A practical guide for precision cancer research using tumor-organoids

The PreCanMed Technical Handbook

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Foreword

Prof. Stefan Schoeftner
Project coordinator

Providing efficient and accessible public health care represents a fundamental goal for the European Union to ensure inclusive growth in member states. Progress in medical care in the past 40 years remarkably increased life expectation in western societies. However, augmented life-span has been found to be accompanied by a progressive increase of aging related pathologies, such as cancer.

Health statistics revealed that in 2012 one out of 4 citizens was at risk of developing cancer. New cancer cases in member states of the European Union are expected to increase from 2,6 mln per year in 2012 to 3,1 mln in 2025. These dynamics represent a significant risk for the sustainability of public health systems.

In order to ensure effective and inclusive patient care, the European Union has launched several initiatives to encourage innovation in health and modernize health services. The pooling of resources and sharing of know-how among both EU member states and trans-border regions represent central pillars of this innovation strategy.

The Interreg V-A Italia-Austria 2014-2020 project 'PreCanMed', funded by the European Regional Development Fund, supported the efforts of 5 research institutions of excellence located in Tyrol and Friuli Venezia Giulia to join competences for implementing an enabling technology for precision cancer medicine in the program region.

In the Italian-Austrian trans-border region, the 'PreCanMed' team established innovative technologies that allow in the laboratory to growth tumors that faithfully resemble a patients' developing cancer. Detailed analysis of these tumor models, also named 'tumor-organoids', allows the design of therapeutic approaches tailored to the individual patient. The outcomes of this project thus represent an important step forward towards more efficient cancer therapies.

'PreCanMed' has generated a series of publicly available experimental protocols and resources for precision cancer medicine, as well as trained new field experts, generated a stable contact with stakeholders and disseminated this new technology to accelerate the development and experimentation of personalized therapies in and beyond the Interreg V-A Italia Austria program region.

This effort has been contributing to raise awareness about this key precision cancer medicine technology among citizens, and to make it widely accessible to experts, ultimately fostering the improvement of patient care and future sustainability of public health systems.

executive summary

Precision medicine is a comprehensive, prospective approach to prevent, diagnose and treat disease by using each person's unique clinical, genetic, genomic and environmental information. This approach is of particular relevance for human cancer, a heterogenous and dynamic disease characterized by patient-specific sets of gene mutations.

Tumor-models that allow the faithful reconstruction of the patients' tumor in the laboratory have primary importance to capture cancer complexity. Tumor-organoids represent an essential building block in the design of precision cancer medicine. Tumor-organoids are 3D cell culture models, generated from surgically resected patient tumor tissues that can be grown in the laboratory. Tumor-organoids precisely recapitulate the characteristics of the original tumor, thus representing a highly promising tool to mimic the original cancer tissue. Performing genomics analyses on tumor tissues and tumor-organiods allows the identification of the dependencies and vulnerabilities of a tumor, on the base of patient specific sets of gene mutations or alterations of gene expression. This open the possibility to test patient specific therapeutic approaches on tumor organoids, in the laboratory, and compare their efficacy to the efficacy of standard cancer therapy regimens. Hence, tumor-organoids can provide oncologists with additional information for designing more efficient cancer therapies in the future.

The introduction of precision medicine requires multi-disciplinary expertise in different fields of cancer research. The required competences can be best covered by collaborative efforts of multiple research institutions.

The Interreg V-A Italia-Austria 2014-2020 project 'PreCanMed' (Creating a platform for precision cancer medicine) coordinated 5 cancer research institutes of excellence in the Italian- Austrian trans-border region to establish a technological platform for precision medicine in colorectal, lung and breast cancer, which represent the most frequent tumor types in the program region.

'PreCanMed' project partners implemented protocols for i) the generation and cryo-conservation of 3D tumor-organoid models from primary tumor tissues ii) the genomics analysis of these models for the identification of genetically driven tumor dependencies and vulnerabilities, iii) the testing of the efficacy of different therapeutic approaches on tumor organoids. Along with this, they generated shared resources of biological material and data for precision cancer medicine.

Our technical handbook for precision cancer medicine contains detailed guide for the implementation of tumor-organoid technology by investigators working in the context of academic or pharmaceutical research. The handbook describes general procedures, strategies and experience obtained during the project period, providing central methodologies as standard operating procedures (SOPs). It allows the efficient reproduction of lung, breast and colorectal tumor organoid technology in the environment of a cancer research laboratory.

The PreCanMed handbook is composed of 10 sections with related SOPs describing sequential steps in the introduction of tumor organoid technology:

1. Bioethics approval and informed consent related to the PreCanMed observational study
2. Communication tools to manage rapid transport of tissues to research laboratories
3. Assessment of tissue material for the generation of normal-tissue and tumor-tissue organoids
4. Processing of patient's tissues to obtain cell material for organoid cultivation
5. Cultivation of tumor-organoids in Matrigel
6. Expansion of organoid cultures
7. Validation of histopathological features on tumor-organoid cultures
8. Genomics analysis of tumor-organoids
9. Drug testing on tumor-organoids
10. PreCanMed Database

The listed contents, together with additional SOPs, are accessible via the PreCanMed project website *www.precanmed.eu*.

Altogether, this handbook aims to make the tumor-organoid technology accessible to researchers in and beyond the Interreg V-A Italia-Austria program region in order to accelerate the development and experimentation of personalized therapies for cancer patients.

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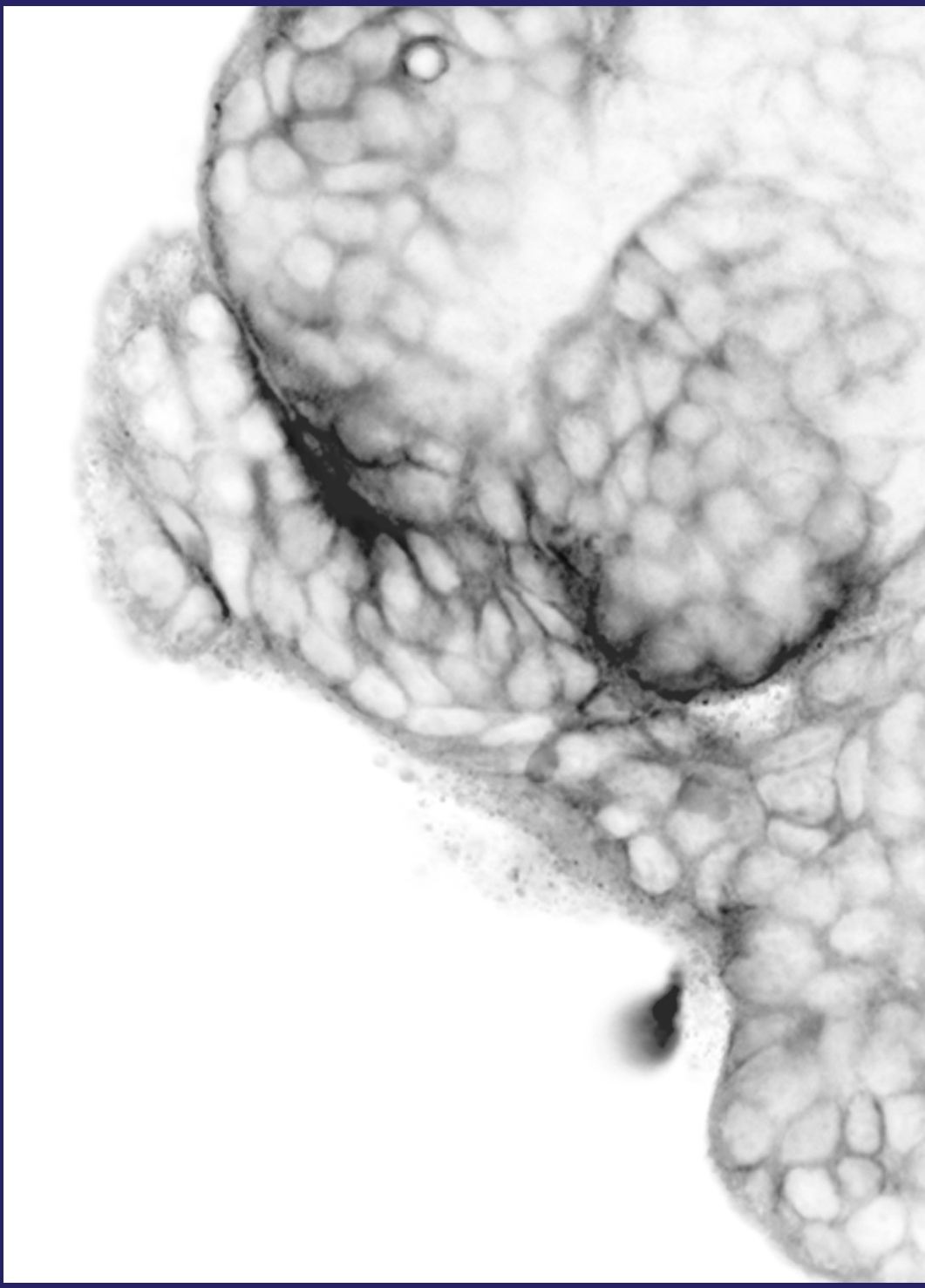

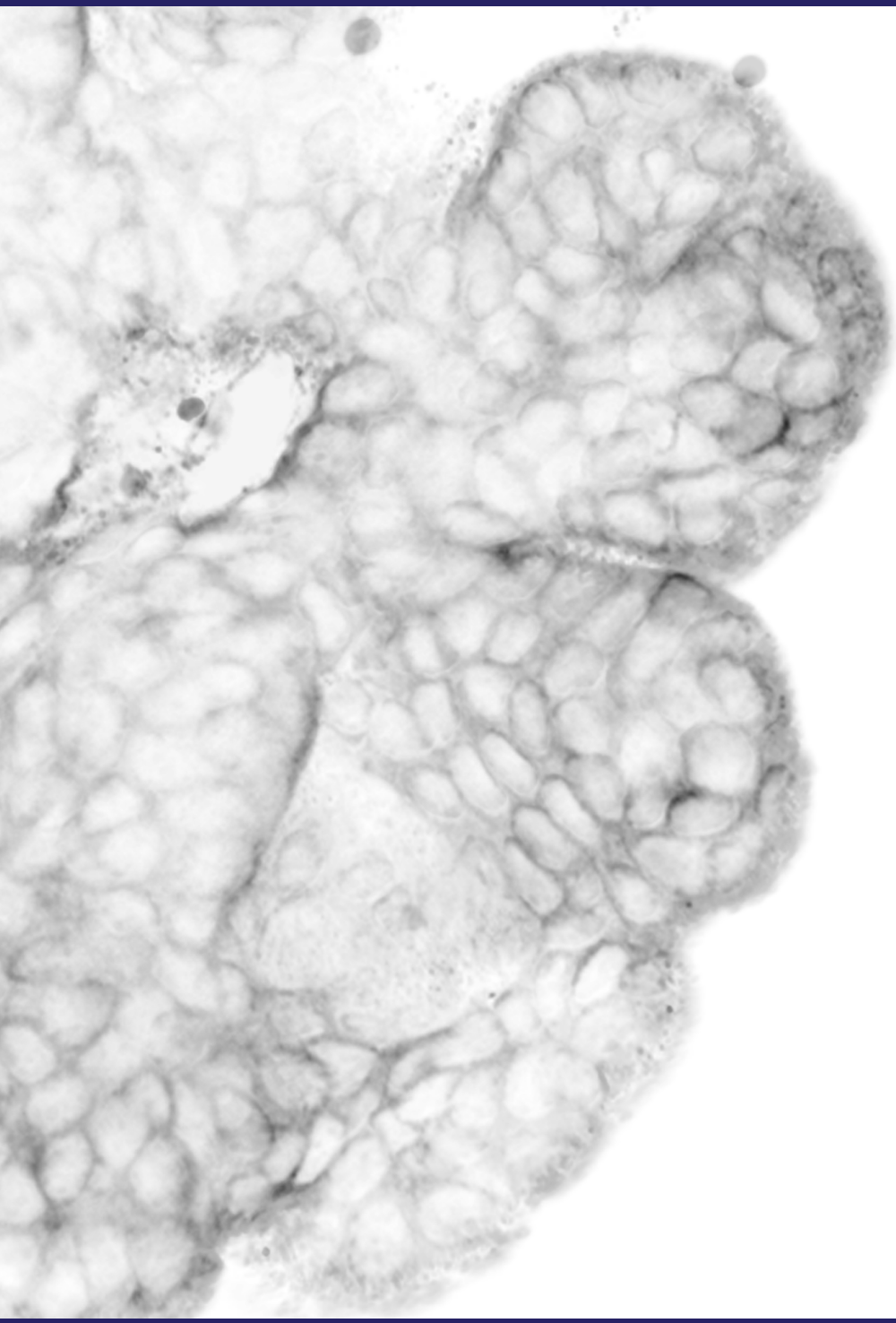

The Project

PreCanMed is a strategic project funded by the European Union, the European Regional Development Fund and the Interreg V-A Italia-Austria Programme 2014-2020. PreCanMed partners work together to the interregional implementation of the patient derived tumor organoid technology, a powerful novel tool allowing investigation of normal and diseased cancer tissues for the development of novel, bespoke treatments for patients.

The project aims to establish a working pipeline for a widespread future use of this technology, and is approved by local Austrian and Italian Bioethic Commitees.

PreCanMed is expected

- *to accelerate the pace of our understanding of multiple facets of the disease,*
- *to unleash new cancer breakthroughs*
- *to help realize the full potential and promise of personalized medicine.*

Tumor organoid technology

The patient derived tumor organoid technology consists of functional, 3D, ex vivo, culture systems of tumor cells from patients.

Defined as effective patient avatars, these systems have emerged as one of the latest frontiers in disease modelling and they represent a very promising experimental tool to reproduce and study a tumor in the laboratory, in a nearly physiological state.

Miniature organs-in-a-dish

The term 'organoid' means 'resembling an organ' and refers to a self-organizing 3D structure that scientists can grow in the lab, under peculiar conditions, from stem cell containing chips of tissue taken from the organism.

A 'tumoroid' or tumor organoid is a similar structure, but derived from a specimen of the cancer tissue resected during surgery or biopsis.

An organoid or tumor organoid is an in vitro system, but it well mirrors the in vivo spatial organization and multi-lineage differentiation of the represented tissue.

Containing an active stem cell (or cancer stem cell) population, it can be propagated and greatly expanded allowing a gamut of investigations.

Culturing organoids

Different tissues have specific needs for organoid development. Culture conditions have to mimic the in vivo signals required for tissue organisation and for the maintenance of the stem cell population. Only under these conditions, cells proliferate in culture and self-organise into 3D structures which can be passaged and indefinitely maintained.

Culture conditions have already been established for generating organoids from various healthy and diseased human organs.

PreCanMed partners work together to integrate existing knowledge and improve it, in the development of patient derived tumor organoids from breast, lung and colon carcinomas and

from mesothelioma. Tumor samples will be collected upon surgery or biopsis and will be subjected to different procedures in order to identify and define the best conditions enabling growth and expansion of the organoid systems.

Powerful model systems

Patient derived tumor organoids are relatively new as preclinical cancer models.

Cancer cell lines and animal models have long been used as model systems. They allow thorough experimentations, but both of them do not fully recapitulate human tumors and their oncogenic processes, as well as their heterogeneity.

Implanting cancer tissue chips into mice (patient derived tumor xenograft) emerged as another advanced model, more closely resembling physiological features of the tumor of origin and predicting tumor clinical responses to therapy. However, xenograft engraftment may occur with low efficiency, the procedure is expensive and time consuming.

Patient derived tumor organoids, instead, are one of the most promising alternatives to mimic the original cancer tissue. They accurately recapitulate tumor histology, molecular subtypes, and response to treatments. They can be generated and propagated with high efficiency, as well as frozen in liquid nitrogen and recovered later for further use. Organoids can be developed from biopsies and/or surgical resections of both normal and cancerous tissues, allowing key matched investigations at the single patient level.

Molecular and drug toxicity profiles

Developed organoids are models incorporating key molecular features of the patient's tumor of origin.

In PreCanMed, each organoid will be characterized with different omics technologies and drug treatments to establish a database linking organoid gene expression information to drug responsiveness.

Molecular analyses

PreCanMed partners focus their investigations onto the protein-coding part of the cancer genome.

The whole exome sequencing method will be applied to detect gene variants that most likely can affect disease progression

and response to therapy. Genomic data will be compared to the whole gene expression profiles obtained by DNA microarray analysis of organoids.

Obtained genetic fingerprints and expression signatures will be correlated with clinical data from patients.

Drug candidates

Bioinformatics analysis of the genomic fingerprint of tumor-organoids may enable the identification of particular biological pathways that mediate a tumor effect.

These pathways may represent new Achilles heels of the tumor and may be targeted by chemical compounds, in a patient specific manner.

On the basis of bioinformatics analysis, PreCanMed partners will identify a panel of potential drugs for each tumor organoid. These drugs will be tested for their potential in limiting the growth of specific tumor organoids.

Living Biobank

One important goal of PreCanMed is the creation of a living biobank of patient derived tumor organoids. Our biobank is the result of the collective effort of PreCanMed clinicians, cancer scientists, omics and technology experts, and bioinformatitians, in the harmonization and standardization of collection, processing, banking and research procedures.

Banked samples

Specimens are collected from patients meeting inclusion criteria of PreCanMed observational study, after obtaining informed consent, and include chips of diseased and matched normal tissues from surgical resections of breast, lung and colon cancers, and mesothelioma.

De-identified, sample matched datasets

PreCanMed biobank collects three categories of data related to the banked, patient-derived organoids:

- patients' health records including clinical information over the course of the disease on diagnosis, molecular and histo-

pathology analyses, prescription drugs and treatments and patient response to therapy,

- genomic data of patient-derived organoids, and
- gene expression profiles of patient-derived organoids.

Clinical data banked by PreCanMed are encrypted, ensuring patient privacy and confidentiality.

