Improved radiation expression profiling by sequential application of sensitive and specific gene signatures

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Running Title: Sequential Radiation Gene Expression Signatures

Number of Figures: 7

Number of Tables: 3

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Abstract (word count = 330)

Purpose. Combinations of expressed genes can discriminate radiation-exposed from normal control blood samples by machine learning based signatures (with 8 to 20% misclassification rates). These signatures can quantify therapeutically-relevant as well as accidental radiation exposures. The prodromal symptoms of Acute Radiation Syndrome (ARS) overlap some viral infections. We recently showed that these radiation signatures produced unexpected false positive misclassification of influenza and dengue infected samples. The present study investigates these and other confounders, and then mitigates their effects on signature accuracy.

Methods. This study investigated recall by previous and novel radiation signatures independently derived from multiple Gene Expression Omnibus datasets on common and rare non-malignant blood disorders and blood-borne infections (thromboembolism, S. aureus bacteremia, malaria, sickle cell disease, polycythemia vera, and aplastic anemia). Normalized expression levels of signature genes are used as input to machine learning-based classifiers to predict radiation exposure in other hematological conditions.

Results. Except for aplastic anemia, these confounders modify the normal baseline expression values, leading to false-positive misclassification of radiation exposures in 8 to 54% of individuals. Shared changes were predominantly in DNA damage response and apoptosis-related gene transcripts in radiation and confounding hematological conditions compromise the utility of these signatures for radiation assessment. These confounding conditions are known to induce neutrophil extracellular traps, initiated by chromatin decondensation, fragmentation and often,

programmed cell death. Ribovirus infections are proposed to deplete RNA binding proteins, inducing R-loops in chromatin which collide with replication forks resulting in DNA damage and apoptosis. To mitigate the effects of confounders, we evaluated predicted radiation positive samples with novel gene expression signatures derived from radiation-responsive transcripts of secreted blood plasma proteins whose expression levels are unperturbed in these confounding conditions.

Conclusions. This approach identifies and eliminates misclassified samples with underlying hematological or infectious conditions, leaving only samples with true radiation exposures. Diagnostic accuracy is significantly improved by selecting genes that maximize both sensitivity and specificity in the appropriate tissue using combinations of the best signatures for each of these classes of signatures.

Keywords: Biodosimetry, Gene expression, False positive reactions, DNA damage response (DDR), Radiation

Introduction

Potential ionizing radiation exposures from environmental exposures including industrial nuclear accidents, military incidents, or terrorism are also threats to public health. There is a need for large scale biodosimetry testing, which requires efficient screening techniques to differentiate exposed individuals from non-exposed individuals and to determine the severity of exposure. Radiation biodosimetry is also useful for biomedical diagnostic and therapeutic applications, where it may be used to monitor and calibrate absorbed radiation levels.

One of the most promising approaches to rapidly quantify absorbed radiation, biodosimetry, is based on responses to combinations of different genes expressed in blood (Dressman et al. 2007; Paul and Amundson, 2008; Ding et al. 2013; Lu et al. 2014). Combinations of genes, termed signatures, can predict ionizing radiation exposure in humans and mice from publicly available microarray gene expression levels (Zhao et al. 2018a). Our approach uses supervised machine learning (ML) with genes previously implicated or established from genetic evidence and biochemical pathways that are altered in response to these exposures. Several groups (Boldt et al. 2012; Budworth et al. 2012; Knops et al. 2012; Ghandhi et al. 2017) have also used signatures to determine radiation exposures. Biochemically-inspired machine learning is a robust approach to derive diagnostic gene signatures for chemotherapy (Dorman et al. 2016; Mucaki et al. 2016; Mucaki et al. 2019; Bagchee-Clark et al. 2020) as well as radiation (Zhao et al. 2018a). Given the limited sample sizes of typical datasets, appropriate ML methods for deriving gene signatures have included Support Vector Machines, Random Forest classifiers, Decision Trees, Simulated Annealing, and Artificial Neural Networks (Rogan, 2019; Boldrini et al. 2019).

Before performing ML, we ranked a set of curated radiation-response genes in radiation-exposed samples by Minimum Redundancy Maximum Relevance (mRMR; Ding and Peng; 2005), which orders genes based on mutual information between their expression and whether the sample was irradiated and the degree to which their expression is dissimilar from previously selected genes. A Support Vector Model (SVM) or signature is generated from the top ranked gene set by adding and removing genes that minimize either model misclassification or log-loss (Zhao et al. 2018a). ML alone is not sufficient to derive useful signatures, since underlying biochemical pathways and disease mechanisms also contribute to selecting relevant and non-redundant gene features (Bagchee-Clark et al. 2020). Signatures were evaluated either by a traditional validation approach or by stratified k-fold validation (which splits the same dataset into k groups reserved for testing and training). The traditional model-centric approach uses a normalized training set which is then used to predict outcome of normalized independent test data. While more susceptible to batch effects than k-fold validation, this approach can incorporate smaller datasets or heterogeneous data sources.

The present study is concerned with the selection of "normal" controls for training and testing signatures, in particular of individuals with clinically overlapping diagnoses, which has a critical but underappreciated impact of accurate molecular diagnostics. Previous studies have revealed gene expression changes in blood from unirradiated individuals with underlying metabolic or confounders, including smokers (Paul and Amundson, 2011) and modulators of inflammation such as lipopolysaccharide of bacterial origin or curcumin (Cruz-Garcia et al. 2018). While investigating the possibility of using radiation gene signatures to differentiate prodromal Acute Radiation Syndrome (ARS) from early stage influenza, we unexpectedly discovered that riboviral infections (influenza and dengue fever) induce similar expression

changes in response to gamma radiation consistent with those present in ARS. Our previous radiation signatures (M1-M4; Zhao et al. 2018a) misclassified a significant proportion of unirradiated blood from influenza and dengue fever patients as radiation-exposed (Rogan et al. 2021 - Section 1 – Table 7). Demonstration of false positive radiation exposure predictions of samples from individuals diagnosed of influenza has also been noted by other investigators (Jacobs et al. 2020 - Supplementary data). The M1-M4 signatures predominantly consisted of genes with roles in DNA damage response, programmed cell death, and inflammation. However, many of these genes were also induced in influenza and dengue virus infected samples. The expression of the DNA damage response gene *DDB2*, for example, increases with radiation, but was also induced in a significant number of viral infected samples which were then classified incorrectly as radiation exposed (Figure 5 of Rogan et al. 2021). *DDB2* is present in many other radiation gene signatures or developed radiation-exposure assays (Paul and Amundson, 2008; Lu et al. 2014; Jacobs et al. 2020).

While this process strictly validates signatures to estimate sensitivity to radiation, the same rigor has not been applied to determining specificity, which we suspected could be impacted by comorbidities in the general population. This study considers other hematological conditions that alter the normal expression of the same genes in blood that are often selected for assessment of radiation exposure and suggests an approach for addressing this issue.

[Figure 1 Near Here]

Methods

Datasets evaluated

We explore whether the presence of other blood-borne conditions (infections, inherited and idiopathic hematological disorders) might also confound prediction with these signatures. We also derive new signatures from additional radiation datasets containing other radiation-responsive genes to investigate whether these confounder effects are reproducible (Figure 1).

A prerequisite for inclusion of any gene in a ML-based signature was that expression data be present in both training and independent validation radiation datasets (Gene Expression Omnibus [GEO; https://www.ncbi.nlm.nih.gov/geo/] identifiers: GSE701 [Jen and Cheung, 2003], GSE1725 [Rieger et al. 2004], GSE6874 [GPL4782; Dressman et al. 2007] and GSE10640 [GPL6522; Meadows et al. 2008]). As a consequence, several well known radiation genes which have appeared in other radiation gene signatures (Paul and Amundson, 2008; Oh et al. 2014; Port et al. 2017; Tichy et al. 2018; Jacobs et al. 2020) were previously not considered and thus not present in any of our derived models. Genes were excluded either because they were: 1) absent from one or more datasets (e.g. *FDXR*, *RPS27L*, *AEN* were missing from GSE10640); 2) mislabeled in the dataset with a legacy name leading to a mismatch between datasets (e.g. *PARP1* appears as *ADPRT* in GSE6874); 3) secondary RNAs, such as micro- or long non-coding-RNA derived from the same gene (e.g. *BBC3* probes also detected multiple microRNAs in GSE1725; *POU2AF1* probes in GSE701 indicated as LOC101928620'); or 4) missing from the set of curated radiation response genes (e.g. *PHPT1*, *VWCE*, *WNT3*).

To address missing genes in radiation response signatures (Zhao et al. 2018a), we developed novel signatures based on more recent radiation datasets, including GEO: GSE26835, GSE85570, GSE102971 and ArrayExpress: E-TABM-90. GSE85570 consisted of pre-treatment blood from 200 prostate cancer patients, with half of each sample exposed to 2 Gy, followed by analysis on HT HG-U133+ PM microarrays (Affymetrix). In GSE102971, peripheral blood (PB)

from healthy volunteers was irradiated ex-vivo at 2, 5, 6 and 7 Gy and analyzed 24 hours after exposure with a custom 4x44K microarray (Agilent). In E-TABM-90, ex-vivo lymphocytes from 50 prostate cancer patients (2 years post-radiotherapy) received either 2 Gy radiation or were unirradiated, and analyzed on HG-U133A microarrays (Affymetrix). In GSE26835, immortalized lymphoblastoid cells at 2 and 6 hours after exposure to 10 Gy were analyzed on HG-U133A microarrays. Previous radiation gene signatures (Zhao et al. 2018a) were derived from GSE6874[GPL4782] and GSE10640[GPL6522] 6 hours post-exposure from healthy donors and patients undergoing total body radiation (~2 Gy), and expression was analyzed with non-commercial, custom microarrays.

To investigate whether other disorders could also confound radiation signatures, we assessed performance of radiation signatures utilized in this study with available gene expression datasets for other blood-borne diseases. These datasets include GEO: GSE117613 (cerebral malaria and severe malarial anemia; Nallandhighal et al. 2019), GSE35007 (sickle cell disease in children; Quinlan et al. 2014), GSE47018 (polycythemia vera; Spivak et al. 2014), GSE19151 (single and recurrent venous thromboembolism; Lewis et al. 2011), GSE30119 (Staphylococcus [S.] aureus infection; Banchereau et al. 2012), and GSE16334 (aplastic anemia; Vanderwerf et al. 2009).

Data Preprocessing

Microarray data from GSE85570 and GSE102971 were pre-processed as described in Zhao et al. (2018a). Briefly, missing gene expression values were imputed (or removed if data for these genes were missing from >5% samples) from nearest neighbours, the expression of patient replicates was averaged, and gene expression was z-score normalized. Genes previously implicated in the radiation response (N=998) were analyzed, including 13 additional radiation

genes described in other studies, including *CD177*, *DAGLA*, *HIST1H2BD*, *MAMDC4*, *PHPT1*, *PLA2G16*, *PRF1*, *SLC4A11*, *STAT4*, *VWCE*, *WLS*, *WNT3*, and *ZNF541* (N=1,011 genes total).

