Mechanism of action of complex drugs: comprehensive clinical exploration for early drug development optimization

1. Background

1.1. Scientific abstract

A precise understanding of the mechanism of action of new drugs is key to optimize their clinical development. The last generation of anticancer drugs, such as antibody-drug conjugates and bispecific antibodies, have shown very promising clinical activity, and are now being increasingly developed. These drugs harbor complex mechanisms of action, both with a complex pharmacology within the tumor cell, and by engaging the immune microenvironment and stroma. Because preclinical models very unfaithfully recapitulate the human tumor cell metabolism and the tumor–immune–stromal cells interactions, translational studies in patients are highly needed. Herein, we propose to use cutting-edge approaches on patients' samples to explore and understand the mechanism of action of the last generation of complex drugs, or of drugs with unknown mechanism of action.

Using single cell technologies and/or spatial transcriptomics on sequential samples from patients treated with complex drugs at the Gustave Roussy Drug Development Department (DITEP), we will characterize, at the single-cell level, signaling and gene expression changes that occur on treatment and, when appliicable, at drug resistance within the tumor cells, immune cells and other cells from the microenvironment. We will further assess pharmacodynamic biomarkers in circulating tumor cells sampled pre- and on-treatment, in order to develop early, non-invasive readouts of drug efficacy.

Altogether, this proof-of-concept program will allow us to investigate and potentially decipher the mechanism of action, in patients, of the last generation of complex drugs, which will further guide the development of biomarkers for patient selection and drug efficacy. The ultimate goal of this high-risk high-gain pilot study is to launch the development of an independent platform that would use single-cell technologies on tumor samples and circulating tumor cells in order to understand, in real life, the mechanism of action of complex drug, thereby allowing to optimize their development for patient's benefit.

1.2. Lay title and abstract

Title: Understanding the mechanism of action of the newest drugs to optimize their development

A precise understanding of the mechanism of action of new drugs is key to optimize their clinical development. The last generation of anticancer drugs uses sophisticated molecules, which are called "complex" drugs because they notably act both on tumor cell and cells from the tumor microenvironment. Although these drugs have shown promising clinical activity and are now being increasingly developed, their precise mechanism of action is still insufficiently understood, mostly because preclinical models do not faithfully recapitulate the human cancer biology. Only the precise analysis of patients' samples can address such question. Herein, we propose to use cutting-edge approaches on patients' samples to explore and understand the mechanism of action of the last generation of complex drugs, or of drugs with unknown mechanism of action.

To do so, we will perform analysis on tumor biopsies and tumor cells that circulate in the blood (called circulating tumor cells, CTCs), sampled sequentially within the same patients who are treated with a "complex" drug; biopsies will be taken pre-treatment, on-treatment and at the end of the treatment. We will analyze these biopsies using cutting-edge technologies called "single cell analyses", which allow to precisely evaluate, at the level of each individual cell (e.g. tumor cell, immune cell, or any other cell) which changes occur on treatment. This will help us in deciphering, in each patient, how the drug works and why it is effective (or not) against the tumor. In parallel, we will collect CTCs and analyze them for specific features (called "biomarkers") of the activity of the drug. As CTCs are detected in the blood, they can be assessed easily in a repeated fashion without inflicting the pain of a biopsy, or be used in patients whose tumor is not accessible to a biopsy.

Altogether, this proof-of-concept program will allow us to investigate and potentially decipher the mechanism of action, in patients, of the last generation of complex drugs. This will further guide the development of specific markers to better select patients who may benefit from them. The ultimate goal of this high-risk high-gain pilot study is to launch the development of an independent platform that would more broadly use such approach, in order to understand, in real life, the mechanism of action of drug, thereby allowing to optimize their development for patient's benefit.

1.3. Scientific and clinical background (current state of research in the field)

Novel classes of anticancer agents, such as antibody-drug conjugates (ADCs), bi-specific antibodies, and epigenetic drugs, harbor complex mechanisms of action. Indeed, these drugs do not only target the tumor cell itself, but also engage cells from the microenvironment, including immune cells and/or stromal cells (Figure 1). For example, **ADCs** are complex molecules consisting of an antibody targeting a specific tumor-associated antigen, which is coupled (through a linker) to several molecules of a cytotoxic chemotherapy ("payload"). ADCs are internalized within the tumor cell, where the cytotoxic drug is released after cleavage of the linker or degradation of the antibody. This allows to increase the therapeutic window and enhance the selectivity for tumor cells as compared to conventional chemotherapy. A dozen of ADCs are currently approved for the treatment of hematological malignancies or solid tumors and many other are being developed. **Bi-specific antibodies** are engineered antibodies that can simultaneously bind two different types of antigens through their Fab domains (e.g. one expressed on a tumor cell and one expressed on an immune cell), thereby bridging the cancer cell to the T-cell and favoring its destruction by the latter. Simultaneously, the Fc part of the antibody can bind to an innate immune cell expressing the Fc receptor, thereby triggering antibody-dependent cytotoxicity and further increasing tumor cell killing. An exponential number of bispecific antibodies are currently being developed, with various chemical structures.
Overall, **the mechanism of action (MoA) of such drugs is currently only partly understood, and deciphering further how these drugs act in human biology and crucial for their adequate development.** Although preclinical studies allow to anticipate which patient populations will most benefit from such therapies, preclinical models have limitations and only partially recapitulate the tumor's and human biology's complexity. Therefore, only **biopsies performed in a sequential fashion (pre-treatment, on-treatment and at resistance) in patients receiving the drug of interest allow a comprehensive and clinically-relevant understanding** of the mechanism of action of complex drugs or drugs of unknown mechanism of action.

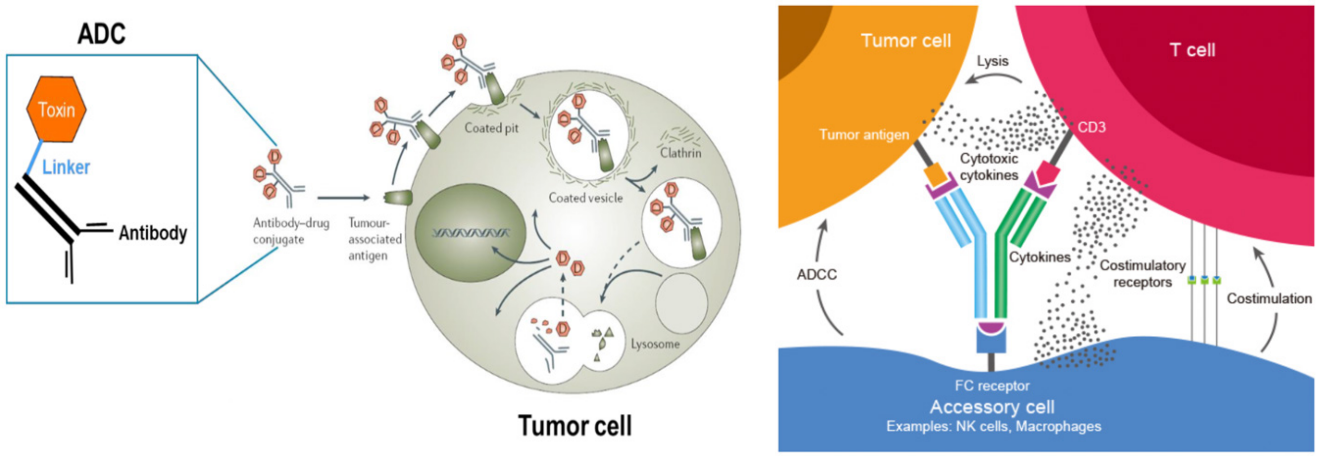


Figure 1: Examples of complex drugs. Left panel: structure and intra-cellular metabolism of an ADC; right panel: structure and mechanism of action of a trivalent bispecific antibody

Early phase trials

Early phase trials are key in the development of novel anticancer compounds, as many important "go / no-go" decisions are made in such phases. Early phase trials now commonly have seamless designs, which include: (i) a dose-escalation / dose-expansion phase (traditional Phase 1 trials), where the optimal dose of the drug is defined based on toxicity, pharmacokinetic and pharmacodynamic parameters; and (ii) a "proof-of-concept" phase (akin phase 2 trials), where the confirmation of the activity of the drug in a particular disease is performed. Because neither dose nor schedule, which can influence drug efficacy, will ever be revisited once the Phase 1 trial is completed, it is absolutely crucial to optimize these at this early step and understand as much as we can about a given drug during them.

Single cell sequencing

Single cell RNA sequencing (scRNA-seq) allows to precisely evaluate, at the cell level, the expression of each gene. This allows to precisely define, for each cell population (i.e. cancer versus immune versus stromal cells), which genes and pathways are expressed or absent before treatment, and how these are changing upon treatment with the drug

of interest (Figure 2). As a complement to scRNA-seq, CITE-Seq (Cellular Indexing of Transcriptome and Epitopes by Sequencing) allows to evaluate the expression of selected cell surface proteins from the cells for which scRNA-seq data is collected. This technology therefore represents a relevant complement to scRNA-Seq, in particular in order to characterize the functional state of immune populations. Because the best results on single cell analyses so far have been obtained using fresh tumor samples, only few cancer centers currently have the ability to perform such studies. Single cell technologies are very powerful and represent extremely promising tools to drive and guide drug development [1]. So far, the vast majority of publications using single cell technologies have described unique cases, or groups of patients who have been pooled according to a characteristic of interest (e.g. sensitivity or resistance to a given drug) [2]. However, because of patient-to-patient variability, such "pooled" approach is much less informative than sequential sampling within the same patient. To date, there is virtually no publication that used single cell sequencing to characterize sequential samples within the same patient; the very few available publications were performed on skin or hematological malignancies – as these are easy to biopsy – and these did not focus on mechanism of action of novel drugs [3-5]. Our approach is therefore highly innovative.

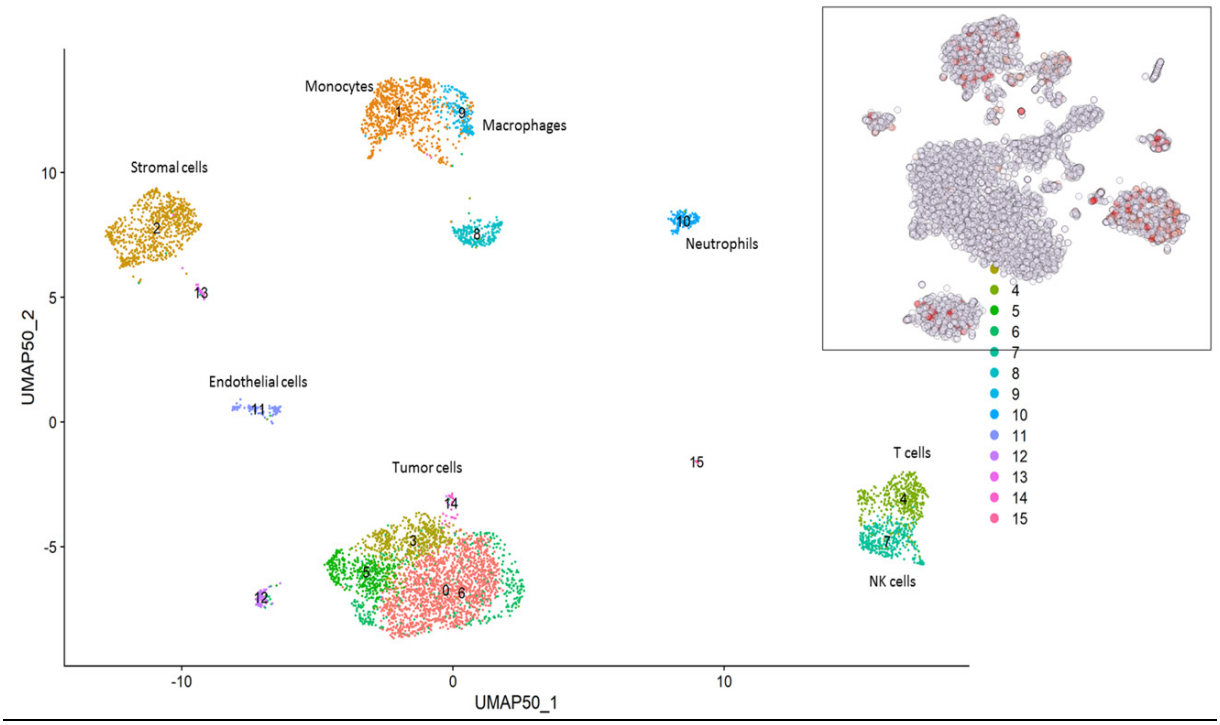


Figure 2: Example of in-house single-cell sequencing result. Different cell populations, together with the several identified clusters per cell population, are depicted; the top right quadrant exemplifies how the expression of a single gene can be assessed in each cell of each cell population (red shows the intensity of expression of that particular gene.

