Protocollo IL2-COVID vers.1 del 23.04.2020

PET/CT with ^{68}Ga-IL2 for imaging IL2R+ cells in COVID-19+ patients
La PET/TC con ^{68}Ga-IL2 per l'imaging delle cellule IL2R+ in pazienti affetti da COVID-19

EudraCT number 2020-001749-38

1.0 Background

Coronavirus disease 2019 (COVID-19) is a severe, often fathal, syndrome emerged in December 2019 in Wuhan, China, and rapidly spread worldwide enough to be declared pandemic by World Health Organization, in March 2020. In few months more than one million one hundred thousand infected patients have been resgistered across the globe and the number of deaths is constantly increasing in many Countries.

Novel coronavirus, called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belongs to a large family of respiratory viruses that can cause a wide spectrum of respiratory diseases and extrapulmonary manifestations. Clinical presentation is extremely variegated ranging from asymptomatic cases or paucisymptomatic cases with fever, dry cough, asthenia and myalgia to acute respiratory distress syndrome (ARDS) due to bilateral interstitial pneumonia that often requires ventilator support in intensive care unit (ICU).

Understanding the underlying mechanisms that affect immune system and identifying the crucial molecules and cells that play a key role in the development of the disease, may explain the differences observed from mild to severe manifestations and may be helpful for selecting patients candidate for most appropriate treatments.

From other viral respiratory diseases, for example the Middle East respiratory syndrome (MERS) that is also substaneid by a coronavirus, and the previous SARS pandemic that emerged at the beginning of this century, it became evident that cytokine dysregulation plays an important role (1). It seems that in the earlier stages of the infection, immune system activation is functional at increasing viral clearance, thus providing a positive response to the virus, but then several other mechanisms, still not elucidated, occour causing an excessive secondary immune response charachterized by an uncontrolled pro-inflammatory cytokines and chemokines release known as "cytokine storm". This cytokine storm is responsible of an initial over-activation of T-cells, enhanced vascular permeability, disseminated intravascular coagulation, T-cell depletion, ARDS and other systemic effects that are usually observed in cytokine release syndrome (CRS) during particular treatments with monoclonal antibodies (1). Therefore, lung injury is the consequence of an exalted immune response rather than a direct viral effect.

Cytokines dysregulation is, indeed, one of the most typical features of SARS as demonstrated by several papers. It induces a down-regulation of antiviral cytokines, for example type I interferons (IFNs) that take part in the prompt immune response in viral infections, and this reduction shows a suppression on T-helper (Th)-1 cells in favour of a Th2 response. Moreover these subjects show an up-regualtion of pro-inflammatory cytokines that may be responsible of T-lymphocytes exhaustion and their depletion from plasma and the accumulation of CD8+ cells in the lungs where they exert their cytotoxic action thus causing immune-mediated tissue injury (1-3). In addition to this, despite the infiltration by CD8+ T cells in pulmonary interstitium is known to play a role in clearing SARS-CoV, these cells become dysfunctional since the lung epithelial cytokines stimulated by the virus, and in particular interleukin (IL)-6 and IL8, can compromise the ability of T-cells to stimulate dendritic cells and

macrophages thus reducing the efficacy of the adaptative immune response and contributing to the immune-mediated lung damage. Indeed, elevated levels of IL6 seem to correlate with the severity of the diasease since they suppress normal T cell activation (4). Tumor necrosis factor (TNF)-α is also up-regulated in SARS patients maybe due to an induction by pulmonary angiotensin-converting enzyme 2 (ACE2) that is present in lungs and seems to represent the portal of entry of SARS-CoV-2.
Lymphopenia is also a typical feature in patients with SARS but its causes are still not well clarified. It might be due to redistribution of lymphocytes in lymphoid tissues or disappearance due to apoptosis or a combination of both mechanisms.
In one study carried in China on 21 confirmed cases with COVID-19, plasma cytokines and chemokines were measured aiming to characterise the effect of coronavirus on their production in the acute phase of disease. They found that levels of IL2, IL2 receptor (IL2R), IL6, IL10 and TNF-α were significantly higher in severe cases. This was associated to a reduction of absolute numbers of total T-cells, CD4+ and CD8+ in the majority of patients and they were markedly lower in severe cases than in moderate ones. Moreover number of regulatory T cells (Tregs) (CD4+, CD25+, $CD127^{low}$+) was lower than normal limits in almost all severe and moderate cases and the expression of IFN-γ by CD4+ was markedly reduced in severe cases (5). Other studies confirmed these findings. In a serie of 41 COVID-19 infected patients studied in China, 63% of them showed lymphopenia and 25% of patients had leucopenia (6). Patients that required ICU showed higher plasmatic levels of IL2, IL7, IL10, TNF-α and other cytokines compared to subjects who presented milder infection. Similarly, in another study, levels of several cytokines were compared among patients with mild, moderate and severe infection and the authors observed a statistically significant higher expression levels IL-2R and IL-6 in the serum of patients belonging to the critical group compared to the other two grups, thus concluding that these two cytokines should always measured since they could predict the severity of COVID-19 infection and prognosis of patients (7). Moreover Tregs (that express IL-2R) exert a critical role in contrasting acute lung injury caused by Th17 and maintaining the balance between $CD4^+CD25^+Foxp3^+$Tregs and Th17 may alleviate lung injury. Therefore to better understand the reason of CD25+ depeletion could be clinically relevant for therapeutic strategies.

