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# Influence of confounding factors on radiation dose estimation in in vivo validated transcriptional biomarkers

**Lourdes Cruz-Garcia**[*], **Grainne O'Brien**[*], **Ellen Donovan**[†], **Lone Gothard**[‡], **Sue Boyle**[‡], **Antoine Laval**[§], **Isabelle Testard**[§], **Lucyna Ponge**[**], **Grzegorz Woźniak**[**], **Leszek Miszczyk**[**], **Serge M. Candéias**[§], **Elizabeth Ainsbury**[*], **Piotr Widlak**[**], **Navita Somaiah**[‡], and **Christophe Badie**[*]

[*]Radiation Effects Department, Centre for Radiation, Chemical & Environmental Hazards Public Health England Chilton, Didcot, Oxfordshire OX11 ORQ United Kingdom [†]Centre for Vision Speech and Signal Processing, University of Surrey, Guildford, GU2 7TE, UK [‡]Institute for Cancer Research/Royal Marsden NHS Foundation Trust, Downs Road, Sutton SM2 5PT, UK [§]CEA, DRF, BIG-LCBM, F-38000 Grenoble, France. CNRS, LCBM, UMR 5249, F-38000 Grenoble, France.Univ. Grenoble Alpes, BIG-LCBM, F-38000 Grenoble, France [**]Maria Sklodowska-Curie Institute – Oncology Center, Gliwice Branch, 44-101 Gliwice, Poland

## Abstract

For triage purposes following a nuclear accident, blood-based gene expression biomarkers can provide rapid dose estimates for a large number of individuals. Ionising radiation responsive genes are regulated through the DNA damage response pathway, including activation of multiple transcription factors. Modulators of this pathway could potentially affect the response of these biomarkers and consequently compromise accurate dose estimation calculations. In the present study, four potential confounding factors, cancer condition, gender, simulated bacterial infection (lipopolysaccharide) and curcumin, an anti-inflammatory/anti-oxidant agent, were selected. Their potential influence on the transcriptional response to radiation of the genes *CCNG1* and *PHPT1*, two biomarkers of radiation exposure ex vivo, was assessed. Firstly both *CCNG1* and *PHPT1* were detected in in vivo blood samples from radiotherapy patients and as such validated as biomarkers of exposure. Importantly, their basal expression level was slightly but significantly affected in vivo by cancer condition. Moreover, lipopolysaccharide stimulation of blood irradiated ex vivo led to a significant modification of *CCNG1* and *PHPT1* transcriptional response in a dose- and time-dependent manner with opposite regulatory effects. Curcumin also affected their response counteracting some of the radiation induction. No differences were observed depending on gender. Dose estimations calculated using linear regression were affected by lipopolysaccharide and curcumin. In conclusion, several confounding factors tested in this study can indeed modulate the transcriptional response of *CCNG1* and *PHPT1* and consequently affect radiation exposure dose estimations but not to a level which should prevent their use for triage purposes.

**Correspondence:** Dr Christophe Badie, Radiation Effects Department, Centre for Radiation, Chemical & Environmental Hazards Public Health England Chilton, Didcot, Oxfordshire OX11 ORQ United Kingdom, Tel: +44(0)1235 822698. Fax: +44 (0)1235 833891. christophe.badie@phe.gov.uk.

**Conflict of interest**

The authors report no conflict of interest.

**Keywords**

confounding factors; gene expression biomarkers; radiation dose estimation; biological dosimetry

## INTRODUCTION

Gene expression biomarkers have become of great interest as potential bioindicators to determine radiation exposure and dose received (Kabacik et al. 2011, Kabacik et al. 2011, Budworth et al. 2012, Manning et al. 2013, Kabacik et al. 2015a, Badie et al. 2016, Manning et al. 2017). The amount of mRNAs of specific genes involved in DNA damage response (DDR) (Kabacik et al. 2011, Manning et al. 2013) as well as non-coding RNAs (Kabacik et al. 2015a) have been proven to show a strong dose-response relationship in blood irradiated ex vivo, making them of great interest for biological dosimetry purposes. In case of emergency, gene expression analysis could offer the advantage of providing quicker results and larger scale analysis compared to other classic assays like dicentric or micronucleus assays (Pernot et al. 2012, Rothkamm et al. 2013, Hall et al. 2017) and this has been successfully tested in exercises with dose assessments of coded samples (Badie et al. 2013, Abend et al. 2016, Manning et al. 2017).

Ionizing radiation (IR) induces genotoxic lesions which activate the transcription of genes involved in cell death, DNA repair, cell cycle arrest and autophagy. The induction of these genes involves the recognition of the DNA damage by the kinases ataxia telangiectasia mutated (*ATM*) and Rad3 related (*ATR*) which phosphorylate and activate transcription factors responsible for inducing mRNA expression (Christmann and Kaina 2013). The main transcription factors regulated by the DDR are *p53*, nuclear factor kappa B (*NF-kappaB*), breast cancer-associated protein 1 (*BRCA1*) and *Ap-1* (Christmann and Kaina 2013). p53 plays a major role activating the transcription of target genes involved in the DDR and is the principal mediator of senescence, apoptosis and cell cycle arrest (Toledo and Wahl 2006, Christmann and Kaina 2013). Inflammatory and anti-inflammatory mediators have been identified to modulate p53 at a transcriptional level (Choudhuri et al. 2002, Li et al. 2015, Odkhuu et al. 2015), which could consequently affect its downstream target genes during DDR. For instance, transcriptional responses to radiation of p53-depenent genes such as *MDM2*, *BBC3*, *FDXR*, and *CDKN1A* have been shown to be affected in the presence of confounding factors such as lipopolysaccharide (LPS) and curcumin (Budworth et al. 2012, Soltani et al. 2016).

Cyclin G1 (*CCNG1*) and phosphohistidine phosphatase 1 (*PHPT1*) are both downstream genes of the transcription factors activated through the DDR and have been identified as genes responsive to IR in whole human blood and in peripheral blood lymphocytes irradiated ex vivo (Paul and Amundson 2008, Kabacik et al. 2011, Manning et al. 2013). Ex vivo analysis of *CCNG1* and *PHPT1* responsiveness to radiation has shown low inter-individual variability in their transcriptional response to radiation, a linear dose-response at low doses (25–100 mGy) for *CCNG1* and high doses (1–4 Gy) for *PHPT1* (Manning et al. 2013), providing high accuracy when estimating the dose received, making them potential biomarkers of IR exposure in vivo. To be able to provide accurate and reproducible dose

estimation using transcriptional markers, the influence of confounding factors, which could affect DDR pathways and might modulate their responses, should be taken into consideration. In the present study this important issue was addressed by studying the response of *CCNG1* and *PHPT1* to several confounding factors potentially able to modulate their radiation-induced transcriptional response. First we assessed the radiation response of these genes in vivo in blood from cancer patients treated with radiation therapy in order to validate them for biological dosimetry purposes. Then, cancer condition, simulation of bacterial infection with LPS and the use of an anti-inflammatory agent (curcumin) were tested as potential confounding factors affecting the response to radiation of these biomarkers. Our findings indicated that both *CCNG1* and *PHPT1* are radiation responsive in vivo. On our ex vivo settings, confounding factors can modify their expression and consequently could affect estimation of the dose but to an extent that should not affect their use in biodosimetry.

