Original research

Repeat measures of DNA methylation in an inception cohort of firefighters

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ABSTRACT

Objectives Firefighters face exposures associated with adverse health outcomes including risk for multiple cancers. DNA methylation, one type of epigenetic regulation, provides a potential mechanism linking occupational hazards to adverse health outcomes. We hypothesised that DNA methylation profiles would change in firefighters after starting their service and that these patterns would be associated with occupational exposures (cumulative fire-hours and fire-runs).

Methods We profiled DNA methylation with the Infinium MethylationEPIC in blood leucocytes at two time points in non-smoking new recruits: prior to live fire training and 20–37 months later. Linear mixed effects models adjusted for potential confounders were used to identify differentially methylated CpG sites over time using data from 50 individuals passing all quality control.

Results We report 680 CpG sites with altered methylation (q value <0.05) including 60 with at least a 5% methylation difference at follow-up. Genes with differentially methylated CpG sites were enriched in biological pathways related to cancers, neurological function, cell signalling and transcription regulation. Next, linear mixed effects models were used to determine associations between occupational exposures with methylation at the 680 loci. Of these, more CpG sites were associated with fire-runs (108 for all and 78 for structure-fires only, q<0.05) than with fire-hours (27 for all fires and 1 for structure fires). These associations were independent of time since most recent fire, suggesting an impact of cumulative exposures.

Conclusions Overall, this study provides evidence that DNA methylation may be altered by fireground exposures, and the impact of this change on disease development should be evaluated.

Key messages

What is already known about this subject?
⇒ DNA methylation is an epigenetic regulator that is responsive to hazardous environmental exposures, including exposures that firefighters face in the workplace.
⇒ Changes in DNA methylation contribute to the development of many diseases including cancers.

What are the new findings?
⇒ DNA methylation changed at 680 sites throughout the genome in new firefighters when comparing baseline samples to samples collected after 20–37 months of working.
⇒ Proxies for fireground exposures were associated with DNA methylation at 140 of these sites.
⇒ These sites were in genes related to cancers, immune function and other disease pathways.

How might this impact on policy or clinical practice in the foreseeable future?
⇒ DNA methylation changes occur before disease develops.
⇒ DNA methylation profiles could be used to inform occupational risk assessment for firefighters.
⇒ They could also be developed into biomarkers to screen for professionals most at risk for adverse health outcomes that would benefit from prevention efforts.

INTRODUCTION

The fire service is an essential workforce, yet firefighters face occupational hazards including chemical exposures, heat stress, circadian disruptions and mental stressors. Some combustion byproducts that firefighters are exposed to are known or suspected human carcinogens.[1 2] There is evidence for increased overall risk of cancer incidence and mortality when comparing US firefighters to the general population.[3 4] In 2010, a working group of the International Agency of Research on Cancer designated the firefighting occupation as a Group 2B carcinogen, indicating it is possibly carcinogenic to humans but evidence to complete the evaluation was limited.[2] Firefighting exposures have also been associated with death from cardiovascular disease, adverse mental health outcomes and injuries.[5–7]

Diseases linked to chronic firefighting exposures may take decades to develop. It is difficult to accurately assess occupational health risks and to implement appropriate prevention and intervention strategies to protect firefighter health given this timeline. Molecular biomarkers, such as epigenetics, reflect subtle biological changes that occur following exposures that contribute to subsequent disease risk and development. The epigenome consists of modifications to DNA and chromatin that do not alter the underlying DNA sequence. One major type of epigenetic regulation is DNA methylation, which is fairly stable across time and often represses gene expression, especially when in the promoter region of genes.[8] Epigenetic

modifications are responsive to the environment, and reproducible epigenetic signatures of exposures such as cigarette smoking have been identified.[9] [10] Epigenetic perturbations are thought to underlie many human diseases.[11] Epigenetic alterations contribute mechanistically to the hallmarks of cancer,[12] and are also one of 10 key characteristics by which carcinogens may exert their effects.[13] Thus, profiling epigenetic marks in firefighters could serve as biomarkers of cumulative exposures and elucidate in which gene pathways DNA methylation is changing.

We have previously shown differential blood DNA methylation in incumbent compared with new recruit firefighters, adjusted for age and other covariates. Differentially methylated loci between the two groups were in genes previously linked to cancer—*YIPF6*, *MPST* and *PCED1B*.[14] However, this study was limited by comparing new recruits and incumbents sampled at one time point each without data on the incumbents' pre-firefighting epigenetic profiles. We also evaluated associations between exposures to perfluoroalkyl and polyfluoroalkyl substances (PFAS), one of the many chemical classes to which firefighters can be occupationally exposed, with DNA methylation among structural firefighters. We reported statistically significant associations between several PFAS with accelerated epigenetic age and altered DNA methylation at cancer-function and immune-function related genes.[15] However, these previous studies were limited by lack of access to the baseline pre-firefighting epigenetic profiles.

