MICRObiota, infLammatory Environment, clinicAl and Radiomic features as predictors of Normal tissue response in radiotherapy for prostatE and head-and-neck canceR. (MICRO-LEARNER)

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BACKGROUND AND STATE OF THE ART

In December 2013 the International Agency for Research on Cancer released its global cancer estimates for 2012. The worldwide number of cancer survivors within five years of diagnosis was estimated to be ~32.6 million for 2012. In Italy cancer of the prostate (PCa) has a forecast of ~50000 new cases in 2020, while ~6400 head-and-neck cancer (HNCa) are foreseen. With increasing life expectancies and improvements in diagnosis and treatment, the number of PCa and HNCa patients and survivors is expected to continue to rise. As the illness increasingly becomes a chronic disease, patients' quality-of-life needs to be addressed in a systematic manner.

The European Society for Radiotherapy and Oncology (ESTRO) recently developed its 2020 vision, and it stated: "Vision 1.2: The majority of patients will live cancer free with minimal toxicity following the use of radical radiation therapy when used as a single curative modality of treatment or when used in combination with surgery, systemic chemotherapy and/or systemic targeted therapeutics. In order to achieve this vision ESTRO will enable and support the following priorities: [...] (c) Innovative research and development on the potential future use of novel biological modifiers of tumour and normal tissue response. To enable the above improvements in clinical care, ESTRO emphases and supports [...] the development of validated predictive models of treatment outcome based on complex databases comprising clinical, biologic, genetic, imaging, dosimetric and population data."

Radiotherapy represents the most effective non-surgical modality in the curative treatment of PCa and HNCa. Around a half of survivors underwent radiotherapy as part of their curative care.

Yet, many patients receiving potentially curative radiotherapy will experience toxicity due to the unavoidable irradiation of surrounding healthy tissue. The toxicity varies in severity from minor to severe and in duration from weeks to a lifetime. Around 7-10% of people suffer with severe long-term side-effects but much more experience mild/moderate chronic toxicity. Such mild/moderate toxicity can have a marked effect on subsequent psychological outcome and the side-effects of radiotherapy have been shown to impair quality-of-life in cancer survivors.

Radiotherapy is an important curative treatment for cancer and side-effects in survivors impact on quality-of-life.

The ability to predict those patients likely to develop toxicity could potentially enable individualization of the treatment including therapy strategy, dose prescription, fractionation choice, use of supportive therapies, which should improve survival and decrease morbidity.

Radioinduced toxicity is a multifactorial problem, related not only to delivered dose, but also to an intrinsic process within tissues responding to cellular injury. Individual biological background and expression pattern, premorbid conditions, as well as the cellular microenvironment, could be important factors in the development of side-effects, although their exact contributions are unknown [1].

In recent years with the increase in data collected, models have been developed to attempt to predict before the start of treatment patients at risk of side-effects [2].

Nevertheless these models are still missing some crucial points. The most important is the <u>capability of describing the individual patient radio-sensitivity</u>. We know that genetics influence a patient's risk of developing side-effects. A number of assays/approaches have been explored, but, at present, none has been proven to be useful in clinical practice. Another issue is related to the <u>lack of understanding normal</u>

tissue radioinduced modifications at the microstructural level, to overcome the approximation of organs as having homogeneous radio-sensitivity. This point is particularly important in the actual scenario of modern image-guided intensity modulated radiotherapy, where steep dose gradients are obtained and reduced fractions of the normal structures are irradiated.

Investigation of approaches to avoid, protect against, ameliorate or treat the long-term side-effects of radiotherapy is also a poorly researched area.
Recent research indicates that microorganisms living in the host have a role in initially driving or controlling inflammation from external causes. A dysbiotic milieu may change the metabolism of the host and provoke increased inflammation [3]. Dysbiosis affects the metabolism of short-chain fatty acids, which are known to have immunomodulatory properties and play a part in radiation-induced toxicity.

The intestinal/salivary bacteria are thus strongly suspected of being very important in mediating the response to inflammation and lesions.

The development of next-generation sequencing technologies and metabolic phenotyping makes stratification of patients at risk of radiotherapy-induced gastrointestinal toxicity a realistic possibility.

Another possibility to be investigated is the role on the inflammatory environment (as measured by levels of inflammatory markers) in modulating the response to radiation. A significant increase in salivary levels of IL1β, IL6, and TNFα, in HNCa patients during radiotherapy or radio-chemotherapy was detected, with cytokines positively associated with the severity of mucosal toxicity. Higher increase of IL1β and IL6 three weeks after treatment initiation was predictive of worse oral mucositis, representing a potential tool for the early identification of patients at risk [4].

