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Review

Smoking and DNA methylation: Correlation of methylation with smoking behavior and association with diseases and fetus development following prenatal exposure

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ABSTRACT

Among epigenetic mechanisms, DNA methylation has been widely studied with respect to many environmental factors. Smoking is a common factor which affects both global and gene-specific DNA methylation. It is supported that smoking directly affects DNA methylation, and these effects contribute to the development and progression of various diseases, such as cancer, lung and cardiovascular diseases and male infertility. In addition, prenatal smoking influences the normal development of the fetus via DNA methylation changes. The DNA methylation profile and its smoking-induced alterations helps to distinguish current from former smokers and non-smokers and can be used to predict the risk for the development of a disease. This review summarizes the DNA methylation changes induced by smoking, their correlation with smoking behavior and their association with various diseases and fetus development.

1. Introduction

Deoxyribonucleic acid (DNA) methylation is a heritable epigenetic mechanism which alters the phenotype, without altering the underlying nucleotide sequence. DNA methylation is critical for the normal development. Its main role is the regulation of tissue-specific gene expression, but it also participates in gene imprinting and X-chromosome inactivation (Suhasni Gopalakrishnan, Beth O. Van Emburgh, 2008).

DNA methylation is a reversible process and occurs at the fifth carbon of a cytosine residue, which is located before a guanine (CpG site), forming 5-methylcytosine. The donor of the methyl-group is S-adenylmethionine (SAM) and the catalytic enzymes participating are the DNA methyltranferases (DNMTs). Most of the CpG sites in the human genome are methylated; however, CpG sites in specific regions, such as promoters of housekeeping genes, are not methylated, allowing for expression to take place. Gene promoters have a high density of CpG sites, called CpG islands, which contain less nucleosomes than other regions, facilitating the binding of transcription factors. Hyper-methylation of gene promoters leads to reduced gene expression. In comparison, intergenic regions are normally methylated to prevent the expression of harmful genetic elements. CpG sites in gene bodies can be either methylated or not, hypo-methylation being associated with increased expression (Moore et al., 2013).

DNA methylation patterns are stable (Gopalakrishnan et al., 2008) and depend on gender, age (Lee et al., 2016a,b) and genetic variations (Qiu et al., 2015). Nevertheless, environmental factors, such as pollutants, lifestyle and smoking, can induce alternations on DNA methylation patterns and thus dysregulation of gene expression, contributing to the development of various diseases (Klebaner et al., 2016).

Smoking is a common habit and despite its known negative health effects (Flouris et al., 2012, 2012b; Metsios et al., 2007; Vardavas et al., 2010), a significant proportion of the population continues to smoke. Tobacco smoke contains ~7000 chemicals, which can influence the human body through DNA damage, inflammation and oxidative stress and lead to cardiovascular disease, chronic obstructive pulmonary disease (COPD), cancer (Zeilinger et al., 2013) and death (Zhang et al., 2016c). Prenatal smoke exposure, through maternal smoking is also a big issue and scientists have designed randomized control studies in order to promote smoking cessation in pregnant women (Loukopoulou et al., 2011). Prenatal smoke exposure can influence the DNA methylation status and development of the fetus, after the first trimester (Küpers et al., 2015). The consequences are stable and persist in the offspring, until childhood and adolescence (Breton et al., 2014). The exact mechanism by which smoking causes various diseases is

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Abbreviation

AACS	Acetoacetyl-CoA Synthetase
ACTA2	Alpha-actin-2
ADM	Adrenomedullin
ADRB3	Adrenoceptor Beta 3
ADX1	Adrenodoxin-like protein 1
AHR	Aryl-Hydrocarbon Receptor
AHRR	Aryl-Hydrocarbon Receptor Repressor
ALPPL2	Alkaline phosphatase, placental-like 2
ANK1	Ankyrin 1
ANKRD33B	Ankyrin Repeat Domain 33B
ANPEP	Alanyl Aminopeptidase
APC	Adenomatous polyposis coli
ARAF	Serine/Threonine-Protein Kinase A-Raf
ARNT	Aryl nuclear receptor translocator
AT	Angiotensin II
ATP9A	ATPase Phospholipid Transporting 9A
Bcl-2	B-cell lymphoma 2
BCOR	B-cell lymphoma 6 corepressor
BID1	BH3 Interacting Domain Death Agonist
BPIFB1	BPI fold containing family B, member 1
C11orf52	Chromosome 11 Open Reading Frame 52
CAD	Coronary artery disease
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CHRNA5	Cholinergic Receptor Nicotinic Alpha 5 Subunit
CHRNB4	Cholinergic Receptor Nicotinic Beta 4 Subunit
CLDN11	Claudin 11
CNTD2	Cyclin N-Terminal Domain Containing 2
CNTNAP2	Contactin Associated Protein Like 2
COPD	Chronic obstructive pulmonary disease
COPX	Regulatory protein cox
CPEB1	Cytoplasmic Polyadenylation Element Binding Protein 1
CRP	C-Reactive Protein
CRYGN	Crystallin Gamma N
CSC	Cigarette smoke condensate
CSE	Cigarettes smoke extract
CSRNP1	Cysteine And Serine Rich Nuclear Protein 1
CST6	Cystatin E/M
CYP2D6	Cytochrome P450 2D6
DCC	Deleted in Colorectal Carcinoma
DMRs	Differentially methylated regions
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltranferases
DPP10	Inactive dipeptidyl peptidase 10
EMILIN2	Elastin Microfibril Interfacer 2
ERK1	Mitogen-Activated Protein Kinase 3
F2RL2	Coagulation Factor II Thrombin Receptor Like 2
F2RL3	Coagulation Factor II Thrombin Receptor Like 3
FCRLA	Fc Receptor Like A
FES	Feline Sarcoma
FGF3	Fibroblast growth factor 3
FKBP5	FK506 Binding Protein 5
FRMD4A	FERM Domain Containing 4A
GALNT2	Polypeptide N- Acetylgalactosaminyltransferase 2
GALR1	Galanin Receptor 1
GATA3	GATA Binding Protein 3
GCH	Guanine Triphosphate Cyclohydrolase
GCLC	Glutamate-Cysteine Ligase Catalytic Subunit
GFI1	Growth Factor Independent Protein 1
GLIS1	GLIS Family Zinc Finger 1
GNA15	G Protein Subunit Alpha 15
GNG12	G Protein Subunit Gamma 12
GNGT2	G Protein Subunit Gamma Transducin 2
GPR15	G Protein-Coupled Receptor 15
GTF2H2	General transcription factor IIH subunit 2-like
HIC1	Hypermethylated In Cancer 1
HOXA4	Homeobox A4
HPA	Hypothalamic pituitary adrenal axis
HR	Hormone receptor
hTERT	Human telomerase reverse transcriptase
ICR	Imprinting Control Region
IFN-γ	Interferon gamma
IGF2	Insulin-like growth factor 2
IL-13	Interleukin 13
ITLN2	Intelectin 2
JAG1	Jagged 1
JAK2	Janus kinase 2
JNK2	c-Jun N-terminal kinase
KLF6	Krueppel-like factor 6
LAYN	Layilin
LCTL	Lactase Like
LEPR	Leptin Receptor
LINC00403/86	Long Intergenic Non-Protein Coding RNA 403/86
LRFN1	Leucine Rich Repeat And Fibronectin Type III Domain Containing 1
LRP5	LDL Receptor Related Protein 5
LRRC2	Leucine Rich Repeat Containing 2
MAOA	Monoamine oxidase A
MAOB	Monoamine oxidase B
MARVELD3	MARVEL Domain Containing 3
MCAM	Melanoma Cell Adhesion Molecule
MEG3	Maternally Expressed 3
MIR	microRNA
MLH1	mutL homolog 1
MRAS	Muscle RAS Oncogene Homolog
MSH	MutS protein homolog
MTHFR	Methylenetetrahydrofolate reductase
MT-RNR1	Mitochondrially Encoded 12S RNA
MTSS1	Metastasis Suppressor 1
MYO1G	Myosin IG
NEUROG1	Neurogenin 1
NFIX	Nuclear Factor I X
NHP2L1	Non-histone protein 2- like protein 1
NISCH	Nischarin
NKX6-2	Homeobox protein Nkx-6.2
NL-20	Immortalized bronchial epithelial cells
Nme2	NME/NM23 Nucleoside Diphosphate Kinase 2
NOS1AP	Nitric Oxide Synthase 1 Adaptor Protein
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1
NTRK	Neurotrophic Receptor Tyrosine Kinase
PAR-4	Protease-activated receptor-4
Pebp1	Phosphatidylethanolamine Binding Protein 1
Pfn1	Profilin 1
PKP3	Plakophilin 3
PLA2	A2 phospholipase
PON3	Paraoxonase 3
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PPEF2	Protein Phosphatase With EF-Hand Domain 2
PPP1R15A	Protein Phosphatase 1 Regulatory Subunit 15A
PRKCZ	Protein Kinase C Zeta
PSAE	Primary small airway epithelial cells
RA	Rheumatoid arthritis
RAR-β	Retinoic Acid Receptor Beta
RARA	Retinoic Acid Receptor Alpha
RIP1	Receptor Interacting Serine/Threonine Kinase 1
RPH3A	Rabphilin-3A
RPS6KA3	Ribosomal Protein S6 Kinase A3
RUNX1	Runt-related transcription factor 1
SAE	Small airway epithelial cells

