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**Identification of a predictive metabolic signature of response to
immune checkpoint inhibitors in non-small cell lung cancer:**

METABO-ICI clinical study protocol

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ABSTRACT

Background: Immune checkpoints inhibitors (ICI) are becoming new standards of care for the treatment of non-small cell lung cancer (NSCLC), both as first (alone or in association with chemotherapy) and second line. However, no powerful predictive biomarker of therapeutic response to ICI has been found to date. It has been recently shown that microbiota composition could influence the ability of patients to respond to ICI. Indeed, the microbiota produces circulating metabolites that will subsequently act on immune system, the investigators hypothesized that plasma metabolic signature, reflecting a global microbiota function, could represent a predictive biomarker of response to ICI.

Methods: Monocentric prospective study. Primary objective is to identify baseline metabolic signature (metabolomics analysis by Mass spectrometry) associated to ICI response. Secondary objectives are to link metabolic signature with microbiota composition (metagenomics analysis RNA 16S) and immune profile, and altogether with clinic response to ICI. The study will include 60 NSCLC patients treated by ICI in 1st, 2nd or 3rd line of treatment at the Grenoble Alpes University hospital (CHUGA) in 18 months. Patients that have received antibiotic or steroid treatment, 2 or 4 weeks before ICI initiation, respectively, will be excluded. Blood and feces will be collected prior to, at 2 months after ICI treatment initiation, and at 6 months or at progression.

Expected results: We expect to highlight a metabolic profile predictive of response to ICI. By identifying factors associated with early progression, we could avoid to treat potential non-responding patients. Moreover, by restoring a favorable microbiota, patients' ability to respond to these treatments might be restored.

KEYWORDS: Non-small cell lung cancer, immunotherapy, metabolism, microbiota

ABBREVIATIONS

CBC : Complete Blood Count

EMA : European Medicines Agency

ICI: Immune Checkpoint Inhibitor

MDSC: Myeloid-Derived Suppressor Cells

NSCLC: Non-Small Cell Lung Cancer

RCC: Renal Cell Carcinoma

1. INTRODUCTION

Immune checkpoint inhibitors (ICI) revolutionized the cancer therapeutic arsenal since the first approval for ipilimumab in 2011 (1) for metastatic melanoma. Nowadays, ICI are approved in many metastatic cancers, notably melanoma, non-small cell lung cancer (NSCLC), clear cell renal carcinoma (RCC), and head and neck cancer. A major limitation of these treatments is the relative low response rate and potential severe side-effects, combined with high costs. For instance, only a subset of NSCLC patients (around 20%) exhibited an objective response and a long-term benefit when used in 2nd line (2–4). On the other hand, ICI synergize with chemotherapy in 1st line setting, improving objective response rate (45.3% to 64.0% (5,6)).

The discovery of a predictive biomarker of response to ICI represents a real challenge that will allow the selection of patients likely to respond. The latter is crucial to determine which patients will not benefit from ICI therapy, to minimize toxicity, prevent side effects and reduce treatment costs. Numerous studies already attempted to identify potential biomarkers. Notably, Myeloid-Derived Suppressor Cell (MDSC) (7), eosinophil (8) and soluble CD25 (9) rates, or tumor infiltrating lymphocytes phenotype or function (10) for ipilimumab, and the expression of Programmed Death (PD)-1/PD Ligand (PDL)-1 within the tumor bed (11) and the tumor mutational burden (12), in response to anti-PD-1/PDL-1. Unfortunately, taken individually, these markers lack robust sensitivity and specificity.

Recently, gut microbiota composition appeared as an important factor influencing ICI responses in melanoma (13,14) or in RCC and NSCLC (15).

Gut microbiota is now well recognized as a critical player in (cancer) immunity modulation throughout life. Indeed, gut microbiota triggers balanced pro- and anti-inflammatory signaling with local and systemic immunomodulatory action. Microbiota can

promote immune modulation through Toll-like Receptors (TLR) recognition of Microbes-Associated Molecular Patterns (MAMPs), and the production of metabolites endowed with immunomodulatory functions (16). Several bacteria families/species have been associated with chemotherapy and ICI responses (13–15,17) and with ICI-associated toxicities (18). These studies clearly highlight the critical role of microbiota in cancer immunotherapy but the identified bacteria are diverse and study- and cohort-dependent, thus limiting the use of these microbiota signatures to anticipate ICI responses.