In terms of other biomarkers, we have identified whole blood microRNA (miRNA) expression at cancer-linked miRNAs when comparing new recruits to incumbents[16] and in a longitudinal design comparing matched baseline to follow-up samples from new firefighters.[17] In general, miRNAs with tumour suppressor activity were decreased and miRNAs with oncogenic activity were increased in incumbent firefighters and in follow-up samples from the new firefighters. In addition, in the follow-up sample analysis in the new firefighters some miRNAs were associated with cumulative measures of fireground exposure and also with time since most recent fire. We have designed the current study to investigate changes to DNA methylation profiles in a similar manner to assess whether the methylation status of any genes are associated with chronic fireground exposures, even within a short time of follow-up. The type of fireground exposure—structure fires compared with vehicle, outdoor or other fire types—may also be important in this association. For example, median urinary concentrations of polycyclic aromatic hydrocarbon (PAH) metabolites measured after a fire response were greatest among firefighters who performed interior operations at structural fires.[18]

In this study, we profiled DNA methylation in non-smoking new recruits prior to live fire training and again 20–37 months later in blood leucocyte samples via the Infinium MethylationEPIC.[19] We compared DNA methylation profiles at baseline and follow-up, and assessed associations between proxies of occupational exposure (cumulative fire-hours and fire-runs for all fires and structure fires-only) at loci that changed over time. We hypothesised that differentially methylated genes would have functions relevant to carcinogenesis or response to environmental exposures and that DNA methylation patterns would exhibit a dose–response relationship with proxies of cumulative exposure.

METHODS
Cohort recruitment and study population
New firefighters ('recruits') with no previous live-fire exposures were enrolled from the Tucson Fire Department (Arizona, USA) between 2015 and 2016.[17] Demographics (age, ethnicity, race), height and weight, and current tobacco usage were assessed via

a questionnaire at study enrolment ('baseline'). A follow-up visit was conducted 20–37 months later, and the time in between is herein referred to as the length of service.

Blood samples for DNA methylation measurements were collected at the baseline and follow-up visits by qualified phlebotomists into dipotassium ethylenediaminetetraacetic acid tubes (K2-EDTA; Beckton, Dickinson and Company, Franklin Lakes, New Jersey, USA), and stored frozen until use.[14]

To be included in the analyses described in this paper, participants had to: (1) be new firefighters at baseline, (2) complete both baseline and follow-up visits, (3) provide data on all key covariates, (4) report no tobacco usage at the baseline visit and (5) provide blood samples at baseline prior to live-fire training and at follow-up for DNA methylation analysis. Out of 90 new recruits from 2015 and 2016, 88 (98%) chose to enrol. Of the 76 who had blood drawn prior to live-fire training at baseline, 17 either did not finish recruit training or left the Tucson Fire Department prior to follow-up, 2 declined follow-up participation and 55 completed their follow-up by the time of DNA methylation analysis and met all other inclusion criteria. Of these, 5 failed quality control for at least one of their samples and were excluded from data analysis for a final set of 50 firefighters with data at baseline and follow-up.

Measures of fireground exposures
We collected data from the fire department's response records to compute cumulative hours at fires (fire-hours) and cumulative number of fires (fire-runs) that each individual experienced between baseline and follow-up.[17] The records include information on type of fire, date, time and duration of fire response (in minutes). Here we define a fire-run as responding to one fire (any type of any duration). We computed fire-hours and fire-runs for both all fire types and structure fires only. Fire-runs and fire-hours have been used as measures of cumulative fireground exposure in previous research.[17] [20] We calculated time since most recent fire (in days), using the date of the last fire-run and the date of sample collection, as a proxy for recent/acute exposures.

DNA methylation analysis
DNA was isolated from blood leucocytes, and concentration was measured via a QuantiFluor dsDNA System (Promega) or a Qubit Fluorometer (Thermo Scientific). DNA methylation was quantified using the Infinium MethylationEPIC array[19] following bisulfite conversion via Zymo EZ-96 DNA Methylation kits. Full details on laboratory procedures for this analysis and preprocessing of the data can be found in online supplemental methods.

Statistical analysis
All data preprocessing and statistical analyses were conducted in the R Project for Statistical Computing (version ≥4.00.3). Descriptive statistics were calculated and compared between baseline and follow-up when appropriate using t-tests or χ^2 tests. Singular value decomposition analysis was performed to identify technical and biological covariates that correlate strongly with variation in the DNA methylation data.[21] Cell type proportions, PC representing technical variation and pack-year residuals strongly correlated with DNA methylation.

