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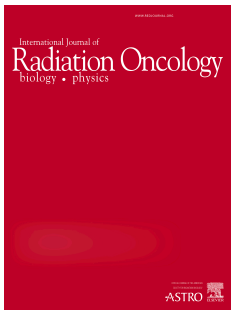

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Intratumoural hydrogen peroxide with radiotherapy in locally advanced breast cancer: results from a Phase I clinical trial

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Running title

Intratumoural hydrogen peroxide with radiotherapy

Additional information

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Data Availability

All data generated and analysed during this study are included in this article (and its supplementary files).

Conflicts of interest

The authors declare no potential conflicts of interest.

Abstract

Purpose

Hydrogen peroxide (H_2O_2) plays a vital role in normal cellular processes but at supraphysiological concentrations causes oxidative stress and cytotoxicity, a property that is potentially exploitable for the treatment of cancer in combination with radiotherapy (RT). We report the first Phase I trial testing the safety and tolerability of intratumoural H_2O_2 + external beam RT as a novel combination in patients with breast cancer, and exploratory plasma marker analyses investigating possible mechanisms of action.

Methods and Materials

12 patients with breast tumours ≥3 cm (surgically or medically inoperable) received intratumoural H_2O_2 with either 36 Gy in 6 twice-weekly fractions (n=6) or 49.5 Gy in 18 daily fractions (n=6) to the whole breast +/- loco-regional lymph nodes in a single-centre, non-randomised study. H_2O_2 was mixed in 1% sodium hyaluronate gel (final H_2O_2 concentration 0.5%) before administration to slow drug release and minimise local discomfort. The mixture was injected intratumourally under ultrasound guidance twice-weekly 1h prior to RT. The primary endpoint was patient-reported maximum intratumoural pain intensity before and 24h post-injection. Secondary endpoints included ≥grade 3 skin toxicity and tumour response by ultrasound. Blood samples were collected before, during and at the end of treatment for cell-death and immune marker analysis.

Results

Compliance with H_2O_2 and RT was 100%. 5/12 patients reported moderate pain following injection (grade 2 CTCAE v4.02) with median duration 60min (interquartile range 20-

120min). Skin toxicity was comparable to RT alone, with maintained partial/complete tumour response relative to baseline in 11/12 patients at last follow-up (median 12 months). Blood marker analysis highlighted significant associations of TRAIL, IL-1β, IL-4 and MIP-1α with tumour response.

Conclusions

Intratumoural H_2O_2 with RT is well-tolerated with no additional toxicity compared to RT alone. If efficacy is confirmed in a randomised Phase II trial, the approach has potential as a cost-effective radiation response enhancer in multiple cancer types where locoregional control after RT alone remains poor.

Keywords
Hydrogen Peroxide, Breast Cancer, Radiotherapy, Radiotherapy drug combinations

Introduction

Breast cancer presents a global challenge, with an estimated incidence of 2 million worldwide, 80% of whom present with locally advanced disease (1,2). In the UK, where women with locally advanced disease represent a minority (7-13%) of the 55,000 new patient presentations, the lifetime morbidity of progressive local disease is significant (3-6). Treatment is challenging in frail or elderly individuals who are unfit for or refuse surgery, and for whom RT +/- endocrine therapy is often the most appropriate option for relief of breast ulceration, bleeding, and pain. Locally advanced inoperable primary or recurrent cancers infiltrating the breast/chest wall and/or axilla, with or without metastases, are typically associated with life expectancies measured in years rather than months and present significant challenges to patients and medical professionals. This represents an area of unmet clinical need, where innovative approaches to enhance response to radiation would be highly beneficial.

An interaction at a cellular level between H_2O_2 and ionising radiation (IR) was first reported in osteosarcoma (HS-Os-1) and prostate cancer (PC-3) cell lines, which demonstrated extreme resistance to either H_2O_2 or 30 Gy alone (7,8). The addition of 0.1 mM H_2O_2 prior to IR resulted in enhanced cytotoxicity without causing DNA double strand breaks that classically mediate cell killing (9,10). A novel mechanism was postulated to involve lysosomal membrane rupture with release of powerful oxidants, including heavy metal ions that permeabilise mitochondria and activate apoptosis (11). *In vivo* use involved a mixture of 0.5% H_2O_2 in 0.83% sodium hyaluronate gel, the Kochi Oxydol-Radiation Therapy for Unresectable Carcinomas (KORTUC) strategy designed to minimise local pain at the

injection site. Intratumoural injection of this H_2O_2 gel mixture into murine tumours prior to 30 Gy IR demonstrated clear evidence of growth delay above that achieved by either modality alone. No toxicity was noted (12).

In this study we report the first systematically conducted Phase I trial testing intratumoural H_2O_2 in combination with RT in locally advanced breast cancer (NCT02757651). The primary objective was assessment of safety and tolerability of H_2O_2 injections with moderately hypofractionated RT. Secondary endpoints included the proportion of patients requiring additional pain medication, incidence of ≥ grade 3 skin toxicity, and tumour response assessment. Exploratory analysis of plasma markers was also performed.

Materials and Methods

Study design

This non-randomised study involved patients with locally advanced or locally recurrent breast cancer (with or without metastases) in whom RT was indicated for loco-regional disease control. Patients were either inoperable due to comorbidities or local disease extent, or else surgery to the breast primary was not appropriate due to presence of metastatic disease.

The single centre study was conducted at XXX (CCR4502). Approval by the Research Ethics Committee (REC) and the Medicines and Healthcare products Regulatory Agency (MHRA) was obtained prior to trial commencement (IRAS 203161, REC 16/LO/1566, EudraCT 2016-

000833-40). Monitoring was undertaken by the Clinical Trials Unit at XXX. The trial schema is shown in Figure 1.

Eligible patients were over 18 years of age, had histologically confirmed breast cancer, and required breast RT for local control and/or palliation of loco-regional symptoms. They had at least one breast tumour measuring ≥3 cm in diameter in a superficial location accessible for injection. Any combination of oestrogen receptor (ER), progesterone receptor (PR) and HER2 expression was allowed. Exclusion criteria included prior RT to the breast and concomitant biological therapies other than trastuzumab, pertuzumab and denosumab. Pregnancy was excluded in female patients of child-bearing age. Patients were excluded if the anatomical location of the breast tumour, such as proximity to blood vessels or the brachial plexus, precluded safe access for intratumoural injection. This precaution minimised the risk of injection into a blood vessel causing embolism, an adverse effect that this has not been described in the literature in relation to intratumoural H_2O_2 (13,14).