Derivation of Radiation Gene Signatures

mRMR ranking was determined for curated, expressed radiation-related genes in the presence or absence of radiation. mRMR first selects the gene with the highest mutual information (MI) with radiation, then corrects for redundancy by selecting the gene candidate with the highest difference between the mutual information and the mean mutual information between all previously selected genes with the candidate gene as a probability vector (Ding and Peng, 2005; Mucaki et al. 2016). Minimizing redundancy results in some subsequent selected gene(s) with orthologous expression patterns relative to the preceding gene(s). These may exhibit significantly lower MI, typical of a weak radiation response. Higher ranked genes exhibit greater MI overall, however. Gene rankings by mRMR and the computed mutual information for each radiation gene in each of the datasets evaluated are provided in Suppl. Table S1.

SVM-based gene signatures were derived by greedy feature selection, including forward sequential feature selection (FSFS), backward sequential feature selection (BSFS) and complete sequential feature selection (CSFS; Zhao et al. 2018a). Software is provided in a Zenodo archive (Zhao et al. 2018b). Both FSFS and BSFS models were derived from the top 50 ranked mRMR genes, in addition to other published radiation responsive genes: *AEN*, *BAX*, *BCL2*, *DDB2*, *FDXR*, *PCNA*, *POU2AF1*, and *WNT3*. SVMs were derived with a Gaussian radial basis function kernel by iterating over box-constraint (C) and kernel-scale (σ) parameters and gene features, minimizing to either misclassification or log loss by cross-validation (Zhao et al. 2018a; Bagchee-Clark et al. 2020). Gene signatures were then assessed with a validation dataset and re-evaluated (by misclassification rates, log loss, Matthews correlation coefficient, or goodness of

fit). This study primarily reports misclassification rates to simplify comparisons of results between radiation-exposed and disease confounder datasets. Those with high misclassification rates in validated radiation datasets (>50%) have been excluded. Variation in radiation signature composition among different source datasets can be attributed to distinct microarray platforms, other batch effects, and inter-individual variation in gene expression which cannot be fully mitigated by normalization. These contribute to MI variability, which both alters mRMR rank and features selected during signature derivation.

The quality of each dataset was assessed based on the dynamic range in responses by signature genes to radiation. This was based on the premise that these responses among different confounding datasets might alter expression of some of the same radiation-responsive genes. MI between gene expression and radiation dose is indicated in Suppl. Table S1. Datasets GSE85570 and GSE102971 both exhibit high MI with radiation exposure (maximum MI > 0.7 bits for both datasets; 77 and 115 genes with > 0.2 bits MI, respectively). By contrast, E-TABM-90 and GSE26835 both exhibited low MI for top ranked genes with radiation exposure. Of the top 50 ranked genes in E-TABM-90, 13 genes had MI values <10% of the MI of the top ranked gene [0.3 bits], and 856 of 860 genes in the complete dataset had MI < 0.2 bits. The maximum MI for GSE26835 was 0.25 bits; 40 of 50 top ranked genes exhibited <10% MI of this value, and the radiation response genes *DDB2*, *PCNA*, *FDXR*, *AEN*, and *BAX*, had unexpectedly low rankings (>100; 2h and 6h post-exposure) and MI < 0.15 bits. The low MIs across all eligible genes indicates that the response to radiation was nearly random in both datasets. They failed to satisfy quality criteria and were excluded from further analyses. Radiation toxicity in E-TABM-90 and cell line immortalization in GSE26835 appears to compromise their radiation response.

Radiation gene signatures derived from the plasma secretome

Global protein synthesis significantly increases 4-8 hr after initial radiation exposures (Braunstein et al. 2009), with some profile changes detectable weeks to months later (Pernot et al. 2012; Hall et al. 2017). Radiation signatures in blood have been derived from proteins secreted into plasma (Wang et al. 2020) and expressed by multiple cell lineages (Ostheim et al. 2021). Radiation-induced short-term changes in the abundance of mRNAs encoding plasma proteins (that correspond to protein concentration changes) could allow steady state mRNA expression to be used as a surrogate for plasma protein levels. Significant correlations between mRNA and protein expression have been shown when the data have been transformed to normal distributions (Greenbaum et al. 2001; Greenbaum et al. 2002). This approach was adopted to derive mRNA signatures from radiation responsive, secretome-encoded genes.

Only genes that encoded proteins present in blood plasma were used to derive an alternate set of gene expression signatures. The initial set of plasma proteins from the Human Protein Atlas "Human Secretome" [http://www.proteinatlas.org/humanproteome/secretome] and the Plasma Protein Database [http://www.plasmaproteomedatabase.org] were cross-referenced to create a list of 1377 shared proteins. The Genotype-Tissue Expression (GTEx) Portal (https://gtexportal.org/home/) was used to determine which genes encoding these secreted proteins are expressed in either leukocytes or transformed lymphoblasts at detectable levels (where Transcripts Per Million (TPM) > 1; N=682). Expressed genes present in radiation datasets GSE6874[GPL4782] (N=428) and GSE10640[GPL6522] (N=325) were used to derive ML models of genes encoding for human plasma proteins using CSFS, BSFS, and FSFS.

Evaluating specificity of radiation gene signatures with expression of genes in confounding hematological conditions

Radiation gene signatures derived in this study and in Zhao et al. (2018a) were used to evaluate datasets of patients with controls for hematological disorders by traditional ML validation (‘*regularValidation_multiclassSVM.m*’; Zhao et al. 2018b). We determined how often these unirradiated individuals were misclassified as radiation-exposed by these models. The confounding effect of the various blood-borne disorders tested was measured by comparing divergence between the FP (false positive) rates in patient vs. control samples. We explored the degree to which specific genes from each signature contributed to misclassification by performing feature removal analysis (Zhao et al. 2018a; Mucaki et al. 2019). Expression levels of radiation responsive genes in confounder datasets of correctly vs. misclassified samples were contrasted using violin plots. These display weighted distributions of the normalized gene expression from each confounder datasets which were either properly (true negative [TN]) and improperly (FP) classified as irradiated by the radiation gene signatures (created in R [i386 v4.0.3] with *ggplot2*). Misclassified sample counts of confounder sub-phenotypes were stratified using Sankey diagrams (SankeyMATIC; http://sankeymatic.com/build/). This analysis delineates FP and TN predictions (at the individual level) of groups of diseased patients and controls from these datasets according to predictions of the designated, specific radiation gene signature.

Results

Initial Evaluation of Candidate Genes in Radiation Gene Expression Datasets for Machine Learning

We derived new gene expression signatures by leave-one-out and k-fold cross-validation from microarray data based on more recent comprehensive gene datasets (GEO: GSE85570 and GSE102971) besides those we previously reported (Zhao et al. 2018a). Only some of the 1,011 curated genes were present on these microarray platforms, including 864 genes in GSE85570 and

971 genes of GSE102971. After normalization, gene rankings by mRMR between GSE102971 and GSE85570 were similar (Suppl. Table S1). In GSE85570, *FDXR* were ranked first, while *AEN* was top ranked in GSE102971 (*FDXR* was ranked 38th). *DDB2* was top ranked in GSE6874 and GSE10640 (Zhao et al. 2018a), datasets which lacked expression for *FDXR* and *AEN*, respectively. Radiation-response genes among the top 50 ranked genes present in all 4 datasets included *BAX*, *CCNG1*, *CDKN1A*, *DDB2*, *GADD45A*, *PPM1D* and *TRIM22*.

ERCC1 was selected by mRMR to be the second-ranked gene in GSE102971, even though its MI was 31-fold lower than the top ranked gene, *AEN* (Suppl. Table S1). MI of the second-ranked genes in GSE6874 (*RAD17*) was 7-fold lower than the first (*DDB2*), while GSE10640 (*CD8A*) showed a 4-fold difference. Six of the top 50 genes in GSE102971 exhibited <10% of the MI of *AEN* (3 genes for GSE6874; none of the top 50 in GSE10640 and GSE88570 were <10% of the top ranked gene). Selection of low MI genes by ML feature selection likely reduces accuracy of gene signatures during validation steps. In the future, signature derivation will set a minimum MI threshold for ranking genes by mRMR.

The overall levels of MI for top ranked genes in GSE85570 (0.72 bits for *AEN*) and GSE102971 (0.82 bits for *FDXR*) were comparable (Suppl. Table S1). In GSE102971, the genes with the highest MI were *AEN*, *DDB2*, *FDXR*, *PCNA* and *TNFRSF10B* (closely followed by *BAX*). While each were found in the top 50 ranked genes, some rankings were decreased to minimize redundant information (*FDXR* and *AEN* are ranked #38 and #41 in the GSE102971 dataset, respectively). MI for the top ranked genes in GSE6874 and GSE10640 were lower by comparison (0.31 and 0.47 bits for *DDB2*, respectively); the depressed maximum MI values in these datasets may, in part, be related to reduced numbers of eligible genes on these microarray platforms.

Radiation Gene Signature Performance in Blood-Borne Diseases

The specificity of previously-derived radiation signatures selected after k-fold validation (KM1-KM7) and traditional validation (M1-M4; Zhao et al. 2018a) was assessed with normalized expression data of patients with unrelated hematological conditions, rather than evaluating unirradiated healthy controls. Signatures M1 and M2 (derived from GSE10640; Table 1) and M3 and M4 (derived from GSE6874; Table 1) were assessed with expression datasets of influenza A (GSE29385, GSE82050, GSE50628, GSE61821, GSE27131) and dengue fever (GSE97861, GSE97862, GSE51808, GSE58278) blood infections (Rogan et al. 2021). FPs for radiation exposure were defined as instances where the misclassification rates of individuals with the disease diagnosis exceeded normal controls. A clear bias towards FP predictions of infected samples relative to controls was evident with all of these radiation gene signatures (Rogan et al. 2020 [extended data – Section 1 Table 7]). Dissection of the ML features responsible implicated 10 genes contributing to misclassification, including *BCL2*, *DDB2* and *PCNA*. These other conditions also confound predictions by radiation signatures derived by k-fold validation (KM1-KM7; Table 1).

[Table 1 Here]

High levels of FP misclassification of viral infections were also evident with these signatures (Supp. Table S2A). KM6 and KM7 (derived from GSE6874) misclassify all influenza and most dengue fever (GSE97861, GSE51808 and GSE58278) datasets of patients at higher rates than uninfected controls. KM3-KM5 exhibited low FP rates in influenza relative to other models, but dengue virus datasets GSE97862, GSE51808 and GSE58278 exhibited higher FP rates in infected samples relative to uninfected controls (Suppl. Table S2A). Interestingly, KM5 is the only gene signature in which *DDB2* is not present, and this gene contributes to high FP

rates (Rogan et al. 2021). KM1 and KM2, which were derived from a third radiation dataset (GSE1725), often misclassified virus infected samples relative to controls (KM1 only: GSE97861; KM2 only: GSE82050, GSE27131, GSE97862 and GSE50628; both KM1 and KM2: GSE51808, GSE58278, and GSE61821). However, in some datasets, these models also demonstrated high FP rates in controls.

Expression levels in patients with latent influenza A and dengue fever stabilize at levels similar to uninfected controls after either convalescence or at the end stage infection. For example, M4 exhibited a 54% FP rate in dengue-infected individuals 2-9 days after onset of symptoms (GSE51808), but samples were correctly classified as unirradiated > 4 weeks after initial diagnosis (Figure 2A). The influenza gene expression dataset GSE29385 longitudinally sampled infected patients after initial symptoms at <72 hours [T1], 3-7 days [T2], and 2-5 weeks [T3]. FPs were significantly decreased at T3 for nearly all models tested (19 infected samples misclassified by M1 at T1 was reduced to 2 cases at T3; Figure 2B). These results clearly implicate these viral infections as the source of the transcriptional changes that affect parallel effects of radiation on these signature genes.

