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

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ABSTRACT

For triage purposes following a nuclear accident, blood based gene expression biomarkers could provide quick results for of a large number of individuals compared to classic cytogenetic methods. Ionising radiation responsive genes are regulated through the DNA damage response including activation of multiple transcription factors. Modulators of these pathways could potentially affect the response of these biomarkers and consequently compromise accurate dose estimation calculations. In the present study, four potential confounding factors, namely cancer condition, gender, simulated bacterial infection (Lipopolysaccharides) and curcumin, an anti-inflammatory/anti-oxidant agent, were selected and their potential influence on the transcriptional response to radiation of the genes CCNG1 and PHPT1, two biomarkers of radiation exposure ex vivo, was assessed. First 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, LPS administration in blood irradiated ex vivo led to a significant modification of their 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 LPS 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.

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. 2015, 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. 2015) have 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 (Pernot et al. 2012, 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 apoptosis, 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 and Rad3 related which will phosphorylate and activate transcription factors responsible of inducing mRNA expression (Christmann and Kaina 2013). The main transcription factors regulated by the DDR are p53, NF-kappaB, breast cancer-associated protein 1 and Ap-1 (Christmann and Kaina 2013). p53 plays a major role activating the transcription of target genes involved in the DDR and being 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 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, p53-depenent genes such as MDM2, BBC3, FDXR, and CDKN1A have shown an affected transcriptional response to radiation in the presence of confounding factors such as lipopolysaccharides (LPS) and curcumin (Budworth et al. 2012, Soltani et al. 2016).

Cyclin G1 (CCNG1) and phosphohistidine phosphatase 1 (PHPT1) have been identified as genes responsive to IR in whole human blood irradiated ex vivo and in peripheral blood lymphocytes (Paul and Amundson 2008, Kabacik et al. 2011, Manning et al. 2013), which are both downstream genes of the transcription factors activated through the DDR. 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 role of confounding factors, which could affect DDR pathways and might module their responses, should be taken into consideration. In the present study we addressed this important issue 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 and that in our ex vivo settings, confounding factors can modify their expression and consequently could affect the estimation of the dose.

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 1h before being either mocked irradiated or exposed to a 2Gy X-rays dose (0.5Gy/min) or just after exposure (LPS only). Blood samples were kept at 37°C in an incubator with 5% CO_2 for either 2h or 24 h after exposure. After the incubation time, the blood was mixed with 1 ml of RNA later and stored at -80°C until being processed for RNA extraction.

On an independent experiment, peripheral blood from 5 healthy donors 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% CO2. One hour after irradiation, 1ng/ml of LPS was added and the RNA was isolated 2h later.

Radiotherapy patient samples

Patients with no previous radio- and/or chemotherapy treatments were recruited for the study. Blood samples from four breast, two endometrial, two lung and one prostate cancer (PC) patients, 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 fraction and the last fraction. The prescribed doses for each patient are described in Table 1.

Another three sub-groups of patients were recruited including seven head and neck squamous cell carcinoma (HNSCC) and six PC patients treated with IMRT using a LINAC and nine PC patients treated with Stereotactic Ablative Radiotherapy (SABR) using a Cyberknife treatment unit (neither surgery nor chemotherapy was applied to enrolled patients). 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.

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 10 healthy donors (eight men and twelve women, ages ranged from 25 to 60 years) was also collected 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 350ng 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 (Macherey and Nagel, Hoerdt, France) kit 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 1µl of the cDNA synthesis reaction together with three different sets of primers and fluorescent probes at 300nM 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 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 3.2×10^{-4} to 8.2×10^{-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 38B4 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 was collected and exposed to a range of X-Ray doses (0.25, 0.5, 1 and 2 Gy at a dose rate of 0.5Gy min^{-1}) and after 24 h post-exposure, RNA was extracted and the cDNA synthesized. 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.

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 reach 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 range of doses.

