

Radiation therapy induces innate immune responses in patients treated for prostate cancers

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Competing interests:

Anatoly Dritschilo, MD, owns equity and serves as CEO of Shuttle Pharmaceuticals, Inc. The remaining authors declare no potential conflicts of interest.

Abstract:

Purpose

Radiation therapy (RT) is a curative therapeutic modality used to treat cancers as a single agent or in combination with surgery and chemotherapy. Advanced RT technologies enable treatment with large fractions and highly conformal radiation doses to effect free-radical damage to cellular DNA leading to cell cycle arrest, cell death, and innate immune response (IIR) stimulation.

Experimental Design

To understand systemic clinical responses after radiation exposure, proteomic and metabolomic analyses were performed on plasma obtained from cancer patients at intervals after prostate stereotactic body radiation therapy. Pathway and multivariate analyses were used to delineate molecular alterations following RT and its correlation with clinical outcomes.

Results

DNA Damage Response (DDR) increased within the first hour after treatment and returned to baseline by one month. IIR signaling also increased within one hour of treatment but persisted for up to three months thereafter. Furthermore, robust IIR and metabolite elevations, consistent with an early proinflammatory M1-mediated innate immune activation, were observed in patients in remission, whereas patients experiencing PSA-determined disease progression demonstrated less robust immune responses and M2-mediated metabolite elevations.

Conclusion/Discussion

To our knowledge, these data are the first report of longitudinal proteomic and metabolomic molecular responses in patients after radiation therapy for cancers. The data supports innate immune activation as a critical clinical response of patients receiving radiation therapy for prostate cancer. Furthermore, we

propose that the observed IIR may be generalized to the treatment of other cancer types, potentially informing multidisciplinary therapeutic strategies for cancer treatment.

Translational Relevance: As a curative or palliative treatment modality, more than 50% of cancer patients receive radiation therapy (RT) during the course of their disease. Although recent studies provide credence to the immune system's role in modulating tumor response to RT, this, however, remains an understudied area in radiation oncology research. Herein, we show that molecular interactions and cellular crosstalk underlying immune responses and DNA damage response activation by RT define at least in part, the underlying biologic basis of tumor response to RT. Validation of this concept can be used for the improvement of therapeutic strategies in RT-based cancer treatment.

Introduction

Prostate cancer is a major cause of death and disability in men, with estimates of 248,530 diagnoses and 34,130 deaths in 2021 in the U.S. (1). Radiation therapy (RT) is an effective modality for curative treatment of prostate cancers as a single agent and in combination with hormonal therapies or after surgery. Efforts to improve the outcomes of RT have focused on advances in imaging, beam shaping, and dose fractionation; however, in all instances, the tumor microenvironment and adjacent normal tissues are irradiated along the radiation beam axis. The development of stereotactic body radiation therapy (SBRT) utilizing a robot mounted linear accelerator to deliver precise, highly conformal radiation therapy to the prostate in large fractional doses has yielded excellent clinical outcomes and shortened the overall treatment time (2,3). For investigation of molecular mechanisms using high throughput technologies, the larger fractional doses amplify biological signals for investigations into cellular and systemic responses to RT.

The immune system has been implicated in patients undergoing RT through observations of “abscopal” cancer responses, as well as improved clinical outcomes in recent clinical trials (4-6). Recent advances in immune directed therapies and personalized medicine have also been extended to treating advanced, metastatic prostate cancers (7). Observed benefits, risks, and late effects in the heterogeneous clinical responses of patients receiving curative doses of radiation therapy (RT) underscore the complexity of clinical therapeutics and the urgent need to understand biology. Integrated responses of tumors and normal tissues following radiation therapy enable the discovery of predictive biomarkers and therapeutic molecular targets.

Although cancers confined to the prostate can be cured by radiation therapy, dose limitations of normal tissues at risk and the potential for undiagnosed metastases underlie treatment failures and cancer recurrences. Recent advances in determining roles for the DNA Damage Response (DDR) and cell cycle arrest after radiation exposure has focused on cancer cell sensitization strategies. In addition, reports of abscopal antitumor immune responses have implicated immune system contribution to both, local tumor control and regression of metastases. Irradiated tumors can recruit monocytes to the injured area, which are differentiated into tumor-associated macrophages (TAMs) (8,9). TAMs are mainly polarized towards the pro-tumoral M2 phenotype and are strongly associated with a poor prognosis in cancer ((10-13). TAMs produce anti-inflammatory cytokines, induce hypoxia, express immunosuppressive mediators, and support tumor growth. These functional characteristics of M2 macrophages have a detrimental effect on CD8 effector T cell function (14,15). Thus, controlling the recruitment or polarization of macrophages in irradiated tumors could be an attractive option to prevent the activation of survival pathways with RT.