The diversity of identified bacteria strongly suggests that beyond species, the microbiota function (*i.e.* metabolic activity) might be a critical parameter. Indeed, it is now well recognized that some metabolites can directly influence immune polarizations, and that the metabolism of immune cells (immuno-metabolism) is critical for driving their functions. We thus strongly believe that microbiota-derived metabolic signature might strongly reflect its activity, beyond species identification, and could represent a “universal” biomarker for identifying responding and non –responding patients. Importantly, it has been recently shown that microbiota-derived Short-Chain Fatty Acids (SCFAs) metabolite production was associated with resistance to ipilimumab (19), highlighting the relevance of such strategy.

We present here our clinical study strategy, aiming to identify a plasma metabolic signature predictive of ICI response in NSCLC. High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS) untargeted metabolomics analysis will be performed. Gut microbiota signature and several immune parameters will also be determined in order to link these parameters with patients’ metabolic profiles using chemometrics and bioinformatics.

2. CLINICAL STUDY PROTOCOL

It is a prospective, observational, and monocentric study conducted at the Grenoble Alpes University Hospital. Protocol has been approved by Personal Protection Committee (07/02/2019). Patients must sign a consent form. Clinical trial ID is NCT04189679.

2.1 Objectives

The aim of this study is to characterize intestinal microbiota composition and function through plasma metabolic profile of NSCLC patients treated with ICI, and to highlight a metabolic profile associated with response to immunotherapy.

Associations between the composition of the intestinal microbiota, the immune status and the response to ICI will be secondary objectives. The evolution of intestinal microbiota composition, metabolic signature, and immune status after ICI initiation will also be studied in order to identify dynamic correlations. Moreover, an eating habit questionnaire will be submitted to study the links between diet habits and microbiota function and composition.

2.2 Study population

All metastatic NSCLC patients, over 18 years old, and newly treated by ICI are included. Patients benefiting from an ICI re-challenge are excluded. Patients could be treated in first, second or third line, and in first line patients could be treated with ICI alone or in association with chemotherapy. Patient with at least one measurable lesion as defined by RECIST are eligible. Patients who have received antibiotic within 2 weeks before the initiation of ICI or corticosteroids (>20mg of prednisolone or equivalent per day) within 4 weeks are excluded.

2.3 Data collection

At baseline, demographic data (sex, date of birth, height, weight, weight loss, performance status), cancer characteristics (histological type, date of diagnosis, stage at diagnosis, anti-tumor treatment received before, number and localization of metastasis) are collected. Patients will also have to complete a questionnaire about their eating habits at initiation of ICI. Throughout the study, we will record treatment characteristics (name of molecule, frequency and dose administered and antibiotics, probiotics, anti-gastric acids or corticosteroids administered within the 2 months prior to inclusion and all along the treatment), response to ICI (RECIST 1.1 criteria) and vital status. A central radiological examination blinded to clinical or biological features will be performed.

Results of biological analyses, usually performed at baseline thus before ICI administration, will also be collected: Complete Blood Count, blood electrolytes, creatinine, urea, SGOT and SGPT transaminases, alkaline phosphatases, γ -glutamyltransferase, bilirubin, lactate dehydrogenase and thyroid hormones.

2.4 Sample collection

Samples will be collected prior to immunotherapy initiation (Baseline M0), 2 and 6 months (M2 and M6) after, or at disease progression (Mx) (Figure 1, Table 1)

Blood sample will be collected and immediately processed to collect the plasma for metabolomics and immunological analyses. Plasma will be frozen at -80°C before analysis.

Stools will be collected 24h before immunotherapy initiation (M0), prior the following cures (M2 and M6 or Mx) and stored at 4°C at home by the patients. Samples will then be brought to the hospital, using ice pack in hermetic bags to ensure a 4°C temperature

maintenance. Stools are then immediately stored at -80°C prior DNA extraction and subsequent sequencing, or prior dilution in sterile water for metabolomics analysis on fecal water samples.

