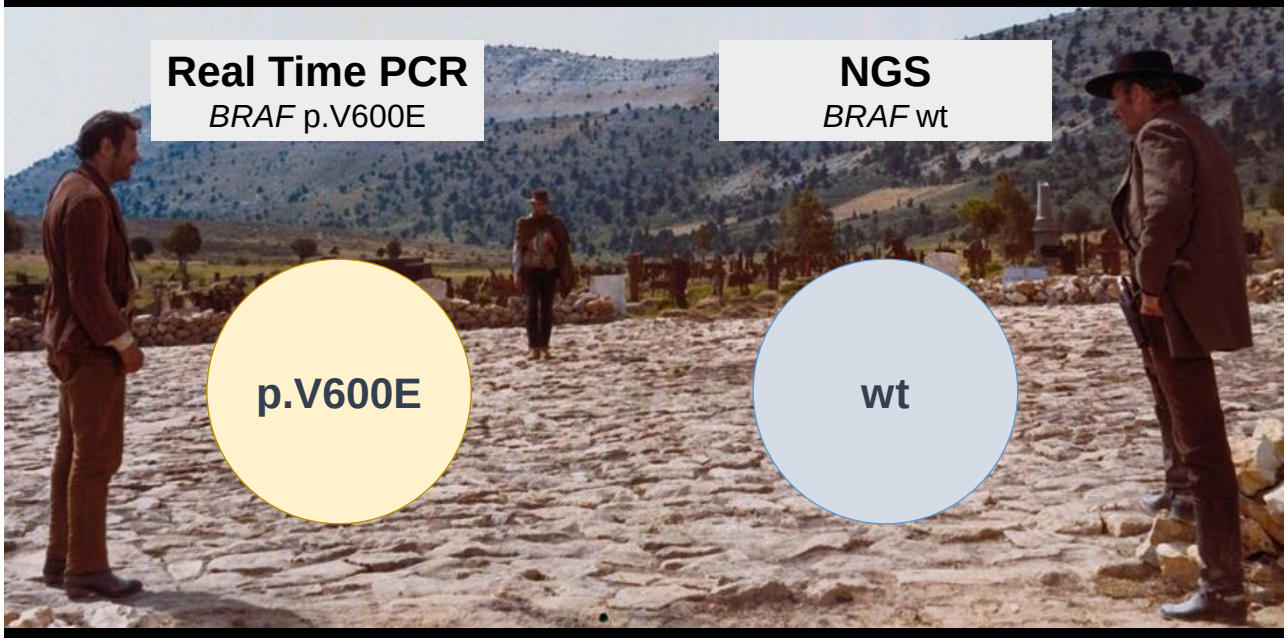
The diagnostics' duel



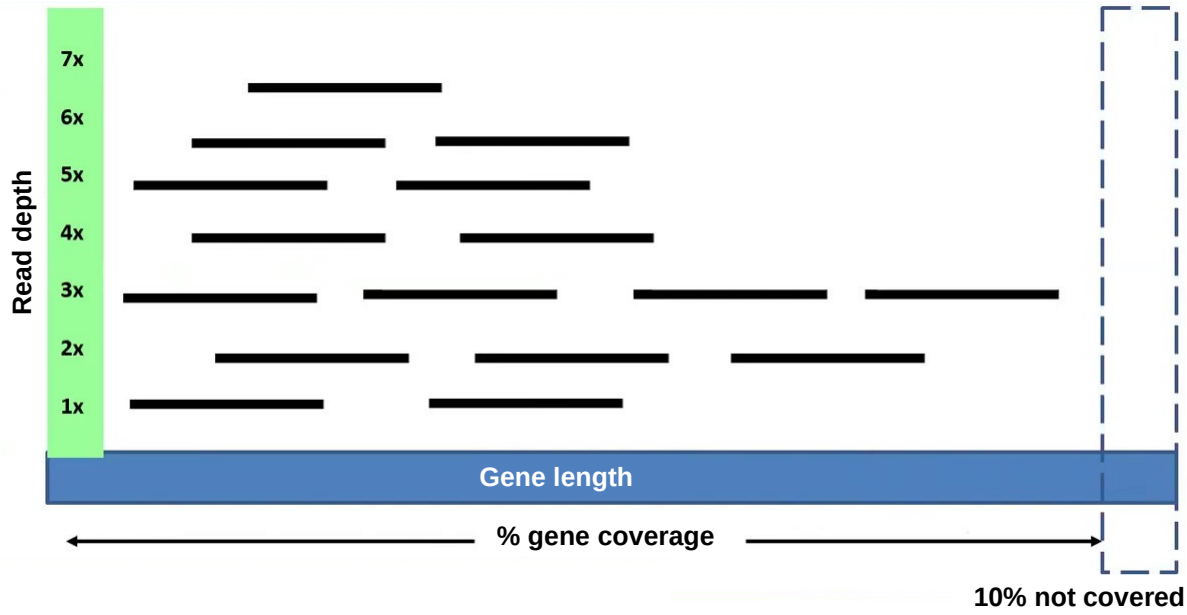
50

The diagnostics' duel



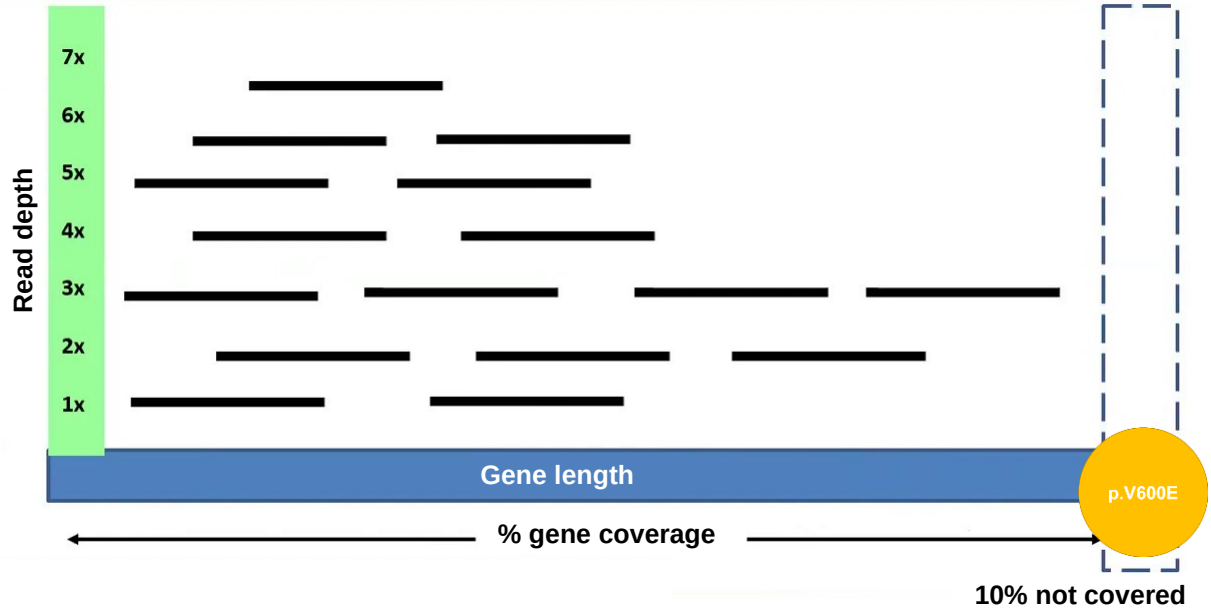
51

NGS technical problems



52

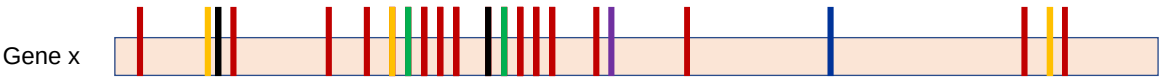
NGS technical problems



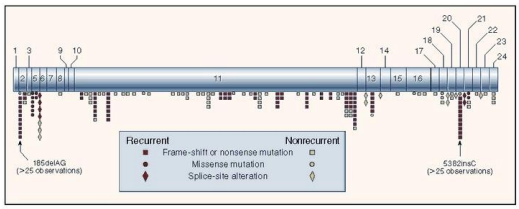
53

Situation 2

We have to test 1 gene with different known and unknown alterations
(mutation/translocation/amplification/deletion)



