Title	Gender Difference in sidE eFfects of ImmuNotherapy: a possible clue to optimize cancEr tReatment (G-DEFINER)
Document type	Protocol
Version N.	5.0
Date	October 12nd 2021

Gender Difference in sidE eFfects of ImmuNotherapy: a possible clue to optimize cancEr tReatment (G-DEFINER)

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 741874.

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GENERAL INFORMATION

Coordinating Center	Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy (INT)
Partner Centers	• Dept of Oncology-Pathology, Karolinska Institutet, Karolinska University Hospital, Sweden (Onk-Pat KI) • Dublin City University, Dublin -St Vincent's University Hospital, Ireland (DCU- SVUH) • Oslo University Hospital – The Radium Hospital Norway (OUH)
Study sponsor	GENDER·NET Plus Promoting gender equality in H2020 and the ERA This project has received funding from the European Union's Horiz 2020 research and innovation programme under grant agreement 741874.

Principal Investigator- Coordinating Center (INT)	**Principal Investigator - Sweden (Onk-Pat KI)**	**Principal Investigator - Ireland (DCU- SVUH)**	**Principal Investigator - Norway (OUH)**
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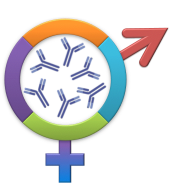
Title	  **Gender Difference in sidE eFfects of ImmuNotherapy: a possible clue to optimize cancEr tReatment (G-DEFINER)**
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ABBREVIATIONS

Acronym	Description
AE	Adverse Events
CRF	Case Report Form
CT	Chemotherapy
CTMR	Centre for Translational Microbiome Research, Dept. of Microbiology, Tumor and Cellbiology, Karolinska Institutet, SciLifeLab, Stockholm, Sweden
DCU- SVUH	St Vincent's University Hospital - Dublin City University, Dublin -, Ireland
F	Female
GDPR	General Data Protection Regulation
IC	Informed Consent
ICI	Immune Checkpoint Inhibitors
ICF	Informed Consent Form
INT	Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
irAE	Immune-related adverse event
M	Male
Onk-Pat KI	Dept of Oncology-Pathology, Karolinska University Hospital, Sweden
OUH	Oslo University Hospital – The Radium Hospital Norway
RCT	Randomized Clinical Trial
SAP	Statistical Analysis Plan
SOP	Standard Operating Procedure

RELATED DOCUMENTS

Type	Title
ANNEX	ANNEX 01 - Informazione per il paziente – ITA ANNEX 02 - Patient information - ENG
ANNEX	ANNEX 03 - Administrative and regulatory details
ANNEX	ANNEX 04 - irAE List
ANNEX	ANNEX 05 - PS CTCAE RECIST
ANNEX FORM	Study CRFs
SOPs	Study specific SOPs for Development, Approval and Review documents, data management, study conduction, AE registration, faeces sampling and microbiota analysis, blood sampling and SNP analysis, blood sampling and gene expression analysis.

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SYNOPSIS

Title of Study	Gender Difference in sidE eFfects of ImmuNotherapy: a possible clue to optimize cancEr tReatment (G-DEFINER)
Type of study	Multicentric prospective observational study
Indication	Melanoma, lung, head and neck, urogenital, breast cancer and, in addition, other solid tumors characterized by the presence of microsatellite instability (MSI-high), treated with immunocheckpoint inhibitors (ICI) irrespective of treatment schedule. It is possible to include patients treated with Immunotherapy in a compassionate use setting. No limitations to previous lines of treatment. ICI therapy may be either as single agent or in combination. Concomitant chemotherapy (CT) and radiotherapy (RT) is allowed.
Sites	• Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy (INT) • Dept of Oncology-Pathology, Karolinska Institutet, Karolinska University Hospital, Sweden (Onk-Pat KI) • Dublin City University, Dublin -St Vincent's University Hospital, Ireland (DCU-SVUH) • Oslo University Hospital – The Radium Hospital Norway (OUH)
Number of patients	Total 400, 200 females (F) and 200 males (M). Stratification : ICI: 100 F/100 M; ICI+ CT/RT: 100 F/100 M.
Study Coordinator	Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy (INT) Principal Investigator: Rosalba Miceli, PhD Unit of Clinical Epidemiology and Trial Organization
Study sponsor	GENDER·NET Plus Promoting gender equality in H2020 and the ERA This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 741874.
Background	The study aim is to investigate the differences between sex and gender in the immune-related adverse events (irAEs) development associated with immune checkpoint inhibitors (ICI) treatment. In common feeling, sex and gender represent the same concept, that is the traditional division of individuals into females (F) and males (M) defined by differential organization of chromosomes, reproductive organs, and sex steroid levels. However, if we go beyond these aspects, which characterize "sex", we can see how the differences between people are also characterized by behaviors and relationships that are the product of the culture and sociality typical of human beings. This is what is called "gender", i.e. the process of social and cultural

