be implicated in tumorigenesis and cancer progression, and the GSK-3-mediated phosphorylation of STK38 may play a role in inhibiting excessive STK38 activation. On the other hand, we found that STK38 knockdown significantly enhanced cell death, when treated with H_2O_2 even at a low concentration (0.1 mM) (Fig. 8), suggesting that STK38 activation may play a physiological role in preventing cell death immediately after exposure to adverse stimuli, giving the cells a chance to recover from transient stresses. We previously reported that STK38 interacts with and negatively regulates some of the JNK kinase kinases [8]. Our data collectively indicate a possible mechanism by which STK38 inhibits H_2O_2-induced cell death, in which STK38 is recruited to several JNK kinase kinases, to negatively regulate JNK signaling (Fig. 9). On the other hand, a recent report showed that the loss of STK38 does not result in major defects in the apoptosis of thymocytes and T-cells [40], which is inconsistent with our findings. STK38's involvement in cell death may depend on the cell type and stimulus. The identification and characterization of natural substrates for STK38 will be required to clarify the molecular mechanism by which it suppresses oxidative stress-induced cell death.

Supplementary materials related to this article can be found online at doi:10.1016/j.freeradbiomed.2011.11.006.

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Evaluation of systemic markers of inflammation in atomic-bomb survivors with special reference to radiation and age effects

Tomonori Hayashi,*,[1] Yukari Morishita,* Ravindra Khattree,†,[2] Munechika Misumi,‡ Keiko Sasaki,* Ikue Hayashi,§ Kengo Yoshida,* Junko Kajimura,* Seishi Kyoizumi,* Kazue Imai,* Yoichiro Kusunoki,* and Kei Nakachi*

*Department of Radiobiology/Molecular Epidemiology and †Department of Statistics, Radiation Effects Research Foundation, Hiroshima, Japan; ‡Department of Mathematics and Statistics, Oakland University, Rochester, Michigan, USA; and §Central Research Laboratory, Hiroshima University Faculty of Dentistry, Hiroshima, Japan

ABSTRACT **Past exposure to atomic bomb (A-bomb) radiation has exerted various long-lasting deleterious effects on the health of survivors. Some of these effects are seen even after >60 yr. In this study, we evaluated the subclinical inflammatory status of 442 A-bomb survivors, in terms of 8 inflammation-related cytokines or markers, comprised of plasma levels of reactive oxygen species (ROS), interleukin (IL)-6, tumor necrosis factor α (TNF-α), C-reactive protein (CRP), IL-4, IL-10, and immunoglobulins, and erythrocyte sedimentation rate (ESR). The effects of past radiation exposure and natural aging on these markers were individually assessed and compared. Next, to assess the biologically significant relationship between inflammation and radiation exposure or aging, which was masked by the interrelationship of those cytokines/markers, we used multivariate statistical analyses and evaluated the systemic markers of inflammation as scores being calculated by linear combinations of selected cytokines and markers. Our results indicate that a linear combination of ROS, IL-6, CRP, and ESR generated a score that was the most indicative of inflammation and revealed clear dependences on radiation dose and aging that were found to be statistically significant. The results suggest that collectively, radiation exposure, in conjunction with natural aging, may enhance the persistent inflammatory status of A-bomb survivors.—Hayashi, T., Morishita, Y., Khattree, R., Misumi, M., Sasaki, K., Hayashi, I., Yoshida, K., Kajimura, J., Kyoizumi, S., Imai, K., Kusunoki, Y., Nakachi, K. Evaluation of systemic markers of inflammation in atomic-bomb survivors with special reference to radiation and age effects. *FASEB J.* 26, 4765–4773 (2012). www.fasebj.org**

Key Words: reactive oxygen species · cytokines · immunosenescence

That atomic bomb (A-bomb) survivors continue to suffer from increased risks of selected cancers and other diseases was a principal finding of the Life Span Study in A-bomb survivors, which commenced in 1950 (1). These late effects pose serious, as yet unanswered, questions about the mechanisms underlying the observation. The list of diseases that reveal radiation-associated increased risks includes many inflammation-associated diseases, such as various cancers, cardiovascular diseases, and stroke (2–6). Recent studies have demonstrated that a great number of plasma cytokines are highly influenced by aging, and that persistently elevated levels of plasma inflammatory cytokines are sometimes associated with chronic diseases, such as cardiovascular diseases and Alzheimer's disease (7–10). Cytokines are involved in the regulation of inflammation and immunity, and they play a key role in the pathogenesis of infectious diseases and immunological disorders (11). Inflammation is characterized by a local reaction that may be followed by activation of a systemic acute-phase reaction (12). Interleukin (IL)-6, a key cytokine in inflammatory responses, induces the synthesis of acute-phase proteins, such as C-reactive protein (CRP), as well as other acute-phase reactants (13), modulating the immune response and participating in the regulation of body temperature (fever) (14). Thus, circulating IL-6 and its downstream CRP are potentially

Abbreviations: A-bomb, atomic bomb; AHS, adult health study; BMI, body mass index; CD, cluster of differentiation; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Ig, immunoglobulin; IL, interleukin; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-κB; RERF, Radiation Effects Research Foundation; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α

[1] Correspondence: Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, 5-2 Hijiyama Park, Minami Ward, Hiroshima 732-0815 Japan. E-mail: tomo@rerf.or.jp

[2] Current address: Department of Mathematics and Statistics, Oakland University, Rochester, MI 48309-4485, USA.

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good indicators of the inflammatory status of the body. Proinflammatory cytokines, typically tumor necrosis factor α (TNF-α), and anti-inflammatory cytokines, typically IL-10, also regulate the inflammatory response (15). Also, IL-4 and IL-10 are viewed as anti-inflammatory cytokines, because, when administered to animals with infection or inflammation, they are found to reduce the severity of the disease and reduce the production of IL-1 and TNF-α (16). Chronic low-grade inflammation may also influence the production of immunoglobulins (Igs) by B cells (17). Clinically, the systemic inflammatory response was evaluated by biochemical or hematologic markers, such as elevated CRP levels, hypoalbuminemia, accelerated erythrocyte sedimentation rate (ESR), and increased numbers of white blood cells and neutrophils, as well as platelet counts (18).

Reactive oxygen species (ROS) generated during inflammation may be key effectors linking inflammation and increasing incidence of various chronic diseases (19–22). ROS are believed to play crucial roles in the disease pathogenesis through different mechanisms, causing damage to important cellular components (*e.g.*, DNA, proteins, and lipids) and/or inducing a series of proinflammatory cytokines and chemokines mediated in part by activation of the c-Jun-N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) cascade (23). Another pathway involved in the induction of cytokines is the activation of the transcription factor, nuclear factor-κB (NF-κB), which regulates immunity, inflammation, and cell survival (24–27). On the other hand, ROS are also known to be induced by TNF-α and other growth factors in some systems (28, 29).

In this study, we investigated the relationship of inflammatory cytokines and markers with the level of past A-bomb radiation exposure and natural aging. We found that A-bomb radiation has possibly contributed to these markers, albeit to a lesser extent than the contribution of natural aging. An inflammatory response is an orchestrated process that involves several protein families and several molecules with similar molecular functions. To gain further understanding of the interrelationships of inflammation-related markers, multivariate statistical analyses (principal factor analysis and principal component analysis) were used to uncover the underlying pathways of the inflammation response. Then, we proposed certain score indexes to identify a set of cytokines/markers most responsive to radiation and aging effects. Collectively, through the techniques of multivariate statistical analyses, the relationships can now be more clearly quantified, thereby implying that the radiation exposure may enhance the persistent inflammatory status of A-bomb survivors in conjunction with natural aging. Our study provides new insights into the interrelationship between radiation and aging-associated inflammation measures and models the inflammation as a function of radiation dose and age along with other epidemiological variables.

MATERIALS AND METHODS

Study populations

Our study subjects were the same A-bomb survivors as used in our previous studies (29, 30), comprising 182 nonexposed (<0.005 Gy) and 260 exposed (≥0.005 Gy) subjects. Briefly, the study subjects were randomly selected from the participants of the Adult Health Study (AHS) in Hiroshima (31), who visited the Radiation Effects Research Foundation (RERF) for a clinical health examination between March 1995 and April 1997 and provided written informed consent for research use of their peripheral blood samples. In the process of subject selection, we had excluded the subjects who carried or had experienced cancer or inflammation- associated diseases (*e.g.*, *current cold, chronic bronchitis, collagen* disease, arthritis) at the time of blood collection. The blood samples were stored and used for measurements of plasma cytokines and some markers in our previous studies (29, 30) and, in this study, for measurement of plasma ROS levels. We took the radiation dose as the γ dose plus 10 times the neutron dose, using the skin doses (as a representative of whole-body irradiation) calculated by dosimetry system DS02 (30). This study was approved by RERF's ethics committee.

Measurements

In this study, we measured plasma ROS levels in duplicate, using the total ROS assay system that we have recently developed (31). We measured plasma IL-6, TNF-α, IL-4, and IL-10 levels in our previous study using an ultrasensitive enzyme-linked immunosorbent assay kit (Quantikine HS, R&D Systems, Minneapolis, MN, USA), as well as plasma CRP levels using a CRP-Latex kit (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) (32). ESR was measured by the Wintrobe method and adjusted for the hematocrit, as described previously (33). We quantitated Ig levels with human Ig quantitation kits (Bethyl Laboratories Inc., Montgomery, TX, USA). The frequencies of cluster of differentiation (CD)4 (helper) and CD45RA-positive naive CD4 in the various lymphocyte fractions were measured as described previously (34). All measurements were performed using plasma samples collected and stored at the same time.

