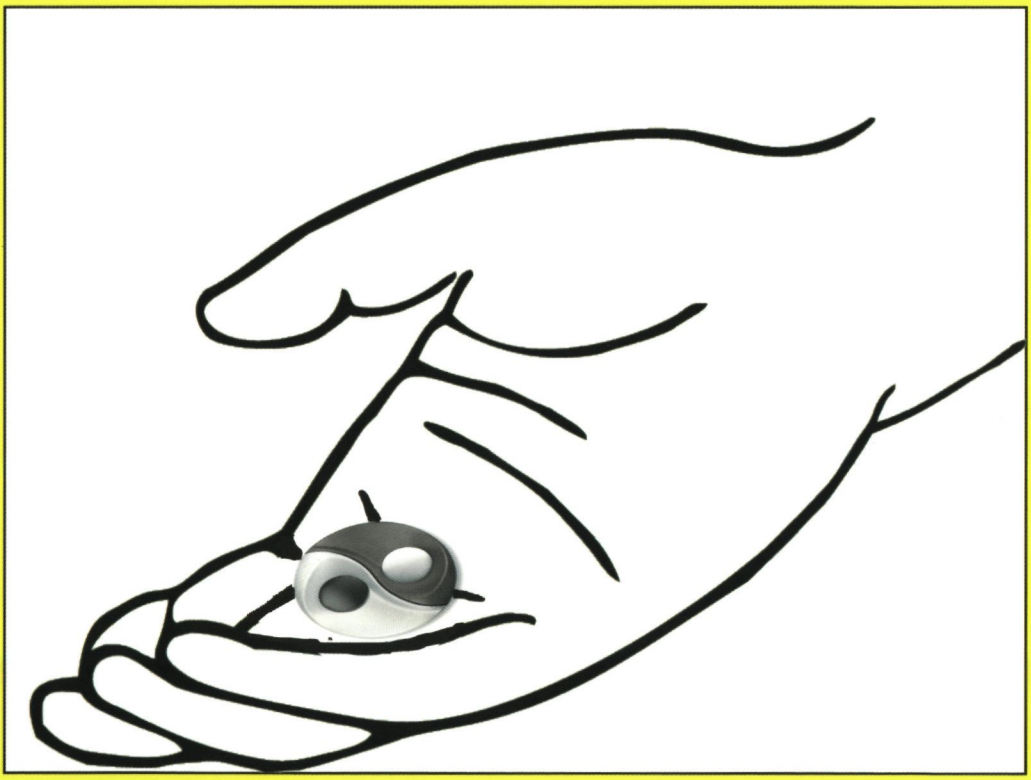
Anti-TNF therapy in rheumatoid arthritis: effects on metabolism and inflammation



Călin Popa

Anti-TNF treatment in rheumatoid arthritis: effects on inflammation and metabolism

Anti-TNF treatment in rheumatoid arthritis: effects on inflammation and metabolism

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Medische Wetenschappen

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Part II: Anti-TNF therapy effect on inflammation

Chapter 1

Introduction

Background

When we encounter a microorganism or suffer from physical trauma, our host defense system reacts and triggers a response that eventually leads to the elimination of the pathogen or assures tissue repair This response can be envisaged as the convergent action of many immune and non-immune cells, each one of them having its particular contribution and a permanent exchange of information with the cells involved The cellular products that mediate this transfer of information are small proteins called "cytokines" Some of the cytokines have chemotactic and pro-inflammatory actions, leading to the activation of host defense, while other cytokines dampen the immune response and restore its homeostasis

Despite this tight regulation, it is not rare that a dysregulated cytokine network fails sometimes to control the inflammatory reaction and results in the development of disease An example is rheumatoid arthritis (RA), a chronic inflammatory disease affecting the joints, in which pro-inflammatory cytokines, such as tumor necrosis factor (TNF), have a crucial contribution to the pathogenesis of the disease This insight led to the development and introduction of a new class of drugs, aimed to block the deleterious effects of TNF, with important positive consequences for both the disease activity of patients with RA, as well as their long-term prognosis

The role of TNF in the host defense against various pathogens, especially intracellular microorganisms, has been strongly documented (Figure 1) Conversely, TNF inhibition has led to a decreased ability to control infections, which was shown both in animal models and in human studies For the host defense against the intracellular pathogens e g , *Mycobacterium tuberculosis*, the implications of TNF have been extensively studied [1] TNF increases phagocytosis, potentiates mycobacterial killing and is important for granuloma formation Mice treated with anti-TNF have delayed and insufficient granuloma formation and an increased susceptibility for mycobacterial infection Given the above-mentioned role of TNF in host defense, it was easy to predict that new therapeutic strategies based on blocking TNF will increase the risk of infections Indeed, several reports describe an association between the TNF-inhibiting treatment and the development of opportunistic infections, especially reactivations of tuberculosis (TB), but also *Salmonella*, fungal and viral infections [2,3] This is particularly apparent in patients receiving treatment with anti-TNF antibodies (infliximab and adalimumab) and mush less so in patients receiving soluble TNF receptor (etanercept) [2] The reasons for this

difference are not entirely clear, but explanations should be sought in pharmacokinetic as well as pharmacodynamic differences.

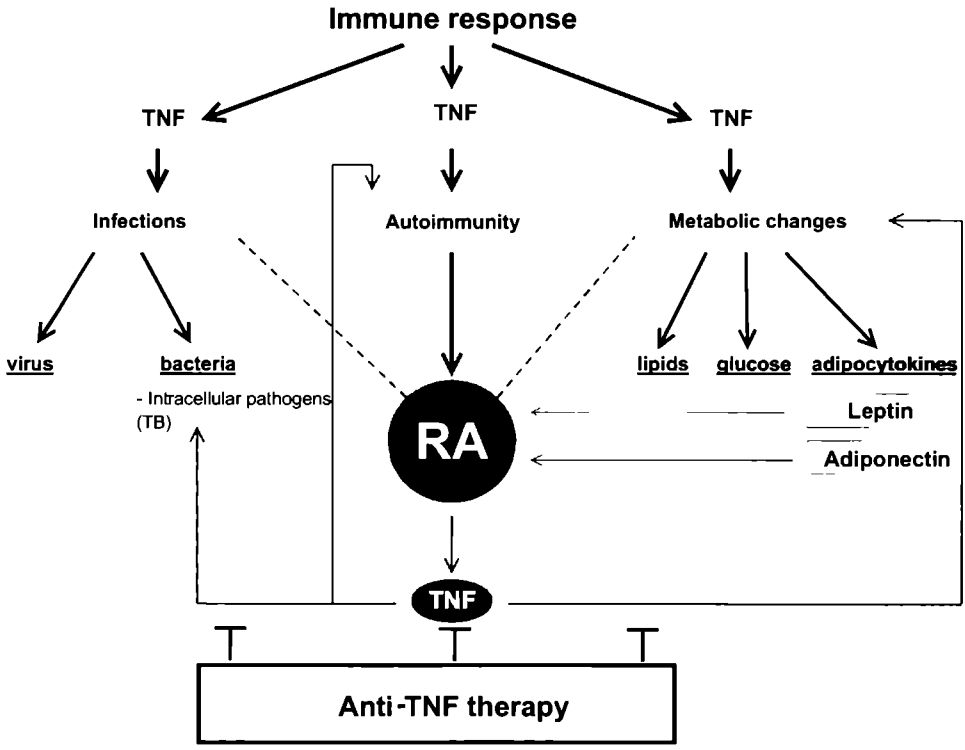

Figure 1 Schematic presentation of the contributions of TNF during immune response and in chronic inflammatory states such as rheumatoid arthritis By blocking this cytokine, anti-TNF drugs are likely to interfere not only with the disease process, but also with the metabolic and anti-infectious pathways

During the immune response the individual reacts through a variety of mechanisms that are meant to reset different homeostatic systems and this may be decisive for the outcome. These adaptations will also involve the intermediary metabolic processes, including lipid and glucose metabolism (Figure 1) [4]. Accordingly, triglyceride (TG) concentrations rise, and plasma cholesterol concentrations fall during the acute phase response. The metabolic changes during the acute phase response are thought to be beneficial for the host. For example, lipoproteins neutralize the toxic effects of lipopolysaccharide (LPS) both in *in vitro* and *in vivo* [5]. The increase in serum TG and the sequestration of cholesterol inside the cells during acute inflammatory conditions may also provide extra nutrients for the elevated metabolic needs of cells involved in host defense and tissue repair. However, when the inflammatory response persists with cytokines such as TNF, changes in both lipid and glucose metabolism arise that are likely to be detrimental for the host. Persistent lipid changes induced by TNF may become pro-atherogenic and sustained increases in glucose and TG plasma concentrations will have an impact on glucose homeostasis, altering glucose tolerance and promoting hyperinsulinemia and an insulin resistance state. Patients with chronic inflammatory diseases, such as RA, display such metabolic disturbances and these are likely to contribute to an increased cardiovascular morbidity and mortality [6;7]. In this light, the exploration of the effects produced by the suppression of inflammation in general, and TNF in particular, on the intermediary metabolism in chronic inflammatory diseases is warranted.

Adipocytes (fat cells) are the main energy store tissue of the human organism, and therefore it is conceivable that during the acute phase response, the homeostasis of these cells will change. Adipocytes are nowadays seen as active endocrine cells, being able to respond to various cytokines (such as TNF) and act as secretory cells. They secrete mediators with effects on other components of the body, including the host defense system [8]. As a result of their resemblance with cytokines and their interference with immune response, these mediators from fat cells are termed "adipocytokines" (Figure 1). Among them, leptin and adiponectin are the best studied. Leptin is the main regulator of energy homeostasis, while one of the main functions of adiponectin is to increase the sensitivity to insulin, thereby interfering with glucose homeostasis. Interestingly, adipocytokines have been suggested to contribute also to the pathogenesis of rheumatoid arthritis. Using different approaches (animal experiments and *in-vitro* and *in-vivo* studies in humans), there is evidence that leptin contributes to the development of arthritis [9;10], through induction of pro-inflammatory cytokines, such as TNF. Unlike leptin, adiponectin was initially demonstrated to have anti-inflammatory properties, e.g. by suppressing TNF production,

and therefore a protective role of this adipocytokine in RA has been initially proposed [11] More recent studies, however, refute this initial hypothesis [12,13] adiponectin has also pro-inflammatory properties and therefore the role of adiponectin in the pathogenesis of RA is a matter of debate Because of their involvement in intermediary metabolism and immune processes, and because of their interrelation with TNF, studies assessing the impact of therapeutic TNF blockade on leptin and adiponectin are needed

The aim of the present thesis is to obtain more insight into the metabolic and immunological changes that take place during therapeutic blockade of TNF in RA patients This insight may not only lead to an increased understanding of the complexity of these therapeutic strategies, but may also prove to be useful for the development of innovative anti-inflammatory strategies to be applied in metabolic disorders

Outline of the thesis

The thesis has been focused on two major areas, one exploring the metabolic effects of anti-TNF therapy and the other one focusing on the modulation of the immune response to different pathogens

In the Chapters 2-4, we addressed the questions to what extent lipid profiles of patients with rheumatoid arthritis during therapy with TNF blockers are being affected According to the traditional cardiovascular risk assessment, the lipid profile in RA has often been described as "pro-atherogenic", based on decreased HDL-cholesterol and increased LDL HDL-cholesterol ratio and lipoprotein (a) plasma concentrations in both active and treated RA [7] Given the effects of TNF on lipids metabolism, we firstly asked the question whether the therapeutical blockade of this cytokine had an impact on plasma lipids concentrations of these patients (Chapter 2) The results observed shortly after the initiation of anti-TNF therapy, prompted us to continue our investigation for an extended period of anti-TNF usage The question we addressed in Chapter 3 was therefore whether these short term effects were sustained over longer periods HDL-cholesterol is protective against cardiovascular disease, but its anti-atherogenic functions seem to be diminished during inflammatory conditions such as rheumatoid arthritis TNF can affect several components of the HDL particle, decreasing its anti-atherogenicity The question we asked in Chapter 4 was whether the administration of anti-TNF drugs was followed by an improvement of HDL anti-atherogenic function

Several studies have previously demonstrated that TNF plays an important role in the pathogenesis of insulin resistance [14] Rheumatoid arthritis patients develop an impaired tolerance to glucose through insulin resistance more often than the general population [6] Given this notion, the question we asked in Chapter 5 was whether anti-TNF therapy is followed by an improvement of insulin sensitivity in RA patients

The next two chapters investigated the influence of anti-TNF therapy on the adipocyte-derived hormones leptin and adiponectin These adipocytokines have been previously shown to interfere with lipids and glucose homeostasis, while probably also contributing to the pathogenesis of RA [9,13,15-17] The question we asked in Chapter 6 was whether short-term anti-TNF therapy can influence circulating leptin concentrations We extended our observations by evaluating the long-term effects of TNF blockade on leptin and adiponectin concentrations in Chapter 7

The ability of anti-TNF therapy to modulate host defence was investigated in the last part of the thesis (Chapters 8-9) Despite the clinical, radiological and functional benefits of TNF inhibitors on RA, safety issues of increased susceptibility to infections, especially due to *Mycobacterium tuberculosis* but also to other intracellular and opportunistic pathogens are a serious concern [2,3] The mechanisms responsible for the enhanced susceptibility are not entirely known, but it is conceivable that host defense mechanisms relying on TNF actions are hampered In this respect, a decrease in the TNF-dependent stimulation of pro-inflammatory cytokines could be envisaged For the study of modulation of pro-inflammatory cytokines in various states, measurement of cytokine concentrations in the circulation alone often gives an incomplete picture and information about the cytokine production capacity by the host cells is needed The question we asked in Chapter 8 was to which extent anti-TNF agents are able to modulate cytokine production by the host cells stimulated with microbial ligands, when assessed by means of a whole blood culture assay In Chapter 9 we addressed the question why are the serious infectious adverse events of anti-TNF drugs more frequently observed in patients treated with monoclonal antibodies (infliximab and adalimumab) than in those treated with soluble receptors (etanercept) We hypothesized that this difference relies mainly on their distinct modulation of cytokine production by the host cells during the course of therapy

A summary of the answers to these questions and the conclusions of the thesis are presented in Chapter 10

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Part I: Anti-TNF therapy effects on intermediary metabolism

Chapter 2

Influence of anti-tumor necrosis factor therapy on the cardiovascular risk factors in patients with active rheumatoid arthritis

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Abstract

Objectives Tumor necrosis factor (TNF) is known to increase the concentrations of IL-6 and CRP and to induce pro-atherogenic changes in the lipid profile and could increase the cardiovascular risk of patients with rheumatoid arthritis (RA) and other inflammatory disorders The aim of this study is to assess whether anti-TNF therapy modifies the cardiovascular risk profile in patients with RA

Methods We investigated the lipoprotein spectrum and the inflammation markers CRP and IL-6 in 33 RA patients treated with human anti-TNF monoclonal antibodies (D2 E7, adalimumab, Humira ®) and 13 RA patients given placebo, before and after two weeks therapy

Results In the anti-TNF treated group, the concentrations of HDL-cholesterol were significantly higher after 2 weeks therapy (0 86 ± 0 30 mmol/L vs 0 98 ± 0 33 mmol/L, $p<0.01$), whereas LDL and triglyceride levels were not significantly changed In addition, a significant decrease in CRP (86 1 ± 54 4 mg/L vs 35 4 ± 35 0 mg/L $p < 0.01$), and IL-6 (88 3 ± 60 5 pg/mL vs 42 3 ± 40 7 pg/mL $p < 0.01$) concentrations was observed in this group No changes in lipid profile, IL-6 or CRP levels were seen in the placebo group

Conclusions TNF neutralization with monoclonal anti-TNF antibodies results in increased HDL-cholesterol levels and decreased CRP and IL-6 levels already after 2 weeks Therefore this therapy may improve the cardiovascular risk profile of patients with RA

Introduction

During the inflammatory response, multiple alterations of the intermediary lipid metabolism do occur. These encompass hypertriglyceridemia and decreased high-density lipoprotein (HDL) cholesterol levels and low-density lipoprotein (LDL) cholesterol levels [1]. Despite the latter, the levels of small dense LDL, a particle believed to be proatherogenic, increase during inflammation [1]. Thus, the pattern of lipid metabolism during inflammation is proatherogenic, and is believed to contribute to atherosclerosis especially in chronic inflammatory diseases such as rheumatoid arthritis (RA) [1]. Tumor necrosis factor α (TNF) is a pro-inflammatory cytokine with pronounced effects on lipoprotein metabolism [2] and this cytokine plays a major role in the pathogenesis of RA.

Patients with untreated active RA have altered lipoprotein and apolipoprotein patterns that may increase the risk of atherosclerosis [3,4]. This is supported by studies showing that the mortality among patients with RA is increased and predominantly due to cardio- and cerebrovascular diseases [4]. Moreover, carotid artery intimae media thickness (IMT), as measured by ultrasound, is increased in patients with RA, suggesting a greater prevalence of subclinical atherosclerosis [5]. In addition, C-reactive protein (CRP) and interleukin (IL)-6 levels are both elevated in patients with active RA and these acute phase reactants have recently been shown to be associated with cardiovascular risk [6]. In RA patients, antifolates therapy was shown to raise homocysteine levels, which was also characterized as an independent risk factor for developing cardiovascular diseases [7].

Therapeutic strategies aimed at TNF neutralization with monoclonal antibodies or TNF receptor fusion proteins, have been shown to reduce the disease activity and structural damage and to improve the quality of life in patients with RA [8]. Moreover blocking TNF does result in a rapid decrease of acute phase reactants [9]. Given these facts, we asked the question whether anti-TNF treatment would also result in changes of the cardiovascular risk profile. To this aim, we assessed the lipid profile and acute reaction markers before and two weeks after the first dose anti-TNF or placebo in patients with active RA enrolled in monotherapy trials with a fully human anti-TNF monoclonal antibody (D2E7, adalimumab, Humira, Abbott Laboratories) at our center.

Patients and Methods

Patients

Patients with active RA included in phase I, double-blind clinical studies with adalimumab monotherapy at our center were studied. Patients fulfilled the 1987 ACR criteria, had an active disease as defined by a disease activity score (DAS) > 3.2 at baseline and underwent a washout period for DMARDs of at least 3 weeks prior to study initiation. Stable dosages of NSAIDs and prednisone (< 10 mg/day) were allowed during the study. Measurements of the parameters studied were done from blood samples collected before the administration of an anti-TNF dose, at baseline and 2 weeks after starting therapy.

Methods

Fasting blood samples were collected in vacutainer tubes (Beckton & Dickinson, Rutherford, NJ) containing K3-EDTA (1 mg/ml), centrifuged at 3600 rpm for 8 min at 4 °C, supplemented with saccharose as a cryoprotectant (final concentration 6 mg/ml) and frozen at -80°C until assay. Cholesterol and triglyceride (TG) were determined by commercially available enzymatic reagents on the Hitachi 747 analyser (Boehringer Mannheim, Germany), while HDL-cholesterol was determined with the phosphotungstate/Mg2+ method [10]. LDL-cholesterol was calculated with the Friedewald formula, which provides reliable values up to a triglyceride concentration of 8.0 mmol/L. IL-6 was determined by a commercial ELISA (BioSource Etten-Leur, The Netherlands), according to the instructions of the manufacturer.

CRP was measured by immunoturbidometry with the Hitachi 747 analyzer using reagents of Roche (#1776371 and #1776428) and the calibrator #BCD1. Sensitivity level was 1mg/L and CV was <2%.

Statistical analysis

Within group comparisons were made using the Wilcoxon signed rank test (for IL-6 and TG) and the paired Student's t-test (for CRP, HDL, LDL and total cholesterol). Significance was set at the 0.05 level. Values are expressed as mean $\pm$ standard deviation (SD).

Results

Within two weeks of anti-TNF administration a 6% increase in total cholesterol concentrations was observed (4.70 ± 1.08 mmol/L vs 5.02 ± 1.16 mmol/L, p = 0.001). This was mainly explained by an increase in the HDL-cholesterol concentrations, (mean of 15%, from 0.86 ± 0.30 mmol/L vs 0.98 ± 0.33 mmol/L, p < 0.0001) (Fig.1A). In contrast, LDL-cholesterol and triglyceride measurements were not significantly changed (3.26 ± 0.88 mmol/L vs 3.37 ± 1.02 mmol/L, mean 3%, Fig.1B, and 1.46 ± 0.60 mmol/L vs 1.38 ± 0.73 mmol/L respectively). The LDL:HDL ratio, as well as total cholesterol:HDL ratio, significantly decreased in the anti-TNF treated group compared with placebo treated patients (4.01 ± 1.85 vs. 4.36 ± 2.16, p=0.017, and 5.76 ± 2.42 vs. 6.24 ± 2.69, p=0.004 respectively). The concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides, as well as LDL:HDL and total cholesterol:HDL ratios, did not change after placebo administration.

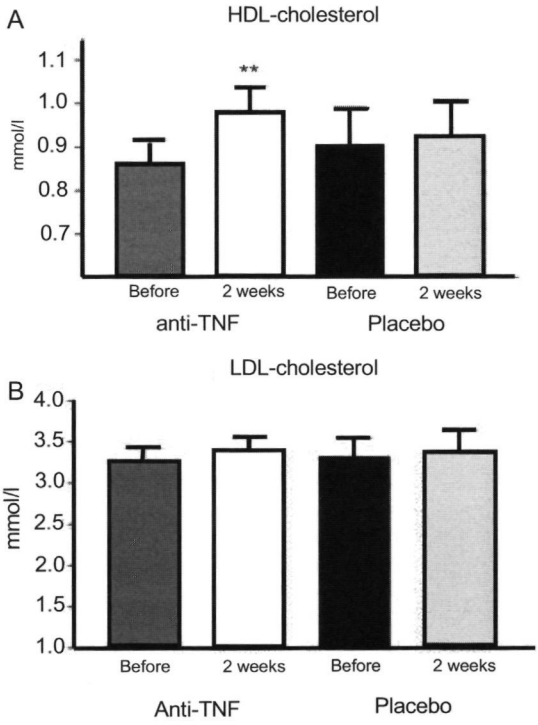

Figure 1. Concentrations of HDL-cholesterol (A) and LDL-cholesterol (B) in 33 RA patients before and after 2 weeks of therapy with a fully human anti-TNF monoclonal antibody or with placebo. (dark grey bars indicate anti-TNF therapy group before; black bars: placebo group before; white bars: anti-TNF therapy group after 2 weeks; light grey bars: placebo group after 2 weeks) *p<0.001; **p<0.0001

As shown in Figure 2, the levels of CRP and IL-6 decreased significantly within 2 weeks of anti-TNF administration ($p < 0.0001$ and $p < 0.001$ for CRP and IL-6 respectively), whereas no changes were observed after placebo. Clinical improvement occurred rapidly after initiation of anti-TNF therapy but not after placebo. This was reflected by a decrease in DAS in the treated and a stable DAS in the placebo group (5.24 ± 1.05 to 4.06 ± 1.14 and 4.8 ± 1.13 to 4.96 ± 1.4 respectively).

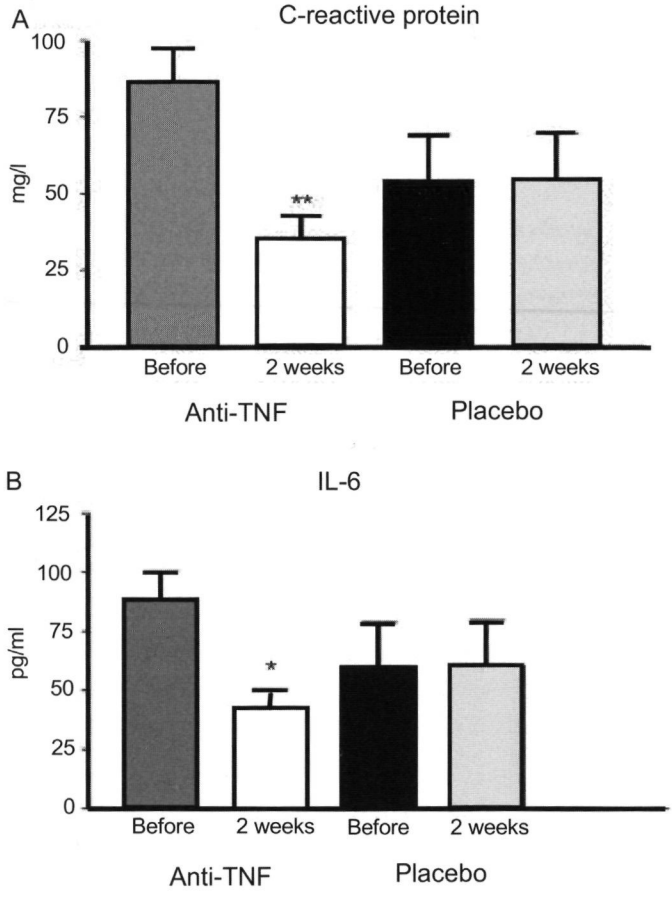

Figure 2. C-reactive protein (A) and IL-6 (B) concentrations in 33 RA patients treated with anti-TNF monoclonal human antibodies and with placebo, before and after 2 weeks therapy. (dark grey bars indicate anti-TNF therapy group before; black bars: placebo group before; white bars: anti-TNF therapy group after 2 weeks; light grey bars: placebo group after 2 weeks) *$p<0.001$; **$p<0.0001$

Discussion

In this study we show that TNF-α neutralization with anti-TNF-α monoclonal antibodies in patients with active RA results in a significant increase in total cholesterol, which is mainly due to enhanced HDL-cholesterol concentrations already within 2 weeks of treatment. In contrast, there were no significant changes in the LDL cholesterol and triglyceride concentrations, while LDL:HDL ratio significantly decreased. These changes are concomitant with a significant decrease in the disease activity and acute phase reactants CRP and IL-6. We therefore hypothesize that if these rapid changes in lipid pattern are maintained, this may also result in a decreased cardiovascular risk in patients with chronic inflammatory disorders.

It is well known that TNF-α induces hypertriglyceridemia in animals and humans [2,11]. This is due to an increased *de novo* fatty-acid synthesis in the liver and esterification into triglycerides, to induction of lipolysis in adipose tissue and to decreased lipoprotein lipase activity [2]. From this perspective, one would expect a decrease in triglyceride concentrations after anti-TNF therapy though this was not the case in our study. This might be explained be the short follow-up period or alternatively, by the lower but still persistent inflammation at week 2.

Besides TNF-α, other cytokines including interleukin 1β (IL-1β), IL-6 and interferon γ (IFN-γ) can modulate the lipid metabolism [2]. TNF is a major inducer of these cytokines and TNF neutralization may result in a decrease of the above-mentioned cytokines. Therefore it is possible that the positive effect of TNF-α blockade on the lipid profile is also mediated by the inhibition of other cytokines.

Previous to the present study, two small studies assessed the effect of TNF neutralization with the chimeric monoclonal antibody infliximab on lipoproteins in patients with rheumatic diseases. Hurlimann et al. also found a slight increase in total cholesterol in 11 patients treated for 12 weeks but no data were provided about HDL, LDL and triglycerides [12]. In another study, Cauza et al. found an increase in triglycerides and a decrease in HDL concentrations in 7 patients with RA after an average of 3 weeks treatment [13]. The discrepancies between the latter study and ours may be explained by the small number of patients tested by Cauza.

In patients with active RA, IL-6 and CRP concentrations are increased and this is most probably mediated by the action of TNF-α. The effect of anti-TNF therapy on the levels of acute phase

reactants observed in this study is similar to those previously described [9,12] IL-6 and CRP, as markers of inflammation, have been recently shown to be positively associated with the cardiovascular risk [6] In the general population, CRP levels much lower than those found in RA are already associated with an increased cardiovascular risk It is tempting to speculate that the decreased CRP, as a mirror of inflammatory status, leads to a decreased risk for atherosclerosis and cardiovascular events in patients with RA treated with TNF-blocking agents, as an association between anti-inflammatory therapy and cardiovascular comorbidity was also previously observed in these patients [14]

In conclusion, we would like to propose that anti-TNF treatment through both improving the lipids pattern (higher HDL-cholesterol and decreased LDL HDL ratio) and decreasing inflammation, improves the cardiovascular risk profile of RA patients. This is strongly supported by a recent study in a large cohort of patients with RA where anti-TNF therapy was associated with decreased incidence of cardiovascular events [15]. Moreover, we are currently testing this hypothesis in our clinic in larger cohorts of patients, with a longer follow-up and using objective measurements of the atherosclerotic process

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Chapter 3

Modulation of lipoprotein plasma concentrations during long-term anti-TNF therapy in patients with active rheumatoid arthritis

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Ann Rheum Dis 2007 66 1503-1507

Abstract

Durable blockade of TNF-α in patients with rheumatoid arthritis (RA) suppresses disease activity and its progression. Cardiovascular diseases are 1.5 to 2 fold more frequent in RA patients than in general population. Although TNF-α has well-established effects on lipid metabolism, long-term effects of TNF-α blockade on lipid pattern are still unclear. In the present study we investigated the effects of one-year therapy with anti-TNF on the lipid profile of RA patients. Methods Disease activity (DAS28) and plasma lipoproteins concentrations (total, HDL and LDL-cholesterol, triglycerides, ApoA, ApoB) were assessed in 55 RA patients and 55 controls. The whole RA group was followed-up for 6 months and 31 of the patients for 1year.

Results In RA patients, DAS28 decreased after 2 weeks from the start of therapy ($p<0.001$) and remained low during the entire study duration. Short-term effects of anti-TNF on plasma lipid concentrations seemed beneficial and anti-atherogenic. However, these changes did not persist: plasma concentrations of total and LDL-cholesterol and the atherogenic index increased after 6 months and one year from the start of therapy. During therapy, the changes in disease activity and inflammatory status inversely correlated with changes in plasma total and HDL cholesterol levels, and positively correlated with the variation of atherogenic index.

Conclusion: We conclude that one-year therapy with infliximab is likely to lead to a more pro-atherogenic pattern of the plasma lipids concentrations. However, the overall impact of these changes on the cardiovascular risk is more complex, considering the strong anti-inflammatory effects of anti-TNF drugs.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of multifactorial etiology. Cardiovascular diseases have been shown to occur 1.5-2 times more often in RA patients than in the general population, leading to increased mortality in this group of patients.[1] Among the traditional cardiovascular risk factors, the lipid profile in RA has often been described as "pro-atherogenic",[2] based on decreased HDL-cholesterol and increased LDL:HDL-cholesterol ratio and lipoprotein (a) plasma concentrations in both active and treated RA.[2-4] Carotid artery intima-medial thickness (IMT), a surrogate marker of atherosclerosis severity, was found to be higher while flow-mediated vasodilatation was lower in RA patients, suggesting a greater prevalence of sub-clinical atherosclerosis in RA.[5] Moreover, insulin resistance is more common in patients with RA than in the general population.[6] However, the increased incidence of cardiovascular events in RA patients cannot be entirely explained by these traditional risk factors.[7] Increased levels of inflammatory markers, including CRP and IL-6, have been shown to be associated with the risk to develop acute cardiovascular events in the general population.[8] Given these observations, chronic inflammation in RA seems to importantly contribute to the development of cardiovascular diseases in these patients.[9,10]

TNF-α is a pleiotropic cytokine with a pivotal role in triggering the host defense against microorganisms. Besides this, TNF-α has also contributes to atherogenesis through a series of mechanisms: it promotes the expression of adhesion molecules on endothelial cells, recruits and activates inflammatory cells and initiates the inflammatory cascade within the arterial wall.[11,12] In addition, TNF-α directly interferes with the metabolic pathways of triglycerides and cholesterol.[13-15] Administration of TNF-α to humans results in an acute elevation of plasma TG with decreased HDL-cholesterol concentrations.[16] TNF-α may also alter the composition of lipoprotein particles.[17] Overall, these changes produced by TNF-α are pro-atherogenic persistence of these modified lipids in the circulation promote the development of atherosclerotic lesions.

