Official journal of
the Society for
Radiological Protection

PAPER • OPEN ACCESS

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To cite this article: Kirsty Josephine Lawrence *et al* 2024 *J. Radiol. Prot.* **44** 011501

IOP Publishing J. Radiol. Prot. 44 (2024) 011501 https://doi.org/10.1088/1361-6498/ad1743

Journal of Radiological Protection

OPEN ACCESS

RECEIVED
17 October 2023

REVISED
11 December 2023

ACCEPTED FOR PUBLICATION
19 December 2023

PUBLISHED
9 January 2024

PAPER

M-FISH evaluation of chromosome aberrations to examine for historical exposure to ionising radiation due to participation at British nuclear test sites

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Keywords: ionising radiation, nuclear test veterans, complex chromosome aberrations, family trio study, de novo germline mutations

Supplementary material for this article is available online

Abstract

Veterans of the British nuclear testing programme represent a population of ex-military personnel who had the potential to be exposed to ionising radiation through their participation at nuclear testing sites in the 1950s and 1960s. In the intervening years, members of this population have raised concerns about the status of their health and that of their descendants, as a consequence. Radiation dose estimates based on film badge measurements of external dose recorded at the time of the tests suggest any exposure to be limited for the majority of personnel, however, only ∼20% of personnel were monitored and no measurement for internalised exposure are on record. Here, to in-part address families concerns, we assay for chromosomal evidence of historical radiation exposure in a group of aged nuclear test (NT) veterans, using multiplex *in situ* hybridisation (M-FISH), for comparison with a matched group of veterans who were not present at NT sites. In total, we analysed 9379 and 7698 metaphase cells using M-FISH (24-colour karyotyping) from 48 NT and 38 control veteran samples, representing veteran servicemen from the army, Royal Airforce and Royal Navy. We observed stable and unstable simple- and complex-type chromosome aberrations in both NT and control veterans' samples, however find no significant difference in yield of any chromosome aberration type between the two cohorts. We do observe higher average frequencies of complex chromosome aberrations in a very small subset of veterans previously identified as having a higher potential for radiation exposure, which may be indicative of internalised contamination to long-lived radionuclides from radiation fallout. By utilising recently published whole genome sequence analysis data of a sub-set of the same family groups, we examined for but found no relationship between paternal chromosome aberration burden, germline mutation frequency and self-reported concerns of adverse health in family members, suggesting that the previously reported health issues by participants in this study are unlikely to be associated with historical radiation exposure. We did observe a small number of families, representing both control and NT cohorts, showing a relationship between paternal chromosome aberrations and germline mutation sub-types which should be explored in future studies. In conclusion, we find no cytogenetic evidence of historical radiation exposure in the cohort of nuclear veterans sampled here, offering reassurance that attendance at NTs sites by the veterans sampled here, was not associated with significant levels of exposure to radiation.

1. Introduction

The British government undertook a series of nuclear tests (NTs) at various sites in the South Pacific between 1952 and 1958. Associated with these atmospheric tests was an experimental programme, conducted largely at Maralinga in Australia, in which radioactivity was dispersed into the environment. This programme ended in 1963 although clean-up operations continued through to 1967 [1]. Additionally, UK personnel participated in a series of American tests based at Christmas Island in 1962. According to the Ministry of Defence (MoD) 22 347 veterans participated in at least one of these British and American tests of which ∼7000 were alive in 2017. Concerns were first raised in the early 1970s that the health of veterans of this testing programme and that of their children may have been adversely affected. Epidemiological studies examining mortality and cancer incidence in test veterans, carried out up to 1998, showed limited evidence of any detectable effect although this has since been revised to show a small excess in mortality ($RR = 1.02$, 90% CI 1.00–1.05, $p = 0.04$), associated with similar increased risks for both cancer and non-cancer diseases [2–4]. Despite this, questions as to whether veterans could have received sufficient radiation exposure to cause harm and, worry about potential genetic risk to future generations of any historical radiation exposure, persist [5].

Dose estimates for British NT veterans were based on film badge measurements of external dose, where available. According to National Radiological Protection Board-R214, only ∼22% of the entire population were monitored of which 8% (1804 participants) recorded a 'non-zero' dose, with 44 NT veterans categorized as receiving between 50 and 100 mSv and 36 as receiving a dose of >100 mSv [6]. Based this, the vast majority of NT veterans were exposed to no or low dose exposures only (low dose defined as less than 100 mSv). The primary health concern for individuals exposed to low-moderate doses is cancer although the debate for non-cancer diseases such as cardiovascular disease and cataracts arising after doses of less than 500 mSv, is ongoing [7]. The psychological impact of real and/or perceived low dose exposure is also of concern [5, 8]. In total, 759 NT veterans were identified by the UK MoD as potentially receiving higher doses to that recorded and categorized into 'special groups', such as those veterans who were involved in air plume sampling, cleaning of 'sampling' aircraft or, crew of HMS Diana who were tasked to sail through a nuclear plume [1]. Many of those present at test sites were involved in support roles, such as construction, transport or catering, however additionally, were directly involved with the actual tests, including working in contaminated areas in the days, weeks and months following each test. Such roles may not have been accounted for by the formal categorization into a special group. Fallout from atmospheric tests (e.g. GRAPPLE series in the South Pacific) and, from radioactivity which was dispersed into the environment during the Maralinga experimental programme in South Australia includes long-lived radionuclides such as Caesium-137, Strontium-90, Uranium-235/238, Plutonium-239, which if inhaled, ingested or otherwise internalized within the body would contribute to chronic radiation exposure with potential relevance for human health risks [9]. Apart from limited autopsy analysis there is no public record of any historical internal dose measurements. For further information on the British atmospheric and experimental testing programme and, on the potential sources and routes of exposure at the varying geographical sites, please refer to [10].

Ionising radiation induces DNA double strand breaks which are the critical lesion for the formation of structural chromosome aberrations [11]. Fluorescence *in situ* hybridisation based techniques, which 'paint' individual chromosomes, enable the detection of structural rearrangements such as reciprocal translocations and has been validated for use in the assessment of radiation doses [12, 13]. As reciprocal translocations are capable of long-term cellular transmission (in otherwise stable cells) their quantification can be informative of historical radiation exposures, including where many decades have passed [14–18]. Damage to DNA is acquired throughout life from a range of endogenous and exogenous sources however meaning that reciprocal translocations will accumulate with age, thus, their quantification reflects a lifetime of all exposures. With the application of M-FISH, where all chromosomes in the genome are uniquely 'painted', a much more complete picture of the complexity of chromosomal interchange 'patterns' are being revealed [19, 20]. Complex chromosome aberrations (rearrangements involving three or more breaks in two or more chromosomes) have been shown to be characteristically induced after exposure to low doses of high-linear energy transfer (LET) radiation, such as α-particle emitters [21]. The frequency and type of chromosome aberrations observed by multiplex *in situ* hybridisation (M-FISH) are thus informative of radiation exposure, dose and radiation quality.

The genetic and cytogenetic family trio (GCFT) study is the first study to obtain blood samples from a group of British NT veterans and their families [10]. The aim was to recruit NT veteran family trios (veteran, child, child's mother) who had 'special group' status and/or who had participated in two or more operations' including the GRAPPLE series and at Maralinga test sites', to ask whether heritable genetic effects could exist

due to historical participation in the British nuclear testing programme. The examination for any differences in the frequency and spectra of de novo germline DNA mutations compared to control veteran families is reported elsewhere [22], whilst future publications will report on the occurrence of chromosomal aberrations in 1st generation adult children of NT and control veterans. Here we report the M-FISH findings to ask firstly, if there is any cytogenetic evidence of historical radiation exposure in the NT veterans and secondly, if there is any relationship between the occurrence of chromosome aberrations in veteran fathers with the de novo germline mutations in these families.

2. Results

2.1. No difference in chromosome aberration frequencies between control and test veteran cohorts

Blood was received from 91 NT and control veterans and processed to collect 1st *in vitro* metaphase cells for analysis. Cultures from five veterans (1 NT and 4 control) either failed to culture or generate sufficient numbers of metaphase cells for M-FISH analysis. In total, we analysed 9379 and 7698 metaphase cells using M-FISH (24-colour karyotyping) from 48 NT and 38 control veteran samples, representing veteran servicemen from the army, Royal Airforce (RAF) and Royal Navy. The number of metaphase cells per sample ranged from 78 to 390 (median = 196); 18 samples had less than 150 cells analysed, with no significant differences detected between the cohorts (figure 1).

We observed stable and unstable simple- and complex-type chromosome aberrations in both NT and control veteran's samples, finding no statistically significant difference in yield of any aberration type between the two cohorts (figure 2, supplementary table 1, supplementary tables 6(a) and (b); $p > 0.2$ unless reported). Specifically, overall frequencies of 1.621 ± 0.167 and 1.585 ± 0.244 simple exchanges/100 cells (mean $\pm$ SEM), and 0.299 ± 0.075 and 0.351 ± 0.079 complex exchanges/100 cells were detected in the NT and control veteran groups, respectively. A total of 8 Robertsonian translocations were found (5 and 3 from NT and control veteran groups, respectively), including a constitutional Robertsonian rob(13;14) in the control group (supplementary table 1).

