Epigenetics in inflammatory liver diseases: A clinical perspective (Review)

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Received November 30, 2021; Accepted December 30, 2021

DOI: 10.3892/etm.2022.11293

Abstract. Inflammatory liver diseases are, nowadays, multifactorial and wide-spread, thus having an important socio-economic impact. Although the therapeutic algorithms are well-known in hepatitis, regardless of etiology, strategies to identify inflammatory hepatic lesions in early stages and to develop new epigenetic therapies should be prioritized. The main entities of inflammatory liver disease are: alcoholic and non-alcoholic fatty liver disease, autoimmune hepatitis, viral hepatitis and Wilson disease. The main epigenetic processes include: DNA methylation/demethylation, which imply changes in DNA tertiary structure; post-translational histone covalent changes (methylation/demethylation, acetylation/deacetylation, ubiquitination), that cause DNA-histone instability; synthesis of small, non-coding RNA molecules, called microRNAs, that modulate translational potential of transcripts (mRNAs) and post-translational modification of polypeptide chains. Consequently, the epigenetic interactions aforementioned, play an important modulatory role in disease progression and response to conventional therapies The present review focused on the main epigenetic changes in inflammatory liver conditions, considering a new perspective: Epigenetic therapy. This approach is more than welcomed, taking into consideration that conventional therapeutic strategies are almost exhausted.

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Key words: epigenetics, acetylation, methylation, microRNA, autoimmune hepatitis, non-alcoholic fatty liver disease, alcoholic fatty liver disease, viral hepatitis, Wilson disease

Contents

1. Introduction

DNA represents the main molecule that incorporates the genetic information of the human cells independent of the organ tissue. It is well known that this molecule, consisting of a specific nucleotide sequence, forms the nuclear chromatin in a cell. The functional unit of chromatin is called a nucleosome, whose structure consists of a DNA double helix and adjacent histone proteins. Furthermore, all individual variations of genes form the genotype, which is the same in all cell types within an organism. The interactions between the genotype and the environment form variable phenotypes, depending on the cell type (1).

As a result, different gene suppression and activation mechanisms determine consistent phenotypic differences between cells from different organs and even within the same organ.

Subsequently, modifying the transcriptional potential of DNA without changing its sequence or genetic information, will change the chromatin tertiary structure, due to histone-DNA interaction. However, this process will not change the amino-acid sequence in the polypeptide chain (this represents the unchanged genetic information). Remodeling of the chromatin conformation, epigenetically, at the nuclear level, results in the synthesis of the mRNA species or in suppressing this transcriptional process (1). Furthermore, in cytoplasm, the mRNA transcripts are controlled by other epigenetic factors: The microRNA (miRNA or miR) species, the transcripts of lesser dimensions (2-22 nucleotides), of the noncoding repetitive DNA. The final product, the polypeptide chain, may therefore be translated or not, depending on the impact of such a post-transcriptional interference RNA network. Finally, there are certain active proteins such as enzymes, for example, which may be formed only

following the post-translational covalent modifications of the polypeptide chains (2).

All these processes are examples of natural, physiological, epigenetic regulations of gene function or expression. Epigenetics studies the variation of gene expression that is independent of genetic information or nucleotide sequences. It refers to gene function control through both nuclear chromatin covalent modification and remodeling and cytoplasmic activity of the interference RNA involving miRNAs along with post-translational covalent modification of the newly synthetized polypeptides (1).

Various epigenetic mechanisms could explain how a static genome interacts with a dynamic environment. According to Cold Spring Harbor Meeting-2009, 'an epigenetic trait' represents a term designed to define 'a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence' (3). Over time, different definitions of epigenetics were suggested. Waddington was the first to introduce the term *epigenetics* in 1942, as a variety of mechanisms which promotes gene expression changes without DNA mutation (4). In 1968, he defined epigenesis as 'the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being' (5).

