Limited evidence for transgenerational chromosomal instability in families with elevated mutation pattern SBS16 in the germline.

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ABSTRACT

Purpose

The transgenerational effects of preconception parental radiation exposure in humans remain unclear. We assessed genomic integrity in adult children of British nuclear test (NT) veterans—a community that has expressed long-standing concerns about adverse health effects, including in their offspring—to investigate for any constitutional chromosomal abnormalities and/or cytogenetic indicators of genomic instability that might be associated with paternal participation at NT sites.

Materials and Methods

Peripheral blood samples were obtained from 86 adult children (45 from nuclear test (NT) and 41 control), all born to veterans from the British Army, Royal Air Force, or Royal Navy.

Results

G-banded karyotyping revealed no constitutional chromosomal abnormalities in any NT sample, including those from families reporting adverse health outcomes. We next assessed for unstable aberrations using conventional Giemsa staining and found some evidence of instability. Specifically, a small subset of NT children (N = 4) showed elevated chromatid aberration frequencies (7.81 ± 4.01 per 100 cells) compared with controls (4.36 ± 0.62; N = 26). To investigate further, we analysed matched veteran father–child pairs observing a weak association between fathers' unstable aberration burden and chromatid aberrations in their children, suggesting a potential transgenerational effect. This positive trend was most pronounced in the small group of families (N=8; 2 control and 6 NT) previously identified as being enriched for mutation signature SBS16 in the germline.

Conclusions

Although based on a small sample size, this observation warrants further investigation to understand the significance of SBS16, if any, including whether it may serve as a potential transgenerational mutational signature of radiation exposure. Overall, and in the context of health concerns raised by NT families, none of the self-reported health-related variables showed any association with unstable aberration burden in either the veteran fathers or their adult children.

INTRODUCTION

Veterans of the British nuclear testing programme comprise a population of ex-military personnel who may have been exposed to ionising radiation through their participation at nuclear testing sites in the 1950s and 1960s. Over the intervening years, members of this community have raised concerns about their own health and that of their descendants, which some believe may have been adversely affected by their involvement at the test sites (Collett et al. 2021). Epidemiological studies examining mortality and cancer incidence in nuclear test (NT) veterans, conducted up to 1998, initially showed limited evidence of any detectable effects (Muirhead et al. 2003; Muirhead et al. 2004). However after longer follow-up to 2017, these findings were revised to indicate a small excess in mortality (RR = 1.02, 90% CI 1.00–1.05, p = 0.04), with similar increases observed for both cancer and non-cancer diseases (Gillies 2022). No formal epidemiological studies have been conducted to examine the health of NT veterans' descendants. This is partly due to the limited epidemiological evidence of adverse effects observed in the veterans themselves, and partly because nationwide registries of birth outcomes were not established until decades after the testing programme ended. However, information gathered from NT families have claimed adverse health effects among veterans' descendants at rates exceeding those in the general population (Busby and Escande de Messieres 2014).

Constitutional chromosomal disorders are defined as alterations in the number or structure of chromosomes present in all cells of an individual at birth and which are typically associated with a distinct set of clinical features. They are known to account for ~60% of first-trimester miscarriages, affect 7.5% of all conceptions and have a live-birth frequency of 0.6%. Genetic damage resulting from radiation exposure to reproductive cells before conception can, in principle, lead to

119 constitutional chromosomal or genetic disorders. However, consistent evidence supporting such
120 effects in human populations is limited with only weak or non-significant associations between
121 parental preconception exposure and adverse outcomes in the offspring reported (Stephens et al.
122 2024; Amrenova et al. 2024; Yamada et al. 2021).

123 Radiation-induced genomic instability is defined as an increased tendency for the accumulation of
124 diverse genomic alterations including DNA mutations, chromosomal aberrations, epigenetic
125 changes and dysregulated gene expression. From a cytogenetic perspective, this may manifest as
126 both stable and unstable chromosomal exchanges—such as reciprocal translocations or dicentrics—
127 as well as chromosome breaks, fragments, chromatid-type and numerical aberrations (Hemminki et
128 al. 2024; Morgan and Sowa 2015). Dubrova and colleagues provided evidence in animal models of
129 radiation- or chemically-induced changes in the germline, along with increased frequencies of
130 mutations and chromosomal aberrations in the offspring, describing the phenomenon as
131 transgenerational genomic instability (TGGI) (Dubrova et al. 2000; Barber et al. 2002). As with
132 constitutional chromosomal aberrations, the evidence for radiation-induced TGGI in humans
133 remains inconclusive. Some studies have reported an excess of DNA mutations or chromosomal
134 aberrations in the children of exposed parents (Aghajanyan and Suskov 2009; Dubrova et al. 2002),
135 while others have found no evidence (Kodaira et al. 2010; Slebos et al. 2004; Tawn et al. 2015).

136 The Genetic and Cytogenetic Family Trio (GCFT) study is the first to obtain blood samples from a
137 group of British NT veterans and their families for the purpose of identifying genetic and/or
138 chromosomal alterations in offspring that may have arisen as a consequence of historical paternal
139 exposure to ionising radiation (Rake et al. 2022). We have previously reported on cytogenetic
140 findings using multiplex fluorescence in situ hybridisation (M-FISH) to assess historical radiation
141 exposure in NT veterans (Lawrence et al. 2024), as well as whole-genome sequencing (WGS)

analyses to determine germline mutation frequencies (Moorhouse et al. 2022). Our findings are largely reassuring in that for the vast majority of NT veterans sampled we find no cytogenetic evidence of radiation exposure above background levels, and no association between paternal chromosomal aberration burden, germline mutation frequency, and self-reported concerns about adverse health outcomes in family members (Lawrence et al. 2024). However, a small number of families—representing both control and NT families—did exhibit a weak statistical relationship between a specific sub-type of paternal chromosomal aberration, known as complex chromosome aberrations (suggestive of internalised radionuclide contamination), and a corresponding germline mutation pattern subtype, referred to as mutation signature SBS16 (Moorhouse et al. 2022). Complex chromosome aberrations are defined as any chromosome exchange involving 3 or more breaks in 2 or more chromosomes.