SAM	S-adenylmethionine
SARS	Seryl-TRNA Synthetase
SDHAP3	Succinate Dehydrogenase Complex Flavoprotein Subunit A Pseudogene 3
Shp2	Tyrosine-protein phosphatase non-receptor type 11 (PTPN11)
SIPA1	Signal-Induced Proliferation-Associated 1
SLC25A24	Solute Carrier Family 25 Member 24
SMG6	Nonsense Mediated MRNA Decay Factor
SMPD3	Sphingomyelin Phosphodiesterase 3
SMUG1	Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase 1
SNP	Single nucleotide polymorphism
SNRPN	Small Nuclear Ribonucleoprotein Polypeptide N
Sort	Sortilin
SOX1	Transcription factor SOX-1
STK32A	Serine/Threonine Kinase 32A
TBX5	T-box 5
TERT	Telomerase Reverse Transcriptase
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TNXB	Tenascin XB
TOM1L2	Target Of Myb1 Like 2 Membrane Trafficking Protein
TRK	Tropomyosin receptor kinase
TRAK1	Trafficking Kinesin Protein 1
Trim27	Tripartite Motif Containing 27
TRIO	Trio Rho Guanine Nucleotide Exchange Factor
TSC22D3	TSC22 Domain Family Member 3
VTI1A	Vesicle Transport Through Interaction With T-SNAREs 1A
WBP1	WW Domain Binding Protein 1
WWTR1	WW domain-containing transcription regulator protein 1
XPNPEP1	X-Prolyl Aminopeptidase 1
ZMIZ1	Zinc finger MIZ domain-containing protein 1
ZNF385D	Zinc Finger Protein 385D

unknown, but altered DNA methylation patterns are widely known to mediate disease etiology (Zeilinger et al., 2013). In this review, we address (1) DNA methylation patterns associated with smoking behavior, (2) smoking-induced DNA methylation changes and diseases, and (3) effect of prenatal smoke exposure on DNA methylation in offspring.

1.1. DNA methylation patterns associated with smoking behavior

Multiple studies have investigated DNA methylation patterns related to smoking behavior. Furthermore, DNA methylation patterns are a way to distinguish current smokers from non-smokers, as well as former smokers from non-smokers, which is difficult to confirm solely by measuring cotinine levels (Shenker et al., 2013b; Zhang et al., 2016b). The degree of DNA methylation changes is related to the intensity of smoking (pack-years) (Ambatipudi et al., 2016; Harlid et al., 2014; Lee et al., 2016a,b; Wan et al., 2015; Wilson et al., 2017). High intensity of smoking can cause alternations in genes that do not appear in lighter smokers. Some of these genes are MTSS1 (cg24838345), NKX6-2 (cg11068946) and a CpG site within SOX1 and LINC00403 (cg15653173) (Su et al., 2016). Differences have also been observed in DNA methylation patterns between the two genders. When the X chromosome was examined, smoking effects were more pronounced in males than in females (Klebaner et al., 2016).

After smoking cessation, methylation is gradually reversed to normal levels (Harlid et al., 2014). The longer the time after smoking cessation, the more the DNA methylation levels resemble to those of non-smokers. It has been found that at least three months are required for methylation levels to approximate those of non-smokers (Sun et al., 2014), but it may take up to 22 years for them to reach levels indistinguishable from non-smokers (Ambatipudi et al., 2016). The reversibility differs for each CpG site and some of them, such as AHRR and F2RL2, never reach the methylation levels of non-smokers, even after 22 (Ambatipudi et al., 2016) or 35 years of smoking cessation (Guida et al., 2015). Smoking cessation has also been shown to reverse the methylation status on 71 and 353 CpG sites. These CpG sites are studied with respect to the methylomic age of an individual and therefore it has been shown that smoking reduction or cessation decelerates aging when measured in terms of methylomic age (Lei et al., 2017).

AHRR (aryl hydrocarbon receptor repressor) is one of the most significant genes related to smoking status (Allione et al., 2015; Dogan et al., 2017, 2015; Elliott et al., 2014; Lee et al., 2016a,b; Philibert et al., 2013; Su et al., 2016; Tsaprouni et al., 2014; Wilson et al., 2017; Zaghlool et al., 2015; Zeilinger et al., 2013), not only in blood DNA, but also in fibroblast DNA and alveolar macrophage DNA (Monick et al., 2013). It contains up to 10 CpG methylated sites (Sayols-Baixeras et al., 2015) that are hypomethylated under smoking conditions, influencing its expression. AHRR functions as a negative feedback regulator by competing with AHR for binding to the aryl nuclear receptor translocator (ARNT). ARNT is part of the xenobiotic pathway, which is responsible for the degradation of hydrocarbons and dioxins that are found in cigarettes (Philibert et al., 2013). A decrease in AHRR methylation is apparent from the very early beginning of smoking, thus its ability to indicate smoking history is limited (Philibert et al., 2017). For the same reason, it is not suitable for distinguishing between heavy and light smokers. Methylation of AHRR is influenced by all levels of smoke exposure and heavy smoking causes a small difference in methylation (Su et al., 2016). It has been reported that smoking-induced hypomethylation of AHRR is associated with hypermethylation of MTHFR. The MTHFR gene contributes to the pathway responsible for the methyl-group availability, so its hypermethylation causes even lower hypomethylation of AHRR. It is suggested that methylation levels both of the AHRR and the MTHFR, should be determined for the correct estimate of smoke exposure (Beach et al., 2017). After smoking cessation, methylation of AHRR is restored relatively fast in the first years, and it is stabilized after some decades, without ever reaching the methylation levels of non-smokers. As a result, it is able to indicate former smoking behavior (Zeilinger et al., 2013). Moreover, hypomethylation of AHRR (cg05575921) can be caused by second-hand smoke exposure and it is related to the intensity of exposure. Nevertheless, the effects are weaker than those caused by active smoking (Reynolds et al., 2017). The AHRR gene, except for being a biomarker for smoking behavior (Zaghlool et al., 2015), can also be a biomarker for mortality, with the intensity of its methylation being inversely associated with increased mortality risk (Zhang et al., 2016c).

Another significant gene related to smoking behavior is F2RL3 (coagulation factor II (thrombin) receptor-like 3) (Qiu et al., 2015; Sun et al., 2014; Allione et al., 2015; Ambatipudi et al., 2016; Besingi and Johansson, 2014; Dogan et al., 2017; Elliott et al., 2014; Guida et al., 2015; Harlid et al., 2014; Tsaprouni et al., 2014; Zeilinger et al., 2013; Zhang et al., 2014, 2016b). This gene encodes protease-activated receptor-4 (PAR-4), which plays a role in platelet activation and cell signaling and is involved in both cardiovascular and neoplastic diseases. Hypomethylation of F2RL3 is inversely associated with the intensity and duration of smoking in current smokers and in former smokers, but methylation levels in former smokers are higher. In former smokers, the increase in methylation is proportional to the time since quitting smoking. However, methylation of F2RL3 approximates the levels inherent to non-smokers after 20 years of cessation, but never reaches them (Zhang et al., 2014).

Additional genes that are significant in terms of smoking are GFI1 (Ambatipudi et al., 2016; Besingi and Johansson, 2014; Dogan et al., 2017; Elliott et al., 2014; Guida et al., 2015; Sayols-Baixeras et al.,

2015; Sun et al., 2014; Wilson et al., 2017; Zhang et al., 2016b), GPR15 (G Protein-Coupled Receptor 15) (Besingi and Johansson, 2014; Dogan et al., 2017, 2015; Harlid et al., 2014; Sun et al., 2014; Tsaprouni et al., 2014), GNG12 (Besingi and Johansson, 2014; Elliott et al., 2014; Guida et al., 2015; Harlid et al., 2014; Sayols-Baixeras et al., 2015; Tsaprouni et al., 2014), RARA (Besingi and Johansson, 2014; Sayols-Baixeras et al., 2015; Su et al., 2016; Tsaprouni et al., 2014), COPX (Besingi and Johansson, 2014; Harlid et al., 2014), ALPPL2 (Ambatipudi et al., 2016; Zeilinger et al., 2013) and CYP2D6 (Tiili et al., 2015). They were all found to be hypomethylated. In MYO1G (Allione et al., 2015; Ambatipudi et al., 2016; Besingi and Johansson, 2014; Elliott et al., 2014; Guida et al., 2015; Philibert et al., 2013; Sayols-Baixeras et al., 2015; Wilson et al., 2017), ZNF385D (Ambatipudi et al., 2016; Tsaprouni et al., 2014) and HIC1 (Peluso et al., 2014) gene hypermethylation was observed. Moreover, the intergenic regions 2q37.1 (Allione et al., 2015; Elliott et al., 2014; Guida et al., 2015; Harlid et al., 2014; Sun et al., 2014; Wilson et al., 2017; Zhang et al., 2016b) and 6p21.33 (Allione et al., 2015; Elliott et al., 2014; Guida et al., 2015; Sun et al., 2014; Zhang et al., 2016b) were found to be hypomethylated in smokers and were inversely associated with mortality (Zhang et al., 2016c).

