EXPERT REVIEWS

Current concepts in the genetic diagnostics of rheumatoid arthritis

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Rheumatoid arthritis (RA) is a systemic, chronic and inflammatory disease of unknown etiology. *HLA-DRB1* and *PTPN22* 1858T gene variants are risk factors of RA, clinical manifestations and rate of progression of joint destruction in this autoimmune disease. Currently, several immunopathogenetic models of other genes (*CTLA4*, *MIF*, *PADI4* and *SLC22A4*) are under debate. The clinical influence of some of the gene polymorphisms associated with RA and the principles of pharmacogenetics applied to different therapies, such as classical disease-modifying anti-rheumatic drugs and new biological agents. Pharmacogenetics is a rapidly advancing area of research that holds the promise that therapies will soon be tailored to an individual patient's genetic profile.

Keywords: biological therapy • genetic diagnostics • pharmacogenetics • rheumatoid arthritis • single-nucleotide polymorphism

Background to rheumatoid arthritis

Autoimmune diseases affect approximately 4% of the Western population [1]. Among autoimmune diseases, rheumatoid arthritis (RA) is one of the most common disorders, affecting 1–1.5% of the population [1]. Concerning the etiology of the disease, genetic (see later) and environmental factors (e.g., tobacco, viral and bacterial infections exposure) should be mentioned; its genetic transmission pattern is unknown. RA is characterized by the inflammation and the destruction of joints, leading to severe damage and limitation of motion. Concerning the pathogenesis of the disease, antigen presentation and T-cell activation seem to be the trigger of an autoimmune response, prompting the investigation of genes associated with RA, several of which are included within the MHC [2]. The MHC (in humans, HLA) is found on chromosome 6p21, containing 252 expressed loci [3] with several genes playing a role in the immune response. The HLA genomic locus is subdivided into HLA class I, II and III loci. These regions contain the largest degree of polymorphism in the whole human genome (there are numerous known loci outside of HLA, see **Table 1**) [4]. The genes of HLA class II were found as the most significant genetic predisposition factor of the autoimmune diseases and RA. Former studies investigating the association of HLA-DR with autoimmune diseases in Type 1 diabetes, indicated a tight connection between the DR3 and DR4 alleles [5–8]. Association with RA is only proven at DRB1; associations with DQB1 are the results of linkage disequilibrium with DRB1 [8–10]. Studies by Gao, and Vignal *et al.*, verified that the association of DRB1 alleles (DRB1*0101, DRB1*0102, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0408, DRB1*1001 and DRB1*1402) was owing to similarities within the DRB1 peptide domain at positions between β70 and β74, and the alleles containing similar sequences were called 'shared epitopes' [11,12]. Amino acid changes within the shared epitopes rendered the differentiation of the predisposing alleles from the protective DRB1*0103, DRB1*07, DRB1*1201, DRB1*1301 and DRB1*1501 alleles [13]. Recent studies on the shared-epitope hypothesis have suggested a new approach, whereby the shared epitope only consists of positions β72–β74, and positions β70 and β71 have been proposed to modulate the association [14,15]. Laiovoranta-Nyman *et al.* confirmed that amino acid changes in the third hypervariable region of the DRB1 molecule both at positions β67 (leucine to isoleucine) and β70 (glutamine to aspartic acid), either alone or in combination, seem to provide protection against RA [16]. Currently, there are some different classifications for the shared epitope hypothesis [13,17–20].

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Table 1. Association of single-nucleotide polymorphisms with rheumatoid arthritis.

Gene	Locus	Polymorphism	SNP	Populations in the study	Association of the polymorphism with RA	p-value and/or OR	Ref.
ADRB2	5q31–q32	Arg16 and Gln27 Gly16 and Glu27	rs1042713; rs35892629 rs1042713; rs1042714	Swedish	Genotype combination GlyGly16–GlnGlu27 had more active disease than other patients	p = 0.005	[155]
CD40	20q12–q3.2	G→T	rs4810485	British	Strong association	$p = 2 \times 10^{-4}$ OR = 0.86	[156]
CD244	1q23.3	A→G	rs6682654	British	No evidence for association	p = 0.06	[156]
CDK6	7q21–q22	C→G	rs42041	British	No evidence for association	p = 0.06	[156]
CTLA4/ICOS	2q33	A→G	rs17268364	Canadian	Association	p = 0.004	[157]
HLA-DRB1	6p21.3	HLA-DRB1*04		Slovakian	Association	$p = 1.2 \times 10^{-13}$ OR = 2.92	[158]
IL-17A	6p12	A→G	rs2275913	Norwegian	Weak association with RA	p = 0.02 OR = 1.17	[159]
MHC2TA	16p13	-168 A→G	rs3087456	Swedish	Association	p = 0.01	[160]
PADI4	1p36.13	89163 G→A 90245 T→C	rs74219785; rs74211904	French	Presence of the AC haplotype had a positive association between RA	p = 0.002	[161]
PTPN22	1p13.3	A→G	rs2476601	British	Association	p = 0.001	[162]
PTPN22		A→G	rs2476601	Slovakian	Association	$p = 9.5 \times 10^{-4}$ OR = 1.67	[158]
PTPN22		A→G	rs2476601	North American	Association	p = 0.003	[163]
RUNX1	21q22.3	C→G	rs2268277	Western European	Not an RA-susceptibility gene	p = 0.08	[164]
RUNX1		C→G	rs2268277	Japanese	Association	p = 0.0035	[165]
STAT4	2q32.2–q32.3	G→T	rs7574865	Slovakian	Association	$p = 9.2 \times 10^{-6}$ OR = 1.71	[158]
STAT4		G→T	rs7574865	Greek	Association	p = 0.002 OR = 1.9	[166]
STAT4		G→T	rs7574865	Colombian	Association	p = 0.008 OR = 1.36	[167]
STAT4	2q32.2–q32.3	G→T	rs7574865	Spanish Swedish, Dutch	Association	p = 0.01	[156]
STAT4		G→T	rs7574865	Japanese	Association	$p = 8.4 \times 10^{-4}$ OR = 1.27	[168]
STAT4		G→T	rs7574865	North American	Association	$p = 2.81 \times 10^{-7}$ OR = 1.32	[169]
STAT4		C→T G→T C→T C→G	rs11889341 rs7574865 rs8179673 rs10181656	Korean	TTCG haplotype carries a significant risk for RA	p = 0.0027 OR = 1.21–1.27	[170]
STAT4		G→T	rs7574865	Swedish	Association	$p = 1.87 \times 10^{-4}$ OR = 1.55	[169]

ADRB2: Adrenergic receptor β2; CDK6: Cyclin-dependent kinase 6; CTLA4/ICOS: Cytotoxic T-lymphocyte-associated protein 4; MHC2TA: Class II, MHC transactivator; OR: Odds ratio; PADI4: Peptidyl arginine deiminase type IV; PTPN22: Protein tyrosine phosphatase nonreceptor type 22; RA: Rheumatoid arthritis; RUNX1: Runt-related transcription factor 1; SNP: Single-nucleotide polymorphism; STAT4: Signal transducer and activator of transcription 4.

