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DNA Methylation and Smoking: Implications for Understanding Effects of Electronic Cigarettes

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Abstract

Purpose of Review Use of electronic cigarettes (e-cigs) has increased sharply recently although understanding of toxicity is limited, particularly target organ effects. Altered DNA methylation is a reversible response to environmental exposures, including smoking, and may be useful as a biomarker of e-cig harm.

Recent Findings Among studies examining DNA methylation in blood by smoking status, there is considerable variability in differentially methylated CpGs identified; certain CpGs are consistently found. These include *AHRR* (aryl hydrocarbon receptor repressor gene), particularly cg05575921, cg0363183 in the F2RL2 gene coding for the protease-activated receptor 4 (PAR-4), and several CpGs in the 2q37.1 genomic region. Differences are found even with short duration and light smoking; effects vary with pack-years and time since quitting among former smokers. For tissues other than blood, data are limited but also indicate altered methylation with smoking.

Summary DNA methylation changes are a consistent biomarker of smoke exposure. Most studies regarding smoke effects on methylation are of blood cells; further evidence regarding effects of smoke, secondhand smoke, and e-cigs on target tissues for smoking-related diseases are needed. Understanding biological effects of e-cigs is critically important to inform regulation; examination of e-cig effects on DNA methylation can significantly add to evidence-based regulation.

Keywords Electronic cigarettes · DNA methylation · Smoking · Toxicity

Introduction

Electronic cigarettes (e-cigs) are battery-powered devices with heating elements. They create a vapor that contains nicotine as well as carrier liquids (vegetable glycerol (VG) and/or propylene glycol (PG)) and flavors [1]. There has been a sharp increase in the use of e-cigs and related products since their introduction into the marketplace in 2007. By 2017, 2.8% of US adults over the age of 18 reported using e-cigs [2•]. Use among younger people is particularly high. Among high school students, report of current use went from 1.5 to 20.8% between 2011 and 2018. During the same period, prevalence of current use among middle school students went from 0.6 to 4.9% [3•]. During the period 2014–2017, 9.2% of middle and high school students reported ever having used e-cigs [4]. More than 37% of current smokers, 1.4% of never smokers, and 43% of former smokers quitting within the past year reported ever using an e-cig. Among current smokers, 3.6% report regular use of e-cigs in the last 30 days [5].

Given these data showing high prevalence of use, and particularly given the rate of increase in use, understanding of the biological impact of use of these devices is critically important. E-cigs may be used both as a tool for smoking cessation as well as by never smokers, particularly young people. Understanding of the toxicity related to e-cig use is needed, in relation to never, current, and former smoking.

E-cigs contain substances which may be harmful including nicotine, ultrafine particulate matter, flavorings, volatile

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organic compounds, and heavy metals. Concentrations are generally considerably lower for these than those in cigarette smoke with the exception of heavy metals where there is evidence that concentrations may be equivalent or higher than those in cigarettes [6]. There is confusion among smokers about the harm from e-cigs and the relative harms of smoking and e-cigs; an understanding of the effects of e-cigs is needed to inform choices [7–10]. An understanding of the public health impact of these devices is urgently required; such an understanding is difficult given the wide variety of products with thousands of different flavors and the rapid change in the products that are available [11–13].

We recently reviewed data regarding inflammation in relation to e-cig use [10]. The focus here is on another potential aspect of e-cig toxicity, namely altered DNA methylation. There is consistent evidence that smoking affects DNA methylation, and that some of the changes in methylation are reversible with smoking cessation. While most of the data regarding altered DNA methylation associated with smoking are examinations of blood cells, there is some for associated changes in other tissues. We review here what is known regarding altered DNA methylation in blood and tissues as a biomarker of smoking toxicity and discuss its potential utility for the assessment of toxicity from e-cig use.

DNA Methylation and Smoking

Epigenetic alterations are an important biological mechanism for an organism to respond to changes in the environment. Among epigenetic changes, DNA methylation plays an important role as a reversible response to environmental exposures [14]. DNA methylation, the addition of a methyl group to a cytosine base on DNA, usually to a cytosine 5′ to a guanine (CpG), plays a role in determining gene expression. The pattern of DNA methylation is not uniform within an individual, differing, at least in part, among tissues. It is also known that DNA methylation can change over time, with aging and in response to exposures such as caloric intake. Alterations in DNA methylation can be observed in response both to exogenous and endogenous exposures, changes which can be locus specific or global across genes.