Spatial transcriptomics

Spatial Transcriptomics (Digital Space Profiler, GeoMX®) is a very recent technology that allows to visualize the spatial architecture and to analyze, in a high-plex and high-throughput profiling fashion, multiple biomarkers in a tissue sample. Therefore, spatial biology brings important complementary information on the location of changes observed in a tissue, on cell-cell interactions, and on tumor heterogeneity. Current panels allow to assess up to 50 proteins and 18000 mRNA transcripts within several regions of interest of a single formalin-fixed paraffin-embedded tissue slide.
Overall, single-cell technologies and spatial transcriptomics are cutting-edge technologies that allow the most in-depth characterization of tumor samples. Applying them on sequential tumor samples of patients receiving the last generation of complex drugs within early phase trials is therefore highly innovative and might be extremely informative for drug development.

Circulating Tumor Cells (CTCs)

CTCs as a liquid biopsy have emerged as a non-invasive and easily accessible alternative to tumor biopsies to assess tumor status and monitoring changes in "real-time". CTCs are derived from primary tumors and/or metastatic sites

and have a causal role in the development of metastasis. They can be found in the blood of a proportion of patients with cancer depending on their clinical stage and the applied CTC detection technology. Because CTCs are likely released from spatially distinct tumor sites, they inform tumor heterogeneity and evolution, and may complement traditional tumor tissue sampling. They also offer the advantage to inform both phenotypical and genomic tumor features and have therefore an undisputable potential for the personalized management of patients with cancer. During the last decade, intense technical efforts have been made to reliably detect, quantify and characterize CTCs at the phenotypical, genomic and functional levels. Studies have reported the prognostic and pharmacodynamic biomarker utility of CTC counts in numerous solid tumors. Our and other groups have reported the clinical interest of CTCs to detect predictive biomarkers of sensitivity and resistance for therapy selection [6-8]. In NSCLC and prostate cancer, we reported genomic profiling of single CTCs which reveal undiagnosed alterations in matched-metastasis biopsy inferring therapeutic resistance pathways [9,10].

Here, we propose: (i) to use CTCs as an early non-invasive biomarker of activity of the novel anticancer compounds in early clinical development, and (ii) to evaluate CTCs as a pharmacodynamic surrogate biomarker in early phase trials. The project involves several constraints and challenges. First, the analysis of protein targets in CTCs poses technical difficulties which result from the rarity, inter- and intra-patient phenotypic and genotypic heterogeneity of CTCs, the absence of a universal detection method, the necessity of working in limited blood volumes (maximum 30 mL) and the impact of fixatives on protein targets. Secondly, CTCs have been mostly studied in epithelial tumors. It will therefore be necessary to set up an approach capable of covering other tumor types such as sarcomas or neuroendocrine tumors. Finally, it will be necessary to screen and validate a large panel of antibodies to detect the wide range of therapeutic targets. Taking into account these constraints and the volume of CTC samples to be processed, we will have to set up a CTC assay adaptable for various types of tumor (epithelial or not). It must include an enrichment step (as usual for CTCs) followed by the detection of a tumor marker and the therapeutic target on candidate CTCs. It must be carried out at baseline ad several time-points on treatment to assess the loss of the target. In the perspective of a dedicated platform to single cells and CTC analysis, it should be usable on a large scale, that is to say, be relatively fast in execution time and analysis.

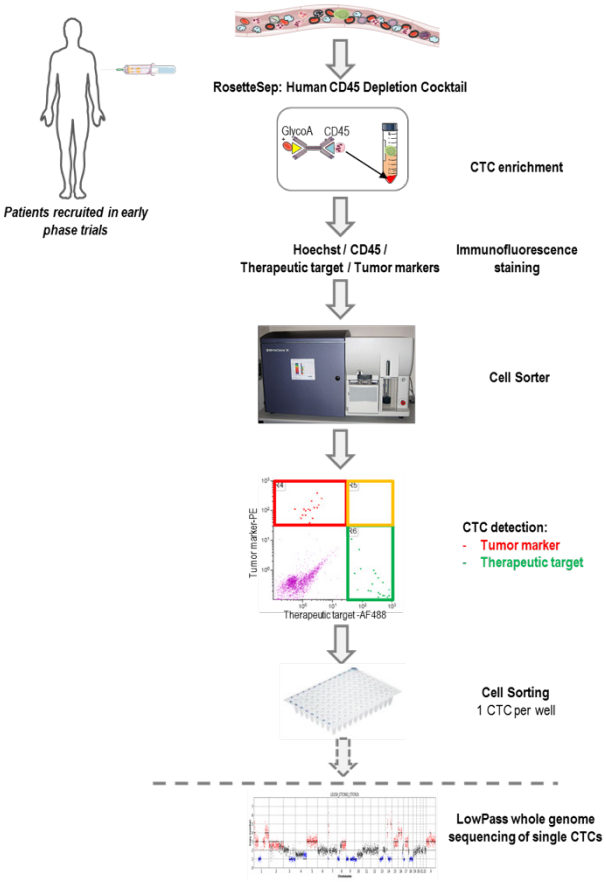


Figure 3: Workflow for CTC detection, single cell isolation and copy number alteration profiling in patients recruited in selected early phase trials.

Based on our experience and recent team's results [9], we propose a workflow which includes a negative selection by the RosetteSep method (loss of the vast majority of red and white blood cells without *a priori* on the phenotype or morphology of CTCs), a quadruple immunofluorescent labeling with Hoechst 33342 (elimination of dead cells/debris), CD45 (exclusion of residual leukocytes), a tumor marker (for example pan-cytokeratins and EpCAM for epithelial tumors) and the therapeutic target marker (Figure 3). The labeling will be followed by FACS analysis and possibly single cell sorting. On a case-by-case basis during treatment monitoring, confirmation of the tumor nature of isolated single CTCs will be carried out by sequencing and copy number alteration (CNA) profiling. The cells will undergo global genome amplification (WGA) followed by Lowpass whole genome sequencing to determine copy number alterations throughout the genome. For years the major drawback of the analysis of very rare events by flow cytometry has been the inability to prove that a "dot" identified on a fluorescence histogram was indeed a real cell and not an artefact. Single cell isolation and sequencing techniques now make it possible to formally demonstrate that a dot displayed on a screen is indeed a true tumoral cellular entity.

➔ In conclusion, we **propose to explore in human tumors the mechanism of action of novel anticancer agents with complex MoA, using notably single-cell sequencing on sequential tumor biopsies and circulating biomarkers profiling**, in order to optimize and impact the development of these drugs. Because the DITEP (Gustave Roussy Drug Development Department), is the largest Phase 1 unit in Europe (and one of the largest worldwide, with approximately 400 patients treated each year and over 80 active clinical trials), and has a long-standing habit of performing tumor and liquid biopsies in collaborations with the INSERM research teams for their analysis, we believe that we are uniquely placed to perform such study (Figure 4).

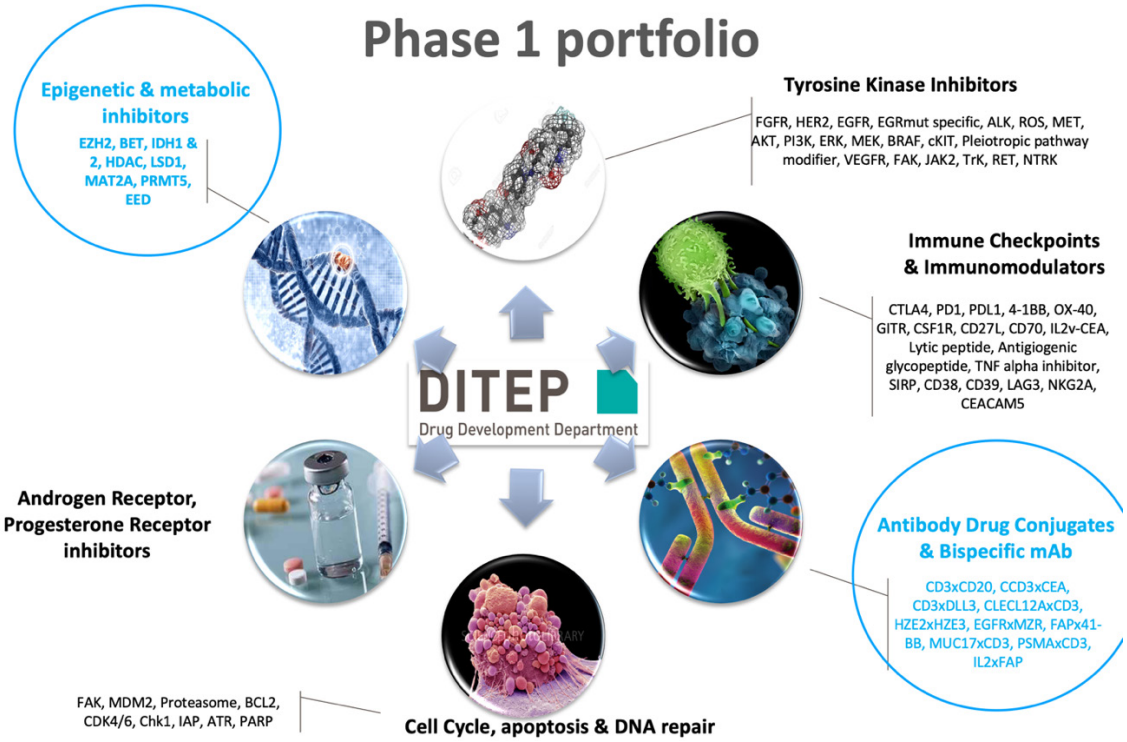


Figure 4: DITEP current portfolio. Complex drugs of specific interest for single cell analysis are highlighted in blue; CTCs analyses will also include the "Tyrosine kinase inhibitors" and "Immune checkpoint" groups of drugs.

<u>Note</u>: The current project is a proof-of-concept study which aims at serving as "catalyzer" to subsequently launch independent single-cell and CTC platforms, which would be funded by academic and industrial grants alongside their respective academic or industrial clinical trials. We believe that such platform would be of high added value and would represent a win-win partnership with pharmaceutical companies. Indeed, it would allow industrials to better understand the mechanism of action of their drugs and subsequently improve their development, and this would allow Gustave Roussy to actively participate to such research and to attract trials that evaluate the best and most innovative complex drugs, for patients' benefit.

1.4. Current state of personal research (main applicant and co-investigator)

1.4.1. Main applicant

Dr Postel-Vinay, physician scientist, is currently the head of the Drug Development Committee and principal or co-investigator of multiple early phase trials. She also leads a research team at the U981 INSERM Unit.

On the laboratory research side, her team recently optimized in-house single cell RNAseq on fresh biopsies for an unrelated project (Hénon et al, CTOS 2019, oral presentation), and managed to process so far 15-20 tumor biopsies from patients included in various clinical protocols or undergoing surgery (Figure 2). More specifically, single cell analysis on sequential biopsies has been technically successfully performed in more than 5 patients enrolled in two academic clinical trials; preliminary bioinformatic analyses are ongoing for these patients.

On the clinical research side, Dr Postel-Vinay has a large expertise in early phase trials and is currently principal investigator of 6 phase 1/2 trials and co-investigator of more than 80 phase 1/2 trials. Patients enrolled in this study would come from any clinical trial run at the DITEP that evaluates “complex” drugs, complex drug combinations, or drugs of unknown mechanism of action. Such candidate trials have already been identified, though these may change as the trial portfolio constantly evolves. As an example, in 2020, nine patients have been enrolled in three trials evaluating ADCs, 39 patients in 12 trials evaluating bispecific antibodies, and 21 patients in 10 trials evaluating epidrugs. Thus, Dr Postel-Vinay would guide the choice of the protocols and patients eligible for this program, whatever the principal investigator of the protocol. This is in line with her position as “head of Drug Development Committee”, i.e. being responsible for the scientific strategy of the Drug Development Department.