Paving the way to personalized therapies

Every single tumor organoid represents a personal, developing model of the disease and enables experimentation at the single patient level in ways that have previously been impossible.

Every single tumor organoid, in fact, enables:

- the characterization of specific molecular traits;
- the testing of chemotherapies and targeted therapies as well as the screening of compounds (combinations and different dosages of one or more drugs can be tested);
- the identification of drugs that specifically exert anti-tumor effects in a specific tumor type and also the mechanism behind;
- the identification of new therapeutic targets and biomarkers to follow disease progression and response to therapies;
- the prediction of the clinical response to treatment.

This will ultimately accelerate the personalization of therapeutic approaches. and this is what PreCanMed will contribute to fulfil.


AGIATA-MENTE
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The PreCanMed Team

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_ **UNIUD** | Università degli studi di Udine
_ **ADSI** | Austrian Drug Screening Institute
_ **MUI** | Medizinische Universität Innsbruck

Consorzio Interuniversitario Biotecnologie

LNCIB

Consorzio Interuniversitario per le Biotecnologie – Laboratorio Nazionale CIB

Trieste, Italy -

Lead Partner

The Consorzio Interuniversitario per le Biotecnologie – Laboratorio Nazionale CIB (LNCIB) was founded in 1992 and focuses on the investigation of biological mechanisms that lead to the formation and advancement of tumors. Today, LNCIB is a competitive and internationally renowned cancer research institution embedded in Area Science Park, the science and technology park in Padriciano, Trieste.

LNCIB represents a centre of excellence in the filed of biotechnology and has been certified as "Highly qualified laboratory for applied research and for functional cancer genomics" by the Region Friuli Venezia Giulia.

A main goal of the institute is to transfer results from basic research to the clinic, but also to act as centre of know-how and training. LNCIB has been training new generations of researchers and research technicians in cancer biology. Each year numerous students in Master and PhD students get enrolled in research projects carried out at the LNCIB.

The institute is also a reference centre, in Friuli Venezia Giulia, for the development of new, enabling technologies in the field of cancer research.

The high level of its research in the field of molecular oncology is granted by the profile and expertise of LNCIB researchers, and also by the network of collaborations with regional Universities and regional, national and international research entities.

LNCIB has a surface of 300 square meters of equipped laboratories, and more than 45 researchers of diverse national and

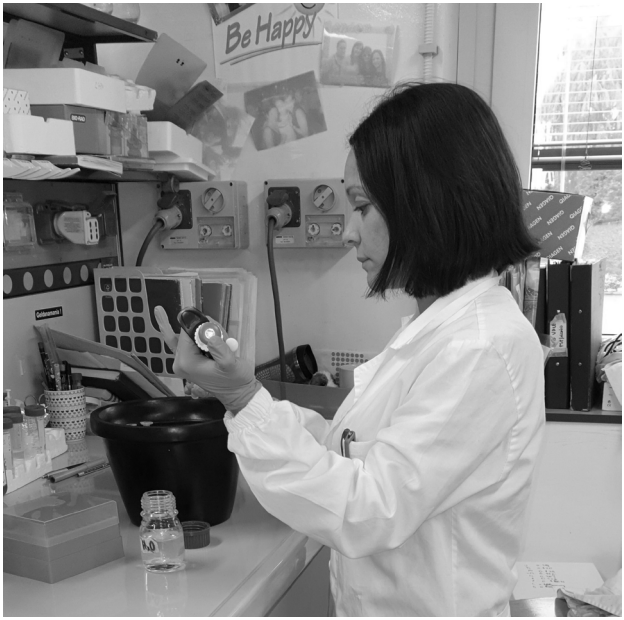
international origin who work in 5 different research groups, on 12 diverse research programs.

The research carried out at LNCIB focuses on the main biological mechanisms of cancer cells. The final goal of this activity is to develop a detailed knowledge on the molecular mechanisms that enable the formation and progression of tumors, in order to define "Achilles heels" of the disease. The operative units working on these themes are:

- the Functional Genomics Unit,
- the Molecular Oncology Unit,
- the Differentiation and Ontogenesis Unit,
- the Onco-epigenetics Unit, and
- the Genomic Stability Unit.

The research activity of these units is supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), Telethon, European Commission, Italian Ministry for Education, Universities and Research, Region Friuli Venezia Giulia, private foundations and donations.

The Consorzio Interuniversitario per le Biotecnologie – Laboratorio Nazionale CIB is Lead Partner in PreCanMed.



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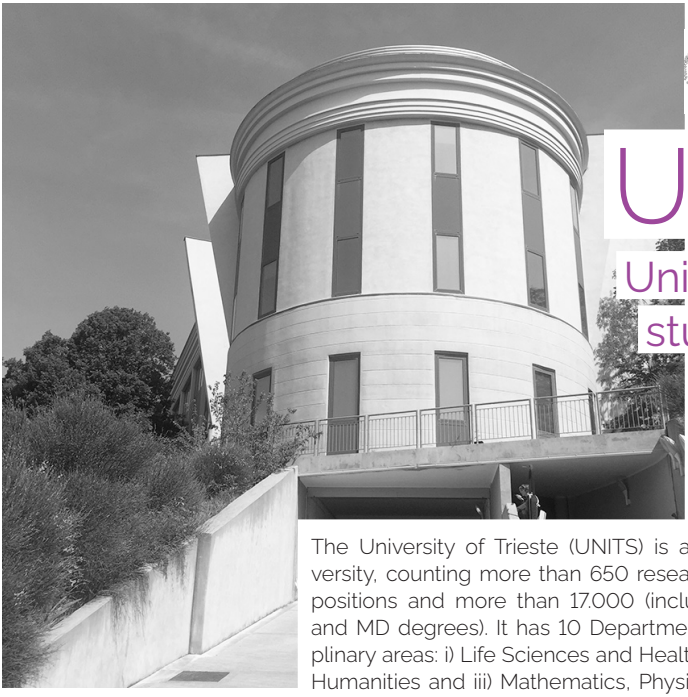


UNIVERSITÀ
DEGLI STUDI DI TRIESTE

UNITS

Università degli studi di Trieste

Trieste, Italy

The University of Trieste (UNITS) is a research-intensive university, counting more than 650 research and teaching tenure positions and more than 17.000 (including Bachelors, Master and MD degrees). It has 10 Departments covering three disciplinary areas: i) Life Sciences and Health, ii) Social Sciences and Humanities and iii) Mathematics, Physical Sciences, Chemistry, Engineering and Earth Sciences.

UNITS is 5th best university in Italy (U.S. News – Best Global Universities Ranking 2017).

It is embedded in a dynamic, international and high-tech environment that comprises several local interrelated research institutions.

Two departments of UNITS are involved in PreCanMed: the Department of Life Sciences and the Department of Medicine, Surgical and Health Sciences.

The **Department of Life Sciences** (DSV) counts 80 faculty and about 200 non-permanent staff members. The activities of DSV span from Biomedicine to Environmental Biology, Psychology and Cognitive Sciences. Research in the area of Biomedicine is particularly active and includes biochemistry of metabolism, pharmacology, molecular oncology, biomaterials for tissue engineering, neurodegenerative diseases and pharmacogenomics.

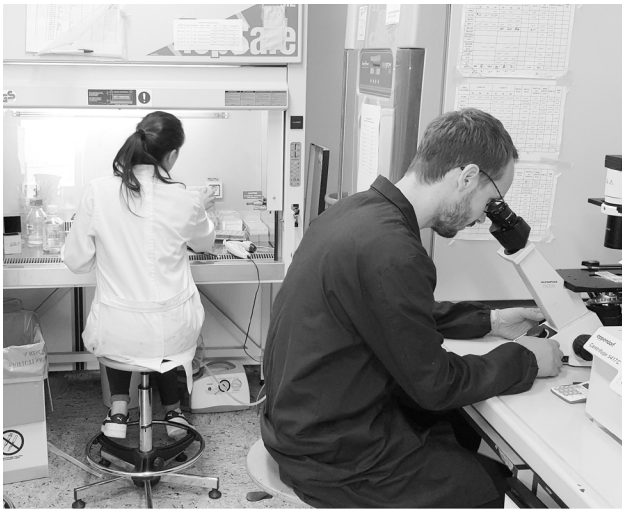
DSV has a strong track record in recruiting, coordinating and managing grants and in carrying out financial, administrative and managerial tasks in European research projects. DSV has participated either as coordinator or as partner in 14 projects financed and co-financed by EU in the last 4 years.

The **Department of Medicine, Surgical and Health Sciences** (DSM) counts 13 full professors, 41 associate professors, 38 researchers and 54 technical-administrative staff members, 47 research fellows and 46 PhD students.

DSM conducts both scientific research and educational as well as healthcare activities, spanning several areas of medicine and integrating different disciplines. DSM activities are carried out in 3 different Poles: Cattinara Hospital, Maggiore Hospital, and IRCCS Burlo Garofolo.

Research at DSM is both basic and clinical. The main research lines deal with the molecular, cell biology and genetic mechanisms underlying the physiopathology and pathogenesis of a gamut of diseases. Aiming at ultimately developing innovative targeted therapies, several research lines addresses, among others, population genetics, molecular biology, methods to culture cells, regenerative medicine, biobanking, and experimental models to study human diseases. In particular, DSM has competence and know-how in tumor molecular diagnostics based on last generation technological platforms. Under a specific institutional agreement, DSM has open access to a clinical study centre for Phase I trials on new drugs targeting solid tumors. Moreover, it has an oncological area and competent personnel to be dedicated to Phase II and III studies on solid tumors.

The DSV and DSM are recognized as Departments for Excellence by the Italian Ministry of Education, University and Research (MIUR) and both cooperate with several institutes and scientists at the regional, national and international level.



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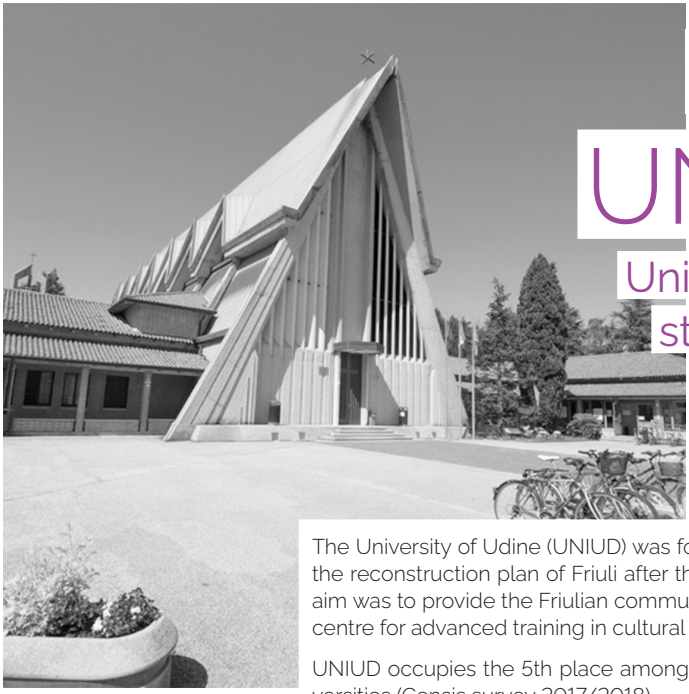

UNIVERSITÀ DEGLI STUDI DI UDINE
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UNIUD

Università degli studi di Udine

Udine, Italy

The University of Udine (UNIUD) was founded in 1978 as part of the reconstruction plan of Friuli after the earthquake in 1976. Its aim was to provide the Friulian community with an independent centre for advanced training in cultural and scientific studies.

UNIUD occupies the 5th place among Italian middle sized universities (Censis survey 2017/2018).

UNIUD mission may be summarised in quattro short sentences:

- higher education;
- research and technology transfer;
- connection with local communities;
- internationalisation;
- These are the main focus areas, all interconnected for a mutual exchange of knowledge, experiences and projects.

In the academic year 2016/2017, UNIUD has offered 36 Bachelor degree courses and 36 Master degree courses. In addition, UNIUD offers postgraduate teaching and research programs, with a broad range of training courses, grad schools (31), masters (15) and doctorate degrees (15). It has 15.385 students and 650 among professors and researchers. Scientific research is performed and coordinated by 8 Departments and 9 Research Centers and supported by a central administrative office.

Udine and its University are a point of reference in a region which is historically a meeting place and crossroads of different worlds and cultures. Geographically situated in the centre of the European Union, the UNIUD plays an active role in a close network of

relations, committed to sharing its knowledge and ideas. Since its establishment, UNIUD has pursued the policy of internationalisation, aimed at preparing students and forging relations and partnerships with universities and institutions in Europe and the rest of the world.

To date, UNIUD has been involved as partner in many different EU research projects

- HORIZON 2020: 19 projects funded,
- FP7: 39 projects,
- other international: 20 projects, 7 of whom coordinated,
- territorial cooperation (Interreg): 47 projects funded, 5 of whom coordinated.

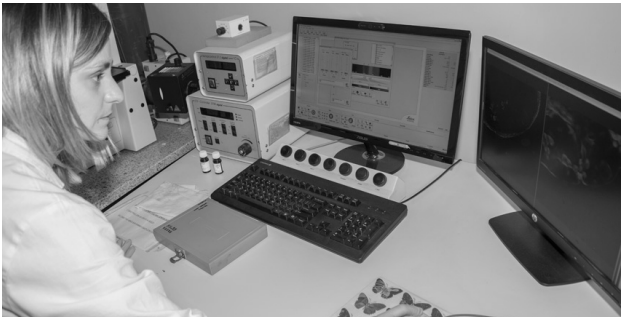
UNIUD collaborates not only with Europe but also across the globe and has long-standing connections with Africa, India and China. UNIUD has a strong link of collaboration with University Medical Hospital of Udine.

UNIUD has competent personnel in administration, economic, legal and planning area. The technical and scientific activities are developed with the support of the research and technology transfer office. This office manages complex projects, protects intellectual property and transfers research results to the local area. The structure is supported by a team of 13 competent people, who have long-time experiences in coordination and planning leadership.

The main features of the technology transfer area are:

- 57.357 publications,
- 100 patents (46 marketed),
- 8 spin off,
- 23 start up.

Since May 2016 UNIUD officially obtained the HR Excellence in Research Award from the European Commission.



Team UNIUD

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adsi
Austrian Drug Screening Institute

ADSI

Austrian Drug Screening Institute

Innsbruck, Austria

The Austrian Drug Screening Institute (ADSI) was founded in 2012 as a research enterprise of the University of Innsbruck (LFU) and offers research services for companies as well as academic research institutes in the fields of drug development, phytopharma, dietary supplements, phytocosmetics, nutrition and related industries.

The ADSI was initiated by the analytical chemist Prof. Dr. Günther K. Bonn, Head of the Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck, and by the cell biologist and physician Prof. Dr. Lukas A. Huber, Head of the Cell Biology Division at the Medical University of Innsbruck. Both Prof. Bonn and Prof. Huber are the scientific directors of the ADSI.

In ADSI, analytical chemistry and cell biology are combined in a unique way. The biological division designs cell-based assays that mimics human tissues in the lab and thus are particularly relevant. On the other hand, the analytical section is able to analyse and read different parameters, no matter if the basic material derives from plants to extract potential active substances or from cell supernatants to detect changes in the regulation of cytokines. Furthermore, a bioinformatics division was developed to integrate the complex readouts and evaluate the resulting data sets. Research services developed at ADSI are the basis of three pillars strategy:

1. screening and product development,
2. mode of action studies and confirmation of cosmetic claims, and
3. quality control analyses for nutrition and dietary supplements industries.

The ADSI employs about 25 persons, mainly highly qualified scientists. The institute is equipped with a state-of-the-art infrastructure. It became an integral part of the Austrian research landscape and works closely with numerous universities and research institutions, as well as with industrial partners in Austria and abroad.

Although the Institute is mainly financed by the state of Austria and the state of Tyrol, revenues from research contracts contribute significantly to the financing of ADSI.

Team ADSI

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MEDIZINISCHE UNIVERSITÄT INNSBRUCK

MUI

Medizinische Universität Innsbruck

Innsbruck, Austria

The Medical University of Innsbruck (MUI) is a public university in Innsbruck. It used to be one of the four historical faculties of the Leopold-Franzens-Universität Innsbruck and became an independent university in 2004.

MUI is one of the largest educational facilities for doctors and medical researchers in Austria and the most important in western Austria. The cooperation with the Tyrolean clinics is very close and University Hospital of Innsbruck with 1600 beds is one of the biggest clinics in Austria.

On average, 1,800 employees work at MUI and over 3,000 students participate in the courses.

Roughly 300 PhD postgraduates take part in one of the 9 PhD programs offered. In addition, a part-time doctoral program – the Clinical PhD – is offered for graduates of the Medicine and Dentistry degree programs.

Like every university, the Medical University of Innsbruck is mostly associated with research, which is one of its three core activities. The research teams achieve remarkable results in both - theoretical departments and clinical practice. Researchers assert themselves well in this extremely competitive field and obtain funding both on a national and international scale. They are involved in many cooperative projects.

Scientific research is carried out in various scientific areas. Main research focuses on the following fields:

- Oncology,
- Neurosciences,

- Genetics, epigenetics and genomics,
- Infectiology, immunology & organ and tissue transplant

The short geographical distances between Medical University, Hospital, further academic and research institutes like ADSI and Oncotyrol, enable fruitful cooperation and simplify data and patient's material exchange.

In 2016 the Comprehensive Cancer Center Innsbruck (CCCI) was established as a partnership between MUI and the Tyrolean Clinics. CCCI is a high-performace centre of modern technology in cancer research and closes the gap between interdisciplinary research and clinical application and accelerates the implementation of know-how in clinical treatment, research and teaching.