Basal CCNG1and PHPT1 expression level 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, fifteen prostate, four breast, two lung and two endometrial cancer patients). Interestingly, the results indicate a significant lower basal transcription level for CCNG1; conversely, an 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 radiotherapies 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, nine patients were recruited including four breast, two lung, two endometrial and one PC patients (Table 1). Peripheral blood from these patients was collected before the start of the treatment, 0.5-2 h after the first fraction, 24 h after the first fraction, before the fifth fraction and before the last fraction. When the data for all patients were analysed together (Fig. 3A), CCNG1 showed an upregulation of expression in vivo at all time points, peaking before the fifth and sixth fraction. Similar CCNG1 expression profile was observed in each cancer group analysed individually (Fig. 3B-E).Interestingly, PHPT1 shows a different expression profile with a significant drop in gene expression after a short period of time after the first fraction (0.5-2h) and before the last fraction (Fig. 3F). As CCNG1, PHPT1 also showed an upregulation at the third sampling point, before the fifth 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 three groups of cancer patients; HNSCC and PC patients treated with IMRT and a third group of PC patients treated with SABR. Peripheral blood from these patients was collected before the start of the treatment, after first/firsts 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 to assess the duration of the radiation-induced transcriptional regulation. The results show a similar profile for CCNG1 and PHPT1 in each group. Both genes are regulated in the IMRT groups after the first 5-7 fractions (Fig. 4), but PHPT1 stays upregulated at the end of the treatment in the HNSCC patients but not CCNG1. In the same group, CCNG1 shows 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) which is more similar 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. Blood was incubated with LPS (1 ng ml^{-1} or 500 ng ml^{-1}) or curcumin (15 µM) 1h before irradiation of a 2 Gy total dose or just after irradiation only for LPS. Expression of both genes was analysed at 2 h and 24 h after irradiation.

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. 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, LPS didn't affect the CCNG1 expression in blood irradiated and non-irradiated anymore (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}). After 24 h, LPS effect was persistent in non-irradiated samples showing a clear dose-dependency. In irradiated samples at 24 h, LPS showed a synergistic effect with irradiation, inducing a higher transcriptional response than the one observed with irradiation alone.

The effect of LPS (1 ng/ml) was also tested at a different time point, 1 h following 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 similar effect when added before irradiation but this was not the case for PHPT1. Significant differences between irradiated samples and irradiated samples in the presence of LPS were observed only at the lowest 50 mGy dose (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. However, curcumin already modulated PHPT1 at 2h post-exposure, showing a more pronounced effect at 24h in the irradiated and non-irradiated blood samples.

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 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 0.5Gy 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. The results indicated that LPS produces a modulation of the response to radiation at 24 h post-exposure mainly for PHPT1. As LPS is lowering the radiation response of CCNG1 at 2 h, the effect at 24 h is not very pronounced but it can be observed a slight underestimation of the dose estimated compared to the effect of irradiation alone for the highest LPS concentration (500 ng ml^{-1}) (Fig. 7A). However, LPS induced a higher gene expression response to radiation in PHPT1 at 24h 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 1h before or just after irradiation. For curcumin, its presence in blood leads to a lower response of both genes to ionizing radiation at 24h 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 act accordingly. Gene expression has proven to be a quick method for large scale of samples and offering 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. 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) and these differences in response 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 partial 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). Unlike total-body irradiated patients, radiotherapy patients in the present study were partially exposed to treat locally their tumours and received a low dose to the blood compared to total-body irradiated patients. So even under these partial 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.

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 70Gy. Although PHPT1 showed similar expression to CCNG1 profile after the first fractions (5-7 fractions, first week of treatment approximately for IMRT and 5 fractions for SABR treatment), differences on type and level of response were observed. PHPT1 presented a higher expression response compared to CCNG1 at different points analysed during the course of the radiation therapy in all cancer and treatment groups. Besides, PHPT1 showed an initial downregulation after a short period of time after radiation exposure (0.5-2h), but its expression rose over the basal level significantly 24 h after the first fractions (Fig. 4). Although we do not have an explanation for this observation, this fluctuation pattern of expression after radiation

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

Their expression wasn't significantly modified after one fraction in the SABR group (7.25 Gy). Surprisingly, CCNG1 showed a lack of response in 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. 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 of an adaptation of 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 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 doses estimates at least based on the data from the cohort of patients studied.