1.4.2. Collaborator 1: Dr Françoise Farace

Dr Françoise Farace is the head of the Rare circulating cell translational platform at the CNRS UMS3655 - INSERM US23 unit and senior group leader at the U981 INSERM unit. The team has a worldwide renewed expertise in circulating tumor cells and has developed innovative technological tools for detecting and characterizing molecular and functional biomarkers in CTCs. Among other approaches, the team has developed specific processes to identify predictive biomarkers of sensitivity and resistance to targeted therapies in particular in NSCLC and prostate cancer CTCs [6-10]. Specific single CTC isolation and sequencing workflows (targeted PCR/NGS with standard and home-maid panels, WES, lowpass whole genome) have been developed for which quality is controlled throughout the processes. These technological efforts have made possible the monitoring of CTCs at the single cell level in several clinical studies to characterize CTC genomic heterogeneity and therapeutic resistance pathways [7-9]. Unique CTC-derived models (CDXs) models have been generated to explore in-depth the biology of CTCs and the phylogenic evolution of tumorigenic CTCs [10,11]. Over the years the team has established numerous collaborations with clinical teams that have resulted in successful translational projects. The team has also worked in collaboration with many research laboratories, in particular in the framework of European programs.

1.4.3. Collaborator 2: Dr Charles-Antoine Dutertre

After spending 7 years as a research scientist in Singapore (Singapore Immunology Network, SIgN – A*STAR) from where he came back in September 2020, Charles-Antoine Dutertre is now working in Institut Gustave Roussy (IGR, INSERM U1015, Florent Ginhoux’s team, Paris, France) and just got a tenured researcher position at INSERM (CRCN position). In IGR, his research activities’ primary aim is to define mononuclear phagocyte (MNP) subsets where he focuses on understanding their pathophysiological involvement and mechanisms leading to their pathogenic reprogramming in human cancer patients. To this end, several high dimensional techniques are employed:

- Single cell multiomics using single cell RNA sequencing (scRNA-seq) combined with the measurement of the expression of cell surface proteins. Protein quantification can be obtained using indexed-SMARseq2 scRNA-seq (full lengh RNA sequencing of the entire transcriptome combined with the flow cytometric quantification of up to 16 proteins, see [12,13] or CITE-seq/10X scRNA-seq (5’-end RNA-seq combined with up to 150 oligo-conjugated CITE-seq antibodies).
- Using our Cytek spectral flow cytometer that allows to simultaneously quantify more than 40 proteins at the single cell level, we are developing the InfinityFlow 2.0 pipeline (see [12] for the InfinityFlow 1.0 pipeline). The upgrade as compare to the version 1.0 is that the InfinityFlow 2.0 method allows to barcode and simultaneously stain PBMC from up to 31 samples/patients for which up to 400 proteins can be measured using a 40 antibody “backbone panel” and the LegendScreen kit (Biolegend). Using the InfinityFlow 2.0 pipeline, which employs

machine learning-based predictions, we generate data files combining real protein measurement dimensions together with predicted protein measurement dimensions for each analyzed cell and from each of the 31 samples analyzed.

These different experimental approaches require technical and analytical (flow cytometry and single cell proteomic/transcriptomic data analysis) know-how which are present within the team of CA Dutertre.

2. Research description

2.1 Detailed research plan

2.1.1 - Objectives:

This program will have two main objectives:

- **AIM1**: to explore and further understand the MoA of complex molecules of interest using notably **single-cell profiling on sequential patient biopsies**
- **AIM2**: to explore the potential for **non-invasive circulating biomarkers** to identify patients who will mostly benefit from these therapies.

2.1.2 - Methodology:

2.1.2.1 – Methodology for AIM1: Single cell sequential profiling on tumor biopsies

Patients will be selected based on

- The possibility to perform sequential biopsies within the same lesion
- The therapeutic protocol in which they participate;

Eligible protocols will have to fulfil the following criteria

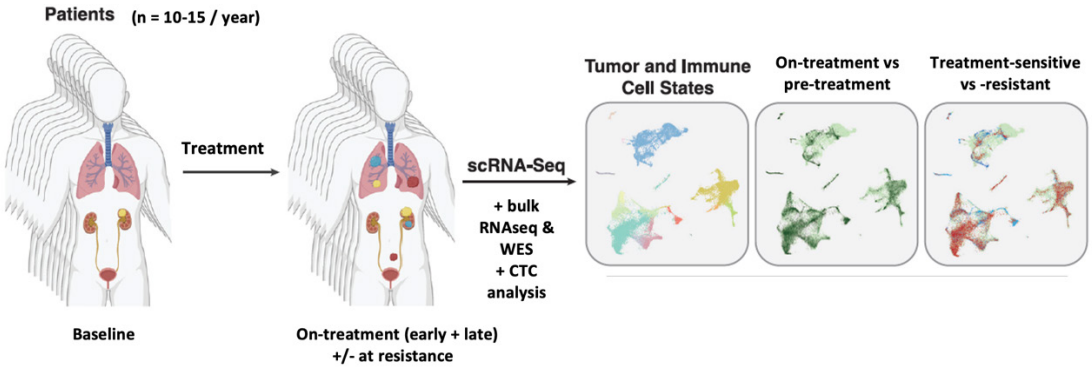
- The protocol evaluates an ADC, bispecific antibody, epigenetic drug, combination of complex drugs or a drug of unknown mechanism of action
- Clinical efficacy has been observed in at least one patient
- Drug is delivered at an active dose

In case no biopsies are planned within the therapeutic protocol, patients will be offered to consent to a non-interventional protocol ("STING" protocol) in parallel of the therapeutic protocol, in order to be within a legal frame which allows to perform the desired biopsies and blood sampling.

We will subsequently perform **sequential tumor biopsies**, which will be collected at **two or three timepoints for each patient**: **prior to starting treatment, on treatment** and **at resistance** (if relevant; Figure 5). These biopsies will be profiled using notably **immunohistochemistry** (IHC) **and single-cell sequencing.** The latter will be performed on fresh tumor samples – which still represents the best approach - until a protocol is adequately optimized to process frozen tissues. This will allow us to precisely identify which signaling changes occur in the tumor cell versus in immune and/or stromal cells, to correlate it with IHC findings, and to explore whether these signaling changes correlate with a specific response to (or toxicity from) the drug. When single cell analysis cannot be performed (for technical or sampling reasons), **spatial biology** will be envisioned as a complementary and/or back-up plan. Spatial biology (GeoMX®) is a cutting-edge technology currently being implemented at Gustave Roussy, which allows to visualize the spatial architecture and analyze multiple biomarkers in a tissue sample. Therefore, this brings important complementary information on the location of changes observed in a tissue and on cell-cell interactions. More details on the processing of the samples and on each technique are provided below and in Appendixes 1-4.

For each timepoint, a minimum of three tissue cores will be sampled:

- one core will be processed immediately for 5' single-cell RNA sequencing (scRNA-Seq) and TCR sequencing, combined with CITE-seq for a subset of patients
- one core will be fresh frozen (FF) and subsequently used for bulk whole exome and RNA sequencing
- one core will be paraffin-embedded (FFPE) and subsequently used for immunohistochemistry (IHC) and/or spatial transcriptomics (GeoMX™).



Tumor biopsy	X	X	(X)
CTC sampling	X	X	X

Figure 5: Overview of the tumor and CTC sampling pipeline, together with corresponding analyses (adapted from [2])

Whatever the technology used for sample profiling, analyses will compare the pre-treatment *versus* on-treatment sample in a longitudinal (intra-patient) fashion, the sensitive *versus* resistant tumors (in an inter-patient fashion), and focus on immune cells versus tumor cells. The following technologies will be used:

2.1.2.1.1. On Fresh samples: 5' single-cell RNAseq and CITE-Seq

A **dedicated post-doc** will be available to immediately collect the fresh biopsy sample at the interventional radiology unit, in order to minimize the time between the biopsy collection and cell dissociation, and thereby maximize cell viability and likelihood of successful scRNA-Seq analysis. Tissue dissociation will be performed immediately. CITE-Seq (for membrane protein analysis) and 5' and TCR scRNA-Seq (for intracellular RNA analysis) will subsequently be performed using the 10X Genomic Chromium® assays. Samples will be processed as follows:

- CITE-Seq: Cells will be stained with oligo-conjugated antibodies directed against 151 membrane proteins (130 using the Biolegend TotalSeq-C cocktail and 21 other Biolegend TotalSeq-C antibodies directed against other MNP markers not included in the cocktail). CITE-Seq will performed in the team of CA Dutertre, who has not so far profiled fresh biopsies from solid tumors using that technique. Because this will be a pilot study, we will therefore limit the number of samples undergoing CITE-Seq to a maximum of six patients, i.e. 12-18 samples.
- 5' and TCR scRNA-Seq: Cells will next be subjected to 10X Genomics 5' scRNA-Seq for the downstream analysis of gene expression and T cells' TCR sequences to evaluate changes in their clonality following treatment, with an objective of 5000 cells sequenced per sample.
  - 5' and TCR scRNA-seq analyses will be performed both at cell and gene levels, using methods derived from [14]. For both 5' gene expression (5'GE) and TCR analyses, after quality control, we will perform read mapping with CellRanger 3.0.
  - For 5'GE single-cell analysis, expression quantification with be performed with CellRanger 3.0 software followed by downstream analyses with the Seurat V4 R package. Following steps will include filtering of cells using % of mitochondrial genes and number of features, log-normalization of the data, detection of variable features and dimensional reduction with principal component analysis (PCA). Clustering of cells subpopulations that will enable to evaluate tumor heterogeneity will be performed using the k-nearest neighbors graph methods. Visualization and exploration of the data will be obtained with the UMAP non-linear dimensional reduction technique. Differential gene expression (DEG) analysis will be performed based on p-value and log fold change using negative binomial models and frequentist models. Gene-set enrichment analysis will be done on the most differentially expressed genes using GeneOntology terms as well as pathway analysis using the Ingenuity Pathway analysis (IPA, Qiagen) tool.
  - For TCR single-cell analysis, we will use scRepertoire to compare the clonotypes between the sequential samples of each patient. Advanced clonal analysis methods such as single-cell clonal homestasis, clonal proportion, overlap analysis and diversity analysis will be evaluated. TCR single-cell data will be integrated into the Seurat objects generated from 5' scRNA-Seq data as described above.

This single cell multi-omics approach will reveal differences in the composition and frequency of cell populations between baseline *versus* on-therapy samples.

2.1.2.1.2. On FFPE samples

A) Immunohistochemistry (IHC) will be performed to assess specific markers of interest, which will be customized for each drug according to the most relevant biomarkers at the time of the analysis. For ADCs, we will notably assess the membrane expression of the target of interest (e.g. HER2 for an anti-HER2 ADC), DNA damage repair biomarkers (e.g. pH2AX and/or RAD51 foci) and immune-related biomarkers (CD8, CD4, Treg and macrophages as a minimum, assessed ideally by multiplex IHC). For bispecific antibodies, we will focus on the drug targets' membrane expression and immune-related biomarkers. More technical details on IHC can be found in Appendix 1.

B) Spatial transcriptomics using the Nanostring GeoMX® plateform. GeoMX™ provides a high-plex and high throughput method that enables spatial RNA and protein detection on FFPE or frozen tissue samples (Figure 6). Briefly, GeoMX™ assay relies upon RNA in situ hybridization or protein antibody hybridization, which are coupled to photocleavable oligonucleotide tags. After hybridization and washes, the oligonucleotide tags are released from target regions using UV exposure. Released tags are then sequenced using nCounter or Next Generation Sequencing (NGS) equipment, while counts are mapped back to previously defined region of interest (ROI) in order to achieve spatially resolved digital profile of protein and/or RNA abundance. Entire methods for GeoMx™ RNA assay can be found in [15].

