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Increased radiological and nuclear threats require preparedness. Our earlier work identified a set of four genes (*DDB2, FDXR, POU2AF1* and *WNT3*), which predicts severity of the hematological acute radiation syndrome (H-ARS) within the first three days postirradiation In this study of 41 *Rhesus macaques* (*Macaca mulatta*, 27 males, 14 females) irradiated with 5.8–7.2 Gy ($LD_{29-50/60}$), including some treated with gamma-tocotrienol (GT3, a radiation countermeasure) we independently validated these genes as predictors in both sexes and examined them after three days. At the Armed Forces Radiobiology Research Institute/Uniformed Services University of the Health Sciences, peripheral whole blood (1 ml) of *Rhesus macaques* was collected into PAXgene® Blood RNA tubes pre-irradiation after 1, 2, 3, 35 and 60 days postirradiation, stored at −80°C for internal experimental analyses. Leftover tubes from these already ongoing studies were kindly provided to Bundeswehr Institute of Radiobiology. RNA was isolated (QIAsymphony), converted into cDNA, and for further gene expression (GE) studies quantitative RT-PCR was performed. Differential gene expression (DGE) was measured relative to the pre-irradiation *Rhesus macaques* samples. Within the first three days postirradiation, we found similar results to human data: 1. *FDXR* and *DDB2* were up-regulated, *FDXR* up to 3.5-fold, and *DDB2* up to 13.5-fold in the median; 2. *POU2AF1* appeared down regulated around tenfold in nearly all Rhesus macaques; 3. Contrary to human data, *DDB2* was more up-regulated than *FDXR*, and the difference of the fold change (FC) ranged between 2.4 and 10, while the median fold changes of *WNT3*, except days 1 and 35, were close to 1. Nevertheless, 46% of the *Rhesus macaques* showed down-regulated *WNT3* on day one postirradiation, which decreased to 12.2% on day 3 postirradiation. Considering the extended phase, there was a trend towards decreased fold changes at day 35, with median-fold changes ranging from 0.7 for *DDB2* to 0.1 for *POU2AF1*, and on day 60 postirradiation, DGE in surviving animals was close to pre-exposure values for all four genes. In conclusion, the diagnostic significance for radiation-induced H-ARS severity prediction of *FDXR*, *DDB2*, and *POU2AF1* was confirmed in this *Rhesus macaques* model. However, *DDB2* showed higher GE values than *FDXR*. As shown in previous studies, the diagnostic significance of *WNT3* could not be reproduced in *Rhesus macaques*; this could be due to the choice of animal model and methodological challenges. © 2024 by Radiation Research Society

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INTRODUCTION

Radiological and nuclear incidents can occur as a result of civil accidents, meltdowns in nuclear power plants, or intentionally via a terroristic attack using a radiological or nuclear dispersion device or the military use of atomic bombs (*1, 2*). In any case, they have the potential to produce mass casualties and even more so-called "worried well" (individuals who did not get harmed or exposed to radioactive contamination or radiation) (*3*). From the human experience with improperly disposed radioactive sources, as in Goiania (*4*), the nuclear meltdown in Chernobyl (*5*), and the use of atomic bombs as in the Castle Bravo test series (*6*), it is likely that thousands of potentially exposed or irradiated individuals must be triaged. The scientific community has been called upon to develop tools for triaging patients that might need immediate and specific medical care and surveillance with regard to development of the acute radiation syndrome (ARS) (*7*). It is not only the absorbed dose that allows prediction of the subsequent course of a patient's ARS; instead, factors such as fractionation, radiation quality, dose rate, external or internal contamination, as well as how the individual was exposed [homogenous vs. inhomogeneous, or total-body (TBI) vs. partial-body (PBI) irradiation], and individual radiosensitivity also play major roles (*8*).

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An early and high-throughput clinical outcome tool for predicting ARS severity would be useful for triage during a large-scale radiological and nuclear event. Gene expression (GE) analysis is a promising approach toward handling the requirements of such a tool (9–20). In previous work, we showed that a set of four genes comprising *FDXR*, *DDB2*, *POU2AF1*, and *WNT3* was able to distinguish non-exposed as well as patients that later developed mild and severe hematologic acute radiation syndrome (H-ARS) (10–13, 21, 22). This corresponds to the METREPOL-Algorithm¨s H-ARS categories (23). Our group previously showed that it is of benefit, when looking at the medical treatment consequences, to break the H-ARS categories down into three groups: H0 represents the unexposed, H0-1 represents those with no or low radiation exposure with sub-clinical H-ARS for which no hospitalization is needed, and H2-4 represents those with mild to severe H-ARS, when early treatment and hospitalization is required (21). In this study, the ionizing radiation dose was comparably high (5.8–7.2 Gy) with a $LD_{29-50/60}$ and might be expected to result in a mild to fatal H2-4 H-ARS severity. The H-ARS severity predicting gene set has already been validated in vitro (12), in vivo in baboons (10, 11, 21), and in irradiated oncology patients (10, 22). The *Rhesus macaques* genome shares 93% of its sequence with the human genome (24). In cooperation with Armed Forces Radiobiology Research Institute (AFRRI), we independently validated and further investigated this model in a new species, *Macaca mulatta*, which is even closer related to humans than baboons. Additionally, due to the design of our study, we could evaluate the GE changes of our four gene-set over a 60-day time course postirradiation, exceeding the 3-day postirradiation measurements of previous studies.

MATERIALS AND METHODS

Animals

Sixty-four healthy *Rhesus macaques* (*Macaca mulatta*, Chinese substrain) were obtained from the National Institutes of Health Animal Center (NIHAC, Poolesville, MD) (Table 1). The animals were quarantined for 6–7 weeks prior to the beginning of the experiment. One *Rhesus macaques* was excluded from the study due to a virus infection, leaving 63 *Rhesus macaques* eligible for this study. Males and females were assigned to dose groups but a lower number of females were assigned due to availability of Rhesus macaques s at a particular time and a lower number of females available to us. Overall, 40 male and 23 female clinically healthy individuals weighing 3.6–8.4 kg were housed at the Armed Forces Radiobiology Research Institute (AFRRI), Bethesda, MA. All animals were kept in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-International. Housing requirements, sensory and dietary enrichment details have been previously described in elsewhere (25, 26). Single housing was utilized for the animals for this study, and the justification for single housing was described earlier (27). All of the procedures performed in this study were in accordance with the animal use protocols approved by the Institutional Animal Care and Use Committee (IACUC, AFRRI) and Department of Defense second-tier approval from the Animal Care and Use Review Office (ACURO). The study was reported in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and with the recommendations made in the Guide for the Care and Use of Laboratory Animals (28).

TABLE 1

Distribution of the Animals over the Different Strata, Sorted by Dose, Sex, Treatment Group, and Survival Status

Radiation dose (Gy)	Sex	Treatment group (GT-3)	Survival status	Number of animals		
				Sex	Treatment	Survival status
5.8	males			20		
		treated			10	
			survivor			7
			non-survivor			3
		untreated			10	
			survivor			7
			non-survivor			3
	females			11		
		treated			6	
			survivor			5
			non-survivor			1
		untreated			5	
			survivor			3
			non-survivor			2
6.5	males			2		
		treated			2	
			survivor			2
	females			3		
		treated			3	
			survivor			1
			non-survivor			2
7.2	males			5		
		untreated			5	
			survivor			3
			non-survivor			2
				27	21	28
			Sum	14	20	13
						41

Notes. The more left and higher number represents the sum of the more specified subgroups (left lower numbers). Numbers in the lower right dark gray box represent the subgroups' sum over all dose groups, from left to right: Sex, treatment and survival. Upper numbers reflect from left to right to male, treated and surviving animals; lower numbers indicate female, untreated and non-survivors.

