ORIGINAL ARTICLE

Exposure to welding fumes is associated with hypomethylation of the *F2RL3* gene: a cardiovascular disease marker

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ABSTRACT

Background Welders are at risk for cardiovascular disease. Recent studies linked tobacco smoke exposure to hypomethylation of the *F2RL3* (coagulation factor II (thrombin) receptor-like 3) gene, a marker for cardiovascular disease prognosis and mortality. However, whether welding fumes cause hypomethylation of *F2RL3* remains unknown.

Methods We investigated 101 welders (median span of working as a welder: 7 years) and 127 unexposed controls (non-welders with no obvious exposure to respirable dust at work), age range 23–60 years, all currently non-smoking, in Sweden. The participants were interviewed about their work history, lifestyle factors and diseases. Personal sampling of respirable dust was performed for the welders. DNA methylation of *F2RL3* in blood was assessed by pyrosequencing of four CpG sites, CpG_2 (corresponds to cg03636183) to CpG_5, in *F2RL3*. Multivariable linear regression analysis was used to assess the association between exposure to welding fumes and *F2RL3* methylation.

Results Welders had 2.6% lower methylation of CpG_5 than controls (p<0.001). Higher concentrations of measured respirable dust among the welders were associated with hypomethylation of CpG_2, CpG_4 and CpG_5 (β=−0.49 to −1.4, p<0.012); p<0.029 adjusted for age, previous smoking, passive smoking, education, current residence and respirator use. Increasing the number of years working as a welder was associated with hypomethylation of CpG_4 (linear regression analysis, β=−0.11, p=0.039, adjusted for previous smoking). Previous tobacco smokers had 1.5–4.7% (p<0.014) lower methylation of 3 of the 4 CpG sites in *F2RL3* (CpG_2, CpG_4 and CpG_5) compared to never-smokers. A non-significant lower risk of cardiovascular disease with more methylation was observed for all CpG sites.

Conclusions Welding fumes exposure and previous smoking were associated with *F2RL3* hypomethylation. This finding links low-to-moderate exposure to welding fumes to adverse effects on the cardiovascular system, and suggests a potential mechanistic pathway for this link, via epigenetic effects on *F2RL3* expression.

What this paper adds

- Exposure to welding fumes has been reported to be associated with increased risk of cardiovascular disease. Tobacco smoke exposure has recently been linked to hypomethylation of the *F2RL3* gene, a marker for mortality and cardiovascular disease prognosis. Both welding fumes and tobacco smoke consist mainly of ultrafine condensation particles.
- We investigated *F2RL3* methylation by pyrosequencing in welders and controls.
- We found that exposure to welding fumes was associated with *F2RL3* hypomethylation, suggesting evidence that low-to-moderate exposure to welding fumes may have adverse effects on the cardiovascular system via epigenetic modifications.

Danish prospective study of welders followed until 2006,[1] and the standardised mortality ratio for ischaemic heart disease was 1.35 (95% CI 1.1 to 1.6) in a Swedish study of welders followed until 1995.[2] Despite these observational data, the mechanisms coupling exposure to welding fumes with harmful cardiovascular events remain unclear.

Low DNA methylation, so-called hypomethylation, of the *F2RL3* gene in blood is a predictor of mortality from CVDs and cancers.[3] F2RL3 hypomethylation can also serve as a marker of CVD prognosis.[4] Recent studies have found that tobacco smoking, a strong risk factor for CVD, is associated with hypomethylation of *F2RL3*.[5–8] Breitling *et al*[5] first reported in a population-based epidemiological study that cg03636183 of *F2RL3* (CpG_2) was significantly hypomethylated in smokers. Later studies have shown that, along with CpG_2, CpG_4 and CpG_5 can serve as biomarkers for current and lifetime smoking,[9] and as strong predictors of CVD-associated mortality.[3] F2RL3 is expressed in different cell types, including circulating leucocytes,[10] and encodes thrombin protease-activated receptor 4 (PAR-4), a cell surface protein.[11] PAR-4 functions in blood coagulation[12] and activation of PAR-4 is important for multiple aspects of immune function, including recruitment of leucocytes, modulation of rolling and adherence of neutrophils

INTRODUCTION

Exposure to welding fumes is associated with an increased risk of cardiovascular disease (CVD); the standardised incidence ratio for acute myocardial infarction was 1.12 (95% CI 1.01 to 1.24) in a

and eosinophils, as well as regulation of vascular endothelial cell activity.[10] [12–14] These physiological events also occur as early steps in inflammatory reactions in the vascular system,[10] [12] [15] and moreover, these events have been observed to occur more frequently in smokers and partly in relation to welding particle exposures.[16–19]

We hypothesised that welding fume exposure is associated with hypomethylation of *F2RL3*, and we analysed the methylation status of *F2RL3* in a group of welders and non-exposed controls.