In the field of characterization of normal tissue response to radiation, a promising emerging field is the use of imaging as source of numeric data describing tissue microstructure that can be analysed by bio-engineering techniques. These techniques, being completely automatic and reproducible, and able to provide quantitative indices of the structural and functional tissue properties, while maintaining information about spatial heterogeneity, could have an impact on investigating tissue response to radiation at the individual level [5].

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PRELIMINARY DATA

(A) Feasibility of collection saliva specimens for microbiome analysis
Fifteen saliva specimens from HNCa patients were collected (Omnigene-oral collection devices, Oragene). Samples were stored at room temperature for 30 days before DNA extraction. Microbial DNA was extracted using QiASymphony DSP pathogen midi kit. DNA was quantified by Qubit. The box plot (figure 1a) shows the total amount (ng) obtained after microbial DNA extraction: median value = 2900 ng (range: 1250-7250 ng). Quality of the material was assessed using the oral microbial DNA qPCR Array (Qiagen) for microbial identification and profiling on 4 specimens. The array is designed using probes detecting 16S rRNA gene for 93 different oral bacteria strains. We were able to detect a range from 59 to 73 out of 93 bacterial strains. The preliminary study shows that: i) total DNA amount is enough for downstream applications including next generation sequencing; ii) the obtained material is suitable for 16S Metagenomics identification and quantification.

(B) Feasibility of results on monitoring the expression of inflammatory molecules during radiotherapy.
Plasma levels of selected inflammatory molecules (IL-1,IL-6,IL-8,TNF,CCL2,PTX3) were measured in prostate cancer patients undergoing radiotherapy. Inflammatory molecules kinetics was determined as a function of radiation dose. Expression of inflammation markers was correlated with patients' characteristics and with acute radio-induced toxicity. Results are presented in figure 1b.

(C) Feasibility of modelling dose-volume response with interaction among organs
Modelling of severe oral mucositis (G3muc) on a population of 132 HNCa patients (toxicity rate 30.3%). G3muc was associated to small volume of the oral cavity receiving high doses and to the mean dose to parotid glands (probability curves in figure 2a). This suggests interaction between the two organs, with a reduced salivary flow entailing a minor protection from inflammation. The result was confirmed in a further study (78 oropharyngeal cancer patients).