Here we report the final phase of the GCFT study, undertaken to examine for any chromosome constitutional disorders and/or any cytogenetic features consistent with a genomic instability phenotype within adult children born to NT veterans. We also aimed to identify any transgenerational relationships in unstable aberration burdens, including within the subsets of families previously identified.

MATERIALS AND METHODS

Study participants and sampling

The study adhered to UK ethical standards and was approved by the UK Health Research Authority (17/LO/0273). Blood samples were obtained as part of the Genetic and Cytogenetic Family trio (GCFT) study from the NT-control family trios of military men (veteran father, mother, child) who were enrolled in the 'UK nuclear test veterans' cohort (Rake et al. 2022). In brief, responding

veteran couples were asked to involve their first child conceived after the veteran's last test site visit. Children with prior chemotherapy for cancer, cytotoxic chemotherapy (such as methotrexate for rheumatoid arthritis), or radiation treatment for any reason, were excluded from the study. This is because such treatments can lead to genetic damage that would interfere with interpretation of the results. After informed consent, sampling kits were sent to families with a request for their GP to collect whole blood and ship to Brunel University of London within 24 hours of sampling. All blood samples were processed on arrival and stored in compliance with Human Tissue Authority guidance. Further details of the GCFT study are given by Rake et al, (Rake et al. 2022).

Cell culture

Blood samples were cultured for the collection of metaphase cells for cytogenetic assessment. For conventional Giemsa staining, 0.4 ml of whole blood was inoculated into 3.6 ml PBMAX Karyotyping Medium with 10µM 5-bromo-2'-deoxyuridine and 10 µl/ml heparin. Cells were incubated at 37°C (95% air/5% CO_2) for a total of 50 h to collect 1st *in vitro* cell division metaphase cells. To arrest cells in metaphase, 50 µg/ml of Colcemid KaryoMAX, was added 4 hrs before harvest.

For G-band analysis, cultures were synchronised with thymidine and deoxycytidine and incubated for a total of 72 h. Colcemid (0.05-0.5ug/ml) was added 25 minutes before harvest.

For both, cells underwent hypotonic treatment (0.075M KCl, 8 mins at 37°C) and repeatedly fixed in ice-cold 3:1 methanol acetic acid until clear, before being stored at -20°C.

Cytogenetic analysis

Harlequin

Slides were Harlequin stained to confirm first-division cells. For this, slides were aged (90°C

for 40 minutes), immersed in Hoechst solution (20µl/ml of Hoechst in dH_20 for 10 minutes), UV-exposed (CL-1000 Ultraviolet Crosslinker) in 2XSSC for 60 minutes, rinsed and Giemsa stained (5% Giemsa, 5 minutes in pH6.8 buffer). Up to 5% second cell divisions was deemed acceptable.

≥200 metaphases/sample were analysed blind by two independent scorers using Zeiss brightfield microscopy (X100). The number of chromosomes and any structural aberration within each metaphase was recorded. Chromosomal abnormalities included dicentrics, double minutes, fragments, rings and discontinuities. Chromatid abnormalities included fragments, breaks, gaps and exchanges.

G-band

Slides were aged (92°C, 40 minutes) immersed in HBSS for 1 minute and treated with Trypsin (0.25%, time dependent on sample) before being stained with Giemsa (5% in pH6.8 buffer for 4-5 minutes). ≥15 metaphases/sample were visualized (x63 oil immersion, Axioplan 2 imaging Zeiss microscope), imaged (Ikarus MetaSystems software) and processed using the Ikarus karyotyping tool. The karyotype for each cell was notated as described in the International System for Human Cytogenetic Nomenclature (ISCN, 2009).

Statistical analysis

Frequencies of unstable chromosome- and chromatid-type aberrations in children of NT veterans and control veterans—both overall and within specific subgroups—were compared using the Kruskal–Wallis test or, where appropriate, Fisher's exact test. For matched data (e.g. father–child pairs), the Wilcoxon Signed-Rank Test was applied. P-values were adjusted for multiple comparisons using the Holm method (step-down Bonferroni procedure).

To account for varying cell counts and confounders, logistic regression models were used to examine the association between chromosome aberration endpoints, a binary "exposure" variable (e.g. representing potential paternal radiation exposure), and additional covariates (e.g. confounders). Overdispersion was accounted for using Williams' method, which estimates a dispersion parameter from the data to appropriately scale the standard errors of the regression coefficients. The models used a logit link function, defined as:

$$logit(p/(1-p)) = \beta_0 + \beta_1 X + z'\theta \quad (1)$$

where p is the probability of observing the event (e.g. a chromosome aberration), β_0 is the intercept parameter, β_1 is the coefficient for the primary predictor variable X ("exposure" variable), and z the vector for covariates with corresponding coefficients in vector θ. Covariates were selected on an endpoint-specific basis. In the case of multicollinearity, the covariate with the most biologically plausible link to both exposure and outcome was retained. To mitigate small sample bias, Firth's penalized maximum likelihood estimation was applied. Model adequacy was evaluated using the Hosmer–Lemeshow goodness-of-fit test. More complex models (nonlinearity, interaction) were ruled out due to the small sample size and the likelihood of overfitting.