MATERIALS AND METHODS
Study population
We enrolled 101 welders from 10 different companies in southern Sweden. The companies were medium sized and produced heavy vehicles, lifting tables, stoves, heating boilers and pumps, and equipment for the mining industry. Detailed characteristics of 8 of the 10 companies which employed 83 welders were recently described.[20] Furthermore, we enrolled 127 controls from seven different companies: participants from six companies were 'blue-collar' worker with the routine task of organising grocery goods and participants from the seventh company were working as gardeners; these unexposed control workers had no obvious exposure to respirable dust at the workplace. All study participants were male and currently non-smokers. Among the previous smokers, one reported smoking in the past 12 months; the remaining previous smokers stopped smoking at least 1 year before study enrolment. Structured questionnaire-based interviews were carried out by a trained nurse to obtain information about age, height and weight (all coded as continuous variables), ethnicity (participants and their parents' nationality), education (five categories: primary school, high school, professional school, university <3 years, university >3 years of education), medical history, personal and family disease history (cancer and CVDs), diet (frequency of intake of fruit, vegetables and fish), physical activity (four levels, from sedentary to regular exercise), previous smoking history (yes or no; if yes, year of start and end), passive smoking (at home and/or at work), alcohol consumption (wine or other alcohol consumption, with six different levels in each), current residence, having a wood burning stove or boiler at home, exposure to wood smoke from the neighbourhood, exposure to traffic (traffic intensity around the residence and time spent in traffic every day), working environment, occupational history, and hobbies with exposure to smoke (eg, working with car engines, etc). The participants were asked whether they had had myocardial infarction, angina pectoris, hypertension, stroke, thrombosis or other CVDs diagnosed by a physician. All study participants answered the same questionnaire, apart from questions regarding work tasks that differed between welders and controls.

Venous blood samples were collected from the participants. This study was approved by the Regional Ethical Committee of Lund University, Sweden, and all study participants gave their informed written consent to take part in the study.

Occupational exposure assessment
Monitoring of exposure
Respirable dust was measured once in each of the welding companies and samples were collected in the breathing zones of welders. For welders who were wearing powered air purifying respirators (PAPRs), air outside the PAPRs was sampled. Exposure to respirable dust was measured by air sampling on pre-weighed 37 mm mixed cellulose ester filters (0.8 μm pore size) fitted in leak-free cassettes (Sure-Seal). Respirable dust

cyclone air samplers of nickel-plated aluminium (BGIL4, BGI Inc) were attached to the filter cassettes. Battery powered sampling pumps (MSA Escort Elf) were operated at a flow rate of 2.2 L/min. The airflow was checked before, during and after the sampling with a primary calibrator (TSI Model 4199, TSI Inc). Most of the air sampling was performed during full-shift work; the average sampling time was 6.8 h (range 2.4–8.6 h). The filter samples were analysed gravimetrically for respirable dust according to a certified method. The limit of detection (LOD) was 0.05 mg/sample.

Parallel measurements of respirable dust were performed to assess the workplace protection factor for the PAPRs. A setup consisting of two parallel sampling systems for respirable dust was used: one for sampling inside and one for sampling outside the PAPR on the shoulder in the breathing zone. Parallel samplings were performed on three workers at different companies and the respirable dust concentrations were at least three times lower inside the PAPRs compared with concentrations outside in the breathing zones.[20]

The exposure to respirable dust was also measured by personal sampling in the breathing zone for 19 workers from two control companies. The average sampling time for the controls was 7.2 h. Stationary measurements of respirable dust were conducted in four other control companies with a direct-reading instrument (Sidepak Model AM510, TSI Inc). In these six companies, the particle number concentrations (size range 20–1000 nm) were also measured with direct-reading, stationary instruments (P-Trak, TSI Inc).