Table 1. Association of single-nucleotide polymorphisms with rheumatoid arthritis.

Gene	Locus	Polymorphism	SNP	Populations in the study	Association of the polymorphism with RA	p-value and/or OR	Ref.
TNF-α	6p21.3	-238 G→A	rs174981	Mexican	Association with juvenile RA in males	p = 0.002	[171]
TNF-α		-308 G→A	rs891308	Mexican	Risk for juvenile RA was greater in females	p = 0.004	[171]
TNF-α		-308 G→A	rs891308	Czech	Association	p = 0.003	[172]

ADRB2: Adrenergic receptor β2; CDK6: Cyclin-dependent kinase 6; CTLA4/ICOS: Cytotoxic T-lymphocyte-associated protein 4; MHC2TA: Class II, MHC transactivator; OR: Odds ratio; PADI4: Peptidyl arginine deiminase type IV; PTPN22: Protein tyrosine phosphatase nonreceptor type 22; RA: Rheumatoid arthritis; RUNX1: Runt-related transcription factor 1; SNP: Single-nucleotide polymorphism; STAT4: Signal transducer and activator of transcription 4.

Immunobiology of RA

Rheumatoid arthritis involves multiple joints in a symmetrical pattern. The predominant symptoms are pain, stiffness and swelling of peripherial joints. At the beginning of the symptoms, joints of the hands, feet and knees are affected, and later, the damage can occur in almost all peripherial joints. Although RA is usually considered a disease of the joints, it can also cause numerous extra-articular manifestations. A hallmark of RA is the synovial inflammation; its severity may vary with the progression of the disease [21].

The genetic association of *HLA-DR1* and *HLA-DR4* with RA suggests that the disorder is partly, or entirely, driven by T cells. Although the pathogenetic role of T cells in RA has not been proven, the success of certain drug treatments (e.g., abatacept) implies that rheumatoid T cells are important in the development of the inflammatory process; therefore, T cells could be targeted in clinical treatment [22]. An HLA-restricted T-cell response to antigen(s) is suggested, since the majority of Caucasian RA patients (75–80%) have a shared epitope conserved across the *HLA-DR1* and *HLA-DR4* haplotypes [23]. T-cell responses to heat-shock proteins and microbial antigens, as well as to collagen type II, have been proven only in a small proportion of RA patients. Rheumatoid T cells maintain a highly activated phenotype, indicated by the expression of CD69 (an immune regulator protein), transferrin receptor (a barrier protein in iron metabolism) and HLA-DR. Simultaneously they are hyporesponsive to antigenic stimulation [24,25]. The pathogenesis of RA is mainly mediated by proinflammatory cytokines (e.g., TNF-α) [26]. Brennan *et al.* verified that spontaneous TNF-α production in the synovium of RA patients was mainly T-cell dependent, suggesting that the regulation of T-cell function is probably essential in controlling the disease [26]. Although antigen-dependent T-cell responses may be important in initiating the inflammatory response during arthritis, there is evidence that antigen-independent responses also play a role in the pathogenesis of RA. Synovial T cells in RA patients can activate the mononuclear phagocyte system, through a contact-dependent way, to induce the expression of inflammatory cytokines, such as TNF-α [27,28]. A crosstalk between natural killer cells and monocytes also results in the sustained stimulation of TNF-α production. Natural killer cells, activated by IL-15, stimulate monocytes to produce TNF-α in a contact-dependent manner and, in turn, monocytic cells induce CD69 expression as well as IFN-γ production in natural killer cells [29].

At present, IL-17 plays an essential role in the immunopathology of RA [30]. This cytokine is produced by Th17 cells, which represent a $CD4^+$ effector T-cell linage. IL-17 influences the function of macrophages, fibroblasts, epithelial and endothelial cells, as well as mesenchymal cells. IL-17 also induces the upregulation of nuclear factor κB, HLA class I, chemokines and cytokines, such as TNF-α, IL-6 and granulocyte–macrophage colony-stimulating factor [30,31]. Also of importance in RA pathogenesis are the effects of IL-17, driving osteoclastogenesis and leading to bone resorption.

The regulation of T-cell apoptosis is essential for lymphocyte homeostasis and immune function. The inhibition of apoptosis in the synovium of patients with RA was first reported in 1995. It was verified that the inhibition of synovial fluid leukocyte apoptosis in a very early phase of RA distinguished this type from other types of arthritides [32,33]. Apoptosis proceeds in two different ways: the intrinsic pathway, which is triggered by cellular stress caused by factors, such as DNA damage or heat shock, and the extrinsic pathway, which is stimulated by molecules released by other cells binding to transmembrane death receptors on the target cell [34].

Tregs inhibit the proliferation and cytokine production of conventional T cells. Their role is essential in controlling the inflammatory response. The volume of Tregs is higher in the synovial fluid than in the blood of RA patients [34].

Recent research with drugs, such as rituximab, has verified that the presence of B cells influences the proinflammatory response in RA. It is known that B cells are the precursors of the autoantibody-producing plasma cells. CD20 is a B-cell surface antigen, expressed only on pre-B cells and mature B cells. The depletion of B cells may inhibit different immune responses, as B cells are capable of internalizing, processing and presenting antigens through the MHC class II molecules to T cells [35]. This procedure leads to T-cell and macrophage activation, as well as TNF-α production.

Numerous autoantibodies have been reported in RA, but only rheumatoid factor (RF), antibodies to citrullinated antigens, and antibodies to immunoglobulin-binding protein, have shown sensitivity and specificity worth mentioning [36]. RF is present in 70–80% of RA patients, but is also detectable in 5–10% of healthy individuals or patients diagnosed with other diseases.