(D) Multi-parametric characterization of penile bulb using MRI images
A preliminary investigation about the variations induced on the penile bulb after radiotherapy was carried out on six patients with prostate cancer treated with RT using multi-parametric MRI images acquired before treatment's start and at the end of RT.
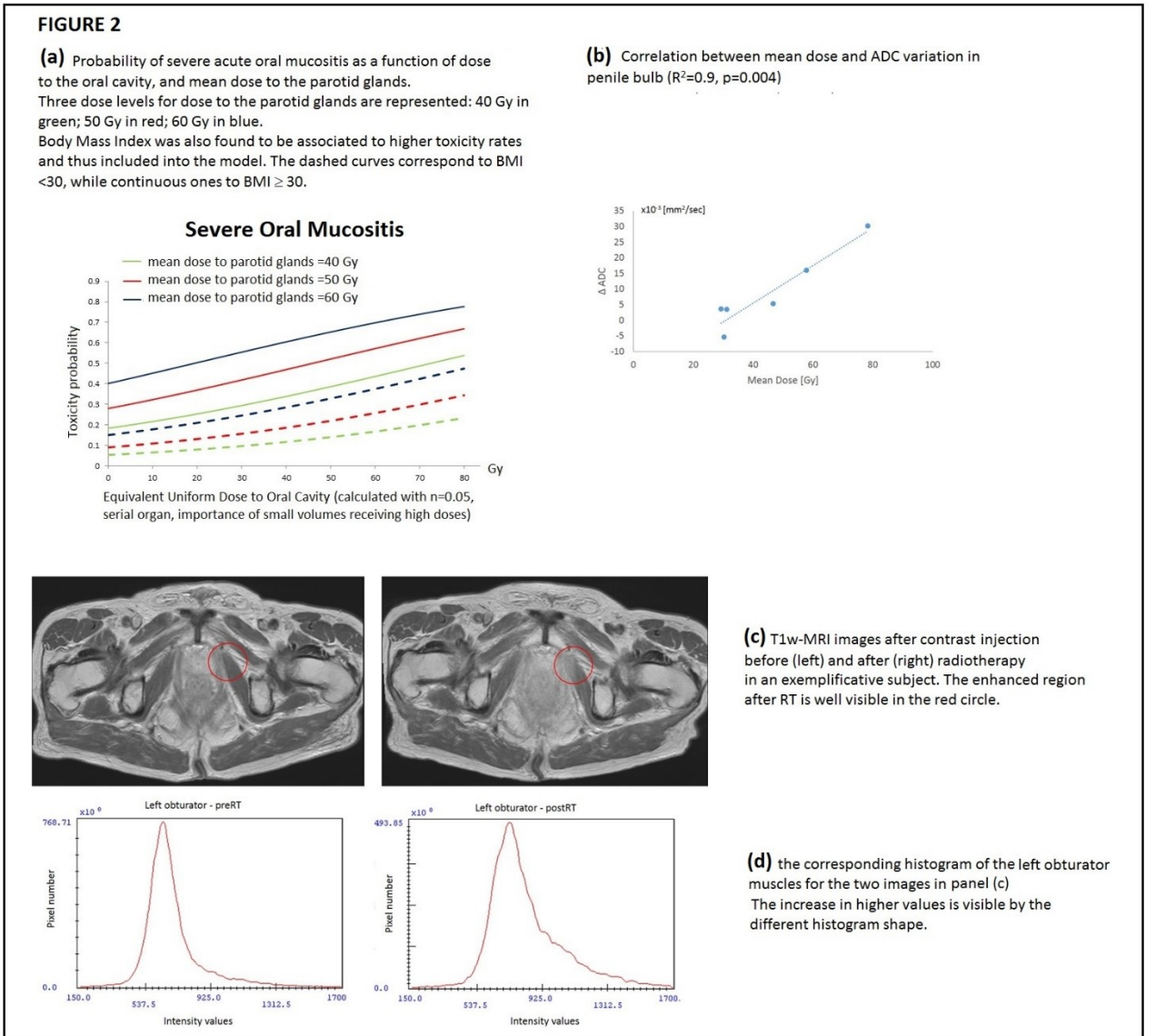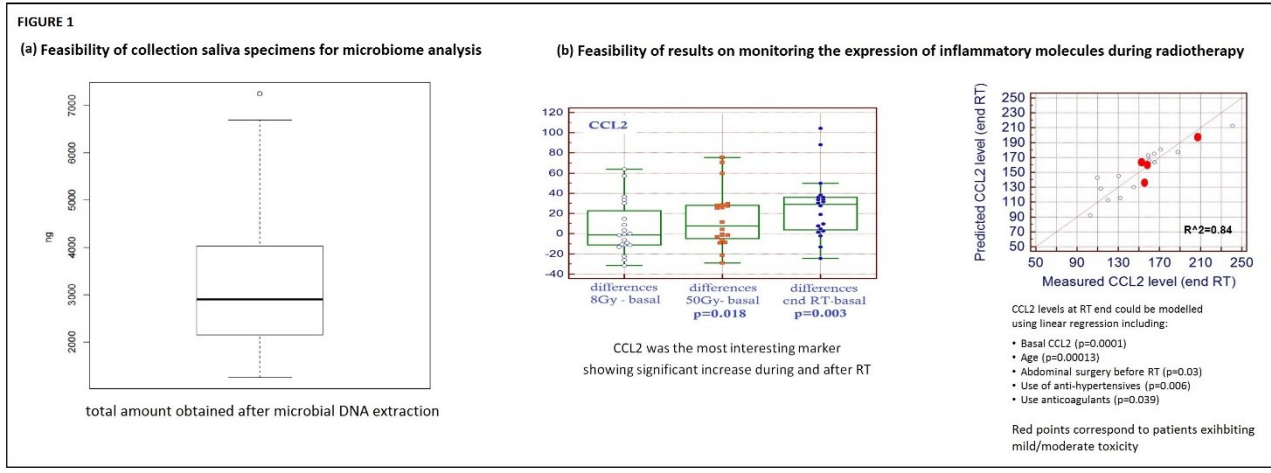We found that ADC value in penile bulb increased and its increment correlated with higher mean doses to (R^2=0.9, p<0.01, Figure 2b). This behaviour could be explained by the inflammatory status that normally follows RT, which is not visible on anatomical images.

(E) Variations on obturator muscles assessed by anatomical MRI
A preliminary investigation about the variations induced on the obturator muscles after radiotherapy was carried out on 13 patients with prostate cancer treated with RT using T2w-MRI and T1w-MRI after contrast injection, acquired before treatment start and at 12 month follow-up. It was found a significant increase in the mean values on both T1w-MRI and T2w-MRI, together with an increase in the number of higher values in the histogram, well visible by the different histogram shape (Figure 2d). This was related to a more enhanced area after RT in the region near the prostate (Figure 2c). A possible explanation of this enhancement can be the inflammatory status of this muscle region, which received the highest dose.