2.0 Rationale

From several preliminary studies it seems clear that IL2 and its receptor (IL2R) play a key role in COVID-19 being elevated in all critical patients. This measurement could be useful to obtain a prognostic information thus allowing the selection of patients that need ICU and to promptly start the most appropriate target therapies. In this optic, the use of monoclonal antibodies (MoAbs), for example Tocilizumab, an anti-rheumatic drug directed against IL6, is under investigation and it is showing promising results in preliminary studies so, several randomized controlled trials are ongoing to futher confirm these results (8).
Moreover the cause of T-lymphocyte depletion, and in particular of CD4+ and CD8+, should also be further investigated since it is not yet completely understood wheter they go to functional exhaustion, apoptosis or wheter they migrate in other organs and, in this case, where?

In another context, it has been described a severe lymphopenia in course of a therapy with a monoclonal antibody (MoAb) directed against CD3 (Visilizumab, Nuvion®). Several authors hypothesised that lymphocutes massively died by apoptosis after binding the mAb on their surface. By using a radiolabelled anti-CD3 MoAb for imaging T-cells, we have demonstrated that the administration of Visilizumab does not cause apoptosis of lymphocytes but, by contrary, massive and

rapid margination of T-cells in peripheral lymphoid tissues, and in particular into the small bowel thus explaining their disappearance from circulation (9).

A similar approach could also be applied for imaging activated T-cells in COVID-19 patients aiming to better understand the mechanisms underlying lymphopenia and bringing new insites into the pathogenesis of the severity of this infection. We aim at studying COVID-19 patients and patients with non-COVID pneumonia, as controls, by using PET/CT with radiolabelled IL2 that binds to IL2R+ cells in tissues.

IL2 is a single chain glycoprotein of 133 amino acids that interacts with a specific receptor expressed by activated T-lymphocytes and macrophages and plays a crucial role in regulating the immune response promoting T cell proliferation, growth and differentiation of B cells, NK cells, monocytes, macrophages, and oligodendrocytes (10).
IL2R+ cells are involved in many pathological conditions characterized by a chronic lymphocytic infiltration and ranging from several autoimmune (AI) diseases (for example Tye 1 diabetes, thyroiditis, coeliac desease, Crohn's disease) to cancer lesions (for example melanoma, hypernefroma, some head and neck tumours). All these different clinical scenarios have been investigated in the last decades by using radiolabelled IL2 with ^{123}I, ^{99m}Tc or ^{18}F (Table 1) and these diagnostic approaches revealed to be an interesting strategy for imaging IL2R+ cells, providing a positive correlation between the entity of infiltrate and clinical outcomes and representing a reliable diagnostic tool for predicting prognosis (11-20).

Table 1
Published studies with radiolabelled-IL2

Reference	Type	Field
Exp Clin Endocrinol 1987;89:301	^{123}I-IL-2	In vitro tests
Lancet 1987;2:537	^{123}I-IL-2	BB rat studies
Prog Clin Biol Res 1990;355:229	^{123}I-IL-2	Autoimmune diabetes
Nuc Med Commun. 1992;13:713	^{123}I-IL-2	NOD mouse
Eur J Endocrinol 1994;131: 431	^{123}I-IL-2	NOD mouse
J Pediatr Endocrinol Metab 1996; 9:139	^{123}I-IL-2	Type 1 diabetes
Nucl Med Biol 1997;24:579	^{99m}Tc-IL-2	In vitro studies
Eur J Nucl Med 2000;27:18	^{123}I-IL-2	Coeliac disease
J Nucl Med 2000;41:242	^{123}I-IL-2	Crohn's disease
Nuc Med Commun 2003;24:305	^{123}I-IL-2	Biochemical studies
Eur J Nucl Med Mol Imaging. 2003;30:374	^{99m}Tc-IL-2	Crohn's disease
J Nucl Med 2004;45:1647	^{99m}Tc-IL-2	Melanoma
Eur J Nucl Med 2006;33:117	^{99m}Tc-IL-2	Vulnerable plaques
Q J Nucl Med Mol Imaging 2007;51:352	^{123}I-IL-2	Hypernefroma
Eur J Nucl Med Mol Imaging 2008;35:281	^{123}I-IL-2	Squamous cell carcinoma
Diabetes Metab Res Rev. 2008;24:115	^{99m}Tc-IL-2	Type 1 diabetes
Eur J Nucl Med Mol Imaging. 2008;35:2286	^{99m}Tc-IL-2	Autoimmune thyroiditis
Mol Imaging Biol. 2010;12:539	^{99m}Tc-IL-2	Labelling procedure
Nucl Med Biol. 2010;37:795	^{99m}Tc-IL-2	Kit production

J Nucl Med 2012;53:679	^{18}F-IL-2	Labelling and in vitro studies
Eur J Nucl Med Mol Imaging 2012;39:1551	^{18}F-IL-2	Animal studies
Eur J Nucl Med Mol Imaging. 2014;41:1710	^{99m}Tc-IL-2	Vulnerable plaques
Diabetes Care. 2015;38:652	^{99m}Tc-IL-2	LADA-IDDM
Oncotarget. 2018;9:30268	^{99m}Tc-IL-2	Melanoma

Since targeting IL2 and T-lymphocytes is becoming more and more attractive in different clinical indications, further alternative radiopharmaceuticals are under investigation in this moment in particular for positron emission tomography (PET) imaging. In addition to ^{18}Fluorine, ^{68}Gallium (^{68}Ga) is routinarly used in nuclear medicine departments for PET studies and it has been recently used for labelling IL2 with excellent results (21).