The current view of epigenetics includes the following processes: i) Active DNA methylation, demethylation, which imply changes in DNA tertiary structure; ii) also post-translational histone covalent changes (methylation/demethylation, acetylation/deacetylation, ubiquitination), that cause broader and complex fluctuations in DNA-histone interactions; iii) synthesis of small, non-coding RNA molecules, called miRNAs, that modulate translational potential of transcripts (mRNAs) at the ribosomal level; and iv) post-translational modification of polypeptide chains (1).

DNA methylation involves the addition of a methyl group to a cytosine major base through a covalent interaction, particularly at 5'-CpG-3' dinucleotide sites in DNA substrates. If this change is symmetrical in both DNA chains, then the DNA conformational structure will change and the replication will be delayed (6). Usually there are specific gene areas rich in 5'-CpG-3' nucleotides, which can be preferentially methylated. These areas are called 'CpG islands', in the case of the housekeeping genes (which have to stay active independent of the cell types and the CpG sites should not be methylated) (7). The extensive methylation of the CpG repetitive regions is linked to chromatin silencing in genes presenting tissue type expression. Over 60% of the genes in the human genome have a high proportion of CpG dinucleotides in the promoter region, thus being potentially influenced by this epigenetic mechanism (8).

DNA methylation is controlled by enzymes that transfer methyl groups from the methyl-donor, S-adenosil-methionine (SAM), to cytosine. They are called DNA N-methyl transferases (DNMT) (Fig. 1).

This epigenetic process is carried out by different isoforms of DNMT: DNMT 1 has a specific role in maintaining the pre-existent methylation pattern, while DNMT 3a and DNMT 3b promote *de novo* DNA methylation (6,9). In addition, environmental factors such as nutrition, exercise and particular chemical substances are able to modify DNMT expression and function with consecutive changes in DNA methylation degree and distribution, and all of these have a variable transcriptional effect upon the gene function (10).

Another epigenetic nuclear mechanism involves histone-DNA interaction and is represented by covalent binding of acetyl groups at lysine residues within histones forming the nucleosome core. Consequently, histone chains around which DNA molecules are wrapped, become more relaxed, easier exposing DNA to transcriptional factors. Conversely, if acetyl groups are removed from the acetylated histones, the nucleosomes will appear more compact and resistant to transcriptional factors (1) (Fig. 2).

The acetylation process is regulated by histone acetyltransferases (HATs), while histone deacetylation is regulated by histone deacetylases (HDACs) (11). At present, histone deacetylation is extensively studied (12). HDACs are classified according to their structural and functional similarities into four classes: class I (HDACs: 1, 2, 3, 8), class IIa (HDACs: 4, 5, 7, 9), class IIb (HDACs: 6, 10), class III (sirtuins 1-7), and class IV (HDAC11). It is well known that different classes of HDACs have specific intracellular locations (HDACs from class I are predominantly located intranuclearly, while class II HDACs shuttle between the cytoplasm and the nucleus) (13).

HATs are represented by a vast family of proteins such as cAMP-response element binding (protein) (CREB) binding protein (CBP), that acts in a phosphorylation dependent manner: Once phosphorylated, a HAT molecule will be activated, while dephosphorylating will lead to HAT inactivation. The equilibrium between HATs and HDACs is termed 'acetylation homeostasis' and will finally dictate the degree of DNA exposure to transcriptional factors inside the nucleosome (14). Histone acetylation and deacetylation regulate cellular processes such as aging and oncogenesis.

Conversely, miRNAs are small non-coding single-stranded RNA species, composed of 15-30 bases, that are involved in the post-transcriptional control of gene function or expression in the cytoplasm (15). Its effect of silencing is achieved by altering mRNA stability and blocking the mRNA elongation, thus terminating protein synthesis (Fig. 3).