Team MUI

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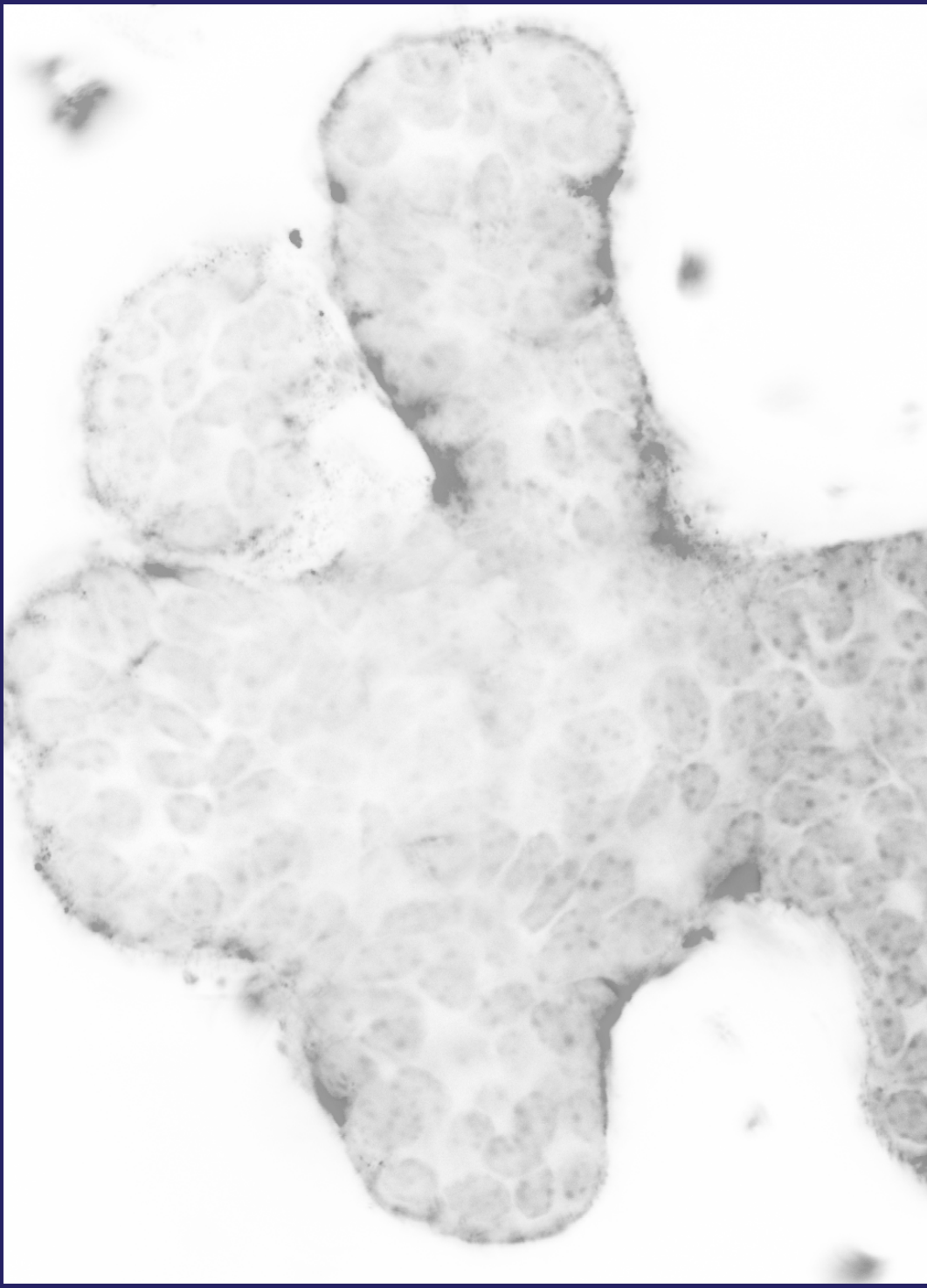
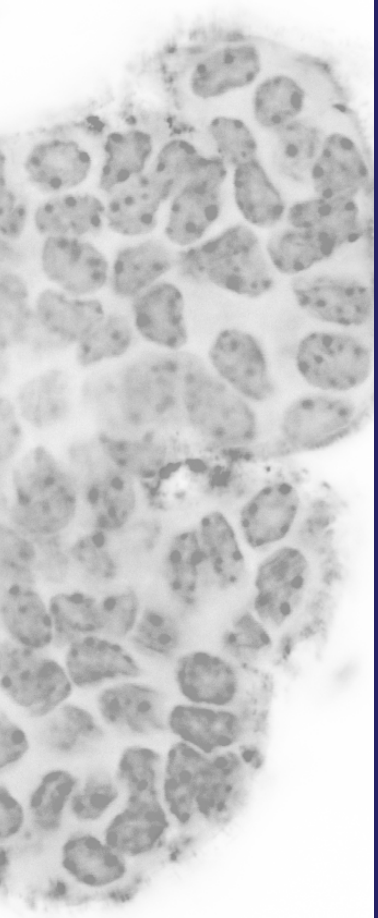
Dr. Arno Amann
Dr. Afschin Soleiman
Dr. Stefan Scheidl
Edith Lorenz



Overview



Personalized medicine is a comprehensive, prospective approach to prevent, diagnose and treat disease by using each person's unique clinical, genetic, genomic and environmental information.

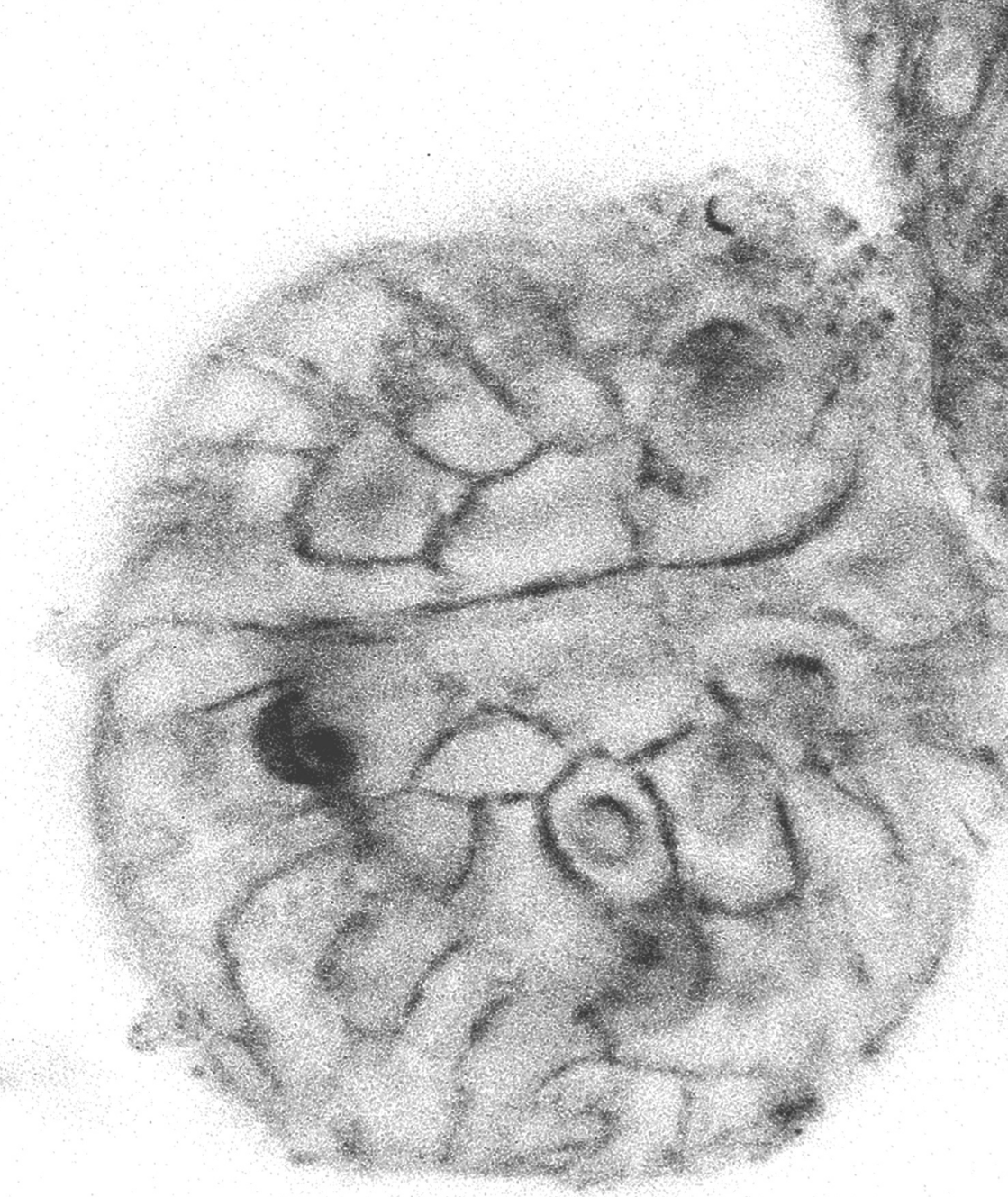
The goal of PreCanMed was to establish a pipeline for the implementation of tumor-organoids in precision cancer medicine. This technical handbook starts with procedures related to bioethics approval of the observational study and embraces procedures ranging from tissue sample preparation, to the establishment and cyro-conservation of organoids cultures, their genomics analysis by RNA Seq and Whole Exome Sequencing, identification of therapeutic targets by bioinformatics analyses as well as setting up procedures for testing drugs on organoid models.

1.
Bioethics approval and informed consent related to the PreCanMed observational study

The generation and cryo-conservation of life-patient derived cell material and storage of genomics data raises complex issues related to bioethics and patient privacy. A key step in the project was the development of protocols for an observational study that received approval from regional Bioethics committees. Procedures comprise policy rules for human biological samples and related data ensuring that the biological material is exclusively obtained from approved sources and has an owner that agrees with the acquisition and the specific use of the biological sample by signing an informed consent document. Biological samples are linked to a custodian, are traceable and are uniquely identifiable by a coding system that protects the donors' identity from experimenters that perform research. The informed patient consent document allows the use of the biological material for non-profit research, allows the withdrawal of consent at any time, regulates use of genomics data and privacy and specifies the period of time the biological material can be retained. Patients obtained detailed information on the observational study in an oral conversation with the treating physician.

- Approval of study of project partner Medizinische Universitaet Innsbruck (**MUI**) and Austrian Drug Screening Institute (**ADSI**): AN2014-0282 341/4.20 342/4.3; Title of study: *'Entwicklung eines Zytotoxizitätsassays mit primären Zellen aus Operationsmaterial/Biopsien und malignen Ergüssen in einem neuen 3D Zellkultursystem'*
- Approval of study of project partner Universty of Udine (**UniUD**): Rregistration number CEUR-2017-PR-048-UNIUD; Title of study *'Sviluppo di metodologie e protocolli per l'isolamento ed il mantenimento di organoidi da campioni di tumore del colon umano'*
- Approval of study of project partner Consorzio Interuniversitario per le Biotecnologie, Laboratorio Nazionale and University of Trieste (**LNCIB, UniTS**): Studio Protocollo n.62/2017; Title of study: *'Sviluppo di metodologie e protocolli per l'isolamento di organoidi da campioni umani di tumore del polmone, mesotelio, mammella'*

Detailed information of the respective ethics approvals can be found on the PreCanMed website (www.precanmed.eu).



2.
Communication tools to manage rapid transport of tissue to research laboratories

Rapid transfer of surgically resected patient tissue from operation room to the experimental laboratory proved to be critical for the successful and reproducible establishment of organoid cultures. Efficient communication channels that are compatible with the hospital routine were established:

- Project partner (PP) MUI/ADSI assured rapid tissue sample processing by dedicating personnel responsible for communication and sample transport between hospital and research laboratories.
- Project Partners (PP) LNCIB and UniTS used a Whatsapp group to update all involved researchers and hospital personnel on the timing of surgeries and transfer of tissue to the hospital's pathology unit.
- Surgeons collaborating with Partner UniUD communicated the timepoint for tissue transport by phone.


TX-100
in PBS

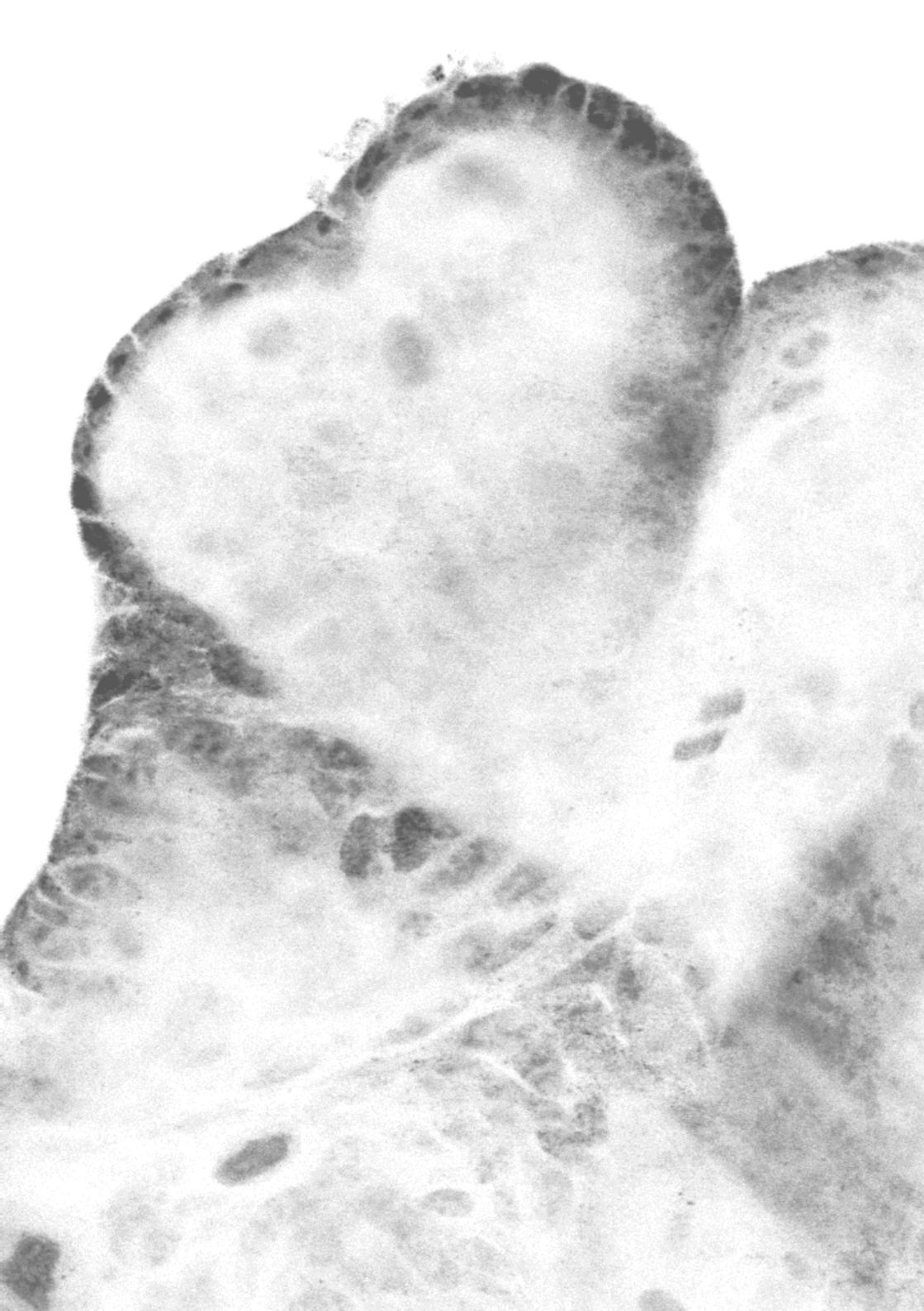
3.
Assessment of tissue material for the generation of normal-tissue and tumor-tissue organoids

Immediately after the arrival of surgically resected tissues to the hospital's pathology unit, pathologists selected relevant regions of tumor- and normal-tissue for routine diagnosis and provided tissue samples for research purpose. Scrape cytology of cell material brushed from tumor-tissue cross section revealed information on tissue quality, abundance and morphology of tumor cells prior to the generation of tumor-organoid cultures. The size of tumor-tissue for tumor-organoid generation should be in a range of 0,4 - 1cm^3. Thus small-size tumors were excluded from the study to ensure the availability of tumor-tissue for obligatory clinical procedures. Resected normal-tissue was obtained from tumor free surgical margins and its morphology was controlled by pathologists. Sections of paraffin embedded normal- and tumor-tissue were reserved for characterization by immunohistochemistry and genomics analyses in the current study. Normal- and tumor tissues was stored in cold storage

medium containing high concentration of antibiotics until pick-up by researchers. The use of antibiotics at high concentration was in particular relevant for colorectal and lung tissues that originate from an organ-context exposed to bacteria and fungi. Storing tissues in this medium for 1-6 hours did not affect cell viability, but, under ideal conditions, the time from surgery to the storage of tissue samples in storage media should be limited to 25 minutes (***Table 1***). Factors that represent high risk of failure to organoid culture establishment are: i) prolonged handling time of tissues at room-temperature, ii) bacterial/fungal contamination and iii) limited availability of tumor/normal-tissue.

