AWARD NUMBER: W81XWH-18-1-0343

TITLE: The MexTAg Collaborative Cross: Understanding Genetic Modifiers in Mesothelioma

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CONTRACTING ORGANIZATION: The University of Western Australia

REPORT DATE: MAY 2022

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MAY 2022	Final	15 JUL 2018 - 31 JAN 2022

4. TITLE AND SUBTITLE

The MexTAg Collaborative Cross: Understanding Genetic Modifiers in Mesothelioma

5a. CONTRACT NUMBER
W81XWH-18-1-0343

5b. GRANT NUMBER
CA170299

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release, Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This is the final progress report for award CA170299. In the last 12 months we have completed all aims as initially intended. In summary we generated, and asbestos exposed over 2500 CC-MexTAg mice, representing 72 genetically distinct groups. Our analysis demonstrated significant variation in disease phenotype, with a 3-fold change in median survival and disease latency between groups, indicating that host genetics does impact asbestos related disease development. We discovered a variety of qualitative trait loci (QTL) for each of 5 traits (phenotypes) assessed and identified candidate genes and regulatory elements associated with each QTL. These data were used to interrogate human mesothelioma datasets, which enabled the identification of human homologues of the candidate genes and their association with mesothelioma development in patient cohorts. We have identified a number of genes that act either individually or in concert to promote or ameliorate mesothelioma development. The role of these genes in mesothelioma pathobiology remains to be fully investigated and this will be the focus of our ongoing and future work.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	24	USAMRDC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

   Mesothelioma is an incurable cancer caused by asbestos exposure. However, why some people develop disease, while others do not, despite similar exposures remains unknown. There is strong evidence that a person's genes can affect their chance of contracting mesothelioma, but the power of conventional human genetic studies are hindered by small sample sizes and various environmental and lifestyle factors associated with this rare cancer, meaning how a person's genetic makeup affects disease development remains unknown. In this project we have combined two powerful mouse models to discover genes (and their associated biological pathways) that prevent, or delay mesothelioma developed after asbestos exposure. To confirm the importance of candidate modifier genes identified in our study we will compare our results against large human mesothelioma genetic data sets.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

   Mesothelioma, asbestos, Collaborative Cross, MexTAg, CC-MexTAg, host genetics, genetic predisposition, asbestos related disease, disease susceptibility, disease resistance.

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

   What were the major goals of the project?
   List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

   Aim 1: Generate CC-MexTAg mice, expose them to asbestos and assess mesothelioma latency, disease progression and survival in CC-MexTAg mice*.*
   Subtask 1: MexTAg mice crossed with CC lines (months 3-12)
   Subtask 2: Expose MexTAg controls and CCMT progeny to asbestos (months 3-23)
   Subtask 3: Local IRB/IACUC Approval (month 3)

   Aim 2: Identify candidate modifier genes associated with these traits.
   Subtask 1: Genotype and haplotype analysis using a combination of collaborative cross-specific bioinformatics programs commonly referred to as the GeneMiner platform (months 18-36).
   Subtask 2: Gene mapping performed by using phenotype traits such as ARD overall survival as a quantitative trait using our GeneMiner pipeline (months 3-36).

   Aim 3: Identify human orthologs and interrogate human mesothelioma datasets.
   Subtask 1: Identify human orthologues using BLAST of DNA sequences encompassing the peak SNPs and/or best candidate causal SNPs identified in Aim 2 (months 36-41).
   Subtask 2: Human orthologues will be interrogated against publicly available mesothelioma data sets (TCGA) and additional human mesothelioma datasets available via CI Bueno (months 36-41).

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Summary of previous progress reports. In the stage 1 report (15th July 2018-14th July 2019) we reported successful completion or initiation of all Aim 1 subtasks; Local IACUC approval was obtained (subtask 3), 50 of 70 CC-MexTAg groups had been successfully bred (subtask 1) and progeny mice exposed to asbestos (subtask 2). At the time of submission limited data was available as the majority of CC-MexTAg groups remained on study with only a few CC-MexTAg mice having reached an experimental endpoint, thus Aims 2 and 3 remained to be implemented. We also acknowledged that we had experienced a significant, unforeseen delay in setting up breeding the remaining 20 CC-MexTAg groups, but had implemented a strategy to overcome this delay, which was progressing as planned. We also noted that consistent with the original timeline, time and effort are heavily weighted toward Aim 1 of this study, *viz*., generation and asbestos exposure of CC-MexTAg groups and subsequent collection of phenotypic data and biological samples.

During the stage 2 reporting period (15th July 2019-14th July 2020), we reported successful completion of Aim 1 subtasks 1, 2, and 3 (generation and asbestos exposure of all proposed 70 CC-MexTAg groups). At the time of submission 55 of 70 asbestos exposed CC-MexTAg groups (including a MexTAg control group on the parental B6 background) had completed the study, for which we have complete survival and phenotypic data; 16 of the last 20 CC-MexTAg groups remaining on study. Data from the 55 completed groups were used in preliminary analyses outlined in Aim 2, subtasks 1 and 2 above and were described in detail in the 2nd progress report (Figure 4, Table 1.2). At submission of the 2nd progress report we expected complete survival and phenotypic data for all 70 CC-MexTAg groups to be available by end of May 2021.

In addition to completion of Aims 1 and 2, we had also initiated work on Aim 3. We received local Human Ethics approval (University of Western Australia) and were awaiting on the US DoD Office of Research Protections (ORP), Human Research Protection Office (HRPO) approval (initial application submitted 19th March 2020), prior to commencing interrogation of human mesothelioma datasets to analyse the relevance of human orthologs of candidate modifier genes identified in data generated from Aim 2. We presumed the delay in response was related to COVID-19, and we finally received HROP human ethics approval in May 2021.

In the period June 1st, 2021, to January 31st, 2022, we successful completed all remaining aims. Specifically, we completed collecting data from animal experiments (Aim 2, subtasks 1 and 2; November 2021) and processed and analysed phenotypic datasets using the GeneMiner bioinformatic portal (custom software used for analyzing data derived from the Collaborative cross). Approval was sought (June 2021) and granted (November 2021) to use these data were used to interrogate human mesothelioma datasets (i.e., TCGA and private datasets associated with CI Bueno; Aim 3, subtasks 1 and 2). Analyses of these data are reported below.

Impact of COVID-19 pandemic.
Impact of COVID-19 on Western Australia.
The COVID-19 pandemic has had a significant global impact, with many countries implementing strict restrictions that have impacted scientific research. Fortunately, Australia, and Western Australia in particular, were not affected to the same degree as the United States or many other European countries, although we did experience an extended period of lock down between March and June 2020 and again in February-April 2021, that slowed our research output. Additionally, Western Australia was effectively 'shut off' from the rest of the world (including other Australian states) from March 2020 to March 2022, with no unauthorized movement of people in or out of the state. While this enabled Western Australia to initially contain rampant community

spread of COVID-19, it had a significant impact on the health and wellbeing of many West Australians. Western Australia officially reopened on March 5th, 2022 and has subsequently experienced a significant wave of Omicron COVID-19 infections. Analysis of this project was affected, particularly in April 2022 as I was restricted to working from home due to COVID-19 infection(s) affecting myself and my family during this period.

Impact of COVID-19 on this project.
COVID-19 had a moderate impact on our CC-MexTAg animal work; daily welfare monitoring and experimental procedures continued as normal, with variation to staff rostering to avoid being in the same place and the same time and implementation of continued social distancing and other Western Australian Government mandated COVID-safe PPE measures being the main significant change to normal operation. We experienced multiple, short-term 'lock down' events that severely restricted access to laboratory work during 2020-2021. Delays to some administrative/reporting procedures (i.e., HRPO approval, approval for external sources, human mesothelioma database approvals etc.) were experienced, and our ability to report findings at local, national and international meetings was significantly affected. However, while COVID-19 delayed many aspects or our work, it did not prevent completion this study. Although we are currently experiencing significant COVID-19 Omicron infections (~15,000/day and increasing), we continue to manage the situation and adapt our research / work structure as required. We will continue to inform the US DoD if any significant delays to the remaining analyses are experienced.