Methylation of the MAOA and MAOB genes was site-specific and differed between genders. The MAOA gene was hypomethylated in current female smokers when compared to non-smokers, but its methylation level was higher in current than former smokers. The MAOB gene was differentially methylated in 2 CpG sites in females smokers, one hypermethylated and one hypomethylated, while in males smokers only one CpG site in MAOB was significantly hypermethylated (Tiili et al., 2017).

When methylation in the X-chromosome of smokers was studied, two genes were significantly differentially methylated, BCOR (cg07764473) and TSC22D3 (cg21380860). They were hypermethylated and hypomethylated, respectively. The effect was higher in males than in females, whereas no CpG site was significantly associated with pack-years (Klebaner et al., 2016).

Smoking-induced DNA methylation changes have also been studied in different ethnic groups. In a comparison made between Europeans and South-Asians, hypomethylation of AHRR was higher among Europeans (Elliott et al., 2014). Furthermore, when comparing African-Americans and European-Americans, hypomethylation of GPR15 was observed only in the second ethnic group (Dogan et al., 2015).

All of the above studies are summarized in Table 1.

1.2. Smoking-induced DNA methylation changes and diseases

1.2.1. Smoking-induced DNA methylation changes in lung cancer

It has been well established that smoking has many negative effects on human health. Smoking-induced DNA methylation changes are known to contribute to the development and progression of various diseases. Furthermore, alternations in DNA methylation in specific regions and genes can be predictive of the risk for presenting with a certain disease. Lung cancer is one of the most common cancers caused by smoking. It has been reported that biomarkers for smoking behavior, such as AHRR (cg05575921) (Baglietto et al., 2017; Bojesen et al., 2017; Fasanelli et al., 2015; Lee et al., 2017) and F2RL3 (cg03636183) (Baglietto et al., 2017; Fasanelli et al., 2015; Lee et al., 2017; Zhang et al., 2015) and intergenic regions 2q37.1 (cg05951221, cg21566642) and 6p21.33 (cg06160421) (Baglietto et al., 2017), also represent biomarkers for lung cancer risk development. All of these markers were found to be more hypomethylated in smokers who had developed lung cancer later in their life. Specifically, a 10% decrease in the methylation of the F2RL3 gene has been correlated with a 33% increase in the risk for lung cancer development (Zhang et al., 2015). Additionally, hypomethylation of the smoking-related genes AHRR and F2RL3 and the intergenic regions 2q37.1 and 6p21.33, shows a stronger association with lung cancer mortality than the methylation status of lung cancer related genes. Therefore, the former might be better biomarkers for lung cancer mortality (Zhang et al., 2016a).

In another report, where the methylation status of lung cancer-related genes was studied, 13 CpG sites located in 8 genes, KLF6 (cg24287110), TERT (cg12324353, cg24908166), MSH5 (cg00640087, cg20640261), ACTA2 (cg19335412), GATA3 (cg10163955, cg11430077, cg22770911), VTI1A (cg03281572, cg07269053), STK32A (cg17928584), CHRNA5 (cg19696491), were associated with smoking behavior. The last two genes were found to be hypermethylated, while the rest were hypomethylated, with a decrease up to 2,4% in DNA methylation. The intensity of smoking (pack-years) was associated with all of them, except for CHRNA5, whilst none of them was associated with time since quitting smoking. Moreover, only the methylation of ACTA2 appears to have significant differences between former smokers and current/never smokers (Gao et al., 2016).

NFIX (cg16200496), WWTR1 (cg25771041), PLA2G6 (cg22515201), NHP2L1 (cg24823993) were hypomethylated and SMUG1 (cg11875268) was hypermethylated in current smokers. All of them were associated with lung cancer and their methylation was proportional to the intensity of smoking (Freeman et al., 2016).

A study in lung adenocarcinoma cell lines identified 5 genes differentially methylated in relation to smoking behavior. CPEB1 and EMILIN2 were hypermethylated in current smokers, in comparison to non-smokers, whereas CST6, LAYN and MARVELD3 were hypomethylated. Notably, the EMILIN2 gene was more methylated in smokers with advanced stage cancer, indicating that smoking-induced hypermethylation of this gene may participate in lung cancer progression (Tessema et al., 2014).

Comparing methylation between smokers and non-smokers, who did or did not have lung adenocarcinoma, 110 differentially methylated regions (DMRs) were identified in lung tissues. Forty eight (48) DMRs were hypomethylated and 62 DMRs were hypermethylated in smokers with lung adenocarcinoma. These DMRs included genes which contribute to significant cellular pathways. For instance, RPS6KA3 and ARAF, which are both involved in regulation of cell proliferation, were hypomethylated, whereas ADRB3 and GALR1 were hypermethylated, the first of which is also involved in cell proliferation and regulation (Tan et al., 2013).

In an attempt to understand how smoking can induce lung cancer, promoter methylation of several genes was studied in two healthy lung cell lines, primary small airway epithelial cells (PSAE) and immortalized bronchial epithelial cells (NL-20), after exposure to cigarette smoke condensate (CSC). Results showed that, in general, promoter methylation increases with concentration and duration of CSC exposure (Lyn-Cook et al., 2014). However, the promoter of hsa-let-7a-3 was highly methylated in both cells lines ($> 90\%$) and in the whole range of CSC concentrations and exposure duration. The hsa-let-7a-3 gene is referred to as a tumor suppressor gene, preventing the development of cancer cells. It has reduced expression in lung cancer patients. Thus, cigarette smoke can enhance lung cancer development via hypermethylation of the hsa-let-7a-3 promoter (Lyn-Cook et al., 2014).

Another tumor suppressor gene, which becomes hypermethylated in its promoter following exposure to cigarette smoke, is NISCH, which is located on chromosome 3p21. Methylation of the NISCH promoter was pronounced in smokers with > 20 pack-years and in former smokers, whereas methylation was not observed in light smokers and non-smokers. Nevertheless, hypermethylation was observed both in heavy and light smokers with lung cancer, indicating that the NISCH promoter methylation can be a good biomarker for lung cancer, even in light smokers (Ostrow et al., 2016).

Promoters of other genes related to lung cancer, were found hypermethylated by smoking. These genes were p16, a tumor suppressor gene (Sun et al., 2015), ANK1, a host gene for tumor suppressor miR-496–5p (Tessema et al., 2017) and RAR-β, which influences the expression of other tumor suppressor genes (Li et al., 2014). In contrast, CHRNB4, which is located on chromosome region 15q25 and encodes

Table 1
The way smoking behavior affects and correlates with DNA methylation.

Genes studied	Analysis Method	Correlation	Effect on DNA methylation	Ref.
MAO-A	Pyrosequencing	Smoking behavior	Hypo-/Hyper-methylation	Tiili et al. (2017)
MAO-B				
CYP2D6			Hypomethylation	Tiili et al. (2015)
AHRR_p1	Bisulfite sequencing	Biomarkers of former exposure to tobacco smoke	Hypomethylation	Shenker et al. (2013b)
6p21				
2q37_p1				
2q37_p3				
CDKN2A, IL-6,	Bisulfite-PCR	Effects of tobacco smoke on DNA methylation	Hypermethylation	Peluso et al. (2014)
HICl promoter,	Pyrosequencing			
Alu,				
TP53, LINE-1				
F2RL3	MALDI-TOF MS	Biomarker of current and life-time smoking exposure	Hypomethylation (reversed after cessation)	Zhang et al. (2014)
AHRR	Illumina HumanMethylation450 Beadchip	Biomarker of secondhand tobacco exposure	Hypomethylation	Reynolds et al. (2017)
MTHFR		Influence of MTHFR methylation on AHHR demethylation induced by smoking	Hypermethylation (reversed after cessation)	Beach et al. (2017)
Global DNA	Illumina HumanMethylation450 Beadchip	Biomarkers of smoking	Hypomethylation	(Philibert et al., 2017, 2013)
		Smoking behavior	Hypo-/Hyper-methylation	Wan et al. (2015)
		DNA methylation changes and reversibility upon smoking cessation	Hypomethylation (reversed after cessation)	Tsaprouni et al. (2014)
		Smoking behavior and differential methylation between Europeans and South Asians	Hypo-/Hyper-methylation	Elliott et al. (2014)
		Comparison of DNA methylation changes between African-Americans and European-Americans	Hypo-/Hyper-methylation	Dogan et al. (2015)
		Association between smoking behavior and mortality outcomes	Hypomethylation	Zhang et al. (2016c)
		Association between DNA methylation and cotinine levels as markers for smoking behavior	Hypo-/Hyper-methylation	Zhang et al. (2016b)
		Smoking-related DNA methylation on the X chromosome	Hypo-/Hyper-methylation	Klebaner et al. (2016)
		Impacts of tobacco smoking and genetic variation in DNA methylation	Hypo-/Hyper-methylation	Qiu et al. (2015)
		Changes in DNA methylation over time in relation to smoking behavior	Hypo-/Hyper-methylation	Wilson et al. (2017)
		Dynamics of DNA methylation following smoking cessation	Hypo-/Hyper-methylation (reversed after cessation)	Guida et al. (2015)
		DNA methylation changes in relation to smoking behavior	Hypo-/Hyper-methylation (reversed after cessation (Ambatipudi et al., 2016))	(Allione et al., 2015; Ambatipudi et al., 2016; Besingi and Johansson, 2014; Dogan et al., 2017; Lee et al., 2016a,b; Monick et al., 2013; Sayols-Baixeras et al., 2015; Zaghlool et al., 2015)
	Illumina 450K arrays and Reduced Representation Bisulfite Sequencing	Smoking behavior	–	Su et al. (2016)
	Illumina Infinium HumanMethylation27K Beadchip Illumina Infinium HumanMethylation450K Beadchip	Smoking-related DNA methylation sites across ethnic groups	Hypo-/Hyper-methylation (reversed after cessation)	(Sun et al., 2014)
	Illumina HumanMethylation27 Beadchip Pyrosequencing Illumina HumanMethylation450 Beadchip	DNA methylation changes during smoking	Hypomethylation (reversed after cessation)	Harlid et al. (2014)
	Illumina HumanMethylation450 Beadchip MALDI-TOF/MS	Impacts of smoking in DNA methylation	Hypo-/Hyper-methylation	Zeilinger et al. (2013)

nicotinic acetylcholine receptor β4 subunit, was found overexpressed in smokers with lung cancer, with its promoter being hypomethylated. On the other hand, its knockdown reduced proliferation, indicating the oncogenic potential of CHRNB4 (Yoo et al., 2014).

