Gene Polymorphisms and Inflammatory Bowel Diseases

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1. Introduction

1.1. What is it a gene polymorphism?

Every protein is coded by a gene and every human gene exists in several allelic variants, which occur due to mutations and are inherited together with other genetic characteristics from parents to children. If a certain allelic variant occurs within the population with at least a 1% frequency, it is known as a common gene polymorphism. However, if the frequency in the given population is below 1%, it is referred to as a rare allelic variant.

An allele having a majority within a population is called a wild type or standard. Allele(s), which are a minority within a population, are known as a variant, non-standard or sometimes a mutant allele.

Percentage frequencies of individual alleles for the same gene polymorphisms in various populations are often significantly different, which makes many clinical studies complicated. For example, variant allele asterisk *10 of gene coding for cytochrome CYP2D6 codes an unstable enzyme with decreasing activity. It can result in the slower metabolism of some drugs, e.g. antidepressants. The frequency of this variant allele is from 1 - 2% in the Caucasian population but 51% in the Asian population. The gene CYP2D6 can also be duplicated or multiplicated. The amount of enzyme is from 2 to 13 times higher compared to the standard gene allele, which leads to ultra rapid metabolism. The occurrence of this polymorphism differs. For example, in Central Europe, the occurrence is 4% and in Saudi Arabia it is 29%.
1.2. Molecular biological basis of gene polymorphism

The molecular biological basis of gene polymorphism is a mutation of the given gene, in which individual variant alleles occur. Mutations can have a form of replacement or substitution when one or more nucleotides in a gene are replaced by another one; or they can have a form of frameshift mutations, including deletions or insertions, when one or more nucleotides in a gene are missing (=deletion) or are redundant (=insertion). Frameshift mutations lead to frameshift changes, and from the position of the mutation other amino acids can be incorporated in the protein chain or a stop codon is generated, which prematurely terminates transcription of the protein chain.

The most common mutation in gene polymorphisms is the replacement of a single nucleotide, or the so-called single nucleotide polymorphism or SNP. SNP represents 90% of all gene polymorphisms in humans. There is a freely accessible database (http://www.ncbi.nlm.nih.gov/SNP/index.html), which lists over 38 million identified and validated SNPs, and their number grows every day. SNPs are also associated into blocks - haplotypes, which are usually inherited together. Therefore, the identification of a single SNP can theoretically identify the whole haplotype.

1.3. Methods of gene polymorphisms detection

Presently, the basic method of molecular diagnostics is a polymerase chain reaction, PCR. A basic PCR reaction contains two primers and serves for the detection of one specific locus in the form of amplicon of a pre-calculated size.

For the purposes of finding simple nucleotide polymorphisms, the products of amplification can be further digested by restriction endonuclease, and this arrangement is marked PCR-REA (PCR-restriction enzyme analysis), PRA (primer restriction analysis) or PCR-RFLP (PCR-restriction fragment length polymorphism) [1]. This method allows for the analysis of any gene up to a length of approximately 5 kbp. Amplicons of the same length are the result of PCR amplification. These amplicons are further digested by a suitable restriction endonuclease and the resulting products analyzed by gel electrophoresis. It is a very simple, fast and highly sensitive detection method for single gene polymorphisms. However, it can only be used for determining gene polymorphism when two alleles in the given locus differ in such a way that one contains a restriction locus for the given endonuclease and the other does not.

DNA sequencing is the determination of the primary structure of nucleotide chain, i.e. the sequence of nucleotides in DNA molecules. It is a final and definite determination of the basic information which DNA carries. Knowledge of the DNA sequence allows to find the reading frames of potential genes, to analyse exons and introns in structural genes, and to determine the sequences of amino acid-coded proteins, and is usable for the detection of regulatory genes and regulatory areas, repetitive sequences and all simple nucleotide polymorphisms. There are two fundamentally different methods which are commonly used for sequencing. The first method is based on terminating DNA chains using chemical substances and is called Maxam-Gilbert Sequencing [2]. The second method uses the inhibition of
the enzymatic synthesis of DNA by dideoxyterminators, and is known as Sanger’s Sequencing Method [3]. Both methods use the same original material, i.e. DNA fragments obtained, for example, by restriction or cloned in vector. They can also be products of polymerase chain reaction. The most recent development in sequencing methods has been the introduction of devices for automatic sequencing. This method uses enzymatic Sanger’s method and allows for the determination and processing of DNA sequences at a much faster rate as well as being more economical than standard techniques [4].

1.4. Clinical consequences of gene polymorphism

The clinical consequences of mutations on the function and the amount of coding protein (phenotype) depend on their localization and can influence the development of certain diseases or a patient’s response to a particular drug regarding their effectiveness, as well as the safety of a chosen pharmacotherapy.

A mutation can be located in the coding areas of a gene (exons), in the regulatory areas (promoter or generally in the 5’ region of gene) or at the exon–intron interface, i.e. in the exon-intron primary transcript splicing site.

Mutation in the exon area (the coding part of a gene), is manifested by the substitution of amino acids or from a change of the amino acid sequence in the protein chain.

If a gene codes for an enzyme, the result of the mutation is a change in the pharmacokinetic parameters at the drug metabolism level, i.e. leading to the altered activity of this enzyme, or to its complete inactivation and consequent acceleration or deceleration of the metabolic processes leading to the degradation of the active substance in the drug or, on the other hand, to the formation of the active substance in case of the “prodrug”. If a gene codes for a receptor protein, the mutation can lead to a change of the receptor’s ability to bind a ligand and to activate the signaling cascade. This can cause changes in the pharmacodynamic parameters of the drug. Membrane carriers, protein and P-glycoprotein transporters coded by a variant allele of a gene can have an altered affinity to its substrates; variant channel proteins can create channels with different electrical and chemical characteristics, and this can result in a change of ionic homeostasis.

Mutation in the regulatory areas of a gene leads to changes in gene expression, i.e. to either upregulation or downregulation. It is manifested by the lack or excess of protein coded by this gene. If the mutation is located in the interface area between intron and exon in the primary transcript splicing site, this can result in the faulty splicing of the primary transcript, and can cause a shortening or lengthening of a protein chain, which leads to the loss or restriction of functions of the coding protein. The splicing site is distinguishable by specific ribonucleoproteins. If the nucleotide sequences in this area differ from the standard gene as a result of mutation, incorrect splicing of primary transcript hnRNA to mRNA occurs.

The presence of variant alleles can have an impact not only on metabolic processes (absorption, distribution and drug elimination), it can also influence the effectiveness and safety of the chosen pharmacotherapy. Furthermore, it represents an increased risk of the development of certain diseases due to the changing structures or functions of several regulatory
proteins, which influence the physiological processes of the body. Disease development is caused by both genetic and environmental factors. Additionally, a patient’s sensitivity to external factors is varied and this variability is again, genetically conditioned.

2. The role of candidate genes in etiopathogenesis of inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gastrointestinal tract. The term covers two specific conditions – Crohn’s disease (CD) and ulcerative colitis (UC), which differ in their anatomic location, intensity and the scope of their affect on the intestinal mucosa. The etiopathogenesis of these two conditions is not fully understood to date. However, several studies have confirmed that there are external factors, together with genetic predisposition, which contribute to the diseases’ onset [5]. The significant role of genetic predisposition is supported by a relatively high familial occurrence of Crohn’s disease, a high concordance in monozygotic twins up to 67% a connection with patients’ ethnic or racial character, and parallel incidences of other rare genetic syndromes. The risk of Crohn’s disease is 3–5 times higher in the first degree relatives, and a familial occurrence was found in 15–20% of cases. However, genetic predisposition is more important in CD than in UC [6–8]. Genes whose products somehow influence the development of the inflammatory reaction are called “candidate genes,” and they are located at different places on the genome marked from IBD1 to IBD9 [9]. These candidate genomic loci include NOD2/CARD15, ICAM-1, CCR5, MDR1, TLR4 and other genes [10–12]. At present, the correlation between the genetic makeup of an individual and the predisposition to contract a disease has been studied as well as its connection with the clinical characteristic of a disease.

2.1. NOD2/CARD15 gene

NOD2 (nucleotide-binding oligomerization domain) is a protein expressed by several immune system cells (monocytes, macrophages and dendritic cells). It is a key molecule that reacts to the intracellular presence of peptidoglycans of bacterial origin [13]. The binding of peptidoglycans initiates the signalling cascade, ending with NF-κB activation and the expression of pro-inflammatory genes including TNFα [14,15]. In the gene for the protein NOD2, several polymorphisms were described. Substitutions at positions 702Arg>Trp, 908Gly>Arg and the frameshift mutation (cytosine nucleotide insertion) at position 1007 (3020fsinsC) are most often linked with the disruption of receptor functions. These three mutations represent 81% mutations of NOD2 in CD patients [16]. The presence of the latter mutation leads to the disruption of the reading frame during the transcription and to the termination of proteosynthesis due to a newly formed stop codon [17]. For the polymorphisms at positions 702 and 908 was described a comparable or just a slightly increased NF-κB induction [18]. Furthermore, the presence of the frameshift mutation at position 1007 limits the structure and function of the NOD2 protein to such an extent that the NF-κB activation in the presence of peptidoglycan is (MDP used as a standard inductor) undetectable [15] and
TNFα expression is decreased [19–21]. The described polymorphisms have a relatively high frequency in the Caucasian population. Carriers of one mutant allele have a 2 to 4 times higher risk of CD outbreak and recessive homozygotes at 20–40 times higher [20]. An association between the occurrence of the 1007fs frameshift mutation in the NOD2 gene and the IBD incidence has also been confirmed. A similar association, somewhat less significant, was also discovered in the 908Gly>Arg polymorphism [21]. Patients with mutations in the gene CARD15/NOD2 also showed a decreased expression of defensines [22]. Mutations can therefore be predisposed to CD not only directly, but indirectly as well, i.e. by obstructing the natural antimicrobial immunity mediated by defensines.

2.2. ICAM-1 gene

Lately, the association between IBD and the ICAM-1 gene has also been intensively studied. This gene codes for the intracellular adhesive molecule ICAM-1 which performs many physiological functions. It controls the migration of inflammatory elements, participates in the presentation of antigen, and because it is expressed by various cell types, it is involved in many signalling cascades [23]. Its significantly increased expression in the intestinal mucosa was observed in inflammatory bowel diseases. In the ICAM-1 gene, at least 20 SNPs were identified. A SNP marked 469Lys>Glu has also been linked with IBD [24]. A substitution of nucleotides leads to the substitution of amino acid in the 5th immunoglobulin domain of ICAM-1, which is important for the adhesion of B cells and dendritic cells [25]. An altered function of the protein ICAM-1 potentially contributes to the genetic predisposition for inflammatory diseases and immunity disorders. The prevalence of the 469Lys>Glu polymorphism has also been linked with multiple sclerosis [26], Behcet’s disease [27], psoriatic and rheumatic arthritis [28] and other chronic inflammatory diseases [29].

