The Effect of Biological Therapies on Epigenetics in Inflammatory Bowel Disease and Rheumatoid Arthritis

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24-04-2024

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Layman's Summary

Chronic inflammatory diseases such as inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) affect millions of people worldwide, and are characterized by chronic inflammation of variable body organs. This inflammation can be a result of the dysregulation of certain immune system components. To treat these diseases, biological therapies are used. These drugs target the immune cell components that are out of balance, and in turn cause a decrease in inflammation.

Epigenetic modifications play an important role in regulating the gene expression of the cell. The term 'epigenetics' includes any modifications to the DNA of the cell that does not alter the sequence. These modifications include the methylation of DNA, modifications to the histones, which are proteins around which the DNA is wrapped, and certain RNAs that can influence gene translation. It was shown that the epigenetics of IBD and RA patients differ from the epigenetics of healthy individuals, although it is not clear how these changes arise or what role they play in the development of disease. Recently, biological therapies have been found to influence the epigenetic modifications in cells, thereby further complicating the relationship between chronic disease and epigenetics.

In this review, multiple studies on epigenetic differences between chronic inflammatory disease patients and healthy individuals will be discussed, as well as studies on the effects of biological therapies on epigenetics in IBD and RA patients. It seems that biological therapies have the ability to alter the epigenetics of a chronically inflamed cell, however the exact effect on disease epigenetics is poorly characterized. Further researching these epigenetic effects of biological therapies, as well as the epigenetics of IBD and RA in general could help in understanding the potential effect of these medications on altering disease epigenetics and their impact on disease progression.

Abstract

Inflammatory diseases such as inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) are characterized by chronic inflammation as a result of immune system dysregulation. Epigenetic modifications of the genome are important for regulating gene expression. Epigenetic mechanisms include DNA methylation, histone modifications and non-coding RNA interference. Biological therapies, which are common drugs for treating IBD and RA, target specific immune system components and have recently been shown to alter the epigenetics of IBD and RA patients. In this review, the differences in epigenetics between healthy individuals and IBD or RA patients are discussed, as well as the epigenetic changes that arise when IBD and RA patients are treated with biological therapies. Furthermore, biological therapies show an ability to alter disease epigenetics. However, the small number of studies on these effects limits drawing conclusions. Increasing our knowledge on epigenetic changes as a result of IBD, RA or biological therapies could improve our understanding of the potential impact of these medications on altering disease progression and could aid in the optimization of treatment for chronic inflammatory disease patients.

Introduction

Biological therapies have gained widespread use in recent years for their effectivity in inducing and maintaining remission in chronic inflammatory diseases (Healy & Galvin, 2024). Worldwide, millions of people are affected by inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD), reducing their quality of life and contributing to financial distress (Kahn-Boesel *et al.*, 2023). Although different in location and pathophysiology, RA and IBD are both characterized by chronic inflammation due to abnormal activation of the immune system. Not only could untreated chronic inflammation be burdensome for the patient, it could also lead to healthy tissue damage and internal scarring.

A big factor in maintaining normal gene expression are epigenetic modifications. Epigenetics has been defined as the heritable changes to the genome that influence gene function and expression without altering the DNA sequence (Waddington, 2012). Heritability in this context alludes to the passing of epigenetic modifications from mother to daughter cell. When a gene is epigenetically modified, its expression is up- or downregulated, either directly through epigenetic actions preventing the gene from being transcribed, or indirectly by blocking mRNA translation (Brettingham-Moore *et al.*, 2015). Common epigenetic mechanisms include DNA methylation, histone modifications and the actions of micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs).

The most commonly researched epigenetic modification is DNA methylation. DNA methylation is the process of attaching a methyl group to cytosine residues in CpG dinucleotide sequences by DNA methyltransferases (DNMTs) (Bird, 1986). CpG methylation generally suppresses transcription, particularly when deposited on gene-regulatory sequences, but can do this in multiple ways (Weber *et al.*, 2007). Methyl groups on DNA can directly block DNA recognition and binding by transcription factors (TFs), or can recruit other cellular factors that subsequently block TFs. When these factors in turn recruit histone deacetylases (HDACs), deacetylation of the DNA-associated histone can promote chromatin condensation, further contributing to gene silencing (Nan *et al.*, 1998). DNA methylation can also have an activating function when it inhibits the recruitment and binding of gene repressors. In this way, DNA methylation functions as a dynamic epigenetic mechanism for gene regulation (Handy *et al.*, 2012).

Histones can be post-translationally modified on their tails by acetylation, methylation, phosphorylation or ubiquitination to influence chromatin structure (Kouzarides, 2007). Combinations of these modifications are suggested to form a ‘histone code’, thereby inducing downstream effects leading to chromatin repacking (Strahl & Allis, 2000). Further details such as nucleosome location on the gene and modification combination specifics add to the complexity of this epigenetic mechanism. Although not fully understood, some of the general mechanisms of histone modifications and their effect on gene expression have been explained. Acetylation by histone acetyl transferases (HATs) of lysines in H3 and H4 tails is associated with chromatin decompression and promoting gene transcription. Alternatively, deacetylation by HDACs promotes an inactive compact chromatin state. Histone lysine methylation is more complex, with mono-, di- and trimethylation showing no apparent pattern in promoting or repressing transcription. Common gene silencing modifications include H3K27me3 and H3K9me; common transcription permissive modifications are H3K4me3 and H3K36me3 (Handy *et al.*, 2012).

In addition to DNA methylation and histone modifications, non-coding RNAs also influence gene expression. Long non-coding RNAs (lncRNAs) commonly have gene silencing effects, through their recruitment of remodeling complexes that induce repressive histone methylation (Rinn *et al.*, 2007). They have also been found to interfere with histone deacetylation through recruitment of RNA-binding proteins (Mercer *et al.*, 2009). MicroRNAs (miRNAs) affect gene expression through RNA-interference pathways (Yao *et al.*, 2019). By binding to the untranslated regions of mRNAs, protein

translation is suppressed. A single miRNA can target multiple mRNAs, which allows for a wide range of effects on gene expression.