2.5 Analyses of biological samples

All biological analyses will be performed at the end of the study, at the same time, to avoid batch-related analysis biases. Plasma and fecal water metabolic profiles will be analyzed by untargeted HPLC-MS/MS metabolomics, while microbiota composition will be determined by 16S RNA sequencing. Circulating immune mediators will also be analyzed, by luminex technology to characterize cytokine profiles and detect relevant soluble immune receptors/ligands, including, IL-2, IL-6, IL-8, IL-10, IL-18, IFN- γ , TNF α , soluble receptor CD25 (IL-2R α) and also a panel of co-stimulatory or inhibitory soluble immune checkpoints, namely soluble PD-1 BTLA, CD27, CD28, CD80/B7-1.

2.7 Statistical plan

2.7.1 Number of patients to include

We plan to include both 1st line and 2nd / 3rd line patients. In first line, objective response rate (ORR) is about 40% among PD-L1>50 (20,21) and about 20% in second line (2–4). About 30% of patients have a tumor with PD-L1 expression \geq 50%. We therefore estimate that 1/3 of patients will be included in the 1st line and 2/3 in the 2nd or 3rd line. As expected, the association of chemotherapy and pembrolizumab obtained EMA authorization at the beginning of the study. ORR were 48% in patients with non-squamous NSCLC (22) and 63% in squamous NSCLC (23). Depending on when this combination of treatments would be

available, it is possible that 2/3 of the patients will be treated in 1st line. But it would not change the primary objective and the number of patients to include.

Our objective is to identify a plasma metabolic profile associated with the response to ICI with a sensitivity greater than 80% [95% CI, 67-93] and a specificity greater than 80% [95% CI, 67-93]. The inclusion of 60 patients (20 in 1st line and 40 in 2nd or 3rd line) should allow us to achieve this objective.

The planned duration of inclusions is 18 months, with an objective of 60 patients included.

2.7.2 Bioinformatics and multi-block chemometrics

As designed, this clinical study will provide a high amount of data spread over several data blocks, namely the Metabolome, Metagenome, “Immunome” and “Clinicome” data blocks. The integration of this collection of data tables is challenging, and requires a multi-block modeling analysis. The purpose of multi-block modeling is to offer an overall picture of all the available data, to assess the relevance of each block and to investigate relationships of variables that may be linked between data (24). The corresponding models offer two levels of analysis: (i) at the individual block level, scores and loading vectors are specifically related to each data table; (ii) at the global scale, common information is derived from the combination of all data tables. A global overview of major trends is thus obtained in order to highlight common sources of variability, but also differences and block-specific information. Similar to standard single-block analysis, both unsupervised (descriptive) and supervised (predictive) approaches can be implemented (25). This type of approach offers suitable data analysis tools when the information available to characterize a set of observations is distributed over several matrices. Extensive statistical validation will be

carried out using resampling procedures. Both cross-validation and permutation tests will be performed to ensure the predictive ability of the models, both in terms of sensitivity and specificity.

2.7.3 Statistical analysis

Patients' characteristics will be described in number and percentages for the qualitative variables and in median (interquartile range 25% -75%) for the quantitative ones. Comparisons between metabolic profiles will be performed using the Wilcoxon test for quantitative variables and Chi square or Fisher exact tests for qualitative variables. Bonferroni correction will be applied for multiple comparisons when comparing variables across groups.

After identification of these profiles, a sensitivity analysis according to the variable "ICI alone or in combination with chemotherapy" will be performed.

All statistical analyses will be performed using SAS 9.4 (SAS Institute, Cary, NC, USA).

3. EXPECTED RESULTS

The link between gut microbiota, host immunity and response to immunotherapy is well established, even if many aspects remain to be understood. Again, all the studies supporting such link also revealed a great diversity of microbes associated to treatment responses (9,13–15,17,27,28). We believe that, beyond bacteria species, their metabolic activity deeply affects host immunity and thus might reflect both microbiota “quality” and subsequent host’s immune status. We have designed this study for addressing these questions, attempting to link these parameters (Figure 2).