## MATERIAL AND METHODS

### Blood collection and irradiation ex vivo

Peripheral blood samples freshly collected from 10 healthy donors (five men and five women; age range: 35–60 years) were incubated with two different concentrations of LPS (1 ng $ml^{-1}$ and 500 ng $ml^{-1}$) or curcumin (15 μM) (Sigma-Aldrich, Irvine, UK). LPS and curcumin were added to 500 μl of blood 1 h before being either mock irradiated or exposed to a 2 Gy X-rays dose (0.5 Gy/min) or just after exposure (LPS only). An HS X-ray system (AGO X-Ray Ltd., Aldermaston, UK) (output 13 mA, 250 KV peak) was used to irradiate the samples. Blood samples were kept at 37 °C in an incubator with 5% $CO_2$ for either 2 h or 24 h after exposure. After the incubation time, the blood was mixed with 1 ml of RNA later (Thermo Fisher Scientific, Loughborough, UK) and stored at −80°C until being processed for RNA extraction. Venous blood was taken at the Centre for Radiation, Chemical & Environmental Hazards Public Health England (Chilton, UK) with informed consent and the ethical approval of the West Midlands - Solihull Research Ethics Committee (REC 14/WM/1182).

In an independent experiment, peripheral blood from 5 healthy donors, purchased from the French Blood Bank (Etablissement Français du Sang, La Tronche, France) under contract #15–2041, was exposed to a range of gamma-irradiation doses ($^{60}Co$ source, doses of 50 mGy, 100 mGy, 2 Gy and 4 Gy with a dose rate of 0.038 Gy $min^{-1}$ or 1.47 Gy $min^{-1}$ for doses below and above 1 Gy, respectively). Blood samples were incubated at 37 °C in an incubator with 5% $CO_2$. One hour after irradiation, 1ng $ml^{-1}$ of LPS was added and the RNA was isolated 2 h later.

### Radiotherapy patient samples

Blood samples from four breast, two endometrial, two lung and one prostate cancer patients (age range: 36–86 years), treated with Intensity Modulated Radiotherapy (IMRT) using a linear accelerator (LINAC) were collected at five different time points during the course of the treatment: before the start of the treatment, 0.5–2 h and 24 h after the first fraction, just before the fifth or sixth fraction and the last fraction. For the last two time points, the blood

was collected 21–28 h after the previous fraction. The prescribed doses for each patient are described in Table 1. Patients did not receive previous radio- and/or chemotherapy treatments except for one of the lung cancer patients who received chemotherapy five weeks before the start of radiotherapy. Blood was collected at the Royal Marsden Hospital and Institute of Cancer Research (Surrey, UK) and was taken with written informed consent from all subjects and the ethical approval by the Health Research Authority (REC 16/SC/0307).

Another three sub-groups of patients were recruited including seven head and neck squamous cell carcinoma (HNSCC) and six prostate cancer (PC) patients treated with IMRT using a LINAC and nine PC patients treated with Stereotactic Ablative Radiotherapy (SABR) using a Cyberknife treatment unit (patients had no previous surgery or chemotherapy). Patient ages ranged from 52 to 75. The blood collection times, dose rates, dose per fraction and total dose received are listed in Table 2. The blood was collected between 1–18 h after the fifth and seventh fractions and after 1–2 h after the last fraction. Blood was collected at the Maria Sklodowska-Curie Institute – Oncology Center (Gliwice, Poland). This study was carried out in accordance with the Bioethical Committee in Maria Sklodowska-Curie Institute, Warszaw, approval number 27/2015 from 18/08/2015.

Blood samples from all different patient groups were collected in PAXgene tubes according to the manufacturer's protocol (Qiagen, PreAnalytiX GmbH, Hilden, Germany).

Blood from 20 healthy donors (eight men and twelve women, ages ranged from 25 to 60 years) was also collected at the Centre for Radiation, Chemical & Environmental Hazards Public Health England (Chilton, UK) in PAXgene tubes to compare the basal expression levels of the target genes from healthy donors and cancer patients.

## RNA isolation and reverse transcription

Total RNA from blood samples exposed ex vivo to X-rays was extracted using a RiboPure™-Blood RNA Purification Kit (Thermo Fisher Scientific, Loughborough, UK). Total RNA from samples collected in PAXgene tubes from radiotherapy patients was extracted with the PAXgene Blood miRNA kit (Qiagen, PreAnalytiX GmbH, Hilden, Germany) using a robotic workstation Qiacube (Qiagen, Manchester, UK). The quantity of isolated RNA was determined by spectrophotometry with a ND-1000 NanoDrop and quality was assessed using a Tapestation 220 (Agilent Technologies, CA, USA). cDNA was prepared from 350 ng of the total RNA using High Capacity cDNA reverse transcription kit (Applied Biosystems, FosterCity, CA, USA) according to the manufacturer's protocol.

For samples exposed to gamma-radiation, total RNA was extracted from 400 μL of whole blood with the Nucleospin RNA Blood kit (Macherey and Nagel, Hoerdt, France) according to the manufacturer's instructions. RNA was converted into cDNA using the Enhanced Avian HS RT-PCR Kit (Sigma-Aldrich, Lyon, France) with oligo-dT priming according to the manufacturer's protocol.

### Quantitative Real-time Polymerase chain reaction

QRT-PCR was performed using a Rotor-Gene Q (Qiagen, Hilden, Germany) with PerfeCTa MultiPlex qPCR SuperMix (Quanta Bioscience, Inc., Gaithersburg, MD, USA). The samples were run in triplicates in 10 μl reactions with 1 μl of the cDNA synthesis reaction together with three different sets of primers and fluorescent probes at 300 nM concentration each. 3′6-Carboxyfluorescein (FAM), 6-Hexachlorofluorescein (HEX) and Atto 680 (Eurogentec Ltd., Fawley, Hampshire, UK) were used as fluorochrome reporters for the probes analysed in multiplexed reactions with 3 genes per run including a housekeeping gene. Primer sequences: *HPRT1* F: 5′ TCAGGCAGTATAATCCAAAGATGGT 3′, R: 5′ AGTCTGGCTTATATCCAACACTTCG 3′, probe: 5′ CGCAAGCTTGCTGGTGAAAAGGACCC 3′; *CCNG1* F: 5′ GGAGCTGCAGTCTCTGTCAAG 3′, R: 5′ TGACATCTAGACTCCTGTTCCAA 3′, probe: 5′ AACTGCTACACCAGCTGAATGCCC 3′; *PHPT1* F: 5′ TCGCTCTCATTCCTGATGTG 3′, R: 5′ TCGTAGATGTCCGCATGGTA 3′, probe: 5′ CTTGTAGCCGCGCACGATCTCCTT 3′. The reactions were performed with the following cycling conditions: 2 min at 95 °C, then 45 cycles of 10 s at 95 °C and 60 s at 60 °C. Data were collected and analysed by Rotor-Gene Q Series Software. Gene target Ct (cycle threshold) values were normalized to hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) internal control. Ct values were converted to transcript quantity using standard curves obtained by serial dilution of PCR-amplified DNA fragments of each gene. The linear dynamic range of the standard curves covering six orders of magnitude (serial dilution from $3.2 \times 10^{-4}$ to $8.2 \times 10^{-10}$) gave PCR efficiencies between 93 and 103% for each gene with $R^2$>0.998.