2.3. CCR5 gene

Another gene, which intervenes in the reactions of the immune system, is the gene that codes for the receptor CCR5, whose ligands are CC chemokines. The endogenous functions of this receptor include the mobilization of the relevant immune system cells and the targeting of their chemotaxis into the inflamed area. The CCR5 receptor is responsible for the transport of chemokine CC and participates in the entering of virus particles of the human immunodeficiency virus (HIV-1) into macrophages [30].

Its regulatory role lies in the preference of the Th1 immune response and suppression of the Th2 immune pathway. It participates, for example, in the immune defence against Mycobacterium tuberculosis, [31]. In the 1990s, it was discovered that the gene for this receptor occurs in various allelic forms in the population. In particular, a 32 base pairs deletion in the gene CCR5 was studied because it leads to the expression of a shorter, and therefore non-functional receptor in the cell membrane and consequently to the disrupted communication between cells of the immune system. This deletion mutation is associated with the onset of pathological inflammatory conditions, such as sarcoidosis [32], rheumatoid arthritis [33] and periodontitis [34]. A cellular immunity disorder caused by the non-functional receptor CCR5 could play some role in IBD development.
2.4. TLR4 gene

The Toll receptor was originally described in Drosophila, where its function lies in the immune defence against fungal and yeast infections in adults. It was found that the intracellular part of this receptor molecule often resembles parts similar to a completely different receptor – for cytokine interleukin-1 (IL-1). In humans, a total of 10 receptors have been described in which their amino acid sequence is similar to the original Toll receptor of Drosophila [35]. Thus, they were named Toll-like receptors. Individual TLR receptors differ in their presence in various cell populations and in their affinity for various ligands. TLR4 in particular is a model ligand of lipopolysaccharide (LPS), which activates the NF-κB expression after binding to the extracellular domain of a receptor, and, in the next step, the expression of pro-inflammatory cytokines (TNFα, IL). The gene for TLR4 is located on chromosome 9, in the area 9q32-33, and is expressed by monocytes, macrophages, mastocytes and immature dendritic cells, as well as by intestinal cells in a small amount in the apical part of epithelium, and also by renal, corneal and pulmonary epithelial cells [36]. A substitution of adenine for guanine at position 896 (896A>G) was detected by direct sequencing. This substitution is manifested on the amino acid sequence level by the substitution of a conservative aspargic acid with glycine at position 299 (299Asp>Gly). This single nucleotide polymorphism (SNP) is located in exon 4 of the gene for TLR4 and results in production of an altered extracellular domain of this receptor.

Another SNP was found at position 399 of the amino acid sequence, where non-conservative threonine was replaced by isoleucine (399Thr>Ile). This mutation cosegregates with mutation 299Asp>Gly [37]. Polymorphisms in the gene for TLR4 are linked with various diseases such as chronic periodontitis [38], COPD [39], Behcet’s disease [40], septic shock [41] or IBD [42] and are associated with the disruption of intracellular processes leading to NF-κB induction and TNFα expression.

2.5. TNFα gene

Several SNPs have been described in the TNFα gene sequence; in the promoter, intron, as well as exon areas. In the promoter area, the following polymorphisms have been described: substitutions at nucleotide positions –1031T>C, –863 C>A, –857 C>A, –851C>T, –419 G>C, –376 G>A, –308 G>A, –238 G>A, –163 G>A, and –49 G>A. In the intron 1 sequence, there is the substitution of 488G>A [43]. The mutation in the gene TNFα results in a changed level of the gene expression and therefore a different amount of active cytokine. As was mentioned before, the physiological or pathological manifestations of its effect are based on TNFα quantity. In this context, the polymorphisms in the promoter area of the gene, which interacts with the transcription factors, are highlighted. Nucleotide polymorphisms at positions –376, –308 and –238 are most often mentioned in connection with the change of the gene expression level.

2.5.1. Polymorphism –376G>A

Transcription factor OCT-1 binds at position –376. It was proved that the transcription factor binds with a higher preference to the variant allele –376A [44].
2.5.2. Polymorphism –308G>A

This polymorphism is most often mentioned in connection with increased TNFα production. At the same time, it is the most closely studied predisposition factor for chronic inflammatory diseases including IBD. Braun et al. [45] as well as Sashio et al. [46] confirmed in vitro a higher transcription activity of the variant allele –308A. In his study of the Czech population, Sykora et al. [47] stated that a statistically significant association existed between the –308G>A polymorphism and the occurrence of UC in child patients. He also discovered that patients who were carriers of at least one variant allele, i.e. carriers of the genotype G/A or A/A, had significantly higher levels of C-reactive protein (CRP) compared with carriers of the standard G/G genotype [47]. A study of the Mexican population confirmed that there was a significantly higher frequency of the variant allele –308A in the gene for TNFα in patients with UC, compared with a healthy population [48,49] found a significantly higher risk of pancolitis in carriers of the variant allele –308A. The risk is 1.91 times higher in comparison with carriers of the standard genotype [49]. A meta-analysis of 27 studies confirmed a significantly higher risk of the onset of ulcerative colitis and Crohn’s disease in carriers of the non-standard A/A genotype polymorphism –308G>A in the European population [50].

2.5.3. Polymorphism –238G>A

Some authors stated that the variant allele –238A is associated with a high expression of TNFα, but other groups of scientists have not confirmed this statement [51,52]. It is also assumed that the levels of the TNFα transcription are influenced by DNA sections situated outside the promoter area.

3. Gene polymorphisms associated with inflammatory bowel diseases: Differences between CD and UC patients

At present, it is generally considered that IBDs have a genetic background and that there are environmental factors which can trigger the disease [53].

The locus IBD1 is the most often linked with a genetic predisposition to IBD. It is situated, along with others, in the gene NOD2/CARD15. In our study, we studied three polymorphisms in this gene (702Arg>Trp, 908Gly>Arg and 1007fs insC) in 101 patients with CD, 35 patients with UC and 78 healthy volunteers. At least one variant allele was found in 56.3% of the patients with CD, whereas in patients with UC it was found in only 14.6%, and in healthy volunteers, 20%. Recessive homozygotes (carriers of two non-standard alleles in the gene NOD2/CARD15) were found only in the group of patients with CD. The most serious clinical impact and therefore the strongest association with CD, was confirmed in the frameshift mutation (Leu1007fs insC) in this gene – see Table 1. Similar results were reported by other authors [5, 54 - 56] and also by our research team in their previous works [21,8].

The second monitored mutation in this gene (702Arg>Trp) was significantly less frequent in patients with UC compared with patients with CD. In the third mutation of the gene, substi-
tution 908Gly>Arg, we did not find any statistical association with CD or UC. The frequency of this allele in Czech and Slovak populations is very low; the allele frequency in the group of healthy volunteers was 2%. Brant et al. [54], unlike our results, found a significant connection between Crohn’s disease and the mutation Gly908Arg in a larger set of patients.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant allele</th>
<th>Patients with CD</th>
<th>Patients with UC</th>
<th>Health volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD2/CARD15</td>
<td>702Trp</td>
<td>9.90•</td>
<td>2.86•</td>
<td>8.97</td>
</tr>
<tr>
<td></td>
<td>908Arg</td>
<td>3.96</td>
<td>1.43</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>Leu1007fs</td>
<td>16.83*</td>
<td>8.57</td>
<td>5.77*</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>469Glu</td>
<td>48.02**</td>
<td>37.14Δ</td>
<td>26.28**, Δ</td>
</tr>
<tr>
<td>CCR5</td>
<td>Δ32</td>
<td>8.91•</td>
<td>17.14•</td>
<td>14.74</td>
</tr>
</tbody>
</table>

Table 1. Frequency (in %) of variant allele of all tested genes and statistically significant differences among groups. Statistical analysis by Fisher test *P<0.01; **P<0.05 - statistical significant differences between group of CD patients and group of health volunteers; ⋆P<0.01; ⋆⋆P<0.05 - statistical significant differences between group of UC patients and group of health volunteers; *P<0.01; **P<0.05 - statistical significant differences between group of CD patients and UC patients.

All present studies imply that the genetic predisposition to IBD is polygenic, i.e. the process of pathogenesis includes more genes, and the presence of individual mutations is cumulative, which means that the occurrence of a larger number of mutations in the same gene increases the probability of a phenotype change, or, i.e., the loss of function of a protein coded by this particular gene. We found a highly significant difference between the group of patients with Crohn’s disease and the control group (P = 0.0019) in the total number of monitored mutations. Our results also confirmed that the average number of mutations in the gene NOD2/CARD15 calculated in one person is significantly (P < 0.05) higher in the group of patients with CD, when compared with UC or the control group. Furthermore, patients with UC revealed a lower frequency of these mutations than the control group, but the result was not significant. It seems that the occurrence of variant alleles in the gene NOD2/CARD15 is really typical for patients with Crohn’s disease [5].

The ICAM-1 gene plays a key role in the migration of neutrophils to the inflammation area and is connected with several inflammatory diseases. Matsuzawa [29] reported a significantly increased frequency of the variant allele for the polymorphism 469Lys>Glu in this gene among Japanese patients with CD. We also confirmed a strong association between the occurrence of a non-standard allele for this polymorphism and CD (P = 0.0002) in our set (patients of Caucasian population). Carriers of two non-standard alleles in this gene were up to 83% in patients with Crohn’s disease, but only 0.6% in patients with UC and less than one percent in healthy individuals. The odds ratio implies that the risk of CD in these recessive homozygotes is 10.6 times (95% CI = 2.9–38.7) higher than in people with standard alleles in the gene ICAM-1, and the risk of UC is 3.1times (95% CI = 0.55–17.35) higher.

Herfarth et al. [57] suggested that the mutation in chemokine receptors CCR5 could play a key role in the regulation of the intestinal immune response in CD. They did not succeed in
finding a higher frequency of the deletion allele Δ32 in patients with CD, but they found out that the polymorphism in the gene for the CCR5 receptor can contribute to the disease progression and its location. The results of our study suggest that patients with CD have a deletion allele Δ32 in the gene CCR5 significantly less often compared with patients having UC. The deletion allele is found in patients with UC insignificantly more often compared with healthy volunteers.

Polymorphism –308G>A in the gene for TNFα is most often mentioned in connection with the increased production of this cytokine, therefore it is studied as a predisposition factor for chronic inflammatory diseases, including IBD. The highest frequency of the non-standard allele of this polymorphism (25%) was found in the group of patients with indeterminated colitis. In the groups of patients diagnosed with CD and UC, the allele frequency was almost identical – 13.96% and 14.29%. To compare the occurrence of the variant allele in the evaluated group of patients, a control group of healthy individuals was used. The overall frequency of the variant allele for the polymorphism –308G>A in the control group of healthy individuals reached 8.46%. When comparing the allele frequencies in both groups, i.e. healthy volunteers to the group of patients with IBD, it is obvious that the variant allele of the monitored polymorphism occurs 1.86 times more frequently in the group of patients compared with the group of healthy individuals, and this probability is highly significant ($P = 0.0002$). In the group of patients with indeterminate colitis, the variant allele occurs 3.95 times more often compared with the group of healthy volunteers.

