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Epigenetic Regulations in Autoimmune Diseases with Special Focus on Rheumatoid Arthritis

Epigenetické regulace u autoimunitních onemocnění se zaměřením na revmatoidní artritidu

Bachelor Thesis

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Declaration:

I declare that I compiled the Bachelor Thesis by myself and I presented all the cited sources of information and literature. This thesis neither its significant parts have been submitted to gain another or the same academic title.

In Prague, 16.5.2014

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Abstract

Exact cause of rheumatoid arthritis, as well as other autoimmune diseases has not been identified yet. In last twenty years, epigenetics showed a new face of immune system. DNA methylation, modification of histones – proteins around which DNA is wrapped, or interference of small RNA sequences – microRNAs, these all are heritable changes outside the DNA sequence that provide another component involved in autoimmunity. Presented epigenetic mechanisms alter gene expression and thus facilitate production of pro-inflammatory factors leading to autoimmune reactions. Moreover, genes regulating apoptosis are also frequently targeted by epigenetic modifications. Not only these mechanisms provide another level of immune defense, they also explain higher female susceptibility to autoimmune diseases and the influence of environment on pathogenesis of these diseases.

Keywords: epigenetics, rheumatoid arthritis, systemic lupus erythematosus, autoimmune disease, DNA methylation, histone modification, microRNA

Abstrakt

Přesná příčina revmatoidní artritidy, jako i dalších autoimunitních onemocnění ještě stále není identifikována. V posledních dvaceti letech epigenetika ukázala novou tvář immunitního systému. Dědičné změny mimo sekvence DNA poskytují další komponentu hrající roli v autoimunitě. Metylace DNA, modifikace histonů – proteinů, kolem kterých je DNA obmotána, či interference malých sekvencí RNA – microRNA, všechny tyto mechanismy ovlivňují genovou expresi a tím umožňují zvýšenou produkci prozánětlivých faktorů vedoucích k autoimunitním reakcím. Kromě toho, geny regulující apoptózu jsou často cílem epigenetických modifikací. Tyto mechanismy nejenže poskytují další úroveň imunitní obrany, ale poskytují také vysvětlení pro vyšší náchylnost žen k autoimunitním nemocím i vliv prostředí na patogenezi těchto nemocí.

Klíčová slova: epigenetika, revmatoidní artritida, systémový lupus erythematodes, autoimunitní onemocnění, metylace DNA, modifikace histonů, microRNA

Contents

1. Introduction

Since times of Waddington, the definition of epigenetics has changed to a great extent. Epigenetics was firstly considered only in terms of developmental biology, when it was perceived as sum of changes in gene expression, where epigenotype was defined as "set of organizers and organizing relations to which a certain piece of tissue will be subject during development make up" (Waddington 1939). The definition of epigenetics has varied since then, until 1996, when Russo *et al.* defined epigenetics as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo *et al.* 1996). Although the definition may still evolve nowadays, there is a general understanding of epigenetics as mechanism of regulation of gene expression by enabling access to transcription mechanism.

Autoimmune disease (AD) is such condition, when body tissues are attacked by its own immune system. ADs are divided into two classes – systemic and organ-specific. The most abundant systemic ADs includes rheumatoid arthritis (RA), affecting mainly joints and surrounding tissues, systemic lupus erythematosus (SLE) affecting skin, joints, kidneys, heart, brain and many other important tissues. The class of organ-specific ADs includes multiple sclerosis (MS), affecting myelination of nerve cells in brain and spine, and type 1 diabetes mellitus (T1DM) affecting pancreatic islets.

In last decades there have been a lot of attempts of finding out the mechanisms causing autoimmune diseases. Researchers carried out genome-wide studies discovering common genetic variants, mostly single-nucleotide polymorphisms (SNPs), predisposing humans to autoimmune diseases. Many of these studies identified human leukocyte antigens (HLA) gene regions playing key role in autoimmune diseases, but they have not yet provided tractable therapeutic targets (Danska and Poussier 2009). Anaya *et al.* tried to investigate whether common genetic basis of ADs exist (Anaya *et al.* 2006). However, as the mechanisms are still not understood precisely, such conclusion cannot be drawn, even when the co-occurrence of multiple ADs is not uncommon. Recently, there have also been mentions of epistasis, masking of disease-causing mutation by genetic interactions, playing role in autoimmune diseases. This matter has been reviewed with emphasizes on SLE and MS (Rose and Bell 2012).

There have been also some successful approaches to treating ADs, for example in cytokine-driven pathway – tumor necrosis factor (TNF) inhibitors in psoriasis (Cauza *et al.* 2002) or by knocking out the gene for Death Receptor 3 (DR3), the TNF receptor superfamily

member. Making the *DR3* gene inoperative causes reduction in number of osteoclasts within areas susceptible to bone erosion (Bull *et al.* 2008).

Still, the exact cause of ADs remains unknown. Therefore, new approaches have been sought, epigenetics being one of them. In fact, epigenetic modifications bringing new insight in origin of ADs have triggered many studies in various research facilities. The aim of the presented thesis is to explore the existent findings in the role of epigenetics in autoimmune diseases, with emphasis on rheumatoid arthritis.

2. Autoimmune Diseases

2.1 Systemic Autoimmune Diseases

Systemic AD is a condition, where multiple organs/tissues are attacked by body's own immune system. This phenomenon is caused not only by disruption in mechanism protecting body from the autoimmune reactions, but also abnormalities in immune response to pathogens. The systemic ADs are characterized mainly by presence of autoantibodies involved in immunopathological activity in connective tissues.

As mentioned above, RA and SLE belong to group of systemic diseases. However, the variety of systemic ADs is broad and it also includes conditions as scleroderma, polymyositis and mixed connective tissue disease. It appears that genes related to these diseases overlap, thus suggesting similar machinery in their pathogenesis. Researchers still find new overlaps of genes responsible for susceptibility to SLE and RA, e.g. in gene coding for B lymphoid tyrosine kinase (Orozco *et al.* 2011), enzyme playing role in B cell receptor signaling. However, the clinical manifestations and their severity differ in patients, indicating existence of multiple pathways in autoimmune pathogenesis. The clinical manifestations and severity may also change in one patient in time.

SLE is an autoimmune inflammatory disease affecting connective tissues all over the body. The cause of this condition is still unknown, however, some factors playing key role have been already discovered. Genetic background of SLE has been highly studied and the research has already indicated that the within HLA genes class II, HLA-DRB1 allelic variations are increasing susceptibility to the disease (Fernando *et al.* 2007). HLA genes code for major histocompatibility complex (MHC) class II, typically expressed on antigen-presenting cells. MHC molecules, binding peptides from outside of the cell, serve as ligands for receptors on T helper cells, which are then activated and able to assist in maturation of B cells and activation of cytotoxic T cells and macrophages.