Father–child associations were evaluated by including the father's aberration frequency ($Freq_{father}$) as a predictor term in the model equation (1), with the total number of paternal cells analysed used as a weighting factor, and the dependent variable p referring to the child's aberration frequency:

$$logit(p/(1-p)) = \beta_0 + \beta_1 Freq_{father} + \beta_2 X + z'\theta \quad (2)$$

This analysis proceeded in two steps. First, a basic model was fit to all 57 family data sets without including the variable X, to assess four potential patterns of association: (i) no association (β_0, β_1 not statistically significant), i.e. unstable chromosome- or chromatid-type aberrations between children

and fathers are unrelated, (ii) constant offset between generations (β_0 significant only), i.e. suggesting a "technical" factor responsible for difference, (iii) linear relationship without offset (β_1 significant only), and (iv) both linear trend and baseline difference (β_0 and β_1 significant).

In the second step, a binary variable *X* (e.g., cohort status, NT service status, reported family health outcomes etc) was added. If the inclusion of *X* improved model fit and the corresponding coefficient β_2 was significant, the variable was deemed relevant for explaining the variation in child aberration frequencies. The coefficient β_2 quantified the average effect of *X* on child outcomes.

To ensure robustness, sensitivity analyses using bootstrap resampling were performed in all regression models to assess the impact of outliers and high-leverage points. Any significant results not supported by sensitivity analyses were flagged as "borderline." A two-sided p-value < 0.05 was considered statistically significant. All analyses were conducted in SAS version 9.3 (SAS Institute, Cary, NC, USA).

RESULTS

Cohorts recruited

Whole blood samples were received from 86 (45 NT and 41 control) adult children born to veteran servicemen from the army, RAF and Royal Navy. Blood samples were processed immediately upon receipt over a 3-year period (arrival periods of NT and control samples were similar over this timeframe). The NT children comprised 25 females and 18 males, and the control group included 21 females and 18 males, with an average age of 51 years for both groups (Supplementary Table 1). Sex information was unavailable for two NT and two control children. The study criteria requested the recruitment of the first child conceived after the veteran returned from their last NT site. In

instances where this was not possible, such as not being alive, unwilling to participate or living abroad, then the next born child was contacted. In the NT cohort, 82% of children recruited were first-born and 18% were second-born, whereas this was 61% and 29% respectively for the controls (10% third or more born). No differences were observed in the total number of children conceived per family between NT and control cohorts. The average interval from potential radiation exposure to conception among the NT veterans was 7 years (range 0-33).

No constitutional chromosomal abnormalities detected in a cohort of NT children.

A total of 76 samples were stained for G-band analysis to identify any constitutional chromosomal aberrations, if present. The majority of cells analysed were between 350-550 banding resolution ("ISCN An International System for Human Cytogenetic Nomenclature" 2024). 10 samples either did not culture or were technical fails.

We found no evidence of any constitutional chromosomal aberrations amongst adult children born to NT veterans, with all displaying a normal constitution of either 46,XX or 46,XY. One sample from the control cohort exhibited a constitutional Robertsonian translocation involving chromosomes 13 and 14, present in all karyotyped cells [n = 15]. The same translocation was identified in the veteran father (Lawrence et al. 2024), confirming it to be familial in origin. Robertsonian translocations are phenotypically normal and those involving chromosomes 13 and 14 are the most common chromosomal rearrangements observed in humans (Wiland et al. 2020). All other control samples displayed a normal chromosomal constitution.

Sub-clonal aneuploidy is defined as two or more cells with the same additional chromosome or three or more cells with the same missing chromosome. Evidence of sub-clonal aneuploidy was observed in two samples in the controls (45,X,-X [3]) and (47,XXX [2]) and one sample within the NT cohort

(45,X,-X [3]) (N.B Square brackets indicate the number of cells the aneuploidy was identified in). These observations all involved chromosome X in women which is a phenomenon known to be associated with ageing (Russell et al. 2007; Machiela et al. 2016). The ages of these three individuals ranged between 53-56 years.

Unstable chromosome aberrations in cohorts of adult children born to control and NT veterans.

Blood samples were cultured to collect 1st division metaphase cells and Giemsa stained for brightfield analysis to detect numerical and unstable structural chromosomal aberrations and, chromatid aberrations as cytogenetic markers of genomic instability. A total of 5897 cells from 33 NT children and 3759 cells from 26 control children were scored. An abnormal cell was defined (and identified) as one containing at least one structural or numerical aberration of any type.

After adjusting for potential confounders, no statistically significant differences were observed in the total frequencies of unstable structural chromosome aberrations between NT and control children (total frequency/100 cells of 1.63±0.28 for control and 1.61±0.24 for NT, respectively; Figure 1 and Supplementary Table 2). Similarly, there was no significant difference in the frequency of chromatid aberrations (total frequency/100 cells of 4.36±0.62 for controls vs. 4.68±0.69 for NT). Analysis of the frequency of aneuploid cells, defined as the loss or gain of one or more chromosomes, also revealed no significant difference between the NT (8.40±0.69/100 cells) and the control (6.42±0.99/100 cells) cohorts (Supplementary Table 3). This was the case also when aneuploidy accompanied by structural chromosome aberrations, or aneuploid with chromatid aberrations, was considered (Supplementary Table 3). The only difference observed was in the frequency of chromosome aberration subtype - chromosome discontinuities - which on average was

nearly threefold higher in children born to NT veterans (0.40±0.09) compared to controls (0.15±0.08) (Supplementary Table 2).