For samples exposed to gamma-irradiation, SYBRGreen qPCR was performed using the LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich, Lyon, France) in triplicate 10 μL reactions containing 2 μM primers and 2 μl of cDNA diluted one-twentieth in water in the following conditions: 20 s at 95 °C, then 40 cycles 5 s at 95 °C and 20 s at 60 °C on a CFX 384 Real Time System thermal cycler (Bio-Rad, Marne-La-Coquette, France). Data were collected and analysed with the CFX Manager 3.1 Software (BioRad). Gene target Ct values were normalized to ribosomal protein lateral stalk subunit P0 (*36B4*) and *HPRT1* internal controls. Primers for these targets were: *36B4* sens: 5′ GAAATCCTGGGTGTCCGCAATGTT 3′, rev: 5′ AGACAAGGCCAGGACTCGTTTGTA 3′; *HPRT1* sens: 5′ ATGGACAGGACTGAACGTCTTGCT 3′, rev: 5′ TTGAGCACACAGAGGGCTACAATG 3′. Amplification efficiency of these primer pairs were 100.1% for *HPRT1* and 98% for 36B4.

### Dose estimation curve

Blood from 10 healthy donors (five men and five women; age range: 35–60 years) was collected and exposed to a range of X-ray doses (0.25, 0.5, 1 and 2 Gy at a dose rate of 0.5 Gy $min^{-1}$) and after 24 h, RNA was extracted using a RiboPure™-Blood RNA Purification Kit (Thermo Fisher Scientific, Loughborough, UK) and the cDNA synthesized using High Capacity cDNA reverse transcription kit (Applied Biosystems, FosterCity, CA, USA). The cDNA from the 10 donors was combined and used as a calibration curve in each multiplexed QRT-PCR run to estimate the dose of the blood samples stimulated with LPS and curcumin

as previously described (Manning et al. 2013). A linear fit was used to construct the dose estimation curve and the increase in expression following irradiation for each sample was entered into the linear equation to give a dose estimate.

### Statistical analysis

Statistical analyses were performed using Minitab software. Data are presented as means ± standard deviation (SD). Comparisons were analysed by an unpaired t-test (student's t-test) or a paired t-test. A significance of $p \leq 0.05$ was applied to all statistical tests performed. Statistical analyses were performed in log transformed data.

## RESULTS

### Gene expression dose-response of *CCNG1* and *PHPT1*

The gene expression profile of *CCNG1* and *PHPT1* in blood exposed ex vivo to doses ranging from 0.25 to 4 Gy was monitored at 24 h post-exposure (Fig. 1A-B). The results showed a dose-dependent upregulation of transcription which reached a plateau phase for doses above 1 Gy for *CCNG1* and 3 Gy for *PHPT1*. When comparing both dose-response curves (Fig. 1C), a higher response to radiation can be seen with *PHPT1* than *CCNG1* for all the different doses.

### Basal *CCNG1*and *PHPT1* expression levels in vivo: Healthy donors and cancer patients

Comparisons between healthy donors and cancer patients were performed in order to see if cancer itself may be a confounding factor by modifying *CCNG1* and *PHPT1* basal expression levels (Fig. 2). *CCNG1* and *PHPT1* expression level in peripheral blood from 20 healthy donors (twelve women and eight men) was compared to 31 cancer patients (seven head and neck, sixteen prostate, four breast, two lung and two endometrial cancer patients). Interestingly, the results indicate a significant lower basal expression level for *CCNG1* in cancer patients; conversely, a significantly increased *PHPT1* expression in cancer patients compared to healthy donors was observed.

### Gene expression profile of *CCNG1* and *PHPT1* in vivo: Cancer patients during radiation therapy

The gene expression profile of *CCNG1* and *PHPT1* was analysed in peripheral blood from cancer patients treated with different external beam radiotherapy regimens for different cancer types. Two independent studies on separate cohorts were performed where blood was collected at different points during the treatment.

In the first study, peripheral blood from nine patients was collected at five different time points during the course of the radiotherapy treatment (Table 1). When the data for all patients were analysed together (Fig. 3A), *CCNG1* showed a significant upregulation of expression in vivo at all time points, peaking before the fifth and sixth fraction. Similar *CCNG1* expression profiles were observed in each cancer group analysed individually (Fig. 3B-E). Interestingly, *PHPT1* shows a different expression profile with a significant drop in gene expression shortly after the first fraction (0.5–2h) and before the last fraction (Fig. 3F). Similar to *CCNG1*, *PHPT1* also showed an upregulation at the third sampling point, 24 h

after the first fraction. Looking at each individual cancer type, *PHPT1* shows a very similar pattern of expression along the sampling time points during the radiation therapy (Fig. 3G-J).

The second study included 22 cancer patients treated with IMRT or SABR. Peripheral blood from these patients was collected before the start of the treatment, after one to seven fractions and at the end of the treatment (Table 2). For this patients' cohort, samples were also collected one month following the end of the treatment allowing assessment of the duration of the radiation-induced transcriptional changes. The results showed a similar profile for *CCNG1* and *PHPT1* in each group. Both genes were up-regulated in the IMRT groups after the first fifth and seventh fractions (Fig. 4), but whereas *PHPT1* stayed upregulated at the end of the treatment in the HNSCC patients, *CCNG1* did not. In the same group, *CCNG1* showed a decrease in expression one month after the last fraction was received. The same profile was observed in the SABR PC group where both biomarkers were upregulated at the end of the treatment (after fifth and last fraction) corresponding more closely to the second time point in the IMRT groups. Apart for the downregulation of *CCNG1* in the HNSCC group, the expression level of both genes went back to basal level one month after the last fraction.

## Effect of LPS and curcumin on *CCNG1* and *PHPT1* gene expression response to ionizing radiation

The effect of two other potential confounding factors was assessed in peripheral blood from healthy donors exposed ex vivo to IR.

LPS modulated the transcription of *CCNG1* and *PHPT1* with a different regulatory effect in a time-dependent manner. *CCNG1* showed a significant LPS dose-dependent downregulation at 2 h. This LPS effect was also observed when the blood was exposed to 2 Gy and LPS counteracted the radiation induction of *CCNG1* expression (Fig. 5A). The administration of LPS before and or just after irradiation showed the same downregulatory effect on *CCNG1* expression (Fig. 5A). However, after 24 h post-irradiation, the effect of LPS on *CCNG1* expression was not detected irrespectively of the irradiation status (Fig. 5B).