Drug formulation

A slow release 0.5% H_2O_2 solution was created by mixing 0.4 ml of 3% H_2O_2 (2.0 ml sterile ampoules supplied by Stockport Pharmaceuticals, UK) with 2.0 ml OSTENIL® (20 mg sodium hyaluronate in a 2.0 ml pre-loaded syringe provided by AAH Pharmaceuticals, UK), the latter licensed for intra-articular injection of arthritic joints (15). The low molecular weight of H_2O_2 (34 g/mol) ensures rapid equilibration of drug within the gel. The mixture is a colourless, viscous solution (pH 6.8-7.8) stored at room temperature and stable for 2 hours following preparation, as determined by viscosity measurements (performed by Stockport

Pharmaceuticals, UK). The gel allows slow release of H_2O_2 over at least 24 hours, as evidenced by generation of oxygen microbubbles upon injection, a feature that provides a strong rationale for twice-weekly administration during RT (16). In the trial, the drug and gel were mixed under aseptic conditions using two syringes connected via a 2-way tap. Once made up, each syringe contained 2.4 ml of 0.5% H_2O_2, the contents of both syringes typically needed for tumours measuring 30-60 mm in diameter.

Radiotherapy

Six patients received 49.5 Gy in 18 daily fractions of 2.75 Gy, and 6 were treated with 36 Gy in 6 twice-weekly fractions of 6 Gy to the whole breast ± locoregional lymph nodes. The equivalent RT dose expressed in conventional 2 Gy fractions (EQD_2) was 57 Gy and 65 Gy for these two schedules respectively (Figure S1). Patients on the 6 Gy twice-weekly schedule, requiring lymph node irradiation in addition to the breast, were treated to a total dose of 30 Gy in 5 twice-weekly fractions of 6 Gy to the nodal regions, in order not to exceed brachial plexus tolerance dose (as per standard institutional guidelines).

The RT schedule was selected according to the patient's performance status and comorbidities, with fitter patients selected for the daily treatment schedule. RT was delivered using a linear accelerator, with 6-10 MV photons, and 3D-planned using data from a CT planning scan, and using standard tangentially opposed fields. Patients were simulated and treated in the supine position on a breast board with both arms abducted. The clinical target volume (CTV) comprised the entire ipsilateral breast, including the deep fascia, but excluding underlying muscle or overlying skin (when not involved with disease). The RT dose

was prescribed to the 100% isodose, ensuring the target volume was within the 95%-107% isodose lines. Organs at risk including the heart, lung and contralateral breast were outlined and standard guidelines for dose tolerances followed. A standard treatment verification protocol was used, consisting of daily imaging for the first 3 days, and subsequent weekly imaging. In cases where there was skin involvement by tumour, treatment included 5 mm wax bolus throughout RT to maximise dose to skin, in keeping with standard practice. In patients treated with 49.5 Gy in 18 fractions, a sequential boost dose to the tumour bed (13.35 Gy in 5 daily fractions using mini-tangential opposed beams or a directly applied electron beam) was allowed, but this needed to be declared at time of trial entry. A tumour bed boost dose increased the EQD_2 to that comparable with 36 Gy in 6 fractions and with dose intensities previously reported in earlier patient cohorts treated with the same drug preparation (17,18).

Intratumoural injections of H_2O_2 in sodium hyaluronate gel

Transdermal intratumoural KORTUC injections were administered twice-weekly commencing in the second calendar week of RT. Each patient received 4 to 6 doses in total (median = 5 injections), the smaller number given to patients prescribed 6 fractions. The rationale for starting KORTUC in the second week was to allow for reduction in tumour interstitial pressure during the first week of RT, enabling technically easier and more tolerable injections for the patient. Injections were performed (23-gauge needle) under ultrasound (US) guidance by a trained radiologist or radiographer after 0.5% lignocaine injection to anaesthetise the skin. For tumours measuring 30-60 mm in size, two syringes (4.8 ml) of 0.5% H_2O_2 in sodium hyaluronate gel was injected at each time point. Three

syringes (7.2 ml) were required for tumours >60 mm in size. Uniform and accurate delivery under US guidance via 2-3 differently angled needle tracks was aided by the immediate appearance of oxygen microbubbles as H_2O_2 degraded to oxygen and water within the tumour (see Figure 2A). The needle tip was positioned at the deepest aspect of the tumour and the gel released slowly whilst withdrawing the needle towards the surface. For smaller tumours, it was possible to achieve even distribution of the H_2O_2 gel mixture within the tumour via a single skin puncture site and by altering the angle of the needle (working from left to right or top to bottom within the tumour). For some larger tumours (for example > 60 mm) it was necessary to inject the tumour via more than one skin entry point from different directions to ensure even distribution of oxygen microbubbles throughout the tumour volume. The number of needle tracks within the tumour and skin entry points were decided by the radiologist during the ultrasound scan, and guided by the extent and distribution of oxygen microbubbles during the injection procedure. If any gel tracked back to the skin surface upon withdrawal of the needle it was promptly wiped away with sterile gauze. If patients had >1 distinct tumour in the breast/axilla, the clinician/radiologist was required to clearly document the injected lesion (usually the largest) to aid response assessment. RT was delivered within 1-2 hours after H_2O_2 injection.

Treatment monitoring

Within each RT group (daily or twice-weekly fractions), a minimum gap of 1 week was stipulated between the first and second patient, during which acute toxicity data associated with intratumoural injections (pain, skin toxicity, and tumour lysis) was reviewed by an Independent Data Monitoring Committee (IDMC). Based on predetermined criteria, the

second and third patients in each group and subsequently the fourth, fifth and sixth patients in each group were allowed to be treated concomitantly.

Primary endpoint

This related to the timing, severity and duration of pain post-injection recorded via a self-reported questionnaire completed by patients at home. An 11-point numerical scale ranging from 0 ("no pain") to 10 ("worst possible pain") recorded severity and duration prior to and over 24 hours after each H_2O_2 injection (Figure S1). Patient-reported scores were used to calculate i) the proportion of patients with pain scores ≥5 points above baseline after any of the intratumoural injections, and ii) the requirement for additional pain medication.