However, genetic aspect of SLE is more complex. Genes like *IRF5* (interferon regulatory factor 5) (Feng *et al.* 2010; Graham *et al.* 2006), *STAT4* (signal transducer and activator of transcription 4) (Zheng *et al.* 2013), *PTPN22* (protein tyrosine phosphatase non-receptor 22) (Shi *et al.* 2013) and *CDKN1A* (p21, cyclin-dependent kinase inhibitor 1) (Kim *et al.* 2009) are associated with increased risk of SLE. Furthermore, *IRAK-1* (IL-1 receptor-associated kinase) gene encoded on X-chromosome has been identified as element in SLE pathogenesis (Jacob *et al.* 2009). The epistatic interaction between *IRF5* SNP

rs77571059 and *STAT4* SNP rs16833215 has been indicated (Kim *et al.* 2013). As if the genetics of SLE was not complicated enough, SNPs in *PTPN22* gene region appear to differ across ethnicities in SLE patients (Namjou *et al.* 2013).

Scleroderma, or systemic sclerosis is another example of systemic autoimmune disease. It primarily affects skin, recruiting fibroblast and immune cells, resulting in thickening of skin by deposits of collagen, created by fibroblasts. Role of HLA genes is also significant in scleroderma. The HLA-DPB1 and DPB2 have been marked as main risk loci in Korean population, confirmed in US patients (Zhou *et al.* 2009). Th17 cells play interesting role in scleroderma. They produce pro-inflammatory cytokine IL-17A, which increases production of matrix metalloproteinase 1 (MMP-1), pro-inflammatory cytokine IL-8 and monocyte chemoattractant protein 1 but, on the other hand, Th17 cells also inhibit collagen type I production (Brembilla *et al.* 2013).

2.1.1 Rheumatoid Arthritis

Rheumatoid arthritis is a chronic inflammatory autoimmune disease resulting in irreversible joint destruction. In patients with RA, hyperplasia of the synovial lining occurs (see Figure 1). The synovial tissue transforms due to activity of immune cells and synovial fibroblasts. Macrophages and synovial fibroblasts of synovial lining in junction of bone and cartilage proliferate from normal 1-3 cell layers up to 13 cell layers (Perlman and Pope 2010). Current prevalence of RA is approximately 0.24 %, what corresponds to 4.8 million patients (Cross *et al.* 2014).

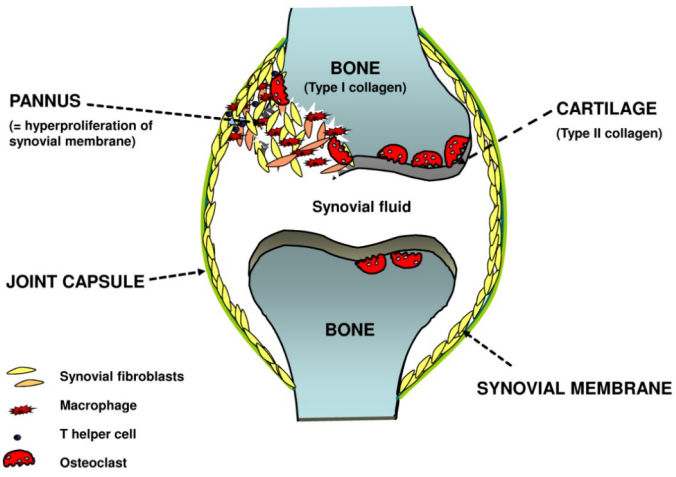

Figure 1: Hyperplasia of synovial lining and joint destruction in RA. Adopted from: (Schurigt 2013)

There have been many histological studies carried out in order to find out the exact mechanism of cartilage destruction. Although a common or regular pattern for the cellular erosion of cartilage has not been found, it has been established that in the contact of the abnormal layer of synovial lining and intraarticular cartilage, there are also cell types other than macrophages and fibroblasts: mast cells, polymorphonuclear leukocytes and displaced chondrocytes. All of the mentioned cell types produce cartilage-degrading enzymes such as metalloproteinases and serine proteinases, regulated by various local mediators (Woolley and Tetlow 1997). In a recent study, two inflammatory axes (four synovial phenotypes) have been indicated: one dominated by B cells, second by macrophages and nuclear factor κB-activating cytokines (e.g. TNF-α). Clinical responses to anti-TNF-α and anti-interleukin-6 (IL-6) receptor treatments differed within these two inflammatory axes (Dennis *et al.* 2014). These results suggest an explanation of drug response heterogeneity observed in RA patients, although further research is necessary.

Treatment using anti-TNF-α drugs has already reported some successful outcomes, though, without any long-term effect. The higher level of TNF-α in synovial tissues was detected already in 1991 (Chu *et al.* 1991). Besides other functions, the TNF-α-dependent pathways play role in inflammation and inhibition of apoptosis in synovial tissue. It has also been shown that pro-destructive function of TNF-α in synovial fibroblasts may be enhanced by other molecules, for example another pro-inflammatory cytokine, IL-33. (Kunisch *et al.* 2012). The serum concentrations of pro-inflammatory IL-6 family cytokines were also elevated in RA patients (Chung *et al.* 2011). However, recent study showed that IL-6 also induces production of anti-inflammatory IL-10 by type 1 regulatory T-cells, which are responsible for maintenance of tolerance (Krämer *et al.* 2013). Similarly to scleroderma, Th17 cells appear to play significant role in RA pathogenesis, inducing activity of IL-6, IL-8, MMP-1 and MMP-3 (van Hamburg *et al.* 2011).

Next to the aforementioned, another aspect of RA pathogenesis has been proposed. The human endogenous retroviruses (HERVs), forming approximately 5-8 % of human genome, are elements and fragments inserted into our genome during evolution from exogenous viruses. Vast scale of processes has led to inactivation of almost every HERV element (Belshaw *et al.* 2004). Inactivation of these elements, however, is not irreversible. It was shown that infection by Epstein-Barr virus transcriptionally activated gene encoding envelope protein of an endogenous retrovirus (Sutkowski *et al.* 2001). The interference of some HERV elements within autoimmune diseases has been indicated in RA (Reynier *et al.*

2009), SLE (Magistrelli *et al.* 1999; Pullmann *et al.* 2008), T1DM (Marguerat *et al.* 2004), MS (Brudek *et al.* 2009) and Sjogren's syndrome (Moyes *et al.* 2005).