1.2.2. Smoking-induced DNA methylation changes in other types of cancer

Smoking appears to affect methylation of genes related to other types of cancer. A study in oral epithelial cells showed that promoters of cancer-related genes, MLH1 and hTERT, were found hypermethylated and site-specific methylated, respectively. Specifically, the hTERT promoter was hypomethylated in one CpG site (hpaII) and hypermethylated in another CpG site (hpaI) as a result of smoking (De Oliveira et al., 2015). Hypomethylation of the Fgf3 gene and subsequent overexpression was found in oral tissues of healthy mice following treatment with dibenzo [def,s]chrysene, a tobacco carcinogen. Overexpression of the Fgf3 gene was also observed in oral squamous cell carcinoma, indicating the effect of tobacco smoking in oral cancer (Sun et al., 2017).

In esophageal cancer, cancer-related genes were more frequently hypermethylated. These genes include TNXB and HOXA4 in Barrett's esophagus, and GFI1 and CLDN11 in esophageal adenocarcinoma. Furthermore, NTRK2 and NTRK3, which regulate the Trk and Shp2 pathways, were hypermethylated. Especially, the NTRK2 promoter was 36% methylated in smokers versus 9% in non-smokers, and the NTRK3 gene body was 85% methylated in smokers versus 62% in non-smokers (Kaz et al., 2016). Moreover, the MSH3 promoter on chromosome 5 was found hypermethylated in esophageal cancer patients, with methylation being more pronounced in smoking patients (Vogelsang et al., 2014).

In prostate cancer, different methylation patterns were observed between smokers and non-smokers, with 99% hypermethylation occurring in smokers. Former smokers had similar methylation patterns to non-smokers, independent of the time elapsed since smoking cessation. Some of the genes, whose expression was affected by smoking-induced hypermethylation, were ADX1 and PON3. The consequence of methylation related changes in expression of these genes was the worse outcomes of prostate cancer (Shui et al., 2016). In contrast with ADX1 and PON3, the CYP1A1 promoter, which is located on chromosome 15q24.1, was hypomethylated at 3 CpG sites in prostate cancer smoking patients in comparison with normal tissues. Overexpression of the CYP1A1 gene, which was caused by its hypomethylation, had negative effects, such as prostate cancer proliferation and reduced survival rate. On the contrary, the reduction of its expression led to increased apoptosis and inhibited cell proliferation (Mitsui et al., 2016).

DCC, HIC1 and MCAM were hypermethylated in bladder epithelial cells, when treated with cigarette smoke extract. This observation suggests that DNA methylation changes, which were caused by cigarette smoke, may lead to development of bladder cancer (Brait et al., 2013).

Smoking can lead to differential methylation in colorectal cancer as well. In tumor samples of current smokers, promoter 1A of tumor suppressor gene, APC, was hypermethylated when compared to tumor samples of never smokers in 6 CpG sites (cg08571859, cg14511739, cg22035501, cg11613015, cg14479889, cg16970232). However, it was not methylated in mucosa adjacent to the tumor. Hypermethylation was associated with smoking duration and was more frequently observed in females (Barrow et al., 2017).

In breast cancer, smoking influenced DNA methylation according to tumor HR (hormone receptor) status. In HR + breast tumors of smokers, hypermethylation was observed, with similar patterns in black and white smokers, but more pronounced in long-term smokers (> 20 years) (Conway et al., 2017). Furthermore, hypomethylation of intergenic region 2q37.1 (cg01940273) in smokers, was strongly associated with the risk for breast cancer development (Shenker et al., 2013a).

1.2.3. Smoking-induced DNA methylation changes in chronic diseases other than cancers

Other than cancer, smoking can affect the development and progression of various other diseases. Only 0,2% of genes analyzed exhibit differential methylation between smokers and non-smokers in small airway epithelium (SAE). Nevertheless, 204 genes were found to be differentially methylated near the transcription initiation start. Most of them were hypomethylated (75%), while a smaller proportion was hypermethylated (25%). The most hypomethylated genes were CYP1A1 and CYP1B1, and the most hypermethylated genes were JAG1 and BPIFB1. The methylation changes influenced the expression of 35 genes. Notably, 14 hypomethylated and 6 hypermethylated genes were associated with increased expression and 10 hypomethylated and 5 hypermethylated genes were associated with decreased expression. Thus, smoking can affect the SAE function, through DNA methylation changes in related genes (Buro-Auriemma et al., 2013).

In lung tissues of patients with chronic obstructive pulmonary disease (COPD) hypermethylated genes were identified, such as NOS1AP (cg26663636) and BID (cg01388022). These genes were also found to be hypermethylated in smokers. Hypermethylation of these genes may affect their expression and may disturb key cellular pathways in which they are involved. These genes are involved in pathways relevant to the pathogenesis of COPD and may be therefore used as predictors of COPD risk development in smokers (Sundar et al., 2017). In addition, the promoter of the oxidative gene GCLC was hypermethylated in smokers (with or without COPD) and in ex-smokers with COPD, when compared to ex-smokers without COPD and non-smokers. Hypermethylation of the GCLC promoter results in reduced expression and reduced GCH (guanine triphosphate cyclohydrolase) synthesis, with the latter being more pronounced in smokers with COPD. These results indicate the effect of an epigenetic-environmental interaction in the initiation and progression of COPD, through oxidant-antioxidant unbalance (Cheng et al., 2016). Hypermethylation of the Bcl-2 promoter was observed in lung tissues of mice, after treatment with cigarette smoke extract (CSE). The hypermethylation caused decreased expression of the Bcl-2 (B-cell lymphoma 2) gene, which encodes for an anti-apoptotic protein. The result was apoptosis of lung cells and subsequent lung dysfunction, which can lead to emphysema, a characteristic of COPD (Zeng et al., 2015).

Coronary artery disease (CAD) is also influenced by smoking via epigenetic pathways. 15 CpG sites within CAD-related genes were identified to be differentially methylated in smokers, compared to non-smokers. Hypomethylation was observed in 12 CpG sites, within TERT (cg24908166, cg12324353), SARS (cg03725309), GNGT2 (cg00980784), SMG6 (cg13916835), SKI (cg09469355, cg05603985), TOM1L2 (cg04324276), SIPA1 (cg25468516), MRAS (cg22907952), CDKN1A (cg15474579), RPH3A (cg18236066). On the contrary, hypermethylation was observed in 3 CpG sites, within LRRC2 (cg20496896) and FES (cg09397246, cg26405020). Hypomethylation of cg05603985 in SKI is associated with decreased expression of a nearby CAD-related gene, PRKCZ. Specifically, PRKCZ participates in proliferation, differentiation and secretion of cardiac myocytes. Moreover, hypomethylation of the SKI gene, a repressor of TGF-beta activity, lead to decreased TGF-beta activity which is associated with atherosclerosis and plaque instability (Steenaard et al., 2015). Hypomethylation of one CpG site (cg05575921) within the AHRR gene, which resulted in reduced AHRR expression, showed a strong association with carotid plaque score in smokers, while no such association was noted in non-smokers. This observation suggests that the AHRR gene might serve as a biomarker for atherosclerosis, induced by smoking (Benton et al., 2016). Differential methylation of CYP11B2, which is located in chromosome 8 and encodes aldosterone synthase, has been associated with essential hypertension and smoking. Specifically, the CYP11B2 promoter was hypomethylated in 3 CpG sites in smokers. Hypomethylation of two of these CpG sites was

associated with a 4.62 fold increased risk for essential hypertension (Gu et al. (2016a,b)).

DNA methylation changes, induced by smoking, have also been associated with inflammatory markers. In particular, hypomethylation of the F2RL3 gene (cg03636183) in smokers has been associated with increased levels of interleukin-18 (IL-18) (Jhun et al., 2017). Hypomethylation of AHRR (cg05575921, cg23576855) and GPR15 (cg19859270) in smokers has been associated with increased levels of IL-6 and CRP (C-Reactive Protein) (Dogan et al., 2014). These observations are indicative of the effect of smoking in inflammatory responses. Rheumatoid arthritis (RA), an inflammatory disease, appears to be affected by smoking, in combination with the genotype. Current smokers, who were carriers of the rs6939349_AA or the rs6933349_GA, but not the rs6933349_GG genotype, had lower methylation levels in one CpG site (cg21325723), when compared to non-smokers. The CpG site cg21325723 has been associated with RA and its smoking-induced hypomethylation increases the risk for RA development (Meng et al., 2017). Furthermore, multiple sclerosis, a chronic inflammatory disease, was also influenced by smoking. Patients with multiple sclerosis, who were smokers, had different DNA methylation patterns in comparison with non-smokers (Marabita et al., 2017).