Secondary endpoints

Secondary endpoints included acute RT-induced skin toxicity, serum biochemistry and tumour response. Skin toxicity was assessed weekly in all patients during and for 4 weeks after RT by a member of the clinical team. Standardised proformas recorded the degree of erythema and desquamation of the skin of the breast. In each of the RT groups, if $no\ more\ than$ one of the first 3 patients had a persistent CTCAE (v4.02) skin toxicity ≥ grade 3 six weeks after RT, the IDMC allowed recruitment to continue for a further 3 patients within that RT schedule. If moist desquamation was seen beyond skin folds, weekly assessments were continued until severity reduced to ≤ grade 1. The proportion of patients with ≥ grade 3 skin toxicity at any time from the start of RT to 4 weeks post-RT, and the worst grade of skin toxicity reported from the start of RT to 4 weeks post RT were recorded

in these cases. However it was recognised that if cancer infiltrated skin, patients would typically experience ≥ grade 3 skin toxicity after RT alone.

In every patient, routine biochemistry including serum potassium, calcium and uric acid were measured 2 days after the first H_2O_2 dose to rule out tumour lysis prior to proceeding with subsequent doses (19).

Tumour response was assessed at 3, 6, and 12 months post-treatment. At each timepoint 3-dimensional US measurements were obtained and the tumour volume calculated on the assumption that breast tumours assume a hemi-ellipsoid shape, as previously demonstrated (20). Maximum tumour dimension alone was not considered an accurate representation of tumour response especially when tumours 'flatten' following radiotherapy. Tumour volumes were compared against pre-treatment measurements applying RECIST-like principles, with complete response (CR) defined as disappearance of the target lesion, partial response (PR) as at least a 30% reduction in tumour volume, and stable disease (SD) as less than a 30% reduction or 20% increase in tumour volume.

Statistical considerations

Based on the previously published data, 30-100% patients experienced pain described as no worse than 'mild' (or CTCAE grade 1) for several hours following injection (17,21-23). A single case of tumour lysis syndrome (mild) was reported in a total of 139 breast cancer patients in the Japanese literature. Given this knowledge of the safety of H_2O_2 plus RT, the

Phase I trial required 12 patients to be recruited. Patients treated with once-daily and twice-weekly fractions of RT were analysed as a single stratum and the study population defined as all patients who registered for the trial and received at least one dose of intratumoural H_2O_2. Tumour volumes were calculated using 3-dimensional measurements obtained from US scans.

Plasma Markers

Blood samples were obtained pre-RT and at the end of the first and last weeks of RT. Blood (two 9 ml K3-EDTA tubes) was obtained by venepuncture and processed within 30 minutes. Plasma and buffy coat were isolated by centrifugation at 1600 x g for 10 minutes at room temperature and frozen in aliquots at -80°C. All the plasma samples subsequently underwent one freeze-thaw cycle prior to assay.

ELISA and Luminex Assay

Frozen plasma samples were thawed on ice and brought to room temperature. They were spun at 10000 x g for 10 minutes and the plasma supernatant assayed either by ELISA or Luminex assay as per manufacturers' protocols. Results were measured against a normal plasma control obtained from a healthy donor (Cambridge BioScience Ltd). The absorbance for ELISA was obtained using a POLAR star Omega plate reader spectrophotometer (BMG LABTECH). The Luminex Human XL Cytokine Discovery Panel (15-plex, #FCSTM18-15, R&D System) was carried out with a minimum of 100 events per bead using a Luminex 200 system with xPONENT v3.1 software (Millipore). The plasma samples were assayed in duplicate wells for individual targets at their corresponding time-points (pre-RT, end of first

week of -RT and post-H_2O_2 + RT) and the log2 fold change calculated. Further information regarding the ELISA and Luminex analytes, their dilutions and kit details used in this study are provided in Table S1.

Exploratory translational endpoints

Exploratory analyses of plasma biomarkers of cell death, inflammation, and immune response were conducted to test feasibility of investigating novel mechanisms of action and biomarkers of response in trial patients. A linear mixed effect model was used to model the random effect of longitudinal data that the markers generated (24). The model was built to study the effect of each marker, and to quantify its significance in terms of association with tumour shrinkage over time. The difference between individual and temporal variability was treated as random effect. The impact of each marker was regarded as a fixed effect. The model used was:

Tumour_volume = marker + time + marker: time + random_effect (time)

The ':' colon sign denotes the variation of each plasma marker with time. Each marker was used to fit a mixed model individually with all patient samples and 20 models were fitted in total. A p-value for the coefficient of each marker was calculated to indicate whether the fixed effect had significance (at the 5% level). All graphs were generated using Graphpad Prism v8.1 (Mac OS), Graphpad Software, La Jolla California USA.

Results

Patient characteristics

Patient demographics, tumour characteristics, prior treatment and RT target volumes are summarised in Table 1. 13 patients (11 female, 2 male) were recruited to the study between February 2017 and August 2018. All patients had locally advanced or recurrent breast cancer, and were inoperable due to co-morbidities, local extent of disease or metastatic disease. One patient withdrew due to clinical deterioration unrelated to the trial before starting RT and received no H_2O_2, so an additional (13^{th}) patient was recruited. Median age was 77 years (range 45-93). Three patients were wheelchair-bound due to co-morbidities and frailty. Breast tumour stage was T2 in 5/12 patients and T4 in 7/12 patients. 6/12 had N0 and 6/12 N1 disease (axillary node involvement). 8/12 patients had distant metastases. Breast tumour size varied from 30 mm to 164 mm (maximum dimension). 10 patients had ER+/HER2- disease and 2 had triple negative disease. There were no patients with inflammatory breast cancer. All patients had received 1-4 previous lines of treatment for their breast cancer, and the majority had progressed on prior systemic treatment. Three patients had prior surgery for breast cancer, but had locally recurrent disease. During RT, 7/12 patients continued taking concurrent endocrine therapy and 2/12 continued bisphosphonate therapy for metastatic bone disease.

Compliance with treatment protocol and follow-up

Compliance with H_2O_2 injections was 100% in all patients, including 1 with needle phobia. All patients received RT within the prescribed 1-2 hours of receiving the H_2O_2 injection, with a

single exception of a patient given 1 RT fraction before H_2O_2 injection in error. Results are reported at a minimum follow-up of 12 months for all patients alive at the time of reporting (range 2-24 months). 11 patients completed 12 months follow up, and the twelfth died of rapidly progressive metastatic disease just under 2 months following RT.