- Forget the «hot spot» option! It requires a large amount of material, is time consuming and has a relatively higher cost.
- NGS is the best option. Need to consider the best NGS approach (RNA- or/and DNA-based)



***BRCA1* - Lots of mutations, lots of dilemmas**
Collins FS – NEJM 1996

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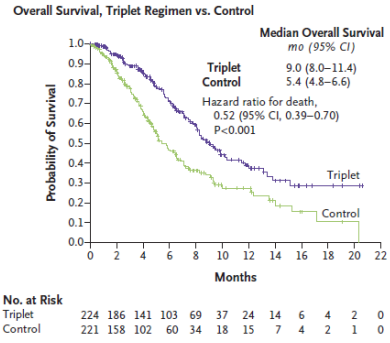
The NEW ENGLAND JOURNAL of MEDICINE

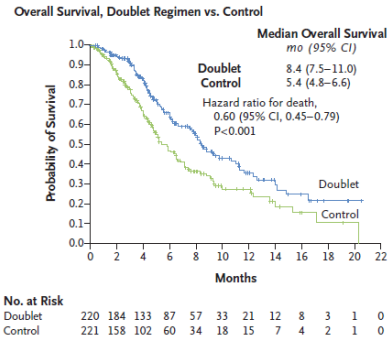
ORIGINAL ARTICLE

Encorafenib, Binimetinib, and Cetuximab in *BRAF* V600E–Mutated Colorectal Cancer

S. Kopetz, A. Grothey, R. Yaeger, E. Van Cutsem, J. Desai, T. Yoshino, H. Wasan, F. Ciardiello, F. Loupakis, Y.S. Hong, N. Steeghs, T.K. Guren, H.-T. Arkenau, P. Garcia-Alfonso, P. Pfeiffer, S. Orlov, S. Lonardi, E. Elez, T.-W. Kim, J.H.M. Schellens, C. Guo, A. Krishnan, J. Dekervel, V. Morris, A. Calvo Ferrandiz, L.S. Tarpgaard, M. Braun, A. Gollerkeri, C. Keir, K. Maharry, M. Pickard, J. Christy-Bittel, L. Anderson, V. Sandor, and J. Tabernero

A combination of encorafenib (anti BRAF), cetuximab, and binimetinib (anti MEK) resulted in significantly longer overall survival and a higher response rate than standard therapy in patients with metastatic colorectal cancer with the *BRAF* V600E mutation.





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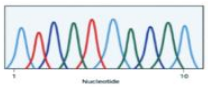
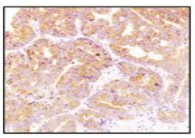
The *BRAF* diagnostic scenario

SANGER SEQUENCING	REAL-TIME PCR	MASS SPECTROMETRY	NEXT-GENERATION SEQUENCING	IMMUNOHISTO-CHEMISTRY (VE1 clone)
				
10-20% AF	1-5% AF	1-5% AF	0.001-5% AF	0.001-5% AF
Low cost High TAT	Low cost Low TAT	Low cost Low TAT	High cost High TAT	Low cost Low TAT
All *BRAF* mutations	Only hotspot *BRAF* mutations	Only hotspot *BRAF* mutations	All *BRAF* mutations	Only ^{V600E}*BRAF* mutations

Angerilli V, *et al.* - Crit Rev Oncol Hematol 2022

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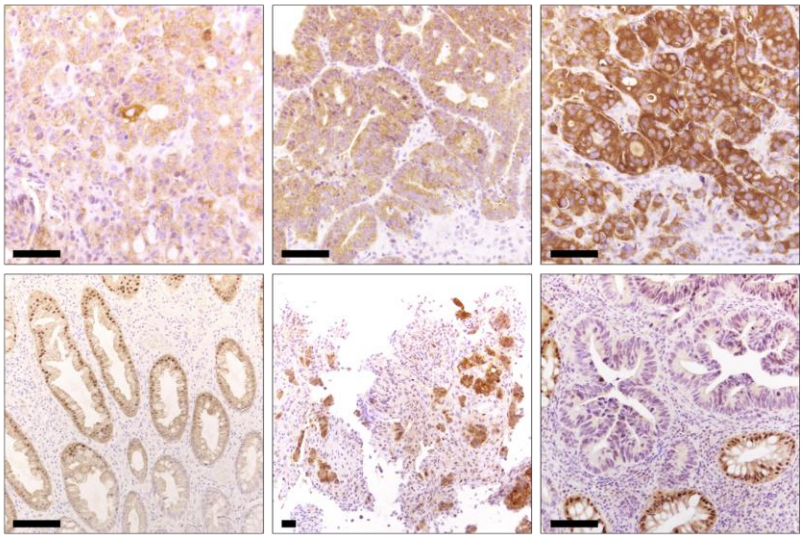
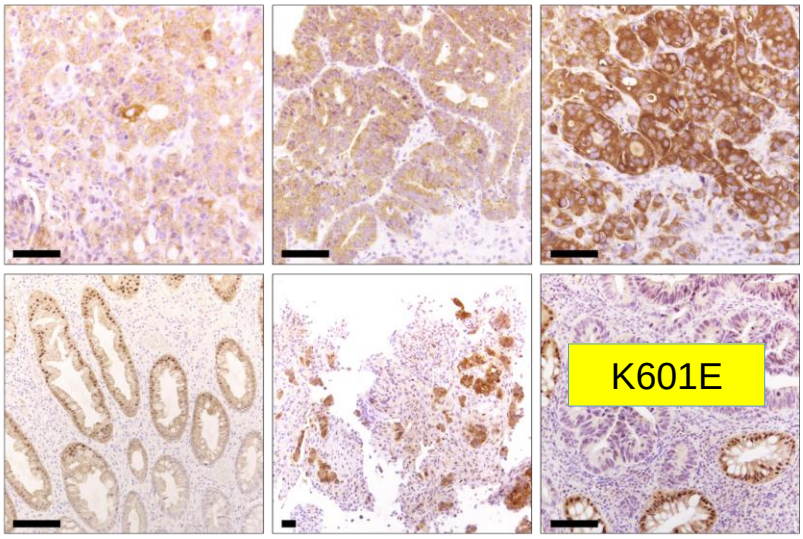
BRAF p.V600E-specific immunohistochemical assessment in colorectal cancer endoscopy biopsies is consistent with the mutational profiling



Galuppini F, *et al*. Histopathology 2017

57

BRAF p.V600E-specific immunohistochemical assessment in colorectal cancer endoscopy biopsies is consistent with the mutational profiling



Galuppini F, *et al*. Histopathology 2017

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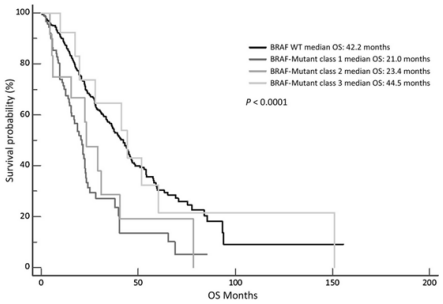
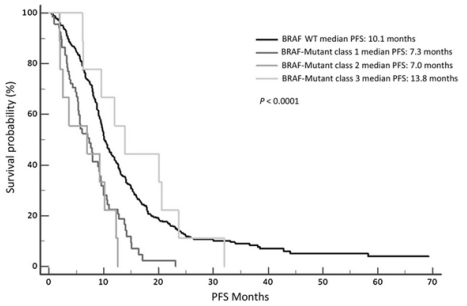
Translational Cancer Mechanisms and Therapy

Clinical Cancer Research

Class 1, 2, and 3 *BRAF*-Mutated Metastatic Colorectal Cancer: A Detailed Clinical, Pathologic, and Molecular Characterization