The etiologies of both IBD and RA are not fully understood, but are generally believed to be caused by a combination of genetic and environmental factors (Guo *et al.*, 2018; Zhang *et al.*, 2018). As gene expression is regulated in part by epigenetic modifications, epigenetics could also play a role in the pathogenesis of inflammatory diseases. Indeed, it has been found that epigenomes of patients differ from healthy individuals, but the epigenetic contribution to pathogenesis remains unclear (*et al.*, 2012; Lin *et al.*, 2012; Toussirot *et al.*, 2013; de Andres *et al.*, 2015; McDermott *et al.*, 2015).

As of yet, there is no cure for either IBD or RA, and so treatments are focused on inducing and maintaining remission of inflammation. This has conventionally been achieved using multiple immunomodulators, including aminosalicylates, corticosteroids and thiopurines. More recently, biological therapies have been approved to target specific immune system components like tumor necrosis factor (TNF) and interleukins, thereby controlling inflammation (Banerjee *et al.*, 2020; Findeisen *et al.*, 2021). Sometimes, a biological therapy is used in combination with an immunosuppressor such as a thiopurine, but different classes of biological therapies are usually not combined. Interestingly, it has been found that biological therapies can alter the epigenetics of the immune system in persons with inflammatory disease (Julià *et al.*, 2022; Lawal *et al.*, 2022; Lin *et al.*, 2023).

In this review, the effects of several biological therapies on the epigenome of IBD and RA patients will be discussed, as well as the implications this could have on the natural history of the disease. Epigenetic differences in IBD and RA patients as compared to healthy individuals have previously been identified, but their role in pathogenesis remains unclear. The epigenome-altering effects of biological therapies are of particular interest, especially the fact that only a few epigenetically altered genes have been confirmed by multiple studies. More research on epigenetics in inflammatory disease and the mechanisms behind epigenetic changes arising from IBD, RA and biological therapies is needed in order uncover the potential impact of epigenetic effects of biological therapies on disease progression, and to optimize personalized medical practices for chronic inflammatory diseases.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) encompasses a group of diseases that cause chronic (remittent) or progressive inflammation due to inappropriate immune responses (Kaser *et al.*, 2010). These diseases affect the gastrointestinal tract (GI), with symptoms ranging from abdominal pain to diarrhea and weight loss (Zeng *et al.*, 2019), and have been associated with an increased risk for developing colonic cancer (Kaser *et al.*, 2010). The etiology and pathogenesis of IBD still remain unclear, but data suggests that genetic, epigenetic, environmental, microbial and immunological factors are all involved (Zhang *et al.*, 2018). The two main subtypes of IBD include Crohn's disease (CD) and ulcerative colitis (UC).

Crohn's Disease

Crohn's disease is defined as a chronic idiopathic inflammatory bowel disease, and one of the two common subtypes of IBD. Crohn's disease affects the entire gastrointestinal tract and individuals with CD often have patchy inflammatory lesions scattered across the GI tract (Kobayashi *et al.*, 2020). Crohn's disease is characterized by periods of remission and flares and is divided into three phenotypes: inflammatory, stricturing and penetrating CD (Feuerstein & Cheifetz, 2017). Inflammatory CD causes transmural inflammation of all layers of the bowel wall and can lead to structuring CD, which is associated with fibrosis and digestive tract narrowing. Penetrating or fistulizing CD is marked by development of a sinus or fistulous tract (Kobayashi *et al.*, 2020; Fan *et al.*, 2023).

The current goal for CD treatment is remission maintenance, and depends on the severity, location and phenotype of the disease (Feuerstein & Cheifetz, 2017). Aminosalicylates, such as mesalamine, are used in mild CD to control inflammation and remission, although their effectivity has been disputed (Lichtenstein *et al.*, 2018). Corticosteroids are used to reduce immune system activity and induce remission, but long-term use is discouraged due to side effects. Therefore they are often used alongside thiopurines: remission is induced with a corticosteroid and afterwards maintained using thiopurines such as azathioprine or mercaptopurine (Present *et al.*, 1980; Chande *et al.*, 2015). Methotrexate has also been used to both induce remission and maintain it (Patel *et al.*, 2014), however as with corticosteroids and thiopurines, the use of this agent often results in multiple side effects (Steinhart *et al.*, 2003). Biological therapies for CD can be used alone or in combination therapies and include infliximab, adalimumab and certolizumab pegol, which are anti-TNFs (Present *et al.*, 1999; Hanauer *et al.*, 2006; Sandborn *et al.*, 2007; Colombel *et al.*, 2010). Anti-integrins such as vedolizumab and natalizumab are also options (Sandborn *et al.*, 2013; Nelson *et al.*, 2018), as well as ustekinumab, which is an anti-interleukin-12 and -23 (Sandborn *et al.*, 2008). Anti-TNF agents are most often used for treatment, as these have been suggested to be most effective and evoke relatively quick responses of less than 6 weeks (Feuerstein & Cheifetz, 2017). Despite these therapies, surgery is common in CD, with 80% of patients requiring a surgical procedure after 20 years of disease activity (Bernstein *et al.*, 2012). Stricturing and fistulizing CD in particular can cause complications that will require surgery. Ultimately, there is no cure for CD yet (Feuerstein & Cheifetz, 2017).

Ulcerative Colitis

Ulcerative colitis, similar to Crohn's disease, is a chronic idiopathic inflammatory bowel disease and one of the two common subtypes of IBD. Ulcerative colitis affects the GI tract, particularly through mucosal inflammation in the rectum, which can expand into the colon in a continuous fashion (Kobayashi *et al.*, 2020). Inflammation in UC is limited to the mucosal wall, resulting in mostly superficial damage, as compared to CD where all layers of the bowel wall are affected. This results in multiple symptoms of which bloody diarrhea is most common. UC severity can differ, and is defined as proctitis when inflammation is limited to the rectum (Kayal & Shah, 2019). When UC extends throughout the colon up until the left (splenic) flexure, it is termed left-sided UC. Inflammation can further expand beyond this point, after which it is referred to as extensive colitis, and can affect the entire colon.