We first used linear mixed models to identify CpG sites differentially methylated between baseline and follow-up samples. Models were fit to beta values (which represent the proportion methylated) for each CpG site separately. We compared results from two models. The first model adjusted

for PCs capturing >90% of the technical variability (batch effects) and a random intercept for each individual. The second model also included estimated cell type proportions (CD4+ T cells, CD8+ T cells, monocytes and neutrophils), Hispanic ethnicity and pack-year residuals in an effort to reduce confounding bias. Results are reported from the second, fully adjusted model. CpG sites associated with follow-up at a false discovery rate adjusted p value <0.05 (q value) are considered statistically significant.[22] As a sensitivity analysis, we ran the model again excluding the samples from the one female participant; effect estimates remained unchanged at the significant CpG sites (correlation coefficient=0.99). We also ran the model excluding the pack-year residuals since all participants are current non-smokers, and estimates for the significant CpG sites were unchanged.

We examined results at specific CpG sites that were identified as differentially methylated between new recruit and incumbent firefighters and/or were predictive of years in the service in our previous study[14]; 13 of the 16 CpG sites were available in our dataset. We also compared statistically significant CpG sites identified in this study to those previously associated with smoking[10] or air pollution[23] in adults.

We conducted a pathway analysis with the differentially methylated genes at follow-up. The CpG sites that changed over time according to the fully adjusted model (q<0.05) annotated to 408 unique genes included in the Ingenuity Knowledge Base of the Ingenuity Pathway Analysis (IPA) software (QIAGEN Inc, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). IPA uses known relationships between transcriptional regulators and their target genes to test for significant enrichment in gene sets linked to functions and diseases.[24] We used the core analysis function and identified canonical pathways and disease gene sets enriched among our results, and considered pathways with a q value <0.05 to be statistically significant.

We examined associations between cumulative fire-hours and cumulative fire-runs for all fires and structure fires only with DNA methylation at the 680 CpG sites that changed over time. Fire-hours and fire-runs were natural log-transformed after adding one, and used as continuous variables in models. Models were adjusted for cell types, batch PCs, pack-years residual, ethnicity and length of service. The latter was included as a proxy for the cumulative impact of time in the fire service. Relationships between each fire-variable and DNA methylation were considered statistically significant at a q value <0.05. We ran the same models again including adjustment for the most recent exposure, approximated by days since most-recent fire-run (for all fires or structure-fires only; natural log-transformed) and compared results to assess the impact of chronic versus acute exposure.[17]

RESULTS

We compared DNA methylation in blood leucocytes across repeat samples from 50 new recruits with an average of 26.5 (±SD 4.5) months between baseline and follow-up (table 1). Participants were non-smokers by design and primarily male (98%), white (94%) and non-Hispanic (88%). Proportion of monocytes was lower at follow-up (0.11±0.08 at baseline and 0.08±0.03 at follow-up, p value=0.005 for paired t-test for unequal variances). Participants accumulated a range of fire-hours with geometric means of 26.4 (range 8.2–53.6) and 13.2 (range 2.4–28.7) for all and structure fires, respectively. The geometric means (ranges) for fire-runs were 48.4 (22–100) for all fires and 16.3 (4–36) for structure fires.

Table 1 Study population characteristics for 50 new recruit firefighters at baseline and follow-up

	n (%)	Mean (SD)
Male	49 (98)	
Female	1 (2)	
Ethnicity		
Hispanic	6 (12)	
Non-Hispanic	44 (88)	
Race		
White	47 (94)	
Black	3 (6)	
BMI (kg/m²) at baseline		26.0 (2.9)

	Baseline visit	Follow-up visit
	Mean (SD)	Mean (SD)
Age (years)	28.0 (5.9)	30.2 (6.0)
Time in between visits (months)	–	26.5 (4.5)
Fire exposure between baseline and follow-up*		
Fire-hours	–	26.4 (0.4)
Fire-runs	–	48.4 (0.4)
Days since most recent fire		17.7 (1.8)
Fire-hours (structure only)	--	13.2 (0.5)
Fire-runs (structure only)	--	16.3 (0.5)
Days since most recent fire (structure only)		28.9 (2.4)
Estimated cell type proportions (%)†		
Neutrophils	0.55 (0.24)	0.58 (0.10)
CD4+ T cells	0.12 (0.07)	0.13 (0.05)
CD8+ T cells	0.11 (0.06)	0.11 (0.04)
Monocytes	0.11 (0.08)‡	0.08 (0.03)‡
B cells	0.06 (0.03)	0.06 (0.02)
Natural killer cells	0.06 (0.03)	0.06 (0.03)
Estimated smoking§		
Pack years	−20.4 (7.1)	0.8 (6.9)
Pack years residual	−21.7 (7.6)	−0.5 (7.3)

*For the fire exposure variables, fire-hours indicates cumulative time at fires since follow-up; fire-runs indicates the cumulative number of fire-runs since follow-up; These are listed for all fire incident types as well as for structural-only. Geometric means and SD are listed as the distributions were skewed.