Class 1: codon 600

Class 2: codons 601 and 597

Poor prognosis

Class 3: codons 594 and 596

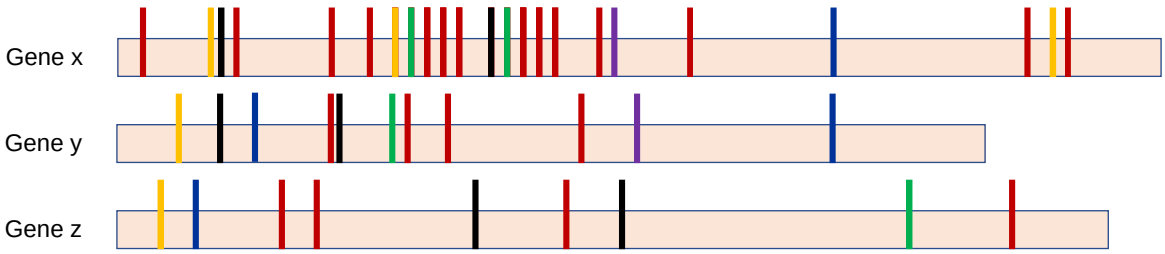
Similar to *BRAF* wt

Schirripa M, *et al.* – Clin Cancer Res 2019

59

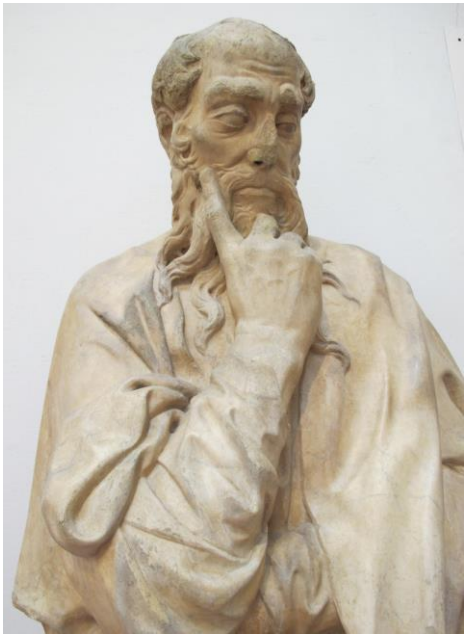
Situation 3

We have to test multiple genes with different known and unknown alterations
(mutation/translocation/amplification/deletion)



- Forget the «hot spot» option! It requires a large amount of material, is time consuming and has a higher cost.
- Comprehensive genomic profiling NGS is the best option. RNA- and DNA-based kits are usually required.

60



More is better?

Is better to use a comprehensive (=larger) or a more sensitive diagnostic NGS panel?

61

Targeted NGS

Limited number of genes with a high diagnostic performance

I know the targetable alteration and I need reliable diagnostic results

CGP NGS (>50 genes)

Large number of genes, higher risk of false negative results

I'm looking for unknown targetable alterations and I can miss something

62

Interpretation and definitions of NGS data!

- missense variants
- nonsense variants
- frameshift deletions/insertions
- splicing variants
- in-frame deletions
- VAF

- pathogenic/likely pathogenic
- uncertain significance variants
- benign/likely benign variants

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The molecular diagnostics' recipe

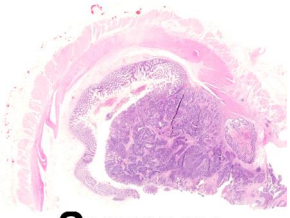
The ingredients
(i.e. the samples)

The kitchen accessories
(i.e. the molecular methods)

64

What a cancer is?

Biopsy

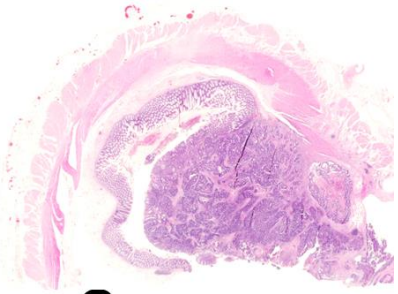


Surgery

The clinical request for molecular testing:

MSI, MMR, *BRAF, FGFR2, TP53, DAXX/ATRX,* TMB, CGP, Methylation, *RAS, ALK, ROS1, BAP1,* chromatin remodeling, *MGMT*, *NTRK*

65



Surgery

- High quantity and good quality of DNA/RNA/tissue sections.
- Most of the methods and diagnostic approaches are applicable.

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Biopsy

- Low quantity of DNA/RNA/tissue sections (usually of high quality).
- Need for tests' prioritization.
- Inadequate sampling/material.

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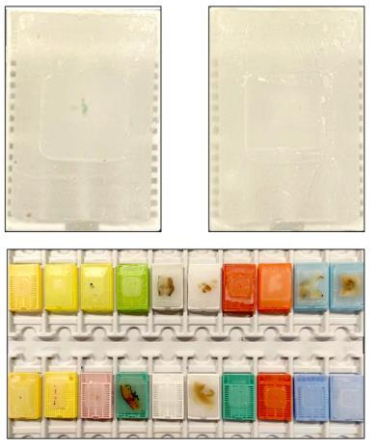
The example of gastroesophageal adenocarcinomas



FFPE tissue blocks **may be inadequate for molecular analysis due to scarcity of material following previous sectioning for diagnostic purposes**. Keep in mind that a tertiary centre receives different types of FFPE tissue specimens obtained with different workflows and processes.

2.6 mm is estimated to be the average diameter of endoscopic biopsies (in reality, it's much lower); a 27G (23G) needle gives a biopsy of 0.42 (0.6) mm of diameter

DIAGNOSIS

- 1 × 4 µm H&E
- 1 × 4 µm Giemsa
- 1 × 4 µm possible IHC (CK)

+ wastage 10–20 µm

Total = around 20–30 µm

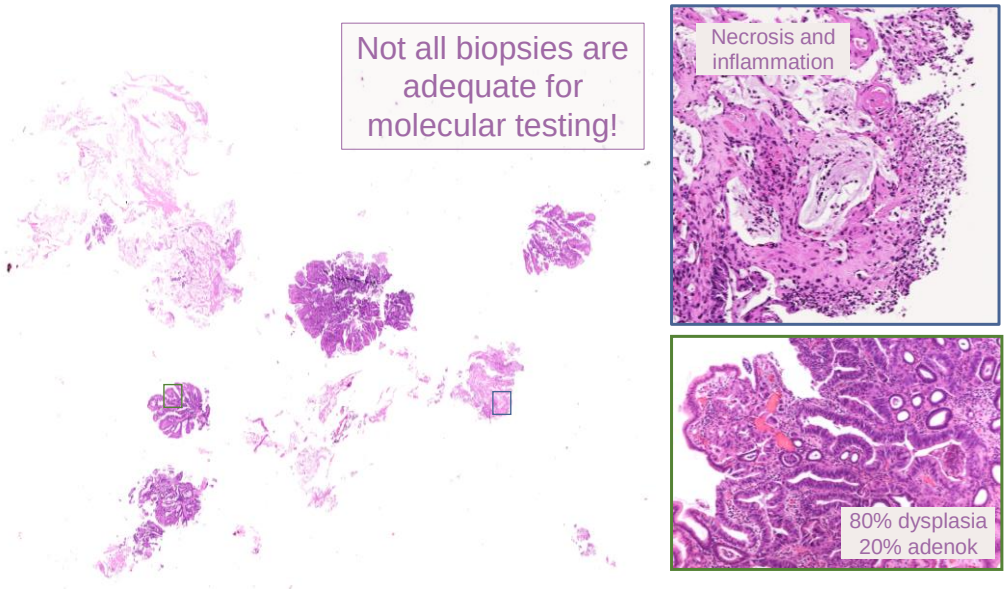
PREDICTIVE BIOMARKERS

- 1 × 4 µm HER2 (plus further 2 sections if 2+)
- 1 × 4 µm PD-L1
- 4 × 4 µm MMR
- 1 × 4 µm EBER

+ wastage 10–20 µm

Total = around 30–50 µm

68



69

Biopsy

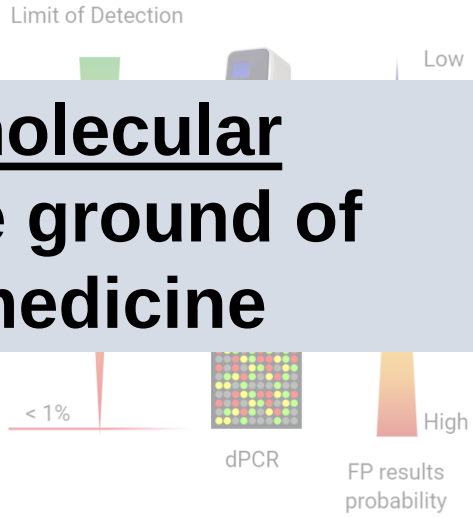
I do not have enough material to perform all my tests:

- Need for tests' prioritization
- NGS: it is possible (quantity/quality DNA/RNA)?
- Liquid biopsy approaches (!!liquid biopsy is not the solution for all our requests!!)?