Therapy and management of UC is similar to DC, with disease severity affecting treatment options (Kobayashi *et al.*, 2020). Aminosalicylates, corticosteroids and thiopurines are conventionally used for inducing and maintaining remission. In recent years, an increase in the use of biological therapies has resulted in combinatorial therapy and therapy based solely on biologicals (Colombel *et al.*, 2010). Anti-TNF agents for UC include infliximab and adalimumab, akin to CD, as well as golimumab (Shealy *et al.*, 2010). Similar to CD, vedolizumab and ustekinumab are used in therapy as anti-integrin and anti-interleukin-12, respectively, as well as the Janus kinase (JAK) inhibitor upadicitinib. For UC, two additional JAK inhibitors have been approved: tofacitinib and filgotinib (Feagan *et al.*, 2021; Flanagan *et al.*, 2010). Cyclosporine, a calcineurin inhibitor, is also used in combinatorial therapy to downregulate certain interleukins and TNFα (Lichtiger *et al.*, 1994; Kayal & Shah, 2020).

Epigenetic Studies on Inflammatory Bowel Disease

Genetic factors explain only a small portion of the disease variance and genetic variance in disease risk in IBD, suggesting other factors play important roles (Lin *et al.*, 2012; Jostins *et al.*, 2012; Zeng *et al.*, 2019). Environmental factors, such as diet, can modulate the gut microbiome by targeting epigenetics (Aleksandrova *et al.*, 2017). In turn, epigenetics could have a significant impact on the pathogenesis of IBD, or could be implicated in the etiology of these diseases. Therefore, it is important to determine whether there are significant differences in the epigenome between patients and healthy individuals to characterize the potential role of epigenetics in the pathogenesis of IBD.

McDermott *et al.* (2015) performed a study on genome-wide DNA methylation changes on DNA extracted from peripheral blood mononuclear cells (PBMCs) of IBD patients. When looking at a regional level, 7 differentially methylated regions (DMRs) in IBD were identified compared to healthy controls, of which 5 DMRs were specific for CD and 2 DMRs were mutual to both CD and UC. One of these mutual DMRs was hypomethylated across 11 CpG sites in the *TRIM39-RPP21* promoter region. When investigating individual CpG sites, including CpGs in the identified DMRs, ~3.2k sites for CD and ~1.5k sites for UC were found differentially methylated as compared to controls. Affected pathways included immune response and T-cell activation regulation. Overlap between these groups of CpG sites was significant: 97% of the UC-associated differentially methylated positions (DMPs) were also differentially methylated in CD. In contrast, only 55% of CD-associated DMPs belonged to this overlapping group. Of interest, the top-ranked DMP for both CD and UC was located in the *TIFAB* gene, and was hypermethylated for both diseases. TIFA-mediated TRAF6 activation can lead to downstream inflammatory cytokine secretion, and this process is inhibited by TIFAB (Wang *et al.*, 2020). Hypermethylation of the *TIFAB* gene, which is a silencing DNA modification, could then contribute to the inflammatory cell phenotype that is characteristic of IBD.

A caveat of this study is that the analyzed DNA originated from PBMCs, whereas IBD largely affects the epithelial tissues of the GI tract. Therefore studies in intestinal tissues and mucosa might provide a more accurate overview of differential epigenetics in CD and UC. Intestinal tissue was used in the study of Lin *et al.* (2012), where the researchers analyzed 1505 CpG sites that represented 807 genes. They found a total of seven loci that had IBD-associated differential methylation, and observed that DNA methylation of these loci differed between CD and UC patients. Genes affected by these DMPs included *STAT5A* and *TNFRSF1A*, which are both associated with inflammatory cytokines. Cooke *et al.* (2012) performed a more wide-spread approach by analyzing DNA from rectal biopsies using genome-wide methylation profiling. By comparing inflamed and noninflamed cases of CD and UC with controls, they were able to identify dozens of differentially regulated genes, with several genes showing overlap between CD and UC patients, including *DOK2* and *COG8* which play roles in interleukin signaling and intracellular trafficking, respectively. Interestingly, noninflamed CD cases were collected from sites with no prior inflammation, and showed no differential methylation compared to controls. In contrast, noninflamed UC cases originated from sites where inflammation had occurred previously, and showed a differential methylation footprint. Due to this, Cooke *et al.* hypothesize that

inflammation could be the cause for methylation changes, and that this methylation could leave the epithelium more vulnerable to future inflammation (Cooke *et al.*, 2012).

Apart from methylation differences, it has been found that some miRNAs are also differentially regulated in IBD compared to healthy individuals (Zeng *et al.*, 2019). For example, IBD patients show an increase in expression of miR-21, which is associated with UC pathophysiological processes including Th2 cell differentiation and the disruption of the intestinal epithelial barrier (Kalla *et al.*, 2015; Zeng *et al.*, 2019). Furthermore, miR-21 is implicated in the IL-23/Th17 signaling pathway, which has been reported to contribute to CD pathogenesis by promoting the production of inflammatory cytokines (Brand, 2009). Autophagy has also been shown to contribute to the IBD pathogenesis, and studies show active roles for miRNAs in this process (Hooper *et al.*, 2017). miR-106b levels are increased in CD patients and this miRNA is claimed to target *ATG16L1* mRNA, leading to suboptimal autophagy-mediated bacteria removal (Lu *et al.*, 2014; Zeng *et al.*, 2019). In addition, miR-196, which is overexpressed in CD patients, disturbs the regulation of *IRGM*, which in turn leads to a decrease in autophagy efficiency (Brest *et al.*, 2011; Zhang *et al.*, 2018). In this way, dysregulation of miRNAs in IBD patients can contribute to disease pathogenesis, and could provide a target for potential therapeutic options.