Smoking has been associated with differential DNA methylation of diabetes-related genes, such as ANPEP (Alanyl Aminopeptidase) (cg23161492), KSNQ1 (cg26963277, cg01744331, cg16556677) and ZMIZ1 (Zinc finger MIZ domain-containing protein 1) (cg03450842) and reduced fasting insulin levels, increasing the risk for development of type-2 diabetes (Ligthart et al., 2016).

Smoking-induced DNA methylation changes were associated with increased levels of 8-isoprostane, a biomarker of oxidative stress. Specifically, the DNA methylation changes that were observed in AHRR (cg05575921), CSRNP1 (Cysteine And Serine Rich Nuclear Protein 1) (cg00501876) and PPP1R15A (Protein Phosphatase 1 Regulatory Subunit 15A) (cg03707168) had a strong association with the 8-iso levels, suggesting that they could serve as new biomarkers for smoking-induced oxidative stress and its negative outcomes (Gao et al., 2017). In addition, the NR33C1 and FKBP5 (FK506 Binding Protein 5) genes were hypomethylated following smoking and caused a dysfunction of the hypothalamic pituitary adrenal axis (HPA). These facts indicate the impact of smoking even on psychiatric disorders (Dogan et al., 2016).

Male fertility is also influenced by smoking via epigenetic pathways. In mice, exposure to tobacco caused hypomethylation of Nme2 (NME/NM23 Nucleoside Diphosphate Kinase 2) (Gu et al. (2016a,b)) and Trim27 (Tripartite Motif Containing 27) promoters (Nie et al., 2016). Hypomethylation of the Nme2 promoter led to its increased expression which can inhibit telomerase activity. The inhibition of telomerase activity leads to the shortening of telomere length and may cause apoptosis of germ cells (Gu et al. (2016a,b)). Hypomethylation of the Trim27 promoter led to overexpression, followed by up-regulation of deubiquitinated RIP1, which caused activation of the TNF apoptotic pathway and germ cells apoptosis (Nie et al., 2016). Furthermore, smokers have an increased risk for infertility through hypomethylation of the imprinting control region (ICR), H19 and SNRPN (Small Nuclear Ribonucleoprotein Polypeptide N) (Dong et al., 2016). Following exposure, hypermethylation of Pebp1 (Phosphatidylethanolamine Binding Protein 1) (Xu et al., 2013) and Sort (Sortilin) promoter (Dai et al., 2016) in mice, influenced spermatogenesis and sperm motility respectively. Specifically, the decreased expression of Pebp1 (induced by its promoter hypermethylation) led to inactivation of ERK1/2 and spermatogenetic damage (Xu et al., 2013), while the downregulation of the Sort gene prevented the sperm from maturation and capacitation through the inhibition of tyrosine phosphorylation in spermatozoa (Dai et al., 2016). In contrast, nicotine-treated mice had increased sperm motility which was likely due to nicotine-induced hypomethylation of Pfn1 (Profilin 1) promoter and Pfn1 overexpression (Dai et al., 2015).

All of the above studies are summarized in Table 2.

1.3. Effects of maternal smoking on DNA methylation

Studies in cord blood showed an association between maternal smoking and alternations in DNA methylation in the fetus. Global hypomethylation was observed in the exposed fetus. However, most of the CpG sites, were hypermethylated in response to maternal smoking. Specifically, 31 CpG sites were significantly altered in 25 genes and 90,3% of these sites were hypermethylated, whereas only 3 CpG sites were hypomethylated, with the most significant CpG site being the cg05727225 in the ADM gene, which is located in the chromosome 11p15.4. ADM contributes to a wide range of biological processes and chronic diseases, such as obesity, diabetes, atherosclerosis and coronary heart disease (Ivorra et al., 2015). Furthermore, CpG sites within GFI1, MYO1G, CYP1A1, RUNX1, LCTL, AHRR, NEUROG1, CNTNAP2, FRMD4A and LRP5 were differentially methylated in exposed fetuses in comparison with unexposed fetuses (Küpers et al., 2015; Rotroff et al., 2016). MYO1G, CYP1A1, NEUROG1 and FRMD4A were hypermethylated and GFI1, CNTNAP2 and LRP5 were hypomethylated, while AHRR was both hypermethylated and hypomethylated in 3 CpG sites (cg23067299, cg22937882, cg01970407) and 1 CpG site (cg05575921), respectively (Küpers et al., 2015). Nine genes (FCRLA, MIR641, SLC25A24, TRAK1, C1orf180, ITLN2, GLIS1, LRFN1, MIR451) were significantly altered. Most of these genes contribute to important biological pathways, cancer, angiogenesis and immune system functions (Rotroff et al., 2016).

In blood obtained from newborns, differential methylation was found in 187 CpG sites, which were located in 110 genes. 43% of these CpG sites were hypomethylated, while 57% were hypermethylated, with a median change in methylation of 2%, which did not correlate with the intensity of exposure. The most notable were MEG3 and FRMD4A, with 3 and 4 hypermethylated CpG sites, respectively, and ATP9A and GALNT2 genes, with 1 and 7 hypomethylated CpG sites, respectively (Markunas et al., 2014).

In fetal lung tissue and placenta, differential methylation was identified following prenatal nicotine exposure. Alternations in ANKRD33B, CNTD2, PKP3 and DPP10 were the most significant in fetal lung tissue, while in placental tissues the most significant alternations were located in GTF2H2C and GTF2H2D. In both tissues, differential methylation was observed in 101 CpG sites. One of the CpG sites, located in JAK2, which is associated with cell growth and differentiation, was hypermethylated both in smokers and in bladder cancer (Chhabra et al., 2014).

A study in tissues from the cortical plate of fetal brains identified differentially methylated regions between exposed and unexposed fetuses to maternal smoking. The results showed a global hypomethylation in 20% of the exposed fetuses, with the most hypomethylated regions located in the SDHAP3 and GNA15 promoters. These smoking-induced changes in DNA methylation led to a decreased number of neurons in fetal brains and alternations in cell-type differentiation (Chatterton et al., 2017).

In rats' offspring, DNA methylation changes were identified following prenatal nicotine exposure. Hypermethylation was observed in the promoters of TBX5 and GATA4, with an 8,2% and 4,8% increase, respectively, leading to cardiac dysfunction. The AT1aR and AT2aR genes, which are related to hypertension and cardiovascular diseases, were found with their promoters hypomethylated and hypermethylated, respectively, in aortic tissues of nicotine exposed rat offspring (Xiao et al., 2014). Furthermore, nicotine exposure of rats during gestation induced hypermethylation of the AT2R promoter in neonatal rat brains, which was associated with increased hypoxic-ischemic encephalopathy, by suspending the binding activity of TATA-binding protein and suppressing AT2R expression in the developing brain (Li et al., 2013).

Following *in utero* exposure to environmental tobacco smoke, 4% global DNA hypomethylation was observed in offspring's lung tissues. Moreover, genes related to inflammation were differentially

Table 2
Association of smoking-induced changes in DNA methylation with pathological conditions and diseases.