(E) Role of HPV status in toxicity prediction for HNCa

A study on 148 HNCa patients, on relationship between patient-reported late dysphagia and clinical/molecular features, highlighted association with gender, HPV status.

The possible role of HPV status in determining tissue response to radiation is also suggested by analysis of MRI features. 23 HNCa cases were considered: tumour ADC mean values were significantly lower for HPV-positive (859 x 10-6 mm2/sec vs 1099 x 10-6 mm2/sec for HPV-negative, p=0.02). This can likely reflect different tumour cellularity and microenvironment among the two patient groups.

FIGURE 1

(a) Feasibility of collection saliva specimens for microbiome analysis

(b) Feasibility of results on monitoring the expression of inflammatory molecules during radiotherapy

total amount obtained after microbial DNA extraction

CCL2 was the most interesting marker showing significant increase during and after RT

CCL2 levels at RT end could be modelled using linear regression including:

• Basal CCL2 (p=0.0001)
• Age (p=0.00013)
• Abdominal surgery before RT (p=0.03)
• Use of anti-hypertensives (p=0.006)
• Use anticoagulants (p=0.039)

Red points correspond to patients exihbiting mild/moderate toxicity

R^2=0.84

FIGURE 2

(a) Probability of severe acute oral mucositis as a function of dose to the oral cavity, and mean dose to the parotid glands.
Three dose levels for dose to the parotid glands are represented: 40 Gy in green; 50 Gy in red; 60 Gy in blue.
Body Mass Index was also found to be associated to higher toxicity rates and thus included into the model. The dashed curves correspond to BMI <30, while continuous ones to BMI ≥ 30.

(b) Correlation between mean dose and ADC variation in penile bulb (R^2=0.9, p=0.004)

Severe Oral Mucositis

— mean dose to parotid glands =40 Gy
— mean dose to parotid glands =50 Gy
— mean dose to parotid glands =60 Gy

Toxicity probability

Equivalent Uniform Dose to Oral Cavity (calculated with n=0.05, serial organ, importance of small volumes receiving high doses)

(c) T1w-MRI images after contrast injection before (left) and after (right) radiotherapy in an exemplificative subject. The enhanced region after RT is well visible in the red circle.

(d) the corresponding histogram of the left obturator muscles for the two images in panel (c)
The increase in higher values is visible by the different histogram shape.

Left obturator - preRT

Left obturator - postRT

HYPOTHESIS

The development of radioinduced toxicity is not solely related to the dose and to the way radiotherapy is delivered. There are some individual characteristics that make some people more susceptible to develop acute and chronic side effects, this is often called the 'consequential effect'.

An important driver of the 'consequential effect' is increasingly believed to be the microbiota. Microbiota are a complex ecosystem of up to 1,000 bacterial species in any one individual. The species vary greatly between individuals but, within each individual, their composition remains stable for the majority of the species over time. The diversity of the microbiota is high in healthy people and low in people with side effects after radiotherapy, a process which very closely parallels findings in people with other (not radioinduced) inflammatory conditions.

The specific hypothesis investigated by this research proposal are:

(A) Gut and salivary microbiota play a role in determining the single patient susceptibility to radiation. Different rates of side-effects can be detected in patients exhibiting differences in the composition of their microbiota. In the longer term, the ultimate hypothesis of the proposed research programme will be to use information on microbiota to find ways of changing the make-up of gut/salivary bacteria to benefit patients.

(B) Information on baseline inflammatory markers (plasma/salivary levels) and on their kinetics in the first two weeks of radiotherapy can be used as indicators of enhanced radiosensitivity. This information can be used for effective adaptive planning to reduce dose in selected radiosensitive patients.

(C) Information on radioinduced microstructural changes in normal tissues, as measured by medical imaging, can help in gaining insight into tissue response. Specifically, local damage can be revealed by both anatomical and functional imaging, measured in an objective way and associated to three-dimensional dose distributions. The role of different tissues/organs and their possible interaction in determining specific side-effects can be investigated. This information (radiomic features) can lead to a more effective planning dose optimization, which is of peculiar importance in the era of intensity modulated image-guided radiotherapy: steep dose gradients can be obtained, provide it is known where optimization should be performed.

(D) Information coming from microbiome measurement, inflammatory marker levels and radiomic features can be included in models for the prediction of radioinduced toxicity, with significant improvement of their performance and capability of identifying patients at high/low risk of side-effects. This will constitute a step forward in radiation oncology, allowing for patient-tailored treatment to modulate toxicity and producing the basic structure of a possible queryable therapeutic algorithm.