To understand molecular events characterizing the global clinical responses in irradiated patients, we analyzed serum from 132 patients receiving stereotactic body radiation therapy (SBRT) for prostate

cancers on an IRB approved protocol collecting serial blood samples and quality of life data prior to and after radiation treatment. Longitudinal plasma protein and metabolite profiles were determined relative to the pre-RT clinical specimens in a time course after SBRT. We then analyzed the global responses using high-throughput proteomics and metabolomics as well as comparisons of cohorts of patients experiencing disease remission to those with cancer progression. Here, we report robust innate immune system activation after radiation therapy for prostate cancer with correlation to clinical outcomes.

Materials and Methods

Patient Recruitment and Study population: Patients with biopsy proven, organ-localized prostate cancer, referred for RT at the MedStar Georgetown University Hospital were offered enrollment into IRB protocol 2012-1175, a quality-of-life clinical trial. Informed written consent was obtained from each patient on the trial and the study was conducted according to the guidelines of the Declaration of Helsinki. Samples were blinded and randomized for downstream multi-omics analysis. Previously published reports of clinical outcomes include GU and GI acute and late effects (2,7,16). Demographics of participants include men of Caucasian, African American, Asian and Hispanic descent, various ethnicities, and age less than 70 years. All protocol-enrolled participants complete informed consent for blood and urine collection and periodic self-reported symptom monitoring. Physical examination and phlebotomy were performed before treatment and follow-up visits (1, 3, 6, 9, and 18 months) after SBRT treatment. Patient inclusion criteria included histologically confirmed adenocarcinoma; Gleason score 2-10; clinical stages T1c-T3c; no clinically or pathologically involved lymph nodes on imaging; no distant metastases on bone scan; measurement of prostate serum antigen (PSA) levels <60 days prior to registration; no history of pelvic radiotherapy, chemotherapy, or radical prostate surgery; no recent (within 5 years) or concurrent cancers and no medical or psychiatric illnesses that would interfere with treatment or follow-up management. Sex as a biological variable was not applicable since this is a

prostate cancer cohort. A power analysis was performed to ensure statistical significance for the proposed analyses. A detailed questionnaire provided annotation of blood samples with a familial cancer history, tobacco use, medication use, occupational history, and socioeconomic status, the 26-item EPIC score (sexual, bowel, and urinary symptoms). Other patient data such as patient de-identifier number, prostatic volume, Gleason's grade, prior hormonal therapy, clinical co-morbidities, age, ethnicity, body mass index etc. were recorded. Blood samples are processed for serum and plasma collection within 4 hours of collection and banked at -80°C .

Proteomic Analysis

Serum samples were analyzed on the proteomic discovery platform described by Gold et al (17). Briefly, this technology uses novel DNA aptamers, which are chemically modified nucleotides, to act as highly specific protein binding reagents, thereby transforming the quantify of each targeted protein into a custom hybridization array. Protein quantities were recorded as relative fluorescent units (RFU), which can be converted to concentrations with standard curves. The samples were batch processed using the SOMAscan Version 3 assays according to the manufacturer's instructions. This assay is commercially available and has been used to investigate other disease systems including lung cancer. In this study, 1129 protein targets were measured in 15 μL of serum for each subject, and all sera were analyzed in a continuous process. For proteomics analysis, the analysts received a blinded data set; the group identities were revealed at the time of analysis. All samples were randomized, normalized and calibrated using standard procedures prior to analysis. The identity of the samples was completely blinded throughout the proteomic analysis process. Pathway analysis was performed using Reactome (18). After data pre-processing and normalization, the proteomics data was log transformed. Unpaired t-tests and linear mixed effect models were used across the study. Benjamini-Hochberg's procedure was applied for multiple testing correction. Statistical analysis was performed by unpaired t-test using R (Version 4.0.2).

