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Gene polymorphisms of cytokines and heat shock protein in inflammatory and immune-related diseases

Ph.D. Thesis

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I. Balog A, Gyulai Z, Boros LG, Farkas G, Takacs T, Lonovics J, Mandi Y. Polymorphism of the TNF-alpha, HSP70-2, and CD14 genes increases susceptibility to severe acute pancreatitis. Pancreas. 2005;30(2):e46-50.

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Abbreviations

ARDS	acute respiratory distress syndrome
ARMS	amplification refractory mutation system
CD	Crohn's disease
CRP	C – reactive protein
CT	calcitonin
CT	computer thomograpy
DMARDs	disease-modifying antirheumatic drugs
DNA	deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAB	French-American-British classification
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte/machrophage colony-stimulating factor
HLA	human leukocyte antigen
HSPs	heat shock proteins
IFN-γ	interferon-γ
IL-1	interleukin
KC	katakalcin
LPS	lipopolysaccharides
MAbs	monoclonal antibodies
MDS	myelodysplastic syndrome
MHC	major histocompatibility complex
NK cells	Natural Killer cells
PBMCs	peripheral blood mononuclear cells
PCT	procalcitonin
PI	propidium iodide
RA	refractory anemia
RAr	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
SIRS	systemic inflammatory response syndrome
SNP	single nucleotide polymorphism
TGF-ß	transforming growth factor-beta
TLR4	toll-like receptor-4
TNF-α	tumor necrosis factor-alpha
US	ultrasound
WBCs	whole blood cultures
WHO	World Health Organization

1. Introduction

1.1. Cytokines

Cytokine is a term applied to any of a rapidly growing number of small, proteins or glycoproteins that serve as messengers between cells and are involved in such processes as cell growth and differentiation, tissue repair and remodelling, and regulation of the immune response. In acute and chronic inflammation, cytokines are instrumental in regulating the magnitude, nature, and duration of the inflammatory response [1]. Cytokines also stimulate or inhibit the development of hematopietic cells.

General properties of cytokines: they are polypeptides produced in response to microbes and other antigens that mediate and regulate immune and inflammatory reactions. Cytokine secretion is a brief, self-limited event. The actions of cytokines are often pleiotropic and redundant. Cytokines often influence the synthesis and actions of other cytokines. Most cytokines act close to where they produced, either on the same cell that secretes the cytokine (autocrine action) or on a nearby cell (paracrine action). When produced in large amounts, cytokines may enter the circulation and act at a distance from the site of production (endocrine action) [2]. Cytokines initiate their actions by binding to specific membrane receptors on target cells. External signals regulate the expression of cytokine receptors and thus the responsiveness of cells to cytokines. The cellular responses to most cytokines consist of changes in gene expression of in target cells, resulting in the expression of new functions and sometimes in the proliferation of the target cells.

In clinical medicine, cytokines are important as therapeutic agents and as targets for specific antagonists in numerous immune and inflammatory diseases [3].

1.1.1. Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α) is produced during immune and host defense responses as a primary mediator of immune regulation and the inflammatory response. The major cellular source of TNF-α is activated mononuclear phagocytes, although antigen-stimulated T cells, Natural Killer (NK) cells, and mast cells can also secrete this protein [4]. In macrophages, TNF-α synthesis can be induced by a wide range of stimuli for example bacterial products such as lipopolysaccharides (LPS), other cytokines: interleukin-1 (IL-1), IL-2, interferon-γ (IFN-γ), granulocyte/machrophage colony-stimulating factor (GM-CSF),

TNF-α itself, complement, X-ray radiation, tumor cells, ischemia and trauma. In other cell types, other stimuli are effective: engagement of the T-cell receptor in T lymphocytes, cross-linking of surface immunoglobulin in B lymphocytes, ultraviolet light in fibroblasts and phorbol esters and viral infections in many other cell types [5, 6]. The principal physiologic function of TNF-α is to stimulate the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes. TNF-α mediates these effects by several actions on vascular endothelial cells and leukocytes [7, 8]. The biology of TNF-α is also characterized by its pathologic activities in many immune-mediated diseases. The net effects of TNF-α are influenced by a complex array of cell- and tissue-specific factors [7]. The diverse role of TNF-α in mediating cellular responses are: the activation and induction of other cytokines such as IL-1, 6, 8, IFN-γ, transforming growth factor-beta (TGF-ß), in monocytes-macrophages [9, 10] and inhibition of differentiation and suppression of proliferation of these cells; increased adherence to extracellular matrix and increasing phagocytic capacity of polymorphonuclear leukocytes; modulation of angiogenesis [11, 12, 13] increasing permeability and enhanced expression of major histocompatibility complex I (MHC I) of vascular endothelial cells. TNF-α also plays an important role in the antitumor activity by inducing cell apoptosis [14, 15, 16]. TNF-α can induce the synthesis of IL-12 and IL-18, two cytokines that are potent inducers of IFN-γ. Therefore TNF-α, by itself and through up-regulation of IL-12 and IL-18, amplifies the $T_{Helper}1$ response, increasing CD4+ T-cell activation and IFN-γ production [17, 18]. The main *in vivo* effects are fever [19], anorexia [20], altered pituitary hormone secretion in central nervous system [21], and shock [22], acute respiratory distress syndrome (ARDS) [23], and capillary leakage syndrome in cardiovascular system [24]. The concentration of TNF-α also influence its biological actions: at low concentrations, TNF-α acts on leukocytes and endothelium to induce acute inflammation; at moderate concentrations, TNF-α mediates the systemic effects of inflammation; at high concentrations, TNF-α causes the pathologic abnormalities of septic shock [25].

1.1.2. Transforming growth factor-beta

Virtually every cell in the body, including epithelial, endothelial, hematopietic, neuronal, and connective-tissue cells produces TGF-ß and has receptor for it. TGF-ß regulates

the proliferation and differentiation of cells, embryonic development, wound healing, and angiogenesis [26]. It is actually a family of closely related molecules encoded by distinct genes, commonly designated TGF-ß1, TGF-ß2, TGF-ß3. Cells of the immune system synthesize mainly TGF-ß1 [27]. The principal action of TGF-ß in the immune system is to inhibit the proliferation and activation of lymphocytes and other leukocytes [28]. Some regulatory T cells produce TGF-ß, and the same cells may also produce IL-10, which, like TGF-ß, has immunosuppressive activities. TGF-ß inhibits the proliferation and differentiation of T cells and the activation of macrophages [29]. TGF-ß also acts on other cells, such as neutrophils and endothelial cells, largely to counteract the effects of proinflammatory cytokines [30]. Mice in which the TGF-ß1 gene has been knocked out develop uncontrolled inflammatory lesions such as cardiac, pulmonary, and gastric inflammation suggesting the immunosuppressive effects of TGF-ß [31, 32]. By these actions, TGF-ß functions to inhibit immune and inflammatory responses. TGF-ß has many diverse actions outside the immune system. TGF-ß is one of the most potent regulators of the production and deposition of extracellular matrix [33]. TGF-ß stimulates fibroblasts and other cells to produce extracellular-matrix proteins and cell-adhesion proteins, including collagen, fibronectin, and integrins and parallel TGF-ß decreases the production of enzymes that degrade the extracellular matrix, including collagenase, heparinase, and stromelysin [34], and increases the production of proteins that inhibit enzymes that degrade the extracellular matrix, including plasminogen-activator inhibitor type I and tissue inhibitor of metalloprotease [35]. TGF-ß directly stimulates angiogenesis *in vivo* [36, 37]. In normal cells, TGF-ß acts as a tumor suppressor by inhibiting cellular proliferation or by promoting cellular differentiation or apoptosis [38, 39]. However, TGF-ß has been suggested to play a dual role, acting as a tumor suppressor in early stages, through its antiploriferative activity, and as a tumor promoter in later stages, by enhancing tumor cell motility and invasiveness [40]. Increases or decreases in the production of TGF-ß have been linked to numerous disease states, including atherosclerosis [41] and fibrotic disease of the kidney [42], lung [43] and liver [44]. Mutations in the genes for TGF-ß, its receptors, or intracellular signaling molecules associated with TGF-ß are also important in the pathogenesis of disease, particularly cancer [45] and hereditary hemorrhagic telangiectasia [46].

1.1.3. Genetic variations in cytokines

Recent work has shown a high degree of polymorphism in the cytokine genes involved in inflammation and immunity [47]. Cytokine gene polymorphisms (deoxyribonucleic acid (DNA) sequence variations) are frequently in regions of the gene that regulate transcription or post-transcriptional events, and so can be functionally significant. The inflammatory response, therefore, is genetically programmed both quantitatively and qualitatively, with some people having a very vigorous response and others a more measured response to the same stimulus [48]. The genetically determined capacity of cytokine production and release may contribute to a wide range of clinical manifestations of inflammatory disease: a patient with peritonitis, for example, may present without symptoms of sepsis and recover within days or may suffer from fulminant septic shock, resulting death in hours [49]. Deduced from pathophysiological related pathways and close metabolic interactions, different clusters of candidate genes are of interest. As a consequence, all genes encoding products for inflammation (i.e., pro-inflammatory and anti-inflammatory cytokines) and for the host defense mechanism (heat-shock proteins) represent potential candidates for inflammatory and immune-related diseases [50].