Briefly, following maturation of pre-miRNA (formed in the nucleus and exported and processed in the cytoplasm) into miRNA by enzymatic cleavage of the hairpin structures, the leading strands of the miRNA are integrated into the protein RNA-induced silencing complex (RISC). The leading strand is thermodynamically unstable relative to the passenger strand and directs the RISC to the complementary strand of mRNA. miRNAs usually recognize binding sites in the 3' untranslated region (UTR) of mRNA transcripts. Perfect base complementarity between miRNA and mRNA cleaves the mRNA by the slicing activity of Argonaute-2 (AGO2), whereas imperfect binding leads to translational suppression and slicer-independent mRNA damage (16).

Since miRNA represents epigenetic markers that modulate terminal cell differentiation and developmental changes, there has been great interest in achieving new miRNA-targeted epigenetic therapies and identifying new epigenetic markers for various clinical paradigms (17). At present, there are numerous miRNA species recognized, being independently and organ-specifically coded which can modulate the protein synthesis and function in health and disease.

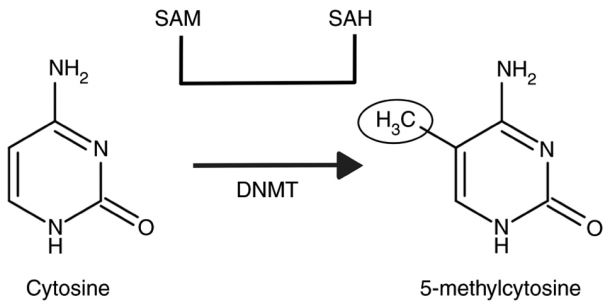

Figure 1. Cytosine methylation. DNMT, DNA methyltransferases; SAM, S-adenosil-metionine; SAH, S-adenosil-homocysteine.

Although the screening tests and therapeutic algorithms are well-established in various hepatic diseases, including liver transplantation as etiologic therapy in end-stage liver disease, strategies to identify reversible lesions in early stages and to develop new epigenetic therapies should be prioritized (18-20).

2. Methods: Selection of studies

A systematic review was performed on articles in English published in databases including PubMed, Elsevier or Scopus until August 2021, using the following key word: 'epigenetics in liver diseases'. Articles referring to any of the following hepatic diseases were included: Non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease (AFLD), autoimmune hepatitis (AIH), viral hepatitis (VH) and Wilson disease (WD). Studies focusing on end-stage liver disease were excluded. The most relevant articles published in the last 15 years were added.

Some relevant articles from the domain of epigenetics, in general, were further included, independent of the year of publication, for a better description of fundamental epigenetic mechanisms. After duplication removal and screening for eligibility, 65 articles consisting of 19 clinical studies, 22 experimental studies and 24 review articles were included.

3. Epigenetics in various liver diseases

Epigenetics in NAFLD. NAFLD represents a serious condition linked with an inappropriate high-fat diet, which is more obvious in developed countries where obesity and unhealthy dietary habits represent public health issues. These liver changes are in correlation with other multisystemic changes such as type II diabetes, various cardiovascular or renal diseases, as consequences of the interaction between the environment (due to various dietary habits) and the organism.

In NAFLD, epigenetic DNA changes have been observed, altering the insulin metabolism or producing dysregulations, at the cellular level of the various metabolic pathways. Ahrens *et al* observed that genes encoding insulin-like growth-factor 1 (IGF-1) and insulin-like factor binding protein 2 (IGFBP-2) could be hypermethylated in NAFLD, inducing gene silencing and consequent impairments in glucose metabolism. Other genes including pyruvate carboxylases and ATP citrate lyase involved in the glucose cycle could also be epigenetically silenced (21).

Histone deacetylation was also observed to interfere with lipid metabolism. Silent information regulator factor 2-related enzyme 1 (SIRT-1) improves hepatic steatosis and circadian rhythm (22,23). Other histone deacetylases such as HDAC-3 and HDAC-8 promote triglyceride metabolism and insulin sensitivity (24). *De novo* liver lipogenesis could also be epigenetically controlled due to certain histone changes: The interaction between host cell factor 1 (HCF-1) and carbohydrate response element binding protein (ChREBP) regulates hepatic lipogenic genes (25).