The allelic frequency of the monitored polymorphism in the entire Caucasian population is generally about 11% [58]. The results indicate a possible role of the variant allele of the polymorphism –308G>A in the gene TNFα as a predisposing factor for the onset of IBD. An increased level of TNFα can lead to a predisposition to a more intense inflammatory reaction and represents one of the risk factors contributing to the development of this disease [49,50].

From the available data it is obvious that genetic factors can determine the IBD character, especially in case of CD. In monozygous twins with CD, 7 out of 9 cases showed a correspondence to the disease location, and in 6 out of 9 cases the disease was diagnosed within 2 years. However, the disease behaviour did not reveal any correspondence [60]. Available data imply that the mutation in the gene CARD15/NOD2 can be connected with the affected ileum, or preferential occurrence of inflammation in the ileocaecal area [6] and with the stenotic form of the disease [59,60]. There has also been a discussion regarding the impact of mutation in this gene on the onset age of Crohn’s disease [61]. Herfarth [57] reported that carriers of the deletion mutation Δ32 in the gene CCR5 are less often affected in the upper part of gastrointestinal tract (GIT), but they are more susceptible to the development of strictures. Due to the absence of CCR5 receptors and decreased ability of a cell to initiate an inflammatory response [57], carriers of the deletion mutation Δ32 experience less often the aggressive progress of the disease (perforating or fistula type of CD) [57].

However, our results revealed that the perforating type of CD occurs significantly more often in patients with the deletion allele Δ32, compared with patients with the non-stenotic form of the disease, in whom the occurrence of this deletion allele was significantly lower. Therefore we did not confirm the results of Herfarth et al. [57].
Statistical analyses of our results confirmed an association between the polymorphism in the gene *ICAM-1* and CD outbreak. The occurrence of the variant allele 469Glu in the gene *ICAM-1*, both in heterozygous and homozygous state, is statistically significantly higher in patients, in whom CD broke out before 16 years of age, compared with patients, in whom the disease broke out between 16 and 40 years of age [8] – see Figure 1. However, we did not confirm the results of Hradský et al. [56] that the frameshift mutation 1007fs in the gene *NOD2/CARD15* occurs more often in child patients with CD than in adult patients. This was probably caused by the fact that the group of patients with a very early clinical manifestation of the disease consisted of only 10 individuals. Nevertheless, even our results imply that the greater the genetic predisposition is, the earlier the age of manifestation and disease diagnosis. For example, three or more mutations in all of the monitored candidate genes were detected in 46.2% of young patients, in whom the clinical manifestations of the disease appeared before 16 years of age, but only in 26.3% of patients did the disease manifested from 16 to 40 years of age and in 0% of patients with CD after 40 years of age [8].

![Figure 1. Influence of ICAM-1 gene mutation on the age of CD manifestation. Note: +/+: standard homozygote (carrier of two wild type alleles); +/-: heterozygote (carrier of one variant allele); -/-: variant homozygote (carrier of two variant alleles).](image)

**4. Pharmacotherapy of inflammatory bowel diseases and genes affecting drug metabolism, the occurrence of adverse effects and response to therapy**

At the present time, there is no curative therapy and hence the pharmacological treatment focuses on inflammation control, eliminating disease symptoms and improving of the quality of life. IBD pharmacology utilises a wide scale of drugs including salicylates, glucocorticoids, and immunosuppressives (e.g. thioguanine derivatives, methotrexate and cyclosporine).

In clinical practice, IBD therapy, using a step-by-step system, is influenced by the intensity of the disease and the effectiveness of previous therapy.
At present, aminosalicylates constitute the substantial part of the conservative treatment of IBD. When this therapy is insufficient, corticoid or immunosuppressive therapy is applied, either separately or combined. The highest level is biological therapy. Many authors indicate that TNFα as a pro-inflammatory cytokine plays a crucial role in the pathogenesis of many diseases, including IBD. The use of monoclonal antibodies, which neutralize TNFα, seems to be a very effective method of treatment. IBD requires long-term treatment. It is also necessary to mention surgical resection of the affected part of the intestine, which is used as an alternative method or pharmacotherapy supplement.

Patients are potentially exposed to the side effects of many drugs. Some toxic effects could be caused by overdose induced by a low metabolism and the elimination of effective substances. However, mechanisms of metabolism and elimination are complex; there are some crucial enzymatic proteins whose activity affects metabolism on an important therapeutical level. Single nucleotide polymorphisms (SNPs) of these genes affect enzyme structure and activity and are therefore, therapeutically significant.

4.1. Aminosalicylates

Aminosalicylates has been used in IBD therapy since the 1940s. Historically, the first preparation in this drug group was Sulfasalazine. It is a prodrug, which is dissociated in the intestine by bacteria into the active substance 5-ASA and pharmacologically inactive sulfapyridine.

During this therapy, adverse effects develop in 5 to 50% of individuals (most common are nausea, vomiting, general asthenia, headache, abdominal discomfort and rarely haemolytic anaemia and rash), in which some patients require termination of the therapy. The cause of these undesired effects is considered to be the sulfonamide component of the substance. Sulfapyridine released in the intestine is absorbed into the systemic circulation and acetylated in the liver by N-acetyltransferase (NAT) into N-acetylsulfapyridine. N-acetyltransferase is a cytosol enzyme with broad distribution into various tissues. The highest activity was observed in liver cells, intestinal epithelium and the urinary bladder. In contrast, an insignificant activity of this enzyme was found in plasma, brain and muscle [62]. N-acetyltransferase exists in two different and independently regulated isoforms: NAT1 and NAT2, which differ in their affinity to various substrates. The NAT2 isoenzyme in the liver is a lot more important for sulfapyridine acetylation than NAT1 present in the liver and gut.

4.1.1. Polymorphisms influencing aminosalicylates therapy

The genes for NAT1 and NAT2 are located on chromosome 8p22. The NAT2 gene is polymorphous and to date, several allelic variants have been identified with various impacts on the acetylation activity of these enzymes. Any population can be divided into fast, intermediate and slow acetylators, where over 50% of the Caucasian population represent the slow acetylator phenotype. Slow acetylators, unlike the fast ones, have higher concentrations of plasma sulfapyridine and its elimination half-time is longer. Sulfapyridine is responsible for various adverse effects; therefore, the NAT2 acetylation phenotype/genotype
could be important for the prediction of toxicity. Several studies confirmed higher incidences of adverse effects in slow acetylators [63–65]. Nevertheless, acetylation status is not the only factor regulating plasma concentration. Sulfapyridine is not only acetylated, it is also metabolised by the hydroxylation of an unknown polymorphous enzyme [66,67] and the high hydroxylation capacity can, in some individuals, compensate for the low level of acetylation. Due to the frequent occurrence of adverse effects, Sulfasalazine has been replaced by the active component alone, i.e. 5-aminosalicylic acid (5-ASA). 5-ASA is acetylated by polymorphous NAT1 into the pharmacologically inactive metabolite, therefore, there is a hypothesis that the increased acetylation capacity of NAT1 could be causing the decreased effectiveness of 5-ASA. Studies in patients with ulcerous colitis did not confirm the correlation between the NAT1 and NAT2 genotypes and the therapeutic response to aminosalicylates [68,69].

4.2. Glucocorticoids

Glucocorticoids (GCs) play an important role in the IBD therapy but like other drug groups, they are now used not in the causal therapy but in the symptomatic therapy. Glucocorticoids (GCs) are broadly used to inhibit common inflammatory and autoimmune reactions of the body. They play a crucial role in the CD therapy, especially during the high activity of the disease. GCs are effective for the treatment of active inflammation but not for the maintaining of remission and prevention of relapses. Despite the massive boom of other drugs, they keep their position of golden standard in the IBD therapy.

Corticosteroids have anti-inflammatory and immunomodulating properties – mainly immunosuppressive. Their anti-inflammatory effect is partly immediate; peaking within 6 hours of the GCs injection application and subsiding within 24 hours. This is connected with quantitative changes in circulating leucocytes. GCs are at high doses affecting B-lymphocytes. Furthermore, they can lead to the decreased production of IgA and IgG 2-3 weeks after the onset of the therapy. Anti-inflammatory effect, which starts later, is a long-term with an impact on T-lymphocytes, especially Th-lymphocytes, and substantially less on Ts-lymphocytes [70]. However, quite limiting factors in the therapeutic use of GCs are various adverse effects during a long-term use, which are mainly connected with their numerous physiological functions in the body. The toxicity of corticosteroids is insignificant and physiological doses are administered during the substitution therapy, adverse effects do not develop. They only occur after administering of high doses during anti-inflammatory and immunosuppressive therapy.

GCs are highly lipophilic substances, which easily penetrate membranes of target cells and bind to specific glucocorticoid receptors. GCs complexes + receptor form homodimers, which are transported into the cell nucleus. Here they bind to specific responsive DNA structures of various genes. The result of this interaction is a changed gene transcription. The inhibition of promoters for the AP-1 gene (activator protein 1) or NF-κB (nuclear factor kappa B), which are transcription factors for pro-inflammatory cytokines, and then induction of IκBα (inhibitor kappa B alfa), which is able to bind and therefore inhibit NF-κB, are important for the immunosuppressive and anti-inflammatory effect of GCs.
Terminating of the corticoidsteroid therapy leads in a part of patients to the disease relapse. To maintain the remission state, their long-term administering is therefore necessary; we talk about the so-called corticodependence. The opposite problem is corticoresistance, i.e. condition, when administering of GCs does not lead to the calming down of the disease or remission. Corticoresistance occurs in 16-20% of patients; corticodependence in 28-36% of treated patients [71,72]. Corticoresistance is not only the problem of Crohn’s disease and has been intensively studied [73].

4.2.1. Polymorphisms influencing glucocorticoid therapy

Corticoresistance is mainly associated with polymorphisms in the \textit{MDR1} gene (multidrug resistance gene). P-glycoprotein (P-gp) coded by this gene is a membrane protein serving as an ATP-dependent transporter of xenobiotics from cells. Polymorphism in this gene was described for the first time in tumour cells in connection with the occurrence of the multidrug resistance of anticancer drugs [74]. However, the \textit{MDR1} gene is also present in normal tissue with excretion functions, i.e. the intestines, liver, kidneys, placenta and testes. It is also formed in brain capillary endothelial cells, where it participates in the function of the blood-brain barrier [75]. P-glycoprotein in these tissues is probably formed as a defence mechanism against xenobiotics. It is an important regulator of the biological activity and tissue distribution of various drugs, and it participates in their transport from cells into the extracellular environment. The increased activity of the P-glycoprotein pump is one of the reasons for the multidrug resistance of cancer cells to cytostatic drugs, and it is assumed that it could cause the non-responsiveness to glucocorticoids.