70

The heterogeneous landscape of diagnostic kits for targeted mutational assessment

	Sanger Sequencing	Real Time PCR	Digital PCR	Next Generation Sequencing
Reference Range	All the mutations present in the analyzed gene regions	Only «hot spot» Mutations (probe based)	Only «hot spot» Mutations (probe based)	All the mutations present in the analyzed gene regions



Personalized molecular diagnostics is the ground of personalized medicine

Passiglia F, *et al.* – J Thorac Oncol 2019

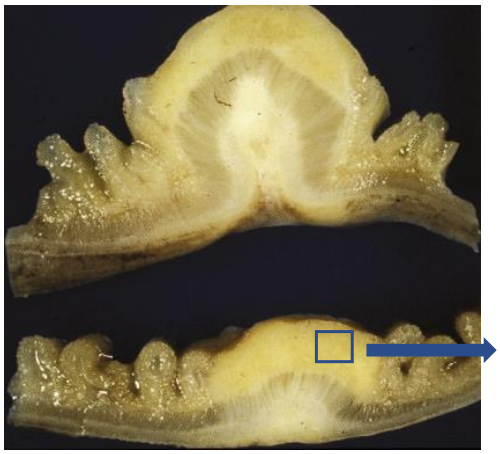
71

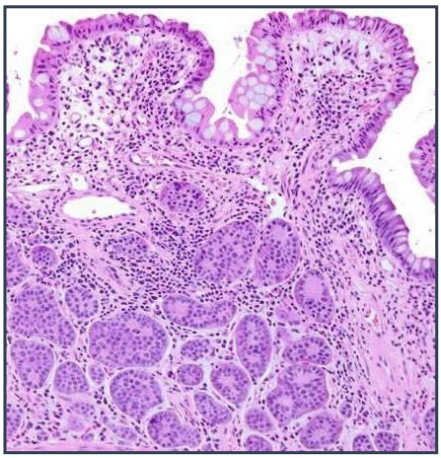
Tissue and molecular diagnostics

- Choice of the right diagnostic approach for the available tissue sample
- Tumor is a tissue and the patologist's evaluation matter!
- Next generation sequencing in old generation laboratories

72

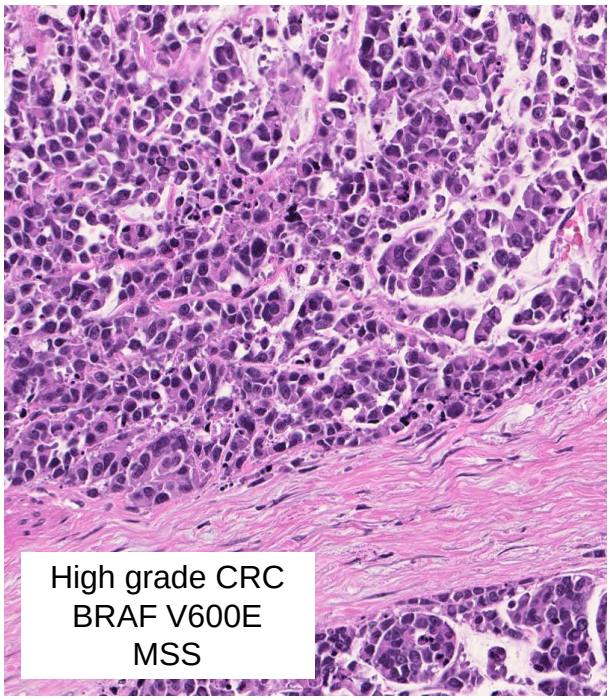
Tumor is a tissue!

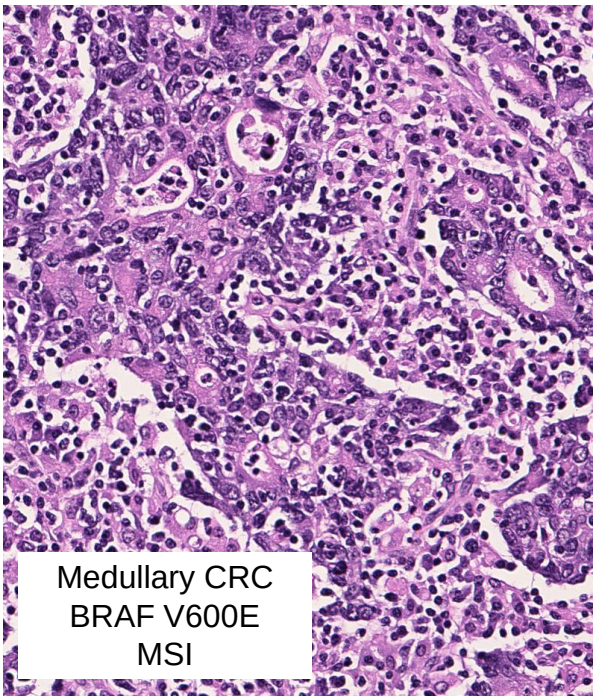




- Tumor cells
- Inflammation
- Stroma
- Normal parenchyma

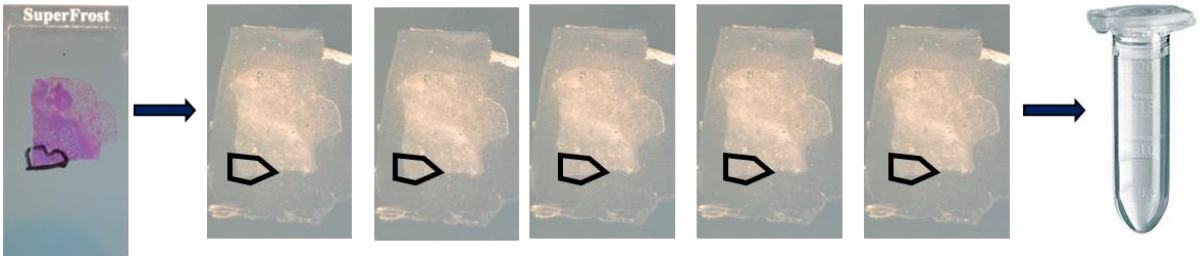
73



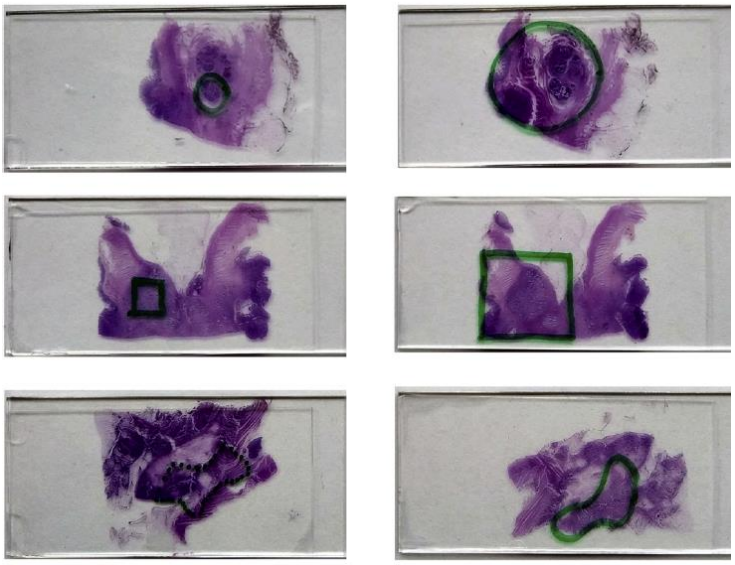


74

Enrichment for cancer cells
(diagnostic sensitivity of molecular testing)