Antibodies to autoantigens modified by citrullination of arginine to citrulline are present in 65–70% of patients with RA, but are rather rare in healthy people (<2%) and in other

inflammatory conditions [37,38]. That is why these antibodies are significant in the diagnostics of RA. It has been verified recently that distinct genetic risk factors are associated with either anticyclic citrullinated peptide (anti-CCP)-positive or anti-CCP-negative diseases [39].

Genetic background of RA

Linkage analysis is a method to identify genomic regions containing genes predisposing to the examined disease by investigating related individuals. It is expected that affected relatives have identical haplotypes in the disease-causing region. Some studies were also conducted to test a linkage analysis in nonrelated individuals. In linkage analysis examinations, the genome can be screened by a whole-genome scan, as well as by multiallelic (microsatellites) or biallelic markers (single-nucleotide polymorphism [SNP]). Several genetic linkage investigations have been performed all over the world, from Europe to the USA, and from Japan to Australia [40–45]. The most important finding was evidence of linkage with the chromosome 6p21 region, which is where the HLA genes are located (Figure 1). Different genomic regions outside the HLA region may also show linkage to RA susceptibility. The HLA region contains 252 expressed loci [3], with several genes playing a role in the immune response. The genes of HLA class II were found as the most significant genetic predisposition factor of the autoimmune diseases and RA.

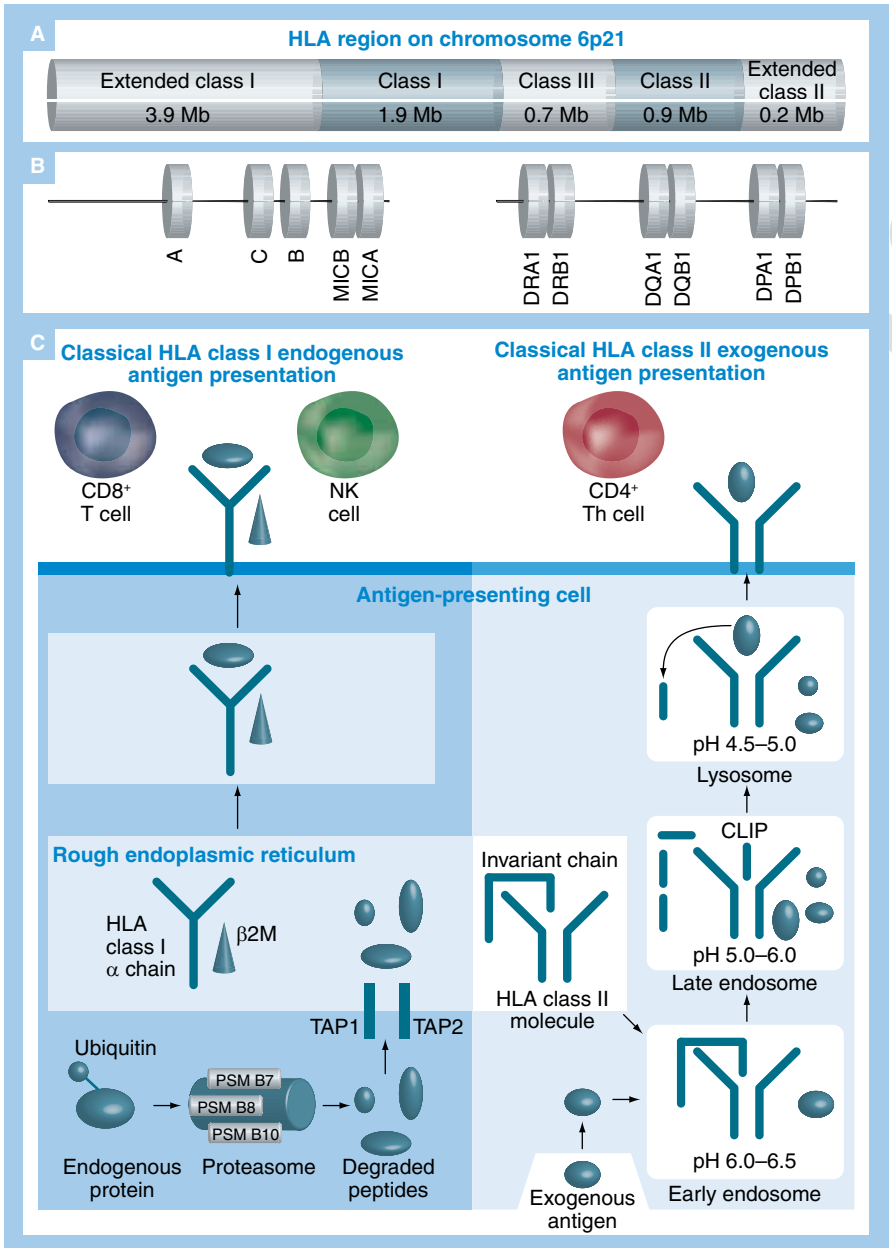

Figure 1. Representation of the HLA region on chromosome 6p21. (A) The HLA genomic locus is subdivided into HLA class I, II and III loci. **(B)** Name of genes coding into HLA class I and II loci. **(C)** Molecular process of the HLA endogenenous and exogenous antigen presentation.
Redrawn from [1].

There might be several loci implicated in the genetics of RA, with sizes less than that of the HLA region. Multiple genetic polymorphisms (e.g., *PTPN22*, *STAT4*, *TRAF1/C5*) are thought to be involved in RA pathogenesis (Table 1) [46].

The *PTPN22* gene is located on chromosome 1p13 [46]. The gene encodes a lymphoid-specific phosphatase (Lyp), an intracellular protein tyrosine phosphatase (PTP), which is involved in presenting spontaneous T-cell activation. The first report on the association of *PTPN22* in Type 1 diabetes was reported in 2004 [47]. Since then, *PTPN22* has become the most important common genetic risk factor for human autoimmune diseases outside the MHC region [48]. *PTPN22* C1858T polymorphism is also associated with systemic lupus erythematosus [49,50,51], juvenile idiopathic arthritis [52], Graves' disease [53,54], generalized vitiligo [55] and other autoimmune disorders. At the same time, it is noteworthy that no significant association with multiple sclerosis [56], celiac disease [57] or inflammatory bowel disease [58] was verified. Geographic differences in the 1858T allele frequencies have been reported. In Northern Europe, in the English and Finnish populations, the frequency of the 1858T allele is approximately 12%, while in Southern Europe, in Spain and Italy, it is approximately 6%. In Asia and among African–Americans, the allele is practically absent [59]. These data suggest that the incidence of the 1858T allele of *PTPN22* gene influences the degree of

predisposition to RA. *PTPN22* suppresses T-cell receptor signaling during thymic development, resulting in the survival of autoreactive T cells, which would have been deleted by selection among people carrying the 1858C/1858C genotype [60]. It is supposed that the 1858C/T (amino acid sequence R620W) polymorphism plays a significant role mainly in autoimmune disorders, with brisk autoantibody production. The protein tyrosine phosphatase encoded by this gene has an inhibitory effect on the proximal T-cell receptor signaling pathways. However, the consequences of carrying this variant, and the mechanism by which it contributes to the development of autoimmunity, are poorly understood. Seropositivity for the RF and anti-CCP autoantibodies have been associated with the 1858T allele [61]. Concerning *PTPN22*, it can be concluded that, in the future, this polymorphism along with other polymorphisms may help to diagnostics of RA (Table 2), probably in combination with nongenetic factors, such as anti-CCP autoantibodies [62].

