Thesis

Effect of in utero exposure to cigarette smoke on DNA- methylation in foetus and offspring

submitted by

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Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 16.08.2022 *Sigrid Gruber m.p.*

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Abstract

Background

Smoking in general and particular during pregnancy is a worldwide problem that, despite of knowledge about its adverse consequences, is not declining in prevalence. Smoking has many well-known and well-studied negative effects on cells and triggers DNA mutations. There are also epigenetic changes that are induced by smoking, and smoking in pregnancy can induce epigenetic changes in the foetus which can affect the offspring's health in long-term. Particularly changes in DNA methylation have been investigated and have been shown to serve as good biomarkers. Maternal smoking is associated with many negative consequences for the offspring, including an increased risk to develop asthma, obesity, cardiovascular disease and cancer later in life. The aim of this thesis is to provide an overview of current research in the area of maternal smoking-associated changes in offspring.

Methods

The online database PubMed was used to research the current literature. Only studies performed in human were used. For this thesis, a total of 127 publications published between 2010 and 2021 were included. In this diploma thesis, the correlations were described, compiled and listed according to the different effects and clinical pictures.

Results

Maternal smoking has been shown to be detrimental to development in utero. Epigenetic changes could be detected in the umbilical cord blood of newborns as well as in various tissue samples, for example the placenta and the lungs. Some of these changes could even be detected over a longer period of time, i.e. years after birth, which illustrates the long-term effect of exposure to cigarette smoke in utero.

Discussion

Smoking not only has negative effects on the intra uterine development and later life of the offspring. Therefore, a healthy lifestyle and the absence of smoking, especially during pregnancy, is particularly desirable and should be more actively promoted and supported in order to carry out primary prevention and to reduce the risk of disease development in the unborn child.

Zusammenfassung

Hintergrund

Das Rauchen ist ein weltweites Problem, dessen Verbreitung trotz des Bewusstseins über die negativen gesundheitlichen Auswirkungen nicht abnimmt. Speziell in der Schwangerschaft kann die Exposition gegenüber Rauchinhaltsstoffen die kindliche Entwicklung beeinflussen. So hat Rauchen viele bekannte und gut untersuchte negative Auswirkungen auf die DNA, da im Rauch mutagene Stoffe vorliegen. Rauchen in der Schwangerschaft führt jedoch auch zu epigenetischen Veränderungen in den Neugeborenen, wie z.B. zu einer veränderten DNA Methylierung, die bei der Zellteilung weitergegeben somit einen Einfluss auf die Entwicklung und spätere Gesundheit haben können. Tatsächlich führt mütterliches Rauchen in der Schwangerschaft zu einem erhöhten Risko für Asthma, Fettleibigkeit, kardiovaskuläre Erkrankungen Risiko und Krebs bei den Nachkommen. Nicht alle diese pathophysiologischen Zusammenhänge sind geklärt. Zusätzlich hat sich aber gezeigt, dass veränderte DNA-Methylierung auch als Biomarker dienen könnten. Ziel dieser Arbeit ist es, einen Überblick über mit dem mütterlichen Rauchen verbundenen, epigenetischen Veränderungen bei den Nachkommen zu geben.

Methoden

Für diese Übersichtsarbeit wurde die Online-Datenbank PubMed verwendet. Es wurden ausschließlich humanorientierte Publikationen verwendet. Für diese Arbeit wurden insgesamt 127 Publikationen, die zwischen 2010 und 2021 veröffentlicht wurden, verwendet. In dieser Übersichtsarbeit wurden die bekannten epigenetischen Veränderungen nach den verschiedenen Wirkungen und Krankheitsbildern gegliedert und aufgelistet.

Ergebnisse

Mütterliches Rauchen ist nachweislich schädlich für die Entwicklung in utero. Epigenetische Veränderungen konnten sowohl im Nabelschnurblut von Neugeborenen als auch in verschiedenen Gewebeproben, zum Beispiel von der Plazenta und der Lunge, nachgewiesen werden. Einige dieser Veränderungen konnten sogar über einen längeren Zeitraum hinweg festgestellt werden, also Jahre nach der Geburt, was die langfristige Wirkung verdeutlicht.

Diskussion

Rauchen hat nicht nur negative Auswirkungen auf die rauchende Person, sondern im Falle einer Schwangerschaft, auch auf das Kind in utero und dessen späteres Leben. Daher ist eine gesunde Lebensweise und der Verzicht auf das Rauchen, insbesondere während der Schwangerschaft, besonders wünschenswert und sollte aktiver gefördert und unterstützt werden, um eine Primärprävention durchzuführen und das Risiko der Krankheitsentwicklung beim ungeborenen Kind zu verringern.

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Abbreviations

ACOX3	Acyl-CoA oxidase 3, Pristanoyl
ACTL9	Actin like 9
ADHD	Attention deficit hyperactivity disorder
ADM	Adrenomedullin
AEDS	Atopic eczema/dermatitis syndrome
AHRR	Aryl hydrocarbon receptor repressor
ALL	Acute lymphatic leukaemia
AluYb8	Alu element Yb8
AML	Acute myeloid leukemia
APBA2	Amyloid beta precursor protein binding family A member 2
ARL4C	Adenosine diphosphate-ribosylation factor-like 4C
ASD	Autism spectrum disorder
ATP10A	ATPase phospholipid transporting 10A
ATP9A	Adenosine triphosphatase phospholipid transporting 9A
AXL	AXL receptor tyrosine kinase
BCL7C	B-Cell CLL/Lymphoma 7 protein family member C
BLCAP	Bladder cancer associated protein
BMI	Body mass index
CD28	Cluster of differentiation 28
CDK6	Cyclin-dependent kinase 6
CHS	Children's Health Study
CMIP	C-maf-inducing protein
CNTNAP2	Contactin associated protein 2
CpG	Cytosine-phosphate-guanine
CYP1A1	Cytochrome P450 family 1 subfamily A member 1
CYP1A2	Cytochrome P450 family 1 subfamily A member 2
DGCR8	DiGeorge syndrome critical region 8
DLGAP2	DLG associated protein 2
DLPFC	Dorsolateral prefrontal cortex
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase

DOHaD	Developmental Origins of Health and Disease
DRD1	Dopamine receptor D1
dsRNA	Double-stranded RNA
ESR1	Estrogen receptor 1
FEV1	Forced expiratory volume in the first second
FRMD4A	FERM domain containing 4A
FTO	Fat mass and obesity- associated protein
GATA3	GATA binding protein 3
GFI1	Growth factor independent 1 transcriptional repressor
GNA15	G protein subunit alpha 15
GNG12	Guanine nucleotide binding protein gamma 12
H1	Histone H1
H19	imprinted maternally expressed transcript
H2A	Histone H2A
H2B	Histone H2B
H3	Histone H3
H4	Histone H4
HAT	Histone-acetyltransferase
HDL	High density lipoprotein
HDM	histone-demethylase
HLA- DOB	HLA- class II histocompatibility antigen- DO beta chain
HMT	histone-methyltransferase
ICR	Imprinting control region
IgE	Immunglobulin E
IGF2	Insulin like growth factor 2
IL- 33	Interleukin-33-receptor
IL- 4R	Interleukin-4-receptor
ITGB7	Integrin subunit beta 7
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
IUGR	Intrauterine growth restriction
LINE-1	Long interspersed nuclear element - 1
lncRNA	Long non-coding RNA
LRP5	Low density lipoprotein receptor-related protein 5
MDS2	Myelodysplastic syndrome 2 translocation associated

miR / miRNA	Micro RNA
mRNA	Messenger RNA
MSI2	Musashi RNA-binding protein 2
mtDNA	Mitochondrial DNA
mtTFA	Mitochondrial transcription factor A
MYO1G	Myosin IG
ncRNA	non-coding RNA
NEST	Newborn Epigenetic Study
NEUROG1	Neurogenin 1
NK	Natural killer cells
NNAT	Neuronatin
NR3C1	Nuclear receptor subfamily 3 group C member 1
NRP2	Neuropilin 2
OPRM1	Opioid receptor mu 1
PAHs	Polycyclic aromatic hydrocarbons
PAI1	Plasminogen activator inhibitor-1
PBX1	PBX homeobox 1/ pre-B-cell leukemia transcription
PDGFB	Platelet-derived growth factor subunit B
PDXK	Pyridoxal kinase
PEG10	Paternally expressed 10
PIM1	Pim-1 proto- oncogene, serine/threonine kinase
PLCG2	Phospholipase C gamma 2
PPARγ	Peroxisome proliferator- activated receptor gamma
pre-mi-RNA	Precursor micro RNA
pri-mi-RNA	Primary micro RNA
PTM	Posttranslational histone modification
PTPRO	Protein tyrosine phosphatasereceptor type O
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RPL3	Ribosomal protein L3
RUNX3	RUNX family transcription factor 3
SDHAP3	Succinate dehydrogenase complex flavoprotein subunit A pseudogene 3
SERPINE1	Serpin family E member 1
SGA	Small for gestational age

SGCE	Sarcoglycan epsilon
SIDS	Sudden infant death syndrome
siRNA	Small interfering RNA
ST2	Soluble interleukin 1 receptor-like 1
SYCE3	Synaptonemal complex central element protein 3
TBX21	T-Box transcription factor 21
TET	Ten-eleven-translocation
TGM1	Transglutaminase 1
TIGIT	T- cell immunoglobuin and ITIM domain
TINAGL1	Tubulointerstitial nephritis antigen like 1
TLR	Toll- like receptor
TRIO	Trio rho guanine nucleotide exchange factor
UTR	Untranslated regions
VPRBP	Vpr-binding protein
WBP1L	WW domain binding protein 1 like

Introduction

This paper discusses the epigenetic modifications and long-term consequences of intrauterine exposure to cigarette smoke on DNA methylation in the foetus and offspring.

