Italian Institute for Genomic Medicine

Scientific Report

IIGM

2022-2023

Introduction

The **Italian Institute for Genomic Medicine (IIGM)** (formerly known as Human Genetics Foundation - Torino) is an operating body of the Compagnia di San Paolo since 2007.

The IIGM is a research center of excellence and training in human genomics, epigenomics, and immunology, and carries out its activities through a model of efficiency and transparency, aiming at maximizing resources for research activities, training, and high-level education.

The Institute shares scientific partnerships and collaborations with University of Turin (UniTo), Polytechnic of Turin (PoliTo) and University of Milan.

In December 2018 IIGM and the Piedmont Foundation for Oncology (FPO) – IRCCS created a joint research platform in the FPO-IRCCS building in Candiolo (To), where IIGM laboratories moved in July 2019.

IIGM has organized the new laboratories in accordance with new operational procedures and structural methods: the spaces on have been organized according to the criterion of "functionality and sharing", so to create more opportunities for interaction between research teams, favoring the sharing of scientific notions and the spring of new ideas and projects.

IIGM's research projects are financed by the Compagnia di San Paolo as well as external funders (i.e. AIRC, Ministry of Health, European Community), and address cancer genomics and bioinformatics, epigenetic modifications related to malignant diseases, genomics instability and tumor immunity, immune-regulation, genomic epidemiology, quantitative and computational biology, immunotherapy. A total of 8 groups are currently hosted at IIGM:

- Salvatore Oliviero (also at UniTo)
- Andrea Pagnani (also at PoliTo)
- Mattea Cereda (also at University of Milan)
- Alessio Gordon Naccarati
- Luigia Pace

- Ilio Vitale
- Tobias Haas (also at University Cattolica of Rome)
- Carlo Campa

IIGM has several scientific collaborations aimed at fostering the basic, translational and clinical research activities, through the activation of joint research projects involving both clinical and laboratory activities.

Operating structure

The Foundation carries out its activities through a streamlined but effective management structure, and through close collaboration with the consortium PR.I.S.MA. and the Compagnia di San Paolo itself.

This model of efficiency and transparency of management allows to maximize the resources for the core activities of research and service to the scientific community.

The ***Board of Directors*** consists of five members, including the ***President***, who is the legal representative of the Foundation, with signing rights and being invested with all the powers for both ordinary and extraordinary administration of the Foundation.

The ***Scientific Director*** manages the ordinary administration delegated to him by the Board of Directors, and collaborates with the President coordinating the institutional activity according to the indications and deliberations of the Board itself.

The ***operating office*** coordinates and optimizes the planning and operational activities of the Foundation in order to make all internal activities effective, performing and functional for the general management of the institute itself.

The Foundation has a ***Control Body***, which exercises the powers and functions provided as stated in art. 2403 *et seq*. of the Civil Code, and a ***Supervisory Body***, which oversees the correct application of the principles provided for by the "Model of organization,

management and control" pursuant to Legislative Decree 231, and has entrusted the task of voluntary auditing of the financial statements to Ernst & Young for the three-year period 2019-2020-2021.

Dr. Sabrina Bertinetti
General Secretariat

Dr. Simonetta Guarrera, MSc.
Scientific Secr. & Lab. Managing

The IIGM pursues a policy of state-of the-art research providing its researchers with innovative and up-to-date instrumentation.

- The IIGM's **Metabolic Facility** hosts the Seahorse XF Analyzer, an *in vivo* metabolic analyzer which allows to study key cellular functions such as mitochondrial respiration and glycolysis. The instrument is operating under the supervision of Dr. B. Pardini, and is available under a "all-inclusive service" or a "collaborative" regimen;
- The **Flow Cytometry and Cell Sorting Facility**, run by Dr. D. Baev, is offering services to researchers from the IIGM, the Candiolo Cancer Institute FPO-IRCCS, the Academia, and other research institutes;
- The **Genomic Facility** was established in 2019 to provide access to the most advanced technologies for genomic analysis to researchers from IIGM, FPO-IRCCS Candiolo, University of Turin, and other institutions in Italy and abroad. The facility is operated by dedicated personnel (Dr. K. Gizzi, Dr. C. Parlato) and is available under a "all-inclusive service" or a "collaborative" regimen.

IIGM operational structure

IIGM Principal Investigators

Cancer Genomics and Bioinformatics Unit

Prof. Matteo CEREDA, PhD

International experiences:
King's College London, London, UK
IEO, Istituto Europeo di Oncologia, Milan, IT
MRC Laboratory of Molecular Biology, Cambridge, UK

Immunotherapy Unit

Dr. Tobias L. HAAS, PhD

Assistant Professor, Catholic University of the Sacred Heart, Rome

International experiences:
Istituto Oncologico del Mediterraneo, Fondazione IOM Ricerca, Catania, IT
Istituto Superiore di Sanità – ISS, Roma, IT
German Cancer Research Center (DKFZ), Heidelberg, Germany

Genetic and Molecular Epidemiology Unit

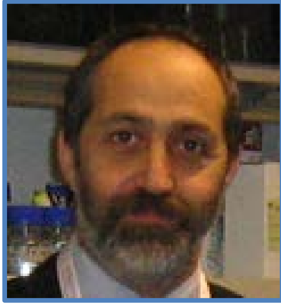
Dr. Alessio Gordon NACCARATI, PhD

International experiences:
Institute of Experimental Medicine (IEM), Czech Academy of Sciences, Prague, Czech Republic

Immuno-Regulation Unit

Dr. Luigia PACE, PhD

International experiences:
Curie Institut, Paris, France
HELMHOLTZ Centre for infection research, Hannover, Germany



Epigenomics Unit

Prof. Salvatore OLIVIERO, PhD

Full Professor in Molecular Biology, University of Turin

International experiences:
Albert Einstein College of Medicine, The Bronx, NY, USA
Harvard Medical School, Boston, USA
EMBL, Heidelberg, Germany

Statistical Inference and Computational Biology Unit

Prof. Andrea PAGNANI, PhD

Full Professor (L.240), Polytechnic University of Turin

International experiences:
University of Paris-Sud ORSAY, Paris, France.

Genomic Instability and Tumor Immunity Unit

Dr. Ilio VITALE, PhD

International experiences:
Gustave Roussy Cancer Campus (INSERM U848), Villejuif, France

Chemical Biology Unit

Dr. Carlo C. CAMPA, PhD

International experiences:
Department of Biosystems Science and Engineering, ETH Zurich, Basel, Switzerland

FACILITIES

Flow Cytometry and Cell Sorting Facility

Dr. Denis BAEV, PhD.

The mission of the IIGM's Flow cytometry and cells sorting Facility is to support the research projects by providing to IIGM, FPO-IRCCS researchers the access to modern multiparametric methods of rapid cell analysis and single cell manipulations.

The facility, run by Dr. D. Baev, was established in 2017 as a shared resource facility for the IIGM (former HuGeF) and UniTo MBC users. Originally the facility had one flow cytometer (BD FACS Canto, 2 laser- 6 FL channels) and the cells sorter (BD Aria III, 2 lasers, 6 FL channels). In 2018, thanks from the generous support from the Compagnia di San Paolo, the IIGM FCCSF acquired 2 analyzers, the BD FACS Celesta (3 lasers, 12 FL channels) and BD LSR Fortessa X20 (3 lasers, 14 FL channels, HTS option) and performed the complete upgrade of the existing Aria III sorter to 3 laser, 14 FL channel configurations, similar to the original optical layout of the X20 instrument.

In 2020 the BD Fortessa X20 instrument was upgraded to 4 laser 18 color configuration and the Beckman Coulter Cytoflex (2 lasers, 6FL channels, 96 well plate option) was adopted as an instrument for the entry-level analyses.

Currently, the IIGM FCCSF provides with the full spectra of flow cytometry and cell sorting services for the IIGM, FPO-IRCCS Candiolo and external customers.

IIGM's Genomic Platform

The IIGM's Genomic Platform was established in 2019 with the goal to provide IIGM researchers, the scientific communities of the FPO-IRCCS Candiolo Cancer Institute, of the University of Turin, and of other institutions in Italy and abroad, with the access to the most advanced technologies for genomic analysis, supporting researchers in their projects.
All instruments were acquired thanks to the contribution of Compagnia di San Paolo.

Genomic Facility: sequencing and microarray analyses

Dr. K. Gizzi, BSc.

Dr. C. Parlato, BSc.

Dr. S. Guarrera, MSc.

The activities of the Genomic Platform include consulting and analyses planning with the users, and laboratory analyses, and are offered as all-inclusive services or as scientific collaboration. The Facility is run by Dr. K. Gizzi and Dr. C. Parlato with the support of Dr. S. Guarrera, and is well equipped with a state-of-the-art NovaSeq6000 massive parallel sequencer (Illumina Inc) which allows a modular and scalable approach to sequencing with the availability of several sequencing formats, providing both the necessary high throughput processing for big-scale sequencing projects, as well as the flexibility to accommodate small and medium size projects at relatively moderate costs. A GridION (Oxford Nanopore Technologies) sequencer for proofreading long-reads sequencing is also available.
Being equipped with a high resolution iScan microarray scanner (Illumina), and with three automatic liquid handlers for the preparation of samples and microarrays, the Genomic Facility provides whole-genome genotyping analyses (microarray genotyping assays) and methylation analyses services (microarray methylation analysis) as well.

Bioinformatic support

Dr. M. Cereda, PhD.

The Genomic Facility is supported by the IIGM's Bioinformatics section, coordinated by Dr. M. Cereda, which provides support for primary data processing and data storage and management until delivery to the user.

Dr. B. Pardini, PhD (Lab managing).

Dr. S. Guarrera, MSc.

The Metabolic Facility of IIGM was set up with the acquisition of a Seahorse XF Analyzer (Agilent) thanks to the kind support of Compagnia di San Paolo.

The facility is supervised by Dr. B. Pardini with the support of Dr. S. Guarrera, and can be accessed in terms of:

- Scientific collaboration regimen: the facility is open to experienced researchers who wish to set up analyses by themselves, upon agreements with IIGM's facility personnel
- Service regimen: for non-experienced users who wish to take advantage of an all-inclusive service.

Cancer Genomics and Bioinformatics Unit

Unit research members:

- Matteo Cereda, principal investigator
- Marco Del Giudice, post-doctoral fellow (till 30/04/2022)
- Serena Peirone, post-doctoral fellow
- Sarah Perrone, PhD student
- Francesca Priante, PhD student

Brief description of the UNIT

The Cancer Genomics and Bioinformatics (CGB) unit studies molecular mechanisms driving tumorigenesis combining computational and molecular biology approaches. Modern biology is facing new challenges caused by the massive production of omics data. Extracting relevant information from these Big Data while taking into account their intrinsic heterogeneity is a crucial issue. This holds particularly true for complex diseases, such cancer, and in light of an RNA-based biology. To face this challenge, we employ the most recent bioinformatics and experimental approaches to develop novel paradigms that can reveal oncogenic mechansims and options for the clincal managmentes of patients.

Our goal is to unravel RNA processing defeatcs that could be instrumental for developing RNA-based therapeutic options. Our research focuses on (i) RNA processing regulation in cancer, (ii) tumor genetics and clonal evolution, and (iii) omics Big Data science. Eventually, all our interests cross at identifying molecular mechanisms that are favorable clinical intervention points.

In the last four years we consolidated tools and strategies to systematically study genomic and transciptomic alteration in cancer and provide the scientific community with candidate actionable targets. By exploiting thousands of sequencing data, we developed computational approaches that highlighted the role of PTEN in modulating the tumor immune composition as well as its impact on disease-free survival of patients (Lauria et al. 2020). We combined bioinformatics and molecular biology approaches to uncover a new role for pioneer transcription factor FOXA1 as the major director of splicing regulation in prostate cancer (Del Giudice et al. 2022). We demonstrated that this regulation influences patient prognosis revealing splicing defeats that are novel biomarkers of disease recurrence. During this time we also contributed: (*i*) to show that an inherited PMS2 splicing mutation in a patient with “POLE-LYNCH” collision syndrome predispose to the development of multiple neoplasms (Berrino, Filippi, et al. 2022); (*ii*) to improve the the ontology of HER2-low breast carcinomas (Berrino, Annaratone, et al. 2022); and (*iii*) to report d a novel lymphocyte-independent antitumor activity of anti–PD-1 antibodies in non–small cell lung cancer (NSCLC) that is capable of inhibiting chemopersistent PD-1+ NSCLC cells (Rotolo, et al. 2022).

RESEARCH QUESTION(S):

Assessing RNA processing defects to identify novel intervention points in adult and pediatric cancer.

MAIN RUNNING FUNDINGS

- Italian Association of Cancer Research (AIRC). My First AIRC Grant. *Deciphering alternative splicing deregulation in cancer to identify novel therapeutic targets. 2017-2023*
- Italian Ministry of Health. Grant Ricerca Finalizzata Giovani Ricercatori - Theory-Enhancing, Italian Ministry of Health. *Targeting alternative splicing neo-junctions as a novel source of neo-antigens in pediatric and adult tumors. Principal Investigator*. GR-2019-12368827. 2021-2023.

- Italian Ministry of Health. Grant Ricerca Corrente 2021. *Advanced Bioinformatics for precision Oncology. Principal Investigator.* FPRC 5xmille 2018 ADVANCE/A-Bi-C. 2021-2023.
- Fondazione Cariparo. Grant Ricerca pediatrica. *Deconvolution of medulloblastoma resistome through high- resolution tracking of drug-tolerant subclones. Co-principal Investigator*. *2021-2023*.

Main achievements in the period covered by the Scientific Report

Deciphering alternative splicing regulation in prostate cancer

(PI: Matteo Cereda, Team: Marco Del Giudice, Serena Peirone, Francesca Priante)

COLLABORATIONS:

- *Dr Prabhakar Rajan, Barts Cancer Institute, Queen Mary University, London*

MAIN OBJECTIVES OF THE PROJECT

Alternative splicing (AS) is dysregulated in prostate cancer and has been linked to transcriptional programs activated by canonical oncogenic transcription factors. However*,* their relative contribution to alternative splicing regulation is yet to be defined. The main objectives of the projects were (1) to define the relative contribution of four key prostate cancer transcription factors on splicing regulation, (2) to evaluate putative epi-trascriptional regulation of AS; and (3) to identified putative actionable targets.

RESULTS ACHIEVED during the reporting period

By integrating omics data of ≥500 primary and metastatic PC patients and cell lines, we found that the pioneer transcription factor FOXA1 finely shapes the mRNA isoform repertoire of prostate cancer (see Del Giudice et al., 2022). We showed for the first time that FOXA1 has a striking preference in actively binding regulatory regions of splicing factors compared to genes in other fundamental biological processes. By controlling these trans-acting factors, FOXA1 lessens the noise of isoform production toward an optimal dominant mRNA product. This regulation reduces the burden of nonsense mediated decay and influences patient prognosis. We revealed the inclusion of FLNA exon 30 as a novel biomarker of prostate cancer disease recurrence. In this study we showed that FOXA1 affects splicing factor expressions to a greater extent than the non-pioneer TFs (*i.e.* AR, MYC). FOXA1 may open different channels to transmit transcriptional signals to splicing factor loci as exemplified by its common pioneer function for AR- and MYC-driven PC transcriptional programs. We are currently assessing whether FOXA1 directs MYC in modulating the expression of splicing factors in neuroendocrine prostate cancer (NEPC), a lethal subtype of AR-independent prostate cancer. We generated RNA-seq and long-read Oxford Nanopore direct RNA sequencing upon FOXA1 and MYC depletions to map the full-length transcriptome of AR^- PC3 cells (Fig.2A-D). We found that FOXA1 and MYC share AS regulatory

programs (Fig.2E-F). FOXA1 and MYC actively and concomitantly bind regulatory regions of oncogenic splicing factors, tuning the expression of distinct productive isoforms (Fig.2F-H). Using long-reads direct RNA we found that FOXA1- and MYC-mediated regulation of splicing factors also occurs via RNA methylation and polyadenylation. We identified m6A sites that were significantly methylated upon FOXA1 and MYC depletion (Fig.2I). These sites were enriched in splicing factors for both transcription factors (Fig.2J). Using MeRIP sequencing data upon FOXA1 depletion we validated that FOXA1 mediates m6A methylation of splicing factors (Fig.2K-M), including demethylation of the productive isoform of the oncogenic spliceosome factor SF3B1. FOXA1 maintains DNA hypomethylation inducing the expression of TET1. Similarly, we found that FOXA1 controls the expression of distinct m6A regulators, including ALKBH5, by actively binding their enhancer regulatory regions (Fig.2O-Q). Furthermore, to assess whether FOXA1-mediated regulation of isoform expression levels correlated with changes in mRNA stability levels, we measured the stabilization of mRNA isoform levels after transcription inhibition with actinomycin D as proposed previously (Fig.3A-B). Overall, downregulated isoforms at 4 hours were less stable in presence of FOXA1 (Fig.3C).

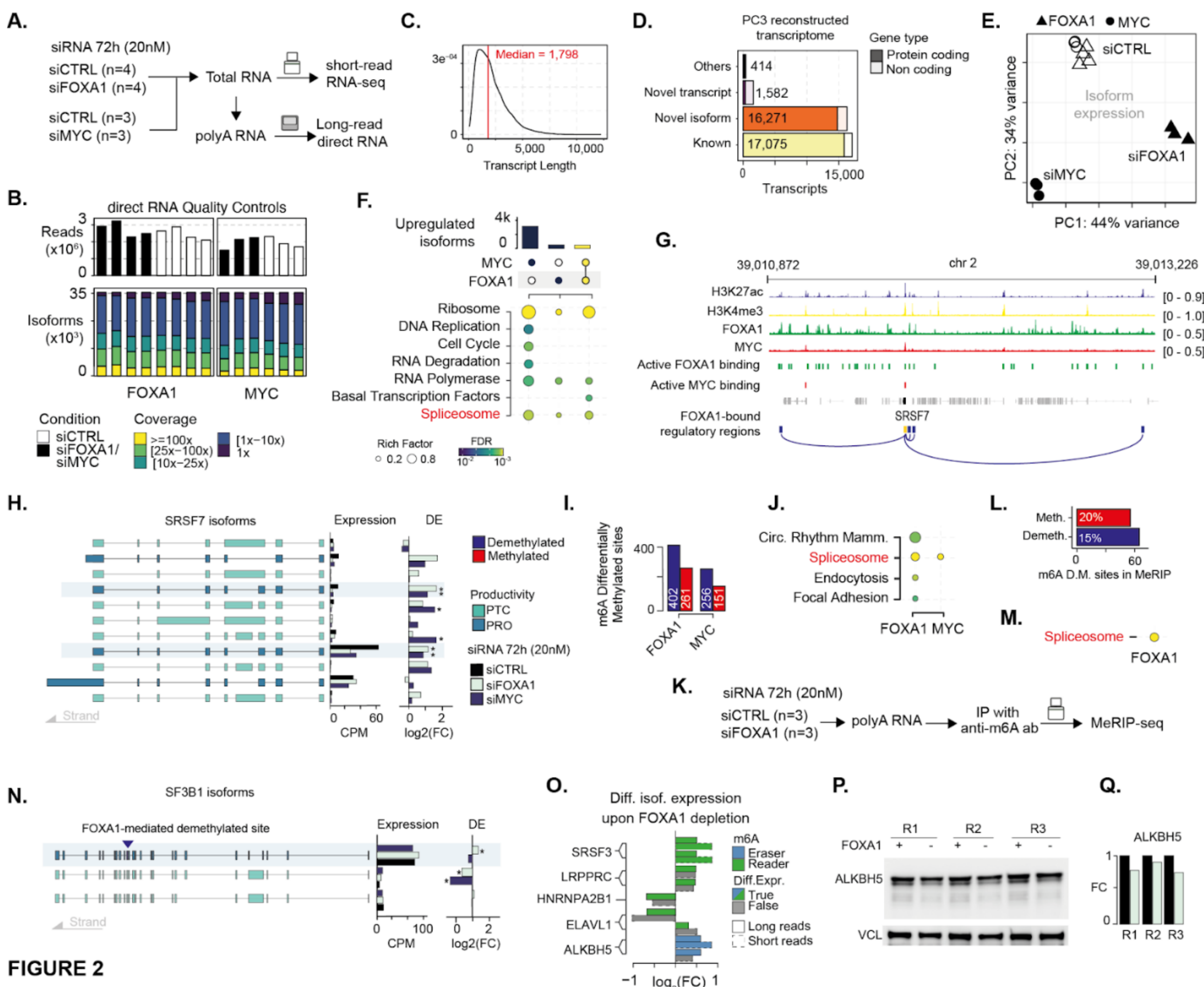
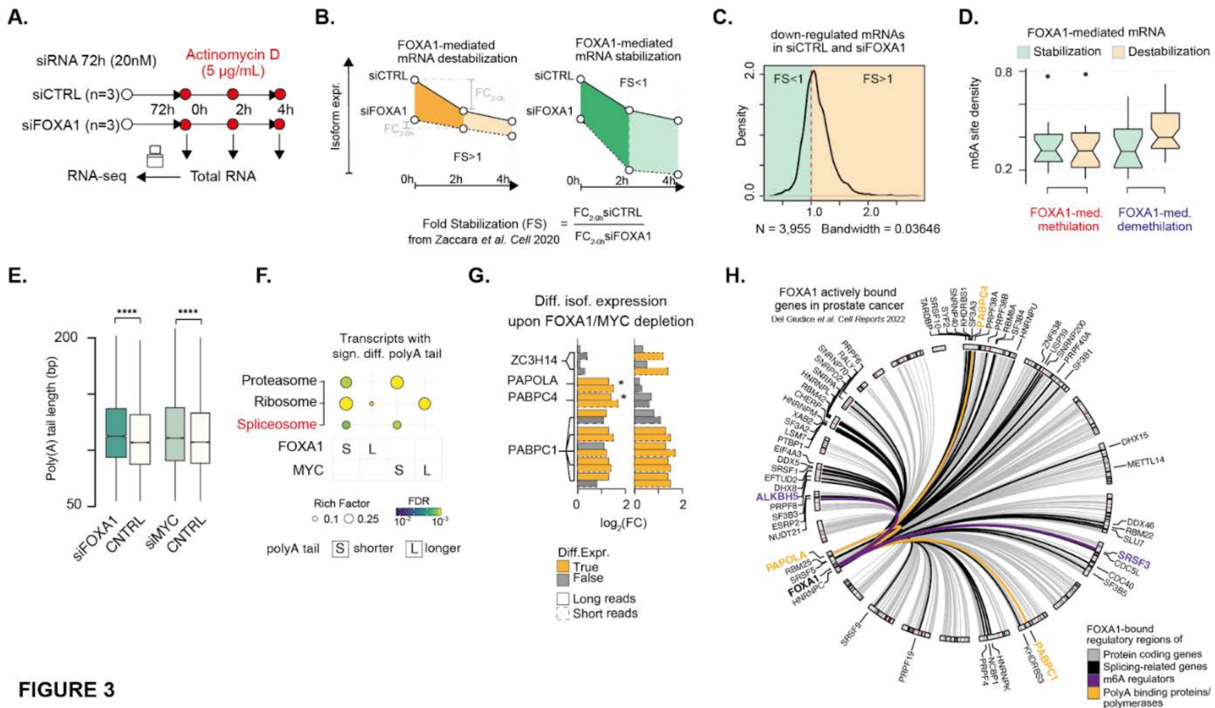


FIGURE 2

Furthermore, the density of significantly demethylated m6A sites by FOXA1 per isoform was higher for FOXA1-mediated destabilized mRNAs (Fig.3D). Thus, we are currently investigating whether FOXA1-mediate m6A demethylation may result in decreased RNA stability levels. Furthermore, alternative polyadenylation

(APA) is affected by transcription factors and chromatin remodeler. Using long-read sequencing data upon FOXA1 and MYC depletions we observed that FOXA1- and MYC-mediated APA resulted in mRNAs with shorter poly(A) tails, which were enriched for splicing factors (Fig.3E-F). FOXA1 and MYC significantly upregulated polyA-binding proteins and polymerase (Fig.3F). We are currently assessing whether these findings could support additional layers (m6A methylation and APA) in transcriptional regulation of splicing factors for FOXA1 (Fig.3H), and MYC, in NEPC.