Recruitment of the NT veterans was group-matched with control veterans based on a number of criteria including age (79.9 and 80.3 years in the NT and control groups, respectively) (supplementary table 2). As shown in figure 3, we find the majority of veterans have similar translocation frequencies to that which is expected based upon their age with only 7 veterans (4 NT and 3 control) identified as having higher frequencies than expected (supplementary tables 3 and 4) [23]. As detailed in the Methods, of those NT veterans recruited, three had a record of dose however none of these individuals corresponded with the higher translocation frequencies observed.

Further, a comparison with related studies examining reciprocal translocation frequencies in NT test veterans (figure 4) shows our 24-colour (full genome) findings to be consistent with full-genome converted frequencies reported in French, but below that of the New Zealand, NT veterans.

The frequencies of dicentrics are, as expected, lower than that of reciprocal translocations with no difference observed between the two veteran groups (0.149 ± 0.053, and 0.143 ± 0.041) dicentrics/100 cells in NT and control, respectively) (supplementary table 1).

For complex chromosome aberrations, indicative of exposure to low doses of high-LET radiation, or high doses of low-LET radiation, frequencies of 0.299 ± 0.075 (range 0–2.78)/100 cells and 0.351 ± 0.079 (range 0–1.69) were seen in both NT and control veteran cohorts (supplementary table 1).

2.2. Higher frequencies of complex chromosome aberrations in a small group of NT veterans

We examined sub-groups of the NT cohort to ask if the broader range of complex exchanges observed in the NT veteran cohort had any association with the exposure rank veterans were assigned or the test site location they attended. Figure 2 and supplementary table 1 shows the average complex aberration frequency of those present at Christmas Island to be similar or no higher (0.256 ± 0.094 and 0.198/100 cells) for veterans assigned into exposure ranks 1&2 combined or rank 3, respectively, than that for all exposure ranks combined (0.299 ± 0.075/100 cells). By contrast, although the numbers of veterans in each sub-group are small (meaning the statistical detection limit for identifying potential group differences were too high) there is a higher average frequency of complex aberrations in NT veterans who were on-board ships (0.350/100 cells for NT veterans on board ships; 3 out of the 4 personnel on HMS Diana) or, present at Maralinga (0.803 ± 0.191/100 cells; 4 of the 5 veterans had at least 1 complex/100 cells) and who were assigned into exposure rank 3, compared to those in respective locations but assigned into lower exposure ranks 1 or 2 (0.117 and 0.126 complex exchanges/100 cells) (figure 2, supplementary table 1).

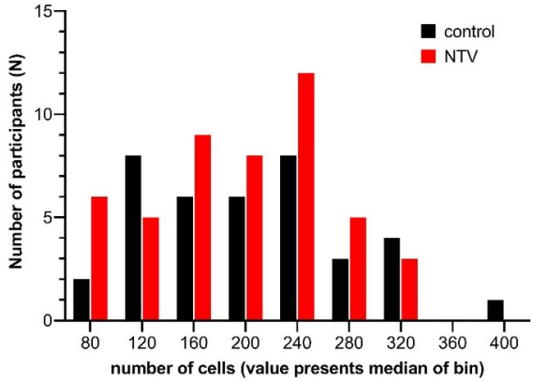

Figure 1. Number of metaphase cells analysed by M-FISH for NT and control veterans.

No differences for any other chromosome aberration type were evident within the NT exposure rank sub-groups. The increase in overall damage burden seen in NT veterans assigned to exposure rank 3 who were onboard ships (5.957 ± 0.442/100 cells) or at Maralinga (6.881 ± 1.527/100 cells), is therefore likely dominated by the excess of complex aberrations noted above. The total damage burden accounts for all breaks necessary to result in the aberration pattern types categorised in supplementary table 1.

2.3. Newly arising unstable chromosome aberrations

Table 1 shows the frequency of stable and unstable exchange types, further categorised according to the completeness of each exchange. The vast majority of all incomplete stable exchanges were defined as incomplete due to unresolved 'ends' being below the limit of detection [26]. Similar to that seen for all aberration types, we find no difference in the frequency of stable or unstable exchanges, or cells, between the two veteran cohorts. When examining by the exposure rank and test site location sub-groups however, some differences are seen with an increase in the proportion of unstable exchanges for those in exposure rank 3 on board a ship (0.467/100 cells) or at Maralinga (0.459 ± 0.183/100 cells respectively), compared to other sub-groups, all exposure ranks combined or the control veterans (table 1). This is also reflected in a higher dicentric equivalent which is the total of all dicentric chromosomes identified in simple and complex exchanges (0.467 and 0.688 ± 0.305/100 cells, for exposure rank 3 onboard a ship and Maralinga respectively) when compared to all other subgroups, all NT veteran ranks combined and controls (table 1).

2.4. Confounding exposures and chromosome aberrations of varying complexity

A similar exercise for examining sub-groups in control veterans to that carried out for the NT veteran groups is not possible, accordingly, more detailed comparisons of pertinent cytogenetic data and, associations between self-reported confounders (medical and occupational exposures etc) and chromosome aberrations observed, were examined for both veteran cohorts.

Firstly, a number of control veterans showed higher levels of complex chromosome aberrations than might be anticipated in non-radiation exposed individuals. To examine for any qualitative differences which may suggest differences in how these complex aberrations were formed, we looked at the complexity of each complex aberration finding the cohorts to be similar (average of 2.8 chromosomes & 2.7 breaks per stable complex and 3.4 chromosomes & 3.5 breaks per stable complex and, 4.0 chromosomes & 4.2 breaks per unstable complex and 4.8 chromosomes & 5.6 breaks per unstable complex, for control and NT veterans respectively). The number of insertions within each complex pattern was also similar averaging at 0.6/0.7 insertions/stable complex and, 0.5/1.0 insertions/unstable complex for control and NT veteran cohorts. To note, based upon the frequency of insertions detected (within either stable or unstable complex aberration types), the only difference seen was for rank 3 NT veterans who were present at Maralinga (frequency of insertions 0.195 ± 0.063, 0.245 ± 0.075 and 0.573 ± 0.247 for control, NT and rank 3 Maralinga veterans, respectively, supplementary table 1).

Secondly, analysis to examine for associations between all known confounders and the chromosome aberrations detected was performed for all veterans, for the control veterans only and, the NT veterans only. Considering only those associations that were identified as highly significant by both statistical approaches ($p < 0.01$) for a trend or difference (supplementary table 7), we detected one out of all 154 associations when considering all 86 veterans: specifically, self-reported exposures to 'other' medical sources of radiation (DEXA, radionuclide, fluoroscopy, or coronary angiogram) was related to higher chromosome aberration

