PUM1 and PGK1 are Favorable Housekeeping Genes over Established Biodosimetry-related Housekeeping Genes such as HPRT1, ITFG1, DPM1, MRPS5, 18S rRNA and Others after Radiation Exposure

Authors: Muhtadi, R., Stewart, S., Bunert, F., Fatanmi, O. O., Wise, S. Y., et al.

Source: Radiation Research, 201(5) : 487-498

Published By: Radiation Research Society

URL: https://doi.org/10.1667/RADE-23-00160.1

RADIATION RESEARCH **201**, 487–498 (2024)
0033-7587/24 $15.00

DOI: 10.1667/RADE-23-00160.1

PUM1 and *PGK1* are Favorable Housekeeping Genes over Established Biodosimetry-related Housekeeping Genes such as *HPRT1*, *ITFG1*, *DPM1*, *MRPS5*, *18S rRNA* and Others after Radiation Exposure

R. Muhtadi,[a,b] S. Stewart,[a,b] F. Bunert,[a,b] O. O. Fatanmi,[c,d] S. Y. Wise,[c,d] C. Gärtner,[e] S. Motzke,[e] C. Ruf,[f] P. Ostheim,[a] S. Schüle,[a] D. Schwanke,[a] V. K. Singh,[c,d,1] M. Port,[a] M. Abend[a,1]

[a] Bundeswehr Institute of Radiobiology, Munich, Germany; [b] Technical University Munich, Munich, Germany; [c] Division of Radioprotectants, Department of Pharmacology and Molecular Therapeutics, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; [d] Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; [e] Microfluidic ChipShop GmbH, Jena, Germany; [f] Department of Urology, Federal Armed Services Hospital Ulm, Ulm, Germany

Muhtadi R, Stewart S, Bunert F, Fatanmi OO, Wise SY, Gärtner C, Motzke S, Ruf C, Ostheim P, Schüle S, Schwanke D, Singh VK, Port M, Abend M. *PUM1* and *PGK1* are Favorable Housekeeping Genes over Established Biodosimetry-related Housekeeping Genes such as *HPRT1*, *ITFG1*, *DPM1*, *MRPS5*, *18S rRNA* and Others after Radiation Exposure. Radiat Res. 201, 487–498 (2024).

In gene expression (GE) studies, housekeeping genes (HKGs) are required for normalization purposes. In large-scale inter-laboratory comparison studies, significant differences in dose estimates are reported and divergent HKGs are employed by the teams. Among them, the *18S* r*RNA* HKG is known for its robustness. However, the high abundance of *18S* r*RNA* copy numbers requires dilution, which is time-consuming and a possible source of errors. This study was conducted to identify the most promising HKGs showing the least radiation-induced GE variance after radiation exposure. In the screening stage of this study, 35 HKGs were analyzed. This included selected HKGs (*ITFG1, MRPS5*, and *DPM1*) used in large-scale biodosimetry studies which were not covered on an additionally employed pre-designed 96-well platform comprising another 32 HKGs used for different exposures. Altogether 41 samples were examined, including 27 ex vivo X-ray irradiated blood samples (0, 0.5, 4 Gy), six X-irradiated samples (0, 0.5, 5 Gy) from two cell lines (U118, A549), as well as eight non-irradiated tissue samples to encompass multiple biological entities. In the independent validation stage, the most suitable candidate genes were examined from another 257 blood samples, taking advantage of already stored material originating from three studies. These comprise 100 blood samples from ex vivo X-ray irradiated (0–4 Gy) healthy donors, 68 blood samples from 5.8 Gy irradiated (cobalt-60) *Rhesus macaques* (RM) ($LD_{29/60}$) collected 0–60 days postirradiation, and 89 blood samples from chemotherapy-(CTx) treated breast tumor patients. CTx and radiation-induced GE changes in previous studies appeared comparable. RNA was isolated, converted into cDNA, and GE was quantified employing TaqMan assays and quantitative RT-PCR. We calculated the standard deviation (SD) and the interquartile range (IQR) as measures of GE variance using raw cycle threshold (Ct) values and ranked the HKGs accordingly. Dose, time, age, and sex-dependent GE changes were examined employing the parametrical t-test and non-parametrical Kruskal Wallis test, as well as linear regression analysis. Generally, similar ranking results evolved using either SD or IQR GE measures of variance, indicating a tight distribution of GE values. *PUM1* and *PGK1* showed the lowest variance among the first ten most suitable genes in the screening phase. *MRPL19* revealed low variance among the first ten most suitable genes in the screening phase only for blood and cells, but certain comparisons indicated a weak association of *MRPL19* with dose ($P = 0.02–0.09$). In the validation phase, these results could be confirmed. Here, IQR Ct values from, e.g., X-irradiated blood samples were 0.6 raw Ct values for *PUM1* and *PGK1*, which is considered to represent GE differences as expected due to methodological variance. Overall, when compared, the GE variance of both genes was either comparable or lower compared to *18S* r*RNA*. Compared with the IQR GE values of *PUM1* and *PGKI*, twofold–fivefold increased values were calculated for the biodosimetry HKG *HPRT1,* and comparable values were calculated for biodosimetry HKGs *ITFG1, MRPS5,* and *DPM1*. Significant dose-dependent associations were found for *ITFG1* and *MRPS5* ($P = 0.001–0.07$) and widely absent or weak ($P = 0.02–0.07$) for *HPRT1* and *DPM1*. In summary, *PUM1* and *PGK1* appeared most promising for radiation exposure studies among the 35 HKGs examined, considering GE variance and adverse associations of GE with dose.

INTRODUCTION

In radiological and nuclear scenarios, providing an early, high-throughput, and point-of-care (POC) diagnosis is crucial to predict the late-occurring hematologic acute radiation syndrome (H-ARS) (*1*). This way, possible risk groups can be identified, sorting the unexposed from radiation-

[1] Co-corresponding authors: Michael Abend, email: michaelabend@bundeswehr.org; Vijay K. Singh, email: vijay.singh@usuhs.edu.

exposed individuals who will develop mild or more severe degrees of H-ARS (*2*, *3*). In such scenarios, examining the transcriptional response by gene expression profiling is a suitable approach (*4*). Quantitative real-time PCR (qRT-PCR) is an appropriate method in GE analysis due to its high sensitivity, high throughput capabilities, and specific detection of target genes (*5*). Previous research from our group indicated that gene expression changes of a radiation-responsive gene set (*FDXR, DDB2, POU2AF1,* and *WNT3*) could predict the severity of H-ARS (*1*, *6*, *7*).

In GE studies, housekeeping genes (HKGs) are required for normalization purposes to adjust RNA yield differences between samples, enabling comparisons (*8*). HKGs are usually necessary for the maintenance of basic cellular functions. Ideally, HKGs should be constitutively expressed in cells of various tissues regardless of their function or state in the organism as well as the influence of different experimental conditions (*9*), including after irradiation or chemotherapy (*10*). A large number of HKGs are identified in the literature. The most commonly used HKG for radiation exposure include *HPRT1*, *ITFG1*, *UBC*, *DPM1*, *MRPS5*, *GAPDH,* and *18S rRNA* (*11*). Despite its prolonged use, *18S rRNA* has some limitations in use. The high abundance of *18S rRNA* copy numbers requires dilution, which is time-consuming and a possible source of dilution error (*12*).

Our study aimed to examine and compare well-known HKGs systematically, including those mentioned above, particularly in the context of radiation-induced gene expression alterations. We also aimed to identify HKGs that do not require dilution steps or have specific biological dependencies to simplify GE analysis. This would result in a ready-to-use normalization across various sample types and gene expression platforms, such as qRT-PCR, microfluidic cards, low-density arrays (LDA), and 12K Open Arrays.

We examined 35 known HKGs in a two-stage study design; all 35 HKGs were systematically investigated in a screening stage employing a pre-designed 96-well platform in addition to other used biodosimetry HKGs followed by an independent validation step using qRT-PCR of the most promising HKGs on another set of biological samples (Fig. 1).

MATERIALS AND METHODS

Stage I: Screening

Thirty-two known HKGs were examined in a screening process employing a pre-designed 96-well qRT-PCR format (TaqMan™ Array Human Endogenous Controls Plate, Fast 96-well, Thermofisher). The set of genes (Supplementary Table S1;[2] https://doi.org/10.1667/RADE-23-00160.1.S1) has been selected by Thermofisher from literature searches. Three additional widely used biodosimetry-related HKGs (*ITFG1*, *MPRS5*, *DPM1*) missing on the pre-designed plate were also examined. The screening stage was performed on different models, such as in vitro cell lines, ex vivo blood samples, and various tissue types [Fig. 1 (top part), and see following section].

[2] Editor's note. The online version of this article (DOI: https://doi.org/10.1667/RADE-23-0016.1) contains supplementary information that is available to all authorized users.

Sample Collection and RNA Isolation

Whole blood samples from three healthy donors (two males, one female, mean age: 33.3 ± 8 years) were collected into: (*1*) PAXgene® Blood RNA tubes (BD Diagnostics, PreAnalytiX GmbH, Hombrechtikon, Switzerland) and (*2*) Vacutainer® EDTA blood tubes (SARSTEDT, Germany), irradiated at room temperature with 0, 0.5, or 4 Gy, incubated for 6 h at 37°C, and then 2.5 ml were pipetted into PAXgene® Blood RNA tubes. The PAXgene tubes were gently inverted (10 times), stored at room temperature overnight, then at –20°C. RNA was isolated before and 6 h postirradiation. Incubation was done at 37°C, to ensure biological response time required for GE changes to occur (*13*) using different chemistries (see below).

RNA from PAXgene® Blood RNA tubes was semi-automatically isolated using the QIAsymphony® SP (Qiagen, Hilden, Germany) following use of the QIAsymphony® PAXgene Blood RNA Kit (Qiagen, Hilden, Germany). In brief, samples in PAXgene® Blood RNA tubes were manually thawed, centrifuged, the supernatant discarded, and pellets resuspended with proteinase K augmented buffers before being placed in the QIAsymphony® SP. Here, RNA binds to the silica surface of MagAttractes magnetic particles. After several washing steps, DNase I and an additional proteinase K digestion, RNA was automatically isolated and eluted in 80 µl buffer BR5. Finally, the RNA tubes were heated to 65°C for 5 min and then stored at –20°C until quantitative and qualitative analysis was performed.