In clinical practice, there are several methods of diagnosis of RA in patients. One of the methods is use of autoantibodies, e.g. rheumatoid factor (Bruns *et al.* 2000) or anti-citrullinated protein antibodies (ACPAs). Autoantibodies are antibodies, which identify body's own tissues as foreign ones. These are tested with second generation anti-cyclic citrullinated peptide (anti-CCP-2) (Peoples *et al.* 2013). The antibody production in peripheral blood mononuclear cells (PBMCs) has been also connected with increase of TNF-like ligand 1A levels in patients with RA. Yet, its function as disease activity marker has not been determined (Sun *et al.* 2013).

Genetic background of RA has been recently evaluated in a genome-wide association study (GWAS) with more than 100,000 subjects (Okada *et al.* 2014). Forty-two non-HLA novel RA risk loci have been discovered. In total, 101 risk loci have been associated with RA susceptibility (Okada *et al.* 2014). These risk loci were cross-referenced with target drugs for RA. Twenty-seven of these drugs were associated with RA risk loci. Okada *et al.* also noted that non-HLA RA risk loci explained 5-6% of heritability in RA (Okada *et al.* 2014). Conducting molecular pathway enrichment analysis, enrichment in T-cell related pathways, B-cell and cytokine signaling pathways have been observed (Okada *et al.* 2014).

Significant association of HLA class II genes with RA has been confirmed. Distinctive HLA class II types have been associated with susceptibility to variety of autoimmune diseases. Mainly increased expression of HLA-DRB1, the most prevalent beta component of MHC class II heterodimer, has appeared to have functional consequences on T cell immune response in RA patients (Kerlan-Candon *et al.* 2001). The *HLA-DRB1* locus has many allelic variants. The hypothesis of "shared epitope" has been proposed in 1987, suggesting that allelic variants have similar amino acid sequence in third hypervariable region, critical for T cell recognition. This hypothesis indicated some allelic variants of HLA-DRB1 sharing similar epitope, which is responsible for RA susceptibility (Gregersen *et al.* 1987). The shared epitope hypothesis, however, has been questioned. Considering different allelic variants with shared epitope having distinct level of risk (Wordsworth *et al.* 1992), the question whether the shared epitope hypothesis is sufficient for explanation of RA susceptibility arose. In a recent study, it was discovered that five amino acid (AA) positions could explain RA susceptibility in seropositive RA. These AAs are situated in HLA-DRB1 in positions 11, 71 and 74, in HLA-B in position 9 and in HLA-DPB1 also in position 9. All these AAs are situated in peptide-binding groove (Raychaudhuri *et al.* 2012).

Numerous studies concerning SNPs have been conducted. For example, it was demonstrated that SNP C1858T in *PTPN22* gene is associated with RA (Song *et al.* 2013), SLE (Lea and Lee 2011), coeliac disease (Santin *et al.* 2008) and T1DM susceptibility (Qu *et al.* 2005). The *PTPN22* gene codes for protein tyrosine phosphatase involved in signaling pathways within immune response. The protein tyrosine phosphatase suppresses signalization of Toll-like receptors in development of T cells in thymus and therefore the autoimmune T cells can survive. It was also indicated that combination of *PTPN22*, *HLA-DRB1* and smoking increased risk of developing RA, however, no multiplication has been recognized (Morgan *et al.* 2009).

2.2 Organ-specific Autoimmune Diseases

Mechanisms of autoimmunity in systemic and organ-specific diseases appear not to differ much, however, the autoreactive immune cells, autoantibodies and pro-inflammatory cytokines are directed against single organ. Such diseases include T1DM, MS, coeliac disease, Crohn's disease, Grave's disease and myasthenia gravis.

Type 1 diabetes mellitus is an autoimmune type of diabetes, when autoimmune processes are aimed towards pancreatic β-cells producing insulin. The lack of insulin results in impairment of carbohydrate metabolism, which may end with several complications. Untreated T1DM may lead to ketoacidosis and organ damage. The tissue specific autoantibodies may be found in T1DM patients: islet cell antibodies (Castro *et al.* 2014) or glutamic acid decarboxylase autoantibodies (GADA). Genetic background of T1DM also includes HLA-related genes. The HLA-DQ8 alleles appear to be responsible for higher risk of T1DM (van Lummel *et al.* 2012), whereas HLA-DQ6 alleles may play role in protection from the disease (Pugliese *et al.* 1995). Other research indicated that combination of HLA-DRB1, HLA-DQA1 and HLA-DQB1 alleles may result in increased susceptibility as well as protection against T1DM (Erlich *et al.* 2008). The non-HLA genes also play significant role in T1DM. SNPs related to T1DM susceptibility were found for example in *INS* gene, coding insulin and *IFIH1* (interferon induced with helicase C domain 1) gene, whose product serves for detecting viral mRNA and long dsRNA, activating type I interferon immune response. T1DM association with *HLA-DRB1* gene, *PTPN22* gene, *INS* gene and the variable numbers of tandem repeats surrounding the *INS* gene was recently confirmed (Portuesi *et al.* 2013). Genes responsible for cytokine signalization in immune system have also been identified as risk loci for T1DM (Barrett *et al.* 2009).

Coeliac disease is another organ-specific AD. The organ affected by this disease is small intestine and patients with this disease cannot process gluten protein. Genetic predisposition to coeliac disease is represented by HLA-DQ2 (Karell *et al.* 2003) and DQ8 (Mazzarella *et al.* 2003) alleles and several non-HLA candidate gene risk loci are suspected. Products of these HLA alleles present the gluten protein to T cells, activating them and causing autoimmune response. Increased expression of MMP-1 and 3 in coeliac disease patients may be responsible for villous atrophy (Daum *et al.* 1999), which is highly associated with this illness. Similar to other autoimmune diseases, the co-occurrence of multiple ADs is not uncommon. The co-occurrence with T1DM (Kota *et al.* 2012) and autoimmune thyroid disorders (Cuoco *et al.* 1999) has been noticed in coeliac disease.

3. DNA Methylation

DNA methylation is one of the most studied mechanisms of epigenetics. Researchers have already conducted wide variety of studies concerning DNA methylation in cancerous growth (Novak 2004), imprinting (Horsthemke 2014) and aging (Florath *et al.* 2014).