\textit{MDR1} gene is located on the 7q chromosome and consists of 28 exons. Farrell et al. proved the increased expression of this gene in T-lymphocytes and in the cells of the intestinal epithelium in patients with UC and CD, who had to undergo an intestine resection after the failure of drug therapy [76]. Therefore, the level of the \textit{MDR1} gene expression significantly influences the therapeutic response to the administered glucocorticoids (GCs). An indirect dependence between the cells’ sensitivity to GCs and MDR1 expression was also described [77].

The presence of polymorphisms in the \textit{MDR1} gene can influence the gene expression, its amount and also the function of the P-glycoprotein pump, based on the mutation place and character. Substitutions of amino acids in the primary protein structure can result in changes of the substrate spectrum and the effectiveness of transport or pump sensitivity to specific inhibitors. These two mechanisms can also be combined.

Polymorphism in the \textit{MDR1} gene was described for the first time in 1989 [78]. \textit{MDR1} variants and their functional impact are still being studied intensively. To date, 50 SNP and 3 deletion and insertion mutations has been described [79,80]. From the perspective of the biological availability of glucocorticoids, mutations in exon 12 (e.g. 1236C>T), exon 21 (2677G>T/A) and exon 26 (3435C>T) have been studied more predominantly [81]. The most frequent polymorphisms have a character of substitution at position 2677, where guanine usually occurs, or possibly thymine and less frequently, alanine (2677G>T/A).
Some authors assume that the reason for the increased transport activity of MDR1 is because of this polymorphism [83]. In contrast, mutation 3435C>T in exon 26 is connected with a low expression of P-glycoprotein in the small intestine. The frequency of this mutation in the Caucasian population reaches 28.6% [83]. Carriers of the homozygous variant genotype TT showed a lower production of intestinal P-glycoprotein, when compared with standard homozygotes of the CC genotype. Several studies imply that the frequency of the CC genotype is significantly higher in African populations, when compared with Caucasian and Asian populations [84,85]. It could be speculated that it might provide the African population with a certain selective advantage against GIT infections, which are endemic in tropical regions, because P-glycoprotein plays a crucial role in the defence against bacterial and viral infections. The physiological role of P-glycoprotein lies in the protection against entering bacterial toxins into the intestinal mucosa. This hypothesis is supported by Panwala [86], who demonstrated that knockout mice are sensitive to the development of severe spontaneous intestinal inflammations. The ambiguous results of the studies focusing on the impact of individual SNPs on the expression or function of the P-glycoprotein pump were brought into consonance by the results of haplotype analysis, which found a correlation between mutations 3435C>T, 2677G>T and 1236C>T. Potocnik [87] demonstrated a significantly higher risk of resistance to glucocorticoids in carriers of the haplotype marked as T/T/T.

The MDR1 gene is also included in the group of so-called candidate genes for IBD. At present, the relationship is being studied between the polymorphism 3435C>T, which results in the decreased expression of P-glycoprotein and the increased susceptibility to the development of IBD... Another cause of corticoresistance is examined on the level of the glucocorticoid receptor (GR). It could be because of the disturbed receptor function or the change of its expression.

Gluocorticoid receptor (GR) exists in two isoforms (GR-α, and GR-β), which are formed by an alternate splicing of hnRNA to mRNA. Only the GR-α isoform, whose expression many times exceeds the expression of GR-β, is able to bind glucocorticoids and mediate their effect. Corticoresistant patients showed a higher expression of the GR-β isoform, but when compared with GR-α, this amount was still very little [88,89]. In-vitro obtained data imply that the formation of GR-β isoform is inducible and can be influenced by cytokines IL-2 and IL-4 [90,91] as well as by glucocorticoids [92]. The question is, how GR-β participates in modulating the sensitivity of an organism to glucocorticoids.

Polymorphisms ER22/23EK, located in exon 2, consist of two point mutations - substitutions in codons 22 and 23. The first mutation is silent, changing codon 22 from GAG to GAA (both code glutamic acid). The consequent mutation in codon 23, AGG→AAG, leads to the substitution of arginine for lysine [93]. ER22/23EK is associated with the decreased sensitivity (relative resistance) to glucocorticoids [94]. Its mechanism probably lies in the changed ratio between the forming translation isoforms GR-α and GR-β, in favour of the weaker transactivator GR-A α [93]. Two other polymorphisms in the gene GR, 363Asn>Ser in exon 2 and Bcl I, are associated with hypersensitivity to GCs [95–97]. A study in 119 IBD patients observed
a more frequent occurrence of the \textit{Bcl} I polymorphism in individuals responsive to GCs therapy [97].

Mutation 641Arg>Val and 729Ile>Val, that lead to the expression of receptors with a decreased affinity for exogenously supplemented corticosteroids, including Dexametasone, were described in the binding domain for the glucocorticoid receptor. The negative feedback induced by glucocorticoid resistance leads to the increased production of cortisol in the adrenal gland. This increased adrenal activity also leads to an increased production of androgens and mineralocorticoids [95,98,99]. In contrast, a polymorphism (363Asn>Ser) in exon 2 leading to an increased sensitivity to dexamethasone was also observed [100]. Yet another potential predictor of glucocorticoids’ effectiveness from the group of polymorphisms of the gene for the corticoid receptor seems to be polymorphism \textit{Bcl} I, in which an association between hypersensitivity and glucocorticoids [97].

Since corticoresistance is most often observed in severely afflicted patients, it is still unclear whether corticoresistance is a singular phenomenon or whether it is caused by an exhaustion of the anti-inflammatory capacity of glucocorticoids due to an excessive production of pro-inflammatory cytokines, which, in turn, is caused by the excessive activity of several intracellular transcription factors that can also reduce the affinity of the glucocorticoid receptor for its intracellular ligands [101]. The results of several studies imply that the relative corticoresistance of T-lymphocytes could possibly be conditioned by their contact with pro-inflammatory cytokines and the consequential decrease of GR affinity for ligands [91]. However, this explanation of corticoresistance is not satisfactory because state of remission can be achieved in a substantial number of patients with a severe form of the disease by cortico-therapy, even though their T-lymphocytes are exposed to the same conditions as the non-responders.

So far, no clinical or genetic marker exists which could be used to predict whether glucocorticoid therapy would lead to the desired therapeutic response, or that the inflammation would be corticoresistant.

4.3. Thiopurines

Thiopurine analogues (azathioprine, 6-mercaptopurine) were therapeutically used as immunosuppressives in the early 1950s for the first time. They are chemical thiol analogues of endogenous purine compounds. Azathioprine is one of the most commonly used drug in this group. The effect of azathioprine, just like its potential toxicity, depends on the amount of its active metabolites or the administered dose. Azathioprine metabolism is so complex that it is obvious that the levels of active and inactive metabolites correlate with the activity of metabolic enzymes. Scientific teams focused on the research of variant alleles of the genes for TPMT, ITPA and XDH, which are key enzymes for the metabolism of thiopurines, in which it is possible to expect an impact on the phenotype or on the decreased enzymatic activity of the native protein. Variant alleles can indirectly influence azathioprine’s effect and incidence of its toxicity manifestations by this mechanism.
Azathioprine has a wide therapeutic spectrum and therefore it is considered to be a relatively safe drug. Despite this, adverse effects are common during therapy. Pharmacovigilance studies have noted them in up to 15-25% of patients [102]. Thiopurines are associated with two types of adverse effects, i.e. reactions, which are independent of the dosage and occur in up to 25% patients (nausea, fever, rash, flu-like syndrome, arthralgia), or adverse effects which are connected with the drug dosage and induced by a different mechanism. The latter is represented mainly by thiopurine-induced myelosupression, which is usually manifested by leukopenia and thrombocytopenia [102]. Basically it is a manifestation of the therapy toxicity caused by a high level of active metabolites when exceeding the therapeutic dose or due to ineffective metabolism and elimination processes. Myelosupression induced by these drugs is reversible; however, for a patient, in whom the therapeutic objective was only immunosuppression, this means a risk of life-threatening infection. Another problem is the fact that the thiopurine-induced myelosupression is manifested after a certain latency period and in most cases it also means termination of the azathioprine medication. However, some works state that a moderate myelosupression is associated with better clinical results from the therapy [103].

4.3.1. Polymorphisms influencing the thiopurines therapy

4.3.1.1. The gene for thiopurine S-methyltransferase (TPMT)

In the 1960s, this enzyme, which catalyzes the methylation of the thiol functional group, was described in several cell populations. Activity of S-adenosyl-L-methionine: thiopurin S-methyltransferase (TPMT; EC 2.1.1.67) was demonstrated in cytoplasm of prokaryotic as well as eukaryotic cells. In humans, the activity of this enzyme was mainly described in erythrocytes, leukocytes, renal parenchyma, hepatocytes and intestinal cells. Its occurrence was also confirmed in mammalian placenta [104].

The human gene for TPMT is located in the short arm of chromosome 6 (6p22.3). It consists of 9 exon and 8 intron regions [105]. Its length (including introns) is about 25 kbp [106]. In the sequence of this gene, there were 21 SNPs identified, which facilitated the formation of 22 variant allelic form of the gene. In compliance with the agreed rules, nomenclature of the non-standard TPMT alleles is marked by a number, or a number and a letter. Variant alleles are listed in Table 2.

Even though the differences in the catalytic activity of the TPMT enzyme has been known for several decades, the first proof of the association of this phenotype with gene polymorphism was provided by Krynetski [107]. It was evidence that a variant allele TPMT*2 is present in individuals with low enzymatic activity [107].