Drug Preparation and Administration

Gamma-tocotrienol (GT-3) was procured from American River Nutrition (Hadley, MA/ExcelVite Sdn. Bhd., Perak, Malaysia) and its preparation and administration have been described earlier (29). The dose used in this study ranged from 37.5–75 mg/kg, and the volume administered to each animal was based on individual animal body weight. At least 24–48 h prior to drug administration, the injection site (dorsal scapular area) was shaved and cleaned to monitor for any skin irritations or abscess formation. GT3 and/or vehicle administrations were performed with a sterile 21–25 gauge needle length of 0.75–1 inch.

Blood Sample Collection

Blood was collected by venipuncture from either the saphenous vein on the caudal aspect of the lower leg or the brachial vein from the upper extremity of the arm. One ml of peripheral blood was drawn into PAXgene Blood RNA tubes (PreAnalytiX, a Qiagen/Becton, Dickinson, and Company, Franklin Lakes, NJ) on either day 1 or 7 prior to irradiation and on days 1, 2, 3, 35 and 60 postirradiation. After collection, the blood was mixed immediately by inverting the tube 10 times. The tubes were maintained at room temperature in the laboratory overnight and were later stored at –80°C until further analysis (30).

Irradiation

Prior to irradiation, dose rate measurements were performed as described earlier (*31, 32*). The animals were fasted for at least 12–18 h prior to irradiation, at approximately 30–45 min before exposure, all animals received appropriate anesthetics. Irradiations were performed using a cobalt-60 source with a dose rate of 0.6 Gy/min. NHPs were fastened in a sitting position within the central beam to perform a total-body irradiation (TBI). All details for the irradiation procedure have been discussed previously (*33*). Three different dose groups of 5.8 Gy (n = 31; males n = 20 and females n = 11), 6.5 Gy (n = 16; males n = 4 and females n = 12), and 7.2 Gy (n = 16 males) were used for this study. After the procedure, once the animals were certified to be in stable condition, they were transported back to the housing cages where they completed their recovery.

Euthanasia

After irradiation, animals were prone to exhibiting clinical ARS-related signs and symptoms, and daily observations were increased to three times a day (no more than 10 h apart) during the critical period (days 10–20 postirradiation) to assess for moribundity. If an animal reached a state of moribundity, the animal was euthanized. Moribundity was used as a surrogate for the mortality assessment of animals, and they were euthanized to minimize pain and distress. All euthanasia criteria and additional details have been provided previously (*34*). In general, euthanized *Rhesus macaques* were defined as non-survivors (n = 22), while all surviving animals on day 60 were euthanized and considered survivors (n = 41).

Sample Selection

Expecting a high H-ARS severity degree, whether the radiation dose was 5.8, 6.5 or 7.2 Gy, sample sets from 41 *Rhesus macaques* were analyzed to keep the remaining samples for other tasks. Also, data appeared very homogenous, so the study could be restricted to the aforementioned number of *Rhesus macaques*. All sample sets of the animals exposed to 5.8 Gy were processed for this study. From those animals exposed to 6.5 Gy and 7.2 Gy, five sample sets each were randomly picked the way that the survivor to non-survivor ratio equals the one of the whole cohort (Table 1). In total, 219 blood specimens were investigated.

RNA-Extraction and Quality-Control

Filled PAXgene® Blood RNA tubes were manually thawed, centrifuged, the supernatant discarded, and pellets resuspended with proteinase K augmented buffers. RNA from PAXgene® Blood RNA tubes was isolated semi-automatically using the QIAsymphony® Blood RNA Kit (QIAGEN, Hilden, Germany) and the QIAsymphony® SP Kit (QIAGEN). The procedure uses the RNA-binding silica surface of magnetic beads. After several washing and digestion steps with DNase I and proteinase K, RNA was isolated automatically, eluted in 80 µl BR5 buffer, heated to 65°C for five min, and stored at –20°C. For quantification, RNA-eluates were measured spectrophotometrically (NanoDrop™, PeqLab Biotechnology, Erlangen, Germany). DNA contamination was precluded via PCR using primers for the β-actin gene. qRT-PCR was performed on all specimens with a ratio of A260/A280nm ≥ 2.0. The quality was addressed by automated electrophoretic integrity measurements (4200 TapeStation System, Agilent Technologies, Santa Clara, CA), and RIN (RNA integrity number) values were calculated. Questionable measurements were confirmed via 18S rRNA-qRT-PCR. Only samples meeting pre-defined quality criteria [e.g., 18S rRNA-raw Ct values (0.01 ng/reaction) ranging between 20 and 28 are expected to be successful qRT-PCR] were further processed, leading to the qRT-PCR.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Aliquots of total RNA (0.5 µg) were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Life Technologies, Darmstadt, Germany). Equal amounts of template cDNA (10 ng) were used per reaction, mixed with the TaqMan®

Universal PCR Master Mix, and gene-specific TaqMan™ Assays for *FDXR* (Hs01031617_m1), *DDB2* (Hs00172068_m1), *POU2AF1* (Hs01573371_m1), and *WNT3* (Hs00902257_m1) were added. The genes were measured in duplicate, and qRT-PCR was performed on a 96-well format using the QuantStudio™ 12K OA Real-Time PCR System (Thermo Fisher SCINTIFIC Inc., Waltham, MA). The raw cycle threshold (Ct) was normalized to the diluted *18S rRNA* (Hs99999901_g1). After normalization, fold change (FC) differences in gene expression were calculated by the $-\Delta\Delta$Ct-approach relative to unexposed samples of the same *Rhesus macaques* used as the calibrator $FC = 2^{-\Delta\Delta Ct_{precep}}$. The fold change refers to several fold of over- or underexpression relative to the calibrator. Genes were assumed to be differentially expressed if $0.5 \geq FC \geq 2$ (*10, 35*).

Statistical Analysis

Results were presented as normalized Ct values, mean fold change and median fold change. Differences in GE were investigated by comparing each day after exposure with the pre-exposure set to the reference. We also checked for varieties in the strata sex, survival, exposure dose, and treatment. Probability distribution was addressed by Shapiro-Wilks and the comparison of variance by Brown Forsythe test. Due to non-normally distributed results, GE changes over time for all individuals were investigated by a Kruskal-Wallis one way analysis of variance (ANOVA) on ranks. For the analysis of the mentioned strata, two-way ANOVAs (Holm-Sidak method) were performed to compare the pre-exposure animals' GE to a reference group (either female, survivor or untreated animals), and the subsequent interactions over time were analyzed. For the stratum exposure dose, the ANOVA was done pairwise stratified by time and exposure dose. P values < 0.05 were defined as significant. Also, univariate linear regression analysis was performed, searching for dose-response relationships in GE. For statistical analyses and graphical presentations, SAS (release 9.4, Cary, NC) and Excel (Microsoft, Redmond, CA), as well as SPW (SigmaPlot, Version 14.5, Jandel Scientific, Erkrath, Germany) and PowerPoint (Microsoft) were used.