3.0 Aim of this study

The objective of this pilot study to perform in vivo mapping of IL2R+ cells in patients affected by COVID-19 pneumonia by PET/CT using ^{68}Ga-IL2 as radiopharmaceutical.
Through the evaluation of the biodistribution of this radiopharmaceutical we will explore, in particular, activated T-cell trafficking in this disease thus providing better understanding of the causes of lymphopenia.
For this purpose we will enroll four newly diagnosed, untreated, patients with COVID-19 related pneumonia and lymphopenia (medium to severe conditions but non requiring external breathing support). As control patients we will study four newly diagnosed untreated patients affected by non-COVID-19 related pneumonia without lymphopenia.
These eight patients will undergo a total body ^{68}Ga-IL2 PET/CT study, in addition to standard of care procedures, in order to compare the biodistribution of this radiopharmaceutical in the two groups.
This pilot study could potentially lay the basis for further clinical researches aiming to explore the possible role of this approach in the selection of patients candidate to target therapies.

4.0 Goals.
Successful completion of this study will generate necessary data to plan a larger prospective study aiming to understand the usefulness of ^{68}Ga-IL2 PET/CT for therapy decision making.

5.0 Patient eligibility.
5.1 Registration – Inclusion Criteria for COVID-19+ group

- Age ≥18 years.
- Diagnosis of COVID-19 infection obtained with positive swab performed in Sant'Andrea Hospital.
- Interstitial pneumonia (either monolateral or bilateral, moderate or severe) diagnosed with CT
- Laboratory values obtained at registration (22):
  - WBC<4x10^3/µL
  - Lymphocytes< 1500/mm^3
- Respiratory distress (22):
  - Respiratory rates ≥30 breaths/min
  - SpO2≤ 93%
  - PaO2/FIO2 ratio ≤300

- Ability to provide informed consent.
- For women of childbearing potential, a negative serum pregnancy test prior to registration.
- Willingness to participate in mandatory imaging studies as well as provide mandatory blood samples for correlative research.

5.2 Registration – Inclusion Criteria for control group

- Age ≥18 years.
- Negative swab for COVID-19 infection.
- Pneumonia (either monolateral or bilateral) from other origin than COVID-19 diagnosed with CT and microbiology
- Laboratory values obtained at registration:
    - WBC count in normal range
    - lymphocyte count in normal range
- Ability to provide informed consent.
- For women of childbearing potential, a negative serum pregnancy test prior to registration.
- Willingness to participate in mandatory imaging studies as well as provide mandatory blood samples for correlative research.

5.3 Registration – Exclusion Criteria for COVID-19+ group .

- Critical patients that need ICU and external breathing support.
- Patients that started immunosuppressive therapies for COVID-19
- Patients in immunosuppressive treatment for other reasons
- Any of the following prior therapies with interval since most recent treatment:
    - Chemotherapy ≤3 weeks prior to registration
    - Biologic therapy ≤3 weeks prior to registration
    - Radiation therapy ≤3 weeks prior to registration
- Any of the following, as this regimen may be harmful to a developing fetus or nursing child:
    - Pregnant women
    - Nursing women
    - Women of childbearing potential or their sexual partners who are unwilling to employ adequate contraception (condoms, diaphragm, birth control pills, injections, intrauterine device [IUD], surgical sterilization, subcutaneous implants, or abstinence, etc.)

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etc.)

6.0 Registration procedures.

- Eligible COVID-19 patients will be selected from first Aid department of S. Andrea Hospital under the supervision of their referring clinicians.
- For COVID-19 patients, the personnel of NM Department will adopt all the precautions able to limit the spread of the infection.
- Eligible non-COVID-19 patients will be selected from the Pneumology Department of S. Andrea Hospital under the supervision of their referring clinician.
- All baseline symptoms must be documented and graded.
- Study drug availability must be checked.

7.0 Protocol procedure.

Routine diagnostic examinations and therapies will be performed, as standard of care, with the addition of ^{68}Ga-IL2 PET/CT and a blood sample (10 cc) for serum collection and evalution of chemokines, lymphoid cells and theis subsets .

7.1 ^{68}Ga-IL2 synthesis and quality controls

Radiolabelled IL-2 will be synthesized in a shielded isolator. Briefly, 740 MBq of a 68GaCl3 solution eluted from a commercially available generator (68Ge/68Ga GalliaPharm, Ecklert Ziegler) will be added to a sterile-glass vial containing lyophilized conjugated IL2. After 15 minutes of incubation at room temperature, a small aliquot will be used for quality controls by RP-HPLC and LAL test. The sample will be analysed using a C18 column and a gradient of H2O:Acetonitrile (A:B) as follows: 5% B for 5 minutes; 5%-95% in 10 minutes; 95% B for 5 minutes; down to 5% B in 5 minutes. Labelling efficiency should be >95%. LAL-test will be performed according to the kit's data sheet (Pierce). If the preparation passes the quality controls a patient's dose can be prepared.