On the contrary, *PHPT1* showed an opposite regulation by LPS. *PHPT1* was upregulated by LPS at 2 h post-exposure in non-irradiated samples for the highest concentration of LPS (500 ng $ml^{-1}$) (Fig. 5C). After 24 h, LPS effect was persistent in non-irradiated samples showing a clear dose-dependency (Fig. 5D). In irradiated samples at 24 h, LPS showed a co-stimulatory effect with irradiation, inducing a higher transcriptional response than the one observed with irradiation alone (Fig. 5D).

The effect of LPS (1ng $ml^{-1}$) was also tested when administered 1 h after exposure to a range of gamma-rays doses (50 mGy, 100 mGy, 2 Gy and 4 Gy) and gene expression of *CCNG1* and *PHPT1* was analysed 2 h post-irradiation. The results indicated that *CCNG1* responds to low doses delivered at a lower dose rate (50 mGy, 100 mGy at 0.038 Gy $min^{-1}$) but not *PHPT1* (Fig. 6). For *CCNG1*, LPS added 1 h after blood irradiation had a downregulatory effect on its response to IR, as observed when added before irradiation (Fig.

5A) but with a slighter effect. Significant differences between irradiated samples and irradiated samples in the presence of LPS were observed only at the lowest 50 mGy dose in the case of *CCNG1* expression (Fig. 6A). The late addition of LPS in blood didn't modulate further the response of *PHPT1* to radiation (Fig. 6B).

Curcumin exerted a similar regulatory effect on both genes but with different kinetics (Fig. 5). Curcumin counteracted the *CCNG1* gene expression induced by irradiation at 24h post-exposure (Fig. 5B). However, curcumin already modulated *PHPT1* at 2 h post-exposure (Fig. 5C), showing a more pronounced effect at 24 h in the irradiated and non-irradiated blood samples (Fig. 5D).

For some of the ex vivo experiments we used five female donors and five male donor in order to assess the role of gender and found no significant differences between males and females neither for *CCNG1* nor *PHPT1* transcriptional response to IR, LPS or curcumin alone and IR with LPS or curcumin (Fig. 5).

### Modulation of dose estimation by confounding factors

A linear regression equation was calculated using the gene expression values of *CCNG1* and *PHPT1* obtained to construct a calibration curve. The calibration curve was performed by exposing blood from 10 healthy donors at a range of X-ray doses (0.25, 0.5, 1 and 2 Gy at 0.5 Gy $min^{-1}$). The gene expression levels of *CCNG1* and *PHPT1* obtained in irradiated samples with LPS or curcumin were used to calculate the dose and assess how they can modify the dose estimated (Fig. 7). The results indicated that LPS produces a modulation of the response to radiation at 24 h post-exposure mainly for *PHPT1* (Fig. 7B). LPS is lowering the radiation response of *CCNG1* mainly at 2 h. At 24 h, LPS slightly affects *CCNG1*, exerting a not very pronounced underestimation of the estimated dose compared to the effect of irradiation alone (Fig. 7A). However, LPS induced a higher gene expression response to radiation in *PHPT1* at 24 h which translate in an overestimation of the dose received compared to irradiation alone (Fig. 7B). Finally, the LPS effect observed is similar when LPS was administered 1 h before or just after irradiation. For curcumin, its presence in blood leads to a lower response of both genes to ionizing radiation at 24 h post-exposure, thus leading to an underestimation of the dose calculations (Fig. 7A-B).

## DISCUSSION

In case of a radiation emergency after a nuclear accident, it is crucial to have a rapid and robust method to assess exposure to radiation and dose received to potentially large numbers of individuals in order to act accordingly (Kulka et al. 2017). Gene expression has proven to be able to provide dose estimates in a short period of time and delivers consistent results between multiple institutions in several countries using different protocols for gene expression (Badie et al. 2013, Abend et al. 2016, Manning et al. 2017). Genes regulated through the DNA damage response have been identified to be good gene expression biomarkers of radiation exposure ex vivo and are promising biomarkers for transcription-based biological dosimetry purposes (Badie et al. 2013, Abend et al. 2016, Manning et al. 2017). Two genes, *CCNG1* and *PHPT1*, were previously identified as showing strong responsiveness to radiation in experiments ex vivo (Manning et al. 2013) and were selected

for this study. Their response to ionizing radiation ex vivo was confirmed by irradiating blood with a range of X-ray doses between 0.25 and 4 Gy (0.5 Gy $min^{-1}$). *PHPT1* showed a higher gene expression response to IR than *CCNG1* as previously observed (Manning et al. 2013). Interestingly, in gamma-irradiated blood at low doses (50 mGy and 100m Gy) at a lower dose-rate (0.038 Gy/min), *CCNG1* but not *PHPT1* showed a significant response to radiation. This lack of PHPT1 response could be attributed to the low dose-rate, since differences in response to dose and dose-rates have been previously observed between radiation responsive genes (El-Saghire et al. 2013, Paul et al. 2013, Ghandhi et al. 2015). The radiation source is unlikely to be the cause of those differences, although differences between X-rays and gamma rays were previously reported for different endpoints (Janatpour et al. 2005, Scott et al. 2013). These differences in response to high and low dose-rates may be of interest in order to determine a gene expression signature providing information on dose and dose-rate.

Being able to validate the radiation responsiveness of *CCNG1* and *PHPT1* in vivo is paramount if they are to be considered as biomarkers for biological dosimetry purposes. In vivo expression profiles of both genes in patients with different types of cancer and treated with different radiotherapy treatments demonstrated that they are regulated after a local body exposure and thus also good biomarkers of exposure in vivo. *PHPT1* and *CCNG1* have been previously identified to respond in blood from total-body irradiated patients (Filiano et al. 2011, Paul et al. 2011). Total-body irradiated patients received 1.25 Gy per fraction and three fractions a day (3.75 Gy) (Paul et al. 2011) or 2 Gy twice a day for three consecutive days (total dose of 12 Gy) (Filiano et al. 2011). Unlike total-body irradiated patients, radiotherapy patients in the present study were partially exposed to treat their tumours and received a lower dose to the blood compared to total-body irradiated patients. Even under these local body irradiations and independently of the body localisation of the radiation exposure (breast, endometrial, lung or prostate), both *PHPT1* and *CCNG1* can be clearly detected above background expression level in blood samples; this was also the case at different point during the course of the treatment. The main difference observed between total and partial body irradiation was the level of response. As expected, *CCNG1* and *PHPT1* showed a higher upregulation in total body compared to partial body patient irradiation.