AIMS

1. To perform a clinical trial (discovering population), including PCa and HNCa patients, allowing prospective evaluation of gut/salivary microbiota before radiotherapy and at the end of treatment. Perform measurements of changes in microbioma composition and determine the association between acute/mid-term toxicity and baseline (changes during treatment) microbiota. Perform validation of findings on an independent population, enrolled with same criteria.

2. To develop predictive models for radioinduced (acute/mid-term) toxicity including dosimetric/clinical/treatment information with the addition of features suggestive of individual sensitivity (microbioma characteristics, inflammatory marker levels and kinetics, tissue features as extracted by radiomic studies on imaging). Evaluation of the performance and clinical utility of these extended models.

3. To develop a complex database of PCa and HNCa patients, comprising clinical, biologic, genetic, imaging, dosimetric and population data. This database will be of value for future studies and for benchmarking/validation of models and results obtained by other research groups. The database will include biobanking of salivary and blood sample for future (genetic) studies.

EXPERIMENTAL DESIGN

Prospective Clinical Trial: discovering population

130 PCa and 130 HNCa consecutive patients will be enrolled in 15-18 months. All patients will receive radiotherapy at radical curative doses at the National Cancer Institute in Milan, and they will receive follow-up visits at the same centre.

Detailed pre-treatment evaluation will include: recording of demographic features, clinical history, comorbidities and habits, evaluation of normal tissue functioning by the health practitioner (CTCAE scoring system), evaluation of normal tissue functioning through validated patient reported outcome (PROs) questionnaires, evaluation of quality of life through validated tools, evaluation of organ functioning by instrumental measures (i.e. baseline swallowing screening with flexible endoscopic evaluation of swallowing – FEES – and unstimulated salivary flow for HNCa patients), biochemical examinations, gut/salivary microbiome measurement, determination of baseline level of plasma/salivary inflammatory markers, baseline multi-parametric magnetic resonance imaging (MRI). Additional salivary and blood samples will be collected and stored for future studies.

Patients will receive radiotherapy and possible adjuvant (hormone or chemo) therapies as foreseen by institutional guidelines. In this aspect the here proposed trial is an observational study, no modification to standard regimens is considered.
Specifically, PCa patients are treated to 78 Gy, 2Gy/fraction, in exclusive setting and to 70 Gy, 2Gr/fraction in the post-prostatectomy setting. Lymph node irradiation is performed (50 Gy, 2Gy/fraction when indicated by risk class). In definitive setting, HNCa patients are treated at a total dose of 70 Gy ,59.4 Gy and 56 Gy in 33 fractions to high risk Planning Target Volume (PTV), intermediate risk PTV and low risk PTV, respectively. In high risk post-operative setting they are treated to 66 Gy (according to histopathological features), 60 and 56.1 to high risk PTV, intermediate risk PTV and low risk PTV, respectively; while, in intermediate risk post-operative case, intermediate risk PTV and low risk PTV irradiated to 60 Gy and 56 Gy, respectively.
Radiotherapy is performed with volumetric arc modulation, with k-voltage cone-beam CT (kCBCT) or Calypso transponder image guidance.

Evaluation during treatment will include weekly assessment of toxicity, as measured by the clinician (CTCAE) and by PROs and biochemical measurements.
Evaluation of inflammatory markers will be performed after a dose of 20 Gy.
For a subpopulation of 60 oropharyngeal cancer patients treated with definitive radiotherapy +/-chemotherapy an additional MRI study during the second week of treatment is foreseen.

Assessment at the end of radiotherapy will include evaluation of toxicity by the health practitioner and by PROs, evaluation of quality of life through validated tools, FEES and unstimulated salivary flow for HNCa patients, biochemical examinations, gut/salivary microbiome measurement, determination of level of plasma/salivary inflammatory markers.

Evaluation at 3, 6 and 12 months will include: evaluation of toxicity by the health practitioner and by PROs, evaluation of quality of life through validated tools, FEES and unstimulated salivary flow for HNCa patients and biochemical examinations.

Minimum follow-up is set to 12 months for the specific purpose of evaluation of acute and mid-term toxicity, which are the endpoints considered in this project. Nevertheless, follow-up will continue until 3 year after the end of radiotherapy (beyond the end of the project) in order to allow evaluation of incidence, prevalence and patterns of late side-effects. After 12 months, follow-up will be performed every 6 months and limited to assessment of toxicity by by the health practitioner and by PROs,

Follow-up MRI studies will be performed at 3, 12 and 24 months for HNCa patients and at 12 months for PCa patients.