Progress July 15th, 2020-January 31st, 2022
Significant outcomes:

To date we have successfully generated and asbestos exposed 71 different CC-MexTAg groups. Furthermore, we have complete data for 72 groups: 71 CC-MexTAg groups and a MexTAg (C57Bl/6) control group. Along with overall survival, we have complete data on four additional phenotypic traits: disease latency (time from asbestos exposure to first signs of disease), disease progression (time from first signs of disease to cull), ascites volume and the total number of mice with tumours for each group. We have also collected tissue samples (ascites, spleen, kidney, liver, diaphragm) and tumour (when present) from each animal as they reached the defined experimental endpoint; either disease development (usually ascites related abdominal distention, or disease related loss of condition), or when animals survived to 18 months from first asbestos exposure (whichever came first). The animal experimental aspect of this project ended on November 26th, 2021. In the last report (May 2021), we applied for, and received a no-cost extension to January 31st, 2022, with the final report due May 31st, 2022, to allow this work to be completed.

A summary of data from all Aims are shown below.

Final Summary
Each mouse was given two intraperitoneal injections of 3 mg asbestos suspended in 0.5 ml PBS at weeks 0 and 4 and survival calculated from the day of injection. We monitored asbestos related disease (ARD) using three metrics: disease latency (time from first injection to first signs of disease; FSD); time to progression (time from first signs of disease to cull); and overall survival (time from first asbestos exposure to cull), which is the sum of the first two measurements (Figure 1A). To date, we have observed considerable variation (three-fold range) in median overall survival between different asbestos exposed CC-MexTAg groups (Figure 1B, C).

Aim 1: Generate CC-MexTAg mice, expose them to asbestos and assess mesothelioma latency, disease progression and survival in CC-MexTAg mice*.*
Subtask 1: MexTAg mice crossed with CC lines (months 3-12)
Subtask 2: Expose MexTAg controls and CCMT progeny to asbestos (months 3-23)
Subtask 3: Local IRB/IACUC Approval (month 3)

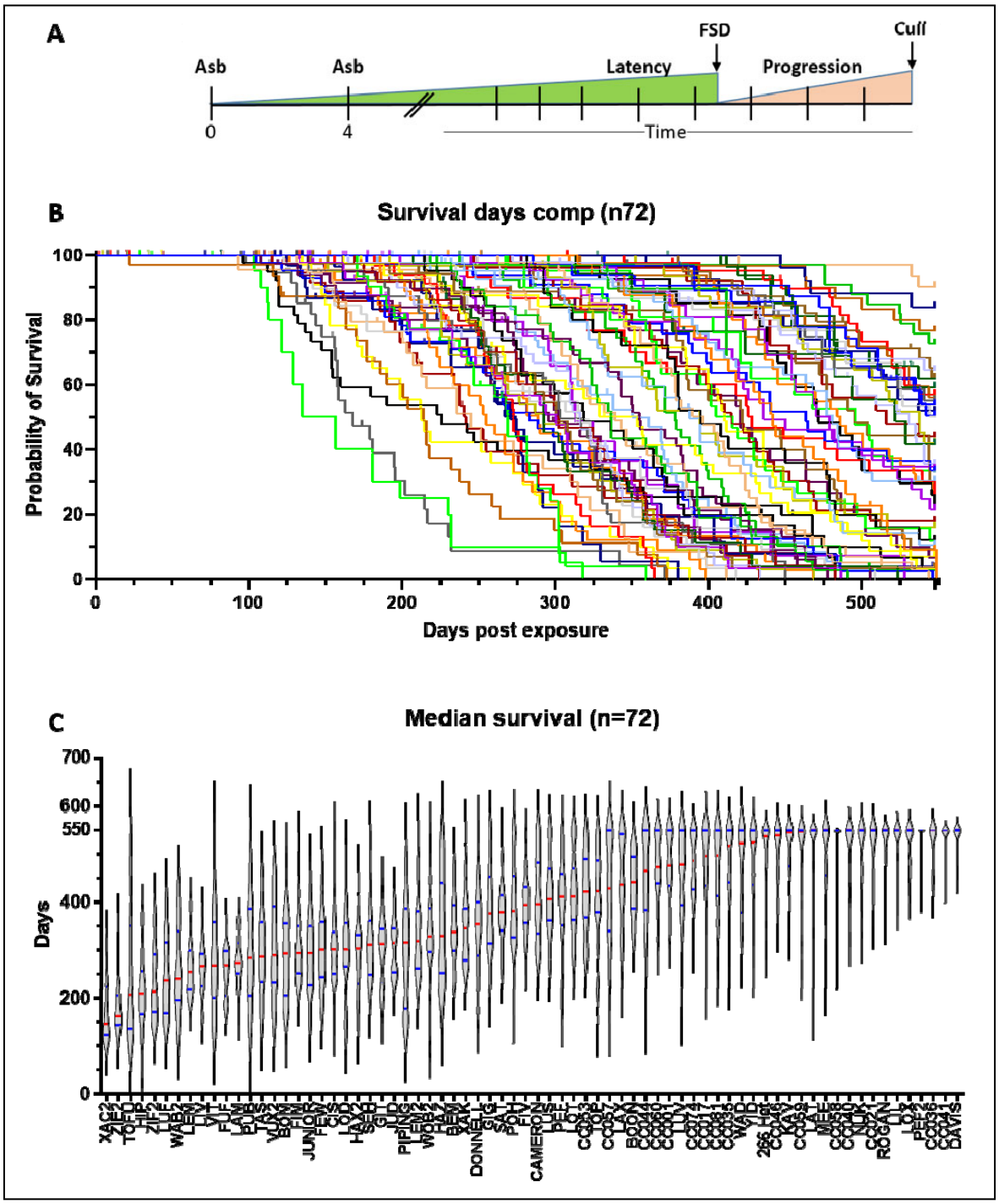


Figure 1: Variation in median survival in asbestos exposed CC-MexTAg mice. Groups of CC-MexTAg mice (n=72, median 36 mice/group) were exposed to a total of 6 mg of asbestos via two consecutive intraperitoneal injections, 4 weeks apart and assessed for disease development over time. (A) Experimental schematic. FSD = first signs of disease. (B) Kaplan Myer survival plot censored for asbestos related disease (ARD). Each line represents a unique asbestos exposed CC-MexTAg group (n=72). (C) Violin plot depicting median overall ARD survival (days; ranked) for each CC-MexTAg group. Red bars = median, blue bars = quartiles.

Further analysis indicated that the observed variation in overall survival was driven by disease latency, but not disease progression (Figure 2A, B). This is supported by the strong correlation between overall survival and latency ($r = 0.9985$, $p < 0.001$), but not overall survival and disease progression ($r = -0.05112$, p = ns; Figure 2).