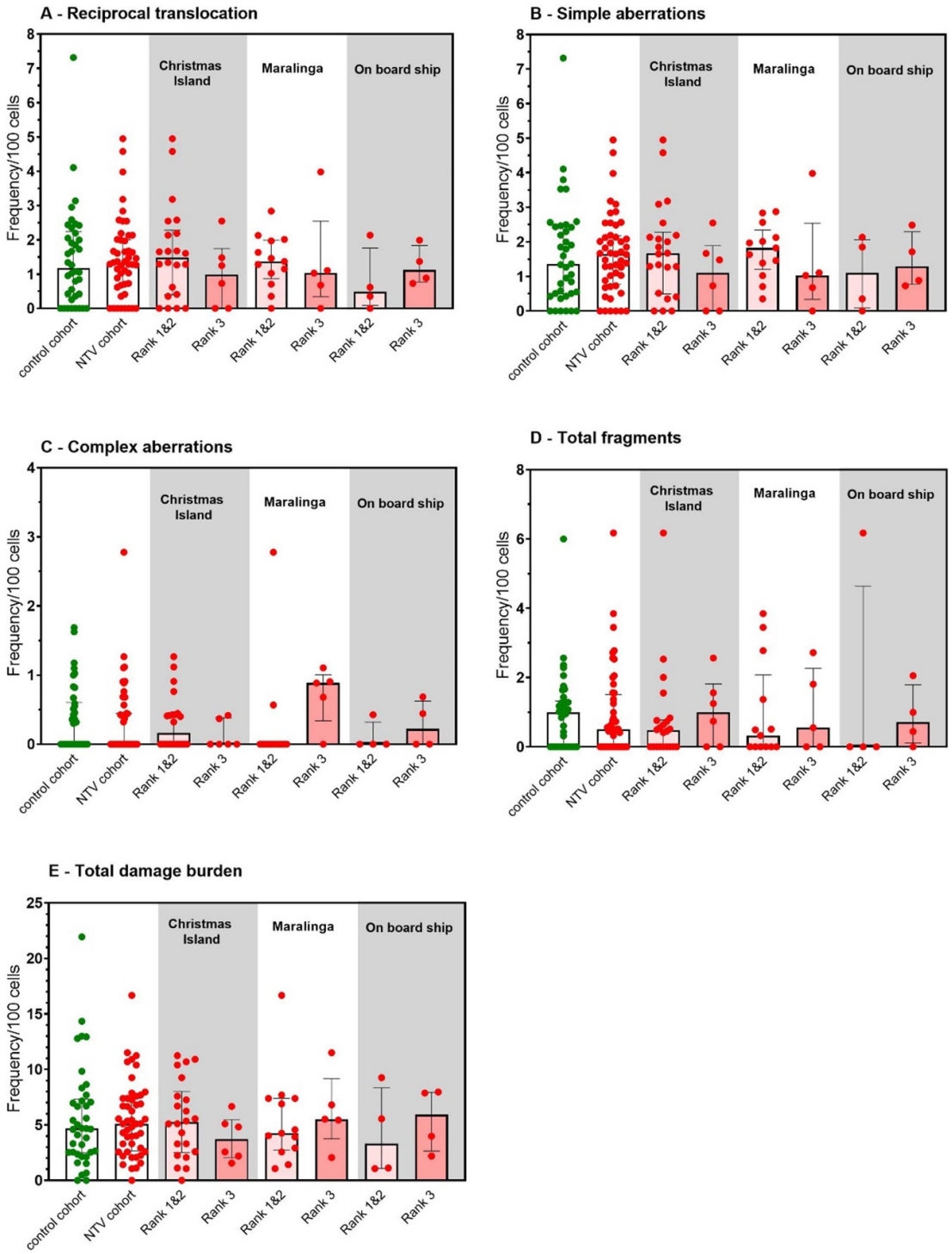

Figure 2. Structural chromosome aberrations observed in control and test veteran cohorts. Frequency of chromosome aberration types per 100 cells reported for control and NT cohorts and, for NT subgroups based upon geographic location (Christmas island, Maralinga range or on board a ship at time of test) and, potential for radiation exposure ranking (allocated blind to cytogenetic data as low [1], medium [2] or high [3] potential) [10]. (A). Total reciprocal translocations (complete and incomplete types), (B). simple exchanges (total reciprocal translocations, Robertsonian translocations and total dicentric chromosomes), (C). complex chromosome exchanges (complete and incomplete types), (D). total fragments (total associated with complex, dicentric, ring or break-only aberrations), E. total damage burden (total number of chromosome breaks irrespective of aberration type). The box-whisker plots show the values for each veteran (dots) together with median values (bar) and the 25%–75% interquartile range (whiskers).

frequencies for total damage burden (supplementary tables 5(a), (b) and 7). When considering only the NT veteran cohort ($N = 48$), none of the potential confounders showed any statistical evidence for a difference or trend (supplementary tables 5(e) and (f)). However, a different picture was seen for the control veterans

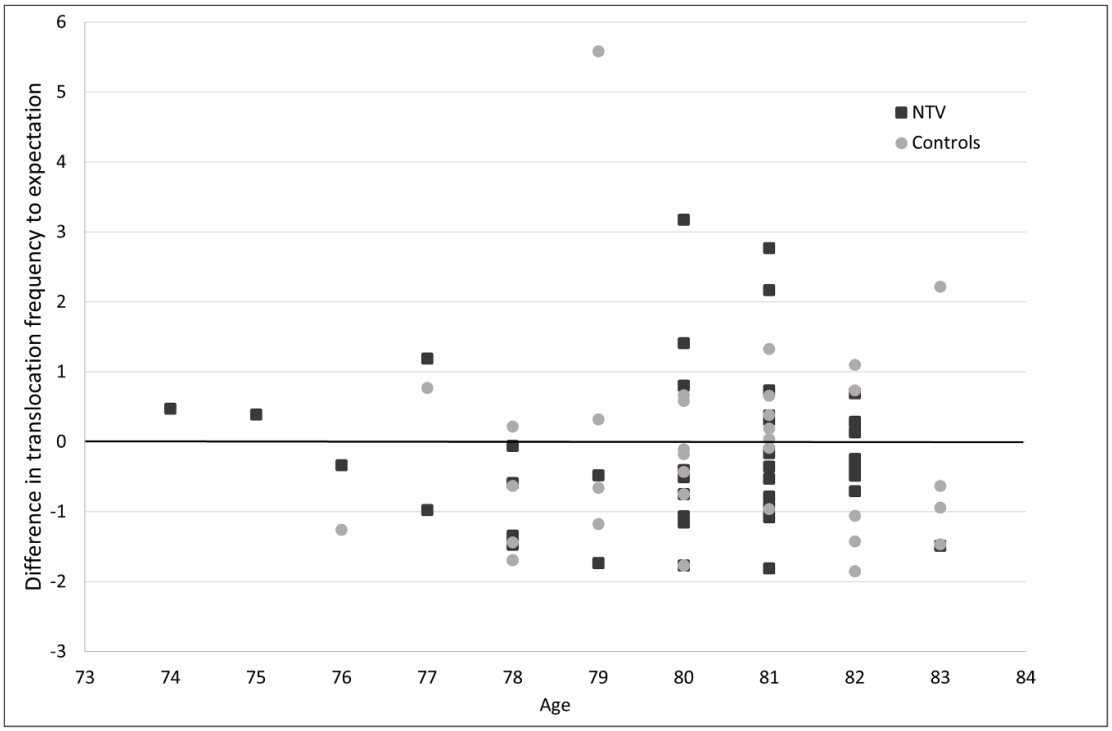

Figure 3. Difference between observed reciprocal translocation frequency with what is expected according to age at time of sampling, for control and NT veterans. Expected frequency as reported by Sigurdsson *et al* [23] is detailed in supplementary tables 3 and 4. Total translocations include complete, incomplete and Robertsonian types.

(supplementary tables 5(c) and (d)) where, despite the relatively small sample size ($N = 38$), we identified a total of 5 associations as being statistically highly significant (associations between alcohol consumption, or exposure to 'other' medical sources of radiation with higher chromosome aberration frequencies (reciprocal translocations and chromosome breaks)). More statistical details can be found in supplementary table 7.

2.5. Examination for association between potential for exposure in veteran father and germline mutation frequency

The frequency of de novo germline DNA mutations was determined for a sub-set of the NT and control veteran family trios (veteran father, mother, biological child) and reported elsewhere [22]. Using this information, we look here to identify any relationship between chromosome aberration frequency in the veteran father and, the germline mutation frequency for the adult child sampled, if such a relationship exists. As shown in figures 5(A) and (B), no evidence for any association between the overall chromosome damage burden or complex chromosome aberrations and DNA germline mutations in their adult child was seen (Spearman correlation coefficient, $p > 0.1$).

We repeated this analysis with a focus on the mutation pattern termed as tumour mutation signature single base substitution (SBS)16. As shown in figures 5(C) and (D), no evidence for any statistically significant trend was observed between the germline single nucleotide variant (SNV) mutations allocated to SBS16 and veteran father's chromosome aberration frequency (Spearman correlation coefficients, $p > 0.1$). However, when those families who were previously identified as having an over-representation of germline SNVs allocated to SBS16, defined as the cluster of >40 SNVs compared to <40, were related to complex aberration groups (none, below and above median frequencies), a potential difference was seen (Kruskal–Wallis test, $p = 0.026$) (figure 5(D)). Specifically, of the 8 families with >40 germline SNVs allocated to SBS16, 2/30, 1/14 and 5/14 veterans have none, below and above medium frequencies of complex chromosome aberrations, respectively.

To explore in more detail, families were further grouped as [1] those who had the highest proportion of SNVs allocated to SBS16 [2], those families who self-reported a health effect and [3], all NT veterans assigned to exposure rank 3 [10]. Table 2 summarises the averages for translocation equivalent in stable cells,