	construction that determines the behaviors that give life to the status of an individual. Gender is therefore learned and not innate. Sex and gender do not constitute two opposing but interdependent dimensions: the process of determining gender identity is triggered on biological characters. The relationship between sex and gender varies according to geographical areas, historical periods and cultures. Sex influences the adaptive immunity, and may influence irAEs types, frequency and severity. Together with genetic and biological differences, the roots of irAEs inequalities between F and M could be linked to psycho-social and behavioral determinants. IrAEs usually develop within the first few weeks to 6 months after treatment initiation; however, they can also present after cessation of ICI therapy. Most studies indicate that prolonged treatment does not result in an increased cumulative incidence of irAEs. Accumulating evidences support the existence of sex-driven differences in immune responses as potential factors contributing to disease outcome and response to therapy. Increasing use of ICI is associated with immune-related adverse events caused by non-specific activation of the immune system. We will conduct a multicenter prospective observational study investigating sex differences in irAEs in relation to clinical factors and genetic, immunological and hormonal profiles. By focusing on biological F/M differences possibly affecting discrepant irAEs incidence, we explicitly address sex inequality, complemented by the exploration of association between gender dimension and irAEs development. Exploring the irAEs occurrence in a "real world" (outside RCT) context will be more easily translated in a ready-to-use personalized approach to irAEs timely diagnosis and treatment.
Study design	This is a multicentric prospective observational study aimed at studying the incidence of irAEs in female and male cancer patients treated with ICI. To allow for balanced sex groups, we will include patients according to the following stratification : ICI: 100 F/100 M; ICI+ CT/RT: 100 F/100 M. Due to the current ICI use in clinical practice we are expecting to mainly populate the ICI strata during the first recruitment period. As the recruitment progresses, the sample will be enriched of ICI+CT and ICI+RT treated patients, since combinations are expanding for many cancers such for instance melanoma, lung and head and neck.
Primary objectives	• To estimate and compare the irAEs incidence in F and M patients, and estimate the incidence according to different clinical features and gender dimensions (behavioral and psychosocial differences associated with being female or male). • To estimate and compare the irAEs incidence in pre- and postmenopausal women.
Secondary objectives	• To develop irAEs predictive tools based on clinical characteristics. • To explore irAEs occurrence in relation to hormonal profiles, exploring the differences in F vs M patients, and in pre- vs post-menopausal F patients. • To explore the role of concomitant medications on irAEs occurrence.
Translational	To explore irAEs occurrence in relation to immune-related genes, germline

objectives	variations and microbioma, exploring the differences in F vs M, and in pre- vs post-menopausal F patients. • Gene-expression analysis. Blood samples will be taken in order to identify immune-related genes in patients with various solid tumors treated with ICI associated with the development of irAEs. • SNPs analysis. Blood samples will be taken in order to perform a preliminary genome wide association study for the identification of germline variations associated with the development of irAEs. with the hypothesis that the individual's genetic makeup may be related to irAEs. • Microbioma analysis. Stool collection will be performed to analyze gut microbiota aiming at performing RNA/DNA sequencing analysis for identifying components associated with the development of irAEs.
Inclusion criteria	• Signed informed consent. • Histologically confirmed diagnosis of one of the following cancers: melanoma, lung, head and neck, urogenital, breast cancer, and, in addition, other solid tumors characterized by the presence of microsatellite instability (MSI-high). It is possible to include patients treated with Immunotherapy in a compassionate use setting. • Any disease stage. • Patients eligible for ICI-containing regimens: o ICI single agent; o Combination of ICIs; o ICI-chemotherapy combination; o ICI-radiotherapy combination. • Any treatment setting (neoadjuvant, adjuvant, advanced disease, maintenance). • Patient age ≥18 years • Eastern Cooperative Oncology Group (ECOG) Performance Status of 0-2. • Adequate bone marrow, liver and renal function. • Life expectancy of at least 12 weeks.
Exclusion criteria	Patients not eligible for ICI-containing regimens.
Treatment schema	As per clinical prescription. Any melanoma, lung, head and neck, urogenital and breast cancers patients, and, in addition, patients with other solid tumors characterized by the presence of microsatellite instability (MSI-high),independently of disease stage and setting, treated with a regimen containing ICI.
Clinical Procedures /Assessments	Patients will undergo physical and laboratory evaluation and disease evaluation with CT and/or imaging scan according to current clinical practice at each study Center. Baseline radiological tumor measurements should be performed preferably within 7 days, but in any case no more than 30 days before entering the study.

	IrAEs of specific interest will be recorded and graded according to most recent Common Terminology Criteria for Adverse Events (CTCAE) . Patients who experience irAEs may continue treatment according to the clinical indications. Response will be evaluated by most recent RECIST according to current clinical practice at each study Center.
Biological samples and sampling times	<table><tr><th></th><th>Type of sample</th><th colspan="3">Times</th><th>N. additional samples required for the study</th></tr><tr><td></td><td></td><td>T0</td><td>T1</td><td>T2</td><td></td></tr><tr><td>Gene-expression profiling for immune assessment</td><td>Blood</td><td>X (3 ml)</td><td>X (3 ml)</td><td>X (3 ml)</td><td>Total 9 ml</td></tr><tr><td>Cytokines</td><td>Blood</td><td>X (7 ml)</td><td>X (7 ml)</td><td>X (7 ml)</td><td>Total 21 ml</td></tr><tr><td>SNPs</td><td>Blood</td><td>X (10 ml)</td><td></td><td></td><td>Total 10 ml</td></tr><tr><td>Microbioma</td><td>Stool</td><td>X</td><td>X</td><td>X</td><td>3 samples</td></tr><tr><td>Hormones</td><td>Blood</td><td>X</td><td>X</td><td>X</td><td>Routine lab, no additional tubes</td></tr><tr><td colspan="6">T0: baseline; T1: at 2nd ICI infusion; T2: at occurrence of the first irAE Grade ≥2</td></tr></table>
Statistical considerations	The incidence of first severe (G≥ 2) irAEs of any type will be estimated in F and M as a proportion of patients developing the event respect to the total number of patients at risk; the corresponding binomial 95% confidence interval (CI) will also be reported. The main comparison F vs M will be performed by estimating the odds ratio (OR) in a univariable logistic model; F/M unbalance for different clinical and gender-related characteristics will be taken into account using the “matching weight” (MW) method; patient weights will be estimated in advance as a function of a model-based balancing score (applying the propensity score methodology). IrAE incidence will also be estimated according to irAE type and grade, tumor site, ICI treatment, patients’ age and gender-based characteristics by sex groups. Logistic models using MW and including the interaction between sex and the different clinical features will be fitted to estimate OR according to different feature categories or values (numerical variables). Considering the large number of statistical tests, correction for multiplicity will be done using the Benjamini–Hochberg false discovery rate (FDR) method. The irAE probability predictive models will be developed after performing variable selection; the predictive model performance will be evaluated examining calibration of predictions and discriminative ability. The same methods described above will be applied for investigating the pre vs post menopausal differences and for developing the related predictive tool. IrAEs will also be tabulated describing the overall number of irAEs and the number of patients reporting irAEs grouped by CTCAE class and grading, and according to sex and menopausal status, ICI treatment, and tumor type. Univariable and multivariable Poisson regression models will also be applied to analyse irAE as