Since then, many gene alleles for TPMT have been discovered. They differ not only in the nucleotide sequence but also in the activity of enzymes transcribed from such sequence. The fact that many variant alleles lead to a decreased enzymatic activity resulted in the belief that gene polymorphisms of this gene substantially influence pharmacokinetic parameters of the drug with a thiopurine structure.
<table>
<thead>
<tr>
<th>Allele</th>
<th>SNP</th>
<th>Substitution of AA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPMT*1</td>
<td>Standard allele - wild type (wt)</td>
<td>474T&gt;C</td>
<td>Silent [108]</td>
</tr>
<tr>
<td>TPMT*1S</td>
<td>474T&gt;C</td>
<td>Silent</td>
<td>[108]</td>
</tr>
<tr>
<td>TPMT*1A</td>
<td>-178C&gt;T</td>
<td>Ala80Pro</td>
<td>[107]</td>
</tr>
<tr>
<td>TPMT*2</td>
<td>238G&gt;C</td>
<td>Ala154Thr</td>
<td>[109]</td>
</tr>
<tr>
<td>TPMT*3A</td>
<td>460G&gt;A</td>
<td>Ala154Thr</td>
<td>[109]</td>
</tr>
<tr>
<td></td>
<td>719A&gt;G</td>
<td>Tyr240Cys</td>
<td></td>
</tr>
<tr>
<td>TPMT*3B</td>
<td>460G&gt;A</td>
<td>Ala154Thr</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*3C</td>
<td>719A&gt;G</td>
<td>Tyr240Cys</td>
<td>[111]</td>
</tr>
<tr>
<td>TPMT*3D</td>
<td>460G&gt;A</td>
<td>Ala154Thr</td>
<td>[110]</td>
</tr>
<tr>
<td></td>
<td>719A&gt;G</td>
<td>Tyr240Cys</td>
<td></td>
</tr>
<tr>
<td></td>
<td>292G&gt;T</td>
<td>Glu92Stop</td>
<td></td>
</tr>
<tr>
<td>TPMT*4</td>
<td>_1G&gt;A (intron 9)</td>
<td>Splicing defect</td>
<td>[111]</td>
</tr>
<tr>
<td>TPMT*5</td>
<td>146T&gt;C</td>
<td>Leu495Ser</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*6</td>
<td>539A&gt;T</td>
<td>Tyr180Phe</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*7</td>
<td>681T&gt;G</td>
<td>His227Gln</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*8</td>
<td>644G&gt;A</td>
<td>Arg215His</td>
<td>[112]</td>
</tr>
<tr>
<td>TPMT*9</td>
<td>356A&gt;C</td>
<td>Lys119Thr</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*10</td>
<td>430G&gt;C</td>
<td>Gly144Arg</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*11</td>
<td>395G&gt;A</td>
<td>Cys132Tyr</td>
<td>[113]</td>
</tr>
<tr>
<td>TPMT*12</td>
<td>374C&gt;T</td>
<td>Ser125Leu</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*13</td>
<td>83A&gt;T</td>
<td>Glu28Val</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*14</td>
<td>1A&gt;G</td>
<td>Met1Val</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*15</td>
<td>_1G&gt;A (intron 7)</td>
<td>Splicing defect</td>
<td>[114]</td>
</tr>
<tr>
<td>TPMT*16</td>
<td>488G&gt;A</td>
<td>Arg163His</td>
<td>[115]</td>
</tr>
<tr>
<td>TPMT*17</td>
<td>124C&gt;G</td>
<td>Gln42Glu</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*18</td>
<td>211G&gt;A</td>
<td>Gly71Arg</td>
<td>[110]</td>
</tr>
<tr>
<td>TPMT*19</td>
<td>365A&gt;C</td>
<td>Lys122Thr</td>
<td>[115]</td>
</tr>
</tbody>
</table>

Table 2. Listing of variant alleles of the gene for TPMT including the location of the nucleotide polymorphism and changes in the protein primary structure.

Variant TPMT alleles occurring in Caucasian population and their phenotype

About 90% of the Caucasian population shows standard enzymatic activity. Medium enzymatic activity correlates with the heterozygous genotype, i.e. the presence of some variant alleles, and is found in less than 10% of the population. Enzymatic activity is virtually unde-
tectable in one of 300 individuals. This condition is related with the occurrence of a homozygous genotype for variant alleles [116]. It is sufficient to administer to these patients just 6-10% of the standard azathioprine dose. In heterozygotes, the activity of an enzyme transcribed according to the matrix of a standard allele is metabolically sufficient. In these patients, however, it is necessary to consider the increased hematotoxicity of the therapy.

Allele TPMT*1 (wt)

Marking TPMT*1 belongs to the standard allele (wild type, wt), which is the most frequent in population. Enzymatic protein, which is expressed on the basis of this nucleotide sequence, has a high, which means standard activity.

Allele TPMT*2 (238G>C; 80Pro>Ala)

Allele TPMT*2 carries a substitution 238G>C (exon 5). This nucleotide transversion leads to a change of the primary structure of the protein, in which the rigid amino acid proline (at position 80) is substituted for alanine, an amino acid which is chemically more flexible. This results in altered intermolecular interactions, a disturbed tertiary structure and also the catalytic activity and stability of the protein [107], and a decreased enzymatic activity of up to a 100 times is expected [116].

Allele TPMT*3A (460G>A, 719A>G; 154Ala>Thr, 240Tyr>Cys)

Allele TPMT*3A includes two nucleotide substitutions in the gene sequence. Substitutions at positions 460G>A (exon 7) and 719A>G (exon 10) lead to the substitution of alanine for threonine at position 154, and tyrosine for cysteine at position 240 of the peptide chain. Based on this mutation, a very unstable protein is formed during the protein synthesis [110]. Enzymatic activity is decreased up to 200 times [116].

Allele TPMT*3B (460G>A; 154Ala>Thr)

Allele TPMT*3B carries only one nucleotide substitution, at position 460G>A, resulting in the change of the primary protein structure due to the substitution of alanine for threonine at position 154. The expressed protein has an extremely unstable structure and is rapidly degraded by cell proteases.

Allele TPMT*3C (719A>G; 240Tyr>Cys)

The nucleotide substitution at position 719 (exon 10) and the consequential amino acid substitution at position 240 have a similar effect on protein stability [111].

The above variant alleles are present in 85-90% of individuals with a low enzymatic activity of the TPMT enzyme. Other gene polymorphisms occur in the Caucasian population with such a low frequency that their low incidence in the population makes them quite difficult to detect, and the possibility to use them in clinical practice is minimal.

Among non-standard alleles of the gene for TPMT, variant alleles TPMT*3A, TPMT*3C and TPMT*2 have the highest frequency. Population testing revealed substantial racial and ethnic differences in the occurrence of variant alleles. For example, the inhabitants of South Asia (India, Pakistan) reveal a smaller incidence of variant alleles – from detected non-
standard alleles, only the TPMT*3A was confirmed [117]. On the other hand, the African population has an overall frequency of variant alleles comparable with Caucasian population, but the highest representation among non-standard alleles has TPMT*3C [118]. This allele (TPMT*3C) represents 50% of variant alleles in Afro-Americans, compared with only 5% in Americans of European origin [104]. The most common variant allele in the Caucasian population is TPMT*3A (some papers state that up to 10% of the population carries the heterozygous genotype; [119]. The occurrence of individual variant alleles in various populations is shown in Table 3.

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>TPMT*2</th>
<th>TPMT*3A</th>
<th>TPMT*3B</th>
<th>TPMT*3C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian - France</td>
<td>0.7</td>
<td>3</td>
<td>0</td>
<td>0.4</td>
<td>[119]</td>
</tr>
<tr>
<td>Caucasian - Germany</td>
<td>0.5</td>
<td>8.6</td>
<td>0</td>
<td>0.8</td>
<td>[113]</td>
</tr>
<tr>
<td>Caucasian - Norway</td>
<td>0</td>
<td>3.4</td>
<td>0</td>
<td>0.3</td>
<td>[120]</td>
</tr>
<tr>
<td>Caucasian - America</td>
<td>0.2</td>
<td>3.2</td>
<td>-</td>
<td>0.2</td>
<td>[112]</td>
</tr>
<tr>
<td>Afro - American</td>
<td>0.4</td>
<td>0</td>
<td>-</td>
<td>2.4</td>
<td>American</td>
</tr>
<tr>
<td>Afro - Kenya</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>5.4</td>
<td>[118]</td>
</tr>
<tr>
<td>African - Ghana</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>7.6</td>
<td>[121]</td>
</tr>
<tr>
<td>Asian - Southeast Asia</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>[122]</td>
</tr>
<tr>
<td>Asian - Japan</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.6</td>
<td>[123]</td>
</tr>
<tr>
<td>Asian - China</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>[124]</td>
</tr>
</tbody>
</table>

Table 3. Occurrence of variant alleles TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C in various populations (in %).
Legend: 0 – occurrence of alleles was not confirmed; - allele was not detected.

An important outcome of these population studies is that clinically significant variant alleles or their polymorphisms are present in all populations; therefore, the same diagnostic methods based on the determination of genotype can be used. [106]. From the phenotype perspective, the overall protein stability of TPMT is the most important. In vitro and in vivo studies confirm that the quantity of expression of variant alleles is comparable with the expression of the variant allele TPMT*1. It is the shorter biological halftime of the protein expressed on the basis of the variant allele which is responsible for the decreased metabolic activity of the enzyme [109]. Experiments assessing protein quality using the Western blot procedure imply that enzymatic protein produced by the transcription of the variant allele has a significantly shorter halftime compared with proteins translated according to the wt allele. Western blot analysis in cells with two copies of a variant allele did not confirm the presence of enzymatic protein. This fact directly correlates with the phenotype feature – undetectable TPMT activity. Tai et al. [109] determined protein quantity in vitro and found that the amount of enzymatic protein at a particular time after the expression decreased the most in the variant allele TPMT*3A, (200 times lower compared to TPMT*1). In the variant allele TPMT*2, the decrease of protein density was smaller by one order (20 times). In the allele
TPMT*3B, in which only one nucleotide substitution is present, the protein density was decreased 4 times; in the variant allele TPMT*3C, the density determined by Western blotting was comparable with the wt allele [109]. Furthermore, the amount of protein determined by Western blotting after 24-hour cultivation also corresponds with the results of degradation halftimes for native proteins. It was confirmed that the halftime of a protein expressed from a wt allele is about 18 hours. A comparable halftime can be observed in case of the protein, if it was expressed from the allele TPMT*3C. Degradation halftimes in the alleles TPMT*3B (6h) and TPMT*2 (0.2h) are significantly shorter. The shortest degradation halftime detected in a protein expressed according to the sequence corresponds with the allele TPMT*3A (0.25h; [109]). These results imply that the lowest enzymatic activity, resulting from the fast degradation of a protein, is connected with the alleles TPMT*3A and TPMT*2. This hypothesis is supported by the phenotype-undetectable metabolic activity of enzymes in patients with the variant alleles TPMT*3A and TPMT*2 in their genotype.

4.3.1.2. The gene for Inosine Triphosphate Pyrophosphatase (ITPA)

Inosine triphosphate pyrophosphatase (ITPA; EC 3.6.1.19) is one of the ubiquitous cytosol enzymes. It catalyzes the reaction of inosine triphosphates hydrolysis to inosine monophosphate. Even though the activity in various types of cells is different and the role of the enzyme in the cell is not clearly understood; it is supposed that it prevents the accumulation of false nucleotides, which could be incorporated in the DNA and RNA molecules instead of standard nucleotides, and therefore damage these macromolecules. Another possible role of these nucleotides can be their competition with GTP during cell signalling processes [125]. The role of ITPA in azathioprine biotransformation lies in the hydrolytic breakdown of potentially hepatotoxic 6-thio-ITP.