Besides automated RNA isolation, RNA was isolated from EDTA blood tubes using a preliminary microfluidic (MF) slide (designed in cooperation with microfluidic ChipShop, Jena, Germany) connected to a peristaltic pump (Peristaltic Pump Relgo Digital, Ismatec Fisher Scientific, Schwerte, Germany). In brief, 250 µL EDTA blood was mixed with Proteinase K and lysis buffer, followed by an incubation time of 10 min prior to loading onto the slide. After several washing steps and DNase I digestion, the RNA was eluted in 80 µL of elution buffer. Afterward, the samples were stored at –20°C until further processing. It is important to note that the same donors were used in both methods to ensure consistency and comparability of the RNA samples.

The Glioblastoma (U118) and the non-small cell lung cancer (A549) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco/Life Technologies, Germany) supplemented with GlutaMAX (Gibco/Life Technologies, Germany) and 10% heat-inactivated fetal bovine serum (Bio&SELL GmbH, Germany). Cell lines were cultured humidified 95% air and 5% CO_2 at 37°C. When cells reached 85% confluence, they were irradiated with 0, 0.5, and 5 Gy and incubated for 6 h to ensure the biological response time required for GE changes to occur (*13*, *14*). RNA was isolated using the mirVana Kit (Life Technologies, Darmstadt, Germany). In brief, after trypsinization (0.05% Trypsin, Gibco/Life Technologies, Germany), washing with Dulbecco's Phosphate Buffered Saline (DPBS, Gibco/Life Technologies, Germany), and centrifugation, cells were lysed (Proteinase K) by adding the Lysis/Binding solution from the mirVana Kit. Total RNA, including small RNA species, was isolated by combining a Phenol-Chloroform (Ambion, Germany) RNA precipitation with further processing using a silica membrane. After several washing steps, DNA residuals were digested on the membrane using the RNAse free DNAse Set (Qiagen, Hilden, Germany). RNA was eluted into a collection tube and frozen at –20°C. Commercially available Human RNA from different tissues was used [liver (Catalogue no. QS0617 and AM7960, ThermoFisher, Germany), kidney (Catalogue no. QS0616, ThermoFisher, Germany), colon (Catalogue no. QS0613 and AM7986, ThermoFisher, Germany) and testis (seminoma biopsies from three males, aged 29–35 years, Table 1, Fig. 1)].

Irradiation

A single X-ray dose was delivered ex vivo at 37°C. X rays were delivered using 3-mm beryllium and 3-mm aluminum filters to give a mean photon energy of 100 keV (Maxishot SPE cabin; Yxlon, Hamburg, Germany). The absorbed doses were measured using a UNIDOS webline 10021 dosimeter (PTW, Freiburg, Germany). The dose rate was approximately 1.0 Gy/min at 13 mA and an accelerating potential of 240 kV (maximum photon energy of 240 keV).

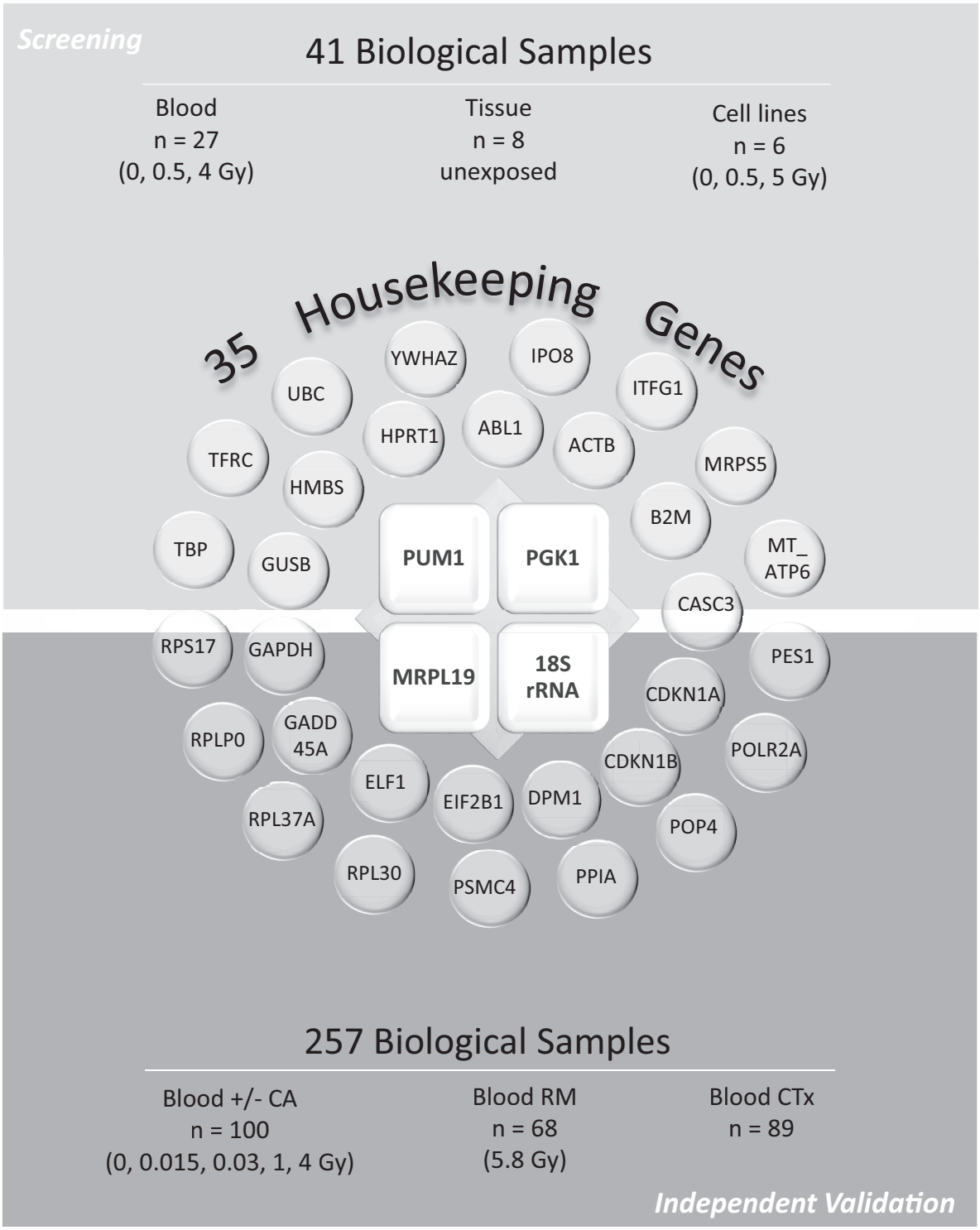

FIG. 1. Study overview, starting with a screening phase (top section), where 35 known HKGs (middle part) were examined in 41 biological samples. Most suitable HKGs are highlighted in the centre of the middle section, and were forwarded for independent validation (bottom section) on another 257 biological samples. Abbreviations: CA = contrast agent; RM = *Rhesus* M*acaques*; CTx = chemotherapy.

RNA Quality and Quantity Control

The quantity and quality of isolated total RNA were measured spectrophotometrically (NanoDrop, PeqLab Biotechnology, Erlangen, Germany). RNA integrity was assessed by the 4200 TapeStation System (Agilent Technologies, Santa Clara, CA), and DNA contamination was inspected by conventional PCR using an actin primer pair. RNA specimens with a ratio of A260/A280 nm $\geq$2.0 and RNA integrity number (RIN) $\geq$8 were processed for qRT-PCR analysis.

cDNA Synthesis and qRT-PCR

Aliquots of total RNA (1 μg) were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems

TABLE 1

Overview of the Examined Genes and Biological Samples as Well as Their Origin Used in the Screening (left side) and the Independent Validation Phase (right side)

Screening (Stage I)	*Validation (Stage II)*
35 genes	**4 genes** (*18S rRNA, PUM1, MRPL19, PGK1*)
41 samples	**257 samples**, total
Blood samples (n = 27)	Blood samples +/- contrast agent (n = 100)
15x 0 Gy	20x 0 Gy +/- contrast agent
6x 0.5 Gy	20x 0.015 Gy +/- contrast agent
6x 4 Gy	20x 0.03 Gy +/- contrast agent
	20x 1 Gy +/- contrast agent
	20x 4 Gy +/- contrast agent
Tissue samples (unexposed, n = 8)	*Rhesus Macaques* , pre & post ionizing radiation (n = 68)
2x liver	13x 7 days pre ionizing radiation
1x kidney	13x 1 day post ionizing radiation
2x colon	13x 2 day post ionizing radiation
3x testis	13x 3 day post ionizing radiation
	8x 35 day post ionizing radiation
	8x 60 day post ionizing radiation
Cell culture samples U 118 & A549 (n = 6)	Breast cancer patients, CTx (n = 89)
2x 0 Gy	46 without CTx
2x 0.5 Gy	43 with CTx
2x 5 Gy	

Abbreviation: +/- with and without; CTx = Chemotherapy.

by ThermoFisher Scientific, Germany). Equal amounts of template cDNA (10 ng per reaction for each gene) were mixed with the TaqMan® Universal PCR Master (Applied Biosystems by Thermo Fisher Scientific, Germany), pipetted in the pre-designed TaqMan™ Express Human Endogenous Control Plate, and measured in triplicates. The template cDNA for the spotted *18S rRNA* HKG (Hs 99999901_s1) had to be diluted to 0.001ng per reaction. Three other housekeeping genes, *ITFG1* (Hs00229263_m1), *MRPS5* (Hs00921602_m1), and *DPM1* (Hs00187270_m1), were examined using the same chemistry and conventional 96-well plates (Thermo Fisher Scientific, Germany). All PCR reactions ran using a conventional amplification protocol (cycling parameters were 2 min at 50°C, 10 min at 95°C then 40 cycles of 1 min at 95°C and 1 min at 60°C) on a QuantStudio™ 12K OA Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA). Raw Ct values representing inverse $\log^2$ transformed RNA copy numbers were calculated.