We expect to find a plasma metabolic signature (metabolome) strongly associated with patients’ responses to immunotherapy (clinicone). Also, we expect to be able to link these plasma profiles with fecal metabolic activity and gut microbiota composition (metagenome). Altogether, these data will not only allow us identifying a predictive signature but should also provide a better understanding of the link between microbiota composition, function and impact on host systemic metabolic profile.

Since the ability to respond to cancer immunotherapy is directly dependent on the host’s immune status, we will assess multiple immune parameters, and notably soluble pro- and anti-inflammatory factors (immunome). We will focus our analysis on soluble immune mediators because their dosage can be easily implemented in clinical routine diagnosis, if required.

The immunome data block will be correlated with all the other blocks in order to reveal the existing links between data sources.

Importantly, when we designed our study, we strongly believed that a multi-block analysis strategy could lead to a powerful predictive algorithm. A recent publication

demonstrated that such integrative strategy allows the identification of critical metabolic pathways (29).

In order to gain further insights into these complexes and dynamic parameters, we also plan to analyze samples at 2 and 6 months post treatment initiation, to better understand the dynamic relationship between all the studied data blocks. This analysis might reveal the direct causality between each block, for instance the influence of the microbiota composition evolution on host metabolism and subsequent immunity (including the ability to respond to immunotherapy).

The perspectives of this work are multiple. First, this clinical study represents a multi-disciplinary clinical proof-of-concept study that will pave the way for a multi-centric prospective clinical study that will fully validate our future findings. Also, one can imagine that patients identified as non-responders (based on metabolic signatures) could be pre-conditioned either by probiotics and/or prebiotics in order to optimize both gut microbiota composition and function prior to immunotherapy initiation and thus to maximize their chance to respond to immunotherapy.

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Conflict of interest

Sarah Sannicolo, Audrey Le Gouellec, Caroline Aspod, Julien Boccard, Laurence Chaperot.

Bertrand Toussaint and Dalil Hannani declare no conflict of interest.

Matteo Giaj Levra declares conflicts of interest with Astra Zeneca, BMS, MSD, Roche; Denis Moro-Sibilot with BMS, MSD, Astra Zeneca, Roche and Anne Claire Toffart with Astra Zeneca, BMS, Boehringer Ingelheim, Novartis, MSD, Pfizer and Roche.