Prospective Clinical Trial: validation population

70 PCa and 70 HNCa consecutive patients will be enrolled in 12 months, starting immediately after the end of enrolment of the discovering population.
The specific aim of this second phase is validation of results on microbiota, specifically on association between selected baseline microbiota profiles and the risk of acute toxicity (for the purpose of this project) and for mid-term/late toxicity (beyond this projects). This result should be the more significant from the clinical point of view, allowing development of a therapeutic algorithm to be used before treatment and permitting introduction ways of changing the make-up of gut/salivary bacteria in patients at high risk of toxicity.

Baseline assessment, treatment and follow-up evaluation of toxicity by the clinician and by PROs will follow the scheme described for the developing population. Microbioma measurement will be only performed at baseline. Imaging and assessment of inflammatory marker levels will not be accomplished in the validation population.

Analysis of gut and oral microbiota

Saliva and stool will be collected using oral- gut-, respectively, OMNIgene devices (Oragene) following manufacture's recommendations. Samples will be stored in a stabilization buffer at room temperature until DNA extraction.

DNA extraction will be carried out using QiASymphony DSP pathogen midi kit starting from 1 ml of saliva and following manufacturer's instructions. DNA extraction for stool specimens will be carried out using the QIAamp DNA Stool Mini Kit (Qiagen) starting from 200 mg of feces after additional incubation at 95 °C for 10 min of the stool sample with the lysis buffer to improve the bacterial cell rupture as detailed in [6]. The concentration will be estimated by Qubit 3.0 Fluorometer (Invitrogen).

Methods of Microbiome detection
the 16S regions will be amplified with 16S Ion Metagenomics Kit ™ (Life Technologies) using 2 separate PCR oligo pools covering V2, V4, V8 and V3, V6-7, V9 hypervariable bacterial 16S rRNA regions. Equal volumes of both amplification reactions per each sample will be combined. Staring from 50 nanograms of combined amplicons DNA libraries will be prepared using Ion Plus Fragment Library Kit and Ion Xpress Barcodes Adapters, 1–16 (Life Technologies). After adapter-ligation and nick-repair, DNA will be amplified as follow: 1 cycle of 95°C for 5 min; 5 cycles of 95°C for 15sec, 58°C for 15 sec, 70°C for 1 min. The final library will be purified using 1.4 volumes of Agencourt AMPure beads (Beckman Coulter) and eluted in Tris-EDTA buffer. Quality check and quantification will be assessed by DNA high sensitivity kit on 2100 Bioanalyzer (Agilent

Technologies). Each sample will be adjusted to 50 pM concentration. Library preparation will be performed by ION Chef System (Life Technologies) according to the manufacturer's instructions. Sequencing of the retrieved templates spheres was carried out on Ion 318™ v2 chip using Ion Torrent PGM™ system and employing the Ion Sequencing 400 kit (Life Technologies). Base calling and run demultiplexing will be performed by Torrent Suite (Life Technologies, Grand Island, NY) with default parameters.

Data processing
BAM files directly obtained from the Ion Torrent PGM output will be processed using Ion Reporter Software (Life Technologies) and the following filtering parameters will be applied: (i) each read will be trimmed tolerating until three errors at primers' sequence, (ii) read with length lower than 165 bp will be discarded, and (iii) a hash table will be created to identify unique sequences and their abundance. The alignment will be performed using MicroSEQ ID and Green Genes databases in order to obtain the taxonomic identification of sequences. To further investigate bacterial biodiversity, operational taxonomic units (OTUs) cluster analysis will be performed based on hypervariable region V4. Sequencing reads will be extracted from the total dataset using cutadapt [7]. Reads will be analyzed using the micca pipeline (version 0.1, http://compmetagen.github.io/micca/). De novo sequence clustering, chimera filtering, and taxonomy assignment were performed by micca-otu-denovo (parameters -s 0.97 -c): operational taxonomic units will be assigned by clustering the sequences with a threshold of 97 % pair-wise identity, and their representative sequences will be classified using the RDP [8] software version 2.8. Finally, a phylogenetic tree will be inferred using FastTree [9] software embedded in micca-phylogeny (parameters: -a template-template-min-perc75).

Assessment of inflammatory marker levels

Plasma and saliva samples (obtained from PCa and HNCa patients, respectively) collected at baseline, during radiotherapy (20 Gy) and at the end of treatment, will be analyzed to prospectively determine baseline levels and changes of selected inflammatory markers. Specifically, for PCa, CCL-2, TGF-β, TNFα, TNFR-1 and PDGF will be considered, while, for HNCa, IL-6, TNFα and IL-1b will be measured.
Evaluation will be carried out using commercially available ELISA kits, according to manufacturer's protocols.