Mass Spectrometry Based Metabolomic Profiling: Metabolite extraction was performed using 25 μ L of plasma sample which was mixed with 175 μ L of 40% acetonitrile in 25% methanol and 35% water containing internal standards (10 μ L of debrisoquine (1mg/mL), 50 μ L of 4, nitro-benzoic acid (1mg/mL), 27.3ul of Ceramide (1mg/ml) and 2.5ul of LPA (4mg/ml) in 10 mL). The samples were incubated on ice for 10 minutes and centrifuged at 14,000 rpm at 4⁰C for 20 minutes. The supernatant was transferred to a fresh tube and dried under vacuum. The dried samples were resuspended in 200 μ L of 5% methanol, 1% acetonitrile, 94% Water. Samples were centrifuged again at 13,000 rpm for 20 minutes at 4⁰C and the supernatant was transferred to MS vials for UPLC-ESI-Q-TOF-MS analysis. Each plasma sample (2 μ L) was injected onto a reverse-phase CSH C18 1.7 μ M 2.1x100mm column using an Acquity G2-QTOF system (Waters Corporation, USA). The gradient mobile phase comprised of water containing 0.1% formic acid solution (A), 100% acetonitrile (B) and 10% acetonitrile in IPA containing 0.1% formic acid and 10mm Ammonium format (D). Each sample was resolved for 13 min at a flow rate of 0.5 ml/min for 8 min and then 0.4 ml/min at 8 to 13 min. The G2-QTOF gradient consisted of 98% A and 2% B for 0.5 min then a ramp of curve 6 to 60% B and 40% A from 0.5 min to 4.0 min, then a ramp of curve 6 to 98% B and 2% A from 4.0 to 8.0 min, then a ramp of curve 6 to 5% B and 95% D from 9.0 min to 10.0 min at 0.4ml/min, followed by 98% A and 2% B from 11.0 min to 13 minutes. The column eluent was introduced directly into the mass spectrometer by electrospray. Mass spectrometry was performed on a performed on a Q-TOF instrument (Xevo G2 QTOF, Waters Corp, USA) operating in either negative (ESI-) or positive (ESI+) electrospray ionization mode with a capillary voltage of 3200 V in positive mode and 2800 V in negative mode, and a sampling cone voltage of 30 V in both positive and negative modes. Pooled quality controls were analyzed throughout the batch to assess chromatographic reproducibility and data consistency.

Metabolomic Data Analysis: The untargeted metabolomics data was initially normalized by internal standard. Following data pre-processing and ion annotation, the m/z values of the measured metabolites are normalized with log transformation that stabilizes variance. Differential expression between various patient groups is assessed using t-test constrained by p-value <0.05. Benjamini-Hochberg's procedure was applied for multiple testing correction. Among these differentially expressed metabolites, each m/z value is scored for annotation against the HMDB, Metlin, MMCD and Lipid Maps databases within a 5ppm mass tolerance. The heat maps were generated for the significant metabolites using the log₂ transformed values of fold changes and hierarchically clustered by Pearson correlation. Statistically significant metabolites were validated using tandem mass spectrometry-based fragmentation matching.

Data availability: All raw data in this study are openly available on the Dryad Digital Repository (<https://datadryad.org/stash/share/ea9YhYVkXzR7klQXqmzTWXb0MiONnhq2nZGmiOIGbyc>) and detailed data analysis results are included in this published article (as Supplementary Information Files).

Results

SBRT is a radiation modality that utilizes advanced image-based technology for precise targeting and delivery of hypo-fractionated RT over an interval of 1 to 2 weeks (2). Radiation dose fraction sizes in this study ranged from 6.5 to 7.25 Gy, doses that are approximately three times greater than conventional daily RT fraction sizes. The clinical outcomes of tumor control and radiation late effects using the Accuray CyberKnife, robot-mounted linear accelerator system at the MedStar Georgetown University Hospital (M-GUH) have been previously reported (3,16,19).

Peripheral blood was drawn before the treatment (Pre), after 1 hour, 24-hour, 1 month, 3 months, 6 months, and 12 months (Figure 1, Panel A). Our patient population included 60% Caucasian and 34% African American males. The clinical data for disease burden assessment, including baseline PSA, biopsy Gleason score, and tumor score were used to assign the subjects to risk categories according to

the D'Amico criteria(20). Briefly, patient mean age was 70 years, mean PSA was 8.6 ng/mL and 28% were high risk, 53% intermediate risk and 16% low risk groups (Figure 1, Panel B). Seventeen patients experienced recurrences as defined by PSA progression [18].

Multi-omics based molecular phenotyping analytics were used to characterize the serum samples.

Proteins were analyzed by SomaLogic, Inc., using the SOMAscan Version 3 proteomic assay. Relative distribution of significantly dysregulated pathways was evaluated using Doughnut charts, which showed discreet differences within 24 hours of RT (top) with persistent changes at 1-month (bottom) that included early onset of changes in immune response, interleukin signaling, PI3K activated AKT signaling, and MAPK signaling pathways upon RT (Figure 1, Panel C). Unpaired t-tests were used to determine overall changes in global protein expression at each time point following RT as compared to pre-treatment (baseline) levels. The results summarizing changes in the expression of proteins by comparing pre-RT (baseline) profiles to each time point after radiation (post-RT) across the entire cohort (N=132) are detailed in Supplementary Table S1. Next, Reactome based longitudinal pathway analyses (18) were performed for all significantly changed metabolites using UniProt ID (Supplementary Table S2).