Taken together, these studies show differential epigenetics between healthy individuals and IBD patients. Both CD and UC are subtypes of IBD and therefore often grouped and studied in parallel, yet results suggest that epigenetics differ between the two. Furthermore, although both diseases share pathophysiological features, their distinction in clinical diagnosis is important for appropriate treatment and long-term prognosis (Farmer *et al.*, 2000). Therefore, it is important to study and discuss CD and UC individually. Here, studies on differential epigenetics between healthy persons and patients with CD or UC will be discussed, as well as the effects of biological therapies on the epigenetics in CD and UC patients that have been identified thus far.

Epigenetic Studies on Crohn's Disease

Many epigenetic studies on Crohn's disease have been compactly summarized in a review by Hornschuh *et al.* (2021). Most epigenetics studies in CD patients have focused on DNA methylation using epigenome-wide association studies (EWAS). DNA methylation in blood samples have included analyses on whole blood samples as well as a range of blood cell types such as peripheral blood mononuclear cells (PBMCs) and T cells. Many DMPs have been found across these studies, affecting multiple genes (Lin *et al.*, 2012; Serena *et al.*, 2020). For example, *TNF* was found both hypomethylated and hypermethylated, and *IL-10* was found hypermethylated in Crohn's patients as compared to healthy controls (Adams *et al.*, 2014; Li Yim *et al.*, 2016; Moret-Tatay *et al.*, 2019). However, comparing studies is often difficult as DNA methylation is affected by age, gender and environmental factors, leading some studies to exclude patients within a certain age range or of a certain sex (Hornschuh *et al.*, 2021).

Furthermore, DNA methylation studies have also been performed on intestinal biopsies as these are the main tissues being affected in CD. Samples from both pediatric and adult patients have been analyzed and compared. Pediatric patient samples are particularly interesting as these patients often don't have a long history with medication as adult patients, and so might reflect the natural disease state more (Hornschuh *et al.*, 2021). In newly diagnosed pediatric patients, several immune-relevant genes were found hypermethylated, including mucin (*MUC2*) and polymeric immunoglobulin receptor (*PIGR*) (Kraiczy *et al.*, 2016). In adult patients, autophagy activating kinase 1 (*ULK1*) has been found in a hypermethylated state (Cooke *et al.*, 2012). In CD, autophagic dysfunctions can play a role in pathogenesis, and ULK1 downregulation could be linked to this (Henderson & Stevens, 2012). Fibrosis-associated complications have also been linked to an altered DNA methylome, with a downregulation of certain WNT-signaling pathway agents (Sadler *et al.*, 2016). Disruption of WNT-

signaling interferes with the promotion of epithelial regeneration by stem cells in intestinal crypts, and thus possibly contributes to chronic inflammation (Koch, 2017).

Other epigenetic alterations such as histone modifications have been less extensively studied. One study found that H3K4 is trimethylated under influence of alterations of the microbiome by CD (Kelly *et al.*, 2018). Affected genes were involved in cytokine signaling, metabolism, homeostasis and reactive oxygen species. Further studies in animal models suggest upregulation of H3K27 and H4 acetylation in inflamed intestinal tissues, although this has not been confirmed in human samples (Tsaprouni *et al.*, 2011; Chen *et al.*, 2019).

Effects of Biological Therapies on Epigenetics in Crohn's Disease

Only a handful of studies have looked into the effects of biological therapies on epigenetics in CD, as many biologicals have only recently become widely used and their disease-modifying effect is not yet widely known or understood. A recent study looked into the effects of anti-TNF agents infliximab and adalimumab on DNA methylation in CD patients (Lin *et al.*, 2023). Whole blood DNA samples were collected at baseline and after 14, 30 and 54 weeks of treatment to determine temporal effects of these drugs on DNA methylation. Patients who experienced primary non-response at week 14 and who were not in remission by week 30 or 54 were compared to patients with a response at week 14 and who were in remission at week 30 or 54. 4999 differentially methylated positions (DMPs) were identified and annotated to 2376 genes. These DMPs were associated with anti-TNF treatment regardless of response. This highlights the DNA methylome profile associated with TNF treatment regardless of therapy response. The top-ranked DMP was located in the *SOCS3* gene, which plays a role in the regulation of the JAK-STAT pathway and in modulating infections and autoimmune diseases. Further implicated biological pathways included the immune response, immune system development, blood cell differentiation and hemopoiesis. SOCS3 has previously been implicated in the pathogenesis of IBD and has been found to be differentially regulated in IBD patients, corroborating these results (Li *et al.*, 2012; McDermott *et al.*, 2015). Interestingly, out of the ~5000 identified DMPs, only 13 were differentially methylated between infliximab and adalimumab treatments, suggesting both drugs exert similar effects on levels of DNA methylation (Lin *et al.*, 2023).

In the study of Lin *et al.*, (2023), the second-most differentially methylated position after anti-TNF treatment was located in the gene of *RPS6KA2*. RPS6KA2 is a ribosomal S6 kinase that phosphorylates various substrates, including MAPK signaling pathway components, and influences cell growth and differentiation as well as autophagy. Interestingly, the methylation status of RPS6KA2 was previously found to be increased in IBD patients in comparison to healthy individuals (Zeng *et al.*, 2019; Hornschuh *et al.*, 2021). Although there is no mention of whether methylation changes of *RPS6KA2* as a result of anti-TNF treatment leads towards a more healthy phenotype or not, it is noteworthy that this gene has been implicated in the differential epigenetics of CD patients. Other genes identified in this study that were previously found to be differentially methylated in IBD include *TOM1L2*, *SBNO2* and *PAG1*, which have functions in protein transport, JAK-STAT signaling and T cell activation, respectively (McDermott *et al.*, 2015; Lin *et al.*, 2023).