75



The Journal of Molecular Diagnostics, Vol. 20, No. 4, July 2018

ELSEVIER

the Journal of Molecular Diagnostics

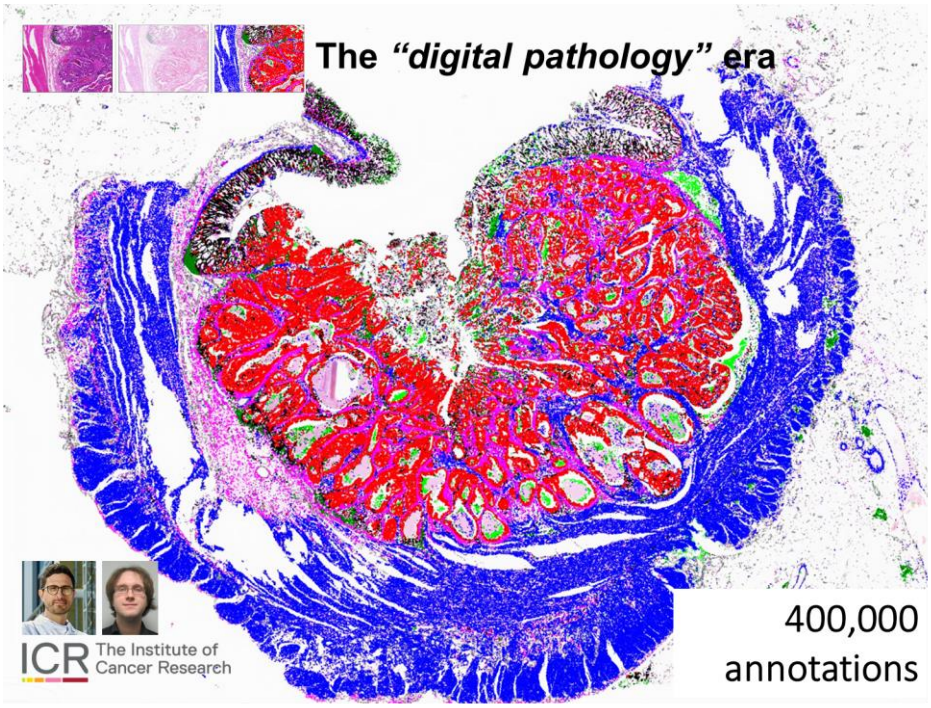
jmd.amjpathol.org

External Quality Assessment Identifies Training Needs to Determine the Neoplastic Cell Content for Biomarker Testing

Kelly Dufraing,*† Gert De Hertogh,‡ Véronique Tack,* Cleo Keppens,* Elisabeth M.C. Dequeker,* and J. Han van Krieken‡

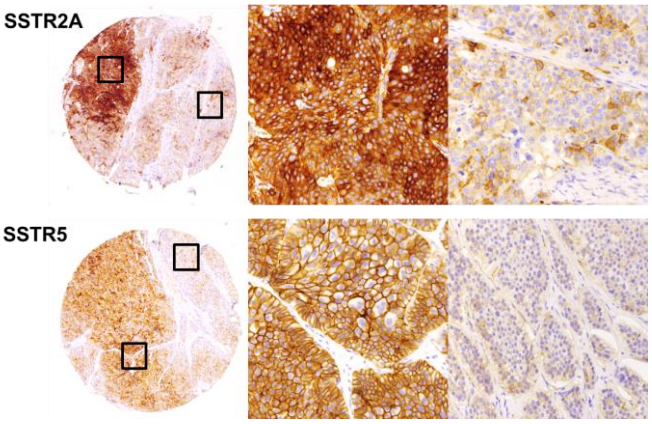
- The selected area was highly variable, and the average difference between the highest and lowest estimation ranged between 51% and 78%.
- The number of overestimations was alarmingly high in samples containing <30% tumor cells.
- **Of concern is that 33 of 105 laboratories reported a wildtype result in a sample without tumor.**

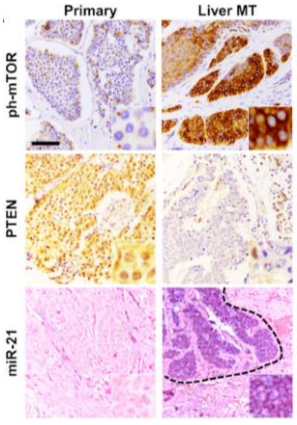
76



77

SSTR/mTOR intratumor heterogeneity



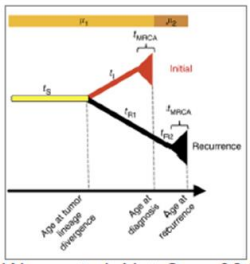


Despite primary and metastatic ileal NETs show a similar molecular landscape, tumor grading and mTOR signaling pathway may diverge in the metastatic setting.

Borga C, *et al*. – Endocr Relat Cancer 2021

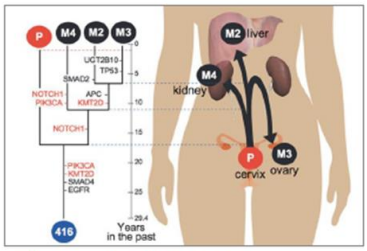
78

GBM



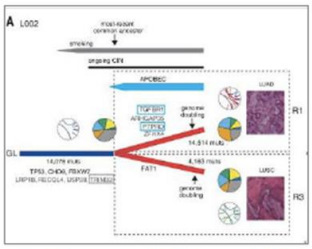
Wang et al. Nat Gen, 2016

Ovarian Cancer



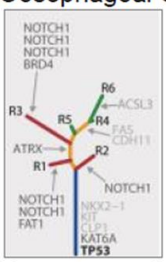
Zhao et al. PNAS 2016

NSCLC



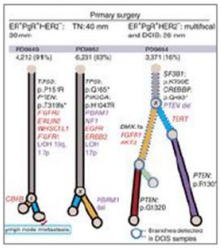
De Bruin et al. Science 2015

Oesophageal Ca



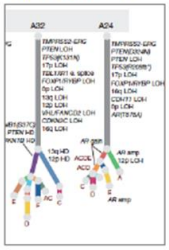
Murugaesu et al. Cancer Disc 2015

Breast cancer



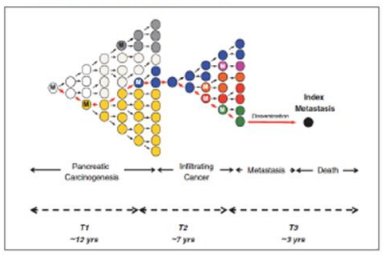
Yates et al. Nat Med 2015

Prostate ca



Gundem et al. Nature 2015

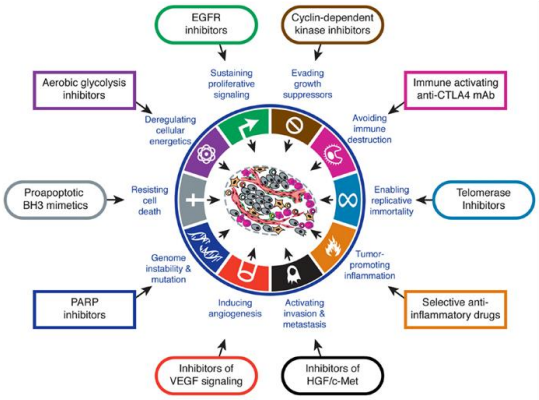
Pancreatic cancer



Yachida et al. Nature 2010

79

Core signaling pathways in human cancer revealed by global genomic analysis



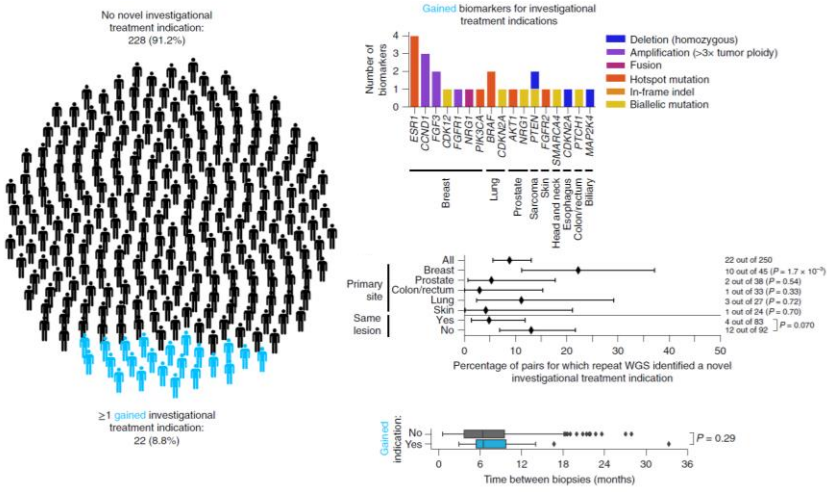
There appears to be only a **limited number of cellular signaling** pathways through which a growth advantage can be incurred

Drugs should target the effect of the altered pathways (i.e. **downstream mediators or key nodal points**) rather than a single gene component!