[Figure 2 Here]

Specificity of radiation signatures using datasets of other hematological conditions

We investigated whether radiation gene signature accuracy was compromised by the presence of other blood borne infections and non-infectious, non-malignant hematological pathologies with publicly-available expression data on patients with adequate sample sets (>10 individuals with corresponding control samples). These included thromboembolism, S. aureus bacteremia, malaria, sickle cell disease, idiopathic portal hypertension, polycythemia vera, and aplastic

anemia. The idiopathic portal hypertension dataset was excluded due to insufficient numbers of samples. We then determined recall levels for signatures M1-M4 and KM3-KM7 evaluated with these datasets, with the expectation that these models would predict all potential confounders as unirradiated (Suppl. Table S2B).

Each radiation gene signature was confounded by some, but not all, blood-borne disorders and infections. S. aureus infected samples were frequently misclassified as FPs by all signatures, except KM7 (Figure 3). High FP rates were observed for: M1 and KM5 – sickle cell and S. aureus; M2 – S. aureus; M3 and M4 – malaria, sickle cell, thromboembolism, polycythemia vera and S. aureus; KM3 and KM4 – malaria, sickle cell and S. aureus; KM6 – thromboembolism, polycythemia vera, S. aureus; KM7 – malaria, thromboembolism and polycythemia vera. The malaria dataset stratified patients with either cerebral malaria or severe malarial anemia (Nallandhighal et al. 2019). The severe malarial anemia subset contains the majority of the FPs (Figure 2C). The thromboembolism dataset (Lewis et al. 2011), which categorized patient diagnoses as either single or recurrent thromboembolism, exhibited similar FP rates for both subsets (Figure 2D). Predictions of radiation exposure by M3, M4, KM6 and KM7 were confounded by transcriptional changes resulting from different blood-borne conditions, while M2 and KM5 are the least influenced by these conditions. Aplastic anemia did not increase FP rates compared to controls for any of the signatures, consistent with our previous findings (Rogan et al. 2021).

[Figure 3 Here]

Predictions by high sensitivity radiation signatures M4, KM6 and KM7 were confounded by multiple viral, blood-borne infections and non-infectious blood disorders. The genes within these signatures with the most profound impact on accuracy were determined by evaluating

differential expression of these genes in samples that were correctly (TN) vs incorrectly (FP) classified. The normalized gene expression distributions of TN and FP samples in malaria, S. aureus, sickle cell disease and thromboembolism, influenza A and dengue fever were visualized as violin plots (Figure 4 and Mucaki et al. 2021). Expression of *BCL2* for the S. aureus, sickle cell disease and malaria samples was significantly lower in FP samples relative to TNs with M4 ($p<0.05$ with Student's t-test, assuming two-tailed distribution and equal variance; Figure 4A [left]), similar to the effect of radiation exposure on expression of this gene (Figure 4A [right]). These same FP individuals have significantly higher *DDB2* expression in both S. aureus and sickle cell disease (Figure 4B). Increased *DDB2* expression was also observed for FPs using KM6 and KM7. For both genes, differences in expression in TN and FP samples were congruent with the changes observed in the radiation exposure datasets. Genes that may also contribute to misclassification include *GADD45A* in M4 (higher expression in diseased individuals vs. controls and induced by radiation exposure), and *PRKCH* and *PRKDC*, respectively, in KM6 and KM7 (decreased expression in FPs and in response to radiation). *BAX*, which is induced by radiation, is similarly expressed in FP and TN samples, and probably does not contribute to misclassification by M4.

[Figure 4 Here]

To determine the extent to which each gene contributes to the FP rates in each signature, gene features were removed individually, the signature was rebuilt, and misclassification rates were reassessed for each confounding condition (Suppl. Tables S3A [M1-M4] and S3B [KM3-KM7]). Removing any gene from gene signatures M1, M3, M4, KM3, KM5 and KM7 did not significantly alter the observed misclassification rates. Elimination of *PRKDC* (DNA double stranded break repair and recombination) and *IL2RB* (innate immunity/inflammation) reduced FP

rates in thromboembolism patients by 10% and 5% for M4, respectively (Figure 5A), which still exceeded the FP rates of controls. Removal of these genes did not improve the FP rate of M4 in S. aureus-infected samples (Figure 5B). Thus, no single gene feature dominated the predictions by these signatures and could account for the misclassified samples. Removal of *DDB2*, *GTF3A* or *HSPD1* from KM4 significantly decreased its FP rate to the malaria dataset (18% to 0-3%; Suppl. Table S3B). Similarly, removal of *DDB2* from M2 and KM6 led to the complete elimination of FPs in both patients and controls. However, the removal of *DDB2* from these models was previously shown to severely reduce the true positive (TP) rate in irradiated samples (Zhao et al. 2018a); these genes cannot be eliminated without affecting the sensitivity of these signatures to accurately identify radiation exposed samples.

[Figure 5 Here]

The contributions of individual signature genes can be assessed by evaluating their impact on overall model predictions for different patients. Expression changes were incrementally introduced to computationally determine the expression level required to change the outcome of the ML model (i.e. the inflection point of the prediction that distinguishes exposed from unirradiated samples). The threshold is visualized in the context of the expression value in the individual superimposed over a histogram of the distribution of expression for all confounders in the dataset. Individual expression values close to this threshold can indicate lower confidence in either the radiation exposure prediction or of misclassification by the model. Expression levels and thresholds of *DDB2*, *IL2RB*, *PCNA* and *PRKDC* for 3 individuals with thromboembolism (GSE19151) predicted as irradiated by M4 (GSM474819, GSM474822, and GSM474828) are indicated in Suppl. Figure 1. Reduction of *DDB2* expression corrected misclassification for all patients, as did decreasing *PCNA* expression in GSM474822 and

GSM474828. Increasing *IL2RB* and *PRKDC* expression of these two patients also corrected their misclassification. These results correspond to the effects of radiation on the expression of these genes in the GSE6874 and GSE10640 datasets, e.g. induction of *DDB2* and *PCNA*, repression of *IL2RB* and *PRKDC* (Mucaki et al. 2021). The expression changes required for *DDB2*, *PCNA* and *PRKDC* in these patients were nominal relative to the dynamic range of the entire dataset, but were sufficient to alter predictions of the signatures. Conversely, changes in expression of *PCNA*, *IL2RB* or *PRKDC* were unable to modify the prediction of M4 in GSM474819. Only a large decrease in *DDB2* expression to levels below that of nearly all thromboembolism patients was able to switch the classification of this individual. This reinforces previous observations about the strong impact of *DDB2* expression levels on prediction accuracy (Zhao et al. 2018a). Nevertheless, the combined expression of most of the genes which constitute the signature ultimately determine the outcome of the classifier. Incorrect classifications where expression values are close to the model's predictive inflection point should be considered when assessing misclassification accuracy.

Misclassification of confounders with radiation gene signatures derived in this study

FSFS- and BSFS-based radiation gene signatures were derived from the top 50 ranked genes of GSE85570 and GSE102791. GSE102791 contained sets of 20 samples, each irradiated at different absorbed energy levels (0 vs 2, 5, 6, and 7 Gy). Different ML models were derived either utilizing the full dataset or based on a combination of 2 and 5 Gy samples. The models derived from either subset of GSE102791 also exhibited very low misclassification (0-1 samples) and log-loss (<0.01). Common genes selected from signatures derived from GSE102791 included *AEN*, *BAX*, *TNFRSF10B*, *RPS27L*, *ZMAT3* and *BCL2* (Table 2A and Suppl. Table S4A). Genes selected in GSE85570-based signatures included *BAX*, *FDXR*, *XPC*, *DDB2* and

TRIM32 (Table 2B and Suppl. Table S4B). All signatures from this dataset exhibited low misclassification rates (<0.5% by cross-validation).

[Table 2 Here]

The radiation gene signatures with the lowest misclassification rates from these datasets were evaluated against the blood-borne disease confounder datasets that compromised the accuracies of the M1-M4 and KM3-KM7 signatures (Zhao et al. 2018a). Misclassification rates were estimated using confounder datasets containing the largest numbers of samples, including thromboembolism, S. aureus infection, sickle cell and malaria. The signature designated M5 (consisting of *AEN* and *BCL2*; Table 2A) showed an elevated FP rate over controls in blood samples from individuals with thromboembolism (18%), S. aureus infection (7%), and malaria infection (33%; Suppl. Table S4A). Misclassification by M5 was increased by 6% in sickle cell disease; a second GSE102791-derived signature containing *AEN* (M8; Table 2A) also exhibited a higher FP rate in sickle cell disease (29%). Removal of genes from M8 significantly increased the FP rate for both controls and diseased individuals, which is a limitation of models based on small numbers of genes (Suppl. Table S5A). M9 (Table 2B) includes *BAX* and *FDXR*, and exhibited increased FP rates in thromboembolism relative to controls (34-38% increased FP). Interestingly, M13 shows increased FPs in thromboembolism (similar to M1-M4), while M11 does not (Table 2B), despite both signatures containing *DDB2*. Removing any of the genes from these models did not substantially alter misclassification, except for a large decrease in FP upon removal of *RPS27L* from M9 (Suppl. Table S5B). Both M11 and M13 exhibited high FPs in malaria samples. BSFS models derived from GSE85570 contained *FDXR*, *BAX* and *DDB2*, and showed high FP in S. aureus, sickle cell disease and malaria samples. These confounders adversely affect the accuracy of gene signatures containing radiation response genes (such as

FDXR and *AEN*) present in both these and other recently derived signatures in the published literature.

Mitigating expression changes arising from confounding blood disorders with secretome-based gene signatures

Signatures that distinguish gene expression levels at different radiation flux rates, levels, and energies also need to differentiate between changes resulting from other hematological conditions. The predicted FPs in unexposed patients with confounding conditions may be the result of changes in expression of DNA damage and apoptotic genes that are shared with radiation responses. We attempted to derive ML-based gene signatures that exclude DNA damage or apoptotic genes which we anticipated would be less prone to misclassifying individuals with confounding blood disorders. Changes in extracellular blood plasma protein levels from radiation exposure tend to exclude intracellular DNA damage or apoptosis response, thereby representing viable candidates for alternative signatures that would not overlap the same gene features found in our previously described predictors. For example, FLT3 ligand (*FLT3LG*) and amylase (AMY; *AMY1A*, *AMY2A*) have previously been established as indicators for radiation exposure in blood serum (Tapio, 2013). AMY levels assess parotid gland damage (Barrett et al. 1982) and FLT3 ligand levels indicate bone marrow effects (Bertho et al. 2001). Genes which encode for secreted proteins were used to derive new radiation gene signatures using our previously described methods (Zhao et al. 2018a).

The plasma protein-encoding gene *GM2A* had the highest MI with radiation in GSE6874 (MI=0.31), while *TRIM24* was highest in GSE10640 (MI=0.27; Suppl. Table S1). Surprisingly, MI of *TRIM24* was low in GSE6874 (MI=0.05) was ranked second to last by mRMR (Suppl. Table S1) and was not differentially expressed (p-value > 0.05 by t-test; Suppl. Table S6C).

GM2A is absent from the GSE10640 dataset. Other top 50 ranked genes by mRMR in both datasets include *ACYP1*, *B4GALT5*, *FBXW7*, *IRAK3*, *MSRB2*, *NBL1*, *PRF1*, *SPOCK2*, and *TOR1A*.