	count variable in relation to clinical variables Sample size considerations for the main sex-related analyses. Given that the events proportion could be even greater than 30%, we simulated scenarios in which the proportion is varying from 20% to 50%. To estimate the proportion by F and M, a sample size of 200 patients (200 F/200 M) will produce a two-sided 95% exact CI with a width as small as 0.115 (CI 0.147 to 0.262) if the proportion is 20%, increasing to 0.143 (CI 0.429 to 0.571) if the proportion is 50%.
Study duration	Patients recruitment: 24 months. Follow-up: 12 months. Total study duration: 36 months.

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1. INTRODUCTION

1.1 Study rationale

Sex, defined by differential organization of chromosomes, reproductive organs, and sex steroid levels, is a biological variable that affects innate and adaptive immunity, acting through genetic, hormonal and environmental factors. [1] Recent studies demonstrate a link among microbioma, sex, and immunity since gut microbiota composition is able to shape the immune response. [2-4]. "Sex-driven dimorphism" in immune functions and responses can lead to differences between female and male in the pathogenesis of infectious diseases, response to viral vaccines, and prevalence of autoimmune diseases.[5-6] Females exhibit higher immune responses to antigenic challenges than males, which can reduce pathogen load and accelerate pathogen clearance but leads to increase in immune-related pathology. [7]

Sex plays a role in cancer incidence, progression, response to chemotherapy (CT) treatments [8,9] and CT adverse events (AE); a recent gastric cancer randomized clinical trial (RCT) showed AE greater risk in women vs men, especially that of nonhematological events.[10] The association between sex and AEs development could be attributed to differences in pharmacokinetics and pharmacodynamics properties of the drugs; in addition, the AE onset and severity are related to the reproductive status, thus being influenced by sex hormones.

Immunotherapy is a promising cancer treatment and immune checkpoint inhibitors (ICIs) have shown benefit in treating a range of cancer types. Accumulating evidence supports the existence of sex-driven differences in immune responses as potential factors contributing to disease outcome and response to ICI. [11,12] X-chromosome linked genes, sex hormones and sex-biased immunophenotype shape different anticancer response in females (F) and males (M) affecting ICIs efficacy. The differential response between F and M could be amplified by the combined use of ICI and CT, as shown by a recent meta-analysis of randomized trials [13] investigated sex hetherogeneity in response to anti-PD1/PD-L1 plus chemotherapy as compared with chemotherapy alone in lung cancer.

However, by increasing the (non-specific) activity of the immune system, ICIs are associated with inflammatory side effects (immune-related adverse events, irAEs). IrAEs usually develop within the first few weeks to 6 months after treatment initiation; however, they can also present after cessation of ICI therapy. Most studies indicate that prolonged treatment does not result in an increased cumulative incidence of irAEs. A related issue is the association between aging and "low-grade pro-inflammatory state", systemic condition characterized by aberrant cytokine production. As demonstrated by studies in mice and humans, [14] the lethal ICI side effects are markedly exacerbated with aging. IrAEs can potentially affect any organ system and the majority are mild to moderate in severity; grade 3-4 irAEs are of major concern as they may cause treatment discontinuation and can occasionally be life-threatening if not promptly treated. Some studies reported a 90% incidence for any-grade irAEs due to single-agent ICI; an RCT meta-analysis indicated an overall incidence <75% with anti-CTLA-4 monotherapy (ipilimumab) (≥ grade 3 in 43% of patients) and ≤30% in trials of anti-PD-1/PD-L1 agents.[15] The incidence of most irAEs with ICI monotherapy appears similar across tumor types, with minor differences for some selected irAEs, and incidence is higher when ICIs are used in combination.

It has been hypothesized, but not yet proven, that irAEs types, frequency and/or severity may vary according to sex. Despite the thorough collection of irAEs data in registrational studies, the sex differentiation has never been published. Exploring irAEs incidence in relation to patients' sex is of clinical

relevance, and developing tools to identify irAEs high risk patients would be of help to optimize patients selection for ICI treatments minimizing toxicity.

In common feeling, sex and gender represent the same concept, that is the traditional division of individuals into females (F) and males (M) defined by biological differences. However, if we go beyond these aspects, which characterize "sex", we can see how the differences between people are also characterized by behaviors and relationships that are the product of the culture and sociality typical of human beings. This is what is called "gender", i.e. the process of social and cultural construction that determines the behaviors that give life to the status of an individual. Gender is therefore learned and not innate. Sex and gender do not constitute two opposing but interdependent dimensions: the process of determining gender identity is triggered on biological characters. The relationship between sex and gender varies according to geographical areas, historical periods and cultures. No studies have been conducted to reveal an association between irAEs and gender specific determinants, such as socio-economic context, exposure to lifestyle and psycho-social factors. An extensive study of gender-based inequalities in health [16] reported that some measures related to structural (e.g. age, family arrangement, occupation), lifestyle (smoking, drinking, physical activity, weight), and psycho-social context (mainly related to stress) are differently distributed between F and M, and most of them are related to health inequalities.

