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Review

Epigenetic Issues. The Link Between Microbiota and Gestational Diabetes.

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Abstract: Gestational diabetes mellitus (GDM) is considered a significant and increasing problem worldwide. The growing body of evidence points out that a hostile intrauterine environment in mothers with GDM via epigenetic mechanisms induces "diabetogenic" and "obesogenic" changes in an offspring's DNA. This sets in motion a vicious intergenerational cycle of metabolic diseases gradually deteriorating the health of the human population. One of the most important players in this process seems to be altered microbiota/microbiome.

There is a chance that the identification of specific epigenetic marks may provide a key for future diagnostic, prognostic and therapeutic solutions/measures in the field of personalized medicine. Given the reversibility of most epigenetic changes, an opportunity arises to improve the long-term health of the human population/race.

In this manuscript, we aim to summarize available data on epigenetic changes among women suffering from GDM and their progeny in association with changes in microbiome.

Keywords: gestational diabetes mellitus; epigenetics; microbiota

1. Introduction

Diabetes mellitus is a global metabolic disease. A significant increase in its incidence rate has been observed for several years. According to data provided by WHO, the total number of people with diabetes has quadrupled in the past 40 years. It is estimated that 425 million people worldwide have diabetes, however, the number of undetected cases remains unknown. In 2025, it is projected that 570 million people will be diabetic, causing 1.6 million deaths [1]. A continuously increasing prevalence of gestational diabetes is also observed [2]. Gestational diabetes mellitus is one of the most common complications of pregnancy. According to the American Diabetes Association (ADA), GDM is defined as diabetes diagnosed during the second or third trimester of pregnancy, provided that overt diabetes was excluded before or at the latest in early pregnancy [3]. GDM is diagnosed in approximately 14-15% of pregnancies [4, 5].

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GDM usually resolves after delivery, but unfortunately, it may induce long-lasting metabolic and cardiovascular complications, such as increased risk for type II diabetes mellitus (T2DM) and cardiovascular disease in the mother, enhanced adiposity or even obesity, impaired glucose metabolism, hypertension, hyperlipidemia and non-alcoholic fatty liver disease in the offspring, as well as pre-term puberty in girls [6].

So far, many risk factors of gestational diabetes have been identified and widely described. The most important of them is overweightness or obesity in pregnant women. Gestational diabetes is more than 3 times more frequent in this group of patients [7, 8]. Increased weight gain in pregnancy during the first or early second trimester also predisposes to the development of GDM in the second half of pregnancy [9]. A family history of type 2 or gestational diabetes is also considered an important predisposing factor [10, 11]. The risk of gestational diabetes has been shown to increase linearly with the age of the pregnant woman [12], as well as being increased by a history of GDM [13] and/or fetal macrosomia in a prior pregnancy [14]. Previously diagnosed insulin resistance (PCOS) predisposes to gestational diabetes in 55% of cases [15].

Although numerous risk factors have been identified and screening in high-risk groups has been introduced, the upward trend in the prevalence of gestational diabetes and its complications still remain. Thus, there is an urgent need to identify sensitive and specific biomarkers for the early detection of GDM in all pregnant women. Molecular studies have been conducted for many years, but to date, none of those proposed have been implemented into clinical practice. The introduction of the whole human genome sequencing (WGS) method has led to increased interest in research on genomics and epigenetics.

Epigenetics is the evaluation of changes in gene expression that are not the result of changes in the nucleotide sequence. Epigenetic modifications occur/appear due to external as well as internal environmental factors, such as microbiota. The human gut microbiota consists of approximately 1,000 trillion microbes: bacteria, viruses, archaea and eukariota microbes [16]. Interestingly, the microbiome composition seems to have significant impact on metabolic processes and thus, on human health [17]. Pathologically altered gut microbiota may contribute to the onset of GDM in the mother, and consequently, an increased risk of epigenetic “diabetogenic” and “obesogenic” changes in her own DNA and in that of the offspring. Exposure of the maternal-fetal unit to the influence of dysbiotic microbiota may result in epigenetic changes, which may manifest themselves immediately or after many years, and/or be passed onto future generations. In our review, we attempted to emphasize that gut microbiota dysbiosis contributes to metabolic complications not only in affected mothers, but also in their offspring. In this way, pathologic changes can be transferred to subsequent generations, increasing the frequency of obesity, diabetes and inflammatory diseases, concerning even younger

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people. Given that most epigenetic changes are reversible, identified epigenetic marks may become important diagnostic, prognostic and therapeutic targets.

2. Pathogenesis and pathophysiology of gestational diabetes

During pregnancy, the mother's organism undergoes a number of physiological changes caused by adaptation to the needs of the developing fetus. The purpose of the metabolic changes occurring at the time of pregnancy is to preferentially provide the growing fetus with an uninterrupted supply of energy and building materials from the mother's body. The pathophysiology of GDM is not fully understood, with the prevailing hypothesis linking abnormal hormone expression from the placenta to maternal metabolic dysfunction and impaired insulin function. Normoglycemia is maintained by a balance between insulin production and tissue insulin sensitivity. Glucose enters the fetal blood stream from the mother's blood according to a concentration gradient, with the participation of the type 1 transporter (GLUT-1). The concentration of fetal glucose is closely correlated with its concentration in the maternal blood. Increased transplacental glucose transfer to the fetal circulation results in overstimulation of the fetal pancreas. Insulin does not pass through the placenta. The fetus pancreas begins to produce insulin around week 9 weeks. Fetal hyperinsulinemia, due to maternal hyperglycemia, drives metabolic disturbances in fetus.

During pregnancy, the sensitivity of tissues to insulin changes. In the early stage of pregnancy, insulin sensitivity increases, but from the 14th week of pregnancy, gradually increasing insulin resistance is observed, reaching up to its 2-fold increase by late pregnancy [18].

During the later stages of pregnancy, the concentrations of local and placental hormones increases, which determine the advancement of the insulin resistance phenomenon. Hormones that affect insulin sensitivity include: placental lactogen, placental growth hormone, estrogens, progesterone, prolactin, adiponectin and leptin. [19-21] This phenomenon promotes endogenous glucose synthesis and the breakdown of fat storage, which intensifies hyperglycemia and increases the level of free fatty acids [22, 23].

Factors promoting insulin resistance also include chronic sub-clinical inflammation, while physiological pregnancy is also considered an inflammatory state. Due to the complex metabolic and biochemical phenomena at the cellular level, genes responsible for the synthesis of pro-inflammatory cytokines, *inter alia*: IL-1b, IL-6 and TNF-alpha are expressed [24-26].