Stage II: Independent Validation of Candidate Genes

Sample collection and RNA isolation. The independent validation of the most promising candidate genes was performed on stored material originating from three previous studies examined for other purposes (*10*) (Table 1; Fig. 1, bottom part):

- In a previous study, an iodinated contrast agent was added to irradiated blood samples to investigate its impact as a confounding factor on radiation-responsive genes used for biodosimetry purposes (Schüle et al., work published in this Focus Issue, 2024). For this study, whole blood samples from ten healthy donors (5 males, 5 females, mean age: 28 ± 2 years) were irradiated with 0, 0.015, 0.03, 1, and 4 Gy with or without the addition of iodinated contrast agent (0.016 ml contrast agent/ml blood, Ultravist®, Bayer Vital GmbH, Leverkusen, Germany) to the blood prior to exposure. Blood samples in EDTA tubes were incubated at 37°C for 8 h post-irradiation, and 2.5 ml aliquots were transferred into PAXgene tubes and stored at –80°C for further analysis. Irradiation was done as mentioned in Stage I.
- In a previous study, irradiated *Rhesus macaques* (*Macaca mulatta*) were examined (for validation purposes of a gene set that predicts the severity degree of the hematological ARS) (Schwanke et al., work published in this Focus Issue, 2024). For this study, whole blood samples from 13 *Rhesus macaques* (10 males, 3 females) were irradiated with 5.8 Gy ($LD_{29/60}$) using the high-level cobalt-60 source. Eleven *Rhesus macaques* were treated with gamma-tocotrienol (a radiation countermeasure) and two with placebo. The irradiation was done at the Armed Forces Radiobiology Research Institute/Uniformed Services University of the Health Sciences, Bethesda, MD (Schwanke et al., work published in this Focus Issue, 2024). Peripheral whole blood (1 ml) of *Rhesus macaques* was collected into PAXgene®-Blood-RNA-tubes preirradiation and 1, 2, 3, 35, and 60 days postirradiation. Subsequently, blood samples were stored at –80°C and then shipped on dry ice to the Bundeswehr Institute of Radiobiology, Germany.
- In a previous study, it was examined whether chemotherapy-treated patients (CTx, Cyclophosphamide 600 mg/m² body surface area, Epirubicin 90 mg/m² body surface area) might be suitable as a surrogate cohort of rare whole body irradiated individuals (*10*) (Schüle et al., work published in this Focus Issue, 2024). For this study, whole blood from 46 female patients (aged 31–79 years)

TABLE 2

Housekeeping Genes (HKGs) Ranked from Lowest to Highest Variance in Gene Expression (GE) Measurements, Represented by Raw Ct Values, Across Various Biological Materials for the Screening, (part A) and Validation (part B) Phases

A) Screening

Ranking	Blood				Cells				Tissues				All in one			
pole #	Genes	IQR	Genes	SD	Genes	IQR	Genes	SD	Genes	IQR	Genes	SD	Genes	IQR	Genes	SD
1	*PSMC4*	0.4	***PUM1***	0.5	***MRPL19***	0.4	***PGK1***	0.5	*ABL1*	0.1	*DPM1*	0.1	***PUM1***	0.5	***PUM1***	0.6
2	***PGK1***	0.6	*ABL1*	0.5	***PGK1***	0.6	*MRPS5*	0.6	*CASC3*	0.1	*ITFG1*	0.2	***PGK1***	0.7	***PGK1***	0.7
3	***PUM1***	0.6	*PSMC4*	0.5	*GUSB*	0.8	***MRPL19***	0.6	*ITFG1*	0.1	*CASC3*	0.2	*UBC*	0.8	*RPL37A*	0.9
4	*RPLP0*	0.6	*ELF1*	0.6	*MT-ATP6*	0.8	*GADD45A*	0.6	*PPIA*	0.2	*ABL1*	0.2	*GAPDH*	1.0	*CDKN1B*	0.9
5	*YWHAZ*	0.6	*PES1*	0.6	*B2M*	0.8	*MT-ATP6*	0.7	*MT-ATP6*	0.2	*PPIA*	0.3	*POLR2A*	1.1	*UBC*	1.0
6	*ABL1*	0.6	*MRPS5*	0.6	*GADD45A*	0.8	*PPIA*	0.7	*DPM1*	0.2	*MT-ATP6*	0.3	*RPLP0*	1.2	*POLR2A*	1.0
7	*ELF1*	0.6	***MRPL19***	0.6	*MRPS5*	0.8	*GAPDH*	0.8	*RPL37A*	0.3	*IPO8*	0.3	*YWHAZ*	1.2	*RPLP0*	1.0
8	*MRPS5*	0.6	***PGK1***	0.7	*PSMC4*	0.9	*HMBS*	0.8	*PES1*	0.3	*MRPS5*	0.3	*CASC3*	1.2	*CASC3*	1.0
9	*DPM1*	0.8	*PPIA*	0.7	*IPO8*	1.0	***PUM1***	0.8	***PUM1***	0.3	*GAPDH*	0.4	*RPL37A*	1.2	***18S rRNA***	1.1
10	*B2M*	0.8	*DPM1*	0.7	*PPIA*	1.0	*PSMC4*	0.9	*IPO8*	0.4	*RPLP0*	0.4	***18S rRNA***	1.2	*MRPS5*	1.1
11	***MRPL19***	0.8	*CDKN1B*	0.7	*CDKN1A*	1.0	*GUSB*	0.9	*TFRC*	0.4	***PGK1***	0.4	*GUSB*	1.3	*DPM1*	1.1
12	*UBC*	0.9	*POLR2A*	0.7	*CDKN1B*	1.1	*RPS17*	0.9	*CDKN1A*	0.4	*TFRC*	0.4	*IPO8*	1.3	*ITFG1*	1.2
13	*ITFG1*	0.9	*RPLP0*	0.7	*RPL30*	1.1	***18S rRNA***	1.0	*GAPDH*	0.4	*ELF1*	0.4	*PSMC4*	1.4	*YWHAZ*	1.2
14	*PPIA*	0.9	*YWHAZ*	0.7	*TFRC*	1.2	*ELF1*	1.0	*RPLP0*	0.4	***PUM1***	0.4	*CDKN1B*	1.4	*GADD45A*	1.2
15	*RPL37A*	0.9	*UBC*	0.7	*ITFG1*	1.2	*CDKN1A*	1.0	*EIF2B1*	0.4	*EIF2B1*	0.4	*ITFG1*	1.5	*CDKN1A*	1.2
16	*POLR2A*	1.0	*CASC3*	0.8	*DPM1*	1.2	*RPL30*	1.0	*MRPS5*	0.4	*HMBS*	0.4	*TBP*	1.5	*GUSB*	1.2
17	*GAPDH*	1.1	*GUSB*	0.8	*GAPDH*	1.3	*CDKN1B*	1.0	*POLR2A*	0.5	*CDKN1A*	0.5	*RPS17*	1.6	*TFRC*	1.3
18	*PES1*	1.1	*GADD45A*	0.8	*HMBS*	1.3	*IPO8*	1.0	*HMBS*	0.5	*PSMC4*	0.5	*PES1*	1.7	*PSMC4*	1.3
19	*TBP*	1.1	*TFRC*	0.8	*POP4*	1.3	*RPLP0*	1.0	***PGK1***	0.5	*RPL37A*	0.5	*DPM1*	1.7	*IPO8*	1.3
20	*CASC3*	1.1	*POP4*	0.9	***PUM1***	1.4	*B2M*	1.1	*ELF1*	0.6	***MRPL19***	0.5	*B2M*	1.8	*EIF2B1*	1.3
21	*GUSB*	1.1	*ITFG1*	0.9	*RPS17*	1.5	*DPM1*	1.1	*CDKN1B*	0.6	*CDKN1B*	0.6	*TFRC*	1.9	*B2M*	1.3
22	*POP4*	1.1	*B2M*	0.9	*ELF1*	1.5	*ITFG1*	1.1	*PSMC4*	0.7	*HPRT1*	0.6	*ELF1*	1.9	*ELF1*	1.3
23	*CDKN1B*	1.2	*RPL37A*	0.9	*UBC*	1.6	*PES1*	1.1	***MRPL19***	0.7	*RPS17*	0.6	*GADD45A*	1.9	*PES1*	1.4
24	*IPO8*	1.2	*GAPDH*	1.0	*RPLP0*	1.7	*UBC*	1.1	*HPRT1*	0.8	*POLR2A*	0.6	*MRPS5*	1.9	***MRPL19***	1.4
25	*GADD45A*	1.3	*HPRT1*	1.0	*PES1*	1.7	*EIF2B1*	1.1	*RPL30*	0.9	*POP4*	0.7	*ACTB*	2.0	*TBP*	1.4
26	***18S rRNA***	1.3	*TBP*	1.0	***18S rRNA***	1.7	*POP4*	1.2	*RPS17*	1.0	*GUSB*	0.7	*EIF2B1*	2.1	*RPS17*	1.4
27	*HPRT1*	1.4	*EIF2B1*	1.0	*CASC3*	1.8	*RPL37A*	1.3	*GUSB*	1.0	*PES1*	0.8	*CDKN1A*	2.1	*ABL1*	1.4
28	*HMBS*	1.4	***18S rRNA***	1.1	*TBP*	1.8	*TFRC*	1.3	*POP4*	1.0	*ACTB*	0.9	***MRPL19***	2.3	*PPIA*	1.5
29	*TFRC*	1.5	*HMBS*	1.1	*POLR2A*	1.8	*ABL1*	1.5	*ACTB*	1.0	*UBC*	0.9	*HPRT1*	2.3	*POP4*	1.6
30	*RPS17*	1.6	*CDKN1A*	1.1	*EIF2B1*	2.0	*YWHAZ*	1.5	***18S rRNA***	1.1	*B2M*	1.1	*POP4*	2.3	*ACTB*	1.7
31	*ACTB*	1.6	*IPO8*	1.1	*YWHAZ*	2.0	*ACTB*	1.5	*UBC*	1.5	*RPL30*	1.1	*MT-ATP6*	2.4	*GAPDH*	1.7
32	*EIF2B1*	1.6	*RPS17*	1.3	*ACTB*	2.3	*CASC3*	1.7	*B2M*	1.8	*GADD45A*	1.2	*ABL1*	2.5	*HMBS*	1.9
33	*CDKN1A*	1.9	*ACTB*	1.6	*RPL37A*	2.3	*POLR2A*	1.7	*TBP*	2.2	*TBP*	1.2	*PPIA*	2.5	*HPRT1*	2.1
34	*MT-ATP6*	2.9	*MT-ATP6*	2.1	*ABL1*	2.7	*HPRT1*	1.9	*GADD45A*	2.2	***18S rRNA***	1.3	*RPL30*	3.0	*MT-ATP6*	2.4
35	*RPL30*	4.7	*RPL30*	2.9	*HPRT1*	3.3	*TBP*	2.4	*YWHAZ*	3.5	*YWHAZ*	2.0	*HMBS*	3.1	*RPL30*	2.4