Exposure assessment of welding fumes
Respirable dust was measured for 53 of 101 welders. For the remaining 48 welders without measurements of respirable dust, their exposure to welding fumes was assessed from exposure data of the 53 welders aforementioned and 17 welders working with similar tasks at the same companies, but not included in the study, as well as exposure data from a previous study.[19] We excluded two participants from the exposure assessment: one welder reported that he worked only with soldering/brazing, which seemed unreasonable and one welder's information about the local exhaust ventilation was missing, making it difficult to correctly estimate his exposure. After excluding these two participants, the total number of welders with data on estimated respirable dust exposure was n=99. In order to calculate the exposure to respirable dust for welders with PAPRs, the respirable dust concentrations were reduced by a correction factor of three to get a better estimate of the exposure inside the PAPRs. The correction factor was based on the results from our parallel respirable dust measurements described above, and literature data on the workplace protection factor of PAPRs.[20–23]

Analysis of DNA methylation
DNA was isolated from venous blood with the QIAamp DNA Blood Midi kit (Qiagen, catalogue nr 51183). The DNA quality was evaluated on a NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 1000) and the DNA showed good quality (260/280 nm >1.80). DNA was bisulfite treated with the EZ DNA Methylation kit (Zymo Research, catalogue nr D5008).

Pyrosequencing assays were designed to quantify the percentage of methylation of *F2RL3* at CpG sites that have previously been linked to tobacco smoke.[5] *F2RL3* is on chromosome 19 and has two exons. The cg03636183 site (number based on Illumina 27 K and 450 K beadchips) is located on a CpG island (http://www.ncbi.nlm.nih.gov/epigenomics/genome/GCF_000001405.13/gene:9002/) in exon two. The cg03636183 site

(labelled CpG_2), based on previous studies[3] [9] [24] and three other CpG sites downstream (labelled CpG_3 to CpG_5) within a total length of 70 bp were analysed. Two pyrosequencing assays covered these four sites: the first assay (amplicon length of 235 nucleotides) encompassed one site and the second assay (amplicon length of 341 nucleotides) encompassed three sites (see online supplementary table S1). The assays were designed by PyroMark Assay Design 2.0 software (Qiagen). The forward primers were biotinylated. PCR was performed using PyroMark PCR reagents (Qiagen, catalogue nr 972807). The PCR product was purified using Streptavidin Sepharose High Performance beads (Amersham Biosciences, catalogue nr 17–5113–01). Pyrosequencing was carried out using the PSQ HS96 Pyrosequencing System (Qiagen). We repeated 21% (N=48) of the samples and found the variation in coefficients (VC) as 5.4%, 3.5%, 2.5% and 3.3% for CpG_2 to CpG_5, respectively. Negative controls were included in each run.

C reactive protein and serum amyloid A measurements

C reactive protein (CRP) was measured in plasma by immuno-turbidimetry, and serum amyloid A (SAA) was measured in serum by immunonephelometry at the Department of Clinical Chemistry in Lund University Hospital using standard protocols.[25]

Statistical analyses

Differences in characteristics and *F2RL3* methylation percentages between welders and controls were compared by the Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables.

General linear models were employed to explore the main research hypothesis, that is, that working as a welder is associated with *F2RL3* methylation, as well as that measured/calculated respirable dust exposure and years working as a welder are associated with *F2RL3* methylation. In addition, the general linear model was employed to evaluate associations between previous smoking and *F2RL3* methylation. Associations between *F2RL3* methylation and CVD were investigated by logistic regression. The methylation levels observed at the CpG_4 were not normally distributed when combining welders and controls; however, it was normally distributed when analysing welders only.

We performed unadjusted, adjusted and full model analyses while investigating the associations between welding versus *F2RL3* methylation. Adjusted analyses included age (continuous) and previous smoking (yes or no), as these two variables were reported to be associated with *F2RL3* methylation. For identification of additional factors to be included in the full model, we considered variables from both published literature and our own data, including individual characteristics, diet, lifestyle, passive smoking and disease history. These variables were tested individually in our main hypothesis, that is, the differences of *F2RL3* methylation between welders and controls. Any variable altering the effect estimate by more than 10% with at least two of four CpG sites was included in the full model. These additional variables were education (low or high), passive smoking (yes or no) and current residence (big city or others). To be consistent, we included the same variables in the full model investigating the associations between welding and methylation, and when investigating the association between previous smoking and methylation. While investigating the association between *F2RL3* methylation and CVD, adjusted analyses included age and body mass index (BMI; continuous). Other possible confounders were considered, but none altered the effect estimate

by more than 10% in the associations between *F2RL3* methylation and CVD.

