Project 8

Name/title of the PhD course	**Public Health and Preventive Medicine**
Name of the PhD coordinator	Prof. Maria Triassi
Name/Title of the PhD project	***Microbiome as predictive factor in cancer immunotherapy***
Department of reference	Department of Public Health
Working conditions, research team, infrastructures, equipment	The Department of Public Health is spread on eight buildings in the medical campus of the University Federico II. It carries out translational activity. Patient care is carried out in a wide range of Clinical Institutes, such as Surgery, Orthopedics, Anatomic Pathology, Endocrinology, Nephrology, Gynecology and Occupational Medicine. Novel therapeutic approaches are developed on mouse and human models. Investigation is carried out in several departmental laboratories, that are fully equipped with luminometers, Bio-Rad imaging systems, Nanodrop and Agilent spectrometers, various Nikon and Zeiss immunofluorescence and confocal microscopes and FPLC. Available instruments include cryostats, tissue embedding station, ten microtomes, as well as several dissecting microscopes for molecular microbiology and molecular cytopathology as well as fully automated next generation sequencing and library preparation systems, Applied Biosystems and Biocartis real time RT-PCRs and N-Counter Nanostring. The human resources of the Department are represented by 16 Full Professors, 20 Associate Professors, 9 Researchers, 11 Assistant Professor Type B, 16 Assistant Professor Type A. Research fields are based on the implementation in clinical practice of novel diagnostic, prognostic and predictive disease biomarkers
Scientific context	The human body represents a really complex ecosystem comprised of 30 trillion cells inhabited by approximately 39 trillion of bacteria, yeast, fungi, protozoa, archaea and viruses that constitute the commensal microbiota. The microbiota is capable of synthesizing or transforming a large number of metabolites, which cannot be otherwise acquired by the host. In addition, the microbiota has also been implicated in modulating the efficacy and toxicity of cancer therapy, including chemotherapy and immunotherapy. Preclinical and early clinical data suggest that a precise characterization of the microbiota could be very useful, in combination with other well standardized biomarkers, such as PD – L1 expression evaluation, to improve the efficacy of cancer patients selection for CTLA-4 and PD-1 immune-checkpoint blockade therapies. The collection of genes within the commensal microbiota is defined as the commensal microbiome and vastly outnumbers human genes and the most common method for taxonomic characterization of complex bacterial communities is based on selective amplification and sequencing of part of the gene encoding the 16S rRNA, part of the small ribosomal subunit in prokaryotes. This is a ubiquitous 1.5 kb gene, containing conserved sequences and hypervariable regions (nine regions: V1-V9), the latter being useful for bacterial taxonomic classification, as originally described by Woese and colleagues. Because bacterial identification is based on a portion of the 16S rRNA gene, species level resolution is usually not feasible with this method and identification is typically limited to family or genus level. Another consideration in 16S analyses is that most bacteria contain multiple copies of the 16S rRNA gene, which can lead to inaccurate quantitation of bacterial cells. Additional bias can be introduced in the amplification step, depending on the choice of primers. Another approach is based on metagenomic shotgun sequencing that generates short reads representing the whole genomic content within an environmental sample and is considered less biased than 16S rRNA gene amplicon sequencing, because it does not contain a PCR amplification step. However, this can result in contamination with human genomic DNA and requires higher sequence coverage to detect bacterial species of low abundance and, in addition, functional capacity can only be inferred indirectly from 16S rRNA amplicon sequencing data. These limits can be overcome by using multiplex digital color-coded barcode hybridization technology (Nanostring Technologies, Seattle, Washington, USA) which gives the possibility to simultaneously analyze in a single-tube multiplexed fashion a broad spectrum of DNA fragments on different biological specimens, across all levels of biological expression. This approach provides a method for direct detection of targets with fluorescent molecular barcoded probes without the need of reverse transcription and/or amplification.In this project, we will aim to design and validate a specific “microbiome panel” for precise characterization of the microbiota, in combination with other well standardized predictive biomarkers to improve the efficacy of cancer patients selection for CTLA-4 and PD-1 immune-checkpoint blockade therapies.
Project Research plan	Four different research and training main phases: A1. Panel Design; A2. In silico validation, A3. In vitro validation A4. Patients sample collection and clinical evaluation. A series of 24 NSCLC cancer patients treated with Pembrolizumab in monotherapy in first line of treatment, with histologically confirmed adenocarcinoma will be characterized for microbiome status. Fecal samples will be collected from each patients and DNA will be extracted by using PureLinkTM Microbiome DNA Purification Kit (PL; Thermo Fisher Scientific) following the manufacturer procedure and analyzed by using the developed and in vitro validated multiplex barcode color code panel. The obtained results will be matched with the clinical data to assess the predictive value of our customized panel. In particular Objective Response Rate and Progression Free Survival at 12 months will be compared with the quantity and quality of Nanostring results, to set a clinical relevant cut – off that will be validated in future prospective studies.