We then address 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 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 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 to simulate a bacterial infection inducing a cascade of immune and inflammatory reactions. 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). 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 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 the 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. 2015). In this study, CCNG1 expression was modulated by the addition of LPS 1h post-exposure, which modulate 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 pathway 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 try 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). In the present study, we were not able to use this regression analysis as it would not have allowed to calculate the doses when assessing the effect of the confounding factors on the response to radiation and we had to use a linear regression analysis

instead. 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 500ng/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 was present in the irradiated blood samples.

Conclusion

In conclusion, our data validated PHPT1 and CCNG1 as biomarkers of exposure in vivo, and they should be considered for gene expression based biological dosimetry tools in future studies. When dose assessments have to be provided in the context of infection/inflammation, presence of anti-inflammatory/anti-oxidant agents and other unknown conditions such as cancer, we have shown 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.

Figure. 1

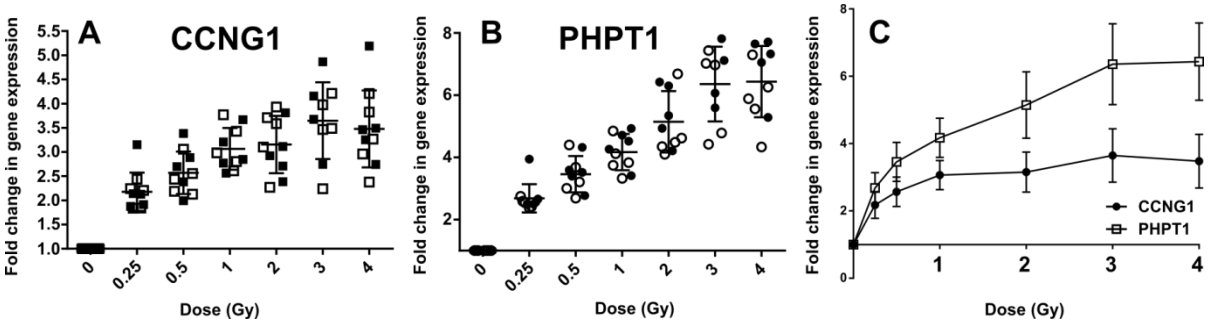


Figure 1. Multiplexed QRT-PCR gene expression changes of CCNG1 (A) and PHPT1 (B) 24h post-exposure in blood samples from 10 healthy donors (white symbols indicate five women and black symbols five men) exposed ex vivo to a range of X-ray doses (0.25, 0.5, 1, 2, 3, 4 Gy of X-rays at 0.5 Gy min^{-1}).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

Figure. 2

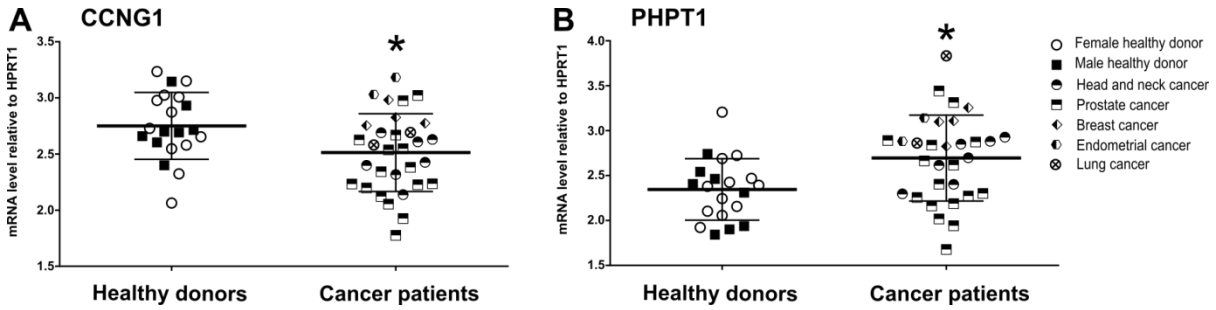


Figure 2. Range of basal gene expression level of CCNG1 and PHPT1 between healthy donors (eight men and twelve woman, ages ranged from 25 to 60 years) and cancer patients (head and neck, prostate, breast, endometrial and lung cancer). The data is presented as individual data points together with the 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 significant differences with the healthy donors group (t-test, $p \leq 0.05$).