1.2. Heat shock proteins

Heat shock proteins (HSPs) are highly evolutionarily conserved proteins found in all prokaryotes and eukaryotes that are expressed in physiologic and pathologic conditions. In normal physiological conditions, HSPs are expressed at low levels and localize intracellular [51]. However, a wide variety of stressful stimuli, including environmental (ultraviolet radiation, heat shock, heavy metals and amino acids), pathological (viral, bacterial or parasitic infections, or fever, inflammation, ischemic injury, malignancy or autoimmunity) or physiological stimuli (cytokines, growth factors, cell differentiation, hormonal stimulation or tissue development), induce a substantial increase in intracellular HSP synthesis, known as the stress response [52, 53, 54]. Its ability to protect cells from the deleterious effects of inflammation is based on refolding and stabilizing denatured protein aggregates (molecular chaperoning) [55, 56], on prevention of DNA strand breaks induced by reactive oxygen species [57], and on transcriptional inhibition of potentially deleterious proinflammatory mediators such as TNF-α and IL-1 [58]. Stress proteins particularly the HSP70 family have a

key role in many diseases [59]. In humans, three members of the HSP70 gene referred to as HSP70-1, HSP70-2 and HSP70-Hom has been mapped [60]. Among them, HSP70-1 and HSP70-2 encode the same protein, which is referred as to inducible HSP70. Recent studies indicate the protective effect of HSP70 in hypoxia-related diseases such as ischaemic heart disease [61], stroke [62], ischaemic acute renal failure [63]. They protect transplanted tissues and organs [64, 65], act against the multiple damage of chronic diseases such as diabetes [66], or neurodegenerative diseases such as Alzheimer's disease [67] and Parkinson's disease [68]. The activation, and role in antigen presentation of stress proteins can be used as an anticancer-therapy [69] and they can also increase longevity [70]. Overexpressed and conserved stressproteins become primary targets of autoimmunity, through infection-induced molecular mimicry. Increased levels of antibodies against the inducible form of the HSPs were found in patients with autoimmune diseases [71, 72]. Recently, exogenous HSP70 was found to bind with high specificity to the surface of human monocytes and acts as a cytokine by stimulating cytokine production through a CD14-dependant pathway [53].

1.3. CD14 receptor

The CD14 receptor is a glycoprotein localized on the cell surface of all myeloid cells, especially on monocytes and macrophages [73]. The LPS-LPS binding protein complex binds to this glycosylphophatidylinositol-anchored membrane protein, membranous CD14 (mCD14), on monocytes and macrophages and activates these cells [74]. CD14 initiates the cytokine cascade by acting as a pattern recognition receptor and exhibiting binding specificity for molecules that allow CD14 to initiate innate immune responses. Soluble CD14 (sCD14), which lacks a glycosylphophatidylinositol anchor, can also be found in plasma. Endothelial cells and smooth muscle cells, lacking their own mCD14, are directly activated by LPS-sCD14 complex [75, 76]. The action of CD14 is exerted through the activation of Toll-like receptor-4 (TLR4), a transmembrane co-receptor to CD14, in response to LPS [77]. Membrane-associated TLRs recognize pathogen-associated molecular patterns [78] and it can be stimulated by highly-purified LPS. However, the major function of CD14 is the recognition of LPS and other microbial products, CD14 has been discovered to be a multifunctional molecule. Furthermore, human CD14 mediates the recognition and phagocytosis of "self" components, such as apoptotic cells [79, 80]. mCD14 in macrophages

and possibly sCD14 contribute to the clearance of apoptotic cells. mCD14 in macrophages may interact with apoptotic cells and bind to apoptotic-cell-associated molecule patterns as well as to pathogen-associated molecular patterns [81]. Interestingly, expression of CD14 on the surface of monocytes seems to promote monocyte survival and antagonize apoptosis, while downregulation of mCD14 parallels apoptosis [82, 83]. sCD14 levels in plasma rise rapidly by 45-75% during endotoxaemia, which is compatible with the criteria for an acute-phase protein [84, 85].

1.4. Single Nucleotide Polymorphisms

1.4.1. Definition and general features of SNP-s

The Single Nucleotide Polymorphism (SNP) is a form of genetic diversity in the population; it can be found anywhere in the genome and each person has a lot of SNPs. A nucleotide change in a given sequence is named as “mutation” if its frequency is less than 1% in the general population and as “polymorphism” if its frequency is higher than 1% [50]. In this context, the term mutation is applied to monogenic diseases in which a genetic variation causes a relatively clear-cut phenotypic effect, whereas the term polymorphisms is almost always used when referring to complex genetic traits such as congenital heart disease, essential hypertension, or insulin dependent diabetes mellitus. In this respect, genetic polymorphisms are considered to be consistently found in ”normal” population samples, even if their allele frequencies differ largely from one sample to another and among different ethnic groups. When referring to case-control studies, we would expect that a given candidate polymorphism is more frequently found in the case than in the control sample if it confers a risk for a given disease, as has been shown for the angiotensin-converting enzyme insertion/deletion polymorphism [86]. On the other hand, the polymorphism might be less frequently found in the case than in the control sample if it conferred a protective function against, for example, myocardial infarct, as has been suggested for the minor allele Pro715 of the P-selectin gene [87]. In contrast to the microsatellites- another form of genetic variability- SNPs are more stable since they differ from each other in only one nucleotide. The international SNP Mapping Workshop is a consortium of the New York Cold Spring Harbor Laboratoy, National Center for Biotechnology Information, Washington, DC, the British Sanger Center, the Washington University, St. Louis, MO, and the Whitehead Center for

Genome Research, Cambridge, MA. The consortium has identified 1.433.393 SNPs so far (September of 2004). On average every 2 kb sequence is containing one SNP.
If a gene is defined as the coding region and 10 kb upstream then 59% of the human genes are containing 5 or more, and 39% are containing 10 or more SNPs. However, there are less SNPs on the sex chromosomes.

One possible use of SNPs is the investigation of the evolution. Comparing different subpopulations under different conditions we can understand how could a definite SNP subsist (what are the advantages or disadvantages carrying a certain SNP form). Another application of SNPs is DNA fingerprints for criminal and for affiliation orders. Third, SNPs are excellent markers for mapping polygenic-features. Fourth is application for genotype-specific treatment of diseases namely pharmacogenomics [88].

1.4.2. SNP of TNF-α gene

The human TNF locus lies in a region recently reclassified as MHC class IV (previously class III) [89] on the short arm of chromosome 6 at the p21.3 locus [90]. There are many SNPs within the TNF-α gene promoter. The most investigated is a polymorphism at position –308 in the promoter region. This polymorphism results in two allelic forms, 1 in which guanine defines the common allele TNF1 and 1 in which an adenosine defines the uncommon allele TNF2 [91]. *In vitro* studies demonstrated that the TNF2 allele is associated with higher TNF-α production [92, 93]. Subjects carrying a TNF2 allele had also significantly higher TNF-α concentrations *ex vivo* than TNF1 homozygotes [94]. Higher frequency of the TNF2 allele in patients with septic shock [95] and a higher mortality rate from septic shock in carriers of a TNF2 allele were described [96]. The TNF2 allele has been also associated with variety of inflammatory disorders, including systemic lupus erythematosus, dermatitis herpetiformis, and celiac disease [97].

1.4.3. SNP of HSP70-2 gene

Besides environmental factors, genetic polymorphisms were also suggested to influence the production of the members of the HSP70 family. HSP70-2 encodes the major heat-inducible HSP70. The gene of HSP70-2 such as TNF-α gene is an MHC-linked gene lies in the central class III region on the short arm of the chromosome 6. HSP70-2 gene has been

shown to be polymorphic [60]. A polymorphic A to G transition *Pst*I site at position 1267 was identified in the coding region of HSP70-2 and individuals homozygous for the HSP70-2 G allele display a decrease in inducible HSP70-2 mRNA expression [98]. Recent studies indicate that HSP70-2 A(1267)G polymorphism is associated with increased risks and poor outcomes of several diseases [99, 100, 101, 102]. The susceptibility to multiple organ damage may also depend on the ability of the organism to express sufficient amount of HSPs.

1.4.4. SNP of CD14 receptor gene

The CD14 gene is located within a cytokine gene cluster in the chromosomal region 5q22-32. A SNP in the promoter of the CD14 gene (C/T at position –159) has been described; this is often referred to as –260 due to numbering from the translation start. The patients with genotypes carrying the T allele at the –159 site of the promoter region of the CD14 gene have higher sCD14 levels than subjects with the non-T genotype [103] and the density of monocyte CD14 receptors was also significantly higher in the TT homozygotes [104]. A large clinical study found this polymorphism associated with the risk for Crohn disease [105] and homozigousity of the T allele was also associated with a history of myocardial infarct in the subgroup of older low-risk patients [106]. The frequency of the TT genotype tended to be increased in subjects with duodenal ulcer and gastric ulcer compared with the *H.pylori*$^{+}$ and *H.pylori*$^{-}$ subjects without peptic ulcer [84]. Thus, the CD14 promoter genotype may affect inflammatory processes.

1.4.5. SNP of TGF-β1 gene

It has been demonstrated that the production of TGF-ß varies from individual to individual and partly depends on the polymorphisms of these genes [107]. The human gene encoding TGF-ß1 is located on chromosome 19q13 [108]. All positions of the TGF-ß1 gene are defined relative to the first major transcription start site (position +1). The first +840 bases are a nontranslated region and codon one begins at position +841 [109]. Several SNPs have been described in the TGF-ß1 gene, including a T-to-C transition at nucleotide 29 at position +869, in the region encoding the signal sequence, which results in a leucine-proline substitution at the 10th amino acid [110]. It has been shown that TT homozygous genotypes are high TGF-β1 producers [111, 112, 113]. The correlation between the TGF-ß1 gene

polymorphism and the disease status has been studied in a diverse range of diseases such as heart diseases [114, 115], acute human liver graft rejection [116], idiopathic pulmonary fibrosis [117], hypertension [118], myocardial infarction [119], atherosclerosis [120, 121, 122] colon, ovarian, breast cancers; diabetic nephropathy, asthma, chronic obstructive pulmonary disease [123], multiple sclerosis and osteoporosis [110].