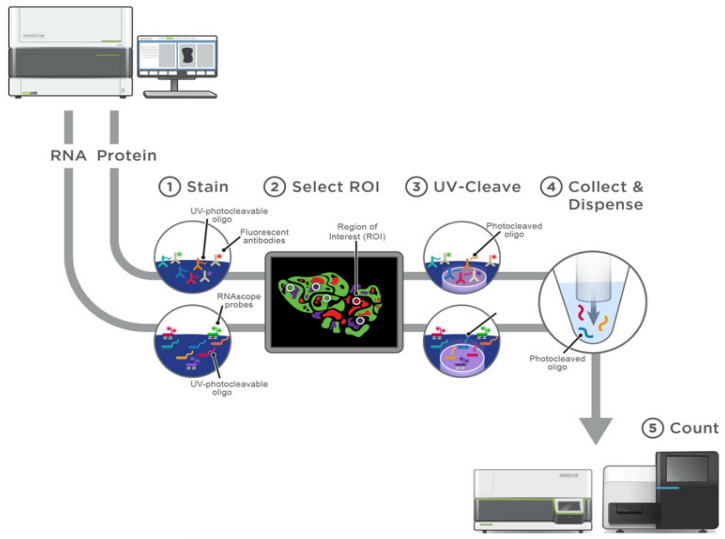


Figure 6. Overview of the GeoMX™ pipeline

GeoMX™ RNA (+/-protein) assays will be performed on FFPE samples of patients whose samples have failed single cell RNA sequencing, or as a complement of single cell analysis for patients of specific interest (e.g. exceptional response). We have not used this technology so far, which is currently being implemented at Gustave Roussy. The exact pipeline can therefore not currently be described, but we anticipate that we will use the Whole Transcriptome Atlas panel (18000 target transcripts coupled to NGS readout, with 32 areas of Interests analyzed per slide) and/or the analysis of 50 proteins (coupled to nCounter readout, with 24 areas of interest analyzed per slide).

Along with SYTOX nuclei staining, up to three potential additional markers will be used whenever technically feasible. For example, one antibody will stain immune cells (CD45), one epithelial tumor cells (pancytokeratin) and one the target of the drug of interest (e.g. HER2 for anti-HER2 therapy). Fluorescent antibodies will be selected and optimized beforehand to ensure compatibility with GeoMX™. Please see Appendix 2 for more details on the anticipated pipeline.

2.1.2.1.3. On FF samples

A) Bulk RNA sequencing (RNAseq)

Bulk RNAseq will be used to assess gene expression, splicing, fusion, immune contexture and neoantigens analyses. RNA sequencing follows a standard procedure detailed in Appendix 3.

B) Whole Exome Sequencing (WES)

WES will be used to assess mutations, tumor mutational burden, Copy Number Variations. DNA sequencing follows a standard procedure detailed in Appendix 4.

2.1.2.1.4. On peripheral blood mononuclear cells: spectral flow cytometry analysis

In the team of CA Dutertre, conventional spectral flow cytometry analyses will be performed on peripheral blood mononuclear cells (PBMC) of patient samples at the different time points. The panel combines 41 fluorochrome-conjugated antibodies, which allows to monitor all PBMC populations with a focus on T cells and MNPs (see Figure 7

for the antibody panel). For selected patients that will or will not respond to therapy, the LegendScreen/InfinityFlow 2.0 pipeline will be used to generate the high dimensional proteomic single cell data described above.

Wavelengh (nm)	UV		Violet		Blue		Yellow Green		Red	
372	UV1									
387	UV2	HLA-DP BUV395								
427	UV3									
428			V1	CD141 BV421						
443	UV4		V2	CD1c SB436						
458	UV5		V3	SLAN VioBlue						
473	UV6	*L/D blue*	V4							
508			V5	CD9 BV480	B1	CD127 BB515				
514	UV7	CD39 BUV496								
525					B2	CD54 FITC				
528			V6							
542	UV8				B3	CD14 SparkB550				
549			V7	HLA-DQ BV510						
571			V8							
577							YG1	CD304 PE		
581	UV9	CD137 BUV563			B4					
594			V9	CD11b BV570						
598					B5		YG2	CD4 Cfl.YG584		
612	UV10	CD38 BUV615								
615					B6		YG3	CD123 PE/Daz594		
618			V10	CD103 BV605						
660					B7	CD1a BB660	YG4		R1	AXL APC
664	UV11	CD169 BUV661	V11	CD3/16/19/20 BV650						
678					B8	*CD45 PercP*	YG5	PD-L1 PE/Cy5	R2	CADM1 + a-chi-AF647
691	UV12		V12							
697					B9	CD206 PP/Cy5.5	YG6		R3	CD45RA Sp.NIR685
717					B10				R4	CD5 APC/R700
720	UV13		V13	FceRIa BV711			YG7			
738					B11				R5	
750	UV14	CCR7 BUV737	V14	PD1 BV750			YG8	CD25 PE/AF700		
760					B12	CD207 BB755			R6	
780	UV15		V15	CD86 BV786			YG9	CD209 PE/Cy7		
783					B13				R7	CD88 APC/Fire750
783										
812	UV16	CD11c BUV805	V16		B14		YG10	HLA-DR-PE/Fire810	R8	CD8 APC/Fire810

Figure 7. Antibody panel for the conventional spectral flow cytometry analysis.

2.1.2.2 – Methodology for AIM2: Circulating Tumor Cells

Taking into account the different MoA of the molecules evaluated, several time-points will be tested. Blood sampling (30 mL) will be performed at baseline, day 1, day 7, day 14 and tumor progression (Figure 8). Depending on the results obtained at day 1, day 7 and day 14 an additional blood sample may be collected at one month to further confirm the treatment impact on the therapeutic target. CTCs will be enriched by hematopoietic cell depletion with the RosetteSep reagent which enables reliable isolation of CTCs without *a priori* on phenotype (Figure 3). Then the enriched cell fraction will be permeabilized, stained by four-color immunofluorescence (Hoechst 33342/CD45/anti-tumor marker/anti-therapeutic target) and isolated as single cells by fluorescence activated cell-sorting (FACS). Whole-genome amplification (WGA) of single CTCs will be performed with the Ampli1 kit (Silicon Biosystems). WGA quality will be controlled by PCR. Copy number alteration (CNA) profiling will be performed on CTC WGA samples by the LowPassGenome technique (Menarini Silicon Biosystems). Control white blood cells (WBC) will be processed according to the same workflow. Germline DNA will be analysed for each patient. All the techniques and bioinformatic methodology for CNA profiling are already established [16].

Epithelial markers (pan-cytokeratins, EpCAM) will be used to assess the tumor origin of cells identified as CTCs in patients with epithelial cancers. In patients with non-epithelial cancer, immunofluorescent staining will be performed with an anti-Hexokinase 2 antibody. Hexokinase 2 is an enzyme involved in glucose metabolism which is expressed in a wide range of cancers of epithelial and non-epithelial origin while being undetectable on normal cells. The panel of antibodies that will be used to cover the wide range of therapeutic targets is under development. A preliminary result of therapeutic target labeling in CTCs from an early phase trial patient is shown in Figure 9.

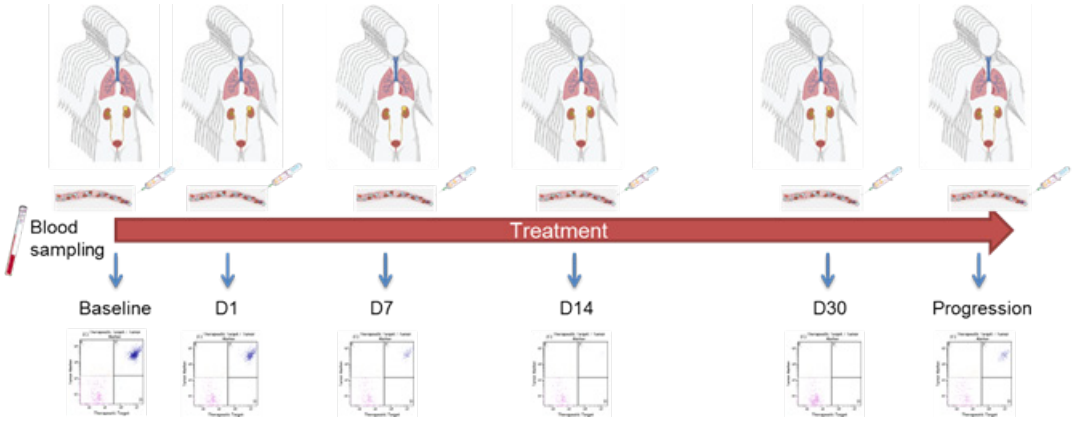
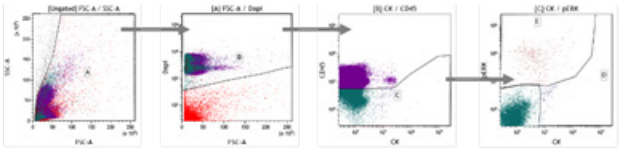
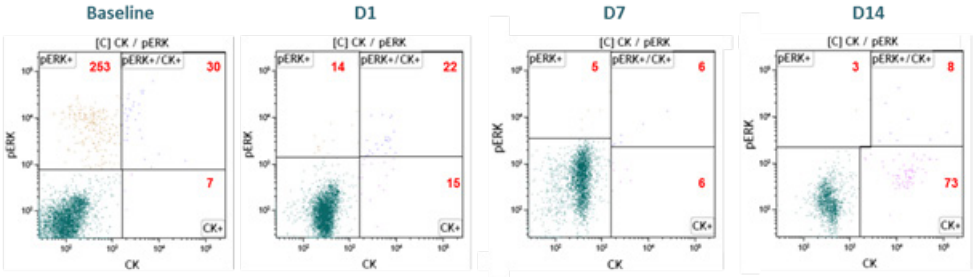


Figure 8. Overview of blood sample collection for CTC analyses. Blood samples will be collected at baseline, day 1, day 7, day 14 and tumor progression. Depending on the results obtained at day 1, day 7, day 14 an additional blood sample may be collected at D30 to further assess the therapeutic target loss.

A. Gating strategy



B. Example of therapeutic target detection



Time-point	Total CTC count	pERK1/2+ CTCs	pERK1/2+/CK- CTCs	pERK1/2+/CK+ CTCs	pERK1/2-/CK+ CTCs
Baseline	290	283	253	30	7
D1	51	36	14	22	15
D7	17	11	5	6	6
D14	84	11	3	8	73

Figure 9. Example of therapeutic target detection in CTCs from a patient with NSCLC included in an early phase trial. (**A**) The gating strategy includes cell selection according to size and granularity, selection of Hoechst 33342-positive elements according to size, selection CD45 negative cells, detection of CTCs according to tumor marker (pan-cytokeratins) and therapeutic target (phosphoERK) expression. (**B**) Therapeutic target detection at different time-points on treatment.

2.1.2.3 - Bioinformatic analyses

Globally, analysis will first be performed as follows: (1) on a per-patient basis and longitudinal fashion for all tumor and CTCs data; and (2) on a per timepoint basis (baseline versus on treatment versus at resistance), notably for bulk RNAseq and CTC data. In tumor samples (bulk analysis, single-cell analysis and IHC), the most variable cell populations or most differentially expressed genes will represent candidates for understanding the mechanism of action of drugs and for biomarker development. In CTCs, the timeframe and magnitude of change in the pharmacological biomarker of interest will be assessed. In all cases, changes will be correlated to patient clinical outcome and tumor response. Because of low numbers and consequent insufficient power of this proof-of-concept study, no formal statistical comparison will be made: best candidates will be identified through an in-depth analysis of each single case. When

relevant, biomarker candidates will be explored and revalidated in larger and more homogeneous cohorts from Phase 2-3 trials.

2.2 Risks and limitations

Based on our previous experience on performing patient biopsies, CTCs analyses and single-cell sequencing, we are confident that at least part of this project will be done as planned. Considering the continuous enrolment of patients at the DITEP and the increase in opening new clinical trials that evaluate complex drugs or complex drug combinations, we do not foresee any difficulty in trials selection or patient accrual. The DITEP also has a long-standing experience in performing tumor biopsies and CTC collection, for which we do not anticipate any particular hurdle.

The main risks and alternative strategies are as follows:

1. **Biopsy failure:** if a biopsy does not pass the quality controls for single cell profiling (low tumor cell number, low cell viability, etc.), we will not proceed to encapsulation and library construction with the 10X platform. Indeed, the 10X Chromium® kits, library construction and sequencing are the costliest steps. In order to mitigate this risk, we will have a specific circuitry with notably: (i) an involvement of the interventional radiologists to explain them the importance of the quality of the biopsy, and (ii) one highly involved and dedicated person (to be recruited specifically for this program) who will immediately handle and process the sample. If the biopsy failure is a pre-treatment biopsy, we will replace the patient; if it involves an on-treatment biopsy in a patient where the pre-treatment biopsy was successful, we will still perform bulk RNAseq, and potentially spatial profiling (GeoMX™), on the corresponding FFPE sample. Indeed, if a drug is active and the patient responds to the therapy, the on-treatment biopsy has a higher likelihood of containing dead cells, thanks to the treatment effect. In order to limit this risk, we will endeavor to perform the on-treatment biopsy as soon as possible (and ethically acceptable) after treatment initiation.