CTLA4 is also a negative regulator of T-cell activation [63], and has been associated with multiple autoimmune disorders, including multiple sclerosis, Hashimoto's thyroiditis and Graves' disease [64,65]. The most widely studied polymorphism of *CTLA4* is +49A/G (rs 231775), located in the first exon of the gene. Together with *PTPN22*, *CTLA4* is also a suspected gene for RA. Associations of this SNP have been reported in Japanese [66], British [67], Irish [62] and German [68] populations. Another polymorphism of *CTLA4* is -318C/T (rs1065442), which has shown no association with RA in Korean [69] and Spanish populations [70]. CT60 is the *CTLA4* polymorphism with the highest functional significance; the G allele of this SNP is associated with lower mRNA levels of soluble CTLA4 isoform, which could increase T-cell activation [62]. Summarizing the recent knowledge on *CTLA4*, it can be concluded that these polymorphisms may facilitate the development of RA, although their significance is limited to determinate clinical subgroups of patients with RA.

PADI4 is a gene encoding an enzyme involved in the post-translational conversion of arginine to citrullin. PADI4 may play a role in granulocyte and macrophage development, leading to inflammation and immune response [71]. The anti-CCP autoantibodies, highly specific for RA, may appear years before the development of the disease [72–74], and PADI4 is thought to play an important role in the pathogenesis of RA. Suzuki *et al*. reported that, in the Japanese population, almost 50% of the SNPs (89163G/A; 90245T/C) in the *PADI4* gene were strongly associated with RA [75]. At the same time, the SNP of *PADI4* did not influence the outcome of RA in European populations [76–78]. Owing to these controversies, the exact role of *PADI4* polymorphisms in the susceptibility to RA remains unclear.

Macrophage migration inhibitory factor (MIF) is a cytokine that has proinflammatory, hormonal and enzymatic activity [79]. It is expressed in macrophages, as well as B and T cells. The presence of a mutation at -173G/C in *MIF* gene was recently shown to be associated with juvenile idiopathic arthritis, as well as RA [80].

Several investigations identified 1p36 as an important locus for predisposition of RA [81,82]. The *TNFR2* gene, which encodes TNF-α receptor 2, is found on this locus. TNF-α plays an essential role in the pathogenesis of RA. This fact suggests that the *TNFR2* gene is a major candidate for RA. An SNP characterized by the substitution of arginine for methionine at position 196 (exon 6), alters the intensity of signal transmission in the cells when TNF-α is bound to TNFR2 [83]. The data concerning the association of this exon 6 polymorphism and RA are conflicting. In a British population, the association proved to be significant, while no association was found among Japanese and Dutch populations [84,85].

SLC22A4 is located at 5q21, and encodes the solute carrier protein family protein 22A4. The biological role of *SLC22A4* is not yet clear, although it is found in the neighborhood of numerous genes involved in the mechanisms of inflammation [86]. In studies investigating the SNPs of *SLC22A4*, it has been verified that RA is associated with an intronic SNP, located in a sequence that contains a binding site for a transcription factor known as RUNX1.

Table 2. Association of single-nucleotide polymorphisms of *PTPN22* with rheumatoid arthritis.

Polymorphism of the *PTPN22* gene	Populations in the study	Association with RA	p-value and/or OR	Ref.
rs247660/1858C>T	Caucasian/ American	Associated with RA risk	p = 0.04 OR = 1.46	[173]
rs247660/1858C>T	Dutch	Association with RA	p = 0.134	[174]
rs247660/1858C>T	Finnish	Association with RA	$p = 3 \times 10^{-7}$ OR = 1.47	[175]
rs247660/1858C>T	Hungarian	Association with RA	p = 0.001 OR = 1.89	[176]
rs247660/1858C>T	Japanese	No association with RA	p = 0.003	[177]
-1123G>C	Norwegian	Association with RA	p = 0.026	[178]
rs247660/1858C>T	Polish	Associations between several clinical manifestations of RA	$p = 4 \times 10^{-4}$ OR = 1.89	[179]
rs247660/1858C>T	Spanish	Associated with the development of RA	$P = 1.6 \times 10^{-6}$ OR = 1.85	[180]
rs247660/1858C>T	South Asian	Association was significant	OR = 5.87	[181]
rs247660/1858C>T	Tunisian	No or minor effect on RA	p = 0.85	[182]
rs2476601/1858C>T	British	Association with RA	p = 0.001	[183]

OR: Odds ratio; RA: Rheumatoid arthritis.

The presence of the T-susceptibility allele may result in greater affinity of RUNX1 for its binding site, which depresses *SCL22A4* transcription. Therefore, decreased SLC22A4 production probably plays a role in the pathophysiology of RA [87].

Pharmacogenetics

Owing to the current 'fixed-dosage strategy' approach to medicine, there are significant differences in individual responses to drugs. Pharmacogenetics is a science that investigates inter-individual variations in the DNA sequence of specific genes affecting drug responses [88–90].

Polymorphisms in drug transporters may change the distribution and excretion of a drug, and the response given to it. Recent advances in molecular research have revealed that many of the genes encoding drug targets demonstrate genetic polymorphism. The sensitivity of the targets to the drug molecule is strongly influenced by these genetic variations.

Comparing different populations, it is clear that the distribution of the common variant alleles of genes that encode drug-metabolizing enzymes, drug transporters and drug targets, can be very different. The real promise of pharmacogenetics is to identify the right drug at the right dose for the right individual. The efficiency of drugs with a narrow therapeutic index is thought to benefit more from pharmacogenetic studies. Owing to the growing significance of pharmacogenomics, personalized medicine seems to be the promise of the near future [90].