In NAFLD, miRNAs species have been identified as epigenetic markers for liver injury. miR-122 is one of the most abundant small non-coding RNAs expressed in the liver. In NAFLD, miR-122 is downregulated. Experimental study models have revealed that the downregulation of this marker promotes lipogenesis and liver inflammation (26). Conversely, upregulation of miR-21 has deleterious effects on the degree of hepatic steatosis and glucose metabolism (27). Other overexpressed liver miRNAs in NAFLD are miR-24, miR-34a and miR-124, which could interfere with lipid metabolism and insulin sensitivity (26,28). miR-155 modulates the crosstalk between adipose tissue and the liver in NAFLD induced by a high-fat diet (29).

In conclusion, NAFLD is a highly epigenetically controlled liver pathology, whose complex mechanisms involve factors associated with dietary habits, alterations in tertiary DNA and histone structure and specific miRNA expression.

Epigenetics in (AIH). AIH exhibits various phenotypes, reflecting the complexity of underlying immune mechanisms. There are two forms: AIH type I, present particularly in middle-aged women and AIH type 2, which is more common in children. AIH type 1 is characterized by an increased titer of antinuclear antibodies, soluble liver antigen/liver pancreas antibodies, and smooth muscle antibodies, while AIH type 2 exhibits large amounts of the liver kidney microsomal 1 antibodies (30). As the main immune mechanism of the disease, the imbalance between pro and anti-inflammatory promoting T-cells is extensively studied. Treg cells are T-cells with anti-inflammatory properties, whose presence is linked with the disease activity and liver inflammation (31).

The data in the literature is rather focused on the miRNA-mediated epigenetic changes in AIH than on DNA or histone changes. Similar to other inflammatory hepatic diseases, miR-122, the most abundant liver miRNA species, is upregulated in AIH as well, serving as a marker of the disease activity (32). Consequently, this epigenetic marker could serve as biomarker for therapy response or disease control. miR-21 is also upregulated and is inversely correlated with the degree of fibrosis (33). miR-223 suppresses proinflammatory liver activity via the NF-κB pathway, inhibiting the macrophage function. In an AIH experimental model, the overexpression of miR-223 was revealed to have a liver-protective effect (34). miR-155 could affect AIH progression as well. The literature offers, however, contradictory data. miR-155 regulates the inflammatory response by influencing the Th17 cells, with no effect on IL-10-mediated Treg response (35).

Other epigenetic markers with an undefined role in AIH are: miR-218, miR-363, miR-518f, miR-628-5p, miR-888, miR-523, miR-141, miR-302b, miR-643 and miR-573 (36).

Although there is insufficient data to characterize epigenetic histone changes in AIH, certain studies have revealed