Untargeted metabolomics was performed in a subset of patients classified as low risk, high risk and recurrence patient cohorts (N= 10 each) and further validated through tandem mass spectrometry (MS/MS) for select metabolites (Supplementary Table S3). Unpaired t-tests were conducted to study overall radiation responses (Supplementary Table S4) while linear mixed effects models were used to identify molecular determinants of tumor response using a retrospective clinical outcome analysis (Supplementary Table S5). Hierarchical clustering-based heat map visualization showed distinctive patterns of metabolic abundance in plasma among low, high and recurrence risk groups as scored by

current clinical criteria, suggesting distinct metabolite types that were worthy of further investigations (Figure 1, Panel D).

DNA Damage Response (DDR) and innate immune response as early indicators of clinical outcomes after RT

We used Reactome analysis to interrogate longitudinal proteomics data to gain insight into pathway perturbations following RT. We observed that DDR, cell cycle arrest, and immune response signaling activated within one hour after RT. DDR and cell cycle activation were relatively short-lived and waned by 1-month after RT while immune activation persisted for up to 3 months (Figure 2, Panels A and B). These observations suggest that robustness of the immune signaling response was greater than that of either DDR or cell cycle arrest. Next, we asked if immune response activation correlated with PSA determined tumor recurrence using linear mixed effects models to identify significantly dysregulated proteins. We used “time” as a random effect and “recurrence” as a fixed effect to determine significant differences between recurrence groups adjusted for time for each protein as an outcome measurement (Supplementary Table S6). Examination of patterns of serum protein abundance revealed increased expression of DDR and immune response proteins including interferon gamma, proteasome subunit alpha and ubiquitin-conjugating enzyme among others within an hour of RT in patients that went into remission while the serum abundance remained relatively unchanged (as compared to baseline) in patients experiencing clinical recurrences (Figure 2, Panel C, Supplementary Table S1). The changes in protein expression related to immune response and DDR were found to be statistically significant in the non-recurrence group while these were non-significant in the sub-group that experienced tumor recurrence after RT. These data suggest that initial triggering of immune response, at least in part, impacts tumor response to radiation. The scatter plots (Figure 2, panel C) for the non-recurrence group (blue) and non-recurrence (red) help visualize the spread and variance of serum protein abundance

within and across groups. Interestingly, we observed that the profiles of some patients in the non-recurrence group showed an overlap with the recurrence group, thereby suggesting that these patients are likely to benefit from increased surveillance and clinical follow up. Since radiation damage to DNA and the tumor microenvironment (TME) underlie molecular and cellular processes that induce DDR, arrest cell cycle progression, and activate the immune system, these results are consistent with the concept that DDR and immune response modulate tumor response following RT. Furthermore, it has been reported that redox-activated signaling events are intricately linked to proceeding of immunological processes, IFN-gamma being one of the key proteins that orchestrates that process (21).

Anti-inflammatory metabolite abundance patterns correlate with biochemical recurrences of prostate cancer

It is known that alterations in metabolism fuels and regulates the maturation of immune responses (22). Hence, we analyzed the metabolomic profiles as orthogonal validation of proteomic data and determined the overall metabolomic alterations and segregation of key small molecule metabolites associated with macrophage metabolism by comparing baseline and post-RT samples (Supplementary Figures S1 and S2). We investigated stratification of patient groups (pre-SBRT, post-SBRT 24-hour, 1 month, and 3-months post-RT) using principal component analysis (PCA) (Figure 3, panel A). Macrophages can be classified according to their inflammatory phenotype into proinflammatory M1 and anti-inflammatory M2, which are known to promote an immunoreactive or an immunosuppressive TME, respectively (23-25). Macrophage phenotypes in the TME correlate with aggressiveness in most types of cancers (26,27). M1 and M2 macrophages exhibit distinct metabolic types; for example, M1 macrophages are glycolytic and break down the amino acid arginine to nitric oxide, while the M2 macrophages produce ornithine and uric acid (28). We found that expression markers like ornithine and uric acid that are indicative of metabolic phenotype of the M2 macrophages are upregulated in prostate cancer patients with

progressive disease after RT (Figure 3, Panel B and Supplementary Table S5) supporting the correlation of immunosuppressive response associated metabolites in predicting prostate cancer recurrence. Plasma levels of metabolites including citric acid, ornithine and uric acid (produced by M2 macrophages) were elevated in high-risk and recurrence groups post-RT as compared to the low-risk groups although baseline levels of these metabolites were comparable in all three groups (Figure 3, Panel C). Since the cohort comprised mostly of elderly patients, based on age, major morbidity was cardiac disease. Androgen deprivation therapy was not found to have a statistically significant correlation with clinical outcomes. Taken together, these data suggest that tumor failure following RT, at least in part, may be attributable to an anti-inflammatory immune-metabolic phenotype.