Hanahan D & Weinberg RA - Cell 2011. Vogelstein B, *et al.* - Science 2013. Hanahan D – Cancer Discov 2022

80

Limited evolution of the actionable metastatic cancer genome under therapeutic pressure

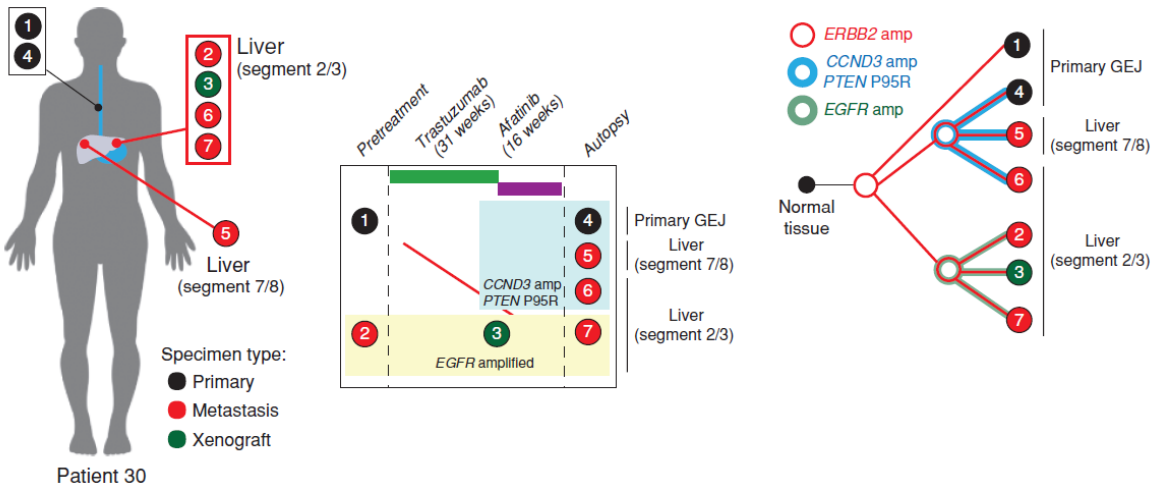


For standard of care genomic biomarkers, we observed <u>full concordance between the first and the second biopsy in 99% of pairs</u>. Of the 219 biomarkers for clinical trial enrollment that were identified in the first biopsies, we recovered 94% in the follow-up biopsies. Furthermore, a second WGS analysis did not identify additional biomarkers for clinical trial enrollment in 91% of patients.

van de Haar J, *et al.* – Nat Med 2021

81

EGFR and *MET* amplifications determine response to HER2 inhibition in *ERBB2* - amplified esophagogastric cancer



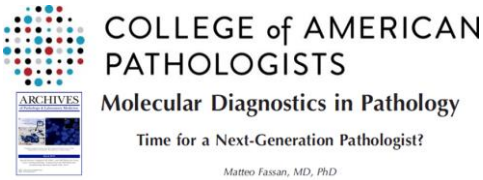
Sanchez-Vega F, *et al.* – Cancer Discov 2019

82

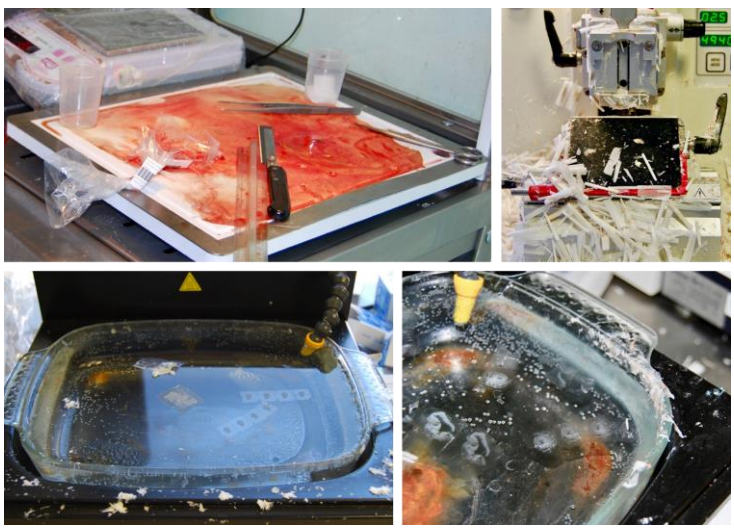
Tissue and molecular diagnostics

- Choice of the right diagnostic approach for the available tissue sample
- Tumor is a tissue and the patologist's evaluation matter!
- Next generation sequencing in old generation laboratories

83

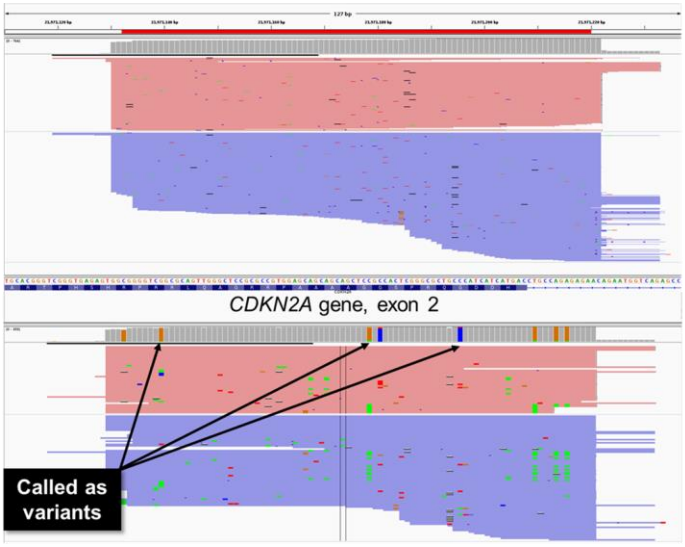


The performance of molecular testing relies not only on the quality of the method itself, but also, profoundly, on the quality of the biospecimen analyzed. Suboptimal material implies suboptimal results in molecular profiling.



84

85



The importance of being FFPE-extracted DNA

Sample A
iCCA (year: **2012**)

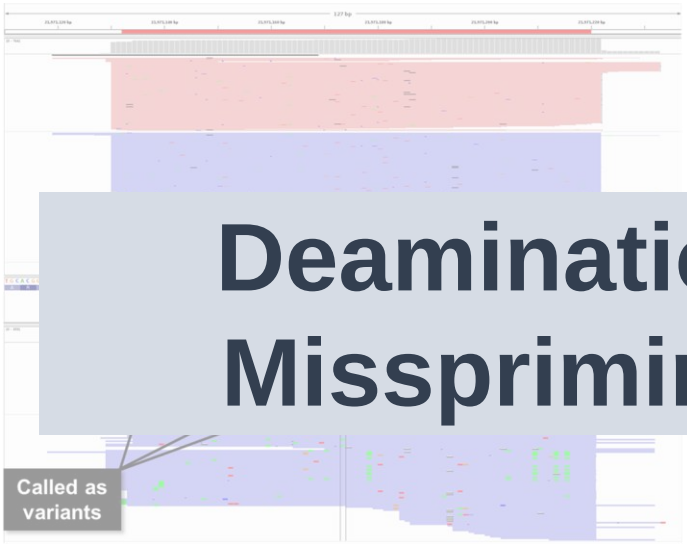
Random G→A or C→T
Changes <1%

Sample B
iCCA (year: **2004**)