In general, *CCNG1* showed an increase in gene expression irrespectively of the cancer type (breast, lung, endometrium, prostate and head and neck) after a short-period of time after the first fraction (0.5 to 2 h time point), and also during and at the end of the radiotherapy treatment with cumulative doses in the range of 36.25 to 70 Gy. Although *PHPT1* showed similar expression to the *CCNG1* profile in the HNSCC and PC patients treated with IMRT and SABR (Fig. 4) after the first fractions (after the fifth and seventh fractions, first week of treatment approximately for IMRT and 5 fractions for SABR treatment), different responses were observed. *PHPT1* presented a slightly higher expression response compared to *CCNG1* at different points analysed during the course of the radiation therapy in most of the cancer and treatment groups. *PHPT1* showed an initial downregulation after a short period of time after radiation exposure (0.5–2 h), but its expression rose over the basal level significantly 24 h after the first fractions (Fig. 3). These differences of *PHPT1* radiation response at different time points could be attributed to fluctuation patterns of expression after radiation exposure

over time as previously observed in irradiated cultures of human T-lymphocytes in other genes also regulated through the DDR (Kabacik et al. 2015a).

The expression of *CCNG1* and *PHPT1* was not significantly modified after one fraction in the SABR group (7.25 Gy). Surprisingly, *CCNG1* showed a lack of response in the HNSCC group at the end of the treatment and this is also true for both genes in the group of PC patients treated with IMRT (Fig. 4). As regulation of gene expression is a very dynamic and temporal process (Yosef and Regev 2011), the high cumulative doses (78 Gy) and number of fractions in these particular groups could be responsible for an adaptation to the stimuli. Persistent stimulation over time could also be implicated in this lack of response or repression of expression. The expression level may also be affected by the modification of the white blood cells analysed between the beginning and the end of the treatment; cell death, cell division and cell renewal may change the global level of expression we observe at different time points.

When comparing the expression profile of the cohorts studied here, differences of response were identified for *PHPT1* after the first fractions and at the end of the treatment (before fifth and last fraction (Fig. 3F) and after fifth-seventh and last fraction (Fig. 4)). Transcriptional responses of genes regulated through the DDR can be transient (Christmann and Kaina 2013) or fluctuate over time (Kabacik et al. 2015a), so the time post-exposure at which samples are analysed has to be taken into consideration when comparing responses. Therefore the differences observed between the two studies could be at least partially attributed to the blood collection time following several radiotherapy fractions; in the first group of patients, blood was collected later after the prescribed fraction dose (21–28 h after the fourth or fifth and the penultimate fraction) compared to the second group of patients (1–18 h after the fifth or seventh fraction and after 1–3 h after last fraction).

When the basal expression level of these genes was compared to normal healthy donors, *CCNG1* showed a significantly lower expression when considering the average of all the cancer patients. During cancer development there are dysregulations of the cell functions and the cell cycle control (Wiman and Zhivotovsky 2017). Since cyclins are the main regulators of the cell cycle transitions, it is not completely surprising that the basal *CCNG1* expression level is modulated in cancer patients compared to a healthy population. On the contrary, *PHPT1* showed a higher expression level in cancer patients than healthy donors. These findings are very interesting, demonstrating the sensitivity of transcription to detect modifications in the body. In the context of this study, these differences in basal expression level could lead to inaccuracies of dose estimation when using these biomarkers. However, a relatively high variability can be seen inside the cancer groups regarding the basal level of expression of these genes and the altered basal expression level is patient dependent and applies only in specific cases. Moreover the modifications of expression are small and would not affect dose estimates at least based on the data from the cohort of patients studied.

We then addressed the role of gender in the transcriptional response to ionising radiation in blood samples exposed ex vivo. With the number of samples studied, we can conclude that gender is not a confounding effect on the IR response of *CCNG1* and *PHPT1* or at least not a major one as we could not detect any significant differences between males and females

irrespectively of the presence of LPS. Regarding the role of the age of the blood donors, we didn't have enough donors per age group to have the statistical power to evaluate if it could be recognised as a potential confounding factor.

The effect of LPS and curcumin as potential confounding factors in the response of biomarkers to IR was analysed in blood samples from 10 donors exposed ex vivo. Ex vivo experiments have been demonstrated to be an excellent model to identify biomarkers of gene expression as their responses are translated in in vivo human blood samples (Paul et al. 2011, Abend et al. 2016). Blood from healthy donors was irradiated and/or incubated with LPS or curcumin for 2 h or 24 h and the transcriptional expression level of *CCNG1* and *PHPT1* was assessed. LPS is a component of the outer membrane of Gram-negative bacteria (Schletter et al. 1995) and it is used as an inflammatory stimulus to mimic bacterial infection. When LPS was present in blood before or just after blood irradiation, it modulated the response of both biomarkers, mainly counteracting the induction of expression mediated by IR for *CCNG1* and conversely increasing the response to IR for *PHPT1*. p53 is a key transcription factor involved in the DDR and LPS has been reported to downregulate its expression (Odkhuu et al. 2015). As *CCNG1* transcriptional response to IR is driven by p53, the negative regulation of p53 expression by LPS could be responsible of the lower response of *CCNG1* to IR. Consistent with the present study, the confounding effect of LPS has been previously observed on radiation-responsive genes like CDKN1A, BBC3 and FDXR (Budworth et al. 2012), all three having a p53-dependent transcriptional expression. LPS also affects other transcription factors regulated through the DDR such as NF-kappaB by increasing its activity (Odkhuu et al. 2015), which, consecutively, could suppress expression of genes dependent on p53 transcriptional activation due to the competition of both transcription factors for transcription co-activators (Webster and Perkins 1999). The mechanisms by how *PHPT1* is regulated through the DDR pathway are to the best of our knowledge not known, but the modulation of expression by transcription factors driving *PHPT1* expression might be influenced by LPS and thus lead in to the increase of response to IR under LPS stimulation. Although for the purpose of this study, we didn't characterise this effect in more details, these opposite regulations mediated by LPS on *CCNG1* and *PHPT1* transcriptional response to radiation suggest that LPS might modulate their response through different pathways and this would certainly deserve further investigations.

In order to characterise further the role of time in these effects, we also assessed the role of LPS when it was administered 1 h post-exposure to irradiation; in this setting, it affected *CCNG1* response to IR but not *PHPT1*. DNA repair such as double-strand break repair occurs extremely quickly and can be observed as early as minutes following irradiation (Badie et al. 1995), and 1 h after irradiation, transcription factors such as p53 are active and induce transcription of their downstream target genes; this has been shown for *CCNG1* and other P53 dependent genes by Kabacik et al. (Kabacik et al. 2015a). In this study, *CCNG1* expression was modulated by the addition of LPS 1 h post-exposure, which modulates the activity of the pathway by which *CCNG1* is regulated when transcription is fully active. The late administration of LPS didn't affect *PHPT1* response to radiation, possibly because the addition of LPS was too late to exert an effect on its transcriptional activation pathway. Overall, these results also support the idea that the effect of LPS in the response to radiation

of both genes happens through different pathways. Interestingly, LPS could potentially be used to reveal specific gene activation pathways following IR exposure.