Discussion

More than half of cancer patients receive radiation therapy (RT) either alone or in combination with surgery or chemotherapy during the course of disease. However, biological and molecular mechanisms underlying tumor responses remain to be fully defined. Ionizing radiations induce molecular, cellular, and biological effects by interacting with DNA or by forming free radical species that damage DNA and other cellular components. In normal and transformed cells, biochemical and molecular signals induce expression of DDR genes, protein modifications, activation of metabolic reactions, generation of pattern recognition receptors (PRRs), and induction of cell surface antigens. In turn, the signaling cascades activate cellular pathways (apoptosis, necrosis or autophagy) and the innate immune system to reshape the composition of the TME (29-31).

The overarching goal of this study was to characterize the overall changes in metabolomic and proteomic profiles based on current patient risk stratification criteria and determine if these were informative of clinical outcomes. Hence, we interrogated longitudinal changes in proteomic profiles using functional pathway analyses and found time dependent changes in innate immune response and

DDR pathways. We asked if these changes correlate with tumor response to RT; and further investigated temporal changes in protein expression related to these pathways. We found statistically significant elevation in serum protein abundance of key proteins including IFN gamma, ubiquitin conjugating enzyme and nucleoside diphosphate kinase in patients who remained tumor free during the follow up period suggesting that early triggering of innate immune response may be an indicator of tumor response to RT. These findings also lend credence to the prevalent notion that RT causes DNA damage that activates the C-GAS STING pathway which in turn triggers the innate immune response although to best of our knowledge we are among the first to report that this sequence of molecular events could then drive clinical outcomes in RT (32). Finally, we asked if metabolomic profiles in these patients were indicative of M1 and M2 macrophage metabolic phenotypes, and further if those corroborated with the clinical outcomes of tumor recurrence. We found that patients that experienced tumor recurrence had statistically significant elevation of M2 specific metabolites including ornithine and uric acid although the baseline levels were similar in all patient groups stratified by current clinical classification criteria (33). Taken together, these findings emphasize that the implication of immune-mediated pathophysiology may be an unappreciated component of tumor response to RT and deserves experimental validation. Our translational investigations of radiation therapy of patients with prostate cancers offers a window into the local regional effects on tumors as well as the systemic immune pathway activation by sampling blood. A better understanding of the role of the immune response informs predictive biomarkers and identifies therapeutic targets to enhance the effort to cure these cancers. Here, we applied state-of-the-art molecular studies and big data analysis to gain insight into cancer and host responses to prostate radiation therapy.

We discovered that activation of the DNA damage response (DDR) following tumor irradiation and the subsequent activation of the innate immune response correlates with clinical outcomes in our cohort of

prostate cancer patients. Others have reported similar observations using murine models (34,35). In this study the radiation volumes are relatively consistent across the patient cohort since this is small field radiation with SBRT that precludes large volume treatment. Additionally, clinical outcomes were considered regardless of risk category. Hence irradiation volume and/or risk category are not likely to confound findings from molecular profiling.

Plasma metabolomics helped corroborate findings suggesting immune-metabolic activation may play a critical role in dictating tumoral responses to RT. Several recent publications have reported that the presence of M2 macrophages is directly associated with poor prognosis in cancer, through enhancement of tumor immune-evading mechanisms (26,27,36-38). Additionally, this association can be extended to patients treated with immunotherapy and targeted therapies (39-41). However, the correlation between the M1/M2 macrophage ratio and improved prognosis in cancer has not been described comprehensively in the context of RT. This study represents a stratified case cohort study where tumor response has been assessed based on longitudinal follow up and clinical outcomes. Recently, statistical models for estimating these quantities using time-to-event data from full cohorts have been proposed that take p-value based outcomes into consideration since the study design allows control of false positives since each patient is his own control. Thus, this study design approach is unique and contrasts with a typical case-cohort design. By design, we then chose to analyze proteomics and metabolomics data as a validation approach using a repeated measure generalized mixed-effects models (GMEM) to help capture the intra-individual correlation; test inter-individual differences in patterns of responses over time and allow for inclusion of all available data regardless of data types as dependent or independent variables.

Recent findings have identified a critical role of the TME cellular composition after exposure to ionizing radiation (42). In this context, RT initially triggers activation of proinflammatory, anti-tumor

M1 macrophages, followed by the recruitment of tumor-associated macrophages (TAMs) that predominantly exhibit the M2 phenotype (43-45). Additionally, macrophages are critical modulators of the metabolic landscape in tumors, a key component of cancer aggressiveness (46,47). Therefore, the ratio of antitumoral M1 and pro-tumoral M2 macrophages (M1/M2) have been proposed as a potential biomarker for various malignancies, including prostate cancer (27,48-50). Thus, our work offers an additional perspective and possibilities to identify more accurate and significant markers of responses pre- and post-SBRT. Variations in clinical sensitivities to radiation have been observed in patients with genetic syndromes, mutations in genes associated with DNA repair processes, cell cycle checkpoints and immunological diseases (51).