Given the effects of TNF-α on both inflammation and the lipid metabolism, one may expect that TNF-α neutralization will have two sets of effects that might lower cardiovascular risk: the anti-inflammatory effects and the anti-atherogenic effects on lipid pattern. Recently, a decrease in the incidence of cardiovascular events was reported in a large group of RA patients after few years of anti-TNF therapy.[18] Moreover, endothelial function can be

restored by anti-TNF agents in terms of improved flow-mediated vasodilatation and reduced expression of adhesion molecules [19,20] However the effect of lipid changes are less clear We have previously shown that short course TNF-α blockade is followed by an improvement of lipids profile and insulin sensitivity on top of the diminished inflammatory status in RA patients receiving anti-TNF [21,22]

The aim of the present study is to investigate the effects of long-term anti-TNF agents on the lipoprotein profile in patients with RA We hypothesize that besides the down regulation of the pro-inflammatory status of RA, in which TNF-α is considered the pivotal cytokine, long-lasting blockade of the pro-atherogenic effects of TNF-α on lipids metabolism will also occur

Patients and Methods

Patients and controls

In our study we prospectively enrolled 67 consecutive patients with RA, who fulfilled the 1987 American College of Rheumatology (ACR) criteria All patients had an active disease (disease activity score (DAS) > 3 2) at baseline and were about to start the therapy with a TNF-α blocker (infliximab) Patients taking lipid-lowering drugs were excluded Patients were attending the outpatient clinic of Sint Maartenskliniek Nijmegen and entered the study after giving their written informed consent The regional medical ethical committee approved the study Infliximab (3mg/kg) was given in infusions at baseline and at 2 weeks, 6 weeks and thereafter every 8 weeks Changes in infliximab doses (5mg/kg) or intervals of administration (6 weeks) were based on patient's response to therapy and were made by the treating rheumatologist, irrespective to the aim of our study Twelve patients who received treatment less than 3 months were excluded because of the short-term therapy In 55 patients data was collected during a follow-up period of 6 months, whereas 31 patients were followed for one-year Stable dosages of DMARDs and oral corticosteroids (CS, prednisone < 10 mg/day) were allowed during the study Disease activity was measured regularly before each infliximab infusion using the DAS28 score [23] Patients' disease duration, body-mass index (BMI), smoking status and other characteristics were recorded at baseline and presented in more detail in Table1 No change in the weight of patients was apparent during therapy Besides the patients group, an age- and gender-matched healthy control group whose characteristics are presented in Table 1 was also assessed

Laboratory measurements

Blood samples were collected before each administration of infliximab. Fasting blood was collected in vacutainer tubes (Beckton & Dickinson, Rutherford, NJ) containing K3-EDTA (1 mg/ml), centrifuged at 3600 rpm for 8 min at 4 °C, supplemented with saccharose as a cryoprotectant (final concentration 6 mg/ml) and frozen at -80°C until assay. Serum levels of plasma total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically on a Hitachi 747 analyser. The normal values for lipoproteins are: total cholesterol 4.7 – 6.5 mmo/l, HDL-cholesterol 0.95 – 1.50 mmol/l (for men) and 1.10 – 1.70 mmol/l (for women), TG 0.8 – 2.0 mmol/l. Low density lipoprotein cholesterol (LDL-C) levels were calculated according to the Friedewald formula, which provides reliable values up to a triglyceride concentration of 4.0 mmol/l. Apolipoprotein B (ApoB) and A-I (ApoA) concentrations were determined by immunonephelometry. The atherogenic index (AI) was calculated as the ratio between total cholesterol and HDL cholesterol plasma concentrations.

Statistical analysis

Between healthy controls and RA patients at baseline, the comparisons were made using the Mann-Whitney test. For non-parametric values within group, comparisons were made using the Wilcoxon signed rank test while the paired Student's t-test was use in the case the values were normally distributed. Correlations between inflammatory status markers and lipids were determined using Spearman test. Friedman's non-parametric test for related samples was used to test for changes in lipids concentrations from baseline during the follow-up period. Significance was set at the level of 0.05. Values are expressed as mean $\pm$ standard deviation (SD), unless otherwise stated.

Results

Characteristics of the patients at baseline and changes in disease activity

As showed in Table 1, we found no important differences regarding the lipid profile between the whole group of RA patients and that of controls. However, male RA patients at baseline had lower HDL-cholesterol concentrations and a higher atherogenic index compared to male controls: 1.18 ± 0.32 mmol/l vs. 1.61 ± 0.44 mmol/l, p = 0.004 and respectively 4.53 ± 0.82 vs. 3.75 ± 1.04, p = 0.025. In addition, female with RA at baseline had higher HDL-cholesterol levels compared to RA men: 1.44 ± 0.28 mmol/l vs. 1.18 ± 0.32 mmol/l, $p<0.01$. This difference was observed at several time-points during the study period (not shown). Interestingly, there was no difference in the lipid pattern at baseline between CS users and

Table 1. The characteristics of control group and patients with RA at baseline

Parameters assessed	Rheumatoid arthritis (N=55)	Controls (N=55)	p-value
General data			
Age (years)	56 ± 11	56 ± 11	N S
Gender (M/F)	16/40	16/40	N S.
Disease duration (years)	9 ± 7	-	-
Rheumatoid factor (+)	64%	-	-
DAS28	5.26 ± 1 25	-	-
Medication			
Oral steroids (N)	9	-	-
Methotrexate (N)		-	-
Cardiovascular profile			
Ever smoking (%)	30%	59%	0 05
CAD history	16%	16%	1 00
BMI (kg/m^2)	26.2 ± 5 2	24 3 ± 3 7	0 08
TC (mmol/l)	5 55 ± 0.99	5.74 ± 0.79	0 18
HDL(mmol/l)	1.37 ± 0.32	1.44 ± 0 39	0.52
LDL(mmol/l)	3.54 ± 0.83	3 56 ± 0 72	0.63
Atherogenic index (TC:HDL)	4.19 ± 1.10	4.24 ± 1.28	0 82
LDL HDL	2.72 ± 0 82	2.66 ± 0 99	0 55
TG(mmol/l)	1.50 ± 0.65	1 77 ± 0.83	0.08
ApoA (mg/l)	1510 ± 350	1537 ± 398	0.46
ApoB(mg/l)	1024 ± 212	1030 ± 219	0.68

RA = rheumatoid arthritis, CAD = coronary artery disease, BMI = body mass index; N.S = not significant

Table 2. Lipid changes during one year anti-TNF therapy in patients with RA.

	Baseline	Δ 2 weeks	Δ 6 months	Δ 12 months	P_1-value	P_2-value	P_3-value
TC	5.55 ± 0.99 mmol/l	**1.07 ± 0.12**	**1.01 ± 0.14**	**1.09 ± 0.16**	**0.001**	**0.79**	**0.01**
HDL	1 37 ± 0.32 mmol/l	**1.08 ± 0.14**	**0.97 ± 0.15**	**1.01 ± 0.31**	**0.001**	**0.06**	**0.54**
LDL	3 54 ± 0 83 mmol/l	**1.08 ± 0.14**	**1.01 ± 0.18**	**0.97 ± 0.38**	**0.001**	**0.94**	**0.11**
AI	4 19 ± 1 10	**1.01 ± 0.14**	**1.09 ± 0.22**	**1.04 ± 0.37**	**0.84**	**0.02**	**0.05**
LDL HDL	2.72 ± 0 82	**0.99 ± 0.20**	**1.06 ± 0.22**	**1.10 ± 0.29**	**0.88**	**0.10**	**0.15**
TG	1 50 ± 0.65 mmol/l	**1.09 ± 0.33**	**1.14 ± 0.37**	**1.28 ± 0.63**	**0.35**	**0.02**	**0.001**
ApoA	1510 ± 350 mg/l	**1.07 ± 0.14**	**0.99 ± 0.14**	**1.00 ± 0.20**	**0.02**	**0.40**	**0.06**
ApoB	1024 ± 212 mg/l	**1.05 ± 0.12**	**1.02 ± 0.15**	**1.01 ± 0.23**	**0.06**	**0.56**	**0.24**
DAS28	**5.26 ± 1.25**	**0.70 ± 0.21**	**0.81 ± 0.30**	**0.75 ± 0.30**	**0.0001**	**0.0001**	**0.0001**

Results are presented as Means ± SD, for 2 weeks, 6 months and 12 months results are expressed as percents from baseline values (1.00 = 100%); P1 compares baseline with 2 weeks levels; P2 compares baseline with 6 months levels; P3 compares baseline with 12 months

non-CS users. As expected, the inflammatory markers were increased in the case of RA patients, but they did not correlate with any of the lipid parameters, although a trend towards an inversely relation between ESR and total and HDL cholesterol could be detected (not shown).

Anti-TNF therapy had an immediate inhibitory effect on the inflammatory status of the patients. After the first infliximab infusion, DAS28 dropped significantly and thereafter remained stable throughout the entire follow-up period (Table 2). According to the EULAR response criteria, 56% of our patients responded to therapy after 6 months while in 21% an important improvement in disease activity could be recorded at this time point. These percentages did not significantly change after one year (60% and respectively 20%). Changes

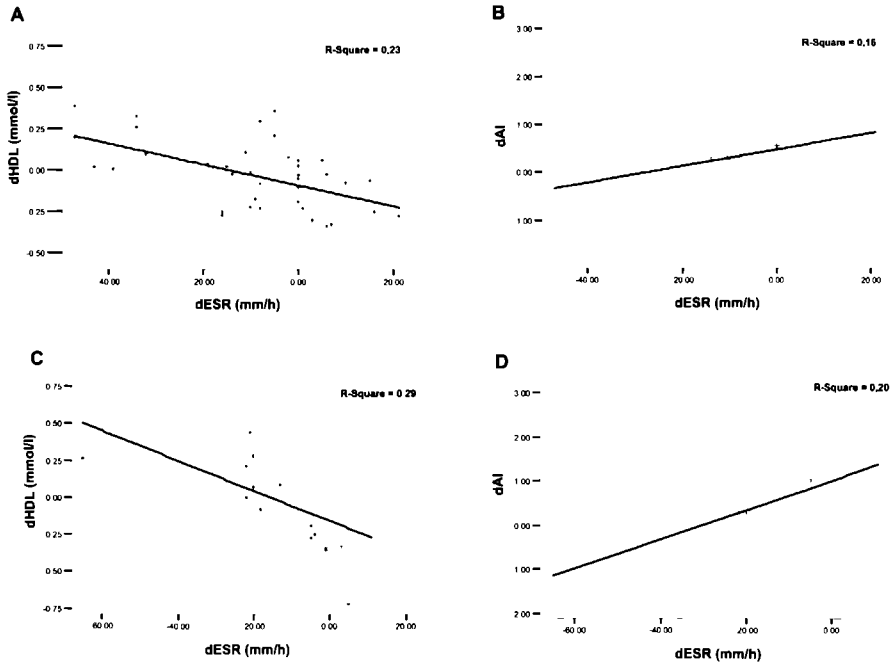

Figure 1. Correlations between inflammatory status, as reflected by plasma ESR concentrations, and HDL-cholesterol (A,C) and atherogenic index (B,D) after 3 months (A,B) and 9 months (C,D) of therapy with infliximab.

of infliximab doses and frequency of administrations occurred in both responders and non-responders patients, but they had little influence on the distribution of the patients between the two subgroups as assessed after 6 and 12 months of therapy

Short-term changes in lipoproteins pattern during anti-TNF therapy

Two weeks after the first anti-TNF infusion, total cholesterol, LDL-cholesterol, HDL-cholesterol and ApoA significantly increased, while the atherogenic index remained unchanged (Table2) The 2-weeks changes on total cholesterol and TG were inversely correlated with changes in DAS28 over the same period r = -0 30 (p = 0 041) and r = -0 34 (p = 0 032), respectively To evaluate whether this initial effects are preserved also in the case of later infliximab infusions, lipid pattern was determined every 2 weeks between two consecutive infusions in a subgroup of 10 RA patients However, no significant changes in lipid profile could be detected during this interval (not shown) Interestingly, after the first 2 weeks of anti-TNF therapy, CS co-medication significantly influenced total cholesterol and HDL-cholesterol levels, which increased more in these patients compared to those not receiving oral CS (p<0 01, between the two subgroups) In contrast, AI changes were not different between the two subgroups during the same interval

Long-term changes in lipoproteins pattern during anti-TNF therapy

After six months of anti-TNF therapy, except for TG, no significant changes in plasma concentrations of lipids fractions assessed could be detected compared to baseline (Table 2) However, the atherogenic index significantly increased during this interval (Table2) In addition, plasma concentration of total cholesterol, LDL-cholesterol and the atherogenic index increased after one year of therapy (Table2) Total-cholesterol, HDL cholesterol and atherogenic index significantly differ over the first 6 months compared to baseline using the Friedman non-parametric test (p<0 002, p<0 001 and respectively p<0 024) The same results regarding total and HDL cholesterol were observed in the one-year follow-up group (p<0 033 and respectively p<0 019)

Interestingly, anti-TNF therapy had a more pronounced effect on lipids profile of male patients compared with female RA patients in male, total cholesterol and LDL-cholesterol increased more markedly after 6 months and even after 1 year of therapy (p<0 04) and AI tended to increase (not shown) However, no trend in the total and LDL cholesterol increase

in men during this period could be noticed These changes were not related to either BMI or CS use

Previous studies have reported the existence of a relation between plasma HDL concentrations and the inflammatory status in RA In our study, the changes in HDL levels were inversely associated with the changes in ESR after 3 months ($p<0.001$, Figure 1A), 6 months ($p<0.01$) and 9 months ($p<0.004$, Figure 1C) of anti-TNF therapy, while only a tendency for the same relation between changes in DAS28 and HDL at the same time points could be detected (not shown) Plasma total cholesterol changes negatively correlated with changes in ESR after 3 ($p<0.032$) and 6 months ($p<0.034$) of therapy and with changes in DAS28 after one year ($p<0.022$) Finally, changes in AI were found to relate with changes in ESR after 3 months ($p<0.03$, Figure 1B), 9 months ($p<0.02$, Figure1D), and 12 months ($p<0.03$) and with changes of DAS28 after 6 months ($p<0.03$) of therapy with infliximab In addition, the atherogenic index had a tendency to increase more in non-responders compared to responders, after 6 months of anti-TNF therapy ($p = 0.089$) No differences could be seen in terms of long-term lipid pattern changes between those taking CS and/or methotrexate as co-medication and those not taking these drugs

Discussion

In the present study we show that TNF- blockade with infliximab modifies plasma lipoprotein concentrations in RA patients While short-term effects seem beneficial and anti-atherogenic, they are not sustained over longer periods The overall decrease of disease activity and inflammatory status are accompanied by changes in lipoprotein profile in these patients

Up to now, several studies have examined plasma lipid concentrations in RA patients compared with control groups Total and LDL cholesterol levels of patients with RA were found elevated in some studies and reduced in others [4,24-26] More constantly, decreased HDL-cholesterol levels were reported in active or untreated RA,[4,24,26] which might augment the cardiovascular risk In our study we also observed a slight decrease in HDL and TG levels in the group of RA patients compared to the controls group In addition, we did not find any important difference between RA and controls regarding the other lipid parameters evaluated One possible explanation may be that the individuals enrolled in our control group were rigorously matched for age and gender, in contrast to other studies, where often the control group was younger Alternatively, this discrepancy between studies regarding lipid

pattern in RA patients might be due to the large heterogeneity between the groups of RA patients studied, in terms of number, disease duration and disease activity

In the present study, short-term therapy with infliximab was followed by important changes of the lipoprotein spectrum that were similar to those previously reported increase of plasma HDL-cholesterol concentrations and no changes of AI during the first 2 weeks of anti-TNF therapy [21,27] However, this short-term probably beneficial effects of TNF-α blockade were not sustained in time total and LDL-cholesterol increased while plasma HDL-cholesterol concentrations did not change after 12 months of therapy In addition, the atherogenic index and LDL HDL-cholesterol ratio also increased during the same interval, suggesting a worsening of lipid pattern and an increase of the atherogenic risk in these patients To date, few studies investigated lipids changes during long-term therapy with infliximab Allanore et al and more recently Seriolo et al have found increased levels of total and LDL-cholesterol but also increased HDL-cholesterol concentrations and no modification of triglycerides and the atherogenic index after approximately six months of anti-TNF therapy [26,28] In another study, 6 months therapy with infliximab was not accompanied by modification in cholesterol-rich lipoproteins, except for a slight increase of triglycerides concentrations [29] Finally, after the same treatment period Rantapaa-Dahlqvist et al observed no changes in HDL-cholesterol levels but noticed an increase in total cholesterol, atherogenic index and LDL HDL cholesterol ratio in RA patients receiving anti-TNF therapy [3] However, the lipid profile present after 6 months of therapy did not further change one and even two years after therapy with infliximab was initiated Given these facts, the results of the present study add to the body of evidence that long-term effects of infliximab therapy do not lead to a more favourable lipoprotein pattern

Previous studies have extensively described the capacity of TNF-α to decrease plasma concentrations of total cholesterol, LDL and HDL-cholesterol in humans [13-16,30] Therefore, the neutralisation of TNF-α in patients with RA is likely to abolish these suppressive effects, leading to an increase in cholesterol-rich compounds as best seen in our study short after the start of therapy In addition, the overall inflammatory status might also influence lipid spectrum in RA This is sustained by studies showing that disease activity and inflammatory markers tend to have inverse relations with HDL-cholesterol levels in these patients [31-33] This might explain the fact that the most significant changes in lipid profile, especially HDL levels, seen after the first 2 weeks from the start of the therapy parallel the

most dramatic decrease of the inflammatory status,[21,26,27] while stable activity of RA as seen afterwards, was often associated with minor or no changes in lipoprotein plasma concentration [3,28,29] Eventually this was reflected in the correlations we found in our study at different time-points between changes in HDL-cholesterol concentrations or AI on one side and changes in ESR on the other side, which is in line with other reports [3,26,28] Although recent studies reported weight-gain after TNF blockade in RA patients, this was not the case in our study [34] Therefore, weight might not essentially contributed to the increase in cholesterol observed in our study Finally, the use of corticosteroids is known to induce an increase in the levels of total and HDL-cholesterol [3,27,29] However, in our investigated group of RA patients we found no important differences in lipid changes during long term TNF-α blockade between CS users and non-users Therefore the simultaneous use of oral CS was unlike to produce the increase in total cholesterol and atherogenic index seen after one year Nevertheless, total and HDL cholesterol increased more in CS users in the first 2 weeks after the start of therapy, which might be also explained by a decrease of IGF-1 levels as recently reported to occur in these patients during the same period [35]

The lipid spectrum is amongst the most important determinants of cardiovascular risk, which is known to be increased in RA compared to general population [1] The present study indicates that long-term anti-TNF therapy may worsen lipids profile, thereby augmenting the cardiovascular risk in these patients However, as dyslipidemia is not the only risk factor present in RA that can be modulated by anti-TNF agents, the impact of lipids changes seen in the present study on the cardiovascular risk profile should be interpreted with caution The increase in total and LDL cholesterol observed in our study after 12 months was 7% (0 38 mmol/l or 14 mg/l) and 8% (0 28 mmol/l or 11 mg/l), respectively Therefore, although statistically significant, the changes in total and LDL-cholesterol plasma concentrations might have a limited contribution to increasing the cardiovascular risk in these patients [7,36] In contrast, the inflammatory status was significantly and constantly depressed by anti-TNF agents, which is likely to diminish the cardiovascular risk [7-10] In addition, several other studies have been indicated that anti-TNF drugs could decrease homocysteine levels, improve endothelial function, increase insulin sensitivity and even reduce the incidence of cardiovascular events in a large cohort of RA patients [19-20,22,37] These data suggest that anti-TNF therapy could either worsen or improve the cardiovascular risk factors in RA and the net effect is difficult to be evaluated only from the results yielded by the current study

In conclusion, the results of the present study indicate that one-year therapy with infliximab is likely to lead to a more pro-atherogenic profile of the plasma lipids concentrations However, the impact of these changes on the cardiovascular risk is complex and should be further evaluated in prospective studies with clinical endpoints

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Competing interests

None

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Chapter 4

Anti-inflammatory therapy with tumor necrosis factor alpha inhibitors improves high-density lipoprotein cholesterol antioxidative capacity in rheumatoid arthritis patients

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Abstract

Objective HDL anti-atherogenic functions seem to be diminished during inflammatory conditions such as rheumatoid arthritis (RA) The aim of this study was to investigate the effects of TNF inhibition on the anti-oxidative capacity of HDL in RA

Methods Plasma lipids and paraoxonase (PON)-1 activity were investigated in 45 RA patients, before and during 6 months of anti-TNF therapy In addition, HDL was isolated and tested for its ability to inhibit copper induced oxidation of LDL in vitro

Results Plasma HDL concentrations did not considerably change after 6 months of therapy However, stable increases of PON-1 activities were observed throughout the same period ($p<0.03$) The increases were more obvious when related to HDL or apolipoprotein A-I concentrations HDL total anti-oxidative capacity significantly improved 6 months after initiation of anti-TNF therapy ($p = 0.015$) The initial improvement of PON-1 activity paralleled a decrease of the inflammatory status, whereas specific TNF blockade was likely to be responsible for the long-term effects

Conclusions Anti-TNF therapy with infliximab has beneficial effects on lipids through changes in HDL anti-oxidative capacity, which might be clinical relevant and contribute to the reported protective effect of anti-TNF on cardiovascular morbidity in RA This emphasizes the importance of HDL anti-atherogenic capacity for the cardiovascular risk in chronic inflammatory conditions

Background

Cardiovascular diseases (CVD) remain the major cause of death in developed countries, despite a recent reported decrease in their incidence [1] A growing body of evidence underlines the crucial role of inflammation in the development and instability of atherosclerotic plaques Consistent with that, patients suffering from chronic inflammatory diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) have increased CVD morbidity It is believed that an adequate control of inflammation in patients with RA or SLE does not only ameliorate disease-related symptoms, but also reduces CVD risk Various epidemiologic studies have recently indicated that anti-TNF therapy is associated with decreases in CV-related mortality in RA [2, 3] However, it is not yet clear whether this effect is mainly due to the blockade of TNF-α itself or if it is more related to the overall suppression and control of inflammation in these patients

An increase in HDL-cholesterol plasma concentrations has been constantly reported shortly after anti-TNF therapy has been initiated and this is likely to mirror an important decrease in the inflammation and acute phase reaction [4] Nevertheless, this increase in HDL was not sustained during long-term TNF-α blockade in RA patients [5] Corroborated with the epidemiologic studies,[2, 3] this ultimately suggests that measuring HDL plasma concentrations would yield limited information concerning the CVD risk in these patients, and therefore, HDL functional qualities may have a greater impact than its concentration in determining the CVD risk
HDL anti-atherogenic properties are represented by its capacity to neutralize oxidized lipids and to extract cholesterol from peripheral tissues, initiating the reverse cholesterol transport (RCT) The HDL component mainly responsible for the anti-oxidative effects is paraoxonase (PON)-1 Compared to healthy controls, PON1 concentrations are decreased in RA patients [6-8] and this probably contributes to the recently reported lower HDL anti-oxidative capacity in these patients [8] In different cohorts, including that of the Framingham study, high HDL-cholesterol concentrations were not always associated with a low CVD risk,[9, 10] suggesting that other HDL-markers, such as those responsible for its anti-oxidative capacity and/or cholesterol efflux from periphery, may be as well of great importance in this respect

The aim of this study was to investigate the effects of anti-TNF therapy on the anti-oxidative capacity of HDL in RA We hypothesized that TNF blockade may also modify the anti-oxidative capacity of HDL and this, combined with the effects on HDL concentration could offer a better

perspective of the CVD protective value of HDL in these patients To test the anti-oxidative capacity of HDL, we have investigated the paraoxonase and arylesterase activities of PON1

Patients and Methods

Patients

Between January 2005 and December 2006 a group of 45 consecutive patients with RA fulfilling the 1987 American College of Rheumatology (ACR) criteria were investigated in this study All patients had an active disease (DAS > 3 2) at baseline and were about to start the therapy with a TNF-α blocker (infliximab) Patients were attending the outpatient rheumatology clinic of the St Maartenskliniek in Nijmegen and entered the study after giving written informed consent The Regional Medical Ethical Committee approved the study Infliximab (3mg/kg) was given in infusions at baseline and at 2 weeks, 6 weeks and thereafter every 8 weeks The mean age of the RA group was 56 ± 11 years and the disease duration was 7 9 ± 6 0 years Among them, 70% were women and 60% were positive for the rheumatic factor (RF) The group was followed for a period of 6 months Seventeen (37%) RA patients were smokers and seven patients (17%) have ever experience an acute cardiovascular event, as documented in the patient clinical charts No changes in body weight could be observed during this period (Table1) Besides infliximab, stable dosages of DMARDs were allowed during the study Patients receiving prednisone or lipid-lowering drugs were excluded from this study Disease activity (DAS28) and laboratory parameters were assessed regularly before each infliximab infusion

Lipids measurements

Fasting blood was collected in vacutainer tubes (Beckton & Dickinson, Rutherford, NJ) containing K3-EDTA (1 mg/ml), and a sample was taken in a tube without anti-coagulant to get serum Tubes were centrifuged at 3600 rpm for 8 min at 4 °C, supplemented with saccharose as a cryoprotectant (final concentration 6 mg/ml) and frozen at -80°C until assay Serum levels of plasma total cholesterol (TC), triglycerides (TG), and HDL-cholesterol were determined enzymatically on a Hitachi 747 analyser LDL-cholesterol levels were calculated according to the Friedewald formula, which provides reliable values up to a triglyceride concentration of 4 0 mmol/L Apolipoprotein A-I (apoA-I) concentrations were determined by immunonephelometry

Analysis of PON1 enzymatic activity

Paraoxonase and arylesterase activities were analyzed spectrophotometrically in flat-bottomed UV transparent 96 well plates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) Serum

paraoxonase hydrolytic activity was determined by addition of 5 µL serum in 0.1 M Tris/HCL, pH 8.5, 0.9 mM CaCl2 and 30 nL diethyl p-nitrophenylphosphate (Sigma Aldrich B.V., Zwijndrecht, The Netherlands) in a final volume of 200 µL. The reaction was monitored for 10 min at 37°C. Serum arylesterase activity was determined by addition of 0.25 µL serum in 0.1 M Tris, pH 8.5, 0.9 mM CaCl2 and 34 nL phenylacetate (Sigma Aldrich B.V., Zwijndrecht, The Netherlands) in a final volume of 200 µL. The reaction was monitored for 5 min at 37°C for the linear increment of absorbance at 280 nm. For the paraoxonase activity the coefficients of variation (CV) were 5% for intra-plate variability and 6% for inter-plate variability. For arylesterase activity the intra-plate CV was 8% and the inter-plate CV was 10%. Paraoxonase and arylesterase activities are expressed as units per litre of serum, where 1 unit equals 1 µmol of substrate hydrolyzed per minute.

In vitro LDL oxidation assay

As a measure of the anti-oxidative capacity of HDL, we assessed the inhibitory effect of isolated HDL on *in-vitro* copper-induced LDL oxidation. LDL was obtained by ultracentrifugation[11] from EDTA-plasma frozen in the presence of saccharose (0.6% (w/v) final concentration) at -80 °C. LDL oxidation (61 µg apolipoprotein/ml) was initiated by the addition of $CuSO_4$ to a final concentration of 18 µM [12] and performed in the presence and absence of HDL (61 µg apolipoprotein/ml) at 30 °C. HDL of the patients was isolated by single-spin density-gradient ultracentrifugation. Protein of LDL and HDL preparations was measured using bovine serum albumin as standard and with chloroform extraction of the color solution to remove turbidity. The kinetics of the oxidation of LDL was determined by monitoring the change of diene absorption in a thermostatic ultraviolet spectrophotometer at 234 nm (Lambda 12, Perkin Elmer GmbH, Rodgau-Jügesheim, Germany). The oxidation characteristics of LDL were determined by the lag time (minutes), the oxidation rate (nmol dienes/mg protein per minute), and the maximal amount of dienes formed during LDL oxidation (nmol/mg LDL protein) as previously described.[13] Within- and between-run coefficients of variation were 3.8% and 6.5% for lag time, 5.8% and 8.2% for oxidation rate, and 2.2% and 5.0% for maximal amount of dienes formed.

Statistical analysis

For non-parametric values comparisons were made using the Wilcoxon signed rank test. The differences between values of variables at baseline and after treatment were done using the paired Student's t-test. Correlations between inflammatory status markers and lipids were

determined using the Spearman test Significance was set at the level of 0 05 Values are expressed as mean ± standard deviation (SD), unless otherwise stated

Results

Anti-TNF effects on lipids concentrations and inflammatory status

At baseline all patients had an active disease ESR = 31 ± 19 mm/h, DAS28 = 5 26 ± 1 24 Six months following infliximab therapy, ESR and the DAS28 had decreased by more than 50% and 30% respectively, from baseline ($p < 0\ 001$ compared to baseline) In addition, lipid concentrations changed during TNF blockade total cholesterol, HDL-cholesterol and apoA-I significantly increased 2 weeks after the therapy was initiated ($p<0\ 01$) (Table 1) Eventually, an increase of the atherogenic index (as expressed by total cholesterol/HDL-cholesterol ratio) was observed after 6 months of therapy, mainly due to a slight decrease in HDL-cholesterol concentrations at this time-point (Table 1)

Table 1. Inflammatory and lipid parameters investigated in the group of rheumatoid arthritis patients during anti-TNF therapy (n=45)

	Baseline	**2 weeks**	**6 months**
DAS28	5 26 ± 1 24	3 82 ± 1 42***	4 23 ± 1 26***
ESR (mm/h)	31 ± 19 2	20 7 ± 17 8***	24 ± 15 1***
BMI (kg/m^2)	25 7 ± 5 5	25 6 ± 5 4	25 7 ± 5 5
Lipids			
Total cholesterol (mmol/l)	5 56 ± 1 03	5 81 ± 1 04**	5 60 ± 0 99
LDL-cholesterol (mmol/l)	3 66 ± 0 87	3 74 ± 0 88	3 60 ± 0 79
HDL-cholesterol (mmol/l)	1 34 ± 0 32	1 43 ± 0 37**	1 29 ± 0 34
Total cholesterol/HDL cholesterol	4 27 ± 1 15	4 34 ± 1 20	4 51 ± 1 27*
LDL cholesterol/HDL cholesterol	2 82 ± 0 91	2 76 ± 1 05	2 94 ± 1 01
Apo-AI (mg/l)	1504 ± 309	1586 ± 299**	1474 ± 299
Triglycerides (mmol/l)	1 38 ± 0 58	1 49 ± 0 63	1 61 ± 0 83*

Results are presented as mean ± SD, DAS28 = disease activity score, ESR = erythrocytes sedimentation rate, *$p < 0\ 05$, **$p < 0\ 01$, ***$p < 0\ 001$ vs baseline

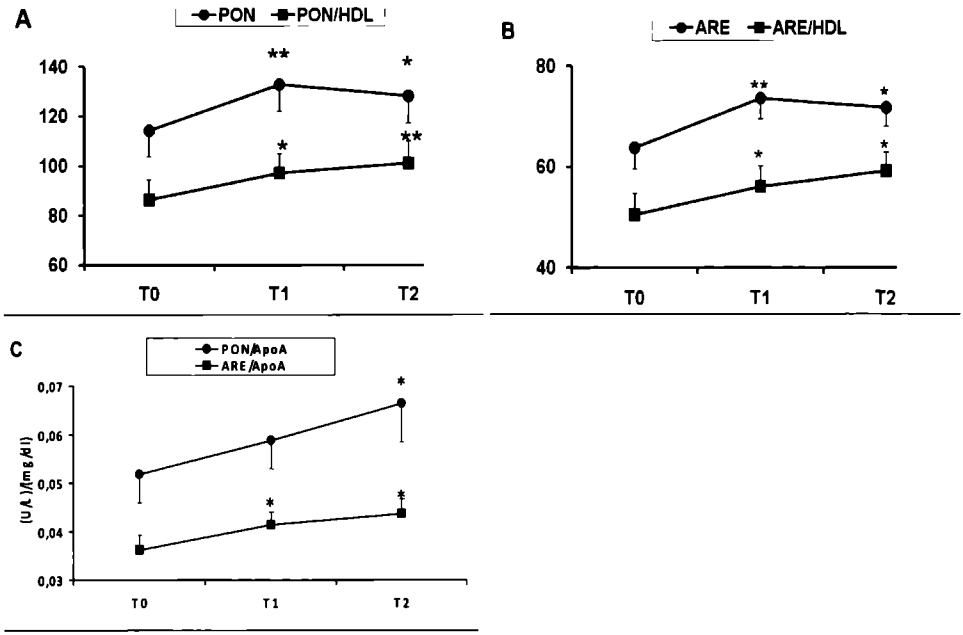

Figure 1. Changes in paraoxonase (PON) activity [PON (U/l), PON/HDL (U/mmol)] [A], arylesterase (ARE) activity [ARE (U/l), ARE/HDL (U/mmol)] [B], and the ratio of them with HDL-cholesterol and respectively with apolipoprotein-AI [C] plasma concentrations during anti-TNF therapy T0 = baseline, T1 = 2 weeks, T2 = 6 months (**p < 0.01, *p < 0.05 vs baseline).