INSERT FIGURE 1 HERE

Genomic instability in a small sub-set of children born to NT veterans

The rationale for exploring for cytogenetic indicators consistent with genomic instability in children of NT veterans, despite the children themselves not being directly exposed to radiation, is to investigate whether any observed instability might reflect transgenerational effects from paternal exposure at NT sites. As has been shown (Kendall et al. 2004; Lawrence et al. 2024), it is likely the majority of the veterans received insufficient radiation dose to cause harm in themselves or be detectable above background levels. Consequently, treating all NT veterans as a homogenous group may obscure potential associations, if present, especially if only a subset of veterans were meaningfully exposed. To address this, and be consistent with the approach taken by Lawrence et al (Lawrence et al. 2024) in their analysis of structural chromosome aberrations in the NT veterans, we stratified the NT children based on two factors: (i) the veteran father's assigned 'potential for exposure' rank , and (ii) the geographical location of their father's nuclear test deployment (Christmas Island, on board ships and Maralinga). To elaborate, the use of a 'potential for exposure' ranking system was necessary given most NT veterans in the UK NTV cohort have no recorded dose (only a limited number were issued with film badges) and no measurement for internal contamination took place. For the GCFT study and as described in Rake et al., and Lawrence et al., the NT veterans were assigned (blind to any results) to a simple three-point rank for the potential of internal/external exposure based on veterans testimony and operation

information drawn from the UK NTV cohort database provided by PHE (now UK HSA) (Rake et al. 2022; Lawrence et al. 2024). Each case was *a priori* assumed to be in the lowest rank, and a higher rank allocated only if sufficient information was given to suggest a higher likelihood for radiation exposure. A defined role in a contaminated or forward area (e.g. aircraft sample retrieval/cleaning) undertaken more than once was considered a higher exposure potential, with activities immediately and up to 3 months after the test where dose and dose rates would be expected to be highest (higher rank) distinguished from those carried out at any time from at least 3 months after the test (medium rank). Geographical location of the test site was also considered relevant. For instance, the potential for a veteran working in a 'forward area' at Maralinga to be exposed to both external and internal radiation was assumed to be higher than a veteran who witnessed an atmospheric test in the safety zone (~40 km from the blast) on Christmas Island (Rake et al. 2022). Thus, although this 'potential for exposure' ranking cannot be considered a substitute for recorded radiation dose, it was employed as a proxy from which sub-groups of the NT cohort could be defined.

When analysis was stratified by these NT veteran subgroups, the elevated frequency of chromosome discontinuities remained statistically significant only among adult children of veterans who served at Christmas Island with exposure potential ranks 1+2 (veterans predominantly RAF but all services represented). In this subgroup, the mean frequency was 0.54±0.19 per 100 cells, significantly higher than that observed in the control group (0.15±0.08/100 cells), and slightly above the average for the entire NT cohort (0.4±0.09/100 cells). Children of veterans classified as exposure rank 3 at Christmas Island (veterans predominantly RAF but all services represented) showed a comparable mean frequency (0.5±0.32/100/cells), however, the small sample size in this group likely limited the statistical power to detect a significant difference (Supplementary Table 2).

An elevated burden of chromatid-type aberrations was identified in a small subgroup of children (N = 4) whose fathers served on board ships (all Royal Navy personnel) and were classified in the highest exposure category (rank 3). This group exhibited a mean frequency of 7.8±4.01/100 cells, which was statistically significant when compared to controls (p=0.02, logistic regression; (Figure 1, Supplementary Table 2). A similar trend was observed for aneuploid cells with additional chromatid aberrations, which were increased in the same subgroup (1.75±1.18/100 cells compared to 0.64±0.17/100 cells in the control, p = 0.02 logistic regression) (Supplementary Table 3), suggesting potential genomic instability associated with paternal service on-board ships. However, while these differences were statistically significant in the initial statistical model, they did not remain robust under sensitivity analyses due to the small sample sizes. Thus, these observations should be interpreted with caution and warrant validation in larger cohorts.

Genomic instability within the sub-set of families enriched with germline mutation pattern SBS16

To further examine potential transgenerational effects, we examined the relationship between the frequencies of unstable aberrations in veteran fathers, as measured by M-FISH in Lawrence et al. (Lawrence et al. 2024), and the frequencies of unstable structural aberrations observed in their adult children. Frequencies were plotted and analysed separately for control and NT family cohorts (Figure 2). Although a slight upward trend is apparent within NT families, no statistically significant associations were detected between the paternal unstable aberration burden and the frequency of either chromosome- (Figure 2A) or chromatid-type (Figure 2B) unstable aberrations in their adult children.

INSERT FIGURE 2 HERE

To investigate for any relationship between aberration frequencies in veteran fathers and their adult children across cohort subgroups, we applied a statistical modelling approach. As an initial step, Wilcoxon Signed-Rank Tests were applied within specific subgroups based on (i) father's cohort status, including NT subgroups (Table 1), and (ii) previously reported family characteristics, such as enrichment of germline mutation signature SBS16 or self-reported health effect in the offspring (Moorhouse et al. 2022; Rake et al. 2022) (Table 2), in order to assess whether mean aberration frequencies differed significantly between fathers and children.

The subsequent modelling of father–child aberration associations, including the potential influence of stratifying variables (i.e. subgroups), applied a basic model (as described in Methods) to all 57 matched father–child data pairs. This was done initially without stratification, and separately for unstable chromosome or chromatid-type aberrations.

For unstable chromosome aberrations, no significant positive or negative association was detected between the individual aberration frequencies of fathers and their children in the basic association model, although the average aberration frequency was slightly higher in children than in their fathers ($p < 0.01$). When potential confounders were included, only a history of CT scans in the children reached statistical significance. Further inclusion of stratification variables (NT sub-groups, Table 1) did not yield any statistically significant improvement to the base model. Similarly, offspring self-reported health parameters and SBS16 status showed no significant association for the father–child frequency association (Table 2).