Epigenetic Studies on Ulcerative Colitis

Epigenetics has been studied quite widely in ulcerative colitis, with most studies focusing on DNA methylation (Yan *et al.*, 2023). Peripheral blood DNA methylation analysis suggests that multiple genes implicated in immune system functions are hypermethylated in UC patients, including *CXCL5*, *CXCL14*, *IL4R* and *IL17C* (Karatzas *et al.*, 2014). Intestinal biopsies also show differences in gene-specific methylation between UC patients and healthy controls for *SPINK4*, a serine protease inhibitor, complement factor *CFI* and adhesion molecule *THY1* (Häsler *et al.*, 2012). A study on treatment-naïve mucosal biopsies found hypermethylated genes related to homeostasis and defense, and hypomethylated genes associated with immune responses (Taman *et al.*, 2018). Interestingly, certain gene-specific methylations have been found to mark early disease progression: abnormal methylation

of the tubulin protein-encoding *TUBB6* gene marks invasive UC (Beggs *et al.*, 2018). Corroborating this, elevated levels of DNA methyltransferase can be observed in early UC-associated colorectal cancer, underlining the importance of DNA methylation in UC pathogenesis (Fujii *et al.*, 2010; Yan *et al.*, 2023).

Other epigenetic mechanisms have been studied to a lesser extent (Yan *et al.*, 2023). However, studies have indicated that certain genes are differentially regulated as a result of histone modifications. One such study found neuropeptide S receptor 1 (*NPSR1*) expression increased due to H3K27 acetylation (Sarvestani *et al.*, 2018). Interestingly, the *SOCS3* gene is inhibited through H3R8 asymmetric demethylation in UC mouse models, whereas in CD SOCS3 has been found to be upregulated (Li *et al.*, 2012; Li *et al.*, 2022). Studies on microRNAs and long non-coding RNAs also show the importance of these molecules in UC pathogenesis. miR-24 was found upregulated in UC patient intestinal biopsies, where it functions by downregulating mRNA and protein expression of cingulin, a tight junction-associated protein (Soroosh *et al.*, 2019). In this way, miR-24 impairs the formation of the intestinal epithelial barrier, thereby contributing to inflammation. UC-related lncRNAs such as *ZFAS1* and *MIR4435-2HG* are possibly regulated by upstream DMRs, and can affect downstream inflammatory immune responses (Fenton *et al.*, 2023).

Effects of Biological Therapies on Epigenetics in Ulcerative Colitis

There are limited studies about the effect of biological therapies on ulcerative colitis. A pilot study on colon biopsy samples assessed whether response to treatment in UC could be associated with differential DNA methylation patterns in mucosal tissue (Shah & Shen, 2020). DNA methylomes of responders were compared to those of non-responders after 14 weeks of treatment with biological therapies. However, no mention was made of which biological treatments were used, and so it could be that the researchers look into methylation changes resulting from multiple agents in this study. Alongside findings on differences in microbiota composition, responders to biological therapies were found to have increased methylation in *P16*, *HOXC5* and *B4GALNT1* genes, as compared to non-responders (Shah & Shen, 2020). These genes are implicated in pathways such as cell cycle control, morphogenesis and angiogenesis, respectively (Seifert *et al.*, 2015; Sen *et al.*, 2017; Yoshida *et al.*, 2020). Another study has looked more specifically into the effect of anti-TNF agents infliximab and vedolizumab (Lawal *et al.*, 2022). DNA methylation differences between responders and non-responders to these drugs were compared and allowed for confirmation of the presence of differentially regulated disease-associated genes, although these were not mentioned within the results. However, subsequent upstream analysis revealed that these genes are regulated by master regulators (MRs), such as chemokine ligands and receptors, and likely regulated through transcription factor-mediated signaling. In addition, the authors also mention significant differences in DNA methylation between UC patients on aminosalicylate maintenance treatment and patients undergoing anti-TNF/immunomodulator treatment against progressive disease (Lawal *et al.*, 2022).

Unfortunately, neither Shah & Shen nor Lawal *et al.* (2022) published full lists of the analyzed genes and their methylation status differences. From the genes mentioned, none matched between the two studies and none have overlapped with UC-associated differentially methylated genes identified in previous studies. Of note, McDermott *et al.* (2015) identified multiple differentially methylated genes in CD patients, of which the methylation status was altered in CD patients after biological therapy treatment. In contrast, although McDermott *et al.* (2015) also identified such genes in UC patients, none of these genes have been found to be altered in their methylation by biological therapies in UC patients. It is likely that biological therapies used to treat UC have the ability to alter methylation, akin to biological therapies used for CD, but that there have been too few studies on this to identify these methylation changes.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affecting primarily synovial joints. The etiology of RA is unknown, but is likely influenced by both genetics and environmental factors (Guo *et al.*, 2018). Inflammation typically starts in peripheral joints and can progress towards proximal joints. If left untreated, joints can be destroyed due to cartilage loss and bone erosion, an irreversible process. Treatment can stop the progression of RA and induce remission, but patients can experience flares. When symptoms of RA last for fewer than six months, it is termed early RA, whereas a longer duration of symptoms is referred to as established RA (Guo *et al.*, 2018; Bullock *et al.*, 2019).

As with IBD, there is no cure for RA but remission is achievable. Conventionally, non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids were used to induce and maintain remission (Findeisen *et al.*, 2021). At the end of the 20th century, conventional synthetic disease modifying anti-rheumatic drugs (csDMARDs) became the standard therapy option and can be used in combinatorial treatment (Padjen *et al.*, 2020). Biological disease modifying anti-rheumatic drugs (bDMARDs) are biological therapies specifically targeting key mediators of inflammation. Anti-TNFs used to treat RA include infliximab, adalimumab, golimumab and certolizumab pegol, which are also used in the treatment of IBD. In addition, etanercept is a TNF receptor which works similar to monoclonal antibodies through competitive inhibition of TNF (Haraoui & Bykerk, 2007). CD80 and CD86 co-stimulation inhibitor abatacept is used to bind CD80 and CD86 on antigen presenting cells, to inhibit T-cell activation (Genovese *et al.*, 2005). Cytokine interleukin-1 can be antagonized using anakinra, and interleukin-6 by tocilizumab and sarilumab (Nishimoto *et al.*, 2007; Mertens & Singh, 2009; Huizinga *et al.*, 2014; Findeisen *et al.*, 2021). B-cells and their antibody production are targeted by the chimeric monoclonal antibody rituximab, which binds CD20 on B lymphocytes (Edwards *et al.*, 2004). JAK inhibitors have also been used to treat RA (Findeisen *et al.*, 2021). Surgeries to improve quality of life can be performed although this does not heal any damage obtained from RA inflammation (Simmen *et al.*, 2008).