For the analysis of measured respirable dust versus methylation, the use of a respirator was also considered. When the number of years working as a welder was evaluated, age was not included in the adjusted analysis and full model, as age and years worked were highly correlated (r=0.75), and including both of them in the same model increased the SE by more than 50% (eg, from 0.032 to 0.049). All statistical analyses were performed using SPSS V.22.0 (SPSS Inc) and statistical significance refers to p<0.05 (two tailed).

RESULTS

Welders were exposed to average levels of 1.2 mg/m^3 (range 0.1–19.3 mg/m^3) of respirable dust, whereas all the control subjects measured had exposure to respirable dust lower than 0.2 mg/m^3. Characteristics of study subjects, including methylation of *F2RL3*, are presented in table 1.

Welders and control subjects did not differ in age, BMI, ethnicity, reported CVD, family history of CVD, number of individuals who reported previous smoking, or hobbies with exposure to dust, gases and fumes. Furthermore, welders and control subjects did not differ in intake of vegetables, fruit or fish, use of snus, consumption of wine, or time spent in traffic (not in table 1). The welders were likely to have shorter education, live in smaller cities and have higher exposure to passive smoking and to respirable dusts compared to controls. CpG_2, CpG_4 and CpG_5 were significantly correlated with each other (Pearson, r$_s$=0.24–0.64). Welders had a significantly lower methylation of CpG_5 and higher methylation of CpG_3 than controls (table 1).

In linear regression analysis, welders showed a significantly lower methylation of CpG_5 compared to controls, also after adjustment for age and previous smoking (table 2).

However, after additional adjustments for passive smoking, education and current residence, the associations became non-significant (p=0.061). Compared to controls, welders had higher methylation in CpG_2 (only in full model) and CpG_3 (table 2).

Measured (N=53 welders) and calculated (N=99) concentrations of respirable dust were associated with DNA methylation (table 3A): higher concentrations of respirable dust (both measured and calculated) were significantly associated with hypomethylation of CpG_2 and CpG_4 (figure 1) and (only measured) CpG_5.

The effect estimates were in general higher for the measured values than the calculated values. Adjustments with age, previous smoking, passive smoking, education, current residence and respirator use marginally changed the estimates, but not the statistical significance, except for calculated respirable dust versus CpG_2 (table 3A). Furthermore, we analysed the association between years working as a welder and methylation of *F2RL3*. Methylation of CpG_4 was inversely associated with working years (β=−0.11, p=0.039, adjusted for previous smoking; table 3B), but this association became non-significant in the full model (β=−0.089, p=0.096). When age was included in the full model, the associations became statistically non-significant, which is most likely due to overadjustments, as age and working years as welders were highly correlated (r$_s$=0.75). Both age and working years as welder were associated with CpG_4 methylation. However, when age and working years were included in the same model, both of them became non-significant, which indicates that the model was suffering from collinearity. In order to distinguish the effect of working years as a welder from the general effect of age on *F2RL3* methylation, we investigated the

Table 1 Characteristics of welders and controls, and comparisons between groups

Characteristics	Welders (n=101)	Controls (n=127)	p Value
Age (year)	41 (23–60)	43 (23–56)	0.90*
BMI (kg/m^2)	27.7 (21.4–34.9)	27.1 (22.3–34.2)	0.48*
Ethnicity: European/others (European, %)†	96/5 (95)	122/5 (96)	0.75‡
Low/high education (low, %)§	94/7 (93)	109/17 (87)	0.032‡
Cardiovascular disease: yes/no (yes, %)¶	23/78 (23)	25/102 (20)	0.63‡
Family history of cardiovascular disease: yes/no (yes, %)**	44/57 (44)	46/81 (36)	0.28‡
Current residence: big city/others (big city, %)††	10/91 (10)	29/98(23)	0.013‡
Previous smoking: yes/no (yes, %)	43/58 (43)	43/83 (34)	0.21‡
Passive smoking: yes/no (yes, %)	29/72 (29)	16/111 (13)	0.0041‡
Hobby with smoke: yes/no (yes, %)	9/92 (9)	5/122 (4)	0.17‡
Respirable dust (mg/m^3)‡‡	1.1 (0.2–8.3)	0.1 (0.0–0.2)	<0.001*
CpG_2 methylation (%)	76.7 (71.5–83.0)	76.5 (70.3–81.2)	0.27*
CpG_3 methylation (%)	78.5 (75.6–81.2)	77.4 (74.1–82.7)	<0.001*
CpG_4 methylation (%)	88.4 (77.0–94.2)	88.9 (78.0–94.2)	0.68*
CpG_5 methylation (%)	87.1 (82.8–97.5)	89.7 (81.8–97.1)	<0.001*