Primary endpoint

The pain scores are summarised in Table 2, with respective grades detailed in Figure S1 (iii). 3/12 patients experienced grade 1 (mild) tumour pain post injection, and 5/12 experienced grade 2 pain (moderate severity, limiting activities of daily living) as per CTCAE v4.02 (25). The remainder did not report any additional pain following intratumoural injection. Median pain duration was 60 minutes with an inter-quartile range of 20-120 minutes.

4/12 patients reported pain ≥5 points above baseline during treatment. One patient was taking opiate analgesia (oral morphine) prior to starting radiotherapy to control pain resulting from a fungating breast tumour. 6/12 patients required additional analgesia to manage their symptoms (paracetamol and codeine-based). In these cases, management included ensuring compliance with pre-existing painkillers and optimising analgesia +/- anxiolytics for the remainder of their treatment.

Secondary endpoints

Skin toxicity and tumour lysis

The highest grade of skin toxicity reported was grade 3 in 5/12, grade 2 in 4/12 patients, grade 1 in 1/12, and grade 0 in 2/12 patients (Table 2 and Figure S1 (iv)). All 5 patients who experienced grade 3 skin toxicity had been treated with bolus during radiotherapy (due to skin involvement by tumour). There was no suggestion of enhancement of erythema due to local leakage of H_2O_2. The acute radiation skin toxicity observed in the trial was comparable to that expected with standard RT alone, including in patients with cancer infiltrating overlying skin (26). There were no cases of tumour lysis syndrome.

Tumour response

Figure 2B (i) and Table S2 detail the tumour response based on US measurements at successive time-points post-treatment. At the last imaging assessment percentage tumour volume reduction was between 50 and 100% as shown in Figure 2B (ii). All evaluable patients in this study maintained loco-regional control in the irradiated target lesion at last clinical follow-up (median 12 months, range 2-24 months). Patient 12 died of metastatic disease 6 weeks after RT and was not evaluable at the 3-month endpoint for tumour response.

As an illustrative example Figure 2C shows tumour extent in patient 10 pre-RT and 12 months post-treatment (patient maintained CR at 18 months). Only 1/12 patients had >1 distinct tumour lesions within the RT treatment volume. In this patient only the tumour injected with H_2O_2 showed maintained PR at 12 months, whereas the 2 lesions receiving the same RT alone showed stable disease (the non-injected lesions acting as internal controls). With regards to tumour response assessment, there were discrepancies in 2 patients

between US and clinical response assessments (Table S2). In patient 9, US measurements between 6 and 12 months suggested an increase in tumour size despite an excellent partial response on clinical examination. Radiology review of the US images at 12 months post-treatment indicated changes consistent with fibrosis rather than active tumour. Similarly, patient 8 demonstrated a complete response on clinical assessment at 12 months, despite the presence of stable measurable disease on US. A staging PET/CT scan performed concurrently confirmed complete metabolic response in the H_2O_2 + RT-treated breast tumour, as shown in Figure 2D.

Exploratory secondary endpoint (post-hoc analysis)

10/12 patients consented to provide blood for research at the time points shown in Figure 3A. The exploratory target panel for ELISA and Luminex assays comprised 21 markers involved in cell death, the immune checkpoint, chemo-attraction, immune regulation and angiogenesis (Table S3). Log2 transformed fold change of targets normalised to their baseline (pre-RT) expression were plotted for all patients, comparing levels after RT alone (at the end of 1st week of treatment) and after H_2O_2 + RT (end of treatment) (Figure 3B and Figure S3). There was no consistent trend when comparing RT alone versus H_2O_2 + RT in this small exploratory cohort. However, upregulation of markers involved in inflammation, immune modulation and Damage Associated Molecular Patterns (DAMPs) was noted (27). Application of a linear mixed effect model identified four significant ($p<0.05$) associations with tumour shrinkage, suggesting TRAIL mediated apoptosis with increased activated T-cell signaling (IL-4, MIP-1α) and macrophage stimulation (IL-1β) (Figure 3C and Table S4).

Discussion

This Phase I study raised no concerns relating to local or systemic toxicity when intratumoural H_2O_2 is delivered with RT doses per fraction up to 6 Gy in patients with locally advanced primary or recurrent breast cancer unsuitable for primary surgery or palliative debulking. The intervention is well tolerated even by the frail, older patients and those with needle phobia. In those patients with pre-existing pain symptoms, it was important and straightforward to optimise pain medication prior to starting treatment.

Commencing H_2O_2 injections in the second calendar week of radiotherapy ensured that there were no technical challenges (i.e. resistance due to tissue turgor) to injecting the prescribed volume of drug in any of the patients. Given that H_2O_2 breaks down to O_2 within the tumour (2 molecules of H_2O_2 degrade to 1 molecule of O_2 and 2 molecules of water), it is hypothesised that this may contribute to reoxygenation of hypoxic areas, thereby alleviating radiation resistance. Therefore, aside from the classical DNA damage effects, another mechanism of synergy between H_2O_2 and RT may result from reoxygenation. This is currently being investigated in the laboratory setting. In tumours that do not reoxygenate spontaneously during fractionated RT, H_2O_2 is expected to be most effective after the second week of RT, where such tumours are likely to be enriched with hypoxic radioresistant subpopulations (28-30) .

Acute skin toxicity was no different to that expected after the same RT alone. As predicted, grade 3 radiation dermatitis occurred only in those patients with tumour involving skin, when

a 5 mm layer of wax 'blanket' ensured 100% prescribed RT dose to skin instead of approximately 70% of prescribed dose in patients without skin involvement (31). Grade 3 skin desquamation managed with standard supportive measures including barrier creams and dressings ensured complete resolution of symptoms in every case. Overall, toxicity and tolerability were entirely consistent with extant literature, and these Phase I data will contribute to an application for regulatory approval if the planned randomised Phase II study confirms efficacy.

In view of the limitations of US in response assessment, MRI has been selected as the imaging modality to monitor tumour response in the forthcoming Phase II trial. In patients with locally advanced breast cancers treated with radiotherapy alone at equivalent doses to those used in this study, local control rates would be expected to be 45-57% at 3 years post-treatment, with lower rates associated with larger tumours (32,33). Although it is impossible to draw conclusions on efficacy in this Phase I trial, anecdotal tumour responses are suggestive of enhanced anti-tumour effect. Since lifetime control of symptomatic locally advanced breast cancer is a major determinant of patient quality of life as well as survivorship, there is potential for an effective treatment to be globally beneficial, where women with inoperable breast cancer often have limited access to effective treatment (34,35). Intratumoural H_2O_2 injections are inexpensive and easy to administer, requiring minimal additional training and infrastructure.