The role of estrogen signaling in tumor development is well understood in breast and ovarian cancer. In melanoma the effect of hormones (estrogen in particular) is supported by the sex differences in patients outcome, observed also after immune therapy, but remains unexplained.[17] The hormones role in anti-tumor immunity has not been extensively studied. Recent studies report hat estrogen signaling is responsible for immunosuppressive effects in the tumor microenvironment across cancer types through both the accumulation and activity of myeloid-derived suppressor cells (MDSCs), a set of immune cells associated with tumors treatment resistance.[18] Sexual hormones could affect autoimmunity via their effects on gene transcription, considering that some genes are thought to be regulated directly by estrogen or androgen receptors.[19] Moreover, since obesity results in increased systemic estrogen levels via adipocyte aromatase activity which converts androgens to estrogen,[17] there is a rationale to examine estrogen (e.g. estradiol) levels in F and also in M patients, given also the estrogen production in men at peripheral fat, and a possible influence on irAEs.

Genes influence the risk of certain autoimmune diseases even in the absence of immune checkpoint blockade.[19] Evidence for genetic predisposition to individual response to ICI treatment and irAEs occurrence is emerging. Several studies have demonstrated an increased probability of clinical benefit when tumors are infiltrated by CD8 T cells, have a high mutation burden or have an interferon gamma signature. Moreover, expression of IFNG, the gene encoding IFN-γ, is associated with clinical response to the immune checkpoint blockade in non-small cell lung cancer, melanoma [20] and head and neck patients. This predisposition may be determined by germline DNA variations, for instance in genes encoding proteins involved in interactions between the immune system and tumors (e.g. in CTLA-4 gene and PD-L1).[21-23] Breunis et al. [21] looked for associations between seven CTLA4 polymorphisms and the occurrence of severe autoimmune reactions (grade 3/4), but they did not find any significant associations. Also, the candidate gene approach is limiting in the analysis of genetic variations predisposing to irAE, since the molecular bases of these side effects are not yet known. Thus, genomewide association studies are needed to establish a relationship between genetic factors and the risk of irAEs. Since several immune response genes localize to sexual chromosomes or are regulated by sex chromosome genes and sexual dimorphism

in both immune response and autoimmunity has been reported [24,25], we hypothesize that genetic germline variations can account for differences in ICI toxicity between F and M.
Microbiota and their collective genomes, referred to as the microbiome, inhabiting the human body play a critical role in human biology, health and disease, acting collectively as a organ integrated in host's metabolism, regulating innate and adaptive immunity, and participating in the control of the energy balance.[26] Recent studies demonstrate a link among microbiota, gender, and immunity since gut microbiota composition is able to shape the immune response [2-4] and shows gender-specific differences.[27-28]. In addition, microbiota can influence the endocrine system developing a cooperative model where signals from both hormones and microbiota are integrated for prevention of disease development.[29]. It has been hypothesized that the microbiologic composition of a patient's gastrointestinal flora could be related to the development of irAEs.[19]. Moreover, in a review by Botticelli et al. [30] it was hypothesized that the identification of different microbiome profiles could help to establish classes of PD-1 and PDL-1 responders, or characterize patients at major risk to develop high grade toxicities.
Finally, since elevated expression of certain cytokines may signal subclinical inflammation that evolves into severe irAEs with treatment [31,32], we believe that experimental efforts should explore the irAEs incidence in relation to patients sex.

1.2 Research questions

The present project focuses on differences between F and M that could affect the incidence of irAEs in cancer patients treated with ICI. We aim at integrating sex dimensions in applied health research on genetic, immunological and hormonal mechanisms related to irAEs, also exploring gender-related and ageing differences.
We hypothesize that there are sex specific profiles that may explain differential occurrence of adverse events. In addition, we hypothesize that the menopausal status in F patients may be associated with specific profiles.
The purpose is to address the unmet clinical need of identifying predictive factors of irAEs. We aim at supporting the development of an innovative culture of a "sex based" approach to modern cancer therapeutic approaches such as ICI. The study results, being obtained in a "real world" (outside experimental clinical trial setting) context, will be more easily translated in a ready to use irAEs timely diagnosis and personalization of treatment approaches.
We expect to be able to improve our clinical practice and decision making in relation to the individualized anticipated toxicity profile, avoiding also inadequate inclusion in clinical trials. By irAEs monitoring, early recognition and timely treatment, we may impact on outcome and quality of life. Tools for irAEs prediction will also be developed.

2. OBJECTIVES

The study aim is to investigate the association between irAEs occurrence and sex and gender characteristics in patients with different tumor types treated with ICI.
We will estimate the irAE incidence in F and M and also in relation to clinical, behavioral and psychosocial, genetic and immunological features. The differences related to menopausal status will also be investigated in the women subset.

By focusing on biological F/M differences possibly affecting discrepant irAEs incidence, we explicitly address sex inequality, complemented by the exploration of association between gender dimension and irAEs development. Exploring the irAEs occurrence in a "real world" (outside RCT) context will be more easily translated in a ready-to-use personalized approach to irAEs timely diagnosis and treatment.

2.1 Primary objectives

- To estimate and compare the irAEs incidence in F and M, and estimate the incidence according to different clinical features and gender dimensions (behavioral and psychosocial differences associated with being female or male).
- To estimate and compare the irAEs incidence in pre- and postmenopausal F patients.