B) Validation

Ranking	Blood sample with/without contrast agent				Breast cancer with/without CTx				*Rhesus macaques* pre/post IR				All in one			
Pole #	Genes	IQR	Genes	SD	Genes	IQR	Genes	SD	Genes	IQR	Genes	SD	Genes	IQR	Genes	SD
1	*PUM1*	0.5	*18S rRNA*	0.4	*PGK1*	0.8	*PGK1*	0.6	*PUM1*	0.8	*18S rRNA*	0.7	*MRPL19*	1	*PGK1*	0.8
2	*MRPL19*	0.5	*PUM1*	0.5	*PUM1*	0.8	*PUM1*	0.7	*PGK1*	0.9	*PUM1*	0.8	*18S rRNA*	1	*MRPL19*	0.8
3	*18S rRNA*	0.5	*PGK1*	0.5	*MRPL19*	0.9	*MRPL19*	0.8	*18S rRNA*	1.0	*PGK1*	0.8	*PGK1*	1	*18S rRNA*	0.9
4	*PGK1*	0.8	*MRPL19*	0.6	*18S rRNA*	1.2	*18S rRNA*	0.9	*MRPL19*	-	*MRPL19*	-	*PUM1*	1.1	*PUM1*	0.9

Notes. Variance measures, such as the interquartile range (IQR) and standard deviation (SD), were computed. The materials used in the screening phase (A, upper part) include combined blood samples (Paxgene, EDTA->Paxgene, and EDTA->MF; N = 27), combined cell culture samples (U118 and A549 sham and irradiated; N = 6), and a combination of various unexposed tissues (liver, kidney, colon, and testis; N = 8). The last column, "All in one" means all different materials [blood, cells, and tissues (N = 41)] combined. In the upper part, the most promising HKGs are shaded in dark grey with HKG names in white letters, while commonly used biodosimetry HKGs are shaded in light grey with HKG names in dark letters. In the validation phase (B, lower part) blood samples with/without contrast agent; N = 100, blood samples pre and postirradiation; N = 68 and blood samples from breast cancer patients with/without CTx; N = 89 were used. The last column, "All in one" means all three groups (N = 257) combined.

was collected into PAXgene® Blood RNA tubes prior to and four days after administration of CTx. Patients were treated with Myelo 001 to mitigate CTx-induced hematological changes or received a placebo over the four days before blood sample collection.

RNA isolation of the three groups was done using QIAsymphony® PAXgene Blood RNA Kit (Qiagen, Hilden, Germany), as mentioned above. Quantity and quality control were assessed as mentioned in the screening stage.

cDNA Synthesis and qRT-PCR

cDNA synthesis and qRT-PCR were performed as in Stage I, using specific TaqMan assays for the detection of four genes, namely *PUM1* (Hs00206469_m1), *MRPL19* (Hs00608519_m1), *PGK1* (Hs99999906_m1), and *18S rRNA* (Hs99999901_s1). The cDNA for the detection of the *18S rRNA* HKG was diluted to 0.001 ng per reaction, whereas the cDNA amount for the other genes was 10 ng per reaction.

All experimental work was performed according to the standard operating procedures implemented in our laboratory since 2008 when the Bundeswehr Institute of Radiobiology became DIN-certified by TÜV Süd München, Germany (DIN EN ISO 9001/2008).

Statistics in Stages I and II

Results were presented as mean values ± standard deviation (SD) (Supplementary Table S2A; https://doi.org/10.1667/RADE-23-00160.1S2). Genes were ranked from the lowest to the highest using two measures of variance: the interquartile range (IQR, representing 50% of the data lying within the 25th–75th quintile) and the standard deviation (including all measurements lying within one SD) (Table 2). Group comparisons were performed employing parametrical (student *t*-test) and non-parametrical tests (Kruskal-Wallis) where applicable. Dose, time, sex, and age-response relationships were examined using linear regression analysis. P values < 0.05 were considered significant, and values between 0.05 and 0.1 were defined as borderline significant (Supplementary Table S2C and Table 4). Undesired associations of gene expression changes were examined, and significant P values would indicate unsuitability as a housekeeping gene. R^2 is a statistical measure of variance used in regression analysis to assess how well the mathematical model depicts the measured data. It reveals values between 0 and 1. An R^2 close to 1 means the model fits the data well, and R^2 close to 0 means the model does not fit the data well.

P values and descriptive statistics [n, min, max, mean, and SD (Supplementary Table 2B; https://doi.org/10.1667/RADE-23-00160.1.S2)] were calculated using SAS (release 9.4, Cary, NC). The

graphical illustrations were created utilizing SPW (SigmaPlot, Version 14.5, Jandel Scientific, Erkrath, Germany) and PowerPoint (Microsoft, Redmond, United States).

RESULTS

Screening Phase

Overall comparison of HKG variance in different tissues and under different treatment conditions. Interquartile range and SD appeared comparable for each HKG, but GE variance increased 11.8-fold from lowest (first rank) to highest (last rank) in blood samples (IQR: 0.4 vs. 4.7, ratio: 11.8), 8.3-fold in cell lines (IQR: 0.4 vs. 3.3, ratio: 8.3) and 35-fold in tissues (IQR: 0.1 vs. 3.5, ratio 35, Table 2). *PUM1* and *PGK1* demonstrated the least variance within the top ten best-matched genes across all biological materials. *MRPL19* exhibited the least variance among the top ten best-matched genes in blood and cells. However, in tissues, *MRPL19* displayed a higher variance compared to *PUM1* and *PGK1*. Consequently, in the "all-in-one" category, *MRPL19* demonstrated a fivefold higher variance than *PUM1* (IQR: 2.3 vs. 0.5) (Table 2). Biodosimetry-related HKGs such as *MRPS5*, *DPM1,* and *ITFG1* performed comparably with slightly higher GE variance in blood and cell lines (e.g., IQR Ct values compared to *PGK1* showed 0.1–0.6 higher values) and slightly lowered GE variance in tissues (e.g., IQR Ct values compared to *PGK1* showed 0.1-0.4 lower values). The biodosimetry-related HKG *HPRT1* showed several-fold higher GE variances and ranked at places 22–35 among all HKGs regarding the examined biological materials (Table 2). Compared to *PUM1*, IQR Ct value of *HPRT1* increased 2.3-, 2.4- and 2.7-fold in blood, cell lines, and tissues, respectively (Table 2). In IQR comparisons, overall biological materials combined (Table 2, second last columns) ranked *PUM1* and *PGK1* in the first and second place (IQR was 0.5 and 0.7, respectively). They were followed by an approximately twofold higher variance of *18S rRNA* (IQR: 1.2), fivefold higher variance of *MRPL19* (IQR: 2.3), and further, up to a 4.6-fold increased variance of biodosimetry-related HKGs such as *ITFG1* (IQR: 1.5), *DPM1* (IQR: 1.7), *MRPS5* (IQR: 1.9) and *HPRT1* (IQR: 2.3).

Detailed examination of the most suitable selected HKG among biological materials. Examinations of *PUM1* revealed mean raw Ct values (averaged over different utilized chemistries for RNA isolation, ±SD) of comparable size in blood (23.7 ± 0.5), cell lines (23.8 ± 0.4) and tissues (23.7 ± 0.8, Fig. 2). The same pattern was identified for *PGK1* with mean raw Ct values (±SD) of 22.7 ± 0.7, 21.8 ± 0.4 and 22.6 ± 0.5 in blood, cell lines, and tissues, respectively, and for *18S rRNA* as well with mean raw Ct (±SD) of 21.6 ± 1.1, 22.2 ± 1.3 and 21.2 ± 1, in blood, cell lines and tissues, respectively (Fig. 2). The mean raw Ct values (±SD) for *MRPL19* were 27.1 ± 0.6 for blood samples, whereas cell lines and tissues showed a decrease for both sample types (24.2 ± 0.5 and 24.7 ± 0.6, respectively). The mean raw Ct values between the biological materials were significantly different for *PUM1* ($P = 0.04$), *PGK1* ($P = 0.005$), and *18S rRNA* ($P = 0.02$), and highly significant for *MRPL19* ($P = 0.0002$), as shown in Table 3B. All other genes (except *POLR2A*) also revealed highly significant associations (median P value = 0.001; Supplementary Table S2C; https://doi.org/10.1667/RADE-23-00160.1.S2).

Detailed examination of the most suitable selected HKG regarding irradiation and time after irradiation. It is important to note that a gene showing a significant linear dose dependence may not be suitable as a HKG. No significant associations of gene expression change with dose and time (using unexposed samples as the reference) within the same biological material were found for *18S rRNA, PUM1,* and *PGK1*, but weak associations were calculated for *MRPL19* ($P = 0.02$–0.09; Table 3). Linear dose-response relationships over all doses were identified for *MRPL19* in irradiated U118 cells only ($P = 0.04$) and were absent for the other three genes. Biodosimetry related HKGs *MRPS5, ITFG1*, and *DPM1* revealed significant dose-dependent group comparisons (using unexposed samples as the reference) within the same biological material. However, the number of significant associations decreased from present in almost all comparisons regarding *MRPS5* ($P = 0.01$–0.07) to significant associations with emphasis on higher doses in all or few materials regarding *ITFG1* ($P = 0.001$–0.06) and *DPM1* ($P = 0.02$–0.1; Table 3B), respectively. *HPRT1* showed no significant associations with dose except for irradiated A549 cells ($P = 0.05$).