Figure.3

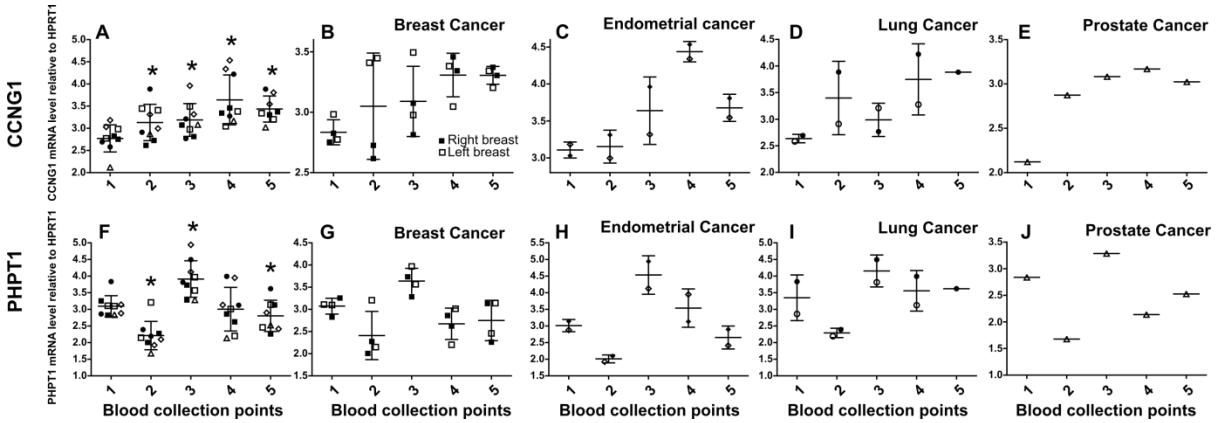


Fig3. Expression levels of CCNG1 and PHPT1 mRNA relative to HPRT1 in blood from four breast, two endometrial, two lung and one PC patients treated with IMRT. Blood was collected at 5 time points: before the start of the treatment (1), 0.5-2h (2) and 24h (3) after the first fraction, before the fifth (4) and 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

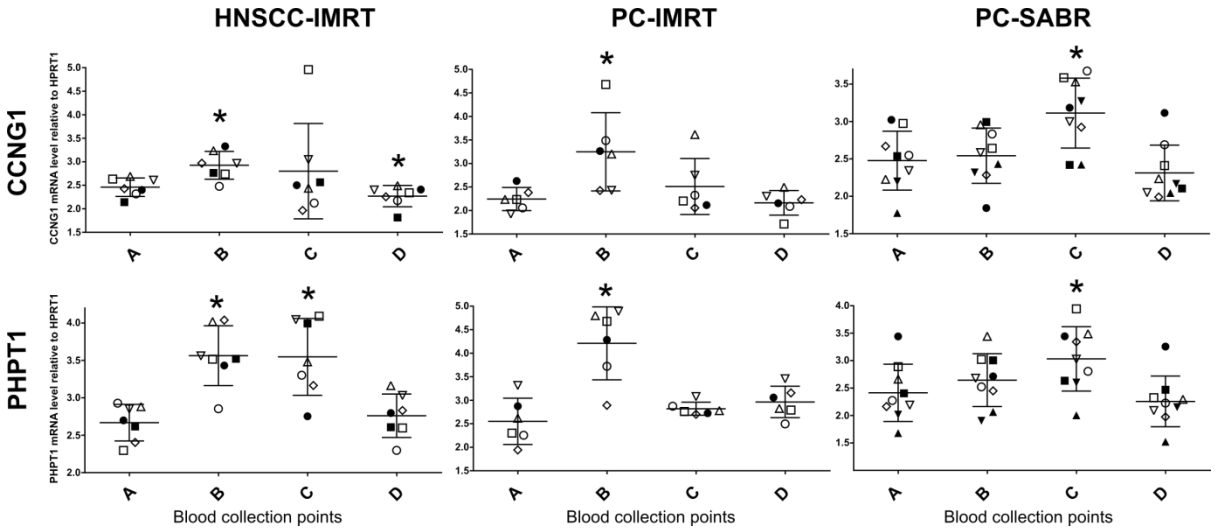


Fig4. Gene expression of CCNG1 and PHPT1 in blood from HNSCC as well as PC patients treated with IMRT and PC patients treated with SABR. Blood was collected before the start of the treatment (A), after 5/7 fractions for the IMRT groups and after the first for the SABR group, after the last fraction (C) 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