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Figure legends

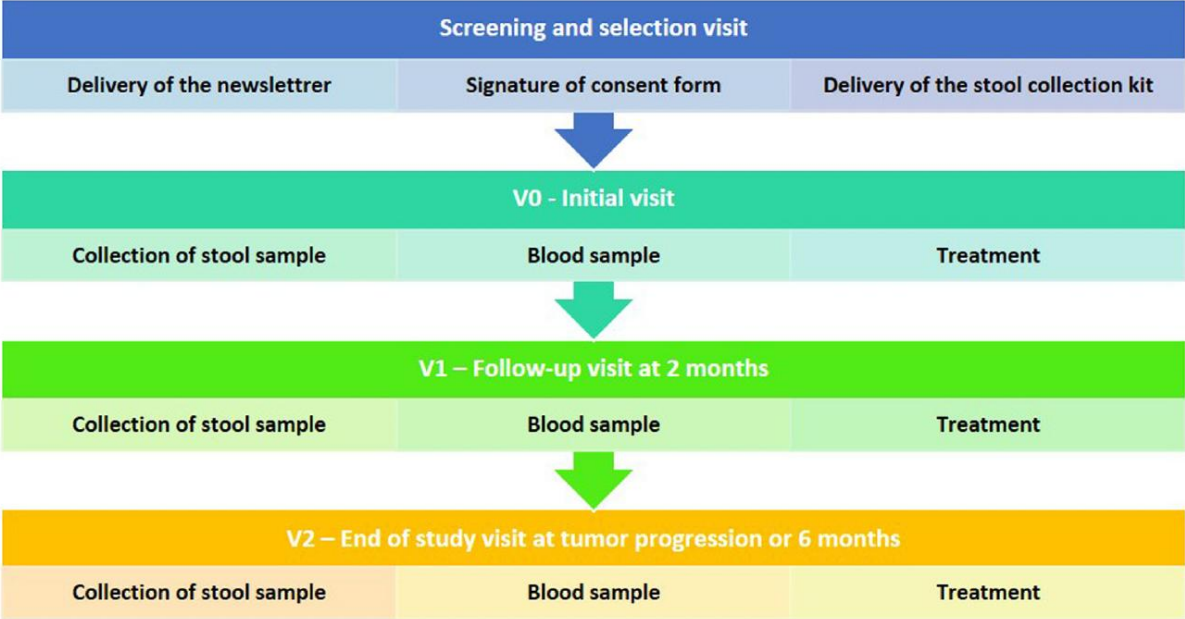


Figure 1. Visits summary table and associated sampling and treatment.

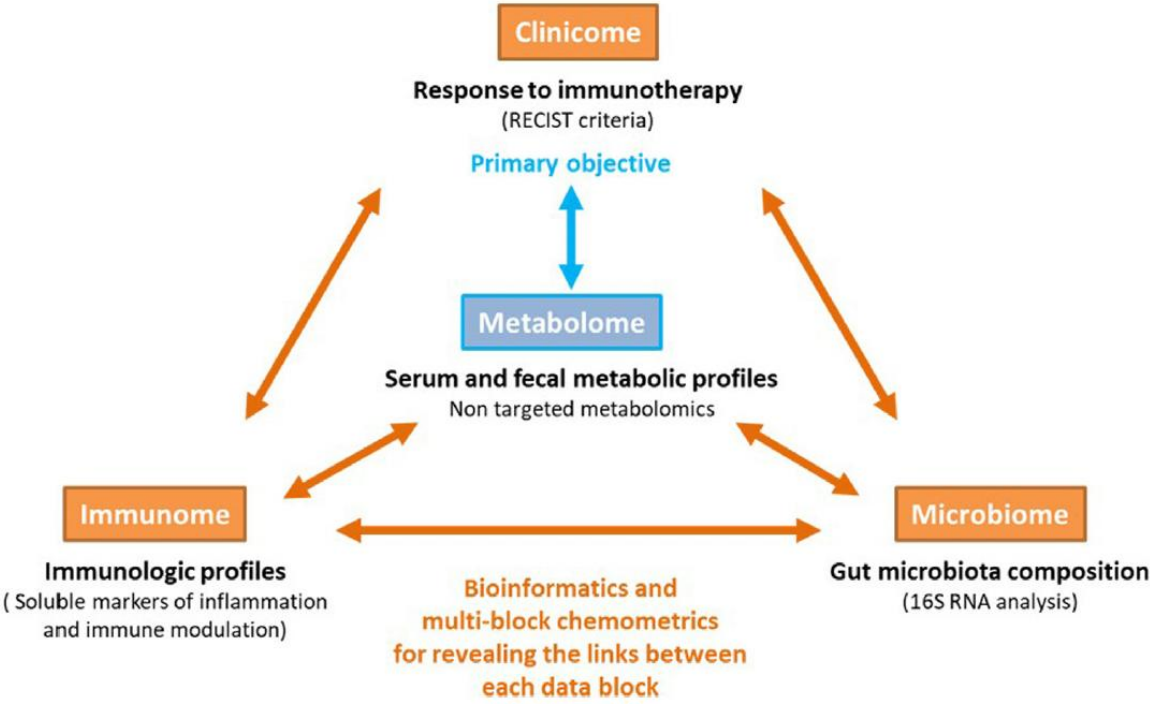


Figure 2: Study principle: Primary objective: to demonstrate a link between the metabolome and response to immunotherapy (“Clinicome”). Secondary objectives: to study the link between microbiota composition (microbiome), its metabolic activity (metabolome), host immunity (immunome) and treatment response (clinicome).

Table 1. Investigations summary table for patient

	Screening	V0	V1	V2
	D-28 to D-1	Start of treatment	M2	M6 or Tumor progression (Mx)
Informed consent	X			
Demography	X			
Previous treatment(s)	X			
Stool collected at home 24 to 48 hours before visit		X	X	X
Blood sample		X	X	X
Weight		X	X	X
ICI		X	X	X (except in case of progression)
Tumor response (RECIST 1.1)		X	X	X
Vital status		X	X	X