Pharmacogenetics in RA

Rheumatoid arthritis is a chronic inflammatory joint disease that is heterogenous in nature. The heterogeneity is reflected by the variation in responsiveness to virtually any treatment method. Our understanding of the molecular mechanisms of RA is incomplete. A promising way to gain insight into the complexity of the disease has arisen from DNA microarray technology, which allows a survey to identify the genes and pathways that are associated with clinically defined condition [89].

Without treatment, RA leads to the development of joint destruction, disability and increased mortality. A better prognosis is available through early diagnosis and adequate treatment. Disease-modifying antirheumatic drugs (DMARDs) are critical in preventing the severe complications of RA. Nowadays, together with DMARDs, therapy with biological agents is also promising, with new perspectives of therapeutic efficiency.

Methotrexate

During the past 15–20 years, methotrexate (MTX) has become the most favored DMARD, owing to its efficacy and safety [91]. MTX is generally administered once a week to patients with RA, in doses between 7.5 and 25 mg per week. It can be administered orally, intramuscularly or subcutaneously. The exact mechanism of action of MTX is supposed to influence intracellular folate and adenosine pathways (Figure 2); it has a complex intracellular metabolism and acts via a number of key enzymes. MTX is a cornerstone for therapy of RA, although it is not universially effective, and up to 30% of the patients fail to respond totreatment. The early use of MTX (within 5 years after disease onset) is clearly associated with improved outcomes. The management of RA should include an early strong suppression of inflammation and continuously a tight control strategy. The pharmacodynamics and kinetics of MTX are still not completely understood [92].

Polymorphisms of the *RFC1*, *ABCB1*, *MTHFR*, *TYMS*, *DHFR* and *AICAR* genes are associated with the mechanisms of MTX for therapy. MTX enters the cells by the reduced folate carrier 1 (RFC1; also known as SLC19A1). MTX is pumped out of the cells through the transporter molecules of the ATP-binding cassette (ABC) transporter family [93]. Intracellular MTX is polyglutamated by

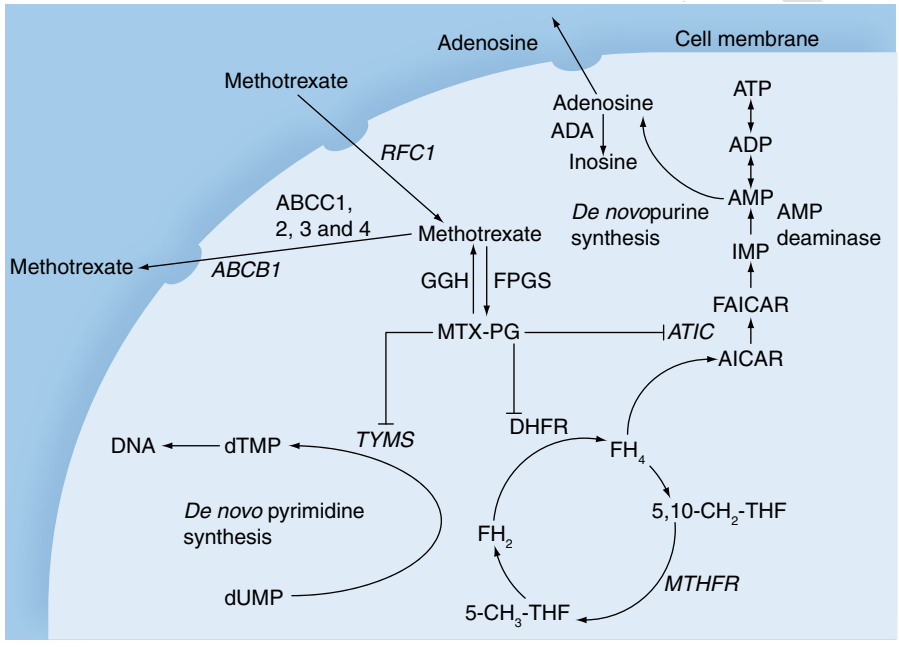

Figure 2. Cellular pathway of methotrexate. Methotrexate (MTX), a folate analogue, is a competitive inhibitor of the enzyme, DHFR. MTX effluxes from the cell by members of the ABCB1 and C1–4, ABCC 1–4. Inside the cell, MTX is polyglutamated by the enzyme, FPGS. Polyglutamation can be reversed by the enzyme GGH, which, by catalyzing the removal of γ-linked polyglutamates, facilitates MTX efflux from the cell. MTXPG has several important functions. It retains MTX within the cell, and inhibits DHFR, which reduces DHF to THF. Polyglutamated MTX also inhibits TYMS, which converts deoxyuridylate to deoxythymidylate in the *de novo* pyrimidine biosynthetic pathway. ABCB1: ATP-binding cassette B1; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; FPGS: Folylpolyglutamate synthase; GGH: γ-glutamyl hydrolase; MTX-PG: Polyglutamated methotrexate; THF: Tetrahydrofolate; TYMS: Thymidylate synthetase.
Reproduced with permission from [13].

the folylpolyglutamate synthase (FPGS) enzyme. This glutamation process can be reversed by γ-glutamyl hydrolase (GGH). The importance of polyglutamation is to inhibit drug efflux from the cells. The polyglutamation process of MTX also inhibits the function of the enzyme dihydrofolate reductase, which reduces dihydrofolate to tetrahydrofolate [94]. Tetrahydrofolate is converted to 5,10-methylenetetrahydrofolate and, subsequently, to 5-methyltetrahydrofolate by methylenetetrahydrofolate-reductase (MTHFR). 5-methyltetrahydrofolate is a biologically active cofactor, functioning as a carbon donor for important intracellular reactions, such as the conversion of homocysteine to methionine [95]. The polyglutamation of MTX also inhibits the functioning of the thymydilate synthase (TYMS) enzyme in the *de novo* pyrimidine pathway [96]. MTX also influences the purine synthetic pathways; the polyglutamation of MTX inhibits aminoimidazole carboxamide ribonucleotide (AICAR) transformylase, which leads to the intracellular accumulation of AICAR. AICAR and its metabolites inhibit two enzymes of the adenosine pathway (adenosine deaminase and adenosine monophosphate deaminase), which results in the intracellular accumulation of adenosine and nucleotides.

Summarizing these molecular mechanisms, it can be seen that the gene polymorphisms of MTX transporters, as well as those of the enzymes in the folate and adenosine pathways, are the focus of recent investigations.