Research and Training Innovative aspects	Currently, the selective amplification and sequencing of part of the gene encoding the 16S rRNA represent the most common method for taxonomic characterization of complex bacterial communities. On the overall, a pair of universal primers targeting conserved sequences flanking a hypervariable region are used to generate an amplicon library, which is then sequenced. Because bacterial identification is based on a portion of the 16S rRNA gene sequencing, species level resolution is usually not feasible with this method. In addition, most bacteria contain multiple copies of the 16S rRNA gene, which can lead to inaccurate quantitation of bacterial cells. Also the amplification step represent an additional bias and is strictly depended on the primers choice. Metagenomic shotgun sequencing is considered less biased than 16S rRNA gene amplicon sequencing, because it does not contain a PCR amplification step. However, this can result in contamination with human genomic DNA and requires higher sequence coverage to detect bacterial species of low abundance. This necessitates additional data storage, computing power, and more sophisticated analysis pipelines. Errors can also be introduced in the downstream analysis at the step of genome assembly or gene prediction. Another important limitation of metagenomic shotgun sequencing is related to the functional capacity definition, in fact functional capacity can only be inferred indirectly from 16S rRNA amplicon sequencing data.Our project proposal will allow the overcome of these limits by using an innovative specific "microbiome panel" for precise characterization of the microbiota based on a multiplex digital colour-coded barcode hybridization technology (NanoString Technologies, Seattle, Washington, USA) approach which gives the possibility to simultaneously analyze in a single-tube multiplexed fashion a broad spectrum of DNA fragments on different biological specimens, across all levels of biological expression. This approach provides a method for direct detection of targets with fluorescent molecular barcoded probes without the need of reverse transcription and/or amplification.
Inter-Multidisciplinary aspects	This project will involve four different area of expertise, in particular: Predictive Molecular Pathology: Design, develop and validation of next generation technology based approach to analyze quality and quantity of microbiome. In particular a multiplex color code barcode technology will be evaluated to assess the microbiome in cancer patients derived samples. Bioinformatics: a dedicated pipeline, based on nSolver 3.0 software will be customized to analyze the data obtained by using the custom multiplex color code barcode probe set.Anatomic Pathology: Cytological or histological confirmed lung adenocarcinoma patients will be recruited for this study.Oncology: Patient treated in monotherapy with Pembrolizumab in first line of treatment with complete clinical records to assess Objective Response Rate and Progression Free Survival evaluated by an experienced oncologist will be recruited for this study..
Secondment opportunities	The **University of Padua,** Department of Medicine DIMED : the role of MSD will be focused on training for the management of clinical data obtained in the step A.4 of this project. Main PI/co-supervisor: **Matteo Fassan**. 3 months. **PANGAEA BIOTECH**, S.L. Quirón Dexeus University Hospital, is a leading European institution in the field of mmunotherapy and target therapy for oncological patients. In particular, PANGAEA BIOTECH, S.L. has a major field of investigation in the application of next generation technologies, such as multiplex colour code barcode technology, that are relevant in the research that has been planned in this application. Main PI/co-supervisor: **Miguel Molina**. 3 months.
Main Supervisor: Prof Giancarlo Troncone (https://www.docenti.unina.it/giancarlo.troncone)	
Brief CV	Full Professor in Anatomic Pathology and Head of the Department of Public Health in School of Medicine, University of Naples Federico II. His research interests include the development, validation, and quality assessment of a wide range of morpho-molecular techniques on cytopathologic specimens in the field of predictive pathology of solid tumors. He is the author of more than 300 papers in peer-reviewed journals (in particular: 1: Vigliar E, J Clin Pathol. 2019;72:412-417. 2: Sgariglia J Clin Pathol. 2017;70:803-806. 3: Malapelle. Br J Cancer. 2017;116:802-810.). He serves on the editorial board of Pathobiology (associate editor), Cancer Cytopathology (associate editor) and Journal of Molecular Pathology (Editor in Chief). He has supervised 8 PhDs.
Publications	- Malapelle U, et al. Reference standards for gene fusion molecular assays on cytological samples: an international validation study. J Clin Pathol. 2021. Epub ahead of print. - Malapelle U, et al. Development of a gene panel for next-generation sequencing of clinically relevant mutations in cell-free DNA from cancer patients. Br J Cancer. 2017 Mar 14;116(6):802-810. - De Luca C, Pepe F, Iaccarino A, et al. RNA-Based Assay for Next-Generation Sequencing of Clinically Relevant Gene Fusions in Non-Small Cell Lung Cancer. Cancers (Basel). 2021;13:139. - Pisapia P, et al. Consistency and reproducibility of next-generation sequencing in cytopathology: A second worldwide ring trial study on improved cytological molecular reference specimens. Cancer Cytopathol. 2019;127:285-296. - Malapelle U, et al. Consistency and reproducibility of next-generation sequencing and other multigene mutational assays: A worldwide ring trial study on quantitative cytological molecular reference specimens. Cancer Cytopathol. 2017;125:615-626.
Projects participation	- "Nuovi Marcatori Molecolari nella Diagnostica Citologica Preoperatoria del Nodulo Tiroideo - TIRNET" - Contributo ANNO 2021 - POR Campania FESR 2014-2020 Progetto "Sviluppo di Approcci Terapeutici Innovativi per patologie Neoplastiche resistenti ai trattamenti (SATIN)"