In collaboration with Prof. Caterina Marchio' and Prof. Sabrina Arena at the IRCCS FPO Candiolo Cancer Institute, we sequenced the transcriptome of 40 fresh-frozen NEPCs to validate our results.

We identified also prognostic and actionable RNA targets in prostate cancers (Del Giudice et al. 2022). One of them featured the progression of pediatric bone sarcomas. We are currently designing SSOs to target *FLNA* exon 30 and *NDRG1* exon 2 and test them in commercially available and patient-derived cell lines.

Molecular characterization of pediatric bone sarcoma transcriptomes

(PI: Matteo Cereda, Team: Serena Peirone, Francesca Priante)

COLLABORATIONS:

- Prof Franca Fagioli, Regina Margherita Children's Hospital, University of Turin

MAIN OBJECTIVES OF THE PROJECT

Pediatric bone sarcomas (BSs) are aggressive diseases with heterogeneous molecular features and few therapeutic targets. Within the national clinical trial SAR_GEN-ITA (NCT04621201) we aimed at characterizing

the transcriptomes of BSs to identify alterations that are shared across tumors, thus could represent putative therapeutic targets.

RESULTS ACHIEVED during the reporting period

As part of the SAR-GEN_ITA clinical trial (ClinicalTrial.gov id:NCT04621201), we have enrolled 28 osteosarcomas (OSs) and 18 Ewing's sarcoma (EWS) pediatric patients. For these patients we generated whole exome and total RNA sequencing data. Genomic profiling confirmed the low mutational rate and revealed high genomic instability of these BS. To expand the number of actionable targets we sought to investigate transcriptomic alterations in the tumors. OSSs and EWSs reveal transcriptomic programs that are shared across samples of the same subtypes. Using data from osteoblast cell lines as control, we found significantly alternatively spliced events (ASEs) in BSs, which were enriched for splicing factors. Amongst other ASEs, we identified the inclusion of *FLNA* exon 30, which we showed to promote PC recurrence (Del Giudice et al. 2022). Using digital droplet PCR on cDNA we validated a higher *FLNA* ex.30 inclusion in BSs compared to controls. Our conventional motifs analysis highlighted splicing factors as candidate AS regulators of BSs. Furthermore, we identified new junctions that could potentially generate targetable neo-epitopes, private to each patient. We are currently assessing these results.

mRNA processing regulation in KRAS-mutated cancers

(PI: Matteo Cereda, Team: Serena Peirone)

COLLABORATIONS:

- Prof C. Ambrogio (Molecular Biotechnology Center (MBC), University of Torino)

MAIN OBJECTIVES OF THE PROJECT

KRAS was considered undruggable until the introduction of $KRAS^{G12C}$ inhibitors (G12Ci) to the clinic. Even if G12Ci demonstrate ~40% response rate in cancer patients, the resistance mechanisms to them are poorly understood. The lack of clonal resistance alterations remains a critical issue for clinical management of patients who progress on G12Ci therapy. We aim at (1) identifying candidate epi-transcriptional regulators and aberrant mRNA isoforms driving clonal resistance in naive or resistant $KRAS^{G12C}$ models, (2) selecting AS-driven adaptive resistance mechanisms that are common to KRAS G12C patients and are suitable for RNA-based therapeutic approaches; and (3) targeting of selected AS-driven resistances with RNA-based therapies in naive and resistant KRAS G12C tumor models.

RESULTS ACHIEVED during the reporting period

In collaboration with Prof. Chiara Ambrogio, we began assessing mRNA processing in KRAS G12C tumor models, either G12Ci treatment naive or resistant, to identify regulators and aberrant mRNA isoforms driving clonal resistance. We used the $KRas^{lox}KRAS^{G12C}$ system generated by the Ambrogio lab. Briefly, we transduced HRas-/-; NRas-/-; KRaslox/lox MEFs with HA-tagged KRAS G12C. Treatment with tamoxifen abolished expression of endogenous wild-type KRAS, thus allowing characterization of a loss of heterozygosity (LOH) phenotype. We then sequenced the transcriptome of KRAS G12C tumor cells, both naive and resistant to G12Ci. Naive and G12Ci-resistant cells showed different gene expression and AS patterns as a function of mutant KRAS allelic frequency, with samples expressing exclusively mutant KRAS G12C having a greater number of aberrant mRNA isoforms triggering nonsense-mediated decay. Amongst other differentially expressed cancer genes in KRAS LOH resistant cells, we found two RNA binding proteins – SF3B1 and BARD1 – for which inhibitors are already approved by the FDA or under clinical trial investigation. Our convention analysis of cis- and trans- acting regulatory elements (Del Giudice et al. 2022) at ASEs revealed the recruitment of different splicing factors across conditions, corroborating a heterogeneous rewiring of transcriptional regulation. We are currently assessing these findings to provide the community with a harmonized dataset of AS events that comprehensively characterize resistance to G12Ci therapy and compounds/SSOs that can revert defective AS.

Development of new computational approaches for omics Big Data analyses

(PI: Matteo Cereda, Team: Marco Del Giudice, Serena Peirone, Sarah Perrone)

MAIN OBJECTIVES OF THE PROJECT

- Provide the scientific community with novel computational methods designed to interpret the complexity of omics Big Data in cancer

RESULTS ACHIEVED during the reporting period

We used available transcriptomic data to develop new computational tools to account for inter-sample tumor heterogeneity and identify the altered biological processes under the phenotype of interest (Lauria et al. 2020; Del Giudice et al. 2021, 2022). We introduced the paradigm shift of “*less is more*” when analyzing large heterogeneous RNA-seq data increasing the accuracy of detecting real defective cellular mechanisms as compared to conventional approaches. We showed that our Gene Set Enrichment Class Analysis (GSECA) algorithm outperformed 'state-of-art' algorithms achieving the highest performance in detecting truly altered biological processes in large heterogeneous cohorts. Appling GSECA to 5,941 samples from 14 different cancer types, we highlighted the role of PTEN in controlling immune-related processes in the majority of cancer type, particularly in those showing a significant alteration of the TIME composition. PTEN loss appears to establish an immunosuppressive TIME through the activation of STAT3 in PC. Furthermore,

low PTEN expression levels have a detrimental impact on disease-free survival in PC patients. With this work we showed that results of our approach can provide means to select clinical conditions that affect patient survival and their management.

Cancer heterogeneity is nowadays explored using single-cell sequencing approaches. However, these data are intrinsically marked by technical noise and sparsity. Therefore, we have adapted our algorithm to handle single-cell RNA-sequencing data, namely scGSECA. Again, our algorithm showed higher performances than other available methods in detecting truly altered biological processes (data not shown). Applied to transcriptomes of ~22.000 cells from cancer and healthy tissues, scGSECA highlighted the proposed bimodal distribution of gene expression profile of a differentiated cell. Interestingly, undifferentiated cells showed unimodal distributions of expression profiles. We are currently assessing whether the type of expression level distribution could be instrumental for the identification of cancer stem cells and guide the reconstruction of tumor evolution.

AWARDS, PATENTS, DISSEMINATION WITH MEDIA

- Member of the Molecular Tumor Board of Piedmont and Valle d'Aosta Italian regions.

NEWFUNDED PROJECT(S)

- Italian Ministry of of Education, Universities and Research (MIUR). Grant FARE2020 Ricerca in Italia. Settore: ERC-2020-COG*. Understanding early steps of KRas-driven oncogenesis in vivo to identify novel actionable targets for cancer therapy.* 2023-2025

Epigenetics Unit

Unit research members:

- Salvatore Oliviero, principal investigator
- Ivan Molineris, researcher
- Valentina Proserpio, researcher
- Andrea Lauria, postdoctoral fellow
- Isabelle Polignano, postdoctoral fellow
- Hassan Dastsooz, postdoctoral fellow
- Guohua Meng, postdoctoral fellow
- Annalaura Tamburrini, PhD student
- Mirko Giuseppe Scrivano, PhD student
- Claudia Vaccari, PhD student
- Chiara Cicconetti, PhD student
- Francesca Anselmi, technician
- Daniela Donna, technician
- Daniele Forte, postdoctoral fellow
- Yegane Feizi, undergraduate student

Brief description of the UNIT

Our research unit combines molecular, cellular, and global genomic approaches to investigate epigenetic and epitranscriptomic modifications that occur in response to environmental or developmental signals and that are deregulated in tumor transformation. Several experimental tests have shown that the factors that bind DNA to specific sequences in collaboration with non-coding RNA and chromatin-modifying enzymes control epigenetic changes. The study of the interaction between these components is necessary to understand the rules that govern gene expression and cell fate. The work of the Unit is aimed at studying the mechanisms that determine these modifications and deciphering the rules to understand, and eventually influence, cell fate. Projects in progress in the laboratory address the study of epigenetic modifications that determine stem cells fate and those involved in tumor transformation. These experiments allowed us to identify and characterize new molecular markers and cellular targets for anticancer therapies.

MAIN RUNNING FUNDINGS

- AIRC, 2023-2027 Epigenetic modifications in cell transformation.

Main achievements in the period covered by the Scientific Report

Role of DNA methylation in stem cells development

Team: A. Lauria, V. Proserpio, M. Maldotti, F. Anselmi, G. Meng.

COLLABORATIONS:

- Prof. Graziano Martello, University of Padova

MAIN OBJECTIVES OF THE PROJECT

1. Regulation of DNA methylase in stem cells by cellular metabolism
2. The role of DNA methylation in stem cell differentiation
3. Regulatory networks of embryonic stem cells (ESCs) self-renewal

RESULTS ACHIEVED during the reporting period

1. Epiblast and embryonic stem (ESC) cells give rise to all adult cells. The cellular plasticity is associated with genome hypomethylation. After fertilization, the zygotic genome is demethylated to allow the differentiation of embryonic cells into different tissues. In the embryo, the Jak / Stat signaling pathway is active by E2.5 and E3.5, as shown by phosphorylation of Stat3 and transcriptional activation of its targets Socs3 and Tfcp2l1.
Our experiments show that LIF / Stat3 signaling induces genomic hypomethylation via metabolic reconfiguration. Stat3 -/- ESCs showed decreased production of alpha-ketoglutarate from glutamine, resulting in reduced expression of Dnmt3a / Dnmt3b and DNA methylation. In particular, genome methylation is dynamically controlled by modulating the availability of alpha-ketoglutarate by Stat3 in mitochondria. Alpha-ketoglutarate binds metabolism to the epigenome, reducing the expression of Otx2 and its Dnmt3a / Dnmt3b targets. Genetic inactivation of Otx2 or Dnmt3a / Dnmt3b causes genomic hypomethylation even in the absence of active LIF / Stat3. Stat3 -/- ESCs showed increased methylation in control regions and impaired expression of transcripts. Single-cell analysis of Stat3 -/- embryos confirmed the dysregulated expression of the Otx2, Dnmt3a / b genes. Several tumors show Stat3 hyperactivation and abnormal DNA methylation, so the molecular modulus we observe could be exploited under pathological conditions.

2. Proper DNA methylation during the early stages of development is essential to determine cell specification. However, the molecular targets as well as the mechanisms that determine the specificity of the methylation mechanism during differentiation are not fully elucidated. The role of DNA methylases in the differentiation of embryonic stem cells was made by differentiation of embryonic stem cells to neuroectoderm and

mesoendoderm. The de novo DNMTs DNMT3A and DNMT3B are strongly induced in the transition from embryonic stem cells to epiblasts. Their level of expression rises considerably from ESCs to Epiblasts with an increase of DNA methylation. DNMT3B knockout shows a strong impairment of DNA methylation in epiblasts. DNMT3A is mainly expressed in neuroectoderm cells while DNMT3B is mostly expressed in the mesoendoderm. Interestingly, the knockout of DNMT3A or DNMT3B show quite different phenotypes, while DNMT3A KO are impaired in neural development DNMT3B KO do not appear to play a role in this developmental pathway. Most of the alterations are observed in intragenic and intergenic regulatory regions. To elucidate the mechanisms underlying the regulation of DNA methylation we generated homozygous DNMT3A and DNMT3B knockout cell lines from the E14 mouse embryonic stem cell (ESCs) line (**Figure 1**). To study the early stages of development and the impact of gene knockout in vitro, ESC Dnmt3a -/- (3AKO), Dnmt3b - /- (3BKO) and wild type (WT) were differentiated. We found that in vitro at epiblast-like cells stage, corresponding to E 6.5 of the mouse embryo that DNMT3B is highly expressed and responsible for the epigenetic priming of epiblast cells to ensure mesoendoderm specification. We identify DNMT3B dependent regulatory elements on the genome which, in 3BKO, impair the differentiation into meso-endodermal (ME) progenitors and redirect EpiLCs towards the neuro-ectodermal lineages. We also identified the differentially methylated regions on the SOX2 superenhancer region that regulate Sox2 expression. Thus, DNMT3B-dependent methylation at the epiblast stage is essential for the
priming of the meso-endodermal lineages and identified key DNMT3B targets on the genome.

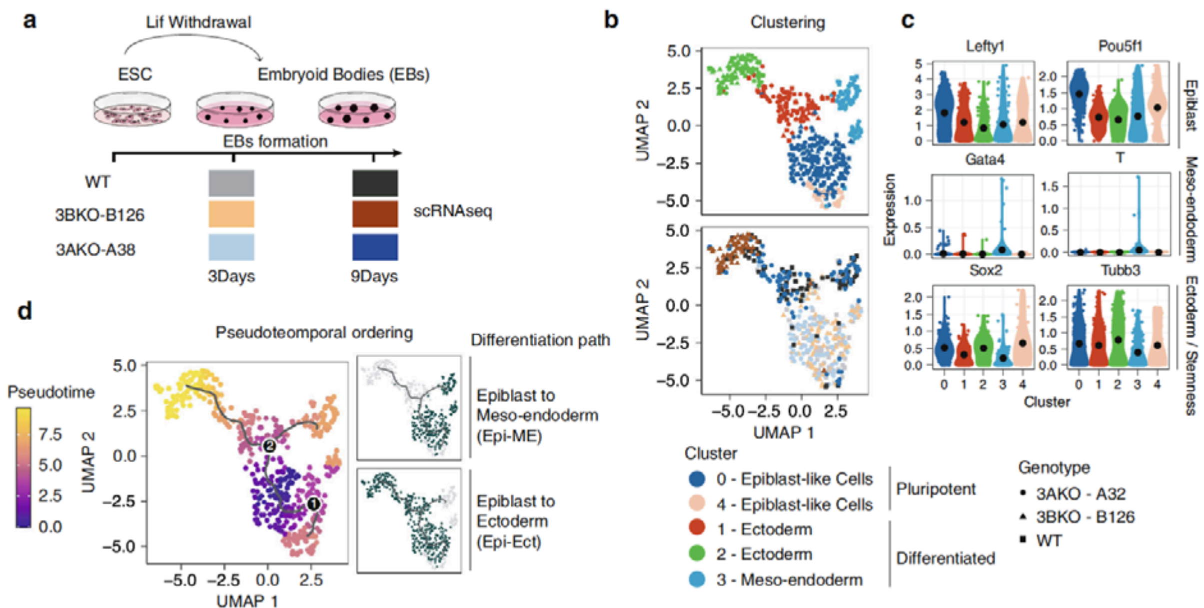


Figure 1. scRNA-seq profiling of Dnmt3a−/− and Dnmt3b−/− differentiating EBs. a Overview of the experimental design and visualisation of collected time points. b UMAP embedding of 487 WT, 3AKO and 3BKO single cell transcriptomes. Cells are coloured by cluster (top panel) and genotype/time of cells' collection (bottom panel). 3AKO = lightblue/darkblue, 3BKO= light orange (3Days)/ dark orange (9Days), WT= light grey (3Days)/dark grey (9Days). c Gene expression levels distribution of representative epiblast

(Lefty1, Pou5f1), meso-endoderm (Gata4, T) and ectoderm markers (Sox2, Tubb3) in the five identified cell clusters.

3. Human embryonic stem cells (hESCs) are kept in culture in the undifferentiated pluripotent state by defined factors. The transforming growth factor-beta1 (TGF-β1) plays an important role in keeping hESCs pluripotency. We addressed, in collaboration with the University of Padova, the downstream effectors of TGF-β1 for the pluripotency. By RNAseq performed in the presence of TGF-□1 or in the presence of its inhibitor SB43 we identified the downstream target of TGF-β1. Among the known regulators involved in pluripotency such as NANOG, KLF7, and MYC we also identified ZNF398, a member of the Krüppel-associated box domain zinc finger proteins (KZFPs), as a new regulator of ECS pluripotency. Ectopic expression of each of these factors could maintain expression of pluripotency markers in presence of SB43. To understand the molecular mechanism by which ZNF398 promotes pluripotency, we performed Chromatin immunoprecipitation (ChIP). Interestingly, ZNF398 binds the genome in close proximity to the transcription factor SMAD3

Identification and characterization of non-coding RNAs in gene regulation

Team: M. Maldotti, F. Anselmi, I. Molineris, A Tamburrini, I. Polignano, M. Scrivano.

MAIN OBJECTIVES OF THE PROJECT

1. Functional role of lncRNAs interaction with p300
2. Functional role of lncRNAs interaction with DNMT3B
3. Analysis of coexisting RNA conformations.

RESULTS ACHIEVED during the reporting period

1. Nuclear lncRNAs control gene transcription by interacting with epigenetic factors. Whole-genome screens have shown that a large fraction of long non-coding RNAs (lncRNAs) play a role in cellular processes and cancers, although the underlying molecular mechanisms remain largely unclear. Starting from the idea that lncRNAs can regulate the activity of epigenetic factors, we performed several screenings looking for lncRNAs that interact with epigenetic modulators. We found that histone acetyltransferase p300/CBP, a general transcriptional coactivator that introduces the H3K27ac modification on mutant enhancer in a large number of tumors, interacts with over hundred lncRNAs. We further analyzed one of them: lncSmad7. We found that l lncSmad7 maintains ESC self-renewal
and it interacts to the C-terminal domain of p300. lncSmad7 also contains predicted RNA-DNA Hoogsteen forming base pairing. By Chromatin Isolation by RNA precipitation followed by sequencing (ChIRPseq) together with CRISPR/Cas9 mutagenesis of the target sites we demonstrated that lncSmad7 binds and

recruits p300 to enhancers in trans, to trigger enhancer acetylation and transcriptional activation of its target genes. Thus, these results unveil a new mechanism by which p300 is recruited to the genome (**Figure 2**). Our data unveil a repertoire of RNAs interacting with p300 and provide new insights into the mechanistic aspects underlying the regulation of enhancer activity.

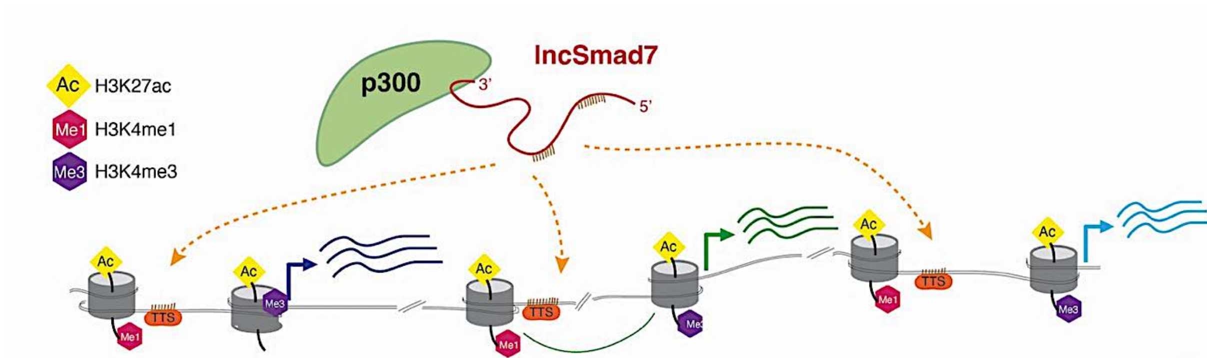


Figure 2. The lncRNA lincSmad7 interacts with the epigenetic factor p300 and recruits it to the genome, resulting in acetylation of enhancers and transcriptional activation of target genes.