In summary, our observations are consistent with a model of RT induced DNA damage activating the DNA damage response (DDR) and DNA repair processes. However, the cellular injuries in the irradiated tumor microenvironment (TME) also trigger activation of the innate immune system and recruitment of an orchestrated pool of immune cells. As such, systematic studies of molecular interactions and cellular crosstalk underlying immune responses, DDR, and TME activation by RT are imperative to understanding the biologic basis of radiation response to inform therapeutic strategies for RT-based cancer treatment. Larger clinical studies will be needed to validate our preliminary findings and the potential for using blood analysis to monitor immune responses after RT. However, the potential application of predictive biomarkers of immune responses may inform personalized medicine in radiation oncology; optimizing radiation treatment planning and multidisciplinary therapeutics based on the individual patient's unique biological signatures.

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Author Contributions:

Conceptualization: AKC, AD; Methodology: SG, EJ, MV, AKC, AD; Investigation: SG, AV, KK, RH, GB, AKC, AD; Data analysis: YL; Funding acquisition: SG, AKC; Project administration: SG served as PI of the NIH SBIR Contract to Shuttle Pharmaceuticals, Inc and AKC served as PI of the subcontract to Georgetown University; Supervision: AKC, SG; Writing – original draft: AKC, AD, YL, AV; Writing – review & editing: All authors contributed to review and edits of the manuscript.

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Figure Legends:

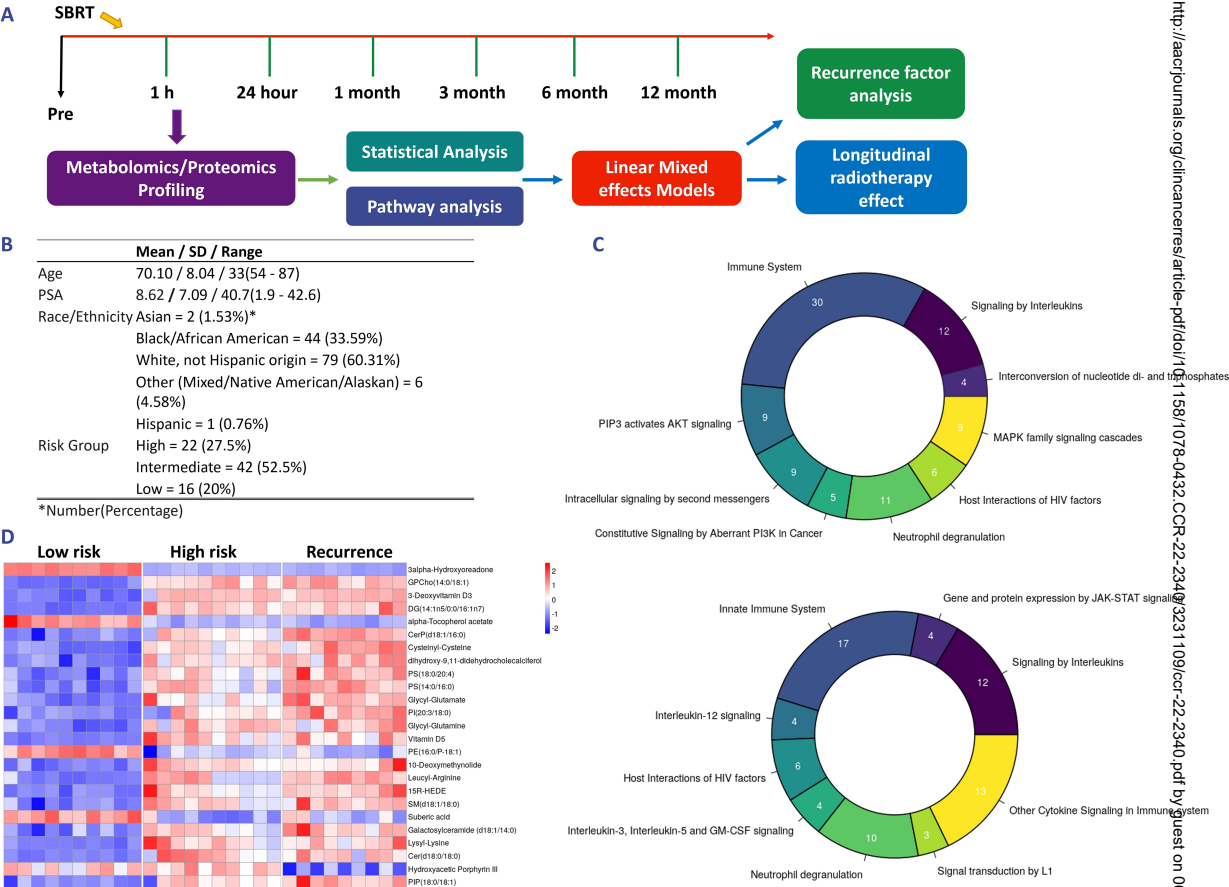
Figure 1. Summary of main findings showing that radiation therapy (RT) induces robust molecular alterations that modulate tumor response in prostate cancer. Panel A. Overall study design. Enrolled patients (N=132) were diagnosed with prostate cancer and elected SBRT radiation treatment. Patient plasma samples were obtained before treatment (pre-RT), after 1 hour, 24-hours, 1-, 3-, 6-, and 12-months post-RT. Tumor response to RT was characterized by performing multi-omics analyses (metabolomics, lipidomics and proteomics) of patient plasma samples. **Panel B.** Clinical characteristics of the prostate cancer cohort that received RT for treatment of prostate cancer. **Panel C.** Doughnut chart with proportions of significantly dysregulated proteomics pathway, based on the number of entities found in each pathway showed discreet differences in the 24 hours vs. baseline (top) and 1-month vs. baseline (bottom) groups. Seventeen patients experienced recurrence episodes. **Panel D.** Hierarchical clustering-based heat map visualization of metabolite patterns that segregate prostate cancer patients based on clinical risk group.