†Cell type proportions are estimated from DNA methylation data according to the established algorithm by Houseman et al.[39]

‡Differed across time points (p<0.05 for paired t-test for unequal variances).

§Lifetime smoking pack-years were estimated from the DNA methylation data using an algorithm by Lu et al.[40] All participants reported being non-smokers at time of the study, but this algorithm also estimates past exposure to smoking; values are relative and not absolute.

BMI, body mass index.

Epigenome-wide approach comparing baseline to follow-up

In the main statistical model, 388 CpG sites had lower and 292 had greater methylation at follow-up (q<0.05; online supplemental table 1 shows all 680 CpG sites and table 2 shows the 60 sites with at least 5% methylation difference). Effect sizes and significance values were similar in the model without cell-type adjustment (not shown). The loci with at least 5% methylation change included two each in the genes *FTO*, *PHACTR1* and *PIK3R1*. At follow-up, DNA methylation was higher in a CpG site within the first exon of *FTO* and lower at a CpG site in an alternate promoter. The two sites with decreased methylation at follow-up in *PHACTR1* are in the second intron of the gene, and other CpG sites included on the array in the same region show a similar relationship. The significant loci in *PIK3R1* are in the promoter region of the gene. Genes with one statistically significant loci included non-coding RNA genes (*LINC01420* and

Table 2 Significantly differentially methylated CpG sites when comparing baseline and follow-up samples from new recruits (with q value <0.05 and absolute difference >5%)