Analysis of images and extraction of radiomic features

Image analysis for the characterization and evaluation of radiation-induced effects on organs at risk will be mainly carried out on multi-parametric MR images (consisting in T2w-MRI, T1w-MRI with and without contrast injection, DW-MRI and DCE-MRI) acquired before treatment's start and during the follow-up period; in the case of head-and-neck cancer treatment, a further MRI acquisition will be available at mid-treatment. Planning CT and daily/weekly CBCT will be eventually considered for anatomical evaluations and for dose deformation from CT to the multi-parametric MRI. The organs at risk considered in the morphological and functional analyses will be the penile bulb and the pelvic floor muscles in the pelvic district, the parotid glands, oral mucosa, larynx and constrictor muscles in the head-and-neck district.

Spatial registration
The first step of the multi-parametric analysis is the spatial registration of all the acquired images to a common reference (generally, the initial T2w-MRI); a non-rigid image registration method, already optimized and validated on a subset of subjects [10], will be applied, 1) to realign DCE-MRI and DW-MRI to

their correspondent T2w-MRI, 2) to deform mp-MRI acquired at different time-points to the reference T2w-MRI, 3) to estimate the deformation between T2w-MRI and planning CT, which will be applied on the corresponding dose map. In this way, it is possible to perform a quantitative image analysis on the same considered volume, in order to extract multiple features from a single region, in terms of both ROI-based and voxel-based analysis. Moreover, using the estimated deformation field, it is possible to quantify in first instance the volumetric modifications of the structures and to propagate organs contours delineate on the first T2w-MRI on the other images, without the need of new manual segmentations.

Analysis and feature extraction

Regarding the image analysis methods, when anatomic MRI (T2w and T1w) are considered, some pre-processing steps are needed. First, T2w-MRI images have to be corrected for magnetic field inhomogeneities by using the non-parametric non-uniform intensity normalization (N3) algorithm [11]. Second, a normalization step has to be performed between T2w-MRI1 and T2w-MRI2 for each patient, in order to reduce possible errors due to the non-quantitative value of signal intensity, using the histogram matching method [12].

After MR signal intensity normalization, advanced quantitative analysis will be performed by extracting different features from anatomic MRI. In particular, textural and fractal features will be estimated to characterize the structural organization of organs tissue. Textural features (e.g. first and second order statistical parameters) describe the histogram distribution and the relationships of gray-level values in the image, related to gray-level frequency and spatial distribution within the region of interest [13]. Fractal analysis can also synthetically describe the spatial heterogeneity of a region of interest by a geometric measure, as the fractal dimension [14]. The same analysis can be applied on CT images, to assess possible correlations between the two imaging modalities.

Regarding the functional images, the diffusion properties will be analyzed by extracting the apparent diffusion coefficient (ADC) [15] following both (1) a ROI-based and (2) a voxel-wise approach. In detail, ADC will be estimated by least-square fitting the mono-exponential model, using different b-values. Finally, the perfusion analysis will be carried out by computing semi-quantitative parameters based on mathematical modelling of the DCE time series (wash-in and wash-out rates, the Integral Area Under the Curve IAUC, signal peak intensity, onset time and time-to-peak) [16], as well as quantitative indices following the extended Tofts model [17] (the volume transfer constant, the volume of extravascular extracellular space EES per unit volume of tissue, and the flux rate constant between EES and plasma), which are inferred using an optimization curve fitting approach. Moreover, texture analysis can be also performed on the parametric maps estimated from functional MRI, thus giving more value to the advanced image analysis protocol. In case dose maps will be present and deformed on MRI images, voxel-by-voxel correlations between dose and parametric maps will be estimated.

Lastly, the best combination of features will be selected to better describe radiation-induced variations in organs of interest and to correlate them with the prescribed dose and toxicity outcomes.

Creation of a large standardized database

Thousands of different types of data will be generated, collected and reported during the experimental process. They include structured data, like PROs or toxicity scoring by the clinician, but also semi-structured and unstructured data, such as dose distributions of radiotherapy treatment plans or CT/MRI images or results of microbial sequencing. A pre-processing and harmonization of data coming from different sources is necessary before proceeding with the analysis. All clinical and radiotherapy treatment data will be uploaded on the VODCA system software (Visualization and Organization of Data for Cancer Analysis, Vodca

Inc) that allows to manage multiple sources of data and is tailored to meet the needs of radiotherapy trials. It allows the extraction of large datasheets that will be employed for the statistical analysis and model building.

Development of advanced numerical tools for data investigation

Advanced numerical algorithms, including supervised or unsupervised learning techniques, will be established and developed for the analysis.