Random G→A or C→T
Changes >**10%**

Mafficini A, *et al.* – Plos ONE 2014
Cappello F, *et al.* – J Pers Med 2022

86



Mafficini A, *et al.* – Plos ONE 2014
Cappello F, *et al.* – J Pers Med 2022

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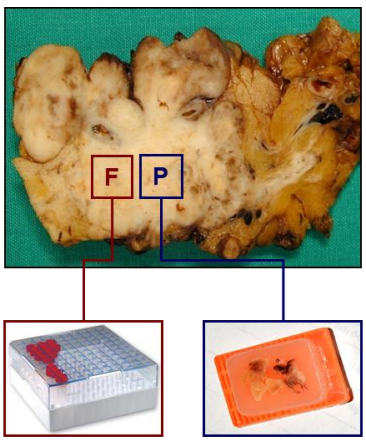
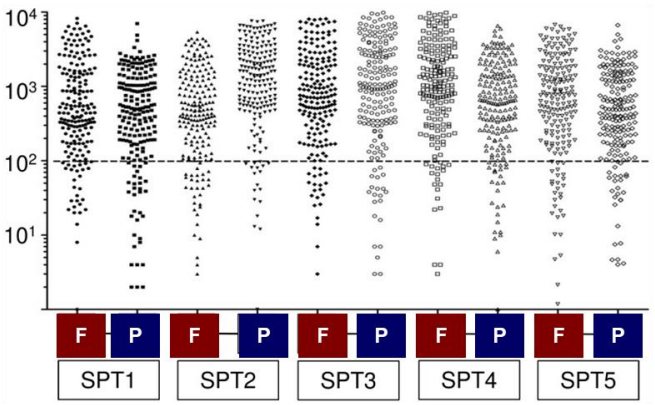
NGS is a good technology to analyze FFPE samples



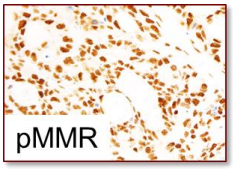


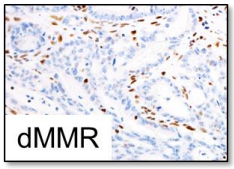
Similar coverage of targeted regions analyzed in 5 matched fresh-frozen (F) and FFPE (P) samples of solid pseudopapillary tumor (SPT).

Mafficini A, Amato E, Fassan M, et al – Plos ONE 2014

88

DNA qualification may impact MSI testing results in mucinous colorectal adenocarcinoma



98.0% | 81.8%

96.0% | 45.4%

…"we demonstrated that preanalytical parameters as neoplastic cellularity and DIN may influence analytical performance for MSI testing.
In particular, a minimum input of **50% of neoplastic cells** is fundamental to correct perform molecular analysis by using Idylla™ system. **DIN < 4** significantly affected TapeStation 4200 results."

Malapelle U, Parente P, *et al* - Cells 2020

89

- FFPE tissue, cytology, plasma
- 1-40 ng DNA/RNA
- 1->500 genes

- Timing/Clinical setting for CGP

DNA-based
- Simplier than RNA analysis
- Limited loss of analyses for low sample's qualification
- May miss translocations/fusions

RNA-based
- 20-25% of samples cannot be analyzed
- Gold standard for translocations/fusions analysis

90

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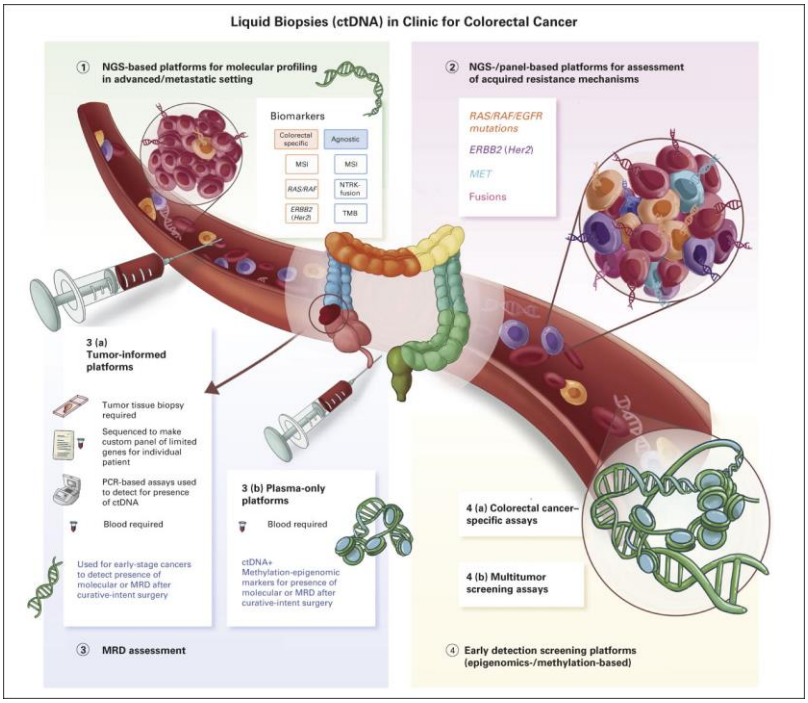


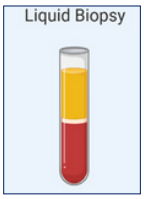
Circulating tumor DNA (ctDNA) analysis through liquid biopsy has proven to be a robust method to tailor personalised treatments for CRC) patient care.

Malla M, *et al.* – JCO 2022

92

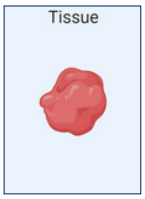
A real-world application of liquid biopsy in metastatic colorectal cancer: the Poseidon study

n= 33 mCRC
(from spoke centers to hub center)



83%

=



7 days

22 days

17 days

Procaccio L, *et al.* – Cancers 2021

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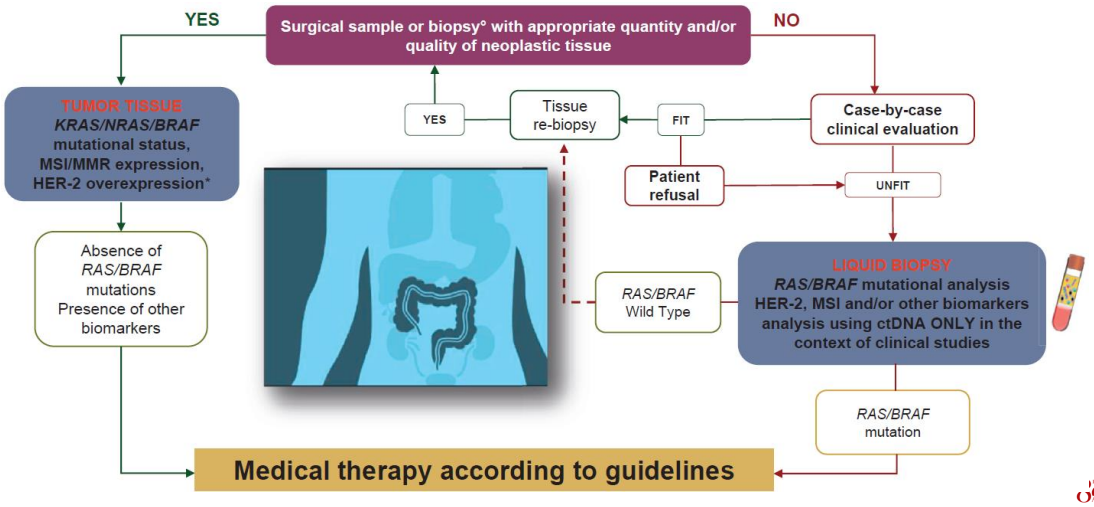
ESMO 2021

ESMO OPEN CANCER HORIZONS

REVIEW

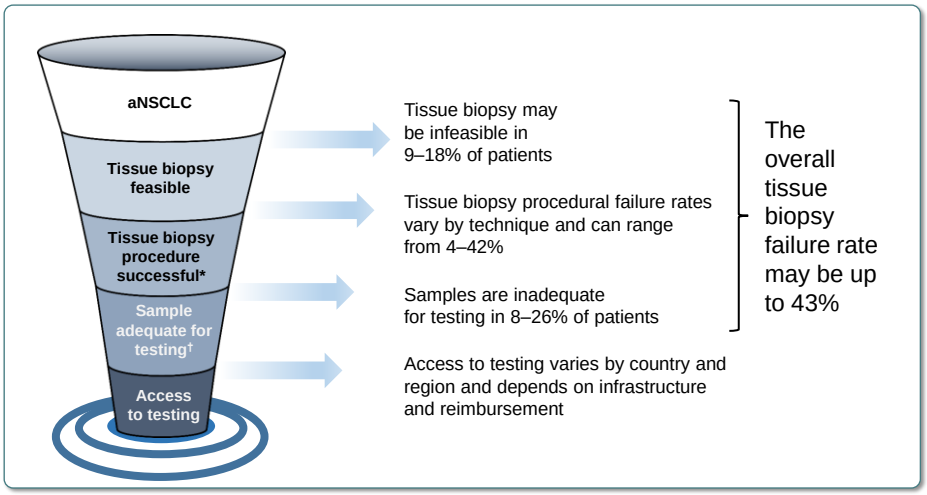
The molecular profiling of solid tumors by liquid biopsy: a position paper of the AIOM–SIAPEC-IAP–SIBioC–SIC–SIF Italian Scientific Societies☆

"...the absence of harmonized procedures corresponds to an unmet clinical need, ultimately affecting the rapid implementation in clinical practice."