In eukaryotes, the DNA methylation occurs mainly on cytosine and guanine-rich regions of DNA. CpG islands are regions of very high CpG dinucleotide density, which mark mainly promoter regions and therefore their primary state is unmethylated, to allow transcription. CpG island hypomethylation is executed by DNA demethylation (Frank *et al*. 1991). Methylation of regions with lower CpG density, which are within 2 kb distance from CpG islands, called CpG island shores appears to play a considerable role in gene expression (Irizarry *et al.* 2009). Methylation of CpG islands is responsible for long-term silencing, including X-chromosome inactivation. The highest occurrence of methylation is on heterochromatin regions of chromosomes, regions with silenced gene expression. The hypothesis that DNA methylation affects the active chromatin formation has already been examined (Keshet *et al.* 1986). In contrast to promoter regions of genes, the gene body has to be methylated for proper transcription. The reason for methylation is to avoid spontaneous initiation of transcription (Ball *et al.* 2009). DNA methylation pattern is not only specific to various regions of DNA, it is also tissue specific (Liang *et al.* 2011). This phenomenon is highly studied in embryogenesis and cellular differentiation.

The enzymes responsible for DNA methylation are called DNA methyltransferases (DNMTs), using S-adenosyl methionine (Magistrelli *et al.* 1999) as donor of methyl group, usually methylating fifth carbon atom of cytosine pyrimidine cycle. Several types of DNMTs have been identified in mammals. The DNMT3a and 3b are responsible for *de novo* methylations of DNA (Xie *et al.* 1999) and DNMT1 is key enzyme responsible for maintenance pattern (Takeshita *et al.* 2011).

The overly enhanced methylation on certain loci – the hypermethylation is responsible for silencing of certain genes. The hypermethylation decreases the affinity for binding of transcription factors and increases recruiting of methyl-CpG binding proteins (MeCPs) to promoter regions, resulting in impaired transcription. Furthermore, the MeCPs show also a link between methylation and histone deacetylation, another epigenetic modification. It was shown that MeCP2 recognizing methylated DNA in nucleosome, recruits Sin3, a transcriptional regulatory protein, which associates with histone deacetylase, resulting in transcriptional silencing (Jones *et al.* 1998). MeCP2 has also been shown to be of importance

in maintaining DNA methylation pattern during cell division, forming complex with DNMT1 and binding to hemimethylated DNA (Kimura and Shiota 2003).

Another important mechanism used in AD that is based on gene regulation, is increase of resistance to apoptosis. The promoter of gene for death receptor 3 (DR3), the member of apoptosis-inducing Fas gene family, has a specific methylation pattern, causing lower expression of DR3 protein in RA synovial cells (Takami *et al.* 2006). This pattern may attributed to apoptosis resistance in synovial cells.

Hypermethylation pattern was discovered also in T1D, but the cause of increased level of methylation appears to be in homocystein metabolism. Homocystein is an intermediate in methionine catabolism and its lower serum levels were detected in T1D patients. The decrease of homocystein serum levels occurs due to increased activation of enzymes by trans-sulfuration (Wijekoon *et al.* 2007).

On the other hand, not only increase in methylation can be found. The mechanism of removing methylation from DNA appears to be a bit complicated than the reverse one. Some research indicates that activation-induced cytidine deaminase may play some role in demethylation and therefore induce gene expression. However, the actual role is not yet known (Isobe *et al.* 2013). Hypomethylation, especially low methylation of promoter causes higher expression of TNF-α. In study conducted by Nakano *et al.*, methylomes, the patterns of DNA methylation, in fibroblast-like synoviocytes (FLS) differed in RA patients compared to control FLSs. Hypo- and hypermethylation was observed in 1859 loci in key genes relevant to RA, such as *STAT3* (Signal transducer and activator of transcription 3), *WISP3* (WNT1 inducible signaling pathway protein), *CHI3L1* (Chitinase 3-like protein 1), *CASP1* (Caspase 1), *MAP3K5* (Mitogen-activated protein kinase 5) and *MEFV* (Mediterranean fever). Hypomethylation was associated with increased gene expression of factors contributing to RA pathogenesis. Increased hypomethylation occurred in several pathways related to cell migration and adhesion (Nakano *et al.* 2013). In RA patients, there has also been observed increased expression of DNMT1 and methyl-CpG-binding domain protein 2 mRNAs (Liu *et al.* 2011). This last finding indicates that regulation of methylation is not that simple and even when hypomethylation occurs in RA patients, the methylation enzyme and MeCPs levels are increased, what implies other level of regulation must be present in DNA methylation.

Though in RA the imbalance between hypo- and hypermethylation occurs, in SLE, hypomethylation appears to be the only abnormal methylation regulation mechanism. For example, the hypomethylation of *ITGAL* gene was detected in T cells (Luo *et al.* 2008).

Product of this gene, integrin α L chain together with β chain, forms leukocyte function-associated antigen (LFA-1). The LFA-1 functions as adhesion molecule in immune cells and plays role in transport to the infection site. In genome-wide SLE study, the hypomethylation was also marked in interferon type I genes (Absher *et al.* 2013). Interferons are glycoproteins produced by various cell types (lymphocytes, fibroblasts, macrophages, etc.) in response to variety of stimuli – e.g. viral infection. Using second messenger signal pathways, expression of anti-viral and immune modulatory genes occurs.

Methylation patterns of genes coding cytokines, important molecules playing role in pathogenesis of inflammatory diseases, were also examined. Promoter region of *IL6* gene, coding IL-6 involved in RA pathogenesis as pro-inflammatory cytokine, displayed hypomethylation of single region, leading to increased serum IL-6 levels (Ishida *et al.* 2012). Furthermore, there has been shown that hypomethylation of proximal CpG motif of promoter of gene encoding IL-10, anti-inflammatory cytokine, may also have a regulatory function in RA (Fu *et al.* 2011). This finding was further examined in study of SLE, where hypomethylation of *IL10* gene together with IL-1 receptor type 2 (*IL1R2*) gene accompanied greater disease activity. Moreover, the detected hypomethylation of *IL1R2* gene was more significant in RA patients than in SLE patients and healthy controls (Lin *et al.* 2012). Further research indicated increased hypomethylation in yet another cytokine pathway, interferon signaling type I in SLE patients (Absher *et al.* 2013).

Not only cytokine activity, but also chemokine activity is increased in RA synovial cells. For example cytokine CXCL12, also known as stromal-derived factor 1, occurs in higher level in patients with RA (Lisignoli *et al.* 2004). This chemokine appears to have multiple functions: attraction and activation of leukocytes, recruiting of macrophages and increase of metalloproteinase expression. Similarly to cytokine genes, the promoter of *CXCL12* gene is regulated by methylation, when the expression of CXCL12 can be increased by demethylation (Karouzakis *et al.* 2011).

Another aspect has been shown to contribute to lowered methylation of DNA in RA patients. The metabolism of polyamines, enhanced in RA synovial fibroblasts, in which SAM molecules are consumed, offers an explanation of hypomethylation (Karouzakis *et al.* 2012). By consumption of SAM molecules, the access to methyl group donor for DNMTs is reduced, therefore hypomethylation occurs.