Smoking during pregnancy is a widespread problem. The ingredients of cigarette smoke are very harmful and have many negative effects directly on the DNA by inducing mutations, as well as on epigenetics, such as changes in DNA methylation, consequently. In fact, smoking during pregnancy leads to a higher level of risk for various non-communicable diseases in the exposed offspring, including respiratory disease, obesity, cardiovascular disease and cancer. Although not all pathophysiological relationships between DNA methylation and the development of these diseases are yet fully understood, methylation differences may further serve as biomarkers.

The aim of this thesis is to provide an up-to-date overview of the research findings in this field.

DOHaD Hypothesis

The DOHaD (Developmental Origins of Health and Disease) model describes the fact that parental factors (environmental factors/endocrine, metabolic and stress related factors) in utero affect the offspring in long term by, "programming" of the genome, i.e. modification of the expression of individual genes, and, in a broader sense, also the risks for the development of chronic, non-communicable diseases in the offspring's later life. These non-communicable diseases include cardiovascular diseases, diabetes, allergies, chronic lung diseases, cancer, osteoporosis and affective disorders and are among the leading causes of death worldwide. Non-communicable diseases are avoidable and prevention, especially in the early stages of development, can dramatically reduce the long-term impact on individuals (1).

Both, experimental studies in the laboratory, as well as epidemiological studies in humans, have shown a link between prenatal stressors and the development of non-communicable disease in affected individuals. Stressors can be of emotional or physical nature, can include hypoxia, drug and alcohol use as well as nutritional factors, such as food excess or deprivation (2,3).

These stressors alter and program gene expression and hence, further development by epigenetic mechanisms. Depending on the triggers, different epigenetic modifications occur which can manifest themselves both positively and negatively in later life. These epigenetic modifications are defined as changes in gene expression and phenotype without changes in genotype. Examples of epigenetic modifications are DNA methylation, histone modification and regulation by non-coding RNAs. Common to these modifications is their heritability, i.e. the fact that they persist throughout mitosis or meiosis. Therefore, epigenetic changes can have long-lasting consequences on the unborn child (2,4).

Because of this, it is important to understand the prenatal mechanisms of programming in order to be able to counteract or prevent these developments (2).

The intrauterine window

Popular studies on the Dutch famine of 1944- 1945 were the first to provide insights into the connections between various environmental influences and the development of diseases in the vulnerable window of pregnancy. For example, effects on birth weight, growth, and the development of cardiovascular diseases, metabolic disorders, diabetes mellitus type II and affective disorders were observed in newborns. These findings were able to show prenatal influences and their effects on the offspring and create an important foundation for any future studies and observations (5).

Introduction to epigenetics

Epigenetics describes the field of research that detects changes in the activity of genes that are not based on a change of sequence within the DNA. These changes hence, affect the phenotype but not the genotype. The regulatory modifications include DNA methylation, histone modification and non-coding RNAs (ncRNAs). For DNA methylation, the mechanism of heritability is well understood (2,6).

Environmental epigenetics is a branch of epigenetics that deals with environmental influences and their effect on gene expression. These environmental factors vary in timing, severity and frequency and influence genome activity throughout life. Examples include trauma, drugs, smoking, stress, and diet. There are three major phases of imprinting in each person's life. These periods of high susceptibility to programming occur during pregnancy, immediately after birth and during puberty (6).

Epigenetic mechanisms

All epigenetic mechanisms modify gene expression. The different mechanisms are discussed in the following sections.

DNA methylation is the best-studied mechanism of programming. It was first described in 1948. DNA methylation is of substantial importance and essential in various processes throughout human development besides programming as it also underlies

imprinting, the inactivation of the second X-chromosome in females, and is responsible for DNA stability (7).

DNA methylation

Within the process of DNA methylation, methyl groups are linked to the base cytosine by the enzyme DNA methyltransferase (DNMT). This occurs mainly in the promoter region of genes. The most effective and important regulatory regions are areas where cytosine nucleotides are located next to guanosine nucleotides. Such sites within the DNA sequence are called CpG dinucleotides. DNA areas containing a larger than expected amount of CpG sites are referred to as CpG islands (2,7). Approximately 29,000 known CpG islands exist in the human genome (8).

An altered amount of methylation or demethylation influences the hydrophobic properties of the chromatin and thus, also affects the accessibility for transcriptional activators or suppressors. Decreased methylation in a promoter sequence causes increased or activated gene expression. Strong methylation leads to the inactivation of genes. (9).

Several DNMTs exist (DNMT1, DNMT2, DNMT3a, DNMT3b), which fulfill different tasks, such as maintenance methyltransferases, RNA catalysis, de novo methylation patterns. On the other hand, methylation can also be removed by the ten-eleven translocation methylcytosine dioxygenases (TET enzymes). This is particularly important in the maintenance of embryonic stem cells. Three TET genes are known: TET1, TET2 und TET3. A reduced function of these enzymes occurs, for example, in hypoxia and is also associated with the development of various malignancies (2).

DNA methylation has a major part to play in the developmental programming of every human being. Studies have shown that the mechanisms for maternal and paternal DNA demethylation differ. The genome of the sperm is actively demethylated after fertilisation, the DNA of the oocyte retains the methylations until the first mitotic division. In addition to this, environmental factors of the mother can influence the methylations at these early stages of development and thus cause potential long-term consequences (7).

Various studies by Jensen Pena et al., Serpeloni et al. and Brunst et al. have shown that stress of all kinds (malnutrition, hyper-hypothermia, protein deficiency and psychological stress) can alter DNA methylation and interfere with developmental programming in order to achieve higher resilience in the offspring (10–12). Moreover, particularly non-communicable diseases such as hypertension, diabetes mellitus type II, cancer, and neurodegenerative diseases are associated with changes in DNA methylation (2).

Histone modifications

Also histone modifications enable the modification of gene expression by regulating the chromatin structure. Similar to DNA methylation changes, parenteral stress can influence histone modifications and promote fetal programming.

Several enzymes are involved in histone modification: Histone acetyltransferases (HAT), histone methyltransferases (HMT), histone deacetylases (HDAC) and histone demethylases (HDM). HMT attaches methyl groups to the histone tails and thereby ensures condensation, i.e. inactivation of the chromatin, which is then termed heterochromatin. The removal of methyl groups occurs via HDM. The attachment of acetyl groups by HAT loosens the chromatin fibre, subsequently referred to as euchromatin. Histone modification also includes the addition of ubiquitin groups and/or phosphate groups. However, the latter have not been researched in detail (2).

Histon Code Hypothesis

Strahl and Allis developed the histone code hypothesis in 2000, which describes that the combination of histone modifications or the sequence of modifications on different/the same histone tails has a certain effect (2).

Posttranslational histone modification (PTM) is an important mechanism of epigenetic programming (13). The lead structure are the nucleosomes, which are formed by eight proteins, the histones. There are several types of histones (H1, H2A, H2B, H3, H4), which form two different dimers (H2A-H2B and H3-H4). By combining four of these dimers, an octamer is formed. About 147 base pairs of DNA are wrapped around an

octamer, which now is referred to as nucleosome. Nucleosomes have a certain momentum and plasticity. This can allow unwinding, twisting and sliding of DNA (2,14).

The linker histone H1 is responsible for chromatin compaction, stability, as well as silencing and has therefore, an effect on gene expression. Different H1 histone variants exist, which differ in their expression patterns. These variants are important for various developmental processes such as oogenesis, spermatogenesis and the differentiation of pluripotent cells (15).

Meanwhile, there are specific histone PTMs patterns known to be are associated with different diseases, e.g. cardiovascular diseases or cancer. Moreover, recent research has identified new histone marks which influence gene regulation in a new and different way. These marks include a group of short-chain Lys-acylations (13).

Micro RNAs

Micro RNAs (miRNAs) are short, 22 nucleotide long non-coding RNAs that bind to messenger RNAs (mRNAs) and either induce mRNA degradation or interfere translation (16). About 400 miRNAs have been identified so far. As miRNA binding to mRNAs does not require complete homology, one miRNA can interact with several mRNAs and it is estimated that 30% of gene expression is regulated by miRNAs (17). miRNAs cannot be inherited throughout mitosis or meiosis, and hence, are no epigenetic regulatory mechanism themselves, but approximately 50% of all yet identified 400 miRNAs contain a CpG site in their promoter region and hence, are regulated by epigenetic mechanisms (2).

miRNA maturation

The two enzymes Drosha and Dicer regulate miRNAs maturation. The first transcript of a miRNA gene is a pri-mi RNA (primary micro RNA), which is formed in the nucleus by the enzyme Drosha and an interacting protein called DiGeorge syndrome critical region 8 (DGCR8). This in turn forms a pre-mi RNA (precursor micro RNA), which is complementarily folded into a double strand. Dicer enzymes now cut the pre-mi RNA and generate the miRNA. This has a length of about 22 nucleotides and binds in the

cytoplasm to an Argonaute protein, which then splits the double-stranded miRNA. The resulting complex of Argonaute protein and single-stranded miRNA is called RNA Induced Silencing Complex (RISC). The RISC complex can interfere with mRNAs and inhibit their expression in a sequence-specific manner. A miRNA can interact with several mRNAs (17–19).