Our study has also established that circulating plasma markers can be successfully quantified using the ELISA and Luminex platforms, providing insights into mechanisms of cell

death following treatment. IR and H_2O_2 induce ROS, inflammatory signaling, DNA damage, senescence and cell death. In addition, IR can modulate the immuno-inflammatory axis through the generation of ROS and DAMPs (36). The wide range of potential mechanisms of interaction informed the choice of 21 markers in our exploratory panel.

The effect of H_2O_2 within the cell is concentration dependent; having a role in signalling and homeostasis at nanomolar concentrations (nM) and triggering cell death at supraphysiological (mM) concentrations (37). The physiological outcome within the cell is modulated by antioxidant enzymes such as catalases, peroxidases and thioredoxin-linked systems (38). By affecting protein kinases and phosphatases, H_2O_2 influences a number of signaling cascades including ERK, JNK, MAPK, p38, TNFα, NFκB, IL-1β, IL-6, IL-8, MCP-1 and MIP (37,39,40). Several publications have demonstrated apoptosis as the principal mode of cell death following H_2O_2 treatment (11,41-43). The intrinsic mitochondrial pathway is thought to be the predominant mechanism of apoptosis (44). One study reported apoptosis induction following exposure to H_2O_2 levels <0.4 mM and upregulation of RIP, a gene associated with necrosis, at higher concentrations (45). Exposure to H_2O_2 can result in increased expression of inflammatory cytokines (46). IL-1 is a key mediator of T-cell and dendritic cell (DC) function. Increased IL-1α levels occur in cells undergoing necrosis, whereas IL-1β signals towards apoptosis (47,48). Another study reported that treating murine splenic T-cells with H_2O_2 resulted in a significant increase in IL-4 production, a key regulator of humoral and adaptive immunity (49). Both IL-1β and IL-4 were significantly associated with tumour shrinkage in our study.

In our plasma analysis, a significant association between TRAIL and tumour shrinkage was also found. Intracellular ROS such as H_2O_2 are thought to mediate apoptosis via death receptor ligands such as TRAIL (44). A study in an astroglial cell line demonstrated an increase in TRAIL gene expression in cells treated with H_2O_2 in a dose-dependent manner up to a concentration of 0.8 mM (50). TRAIL-dependent apoptosis regulates the priming of $CD8^+$ memory T-cells by $CD4^+$ T_H1 cells (51). In a study using a murine macrophage cell line (B10R), exposure to H_2O_2 increased the transcription of the chemokines MIP-1α, MIP-1β, MIP-2 and MCP-1 (52). MIP-1β was undetectable in 7/10 patients but MIP-1α expression was detected to a varying degree in all cases, suggesting activation of $CD8^+$ T lymphocytes. In summary, the analyisis of blood biomarkers showed correlations with clinical tumour response and suggest an inflammatory/immune response associated with apoptotic cell death. These mechanisms of action are potentially relevant to an interaction between IR and drug. However, in this cohort, all of whom received H_2O_2 + RT, it is difficult to distinguish the contribution of H_2O_2 over and above that of RT alone. The plasma analyses have been valuable in informing the selection of markers to investigate in a subsequent trial.

In conclusion, the results from this Phase I trial confirm intratumoural H_2O_2 in combination with RT is a safe and simple intervention with the potential for high global impact if efficacy is confirmed in the forthcoming randomised Phase II trial. Proof of concept in breast cancer could lead to rapid evaluation in other challenging and accessible primary sites, including cancers of the head and neck, cervix uteri and soft tissue sarcomas, where loco-regional control with RT alone is poor.

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Figure Legends

Figure 1. Phase I trial schema

A non-randomised study design testing intratumoural H_2O_2 in sodium hyaluronate gel in combination with two radiotherapy fractionation schedules in patients with locally advanced breast cancer, and corresponding follow up schedule. #- radiotherapy fraction; US- ultrasound; RT- radiotherapy.

Figure 2. Intratumoural H_2O_2 administration and tumour response

(**A**) Sequence of ultrasound images of a breast tumour showing H_2O_2 administration, red arrow indicating (**i**) Needle entering under ultrasound guidance (**ii**) H_2O_2 + sodium hyaluronate gel mixture being injected intratumourally (**iii**) Breakdown of H_2O_2 with formation of echogenic oxygen microbubbles (white) within the tumour (**B**) Tumour volume changes (**i**) Box plot showing the cumulative fold decrease (log_2 transformed) for all 12 patients, at the indicated time points post-RT (**ii**) waterfall plot showing % tumour volume change up to 12 months post-RT normalized to baseline tumour measurement (data represent tumour measurements at 9 and 12 months post-RT for 3 and 8 patients respectively). (**C**) Clinical photographs of patient 10 (**i**) Left breast with fungating tumour (baseline) (**ii**) 12 months post-treatment with H_2O_2 + RT. (**D**) ^{18}F-FDG PET scans of patient 8 (**i**) High tracer uptake in left breast tumour at baseline (**ii**) Complete metabolic response at 12 months post-treatment.

Figure 3. Analysis of Phase I trial plasma markers

(**A**) Scheme showing clinical imaging and blood sampling performed in this study. #-radiotherapy fraction (**B**) Box & Whisker plot depicting log_2 transformed fold change of analysed targets from individual patients. Values are normalized to their baseline expression for all 21 targets. (**C**) Plot shows the significant markers associated with tumour volume shrinkage, with multi-comparison adjusted p-value < 0.05. The bar shows the fixed effect of each marker with error bars, ranked by p values. A negative coefficient means that the marker is positively correlated with tumour shrinkage.