In comparisons of current smokers to never smokers, there is consistent evidence of differences in DNA methylation; most of these studies are focused on methylation in blood cells. These findings are consistent for men and women and among adults, varying by age. At most of the differentially methylated sites, there is hypomethylation for smokers [14–23]. For example, in one study, 85% of the differentially methylated CpGs were lower for smokers [24].

As noted above, DNA methylation contributes to tissue differentiation and therefore varies by tissue type. Examination of the tissue of interest is critical to understanding the impact of an exposure on methylation [25]. Much of the existing literature regarding effects of cigarette smoking on DNA methylation is focused on DNA methylation from blood cells [15–23]. While these studies potentially provide insight into systemic effects of smoking, they may not provide a full picture of the impact of smoking on particular tissues, especially target organs. Further, in studies of differences in DNA methylation in blood cell DNA, the distributions of cell type may affect results. In some studies comparing blood DNA methylation for smokers and nonsmokers, findings are adjusted for individual white blood cell type percentage [16, 21, 26••]; most studies do not account for these differences, making it difficult to separate alterations as a result of the smoking and those as a result of differences in blood cell types.

Among studies examining differences in blood cell DNA methylation by smoking status, a large number of CpGs have been identified as differentially methylated, with considerable variability among studies. In a meta-analysis of almost 16,000 participants in 16 cohorts, comparing current to never smokers, 185 differentially methylated CpGs were identified [27••]. In another study, 192 CpGs were determined that had been reported in more than one of 16 published studies [24]. In another, examining studies published before June 2015, there were 320 genes identified as differentially methylated in more than one CpG position or in more than one study [28]. Differences in findings among studies are likely related to differences in the analytic methods, differences in the populations under study, including in their smoking habits, differences in the other exposures of the populations, and differences in population genetics. Some observed differences may also result from random noise. Nonetheless, there are CpGs that are identified as associated with smoking status with considerable consistency. These include the aryl hydrocarbon receptor repressor gene (*AHRR*), particularly cg05575921, identified as the CpG that is the most strongly or one of the most strongly associated with smoking status (that is, lower methylation in smokers compared to nonsmokers) in many studies [16, 19, 20, 23, 24, 26••, 27••, 28–31, 32•, 33•, 34–42] (Table 1). In one study, examining methylation of this single CpG, the receiver operating characteristic (ROC) area under the curve (AUC) was 0.99 for the classification of smoking status, comparing current smokers to lifetime never smokers [55•]. Other CpGs in the *AHRR* gene have also been found to be associated with smoking status in a number of studies examining DNA methylation in blood [16, 20, 21, 23, 26••, 31, 48, 50]. Additionally, cg0363183in the F2RL2 gene coding for the protease-activated receptor 4 (PAR-4) [17, 19–24, 26••, 27••, 29–31, 36–40, 42, 45, 49, 50, 54] and CpGs (cg21566642, cg05951221, cg21566642, cg01940273, cg06644428, cg21566642, and cg05951221) in the 2q37 region are frequently identified as differentially methylated [20, 26••, 30, 31, 36, 40, 42, 48, 54].