Table 1: Summary of optimized procedures for the assignment of tissue originating from surgically resected tumors, established by PreCanMed project partners. For details see also SOPs on www.precanmed.eu

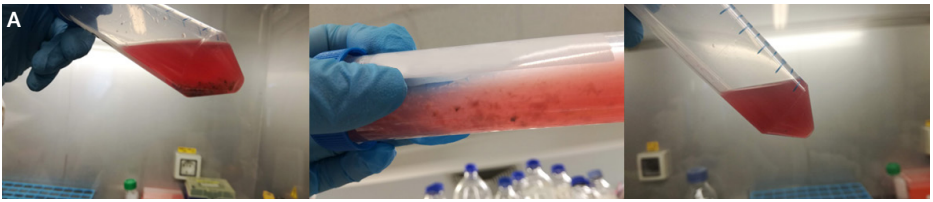
Post Surgery Procedures	Breast tissue (UniTS)	Lung tissue (LNCIB)	Colorectal tissue (MUI, ADSI, UniUD)
Transport Operation theatre to pathology unit	max. 10 minutes	max. 10 minutes	max. 15 minutes
Selection of tissue for research purpose	max. 10 minutes	max. 10 minutes	max. 10 minutes
Storage of tissue samples	max. 6 hours without loss of tissue quality (4°C)	max. 3 hours without loss of tissue quality (4°C)	max. 1 hour without loss of tissue quality (4°C)
Transport time to research laboratory	15 minutes	15 minutes	5-15 minutes
Tissue storage media	Medium + antibiotics	Medium + antibiotics	0,9% NaCl



CaCl2 0,1M
40
35
30
25
20

4.
Processing of patient tissue to obtain cell material for organoid cultivation

Normal- and tumor-tissue environments contain collagen fibers, reticular fibers and other elastic fibers in the extracellular matrix. Liberating normal and tumor cells from connective fibers without impairing cell viability represents a key step for the establishment of organoid cultures. Project partners have combined mechanical dissection (mincing tissue using forceps, scalpel blades or scissors) followed by tightly controlled collagenase treatment of 1-3mm^3 tissue fragments for 1-2 hours (***Table 2***; ***Figure 1***). Prolonged collagenase treatment or the use of general proteases such as Protease type XIV (from Streptococcus) significantly impaired cell viability. After enzymatic treatment cells were separated from residual tissue fragments using cell strainers and plated in commercially available matrix (Matrigel), a reconstituted basement membrane preparation that is reported to mimic the tumor-environment.



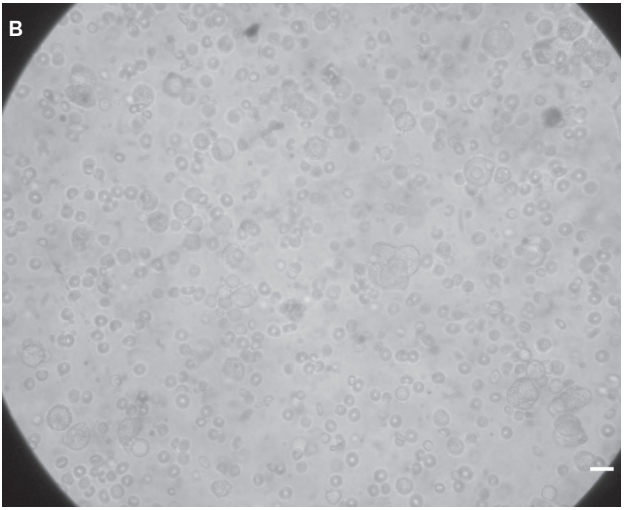


***Figure 1**: Representative images of processing of mechanically dissected lung cancer. Images show progressive digestion after 0, 1 and 2 hours (left to right). (B) Obtained cell material for lung tumor tissue plated in Matrigel for organoid cultivation. (C) Matrigel drops containing tumor cells placed in a 24 well plate.*

Tissue processing	Breast tissue (UniTS)	Lung tissue (LNCIB)	Colorectal tissue (MUI, ADSI, UniUD)
Minimal tissue size	1 cm^3	1 cm^3	*Tumor-tissue:* > 0,4 cm^3 *Normal-tissue:* > 0,5 cm^3
Mechanical dissection	*Normal- and tumor-tissue:* Scalpels until tissue piece size of ca. 1 mm^3	*Normal- and tumor-tissue:* Scalpels until tissue piece size of ca. 1 mm^3	*Tumor-tissue:* sterile scissors, scalpels and forceps; final size of ca. 1 mm^3 *Normal-tissue:* sterile forceps and scalpel; epithelium is pulled by from the connective tissue; final size pieces of 2-3 mm^3
Collagenase treatment	2g of tissue treated with 1,5 mg/ml Collagenase A in 5 ml, for 2 hours at 37°C; shaking at 200 rpm - until obtaining cells aggregates separated from stroma; remove debris	3g of tissue treated with Collagenase A; 1,5 mg/ml in 10ml, for 2 hours at 37°C; shaking at 200 rpm - until the observation of cells aggregates separated from stroma; remove debris.	*Tumor-tissue (1g):* Liberase (collagenase I and II); 50 µg/ml (0.26 units/ml) in 10ml for 1 hour at 37°C; shaking at 250 rpm; remove debris. *Normal-tissue (1g):* 10 ml of 10mM EDTA in PBS; remove debris
Seeding in Matrigel	Cells aliquots suspended in 50 µl Matrigel drop.	300.000 cells suspended in 30 µl Matrigel.	*Tumor-tissue:* 100.000 – 200.000 cells per 30 µl Matrigel drop, *Normal-tissue:* 50-100 crypts per 30 µl Matrigel drop.

Table 2: *Overview of tissue processing procedures developed by PreCanMed project partners (for SOPs see also www.precanmed.eu)*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5.
Cultivation of tumor-organoids in Matrigel

Signalling molecules and specific extracellular matrix (ECM) conditions are key for the formation of self-organized 3D tumor-organoids with architecture recapitulating the original tumor[1]. At the start of the PreCanMed project, protocols for the generation of 3D organoid cultures were available in the literature for colorectal cancer[2], pancreatic cancer[3], gastric cancer[4] and for normal mammary tissue of mouse origin[5,6]. Protocols for the establishment of breast cancer organoid culture[7] as well as for colon[8] and lung[9] normal-tissue organoid cultures were made available during the project period. Project partners working on colorectal cancer, expanded and improved available protocols by developing procedures for multiple media establishment of parallel tumor-organoid cultures. Project partners working on breast and lung cancer concentrated on defining media composition for organoid cultivation, according to published protocols for mammary (normal/tumor) tissue organoid cultivation[5–7] and normal-tissue lung organoid cultivation[10].

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[2] van de Wetering, M. et al. Prospective Derivation of a Living Organoid Biobank of Colorectal Cancer Patients. Cell 161, 933–945 (2015).

[3] Boj, S. F. et al. Organoid models of human and mouse ductal pancreatic cancer. Cell (2015). doi:10.1016/j.cell.2014.12.021

[4] Yan, H. H. N. et al. A Comprehensive Human Gastric Cancer Organoid Biobank Captures Tumor Subtype Heterogeneity and Enables Therapeutic Screening. Cell Stem Cell (2018). doi:10.1016/j.stem.2018.09.016

[5] Shackleton, M. et al. Generation of a functional mammary gland from a single stem cell. Nature (2006). doi:10.1038/nature04372

[6] Jamieson, P. R. et al. Derivation of a robust mouse mammary organoid system for studying tissue dynamics. Development (2016). doi:10.1242/dev.145045

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[8] Crespo, M. et al. Colonic organoids derived from human induced pluripotent stem cells for modeling colorectal cancer and drug testing. Nat. Med. (2017). doi:10.1038/nm.4355

[9] Choi, J., Iich, E. & Lee, J. H. Organogenesis of adult lung in a dish: Differentiation, disease and therapy. Developmental Biology (2016). doi:10.1016/j.ydbio.2016.10.002

[10] Barkauskas, C. E. et al. Lung organoids: current uses and future promise. Development (2017). doi:10.1242/dev.140103

5.1 Colorectal tumor (CRC) and normal colon tissue organoids (MUI, ADSI, UniUD)

Experience from PP UniUD/ADSI/MUI and published literature suggest that failure in the establishment of tumor-organoids may be due to patient-specific genetic alterations that are in conflict with growth factors present in the standard organoid cultivation medium. Accordingly, these problems may be resolved by using growth factors combination that are adapted to the genetic make-up of the primary tumor. Given that a complete picture of genetic alterations of the resected tumor was not available at the start of organoids cultivation, parallel multiple media approaches were developed to i) demonstrate patient specific requirements for growth factor combinations and ii) prove if this strategy increases the overall-efficiency of tumor-organoid establishment.

Project Partners ADSI/MUI and UniUD enrolled 19 patients and 21 patients in the study, respectively. Patient tissues from 8 (ADSI, MUI) and 11 (UniUD) patients were subjected to a multi-media approach to test the impact of Wnt3a (WCM), EGF, ALK5 inhibitor (A83-01), p38 MAP Kinase inhibitor (SB202190) and PGE2 (ProstaglandinE2) on tumor-organoid formation (***Figure 2***, ***Table 3***).

Remarkably, it became apparent that all UniUD CRC organoids (11/11) grew in complete media containing all listed growth factors/inhibitors (WRENAS=M6 media); 64% (7/11) did grow in the absence of Wnt3a and only in 36% of cases (4/11) proliferation was improved by the supplementation of media with Wnt3a (***Table 4***). This suggested that a significant proportion of CRC adenocarcinomas may show a genetic make-up that confers independence to Wnt3a cell signalling. However, processed CRC tissue may contain a mix of Wnt3a dependent and independent cancer cells but only Wnt3a independent tumor cells give rise to organoid cultures in RENAS medium lacking Wnt3a (***Table 4***). This 'polyclonal' theory of tumor organoid populations has important impact on designing therapeutic approaches, which could then be unveiled by Whole Exome Sequencing of matched organoids grown in RENAS or WRENAS media.

Work form ADSI/MUI provided additional evidence for diversified growth factor requirements for tumor-organoid cultivation by showing that out of 8 processed CRC tissues 7 organoid cultures required specific growth factor combinations. Among the 8 patient tissues, 5 produced tumor organoids when only adding the ALK5 inhibitor A83-01 to the basic medium (TPIO-16, -17, -18, -19 – 21). Interestingly, when A83-01 was not sufficient to sustain tumor organoid cultivation, the addition of EGF and p38 MAP kinase inhibitor (SB202190) was sufficient to rescue tumor organoid formation (TPIO-20, - 22, -23; ***Table 5***). The results demonstrate that tumor-organoid proliferation can be sustained by a

minimal set of signalling stimulants/inhibitors. This raises the possibility that additional stimulation by non-essential components in the medium may trigger alterations in cancer's cell biology or promote the expansion of under-represented tumor cell populations. This is of special importance for the careful identification of patient-specific therapeutics using organoid models.

In line with data from UniUD, Wnt3a was found to have a positive effect on tumor-organoid proliferation in only 3 out of 8 organoid cultures. For both ADSI/MUI and UniUD normal-tissue organoids were obtained from all patients (WRENAS/M6 media).

Implementing a multiple media regime for successful tumor-organoid cultivation increases workload and reagent costs. To circumvent these negative 'side-effects', a real-time metabolic activity assay (RealTime-Glo™ MT Cell Viability Assay, PROMEGA) was adapted and applied for Matrigel cultures. This allowed to identify ideal media composition by measuring cell viability in a time window of 24-48 hours post-seeding (***Figure 3***, ***Figure 4***) and also the efficient omission of non-appropriate media from further organoid cultivation procedures.

Together, these results demonstrate that multimedia approaches are instrumental for the establishment of tumor-organoid cultures in media containing a 'minimal sufficient' number of growth-factors, when the genetic profile of the primary tumor remains unknown prior to cultivation.

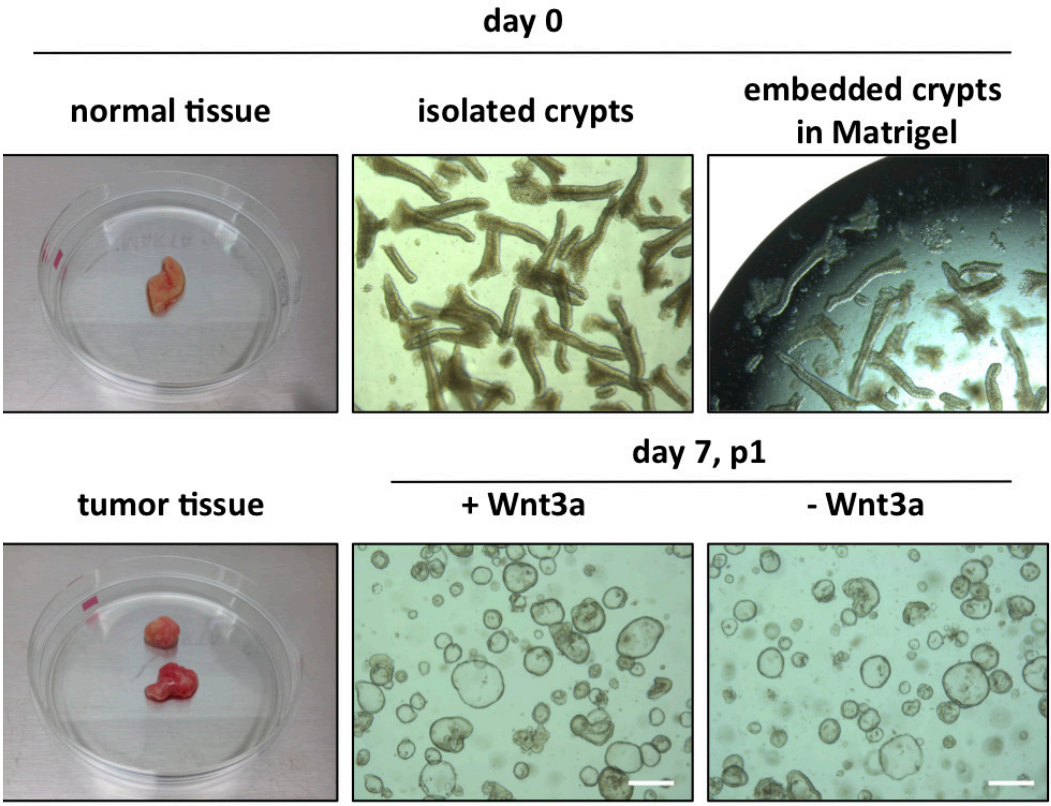
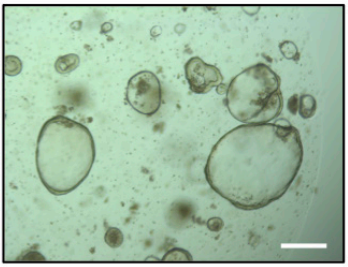


Figure 2. Organoid cultured from normal tissue (top panels) and colorectal cancer tissue (bottom panels) obtained from the same patient. Tumor-tissue supported the formation of organoids in the presence and absence of Wnt3a (bottom panel); scale bar (see also SOPs www.precanmed.eu).

day 7, p1



	ADSI/MUI	ADSI/MUI	ADSI/MUI	ADSI/MUI	ADSI/MUI or UniUD	UniUD
Media composition	Medi-umM2	Medium M3	Medium M4	Medium M5	Medium M6 and Medium 'WRENAS'	Medium 'RENAS'
Basal media	x	x	x	x	x	x
R-Spondin conditioned media	x	x	x	x	x	x
Noggin conditioned medium	x	x	x	x	x	x
Nicotinamide	x	x	x	x	x	x
N-acetylcysteine	x	x	x	x	x	x
B27 supplement	x	x	x	x	x	x
Rock kinase inhibitor (Y-27632)	x	x	x	x	x	x
Gastrin	x	x	x	x	x	x
Primocin	x	x	x	x	x	x
Wnt3a conditioned medium	-	-	-	-	x	-
EGF	-	x	-	x	x	x
A83-01 (ALK 5 inhibitor)	-	-	x	x	x	x
SB202190 (p38 MAP Kinase inhibitor)	-	x	-	x	x	x
ProstaglandinE2 (PGE2)	-	-	-	-	x	x

Table 3: Composition of media used by project partners in the multiple media tumor-organoid cultivation approach. Note: 'WRENAs media from UniUD: Violet: critical growth factors/inhibitors addressed in the multiple media approach (see also SOPs on www.precanmed.eu).

Table 4: Tumor-organoid cultivation efficiency in WRENA and RENA medium (Project partner UniUD). -, no proliferation; +, ++ poor/good proliferation; +++, excellent proliferation capacity - sufficient to perform genomics analysis and functional tests.

For detailed information see www.precanmed.eu.

Patient-ID (UniUD)	Growth potential in WRENAS (+Wnt3a)	Growth potential in RENAS (no Wnt3a
P6 - Tubulovillous Adenoma	++	+
P7 - Adenocarcinoma	++	-
P8 - Adenocarcinoma	+++	-
P12 - Adenocarcinoma	+++	+++
P13 - Adenocarcinoma	++	-
P14 - Adenocarcinoma	++	+++
P15 - Adenocarcinoma	+	-
P16 - Adenocarcinoma	++	+++
P17 - Adenocarcinoma	+	++
P20 - Adenocarcinoma	+	+++
P21 - Adenocarcinoma, mixed type	+	+

Table 5: Multiple media approach to assess patient specific requirements for the generation of tumor-organoids (Project partner MUI/ADSI). Growth factor combinations that support tumor-organoid proliferation are indicated. '-', no efficient proliferation; '+' efficient proliferation.

For detailed information see www.precanmed.eu.