Unlike small interfering RNAs (siRNAs), and despite their similarity to them, miRNAs do not cause degradation of mRNAs. They bind to the 3' untranslated regions (UTR) of mRNAs and thus, inhibit gene expression. siRNAs and miRNAs show hardly any difference functionally and biochemically. They only differ in their origin: siRNAs are formed from a double-stranded RNA and miRNAs from a region of a double-stranded precursor RNA, a so-called hairpin RNA (18).

Chang et al. have shown that miRNAs can cross the placental barrier, either bound to proteins or via exosomes (20). Thus, the range of miRNAs is increased and maternal miRNAs can potentially also regulate gene expression in placenta and foetus. Vice versa, placental miRNAs may enter the maternal circulation, which may be associated with the development of pre-eclampsia, hypertensive disorders or fetal growth restriction (2).

Laboratory studies have shown that miRNAs have an essential part in the development of the heart, brain, neuronal functions and various endocrine organs (2,16). During pregnancy, environmental changes in the expression of miRNAs in the umbilical cord blood have also been identified (2). For instance, nutritional status during pregnancy affects miRNA expression and may be associated with the development of cardiometabolic diseases (21). There is also a link between prenatal stress and epigenetic changes in miRNAs, which is associated with the development of neurological conditions such as bipolar affective disorder, schizophrenia and multiple sclerosis (22).

Research on miRNAs, their activity and function may offer new therapeutic options for various diseases in the future. The regulation of specific miRNAs has already shown a promising lead in experiments with animals suffering from cardiovascular disease (23).

Smoking during pregnancy and the effects on the foetus

Cigarette smoke contains far more than 4000 ingredients in total, of which 250 are considered toxic and more than 40 are known carcinogens.

Tobacco smoke is associated with a very large number of diseases. Studies have shown that when people are being exposed to tobacco smoke, it can cause epigenetic changes in addition to many well-known genetic mutations (6,24). For instance, smoking leads to endothelial dysfunction, due to the release of free radicals, the increase of various inflammatory parameters and the reduced amount of nitric oxide, which is normally responsible for the dilatation of blood vessels. This increases vascular stiffness and promotes the development of cardiovascular diseases (25).

Since the degradation products of tobacco pass through the placenta, these compounds can enter the fetal blood and pass into the amniotic fluid and affect the foetal development. Moreover, tobacco degradation products and are present in the urine of the newborn baby (26). It is well known that smoking has many negative and damaging effects on the unborn child. It is harmful to the foetus in all trimesters of pregnancy and is a very important preventable risk factor (24).

Known effects can be grouped under the term foetal tobacco syndrome. These include impaired growth (small for gestational age), impaired differentiation of foetal and placental cells, effects on the development of organ systems, effects on the immune, nervous, respiratory and cardiovascular systems, which can lead to death of the infant, miscarriage, higher risk of spontaneous abortion, abruption of the placenta, premature birth, premature lung ageing and chronic obstructive lung disease (6,27,28).

Also in the placenta morphological changes can be observed after cigarette smoke exposure, such as a thickened basement membrane, an increased proliferation of tissue, as well as a reduced number of blood vessels, with all of these changes having the potential to reduce placental transport function and thus, contribute to the incidence of small for gestational age, miscarriage and stillbirth (24).

Prevalence of smoking during pregnancy

It is difficult to determine the prevalence of smoking during pregnancy, since the truth is often not revealed during interviews. This brings an increased risk of falsification of the results in studies that are carried out. One way to rule this out is online questionnaires where participants are more willing to confess if they smoke (24).

In General, studies have shown that smoking mothers have a lower level of education and a higher unemployment rate. Furthermore, in the respective groups of smokers, the number of previous abortions was higher in percentage terms, and the age was lower than that of non-smoking mothers (29). Further factors associated with an increased prevalence of smoking during pregnancy are a low socioeconomic status, a low income and the absence of a partner. The willingness to try smoking or to start smoking is reported to be higher in children whose mothers have smoked in pregnancy than in unexposed children (24).

It is clear that maternal smoking is disliked in society. Nevertheless, the prevalence is alarmingly high. In the United States, 7.2% of women who delivered a baby in 2016 had smoked during their pregnancy. In Europe, the prevalences were 10.7% in the 20-24 age group, 8.5% in the 15-19 age group and 8.2% in the 25-29 age group (30). In some cases, a decrease in prevalence rates with the progression of pregnancy could be observed. Yet, the overall prevalence has probably decreased since the 1990s. However, the data varies greatly (24). Lange et al. estimated the global prevalence of smoking in pregnancy at 1.7%. In Europe, the prevalence is 8.1%, in the Americas 5.9%, in Southeast Asia and the Western Pacific Region 1.2%, in the Eastern Mediterranean 0.9% and on the continent of Africa 0.8%. Among the smoking pregnant women interviewed in this study, 72.5% smoked daily and 27.5% smoked occasionally. Furthermore, 51.8% were light smokers, 34.8% moderate smokers and 13.5% heavy smokers. 52.9% of the daily smokers continued smoking during pregnancy (31). A study by Kondracki estimated the prevalence of smoking during pregnancy in the USA at 9.5% (32).

Smoking during pregnancy in Europe and Austria

This is data that was collected specifically for Europe. The smoking prevalence of pregnant women at the time of delivery was found to be between 7% (in Bulgaria) and 52.5% (in Ireland) in a study published in 2006 including six European countries (Germany, Ireland, Portugal, Greece, Belgium, Bulgaria). The mean prevalence for smoking during pregnancy in this study was 19.6% (33). However, the prevalence of smoking during pregnancy in Ireland was only 9-10% in 2015 (31). Another study from Spain found a smoking prevalence in pregnancy of 32%. In Great Britain, a smoking rate of 36% was recorded in 2000 but in 2021, the rate of smoking mothers at birth was only 9.1% (34). In Germany the prevalence was 21% (2008) and in 2010 in Austria the prevalence was between 20-30%. Likewise, the prevalence of smoking in the general population in Austria is very high at 34% overall (28).

The study by Schultze et al. even showed a prevalence of smoking during pregnancy of 18.1 % in Austria. The prevalence measured during this 6-year study period, decreased to 15.6% at the end of the study (2012). Smoking rates are particularly high among mothers under 20 years of age (43.7%) and those aged 20-25 years (28.8%) (28).

Passive smoking

Unfortunately, it is very likely that a large number of women are also exposed to passive smoking during pregnancy. This exposition to second-hand smoke can occur in public areas, in homes, in workplaces and vehicles. Passive smoking also results in adverse effects on the unborn child, as passive smoking is as harmful as active smoking (6).

But why do pregnant women continue smoking?

There is often a lack of awareness about the negative effects of tobacco consumption during pregnancy on the foetus. Further influences can be the social status, smoking before pregnancy, influence by others or by the partner, stress factors before and during pregnancy, depression or not being able to avoid passive smoking (27).

Furthermore, nicotine and cotinine clearance is increased during pregnancy, which makes it more difficult to stop smoking in consideration of the addictive characteristics (24).

Electronic cigarette use – Prevalence among pregnant women

Another exciting aspect is the use of e-cigarettes during pregnancy, as the popularity as well as prevalence of electronic cigarettes has increased significantly in recent years. In animal studies, exposure to electronic cigarettes was found to have adverse effects similar to exposure to conventional cigarettes. These changes showed increased methylation levels of DNA with negative developmental consequences, increased cell death, decreased cognitive abilities and malformations in the offspring (35).

In the study by Wagner et al., 445 pregnant women were surveyed, 5.62% used tobacco cigarettes, 6.5% used e-cigarettes, 8.5% used tobacco cigarettes and e-cigarettes, and 79.3% used neither of them. 74.6% of the women who used e-cigarettes reported that they switched to using e-cigarettes when they became aware of their pregnancy. Furthermore, e-cigarette users were on average younger than users of ordinary cigarettes. In addition, the factor of advertising should not be overlooked, which has a considerable impact and possibly contributes to the fact that electronic cigarettes are considered safer compared to tobacco cigarettes. It is interesting to note that 64.27% of the participants believed that electronic cigarettes are generally safer than traditional tobacco cigarettes. Furthermore, a total of 35.28% believed that e-cigarettes are safer also for pregnant women than tobacco cigarettes. In particular, compared to non-smoking pregnant women, women who reported both types of tobacco use during pregnancy considered electronic cigarettes to be safer both in general and during pregnancy (36). Of the participants who switched to or started using e-cigarettes when they became pregnant, 46% said they did so in the belief that they would harm their child less or that they were safer than tobacco cigarettes. Another 18% said they switched to e-cigarettes to quit smoking (36).

Methods

The purpose of this paper was to summarize and review available literature about the effects of in utero exposure to cigarette smoke and air pollution on DNA- methylation in foetus and offspring by using the online data base PubMed.