Genes studied	Analysis Method	Correlation	Effect on DNA methylation	Ref.
AHRR, F2RL3	Bisulfite sequencing PCR	Biomarker of smoke exposure and risk of lung cancer	Hypomethylation	Lee et al. (2017)
Bcl-2 promoter		Emphysema	Hypermethylation	Zeng et al. (2015)
F2RL3	MALDI-TOF/MS	Biomarker for the risk of lung cancer	Hypomethylation	Zhang et al. (2015)
Promoters of several genes	DNA methylation PCR	Lung cancer	Hypermethylation	Lyn-Cook et al. (2014)
CHRNB4 promoter	Methylation- specific PCR		Hypomethylation	Yoo et al. (2014)
RAR-β promoter			Hypermethylation	Li et al. (2014)
P16 promoter		Lung adenocarcinoma	Hypermethylation	Sun et al. (2015)
MSH3 promoter		Esophageal carcinogenesis	Hypermethylation	Vogelsang et al. (2014)
AHRR	Illumina HumanMethylation450	Biomarker for the risk of atherosclerosis	Hypomethylation	Benton et al. (2016)
NR3C1, FKBP5	Beadchip	Association of tobacco smoking and alcohol with psychiatric disorders	Hypomethylation	Dogan et al. (2016)
ANK1		Lung tumor	Hypermethylation	Tessema et al. (2017)
F2RL3, GPR15, HNRPUL1, LIM2, AKT3	Illumina HumanMethylation27 Beadchip	Association between smoking and inflammation	Hypomethylation	Jhun et al. (2017)
Global DNA, AHRR, F2RL3, 2q37, 6p21.33	Illumina HumanMethylation450 Beadchip Bisulfite pyrosequencing	Cancer risk	Hypomethylation	Shenker et al. (2013a)
Pebp1 promoter	Bisulfite sequencing	Spermatogenesis	Hypermethylation	Xu et al. (2013)
Nme2 promoter		Germ cell apoptosis	Hypomethylation	Gu et al. (2016a,b)
Trim27 promoter	Bisulfite sequencing		Hypomethylation	Nie et al. (2016)
Sord	Methylation-Specific PCR	Sperm maturation	Hypermethylation	Dai et al. (2016)
Pfn1 promoter		Sperm mortality	Hypomethylation	Dai et al. (2015)
CYP1A1 promoter		Prostate cancer	Hypomethylation	(Mitsui et al., 2016)
AHHR	Pyrosequencing	Biomarker for smoking behavior and related morbidity and mortality	Hypomethylation	Bojesen et al. (2017)
GCLC, GSTM1, GSTP1, SOD3 promoters		Pulmonary disease	Hypermethylation	Cheng et al. (2016)
H19, SNRPN		Male infertility	Hypomethylation, Hypermethylation	Dong et al. (2016)
CYP11B2 promoter		Essential hypertension risk	Hypomethylation	Gu et al. (2016a,b)
NISCH	Quantitative-fluorogenic real-time PCR	Biomarker for smoke-induced lung cancer	Hypermethylation	Ostrow et al. (2016)
MLH1, hTERT, TP53 promoters	Methylation-sensitive restriction enzymes (MSRE) PCR	Effect of smoking on DNA methylation of cancer-related genes	Hypo-/Hyper-methylation	De Oliveira et al. (2015)
Global DNA	Illumina HumanMethylation450 Beadchip	Esophagus pathogenesis	Hypo-/Hyper-methylation	Kaz et al. (2016)
		Differential DNA methylation patterns associated with smoke exposure in lung neoplasm	Hypo-/Hyper-methylation	Freeman et al. (2016)
		Lung cancer	Hypo-/Hyper-methylation	Gao et al. (2016)
		Colorectal cancer	Hypo-/Hyper-methylation	Barrow et al. (2017)
		Oxidative stress	Hypo-/Hyper-methylation	Gao et al. (2017)
		Coronary artery disease	Hypo-/Hyper-methylation	Steenaard et al. (2015)
		Lung cancer risk	Hypomethylation	(Baglietto et al., 2017; Fasanelli et al., 2015)
		Diabetes risk	Hypo-/Hyper-methylation	Ligthart et al. (2016)
		Prostate cancer outcomes	Hyper-methylation	Shui et al. (2016)
		Lung cancer mortality	Hypo-/Hyper-methylation	Zhang et al. (2016a)
		Association between smoking-induced DNA methylation and DNA methylation in COPD patients	Hypermethylation	Sundar et al. (2017)
		Inflammation in long-term smokers	Hypo-/Hyper-methylation	Dogan et al. (2014)
		Multiple sclerosis	Hypo-/Hyper-methylation	Marabita et al. (2017)
		Interaction of smoking and genotype on DNA methylation and rheumatoid arthritis	Hypomethylation	Meng et al. (2017)
	Illumina HumanMethylation27 Beadchip	Lung adenocarcinoma	Hypo-/Hyper-methylation	Tan et al. (2013)
	Illumina HumanMethylation27K Beadchip Quantitative methylation-speciefic PCR	Biomarkers for smoke-induced urothelial cell carcinoma	Hypo-/Hyper-methylation	Brait et al. (2013)
	Illumina GoldenGate Cancer Panel I methylation array	Smoking behavior and breast cancer	Hypo-/Hyper-methylation	Conway et al. (2017)
	CoBRA Methylation-specific PCR Bisulfite sequencing	Lung adenocarcinoma	Hypo-/Hyper-methylation	Tessema et al. (2014)
	Bisulfite sequencing	Oral cancer	Hypo-/Hyper-methylation	Sun et al. (2017)
	HELP assay	Impact of smoking on small airway epithelium	Hypo-/Hyper-methylation	Buro-Auriemma et al. (2013)

Table 3
Effect of maternal smoking on DNA methylation in the fetus and newborn. Details on most of the genes studied concerning the relative CpG locus, chromosome position and gene region are summarized in Table 4.

Genes studied	Analysis Method	Correlation	Effect on DNA methylation	Ref.
Global DNA	Illumina HumanMethylation450 Beadchip	Reduced neuronal content in the developing fetal brain by maternal smoking	Hypomethylation	Chatterton et al. (2017)
		DNA methylation changes in newborns related to maternal smoking	Hypo-/Hyper-methylation	(Markunas et al., 2014), (Rotroff et al., 2016)
		Effects of maternal smoking on DNA methylation of offspring	Hypo-/Hyper-methylation	Ivorra et al. (2015)
		Effect of maternal smoking on DNA methylation in fetal lung and placental	Hypo-/Hyper-methylation	Chhabra et al. (2014)
		Postnatal stability of offspring DNA methylation changes related to maternal smoking	Hypo-/Hyper-methylation	(Lee et al., 2015; Richmond et al., 2015; Rzehak et al., 2016)
	Illumina HumanMethylation27K Beadchip			Breton et al. (2014)
	Illumina HumanMethylation450 Beadchip	Effect of maternal smoking on birthweight of the offspring	Hypo-/Hyper-methylation	Küpers et al. (2015)
	Illumina HumanMethylation450 Beadchip Bisulfite pyrosequencing		Hypo-/Hyper-methylation	Morales et al. (2016)
	Illumina HumanMethylation27K Beadchip Bisulfite pyrosequencing	DNA methylation patterns in the placenta related to maternal smoking and gestational age	Hypo-/Hyper-methylation	Maccani et al. (2014)
	Pyrosequencing	Effects of prenatal environmental tobacco smoke exposure in the offspring	Hypo-/Hyper-methylation	Lee et al., 2016a,b
	ELISA Bisulfite pyrosequencing	Effect of maternal smoking during the first trimester on fetal global DNA methylation	–	Fa et al. (2016)
	Whole-genome bisulfite sequencing	Effects of maternal smoking on DNA methylation in children	Hypo-/Hyper-methylation	Bauer et al. (2016)
AHHR, GFI1, MYO1G	SEQUENOM MassARRAY EpiTYPER platform	Effect of maternal smoking on AHHR methylation	Hypomethylation	Novakovic et al. (2014)
AHHR, CYP1A1 promoter	Pyrosequencing	Effect of maternal smoking on DNA methylation of AHHR and CYP1A1 during the first trimester of pregnancy	Hypermethylation	Fa et al. (2018)
IGF2, NR3C1 promoter		Effects of maternal smoking	Hypo-/Hyper-methylation	Drake et al. (2015)
26 CpG loci	Illumina HumanMethylation450 Beadchip	DNA methylation changes in childhood as biomarkers for prenatal smoke exposure	Hypo-/Hyper-methylation	Ladd-Acosta et al. (2016)
LEPR/LEPROT genes		Effect of maternal smoking on leptin levels in offspring	–	Yousefi et al. (2013)
PPARγ promoter	Methylation-specific PCR	Alveolar fibroblast differentiation	Hypermethylation	Gong et al. (2015)
AT_1R and AT_2R promoters	Quantitative methylation-specific PCR	Perinatal smoking exposure and heightened vascular contraction	Hypo-/Hyper-methylation	Xiao et al. (2014)
MT-RNR1 CYP1A1	Bisulfite pyrosequencing	Effect of maternal smoking on birthweight and newborn length	Hypo-/Hyper-methylation	Janssen et al. (2017)
AT_2R promoter	Quantitative methylation-specific PCR	Effects of perinatal smoke exposure on developing brain	Hypermethylation	Li et al. (2013)
GFI1 promoter	Bisulfite sequencing	Maternal smoking related to sudden infant death syndrome	Hypomethylation	Schwender et al. (2016)
AACS promoter	Bisulfite sequencing PCR	Prenatal tobacco smoking and the relationship to fetal growth and birthweight	Hypermethylation	Wu et al. (2016)
H19, IGF2-DMR	PCR Mass spectrometry		Hypomethylation	Bouwland-Both et al. (2015)

Table 4
Relative CpG locus, chromosome position and gene region of the studied genes.