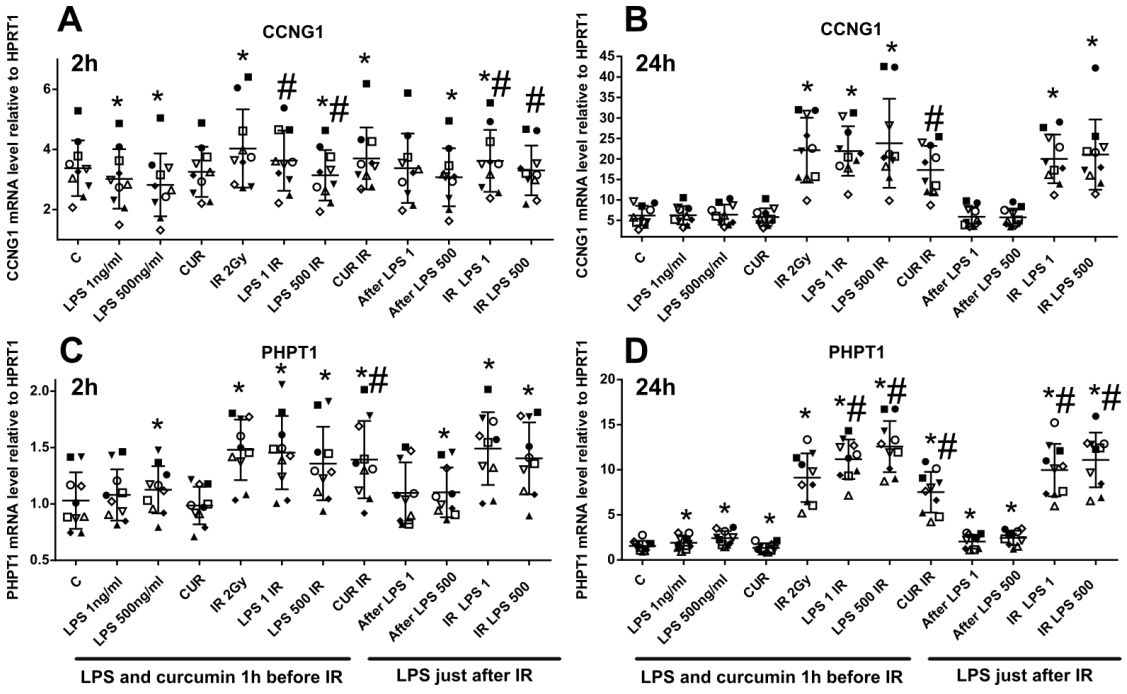


Figure 5. Gene expression of CCNG1 and PHPT1 in human blood irradiated and/or stimulated with LPS and curcumin ex vivo. Blood from 10 donors was incubated with two different concentration of LPS (1 or 500 ng ml^{-1}) or curcumin (15 µM) 1 h before irradiation (2Gy) or just after irradiation (only for LPS). Transcriptional expression of CCNG1 was analysed at 2h (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.