Table Legends

Table 1. Summary of patient demographics, tumour characteristics, previous lines of treatment and RT treatment volumes. Patients 1, 3, 4, 5, 11 and 13 received 36Gy/6 fractions, and 2, 6, 8, 9, 10 and 12 received 49.5Gy/18 fractions. *Performance status (Eastern Cooperative Oncology Group)

Table 2. Summary of pain scores and RT acute skin toxicity scores

Pain intensity scored from 0-10 via patient self-assessment questionnaires (median calculated from difference in pain score pre- and post-H_2O_2 injection for each patient throughout treatment course (4-6 injections in total for each patient)); RT acute skin toxicity scored from 0-5 using CTCAE V4.02 by clinicians

*patient with needle phobia; ¶patient had significant breast pain prior to H_2O_2 injection and poor compliance with analgesia; ¥ patient with significant breast pain and was taking opiate analgesia prior to RT, explaining the pain score of 0. ADL – Activities of daily living.

Patient	Age	Sex	PS* (ECOG)	Baseline TNM stage	Tumour Phenotype	Prior treatment (no. of lines of therapy)	RT target volume
1	77	F	1	T2N1M1	$ER^+/HER2^-$	Endocrine(3)	Breast + axillary LN levels I-IV
2	69	F	0	T4N0M1	$ER^+/HER2^-$	Endocrine(2)	Breast
3	79	F	3	T4N0M0	$ER^+/HER2^-$	Endocrine(3)	Breast
4	80	M	2	T4N0M0	$ER^+/HER2^-$	Endocrine(2)	Breast
5	89	F	3	T2N0M0	$ER^+/HER2^-$	Endocrine(1)	Breast
6	78	F	2	T4N1M1	$ER^+/HER2^-$	Endocrine(2) Chemotherapy(1)	Breast + axillary LN levels I-IV
8	53	M	0	T2N0M1	$ER^-/HER2^-$	Surgery Endocrine(3) Chemotherapy(4) RT (contralateral)	Breast
9	53	F	2	T2N1M1	$ER^+/HER2^-$	Surgery Endocrine(3) Chemotherapy(2) RT (contralateral)	Breast + axillary LN levels I-IV
10	45	F	0	T4N1M1	$ER^+/HER2^-$	Chemotherapy(1)	Breast + axillary LN levels I-IV
11	75	F	3	T4N1M1	$ER^+/HER2^-$	Surgery Endocrine(3)	Breast
12	45	F	1	T4N1M1	$ER^-/HER2^-$	Chemotherapy(2)	Breast + axillary LN levels I-IV
13	93	F	3	T2N0M0	$ER^+/HER2^-$	None	Breast

Table 1. Summary of patient demographics, tumour characteristics, previous lines of treatment and RT treatment volumes. Patients 1, 3, 4, 5, 11 and 13 received 36Gy/6 fractions, and 2, 6, 8, 9, 10 and 12 received 49.5Gy/18 fractions. *Performance status (Eastern Cooperative Oncology Group).

Patient number	Maximum pain intensity		Extra analgesia required	Median difference in pain score (pre- and post RT)	Effect of pain on ADLs	Maximum RT acute skin toxicity score	Bolus during RT
	Score	Period					
1	4	2 hrs	N	3	Y- housework, shopping	3	Y
2	0	0 mins	N	0	N	3	Y
3	3	30 mins	N	2.5	N	2	N
4	4	30 mins	Y	0.5	N	2	N
5	0	0 mins	N	0	N	0	N
6*	10	6 hrs	Y	5	N	1	N
8†	10	6 hrs	Y	6	Y-driving	2	N
9	6	5 hrs	N	4	N	2	N
10	8	2 hrs	Y	7	Y-housework	3	Y
11	0	0 mins	N	0	N	3	Y
12‡	0	0 mins	Y	0	N	3	Y
13	6	1 hr	Y	5	N	0	N

Table 2. Summary of pain scores and RT acute skin toxicity scores

Pain intensity scored from 0-10 via patient self-assessment questionnaires (median calculated from difference in pain score pre- and post-H2O2 injection for each patient throughout treatment course (4-6 injections in total for each patient)); RT acute skin toxicity scored from 0-5 using CTCAE V4.02 by clinicians

*patient with needle phobia; †patient had significant breast pain prior to H_2O_2 injection and poor compliance with analgesia; ‡patient with significant breast pain and was taking opiate analgesia prior to RT, explaining the pain score of 0. ADL – Activities of daily living.