Statistical analysis

For the statistical analysis of our data, we have made use of radiation dose data of individual subjects. Initially, 8 markers and cytokines, namely, ROS, IL-6, TNF-α, CRP, ESR, IL-4, IL-10, and Igs, were considered as the dependent variables to be analyzed after taking the logarithm at the base 10. This logarithmic transformation was applied to ensure the normality of the error distribution. We first investigated whether there were differences in 8 cytokines and markers by radiation exposure status (*i.e.*, nonexposed and exposed). Then, we fitted multiple linear regression models (35) for each of the 8 markers. Age at the time of blood collection, gender of the subject, smoking status, and body mass index (BMI) values were taken as the covariates. The model considered for the logarithm of any of the markers (dependent variable, generically denoted, after taking the logarithm, by y) is given by Eq. 1:

$$y = \beta_0 + \beta_1 * \text{dose} + \beta_2 * \text{gender} + \beta_3 * \text{age} + \beta_4 * \text{smoking} + \beta_5 * \text{BMI} + \text{error} \quad (1)$$

Since these markers and cytokines were correlated with each other, and to elucidate the effects of radiation and

TABLE 1. *Inflammation marker values for subjects selected from A-bomb survivors in Hiroshima, Japan*

Variable	Nonexposed, $n = 182$	Exposed, $n = 260$	P
Age (yr)	68.1 ± 8.1	68.3 ± 8.3	0.869
Female/male ratio	0.53	0.57	0.394
BMI (kg/m^2)	22.8 ± 2.8	22.6 ± 2.6	0.576
Smokers (%)	25.3	22.7	0.607
Log(ROS)	2.24 ± 0.10	2.26 ± 0.10	0.234
Log(IL-6)	0.13 ± 0.26	0.17 ± 0.28	0.156
Log(TNF-α)	0.18 ± 0.27	0.19 ± 0.27	0.690
Log(CRP)	−1.32 ± 0.65	−1.19 ± 0.62	0.031
Log(ESR)	1.19 ± 0.32	1.29 ± 0.30	0.001
Log(IL-4)	1.37 ± 0.42	1.45 ± 0.44	0.055
Log(IL-10)	0.45 ± 0.20	0.46 ± 0.24	0.412
Log(Igs)	1.15 ± 0.09	1.17 ± 0.11	0.048

Values are means ± SD or as indicated. Nonexposed, subjects with radiation exposure <5 mGy; exposed, subjects with radiation exposure ≥ 5 mGy. P values determined by χ^2 test for age, female/male ratio, BMI, and smokers; t test comparing means between radiation groups in others.

aging on the pathways among these variables, we performed a principal factor analysis (36) on all 8 cytokines and markers. This analysis identifies latent factors, each of which is expressed as a linear combination of selected cytokines and markers. As part of this analysis, we performed a Varimax rotation with 3 underlying factors to add to the interpretability of the results by maximizing the extent to which each marker or cytokine was associated with only one factor. After we identified 3 latent, or unobservable, factors reflecting certain biological inflammation pathways or other connections (*e.g.*, gene-gene interactions, use of common transcription factors) among certain subsets of the markers and cytokines, we used a principal component analysis to obtain a set of coefficients for the cytokines/markers in each of the three factors which maximize the amount of variation explained by that factor (37). The corresponding construct is commonly referred to as the first principal component. In all, we obtained three sets of the first principal components (henceforth referred to as the 1st_score, 2nd_score, and 3rd_score) corresponding to the 3 principal component analyses. We then investigated the effects of radiation, aging, and other epidemiological variables listed above on each of these first principal components by performing, in each case, a multiple regression analysis with the first principal components or scores as response variables. All of the above analyses were performed using SAS 9.2 software (SAS Institute, Cary, NC, USA).

RESULTS

Characteristics of subjects

Table 1 shows the characteristics of subjects by radiation exposure status and the means of inflammation markers after taking the logarithms. Corresponding P values of the 2-sample t tests show that for most of the markers, solely on the basis on means, the nonexposed and exposed groups cannot be distinguished.

Linear regression analysis for each inflammation marker

The results of multiple linear regression analysis for each marker are presented in **Table 2**. Statistically significant associations of cytokines or markers to radiation dose were observed, except for IL-4. However, the coefficient of determination (R^2) in each regression was small. Four graphs given in **Fig. 1** indicate the scatter plots of the predicted values of y, where y is the logarithm of ROS, IL-6, CRP, or ESR, respectively, as a function of radiation dose; at least visually, we could see little relationship to radiation dose, and these should not be concluded as biologically meaningful relationships. There was no improvement of the regression fit even after we included the interactions of covariates in the corresponding models.

TABLE 2. *Regression models for individual markers*

Response	Factor	Estimate	95% CI	R^2
Log(ROS)	Dose (Gy)	0.011	(0.001, 0.021)	0.081
	Gender	0.033	(0.013, 0.053)	
	Age (10 yr)	0.020	(0.012, 0.029)	
	Smoking	0.041	(0.018, 0.063)	
	BMI	−0.001	(−0.003, 0.002)	
Log(IL-6)	Dose (Gy)	0.047	(0.021, 0.072)	0.174
	Gender	−0.028	(−0.080, 0.024)	
	Age (10 yr)	0.101	(0.078, 0.124)	
	Smoking	0.087	(0.029, 0.145)	
	BMI	0.006	(−0.001, 0.013)	
Log(TNF-α)	Dose (Gy)	0.031	(0.004, 0.057)	0.083
	Gender	0.063	(0.009, 0.116)	
	Age (10 yr)	0.062	(0.039, 0.086)	
	Smoking	0.011	(−0.049, 0.071)	
	BMI	0.005	(−0.002, 0.012)	
Log(CRP)	Dose (Gy)	0.111	(0.050, 0.173)	0.094
	Gender	0.018	(−0.108, 0.144)	
	Age (10 yr)	0.103	(0.048, 0.159)	
	Smoking	0.082	(−0.059, 0.223)	
	BMI	0.043	(0.026, 0.061)	
Log(ESR)	Dose (Gy)	0.065	(0.037, 0.093)	0.248
	Gender	0.235	(0.178, 0.291)	
	Age (10 yr)	0.069	(0.044, 0.094)	
	Smoking	−0.026	(−0.090, 0.037)	
	BMI	−0.005	(−0.013, 0.003)	
Log(IL-4)	Dose (Gy)	−0.018	(−0.061, 0.026)	0.030
	Gender	−0.063	(−0.151, 0.026)	
	Age (10 yr)	0.057	(0.019, 0.096)	
	Smoking	v0.066	(−0.165, 0.032)	
	BMI	0.005	(−0.007, 0.017)	
Log (IL-10)	Dose (Gy)	0.025	(0.003, 0.048)	0.048
	Gender	−0.039	(−0.085, 0.007)	
	Age (10 yr)	0.035	(0.015, 0.056)	
	Smoking	0.016	(−0.035, 0.067)	
	BMI	0.007	(0.000, 0.013)	
Log(Igs)	Dose (Gy)	0.015	(0.004, 0.025)	0.056
	Gender	0.021	(−0.001, 0.042)	
	Age (10 yr)	0.012	(0.002, 0.021)	
	Smoking	−0.019	(−0.042, 0.005)	
	BMI	−0.001	(−0.004, 0.002)	

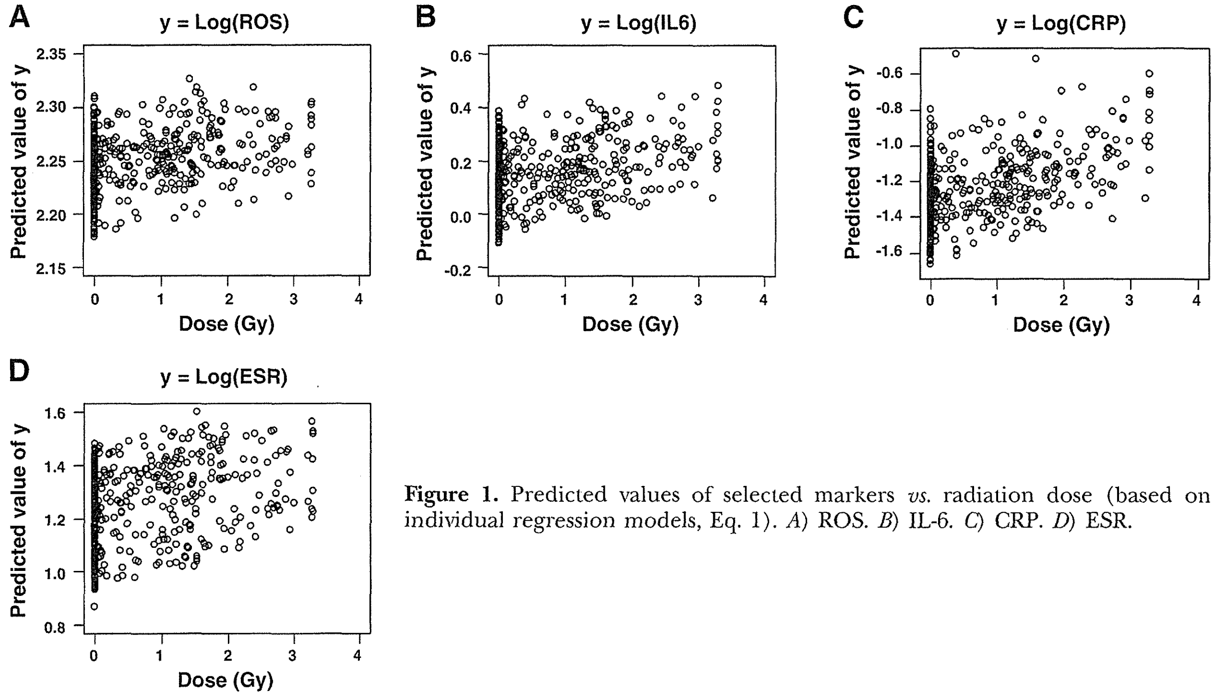

Figure 1. Predicted values of selected markers *vs.* radiation dose (based on individual regression models, Eq. 1). *A*) ROS. *B*) IL-6. *C*) CRP. *D*) ESR.