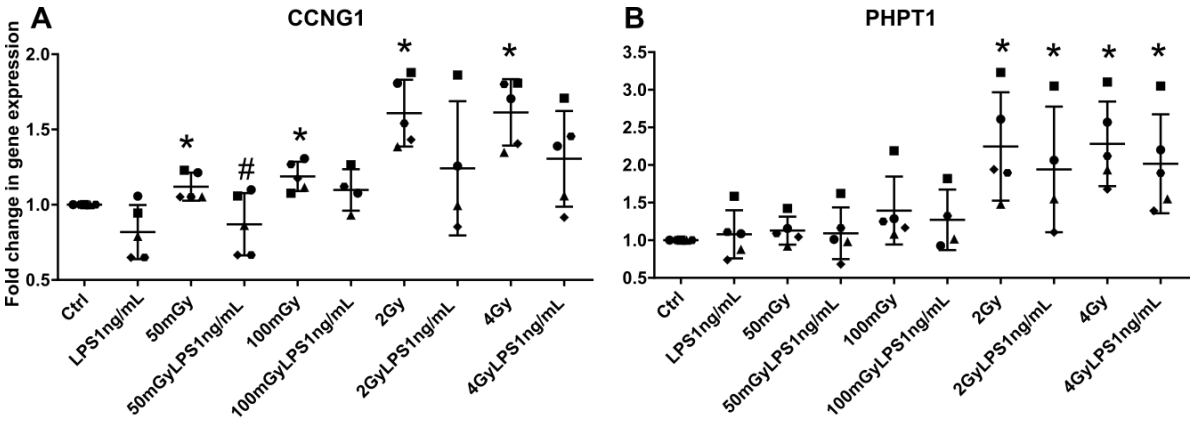
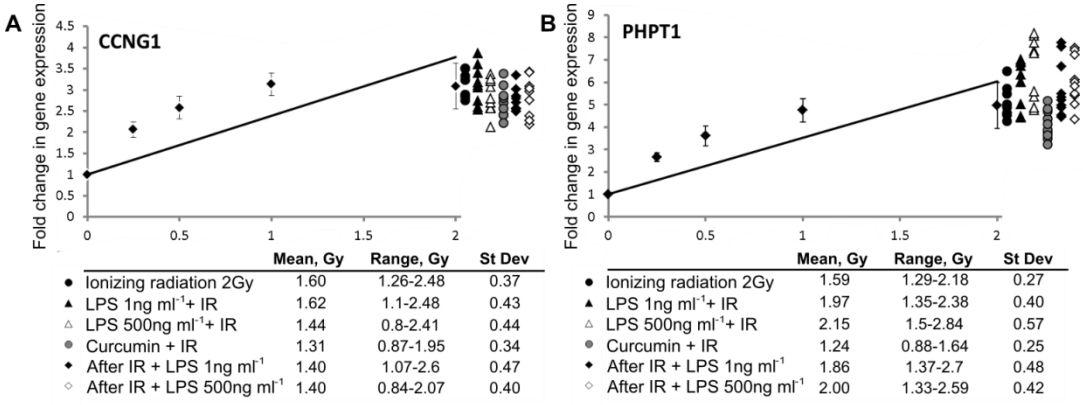


Figure 6. Gene expression of CCNG1 and PHPT1 in human blood 2h after exposure to a range of gamma-irradiation doses (^{60}Co source, doses of 50mGy, 100 mGy, 2 Gy and 4 Gy with a dose rate of 0.038 Gy min^{-1} or 1.47 Gy min^{-1} for doses below and above 1 Gy, respectively) and stimulated with LPS (1 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, $p \leq 0.05$).

Fig 7.



	Mean, Gy	Range, Gy	St Dev
Ionizing radiation 2Gy	1.60	1.26-2.48	0.37
LPS 1ng ml^{-1}+ IR	1.62	1.1-2.48	0.43
LPS 500ng ml^{-1}+ IR	1.44	0.8-2.41	0.44
Curcumin + IR	1.31	0.87-1.95	0.34
After IR + LPS 1ng ml^{-1}	1.40	1.07-2.6	0.47
After IR + LPS 500ng ml^{-1}	1.40	0.84-2.07	0.40

	Mean, Gy	Range, Gy	St Dev
Ionizing radiation 2Gy	1.59	1.29-2.18	0.27
LPS 1ng ml^{-1}+ IR	1.97	1.35-2.38	0.40
LPS 500ng ml^{-1}+ IR	2.15	1.5-2.84	0.57
Curcumin + IR	1.24	0.88-1.64	0.25
After IR + LPS 1ng ml^{-1}	1.86	1.37-2.7	0.48
After IR + LPS 500ng ml^{-1}	2.00	1.33-2.59	0.42

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 estimate 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. Prescribed doses, doses per fraction and number of doses for breast, lung, endometrial and PC patients,

Cancer type	Total dose, Gy	Fractions	Dose per fraction, Gy
Right Breast	40.05	15	2.67
Left Breast	40.05	15	2.67
Lung	55	20	2.75
Endometrium	45	25	1.8
Prostate	60	20	3

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

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