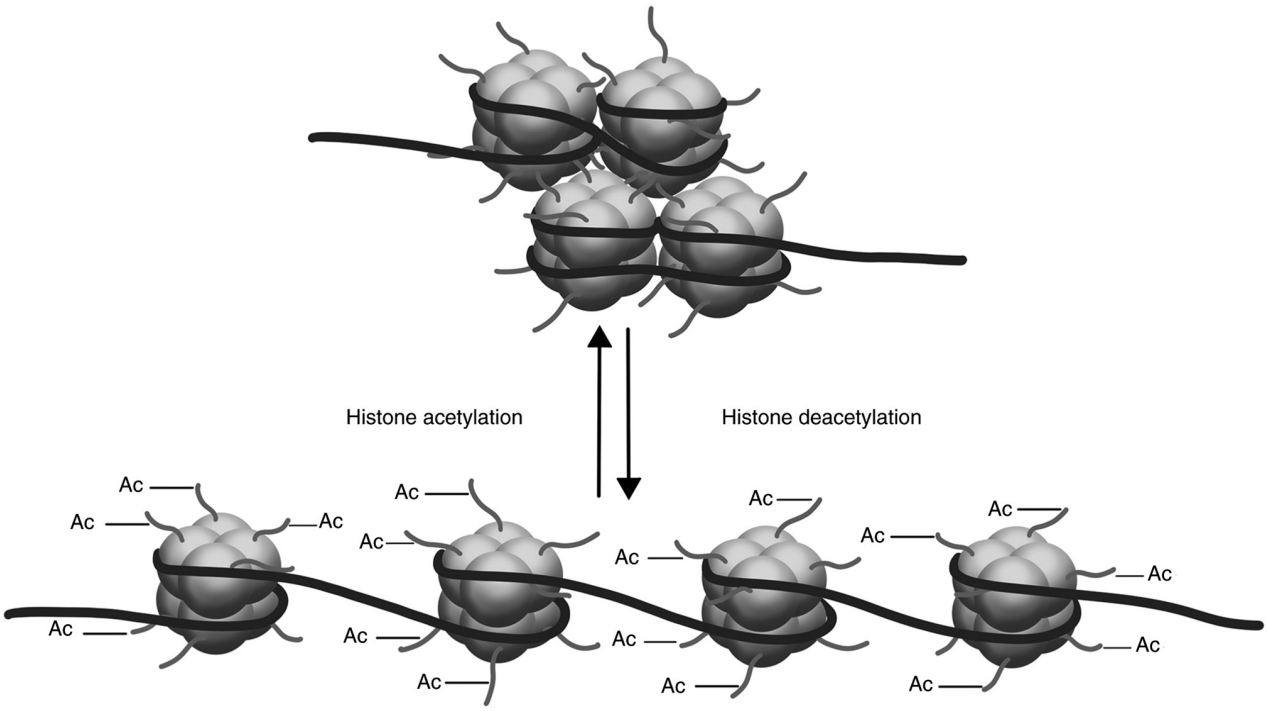

Figure 2. Histone acetylation and deacetylation.

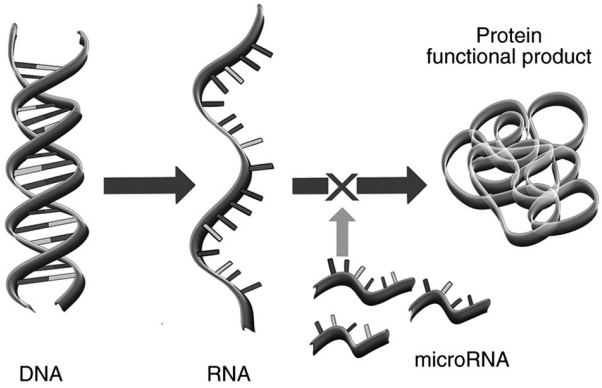

Figure 3. miRNA regulatory mechanism.

anti-histone auto-antibodies in AIH, whose interaction with the histones could be reduced due to the epigenetic aforementioned potential structural change (37). This paradigm should be, however, further explored.

A previous clinical study reported a correlation between the DNA methylation status in certain immune cells such as $CD4^+$ and $CD19^+$ lymphocytes and disease activity. Altered expression of enzymes involved in DNA methylation, TET1 and DNMT3A, characterizes lymphocytes in AIH (38). Further studies are required in order to confirm this association.

From an epigenetic perspective, AIH still represents an open field for research: To date, miRNAs represent the only area which have offered a perspective regarding epigenetic modulating mechanisms in this disease.

Epigenetics in VH. The interplay between a hepatic virus and the liver leaves, in the majority of cases, an epigenetic signature. Due to complex modulatory mechanisms, these signatures act either as a prognostic tool or as a therapeutic response (39). Subsequently, epigenetic therapies are evoked as novel therapies against these viruses. For example DNMT inhibitors could be useful in VHC-associated HCC, while HDAC inhibitors could reduce the VHB replication (39).