In the course of GDM, however, there is an increased concentration of pro-inflammatory markers, such as: CRP, TNF-alpha, IL-6. In the course of GDM, an increased concentration is observed of pro-inflammatory markers, such as: CRP, TNF-α, IL-. The concentration of TNF-α, which is mainly produced by the placenta, reaches its maximal

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value in the third trimester of pregnancy. By increasing the serine phosphorylation of IRS-1, TNF-α impairs the binding of insulin to the receptor [21].

Increased concentrations of prodiabetogenic hormones and pro-inflammatory changes at a cellular level contribute to an overall increase in insulin resistance, which is aimed at (as mentioned above) ensuring an adequate supply of glucose to the fetus. Normoglycemia is maintained by a compensatory increase in maternal insulin production.

Increased production and secretion of insulin is possible due to the hypertrophy and proliferation of pancreatic beta β-cells [27]. This phenomenon allows glucose-stimulated insulin production to be increased. Approximately 40% of the absolute increase in pancreatic β-cell islets during pregnancy has been demonstrated [28]. The impaired adaptation of pancreatic β-cells to altered metabolic conditions appears to be a significant contributor to the development of gestational diabetes [29]. Reduced *β*-cell mass, reduced *β*-cell number, *β*-cell dysfunction (or a mix of all 3), together with tissue insulin resistance, are critical, but not the only causes of GDM development.

Increased body fat, chronic low-grade inflammation and oxidative stress have also been identified as contributing factors [30-32]. More recently, environmental factors, and among them altered microbiota, have been shown to affect metabolic processes in GDM. The gut microbiota may be a potential marker of impaired glucose metabolism during pregnancy and a promising target allowing to improve health outcomes in women with GDM and their offspring [33-38].

3. The role of microbiota in GDM development

Microbiota mean all microorganisms, i.e. bacteria, viruses, fungi and/or archaea that inhabit the human body. The microbiome is found in specific places such as the mouth, skin, digestive system, upper respiratory tract and the reproductive system. It is estimated that the absolute mass of the microbiome in the human body reaches approximately 2 kilograms [39]. The gut microbiome is the most numerous and active. Microbiota have numerous functions in the body, including participation in the synthesis of vitamin K, biotin, folic acid, and also in the absorption of magnesium, iron and calcium ions. The microbiome induces the synthesis of mucins that protect the epithelium against pathogens, being a source of butyric acid that stimulates the maturation of colonocytes. Moreover, by occupying an ecological niche and stimulating the immune system, commensal bacteria undertake a protective role against the multiplication of potential pathogens [40]. The gut microbiome composition is predominantly shaped by environmental factors. Rothschild, D. et al. have demonstrated significant similarities in the microbiome compositions of genetically unrelated individuals sharing the same household, and over 20% of the observed variability was associated with diferences in diet, drugs and anthropometric measurements [41].

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Firmicutes and bacteroidetes account for 80–90% of the intestinal bacterial microbiome. Proteobacteria, verrucomicrobia, actinobacteria and fusobacteria are also included in the bacteriome. Human gut microbiome composition alters from early life to od age, being shaped by multiple external and internal factors. In order to ensure homeostasis, the microbiota constantly adjust and react vigorously to a variety of external and internal stimuli [42 ,43].

The growing interest in the influence of the microbiome on human health resulted in the commencement of the Human Microbiome Project, carried out by the National Institute of Health since 2008. The project was aimed at detecting differences in the human microbiome, depending on the studied population, their genotype, health condition, age, nutrition, treatment used, living environment and social factors [39]. The methods for studying the human microbiome were based on the analysis of the 16S rRNA sequence and sequencing of the metagenome [44]. Recently, more and more evidence proves the influence of microbiota on specific disease entities, such as: inflammatory bowel diseases [45-47], functional disorders of the digestive tract [48], obesity [49], colorectal cancer [50], allergies [51], autism [52], Parkinson's disease [53] as well as metabolic diseases [54,55, 34]

There is a lot of available evidence concerning the importance of microbiota in infectious [56], immune-dependent [57, 58], pulmonary [59], cardiovascular [60, 61]. In the literature, there is much evidence regarding the influence of microbiota on the development of metabolic syndrome, obesity and diabetes mellitus.

It is believed that metabolites play a key role in the microbiota-host axis [62]. Moreover, the influence of lipopolysaccharide and trimethylamine on the development of cardiovascular diseases has been proven [27, 63]. The influence of short-chain fatty acids on the development of obesity and metabolic syndrome is also known [64, 65].

During pregnancy, changes in the composition and functioning of the microbiome occur. The gut microbiota alters during each trimester of pregnancy. The number of bacteria in the intestinal microbiota increases while its composition changes [66]. To allow normal fetal growth, an incremental shift is observed towards microorganisms involved in energy production and accumulation. The increase in *Akkermansia*, *Bifidobacterium* and firmicutes occur parallel to the rising energy storage, while the rise in proteobacteria and actinobacteria helps protect the feto-maternal unit from external infection [43]. Haro et al. proved that the numbers of actinobacteria and proteobacteria taxomes increases during pregnancy, while the number of *Faecalibacterium* taxomes and other producers of short-chain fatty acids decreases [67]. *Faecalibacterium* is a bacterial taxa responsible for the production of butyric acid, with proven anti-inflammatory properties [68]. As the gestational age increases, firmicutes begin to dominate, similarly to the microbiota of obese people [55].

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From the beginning of research on the intestinal microbiota in pregnant women, it has been known that in the subsequent stages of pregnancy, changes in the microbiota occur.

Koren et al. have analyzed stool probes from 91 pregnant women, previously recruited for a prospective, randomized, mother-infant nutrition study in Finland. They described a significant alteration of the gut microbiota between the first and third trimester. Although they found no differences between either the microbiota of the pregnant women in the first trimester or that compared to their normal healthy controls, the differences were significant in the third trimester. The between-subject diversity has greatly expanded, and enrichment of proteobacteria and actinobacteria has been observed in the majority of pregnant women. In the third trimester, in most women, an increase in proteobacteria level has been previously reported in the case of inflammation-related dysbiosis. *Faecalibacterium*, on the other hand, being an anti-inflammatory butyrate producer, was - on average - less abundant in late pregnancy [69]. They also transferred gut microbiota from the first and third trimester from women to germ-free recipient mice and noticed that the latter induced grater adiposity insulin resistance as well as inflammatory response. Increased concentration of pro-inflammatory cytokines, such as: IL-1β, IL-2, IL-5, IL-6 and GM-CSF, was observed [69].