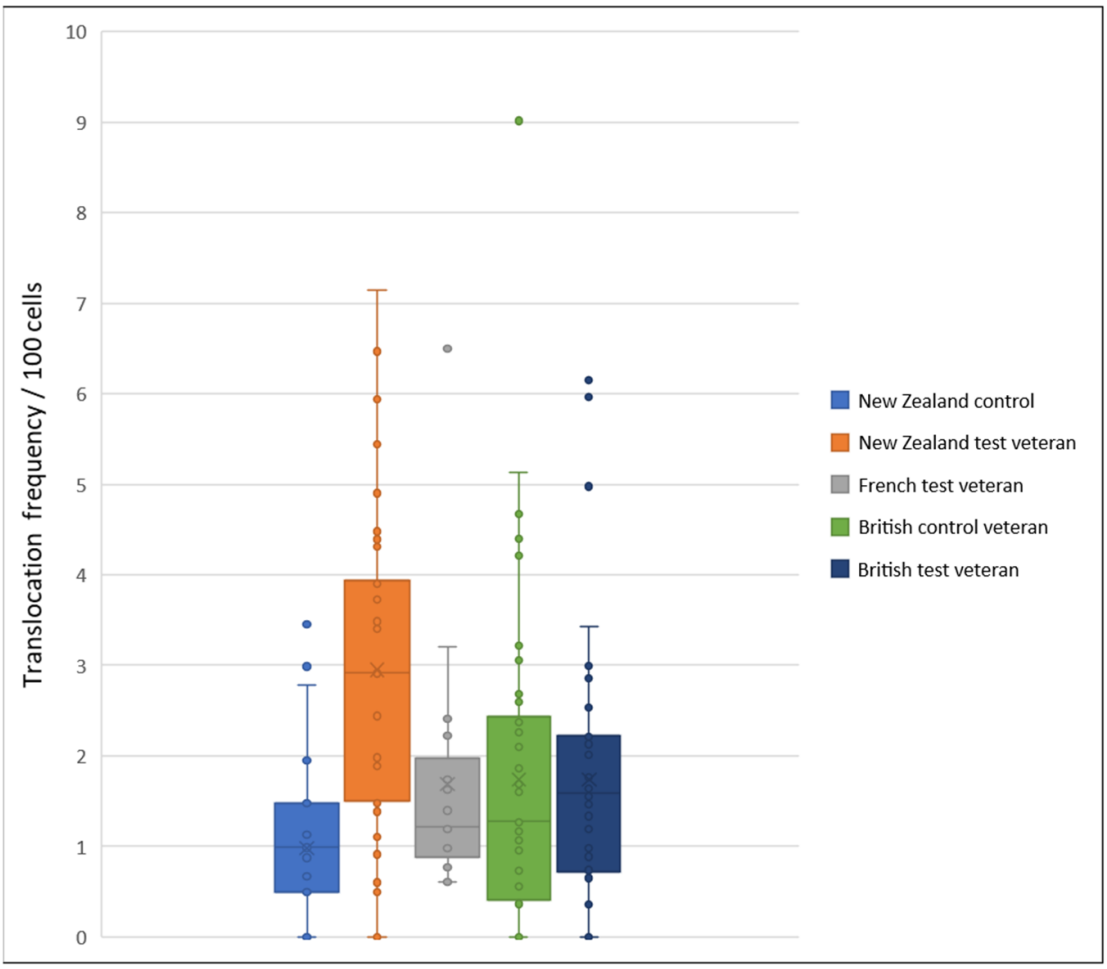

Figure 4. Reciprocal translocation frequencies in nuclear test cohorts. New Zealand control and NT veteran [24], French NT veteran (study lacked control group) [18] and GCFT British control and NT veteran's studies shown. All data presented as full genome (from 24-colour FISH) or full genome conversion (3-colour FISH data from Gregoire [18] converted based on [25]. British control and test veteran translocation equivalent frequency shown. Box-whisker plots shows the median value, the quartiles (bar), max/min values (whiskers) and outliers.

complex aberration frequency and, the overall damage burden detected by M-FISH in the veteran father with the DNA germline mutation averages (total mutations, SNV, indels, SV, clustered and mutations allocated to SBS16) for these family groupings. Chromosome aberration frequencies for this smaller sub-set of control ($N = 28$) veterans is also shown and is consistent with those derived from the analysis of 91 veterans (supplementary table 1). As expected, those families categorised as having the highest SBS16, average around 2–3 times higher for this signature (46.3) than all other family categories examined here (∼15–16). Further, both the total of all de novo mutations (88.5) and all SNVs (79) are raised compared to any other sub-group, whilst the frequencies of complex aberration (0.686 ± 0.315/100 cells) and overall damage burden (7.546 ± 1.82/100cells) in the veteran father are also raised, again relative to any other sub-group examined. Families of veterans who were assigned into exposure rank 3 were associated with a slightly higher proportion of SNVs allocated to SBS16 (24.3 compared to average ∼15–16 for e.g. exposure ranks 0 (control), 1 and 2). For those families who self-reported a health effect, no elevation in aberration burden in the veteran father or germline mutations relative to those families who did not, was seen. No other differences were evident although it is noted that significantly more veterans who were allocated into exposure rank 3, reported health concerns compared to all other exposure ranks (0.014 (5/48 families), 0.31 (11/35 families) and 0.429 (6/14 families) reporting at least one child/grandchild with health issues for exposure rank 0 (control), 1 + 2 combined and rank 3, respectively, $p < 0.1$) [10].

Table 1. Stability and completeness of simple and complex exchanges.

		Stable aberrations frequency per 100 cells (number)						Unstable aberrations frequency per 100 cells (number)						
		Reciprocal translocation		Stable complex		Total		Dicentric		Unstable complex		Total		
Cohorts ($N = 86$)	Cells scored	*C*	*I*	*C*	*I*	Stable aberrations	Stable cells	*C*	*I*	*C*	*I*	Unstable aberrations	Dicentric equivalents	Unstable cells
Control ($N = 38$)	7698	1.000 ± 0.170 (77)	0.390 ± 0.086 (30)	0.156 ± 0.049 (12)	0.065 ± 0.035 (5)	1. 611 ± 0.267 (124)	1.403 ± 0.250 (108)	0.065 ± 0.030 (5)	0.078 ± 0.028 (6)	0.039 (3)	0.091 ± 0.036 (7)	0.273 ± 0.063 (21)	0.260 ± 0.072 (20)	1.234 ± 0.179 (95)
BNTV ($N = 48$)	9379	1.152 ± 0.140 (108)	0.309 ± 0.066 (29)	0.128 ± 0.039 (12)	0.043 ± 0.032 (4)	1.631 ± 0.185 (153)	1.461 ± 0.172 (137)	0.075 ± 0.044 (7)	0.075 ± 0.0.25 (7)	0.032 (3)	0.096 ± 0.044 (9)	0.277 ± 0.078 (26)	0.288 ± 0.083 (27)	1.034 ± 0.171 (97)
NTV by exposure rank														
Christmas Island ($N = 28$)														
Rank 1 ($N = 21$)* & Rank 2 ($N = 1$)	4685	1.174 ± 0.250 (55)	0.299 ± 0.096 (14)	0.149 ± 0.066 (7)	0.064 ± 0.033 (3)	1.686 ± 0.325 (79)	1.588 ± 0.319 (73)	0.064 (3)	0.064 (3)	0.021 (1)	0.043 (2)	0.192 ± 0.086 (9)	0.171 ± 0.085 (8)	0.832 ± 0.235 (39)
Rank 3 ($N = 6$)*	1010	0.693 ± 0.278 (7)	0.495 ± 0.224 (5)	0	0	1.188 ± 0.398 (12)	0.990 ± 0.405 (10)	0	0.099 (1)	0.099 (1)	0.099 (1)	0.297 (3)	0.297 (3)	1.089 ± 0.369 (11)
Onboard ship ($N = 8$)														
Rank 1 ($N = 4$)*	858	0.699 ± 0.369 (6)	0.117 (1)	0.117 (1)	(0)	0.932 ± 0.574 (8)	0.816 ± 0.471 (7)	0.117 (1)	0.117 (1)	(0)	(0)	0.233 (2)	0.233 (2)	1.166 ± 1.164 (10)
Rank 3 ($N = 4$)*	856	1.051 ± 0.407 (9)	0.234 (2)	0.117 (1)	(0)	1.402 ± 0.308 (12)	1.051 ± 0.165 (9)	0.117 (1)	0.117 (1)	(0)	0.234 (2)	0.467 (4)	0.467 (4)	2.103 ± 0.683 (18)
Maralinga ($N = 18$)														
Rank 1 ($N = 9$)* & Rank 2 ($N = 4$)	2376	1.094 ± 0.143 (26)	0.337 ± 0.147 (8)	0.042 (1)	0.042 (1)	1.515 ± 0.210 (36)	1.389 ± 0.151 (33)	0.126 (3)	0.0842 (2)	(0)	0.042 (1)	0.253 ± 0.232 (6)	0.253 ± 0.232 (6)	1.010 ± 0.391 (24)
Rank 3 ($N = 5$)	872	1.38 ± 0.600 (12)	0.115 ± 0.089 (1)	0.344 (3)	(0)	1.835 ± 0.848 (16)	1.606 ± 0.683 (14)	(0)	(0)	0.115 (1)	0.344 (3)	0.459 ± 0.183 (4)	0.688 ± 0.305 (6)	0.803 ± 0.399 (7)

C = Complete; *I* = incomplete; * includes veterans who attended more than one location. Frequencies are expressed as relative number of aberrations per total cells analysed, corresponding uncertainty is calculated as SEM (for $N > 4$ and with veteran as statistical unit) and absolute count number is provided in brackets.