As mentioned previously, RFC1 is the transporter of MTX towards the cells. Polymorphisms affecting the gene of RFC1 influence MTX transport (Table 3) [97,98]. The *RFC1* gene is located on chromosome 21 (21q22.3). The G80A polymorphism of the RFC1 protein (substitution of arginine for histidine at codon 27 in the first transmembrane domain), and a 61-bp-repeat polymorphism in the promoter region of RFC1, increase the transcriptional activity of the gene. Stimulated transcription also has an essential effect on MTX transport [99]. A significant study about the effect of the G80A SNP has revealed that patients homozygous for the RFC SNP 80A/A have a greater response to MTX than patients carrying the wild-type 80G/G SNP [100].

The *ABCB1* gene, located on chromosome 7 (7q21.1), produces the P-glycoprotein, which is an important membrane transporter, participating in the transport of numerous drugs [101]. The SNPs of this gene influence the cellular transport of MTX, leading to different therapeutic effects (Table 4) [102].

The *MTHFR* gene is located on chromosome 1 (1p36.3). *MTHFR* gene variants, associated with reduced enzyme function and hyperhomocysteinemia, may affect MTX sensitivity and contribute to toxicity. The two most common polymorphisms of this gene (C677T and A1298C) have also been studied for their effect on the efficacy of MTX therapy (Table 4). The C677T polymorphism leads to a thermolabile variant of MTHFR, with a subsequent decreased enzyme activity [103]. The A1298C polymorphism has a similar decreasing effect on enzyme activity [104]. As a consequence of decreased MTHFR activity, the level of homocysteine in the plasma elevates, which facilitates the toxic effect of MTX. The most severe complications seem to affect the GI tract [105].

The TYMS enzyme is important for the *de novo* synthesis of pyrimidines. It converts deoxyuridine monophosphate to deoxythymidine monophosphate, and is a direct target of the polyglutamated MTX. The gene of TMYS is located on chromosome 18 (18p11.32). A 28-base pair polymorphic tandem-repeat sequence has been developed in the 5′ untranslated region of the *TYMS* gene, with a variable number of repeat elements [106]. These repeat elements may enhance the expression of *TYMS* mRNA, as well as enzyme activity [107]. Patients homozygous for the triple-repeat allele have higher *TYMS* mRNA expression than those who are homozygous for the double-repeat allele (Table 3).

Table 3. Gene polymorphisms of the *RFC1* and *TYMS* genes influence the methotrexate transport.

Gene	Polymorphism	Population	Function	Ref.
TYMS	5′ UTR repeat	Indian	Does not show any association	[192]
	3′ UTR deletion	Indian	Does not show any association	[192]
	28-bp tandem repeats in the 5′ UTR	Japanese	Allelic frequencies may associate with the difference in the effects of methotrexate in rheumatoid arthritis patients	[193]
	6-bp deletion/insertion in the 3′ UTR	Japanese	Allelic frequencies may associate with the difference in the effects of methotrexate in rheumatoid arthritis patients	[193]
	3′ UTR 28	Japanese	Significantly different in Japanese patients from that in Caucasians	[189]
RFC1 (*SLC19A1*)	Three tandem repeats (49–61 bp)	American	Polymorphism increases promoter activity and may contribute to interpatient variations in human reduced folate carrier expression	[197]
	G80A	Spanish	Other disease: osteosarcoma cells become resistant to methotrexate	[195]
		Japanese	Other disease: polymorphisms may serve as predictors of toxicity during maintenance chemotherapy.	[196]
		Slovenian	The polymorphism increased the risk for overall MTX toxicity	[186]
		American	Other disease	[99]
	C696T	American	Other disease: phenotypically silent	[99]
	C1242A	American	Other disease: phenotypically silent	[99]

Table 4. Polymorphisms of the *ABCB1*, *DHFR* and *MTHFR* genes and their influence on methotrexate transport.

Gene	Polymorphism	Population	Function	Ref.
ABCB1	C3435T	Japanese	Genetic diagnosis of ABCB1 C3435T can be applied to determine MTX sensitivity for the treatment of RA patients	[184]
		Polish	Not an important genetic risk factor for RA susceptibility, but may have an influence on the activity of the disease and its response	[185]
		Slovenian	Increased the risk for overall MTX toxicity	[186]
DHFR	G473A	Dutch	Were not found to be associated with efficacy	[187]
	G35289A	Dutch	Were not found to be associated with efficacy	[187]
MTHFR	C677T	Japanese (female)	Does not appear to be a clinically useful marker for predicting fracture risk in Japanese female RA patients	[188]
		Japanese	Showed no association with MTX-related toxicity or efficacy	[189]
		American	Associated with increased toxicity	[190]
		Polish	May be associated with an increased rate of RA remission in patients treated with MTX receiving high doses of folic acid supplementation	[191]
	A1298C	Japanese (female)	Does not appear to be a clinically useful marker for predicting fracture risk in Japanese female RA patients	[188]
		American	Was not associated with increased toxicity	[190]
		Slovenian	Had a protective effect on overall MTX toxicity	[186]
		Polish	May be associated with an increased rate of RA remission in patients treated with MTX receiving high doses of folic acid supplementation	[191]
		Indian	'C'-allele incidence among RA patients was significantly higher	[192]
		Japanese	Showed no association with MTX-related toxicity or efficacy	[189]

ABCB1: ATP-binding cassette B1; DHFR: Dihydrofolate reductase; MTHFR: Methyl-tetrahydrofolate reductase; MTX: Methotrexate; RA: Rheumatoid arthritis.

Dihydrofolate reductase reduces dihydrofolate to tetrahydrofolate in the intracellular folate metabolism. The *DHFR* gene, located on chromosome 5 (5q11.2–q13.2), is directly inhibited by polyglutamated MTX. *In vitro* studies in lymphocytic leukemia suggest that low-level gene amplification of *DHFR*, or mutations in the gene of the enzyme, may provide a mechanism of resistance to MTX [108].