Mor and Cardenas proved that the increased concentration of circulating pro-inflammatory cytokines has positive impact on the development of insulin resistance [70]. In a study conducted by Vijay-Kumar et al., it was confirmed that physiological dysbiosis occurring in the third trimester of pregnancy has a direct, positive influence on the formation of low- inflammatory processes, hyperglycemia, excessive weight gain and insulin resistance [1]. This suggests the direct influence of microbiota on the initiation of metabolic and immunological changes.

Dysbiosis, inflammation and weight gain increase are known as risk factors for type 2 diabetes. Similar changes seem to be crucial in normal pregnancy, as they promote energy storage and fetal growth. The authors suggest that, on the one hand, the gut microbiota affect host metabolism but, on the other, the host can manage/control the gut microbiota to promote metabolic changes [69].

The presence of dysbiosis in diabetic patients has been demonstrated in numerous publications. [72, 73]. However, proof of the relationship between particular taxonomic classes and GDM is so far lacking [74,73].

However, the proof of the relationship between particular taxonomic classes and GDM is so far lacking [74,73].

In the literature, various potential mechanisms are discussed regarding the influence of intestinal microbiota on the development of glucose intolerance, diabetes mellitus 2 and

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GDM. The phenomenon of higher numbers of Gram-negative bacteria and disturbance of the ratio of Gram-negative/-positive bacteria is widely discussed. Lipopolysaccharides (LPS) constitute a large proportion of the cell wall of Gram-negative bacteria. Sub-acute inflammatory processes, changes in the permeability of the intestinal epithelium and insulin resistance influence the development of diabetes. These relationships have been widely studied in animal models [75-77, 16].

In the research by Amar et al., the effects of a high-fat diet on adherence of bacteria to the intestinal epithelium, the formation of inflammatory processes and insulin resistance, have been proven in a mouse model. A greater proportion of Gram-negative bacteria and LPS levels in the intestines of the tested subjects was demonstrated. It has been proven that the translocation of ampicillin-resistant strains of *E. coli* (GFP-*E. coli*) is closely dependent on Toll-like (TLR/CD14) and Nod-like receptors (Nod-1), as well as adapter proteins. The role of the dendritic cells (DC) responsible for phagocytosis and translocation of the pathobiom is also important [77].

The "leaky gut phenomenon" is a widely-studied hypothesis that can explain the mechanisms of pathobiom invasion into the mesentery and blood vessels. In addition to phagocytosis and receptor-dependent active mechanisms, there are also mechanical possibilities dependent on defects in the mucosa and tight junction proteins. There is ample evidence suggesting the involvement of *Prevotella spp.* in the degradation of the mucin covering the intestinal villi cells [78, 79]. In the work by Cani et al. and Bagarolli et al., it was also proved that the change of the intestinal microflora negatively influences the expression of adherent proteins - ZO-1 and occludin, increasing the mechanical ability of the pathobiom leak [80, 75]. In these studies, the influence has been emphasized of a high-fat diet on the negative regulation of intestinal tightness, depending on the mechanisms of the endocannabinoid system (eCB) [81-83]. In a study carried out by Bawah et al., a close relationship has been described between the increase in plasma zonulin levels in pregnant women in the first trimester of pregnancy as a modulator of tight intercellular junctions and the risk of GDM [84, 16].

The increase in the level of LPS in the intestinal microflora contributes to the induction of metabolic endotoxemia and the production of low-grade inflammation [85]. In animal models, an increase in the activity of Toll-like receptors (TLRs) and subsequent mobilization of inflammatory vectors has been demonstrated. Several pro-inflammatory pathways have been identified which are mediated by the interleukin receptor associated kinase (IRAK), TGF-1 related kinase, NFkB, IKK-β, INK. etc. This leads to the development of low-grade inflammation, infiltration of macrophages and an increase in the concentration of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α [85-90].

Pro-inflammatory cytokines induce a state of insulin resistance in nephralgic locations – the liver, muscles and adipose tissue. In adipocytes, phosphorylation of serine

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residues occurs at the level of insulin receptor substrate proteins (IRS) by the kinase activated via the mutation of the p38 gene (MAPK). In microtubules, the process of phosphorylation takes place at the serine residue - 307 (Ser307) by MAPK and kB inhibitor kinase [91]. Interleukin 1β significantly influences the failure of β cells in pancreatic islets. During glucose stimulation, insulin production in β cells is intensified and inflammatory processes dependent on interleukin 1β and interleukin receptors (IL-1R) are intensified. These processes lead to the dysfunction and apoptosis of pancreatic islet β cells [92, 93]. In recent years, clinical trials have been carried out on the use of IL-1 receptor antagonists (IL-1R) in the treatment of diabetes. Test substances include canakinumab and gevokizumab, known as XOMA 052 [94-96]. For unarguable reasons, there are no studies on the use of IL-1R antagonists in pregnant women.

A series of complex, biochemical, pro-inflammatory and microbiological mechanisms lead to insulin resistance and the development of GDM in pregnant women. As mentioned above, this phenomenon is based on a number of changes in the intestinal microbiota of a pregnant woman.

GDM diagnosed in the third trimester of pregnancy is associated with a disturbance in the composition of the mother's microbiota. This altered microbiota is similar to that of people with type 2 diabetes [34]. Moreover, the maintenance of a changed composition of the microbiota after childbirth was observed [34]. Disturbed composition of the microbiota in early pregnancy has been shown to be associated with the development of GDM in later pregnancy [97]. Also, Mokkala et al. presented the relationship between an increase in the relative abundance of *Ruminococcaceae* in early pregnancy and the later development of GDM [98].

Both in normal pregnancy and that complicated with GDM, the abundance of *Blautia* and *Collinsella* increase [99]. In GDM, the firmicute/bacteroides ratio increases compared to healthy pregnant women [100]. Crusell et al. presented actinobacteria, *Collinsella*, rothia, actinomyces, *Desulfovibrio*, leuconostoc, *Granulicatella*, and *Mogibacterium* as GDM biomarkers, while *Marvinbryantia*, *Acetivibrio*, and *Anaerosporobacter* were presented as markers of normal carbohydrate metabolism in pregnancy [34]. In another study, bacteroides, dialister, and *Campylobacter* were indicated as biomarkers of GDM, while *Gemminer* and *Bifidobacterium* were noted as markers of normal blood glucose levels during pregnancy [101]. In women with GDM in the third trimester of pregnancy, increases in the number of *Bacteroides caccae*, *Bacteroides massiliensis* and *Bacteroides thetaiotaomicron*, as well as a reduction of *Bacteroides vulgatus*, *Eubacterium eligens*, *Lactobacillus rogosae* and *Prevotella copri*, have been observed [102].