Patient ID (MUI/ADSI)	Media supporting tumor-organoid proliferation
TPIO-16 Adenocarcinoma	M2(no factors) ➞ + M3 ➞ - M4 (A83-01) ➞ + M5 ➞ - M6 ➞ -
TPIO-17 Adenocarcinoma	M2(no factors) ➞ - M3 ➞ - M4 (A83-01) ➞ + M5(EGF, A83-01, SB202190) ➞ + M6 ➞ -
TPIO-18 Adenocarcinoma	M2 (no factors) ➞ + M3 ➞ - M4(A83-01) ➞ + M5 ➞ - M6 (Wnt3a, EGF, A83-01.SB202190, PGE2) ➞ +
TPIO-19 Adenocarcinoma	M2 ➞ - M3 ➞ - M4 (A83-01) ➞ + M5 ➞ - M6 (Wnt3a, EGF, A83-01.SB202190, PGE2) ➞ +
TPIO-20 Adenocarcinoma	M2 ➞ - M3 ➞ - M4 ➞ - M5(EGF, A83-01, SB202190) ➞ + M5 ➞ - M6 ➞ -
TPIO-21 Adenocarcinoma	M2 ➞ - M3 ➞ - M4(A83-01) ➞ + M5 ➞ - M6 ➞ -
TPIO-22 Adenocarcinoma	M2 ➞ - M3 ➞ - M4 ➞ - M5(EGF, A83-01, SB202190) ➞ + M6 ➞ -
TPIO-23 Adenocarcinoma	M2 ➞ + M3 (EGF, SB202190) ➞ + M4 ➞ - M5 (EGF, A83-01, SB202190) ➞ + M6 (Wnt3a, EGF, A83-01, SB202190, PGE2) ➞ +

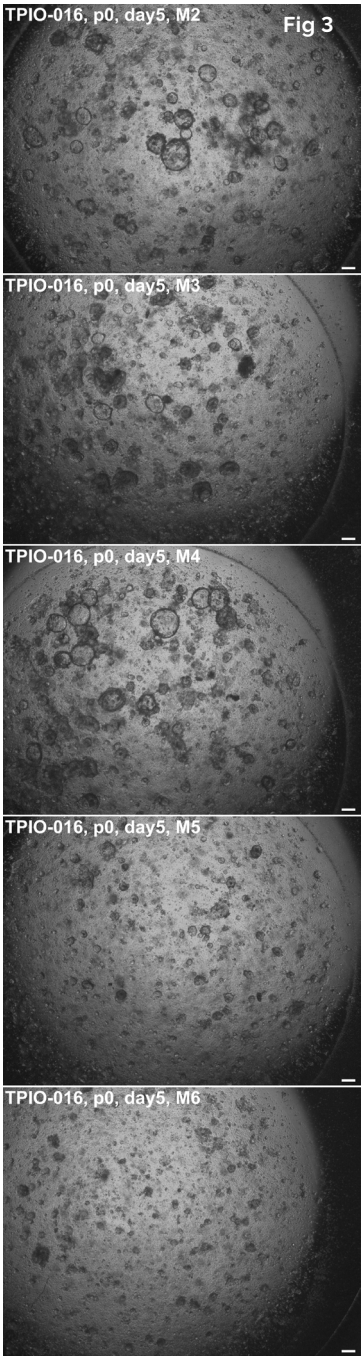


Figure 3: Cultivation of CRC organoids in a multiple-media approach. Colorectal tumor tissue from patient was used to establish tumor-organoid cultured in different media conditions (M2-M6). Representative photographs with tumor-organoids were taken at day 5 of cultivation. Medium M2 and M4 supported efficient proliferation of organoids. Scale bar indicates 50µm.

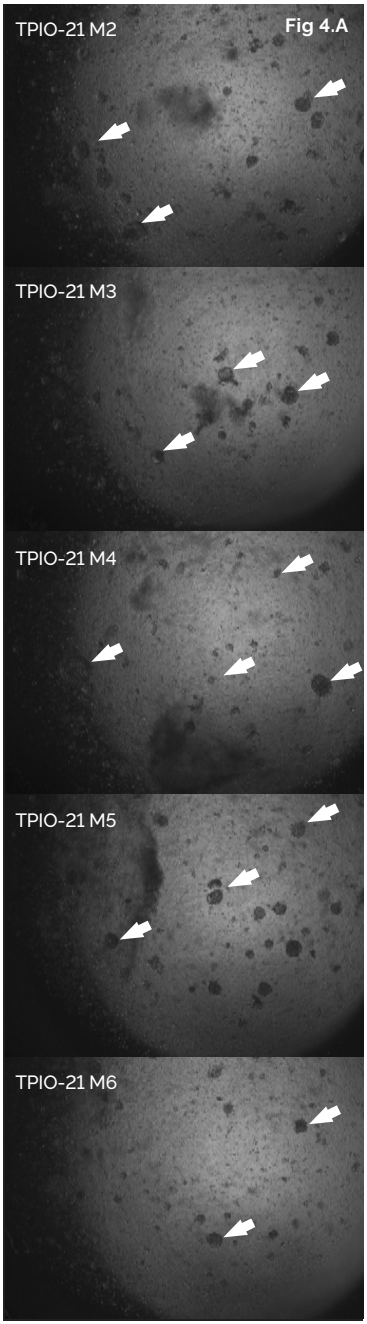
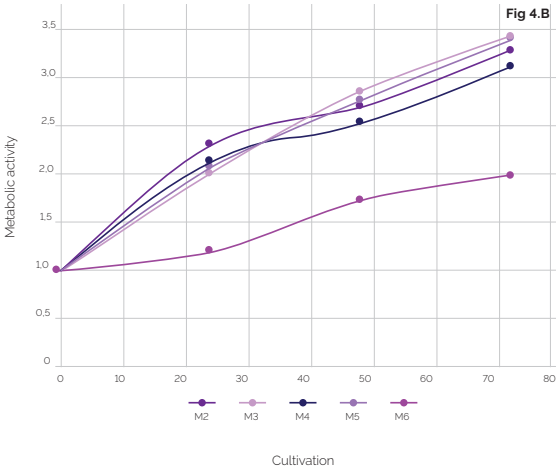


Figure 4: Real-time measurement of metabolic activity to identify favorable media condition for CRC organoid cultivation. Tumor tissue obtained from patient supports organoids formation in M2, M3, M4, M5, M6. (A) Representative pictures of organoids after 72hrs of cultivation. (B) Quantification of metabolic activity at indicated timepoints. Scale bar indicates 50µm.



5.2 Efficiency of breast tumor- and normal-tissue organoids (UNITS)

Media reported to support the establishment of mouse mammary tissue organoids was not applicable for human tissues as these conditions did not support growth of 13 breast cancer tissues tested. Changing media composition, adding R-Spondin, Heregulin ß1, Noggin, FGF-7, FGF-10, as well as ALK5 inhibitor (A83-01), p38 MAP Kinase inhibitor (SB202190) and Rock inhibitor (Y-27632) significantly improved the efficiency of tumor organoid cultures[7] (***Table 6***). Under these conditions 33 patient tissue samples were processed. 48% of invasive ductal carcinomas (13/27) and 50% of invasive lobular carcinomas (3/6) resulted in viable tumor organoid cultures. Finally, 26% of all invasive ductal carcinoma (7/27) and 16% of invasive lobular carcinoma tissue (1/6) gave rise to tumor-organoids with strong proliferative potential. In addition to histological classification, breast cancers are also stratified according to the expression of molecular markers into luminal A, luminal B, triple negative and Her2 like subclasses. Out of 33 enrolled patients, viable breast cancer organoids could be obtained from 47% of luminal A, 75% of luminal B, 38% of triple negative and 50% of Her2-like tumors. Remarkably, the viable organoid culture proliferation rates amongst those samples appeared to increase with augmented tumor aggressiveness: 22% of luminal A (2/9), 67% of luminal B (2/3), 100% of triple negative (3/3) and 100% of Her2-like (1/1) tumors produced efficiently proliferating organoid cultures that could be used for functional analyses (***Figure 5***). Together, these data confirm that the used growth media represent good conditions for the establishment of breast tumor organoid cultures. However, based on the insights from CRC organoid culture, we believe that the broad growth factor/inhibitor combination may not be compatible with a fraction of breast cancers, explaining the relatively high failure rate of organoid culture establishment for some breast cancer subclasses.

Due to the small size of resected tissue and the massive overrepresentation of adipocytes and fibrotic tissue compared to lobular and ductal cells it appeared difficult to generate a larger number of normal-tissue breast organoids. This limitation may only be overcome by recruiting patients affected by large tumors or subjected to mastectomy with esthetical reconstruction. However, it was not possible to recruit such patients during the project period.

[7] Sachs, N. et al. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. Cell (2018). doi:10.1016/j.cell.2017.11.010

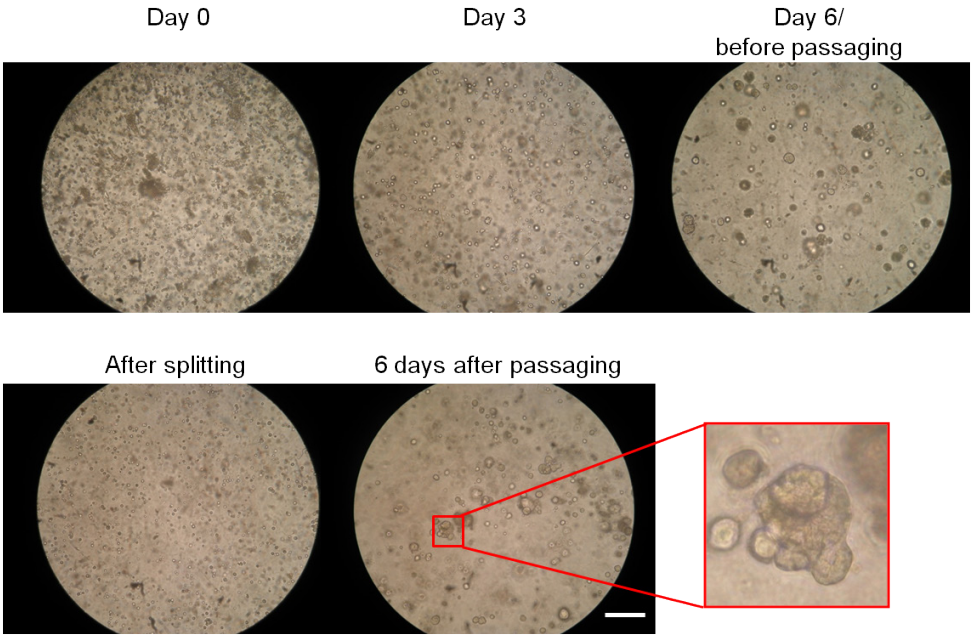


Figure 5: Cultivation of breast cancer organoids. Top panels: phase contrast microscope images of seeded breast tumor cells (Day 0) and tumor-organoids after day 3 and day 6 of cultivation. Bottom panels, tumor-organoids after passaging (left) and subsequent cultivation for 6 days (center). Scale bar (200 µm) is indicated. Right panel: tumor-organoid structure at higher magnification.

Table 6: Composition of medium used by project partners for the establishment of breast tumor-tissue organoid cultures[7]. See also SOPs on www.precanmed.eu.

[7] Sachs, N. et al. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. Cell (2018). doi:10.1016/j.cell.2017.11.010

Breast tumor-organoid medium composition
Ad-DF basal medium
B27 supplement
Nicotinamide
N-Acetylcystein
R-Spondin 3
Heregulin ß-1
Noggin
FGF-7
FGF-10
EGF
A83-01 (ALK5 inhibitor)
SB202190 (p38 MAP Kinase inhibitor)
Y-27632 (Rock inhibitor)
Antibiotics

Patient	Diagnosis	Proliferative potential
1	Luminal A	-
2	Luminal A	-
3	Luminal A	-
4	Luminal A	-
5	Luminal A	-
6	Luminal A	-
7	Luminal A	++
8	Luminal A	-
9	Luminal A	-
10	Luminal A	+
11	Luminal A	+
12	Luminal A	+
13	Luminal A	-
14	Luminal A	+++
15	Luminal A	++
16	Luminal A	++
17	Luminal A	+
18	Luminal A	+++
19	Luminal A	-
20	Luminal B	-
21	Luminal B	+
22	Luminal B	++
23	Luminal B	+++
24	Triple negative	-
25	Triple negative	++
26	Triple negative	-
27	Triple negative	-
28	Triple negative	-
29	Triple negative	+++
30	Triple negative	-
31	Triple negative	+++
32	Her-2 like	-
33	Her-2 like	+++

Table 7: Proliferative potential of breast tumor organoids obtained from individual patients enrolled into the study (-, no proliferation; +, ++ poor/good proliferation; +++, excellent proliferation capacity/ sufficient to perform genomics analysis and functional tests). For detailed information see www.precanmed.eu.

5.3 Lung tumor- and normal-tissue organoids (LNCIB)

Initial protocols for long term tumor organoids culture were based on published protocols used for the generation of normal tissue lung airway protocols that contain a limited set of growth factors and inhibitors such as EGF or Rock inhibitor (Y-276325), ALK5 inhibitor (A83-01), LIF (Leukemia inhibitory factor) and FGF10. This combination was previously demonstrated to mimic the microenvironment of lung epithelial stem cells[10,11]. This medium generated viable 3D cell structures in 25% of processed tissue samples (4/20). However, the medium did not fulfil requirements for long-term proliferative capacity. Tumor-organoid formation and growth potential was substantially improved by the addition of FGF7, p38 MAP Kinase inhibitor (SB202190), R-Spondin3 and Noggin, as reported by studies that were published during the project period[12–14] (***Table 8***). Under these conditions out of 21 processed patient samples only 4 failed to form 3D cell aggregates (19%); 81% initiated the formation of 3D cell structure (17/21). A total of 67% of organoids showed good initial organoid growth (14/21) that however ceased after a 1-2 weeks cultivation period. Finally, a total of 20% of tumor-tissues (4/21) gave rise to tumor-organoid cultures with high proliferative potential, suitable for the initiation of genomics analyses and functional tests (***Table 9***, ***Figure 6***). Remarkably, the generation of normal-tissue organoids was possible only in one case. At current we were not able to identify a lung cancer histotype that allows tumor-organoid establishment at especially elevated efficienwcy.

[10] Barkauskas, C. E. et al. Lung organoids: current uses and future promise. Development (2017). doi:10.1242/dev.140103

[11] Hegab, A. E. et al. Mimicking the niche of lung epithelial stem cells and characterization of several effectors of their in vitro behavior. Stem Cell Res. (2015). doi:10.1016/j.scr.2015.05.005

[12] Heo, I. et al. Modelling Cryptosporidium infection in human small intestinal and lung organoids. Nat. Microbiol. (2018). doi:10.1038/s41564-018-0177-8

[13] Dijkstra, K. K. et al. Generation of Tumor-Reactive T Cells by Co-culture of Peripheral Blood Lymphocytes and Tumor Organoids. Cell (2018). doi:10.1016/j.cell.2018.07.009

[14] Sachs, N. et al. Long-term expanding human airway organoids for disease modeling. EMBO J. (2019). doi:10.15252/embj.2018100300

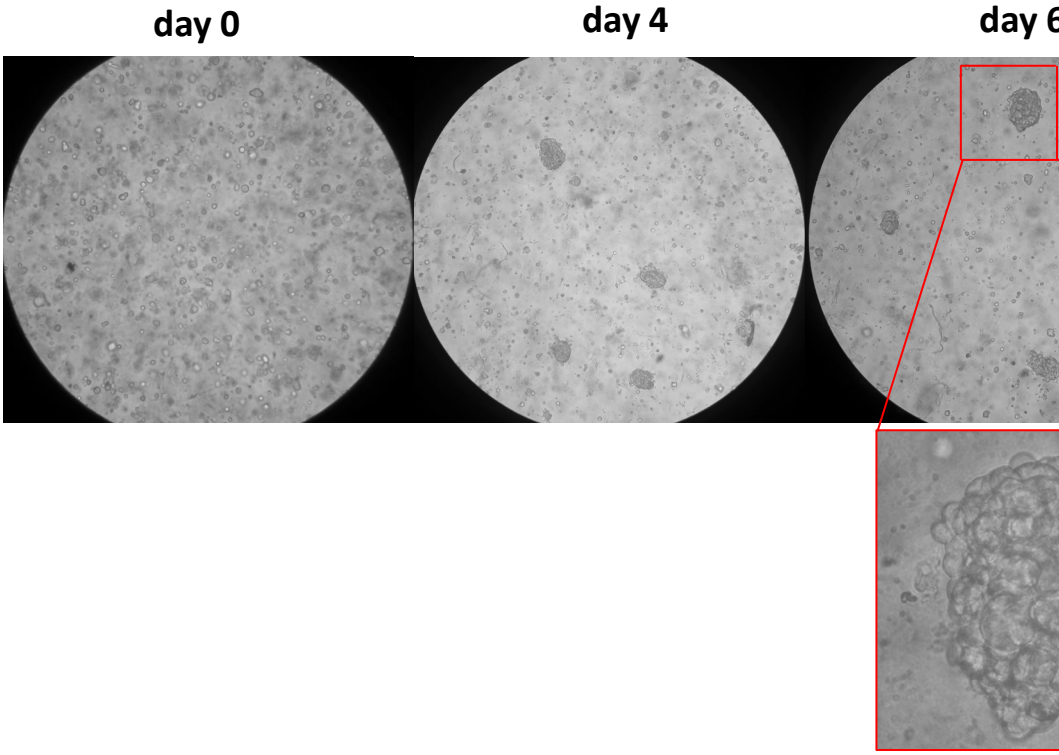
Table 8: Composition of media used by project partners for the establishment of lung tumor tissue organoid cultures See also SOPs on www.precanmed.eu.

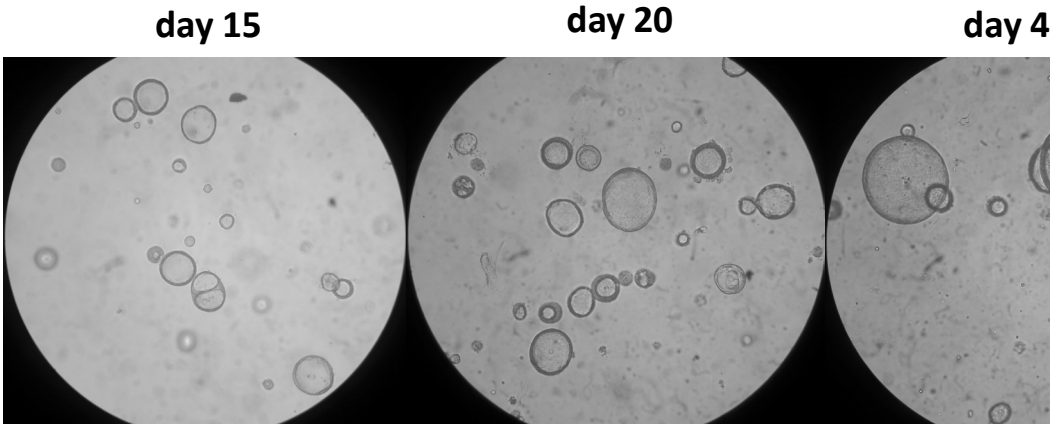
Lung tumor-organoid medium composition
DMEM
Ham's F-12
R-Spondin 1
FGF 7
FGF 10
Noggin
A83-01 (ALK5 inhibitor)
Y-27632 (Rock inhibitor)
SB202190 (p38 MAP Kinase inhibitor)
B27 supplement
N-Acetylcysteine
Nicotinamide
Penicillin / Streptomycin
Antibiotics

Together these data confirm that FGF7, FGF10, p38 MAP Kinase inhibitor (SB202190), R-Spondin and Noggin, Rock inhibitor (Y-276325) and the ALK5 inhibitor (A83-01) represent a good growth factor/inhibitors combination for the establishment of lung tumor-organoid cultures. Based on the insights from CRC organoid culture efforts, we believe that the broad growth factor/inhibitor combination may not be compatible with a fraction of lung cancers, explaining the relatively high failure rate of organoid culture establishment for lung cancer.