We derived 5 independent plasma protein-encoded radiation gene signatures ('secretome' models) that showed the lowest cross-validation misclassification accuracy or log-loss by various feature selection strategies (labeled SM1-SM5 [Secretome Model 1-5] in Table 3 and Suppl. Table S6A). SM5 feature selection was limited to the top 50 genes ranked by mRMR. This pre-selection step was not applied when deriving SM2 and SM3, whereas SM1 and SM4 were derived by CSFS feature selection which obtains genes sequentially by mRMR rank order without applying a threshold. Significantly upregulated genes and models consisted of *SLPI* (SM1, SM2), *TRIM24* (SM3, SM4, SM5), *TOR1A* (SM3, SM4), *GLA* (SM4), *SIL1* (SM4), *NUBPL* (SM4), *NME1* (SM4), *IPO9* (SM4), *IRAK3* (SM5), *MTX2* (SM5), and *FBXW7* (SM5). Downregulated genes included *CLCF1* (SM1, SM2), *USP3* (SM1, SM2), *TTC19* (SM2), *PFN1* (SM3, SM4, SM5), *CDC40* (SM4), *SPOCK2* (SM4), *CTSC* (SM4), *GLS* (SM4), and *PPP1CA* (SM5; Suppl. Table S6C). The models exhibited 12-39% misclassification (by k-fold validation) when validated against the alternative radiation dataset. The GSE6874 dataset was not suitable for signature derivation or validation, since data for *LCN2*, *ERP44*, *FN1*, *GLS*, and *HMCN1* were missing; these genes are present in models SM3 and/or SM4 (Table 3). The performance of the derived signatures was also assessed by inclusion of *FLT3* or *AMY*, either individually or in combination. These genes did not improve model accuracy beyond the levels of the best performing signatures that we derived.

[Table 3 Here]

The specificity of secretome signatures was then evaluated with expression data from unirradiated individuals with blood-borne diseases and infections (Suppl. Table S6B). SM3 and SM5 correctly classified nearly all samples in each dataset as unirradiated and maintained a FP rate <5% in all datasets (Figure 6). SM3 and SM5 contain <10 genes, were derived from GSE10640 and share the genes *TRIM24* and *PFN1* (ranked #1 and #21 by mRMR). Both genes are significantly differentially expressed after radiation exposure, as is *TOR1A* in SM3 and *IRAK3*, *PPP1CA*, *MTX2*, *FBXW7* and *CTSC* in SM5 (Suppl. Table S6C). SM3 and SM5 have the highest fraction of genes found significant by Student's t-test, which may explain its superior specificity relative to the other secretome signatures. Thromboembolism could only be evaluated with SM3 and SM5 due to missing genes from the SM1, SM2 and SM4 signatures. Conversely, SM1, SM2 and SM4 accuracy was compromised by expression changes of genes in one or more blood-borne diseases. Malaria (28%) and S. aureus (19%) infected patients were misclassified by SM1 as FPs (with 0.5% and 6.1% FP in controls, respectively), indicating that SM1 accuracy was affected by these underlying infections. SM4 accuracy was also impacted by S. aureus infection.

[Figure 6 Here]

SM3 and SM5 exhibited high specificity for radiation exposure (low false positivity for confounders) but were less sensitive than M1-M4 and KM3-KM7 (Table 3). Accurate identification of radiation exposed individuals should be feasible with a sequential strategy that first evaluates blood samples with suspected radiation exposures with signatures known to exhibit high sensitivity (e.g. M4; 88% accuracy to radiation exposure), followed by identification of FPs among predicted positives with SM3 and/or SM5 (which were not influenced by confounders; Suppl. Table S6B and S6D). By identifying and removing misclassified,

unirradiated samples with the secretome-based radiation signatures, sequential application of both sets of signatures would predict predominantly TP samples.

Discussion

We demonstrated high misclassification rates of radiation gene expression signatures in unirradiated individuals with either infections or blood borne disorders relative to normal controls. This was confirmed with a second set of k-fold validated radiation signatures from our previous study (Zhao et al. 2018a). Similar results were obtained with expression data from unirradiated individuals exhibiting other hematological conditions, which extended the spectrum of other abnormalities misclassified as exposed to radiation. Some of the same genes that are induced or repressed by radiation exhibit similar changes in direction and magnitude in infections and hematological conditions (for example, *DDB2*, *BCL2*). Signatures derived from more recent microarray platforms that contain key radiation response genes missing in our previous study (e.g. *FDXR*, *AEN*) were also prone to misclassifying hematological confounders as false positives. By assessing the performance of each model and rejecting signatures with a high rate of false radiation diagnoses in confounding conditions, many individuals with these comorbidities might be ineligible for these radiation gene signature assays.

Given the similarities between the changes in genes expressed by radiation and by confounding hematological disorders, this raises the question as to whether therapeutic radiation in individuals with these conditions would be contraindicated, since they might exacerbate, increase severity, or compromise treatment outcomes. Indeed, there is already some evidence of interaction of these comorbidities with radiation exposure. A side effect of therapeutic radiation, radiodermatitis, is associated with S. aureus infections (Hill et al. 2004). In a large meta-analysis,

radiation therapy was contraindicated for individuals with venous thromboembolism (Guy et al. 2017).

The symptoms of prodromal influenza and ARS significantly overlap. During influenza outbreaks, this could impact accurate and timely diagnosis of ARS. Expression-based bioassays might not improve this diagnostic accuracy, since traditional radiation signatures maximize sensitivity without accounting for the diminished specificity due to underlying hematological conditions. Other highly specific tests for radiation exposure, such as the dicentric chromosome assay, require longer to perform, but analysis times are now as fast or faster than commercial gene expression assays, less variable, and can be more accurate (Rogan et al. 2016; Liu et al. 2017; Shirley et al. 2017; Li et al. 2019, Shirley et al. 2020). Existing gene expression assays will need to address the false positive results obtained for individuals with hematological conditions before they can be used in general populations, who may not have a history of these conditions or who may have been pre-screened as a precondition to military or space deployment.

Use of matched, unirradiated controls provides a measure of sensitivity and dynamic range of the derived radiation gene signature. Given the shared responses of different hematopathologies by leukocytes, the specificity of the signature for radiation exposure would, under ideal circumstances, be expected to exclude detection of other pathologies. Negative controls are typically people who are currently without disease or mild symptoms. In a nuclear incident or accident, the exposed population will include many individuals with underlying comorbidities. Application of radiation signatures derived by maximizing sensitivity in this population could lead to inappropriate diagnosis, and possibly treatment for ARS. The sequential gene signature assay design should improve the specificity of radiation gene expression assays in these individuals, and across the general population.

The cumulative incidences of these confounders are not rare, especially influenza which affected approximately 11% of the US population during the 2019-2020 season (11,575 per 100,000; https://www.cdc.gov/flu/about/burden). The frequency of dengue fever was also high in the Caribbean (2,510 per 100,000), Southeast Asia (2,940 per 100,000) and in South Asia (3,546 per 100,000; based on cases from 2017 [Zeng et al. 2017]). The annual prevalence of S. aureus bacteremia in the US is 38.2 to 45.7 per 100,000 person-years (El Atrouni et al. 2009; Rhee et al. 2015), but is higher among specific populations, such as hemodialysis patients. There are between 350,000 and 600,000 cases (200 per 100,000) of deep vein thromboembolism and pulmonary embolism that occur in the US every year (Anderson et al. 1991). Furthermore, there are over 100,000 individuals with sickle cell in the US (33.3 per 100,000; Hassell, 2010). Malaria is also common in sub-Saharan Africa in 2018 (21,910 per 100,000; World Health Organization, 2018). The prevalence of these diseases makes it clear that they could very well have a severe impact on assessment in a population-scale radiation exposure event.

Exploring the basis of these confounding disorders could facilitate strategies that minimize FPs suggesting radiation exposures. Riboviral infections have been proposed to sequester host RNA binding proteins, leading to R-loop formation, DNA damage responses, and apoptosis (Rogan et al. 2021). This study suggested that expression of some key radiation signature genes appear to be altered by such infections. We also suggest that neutrophil extracellular traps (or NETs; Qi et al. 2020) may activate biochemical pathways that are present in early radiation responses. An early step in the formation of these structures is chromosome decondensation followed by the fragmentation of DNA which act as extracellular fibers which bind pathogens (such as S. aureus) in a process similar to autophagy in neutrophils (NETosis). This process would likely activate DNA damage in neutrophils, and some of the same DNA

damage response genes that are activated (*DDB2*, *PCNA*, *GADD45A*) and repressed (*BCL2*) after radiation exposure are also similarly regulated after infections such as S. aureus. NETosis also contributes to the pathogenesis of numerous non-infectious diseases such as thromboembolism (Demers and Wagner, 2014; Collison, 2019) and sickle cell disease (Hounkpe et al. 2020), in addition to autoimmune disease (He et al. 2018) and general inflammation (Delgado-Rizo et al. 2017). If the origin of the FPs is confined to this lineage, then a comparison of the predictions of our traditionally validated signatures using data from the granulocyte versus lymphocyte lineages in individuals with these conditions should reveal whether NETosis is the likely etiology of the confounder expression phenotypes, or possibly even in radiation treated cells. To do this for radiation exposed cells, would require RNASeq data from these isolated cell populations (Ostheim et al. 2021). We would expect FPs in the confounder populations using signatures derived from myeloid-derived lineages, which include neutrophils.

The discovery of blood-borne conditions which lead to high FPs raises the question of whether other hematological conditions could also increase misclassification by radiation gene signatures. Such datasets are either unavailable or not suitable for analysis. Some studies consist with too few samples (e.g. GSE69601 [idiopathic portal hypertension] has 6 samples total) or lack the control samples necessary to perform a proper comparison (e.g. GSE33812 [aplastic anemia]). Although the available gene expression datasets covered a broad range of hematopathologies, additional testing of the sequential gene signatures will be required to exclude FPs due to underlying changes in gene expression from other confounders.

Confounding conditions will affect the precision of other assays and biomarkers that are routinely used to assess radiation exposure. Indeed, some of these are well known in the published literature. For example, elevated levels of γ-H2AX occurs in melanoma (Warters et al.

2005), cervical cancer (Banath et al. 2004; Yu et al. 2006), colon carcinoma, fibrosarcoma, glioma, osteosarcoma, and neuroblastoma (Sedelnikova and Bonner 2006). The DNA damage detected by this marker is characteristic of the development of cancer (Banath et al. 2004; Warters et al. 2005; Sedelnikova and Bonner 2006; Yu et al. 2006). Colonocytes from patients with ulcerative colitis also have elevated *γ*-H2AX (Risques et al. 2008). Increased expression of *γ*-H2AX is such a reliable biomarker in this context, that it has been suggested for early cancer screening and cancer therapy monitoring (Sedelnikova and Bonner 2006), which would make its use to assess radiation exposure problematic in such patients. Aside from its application in radiation damage assessment, the cytokinesis block micronucleus assay (Fenech 2010) is actually a multi-target endpoint for genotoxic stress from exogenous chemical agents (Kirsch-Volders et al. 2011; Fenech et al. 2016, Kirsch-Volders et al. 2018) and deficiency of micronutrients required for DNA synthesis and/or repair (folate, zinc; Beetstra et al. 2005; Sharif et al. 2012). Zinc depletion/restriction also increased γ-H2AX (Mah et al. 2010) suggesting increased DNA breakage, which has been confirmed by the comet assay (Song et al. 2009).