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Reports from recent research have allowed to identify various microorganisms within the intestinal microbiota of women with GDM. Ordinarily, in these studies, the composition of the microbiota among a control group is compared with women at a high risk of or diagnosed with GDM.

An overview of the research results from recent years is presented in Table 1.

Table 1. Recent studies showing the dominant taxonomies in the group of women from GDM

Study	Number of women surveyed GDM/Control (Total)	Gestational age (weeks)	Methods	Prevailing taxonomies in the group of women with GDM
Su et al. (2021)	21/32 (53)	24-28	16s rRNA sequencing	*Bacteroidetes, Bacteroides spp., Weisella spp., Fusicatenibacter spp., Parabacteroides, Roseburia, Flavonifractor*
Silias et al. (2021)	49/39 (88)	24-28	16s rRNA sequencing	*Bacteroidetes, Enterobacteriaceae, Clostridiales, Firmicutes/Bacteroidetes ratio*
Wei et al. (2021)	15/18 (33)	24-28	16s rRNA sequencing	*Ruminococcus bromii, Clostridium colinum, Streptococcus infantis*
Hu et al. (2021)	201/201 (402)	6-25 & 24-28	16s rRNA sequencing	*Enterobacteriaceae, Ruminococcaceae spp., Veikkonellaceae*
Chen et al. (2021)	30/28 (58)	28	16s rRNA microarray	*Corynebacterium spp., Lactobacillus spp., Blautia hydrogenotrophica*
Chen et al. (2020)	110/220 (330)	25-26	16s rRNA sequencing	*Bacteroidetes spp., Dialister spp., Campylobacter spp., Enterococceae spp.*
Ma et al. (2020)	70/70 (140)	10-14 & 42 days after delivery	16s rRNA sequencing	*Eisenbergiella, Tyzzerella, Lachnospiraceae NK4A136*
Ye et al. (2019)	36/16 (52)	24-28	16s rRNA sequencing	*Blautia, Eubacterium halli*
Cortez et al. (2018)	26/42 (68)	28-36	16s rRNA sequencing	*Ruminococcus, Prevotella*
Crusell et al. (2018)	50/157 (207)	27-33	16s rRNA sequencing	*Desulfovibrio, Rothia spp.*

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Kuang et al. (2017)	43/81 (124)	21-29	Whole meta-genome shotgun sequencing	*Klebsiella varicolla*

Interesting findings were noted by Hu et al. in their study based on measuring the quality of microbiota in women, comparing the first trimester (6-15 Hbd) with the third (24-28 Hbd). The study was conducted on a numerically equivalent group of women with GDM and controls. Strong dependence on the prevalence of the dominance of *Enterobacteriaceae, Ruminococcaceae spp.* and *Veillonellaceae spp.* species in the group of women with GDM has been demonstrated [103].

In a study by Ma et al., the microbiota of women with GDM in the first trimester of pregnancy (10-14 Hbd) and after the post-partum period was compared in 2 equal groups. The *Eisenbergiella, Tyzzerella* and *Lachnospiraceae NK4A136* species were highly prevalent in women with GDM, also 42 days after delivery [36].

The available studies also allow to note depletion in the presence of some bacterial taxomes in the microbiota of women with GDM, compared to pregnant women with normoglycemia. This concerned the *Faecalibacterium* [104], *Bifidobacterium spp., Eubacterium spp.* [33], *Marvinbryantia, Acetivibrio, Anaerosporobacter* [34] and *Bacteroides spp* groups [100]. These taxa can be potential predictors of normoglycemia.

The observations discussed above are summarized in Table 2.

Table 2. Recent studies showing the dominant taxonomies in the group of women withoud GDM

Study	Number of women surveyed GDM/Con-trol (Total)	Gestational age (weeks)	Methods	Prevailing taxonomies in the group of normoglycemic women
Ye et al. (2019)	36/16 (52)	24-28	16s rRNA sequencing	*Faecalibacterium spp.*
Cortez et al. (2018)	26/42 (68)	28-36	16s rRNA sequencing	*Bacterioides spp.*

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Crusell et al. (2018)	50/157 (207)	27-33	16s rRNA sequencing	*Marvinbryantia spp., Acetivibrio, Anaerosporobacter*
Crusell et al. (2018)	43/81 (124)	21-29	Whole meta-genome shotgun sequencing	*Bifidobacterium spp., Eubacterium spp.*

In the previous chapter, differences are shown with regard to microflora profile in pregnant women with GDM and NDM (non-diabetic mothers). The range of taxomes prevalent in the groups of patients with GDM is positively correlated with the glucose level in pregnant women [98].

Although the relationship between the intestinal microflora and GDM has been proven, the molecular mechanisms of the interaction of the intestinal microflora on the development of GDM are still largely unknown [105].

As mentioned above, it is now suspected that microbiota metabolites play a main part in maternal epigenetic regulation of GDM and in programming the child's metabolite profile. In the publication by Zhao et al. [106], the relationship between maternal faecal matabolome and neonatal blood metabolome was investigated. The authors observed a negative prevalence in the presence of 4 different fecal metabolites in mothers with GDM compared to the control group - lysine, putrescine, guanidinoacetate and hexadecanedioate. These substances are known to play a protective or indicative role in the development of glucose metabolism disorders [36, 107, 108]. Zhao et al. also proved an increased level of biotin metabolism in patients with GDM. These observations may also provide evidence of the significant contribution of maternal microbiota metabolites to inborn errors of metabolism (IEMs).

It seems likely that the metabolites of the gut microbiota may become recognized biomarkers of GDM in the future. The development of screening tests would allow for earlier identification of GDM and IEM risk and to take preventive measures [109].

In the literature on the subject, the hypothesis concerning the influence of internal (microbiota) and external (environment) factors on the regulation of gene expression within the context of the development of glucose metabolism disorders is currently being discussed. Still, there is a lack of publications on the direct influence of microbiota or its metabolites on specific genome sequences.