Patient	Diagnosis	Proliferative potential
1	Adenocarcinoma	++
2	Adenosquamous carcinoma	+
3	Adenocarcinoma	+
4	Squamous carcinoma	-
5	Adenocarcinoma	+++
6	Neuroendocrine carcinoma	+
7	Adenocarcinoma	-
8	Adenocarcinoma	+
9	Adenocarcinoma	+++
10	Adenocarcinoma	-
11	Adenocarcinoma	++
12	Squamous carcinoma	+
13	Adenocarcinoma	+
14	Squamous carcinoma	++
15	Mucinous adenocarcinoma	+++
16	Adenocarcinoma	-
17	Squamous carcinoma	-
18	Adenocarcinoma	+
19	Adenocarcinoma	+++
20	Adenocarcinoma	-
21	Squamous carcinoma	+

Table 9: Table 9: Proliferative potential of lung tumor-organoid obtained from individual patients enrolled into the study (-, no proliferation; +, ++ poor/good proliferation; +++, excellent proliferation capacity – sufficient to perform genomics analysis and functional tests. For detailed information see www.precanmed.eu.


day 0
day 4
day 6


day 15
day 20
day 4

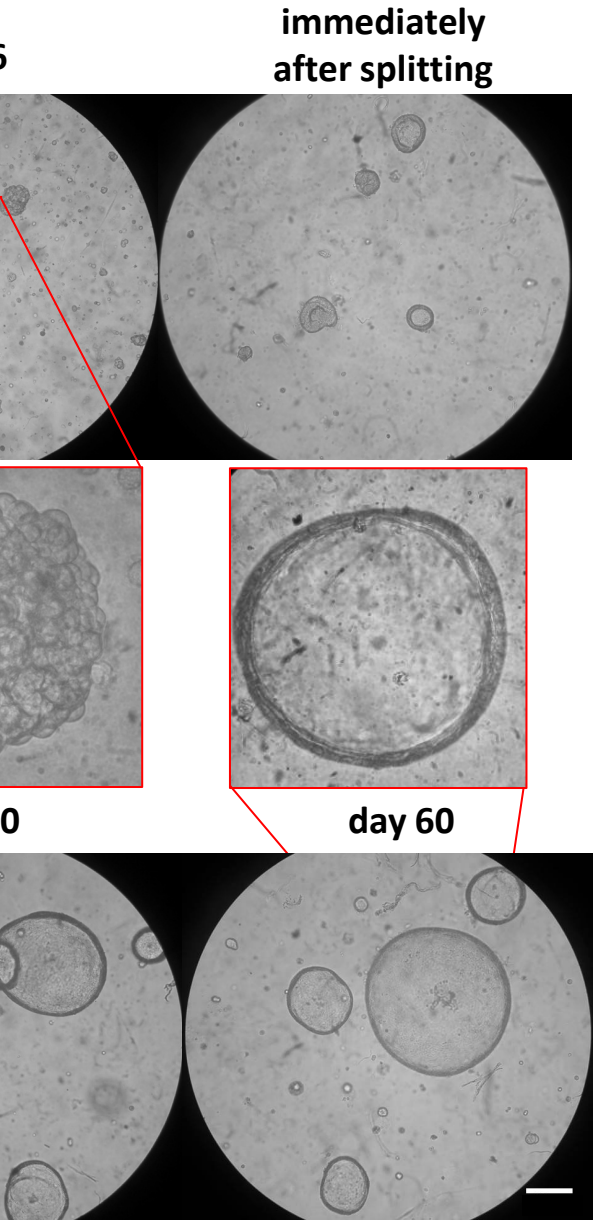


Figure 6: Cultivation of lung cancer organoids. Top panels: phase contrast microscope images of seeded lung tumor cells (Day 0) and tumor organoids after day 4 and day 6 of cultivation and the day after splitting. Bottom panels, continuous tumor organoids cultivation until day 60. Note, after splitting grape shaped organoids form spheroid structures (zoomed imaged at day 6 and day 60). Scale bar (50 µm) is indicated. See also SOPs on www.precanmed.eu.

6. Expansion of organoids cultures

Organoids grow in 3D cell structures that arrange in round-shaped and/or grape-like structures (***Figure 3, 5, 6***)[2,7,14]. Prolonged cultivation results in the formation of large structures that do not allow the access of growth factors and nutrients to centrally located cells, resulting in cell death and reduced tumor-organoid viability. Consequently, organoid structures need to be disintegrated into smaller cell aggregates to allow the continuous propagation of organoid cultures. Several strategies for the separation of organoids from breast, colorectal and lung tissues into smaller subunits or even single cells, without impairing proliferative potential, were tested. Best results were obtained by performing gentle mechanical separation of organoid structures, combined with enzymatic degradation of protein-based cell-cell contacts. For an overview see ***Table 10*** and SOPs on the project website www.precanmed.eu.

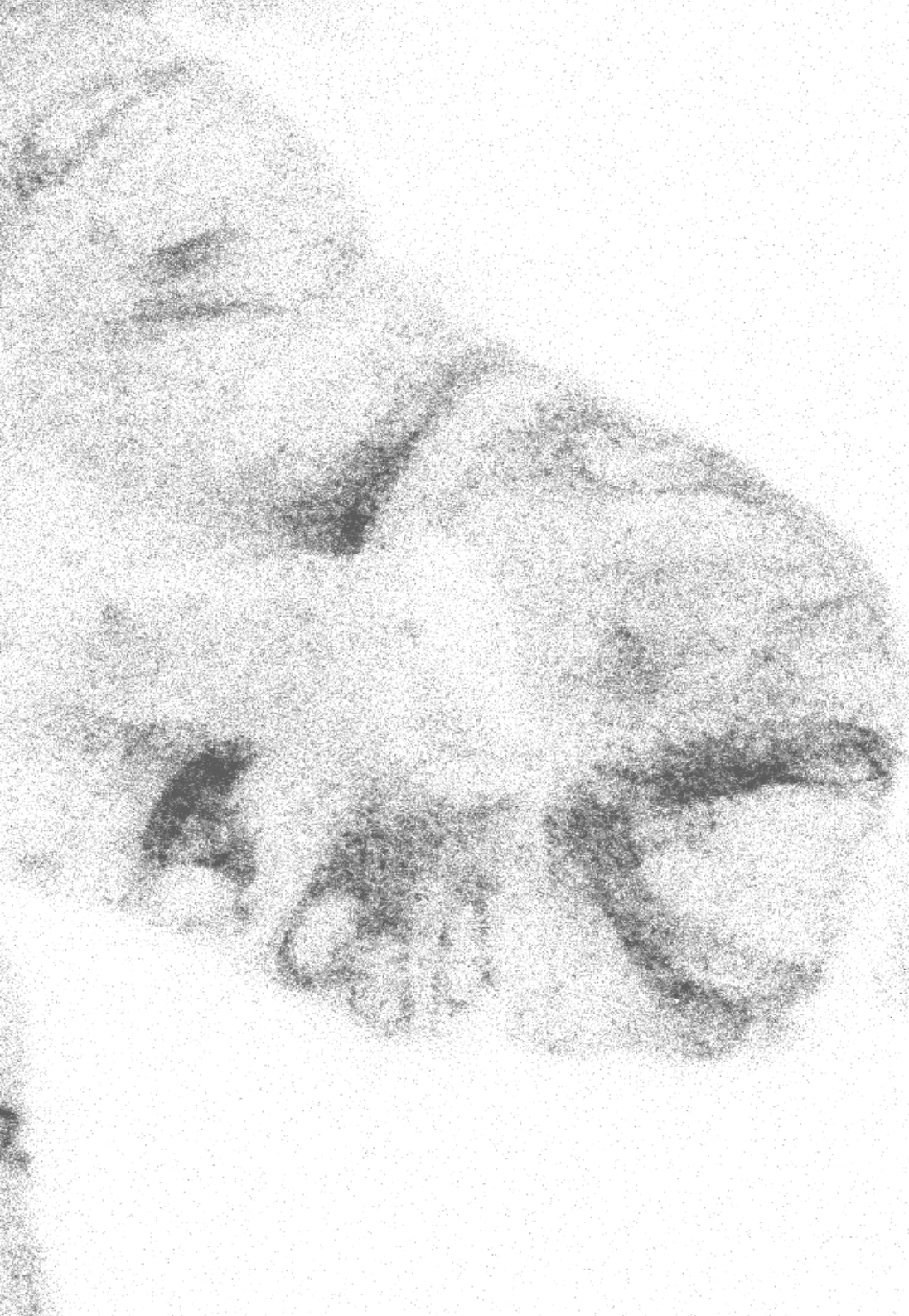
[2] van de Wetering, M. et al. Prospective Derivation of a Living Organoid Biobank of Colorectal Cancer Patients. Cell 161, 933–945 (2015).

[7] Sachs, N. et al. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. Cell (2018). doi:10.1016/j.cell.2017.11.010

[14] Sachs, N. et al. Long-term expanding human airway organoids for disease modeling. EMBO J. (2019). doi:10.15252/embj.2018100300

Splitting procedures for expansion of organoid cultures	Breast tissue (UniTS)	Lung tissue (LNCIB)	Colorectal tissue (MUI, ADSI, UniUD)
Optimal conditions	Resolving matrigel, enzymatic digestion of recovered organoids with trypsin replacement protease (5 minutes at 37°c)	*Small organoids:* Resolving matrigel and mechanical disruption of recovered organoids, followed by elimination of residual matrigel using dispase *Large organoids:* Resolving of matrigel, elimination of residual matrigel from organoids using dispase; enzymatic digestion with trypsin replacement protease (5 minutes at 37°c)	Resolving matrigel, enzymatic digestion of recovered organoids with trypsin protease. Fast growing organoids: digestion to single cells Slow growing organoids: digestion to cell clusters

Table 10: Overview on conditions for splitting of organoids derived from lung, colorectal and breast tissue. See also related SOPs on www.precanmed.eu.

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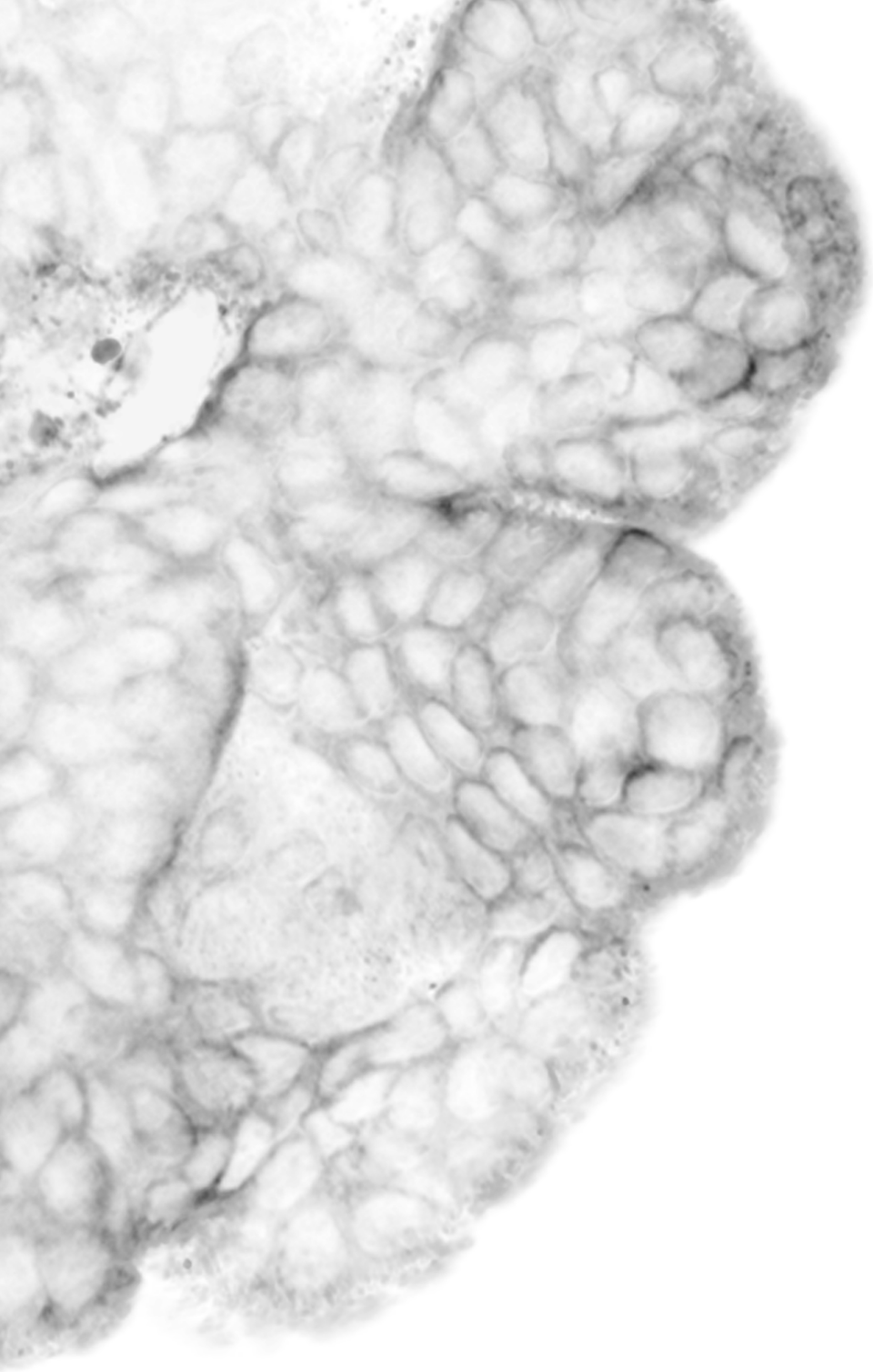
7.
Cryo-conservation of organoid cultures

Cryo-conservation of patient derived organoid culture is a central issue for the long-term use of tumor-organoids in translational and clinical research. During the course of the project it became apparent that the viability of organoids was highly sensitive to freezing conditions defined by the medium compositions and cooling rates. Organoids with robust proliferative potential showed good tolerance to freeze-thaw cycles. Freezing a high number of cells assured the re-establishment of organoid cultures after a freeze-thaw cycle. After tissue type dependent processing of organoids, best results were obtained by freezing cells down to -80˚C at controlled freezing rate of 1˚C/minute, followed by cryo-conservation in liquid nitrogen. (***Table 11***). These procedures ensured successful maintenance of organoids in 50-80% of cases. Average cell lethality during a freeze-thaw cycle was in the range of 20-50% (***Table 11***).

Freezing conditions for organoid cultures	Breast tissue (UniTS)	Lung tissue (LNCIB)	Colorectal tissue (MUI, ADSI, UniUD)
Optimal conditions	Resolve Matrigel; freeze obtained cells in FBS/10% DMSO	Resolve Matrigel, freeze obtained cells in FBS/10% DMSO	*Option 1:* Resolve Matrigel and directly freeze in Recovery™ Cell Culture Freezing Medium *Option 2:* Resolve Matrigel, digest organoids with Trypsin to single cells and freeze in Recovery™ Cell Culture Freezing Medium
Freezing rate	-1°C/minute to –80°C; transfer to N_2	-1°C/minute to –80°C; transfer to N_2	-1°C/minute to –80°C; transfer to N_2
Successful freezing of cultures	50%	50%	80%
Rate of cell death after thawing	50%	50%	20%

Table 11: Overview on conditions for freezing of organoids derived from breast, lung and colorectal tissue. See also related SOPs on www.precanmed.eu



8. Validation of histopathological features on tumor-organoid cultures

Immuno-histopathological (IHC) analysis of primary tumor-sections represents key information for tumor-classification and for the selection of post-operative therapeutic treatments. A complete set of main IHC markers used for the classification of breast, lung, and colorectal cancer is listed in ***Table 12***. Organoid cultures derived from tumor tissue maintain histopathological and molecular features of the primary tumor[2,7,14]. Immunohistochemistry on tumor-organoid cultures was performed to validate primary tumor properties in tumor-organoids prior to detailed genomics analysis. Organoids (e.g. 6 wells of a 24 well plate) were formaldehyde fixed and embedded in agarose. Agarose plugs were embedded in paraffin and sections were generated by trained personnel. The localization of organoid-structures in paraffin blocks is challenging and can be best achieved by periodic H&E staining in intervals of 10 sections. To this end, we found that highly proliferative organoids obtained from breast and lung

[2] van de Wetering, M. et al. Prospective Derivation of a Living Organoid Biobank of Colorectal Cancer Patients. Cell 161, 933–945 (2015).

[7] Sachs, N. et al. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. Cell (2018). doi:10.1016/j.cell.2017.11.010

[14] Sachs, N. et al. Long-term expanding human airway organoids for disease modeling. EMBO J. (2019). doi:10.15252/embj.2018100300

tissues recapitulated the expression of IHC markers, suggesting that organoid cultures maintain a set of classic parameters for tumor classification (***Table 13-14, Figure 7-8***).