Recently, we developed a new ready-to-use kit to radiolabel IL2 with gallium-68 at room temperature. The protein is conjugated to a chelator for radiometals, purified and lyophilized under sterile conditions. The precursor is dissolved in a sterile ammoniun acetate buffer (pH 4.5) prior adding the eluate of a 68Ge/68Ga generator (GalliaPharm, Ecklert Ziegler). The reaction is carried out at room temperature for 15 minutes prior performing quality controls like visual inspection, RP-HPLC, pirogenicity and sterility tests.

RP-HPLC

Routine quality controls can performed by both reverse phase HPLC (RP-HPLC) and size exclusion HPLC. RP-HPLC can be performed using a kinetex C18 column (Phenomenex) and a gradient of H2O and ACN as mobile phases (0-5 min 5% ACN; 5-15 50% ACN; 15-25 95% ACN; 25-35 5% ACN). Size exclusion HPLC can be performed using a Yarra column (Phenomenex) and 0.1 M phosphate buffer as mobile phase (isocratic). Stability of the radiopharmaceutical has been tested against 0.9% NaCl solution and human serum donated by normal volunteers after signing written consent up to 3 h. Radiochemical purity is determined using ITLC-SG strips developed with 0.1M HCl to quantify levels of ionic 68Ga, and ITLC-SG strips developed with 1:1 MeOH:1 M NH4OAc to quantify colloidal 68Ga-hydroxide plus ionic 68Ga. The strips can be analyzed with a Bioscan AR-2000 radiochromatogram scanner fitted with high-resolution collimator. Negligible amount of free or colloidal 68Ga should be observed (<5%).

The radiolabelled protein has also been characterized by in vitro binding assays on activated T cells.

Saturation binding assay

In order to verify the receptor binding capacity of radiolabelled IL2, T-cells were isolated from peripheral blood mononuclear cells (PBMNCs) from healthy donors (after signing written consent) by centrifugation on a standard Ficoll/Hyplaque density gradient. Cells were cultured for 48 to 72 h at 106/mL in complete culture medium and 1 kg/mL purified phytohemagglutinin (PHA) (Mirux) at 37°C. Before the assays, cells were incubated for 60 min at 37°C in RPMI medium to remove endogenous IL-2 from the cell-surface IL-2R and then washed twice and resuspended in iced 1% BSA phosphate-buffered saline (PBS) containing 0.01% sodium azide (4°C).

Then, $3x10^6$ cells were placed in triplicate in Eppendorf vials and incubated with decreasing concentrations of ^{68}Ga-IL2 for 1 hour at 4°C, to calculate total binding curve. The same experiment was performed in the presence of a 100 fold molar excess of unlabelled IL2 to each vial, to calculate non-specific binding. At the end of the incubation time, the cells were washed twice with 0.5 ml of PBS. After centrifugation, cell pellets and the supernatants were counted separately in a single-well gamma counter. Data were analysed using Prism Graphpad software, as shown in figure 1, and revealed a Kd value of 1.79 nM.

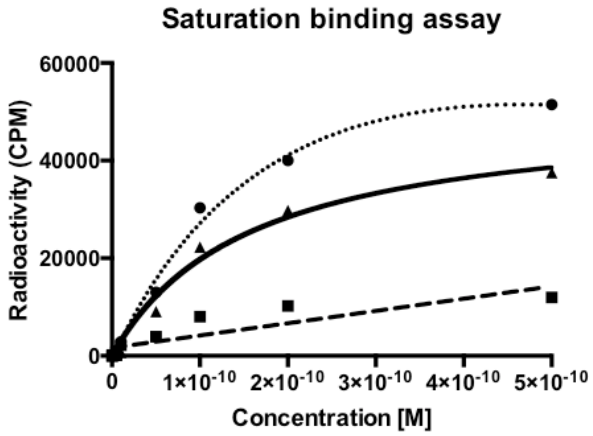

Figure 1. *Saturation binding assay of radiolabeled IL2 on activated T cells; the graph shows total binding (circle), non-specific binding (square) and specific binding (triangle) to IL2 receptor.*

Immunoreactive fraction (IRF) assay

IRF assay was performed as described by Lindmo et al. Briefly, cells were seeded in eppendorf vials (from 8x106/mL to 0.4x106/mL) and in each vial 68Ga-IL2 was added at constant concentration (10 nM). After 1 h incubation at 4°C, the vials were centrifuged at 13000 rpm (5000 g) for 3 minutes and the supernatant was collected. This step was repeated after washing the pellet with 0.5 ml of PBS.

Radioactivity associated with pellets and supernatants was then determined by counting each vial with a single-well gamma counter. Data were analysed using Prism Graphpad software and an IRF of 78.4% was obtained, as shown in figure 2, demonstrating that the majority of IL2 is radiolabelled and capable to bind to its receptor.