Most of the research is conducted in patients with already diagnosed autoimmune disease. Therefore, the primary impulse leading to autoimmunity remains unclear. In T1DM,

it was detected that variable positions of DNA methylation are enriched even before disease diagnosis (Rakyan *et al.* 2011).

The effect of methylation has also been demonstrated in promoters of *CD40L* gene, located on X chromosome. The gene product, ligand of CD40 receptor (CD40L), causes multiple immune cell activation. The hypomethylation in CD40L promoter has been shown in female scleroderma patients (Lian *et al.* 2012) , as well as in RA female patients. The hypomethylation led to increased expression of CD40L in CD4(+) T cells of female RA patients (Liao *et al.* 2012). In SLE, hypomethylation in *CD40L* gene doubled the CD40L expression in women (Lu *et al.* 2007). Authors of this study explained that main reason for such findings is that female genome includes two X chromosomes and therefore hypomethylation of this gene activates gene expression on both of these chromosomes. The effect of increased CD40L expression is therefore responsible for increased immune cell activation, which may lead to autoimmunity. Findings in these studies indicate that CD40L may give a possible explanation for higher level of female susceptibility in some autoimmune diseases. The overexpression of CD40L in women, compared to men was recently confirmed in lupus patients (Hewagama *et al.* 2013).

There is still no exact explanation of gender-specific susceptibility. ADs appear to have multiple levels of regulation; thus, existence of a single explanation is improbable. Still, X-chromosome inactivation is perceived as the main reason for this phenomenon. The example of CD40 ligand indicates that this viewpoint could be worth further research. The inactivation of most genes on one chromosome in females is believed to occur due to dosage compensation of products of X-linked genes. The dosage compensation theory proposes that the X-chromosome inactivation occurs due to need of equal gene expression in both sexes, as the X-chromosome has approximately 2.6 times more base pairs. Thus, X-chromosome inactivation equals the expression between sexes. The increased methylation, causing inactivation of chromatin, was observed in genome study of DNA methylation profiles on X-chromosomes (Sharp *et al.* 2011).

4. Histone Modifications

Histones, package proteins around which DNA is wound, were discovered in 1884 by Albrecht Kossel. There are several types of histones forming chromatin structural units – nucleosomes. The basic classification of histone proteins separates them into two groups: core proteins, forming octameric nucleosome core, around which DNA is wound, and linker histones sustaining the structure of nucleosomes. Among the former, there are four families of proteins: H2A, H2B, H3 and H4. The latter group of histones is formed only by H1 family of histone proteins. There are many types of modifications, which can occur at histones. Several modifications can occur at one promoter or enhancer region at the same time (Wang *et al.* 2008). These modifications thus create some kind of 'code' read by binding proteins, which include transcription machinery. Because of various combinations of modification, there are plenty possible variations in transcription.

4.1 Acetylation

Histone acetyltransferases (HATs), enzymes responsible for histone acetylation, neutralize the positive charge on amino acid lysine in sequence of histone protein, by transferring acetyl group from acetyl coenzyme A. This causes loosening of DNA-histone interaction, allowing access to transcription machinery. Besides RNA polymerase, the transcription machinery includes various transcription factors, what results in enhancement of transcription. The histone acetylation mechanism, however, appears to be connected with methylation status of DNA. Heterochromatin regions of DNA are not only methylated, but histone deacetylation occurs. The euchromatin regions are demethylated and histone tails are acetylated (see Figure 2).

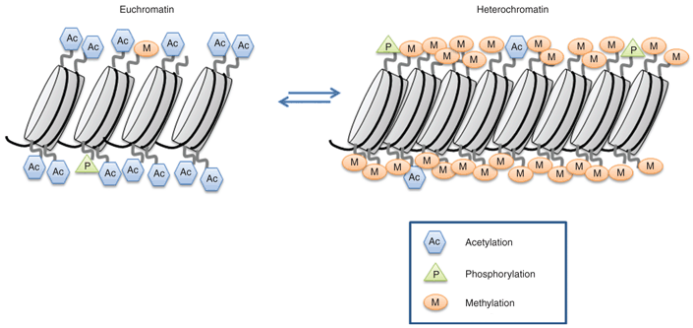

Figure 2: Epigenetic modifications in euchromatin and heterochromatin. Adopted from: (Schroeder *et al.* 2012)

The acetylation of histones appears to regulate activity of transcription factor FOXP3 (scurfin), which plays key role in regulation of development and function of regulatory T cells (van Loosdregt *et al.* 2010). The regulatory T cells expressing FOXP3 are involved in immune self-tolerance mechanisms, thus abnormal function of FOXP3 may lead to autoimmune reactions. Hyperacetylation of histones in *FOXP3* locus turned out to prevent polyubiquitination, therefore inhibiting protein degradation and disrupting stability in FOXP3 level (van Loosdregt *et al.* 2010).

Abnormal cytokine regulation is also examined in histone modifications. The higher level of H3 histone acetylation in promoter region of *IL6* gene has been detected in RA synovial fibroblasts compared to non-autoimmune osteoarthritis. This hyperacetylation indicates loosened state of promoter, accessible for transcription (Wada *et al.* 2014). Expression of this pro-inflammatory cytokine is increased by combination of epigenetic mechanisms and therefore IL-6 is a possible target in developing new treatment of autoimmune inflammatory diseases.

Hyperacetylation of histones also occurs in *INFG* gene, coding for interferon γ (Zhou *et al.* 2004). Interferon γ (type II interferon) plays substantial role in immunoregulation and is secreted by variety of immune cells – macrophages, type 1 helper T cells, cytotoxic T cells, natural killer cells and others.

Acetylation of histones may not only serve as regulator of access for transcription machinery. In SLE, nucleosomes have been identified as autoantigens (Bruns *et al.* 2000). Using monoclonal antibodies, apoptosis-induced acetylation of H2BK12 (van Bavel *et al.* 2009) and acetylation of three lysine residues (from 1st to 22nd residue position) on H4 (Dieker *et al.* 2007) was presented as target epitope for autoantibodies in SLE patients.

Histone acetylation seems to be a component in autoimmune processes involved in multiple sclerosis. It is a condition, where myelin sheath of nerve cells is damaged by autoimmunization. The acetylation of promoter region of H3 has been detected in NogoA(+) oligodendrocyte in white matter. This acetylation was associated with increased level of oligodendrocyte differentiation transcriptional inhibitors and high expression of HAT mRNAs. However, the acetylation decreased in cells with active demyelination and remyelination (Pedre *et al.* 2011). Acetylation of H3 histone may provide an epitope responsible immunoreactivity in MS. Interestingly, hypoacetylation pattern of H3 and H4 was found in SLE patients (Hu *et al.* 2008).