1.5. Cytokines, HSP70-2, CD14 receptor in diseases

The pleiotropic effects of cytokines and their essential role together with the heat shock proteins can't be proved better than realizing their importance in diseases with seemingly completely different etiology.

1.5.1. Acute pancreatitis

Acute pancreatitis is a relatively common disease with an annual incidence of 10-20 cases per 100,000 population in the Western world. The majority of cases are alcohol related or due to gallstones [124]. Although many etiological factors are known to be involved in triggering acute pancreatitis, however, once the inflammatory process has been initiated, the ultimate outcome is relatively independent of the causative agent. Furthermore, acute pancreatitis has a wide spectrum of clinical manifestations, which ranges from a mild edematous, self-limited disease with a fair prognosis to severe necrotizing inflammation with a fatal outcome [125]. The overall mortality rate is about 10%, but in its most severe form, which is characterized by pancreatic necrosis, 20%-30% of patients die. The cause of death in most patients does not seem to be related specifically to the pancreatic inflammation or even to the infection of the necrotic pancreas or peripancreatic tissue that may occur. Rather death is often the result of multiorgan system failure [126]. In fact, multiple organ failure and septic complications in acute pancreatitis do not differ from the systemic complications of other diseases such as sepsis itself, trauma, or burn, which are included in a special group of diseases, namely the systemic inflammatory response syndrome (SIRS). The symptoms in different SIRS diseases might be very similar in consequence of tremendous activation of the cytokine cascade and inflammatory reactions.

The systemic manifestations of acute pancreatitis are now believed to be owing to the local and systemic actions of specific inflammatory cytokines [127]. TNF-α, the early cytokine to be released, is a principal mediator of immune responses to endotoxin and other

stimuli. It can be produced in large amounts in several organs during acute severe pancreatitis and is also believed to mediate pathophysiological changes [128, 129]. TNF-α can mirror the clinical signs of septic shock: hypermetabolism, fever, coagulopathies, increases vascular permeability, and vasodilatation [130]. Systemic release of TNF-α is associated with septic shock and fatal outcome. TNF-α levels are increased in patients with acute severe pancreatitis and septic shock and appear to correlate with clinical outcome [131]. The systemic manifestations are responsible for the majority of pancreatitis-associated morbidity and mortality and are due to the actions of specific proinflammatory cytokines such as TNF-α, IL-1, IL-8. It has been suggested that the clinical course of an acute inflammatory illness such as acute pancreatitis may have a genetic basis because certain genetic polymorphisms may operate functional differences and hence the outcome of the inflammatory process.
The preinduction of pancreatic HSPs expression has been shown to have a protective effect in different experimental acute pancreatitis model [132].

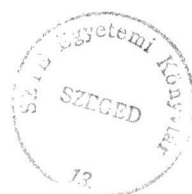
1.5.2. Myelodysplastic syndrome

The myelodysplastic syndrome (MDS) comprises a distinct, albeit heterogeneous group of hematopoietic disorders characterized by the patients' generally elderly ages, hematopoietic insufficiency associated with cytopenias leading to potentially serious morbidity (transfusion-dependent anemia, bleeding manifestations) and mortality (death from infection in the setting of neutropenia), plus the additional risk of leukemic transformation. The French-American-British (FAB) classification initially categorized patients morphologically for the diagnostic evaluation of MDS [133]. MDS patients have been classified by FAB as having 1 of 5 subtypes of disease: Refractory anemia (RA): $< 5\%$ marrow blasts; RA with ringed sideroblasts (RARS): $< 5\%$ blasts plus $\geq 15\%$ ringed sideroblasts; RA with excess of blasts (RAEB): 5-20% marrow blasts; RAEB in transformation (RAEB-t): 21-30% marrow blasts; and chronic myelomonocytic leukemia (CMML): $\leq 20\%$ marrow blasts plus monocytosis $> 1000/mm^3$ [134]. Although a substantial proportion of MDS cases evolve to acute myeloid leukemia (AML), the natural history of these syndromes ranges from more indolent forms of disease spanning years to those with a rapid evolution to AML. Thus, MDS is best considered a preleukemic disorder in which the neoplastic clone that has been established may or may not fully progress to acute leukemia

[135]. Cytogenetic and molecular studies support the concept that the stepwise accumulation of genomic damage in the hematopoiesis is central in the natural disease course of MDS, but it is generally accepted that the hematopoietic microenvironment is also active in the hematopoietic failure. Abnormal cytokine production, autoreactive T-lymphocytes and an altered interaction between the progenitor cells and the extracellular matrix can all promote apoptosis. The high rate of apoptosis in bone marrow of MDS mainly RA and RARS patients was described, and this [136] excessive apoptosis in patients with "early stage" myelodysplasia (RA, RARS) is a major characteristic of MDS. Excessive production of growth-inhibitory cytokines such as TNF-α was demonstrated in bone marrow plasma of patients with MDS [137, 138]. Transforming growth factor –ß (TGF-beta) is another cytokine which is generally considered to be a key negative regulator of hematopoietic stem and progenitor cells. It was shown that the inhibitory effect of TGF-ß on human hematopietic progenitor cells is clearly mediated through the Smad5 gene [139, 140].The bone marrow apoptotic rate was found 7-9.5 times higher among MDS patients than in controls [136, 141]. Moreover, one of the main new directions in the treatment of MDS is the use of compounds which exert inhibitory activity on proapoptotic cytokines [142, 143, 144]. In spite of the obvious importance of cytokines in MDS, there are only few genetic data on polymorphisms of TNF-α but there are no genetic data of TGF-ß, whether they influence occurrence of MDS or response to immunosuppressive therapy in this disease [145].

1.5.3. Rheumatoid arthritis

Rheumatoid arthritis (RAr) is a common human autoimmune disease is characterized by chronic inflammation of the synovial joints and infiltration by blood-derived cells, chiefly memory T cells, macrophages, and plasma cells, all of which show signs of activation [146]. This leads in most cases to progressive destruction of cartilage and bone, which occurs after invasion of these tissues by the cellular synovial tissue, and is believed to be mediated by cytokine induction of destructive enzymes, such as matrix metalloproteinases. Much clinical and experimental evidence exists for the pivotal role of TNF-α in RAr pathogenesis [147]. TNF-α is found at elevated levels in the pannus, the region of tissue damage in the RAr joint [148]. TNF-α, synergistically with IL-1, induces a cytokine cascade which results in the production of matrix metalloproteinases [149]. Furthermore, overexpression of human TNF-α



alone in transgenic mice can induce a polyarthritis similar to RAr [150]. A role for HSPs in RAr has been suggested because they are a major target of the immune response and are common autoantigens in autoimmune diseases [151]. However, the precise etiology and pathogenesis of RAr is still incompletely understood, many genes interact to produce the final clinical phenotype of RAr and other non-genetic factors may also influence the outcome of the disease. The association of RAr with genes of the Human Leukocyte Antigen (HLA) class II region is well documented, and several DRB1 alleles (in particular HLA-DRB1*04 and *01 alleles) have been associated with RAr susceptibility or severity [152, 153]. The important physiological role TNF-α, and the possible influence of HSPs in RAr and the location of their genes within the MHC has led to speculation about the role the TNF-α gene and HSP70-2 gene itself may play in RAr.

1.5.4. Anti-TNF therapy

The central role of TNF-α in the pathogenesis of RAr and Crohn's disease (CD) has been well documented [154, 155], and TNF-α is therefore an excellent therapeutic target. Therapeutic strategies for reducing the effects of proinflammatory cytokines can be considered to be either specific or nonspecific. A specific anticytokine therapy targets only one cytokine or closely related members of a single cytokine family. The most specific anticytokine is a neutralizing monoclonal antibody [156]. One of the most effective biological agents of them, infliximab, a chimeric anti-TNF antibody, has been shown to be highly effective for the treatment of both patients with CD [157] and patients with RAr [158, 159]. Despite the generally dramatic efficacy of infliximab, some of the patients did not display a clinical response, and the precise mechanism of action of infliximab is only partly understood [159, 160]. Adverse events with TNF inhibitors, including infections and cases of sepsis were also described [161, 162, 163]. We hypothesized that a genetic predisposition might be involved in the level of responsiveness, and some biological changes causing by infliximab were also investigated.