2. We previously demonstrated that de novo DNMTs show target specificities. However, a clear understanding of how DNA methyltransferases recognize their target sites, and which are the upstream and downstream events contributing to their tumorigenic processes, is still missing. DNMT3B is highly expressed in colorectal cancer (CRC) and the major driver of altered DNA methylation. The mechanisms of its deregulation and of its altered targeting in CRC have not yet been clarified. To understand the contribution of lncRNAs to the DNMT3B function we screened the

DNMT3B RNA interactome in the HCT116 cell line by UV photoactivatable ribonucleoside enhanced crosslinking and immunoprecipitation followed by sequencing (PAR-CLIP-seq) from two biological replicates. After removing potential PCR duplicates by using a Unique Molecular Identifier (UMI) the DNMT3B-interacting lncRNAs were identified. These lncRNAs are highly expressed in CRCs with respect to normal colon tissue. Of them 41 were coexpressed with DNMT3B in PDX and enriched in the CRC subtype CRIS-D, characterized by WNT activation, a bottom crypt phenotype and expressing LGR5 **Figure 3).**

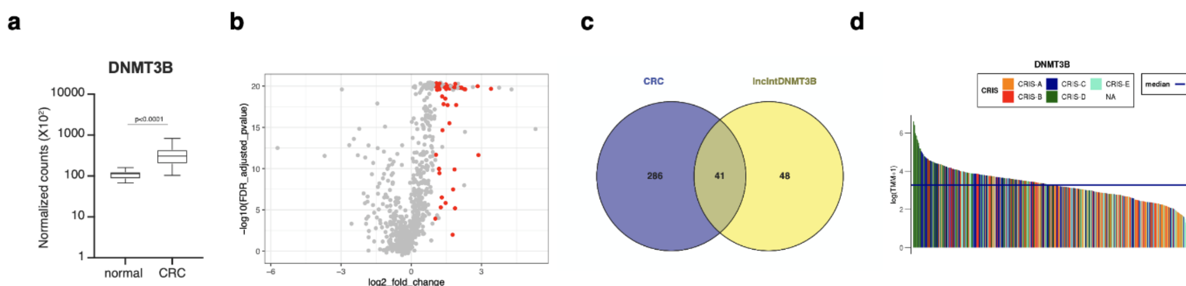


Figure 3. DNMT3B interacting lncRNAs are expressed in CRC. a. DNMT3B expression in normal colon and in CRCs. b. Vulcano plot showing the level of expression of the identified lncRNAs in comparison lncRNAs expressed in normal and colon tumours. c. Venn diagram showing the identified lncRNAs interacting with DNMT3B (lncIntDNMT3B) and lncRNAs expressed in PDX (CRC). d. DNMT3B expression in the different CRC subtypes. The lncRNAs are enriched in the CRIS-D subtype.

Analysis of cell-specific transcriptome from a collection of 244 CRC tumours of patient-derived xenograft (PDX) ordered by DNMT3B expression revealed a correlation with a previously defined CRISd subtype (characterized by chromosomal instability, activation of the WNT pathway and a LGR5 positive stem cell signature with a bottom crypt phenotype.

3. RNA structural analysis by means of chemical probing is powerful but it suffers from the intrinsic limitation of providing only an averaged measurement of the base reactivities of all coexisting conformations that are simultaneously sampled by an RNA species in biological samples. In collaboration with a formed post-doctoral fellow of the lab we developed DRACO, a method for the deconvolution of alternative RNA conformations from mutational profiling experiments. Analysis of the SARS-CoV-2 genome using dimethyl sulfate mutational profiling allowed us to identify multiple regions that fold into two mutually exclusive conformations, including a conserved structural switch in the 3′ untranslated region.

Statistical Inference and Biological Modeling Unit

Unit research members

- Andrea Pagnani, principal investigator (full professor PoliTo)
- Andrea Gamba, researcher (associate professor PoliTo)
- Alfredo Braunstein, researcher (associate professorPoliTo)
- Luca Dall'Asta, researcher (associate professor PoliTo)
- Andrea De Martino, researcher (tenure track researcher PoliTo)
- Carla Bosia, researcher (tenure track researcher PoliTo)
- Guido Uguzzoni, researcher (IIGM)
- Louise Budzyinski, researcher (IIGM)
- Elsi Ferro (PhD student PoliTo)
- Matteo De Leonardis (PhD student PoliTo)
- Anna Paola Muntoni (researcher PoliTo/ IIGM)
- Luca Sesta (PhD student PoliTo)
- Francesco Caredda (PhD student PolTo)
- Elisa Floris (PhD student PoliTo)
- Andrea Piras (PhD student UniTo)
- Roberto Mulet, visiting scientist, (University of Havana)
- Bárbara Ariane Pérez Fernández, visiting scientist, (University of Havana)
- José Antonio Pereiro Morejón, visiting scientist, (University of Havana)
- Alberto Batista, visiting scientist (University of Havana)
- Lisandra Calzadilla Rosado, visiting scientist, (Centro de Imunlogia Molecular, Cuba)

- Candela Szischik, visiting scientist, (CONICET Buenos Aires)
- Ariel Scagliotti, visiting scientist (University General Sarmiento Buenos Aires)
- David Ippolito Margarit, visiting scientist (University General Sarmiento Buenos Aires)

Brief description of the UNIT

Advances in sequencing and other high-throughput experimental technologies have fueled the genomic revolution and deeply transformed the world of biological research over the two decades. Remarkable examples of data obtained using such technologies include more than one thousand fully sequenced genomes (roughly doubling every two years), genome-wide measurements of gene expression or methylation profiles, experimental determination of genome-scale protein-protein interaction networks, and whole organism metabolic reconstruction. Alongside high-throughput measurements, this paradigmatic shift is observable also in the "systematization" of our knowledge into databases and templates, gathering all information currently available (ontological, functional, compartmental, etc.). Databases such as KEGG, Biocyc, Human Proteome Atlas, Pfam, etc. provide a collection of elementary components and their connections in a growing number of different contexts. Due to the nature of the problems involved, however, the pace of progress of experimental and bioinformatics techniques has often been unequal: analyzing optimally a large amount of experimental data requires in many cases the solution of hard problems which are intractable by conventional computational tools.

The perimeter of each research activity in our group is delimited by: (a) the research themes characterized by the toolbox and methods developed, and (b) the choice of the application domains. Each research project is a puzzle piece of the wider research project. Principal research themes covered by the group are: (i) the inference of interaction networks from data, and (ii) the analysis of static and dynamical processes on networks. Application domains can be broken down into four main areas: (i) the inference and modeling of multi-scale biological networks, (ii) the rational design of biological molecules, (iii) the quantitative study of cell energetics in proliferative regimes, (iv) quantitative biological modeling.

RESEARCH QUESTIONS

The design of novel proteins with desired functions is a complex problem with enormous repercussions in the pharmaceutical, biomedical, and industrial sectors (e.g. monoclonal antibodies, synthetic enzymes, engineered cytokines, etc...) However, designing new proteins is a difficult task, due to the huge combinatorial complexity of the sequence space and the many structural constraints that must be satisfied. For example, a small protein of 100 amino acids has 10^{130} possible variants, more than the atoms in the universe, the overwhelming majority of which are non-functional. To find the best variant for a given purpose, it is necessary to employ sophisticated experimental solutions (e.g., Phage Display, Selex, Directed Evolution, Deep Mutational Scanning) combined with advanced computational approaches.

Metabolic networks encode the thousands of chemical reactions through which cells break down nutrients to fuel biosynthesis, growth, and replication. Mathematical models of metabolism, most notably genome-resolution ones, are highly developed and widely used for purposes ranging from the optimization of biotechnological processes to the discovery of new drug targets. While remarkably successful in predicting cell viability, these methods mostly fail to capture the heterogeneity of cellular behavior. This is a key issue as, for instance, not all cells in a population respond to drugs in the same way, and the persistence of non-

responsive cells is the main source of resistance to treatment. A possible route to understanding cellular diversity consists of inverse-modeling metabolism using experimentally measured reaction rates. Unfortunately, experiments only probe a very small part of the network, and reliably inferring the activity of the entire network from such a small sample inevitably requires the use of advanced inference methods.

Protein sorting plays a vital role in ensuring the appropriate localization of specific molecules within cells. Misregulation of protein sorting can result in severe pathologies. In particular, protein mislocalization has been identified as a significant contributor to the development and progression of cancer. While significant progress has been made in understanding the biochemistry of protein sorting, a systemic understanding of the process remains elusive. One difficulty is that the process is highly dynamic and involves the complex interplay of protein aggregation and lipid membrane bending. Using a statistical physics approach, we have recently proposed a dynamic model of protein sorting whose predictions compare favorably with experimental observations. The model provides a useful framework for interpreting experimental data and investigating the possibility of interfering with the process for therapeutic purposes.

The entire stochastic process that leads a DNA molecule to be transcribed into RNA and then eventually translated into proteins is tightly controlled and regulated. MicroRNAs, small non-coding RNA molecules often involved in crucial biological processes in higher eukaryotes and plants, fine-tune this regulation at the post-transcriptional level. Together with transcription factors, microRNAs form a complex and highly interconnected regulatory network where transcriptional and post-transcriptional regulators, as well as their targets, may compete for shared resources (ribosomes, RNAses, etc.) and crosstalk with each other. By combining wet-lab experiments and theoretical modeling, we contributed to characterizing the microRNA-mediated cross-correlation effects amongst different targets that compete for binding to the same pool of microRNAs at the steady state. We discovered that microRNA-mediated regulation is always related to noise control of target RNAs and proteins and can convey and couple fluctuations in asymmetric manners in networks of competing targets. However, how different levels of competition translate into out-of-equilibrium behaviors is unknown, an open point related to cell timing (i.e., for cells the time necessary to reach a precise level of protein expression can be of vital necessity).

MAIN RUNNING FUNDINGS

INFERNET, “New algorithms for inference and optimization from large-scale biological data” H2020, Funded by the European Union’s H2020 research and innovation program under the Marie Sklodowska-Curie grant agreement number 734439 for 900.000 Euros. The project ended on December 31st, 2022. Project Scientific Coordinator: Andrea Pagnani.

Main achievements in the period covered by the Scientific Report

Computational Methods for Optimizing the physicochemical properties of Proteins

PI: A. Pagnani – Team : G. Uguzzoni, A.P. Muntoni, L. Sesta, L. Budzynski, M. De Leonardis, F. Caredda

COLLABORATIONS:

- Martin Weigt (Sorbonne Université de Paris),
- Francesco Zamponi (Ecole Normale de Paris),
- Olivier Rivoire, and Clement Nizak (Sorbonne Université / College de France),
- Jorge Fernandez De Cossio Diaz (Paris Sciences et Lettres University).

MAIN OBJECTIVES OF THE PROJECT

The main objective of the project is to develop innovative machine-learning methods that, starting from the sequencing samples of a screening experiment, produce an accurate statistical mapping of the genotype-phenotype association of the protein that can be used for the generation of new amino acid sequences with biochemical functions of interest. The approach can be used in an iterative cycle with Directed Evolution experiments, where previously tested variants are used to learn a computational model that provides a data-driven rational design of the new variant candidates to be tested. At each iteration, the in-silico model is refined, it gains from collected data from previous rounds and performs an optimal exploration of the space of possible molecule candidates.

Antibodies, and other binding proteins, are the subjects of intense interest in biological and pharmaceutical research, with an expanding market of products and services. The present project relates to the general problem of the in-silico design of proteins with high affinity with a specific target molecule. Despite the intense research work from an experimental and theoretical point of view, the problem of the prediction of amino acid sequences that generate a specific behavior remains a difficult problem. This is particularly true for applications that require a high performance in terms of specificity, as in the case of industrial enzymes, or proteins with therapeutic purposes such as monoclonal antibodies. The experimental process of direct evolution is a powerful approach to protein engineering. This approach has been applied to the optimization of enzymes and antibodies for chemical synthesis. In the last decade, thanks to the spread of a new type of experimental biochemistry analysis technique called "deep mutational scanning", they have combined large-scale selection of a combinatorial library of mutants specific to directed evolution, with high-throughput sequencing techniques, to quantify the functionality of ~ 10^6 variants of a given protein (**Figure 1**).

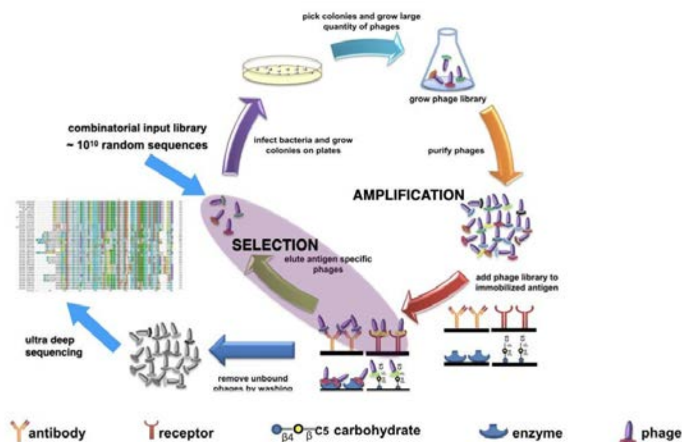


Fig 1: Experimental and computational pipeline

RESULTS ACHIEVED during the reporting period

Concerning the Deep Mutational Scanning type of experiments, the computational pipeline we developed consists of probabilistic modeling of the three phases of each experiment panning cycle: 1) selection, 2) amplification, and 3) sequencing. In brief, what we observe are the sequenced reads from the library at any specific time step. The other phases are described in terms of latent variables referring to the number of amplified and selected mutants. The probability that a mutant is selected (e.g., by physical binding to the target) depends on the specific mutant sequence composition. In [3] we showed how the inference strategy can predict the selectivity of artificially generated sequences with unprecedented accuracy. A key feature of the inference of mutational landscapes is the ability to efficiently generate artificial sequences with a given target specificity. Different computational strategies and specific modeling have been devised for this aim. Generating artificial sequences, from our standpoint, means being able to efficiently generate a set of sequences with indistinguishable statistical characterizations from the training set. Of course, in these cases, a matter of concern is that the artificial set generated is very different from the training set. All our proposed computational strategies generate sequences that are very different from the natural ones [2].

Metabolic Networks

co-PI Andrea De Martino – Team: Alfredo Braunstein, Anna Paola Muntoni, Andrea Pagnani; Alberto Batista, Lisandra Calzadilla Rosado, Roberto Mulet, Bárbara Ariane Pérez Fernández, José Antonio Pereiro Morejón

COLLABORATIONS:

- Daniele De Martino, Instituto Biofisika, Bilbao;
- Loretta L. Del Mercato, CNR-NANOTEC (Lecce)

MAIN OBJECTIVES OF THE PROJECT

The main goal is to address the problem of inverse modeling metabolic networks from strongly undersampled population-level data on reaction rates. In brief, given empirical data for (i) the fluxes through a small number of metabolic reactions quantified via bulk methods (e.g. Mass Spectrometry, nanoSIMS), (ii) the distribution

of a macroscopic observable (e.g. the growth rate or the carbon incorporation rate) at single-cell resolution across a population of cells, or (iii) the (spatially resolved) concentration of a key signaling molecule in cell cultures, we want to find the most likely distribution of the fluxes through all intracellular metabolic reactions (including the vast majority that is experimentally inaccessible) across the whole population.

RESULTS ACHIEVED during the reporting period

The huge difference between the number of reactions for which experimental information can be obtained (usually a few tens) and the number of variables to be inferred (thousands for a genome-scale reconstruction) would seem to make inference hopeless. There is however a mitigating factor: variables are topologically interconnected, i.e. they form a network that can be reconstructed at genome-level detail. Together with the assumptions of homeostasis and time scale separations between metabolism (fast) and regulation (slow), this implies that feasible flux configurations are non-equilibrium steady-state material flows across a known network, driven by the import of nutrients from the environment. If S represents the NxM matrix of the stoichiometric coefficients of metabolic reactions (with N the number of reactions and M that of compounds), feasible flux vectors v, therefore, satisfy the mass-balance conditions $Sv = 0$. As v is continuous, when ranges of variability for each flux are supplied, solutions span a convex polytope F of dimension at least equal to N-rank(S). The inference problem to be solved therefore consists in finding the probability distribution P(v) over F that is the most likely generator of the empirical data. Because the dimension of F for a typical genome-scale network makes exact computational methods (e.g., Monte Carlo) impracticable, developing controlled approximations is the first essential step. In this context, we have shown that Expectation Propagation (EP), a message-passing algorithm for approximate Bayesian inference, allows for high-quality, scalable, and high-efficiency reverse modeling.

On a different (related) topic, we have developed an inference pipeline for the online reconstruction of the cell-to-cell proton signaling network in cancer cell cultures from (high-resolution) spatial pH measurements. When microenvironmental pH inhomogeneities are induced by high cellular metabolic activity, diffusing protons are known to act as signaling molecules, driving the establishment of exchange networks sustained by the cell-to-cell shuttling of overflow products such as lactate. We have provided direct experimental characterization of such networks by devising a method to quantify single-cell fermentation fluxes over time through the integration of high-resolution pH microenvironment sensing via ratiometric nanofibers with constraint-based inverse modeling (**Figure 2**).

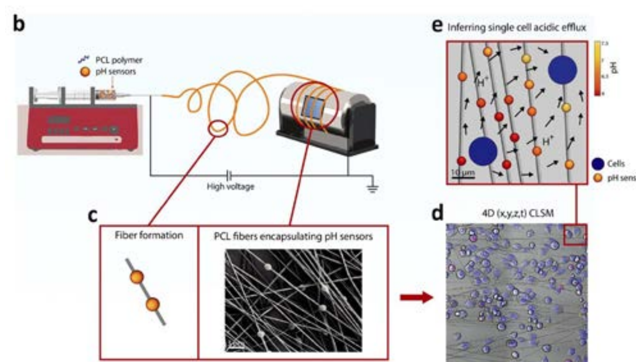


Fig 2: Experimental and inference pipeline

This addresses issues ranging from the homeostatic function of proton exchange to the metabolic coupling of cells with different energetic demands, allowing for real-time noninvasive single-cell metabolic flux analysis.

Dynamical modeling of protein sorting

Co-PIs: Andrea Gamba, Luca Dall'Asta - Team: Elisa Floris, Andrea Piras

- *COLLABORATIONS:* Guido Serini, Department of Oncology, University of Torino, and IRCCS Candiolo, Candiolo;
- Marco Zamparo, Dipartimento di Fisica, Università degli Studi di Bari;
- Igor Kolokolov and Vladimir Lebedev, Landau Institute for Theoretical Physics, Moscow;
- Emilio Hirsch, Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Torino;
- Carlo Campa, Italian Institute for Genomic Medicine, Candiolo;
- Francesco Saverio Pezzicoli, Laboratoire Interdisciplinaire des Sciences du Numérique, Université Paris-Saclay, Gif-sur-Yvette.

MAIN OBJECTIVES OF THE PROJECT

The main objectives of this project are to develop and extend a comprehensive dynamical model of molecular sorting using statistical physics methods, apply the model to the interpretation of experimental data, and investigate the possibility of interfering with the molecular sorting process for therapeutic purposes.

RESULTS ACHIEVED during the reporting period

We have developed a theoretical model for protein sorting that describes the dynamic behavior of molecules on a cell membrane region [6]. Our model is based on the assumption that protein sorting arises from the formation of sorting domains through phase separation, in conjunction with the domain-induced bending of lipid membranes. The model considers the rate of insertion of proteins into the membrane (*kI*), their diffusion rate (*kD*), their aggregation in sorting domains (with an aggregation coefficient *g*), and their extraction rate (*kE*) when a domain reaches a characteristic size (Fig. 3). The model predicts the size distribution of sorting domains and demonstrates the existence of an optimal parameter region where the sorting process is most efficient. Our model's predictions are in agreement with experimental measures of endocytic sorting of low-density lipoproteins in primary human endothelial cells (Fig. 3d,e). These findings suggest that the observed process of molecular sorting operates in close proximity to the predicted optimal regime and support the hypothesis that phase separation is a primary driver of protein sorting.

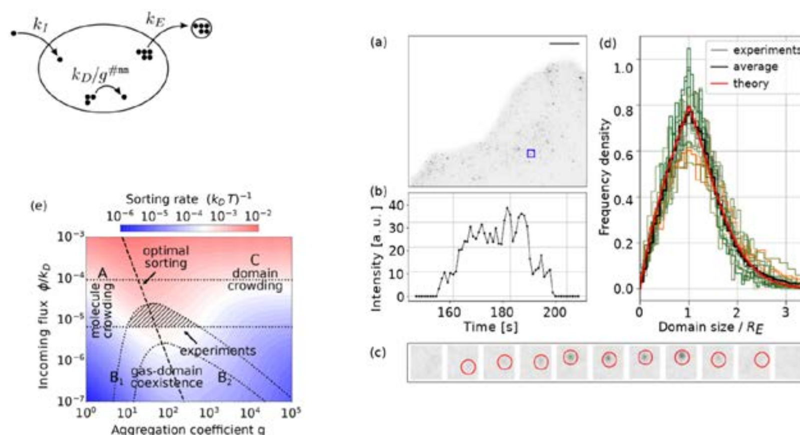


Fig. 3: Dynamical modeling of protein sorting

According to the physical theory of phase separation [7], there is a critical size, such that smaller phase-separated domains have short lifetimes and tend to dissolve, while larger domains can grow indefinitely. Based on the success of our model in describing the statistical properties of endocytic sorting [6], we reasoned that if molecular sorting is driven by phase separation, the existence of a critical domain size would lead to the observation of transient sorting domains that are never extracted from the membrane system, and of larger domains that instead grow to the point where they can bend the lipid membrane and ultimately become engulfed into a newly-formed lipid vesicle. Our analysis of quantitative experiments on endocytic sorting confirms this hypothesis [16]. Two classes of sorting domains ("unproductive", or "abortive", and "productive") are observed in the experiments, and their statistical properties can be measured. To further validate our predictions, we fitted the theoretical curves predicted by the model with the experimental statistical distribution of productive and unproductive sorting domains in the endocytic sorting of transferrin receptors obtained by the groups of Sandra Schmid and Gaudenz Danuser [eLife 9, e53686 (2020)], finding very good agreement between theory and observations [16]. This provides further support to the hypothesis that the formation of sorting domains is driven by a phase separation process.

microRNA-target interaction out-of-equilibrium

PI: Carla Bosia – Team: Elsi Ferro, Candela Szischik, Andrea De Martino.

COLLABORATIONS:

- Alejandra Ventura (Universidad de Buenos Aires and CONICET),
- Carlo Cosimo Campa (Italian Institute for Genomic Medicine - IIGM),
- Mario Matteo Modena (ETH Zurich), Chiara Enrico Bena (French National Institute for Agriculture, Food and Environment - INRAE),
- Velia Siciliano (Istituto Italiano di Tecnologia - IIT),
- Francesca Ceroni (Imperial College London),
- Silvia Grigolon (Laboratoire Jean Perrin at Sorbonne Université and CNRS).