This paper is a summary of previously published results. The information summarised and processed in this paper was retrieved from the Pubmed archive under the search criteria "((smoking) OR (nicotine abuse)) AND (pregnancy)) AND ((dna methylation) OR (epigenetics))". This search yielded a total of 391 studies from 1993 to 2021. The studies found were screened and initially reduced to 233 studies by excluding studies that did not fit the content or were not conducted in human. This left 129 studies that formed the basis of this work. Secondary literature was often cited to obtain more detailed information.

These studies were further supplemented by studies on epigenetic mechanisms (DOHaD, DNA methylation, histone modification, miRNA).

Results

Timing and dose

The dose makes the poison, as the saying goes. Thus, timing and dose are important influencing factors when it comes to the effects of smoking during pregnancy.

In the following study, 1,391 CpG islands associated with sustained maternal smoking during pregnancy were analysed in 1,261 neonates. The cord blood DNA methylation of newborns born to mothers who smoked was compared with newborns of non-smoking mothers. If the mothers continued to smoke throughout pregnancy, the investigated CpGs showed a 67.6 % lower methylation rate, i.e. hypomethylation, than the control group. However, if mothers smoked before pregnancy or quitted early in pregnancy, no changes in DNA methylation were detected. Unfortunately, no precise definition of early smoking cessation was given. The covariates were BMI as well as parental age, birth weight and sex of the child (37).

In the same study, the dose of smoking was evaluated in the third trimester, ranging between less than 1 and more than 20 cigarettes per day. It was observed that smoking more than 5 cigarettes per day was associated with changes in DNA methylation at seven CpG sites. The altered gene segments included *AHRR, MYO1G, ARL4C, ATP9A, ACTL9* and *BCL7C*. *AHRR* (Aryl Hydrocarbon Receptor Repressor) encodes a transcription factor that plays an important role in the degradation of toxic products (polyaromatic hydrocarbons and dioxin) present in cigarette smoke (37).

In a study with 2,504 participants, time- and dose-dependent effects of smoking and the influence of an early cessation of smoking (before the 15th week of pregnancy) during pregnancy were observed. Thus, non-smokers and mothers who stopped smoking before the 15th week of pregnancy had similarly high probabilities for small newborns at gestational age, the occurrence of spontaneous premature births and an uncomplicated course of pregnancy in comparison to mothers who smoked throughout the entire pregnancy. Hence, the effects of early smoking in pregnancy seem to be largely reversible if stopped in time (38).

The effects of smoking in pregnancy on the were investigated in an European study involving 1203 children. It revealed that smoking during pregnancy causes methylation changes in the blood of the newborns at 39 CpGs. The methylation of some of these CpG sites described were dependent on the dose and duration of smoking during pregnancy, but no more precise details were described. (39).

IUGR - Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR) is a pathological fetal growth development during pregnancy. It can be defined as a sex- and age-matched fetal weight below the 10th percentile. The causes of growth restriction can be manifold, and maternal smoking is one of them (40). Smoking leads to endothelial dysfunction, due to the release of free radicals, the increase of various inflammatory parameters and the reduced amount of nitric oxide, which is normally responsible for the dilatation of blood vessels. This increases vascular stiffness and promotes the development of cardiovascular diseases (25). A strong correlation could be found depending on the dose of smoking as well as the age of the mother. Thus, a higher risk of IUGR was noticed with a consumption of more than 10 cigarettes per day as well as for a maternal age above 31 years (41). Notably, there was no difference in weight between the children of women who quit smoking while they were pregnant and those whose mothers did not smoke during their pregnancy. No detailed data on smoking cessation during pregnancy was available (42).

IUGR is associated also with other health problems: Foetuses with IUGR and reduced lung maturation have been found to have an increased risk of premature birth with more complications and, subsequently, an elevated chance of developing asthma (43). Several studies have shown that there is a causal relationship between smoking during pregnancy and reduced birth weight of babies. This difference in weight compared to babies whose mothers did not smoke during pregnancy is about 170-260g. A dose dependence could also be determined here (44).

The exposure to tobacco during pregnancy appears to be associated with fetal hypoxia. Nicotine is responsible for vasoconstriction in the placenta, leading to increased cell death of the syncytiotrophoblast and, consequently, to growth retardation. In cells of the umbilical cord, a lower production of vasodilative substances could be found, which also contribute to hypoxia (44). It is well known that inadequate blood supply to the uteroplacental unit through reduced capillary volume and associated intrauterine nutrient and oxygen deficiency can lead to growth restriction as well as metabolic, neurological and cardiovascular diseases (45).

The sudden infant death syndrome (SIDS) is a syndrome which, among other causes, is triggered by smoke exposure in utero and, is also associated with fetal growth retardation. This was shown in a British study of 104 SIDS cases in children. Authors assumed that nicotine causes a constriction of the placental blood vessels and a hypoxia of the foetus due to the destruction of the syncytiotrophoblasts, which leads to growth retardation. Moreover, the production of nitric oxide was reduced in endothelial cells of the umbilical veins of pregnant women who smoke. Nitric oxide is responsible for the vasodilation of blood vessels, so a reduced production leads to a reinforcement of the pre-existing hypoxia (44). Furthermore, intrauterine growth retardation is associated with reduced lung function, as well as lower forced expiratory volume (FEV1) (46).

Another study, involving 954 newborn-mother pairs, also found a relation between the birth weight of the foetus and a history of smoking during pregnancy. The affected newborns were on average 258 grams lighter than newborns who were not exposed to cigarette smoke. In cord blood, seventeen CpG sites were found differentially methylated in the genes *GFI1*, *AHRR*, *CYP1A1* and *CNTNAP2*. For 8 of these CpG islands in the *GFI1*, *AHRR* and *CYP1A1* genes, an association between birth weight and smoking during pregnancy could be identified (47).

A study by Cardenas et al. included 441 mother-child pairs and examined DNA methylation at a total of 720,077 CpG sites between 2010 and 2014. 71 differentially methylated CpGs were identified in the placenta, which were linked to smoke exposure. Of these, a correlation between birth weight and maternal smoking was shown for 7 CpGs through changes in DNA methylation of some genes (*MDS2*, *PBX1*, *CYP1A2*, *VPRBP*, *WBP1L*, *CD28* and *CDK6*). Maternal smoking was associated with a reduced birth weight of 175g on average. The greatest impact was observed for the

PBX1 gene in CpG cg22638236. This gene is involved in skeletal patterning and programming as well as in osteoblast differentiation. . However, similar methylation changes as in the placenta were not found in cord blood (48).

A study by Witt et al. was able to show interesting effects of smoking during pregnancy in connection with weight reduction through epigenetic changes in *PIM1*, *ITGB7* and *CNTNAP2*. A reduced birth weight of > 200g was observed in smoke-exposed foetuses. Umbilical cord whole blood samples were used from a total of 25 newborns exposed to maternal smoking during pregnancy. The contactin associated protein like 2 gene (*CNTNAP2*) is responsible for cell adhesion and belongs to the neurexin gene family. It is associated with body weight on the one hand and with the development of neurological disorders on the other. A lower methylated CpG in the *CNTNAP2* gene is associated with maternal smoking exposure. This means that the gene is expressed more strongly in people who are exposed to smoke. It has been previously revealed that the loss of *CNTNAP2* has a negative effect on the transmission of nerve signals. Thus, the increased expression could be a defensive mechanism of the body. The *PIM1* gene encodes a serine/threonine protein kinase that counteracts programmed cell death and is responsible for cell differentiation and growth. Thus, increased expression of the *PIM1* gene protects against apoptosis, which is indicated by smoking. A CpG segment of this gene has been found to be less methylated in smokers, leading to increased expression in lung tissue. This could be a protective mechanism of the body (49). The *ITGB7* gene (Integrin subunit beta 7) is responsible for cell adhesion and signal transduction. It has been shown that decreased expression of this gene can contribute to the development of cancer through increased methylation when exposed to smoke. Witt et al. showed that methylation and the associated mediation effects can be gender-specific. For example, the impact of the differentially methylated CpG site in the *CNTNAP2* gene had no influence in female newborns, but only affected male newborns (49).

Also Küpers et al. observed that the average birth weight of smoke-exposed newborns was 281 g lower. They found 23 CpG sites on eight genes that showed differential methylation among the smoke-exposed infants. These genes were *GFI1, AHRR, CYP1A1, CNTNAP2*, *MYO1G, FRMD4A, NEUROG1* and *LRP5*. In particular, three CpG sites of the *GFI1* gene showed a remarkable connection (12 - 19 %) with weight reduction (50).

Differentially methylated regions were also found in the *IGF2* gene after smoking exposure during pregnancy. This gene encodes an important growth factor driving intrauterine growth. Interestingly, the three differentially methylated CpGs affected were only significantly hypermethylated in male offspring (51).

Pre-eclampsia and premature birth

A Swedish study showed a link between smoking during pregnancy and the risk of spontaneous preterm birth and very premature birth. An amount of more than 10 cigarettes each day has been considered a major risk factor. However, the exact underlying mechanisms have not yet been fully understood (52). Smoking leads to an acute inflammatory reaction in the placenta as well as in the umbilical cord and affects the mother's immune system. Furthermore, women who smoked during pregnancy have increased prostaglandin levels. Prostaglandins promote labor and are thus associated with preterm birth (53). Moreover, smoking during pregnancy increases the risk of premature rupture of the amniotic sac by a factor of 2 (44).