Many radiation response genes were frequently selected as features for multiple signatures, and includes genes with roles in DNA damage response (*CDKN1A*, *DDB2*, *GADD45A*, *LIG1*, *PCNA*), apoptosis (*AEN*, *CCNG1*, *LY9*, *PPM1D*, *TNFRSF10B*), metabolism (*FDXR*), cell proliferation (*PTP4A1*) and the immune system (*LY9* and *TRIM22).* In general, the removal of these genes did not significantly alter the FP rate against confounder data. However, the removal of *LIG1*, *PCNA*, *PPM1D*, *PTP4A1*, *TNFRSF10B*, and *TRIM22* could partially decrease misclassification of influenza samples in some models, as well as *DDB2* for dengue (in addition to S. aureus and Polycythemia Vera). Many of these genes in our models are also present in other published radiation gene signatures and assays (Paul and Amundson, 2008; Lu et

al. 2014; Oh et al. 2014; Port et al. 2017; Tichy et al. 2018; Jacobs et al. 2020). Paul and Amundsen (2008) developed a 74-gene radiation signature comprised of 16 genes present in the human signatures reported in Zhao et al. (2018a), including *CDKN1A*, *DDB2* and *PCNA*. Similarly, three of the 5 biomarkers implicated in Tichy et al. (2018) were also commonly selected (*CCNG1*, *CDKN1A*, and *GADD45A*), as were 5 of the 13 genes in the radiation assay described in Jacobs et al. (*BAX*, *CDKN1A*, *DDB2*, *MYC* and *PCNA*). While we cannot determine the impact on the accuracy of their signatures for confounders, it is evident that some genes that are included in these and other gene signatures (such as *DDB2*) can have a profound impact on the misclassification of individuals with confounding conditions.

[Figure 7 Here]

The proposed sequential approach that combines highly sensitive predictors (affected by confounders) with high-specificity signatures could improve the accuracy of predicting TP exposures (Figure 7). After assessment with a sensitive signature (e.g. M4), all predicted positive samples would be re-evaluated with a high specificity signature (eg. SM3 or SM5) to remove misclassified FP samples resulting in a higher performance assay that predominantly or exclusively labels truly irradiated samples. The high specificity signatures, SM3 and SM5, are not as sensitive to radiation as M1-M4 and KM1-KM7. Therefore, a proportion of radiation-exposed individuals could be misclassified as FNs. Besides radiation exposure, the application of sequential gene signatures, each optimized respectively to maximize sensitivity and specificity, may turn out to be a general strategy for improving accuracy of molecular diagnoses for a wide spectrum of disease pathologies.

Differential molecular diagnoses based on gene signatures would evaluate predicted radiation positive samples by individual gene signatures, each trained on different confounders

(e.g. one model for influenza infection, another for thromboembolism, etc). This approach would explicitly exclude FPs for radiation response while identifying the underlying condition. Separate signatures for sensitivity and specificity might also be avoided by training adversarial networks (Goodfellow et al. 2014) that contrast radiation-exposed samples with one or more datasets for confounding conditions, with emphasis on samples predicted to be FPs from the current radiation signatures. These resultant signatures would select radiation responsive genes which are resistant to the effects of confounders. Finally, ensuring that both the positive test and negative control samples in training sets properly account for the population frequencies of confounding diagnoses would also be expected to improve the performance of radiation gene signatures.

Acknowledgements

This work was supported by the University of Western Ontario and CytoGnomix Inc. The authors thank Dr. Ruth Wilkins for her constructive comments on this article.

Disclosure of Interest

Ben C. Shirley is an employee and Peter K. Rogan is a cofounder of CytoGnomix Inc. Biochemically-inspired machine learning is patent pending.

Data Availability Statement

A Zenodo data repository has been created for this study (DOI: doi.org/10.5281/zenodo.5009008). This archive provides additional violin plots which illustrate the expression of genes in models M1-M4, KM3-KM7 and SM1-SM5 for patients with a bloodborne condition or RNA viral infection.

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Figures

Figure 1: Evaluation of Conditions Confounding Radiation Gene Signatures

The traditional validation approach was used to evaluate unirradiated datasets for hematological conditions to assess the performance of radiation gene signatures derived in Zhao et al. (2018a). With this approach, we can identify and then reject models with high rates of FP radiation diagnosis in confounding conditions while identifying what confounders could make individuals ineligible for a radiation gene signature assay. Alternatively, new radiation gene signatures could be derived that show improved FP rates in both controls and test subjects.

Figure 2: Performance of Traditionally-Validated Radiation Signatures on Confounders Stratified by Sub-phenotypes

Sankey diagrams delineate what fraction of disease patients and controls were properly (TN) and improperly classified (FP) by a radiation gene signature. A) The radiation signature M4 incorrectly classified 53% of dengue-infected patient as irradiated, however all convalescent patients were properly classified (0% FP). B) Similarly, the FP rate of M1 decreased considerably after patient recovery (27% FP rate [N=19] against samples <3 days after symptoms; 3% [N=2] after 2-5 weeks). C) The FP rate of M3 was higher for severe malarial anemia patients versus those with cerebral malaria, suggesting that the differential expression caused by the two infection types may diverge in such a way that is measurable by M3. D) Conversely, the FP rate of venous thromboembolism patients by M4 was not influenced on whether the disease was recurrent.

Figure 3 - Misclassification Rates of Radiation Models in Unirradiated Blood-borne Disorders

Radiation gene signatures M1-M4 and KM3-KM7 performed well when predicting radiation exposure (grey bar; presented in order of descending accuracy and grouped by validation type). However, many of these models falsely predicted individuals with blood-borne disorders (thromboembolism [top-left] and sickle cell disease [bottom-left]) and infectious diseases (S. aureus [top-right] and malaria [bottom-right]) as irradiated (%FP provided for individuals with the indicated disease [dark grey; top value], and controls [light grey; bottom value]). In general, the FP rate was high for all traditional validated (M1-M4) and most k-fold validated models (KM4, KM6 and KM7). Models KM3 and KM5 had a low FP rate across all datasets tested.

Figure 4: *DDB2* and *BCL2* Expression in Hematological Disorders with Radiation-Exposed Control Datasets

Normalized distribution of gene expression of confounder datasets (VTE: Venous Thromboembolism [orange]; SAu: S. aureus [teal]; Sic: Sickle Cell [yellow]; and Mal: Malaria [dark green]) for the genes **A)** *DDB2* and **B)** *BCL2* are presented as violin plots, where the expression of individuals with these conditions are divided by those predicted as irradiated (FP; left) or unirradiated (TN; right) by signature M4. Control expression of radiation-exposed (Irr.) and (Non) unirradiated individuals are indicated by distributions labeled with light (GSE6874) and dark (GSE10640) red outlines on the right side of each panel. Significant expression differences between FP and TN samples from the same dataset predicted with signature M4 are indicated by brackets above the corresponding pair of predictions.

Figure 5: Multiple Genes Contribute to Misclassification of Confounding Datasets

Accuracy of M4 was significantly influenced by hematological confounders such as venous thromboembolism (top) and S. aureus infection (bottom). M4 misclassified diseased individuals (orange circles) far more often than controls (blue squares). Feature removal analysis of M4 determines if a particular gene was contributing to the FP rate by observing how accuracy changes when a gene is removed. While M4 accuracy improved with the removal of *PRKDC*, *IL2RB* and *LCN2*, no individual gene restored misclassification back to control levels suggesting multiple genes are confounded by these diseases.

Figure 6 - Secretome-Derived Gene Signatures Reduce Misclassification in Unirradiated Confounder Phenotypes

Radiation signatures which consist exclusively of genes encoding plasma secreted proteins were derived following the same basic approach of Zhao et al. (2018a). These models showed generally favorable performance when tested against an independent radiation dataset by k-fold validation (grey bar; in order of descending accuracy). Five secretome radiation signatures were derived consisting of 7-75 genes (SM1-SM5). Two models (SM3 and SM5) show high specificity across all hematological conditions tested.

Figure 7: Sequential Application of Radiation-responsive and Secretome Gene Signatures Identifies Exposed Individuals

False positive predictions due to differential expression caused by confounding conditions could be mitigated by following a sequential approach where samples are evaluated with both a highly sensitive radiation gene signature and a second signature with high specificity. M4, for example, is highly sensitive when validated against radiation dataset GSE10640[GPL6522] (88% accuracy), where all incorrect classifications were due to FP predictions (zero false negatives [FN]). Predicted irradiated samples could then be evaluated with a highly specific model such as SM3, which would identify and remove any misclassified unirradiated samples remaining in the set and leave only TPs.

Supplemental Figures

Supplemental Figure 1: Threshold Mapping of Venous Thromboembolism Patients Predicted Irradiated by Model M4

The expression of *DDB2*, *PCNA*, *IL2RB* and *PRKDC* from all venous thromboembolism patients (blue) and controls (orange) are presented as a histogram (non-normalized expression in 0.1 bins). The expression for patients GSM474822 (A), GSM474828 (B) and GSM474819 (C), each of which having been improperly classified as irradiated by M4, are indicated with a blue arrow. The vertical dashed blue line designates the inflection point where expression of the gene will alter the prediction of the model. If the inflection point is absent, no change in gene expression could correct patient misclassification, which may indicate that the gene does not strongly contribute to the FP prediction of that individual.

Tables

Table 1 – Traditional and K-Fold Validated Radiation Gene Expression Signatures M1-M4 and KM1-KM7

	Signature	FS. Algorithm
	a) Derived from Radiation Dataset GSE1725	
KM1	*GADD45A DDB2*	FSFS
KM2	*PPM1D DDB2 CCNF CDKN1A PCNA GADD45A PRKAB1 TOB1 TNFRSF10B MYC CCNB2 PTP4A1 BAX CCNA2 ATF3 LIG1 CCNG1 FHL2 PPP1R2 MBD4 RASGRP2 UBC NINJ1 TRIM22 IL2RB TP53BP1 PTPRCAP EEF1D PTPRE RAD23B EIF2B4 STX11 PTPN6 STK10 PSMD1 BTG3 MLH1 RNPEP HSPD1 UNG PTPRC PTPRA BCL2 GSS SH3BP5 TPP2 IDH3B CCNH STK11 EIF4EBP2 HSPA4 FADS2 RPA3 GZMK ANXA4 ICAM1 PPID LMO2 PPIE NUDT1 FUS POLR2A LY9 RPA1 PTS TNFRSF4 RPA2 PSMD8 GCDH MAN2C1 PTPN2 RUVBL1 ATP5H GK CD79B MAP4K4 POLE3 PRKCH AKT2 MOAP1 CCNG2 ALDOA SRD5A1 HAT1 XRCC1 EIF2S3 RAD1 UBE2A ZFP36L1 CD8A TALDO1 GPX4 SSBP2 ERCC3 ATP5O PEPD EIF4G2 ACO2 HEXB UBE3A ARPC1A PSMD10 PRCP PPIB ZNF337 CETN2 RPL29*	CSFS
	b) Derived from Radiation Dataset GSE10640[GPL6522]	
M1	*DDB2 HSPD1 MAP4K4 GTF3A PCNA MDH2*	FSFS
M2	*DDB2 GTF3A TNFRSF10B*	FSFS
KM3	*DDB2 RAD17 PSMD9 LY9 PPIH PCNA MDH2 MOAP1 TP53BP1 PPM1D ATP5G1 BCL2L2 ENO2 PTP4A1 PSMD8 LIG1 FDPS OGDH CCNG1 PSMD1*	BSFS
KM4	*DDB2 HSPD1 ICAM1 PTP4A1 GTF3A LY9*	FSFS
KM5	*RAD17 TNFRSF10B PSMD9 LY9 PPIH PCNA ZNF337 MDH2 TP53BP1 PPM1D ZFP36L1 ATP5G1 ALDOA BCL2L2 ENO2 GADD45A PTP4A1 PSMD8 LIG1 ATP5O FDPS OGDH PSMD1*	BSFS
	c) Derived from Radiation Dataset GSE6874[GPL4782]	
M3	*DDB2 CD8A TALDO1 PCNA EIF4G2 LCN2 CDKN1A PRKCH ENO1 PPM1D*	BSFS
M4	*DDB2 CD8A TALDO1 PCNA LCN2 CDKN1A PRKCH ENO1 GTF3A IL2RB NINJ1 BAX TRIM22 PRKDC*	BSFS