MAIN OBJECTIVES OF THE PROJECT

Environmental fluctuations, henceforth called extrinsic noise, can be a source of noise in molecular networks. Together with intrinsic fluctuations due to the probabilistic nature of chemical reactions, extrinsic noise shapes gene expression and may lead to cell differentiation. Both theoretical and in vitro studies have hypothesized that miRNAs, under particular stoichiometric conditions, can induce bimodality in the expression of their targets simply due to stochastic effects related to their peculiar titrative interaction and not due to bistable systems. We are interested in the dynamic aspects of such interactions in a context of competition for intracellular resources (also known as gene expression burden). In particular, we aim to answer questions like (i) are there preferential microRNA expression frequencies that optimally repress microRNA-target over time? (ii) How does this combine with the expression of other targets of the same microRNAs over time? (iii) How intracellular resources (ribosome, RNAses, etc…) are consequently redistributed?

RESULTS ACHIEVED during the reporting period

We studied the role of extrinsic noise in miRNA-mediated bimodal gene expression, both in silico (Gillespie simulations) and in vitro (transfection of synthetic constructs into immortalized cells), and with stochastic

models of gene regulation developed by the unit, to link theoretical predictions to experimental contexts of interest. The temporal aspect of these types of interactions is of increasing interest. In collaboration with C.C. Campa we produced single-cell time trajectories for a miRNA target and a control within the same cell. Depending on the specific synthetic construct, the miRNA-target has been engineered with an increasing miRNA-target interaction strength. These data will be used to calibrate the resource-aware model and infer biological parameters of interest.

In collaboration with Prof. A. Ventura (Universidad de Buenos Aires and CONICET) and her Ph.D. student C. Szischik, we have developed the theoretical framework required to interpret data produced by the unit when microRNA's level of expression changes over time with specific frequencies. We engineered an optogenetic microRNA gene whose expression levels, responsive to light pulses of a particular wavelength, will be varied over time by a time-varying light pulse. The temporal responses of the targets of this miRNA and the physiological repercussions on the cells under investigation in response to different input signals will then be studied. Experimental data are produced with the support of an optogenetic platform provided by M. M. Modena from ETH Zurich. Overall, the results achieved during the reporting period demonstrate significant progress toward the project's main objectives and may have relevant implications for our understanding of molecular sorting and its potential therapeutic applications.

Immune Regulation Unit

Unit research members

- Luigia Pace (PI)
- Carlo De Intinis PhD, post-doc fellow (IIGM)
- Ines Elia PhD, post-doc fellow (IIGM)
- Stefano Carello, post-doc fellow (IIGM)
- Valentina Russo d Stabile PhD student (IIGM, UniTo)
- Nadia Brasu, Stabile PhD student (IIGM, UniTo)
- Gaia Montecchiesi Stabile PhD student (IIGM, UniTo)
- Simona Aversano Stabile PhD student (IIGM, UniTo)
- Simone Attisani master student (UniBicocca)
- Shahrzad Shiran master student (UniTo)

Brief description of the UNIT *(half a page minimum, 1 page maximum)*

The immuno-regulation Unit was started at the beginning of 2018. The research activities of the new laboratory aim at understanding the fundamental mechanisms underlying the development of the immune responses, especially the long-term immunological memory processes in the context of tumours and infections.

RESEARCH QUESTIONS:

The research program is developed on three major axes:

1) defining how T lymphocytes integrate complex environmental stimuli and transform these signals into specific epigenetic and differentiation programs;

2) to determine the ontogenesis and function of the T lymphocyte subpopulations involved in the antitumor response.

3) to unravel the diversity of memory CD8+ T and B cell responses following SARS-CoV-2 infection and after mRNA vaccination.

These studies will allow acquiring new knowledge on the functional identity of T lymphocytes and on epigenetic memory mechanisms, thus opening to new perspectives in the manipulation of the immune responses in the promising field of immunotherapy, as well as in the development of new vaccines.
The strategy of the laboratory is based on multidisciplinary approaches, at the crossroads of immunology, epigenetics, cancer biology and bioinformatics. To this end, our research group use a wide variety of methodologies, including immunological models relevant to the study of cancer and infection, genetic and genomic techniques, analysis of epigenetic markers and chromatin structure, new algorithms for bioinformatics analysis and modelling.

MAIN RUNNING FUNDINGS

- Armenise-harvard Carrer Development Award, 2018-2023, Role of Heterochromatin in T Lymphocyte Memory Development.
- AIRC, 2020-2025, deciphering the molecular framework regulating stemness and cytotoxicity of t lymphocytes to potentiate immunotherapy.

Main achievements in the period covered by the Scientific Report

Study of the epigenetic control during T cell fate commitment

MAIN OBJECTIVES OF THE PROJECT

This project aims at understanding the **mechanisms of immunological memory**, focusing on elucidating the **contribution of chromatin structure and dynamics** to lineag diversification, functional specialization and long-term survival of memory T lymphocytes.
The main objectives are:
1. Examine the role of heterochromatin factors during memory T cell differentiation.
2. Analysis of heterochromatin factors during the anti-tumor immune response.

ACHIEVED RESULTS:

Understanding the mechanisms of the epigenetic regulation involved in the effector and memory T lymphocyte development has considerable implications in multiple fields of medicine, including cancer immunotherapy. In cancer, in fact, the T lymphocytes differentiate to a dysfunctional state, known as exhaustion, characterized by a poor ability to functionally respond to tumour antigens. How the exhaustion processes are established, which mechanisms can inhibit this dysfunctional state are subject of intense research.
Although multiple studies reveal an enormous complexity of T lymphocyte subsets, the low number of recovered cells has often represented a limiting factor for a detailed molecular analysis of both epigenetics and nuclear lymphocyte dynamics. To overcome this difficulty, we have developed a new ChIP-seq protocol for epigenetic markers, including H3K9me3, starting from a low number of lymphocytes.
Although H3K9me3, is a highly conserved epigenetic mark, it remains to be established how this silencing marker regulates the expression of our genome, and how its enrichment correlates with the state of differentiation of T lymphocytes. In this project, the profiles of H3K9me3 have been analyzed by ChIP-seq analysis both in T lymphocytes and in stem cells, applying a new segmentation model of the immunoprecipitation / input signals, followed by data mapping, filtering and normalization of read counts. The genome regions enriched for H3K9me3 have been annotated to the most proximal gene in order to evaluate the effect of H3K9me3 on on lineage specific transcriptional programs.
Several epigenetic factors could be involved in the process of lymphocyte differentiation. In order to understand the key factors involved in the immune response, the Unit is developing a CRISPR/CAS9 screening for several epigenetic factors involved in heterochromatin architecture.

Memory CD8+ T cell diversity and B cell responses correlate with protection against SARS-CoV-2 following mRNA vaccination

MAIN OBJECTIVES OF THE PROJECT

Study of protective immunity and immunological memory in healthy subjects and cancer patients following mRNA vaccination against the SARS-CoV2 virus.

RESULTS ACHIEVED during the reporting period

In the last years, infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused remarkable morbidity and mortality. Memory B cells, CD4+ T cells and CD8+ T cells elicited by SARS-CoV-2 mRNA vaccination have a critical role in the protection against infection and represent key determinants in

the vaccine boost. Both Pfizer/BioNTech and Moderna mRNA vaccines have showed 91% and 93% protective immunity over several months, respectively, and up to 95% efficacy in preventing symptomatic COVID-19 disease caused by the wild-type (WT) SARS-CoV-2 and delta and omicron VOCs. So far, understanding immune responses to SARS-CoV-2 messenger RNA (mRNA) vaccines is of great interest, principally because of the poor knowledge about the mechanisms of protection.
In the present study, we enrolled 400 subjects, and we analyzed longitudinally B cell and T cell memory programs, against the spike (S) protein derived from ancestral SARS-CoV-2 (Wuhan-1), B.1.351 (beta), B.1.617.2 (delta) and B.1.1.529 (omicron) variants of concern (VOCs) after immunization with an mRNA-based vaccine (Pfizer). According to the magnitude of humoral responses 3 months after the first dose, we identified high and low responders. Opposite to low responders, high responders were characterized by enhanced antibody-neutralizing activity, increased frequency of central memory T cells and durable S-specific CD8+ T cell responses. Reduced binding antibodies titers combined with long-term specific memory T cells that had distinct polyreactive properties were found associated with subsequent breakthrough with VOCs in low responders. These results have important implications for the design of new vaccines and new strategies for booster follow-up (*Brasu et al, Nature Immunology, 2022;* **Figure 1**).

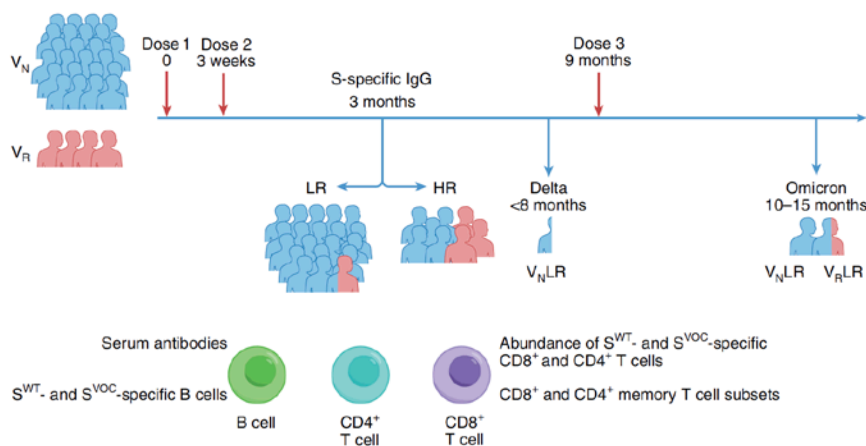


Figure 1: Assessing the effect of infection-elicited immune memory on responses to SARS-CoV-2 vaccination. Members of the cohorts differed first in their infection history before vaccination (VN, naïve before vaccination; VR, infection-recovered before vaccination) and later in the magnitude of their early antibody response to vaccination (LR, low responders; HR, high responders). PBMC samples were obtained at the indicated sampling times and subjected to detailed analyses to follow SARS-CoV-2 specific CD4+ and CD8+ T cell subsets and effector function and spike-specific B cells over time. The members of the cohort were also tracked for more than a year for breakthrough infection. Modified from A. Sant, et al. Nature Immunol 2022).

To determine the ontogenesis and function of the T lymphocyte subpopulations involved in the anti-tumor response following therapeutic vaccination

MAIN OBJECTIVES OF THE PROJECT

Analysis of the role for adenoviral-based vectors to promote broadening, expansion, and differentiation of T cells to sustain an antitumor response in mouse and humans.

ACHIEVED RESULTS:
After chronic exposure to cancer or viral infection, CD8+ T cells acquire an exhausted phenotype, characterized by a poor ability to respond to antigenic stimulation. Tumor-reactive lymphocytes progressively become exhausted and rarely acquire memory features. Conversely to exhausted T cells, the Tcf1+ stem-like CD8+ T (T_{STEM}) progenitors of exhausted cells resemble self-renewal memory stem-like cells

and proliferate after blockade of the PD-1 inhibitory pathway before differentiating into terminally exhausted CD8+ T cells. In line with their capacity to respond to immunotherapy, an increased percentage of Tcf1+ CD8+ T cells has been correlated with prolonged duration of responses to checkpoint blockade therapy in patients with cancer. As a consequence, understanding the functional programs to sustain Tcf1+ CD8+ T cell differentiation represents a critical step for the improvement of immunotherapy, aimed at increasing their number and maintaining their self-renewal capacity, thus overcoming resistance to checkpoint blockade treatment in cancer.

Several studies have shown that the clinical efficacy of PD-1 checkpoint blockade depends on the magnitude, quality, and tumor-infiltrating properties of CD8+ T cells targeting mutation-associated neoantigens. As opposite to self-antigens, neoantigens are self-mutated peptides, without preexisting central tolerance and with the potential of inducing stronger immune response and effective antitumor activity. As a consequence, neoantigens have become of great interest as targets of cancer vaccines. Adenovirus (Ad)-vectored vaccines encoding tumor neoantigens have been shown to eradicate large tumors when combined with anti-programmed cell death protein 1 (anti-PD-1) in murine models; however, the mechanisms and translational potential have not yet been elucidated.

We have recently showed that gorilla Ad vaccine targeting tumor neoepitopes enhances responses to anti-PD-1 therapy by improving immunogenicity and antitumor efficacy. Single-cell RNA sequencing demonstrated that the combination of Ad vaccine and anti-PD-1 increased the number of murine polyfunctional neoantigen-specific CD8+ T cells over anti-PD-1 monotherapy, with an accumulation of Tcf1+ stem-like progenitors in draining lymph nodes and effector CD8+ T cells in tumors. Combined T cell receptor (TCR) sequencing analysis highlighted a broader spectrum of neoantigen-specific CD8+ T cells upon vaccination compared to anti-PD-1 monotherapy. The translational relevance of these data is supported by results obtained in the first 12 patients with metastatic deficient mismatch repair (dMMR) tumors vaccinated with an Ad vaccine encoding shared neoantigens. Expansion and diversification of TCRs were observed in post-treatment biopsies of patients with clinical response, as well as an increase in tumor-infiltrating T cells with an effector memory signature. These findings indicate a promising mechanism to overcome resistance to PD-1 blockade by promoting immunogenicity and broadening the spectrum and magnitude of neoantigen-specific T cells infiltrating tumors (*D'Alise M, et al. Science Translational Medicine, 2022;* **Figure 2**).

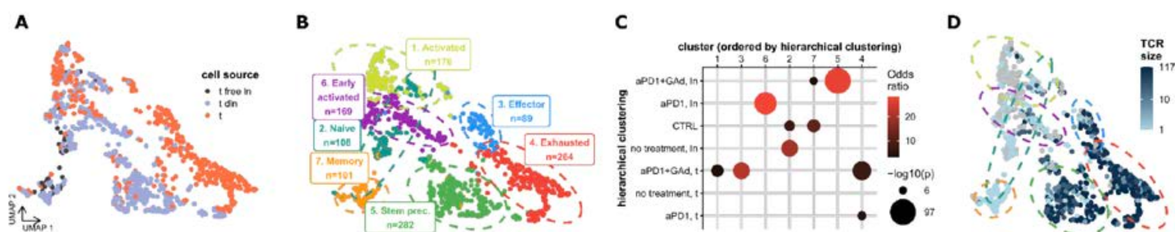


Figure 2: GAd-7+aPD-1 leads to accumulation of TPEX in lymph node. (A) Microenvironment distribution of neoepitope specific Adpgk+ CD8+ T cells (t = tumor, ln = lymph node). (B) Adpgk+ CD8+ T cells are distributed in distinct clusters. (C) Fisher's exact test of cell frequencies found in each cluster displays accumulation of TPEX in lymph nodes of mice treated with aPD-1+GAd-7 combined therapy. (D) TCR analysis highlights clonal expansion in TPEX, effector and exhausted phenotypes (adapted from D'Alise M et al., Science Transl. Medicine, 2022).

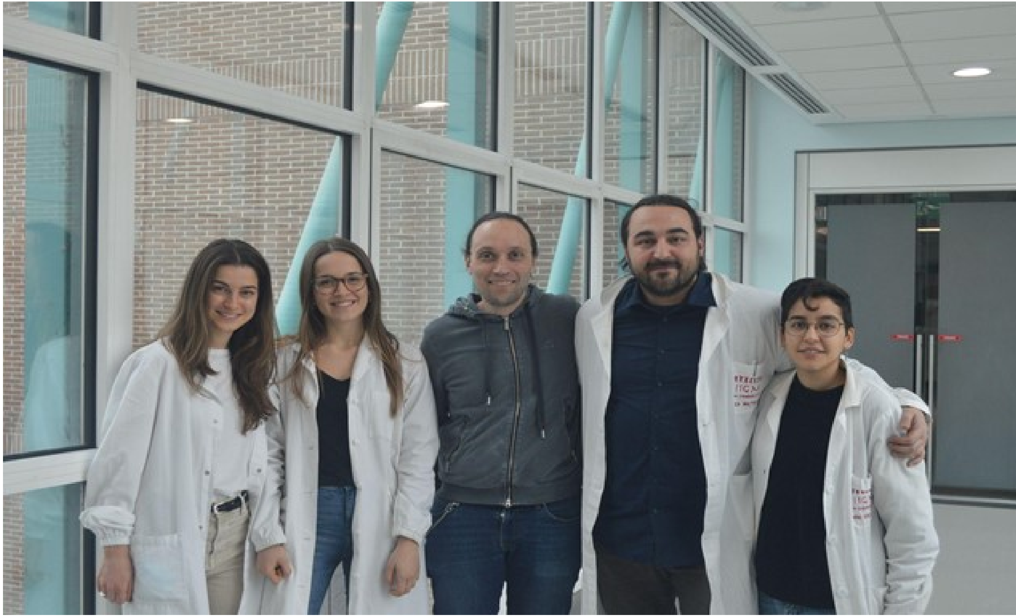
Awards, patents, dissemination

-Interviews with journalists for the dissemination and promotion of research activity.

-Patent: "Method for *in vitro* evaluation of the immune response after vaccination with a mRNA vaccine",

2022.

Genomic Instability and Tumor Immunity Unit



Unit research members

- Ilio Vitale, principal investigator
- Gwenola Manic, researcher
- Luca Mattiello, postdoctoral fellow
- Alessandro D'Ambrosio, postdoctoral fellow
- Andrea Guarracino, PhD student until 31/05/2021
- Sara Soliman Abdel Rehim, PhD student
- Ginevra Campia, fellow
- Federica D'Amico, undergraduate student

Brief description of the UNIT *(half a page minimum, 1 page maximum)*

The main research interests of the Unit concern the mechanisms regulating genomic stability and the processes governing tumor immunity. We place a particular focus on cancer stem cells (CSCs), the subpopulation of malignant cells with pronounced tumor-initiating, tumor-propagating and tumor-disseminating potential and elevated resistance to currently used therapies. CSCs are resilient to adverse

microenvironmental conditions and have been associated with therapeutic failure and tumor relapse. It is thus fundamental to develop novel (immuno)therapeutic approaches specifically targeting CSCs.

Our research activity proceeds in two parallel and interlinked fashions. In a first line of investigation , we analyze the mechanisms regulating DNA replication and chromosome segregation in CSCs, with the objective of identifying specific vulnerabilities and targets for prophylactic and therapeutic purposes. In a second line of investigation, we explore the mechanisms adopted by CSCs to evade the surveillance of the immune system with the aim of developing novel approaches to increase CSC immune visibility and attack.

To explore these aims, we have developed a series of experimental methodologies to profile the mutational, transcriptomic, proteomic, and antigenic landscape of patient-derived models and to quantify dynamic parameters such as chromosomal instability (CIN), tumor heterogeneity, immunogenicity and (clonal) response to therapies. Current approaches include NGS studies, karyotypic and functional analyses, videomicroscopy, microfluidics, algorithm development, murine models of cancerogenesis, syngeneic murine models, and humanized mice.

We believe that the identification of the mechanisms regulating genomic stability and governing tumor immunity can guide the design of novel immunotherapeutic regimens.

RESEARCH QUESTION(S):

How frequent and relevant are pre-mitotic and mitotic defects associated with CIN in CSCs?

Can the DNA damage response constitute a specific vulnerability of CSCs?

Do replication stress and mitotic stress activate a specific cellular response impacting on therapeutic resistance and immunogenicity of CSCs?

What are the principal mechanisms adopted by CSC to evade surveillance of the immune system?

Is boosting chromosomal instability an effective strategy to increase the immunogenicity of CSCs?

MAIN RUNNING FUNDINGS

- Associazione Italiana per la Ricerca sul Cancro (AIRC). Project title: "*Exploiting karyotypic aberrations and chromosomal instability in cancer stem cells for precision immunotherapy*" (PI: I. Vitale): € 149.150

Main achievements in the period covered by the Scientific Report

Mechanisms regulating genetic and chromosomal stability in CSCs

(Team: G. Manic, A. Guarracino, S. Soliman, L. Mattiello, G. Campia)

COLLABORATIONS:

- Prof Livio Trusolino, Candiolo Cancer Institute - FPO, IRCCS, Candiolo, Italy
- Dr Mauro Biffoni, ISS, Rome, Italy
- Dr Lorenzo Galluzzi, Weill Cornell Medical College, New York, NY, USA

MAIN OBJECTIVES OF THE PROJECT

1. Characterize the response to replication and mitotic stress in cancer cells and CSCs.
2. Identify the intracellular pathways and extracellular signals linked to the response to replication and mitotic stress in cancer cells and CSCs.
3. Develop novel therapeutic strategies directed against CSCs.

RESULTS ACHIEVED during the reporting period

We previously established a panel of spheroids and organoids derived from colorectal (CRC) cancer-patients, which are two experimental models to enrich and study CSCs. Taking advantage of these models, we demonstrated that CSCs tend to undergo DNA replication errors but have a high tolerance to replication stress (RS) due to a very robust DNA damage response. In this period of research activity, we provided evidence that a high level of constitutive RS is a marker of the sensitivity of CSCs from CRC to DNA damaging agents, including 5-FU, oxaliplatin and irinotecan (which are conventionally used in the clinics against CRC) as well as to inhibitors of the RS response, including ATR-CHK1 inhibitors. We also identified a non-genetic mechanism of resistance of CSCs to these agents based on the upregulation of PARP1, one crucial player of the DNA damage response also involved in the management of RS. We showed that PARP1 determines resistance to RS inducers by decelerating the DNA replication process (**Figure 1A**). This role of PARP1 results in an enhanced DNA replication fidelity with consequent decrease in the level of RS**.** We also provided evidence that PARP1 inhibition with so-called PARP1 trappers sensitizes cancer cells and CSCs to RS inducers such as irinotecan, also fostering their pro-immunogenic potential (**Figure 1B**).

Using our panel of primary spheroids and organoids, we also showed that a considerable fraction of CSCs displays a weak mitotic checkpoint, a surveillance mechanism best known as spindle assembly checkpoint (SAC) that monitors and ensures correct sister chromatid segregation. We finally performed a drug screening on cell lines representative of SAC functionality, demonstrating that SAC deregulation increases the resistance to therapy and the immunogenicity of CSCs (**Figure 1C**).