RESULTS

Time Course of Gene Expression Changes

In all animals, for *FDXR* we saw an increase in gene expression after irradiation relative to unexposed animals, with the increase becoming significant on day 2 postirradiation (P < 0.001, Fig. 1 and Table 2) with a median fold change of 2.3. GE of *FDXR* increased until day 3 up to a median fold change of 3.5 (P < 0.001). On day 35, we saw decreased GE (median FC = 0.5) but this change was not significantly different. On day 60, GE was nearly that of unexposed animals. Expression of *DDB2* was already significantly increased one day postirradiation (median FC = 3.4, P < 0.001) and increased further after 2 days (median FC = 5.4), up to a median fold change of 13.5 through day 3 (Fig. 1 and Table 2). On days 35 and 60, GE for *DBB2* was similar to *FDXR*. GE of *POU2AF1* declined after irradiation (Fig. 1). The approximate 8–10 fold decrease in GE reached significance on day 1 postirradiation (P < 0.001, median FC ≈ 0.13 to 0.09, Fig. 1 and Table 2) and remained decreased until day 35. As observed for *FDXR* and *DDB2*, the GE of *POU2AF1* on day 60 resembled pre-exposure GE values. *WNT3* showed a high variance in GE levels 1–3 days and 60 days postirradiation. Only on day 35 postirradiation was a significant downregulation observed.

If we consider a twofold up- or downregulation to represent a robust change in GE, Table 3 shows that between the first three days postirradiation, 90–100% of the irradiated animals
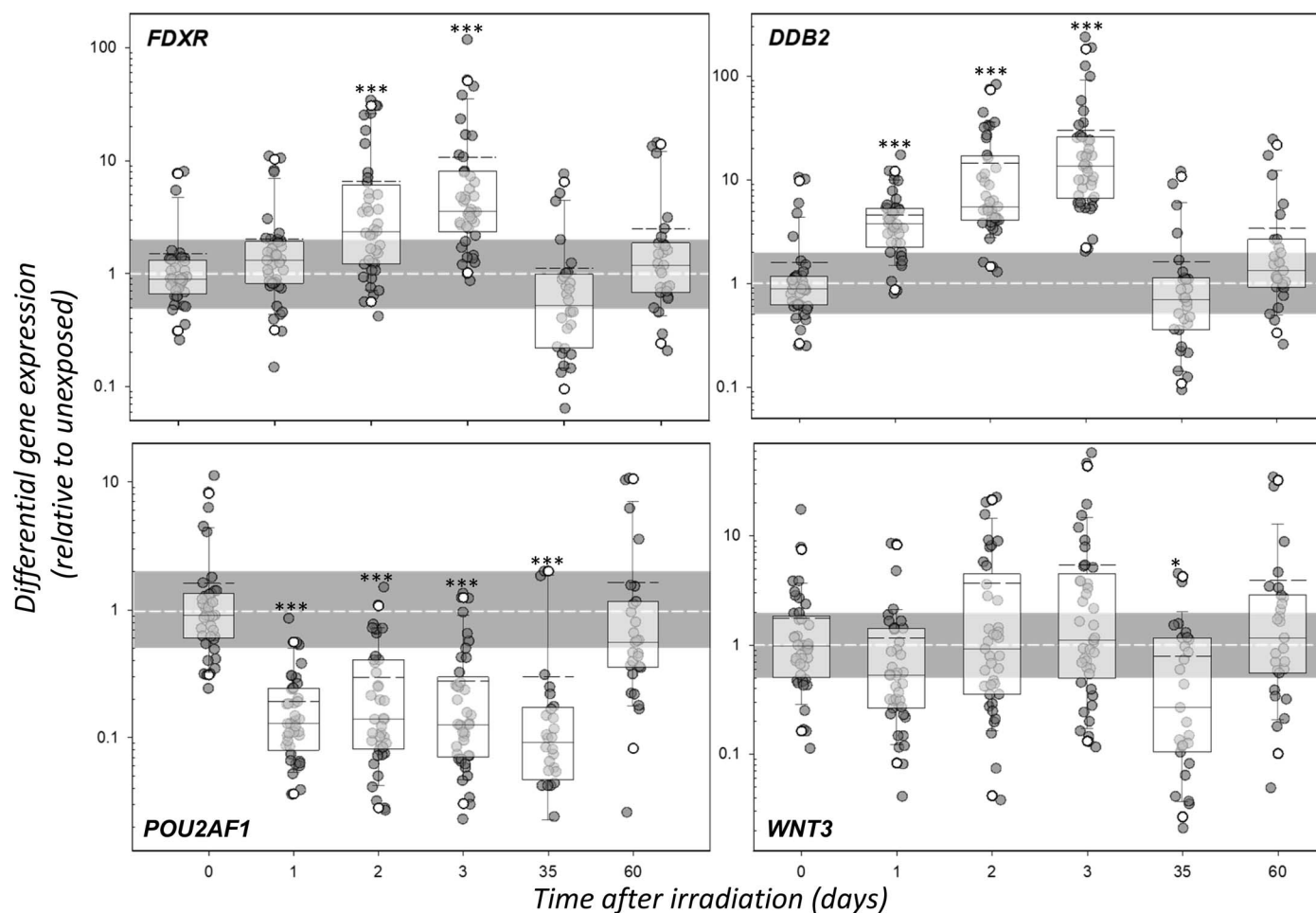
FIG. 1. Boxplots superimposed over corresponding jitter plots reflecting differential gene expression (DGE) for each animal and the four genes over time. Boxes contain 50% of the data (25–75th percentiles). The error bars represent the 10th and 90th percentiles, while the white circles represent the 5th and 95th percentiles. Some outliers are not shown. The continuous horizontal lines indicate the position of the median, while the interrupted one indicates that of the mean. Significant changes in GE relative to unexposed animals are marked with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001). The gray area in the background adjusts for methodological variance and is defined for fold changes between 0.5 and 2. The interrupted white line refers to FC = 1 as a reference for GE prior to exposure.

could have been identified as exposed individuals *based on GE of DDB2 and POU2AF1*. In a receiver-operator-characteristics (ROC) analysis, *DDB2* and *POU2AF1* showed a high diagnostic strength with an area under the curve ranging from 0.88–0.97 over the first 3 days postirradiation (Fig. 2). A bivariate model of *DDB2* and *POU2AF1* reached an area under the curve of 1.0 on days 1–3 postirradiation (Fig. 2). While the percentage of correctly identified animals, based on *FDXR* and *DDB2* GE, increased from day 1 to day 3 from 22–78% and from 78–100%, respectively, *WNT3* was reduced in its diagnostic strength, decreased from at least 46% on day 1 down to 12% on day 3. Based on *POU2AF1* GE, it was possible to identify 90% of the exposed individuals as early as day one; this represented the highest diagnostic reliability of the four genes on day one. *POU2AF1* GE remained around tenfold downregulated up to day 35 postirradiation. Between 80 to 89% of the irradiated animals could have been identified as having been exposed over this longer period.

GE Differences by Sex

For *FDXR* GE, we saw a tendency toward a higher median fold change for females (e.g., 5.0 vs. 2.2 on day 2, and 6 vs. 3.5 on day 3 postirradiation, Table 2), but there was no significant difference for any time-point after exposure. For *POU2AF1* GE, we observed a tendency toward a more pronounced, but insignificant, downregulation after exposure regarding the median fold change for males for each single time point (0.2 vs. 0.1 on days 1 and 3 and 0.3 vs 0.1 on day 2 postirradiation, Table 2). For *DDB2* and *WNT3 GE*, no other sex-specific effects could be observed.

GE Differences by Survival Status

For *FDXR*, *DDB2 POU2AF1*, and *WNT3*, no GE differences between survivors and non-survivors could be found, except for *WNT3* on day 2 postirradiation (median FC = 1.3, P = 0.04, Table 2).