Principal factor analyses of cytokines and markers

Principal factor analysis revealed that 3 factors explained different characteristics among cytokines and markers. The most important factor explained 31% of the total variation and had large coefficients for Log(ROS), Log(IL-6), Log(CRP), and Log(ESR); the second factor explained 14% of the total variation and had large coefficients for Log(TNF-α), Log(ESR), and Log(Igs); and the third factor explained 13% of the total variation and had large coefficients for Log(TNF-α), Log(IL-4), and Log(IL-10). This implies the presence of some latent factors possibly related to different immunological pathways or mechanisms, each involving a unique set of the cytokines and markers (that is, those with large coefficients for that particular factor). Then, the principal component analysis was conducted separately for the aforementioned cytokines and markers strongly associated with each of the first, second, and third factors to create scores quantifying the information of that latent factor. **Tables 3–5** show these 3 correlation matrices used in the principal component analyses. All correlations are positive and lie between 0.12 and 0.42, indicating a low to moderate interrelationship among the variables (37).

TABLE 3. *Correlation matrix for logarithms of ROS, IL-6, CRP, and ESR*

Variable	Log(IL-6)	Log(CRP)	Log(ESR)
Log(ROS)	0.329	0.254	0.286
Log(IL-6)	–	0.410	0.327
Log(CRP)	–	–	0.312

Regression model for inflammation scores

Only the first principal component of each of the 3 principal component analyses contained more information than the original single cytokines and markers. We call those first principal components the 1st_score, 2nd_score, and 3rd_score, which quantify the first, second, and third unobserved latent inflammation mechanisms implied by factor analysis, respectively. These scores are obtained by Eqs. 2–4:

$$\text{1st\_score} = 0.463 * \text{s\_Log(ROS)} + 0.539 * \text{s\_Log(IL-6)} + 0.509 * \text{s\_Log(CRP)} + 0.486 * \text{s\_Log(ESR)} \quad (2)$$

$$\text{2nd\_score} = 0.527 * \text{s\_Log(TNF-}\alpha) + 0.601 * \text{s\_Log(ESR)} + 0.601 * \text{s\_Log(Igs)} \quad (3)$$

$$\text{3rd\_score} = 0.636 * \text{s\_Log(TNF-}\alpha) + 0.445 * \text{s\_Log(IL-4)} + 0.630 * \text{s\_Log(IL-10)} \quad (4)$$

where the prefix s is used to indicate that individual (logged) variables have been standardized to have 0 means and SD of 1. We view these scores as single multivariate indices of inflammation, each of which incorporates the inflammation characteristics of the associated group of variables into a single variable. With

TABLE 4. *Correlation matrix for logarithms of TNF-α, ESR, and Igs*

Variable	Log(ESR)	Log (Igs)
Log (TNF-α)	0.300	0.301
Log(ESR)	–	0.421

TABLE 5. *Correlation matrix for logarithms of TNF-α, IL-4, and IL-10*

Variable	Log (IL-4)	Log (IL-10)
Log (TNF-α)	0.133	0.277
Log (IL-4)	–	0.125

y as scores defined in Eqs. 2–4, we fit the model Eq. 1, resulting, in each case, in a prediction equation. As shown in **Table 6**, all 3 models are statistically significant (with $P<0.001$), with R^2 = 19.0, 19.1, and 8.8%, respectively: considerably better than models for most of the individual markers, thereby implying superior fits and better predictability. More important, the inflammation scores are independent of each other. The associations of the 1st_score, 2nd_score, and 3rd_score with the explanatory variables; *i.e.*, radiation, gender, age, smoking, and BMI, in terms of the regression model Eq. 1, are also shown in Table 6. All the variables in the model of the 1st_score are statistically significant, with very small P values, an exception being the BMI, for which the statistical significance is somewhat marginal. More specifically, radiation, gender, and age are statistically significant in both models for the 1st_score and 2nd_score. Also, radiation and age are significant for the 3rd_score. In addition, this score may be closely associated with BMI, but not with gender and smoking. Standard error values, also presented in Table 6, of all the variables are small, thereby supporting, as desired, the good precision of the estimates of the respective slopes. **Figure 2** shows the plots of predicted values of the 1st_score (Fig. 2*A, B*), 2nd_score (Fig. 2*C, D*), and 3rd_score (Fig. 2*E, F*) among study subjects against their given dose and age at the time of collecting plasma samples, respectively. In each of the 6 images, an increasing trend for the score is self-evident. However, in terms of the scatter in these plots, the effect of aging is more pronounced than the effect of radiation, since points are more tightly clustered around the trend in the former case.

Inflammation scores and CD4 or naive CD4 T-cell frequencies

We also investigated the association between the inflammation scores and CD4 or naive CD4 T-cell frequencies, respectively, by adding CD4 T-cell frequencies and naive CD4 T-cell frequencies, separately, into the regression models mentioned above (**Fig. 3**). The estimates of the regression coefficient in each model were −0.022 (CD4, $P<0.001$) and −0.016 (naive CD4, $P=0.008$) for the 1st_score, −0.017 (CD4, $P=0.005$) and −0.015 (naive CD4, $P=0.010$) for the 2nd_ score, and −0.006 (CD4, $P=0.24$) and −0.005 (naive CD4, $P=0.38$) for the 3rd_score. There were negative correlations between inflammation scores and helper T-cell measurements, while only the associations of the 1st_score and 2nd_score were statistically significant.

Radiation effects on age-dependent inflammation

The regression slope coefficients in the fitted model shown in Table 6 can also be used to compare the effect of radiation dose with the corresponding effect of aging. Specifically, for the inflammation pathway quantified by the 1st_score, where $\beta_{dose} = 0.33$ and $\beta_{age} = 0.48$, when viewed in the context of the inflammation explained by the 4 markers in the 1st_score, 1 Gy of radiation exposure is approximately equivalent to $\beta_{dose}/\beta_{age} = 0.33/0.48 = 0.69$ decade of aging, or an age increase of 6.9 yr. In view of the small standard error values of both slope coefficients, this estimate is also expected to be reasonably accurate. Similarly for the inflammation pathways quantified by the 2nd_score and 3rd_score, 1 Gy of radiation exposure is approximately equivalent to $0.27/0.33 = 0.82$ and $0.12/0.30 = 0.40$ decades of aging, respectively. For the 2nd_score and 3rd_score, we calculated these ratios using the coefficients of models chosen with minimum Akaike's information criterion (AIC). The percentage increments in each inflammation score for the unit change in dose or age can be measured as $100 * (\exp(\beta_{dose}) - 1)$ and $100 * (\exp(\beta_{age}) - 1)$, respectively. Accordingly, corresponding to 1 Gy of radiation exposure, the percentage increments in 1st_score, 2nd_score, and 3rd_score are approximately 39, 31, and 13%, respectively, while for one decade of extra age, the score increments are approximated as 62, 39, and 35%, respectively.

DISCUSSION

In this study, we measured plasma ROS levels in A-bomb survivors and used those results and previously measured results of plasma markers and cytokines for evaluation of radiation and aging effects on immune

TABLE 6. *Regression model for scores of inflammation pathways (based on leading principal components as response)*

Factor	Estimate	95% CI	P	R^2
1st_score				
Dose (Gy)	0.332	(0.204, 0.461)	<0.001	0.190
Gender	0.475	(0.213, 0.737)	<0.001	
Age (10 yr)	0.482	(0.367, 0.597)	<0.001	
Smoking	0.383	(0.091, 0.676)	0.011	
BMI	0.037	(0.001, 0.073)	0.045	
2nd_score				
Dose (Gy)	0.267	(0.148, 0.386)	<0.001	0.191
Gender	0.689	(0.446, 0.932)	<0.001	
Age (10 yr)	0.304	(0.214, 0.427)	<0.001	
Smoking	0.001	(−0.406, 0.136)	0.329	
BMI	0.035	(−0.039, 0.028)	0.745	
3rd_score				
Dose (Gy)	0.124	(0.011, 0.238)	0.032	0.088
Gender	−0.025	(−0.257, 0.207)	0.836	
Age (10 yr)	0.304	(0.203, 0.406)	<.001	
Smoking	0.001	(−2.257, 0.260)	0.991	
BMI	0.035	(0.004, 0.067)	0.030	

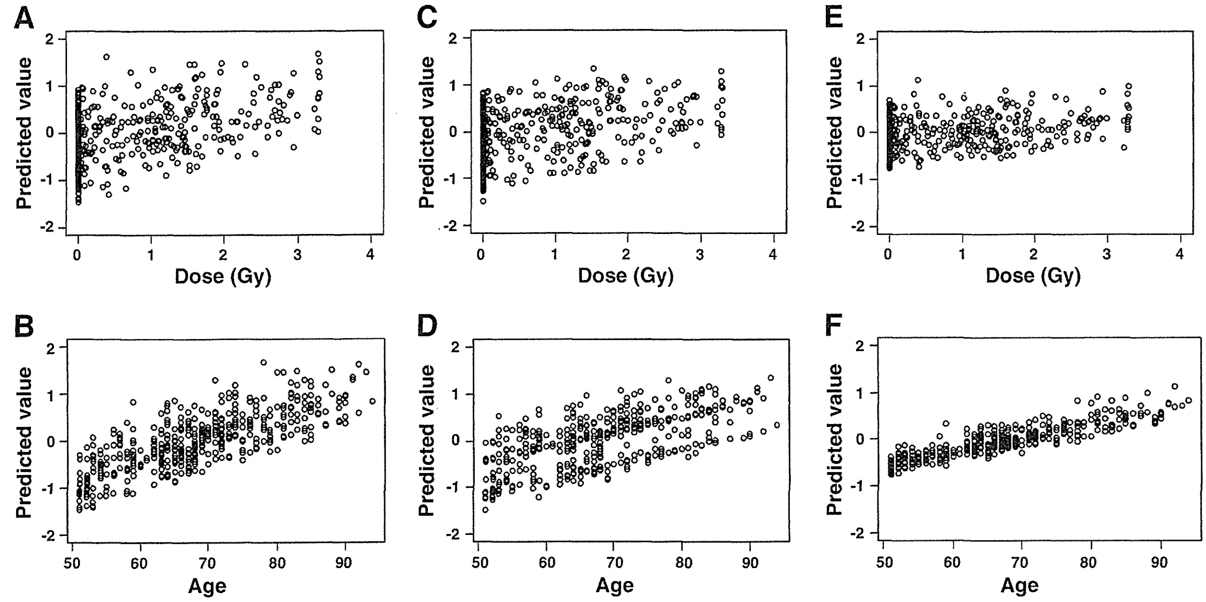

Figure 2. Predicted inflammation scores as functions of radiation dose and age (as calculated for all study subjects by the 1st, 2nd, and 3rd scores, comprised of ROS, IL-6, CRP, and ESR; TNF-α, ESR, and Igs; and TNF-α, IL-4, and IL-10, respectively). *A*) 1st_score *vs.* dose. *B*) 1st_score *vs.* age. *C*) 2nd_score *vs.* dose. *D*) 2nd_score *vs.* age. *E*) 3rd_score *vs.* dose. *F*) 3rd_score *vs.* age.