Table 1 Summary of studies of DNA methylation and smoking status

Reference	Study design	Sample	Smoking measures	Assessment of methylation	Tissue measured	Main findings	Largest DM
Zöchbauer-Müller et al. 2003 [43]	Cross-sectional	Canada: bronchoscopy: healthy current ($n = 81$) and former smokers ($n = 26$), age 42–75 Sputum: additional 30 never smokers, age 21–64	Self-report	Methylation-specific PCR of *retinoic acid receptor β-2*, *CDH13*, *p16^{INK4a}*, *RASSF1A*	42 oropharyngeal brushes, 103 sputum samples, 87 bronchial brushes and 43 bronchioloalveolar lavage (BAL) samples	At least one gene in one sample methylated in 48% of samples; *RARβ-2* methylation most frequent	NA, candidate gene study
Tessema et al. 2009 [44]	Cross-sectional	USA: lung cancer patients (37 current smokers, 59 former and 75 never smokers)	Not indicated	26 candidate genes by combined bisulfite modification and restriction analysis	Lung adenocarcinomas	Three genes differentially methylated for never smokers compared to smokers	*TNFRSF10C* *BHLHB5* *BOLL*
Breitling et al. 2011 [22]	Cross-sectional	Germany: 177 participants in ESTHER cohort, ages 50–60; 51% male, 49% female	Self- reported status	Illumina Infinium Human Methylation27K	Peripheral blood cells	1 differentially methylated site; evidence of dose–response with pack-years smoked, time since cessation among former smokers	cg03636183 *F2RL3*
Philibert et al. 2012 [32•]	Cross-sectional	USA; 399 young African Americans, average age 19	Self-report	Illumina Infinium Human Methylation450K	Lymphocytes	Comparison of non-smokers, < 1/2 pack-year smoking, > 1/2 pack-year smoking, *AHRR* methylation different by smoke exposure; no other CpGs significantly different for males; trend but not significant for females	*c*g05565921 *AHRR*
Wan, et al. 2012 [45]	Cross-sectional	USA: Two family-based studies of COPD, and their siblings, all Caucasian ancestry, age 45–65, with ≥ 5 pack-years of smoking, airflow limitation	Self-report	Illumina Infinium Human Methylation27K	White blood cells	15 differentially methylated sites associated with smoking status, 2 with pack-years, among former smokers, 3 with time since cessation	Associated with smoking status: cg03636183 *F2RL3* cg19859270 *GPR15* cg09837977 *LRRN3* cg01500140 *LIM2* cg13247990 *MYLK* Associated with pack-years: cg03636183 *F2RL3* cg19859270 *GPR15*
Buro-Auriemma et al. 2013 [46]	Cross-sectional	USA: 20 smokers, 19 nonsmokers	Self-report with urinary nicotine and cotinine verification of recent status	HELP (Hpa II tiny fragment enriched by ligation-mediated PCR) assay	Small airway epithelial cells by bronchoscopy	204 unique differentially methylated genes	CYP1B1, CYP1A1, ALDH3A1, SFRP2
Ostrow et al. 2013 [47]	Cross-sectional	USA: 20 healthy never smokers, 13 never smokers with lung cancer, 85 healthy heavy smokers	Self-report	*NISCH* methylation by quantitative fluorogenic real-time PCR	Plasma DNA	NISCH methylation in 68% of heavy smokers, 69% of light smokers with lung cancer, 0 healthy light smokers	*NISCH*
Shenker et al. 2013 [48]	Cross-sectional	Italy: 374 participants from Italian EPIC cohort, both men and women, both breast and colon cancer cases and	Self-reported smoking status	Illumina Infinium Human Methylation450K	White blood cells	20 differentially methylated loci in comparisons of smokers to never and former smokers; 8 for both breast and colon groups; 9 among breast ca-co	Comparing smokers to former and never smokers Breast ca and co:

Table 1 (continued)

Reference	Study design	Sample	Smoking measures	Assessment of methylation	Tissue measured	Main findings	Largest DM
		healthy controls (prospective blood collection for cancer ascertainment)				and 11 among colon ca-co comparisons only	cg05575921 *AHRR* cg05951221 2q37.1 cg21566642 2q37.1 cg06126421 6p21/33 cg03636183 *F2RL3* Colon ca and co: cg05575921 *AHRR* cg01940273 2q37.1 cg21566642 2q37.1 cg5951221 2q37.1 cg03636183 *F2RL3*
Shenker, et al. 2013 [20]	Cross-sectional	Italy: 81 healthy participants in Italian cohort of the EPIC study for the test component; 180 healthy women from the cohort for validation	Self-reported smoking status	Bisulfite pyrosequencing of four predetermined loci	Peripheral blood cells	Methylation index of four CpGs had high sensitivity and specificity for smoking status	cg23576855 *AHRR* cg06644428 2q37 cg21566642 2q37 cg06126421 6p21.33
Sun et al. 2013 [17]	Cross-sectional	USA: GENOA study, 972 African Americans in discovery sample, 239 African Americans in replication	Self-report of smoking within the past year (y/n); ever/never smokers	Illumina Infinium Human Methylation27K	Leukocytes	15 differentially methylated CpGs in comparisons of current smokers and current non-smokers	cg03636183 *F2RL3* cg19859270 *GPR15* cg04983977 *GPR25* cg13668129 *HNRPUL1* cg13500388 *CBFB*
Zeilinger et al. 2013 [21]	Cross-sectional	Germany: KORA cohort, 1814 healthy individuals, ages 32–81, 479 in a replication	Self-report	Illumina Infinium Human Methylation450K	Whole blood, models adjusted for white blood cell proportions	972 differentially methylated CpGs; 187 replicated	cg05575921 *AHRR* cg21566642 *ALPPL2* cg01940273 *ALPPL2* cg21161138 *AHRR* cg03636183 *F2RL3*
Besingi and Johansson et al. 2014 [29]	Cross-sectional	Sweden: 421 (223 females, 198 males)	Self-report	Illumina Infinium Human Methylation450K	Peripheral blood cells	95 differentially methylated sites; all but 3 hypomethylated for smokers	cg04885881 cg25189904 *GNG12* cg09935388 *GFI1* cg11231349 *NOS1AP* cg20295214 *AVPR1B*
Dogan et al. 2014 [16]	Cross-sectional	USA: adult females, African Americans from the Family and Community Health Study longitudinal study, smokers ($n = 50$), non-smokers ($n = 61$), average age 48	Self-report	Illumina Infinium Human Methylation450K	Peripheral mononuclear cells, adjusted for cell type	910 differentially methylated loci	cg05575921 *AHRR* cg23576855 *AHRR* cg19859270 *GPR15*
Elliott et al. 2014 [30]	Cross-sectional	UK: 192 men, aged 40–55 from the SABRE cohort of South Asian migrants and people of European origin living in West London	Self-report	Illumina Infinium Human Methylation450K	Peripheral blood cells	29 differentially methylated CpGs	cg05575921 *AHRR* cg21566642 *2q37.1* cg03636183 *F2RL3* cg22132788 *MYO1G* cg06126421 *6p21.33*
Harlid et al. 2014 [19]	Cross-sectional	USA: 1108 women from the Sister Study cohort, with a	Self-report	908: Illumina Infinium HumanMethylation27 BeadChip	Peripheral blood cells	12 differentially methylated CpGs	cg03636183 *F2RL3* cg19859270 *GPR15* cg09837977 *LRRN3*