Aims

The aims of this study were to investigate the genetic polymorphisms of TNF-α and/or TGF-ß1 in different disorders. We focused on such gastrointestinal, autoimmune, hematological diseases, where the overproduction of these cytokines might be a predominant factor in the pathomechamisms, or where anti-TNF therapy has been introduced. In a close connection the SNPs of CD14 and HSP70-2 genes were also analyzed. The present study was designed to address the following aims:

Aim 1. To determine the correlation of TNF-α, HSP70-2, and CD14 gene polymorphisms and the severity of acute pancreatitis

Aim 2. To determine the prevalence of TNF-α and HSP70-2 gene polymorphisms in a family with rheumatoid arthritis

Aim 3. To determine the prognostic value of TNF-α gene polymorphism among patients treated with anti-TNF-α therapy, and the effect of anti-TNF-α therapy on TNF-α production and apoptosis

Aim 4. To determine the association between TNF-α and TGF-ß1 gene polymorphism and the susceptibility to myelodysplastic syndrome (MDS) and the progression of the disease among patients with MDS belonging to the refractory anemia subtype

2. *Patients and Methods*

2.1. *Patients*

2.1.1. *Patient group with acute pancreatitis*

Seventy-seven patients with acute pancreatitis enrolled in a prospective study. The criteria for diagnosis of acute pancreatitis were the clinical history consistent with the disease, radiological evidence and a serum amylase level greater than 660 U/L. Patients entered this prospective study at the Department of Surgery and the I.st Department of Internal Medicine of Albert Szent-Györgyi Medical Center of Szeged University during the time from 03/2003 - 02/2004. All patients were classified as having mild or severe pancreatitis according to the original criteria of Ranson [164]: mild pancreatitis, patients with fewer than three positive prognostic signs (n=29); and severe pancreatitis, patients with three or more positive prognostic signs (n=48). Patients with severe acute necrotizing pancreatitis were divided into aseptic (n=28) or infected (n=20) groups based on bacterial cultures of the necrotic pancreatic tissue sampled during surgery or US- or CT-guided biopsies (Paper I / Table 1).

2.1.2. *Patient group in a family with rheumatoid arthritis*

Twenty-nine members of a family including 5 sisters with RAr enrolled in this study (Paper III / Figure 1). All RAr patients were diagnosed by using the revised 1987 American College of Rheumatology (ACR) criteria [165]; they took part in regular control examination. This family entered this study at the Department of Rheumatology, County Hospital in Kecskemét.

2.1.3. *Patient group treated with anti-TNF-α therapy*

Nine patients with DMARD-refractory RAr were diagnosed by the revised 1987 ACR criteria [165]. The infusion regimen of anti-TNF-α therapy was as follows: 3 mg/kg infliximab (Schering Plough) administered at 0, 2 and 6 weeks and then in every 8 weeks for 1 year.

The 14 patients with chronic active CD unresponsive to standard therapy were diagnosed in accordance with the clinical guidelines and on the basis of conventional radiological, endoscopic and histopathologic examinations [166]. Fistulas were a common complication. The infusion regimen of anti-TNF-α was as follows: 5 mg/kg infliximab administered at 0, 2 and 6 weeks.

Patients with RAr entered this prospective study at the Department of Rheumatology, County Hospital, in Kecskemét, and patients with CD entered this study at the I.st Department of Internal Medicine of Albert Szent-Györgyi Medical Center of Szeged University.

All patients took part in regular control examinations.

Following the infliximab therapy, the RAr patients and CD patients were divided into responding and nonresponding groups. (Paper IV / Table 1)

2.1.4. Patient group with MDS belonging to the refractory anemia subtype

Fifty cases with refractory anemia (RA) documented by marrow biopsy were enrolled (12 men and 38 women, age 72.48 +-1.02, 55 to 87). The diagnosis of MDS was based on the FAB criteria [167]. In 4 of these cases, hypoplastic MDS was documented; in all of the other patients, the bone marrow was normocellular or hypercellular, satisfying the morphologic features of RA according to the FAB and WHO criteria: <5% blasts, <15% ringed sideroblasts. Neither a fibrotic form nor secondary MDS was diagnosed. Cytogenetic analysis revealed a normal karyotype in 38 cases, no mitosis in 5 cases, 5q- in 1 case and 8 chromosome abnormalities in 6 cases. The median follow-up was 34.5 months (14-62 months). None of the patients had received specific therapeutic agents prior to the study. They were supported only by red blood cell transfusion. Patients were devided into two groups: mild group containing patients with only anemia (hemoglobin<100 g/l) (n=30), and severe group containing patients with bi- or pancytopenia (neutrophil count <1 G/l, platelet count <100 G/l) (n=20). Leukemic transformation occurred in 3 patients; another 4 patients died due to the MDS, without leukemic transformation. Patients entered this prospective study at the 2nd Department of Medicine and Cardiology Center of Albert Szent-Györgyi Medical Center of Szeged University and the I.st Department of Internal Medicine of Faculty of Medicine, University of Pécs during the time from 05/2001 -01/2004.

2.1.5. Control group

The control cohort consisted of a random, unrelated population sample involving n=[71-100] healthy blood donors. Involving controls into the study was continuous, that is why the number of controls is different. All patients and controls were of Hungarian ethnic origin and resided in Hungary.

Ethical committee

These studies were performed in accordance with the ethical standards laid down in the most recent version of the 1964 Declaration of Helsinki. The ethical committee of the participating university had approved the study. Informed written consent was obtained in advance from each of the patients. With the permission of parents from the underage relatives, blood was taken and genetic investigations were performed in the family with RAr.

2.2. *DNA extraction*

For the examination TNF-α, HSP70-2, TGF-ß1 and CD14 polymorphisms, genomic DNA purified from peripheral blood was used. Leukocyte DNA was isolated using the High Pure PCR Template Preparation Kit according to the manufacturers' instructions (Roche Diagnostic GmbH, Mannheim, Germany) and the genomic DNA was stored at –20°C until further use.

2.3. *Determination of the TNF-α -308 G→A polymorphism*

The G (TNF1) to A (TNF2) transition at position –308 in the promoter region is associated with an elevated expression of TNF-α. This SNP of TNF-α at position –308 in the promoter region was analyzed by PCR-RFLP (restriction fragment length polymorphism) [5]. The amplified product was digested with the endonuclease *NcoI* and analyzed on a 12% polyacrylamide gel under UV illumination. The TNF G allele gives two fragments, of 87 bp and 20 bp, while the TNF A allele gives a single, 107 bp fragment (Figure 1).

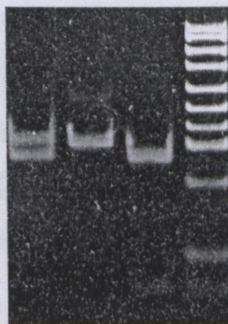


Figure 1. Representative results of typing of patients for SNP of TNF-α gene. Lane 1, TNF 1/2; lane 2, TNF 2/2; lane 3, TNF 1/1; lane 4, molecular weight markers (pUC19DNA/MspI (HpaII) Fermentas)

2.4. *Determination of the TGF-ß1 T^{29}→C polymorphism*

The defined single-nucleotide polymorphism T^{29}-C in exon 1 of the human TGF-ß1 gene was determined with an amplification refractory mutation system (ARMS) with a

generic primer (sense), (5'-TCCGTGGGATACTGAGACACC-3'); and with two allele-specific antisense primers, differing from each other in only one base at the 3'-end- primer C: 5'-GCAGCGGTAGCAGCAGCG-3' and primer T: 5'-AGCAGCGGTAGCAGCAGCA-3' [6]. The expected size of the specific amplification product was 241 bp. Samples from 2 known homozygotic individuals and 1 heterozygotic individual, confirmed by sequencing, were included in each reaction. Sequencing was performed with an automated sequencer (ABI Prism; Applied Biosystems, CA, USA) (Figure 2).

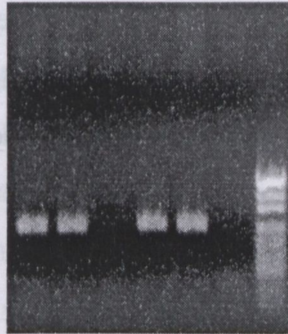


Figure 2. Representative results of typing of patients for SNP of TGF-ß1 gene. Lane 1-2, TGF T/C; lane 3-4, TGF C/C; lane 5-6, TGF T/T; lane 7, molecular weight markers (pUC19DNA/MspI (HpaII) Fermentas)

2.5. *Determination of the HSP70-2 1267 A→G polymorphism*

HSP70-2 RFLP was assessed by means of a PCR-RFLP procedure using the following primers (sense: 5'-TCCGAAGGACTGAGCTCTTG-3' and antisense: 5'-CAGCAAAGTCCTTGAGTCCC-3') spanning the polymorphic *Pst*I site (nucleotide 1267) [7]. The presence of a *Pst*I site (G allele) was indicated by cleavage of the 2075 bp amplified PCR product to yield fragments of 1159 bp and 916 bp (Figure 3).

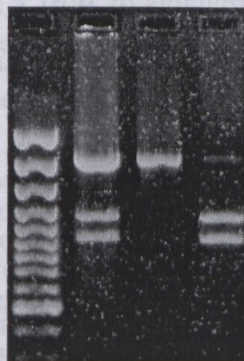


Figure 3. Representative results of typing of patients for SNP of HSP70-2 gene. Lane 1, molecular weight markers (Gene Ruler™ 100bp DNA Ladder Plus Fermentas); lane 2, HSP70-2 A/G; lane 3, HSP70-2 A/A; lane 4, HSP70-2 G/G

The analysis of the TNF-α, HSP70-2 and TGF-ß1 polymorphisms was based on polymerase chain reaction (PCR) techniques performed in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City CA, USA).

2.6. Determination of the CD14 -159 C→T gene-polymorphism

Genotyping for CD14 -159 C→T polymorphism was based on a real-time polymerase chain reaction (RT PCR) assay using specific fluorescence-labeled hybridization probes, and by melting point analysis [8]. Based on the derivative melting curves [-(dF/dT) vs T], a sample was classified as genotype TT, CT or CC. The thermocycler was a LightCycler instrument (Roche Diagnostics) (Figure 4).