and systemic markers of inflammation. The analyses included both inflammatory cytokines and markers (ROS, IL-6, TNF-α, CRP, ESR, and Igs) and antiinflammatory cytokines (IL-4 and IL-10). IL-4, IL-6, TNF-α, and, CRP play a central role in the coordination of the inflammatory response as key proinflammatory cytokines. In the previous study, plasma levels of inflammatory cytokines and biomarkers (IL-6, TNF-α, CRP, and ESR) increased significantly with radiation dose or age (32, 38). We demonstrated here that 7 of these 8 cytokines and markers, including plasma ROS levels, were correlated with increasing age and radiation dose. However, the coefficient of determination in each regression was small. Then, to assess the biologically significant relationship between inflammation and radiation exposure or aging, we used multivariate statistical analyses and evaluated the systemic markers of inflammation as scores calculated by linear combinations of selected cytokines and markers. Principal factor analysis revealed that 3different factors explained the

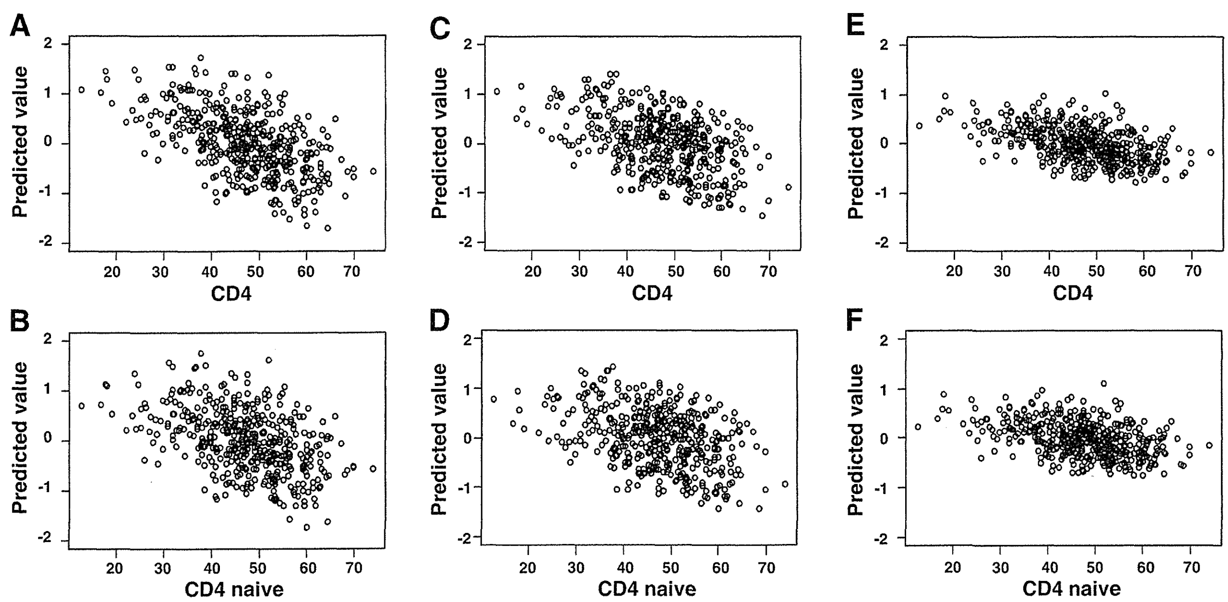

Figure 3. Association between the inflammation scores and CD4 or CD4 naive T cell frequency. *A*) 1st_score *vs.* CD4 T cells. *B*) 1st_score *vs.* naive CD4 T cells. *C*) 2nd_score *vs.* CD4 T cells. *D*) 2nd_score *vs.* naive CD4 T cells. *E*) 3rd_score *vs.* CD4 T cells. *F*) 3rd_score *vs.* naive CD4 T cells.

correlation structure among cytokines and markers. The first, second, and third factors had large coefficients for ROS, IL-6, CRP, and ESR; TNF-α, ESR, and Igs; and TNF-α, IL-4, and IL-10, respectively. These factors imply the presence of some latent variables possibly related to immunological pathways or mechanisms, each involving the cytokines/markers with large coefficients for that factor. In the association of the 1st_score, 2nd_score, and 3rd_score with the explanatory variables, radiation, gender, and age were statistically significant in the models of the 1st_score and 2nd_score. These results indicate that linear combinations of ROS, IL-6, CRP, and ESR and TNF-α, ESR, and Igs generated such a score that was the most indicative of inflammation and revealed clear dependences on radiation dose and aging and that there may be two inflammation pathways related to radiation. One pathway is involved in an ROS-dependent pathway related to IL-6 and CRP. The other is in an ROS-independent pathway related to TNF-α and Igs. Our results indicated that the systemic markers of inflammation might be accelerated by these ROS-dependent and -independent pathways.

The ROS measured in the plasma at the time of sampling indicates an equilibrium level that is constitutively produced by cells and reflects the oxidative state of the body. It has been known that ROS perform essential roles in inflammation and immune responses to pathogens, including bacterial killing through the production of superoxide by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases during the respiratory burst in activated macrophages and neutrophils (39,40). One possibility is that the pathways related to these NADPH oxidases may be involved in ROS generation and modulate by aging and radiation. Evidence that high serum concentrations of cytokines and inflammatory proteins were associated with high levels of ROS and low levels of superoxide dismutase and glutathione peroxidase is also of particular interest (41). Circulating levels of TNF-α and IL-6 increased with age in the general population (42); aging was also associated with increased levels of acute-phase proteins, such as CRP and serum amyloid A (43). Moreover, whereas IFN-α and IFN-γ production decreased in the elderly, IL-4 and IL-10 production increased (44), as did production of proinflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α (45). Aging is also found to cause the increased levels of inflammatory cytokines and ROS, both of which have been associated with obesity, insulin resistance, and atherosclerosis (46, 47). Inflammation-induced formation of ROS is often indicative of the development of cardiovascular disease, diabetes, and cancer, and its implications for aging provide strong support for the hypothetic involvement of interrelated inflammation and oxidative stress in aging processes.

It has been reported that vascular endothelial cells might also be involved in production of many inflammation-related cytokines and ROS in plasma and that their extensive production has been implicated in diseases such as atherosclerosis through the induction of chronic activation of the vascular endothelium and components of the immune system (48). It is likely that elevated plasma levels of CRP and IL-6 are risk factors for cardiovascular disease (49). Indeed, human CRP and complement activation are major mediators of ischemic myocardial injury (50). Experimental and clinical studies have suggested increased production of ROS both in animals and patients with acute and chronic heart failure (51–55). Our results indicate that a linear combination of ROS, IL-6, CRP, and ESR (Eq. 2) generated such a score that was the most indicative of inflammation and revealed clear dependences on radiation dose and aging. These results suggest that, collectively, radiation exposure may enhance the persistent inflammatory status of A-bomb survivors in conjunction with natural aging. Given the potential implications of our findings, a follow-up study with an increased number of subjects or retrospective study with the use of stored plasma samples, in association with the development of various inflammation-associated diseases, is warranted to confirm the clinical benefit of these scores.

It has been reported that a regression analysis indicates statistically significant association between radiation dose and leukocyte counts but not neutrophil counts (33). In addition, long-term immunological studies of A-bomb survivors have revealed that the percentages of CD4 (helper) T-cell populations, especially those of $CD45RA^+$ (naive) CD4 T cells, decreased in peripheral blood as radiation dose or age increased (34, 56). In our present studies, score values correlated negatively with the percentages of CD4 and naive CD4 T cells. We therefore suppose that elevated inflammatory score values indicate certain mechanisms which play a role in the attenuation of T-cell immunity in A-bomb survivors. Furthermore, we have previously reported that the percentages of $CD4^+$ T cells in peripheral blood lymphocytes decreased significantly in A-bomb survivors who had a history of myocardial infarction, indicating that such an immunological modification may be related to the increased risk of the disease (57). Taken together, this immunological balance impaired by aging and radiation exposure might result in acceleration of inflammatory status related to those multiple ROS-dependent and -independent inflammatory pathways.

To obtain further support of the hypothesis that A-bomb radiation has accelerated immunological aging, which, in turn, might lead to long-lasting inflammation, we are conducting a longitudinal analysis that assesses changes in immunological and clinical status with radiation exposure and aging in the A-bomb survivor population, on the basis of a number of immunological and inflammatory biomarkers interacting with each other. We have also begun to analyze genotype-phenotype associations concerning immune-related genes to take into account the genetically-regulated production and function of inflammatory cytokines and chemokines, which may in part explain the interindividual variation in inflammatory response. Based on the studies mentioned above, it is expected that we will be able to derive the basis for the development of an integrated scoring system to verify our hypothesis of acceleration of immunosenescence due to radiation exposure. FJ

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METHODOLOGY

Conventional case–cohort design and analysis for studies of interaction

John Cologne,[1]* Dale L Preston,[2] Kazue Imai,[3] Munechika Misumi,[1] Kengo Yoshida,[3] Tomonori Hayashi[3] and Kei Nakachi[4]

[1]Department of Statistics, Radiation Effects Research Foundation, Hiroshima, Japan, [2]Hirosoft International Corporation, Seattle, WA, USA, [3]Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, Hiroshima, Japan and [4]Consultant, Radiation Effects Research Foundation, Hiroshima, Japan

*Corresponding author. Department of Statistics, Radiation Effect Research Foundation, 5-2 Hijiyama Park, Minami-ku, Hiroshima 732-0815 Japan. E-mail: jcologne@rerf.jp

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Background The case–cohort study design has received significant methodological attention in the statistical and epidemiological literature but has not been used as widely as other cohort-based sampling designs, such as the nested case–control design. Despite its efficiency and practicality for a wide range of epidemiological study purposes, researchers may not yet be aware of the fact that the design can be analysed using standard software with only minor adjustments. Furthermore, although the large number of options for design and analysis of case–cohort studies may be daunting, they can be reduced to a few simple recommendations.