Probe ID	Genomic location*	Gene name	Estimate (SE)†	P value	q value
Decreased methylation at follow-up					
cg11835347	chr1: 113248232	RHOC	−0.054 (0.011)	1.09E-05	0.035
cg16265542	chr1: 152814831	LCE6A	−0.058 (0.012)	8.23E-06	0.032
cg21721331	chr1: 157670877	FCRL3	−0.061 (0.013)	2.13E-05	0.043
cg02928345	chr1: 224411698	NA	−0.065 (0.014)	8.08E-06	0.032
cg15591386	chr1: 76265299	MSH4	−0.054 (0.012)	2.67E-05	0.044
cg03740864	chr10: 120660492	NA	−0.054 (0.01)	8.46E-07	0.023
cg02884943	chr10: 72725905	NA	−0.059 (0.013)	1.71E-05	0.040
cg10967759	chr10: 73555025	CDH23	−0.071 (0.015)	7.81E-06	0.032
cg23024158	chr10: 78011952	C10orf11	−0.051 (0.011)	2.36E-05	0.043
cg03762984	chr11: 29355671	NA	−0.081 (0.016)	4.70E-06	0.027
cg01078446	chr12: 115113212	TBX3	−0.05 (0.01)	1.31E-05	0.037
cg18956104	chr12: 119621039	HSPB8	−0.059 (0.012)	3.68E-06	0.026
cg12248652	chr12: 132703589	GALNT9	−0.056 (0.011)	3.64E-06	0.026
cg02121135	chr14: 24858912	NA	−0.073 (0.015)	4.82E-06	0.027
cg02938807	chr14: 63785523	GPHB5	−0.069 (0.015)	2.72E-05	0.044
cg02428178	chr15: 49104237	CEP152	−0.053 (0.011)	9.69E-06	0.034
cg04245616	chr15: 59543693	MYO1E	−0.072 (0.017)	4.20E-05	0.048
cg06297541	chr16: 22384949	CDR2	−0.053 (0.011)	1.35E-05	0.037
cg12891543	chr16: 53958626	FTO	−0.052 (0.012)	3.09E-05	0.045
cg07280206	chr16: 56554249	BBS2	−0.063 (0.01)	1.61E-07	0.016
cg03432196	chr18: 9643805	NA	−0.063 (0.013)	6.43E-06	0.029
cg19995259	chr19: 53795827	BIRC8	−0.056 (0.013)	3.53E-05	0.046
cg16903817	chr2: 114655480	ACTR3	−0.09 (0.019)	7.62E-06	0.031
cg16262572	chr2: 122479703	NIFK-AS1	−0.052 (0.01)	2.53E-06	0.026
cg25296103	chr2: 145268533	ZEB2	−0.06 (0.012)	5.25E-06	0.028
cg00441550	chr20: 29955998	DEFB118	−0.06 (0.013)	2.21E-05	0.043
cg16145176	chr21: 25596030	NA	−0.06 (0.013)	2.32E-05	0.043
cg17885233	chr3: 132450221	NPHP3-AS1	−0.051 (0.011)	1.84E-05	0.041
cg13166622	chr3: 150904863	MED12L	−0.081 (0.016)	1.64E-06	0.025
cg03520471	chr3: 97753623	GABRR3	−0.054 (0.012)	2.30E-05	0.043
cg16350196	chr4: 137426420	NA	−0.052 (0.011)	2.36E-05	0.043
cg20300412	chr4: 65749341	NA	−0.064 (0.014)	3.07E-05	0.045
cg21359538	chr4: 76440740	RCHY1	−0.062 (0.014)	3.05E-05	0.045
cg03498271	chr5: 38427885	EGFLAM	−0.055 (0.012)	1.12E-05	0.036
cg06887251	chr5: 67576008	PIK3R1	−0.054 (0.012)	2.83E-05	0.045
cg10874300	chr5: 67588385	PIK3R1	−0.066 (0.011)	4.36E-08	0.014
cg04708601	chr6: 101880078	GRIK2	−0.051 (0.011)	2.19E-05	0.043
cg24105728	chr6: 13007033	PHACTR1	−0.054 (0.011)	6.61E-06	0.029
cg18742528	chr6: 13053520	PHACTR1	−0.055 (0.011)	5.83E-06	0.029
cg27509293	chr6: 14919597	NA	−0.057 (0.013)	3.36E-05	0.046
cg08188318	chr6: 34984953	ANKS1A	−0.093 (0.02)	1.24E-05	0.037
cg10420626	chr6: 43741154	VEGFA	−0.057 (0.013)	2.72E-05	0.044
cg00325383	chr6: 85404488	LOC102724201	−0.057 (0.012)	9.43E-06	0.034
cg19989663	chr7: 115981565	NA	−0.056 (0.013)	4.46E-05	0.050
cg03083251	chr7: 135408513	SLC13A4	−0.076 (0.016)	6.70E-06	0.029
cg12018852	chr7: 138777233	ZC3HAV1	−0.052 (0.01)	4.63E-06	0.027
cg05753918	chr7: 14899040	NA	−0.065 (0.013)	2.32E-06	0.026
cg04946588	chr7: 86271783	GRM3	−0.052 (0.011)	1.66E-05	0.040
cg21637741	chr8: 69834365	LINC01592	−0.052 (0.011)	2.40E-05	0.043
cg15728672	chr8: 89189971	MMP16	−0.059 (0.011)	1.64E-06	0.025
cg01761595	chr8: 9821723	NA	−0.069 (0.015)	1.70E-05	0.040
cg14611112	chr9: 139643351	LCN6	−0.053 (0.011)	6.49E-06	0.029
cg03779244	chrX: 56771607	LINC01420	−0.085 (0.016)	1.60E-06	0.025
Increased methylation at follow-up					

Table 2 continued

Probe ID	Genomic location*	Gene name	Estimate (SE)†	P value	q value
cg19190900	chr2: 37553621	NA	0.053 (0.012)	2.23E-05	0.043
cg25229577	chr3: 58367583	PXK	0.062 (0.013)	6.81E-06	0.029
cg00367499	chr5: 170634447	RANBP17	0.062 (0.014)	3.81E-05	0.048
cg14396995	chr7: 42278089	GLI3	0.06 (0.013)	1.86E-05	0.041
cg21156439	chr9: 94716496	NA	0.051 (0.011)	1.57E-05	0.039
cg00625110	chr16: 53741731	FTO	0.069 (0.014)	3.35E-06	0.026
cg01154210	chr21: 27493310	APP	0.102 (0.022)	1.63E-05	0.039

NA means the CpG site is not within a gene or within a known feature (eg, promoter) of a specific gene.
*Genomic location is according to genome build GRCh37/hg19.
†Effect estimates represent the proportion of methylation difference at follow-up compared with baseline, adjusted for batch, cell type estimates, Hispanic ethnicity, estimated smoking pack-years and a random intercept for individual to account for the repeated sampling design.

LINC01592), genes involved in neurological function (*GRIK2, APP, ACTR3, GABARR3, ANKS1A, GRM3, PXK, GLI3*), immune function (*GPHB5, DEPB118, ZC3HAV1*), and genes that have been linked to multiple cancers (*MM16, PIK3R1, RCHY1, ZEB2, CEP152, HSPB8, TBX3, FCLR3* and *RHOC*).