The remaining examined HKG fell into two categories: 1. significant dose-dependent group differences (using unexposed samples as the reference) within the same biological material in almost all group comparisons (*ABL1, ACTB, CDKN1A, ELF1, GADD45A, PES1, POP4, PSMC4,* and *RPL37A*), and 2. significant associations with an emphasis on higher doses in few materials and comparisons only (*CASC3, CDKN1B, EIF2B1, GAPDH, GUSB, HMBS, IPO8, MT-ATP6, PPIA, RPL30, PRLP0* and *PRS17*; Supplementary Table 2C; https://doi.org/10.1667/RADE-23-00160.1.S2)). Linear dose-response relationships over all doses and almost all materials were identified for *B2M* ($P = 0.04$–0.06), *CDKN1A* ($P = <0.0001$–0.07), and *GADD45A* ($P = 0.03$–0.06). Taken together, *PUM1, PGK1,* and *MRPL19* appeared to be the most suitable candidate genes and were forwarded for independent validation in Stage II. The *18S rRNA* HKG was used as a reference.

Validation Phase

Blood samples with/without contrast agent. The application of contrast agent had no significant effect on the mean raw Ct values of all four HKGs in either stratified or unstratified analysis regardless of the radiation dose (data not shown). Differences in mean Ct values (with and without contrast agent combined) between radiation doses did not exceed 0.26 Ct values for all four HKGs. Corresponding min/max Ct values for *18S rRNA,* M*RPL19, PGK1* and *PUM1* were 20.9/21.2, 27.5/27.7, 24.0/24.2 and 24.5/24.7, respectively (Fig. 3A). Significant ($P < 0.0001$) sex-dependent differences (approximately 1 Ct value higher in

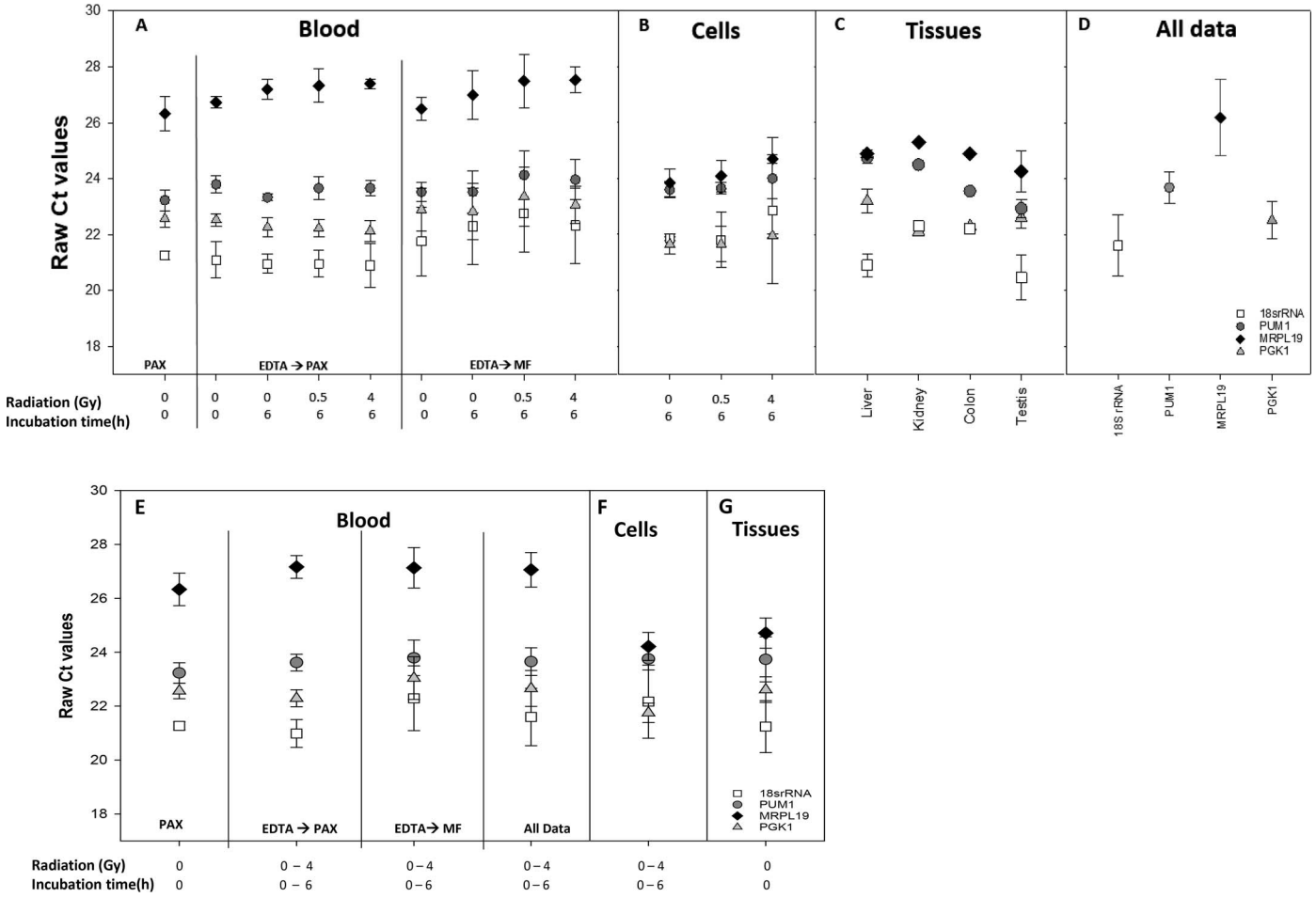

FIG. 2. Results for the most suitable HKGs; *PUM1*, *PGK1*, *MRPL19*, and *18S rRNA* from the screening phase for all examined materials. Panel A: Ct values from blood samples plotted against radiation doses and incubation time. Blood was drawn in different tubes (PAXgene or EDTA), and different chemistries were used for RNA isolation. Vertical lines visualize these different workflows. A shortened syntax such as PAX, EDTA → PAX, EDTA → MF refers to workflow differences with more details provided in the Materials and Methods section. Panel B: Associations of raw Ct values from cell lines vs dose and time after irradiation. Panel C: Associations of raw Ct values from non-irradiated tissues (liver, kidney, colon, and testis). Panel D: Mean Ct values, averaged for each HKG across all biological materials. Panel E: Mean Ct values for the different chemistries used for RNA isolation. Panel E: Mean Ct values for all blood samples. Panels F and G: Mean Ct values for cells and tissues, respectively. Symbols represent averaged raw Ct values, and error bars the standard deviation. Abbreviations: PAX = PAXgene tubes; EDTA = Ethylenediaminetetraacetic acid tubes; MF = microfluidic card; Ct = cycle threshold.

males) were observed for *PGK1* for each radiation dose and overall doses combined, and were absent for the other three HKG. Regarding age, only *18S rRNA* showed a significant (P = 0.001–0.09) association for each radiation dose or overall doses combined.

Rhesus macaques, preirradiation and postirradiation. Relative to unexposed samples, Ct values of all three HKGs increased continuously over the first 3 days (*18S rRNA*) and 35 days (*PGK1* and *PUM1*) postirradiation and declined to control values at days 35 and 60 after exposure (Fig. 3B, Table 4). In all three HKGs, these Ct value differences were highest in females and twofold lower in males (Table 4). The Ct value differences over time after irradiation were highly significant for *18S rRNA* and less or insignificant for *PGK1* and *PUM1*, respectively (Table 4). Sex-dependent differences were observed for *PUM1* only, and solely on days 2, 3, 35, and 60; female Ct values constantly were 1–2 Ct values greater than the male Ct values (Table 4).

Chemotherapy-treated breast cancer patients. Pre- and post-CTx mean raw Ct values of all four HKGs differed less than 0.5 Ct values from each other (Fig. 3C). These differences were highest (0.49 Ct values) for *18S rRNA* (mean pre- vs. post-CTx Ct values of 21.6 vs. 22.1). Lowest differences (<0.1 Ct values) were observed for *PGK1* (mean pre- vs. post-CTx Ct values of 23.6 vs. 23.5) as well as for *PUM1* (mean pre- vs. post-CTx Ct values of 25.7 vs. 25.6, Fig. 3C). This difference reached significance for *18S rRNA* (P = 0.01) only. Treatment with placebo but not Myelo 001 reached significant differences regarding pre- vs. post-CTx mean *18S rRNA* Ct values (P = 0.04) and was absent for the other three HKGs (data not shown).

DISCUSSION

Radiological or nuclear scenarios require an early and high-throughput diagnosis to identify and discriminate unexposed

TABLE 3
Descriptive and Analytical Statistics Comparison of the Three Gene Groups in Biodosimetry