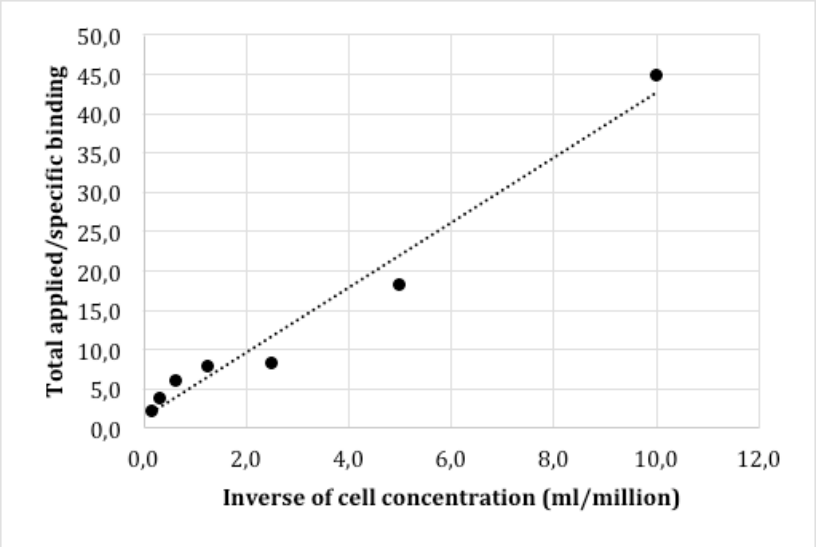

Figure 2. *Immunoreactive fraction assay of radiolabeled IL2 on activated T-cells; inverted plot showing the formula to calculate the %IRF.*

7.2 ^{68}Ga-IL2 injection to the patient

A maximun of 74 MBq (2 mCi) of radiolabelled IL2 (avarage 10 µg per patient; 50 µg maximum dose) will be intravenously (i.v.) injected using an indwelling cathether or butterly (to ensure good venous access) connected to a 100 cc vial of physiological solution in order to ensure idratation of the patient. By using the same venous access, 10 cc of blood will be collected for the evalution of chemokines, lymphoid cells and theis subsets (see Section 7.4) before the injection of ^{68}Ga-IL2.

After, blood sample and the injection, the patients will lay down approximately 1 hour in a single waiting room before the scan in order to ensure a good biodistribution of the radiopharmaceutical.

Personnel preposed to injection will stay in the waiting room only at the moment of i.v. administration of ^{68}Ga-IL2 and at the end of administration to move the patient from waiting room to PET-SCAN room, using appropriate individual protective devices.

7.3 PET/CT scan

After 1 hour from injection, a whole body scan will be acquired with a dedicated hybrid PET/CT tomograph (Siemens Biograph Flow Edition) that is close to waiting room in order to limit the exposure to radiations (and to COVID-19). After a scout CT for the definition of field of study, a low-dose CT scan, without contrast, will be acquired for attenuation correction and anatomic localization and PET images will be then acquired at speed of 0.9 mm/sec minutes per bed position from head to mid-thigh at the end of low-dose CT scan. After the acquisition, the attenuation corrected PET images will be automatically fused with CT images and displayed in maximum intensity axial, coronal and sagittal plane projections (MIP). Low-dose CT scan is not diagnostic since it will be used only for attenuation correction and anatomic location therefore it can not replace the chest CT (with contrast) that the patient need to perform for the diagnosis and evaluation of pneumonia at his/her admission at the hospital. The lenght of the scan will be approximately of 30 minutes (see Appendix I for PET acquisition SOP and Instrument specifications).

7.4 Blood sample

For purposes of the study all patients will undergo a 10 cc of blood collection in NM Department, before the injection of ^{68}Ga-IL2 in order to evaluate:

- IL2, soluble IL2R, IL6 and TNF-α.
- T, B and NK cells count and subsets: serum and cells will be frozen and analysed using commercial fluorescently labeled monoclonal antibodies (flow cytometry). Samples will be analyzed for: CD3, CD4, CD8, CD25, CD16, CD56 and CD20 (R&D Systems, Inc., Minneapolis, MN). Immunophenotyping will be performed using manufacturer's instruction in batch samples of the same patients analyzed on the same day. The stained samples will be analyzed by flow cytometry (FACScan and CellQuest software (BD Biosciences, San Jose, CA).

7.5 Follow-up

Once performed ^{68}Ga-IL2 PET/CT the patients will leave the NM Department and admitted in the appropriate unit depending on their clinical conditions.

For each patient a follow-up of two months from the PET/CT scan will be applied. No follow-up visits are required for this project in order to limit the direct contact with infected patients. But all the diagnostic examinations and blood samples that patients will perform, according to normal clinical practice, together with therapeutic strategies and clinical conditions, will be collected and recorded by personnel involved in this project, through communication with referring clinicians.

The safety of ^{68}Ga-IL2 will be evaluated with a conference call with the patients and his/her referring clinicians.

A visist in NM Unit could be planned with the patients once they definitely recovered from infection.