Anti-TNF effects on PON-1 activities

PON-1 was further investigated as the main enzyme situated on HDL responsible for its anti-oxidative capacity. In our study population, the paraoxonase activity of PON-1 significantly increased after two weeks from the first infusion (p = 0.002) and remained high also after 6 months (p = 0.018) (Figure 1A). The same trend was observed in arylesterase activity after 2 weeks and 6 months (p = 0.003 and p = 0.022, respectively) (Figure 1B). To better assess the capacity of one HDL molecule to neutralize the effects of oxidized lipids, we investigated the ratios between paraoxonase activity and HDL on the one hand, and arylesterase activity and HDL on the other hand, respectively. A higher value of this ratio would indicate a higher anti-oxidative capacity of one single HDL molecule. Both paraoxonase/HDL and arylesterase/HDL ratios had increased significantly already two weeks after the initiation of therapy with infliximab (Figure 1A) and, unlike paraoxonase and arylesterase activities, continued to increase slightly up to 6 months of therapy (p = 0.008 for PON/HDL ratio and p = 0.011 for ARE/HDL

ratio, respectively) (Figure 1B). The same results were seen when the enzyme activities were related to apoA-I concentrations (Figure 1C). The ratio between paraoxonase and arylesterase remained stable throughout the study period (not shown).

Anti-TNF effects on total HDL anti-oxidative capacity

In order to test the impact of PON1 activities changes on HDL overall anti-oxidative capacity, we isolated HDL of the last 15 patients consecutively enrolled in the study and tested its capacity to inhibit LDL oxidation in vitro. Due to our initial observations indicating no effect of long-term therapy with infliximab on plasma HDL concentrations, we only tested the anti-oxidative capacity of HDL before and 6 months after anti-TNF therapy was initiated. We observed an increased lag-time of LDL oxidation in the presence of HDL obtained 6 months after anti-TNF has been initiated compared to baseline HDL($p = 0.015$) (Figure 2). LDL oxidation rate and maximal amount of dienes formed did not significantly differ between both conditions. These findings suggest that the overall HDL anti-oxidative capacity improved after long-term infliximab therapy.

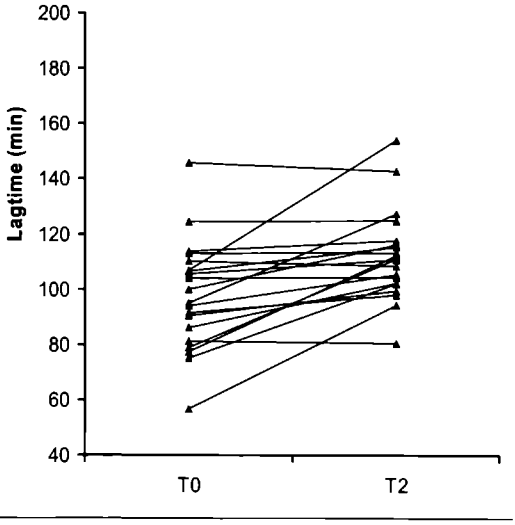

Figure 2. Changes in HDL anti-oxidative capacity as measured by means of an *in-vitro* LDL oxidation assay. Black horizontal lines represent the means T0 = baseline, T2 = 6 months (*$p < 0.05$ vs baseline).

Inflammatory markers and PON-1 activity

In order to investigate whether these changes in PON-1 activities were due to specific TNF inhibition or an overall decrease of inflammation, correlations between PON-1 activities and ESR and DAS28 were performed (Table 2). Inverse correlations with ESR were observed for paraoxonase (r = - 0.37, Figure 3A) and for arylesterase activities (r = - 0.58, Figure 3B) 2 weeks after the start of the therapy, but these relations became weaker after a half-year of TNF blockade (Table 2).

Tabel 2. Correlations between DAS28/ESR and PON-1 activities during TNF blockade

	Baseline		**2 weeks**		**6 months**	
	PON	**ARE**	**PON**	**ARE**	**PON**	**ARE**
DAS28	-0 18	-0 39*	-0 31**	-0.47**	-0 12	-0 10
ESR (mm/h)	-0 36*	-0 36*	-0 37*	-0 58***	-0 22	-0.16
	Δ PON	**Δ ARE**	**Δ PON**	**Δ ARE**	**Δ PON**	**Δ ARE**
Δ DAS28	-	-	-0 07	-0 35*	-0 02	-0.02
Δ ESR (mm/h)	-	-	-0 28	-0 15	0 00	0 02

DAS28 = disease activity score, ESR = erythrocytes sedimentation rate, PON = paraoxonase activity; ARE = arylesterase activity; *p < 0 05, **p < 0.01, ***p < 0 001

Discussion

In the present study we report for the first time that in RA patients therapy with infliximab causes sustained increases of paraoxonase and arylesterase activities of PON1 on HDL-cholesterol molecules, while it only transiently raises the concentrations of HDL-cholesterol. We suggest that by increasing the anti-oxidative capacity of HDL, infliximab may improve HDL anti-atherogenic capacity.

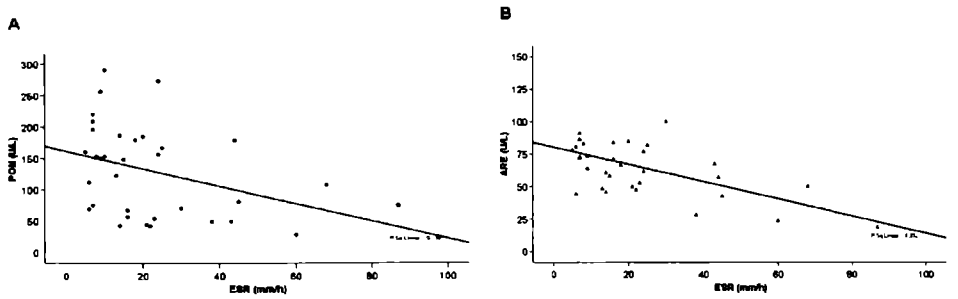

Figure 3 Correlations between ESR and HDL-paraoxonase activity [A] (R Sq = 0 138) and HDL-arylesterase activity [B] (R Sq = 0.332) at 2 weeks after the initiation of therapeutic TNF blockade

RA patients treated with anti-TNF therapy were recently reported to show a decreased incidence of CV events compared with those on other anti-rheumatic medication.[2, 3] HDL is one of the most stable clinical predictors of future CVD events. Two mechanisms are mainly responsible for its the anti-atherogenic effects: 1) its anti-oxidative capacity, neutralising oxidized lipids and 2) its capacity to promote cholesterol efflux from vessels and to transport it to the liver for excretion.[14] In order to exert its anti-oxidative function, the HDL particle harbours the anti-oxidative enzyme PON-1, which is one of the most potent circulating enzymes in counteracting the pro-atherogenic effect of oxidized lipids.[15] In our study we show for the first time that therapeutic TNF blockade using infliximab is able to robustly improve HDL anti-oxidative capacity by increasing its PON-1 activities. This was further emphasized and corroborated in an additional smaller group of patients, using a direct cell-free assay. Besides the increase in PON-1 activities, this therapy induced a significant improvement of the whole-HDL anti-oxidative capacity in this group. Proinflammatory cytokines, including TNF have previously been shown to suppress the hepatic synthesis of PON-1.[16, 17] Although we have not directly assessed the circulating PON-1 levels, it is likely that they may increase when TNF is blocked. Therefore, we hypothesize that the increase in PON-1 activities during anti-TNF can be explained by an increase in hepatic PON-1 synthesis, which is now free of TNF inhibitory effects. Additionally, the decrease in other TNF driven pro-inflammatory cytokine concentrations could also have the same effect on hepatic PON-1 synthesis during therapy.

We tried to elucidate whether these changes in PON-1 activities are due to the overall decrease in inflammation or to the therapy itself. Our results suggest that early after therapy was initiated the

overall decrease of inflammatory status plays an important role in the HDL anti-oxidative capacity. However, this role appears to have diminished after six months of therapy. This is mainly suggested by the fact that between 2 weeks and 6 months HDL anti-oxidative capacity, as assessed by PON/HDL, ARE/HDL, PON/apoA-I and ARE/apoA-I ratios, preserved its tendency to increase, whereas inflammatory status changed its initial decreasing trend into a slight increase. These findings would further suggest that prevention of TNF-induced suppression of hepatic synthesis of PON-1 by anti-TNF antibodies is also important in improving HDL anti-oxidative capacity, especially later in the course of therapy. Increasing the capacity of HDL to dampen the atherogenic effects of oxidized lipids could represent one mechanism through which infliximab might be anti-atherogenic and contributes to the recently reported reduction of the incidence of CVD in RA patients treated with these drugs.[2, 3] However, one note of caution should be emphasized, as it is not clear whether this increase of HDL anti-oxidative capacity reported here contributes to the diminished CVD morbidity, because TNF blockade has been shown previously to improve also other CVD risk factors in RA, including endothelial function and insulin sensitivity.[18-20] On the other hand, the increase in atherogenic index following long-term therapy with infliximab suggests that lipid concentrations pattern in RA patients appears to be less predictive for CVD risk than in the general population. This would also explain the progression of subclinical atherosclerosis found by some investigators in patients on long-term anti-TNF therapy.[21]Conversely, others have indicated neutral long-term effects of TNF blockers on lipid concentrations of RA patients [22], with factors related to disease activity, (co)medication, dietary intake and physical activity being most probable responsible for that.

In our study, the immediate effects of the overall decrease of inflammation were difficult to delineate from those produced by the specific blockade of TNF effects on circulating lipids pattern. Although it is very likely that the anti-oxidative effects observed with infliximab in the present study would occur also in response to the other anti-TNF agents, further studies are needed in order to prove this hypothesis.

In conclusion, our study shows a beneficial effect of infliximab through changes in the composition of the HDL particle leading to improved anti-oxidative properties. Such changes are clinically relevant because they could contribute to the protective effect of anti-TNF therapy on CVD morbidity in RA. Furthermore, our results underline the importance of evaluating HDL

anti-oxidative properties in addition to HDL concentrations, especially in those populations where the predictive value of traditional cardiovascular risk factors is limited.

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Chapter 5

Improved insulin sensitivity by anti-TNFα antibody treatment in patients with rheumatic diseases

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Ann Rheum Dis 2007, 66 558-559

Abstract

Various animal studies suggest a role for endogenous tumor necrosis factor alpha (TNF-α) in the development of insulin resistance This study assessed the effect of infliximab, an anti-TNF-α-antibody with prolonged activity, on insulin sensitivity (IS) in nondiabetic subjects with a rheumatic disease

Methods IS was assessed by hyperinsulinemic euglycemic clamp technique in 8 patients with rheumatic disorders, before the first and third infusion (6 weeks later) of infliximab (3 mg/kg) Simultaneously, blood samples were drawn for determination IL-1β, IL-6 and IL-10 levels, both basal and after 24 hr ex vivo whole-blood stimulation with E coli lipopolysaccharide (LPS) or phosphate buffered saline (PBS) as control

Results The therapeutical blockade of TNF was accompanied by an improvement of IS (p=0 05) The relative changes in IS were negatively correlated with the initial body mass index BMI (R = -0 9, p<0 01) The basal production of IL-6 and IL-1β after incubation with medium was reduced by the therapy with infliximab, whereas the LPS stimulated cytokines remained unchanged

Conclusion TNF-α blockade in patients with chronic inflammatory conditions improved insulin sensitivity assessed by hyperinsulinemic-euglycemic clamp This was accompanied by the reduction of the systemic inflammatory status of these patients

Introduction

Insulin resistance is a key-factor in the pathogenesis of the metabolic syndrome and type 2 diabetes, and yet, the mechanisms responsible for it remain poorly understood Recently, many investigators have reported the presence of a low-grade inflammatory state in obese compared with lean subjects [1] Accordingly, adipocytes were demonstrated to secrete a variety of bioactive molecules, termed adipokines (adipocytokines), including TNF-α, IL-6, leptin and adiponectin [2] These adipokines play pivotal roles in energy homeostasis by affecting insulin sensitivity, glucose and lipid metabolisms, food intake and inflammation Among these molecules, further investigations yielded evidences for important negative effects of TNF-α on insulin-mediated glucose uptake, which eventually led to the development of insulin resistance and type2 diabetes [3] However, TNF-alpha neutralization over a period of 4 weeks failed to improve the insulin sensitivity in obese non-insulin dependent diabetes subjects [4]

Rheumatoid arthritis (RA) and ankylosing spondylitis (AS) are chronic inflammatory diseases and TNF- α is a central mediator involved in the pathogenesis of these diseases Furthermore, the therapeutical blockade of this cytokine is very beneficial in patients with RA as well as in other chronic inflammatory diseases [5,6] In addition, previous studies have indicated that impaired glucose tolerance is more prevalent in patients with RA than in the general population [7], and may contribute to the increased cardiovascular risk seen in these patients Giving these facts, one may expect a change in the glucose homeostasis during therapeutical neutralization of TNF- α in these patients

In the present study we investigated the influence of anti-TNF-α medication on insulin resistance in regularly treated rheumatic patients in an open prospective study, using the euglycemic clamp technique Giving the mounting role of inflammation in the pathogenesis of insulin resistance and metabolic syndrome, the effect of anti-TNF-α therapy on the general inflammatory status was studied simultaneously

Subjects and Methods

Patients

The patients were recruited from the outpatient clinic of the Sint Maartenskliniek Written informed consent was obtained from 8 non-diabetic patients, suffering from rheumatoid arthritis (n=6), disease of Whipple (n=1) and ankylosing spondylitis (n=1) Fasting glucose levels, body-mass index (BMI) and disease activity (DAS28 score) were recorded at both the beginning and

the end of the study. Patient characteristics are depicted in Table 1. Patients started the therapy with TNF-α antibodies (infliximab 3mg/kg) and were followed-up for 6 weeks. During this interval the additional anti-rheumatic medication remained unchanged. None of the patients were following dietary advice neither they changed their degree of physical activity during the study period. Insulin sensitivity was measured using an euglycemic hyperinsulinemic clamp and blood samples for cytokine profile evaluation were drawn prior to the first and third intravenous administration of the TNF-α-antibody. The regional medical ethical committee approved the study.

Hyperinsulinemic-euglycemic clamp

All participants completed two experiments performed 6 weeks apart. Subjects arrived at 8.00 AM after an overnight fast, also abstaining from nicotine. Intravenous catheters were inserted in forearm veins for insulin and glucose infusion in one arm and blood sampling in the contralateral arm. A stepped hyperinsulinemic (60 $mU.m^{-2}.min^{-1}$) euglycemic clamp was initiated thereafter, using 20%glucose infusion to clamp the plasma glucose levels at baseline level, sampled at 5-min intervals. Steady state glucose level was obtained in arterialized blood, using a heated gel pad, after 60 – 90 min in all patients and was maintained at this plateau for 30 min. Plasma glucose was measured in duplicate by the glucose oxidation method (Glucose Analyzer 2; Beckman, Fullerton, CA) in arterialized blood samples and immediately centrifuged for 10 s after withdrawal. M-value was calculated using the average glucose infusion rate during the last 30 minutes, the plateau phase, of the clamp.

Whole blood cytokine production

Venous blood was collected from the cubital vein in 4-ml lithium heparin tubes. Whole blood was stimulated with *E coli* LPS 10ng/ml and phosphate buffer saline (PBS). After incubation for 24h at 37°C, supernatants were obtained by centrifugation and stored at -80°C until assay. Concentrations of TNF-α, IL-6 (Sanquin, Amsterdam, The Netherlands) and IL-1β (R&D Systems, Minneapolis, USA) were measured in the supernatants using commercial ELISA kits.

Statistical methods

Statistical analysis was done using Mann-Whitney U test for unpaired samples and Wilcoxon test for paired samples. For calculations and statistical analysis, the SPSS personal computer software package was used, and $P \leq 0.05$ was considered statistical significant. Results are expressed as mean ± standard error (SE).

Tabel 1 Characteristics of study participants

Patient no	1	2	3	4	5	6	7	8	Mean ± SEM
Age (years)	65	38	70	57	54	34	**61**	**43**	**53 ± 5**
Gender	M	M	M	M	M	F	**M**	**M**	**M:F = 7:1**
Diagnosis	RA	RA	RA	RA	Wd	RA	**AS**	**AS**	
Disease duration (years)	7	4	14	10	3	8	**26**	**22**	
RF	+	-	+	+	n a	+	**n.a.**	**n.a.**	
DAS28 I	5 26	4 28	7 65	3 75	4 25	3 00	**n.a.**	**n.a.**	**4.7 ± 0.7**
DAS28 II	2 75	1 09	5 40	2 68	4 43	2 25	**n.a.**	**n.a.**	**3.1 ± 0.6**
ESR I (mm/h)	36	14	43	37	38	10	**67**	**14**	**32 ± 7**
ESR II (mm/h)	26	3	25	18	56	6	**15**	**5**	**19 ± 6**
Prednisone	yes	no	yes	no	yes	yes	**no**	**yes**	
DM2 in family	no	yes	no	no	no	yes	**no**	**yes**	
Glucose (mmol/l)	7 5	5 2	4 9	4 3	4 6	5 7	**4.9**	**4.8**	**5.2 ± 0.4**
BMI (kg/m^2)	27 0	25 0	24 3	22 0	28 1	22 4	**26.0**	**24.5**	**24.6 ± 0.7**
ΔM(mg kg^{-1} min^{-1})	0 12	2 79	1 33	4 28	0 06	3 46	**-1.13**	**0.17**	**0.75(5.41)**

M = male; **F** = female; **RA** = rheumatoid arthritis; **Wd** = Whippel's disease; **AS** = ankylosing spondylitis; **RF** = rheumatoid factor; **DAS28 I /II** = disease activity score at baseline **(I)** and after 6 weeks **(II)**; **ESR I/II** = erythrocytes sedimentation rate at baseline **(I)** and after 6 weeks **(II)**; **DM2** = type2 diabetes mellitus; **BMI** = body mass index; **ΔM** = variation of insulin sensitivity, as assessed by M-value

Results

Insulin sensitivity assessment

All subjects were non-diabetic (normal glucose levels and normal M-value), suffering from a rheumatic inflammatory disease. Fasting glucose concentrations were 5.3 ± 1.0 mmol/l at baseline and did not change throughout the study: 5.3 ± 0.3 mmol/l after 6 weeks of therapy. The M-values improved in 7 subjects (Fig 1A). This improvement was not correlated with changes of ESR, TNF-α, IL-1β and IL-6 levels. However, the relative changes in M-values were negatively correlated with the BMI (Fig 1B, $R = -0.83$, $p < 0.01$).

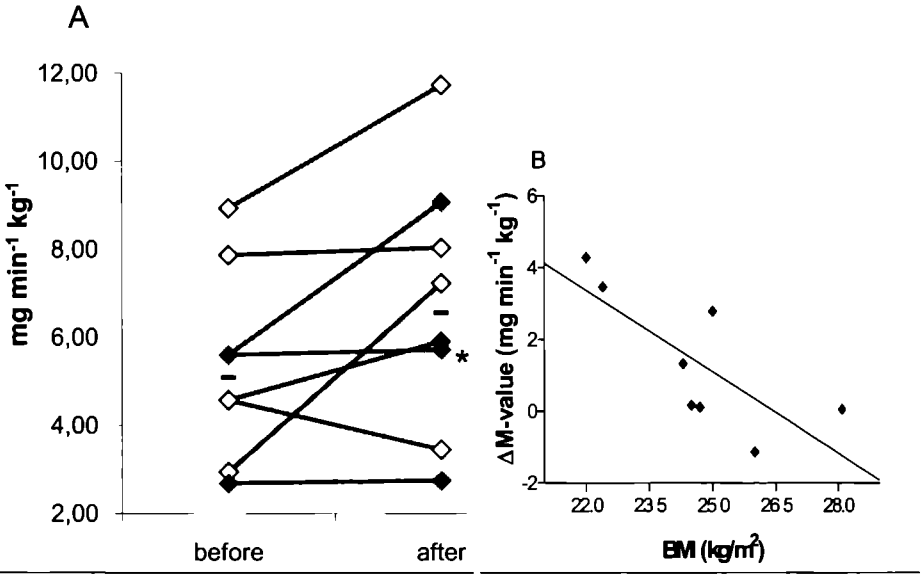

Figure 1. [A] Effects of anti-TNF therapy on insulin sensitivity M-values were calculated as the average glucose infusion rate during the last 30 minutes, the plateau phase, of the clamp The lowest limit of normal values interval was depicted from previous studies of our group in healthy volunteers [7,8] and corresponds to 5,80. Black lines represent the median, while the dotted line represents the lowest normal limit. Darker diamonds represent patients on concomitant oral corticosteroids, (Wilcoxon test was used *$p < 0.05$); [B] Relationship between BMI at baseline and the changing in insulin sensitivity after therapeutic TNF-α blockade. ΔM = variation of M-value between the two time points investigated (Spearman's test was used).

General inflammatory status

Clinical and inflammatory markers, as assessed by the DAS28 score and ESR, improved significantly during the infliximab therapy in all patients, except one that later appeared having Whipple disease (Table 1). To have a better insight of the systemic inflammatory status, we sought to investigate the capacity of whole blood cells to produce proinflammatory cytokines under various conditions. Interestingly, the basal production of IL-1β (207 ± 67 pg/ml vs. 69 ± 36 pg/ml, p = 0.09) and IL-6 (6809 ± 3025 pg/ml vs. 580 ± 346 pg/ml, p = 0.06) from whole blood cultures incubated with PBS decreased after 6 weeks of TNF-α blockade, although it narrowly failed to reach statistical significance (Fig 2A and 2B). Incubation of the whole blood

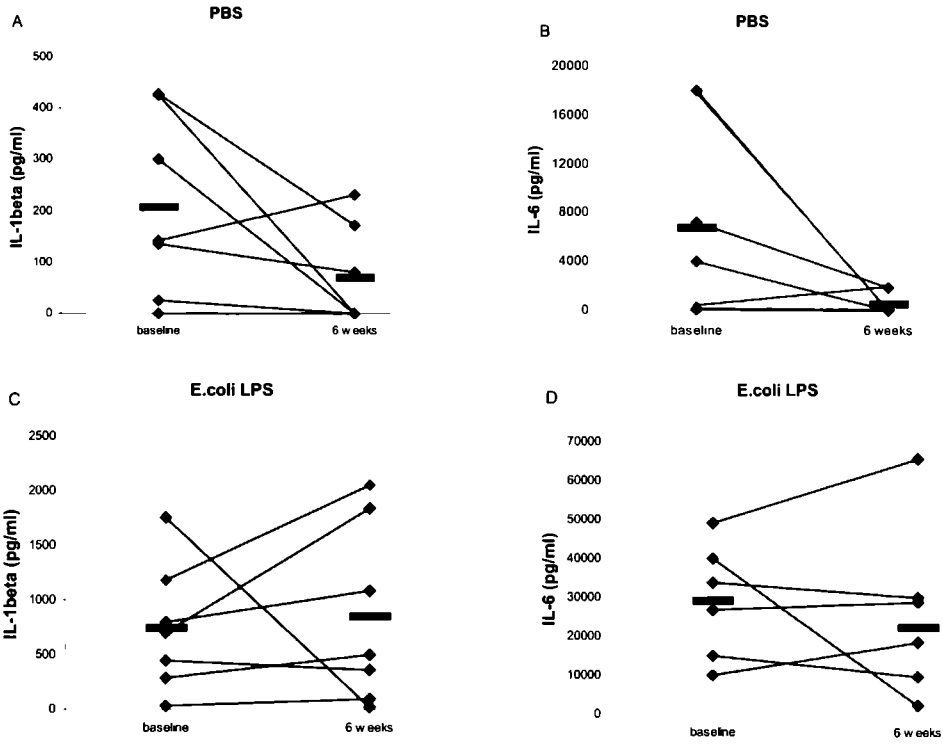

Figure 2. Basal (PBS) and stimulated (LPS) interleukin-1 [A,C] and interleukin-6 [B,D] production from whole-blood cultures of anti-TNF treated patients before and 6 weeks after the start of therapy. Black lines represent the means.

with LPS resulted in stimulation of pro-inflammatory cytokine production as assessed by the measurements of IL-1β, IL-6 and TNF-α in the supernatants. Short-course anti-TNF therapy did not significantly influence these parameters, although a tendency towards higher cytokine productions could be noticed: 742 ± 219 pg/ml vs. 852 ± 312 pg/ml for IL-1β and 29098 ± 6112 pg/ml vs. 29532 ± 7152 pg/ml for IL-6 (Fig 2C and 2D). Giving the higher probability of TNF-α/anti-TNF-α complexes, that are formed after the start of therapy, to interfere with our ELISA assay, we could not provide the data regarding TNF-α concentrations in the samples collected 6 weeks after the start of anti-TNF treatment.

Discussion

In the present study we report an improvement of insulin sensitivity after a short-course therapeutical blockade of TNF-α in a small group of patients with rheumatoid diseases. To our knowledge, this is the first study to use hyperinsulinemic-euglycemic clamp to assess the effect of anti-TNF agents on insulin resistance. Our findings were accompanied by an important attenuation of the disease activity together with the decrease of systemic inflammatory status of these patients.

Persistent metabolic disturbances can lead to disturbances in the normal body homeostasis, including the activation of an inflammatory response. In line with this, the role of inflammation, and particularly TNF-α, in the pathogenic cascade of insulin resistance was recently shown to be central. RA inflammation is characterized by increased circulating levels of TNF-α, which besides the joints are likely to exert generalized effects, which affect skeletal muscle tissue and adipose tissue. We therefore hypothesized that in patients with RA persistently increased plasmatic TNF-α concentrations represents the main mechanism responsible for the decrease in insulin sensitivity reported in these patients.

The insulin sensitivity in our group of patients was within the normal range. Other studies have previously reported a decreased insulin resistance in RA patients with active disease [9]. However, during the therapy with infliximab the insulin sensitivity in our group of patients improved significantly. This is in line with previous reports that hypothesized a beneficial role of the anti-TNF agents in correcting the possible disturbances in glucose metabolism in patients with RA [10-12].

Because none of the patients in our study group has followed dietary advices during the study period [13], and giving the central role of inflammation and TNF-α in the regulation of insulin sensitivity, the improvement of insulin sensitivity observed in our study is likely to be due to TNF-α neutralisation Indeed, TNF-α neutralisation was accompanied by a significantly decrease of DAS28 and ESR after 6 weeks therapy with infliximab This was further translated into decreased spontaneous production of pro-inflammatory cytokine from whole-blood cultures However, no significant correlation between disease activity or other inflammatory markers and insulin sensitivity at both time points could be detected

In our study, the improvement in insulin sensitivity correlated negatively with the BMI, which is line with other studies [12], although not all the studies reported the same relation [10] Possibly, the smaller BMI reflects a more chronic catabolic state due to more severe chronic inflammation in the subjects that were resistant to conventional antirheumatic agents, and therefore selected for a therapy with biologicals In contrast to healthy individuals, we previously reported a negative correlation between leptin and inflammatory markers in a group of RA patients who were about to start anti-TNF therapy [14] These results, corroborated with those from the present study, suggest that the inflammatory status rather than obesity has a strong influence on metabolic processes in patients with RA and might be a strong determinant of insulin sensitivity in these patients

Limitations of the present study include the small number of patients and the relative short follow-up period, and larger studies are warranted

In conclusion, the results of our study suggest that, besides the reduction of the inflammatory status, TNF-α blockade in patients with chronic inflammatory conditions is able to improve insulin sensitivity assessed by hyperinsulinemic-euglycemic clamp This may further contribute to a decrease in the cardiovascular risk in RA

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Chapter 6

Markers of inflammation are negatively correlated with serum leptin in rheumatoid arthritis

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Abstract

Objective: Leptin regulates food-intake and plays a modulator role in immunity and inflammation. A positive feed-back mechanism has been described between tumor necrosis factor (TNF) and leptin, and leptin has been suggested to potentiate inflammation in RA patients. The aim of this study was to assess whether inflammation correlates with leptin concentrations in patients with RA, and whether anti-TNF treatment modulates leptin concentrations in these patients.

Methods: Leptin, IL-6 and CRP were measured in the blood of 31 RA patients starting either anti-TNF therapy or placebo (at baseline and after 2 weeks of therapy) and in 18 healthy controls.

Results: In RA patients, plasma leptin concentrations at baseline were inversely correlated with the degree of inflammation as assessed by CRP ($r^2 = 0.21$, $p<0.01$), or IL-6 concentrations ($r^2 = 0.22$, $p<0.008$). Leptin concentrations did not differ between RA patients and controls (5.97 + 4.55 vs. 4.22 + 2.77 ng/mL in males; 15.05 + 7.91 vs. 13.37 + 5.16 ng/mL in females). Short-course anti-TNF therapy for 2 weeks did not modify leptin concentrations, despite significant reduction of CRP and IL-6.

Conclusion: A significant inverse correlation between inflammation and leptin concentrations was observed in active RA patients, although plasma leptin concentrations did not significantly differ from that in healthy controls. This suggests that active chronic inflammation may lower plasma leptin concentrations. A short 2-weeks treatment with anti-TNF did not influence plasma leptin concentrations and longer therapy may be needed to see an effect on leptin.