When analysing chromatid aberrations**,** the basic model revealed a highly significant baseline shift, indicating that aberration frequencies measured in adult children were consistently higher than

those in their fathers ($p < 0.01$; Tables 1 and 2). Additionally, a significant positive trend was observed whereby higher aberration frequencies in veteran fathers were associated with higher frequencies in their children, suggesting a potential transgenerational relationship. Among the limited confounding variables available for evaluation, only the number of reported X-rays in children showed a significant (inverse) association. Inclusion of stratification and confounder variables revealed that SBS16 mutation status had a modest but statistically significant effect ($p = 0.02$), improving overall model fit. Specifically, for a given veteran father aberration frequency, predicted values were higher in children from the high-SBS16 group compared to those from the low-SBS16 group (Table 2). However, the statistical significance for the general positive trend in father-child aberration frequencies (i.e. $\beta_1 > 0$) was attenuated ($p = 0.06$), suggesting that the trend observed in the unstratified model may have been primarily driven by SBS16 mutation status. None of the health-related stratifications, such as reported family health concerns; congenital conditions, or cancer diagnoses, significantly improved the model (Table 2). Likewise, neither NT veteran cohort status nor radiation exposure-related subgroups had any measurable effect on the father–child aberration frequency association (Table 1).

INSERT TABLE 1 HERE

INSERT TABLE 2 HERE

DISCUSSION

Given the anecdotal evidence of increased adverse health effects in NT offspring and the range of

conditions reported, this final phase of the GCFT study aimed to investigate the presence of any chromosome constitutional disorders and/or cytogenetic indicators of genomic instability in adult children that may be of relevance. Notably, within the recruited GCFT cohort, a significantly higher number of NT families self-reported congenital abnormalities in their children or grandchildren compared to the control group (Rake et al. 2022). This likely reflects heightened concern within the NT population and may have served as a motivating factor for participation.

Congenital anomalies, defined as conditions present at birth, include disorders such as neural tube defects and congenital heart defects. Although approximately 50% of these lack a specific cause, some may arise from chromosomal abnormalities. In this study, we examined adult children born to nuclear test (NT) veterans for constitutional chromosomal abnormalities, finding all individuals to exhibit apparently normal karyotypes—46,XX or 46,XY—including those from families who self-reported adverse health effects. High-resolution G-banding was used; however, it is acknowledged that most constitutional abnormalities identified in adults likely involve small structural alterations or balanced exchanges which may escape detection. In light of this, and for completeness, we re-examined WGS germline data (Moorhouse et al. 2022) but again found no evidence of genetic variants at loci potentially relevant to the conditions reported at the time of the interviews (Rake et al. 2022). An objective of the GCFT study was to recruit and obtain blood samples from the first-born child conceived after the veteran's last test site participation (Rake et al. 2022). This was to both minimise the interval between potential paternal exposure and conception (time is one explanation for the differences seen between species where unlike human data, animal data shows strong evidence for radiation effects across the generations (Little et al. 2013)), and to reduce bias. However, consequently, most health conditions reported were present in siblings rather than in the sampled child. Nonetheless, no constitutional abnormalities were observed in any individual from

435 the NT cohort. Additionally, there was no evidence of an association between the chromosomal
436 aberration burden in veteran fathers and the presence of these reported health concerns in children
437 (Table 2; (Lawrence et al. 2024)).

438 Somatic (non-clonal) chromosomal aberrations are induced throughout life due to various lifestyle
439 and environmental factors. Aberration types that are stable through cell division are expected to
440 accumulate over time, contributing to an increased aberration burden with age (López-Otín et al.
441 2013). The technique used in this study—conventional Giemsa—effectively detects unstable
442 chromosomal and chromatid aberrations, which typically do not accumulate with age. Accordingly,
443 an increased occurrence may indicate underlying genomic instability. Overall, we found only
444 limited evidence of genomic instability in adult children of NT veterans compared to controls.
445 Specifically, a higher frequency of chromosome discontinuities (i.e., chromosome breaks) among
446 children of Christmas Island veterans (exposure ranks 1+2) and, elevated chromatid aberrations—
447 both in complete and aneuploid cells—in adult children of veterans who had served on ships
448 (exposure rank 3). The statistical support for this latter finding was weak however, which crucially,
449 limits its interpretability.

450 Although adjusted for potential confounders, limitations in the available data for adult children and
451 the potential for recall bias should be noted. As described in Rake et al, data were collected via
452 telephone interview at recruitment, providing self-reported numbers of X-ray, CT, and other
453 diagnostic scans (Rake et al. 2022). No information was collected on occupational exposures,
454 smoking history, or other lifestyle factors, and details such as the anatomical site of the scan were
455 not recorded. The variables included as potential confounders in the statistical models used here
456 were: (i) maternal and paternal age at conception, (ii) interval (in years) between the father's last
457 potential radiation exposure and conception (NT only), (iii) number of X-rays (none, 1–5, 6–10,