Much more studied mechanism in histone acetylation is inhibition of enzyme with antagonistic function compared to the HATs - histone deacetylases (HDACs). Mechanism of their functioning is removing acetyl group from acetyl-lysine, leading to stabilization of chromatin and silencing respective genes.

As the mechanism of HDACs is inhibition of transcription, according to previous findings, low expression is expected in autoimmune patients. In RA patients, reduction in the HDAC activity compared to non-autoimmune osteoarthritis patients and healthy controls occurred in synovial tissue specimen (Huber *et al.* 2007a). However, higher expression of HDAC1 and the induction of HDAC1 activity by TNF-α stimulation in synovial fibroblasts have been detected in RA patients (Kawabata *et al.* 2010). Cell proliferation and survival of activated synovial fibroblasts in RA was impaired by HDAC1 knockout and suppression of MMP production was also detected (Horiuchi *et al.* 2009). Decreased recruitment of HDAC1, probably causing H3K18 hyperacetylation together with low recruitment of DNMT3a, possibly resulting in H3K27 hypomethylation, was observed in *IL17A* gene promoter. These modifications allow binding of (CREM)α transcription factor, which induces pro-inflammatory IL-17A expression (Rauen *et al.* 2011). All these findings indicate that HDAC1 activity may play role in far more complicated pathway than understood nowadays.

The main support for acetylation of histones playing role in autoimmune diseases has been shown in treatment using inhibitors of HDACs. This matter will be discussed in chapter on current and potential treatment.

4.2 Methylation

Unlike acetylation and deacetylation, histone methylation does not change the charge of any amino acid. It occurs usually at lysine, arginine on H3 and H4 types of histones. The knowledge of histone methylation effects in autoimmune diseases is very limited. Most of the research on topic of histone methylation occurs in cancer studies. The enzymes responsible for histone methylation are divided into two groups. First group unites histone lysine methyltransferases, the second group contains histone arginine methyltransferases. As well as DNA methyltransferases, they use SAM as methyl group donor. Antagonist function is provided by histone demethylase.

Genome-wide study of SLE H3K4 trimethylation in PBMCs indicates that there were significant alterations in H3K4me3 in SLE-related genes *PTPN22* and *LRP1B* (LDL receptor-related protein B1), compared to healthy controls and RA patients (Dai *et al.* 2010). Increase

in recruitment of the H3K4 methyltransferase SET7/9 was observed in murine diabetes model (Li *et al.* 2008). Another research indicated that SET7/9 may prevent histone deacetylation by sirtuin 1 and therefore lead to type II collagen α1 expression (Oppenheimer *et al.* 2014). Not only histone methylation alone, bud combination of histone methylation and acetylation in gene for CD70 appears to contribute to development of SLE (Zhou *et al.* 2011). CD70 is protein from TNF superfamily and serves as ligand for receptor CD27. The ligation of this receptor induces NK cell proliferation and production of interferones, serves as co-stimulus for effector T cell production and also contributes to B cell maturation. Besides histone modifications, hypomethylation of CD70 promoter appears to contribute to autoimmune response. Promoter hypomethylation leading to CD70 overexpression was detected in scleroderma (Jiang *et al.* 2012). In RA patients, H3K27 histone trimethylation repressed expression of pro-fibrotic factor fra-2. Therefore, inhibition of histone trimethylation on 27^{th} lysine residue in H3 histone contributes to inhibition of fibroblast activation (Krämer *et al.* 2013). The methylation of 4^{th} lysine of H3 by Ash1l methyltransferase indicated the suppression of IL-6 production, therefore having anti-inflammatory effect (Xia *et al.* 2013).

4.3 Deimination

Human peptidylarginine deiminases, especially PAD2 and PAD4 have been shown to be expressed in synovial tissue of RA patients. PADs catalyze posttranslational modification of peptidylarginine into peptidylcitrulline. PAD4 citrullinates proteins, contributing to creating of autoantibody specific epitopes (Schellekens *et al.* 1998). The hypercitrullination of histones by PAD4 was detected in neutrophils. This increase in citrullination was linked to decondensation of chromatin, used in creation of neutrophil extracellular traps, which play role in immune response to bacterial infection (Leshner *et al.* 2012; Wang *et al.* 2009).

4.4 Oxidative Modification

Besides rather ‘classical’ histone modifications, there are plenty substances capable of modifying protein structure. One of these compounds is peroxynitrite with powerful nitrating and oxidizing effect. It has been shown that autoantibodies in SLE bind preferably to histone H1 modified by peroxynitrite (Khan *et al.* 2014). This preference of autoantibodies towards oxidatively modified histone proteins was also demonstrated in RA when H2A histone protein was modified with peroxynitrite (Khan *et al.* 2012).

5. MicroRNAs

MicroRNAs or micro ribonucleic acids are small, 18 to 23 base-pair long non-coding molecules that serve as post-transcriptional regulators of gene expression. Their first link to autoimmune diseases occurred in 2006, when connection between autoantibodies and RNA interference was observed (Jakymiw *et al.* 2006). The usual mechanism of microRNA interference is its binding to 3' untranslated region (UTR) of target mRNA with assistance of RNA-induced silencing complex (RISC) (Gregory *et al.* 2005). This process results in mRNA destabilization and/or inhibition of translation.

The upregulation of some microRNAs has been discovered in patients with RA. Elevated miR-146a, miR-155, miR-132 and miR-16 expression in PBMCs was detected. Despite increased expression of miR-146a, levels of its two targets – TNF receptor-associated factor 6 (TRAF6) and IRAK-1 did not differ in RA patients and healthy individuals. However, the repression of *TRAF6* and *IRAK-1* genes in THP-1 cells (derived from acute monocytic leukemia and used in immunohistochemistry to mimic native monocyte-derived macrophages) reduced TNF-α production (Pauley *et al.* 2008). *In vitro* studies also showed that TNF-α upregulated miR-146a expression (Li *et al.* 2010), therefore TNF-α appears to regulate its own level with help of miR-146a. Furthermore, the overexpression of miR-146a was associated with suppression of T cell apoptosis (Li *et al.* 2010). Another target of miR-146a activation, signal transducer and activation transcription 1 (STAT1), was suggested to facilitate a pro-inflammatory regulatory T cell phenotype. This study was conducted in patients with active RA, where decreased expression of miR-146a was detected (Zhou *et al.* 2014).

Level of miR-124a was found to be lower in RA patients. There are indications of this microRNA functioning as suppressor of cyclin-dependent kinase 2 and monocyte chemoattractant protein 1 production, having binding site in 3' UTR of their mRNA (Nakamachi *et al.* 2009).