The response of *CCNG1* and *PHPT1* to IR was also tested under the presence of the natural dietary polyphenol, curcumin. Curcumin has been associated with antioxidant, anti-cancer, anti-inflammatory and anti-microbial properties (Hussain et al. 2017). In the present study, curcumin counteracted the upregulation of *CCNG1* and *PHPT1* by IR, showing an earlier effect on *PHPT1* than on *CCNG1*. This counteractive effect of IR by curcumin has been reported on CDNK1A and BBC3 (Soltani et al. 2016). Transcription factors involved in the DDR like p53 and NF-kappaB have been previously identified as targets of curcumin (Brennan and O'Neill 1998, Moos et al. 2004), both presenting impairment of functions mediated by curcumin action. Since the response of *CCNG1* and *PHPT1* to IR depends on DDR pathways, it is not surprising that curcumin modified to some extend their responses.

Finally, we wanted to quantify the importance of these modifications on dose estimations which is crucial for biological dosimetry purposes. In a previous study, we used a polynomial regression for the dose calibration curve as it fitted better the dose-response observed for different genes (Manning et al. 2013). However, in the present study, a linear regression offered a better fit than a non-linear for the effect of the confounding factors and was therefore used in order to obtain dose estimates. LPS mainly increased the response of *PHPT1* to radiation, with the higher overestimation being seen at the highest concentration (calculated dose 2.15 Gy for LPS at 500 ng/ml while the calculated dose without LPS was found to be 1.59 Gy, for a physical dose delivered to the blood of 2 Gy). Further research is ongoing to better fit the data and provide a better curve for the estimation of the doses. Nevertheless, using a linear regression, we showed an effect, although mostly moderate on the dose calculated when LPS and curcumin were present in the irradiated blood samples.

## CONCLUSION

In conclusion, first, our data validated *PHPT1* and *CCNG1* as biomarkers of radiation exposure in vivo, and they should be considered for gene expression based biological dosimetry tools in future studies. Second, when dose assessments have to be provided in the context of infection/inflammation, presence of anti-inflammatory/anti-oxidant agents or cancer, we showed that these factors can modulate the response of these transcriptional biomarkers hence affecting dose estimation calculations although not to a level which should prevent the use of these genes for triage purposes. These findings highlight the fact that some confounding variables may need to be taken into consideration when estimating the dose received and that information on a known infection at sampling time should allow more accurate dose estimates.

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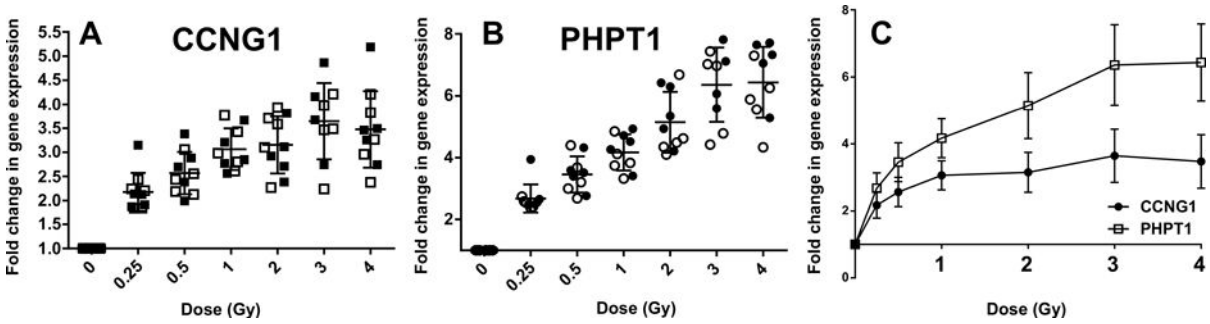


**Figure 1.**

Multiplexed QRT-PCR gene expression fold changes of *CCNG1* (A) and *PHPT1* (B) 24 h post-exposure in blood samples from 10 healthy donors (white symbols indicate five women and black symbols five men) exposed ex vivo to a range of X-ray doses (0.25, 0.5, 1, 2, 3, 4 Gy; 0.5 Gy $min^{-1}$). The expression profile of these genes has been compared in C using the mean +/− SD of the 10 donors (C).

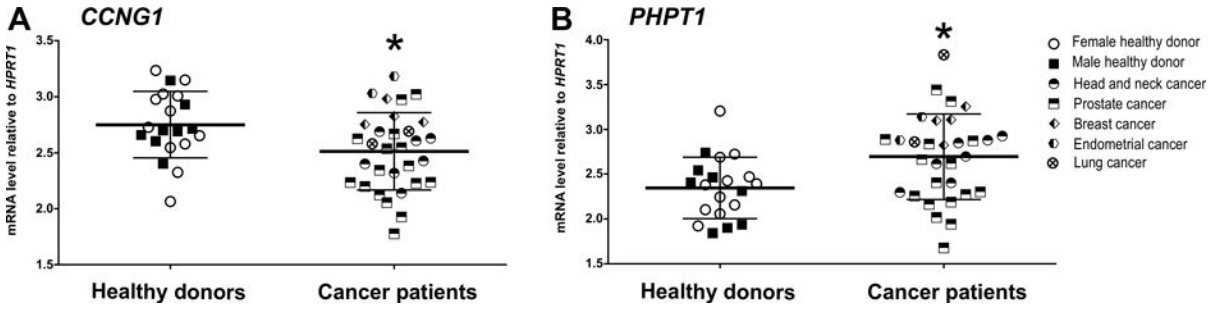


**Figure 2.**

Range of basal gene expression levels of CCNG1 and PHPT1 in healthy donors (eight men and twelve woman, ages ranged from 25 to 60 years) and cancer patients (head and neck, prostate, breast, endometrial and lung cancer, ages ranged from 36 to 86 years). The data is presented as individual data points together with the mean ± SD (n=20 for the healthy donors group and 31 for the Cancer patients group). Statistical analyses were performed in log transformed data. Asterisk (*) indicates statistically significant differences with the healthy donors group (t-test, $p \leq 0.05$).