The gene coding for ITPA is situated on the short arm of chromosome 20 (20p13) and contains 8 exon sequences [126]. Several nucleotide polymorphisms were identified in its sequence; in 5 of these polymorphisms, a correlation with a low activity of the native protein was proven [127]. The lowest enzymatic activity was discovered in individuals who carry the nucleotide substitution of cytosine for adenine at position 94 in the sequence of the exon 2 (94C>A). At the protein primary structure level, this substitution is manifested by the substitution of proline for threonine at position 32, which decreases its affinity for the production of active dimer in ITPA proteins, decreases the protein stability and leads to a non-specific mRNA splicing [128,125,129]. The heterozygous genotype for the variant allele 94A is from the phenotype perspective manifested by the decrease of ITPA activity up to 22.5% of the activity of a standard form. ITPA activity was not detectable in individuals who are homozygous for this variant allele [128]. The frequency of the variant allele 94A in the ITPA gene is 5–7% in the Caucasian population [130–133]. Another nucleotide substitution in which an impact on the enzymatic activity was confirmed, is the substitution of adenine for cytosine at the sequence of intron 2 (IVS2+21A>C). The presence of this substitution leads to a faulty mRNA splicing during post-transcriptional modifications and the formation of a protein, which reaches 61% activity in heterozygous individuals, when compared to the native form of the enzyme [134,125]. Homozygous carriers of this variant allele have their en-
zymatic activity at 30% of the activity of a standard form [132]. The frequency of this variant allele in the Caucasian population reaches 10-13% [128,134]. Individuals who have the variant allele 94A in their genome as well as the variant allele IVS2+21C, showed 10% activity of ITPA [128]. A connection between the decreased ITPA activity and the occurrence of pancreatitis, a rash, a flu-like syndrome and leukopenia was identified in patients treated by azathioprine [135,136].

4.3.1.3. The gene for Xanthine Dehydrogenase (XDH)

Xanthine dehydrogenase (XDH, XO; EC 1.17.1.4) also belongs among cytosol enzymes, which participate in the metabolism of endogenous purine and pyrimidine nucleotides, as well as exogenous substances with this chemical structure [137]. The enzyme activity has been observed mainly in hepatocytes, enterocytes and renal parenchyma cells. XDH catalyses the oxidation of hypoxanthine to xanthine and consequently to uric acid. This enzyme is a crucial element of the biological availability of drugs containing the thiopurine molecule [138]. XDH activity significantly fluctuates depending on the ethnicity, sex and tissue type [139–141]. The administering of the competitive XDH inhibitor allopurinol leads to a higher biological availability of 6-MP (6-mercaptopurine) and in some cases to a better therapeutic response to thiopurine therapy [138,142].

The gene coding for the enzyme XDH is situated on the short arm of chromosome 2 (2p23). In humans, several polymorphisms have been identified, which facilitate the formation of 21 allelic variants [143]. Several of these allelic variants are associated with the occurrence of adverse effects during azathioprine therapy and with higher levels of toxic metabolites 6-MP [133,144]. Several of the noted single nucleotide polymorphisms influence XDH enzymatic activity. However, many of these variant alleles have only been discovered within the Japanese or Asian populations. A potential clinical importance can be expected in example of polymorphisms 837C>T, which has a 6% frequency in the Caucasian population. At the primary protein structure level, there is no amino acid substitution. Nevertheless, this polymorphism somehow influences enzymatic activity and its occurrence correlates with higher levels of thiopurine metabolites. A possible explanation may be the coincidence with an unknown polymorphism [144] or RNA interferency. During the reaction of the catalyzed XDH, free oxygen radicals are generated, which are expected to participate in the hepatotoxic effect of thiopurines [145].

4.4. Biological therapy

Biological therapy represents a completely new therapeutic approach to chronic inflammatory diseases. This therapeutic approach has proved useful not only in IBD therapy, but also in the therapy of other chronic inflammatory diseases, such as rheumatoid arthritis, ankylosing spondylarthritides and psoriasis. In IBD, the most common drugs intervene in the TNFα cytokine-mediated signalling at the molecular level (infliximab, adalimumab and etanercept). The main mechanism of these “anti-TNFα” antibodies is the binding of a drug to the TNFα soluble form (sTNFα) and blocking biological functions, which are mediated by it. Some studies demonstrated that individual antibodies have a different neutralisation poten-
tial and that this ability is also dependent on the TNFα concentration. At high sTNFα concentrations, the neutralising potential of infliximab, adalimumab and etanercept is comparable [146]. However, if the sTNFα concentration in the tissue is small, etanercept is about 20 times more effective than other antibodies. Two mechanisms are employed during the binding of the antibody to the membrane-bound TNFα form (tmTNFα). The first is antagonism, preventing the interaction between tmTNFα and its receptor (TNFR). The second is agonism; the binding of the drug to tmTNFα activates the reverse signalling cascade which leads to the suppression of TNFα production, other pro-inflammatory cytokines and apoptosis [147].

The cytotoxicity of anti-TNFα antibodies is caused by two mechanisms. The first lies in the binding of antibodies to tmTNFα and the apoptosis activation. The second mechanism is based on the initiation of complement-dependent cytotoxicity through the antibody Fc fragment. The antibody Fc fragment has the function of effector mediated by the Fc receptor in immunocompetent cells of monocytes and macrophages, NK cells and some types of T lymphocytes. Activation initiates a cascade of intracellular processes leading to phagocytosis, degranulation, activation of the complement and cytokines release [148]. Monoclonal substances with an Fc fragment in its structure (infliximab, adalimumab, and etanercept) have the ability to interact with these receptors. The antibody or the complex anti-TNFα Ig-TNFα can bind to the receptor [147]. One of the major risks of biological therapy is the production of antibodies against the drug. No of anti-TNFα drugs, in which the sequences are fully human, have this undesired property suppressed. Infliximab is the most immunogenic chimeric antibody [149]. The production of antibodies targeted against the drug decreases its therapeutic effect and increases the drug clearance. Due to this, the anti-TNFα therapy is supplemented by immunosuppressives (MTX, AZA) with the goal to prevent the induction of immune reactions [150].

4.4.1. Polymorphisms, which influence the anti-TNFα antibodies therapy

Mutations in the gene TNFα, especially mutations in the promoter area of this gene, lead to an altered expression of the gene and subsequently to a change in the amount of active TNFα. Its increased level result not only in pathological manifestations of chronic inflammation, but also in the effectiveness of the biological therapy, which at the molecular level influence the TNFα cytokine-mediated signalling (infliximab, adalimumab and etanercept). Therefore, the gene coding for the cytokine TNFα is classified among candidate genes as well as therapeutic genes, whose protein products influence the effectiveness and safety of drug therapy. More information on polymorphisms in this gene can be found in Chapter 2.5.

TNFα production is strictly controlled. Most of the regulatory mechanisms are embedded into the post-transcription processes [148]. The amount of the TNFα active form (both s and tm forms) is therefore dependent not only on the presence of the above mentioned SNPs, but also on the stimulation of cytokine-producing cells, TACE activity (TNF-α-converting enzyme) and intracellular regulatory processes and mRNA stability [151]. The initiating factor is the presence of gram-negative and gram-positive microorganisms (lipopolysaccharide, LPS, is one of the strongest stimulators), viral antigens or tumour-transformed cells.
Receptor molecules such as NOD2/CARD15 (receptor for muramyl dipeptide, MDP) and transmembrane receptor structures of the TLR family (receptor for lipopolysaccharides, LPS) play a key role in this step. The system of gradual activation of intracellular elements of signal pathways leads to the activation of NF-κB, which is common for both of these pathways and leads to the activation of the expression of TNFα and other pro-inflammatory cytokines. Another area, which influences the level of TNFα expression, is the promoter area of the gene coding for this cytokine.

There are also other cytokines which influence TNFα production (e.g. IL-1, IL-17, GM-CSF and interferon-γ), the antigen-antibody complexes and complements. TNFα release is also stimulated by pathological damage to tissue due to trauma or ischemia/hypoxia [148].

Factors which quantitatively influence the TNFα expression as well as molecules, which are inducers of this expression, seem to be potential predictors of the therapeutic response to biological therapy. One of the key molecules which regulate the gene expression of many cytokines is the nuclear factor κB (NF-κB) [14,15]. Its activation is closely linked with complex intracellular processes, by which the immune system recognizes bacterial antigens and reacts to their presence. In mammalian cells, there are NOD proteins and Toll-like receptors (also the so-called pattern recognition receptors, PRRs), which recognize these bacterial structures. More information about polymorphisms in the NOD2/CARD15 and TLR4 genes is given in Chapters 2.1 and 2.4.

During the infliximab therapy, the growth of apoptosis was confirmed in immune cells. As described above, it is one of the effect mechanisms which lead to the suppression of the immune reaction. A Belgium group of scientists led by Mr. Hlavaty [152] based their research on this finding and demonstrated the possible impact of polymorphisms in genes for individual proteins of the pro-apoptotic pathway (Fas-ligand and caspase 9) on the infliximab therapy effectiveness in patients with Crohn's disease. Apoptosis can be induced in cells by the “extrinsic pathway”, where TNFα molecules, Fas ligand and TRAIL (TNF-related apoptosis-inducing ligand) work as inducers of the apoptosis. There is also an intrinsic pathway, which includes the release of cytochrome c from mitochondria in a reaction to DNA damage. Both of these pathways are connected in the area of activation of cysteinyl-aspartate-specific protease (Caspase 3). Fas ligand is expressed in many immune system cells and, via the apoptosis induction, maintains the balance between the production and death of T lymphocytes and B lymphocytes. In this manner it contributes to immune tolerance [153]. The gene for Fas ligand is situated on the long arm of chromosome 1 (1q23). It consists of 4 exons and its length is approximately 8kb [153]. Polymorphism located in the promoter area (−844C>T) is at a position where a transcription enhancer has bonded. Variant allele T is responsible for the decreased binding affinity of this transcription factor. Individual homozygous for variant T allele have a decreased expression activity and a decreased quantity of the native protein in the membrane [154]. Patients with such polymorphism have a decreased activation of the pro-apoptotic pathway.

The intrinsic pathway for apoptosis activation is formed by individual cytosol proteases, which are activated in cascades. Caspase 9 (Casp9) is one of them. The primary stimulus for the activation of intrinsic pro-apoptotic pathway is damage to mitochondria and cyto-
chrome c release. This, after binding to APAF-1, forms a complex with caspase 9 [155]. The consequential chain of reactions results in the activation of other proteases - caspase 3, 6 and 7, which cause the disruption of the nuclear membrane, the fragmentation of nucleic DNA and the production of apoptotic vesicles [156]. Even though there has been some research focusing on the possible association between the 93C>T polymorphism and infliximab therapy effectiveness or the predisposition to Crohn’s disease and the disease activity [157], its role at the molecular level has not been exactly understood to date. However, it was demonstrated that this polymorphism does not result in the amino acid substitution at the level of the protein primary structure.