In the work by Kumar et al [110], the effect was investigated of the *Bacteroides, Firmicutes, Proteobacteria* taxomes (present in the intestines of pregnant women) on DNA methylation of genes in inflammatory response, obesity and cardiovascular diseases. The pres-

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ence of up- and down-regulation of many genes responsible for the development of metabolic disorders has been proven. Interestingly, several of these genes are also methylated in GDM patients - e.g. KCNIP3/4.

In the publication by Van der Vossen et al. [111], concerning fecal microbiota transplants (FMT), *Prevotella* spp. involvement in AFAP1 methylation was proven. This gene is responsible, *inter alia*, for insulin sensitivity and peripheral insulin resistance. However, there is no direct evidence of its involvement in the pathogenesis of GDM.

In the study by Liu et al. [112], conducted on whole genome research using the EWAS method, the relationship was examined between guanidine cytosine phosphate methylation and GDM. Nine sites of different CpG methylation have been identified that may be used in the future as biomarkers in the early diagnosis of GDM patients.

3. Overview of epigenetics

For over 70 years, in the development of epigenetics as a separate field of science, many mechanisms of genome regulation have been recognized and described. The term "epigenetics" was first used by C. H. Waddington in 1939 to explain the differentiation of embryonic cells with identical genetic material into functionally distinct cells and tissues.

Recent years and advances in molecular biology have made it possible to distinguish epigenetics as a separate field of science. Conventionally, epigenetics is concerned with the study of changes in gene expression caused by mechanisms other than changes in the nucleotide sequence of DNA [113,114].

Epigenetic modifications are defined as alterations in gene activity unrelated to changes in nucleotide sequence. Two major periods of epigenetic reprogramming (erasure and re-establishment) are known: in primary germ cells (PGCs) and from fertilization to the pre-implantation stage. This is a period particularly vulnerable to the disruptive effects of internal and external factors. The then-created phenotypes and/or epimutations can be passed down through subsequent generations, thus, making them significant underlying pathogenetic mechanisms for the development of complex multifactorial diseases. In this context, we refer to intergenerational epigenetic inheritance when direct exposure to a particular stressor cannot be excluded, and transgenerational inheritance of phenotypes and/or epimutations are maintained without any direct exposure to the stressor. In the maternal lineage, exposure during gestation implies direct exposure of the mother and the fetus (intergenerational), and their developing primary germ cells (transgenerational inheritance) [115].

The genome of a living organism is located in the cell nucleus. It is in the form of a long chain of DNA packed into nucleosomes. Chromatin is wound up on a complex of stabilizing proteins called histone octamers, between which a linker DNA fragment is located. Depending on the degree of packing or loosening of the chromatin structure, the

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degree of its availability for the transcriptional apparatus and epigenetic mechanism changes.

Loosened fragments of chromatin, readily-available for transcriptional mechanisms, are called euchromatin, while tightly packed fragments that are difficult to access for biological processes are called heterochromatin [116,117]. Changes in the chromatin structure directly lead to a change in the functional state of specific genes encoded in a given fragment of the genome. The site of the interaction of epigenetic mechanisms is most often the site of transcription initiation or regulatory sequences near a specified gene [118].

It is believed that the essential function of epigenetics in the genome environment is to provide a response to internal and external environmental factors through dynamic, mostly reversible changes in chromatin structure and gene expression. The basic epigenetic processes are considered to be : DNA metylation, histone modifications, noncoding RNA regulation and chromatin remodeling.

The most frequently studied mechanism is the methylation of cytosine nitrogenous bases in the DNA chain. The DNA methylation reactions are catalyzed by DNA methyltransferases (DNMT). It is well known that methylation is directly related to the inhibition of gene expression. The spots of increased methylation are CG dinucleotides within the cytosine bases, known as the CpG regions. It has also been proven that functionally active genes are hypomethylated, which enables the synthesis of proteins suitable for the metabolic tasks of a specified cell. Methylation, in the promoter region of a gene, reduces its expression, and methylation within the repressor of a given gene positively influences the expression of this particular gene. A number of consequences concerning disturbed cellular homeostasis have also been discovered, resulting in selective or global defects of genome methylation, including carcinogenesis processes [119, 120].

Histone tail methylation may affect DNA methylation processes, and vice versa. The role of lysine aminoacid trimethylation in histone H3 (H3U9, H3U27) and histone H4 (H4U20), as a condition of subsequent DNA methylation, has been demonstrated [121]. This problem remains the subject of on-going research.

Another mechanism for modifying the chromatin strand and regulating gene expression is the modification of histones. The most widely-described mechanisms are histone acetylation and deacetylation. These processes are catalyzed by histone acetyltransferases (HAT) and histone deacetylases (HDACs), respectively [122].

The degree of chromatin folding is tightly regulated by the acetylation of lysine residues. Acetylation affects electrostatic relaxation between histones and phosphate residues. Therefore, this process leads to increased availability of the genome for the transcriptional apparatus and epigenetic mechanisms [123]. It has also been proved that the process of lyisine deacetylation in histone 4 (H4U16) strongly influences folding of the chromatic thread [124].

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Phosphorylation of serine residues in regions close to the sites prone to methylation, i.e. H3K9 and H3K27, affects the possibility of epigenetic regulation in the area of lysine residue methylation [125].

Other mechanisms of epigenetic regulation include complex ATP-dependent processes of chromatin remodeling [126]. Gene expression is noted by modifying histones via non-coding RNA - miRNA [127].

Disturbances in mitotic and meiotic inheritance, as well as a simultaneous disturbance in genetic balance of the cell, may lead to the development of multifactorial diseases, which include gastrointestinal neoplasms, arterial hypertension, as well as metabolic and hematological diseases. Recent years have also brought to light many novel insights into epigenetic mechanisms regarding the development of gestational diabetes [128]. Figure 1 shows interactions between environmental factors, GDM, microbiota and epigenetics.