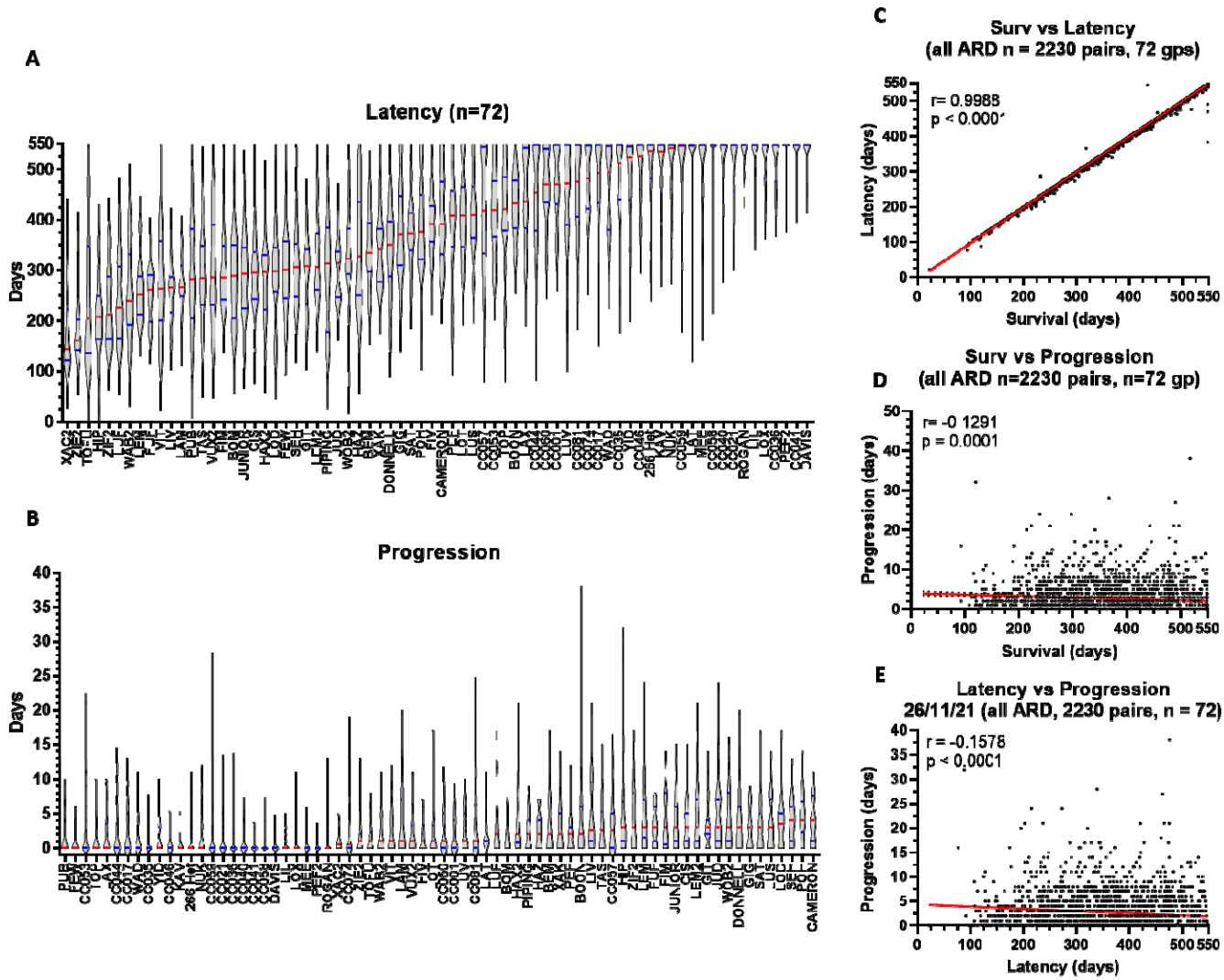


Figure 2: Asbestos related disease latency drives variation in overall survival in asbestos exposed CC-MexTAg mice. (A-B) Violin plots depicting variation in ARD latency and progression (days, ranked) respectively (median, red line and interquartile range, blue lines) for each of the 72 asbestos exposed CC-MexTAg groups. (C-E) Scatter plots depicting correlations (Pearson's) between individual phenotypes for individual mice. Dots represent individual mice.

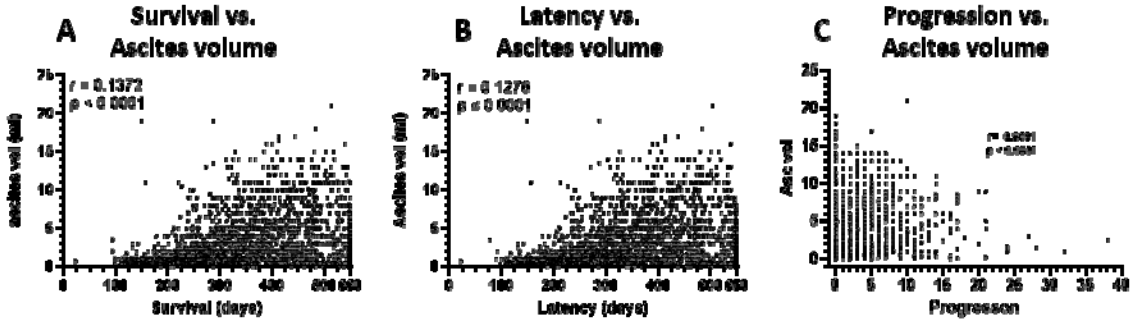


Figure 3: Asbestos related disease phenotype correlations. (A-C) Plots depicting Pearson's correlation analyses between survival, latency and progression relative to ascites volume for individual mice.

Taken together, overall survival and disease phenotype data suggest that in asbestos exposed CC-MexTAg mice, as with the human disease, ARD manifests over time with a strong, significant correlation between overall survival and ascites volume. Furthermore, the discordance between disease progression and all other ARD phenotypes suggests that the influence of host genetics on ARD occurs during disease latency i.e., prior to disease manifestation, but has limited influence on survival once ARD is established.

Histological confirmation of disease and establishment of a of tissue sample biobank. Throughout Aim 1 we collected tissue and tumour samples from individual mice as they complete the study. A list of samples from the 72 different asbestos exposed groups are shown in Table 1. We have collected, prepared and continue to analyse 2558 histological blocks each containing the kidney, spleen, liver and diaphragm from a single animal. The process of cutting and staining sections continues as outlined in the original grant application, with a focus on the CC-MexTAg groups located in the upper and lower 10% of survival. A brief summary of histological finding is shown in Figure 4. A detailed analysis will be included in our final publications. In the absence of overt tumour, histological confirmation of disease was essential prior to GeneMiner analyses.

Table 1 also shows the number of samples collected from the first 72 asbestos exposed groups, including the number of ascites derived cells lines made from each group and the total number of histology samples in each group. To date, we have generated a total of 951 ascites derived cell lines, 51 tumour derived cell lines and 374 tumour samples for RNA sequencing and immunofluorescence / histological analyses. The wealth of data from this program is a significant resource that will undoubtedly prove valuable in future research into ARD. Indeed, the CC-MexTAg sample biobank is currently being exploited as part of a separate Australian Commonwealth National Health and Medical Research Council (NHMRC) funded grant (2019_APP1163861) investigating how the inherent variation in host genetics between different CC-MexTAg groups affects tumour gene expression and immune cell infiltration within the tumour microenvironment.

Table 1: Samples generated from the CC-MexTAg study. Data indicates the number of mice per group for each type of sample. Asc = ascites, PE = pleural effusion. TC = tissue culture, Tum = tumour.

Group	Name	Full CCMT strain name	No# Tum in RNA later	No# Tum TC stocks	No# Asc TC stocks	No# histology samples
C2	266 Het	266 MexTAg Het	4	0	17	26
7	BEM	266-BEM_AG	1	4	7	37
42	BOM	266-BOM_GB	1	0	21	34
52	BOON	266-BOON_HF	23	0	25	28
36	CAMERON	266CAMERON_GA	6	0	23	25
35	CIS	266-CIS_AD	8	3	31	32
23	DONNELL	266-DONNELL_HA	4	5	20	38
28	FEW	266-FEW_FD	0	4	4	29
17	FIM	266FIM-DF	0	1	4	33
21	FIV	266-FIV-AC	0	0 Tum +1PF	7	40
37	FUF	266-FUF_HE	2	1	20	27
50	GIG	266-GIG_EF	20	0	28	32
34	GIT	266-GIT_GC	5	3	34	37
8	HAX2	266-HAX2_EF	0	0	16	34
25	HAZ	266-HAZ_FE	1	0	13	36
12	HIP	266-HIP_GA	0	1	2	28
18	JUD	266-JUD_EF	0	1	12	31
22	JUNIOR	266-JUNIOR_GB	0	0	4	33
53	KAV	266-KAV_AF	14	0	19	27
14	LAM	266-LAM_HD	0	0	15	30
59	LAT	266-LAT_AC	13	0	18	29
47	LAX	266-LAX_FC	16	0	23	33
20	LEM	266-LEM_AF	0	0	13	34
11	LEM2	266-LEM2_AF	0	0	7	35
2	LIL	266-LIL_AF	4	3	6	35
15	LIV	266-LIV_DA	0	1	11	20