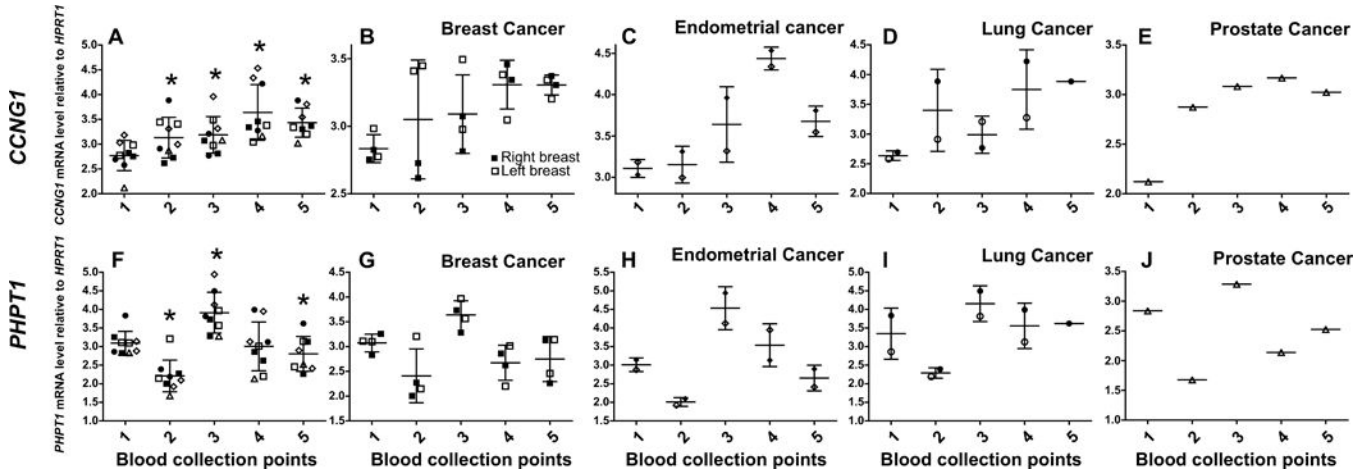


**Figure 3.**

Expression levels of *CCNG1* and *PHPT1* mRNA relative to HPRT1 in blood from four breast, two endometrial, two lung and one prostate cancer patients treated with IMRT. Blood was collected at 5 time points: before the start of the treatment (1), 0.5–2 h (2) and 24 h (3) after the first fraction, before the fifth or sixth (4) and before last fractions (5). Data are shown as individual data points for all patients together with the mean ± SD (A and F). Each individual cancer group was also represented (B, C, D, E and G, H, I, J). Statistical analyses were performed in log transformed data. Significant differences (Paired-T-test, $p \leq 0.05$) with the control (blood collection point 1) were indicated with an asterisk (*).

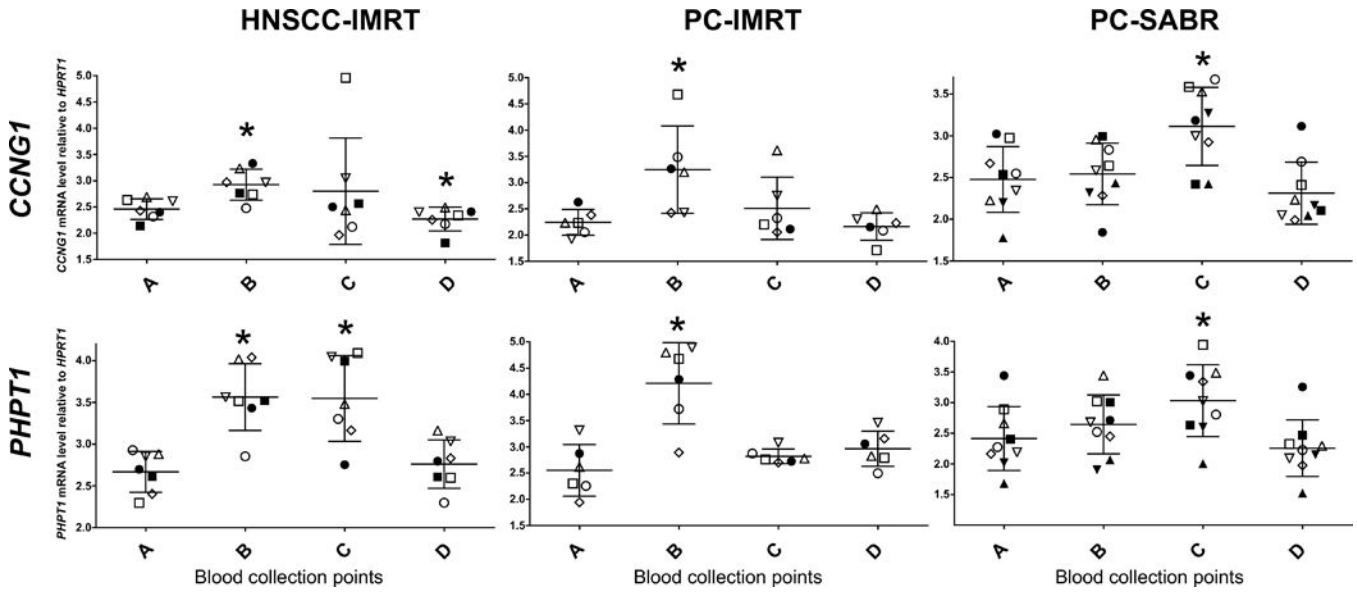


**Figure 4.**

Gene expression of *CCNG1* and *PHPT1* in blood from HNSCC as well as PC patients treated with IMRT and PC patients treated with SABR. Blood was collected before the start of the treatment (A), after 5/7 fractions for the IMRT groups and after the first fraction for the SABR group (B), after the last fraction (C) and one month after the last fraction (D). Data are shown as individual data points together with the mean ± SD (n=7 for HNSCC, n=6 for prostate-IMRT and n=9 for prostate-CK). Statistical analyses were performed in log transformed data. Significant differences (Paired-T-test, $p \leq 0.05$) with the control (blood collection point A) were indicated with an asterisk (*).

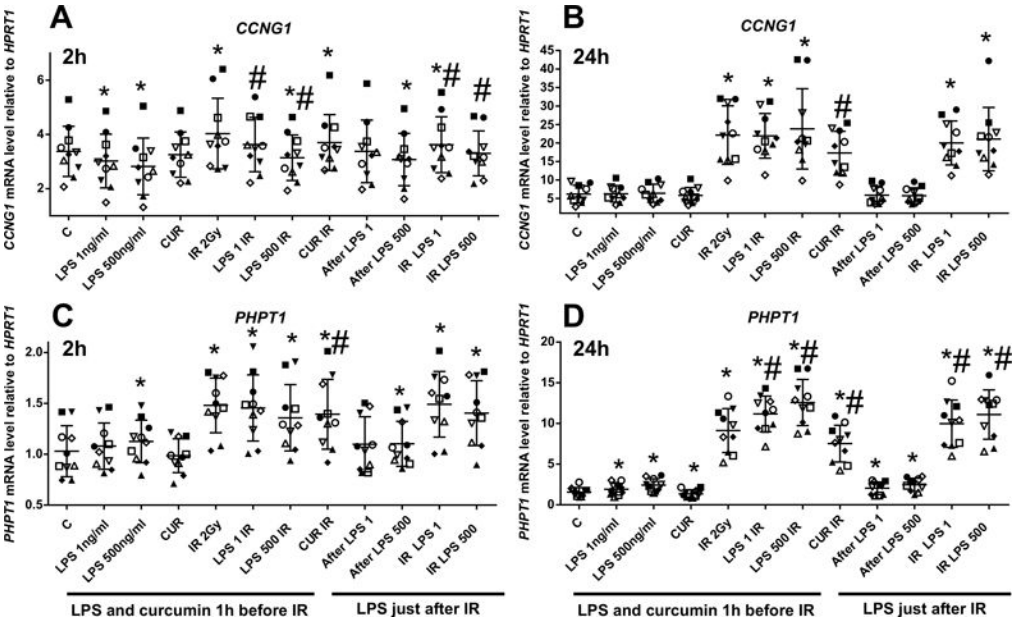


**Figure 5.**

Gene expression of *CCNG1* and *PHPT1* in human blood irradiated and/or stimulated with LPS and curcumin ex vivo. Blood from 10 donors was incubated with two different concentrations of LPS (1 or 500 ng $ml^{-1}$) or curcumin (15 μM) 1 h before irradiation (2 Gy) or just after irradiation (only for LPS). Transcriptional expression of *CCNG1* was analysed at 2 h (A) and 24 h post-irradiation (B) as well as for *PHPT1* (C,D). Data are shown as mean ± SD (n=10, white symbols indicate five women and black symbols five men). Statistical analyses were performed in log transformed data. Significant differences (Paired-T-test, $p \leq 0.05$) with the control were indicated with an asterisk (*) and with a hash (#) differences with IR (only for IR groups).