Medians (5–95%) are presented, if not indicated otherwise.
*Between welders and controls (Mann-Whitney U test).
†Parents of the participants from Europe/others (%).
‡Between welders and controls (Fisher's exact test).
§Participants with high school or lower/university education, or higher.
¶Participants reported whether they had had myocardial infarction, angina pectoris, hypertension, stroke, blood clot or any other cardiovascular diseases diagnosed by their doctors.
**Participants were asked whether their parents or siblings were diagnosed by doctors for having myocardial infarction, stroke and/or hypertension before they were 65 years old.
††Big city/small city, villages.
‡‡Nineteen of the controls and 53 of the welders were monitored.
BMI, body mass index.

association between age and CpG_4 in controls as well. The effect estimate was 23% lower than in the welders, and the p value was rather close to the significance threshold (data not shown). The difference of the effect of age in welders and in controls suggests that the welders may be influenced by other factors strongly correlated with age, such as working years as a welder. Moreover, in another sensitivity analysis, we also adjusted the models presented in tables 2 and 3A, B with CRP and SAA as markers for acute inflammatory response, but these adjustments did not affect the associations substantially (data not shown).

We also investigated the association between previous smoking and F2RL3 methylation, and F2RL3 methylation and CVD (table 4A, B).

Previous smokers had a significantly lower methylation in CpG_2 (1.5%), CpG_4 (4.7%) and CpG_5 (2.1%) compared to never-smokers. These associations remained significant after adjustments. We found that previous smokers had lower methylation compared with never-smokers both among welders and non-exposed controls, for example, previous smokers showed a 1.6% lower methylation of CpG_2 compared to non-smokers in both groups. For CpG_4, previous smokers showed a 4.0% and

5.3% lower methylation in welders and controls, respectively (data not shown). We also performed logistic regression analysis between CpG methylation and self-reported CVD. None of the CpG sites were significantly associated with history of CVD, although a non-significantly lower risk with more methylation was observed for all CpG sites.

DISCUSSION

This study shows that exposure to welding fumes, similar to tobacco smoke, is associated with hypomethylation of F2RL3, a risk marker for CVD. The effects on F2RL3 methylation were found at low-to-moderate levels of exposure to welding fumes and suggest that welders might be at risk for CVD, despite precautions taken at lower exposure levels. The welders we studied had a median exposure level of 1.2 mg/m^3 welding fumes over a shift, and the occupational exposure limit (8 h time weighted average) set by the Swedish Work Environment Authority is 5 mg/m^3.[26] The fact that welding fumes and tobacco smoke were linked to hypomethylation of F2RL3 suggests that a common factor may cause the observed associations. One common factor is ultrafine particles, and indeed we found strong associations between individual exposure to respirable

Table 2 Association between occupation as a welder and methylation of CpG sites in F2RL3

Variable	Unadjusted analysis		Adjusted analysis		Full model	
	β (95% CI)	p Value	β (95% CI)	p Value	β (95% CI)	p Value
CpG_2	0.70 (−0.23 to 1.6)	0.14	0.86 (−0.054 to 1.8)	0.065	1.1 (0.094 to 2.0)	0.031
CpG_3	0.86 (0.26 to 1.5)	0.0053	0.90 (0.30 to 1.5)	0.0036	0.99 (0.37 to 1.6)	0.0020
CpG_4*	−0.15 (−1.6 to 1.4)	0.85	0.24 (−1.2 to 1.6)	0.74	0.83 (−0.62 to 2.3)	0.26
CpG_5	−1.8 (−3.0 to −0.59)	0.0036	−1.6 (−2.8 to −0.43)	0.0079	−1.2 (−2.4 to 0.055)	0.061

The β values presented for occupation as a welder versus control are derived from general linear models. Unadjusted analyses: F2RL3 methylation=intercept + β (occupation); adjusted analyses: F2RL3 methylation=intercept + β (occupation) + β1 (age) + β2 (previous smoking); and full model: F2RL3 methylation=intercept + β (occupation) + β1 (age) + β2 (previous smoking) + β3 (passive smoking) + β4 (education)+ β5 (current residence).
*The residuals for methylation levels for CpG_4 were not normally distributed when comparing welders and controls and log transformation did not improve the distribution.