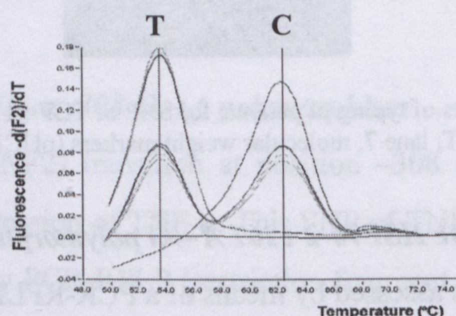


Figure 4. Representative results of typing of patients for SNP of CD14 gene.

2.7. Detection of TNF-α in supernatant of whole blood cultures (WBCs)

TNF-α concentrations in the supernatants of WBCs samples of the 9 patients with RA were determined by bioassay, applying the WEHI 164 mouse fibroblast cell line [9]. Human recombinant TNF-α (Amersham) was used as a standard in the same assay. The specificity of the effect of TNF-α in the supernatants was confirmed via the neutralizing effect of monoclonal anti-TNF-α antibody, Clone TA-31 (Sigma product No. T1549). The amounts of TNF-α are expressed in U/ml. In control experiments TNF-α concentrations in the supernatants of WBCs samples were also determined by using TNF-α ELISA kit (BIOSOURCE), according to the instructions of the manufacturer.

2.8. *Isolation of human mononuclear cells*

Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient purification (Ficoll-Paque Sigma) from heparinized venous blood samples from healthy volunteers and from RA patients treated with infliximab and the 95% cell viability was then confirmed by trypan blue staining. Subsequently, 5×10^6/ml PBMCs suspended in the medium were used in all the experiments. Isolated cells were incubated in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, antibiotics and glutamine.

2.9. *Intracellular staining of TNF-α*

PBMCs were incubated in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, antibiotics and glutamine for 24/48 h in the presence or absence of infliximab (100 μg/ml). They were then stimulated with heat-killed (90 °C 10 min adjusted to 10^8/ml) [10] *Staphylococcus aureus* (SA) as TNF inducer in the presence of brefeldin-A (10 μg/ml) for 4 h. After centrifugation, the cells were permeabilized with Cytofix-Cytoperm solution (Becton-Dickinson) for 20 min, thereafter, fluorescein isothiocyanate (FITC)-labeled human TNF-α specific monoclonal antibodies (MAbs) were added. Flow cytometric analysis was subsequently performed with a FACStar plus fluorescence-activated cell sorter (Becton-Dickinson) at 488 nm excitation to estimate intracellular TNF-α in the peripheral mononuclear cells. Cells were gated for CD14-positive cells.

2.10. *Detection of apoptosis with annexin V*

Cell apoptosis was assessed by annexin V – FITC staining with the flow cytometric apoptosis detection kit (PharMingen, Becton Dickinson, Catalog no 556420).
The PBMCs were incubated for 24 to 48 h in the presence or absence of infliximab (100 μg/ml).Those cells that were negative for both dyes were considered to be live cells; necrotic cells were positive for both fluorochromes or only for propidium iodide (PI); and apoptotic cells were positive only for annexin V - FITC and negative for PI.

2.11. *Measurement of intracellular procalcitonin by flow cytometric analysis*

The intracellular procalcitonin (PCT) content of cells was estimated via the PCT components calcitonin (CT) and katakalcin (KC) after incubation for 18 h in the presence or absence of heat killed *S. aureus*. Mononuclear cells, granulocytes and U937 cells were permeabilized by Cytoprem solution (Becton-Dickinson). For staining, an indirect immunofluorescence technique was used, applying two MAb to CT and KC (BRAHMS Diagnostica, Berlin). Flow cytometric analysis was performed thereafter with a FACStar plus fluorescence-activated cell sorter (Becton-Dickinson) at 488nm excitation to estimate intracellular CT and KC in peripheral monocytes and granulocytes.

2.12. *Statistical analysis*

Statistical analyses for comparison of allele and genotype frequencies between groups were made by using the χ^2 test, and Fisher's exact test if one cell had $n<5$. For comparison of age and sex between the patients and the controls, the Mann–Whitney *U* test and the Fisher's exact test was used. The genotype frequencies for each polymorphism were tested for deviation from the Hardy-Weinberg equilibrium by means of the χ^2 test, with one degree of freedom used. The levels of intracellular TNF-α production according to the SA inducer and the experimental results on apoptosis were compared by means of one-way ANOVA. The Student-Newman-Keuls test was used for *post hoc* pairwise multiple comparisons. In all tests, an α level of $p<0.05$ was taken as an indication of statistical significance. All statistical calculations were performed with the GraphPad Prism4 statistical program.

3. *Results*

3.1. *Correlation of TNF-α, HSP70-2, and CD14 gene polymorphisms and the severity of acute pancreatitis*

The distributions of genotypes and alleles for the different polymorphisms were consistent with those predicted by the Hardy-Weinberg equilibrium in the patients and in the controls.

3.1.1. *The TNF-α -308 G→A polymorphism*

Overall, there was no significant difference in the distribution of the TNF -308 genotype between the patients with acute pancreatitis and the healthy controls. However, when the patients were stratified according to disease severity, there was a higher frequency of the TNF 1/2 genotype among patients with the severe disease as compared with the patients with mild pancreatitis. Carriage of the high secreting allele (TNF2) was significantly different between the two groups, although the number of homozygotes itself was very low. Likewise, the frequency of the TNF 1/1 genotype was significantly higher in the patients with mild disease as compared with patients with the severe form of pancreatitis (Paper I / Table 2).

3.1.2. *The HSP70-2 1267 A→G polymorphism*

The genotypic frequencies were significantly different between severe and mild pancreatitis (Table 1). This was due to over-expression of the HSP70-2 G allele in the severe population. The frequency of AG genotype was also significantly higher in the severe group. No significant difference was found however in the frequency of either the A or the G allele between the pancreatitis patients overall and healthy controls. This connotes that the HSP70-2 alleles are not directly implicated in disease predisposition, however patients carrying the G allele might be at higher risk of the severe form of acute pancreatitis. Alternatively, the "protective" AA genotype is markedly more frequent among the patients who have only a mild form of pancreatitis. The frequency of the AA genotype was significantly higher in patients with mild disease, than in the severe form of acute pancreatitis, or even in healthy donors (Figure 5).

	AA	AG	GG	G%
Mild (n=29)	20/29	7/29	2/29	18.9 (11/58)
Severe (n=48)	7/48	31/48	10/48	53.1 (51/96)
	P < 0.0001	P = 0.0009		P < 0.0001
Total (n=77)	27/77	38/77	12/77	40.2 (62/154)
Control (n=71)	32/71	30/71	9/71	35.9 (51/142)
	ns vs. total	ns vs total		ns vs. total

Table 1. HSP70-2 genotype and G allele frequencies (%) in patients with acute pancreatitis and controls.

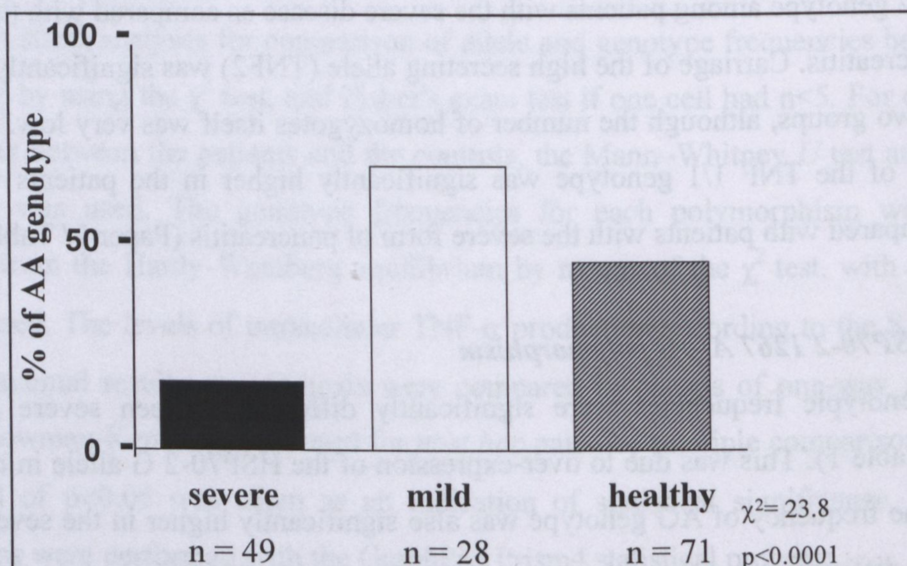


Figure 5. Bars indicate the percentage of patients carrying the AA (wild type, "protective") alleles. Genotypic frequencies were compared by χ^2 test. Statistical significance was taken at the p=0.05 level.

3.1.3. The CD14 -159 C→T gene-polymorphism

We did not find correlation between CD14-159 promoter polymorphism and any forms of the acute pancreatitis investigated herein. Furthermore, there were no significant differences in CD14-159 genotype frequencies between patients and the healthy control group.