An interesting relation exists between pre-eclampsia and smoking: smoking reduces the risk of pre-eclampsia by up to one-third. Thus, a lower incidence of pre-eclampsia is present in women with higher cotinine levels of > 200 ng/ ml in their sputum. However, the neonates also had a lower average birth weight and a lower gestational age. Other studies have shown that the consequences of smoking for pregnant women with pre-eclampsia are less favourable compared to non-smoking pregnant women (44).

In general, premature babies show reduced maturation, as well as reduced lung function. These newborns often require assisted ventilation and administration of glucocorticoids. Early cortisone therapy and prematurity itself is associated with a greater risk of developing respiratory diseases, such as asthma, later in life (24).

Immune system / Infections

The maturation of the immune system is affected by a great number of factors, even in prenatal life. These factors include maternal smoking during pregnancy, air pollution, fine particle pollution, exhaust fumes of cars, heavy metals, antibiotics and estrogens (24).

In fact, maternal smoking in pregnancy is associated with changes in the immune response in umbilical cord blood cells., i.e. lower number of CD4+ and CD25+ T-cells as well as an increase in the concentration of particulate matter (54). Moreover, smoking during pregnancy increases morbidity and the risk of viral respiratory diseases such as whooping cough, as found in a study involving a total of 101,245 infants (55).

A protective factor against infections as well as against the development of asthma is breastfeeding (24). This was demonstrated in a study involving 1,456 children (56). Nevertheless, it has been observed that mothers who smoke breastfeed their children less frequently (24).

Estrogen is important in the development of the foetal immune system. Smoking mothers have low levels of estrogen in their umbilical cord blood. Thus, smoking has an anti-estrogenic effect, which may contribute to the impaired immune development (45).
Fine dust and cigarette smoke also have an effect on the immune system. Increased IgE levels are present in the umbilical cord blood if pregnant women were exposed to smoke and fine dust. Furthermore, an increased expression of mRNA encoding IL6, CD56 (marker for NK cells) and CD68 (marker for macrophages) was observed in smokers' decidual tissue. This correlation was linked to the amount of smoked cigarettes. An increase in mRNA of *GATA3* and markers for M1 macrophages was observed with consumption of more than 10 cigarettes per day (45). In pregnant women exposed to smoke, a reduced number of regulatory T-cells was found in the peripheral blood (57). Smoke-exposed babies had reduced reactions of TLRs (Toll-like receptors) compared to non-exposed babies (58). Altogether, the data indicates a slower maturation of the immune system. Unfortunately, no information on the cohort sizes was given (45).

Asthma

Asthma is the most common chronic disease in childhood The increasingly poorer air quality due to industrial and pollutant emissions as well as tobacco smoke play an important role in the higher prevalence (24).

Exposure to tobacco smoke during pregnancy increases the risk of developing wheeze by 41% in the first two years of life (59). A meta-analysis of 79 studies showed that prenatal exposure to smoke increased the risk of wheezing by 30 - 70% and the incidence of asthma by 21 - 85%, with a lengthening of the airway and a decrease in airway diameter in newborns (60).

Prenatal influences such as cigarette smoke exposure as well as air pollution like diesel exhaust have been shown to have a negative impact on the maturation of the immune system. It is suspected that these toxic substances can regulate genes of the immune system and thus have a great influence on the development of diseases (24). As a result, there is an imbalance of type 1 and type 2 T-helper cells in the sense of a type 1 hypersensitivity disorder and an increased type 2 reaction (61). Some methylations of certain gene loci, which were altered by maternal smoking, persisted into childhood (62). The effects of smoking during pregnancy appear to bear an increased risk of developing asthma across generations (24).

Oxidative stress is a major risk factor for the development of allergies or asthma. Oxidative stress can result from free radicals and can be caused by high levels of air pollution or cigarette smoke. Fine dust consists of polycyclic aromatic hydrocarbons and metals. Animal studies have shown that smoking during pregnancy reduces the body's own antioxidants and produces more oxygen radicals (45). Free radicals, in turn, promote lipid peroxidation, which produces fatty acid radicals that damage the cell membrane (63).

Vitamin C, vitamin E, selenium and B-carotene are important antioxidants in human physiology (63). Vitamin C has a preventive effect on lung function and is also involved in the development of asthma (45). Lung function of children of smoking mothers who took vitamin C during pregnancy was better than that of children whose mothers took a placebo. The intake of vitamin C had also reduced the incidence of wheezing at the age of one year (64).

Cotinine as well as polycyclic aromatic hydrocarbons were detected in lungs and blood of the foetuses exposed to smoking in pregnancy, suggesting that these compounds exert an effect early in lung development (45).

The differentially methylated regions at CpG sites caused by cigarette smoke and particulate matter and their effects on gene expression suggest that these exposures are associated with the development of asthma. In addition, epigenetic changes in maternal mitochondrial DNA and its inheritance may play an important role in the development of asthma (45).

Two studies identified increased methylation of the *AXL* gene by maternal smoking in pregnancy, i.e. the CHS (Children's Health) Study (65) and the NEST (Newborn Epigenetic) Study (66). The Children's Health Study included 799 participants and the Newborn Epigenetic Study 592. *AXL* encodes a receptor tyrosine kinase which is important in the development of inflammation and the regulation of Toll-like receptors. AXL is post-transcriptionally regulated by the microRNA miR-199a1. The expression of miR-199a1 is lower in offspring exposed to smoke during pregnancy. These two studies showed similar methylation levels of the *AXL* gene and were able to demonstrate this independently of the sex and ethnicity of the participants (43).

In summary, the epigenetic effects of smoking during pregnancy were shown to be associated with a higher risk of bronchitis in children (67).

Fine dust, like cigarette smoke, affects the DNA methylation of various genes involved in the presentation of antigens, these include HLA- DOB (HLA class II histocompatibility antigen- DO beta chain) and cytokines. In umbilical cord blood of newborns exposed to particulate matter, an increased number of B and natural killer cells and a reduced number of T lymphocytes were detected (43).

An agricultural environment has an protective effect against the development of asthma or atopic diseases. Contact with animals and stables results in an increased expression of genes of the innate immune system, such as *TLR2*, *TLR4*, *CD14* and protection against the development of atopic diseases (43).

Allergies

The first step in the development of an allergic disease is sensitization to the affected allergen through an initial contact. In the following course of an allergic reaction, the immune system becomes activated and an immune reaction against foreign substances occurs. The allergens can be aeroallergens such as air pollutants as well as cigarette smoke, which can cause respiratory or skin damage. Cigarette smoke leads to increased oxidative stress, increased expression of Toll- like receptors and cytokines such as interleukins (IL 1, 25, 33). Furthermore, dendritic and lymphoid cells become activated. In a study of the LINA cohort (Lifestyle and environmental factors and their influence on Newborns Allergy risk) with 622 mother child couples, a reduced number of regulatory T-cells in the umbilical cord blood, as well as an increased expression of miRNA-223 was shown (59). This miRNA plays an important role in haematopoiesis; in the differentiation of the myeloid (68,69), as well as in the differentiation of the granulocytic cell line (70). It is also involved in the in suppressing erythrocytic differentiation (71). Diverse regulation of this miRNA is connected to the development of a variety of diseases such as carcinomas (72,73), rheumatoid arthritis (74) or type 2 diabetes mellitus (75). Furthermore, an enhanced risk of a food allergy developing in the first year of life and atopic dermatitis later in life was observed. These increased susceptibility appeared to persist during the first three years of life (59).

A history of atopy of the parents was also associated with a reduced number of regulatory T-cells in umbilical cord blood. Offspring with reduced regulatory T-cells had an increased risk of developing food allergy and atopic dermatitis within their first 12 months of life (76).

A meta-study combining results of a in total 15,662 participants revealed a 12 % increased risk for the presence of IgE antibodies as well as for a positive skin prick tests in children under 7 years of age who were exposed to smoke. Unfortunately, cotinine was rarely used as a marker in studies and also provided inconsistent results (59).

Atopic dermatitis

An analysis of 58 studies on passive smoking and 33 studies on active smoking revealed an association linking the development of atopic dermatitis and smoke exposure in the general population. In 19 of these studies, maternal smoking during pregnancy was investigated. However, no association was found between maternal smoking and the development of atopic dermatitis in the offspring (77). Nevertheless, a combination of prenatal exposure to particulate matter and perinatal exposure to cigarette smoke showed an increased risk for the development of atopic dermatitis. (59).

In a Japanese study with 1,177 mother-child pairs, the effect of nicotine exposure at different time points in pregnancy on the development of atopic eczema/dermatitis syndrome (AEDS) was investigated. A significantly increased cumulative incidence of AEDS was observed in offspring when exposed in the 3rd trimester of pregnancy. The authors suspected an epigenetic process in the regulation of the immune system (78).

Allergic rhinitis

Of a total of 97 studies, 34 on active smoking and 63 on passive smoking, 11 studies investigated maternal smoking during pregnancy. Both active and passive smoking have been linked to the development of allergic rhinitis. However, no evidence of an association with allergic rhinitis in the offspring could be found for smoking during pregnancy (77).