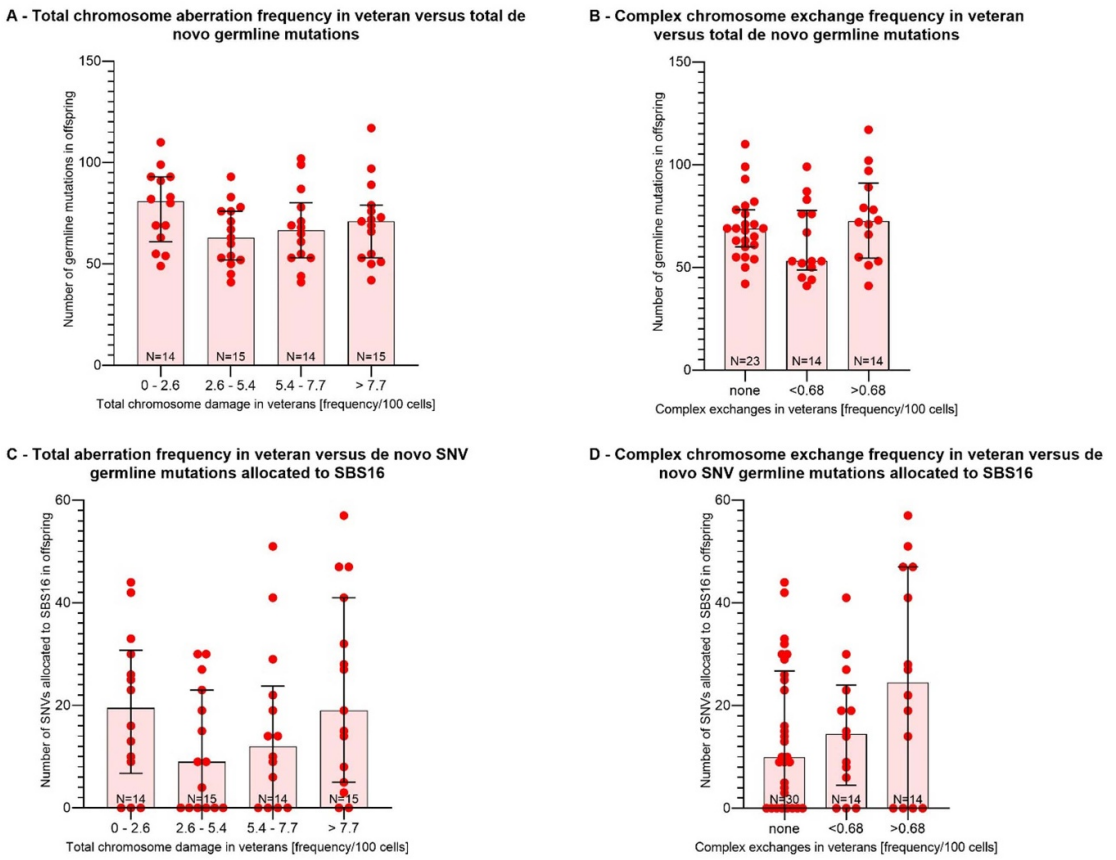

Figure 5. Association between veteran father's chromosome aberration frequency and germline de novo mutations. Analysis was carried out for all families where both M-FISH (as reported here) and WGS data [22], was available (28 control and 30 NT families). (A). Total chromosome aberration frequency in veteran verses total de novo germline mutations, (B). complex chromosome exchange frequency in veteran verses total de novo germline mutations, (C). total aberration frequency in veteran verses de novo SNV germline mutations allocated to SBS16, (D). complex chromosome exchange frequency in veteran verses de novo SNV germline mutations allocated to SBS16. Total chromosome damage frequencies were categorised according to their quartiles, and complex exchanges according to their median (0.68) and a separate zero group, for all veterans (control and NT). The box-whisker plots show the values for each offspring (dots) together with median values (bar) and the 25%–75% interquartile range (whiskers).

3. Discussion

The GCFT study is the first study to obtain blood samples from a group of British NT veterans and their families for the purposes of identifying genetic alterations in offspring which may have arisen as a consequence of historical paternal exposure to ionising radiation [10]. The available information on radiation dose received by veterans, if any, is limited due to approximately only ∼22% being monitored at the time, therefore, the purpose of the work carried out in this part of the study is to ascertain if there is any cytogenetic evidence of historical exposure to ionising radiation in veterans of the nuclear testing programme. For this, 24-colour karyotyping M-FISH was used to detect the occurrence of stable and unstable chromosome exchanges of varying complexity [27]. By doing this, aberrations which have persisted over time and those which may be more recently induced, arising as a consequence of lifestyle/medical/occupational factors, ongoing internalised radiation exposure or through other mechanisms including delayed genomic instability, may be compared between the NT and control veteran cohorts. As shown in figure 2, table 1 and supplementary table 1, we observed stable and unstable chromosome type aberrations of varying complexity to occur in both cohorts, however for all aberration types, no difference in frequencies between the NT and control veteran's cohorts, was seen.

Those exposed to radiation, even in the distant past, may be expected to have more aberrations (particularly stable types) than someone not exposed to radiation. For instance, the occurrence of reciprocal translocations after radiation exposure is routinely applied for the retrospective assessment of radiation dose [12]. However, other factors including occupational, medical and lifestyle exposures also contribute to the induction of translocations and as a consequence, a person's translocation burden increases with increasing age [23]. As it is not possible to distinguish translocations induced by radiation from those arising by other

Table 2. Summary of chromosome aberration burden in veterans observed by M-FISH and de novo germline mutations detected by whole-genome sequencing (WGS) analysis.

	Veteran M-FISH data Frequency/100 cells (number)			Germline mutation frequency/offspring						
	Cells (stable)	Complex	Total damage burden	Translocation equivalent in stable cells	Total	SNV	InDel	SV	Cluster (10bp /100bp)	SBS16
SBS16[1]										
Families with >40 SNV mutations allocated to SBS16 ($N = 8$)	1312 (1288)	0.686[2] ± 0.315 (9)	7.546 ± 1.82 (99)	2.562 ± 0.717 (33)	88.5	79	8.1	1.4	0.9/1.4	46.3
Families with <40 SNV mutations allocated to SBS16 ($N = 50$)	9885 (9772)	0.303 ± 0.061 (30)	5.53 ± 0.573 (547)	1.770 ± 0.265 (175)	67	59.76	6.26	0.98	0.8/1.2	12.5
Families who self-reported health effect in offspring[3]										
Families reporting effect ($N = 16$)	3355 (3318)	0.268 ± 0.107 (9)	4.978 ± 0.731 (167)	1.567 ± 0.299 (52)	71.5	62.44	7.75	1.3	0.8/1.4	14.5
None ($N = 42$)	7844 (7742)	0.382 ± 0.089 (30)	6.107 ± 0.711 (479)	2.015 ± 0.320 (156)	69.4	62.4	6.05	0.93	0.8/1.2	18.2
Veterans allocated to Rank 3[3]										
Rank 3 ($N = 11$)	2037 (2005)	0.540 ± 0.156 (11)	6.284 ± 0.900 (128)	1.546 ± 0.535 (31)	70.1	62.2	6.9	1	0.6/1.1	24.3
Ranks 1 + 2 ($N = 19$)	3525 (3493)	0.312 ± 0.163 (11)	5.702 ± 0.952 (201)	2.262 ± 0.430 (79)	69.5	62.3	6.21	0.95	0.63/0.89	16.05
Rank 0 (controls) ($N = 28$)	5637 (5562)	0.302 ± 0.085 (17)	5.624 ± 0.913 (317)	1.762 ± 0.370 (98)	70.3	62.6	6.57	1.11	0.93/1.5	15.2
Ranks 0,1 + 2 ($M = 47$)	9160 (9055)	0.306 ± 0.083 (28)	5.655 ± 0.200 (518)	1.932 ± 0.279 (177)	69.9	62.5	6.43	1.04	0.81/1.3	15.5

Includes all families (NT and control combined) where both veterans father M-FISH data and adult child's whole genome sequence data [22], were available.[1] Moorhouse *et al* [22], [2]statistical significance for difference: $p = 0.054$ (Wilcoxon rank-sum/KruskalWallis test) and $p = 0.032$ (Negative binomial regression), [3] Rake *et al* [10].