3.1.4. *TNF-α, PCT and acute pancreatitis*

Pancreatitis obviously can be regarded as a prominent disease belonging to SIRS. TNF is a key cytokine orchestrating even the cytokine cascade in SIRS, but half-life of TNF-α is extremely short in the circulation. Even therefore, a relatively new diagnostic marker, procalcitonin (PCT) has recently attracted attention as a possible marker of systemic inflammatory response to infection. The rapid induction of PCT after endotoxin stimulation and its relation to cytokines such as TNF-α and IL-6 suggest that the stimulation of PCT is closely correlated with the induction of proinflammatory cytokines. In our previous study we found that PCT level was significantly higher [8.5 ± 4.8 ng/mL] in patients with infected pancreatic necrosis [174]. PCT is an excellent, sensitive marker, and a valuable, rapid laboratory test for discriminating infected pancreatic necrosis from sterile pancreatic necrosis. Thus, we further investigated the possible cellular sources of PCT, and the role of TNF in PCT induction. The intracellular expression of PCT was investigated by flow cytometric analysis with intracellular staining with antibodies to the PCT components calcitonin and katacalcin. Both human peripheral monocytes and granulocytes expressed PCT, and increased intracellular amounts of the PCT components were demonstrated after stimulation with *Staphylococcus aureus* as TNF-α inducer (Paper II / Figure 2). We could demonstrate that TNF-α is the main mediator in the *S. aureus* induced stimulation of PCT production in monocytes and granulocytes, as the effect could be almost totally abrogated when the cytokine was neutralized by anti-TNF. The TNF-inducing ability of the bacterial cells is responsible for the elevation of the intracellular PCT content.

3.2. *Prevalence of TNF-α and Heat-shock protein 70-2 gene polymorphisms in a family with rheumatoid arthritis*

Two of the 29 members were homozygotes for TNF 1, 18 of them were heterozygotes TNF 1/2 and 9 of them were homozygotes for TNF 1. Nineteen of the 29 were heterozygotes and none were homozygotes for allele A (HSP70-2), 10 of them were homozygotes for allele G (HSP70-2) (Paper III / Figure 2). Four of the 5 RA patients in this family carried the TNF 2 allele; all 5 were heterozygotes for HSP70-2 genotypes (Paper III / Table 1). Further, among the 19 members of the family who carried the TNF 2 allele, 15 were heterozygotes for HSP70-2, and only 5 were homozygotes for the HSP70-2 G allele. None of the family members were homozygotes for the HSP70-2 A allele (Paper III / Figure 1). The family

members are by definition not genetically independent; therefore the increase in TNF 2 can not be taken to reflect a significant effect of the TNF-α polymorphism, but the accumulation of TNF 2 in this family is striking in contrast with the frequencies of TNF-α alleles among healthy blood donors.

3.3. The prognostic value of TNF-α gene polymorphism among patients treated with anti-TNF-α therapy, and the effect of anti-TNF-α therapy on TNF-α production and apoptosis

3.3.1. The TNF-α -308 G→A polymorphism in patients treated with infliximab

The frequency of TNF2 carriers in the patient group (9 patients with RA and 14 patients with CD) was higher as compared with the healthy controls. Almost all of the 9 patients carrying TNF2 allele (8 patients) were non responders (4 RA and 4 CD non responder patients). Eight of the 12 non responder CD or RA patients carried the TNF2 allele (Paper IV / Table 1). Statistical analysis was not applied because of the relatively small number of patients receiving infliximab therapy. However, the high number of TNF2 carriers in the nonresponding group is very striking.

3.3.2. Effect of infliximab therapy on TNF-α production

3.3.2.1. Effect of infliximab therapy on in vitro TNF-α production by WBCs

The *in vitro* TNF-α production by WBCs was determined at different times after infliximab therapy. The TNF-α concentration in the supernatants of the WBCs before infliximab treatment was considerable 527.8±363.2 U/ml. It is noteworthy that those patients who carried the TNF2 alleles exhibited the highest *in vitro* TNF production (data not shown). After the 3rd infusion, the *in vitro* TNF-α production was only 33.0±12.2 U/ml, and after the 4th infusion it was below the detection limit of the assay. When we washed out the infliximab from the WBCs, a marked TNF-α concentration of 319.4±141.3 U/ml was measured even after the 4th infusion (Paper IV / Figure 1). It is very likely that the anti-TNF antibody in the supernatants of the WBCs could neutralize the cytotoxic effect of TNF-α. The reduced TNF simply be due to a TNF-α trapping by infliximab, which is then not recognized either by bioassay or by ELISA.

3.3.2.2. Effect of infliximab on intracellular TNF-α content of monocytes

Following SA stimulation, the *in vitro* TNF-α production by monocytes from the healthy controls was increased significantly, as revealed by an intracellular TNF-α staining method. Pretreatment of mononuclear cells with 100 μg/ml of infliximab for 24 or 48 h did not result in a loss of their TNF-producing ability; the mean fluorescence intensity data for the control and the SA-stimulated cells were significantly different in each experiment, as assessed by the ANOVA test. The same tendency was observed for the mononuclear cells of the RA patients 48 h after the 4th infusion of infliximab (Paper IV / Figure 3). Accordingly, we conclude that the infliximab therapy did not inhibit the ability of these cells to respond to the bacterial inducer with TNF-α production.

3.3.3. Effect of infliximab on apoptosis of PBMCs

The effect of infliximab on the apoptosis was investigated after different time points following the *in vitro* infliximab pretreatment of PBMCs from the healthy blood donors. The results were compared with the apoptosis of PBMCs isolated from the patients participating in infliximab therapy 48 h after the 4th infusion. *In vitro* infliximab treatment (100 μg/ml) of the PBMCs for 48 h resulted in an increase in the percentage of apoptotic cells. The kinetic study revealed that the spontaneous apoptosis was slightly elevated following a 48-h incubation of the PBMCs, but it was not significant according to the ANOVA test. There was a significant increase in apoptosis, however, as a result of a 48-h *in vitro* treatment of the PBMCs with infliximab. In comparison with this *in vitro* infliximab treatment, in *ex vivo* samples investigated directly from the RA patients undergoing infliximab therapy, the degree of apoptosis was not considerable. Hence, we conclude that this infliximab therapy itself did not result in as high a rate of apoptosis as was observed on the *in vitro* treatment of PBMCs for 48 h (Figure 6).

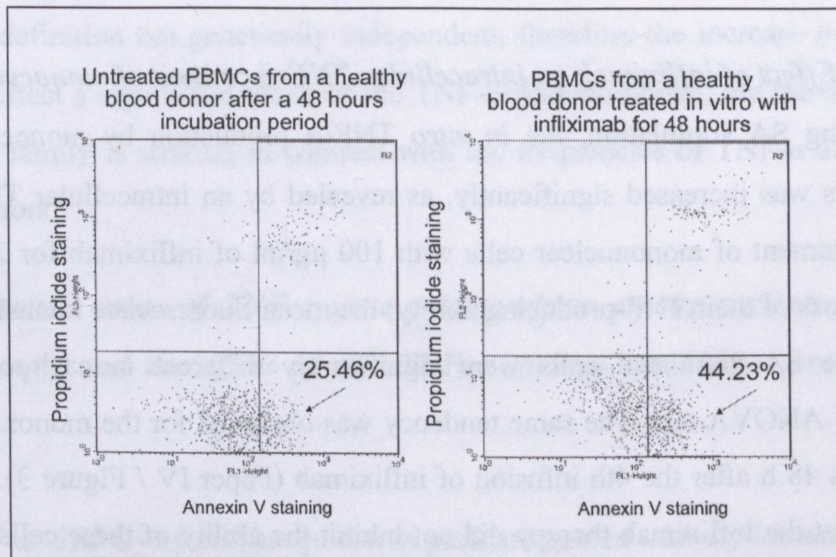


Figure 6. Effect of in vitro infliximab treatment on the apoptosis of PBMCs. PBMCs were labelled with annexin V-FITC and PI. Surviving cells (low signals for both annexin V and PI) appear in the lower-left quadrant, early apoptotic cells (high annexin V signal, but low PI signal) in the lower-right quadrant, late apoptotic cells and necrotic cells (high signals for both annexin V and PI) in the upper-right quadrant, and necrotic cells in the upper-left quadrant (high PI signal).

3.4. TNF-α and TGF-β1 gene polymorphism among patients with MDS belonging to the refractory anemia (RA) subtype

The distributions of genotypes and alleles for the different polymorphisms were consistent with those predicted by the Hardy-Weinberg equilibrium in the patients and in the controls.

3.4.1. The TNF-α -308 G→A polymorphism

There were no significant differences in the TNF-α -308 promoter genotypic distribution between the patients with RA. Similarly, there were no differences in the distribution of TNF-α -308 G-to-A genotypes between the RA patients only with anemia (mild group) and those with bi- or pancytopenia (severe group) (Paper V / Table 1).

3.4.2. The TGF-β1 T^{29}→C polymorphism

No significant difference in genotypic distribution was found between the refractory anemic patients overall and the healthy controls. A significant difference was observed, however, when the genotypes were taken into consideration within the two subgroups of RA.

To elucidate the reason for this significance, we compared the number of TT homozygotes (high TGF-β-producing phenotype) and C carriers (CT and CC) among the patients with mild and severe cytopenia forms of RA and the healthy controls. A significant difference was observed when the TT homozygotes and C carrier genotypes were compared within the two subgroups of RA together with the healthy controls. There was also a considerable difference in frequency of the TT genotype between the two subgroups of RA. This was due to overpresentation of the TT genotype in the patients with bi- or pancytopenia (severe group). Alternatively, the C carrier status (representing the CT and CC genotypes) was markedly more frequent among the RA patients with only anemia (mild group) (Paper V / Table 2). Since we had only 4 patients with the hypoplastic form of MDS, it was not meaningful to perform any statistical analysis concerning the marrow cellularity. Nevertheless, the genotypic analysis revealed that 2 of these patients had the TT homozygote genotype. The results suggesting that the gene polymorphisms of TGF-ß1 may provide a rational indicator of the disease susceptibility or prognosis in MDS.

Table 1. Distribution of TGF-ß T^{29}-C (Leu→Pro) genotypes between the MDS patients belonging to the RA subtype and the healthy controls.