2. **Technical challenges on CITE-Seq:** looking at the membrane proteins at the cell level (CITE-Seq) adds a technical time for sample processing, after the tumor sample has been dissociated and prior to cell encapsulation for scRNA-Seq at the 10X® machine. This additional time may affect cell viability. This step will be optimized with the team of C. A. Dutertre, who has not yet used such technique on solid tumors. We have therefore limited the number of patients whose samples will undergo CITE-Seq to six (i.e. 12- 18 samples maximum). In case this extra-time affects cell viability, we will not proceed further with CITE-Seq and perform only scRNA-Seq (i.e. looking at the whole transcriptome at the unique cell level, and not evaluating the membrane protein expression) or complete with GeoMX™.

3. **Heterogeneity in patients and treatments:** our study will evaluate various drugs and, by definition, phase 1 trials include a low number of highly-selected patients with diverse malignancies. We therefore plan to apply a stringent trial selection for patients profiled by scRNA-Seq, with an approximate number of 5 (and maximum 10) trials evaluating molecules with promising activity being selected. For CTCs, as the number of patients will be higher, we plan to evaluate a higher number of trials. In any case, the plan of this program is to make a proof-of-concept with a very in-depth characterization of a limited number of patients, rather than building a large homogeneous cohort. This “heterogeneity” risk is therefore inherent to the nature of the project.

4. **CTCs staining optimization:** F. Farace and her team are worldwide-renowned experts in CTC processing and analysis. Some stainings that will be used in this project have already been optimized, while others are being optimized to match the trials pharmacodynamic targets. There is therefore a possibility that not all desired markers will be successfully optimized, for technical reasons. We will therefore focus on trials for which we have validated biomarkers that will allow a reliable assessment of the drug’s pharmacodynamics.

5. **Refusal from the drug company** for us to perform such analyses on biopsies: although drug companies are traditionally very favorable to translational studies performed by investigators, we cannot rule out that one may be against such program (e.g. extra-biopsy, IP, etc.); considering the large number of molecules valuated at the DITEP which are developed by multiple drug companies, we are confident that such program will be feasible with at least some of the compounds of interest.

2.3. Time frame / calendar

		Year 1		Year 2		Year 3	
		S1	S2	S1	S2	S1	S2
Single cell / spatial transcriptomics (2-3 timepoints per patient)	Inclusion of 10 patients						
	Analysis of the first 10 patients						
	Inclusion of all patients						
	Analysis of all patients						
	IHC, RNAseq and WES on archived samples						
	Analysis of IHC, RNAseq and WES						
	Final analysis with integration of patient outcome and clinical data						
Peripheral immunophenotyping	CYTEK on patients with single cell						
Circulating tumor cells (3-5 timepoints per patient)	Inclusion of 30 patients						
	Analysis of the first 30 patients						
	Inclusion of 60 patients						
	Analysis of all patients						
	Final analysis with integration of patient outcome and clinical data						
	Biomarker optimisation (if relevant)						
	Scientific valorisation (congres, publication)						

2.4. Significance of the planned work or why the project can make the difference

Such program is quite unique and has not been performed by any other large cancer center or drug development department. It is highly innovative, and uses cutting-edge technologies applied to the newest anticancer drugs, in one of the world largest drug development departments. The aim of this project is to be a proof-of-concept pilot study, which will serve as catalyzed to launch larger programs in collaboration with the pharmaceutical industry and /or academic funding.

Expected results: at the end of this project, we aim at having:

- explored and further deciphered the MoA of complex drugs in patients using cutting-edge technologies, such as single-cell RNA sequencing and spatial biology;
- correlated these findings with peripheral biomarkers of interest on circulating tumor cells, which may allow to better select patients and identify early patients who do (or not) respond to a given drug
- made the proof-of-concept of the feasibility and the added-value of such approach to the drug development process, with the ultimate aim of launching a larger and independent program.

Short and long-term impact: Despite some anticipated heterogeneity among studied cases, we believe that our program will bring essential information in deciphering the MoA of complex drugs, which will allow to optimize their development and be hypothesis-generating for clinically-relevant biomarkers to be validated in larger later phase trials. Only such integrated clinical-scientific approach can generate impactful results for drug development, and trigger a long-term win-win partnership with industry that develop new drugs. Indeed, we hope that our program will help the pharmaceutical companies to understand the MoA of new drugs and subsequently improve their development; reciprocally, this will allow Gustave Roussy to actively participate in such drug development-associated translational research and, importantly, to become even more attractive for participating in trials that evaluate the best and most innovative complex drugs: this will ultimately benefit to patients who will be offered such trials.

3. Partnerships / Team structure

3.1. Other partners involved (scientific, academic, social, political, etc.)

The main partners involved have been described in section 1.4. Other partners involved will include:

- DITEP medical staff: investigators and co-investigators
- DITEP clinical research staff: research nurses, clinical trials coordinators, data managers, etc.
- Members of S. Postel-Vinay, F. Farace and CA Dutertre teams (U981 INSERM)
- Interventional radiologists
- Gustave Roussy sequencing platform
- Indirectly: academic collaborators and pharmaceutical companies that will be developing the drugs of interest and sponsoring the corresponding trials

3.2. CVs and biography of scientists involved

POSTEL-VINAY Sophie, MD-PhD
Gustave Roussy - Drug Development Department (DITEP) – U981 INSERM — CNOM 17919
114 rue Edouard Vaillant – 94805 Villejuif — RPPS 10100526960
sophie.postel-vinay@gustaveroussy.fr — GMC 6161446

EDUCATION

School / University	*Diploma*	*Year*	*Specialty*
Lycée Louis-le-Grand, Paris	Baccalauréat	1998	Mathematics and Sciences
Université Paris-Sud XI	Bachelor of Science	2009	Oncology
Université Paris-Sud XI	MD	2010	Medicine
Université Paris-Sud XI	PhD	2013	Oncology
Université Paris-Sud XO	HDR	2020	Oncology

PROFESSIONNAL EXPERIENCE

Mar 20 – current	**Head of the Drug Development Committee** – Gustave Roussy
Jan 18 – current	**ATIP-Avenir Young Group Leader INSERM – U981 INSERM**
Feb. 16 – current	**Physician scientist - Gustave Roussy**; DITEP (Dr C. Massard) – **U981 INSERM** (Pr F. André)
Nov. 13 – Jan. 2016	**Assistant Professor – Gustave Roussy**; DITEP (Pr J.C. Soria)
July 11 – Oct. 13	**PhD – The Institute of Cancer Research (London) -** Pr A. Ashworth, Pr J.C. Soria, Pr C. Lord
Nov. 09 – May 11	**Medical Oncology Fellow (**Gustave Roussy & Institut Curie)
Nov. 08 – Nov. 09	**Master 2 (Bachelor of Science) – Institut Curie** (Dr O. Delattre)
May 07 – Nov. 08	**Clinical Fellow Drug Development Unit – Phase 1 trials**; Royal Marsden Hospital (London, UK)
Nov. 06 – May 07	**Medical Oncology Fellow** (Gustave Roussy, St Louis)

DIPLOMAS

2020	**Ability to supervise research (Habilitation à diriger les recherches)**
2017	**Specialist oncologist of Cancer Centres**
2013	**PhD** (with congratulations of the jury), Paris-Sud XI
2010	**Medical thesis and Medical Oncology Specialty Graduation** (silver medal), Paris-Sud XI
2009	**Bachelor of Science – Biology of the cancer cell**, Paris-Sud XI
2004	**Examen National Classant**
2001	**Four Master 1: Clinical genetics; Immunology; Biostatistics; Molecular biology**
1999	**Received first** at the medical school first year competitive exam - Paris V
1998	**Baccalauréat in Science, « mention Très Bien »**, Lycée Louis-Le-Grand

PUBLICATIONS (10 among 91 publications; 17 as **first** and **20** as **last author)**

- Citations = 7057; H-index = 39; i10-index = 77 (google scholar, accessed 27 Aug 2021)

1. *Targeting the DNA damage response in immuno-oncology: Developments and opportunities.* Chabanon RM, Rouanne M, Lord CJ, Soria JC, Pasero P and **Postel-Vinay S. Nature Reviews Cancer** *(Online ahead of print)*
2. *PBRM1 deficiency in cancer is synthetic lethal with DNA repair inhibitors.* Chabanon RM, Morel D, Eychenne T, Colmet-Daage L, Bajrami I, Dorvault N, Garrido M, (…) Massard C, Pettitt SJ, Margueron R, Choudhary JS, Almouzni G, Soria JC, Deutsch E, Downs JA, Lord CJ, **Postel-Vinay S. Cancer Res.** 2021 Jun 1;81(11):2888-2902
3. *Sustained cancer clinical trial activity in a French hospital during the first wave of the COVID-19 pandemic.* Bayle A, Baldini C, Martin-Romano P, Michot JM, Champiat S, Bahleda R, Marabelle A,(…), Albiges L, Besse B, Soria JC, Massard C, Barlesi F, **Postel-Vinay S. Cancer Cell.** 2021 Aug 9;39(8):1039-1041
4. *Epigenetic drugs in solid tumours: lessons learned and future promises?* Morel D, Jeffery D, Aspeslagh S, Almouzni G, **Postel-Vinay S. Nature Reviews Clinical Oncology.** 2020 Feb;17(2):91-107
5. *ERCC1-deficiency exacerbates tumor cell-intrinsic immunity in response to PARP inhibitors in non-small cell lung cancer.* Chabanon RM, Muirhead G, Krastev DB, Adam J, (…), Ashworth A, Pettitt SJ, Haider S, Marabelle A, Tutt ANJ, Soria JC, Lord JC, **Postel-Vinay S**. **The Journal of Clinical Investigation**. 2019 Mar 1;129(3):1211-1228

6. *DNA repair deficiency sensitizes lung cancer cells to NAD+ biosynthesis blockade.* Touat M, Sourisseau T, Dorvault N, Chabanon RM, Garrido M, Morel D, (…), Bouillaud F, Pierron G, Ashworth A, Lombès A, Lord CJ, Soria JC, **Postel-Vinay S**. **The Journal of Clinical Investigation** 2018 – Apr 2;128(4):1671-1687
7. *High-throughput screen identifies PARP1/2 inhibitors as novel therapeutic targets for ERCC1-deficient Non-Small Cell Lung Cancer*. **Postel-Vinay S,** Bajrami I, Friboulet L, Elliott R, (…), André F, Soria JC, Lord CJ, Ashworth A. – **Oncogene**. 2013 Nov 21;32(47):5377-87.
8. *DNA repair in Non-Small Cell Lung Cancer: the potential for treatment optimization and the design of novel therapeutic approaches***. Postel-Vinay S,** Vanhecke E, Olaussen K, Lord CJ, Ashworth A, Soria JC – **Nature Reviews Clinical Oncology,** 2012 Feb 14;9(3):144-55
9. *Phase I trials of molecularly targeted agents: should we pay more attention to late toxicities?* **Postel-Vinay S**, Gomez-Roca C, Molife R, Anghan B, (…) , Soria JC, Kaye S, Paoletti X; **J Clin Oncol.** 2011 May 1; 29(13):1728-35
10. *Variants at TARDBP and EGR2/ADO loci associated with Ewing sarcoma susceptibility.* **Postel-Vinay S,** Véron A, Tirode F, Pierron G, (…), Chanock SJ, Thomas G, Cox DG, Delattre O. – **Nature Genetics,** 2012 Feb 12;44(3):323-7

GRANTS and AWARDS

2019	**Prix de l'Académie des Sciences – Prix Irène Joliot-Curie –** National Science Academy Award
	Prix de l'Académie de Médecine – Prix Gallet et Breton - National Medicine Academy Award
	Labellisation ARC Recherche Fondamentale – 3 years fundamental research grant and ARC label
2018	**Prix des Sœurs Lucie et Olga Fradiss**
	SIRIC (Co-leader for workpackage 1)
	Société Française de Cancérologie de l'enfant
2017	**ATIP-Avenir INSERM-CNRS** (Young Group Leader)
	Emergence INCa
2011 & 2012	**INCa** "soutien à la formation à la recherche translationnelle" (PhD)
	ESMO translational research fellowship
	ASCO merit award
2010	**AACR** *« Scholars in training »* Finalist
2008	**INCa** : « bourse jeunes chercheurs » (Master 2)