2.2 Secondary objectives

- To develop irAEs predictive tools based on selected clinical characteristics.
- To explore irAEs occurrence in relation to hormonal profiles, exploring the differences in F vs M patients, and in pre- vs post-menopausal F patients.
- To explore the role of concomitant medications on irAEs occurrence.

2.3 Translational objectives

To explore irAEs occurrence in relation to immune-related genes, germline variations and microbioma, exploring the differences in F vs M, and in pre- vs post-menopausal F patients.

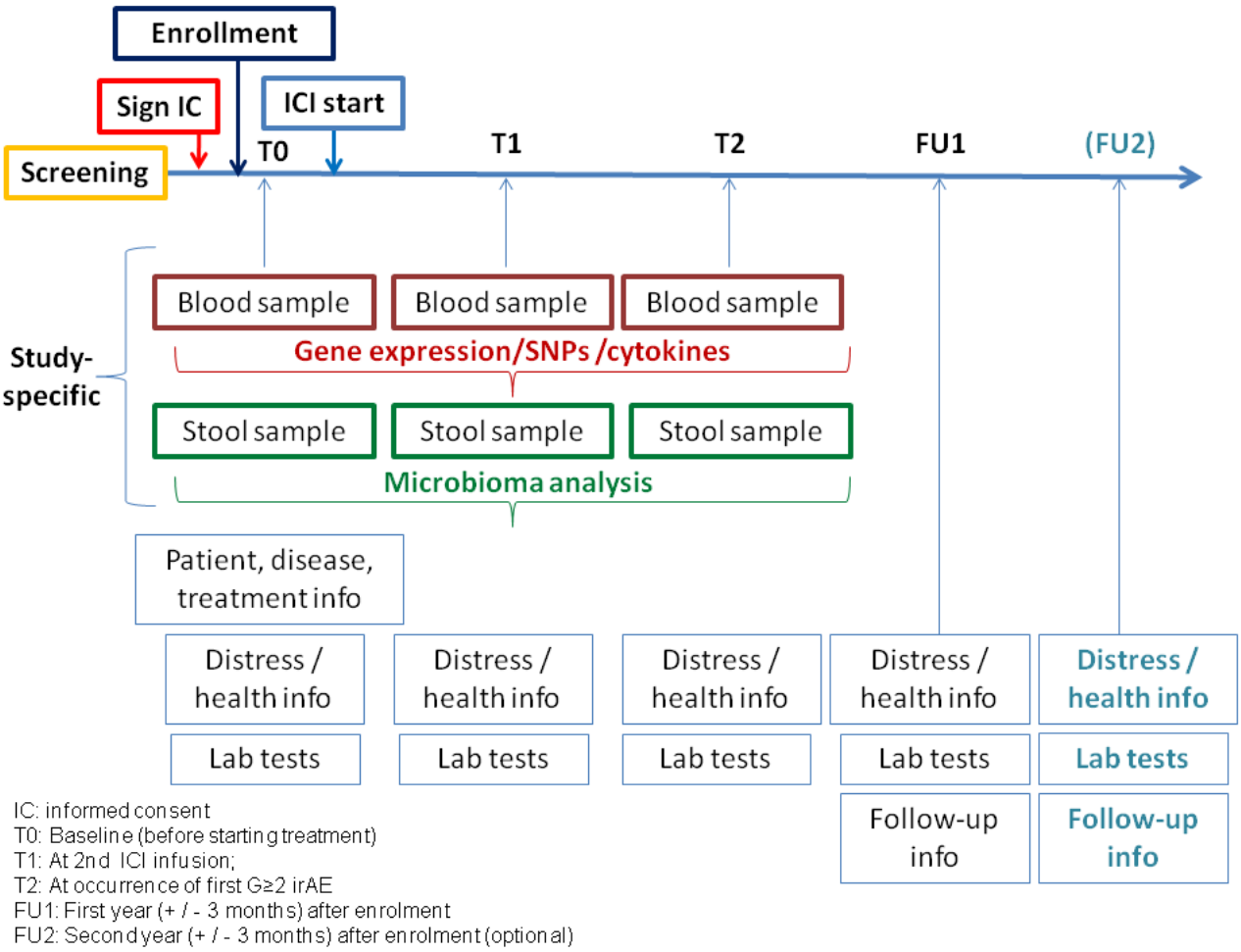
- Gene-expression analysis. Blood samples will be taken in order to identify immune-related genes in patients with various solid tumors treated with ICI associated with the development of irAEs.
- SNPs analysis. Blood samples will be taken in order to perform a preliminary genome wide association study for the identification of germline variations associated with the development of irAEs. with the hypothesis that the individual's genetic makeup may be related to irAEs.
- Microbioma analysis. Stool collection will be performed to analyze gut microbiota aiming at and performing RNA/DNA sequencing analysis for identifying components associated with the development of irAEs.

3. STUDY DESIGN

3.1 Overview of study design

This is a multicentre prospective observational study. A total number of 400 patients will be consecutively recruited in 4 centers, 200 F and 200 M. To allow for balanced sex groups, we will include patients according to the following stratification : ICI: 100 F/100 M; ICI+ CT/RT: 100 F/100 M. Due to the current ICI use in clinical practice we are expecting to mainly populate the ICI strata during the first recruitment period. As the recruitment progresses, the sample will be enriched of ICI+CT/RT treated patients, since combinations are expanding for many cancers such for instance melanoma or head and neck. Recruited patients will be treated with ICI according to usual clinical practice. Study duration: 24 months recruitment and 12 months follow-up ; total study duration: 36 months.