Food allergies

In a research study, the results of six studies investigating the effect of passive smoking and one study investigating the effect of active smoking on food allergies, no correlation was observed between the development of food allergies and prenatal smoke exposure (77).

Inflammatory bowel disease

In a study with a total of 2,821 participants aged between 16 and 48 years, differentially methylated sites were found in 69 CpGs as a result of maternal exposure to smoke in pregnancy. Among these CpGs, three were associated with the development of inflammatory bowel disease: cg15578140 in microRNA 548f-3, cg09935388 in Growth Factor Independent Protein 1 (*GFI1*) and cg04598670 in an yet unknown gene. The methylation levels were correlated with the amount of cigarettes smoked and the methylation changes persisted even over several decades (79).

Neurological effects

Infant Neurobehavior

A study investigating 45 mother-child couples revealed increased cortisol levels in the umbilical cord blood of newborns who were exposed to maternal smoking during pregnancy. Moreover, offspring were at higher risk to develop addiction to nicotine, probably due to an altered programming of glucocorticoid signalling pathways. Within the promoter of the placental *NR3C1* gene, encoding the glucocorticoid receptor, methylation changes were associated with neurobehavior: Higher levels of methylation of CpGs 3, 4 and 7 were associated with a positive influence on neurobehaviour in the offspring, which showed reduced lethargy and increased attention. Decreased methylation of these CpGs, as well as increased cortisol levels in the umbilical cord blood, were observed in newborns of smoking mothers. Postnatal behavioral changes were observed in newborn babies who had been exposed to smoke during pregnancy (80). In other studies, even postnatal similarities with a withdrawal syndrome was observed (81–83).

Nervous system

In a meta-analysis of 62 studies, Rumrich et al. summarized the negative effects of smoking on the nervous system. Combined data revealed that maternal smoking is associated with an increased risk for certain types of tumors in the nervous system. For instance, they discovered a 30 per cent higher risk for developing a neuroblastoma (84).

Brain

Chatterton et al. investigated the effects of smoking during the second trimester of pregnancy in relation to DNA methylation in the dorsolateral prefrontal cortex (DLPFC) in 14 exposed and 10 nonexposed foetuses. Samples were obtained from second-trimester elective abortions for non-medical reasons. The most prominent regions were hypomethylated and found in CpG islands of the *GNA15* and *SDHAP3* genes (85). In fact, previous studies reported differential methylation of these two genes in patients with autism spectrum disorder (ASD) and schizophrenia (86,87).

ADHD

In a study with 1,079 patients and the same number of control participants, Sourander et al. identified a significant, dose dependent correlation between the cotinine levels of smoking mothers during pregnancy and the development of ADHD in their offspring. Authors assumed that this connection is due to epigenetic changes in DNA methylation and miRNAs (88).

A study by Miyake and Miyashita et al. containing 1,150 mother-child couples selected from the Hokkaido study, were able to show a significant correlation between maternal smoking during pregnancy and the development of ADHD in childhood. They showed that methylation in the *GFI1* gene was responsible for almost 50 percent of the association. *GFI1* was hypomethylated and, in addition to the observation of ADHD symptoms, also led to a lower birth weight. The *GFI1* gene is also part of the regulation of the immune response (T helper types 1 and 2) as well as oncogenesis and haematopoiesis. Interestingly, an association between ADHD and the development of

atopic disease patterns could also be demonstrated. These effects were only present when women were active smokers, the correlation did not exist in women with passive smoke exposure. (89).

Autism spectrum disorder

Caramaschi et al. investigated the connection between maternal smoking during pregnancy and the development of autism. Analyzing data from 11,946 participants in the Avon Longitudinal Study of Parents and Children, they discovered no association between maternal smoking and the development of autism (90).

Persistence of maternal smoking-related methylation changes until the age of 17 years was observed within the *CNTNAP2* gene. This gene is the largest gene in the human genome, encodes a protein of the neurexin family and plays an important role in cell adhesion and signal transduction in the nervous system. Alterations in this gene are associated with an increased risk of developing ASD (91).

Schizophrenia

In a meta-analysis involving data from 1,366 adolescents and 1,455 adults aged 16 - 48 years, 69 CpGs were found to be differentially methylated in 36 gene loci as a result of maternal smoking exposure during pregnancy. Data were taken from genome-wide studies. The methylation changes of the CpG site cg25189904 in the *GNG12* gene (Guanine Nucleotide Binding Protein Gamma 12) have been linked to the development of schizophrenia. These methylation changes even appeared to persist over several decades, i.e. were present in adolescents as well as in the adult group, and also had a dose-response correlation with the amount of cigarettes smoked (79).

Ageing

The epigenetic clock is a biomarker and was used in this study to determine the epigenetic age of cells. Here, methylations at 353 CpG sites are used for evaluation. Observations in a total of 613 data sets from cord blood revealed that cigarette smoke

exposure in utero was associated with a faster ageing process of the newborn. This correlation could be shown by the shorter length of the telomeres depending on the number of cigarettes smoked. A decrease in the number of naïve T-cells could also be a sign of the faster ageing of the immune system in smoke-exposed foetuses (92).

A study of 207 placental and lung epithelial samples showed that smoking in early pregnancy causes shorter telomere lengths in the developing lungs of exposed foetuses. The samples were obtained from elective abortions. Shortened telomere lengths are generally associated with the ageing of cells or the development of diseases (93).

Oncology

The *RUNX3* gene is a tumor suppressor gene and is involved in the development of the immune system, the differentiation of various immune cells, such as T cells, macrophages, dendritic cells, neuronal cells, as well as in cancer development. In this study with a sample size of 206 subjects, an increased methylation of a CpG site of the RUNX3 gene in the placenta was identified after smoke exposure during pregnancy. Placental hypermethylation of this gene was associated with the development of various cancers such as breast cancer, gastric cancer, lung cancer, prostate cancer and urothelial carcinoma (94). The inactivation of this gene due to hypermethylation can lead to the development of hepatocellular cancer (95). Moreover, reduced expression of *RUNX3* is associated with the development of clear cell renal cell carcinoma (96). Furthermore, four altered CpGs were detected in *APBA2*, *ATP10A* and *PTPRO*, which are also associated with maternal smoking. Hypermethylation of *APBA2* is associated with the development of pancreatic cancer, oral cavity cancer and hepatocellular carcinoma (94). Loss of the ATPase encoded by *ATP10A* has been observed in individuals diagnosed with Angelman syndrome (97). The *PTPRO* gene encodes a protein tyrosine phosphatase, acts as a tumor inhibitor and has an important part in cell development (94).

Lung

A study with twenty pairs of mothers and newborns revealed lower global DNA methylation in the whole blood of neonates exposed to cigarette smoke. in. Differential methylation was found at 31 CpG sites associated with 25 genes. Although smoking is generally associated with genome hypomethylation, these 31 CpG regions were mainly hypermethylated. The most remarkable hypermethylated CpG site was identified in the *ADM* gene. The *ADM* gene has diverse functions including cell growth, hormone secretion, natriuresis as well as vasodilation, and is associated with various chronic diseases such as atherosclerosis, diabetes mellitus, coronary heart disease and obesity. The pathogenesis of these diseases, as well as the expression of *ADM*, is linked to exposure to cigarette smoke in utero. Higher expression of the *ADM* and *AHRR* genes were found in lung tumour patients, with the *AHRR* gene being responsible for the expression of the *ADM* gene. This suggests that there is a link between *ADM* and the development of cancer (98).

Based on their own findings (99) and a study by Richmond et al., (100), Ryan and Robles assume, that methylation changes in the *DRD1* gene (Dopamine receptor D1) caused by in-utero smoke exposure are associated with the development of lung cancer. Methylation was examined in the peripheral blood of the descendants (100). Furthermore, germline mutations of this gene could influence the risk of the development of lung cancer (99). Dugué et al. were also able to observe a connection between maternal smoking and later development of lung cancer in their study by examining a variety of methylation scores on 28 CpGs in a study population of 327 participants (26).

Urothelial carcinoma

Analyzing differentially methylated regions using self-generated scores in a cohort of 404 cancer patients, the study by Dugué et al. revealed a positive association between prenatal smoke exposure and the development of urothelial carcinoma in adulthood (26).

Lymphomas

Smoking in pregnancy increases the risk for lymphomas. For instance, Rumrich et al. identified an increased risk for developing non-Hodgkin's lymphoma in childhood if the mothers smoked during pregnancy They discovered this through a literature search and analyzed 6 papers for this particular data (84). Moreover, a weak correlation between maternal smoking exposure and the development of certain subtypes of B-cell lymphomas (high-grade Non- Hodgking- lymphomas) was identified in a study with a sample size of 426 cancer patients using 15 CpGs (26).

Leukaemia

Whilst a study by Rumrich mentioned above showed no significant relation between smoking during pregnancy and the development of AML (acute myeloid leukaemia) or ALL (acute lymphatic leukaemia), such correlation was demonstrated by de Smith et al. in a cohort of 599 participants (84). In fact, this study identified a specific CpG site in the *AHRR* gene that was less methylated when exposed to smoke in utero. This differential methylation was associated with a greater number of gene deletions in B-cell ALL. Moreover, there was a dose dependence as well as a pre- and postnatal association between smoke exposure and deletions. Interestingly, this association was stronger in male offspring. The effect of paternal smoking was also investigated, and authors revealed that DNA damage occurred already in the sperms, which was associated with a higher risk of leukaemia in the offspring (101).