	GADD45A MOAP1 ARPC1B LY9 LMO2 STX11 TPP2 CCNG1 GABARAP BCL2 GSS FTH1	
KM6	*DDB2 PRKDC PRKCH IGJ*	FSFS
KM7	*DDB2 PRKDC TPP2 PTPRE GADD45A*	FSFS
Performance metrics for these radiation gene signatures are available in Zhao et al. (2018a) Tables 2 (k-fold validated signatures [KM1-KM7]) and 3 (traditionally validated signatures [M1-M4]); FS – Feature Selection metrics		

Table 2 – Radiation Gene Expression Signatures derived from GSE85570 and GSE102791 Radiation Datasets

Signature		FS. Algorithm	FS. Misclass	FS. Log Loss	% FP by Confounder (Disease / Controls)			
					Thrombo-embolism	S. Aureus	Sickle Cell	Malaria
a) Derived from Radiation Dataset GSE102791								
M5	*AEN BCL2*	FSFS*	-	8.1E-15	0.57 / 0.40	0.48 / 0.41	0.49 / 0.43	0.41 / 0.08
M6	*RPS27L ZMAT3*	FSFS	-	5.2E-15	0.78 / 0.69	0.49 / 0.46	0.50/ 0.43	0.71 / 0.25
M7	*AEN ERCC1 BAX*	CSFS	0%	-	0.60 / 0.83	0.71 / 0.66	0.66 / 0.72	0.68 / 0.42
M8	*AEN TNFRSF10B*	FSFS	-	5.2E-15	0.00 / 0.00	0.00 / 0.00	0.56 / 0.26	1.00 / 1.00
b) Derived from Radiation Dataset GSE85570								
M9	*BAX FDXR*	FSFS	0%	-	0.64 / 0.27	0.46 / 0.64	0.52 / 0.33	0.44 / 0.75
M10	*BAX FDXR XPC*	FSFS	0%	-	0.80 / 0.46	0.84 / 0.65	0.67 / 0.69	0.68 / 1.00
M11	*BAX DDB2*	FSFS	0%	-	0.34 / 0.76	0.52 / 0.48	0.48 / 0.49	0.59 / 0.17
M12	*BAX DDB2 SLC7A6*	FSFS	0%	-	0.51 / 0.31	0.40 / 0.23	0.41 / 0.47	0.44 / 0.20
M13	*RPS27L DDB2 ARL6IP1 TRIM32*	FSFS	0%	-	0.77 / 0.40	0.63 / 0.55	0.54 / 0.67	0.68 / 0.25
* Gene Signatures derived using 0Gy, 2Gy and 5Gy samples only (excludes 6Gy and 7Gy samples from GSE102791); Additional Models can be found in Suppl. Tables S4A and S4B; FS – Feature Selection metrics								

Table 3 – Radiation Gene Signatures including only Secreted Genes derived from GSE6874 and GSE10640 Datasets

	Signature	FS. Algorithm	Validation Misclassification	
			K-Fold [1]	Traditional
a) Derived from Radiation Dataset GSE6874[GPL4782] and Validated on GSE10640[GPL6524]				
SM1	*PDE7A FBXW7 CLCF1 ALB IDUA USP3 SLPI COASY MFAP4 LTBP1 VPS37B VEGFA IRAK3*	CSFS	0.12	0.25
SM2	*PDE7A FBXW7 CLCF1 ALB IDUA USP3 SLPI COASY MFAP4 LTBP1 VPS37B VEGFA IRAK3 MZB1 DHH GRN AEBP1 CNPY3 NUCB1 RDH11 CXCL3 POFUT1 CST1 ARCN1 PLA2G12A ERAP2 GOLM1 B3GAT3 ADAMTS9 FKBP9 ALDH9A1 LY86 HARS2 PRSS21 RETN C1GALT1 MGAT2 FUCA1 TTC19 MANF LUM GALNT15 APOM NME1 ATMIN GPX4 POLL LY6H SMARCA2*	BSFS	0.12	0.27
b) Derived from Radiation Dataset GSE10640[GPL6522] and Validated on GSE6874[GPL4783]				
SM3	*TRIM24 TOR1A GRN HP RBP4 PFN1 FN1*	FSFS	0.32	0.49
SM4	*XCL1 CDC40 PTGS2 DHX8 NENF PTX3 WNT1 CTSW TINF2 AOAH VPS51 TOR1A HINT2 CRTAP SUCLG1 TF EDEM2 LAMA5 AGPS TFPI WFDC2 SRGN SIL1 PPOX AMY2A NUBPL GARS LRPAP1 VPS37B PNP C3orf58 HP SPOCK2 NME1 GRN TRIM24 MRPL34 SRP14 THOC3 RNASE6 RBP4 MSRB2 RNASET2 TGFBI PRDX4 GLA GLB1 PFN1 GDF15 VCAN TRIM28 TAGLN2 TIMP1 IPO9 CPVL MANBA CEP57 RNF146 PF4 RETN HCCS DPP7 RNASE2 QPCT AHSG CTSC LYZ B2M EMILIN2 STOML2 LCN2*	CSFS	0.39	0.32
SM5	*TRIM24 IRAK3 PPP1CA MTX2 FBXW7 PFN1 SDHB CTSC MSRB2*	FSFS[2]	0.33	0.38
Additional Metrics for these signatures can be found in Suppl. Table S6A; [1] Tested using K-Fold validation methods (where K=5) [2] Derived from the top 50 genes by ranked mRMR (Suppl. Table S1); FS – Feature Selection metrics				

Supplementary Tables

Supplemental Table S1: mRMR Rankings of Radiation Response Genes in Ex-Vivo Control and Radiation Exposed Paired Datasets

Supplemental Table S2A: Influenza and Dengue Infection Increase False Positives by Radiation Gene Signatures

Supplemental Table S2B: Infectious, Inherited and Non-Inherited Blood-borne Disorders Increase False Positives by Radiation Gene Signatures

Supplemental Table S3A: False Positive Rate after Feature Removal of M1-M4 Against Blood Disease Pathologies

Supplemental Table S3B: False Positive Rate after Feature Removal of KM3-KM7 Against Blood Disease Pathologies

Supplemental Table S4A: Radiation Signatures derived from GSE102971 with Increased FPs evaluating Blood Disease Pathologies

Supplemental Table S4B: Radiation Signatures derived from GSE85570 with Increased FPs evaluating Blood Disease Pathologies

Supplemental Table S4C: Radiation Signatures derived from GSE6874 with Increased FPs evaluating Blood Disease Pathologies

Supplemental Table S4D: Radiation Signatures derived from GSE10640 with Increased FPs evaluating Diseased Blood Disease Pathologies

Supplemental Table S5A: Feature Removal Analysis Gene Signatures derived from Radiation Dataset GSE102971

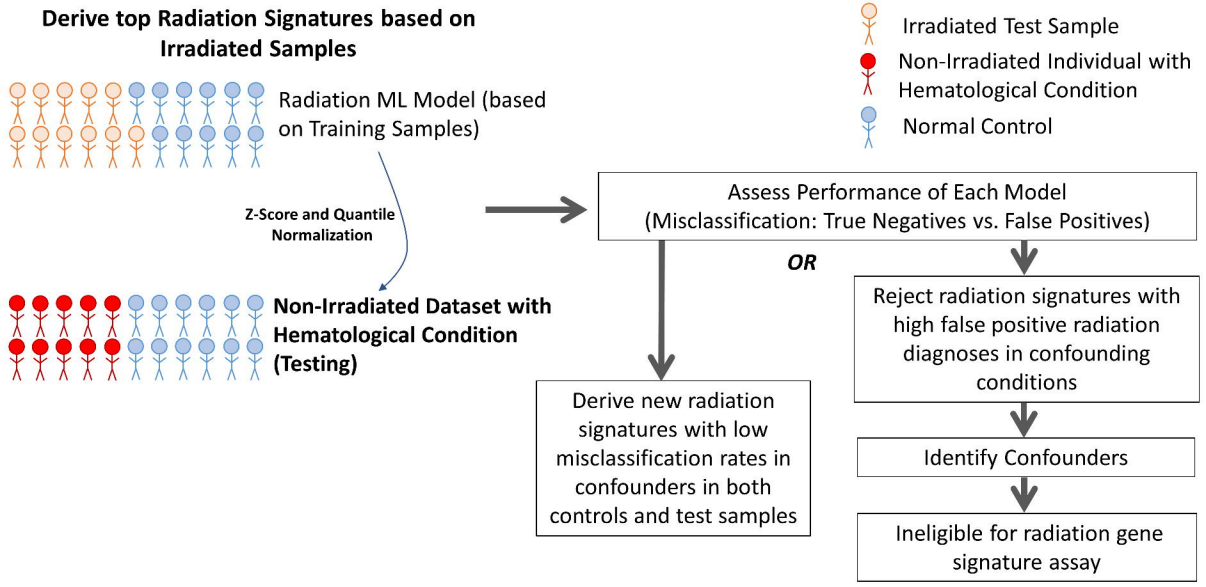
Supplemental Table S5B: Feature Removal Analysis Gene Signatures derived from Radiation Dataset GSE85570