13	LOD	266-LOD_AE	0	0	11	26
3	LOT	266-LOT_FC	0	1	15	35
51	LOX	266-LOX_GF	18	1	21	30
19	LUF	266-LUF_AD	0	0	8	27
39	LUS	266-LUS_AH	8	1	25	29
5	LUV	266-LUV_DG	0	1	6	39
58	MEE	266-MEE_AG	7	0	1	31
1	NUK	266-NUK_AC	0	0	4	30
4	PEF	266-PEF_EC	0	0	12	29
57	PEF2	266-PEF2_EC	9	0	0	30
26	PIPING	266-PIPING_BD	5	3	19	35
43	POH	266-POH_DC	8	0	26	29
49	PUB	266-PUB_CD	2	0	27	41
48	ROGAN	266-ROGAN_CF	15	0	18	34
24	SAT	266-SAT_GA	3	3	20	39
10	SEH_	266-SEH_AH	0	0	8	19
41	TAS	266-TAS_FE	10	0	31	37
46	TOFU	266-TOFU_FB	2	0	15	34
9	TOP	266-TOP_DA	0	0	13	33
33	VIT	266-VIT_ED	2	1	21	27
31	VUX2	266-VUX2_HF	4	3	24	34
38	WAB2	266-WAB2_DH	3	0	23	27
55	WAD	266-WAD_HG	2	0	0	13
32	WOB2	266-WOB2_DH	2	2	31	39
6	XAC2	266-XAC2(LID)_HG	0	0	4	20
16	XAK	266-XAK(LOY)_AG	0	4	14	39
45	YID	266-YID_FH	26	1	33	37
27	ZIE2	266-ZIE2_AH	0	0	2	26
30	ZIF2	266-ZIF2_FC	0	0	6	31
62	IL57	266-CC040/TauUnc	6	0	1	32
61	IL3912	266-CC059/TauUnc	6	0	3	30
60	OR5346	266-CC046/Unc	3	0	0	29
64		266-CC044/Unc	14	0	33	33
63		266-CC057/Unc	8	0	0	31
66		266-CC058/Unc	7	0	0	29
65	OR3140	266-CC001/Unc	13	0	14	26
67	OR3015	266-CC074/Unc	13	0	2	40
68		266-CC060/Unc	8	1	17	27
69	DAVIS_BA	266-DAVIS_BA	2	1	0	25
74		266-CC017/Unc	9	1	2	27
75		266-CC021/Unc	7	0	11	26
71		266-CC053/Unc	7	0	2	30
70		266-CC035/Unc	5	0	1	25
73		266-CC036/Unc	14	0	0	27
76	IL4141	266-CC041/TauUnc	1	0	2	31
77		266-CC081/Unc	3	0	25	21
Total		**72**	**374**	**51**	**951**	**2212**

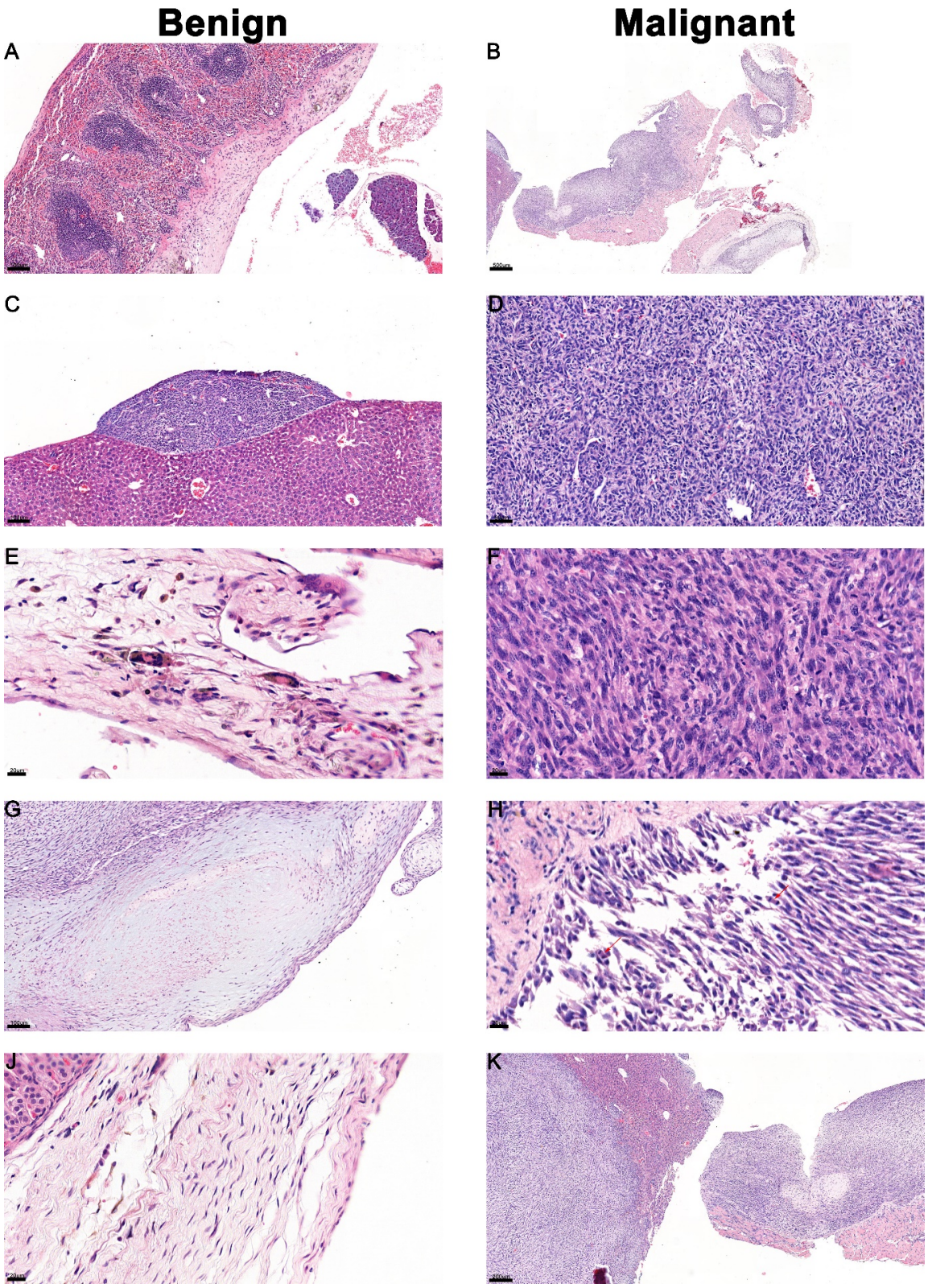


Figure 4: Summary of histological findings: Figure 1: Representative images of benign (A, C, E, G, J) and malignant (B, D, F, H, K) histological features. Benign features include thickening (A), plaque presence (C), Giant cell presence (E), paucicellular regions (G) and regular nuclei (J). Malignant features include overt tumour (B), hypercellularity (D), nuclear atypia (F), mitotic figures (H) and invasion (K). Scale bars 20 µm (E, F, H & J), 100 µm (A, C, & G), 200 µm (K) and 500 µm (B & D).

Aim 2: Identify candidate modifier genes associated with these traits

Subtask 1: Genotype and haplotype analysis using a combination of collaborative cross-specific bioinformatics programs commonly referred to as the GeneMiner platform (months 18-23).

Subtask 2: Gene mapping performed by using phenotype traits such as ARD overall survival as a quantitative trait using our GeneMiner pipeline (months 3-21).