	TGF-ß (T^{29}-C) genotypes			*P*
	TT n (%)	**CT** n (%)	**CC** n (%)	
Patients with RA (n=50)	24 (48.0)	19 (38.0)	7 (14.0)	0.031[a]
mild (n=30)	9 (30.0)	16 (53.3)	5 (16.7)	0.007[b]
severe (n=20)	15 (75.0)	3 (15.0)	2 (10.0)	0.003[c]
Controls (n=74)	33 (44.6)	28 (37.8)	13 (17.6)	

[a] χ^2= 10.58, d.f. = 4; comparisons between the mild group (patients with anemia only), the severe group (patients with bi- or pancytopenia) and the controls.
[b] χ^2= 9.923, d.f. = 2; statistical analysis was performed between the TT and CT + CC genotypes; comparisons between the mild group, the severe group and the controls.
[c] Fischer's exact test: odds ratio = 6.99; statistical analysis was performed between the TT and CT + CC genotypes; comparison between the mild group and the severe group.

4. *Discussion*

4.1. *TNF-α, HSP70-2, and CD14 gene polymorphisms in acute pancreatitis*

CD14 We found no correlation among CD14 promoter polymorphism, disease severity and susceptibility to acute pancreatitis. The activation of CD14 by LPS or by other bacterial products could still be extremely important in cases of pancreatic necrosis associated with infection. Although we did not find significant differences in CD14 polymorphism associated with infected or aseptic pancreatic necrosis (data not shown), the low number of cases examined and reported to date warrants further investigations among cases of infected pancreatic necrosis to find a meaningful correlation if it exists.

HSP70-2 Developmental studies suggest that heat shock proteins play an important role in pancreatitis [132, 175, 176, 177, 178, 179]. Although previous studies clearly demonstrate the importance of HSP70-2 in an increased risk for hypoxia related diseases [180], data have not been reported in connection with acute pancreatitis. There was the first to demonstrate that the HSP70-2 G allele, which has been associated with a low HSP70-2 expression, is more prevalent in severe pancreatitis than in mild disease or in the healthy population. Conversely, patients with the "protective" AA genotype are less vulnerable to the disease and have a better prognosis by surviving pancreatitis with far fewer complications.

TNF-α Previous studies of pro-inflammatory cytokine gene variations have failed to reveal their disease predicting role and close relationship with the development of a severe attack of acute pancreatitis [181, 182, 183]; however, our results suggest that the carrying of the TNF2 allele may be a significant factor in process. There are many reasons for considering that TNF-α is involved in the pathogenesis of severe acute pancreatitis, as it mediates many symptoms such as fever, hypotension, shock, and multiple organ dysfunction, all which are present during a severe attack [184, 185]. High TNF-α producer genotypes proved to be more frequent among the patients with severe pancreatitis. The difference was significant only when the two groups of patients were compared; a significant difference was not observed between the patients and healthy donors. This draws attention to the fact carrying of the TNF2 allele itself is not a factor predisposing to acute pancreatitis, but once it has developed, the higher TNF-α producing genotype exacerbates inflammatory processes with more severe consequences of the disease.

Our results on TNF-α and HSP70-2 gene polymorphisms allow the speculation that the constellation of a high TNF-α producer and the low HSP70-2 producer genetic variant increases the risk of developing severe acute pancreatitis. We found that individuals demonstrating the simultaneous presence of these polymorphisms (with 2 "high-risk" genotypes) may be predisposed to the most severe complications of acute necrotizing pancreatitis (Figure 7). However, the relatively small size of the subgroup cohorts warrants further investigations.

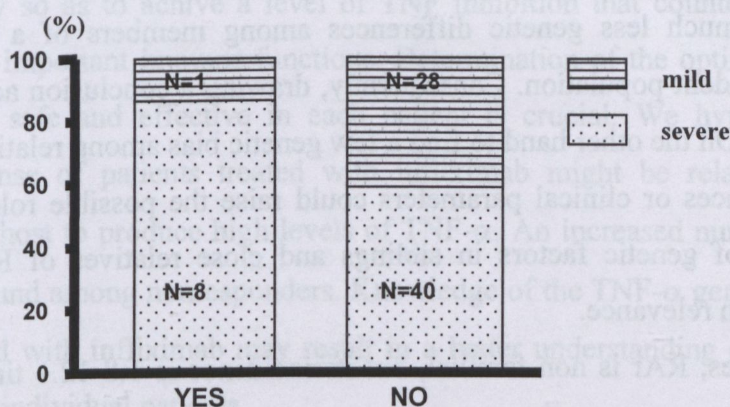


Figure 7. The common presence of TNF2 allele and HSP70-2 G allele (YES) in patients with acute pancreatitis.

Besides the role of the of TNF-α of the initiation of the cytokine cascade, these results provide evidence of the importance of heat shock proteins in the pathomechanism of acute pancreatitis, thereby suggesting the clinical value of genotype assessment as an important prognostic tool to predict disease severity. Genotype assessments therefore provide important clinical evaluation criteria to guide treatment or to identify risk populations for severe acute pancreatitis.

TNF-α and PCT In our previous studies elevated levels of PCT were observed in patients with severe AP, which could be the consequence of TNF-α induction by bacterial stimuli (infected form of AP) [174]. As concerns the kinetics after endotoxin injection in experimental animals and humans, where TNF peaks 2-5 h before PCT, it is presumed that PCT is produced in response to a massive release of TNF [186, 187]. During the course of our in vitro experiments we presumed that TNF was the inducing agent of PCT after stimulation

of peripheral white blood cells with *S. aureus.* We applied *S. aureus* because it has been accepted as an excellent TNF inducer [188, 189]. Our results demonstrate that not only monocytes but also PMNs can be the source of PCT. It is very likely, that monocytes and granulocytes might be the main source of PCT in AP. It is tempting to speculate whether it would be any correlation between TNF-α polymorphism and PCT levels in severe form of AP. Further investigations would be necessary with a high number of patients.

4.2 TNF-α and Heat-shock protein 70-2 gene polymorphisms in a family with rheumatoid arthritis

There are much less genetic differences among members of a family than in a genetically independent population. Consequently, drawing a conclusion according to family studies are limited On the other hand to find a few genetic bias among relatives which parallel phenotypic differences or clinical parameters could raise the possible role of these genetic factors. The role of genetic factors in siblings and close relatives of RAr patients is of appreciable research relevance.

In most cases, RAr is non-familial, but its incidence is 1.8-12.1 times higher among first-degree relatives. Familial RAr has been reported but its extreme rarity to observe 5 female siblings all suffering from RAr. Four of the 5 RAr patients participating in full-dose triple combination therapy carried the TNF2 allele. There was one extremely good responder (Paper III/ Table 1 [no.5]) who was not a TNF2 carrier. Patient 4, who carried the TNF2 allele displayed the most severe clinical status. Moreover, she was the only rheumatoid factor-positive case. However, the family members are by definition not genetically independent, the accumulation of TNF2 in this family is striking.

Individuals being homozygotes for the HSP70-2 G allele consistently display a lower HSP70-2 mRNA expression than those for heterozygotes and HSP70-2 A homozygous individuals [98]. None of the RAr patients were homozygotes for the HSP70-2 G allele. However, it is tempting to speculate whether the 5 family members with HSP70-2 GG genotypes would have the same risk of development of disease in the future as those who carry the A allele together with the TNF2 allele and/or shared epitope. These members of the family need to be closely followed.

4.3. The prognostic value of TNF-α gene polymorphism among patients treated with anti-TNF-α therapy, and the effect of anti-TNF-α therapy on TNF-α production and apoptosis

The clinical efficacy of the biological response modifier infliximab proved to be impressive. One of the crucial questions that arises is how to select the optimal rate of TNF inhibition for each patient [190]. The degree of TNF inhibition required to control the disease in an optimal manner may vary from patient to patient. It may depend on the patient's weight or the drug metabolism, but also on each patient's innate TNF production. We should learn to titrate our therapy so as to achive a level of TNF inhibition that counters the disease, but without crippling important immune functions. Determination of the optimal degree of TNF inhibition that is safe and effective in each patient is crucial. We hypothesized that the therapeutic response of patients treated with infliximab might be related to the genetic propensity of the host to produce high levels of TNF-α. An increased number of the TNF A allele has been found among nonresponders. Knowledge of the TNF-α gene polymorphism in all patients treated with infliximab may result in a better understanding of how to optimize TNF inhibition in individual patients.

Another important feature of our study is the demonstration that the cytokine-producing ability of PBMCs is not irreversibly impaired by infliximab therapy, as revealed by *ex vivo* experiments in which washed WBCs were used. Stimulation of these white blood cells with killed SA led to TNF-α production, which could be detected in a bioassay when the anti-TNF antibodies were washed out in the supernatant of the cell cultures. The retained TNF-α-producing ability was further proved in experiments in which intracellular TNF-α was detected in monocytes. Infections and cases of sepsis have been reported in patients receiving infliximab [162, 163]. Tuberculosis seems to be particularly common [161]. Our observation demonstrated that the host keeps its ability to produce an appropriate TNF-α and Th1 cytokine profile, which appears to be vital in keeping infections localized. It is important to stress that a rigorous follow-up of patients receiving infliximab is essential, however, in order for possible toxic effects and infections to be recognized in time, so that alternative medication can be applied [190].