An innovative method based on in-silico experimentation and aimed at identifying the best predictors of a binary endpoint will be applied for identification of leading robust predictors of toxicity and minimization of the influence of noise. The well-known logistic regression is the core of the method, whose results are simpler to interpret, with respect to more recent and complex methods. In this approach, logistic regression is enhanced with upstream and downstream data processing to find stable predictors, even when the number of available cases is limited or when the endpoint is not balanced, i.e. when there are many more negative than positive cases, as it usually happens in clinical research. For upstream and downstream data processing, the idea is borrowed by business analytics, like market basket analysis, as well as social network analysis. This method was tested with satisfactory results in previous work [18].

METHODOLOGIES AND STATISTICAL ANALYSIS

R statistical computing programming language [www.r-project.org] and KNIME data mining environment [KNIME GmbH, Germany] will be used for statistical analyses.

For analysis of the association between presence/absence of acute/mid-term toxicity and microbiome profiles/inflammatory marker levels, non-parametric tests (Wilcoxon, Kruskal Wallis and Mann-Whitney test) will be performed. The first step will be comparison of microbiome profiles/inflammatory marker levels at baseline and during/after radiotherapy (paired sample analysis), thus identifying the effect of radiation modulation. Then comparison of microbiome profiles/inflammatory marker levels in different groups of patients (exhibiting/not exhibiting toxicity) will be completed, for the identification of profiles potentially predictive of the individual response to radiation, both before and (during) after treatment.

Another important issue when developing predictive models in this type of observational studies, is the possible number of predictors that can be safely included into multivariate analysis. In these cases, being the number of predictors unknown a priori, an important rule of thumb to be considered is that the events per variables to assess each predictor is around 10 [18]. Then, seeing the estimated number of moderate/severe acute toxicity events, for PCa, with a sample size of 130 patients and 15-20% toxicity rate, 2-3 variable can be included into model, while for HNCa models with inclusion of at least 4-6 variables are expected to be developed (toxicity rates even more than 30%).

The starting point for predictive model development will be classical multivariable logistic analysis (MVA), followed by nomogram translation of MVA results. In a first step information on clinical/dosimetric/radiomic features will be included into models.
Data will be then analyzed in the frame of radiobiological normal tissue complication probability (NTCP) models, with inclusion of clinical risk factors.
An innovative method based on in-silico experimentation and aimed at identifying the best predictors of a binary endpoint will be applied for identification of leading robust predictors and minimization of the influence of noise. The well-known logistic regression is the core of the method, whose results are simpler to interpret, with respect to more recent and complex methods. In this approach, logistic regression is enhanced with upstream and downstream data processing to find stable predictors, even when the number of available cases is limited or when the endpoint is not balanced, i.e. when there are many more negative than positive cases, as it usually happens in clinical research. For upstream and downstream data processing, the idea is borrowed by business analytics, like market basket analysis, as well as social network analysis. This method was tested with satisfactory results in previous work [20].

An imperative point will be the need to develop suitable model visualization tools in order to apply modeling techniques in the medical domain and to make the results available as user-friendly tools to both the clinicians and the patients.

Information on microbiome profiles/inflammatory marker levels will be added to the significant variables obtained at the previous step in order to verify if the predictive ability of the models is improved.
Several performance measures will be considered to evaluate improved predictive ability: Brier score, likelihood, calibration, reclassification table, net reclassification improvement and clinical usefulness.

Power calculation

In order to analyze the association between presence/absence of acute/mid-term toxicity and microbiome profiles/inflammatory marker levels, we are going to use non-parametric tests (Mann-Whitney, Wilcoxon matched pairs and Kruskal Wallis). These tests will be used because we are not sure that microbiome profiles/inflammatory marker data come from a Gaussian distribution. On the contrary, we foresee that radiosensitive/radioresistant patients have anomalous microbiome profiles/inflammatory marker levels (placed in the tails of distributions). We can estimate that a sample of 130 patients will allow to detect a large effect size, which would be clinically relevant for identification of radiosensitive/radioresistant patients (OR≈1.8-2.5, Cohen's d=0.8), with statistical power 0.8 and significant level p=0.01 and medium effect size (OR≈1.3-1.5, Cohen's d=0.5) with statistical power 0.8 and significant level p=0.05 (two-tailed hypotheses in all cases).

When considering development of models, a sample size of 130 patients will have the power to detect a clinically relevant odds-ratio equal to ≈2 for a proportion of toxicity endpoint in the range 15-20%, which can be foreseen for acute toxicity after radiotherapy for PCa (with an alpha error=0.05 and beta error=0.2). For HNCa treatment higher toxicity rates can be forecast (even more than 30%) thus allowing detection even of less clinically relevant odds-ratios (≈1.7).

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