5. Gene polymorphisms and adverse effects of azathioprine treatment

During azathioprine therapy, about 10-28% of patients experience adverse effects [158–160]. The most serious of these is myelosupression, which is most often manifested as leukopenia. In their study, Katsanos [161] found the myelosupression incidence totalling 7%. Other common adverse effects, which may result in the termination of the therapy, are hepatotoxicity (9.7%), pancreatitis (10.5%) and digestive intolerance (17.5%), and to a lesser degree, extended infections (6.1%) or flu-like symptoms (5.7%) [162]. Most of these adverse effects are dose-dependent reactions.

The occurrence of myelosupression correlates with high levels of active azathioprine metabolites (6-thioguanine nucleotide, 6-TGN) [163], which result from a deficient azathioprine metabolism in individuals with a variant \textit{TPMT} genotype. There are many experiments which confirm this dependence [136,164–167].

We tested a group of 188 patients treated with azathioprine for inflammatory bowel disease (IBD). During the therapy, 34 individuals experienced leukopenia (WBCs count <4 x 10^9/L), hepatotoxicity (alanine aminotransferase or aspartate aminotransferase > 3times upper limit of normal) was confirmed in 4 patients, digestive intolerance was revealed in 4 individuals (nausea, vomiting, abdominal pain associated with treatment; ceased after AZA withdrawal) and pancreatitis developed in 2 patients. The variant \textit{TPMT} allele was confirmed in 8 (23.5%) patients with leukopenia. Within the group of patients who did not experience leukopenia, the variant \textit{TPMT} allele was confirmed in 8 people (5.2%). Fisher test confirmed an association between the variant \textit{TPMT} genotype and leukopenia ($P=0.003$) – see Figures 2, and 3.

Patients with a variant \textit{TPMT} genotype (heterozygotes) were five times at risk of developing leukopenia than patients with a standard genotype ($P = 0.003$, CI 95%, 1.8058–13.8444) [168]. These findings comply with previously published findings [136,164–167].

The FDA recommends a large-scale \textit{TPMT} genotype testing prior to the azathioprine therapy [169]. Nevertheless, the presence of the variant \textit{TPMT} genotype does not imply an absolute azathioprine contraindication in these patients. There were even earlier works, which reported the desired therapeutic effects without the manifestation of myelotoxicity in pa-
patients with variant TPMT genotypes achieved by a proportionate decrease of the dose [170]. Carriers of variant TPMT alleles were recommended to decrease the AZA dosing to 30-70% or to 10% of standard doses in heterozygotes and in variant homozygotes, respectively [165,171]. In these patients, the dosage should be carefully subjected to titration and regular checking of WBCs counts [170,172–174].

**Figure 2.** Occurrence of leukopenia in patients with different genotypes. Note: +/+: carrier of two wild type alleles TPMT*1; +/-: carrier of one variant allele: TPMT*2 or *3A, *3B or *3C.

**Figure 3.** Frequency of variant alleles in patient with/without adverse effects

A similar association between deficient azathioprine metabolism and the occurrence of leukopenia can be expected in the case of the polymorphism in the gene for XDH. Even though our set revealed a tendency for a higher frequency of the variant allele 837T in patients with
leukopenia (4.4%) compared with patients without this adverse effect (2.6%), this difference was not statistically significant [168,175]. Unlike the TPMT enzyme, whose activity is well described and quantified in individuals with standard and variant genotypes, the relationship between the genotype and phenotype activity of XDH is not so clear. It is supposed that despite the decrease of metabolic activity, this decrease is not as striking as in case of TPMT. Furthermore, the decreased XDH activity can be better compensated for by the activity of other enzymes, which participate in azathioprine metabolising. However, the situation is different if XDH is inhibited by allopurinol. In this case, there is probably an extensive competition that would result in the 6-TGN levels having a toxic effect (343 pmol/8x10⁸) [163].

To date, no pathological condition connected with decreased ITPA activity has been described. Similarly, available data regarding the association between polymorphisms in the gene for this enzyme and the occurrence of adverse effects of azathioprine therapy are contradictory. Some studies describe the association between decreased ITPA activity and the occurrence of pancreatitis, rash, flu-like syndrome and leukopenia [135,136]. There are no other studies confirming the association between the ITPA polymorphism and the occurrence of adverse effects [166,176,177]. Our test group included 4 patients who experienced digestive intolerance during azathioprine therapy; 2 of these patients were heterozygous for the variant allele 94A in the gene ITPA. The frequency of the variant allele in patients with digestive intolerance reached 25% (compared with 6.5% in patients without digestive intolerance) but due to a low number of individuals its statistical interpretation is difficult – see Figure 3. However, we confirmed that patients, who are carriers of the variant allele, have a 3.33 times greater probability of digestive intolerance (CI 95%, 1.09-13.5) than patients with a standard allele [168,175]. The association between the variant allele 94A and the occurrence of digestive intolerance must be confirmed in a larger set of patients. The occurrence of pancreatitis was similarly rare (n=4) in our set. In these patients, the variant allele XDH 837T had a frequency of 25% (compared with 2.7% in patients without pancreatitis). It is expected that the presence of this variant allele leads to the decreased activity of the XDH enzyme [168,175] – see Figure 3. Because of this, 6-MP is subsequently metabolised and the levels of its cytotoxic metabolites increase, which can cause pancreatitis [133]. In the group of individuals, in whom the variant genotype was confirmed, there were 12.1 individuals with pancreatitis per one individual without this adverse effect (CI 95%; 1.15-126.4). However, even in this case it is necessary to confirm or disprove these results in a larger set of patients. The most important polymorphisms with a significant impact on the azathioprine therapy are SNPs in the gene for TPMT. The impact of other genetic variants must be verified in a larger set of patients.

6. Gene polymorphisms as predictors of the effectiveness of infliximab therapy

Infliximab, a chimeric monoclonal antibody, was the first biological agent registered for the treatment of CD by FDA in 1998. Presently, infliximab, adalimumab and cetrelizumab pegol
are used in the therapy of CD. Subsequently, the effectiveness of biological therapy was also confirmed in moderate to severe active UC [178–180].

The effect of biological therapy is based on the suppression of the anti-inflammatory effect of cytokines, which are inflammation mediators. In patients with IBD, it is assumed that TNFα has the main role in the induction and prolonging of the inflammatory reaction. The use of monoclonal antibodies inhibiting this cytokine has been a great therapeutic benefit to the therapy. Biotechnologically produced monoclonal antibodies have already demonstrated its significant position in the therapy of IBD as well as other chronic inflammatory diseases. Infliximab was firstly used for Crohn’s disease therapy and later Rutgeerts et al. confirmed its effect in patients with UC [179]. Infliximab as a monoclonal antibody has an affinity for both soluble and membrane-bound forms of TNFα. Binding to soluble cytokine inhibits its pro-inflammatory effect, and the affinity for the membrane form of TNFα induces apoptosis. Today, biological therapy in IBD has a significant position in the treatment of patients who do not respond to the conventional pharmacological approach, or in corticoid-dependent patients and in patients with severely active Crohn’s disease with fistulas and in serious active ulcerative colitis. The high therapeutic effectiveness of infliximab was confirmed by large-scale clinical studies [179,181,182].

During infliximab therapy, several adverse effects were described. However, except for the allergic reaction, which occurs immediately after the infliximab is administered, they occur with low frequency (183). Biological therapy can therefore be considered highly effective. Still, there are about 20-30% of patients in whom no therapeutic effect occurs. At first, it was thought that the reason for the absence of any clinical effect was the presence of autoantibodies against infliximab [184]. However, this hypothesis has not been fully confirmed. Other possible causes are the subject of the study [185,186]. Factors which quantitatively influence TNFα expression seem to be among the potential predictors for the therapeutic response. They are molecules, which participate in the activation of NF-κB, the inductor of the TNFα expression. Nuclear factor κB (NF-κB) is a key component in the regulation of expression in the area of genome-carrying genes for cytokines [14,15]. Its activation is closely connected with complex intracellular processes, by which the immune system responds to the presence of bacterial antigens. For example, the presence of muramyl dipeptide (MDP) is detected by the interaction with NOD2. This cytosol protein is a component of cascade, which leads to local activation of the immune system in response to the presence of pathogen, and consequently to the expression and release of cytokines including TNFα. The gene for NOD2 lies in the area of the genome, which was earlier associated with incidences of IBD and which is linked to the occurrence of IBD [21,8]. In the Caucasian population, there are two single nucleotide polymorphisms marked 702Arg>Trp and 908Gly>Arg and a frameshift mutation 1007fs (3020fsinsC), which occur with a relatively high frequency. In vitro studies confirmed that a cell, which carries in its genome an allelic form corresponding to variant alleles 702Trp and 908Arg, is able to release TNFα after the induction by muramyl dipeptide in a quantitatively comparable amount like a cell with wild alleles in these polymorphisms [187–189]. These nucleotide substitutions lead to the change of the primary structure, but not so significant a change at the level of the secondary and tertiary protein
structures. Therefore, the impact on the biological functions of the coded protein is minimal [188]. The insertion of cytosine nucleotide in the case of the frameshift mutation leads to the shift of the reading frame during the translation and the formation of a premature stop codon [190]. The transcription of the variant allele results in the production of a protein, which is shorter by the area coded behind the stop codon. Due to this, the biological activity of the shortened protein NOD2 is strongly disturbed, which was confirmed by in vitro and in vivo studies, which discovered that the production of TNFα was not detected after the stimulation of MDP [187–189]. The presence of the frameshift mutation therefore results in the absence of the TNFα expression via NF-κB [15].

If we accept the hypothesis that a higher effectiveness of infliximab therapy correlates with lower levels of TNFα, then carriers of the alleles, which are connected with the lower expression of this cytokine, should also respond favorably to the therapy. Our results confirm this hypothesis, even though we did not study the phenotype (plasma or tissue levels of TNFα) but only the genotype. The frequency of variant alleles, in which the significant influence on the quantity of the TNFα expression (702W and 908R) was not confirmed in vitro, was comparable in patients with a therapeutic response and in patients resistant to the therapy. On the other hand, the frequency of the frameshift mutation, which leads to a significant decrease of the TNFα expression after the MDP induction, was statistically and significantly higher (15.2%) in patients who reacted positively to the therapy, compared to patients that were resistant to the therapy (3.6%). Patients, who are carriers of the frameshift mutation, have a 4.25 times higher probability that the infliximab therapy will be effective (CI 95%; 1.06-17.07). The frequency of the frameshift mutation (variant allele 1007fs) was virtually identical in patients who responded to the infliximab therapy by mucosal healing, and in patients who revealed only clinical improvement of parameters (15.15% and 15.22%, respectively).