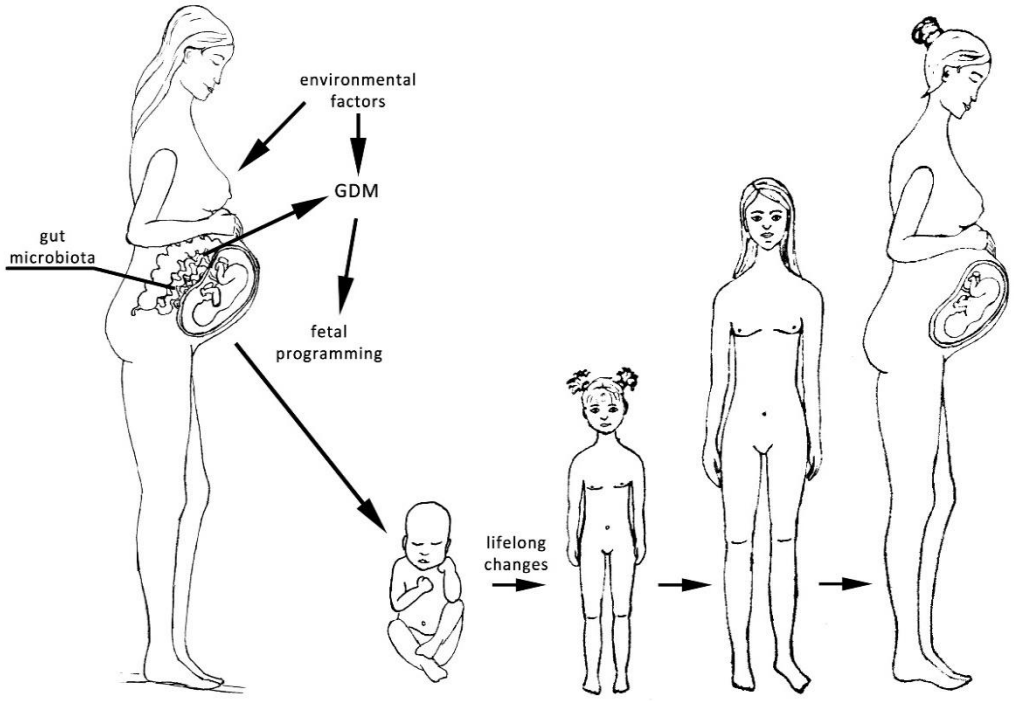

Figure 1. Interactions between environmental factors, GDM, microbiota and epigenetics.

4. The role of epigenetics in the pathogenesis of GDM

During normal pregnancy, the maternal organism experiences a number of physiological adjustments to meet the demands of the developing fetus. They involve the cardiovascular, renal, hematological, respiratory and metabolic systems. In the first and second trimester, anabolic processes dominate, insulin secretion in response to glucose increases, insulin

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sensitivity does not change at all or only slightly rises. In the third trimester, however, catabolic processes dominate, lipolytic activity rises, insulin resistance and insulin production increases. In most women, the increase in insulin requirements is compensated for by enhanced insulin production. In GDM, the compensatory mechanism of increased insulin production fails, which is most likely related to insufficient adaptation of pancreatic ß-cells to progressive insulin resistance, making it impossible to maintain normoglycemia [18, 21, 129, 130].

In the case of elevated maternal blood glucose values, fetal homeostasis in the regulation of carbohydrate metabolism is disturbed [131-133]. Disturbances in the intrauterine environment are the cause of aberrant fetal programming and predispose to a number of consequences for the child in adulthood. The impact of maternal GDM on the later occurrence of obesity, cardiovascular disease and the development of type 2 diabetes in the offspring has been widely demonstrated [134-137].

Epigenetic modifications have been considered as a probable link between the impact of a disrupted intrauterine environment and detrimental consequences in the progeny [138,139, 140 , 141].

Disruption of carbohydrate metabolism affects the modulation of gene expression through epigenetic mechanisms. In recent research, the role has been proven of DNA methylation, histone modification and miRNA gene silencing mechanisms in the pathogenesis of GDM. It is worth noting that these mechanisms may be hereditary and transgenerational, which could further predispose the offspring to the occurrence of metabolic disorders in later years of life. This phenomenon allows to confirm the theory of "fetal programming'' [142, 143].

The role of methylation in the insulin receptor promoter gene has been reported in literature on the subject. In a study by Ott et al., the authors proved the variable nature regarding methylation of the IR transcription initiation regions within chromosome 19. A significant increase in methylation within region 1 and intron 1 was demonstrated. Moreover, the authors demonstrated the highest level of DNA methylation in CpG in introns 1 in umbilical cord blood, then in maternal blood, visceral adipose and subcutaneous adipose tissue. Regarding region 1, methylation levels were relatively similar in all of the analyzed tissues. This study allowed to prove the inverse relationship of methylation concerning the CpG4-GRE2, CpG3-AP-2 and SP1 regions with the expression of the IR gene in subcutaneous adipose tissue. The authors also showed that higher glucose levels were positively correlated with CpG2-AP-2 and SP1 methylation, while negatively associated with methylation of the CpG3-GRE1 region in umbilical cord blood cells of GDM-affected mothers [144].

The role of promoter region methylation regarding the IR and IGF-1 receptor genes has also been proven in an animal model. In a study by Nihoshkov et al., a higher level of

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methylation concerning selective CpG regions has been demonstrated in myocardial and muscle tissue among mice with DM [145].

In the research by Haertle et al., the methylation level of many genes that may be associated with GDM was analyzed. Significantly lower CpG2 region methylation levels of the gene encoding the alpha subunit of ATP- synthase F1 (ATP5F1A) were demonstrated. This study also allowed to note significant changes in methylation levels for the CpG4 regions for the MFAP4, CpG1, CpG2 and CpG3 gene in the PRKCH gene and in the CpG5, CpG6, CpG10 and CpG11 regions of the HIF3A gene. This significantly proves the existence of epigenetic mechanisms at mitochondrial and extracellular levels in the epidemiology of GDM [141].

The above-observations have been confirmed by other extensive genetic studies on the role of DNA methylation in GDM. In the trial by Wu et al., 100 CpG islands with significantly variable methylation were identified and the significant effect of the CpG variable methylation was proven for the genes of COPS8, PIK3R5, HAAO, CCDC124, C5orf34 proteins [146].

The subject of research was also the expression of the PGC-1alpha (PPARGC1A) protein gene in the offspring of women with GDM. Kelstrup et al. showed lower expression of this gene in the muscle tissue of the offspring of GDM mothers. The negative influence of PPARGC1A gene expression on HOMA-IR expression in subcutaneous adipose tissue has also been proven. This suggests the influence of PPARGC1A expression on the development of insulin resistance and the influence of maternal overweightness on the onset of GDM [147]. These convergences were confirmed in studies on the expression of PPAR gamma and PGC-1alpha carried out by Ruschle et al [148].

In a cohort study conducted by Reichetreder et al., 1,030 samples were tested for global methylation of placental DNA. A very strong correlation was found between maternal GDM and global DNA methylation (p=0.0009). Variations related to clinical history, BMI, GDM predisposition and ethnicity were separated in this study. This allows to suggest that GDM independently influences placental DNA methylation [149].