Epigenetic Studies on Rheumatoid arthritis

Compared to healthy controls, blood samples of RA patients have previously been found to have global DNA hypomethylation in T-cells and monocytes, and altered DNA methylation in B-cells (de Andres *et al.*, 2015; Glossop *et al.*, 2016; Nemtsova *et al.*, 2019). DMPs in B lymphocytes were located in multiple genes, including *CD1C*, *TNFSF10* and *PARVG* (Julià *et al.*, 2017). More specifically, Liu *et al.* (2013) found 10 DMPs within the gene region of the major histocompatibility complex (*MHC*). This suggests that DNA methylation could affect the susceptibility of individuals to RA. Alongside blood samples, fibroblast epigenetics have been studied in quite some detail due to their importance in RA pathogenesis: these cells in particular are the cells that maintain the chronic inflammation found in RA (Turner & Filer, 2015). Hypomethylation was observed for DMPs located in genes like *CHISLI*, *CASP1* and *STAT3*, and hypermethylation in genes *TGFBR2* and *FOXO1* (Nakano *et al.*, 2013). Analyzing the implicated pathways, these differentially methylated genes affected cell migration, adhesion, trans-endothelial penetration and extracellular matrix interactions (Nakano *et al.*, 2013). Interestingly, data suggest that RA fibroblast DNA methylomes are altered during disease development and in early to chronic stage disease progression (Whitaker *et al.*, 2013).

Not many studies have looked into histone modification in RA, but it has been suggested that histone deacetylases are expressed at a higher level in PBMCs of RA patients compared with healthy controls (Toussirot *et al.*, 2013; Nemtsova *et al.*, 2019). This was corroborated in RA synovial tissue, where *HDAC1* expression was found upregulated (Huber *et al.*, 2007). Surprisingly, a different study found that *HDAC1* and *HDAC2* gene expression in synovial tissue is decreased, resulting in reduced HDAC levels and activity (Kawabata *et al.*, 2010). Along with acetylation marks, evidence for trimethylation of H3K4 and H3K27 has also been observed, leading to differential regulation of matrix

metalloproteinases (MMPs) (Maciejewska-Rodrigues *et al.*, 2010). These MMPs have also been found to be influenced by miRNA-146 and miRNA-155, which are overexpressed in fibroblasts and synovial fluid of RA patients (Long *et al.*, 2013). Other miRNAs have also been found to be increased in RA fibroblasts, affecting the JAK-STAT and WNT-signaling pathways (Miao *et al.*, 2015; Zhou *et al.*, 2015). lncRNA *ZFAS1* levels are increased in RA synovial tissue and affects miRNA-27a, thereby indirectly affecting RA fibroblast migration and invasion (Ye *et al.*, 2018).

Effects of Biological Therapies on Epigenetics in Rheumatoid Arthritis

As with IBD, not a lot of studies have looked into the effects of biological therapies on epigenetics in RA. Julià *et al.* (2022) performed DNA methylation studies on RA patients before and during anti-TNF treatment with either adalimumab, certolizumab, etanercept, golimumab or infliximab. A pathway analysis was performed on biological processes associated to RA. These included T-cell activation, GTPase signaling and actin cytoskeleton changes. Of the 246 associated processes, 144 were found differentially methylated after 12 weeks of anti-TNF treatment, with additional differences in 56 pathways between responders and non-responders. Of importance, all methylation changes were found to be towards the direction of healthy individuals, suggesting a possible mechanism of disease-related methylation reversal for anti-TNF agents (Julià *et al.*, 2022).

In another study, the effect of ruxolitinib on histone modifications in juvenile idiopathic arthritis (JIA) synovial fluid monocytes was investigated (Peeters *et al.*, 2023). These monocytes can be used as a model for studying inflammatory arthritis, which includes RA. Treatment of these monocytes with ruxolitinib, a JAK inhibitor, resulted in ~450 significant changes in the H3K27 acetylation epigenome, with most changes reducing this acetylation signal. Genes associated with this acetylation decrease are involved in IFN as well as JAK-STAT signaling. These genes included *STAT3*, and *TNF*, which were previously found to be differentially methylated in RA patients (Nakano *et al.*, 2013). To extend their observations to RA the authors assessed RA synovial fluid monocytes through gene set enrichment analysis, which revealed that the inflammatory phenotype of these cells is inhibited and that genes associated with an increased H3K27ac signal in inflammatory monocytes are decreased by ruxolitinib (Peeters *et al.*, 2023). Ruxolitinib is currently not used as an agent against RA, but has been approved for use in treating atopic dermatitis, a chronic inflammatory disease that affects the skin (Melki & Frémond, 2023). A clinical trial on the use of ruxolitinib for RA has been performed but has not been published (Bonelli *et al.*, 2024). Although ruxolitinib is not actively used as an agent against RA in clinical settings, Peeters *et al.* show that JAK inhibitors could have epigenetic modulation potential in patients with RA.