7.6 Study duration

Once this protocol will be approved, we will give the priority to the enrollment of 4 COVID-19+ patients (within 2 months from approval), then other 4 patients with non COVID-19 pneumonia will be recruited (within 4 months). For each patient a follow-up of 2 months from the PET/CT scan will be applied, therefore we aim at concluding the study within 6 months from its start.

8.0 Statistical considerations and methodology

Present project is a pilot study based on a small sample size of 8 subjects (4 COVID-19+ patients and 4 controls) aiming to assess whether the ^{68}Ga-IL2 PET/CT is feasible in studying T-cell traffiking in COVID patients and aiming to define an appropriate sample size for further prospective studies.

Data obtained from this study will be analysed with descriptive statistic.

9.0 Risk-benefit considerations

The methodology that will be used in the present protocol is non-invasive, unharmful diagnostic technique. The risk of developing a secondary malignancy is negligible due to the low radiation burden to the patient (approximately 10 mSv). This very low risk is totally overcome by the benefits, since the possibility to better understand the mechanisms underlying lymphopenia and to bring new insites into the pathogenesis of the severity of this infection. This approach could be able to predict wheter the patient will show a more severe manifestation of the disease thus allowing to promptly select the most appropriate treatment.

10.0 Preliminary studies

Over the past decade, studies by Signore et al. have resulted in the development of a methodology for clinical visualization of T lymphocyte organ infiltration using radioactively labeled clinical grade interleukin-2 (IL2) (11). In their initial work, IL2 was labeled with iodine-123 (^{123}I) demonstrating clinical visualization of lymphocyte infiltration in the bowel of patients affected by Celiac disease (11-14). Due

to high costs and low availability of radioactive iodine, we developed a technetium-99m (^{99m}Tc) labelled interleukin-2 (^{99m}Tc-IL2) and published the results of several studies in Crohn's disease and autoimmune thyroiditis (15-17).
In the following years, and thanks to international collaborations with the University of Ghent (Belgium), University Medical Centre Groningen (The Netherlands) and Mayo Clinic (Rochester, USA) we extended our studies with ^{99m}Tc-IL2 in oncological patients with hypernefroma, head and neck cancer and melanoma (18-20). In all these studies radiolabelled IL2 demostrated a reliable tool for imagin of CD25+ cells without any side effect reported by the patients given the low amount of IL2 used (average of 10 µg per patient; 50 µg maximum dose).

10.0 Dosimetry to the patient, safety and adverse events evaluation

10.1 Dosimetry to the patient

Effective dose equivalent is 7.3 µSv/MBq; for a typical diagnostic activity of 74 MBq: 1.35 mSv.
Reported doses to major organs for radiolabelled IL2 are: kidneys 58.4 µGy/MBq, liver 11.3 µGy/MBq and spleen 12.7 µGy/MBq.

10.2 Toxicity of IL2

Radiolabelled IL2 has been safely used in over 1000 patients all over the word without reported adverse events. Indeed the Clinical toxicity of radiolabelled IL2 is virtually the same reported for native IL2 (see attachment X). Injected amount of the biologically active protein is negligibile ($7x10^{-4}$ mg/Kg, injected once, versus $3.7x10^{-2}$ every 8 h - max 14 times). The radiolabelling procedure is safe and does not show any modification of the protein's pharmacokinetics and metabolism.

10.3 Management of adverse events

10.3.1 Regulations

The management and report of adverse events will be performed according to the following regulations:

- Decreto Legislativo 24 giugno 2003, n. 211 - Attuazione della direttiva 2001/20/CE relativa all'applicazione della buona pratica clinica nell'esecuzione delle sperimentazioni cliniche di medicinali per uso clinico.
- Decreto Ministeriale 17 dicembre 2004 - Prescrizioni e condizioni di carattere generale, relative all"esecuzione di sperimentazioni cliniche con i medicinali, con particolare riferimento a quelle ai fini del miglioramento della pratica clinica, quale parte integrante dell"assistenza sanitaria.
- European Commission - Detailed guidance on the collection, verification and presentation of adverse event/reaction reports arising from clinical trials on medicinal products for human use („CT-3").
- ICH – Guideline on development safety update report E2F (EMA/CHMP/ICH/309348/2008).
- Determinazione AIFA n.9 del 20 settembre 2012 - Adozione delle line guida CT-3 (giugno 2011) della C.E. di attuazione della Direttiva 2001/20/CE, delle line guida ICH E2F (settembre 2011) e istituzione di una banca dati nazionale relativa al monitoraggio della sicurezza dei medicinale in sperimentazione clinica.
- https://eudravigilance.ema.europa.eu

10.3.2 Definitions

- An adverse event (AE) is any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment.

It can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product.

- An adverse drug reaction (ADR) comprises all noxious and unintended responses to a medicinal product are related to any dose of the drug.