In another study, by Gague-Ouellet, focus was on the level of DNA methylation at the lipoprotein lipase (LPL) gene locus. The authors demonstrated a significant decrease in methylation in the CpG1 and CpG2 regions of the LPL gene in GDM-affected patients. A negative correlation was been demonstrated between the methylation of the placental DNA of the LPL gene and its transcriptional activity [150].

Modification of histones in the genome also plays an important role in the mechanisms of epigenetic variation. Hepp et al. investigated the level of lysine 9 acetylation and lysine 4 trimethylation within histone H3 (H3K9ac and H3K4me3, respectively) in the placental tissue of women with GDM. In this study, a significant reduction was demonstrated in H3K9ac expression in the syncytiotrophoblast, cells of the

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trophoblast, extracellular placental tissue and fetal endothelial cells in tissues derived from GDM-complicated placenta, in comparison to the control group. No similar relationships were found in terms of H3K4me3 expression [151].

Chang et al. proved that deacetylation of H3 and H4 histones, H3K4 demethylation and H3K9 methylation negatively affect the expression of PDX1 (IPF-1) [152]. IPF-1 deficit causes disorders in pancreatic beta cell maturation, leading to insulin secretion disorders [153]. In studies on animal models, the possibility has been proved of generation inheritance regarding disorders related to the dysfunction of PDX1 expression [154].

Another mechanism of epigenetic regulation is the interaction of short, non-coding RNA, known as miRNA sequences. Zhao et al. proved the protective role of miRNA-221 interacting with PAK1 towards pancreatic beta cells. In an animal model, the effect of miRNA-221 on insulin sequence, proliferation and inhibition of apoptosis concerning pancreatic islet cells was demonstrated. In the tested control group, a significantly reduced expression of miRNA-221 was noted in individuals with GDM [155].

In the work by Stirm et al., a higher level of miRNA-340 expression was observed in the blood lymphocytes of mothers with GDM (p=0.02) [156]. In other studies, an increased expression of miRNA-7-5p in women with GDM was also proved [157]. Moreover, studies on animal models allow to prove the increased expression of miRNA-143 and the possible generation-inheritance of glucose metabolism disorders [158].

In the human gut microbiota phyla such as bacteroidetes, firmicutes, and actinobacteria are major components other such proteobacteria, whichare a smaller but also significant. Alterations in microbial composition may have great impact on metabolism. Firmicute-dominant microbiota has been found to dominate in overweightness, obesity and metabolic syndrome [110].

The molecular mechanisms behind the effects of the microbiota on metabolism remain largely unknown, although recent evidence suggests that the gut microbiota plays an pivotal role in human metabolism and may be a significant environmental factor affecting our epigenome. Among many important compounds synthesized by the gut microbiota, we also find liposacharydes, folate, polyamines and enzymes such as: methyltransferases, acetyltransferases, deacetylases, BirA ligases, phosphotransferases, which may act as epigenetic modulators taking part in DNA methylation and histone modification. [159,160].

To date, there has been only little research among humans, evaluating the correlation between microbiota and epigenetic modifications.

In a pilot study conducted among pregnant women, Kumar et al. investigated the association between gut microbiota and epigenetic changes, and found a strong correlation between blood DNA methylation patterns and gut microbiota profiles. Clustering analysis of DNA methylome data revealed a clear correlation between the whole-blood epigenetic

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profile and the composition of the gut microbial population of the mothers with a predominance of either bacteroidetes and proteobacteria (High Bact) or firmicutes (High Firm). In mothers with higher firmicute levels (High Firm), the authors found that promoters of 568 genes were more methylated, while the promoters of 245 genes were less methylated than in mothers with greater bacteroidete and proteobacteria levels. Among affected genes, 82 are known to be associated with the risk of cardiovascular disease, 72 with lipid metabolism, 23 with obesity and 85 with inflammatory response. The most significant difference between the 2 groups was observed in methylation of the promoter region of SCD5, which was more highly methylated in the High Firm group and had undetectable methylation in the High Bact group (p=0.00208). Lipopolysaccharide (LPS) was one of the upstream regulators of genes identified in the network, which according to the authors, further strengthens the role of microbial molecules in epigenetic modifications [110].

In their work, Ramos-Molina et al. analyzed DNA of gut microbiota composition in stool samples from 45 obese subjects by 16S ribosomal RNA (rRNA) gene sequencing. Twenty patients were selected based on their bacteroidete-to-firmicute ratio (BFR). The authors found that in adipose tissue, both *HDAC7* and *IGF2BP2* were hypomethylated and over-expressed in the low BFR group compared with the high BFR one. They demonstrated that the DNA methylation status was correlated with gut microbiota composition in obese subjects and that the expression levels of candidate genes involved in glucose and energy homeostasis (e.g., *HDAC7* and *IGF2BP2*) could be epigenetically regulated by gut bacterial populations in adipose tissue. They further noted that hypomethylation in the *HDAC7* promoter, in both blood and fat tissue, is also related to impaired glucose metabolism, as distinct differences in glucose and HbA1c levels were observed in both study groups. This implies that alterations in the methylation profile of the HDAC7 gene are linked not only to the structure of the gut microbiota, but also to the metabolic status of the subjects, at least in the blood and adipose tissue [160].

In the study conducted by Tachibana et al., it was found that changes in the UBE2E2 and KCNQ1 methylation rates among umbilical cord samples were associated with the proportion of firmicutes in the maternal gut, although with marginal correlations after adjusting for age and body mass index. These results may suggest a link between fetal diabetes-related gene methylation in fetuses and maternal microbiota components during pregnancy, but a limitation of this study is its small sample size [161].

In a randomized trial by Vähämiko et al., the authors aimed to assess whether probiotic supplementation throughout pregnancy may modify the DNA methylation status of gene promoters linked to obesity and weight gain in mothers and their children. They have analyzed DNA methylation status of certain obesity promoters (623genes) and weight gain-related genes (433 genes) in mothers as well as their offspring, and have

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concluded that probiotic supplementation resulted in markedly reduced DNA methylation levels in 37 gene promoters and increased DNA methylation levels in 1 gene promoter of women. In children, 68 gene promoters were significantly affected, with DNA methylation levels lower in the probiotic-treated group. They identified alterations in the epigenetic regulation of some components of insulin signalling pathways in response to the probiotic intervention, which may partly explain the favorable impact of probiotics on glucose metabolism. Remarkably, the promoter for insulin-like growth factor binding protein 1 (IGFBP1) was found to be less methylated in both mothers and their children in the probiotic group. IGFBP1 encodes a protein that binds insulin-like growth factors I and II, and low levels of this protein have previously been linked to insulin resistance and diabetes.