Table 1 (continued)

Reference	Study design	Sample	Smoking measures	Assessment of methylation	Tissue measured	Main findings	Largest DM
		sister with breast cancer, ages 35–75		200: Illumina Infinium Human Methylation450K			cg26764244 *GNG12* cg16254309 *CNTNAP2* *ranked by FDR*
Tsaprouni et al. 2014 [39]	Cross-sectional	France, Germany, and UK: 464 participants of European ancestry, age 38–67, some healthy, some with coronary artery disease (22 current, 263 former, 179 never smokers); replication in a female twin cohort (41 current, 104 former, 211 never smokers)	Self-report	Illumina Infinium Human Methylation450K	Peripheral blood cells	30 differentially methylated probes	cg05951221 *ALPPL2* cg05575921 *AHRR* cg01940273 *ALPPL2* cg21566642 *ALPPL2* cg06126421 *IER3*
Zhang et al. 2014 [49]	Cross-sectional	Germany: ESTHER cohort, healthy individuals aged 50–75, $n = 3588$	Self-report	MALDI-TOF quantitation of DNA methylation in a region of *F2RL3*	Whole blood	F2RL3 differentially methylated with smoking status, also associated with current smoking intensity and pack-years of smoking, among former smokers, associated with time since quitting	NA, candidate gene study
Guida et al. 2015 [50]		Europe: women, from nested case–control study of breast and colon cancer in EPIC Italy ($n = 451$), ages 35–70, and Norwegian Women and Cancer Study ($n = 333$), ages 46–63	Self-report	Illumina Infinium Human Methylation450K	Leukocytes	461 differentially methylated CpGs	cg22132788 *MYO1G* cg12803068 *MYO1G* cg03604011 *AHRR* cg26718213 *SNED1* cg11207515 *CNTNAP2* (ranked by beta)
Ottini et al. 2015 [51]	Cross-sectional	Italy: 21 pairs of monozygotic twins with discordant smoking status	Self-report	Promoter methylation of *p16*, *FHIT*, *RAR*, *CDH1*, *DAPK1*, *hTERT*, *RASSF1A*, *MGMT*, *BRCA1*, and *PALB2* genes by methylation-sensitive high-resolution melting (MS-HRM)	Peripheral blood cells	No significant differences by smoking	NA, candidate gene study
Philibert et al. 2015 [34]	Cross-sectional	USA: drinkers in treatment for alcohol and non-drinking controls, mostly male and white ($n = 61$)	Self-report, validated with serum cotinine	Illumina Infinium Human Methylation450K; analysis of 5 CpGs found associated with smoking in other studies	Lymphocyte DNA	Sensitivity and specificity of 5 CpGs in relation to smoking intensity; cg05575921 *AHRR* most strongly associated, also cg01940273 2q37.1, cg21566642 2q37.1, cg05951221 2q37.1	NA, candidate gene study
Sayols-Baixeras et al. 2015 [37]	Cross-sectional	Spain: 645 participants in the REGICOR cohort, both men and women	Self-reported smoking status	Illumina Infinium Human Methylation450K	Whole blood	66 differentially methylated CpGs associated with smoking, in most former smokers' values approached never smokers	cg04885881 cg27537125 cg25189904 *GNG12* cg09662411 *GFI1* cg06338710 *GFI1*
Reynolds et al. 2015 [35]	Cross-sectional	USA: 495 never smokers, 411 former smokers from Multi-Ethnic Study of Atherosclerosis,	Self-report of second-hand	Illumina Infinium Human Methylation450K for methylation of *AHRR*	CD14+ blood monocytes	AHRR methylation inversely associated with SHS exposure	NA, candidate gene study