Loci identified as differentially methylated between new recruits and incumbents in our previous cross-sectional study were not among the CpG sites identified with q<0.05 in the present study.[14] Four had raw p values <0.05, but the association was not always in the same direction as the previous results (online supplemental table 2). While results did not replicate, this study is comparing short-term changes among the same individuals while the previous study compared two separate groups of firefighters with 14 years difference in experience, on average.

In the pathway analysis of the 680 CpG sites with differential methylation, six canonical pathways were significantly enriched (q<0.05; table 3). In the disease and functions analysis, 123 annotations were enriched among the differentially methylated genes (q<0.05, online supplemental table 3) within 27 disease categories—11 of which are directly cancer related (table 4). The rest of the enriched categories relate to cardiovascular disease, cell signalling and movement, and gene expression.

Fire-exposures analysis
There were linear relationships, suggestive of dose–response, between fire variables and 140 of the 680 loci that changed over time (q<0.05, online supplemental table 4). DNA methylation at 108 and 78 CpG sites were associated with fire-runs for all and structure fires, respectively (figure 1). For fire-hours, 27 CpG sites were associated with all fire-hours and one CpG site with structure fire-hours only. There were 22 CpG sites that were associated with at least three of the fireground exposure variables, including one within the promoter region of *IQCC* that was positively associated with all four. Among the 32 sites

only associated with structure fire-runs, 15 of them may reflect a unique association with structure fires since the q value was between 0.05 and 0.1 with all fire-runs or hours for the other 17 loci.

There was limited evidence for the associations being driven by acute versus cumulative exposure during the follow-up period. The effect estimates at the 680 CpG sites correlated strongly (correlation coefficients 0.92–0.99) when comparing results from the models with and without adjustment for acute exposure. Days since most recent fire was associated with one CpG site (probe ID cg26723045 in the gene *SCUBE2*; effect estimate (SE) −0.012 (0.003), q=0.046) in the model with fire-hours. Days since most recent fire was not significantly associated with DNA methylation at any other loci.

DISCUSSION
In this pilot study of new firefighters, we observed differences in DNA methylation at 680 CpG sites when comparing samples taken before any fireground exposures to matched samples collected after 20–37 months in the service, including 60 CpG sites that differed by at least 5%. These sites were enriched in pathways involved in cancers, neurological function, cell signalling, cell movement and transcriptional regulation. When assessing the relationship with proxies for cumulative fireground exposures, there were linear associations between fire-hours or fire-runs for all fires or structural fires only with 140 of these CpG sites, including several that were uniquely associated with structural fire-runs. These associations did not depend on time since most recent fire-run, and therefore may reflect cumulative exposures since joining the fire department. These results provide preliminary evidence for epigenetic mechanisms that may underlie multiple adverse health outcomes firefighters face after years in the service. Alternatively, differentially methylated loci could serve as biomarkers of cumulative firefighting

Table 3 Canonical pathways enriched among the genes differentially methylated between baseline and follow-up

Ingenuity canonical pathways*	Total genes in pathway (n)	q value for enrichment	Genes in pathway with less methylation at follow-up (n)	Genes in pathway with more methylation at follow-up (n)
Molecular mechanisms of cancer	437	0.0011	12	11
Colorectal cancer metastasis signalling	262	0.0011	11	6
ERBB signalling	93	0.0044	5	4
UVB-induced MAPK signalling	51	0.0044	3	4
Reelin signalling in neurons	125	0.0069	5	5
Production of nitric oxide and reactive oxygen species in macrophages	187	0.0098	6	6

*These pathways were identified with IPA software (Qiagen). Pathways significantly enriched at a cut-off of q<0.05 are shown here.

Table 4 Enriched gene sets annotated to disease and disease-related functions in IPA among differentially methylated genes comparing baseline to follow-up (q<0.05)

Disease category	Enriched disease annotations (gene-sets) in this category (n)
Cancer, cell cycle, organismal injury and abnormalities	3
Cancer, dermatological diseases and conditions, organismal injury and abnormalities	1
Cancer, endocrine system disorders, gastrointestinal disease, organismal injury and abnormalities	5
Cancer, gastrointestinal disease, organismal injury and abnormalities	32
Cancer, gastrointestinal disease, organismal injury and abnormalities, tumour morphology	1
Cancer, haematological disease, immunological disease, organismal injury and abnormalities	3
Cancer, neurological disease, organismal injury and abnormalities	19
Cancer, organismal injury and abnormalities	23
Cancer, organismal injury and abnormalities, reproductive system disease	4
Cancer, organismal injury and abnormalities, respiratory disease	5
Cancer, organismal injury and abnormalities, tumour morphology	1
Cardiovascular disease, cardiovascular system development and function, organ morphology, organismal development, organismal injury and abnormalities, skeletal and muscular disorders	1
Cardiovascular system development and function, cellular development, cellular growth and proliferation	1
Cardiovascular system development and function, organ development, organ morphology	1
Cell cycle	3
Cell-to-cell signalling and interaction	1
Cell-to-cell signalling and interaction, haematological system development and function	1
Cellular development	1
Cellular development, cellular growth and proliferation, haematological system development and function, haematopoiesis, tissue development	1
Cellular movement	4
Cellular movement, nervous system development and function	1
Connective tissue disorders, inflammatory disease, inflammatory response, organismal injury and abnormalities, skeletal and muscular disorders	1
Dermatological diseases and conditions, organismal injury and abnormalities	1
Developmental disorder, hereditary disorder, neurological disease, organismal injury and abnormalities	1
Gene expression	6
Haematological system development and function, haematopoiesis	1
Neurological disease, organismal injury and abnormalities	1