10.3.3 Classification of AEs

- Seriousness:
    - serious adverse event (SAE) or serious adverse drug reaction (SAR) are the events resulting in death, requiring hospitalization or prolonged hospitalization, life-threatening, resulting in persistent or significant disability/incapacity, a congenital anomaly/birth defect or medically important condition.
    - Suspected Unexpected Serious Adverse Reactions (SUSARs) are any events suspected to be caused by an IMP, but which are not consistent with information about the IMP (these are the most serious of events and are subject to expedited reporting procedures).
    - The rest adverse events would fall under the category of non-serious events.
- Expectedness: AEs must be assessed as to whether they were expected to occur or unexpected, meaning not anticipated based on current knowledge found in the protocol, investigator brochure, product insert, or label.
    - Unexpected adverse reaction is an adverse reaction, the nature or severity of which is not consistent with domestic labelling or market authorization, or expected from characteristics of the drug.
    - Expected adverse reaction is an adverse reaction which is known to be associated with the drug under study.
- Assessment of Attribution: when assessing whether an adverse event is related to a medical treatment or procedure, the following attribution categories are utilized:
    - Definite - The adverse event is clearly related to the agent(s).
    - Probable - The adverse event is likely related to the agent(s).
    - Possible - The adverse event may be related to the agent(s).
    - Unlikely - The adverse event is doubtfully related to the agent(s).
    - Unrelated - The adverse event is clearly NOT related to the agent(s).

  Events determined to be possibly, probably or definitely attributed to a medical treatment suggest there is evidence to indicate a causal relationship between the drug and the adverse event.
- Severity: Based on the severity, the AEs can be classified as:
    - Mild: Awareness of signs or symptoms, but easily tolerated and are of minor irritant type causing no loss of time from normal activities. Symptoms do not require therapy or a medical evaluation; signs and symptoms are transient.
    - Moderate: Events introduce a low level of inconvenience or concern to the patient and may interfere with daily activities, but are usually improved by simple therapeutic measures; moderate experiences may cause some interference with functioning.
    - Severe: Events interrupt patient's normal daily activities and generally require systemic drug therapy or other treatment; they are usually incapacitating.

10.3.4 Operative modalities

Principal investigator (PI)/promoter has to:

1. Record in detail all the AEs of the study in a special register;

2. Evaluate the severity (seriousness) of the event in accordance with the definitions provided by Art. 2 del DL 211/2003;
3. Evaluate the causality between experimental drug (IMP) and / or concomitant therapy and the adverse event;
4. Evaluate the reliability of the event: it assesses whether it is expected or not expected with respect to the reference document indicated in the protocol (IB / RCP). An adverse reaction is considered unexpected if the nature, severity, intensity, outcome of the reaction do not correspond to the information contained in the product reference document (IB / RCP);
5. report AEs and reactions to the marketing authorization holder or to the drug development manager;
6. send appropriate SAE forms and CIOMS (Council for International Organizations of Medical Sciences) forms dated and signed to the e-mail address aousa.studiclinici@ospedalesantandrea.it and in original to ULSC (Local Office for Clinical Trials), within 24h from the moment of onset of the same.

ULSC must transmit any serious and unexpected adverse reactions (SUSAR) communicated by the Principal Investigator within the established times, to the Competent Authorities and to the competent Ethical Committees.

The ULSC will regularly check the e-mail address aousa.studiclinici@ospedalesantandrea.it and the reports received will be saved in the specific electronic folders for each clinical trial. The original copy will be kept in the study reference dossier at the ULSC.

10.3.5 Validation criteria of SAE form

The SAE for will report the following data:

- Patient information:
  - Patient identification code (initials or identification code)
  - Date of birth or age of the patient
  - Sex
- Information relating to the signaler:
  - Name and surname
  - Report sending date
  - Sign

In the form there must be a detailed description of the adverse reaction that is being reported with its temporal information.

In case of incomplete data, the ULSC will request the integration of the missing information related to the SAE on:

- Initial or follow-up
- Onset of the reaction
- Administered drug
- Dosage of the drug
- Date of last administration
- Correlation

The incomplete form will be replaced once the complete form is received.

If the reaction is still ongoing, follow up will be requested until the reaction is resolved.

10.3.6 Classification of reported AEs and their management

Depending on the classification provided by the PI to the reaction, the ULSC will manage the reports as follows:

a) known/unknown reaction but unrelated to the study - SAE: the dossier related to the SAE will be archived. If the reaction is ongoing, follow-up will be requested.
b) related known reaction - SAR: the documentation related to the SAE will be archived. If the reaction is ongoing, follow-up will be requested.
c) unknown and correlated reaction - SUSAR: the Community guidelines stated that the notification by urgent procedure of the SUSARs to the authorities is made in electronic format. The ULSC will include the SUSARs, notified by the PI, in the EVCT form of the European EMA database, EudraVigilance. In this way, the obligations towards the Member States concerned, the EMA and also the Member State where the SUSAR occurred, will be satified.

- If the SUSAR determines life-threatening, or hospitalization or death of the patient, insertion in Eudravigilance will be carried out within 7 days of receipt. In case of a follow-up request, the insertion can be made after a further 8 days. After the communication, the ULSC will file the SUSAR with its CIOMS, the receipt of insertion in EV and the receipt of reading (acknowledgment).
- In case of serious SUSAR, the insertion in Eudravigilance will be made within 15 days of receipt. In case of a follow-up request, the insertion can be made after a further 8 days.