Flow-chart:



3.2 Study centers

- Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy (INT)
- Dept of Oncology-Pathology, Karolinska University Hospital, Sweden (Onk-Pat KI)
- Dublin City University, Dublin -St Vincent's University Hospital, Ireland (DCU- SVUH)
- Oslo University Hospital – The Radium Hospital Norway (OUH)

3.3 Study population

3.3.1 Inclusion criteria

Inclusion criteria for ICI administration are well established according to clinical practice. Patients fulfilling the following criteria will be enrolled:

- Signed informed consent.
- Histologically confirmed diagnosis of one of the following cancers: melanoma, lung cancer, head and neck cancer, urogenital cancer, breast cancer, and, in addition, other solid tumors characterized by the

presence of microsatellite instability (MSI-high). It is possible to include patients treated with Immunotherapy in a compassionate use setting.

- Any disease stage.
- Patients eligible for ICI-containing regimens irrespective of treatment schedule:
  - ICI single agent.
  - Combination of ICIs.
  - Combination of ICI and chemotherapy.
  - Combination of ICI and radiotherapy
- Any treatment setting (neoadjuvant, adjuvant, advanced disease, maintenance). No limitations to previous lines of treatment.
- Patient age ≥18 years
- Eastern Cooperative Oncology Group (ECOG) Performance Status of 0-2.
- Adequate bone marrow, liver and renal function.
- Life expectancy of at least 12 weeks.

3.3.2 Exclusion Criteria

Patients not eligible for ICI-containing regimens. Exclusion criteria for ICI administration are well established according to clinical practice.

4. DATA SOURCES/DATA COLLECTION PROCESS

The process for data management and CRF completion is outlined in the "**SOP 02 Data management – CRF completion"**.

4.1 Investigators

- *Clinical investigator.* Each Center will independently appoint eligible physicians, which will participate in the study as CI. The participating physicians will be responsible for the accuracy, completeness, legibility and timeliness of the data documented in the CRFs & eCRF's and all required reports.
- *Data Manager.* Each Center will appoint eligible data manager/s responsible for data collection and CRF/eCRF completion

4.2 Patient clinical data

Data collection/reporting will be conducted in a consistent way among different Centers to avoid bias in the data collection process. Data will be entered by all sites onto CRF/eCRFs specifically designed for the study.
The study will collect data primarily from Oncology Units treating patients deemed to be eligible for the study in the participating Centers. Paper and electronic case report forms will be used by the study investigators to enter data.
The greater part of the data that will be collected in this study are those usually collected at appointments for routine checks. Additional data will be collected to characterize gender aspects, and additional blood and stool samples will be taken to study genetic, immunological and hormonal profiles (par. 4.3). As regards study-specific gender related data, based on the results shown in [16] we will examine some measures related to structural factors (e.g. age, family arrangement, social support, occupation, socio-economic),

lifestyle factors (e.g smoking, drinking, physical activity, weight), and psycho-social factors (e.g. practical, family, emotional, and physical problems; stress). Moreover, we will investigate general health levels (mobility, self care, usual activities, pain anxiety).

4.3 Patient biological data

Biological samples will be collected to derive data for translational studies aimed at studying irAEs occurrence in relation to immune-related genes, germline variations and microbioma, exploring the differences in F vs M, and in pre- vs post-menopausal F patients.

Biological samples and timing:

	Type of sample	Times			N. additional samples required for the study
		T0	T1	T2	
Gene-expression profiling for immune assessment	Blood	X (3 ml)	X (3 ml)	X (3 ml)	Total 9 ml
Cytokines	Blood	X (7 ml)	X (7 ml)	X (7 ml)	Total 21 ml
SNPs	Blood	X (10 ml)			Total 10 ml
Microbioma	Stool	X	X	X	3 samples
Hormones	Blood	X	X	X	Routine lab, no additional tubes

T0: baseline; T1: at 2nd ICI infusion; T2: at occurrence of the first irAE Grade ≥2.

- Gene expression profiles for immune assessment. Blood samples will be collected in order to identify immune-related genes associated with the development of irAEs. Each center will ship the samples to the Piattaforma di Biologia Integrata at INT, where the analyses will be carried out on T0 samples. T1 and T2 samples will be stored for future studies.
- SNPs. Blood samples will be collected in order to perform a preliminary genome wide association study for the identification of germline variations associated with the development of irAEs with the hypothesis that the individual's genetic makeup may be related to irAEs. Each center will ship the samples to the Genomic Core facility at OUH, where whole genome SNP genotyping will be carried out.
- Microbioma. Stool collection will be performed to analyze gut microbiota aiming at the identification of components associated with the development of irAEs. Each center will ship the samples to CTMR Karolinska Institutet, Sweden, where extraction and analyses will be centralized and performed for all centres participating in the G-DEFINER project.
- Hormones. Blood samples will be taken in order to explore irAEs occurrence in relation to hormonal profiles. We will collect data Routine lab, no additional tubes are requested for the study.
- Cytokines. Additional blood collected will be taken and stored for future studies aimed at identify cytokines associated with the development of irAEs.

Blood samples will be collected at medical examinations by applying routine drawing procedure. Stool samples will be taken from patients at home; patients will be provided with special kits.

5. TREATMENT AND CLINICAL PROCEDURES

Treatment will be as per clinical prescription for melanoma, lung cancer, head and neck cancer, renal cancer, urothelial cancer, breast cancer, and for other solid tumors characterized by the presence of microsatellite instability (MSI-high). It is possible to include patients treated with Immunotherapy in a compassionate use setting.

Any ICI-containing regimen can be used in this study:

- ICI single agent;
- Combination of ICIs;
- ICI-chemotherapy combination;
- ICI-radiotherapy combination.

Treatment may be administered in any setting: neoadjuvant, adjuvant, advanced disease, maintenance.