Gene	CpG locus	Position	Chromosome position	Gene region	Ref.
AHRR	cg05575921	373378	5	Body	(Allione et al., 2015; Ambatipudi et al., 2016; Baglietto et al., 2017; Besingi and Johansson, 2014; Bojesen et al., 2017; Dogan et al., 2017, 2015; Elliott et al., 2014; Fasanelli et al., 2015; Küpers et al., 2015; Lee et al., 2016a,b; Monick et al., 2013; Philibert et al., 2017, 2013; Sayols-Baixeras et al., 2015; Su et al., 2016; Tsaprouni et al., 2014; Wilson et al., 2017; Zaghlool et al., 2015; Zeilinger et al., 2013; Zhang et al., 2016b)
	cg14817490	392920			(Ambatipudi et al., 2016; Dogan et al., 2017; Monick et al., 2013; Sayols-Baixeras et al., 2015; Tsaprouni et al., 2014; Zhang et al., 2016b)
	cg21161138	399360			(Ambatipudi et al., 2016; Dogan et al., 2017, 2015; Philibert et al., 2013; Sayols-Baixeras et al., 2015; Tsaprouni et al., 2014; Wilson et al., 2017; Zeilinger et al., 2013; Zhang et al., 2016b)
	cg23576855				(Ambatipudi et al., 2016; Dogan et al., 2017, 2015; Wilson et al., 2017; Zhang et al., 2016b)
	cg 23916896	368804			(Sayols-Baixeras et al., 2015; Zhang et al., 2016b)
	cg25648203	395444			(Allione et al., 2015; Dogan et al., 2017; Sayols-Baixeras et al., 2015; Tsaprouni et al., 2014; Wilson et al., 2017; Zeilinger et al., 2013; Zhang et al., 2016b)
	cg26703534				(Dogan et al., 2017, 2015; Philibert et al., 2013; Wilson et al., 2017; Zaghlool et al., 2015; Zeilinger et al., 2013; Zhang et al., 2016b)
	cg11554391				(Ambatipudi et al., 2016; Dogan et al., 2015; Wilson et al., 2017; Zhang et al., 2016b)
	cg09338136				Wilson et al. (2017)
	cg03991871	368447			(Dogan et al., 2017; Monick et al., 2013; Sayols-Baixeras et al., 2015; Tsaprouni et al., 2014; Zeilinger et al., 2013)
	cg12806681	368394			(Dogan et al., 2017; Sayols-Baixeras et al., 2015)
	cg14647125				Zaghlool et al. (2015)
	cg23067299	323907			Küpers et al. (2015)
	cg22937882	405774			
	cg01970407	323320			
F2RL3	cg03636183	17000585	19	Body	(Allione et al., 2015; Ambatipudi et al., 2016; Baglietto et al., 2017; Besingi and Johansson, 2014; Dogan et al., 2017; Elliott et al., 2014; Fasanelli et al., 2015; Guida et al., 2015; Harlid et al., 2014; Qiu et al., 2015; Sayols-Baixeras et al., 2015; Su et al., 2016; Sun et al., 2014; Tsaprouni et al., 2014; Wilson et al., 2017; Zeilinger et al., 2013; Zhang et al., 2016b)
GFI1	cg09935388	92947588	1	Body	(Besingi and Johansson, 2014; Dogan et al., 2017; Elliott et al., 2014; Küpers et al., 2015; Su et al., 2016; Wilson et al., 2017; Zeilinger et al., 2013; Zhang et al., 2016b)
	cg18316974	92947035			(Ambatipudi et al., 2016; Dogan et al., 2017; Küpers et al., 2015; Sayols-Baixeras et al., 2015; Zhang et al., 2016b)
	cg18146737	92946700			(Ambatipudi et al., 2016; Dogan et al., 2017; Küpers et al., 2015; Sayols-Baixeras et al., 2015)
	cg12876356	92946825			(Dogan et al., 2017; Küpers et al., 2015; Sayols-Baixeras et al., 2015; Zeilinger et al., 2013)
	cg09662411	92946132			(Dogan et al., 2017; Küpers et al., 2015; Sayols-Baixeras et al., 2015)
	cg06338710	92946187			Sayols-Baixeras et al. (2015)
	cg14179389	92947961			Küpers et al. (2015)
	cg04535902	92947332			
	cg10399789	92945668			
GPR15	cg19859270	98251294	3	1st exon	(Besingi and Johansson, 2014; Dogan et al., 2017, 2015; Harlid et al., 2014; Sayols-Baixeras et al., 2015; Su et al., 2016; Sun et al., 2014; Tsaprouni et al., 2014; Wilson et al., 2017; Zhang et al., 2016b)
GNG12	cg25189904	68299493	1	TSS1500	(Besingi and Johansson, 2014; Elliott et al., 2014; Guida et al., 2015; Sayols-Baixeras et al., 2015; Tsaprouni et al., 2014; Wilson et al., 2017; Zeilinger et al., 2013; Zhang et al., 2016b)
	cg26764244				Harlid et al. (2014)
	cg13184736				Wilson et al. (2017)
GPX1	cg18642234		3	3′UTR	Zhang et al. (2016b)
MYO1G	cg22132788	45002486	7	Body	(Besingi and Johansson, 2014; Elliott et al., 2014; Küpers et al., 2015; Philibert et al., 2013; Rzehak et al., 2016; Sayols-Baixeras et al., 2015; Zeilinger et al., 2013; Zhang et al., 2016b)
	cg12803068	45002919			(Allione et al., 2015; Ambatipudi et al., 2016; Küpers et al., 2015; Lee et al., 2015; Philibert et al., 2013; Rzehak et al., 2016; Sayols-Baixeras et al., 2015; Zeilinger et al., 2013)
	cg07826859				Ambatipudi et al. (2016)
	cg04180046	45002736			Küpers et al. (2015)
	cg19089201	45002287		3′UTR	(Küpers et al., 2015; Rzehak et al., 2016)
GNTNAP2	cg25949550	145814306	7	Body	(Küpers et al., 2015; Lee et al., 2015; Rzehak et al., 2016; Sayols-Baixeras et al., 2015; Zhang et al., 2016b)
LPR15	cg21611682		11	Body	(Ambatipudi et al., 2016; Zhang et al., 2016b)
RARA	cg10062919		17		Wilson et al. (2017)
	cg19572487	38476024			(Besingi and Johansson, 2014; Guida et al., 2015; Sayols-Baixeras et al., 2015; Tsaprouni et al., 2014; Zeilinger et al., 2013)
	cg08446900				Su et al. (2016)

(*continued on next page*)

Table 4 (*continued*)

Gene	CpG locus	Position	Chromosome position	Gene region	Ref.
ALPPL2	cg21566642	233284661	2		(Ambatipudi et al., 2016; Su et al., 2016; Zeilinger et al., 2013)
	cg05951221	233284402			Ambatipudi et al. (2016)
	cg01940273	233284934			
ZNF385D	cg03274391				
	cg23480021	22412746			(Ambatipudi et al., 2016; Tsaprouni et al., 2014)
	cg15693572				Ambatipudi et al. (2016)
	cg17024919	21792248			(Tsaprouni et al., 2014)19-(30)
2q37.1	cg05951221	233284402	2		(Allione et al., 2015; Baglietto et al., 2017; Guida et al., 2015; Wilson et al., 2017)
	cg03329539				Wilson et al. (2017)
	cg21566642	233284661			(Allione et al., 2015; Baglietto et al., 2017; Besingi and Johansson, 2014; Elliott et al., 2014; Guida et al., 2015; Wilson et al., 2017)
	cg01940273	233284934			(Allione et al., 2015; Baglietto et al., 2017; Elliott et al., 2014; Guida et al., 2015; Sayols-Baixeras et al., 2015; Wilson et al., 2017)
6p21.33	cg06126421	30720080	6		(Allione et al., 2015; Baglietto et al., 2017; Elliott et al., 2014; Guida et al., 2015; Sayols-Baixeras et al., 2015; Wilson et al., 2017)
	cg14753356	30720108			(Sayols-Baixeras et al., 2015; Wilson et al., 2017)
MTSS1	cg24838345				Wilson et al. (2017)
within SOX1 & LINC00403	cg15653173				
NKX6-2	cg11068946				
CYP1A1	cg05549655	75019143	15	TSS1500	Küpers et al. (2015)
	cg11924019	75019283			
	cg22549041	75019251			
	cg18092474	75019302			
	cg12101586	75019203			
	cg23680900	75017924		TSS200	
NEUROG1	cg11429111	134813329	5	–	
	cg01952185	134813213			
FRMD4A	cg15507334	14372913	10	TSS200	(Küpers et al., 2015; Markunas et al., 2014)
	cg25464840	14372910			
	cg11813497	14372879			(Küpers et al., 2015; Markunas et al., 2014; Rzehak et al., 2016)
	cg20344448	14372431		–	Markunas et al. (2014)
LRP5	cg21611682	68138269	11	Body	Küpers et al. (2015)
MEG3	cg08698721	101294147	14	–	Markunas et al. (2014)
	cg04291079	101294430			
ATP9A	cg07339236	50312490	20	–	
GALNT2	cg16517298	230413174	1	–	
	cg19727396	230415185			
	cg24591105	230415225			
	cg00589617	230415343			
	cg05697274	230415377			
	cg24250902	230415547			
	cg03144619	230415668			
ANKRD33B	cg25612428		5		Chhabra et al. (2014)
CNTD2	cg19605788		19		
PKP3	cg17165241		11		
DPP10	cg22670147		2		
GTF2H2C; GTF2H2D	cg27324075		5		
	cg17290897				
	cg06966320				
	cg02299136				
	cg23813556				
JAK2	cg02405213		9		
KLF6	cg24287110		10	Body	Gao et al. (2016)
MSH5	cg00640087	31707203	6	TSS1500	
	cg20640261	31707020			
ACTA2	cg19335412	90694875	10	3′UTR	
GATA3	cg10163955	8101402	10	Body	
	cg11430077	8099019			
	cg22770911	8101307			
VTI1A	cg03281572	114502318	10	Body	
	cg07269053	114497612			
STK32A	cg17928584	146614458	5	TSS200	
TERT	cg12324353	1269197	5	Body	(Gao et al., 2016; Steenaard et al., 2015)
	cg24908166	1268801			
CHRNA5	cg19696491	78857125	15	TSS1500	Gao et al. (2016)
NFIX	cg16200496	13107141	19		Freeman et al. (2016)
WWTR1	cg25771041	149376042	3		
PLA2G6	cg22515201	38577827	22		
NHP2L1	cg24823993	42085003	22		
SMUG1	cg11875268	54576025	12		

(*continued on next page*)

Table 4 (*continued*)