Methods We review conventional methods for the design and analysis of case–cohort studies and describe empirical comparisons based on a study of radiation, gene polymorphisms and cancer in the Japanese atomic bomb survivor cohort.

Results Stratified, as opposed to simple, random subcohort selection is recommended, especially for studies of gene–environment interaction, which are notorious for lacking statistical power. Methods based on the score-unbiased exact pseudo-likelihood (or its analogue with stratified case–cohort data) are recommended for use in conjunction with the asymptotic variance estimator.

Conclusions We present an example of how to implement case–cohort analysis methods using SPSS, a popular statistical package that lacks some of the features necessary to directly adapt and implement published methods based on other software platforms. We also illustrate case–control analysis using Epicure, which provides greater risk-modelling flexibility than other software. Our conclusions and recommendations should help investigators to better understand and apply the case–cohort design in epidemiological research.

Keywords case–cohort study, pseudo-likelihood, stratification, statistical interaction, gene–environment interaction

Introduction

A recent explosion in molecular genetic methods facilitates detailed investigation of mechanisms of radiation-related cancer, which is one of the most well-documented outcomes of radiation exposure in the Japanese atomic bomb survivors.[1] Using stored leukocytes obtained from survivors who attended clinical examinations in the Adult Health Study[2] conducted at the Radiation Effects Research Foundation, studies are being conducted to investigate various components of the immunogenome and cancers at several sites.[3,4] The standard approach to assessing risk is through the effect of radiation exposure on rates of disease or death using cohort follow-up. Analysis may be based on the proportional hazards model fit via the partial likelihood (PL).[5] Extensions to the proportional hazards model involving more general risk functions other than proportional hazards, such as excess relative risk (ERR) or excess absolute rates, are often used.[6]

Cohort studies involving costly or time-consuming methods to obtain covariate information, such as interviews, use of precious biological specimens, collecting hospital or employment records, or genotyping, can be carried out efficiently using sampling from the cohort non-cases because the cases are more influential on risk estimates and typically represent a relatively small fraction of the total cohort.[7] Selecting subjects from the cohort using the counter-matched nested case–control study design[8] can be effective, especially when studying interactions.[9,10] However, with separate studies for multiple outcomes (e.g. multiple sites of cancer to be studied individually) selecting control subjects separately for each outcome wastes resources and can be inefficient. An appealing alternative is the case–cohort design,[11] in which one selects a single subcohort from the initial cohort at its inception with a pre-specified sampling fraction, either randomly or using stratified random sampling, and adds in all cases that occur in the cohort outside the subcohort. Several discussions of the relative merits of case–cohort and nested case–control designs are available,[12–15] and both designs have been shown to be cost-effective for gene–disease association studies.[16] In this review, we address case–cohort study design and analysis focusing, in particular, on exposure-based stratified random sampling from the cohort as a means to increase statistical efficiency relative to simple random sampling.

Because the subcohort is a random or stratified random sample from the cohort, it can be analysed by itself as a cohort study using the ordinary PL, but this is clearly inefficient because of the loss of cases that occur outside the subcohort. Thus, the key feature of the case–cohort design is the inclusion of all cases that occur in the cohort regardless of whether they are in the selected subcohort. Because of such biased sampling with regard to case status, risk estimation using the ordinary PL is not appropriate, one reason being that asymptotic distribution theory for the PL estimators breaks down because cases outside the subcohort induce non-nesting of the so-called sigma fields, or sets on which the counting process probability measure is defined.[17] However, that problem can be overcome using straightforward adjustments. Most importantly, naïve estimates of parameter standard errors output by ordinary proportional hazards regression programmes will be incorrect because of the statistical dependencies between elements of the estimating equations that inflate the variance, but those can also be handled in a straightforward manner using influence residuals available in most regression programmes. The upshot of this is that, although the synthetic nature of a case–cohort design may seem baffling to researchers, there is no impediment to obtaining appropriate estimates of risk and standard errors as long as appropriate methods of analysis are applied, and there are methods for performing such analyses using standard software.

Conventional options for analysing randomly sampled case–cohort data include the exact pseudo-likelihood (PsL)[11,18] and approximate PsL,[17,19] with various adjustments to parameter standard error estimates. In addition, more recent approaches based on considering the case–cohort sample as a cohort study with missing data or with alternative weighting[20–23] or as the second phase in a two-phase design[24] have been proposed. A generalized approach to analysing various designs involving sampling from a cohort has also been proposed.[25] Although the conventional approaches have been available for some time and have been studied in some detail, the more recent approaches still require further evaluation and comparison among themselves and with the more conventional methods. Therefore, we believe it is possible to draw generalizations and conclusions regarding choice of design and method among conventional approaches, and that is the focus of this review.

The exact PsL is score unbiased and has been shown to have the best small-sample properties in simulation studies.[26,27] However, there was little difference among the various PsL estimators when subcohort size was not small (>15% sampling fraction relative to the full cohort).[26] Procedures for adjusting the standard errors of estimated parameters include the asymptotic method (based on the pseudo-score, analogous to likelihood-based methods) and the robust (empirical or 'sandwich') types of estimators. Both methods use delta–beta statistics (influence residuals) that can be obtained from most Cox regression software, and they are asymptotically equivalent. However, the robust variance estimates can be conservative (too large) in finite samples.[28]

If there are risk factors whose values are known in the entire cohort, an alternative approach is to construct the subcohort using stratified sampling. In that case, the methods appropriate for randomly selected subcohorts require further modification to achieve unbiased parameter estimates and correct variance estimates. Borgan *et al.*[29] described three estimation methods, analogous to those for randomly sampled subcohorts. Their method III, known as the 'swapper' method because a case not in the subcohort swaps places with a member of the subcohort in the same sampling stratum, is the analogue of the exact PsL and is the only score-unbiased procedure among the three. Score unbiased means that the estimating equations have expected value zero; otherwise they can result in finite-sample bias even though they are statistically consistent (unbiased asymptotically). Although the computational complexity involved in analysing data from a stratified case–cohort study is not great,[18] it nevertheless requires some effort, and it is not clear what magnitude of efficiency gains may be achieved by stratification to guide investigators in choosing between simple and stratified subcohort selection. In addition, the score-unbiased swapper method for stratified data does not seem to be used, despite mention of a stratified design in some published works,[27] and it is not implemented in the case–cohort function 'cch'[30] in the R package 'survival'.[31]

The present report describes the conventional simple and stratified random-sampling approaches to case–cohort study design and compares them using actual data from a study of epithelial growth factor receptor (EGFR) gene repeat length polymorphism and radiation joint effects on lung cancer in atomic bomb survivors.[4] We provide simple recommendations as to which methods of analysis should be used, and we present examples of how to implement the methods using Epicure and SPSS, which are popular statistical packages among epidemiologists. We also provide S-plus code for implementing the swapper method for stratified case–cohort data, which makes critical use of a built-in function not available in R.

Review of conventional case–cohort methodology

Early work focused on estimation of relative risk based on the analogy of a case–control comparison.[32–34] Subsequent analytical methods capitalized on the analogy with cohort follow-up and PL, although the unique sampling approach (over-selecting cases) leads to a PsL rather than the usual PL. The PsL may be used to fit the analogue of a PL analysis (e.g. the proportional hazards model). Prentice noted that parametric functions other than log-linear risk functions could be used, and the PsL can be extended to accommodate absolute rate differences and other non-proportional hazard models for estimating the hazard rate ratios for covariates.[11] However, with the exception of Epicure,[6] such models are not generally available in case–cohort analysis software.

Analyses of the case–cohort sample must adjust for bias introduced in the distributions of covariates used in calculating the denominator of the PsL, as the case–cohort sample is not population-based because of over-sampling of cases. Bias incurred by including cases outside the subcohort is corrected by not allowing those cases to contribute to risk sets other than their own (risk sets comprise cohort subjects still under observation and at risk at the times cases occur). Two approaches exist based on whether cases outside the subcohort are allowed to enter the denominators of the PsL for their own risk sets. With Prentice's original method,[11] cases outside the subcohort appear in their own risk set denominators; this method is called the exact PsL. The method on which the asymptotic variance estimate was originally based[17] excludes cases not in the subcohort even from the denominators of their own risk sets; this method is called the approximate PsL. These inclusions or exclusions can be carried out by weighting the cases. With the exact PsL, cases outside the subcohort are given Weight 1 in their own risk set and Weight 0 otherwise; with the approximate PsL, cases outside the subcohort are given Weight 0 in all risk sets, including their own, although those cases still contribute to the numerators of the PsL in their own risk sets by virtue of being cases (defining the risk sets). In practice, this means that weighting must be accomplished by the use of offsets rather than prior weights because the latter will exclude non-subcohort cases altogether. The exact PsL is score unbiased, whereas the approximate PsL is not score unbiased and can result in some small-sample bias, particularly if the subcohort risk sets are small.[18]

In addition to case weighting, all subjects can be weighted using risk set weights based on follow-up time and cohort attrition: who among the subcohort subjects are still at risk as time passes. This type of weighting, proposed by Barlow, uses the inverse of the sampled fraction as a weight.[35] The original proposal updates the weights at each failure time (case occurrence or risk set) using time-dependent weights according to the surviving fraction in the sampled subcohort vis-à-vis the surviving fraction in the full cohort. This approximates the likelihood function denominator contribution that would pertain in the full cohort analysis and is intuitive based on counting process theory.[36]

Barlow's robust variance estimate, a jackknife estimate related to the influence function, was shown to have good performance and said to be easier to calculate than either the bootstrap estimate[37] or the asymptotic variance estimate, the latter requiring calculation of the correlations among components of the scores (estimating equations). An advantage of the robust variance vis-à-vis the asymptotic variance is that the latter is difficult to compute in open cohorts where there is staggered entry.[15] Practical computation was via SAS using the PHREG procedure with non-subcohort cases defined to enter the study just before their failure times and variances derived from the delta–beta residuals[15] (originally using martingale residuals[35]). Similar computations may be done in S-plus[36] or R. If the data are partitioned into risk sets (if there are time-dependent covariates, for example), the subject-specific residuals must be summed up separately for each subject over all risk sets in which the subject appears.