Whole blood samples of RA patients have also been analyzed for differential levels of miRNAs (Castro-Villegas *et al.*, 2015). Following combination treatment with anti-TNFs infliximab, etanercept or adalimumab, the expression of 75 miRNAs was increased, and of 9 miRNAs expression was decreased. Targets of these molecules included mRNAs involved in immune and inflammatory responses and pathways of STAT3 and IL-6 signaling. This altered miRNA regulation was only seen in responders, and was paired with a decrease in levels of multiple cytokines, including TNFα, IL-6 and IL-7 (Castro-Villegas *et al.*, 2015). In addition, direct targets of identified miRNAs included *PRKCE* and *BCL-2*, which were found to be differentially methylated in RA and are involved in cell death, among other pathways (Nakano *et al*., 2013).

One of the miRNAs found to have differential expression was miR-146a (Castro-Villegas *et al.*, 2015). After 6 months of anti-TNF treatment, levels of miRNA-146a were increased in responders versus non-responders. Previous research has indicated miRNA-146a is downregulated in RA patients, leading to regulatory T cells to adopt a more pro-inflammatory phenotype and thereby contributing to RA inflammation (Long *et al.*, 2013; Zhou *et al.*, 2015). Consequently, anti-TNF treatment could influence inflammation through modulating the expression of certain miRNAs in some way.

One of the direct targets of the identified miRNAs affected by infliximab treatment in the study of Castro-Villegas *et al.* (2015) is IL6R, involved in cytokine signaling. IL6R levels were also found to be affected by histone modifications upon ruxolitinib treatment (Peeters *et al.*, 2023). Here, ruxolitinib reduced H3K27 acetylation, which is a marker for DNA that is actively being transcribed. One of the genes affected by this downregulation of histone acetylation was *IL6R*, which in turn could lead to reduced cytokine signaling and reduced inflammation. As miRNAs often have repressing effects on the mRNAs of their target genes, the specific miRNAs and histone modifications found here could work *in tandem* to reduce the expression of *IL6R*. Interestingly, ruxolitinib is a JAK inhibitor whereas infliximab is an anti-TNF agent. Although they target different immune system components, they both seem to cause changes in the gene expression of *IL6R*. This suggests that different biologicals can modulate disease in a similar fashion by affecting the same genes.

Discussion

Multiple studies have shown that epigenomes differ between IBD or RA patients and healthy controls (Nemtsova *et al.*, 2019; Zeng *et al.*, 2019; Hornschuh *et al.*, 2021). In addition, treatment of these patients with biological therapies shows epigenome altering, suggesting that these treatments could impact the natural history of the disease through modifying the disease course (Castro-Villegas *et al.*, 2015; Shah & Shen, 2020; Julià *et al.*, 2022; Lin *et al.*, 2023). However, there are currently not a lot of studies to corroborate this, nor is there a general understanding of the role of epigenetics in IBD and RA: whether epigenetic differences between healthy individuals and inflammatory disease patients underlie disease pathogenesis or are a result of the disease remains unclear. Nevertheless, if there is a dysregulation on an epigenetic level that characterizes the disease, therapeutic correction of this epigenetic profile back to a normal condition, regardless of how these disease-related epigenetic alterations arose, is an interesting goal to achieve. This could result in a less aggressive disease phenotype and less flares. As epigenetic modifications are heritable, treatment on an epigenetic level could change how the disease manifests itself.

The mechanism of how epigenetics contribute to the pathology of IBD and RA remains unclear, but can be speculated upon. If the epigenome influences the pathogenesis of IBD and RA, individuals can possess epigenomes that are predisposed towards developing an inflammatory phenotype, and possibly subsequently IBD or RA. In this way, the epigenome plays an active role in the development of the disease. It could also be that patients display epigenomes that differ from healthy individuals as a result of the disease altering the epigenome. For instance, prolonged or chronic inflammation can lead cells to alter their gene expression through epigenetic mechanisms, resulting in altered epigenomes. This seems to be a plausible explanation, as Cooke *et al.* (2012) found that noninflamed CD cases collected from sites with no previous inflammation showed no differential methylation compared to controls, whereas noninflamed UC cases collected from sites with prior inflammation did show differential methylation. To confirm this, a study on noninflamed cases of CD and UC from sites with and without prior inflammation could be performed.

As with the mechanism of how epigenetics contribute to pathogenesis, it is also unclear how the biological drugs discussed here influence the epigenetics in patients with IBD or RA. These biological therapies target certain immune system components, most often by binding and sequestering these molecules to disrupt signaling cascades and thereby reducing inflammation. It could be that in response to this reduction in inflammation, the cell changes its gene expression and the regulation of this expression. This would include changes in epigenetics, leading biologicals to influence epigenetics in an indirect manner. If this were to be true, biological therapies would likely only alter the epigenome in patients who respond to the treatment and experience a reduction in inflammation. However, Lin *et al.* (2023) show that epigenetic changes upon infliximab or adalimumab treatment occur in both responders and non-responders, suggesting that there is more at play here. In order to understand exactly how these biological drugs function, the mechanism of epigenetic altering should be further investigated.

Not only is there not much known about the mechanism underlying epigenetic effects of biological therapies, it is also unclear whether altering the epigenome through biological therapy functionally contributes to the therapeutic efficacy of these drugs. One study reports biological drug-associated epigenetic alterations back towards an epigenome that resembles the epigenome of a healthy individual (Julià *et al.*, 2022). Whether this contributes to a less aggressive disease manifestation remains unclear, but it was reported that this epigenome 'reversal' was only observed in responders to the treatment used. This suggests that the transformation of the epigenome towards a more healthy state is paired with a more remission-like disease phenotype, and thus possibly influences the disease phenotype. However, it should not be forgotten that some studies have also found biological therapies to alter the epigenome in patients that were classified as non-responders.

In addition, whether biological therapy-associated alterations to the epigenome are stable and inherited from cell to cell is unclear, and should be further researched. For example, changes in the epigenome as a result of biological therapies could remain stable over time, or could convert back to a pre-treatment state. Similarly, it could be that during treatment the disease might show a remission-like phenotype, but stopping the treatment could result in a big flare and a persistent phenotype of the disease.