Background

Leptin was initially described as a hormone that regulates food intake and energy balance (1) Later, it became apparent that leptin has an important role in regulating neuroendocrine and immune functions Leptin and its receptors (OB-R) share structural and functional similarities with cytokines of the interleukin-6 family and their receptors (2) During acute inflammations, proinflammatory cytokines increase circulating leptin concentrations (3) and leptin, in turn, potentiates cytokine release from monocytes/macrophages (4) In addition, leptin stimulates T-cell mediated immunity and is able to induce the proliferation and differentiation of hemopoietic cells (3) The involvement of leptin in regulating immune functions in humans is strongly sustained by the increased incidence of severe infections in subjects with genetic leptin deficiency (5) and by the deficiencies of the immune system during starvation and malnutrition, when concentrations of leptin are low (3)

Rheumatoid arthritis (RA) is a chronic inflammatory condition characterized by polyarthritis and high concentrations of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8 and IFN-γ especially in the synovial fluid, but also in the circulation A dual effect of inflammation on leptin production has been suggested On the one hand, a positive feed-back between leptin and proinflammatory cytokines has been reported (4), and immunized leptin-deficient mice (ob/ob) were shown to develop less severe arthritis than control mice (6) Recently, the relation between leptin and arthritis was further sustained by studies showing that human chondrocytes express the leptin receptor OB-Rb and, when acting together with IFN-γ, leptin was able to stimulate nitric oxide (NO) production in the joint cavity (7) This suggests a possible direct implication of leptin in the pathogenesis of RA On the other hand, studies assessing leptin concentrations in RA patients have provided controversial results (8-12) In addition, chronic inflammation has been suggested to downmodulate leptin production, which in turn may lead to an impaired antimicrobial defense (13)

The aim of our study was to investigate circulating leptin concentrations in a group of RA patients and to assess whether leptin concentrations correlate with systemic inflammation In addition, we were also interested to find out whether anti-TNF therapy modulates plasma leptin concentrations, as TNF has been shown to directly stimulate leptin production

Patients and Methods

Patients and controls

We analyzed samples from 31 patients (mean age 61, M:F = 11:20) with active RA included in phase I, double blind placebo controlled clinical study with the humanised anti-TNF antibody adalimumab (Humira, Abbott Laboratories) monotherapy in our center. Patients fulfilled the 1987 ACR criteria for RA, had an active disease as defined by a disease activity score (DAS) > 3.2 at baseline and underwent a washout period for DMARDs of at least 3 weeks prior to study initiation. Stable dosages of NSAIDs and prednisone (< 10 mg/day) were allowed during the study. 18 healthy controls (mean age 38.4, M:F = 9:9) were also included in this study. Due to the existing gender differences on leptin concentrations in humans and because sex ratio was not similar in patients (M:F = 1:1.8) and controls groups (M:F = 1:1), we divided each group according to gender and compare them thereafter. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2) and was used to better compare leptin concentrations between groups.

To investigate the effect of TNF blockade on circulating leptin concentrations, we compared the samples from 23 RA patients treated with anti-TNF and 8 RA patients receiving placebo at baseline and after 2 weeks of therapy.

Leptin, IL-6 and CRP

Fasting blood samples were collected in vacutainer tubes (Becton & Dickinson, Rutherford, NJ) containing K3-EDTA (1 mg/ml), centrifuged at 3600 rpm for 8 min at 4 °C, supplemented with saccharose as a cryoprotectant (final concentration 6 mg/ml) and frozen at -80°C until assay. Leptin and IL-6 were determined using a commercial ELISA (Biosource, Etten-Leur, The Netherlands, detection limit 32pg/mL, respectively 1,5pg/mL), according to the instructions of the manufacturer. CRP was measured by immunoturbidometry with the Hitachi 747 analyzer using reagents of Roche (#1776371 and #1776428) and the calibrator #BCD1. Sensitivity level was 1mg/L and CV was <2%.

Statistical analysis

Groups were compared using Mann-Whitney non-parametric U test and Student T-test for parametric data. Paired group comparisons during therapy were made using the Wilcoxon signed rank test. Correlation was calculated using Spearman non-parametric test. The relationship between plasma leptin concentration and CRP/IL-6 was analyzed by linear regression. Results are expressed as means $\pm$ standard deviation (SD). The significance level was set at 0.05.

Results

Circulating leptin concentrations do not differ between RA patients and healthy controls

To compare our groups of RA patients and controls regarding leptin concentrations, we divided each group according to gender. We found no significant differences in circulating leptin concentrations between RA patients and controls both in males (5.97 ± 4.55 ng/mL and 4.22 ± 2.77 ng/mL) and females (15 05 ± 7.91 ng/mL and 13.37 ± 5.16 ng/mL) (figure 1). Similar results were obtained when comparing leptin concentrations adjusted to BMI: 0.23 ± 0.14 ng/mL vs 0.17 ± 0.1 ng/mL in males and 0.65 ± 0.29 ng/mL vs 0.58 ± 0.17 ng/mL in females. However, BMI was significantly higher in RA patients then in controls both in males (25.5 ± 2 88 kg/m² and 23.12 ± 2.35 kg/m², $p < 0.032$) and females (26.19 ± 4.89 kg/m² and 22.4 ± 2.56 kg/m², $p < 0.041$).

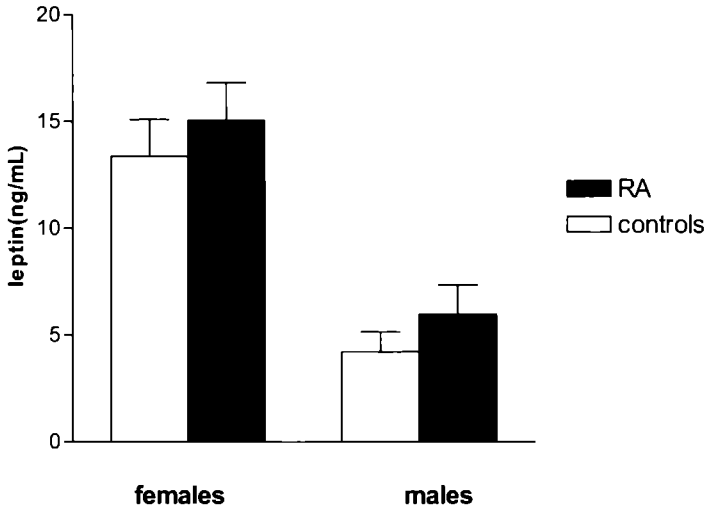

Figure 1 Circulating leptin concentration in RA patients (n=31) and controls (n=18) No significant differences could be found for males as well as for females

Inflammation is negatively correlated with plasma leptin concentration.

Leptin concentrations in plasma are mainly regulated by the body fat mass (BF%), and correlate with it and with BMI in healthy individuals (21). In our study, circulating leptin concentrations correlated with BMI in healthy males ($r = 0.88$ and $p = 0.003$) and females ($r = 0.70$ and $p = 0.043$). Interestingly, no such correlation was observed in RA males ($r = 0.17$, p = N.S.) or females ($r = 0.15$ p = N.S.) before starting anti-TNF therapy.

Besides the body fat mass, inflammation is also known to be involved in regulating plasma leptin concentrations. In RA group, we measured CRP and IL-6 as markers of inflammation and found them to be higher than normal. To analyze the relation between plasma leptin concentration and CRP/IL-6, we used the linear regression test. In RA patients, before starting anti-TNF therapy, plasma concentrations of leptin inversely correlated with CRP (figure 2A) and IL-6 levels (figure 2B). The same results were obtained after adjusting leptin concentrations to BMI: $r^2 = 0.22$, $p<0.008$ when correlated with CRP concentrations, and $r^2 = 0.20$, $p<0.01$ when correlated with IL-6 concentrations at baseline.

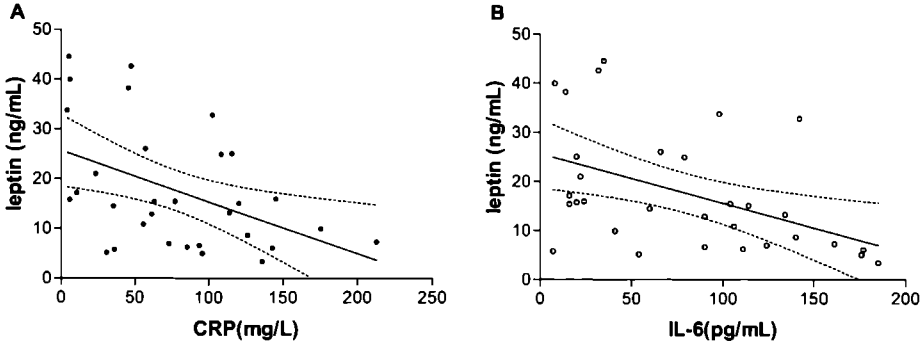

Figure 2. Circulating leptin concentrations in RA patients (n=31) are negatively correlated with CRP ($r^2 = 0.21$, $p<0.01$) (A) and IL-6 ($r^2 = 0.22$, $p<0.008$) (B). Linear regression test was used.

The duration of the disease in our RA group varied between 3 and 26 years, with an average of 11 years, and it was not associated with serum leptin concentrations We did not find also any relations between the previous use of DMARDs and serum leptin concentrations at baseline

Effects of anti-TNF treatment on inflammatory markers and plasma leptin concentrations

As already reported in our previous studies (14), the levels of CRP and IL-6 decreased significantly within 2 weeks of anti-TNF administration, whereas no changes were observed after placebo (Table 1)

Table 1 Evaluation of the inflammatory status and plasma leptin concentrations in RA patients 2 weeks after initiation of the anti TNF therapy (n = 23) or placebo (n = 8)

	week 0	week 2	*P* value
Leptin (ng/mL)			
male (n=10)	6 21 ± 5 00	5 67 ± 3 48	n s
female (n=13)	15 35 ± 8 65	16 71 ± 9 89	n s
C-reactive protein (mg/mL)			
anti-TNF	86 11 ± 54,42	35 42 ± 35 55	<0,0001
placebo	53 72 ± 49 22	53 91 ± 50 0	n s
Interleukin-6 (pg/mL)			
anti-TNF	88 32 ± 60 53	42 31 ± 40 72	<0 001
placebo	60 00 ± 59 64	60 83 ± 60 21	n s

After 2 weeks of anti-TNF treatment, plasma leptin concentrations in RA patients were similar to those at baseline both in males and females (Table 1) Moreover, no significant differences were seen in placebo-treated group

Discussion

Inflammatory mediators, such as cytokines TNF-α and IL-1β, decrease energy intake and may lead to the wasting described in RA patients Wasting, in turn, affects the inflammatory response and may suppress cellular immunity In this complex relationship, leptin is a possible mediator In this study, we show that in RA patients, both circulating leptin concentrations and leptin adjusted to BMI, inversely correlated with the inflammatory status of the patients, as assessed by the inflammatory markers CRP and IL-6 These results are supported by the observations that long-term in-vitro stimulation of adipose tissue by TNF-α

or IL-1β leads to inhibition of leptin and leptin mRNA production (15). Similarly, in patients suffering from tuberculosis, another chronic inflammatory condition, inflammation negatively correlates with leptin concentration (13). In RA patients, plasma leptin concentrations were not correlated with BMI, suggesting that leptinemia of RA is under a complex regulation, and weight is not the only major regulator of leptin concentrations in the blood of these patients. These facts led us to hypothesise that in RA chronic inflammation, probably through proinflammatory cytokines (e.g. TNF, IL-1, IL-6), is an important determinant of plasma leptin concentration and has an inhibitory effect on leptin production.

In addition, we report that plasma leptin concentrations in RA patients do not differ from those observed in healthy controls. This is in line with two earlier studies (8,9). In contrast, Bokarewa et al found higher plasma leptin concentrations in a group of RA patients (11). Theoretically, one could expect increased leptin concentration due to the proinflammatory status of RA and to the stimulatory activity of TNF-α and IL-1β on leptin release (3,15). Similarly, patients with sepsis and major surgery, two situations also characterised by increased TNF-α and IL-1β concentrations, exhibit elevated serum leptin concentrations (16). However, as shown above, the chronic inflammation in RA patients had inhibitory effects on leptin concentrations in the blood, in contrast with the acute inflammation of sepsis and surgery. Recently, Harle et al found almost three times lower serum leptin concentrations in a group of RA women than in the healthy women group (12). In addition, the body compartment in which leptin is measured may be of importance. Whereas blood concentrations did not differ significantly between RA patients and controls, concentrations in the synovial fluid may be of importance (7,11).

The lack of difference between plasma leptin concentrations in RA patients and healthy controls may seem in contrast with the inverse correlation of leptin and inflammation in these patients, which would have predicted lower leptin concentration in RA patients. The cause of this discrepancy is likely due to a combination of factors: a significant percentage of the RA patients did not display very high inflammatory parameters at the time of investigation; the BMI of the RA patients in our group was slightly higher than that of control volunteers; and some of the inhibitory effects of chronic inflammation might have been counterbalanced by potential stimulatory actions of acute inflammatory reactions during RA exacerbations.

The duration of the disease was also evaluated in our study in respect to plasma leptin concentrations, but no direct relation between these two parameters was found These results are in line with those of Anders et al (8), while Bokarewa et al (11) showed a gradually increase of leptin concentrations with the duration of RA

To explain our results better we evaluated the influence of previous therapy with DMARDs on serum leptin concentration in our RA group We were unable to establish a relation between leptin concentrations at baseline and this therapy, no matter the type of medication or the dosage that were used Bokarewa et al found higher leptin concentrations in the methotrexate treated RA group than in the group receiving other DMARDs, but in the same time, these concentrations were similar with those found in the group that was not treated with any DMARDs Consecutively, there was no difference in serum leptin concentrations between RA patients treated and not treated with glucocorticoids (11) Sulphasalazine was also proved to have no influence on leptin release from adipose tissue and skeletal muscle (17) The above-mentioned studies point out to a lack of a specific influence of one DMARD to serum leptin concentrations Moreover, a washout period for DMARDs was performed on every patients included in our study 3 weeks prior the entry, thus prior to the time that blood was collected for leptin determination

Leptin is known to have stimulatory effects on T-cell mediated immunity In the case of septic shock, mortality is associated with decreased plasma leptin levels (18), while genetic leptin deficiencies increase the severity of infections in humans (5) In addition, severe infections have been reported to occur more often in RA patients than in the general population (19) especially in patients receiving anti-TNF drugs (20) These data suggest that suppression of leptin concentration by chronic inflammation may contribute to the susceptibility of RA patients to infections

An additional aim of our study was to investigate whether a short-course of anti-TNF therapy can influence leptin concentrations To date, no study investigated the effect of in-vivo TNF-α blockade upon circulating leptin levels We found that a short course of anti-TNF therapy for 2 weeks did not change plasma leptin concentrations, despite decreasing of the acute phase reactants Therefore, a short course therapy with anti-TNF does not modulate leptin concentrations in RA patients, and studies investigating long-term treatment effect on leptin concentration are warranted

In conclusion, our study reveals that circulating leptin concentrations are inversely correlated with the inflammatory status in patients with RA. We suggest that in RA, chronic inflammation down-regulates leptin production, which may indirectly contribute to the susceptibility to infections seen in these patients. The precise role of leptin in RA remains uncertain, but it is possible that local actions, through synovial leptin, are involved in the pathogenesis of the disease, while decreased circulating leptin contribute to impaired host defense to infections. A short course of anti-TNF therapy does not modify plasma leptin concentrations and therefore longer follow-up studies are needed to further assess this issue.

Competing interests: none declared.

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Chapter 7

Circulating leptin and adiponectin concentrations during TNF blockade in patients with active rheumatoid arthritis

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Abstract

Adipocytokines, including leptin and adiponectin, may play an important role in the pathogenesis of rheumatoid arthritis (RA) The effects of long-term therapeutic TNF blockade on adipocytokines levels in patients with RA are poorly investigated

Methods 58 RA patients starting anti-TNF therapy and 58 healthy controls matched for age, gender and BMI were investigated Fasting blood samples were drawn at baseline, 2 weeks and 6 months after the start of anti-TNF therapy and serum levels of leptin and adiponectin were measured

Results Patients with RA had increased adiponectin ($p < 0\ 001$) and similar leptin concentrations compared with the group of healthy volunteers Leptin concentrations were significantly higher in patients with high BMI ($p < 0\ 001$) and positively correlated with BMI at all time-points ($r > 0\ 75$) In contrast, serum adiponectin tended to be higher in lean RA patients and did not relate with BMI at any time point There were no clear correlations between serum concentrations of adipocytokines and disease activity (DAS28) Short- or long-term TNF blockade alone had no influence on circulating leptin and adiponectin concentrations Patients treated with anti-TNF and concomitant corticosteroids on a stable dosis showed a significant decrease in adiponectin levels after 6 months of therapy ($p < 0\ 025$)

Conclusion In RA patients, chronic inflammation and its suppression during anti-TNF therapy have limited influence on plasma leptin concentrations, while significantly decreasing circulating adiponectin levels in patients receiving concomitant corticosteroids Our findings question the suggested key role of inflammation in regulating adipocytokine pattern in RA

Introduction

Leptin and adiponectin are two adipocyte-derived hormones, which play a central role in the homeostasis of the energy and glucose metabolism, respectively [1] Leptin was initially described as a hormone that regulates food intake and energy balance [2] Later studies have shown that leptin also stimulates the T-cell mediated immunity, cytokine release from monocytes/macrophages and the differentiation of haematopoietic cells [3,4]. The role of leptin as immunomodulator in humans is strongly sustained by the increased incidence of severe infections in subjects with genetic leptin insufficiency [5] and by the immune system deficiency during starvation and malnutrition, when leptin concentrations are low [3] Adiponectin is also synthesized by adipocytes and one of its main actions is to improve insulin sensitivity [6] Serum levels of adiponectin are markedly decreased in individuals with visceral obesity and states of insulin resistance [7] Both hormones are also regulated by central mechanisms through hypothalamus [8] Like leptin, adiponectin can also modulate inflammatory processes Earlier studies have indicated that adiponectin has anti-inflammatory effects, through the inhibition of NF-kB activation in endothelial cells and macrophages [9], inhibition of tumor necrosis factor (TNF) production and phagocytic activity of macrophages [10], and by inducing the production of anti-inflammatory cytokines interleukin (IL)-10 and IL-1 receptor antagonist (IL-1RA) by human monocytes macrophages and dendritic cells [11] Nevertheless, certain situations in which adiponectin might have pro-inflammatory actions have been recently reported Accordingly, adiponectin can increase IL-6 production from endothelial cells, monocytic cells, and respectively from synovial fibroblasts [12] These effects are likely to be strongly related to its different molecular species, with low-molecular weight adiponectin being anti-inflammatory, whereas high-molecular weight and globular adiponectin pro-inflammatory [13]

Recently, *in vitro* [4;12] and *in vivo* [14-16] studies have suggested that leptin and adiponectin may play a role in the pathogenesis of RA. In addition, they may also interfere with atherosclerosis [17], which develops more frequent in RA compared to the general population Nonetheless so far, the studies questioning the roles of adipokines in RA have had mainly a cross-sectional character [14,16,18-20] and little is known about how these hormones behave during the course of the disease or about the effects of therapy with anti-rheumatic agents, i e anti-TNF drugs, on the homeostasis of leptin and adiponectin [21,22] Given these facts, our study aimed to investigate potential relations between circulating leptin and adiponectin concentrations and RA disease activity and body weight in a prospective manner. In addition, since TNF is an important determinant of the production of leptin and

adiponectin, we investigated whether long-term TNF neutralisation therapy modulates the circulating concentrations of these adipokines

Patients and Methods

Patients and controls

58 consecutive patients with active RA and the same number of age, gender and BMI matched healthy controls have been enrolled in this study All patients were attending the Sint Maartenskliniek in Nijmegen, The Netherlands, and were about to start with TNF neutralizing therapy with infliximab Patients had failed to at least 2 DMARDs before starting anti-TNF All patients fulfilled the ACR criteria, had given written informed consent, and had an active disease as defined by an disease activity score (DAS28) > 3 2 Patients on therapy with lipid lowering drugs were excluded from the study because this medication may interfere with several adipocytokines activities Infliximab at a dose of 3mg/kg was administered at baseline, at 2 and 6 weeks, and thereafter every 8 weeks Data for this study were collected before, 2 weeks and 6 months after the start of therapy Stable dosages of DMARDs and concomitant prednison (< 10 mg/day, n = 11) were allowed during the study There were no other DMARDs then methotrexate used, except for two patients, who took salazopyrine Disease activity was measured using the DAS28 score [23] before each infliximab infusion Demographic and disease characteristics were recorded at baseline and the body-mass index (BMI) was determined at each visit According to their BMI, patients were classified as lean or normal weight (BMI < 25), overweight (BMI = 25-30) and obese (BMI > 30) The Regional Medical Ethical Committee approved the study

Laboratory measurements

Fasting blood samples were collected before each administration of infliximab using in vacutainer tubes (Beckton & Dickinson, Rutherford, NJ) containing K3-EDTA (1 mg/ml) Blood was centrifuged at 3600 rpm for 8 min at 4 °C, supplemented with saccharose as a cryoprotectant (final concentration 6 mg/ml) and frozen at -80°C until assay Serum levels of leptin and adiponectin were measured using commercial ELISA kits (R&D, Minneapolis MN) according to the instructions of the manufacturer

Statistical analysis

For non-parametric values within group, comparisons were made using the paired Wilcoxon signed rank test, while the paired Student's t-test was use in the case the values were normally

distributed. Correlations between inflammatory status markers and adipocytokines levels were determined using Spearman test Friedman's non-parametric test for more than two related samples was used to test for changes in adipocytokine concentrations from baseline during the follow-up period. Significance was set at the level of 0.05. Values are expressed as mean ± standard deviation (SD), unless otherwise stated.

Table 1. The characteristics of control group and patients with RA at baseline

	Rheumatoid arthritis (N=58)	**Controls (N=58)**	**p-value**
General data			
Age (years)	56 ± 11	55 ± 13	N.S.
Gender (M/F)	16/40	16/40	N.S.
Disease duration (years)	9 ± 7	-	-
Rheumatoid factor (+)	64%	-	-
DAS28 - baseline	5.26 ± 1.25	-	-
- 2 weeks	3.86 ± 1.32	-	P<0.001*
- 6 months	4.14 ±1.22	-	P<0.001*
ESR (mm/h) - baseline	26(4;85)	-	
- 2 weeks	16(4;87)	-	P<0.0001*
- 6 months	23(5;73)	-	P<0.02*
BMI (kg/m^2) - baseline	25.7 ± 5.5	25.5 ± 5.1	N.S.
- 2 weeks	25.6 ± 5.4	-	N.S.*
- 6 months	25.7 ± 5.5	-	N.S.*
Medication			
Oral steroids (N)	11	-	-
Methotrexate (N)	27	-	-

DAS28 = disease activity score; BMI = body mass index; N.S. = not significant compared to controls; * = compared to baseline levels; data are expressed as mean ± SD, except for ESR which is expressed as median(min;max).

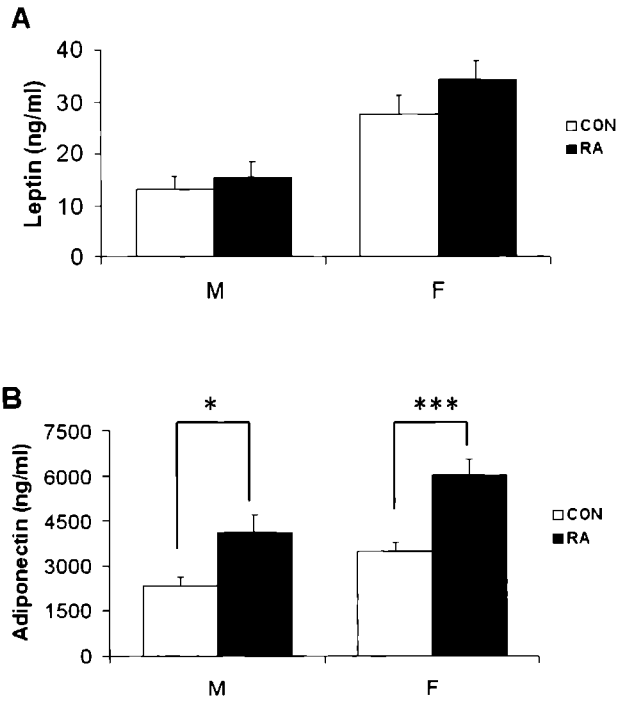

Figure 1. Circulating levels of leptin (A) and adiponectin (B) in RA patients (N=58) and healthy controls (N=58). No differences were found in leptin concentrations, while adiponectin was increased in both men and women with RA. Error bars represent SEM. (*p<0.05, ***p<0.0001)

Results

Characteristics of the patients at baseline and changes in disease activity

The baseline characteristics of RA patients and healthy controls are presented in Table 1. As shown, patients with RA had almost twofold higher circulating adiponectin concentrations than healthy controls: 4116 ± 598 ng/ml vs. 2352 ± 266 ng/ml for men (p<0.02) and 6017 ± 524

ng/ml vs 3487 ± 298 ng/ml for women (p<0 0001) (fig1A), whereas leptin concentrations did not differ between RA patients and controls (fig1B) As expected, the DAS28 and ESR were high in RA patients at baseline and decreased already after the first infliximab infusion, remaining low throughout the entire follow-up period (p<0 001) We tested whether disease activity and markers of inflammatory status relate to adipocytokines concentrations in our group of RA patients We found no correlation of adiponectin, leptin and leptin adjusted to BMI with DAS28 and ESR, even after correction for patients' gender between DAS28 and adiponectin r = 0 19 (p = 0 26), leptin r = -0 02 (p = 0 88), leptin/BMI r = -0 30 (p = 0 11) and respectively between ESR and adiponectin r = 0 21 (p = 0 19), leptin r = -0 06 (p = 0 68), leptin/BMI r = -0 18 (p = 0 36) Nevertheless, a sub-analysis performed in women with active disease (DAS28 > 3 2) revealed a trend towards an inverse relation between leptin and leptin/BMI on one hand, and DAS28 and ESR on the other hand, at the majority of time-points studied (Table2) Of note, no relation between leptin concentrations at baseline and treatment with methotrexate or glucocorticoids could be established

Table 2. Correlations between leptin and leptin adjusted to BMI levels and DAS28 and ESR in women with active disease (DAS28>3 2)

	DAS28			ESR (mm/h)		
	baseline	**2 weeks**	**6 months**	**baseline**	**2 weeks**	**6 months**
Leptin	r = -0 16	r = -0 33	r = -0 32	r = -0 39	r = 0 56	r = 0 48
	(p = 0.41)	(p = 0 15)	(p = 0 15)	(p = 0 03)*	(p = 0 004)*	(p = 0 02)*
Leptin/BMI	r = -0 21	r = -0 37	r = -0 20	r = -0 30	r = -0 43	r = 0 32
	(p = 0 27)	(p = 0 12)	(p = 0 37)	(p = 0 11)	(p = 0 04)*	(p = 0 14)

r = correlation coefficient (Spearman test was used),* = correlations are statistically relevant

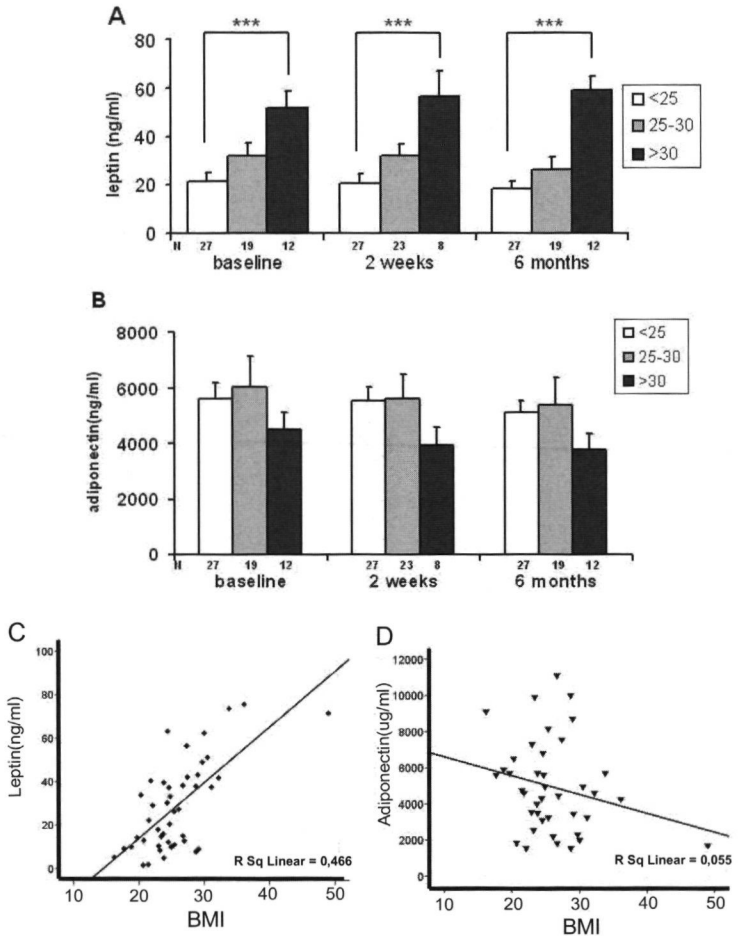

Figure 2. Circulating leptin concentrations are elevated in obese RA patients compared to lean RA individuals (A). Serum leptin is strongly correlated with BMI at all time points, including 6 months after the start of anti-TNF therapy (C). Circulating adiponectin tends to be decreased in obese RA patients (B) but shows no relation with BMI at any of the time-points studied, including 6 months after the start of TNF blockade (D). White bars represent patients with BMI < 25 kg/m^2, gray bars patients with BMI = 25-30 kg/m^2, while black bars patients with BMI > 30 kg/m^2. Data are presented as means ± SEM.(***$p<0.001$)

Influence of BMI on adipocytokines levels

We tested the relations between BMI and adipocytokines concentrations measured at several time-points during the study Using the BMI classification, 59% of our patients had normal weight, 24% overweight and 17% were obese There was no variation in these percentages throughout the follow-up We found that leptin concentrations were significantly higher in obese patients compared with normal weight RA patients, at all time-points (fig2A) Although leptin concentrations were higher in women, the proportion of women and men was similar in all BMI subgroups, and therefore the gender cannot explain these results In addition, in the whole RA group leptin serum concentrations positively correlated with BMI at all time-points ($r > 0\ 62$, $p < 0\ 001$) (fig2C) This relation was even more consistent when evaluated separately for men and women $r > 0\ 74$ ($p < 0\ 002$) and $r > 0\ 78$ ($p < 0\ 0001$), respectively Leptin positively correlated with BMI also in the healthy controls group ($r = 0\ 75$, $p < 0\ 0001$), both in men ($r = 0\ 69$, $p < 0\ 006$) and women ($r = 0\ 89$, $p < 0\ 0001$) In contrast to leptin, serum adiponectin levels were not significantly increased in lean RA patients compared with obese patients (fig2B) In addition, in RA patients there was no correlation between adiponectin levels and BMI at any time point studied (fig2D), whereas a negative correlation was observed in healthy controls group ($r = -0\ 54$, $p < 0\ 0001$)

Serum adipocytokines levels during TNF blockade

Anti-TNF therapy was initiated in these patients and the short-term as well as long-term effects on serum adipocytokines concentrations were assessed after 2 weeks and 6 months of medication, respectively Serum leptin concentrations were not modified throughout the entire follow-up period (fig3A), both in men and women (Table3) Adiponectin serum concentrations significantly dropped after 6 months of anti-TNF therapy (fig3B) Further analysis showed that this was only the case among patients with concomitant corticosteroid therapy ($n = 11$) and not in the rest (fig3B) In contrast, leptin and leptin/BMI ratio were not affected by concurrent corticosteroid therapy (fig 3A) Of note, no association between baseline levels of adipocytokines and the response to anti-TNF therapy could be found