Similarly, the MSRA (methionine sulfoxde reductase A) gene promoter was found to be less-methylated in the probiotic mother-children pairs . In an experiment on mice, animals lacking the MSRA gene showed reduced physiological insulin response in comparison to wild-type mice [31]. On this basis, the authors hypothesised that the reduced methylation of IGFBP1 and MSRA may be a possible mechanism for providing health benefits to both women and their children by diminishing the risk of impaired glucose metabolism.

The authors have suggested that probiotic supplementation during pregnancy, by targeting specific gene promoters, may provide beneficial long-term health effects. Nonetheless, a significant limitation of this study was the small size of the study group [162].

Further research is required to fully understand the epigenetic mechanisms influencing GDM. Their results may be important in clinical and experimental practice.

In Table 3, the results obtained by the discussed researches are presented.

Table 3. Studies proving the epigenetic mechanisms influencing GDM.

Ott et al. (2018)	• variable levels of DNA methylation in the promoter regions of the IR gene in SAT, VAT, CB and MB; • negative correlation between CpG4-GRE2, CpG3-AP-2 and SP1 methylation and the expression of the IR gene in SAT; • expression of the IR gene in CB positively related to methylation of the CpG2-AP-2 and SP1 region and negatively related to methylation of the CpG3-GRE1 region of the promoter of the IR gene.
Haertle et al. (2017)	• lower level of methylation in the CpG2 region of the ATP5F1A gene in women with GDM; • variable methylation levels of several CpG regions for MFAP4, PRKCH and HIF3A genes in women with GDM.

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Wu et al. (2016)	• variable methylation levels of CpG regions for COPS8, PIK3R5, HAAO, CCDC124, C5orf34 genes in women with GDM;
Kalstrup et al. (2016)	• lower level of PPARGC1A gene expression in the muscle tissue of the offspring of women with GDM - a mechanism probably different than variable CpG methylation for the PPARGC1A gene. • negative correlation between the expression of the PPARGC1A gene and the expression of the HOMA-IR gene in subcutaneous adipose tissue in children of mothers with GDM.
Reichetzeder et al. (2016)	• GDM strongly influences the level of placental DNA methylation in a way that is probably independent of other clinical factors;
Gagne- Ouellet et al. (2017)	• decrease in the level of methylation in the CpG1 and CpG2 regions of the LPL gene in the placental tissues in patients with GDM; • negative correlation between methylation at the LPL gene locus and its transcriptional activity.
Hepp et al. (2018)	• decreased level of lysine acetylation in H3K9 in placental tissues during pregnancy complicated by GDM
Chang et al. (2016)	• H3 and H4 deacetylation, H3K4 demethylation and H3K9 methylation negatively correlate with the expression of the PDX1 gene (IPF-1);
Zhao et al. (2019)	• GDM negatively correlates with the level of miRNA interacting with PAK1 on the beta cells of the pancreatic islets.
Stirm et al. (2018)	• higher levels of miRNA-340 expression in peripheral blood lymphocytes in women with GDM.
Balci et al. (2020)	• higher level of miRNA-7-5p expression in the blood serum of mothers with GDM.

5. Conclusions

The gut microbiota appears to play an important role in human metabolism and can be a significant environmental factor affecting our epigenome.

In literature on the subject, the complex influence is emphasized of epigenetic mechanisms, including the effects of the gut microbiota on the formation and development of GDM in pregnant women. Currently, several dozen mechanisms of gene modulation at the mitochondrial and extracellular levels have been described. However, the authors of various studies highlight the lack of knowledge on the direct and indirect influence of epigenetic mechanisms on each other. An important question remains whether one regulation mechanism may affect the others in a cascade manner - the so-called "trigger effect". The development of molecular techniques, e.g. RNA sequencing of the intestinal microbiome, will, in the future, allow to find answers to many questions about epigenetic mechanisms of genome regulation in pregnant patients. There is also no answer to the question regarding the risk scale of intergenerational inheritance in terms of the effects of

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epigenetic mechanisms. A limitation of many available studies is the failure to standardize patient groups in terms of BMI, race and origin, socioeconomic status and the number of offspring.

The currently-available literature and the evidence cited therein, point to a strong role of epigenetic mechanisms and the gut microbiota profile on the pathogenesis of GDM and human health programming in future ectopic life. The impact of epigenetic mechanisms on the pathophysiology of GDM and their long-term consequences for ectopic life require many years of wide-spectrum and complex research, including careful selection of patients, molecular analysis of the material and several years of follow-up.

Whole-genome, sequence-based metagenomic analyses and 16S rRNA gene sequencing have made it possible to define the richness and diversity of bacterial species. The link between the gut microbiome and epigenome can be used as effective targets for the diagnosis and treatment of diseases, e.g. to reduce the risk of GDM dietary interventions changing microbial composition could be used. The composition of the gut microbiota may help understand the risk of developing GDM and therefore, increase the chance of detecting, preventing and treating this disease. There is an urgent need for further multi-directional, interventional and longitudinal studies on epigenetic modifications induced by microbiota alterations. Given that these studies are complex and costly, while awaiting their results, it seems valuable to attempt a broader introduction of recommendations that may serve to maintain eubiosis, especially among women of child-bearing age and, in particular, pregnant ones. The periconceptional period as well as intrauterine development is a unique times in the formation of the gut microbiota for the mother, but especially, for the fetus and thereby, they are essential for the programming of future human health. As epigenetic changes are mostly modifiable, there is a possibility to limit the intergenerational inheritance of metabolic traits and to reverse the unfavourable trend of the rising incidence regarding a broad spectrum of metabolic diseases and their serious long-term consequences. Undoubtedly, the reversibility of epigenetic changes should be taken into account, particularly within the context of GDM development. After analyzing the scientific evidence, it seems particularly important to develop the aspect of social education and incorporate the conclusions of the currently-available research into the protocols of perinatal counseling.

Undoubtedly, it is necessary to consider the fact that the effects of epigenetic mechanisms are temporarily reversible within the context of GDM development . After analyzing the scientific evidence, it seems necessary to develop the aspect of social education and incorporate the conclusions of the currently-available research into the protocols of perinatal counseling.

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