A
Descriptive statistics

Material	Incubation time (h)	Dose (Gy)	Most suitable HKGs												Established biodosimetry HKGs												Known radiation-induced HKGs					
			18S rRNA			*PUM1*			*PGK1*			*MRPL19*			*HPRT1*			*ITFG1*			*MRPS5*			*DPM1*			*CDKN1A*			*GADD45A*		
			N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
PAXgene	0h	0	3	21.3	0.2	3	23.2	0.4	3	22.6	0.3	3	26.3	0.6	3	27.2	0.5	3	26.2	0.4	3	26.1	0.4	3	26.5	0.9	3	26.6	0.3	3	29.5	0.4
EDTA -> PAXgene	0h	0	3	21.1	0.7	3	23.8	0.3	3	22.5	0.3	3	26.7	0.2	3	27.5	0.7	3	26.6	0.1	3	26.8	0.1	3	27.0	0.3	3	26.6	0.2	3	29.4	0.2
EDTA -> PAXgene	6h	0	3	21.0	0.3	3	23.4	0.1	3	22.3	0.4	3	27.2	0.3	3	27.6	0.6	3	27.1	0.2	3	27.0	0.2	3	27.5	0.3	3	25.7	0.2	3	28.9	0.2
EDTA -> PAXgene	6h	0.5	3	21.0	0.5	3	23.7	0.4	3	22.2	0.3	3	27.3	0.6	3	28.3	1.3	3	27.4	0.4	3	27.3	0.4	3	27.6	0.5	3	24.7	0.4	3	28.6	0.7
EDTA -> PAXgene	6h	4	3	20.9	0.8	3	23.6	0.3	3	22.1	0.4	3	27.4	0.2	3	27.7	0.8	3	27.6	0.2	3	27.3	0.2	3	27.6	0.1	3	23.8	0.7	3	27.9	0.7
EDTA -> MF	0h	0	3	21.8	1.3	3	23.5	0.4	3	22.9	0.7	3	26.5	0.4	3	27.8	1.5	3	26.8	0.5	3	26.4	0.3	3	26.6	0.4	3	26.7	0.6	3	28.8	1.1
EDTA -> MF	6h	0	3	22.3	1.4	3	23.6	0.8	3	22.8	1.0	3	27.0	0.8	3	27.9	1.0	3	27.6	1.1	3	27.0	0.9	3	27.4	1.0	3	26.1	0.4	3	28.9	0.8
EDTA -> MF	6h	0.5	3	22.8	1.4	3	24.1	0.9	3	23.4	1.0	3	27.5	1.0	3	28.4	1.3	3	28.2	1.1	3	27.4	0.8	3	27.7	0.9	3	25.0	0.2	3	28.2	0.7
EDTA -> MF	6h	4	3	22.4	1.3	3	24.0	0.7	3	23.1	0.7	3	27.5	0.5	3	28.5	1.5	3	28.4	1.0	3	27.5	0.7	3	27.8	0.8	3	24.0	0.2	3	27.6	0.3
Humane RNA - Liver	-	-	2	20.9	0.4	2	24.8	0.2	2	23.2	0.4	2	24.9	0.2	2	24.1	3.6	2	26.6	0.1	2	25.5	0.1	2	26.6	0.1	2	26.0	0.2	2	26.5	0.3
Humane RNA - Kidney	-	-	1	22.3	.	1	24.5	.	1	22.1	.	1	25.3	.	1	25.5	.	1	26.5	.	1	25.5	.	1	27.1	.	1	24.0	.	1	26.5	.
Humane RNA - Colon	-	-	2	22.2	0.0	2	23.5	0.1	2	22.3	0.0	2	24.9	0.0	2	25.8	0.1	2	25.8	0.0	2	24.6	0.0	2	25.8	0.0	2	23.9	0.0	2	27.3	0.2
Humane RNA - Testis	-	-	3	20.5	0.8	3	23.0	0.3	3	22.6	0.4	3	24.3	0.7	3	23.3	1.5	3	24.5	1.0	3	24.6	0.5	3	24.7	0.9	3	24.1	0.7	3	26.5	0.9
U118	6h	0	1	21.9	.	1	23.4	.	1	21.9	.	1	24.2	.	1	24.7	.	1	25.6	.	1	25.1	.	1	25.1	.	1	23.9	.	1	25.9	.
U118	6h	0.5	1	21.1	.	1	23.5	.	1	22.1	.	1	24.5	.	1	25.1	.	1	25.8	.	1	25.2	.	1	25.3	.	1	24.1	.	1	26.1	.
U118	6h	5	1	21.0	.	1	23.5	.	1	22.1	.	1	24.8	.	1	24.6	.	1	25.7	.	1	25.2	.	1	25.2	.	1	23.8	.	1	26.2	.
A549	6h	0	1	21.9	.	1	23.8	.	1	21.4	.	1	23.5	.	1	23.4	.	1	25.8	.	1	24.4	.	1	25.1	.	1	24.1	.	1	28.1	.
A549	6h	0.5	1	22.5	.	1	23.8	.	1	21.2	.	1	23.7	.	1	23.7	.	1	25.9	.	1	24.6	.	1	25.0	.	1	23.6	.	1	28.4	.
A549	6h	5	1	24.7	.	1	24.5	.	1	21.9	.	1	24.6	.	1	24.1	.	1	26.1	.	1	25.1	.	1	25.3	.	1	22.9	.	1	28.5	.

B
Analytical statistics

Aplied statistic & Material	Incubation time (h)	Dose (Gy)	*18S rRNA*	*PUM1*	*PGK1*	*MRPL19*	*HPRT1*	*ITFG1*	*MRPS5*	*DPM1*	*CDKN1A*	*GADD45A*
Pairwise comparisons, t-test												
Pax vs EDTA												
Pax	0	0	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
EDTA	0	0	0.69	0.12	0.81	0.33	0.60	0.14	***0.06***	0.44	0.73	0.87
	6	0	0.24	0.53	0.30	***0.09***	0.42	***0.02***	***0.03***	0.16	***0.01***	***0.10***
	6	0.5	0.35	0.22	0.21	0.11	0.23	***0.02***	***0.03***	0.14	***0.002***	0.11
	6	4	0.47	0.18	0.17	***0.04***	0.41	***0.004***	***0.01***	0.18	***0.003***	***0.03***
EDTA only												
EDTA	0	0	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
	6	0	0.79	0.11	0.37	0.12	0.86	***0.02***	0.13	***0.10***	***0.005***	***0.05***
	6	0.5	0.78	0.71	0.26	0.18	0.38	***0.02***	***0.09***	0.11	***0.002***	***0.09***
	6	4	0.74	0.62	0.21	***0.02***	0.75	***0.001***	***0.01***	***0.02***	***0.003***	***0.02***
Pax vs MF												
Pax	0	0	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
MF	0	0	0.56	0.33	0.49	0.65	0.54	0.16	0.35	0.95	0.87	0.32
	6	0	0.33	0.53	0.68	0.32	0.34	0.11	0.19	0.35	0.11	0.36
	6	0.5	0.14	0.18	0.27	0.14	0.22	***0.04***	***0.06***	0.19	***0.001***	***0.06***
	6	4	0.29	0.16	0.32	***0.05***	0.22	***0.02***	***0.05***	0.14	***0.0001***	***0.003***
MF only												
MF	0	0	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
	6	0	0.66	0.99	0.92	0.43	0.92	0.30	0.33	0.27	0.19	0.83
	6	0.5	0.42	0.36	0.57	0.17	0.64	0.10	***0.10***	0.12	***0.01***	0.51
	6	4	0.60	0.39	0.81	***0.05***	0.58	0.53	***0.07***	***0.07***	***0.001***	0.14
Materials												
PAX	0	0	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
liver			0.28	***0.01***	0.11	***0.05***	0.45	0.25	0.17	0.88	***0.06***	***0.003***
colon			***0.01***	0.35	0.26	***0.06***	***0.04***	0.19	***0.02***	0.37	***0.003***	***0.01***
testis			0.17	0.44	0.96	***0.02***	***0.02***	***0.06***	***0.02***	***0.08***	***0.004***	***0.01***
Grouped comparison, Kruskal Wallis test												
PAXgene, EDTA-PAXgene, EDTA-MF, liver, colon, testis, U118, A549			***0.0177***	***0.0399***	***0.0049***	***0.0002***	***0.0005***	***0.0002***	***0.0001***	***0.0007***	***0.007***	***0.001***
PAXgene, EDTA 6h,0Gy. EDTA 6h,0.5Gy. EDTA 6h,4Gy			0.97	0.24	0.53	0.15	0.79	***0.04***	***0.05***	***0.10***	***0.02***	***0.07***
PAXgene, EDTA-MF 6h,0Gy. EDTA-MF 6h,0.5Gy. EDTA-MF 6h,4Gy			0.67	0.60	0.87	0.18	0.91	0.12	0.11	0.18	***0.02***	0.15
Linear regression analysis												
PAX-EDTA	6	0, 0.5,4	0.84	0.25	0.64	0.55	0.89	0.06	0.18	0.67	***0.001***	***0.05***
PAX-MF	6	0, 0.5,4	0.94	0.51	0.76	0.41	0.54	0.32	0.45	0.53	***<0.0001***	***0.03***
U118	6	0, 0.5,5	0.27	0.27	0.56	***0.04***	0.90	0.67	0.33	0.67	0.73	***0.06***
A549	6	0, 0.5,5	0.22	0.31	0.46	0.21	***0.05***	0.12	0.15	0.55	***0.07***	0.14

Notes. Part A of Table 3 shows the descriptive statistics of the three groups obtained from the 35 HKGs: most suitable candidate genes, established genes in biodosimetry and known radiation induced genes. The lower part B reflects the results of analytical statistics for each experiment and section for the same groups in part A. P = 0.06–0.1 are written in bold italics, and P < 0.05 are written in bold italics and highlighted in gray. Abbreviation: N = number of repetitions; SD = standard deviation, HKGs = housekeeping genes.

from highly exposed individuals who might develop acute life-threatening symptoms of ARS (*1*, *15*). Early diagnosis facilitates an early identification of individuals needing hospitalization and immediate onset of treatment to improve their prognosis (*2*).

Radiation-induced GE changes have been intensively investigated in recent decades, confirming their suitability for early and high-throughput retrospective biodosimetry and clinical outcome prediction purposes of the ARS (*6*, *16–18*). Large-scale biodosimetry exercises consistently revealed undesired significant differences in GE-based dose estimations by different laboratories (*11*, *14*, *19*). The underlying reasons remain unclear, but different HKGs such as *HPRT1, ITFG1, DPM1, MRPS5,* and *18S rRNA* were utilized. In particular, *18S rRNA* proved its reliability in many studies, but the high copy number requires dilution and facilitates dilution errors (*8*, *9*). The purpose of this study was to compare HKGs and identify the most suitable of these, characterized by low variance in GE and no association with