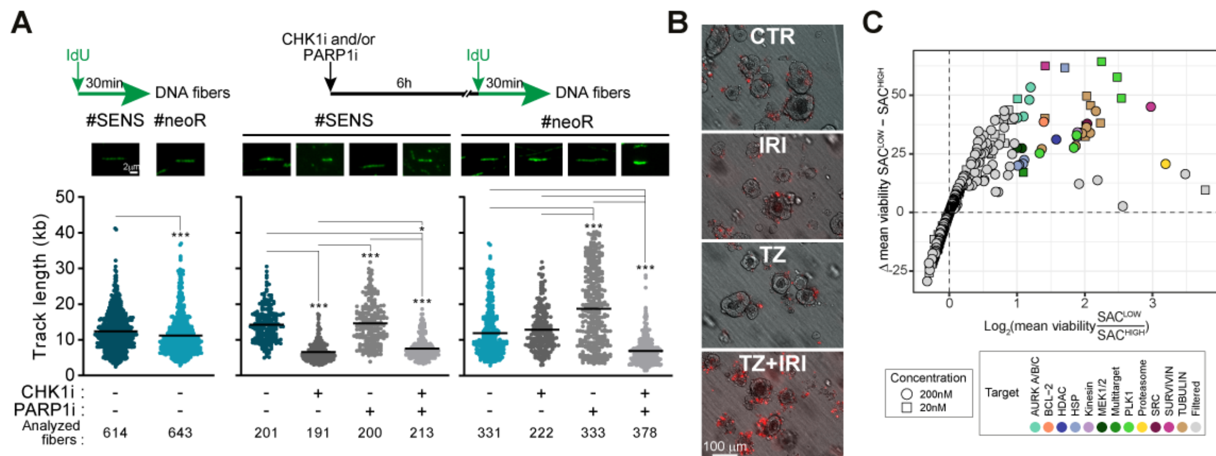


Figure 1. Role of PARP1 in the RS response of CSCs. A. Primary spheroids sensitive or insensitive to RS induction were treated as reported (I, inhibitor) and then subjected to DNA fiber assay upon incubation with IdU. Fiber lengths are illustrated. Mann–Whitney test (left) and Kruskal–Wallis ANOVA followed by Dunn's post-hoc test (center/right). **B**. Primary spheroids treated as reported (CTR, Control; IRI, Irinotecan; TZ, Talazoparib) were analyzed by live microscopy upon staining with the vital dye propidium iodide (PI, in red). **C**. Primary spheroids with distinct SAC robustness (strong: SACHIGH; weak: SACLOW) were administered for 72h with 20 or 200 nM of a library of 349 cancer-relevant compounds, prior to the assessment of cell proliferation/survival by CellTiter GLo® Luminescent Cell Viability assay**.** Grey points in the scatterplot indicate filtered out cases (log2/mean viability < 1 or Δ < 20).

We are now conducting multi-omic studies with the objective of identifying modulated targets/pathways of the response of CSCs to RS and SAC deregulation.

Mechanisms of immune evasion by cancer stem cells

(Team: G. Manic, A. Guarracino, S. Soliman, L. Mattiello, G. Campia)

COLLABORATIONS:

- Dr Antonella Sistigu (Università Cattolica del Sacro Cuore, Roma)
- Prof Ruggero De Maria (Università Cattolica del Sacro Cuore, Roma)

MAIN OBJECTIVES OF THE PROJECT

1. Investigate the impact of immunogenic therapy on cancer stemness.
2. Identify the strategies adopted by CSCs to evade immune surveillance.
3. Develop novel strategies to sensitize CSCs to immunogenic therapy.

RESULTS ACHIEVED during the reporting period

In this period of activity, we optimized murine models of CSCs to explore the immune escape of CSCs and their resistance to immunotherapy. We demonstrated that the treatment with inducers of immunogenic cell death (ICD, a type of regulated cell death activating an adaptive immune response in immunocompetent

hosts) favors the emergence and selection of subpopulations of therapy-resistant cells with phenotypic and functional properties of CSCs. We confronted breast CSC and non-CSC fractions with histocompatible splenocytes in microfluidic devices. By videomicroscopy we showed that non-CSCs attracted and stably interacted with splenocytes at 24h, while CSCs failed to do so, migrating towards splenocytes and starting a transient interaction only upon 48h (**Figure 2A**). Using a syngeneic murine model, we observed that CSCs induced by immunogenic therapy display high aggressiveness and low immunogenicity. This was demonstrated by the high percentage of immunocompetent mice developing tumors (**Figure 2B**) and lung metastasis (not shown) once xenografted with syngeneic CSCs.

In following epigenetic analyses, we revealed a key role of the demethylase KDM1B in promoting and maintaining cancer stemness. Through ATAC-Seq studies in CSCs and non-CSCs isolated from cancer cells subjected to immunogenic therapy, we observed that CSCs display a higher chromatin accessibility in stemness associated genes as well as in the gene encoding KDM1B (**Figure 2C**). Importantly, in *in vivo* studies, we observed that the treatment with the KDM1B inhibitor tranylcypromine (TCP) reduced the fraction of CSCs induced by ICD inducers (**Figure 2D**), an effect that was associated with an enhanced antitumor efficacy of immunogenic therapy (**Figure 2E**).

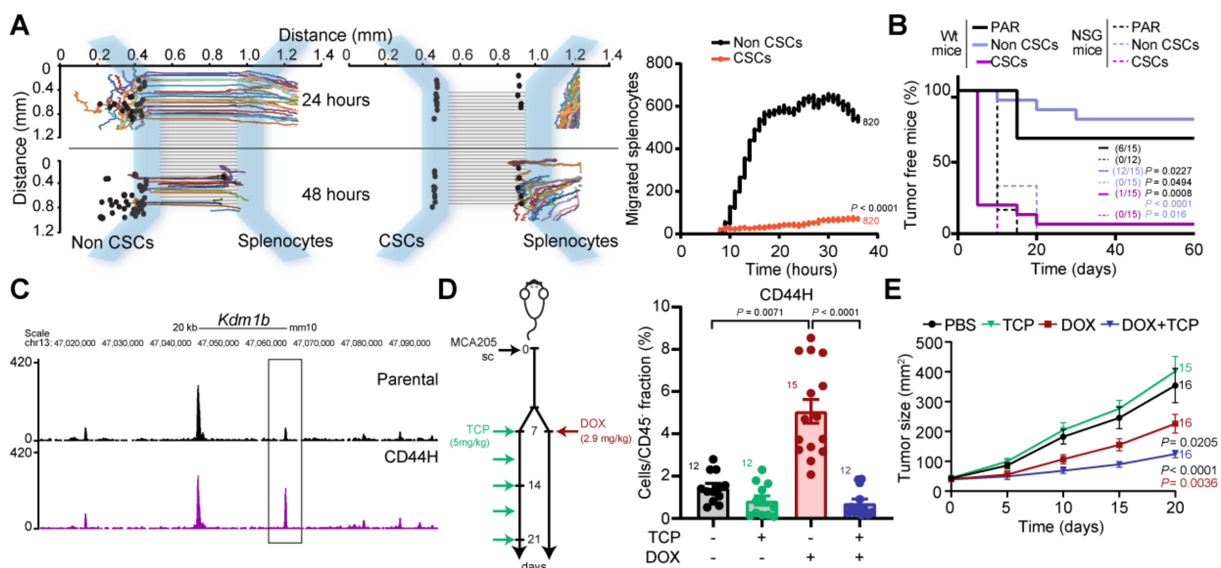


Figure 2. CSC immunogenicity. A. Time-lapse analysis of splenocyte migration towards AT3 cell-isolated CSC and non-CSC fractions in microfluidic devices. Plots represent trajectories of individual splenocyte towards target cancer cells (black spots). Quantification of interaction times are also shown. **B.** *In vivo* evaluation of the tumorigenicity of parental MCA205 cells (PAR) and MCA205-isolated CSCs and non-CSCs in C57Bl/6J (Wt) or NSG mice. The percentage of tumor-free mice is shown. **C.** Representative Kdm1b loci from ATAC-seq studies in PAR and CSCs. **D**,**E.** Ex vivo multiparametric flow cytometry analysis of CSCs (CD44H) percentages (**D**) and *in vivo* MCA205 tumor growth control in mice treated as illustrated (**E**). Tumor growth curves are reported. DOX, doxorubicin; OXP, oxaliplatin; TCP, tranylcypromine. Ordinary two-way RM ANOVA test with Bonferroni's correction (**D**) and Log-rank (Mantel-Cox) test (**E**).

We are now exploring the mechanisms behind the low immunogenicity of CSCs. We are also trying to validate results on KDM1B to unveil whether epigenetic therapy can improve immune-mediated targeting of CSCs.

- Title of the project “**Chromosomal instability and immunogenicity of CSCs”**

(Team: G. Manic, A. Guarracino, S. Soliman, L. Mattiello, G. Campia)

COLLABORATIONS:

- Dr Antonella Sistigu (Università Cattolica del Sacro Cuore, Roma)
- Dr Lorenzo Galluzzi (Weill Cornell Medical College, New York, USA)
- Prof Matteo Cereda (IIGM, Candiolo)

MAIN OBJECTIVES OF THE PROJECT

1. Explore the immunogenic potential of CSCs with an increased chromosome number.
2. Determine the mutational, antigenic, transcriptomic, and proteomic profiles of CSCs with an increased chromosome number.
3. Develop novel strategies to increase the immune visibility and immune attack of CSCs.

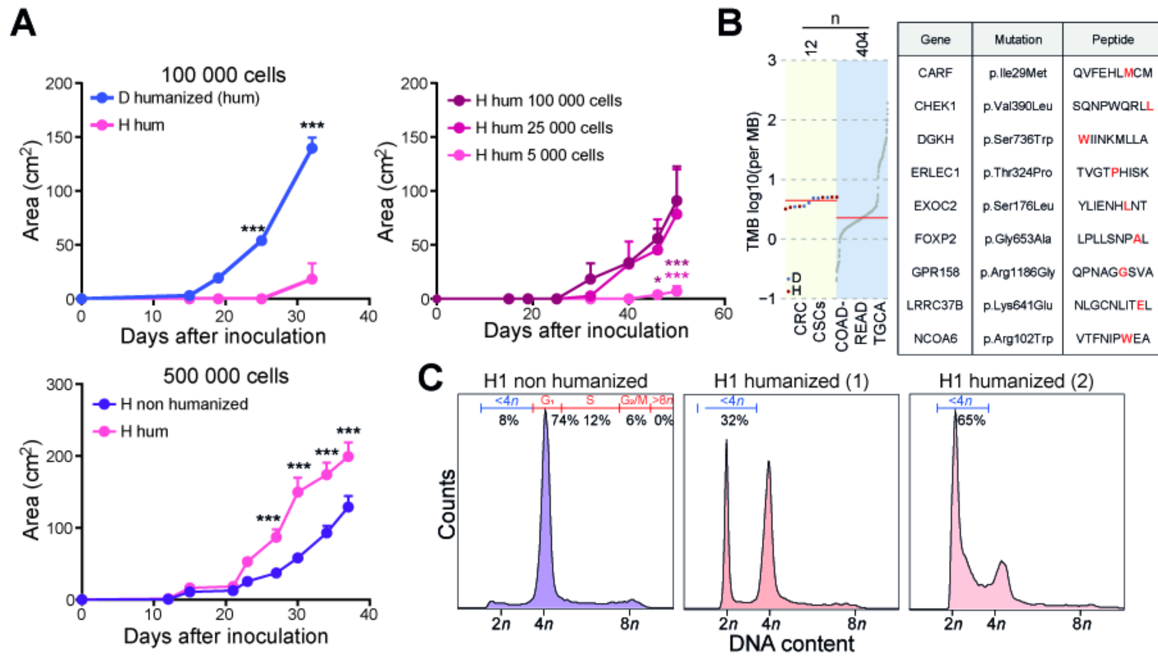
RESULTS ACHIEVED during the reporting period

In this period of activity, we demonstrated that an increase in ploidy due to whole-genome duplication boosts the immunogenic potential of CSCs, both *in vitro* and *in vivo*. We used a model of humanized mice, i.e., immunodeficient NSG mice subjected to the protocol of humanization through injection of peripheral blood mononuclear cells (PBMCs) from histocompatible healthy donors. Humanized mice were xenografted with primary colorectal CSCs with different ploidy and tumor development and growth were followed over the time. We observed that hyperploid CSCs (H-CSCs, i.e., CSCs with a redoubled genome content) grew less efficiently than their pseudo-diploid counterparts (D-CSCs) when injected in humanized mice (**Figure 3A**). This evidence supports the existence of an immunosurveillance mechanism directed against CSCs that underwent whole-genome duplication.

To characterize the immunogenicity of H-CSCs, we profiled the mutational and antigenic landscape, by whole-exome sequencing (WES, also including healthy tissue) and RNASeq. By integrating data on the tumor mutational burden (TMB), copy-number and mutation multiplicities, we revealed a higher number of mutations (but not of distinct mutations) in H-CSC clones than D-CSC clones (**Figure 3B**). We could also identify a panel of unique mutations and, using prediction algorithms, a list of potential unique neoantigens present in H-CSCs (**Figure 3B**). We are now performing antigen validation and exploring deregulated pathways associated with H-CSC immunogenicity.

We finally revealed a mechanism of immunoediting of H-CSCs. In humanized mice, upon initial antitumor effects, the immune response against H-CSCs resulted in the selection of CSCs with a reduced ploidy and increased capability to escape immune surveillance and grow as tumors (**Figure 3D**). We ascribed such an effect to the high CIN levels of H-CSCs. This evidence indicates that CIN has a duplex, antithetic effect in CSCs,

as it increases CSC immunogenicity but eventually promotes the generation of a progeny with high potential to evade immune surveillance.



Gene	Mutation	Peptide
CARF	p.Ile29Met	QVFEHLMCM
CHEK1	p.Val390Leu	SQNPWQRLL
DGKH	p.Ser736Trp	WIINKMLLA
ERLEC1	p.Thr324Pro	TVGTPHISK
EXOC2	p.Ser176Leu	YLIENHLNT
FOXP2	p.Gly653Ala	LPLLSNPAL
GPR158	p.Arg1186Gly	QPNAGGSVA
LRRC37B	p.Lys641Glu	NLGCNLITEL
NCOA6	p.Arg102Trp	VTFNIPWEA

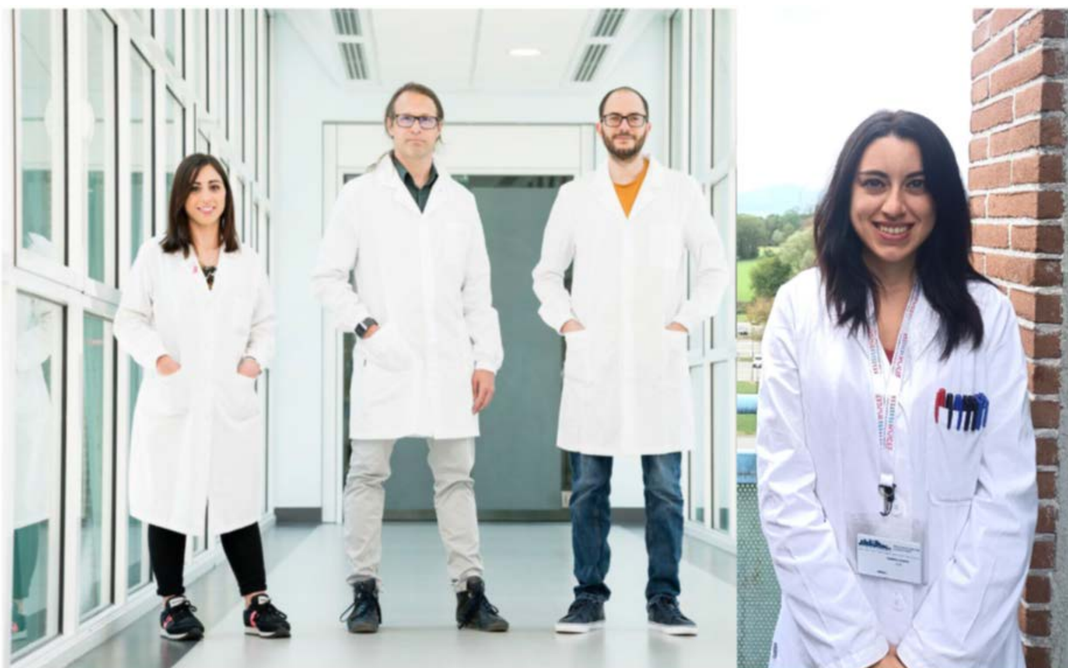
Figure 3. Ploidy increase and CSC immunogenicity. A. Tumor growth analysis of pseudo-diploid (D) and hyperploid (H) CSCs as reported**.** Mann–Whitney test (left, below), and Kruskal–Wallis ANOVA followed by Dunn’s post-hoc test (right). **B.** Determination of the tumor mutational burden (TMB) of CSCs and lists of potential antigens of H-CSCs identified by integration of WES+RNASeq and prediction algorithm. **C.** Flow cytometry-mediated analysis of cell cycle profiles on explanted H-CSC-derived tumors stained with DNA dye.

Our future research will try to identify specific strategies to limit ploidy reduction, keeping up the immunogenicity of H-CSCs.

NEWFUNDED PROJECTS

- Associazione Italiana per la Ricerca sul Cancro (AIRC). Project Title: "*Harnessing replication stress for effective therapies against microsatellite and chromosomal unstable colorectal cancers*" (PI: I. Vitale). 2023-2027
- Associazione Italiana per la Ricerca sul Cancro (AIRC). Project Title: "*Exploring the impact of the mitotic checkpoint on clonal response and therapy resistance of colorectal cancer*" (Fellowship for L. Mattiello under supervision of I. Vitale). 2023-2024
- IIGM - Italian Institute for Genomic Medicine. Project Title: "*Identifying the mechanisms and biomarkers of resistance to PARP1 inhibitors in cancer stem cells*". (PI: I. Vitale IIGM and Dr P. Pichierri INBB). 2023.
- Ministero della Salute. Project Title: "*Deconstructing cancer therapy resistance: integration of advanced in vitro, in vivo and in silico models to dissect patient-specific mechanisms of chemo/immunotherapy resistance, identify novel therapeutic vulnerabilities and generate personalized strategies to target relapse-inducing cancer cells*". (PI: I. Vitale). 2023-2025
- Ministero della Salute. Project Title: "*Investigating the mitotic checkpoint in patient-derived models for effective (immuno)therapy against colorectal cancer*" (PI: G. Manic). 2023-2026

Immunotherapy Unit



Unit research members

- Tobias Longin Haas, head of Unit (IIGM), assistant professor (UCSC)
- Jolanda Magri, Ph.D. student (UCSC)
- Roberta Giampà, fellow technician (IIGM)
- Matteo Menotti, Ph.D., post-doctoral fellow (IIGM) until 31/08/2022
- Alessandro Abbati, fellow technician (IIGM) until 31/09/2021

Brief description of the UNIT

The Immunotherapy unit is composed of a small group of young and early-stage scientists following a line of research that emerged from a high throughput screening for antibodies binding to surface molecules of primary colorectal cancer cells performed at the UCSC in Rome. The unit was established at IIGM at the beginning of 2020 and dedicates its activities to generating novel strategies to involve natural immunity in fighting and eradicating solid tumors.

In the last years, it became evident that supporting the anti-tumor activities of the immune system can provide a very effective weapon in the fight against cancer. Starting from this assumption, immune checkpoint inhibitors (ICIs) were developed. This new class of drugs can unleash forces of T cells that are

usually suppressed by the tumor or by the tumor microenvironment and result in remarkable clinical outcomes in several solid tumors including lung cancer and melanoma.

One of the main findings of these studies was that tumors with a high mutational load that have an immunologically active (or immunologically hot) microenvironment are more susceptible to ICI treatment, while tumors with lower mutation rates, containing few infiltrated immune cells have an immunologically cold tumor microenvironment and do not respond as well. The aim of the research done in this unit is to boost the intrinsic anti-tumor activity of T cells and to turn an immunologically cold into an immunologically hot tumor (e.g., colorectal cancer (CRC)).

The main strategies to reach these aims are based on proprietary monoclonal antibodies specifically binding surface structures on primary tumor cell cultures developed in collaboration with Prof. De Maria at the UCSC. The sequence information of these antibodies was used to generate scFvs enabling the generation of novel "bispecific T cell engagers" (BiTEs) and "chimeric antigen receptor T cells" (CAR T cells). In the projects performed at IIGM novel formats of the BiTEs and innovative fourth-generation CAR T cells are developed, not only targeting the tumor cells but also aimed at fueling an inflammatory response within the tumor microenvironment.

RESEARCH QUESTION(S):

Project 1:

In the first project, we investigate if it is possible to generate therapeutically effective bi-specific T cell engagers (BiTEs) based on proprietary tumor-targeting antibodies identified in a high throughput hybridoma screening. Because solid tumors are very hard to treat with this kind of approach, we opted for a combinatorial approach creating BiTEs activating the T cell receptor complex and BiTEs that bind and activate co-stimulatory receptors on the surface of the T cells.

Project 2:

Heterogeneity in the expression of tumor antigens is one of the main obstacles in treating solid tumors. Thus, we set up the second project to answer the question of whether modified 4th generation CAR T cells may be a suitable vector to deliver "danger signals" into a solid tumor and thus exert a stronger anti-tumor response by activating or reprogramming tumor-associated antigen-presenting cells.

MAIN RUNNING FUNDINGS

- Period: 02/01/2020-01/01/2025: Tobias Haas is co-PI of the project entitled: "Development of therapeutic antibodies and CAR T cells for the treatment of advanced colorectal cancer and glioblastoma" (AIRC Investigator Grant – IG 2019)
- Period: 02/01/2020-01/01/2023: Tobias Haas is co-PI for the research carried out at the IRCCS Fondazione Policlinico Universitario A. Gemelli in work packages 2 and 3 of the project: "Progetto di ricerca sulle cellule T CAR per i tumori ematologici maligni e solidi". (Ministero della Salute RCR-

2019-23669115)

Main achievements in the period covered by the Scientific Report

Generation and validation of novel BiTEs antibodies

(Team: **Matteo Menotti**, Roberta Giampà, Alessandro Abbati)

COLLABORATIONS:

- Dr U. Warnken (German Cancer Research Center, Heidelberg, Germany)
- Prof R. De Maria (Università Cattolica del Sacro Cuore, Roma)
- Dr Eleonora Ponterio, Istituto Superiore di Sanità, Roma)

MAIN OBJECTIVES OF THE PROJECT

1. Design, manufacturing, and purification of bispecific antibodies (BiTEs) activating human and murine T cells based on the sequence information of the TAA-specific 13C4 antibody.
2. Enhance the immunological potential of T lymphocytes against tumor cells by using BiTEs targeting co-stimulatory receptors on the T cells.
3. Test the ability of antibodies to activate T lymphocytes against tumor cells *in vitro* and *in vivo.*

RESULTS ACHIEVED during the reporting period

Using recombinant DNA technologies, we generated bispecific T cell engagers (BiTEs). Canonical BiTEs crosslink the endogenous T cell receptor complexes in the presence of tumor cells expressing a respective tumor-associated antigen leading to the activation of the lymphocyte.