Table 1 (continued)

Reference	Study design	Sample	Smoking measures	Assessment of methylation	Tissue measured	Main findings	Largest DM
		approximately half women, includes whites, Hispanics, and blacks	smoke exposure				
Ambatiputi et al. 2016 [23]	Cross-sectional data from nested case–control study of breast cancer	Europe: 910 women, 41% pre- and 59% postmenopausal (from EPIC cohort)	Self-report of status, cigarettes per day, time since quitting (former smokers)	Illumina Infinium Human Methylation450K	Leukocytes	748 CpG differentially methylated; altered methylation reversible with smoking cessation though some changes persist after more than 20 years; 450 hypo- and 298 hypermethylated for smokers compared to non-smokers	Most hypomethylated in smokers: cg05575921 *AHRR* cg23576855 *AHRR* cg21566642 *ALPPL2* cg0363183 *F2RL3* cg06126421 *IER3* Most hypermethylated in smokers: cg03274391 *ZNF385D* cg23480021 *ZNF385D* cg12803068 *MYO1G* cg15693572 *SNF385D* cg23126342 *PCDH9*
Georgiadis et al. 2016 [31]	Cross-sectional data from nested case–control study of breast cancer and B cell lymphoma	Italy and Sweden: 649 current, former, and never smokers from 2 cohorts: Northern Sweden Health and Disease Study and EPIC Italy, average age 52	Self-report	Illumina Infinium Human Methylation450K	Leukocytes	1273 differentially methylated CpGs	cg05575921 *AHRR* cg21566642 2q37.1 cg03636183 *F2RL3* cg01940273 2q37.1 cg05951221 2q37.1
Joehanes et al. 2016 [27]	Cross-sectional	USA and Europe: 15,907 from 16 cohorts	Self-report	Illumina Infinium Human Methylation450K	Blood cells, $CD4^+$ T or monocytes, depending on study	2623 differentially methylated CpGs	cg16145216 *HIVEP3* cg19406367 *SGIP1* cg05603985 *SKI* cg14099685 *CUGBP1* cg12513616
Lee et al. 2016 [24]	Cross-sectional	Korea: 60 COPD patients, 40 no COPD (31 current, 30 former, and 39 never smokers)	Self-report confirmed with urinary cotinine	Illumina Infinium Human Methylation450K	Peripheral blood cells	108 differentially methylated probes comparing current to never smokers; 104 of these in comparison of former to never smokers	cg05575921 *AHRR* cg10664184 *DDA1* cg20723792 *FAM53B* cg24780263 *ALDOA* cg05951221 12,850 base pair from *ALPPL2*
Philibert et al. 2016 [33•]	Prospective	USA: 35 current smokers planning to quit smoking, followed for 6 months, mostly female and white	Self-report validated with serum cotinine and exhaled carbon monoxide	Quantitative PCR of cg05575921	Peripheral blood cells	DNA methylation cg05575921 increased with decreased smoking and smoking cessation	NA, candidate gene study
Chatterton et al. 2017 [52]	Cross-sectional	USA: fetal brain tissue, maternal smoke exposure, 14 smoke exposed, 10 non-exposed	Maternal self-report	Illumina Infinium Human Methylation450K	Fetal dorsolateral prefrontal cortex	No differentially methylated regions; interaction of gestational age and smoking status	

Table 1 (continued)