Higher expression of miR-155 in RA patients was associated with repression of MMP-3 synthesis, thus suggesting involvement in destruction of extracellular matrix, mainly collagen in joints (Stanczyk *et al.* 2008). The upregulation of miR-155 appeared also in MS, where it targeted two sites in 3' UTR in CD47 mRNA. The CD47 serves as "don't eat me" signal for macrophages. Deregulation of this inhibitory signal thus allows destruction of myelin, carried out by macrophages (Junker *et al.* 2009). In model of autoimmune encephalomyelitis, knockdown of miR-155 expression resulted in decrease in number of Th1

and Th17 cells (Zhang *et al.* 2014). Suggesting similar function in MS, the miR-155 regulation has potential in MS therapy. Matrix remodeling in coeliac disease, another autoimmune disease, which affects small intestine, appears to be connected with regulation of cluster of microRNAs – miR-192/194 (Vaira *et al.* 2014).

Regulation function of microRNA in other epigenetic modifications is emerging in recent research. To achieve hypomethylation effect, several microRNAs are involved. The downregulation of DNMT1 level has been detected in correlation to increased levels of miR-21, miR-148a (Pan *et al.* 2010) and miR-126 (Zhao *et al.* 2011). However, these three microRNAs decrease DNMT1 level in their own way. Indirect increase in DNMT1 level is performed by miR-21, targeting autoimmune gene *RASGRP1*, whereas miR-148a and miR-126 target directly DNMT1 mRNA (see Figure 3).

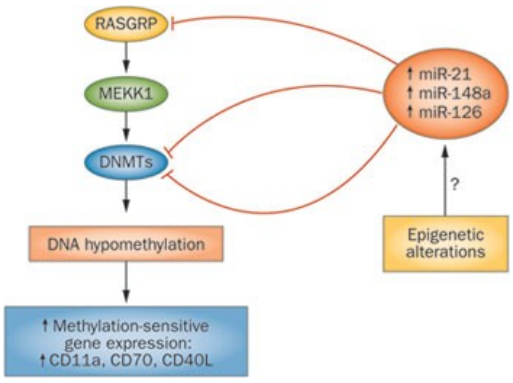

Figure 3: Regulation of DNMT function by microRNAs. Adopted from: (Shen *et al.* 2012)

In last few years, research of microRNA function in autoimmune diseases brought variety of deregulations in wide range of microRNA molecules; however, the targets of their regulation in autoimmune diseases are still unknown. Such examples may be seen in downregulation of miR-363 and miR-498 in CD4(+) T cells of RA patients (Li *et al.* 2010).

Not that all the epigenetic machinery was complicated enough, the different expression of microRNAs was shown in SLE patients with distinct types of autoantibodies. The patients with autoantibodies against dsDNA expressed higher levels of miR-155 and miR-146a, whereas patients with autoantibodies against RNA-associated antigens expressed miR-493 and miR-939 in higher levels (Chauhan *et al.* 2014).

6. DNA vs. Environment

The question of nature vs. nurture – their influence on human phenotype has been a dispute in scientific circles for a long time. The pattern of heredity discovered by Gregor Mendel, although brought to light 16 years after his death, inclined the general scientific opinion towards the "nature". Nevertheless, the concept of "nurture" influencing human development did not diminish. Numerous twin, starvation, viral and smoking studies have been carried out, indicating the environment playing an important role in human phenotype and diseases.

Although there has been evidence of correlation between the environmental factors and phenotype, an exact mechanism how external factors execute the modifications was missing until the discovery of epigenetics, which provides a new approach.

The concept of healthy diet has been part of general knowledge for a long time. Nowadays, besides effects of vitamins and various inorganic compounds in diet, epigenetics reveals new mechanisms of food affecting disease susceptibility. When administrating methyl donor-rich food to pregnant *Agouti* mice, increase of DNA methylation on long terminal repeat in viable yellow allele of *Agouti* gene occurs. This results in increase of darker brown coat phenotypes in offspring. The higher the methylation supplement, the higher number of dark brown coat phenotypes (Cooney *et al.* 2002). Methyl-deficient diet also appears to influence genes with oncogenic functions. The higher expression of genes encoding IGF2 (insulin-like growth factor 2) and H19 (small non-coding RNA) was associated with significant decrease in histone methylation of promoters of given genes (Dobosy *et al.* 2008).

Cruciferous vegetables contain sulforaphane, HDAC inhibitor, whose activity leads to cancer prevention (Horiuchi *et al.* 2009). Sulforaphane also appears to inhibit hyperplasia in synovial cells, T cell activation and TNF and IL-17 production in CD4(+) T cells in RA patients (Kong *et al.* 2010). Another HDAC inhibitor, butyrate, led to inhibition of cell proliferation in breast cancer. Vitamin A enhanced this effect significantly (Andrade *et al.* 2012). Curcumin, a substance found in plant *Curcuma longa* is often used in spice in Indian cuisine. Curcumin modulates transcription factor T-bet that plays role in differentiation of pro-inflammatory Th1 cells. Therapeutic effect of curcumin in T1DM was detected in murine model (Castro *et al.* 2014). Curcumin functions as HAT inhibitor and recently, its reducing effect on acetylation of H3 in *IL6* promoter was detected (Wada *et al.* 2014).

Not only diet, but also other environmental influences affect our susceptibility to diseases. Tobacco smoking, for example, lowers methylation in single locus of *F2RL3* gene

(Breitling *et al.* 2011). This gene encodes protease activated receptor 4 (PAR-4). Signaling through this receptor was detected in several clotting mechanisms, therefore its increased expression may lead to increase of cardiovascular disease.

As we can see, environment plays role in epigenetic changes in our body. While our bodies are subjected to outside world, our cells also undergo aging. The 52 % increase in methylation was detected in age-associated CpGs (Florath *et al.* 2014).

7. Current and Potential Treatment

Since the classification of the ADs, there have been numerous efforts to create an ultimate treatment to cause long-term remission in ADs. Such treatment is still unavailable, but new approaches as epigenetic treatment may lead towards the success in this goal.

The most frequent treatments used currently in RA patients include disease-modifying anti-rheumatic drugs (DMARDs), which function rather on cellular and cytokine level, reducing synovitis and systemic inflammation and appear not to have effect on epigenetic modifications. The most aggressive DMARD is methotrexate, which can be combined with other drugs to improve function. Methotrexate inhibits dihydrofolate reductase that is important component in *de novo* purine synthesis. Recently, a clinical trial showed that use of tofacitinib, a Janus kinase inhibitor, together with methotrexate, improved signs and symptoms of RA in 6 months with relatively manageable safety (Burmester *et al.* 2013). As the mechanism of methotrexate function includes inhibition of DNA and RNA synthesis, severe side-effects are part of therapy. It was originally developed as chemotherapeutic agent, so suppression of immunity is part of the treatment (Herman *et al*. 2004).