*These gene-sets were identified with IPA software (Qiagen). Summary is shown of enriched gene-sets that fall under broader categories of disease functions. For full list of statistically significant gene-sets, see online supplemental table 3.

exposures. Further work is needed to determine which of the many exposures firefighters experience contribute to these associations.

This study builds on our previous research comparing new recruits to incumbents in a cross-sectional study[14 16] by comparing epigenetic data from matched samples pre-employment and postemployment in the fire service. In the previous study, microRNA expression and DNA methylation differed between new recruits and incumbents. However, since these were one-time collection of samples, the extent to which occupational exposures and hazards contributed to these differences is uncertain. The inception cohort design used in the current study enables us to infer whether differences between baseline and follow-up are attributed to occupational exposures. We recently reported miRNA differences in this same set of firefighters with longitudinal follow-ups and reported changes in multiple miRNAs associated with time to follow-up, as well as association of some miRNAs with measures of cumulative fireground exposure and most recent fire.[17] Overall, the miRNA study and the DNA methylation results reported here provide evidence that even a short time (~2–3 years) in the fire service may be associated with an altered epigenome. It is unlikely these loci changed because of impacts from short-term ageing during the time period as only one of the 680 loci are among loci known to

predict chronological age.[25] Furthermore, widespread changes by ageing tend to occur over decades instead of years.[26 27]

Our results could serve as biomarkers of exposure and may have implications for future disease risks. Firefighters have increased incidence and mortality for specific cancers, including those of the digestive tract, bladder, prostate, testicles, thyroid, brain and blood, among others.[3 4 28 29] Disease functions enriched among the differentially methylated genes included several types of gastrointestinal, brain and blood cancers (online supplemental table S3). Cardiac events are also a leading cause of death in firefighters,[30] consistent with the identification of enriched pathways involving cardiovascular disease. There is also evidence that firefighter exposures such as shift work could lead to neurocognitive decline,[31] consistent with the findings of the current study involving enriched gene pathways for neurological functions.

The associations between crude proxies of fireground exposure and DNA methylation suggest that exposure to products of combustion may be responsible. We previously measured increased concentration of PAH metabolites, proxies of combustion byproducts, in the urine of Tucson firefighters following fireground incidents.[32] PAH exposures have been associated with altered DNA methylation,[33] as have other chemicals such as formaldehyde[34] which are commonly measured at the fireground. Hypomethylation of the *DUSP22* promoter in a
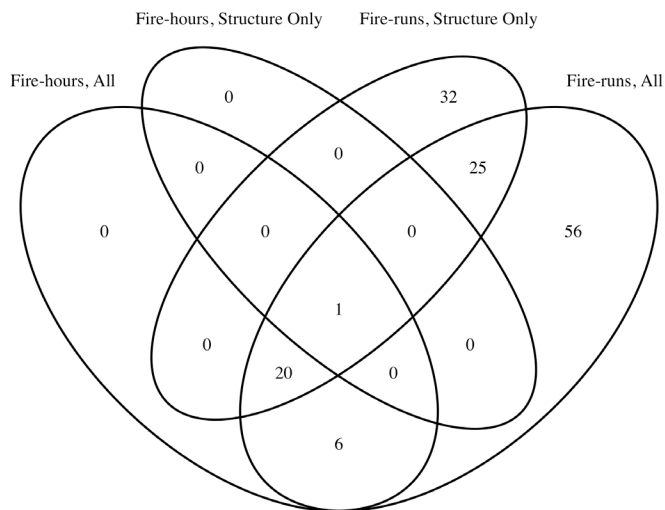
Figure 1 Overlap of CpG sites associated with proxies of fireground exposures. When comparing DNA methylation in blood leucocyte samples from new recruits with a repeat sample collected approximately 2 years later, 680 CpG sites had altered methylation in adjusted linear mixed models (q<0.05). We tested the association of cumulative fireground exposures from all fires and structure-fires only within this time period with DNA methylation at these 680 loci. 140 of them were associated with at least one exposure variable (q<0.05), with overlap indicated in the Venn diagram.

candidate gene study comparing firefighters to controls was previously reported, and treatment of cells with benzo-a-pyrene reduced methylation at the same gene demonstrating biological plausibility for PAH exposures as epigenetic modifiers.[35] In our study, DNA methylation at 11 out of 12 CpG sites included on the array near the transcription start site of *DUSP22* had decreased methylation at follow-up compared with baseline, yet results were not statistically significant.