Polygenic analyses of the MTX pathway genes have also been performed. AICAR transformylase (ATIC) converts AICAR to 10-formyl AICAR, and is directly inhibited by MTX. Owing to this inhibition, AICAR and adenosine accumulate. Both have anti-inflammatory properties; adenosine may be an important mediator of the anti-inflammatory effect of MTX [109]. The *ATIC* gene is located on chromosome 2 (2q35). The C347G SNP has been reported in this gene. In a few studies, attempts have been made to evaluate the common effect of C347G SNP in ATIC, the double-repeat allele in *TYMS*, and the G80A polymorphism in *RFC1*, on the efficacy of MTX [110]. A pharmacogenetic index was calculated from the sum of the homozygous variant genotypes. Based on the value of the index, patients were divided into groups of MTX responders and nonresponders. A higher pharmacogenetic index correlated with an increased level of polyglutamated MTX and, also, an increased response to MTX [110].

Differences in folate metabolism based on racial differences have been investigated in several studies. Different folate metabolism may also mean different efficacy of MTX [111]. The lower frequency of the thermolabile 677T variant of MTHFR among African–Americans is also supposed to present a lower toxicity of MTX than among Caucasians [112]. Therefore, it can be concluded that ethnicity may have a strong influence on pharmacogenetic associations [113].

Azathioprine

Azathioprine is a drug used in different rheumatic diseases, such as systemic lupus erythematosus and RA. Owing to the high frequency of eventual side effects (e.g., severe allergic reactions, fever and dizziness), 20–30% of RA patients interrupt receiving azathioprine therapy [114]. Actually, azathioprine is a prodrug, which is converted into 6-mercaptopurine (6-MP) after oral intake. 6-MP is also a prodrug and has to be converted by the anabolic purine pathway into active thiopurine nucleotides before exerting cytotoxicity. Concerning its metabolism, 6-MP is converted to 6-thioguanine nucleotides by the hypoxanthine phosphoribosyltransferase (HPRT) enzyme. Another possible metabolic pathway is the inactivation of 6-MP by thiopurine

methyltransferase (TPMT). This inactivation results in the formation of 6-methylmercaptopurine or (by xanthine oxidase) thiouric acid. When TPMT is deficient, cytotoxic thioguanine nucleotides will accumulate, and the toxicity of azathioprine will increase [115–117].

The gene encoding TPMT is located on chromosome 6 (6p22.3); its allelic variant can determine enzyme activity in erythrocytes. Population studies verified that 90% of the population has high enzyme activity, almost 10% shows medium activity and only 0.2–0.3% has low activity [118]. For patients with low TPMT activity, a standard dose of azathioprine may lead to severe toxicity and, thus, they require lower doses. Among the symptoms of toxicity, leucopenia, disorders of liver function and gastrointestinal problems are worth mentioning. Three different allelic variants, TPMT*2, TPMT*3A and TPMT*3C, may lie in the background of low enzyme activity (Table 5) [119–121]. These allelic variants show differences in their frequency in different populations [122].

Sulfasalazine

Sulfasalazine (SSZ) is a DMARD often used in the therapy of RA. It is a combination of sulfapyridine and 5-aminosalicylic acid (5-ASA), into which SSZ is split in the bowels. 5-ASA remains in the colon, while most of the sulfapyridine will be absorbed and acetylated, hydroxylated and glucuronidated in the liver. Acetylation is performed by the N-acetyltransferase 2 (NAT2) enzyme. The gene encoding NAT2 is located on chromosome 8 (8p22) and can show polymorphism (Table 6). Based on NAT2 polymorphisms, individuals can be classified as slow and fast acetylators. Slow acetylators have been shown to be more prone to the toxicity of SSZ; patients complain of nausea, abdominal pain, rash and headache more often than fast acetylators [123,124]. Two outstanding studies evaluated the effects of NAT2 polymorphisms on the toxicity of SSZ. The first reported that, on a daily dose of SSZ 500–1500 mg, slow acetlyators lacking the NAT2*4 allele experienced adverse side effects more commonly than fast acetylators with one or two NAT2*4 alleles. The authors concluded that a *NAT2* genotype and SSZ toxicity showed a significant association with each other [125]. The other study genotyped the sample population for five allelic variants: NAT2*5A, NAT2*5B, NAT2*5C, NAT2*6 and NAT2*7. Investigations have shown a close relationship between the acetylator status and the frequency of side effects [126].

Biological agents in the therapy of RA

The importance of biological agents in the treatment of RA has dramatically increased. They not only reduce the symptoms, but also slow x-ray progression of the disease [127]. It is a great disadvantage that 25–30% of patients with RA fail to respond to biological agents, and the therapy is rather expensive [127,128]. Studies based on the investigation of the inflammatory process mediated by TNF and IL-1 have led to the development of TNF blockers. Some of them, for example, etanercept, infliximab, adalimumab and leflunomide, are used in the treatment of RA. Genome-wide association scan studies have proven that certain genetic polymorphisms are associated with different responses to anti-TNF treatment in RA. Multiple SNP markers show significant association with anti-TNF treatment (e.g., IFN type I gene, paraoxonase I gene and IL-10 promoter SNP [rs 1800896]).

Etanercept (ETN) is a protein that joins the human p75 TNF receptor at the FC domain of IgG1. The drug consists of 934 amino acids. In clinical practice, it is used either in monotherapy or as part of combined therapy, together with MTX [130]. After 24-month therapy, the radiographic progression of the disease decreases significantly [131]. Monotherapy yields a better therapeutic effect than combined treatment with MTX. The possible side effects are opportunistic infections, cardiac insufficiency and lymphoma. The use of MTX for therapy increases the probability of severe side effects [132–134].

Infliximab is a monoclonal antibody, which neutralizes the activity of TNF-α. This drug was the first TNF blocker used in the treatment of RA [135]. The most important benefits of the drug are a better quality of life, bone repair and structural articular damage. The drug is used in the treatment of RA, Bechterew's disease and Crohn's disease, either in monotherapy or in combination with MTX. The eventual side effects of infliximab are similar to those of etanercept (e.g., infusion reactions and opportunistic infections [tuberculosis]) and, in addition, there may be an increased risk of lymphoma development [136]. Cardiac insufficiency and neurological problems, such as demyelination, are also possible [137–139].

Adalimumab is a human IgG1 antibody, with a similar mechanism of action to that of infliximab. Adalimumab inhibits the interaction of cell-surface TNF receptors p55 and p75 with TNF-α. The drug also reduces the biological responses induced by TNF, and decreases the level of IL-6. It can be applied either in monotherapy or together with MTX [140–142]. Adalimumab may inhibit the progression of articular damage, even in cases with insufficient DMARD therapy. The side effects are similar to those of etanercept and infliximab (i.e., opportunistic infections, demyelinating processes, autoimmune disorders and cardiac insufficiency may develop) [143].