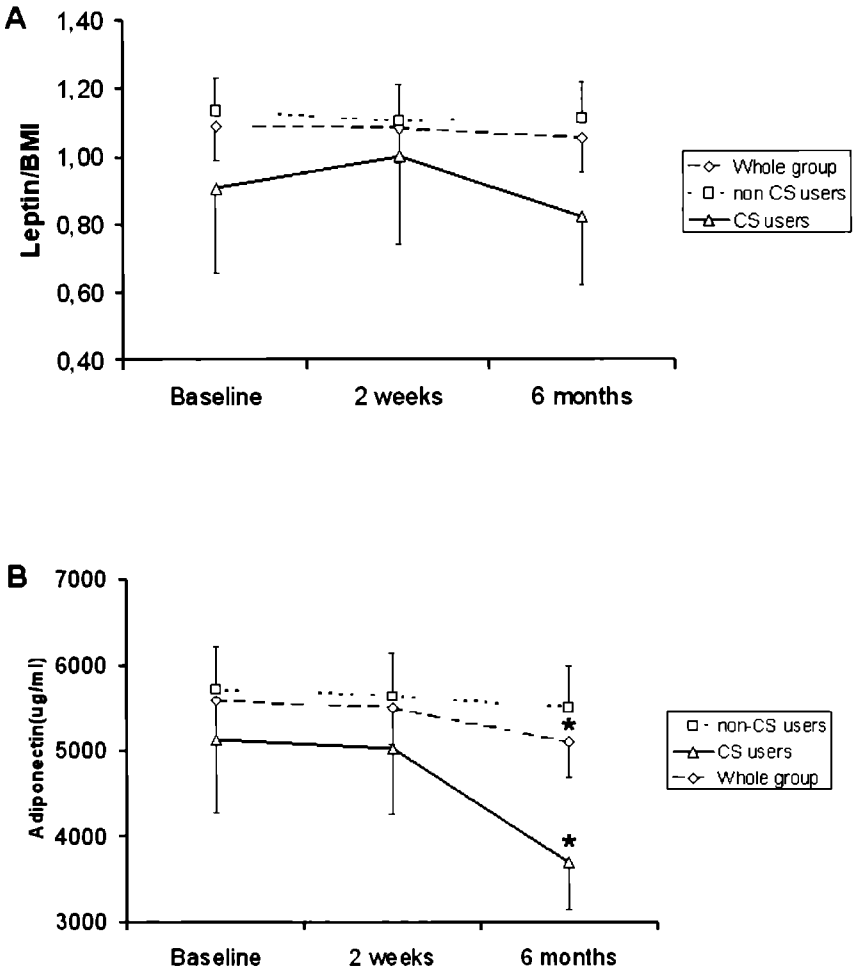

Figure 3. Course of serum levels of leptin (A) and adiponectin (B) in patients with RA treated with TNF blockers with (black triangle symbol) and without (white square symbol) concurrent corticosteroid therapy. Data are presented as means ± SEM. (* $p<0.025$ compared to baseline)

Tabel 3 Serum concentrations of leptin, adiponectin and leptin/BMI during 6 months of anti-TNF therapy in men and women with RA

		Baseline	2 weeks	6 months	p-value
Leptin (ng/ml)	F	33 6 ± 20 3	34 2 ± 20 7	33 3 ± 20 8	0 86
	M	15 8 ± 11 7	14 1 ± 10 3	14 7 ± 12 8	0 54
Leptin/BMI	F	1 34 ± 0 62	1 34 ± 0 61	1 29 ± 0 61	0 54
	M	0 55 ± 0 36	0 51 ± 0 32	0 54 ± 0 39	0 76
Adiponectin (ng/ml)	F	6205 ± 2850	6079 ± 2748	5653 ± 2906	0 05
	M	4116 ± 2070	4083 ± 2010	3768 ± 1506	0 29

Results are expressed as mean ± SD, BMI = body mass index, p-values express differences between baseline and 6 months values

Discussion

We report here for the first time in a larger group of RA patients that long-term TNF blockade using infliximab does not alter circulating levels of leptin and adiponectin, except for the patients using stable dosage of corticosteroids, in which long-term TNF blockade significantly decreased adiponectin concentrations In addition, we found that BMI is an important determinant of leptin levels in RA patients, while inflammation and disease activity have no clear association with serum concentrations of the adipocytokines studied

Previous studies have suggested that leptin is a pro-inflammatory mediator that favors the damaging processes characteristic to RA Initially, leptin has been found to be higher in RA compared to healthy volunteers, both in the circulation and synovial fluid [14], and a positive correlation with disease activity and inflammatory markers has been suggested [19,24] However, as shown here, chronic inflammation in patients with RA does not have stimulatory effects on serum leptin levels, in contrast with the acute inflammation of sepsis and surgery [25] Accordingly, we show here that RA patients and healthy controls have similar plasma concentrations of leptin, despite an increased inflammatory status in the first group In addition, there were no clear relations between plasma leptin and inflammatory status, as assessed by disease activity and ESR This was further sustained by the fact that anti-TNF therapy had no effect on plasma leptin concentrations, while importantly decreasing the inflammation Given these results and the fact that they are in line with several later reports from our group and other investigators [18,21,22,26,27], we argue that circulatory leptin reflects the role of leptin in RA development, as previously suggested In line with this, plasma leptin can inversely relate to inflammation in RA [22], as observed here in a subgroup of patients with active disease In

addition, the compartment where leptin is produced seem to be of importance, with locally (intraarticularly) produced leptin being likely to be more important for RA pathogenesis [14;16]. BMI and thus most likely fat tissue, remains the major determinant of plasma leptin concentrations, which is confirmed by previous studies in RA [18] but also in other chronic inflammatory conditions [6;28;29]. Therefore, we hypothesize that in RA circulating leptin does not reflect and has a limited implication on the intra-articular inflammation. We further consider that in these patients plasma leptin concentrations should be better coupled to the energetic metabolism (e.g. nutritional status, cachexia) and susceptibility to infections.

In our study, adiponectin plasma concentrations were higher in the RA patients group than in healthy controls. In contrast to the initial hypothesis stating that chronic inflammation associated with obesity inhibits adiponectin production, increased adiponectin levels have been observed during chronic inflammatory conditions that are unrelated to increased adipose tissue mass [20;24]. This can be explained by the presence of inflammation-induced catabolic responses, which may raise adiponectin in these patients. Moreover, these levels may be positively associated with CRP concentrations [24] and exert pro-inflammatory actions in a TNF-dependent manner, including stimulation of matrix metalloproteinase 1 (MMP-1) synthesis in human synovial fibroblasts and monocyte chemoattractant protein 1 (MCP-1) expression in osteoarthritis chondrocytes [12;30]. However, the pro-inflammatory activities are related only to high-molecular weight and globular adiponectin, whereas low-molecular weight adiponectin has anti-inflammatory effects [31;32]. Since no study has previously assessed the presence and the ratios between these three forms in patients with RA, the question whether the overall effects of adiponectin in RA are either pro- or anti-inflammatory remains open and awaits further investigations.

In the present study, therapeutic TNF blockade during 6 months was not able alone to produce changes in adiponectin circulating levels. However, adding anti-TNF to patients that already received stable doses of corticosteroids resulted in a significant decrease of adiponectin 6 months after TNF blockade was initiated. These results differ from a recent report of Härle et al., who reported a constant decreased adiponectin concentration in patients receiving corticosteroids already from baseline and observed no additional potential of anti-TNF agents to further diminish these levels [21]. In addition, Serelis et al. found in a small group of women with RA that anti-TNF agents raised adiponectin levels after one year of follow-up [33]. A different anti-TNF agent and a longer exposure to either drug may account for these differences. The

combined effects of prednisolone and TNF blockade on adiponectin may be of increased importance since data regarding the effects of corticosteroids alone on adiponectin are still contradictory, with suppressor activities *in vitro* [34] and no effects *in vivo* [35,36]

In conclusion, the results of our study suggest that in RA chronic inflammation and its suppression during anti-TNF therapy has limited influence on plasma leptin concentrations and therefore, they question the importance of circulating leptin in RA pathogenesis While circulating adiponectin is higher in RA, its concentrations significantly dropped 6 months after the initiation of TNF blockade in patients on concurrent corticosteroid therapy Dependent on which molecular form of adiponectin is diminished, this may have either detrimental effects for the disease itself and by increasing the risk for atherosclerosis and cardiovascular diseases [17,37] (if anti-inflammatory low-weight adiponectin is affected), or beneficial consequences, if pro-inflammatory high-weight and globular adiponectin drops Finally, our findings suggest the possibility that other mechanisms rather than inflammation might be of greater importance for regulating circulating adipocytokine pattern in RA

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Chapter 8

The role of TNFα in chronic inflammatory conditions, intermediary metabolism and cardiovascular risk.

Discussion Part I

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Abstract

The recent insight that inflammation contributes to the development of atherosclerosis and type 2 diabetes mellitus constitutes a major breakthrough in understanding the mechanisms underlying these conditions. In addition, it opens the way for new therapeutic approaches that might eventually decrease the prevalence of these public health problems. TNFα has been shown to play a key role in these processes and thus might be a potential therapeutic target. Increased concentrations of TNFα are found in acute and chronic inflammatory conditions (e.g. trauma, sepsis, infection, rheumatoid arthritis), in which a shift towards a pro-atherogenic lipid profile and impaired glucose tolerance occurs. While therapeutical blockade of TNFα worsens the prognosis in patients with abscesses and granulomatous infections, this strategy is highly beneficial in the case of chronic inflammatory conditions including rheumatoid arthritis. Current investigations assessing the impact of anti-TNF agents on intermediary metabolism suggest that TNFα blockade may improve insulin resistance and lipid profiles in patients with chronic inflammatory diseases. The effects of therapeutic TNF blockade on several adipocytokines, e.g., leptin and adiponectin, are controversial and need to be further clarified.

1. Introduction

The metabolic syndrome, also known as the syndrome X, represents a constellation of metabolic abnormalities that includes central obesity, insulin resistance, glucose intolerance, dyslipidemia, and hypertension [1]. Each of these features is known to augment the risk of developing diabetes mellitus and cardiovascular disease. For decades, both exogenous factors such as diet, sedentarism and alcohol consumption and genetic background, were considered to constitute the major determinants of disturbances in intermediary metabolism. Consequently, lifestyle changes and genetic familial screening were advocated in order to combat the onset and development of metabolic syndrome and those of diabetes and cardiovascular disease. However, the appearance of metabolic syndrome in people with normal dietary habits and without a particular genetic background raised the possibility that other pathogenetic factors contribute to the development of this syndrome. Further studies indicated that inflammation constitutes the "missing puzzle piece" in the pathogenesis of the metabolic syndrome.

Evidence pointing to a link between inflammation and lipid metabolism was provided by studies showing dyslipidemia and insulin resistance during acute inflammation, as occurs in septic shock or trauma [2-5]. In turn, hyperlipidemia was shown to inhibit the acute inflammatory response [6]. In addition, patients with chronic inflammatory diseases such as rheumatoid arthritis (RA) were often described to have a dyslipidemic profile and an altered glucose tolerance [7,8]. However, not until the past decade, did the role of inflammation in the development of the metabolic syndrome become documented or its importance accepted [9]. Besides, the contribution of inflammation to the development of both early and late atherosclerotic lesions led Ross to affirm that atherosclerosis is an inflammatory disease [10]. Additionally, evidence came from studies showing that adipose tissue secretes inflammatory cytokines, which in turn contribute to impaired glucose tolerance, insulin resistance and type 2 diabetes [11,12]. Finally, the level of circulating inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) independently was found to predict the risk of future cardiovascular events in the general population [13,14].

Among many inflammatory markers, TNFα emerged as a key cytokine which influences intermediary metabolism. TNFα was originally described as cachectin, a protein that was demonstrated to be involved in the development of cachexia [15]. Later studies described the pro-atherogenic changes in lipid metabolism induced by this cytokine [4,16]. In animal models, administration of TNFα led to severe impairment of glucose tolerance and insulin

sensitivity [17]. Taken together, TNFα might serve as a therapeutic target in these disorders. Treatment of sepsis with agents that block TNFα actions was proven to worsen these conditions [18], while in the case of chronic inflammation, such as that in the joints of RA patients, this therapy was highly beneficial [19,20]. Given these facts, the evaluation of metabolic markers in patients with chronic inflammatory conditions during anti-TNF therapy is warranted.

This review will focus on the role of TNFα in the development of dyslipidemia and insulin resistance as important features of the metabolic syndrome, which may eventually augment the risk of cardiovascular diseases and type 2 diabetes mellitus (DM). Patients with RA have 1,5-2 times higher risk of cardiovascular morbidity and mortality and chronic systemic inflammation is likely to play a crucial role in this respect. Therefore, the impact of anti-TNF strategies on intermediary metabolism in these patients will be further discussed.

2. Intermediary metabolism: acute vs. chronic inflammatory state

During acute conditions the organism reacts quickly through a variety of mechanisms that are meant to set the different homeostatic systems at new thresholds that are eventually decisive for the outcome. These modifications also include changes in lipid metabolism [2-4] that eventually have beneficial consequences for the host. Accordingly, an increase in lipoprotein concentrations during the acute phase response was shown to neutralize the toxic effects of lipopolysaccharide (LPS) both in *in vitro* and *in vivo* models, conferring on them a crucial role in host defense during endotoxemia [4,21,22]. Although LPS binds and activates monocytes more rapidly than lipoprotein binding and neutralization occurs, the infusion of lipoproteins was indicated to accelerate the kinetics of the neutralization of LPS, providing some advantage [23]. Interestingly, the phospholipid content was reported to correlate with the ability of lipoproteins to neutralize LPS. The increase in serum triglycerides (TG) and glucose during acute inflammatory conditions may also provide extra nutrients for the elevated metabolic needs of cells involved in host defense and tissue repair. In contrast, hyperglycemia, even acutely, has been extensively demonstrated to be associated with an impairment of host defense, including decreased polymorphonuclear (PMN) mobilization, chemotaxis, and phagocytic activity [5].

Despite all these beneficial acute effects, the longer the persistence of inflammatory markers, such as TNFα, the more will they induce changes in both lipid and glucose metabolism that are

Figure 1

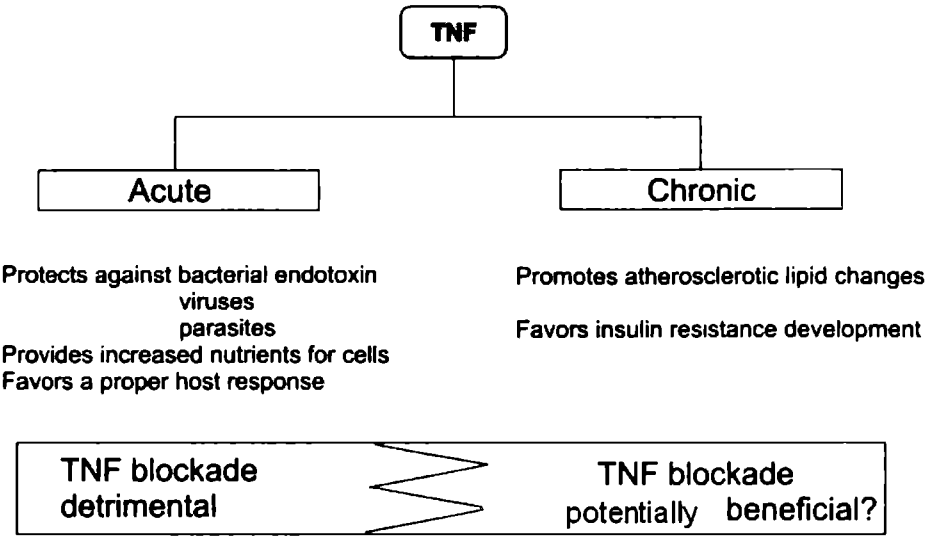

Figure 1. The acute and chronic effects of TNFα on intermediary metabolism and their consequences for the host Although anti-TNF agents have been proved to be detrimental in acute conditions, they might be beneficial in combating metabolic syndrome features in the case of chronic inflammatory diseases

likely to have detrimental consequences for the host (**figure 1**). The lipid changes induced by TNFα are pro-atherogenic in terms of both quality and quantity, and therefore the persistence of these modified lipids in the circulation will promote the development of atherosclerotic lesions. The sustained increase in glucose and TG plasma concentrations will have important consequences for glucose homeostasis, altering glucose tolerance and promoting hyperinsulinemia and an insulin resistance state.

Chronic inflammatory conditions have been shown to be associated with a pro-atherogenic lipid pattern and altered glucose tolerance. Patients with inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) have lower HDL and apoA1, and higher apoB, total cholesterol/HDL-cholesterol ratio and lipoprotein (a) plasma concentrations [7,24,25].

Consequently, they have more atherosclerotic lesions, impaired endothelial function and elevated carotid IMT, which is associated with a higher mortality due to cardiovascular diseases than in the general population [24,26,27] In addition, it was recently found that especially RA patients show an impairment of glucose handling and an enhanced insulin response after intravenous glucose loading [8] In this light, an exploration of the contributions of inflammation in general and TNFα in particular to the modified intermediary metabolic pattern of chronic inflammatory diseases is warranted

3. TNFα

TNFα is a member of a growing family of peptide mediators comprising at least 19 cytokines, including lymphotoxin-α (LTα), Fas ligand (FasL) and CD40 ligand (CD40L) TNFα has important proinflammatory properties, which play crucial roles in the innate and adaptive immunity, cell proliferation and apoptotic processes The cytokine is produced by different kind of cells, including macrophages, monocytes, T cells, smooth muscle cells, adipocytes and fibroblasts Biological responses to TNFα are mediated by ligand binding via two structurally distinct receptors type I (TNF-RI, p55) and type II (TNF-RII, p75), which are present on the membrane of all cell types except erythrocytes The two receptors differ significantly in their binding affinities as well as in their intracellular signalling pathways Upon stimulation, the intracellular domain of TNF-RI binds to the TNF-receptor associated death domain (TRADD) protein, which can further activate either the apoptotic pathway, via the Fas associated death domain (FADD) protein, or the proinflammatory pathway, via TNF receptor associated factor 2 (TRAF2) and receptor-interacting protein (RIP), resulting in the activation of NF-kB In contrast to TNF-RI, TNF-RII is unable to activate the TRADD/FADD pathway and signals only through the TRAF2 associated pathway Of note, studies have indicated the presence of an important crosstalk between the two receptors, which is likely to be responsible for the net response of a cell upon TNFα stimulation [28] Accordingly, TNF-RI is mainly responsible for mediating the inhibitory effects of TNFα on the insulin receptor signaling pathway [29,30], whereas TNF-RII deficiency alone does not affect insulin sensitivity but may potentiate the effects of TNF-RI deficiency in animals lacking both TNFRs [31] In addition, the lipolytic effect of TNFα on triglycerides is mainly mediated via TNF-RI [32] Besides membrane expressed TNFRs, plasma soluble TNFRs can also modulate the actions of TNFα In line with this, sTNF-RII is likely to play an important role in human obesity by neutralizing TNFα actions and therefore it was suggested to be the best predictor of adipose TNFα activity in these subjects [33] Moreover, patients with peripheral vascular disease or myocardial infarction survivors were

found to have increased plasma TNF-RII concentrations [34]. Therefore, the measurement of soluble TNF-RII might be of relevance when assessing the contribution of TNFα to the pathogenesis of these conditions, while TNF-RI remains the main membrane-bound receptor to signal TNFα actions.

4. TNFα and lipid metabolism

TNFα is a pleiotropic cytokine and its role in inflammation and metabolism is complex. Together with other proinflammatory cytokines, chemokines and various immune cells, TNFα has emerged as an important contributor to the development of atherosclerotic lesions by promoting the expression of adhesion molecules on endothelial cells, the recruitment and activation of inflammatory cells and the initiation of the inflammatory cascade inside the arterial wall [10,35]. TNFα has been demonstrated to directly interfere with the metabolic pathways of triglycerides and cholesterol [4,16,36], which will be further discussed in detail. Taken together, TNFα may gain a special importance when referring to atherosclerotic lesion development and the risk of acute cardiovascular events.

TNFα and triglyceride metabolism

Patients with acute inflammatory disorders and sepsis, in which elevated TNFα concentration occur, have been shown to have increased TG concentrations early during the acute episode of the disease [37,38]. In addition, hypertriglyceridemia has also been observed in cancer and chronic infections such as AIDS, conditions in which elevated concentrations of TNFα may be present [39,40]. Finally, the administration of TNFα and endotoxin (LPS) to mice and humans results in an acute elevation of plasma TG concentration of approximately 85% [36,41,42]. The effects of TNFα on plasma TG occur through effects on both adipose tissue and liver TG metabolic pathways. Accordingly, TNFα raises plasma TG by increasing the concentration of free fatty acids (FFA) [43], the substrate for TG synthesis, and by diminishing the clearance of TG-rich lipoproteins (VLDL) from circulation [42] (**figure 2**).

TNFα increases the FFA production from both adipose tissue and liver. In human adipose tissue, *in vitro* studies demonstrated that TNFα stimulates lipolysis. This effect is mainly mediated via TNFR-I [32], and involves the activation of several kinases of the mitogen-activated protein kinase (MAPK) family of signalling kinases, including extracellular signal-related kinase (ERK1/2 or p44/42) and c-jun-NH_2 -terminal kinase (JNK) [44]. There are several pathways through which TNFα exerts its lipolytic effects. Firstly, following activation of ERK1/2 kinase,

an increase in the intracellular concentrations of cAMP occurs that further activates protein kinase (PK) A, which in turn phosphorylates hormone-sensitive lipase (HSL) and perilipins, adipocyte proteins situated on the surface of the lipid droplet [45] Perilipins were recently shown to play a crucial role in the induction of lipolysis by regulating the substrate accessibility for HSL and adipocyte triglyceride lipase (ATGL), the main lipolytic enzymes [45-47] After their phosphorylation, which may also occur directly via JNK and p44/42, perilipins translocate away from the lipid droplet allowing access for HSL and ATGL to hydrolyze the triglycerides inside While ATGL exerts its hydrolyzing activity only on TG, HSL has a ten-fold higher activity on diglycerides (DG) resulting from the previous lipolytic step Secondly, TNFα down-regulates the expression of perilipins [46], which may further enhance the lipolysis in adipocytes In addition, TNF-α can suppress the expression of HSL [48] and ATGL [49] but without changes in its net lipolytic effects [50] Finally, through activation of the p44/42 kinase, TNFα may inhibit early insulin receptor signaling, thereby counteracting the anti-lipolytic role of the hormone [51] In addition, in rodents TNFα may inhibit the expression of Gi-protein-coupled adenosine receptors present on the surface of adipocytes, thereby suppressing the anti-lipolytic effect of adenosine [52] However, this effect could not yet be evidenced in human fat cells [53] As a result of these actions, FFA are released from adipocytes into the circulation and may further constitute the substrate required for TG synthesis in the liver Of note, hypertriglyceridemia constitutes an important pre-step in the development of glucose intolerance and insulin resistance, which will be addressed later in this review

Hepatic triglyceride production is increased in both human and murine studies, as demonstrated by the increase of TG-containing VLDL particles after TNF administration [36,41,42] However, insights into the mechanisms responsible for this effect were based only on animal models Such mechanisms are represented by an increase in the availability of FFA released by stimulated lipolysis in peripheral adipose stores as well as de-novo fatty acid synthesis in the liver Cytokines can stimulate hepatic TG synthesis by various mechanisms TNFα, IL-1β and IL-6 acutely increase hepatic levels of citrate, an allosteric activator of acetyl-CoA carboxylase, the rate-limiting enzyme in FFA synthesis [54,55] In contrast, IFNα, using an unknown mechanism, activates acetyl-CoA carboxylase without increasing citrate levels, and has a synergistic effect with TNFα, IL-1β and IL-6 on the FFA production [4,55] This suggests that TNFα can induce these changes either directly, or indirectly by increasing the level of other pro-inflammatory cytokines, such as IL-1β and IL-6 Finally, the hepatic enzymes involved in the esterification of

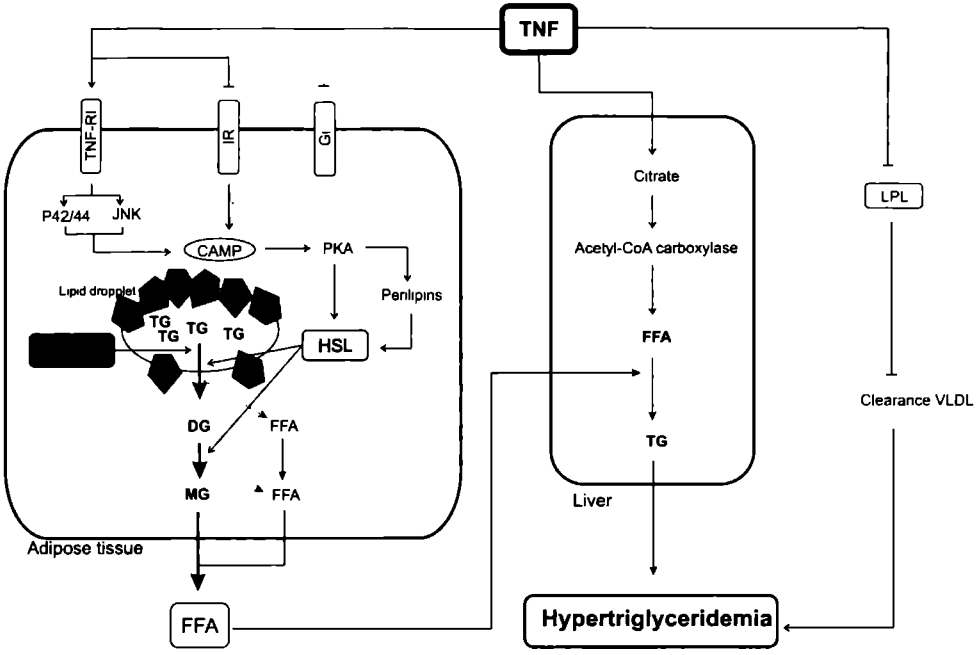

Figure 2. TNFα induced hypertriglyceridemia. The different pathways are represented. The Gi-dependent pathway (dotted lines in the figure) has up to now been evidenced only in rodents. TNF-RI = tumor necrosis factor receptor type I; IR = insulin receptor; Gi = Gi-protein-coupled adenosine receptor; p44/42 (ERK1/2) = extracellular signal-related kinase 1/2; JNK = c-jun-NH_2 –terminal kinase; PKA = protein kinase A; HSL = hormone sensitive lipase; ATGL = adipose tissue triglyceride lipase; G = glycerol; TG = triglycerides; DG = diglycerides; MG = monoglycerides; LPL = lipoprotein lipase;

fatty acids with glycerol are not increased after TNFα treatment suggesting that hepatic TG content is driven by the availability of the chief substrate, the fatty acids.

Another mechanism through which TNFα can raise plasma TG concentrations in both human and rodents is the inhibition of lipoprotein lipase (LPL) activity [4,16,42]. The inhibitory effect is seen at both transcriptional and post-transcriptional levels, leading to a decrease in the clearance of TG-rich lipoproteins, thereby contributing to hypertriglyceridemia. In addition, TNFα can decrease apolipoprotein E (apoE) mRNA in rat hepatocytes and consequently reduce the receptor-mediated uptake of TG-rich lipoproteins, which will therefore remain longer in the circulation [56].

Besides raising the concentration of TG-rich VLDL particles, TNFα may also alter their composition Accordingly, the VLDL content of sphingolipids has been shown to increase [57] Sphingolipids and sphingolipid metabolizing enzymes may play important roles in atherogenesis, not only by altering the composition of lipoproteins but also by mediating a number of cellular events, which are believed to be crucial in the development of the vascular lesions such as proliferation or cell death [58] In addition, the modified VLDL particle has a decreased clearance and may interact with the LDL receptor on macrophages, enhancing foam cell formation Thus, the TNFα induced changes in TG and VLDL metabolism are the same in both humans and rodents and they can be considered to be proatherogenic

TNFα and cholesterol metabolism

Besides the modifications that occur in TG metabolism, TNFα may also interfere with cholesterol metabolic pathways Whereas TNFα-induced changes in TG metabolism are similar in all species, the effects on cholesterol metabolism differ between rodents and primates Whereas the administration of TNFα in rodents is followed by a delayed increase in serum concentrations of total cholesterol and hepatic cholesterol synthesis [4,16,36,55], non-human primates and humans show either no change or a decrease in serum cholesterol and LDL-cholesterol levels [38,41] The mechanisms underlying this species difference are not known In primates TNFα was indicated to decrease HDL concentrations [41] In addition, the composition of lipoproteins can be altered upon the action of TNFα The mechanisms through which TNFα exerts its effects on cholesterol metabolism are complex and to take place at different levels, including the hepatocytes and peripheral cells, such as endothelial cells (**figure 3**)

In rodents, TNFα may increase hepatic cholesterol synthesis by stimulating the HMG-CoA reductase activity, the rate-limiting enzyme in the cholesterol biosynthetic pathway [36] The effect is specific, as other enzymes implicated in cholesterol synthesis in the liver are not activated, and is likely to be independent of dietary regulation [59] Despite a marked increase in HMG-CoA reductase activity, TNFα produces only a modest increase in hepatic cholesterol synthesis and the circulating cholesterol concentrations This is due to an inhibitory effect of TNFα on the production and activity of squalene synthase [60], the first committed enzyme in the cholesterol synthesis located at a branch point in the mevalonate pathway The enzyme plays an important role in regulating the flux of metabolic intermediates to the sterol pathways Thus the effects of TNFα on the cholesterol biosynthetic pathway are likely to maintain an adequate

cholesterol synthesis while redirecting a proportion of the mevalonate metabolites into the non-steroidal pathways.

In contrast to the situation in rodents, a decrease of 7% in total cholesterol and of 43% in HDL cholesterol concentrations was observed in cancer patients, after administration of recombinant human TNFα as a five-day continuous infusion [41]. Moreover, plasma cholesterol concentration was shown to be constantly depressed in all types of acute conditions [2-4,37,38], that are normally associated with high levels of TNFα. The cholesterol content was reduced in both LDL and HDL particles. The mechanisms responsible for these effects in humans and primates have not been thoroughly studied using *in vivo* experiments. However, using human hepatoma HepG2 cells, it was shown that TNFα is capable of decreasing the secretion of ApoA-I and ApoB in a dose-dependent manner [61]. Therefore, the decrease in apolipoprotein secretion might account at least in part for the hypocholesterolemia seen during acute and chronic inflammatory conditions.

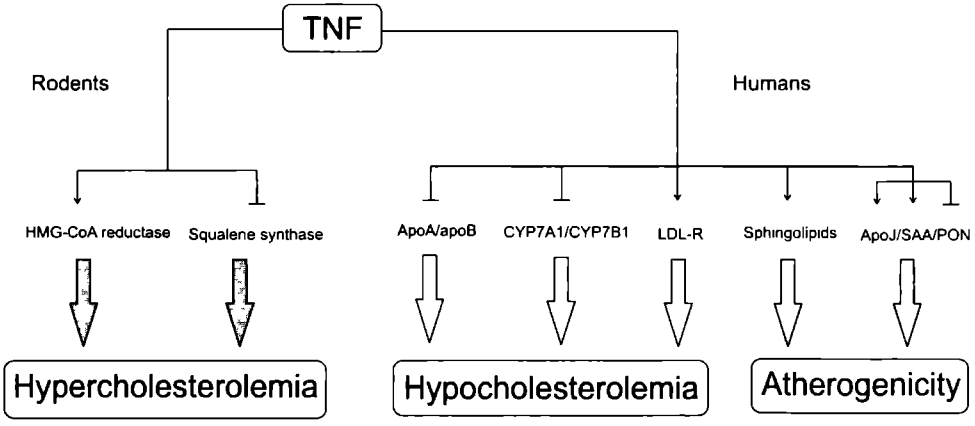

Figure 3. The effects of TNFα on cholesterol metabolism in rodents and humans. CYP7A1 = cholesterol-7α-hydroxylase; CYP7B1 = oxysterol 7α-hydroxylase; LDL-R = low density lipoprotein receptor; SAA = serum amyloid A; PON = paraoxonase.