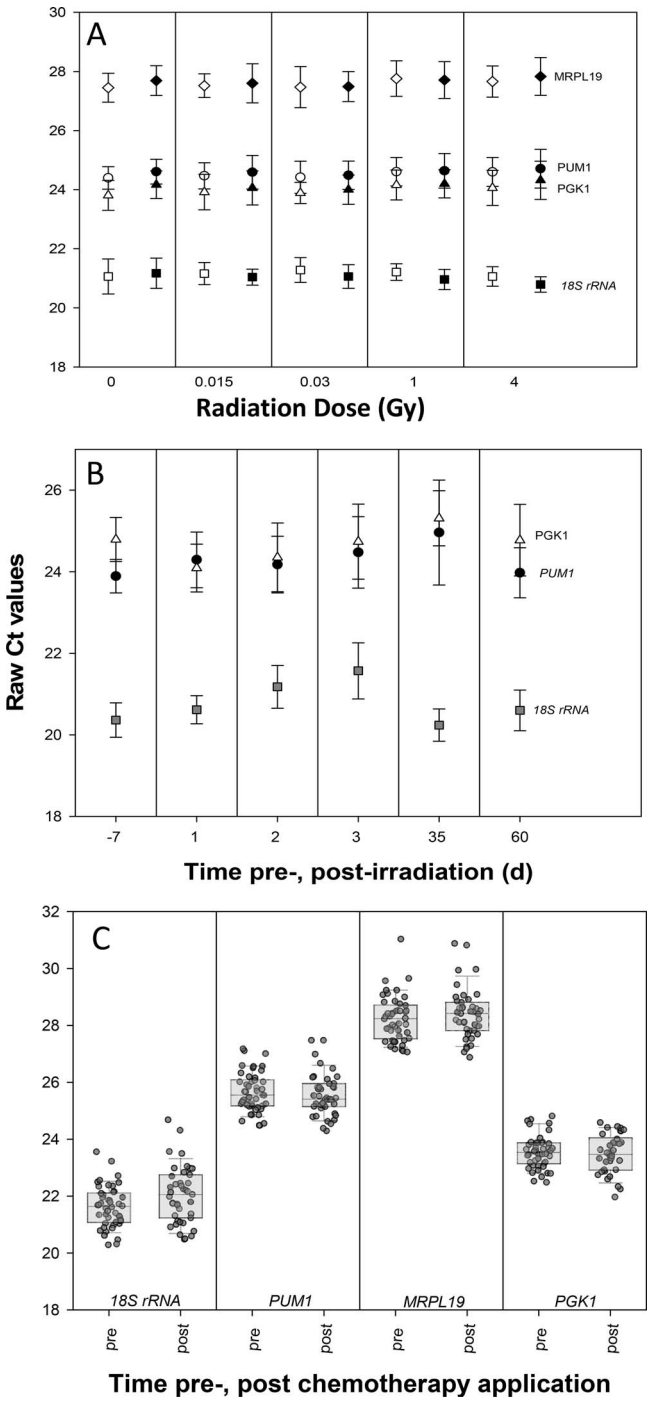

FIG. 3. Summary of the results from the independent validation using the four most suitable HKGs (*MRPL19*, *PUM1*, *PGK1*, and *18S rRNA*) from the screening phase. Panel A: Averaged raw Ct values from ex vivo irradiated blood samples of healthy donors, which are plotted against dose and blood samples administered with (solid symbols) and without (open symbols) iodinated contrast agent prior to exposure. Panel B: Averaged raw Ct values of in vivo irradiated *Rhesus macaques* at indicated time points after irradiation. Symbols in both graphs represent averaged raw Ct values and error bars for the standard deviation. Panel C: Raw Ct values from chemotherapy (CTx) treated breast cancer patients. Individual measurements before (pre) and after (post) CTx are presented as a jitter plot and superimposed by a corresponding box plot. Abbreviations: d = days; Ct = cycle threshold.

radiation exposure. A commercially available pre-designed 96-well platform comprising referenced HKGs was used. In this study, we extended the HKG panel by including HKGs established in the field of biodosimetry, which were not included on the pre-designed plate. GE examinations of peripheral blood samples employing different chemistries are very common (*13*, *14*, *17*, *20*, *21*). PAXgene tubes are widely used because of their RNA stabilizing abilities, and are a gold standard in GE studies (*20–23*). However, these tubes are less common in hospitals and are expensive. EDTA tubes are widely used in hospitals, and future use of microfluidic cards for point-of-care RNA isolation representing alternative techniques/chemistries was considered in this as well as in another previous study (Stewart et al., work published in this Focus Issue, 2024). To encompass multiple biological entities and for convenience reasons (it would be desirable to identify one HGK revealing the same baseline copy number in different materials), HKGs were examined in cell lines and tissues of different origins as well.

GE variance was examined using both IQR and SD. Values of both measures of GE variance were mostly similar in all examined genes and materials (Table 2). Also, expected dose-dependent GE changes of known radiation-modulated genes controlling the cell cycle and the DNA-repair response (*CDKN1A* and *GADD45A*) and spotted as HKGs on the commercial 96-well platform could be found (Table 3). These results indicate a high consistency and reliability of our data set.

In terms of the most suitable HKGs in blood *PUM1, PGK1*, and M*RPL19* exhibited the lowest GE variance. While *MRPL19* showed some weak associations with dose, no dose-dependent associations could be shown for *PUM1* and *PGK1* in both the screening and validation phases of our study. *MRPS5* and *DPM1* appeared comparable regarding the GE variance, but associations with radiation exposures could be shown. *ITFG1* and *HPRT1* showed 2–3 times larger GE variance in blood and undesired associations with dose in many (*ITFG1*) or a few (*HPRT1*) comparisons. In particular, the GE variance of the widely used biodosimetry HKG *HPRT1* was high, shifting the gene to the 22nd to 35th lowest rank among all 35 genes examined. These results appeared significant and could explain differences in dose estimates observed among different laboratories in large-scale biodosimetry exercises that received RNA aliquots from the same source so that similar dose estimates would be expected (*19*). Compared to our reference *18S rRNA*, both most promising HKGs (*PUM1* and *PGK1*) revealed either reduced (screening phase) or comparable GE variance (validation phase) and no dose-dependent associations in all performed comparisons. This finding is important because it will simplify and improve the robustness of the workflow. In the absence of an additional dilution step (as required for *18S rRNA*), it also allows the easy implementation of the most promising HKG on high-throughput platforms such as LDA or the 12k Open Array platforms, or currently developed microfluidic systems for point-of-care RNA isolation purposes.

TABLE 4
Descriptive Statistics and Sex Differences Analysis in the *Rhesus Macaque* Samples

					Comparison per time point		Comparison over time	
Days after irradiation	Sex	N	Mean Ct-values	Standard deviation	Difference	P-value	Difference females/males/combined	P-value females/males/combined
18S rRNA								
-7	female	3	20.02	0.12	-0.44	0.11		
-7	male	10	20.46	0.42				
1	female	3	20.41	0.45	-0.26	0.26		
1	male	10	20.67	0.31				
2	female	3	21.38	0.65	0.27	0.46		
2	male	10	21.11	0.5				
3	female	3	21.97	0.52	0.52	0.27		
3	male	10	21.45	0.71				
35	female	3	20.04	0.39	-0.29	0.33		
35	male	5	20.33	0.36			1.95/	0.036/
60	female	3	20.21	0.29	-0.59	0.1	1.12/	0.001/
60	male	5	20.8	0.47			1.45	<0.0001
PGK1								
-7	female	3	24.95	0.19	0.19	0.61		
-7	male	10	24.76	0.6				
1	female	3	24.52	0.22	0.58	0.14		
1	male	10	23.94	0.6				
2	female	3	24.72	1.07	0.48	0.41		
2	male	10	24.24	0.8				
3	female	3	25.43	0.49	0.91	0.13		
3	male	10	24.52	0.91				
35	female	3	25.82	0.89	0.8	0.11		
35	male	5	25.02	0.34			1.3/	0.123/
60	female	3	25.5	0.19	1.18	0.06	1.1/	0.07/
60	male	5	24.32	0.86			1.1	0.013
PUM1								
-7	female	3	24.16	0.59	0.35	0.22		
-7	male	10	23.81	0.35				
1	female	3	25.22	0.77	1.22	0.1		
1	male	10	24	0.33				
2	female	3	25.07	0.49	1.18	0.004		
2	male	10	23.89	0.5				
3	female	3	25.62	0.43	1.48	0.003		
3	male	10	24.14	0.62				
35	female	3	26.2	1.32	2	0.017		
35	male	5	24.2	0.44			2.0/	0.09/
60	female	3	24.57	0.3	0.93	0.018	0.6/	0.24/
60	male	5	23.64	0.44			1.2	0.15

Notes. Descriptive statistics (left part of the table) of the Macaque study related to the graphical presentations of Fig. 3B. Examinations of significant sex differences for each day after irradiation and overall days after irradiation are presented in the middle and right parts of the table, respectively. The term "Differences" refers to the difference of mean Ct values between females and males per time point and overall time points, but based on the lowest and highest mean Ct values. P values are calculated using the t-test statistic.

Examinations of other biological materials, such as cell lines and tissues, confirmed the results as shown for GE measurements in blood; the two most promising HKGs demonstrated gene expression variance comparable to widely utilized biodosimetry HKGs such as *MRPS5*, *ITFG1*, and *DPM1,* while *HPRT1* revealed the highest GE variance. However, in contrast to the established HKGs, these two promising HKGs did not exhibit any correlations with radiation dose. We interpret these findings as another indication for more general applicability of the two most promising HKGs we identified for other radiation studies.

Among all 35 HKGs, only *PUM1* and *18S rRNA* revealed weak significant GE differences across all examined materials, but strong associations were found for other HKGs (Supplementary Table S2C; https://doi.org/10.1667/RADE-23-00160.1.2). Hence, for all HKGs (but less pronounced for *PUM1* and *18S rRNA*), RNA copy numbers differ significantly in each material, and this has to be considered for quality assurance of qRT-PCR data.

Examinations of the selected four HKGs in the validation phase showed dependency on sex, age, and time after irradiation which appeared more pronounced (significant) for *18S rRNA*; less or missing associations regarding *MRPL19, PGK1,* and *PUM1* were found (Fig. 3, Table 4). Due to the small number of examined females in the Macaques (n = 3) and the contrast agent studies (n = 5) and data inconsistencies (e.g., *PGK1* showed a sex dependency in the contrast agent study which was missing in the Macaque study), these results should be interpreted carefully and certainly require further examinations. Also, other than *18S rRNA*, no age dependency was identified for *MRPL19, PGK1,* and *PUM1*. Age and sex dependencies were also studied by Agbenyegah et al., who examined 200 healthy donors and showed that these dependencies contribute less than 20–30% to the inter-individual variance (*24*).

Furthermore, *MRPL19, PGK1,* and *PUM1* showed approximately a fivefold lower variance regarding pre- and post-CTx mean raw Ct values, which again underlines their preferable applicability. All four HKGs showed no significant associations with contrast agents, making them suitable for situations where radiological diagnostics using contrast agent are employed. Weak significant associations of *18S rRNA* with placebo but not Myelo 001 in pre- vs. post-CTx Ct values (P = 0.04) should be interpreted cautiously, and the absence of a significant association of the other three HKGs argues more in favor of their use in CTx patients as well. This is of relevance since CTx patients are considered as a surrogate cohort for whole body irradiated individuals (*10*) (Schüle et al., work published in this Focus Issue, 2024). However, relative to unexposed *Rhesus* M*acaques* samples, increased Ct values for *18S rRNA* was observed from days 1–3 and on day 35 for *PGK1* and *PUM1* after irradiation (Fig. 3B). Again, this effect appeared more pronounced (significant) for *18S rRNA*. For *PGK1* and *PUM1,* a Ct value difference of one (calculated between the lowest and highest Ct value) was well within the acceptable variance of Ct values considering methodological variance inherent to this methodology (Table 4) (*25*).