Table 12: List of immunohistochemistry markers used for the validation of histopathological features of lung, colorectal, breast and lung tumor-organoids

Colorectal cancer	Breast cancer	Lung cancer
MLH1	Estrogen receptor	Ki-67
MSH2	Progesteron receptor	CK5/6
MSH6	Androgen receptor	CK7
PMS2	Her2/neu	p63
Her2/neu	Ki67	Napsin A
	p53	TFF-1
	E-cadherin	p40
	Cytokeratin 5/6	Calretinin
	Cytokeratin 7	ESA/ BEREP4
	Cytokeratin 14	EMA
	Cytokeratin 19	CEA
	p63	Desmin
	p120	WT-1
		Vimentin
		CK AE1/ AE3
		CD56
		Cromogranin A
		Synaptophysin
		panCK
		TG
		ERG
		PSA
		CK20
		CDX-2

Patient	CK5/6	CK7	P40	TTF1	Napsin	Post-operatory therapy
Patient 5– Lung Adenocarcinoma	Match	Match	Match	Match	N.A.	None
Patient 9– Lung Adenocarcinoma	N.A.	Match	N.A.	N.A.	N.A.	None
Patient 15 – Mucinous Adenocarcinoma	N.A.	Match	No match	N.A.	Match	None
Patient 19– Lung Adenocarcinoma	TBD	TBD	TBD	TBD	TBD	TBD

Table 13: Overview on the maintenance of immunohistochemistry markers of lung tumor-organoids and original primary tumor-tissues. Samples selected for genomics analysis are shown. Match: IHC staining of primary tumor matches that of tumor-organoids. No match: IHC of tumor-organoids doesn't match primary tumor. N.A., not analyzed. TBD: to be determined. For detailed data see also www.precanmed.eu.

Patient	ER	PR	Cytokeratin	E-cadherin	Post-operatory therapy
Patient 14 – Luminal A breast cancer	Match	Match	Match	Match	Hormonal
Patient 23 – Luminal B breast cancer	Match	Match	Match	Match	Epirubicin + Cyclophosphamide + paclitaxel + hormonal
Patient 29 – TNBC	Match	Match	Match	No match	None
Patient 31 - TNBC	Match	N.A.	Match	Match	None
Patient 33 - Her2 breast cancer	No match	Match	Match	Match	Docetaxel + Cyclophosphamide + Trastuzumab

Table 14: Overview on the maintenance of immunohistochemistry markers of breast tumor-organoids and original primary tumor-tissues. Samples selected for genomics analysis are shown. Match: IHC staining of primary tumor matches that of tumor-organoids. No match: IHC of tumor-organoids doesn't match primary tumor. TBD: to be determined. For detailed data see also www.precanmed.eu.

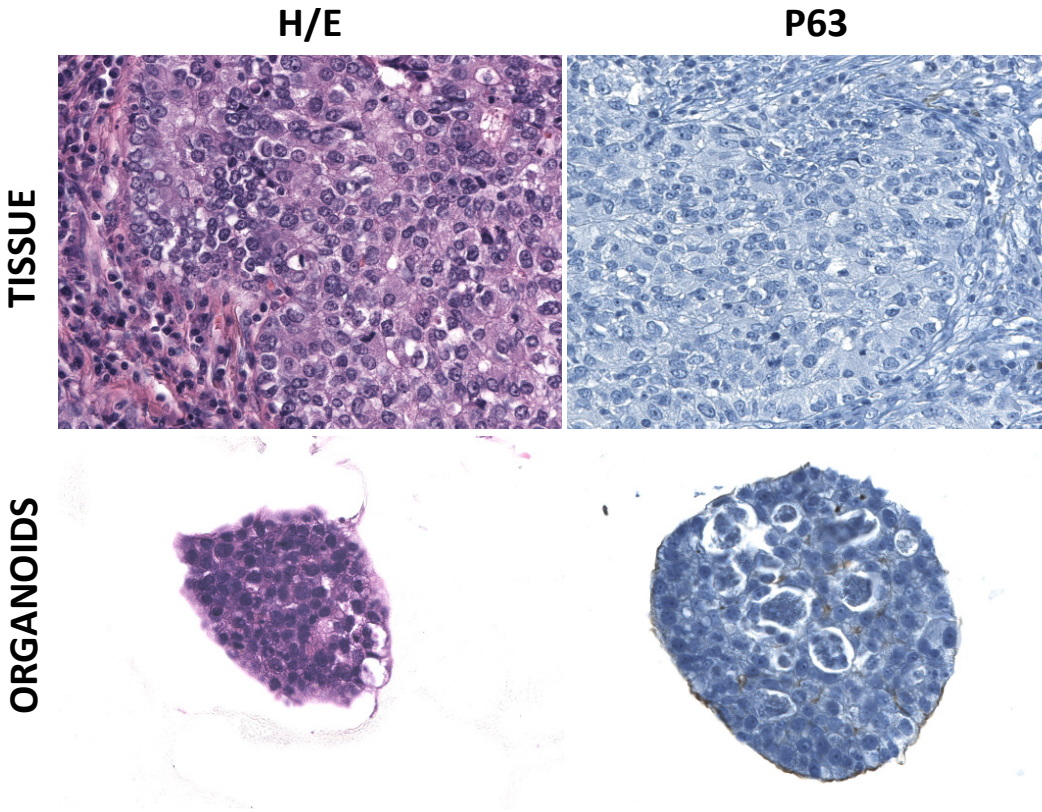
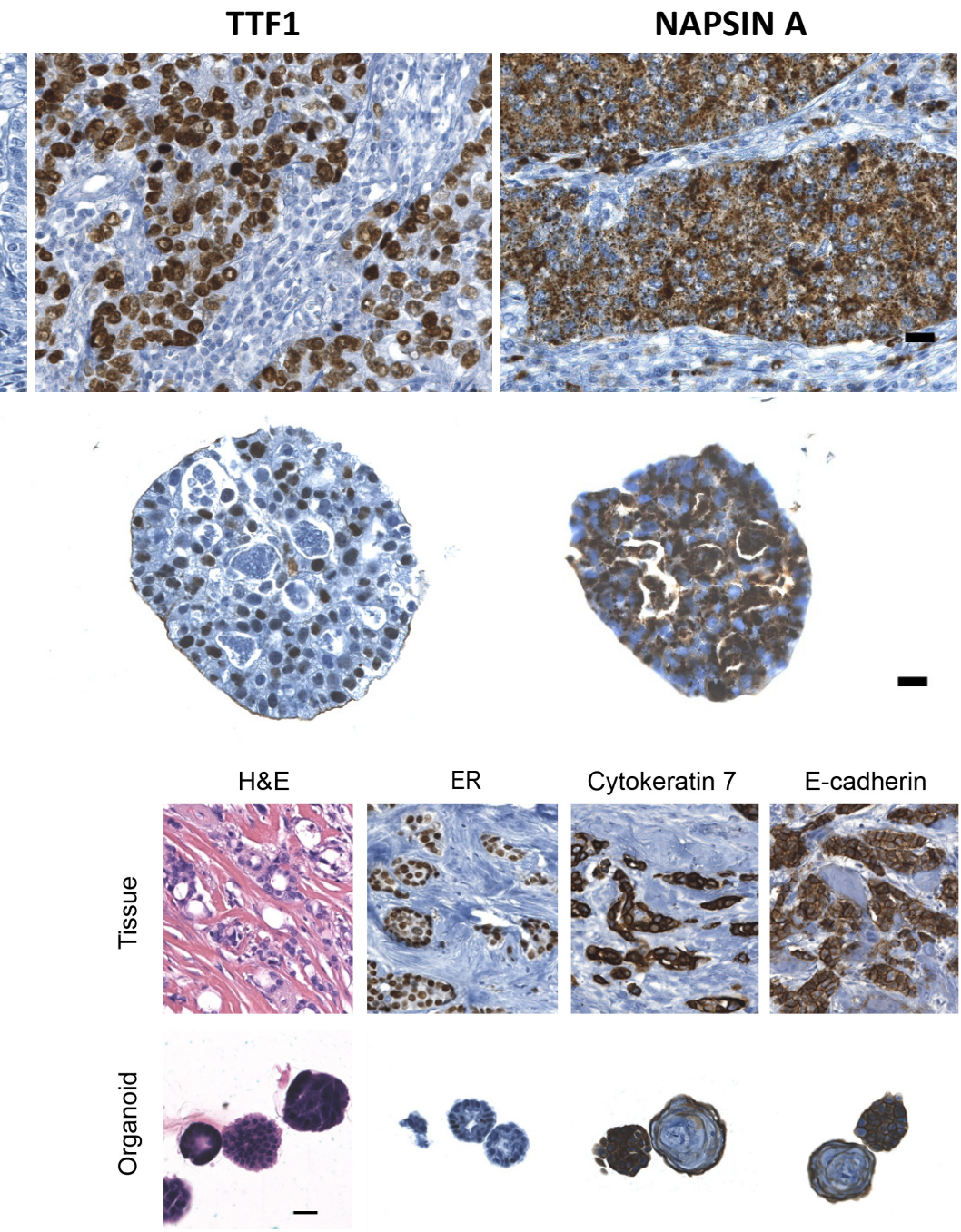


Figure 7: Organoids recapitulate histological features of primary lung tumors (A) Exemplary H&E and immunohistochemistry on sections of the primary tumor of a patient (top panel) and matched tumor-organoid (bottom panel). Immunohistochemistry was performed using antibodies for P63, TTF1 and Napsin A. The status of original lung cancer adenocarcinoma is maintained in tumor-organoid cultures. Scale bars indicate 50 µm.

Figure 8: Organoids recapitulate histological features of the original tumor. Exemplary H&E and immunohistochemistry on sections of a Luminal A invasive ductal breast carcinoma (top panels) and of the corresponding tumor organoids (bottom panels). Immunohistochemistry was performed using antibodies raised against Estrogen Receptor (ER), Cytokeratin 7 and E-cadherin. The status of the original breast carcinoma is maintained in tumor-organoid cultures. Scale bar 10 µm.


TTF1
NAPSIN A
H&E
ER
Cytokeratin 7
E-cadherin
Tissue
Organoid

9.
Genomics analysis of tumor-organoids

Tumor-organoids represent an essential building block towards the development of precision cancer medicine. Producing expression and mutation profiles from tumor-organoids and primary tumors by omics technologies is expected to enable drug discovery for precision cancer medicine. Here, we subjected a limited number of patients to RNA and Whole Exome sequencing to test the stability of organoid cultures and perform in silico drug prediction for personalized therapy.

9.1 RNA sequencing of organoid cultures

RNA from 3 replicates of paired tumor and normal colorectal organoids (UniUD) underwent TruSeq 2×100bp stranded library construction (***Table 15***). A minimum of 30 million reads per sample was required for downstream differential expression analysis. This included raw read quality control, the reference-based read alignment and the gene expression quantification (see SOP collection on www.precanmed.eu) (***Figure 9***). Then, pairwise comparison of the read counts between the matched tumor and normal organoids at different passage number was performed using the likelihood ratio tests, with a false discovery rate threshold (FDR)=0.01. As expected, the multi-dimensional scaling plot of the RNA-seq data revealed differences between the expression profiles of normal tissue and tumor tissue organoids in two dimensions (***Figure 10***). Dimension 1 separated the tumour from the normal organoid models, confirming the paired nature of the samples; dimension 2 corresponded to different passage number. Replicate samples from both tumour and normal organoids and at the same passage number, clustered together (Spearman's ρ correlation coefficients >0.92), while the samples 'Late passage' and 'Freeze/Thaw' were more similar in their expression profiles with respect to 'Early passage' (***Figure 10***). This suggested that overall gene expression was subjected to alteration during prolonged periods of cultivation and freeze/thaw circles. The analysis revealed the number of significantly Differentially Expressed up-regulated and down-regulated Genes (DEGs) at each time points (***Table 16***). Addressing colon cancer-related gene expression pathways we found that a common set of pathways including the 'Wnt signalling pathway' and 'Signaling pathways regulating pluripotency of stem cells', were altered in 'Early, Late and Freeze/Thaw' samples when compared to corresponding colon normal-tissue organoids. In order to investigate any transcriptomics adaptation through time, the expression comparison between the different time points was performed. A total number of 1,101 up-regulated and 1,326 down-regulated genes were observed when comparing 'Late passage' vs. 'Early passage' and 1254 and 935 in the 'Freeze/Thaw' vs. 'Late passage' (***Table 16***, ***Figure 11***). The functional enrichment analysis of DEGs especially unveiled pathways biologically relevant to colorectal cancer progression, including 'PI3K-Akt signalling pathway' and 'TNF signalling pathway', in the comparison between 'Late passage' and 'Early passage' (FDR≤5%, ***Table 17***).

Altogether, these results suggest that long-term passaging/freezing can lead to alterations in gene expression that result in the perturbation of biological pathways. Whole exome sequencing will provide information whether this is due to cell intrinsic effects or selection of subpopulations during long-term cultivation.

In order to translate patients' specific gene expression profiles to novel therapeutic approaches, computational screening of drugs was carried out by the connectivity method CMap (Connectivity Map)15 to identify compounds that could reverse the query cancer phenotype. The top 100 up- and down-regulated genes in tumor organoids at 'Early passage' (more similar in their expression profiles to primary tumor) were used as query signature for the CMap analysis. The performed analysis revealed a panel of candidate therapeutic agents for small scale drug-testing, including RAF kinase inhibitors, EGFR inhibitors and MAP kinase inhibitors (***Table 18***).

Patient12 (Adeno-carcinoma-MSS stable)	**Early Passage (3 weeks cultivation)**	**Late passage (13-14 weeks cultivation)**	**Freeze /Thaw**
Normal colon tissue organoids	Early passage (passage 3)	Late passage (passage 12)	Freeze at p3 + 7 weeks cultivation
Colorectal tumor-tissue organoids	Early passage (passage 3)	Late passage (passage 12)	Freeze at p3 + 7 weeks cultivation

Table 15: RNA seq analysis performed on organoids generated from colorectal patient 12 at different passage number and after freeze-thaw cycle. MSS, microsatellite stable. (Project partner UniUD).

Figure 9: Pipeline for RNA-seq analysis of organoids generated from colorectal cancer patients.

Figure 10: Multi-dimensional scaling (MDS) plot of RNA-seq libraries from Tumor (-TO) and Normal (-NO) Organoids at each time point (EARLY, LATE and FREEZE/THAW). Capital letters indicates the sample replicates. 'LATE': late passage organoids; 'EARLY', early passage organoids; 'FREEZE/THAW', organoids after freeze-thaw cycle.

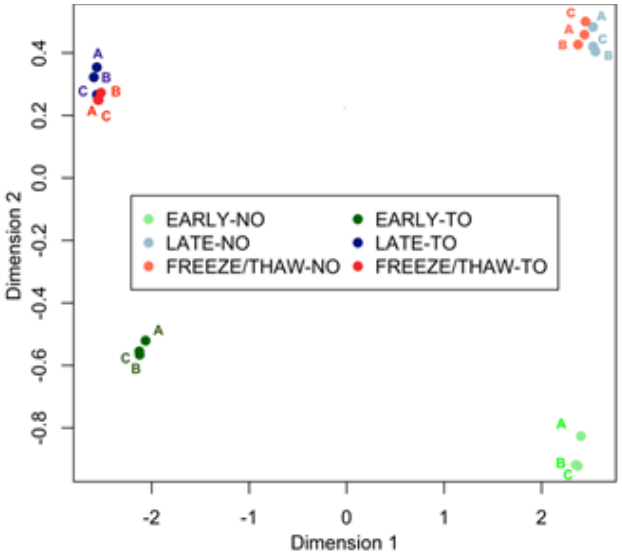
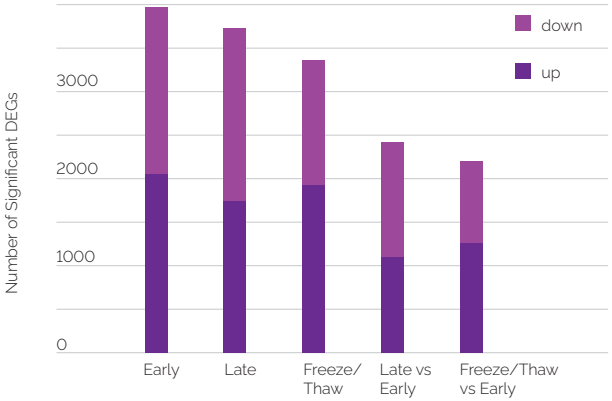


Figure 11: The number of the significant up- and down-regulated genes in tumor vs paired normal-organoids at and between ('LATE vs. EARLY' and 'FREEZE/THAW vs. EARLY') the different time points (fold-change≥2, adjusted FDR≤0.01). 'LATE': late passage organoids; 'EARLY', early passage organoids; 'FREEZE/THAW', organoids after freeze-thaw cycle.



Time point	Up-regulated	Down-regulated
EARLY	2022	1955
LATE	1737	2000
FREEZE/THAW	1909	1443
LATE vs. EARLY	1101	1326
FREEZE/THAW vs. EARLY	1254	935

Table 16: The number of the significant up- and down-regulated genes in tumor organoids versus the normal tissue organoids, at and between each time point (fold-change≥2, adjusted FDR≤0.01). 'LATE'; late passage organoids; 'EARLY', early passage organoids; 'FREEZE/THAW', organoids after freeze-thaw cycle.

Table 17: List of significantly enriched KEGG pathways of the most significantly up- and down-regulated genes when comparing the gene expression of the LATE and FREEZE/THAW time points vs the EARLY ones. The KEGG identifier (ID) and description (Description), the number of genes in the analyzed dataset associated with a pathway (Count) and the FDR value are reported for each pathway. 'LATE'; late passage organoids; 'EARLY', early passage organoids; 'FREEZE/THAW', organoids after freeze-thaw cycle.