458 and >10), (iv) CT scans (yes/no), (v) other diagnostic scans (yes/no), and (vi) child sex (Table S1).
459 Among these, a history of CT scans in the adult child emerged as a strong predictor of elevated
460 chromosomal aberration frequencies. Conversely, a higher number of reported diagnostic X-rays
461 was inversely associated with chromatid-type aberration frequency, suggesting a negative
462 relationship. This aligns with chromosome-type — rather than chromatid-type aberrations — being
463 more typical of ionising radiation, including from diagnostic imaging, and may also explain the
464 observed increase in chromosome discontinuities (Table S2) (Bhatti et al. 2008). By contrast, the
465 elevated (albeit statistically weak) frequency of chromatid-type aberrations observed in a small
466 group of adult children of ship-based veterans is consistent with a phenotype of ongoing genomic
467 instability. This finding is based on a very small sample (N = 4) and cannot be generalised to other
468 children born to ship-based NT veterans. Furthermore, veteran fathers' lifestyle and occupational
469 confounding exposures were not considered here meaning we cannot rule out any effect from
470 agent/s other than ionising radiation. Indeed, given the lack of actual dosimetry, we cannot formally
471 associate any observations reported here to paternal exposure to ionising radiation. In stating this, it
472 is pertinent to note that no confounders were found which explain the elevated chromosome
473 aberrations detected in NT veterans themselves (Lawrence et al. 2024).

474 When matched veteran father–adult child pairs were examined, we observed a non-significant
475 upward trend between paternal unstable aberration burden and the frequency of either unstable
476 chromosome- or chromatid-type aberrations in the adult children of NT, but not control, families.
477 To investigate further, we applied a more complex statistical modelling approach, stratifying the
478 data by paternal cohort subgroup and previously reported family characteristics, such as enrichment
479 of mutation signature SBS16 or self-reported health effects in offspring (Lawrence et al. 2024).
480 This analysis revealed a significant positive trend for chromatid aberrations—but not chromosome-

type aberrations—suggesting that higher aberration frequencies in veteran fathers were associated with higher frequencies in their children inferring a potential transgenerational effect. Notably, within the small group of families characterised as high-SBS16, this association was stronger: a given aberration frequency in the veteran father predicted a higher aberration frequency in the child compared to the low-SBS16 group (Table 2). This raises the possibility that the overall association may be primarily driven by SBS16-associated mutation processes or by another unidentified factor within this subgroup.

In Moorhouse et al. (Moorhouse et al. 2022), we reported an enrichment of germline SNV mutations associated with mutation signature SBS16 in a small group of eight families (2 controls and 6 NT; subsequently termed as the high SBS16 subgroup). SBS (and other) signatures are detectable 'patterns' of mutation which remain in the DNA sequence following damage and repair. SBS16 is thought to arise via transcription-coupled nucleotide excision repair of bulky DNA lesions (Alexandrov et al. 2013; Alexandrov et al. 2020) and although the aetiology remains unknown it is seen in alcohol-associated liver cancers (Letouzé et al. 2017). In Lawrence et al., (Lawrence et al. 2024), we found a weak statistical association between the high-SBS16 subgroup and complex chromosomal aberrations, which are potentially indicative of internalised long-lived radionuclide exposure. Although interpretation is complicated by the presence of control families, this raised the possibility that SBS16 could reflect molecular processing of radiation-induced damage and, as such, serve as a transgenerational biomarker of paternal radiation exposure.

Our current findings add to this by revealing a significant positive association between increased unstable aberration burden in veteran fathers—including unstable complex aberrations —and increased chromatid aberration frequencies in their adult children within the high-SBS16 subgroup. Although this observation implies a relationship between cytogenetic markers of radiation exposure

in the father (complex aberrations) and markers of effect (genomic instability) in their adult child, cautious interpretation is required. The many caveats already highlighted (small subgroups, presence of controls in subgroup, lack of radiation dosimetry), all downplay the confidence of this finding. Indeed, a pilot study measuring $^{239/240}$Plutonium in urine for seven of the eight veterans in this high-SBS16 sub-group found both mass and activity of these long-lived radioisotopes to be below the limit of detection (Jerome et al., in preparation, personal communication). What can be stated is that four of the eight families within the high-SBS16 subgroup include veterans classified in the highest exposure category (rank 3), including two with recorded doses of <1.5 mSv. Additionally, three of the NT and control families in this subgroup self-reported a congenital condition.

In the broader context of concerns raised by NT families regarding adverse health outcomes, we observed no significant associations between any reported health-related variables and unstable aberration burden in either veteran fathers or their adult children. As mentioned above, the interval between exposure and conception may be relevant, given that sperm maturation from sperm stem cells in humans is ~64 days (Johnson et al, 2000). Thus, directly exposed sperm cells have only this timeframe to fertilise an egg (or for a veteran to conceive) for any effects in the germline to manifest, although this would be longer if damage is within the stem cell pool, given their ability to self-renew. Most of the children sampled here were conceived months or years after their veteran father's return from the final test site, with an average lag of seven years. This may have impacted the study's ability to detect transgenerational effects. However, and similar to Yeager et al., 2021 who observed no increase in germline mutations in the year following the Chernobyl accident (Yeager et al. 2021), we found no trend with respect to chromosome or chromatid aberration

frequency, sub-group status (high-SBS16 or adverse health in family) and, interval between last test site and conception.

In conclusion, we found no evidence of constitutional chromosomal abnormalities in adult children born to NT veterans, and no evidence of genomic instability in the vast majority—including those from families who self-reported adverse health effects in one or more children. These findings are consistent with our previous findings, which showed no relationship between paternal chromosome aberration burden, germline mutation frequency, and self-reported concerns about adverse family health outcomes (Lawrence et al. 2024) and should reassure concerned families, as we observed no genetic effects or elevated aberration burdens in veteran fathers attributable to historical participation at nuclear test sites. The previously reported weak association between complex chromosomal aberrations in veteran fathers and an over-representation of germline mutations with the mutation signature SBS16 now appears to be linked with potential transgenerational genomic instability in a small subset of families. While the data is limited and preliminary, these multiple observations in the high-SBS16 subgroup provide a rationale for further investigation including in other human populations with known radiation exposure and estimated doses—especially those internally contaminated with alpha-emitters. These results underscore the importance for future genomic studies to move beyond mutational burden and examine the full spectrum of genomic (and emerging epigenomic) alterations. Finally, the GCFT study highlights the value of trio-based designs for assessing genetic effects of preconceptional radiation exposure. Such studies are increasingly important given rising medical radiation use, the threat of nuclear conflict, and potential population-level exposures to ionising radiation.