Both newly identified promising HKGs for radiation studies are coding for general processes such as post-transcriptional regulation and glycolysis. *PUM1* (Pumilio proteins) negatively regulates GE by repressing the translation of mRNAs to which they bind (*26*). *PGK1* (phosphoglycerate kinase-1) catalyzes the reversible conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate during glycolysis, generating one molecule of ATP (*27*). To the best of our knowledge, neither HKG has been used in radiation experiments before. However, the use of *PUM1* as a HKG for different cancer and normal cell lines was proposed by Ayakannu et al. (*28*). Falkenberg et al. identified *PGK1* as a stable HKG for GE measurements in human blood RNA (*29*).

Our study has its strengths and limitations. A two-stage study design was chosen, allowing for independent validation of the most promising HKGs on samples not used during the screening phase. A large sample size (257) was used for an extensive validation, which was performed employing *in* v*ivo* irradiated cohorts. Those included non-human primates and CTx-treated breast cancer patients. Based on previous work, we examined whether CTx patients could be used as a suitable surrogate cohort of rare whole-body radiation-exposed individuals. Pathomechanism and biological effects following radiotherapy and chemotherapy (in this case, cyclophosphamide and epirubicin) differ, but they share certain common features, such as the formation of free radicals and the induction of apoptosis. Similar deregulations of a four gene set predicting the radiation-induced H-ARS severity degree indicated the suitability of CTx-treated breast cancer patients for that purpose (*10*) (Schüle et al., work published in this Focus Issue, 2024). The absence of a significant association of our two promising HKGs with CTx treatment confirms the use of both most promising HKGs in this patient cohort. The sample size of 41 in the screening phase was low, but due to the integrity of the data set, it was sufficient to select the correct HKG to be extensively validated in the validation phase. *MRPL19* exhibited less consistency and was not detectable in *Rhesus macaques*, which might be attributed to the use of a human TaqMan assay. However, the applicability in human samples was the major goal of our study.

In summary, for radiation exposure studies, *PUM1* and *PGK1* appear to be the most promising among the 35 examined HKGs when considering GE variance and undesired associations of GE with dose.

SUPPLEMENTARY MATERIALS

Supplementary Table S1. The 35 examined housekeeping (HKGs) with their respective official symbol, official full name and the utilized TaqMan assay ID for qRT-PCR. The first 32 HKGs originate from a commercially available predesigned TaqMan® Express Human Endogenous Control Plate. The three remaining HKGs, *ITFG1*, *MRPS5*, and *DPM1*, were examined using a conventional 96-well plate.

Supplementary Table S2. Panel A: Raw Ct value measurements of the 35 housekeeping genes from the screening phase for each donor, material used, incubation time, and

dose. Genes are categorized into sections as indicated; Panel B: Corresponding descriptive statistics for each experiment; Panel C: Results of analytical statistics for each experiment and section. P = 0.06–0.1 (bold–italics); P < 0.05 (bold–italics highlighted in gray): Abbreviations: N = number of repetitions; SD = standard deviation, SE = standard error.

ACKNOWLEDGMENTS

We are very thankful for the sophisticated technical support from Sven Doucha-Senf, Oliver Wittmann, and Thomas Müller. The authors gratefully acknowledge the research support from the Congressionally Directed Medical Research Programs (W81XWH-15-C-0117, JW140032) of the United States Department of Defense to VKS. The opinions or assertions contained herein are the private views of the authors and are not necessarily those of the Uniformed Services University of the Health Sciences or the Department of Defense.

Received: August 3, 2023; accepted: November 16, 2023; published online: March 13, 2024

REFERENCES

1. Port M, Ostheim P, Majewski M, Voss T, Haupt J, Lamkowski A, et al. Rapid High-Throughput Diagnostic Triage after a Mass Radiation Exposure Event Using Early Gene Expression Changes. Radiat Res 2019; 192:208-18.
2. Port M, Hérodin F, Drouet M, Valente M, Majewski M, Ostheim P, et al. Gene Expression Changes in Irradiated Baboons: A Summary and Interpretation of a Decade of Findings. Radiat Res 2021; 195(6): 501-21.
3. Friesecke I, Beyrer K, Fliedner TM. How to cope with radiation accidents: the medical management. *Br* J R*adiol* 2001; 74:121-2.
4. Singh VK, Newman VL, Romaine PLP, Hauer-Jensen M Pollard HB. Use of biomarkers for assessing radiation injury and efficacy of countermeasures. Exp Rev Mol Diag 2016; 16:65-81.
5. Cherubini, A, Rusconi, F, Lazzari, L. Identification of the best housekeeping gene for RT-qPCR analysis of human pancreatic organoids. PLoS One 2021; 16:e0260902.
6. Ostheim P, Amundson SA, Badie C, Bazyka D, Evans AC, Ghandi SA, et al. Gene expression for biodosimetry and effect prediction purposes: promises, pitfalls and future directions–key session ConRad 2021. Int Journal Radiat Biol 2022; 98:843-54.
7. Port M, Abend M. Diagnostics for radiation injuries in large scale radio-nuclear accidents: Recent developments for a medical-management focused approach. Envir Adv 2022; 8: 100233.
8. Port M, Schmelz HU, Stassen T, Müeller K, Stockinger M, Obermair R, et al. Correcting false gene expression measurements from degraded RNA using RTQ-PCR. Diag Mol Pathol 2007; 16:38-49.
9. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 2004; 37(1):112-4, 6, 8-9.
10. Schüle S, Bristy EA, Muhtadi R, Kaletka G, Stewart S, Ostheim P, et al. Four Genes Predictive for the Severity of Hematological Damage Reveal a Similar Response after X Irradiation and Chemotherapy. Radiat Res 2023; 199:115-23.
11. Badie C, Kabacik S, Balagurunathan Y, Bernard N, Brengues M, Faggioni G, et al. Laboratory intercomparison of gene expression assays. Radiat Res 2013; 180:138-48.
12. Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet 2013; 54:391-406.
13. Ostheim P, Coker O, Schüle S, Hermann C, Combs SE, Trott K, et al. Identifying a Diagnostic Window for the Use of Gene Expression Profiling to Predict Acute Radiation Syndrome. Radiat Res 2021; 195:38-6.
14. Abend M, Amundson SA, Badie C, et al. RENEB Inter-Laboratory Comparison 2021: The Gene Expression Assay. Radiat Res 2023; 199:598-615.
15. Abend M, Ostheim P, Port M. Radiation-induced gene expression changes used for biodosimetry and clinical outcome prediction: challenges and promises. Cytogenet Genome Res 2023. doi: 10.1159/000530947.
16. Port M, Abend M. Clinical Triage of Radiation Casualties-The Hematological Module of the Bundeswehr Institute of Radiobiology. Radiat Prot Dosimetry 2018; 182:90-2.
17. Ostheim P, Don Mallawaratchy A, Müller T, Schüle S, Hermann C, Popp T, et al. Acute radiation syndrome-related gene expression in irradiated peripheral blood cell populations. Int J Radiat Biol 2021; 97:474-84.
18. Port, M, Ostheim, P, Majewski, M, Voss T, Haupt J, Lamkowski A, et al. Rapid High-Throughput Diagnostic Triage after a Mass Radiation Exposure Event Using Early Gene Expression Changes. Radiat Res 2019; 192:208-18.
19. Abend, M, Amundson, SA, Badie, C, Brzoska K, Hargitai R, Kriehuber R, et al. Inter-laboratory comparison of gene expression biodosimetry for protracted radiation exposures as part of the RENEB and EURADOS WG10 2019 exercise. Sci Rep 2021; 11:9756.
20. Fang F, Yu X, Wang X, Zhu X, Liu L, Rong L, et al. Transcriptomic profiling reveals gene expression in human peripheral blood after exposure to low-dose ionizing radiation. J Radiat Res 2022; 63:8-18.
21. Abend M, Blakely WF, Ostheim P, Schule S, Port M. Early molecular markers for retrospective biodosimetry and prediction of acute health effects. J Radiol Prot 2022; 42(1).
22. Tang R, She Q, Lu Y, Yin R, Zhu P, Zhu L, et al. Quality Control of RNA Extracted from PAXgene Blood RNA Tubes After Different Storage Periods. Biopreserv Biobank 2019; 17:477-82.
23. Stephenson NL, Hornaday KK, Doktorchik CTA, Lyon A, Tough SC, Slater DM. Quality assessment of RNA in long-term storage: The All Our Families biorepository. PLoS One 2020; 15:e0242404.
24. Agbenyegah S, Abend M, Atkinson MJ, Combs SE, Trott KR, Port M, et al. Impact of Inter-Individual Variance in the Expression of a Radiation-Responsive Gene Panel Used for Triage. Radiat Res 2018; 190:226-35.
25. Port, M, Hérodin, F, Drouet, M, Valenti M, Majewski M, Ostheim P, et al. Gene Expression Changes in Irradiated Baboons: A Summary and Interpretation of a Decade of Findings. Radiat Res 2021; 195:501-21.
26. Lee, S, Kopp, F, Chang, TC, Sataluri A, Chen B, Sivakumar S, et al. Noncoding RNA NORAD Regulates Genomic Stability by Sequestering PUMILIO Proteins. Cell 2016; 164:69-80.
27. Fujii, H, Krietsch, WK, Yoshida, A. A single amino acid substitution (Asp leads to Asn) in a phosphoglycerate kinase variant (PGK München) associated with enzyme deficiency. J Biol Chem 1980; 255:6421-3.
28. Ayakannu T, Taylor AH, Willets JM, Brown L, Lambert DG, McDonald J, et al. Validation of endogenous control reference genes for normalizing gene expression studies in endometrial carcinoma. Mol Hum Repro 2015; 21:723-35.
29. Falkenberg VR, Whistler T, Murray JR, Unger ER, Rajeevan MS. Identification of Phosphoglycerate Kinase 1 (*PGK1*) as a reference gene for quantitative gene expression measurements in human blood RNA. BMC Res Notes 2011; 4:324.