Identification of candidate modifier genes associated with asbestos related disease development was achieved using the GeneMiner analysis platform developed by Professor Grant Morahan at The University of Western Australia. Upgrades to the GeneMiner platform to enable incorporation of the additional 20 CC strains obtained from UNC were completed in November 2021. This allowed full analysis of all phenotypic parameters associated with the 72 asbestos exposed CC-MexTAg. A comprehensive analysis of all 72 CC-MexTAg groups is detailed below.

GeneMiner analysis identified multiple 'suggestive' qualitative trait loci (QTL) observed for each asbestos related disease (ARD) trait/ phenotype. The QTL are considered suggestive at this stage as a LOD score of > 7 is required for statistical significance. Analysis for the ARD 'overall survival' phenotype (i.e., first asbestos exposure to cull) is shown in Figure 4, with complete data for all ARD phenotypes summarised in Table 2.

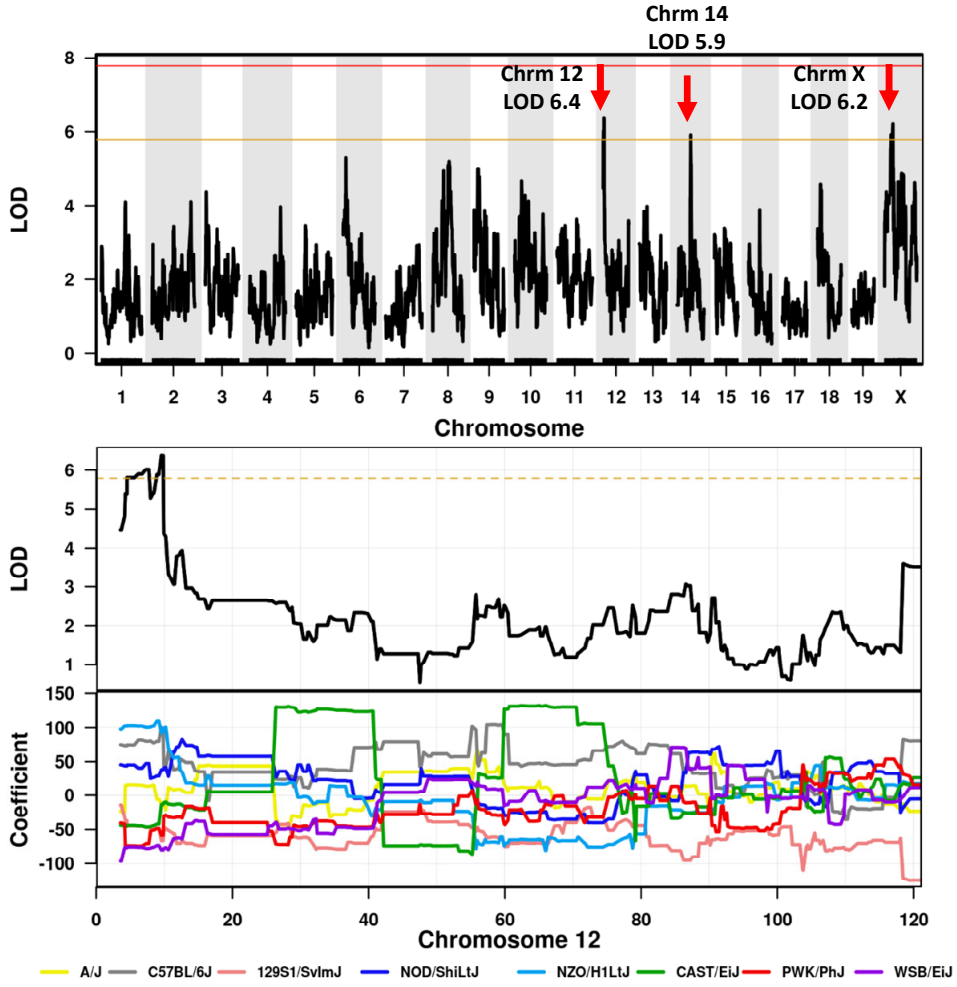


Figure 5. Identification of qualitative trait loci (QTL) associated with overall survival in asbestos exposed CC-MexTAg mice. QTL analysis performed using the GeneMiner platform was used to analyse overall survival data from 72 completed CC-MexTAg groups. **(Top)** Genome wide scan comparing overall survival between different CC-MexTAg groups. The X-axis shows chromosome position and the Y-axis shows the logarithm of odds values (LOD = -log10(P), where P values were derived from CC linkage haplotype data). The gold line indicates 'suggestive' threshold, red line indicates 'highly significant'. Suggestive QTLs were identified on chromosomes 12, 14 and X. **(Middle) Founder coefficient plot. (**top half) The -log10(P) values across chromosome 12. **(Bottom)** The plot of the calculated log odds ratio of eight founder alleles over chromosome 12, where the founders are color-coded.

The genome wide scan on data obtained from 72 completed CC-MexTAg groups indicated 3 suggestive QTL associated with median survival as the phenotypic trait with LOD scores greater that 5.9 on: chromosome 12 (peak QTL position 9.5 mega base (Mb) to 9.8 Mb (LOD 6.4); chromosome 14 (peak QTL position 66.8Mb, range 66 Mb to 68 Mb, LOD 5.9) and the X chromosome (peak QTL position 35.293 Mb (LOD 6.2), with a large area of high LOD score (5.9) between 23.523Mb to 34.329 Mb (Figure 5 top panel). Closer analysis of

each QTL was performed to identify exactly where each peak QTL was positioned along the respective chromosome. Focusing on Chromosome 12, our analysis indicated that genes associated with the peak QTL were located within a 300-kilo base (kb) region between nucleotide positions 9.5 Mb and 9.8 Mb on chromosome 12 (Figure 5 middle panel). The probability that gene variants (alleles) from any particular CC founder stain contributes to the peak QTL is represented as a greater deviation from 0 in founder coefficient plot, within the peak QTL region (Figure 5 bottom panel, deviation from midline '0'). These data indicate that alleles from CC founder stains NZO (light blue line) and C57BL/6 (grey line) have a strong positive association with median survival (founder coefficient >100 on chromosome 12 between 9.5Mb and 9.8 Mb), while the alleles from CC founder stain WSB (purple line) are negatively associated with survival (founder coefficient <100).

Table 2: ARD associated peak QTL from 72 asbestos exposed CC-MexTAg groups

Phenotype/trait	Peak QTL location (chromosome) LOD >6
Overall median survival	12, 14, X
Latency	12, 14, X
Progression	none
Ascites volume	3, 6, 8, 9 and 12*

Table 2 summarises the quantity and location of peak QTL identified for each phenotypic trait for asbestos exposed CC-MexTAg mice. Consistent with earlier data, both survival and latency demonstrate identical QTL locations, further suggesting that the observed variation in overall survival was driven by disease latency. A statistically significant QTL (*LOD >7) was identified on chromosomes 12 when 'ascites volume' was used as the phenotypic trait. Although, this QTL seems unrelated to the other QTL identified for median survival / latency located on chromosome 12 as they occur at different chromosomal locations. We next used the publicly available mouse genome informatics database (http://www.informatics.jax.org/) to identify both coding (known and predicted genes) and regulatory elements (regions that affect or regulate gene expression) associated with each peak QTL. These analyses have identified potential candidate modifier genes and regulatory elements associated with asbestos related disease development.

A summary of known potential modifier genes (*viz*., known/predicted protein coding genes or pseudogenes) associated with each phenotypic trait are shown in Table 3 ('other regulatory elements' are not shown in this report for brevity). Hear we have identified two known protein coding genes located at the peak QTL on chromosomes 12 and 14 associated with median survival / latency as the phenotypic traits. Expanding the QTL region to either side of the peak QTL identified an additional 80 candidate modifier genes located on chromosomes 14 and X. Likewise, 91 potential pseudogene sequences were identified across the three QTL regions. Assessment of potential candidate genes associated with ascites volume as the phenotypic trait are ongoing.

These data provide potential starting points with which to interrogate human mesothelioma genomic datasets for potential human gene homologues that may act as modifier genes for asbestos related disease (Aim 3 below).