Adding to this, there is still an unclear relation between responders and non-responders to biological therapies. Castro-Villegas *et al.* (2015) and Julià *et al.* (2022) observed differences between responders and non-responders, as well as between these patients and healthy individuals. In contrast, Lin *et al.*, (2023) found biologicals to alter epigenetics regardless of whether there was a response to the treatment. This is significant, as Shah & Shen (2020) rely on the distinction between responders and non-responders in their analyses. They found differences between these two patient groups, but did not put this in context against healthy controls. This difference between responders and non-responders once again brings into question the mechanism of how epigenetic differences between patients and healthy persons arise: do these biological drugs have an effect on the epigenetics, or are the epigenetics already different and potentially a factor in whether a patient is a responder or non-responder?

This review has looked into independent studies on both epigenetic alterations found in patients of inflammatory diseases in comparison to healthy individuals, and epigenetic alterations found in patients after biological therapy treatment in comparison to before treatment. Although individual genes have been identified in both types of studies, few genes overlap between the two. For CD, only *RPS6KA2* was found altered in multiple epigenetic studies and after treatment with anti-TNF agents (Zeng *et al.*, 2019; Hornschuh *et al.*, 2021; Lin *et al.*, 2023). For UC no overlapping genes were found between genes identified in studies on epigenetic changes between UC patients and healthy controls and genes identified in studies on patients treated with biological therapies. In RA, miRNA-146a was associated with RA and was altered following biological treatment, as well as one of its targets, namely *IL6R* (Castro-Villegas *et al.*, 2015; Peeters *et al.*, 2023). It is unclear why so little overlap is found between the studies. As many epigenetic alterations in inflammatory disease patients are found to contribute to inflammation, it would be expected that epigenetic alterations associated with a biological treatment that induces remission also affect these types of genes to bring inflammation back down. However, very little overlap between these genes suggests this might not be the case. Instead, the genes affected by epigenetic alterations in both situations might belong to a more random selection. It is important to mention that some studies have not published full lists of genes that were found altered, making it impossible to perform full analyses on these studies.

The group of genes identified in studies on epigenetics in IBD and RA show little overlap with the group of genes identified in studies on the epigenetic effects of biological therapies in these diseases. Similarly, there is little overlap between the genes found in the different studies on the epigenetic effects of biological therapies. This makes it hard to conclude whether the changes induced by biological drugs are common to all patients, as the studies mentioned here do not show genes affected in all studies, nor are there more studies to confirm the findings of the few reported studies. Although more studies are needed to look into this, one possible explanation is that epigenetic altering effects of biologicals are not targeted towards certain genes, but instead are a result of a more global influence on the epigenetic mechanisms of the cell that then results in a seemingly random selection of genes of which the epigenetics are altered.

Complicating the comparison between studies on the epigenetic altering effects of biologicals is the fact that they use different methods. Looking at DNA methylation in particular, a lot of DNA methylation methods only analyze a small percentage of the genome. Not only can the percentage of the genome that is analyzed vary between studies, which exact parts are analyzed can also differ. It

could be that one study looks at different genome regions, identifying genes that do not overlap with genes identified by a second study, simply because the first study did not look at any of the genes analyzed by the second study. For example, Julià *et al.* examine only ~2% of the genome by examining one or more CpG per gene, meaning that a large portion of the genome is simply not looked into, even though relevant changes in methylation could reside in these regions. This in turn could explain why the studies discussed in this review do not show much overlap in the genes for which the researchers have identified a difference in methylation status. A possible method to overcome this issue is whole genome bisulfite sequencing, although this might not be cost-effective and therefore not preferred when designing experiments. Adding to the difficulty in comparing studies is the biological drug the study is investigating. The specific biological that is inducing these changes in the epigenetics of the identified genes could also play an important role: it is possible that each biological drug has a specific group of genes that are more prone towards being altered in their epigenetics, likely due to the mechanism of action of that particular biological. However, ruxolitinib, a JAK inhibitor, and infliximab, an anti-TNF agent, have been shown to induce different epigenetic changes in RA resulting in a change of IL6R levels, suggesting that multiple different biologicals can have an impact on the epigenetics of a singular gene (Castro-Villegas *et al.*, 2015; Peeters *et al.*, 2023). Still, there are little studies on the epigenetic changes induced by biologicals that look into the same drug, and so making any conclusions on this is difficult.

Although a lot is still unclear in the effects of biological therapies on epigenetics, knowing more about this could aid in improving the treatment of inflammatory disease patients. In fact, quite some studies have looked into the use of epigenetic changes in inflammatory diseases as predictive biomarkers for drug response (Plant *et al.*, 2016; Joustra *et al.*, 2022; Joustra *et al.*, 2023). Here, methylation changes are investigated in order to identify DNA methylation patterns associate with a certain response to a drug. Patient samples can be analyzed, methylation patterns identified and consequently treatment can be adjusted towards biological therapies that have been shown to have a higher chance of response in patients with similar methylation patterns. In addition to this, analyzing the epigenome of patients might also be useful in choosing a certain biological when looking at specific genes. If for example *STAT1* is hypomethylated, the JAK-STAT pathway might be hyperactivated, resulting in inflammation. Treatment with a JAK inhibitor could reduce this inflammation. In this way, investigating the epigenetics of inflammatory diseases and epigenetic effects of biological therapies could help in optimizing treatments for inflammatory disease patients.

In this review, multiple studies have been discussed on the epigenetic differences between inflammatory disease patients and healthy individuals, as well as the epigenetic-altering properties of biological therapies that are used to treat inflammatory diseases. Although epigenomes have been shown to differ between patients and healthy controls, the mechanism on how these changes arise is unclear, nor is there much known about the effects these epigenetic changes have on the pathogenesis of IBD and RA. Biological treatments were further found to complicate inflammatory disease epigenetics, as these drugs seem to have epigenetic-altering potential. How these drugs alter epigenomes and in what way these epigenetic changes might functionally contribute to induce remission in patients remains unknown, and should be investigated in further studies.

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