Table 3 (A) Association between respirable dust (measured and calculated) exposure and CpG sites methylation in *F2RL3* in welders and (B) linear regression analysis of working years as welder and *F2RL3* methylation

Panel A

Variable	Measured respirable dust (N=53)				Calculated respirable dust (N=99)			
	Unadjusted analysis*		Full model†		Unadjusted analysis*		Full model‡	
	β (95% CI)	p Value	β (95% CI)	p Value	β (95% CI)	p Value	β (95% CI)	p Value
CpG_2	−0.51 (−0.85 to −0.16)	0.0045	−0.49 (−0.88 to −0.10)	0.014	−0.44 (−0.85 to −0.036)	0.033	−0.38 (−0.79 to 0.025)	0.065
CpG_3	−0.12 (−0.31 to 0.076)	0.23	−0.059 (−0.28 to 0.16)	0.59	−0.19 (−0.39 to 0.0057)	0.057	−0.17 (−0.38 to 0.039)	0.11
CpG_4	−1.4 (−2.0 to −0.80)	<0.001	−1.1 (−1.7 to −0.46)	0.0011	−1.0 (−1.7 to −0.35)	0.0032	−0.71 (−1.4 to −0.062)	0.032
CpG_5	−0.64 (−1.1 to −0.15)	0.012	−0.64 (−1.2 to −0.069)	0.029	−0.25 (−0.81 to 0.32)	0.39	−0.089 (−0.66 to 0.48)	0.76

Panel B: Working years as welder (N=100)

Variable	Unadjusted analysis*		Adjusted analysis§		Full model¶	
	β (95% CI)	p Value	β (95% CI)	p Value	β (95% CI)	p Value
CpG_2	−0.027 (−0.091 to 0.037)	0.41	−0.019 (−0.082 to 0.044)	0.56	−0.011 (−0.075 to 0.053)	0.73
CpG_3	−0.020 (−0.051 to 0.011)	0.20	−0.020 (−0.051 to 0.011)	0.21	−0.018 (−0.051 to 0.015)	0.28
CpG_4	−0.13 (−0.23 to −0.021)	0.019	−0.11 (−0.21 to −0.0058)	0.039	−0.089 (−0.19 to 0.016)	0.096
CpG_5	−0.049 (−0.14 to 0.037)	0.26	−0.047 (−0.13 to 0.041)	0.29	−0.033 (−0.12 to 0.055)	0.46

The β values are derived from linear regression models.
*Unadjusted analyses: *F2RL3* methylation=intercept + β (respirable dust/calculated respirable dust/years working as welder).
†Full model: intercept + β (respirable dust) + β1 (age) + β2 (previous smoking) + β3 (passive smoking) + β4 (education) + β5 (current residence) + β6 (respirator use).
‡Full model: intercept + β (calculated respirable dust) + β1 (age) + β2 (previous smoking) + β3 (passive smoking) + β4 (education) + β5 (current residence). For the calculated respirable dust, use of a respirator was included in the calculation as described in the Materials and methods section.
§Adjusted analysis: intercept + β (years working as welder) + β1 (previous smoking).
¶Full model: intercept + β (years working as welder) + β1 (previous smoking) + β2 (passive smoking) + β3 (education) + β4 (current residence) + β5 (respirator use).