The TNF family consisting of TNF-α, lymphotoxin A (LTA) and lymphotoxin B (LTB) are basic cytokines in human immune

Table 5. Gene polymorphisms in azathioprine metabolism.

Polymorphism	Population prevalence (%)	Possible effect of polymorphism	Clinical effects
TPMT*2 (G238C)	0.2–0.5	Enzyme activity decreased due to enhanced degradation of thiopurine methyltransferase	Hematologic and gastrointestinal toxicity
TPMT*3A (G460A; A719G)	3.2–5.7	Enzyme activity decreased due to enhanced degradation of thiopurine methyltransferase	Hematologic toxicity
TPMT*3C (A719G)	0.2–0.8	Enzyme activity decreased due to enhanced degradation of thiopurine methyltransferase	Hematologic toxicity

Table 6. Gene polymorphisms in sulfasalazine metabolism.

Polymorphism	Possible effect of polymorphism	Clinical effects
NAT2*5A	Slow acetylator status (decreased activity of NAT2 enzyme)	Fever, agranulocytosis and rash
NAT2*5B	Slow acetylator status (decreased activity of NAT2 enzyme)	Fever, agranulocytosis and rash
NAT2*5C	Slow acetylator status (decreased activity of NAT2 enzyme)	Fever, agranulocytosis and rash
NAT2*6	Slow acetylator status (decreased activity of NAT2 enzyme)	Fever, agranulocytosis and rash
NAT2*7	Slow acetylator status (decreased activity of NAT2 enzyme)	Fever, agranulocytosis and rash

regulation. The gene encoding TNF-α is in the MHC III region on chromosome 6. At the TNF locus, *TNF*, *LTA* and *LTB* genes are arranged in tandem, just in the neighborhood of the HLA-B and MHC III-DR regions. The responses to TNF-antagonists have been studied through the investigation of DNA microsatellites, and analyzing the polymorphisms in the *TNF* and *TNFR* genes. The most common polymorphisms of the *TNF* gene are -308, -238 and +489. It is reported that polymorphisms in the promoter region elevate the transcriptional activity of the gene [144,145].

The polymorphisms of the TNF-α receptors are also of great importance: p55 (CD120a; TNFRSF1A) and p75 (CD120b; TNFRSF1B) are the two transmembrane receptors of TNF-α. The gene *TNFRSF1B* is located on chromosome 1 and consists of ten exons and nine introns [146]. The SNP 196T/G has been described in exon 6, which leads to an amino acid substitution (methionine for arginine) in the fourth extracellular domain of *TNFRSF1B*. The 196R allele may increase the production of IL-6, and express a strong inflammatory effect compared with the 196M allele [147].

The TNF-308 polymorphism may be predictive of the patient's response to infliximab therapy [148]. It has been confirmed that patients of genotype *TNFRSF1B* 196G/G more often suffer from severe RA, than patients of another genotype. Patients of the 196 T/T genotype are much better responders to RA therapy than those of the TG or GG genotypes [149].

The region of the loci of *TNF* and *HLA-B* and *HLA-DR* genes on chromosome 6, and the strong association between *HLA-DRB1* alleles and susceptibility to RA, suggest that MHC gene polymorphism probably influences the response to anti-TNF therapy [150]. Several studies examined this association and two of them have reported encouraging results. The first one, based on the examination of 78 RA patients, verified that single alleles of SNP TNF-308 did not reveal an association with the response to anti-TNF therapy [151]. In the second study of 457 patients, certain association between polymorphic *HLA-DRB1* alleles and the response to the applied therapy was proven. SNPs in *TNF*, *TNFRSF1A* and *TNFRSF1B* showed no correlation with response to treatment [152].

Personalized medicine in RA

Physicians have a continuously increasing number of therapeutic agents available for the treatment of RA. The real aim of the studies in pharmacogenetics is to be able to find the optimal tailor-made therapy for each RA sufferer. There are many difficulties in individualizing medication for RA patients. The first big problem emerges in association with the pathogenesis of the disease, since it is not entirely understood yet. The drug-response phenotype is very complex, mainly owing to the use of DMARDs (e.g., MTX). The available databases of RA patients contain a standardized treatment and, also, standardized outcome measures [153].

Until researchers get access to tests that provide an accurate and fast result regarding the patient's genetic condition, testing to classify patients based on the likelihood of treatment response will not be commonly used in the daily clinical practice. Before clinical application, clear and convincing tests are required [154].

In summary, it can be concluded that the perfect drug against RA has not yet been found. At the same time, the aim of identifying genetic and clinical factors to profile individuals for predicting the optimal treatment is realistic and worth further study.

Expert commentary

Current technologies of RA genetic diagnostics continue to develop rapidly. The *HLA-DRB1* gene variants (HLA-DR1, -DR4 and -DR10), and 1858T SNP of the *PTPN22* gene, are associated with RA, and can be used to help diagnose RA. The polymorphisms of other genes (e.g., *CTLA4*, *MIF*, *PADI4*, *SLC22A4* and *TNF*-α) may facilitate the development of RA but, generally, it can be concluded that the genotyping of these genes are not used for genetic diagnostics. Pharmacogenetics is also promising, with new perspectives of DMARD therapy, and the pharmacogenetic tests help for individual RA therapy. The real aim of studies in pharmacogenetics is to find the optimal tailor-made therapy for each RA patient.

Five-year view

During the last few years, microarray technologies opened new directions for a more detailed analysis of biological systems. Nowadays, it is possible to monitor thousands of genes in one single experiment. The molecular profiling procedures, together with standardized clinical examinations, allow an outstanding analysis of the patient's phenotype, and may lead to therapeutic protocols tailored to the patient's individual needs and demands. These individually designed methods may significantly increase the efficacy of the treatment, as well as yield a better prognosis for the patient.

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Key issues

- HLA-DRB1*01 (DR1), HLA-DRB1*04 (DR4), HLA-DRB1*10 (DR10) and *PTPN22* 1858T alleles are the most important common factors for the genetic diagnostics of rheumatoid arthritis (RA).
- Polymorphisms of several genes (*CTLA4*, *MIF*, *PADI4*, *SLC22A4* and *TNF*-α) probably play a role in the pathophysiology of RA but, currently, they are not used in the genetic diagnosis of RA.
- Pharmacogenomic experiments promise new perspectives on the therapeutic efficiency of disease-modifying antirheumatic drugs.
- At present, the aim of predicting optimal treatment is the identification of genetic and clinical factors to profile individuals.

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