The TNFα expression via NF-κB occurs also after the binding of a specific ligand, e.g. lipopolysaccharide (LPS), to the membrane TLR4 receptor. The activation of the intracellular signalling cascade and the expression of pro-inflammatory cytokines occur in cooperation with the molecule CD14 [191]. In the TLR4 gene, there are nucleotide polymorphisms, which influence its biological functions [192,193]. One of these is a substitution of adenine by guanine at the position 896 of the nucleotide chain. The variant allele 896G has a lower ability to induce TNFα production in reaction to LPS stimulation in the in vitro experiments [192,193]. Analogically to the variant genotype NOD2, there is a possible hypothesis that the variant allele 896G is associated with a better therapeutic response. The occurrence of variant allele in our set was the highest in patients who responded best to infliximab therapy, i.e. those who showed both a clinical and morphological response to the therapy (9.9%). In patients with only a clinical response, the frequency of the variant allele was lower (3.6%), and among patients resistant to the therapy, there were only 1.8% of carriers of the variant allele. We can describe a certain tendency that a carrier of the variant allele has a greater probability that infliximab therapy will be effective. However, differences in the frequencies of the variant allele 896G in the TLR4 gene between individual groups of patients were not statistically significant. According to the odds ratio, there are 2.59 patients with both a clinical and
morphological reaction per one patient, with a clinical reaction in the group of patients with a variant allele 896G in the gene TLR4 (CI 95%; 1.01-8.39) [175].

Several polymorphisms influencing the expression degree were described in the promoter area of the gene coding for TNFα. A single nucleotide substitution –308G>A is often mentioned. Production of TNFα can be increased by this mechanism even under the standard stimulation conditions. The variant allele –308A occurred with a 14.03% frequency in the set of patients. Even though according to the in vitro testing, this variant allele means a higher production of TNFα due to a higher transcription activity of the gene [194], it could be expected that it will be more frequent in patients resistant to the therapy. Our results did not confirm statistically significant differences between individual groups of patients.

Based on the above information, it can be concluded that a patient who is a carrier of a genotype containing variant allele 1007fs in the gene NOD2, variant allele 896A in the gene TLR4 and the standard allele –308A in the gene TNFα, they will have the greatest probability that the infliximab therapy will be effective for them. Individuals who are homozygous for the standard (wild type) alleles of the listed polymorphisms in the gene NOD2, TLR4 and TNFα, are more often resistant to the infliximab therapy. This standard genotype was in our study of 163 infliximab-administered patients confirmed in up to 75% of resistant patients, compared with 46.7% in the group with a therapeutic response. In patients with the best response to infliximab, (i.e. both clinical and morphological) the frequency of this allele combination was even lower (43.9%) – see Figure 4.

![Figure 4](http://dx.doi.org/10.5772/53465)

**Figure 4.** Occurrence (in %) of genotype consisted from wild type alleles in polymorphisms NOD2:1007fs, TLR4: 896A>G and TNFα: –308G>A in groups of patients with different response to infliximab therapy. Legend: C - M response - patients with clinical and morphological response to infliximab therapy; C response - patients with clinical response, but without morphological response; Therapeutic response - total number of patients in both groups (CM + C); Resistant patients = patients without clinical and without morphological response to infliximab therapy.
Patients who are carriers of the standard genotype, are 2.13 times more probable to be resistant to the infliximab therapy (CI 95%; 1.10-4.13) compared with patients with other allele combinations [175].

In the group of patients with the genotype containing standard alleles of the monitored polymorphisms, there were 3.43% of individuals resistant to the therapy per one individual with a therapeutic response (CI 95%; 1.37-8.60). Patients, in whom the infliximab administering resulted in a therapeutic response, were also most often carriers of the combination 1007fs mut in NOD2 gene, wt allele in TLR4 gene and wt allele in TNFα gene (17%). The genotype containing standard alleles (wt) of NOD2 and TLR4 genes, i.e. the genotype corresponding to the standard expression activity of TNFα, was significantly more often presented in individuals resistant to the therapy (89.3%). The genotype composed of at least one variant allele responsible for the lower expression activity of TNFα was significantly more common in patients with a therapeutic response (32.6%; \( P=0.02 \)).

In the group of patients with the greatest therapeutic benefits (clinical and morphological response), they most often represented carriers of at least one of the monitored variant alleles (39.4%) [175].

7. Conclusion of chapter – The potential of genotyping of IBD patients in clinical practice: The importance of gene polymorphisms for individualisation of IBD pharmacotherapy

Pharmacogenetics is a relatively young branch of pharmacology, which deals with the research of genetic backgrounds of individual differences in the patient’s response to therapeutic drugs, both from the perspective of drug effectiveness and its safety. Its origin and development is closely connected with new knowledge in the area of the human genome and the development of molecular biology methods, which are used in this branch. The first, partly euphoric visions regarding the use of pharmacogenomic findings for pharmacotherapy individualisation and personalised medicine were later revised thanks to the empirical experience from applying theoretical hypotheses into medical practice. The difference between theoretical hypothesis and empirical findings results from the complicated biological system both at the level of genome and its regulatory mechanisms, and at the level of proteosynthesis, signalling pathways and interactions of intracellular molecules. The more we know about the human genome, the more we realise that we can only guess how many of its regions are activated in a decisive moment, and how complex the regulatory mechanisms are which influence the final effect, and resulted in phenotype.

The clinical application of pharmacogenetic findings can be limited by the existence of non-genetic factors. Even though genome cannot be influenced by external factors, the drug to drug interaction which occurs in the organism can influence not only the onset and development of a disease, but also the patient’s response to the drug. Furthermore, it is necessary to remember that the patient’s sensitivity to external factors is also a variable and that it is also
genetically conditioned. Because of this, pharmacogenetic information alone cannot predict exactly the effectiveness or safety of a drug. Despite this, pharmacogenetic studies have provided much significant information regarding the different rate of drug metabolisation in individuals as well as in populations, the effectiveness of various therapies and the development of serious adverse effects. Pharmacogenetics is a promising tool in the individualisation of the doctor-patient approach.

The characteristic presence of some polymorphisms in the “candidate genes” is connected with a predisposition to IBD. It seems that Crohn’s disease is more genetically conditioned than ulcerative colitis. Some polymorphisms are typical for CD, others for UC. In patients with CD, the frameshift mutation is statistically more often present in the gene NOD2/CARD15 (Leu1007fs insC) and the substitution mutation 469Lys>Glu in the gene ICAM-1 than in a healthy population. Unlike patients with UC, they less often carry the substitution mutation 702Arg>Trp in the gene NOD2/CARD15. All three studied mutations in the gene NOD2/CARD15 (Leu1007fs insC, 702Arg>Trp and 908Gly>Arg) are statistically more frequent in patients diagnosed with CD than in patients with UC. Genetic analysis could therefore help find individuals with a genetic predisposition that would require more frequent check-ups, or to inform physicians when to use additional endoscopic and laboratory tests or to help specify ambiguous diagnosis.

IBD therapy is lengthy; therefore it requires an individual choice of a drug with an aim to reduce or completely eliminate adverse effects, while at the same time maintain the therapy effectiveness. The aim is to achieve a maximum pharmacotherapeutic effect as well as the effective use of finance. The presence of variant alleles of polymorphisms in “therapeutic genes” can be of significant help in predicting the adverse effects of a given therapy. The relationship between myelosupression and the presence of variant alleles in the gene for TPMT is of the greatest interest. The frequency of variant alleles is significantly higher in patients who experience leukopenia during azathioprine therapy, than in patients in whom leukopenia does not develop. TPMT genotyping can therefore serve as a marker which will notify clinicians of a significantly increased potential risk in patients with a certain genotype, and on the necessity of increased surveillance over the patient or the need to adjust the dosage. In clinical practice, it is now common to determine variant alleles in the TPMT gene prior to the azathioprine therapy. This allows individualisation of the therapy and prevents serious complications.

Another desired goal is to find genetic markers which can potentially influence the effectiveness of pharmacotherapy, especially a therapy which is costly, such biological therapy for example. Our experimental data imply that patients who are carriers of a genotype composed from the variant allele 1007fs in the gene NOD2, variant allele 896A in the gene TLR4 and standard allele –308A in the gene for TNFα will have the highest probability that infliximab therapy will be effective. On the other hand, individuals who are homozygous for standard (wild type) alleles of the given polymorphisms in the gene NOD2, TLR4 and TNFα, are more often resistant to infliximab therapy. This standard genotype was confirmed in up to 75% of patients resistant to the therapy. Patients who are carriers of the standard genotype are 2.13 times more probable to be resistant to infliximab therapy (CI 95%; 1.10-4.13)
than patients with different allelic combinations. A more complex genotyping of a larger set of patients could help define the “risk genotype”, whose carriers have statistically and significantly decreased the probability of a full morphological response to anti-TNF therapy, or in contrast, to define a “positive genotype”, where the probability of a successful therapy would be very high.

**Abbreviations**

AA - Amino acid  
AP-1 - Activator protein 1  
APAF-1 - Apoptotic protease activating factor 1  
5-ASA - 5-aminosalicylic acid  
AZA - Azathioprine  
CARD15 - Caspase recruitment domain-containing protein 15  
CCR5 - C Chemokine receptor type 5  
CD - Crohn’s Disease  
CD-14 - Cluster of differentiation 14  
COPD - Chronic obstructive pulmonary disease  
CRP - C-reactive protein  
DNA - Deoxyribonucleic acid  
FDA - Food and Drug Administration  
GCs - Glucocorticoids  
GM-CSF - Granulocyte macrophage colony-stimulating factor  
GR - Glucocorticoid receptor  
GTP - Guanosine-5’-triphosphate  
hnRNA - Heterogeneous nuclear RNA  
IBD - Inflammatory Bowel Disease  
ICAM-1 - Intercellular Adhesion Molecule 1  
IκBα - Inhibitor kappa B alfa  
IL-1 - Interleukin 1  
ITPA - Inosine triphosphatase (nucleoside triphosphate pyrophosphatase)
LPS - Lipopolysaccharide
MDP - Muramyl dipeptide
MDR1 - Multidrug resistance protein 1
6-MP - 6-mercaptopurine
mRNA - Messenger RNA
MTX - Methotrexate
NAT - N-acetyl transferase
NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD2 - Nucleotide-binding oligomerization domain
OCT-1 - Organic cation transporter 1
P-gp - P-glycoprotein
PCR - Polymerase chain reaction
PCR-REA - PCR-restriction enzyme analysis
PCR-RFLP - PCR-restriction fragment length polymorphism
PRA - Primer restriction analysis
SNP - Single-nucleotide polymorphism
sTNFα - Soluble TNFα
TACE - TNF-alpha converting enzyme
6-TGN - 6-thioguanine nucleotide
TLR4 - Toll-like receptor 4
tmTNFα - Transmembrane TNFα
TNFα - Tumor necrosis factor alpha
TNFR - Tumor necrosis factor alpha receptor
TPMT - Thiopurine S-methyl Transferase
TRAIL - TNF-related apoptosis-inducing ligand
UC - Ulcerative Colitis
WBCs - White blood cells
wt allele - Wild type allele
XDH - Xanthine dehydrogenase
XO - Xanthine oxidase
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