A simplification of the Barlow weighting approach is to use the single, original subcohort sampling fraction at all failure times (i.e. weights are fixed, not depending on risk set). This has the practical advantage that multiple records (one record for each subject for each risk set in which she or he is at risk) do not have to be constructed, as the number of records in the analysis data set can significantly increase if there are many risk sets (many cases of disease or death). As with the exact PsL estimator, the Barlow method weights each case with Weight 1 at its failure time whether or not it is in the subcohort. Therneau and Li suggested that cases in the subcohort be weighted by the appropriate risk set fraction just as the non-cases, rather than converting their weight to 1 at their time of failure, to reflect the fact that they are legitimate risk set members in the sampled data.[19]

Details of variance estimation are described in a number of publications.[17,19,35,38] The asymptotic variance consists of within-risk set variances and between-risk set covariances, the latter arising from correlations among score contributions (components of the estimating equations). Briefly, the problem is solved by adding to the usual PL parameter covariance matrix a weighted product of influence residuals for the subcohort data. One approach uses weights based on the subcohort sampling fraction.[29] Another uses as weights the proportion of cases actually sampled in the subcohort.[19] With a large amount of data, the two types of weights will be similar because the expected fraction of cases in the subcohort approaches the subcohort sampling fraction as the subcohort size increases. However, due to random sampling variation, the actual proportion of cases in the subcohort may deviate from the expected value. Essentially all of the so-called 'robust' variance estimators that have been proposed can be expressed as special cases of a general influence function variance estimator that is robust to misspecification of the model relating the risk parameters to the underlying distribution function.[38]

All of the aforementioned methods consistently estimate the risk and variance asymptotically (with large studies), but they differ in their efficiency (estimates of the parameter variances) and small- or finite-sample bias (in the case of the approximate PsL). Therefore, a major concern is how well they perform in studies of the size typically encountered in epidemiological practice. According to Onland-Moret *et al.*,[26] the 'most simple approach'—Prentice's original, exact PsL method—provides estimates and standard errors closest to the full-cohort results (i.e. using all subjects with no case–cohort sampling) when the cohort is small (about 1000 subjects or less) or when the subcohort is small (sampling fraction 0.15 or less with a cohort size of 1000 subjects). Those authors also demonstrated that the approximate PsL is inferior to the exact PsL method. However, they did not implement Barlow's method as originally proposed: they used the initial sampling fraction rather than time-dependent weights. Langholz and Jiao[18] reported that the approximate PsL estimator does not perform as well as the exact estimator when risk set sizes are small (e.g. when there are large numbers of cases and a small subcohort sampling fraction). The approximate PsL estimator is also not score unbiased in finite samples,[29] which means that it can result in small-sample bias.

From a practical standpoint, either the exact or approximate PsL involves about the same amount of data manipulation, and both require constructing duplicate data records for subcohort cases to accommodate the case weighting described earlier. The approximate method requires one set of records for all subcohort members (including subcohort cases) having Weights 1, and a second set of records for all cases (including both subcohort and non-subcohort cases) having Weights 0. Weight 0 is achieved by including a large, negative offset, -100 say, which when included in the proportional hazards model becomes $\exp\{-100\}$ or a number essentially equal to zero to the level of machine precision. A weight of one is achieved by using an offset of zero ($\exp\{0\} = 1$). This means that cases not in the subcohort will define risk sets but will not enter into the denominators of the PsL calculations, whereas subcohort cases are included in all risk set denominators up to (in time) and including their own. This data setup was illustrated by Therneau and Li, who also described how to calculate the asymptotic variance estimates, using SAS and S-plus.[19] Their S-plus routine calculates the variances directly, whereas their SAS routine requires the IML procedure and an additional calculation by hand.

With the exact PsL, non-subcohort cases should be included in the denominators of their own risk sets. Instead of calculating offsets to down weight the cases, it is easier to specify entry and exit times in such a way as to prevent non-subcohort cases from contributing to the overall subcohort follow-up while allowing them to contribute to their own risk sets. One record is constructed for each case with entry time set to a small increment before its onset time. The increment should be much smaller than the differences between successive failure times so as to avoid overlap of risk sets. Another record is constructed for each subcohort subject (including subcohort cases) with exit time set to a small increment before its onset or censoring time. This ensures that subcohort cases contribute to all risk sets before their time of onset. Langholz and Jiao[18] described SAS commands to set up the data for the exact PsL. They also described fitting the proportional hazards model and computing asymptotic and robust variance estimators in SAS using the same data setup, although (as with the Therneau and Li[19] SAS variance estimate) the asymptotic variance estimate requires an additional calculation by hand.

The exact PsL with asymptotic variance estimate is also available in the Epicure survival analysis module 'Peanuts'.[6] In Epicure, the user only need specify, in addition to the usual PL variables (entry time, exit time and failure indicator), an indicator of which cases are outside the subcohort (CSCHRT) and the subcohort sampling fraction; thus, substantially less setup is required than with SAS or S-plus. Furthermore, Epicure allows fitting general risk models, including absolute rates; therefore, it is our method of choice for unstratified case–cohort analyses.

Up to this point, we have focused on case–cohort studies involving simple random selection of the subcohort. Stratification (stratified random sampling) can be used to increase efficiency ('exposure stratification') and to deal with confounding ('confounder stratification').[18] In the present work, we are concerned with efficiency of risk estimation when exposure is known in the entire cohort and interest lies in making inference about interactions with exposure (exposure risk modification). Thus, we focus on exposure stratification. Stratified sampling in case–cohort studies merely involves randomly selecting subcohort subjects separately within exposure strata. As with all stratified sampling scenarios, the optimal number of strata and optimal numbers of persons to sample from each stratum are issues worth consideration,[29] but we do not pursue them here as they are difficult to generalize.

Borgan *et al.*[29] defined three methods for handling stratified case–cohort data. The first of their estimators (method I) is analogous to the approximate PsL estimator in the unstratified situation in that a case outside the subcohort does not enter into the denominator, even in its own risk set. The second estimator (method II) is somewhat analogous to the exact PsL, but non-cases are weighted according to their respective stratum proportions. The third estimator (method III) weights all subjects in the subcohort according to their respective stratum proportions and further deals with cases not in the subcohort by randomly selecting one member of the subcohort in the same stratum to be removed or 'swapped'. The reason for this approach is that the exact PsL for stratified data remains score unbiased if non-subcohort cases are excluded from the denominators of their likelihood terms, but such exclusion is inefficient.[29] Swappers may be chosen on a risk set basis if the analysis includes time-dependent covariates; otherwise, the swapper is selected from the initial subcohort strata, and if the randomly chosen swapper has been censored as of the time of the current risk set, swapping for the corresponding non-subcohort case is simply ignored.

The swapper method (method III) is our recommended method for stratified case–cohort data, as it is the only score-unbiased method. Unfortunately, it is not included among the procedures for stratified case–cohort data analysis in the cch function of the R survival package. We therefore adapted to S-plus the SAS macros of Langholz and Jiao.[18] The script is provided in the Supplementary Appendix A1 available at *IJE* online, but cannot be directly implemented in R due to the lack of a necessary function—match.data.frame—that is included with S-plus, but not with R.

Mark and Katki[38] stated that variance estimation with the stratified case–cohort design might be handled using their influence function variance estimator starting with a stratified proportional hazards model, but did not provide details except to suggest that one approach is to treat cases with missing information as if they occurred outside the subcohort. Additive models may also be entertained in the stratified case–cohort design,[39] as may frailty models for cluster data.[40] Many other options and proposals exist in the literature, but they are too numerous to describe in detail. Table 1 presents an overview of the evolution of conventional methods for case–cohort study design and analysis. We conclude that score-unbiased methods with asymptotic variance are preferable based on both theoretical and small-sample properties. In particular, the robust variance cannot be implemented with stratified data, and risk-set (time-dependent) weights do not seem to be necessary with PsL estimators (although they may be related to efficiency with other types of estimators[22]). We therefore recommend the use of the exact PsL (using its analogue, the swapper method, in the stratified case) and the asymptotic variance estimates. We restrict our attention to that approach in the remainder of this work.