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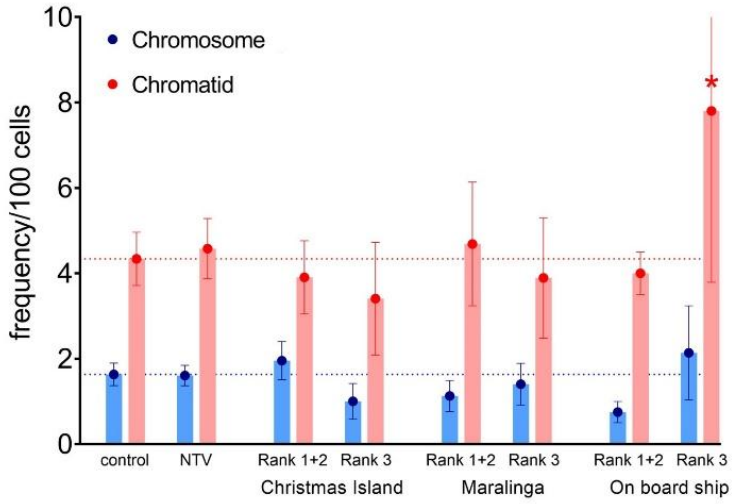

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691 **Figure 1: Unstable structural chromosome and chromatid-type aberrations in adult children**
692 **born to control and NT veterans.** Frequency of aberration types per 100 cells are grouped
693 according to the veteran father's status (control (N=26) and NT (N=33) with NT subgroups
694 reported as geographic location (Christmas island (N=17), Maralinga (N=13) or on board a ship
695 (N=6) at time of test) and, NT veteran's potential for radiation exposure ranking as lower (1),
696 medium (2) or higher (3) potential (Rake et al. 2022). Chromosomal-type aberrations (dicentrics,
697 double minutes, fragments, rings and discontinuities) and chromatid-type aberrations (fragments,
698 breaks, gaps and exchanges). Error bars represent the SEM (for N>4 and with participant as
699 statistical unit). Where statistical analysis was possible; *significance for difference ($p<0.05$,
700 confounder-adjusted logistic regression model accounting for overdispersion using the Williams
701 method).

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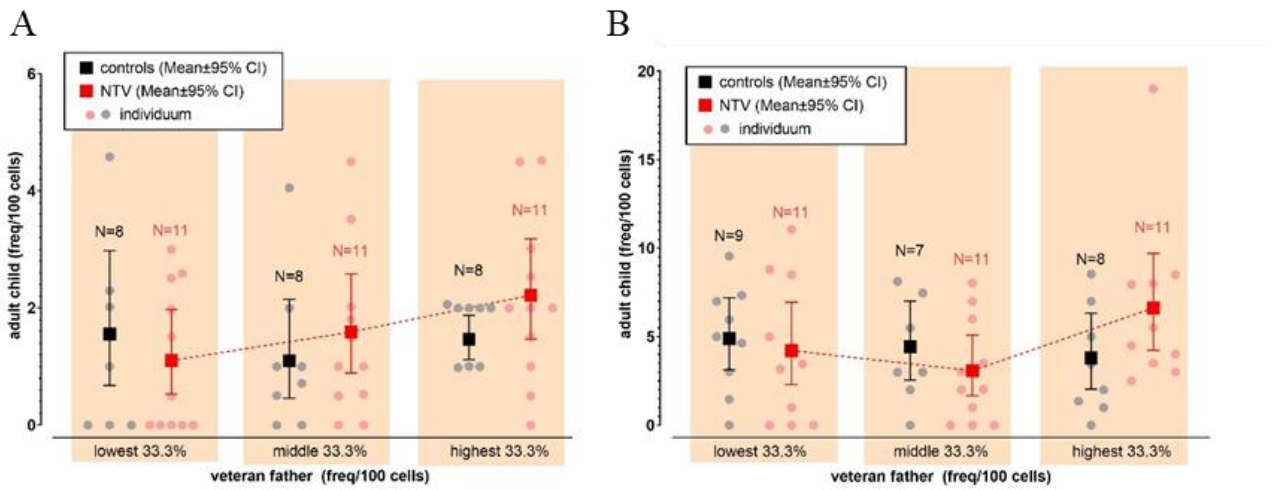

Figure 2: Association between veteran father and adult child aberration frequencies. Analysis was carried out for all veteran father-adult child samples where both veterans M-FISH data (Lawrence et al. 2024) and adult child Giemsa solid stained data (as reported here) was available (24 control and 33 NT). (A) Unstable chromosome aberrations and (B) chromatid aberrations. For (A) veteran fathers, unstable chromosome aberrations represent the total of the dicentric equivalent plus all fragments from simple, complex or breaks as detected by M-FISH while (B) chromatid aberrations were determined from the DAPI stained metaphase for each cell (Lawrence et al. 2024). For both (A) and (B) veteran fathers' frequencies are categorized into tertiles, representing the lowest, medium, and highest thirds (each comprising approximately 33% of the data). For the adult children, the total aberration frequencies shown with mean values and 95% confidence intervals (CI) estimated using logistic regression model, accounting for overdispersion using the Williams method.