Patients will undergo physical and laboratory evaluation and disease evaluation with CT and/or imaging scan according to current clinical practice at each study Center.

Baseline radiological tumor measurements should be performed preferably within 7 days, but in any case no more than 30 days before entering the study.

IrAEs of specific interest will be recorded and graded according to most recent Common Terminology Criteria for Adverse Events (CTCAE) v. 5.0. Patients who experience irAEs may continue treatment according to the clinical indications.

Response will be evaluated by most recent RECIST according to current clinical practice at each study Center.

6. STUDY CONDUCTION

The process for the study conduction is outlined in the "**SOP 03 - Study conduction"**.

Being G-DEFINER an observational study, patients will not modify their course of treatment according to oncologists prescriptions, nor visits or instrumental examinations additional to those planned for the treatment of their disease. The decision for discontinuation from ICI lies with the treating physician in agreement with the patient's decision and is not regulated by this protocol.

7. STATISTICAL ANALYSIS

This section presents an overview of the statistical analysis planned. Details will be presented in the statistical analysis plan (SAP). Patients recruitment will be periodically monitored. All patients who fulfill the study entry criteria will be included in the data set for analyses. The statistical analyses will be performed with SAS (Cary, NC, USA) and R software [R Core Team (2016): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.]

7.1 Clinical data analyses

All collected data and endpoint variables will be summarized. The variables will be described using standard statistical analyses to gain an understanding of the qualitative and quantitative nature of the data collected and of the characteristics of the sample studied. Results will be displayed using tables, listings, and/or figures.

The incidence of first severe (G≥ 2) irAEs of any type (below referred to as "*event"*) will be estimated in F and M as a proportion of patients developing the event respect to the total number of patients at risk; the

corresponding binomial 95% confidence interval (CI) will also be reported. The main comparison F vs M will be performed by estimating the event odds ratio (OR) and its CI in a univariable logistic model; F/M unbalance for different clinical and gender-related characteristics will be taken into account using the "matching weight" (MW) method.[33] Patient weights will be estimated in advance as a function of a model-based balancing score by applying the propensity score methodology. To develop the event predictive signature on the bases of sex, gender, and other patients, disease and treatment characteristics we will perform variable selection in multivariable setting (e.g. by means of a random forest (RF) [34] for binary response or LASSO methods in binary logistic models [35]). The same methods described above will be applied for investigating the pre vs post menopausal differences and for developing the related predictive signature.

IrAEs will also be tabulated describing the overall number of irAEs and the number of patients reporting irAEs grouped by CTCAE class and grading, and according to sex and menopausal status, ICI treatment, and tumor type. Univariable and multivariable Poisson regression models will also be applied to analyse irAE as count variable in relation to clinical variables.

7.2 Translational analyses

The analyses will be performed separately in the F and M groups to identify the features associated with event (defined above) occurrence.

Hormonal, gene expression, and microbioma data analyses. For each type of analysis, baseline features data will be categorized according to their distribution quartiles and irAE event incidence and corresponding 95% confidence intervals will be estimated in the 4 strata defined by the combination ICI/sex (i.e. ICI F, ICI M, ICI+ CT/RT F, ICI+ CT/RT M). Estimation will also be performed according to ICI type and grade, tumor site, ICI treatment, patients' age and gender-based characteristics. Sex-specific signatures to predict the event will be developed by performing class comparison (univariable) and class prediction (multivariable) analyses. In class comparison analysis, the features differentially expressed in patients with or without event will be identified by applying the Wilcoxon-Mann-Whitney (WMW) test. The features will be selected based on their 5% significance at class comparison analysis. The control of the False Discovery Rate (FDR) due to multiple testing will be done by adjusting the p-values with the Benjamini-Hochberg procedure [36]. In class prediction analysis, since the number of features to be investigated will be high, even after class comparison pre-filtering, variable selection techniques (e.g. [34] or [35]) will be used. The event predictions will be obtained by using statistical models for binary response, such as logistic regression, including the selected features. The same methodology will also be applied for the menopausal status-specific signatures to predict the event. As regards microbioma data, the statistical methods will take into account their peculiarities (e.g. over-dispersion and zero-inflation);[37] moreover, as in addition to baseline, samples will be taken at 2 other later times, models for longitudinal data will be applied.

SNPs analysis. This has to be regarded as a very exploratory analysis, given that, after applying the FDR method [36] to adjust for test multiplicity, the statistical power of bioinformatical analyses will be sufficiently higher (>70%) only with irAE proportion> 30% and the OR associated to a single feature > 5. SNP allele frequencies will be tested in F and M for association with irAEs adjusting for confounders as ICI treatment, age, smoking habit, tumor type and stage. SNPs analyses will be performed separately in the F and M groups to identify the features associated with irAE event occurrence. The features differentially expressed in one group but not in the other one will also be selected. These analyses will be done by

logistic regression using PLINK software. The FDR method [36] will be applied for test multiplicity correction.