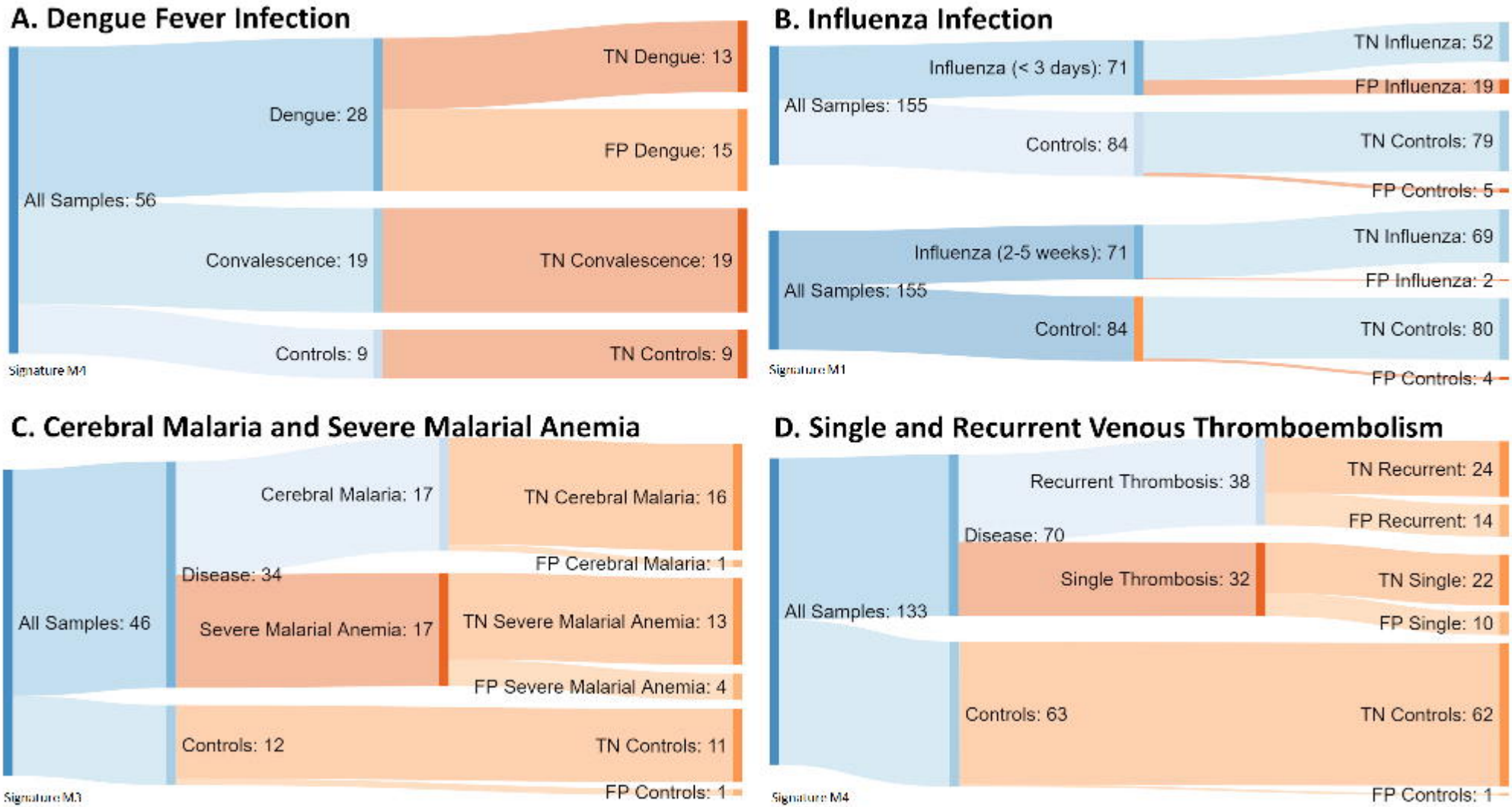
Supplemental Table S6A: Derivation of Radiation Models based on Secreted Genes

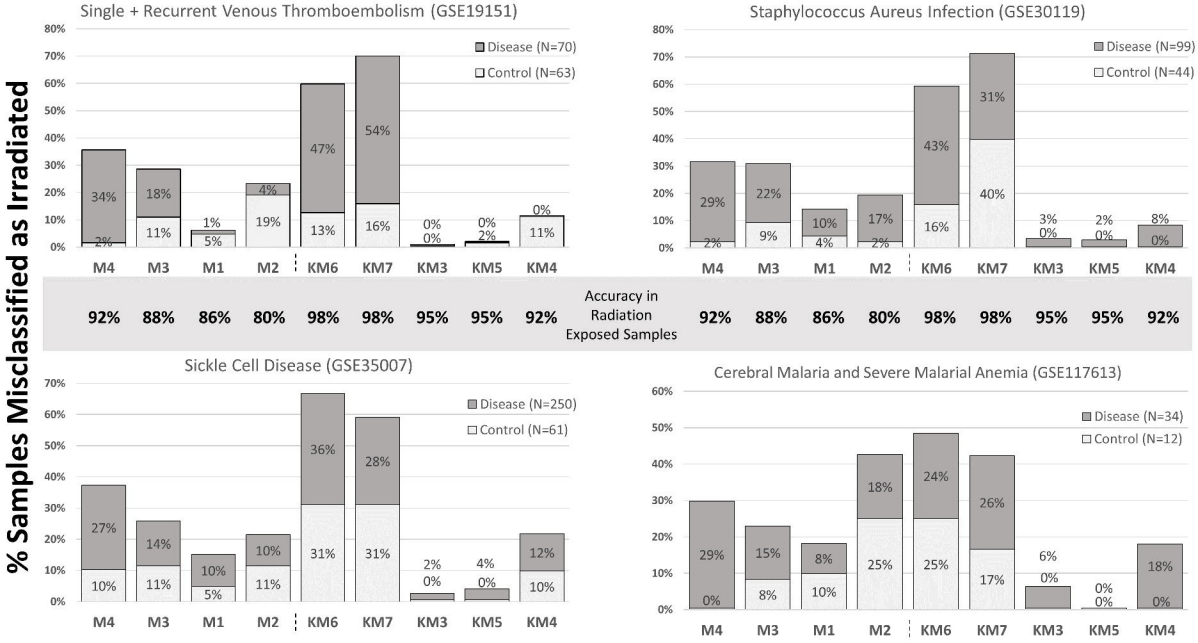
Table S6B: Testing Secreted Gene Radiation Signatures against Blood-Borne Diseased Patients

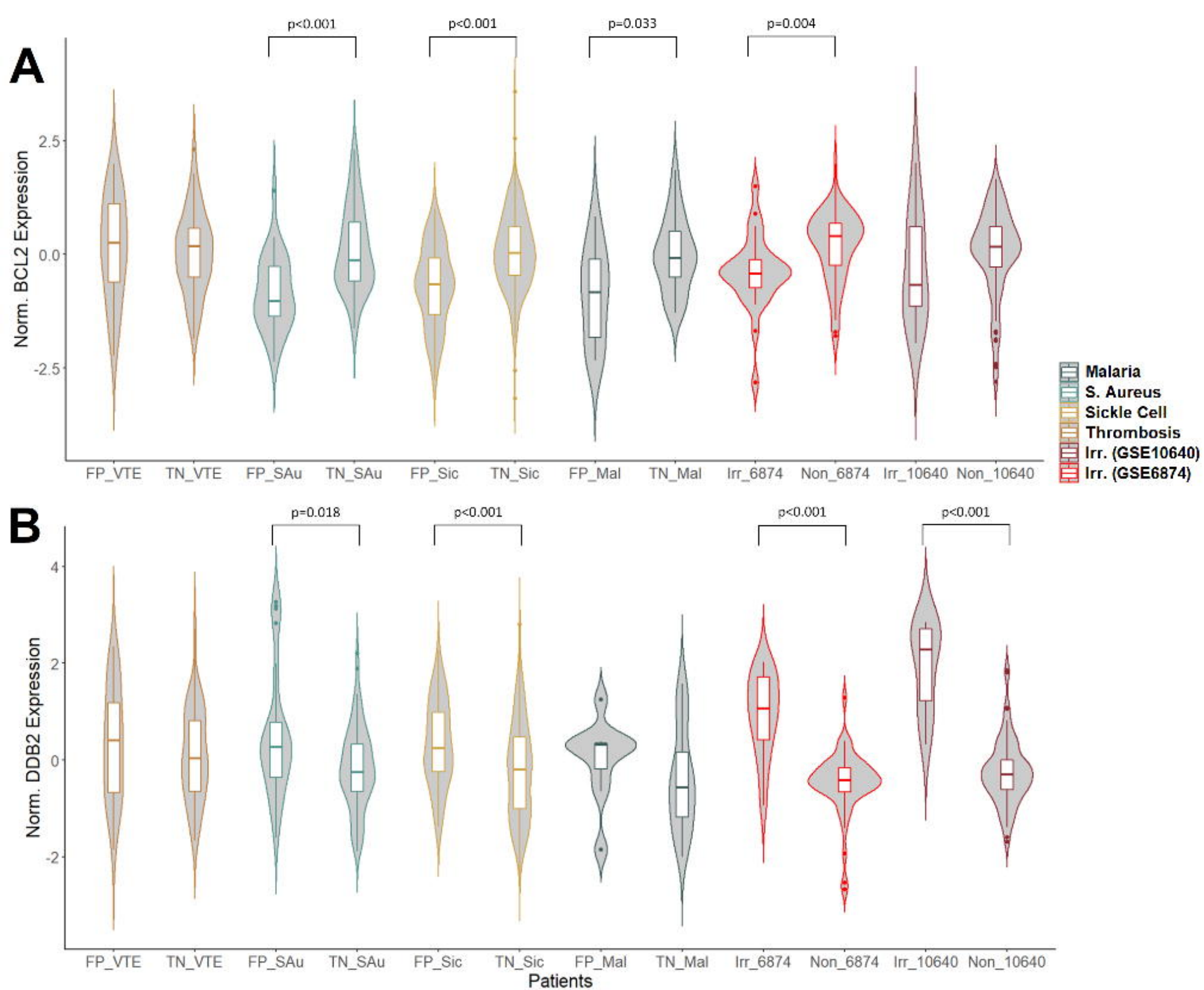
Supplementary Table S6C: Normalized change in mRNA expression of plasma protein encoded gene signature components after Radiation Exposure

Supplemental Table S6D: Testing Secreted Gene Radiation Signatures against Influenza and Dengue Infection


Derive top Radiation Signatures based on Irradiated Samples
Irradiated Test Sample
Non-Irradiated Individual with Hematological Condition
Normal Control
Radiation ML Model (based on Training Samples)
Z-Score and Quantile Normalization
Non-Irradiated Dataset with Hematological Condition (Testing)
Assess Performance of Each Model (Misclassification: True Negatives vs. False Positives)
OR
Reject radiation signatures with high false positive radiation diagnoses in confounding conditions
Identify Confounders
Ineligible for radiation gene signature assay
Derive new radiation signatures with low misclassification rates in confounders in both controls and test samples


A. Dengue Fever Infection
All Samples: 56
Dengue: 28
Convalescence: 19
Controls: 9
TN Dengue: 13
FP Dengue: 15
TN Convalescence: 19
TN Controls: 9
Signature M1
B. Influenza Infection
All Samples: 155
Influenza (< 3 days): 71
Controls: 84
TN Influenza: 52
FP Influenza: 19
TN Controls: 79
FP Controls: 5
All Samples: 155
Influenza (2-5 weeks): 71
Control: 84
TN Influenza: 69
FP Influenza: 2
TN Controls: 80
FP Controls: 4
Signature M1
C. Cerebral Malaria and Severe Malarial Anemia
All Samples: 46
Disease: 34
Controls: 12
Cerebral Malaria: 17
Severe Malarial Anemia: 17
TN Cerebral Malaria: 16
FP Cerebral Malaria: 1
TN Severe Malarial Anemia: 13
FP Severe Malarial Anemia: 4
TN Controls: 11
FP Controls: 1
Signature M3
D. Single and Recurrent Venous Thromboembolism
All Samples: 133
Disease: 70
Controls: 63
Recurrent Thrombosis: 38
Single Thrombosis: 32
TN Recurrent: 24
FP Recurrent: 14
TN Single: 22
FP Single: 10
TN Controls: 62
FP Controls: 1
Signature M4