Hepatitis B virus (HBV) is a DNA virus, whose genetic structure consists of covalently closed circular (ccc)DNA incorporated into hepatocytes. The translational process is realized using the host nuclear enzymes. The cccDNA methylation in the region of GpG islands could, however, reduce the translational potential of the viral DNA. There are three CpG regions defined in the viral DNA: i) The start site of the S gene; ii) the region surrounding enhancer I, the HBx gene promoter (Xp), and the core promoter (Cp); and iii) the region harboring the Sp1 promoter and the start codon of the Pol gene (40). According to Zhang *et al*, the start site of the S gene is variably methylated among the different HBV genotypes, while the other two regions are more stably methylated (41). The methylation of the second island is linked with a decreased viremia, while the methylation of the third island influences carcinogenesis (41). This epigenetic change is observed mainly in the nuclear cccDNA, integrated in the host cells and not in the histone-free cytoplasmic DNA or circulating virions. The role of DNMTs in HBV infection is not fully understood. According to the literature, DNMT1, DNMT2 and DNMT3, are upregulated in HBV infection leading to hypermethylation in host cells and, consequently, to a reduction in virus replication (42). cccDNA replication is also modulated by epigenetic changes of the histones. Hypomethylation of H3 and H4 histones and the recruitment of HDAC 1 nearby cccDNA, could reduce the replication potential of the virus (43). Furthermore, epigenetic therapies targeting upregulation of DNMTs and histone hypomethylation linked with immunomodulatory therapy could represent the future in chronic HBV infection.

Considering the miRNA species as potential epigenetic biomarkers in HBV, miR-146 predicts the evolution to fibrosis in HBV-infected patients (44).

Conversely, hepatitis C virus (HCV) virus is an RNA virus, whose particular epigenetic signature reveals the risk of carcinogenesis even in the presence of the sustained viral response. Specific histone changes, H3K4Me3 and H3K9Ac, promote the persistence of the virus following successful direct antiviral therapy, acting as an epigenetic signature (45).

DNA methylation is another epigenetic change, influencing carcinogenesis in HCV-infected patients. The two most common repetitive elements in humans, long interspersed nuclear element-1 (LINE-1) and Alu element (Alu), have been linked with carcinogenesis. HCV may cause hepatocellular carcinoma (HCC) by suppressing host defenses through DNA methylation that controls the mobilization of repetitive elements (46).

Certain miRNA species are used as prognostic tools, being particularly associated with the risk for HCV patients to develop various complications such as HCC, fibrosis or cirrhosis. miR-122-5p, miR-486-5p and miRNA-142-3p could predict the development of HCC in HCV-infected patients (47).

According to Shrivastava *et al*, miR-20a and miR-92a are epigenetic biomarkers, which promote the evolution from an acute to a chronic state (48). Let-7c is another epigenetic biomarker, which could predict the evolution to fibrosis (49).

miR-494 is associated to the therapeutic response, while miR-34a is upregulated in fatty liver compared with chronic HCV (50,51).

Epigenetics in alcoholic fatty liver disease (ALFD). Diet-induced epigenetic changes are common and one of the first described modulatory factors which could lead to epigenetic changes according to the target tissue. The most exposed tissues developing epigenetic modulatory mechanisms secondary to various dietary factors are the brain, hematopoietic system or liver (52,53). Alcoholic liver disease (ALD) has a significant influence on the life quality, having a very important impact on various health systems.

Alcohol-induced oxidative stress in hepatocytes interferes with all main mechanisms of chromosomal epigenetic control including DNA hypomethylation, histone acetylation and phosphorylation and miRNA alteration.

Histone H3 acetylation at Lys 9 (H3AcK9) in alcohol-exposed hepatocytes was observed in experimental studies (54,55). HDAC inhibition, particularly SIRT1, and HAT enzyme activation are responsible for this change (54). Phosphorylation of the histone H3 at serine residues could synergistically influence the epigenetic signature in hepatocytes exposed to alcohol (55). Another experimental study revealed a differential methylation pattern of the histone H3 and H4 in alcohol-exposed hepatocytes (56). Whether chronic or acute exposure to alcohol could induce these histone alterations is, however, controversial.