Reference	Study design	Sample	Smoking measures	Assessment of methylation	Tissue measured	Main findings	Largest DM
Conway et al. 2017 [53]	Cross-sectional	USA: Breast cancer cases, ages 20–74, $n = 517$, 125 current, 124 former, 268 never smokers	Self-report	Illumina GoldenGate Cancer Panel I methylation bead array (1505 CpG loci)	Breast tumors	107 differentially methylated by current compared to never smokers in hormone receptor negative tumors (HR−), 22 in HR+ tumors	HR− cg23305046 *CDKN1A* cg18307303 *IL12B* cg19728002 *MCC* cg05794098 *FLJ20712* HR+ cg27650434 *MYCL1* cg27186533 *CCNA1* cg01872931 *FRZB* cg09229893 *BMP4*
Reynolds et al. 2017 [36]	Cross-sectional	934 participants in Multi-Ethnic Study of Atherosclerosis, approximately half women, includes whites, Hispanics, and blacks	Urinary cotinine	Illumina Infinium Human Methylation450K	$CD14^+$ monocytes from blood	176 CpGs associated with urinary cotinine	cg05565921 *AHRR* cg21566642 2q37.1 cg06126421 *6p21.33* cg03636183 *F2RL3* cg009935388 *GFI1* cg21322436 *CNTNAP2* cg05284742 *ITPK1*
Stueve et al. 2017 [38]	Cross-sectional	Italy: 237 lung cancer patients (121 current smokers, 106 former smokers, 10 never smokers)	Not indicated	Illumina Infinium Human Methylation450K	Non-tumor lung tissue	Seven differentially methylated CpGs in comparisons of current smokers and current non-smokers; methylation inversely associated with smoking duration, pack-years, positively associated with years since cessation among former smokers	cg17113147 *NXN* cg05575921 *AHRR* cg07992500 *CDC42EP3* cg14120703 *NOTCH1* cg11152412 *EDC3*
Wilson et al. 2017 [26••]	Cross-sectional and prospective cohort analyses	Germany: KORA cohort, Germany, 1344 healthy individuals	Self-report at 2 time points 7 years apart	Illumina Infinium Human Methylation450K	Whole blood models adjusted for white blood cell proportions	Cross-sectional: 590 differentially methylated sites associated with smoking status Longitudinal: 52 differentially methylated in comparison of former to never smokers; most altered methylation with cessation within 20 years	Cross-sectionally associated with smoking status: cg05575921 *AHRR* cg21566642 2q37.1 cg01940273 2q37.1 cg03636183 *F2RL3* cg05951221 2q37.1 Longitudinally associated with cessation: cg26703534 *AHRR* cg05575921 *AHRR* cg14817490 *AHRR* cg01940273 2q37.1 cg23576855 *AHRR*
Li et al. 2018 [40]	Cross-sectional	Australia: cohort study of 66 monozygotic twin pairs, 66 dizygotic twin pairs, 215 sisters	Self-report	Illumina Infinium Human Methylation450K	Dried blood spots on Guthrie cards	39 differentially methylated sites associated with smoking status	cg05575921 *AHRR* cg05951221 2q37.1 cg01940273 2q37.1 cg03636183 *F2RL3* cg06126421 6p21.33
Prince et al. 2018 [41]	Cross-sectional	UK: 932 adolescents, ages 14–16, ALPAC cohort	Self-report, blood cotinine	Illumina Infinium Human Methylation450K; studied 2620 CpGs previously identified in	Peripheral blood cells	11 differentially methylated in association with smoking status	cg05575921 *AHRR* cg26703534 *AHRR* cg08331398 *PSMB8*

Table 1 (continued)

Reference	Study design	Sample	Smoking measures	Assessment of methylation	Tissue measured	Main findings	Largest DM
				a study of smoking and methylation			cg09935388 *GFI1* cg02512902 *KSR1*
Tsai et al. 2018 [54]	Cross-sectional	UK: healthy, female twins from the TwinsUK cohort, 54 current, 291 non-smokers, 84 mono-, 112 dizygotic twins, 150 unrelated	Self-report	Illumina Infinium Human Methylation450K	Adipose tissue	42 differentially methylated sites	cg05951221 2q37.1 cg21566642 2q37.1 cg23680900 *CYP1A1* cg14120703 *NOTCH1* cg26516004 *CYP1A1*

DNA methylation may be altered even with relatively low smoke exposures. In a study of young people with relatively short histories of light smoking, comparing never smokers, smokers with less than one-half and those with more than one-half of pack-year history, *AHRR* cg05575921 methylation for the males differed by group. The number of females in the study was smaller and did not reach statistical significance [32•] (Table 1). Environmental tobacco smoke exposure may also impact DNA methylation. Exposure to environmental smoke within the previous week was associated with cg05575921 methylation [56] in a study of never and former smokers. In a study of breast tumor DNA methylation, environmental tobacco smoke exposure was associated with differences in methylation [57]. In utero exposure to maternal smoking has also been shown to affect offspring methylation [14].