TABLE 2
Descriptive Statistics [n – Number of Evaluable Animals in the Subgroup, Mean and Median Fold Change and Standard Deviation (Std Dev)], as well as P Values for Comparisons to Pre-Exposure Status and in-between Subgroups for each Day and each Gene

Gene Strata / Subgroup	Preexp n	Preexp Mean FC	Preexp Median FC	Preexp Std Dev	Preexp p-value Group comp.	D1 n	D1 Mean FC	D1 Median FC	D1 Std Dev	D1 p preexp vs. d1	D1 p Group comp.	D2 n	D2 Mean FC	D2 Median FC	D2 Std Dev	D2 p preexp vs. d2	D2 p Group comp.	D3 n	D3 Mean FC	D3 Median FC	D3 Std Dev	D3 p preexp vs. d3	D3 p Group comp.	D35 n	D35 Mean FC	D35 Median FC	D35 Std Dev	D35 p preexp vs. d35	D35 p Group comp.	D60 n	D60 Mean FC	D60 Median FC	D60 Std Dev	D60 p preexp vs. d60	D60 p Group comp.
FDXR – All	41	1.5	0.9	2.0		41	2.0	1.3	2.6	0.6		40	6.6	2.3	9.5	**<0.001**		41	10.7	3.5	20.6	**<0.001**		28	1.1	0.5	1.7	0.5		27	2.5	1.2	3.9	1.0	
Sex																																			
Females	14	1.7	0.9	2.1		14	2.7	1.6	3.0	0.5		13	9.8	5.0	11.4	**0.001**		14	17.5	6.0	30.6	**<0.001**		10	1.7	0.7	2.5	0.6		9	2.3	0.8	3.6	0.7	
Males	27	1.4	0.9	1.9	0.60	27	1.7	1.1	2.3	0.6	0.1	27	5.0	2.2	8.2	**0.004**	0.05	27	7.2	3.5	12.2	**<0.001**	0.1	18	0.8	0.5	1.0	0.08	0.2	18	2.6	1.4	4.2	0.5	1.0
Survival																																			
Survivor	27	1.6	0.8	2.2		27	2.4	1.3	3.1	0.2		27	8.0	2.8	11.0	**<0.001**		27	13.6	3.7	24.8	**<0.001**		27	1.1	0.5	1.8	0.6		27	2.5	1.2	3.9	1.0	
Non-survivor	14	1.3	0.9	1.2	1.0	14	1.2	1.2	0.6	0.8	0.5	13	3.5	2.2	3.7	0.06	0.4	14	5.3	3.3	5.0	**0.001**	0.4	1	0.9	0.9	-	-	-	0	-				
Dose																																			
5.8 Gy (Group Comp. 5.8 vs. 6.5 Gy)	31	1.7	0.9	2.2	0.8	31	2.0	1.3	2.4	0.8	0.2	30	5.3	2.3	8.7	**0.01**	**<0.001**	31	10.9	3.5	22.8	**<0.001**	**<0.001**	23	1.2	0.5	1.9	0.4	0.09	22	2.7	1.1	4.3	0.9	0.4
6.5 Gy (Group Comp. 6.5 vs. 7.2 Gy)	5	0.9	0.9	0.3	0.6	5	3.6	2.3	4.2	0.5	0.2	5	19.1	18.4	9.3	**<0.001**	**<0.001**	5	18.1	16.5	13.2	**<0.001**	**0.007**	3	0.9	1.0	0.3	1.0	0.3	3	2.1	2.1	0.4	0.7	0.3
7.2 Gy (Group Comp. 7.2 vs. 5.8 Gy)	5	0.6	0.6	0.2	0.5	5	0.8	0.8	0.4	1.0	0.4	5	1.5	1.3	0.7	0.2	0.2	5	2.2	2.2	0.7	**0.03**	0.1	2	0.4	0.4	0.5	1.0	0.3	2	0.7	0.7	0.5	1.0	0.4
Treatment																																			
Untreated	20	0.8	0.8	0.3		20	1.0	0.9	0.5	0.8		20	1.5	1.2	0.8	0.08		20	2.4	2.6	1.0	**<0.001**		12	0.4	0.3	0.4	**0.02**		12	0.8	0.7	0.4	0.9	
Treated	21	2.2	1.1	2.6	**0.01**	21	3.0	1.7	3.3	0.2	**0.002**	21	11.6	5.8	13.0	**<0.001**	**<0.001**	21	18.6	7.9	26.7	**<0.001**	**<0.001**	16	1.7	0.8	2.1	0.3	**<0.001**	15	3.9	1.8	4.9	0.3	**<0.001**
DDB2 – All	41	1.6	0.9	2.3		41	4.6	3.7	3.4	**<0.001**		40	14.4	5.4	18.7	**<0.001**		41	29.8	13.5	48.9	**<0.001**		28	1.6	0.7	2.8	0.5		27	3.4	1.3	5.6	1.0	
Sex																																			
Females	14	1.7	1.0	1.7		14	4.4	3.6	3.2	**0.02**		13	15.0	9.6	12.8	**<0.001**		14	29.4	12.7	37.8	**<0.001**		10	2.3	0.7	3.8	0.5		9	2.7	1.1	3.4	0.7	
Males	27	1.5	0.9	2.6	0.4	27	4.7	3.7	3.6	**<0.001**	0.9	27	14.1	5.1	21.2	**<0.001**	0.4	27	30.6	13.5	54.4	**<0.001**	1.0	18	1.3	0.6	2.1	0.3	0.4	18	3.7	1.6	6.5	0.1	1.0
Survival																																			
Survivor	27	1.8	0.9	2.7		27	5.1	4.2	4.1	**<0.001**		27	16.4	6.3	21.6	**<0.001**		27	34.2	13.6	57.5	**<0.001**		27	1.6	0.7	2.8	1.0		27	3.4	1.3	5.6	0.705	
Non-survivor	14	1.2	1.0	1.1	0.9	14	3.6	3.6	1.2	**<0.001**	0.8	13	10.3	5.0	10.3	**<0.001**	0.6	14	21.3	11.3	25.1	**<0.001**	0.9	1	0.9	0.9	-	-	-	0	-				
Dose																																			
5.8 Gy (Group Comp. 5.8 vs. 6.5 Gy)	31	1.8	1.0	2.6	0.8	31	4.7	3.8	3.6	**<0.001**	0.9	30	13.1	4.4	19.9	**<0.001**	**0.01**	31	31.4	12.8	54.3	**<0.001**	0.3	23	1.8	0.6	3.1	0.2	0.6	22	3.7	1.2	6.2	0.2	0.4
6.5 Gy (Group Comp. 6.5 vs. 7.2 Gy)	5	1.0	1.0	0.2	0.8	5	5.0	3.0	4.1	0.1	1.0	5	29.8	32.1	13.0	**<0.001**	0.06	5	38.5	26.7	35.4	**<0.001**	0.3	3	1.0	1.1	0.3	1.0	0.5	3	3.1	2.7	1.4	0.5	0.4
7.2 Gy (Group Comp. 7.2 vs. 5.8 Gy)	5	0.7	0.6	0.1	0.6	5	3.6	3.7	1.2	0.07	0.9	5	7.2	5.5	2.9	**0.003**	1.0	5	11.3	11.9	3.2	**<0.001**	0.6	2	0.6	0.6	0.7	0.8	0.4	2	0.8	0.8	0.4	0.8	0.5
Treatment																																			