7.3 Sample size considerations for the main sex-related analyses

Considering Puzanov et al. data,[15] the events proportion associated to 3 ICIs (CTLA-4,PD-1, PD-L1) considering all sites could be even greater than 30%. We simulated scenarios in which the proportion is varying from 20% to 50%. To estimate the proportion by F and M, a sample size of 200 patients (200 F/200 M) will produce a two-sided 95% exact CI with a width as small as 0.115 (CI 0.147 to 0.262) if the proportion is 20%, increasing to 0.143 (CI 0.429 to 0.571) if the proportion is 50%. In the multivariable logistic model analysis of association between the event and sex, given that using MW each patient weights less than 1, the effective sample size is reduced. However, we foreseen that the number of events will be still adequate to the model degrees of freedom (DF).[38] In fact, even with a proportion of irAE as low as 20% and with an effective sample lowered to 200 patients (100 F/100 M) we would have 40 events, sufficient to reliably estimating the sex OR in a univariable model, or in a multivariable model with interaction sex x another covariate with 2 DF. In the multivariable logistic model analysis of association between first G≥2 irAEs and gender, a sample of 400 patients (200 females, 200 males) will produce a power ≥80% at a two-sided Wald test with α=5% in the following scenarios:

R^2	P_0	OR	R^2	P_0	OR	R^2	P_0	OR
0.5	20%	≥2.40	0.6	20%	≥2.70	0.7	20%	≥3.05
0.5	25%	≥2.35	0.6	25%	≥2.55	0.7	25%	≥2.90
0.5	30%	≥2.30	0.6	30%	≥2.50	0.7	30%	≥2.85
0.5	≥35%	≥2.25	0.6	≥35%	≥2.45	0.7	≥35%	≥2.85

R^2: correlation between gender and other model covariates; P_0: baseline proportion of irAE; OR: testable gender odds ratio.

8. ADMINISTRATIVE AND REGULATORY DETAILS

Administrative and regulatory details are described in “ANNEX 03 - Administrative and regulatory details”. By signing this protocol, the Consortium Investigators participating to the G-DEFINER study agree to be responsible for implementing and conducting the study according to the all the principles described in the “ANNEX 03 - Administrative and regulatory details”.

Title	**Gender Difference in sidE eFfects of ImmuNotherapy: a possible clue to optimize cancEr tReatment (G-DEFINER)**
Document type	**Protocol**
Version N.	**5.0**
Date	**October 12nd 2021**

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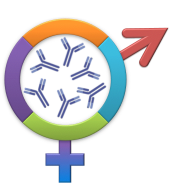
Title	  **Gender Difference in sidE eFfects of ImmuNotherapy: a possible clue to optimize cancEr tReatment (G-DEFINER)**
Document type	**Protocol**
Version N.	**5.0**
Date	**October 12nd 2021**

13. Document history- amendments

Version No.	Date	Reviewer	Details of changes
0.1	26.03.2019	RM	Very first version
0.2	27.05.2019	RM	Synopsis, added references to forms and SOPs
1.0	29.05.2019	RM, SA, GLR	Substantial modifications; main: 1) Synopsis; 2) par. 2.3, 3) par. 3.3.1 and 3.3.2 (eligibility); 4) timing: T1: 2nd infusion instead of 6th week; 5) eliminated eligibility=biological make-up as male/female. 5) bib citation to be included: Conforti F 2019 in introduction.
1.1	05.06.2019	RM	Inclusion annex 03 and SAP, modification statistical analysis section
1.2	07.06.2019	RM	Corrections by GLR
1.3	10.6.2019	RM	Specified 10 ml for blood tubes.
2.0	19.6.2019	RM	Modifications: 1) Participating Units at INT (no person names), 2) incorporated corrections SA/LL mail 16/06/2019 and GL; 3) flow chart added
3.0	21.10.2019	RM	**Amendments** post meeting with partners. As version 2 of the protocol was approved by the INT Ethical Committee, INT has to present protocol amendments **(Em1.0-21.10.2019-Prot3.0)** . **List of Amendments:** 1) substantial: exclusion criteria: eliminated all the criteria, because implicit for patients treated with ICI; patients excluded are those not eligible for ICI-containing regimens. Inclusion criteria: the partner established that patients with curative RT can be included (sections inclusion/exclusion criteria). 2) substantial: new biological sampling scheme, with addition of 2 blood samples at times T1 and T2 for gene expression. Nonetheless, the total amount of blood for all the analyses is less than in version 2 of the protocol. Par. 4.3 modified accordingly and it was specified that gene expression analysis is performed on T0 samples in the present study; T1/T2 samples will be analysed in future studies.Par 4. of patient information was modified accordingly. 3) substantial: Patients informed consent: patients who do not accept to participate to the study can give a separate consent to register their age, sex, disease type and reason for refusal to participate. 4) not substantial: flow chart: added "sign consent" before enrollment and added cytokines among study-specific blood samples. 5) not substantial: eliminated section 9 "flow chart" because of redundancy with flow chart picture in pag. 3.1. 6) not substantial: par 5 patient information: specified that additional 10 ml of blood will be taken in case of serious adverse event. 7) not substantial par. 6 patient information: more clearly specified that after 1 year no data will be directly requested to the patient, and only clinical chart data related to disease evolution will be registered.
4.0	12.06.2020	RM	**Amendment:** possibility to include patients with solid tumors characterized by the presence of microsatellite instability (MSI-high).
5.0	12.10.2021	RM	**Amendments** post meetings with partners. **List of Amendments:** 1) substantial: Synopsis, sections "Indications" and "Inclusion criteria", par. "3.3.1 Inclusion criteria", par "5. TREATMENT AND CLINICAL PROCEDURES": Possibility to include patients patients treated with Immunotherapy in a compassionate use setting (to comply DCU- SVUH request). 2) Substantial: Synopsis, section "Statistical considerations", Flow-chart par. "3.1 Overview of study design", table par. "4.3 Patient biological data": first severe irAEs defined as G≥ 2, instead of G≥3, is estimated as main end-point. This because the observed proportion of events G≥3 is low because patients are eligible if treated with ICI for the first time, and many patients are treated with single ICI. Considering G≥2 fits well with the scenarios hypothesized in section "7.3 Sample size considerations for the main sex-related analyses", where we simulated scenarios in which the irAEs proportion was varying from 20% to 50%.