Figure 2. Radiation induced early immune response is associated with tumor response in prostate cancer. Panel A. Reactome based longitudinal pathway analysis of the plasma proteomics data set. A robust immune signaling response was observed within an hour of SBRT and the duration of signaling extended longer than DDR or cell cycle signaling. Analysis of selected markers of immune response shows a more muted innate immune response in patients with cancer recurrence. **Panel B.** Reactome based pathway analysis shows activation of DDR and cell cycle within 1 hour after RT, attenuation of the response by 24 hours and return to pre-RT baseline by 1 month. **Panel C.** Trend lines showing differential pattern of protein expression changes over time for immune response (sub-panels i - iv), DDR and cell cycle (sub-panels iv - viii), in patients undergoing remission (blue) as compares to the recurrence group (red). Proteins showing a statistically significant change are marked with an asterisk*.

Figure 3. Metabolomic profiles can segregate PC patients based on recurrence. Panel A. 2D-PCA for metabolomics data showing separation for Pre, 24-hour, 1 month, and 3-month samples. **Panel B.** Linear mixed effects models for longitudinal metabolomics using “time” as a random effect and tumor recurrence as a fixed effect, to see are there any significant differences between Recurrence groups adjusted for time for each protein as outcome measurement. Metabolites associated with immune response are significantly dysregulated. **Panel C.** Box plots showing metabolites associated with M2 macrophage phenotype are upregulated in patients that experienced recurrence.

Fig. 1. Summary of main findings showing that radiation therapy (RT) induces robust molecular alterations that modulate tumor response in prostate cancer.