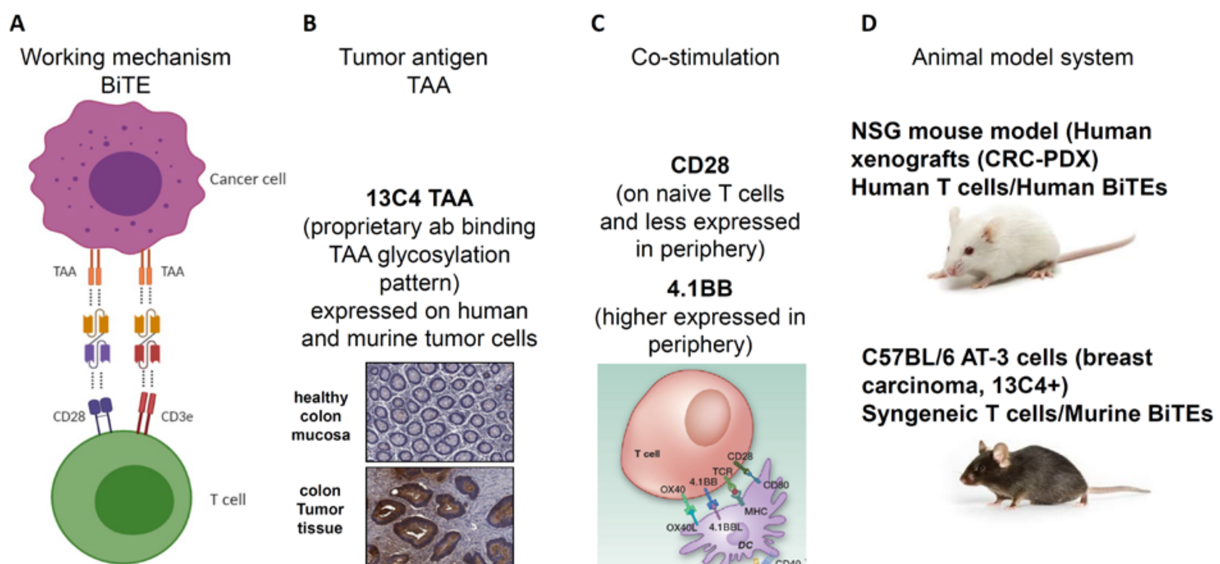


Figure 1: (A) The strategy of activating T cells in the presence of tumor cells. (B) description of the TAA identified by high content hybridoma screening, (C) Co-stimulatory BiTEs used. (D) *In vivo* model systems to be used to test the BiTE function in mouse models.

The BiTEs designed and generated at IIGM consist of two monoclonal antibody (mAb)-derived antigen-binding domains, that are expressed as single chain fragments of the variable chains (scFvs) linked by a flexible amino acid sequence. The activation of the T cells is obtained by the binding of the BiTE to the CD3 epsilon receptor (CD3e), within the T-cell receptor (TCR) complex. We generated a second BiTE acting as a co-stimulatory signal (**Figure 1A**). The activation of the T cell will cause the release of perforin and granzyme B and the subsequent apoptotic death of the tumor cell expressing the tumor-associated antigen (TAA) that was found to be specifically expressed on the surface of CRC (see **Figure 1B**). To obtain a stronger T cell activation we have also designed two more BiTEs to be used in combination with the previous one that has, instead of the CD3e scFv, another sequence that can bind and activate the CD28 co-receptor or 4.1BB on T lymphocyte. Using the TAA/CD28-BiTE in combination with the TAA/CD3e-BiTE will allow us to support the activation via CD3e and to activate naïve T-cells, which are usually not fully activated by a single stimulation. While using the TAA/4.1BB-BiTE will co-activate the tissue-resident memory T cells (**Figure 1C**). The function of these BiTE formats will be tested in xenograft models using CRC PDX-derived tumors and furthermore in syngeneic mouse models. To this end, we designed 5 independent BiTEs (TAA/huCD3; TAA/HuCD28; TAA/hu4.1BB; TAA/muCD3 and TAA/muCD28, **Figure 1D**).

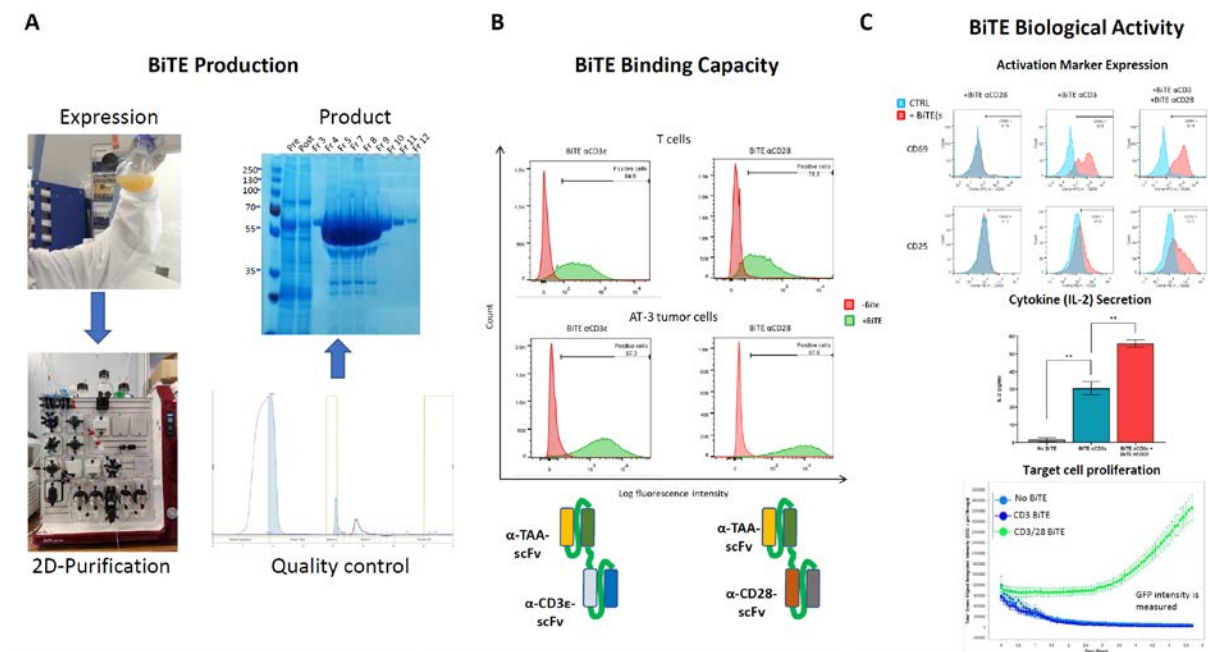


Figure 2: (A) Workflow for the expression of the recombinant BiTEs. (B) Evaluation of the surface binding on T cells and tumor cells by flow cytometry. The binding was visualized with an anti-V5 antibody that binds the C-terminus of the BiTE. Functional analyses of the BiTE (upper panel: increase of the T cell activation markers CD25/CD69, middle panel: release of IL-2 upon co-culture of T with target cells. Lower panel: the real-time proliferation of the EGFP-positive target cells in the presence of T cells +/- the BiTEs indicated.

In the last couple of years, we set up a functional workflow of cloning, expressing, and purifying the BiTEs described above (**Figure 2A**). For all the BiTE formats we tested the binding to both, the tumor-associated antigen (TAA) recognized by our proprietary scFv derived from 13C4 and the second target (CD3e, CD28, and 4.1BB) expressed on the surface of human or murine T cells. As exemplified in **Figure 2B**, we obtained BiTEs that showed binding on the surface of tumor cells via the 13C4 scFv portion and binding to the surface of T cells via the CD3e-targeting scFv, or the CD28-targeting scFv, respectively. Furthermore, we evaluated the ability of our BiTEs to activate lymphocytes in the presence of tumor cells by measuring the abundance of the T cell activation markers CD69 and CD25. As expected, T cells enhanced the expression of both markers when cultured with primary colon cancer cells and the BiTE TAA/CD3 (**Figure 2C**, upper panel, center histogram), the second BiTE TAA/CD28 alone does not affect lymphocyte activation (left histogram) but amplifies the effect of BiTE TAA/CD3 when combined with it (right histogram). A similar effect was observed for the secretion of the pro-inflammatory cytokine IL2. (Figure 11C middle panel). While the coculture of human T cells with tumor cells and the TAA/CD3-BiTE activated the cells as expected, the addition of the TAA/CD28 BiTE enhanced the cytokine secretion and thus the activation of the T cells. To monitor the anti-tumoral activity of the T cells in the presence of the BiTEs over time, we used primary CRC tumor cells engineered to express an enhanced green fluorescent protein (EGFP) and performed real-time proliferation assays using the IncuCyte system. As shown in **Figure 2C** (lower panel) the number of cells expressing the EGFP is blunted in the presence of the TAA/CD3 BiTE and the combination of TAA/CD3 and TAA/CD28 BiTE. Thus, we can deduce that lymphocytes have also gained cytotoxic activity and eliminated the targets. Recent results showed that by using suboptimal doses of the TAA/CD3-BiTE, the addition of the TAA/CD28 BiTE results in stronger anti-tumor activity *in vitro* (data not shown). The next steps of this project will be to evaluate the ability of BiTEs to perform their function also *in vivo* using immunocompromised mice with subcutaneous tumors. Because our 13C4 scFv recognizes and binds to both human and murine tumor cells, we have also produced two other BiTEs that are able to activate murine lymphocytes (data not shown). We will use these BiTEs to test their preclinical validity in an immuno-competent mouse model (C57BL/6 mice).

Generation of 4th generation CARs targeting TAAs in solid tumors

(Team: **Jolanda Magri**, Matteo Menotti, Roberta Giampà)

MAIN OBJECTIVES OF THE PROJECT

1. Design of fourth-generation CAR T cells.
2. Selection of the best promoter that allows secretion of the entolimod protein upon CAR T cell activation.
3. Validation of the antitumor efficacy of CAR T cells *in vitro* and *in vivo.*

RESULTS ACHIEVED *during the reporting period*

The second project is based on the recent finding that vaccination approaches targeting tumor-associated antigens (TAAs) in solid tumors can help to break the immunological tolerance. It was shown that intratumoral administration of immunomodulators can improve the immunogenicity of cancer vaccines. However, even if some adjuvants are licensed for clinical use, intratumoral injection is not always feasible, considering metastatic disease and patient compliance. On the other hand, a weakness of the current CAR T cell therapy is that, due to tumor heterogeneity, antigen loss is commonly observed, this permits the outgrowth of antigen-negative cells and hampers the long-term response to the treatment.

To overcome these limitations, we have engineered CAR T cells that, following their activation, not only cause the direct lysis of the target cells but also secrete a modified version of a toll-like receptor 5 (TLR5) ligand, leading to the activation of the innate immune system. Our hypothesis is that this construct will get expressed and secreted by CAR T cells when they infiltrate the tumor mass and get in contact with the TAA recognized by the CAR. In turn, the TLR5 ligand will then activate innate immune cells present in the tumor microenvironment (TME). Once activated, these cells will take up tumor antigens derived from tumor cells killed by the CAR T cells and migrate into the lymph nodes to present these peptides which results in the expansion of new tumor-specific T cells. These will subsequently migrate back into the tumor, as well as into possible metastases, contributing to their elimination independent of the TAA recognized by the CAR T cells (Figure 3). Given the fast-moving field of CAR T cell therapies, we are confident that in case we see efficacy *in vitro* and in animal models, this treatment regime has some potential to be translated into the clinical setting.

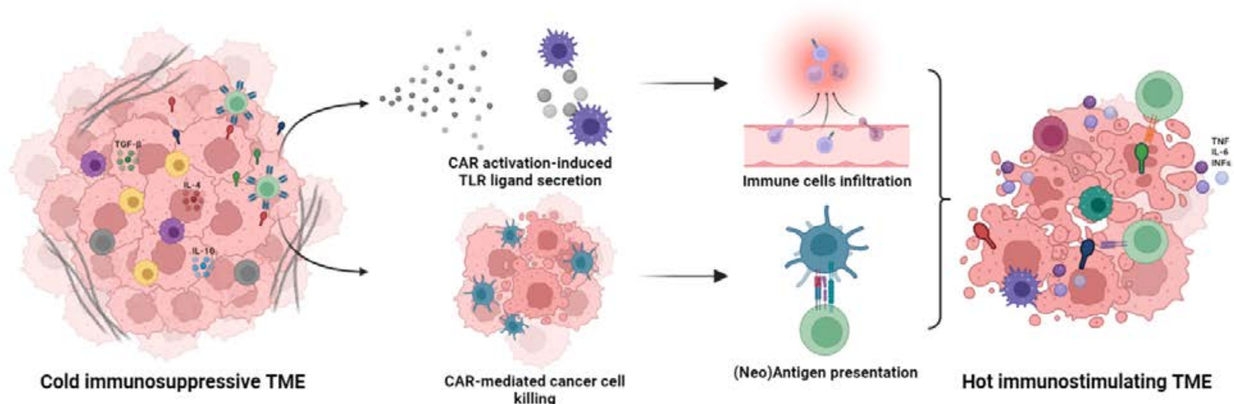


Figure 3: Scheme of the hypothesis

To develop a tumor model in which the percentage of TAA-positive and -negative tumor cells can be tuned (see **Figure 4A**), we generated murine CRC tumor cells (MC38) expressing a non-functional, truncated form of the human epidermal growth factor receptor (trEGFR, **Figure 4B**). These cells can be co-injected with trEGFR-negative tumor cells in syngeneic C57BL/6 mice to establish a tumor consisting of TAA-positive and negative cells. Furthermore, this will allow us to work with a fully immunocompetent model to study the effects of CAR T cells on the TME.

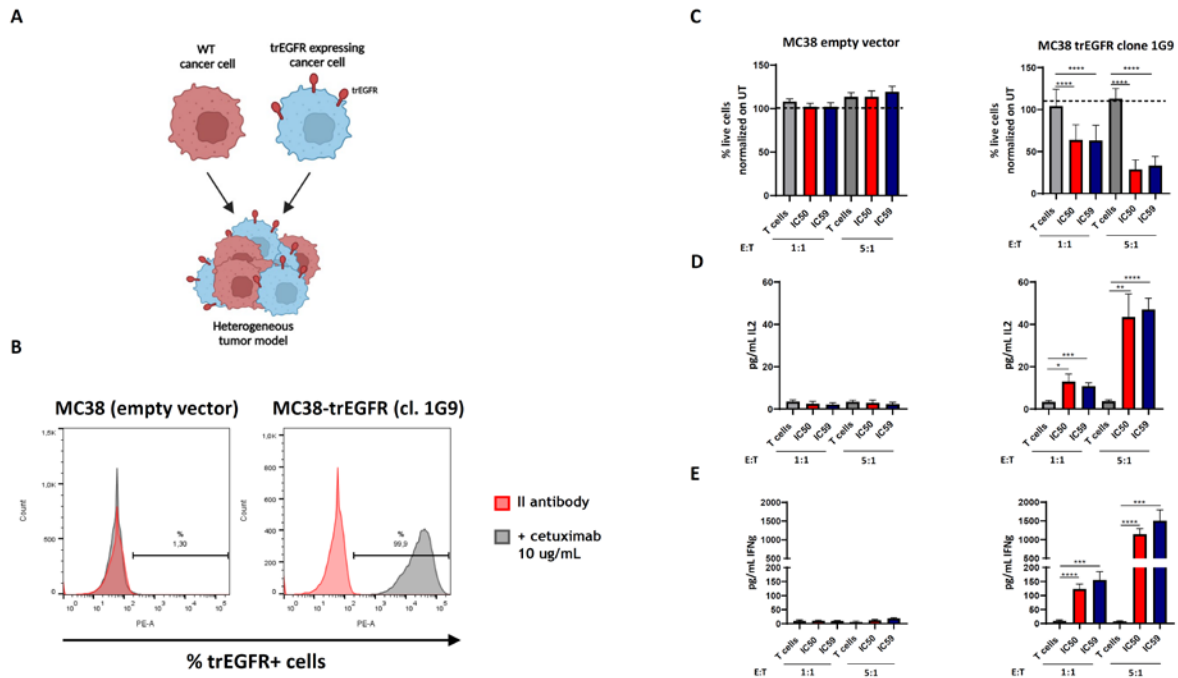


Figure 4: (A) The system used to create a heterogenous expression of the TAA. (B) Expression of trEGFR recognized by the cetuximab scFv in MC38 cells stably transfected with a pCDNA3.1 vector encoding for the gene of interest. (C) Killing ability and (D+E) cytokine production was measured by ELISA of CAR-T cells (IC50 and IC59) co-cultured with target cells for 48 h at different E:T ratios. The viability of MC38 target cells expressing luciferase was measured through luminescence detection. Data are represented by at least 3 independent experiments. * p-value <0,05.

The variable portion of the commercial antibody Cetuximab, able to recognize trEGFR, has been used to design the extracellular part of the chimeric receptor expressed on T cells. We transduced primary murine T cells with two independent CAR-expressing vectors (IC50 and IC59), differing only in the presence of a puromycin resistance cassette, and tested their killing ability, cytokine production, and activation status of the CAR T cells in coculture with target cells. Indeed, after 48 hours of coculture, the viability of target cells measured by their expression of a luminescent signal was significantly reduced (**Figure 4C**). The killing of target cells was accompanied by secretion of the activation-induced cytokines IL2 (**Figure 4D**) and IFNγ (**Figure 4E**), and upregulation of the activation markers CD25 and CD69 (data not shown). These results indicated that the CAR T got activated and killed selectively trEGFR-positive target cells *in vitro*, laying the foundation for *in vivo* experiments.

To achieve the synthesis and secretion of the entolimod protein (TLR5-ligand) by T cells, we designed several DNA constructs carrying entolimod together with sequences of signal peptides for secretion into the supernatant. These include the immunoglobulin K signal peptide (IgK SP) or the so-called "cell-penetrating peptides" (CPPs) capable of passing through the cellular lipid membrane, namely transportan, an amino acid repeat of arginine (polyR) and a protein sequence belonging to homeoproteins termed SecPen (Secretin-Penetratin). These constructs were cloned into a plasmid vector under a constitutive promoter. We tested the production and secretion of 8 different entolimod constructs shown in **Figure 5A** using transiently

transfected HEK293T cells and detected activity in all supernatants (data not shown). By western blot, however, we observed different levels of protein production and secretion among the various constructs (data not shown). Based on these data, we chose the IgK SP alone or the IgK SP in combination with polyR as our "lead constructs" for further analyses. Then we generated 3T3 murine fibroblasts stably expressing entolimod to assess the continuous production of our ligand and possible related toxic effects. We confirmed the production and secretion of both entolimod constructs in supernatants of transduced 3T3 cells by western blot (**Figure 5B**) and validated its ability to stimulate TLR5 in a sensor cell line (**Figure 5C**). Moreover, we were able to detect TLR5 stimulation upon a permeability assay that suggested the ability of entolimod to diffuse through cell membranes and possibly through the tumor mass (**Figure 5D**).

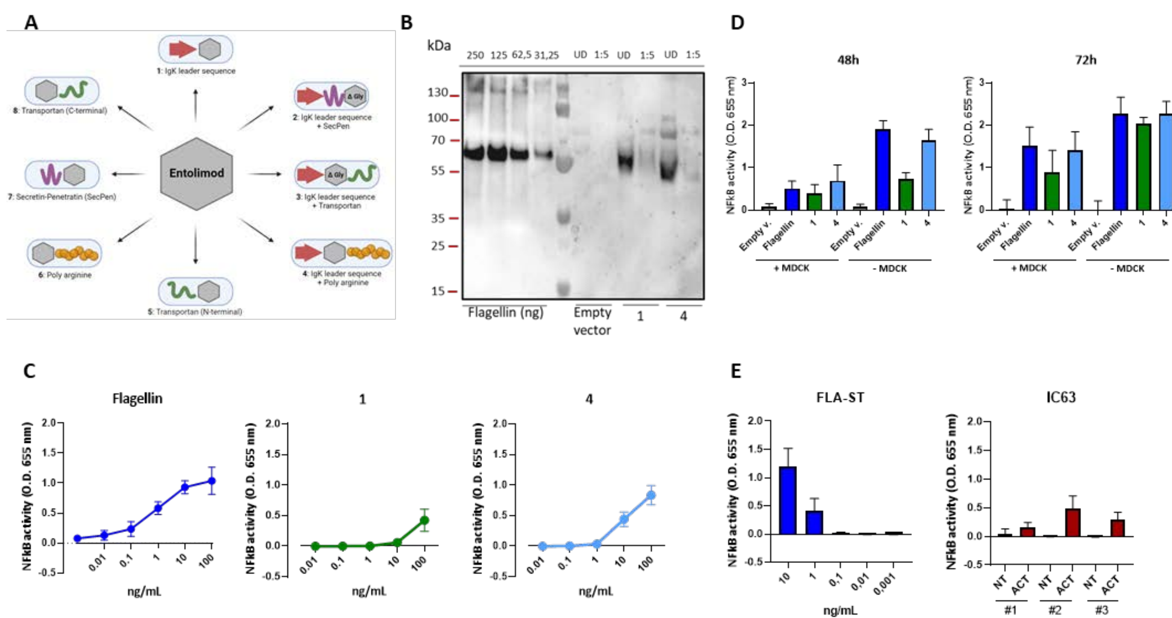


Figure 5: (A) Representative scheme of entolimod constructs cloned in a pCDNA3.1 vector for production and secretion by cells. (B) Western Blot analysis of supernatants of 3T3 murine fibroblasts stably producing entolimod in constructs 1 and 4. (C) TLR5 binding and stimulation by entolimod and flagellin (control) measured through a colorimetric-based assay using supernatants of 3T3 entolimod-producing cell lines. (D) Diffusion assays through MDCK cell impermeable monolayer of entolimod constructs 1 and 4 and measurement of TLR5 stimulation. (E) TLR5 stimulation by flagellin (control) and entolimod produced under the control of the inducible 6xNFAT promoter upon CD3/CD28 stimulation of transduced primary murine T cells.