Monocytopenia is commonly observed after treatment with infliximab. Treatment with infliximab at therapeutic concentrations resulted in apoptosis of monocytes in patients with

chronic active CD who were receiving a dose of $\geq$ 5 mg/kg [191]. We investigated the role of infliximab-induced of PBMCs in RA patients receiving 3 mg/kg infliximab. A 48-hour treatment of PBMCs *in vitro* resulted in a significant increase in the level of apoptosis. However, significant apoptosis was not observed in a direct analysis of the PBMCs of RA patients treated with infliximab. Monocytopenia/leukopenia was not observed in either our RA patients or our CD patients.

Treatment of TNF inhibitors may be accompanied by adverse events, and the precise mechanism of the infliximab therapy remains unclear. In our study, the potentially TNF-α-producing PBMCs retained their host defense function and their number was not decreased statistically by apoptosis. In view of the high costs of infliximab therapy, it appears necessary to determine the TNF genotype before such therapy is initiated, so as to achieve a better prognosis.

4.4. TNF-α and TGF-β1 gene polymorphism among patients with MDS belonging to the refractory anemia subtype

The main features of MDS are an increased apoptosis of hematopietic progenitor cells, and aberrant hematopiesis. During the past few years, major progress has been made toward an understanding of the pathological processes of MDS. Genetic abnormalities or altered gene expressions leading to the suppression of hematopoiesis may also be responsible for the development of cytopenia. One of the genetic factors may be an aberration of cytokine regulation and cytokine production, which can lead to an increased apoptosis of hematopoietic progenitor cells, and aberrant hematopiesis. Both TNF-α and TGF-ß1 inhibit myeloid, erythroid, megakaryocyte and multilineage colony formation and their excessive production has been also described [26, 136, 139, 140, 192, 193]. Accordingly, it appeared plausible to hypothesize that polymorphisms of the TNF-α or TGF-ß1 genes may be correlated with MDS or the severity of the disease. However, no significant difference in -308 TNF-α was observed as regards the G or A alleles when any of the forms of RA were compared with each other or with the controls. On the other hand, we have shown that in patients with RA the TGF-ß "high-producer genotype" TT causes about a 7-fold risk of bi- or pancytopenia as compared with C-carriers. Patients carrying the C "protective" allele, with a concomitantly lower TGF-ß1 production, have only anemia. As TGF-ß1 is a member of the proapoptotic cytokines, it is

very likely that patients with the low-producing genotypes (CC and CT) may have a better chance of a less severe cytopenia in MDS in this way the polymorphism of TGF-ß1 $T^{29} \rightarrow C$ gene may have relevant in the outcome of the disease.

5. *Summary: conclusions and potential significance*

- The risk of severe pancreatitis is increased in patients carrying the TNF2 allele at the -308 promoter site of TNF-α, and even more the G allele of HSP70-2.
- It has shown that TNF-α is the main inducing agent of PCT and that not only monocytes but also PMNs can be the source of PCT in acute pancreatitis.
- The AA genotype of the HSP70-2 gene is associated with a less severe form of acute pancreatitis.
- There is no correlation among CD14 promoter gene polymorphism, disease severity and susceptibility to acute pancreatitis.
- The cumulation of TNF2 allele in this multiplex RAr family draws the attention to the possible role of the -308 TNF-α gene polymorphism both in the susceptibility and the severity of RAr.
- There was a connection between the therapeutic response to infliximab and the -308 TNF-α gene polymorphism in RA and CD patients. There was a strong tendency for a much higher frequency of carriers of the TNF2 allele among nonresponders.
- The neutralizing effect of anti-TNF-α therapy does not result in irreversible inhibition of the TNF-α producing ability of mononuclear cells.
- A 48-hour *in vitro* treatment of PBMCs with infliximab resulted in a significant increase in the level of apoptosis, but no significant apoptosis was observed in a direct analysis of the PBMCs of RAr patients treated with infliximab
- There is no correlation among the polymorphism of the TNF-α -308 promoter region and degree of cytopenia in MDS patients with RA.
- The risk of bi-or pancytopenia is increased significantly higher in TT homozygous at T^{29}-C of TGF-ß1 gene in MDS patients with RA.

6. *Epilogue*

Though, by the end of 2004 more than 99% of the human genome was sequenced, and the decoding of life was accomplished, it doesn't mean the end of the genetic research. Moreover, we can not say that we know the whole human genome. The basic premise that there is one SNP in every 1000 bases [194] leads to an estimate that any two individuals differ by up to three million SNPs. Most of these SNPs are certainly not involved in the coding sequence, but it doesn't mean that they wouldn't have any functional role.

The next important enterprise after the Human Genome Project (HUGO) is the HapMap project concerning the distribution of haplotypes in different ethnic groups. 600 thousands SNP-s of 270 different persons with different ethnic origins have been compared in the HapMap project. The knowledge of the human gene sequence within SNPs more and more precisely could open many new perspectives in the medicine. Nowadays it is possible to knock-out or trans-in either genes or gene fragments containing SNP in the different model organisms. The intensive development of the genetic research has led to the knowledge of more than 1600 monogenic diseases. Genetic susceptibility to complex traits is not caused by specific, relatively rare mutations, rather, the inheritance pattern of these common disorders results from genetic variations that are relatively common in the general population, with many genes each contributing a small quantity of the overall disease susceptibility. For instance, the role of at least 17 different genes has been showed in the pathogenesis of diabetes mellitus. The next example demonstrates the difference between SNP-s and mutations: There are three different mutations known at present, which any of them causes surely Alzheimer-disease. However, these mutations are present only in the 5% of all patients (presenilin-1,-2, amiloid precursor protein). The APO-E ε4 allele has a smaller effect in causing Alzheimer-disease but it can be found in more than the 20% of the patients.

It can be clearly seen, that the presence or the knowledge of one SNP can't either explain the development of a disease or it can't be used as a 100% secure diagnostic method, rather a "SNPs network" could give us valuable information. Choosing the ideal candidate genes is a vital point. For example in sepsis both pro- and anti-inflammatory responses contribute to the outcome of the disease; thus all genes that encode proteins involved in the transduction of inflammatory processes are candidates. This group of genes is not restricted to

those encoding cytokines, but also includes genes for numerous other effector molecules, such as components of the coagulation system, heat shock proteins, and signal transduction molecules. As more genetic information on different markers from different candidate genes becomes available, studies of combined gene effects and gene-gene interactions will become an essential tool for studying genetic predisposition. A combination of TNF high responders and IL-10 low responders to an inflammatory stimulus was found to have the highest death rate in a study of patients with acute renal failure [195, 196]. One of our observations also draws the attention to the fact that not only one SNP, but their combination could be more informative: the simultaneous presence of high TNF-α producer and the low HSP70-2 producer genetic variant may be predisposed to the most severe complications of acute necrotizing pancreatitis.

There is also a big expectation on the part of the pharamaceutical industry, and this is the question of pharmacogenetics. The previous gold standard in the medication used to be the principle: the same dosage for everybody. In spite of this medication habit there is more than 100 thousands deaths in a year because of the drugs adverse-side effects only in the USA. Another important problem is the relatively low effectiveness of many drugs: only 60% of the patients with asthma, 57% in DM, 52% in migraine, and 48% in osteoporosis can be called as good responders. The currently used drugs have about 500 targets, but the number of possible targets may be 5-10 thousands. It is also a big challenge to the pharmacogenetic researchers to find these possible targets. SNPs also may be the key to understanding the genetic factors that predict individual variability to drug response because response to medication in terms of both benefical response and adverse reaction is also a complex trait governed by a large number of genes. Armed with this knowledge, it is hoped that even medications that cause some people significant side-effects can be developed for a subset of patients who will derive therapeutic benefits from them [197]. This approach of targeting drugs to each unique genetic profile is the premise of the new field of pharmacogenomics [198, 199]. The final goal of the pharmacogenetic research should be "the right patients getting the right drugs at the right time".

Francis S. Collins is the director of the National Human Genome Research Institute (NHGRI) at the National Institutes of Health (NIH). He led the successful effort to complete Human Genome Project, a complex multidisciplinary scientific enterprise directed at mapping

and sequencing all of the human DNA, and determining aspects of its function. He and his co-workers also in the HapMap project believe that the results of their and the related projects will change the whole diagnostic and therapeutic approach. SNP libraries will shorten the disease-gene discovery process and initiate the era of personalized medicine. Human geneticists will have at their disposal a super-dense genetic map to identify genes contributing moderate effects on complex traits. Drug companies will be able to determine genetic profiles that will tell them whether an individual patient will benefit or suffer adverse side-effects when given a particular drug. Physicians will be able to prescribe the most effective and safe medication for their patients. Every neonate will be tested for his or her disease expectancy. Eventually, individuals will be able to use their own genetic profiles to find out what diseases they are susceptible to. This might allow them to take preventive measures to maintain their health. Naturally, we have to find the right use of the patient's genetic information even more the whole population including healthy people. Many ethical questions come up at the same time. There is an essential role of the clinical medicine to determine the real importance of SNPs. The value of genomic risk markers as SNPs needs to be verified by prospective, full particular outcome studies. Moreover, the question of whether groups of patients identified as high risk by genomic markers may benefit from individual therapeutic strategies needs to be answered. If this is the case, genotype information may be used as inclusion criteria for future therapeutic trials.

It is also important that the technical backgrounds of these genetic analyses have to be very reliable, repeatable. They should take place on the basis of reference materials and quality control from that time they would become the part of the daily diagnosis. Maybe carrying a subcutaneous silicon chip, which identifies our genotypic profile, will become natural. The chip will be scanned and our allelic variants will determine the protocol of the therapy.

7. *References*

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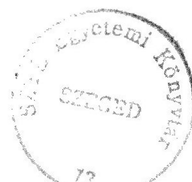

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