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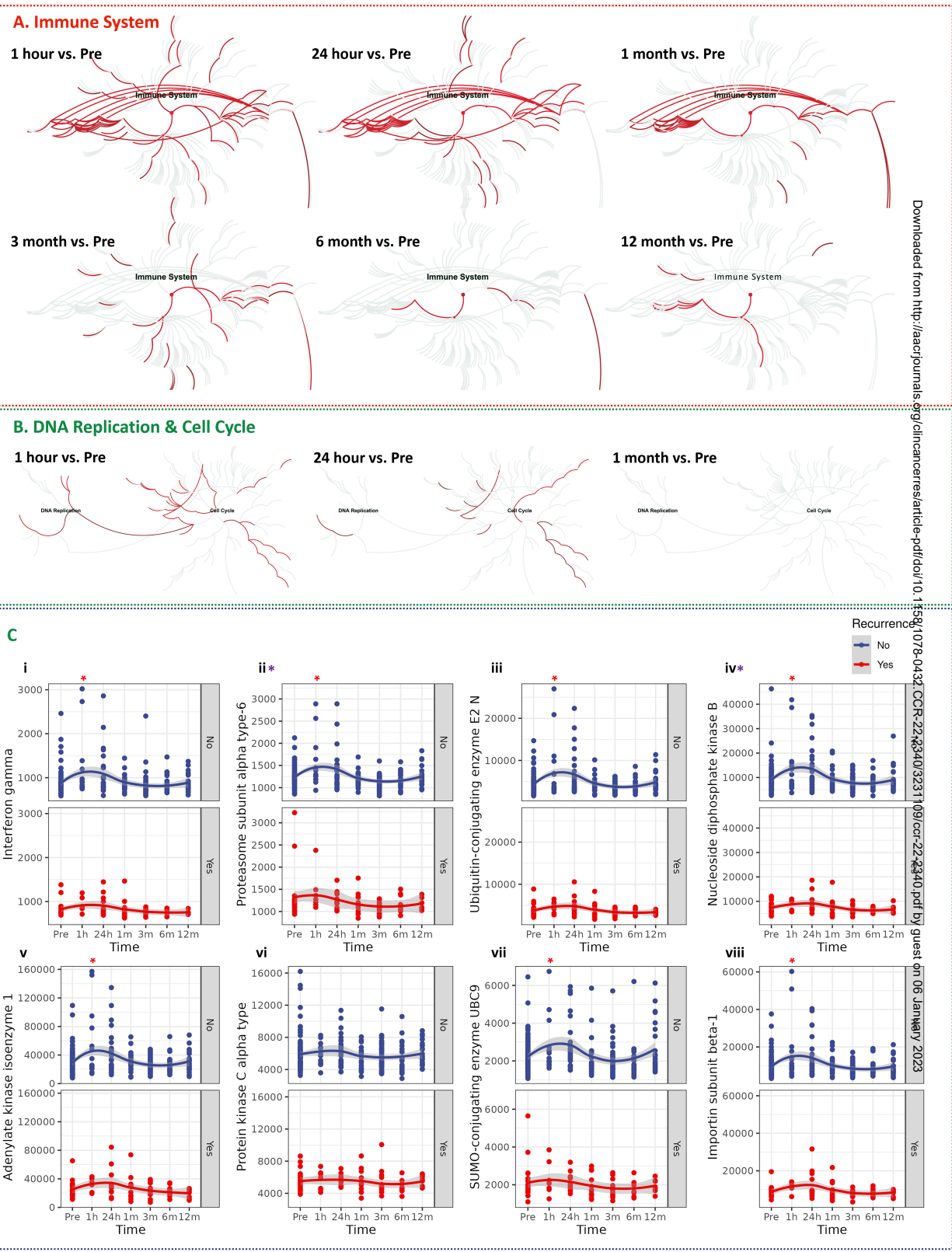


Fig. 2. Radiation induced early immune response is associated with tumor response in prostate cancer.

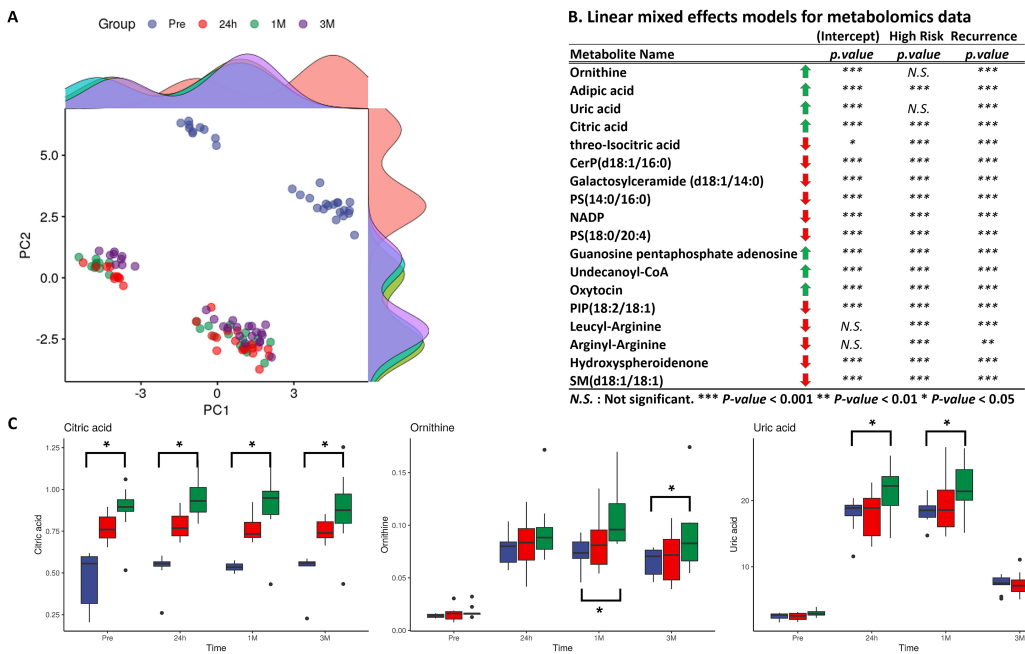


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