To achieve inducible production of entolimod by T cells, we designed an inducible promoter based on six repetitions of the consensus sequence bound by the nuclear factor of activated T cells (NFAT) transcription factor, which is recruited upon T cell activation. Indeed, we detected TLR5 stimulation by the supernatants of CD3/CD28 activated T cells transduced with the inducible construct and not in control cells, indicating inducible secretion of entolimod (**Figure 5E**).

We are currently improving transduction efficiencies and expansion of primary murine T cells for the *in vivo* validation of our 4th generation CAR T cells. Moreover, the ongoing RNAseq experiments performed on T cells and macrophages cocultured in the presence or absence of entolimod will unravel genomic signatures that

will be validated in further experiments.

Awards:

Tobias Haas is PI of the project entitled "Development of Monoclonal Antibody-based Therapeutics for the Treatment of Cancer, in Particular Colorectal Cancer" receiving a £100.000 award from LifeArc for preclinical development of a monoclonal antibody inhibiting CRC growth.

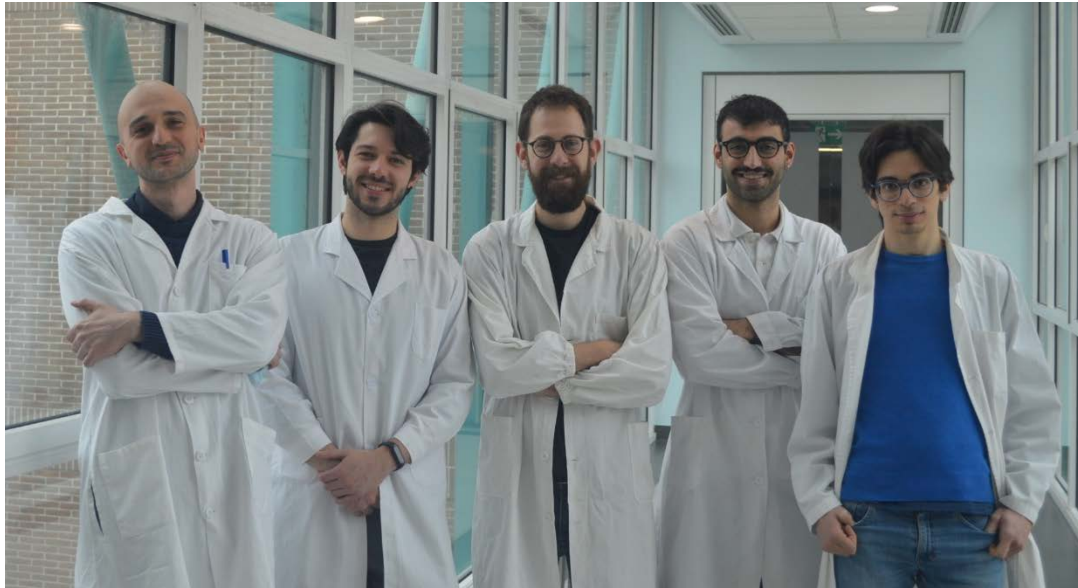
Linea PREMIO, Premio pubblicazione di alta qualità (2019)
Università Cattolica del Sacro Cuore

Linea PREMIO, Premio pubblicazione di alta qualità (2017)
Università Cattolica del Sacro Cuore

Patents:

Tobias Haas is co-inventor of patent application WO2014072940A1; PCTIB2013/059993: Inhibitor of integrin for treatment or prevention of tumours.

Chemical Biology Unit



Unit research members

- Carlo Cosimo Campa, Principal investigator
- Luca Gozzelino, Post-doctoral fellow
- Giampaolo Placidi, PhD student
- Simone Tealdi, PhD student
- Aastha Jaipuria, Undergraduate student
- Stefano Romanazzi, Undergraduate student

Visiting scientist over the year

- Gabriele Rigano, Undergraduate student, Polytechnic school of Turin
- Paulius Gibieža, Post-doctoral fellow, Lithuanian University of Health Sciences

Brief description of the UNIT

Despite significant advances in human genetics over the past two decades, the percentage of the human proteome that is targeted by small molecules remains quite small compared to the total number of genes found to be associated with a disease phenotype. In part, this is due to the lack of technologies that can systematically address the vulnerability of uncharacterized disease-relevant proteins or pathways and the associated scientific and commercial opportunities.

Alterations in membrane trafficking pathways are associated with many human diseases, including cancers, neurodegenerative disorders, and more than 340 distinct Mendelian diseases, reflecting new opportunities for target discovery. However, current approaches do not systematically address the vulnerability of membrane trafficking pathways to pharmacological inhibitors.

The Chemical Biology unit aims to develop and validate enabling technologies that have the potential to drive a sea change in the ability of basic scientists to achieve relevant clinical and health goals. Particular attention is paid on pathways that enable the internalization and delivery of biomolecules and biopharmaceuticals into cell's intracellular compartments.

Our research program has three main goals:
1) to develop new technologies for the analysis of membrane trafficking pathways.
2) to understand the structure-function relationships of membrane trafficking networks.
3) to identify drugs that can reverse disease phenotypes caused by alterations in membrane trafficking.

The research unit's activities include the development of biosensors and gene editing tools for high-throughput identification of novel biomarkers and drug inhibitors that, by acting on specific intracellular trafficking pathways, have the potential to transform the therapeutic treatment of incurable diseases.

MAIN RUNNING FUNDINGS

- Italian Association for Cancer Research (AIRC), "Mixing of Rab GTPase membrane domains reduces glioblastoma response to immunotherapeutic agents", 2021-25.

Main achievements in the period covered by the Scientific Report

Visualize feedback loops on the surface of a single endosome using correlation analysis

COLLABORATIONS:

- Dr Carla Bosia and Elsi Ferro (Polytechnic of Turin and IIGM)

MAIN OBJECTIVES OF THE PROJECT

The ability to parse out biochemical signalling events in time and space would help to identify both network topology and activity patterns characterizing biomolecular systems. However, current approaches do not allow to resume the relationship between molecular signalling components into a single estimate.

RESULTS ACHIEVED during the reporting period

We demonstrate that both the direction and the extent feedback loops in biochemical circuits can be quantified by the spatial correlation. We combine both FRET-based imaging and statistical approaches to the analysis of the information signalling flow occurring at the surface of endocytic organelles.

By measuring the association between the active fraction of signalling molecules and changes in their molecular level, we report unprecedented observations of feedbacks and putative crosstalk between Rab5 and Rab11 biomolecular switches on the membrane surface of a single endosome (**Figure 1**). Theoretical prediction and experimental data provide clues on Rab protein stoichiometry in controlling feedback signal and support a role for Zfyve26 as a Rab5-Rab11 signalling regulator. Our spatial mapping provides a powerful system to visualize and understand the information flow in biochemical signalling networks even at the submicrometric scale.

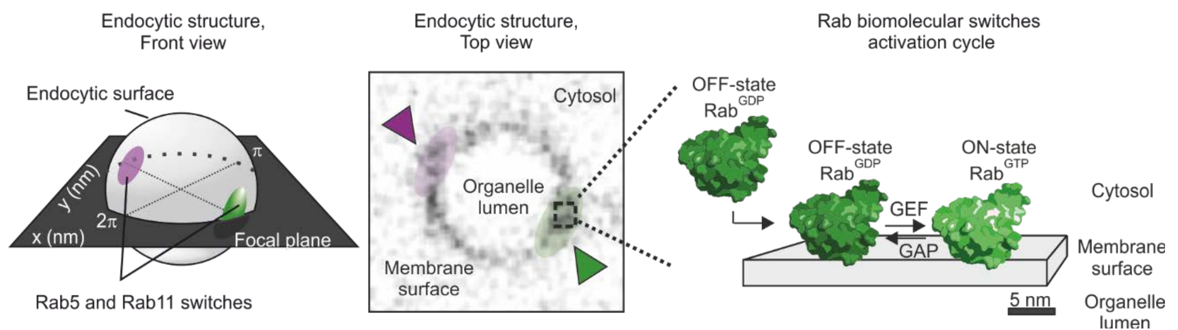


Figure 1. Schematic of the imaging system used for the investigation of feedback loops at the surface of endocytic vesicles. The activation mechanisms and abundance of both Rab5 (green) and Rab11 (purple) molecular switches was employed to obtain information about feedbacks and functional crosstalk's.

Engineering Rab GTPase biosensors for drug discovery

(Team: Luca Gozzelino)

MAIN OBJECTIVES OF THE PROJECT

The ability to visualize signalling activities of membrane trafficking pathways would help to identify both molecular components of biological signalling networks and chemical compounds capable of rescuing disease phenotypes caused by membrane trafficking alterations. However, current technologies do not allow the estimation of membrane trafficking pathway activity using high-throughput platforms.

RESULTS ACHIEVED during the reporting period

We demonstrate that genetically-encoded Rab small GTPase indicators are powerful tools for detection of membrane trafficking pathways activity at high-throughput.

Inspired by proteins that fluorescence in response to chemical ligands, we have developed a green emitting fluorescent Rab activity reporter that exhibits a high molecular brightness and a linear response to Guanine Nucleotide Exchange Factors (GEFs) and GTPase Activating proteins (GAPs). We demonstrated the utility of this genetically encoded biosensor for the identification of novel Rab11 regulators. Our biosensor technology provides a powerful system to quantify the signalling activity of membrane trafficking routes even in complex experimental settings take advantage of size reduction to minimize research costs.

Perturbation of membrane trafficking pathways with small molecules in glioblastoma

(Team: Luca Gozzelino, Simone Tealdi, Aastha Jaipuria, Giampaolo Placidi)

MAIN OBJECTIVES OF THE PROJECT

The ability to interfere with membrane trafficking pathways would help control the cell surface proteome and, consequently, the ability of cells to sense and respond to environmental changes. However, it is unclear whether disruption of intracellular transport could be a strategy to improve antibody-based cancer treatments.

RESULTS ACHIEVED during the reporting period

We demonstrated that the endocytic recycling trafficking pathway is involved in the control of EGFR cell surface expression in a human model of glioblastoma (GBM), a brain tumour resistant to EGFR monoclonal antibody therapies.

Using a Rab11 biosensor-based approach, we found that chemical inhibition of phosphatidylinositol 4,5-bisphosphate synthesis, a membrane lipid constituent, reduced Rab11 activation and simultaneously decreased EGFR surface expression in GBM cells. This effect was not rescued by overexpression of Rab11 isoforms (RAB11A, RAB11B, RAB25), although these were found to control the protein level of EGFR. These

data suggest that PIP5K2C blocks EGFR in intracellular compartments and reduces receptor surface expression.

To understand the impact of targeting the Rab11 recycling axis in GBM, we searched for Cetuximab-resistant patient cell lines. We found 4 distinct GBM clones derived from patients with different mutational signatures characterized by high resistance to Cetuximab. In these cells, pharmacological inhibition of phosphatidylinositol 4,5-bisphosphate synthesis reduced cell proliferation in a dose-dependent manner, thus demonstrating that blocking receptor recycling could be a strategy to target tumour cells resistant to antibody-based treatment. Notably, we did not observe an additive effect between Cetuximab and phosphatidylinositol 4,5-bisphosphate reduction, as the combined treatment did not significantly increase the percentage of dead cells compared with the single agent. This could be explained by the downregulation of EGFR induced by chemical inhibition of phosphatidylinositol 4,5-bisphosphate synthesis, which decreases the availability of EGFR, the target of Cetuximab, from the cell surface.

Generation of glioblastoma mouse models for identification of novel therapeutic targets

(Team: Luca Gozzelino, Stefano Romanazzi)

COLLABORATIONS:

- Dr L. Avalle (University of Turin)

MAIN OBJECTIVES OF THE PROJECT

The ability to identify genes that are fundamental during the interaction between cancer cells and their immune microenvironment would help identify therapies that could synergize with signals occurring in the tumour niche. However, current genomic engineering technologies do not allow the modelling of transient gene interactions that might be crucial only at the tumour niche.

RESULTS ACHIEVED during the reporting period

We demonstrate that our CRISPR platform, designed to perform multiple genome engineering when encoded in a single transcript, can be optimized to achieve better transcriptional regulation and inducible response to stimuli characterizing the glioblastoma microenvironment. In our experimental design, the use of this optimized genomic engineering tool will allow the generation of syngeneic mouse model of glioblastoma that will enable discovery of novel immunotherapy targets for tumours characterized by an immunosuppressive microenvironment. We encoded both Cas12a and CRISPR RNAs (crRNAs) downstream of a Polymerase type II promoter to facilitate simultaneous Cas protein and crRNA expression in a single transcript. We tested various combination of both promoter, polyadenylation and posttranscriptional regulatory element sequences to identify the best construct able to repress transcription of a fluorescent reporter. In addition,

we benchmarked several repressor domains to find the best variant that could allow the size constraints imposed by lentiviral vectors to be met.

To improve the stability of crRNAs, we split the single-transcript CRISPR system, thus generating two mRNAs, the first containing the Cas enzyme and the second harbouring crRNAs. Notably, to combine this system into a lentiviral vector, we fused the promoters in a bidirectional orientation. Unfortunately, this system did not provide increased transcriptional repression efficiency. Lastly, to enable transcriptional repression of genes in the presence of specific environmental stimuli, we designed novel promoters responsive to tumour cytokines found abundant in "cold" glioblastoma tumours. Using this trial-and-error approach, we generated a novel CRISPR platform specifically designed for *in vivo* discovery of novel immunotherapy targets.

Generation of glioblastoma mouse models for identification of novel therapeutic targets

(Team: Luca Gozzelino, Simone Tealdi, Stefano Romanazzi)

COLLABORATIONS:

- Dr L. Avalle (University of Turin)

MAIN OBJECTIVES OF THE PROJECT

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NEWFUNDED PROJECT

- Cariplo foundation and Telethon Foundation “Disease mechanism and pharmacological approaches for TBC1D8B-induced steroid-resistant nephrotic syndrome.”, 2023-2025

Unit Genetic and molecular epidemiology

Unit research members

- Alessio Gordon Naccarati, principal investigator
- Barbara Pardini, senior researcher
- Sonia Tarallo, senior researcher
- Paolo Vineis, senior researcher (honorary member)
- Francesca Cordero, researcher at UniTO (visiting scientist)
- Giulio Ferrero, researcher at UniTO (visiting scientist)
- Amedeo Gagliardi, PhD student
- Giulia Francescato, PhD student
- Carla Di Battista, post-graduate fellow
- Virginia Alberini, post-graduate fellow

Former Members over the report years:

- Antonio Francavilla, post doc fellow until October 2022
- Giulia Piaggeschi, post doc fellow until December 2022
- Cecilia Martuzzi, bachelor thesis until May 2022
- Laura Zanatto master thesis until April 2021
- Elton Jalis Herman master thesis until October 2021

VISITING SCIENTISTS Over the report years:

- Agata Sofia Asuncao Carreira PhD student (CIBIO, Trento)

- Arda Keles Phd student on Cost Action STSM (Turkey)
- Barbora Stefikova Erasmus intership (Czech Rep.)
- Beatriz Garcia Fontana EMBL postdoc fellow (Spain)
- Cristina Garcia Fontana, EMBL postdoc fellow (Spain)
- Egeria Scoditti, researcher (CNR, Lecce)
- Katerina Vyklicka PhD student Erasmus intership (Czech Rep.)

Brief description of the UNIT

The Unit follows a research program focused on the integration of data from the clinics (diseases and intermediate phenotypes) and the environment (exposures, lifestyles, dietary habits) with epigenomic and other omics analyses collected in large observational studies. The program of the Unit has three main objectives: (a) to develop and apply new laboratory techniques for the identification of "fingerprints" left on molecules (RNA, DNA, proteins) by environmental exposures in human populations; (b) to identify novel markers in blood and other body fluids that can be used for an early and possibly non-invasive diagnosis of complex diseases such as cancer; (c) to develop and apply cutting-edge methods for statistical analysis and biological interpretation of data from omics technologies.

This Unit work combines population research with the development of molecular tests designed to be transferred into the practice of early diagnosis and primary prevention. The parallel development of high-throughput laboratory technologies, the collaboration with a vast network of researchers (often with a driving function of our Unit in terms of study design and strategic development), and the multidisciplinary approach have made our Unit a unique example in the system of research. The main translational impact derives from the collaboration with several national and international hospitals and institutes; in particular, with the Piedmont Center for Cancer Prevention, we share several lines of research, and the CRC Screening Programs and Molecular Analyses Network.

RESEARCH QUESTION(S):

- To investigate the role of non-coding RNAs (microRNAs, PIWI-interacting RNAs, long non-coding RNAs, circular RNAs, etc.) and gut microbiome in molecular pathology of solid cancers and their potential as innovative diagnostics and novel therapeutic targets in oncology.
- To perform comprehensive and high-throughput analyses of non-coding RNAs, mainly RNAseq and small RNAseq, in tumor tissue or patient’s body fluids for the identification of potential susceptibility, diagnostic, prognostic, and predictive biomarkers to be applied in the clinics.
- To design and coordinate multi-centric prospective studies for the validation of the biomarkers identified in retrospective studies and collaborate on the development of diagnostics to enable higher level

of individualization in clinical management of solid cancer patients (mainly gastrointestinal and urologic cancers).

MAIN RUNNING FUNDINGS

- AIRC Associazione Italiana per la Ricerca sul Cancro. Principal Investigator of the project: “Fecal non-coding RNA markers for a precision medicine approach: how they reflect colorectal cancer onset and progression” funded by (IG 2020 ID 24882). From 01/01/2021 to 31/12/2025.
- EU commission. Partner in the project “Gut OncoMicrobiome Signatures (GOMS) associated with cancer incidence, prognosis and prediction of treatment response” (Proposal number SEP-210511940 Call H2020-SC1-BHC-2018-2020, PI Prof Laurence Zitvogel). From: 01/01/2019 to 30/06/2024.
- AIRC Associazione Italiana per la Ricerca sul Cancro. Collaborator in the project " Combining faecal biomarkers to improve prediction of individual’s risk of pre-invasive and invasive colorectal lesions" funded by (IG2019 N. 23473, PI Dr. Carlo Senore). From 01/01/2020 to 31/12/2024.
- Compagnia di San Paolo. Partner in the project “DP3: Diagnosi precoce e prognosi del tumore prostatico: un modello integrato tra biologia e tecnologia”. From 01/01/2020 to 31/12/2023

Main achievements in the period covered by the Scientific Report

Fecal microRNA and microbial markers for a precision medicine approach: how they reflect colorectal cancer onset and progression and possible application to screening

(co-PI: A.G. Naccarati – Team: B. Pardini, S. Tarallo, G. Ferrero, G. Francescato, C. Di Battista)

COLLABORATIONS:

- Dr P. Vodicka, (Institute of Exp. Medicine, Czech Academy of Sciences, Prague, Czech Rep.)
- Prof N. Segata (Center for Integrative Biology, Cibio, University of Trento, Trento, Italy)
- Prof E. Budinska (Masaryk University, Brno, Czech Rep.)
- Dr C. Senore (Unità di Epidemiologia, Screening e Registro Tumori, Torino)
- Dr R. Sinha (National Cancer Institute, Bethesda, USA)
- Prof P. Costelli, Prof. F. Penna (Department of Clinical and Biological Sciences, University of Torino)
- Prof. A. Boleij (Radboud University Medical Center, Nijmegen, The Netherlands)
- Dr M. Gariboldi (Fondazione IRCCS Istituto Nazionale dei Tumori, Milano)
- Dr Trine Rounge (Cancer Registry of Norway, Oslo, Norway)

MAIN OBJECTIVES OF THE PROJECT

Current screening programs for non-invasive detection of colorectal cancer (CRC) are based on fecal tests with limited accuracy for early malignancies or precancerous lesions. Evaluating novel biomarkers in stool could improve screening strategy. The hypothesis is that specific stool biomarker profiles (microRNAs and gut microbiome) can accurately characterize patients with sporadic CRC. The main aim of this project is to investigate microRNA expression profiles by deep sequencing in fecal samples in relation to cancer or precancerous lesions. A further objective of the study is to study other small non-coding RNAs detectable with small RNA sequencing and relate miRNA profiles to microbiome composition. All biomarkers are finally tested in the CRC screening setting with leftover from fecal immunochemical test (FIT).

RESULTS ACHIEVED during the reporting period

We comprehensively evaluated stool microRNA (miRNA) profiles as biomarkers for non-invasive CRC diagnosis. A total of 1,273 small RNA sequencing experiments were performed in multiple biospecimens. In a cross-sectional study, miRNA profiles were investigated in fecal samples from an Italian and a Czech cohort (155 CRC, 87 adenomas, 96 other intestinal diseases, 141 colonoscopy-negative controls). A predictive miRNA signature for cancer detection was defined by a machine learning strategy (**Figure 1 A**).