Gene	CpG locus	Position	Chromosome position	Gene region	Ref.
APC	cg08571859	112073350	5	TSS1500	Barrow et al. (2017)
	cg14511739	112073373		TSS200	
	cg22035501	112073426			
	cg11613015	112073433			
	cg14479889	112073426			
	cg16970232	112073433			
SARS	cg03725309	1268800	5	Body	Steenaard et al. (2015)
GNGT2	cg00980784	47287577	17	TSS1500	
SMG6	cg13916835	2025181	17	Body	
SKI	cg09469355	2161886	1	Body	
	cg05603985	2161049		1st exon	
TOM1L2	cg04324276	17817462	17	Body	
SIPA1	cg25468516	65408028	11	5′UTR	
MRAS	cg22907952	138121287	3	3′UTR	
CDKN1A	cg15474579	36753790	6	Body	
RPH3A	cg18236066	113293823	12	Body	
LRRC2	cg20496896	46579532	3	Body	
FES	cg09397246	9427361	15	TSS1500	
	cg26405020	9427363			
ANPEP	cg23161492	90357202	15		Ligthart et al. (2016)
KSNQ1	cg26963277	2722407	11		
	cg01744331	2722358			
	cg16556677	2722401			
ZMIZ1	cg03450842	80834947	10		

methylated. The IFN-γ promoter showed a 3% increase in methylation, whereas the IL-13 promoter showed a 2% decrease in methylation, increasing the risk for pulmonary inflammation development and airway hyperactivity in infants (Lee et al., 2016a,b). Moreover, the PPAR-γ promoter was hypermethylated in lung alveolar fibroblast cells of rat fetuses following nicotine exposure. Hypermethylation of the PPAR-γ promoter caused reduction in PPAR-γ gene expression levels, affecting the alveolar fibroblast differentiation (Gong et al., 2015).

The promoter of the GFI1 gene, which participates in inflammatory response, was shown to be hypomethylated in the blood of infants with sudden death syndrome whose mothers were smoking during pregnancy. The mean decreased methylation rate was 16%, while in a specific transcription factor binding site the hypomethylation was up by 20%. It is possible that the decreased methylation led to increased GFI1 expression, resulting in dysregulations of hematopoiesis and immune responses (Schwender et al., 2016).

In placental tissue, 50 CpG sites were differentially methylated through maternal smoking. Some of the most significant CpG sites found in placental tissue, were two CpG sites located in TRIO gene (cg25585967 and cg12294026). They were 6,8% and 7,4% hypermethylated, whereas another two CpG sites were hypomethylated by 9,3 and 8,7%, where the first one (cg27402634) was located between LINC00086 and LEKR1 and the second one (cg20340720) was found in WBP1L. Hypomethylation of cg27402634 and hypermethylation of cg25585967 could explain the reduction by 36% and 5,1%, respectively, of the birthweight of newborns exposed prenatally to smoke (Morales et al., 2016). Hypomethylation of GFI1 and NEUROG1 genes in cord blood showed association with birthweight, as well. In particular, hypomethylation of GFI1 (cg09935388, cg14179389, cg12876356) could explain the 12–19% decrease in birthweight (Küpers et al., 2015). Alternations in mitochondrial DNA, especially hypermethylation of the MT-RNR1 gene, were also associated with maternal smoking during pregnancy, lower birthweight and length of the newborns (Janssen et al., 2017). Hypermethylation in the AACS promoter and reduction of the AACS gene expression in fetal rat adrenals after *in utero* nicotine exposure caused reduction in cholesterol levels, leading to decreased synthesis of steroid hormones and lower fetal weight and length (Wu et al., 2016). Furthermore, maternal tobacco smoking induced hypermethylation of cg04757093 within the body of the RUNX3 gene, which has been associated with premature birth. Specifically, increased methylation by one logit, increases 10 times the risk of premature birth (Maccani et al., 2014).

IGF2 differentially methylated region was hypomethylated in the blood of newborns exposed to prenatal parental tobacco smoking (Bouwland-Both et al., 2015) and was related to fetal growth, with more pronounced hypomethylation being observed in females. It was also hypomethylated in female fetal liver, with decreased expression only in males, indicating gender-specific alternations. Moreover, one CpG site in the imprinting control region H19 was hypermethylated only in male newborns, and the NR3C1 promoter had slight DNA methylation changes, which were distinct in males versus females (Drake et al., 2015).

Generally, alternations in DNA methylation are significant with sustained maternal smoking after the first trimester of gestation, while maternal smoking until the first trimester or before pregnancy seems to not affect the DNA methylation of the fetus (Bouwland-Both et al., 2015; Fa et al., 2018, 2016; Novakovic et al., 2014). Instead, sustained maternal smoking, even after the first trimester, has consequences for the infant's DNA methylation, potentially affecting the offspring throughout life. Specifically, hypermethylation of MYO1G, FRMD4A and CYP1A1 genes and hypomethylation of AHRR, CNTNAP2 and GFI1 genes have been shown to be persistent both in childhood (Bauer et al., 2016; Breton et al., 2014; Ladd-Acosta et al., 2016; Rzehak et al., 2016) and adolescence (Lee et al., 2015; Richmond et al., 2015). Moreover, 5–12 year old children with asthma, exposed to maternal smoking during pregnancy, had differential methylation in XPNPEP1, PPEF2, FRMD4A, C11orf52, SMPD3 and CRYGN genes compared to unexposed children. In addition, a 10% decreased methylation of the JNK2 gene, caused by prenatal smoke exposure, led to a 40% increased risk of wheezing in children (Bauer et al., 2016). In addition, differential methylation of the LEPR gene in blood from 18 year old females, affected by SNP genotype, was more intense in cases with prenatal smoke exposure. Hypermethylation and hypomethylation of CpG sites within both the LEPR promoter and body region have been shown to influence leptin levels and body mass index, indicating long term effects of maternal smoking during pregnancy (Yousefi et al., 2013).

All of the above studies are summarized in Table 3.

2. Discussion

In summary, it has been well established that smoking affects DNA methylation patterns. Current smokers have different methylation levels compared to non-smokers, while former smokers tend to have intermediate methylation levels, depending on the time since quitting smoking, due to the reversible dynamic of the methylation process. Moreover, smoking influences DNA methylation of specific genes, which contribute to various diseases. This effect is even encountered in fetuses exposed to maternal smoking. Thus, it is possible to quantify smoking exposure and evaluate the risks of smoking-induced diseases by determining the extent of DNA methylation.

The small number of volunteers in some of the studies described above could pose a limitation since the bigger the size of the study, the more conclusive the results are (Dong et al., 2016; Jhun et al., 2017; Wan et al., 2015). However, it is indeed challenging to find a satisfying number of human volunteers who fulfill all the requirements of a study and would be willing to be tested upon, repeatedly in some cases. Moreover, in many cases, the statistical evaluation is based on self-reported smoke exposure levels (Shui et al., 2016; Zhang et al., 2014). In these cases, the researcher relies on the participants' sincerity and correct estimation of exposure, without being able to apply a control mechanism or adjust the results for incorrect estimation. This could only be avoided by simultaneous analysis for biochemical indicators of tobacco exposure which would reflect on the true exposure levels (Karabela et al., 2011; Matsunaga et al., 2014; Misailidi et al., 2014; Tzatzarakis et al., 2012; Vardavas et al., 2006). Although the results in most cases are adjusted for factors such as age and sex, inter-individual genetic variations, that could also obscure the results, should be considered in the study design (Qiu et al., 2015; Zaghlool et al., 2015). Thus, studies could include individuals from different demographic populations with alternative lifestyle habits, accounting in this way for the genetic variability imposed by environmental factors. In the same manner, differences in the DNA methylation levels between different tissues (e.g. blood vs lung) or different cell types within the same tissue should also be considered (Sayols-Baixeras et al., 2015). Scientists should examine whether testing the whole tissue or specific cell types would prove more suitable, depending on the study. Most of the research that has been carried out on the effects of smoking on DNA methylation has not included the effects on gene expression which may or may not occur, since an altered DNA methylation state could also lead to histone alterations and even DNA instability (Steenaard et al., 2015). Having this knowledge would lead towards a better understanding of the mechanisms underlying the onset and progression of various diseases. Last but not least, even a small and statistically insignificant change in DNA methylation could have a significant effect when studied in the course of a lifetime (Ivorra et al., 2015). A follow-up, when possible, should be considered to examine the effects within longer time periods.

3. Conclusion

Overall, altered DNA methylation status caused by smoking has been linked to various disease states, such as cancer, and effects of smoke exposure, both actively and passively, for example through in uterus maternal exposure. However, DNA methylation is a reversible process. It has been shown, mainly with respect to cancer, that certain demethylating agents, such as curcumin, azacytidine, decitabine and epigallocatechin-3-gallate, can restore the methylation levels of certain gene promoters, providing a new treatment potential. Treatment of smokers with such demethylating agents can perhaps reverse the effects of smoking in those cases that the DNA methylation status is not restored after smoking cessation (Kumar et al., 2017; Morris et al., 2016; Wolff et al., 2017).

The knowledge of the effects of smoking on the epigenome and its consequences for human health could be very useful for the construction of an individualized patient profile and for the prediction of smoking-associated negative outcomes. Nevertheless, more studies with a more global approach are required to fully understand the mechanisms which participate and link smoking, DNA methylation and diseases, laying out the foundation for more targeted drug development and advanced therapies.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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