causes, detectable frequencies potentially attributable to radiation, in a controlled study such as this, need to be higher than would be expected for their respective age. In this study, translocation frequencies of 1.461 ± 0.166 and 1.416 ± 0.234/100 cells were detected for NT and control cohorts respectively, showing no differences between these age-matched cohorts nor, any differences (for the majority of veterans) with expected frequencies based upon their individual age (figure 3, supplementary tables 3 and 4) [23, 28]. Of the seven (representing three control and four NT) veterans who did show an excess occurrence of translocations, six were smokers (supplementary tables 3 and 4) [23]. Wahab *et al* who assayed New Zealand (NZ) NT veterans who had been on-board ships at the time of atmospheric tests together with an age-matched land-based control group using M-FISH, showed a control frequency of 1.005/100 cells consistent with the NZ population being ∼20 years younger than those studied here [24]. However, the 3-fold increase in translocations (2.938/100 cells) reported for NZ NT veterans, is markedly different to what we see, indeed the frequency of translocations in just those British NT veterans who were on-board ships in this study, although small ($N = 8$), is slightly lower than the averages for all exposure ranks combined (0.816 ± 0.471 and 1.285 ± 0.284 translocations/100 cells for those on-board ships allocated to exposure ranks 1 and 3 respectively) (supplementary table 1). Of the potentially relevant confounders, only smoking was reported by Wahab *et al*, meaning we cannot directly compare confounder profiles between both studies. It is noted from a technical perspective however that blood was cultured for longer than is standard and further, that a higher frequency of Robertsonian translocations and incomplete (one-way) reciprocal translocations compared to what we observed, were reported [24]. The distinction between satellite fusions between acrocentric chromosomes which occur in normal populations and, true Robertsonian translocations can be difficult particularly when assessing with the M-FISH technique due to the lack of centromeric probes. Thus, further understanding of the process for analysis and reporting and also, the management of sampling and time before processing for the control and NT veteran blood samples are necessary before clarity on the disparity between our two studies can be reached. Instead, our findings are more consistent with those in French NT veterans reported by Gregoire *et al* [18]. Thus, from this, we find no evidence to support the notion that, as a cohort, the NT veterans sampled in this study were exposed to radiation at or above the detectable limits of this study [18]. This should reassure veterans, whereby the concern that being present at test sites and witnessing nuclear operations, irrespective of role undertaken, resulted in significant radiation exposure, is not supported by the chromosomal evidence presented here [29–31]. In reporting this, we do not preclude the possibility that our findings reflect the sampling of veterans who remained alive into their 80 s, nor does it infer the same or different outcome would have been seen if veterans who have since passed away had been examined.

Due to the absence of dose information, we assigned NT veterans using a simple 3-point exposure ranking system and employed this as a proxy for dose, whereby those allocated into exposure rank 3 had the highest potential for exposure [10]. 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 is assumed to be higher than a veteran who witnessed an atmospheric test in the safety zone (∼40 km from the blast) on Christmas Island [10]. From this, sub-groups of the NT cohort were defined (blind to any cytogenetic data) allowing us to examine for any differences each of these factors may have on aberration frequencies. Similarly to above, no indication for statistical differences between both cohorts was seen (figure 2, supplementary tables 6(a) and (b)). However, higher average frequencies of complex aberrations were seen for just those veterans who were assigned into exposure rank 3 and who were either onboard ships (0.350/100 cells (3 out of the 4 personnel were on HMS Diana which sailed through a nuclear plume)) or, at Maralinga (0.803 ± 0.191/100 cells) (supplementary table 1). These were associated with an increase in the proportion of unstable exchanges which was also reflected in a higher dicentric equivalent, suggesting their formation to be relatively recent events (table 1). The background level of complex aberrations is reported to be 0.128 and 0.193/100 cells for individuals aged 70–80 yrs and 80+ yr respectively [32], with the differences seen potentially reflecting general versus military populations. However, the increased frequencies of complexes are within ranges reported in nuclear workers known to be chronically exposed to internalised plutonium (high-LET α-particle emitter), for instance, frequencies of ∼0.200–∼1.00/100 cells have been detected by M-FISH, rising to >2/100 cells in highly exposed workers [16, 17, 33, 34]. Complex aberrations of the pattern, size and complexity (number of chromosomes and breaks involved) as detected here are characteristic of induced aberrations seen after exposure to low doses of high-LET radiation, or high doses of low-LET radiation [19, 21, 35], rather than from any ongoing instability to the genome. Thus, given the potential for exposure in these exposure rank 3 groups, the origin of the complex aberrations may be associated with ongoing exposure to internalised radionuclides due to contamination with nuclear fallout. Indeed, the MoD state that the potential for internalised radionuclide exposure may have arisen in Maralinga and HMS Diana. Wahab *et al* [24, 36] also reported an excess of 'very complex' chromosome aberrations in NZ NT veterans' that were not seen in the control group.

Despite the measures taken to reduce bias in the recruitment methods, the response rates for providing blood samples were low and so it is possible that those taking part had a particular interest in the study [10]. For instance, a higher proportion of control veterans reported occupational exposure to both radiation ($p = 0.033$) and chemicals ($p = 0.021$) compared to NT veterans (supplementary table 3). Indeed, an analysis to examine for associations between all known confounders and chromosome aberrations revealed that some associations were present only in the control cohort, suggesting that the confounder profiles were different between both cohorts. This was not accounted for by the reported occupational exposures however, rather by the number of medical radiation exposures received by these aged veterans. A simple system for assigning estimated doses, based upon known average doses received after a chest x-ray or head CT scan for instance and, the information provided during telephone interview with the veteran, was used to enable assessment [10]. No statistically significant associations (defined as $p < 0.01$ by two different statistical tests) were judged for the NT veterans, however, upward trends showing some association between medical exposures, particularly those grouped as 'other' (e.g. fluoroscopy) and simple (e.g. reciprocal translocations) chromosome aberration types in control veterans were seen (supplementary tables 5(A)–(F)). Although, no confounder associations with complex aberrations were identified, it should be noted that complexes have recently also been detected after partial body exposure to low occupational exposures received during fluoroscopic procedures [37], therefore, it cannot be ruled out that the complex aberrations seen in both the control and, the NT cohorts, could in part have arisen due to medical (non-therapeutic) exposures.

Similar to the arguments above whereby some control veterans may have had a particular interest in participating, the NT veterans may also have been more likely to take part if they believed they had been highly exposed to radiation during the tests or, that their family had been adversely affected. During the GCFT study recruitment interview, participating veterans were asked whether they were aware of any birth defects, genetic disorders, inherited diseases or cancers that had affected their children or grandchildren [10]. The limited information generated from this revealed one fifth of the NT veterans recruited reported a congenital abnormality among at least one of their children or grandchildren, which was higher than that reported by the control families (Fisher's exact, $p = 0.03$) [10]. Analysis of a sub-set of these veteran family groups, carried out in parallel to the data generated here, found no difference in germline mutation rates between the 30 control and 30 test veteran family cohorts [22]. To examine in more detail and for the first time, we integrated all of this information with the M-FISH data to look for any association between the veteran father's chromosome aberration burden and the frequency of de novo germline mutations in their respective children. Analysis was carried out for all those families where both M-FISH and WGS data was available (28 control and 30 NT families). When examined as either the NT or control family cohorts, no evidence for any association between the overall chromosome damage burden or complex chromosome aberrations and, DNA germline mutations was seen (figures 5(A) and (B)). However, when those families who were previously identified as having the highest number (>40) of germline mutations assigned to tumour mutation signature SBS16 were grouped and compared against the group of the remaining families [22, 38, 39], a weak relationship between increased frequencies of complex aberrations was seen (figure 5(D), table 2) ($p = 0.054$ by Wilcoxon rank-sum and $p = 0.032$ by negative binomial regression). SBS (and other) signatures are detectable 'patterns' of mutation which remain in the DNA sequence after DNA damage and repair processing whereby assignment into one of the 60 currently verified SBS signatures involves a mathematical process of 'fitting'. As a consequence, false-assignment of mutations to SBS signatures cannot be ruled out. Indeed, whether the higher representation of SBS16 in a small number of families reported by Moorhouse *et al* is simply a consequence of the increased total germline mutation frequency in these families remains unclear, although it is noted that the occurrence of indels and SVs are also in the upper range (table 2) [22]. The mutational process thought to be associated with SBS16 is repair of bulky DNA lesions via transcription-coupled nucleotide excision repair mechanisms [38, 39]. Whether this represents a mutational pattern consistent with paternal high-LET radiation exposure which is detectable in the germline remains to be established, including by further examination to that already carried out for any clustering of mutations [22, 40, 41]. When the self-reported information on clinical conditions of veterans' children or grandchildren is considered, three (representing both NT and control families) of the 8 families with an SBS16 mutation count of >40 self-reported adverse health in one of their descendants. Overall, however, we observe no relationship between veteran's chromosome aberration burden and germline mutation frequency in those families who report a descendant health concern (table 2).

In conclusion, we find no cytogenetic evidence of historical radiation exposure in the cohort of NT veterans sampled here, offering reassurance that attendance at NTs sites per se was not associated with significant levels of exposure to radiation. We do observe complex aberrations to be raised in a very small number of veterans who were previously identified as having a higher risk of exposure, which although not statistically significant, may suggest internalised contamination from fallout. A pilot analysis to measure long-lived radionuclides, if present, in urine is underway to examine for this possibility. Lastly, by integrating

information obtained from across the GCFT study, we find a small number of families from both control and NT cohorts where a possible relationship between paternal aberrations (specifically complex aberrations) and germline mutation sub-types is seen. This may represent a transgenerational biomarker of paternal exposure and warrants further investigation. However, we find no relationship between paternal chromosome aberration burden, germline mutation frequency and self-reported concerns of adverse health in descendants, suggesting that the reported health issues in these families are unlikely to be associated with historical radiation exposure.