Empirical investigations

The initial design of the immunogenome and cancer case–cohort study included two subcohorts: one using simple random sampling and one using stratified random sampling with stratification on radiation dose. Five strata were defined approximately as quintiles, but with round numbers as the cutpoints (Table 2). Both subcohorts were constructed using a sampling fraction of 0.5 to reduce genotyping effort by about one-half while retaining most of the full-cohort power.[3] To minimize the effort involved in molecular analyses and to facilitate direct comparison between the two sampling procedures, the two subcohorts were selected with the greatest amount of overlap possible between them. This was done by modifying the simple random subcohort, randomly adding to or excluding from each stratum the

Table 1 Overview of developments in casecohort study design and analysis

Reference	General approach	Special characteristics
Prentice[11]	Initial proposal of case-cohort design based on time-to-event analysis[a]	Defined exact pseudolikelihood (PsL) analogous to partial likelihood (PL)
Self and Prentice[17]	Asymptotic distribution of approximate PsL	Asymptotic normality of pseudolikelihood estimate and consistency of Prentice's variance
Wacholder et al[37]	Variance estimators derived under superpopulation (sampling without replacement) model or using the bootstrap	Superpopulation variance valid under $\beta = 0$ Bootstrap method non-standard due to lack of knowledge of covariate information for all cohort members
Barlow[35]	Robust variance estimator based on influence functions Weights based on subcohort membership within risk sets	Analogous to Lin and Wei[41] robust variance for the full-cohort proportional hazards (PH) model Preserves correct expectation of PsL denominator in each risk set
Lin and Ying[20]	Missing-data approach to estimation with proportional hazards model	Variance estimators more readily derived with large data than those of Self/ Prentice or Wacholder et al.
Barlow et al[15]	Jackknife variance estimator computed via delta-beta residuals	SAS macros available through statlib
Therneau and Li[19]	Intuitive description of fitting PH model with approximate pseudolikelihood and asymptotic variance estimation	SAS and S-plus examples provided
Chen and Lo[21]	More efficient use of case and cohort information in the estimating functions	Derived three methods depending on extent of knowledge of case proportion in the population
Borgan et al[29]	PsL estimators for stratified case-cohort data	Score-unbiased 'swapper' method analogous to unstratified exact PsL
Mark and Katki[38]	Generalized influence function approach to variance computation	Dealing with missing data among subcohort or case subjects
Scheike and Martinussen[23]	Maximum likelihood via EM algorithm (including non-sampled cohort members who do not fail)	Efficiency gain related to disease intensity Variance estimated by profile likelihood
Kulich and Lin[22]	Doubly weighted estimation utilizing covariate information available on the full cohort	Efficiency gain greatest for continuous covariates, less for binary covariates
Nan[42]	Estimation via efficient scores	Relaxes some assumptions underlying the proportional hazards model
Langholz and Jiao[18]	Computational methods for random and stratified designs using exact PsL and asymptotic variance	SAS code provided for data construction, proportional hazards model fitting and variance estimation
Samuelsen et al[28]	Intuitive methods analogous to those of Therneau and Li[19] for the asymptotic variance	Script provided for asymptotic variance estimation using S-plus/R Compared asymptotic and robust variance estimators
Kulathinal et al[27]	Design options and overview of analysis methods	SAS and R command examples
Moger et al[40]	Case-cohort methods for cluster-based sampling	Extended pseudolikelihood approach to gamma frailty and copula failure models

[a]Prentice[11] attributes the earliest ideas similar to the case-cohort design to Kupper et al[43] and Miettinen[32], but seems to be the first to describe the link to partial likelihood and use of continuous explanatory factors

Table 2 Numbers of subjects in the immunogenome study cohort and case–cohort subcohorts

	Radiation dose stratum (whole-body dose, weighted milliGray)					
	0⩽1	1⩽5	5⩽100	500⩽1500	1500<	Total
Number of cohort subjects	1433	516	1110	1005	618	4682
Number of lung cancer cases	29	12	28	27	27	123
Random subcohort						
Total subcohort subjects	655	238	502	454	277	2126
Subcohort lung cases	17	6	10	16	13	62
Non–subcohort lung cases	12	6	18	11	14	61
Stratified subcohort						
Total subcohort subjects	428	421	425	423	428	2125
Subcohort lung cases	11	10	7	16	20	64
Non–subcohort lung cases	18	2	21	11	7	59

necessary number of individuals to obtain a stratified sample.

The first study performed using the immunogenome and cancer case–cohort design, a study of lung cancer and *EGFR* gene, was originally conducted using the simple random case–cohort sample.[4] However, the *EGFR* gene was ultimately assessed on the full cohort (this would not generally be the case with a case–cohort study). Hence, the two designs (simple and stratified) can be directly compared and various sampling fractions (i.e. <50%) can be evaluated via Monte Carlo simulation drawing from the full cohort. We performed such simulations analysing the data using the exact PsL (or its analogue with stratified data, the swapper method) and asymptotic variance adjustment, the methods of choice noted earlier.

Relative efficiency (efficiency of the case–cohort sample relative to that of the full cohort) was calculated as the inverse ratio of standard errors. Analyses were conducted using S-plus (version 6.2 for Windows) or R (version 2.10.0 for Windows). Analyses of simple case–cohort data were made using the cch function in the R survival package with the default 'Prentice' option (exact PsL). Analyses of stratified case–cohort data were performed using method I or II in the R survival package cch function. For method III, we adapted the SAS programs described by Langholz and Jiao[18] in S-plus using the coxph function (Supplementary Appendix A1 available at *IJE* online). Asymptotic standard errors were based on Cox regression delta–beta statistics [S function residuals.coxph(type = "dfbeta")]. Residuals for the non-failures were used and summed over risk sets within individuals using the aggregate function in S-plus. Age was used as the primary time scale in proportional hazards regression with left truncation at entry age.[44] Covariates were as follows: city of residence, gender, year of birth (centred at the median, 1927, and divided by 5 years), smoking frequency (number of cigarettes smoked per day divided by 10), whole-body radiation dose (wGy: weighted Gray skin dose, using weights 10 for neutron dose and 1 for gamma dose), an indicator of *EGFR* gene CA repeat length < 38 (short-repeat genotype) and the product of radiation dose and EGFR CA repeat length indicator. Confidence intervals (95%) and tests were based on Wald statistics. Results obtained using SPSS and Epicure are provided along with relevant commands in the Supplementary Appendix A1 available at *IJE* online.

Results

Table 3 shows results of analyses of the actually selected simple and stratified 50% case–cohort designs. There was little practical difference between the two designs in terms of estimated parameters and standard errors, consistent with the expectation that a large case–cohort sample should provide close to full-cohort efficiency. Thus, the original analysis,[4] which was based on the simple random subcohort with 50% sampling fraction, should be reasonably efficient. The exact PsL method with asymptotic variance for the random case–cohort sample was programmed in S-plus and gave results identical (to three decimal places) to those obtained with the R cch function (data not shown). The three methods for a stratified case–cohort sample produced nearly identical results. How the methods compare with smaller sample sizes is addressed below.

Proportional hazards imply multiplicative effects; therefore, the negative interaction suggests a submultiplicative joint effect of radiation and CA repeat length polymorphism. What this implies about biological interaction, however, cannot be assessed without examining other scales of the joint effect, such as a purely additive statistical model.[45] An additive ERR model for the joint effects of radiation and repeat length polymorphism (which can only be fitted using Epicure with the simple case–cohort sample; see Supplementary Appendix A1 available at *IJE* online) produced virtually the same result as the multiplicative

Table 3 Proportional-hazards model fits to random and stratified subcohorts with 50% sampling fraction [coefficient (SE), Wald *P*-value]

Parameter	Case–cohort sampling method			
	Random	Stratified		
	(exact method)	Method I	Method II	Method III (swapper)
Year of birth (5-year difference; centred at 1927)	0.61 (0.12)	0.62 (0.12)	0.62 (0.12)	0.62 (0.12)
P-value	<0.001	0.001	0.001	0.001
City (Nagasaki: Hiroshima)[a]	0.01 (0.21)	−0.10 (0.21)	−0.11 (0.21)	−0.10 (0.21)
P-value	0.96	0.65	0.69	0.63
Gender (female: male)	−0.61 (0.23)	−0.62 (0.22)	−0.63 (0.22)	−0.62 (0.22)
P-value	0.008	0.005	0.005	0.005
Number of cigarettes smoked per day (increment of 10)	0.45 (0.08)	0.42 (0.07)	0.42 (0.07)	0.42 (0.08)
P-value	<0.001	<0.001	<0.001	<0.001
Radiation dose (Gray)	0.40 (0.10)	0.37 (0.10)	0.38 (0.10)	0.37 (0.10)
P-value	<0.001	<0.001	<0.001	<0.001
EGFR repeat polymorphism (indicator of length <38)	0.60 (0.24)	0.52 (0.24)	0.51 (0.24)	0.52 (0.24)
P-value	0.012	0.029	0.034	0.027
Interaction between radiation and EGFR polymorphism	−0.40 (0.19)	−0.37 (0.18)	−0.37 (0.18)	−0.37 (0.18)
P-value	0.036	0.041	0.041	0.041

[a]Although not statistically significant, city of residence was included to avoid possible confounding of the gene–radiation interaction.

proportional hazards model, except that the radiation dose response is linear rather than log linear. The ERR for radiation was 1.00/wGy (RR = 2.00 at 1 wGy) compared with the log-linear relative risk model RR = exp{0.396} = 1.49 at 1 wGy. With either the multiplicative relative risk or the additive ERR model, the effect of the short CA repeat length polymorphism is to approximately double the cancer risk: the ERR for the short repeat length polymorphism was 1.06 (RR = 2.06) compared with the log-linear model RR = exp{0.601} = 1.82. With both models, statistical interaction between radiation dose and repeat length polymorphism essentially negated the radiation dose–response main effect. Interaction on the additive ERR scale was −1.09, which effectively cancels the radiation ERR of 1.00. On the multiplicative scale, interaction parameters were close to equal but opposite in sign compared with the radiation main effect (Table 3). Thus, there is apparently no practical radiation effect among individuals with the short CA repeat length polymorphism.[4]

Results of Monte Carlo sampling from the full cohort with sampling fractions varying from 0.5 down to 0.01 are shown in Figure 1 and Table 4. Relative efficiency decreased with decreasing sampling fraction. Relative efficiency of the simple case–cohort sample declined more dramatically than that of the exposure-stratified sample—that is, the stratified design becomes relatively more efficient with smaller subcohort fractions (Figure 1).