Untreated	20	0.8	0.7	0.6		20	4.3	4.2	2.0	**<0.001**		20	5.2	4.4	2.9	**<0.001**		20	10.3	10.0	5.7	**<0.001**		12	0.5	0.4	0.3	**0.04**		12	1.0	1.0	0.5	**0.03**	
Treated	21	2.3	1.1	3.0	**0.02**	21	4.8	3.0	4.4	**<0.001**	0.7	21	23.6	16.8	23.1	**<0.001**	**<0.001**	21	48.4	25.5	63.3	**<0.001**	**<0.001**	16	2.5	1.0	3.5	0.8	**<0.001**	15	5.3	2.2	7.0	0.57	**<0.001**
POU2AF1 – All	41	1.6	0.9	2.2		41	0.2	0.13	0.2	**<0.001**		40	0.3	0.14	0.3	**<0.001**		41	0.3	0.13	0.3	**<0.001**		28	0.3	0.09	0.6	**<0.001**		27	1.6	0.6	2.9	0.8	
Sex																																			
Females	14	1.8	1.1	1.8		14	0.2	0.2	0.2	**<0.001**		13	0.4	0.3	0.3	**<0.001**		14	0.4	0.2	0.4	**<0.001**		10	0.5	0.1	0.8	**<0.001**		9	1.7	0.5	3.4	0.14	
Males	27	1.5	0.9	2.4	0.3	27	0.2	0.12	0.2	**<0.001**	0.3	27	0.3	0.12	0.4	**<0.001**	0.1	27	0.2	0.11	0.3	**<0.001**	0.09	18	0.2	0.07	0.4	**<0.001**	0.1	18	1.6	0.6	2.7	0.4	1.0
Survival																																			
Survivor	27	1.8	0.9	2.7		27	0.2	0.11	0.2	**<0.001**		27	0.3	0.14	0.4	**<0.001**		27	0.3	0.13	0.4	**<0.001**		27	1.6	0.6	2.9	**<0.001**		27	1.6	0.6	2.9	1	
Non-survivor	14	1.2	1.1	0.9	0.9	14	0.2	0.13	0.1	**<0.001**	0.9	13	0.2	0.11	0.2	**<0.001**	0.3	14	0.2	0.12	0.3	**<0.001**	0.4	1	0.1	0.1	-	-	-	0	-				
Dose																																			
5.8 Gy (Group Comp. 5.8 vs. 6.5 Gy)	31	1.9	1.0	2.5	0.6	31	0.2	0.15	0.1	**<0.001**	1.0	30	0.3	0.14	0.3	**<0.001**	**0.02**	31	0.3	0.13	0.4	**<0.001**	0.2	23	0.3	0.1	0.6	**<0.001**	0.8	22	1.8	0.5	3.1	0.6	0.6
6.5 Gy (Group Comp. 6.5 vs. 7.2 Gy)	5	1.0	0.9	0.4	0.4	5	0.3	0.1	0.3	0.1	0.3	5	0.6	0.7	0.2	**<0.001**	**<0.001**	5	0.3	0.3	0.2	**<0.001**	0.05	3	0.1	0.1	0.1	0.09	0.9	3	0.9	1.1	0.3	1.0	0.7
7.2 Gy (Group Comp. 7.2 vs. 5.8 Gy)	5	0.4	0.4	0.1	0.07	5	0.07	0.05	0.04	**0.04**	1.0	5	0.06	0.06	0.03	**0.02**	**0.02**	5	0.05	0.06	0.02	**0.02**	**0.03**	2	0.04	0.04	0.06	**0.003**	**0.02**	2	0.4	0.4	0.3	1.0	0.7
Treatment																																			
Untreated	20	1.0	0.8	0.9		20	0.2	0.14	0.1	**<0.001**		20	0.1	0.09	1.0	**<0.001**		20	0.10	0.07	0.09	**<0.001**		12	0.06	0.05	0.05	**<0.001**		12	0.5	0.4	0.4	**0.02**	
Treated	21	2.2	1.0	2.9	**0.04**	21	0.2	0.13	0.2	**<0.001**	0.7	20	0.5	0.4	0.4	**<0.001**	**<0.001**	21	0.4	0.3	0.4	**<0.001**	**<0.001**	16	0.5	0.13	0.7	**<0.001**	**<0.001**	15	2.6	1.0	3.6	0.6	**<0.001**
WNT3 – All	41	1.8	1.0	2.9		40	1.1	0.5	1.9	0.2		41	3.7	0.9	6.0	1.0		41	5.4	1.1	11.6	1.0		27	0.8	0.3	1.1	**0.01**		27	3.9	1.2	8.2	1	
Sex																																			
Females	14	1.8	1.0	2.0		14	1.8	0.5	2.9	0.8		14	6.1	1.3	8.7	0.8		14	8.1	1.4	15.4	0.7		10	1.2	0.5	1.6	0.5		9	5.4	1.7	11.0	0.6	
Males	27	1.7	1.0	3.3	0.8	27	0.8	0.4	1.0	0.3	0.5	27	2.5	0.8	3.7	0.9	0.3	27	4.0	1.1	8.9	0.8	0.4	17	0.5	0.3	0.5	**0.01**	0.3	18	3.2	1.0	6.6	0.9	0.6
Survival																																			
Survivor	27	1.9	0.9	0.8		27	1.4	0.6	2.2	0.4		27	4.3	1.3	6.3	0.3		27	5.2	1.1	9.5	0.4		26	0.8	0.2	1.1	**0.01**		27	3.9	1.2	8.2	0.5	
Non-survivor	14	1.4	1.1	1.1	0.8	13	0.6	0.4	0.6	0.26	0.5	14	2.5	0.5	5.6	0.4	**0.04**	14	5.7	1.0	15.0	0.8	0.5	1	1.2	1.2	-	-	-	0	-				
Dose																																			
5.8 Gy (Group Comp. 5.8 vs. 6.5 Gy)	31	1.9	0.9	3.3	0.9	31	1.0	0.4	1.6	0.3	0.2	31	2.3	0.7	4.3	0.9	**<0.001**	31	4.0	0.9	8.9	1.0	**0.004**	22	0.8	0.2	1.2	**0.03**	0.6	22	4.2	0.9	9.1	0.9	0.5
6.5 Gy (Group Comp. 6.5 vs. 7.2 Gy)	5	1.7	1.2	1.0	0.8	5	2.7	1.7	3.3	0.9	0.3	5	14.6	15.6	7.3	0.1	**0.003**	5	17.2	5.3	23.1	0.3	0.06	3	0.6	0.4	0.6	0.8	0.7	3	3.3	2.9	1.2	0.9	0.7
7.2 Gy (Group Comp. 7.2 vs. 5.8 Gy)	5	1.1	0.8	0.7	0.9	5	0.6	0.4	0.5	1.0	1.0	5	1.2	0.8	1.3	1.0	0.9	5	2.1	1.2	2.1	1.0	0.7	2	0.6	0.6	0.8	0.8	0.7	2	1.9	1.9	2.0	1.0	0.8
Treatment																																			
Untreated	20	1.1	0.8	1.0		20	0.6	0.3	0.5	0.3		20	0.7	0.4	0.9	0.4		20	1.2	0.7	1.4	0.9		11	0.2	0.1	0.4	**<0.001**		12	0.9	0.6	0.9	0.7	
Treated	21	2.4	1.0	3.8	0.2	20	1.7	0.6	2.5	0.2	0.08	21	6.5	2.8	7.4	0.2	**<0.001**	21	9.3	3.8	15.1	0.1	**<0.001**	16	1.2	0.9	1.3	0.3	**<0.001**	15	6.4	2.4	10.5	0.3	**<0.001**

Note. P < 0.05 are indicated in bold letters.