S-adenosil-methionine (SAM) is an important methyl donor involved in CpG island DNA methylation. According to Dannenberg *et al*, SAM concentration is reduced in ALD, which consequently interferes with the DNA methylation process (57). This hypomethylation state promotes DNA damage and strand breaks in ALD (57). DNA hypomethylation also disturbs alcohol-metabolizing enzymes, such as alcohol dehydrogenase 1B (ADH1) (58). An interplay between histone acetylation and DNA methylation is also possible in ALD: DNA hypomethylation in promoter regions triggers histone deacetylation. The exact effect of this epigenetic crosstalk is, however, unknown.

miRNA species represent other epigenetic markers in ALD. miR-155 is increased in liver macrophages secondary to alcohol intake (59). miR-212 is upregulated secondary to alcohol intake, affecting the intestinal permeability by downregulating tight junction protein zonula occludens 1 (60). Further studies are required, in order to understand the exact prognostic and therapeutic potential of these epigenetic markers in ALD.

Epigenetics in WD. WD is an autosomal recessive disease characterized by accumulation of copper in the liver and brain. The key gene causing this disease is the copper-transporting gene ATP7B, which blocks copper extraction through the biliary tract. The knowledge of the disease physiopathology is continuously evolving. At present, it is widely accepted that alteration of this gene interferes with at least eight transmembrane active transporters of copper from the hepatocytes (61).

There is a large variability in WD considering the onset, sex, severity, response to treatment and target organ (the liver vs. the brain). This disease inconsistency is partially explained through certain epigenetic modulatory mechanisms. WD impairs the methionine metabolism, which is the main methyl supplier for DNA and histone methylation. The aberrant SAM/S-adenosil-homocysteine (SAH) ratio impairs the methylation process (62).

Furthermore, traces of heavy metals impair the mitochondrial metabolism, thus increasing the amount of reactive oxygen species (ROS). This metabolic change dysregulates the activity of TET enzymes, inhibiting the DNA demethylation (63).

In animal models, the hepatic accumulation of copper is inversely correlated with DNMT3a and DNMT3b levels, impairing the DNA methylation process (64). This alteration is particularly important *in utero*. Consequently, choline and penicillamine treatment, differentially modify the methylation status of the DNA in mice according to the sex (65).

4. Final considerations

Liver pathology is a vast field with incomplete knowledge, which requires profound expertise. The outcome in advanced and irreversible chronic hepatic injury in the absence of a liver transplant (cirrhosis and end-stage liver disease) is poor, with short and long-term socio-economic consequences. Therefore, new strategies to identify, stratify and treat these hepatic diseases while still being in a reversible state are highly required.

The epigenetic approach of all these diseases is more than welcomed, taking into consideration that conventional therapeutic strategies are almost exhausted. This is generally valid for ALD and NAFLD, viral hepatitis, AIH as well as other metabolic conditions.

Analysis of gene function and expression independent on the 'heritable' character of the genome, consisting in analysis of histone-DNA interactions and small non-coding RNA

synthesis, is an extremely valuable tool for future diagnostic and therapeutic strategies of hepatic diseases, whose molecular etiologies are far from being completely elucidated.

Acknowledgements

The authors would like to thank Ms Irina Matache, researcher at the Department of Physiology II and Neurosciences of 'Carol Davila' University of Medicine and Pharmacy (Bucharest, Romania), for her help with manuscript reviewing.

Funding

No funding was received.

Availability of data and materials

Not applicable.

Authors' contributions

TI contributed to literature research, manuscript writing and reviewing. SI contributed to manuscript writing and reviewing. RR contributed to manuscript reviewing and design of figures. MC contributed to manuscript reviewing. LI coordinated and reviewed the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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