CLINICAL RESEARCH (over the last 3 years)

• **Head of the drug development committee** and referee for the Drug Development Unit INCa agreement
Principal Investigator of **8 phase 1 trials** and **2 phase 2** trials; Co-investigator of more than 80 phase 1 trials
• **Writing and opening** of **two academia-sponsored multicentric phase 2** trials

CLINICAL TEACHING and MENTORING

2013 – current	**Chemotherapy course– Gustave Roussy**
	Teaching to clinical fellows / medical students
	Master 2 of Oncology (Paris-Sud XI)
	Diploma of molecular medicine – Gustave Roussy, HEGP, Institut Curie
	Clinical supervision: 16 clinical fellow's projects and one pharmacy thesis resulting in eleven international presentations and four publications – some projects are still ongoing
2015 & 2016	**Faculty for the FLIMS workshop**
	Creation of weekly teaching sessions at the Drug Development Department

FUNDAMENTAL RESEARCH ACTIVITIES and SUPERVISION (U981 INSERM Unit – INSERM/CNRS ATIP Avenir)

2016 – current	**Physician Scientist and independent ATIP-Avenir Young Group Leader** studying Chromatin remodeling deficiencies in solid tumors; Supervision of 2 BSc, 4 PhD students, 1 technician, 1 engineer, 2 postdoctoral fellow and 1 bioinformatician, resulting in the publication of 5 reviews (1 in *Nat Rev Cancer* and 1 in *Nat Rev Clin Oncol.;* 2 in *Ann. Oncol;* 1 in *Clin Cancer Res*) and 2 original manuscripts (*Cancer Research; The J. Clin Invest*) as senior author
2013 – 15	**30%-time laboratory activity – 70% Assistant Professor clinical activity;** Supervision of 3 BSc, resulting in the publication of 2 original manuscripts (1 in *The J. Clin Invest*) as senior author

INTERNATIONAL COMMUNICATIONS (oral only since 2016; total of 33 communications as first or last author)

- 2021: TAT – Invited Speaker
- 2020: Gordon Conference, TAT, ESMO– Invited Speaker; AACR: Oral presentation
- 2019: Keystone Symposia DNA replication and genome instability – Invited speaker
- 2018: MACC – Invited speaker
- 2017: Advances in Drug Discovery – Invited speaker
- 2016: EORTC-NCI-AACR– Invited Speaker

CONGRES (Organisation)

2017 - 21 Targeted Anticancer Therapies; post-TAT; Gustave Roussy research days

EDITORIAL ACTIVITIES

- **Reviewer** *ad hoc* (e.g. J Clin Invest; Ann Oncol; Lancet Oncol; Nat Comm); national and international grants
- **Editorial** board (Annals of Oncology; Journal of Immunology and Personalized Oncology)

Françoise FARACE, PhD, HDR
Email: françoise.farace@gustaveroussy.fr

Professional Career

- Since 2005, Head of the Rare Circulating Cells Translational Unit, CNRS UMS3655 – INSERM US23 AMMICa "Analyse moléculaire, modélisation et imagerie de la maladie cancéreuse" Gustave Roussy, Villejuif
- Since 2010, Senior group leader of the Circulating Tumor Cell team, INSERM Unit U981 "Predictive biomarkers in new therapeutic targets in oncology" Gustave Roussy Institute, Villejuif
- 2002 – 2005, President of the Board and Chief Scientific Officer (CSO) of C.I.L Technologies (biotech company, main shareholders: INSERM Transfert and Free University of Brussels), Free University of Brussels, Brussels, Belgium and Medical School of Cochin-Port Royal, Paris
- 1999 - 2002, Researcher permanent position, Cellular therapy laboratory, INSERM Unit 362 (Hematopoiesis and Stem cells) Gustave Roussy Institute, Villejuif
- 1990 –1999, Researcher permanent position, Translational laboratory of cellular immunology, INSERM Unit 333 (Tumor Immunology) Gustave Roussy Institute, Villejuif
- 1990, Post-doctoral visitor, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland
- 1988 - 1990, Post-doctoral position INSERM Unit 333 (Tumor Immunology) Gustave Roussy Institute, Paris

Education

- 2011, **Ability to supervise research (Habilitation à diriger les recherches),** Paris University XI
- 1988, PhD in pharmacology, Paris University VI
- 1984, DEA (diplôme d'études approfondies) in pharmacology Paris University VI
- 1983, Master degree in biochemistry, Saint Charles, Aix Marseille University.
- 1982, License of biochemistry, Saint Charles, Aix Marseille University.
- 1978/81, Graduate School of Chemistry, Saint Jérôme, Aix Marseille University

Main Teaching Activities

- Since 2010, internships in medical biology, diplomas of thoracic oncology and of therapeutic innovation, Paris Saclay University
- Since 2017, Licence-Master-PhD course « Etude des Mécanismes Cellulaires et Moléculaires des Processus Cancéreux », Faculté de Médecine Sorbonne Université, Paris.

Research Activities

- Supervision of more than 25 post-docs, PhD and master 2 students
- Coordination or participation to more than 30 national or European academic research projects or in collaboration with pharmaceutical/biotech companies

Main funded academic research projects (selected):

- European Innovative Medicine Initiative (IMI) CANCER-ID project "Developing liquid biopsy to diagnose cancer" (2014-2019)
- INCA PRTK CELLTRACE project (coordinator): "Mechanism of resistance to targeted therapies through circulating tumor cell (CTC) analysis in *ALK*-positive NSCLC patients" (2015-2018)

- ANR CTC-ID (coordinator): Molecular and functional characterization of CTCs.. to analyze resistance mechanism to tyrosine kinase inhibitors in NSCLC patients… (2015-2019)
- UNICANCER/FFCD PRODIGE 28, ancillary study (2017-ongoing)
- ERA PerMed RAD51 Predict Project (2020-2024)
- INCA PRTK ARIANES – CTC project (S. Postel Vinay coordinator) (2021-2023): Development of circulating predictive biomarkers of response to PARP inhibitors +/- anti-PD-L1 therapy: a proof-of-concept pilot study

Selected publications (out of 105 scientific publications)

1. Oulhen M, Pawlikowska P, Tayoun T, Garonzi M, Buson G, Forcato C, Manaresi N, Aberlenc A, Mezquita L, Lecluse Y, Lavaud P, Naltet C, Planchard D, Besse B, **Farace F**. *Circulating tumor cell copy-number heterogeneity in ALK-rearranged non-small-cell lung cancer resistant to ALK inhibitors.* **NPJ Precis Oncol.** 2021 Jul 16;5(1):67. doi: 10.1038/s41698-021-00203-1.PMID: 34272470V
2. Faugeroux, E Pailler, M Oulhen, O Deas, L Brulle-Soumare, C Hervieu, V Marty, K Alexandrova, KC Andree, N H Stoecklein, DTramalloni, SCairo, C Nicotra, LWMM Terstappen, N Manaresi, V Lapierre, K Fizazi, JY Scoazec, Y Loriot, JG Judde, **F Farace.** *Genetic characterization of a Unique Neuroendocrine Transdifferentiation Prostate Circulating Tumor Cell - Derived eXplant Model.* **Nature Com 2020** 11, 1-16 doi:10.1038/s41467-020-15426-2
3. Pailler E, Faugeroux V, Oulhen M, Mezquita L, Laporte M, Honoré A, Lecluse Y, Queffelec P, NgoCamus M, Nicotra C, Remon J, Lacroix L, Planchard D, Friboulet L, Besse B, **Farace F**. *Acquired Resistance Mutations to ALK Inhibitors Identified by Single Circulating Tumor Cell Sequencing in ALK-Rearranged Non-Small-Cell Lung Cancer.* **Clin Cancer Res. 2019** Aug 22. doi: 10.1158/1078-0432.CCR-19-1176.
4. Faugeroux V, Lefebvre C, Pailler E, Pierron V, (…) Oulhen M, Vielh P, Rameau P, NgoCamus M, Massard C, Laplace-Builhé C, Tibbe A, Taylor M, Soria J-C, Fizazi K, Loriot Y, Julien S and **Farace F.** *An Accessible and Unique Insight into Metastasis Mutational Content through Whole-Exome Sequencing of Circulating Tumor Cells in Metastatic Prostate Cancer.* **Eur Urol Oncol 2019**, Jan 4. pii: S2588-9311(18)30213-X. doi: 10.1016/j.euo.2018.12.005.
5. C. R. Lindsay, V. Faugeroux, S. Michiels, E. Pailler, F. Facchinetti, D. Ou, M. V. Bluthgen, (…) C. Caramella, F. Billiot, J. Remon, D. Planchard, J.-C. Soria, B. Besse. **F.Farace**. *A Prospective Examination of Circulating Tumor Cell Profiles in Non-Small Cell Lung Cancer Molecular Subgroups.* **Ann Oncol 2017** Jul 1;28(7):1523-1531.
6. Pailler E, Oulhen M, Borget I, Remon J, Ross K, Auger N, (…) Lindsay CR, Planchard D, Soria JC, Besse B, **Farace F**. *Circulating Tumor Cells with Aberrant ALK Copy Number Predict Progression-Free Survival during Crizotinib Treatment in ALK-Rearranged Non-Small Cell Lung Cancer Patients.* **Cancer Res. 2017** May 1;77(9):2222-2230.
7. Pailler, E, Auger, N, Lindsay, CR, Vielh, P, Islas-Morris-Hernandez, A, Borget I, Ngo-Camus, M, Planchard, D, Soria, J-C, Besse, B, **Farace, F**. *High Level of Chromosomal Instability in Circulating Tumor Cells of ROS1-Rearranged Non-Small-Cell Lung Cancer.* **Ann Oncol 2015** 26 :1408-15.
8. C. Massard, M. Oulhen, S. Le Moulec, N. Auger, S. Foulon, A. Abou-Lovergne, F. Billiot, A. Valent, V. Marty, Y. Loriot, K. Fizazi, P. Vielh, **F. Farace**. *Phenotypic and genetic heterogeneity of tumor tissue and circulating tumor cells in patients with metastatic castration-resistant prostate cancer: a report from the PETRUS prospective study.* **Oncotarget 2016** Aug 23 ;7(34):55069-55082.
9. Pailler, E., Adam, J., Barthélémy, A., Oulhen, M., Auger, N., Valent, A., Borget, I., Planchard, D., Taylor, M., André, F., Soria, J.C., Vielh, P., Besse, B. and **Farace, F**. *Detection of Circulating Tumor Cells Harboring a Unique ALK-Rearrangement in ALK Positive Non-Small-Cell Lung Cancer.* **J Clin Oncol 2013**, 31(18):2273-81.
10. Taylor, M., Billiot, F., Marty, V., Rouffiac, V., Cohen, P., Tournay, E., Opolon, P., Louache, F., Vassal, G., Laplace-Builhe, C., Vielh, P. Soria, J.C and **Farace, F.** *Reversing resistance to vascular-disrupting agents by blocking late mobilization of circulating endothelial progenitor cells.* **Cancer Discov 2012**, 2: 434-449.

Charles-Antoine DUTERTRE, PhD, CRCN INSERM

INSERM UMRS1015, Villejuif, France (Institut Gustave Roussy), Dr Florent Ginhoux's team
114 rue Edouard Vaillant – 94805 Villejuif
e-mail: charles-antoine.dutertre@inserm.fr, Charles.DUTERTRE@gustaveroussy.fr

EDUCATION:

***2004-2008*: PhD in Immunology**, **CIFRE fellowship** [INSERM UMRS 872 team / Laboratoire français du Fractionnement et des Biotechnologies (LFB)], University Paris 7/D. Diderot, summa cum laude (mention très honorable). http://www.theses.fr/2008PA077132

***2002-2003*: Post-master professional certificate** "**Mastère Spécialisé Management de l'Innovation technologique dans les Agroactivités et les Bioindustries"** (**MASTERNOVA**) (INA-PG/AgroParisTech, Reims Management School, France).

***2001-2002*:** "**Magistère de Génétique"** (University Paris 7/D. Diderot) (Engineer in genetics)
Graduate studies in oncology at the D.E.A. "Bases Fondamentales de l'Oncogenèse" (University Paris 7 – Denis Diderot), with honors (mention Assez bien).