Table 3: Concise summary of identified murine modifier genes associated with asbestos related disease median survival / latency.

Phenotypic trait	Suggestive QTL Chrm No# (position)	Feature type	DNA segment	protein coding gene	pseudogene	QTL
Median overall survival / Latency	12 (LOD 6.4) 9.5Mb-9.8Mb	No# @ QTL	0	**1 (*Osr1*)**	3	**1**
		No# in QTL range	0	0	1	0
	14 (LOD 5.9) 66Mb-68Mb	No# @ QTL	0	**1 (*Adra1a*)**	0	0
		No# in QTL range	13	**28**	8	**2**
	X (LOD 6.2) 35.293 Mb	No# @ QTL	1	0	1	**1**
	X (LOD >5.9) 23.52 Mb-34.32 Mb	No# in QTL range	0	**52**	78	**2**

No# @= number at. QTL = qualitative trait loci

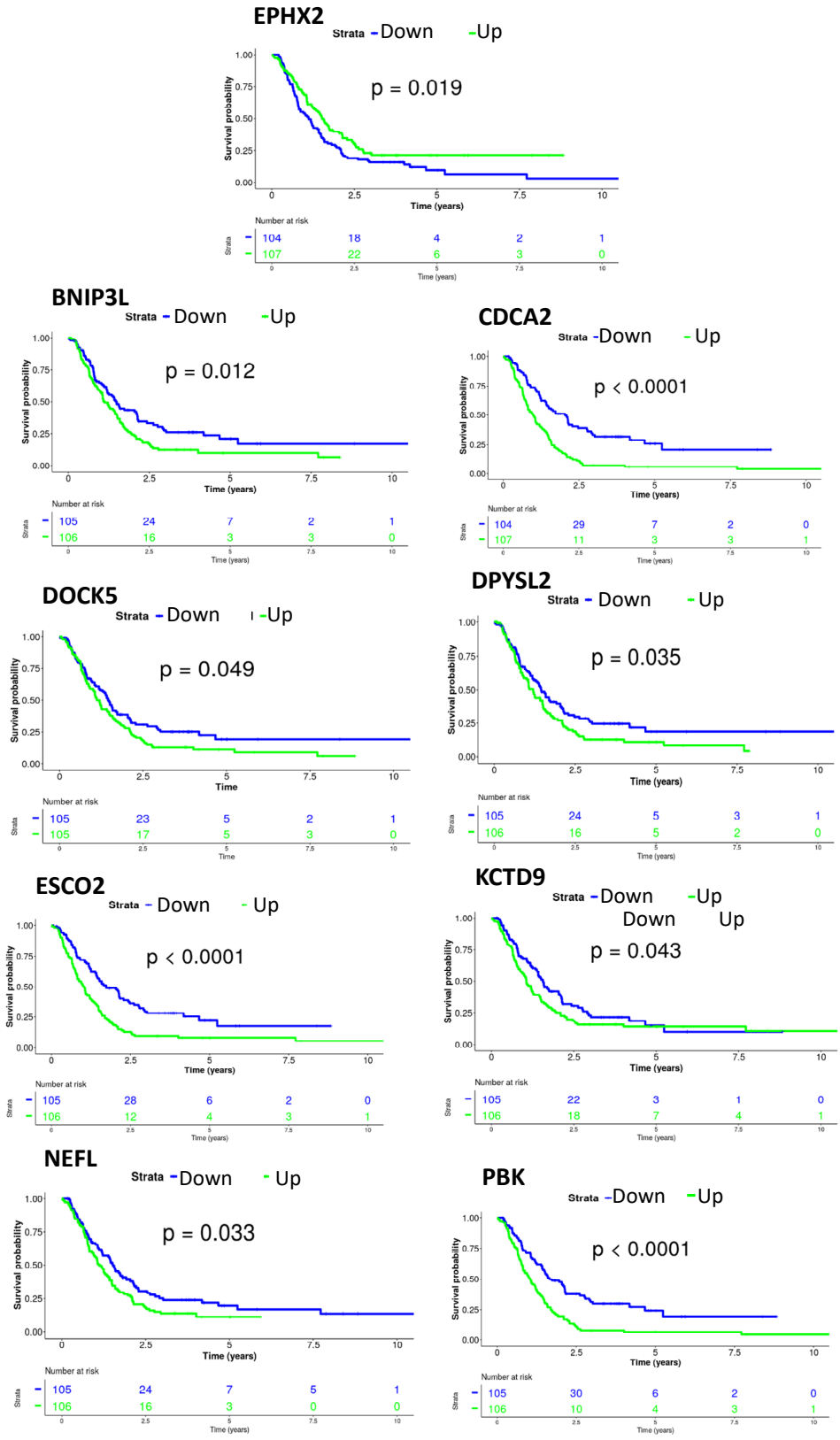
Aim 3: Identify human orthologs and interrogate human mesothelioma datasets.

Subtask 1: Identify human orthologues using BLAST of DNA sequences encompassing the peak SNPs and/or best candidate causal SNPs identified in Aim 2 (months 21-24).

Subtask 2: Human orthologues will be interrogated against publicly available mesothelioma data sets (TCGA) and additional human mesothelioma datasets available via CI Bueno (months 22-24).

Work on Aim 3 was significantly delayed due to issues associated with obtaining US DoD Office of Research Protections (ORP), Human Research Protection Office (HRPO) approval; the initial application was submitted 19th March 2020, final approval granted 19th May 2021. We are not sure why approval took so long and can only assume the delay was COVID related. None-the-less, we have since been able to obtain access to de-identified / anonymized human mesothelioma datasets and have performed preliminary analyses. We experienced an additional delay (May 2021-November 2021) in obtaining access to data derived from Prof Raphael Bueno mesothelioma tumour database as it was revealed that access to these data were controlled by Genentech. Subsequent to Genentech approval, the datasets were found to be corrupt (January 2022), and it took and additional 2 months before Genentech rectified the issues (March 2022). None-the-less, we now have these data and preliminary outcomes are discussed below, while in-depth analyses remaining ongoing. Additionally, we have accessed publicly available cancer databases such as The Cancer Genome Atlas Program (TCGA, n=72 mesothelioma samples) supported by the National Cancer Institute (NCI, USA). Initial interrogation of these databases identified human orthologs (i.e., the human version of mouse genes) of many of the murine candidate genes or regulatory regions that we identified in Aim 2. Data from these preliminary analyses are shown in Figure 6 below.

To interrogate human mesothelioma datasets, we focused on the top twenty-six (26) human homologues of the 82 murine candidate modifier genes associated with ARD related QTL identified during GeneMiner analysis (Aim 2). We assessed the potential for each candidate gene to affect survival using the publicly available TCGA mesothelioma dataset and a private dataset of 211 mesothelioma patient RNAseq samples from Professor Raphael Bueno. First, we performed univariate analyses of the individual expression profile of each candidate gene and assessed how their expression (above or below the median) correlated with patient survival. Interestingly, human homologs of the two murine genes located at the peak QTL on chromosomes 12 and 14 in the CCMT GeneMiner analyses (*Osr-1* and *Adra1a* respectively) did not significantly correlate with patient survival. However, we identified eight candidate genes (*PBK, CDCA2, ESCO2, NEFL, DPYSL2, KCTD9, DOCK5 and BNIP3L*) in which higher expression correlated with poorer survival, while higher expression of



one gene, epoxide hydrolase 2 (EPHX2) correlated with improved survival (Figure 6). Expression levels of the remaining genes had no significant impact on survival outcome (data not shown). Human homologues of 5 of the 26 murine candidate modifier genes (STMN4, ADAM2, ADAM7, NEFL and EBF2) could not be assessed as they were absent from the TCGA dataset (data not shown). However, we did identify four genes (DOCK5, KCTD9, ESCO2 and CDCA2) in both datasets that impacted patient survival.