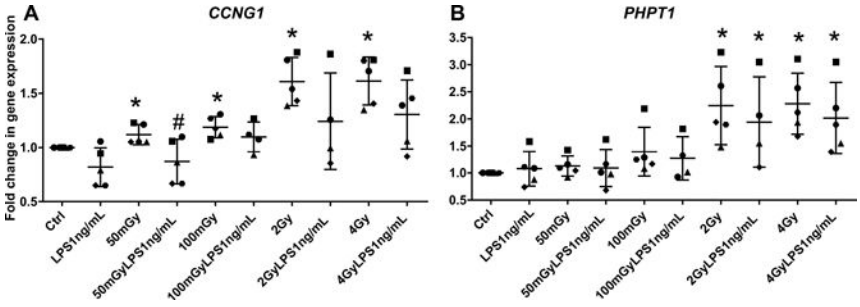


**Figure 6.**

Gene expression of *CCNG1* and *PHPT1* in human blood 2 h after exposure to a range of gamma-irradiation doses ($^{60}$Co source, doses of 50m Gy, 100 mGy, 2 Gy and 4 Gy with a dose rate of 0.038 Gy min$^{-1}$ or 1.47 Gy min$^{-1}$ for doses below and above 1 Gy, respectively) and stimulated with LPS (1 ng ml$^{-1}$) 1 h after exposure. The data is presented as individual data points together with the mean ± SD (n=5). Asterisk (*) indicates significant differences with the control and # with their irradiated control for the samples incubated with LPS (t-test, $p \leq 0.05$).

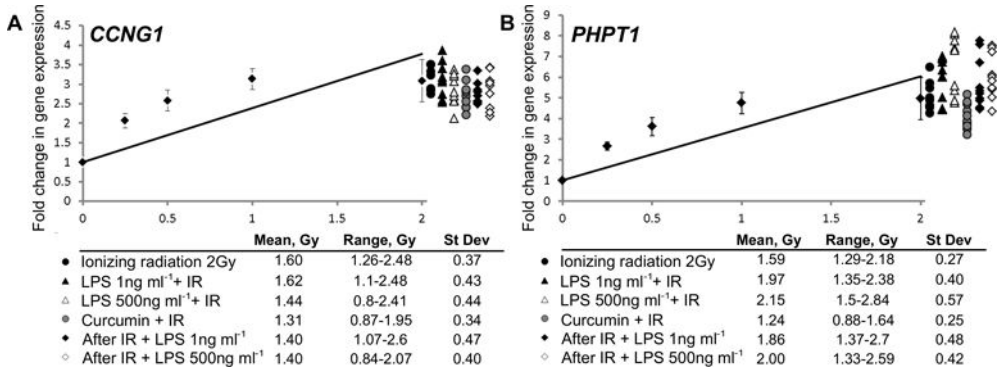


**Figure 7.**

Dose estimation curves for *CCNG1* and *PHPT1*. A linear fit was used with the corresponding equation and $R^2$ values being respectively y=1.3854X+1, $R^2$=0.25 and y=2.5231X+1 with $R^2$=0.5 for *CCNG1* and *PHPT1*. The fold of change for each condition tested is shown next to the 2 Gy dose point. Dose estimates for each condition (with or without LPS or Curcumin after 24 h post-exposure) were obtained using the linear equations above. Mean dose estimates, standard deviation and range of values for six conditions per donor are presented in the tables.

**Table 1**

Blood collection sampling times and prescribed doses, doses per fraction and number of doses for breast, lung, endometrial and prostate cancer patients.

| Cancer type | Radiotherapy scheme | Fractions | Total dose, Gy | Blood collection sampling times | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 |
| **Right Breast** | IMRT | 15 | 40.05 | Before first fraction | 0.5–2 h after first fraction 2.67 Gy | 24 h after first fraction 2.67 Gy | Before 5–6 fractions (10.68–13.35 Gy) | Before last fraction (37.38 Gy) |
| **Left Breast** | IMRT | 15 | 40.05 | Before first fraction | 0.5–2 h after first fraction 2.67 Gy | 24 h after first fraction 2.67 Gy | Before 5–6 fractions (10.68–13.35 Gy) | Before last fraction (37.38 Gy) |
| **Lung** | IMRT | 20 | 55 | Before first fraction | 0.5–2 h after first fraction 2.75 Gy | 24 h after first fraction 2.75 Gy | Before 5–6 fractions (11–13.75 Gy) | Before last fraction (52.25 Gy) |
| **Endometrium** | IMRT | 25 | 45 | Before first fraction | 0.5–2 h after first fraction 1.8 Gy | 24 h after first fraction 1.8 Gy | Before 5–6 fractions (7.2–9 Gy) | Before last fraction (43.2 Gy) |
| **Prostate** | IMRT | 20 | 60 | Before first fraction | 0.5–2 h after first fraction 3 Gy | 24 h after first fraction 3 Gy | Before 5–6 fractions (12–15 Gy) | Before last fraction (57 Gy) |

**Table 2**

Blood collection sampling times and prescribed doses, doses per fraction and number of doses for HNSCC and PC patient groups.

| Patient subgroup | Radiotherapy scheme | Dose rate | Blood collection sampling times | | | |
|---|---|---|---|---|---|---|
| | | | A | B | C | D |
| **HNSCC** | IMRT (LINAC; 6 or 20 MV photons) | 3 Gy/min | Before first fraction | After 5–7 fractions (1.6–3.0 Gy each): total 8–15 Gy | After last fraction (17–35 fractions): 51–70 Gy total dose | 4–6 weeks after last fraction |
| **PC** | IMRT (LINAC; 6 or 20 MV photons) | 3 Gy/min | Before first fraction | After 4–6 fractions (2 Gy each): total 8–12 Gy | After last fraction (39 fractions): 78 Gy total dose | 4–5 weeks after last fraction |
| **PC** | SABR (CyberKnife) | 9 Gy/min | Before first fraction | After first fraction: 7.25 Gy | After last fraction (5 fractions): 36.25 Gy total dose | 4–5 weeks after last fraction |