dust, which in a welding setting predominantly consists of ultra-fine particles, and hypomethylation. However, it should be mentioned that other common factors, such as different metals, present in both tobacco smoke and welding fumes, may also cause the association with *F2RL3* methylation. We previously reported that welders are exposed to high levels of respirable manganese from welding fumes.[20] Apart from manganese, iron is also a major component of black steel, and both iron and manganese may adversely affect cardiovascular health.[27 28]
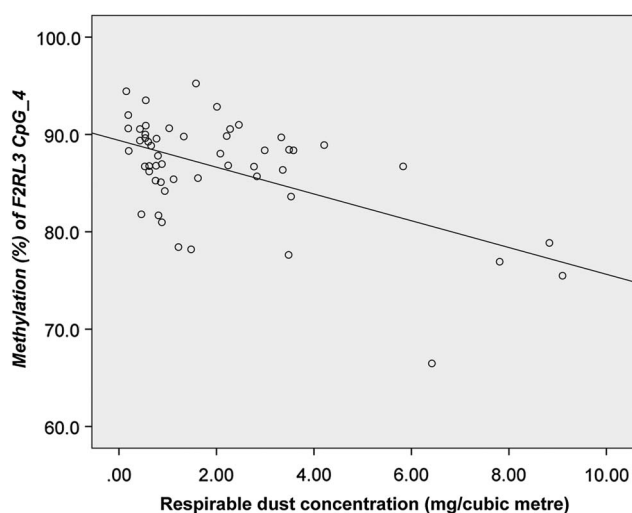
Figure 1 Association between *F2RL3* methylation and respirable dust exposure. Scatterplot of *F2RL3* CpG_4 methylation (%) versus measured respirable dust in welders (n=53). The DNA methylation was measured using pyrosequencing in DNA isolated from peripheral blood and exposure to respirable dust was measured by air sampling close to the breathing zone on pre-weighed mixed cellulose ester filters. The fit (R^2) of the line is 0.31.

F2RL3 is a key gene for recruitment and behaviour of immune cells and blood coagulation.[10 12–14 29] Hypomethylation is often linked to increased gene expression, and if this is the case for *F2RL3*,[7] increased expression of this gene could result in increased inflammation and possibly coagulation. Zhang *et al*[9] reported that hypomethylation of the CpG_4 site was most strongly associated with smoking behaviour, with an average difference of 5% between previous smokers and never-smokers, which is in accordance with what we found in our study. We also found that hypomethylation of CpG_4 had the strongest association with exposure to respirable dust, indicating that this particular CpG site is a main target for the effect of different types of exposure to small particles. The other CpGs showed less consistent associations with occupational exposure to welding fumes. More studies on other types of occupational and environmental particle exposure and methylation of *F2RL3* are warranted to identify if aberrant methylation of this gene is a common change in response to exposure to ultrafine particles and, further, the effects of this altered methylation on *F2RL3* expression and the cardiovascular system.

Moreover, this study identified significant associations between reported CVD and hypomethylation of sites in *F2RL3*, despite the fact that the study was rather small and cross-sectional. These findings further support the link between this gene and CVD.

Previous publications did not report results for CpG_3, since the methylation of this CpG could not be well characterised by the matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF) assay used which showed high technical variability.[3 5 24] However, the pyrosequencing assay we developed identified CpG_3 methylation reliably (VC 3.5% for repeated tests). Surprisingly, we found that welders showed hypermethylation of CpG_3 compared to the non-exposed controls, but we did not find any association of CpG_3 methylation with previous smoking or CVD prevalence. This could indicate that this site is specific for exposure to welding

Table 4 (A) Association between previous smoking and CpG sites methylation in *F2RL3* in welders and controls (N=228) and (B) Association between *F2RL3* methylation and cardiovascular disease by logistic regression (N=228)

Panel A

Variable	Unadjusted analysis*		Adjusted analysis†		Full model‡	
	β (95% CI)	p Value	β (95% CI)	p Value	β (95% CI)	p Value
CpG_2	−1.5 (−2.5 to −0.60)	0.0014	−1.5 (−2.5 to −0.55)	0.0022	−1.5 (−2.5 to −0.52)	0.0028
CpG_3	−0.12 (−0.75 to 0.50)	0.70	−0.049 (−0.69 to 0.59)	0.88	−0.058 (−0.70 to 0.59)	0.86
CpG_4	−4.7 (−6.1 to −3.2)	<0.001	−4.2 (−5.7 to −2.8)	<0.001	−4.2 (−5.7 to −2.8)	<0.001
CpG_5	−2.1 (−3.3 to −0.85)	0.0010	−2.0 (−3.2 to −0.69)	0.0026	−2.1 (−3.3 to −0.80)	0.0014