% Samples Misclassified as Irradiated
Single + Recurrent Venous Thromboembolism (GSE19151)
Disease (N=70)
Control (N=63)
M4 34% 2%
M3 18% 11%
M1 1% 5%
M2 4% 19%
KM6 47% 13%
KM7 54% 16%
KM3 0% 0%
KM5 0% 2%
KM4 0% 11%
Staphylococcus Aureus Infection (GSE30119)
Disease (N=99)
Control (N=44)
M4 29% 2%
M3 22% 9%
M1 10% 4%
M2 17% 2%
KM6 43% 16%
KM7 31% 40%
KM3 3% 0%
KM5 2% 0%
KM4 8% 0%
Accuracy in Radiation Exposed Samples
92% 88% 86% 80% 98% 98% 95% 95% 92%
92% 88% 86% 80% 98% 98% 95% 95% 92%
Sickle Cell Disease (GSE35007)
Disease (N=250)
Control (N=61)
M4 27% 10%
M3 14% 11%
M1 10% 5%
M2 10% 11%
KM6 36% 31%
KM7 28% 31%
KM3 2% 0%
KM5 4% 0%
KM4 12% 10%
Cerebral Malaria and Severe Malarial Anemia (GSE117613)
Disease (N=34)
Control (N=12)
M4 29% 0%
M3 15% 8%
M1 8% 10%
M2 18% 25%
KM6 24% 25%
KM7 26% 17%
KM3 6% 0%
KM5 0% 0%
KM4 18% 0%


A
p<0.001
p<0.001
p=0.033
p=0.004
Norm. BCL2 Expression
2.5
0.0
-2.5
FP_VTE
TN_VTE
FP_SAu
TN_SAu
FP_Sic
TN_Sic
FP_Mal
TN_Mal
Irr_6874
Non_6874
Irr_10640
Non_10640
Malaria
S. Aureus
Sickle Cell
Thrombosis
Irr. (GSE10640)
Irr. (GSE6874)
B
p=0.018
p<0.001
p<0.001
p<0.001
Norm. DDB2 Expression
4
2
0
-2
FP_VTE
TN_VTE
FP_SAu
TN_SAu
FP_Sic
TN_Sic
FP_Mal
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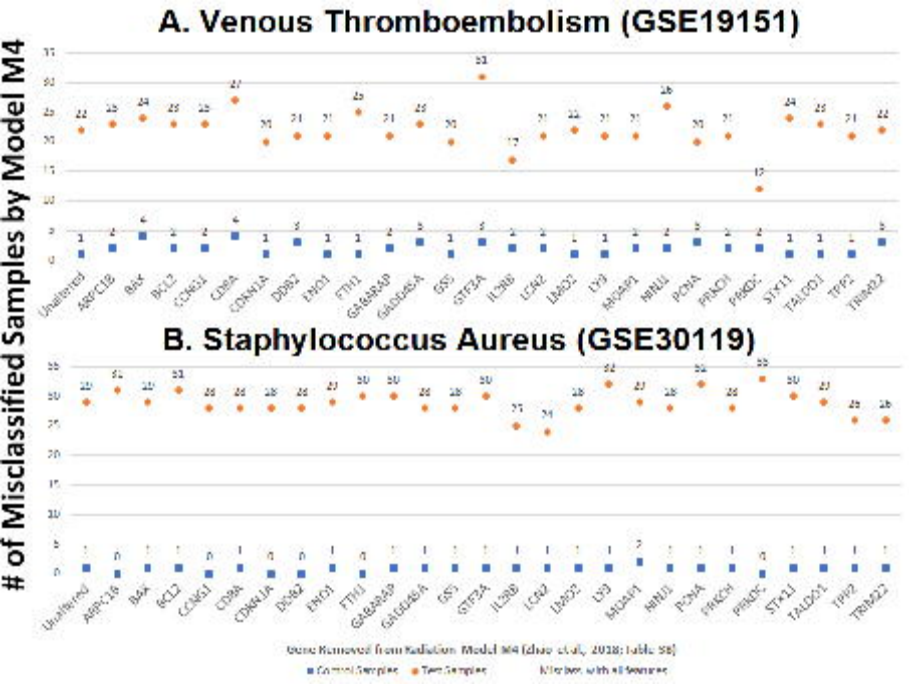
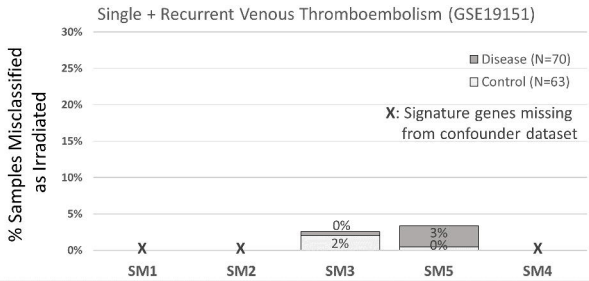
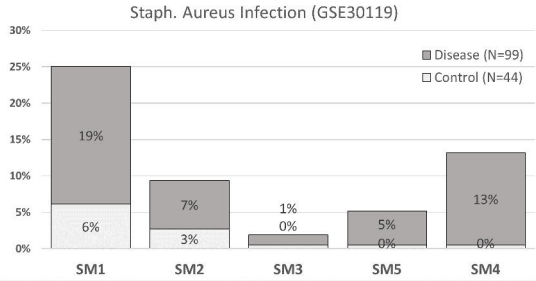
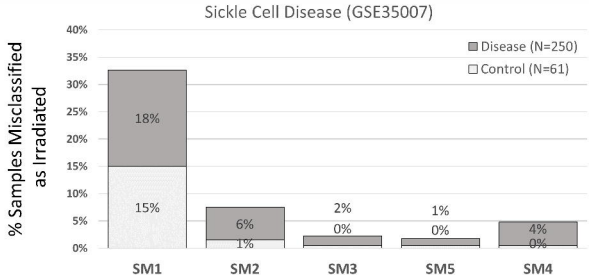
Irr_6874
Non_6874
Irr_10640
Non_10640
Patients

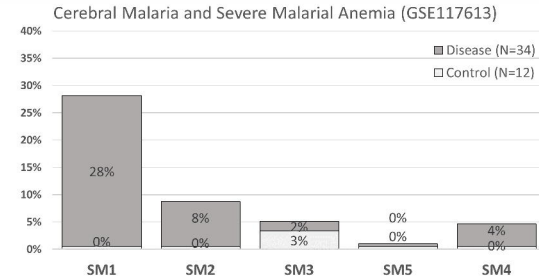

A. Venous Thromboembolism (GSE19151)
B. Staphylococcus Aureus (GSE30119)
# of Misclassified Samples by Model M4
Unaltered
ARPC1B
BAX
BCL2
CCNG1
CDBA
CDKN1A
DDB2
ENO1
FTH1
GABARAP
GADD45A
GSS
GTF3A
IL2RB
LCN2
LMO2
LY3
MOAP1
NINJ1
PCNA
PRKCH
PRKDC
STX11
TALDO1
TPP2
TRIM22
Gene Removed from Radiation Model M4 (Zhao et al., 2018; Table S8)
Control Samples
Test Samples
Mistakes with all features

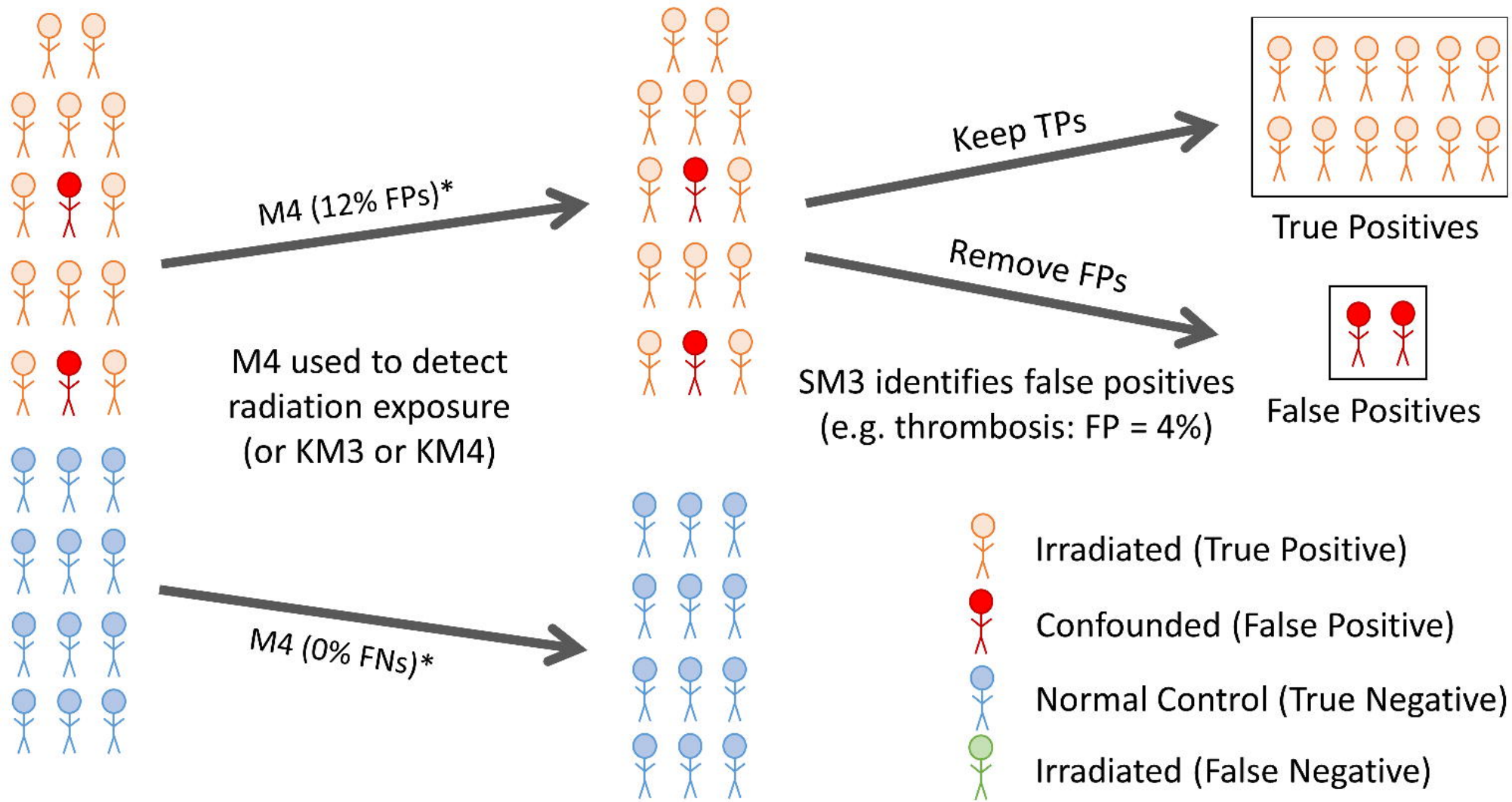




Irradiated Samples	SM1	SM2	SM3	SM5	SM4	SM1	SM2	SM3	SM5	SM4
Accuracy:	88%	88%	68%	67%	61%	88%	88%	68%	67%	61%
Log loss:	0.36	0.27	0.70	0.65	0.74	0.36	0.27	0.70	0.65	0.74






M4 (12% FPs)*
M4 used to detect radiation exposure (or KM3 or KM4)
Keep TPs
True Positives
Remove FPs
SM3 identifies false positives (e.g. thrombosis: FP = 4%)
False Positives
M4 (0% FNs)*
Irradiated (True Positive)
Confounded (False Positive)
Normal Control (True Negative)
Irradiated (False Negative)