Figure 1

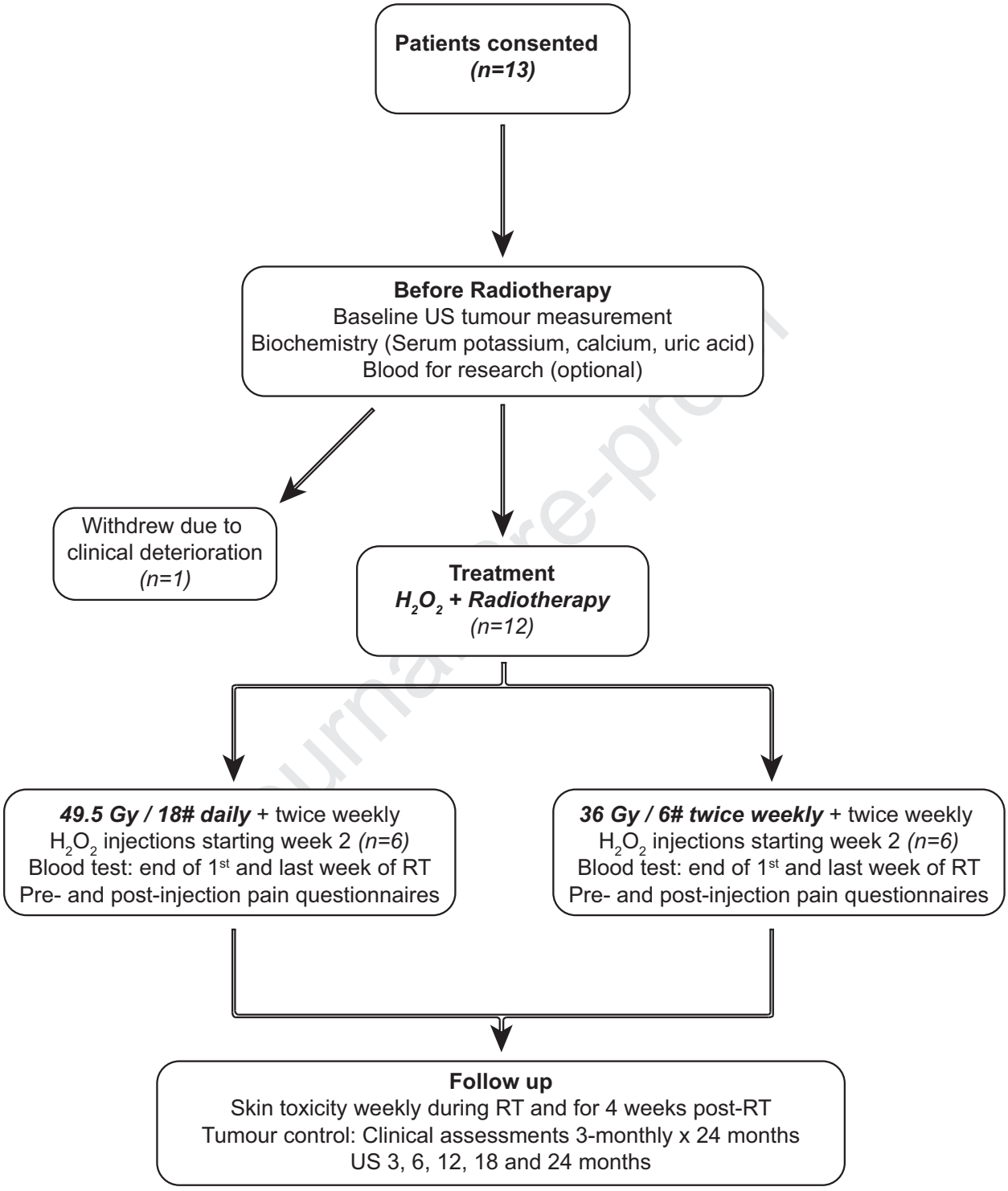
Patients consented
(n=13)
Before Radiotherapy
Baseline US tumour measurement
Biochemistry (Serum potassium, calcium, uric acid)
Blood for research (optional)
Withdrew due to clinical deterioration
(n=1)
Treatment
H2O2 + Radiotherapy
(n=12)
49.5 Gy / 18# daily + twice weekly H2O2 injections starting week 2 (n=6)
Blood test: end of 1st and last week of RT
Pre- and post-injection pain questionnaires
36 Gy / 6# twice weekly + twice weekly H2O2 injections starting week 2 (n=6)
Blood test: end of 1st and last week of RT
Pre- and post-injection pain questionnaires
Follow up
Skin toxicity weekly during RT and for 4 weeks post-RT
Tumour control: Clinical assessments 3-monthly x 24 months
US 3, 6, 12, 18 and 24 months