Panel B

Variable	Unadjusted analysis§		Adjusted analysis¶	
	OR (95% CI)	p Value	OR (95% CI)	p Value
CpG_2	0.92 (0.84 to 1.001)	0.054	0.93 (0.85 to 1.01)	0.083
CpG_3	0.88 (0.76 to 1.016)	0.084	0.90 (0.77 to 1.04)	0.14
CpG_4	0.96 (0.91 to 1.011)	0.13	0.98 (0.93 to 1.04)	0.46
CpG_5	0.96 (0.89 to 1.024)	0.20	0.97 (0.90 to 1.04)	0.32

The β values are derived from linear regression models.
*Unadjusted analyses: *F2RL3* methylation=intercept + β (previous smoking).
†Adjusted analysis: intercept + β (previous smoking) + β1 (age)+ β2 (passive smoking).
‡Full model: intercept + β (previous smoking) + β1 (age) + β2 (passive smoking) + β3 (education) + β4 (current residence).
§Unadjusted analyses: logistic regression, cardiovascular disease=intercept + β (*F2RL3* methylation).
¶Adjusted analysis, logistic regression: intercept + β (*F2RL3* methylation) + β1 (age)+ β2 (body mass index).

fumes. However, we did not find any association of CpG_3 methylation with welding fume exposure or length of time working as a welder, suggesting that this is unlikely. The significance of hypermethylation of CpG_3 in welders needs further investigation.

A major advantage of this study was the individual measurements of exposure to welding fumes in a large group of workers, and estimated exposure for the rest of the workers. The higher effect estimates in the analyses restricted to workers whose exposure was individually monitored indicate that this substantially reduced exposure misclassification. A further strength of this study was that the participants were currently non-smokers, as this avoids any masking effect of current smoking on *F2RL3* methylation observed in previous studies.[6 9] Indeed, previous smoking was here associated with hypomethylation of CpG_2, CpG_4 and CpG_5, and this was adjusted for in the regression models. Moreover, we gathered detailed data about other potentially influential factors. However, this study also had some limitations. CpG methylation was measured in the DNA isolated from peripheral blood and not from individual cell populations. DNA methylation patterns may vary among different blood cell types,[30] and we cannot rule out the possibility that the observed effect on DNA methylation was due to the differences in cell populations. However, we did adjust our data for acute phase response proteins in blood (CRP and SAA), as a proxy for alterations of blood cell composition due to infection or inflammation, but these sensitivity analyses did not change the effect estimates or p values substantially. Metal fume fever, an acute response to exposure to freshly generated and relatively high concentrations (320–580 mg zinc/m^3) of metal-rich particles,[31] might potentially influence our results by altering the cell composition in blood. Although the welders in our study who were exposed to low-to-moderate levels of welding fumes did not report any fever, and the cytokine levels in welders were mostly similar to the levels in the controls,[25] we cannot rule out the possibility that the epigenetic effects observed were due to subtle differences in the subpopulation of

immune cells caused by particle exposure. The study design is cross-sectional, which limits the conclusions with regard to causality of the observed associations. Some workers used protective devices to protect against inhalation of smoke and we could not measure the respirable dust inside the respirators. Therefore, the measured respirable dust could overestimate the true exposure to inhalable particles. Use of protective masks was, however, adjusted for in the models and this did not change the results significantly. Self-reporting of the personal and family history of CVD was also a limitation of our study and might have blurred the association between *F2RL3* methylation and history of CVD. There is an issue of multiple comparisons, as there were four different CpG sites in the linear regression analysis and both measured and calculated data on respirable dust. However, the CpG sites were partly correlated (ie, they were not independent), and therefore we did not make corrections for multiple testing.

CONCLUSION

We identified that low-to-moderate levels of exposure to welding fumes were consistently associated with hypomethylation of *F2RL3*. With the observed similarity of the effects of tobacco smoke and welding fumes on *F2RL3*, one can hypothesise that ultrafine particles are the causative agents. Our finding suggests a potential mechanistic pathway for the link between welding fumes and CVDs via possible epigenetic effects on *F2RL3* expression.

Contributors MBH performed the epigenetic and statistical analyses and drafted the paper. HL recruited the study participants and performed statistical analyses. MH performed the exposure measurement and assessment. HT and MA contributed to the study design and HT supervised the exposure measurement and assessment. KB designed and supervised the project.

Competing interests None declared.

Patient consent Obtained.

Ethics approval Regional Ethical Committee of Lund University, Sweden.

Provenance and peer review Not commissioned; externally peer reviewed.

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