There are only a small number of studies which have examined smoking effects in target organ tissues, including the lung. There may be systemic effects of smoking such that the sites of consistently altered DNA methylation are found not only in blood but in other tissues. Altered methylation of the *AHRR* cg05575921 was found in non-tumor lung tissue from smokers with lung cancer for smokers compared to non-smokers [38]. Further, in another study of normal tissue collected during a lung tumor resection where the normal lung tissues were checked for abnormal pathology, there were similar differences in *AHRR* cg05575921 methylation by smoking status [42]. In a study examining adipose tissue, there were differences in DNA methylation by smoking status including two CpGs in the 2q37.1 region [54]. There have been a few studies examining tumor DNA methylation, showing differences by smoking status, with some overlap with the CpGs found in studies of normal tissues [53, 57, 58]. Because of the importance of smoking-related lung diseases, understanding of effects in the lung are particularly important. There is some evidence from sputum of altered DNA methylation with smoking status—findings that likely reflect changes in the lung [18, 59, 60]. There are a small number of studies directly examining lung biospecimens by bronchoscopy [18, 43, 58, 61]. As for the studies of blood, there is a finding in the lung of consistent differences in methylation for smokers and never smokers, including some overlap between lung and blood in the locations of altered methylation [43, 60]. While these studies are useful, more data regarding effects on target tissues are needed to understand biologic effects in particular organs.

Former Smokers: Time Since Smoking

Differential DNA methylation can be used as a biomarker of progress toward smoking cessation [33•] and of past exposure to smoking [50]. Many, but not all, smoking-associated

changes in DNA methylation are reversible. Blood cell DNA methylation for former smokers is generally intermediate between that for smokers and never smokers, with former smokers generally showing a pattern more similar to never smokers [19, 21, 23, 27••, 31, 33•, 37, 48, 50]. In studies of particular CpGs (e.g., in the *AHRR* gene, consistently identified as differentially methylated for smokers at one or more CpGs) or in genome-wide studies, DNA methylation is correlated with both pack-years of smoking and with time since smoking cessation among former smokers [15, 21, 32•, 45, 49, 62]. There are a limited number of studies examining sputum and lung cells of former smokers; these show a similar pattern—former smokers' methylation is more similar to never than to current smokers [18, 59]. There are no human data regarding DNA methylation for e-cig users.

There are few studies regarding the speed of the changes in methylation with smoking cessation. Most studies of former smokers are of individuals who have not smoked for periods on the order of 5 years or longer [17, 19, 21, 22, 26••, 30, 45, 62]. However, a few studies have examined changes over shorter time periods [33•, 39, 49]. In a study following smokers in the process of smoking cessation, there was evidence of increased blood cell DNA methylation of cg05575921 in the *AHRR* gene after 1 month [33•]. In another study, there were alterations in DNA methylation detectable within 3 months [39]. With respect to timing of methylation changes, in cell culture studies, effects on gene transcription and DNA methylation have been demonstrated in very short time periods [63–65]. In a study of malignant transformation of a human cell line in culture, there were both genome-wide and site-specific alterations in methylation within 10 min following exposure to cigarette smoke [64]. In another study, DNA methylation was altered for cell cultures exposed to cigarette smoke condensate after periods of as little as 1 day [63].

DNA Methylation and Lung Disease

Smoking-related identified DNA methylation alterations frequently map to genes with significance for lung function, lung diseases, and inflammation [15, 16, 27••, 59]. DNA methylation has been shown to play a critical role in chronic obstructive pulmonary disease (COPD) [24, 62, 66–75]; differentially methylated genes that are associated with smoking have also been shown in studies of blood to be associated with risk of COPD [69, 71, 75, 76]. Altered methylation has been found to be associated with lung function [75, 77]. In sputum from smokers, altered DNA methylation was associated both with lung function and odds of COPD [71, 78]. Further, DNA methylation profiles may predict response to treatment of acute exacerbations of COPD [69]. DNA methylation has been shown to be associated with lung and other cancers [18, 28, 47, 58, 79••, 80–82, 83, 84]. For two of the genes where there is consistent evidence of hypomethylation among smokers, *AHRR* and *F2RL3*, altered methylation has been shown in several cohort studies to be strongly predictive of lung cancer risk, independent of smoking history [79••, 80]. In addition, altered methylation is associated with cardiovascular disease [22, 35, 49], inflammation [85], and overall mortality [22, 49, 80], as well as older adult frailty [86] and age acceleration [87].