Figure 2A

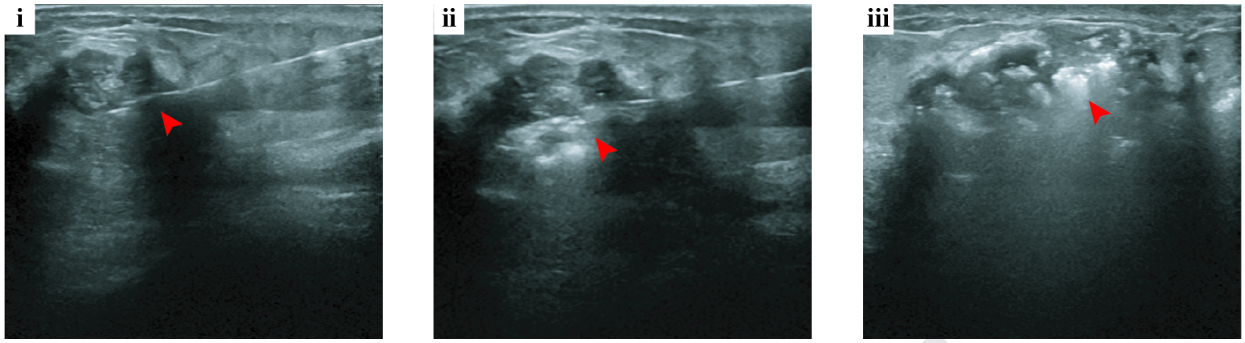

Figure 2B

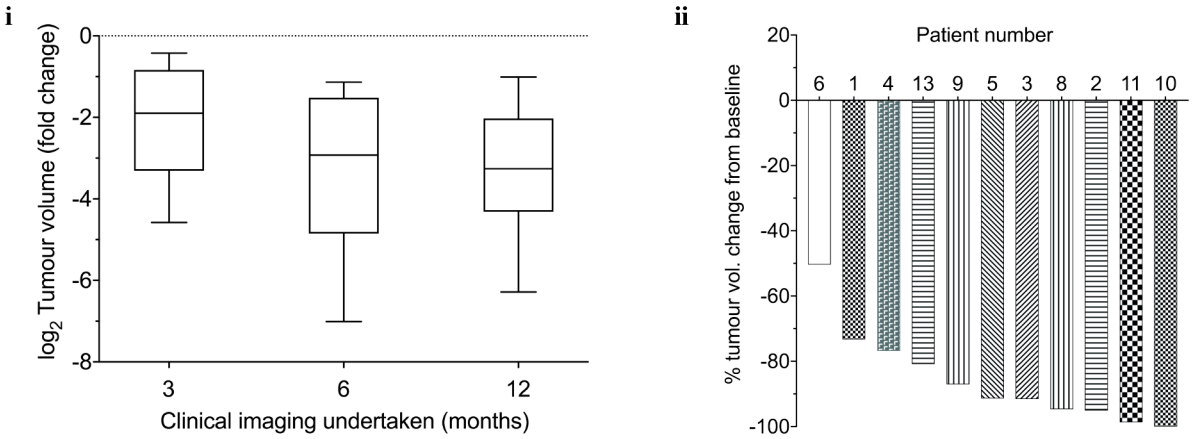

Figure 2C

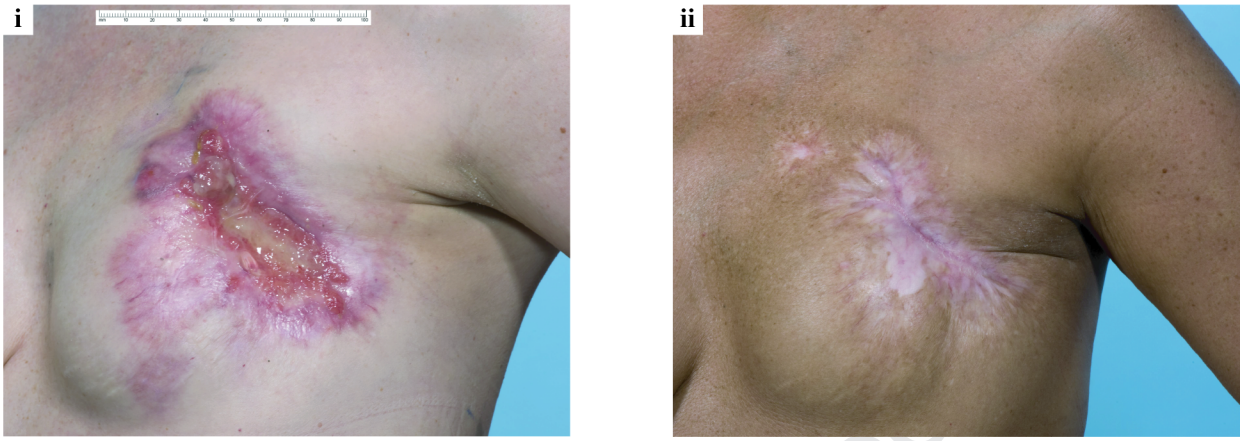

Figure 2D

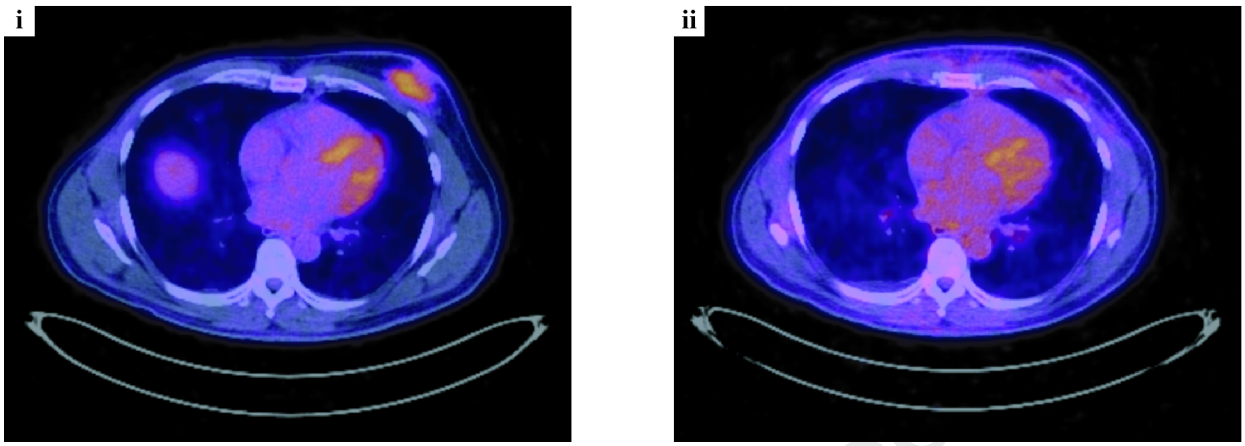

Figure 3A

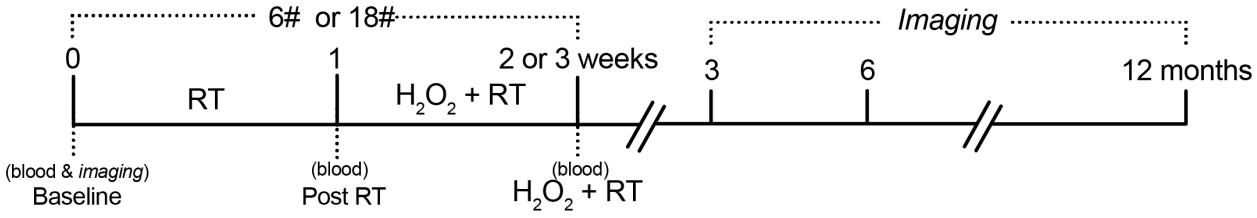

Figure 3B

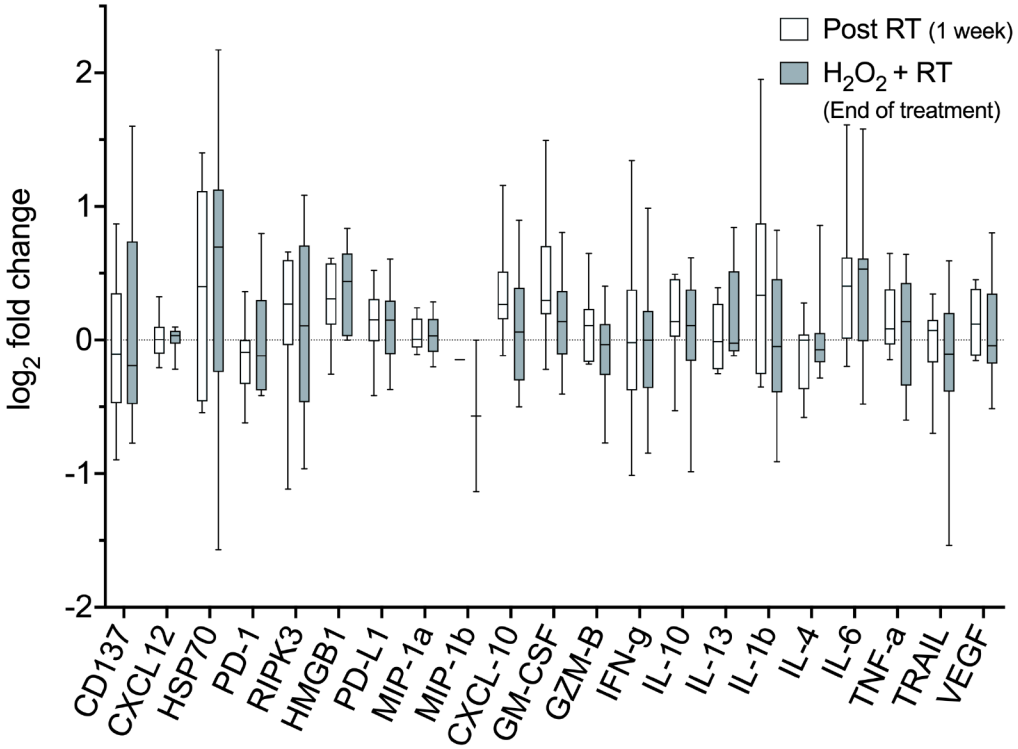

Figure 3C