Table 1: Unstable aberrations in veteran fathers and their adult children, stratified by control and NT sub-groups

	Veteran father (frequency/100 cells)[1]					Adult child (frequency/100 cells)			Germline SNV mutations
Cohort	Cells	Dicentric equivalent	Total fragments	Total unstable chromosome	Total chromatid	Cells	Total unstable chromosome	Total chromatid	SBS16[2]
Control (N=24)	4876	0.26±0.09	1.07±0.27	1.33±0.30	1.10±0.31	3469	1.41±0.24	4.23±0.60**	15.59
NT (N=33)	6283	0.43±0.11	1.15±0.25	1.58±0.33	1.23±0.20	5897	1.58±0.24	4.58±0.71**	19.75
Veteran Fathers potential for exposure ranking‡									
Christmas Island, Rank 1+2 (N=12)	2344	0.35±0.14	1.25±0.55	1.60±0.67	1.07±0.31	2067	1.87±0.47	3.91±0.86*	16.66
Christmas Island, Rank 3 (N=5)	740	0.17±0.17	1.07±0.49	1.24±0.53	1.20±0.42	799	1.00±0.42	3.41±1.32	33.04
On board ship, Rank 1+2 (N=2)	341	0.587	3.226	4.012	1.147	400	0.25	4	22.16
On board ship, Rank 3 (N=4)	856	0.467	1.051	1.280	1.511	688	2.137	7.804	26.05
Maralinga, Rank 1+2 (N=8)	1608	0.55±0.35	1.12±0.47	1.66±0.78	1.28±0.42	1442	1.13±0.36	4.69±1.45*	20.32
Maralinga, Rank 3 (N=5)	872	0.65±0.30	1.02±0.54	1.67±0.84	1.10±0.52	1001	1.40±0.49	3.89±1.41	17.57

Includes all families for which both M-FISH data from the veteran fathers [1](Lawrence et al. 2024) and solid stain data from their adult children were available. ‡Includes veterans who attended more than one location. Mean ± SEM frequency of aberrations per cell (calculated where N > 4, using participant as the statistical unit). [2] Number of germline mutations assigned to SBS16 reported in (Lawrence et al. 2024) using data from (Moorhouse et al. 2022). *p-value<0.05 (Wilcoxon Signed-Rank Test), **p-value<0.01 (Wilcoxon Signed-Rank Test).

Table 2: Unstable aberrations in veteran fathers and their adult children, stratified by family groups of interest

	Veteran father (frequency/100 cells)[1]					Adult child (frequency/100 cells)			Germline SNV mutations
Cohort	Cells	Dicentric equivalent	Total fragments	Total unstable chromosome	Total chromatid	Cells	Total unstable chromosome	Total chromatid	SBS16[2]
SBS16[1]									
Families with >40 SNV mutations allocated to SBS16 (N=8)	1312	0.50±0.34	2.11±0.64	2.61±0.74	2.07±0.57	1617	1.88±0.47	7.75±1.97*	46.06
Families with <40 SNV mutations allocated to SBS16 (N=49)	9849	0.34±0.07	0.95±0.18	1.29±0.23	1.03±0.17	7749	1.44±0.19	3.89±0.42**	12.22
Families who self-reported health effects in their offspring[3]									
None (N=40)	7707	0.38±0.09	1.16±0.23	1.54±0.28	1.10±0.19	6286	1.56±0.20	4.89±0.46**	19.49
Effect (N=17)	3454	0.31±0.13	1.01±0.28	1.32±0.40	1.36±0.37	3080	1.37±0.37	3.37±1.14*	14.77
Congenital (N=10)	2073	0.39±0.17	1.18±0.38	1.57±0.53	1.60±0.43	1777	1.68±0.54	3.71±1.81	21.02
Non-cancer (N=52)	759	0.39±0.08	1.17±0.19	1.57±0.24	1.13±0.17	902	1.01±0.18	4.46±0.51**	4.62
Cancer (N=5)	1231	0	0.52±0.40	0.52±0.40	1.61±0.92	899	1.32±0.53	4.14±1.41	6.96

Includes all families for which both M-FISH data from the veteran fathers [1](Lawrence et al. 2024) and solid stain data from their adult children were available. Mean ± SEM frequency of aberrations per cell (calculated where N > 4, using participant as the statistical unit). [2] Number of germline mutations assigned to SBS16 reported in (Lawrence et al. 2024) using data from (Moorhouse et al. 2022). [3](Rake et al. 2022). *p-value < 0.05 (Wilcoxon Signed-Rank Test), **p-value < 0.01 (Wilcoxon Signed-Rank Test).

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AUTHOR CONTRIBUTIONS: RA acquired the funding, conceptualized the study and supervised the work; RA and MS devised the methodology; CR recruited the cohort, JS, SE, CS, MS and RA performed the sample processing and analysis; RA performed project administration; RA and JS wrote the manuscript with contributions from MS, SE, CR and CS.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AVAILABILITY OF DATA AND MATERIALS

752 The dataset generated during this current study are available as supplementary materials.

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754 **ETHICAL APPROVAL AND CONSENT TO PARTICIPATE**

755 The Genetic and Cytogenetic Family Trio study and all methods conducted in this manuscript were
756 performed in accordance with the relevant guidelines and regulations of the UK ethical framework
757 and were approved by the UK Health Research Authority (17/LO/0273).

758

759 **CONSENT TO PARTICIPATE**

760 Written informed consent was obtained from all subjects.

761 **SUPPLEMENTARY MATERIALS**

762 Supplementary Tables 1-3.
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