Besides the effects on the apolipoprotein synthesis pathway, TNFα may decrease hepatic cholesterol catabolism and excretion The elimination of cholesterol from the body is achieved mostly through its conversion into bile acids, a pathway regulated by the enzyme cholesterol-7α-hydroxylase (CYP7A1) Several cytokines, including TNFα, were shown to inhibit the expression and activity of CYP7A1, the rate-limiting enzyme in the classic pathway of bile acids synthesis [62] The mechanism involves the activation of a MAPK-dependent signaling cascade which eventually dampens the hepatic nuclear factor-4 HNF-4 mediated activation of CYP7A1 [62] In addition, the activity of mitochondrial sterol 27-hydroxylase (CYP27A1) and oxysterol 7α-hydroxylase (CYP7B1), the rate-limiting enzymes in the alternative pathway of bile acids synthesis, are also down regulated by TNFα and other inflammatory cytokines in experiments using human hepatoma cell lines [63] These data suggest that in both humans and animals, TNFα limits the cholesterol elimination from the body while increasing its availability for other hepatic processes that operate during the acute phase response

TNFα can increase LDL binding to HepG2 cells in a dose-responsive manner, paralleled by increased steady-state levels of LDL receptor mRNA [64] Other cytokines including IFNγ, macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) do not affect the LDL binding to such cells [64] This mechanism may increase the clearance of the LDL particles from circulation and therefore may contribute to the low cholesterol concentrations in blood observed after the TNFα administration in humans Despite the decrease in LDL plasma concentrations, TNFα is likely to induce changes in the LDL composition that eventually increase the atherogenicity of this particle In patients with AIDS, a decrease in LDL concentrations was associated with a decrease in particle size, resulting in small dense LDL [65], which is more pro-atherogenic Moreover, TNFα increased the concentration of secretory phospholipase A2 (sPLA2) [66] The sPLA2 hydrolyzes phospholipids in LDL generating FA that can further contribute to oxidized LDL formation [67] Finally, the LDL content in sphingolipids, including sphingomyelin and ceramide, increases [57] All these changes in the LDL composition render the particle more atherogenic

The reverse cholesterol transport (RCT)

There are several mechanisms through which HDL protects against atherogenesis One of the most extensively studied and accepted hypotheses suggests that HDL plays a role in removing excess cholesterol from peripheral cells and returning it to the liver for excretion This

mechanism is called reverse cholesterol transport (RCT) and it plays a crucial role in preventing or reversing the development of atherosclerotic lesions [68,69] (**figure 4**) This process is initiated by the efflux of cholesterol from arterial wall cells onto lipid-poor apoA-I or pre-β HDL particles, and is regulated by ABCA1 and ABCG1 (ATP binding cassette A1 and G1 respectively) [70] Subsequently, lecithin-cholesteryl acyltransferase (LCAT) esterifies free cholesterol in HDL, a process that is essential for HDL to efficiently remove cholesterol from cells and tissues, thus contributing to the anti-atherogenic properties of HDL Cholesteryl ester transfer protein (CETP) further transfers cholesteryl esters from HDL to TG-rich lipoproteins, while phospholipid transfer protein (PLTP) transfers phospholipids from TG-rich lipoproteins to HDL Finally, hepatic lipase (HL) hydrolyzes TG and phospholipids in large α-HDL, generating small pre-β HDL particles that begin a new cycle in the RCT process [68,69] In addition, the scavenger receptor class B type I (SR-BI) plays a key role in the selective uptake of cholesteryl ester into hepatocytes [71] TNFα has been demonstrated to induce a reduction in RCT attributable to multiple changes at each step in this pathway

Hepatic synthesis and plasma activity of LCAT is decreased by TNFα in primates [72], resulting in decreased HDL-cholesterol concentrations, similar to what is found in humans or animals with mutations in the LCAT gene [73] This might partly account for the decrease in cholesterol concentrations seen after TNF infusion CETP activity is decreased upon TNFα action in rodents [74] Interestingly, Japanese populations with CETP deficiency exhibited high levels of HDL Given these facts, CETP has recently been indicated as a new therapeutic target and CETP-blocking agents have been developed to test their potential in raising HDL and decreasing cardiovascular risk [69,75-77] TNFα is also able to decrease SR-BI mRNA in the liver and in Hep3B hepatoma cells, resulting in an impaired cholesterol uptake and excretion [71] Thus besides reducing hepatic Apo-AI, TNFα is likely to affect the level of cholesterol removal from peripheral cells, transfer between particles and uptake by the liver Although an initial decrease of reverse cholesterol transport during the acute phase response may be beneficial as it redirects cholesterol towards macrophages for host defense, a prolonged or sustained inflammatory response, as seen in chronic infection and inflammation, may continually impair RCT, thus leading to cholesterol deposition in macrophages and promoting atherosclerosis

Besides decreasing circulating HDL-cholesterol concentrations, TNFα may also alter HDL composition The content of apoJ and apoSAA in HDL increases, while that of apoA-I may decrease [4,78,79] SAA rich HDL particles are rapidly cleared from the plasma, and thus the

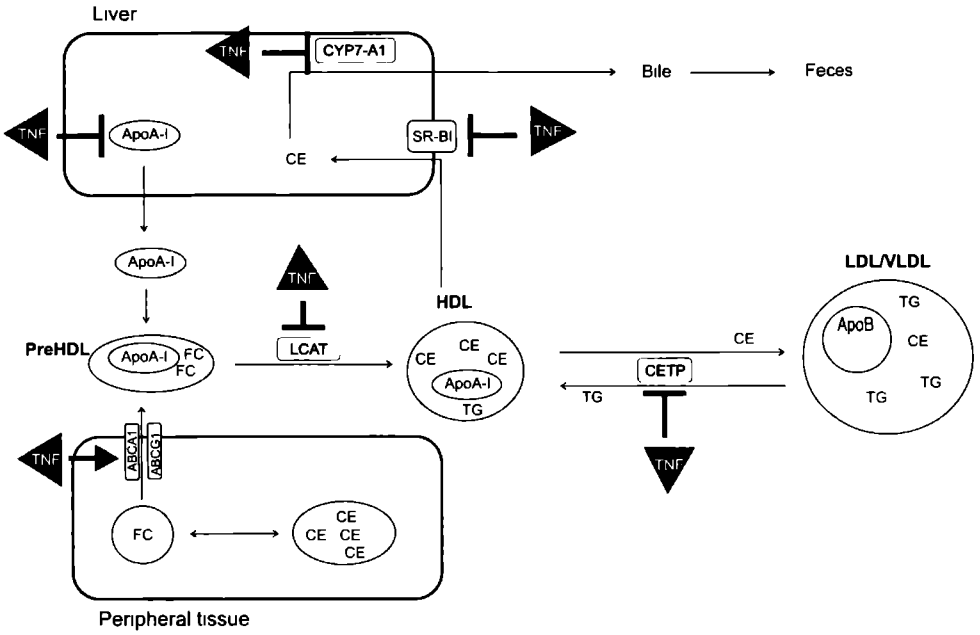

Figure 4. Reverse cholesterol transport TNFα can interfere at several levels with the major mechanism responsible for the removal of cholesteryl-esters from peripheral tissues. FC = free cholesterol, CE = cholesteryl esters, ABCA1/ABCG1 = ATP binding cassette A1 and G1, respectively; CETP = cholesteryl ester transfer protein; LCAT = lecithin-cholesteryl acyltransferase; SR-BI = scavenger receptor class B type I

increase in SAA could also contribute to a decrease in HDL concentrations [80]. In addition, SAA-rich HDL has a decreased affinity for hepatocytes and an increased affinity for macrophages, which may result in a redirection of the HDL metabolism. Further, the decrease in apoA-I may alter the capacity of pre-β HDL particles to attract cholesterol from peripheral cells in a step that initiates the RCT. Several HDL-associated proteins including paraoxonase-1 (PON1) possess antioxidant activities and help HDL to exert its role in protecting LDL against oxidation. On HepG2 cells TNFα downregulates the expression of PON1, which results in a depletion of HDL antioxidant properties [81]. This will eventually convert HDL into a proatherogenic particle, enhancing the atherogenic process. Accordingly, a recent study documented the presence of proinflammatory HDL in patients with systemic lupus erythematosus and rheumatoid arthritis and suggested that it may serve as a biomarker for atherosclerosis in these chronic inflammatory conditions [82].

Several recent publications have indicated that TNFα may also exert anti-atherogenic effects, for instance through the inhibition of atherosclerotic plaque development These assumptions are based on the observations that TNF-RI-deficient mice develop more severe atherosclerotic lesions compared to normal littermates when fed an atherogenic diet [83] In the TNF receptor knock-out mice this is due to an upregulation of the scavenger receptor on the macrophages followed by an increase of the cholesterol uptake and foam cell formation Interestingly, plasma lipid concentrations did not differ between the strains Assuming that TNFα has anti-atherogenic properties, Gerbod-Giannone et al have recently indicated that ABCA1, a member of the ATP-binding cassette (ABC) transporter superfamily, is upregulated by TNFα in a dose-dependent manner, through a mechanism involving the activation of nuclear factor-kB (NF-kB) [84] As previously mentioned, ABCA1 plays an important role in the RCT promoting the efflux of cholesterol from peripheral cells into the lipid-poor apoA-I or pre-β HDL particles These findings in the TNFR knock-out mice are puzzling since the TNFα gene disruption has been shown to diminish the development of atherosclerosis in the ApoE deficient mice [10,35,85] However, in another study, loss of TNFα did not alter the development of the lesions in mice fed an atherogenic diet [86] In addition, loss of the TNFR II had no influence on lesion growth in the same mice [86] Taken together, these data illustrate the complexity of TNF ligand and receptor interactions in the atherogenesis pathways, with disparate actions depending on the targeted cell type, signaling pathways, duration of action and, in the case of murine experiments, the genetic background of the rodent In addition, some discrepancies regarding TNFα actions might be due to the fact that an interchangeability of data from whole organisms and cell culture studies may not always be valid The implication of other undefined members of the TNF ligand or receptor-signaling pathways in regulating atherogenesis could not be excluded To conclude, TNFα has dissimilar effects on cholesterol levels in rodents and humans, with inhibitory actions on the Apo-AI hepatic synthesis and the cholesterol reverse transport in humans

TNFα blockade and lipid metabolism

The effects of TNFα blockade on the circulating lipid pattern were recently explored in patients with chronic inflammatory conditions and elevated TNFα plasma concentrations We evaluated the influence of short-term therapy with adalimumab, a fully human anti-TNF monoclonal antibody, on the lipoprotein profile and on markers of inflammation in 33 patients with active RA Plasma HDL-cholesterol concentrations significantly increased 2 weeks after the start of the therapy with an average of 0 12 mmol/l, while no differences were seen in the placebo treated

group. In addition, the atherogenic index decreased [87]. Our results were later confirmed in another study in a larger RA group, indicating that infliximab, a chimeric anti-TNF monoclonal antibody, increases plasma HDL-cholesterol concentrations by about 0.10mmol/l after 2 weeks of therapy, and that the effect is still sustained after 6 weeks of therapy [88]. While short-term effects of anti-TNF agents on lipoproteins are likely to raise the HDL-cholesterol and decrease the atherogenic index, the immediate effects seem to yield opposite results. One study, investigating the effects of infliximab on plasma lipoproteins concentrations 24 hours after each infusion, showed that the total cholesterol, HDL-cholesterol and ApoAI levels significantly dropped, while the atherogenic index increased the day after the infusion. However, between infusions, a slight increase in HDL-cholesterol concentrations and a decrease in the atherogenic index after 2 and 6 weeks of therapy were observed compared to baseline [89]. Whether the non-atherogenic lipid profile persists and indeed results in less atherogenesis in the long term is the subject of future studies.

5. TNFα and glucose metabolism

The development of the concept that type2 DM is an inflammatory condition is an exciting and novel approach to the understanding of this condition. Recent work in the area of obesity has confirmed that obesity is a state of low-grade chronic inflammation, as indicated by the increased concentrations of CRP, IL-6 and other inflammatory markers identified in the plasma of obese individuals [11,90]. This concept has raised the possibility that type2 DM, another closely related insulin resistant state, might be also an inflammatory condition. Indeed, proinflammatory cytokines (TNFα, IL-18, IL-6) and sialic acid were found to be elevated in patients with type2 DM [91,92]. Moreover, inflammatory markers such as CRP and IL-6 are even likely to predict the development of type2 diabetes in white non-smoking adults [93]. There are now ample data to regard inflammation as a link between the insulin resistance, obesity and diabetes.

Among inflammatory markers, TNFα was first demonstrated to be involved in the pathogenesis of insulin resistance. In 1993, Hotamisligil et al. published the first evidence of constitutive TNFα expression in adipocytes and further demonstrated that adipocytes from the obese animals (*ob/ob* mouse, *db/db* mouse and *fa/fa* Zucker rat) express markedly increased amounts of TNFα. In addition, neutralization of TNFα using soluble receptors was followed by an improvement of the insulin sensitivity in these animals [17]. Later data have shown that TNFα is also expressed in human adipose tissue and its plasma concentration in obese subjects has fallen after weight

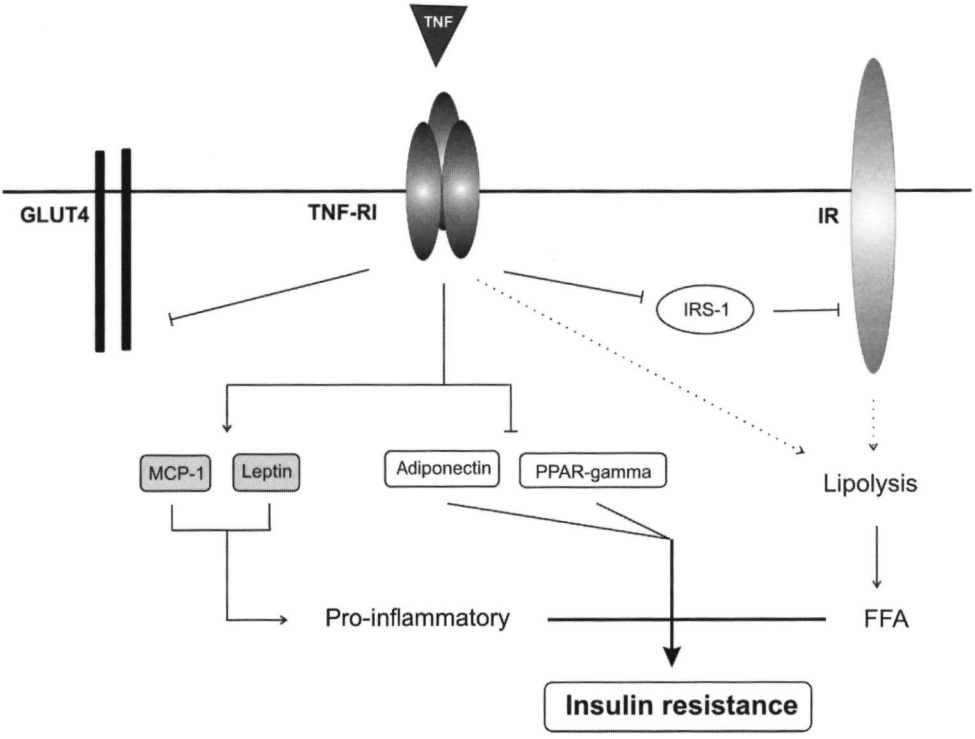

Figure 5. The mechanisms used by TNFα to exert its effects on glucose metabolic pathways. G = glucose; TNF-RI = TNFα receptor type I; IR = insulin receptor; GLUT4 = glucose transporter4; IRS-1 = insulin receptor substrate-1; MCP-1 = monocyte chemotactic protein-1.

loss [94,95]. In addition, there has been a significant positive correlation between the changes in circulating TNFα concentrations and body mass index (BMI). Adipocyte-derived TNFα is thought to function predominantly in an autocrine/paracrine manner in adipose tissue and it has been postulated to play a crucial role in the development of insulin resistance and glucose metabolism abnormalities that link obesity to type2 DM [11,96].

Indeed, TNFα and TNF-RI deficient mice have lower body weights than wild type mice and their sensitivity to insulin is increased; they are protected against obesity-induced insulin resistance [97,98]. *In vitro* studies on human cell-lines have confirmed that when exposed to TNFα, adipocytes become insulin-resistant [12]. In addition, human skeletal muscle cells overexpress TNFα, which therefore may contribute to the development of a generalized

insulin resistant state during inflammation [99] In contrast to TNF-induced changes in lipid metabolism, there are no significant differences between murine and humans regarding the TNF effects on insulin sensitivity Several mechanisms have been proposed to explain how TNFα induces the insulin resistance in adipocytes as well as systemically (**figure 5**) Firstly, TNFα has the ability to inhibit the insulin-stimulated tyrosine kinase activity of the insulin receptor (IR) and the insulin receptor substrate-1 (IRS-1) by inducing a serine phosphorylation of IRS-1 and thus converting IRS-1 into an inhibitor of the insulin receptor tyrosine kinase in vitro [12] This effect is mainly mediated via the TNF-RI [29-31], and involves the activation of the inhibitor kB kinase-β (IKK-β) [100] Secondly, TNFα stimulates the lipolysis in the adipose tissue, thus increasing the plasma concentration of the FFA that eventually contribute to the development of the insulin-resistant phenotype [42] Accordingly, the hepatic glucose production increases whereas the glucose uptake and metabolism in the muscles decrease In adipocytes, TNFα down-regulates the expression of several proteins implicated in the insulin receptor pathway, including IRS-1, GLUT4 glucose transporter, peroxisome proliferator activated receptor –γ (PPAR-γ) and adiponectin [12,101-103] In particular, adiponectin plays an important role in the mechanisms maintaining the sensitivity to insulin and its plasma concentration is inversely related with the degree of insulin resistance [104] Moreover, TNFα up-regulates the production of leptin, which is known to regulate energy homeostasis, to reduce pancreatic insulin secretion and to promote insulin resistance [105] Therefore, TNFα may also indirectly contribute to an insulin resistant state, by inhibiting adiponectin and by stimulating leptin actions on the glucose metabolic pathways In addition, MCP-1 (monocyte chemotactic protein-1) expression and production can be stimulated by TNF [106], increasing the recruitment of macrophages into the adipose tissue, which will augment the inflammatory state and will trigger the resistance to insulin

Pathological situations associated with a high TNFα production such as endotoxemia, cancer and trauma were indicated to present a state of peripheral insulin resistance The administration of TNFα to healthy humans was reported to reduce insulin sensitivity, inducing hyperglycemia without lowering insulin levels [107] In humans, the presence of a promoter polymorphism of TNFα (G-308A) is associated with increased plasma TNFα concentrations and a 1 8 higher risk of developing diabetes compared to non-carriers [108]

Finally, considerable attention is now focused on the mechanisms by which TNF induces resistance in the cascade of insulin signal transduction and the possibility that interference

with this pathway could be a new therapeutic approach to abrogate insulin resistance and thereby obesity-induced diabetes Although therapy with soluble TNFα receptors in diabetic insulin-resistant patients failed to prove its hypothesized beneficial effect [109,110], several recent reports support a favorable action of the anti-TNFα antibodies on insulin sensitivity [111,112]

6. TNF and adipocytokines

Adipocytokines such as leptin and adiponectin have been primarily described to be involved in the regulation of energy homeostasis and intermediary metabolism, including insulin sensitivity [113] Recently, leptin and adiponectin have been shown to interact with the immune system, with leptin bearing pro-inflammatory actions, whereas adiponectin seems to promote anti-inflammatory responses [114,115] Given their modulatory role in both metabolic and inflammatory processes, studies have been performed investigating leptin and adiponectin effects in rheumatoid arthritis (RA), a disease which is characterized by both chronic inflammation and disturbances of the intermediary metabolism Interestingly, the evaluation of leptin in RA has been focused until now on its relation with the inflammation, whereas adiponectin has been mainly investigated in the context of altered insulin sensitivity translated into a higher cardiovascular risk, known to be present in patients with RA

The studies exploring the role of leptin in the pathogenesis of RA have yielded controversial results *In vitro,* leptin can stimulate the production of pro-inflammatory cytokines, such as TNF [116], and leptin concentrations have been found by several investigators to be increased in both the blood and synovial fluid of patients with RA Leptin concentrations correlated with disease activity and the degree of inflammation [117-119] In line with this, mice bearing a mutated leptin gene (ob/ob) develop less arthritis than their wild-type littermates [120] These studies all support the hypothesis of a pro-inflammatory, pathogenic role of leptin in RA However, these data have been brought into question by more recent studies Firstly, *in-vitro* experiments have shown that repeated stimulation of cells with TNF, as it is likely to happen inside RA patients joints, is followed by a downregulation of leptin gene expression and production [116] Secondly, the milder arthritis observed in the *ob/ob* mice might not be directly due to the absence of leptin in the inflamed joints during antigen-induced arthritis (AIA) model Ob/ob mice are likely to have impaired thymic development and therefore may react in a milder manner when challenged with antigens in the case of AIA [121] Moreover, ob/ob mice have higher concentrations of the anti-inflammatory hormone corticosterone than wild type littermates,

which may lead to less severe arthritis [122]. Finally, we and others have found that in patients with RA, leptin concentrations are similar to those of age- gender- and BMI-matched healthy volunteers [123-127]. In addition, the chronic inflammation in RA and its suppression by anti-TNF agents have a limited influence on plasma leptin concentrations [125, 127].

Adiponectin has been mainly investigated in RA patients in relation to its role in enhancing the sensitivity of cells to insulin, thereby bearing favorable effects on cardiovascular risk. In addition, adiponectin has been initially shown to exert anti-inflammatory effects [115, 128]. Recent studies, however, have underlined that this adipocytokine may be also pro-inflammatory, especially in the joints [128-131]. This seems to be dependent on the molecular weight (MW) of adiponectin: high-MW together with the globular form of the molecule exerts pro-inflammatory effects, whereas the low-MW acts anti-inflammatory [132, 133].

Plasma adiponectin concentrations are higher in RA patients than in healthy individuals [119]. However, it is not known which of the above-mentioned molecular forms predominates in patients and controls. This may be crucial to the understanding of the studies on plasma adiponectin concentrations in RA. One interpretation would be that the higher adiponectin concentrations in RA may be the result of a counter-regulatory mechanism aimed to maintain insulin homeostasis in these patients at normal levels, with beneficial effects for the cardiovascular risk. Additionally, adiponectin may have predominantly anti-inflammatory actions, counteracting the pro-inflammatory mediators in RA. In line with this, an increase of adiponectin concentrations after TNF blockade, as recently reported by some authors [134,135], would only add to the above-mentioned beneficial effects of adiponectin in these patients.

Conversely, higher adiponectin concentrations based on the pro-inflammatory molecular forms would be unfavorable in these patients. Raising these concentrations would therefore be even more detrimental, whereas a decrease in adiponectin concentrations might signal the return of the system to a situation similar to that encountered in healthy individuals [Chapter 7 of this thesis]. Not only adiponectin molecular isoforms, but also patients' demographics and/or ethnicity, make the interpretation of the role of adiponectin in RA more difficult. Further studies are needed to elucidate this complex issue.

7. Concluding remarks

Inflammation plays a pivotal role in the development of metabolic syndrome features, including dyslipidemia and altered glucose tolerance These metabolic changes constitute the substrate for the subsequent development of atherosclerotic plaque and insulin resistance Among inflammatory markers, TNFα seems to be a crucial element in the pathogenesis of these conditions While lipid changes are beneficial to the host in the case of acute circulatory TNFα raising conditions, prolonged TNF-induced lipid modifications will increase cardiovascular risk and subsequent morbidity and mortality In this light, chronic inflammation in general and TNFα in particular are likely to represent the driving force connecting RA, atherosclerosis and the impaired insulin sensitivity that may occur simultaneously in one individual Firstly, TNFα plays a key role in the pathogenesis of RA, as we know from the pharmacological effects of TNFα blocking agents in the therapy of RA [19,20] Secondly, TNFα, as part of the inflammatory cascade, plays in this regard a crucial role in the development of atherosclerotic lesions [10,35,85]. In addition, TNFα is able to induce pro-atherogenic lipoprotein changes Finally, TNFα, by decreasing insulin sensitivity, contributes to the development of the glucose metabolism disturbances [17,94,98]

Given these facts, TNFα might emerge as a therapeutic target to combat the development and progression of the metabolic syndrome features, at least in RA and other chronic inflammatory conditions. Indeed, an anti-inflammatory therapy seems reasonable in the case of patients displaying markers of the metabolic syndrome since statins and PPARγ agonists have been recently proven to improve the lipid and glucose metabolic parameters using anti-inflammatory mechanisms [136,137] Several studies have explored the effectiveness of TNFα blockade in combating different features of the metabolic syndrome but the results obtained are not yet substantial [87-89,109-112] However, given the increasing incidence of metabolic disturbances and their associated pathology, especially in developed countries, studies that will further explore the feasibility of a TNFα blocker in these pathologic conditions, either as monotherapy or in combination with other drugs, are warranted

Further on, we found that chronic inflammation in RA and its suppression during anti-TNF therapy has limited influence on plasma leptin concentrations This casts doubts on the importance of leptin in RA pathogenesis Our findings further suggest that inflammation-induced metabolic abnormalities present during the course of RA, rather than inflammation *per se,* are the main modulators of circulating adiponectin concentrations in RA patients

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Abbreviations

ABCA1, ATP binding cassette A1; ABCG1, ATP binding cassette G1; AIDS, acquired immunodeficiency syndrome; BMI, body mass index; CEPT, cholesteryl ester transfer protein; CRP, C-reactive protein; CYP7A1, cholesterol-7α-hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; DM, diabetes mellitus; ERK (p44/42), extracellular signal-related kinase; GLUT4, glucose transporter4; Gi, Gi-protein-coupled adenosine receptor; GM-CSF, granulocyte macrophage-colony stimulating factor; HL, hepatic lipase; HSL, hormone-sensitive lipase; IL-1, interleukin-1; IL-6, interleukin-6; IL-18, interleukin-18; IR, insulin receptor; IRS-1, insulin receptor substrate-1; IKK-β, inhibitor kB kinase-β; JNK, c-jun-NH_2 –terminal kinase; LPL, lipoprotein lipase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage-colony stimulating factor; NF-kB, nuclear factor-kB; PK, protein kinase; PON1, paraoxonase-1; PLTP, phospholipid transfer protein; PPAR, peroxisome proliferator activated receptor; RA, rheumatoid arthritis; RCT, reverse cholesterol transport; SAA, serum amyloid A; SLE, systemic lupus erythematosus; sPLA2, secretory phospholipase A2; SR-BI, scavenger receptor class B type I; TG, triglycerides; TNF, tumor necrosis factor; TNF-RI, tumor necrosis factor receptor type I

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Part II: Anti-TNF therapy effects on inflammation

Chapter 9

Cytokine production of stimulated whole blood cultures in rheumatoid arthritis patients receiving short-term infliximab therapy

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Abstract

Patients with rheumatoid arthritis (RA) treated with anti-tumor necrosis factor (TNF) strategies have an increased susceptibility to infections, especially those caused by intracellular pathogens. In this study we assessed the cytokine production capacity in patients with RA and we further investigated whether anti-TNF therapy modulates the production of pro-inflammatory cytokines involved in the resistance against infections.

Methods: Whole blood cultures from 10 RA patients and 10 healthy controls were stimulated with heat-killed *Candida albicans, Salmonella typhimurium, Staphyloccocus aureus, Aspergillus fumigatus* or *Mycobacterium tuberculosis* and production of IL-1β, IL-6, IL-10, IFN-γ and TNF-α was measured.

Results: Before anti-TNF therapy, whole blood cultures from RA patients released significantly less IFN-γ than healthy controls after stimulation with all tested microorganisms. Short-term anti-TNF therapy did not have an inhibitory effect on the release of the cytokines tested.

Conclusion: We conclude that cells of patients with RA have a strongly reduced production capacity of IFN-γ after bacterial challenge. Although short-term therapy with anti-TNF agents did not further decrease the release of other proinflammatory cytokines, the combination of defective IFN-γ production in basal conditions and TNF neutralization during anti-TNF therapy is likely to be responsible for the higher susceptibility to infections in patients with RA.

Introduction

Tumor necrosis factor (TNF) is a proinflammatory cytokine that plays an important role in the pathogenesis of rheumatoid arthritis (RA) [1] TNF-α blockade with monoclonal antibodies and receptor fusion proteins has proved beneficial in RA [2-5] Despite the clinical, radiological and functional benefits of TNF inhibitors, safety issues of increased susceptibility to infections [6-8], especially due to *Mycobacterium tuberculosis* but also to other intracellular and opportunistic pathogens including *Pneumocystis carinii Histoplasma capsulatum Aspergillus fumigatus Listeria monocytogenes* and *Salmonella typhimurium* [6,8-11], are a serious concern

Cellular recognition of different microorganisms leads to release of proinflammatory cytokines, such as TNF, IL-1β, IL-18 and IFN-γ, which activate host defense [12] In-vitro stimulation of monocytes and macrophages with mycobacteria or mycobacterial products induces the production of TNF-α, which in turn increases phagocytosis, potentiates mycobacterial killing and is central in granuloma formation [13,14] In *in-vivo* models, mice treated with anti-TNF have delayed and insufficient granuloma formation and an increased susceptibility for mycobacterial infection

TNF also induces the production of cytokines such as IL-1β, IL-6 and chemokines and increases the expression of adhesion molecules, all of which are involved in cell recruitment and in the immune response towards different microorganisms [12] Besides TNF, IFN-γ plays a key role in the killing of microorganisms by macrophages, inducing the production of reactive oxygen (ROI) and nitrogen (RNI) intermediates [16] IFN-γ knockout mice are highly susceptible to *M tuberculosis* [17] and individuals lacking receptors for IFN-γ suffer from recurrent and sometimes lethal mycobacterial infections [18]

TNF-α neutralisation in RA results in decreased circulating levels of IL-6, IL-8, soluble adhesion molecules and reduces leukocyte traffic into inflamed joints, but little is known about its effect on cytokine production profile, after bacterial stimulation the latter may have consequences for the development of infections Recently, we have reported that whole blood cultures from patients with RA treated with anti-TNF agents that developed infections exhibit a significantly lower IFN-γ production than controls after stimulation with intracellular organisms [11] We suggested that this may contribute to the increased susceptibility of these patients to infections with intracellular organisms

In the present study our aim is to extend these observations in a group of non-treated RA patients and to assess the effect of anti-TNF therapy on cytokine production capacity

Materials and Methods

Patients and controls

Venous blood was collected from 10 healthy volunteers and 10 RA patients, matched for gender and age, at baseline and after 2, 6 and 14 weeks after initiation of anti-TNF therapy with the anti-TNF antibody infliximab (Schering-Plough) Informed consent was obtained from all study subjects

Whole blood cytokine production

Venous blood was collected from the cubital vein in 4-ml lithium heparin tubes Whole blood was diluted 1 5 with RPMI 1640 in 24-wells plates and stimulated with $1x10^7$ microorganisms/mL, as previously described by our group [26] The pathogens studied consisted of heat-killed *Candida albicans, Staphylococcus aureus Salmonella typhimurium Mycobacterium tuberculosis* and *Aspergillus fumigatus* Incubation with RPMI was used as negative control After incubation for 24h or 48h at 37°C, supernatants were obtained by centrifugation and stored at -80°C until assay In pilot experiments, TNF-α production from stimulated PBMCs reached the maximum level after approximately 8 hours and the highest IL-6 production was seen in the first 24 hours after stimulation, while IL-10 and IFN-γ reached the maximum concentration in the supernatants later, in approximately 48 hours Subsequently we have chosen 24 hours and 48 hours to measure the production of the above-mentioned cytokines Concentrations of TNF-α, IL-6 (after 24h of stimulation) and IFN-γ, IL-10 (after 48h of stimulation) were measured using commercial ELISA kits (Pelikine, CLB Amsterdam, The Netherlands), while IL-1β was measured using specific RIA

Statistical analysis Differences between groups were assessed using Mann-Whitney U-test Differences within groups were assessed using paired Wilcoxon test Unless otherwise stated, results are expressed as means ± standard deviation (SD)

Results

Cytokine production capacity in RA patients at baseline

Stimulation of whole blood cultures with each of the microbial stimuli resulted in profound inhibition of IFN-γ production in patients with RA as compared to healthy controls 0 13 ± 0 28

ng/mL vs. 2.8 ± 4.5 ng/mL when stimulated with *C. albicans* ($p<0.002$); 0.42 ± 0.12 ng/mL vs. 4.0 ± 4.2 ng/mL with *S. aureus* ($p<0.002$); 0.48 ± 1.1 ng/mL vs. 5.8 ± 3.8 ng/mL with *S. typhimurium* ($p<0.0004$); 0.010 ± 0.011 ng/mL vs. 0.09 ± 0.11 ng/mL with *A fumigatus* ($p<0.045$); 0.017 ± 0.02 ng/mL vs. 0.19 ± 0.36 ng/mL with *M. tuberculosis* ($p<0.1$). In contrast, production of IL-1β, TNF-α and IL-6 as well as IL-10 did not differ between RA and healthy controls, regardless of the stimulus used (fig.1).