DNA Methylation Affects Gene Expression and Inflammation in Smokers

It is known that changes in DNA methylation can affect gene expression [88, 89]; there is more limited evidence regarding changes in gene expression as a result of smoking-related altered DNA methylation specifically [37, 46, 60]. In one study, *AHRR* methylation was related to gene expression in pulmonary macrophages from smokers [61]. Altered methylation is associated with increased inflammation [90–92] as well as inflammation affecting methylation. Cytokine expression has been found to be controlled, in part, by DNA methylation and other epigenetic mechanisms [93]. Understanding the interplay of smoking with biological effects including methylation, gene expression, and inflammation could potentially provide new insight into the effects of smoking and potentially of e-cigs. e-Cig users inhale a variety of constituents and their breakdown products in the vapor produced by these devices; many of these compounds are known to be irritants and to provoke inflammation. The toxic effects of these exposures need to be determined.

E-cigs and DNA Methylation

There are no human studies examining effects of e-cigs on DNA methylation. While it is plausible that there would be changes in DNA methylation for smokers who switch to e-cig use, at least somewhat similar to changes observed for former smokers, direct evidence is needed.

Nicotine present in e-cigs is a bioactive compound that impacts cell proliferation, apoptosis, angiogenesis, and inflammation [10]. There are just a few studies regarding the specific effects of nicotine on DNA methylation. There are animal studies showing maternal nicotine exposure affects DNA methylation in her offspring [94–96]. In cell culture studies, the effects of e-cig vapors on transcription differ for devices with and without nicotine [64, 97]. In a mouse study, the biologic response to exposure to e-cigs was different depending on whether or not they contained nicotine; effects of the nicotine-containing e-cigs were more similar to those related to COPD development [97]. There are few studies regarding nicotine exposure effects on DNA methylation in humans [98–100].

Staudt et al. [100] saw acute differences in transcription following e-cig exposure with and without nicotine in never smokers; they did not examine DNA methylation. In a study of *MAOA* methylation, nicotine dependence was associated with methylation in women, but not in men [101].

The other e-cig constituents could also impact methylation. In addition to nicotine, e-liquids are composed mostly of vegetable glycerol (VG; also known as glycerin) and/or propylene glycol (PG) and flavorings. The FDA has designated these constituents as "generally regarded as safe" when used in foods and skin products [102, 103]. However, it is unknown what happens to exposed tissues such as the lung when these constituents are heated and inhaled. In e-cigs, PG can be converted to propylene oxide [1, 104], an irritant and an International Agency for Research on Cancer group 2b carcinogen [105]. Heated VG and PG can be converted to acrolein, acetaldehyde, and formaldehyde, also strong irritants [106–108]. In one study, there were 31 chemical constituents identified in e-cig aerosols, including glycidol, acetol, and diacetyl [109]. E-cig aerosols have also been reported to contain other potentially harmful chemicals, including tobacco-specific nitrosamines, aromatic hydrocarbons, acetone, and volatile organic compounds (VOC) (e.g., benzaldehyde, propionaldehyde, crotonaldehyde) [1, 7, 108, 110–128]. A recent study using mass spectroscopy identified over 115 VOCs, many that were not present in the unheated liquids [111], and another identified trace quantities of benzene, methyl ethyl ketone, toluene, xylene, styrene, and acetic acid [128]. Nonetheless, the presence of many of these compounds is substantially less than for cigarette smoke; heavy metal concentrations may be the same or higher for e-cigs compared to cigarettes [6]. It is anticipated that the effects of e-cigs will likely be less than for cigarettes. Direct data regarding toxicity from e-cig exposure in humans are required; data from human biomarker studies can provide insights into this important question.

Conclusions

Understanding of the biological effects of e-cigs on all tissues, particularly target tissues for smoking-related disease, is critically important, and a public health problem of considerable significance. There is a pressing need for more information to inform regulation. Understanding of how e-cig use affects DNA methylation, including among the different kinds of users, those who are never smokers, former smokers and dual users, can significantly add to this evidence-based regulation.

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Compliance with Ethical Standards

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the FDA.

Conflict of Interest Jo L. Freudenheim reports grants from Prevent Cancer Foundation, during the conduct of the study.

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