Twenty-five miRNAs showed altered levels in stool of CRC patients in both stool cohorts (adj. $P<.05$) (**Figure 1B**). A five-miRNA signature, including miR-149-3p, miR-607-5p, miR-1246, miR-4488, and miR-6777-5p, distinguished patients from controls (AUC=0.86, 95% CI=0.79-0.94) and was validated in an independent

cohort (141 CRC and 80 healthy volunteers; AUC=0.96, 95% CI=0.92-1.00; **Figure 1C**). The signature classified controls from low-/high-stage tumors, and advanced adenomas (AUC=0.82, 95% CI=0.71-0.97).

miRNA profiles in stool were compared with those of 132 tumor/adenoma paired with adjacent mucosa, 210 plasma extracellular vesicles samples, and 185 FIT leftover samples. Tissue miRNA profiles mirrored those of stool samples, while fecal profiles of different gastrointestinal diseases highlighted miRNAs specifically dysregulated in CRC. miRNA profiles in FIT leftover samples showed good correlation with those of stool collected in preservative buffer and their alterations can be detected in adenoma or CRC patients. Our comprehensive fecal miRNome analysis identified a signature accurately discriminating cancer aimed at improving a non-invasive diagnosis and screening strategies *(Pardini et al, 2023 in press).*

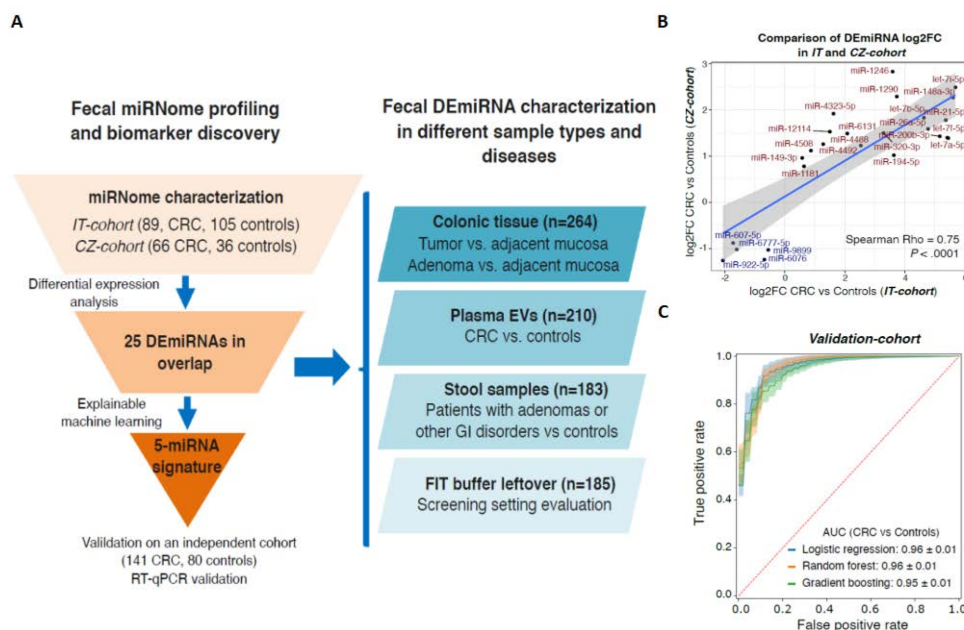

FIGURE 1 A. Study design and workflow. B High correlation between overlapping differentially expressed miRNAs in stool samples of the Italian cohort and Czech cohort investigated. C Performance of the identified stool miRNA signature for the classification of CRC patients with respect to colonoscopy-negative controls.

In the same samples, microbiome composition has also been investigated in collaboration with Prof. Segata's lab in the frame of the Oncobiome project. A study focused on the relationship between gut microbiome composition and CRC staging is currently in progress and a manuscript is in preparation. Shotgun metagenomics was also performed in 1300 FIT leftover samples (including subjects with cancer/precancer lesions matched with healthy controls) collected in collaboration with the Piedmont screening and whose clinical/demographic data will be analysed in collaboration with Dr Sinha's group. Both miRNAs and microbiome are investigated also in Lynch syndrome (*Francescato et al. in preparation*) and FAP subjects (*Pomposo et al., submitted*) in the context of the Oncobiome project and compared with results from sporadic CRC, precancer lesions, and other gastrointestinal diseases.

Host/tumor integrated omics analyses to characterize colorectal cancer heterogeneity

(PI: B. Pardini– Team: A.G. Naccarati B. Pardini, S. Tarallo, G. Ferrero, A. Gagliardi, C. Di Battista)

COLLABORATIONS:

- Dr P. Keski-Rahkonen (International Agency for Research on Cancer, Lyon, France)
- Prof N. Segata (Center for Integrative Biology, Cibio, University of Trento, Trento)
- Prof E. Budinska (Masaryk University, Brno, Czech Rep.)
- Dr E. Berrino (Candiolo Cancer Institute, Torino)

MAIN OBJECTIVES OF THE PROJECT

This project follows in parallel the development of the previous one and is based on the possibility to better characterize the CRC cohort investigated for the stool molecular markers, thanks to the possibility to collect multiple biospecimens and a perform several different omics (**Figure 2**). The main objective is to assess if data resulting from different omics could be integrated in one or more models to have a better view of CRC heterogeneity.

To investigate the relationship between plasma metabolomics, gut microbiome, and gut transcriptome in relation to CRC subtype classification and mutational/genomic status in CRC and pre-cancer lesion. To evaluate the possibility to integrate various omics relevant for CRC in two longitudinal studies on CRC patients.

RESULTS ACHIEVED during the reporting period

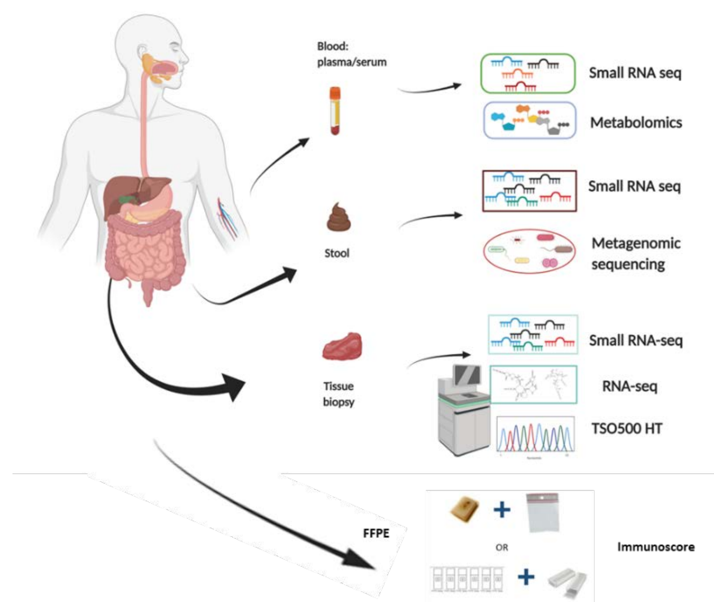

Figure 2 Omics analyses investigated according to different collected biospecimens

We have performed a comprehensive description of CMS subtypes of our cohorts by integrating RNAseq with target genomic sequencing, small RNAseq and immune infiltrate profile and we have investigated how each CMS reflects in the stool miRNome and gut metagenomic landscape to identify possible CMS-specific signatures in surrogate tissue, allowing a non-invasive classification of CRC.

Plasma metabolomic was explored in samples from 265 subjects from the Italian (n=118) and the Czech cohort (n=147), including 72 colonoscopy-negative subjects, 101 and 52 patients with CRC or colorectal adenomas, respectively. The analysis showed 364 metabolite clusters that were quantified consistently in both cohorts. Among them, 67 were observed as associated with altered levels in CRC patients with respect to control subjects by a regression analysis corrected by sex, age, BMI, and smoking status. These features include human and microbiome-specific metabolites and an integrative analysis between their levels and the prevalence of specific microbial metabolic pathways is ongoing.

The metagenomic data of the Italian and Czech cohorts are currently investigated in relationship to the extent of the tumor immune-infiltrate by deconvolution analysis of RNA-Seq and by the analysis of immunoscore.

Study of the microRNA expression profiles and intestinal microbiota composition in relation to different dietary habits and lifestyle

(co-PI: S. Tarallo – Team: A.G. Naccarati, B. Pardini, G. Francescato, G. Ferrero)

COLLABORATIONS:

- Dr M. Cahova (Institute for Clinical and Experimental Medicine, Prague - Czech Republic)
- Prof D. Ercolini (Università di Napoli Federico II, Napoli).
- Prof V. Krogh (Fondazione IRCCS Istituto Nazionale dei Tumori, Milano)

MAIN OBJECTIVES OF THE PROJECT

Investigating fecal molecular markers in relation to different gastrointestinal diseases, the Unit naturally evolved an interest in study their profiles in relation to different dietary habits and particular lifestyle to explore how these factors contribute to the inter- and intra-individual diversity and may influence results on clinical investigations. Thus, the aims of the present project are to explore fecal miRNome and microbiome, as well as other omics, in relation to vegan and vegetarian diet, or in concomitance with a one-month ketogenic diet administered to obese subjects before undergoing bariatric surgery.

RESULTS ACHIEVED during the reporting period

We performed small-RNA and shotgun metagenomic sequencing in fecal samples from 120 healthy volunteers with dietary recording, equally distributed among vegans, vegetarians and omnivores and matched for gender and age. We found 49 miRNAs differentially expressed among vegans, vegetarians and

omnivores (adj.p<0.05) (**Figure 3A**). All the altered miRNAs confirmed their trends of expression levels in vegans and vegetarians when compared to an independent cohort of 45 omnivores. Two miRNAs related to lipid metabolism, miR-636 and miR-4739, were inversely correlated to the non-omnivorous diet duration, independently of subjects'age (**Figure 3B**). Seventeen miRNAs correlated (|rho|>0.22, adj.p<0.05) with the estimated intake of nutrients, particularly animal proteins, phosphorus and, interestingly, lipids. In omnivores, higher *Prevotella* and *Roseburia* and lower Bacteroides abundances than in vegans and vegetarians were observed. Lipid metabolism-related miR-425-3p and miR-638 expression levels were associated with increased abundances of microbial species, such as *Roseburia*, *SpCAG182*, and *Akkermansia muciniphila*, specific of different diets (**Figure 3C**). An integrated analysis identified 25 miRNAs, 25 taxa, and 7 dietary nutrients that clearly discriminated (AUC=0.89) the three diets. Stool miRNA profiles are associated with specific diets and support the role of lipids as a driver of epigenetic changes and host-microbial molecular interactions in the gut *(Tarallo et al., Gut 2022)*.

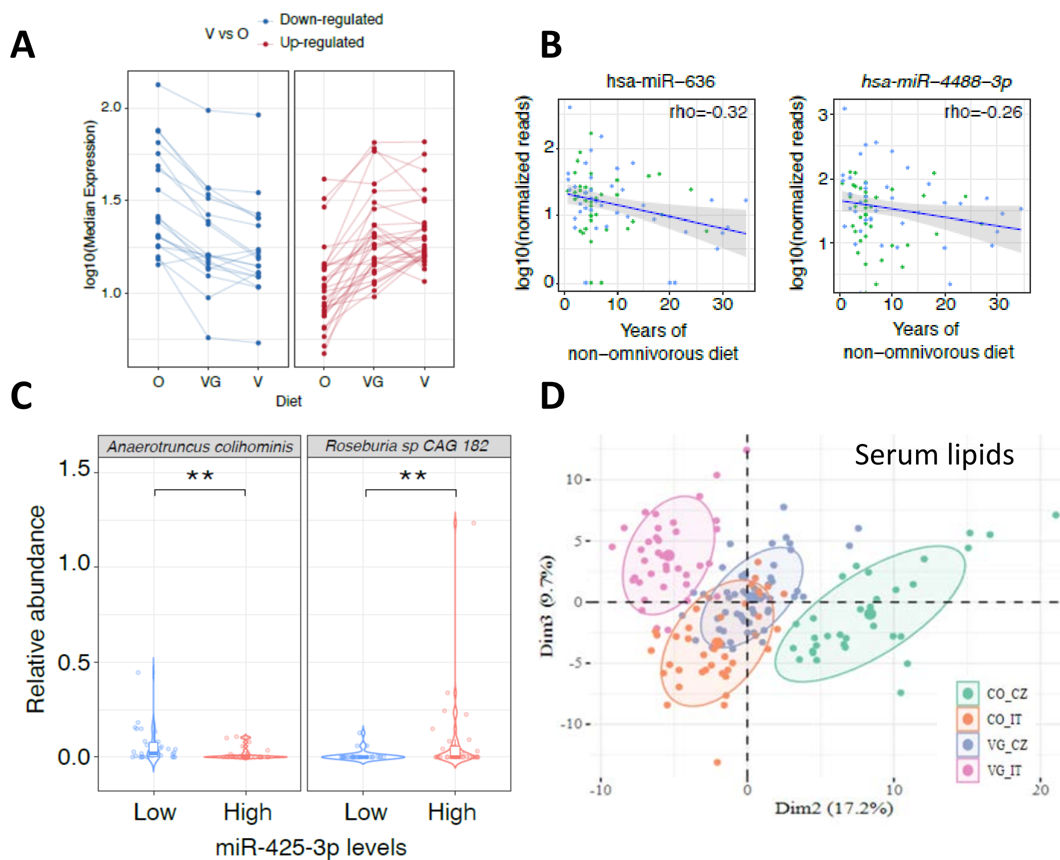

Figure 3 A) Median levels of the differentially expressed miRNAs in stool according to different diet, B) miRNA profiles in relation to the years of non-omnivorous diet, C) differential expression of miR-425-3p according to relative abundance of specific microbial species; D) PCA analyses showing lipid profiles in the investigated cohorts stratified according to the diet.

In a recent study, we sought to determine whether diet-dependent markers explaining the observed health benefits of a vegan diet could be identified in gut MIcrobiome and serum Metabolome (MIME) of vegans from different geographic regions. Lean, healthy vegans (102) and omnivores (73) from two different

geographical areas (Czech Republic, Italy) participated in this cross-sectional study. We investigated their glucose and lipid metabolism and used an integrated multi-omics approach (16S rRNA sequencing, serum metabolomics and lipidomics profiling) to identify country- and diet-specific MIME markers. Czech and Italian vegans exhibited different lipid profiles compared to omnivores characterized by decreased serum concentration of sphingomyelins, ceramides, cholesterol esters and lipid species containing saturated fatty acid compared with omnivores. Using a machine learning approach, we were able to discriminate between vegans and omnivores based on separate omics datasets, regardless of the country of origin. By combining all MIME features, we identified a vegan diet-specific multi-omics signature independent of geographical influence that allows classification of vegans and omnivores with a 93% accuracy. This signature includes 29% of non-lipid metabolites in serum, 16% of serum lipids, and 13% of gut bacterial taxa (**Figure 3D**). Most of the MIME markers that are down-regulated in vegans are predominantly associated with adverse health outcomes, whereas those that are up-regulated are associated with a healthy phenotype and low risk of NCDs. Our findings support the use of a plant-based diet in the treatment of metabolic disorders (*Cahova et al. submitted*)

Analysis of the expression of microRNAs and other small non-coding RNAs in subjects on a gluten-free diet and in pediatric patients with suspected celiac disease

(co-PI: A.G. Naccarati (former co- PI A. Francavilla) – Team: B. Pardini, C. Di Battista, S. Tarallo)

COLLABORATIONS:

- Dr. P. Calvo, Dr. I. Giraudo (Ospedale Infantile Regina Margherita, Torino)
- Dr D. Ribaldone and Dr G.P. Caviglia (Università di Torino)

MAIN OBJECTIVES OF THE PROJECT

Celiac disease (CD) is a complex autoimmune disease and the diagnosis is based on the positivity of serum tissue-transglutaminase 2 (TG) IgA antibodies, confirmed by histopathological analyses of duodenal biopsy. This approach presents some limitations. The only treatment for CD is to adhere to a lifelong gluten-free diet (GFD). Novel biomarkers for CD diagnosis and monitoring of the GFD adherence/effects are needed. The aim of the initial project was to explore specific molecular patterns related to a strict dietary regime (with negative TG serology) or a not strict/short time adherence to GFD (with related gastrointestinal symptoms and positive TG serology) in adults. In the running project the fecal molecular markers and other markers are investigated in newly diagnosed pediatric celiac disease.

RESULTS ACHIEVED during the reporting period

In the observational study on the adults, we performed small RNA and shotgun metagenomic sequencing in stool from 63 treated CD (tCD) and 3 untreated subjects as well as 66 sex- and age-matched healthy controls. tCD included 51 individuals on strict GFD and with negative transglutaminase (TG) serology (tCD-TG-) and 12 symptomatic with not strict/short-time of GFD adherence and positive TG serology (tCD-TG+). Samples from additional 40 healthy adult individuals and a cohort of 19 untreated pediatric CD subjects and 19 sex/age matched controls were analyzed to further test the outcomes. Several miRNA and microbial profiles were altered in tCD subjects (adj. $p<0.05$). Findings were validated in the external group of adult controls. In tCD-TG-, GFD duration correlated with five miRNA levels ($p<0.05$): for miR-4533-3p and miR-2681-3p, the longer the diet adherence, the less the expression differed from controls. tCD-TG+ and untreated pediatric CD patients showed a similar miRNA dysregulation. Immune-response, trans-membrane transport and cell death pathways were enriched in targets of identified miRNAs. *Bifidobacterium longum*, *Ruminococcus bicirculans* and *Haemophilus parainfluenzae* abundances shifted (adj. $p<0.05$) with a progressive reduction of denitrification pathways with GFD length. Integrative analysis highlighted 121 miRNA-bacterial relationships (adj. $p<0.05$). Specific molecular patterns in stool characterize CD subjects, reflecting either the long-term GFD effects or the gut inflammatory status, in case of a not strict/short-time adherence. Our findings suggest novel host-microbial interplays and could help the discovery of biomarkers for GFD monitoring over time *(Francavilla et al, Gut microbes 2023)*.

Awards, patents, dissemination

- 2023 AACR-PEZCOLLER FOUNDATION SCHOLAR-IN-TRAINING AWARDS to Giulia Francescato
- 2022 EACR Travel Grant to Amedeo Gagliardi.
- Bando Ricerca Locale 2022 – support for publications of young researchers to Giulia Francescato for the article: Gagliardi A, Francescato G, et al. The 8q24 region hosts miRNAs altered in biospecimens of colorectal and bladder cancer patients. Cancer Med 2023;12(5):5859-5873.
- Christian Boulin Fellowship 2022 to visit the Genomics Core Facility at the EMBL Heidelberg to Sonia Tarallo and Barbara Pardini
- The Unit is part of the ColoMARK (Grant agreement ID: 101072448) initiative funded under the Marie Skłodowska-Curie Actions programme for Doctoral Networks. Carla Di Battista has been accepted for a PhD position in Barcelona in this highly competitive and prestigious programme.

TECHNOLOGICAL UNITS

The IIGM pursues a policy of state-of the-art research providing its researchers with innovative and up-to-date instrumentation.

IIGM laboratories are equipped with advanced instruments for Next Generation Sequencing (NGS), flow cytometry and cell-sorting, and "*in vivo*" metabolic analyses, managed by dedicated Technological Units.

The equipment is available as an internal facility to IIGM researchers and to scientists from our institutional partner FPO-IRCCS Candiolo. Staff from the IIGM Technological Units cooperate with the "Sequencing" and "Flow Cytometry (FLOCC)" facilities at the FPO-IRCCS, taking advantage of the reciprocal instruments and expertise. Joint activities are coordinated in periodic workgroup meetings.

Access to the facilities is also available to external users as part of scientific collaborations with the IIGM, or as a customer service.

Three facilities are currently active in IIGM:

- Genomic Platform
- Flow Cytometry and Cell Sorting Facility (FCCSF)
- Seahorse

The activities of the facilities include both consulting in the planning phase of the study and the subsequent technical execution of the experiments.

Genomic Platform

Two sequencers are currently active in IIGM:

- the GridION (Oxford Nanopore Technologies) sequencer, which enables proofreading long read sequencing;

- the NovaSeq6000 (Illumina Inc.), a state-of-the-art, high-throughput sequencer that provides the flexibility to accommodate small and medium size sequencing projects, as well as the necessary high throughput processing for big-scale sequencing projects at relatively moderate costs.

For smaller sequencing projects, two Illumina MiSeq sequencers and one Illumina NextSeq500 sequencer (all from Illumina Inc.) are also available in the context of the collaboration with the FPO-IRCCS Sequencing Facility.

The Genomic Platform is also equipped with the high-sensitivity microarray scanner iScan (Illumina Inc.) for large-scale genotyping and methylation analysis projects.

A Beckman Coulter Biomek i7 automated liquid handler for NGS library preparation was recently acquired and is currently in a set-up phase.

The Genomic Platform Technical Unit provides the following analyses:

- Bulk RNA sequencing: Total RNA-Seq
- Transcriptome sequencing: mRNA-Seq.
- Targeted enrichment: exome sequencing; selected gene-panels (eg. TruSight Oncology 500 Assay)
- Genome and metagenome sequencing
- ChIP-Seq

Implementation of the following techniques is on-going:

- Discovery of small non-coding RNAs: sRNA-Seq.
- Single-cell genomic applications (scRNA-Seq, scDNA-Seq).
- Genome-wide DNA methylation analysis: Methyl-Seq.

IIGM staff personnel collaborate with several companies (eg. Promega, Illumina, New England Biolabs), involved in developing MGS-related products, eg. acting as Beta-tester for newly launched products and fine-tuning existing protocols.

Staff Personnel

Simonetta Guarrera (supervisor and scientist), Katiuscia Gizzi (technician), Caterina Parlato (technician), Barbara Pardini (scientist)

Main Projects

Since its establishment in 2020, the Genomic Platform provided technical and scientific support as collaborator or service provider to several sequencing projects.

Currently, IIGM is partnering an ambitious pilot project aiming at the genomic characterization of about 6,000 newborns in the Regina Margherita Hospital of Turin in the years 2023-2024. The Genomic Unit is involved in the project setup and methods testing since the inception of the project.

Flow Cytometry and Cell Sorting

The mission of the Flow Cytometry and Cell Sorting Facility" (FCCSF) is to support researchers with access to modern multiparametric methods of rapid cell analysis and single-cell manipulation.

Being equipped with three cytometers and one cell sorter, the IIGM FCCSF provides the full spectra of flow cytometry and cell sorting services:

- Beckton Dickinson FACS Celesta BVR (3 lasers, 12 FL channels)
- Beckton Dickinson LSR Fortessa X20 (4 lasers, 18 FL channels, HTS option)
- Beckman Coulter Cytoflex (2 lasers, 6FL channels, 96 well plate option), as an instrument for the entry-level analyses.

- Beckton Dickinson FACS Aria III high-speed cell sorter (3 laser, 14 FL channel configurations)

Staff Personnel

Denis Baev (scientist)

Seahorse

The Seahorse Core Facility provides access to the Seahorse XF technology, and operates a state-of-the-art Seahorse XFe96 Extracellular Flux Analyzer (Agilent Biotechnologies), which allows parallel analysis of mitochondrial respiration and glycolysis in real time.

Staff Personnel

Barbara Pardini (supervisor and scientist), Simonetta Guarrera (scientist)

Main Projects

The Seahorse facility staff supported the metabolic experiments in the context of projects addressing:

- Mitochondrial Pyruvate Carrier 2 inhibition and metabolic adaptation to NAD(H) shortage in breast cancer (Collaboration with the University of Trento)
- The role of the adipocyte-derived extracellular vesicles in the survival and function of pancreatic β cells (Collaboration with the University of Turin)
- Metabolism of mesothelioma cells in presence of vitamins and other micro and macronutrients (Collaboration with the University of Turin)

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