***2000-2001*: Master's degree of "Génétique Approfondie"** (Genetics) (University Paris 7/D. Diderot), with honors (mention Assez bien).

***1999-2000*: Bachelor's degree of "Biologie Cellulaire et Physiologie"**, genetics specialty, (University Paris 7 – Denis Diderot), with honors (mention Assez bien).

***1997-1999*: Associate's Degree of Biochemistry** (University Paris 6/UPMC), with honors (mention Assez bien).

PROFESSIONNAL EXPERIENCES:

Mar 2021 – onwards: INSERM UMRS1015, Villejuif, France (Gustave Roussy), **Dr F. Ginhoux**'s team (***INSERM CRCN***)

Sep 2020 – Feb 2021: INSERM UMRS1015, Villejuif, France (Gustave Roussy), **Dr F. Ginhoux**'s team (***Res. engineer***)

Dec 2013 – Aug 2020:* Duke-NUS Medical School** & **SIgN (A*STAR)**, Singapore, **Dr F. Ginhoux**'s team (Research Scientist***)

Aug 2012 – Aug 2013:* INSERM UMRS698 team 4**, Paris (X. Bichât hospital), **Pr A. Nicoletti laboratory** (Post Doctorat***)

Mar 2009 – Jul 2012:* INSERM U1016 team 34**, Paris (Cochin Institute), **Dr A. Hosmalin laboratory** (Post Doctorat***)

Feb 2004-Feb 2009:* INSERM UMRS 872 team 14**, Paris (Cordeliers Research Center), **Dr. JL Teillaud** team. (PhD***)

***Mar 2003-Jul 2003:* CRITT Chimie-Environnement (*MASTERNOVA internship*)**

Sep 2001-Aug 2002:* INSERM U434**, Saint-Louis Hospital, Paris, France, **Dr. M. Giovannini laboratory**. (DEA***)

Mar-Jul 2001: Genetics Department **(University of Leicester, Leicester, England**), **Pr. Sir Alec Jeffreys**. (***ERASMUS***)

Jul-Aug 2000: Human Genetics Department (**RamBam Hospital, Haifa, Israel**), **Dr. R. Bachour-Guershoni**. (***Bachelor's degree)***

Jul-Aug 1999:* Molecular and Cellular Biophysics Laboratory of Paris 6 University**, **Pr. JY Turpin**. (Associate's deg.)***

RESEARCH MANAGEMENT:

1. **Annalisa Derosa**, **medical extern from La Sapienza University, Rome, Italy**; One-year internship (2009-2010)
2. **Matthieu Goguet**, Bachelor's degree student from the **École Supérieure Technique de Biologie Appliquée (ESTBA)**; One-year internship (2009-2010)
3. **Jean-Pierre Jourdain**, Bachelor's degree student from the **École Supérieure Technique de Biologie Appliquée (ESTBA)**; One year internship (2010-2011), followed by 2 years internship for the Master's degree from the **Ecole Pratique de Hautes Etudes (EPHE, 2011-)**
4. **Sonia Amraoui**, **Graduate student for the Master2 Physiopathologie de la réponse immune (PRI, Université Paris 6)**; One year internship (2010-2011), followed by her PhD (**Thèse de doctorat, Université Paris 6, 2011-)**
5. **Edouard Lefèvre**, Medical doctor, **Graduate student for the Master2 Physiopathologie de la réponse immune (PRI, Université Paris 6)**: One-year internship (2011-2012)
6. **Sergio Irac Erdal**, Research Assistant hired under the NMRC BNIG14MAY009 Grant. (2015-2017)
7. **Alfonso Tan-Garcia**, Medical student, **PhD (Duke-NUS Medical School)**; (2014-2017)
8. **Pei Yi Ng**, A-star scholar intern **(SIgN, A-Star)**; September 2017 – June 2018
9. **Shabnam Khalilnezhad**, Foreign intern student **(SIgN, A-Star)**; March 2018 – ongoing
10. **Ahad Khalilnezhad,** Singa fellow PhD student **(SIgN, A-Star)**; August 2018 – ongoing
11. **Rachel Ee**, A-star scholar student **(SIgN, A-Star)**; September 2018 – January 2019
12. **Cécile Piot**, SIGPA intern student (**SIgN, A-Star**); January 2019 – August 2019
13. **Sergio Irac Erdal**, Research fellow hired under the NMRC BNIG14MAY009 Grant. (2015-2017)
14. **Wan Ting Kong**, Research Officer (**SIgN, A-Star**); May 2020 – September 2020
15. **Evelyn Halitzki**, Foreign intern student (**Limes Institute, Bonn, Germany**); July 2020 – September 2020
16. **Kevin Mulder**, SIGPA intern student (**SIgN, A-Star**); January 2020 – August 2020
17. **Amit Patel**, Post-doctorate researcher (**Inserm UMRS1015, IGR, Paris, France**); Sep 2020 – ongoing
16. **Kevin Mulder**, Philanthropia fellowhip awardee, PhD student (**UMRS1015, IGR, France**); Nov 2020 – ongoing

TEACHING:

January 2013: Extensive Flow Cytometry workshop NV602, Master2 of Immunology (40h, Paris 7 University)
2016: Duke-NUS Medical School (GMS6904/Y2016). Dendritic cell biology
2017: Duke-NUS Medical School (GMS6904/Y2017). Dendritic cell biology

GRANT:

April 2015 – February 2018: NMRC BNIG14MAY009 Grant (NMRC/BNIG/2026/2014). **173,800 SGD awarded**. Evaluation of Vaccibodies® as a novel dendritic cell targeting strategy to protect against Hepatitis B virus.

PATENTS and technology disclosures:

Hosmalin A, **Dutertre CA**. A M-DC8+ MONOCYTE DEPLETING AGENT FOR THE PREVENTION OR THE TREATMENT OF A CONDITION ASSOCIATED WITH A CHRONIC HYPERACTIVATION OF THE IMMUNE SYSTEM. **PATENT application: # PCT/EP2012/07816.**

Dutertre CA, Florent Ginhoux. Cytometry by Time of Flight (CyTOF) antibody panel to study human mononuclear phagocytes. **RI Technology Disclosure No: SIGN/TDF/082.**

Dutertre CA, Florent Ginhoux. cDC2 subset comprising of conventional DC2 and DC3 as biomarkers of inflammation and potential therapeutic targets in inflammatory diseases. **Singapore Patent Application Number: 10201905956V.**

PUBLICATIONS (10 among 61 publications; 12 as first and 4 as last author):

Citations = 5762; h-index = 29; i10-index = 43 (Google Scholar, accessed 31 Aug 2021)

1. **Dutertre CA**, Amraoui S, DeRosa A, Jourdain JP, Vimeux L, Goguet M, Richard Y, Liovat AS, Müller-Trütwin M, (…), Viard JP, Garderet L, Decroix N, Launay O, Goujard C, Deveau C, Meyer L, Hosmalin. Pivotal role of M-DC8+ monocytes in the TNFα over-production of viremic HIV-infected patients. ***Blood***, 2012, 120, 2259-2268.
2. **Dutertre CA**, Jourdain JP, Rancez M, Amraoui S, Fossum E, Bogen B, (…), Richard Y, Dalod M, Feuillet V, Cheynier R, Hosmalin A. TLR3-Responsive, XCR1+, CD141(BDCA-3)+/CD8α+-Equivalent Dendritic Cells Uncovered in Healthy and Simian Immunodeficiency Virus-Infected Rhesus Macaques. ***J Immunol***, 2014, 192, 4697-708.
3. Guilliams M*, **Dutertre CA***, Scott CL, McGovern N, Sichien D, Chakarov S, Van Gassen S, Chen J, Poidinger M, De Prijck S, (…), Henri S, Saeys Y, Newell EW, Lambrecht BN, Malissen B, Ginhoux F. Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. ***Immunity***, 2016, 20, 669-684. ***co-1st authors**
4. Zhou P, Chionh YT, Irac SE, Ahn M, Jia Ng JH, Fossum E, Bogen B, Ginhoux F, Irving AT**, **Dutertre CA*,****, Wang LF*. Unlocking bat immunology: establishment of Pteropus alecto bone marrow-derived dendritic cells and macropages. ***Scientific Reports***, 2016, 6, 38597. ***co-senior authors, **corresponding author**
5. See P*, **Dutertre CA***, Chen J*, Günther P, McGovern N, Irac SE, Gunawan M, Beyer M, Händler K, Duan K, Bin Sumatoh HR, Ruffin N, Jouve M, Gea-Mallorquí E, (…) Larbi A, Poidinger M, Chan JKY, Chen Q, Renia L, Haniffa M, Benaroch P, Schlitzer A, Schultze JL, Newell EW, Ginhoux F. Mapping the human DC lineage through the integration of high dimensional techniques. ***Science,*** 2017, 356, 6342. ***co-1st authors**
6. Tan-Garcia A, Wai LE, Zheng D, Ceccarello E, Jo, (…), Ginhoux F, Chen Q, Bertoletti A*, **Dutertre CA*,****. Intrahepatic CD206+ macrophages accumulate and drive inflammation in advanced viral-related liver disease, ***J Hepatol***, 2017*, 67, 490*. ***co-senior authors, **corresponding author**
7. **Dutertre CA**, Becht E, Irac SE, Khalilnezhad A, Narang V, Khalilnezhad S, Ng PY, van den Hoogen LL, Leong JY, Lee B, Chevrier M, Zhang XM, (…), Karagianni P, Tzioufas AG, Malleret B, Brody J, Albani S, van Roon J, Radstake T, Newell EW, Ginhoux F. Single-Cell Analysis of Human Mononuclear Phagocytes Reveals Subset-Defining Markers and Identifies Circulating Inflammatory Dendritic Cells, ***Immunity***, 2019, pii: S1074-7613, 30334-6.
8. Tan-Garcia A, Lai F, Yi NP, Sheng Yeong JP, (…) Gill US, Kennedy PTF, Ginhoux F, Bertoletti A, Chen Q*, **Dutertre CA*,****. GM-CSF neutralisation suppresses pro-inflammatory CD206+ macrophage accumulation in viral-related liver disease, ***J Hepatol Reports***, 2019, 2, 100062. ***co-senior authors, **corresponding author**
9. Sharma A, Seow JJW*, **Dutertre CA***, Pai R, Blériot C, Mishra A, Wong RMM, (…) Chung CP, Ginhoux F, DasGupta R. Onco-fetal Reprogramming of Endothelial Cells Drives Immunosuppressive Macrophages in Hepatocellular Carcinoma. ***Cell***, 2020, DOI:https://doi.org/10.1016/j.cell.2020.08.040. ***co-2nd authors**
10. Mulder K*, Patel AS*, Kong WT*, Piot C, Halitzki E, Dunsmore G, Khalilnezhad S, (…), Zhang XM, Tam JKC, Lim TKH, Wong RMM, Pai R, Khalil AIS, Chow PKH, Chan JKY, Bertoletti A, Albani S, Sharma A, Blériot C, **Dutertre CA**** and Ginhoux F**. The MoMac-VERSE: Dissecting the diversity of human monocytes and macrophages in health and disease ***Immunity***. 2021 Aug 10;54(8):1883-1900.e5. *co-1st authors, ****co-senior authors.**

4. Finance

4.1. Detailed budget asked to Philanthropia:

Because we had no information on the potentially available funding, we present below a "modular" budget which has been adapted according to the number of patients enrolled and number of samples processed per patient. We aim at processing 2 to 3 tumor samples per patient (baseline, on-treatment, +/- resistance when a response is observed) and 5 CTC samples, but because we do not know in advance which patients will respond to thee drug, this number may vary. GeoMX is shown in grey (not counted) as it is an alternative solution to scRNAseq.