Comparison	Expression	ID	Description	Count	FDR
LATE vs. EARLY	up-regulated	hsa04510	Focal adhesion	33	0.00
		hsa05205	Proteoglycans in cancer	30	0.01
		hsa04360	Axon guidance	22	0.03
		hsa04151	PI3K-Akt signaling pathway	38	0.68
		hsa04668	TNF signaling pathway	17	0.95
		hsa05200	Pathways in cancer	41	1.16
		hsa05132	Salmonella infection	14	1.89
		hsa04512	ECM-receptor interaction	14	2.91
	down-regulated	hsa05162	Measles	20	0.35
		hsa04068	FoxO signaling pathway	18	2.74
FREEZE/THAW vs. EARLY	up-regulated	hsa04360	Axon guidance	24	0.01
		hsa01230	Biosynthesis of amino acids	17	0.02
		hsa05205	Proteoglycans in cancer	29	0.23
		hsa04510	Focal adhesion	29	0.37
		hsa05219	Bladder cancer	11	0.40
		hsa05200	Pathways in cancer	45	0.73
		hsa05166	HTLV-I infection	32	1.18
		hsa04320	Dorso-ventral axis formation	8	2.19
	down-regulated	hsa03030	DNA replication	13	0.00
		hsa04110	Cell cycle	23	0.00

Table 18: List of the perturbagens reversing (negative score) the expression of the tumor-organoids at passage 1 by CMap method. The score value, name, description and target genes are reported for each compound.

Score	Name	Description	Target
-99.4	vemurafenib	RAF inhibitor	BRAF, CYP2C19, CYP3A4, CYP3A5, RAF1
-97.43	PD-198306	MAP kinase inhibitor	MAP2K1, MAP2K2, MAPK1, MAPK3
-96.18	brefeldin-a	Protein synthesis inhibitor	ARF1, ARFGEF1, ARFGEF2, CYTH2, GBF1, SAR1A
-96.05	SA-792709	Retinoid receptor agonist	RARA, RARB
-95.99	tyrphostin-AG-1478	EGFR inhibitor	EGFR, MAPK14
-94.82	TPCA-1	IKK inhibitor	IKBKB
-94.76	GSK-3-inhibitor-II	PKC inhibitor	GSK3B

9.2 Whole exome sequencing of organoid cultures

Whole Exome Sequencing (WES) was performed on normal-tissue, primary tumor-tissue and organoids to identify tumor-specific somatic mutations in protein encoding regions. This allows validating the genetic descendance of tumor-organoids from the primary tumor-tissue and identifying genetic alterations that confer tumor vulnerabilities and dependencies that may pave the way to the novel therapy design. Finally, WES may unveil possible selection of tumor-organoid sub-populations defined by genetic variants that accumulate during long-term passaging or cultivation in different cultivation media. Genomic DNA was extracted from the organoids and paraffin embedded normal- and tumor-tissues and then subjected to the construction of paired 2×150bp libraries (SureSelect Human All Exon V6 kit), and to WES on an Illumina NovaSeq platform. A custom pipeline was applied to analyse the obtained data, catalogue tumor-specific somatic mutations and identify those with clinical relevance (***Figure 12***; see also SOP collection www.precanmed.eu). Specifically, high-quality reads were aligned to the GRCh37 human reference genome after adapters and PCR duplicate removal. The germline and tumor-specific somatic mutations were identified by comparison of the tumor *versus* the normal samples (tissues and organoid cultures). The somatic non-synonymous mutations identified in the tumor samples, were prioritised when showing a variant allele frequency (VAF)>0.1 and potentially deleterious impact on the protein function, as predicted by the *in silico* pathogenicity prediction tools. Polymorphic variants with an AF>0.01 in ExAC[16] were filtered out.

[16] Lek, M. et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature (2016). doi:10.1038/nature19057

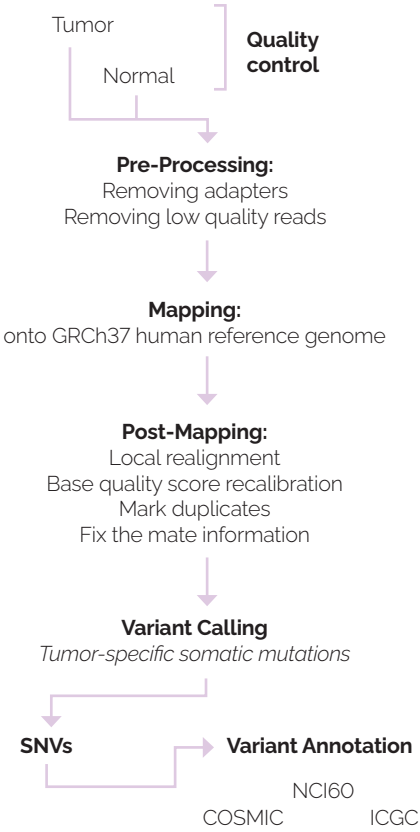


Figure 12: Pipeline for WES analysis of organoids generated from cancer patients.

COSMIC= Catalogue Of Somatic Domatic Mutations In Cancer
NCI60= national Cancer Institute (NCI) 60 human tumour cell
ICGC= International Cancer Genome Consortium version 21

DNA from normal- and tumor-tissues and normal- and tumor-organoids at early and late passages, are subjected to WES sequencing (more details in ***Tables 19 -22***).

9.2.1 Lung, Breast and Colorectal cancer (UniUD, UniTS, LNCIB)

The goal of the analysis is to identify tumor-specific somatic mutations in primary tumors and validate the presence and stability of these sequence variants over time in the organoids (***Tables 19-22***). In a subsequent step patient-specific genomic data will be used to identify tumor vulnerabilities and dependences that will be tested by in vitro drug testing on tumor-organoids (see also 10. Drug testing on tumor-organoids).

9.2.2 Colorectal cancer (ADSI, MUI)

To extend information on the stability of colorectal tumor-organoids cultures in multiple media approaches, WES on normal-tissue, primary tumor-tissue and tumor-organoids will be performed to address an eventual selection of tumor-organoid subpopulations with distinct genetic makeup (***Table 22***).

Patient	Normal-tissue	Primary tumor-tissue	Tumor-organoids
Patient 14 – Luminal A breast cancer	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	Passage p5; 6 weeks of cultivation
Patient 23 – Luminal B breast cancer	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	Passage p8; 10 weeks of cultivation
Patient 29 - TNBC	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	Passage p5; 5 weeks of cultivation
Patient 31 - TNBC	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	Passage p6; 4 weeks of cultivation
Patient 33 - Her2 breast cancer	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	Passage p4; 4 weeks of cultivation

Table 19: Breast cancer samples analysed by WES (Project Partner UniTS).

Patient	Normal-tissue	Primary tumor-tissue	Tumor-organoids (early passage)	Tumor-organoids (late passage)
Patient 5 – Lung Adenocarcinoma	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	Passage p2; 3 weeks of cultivation	Passage p3; 5 weeks of cultivation
Patient 9 – Lung Adenocarcinoma	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	Passage p2; 2 weeks of cultivation	Passage p5; 6 weeks of cultivation
Patient 19 – Lung Adenocarcinoma	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	N.A.	Passage p6; 8 weeks of cultivation
Patient 15 – Mucinous Adenocarcinoma	Tissue sections of paraffin blocks	Tissue sections of paraffin blocks	Passage p3; 7 weeks of cultivation	Passage p5; 11 weeks of cultivation

Table 20: Lung cancer samples analysed by WES (Project partner LNCIB); N.A.= not analysed.

Patient	Normal-tissue	Normal-tissue organoids	Tumor-tissue	Tumor-tissue organoids (WRNAS medium with Wnt3a)	Tumor-tissue organoids RNAS medium; no Wnt3a)
1 – patient 12; Adenocarcinoma, microsatellite stable	Sections of paraffin blocks	early passage (p4; 6 weeks cultivation) and late passage (p13-13 weeks cultivation)	Sections of paraffin blocks	early passage (p4; 8 weeks cultivation)	early passage (p4; 8 weeks cultivation) and late passage (p12; 12 weeks cultivation)
2 – patient 14; Adenocarcinoma, microsatellite stable	Sections of paraffin blocks	early passage (p5-7 weeks cultivation)	Sections of paraffin blocks	early passage (p5; 9 weeks cultivation) and late passage (p12; 16 weeks cultivation)	early passage (p5; 9 weeks cultivation) and late passage (p12; 13 cultivation)

Table 21: Colorectal cancer samples analysed by WES (Project Partner UniUD).

Patient	Normal -tissue	Normal-tissue organoids	Tumor-tissue	Tumor-tissue organoids (Medium 4)	Tumor-tissue organoids (Medium 5)	Tumor-tissue organoids (Medium 6)
TPIO-19; Adenocarcinomamicrosatellite unstable	Sections of paraffin blocks	late passage p11/p3	Sections of paraffin blocks	early passage p4/p3/p5 and late passage p5/p10/p3	N.A.	early passage p8/p3 and late passage p13/p4
TPIO-18 – patient 14; Adenocarcinomamicrosatellite stable	Sections of paraffin blocks	early passage p2/p3	Sections of paraffin blocks	late passage p9/p4/p2	N.A.	early passage p2/p3 und late passage p2/p7/p3
TPIO-23 – patient 14; Adenocarcinoma, microsatellite stable	Sections of paraffin blocks	N.A.	Sections of paraffin blocks	N.A.	N.A.	early passage p4

Table 22: Colorectal cancer samples analysed by WES (Project Partner ADSI/MUI). N.A.= not analysed.

10.
Drug testing on tumor-organoids

Tumor organoids represent powerful future model-systems to test the efficiency of classic therapeutic treatments and evaluate alternative therapeutic strategies, prior to the start of patient therapy. In addition, organoids can act as advanced preclinical cancer models to test candidate drugs resulting from research based on low-complexity monoclonal cancer cell lines.

The proliferative potential of human tumor-organoids varies among patients turning the availability of cell material for drug testing into a critical issue. This limitation could be compensated by the use of assays that allow a fast and precise readout of cell viability using a low number of experimental cells. Therefore, time-course assaying of metabolic activity of tumor-organoids allowed a reliable read-out for the response of organoids to therapeutic treatments in vitro. Normal- and tumor-tissue organoids generated from a colorectal cancer patient were treated with inhibitors of cyclin dependent kinases (Dinaciclib and an

additional kinase inhibitor, under development(iCDKu)). Dinaciclib is a pyrazolo[1,5-a]pyrimidine with potential antineoplastic activity that inhibits CDK1, CDK2, CDK5, and CDK9; inhibition of CDK1 and CDK2 may result in cell cycle repression and tumor cell apoptosis[17]. Measurement of metabolic activity after 1, 24, 48 and 72 hours of treatment revealed reduced cell metabolic activity of tumor-organoids. Remarkably, metabolic activity of normal-tissue organoids remained unchanged indicating a tumor cell specific effect triggered by both kinase inhibitors (***Figure 13***). To confirm the reliability of the organoid culture as a preclinical cancer model system, the efficiency of Docetaxel, one of the components of the cancer therapy regimen for triple negative breast cancer patients, was evaluated *in vitro* using triple negative breast tumor-organoids. Treatment with increasing concentrations of Docetaxel, efficiently reduced metabolic activity of tumor-organoids (***Figure 14***). This confirms the effectiveness of real-time measurement of metabolic activity as fast and easy readout to determine the efficiency of therapeutic strategies. Future experiments will test panels of drugs revealed by computational drug screening on genomics data from patient-derived organoids.

[17] Kumar, S. K. et al. Dinaciclib, a novel CDK inhibitor, demonstrates encouraging single-agent activity in patients with relapsed multiple myeloma. Blood (2015). doi:10.1182/blood-2014-05-573741

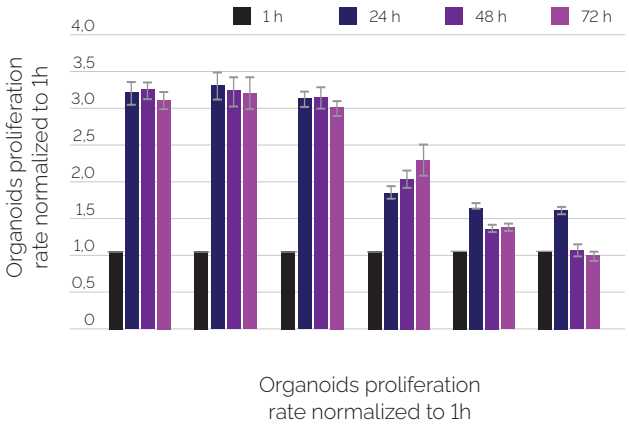


Figure 13: Real-time metabolic activity assay to determine viability of normal-tissue and tumor-tissue organoids upon pharmacological treatment with the cyclin dependent kinase (CDK) inhibitors (Dinaciclib and a CDK inhibitor currently under development, iCDKu) at the indicated final concentrations. CDK inhibitors specifically reduce metabolic activity of colon tumor but not normal-tissue organoids. Project Partner ADSU/MUI)

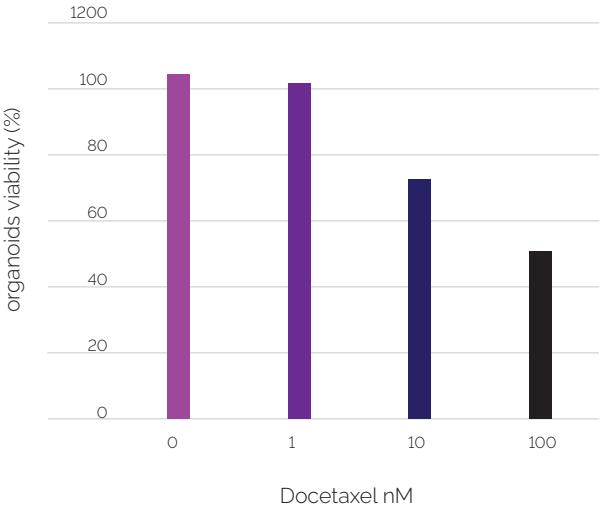


Figure 14: Real-time metabolic activity assay determining viability of tumor-organoids obtained from tumor-tissue of a patient diagnosed with TNBC. Treatment with Docetaxel, was validated on tumor-organoids. 100 nM Docetaxel reduced organoids viability to 50%. (Project Partner UniTS)

11.
PreCanMed Database

The management and interpretation of data from organoids and omics technologies, produced and collected in this project, is highly relevant to support investigators working on precision oncology. To this aim, a platform to catalogue and share the curated multi-omics, biobank and clinical annotations was implemented. The 'Informatics for integrating biology and the bedside' (i2b2)[18,19] an NIH-funded open-source platform used at more than 200 sites worldwide, was the chosen software for hosting multi-sourced data. It supports investigators in data querying and privacy-preserving sharing. The platform specifically comprises i) a database storing data from multiple experimental platforms, i.e. immunohistochemistry data (IHC), clinically relevant mutations (by WES), ii) the in silico drug sensitivity data associated with expression profiles (by RNA-seq) involved in the cancer-relevant pathways, and iii) the results from the drug testing assays on organoids. The i2b2 ontology, namely the hierar-

[18] Murphy, S. N. et al. Serving the enterprise and beyond with informatics for integrating biology and the bedside (i2b2). J. Am. Med. Informatics Assoc. (2010). doi:10.1136/jamia.2009.000893

[19] Murphy, S. et al. Architecture of the open-source clinical research chart from Informatics for Integrating Biology and the Bedside. Symp. A Q. J. Mod. Foreign Lit. (2007).

chical vocabularies describing the multi-level data, i.e. patient, biotype (tissue, organoid), omics and drug levels, is customized to organoids models, with the aim to provide the query terms. 'Biobank', 'Drug screening' ('Virtual screening', 'Drug response'), 'WES data' and 'transcriptomics', are examples of terms adapted to an organoid-centric translational database. The translational platform implemented with this project allows the comprehensive summary of the relevant clinical and omics information and represents an accessible tool for data sharing among hospitals and research institutions, preserving their confidential nature.

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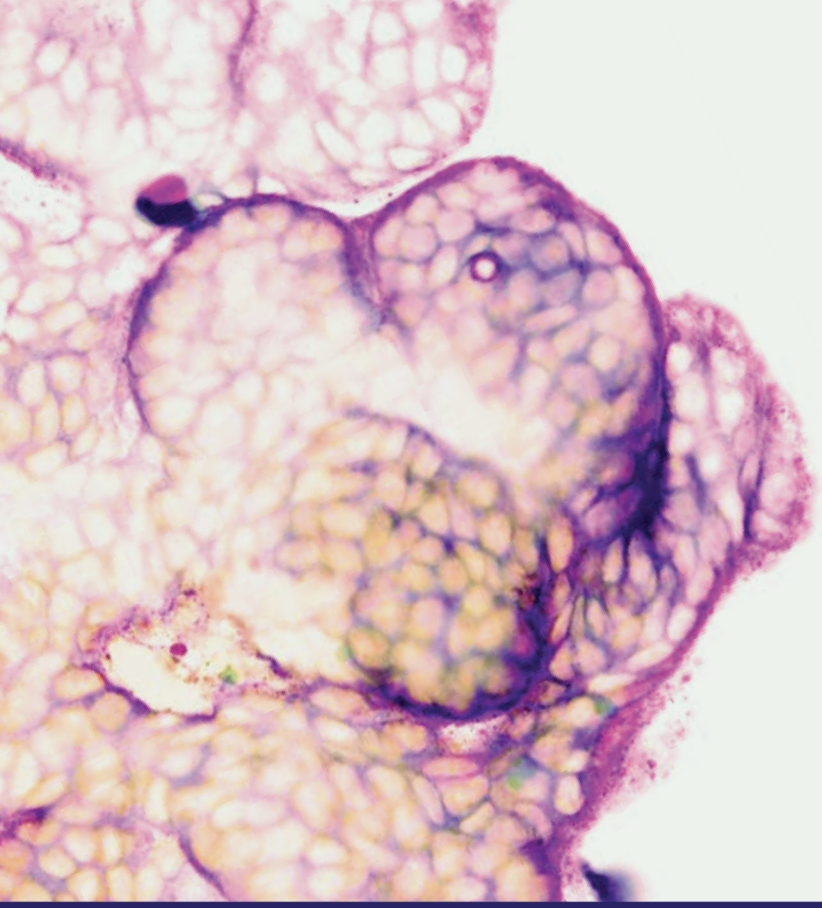

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