We compared statistically significant results with those from epigenome-wide associations of cigarette smoking and air pollution exposure among adults to assess whether common signals emerged. A meta-analysis of nearly 16 000 adults from 16 cohorts with blood derived DNA methylation data from the Infinium 450K array identified 18 760 and 2623 CpG sites with differential methylation (q<0.05) when comparing current and former smokers to controls, respectively.[10] 305 of the 680 differentially methylated sites in our study were covered on the 450K array. Among these, 25 were reported as differentially methylated in current smokers compared with non-smokers including four that also differed in former smokers (online supplemental table S1).[10] A systematic review of epigenome-wide association studies focused on adult air pollution exposure compared results across eleven studies using the Infinium 450K or EPIC arrays, and reported 201 CpG sites that were associated with component(s) of air pollution.[23] None of the CpG sites reported here that changed in firefighters were among the air pollution-related sites. However, there was heterogeneity across studies in component of air pollution measured and duration of exposure, and the 201 CpG sites did not replicate across air pollution studies either. This comparison does not provide evidence for a

common epigenetic signature of smoke/combustion byproduct exposure in firefighters and the general population.

We hypothesised that structural fire exposures would have different associations than other types of fires. Structural fires are more likely to involve interior responses by firefighters, and interior responses are associated with increased levels of urinary PAH metabolites compared with exterior responses.[32] There were 15 CpG sites that were associated with fire-runs only for structural fires (q<0.05) with little evidence for an association with total fire runs or hours (q>0.1). The most notable genes represented among these sites were *MAPK9* which increases the stability of the tumour suppressor gene *TP53*,[36] and *PEAR1* which has been linked to endometrial, renal, and lung cancers.[37]

This study had several strengths. This is the first longitudinal analysis of DNA methylation in firefighters, with an inception cohort design. We enrolled non-smoking new recruits prior to any occupational exposure and compared epigenetic profiles after live-fire training. The repeat sampling design reduces confounding bias by a variety of demographic and non-occupational exposures. Our two-tiered analysis plan included an epigenome-wide approach, and then assessment of associations with proxies of fireground exposures. This approach has advantages but may have limited our ability to find all CpG sites associated with specific fireground exposures, given adjustment for multiple comparisons. Other limitations of this study include the sample size which limits ability to detect all true associations, especially those with small effect sizes. Given the design, it is possible that epigenetic changes reported here reflect ageing over the 2 years of follow-up instead of occupation. However, this is unlikely as the top CpG sites were not among those known to correlate with chronological age in adults.[25] Our fireground exposure metrics were not specific (ie, we did not measure exposure biomarkers of specific chemicals). The majority of study participants were male, white, non-Hispanic, and came from one location; thus sex, regional or race/ethnicity-specific associations could not be assessed. Our study lacked a non-firefighter control group with repeat measures of DNA methylation. Since we previously reported epigenetic differences by Hispanic ethnicity among firefighters,[38] epigenetic biomarkers should be profiled in a diverse cohort (eg, by geography, race, ethnicity and sex) in order to determine generalisability.

CONCLUSION

In conclusion, this pilot study provides evidence consistent with previous research that molecular biomarkers including DNA methylation may change from the cumulative burden of exposures encountered when firefighting. This is important because DNA methylation could be used as a biomarker of exposures and could inform occupational health risk assessment for firefighters. The study findings merit further investigation as epigenetics alterations as associated with many diseases states and constitute one of the key characteristics by which carcinogens exert their effects.[13]

study. JMG conducted statistical analysis and drafted the manuscript with critical intellectual input from AMJ, MAF and JLB. JLB is the guarantor for the research described in this publication. All authors approve the final version of the manuscript and accept responsibility for the accuracy and integrity of reported results.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the University of Arizona Institutional Review Board (IRB approval No. 1509137073). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. Data requests will be reviewed by the study's Oversight and Planning Board to address firefighter concerns prior to determination of sharing de-identified data which may include de-identified epigenomic (DNA methylation) data. This study is not a clinical trial. Contact the corresponding author, JLB, with requests.

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