Placenta

The placenta plays a vital role for the developing child in the uterus. It is responsible for the supply of nutrients, serves as protection and helps to filter various substances, including toxins. Furthermore, it is important for the production of hormones and therefore for the maintenance of the pregnancy. However, these central functions can be affected by epigenetic changes due to environmental influences. This can have negative consequences for the development of the foetus (5).

Smoking during pregnancy affects genome-wide and site-specific DNA methylation and gene expression in the placenta. Mutagens in cigarette smoke are involved in the formation of DNA adducts, which reach the foetus via the placenta or are deposited in the placenta. These adducts are metabolic products of polycyclic aromatic hydrocarbons. Consequently, the expression of enzymes that are able to break down the toxic metabolites is altered by maternal smoking during pregnancy. Analyzing genome wide and site-specific DNA methylation in placentas of 18 smoking and 18 non-smoking women, Suter et al. did not observe changes in genome-wide DNA methylation. However, there was a change in CpG-specific methylation: 6 CpG sites with altered methylations were associated with reduced birth weight and maternal smoking (102).

A Canadian study mentioned above, which was carried out on 441 mother-child pairs between 2010 and 2014, showed effects of smoking during pregnancy on the development and growth of the newborn and on the methylation of certain genes in the placenta (48). Interestingly, the study demonstrated that these DNA methylation changes caused by maternal smoking could be reversed after vitamin C intake. Smokers, and consequently their foetuses, have reduced levels of vitamin C in their blood. In a randomised, double-blind study, vitamin C was substituted in a dose of 500mg daily in pregnant women who smoked. Vitamin C supplementation showed a decrease or reversal of the methylation changes caused by smoking. Overall, it was found that the offspring's pulmonary function had improved and the wheezing episodes by the age of one year were reduced. Samples from a total of 41 subjects were used, with samples coming from the placenta (27 samples), cord blood (27 samples) or buccal mucosa swabs (22 samples) (103).

Besides methylation changes, also miRNAs war altered in the placenta of 25 smoking mothers. Specifically, miR-16, miR-21, miR-146a were reduced, and miR-182 was increased (104). These miRNAs are postulated to be responsible for growth and development of the placenta. Interestingly, lower expression of miR-146a was also found as an effect of nicotine and benzoapyrene exposure in TCL-1 cells (51).

In pregnant women who smoke, a 21.6% lower content of mitochondrial DNA (mtDNA) was found in the placental tissue, as well as higher methylation at the *MT-RNR1* gene of the mtDNA. In smokers, the promoter region of the *CYP1A1* gene was also less methylated in the placental tissue. Furthermore, it could be shown that smoke

exposure during pregnancy changed the gene expression of 623 genes and the methylation of 1,024 CpGs in the placentas. For 438 of these genes, a connection between their expression and methylation could be established. The affected genes included the function of the mitochondria (105).

Similar results could be discovered with exposure to particulate matter in late pregnancy. An increase in particulate matter exposure of 10 µg/m^3 showed a decrease of 16 % in mtDNA content of the placenta. Similarly, an increase in distance from busy roads showed a 4 % increase in mtDNA content. The number of tissue samples from placentas was 174 (106).

In another study involving 568 pregnant women, 203 differentially methylated regions (DMRs) were found in placentas affected by active smoking during pregnancy. In this study, 152 out of the 203 DMRs were found in the placenta of active smokers during pregnancy, which were not present in former smokers before pregnancy or in non-smokers. These methylations were therefore regarded as reversible. In contrast, 26 differentially methylated regions were found that were already altered in the placental tissue during smoking exposure prior to pregnancy and remained so during pregnancy (107). In this total of 203 DMRs, 1,023 CpGs were identified, 9 of which were also associated with methylation changes due to smoke exposure during pregnancy in another study by Morales (108). These were found in those genes: *TINAGL1, TRIO*, *TGM1, CMIP*/*PLCG2*, *ACOX3*, *PDGFB*/*RPL3 and PDXK*. With the exception of the *PDXK* gene, the methylations triggered by smoke exposure even seemed to be reversible (107).

In women who smoked during pregnancy, hypomethylation of the gene *TINAGL1* (tubulointerstitial nephritis antigen like 1) and higher methylation in *PDGFB* (platelet-derived growth factor subunit B) were identified. These genes play act as pro-angiogenic factors, suggesting a possible link between smoke exposure and vascularisation of the placenta. Nicotine is also known to have a pro-angiogenic effect. Also, the *TINAGL1* gene was found to be less active in pregnant women with pre-eclampsia than in pregnant women without pre-eclampsia symptoms. However, a concordance of epigenetic marks in blood cell samples from the umbilical cord and placenta could not be demonstrated. At the DMRs associated with smoking exposure, changes were found in the post-translational modification marks (PTM) of histone H3

leading to increased translation (H3K4me1 and H3K27ac). It is known that methylation of the *LINE-1* and *AluYb8* regions is less pronounced in the placenta. The study of Rousseaux et al. revealed that smoke exposure decreases methylation of the *LINE-1* gene, but has no effect on the *AluYb8* gene (107). However, other studies could only provide contradictory results on the methylation of these genes in connection with smoking and the associated changes in DNA methylation also had effects on specific imprinting control regions (ICRs) (109,110). For example, incomplete removal of germline DNA methylation and specific methylations of certain alleles altered the expression of imprinted genes in the placenta. Here, three of the previously discussed DMRs were in close proximity to three of these ICRs, i.e. *NNAT*/*BLCAP* on chromosome 20, *SGCE*/*PEG10*, on chromosome 7 and *H19*/miRNA 675 on chromosome 11. Increased expression of the first locus was associated with a deviation in the gestational age of the foetus. Altered methylation (in cord blood as well as lung epithelium) of *SGCE*/*PEG10* was associated with birth weight changes and. The changes at these two gene loci were considered reversible. The third gene locus *H19*/miRNA 675 was irreversible. Again, associations were found between the degree of methylation in the placenta or cord blood and gestational age/birth weight (107).

Cardiometabolic risk

A study by S. Rauschert et al. using 995 participants, demonstrates a significant, dose-dependent relation between smoking during pregnancy and the DNA methylation of the infants. Authors identified 23 CpGs that were hypermethylated and linked to the genes *AHRR, FTO, CYP1A1, MYO1G, CNTNAP2, FRMD4A*. These genes play important roles in the development of cancer, obesity, detoxification, cell signalling, various developmental processes and nicotine dependence. Moreover, there were associations between the differentially methylated regions and cardiovascular risk, as represented by BMI (body mass index), blood pressure and blood lipids, such as triacylglycerols and HDL (high density lipoprotein) cholesterol (111).

Frayling et al. identified a correlation between smoking during pregnancy and hypermethylation of the *FTO* gene on chromosome 16 and the developing of obesity and diabetes, as well as the birth weight of the neonates. The differential methylation of *FTO* and *CYP1A1* also correlated with blood lipids and blood pressure (112). A meta-analysis of Parmar et al. (113) revealed an association between waist

circumference, blood lipid levels as well as blood pressure and methylation levels in the CpGs within the *GFI1* gene. These findings were obtained from 5 studies (Avon Longitudinal Study of Parents and Children, Northern Finland Birth cohort 1966 - 31 years, Northern Finland Birth cohort 1966 - 46 years, Northern Finland Birth cohort 1986, Western Australian Pregnancy Cohort - RAINE) with a total of 4,230 participants aged 16 - 81 years using samples from whole blood. Three CpGs (cg09935388, cg18316974, cg18146737) within the *GFI1* gene were found to have reduced methylation associated with maternal smoking exposure during pregnancy (113). These methylation patterns showed consistency and persistence into adolescence and thereby influence the long-term health of the individuals (111). Other studies (114,115) showed an association between altered CpGs of the *AHRR* gene (cg05575921, cg21161138), the *F2RL3* gene (cg03636183) and CpGs cg21566642, cg01940273 and cg06126421 and an increased overall and cardiovascular mortality. For this research, DNA samples from the blood of 1,000 adult study participants were analysed. (116).

Obesity

A causal relationship between smoking during pregnancy and the development of obesity later in life of the offspring, was described in a review paper by Fernandez-Twinn (117). Albers et al. summarised data from 26 studies with a total of 238,340 mother-child couples and discovered a linear relationship between obesity and the number (1- 15) of cigarettes smoked per day (118).

McConnell et al. identified a relation between maternal smoking and an increased BMI (Body Mass Index) in childhood. Authors were also able to show such relation between BMI and air pollution in the vicinity of roads. Among other things, they attributed this to an epigenetically altered expression of the *PPARγ* gene (peroxisome proliferator activated receptor), which has an important part in the metabolism of fats (119).

An association between maternal smoking during pregnancy and the development of obesity was also demonstrated by the methylation of various other genes, including *MSI2* (Musashi RNA-Binding Protein 2). The methylation of this gene showed a positive association with the serum level of leptin, as well as a correlation with BMI,

even after two generations. This was demonstrated in 1,192 participants aged 17 years in the Raine trial (120).