Figure 6: Univariate modifier gene expression affects patient survival. Kaplan Myer plots of candidate modifier genes that had a significant impact on patient survival (n=211 RNAseq samples from RB dataset). Green line above median expression. Blue line below median expression. Only expression of EPHX2 improved patient survival.

We next performed multivariate Cox proportional analysis on 24 human homologues of murine modifier candidate genes in association with patient age, sex and histological subtype to identify independent predictors of survival (Figure 7). Multivariate analysis indicated that upregulation of four genes; *ADAMDEC1, CDCA2, DPYSL2,* and *STMN4* were associated with poor survival, while upregulation of *SCARA3* was associated with improved survival. Interestingly, only *CDCA2* and *DPYSL2* remained significant following multivariate analysis, whereas individual expression of *ADAMDEC1*, *STMN4* and *SCARA3* did not have a significant influence of patient survival.

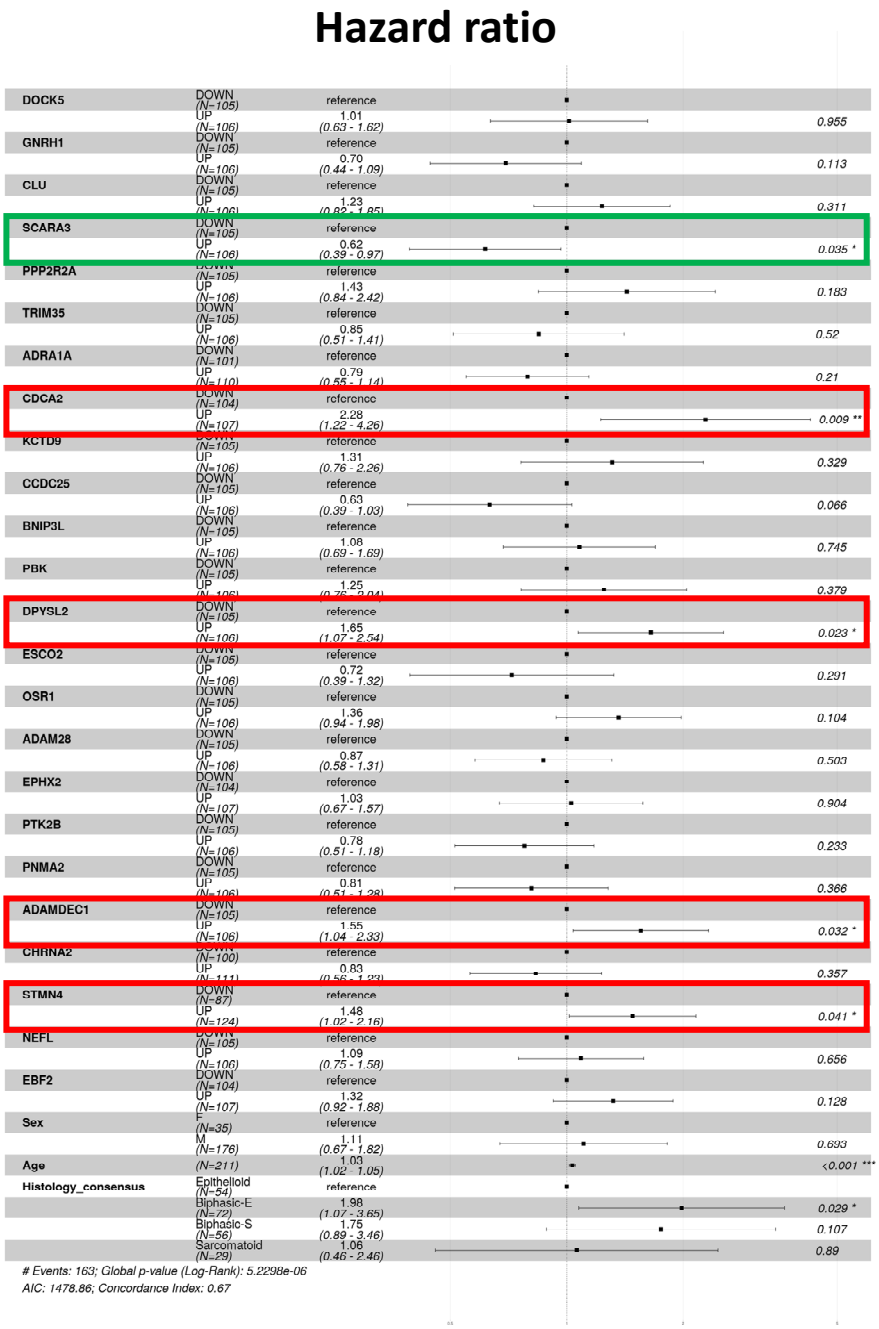


Figure 7: Multivariate cox proportional analysis for identifying independent predictors: Forrest plot of hazard ratios for overall survival with age, sex and histology as covariables. Estimated hazard rations are shown by black boxes and 95% confidence intervals are shown in whiskers. Wald test q-values are shown on the right. DOWN Correspond to gene expression below median, Up correspond to expression above median. DPYSL2, CDCA2, SCARA3, ADAMDEC1 and STMN4 were identified as independent predictors of survival. Upregulation of CDCA2, DPYSL2, ADAMDEC1 and STMN4 (red boxes) are associated with poor prognosis (red boxes). SCARA3 upregulation is associated with better prognosis (green box).

Taken together, these data demonstrate the complex relationship between host genetics and asbestos induced mesothelioma development. We have identified a number of target genes that we are continuing to investigate in relation to their role (individually or in concert with other identified genes) in asbestos induced disease development. Future work will assess the biological pathways/network that these genes affect and their role in mesothelioma development. These data are essential to better understand the underlying pathobiology of asbestos related disease development and will provide the scientific rationale for the development of new and improved treatment options.

Publications & presentations

Throughout the duration of this research program, we have had numerous opportunities to present our research data at local, national and international meetings.

2022: Invited speaker. UWA Cancer Research Cluster seminar series Perth, Australia. (Sept 2022)
2022: Invited speaker. Telethon Kids Institute seminar series, Perth, Australia (August 2022)
2021: Invited speaker. Lung Club (Local), Perth, Western Australia.
2021: Invited speaker. 15th International Mesothelioma Interest Group (iMig). Brisbane. Australia.
2019: Invited speaker. Asbestos safety and eradication agency (ASEA) conference. Perth. Australia.
2019: Invited speaker. Aust. & New Zealand Laboratory Animal Association. Perth, Australia.

2019: Invited speaker. UWA Animal Care Services Seminar series, Perth. WA.
2018: Invited speaker. 14th International Mesothelioma Interest Group (iMig). Ottawa. Canada.

With respect to publications, an invited article for a special edition of Frontiers in Oncology focusing on the immune-tumour microenvironment of thoracic cancers has been published (Behrouzfar K, Burton K, Mutsaers SE, Morahan G, Lake RA and Fisher SA (2021) How to Better Understand the Influence of Host Genetics on Developing an Effective Immune Response to Thoracic Cancers. Front. Oncol. 11:679609. doi: 10.3389/fonc.2021.679609). Additionally, a manuscript reporting on data form the first 30 CC-MexTAg group is currently in preparation (submit June 2021) and progress on the full CC-MexTAg study is scheduled for August 2022.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

This project has allowed the training of 2 research assistants (RAs) and a number of both Honours (a short 10-month post BSc research program) and PhD students with respect to *in vivo* (animal welfare monitoring / vivisection) and *in vitro* (cell culture, RNA sequencing and immunofluorescence) related work under the supervision of Dr Scott Fisher.
We currently have 1 PhD student working on the NHMRC grant that utilised the CC-MexTAg biobank samples that were generated from this study. Additional grants utilizing the CC-MexTAg biobank samples are also in preparation.

Professional Development
All personnel associated with the daily running of this project (i.e. the students, RAs and Dr Fisher) have been afforded the opportunity for professional development primarily via presentation of work from this project at numerous local, national and international symposia and conferences (some listed above).