GE Differences by Dose

A significant association between GE and dose (P = 0.007, P < 0.001) was observed for *FDXR*, *DDB2* and *POU2AF1*, but this association was absent for *WNT3* and FDXR on day 1 (Fig. 3). These associations became insignificant when omitting the pre-exposure data set (data not shown).

GE Differences by Treatment Status

For all four genes and examined time points, and even for pre-irradiation conditions, treated *Rhesus macaques* revealed higher GE values than untreated *Rhesus macaques* (Fig. 4, Table 2). These GE differences were significant in most comparisons, and median fold change of up to 25.5 vs. 10.0 at day 3 for *DDB2* was observed (Fig. 3, Table 2).

DISCUSSION

As the war in Ukraine raises the threat of large-scale radiological and nuclear incidents, it is essential to be prepared. Early and high-throughput diagnostics for triage purposes are required to supply limited resources to those in need of it (*7*). Early prediction of the clinical outcome, namely the severity of the life-threatening ARS, allows for early treatment decisions to improve prognosis and selection of those individuals requiring limited treatment resources and rare clinical infrastructure such as intensive care units (*21*).

This study aimed to validate a set of four genes which previously showed promising results for predicting H-ARS in baboon and human in vivo and ex vivo studies (*9, 10, 12–14, 21, 22, 36*). Furthermore, the current study followed irradiated *Rhesus macaques* up to 60 days postirradiation. Therefore, we showed that GE changes of these genes could be examined over a prolonged period of time exceeding the 3-day period postirradiation in during which these changes were examined in previous baboon and human studies (*9, 10, 12–14, 21, 22, 36*).

Previous studies indicated a mild-to-severe hematological severity degree (*2–4*) of the ARS when *FDXR* and/or *DDB2* exceeded a twofold threshold in GE relative to unexposed in combination with other genes (*21*). This could be shown for *DDB2* on the first day, and for *FDXR* starting on the second day in our current study on *Rhesus macaques* (Fig. 1, Table 2). GE values increased up to 3 days postirradiation, thus confirming both genes could be used for diagnostic purposes of H-ARS severity prediction in this model as well (Fig. 1, Table 2). However, previous studies gave reasons to expect

TABLE 3
Frequency Distribution of the Four Genes Grouped by FC ≥ 2, 2 > FC > 0.5, and FC ≤ 0.5 for each Day (Bold Numbers Left Part of the table) after Irradiation

Days after Irradiation	Total (n)	Gene	FC ≥ 2		2 > FC > 0.5		FC ≤ 0.5	
			n	(%)	n	(%)	n	(%)
1	41	*FDXR*	**9**	22.0	27	65.9	5	12.2
	41	*DDB2*	**32**	78.0	9	22.0	**0**	0.0
	41	*POU2AF1*	0	0.0	4	9.8	**37**	90.2
	41	*WNT3*	4	9.8	18	43.9	**19**	46.3
2	40	*FDXR*	**23**	57.5	16	40.0	1	2.5
	40	*DDB2*	**37**	92.5	3	7.5	**0**	0.0
	40	*POU2AF1*	0	0.0	8	20.0	**32**	80.0
	41	*WNT3*	13	31.7	13	31.7	15	36.6
3	41	*FDXR*	**32**	78.0	9	22.0	0	0.0
	41	*DDB2*	**41**	100.0	0	0.0	**0**	0.0
	41	*POU2AF1*	0	0.0	7	17.1	**34**	82.9
	41	*WNT3*	19	46.3	17	41.5	**5**	12.2
35	28	*FDXR*	**3**	10.7	11	39.3	14	50.0
	28	*DDB2*	**4**	14.3	13	46.4	11	39.3
	28	*POU2AF1*	2	7.1	1	3.6	**25**	89.3
	27	*WNT3*	2	7.4	10	37.0	**15**	55.6
60	27	*FDXR*	**6**	22.2	17	63.0	4	14.8
	27	*DDB2*	**8**	29.6	17	63.0	2	7.4
	27	*POU2AF1*	4	14.8	10	37.0	**13**	48.1
	27	*WNT3*	10	37.0	11	40.7	**6**	22.2

Notes. Bold numbers in the right part of the table represent the number of animals per group. The corresponding percentage of all evaluable animals per time point and gene is written behind.

FIG. 2. Sensitivity vs. 1-specificity of a receiver-operator characteristic curve (ROC) for days 1 to 3 after irradiation for *DDB2, POU2AF1,* and a bivariate model of these two genes. Area under the curve describes the area under the curve for each gene and the bivariate model.