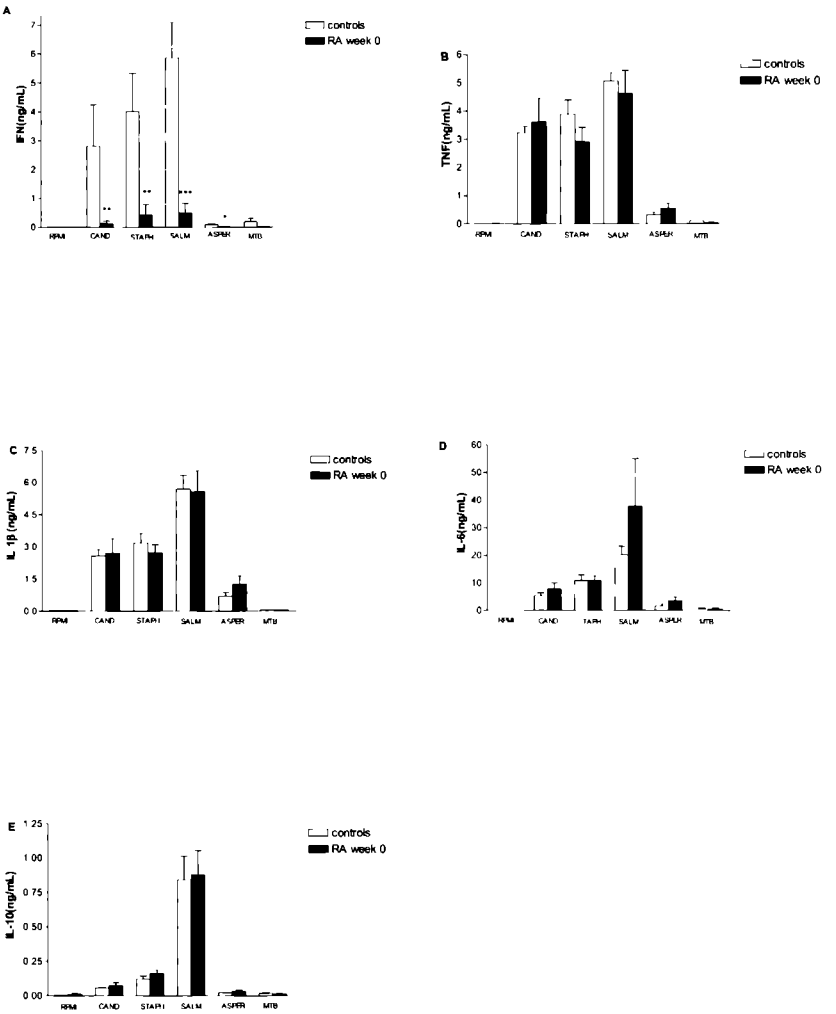

Figure 1 Production of IFN-γ (A), TNF-α (B), IL-1β (C), IL-6 (D) and IL-10 (E) from stimulated whole blood cultures in healthy controls (white bars) and RA patients (black bars) prior to receiving infliximab. Values are expressed as means ± SD *p* values, calculated using Man-Whitney U-test, are as follows *p<0.05, **p<0.002, ***p<0.0004.

The effect of anti-TNF treatment on cytokine production

Cytokines were measured in whole blood cultures where the presence of the therapeutic anti-TNF antibodies prevented us to reliable measure endogenous TNF production Therefore, during therapy, the capacity to produce TNF-α under microbial stimulation could not be assessed, although its concentrations measured in our samples were lower than prior the treatment (not shown) The production capacity of IL-10 after 2 weeks of anti-TNF therapy was slightly decreased when the blood was stimulated with *C albicans* and *S typhimurium*, but returned to basal levels at week 14 (fig 2) Anti-TNF therapy did not influence the capacity of whole-blood from RA patients to release IFN-γ (fig 2), IL-6 and IL-1β (not shown)

Discussion

In the present study, we show that RA patients at baseline have a significantly decreased production of IFN-γ compared to healthy controls, while the production of TNF-α, IL-6, IL-1β and IL-10 did not differ between the two groups Short-term treatment of RA patients with anti-TNF antibodies scarcely influenced IFN-γ, IL-6, IL-1β and IL-10 production capacity of these patients

Stimulation of whole-blood cultures with Gram-positive *S aureus C albicans* and intracellular microorganisms (*M tuberculosis* and *A fumigatus*) resulted in an impaired IFN-γ production in RA patients when compared to healthy controls IFN-γ is produced mainly in T cells and NK cells and its production is strongly modulated by other cytokines such as IL-12, IL-18 The decreased IFN-γ production might be due to a decreased in number of Th1 cells in the peripheral blood of RA patients [19-23] Due to the central role of IFN-γ for the activation of cellular immunity, we speculate that a decreased IFN-γ production in RA patients contributes to an increased susceptibility of these patients to infections The types of infection of patients taking anti-TNF drugs (tuberculosis, salmonelosis, candidosis) are compatible with decreased cell immunity and decreased IFN-γ [16] We have reported previously that RA patients treated with anti-TNF have a lower IFN-γ production, compared with RA patients that were not treated with anti-TNF agents [11] In the present study, RA patients had a much lower IFN-γ production before start of anti-TNF, compared to non-treated patients in the initial report This discrepancy might be explained by the different duration of disease, disease activity and response to therapy between the two groups recently diagnosed, mild RA responding to standard therapy in one group [11], and long-lasting RA, relatively severe disease where usual disease modifying drugs failed to control the disease, in the anti-TNF treated group of the present study

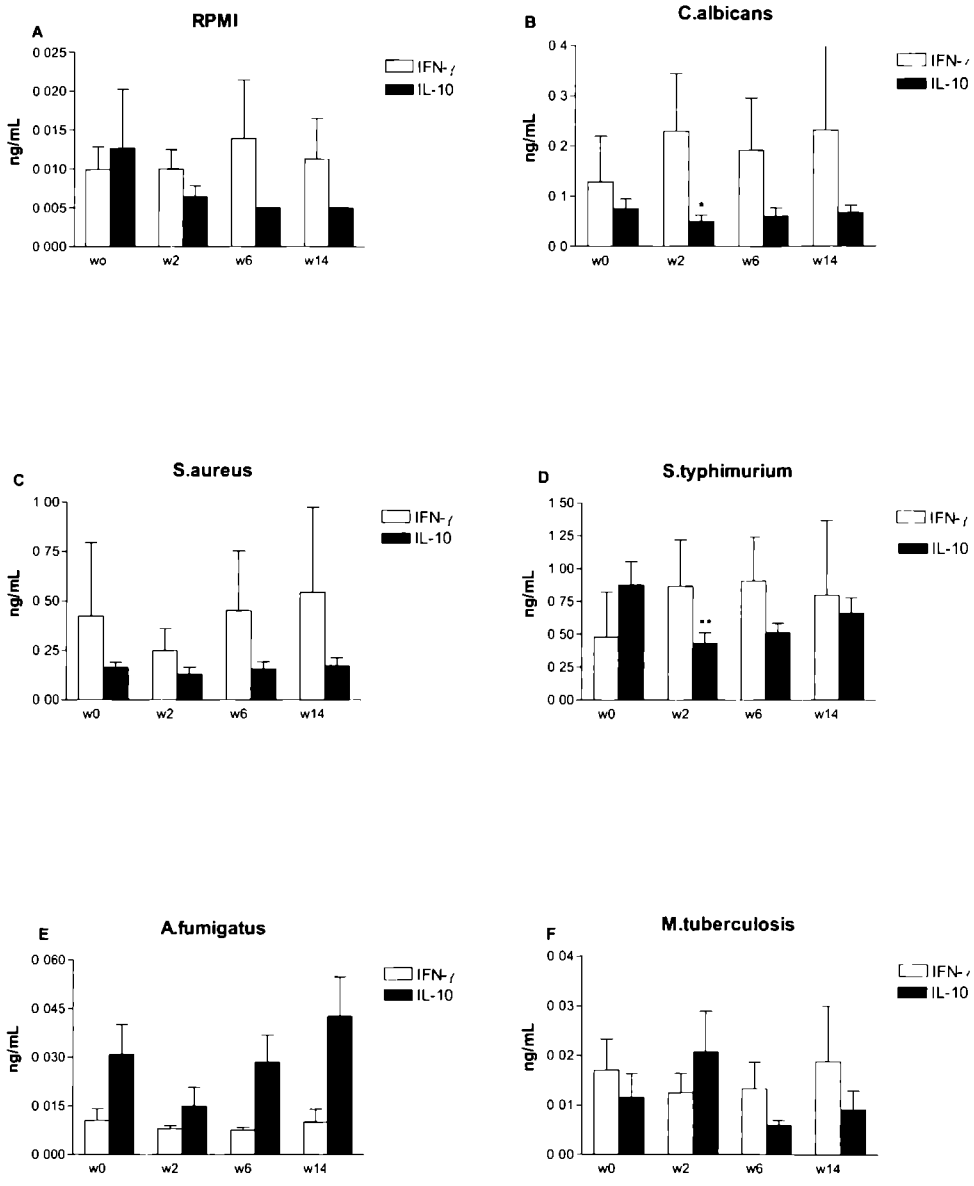

Figure 2 IFN-γ and IL-10 production from stimulated whole blood cultures in RA patients at baseline and after starting therapy with infliximab (w2, w6 and w14 represent week2, 6 respectively 14 of therapy) Values are expressed as means ± SD *p* values, calculated using Wilcoxon paired t-test are as follows *p<0 05, **p<0 005 The following stimuli were used RPMI (A), *C albicans* (B), *S aureus* (C), *S typhimurium* (D), *A fumigatus* (E), *M tuberculosis* (F)

From the present study, it is clear that the production of IFN-γ in RA is already disturbed at baseline and not influenced by short-term therapy with anti-TNF agents. This was also true for cytokines such as IL-1β and IL-6. TNF-α is a proinflammatory cytokine that plays a critical role in the regulation of the inflammatory processes during infections. TNF-α inhibition has led to a decreased ability to control infections, which was demonstrated both in animal models and in human studies [14,15,25]. We could not assess TNF-α production capacity due to the interference of the therapeutic anti-TNF antibodies in our whole blood cultures with the immunoassay.

In addition, IL-10 production from stimulated whole blood cultures after short-term therapy with infliximab for 14 weeks was scarcely influenced when compared with the one prior to therapy. Recently, Schuerwegh et al. have assessed TNF-α, IL-1β and IL-6 production of PBMCs immediately after the first dose of infliximab and 6 month after starting the therapy, and found the production of these cytokines significantly decreased at both time points after stimulation with E.coli LPS [24]. LPS from Gram-negative bacteria is known to trigger the cytokine production through binding toll-like receptor-4 (TLR-4). In a previous study, we showed that anti-TNF antibodies have significantly reduced the percentage of TLR-4 positive dendritic cells in RA patients [11] and this might explain the lower levels of TNF-α, IL-1β and IL-6 seen after PBMCs stimulation with LPS. Heat-killed microorganism, however, are much more complex structures than LPS and they may activate cells through interactions with different receptors, both TLRs and non-TLRs. From this perspective, evaluating cytokine production pattern after stimulation with heat-killed microorganisms known to be involved in the infectious complications of RA patients treated with anti-TNF agents, is much more relevant clinically. For the same reason, to mimic as close as possible the in-vivo situation, we have chosen to use a whole-blood stimulation method for the assessment of cytokine production, instead of purified cell population. The advantage of a whole-blood assay is that it contains all relevant cell-populations from blood that come in contact with the invading pathogen and it has all the circulating plasma components.

In conclusion, we suggest that the combination of a preexisting severely depressed IFN-γ production together with neutralisation of TNF-α induced by anti-TNF treatment is very likely to play a major role for the impaired host defence against intracellular and fungal microorganisms observed in RA patients undergoing this kind of therapy. Further investigations need to be done

on a large number of patients and for longer periods in order to assess the effects of anti-TNF therapy on the long-term.

Abbreviations

RA = rheumatoid arthritis; TNF = tumor necrosis factor; IL = interleukin; IFN = interferon; ROI = reactive oxygen intermediates; RNI = reactive nitrogen intermediates; SD = standard deviation; ELISA = enzyme-linked immunosorbent assay; RIA = radio-immuno assay; PBMC = peripheral blood mononuclear cell; TLR = toll-like receptor; LPS = lipopolysaccharide

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Chapter 10

Cytokine production from stimulated whole blood cultures in rheumatoid arthritis patients treated with various TNF blocking agents

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Eur Cytokine Netw 2008 [conditionally aceepted]

Abstract

Infectious complications are not rare in rheumatoid arthritis (RA) and the susceptibility to infections is increased during treatment with TNF blocking agents As a possible mechanism contributing to that, we assessed the modulation of cytokine production induced by TNF neutralization

Methods Whole blood cultures from 6 healthy volunteers and 13 RA patients starting therapy with either adalimumab (N = 7) or etanercept (N = 6) were stimulated with heat-killed *Salmonella typhimurium*, *Staphylococcus aureus* or with *S typhimurium* lipopolysaccharide (LPS) The production of interleukin (IL)-1β, IL-6, IL10, IL-17, TNF and IFN-γ was measured by specific immunoassays

Results Stimulation with *Salmonella* LPS resulted in a significant lower production of IL-1β, TNF and a trend towards lower IL-6 and IFNγ production in RA patients compared to healthy volunteers Therapy with either of the agents did not significantly change the cytokine production capacity, with the exception of a lower IFNγ production in patients treated with adalimumab and stimulated with *Salmonella* LPS, and lower IL-6 production in those treated with etanercept

Conclusion The detrimental effects of anti-TNF agents on immune response can largely vary, from severe to mild As reported in this study, we did not find major differences in the effects of adalimumab and etanercept on cytokine production, arguing that the direct TNF-mediated effects, rather than indirect action on other proinflammatory cytokines, are responsible for their differential influence on susceptibility to infections However, caution should be constantly exercised to prevent the development of severe infections when therapy with anti-TNF is started in RA patients

Introduction

Treatment strategies that modulate pro-inflammatory cytokines such as tumor necrosis factor (TNF) – α and interleukin-1 (IL-1) -β constitute a breakthrough in the treatment of rheumatoid arthritis (RA) and other inflammatory diseases including juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, and Crohn's disease Agents including anakinra, adalimumab, etanercept, and infliximab yield substantial improvement in symptoms, disability, and quality of life and prevent joint damage in early and long-standing RA However, safety issues of increased susceptibility to infections in individuals receiving these treatments, particularly with intracellular pathogens such as *Mycobacterium* species [1], represent a serious concern

Interestingly, the rate of severe infections is lower in patients treated with the soluble receptor etanercept than in those treated with monoclonal anti-TNF agents, such as adalimumab and infliximab [2-5] This may be due to differences in the capacity of these drugs to interact with soluble and membrane-bound TNF, to activate complement and to induce cytolysis [6,7], but the exact mechanisms are not completely known

Cellular recognition of pathogens involves binding to pattern recognition receptors (PRRs), including toll-like receptors (TLR), which ultimately leads to the release of proinflammatory cytokines, such as TNF, IL-1β and IFN-γ, and activation of host defense *Salmonella typhimurium* and *Staphylococcus aureus* are two microorganisms that have been previously reported to be able to cause severe infections in RA patients receiving anti-TNF drugs [8-11] TNF neutralisation in RA results in a marked decrease of circulating acute phase reactants, IL-6, IL-8 and soluble adhesion molecules but does not affect white blood cells (WBC) counts and differentiation [12] With the exception of infliximab, which seems to have no effect on the capacity of blood cells to produce IL-10 and IFN-γ after challenged with microbial agents [10,13], little is known about the effect of the other anti-TNF agents on cytokines production capacity and the latter may be crucial for preventing infections In the present study we assessed the effect of adalimumab and etanercept on cytokine production capacity after microbial challenge

Patients and Methods

Patients and controls

Six healthy controls and 13 RA patients that were about to start anti-TNF treatment with either adalimumab (N= 7) or etanercept (N= 6) and attending our outpatient clinic were enrolled in the study, after giving written informed consent. Adalimumab was given in subcutaneous injections in a dose of 40mg every other week, while etanercept was delivered in doses of 25mg twice weekly. Stable dosages of disease-modifying anti-rheumatic drugs (DMARDs) and oral corticosteroids (CS, prednisone < 10 mg/day) were allowed during the study. Patient characteristics are presented in more detail in Table 1. Patients received anti-TNF drugs for a period of at least 3 months. The regional medical ethics committee approved the study.

Whole blood cytokine production

Cytokine production in whole blood cultures has been investigated as previously described [13]. In short, venous blood was collected from the cubital vein in 4-ml lithium heparin tubes. Whole blood was diluted 1:5 with RPMI 1640 in 24-wells plates and incubated at 37°C with heat-killed *Salmonella typhimurium* (10^7 microorganisms/ml), *Staphylococcus aureus* (10^7 microorganisms/ml), and *S. typhimurium* LPS (μg/ml). Incubation with RPMI was used as negative control. TNF, IL-6, IFN-γ, IL-10, IL-1β and IL-17 production was measured in the supernatants using commercially available kits (Bio-Rad) according to the manufacturer's instructions. Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad). The sensitivity of the cytokine assay was < 5 pg/ml for all cytokines measured.

Statistical analysis. Differences between groups were assessed using Mann-Whitney U-test. Differences within groups were assessed using paired Wilcoxon test. Unless otherwise stated, results are expressed as means ± standard error of the mean (SEM).

Table 1 Characteristics of patients

	Controls	RA patients (total)	RA patients (adalimumab)	RA patients (etanercept)
Age (years)		61 ± 13	61 ± 15	60 ± 10
Gender (F:M)		8 5	5 2	3 3
WBC ($x10^9$/l)				
baseline		6.98 ± 2 35	8 10 ± 1 46	5 68 ± 2 62
3 months		6.48 + 2 14	7 57 ± 1.56	5 22 ± 2.13
DAS28				
baseline		4.29 ± 1 79	4 29 ± 1 79	4 61 ± 1.16
3 months		2.65 ± 1 75*	2 65 ± 1.75*	3 61 ± 0 58*
ESR (mm/h)				
baseline		22 ± 24	16 ± 13	28 + 32
3 months		15 ± 21*	9 + 6*	22 ± 31*
CRP (mg/l)				
baseline		21 ± 27	18 ± 17	24 + 38
3 months		9 ± 12*	6 ± 3*	12 ± 18*
Medication (N)				
Methotrexate		3	2	1
Corticosteroids		2	2	

WBC = white blood cells count, DAS28 = disease activity score; ESR = erythrocytes sedimentation rate, CRP = C-reactive protein, Results are expressed as mean ± SD, *p < 0 05

Results

Cytokine production capacity in RA at baseline

Stimulation of whole blood cultures with *Salmonella* LPS resulted in a significant lower production of IL-1β (567 ± 119 pg/ml vs. 289 ± 49 pg/ml, p = 0.042) and TNF (417 ± 77 pg/ml vs. 198 ± 36 pg/ml, p = 0.015) (Figure 1A) in RA patients compared to healthy controls. The production of IL-6 and IFN-γ was also lower in RA patients though this did not reach statistical significance: 4992 ± 596 pg/ml vs. 3609 ± 492 pg/ml (p = 0.06) for IL-6 and 382 ± 53 pg/ml vs.

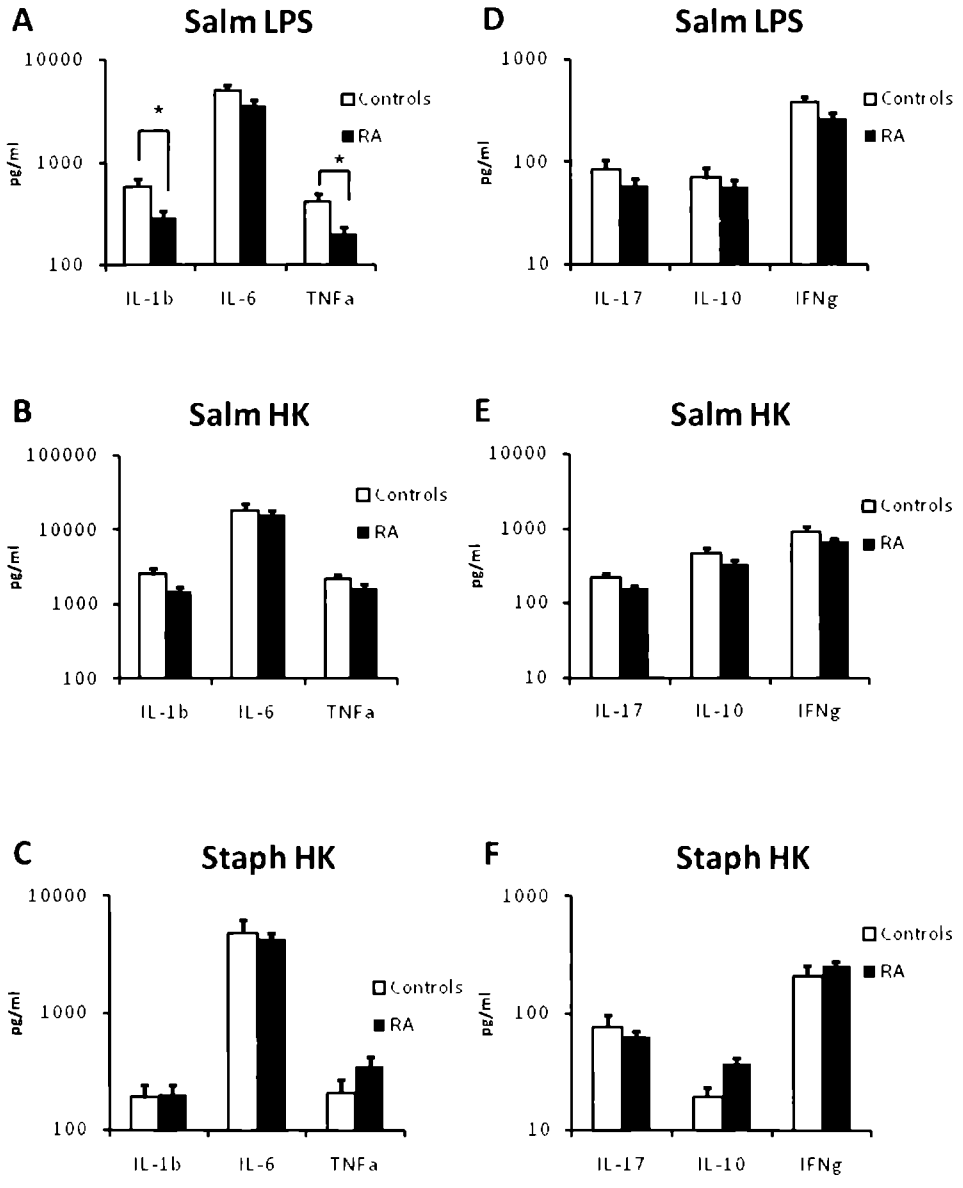

Figure 1 Production of cytokines in healthy controls (white bars) and RA patients prior to receiving anti-TNF therapy(black bars) Whole blood cultures were stimulated with *Salmonella typhimurium* lipopolysaccharide (Salm LPS) (A,D), heat-killed *Salmonella typhimurium* (Salm HK) (B,E) and heat-killed *Staphyloccocus aureus* (Staph HK) (C,F) Values are expressed as means ± SEM p value calculated using Mann-Whitney U-test, *$p < 0.05$

Figure 2

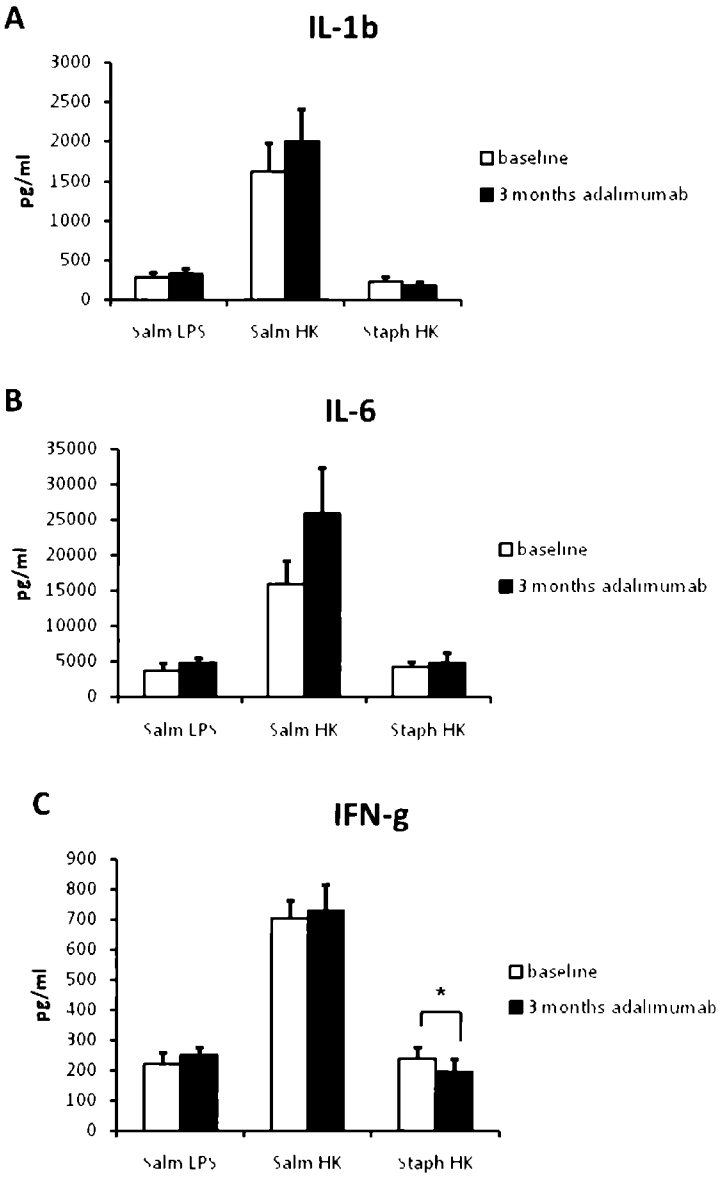

Figure 2 IL-1β (A), IL-6 (B) and IFNγ (C) production from stimulated whole blood cultures in RA patients treated with adalimumab The following stimuli were used *Salmonella typhimurium* lipopolysaccharide (Salm LPS) heat killed *Salmonella typhimurium* (Salm HK) and heat-killed *Staphylococcus aureus* (Staph HK) Values are expressed as means ± SEM *p* value calculated using Wilcoxon paired *t*-test *p < 0 05

264 ± 38 pg/ml (p = 0 06) for IFN-γ, respectively (Figure 1A,D) Interestingly, cytokine production in RA and controls did not differ after stimulation with the whole *S typhimurium* microorganisms (Figure 1B,E) or with heat-killed *S aureus* (Figure 1C,F)

Effects of anti-TNF therapy on cytokine production

Cytokines were measured in the whole blood cultures, where the presence of the therapeutic anti-TNF drugs prevented us to reliable measure endogenous TNF production Therefore, during the therapy, the capacity to produce TNF under microbial stimulation could not be assessed In RA patients treated with adalimumab, the production capacity of IFNγ after 3 months of treatment was slightly decreased when blood was stimulated with *S aureus* (240 ± 39 pg/ml vs 194 ± 44 pg/ml, p < 0 05) (Figure 2), whereas IFNγ production capacity remained unchanged in etanercept users (Figure 3) IL-6 production after stimulation with *S typhimurium* decreased in etanercept users (15049 ± 5791 pg/ml vs 8966 ± 1640 pg/ml, p < 0 05) (Figure 3), but showed a trend to increase in adalimumab users (15859 ± 3321 pg/ml vs 25984 ± 6472 pg/ml, p = 0 06) (Figure 2) IL-1β production did not change within three months of therapy with any of the agents tested (Figure 2 and 3) These observed changes in cytokine production were not explained by changes in WBC count since these remained stable during the present study (Table 1)

Discussion

In the present study we show that rheumatoid arthritis patients react differently to microbial stimuli compared to healthy individuals in terms of cytokine production capacity in a whole-blood stimulation model Therapeutic blockade of TNF with adalimumab or etanercept had a limited influence on cytokine production capacity of these patients, with only subtle differences between the anti-TNF agents used by the patient

Previous studies from our group have indicated that especially the capacity of immune cells to produce IFNγ in response to bacterial stimuli is impaired in patients with RA compared to healthy volunteers [10,13] In one study, anti-TNF therapy with infliximab was suggested as the main factor responsible for that, since the capacity of cells to produce IFNγ was restored three weeks after the drug was discontinued [10] Alternatively, long-lasting and relatively severe disease together with the failure of other DMARDs to control this process were likely to determine a lower IFNγ production capacity in RA patients prior to infliximab therapy [13] In contrast to infliximab, adalimumab at the lowest recommended doses and etanercept have been

Figure 3

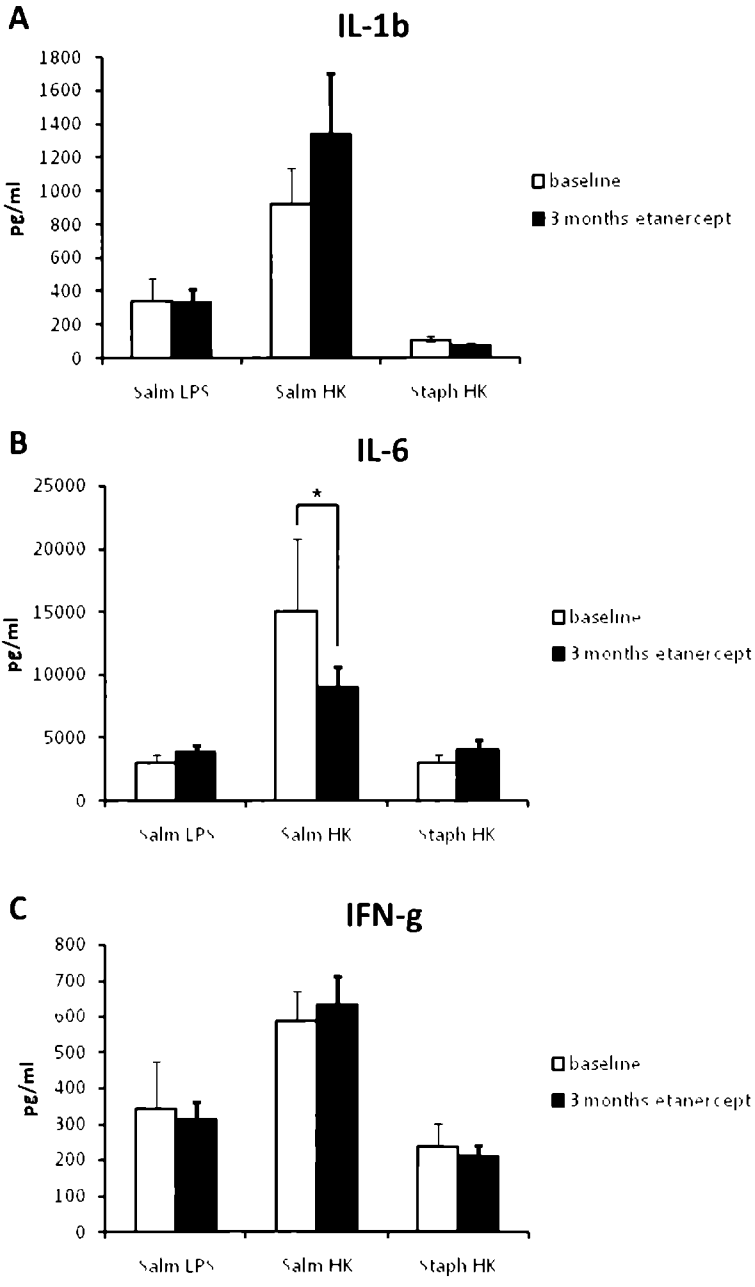

Figure 3 IL-1β (A), IL-6 (B) and IFNγ (C) production from stimulated whole blood cultures in RA patients treated with etanercept The following stimuli were used *Salmonella typhimurium* lipopolysaccharide (Salm LPS), heat-killed *Salmonella typhimurium* (Salm HK) and heat-killed *Staphylococcus aureus* (Staph HK) Values are expressed as means ± SEM *p* value calculated using Wilcoxon paired *t*-test, *p < 0 05

previously indicated to have a lower risk for inducing the development of severe infections [2-4]. In the present study, a small but reproducible inhibitory effect of adalimumab on IFNγ production capacity was observed after 3 months of treatment, whereas etanercept did not influence IFNγ release. Nevertheless, the extent of this decrease makes it improbable that this inhibition has major effects on susceptibility to infections. However, it cannot be excluded that higher doses of adalimumab may lead to stronger decrease of IFNγ production at levels similar to those induced by infliximab[10], in line with the dose-dependency of adalimumab infectious side-effects reported by epidemiological studies [3]. In addition, therapy with either of the drugs did not affect the capacity of immune cells to produce IL-1β, while the only difference in IL-6 release was observed in blood of the RA patients treated with etanercept. Therefore, the similar capacity of immune cells to respond to bacterial products during therapeutic TNF blockade with these agents may be of clinical relevance and explain the lower incidence of infections in patients treated with these agents.