First approved anti-TNF drug is adalimumab. It is a monoclonal antibody, which binds to TNF-α, thus inhibiting pro-inflammatory reactions. There are now two other antibody-based anti-TNF drugs used in clinical practice - golimumab and infliximab. Adalimumab and golimumab are human immunoglobulins, whereas infliximab is a chimeric antibody from mice and human. Another chimeric monoclonal antibody rituximab, drug primary developed for cancer treatment, was repurposed for RA (Okada *et al.* 2014). Rituximab is an antibody recognizing B-lymphocyte antigen CD20, and the treatment ends up with elimination of B cells, highly activated in autoimmune response.

In addition to methotrexate and antibodies, non-epigenetic treatment includes several other approaches. One of them is based on inhibition of cyclin-dependent kinases (CDKs), which play role in cell cycle, division and differentiation of blood cells, including lymphocytes. Flavopiridol, a CDK6/4 inhibitor suppresses synovial hyperplasia and joint destruction in mouse models (Sekine *et al*. 2008). Besides the aforementioned, symptomatic treatment by steroidal and non-steroidal anti-inflammatory drugs is used in clinical practice.

Epigenetic treatment is yet in its beginnings and development of these drugs is still in trial phase. Inhibition of HDACs was mentioned above as mechanism of hyperacetylation in cells. Vorinostat (suberanilohydroxamic acid – SAHA) is HDAC inhibitor associated with NF-kappa B subunit p65 hyperacetylation. Its administration in non-obese diabetic mouse

(murine model of T1DM) reduced diabetes incidence by 38 % and increased pancreatic insulin content by 200 %. The study indicated immune response involving increase of transcription factors Gata3 and FOXP3, and decrease of pro-inflammatory cytokines IL-6, IL-12 and TNF-α (Christensen *et al.* 2014). Another HDAC inhibitor, entinostat (MS-275) has been shown to reduce inflammation reaction in rats with experimental autoimmune neuritis (Dai *et al.* 2010). These two HDAC inhibitors appeared to be related to suppression of p38 mitogen-activated protein kinase (MAPK) signaling pathway and chemotaxis (Choo *et al.* 2013). Selective HDAC6 inhibitor, tubastatin, also showed anti-inflammatory and anti-rheumatic effects in mice models, including inhibition of IL-6 production (Vishwakarma *et al.* 2013). Givinostat is another member of HDAC inhibitor family. Its activity was examined in experiments, trying to explore the mechanism of induction of T1DM by viral infection. The combined administration of givinostat and Killham rat virus in rat models resulted in protection from T1DM by preventing viral infection of pancreatic islets (Hara *et al.* 2014). The production of extracellular matrix, mainly collagen, was reduced by another HDAC inhibitor, trichostatin A in mouse model (Huber *et al.* 2007b). Trichostatin A also reduced hypoacetylation on H3 and H4, improving disease phenotype in murine lupus model (Garcia *et al.* 2005). Another effect of trichostatin A was detected in FLS and macrophages, where it significantly weakened IL-6 transcript stability (Grabiec *et al.* 2012).

Recently, hypomethylation agent 5-aza-2'-deoxycytidine exhibited therapeutic effects in murine models of multiple sclerosis. Treatment with this agent also caused hypomethylation in CpG island of *FOXP3* gene (Mangano *et al.* 2014).

The development of microRNA targeting drugs is highly in demand. The first drug of this kind, miravirsen was discovered targeting miR-122 expressed in liver. Use of miravirsen is currently in stage of clinical trials for treatment of infection by hepatitis C virus (Lindow and Kauppinen 2012). Therefore, drugs targeting microRNAs provide highly potential treatment opportunities in autoimmune diseases.

8. Conclusion

Understanding of autoimmune diseases is currently perceived in multiple levels. Epigenetic modifications appear to play significant role in pathogenesis of autoimmune disease. Impaired function of methylation enzymes provides accessibility for transcription machinery, what allows higher expression of cytokines, microRNAs and multiple transcription factors playing role in pathogenesis of autoimmune diseases. Not only demethylation is responsible for increased expression. Histone acetylation is another epigenetic mechanism contributing to pathogenesis. Higher susceptibility to autoimmune diseases in women appears to be caused by pathogenous factors encoded on X chromosome. Its double count in women presents double opportunity for epigenetic modifications activating their transcription. Furthermore, acetylation together with other histone modifications provides epitopes for autoantibody recognition. Variety of microRNAs has been discovered to be deregulated in autoimmune diseases. Although their function is still unclear, microRNAs appear to affect all levels of regulation. This relationship is, however, mutual. All epigenetic mechanisms seem to co-operate, creating a destructive autoimmune machinery complicating lives of millions of people. Although the level of regulation in autoimmune diseases is complex, epigenetics appears to explain relation of diet, smoking and other environmental factors to these diseases.

9. List of Used Abbreviations

ACPA	anti-citrullinated protein antibody
CD (e.g. CD40)	cluster of differentiation (e.g. cluster of differentiation 40)
CD40L	ligand to CD 40 receptor
CDK	cyclin dependent kinase
CXCL12	CXC-motif chemokine 12, stromal cell-derived factor 1
DMARD	disease modifying antirheumatic drug
DNMT	DNA methyltransferase
DR3	death receptor 3
FLS	fibroblast-like synoviocytes
FOXP3	forkhead box P3 - transcription factor scurfin
H2A/2B/3/4	histone type 2A/2B/3/4
H2BK12	lysine residue on position 12 on H2B type histone
H3K4me3	trimethylation on lysine residue on position 4 on H3 histone
HAT	histone acetyltransferase
HDAC	histone deacetylase
HERV	human endogenous retrovirus
HLA-DRB1/DPB1	human leukocyte antigen type DRB1/DPB1
IL (e.g. IL-6)	interleukin (e.g. interleukin-6)
IRAK-1	IL-1 receptor-associated kinase 1
IRF	interferon regulatory factor
MAPK	mitogen-activated protein kinase
MeCP	methyl-CpG binding protein
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
PAD	peptidylarginine deiminase
PBMC	peripheral blood mononuclear cell
PTPN22	protein tyrosine phosphatase non-receptor 22
SAM	*S*-adenosyl methionine
STAT	signal transducer and activator of transcription
Th1	T helper cell 1
TNF	tumor necrosis factor
UTR	untranslated region

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