Staff					
Bioinformaticien	Single cell, WES, RNAseq	74 250 €	56 250 €	56 250 €	45 000 €
Post-doctoral fellow	single-cell + CTCs + coordination of the whole project	212 400 €	212 400 €	212 400 €	212 400 €
Pathologist	IHC	16 009 €	12 807 €	12 807 €	9 606 €
Single cell		**30 patients with single cell at 3 timepoints**	**20 patients with single cell at 3 tiimepoints**	**30 patiients with single cell at 2 timepoints**	**20 patients with single cell at 2 timepoints**
Preanalytics (AN extraction, Tumor sections)		14 400 €	9 600 €	9 600 €	6 400 €
IHC (10 stainings with some multiplex)		54 000 €	36 000 €	36 000 €	24 000 €
Sampling		3 150 €	2 100 €	2 100 €	1 400 €
Extraction		2 700 €	1 800 €	1 800 €	1 200 €
Banking		12 600 €	8 400 €	8 400 €	5 600 €
RNAseq		40 500 €	27 000 €	27 000 €	18 000 €
WES		55 080 €	36 720 €	36 720 €	24 480 €
5' Single-cell RNASeq		366 300 €	244 200 €	244 200 €	162 800 €
CITE-Seq (max. 6 patients)		77 040 €	51 360 €	51 360 €	51 360 €
Data storage		10 000 €	9 000 €	9 000 €	8 000 €
Immunomonitoring	immunophenotypin	18 000 €	12 000 €	18 000 €	12 000 €
GeoMX	Protein	95 310 €	63 540 €	63 540 €	42 360 €
	RNA (WTA)	204 120 €	136 080 €	136 080 €	90 720 €
	Sequencing	180 000 €	120 000 €	120 000 €	80 000 €
Circulating Tumor Cells		**30 patients wiith CTCs at 5 timepoints; sequencing 2 timepoints**	**30 patients wiith CTCs at 5 timepoints; sequencing 2 timepoints**	**30 patients wiith CTCs at 5 timepoints; sequencing 1 timepoint**	**30 patients wiith CTCs at 5 timepoints; sequencing 1 timepoint**
RosetteSep + Staining + FACS	(5 samples)	51 600 €	51 600 €	51 600 €	51 600 €
WGA + QCs	(6CTCs + 1 CD45 / sample + 1 gDNA)	27 720 €	27 720 €	13 860 €	13 860 €
LowPassGenome Sequencing	(4 CTCs / sample + 1 CD45 + 1 gDNA)	45 000 €	45 000 €	22 500 €	22 500 €
NGS		60 000 €	60 000 €	30 000 €	30 000 €
VAT		36 864 €	36 864 €	23 592 €	23 592 €
Management fees	0.1 of total	117 761 €	75 650 €	88 839 €	45 679 €
Grand total		**1 295 375 €**	**1 016 471 €**	**977 230 €**	**783 929 €**

4.2. Others sources of funding

- Costs at the DITEP = cost per patient (on average 23700€ / patient); staff for organizing and performing the tumor biopsy; clinical investigators time
- F. Farace: 50k€ from Gustave Roussy Fundation for optimizing the stainings on CTCs and for a small pilot experiment
- CA Dutertre: 1.5M from Agence Nationale pour la Recherche (ANR): grant for setting his own lab (for independent projects), but his staff would contribute to the analysis of the CYTEK and CITE-Seq results

As mentioned above (please see Note on page 5), the funding asked to Philanthropia would be used to optimize to perform some pilot "proof-of-concept" experiments and help in launching such program, which ultimately aims at becoming independently funded.

References

1 Yofe, I., Dahan, R. & Amit, I. Single-cell genomic approaches for developing the next generation of immunotherapies. *Nature medicine* **26**, 171-177, doi:10.1038/s41591-019-0736-4 (2020).

2 Bi, K. *et al.* Tumor and immune reprogramming during immunotherapy in advanced renal cell carcinoma. *Cancer cell* **39**, 649-661 e645, doi:10.1016/j.ccell.2021.02.015 (2021).

3 Sade-Feldman, M. *et al.* Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell* **175**, 998-1013 e1020, doi:10.1016/j.cell.2018.10.038 (2018).

4 Zhang, S. *et al.* Longitudinal single-cell profiling reveals molecular heterogeneity and tumor-immune evolution in refractory mantle cell lymphoma. *Nature communications* **12**, 2877, doi:10.1038/s41467-021-22872-z (2021).

5 Sharma, A. *et al.* Longitudinal single-cell RNA sequencing of patient-derived primary cells reveals drug-induced infidelity in stem cell hierarchy. *Nature communications* **9**, 4931, doi:10.1038/s41467-018-07261-3 (2018).

6 Pailler, E. *et al.* Detection of circulating tumor cells harboring a unique ALK rearrangement in ALK-positive non-small-cell lung cancer. *J Clin Oncol* **31**, 2273-2281, doi:10.1200/JCO.2012.44.5932 (2013).

7 Pailler, E. *et al.* Circulating Tumor Cells with Aberrant ALK Copy Number Predict Progression-Free Survival during Crizotinib Treatment in ALK-Rearranged Non-Small Cell Lung Cancer Patients. *Cancer research* **77**, 2222-2230, doi:10.1158/0008-5472.CAN-16-3072 (2017).

8 Pailler, E. *et al.* High level of chromosomal instability in circulating tumor cells of ROS1-rearranged non-small-cell lung cancer. *Ann Oncol* **26**, 1408-1415, doi:10.1093/annonc/mdv165 (2015).

9 Pailler, E. *et al.* Acquired Resistance Mutations to ALK Inhibitors Identified by Single Circulating Tumor Cell Sequencing in ALK-Rearranged Non-Small-Cell Lung Cancer. *Clin Cancer Res* **25**, 6671-6682, doi:10.1158/1078-0432.CCR-19-1176 (2019).

10 Faugeroux, V. *et al.* An Accessible and Unique Insight into Metastasis Mutational Content Through Whole-exome Sequencing of Circulating Tumor Cells in Metastatic Prostate Cancer. *Eur Urol Oncol* **3**, 498-508, doi:10.1016/j.euo.2018.12.005 (2020).

11 Faugeroux, V. *et al.* Genetic characterization of a unique neuroendocrine transdifferentiation prostate circulating tumor cell-derived eXplant model. *Nature communications* **11**, 1884, doi:10.1038/s41467-020-15426-2 (2020).

12 Dutertre, C. A. *et al.* Single-Cell Analysis of Human Mononuclear Phagocytes Reveals Subset-Defining Markers and Identifies Circulating Inflammatory Dendritic Cells. *Immunity* **51**, 573-589 e578, doi:10.1016/j.immuni.2019.08.008 (2019).

13 Mulder, K. *et al.* Cross-tissue single-cell landscape of human monocytes and macrophages in health and disease. *Immunity* **54**, 1883-1900 e1885, doi:10.1016/j.immuni.2021.07.007 (2021).

14 Luecken, M. D. & Theis, F. J. Current best practices in single-cell RNA-seq analysis: a tutorial. *Mol Syst Biol* **15**, e8746, doi:10.15252/msb.20188746 (2019).

15 Merritt, C. R. *et al.* Multiplex digital spatial profiling of proteins and RNA in fixed tissue. *Nature biotechnology* **38**, 586-599, doi:10.1038/s41587-020-0472-9 (2020).

16 Oulhen, M. *et al.* Circulating tumor cell copy-number heterogeneity in ALK-rearranged non-small-cell lung cancer resistant to ALK inhibitors. *NPJ Precis Oncol* **5**, 67, doi:10.1038/s41698-021-00203-1 (2021).

Appendix 1 – Detailed Material and Methods for immunohistochemistry

- Technical approach: fresh tumor samples will be fixed in formalin and embedded with paraffin prior to slide sectioning (5 µm sections using a microtome). FFPE sections will be prepared for staining using standard protocols with xylene and alcohol gradient for deparaffinization, rehydration and antigen retrieval procedures. Hematoxylin and eosin stained FFPE sections will be analyzed by a senior expert pathologist to select the most representative slide and zones of interest. One representative slide of each sample will be selected for IHC. In total, we anticipate that we will require six to ten slides for each sample. Slides will be scanned either on Olympus V120 for chromogenic stainings, or on Vectra 3 scanner (Akoya/ PerkinElmer) for fluorescent stainings.

- Analytical approach: Chromogenic staining will be quantified automatically using Definience software, except for immune checkpoint markers which will be read by expert pathologist. Automatic quantification of fluorescent stainings will be done using Inform software which automatically performs spectral decomposition. The percentage of stained cells will be quantified for each multiplex or monostaining assay. Percentage of positively stained cells values will be analyzed using the appropriate statistical model.

Appendix 2 – Detailed Material and Methods for GeoMX™

(i) Slide preparation: FFPE samples will be sectioned into 5 µm sections, before slide preparation according to GeoMx Slide Preparation User Manual on Leica Biosystems BOND RX. Briefly, slides will be baked, followed by deparaffinization, epitope retrieval, +/- proteinase K digestion and post fixation steps for RNA assays. For protein assay, after automated slide preparation, both detection primary antibodies and fluorophore-labeled morphology markers will be incubated overnight before post fixation and nuclei staining. For RNA assay, WTA panel probes will be then used for overnight In Situ hybridization, followed by stringent washes to remove off-target probes and finally fluorophore-labeled morphology markers stainings.

(ii) Imaging and ROIs/AOIs definition: Slide imaging will enable the proper selection of tissue locations of interest, using fluorophore-labeled morphological antibodies. After image scanning with acquisition at 20X magnification on GeoMX™ DSP instrument, ROIs will be defined for a maximum of 11 ROIs per slide, divided into 2-4 zones. Within each ROI, area of interests (AOIs) will be segmented using the precited morphological markers.

(iii) GeoMX DSP instrument: Once AOIs are defined, the latter will be exposed to 385 nm light (UV) to release indexing oligonucleotides that will be collected via microcapillaries and distributed into 96-well plates for subsequent processing.

(iv) nCounter and NGS readout: For nCounter readout, after drying of aspirates and rehydration, the latter will be transferred on hybridization plate into GeoMX Hyb Code Pack reagents before library pooling and loading into nCounter instrument. For NGS readout, the DSP plate aspirates will be mixed with GeoMX Seq Code PCR Master Mix and GeoMX Seq Code Pack Plate reagents for library preparation. After pooling of libraries, AMPure clean-up and Bioanalyzer quality control, libraries will be sequenced using Illumina Nextseq equipment using paired-end and dual index workflow. The sequencing depth will be defined according to the total AOI area in μm^2.

(v) Data Analysis: Protein assay data will be analyzed using the nCounter Data Analysis Manual in order to evaluate the expression level of the target of interest and composition of the tumor microenvironment according to the latter expression, pre- and on-treatment. GeoMX WTA RNA assay coupled with NGS readout will be analyzed as follows. After trimming using Trim Galore, paired-end reads will be stitched before extraction of UMI sequence and alignment using bowtie2. Subsequent filtering will include PCR duplicates removal and outlier tags removal. Count for each target transcript will be calculated as the mean of corresponding probes after normalization of target transcript counts across AOIs. The variation in target expression, in the associated activated pathways, and in the microenvironment composition pre- and on-treatment will be analyzed.

Appendix 3 – Detailed Material and Methods for RNA sequencing

- Technical approach: extraction will be performed using RNeasy tissue kit (Qiagen). RNA quality will be verified by spectrophotometry on Nanodrop and by electrophoresis on BioAnalyzer. Paired-end Illumina-compatibles libraries will be built using adapted kit and sequencing will be performed at 100X using on NovaSeq platform.

- Analytical approach: pre-processing of reads will include quality controls with FASTQC and adapter trimming with TrimGalore. Following alignment to hg38 with STAR, quality of RNA-seq data will be evaluated with RSeQC (BAM stat, junction saturation, RPKM saturation, read duplication, Inner distance). FeatureCounts will be used to quantifies expression relative to the transcriptome. Using R packages DESeq2, the matrix of count will be analyzed to perform differential expression through negative binomial generalized linear models; the dispersion and logarithmic fold changes will be estimated using the latter distributions. Downstream analysis will include enrichment and signature analysis with GSEA (Gene Set Enrichment Analysis).

Appendix 4 – Detailed Material and Methods for DNA sequencing

- Technical approach: DNA extraction will be done using DNeasy blood and tissue kit (Qiagen). DNA quality will be verified by spectrophotometry on Nanodrop and by electrophoresis on BioAnalyzer. Sequencing will be performed at 100X using Illumina NovaSeq technology.

- Analytical approach: Following alignment to hg38 with BWA allowing up to 4% of mismatches, bam files will be cleaned according to the Genome Analysis Toolkit (GATK) recommendations, namely duplicates marking and base quality score recalibration. WES variant calling of SNVs and small indels will be performed using GATK HaplotypeCaller, Strelka2, FreeBayes and GATK Mutect2. Structural variants will be explored with Manta, TIDDIT, ASCAT and Control-FREEC. SnpEff and VEP will be used for annotations and functional prediction