4. Methods

4.1. Study participants and sampling

4.1.1. Selection

The study was conducted in accordance with UK ethical framework and approved by the UK Health Research Authority (17/LO/0273). Blood samples were obtained as part of the GCFT study from the NT-control family trios of military men (veteran father, mother, child) who were enrolled in the 'UK NT veterans' cohort [10]. This involved gaining information on test veterans who were born 1935 or later, thought to be alive and cancer-free by the custodians of the UK NT veteran cohort (PHE, now UK HSA). Information included service (RAF, Navy, Army), location of test site, years attended and any special group status. Special groups included record of dose, noted in health physics records and categorization into specialized roles deemed by the MoD, UK as having a higher likelihood of exposure such as aircraft handling crew. A total of 5,818 veterans were provided, of which only ∼6% had a record of dose, the majority of which were below 10 mSv (<1 mSv (293), 1–10 mSv (67), 10–50 mSv [13] and >50 mSv [4]). Given that only 22% of the entire 22 000 test veterans were issued with a badge and concerns that exposure was not limited to just those issued with film badges, selection was based upon the potential for exposure through attendance at multiple operations and/or allocation into a special group.

4.1.2. Recruitment

Veterans were selected if they were currently ⩽80 years old and had participated in two or more operations which included the GRAPPLE *X, Y, Z* series, Maralinga test sites and/or those who had special group status. In addition, a small number ($n = 42$) of veterans aged ⩽82 who were part of the crew of Diana, active handling flight or aircrew sampling plumes special groups were also included. A long-list of 1459 veterans was generated, which reduced to 908 veterans after flagging with National Health Service (NHS) Digital (mainly due to death, diagnosis of cancer or no GP contact detail). NHS Digital provided general practitioner (GP) contact details for 908 test and 3796 control veterans; all 908 test and 2,741 control GPs were contacted with the request to forward invitation packs. Invitations to participate were subsequently carried out in batches with veterans with the highest potential for exposure being prioritized (according to a ranking algorithm identifying those attending multiple tests with special group status). From this, GP practices forwarded the invitation packs to a total of 405 test veterans and 1028 control veterans, group-matched on age, service (RAF, Royal Navy, Army) and period of service in tropical regions. Responding veterans were screened by telephone to confirm eligibility and gain written informed consent. Military service details and other potential clastogenic exposures were collected from veterans using a structured questionnaire. Veterans were excluded if they ever had cancer (other than non-melanoma skin cancer), or if they were known to have had cytotoxic chemotherapy or radiation treatment for any reason (such as methotrexate for rheumatoid arthritis) as this could cause genetic damage and interfere with interpretation of the study. Supplementary table 2 shows the characteristics of the veterans recruited. Further details of the GCFT study are given by Rake [10].

4.1.3. Sampling

Upon receipt of written consent, study packs for sampling whole blood were delivered to the family with a request for their GP to sample and ship to Brunel University London within 24 h, where all samples were stored in compliance with Human Tissue Authority guidance. Lithium heparin blood samples were immediately processed for cytogenetic analysis with isolation of peripheral blood lymphocyte (PBL) for long-term storage where possible.

4.1.4. Exposure rank

The majority of test veterans in the UK NTV cohort have no recorded dose as only a limited number were issued with film badges, mainly accounting for those identified in special groups and, no measurement for internal contamination took place. Based on the testimony and verified operation attendance and, blind to any results, the test veterans were assigned independently by two of the authors to a three-point rank for the

potential of internal/external exposure [10]. The information used included service history, dates and sites attended, number of tests witnessed, normal role and roles carried out pertaining to tests both immediately after and in the months/years after tests. Each case was *a priori* assumed to be in the lowest rank (exposure rank 1), 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, and here we distinguished between activities immediately and up to 3 months after the test where dose and dose rates would be expected to be highest (higher rank, exposure rank 3) or, at any time from at least 3 months after the test (medium rank, exposure rank 2).

Fourteen of the 48 NT veterans (29%) for which M-FISH data was obtained were assigned to the highest exposure group. These included those previously identified with 'special group' status such as being aboard the HMS Diana (Montebello) which sailed through plumes and RAF active handling flight crew at both Maralinga and Christmas Island sites. Other veterans assigned with the highest potential for exposure were involved in cleaning aircraft or vehicles, clean-up operations and/or supporting collecting samples in forward areas. Subsequent to this allocation, information was linked to those veterans (3 in total) who had a record of dose (doses of 0.4 (0.2 on 2 tests), 1.4 and 6.5 mSv). Five veterans were assigned a medium potential for exposure and all accessed forward areas and cleaned aircraft or vehicles but sometime after the tests or less regularly as those assigned the highest rank. The remaining 29 veterans (60%) were assigned the lowest potential for exposure.

4.2. Cell culture

Upon arrival, the blood samples were immediately processed for culture and the collection of 1st *in vitro* cell division metaphase cells for cytogenetic assessment. For each sample, two whole blood cultures were set-up. For this, 0.4 ml of whole blood was used to inoculate 3.6 ml of freshly prepared media (PBMAX Karyotyping Medium (ThermoFisher, cat. number 12557021)), 10 μM 5-bromo-2′-deoxyuridine (BrdU Sigma-Aldrich product details), 10 μl ml^{-1} heparin (Sigma-Aldrich cat. number 9041-08-1) and cultured in a humidified incubator at 37 °C (95% air/5% CO_2), at a 45° angle, and with the cap left slightly open to allow gaseous exchange. Cultures were set up to maximise the yield of 1st cell division of PBLs and harvested using standard cytogenetic techniques after a total of 50 h. To arrest cells at the metaphase stage of the cell cycle, 50 μg ml^{-1} of Colcemid KaryoMAX (ThermoFisher, cat. number 1521012), a tubulin inhibitor, was added 3 h prior to harvest. After this time, the cultures were centrifuged at 200 g for 10 min and the cell pellet re-suspended before the addition of 0.075 M KCl hypotonic solution (Fisher Scientific cat. number10575090) for 8 min at 37 °C. Cells were then centrifuged at 200 g for 10 min and fixed in 3:1 methanol (Thermo Fisher catalogue number 15654570) acetic acid (Thermo Fisher catalogue number 1743468) on ice. The fixation process was repeated until the samples appeared clear (∼5 times), before being stored −20 °C.

4.3. M-FISH analysis

4.3.1. M-FISH assay

Fixed preparations were dropped onto 'grease-free' slides to obtain quality metaphase spreads. Slides with ∼>200 metaphases of which <5% were in their 2nd *in vitro* cell division, determined by Harlequin staining, were selected for painting. M-FISH was carried out utilizing 24XCyte staining probe (Metasystems Probe cat num D-0125-600-DI) as per manufacturer protocol. In brief, slides were incubated in 2xSSC at 70°C (±1°C) for 30 min. After this time, the cooled slide was transferred into 0.1xSSC at RT for 1 min. Chromosomes were then denatured in 0.07 M NaOH at RT for 1 min followed by 1 min incubation in 0.1xSSC, then 2xSSC at 4°C, before being dehydrated through a series of alcohol solutions (70%, 95% and 100%). The 24Xcyte probe was denatured by incubating at 75°C (±1°C) for 5 min, placed on ice briefly and then incubated at 37°C for 30 min. The probe was overlaid on to the slide and left to hybridize in a humidified chamber at 37°C (±1°C) for 2–3 d. Slides were then washed in 0.4xSSC preheated to 72°C (±1°C) for 2 min incubated in 2xSSCT (containing 0.05% Tween20) for 30 s. For counterstaining, the slide was rinsed in double distilled water and left to air dry before application of DAPI/antifade and sealing. Slides were visualised utilizing an automated 8-position fluorescence microscope (Axioplan 1) containing individual filter sets for 24XCyte probe cocktail plus DAPI (FITC, Spectrum Orange, Texas red, Cy5, DEAC and DAPI). Metaphase cells were imaged under x63 oil immersion and captured by Cool Cube camera driven by Metafer4 (version 3.11.6) and ISIS v5.5.6 software. The image files were exported and karyotyped in ISIS.

4.3.2. Chromosome aberration classification

All analysis was carried out blind to control/test status. A minimum of 10% of all apparently normal cells and, all abnormal cells were cross-checked by an experienced analysist. A cell was classified as being apparently normal if all 46 chromosomes were present and contained the appropriate fluorophore combination along

their entire length. Metaphase cells with up to three chromosomes missing were also included for analysis due to the high number of cells with aneuploidy, likely a consequence of the advanced age of the participants.

Chromosomal aberrations were identified by colour junctions along the length of each individual chromosome and/or by the presence of chromosomal break or fragment. A chromosome interchange involving up to two breaks in two chromosomes was categorised as a simple exchange (reciprocal translocation, Robertsonian or dicentric ± acentric fragment). Ring chromosomes, which involve two breaks in one chromosome were also classed as simple. Exchanges involving three or more breaks in two or more chromosomes were classed as complex and assigned the minimal number of chromosomes, arms and breaks involved [42]. The presence of insertion-type rearrangements was noted. Chromosomes breaks not involving any additional chromosomes were classed as chromosome breaks, with further categorisation as truncated ± associated fragment. When classifying cells with multiple aberrations, all aberrations were recorded as independent events. Where homologous chromosomes were involved, efforts were made to establish whether the homologues were in the same event or in different independent events, mainly by consideration of chromosome length. All exchanges were recorded as either complete (all break-ends re-joined), true incomplete (where one or more break-ends fail to find an exchange partner) or one-way incomplete (where one or more elements appear to be missing due to unresolved 'ends' being below the limit of detection).