94

Limitations of tissue biopsy



*I.e. tissue sample successfully extracted from target lesion; †Molecular diagnosis and/or histological diagnosis. aNSCLC, advanced NSCLC.

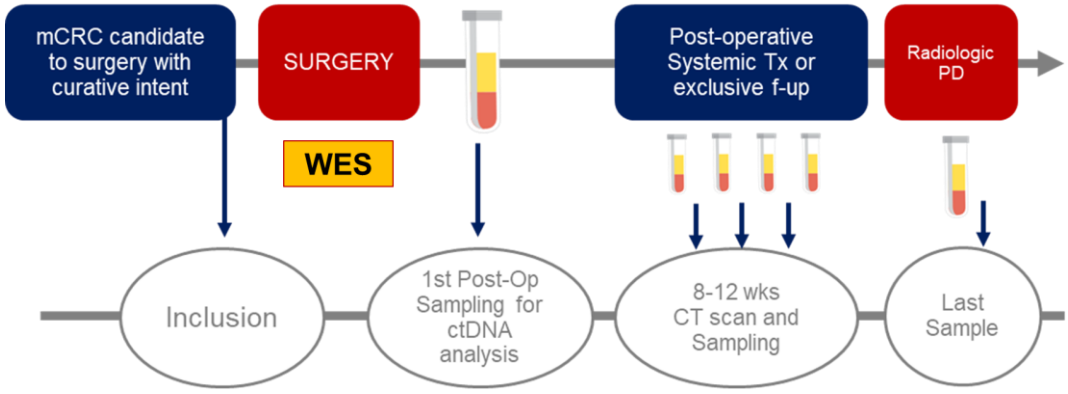
Malapelle U, et al. - J Mol Pathol 2021

95

JCO® Precision Oncology 2021

BIOMARKERS

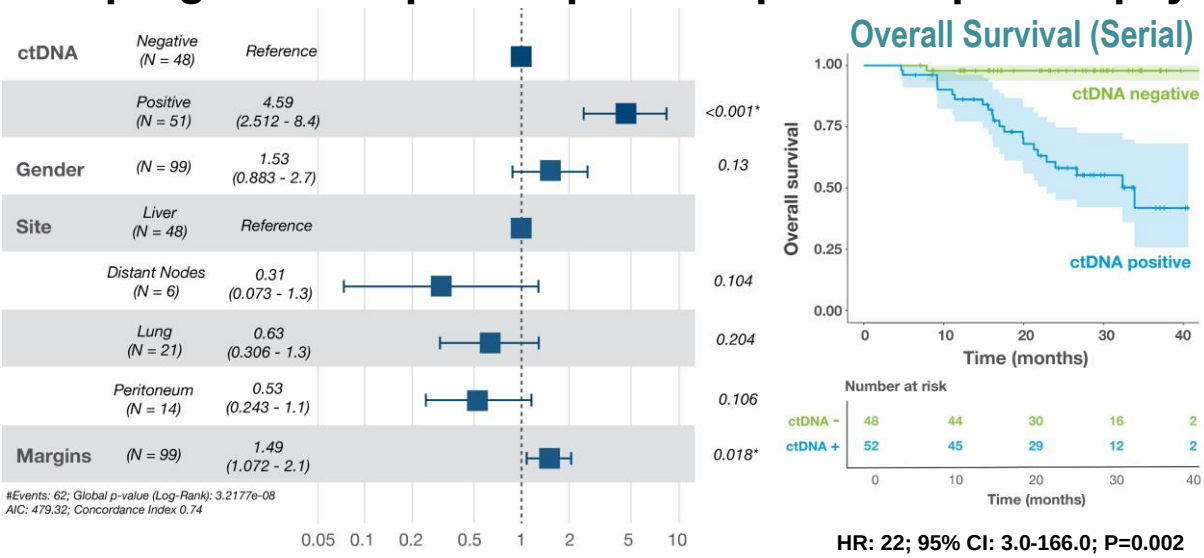
Detection of Molecular Residual Disease Using Personalized Circulating Tumor DNA Assay in Patients With Colorectal Cancer Undergoing Resection of Metastases



Loupakis F, *et al.* – JCO Prec Oncol 2021

96

The prognostic impact of patient-specific liquid biopsy

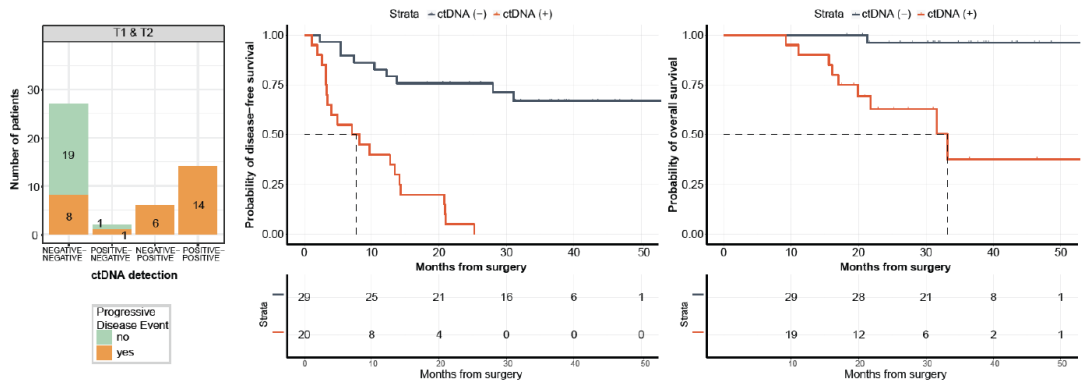


HR: 22; 95% CI: 3.0-166.0; P=0.002

Loupakis F, *et al.* – JCO Prec Oncol 2021

97

Comprehensive Genomic Profiling (CGP)-informed personalized molecular residual disease (mrd) detection: an exploratory analysis from the predator study of mCRC patients undergoing surgical resection



Tissue CGP identified potentially actionable alterations in 54% (37/69) of patients. MRD-positivity was significantly associated with lower disease-free survival (DFS) (HR: 4.97, 95% CI: 2.67–9.24, p < 0.0001) and overall survival (OS) (HR: 27.05, 95% CI: 3.60–203.46, p < 0.0001).

Lonardi S, *et al.* – IJSM 2022

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Take home messages

- The introduction of TCGA/ICGC data into clinical practice
- The clinical impact of the pathological report (educational programs)
- The diagnostic performance of the different technologies in the therapeutic management of the neoplastic patient
- The need of molecular test prioritization (TMB's role)

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Is the right time for next generation histopathological diagnostics?

100

Veneto Institute of Oncology – IOV
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The Institute of Cancer Research, Sutton, UK
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Chiara Braconi
Andrea Sottoriva

UniPD – Department of Molecular Medicine
Stefano Piccolo

UniPD – Department of Biology
Marina de Bernard

Padua University Hospital
Marco Scarpa

Fondazione Città della Speranza
Marco Agostini

The Ohio State University
Carlo M. Croce

University of Verona
Aldo Scarpa
Claudio Luchini

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Luciano Cascione

Cancer Research UK – Manchester Institute
Michela Garofalo

Semmelweis University Budapest
Andras Kiss

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