A study of 956 adolescents analyzed DNA methylation in peripheral blood and demonstrated an association between prenatal exposure to maternal smoking during pregnancy, and fat and carbohydrate intake depending on the expression of a specific allele of the *OPRM1* (opioid receptor mu-1) gene. *OPRM1* encodes a receptor expressed in the brain's reward centre and was linked to the development of obesity. A variant of the *OPRM1* gene with a specific allele (T- allele rs2281617) is protective against weight gain, as this variant is known to be related to a lower fat intake and thus a lower body weight. This protective T- allele appeared to be muted in adolescents exposed to smoke. Thus, exposed adolescents were more likely to consume more fat and less carbohydrates than the unexposed participants. In addition, smoking during pregnancy was linked to lower DNA methylation over a number of CpGs in *OPRM1* in individuals exposed to it (121).

Transgenerational Inheritance

Transgenerational inheritance of epigenetically acquired changes has already been demonstrated in several animal and human studies. In fact, DNA methylation and its heritability are already well studied. However, the heritability of other epigenetic changes such as genomic imprinting, histone modifications, and allelic silencing is not yet well understood (43,91).

In this context, grandmother's smoking has been studied. An increased risk for the development of third-generation asthma or allergies was discovered with grandmotherly smoking during her pregnancy with the mother. The risk of the offspring to develop asthma was independent on whether the mother had asthma or not (122).

The prevalence of grandchildren developing myopia was studied using data from the Avon Longitudinal Study of Parents and Children (ALSPAC) with a sample size of 13,988 children. The risk for developing myopia was reduced when the paternal grandmother smoked during pregnancy This effect seemed to be stronger in male offspring. If the maternal grandmother smoked when she was pregnant, the prevalence

of grandchildren developing myopia was lower. The samples were taken from peripheral blood or from the umbilical cord and were examined at three points in time: at birth, in the age of seven and at the age of 15. No changes were found in the period from 7-15 years. However, in the period of 1-7 years, a methylation change of the CpG cg13403566 near the *RASGRF1* gene was observed. This gene has a key role in the process of transmitting neuronal signals in the brain. It seems to indicate that the expression of this gene in the placenta is expressed from the paternal side (123).

Moreover, grandmotherly smoking in the family from the mother's side is linked to the development of behaviors in the grandchildren, which were later associated with the diagnosis of autism (91).

Influence of paternal smoking

Although this thesis focuses on the intrauterine effects of maternal smoking and subsequent epigenetic changes, a brief insight into the paternal influence will be provided. For instance, an increased risk for the development of asthma in the newborn was found with paternal smoking during puberty. The body fat percentage was equally increased in male adolescents whose fathers smoked as teenagers. During adolescence, important maturation processes take place in the germ line and it is particularly susceptible to mutations at this time (122). Altered mRNA profiles were found in sperm of smoking men compared to sperm from non-smokers. Specifically, 781 genes were differentially expressed under the influence of smoking exposure. Furthermore, cell apoptosis appeared to be inhibited in smokers (124). This could possibly be inherited via the germ line or transferred to the egg cell during fertilisation and could have an influence on the expression of various genes in the offspring (122).

In a study analysing 195 female and male participants aged 11 to 54 years, six significantly differentially methylated regions were identified that were associated with the father's exposure to smoking. These regions were related to neuronal system development, to innate and acquired immune system, and to fatty acid synthesis. Moreover, authors assumed that these methylation changes were associated with behavioral disorders and addictive behavior (125).

Mitochondrial DNA

Mitochondrial DNA is probably epigenetically modified by environmental factors in the same way as DNA in the cell nucleus. It is actually even more damage susceptible than the nuclear DNA. However, it is assumed that the mechanisms of epigenetic changes in mtDNA may differ from the mechanisms of DNA methylation in the cell nucleus. As an example, increased DNA methylation of mitochondrial transcription factor A (mtTFA) due to cigarette smoke exposure led to its inactivation and subsequently to a disruption of the mitochondrial respiratory chain. This can cause an increase in oxygen free radicals, which can, in turn, cause damage to nuclear DNA and can lead to an increased production of mtDNA, which may represent a compensatory effect for the lower production of energy (126).

Prevention

Smoking or the ingredients of tobacco cause oxidative stress in various tissues. Studies in humans and animals have shown that the administration of vitamin C as antioxidant can minimize potential negative consequences for the unborn child.

Vitamin C supplementation has been shown to improve hormone levels of serotonin and norepinephrine, as well as lung function, which are negatively affected by smoking during pregnancy. However, an imbalance in normal development has also been observed with antioxidant supplementation, so this should be considered very carefully (24,127).

In general, there is a need for greater awareness of the harmful effects of smoking during pregnancy and for more public education. In addition, a ban on public smoking could lead to a decrease in the overall prevalence of smoking and thus lead to a lower prevalence of smoking in pregnancy (24).

Discussion

General

Smoking in general and smoking in pregnancy in particular are, although a well-known problem worldwide, widespread in our society. Despite the fact that the overall prevalence has declined in recent decades, the prevalence of smoking in pregnancy remained almost unchanged. This has far-reaching consequences for the developing offspring as consequences manifest in various clinical conditions and can even have long-term effects.

Environmental influences and the period of their exposure can affect the development of non-communicable diseases later in life of the individual. This is being described on the basis of the DOHaD concept (Developmental Origins of Health and Disease). The DOHaD concept portrays the effects of various in utero environmental influences, as well as endocrine, metabolic and other stress-related factors that affect the offspring in the long term. This occurs through epigenetic changes in gene expression and results in increased risks for the development of chronic diseases later in life. The diseases we are talking about here are non-communicable diseases. These include some cancers, allergies, diabetes, affective disorders and diseases of the cardiovascular system and lungs. These are among the most common causes of death. Non-communicable diseases are preventable and their development can be prevented through proper measures and prevention. This aspect is significant for health policy and offers great potential for a high improvement (1).

Pregnancy is one of the most vulnerable phases of developmental imprinting of an individual and can thus have a great and long-lasting effects. Thus, smoking in pregnancy can cause changes in the methylation of the DNA, which have been shown to be inheritable and potentially responsible for the development of various diseases.

The epigenetic changes that take place are DNA methylation, histone modification and non-coding RNAs. These can be caused by various environmental influences (toxic substances) and can be initiated by the mother (caloric excess, lack of proteins or nutrients), the father or by the placenta. The resulting new changes can have effects on the functions of genes, as well as on the function and development of cells (2).

Effects

The effects of smoking during pregnancy are diverse and versatile. Maternal smoking in general seems to be responsible for an accelerated process of ageing in newborns (52). Smoking also causes growth restriction and reduction in birth weight, which results from reduced blood supply, hypoxia and nutrient deficiency, and can lead to neurological, cardiovascular and metabolic diseases later in life. Reduced lung function and an association with the development of SIDS have been shown in association with maternal smoking. Smoking during pregnancy also increases the risk of preterm birth and premature rupture of the bladder (33,34). The development of the immune system is equally affected by maternal smoking as it matures slowly. Moreover, offspring of smoking mothers and are more likely to develop respiratory diseases such as asthma and whooping cough (22,40,41). An increase in atopic diseases such as allergies, food allergies and atopic dermatitis was observed in the newborns as well (40,43).

Postnatal behavioral changes were identified in the newborns, as well as a tendency to develop neurological disorders such as schizophrenia, ADHD and autism later in life (45,48). Maternal smoking also has a significant influence on both carcinogenesis and the regulation of gene expression of various tumour suppressor genes in the unborn child. Thus, a higher incidence of tumours of the lung and urothelium as well as a higher probability of developing leukaemia and neuroblastoma were observed (46,53,54,56,57).

The placenta and umbilical cord blood are easy accessible fetal tissues. Thus, in the placenta as well as in cells present in umbilical cord blood, several differentially methylated regions were identified and served as important epigenetic markers for cigarette smoke-associated changes.

Most exciting, however, is the transgenerational inheritance of epigenetic marks, which has been demonstrated in a number of studies, but the specific mechanisms are not yet fully understood. Genetic imprinting, histone modification and gene silencing play an important role in this context. Thus, the effects of the grandparent generation on the offspring could be shown. This provides an exciting outlook and anticipation for future research in this area (38,51).

Purpose and limitations

The aim of this review was to examine the current literature on PubMed and to gather important information from the studies, reviews and papers. Of course, various limitations were encountered in the writing of this paper. The literature search identified several studies using animal models, and often genetic changes instead of epigenetic changes. These were then sorted out during the rescreening process.

Studies also had limitations in terms of different sample sizes and timing of the sampling. For example, sample sizes that were too small can produce biased results, and it was often difficult to find out at which time in pregnancy or in postnatal life the screening took place. Regarding the smoking history, many studies relate on the honesty of the participants, since often, smoking status was not verified by cotinine analysis of maternal blood. Various other influencing factors before or at the beginning of the pregnancy were also difficult to record or adjust for, such as the exposure to passive smoking.

Smoking is a severe health problem and it seems that it will remain so also in future generations. To reduce its prevalence, prevention must start early and education must be provided to all levels of society. In addition, for a healthy lifestyle, sufficient physical activity as well as healthy nutrition is of great importance to successfully achieve disease prevention. However, it will take some time before the DOHaD concepts are implemented and their effects can be felt.

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