The potential transmissibility of exchanges through cell division was recorded, where a stable (transmissible) exchange is defined as complete (or assumed to be complete) with no evidence of unstable elements e.g. dicentric or acentric fragments. Metaphase cells were categorised as stable (and therefore capable of long-term transmission) only if all the aberrations detected within that spread were classified as stable. Unstable complex chromosomal exchanges containing dicentric chromosomes were broken down in to their dicentric equivalents, and included with simple dicentrics to produce the dicentric equivalent. The same process was carried out for translocations within stable complexes in stable cells, which combined with reciprocal translocation and Robertsonians in stable cells, to produce the translocation equivalent in stable cells. A clone was defined as two or more abnormal cells containing an identical structural rearrangement and recorded as a single occurrence for frequency purposes.

4.4. Statistical analysis

The frequencies of aberrations in NT and control veterans, both overall and in subgroups, were compared by the Kruskal–Wallis test or, if appropriate, Fisher's exact test, in combination with multiple p-value adjustment by the Holm (Step-down Bonferroni). To evaluate the influence of potential confounders and to account for differences in total number of cells analysed per sample, the strength of the association between the chromosome aberration endpoints, variables for a potential radiation exposure ('exposure' variable) and potential confounder covariates were described by the negative binomial regression model (NB regression). This model was found to be superior to the Poisson model in describing the aberration endpoints (judged by the Akaike information criterion). The NB model is a generalization of Poisson regression and used for modelling over-dispersed count variables while adjusting for one or more covariates, and operates on a log link function $g(.)$, given by

$$g(\mu) = \beta_0 + \beta_1 X + z'\theta$$

where μ is the mean count of the chromosome aberration endpoint, β_0 the intercept parameter, β_1 the model parameter for the predictor variable X ('exposure' variable), and z the vector for covariates with corresponding parameters in vector θ. Differences between the total number of cells analysed per sample were accounted in the NB model by including an offset variable. Potential covariates in the study were included as predictor in the final regression analysis on an endpoint-by-endpoint basis. In case of highly correlated covariates, we selected the one with the most biologically plausible covariate–exposure and covariate–outcome association. The association between an aberration endpoint and predictor for radiation exposure was also analysed with and without confounder variables, and both outcomes are reported individually only if conflicting statistical significance outcomes between both were observed. Statistical significance refers always to the model parameter of the predictor variable of interest, and a p-value below 0.05 was chosen as cut-off for deciding statistical significance ($\alpha = 5\%$). Due to the huge number of multiple comparisons and statistical tests it is very likely that false-positive decisions have occurred, which usually can be accomplished by a p-value adjustment towards a lower α as cut-off to maintain a global $\alpha = 5\%$. However, in this study it was unclear what a testing family for multiplicity constitutes and how many comparisons within a testing family should be considered, and therefore a p-value adjustment were not explicitly done, but only test decisions were considered as significance for p-values below 0.01. All statistical analyses were done using SAS 9.3 (SAS Institute, Cary NC).

4.4.1. Potential variables for radiation exposure and confounders

The main radiation exposure variable was the NT or control status. Further 'exposure' variables for subgroup analyses were developed for the NT veterans, and included (i) number of atmospheric tests witnessed, (ii) present at activities that involved cleaning of aircraft or vehicles used in tests (YES/NO), (iii) involved in clean-up operations after tests (YES/NO), (iv) accessed forward test area (YES/NO), (v) supported collecting samples from tests (YES/NO), (vi) aboard the HMS Diana (YES/NO), (vii) present at Maralinga (YES/NO), (viii) present at Christmas Island (YES/NO), (ix) duration at test sites (years), and (x) the exposure rank with three likelihood categories [1–3]. Due to the low sample size for category 2 ($N = 5$) the two lowest ranks were merged into one category for data analysis.

The following variables were considered as potential confounders in data analysis: (i) year of birth, with age (years) related to the time at which the blood sample was taken, (ii) alcohol consumption (YES/NO), (iii) smoking exposure, expressed as ever smoked (YES/NO) and further quantified in smoking pack years (one pack year equivalent to 20 cigarettes smoked daily for one year, a cigarillo equivalent to two cigarettes, a cigar to four cigarettes, and one pipe equivalent to $2^1/_2$ cigarette; www.smokingpackyears.com/), (iv) occupational chemical exposure (e.g. asbestos, pesticides, solvents, dyes, coal/gas, wood dust), expressed as ever in contact (YES/NO) and further selected those with a high likelihood for a chronic exposure (at least one year in a working environment with potential contact to mutagenic and carcinogenic chemicals on a regular basis) (YES/NO), (v) occupational radiation exposure, expressed as ever in contact (YES/NO) and further classified into three groups of likely radiation exposure according to the evidence provided (0: unlikely, 1: radar, 2: potential for exposure), and (vi) medical/diagnostic radiation exposure, expressed as number of x-ray scans in lifetime (three categories, 0–4 x-ray scan, 5–9 x-ray scan, $\geqslant$10 x-ray scan), CT scan in lifetime (YES/NO), other medical scans (e.g. DEXA, radionuclide, fluoroscopy, coronary angiogram) (YES/NO). From the questionnaire data on a potential medical/diagnostic radiation exposure, a quantification to roughly estimate the total lifetime dose was conducted for all veterans. This was performed using average patient dose information for such procedures (Patient dose information: guidance-GOV.UK (www.gov.uk) and recorded in units of mSv and Gy $cm^{-1} > 2$).

4.4.2. Sample size

The target for the GCFT study was to recruit 50 NT and 50 matched controls for analysis [10]. Original statistical power calculations, conducted on the basis of Wahab *et al* for the frequency of reciprocal translocations, were performed for varying sample sizes [24]. These showed that a sample size of 30 NT and 30 control veterans with a minimum of 50 metaphase cells from each veteran would be sufficient to identify 1.5 counts per 100 cells above the control background as statistically significant (number of cells with translocations; 0.9% in control group vs. 2.4% in NT veterans according to Wahab [24]. Further, analysis of 50–200 metaphase cells from each sample was deemed as sufficient to identify statistically significant differences above control. In this study, the number of metaphase cells per sample ranged from 78 to 390 (median = 196), with less than 150 cells analysed in 18 samples (figure 1). The variation in aberration frequencies in the control veterans was higher than expected which violated the assumptions of the original sample size calculations. None of the chromosome aberrations types provided any indications for a potentially overlooked cohort-difference as a consequence of an underpowered study: e.g. by increasing the study sample size of 38 control and 48 test veteran samples to 76 control and 96 test veteran samples by simple doubling the existing M-FISH data would have not changed the statistical outcomes.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Acknowledgments

We thank all veterans for their participation in this project and, all families for their support, patience and involvement in the wider GCFT study. Given the scale of the work undertaken we relied upon the technical support, particularly with the processing and initial analytic stages of M-FISH painted cells, from post-graduate and undergraduate students within the Department of Life Sciences at Brunel University London. Our thanks go to Aqeela Amanullah, Ninoshka Barros, Siobhan Casha, Afsana Chowdhury, Daniel Horton, Zurka Jabar, Rachel Kerslake, Felicia Kruse, Enny Ocwee, Shania Susumaran, and Samiha Quiyum. We are also extremely appreciative of the time given by Prof Dudley Goodhead OBE and Dr Mark Hill who have acted as independent experts to offer oversight for this project and for their useful comments on this manuscript. Lastly, I would like to acknowledge Prof Yuri Dubrova who sadly passed away earlier this year. Yuri, together with his team at the University of Leicester, worked with us to generate germline mutation

frequencies of families recruited to the GCFT study. It was through Yuri's work that our examination into mutational patterns, as described in Moorhouse *et al* [22] and further discussed in this manuscript, first began.

Funding

This work was, in part, supported by the Nuclear Community Charity Fund (NCCF) through funds received by The Armed Forces Covenant Fund Trust under the Aged Veterans Fund Grants AVF15A and AVF16. The funding organization had no role in the design and conduct of the study; in the collection, management, analysis and interpretation of the data; or in the preparation, review or approval of the manuscript.

Author contributions

R A and J P acquired the funding, conceptualized the study and supervised the work; R A, J P, C G, C R and M S devised the methodology; K L, J S, F D, E A, K C, M S and R A performed the sample processing and analysis; R A performed project administration; R A, K L and M S wrote the manuscript with contributions from C G and J P.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval and consent to participate

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

Consent to participate

Written informed consent was obtained from all subjects.

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