Serious and unexpected adverse reactions (SUSAR) will also be communicated to the competent Ethics Committees.
At the end of the management, the ULSC will carry out the detailed registration and the insertion of each report (SAE and SUSAR) within a special register, in which the data relating to the follow up requests will also be reported.
d) unrelated deaths: deaths due to disease progression or other causes unrelated to the experimental treatment will be included in a separate section of the register kept by the Principal Investigator

11.0 Discontinuation of the study
Criteria for discontinuation of protocol imaging procedure include:

- Request by the patient to discontinue;
- Allergic reaction;
- Intercurrent illness that would, in the judgment of the investigator, affect assessments of clinical status to a significant degree;
- Death of patient.

12.0 Budget considerations
The costs of materials for synthesis and IL2 labelling, analysis of CKs and cells subtypes and insurance will be covered by research funds as specified in the financial form.

13.0 Investigation materials
All the acquired data will be reported in Case Report Form (CRF). Investigators are responsible for ensuring the quality of the data reported as planned in this document.

14.0 Ethic Committee
The investigator will provide the ethic committee (EC) the appropriate material, including the informed consent document, in accordance with local regulations.
The study will not start until the EC has approved the protocol and the informed consent document. In addition, all documentation will be submitted to other competent authorities based on local jurisdictions.

Before starting the enrollment, the investigator must provide written confirmation that his ethical and legal responsibilities have been observed. Both the EC and the other regulatory authorities must be informed of any amendments to the protocol according to the local modalities required. The investigator will provide the CE the appropriate reports regarding the progress of the study in accordance with applicable regulations.
This study will be conducted in accordance with the ethical principles deriving from the Helsinki declaration, and will follow the rules of the Good Clinical Practice (GCP), the regulatory requirements requested by this country in accordance with the guidelines "International Conference on Harmonization (ICH) and the applicable Standard Operating Procedures (SOPs).
Specifically, the trial will be conducted following a protocol analyzed and approved by the Ethics Committee; the trial will be conducted by qualified medical and scientific personnel; the benefits of the study are proportional to the risks; the patient's rights and well-being will be respected; the doctors conducting the trial found that the risks do not outweigh the potential benefits; each patient will provide their informed consent before the start of each study examination or evaluation.

15.0 Patient's privacy

Patient's names will not be recorder; each patient enrolled will be assigned to a sequential identification number. This number will identify the patient and must be included in all Case Report Forms.
In order to avoid errors in identification, the patient's date of birth will also be reported in the Case Report Form. The investigator will guarantee the confidential nature of the information concerning the patient. All the personnel involved in this experimental study will maintain confidentiality in order to ensure that the privacy of the patient or family members is violated; Adequate measures will be taken to avoid access to the clinical data of unauthorized persons.
The use of the personal data of enrolled patients, and in particular the data regarding consent, must respect the local privacy laws (D.Lgs 196/03).
Patients will be informed in writing that the EC or regulatory authorities can view their medical records to verify the information reported, and that all available personal information will be handled confidentially and in accordance with local data protection laws.
If the results of the study are published, the identity of the subject will remain confidential.
The investigator will maintain a list which will allow to identify the enrolled patients. The Principal Investigator will be responsible for respecting confidentiality and privacy.
The collection and analysis of personal data of patients enrolled in the study will be limited to only the data necessary to evaluate the efficacy, safety, quality and usefulness of the Investigational Product (IP) used in the study.

16.0 Publication policy

The results are expected to be published in scientific journals and to be presented in congresses. All data and results and all property rights concerning the data and results of the study belong to University Hospital of Sant 'Andrea in Rome. The investigator will use the study data for scientific purposes and publish the results after the conclusion of the study.

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APPENDIX I

PET Image Acquisition SOP:

Registration of patient's personal data on the PET workstation	O	h
Registration of body weight and height of the patient on the PET workstation	O	h
I.v. injection of a maximum of 74 MBq (2 mCi) of ^{68}Ga-IL2 and connect the patient to a 100 cc vial of physiological solution in the wainting room	O	h Activity: … MBq
After 1 hour, move the patient from the waiting room to PET-scan room	O	h
Put the patient in supine position on camera bed with arms up	O	h
Set the machine for starting scout CT scan (130 Kv, 25 mA) for the definition of field of study	O	h
After the scout, start with whole body low-dose CT scan, without contrast, from head to mid-thigh with the following pre-imposted parameters (110 kV, 80 mA, pitch 1.2, axial slice thickness 3 mm) for correction attenuation purpose	O	h
At the end of the CT scout, start with PET acquisition (speed: 0.9 mm/sec) from head to mid-thigh	O	h
At the end of the scan, evaluate the fused images and dismiss the patient	O	h

Instrument Specifications
Images will be acquired by using a hybrid PET/CT tomograph (Siemens Biograph Flow Edition) with the following characteristics:

- 3rd generation spiral CT multislice (16 slices), 130 KV, 400 mA maximum with care dose 4D, tube rotation: 0.6/sec-1.5/sec, thickness: 3 mm
- PET crystal: Lutetium orthosilicate (LSO)PET

PET/CT acquisition will be performed with the following parameters:

- scout CT-scan (130 kV, 25 mA)
- whole body low-dose CT-scan, without contrast (110 kV, 80 mA, pitch 1.2, axial slice thickness 3 mm)
- PET scan (0.9mm/sec)