a peak in GE at 24 h postirradiation, but decreasing GE of *FDXR* and *DDB2* from 24–72 h postirradiation (*12, 37*). The response of *FDXR* in *Macaca mulatta* appears more subdued and delayed compared to numerous human studies. The same is true for *DDB2*. Interestingly, in our *Rhesus macaques* study, *DDB2* throughout revealed several-fold higher GE changes than *FDXR*, but in human in vivo and ex vivo models, *FDXR* appeared stronger deregulated compared to *DDB2* (*12*). However, *FDXR* in baboons was even down regulated in contrast to e.g., 30-fold upregulation, as cited in many publications using irradiated human blood (*12*). *POU2AF1* showed a pronounced downregulation after 24 h which, in contrast to baboon and human data (*10, 12*), lasted until day 3 and was found at day 35 as well. This indicates species-specific differences. However, the diagnostic significance of our study remained unaltered, as indicated by the almost complete separation of unexposed (H0) from H2-4 ARS severity groups using logistic regression analysis (Fig. 2). In our *Rhesus macaques* study, *DDB2,* combined with *POU2AF1*, showed a complete separation of both groups (Fig. 2). However, although marginally present, no significant sex-dependent GE changes were found, consistent with other studies (*14, 38*). Furthermore, survival status and treatment did not affect discrimination between the two groups.
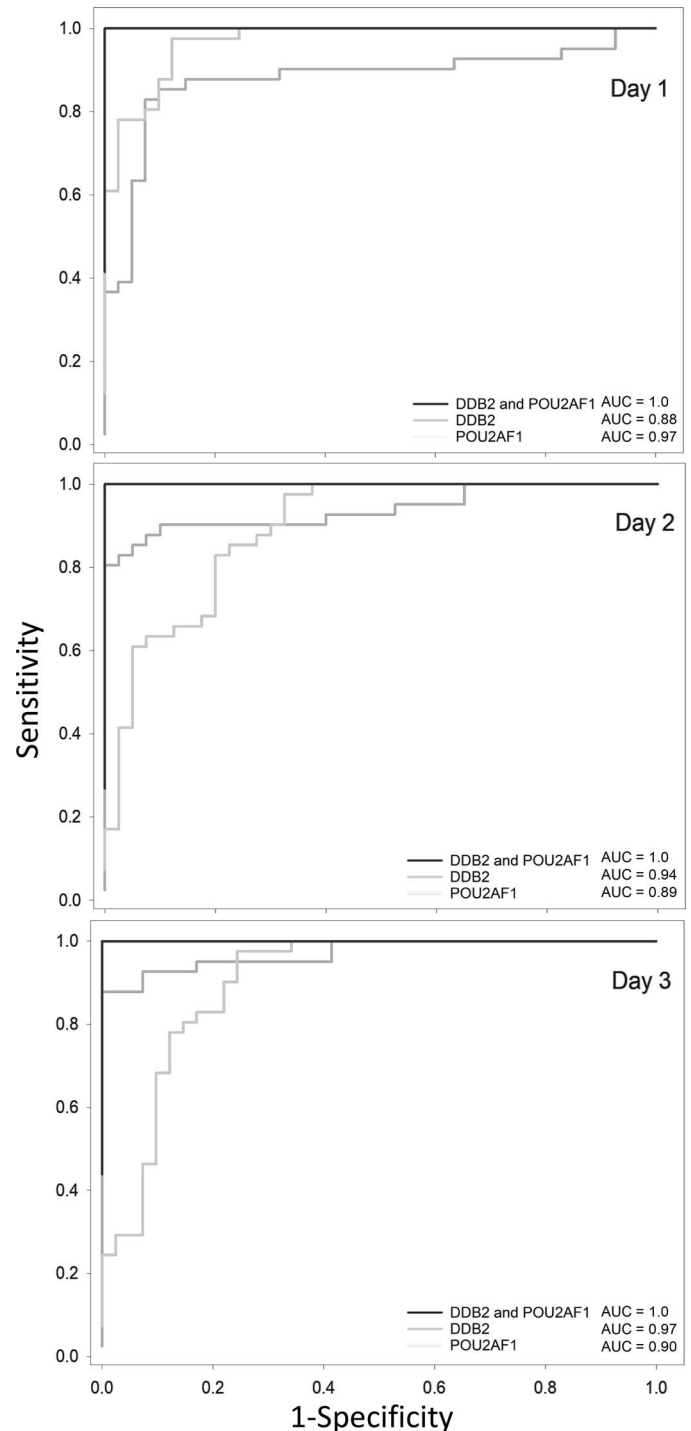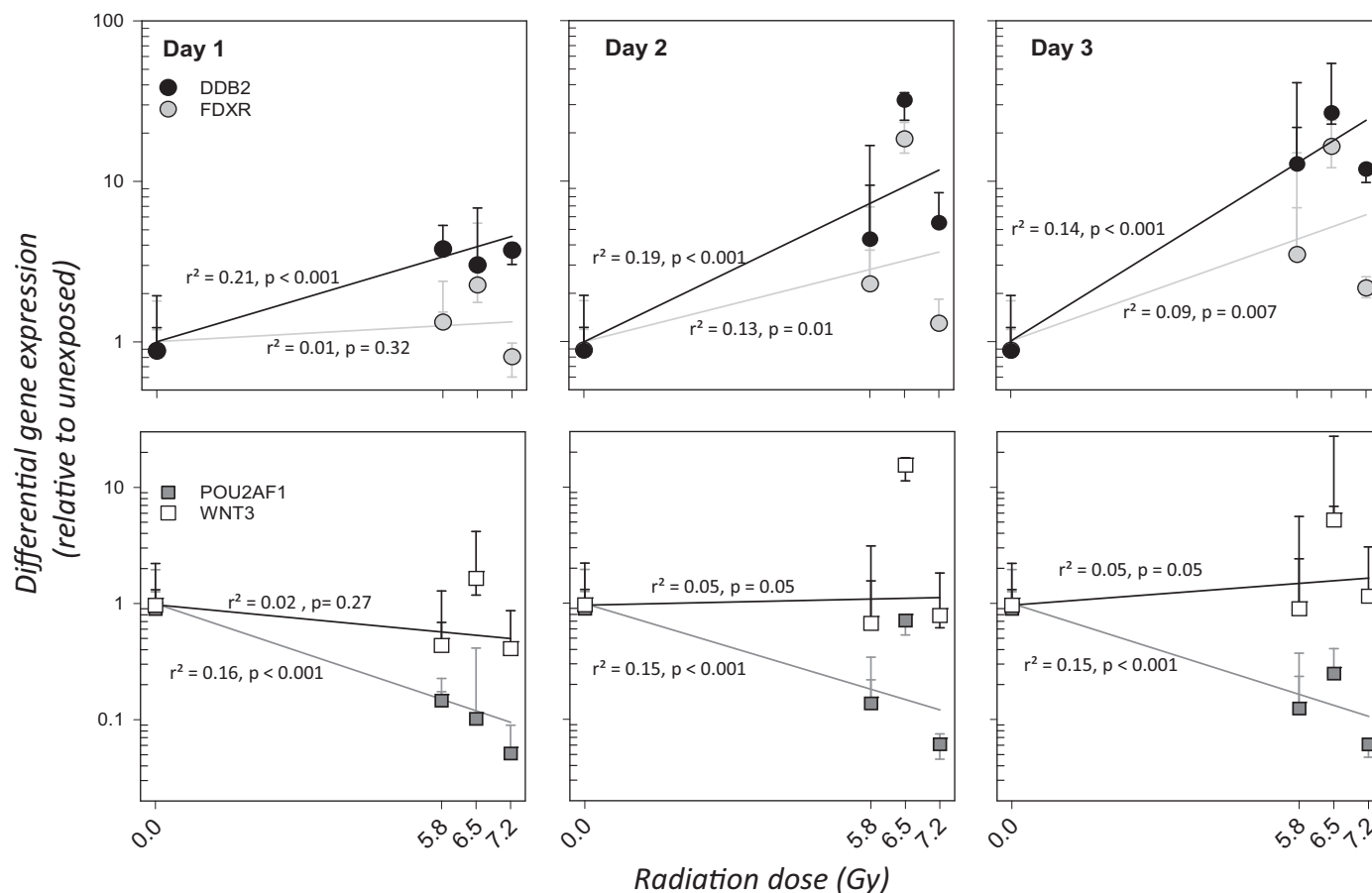
In previous studies, a downregulation of *POU2AF1* and/or *WNT3* that was ≥ twofold in combination with a ≥twofold upregulation of *FDXR* and/or *DDB2* indicated an H-ARS 2-4 severity degree (*21*). In our *Rhesus macaques* study, *POU2AF1* responded as known from previous human studies (*11*), and median GE values appeared tenfold down regulated,

FIG. 3. GE dose-response curves for each gene 1–3 days postirradiation, displayed as the median fold change relative to unexposed animals (black, gray, and white circles) for three dose groups as well as unexposed animals. The upper three graphs contain the GE for *DDB2* and *FDXR*, and the lower ones *POU2AF1* and *WNT3*. Error bars represent the standard error of the mean. r^2 specifies the corresponding correlation coefficient. P values refer to the slope of the assumed graph.

independent of sex, survival, or treatment. These pronounced GE changes were found on the first day after irradiation and, if used as a single criterion, would already identify 90% of the Rhesus macaques which later developed an aggravated H-ARS 2-3 severity degree (Table 3). Other than *POU2AF1*, no pronounced downregulation of *WNT3* could be found, as expected due to previous studies conducted on baboons, human in vivo and ex vivo blood studies, as well as CTx-treated breast cancer patients used as a surrogate cohort for rare whole-body irradiated patients (*22, 36*). Based on *WNT3* GE changes on day 1 after irradiation, 46% of the exposed *Rhesus macaques* would have been correctly categorized as H2-4 H-ARS severity degree (Table 3). However, this effect was inconsistent over time; therefore, the diagnostic value of *WNT3* in this animal model could not be reproduced. Detection of radiation-induced lowered *WNT3* copy numbers appears challenging, as already noted in human ex vivo studies (*12, 21*). Baseline raw Ct values close to the upper limit of the qRT-PCR linear dynamic range restrict the identification of down-regulated *WNT3* copy numbers since raw Ct values increase with decreasing RNA copy numbers. Therefore, a downregu-lation cannot be detected in some individuals with high baseline raw Ct values. This could be observed in our

Rhesus macaques study as well. To overcome this limita-tion and to increase the robustness of our H-ARS predic-tive gene set, we introduced the redundant application of two genes (*POU2AF1* and *WNT3*) based on previous stud-ies. We interpreted the pronounced downregulation of one or both genes as an indication for a later developing H-ARS 2-4 severity degree in consideration with an upregu-lation of *FDXR* and/or *DDB2* (*21*). For improved *WNT3* detec-tion, we also added five times more cDNA into the qRT-PCR reaction compared to the other genes, but that did not sufficiently adjust for the limitations as described.