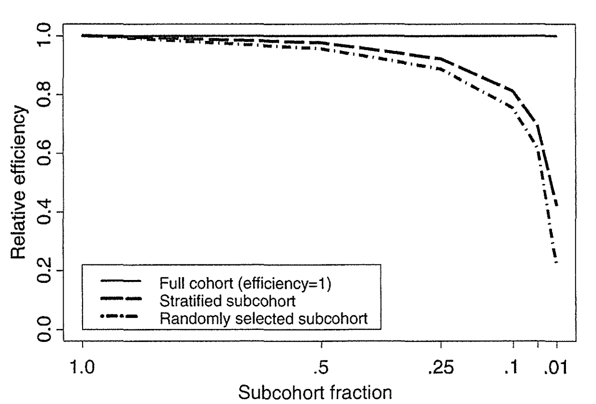

Figure 1 Relative efficiency of case–cohort analysis of gene–radiation interaction as a function of subcohort fraction: simple vs stratified sampling

To compare by simulation the three methods for stratified data, we ran the S-plus swapper function in R by importing without modification the S-plus match.data.frame function. All three methods produced similar results with subcohort fractions down to ~10%, but with sampling fraction of 5% or 1% method I had greater bias and larger standard error than methods II and III (Table 4). Overall, method III (the swapper method) performed the best, having the least bias and smallest standard errors, although differences between methods II and III were trivial.

Table 4 Mean and average standard error (SE) of estimates of the radiation–gene interaction with stratified case–cohort data using simulation from the full cohort[a]

Subcohort fraction[b] (%)	Method I Mean (SE)	Method II Mean (SE)	Method III (swapper) Mean (SE)
50	−0.43 (0.18)	−0.43 (0.18)	−0.43 (0.18)
25	−0.43 (0.19)	−0.43 (0.19)	−0.43 (0.19)
10	−0.45 (0.22)	−0.45 (0.21)	−0.45 (0.21)
5	−0.47 (0.26)	−0.46 (0.25)	−0.45 (0.25)
1	−0.72 (0.64)	−0.48 (0.44)	−0.43 (0.41)

[a]Results are based on 2000 simulations at each subcohort fraction.
[b]For comparison, the estimate and standard error with the full cohort data were −0.42 and 0.17, respectively.

Discussion and conclusions

The case–cohort design is not used as frequently as it might be given the advantages it offers epidemiologists, perhaps because the design is misunderstood given its synthetic nature. It is hoped that the present review will serve to heighten investigators' awareness of the definition and advantages of the case–cohort design and provide guidance to facilitate its implementation and analysis. Issues that can be resolved in a fairly general manner include: whether to stratify, whether to perform exact or approximate PsL analysis, whether to use asymptotic or robust variance estimates and whether/how to weight the observations in the analysis.

Our results suggest that selecting the subcohort using stratified, rather than simple, random sampling can be beneficial in terms of statistical efficiency, especially for studying interaction, when the sampling fraction is substantially less than one-half. Although relatively small subcohort fractions are typical, subcohorts as large as 50% (such as in the immunogenome and cancer study described here) are not beyond imagination. Even such a large study reduces by almost half the amount of covariate collection effort yet retains efficiency close to that of the full cohort. If an investigator is able to conduct a study with large subcohort fraction, there may be little benefit from the stratified sampling. However, there is little additional effort involved in selecting and analysing a stratified subcohort; therefore, if there are factors for which it makes sense to stratify, we recommend doing so, regardless of the sampling fraction.

Simulation studies conducted by others have demonstrated that the exact PsL is preferable to the approximate PsL in small samples, even though the two are asymptotically equivalent. Because there is little difference in the data preparation required for analysis, we recommend that the exact PsL be used. Borgan *et al.*[29] compared methods I, II and III for stratified case–cohort data, using simulations with a 10% subcohort fraction and did not note any major differences among them. However, from our Table 4, it is apparent that method I can incur more small-sample bias and lose efficiency more rapidly than the other methods with decreasing subcohort sampling fractions <10%. Method III produced the best results overall. These new findings support our conclusion that the swapper method (method III) is the method of choice for stratified case–cohort data.

Other authors have noted that the robust variance estimate can perform poorly in small samples, and the robust variance estimate cannot be used with stratified data. We therefore recommend use of the asymptotic variance estimate with both the simple (unstratified) and stratified designs.

Because risk set weighting is related to the counting-process theory underlying PL analysis, it is expected to perform well in theory. The results of Onland-Moret *et al.*[26] did not resolve its performance vis-à-vis other methods because they used the simplified version with constant weight.[26] In the simulations of Borgan *et al.*,[29] time-dependent weights only slightly improved efficiency of the stratified design. We conclude that gains from time-dependent weighting probably are not sufficient to justify the additional data construction effort with unstratified data. With stratified data, the data construction facilitates time-dependent weighting, but a variance estimator is not yet available. It has been noted that choice of appropriate weights might be affected when specimen storage duration or analytical batch effects are a concern (i.e. for cases outside the subcohort in a prospectively conducted case–cohort study[46]); this would not be a problem with retrospectively conducted case–cohort studies. In fact, although we are not aware that it has been proposed before, it should be possible to perform risk set subsampling of nested control subjects from the subcohort, if the timing of case ascertainment could lead to biases due to storage or batch effects.

Missing data are an important consideration in observational studies. Regardless of design, measurements made on biological specimens can be missing for a number of reasons. Mark and Katki[38] suggested that, as long as missingness is not related to covariate values, case–cohort analyses without time-varying weights should remain valid if cases with missing covariate information are simply ignored.

One advantage of the nested case–control and case–cohort designs as compared with the traditional case–control study design is their ability to estimate absolute risk difference (as opposed to relative risk) and survival probability. However, when there is sampling of cases or missing case data, estimators of those quantities require modification to avoid bias.[47] Another advantage of the cohort-based sampling designs is that the reference group can be used to study population characteristics, such as allele frequencies or Hardy–Weinberg equilibrium; the control subjects for a traditional case–control study are not population based, being left over after cases have been removed from the population.

The ability to study multiple outcomes individually without having to obtain separate sets of comparison subjects is often cited as one of the benefits of the case–cohort design, but this aspect is seldom illustrated or examined in the literature. In particular, the use of the same comparison subjects induces correlations among results for multiple outcomes. Sørensen and Andersen[48] investigated this via asymptotic theory and simulations and, using competing risks and martingale theory, derived a consistent estimate of the asymptotic covariance matrix. They found that correlations increase with smaller subcohort sampling fractions or, equivalently, with larger cohorts and a fixed subcohort size. For example, with a cohort of size 500 and subcohort sampling fraction of 10%, correlations were substantially larger than 0.5 for the scenarios they studied. With a subcohort sampling fraction of ~50%, they estimated correlations of 0.1–0.2 for their scenarios. In the immunogenome and cancer study described here, where the subcohort sampling fraction was 50%, correlations between effect estimates for multiple cancer outcomes should therefore not be a problem (studies involving cancers at sites other than the lung are ongoing). However, such correlations could be a problem with more typical designs (25 or 10%), which may be used if more expensive procedures, such as larger numbers of loci or whole-genome sequencing, are used.

Another interesting aspect of the case–cohort design is that outcomes occurring more frequently have smaller effective control:case ratios given that the number of comparison subjects is fixed. Kulathinal *et al.*[27] noted that choosing a subcohort size suitable for more common outcomes would provide flexibility for including additional, less common outcomes after the fact of subcohort selection. With common outcomes, sampling of cases may be preferable to using all cases in terms of reducing effort without seriously affecting statistical efficiency.[49]

Even though ascertainment of some covariates, such as genotypes, may be conducted only on the case–cohort sample, efficiency may be lost because analysis based on the case–cohort sample alone wastes other information available in the entire cohort. For example, if modelling adjustment is made for important factors or potential confounders that are known in all cohort subjects, that adjustment is inefficient if restricted only to the case–cohort sample. Further efficiency gains may be obtained by considering the case–cohort sample as the second phase in a two-phase study design by weighting the analysis of the case–cohort sample through post-stratification or calibration.[24,50] A similar strategy can be applied to nested case–control studies,[51] and more general multi-phase methods can be considered.[52] Alternatively, efficiency might be improved by considering the case–cohort study as a full cohort with missing data using, for example, inverse probability of sampling weighted methods.[53] We do not pursue those methods in this work, as they are beyond the scope of traditional case–cohort analysis, and we believe that more comparative work needs to be done to fully understand the relative merits of these new approaches vis-à-vis the conventional design and analysis of case–cohort studies. We are currently exploring the two-phase approach in comparison with the conventional methods recommended in the present review.

The typical case–cohort analysis is based on a restricted model for the hazard rate (incidence or mortality rate) as a log-linear function of explanatory covariates. Kong and Cai[54] described how to fit accelerated failure time models to case–cohort data. The accelerated-failure-time model does not require the assumption of proportional hazards, although that assumption can also be relaxed in standard proportional hazards models by including interactions of covariates with the basic time variable. Apart from Langholz and Jiao[18] describing how to estimate absolute risks, a frustrating aspect of many current software implementations of case–cohort analysis methods is the restriction to standard proportional hazards models. In radiation risk assessment, more general models are routinely used.[1] A Bayesian full-likelihood approach[55] may allow more general risk models. At present, Epicure only allows fitting general risk models with unstratified case–cohort data, but a procedure to accommodate stratified data should become available in the near future.

Because we have focused on gene–environment interaction, a few words about such tests are appropriate. Failure to adequately model the main effect of an environmental risk factor can lead to inflated type I errors (false positives) in tests of gene–environment interaction involving that factor.[56] This result emphasizes the need for use of general risk models, such as the ERR model used in our example. In our opinion, models for main effects of genotype have often not been given due consideration. Perhaps this is due to the popularity of the Cochran-Armitage trend test, which is applied under the assumption of a linear (co-dominant) genomic model. It is unfortunate that one of the major software packages for genomic analysis (R haplo.stats[57]) does not include the arbitrary two-parameter genomic model which we deem an important starting point (e.g. for testing homogeneity with biallelic loci). The case-only design can be powerful when gene and environmental factor are independent, and hybrid methods address the fact that such independence often cannot be verified.[58] However, the case-only design is limited to a multiplicative test of statistical interaction, whose use has been over-emphasized.[59] Furthermore, the subcohort in a