Dr Fisher has also undergone professional development with Prof Morahan's group with respect to the use and interpretation of the GeneMiner bioinformatics analysis platform.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Throughout the duration of this project and especially in the 12 months between July 2020-July 2021, we have had the opportunity to present updates on this project at both local (academic departments within the University of Western Australia and related Medical research institutions), and national meetings (Australian and New

Zealand Laboratory Animal Association (ANZLAA), Sept. 2019 and the Asbestos Safety and Eradication Agency (ASEA), Nov 2019), and at the 15th Meeting of the International Mesothelioma Interest Group (IMIG) in May 2021 (held virtually due to the initial conference scheduled in March 2020 being affected by COVID-19 restrictions). Additional presentations of the completed work will be given in August and September 2022 (see above). In addition to the above presentations, we are fortunate to be able to disseminate our work to a number of asbestos consumer/advocacy groups. These are often associated with the Asbestos Diseases Society of Australia (ADSA) and are mostly a non-scientific forum aimed at informing the general public and asbestos affected individuals and their families about the current progress of research related to mesothelioma and asbestos related diseases. This is usually via an annual event (Perth Mesothelioma symposium, held in October/November each year), but may also include short, invited talks at local ADSA meetings/branches on an ad hoc basis. We are also able to disseminate research updates to the asbestos consumer/advocacy groups via our monthly National Centre for Asbestos Related Diseases (NCARD) newsletter, website www.NCARD.org.au and other social media platforms such as Twitter (@NCARD_research) and Facebook (NationalCentreforAsbestosRelatedDiseases).

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

As this is the final report, we have nothing to report in this section.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Identification of modifier genes affecting mesothelioma development provided by this study will advance our base knowledge of mesothelioma biology. Furthermore, the CC-MexTAg biobank comprised of tumour and tissue samples taken from over 2500 CC-MexTAg mice, representing 72 genetically distinct CC groups with differential disease development (i.e. short vs. long overall survival, indolent vs. rapid tumour development, variable ascites development) provides a unique biological resource and dataset that will form the foundation for a long-term, asbestos induced mesothelioma research program. Indeed, the successful funding for this US Dept. Defense Ideas Award, we (CIs Lake, Morahan and Fisher) has already led to additional funding from the Australian National Health and Medical Research Council (NHMRC) for phase 2 of this study, in which we are undertaking comprehensive gene expression analysis and multiplex histological profiling on CC-MexTAg derived tumour samples. Collection of solid tumour samples from asbestos exposed mice with variable genetic backgrounds is an invaluable resource for a variety of future genetic based studies where we can assess the association between various genomic data (whole genome sequencing, somatic mutations, transcriptome, methylome etc.) with respective disease phenotypes (survival, latency, progression, ascites volume and presence of tumour.). Together, these data will provide the necessary information to better understand the biological pathways associated with mesothelioma susceptibility and progression; knowledge that is fundamental for the

rational development of new diagnostic and therapeutic strategies for mesothelioma and which complements the molecular studies that are currently being pursued using clinically obtained human mesothelioma samples.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

This project brought together key disciplines including systems genetics and cancer biology to advance our understanding how an individual's genetic background affects the underlying biological processes associated with mesothelioma. To date we have established a unique animal model, in a 'first of kind' study for mesothelioma, to rapidly identify disease associated modifier genes; something that cannot be achieved using any other animal model. The unique approach of this study and the data and resources it generated will enhance our knowledge in the fields of both mesothelioma and systems genetics as well as providing unique genetic and phenotypic information for publicly available databases (i.e. mouse phenome database).

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Currently there is nothing to report. However, we will make all reasonable attempts to have the phenotypic data produced from this study entered into publicly accessible data repositories such as the mouse phenome / mouse genome informatics databases when / where appropriate.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report as yet. However, there is potential for identified modifier genes to be used in future screening of asbestos exposed individuals to assess the relative risk of developing mesothelioma.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

   Apart from minor disruptions to our usual work environment due to COVID-19 related issues, there have been no significant delays that have impacted our ability to complete or perform this study as outlined in the original application and funding agreement. All challenges/problems that we encountered during this project we're resolved adequately, and we were able to complete all aims.

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

During this project we requested and received approval for two 'no cost extension' to carry over grant funding until January 31st 2022. These requests were based on; a) the delay to breeding the last 20 CC-MexTAg groups that we experience in early 2019 (during the previous reporting period), and b) delays to aim 3 associated with obtaining HPRO approval for use of anonymized human datasets and delays associated with obtain access to non-corrupt dataset from Genentech. The funding extensions allowed payment of animal husbandry and agistment costs associated with completion of breeding and experimental work related to the last 20 CC-MexTAg groups (to Nov 2021) and all Aim 3 costs.

All aims are now complete as setout in our original application. We are grateful to the US DOD for supporting this work as the data generated in Aim 3 is vast, and will allow us to continue to interrogate human mesothelioma datasets and utilize our findings to inform our current and future research.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

There haven't been change that have had a significant impact on expenditure, other than 'delays' associated with receiving invoices for animal related work (agistment, etc.). There is usually a 2 to 3 month delay in receiving invoices from UWA Animal Care Services although payment is promptly made when received.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

We experienced two relatively significant delays related to the use of human data sets. The first being delays associated with obtaining HRPO approval for use of anonymized human mesothelioma patient datasets. Our

initial application was submitted March 2020 and final approval was not granted until May 2021. We can only presume COVID-19 related issues in the US delayed to processing of our application. The second was an unexpected delay in obtaining initial approval to access Prof Raphael Bueno's private mesothelioma patient dataset (curated by Genentech; a process that took longer than expected, despite reassurance that it was 'simple') and then releasing that the datasets provided were corrupt, which required extra time for Genentech to 'fix' the problem. None-the-less, once access was approved and correct datasets provided, work progressed smoothly.

Significant changes in use or care of vertebrate animals

The only significant change in animal ethics during this research project was that we had to update the breeding and experimental animal ethics protocols related to the CC-MexTAg work. This was required as the local IACUC approval (UWA AEC protocols) had reached the 5-year expiry limit. The new CC-MexTAg breeding (RA/3/300/131) and experimental protocols (RA/3/100/1730) have received local IACUC and US DoD ACCURO approval.

Significant changes in use of biohazards and/or select agents

None to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  In the last 12 months we have published one review article and have at least 3 manuscripts in preparation related to the US DoD funded CC-MexTAg project. Additional manuscripts associated with a PhD project that utilized the CC-MexTAg biobank (NHMRC funded work) are also in preparation for publication H2 2022/ H1 2023.

  Published
  Behrouzfar K, Burton K, Mutsaers SE, Morahan G, Lake RA and Fisher SA (2021) How to Better *Understand the Influence of Host Genetics on Developing an Effective Immune Response to Thoracic Cancers*. Frontiers in Oncology. 11:679609. doi: 10.3389/fonc.2021.679609

  In preparation*:*
  Asbestos related disease as a complex trait: host genetics affects disease onset after asbestos exposure. Scott A. Fisher, Kimberley Burton, Elly Marcq, Tracy Hoang, Pristina Goh, Sylvia Young, Anna K. Nowak, W. Joost Lesterhuis, Bruce W.S. Robinson, Grant Morahan, and Richard A. Lake.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title*

of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

None to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

None to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  www.NCARD.org.au (Lab website, limited scientific data; URL links to published work).

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  None to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  None to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - Biobank of CC-MexTAg derived histological (tissue) and tumour samples, cell lines, RNA derived from CC-MexTAg tumours.

  - CC-MexTAg phenotype and GeneMiner analysis databases are generated as the study progresses.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person

month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Professor Richard Lake	No Change
Professor Grant Morahan	No Change
Dr Scott Fisher	No Change
Dr W. Joost Lesterhuis	No Change
Professor Anna Nowak	No Change
Professor Raphael Bueno	No Change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

No Change.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc.,*

available to project staff);

- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

Not applicable. Nothing to report.

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

Not applicable. Nothing to report.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Nil