Our *Rhesus macaques* study showed a downregulation of all four genes at day 35 postirradiation, and all GE values at 60 days postirradiation revealed values in the control range (Fig. 1). This effect was independent of sex, survival status, and treatment and might indicate an active response of the irradiated *Rhesus macaques* since GE values returned to normal values at day 60. The downregulation on day 35 postirradiation might have diagnostic implications, but the underlying mecha-nism, as well as missing data filling the 4–34 daytime gap after irradiation, requires further investigation. A persistent downregu-lation of almost all 27 evaluable genes out of 34 genes that were examined in lethally irradiated *Rhesus macaques* pre-mortem
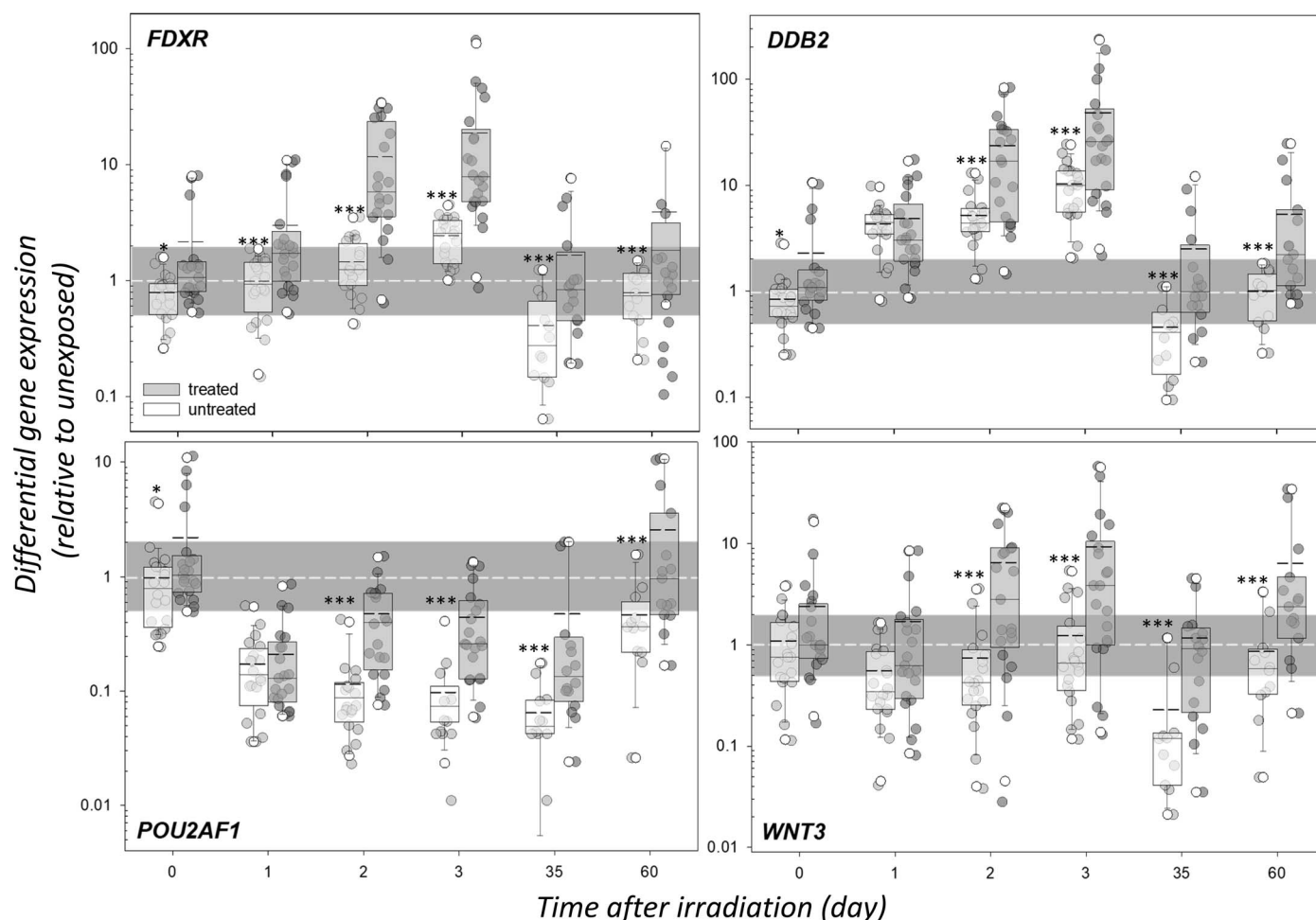
FIG. 4. Differences in DGE between untreated (white squares) and treated animals (light gray squares) over time postirradiation. Squares contain 50% of the data (25–75th percentiles). The error bars represent the 10th and 90th percentiles, respectively, while the white circles represent the 5th and 95th percentiles. Some outliers are not shown. The continuous horizontal lines indicate the position of the median, while the interrupted one indicates that of the mean. Significant changes in gene expression (GE) between untreated and treated animals are marked with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001). The gray area in the background adjusts for methodological variance and is defined for fold changes between 0.5 and 2. The interrupted white line refers to FC = 1 as a reference for GE prior to exposure.

was recently published (*39*). However, the observed transient downregulation in our *Rhesus macaques* study might indicate another response. To our knowledge, no further GE examinations of our gene set on day 35 postirradiation are published.

All genes in our study revealed an expected and significant association with dose (Fig. 3). However, this association was driven by unexposed samples because GE values in the dose range of 5.8–7.0 Gy persisted and showed no significant differences for all four genes. A saturation in GE values with doses exceeding 4 Gy has been independently demonstrated in several large-scale inter-laboratory comparison exercises, including up to eight different laboratories (*40–42*). Hence, our observations are in line with cited work.

Although of no impact regarding the diagnostic value of our four-gene set, higher GE values in treated compared with untreated *Rhesus macaques* were consistently observed for all genes and at each time point before and after irradiation (Fig. 3). An effect related to the administered vehicle (olive oil) or GT3 treatment seems most likely, but the later different

treatment groups already differ significantly in GE before irradiation and before neither GT3 nor vehicle was administered. However, for this study, new vendors of GT3 were selected and the specific formulation used in this study was found not to be efficacious (probably caused by unstable emulsion of GT3). Since this effect was present in all genes and observed even pre-exposure and aggravated over time, it probably reflects unknown and uncontrolled aspects inherent to our animal model. Further research in this regard is required and represents another limitation of our study.

In conclusion, the diagnostic significance for radiation-induced H-ARS severity prediction of *FDXR*, *DDB2,* and *POU2AF1* could be confirmed in this *Rhesus macaques* model as well, except that *DDB2* showed higher GE values than *FDXR*. The diagnostic significance of *WNT3,* as demonstrated in previous studies, could not be reproduced in Rhesus macaques, although this may be due to the animal model and methodological challenges.

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