Our findings suggest that RA patients treated with etanercept or adalimumab have a preserved capacity to release cytokines when stimulated with whole bacteria, despite a tendency to produce less TNF and IL-1β when challenged with *S. typhimurium* LPS, a TLR4 stimulus. Recent systematic and vigorous screening for latent tuberculosis infection in RA patients before starting anti-TNF therapy is likely to account for a different immunologic background of the RA patients in this study compared with previous investigations, rendering them to be more immuno-competent compared to the patients in previous studies [3]. Alternatively, the limited number of patients investigated might contribute to our results and therefore reflect a large variation of the immune response to infectious agents which occur in RA population.

The results of our study suggest that the detrimental effects of anti-TNF agents on the immune response can vary quite largely, from very serious to limited effects, as reported in this study for etanercept and adalimumab. Although the medical history of every patient has a decisive role in selecting the appropriate treatment, when therapy with anti-TNF is started caution should be constantly taken in order to prevent the development of severe infections.

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Chapter 11

Anti-TNF therapy in rheumatoid arthritis and the susceptibility to infections.

Discussion Part II

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Neth J Med 2005, 63 78-80

Treatment strategies interfering with the bioactivity of proinflammatory cytokines such as tumour necrosis factor alpha (TNF) and interleukin-1 (IL-1) constitute a breakthrough in the treatment of rheumatoid arthritis and other inflammatory disorders. However, before their introduction in clinical practice, it was demonstrated in experimental models that treatment with antibodies against TNF was deleterious in mycobacterial infections, fungal infections and abscesses. The exact mechanisms by which interference with TNF produced these results were not entirely clear, but it was concluded that containment of microorganisms within granulomas and abscesses was not achieved or maintained properly [1-3]. Based on these observations, it was easy to predict that large-scale and prolonged anti-TNF treatment in humans would also lead to an increased susceptibility to infections, especially by organisms that lead to a granulomatous response. Indeed, such complications (especially mycobacterial and salmonella infections) were readily encountered, but still seemed to come as a surprise to the medical community.

The interpretation of safety data relating to the use of anti-TNF agents may be influenced by numerous factors. Restricted entry into clinical trials, based on comorbidities and concomitant medications, results in an unique population with lower risks of adverse events than in the general population. When referring to post-approval studies, the data emerging from these studies are limited by other factors, i.e. underreporting, incomplete and unverifiable data

Table 1 The main differences between the three anti-TNF agents currently on the market

	Infliximab	**Adalimumab**	**Etanercept**
Binding to sTNF	+	+	+
Binding to LTα	-	-	+
Binding to mTNF	+*	+*	+
CDC	+	+	-
ADCC	+	+	+
Apoptosis	+	+	-
Half-life	+++	++	+

sTNF = soluble TNF, mTNF = transmembrane TNF, LTα = lymphotoxin α, CDC = complement dependent cytotoxicity, ADCC = antibody-dependent cell-mediated cytotoxicity, * = binding is stable compared to etanercept, which dissociates very quickly

acquisition, availability of drugs, and ascertainment bias Nonetheless, conclusions regarding the safety of therapeutic TNF blockade can be drawn and are further discussed in this chapter

One of the first observations to be made is that the risk for infection is greater with the monoclonal antibodies against TNF, infliximab and adalimumab, than with the TNF receptor construct, etanercept When referring to tuberculosis (TB), the most frequent serious infectious side-effect of these drugs, the initial analysis of patients reported to have developed TB during anti-TNF therapy demonstrated a predictable pattern of disease for patients who are immunosuppressed [4] Most of the patients developed extrapulmonary TB disease, and nearly a quarter had disseminated disease This is in contrast with 15% extrapulmonary disease and <1% disseminated disease in immunocompetent persons [5] In one recent study comparing background TB incidence rates between RA patients treated and not with infliximab in the US, the authors found that the background TB rate was 6 2 cases per 100 000 patients/year in the group of RA patients not receiving infliximab, compared with the infliximab-treated RA reported TB rate of 52 2 per 100 000 patient-years of exposure [6] While similar to the US data, EU post-approval studies have estimated a higher rate of TB in infliximab-treated patients compared with the background rate of TB in RA [7] In the case of adalimumab, a dose-response association between the drug and TB was seen, more patients reactivated TB on a higher dose of the agent, and less TB reactivation was observed in patients on a lower dose of adalimumab if no prior screening for TB had been performed [8] In contrast, fewer cases of TB have been reported after use of etanercept [9,10] The same differences between these anti-TNF drugs are also observed in the case of overall risk for infections during infliximab therapy the risk is estimated to be 200 infections per 100000 treatments, with etanercept the risk is 9 infections per 100000 [4] These observations initiated a series of studies investigating the mechanisms of action that might explain the different incidence of infections during therapy with various TNF-blockers

The precise mechanism through which anti-TNF drugs impair the host defense in various degrees in patients is still unknown, but their binding properties, pharmacokinetics and function are most likely to account for this variation (Table1) In the effort to explain the different incidence of infections between patients using the two classes of anti-TNF agents, namely antibodies and soluble receptors, understanding the interaction of these drugs with the transmembrane form of TNF becomes crucial *In-vitro* experiments have suggested that cells expressing transmembrane TNF are vulnerable to death by either complement-mediated cell lysis or antibody-dependent cytotoxicity when exposed to anti-TNF antibodies [10-12] However, clinical studies reveal a different mechanism of cell death associated with

immunoglobulin G1 (IgG1) antibodies [13] By mechanisms that are presently unclear (although likely due to reverse signaling), a cascade is initiated by membrane-associated TNF that is engaged by infliximab or adalimumab, which further activates caspase-8 and finally results in activation of caspase-3 This will stimulate the release of reactive oxygen species from mitochondria, which in turn bring about the disintegration of nuclear DNA and will initiate the process of apoptotic cell death [14-16] Although etanercept is constructed with the complement receptor domains of human IgG1, one of the CH_2 groups at the hinge region of the fusion of the Fc chain to the p75 extracellular domain of the TNF receptor is missing To what extent the deleted CH_2 could explain the different binding properties of etanercept and infliximab or adalimumab is unclear, but etanercept is not able to activate complement and does not lyse cells expressing transmembrane TNF

Interestingly, it is especially $CD4^+$ and $CD8^+$ T cells that have the ability to express membrane-bound TNF and therefore treatment with monoclonal anti-TNF antibodies primarily affect the number of peripheral $CD4^+$ and $CD8^+$ T cells [17] A reduction of Th1 response and interferon-gamma (IFNγ) production was observed during infliximab therapy in patients with rheumatic diseases [18], despite a defective IFNγ production background observed in anti-TNF-naive RA patients [19] Therefore, therapeutic blockade of TNF using monoclonal antibodies is likely to affect crucial players in the formation and maintenance of granuloma, this may partly explain the higher incidence of TB reactivation reported in these patients, when compared with those receiving etanercept [17,20]

We have demonstrated in RA patients receiving infliximab who developed serious Salmonella infections, that IFNγ production was strongly inhibited [18] The role of the latter cytokine in host defense against intracellular bacteria is well-known a deficient response to this cytokine has been shown to lead to serious infections [18,21,22] However, our recent findings suggest that RA patients treated with adalimumab or etanercept have a preserved capacity to release cytokines when stimulated with whole bacteria, with only subtle differences between the anti-TNF agents used [23] Another important difference between these TNF blocking agents relates to their circulation half-life Shorter-acting agents such as the soluble TNF receptor etanercept will allow more rapid restoration of the host defense functions, compared with the long-acting antibodies infliximab and adalimumab Although not interfering with the overall incidence of infections, the different elimination half-life might yet contribute to the decreased incidence of

tuberculosis infection in etanercept-treated patients compared with patients receiving anti-TNF antibodies

The development of infections, especially TB, in patients receiving TNF blockers represents a serious concern Guidelines for assessing risk and managing TB infection in patients due to start anti-TNF treatment have been therefore developed Screening for latent TB infection, which includes taking a history of exposure to *Mycobacterium tuberculosis*, a tuberculin skin test, and a chest radiograph, is advised in all patients before the use of TNF blockers [24] The impact of screening for latent TB infection prior to initiating TNF blocker therapy has been reported recently Perez et al [25], and Gomez-Reino et al [26] have suggested a consistent reduction in TB reactivation after screening according to these guidelines

In conclusion, as with any immunosuppressive agent, safety considerations remain an important issue when anti-TNF therapy is employed in patients with RA or Crohn's disease However, despite their side effects, anti-TNF agents have a very good risk/benefit ratio Caution is warranted when these drugs are given to patients who already have an increased risk of developing any of the complications seen with these agents

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Chapter 12

Summary and conclusions

For the last decade, anti-TNF agents have been considered a breakthrough in the therapeutical armamentarium of chronic inflammatory diseases, such as rheumatoid arthritis (RA). Numerous studies have proved that monoclonal anti-TNF antibodies and TNF-soluble receptor constructs are equally efficient in reducing disease activity and improving the life span of patients with RA. However, because TNF is a pleiotropic cytokine, it was not difficult to envisage that anti-TNF agents would also interfere with other pathophysiological processes controlled by TNF in these patients, besides those related to joints' inflammation. Indeed, a higher number of serious infectious side-effects have been reported in RA patients taking TNF blockers compared to anti-TNF naïve RA patients, highlighting that host defense is impaired by these drugs. The understanding of the underlying mechanisms responsible for this major side-effect is essential in our attempt to diminish the rate of serious infections in these patients. Cytokine production capacity of immune cells may be of crucial importance in this regard.

During inflammation, high TNF concentrations are interfering with the intermediary metabolism, resulting in changes of lipids pattern and glucose/insulin homeostasis. Although favoring the acute immune response, these changes are detrimental if lasting for longer periods corresponding to persistent high TNF concentrations, as in the case of patients with RA. Indeed, an altered lipid pattern, an impaired glucose tolerance and accelerated atherosclerosis have been shown to occur in RA patients, resulting in a higher cardiovascular risk than in the general population. Therefore, it was tempting to hypothesize that therapeutical TNF blockade may also have "favorable side-effects" and improve lipids pattern, glucose tolerance and cardiovascular risk in these patients. The aim of this thesis was to gain more insight into the modulation of intermediary metabolism pathways, as well as the immune response, by patients with RA treated with anti-TNF agents.

The first part of the thesis assessed the capacity of anti-TNF antibodies to influence lipids concentrations. In chapter 2, the effects of short-term anti-TNF therapy on plasma lipoprotein concentrations has been investigated. It has been previously indicated that TNF could increase triglycerides and decrease total and HDL cholesterol concentrations. Two weeks after administration of adalimumab, a fully human anti-TNF monoclonal antibody, we observed a significant increase of HDL cholesterol concentrations together with a decrease in total:HDL-cholesterol ratio. In contrast, no changes were observed in a placebo treated group. We therefore concluded that shortly after it has been initiated, TNF blockade in RA patients is accompanied by an anti-atherogenic effect, due to both a decrease of inflammatory status and an improvement of

lipid pattern. However, to be of clinical significance these results had to be confirmed after prolonged treatment with these agents.

This question was addressed in chapter 3, in which we studied whether the effects observed short after anti-TNF therapy has been initiated are sustained during longer periods of treatment. To this aim we investigated a group of RA patients starting the therapy with infliximab, a chimeric anti-TNF monoclonal antibody. In this study we were able to confirm the short-term effects of anti-TNF on lipid pattern that were previously observed. However, 6 months and even 12 months after the initiation of this therapy, we noticed an increase in total cholesterol and total:HDL-cholesterol ratio, compatible with a worsening of the lipids pattern. It is therefore concluded that long-term TNF blockade worsens plasma lipid profile in RA. However, the impact of this finding on the global cardiovascular risk is difficult to estimate, due to lipid-independent beneficial effects of anti-TNF drugs, namely reduced homocysteine levels, improved insulin sensitivity and reduced inflammatory status.

In the light of the recent literature suggesting that inflammation may modulate the anti-atherogenic mechanisms of HDL, we investigated in chapter 4 whether and how does TNF blockade interfere with this process. The anti-oxidative capacity of HDL has been assessed by measuring the activity of paraoxonase (PON)-1, the main enzyme responsible for the anti-oxidative capacity of HDL. In addition, an *in vitro* assay to test the global capacity of HDL to directly neutralize oxidized-LDL has been developed and used for the same purpose. Although plasma HDL-cholesterol concentrations have not been affected 6 months after anti-TNF therapy has been initiated, the anti-oxidative capacity of HDL significantly improved during the same interval. Our results suggest that the overall effects of anti-TNF on circulating lipids may be yet beneficial and of clinical relevance, since anti-TNF treatment has been recently shown to decrease the incidence of myocardial infarction in RA patients responding to this therapy.

The effects of anti-TNF therapy on insulin sensitivity have been investigated in Chapter 5. We observed that short-course TNF blockade has been able to improve insulin sensitivity assessed by the hyperinsulinaemic-euglycaemic clamps . This was associated with a decrease in the inflammatory status of these patients. Our findings are in line with previous studies reporting that anti-TNF corrected the disturbances in glucose metabolism in rheumatoid arthritis patients and support a beneficial effect of TNF blockers on their cardiovascular risk. Interestingly, despite the encouraging findings in animal models and patients with chronic inflammatory diseases, TNF

blockade has not been shown to be beneficial in diabetic patients yet The doses and the agent itself might be responsible for the lack of beneficial effects in these patients

The influence of anti-TNF on the circulating concentrations of leptin and adiponectin have been investigated in Chapter 6 and 7 We found that the chronic inflammation of RA and its suppression during anti-TNF therapy have limited influence on plasma leptin concentrations, while significantly affecting circulating adiponectin levels RA patients displayed higher adiponectin concentrations than controls, and these were lowered by anti-TNF therapy Body mass index (BMI) and thus most likely fat tissue, remained the major determinant of plasma leptin concentrations, which is in agreement with previous studies in RA and other chronic inflammatory conditions The results of our studies may therefore cast doubt on the detrimental role of circulating leptin on RA development The suppressive effects of concomitant anti-TNF and oral corticosteroids on plasma adiponectin concentrations were not previously described and will be investigated in future studies

A second major effect of anti-TNF treatment is exerted on host defense mechanisms The effects of therapeutical TNF blockade on the immune response towards various microbial agents was investigated in chapter 9 and 10 We initially observed that especially the capacity of immune cells to produce IFNγ in response to bacterial stimuli is impaired in patients with RA compared to healthy volunteers Interestingly, anti-TNF therapy with infliximab was suggested as the main factor responsible for that, since the capacity of cells to produce IFNγ restored three weeks after the drug was discontinued In contrast to infliximab, a small but reproducible inhibitory effect of adalimumab on IFNγ production capacity was observed after 3 months of treatment, whereas etanercept did not influence IFNγ release Nevertheless, the extent of this decrease makes it improbable that this inhibition has major effects on susceptibility to infections, unless the effect is stronger at the tissue level. Therapy with either of the drugs did not affect the capacity of blood cells to produce IL-1β, while the only difference in IL-6 release was observed with blood cells of the RA patients treated with etanercept Thus, during therapeutic TNF blockade with low-dose adalimumab and etanercept white blood cells respond to bacterial products with similar productions of proinflammatory cytokines and may explain the lower incidence of infections in patients treated with these agents as compared to infliximab

Conclusions and future perspectives

In this thesis the effects of anti-TNF treatment on intermediary metabolism on the one hand, and on the host defense mechanisms on the other hand, have been investigated The data presented in chapters 2-4 clearly demonstrates that anti-TNF agents are able to modulate lipids pattern in RA patients receiving this treatment These effects are echoed not only at the level of plasma concentrations (LDL, HDL and triglycerides), but also at the functional level (HDL anti-oxidative capacity) One of the main conclusion of these studies is therefore that anti-TNF therapy modulates plasma lipoprotein profiles, while improving the anti-oxidative capacity of HDL Another conclusion drawn from Chapter 5 is that anti-TNF is able to improve insulin sensitivity in patients with RA From the results in chapter 6 and 7 we conclude that inflammation and its suppression during anti-TNF blockade has an important impact especially on adiponectin plasma concentrations of RA patients Finally, the investigation of immunomodulatory capacity of anti-TNF agents in chapters 9 and 10 has led to the conclusion that this therapy also influences the immune response to various microorganisms

An important point of discussion especially when analyzing our metabolic data was whether the effects observed under TNF blockade are indirectly determined by changes of inflammatory status in these patients This would imply that any other anti-rheumatic drug showing an important anti-inflammatory effect would have similar effects on lipids as anti-TNF agents do This is an important question which deserves to be investigated in future studies involving patients with RA treated with anti-TNF and those treated with other therapeutic regimens A positive answer to that question would have important implications not only for RA but also for patients with cardiovascular diseases in general, highlighting again the crucial role of inflammatory process in the pathogenesis of acute cardiovascular events In addition, it would be interesting to study whether the effects observed by us may be generalized to all TNF blockers The beneficial effects of TNF blockade on insulin sensitivity observed in patients with RA are not similar to that reported in patients with diabetes The mechanisms responsible for this difference are not known, although differences between the tissue source of TNF in diabetes versus RA may have been one cause, and may be subject to future investigation Our data regarding leptin cast doubt on the pro-inflammatory and pathogenetic role of circulating leptin in RA, as chronic inflammation showed inhibitory effects on leptin concentrations Regarding adiponectin, some controversies still remain and should be addressed in future studies, especially related to the significance of a higher or lower plasma concentration and its translation into the cardiovascular risk and/or pathogenetic process characteristic to RA

It is likely that an impaired cytokine production capacity of immune cells has an important contribution to the susceptibility to infections of RA patients. However, little is known about the distribution among various types of immune cells of anti-TNF effects on cytokine production capacity. This is important for the further understanding of the mechanisms involved in the development of severe infectious side-effects in these patients, and should therefore be considered for investigation in further studies. Additionally, this might increase our understanding of the differences in therapeutic spectrum between anti-TNF antibodies and TNF soluble receptors.

Samenvatting en conclusies

Gedurende het laatste decennium werden anti-TNF middelen beschouwd als een doorbraak in het therapeutisch armamentarium van chronische inflammatoire ziekten, zoals reumatoide artritis (RA) Verscheidene studies hebben aangetoond dat monoclonale anti-TNF antilichamen en TNF-oplosbare receptor concepten in gelijke mate efficient zijn in het verlagen van de ziekteactiviteit en het bevorderen van de levensduur van patienten met RA Echter, omdat TNF een pleiotropische cytokine is, was het niet moeilijk je voor te stellen dat anti-TNF middelen ook zouden interfereren met andere pathofysiologische processen gecontroleerd door TNF in deze patienten, naast deze gerelateerd aan gewrichtsontsteking Het aantal ernstige infectieuze bijwerkingen gerapporteerd bij RA patienten die TNF blokkerende middelen gebruiken vergeleken met anti-TNF naieve patienten is groter, wat er op wijst dat de afweer van de gastheer wordt verzwakt door deze medicijnen Het begrip van de onderliggende mechanismen verantwoordelijk voor deze belangrijke bijwerking is essentieel in onze poging het aantal van deze serieuze infecties te verminderen bij deze patienten Cytokine productiecapaciteit van immuuncellen kan in dit opzicht van cruciale importantie zijn

Tijdens inflammatie interfereren hoge TNF concentraties met het intermediaire metabolisme, resulterende in veranderingen van lipidenpatronen en glucose/insuline homeostase Hoewel ze de acute immuunreactie in de hand werken zijn deze veranderingen ongewenst als ze voor langere tijd blijven bestaan corresponderend aan persistente hoge TNF concentraties, zoals het geval is bij RA patienten Van een verhoogd lipidenpatroon, een verminderde glucose tolerantie en geaccelereerde atherosclerosis is inderdaad bewezen dat zij voorkomen bij RA patienten, resulterende in een hoger cardiovasculair risico dan in de gewone populatie Bijgevolg was het verleidelijk om te veronderstellen dat therapeutische TNF blokkade ook "gunstige bijwerkingen" zou kunnen hebben en lipidenpatroon, glucosetolerantie en cardiovasculair risico bij deze patienten zou verbeteren Het doel van deze thesis was het verkrijgen van meer inzicht in de modulatie van intermediaire metabolisme, zowel als de immuunrespons, bij patienten met RA die worden behandeld met anti-TNF middelen

Het eerste deel van de thesis benadert de capaciteit van anti-TNF antilichamen om lipidenconcentraties te beinvloeden In hoofdstuk 2 zijn de effecten van kortdurende anti-TNF therapie op plasma lipoproteine concentraties onderzocht Voorheen zijn aanwijzingen gevonden dat TNF in staat is de concentratie triglyceriden te verhogen en concentraties totaal en HDL cholesterol te verlagen Twee weken na het voorschrijven van adalimumab, een volledig humaan anti-TNF monoclonaal antilichaam, was een significante toename van HDL cholesterol

concentraties samen met een daling in totaal:HDL-cholestrol ratio waarneembaar. In tegenstelling, werden er geen veranderingen gezien in een met placebo behandelde groep. Daaruit concluderen wij dat kort na de initiatie, TNF blokkade bij RA patiënten gepaard gaat met een anti-atherogeen effect, dankzij een afname van inflammatoire status en een verbetering van het lipidenpatroon. Echter, om dit als klinisch significant te beschouwen, hadden deze resultaten bevestigd moeten worden na een verlengde behandeling met deze middelen.

Deze vraag is besproken in hoofdstuk 3, waarin we bestudeerd hebben of de effecten waargenomen kort na initiatie van anti-TNF therapie, aanhouden gedurende langere perioden van behandeling. Met dit doel onderzochten we een groep RA patiënten die therapie met infliximab, een chimerisch anti-TNF monoclonaal antilichaam, startten. In deze studie waren we in staat om de korte termijn effecten van anti-TNF op lipidenpatronen die eerder werden waargenomen, te bevestigen. Echter, 6 maanden en zelfs 12 maanden na het starten van deze therapie, bemerkten we een toename in het totaal cholesterol en totaal:HDL-cholesterol ratio, overeenkomend met een verslechtering van het lipidenpatroon. Daarom werd er geconcludeerd dat lange termijn TNF blokkade het plasma lipidenprofiel bij RA patienten verslechterd. Echter, de impact van deze bevinding op het globale cardiovasculaire risico is moeilijk in te schatten, te wijten aan lipiden onafhankelijke heilzame effecten van anti-TNF middelen, namelijk verlaagde homocysteïne waarden, verbeterde insuline sensitiviteit en verlaagde inflammatoire status.

In het licht van recente literatuur suggererende dat inflammatie mogelijk anti-atherogene mechanismen van HDL moduleert, onderzochten wij in hoofdstuk 4 of en hoe TNF blokkade interfereert met dit proces. De anti-oxidatieve capaciteit van HDL is beoordeeld door middel van het meten van de activiteit van paraoxonase (PON)-1, het voornaamste enzym verantwoordelijk is voor de anti-oxidatieve capaciteit van HDL. Bovendien ontwikkelden is een *in vitro* analyse om de te toetsen wat de globale capaciteit van HDL is om geoxideerde-LDL direct te neutraliseren ontwikkeld en gebruikt voor hetzelfde doeleinde. Alhoewel plasma HDL-cholesterol concentraties niet zijn beïnvloed 6 maanden na de initiatie van anti-TNF therapie, is de anti-oxidatieve capaciteit van HDL significant verbeterd gedurende hetzelfde interval. Onze resultaten suggereren dat het totale effect van anti-TNF op circulerende lipiden mogelijk toch voordelig is en van klinische relevantie, omdat recent gebleken is dat anti-TNF behandeling de incidentie van myocard infarct bij RA patiënten die reageren op deze therapie verlaagt.

De effecten van anti-TNF therapie op insuline sensitiviteit zijn onderzocht in hoofdstuk 5. We namen waar dat een kortdurende TNF blokkade in staat was om insuline sensitiviteit gemeten met hyperinsulinemische-euglycaemische klemmen, te verbeteren. Dit was geassocieerd met een daling in e inflammatoire status van deze patiënten. Onze bevindingen komen overeen met voorgaande studies die rapporteren dat anti-TNF de verstoringen in glucose metabolisme bij reumatoïde artritis patiënten corrigeert en ondersteunen een voordelig effect van TNF blokkers op het cardiovasculaire risico. Interessant is echter dat ondanks de bemoedigende bevindingen in diermodellen en patiënten met chronische inflammatoire ziekten, anti-TNF blokkade niet voordelig lijkt te zijn bij diabetespatiënten to dusver. De dosis en het middel zelf zijn mogelijk verantwoordelijk voor het ontbrekende positieve effect bij deze patiënten.

De invloed van anti-TNF op de circulerende concentraties van leptine en adiponectine zijn onderzocht in hoofdstuk 6 en 7. We vonden dat chronische inflammatie van RA en de onderdrukking hiervan gedurende anti-TNF therapie een beperkte invloed hebben op plasma leptine concentraties, terwijl het de circulerende adiponectine waarden significant beïnvloedt. RA patiënten toonden hogere adiponectine concentraties dan controles, en deze werden verlaagd door anti-TNF therapie. Body mass index (BMI) en dus meest waarschijnlijk vetweefsel, bleef de voornaamste determinant van plasma leptine concentraties, wat in overeenstemming is met voorgaande studies bij RA en andere chronische inflammatoire aandoeningen. De resultaten van onze studies kunnen daardoor twijfels opwekken over de nadelige rol van circulerend leptine op RA ontwikkeling. De suppressieve effecten van samengaand anti-TNF en oraal corticosteroïden op plasma adiponectine concentraties zijn voorheen niet beschreven en zullen worden onderzocht in toekomstige studies.

Een tweede groot effect van anti-TNF behandeling treedt op in de afweermechanismen van de gastheer. De effecten van therapeutische TNF blokkade op de immuunrespons jegens verscheidene microbiële middelen is onderzocht in hoofdstuk 9 en 10. In eerste instantie werd waargenomen dat met name de capaciteit van immuuncellen om IFNγ te produceren als reactie op bacteriële stimuli verzwakt is bij patiënten met RA vergeleken met gezonde vrijwilligers. Interessant is ook dat anti-TNF therapie met infliximab werd gesuggereerd als de voornaamste factor verantwoordelijk hiervoor, omdat de capaciteit van cellen om IFNγ te produceren drie weken nadat het medicijn werd stopgezet herstelde. In tegenstelling tot infliximab, werd een klein maar reproduceerbare inhibitie van adalimumab op de IFNγ productiecapaciteit waargenomen na 3 maanden behandelen, daarentegen werd de afgifte van IFNγ niet door

etanercept beinvloed Niettemin maakt de mate van deze daling het onwaarschijnlijk dat de inhibitie grote effecten heeft op de vatbaarheid voor infecties, tenzij het effect sterker is op weefselniveau Therapie met een van beide medicijnen heeft de capaciteit van bloedcellen om IL-1β te produceren niet beinvloed, terwijl het enige verschil in IL-6 afgifte werd waargenomen met bloedcellen van RA patienten behandeld met etanercept Aldus reageren witte bloedcellen gedurende therapeutische TNF blokkade met lage-dosis adalimumab en etanercept op bacteriele producten met dezelfde productie van proinflammatoire cytokines en verklaren mogelijk de lagere incidentie van infecties bij patienten behandeld met deze middelen in verhouding met infliximab

Conclusies en toekomstperspectief

In deze thesis is het effect van anti-TNF behandeling op intermediaire metabolismen aan de ene kant, en het afweermechanisme van de gastheer aan de andere kant, onderzocht De data gepresenteerd in de hoofdstukken 2-4 laten duidelijk zien dat anti-TNF middelen in staat zijn lipidenpatronen bij RA patienten die deze behandeling ondergaan moduleren De effecten worden nagebootst niet alleen op het niveau van plasma concentraties (LDL, HDL en triglyceriden), maar ook op functioneel niveau (HDL anti-oxidatieve capaciteit) Een van de voornaamste conclusies uit deze studies is daarom dat anti-TNF therapie plasma lipoproteine profielen moduleert, terwijl het de anti-oxidatieve capaciteit van HDL verbetert Een andere conclusie die getrokken kan worden uit hoofdstuk 5 is dat anti-TNF in staat is om insuline sensitiviteit bij RA patienten te verbeteren Van de resultaten in hoofdstuk 6 en 7 concluderen we dat inflammatie en de onderdrukking hiervan gedurende anti-TNF blokkade een belangrijke rol speelt met name in adiponectine plasma concentraties van RA patienten Ten slotte heeft het onderzoek van de immunomodulatoire capaciteiten van anti-TNF middelen in hoofdstuk 9 en 10 geleid tot de conclusie dat deze therapie ook invloed heeft op de immuunrespons tegen verscheidene micro-organismen

Een belangrijk discussiepunt met name wanneer we onze metabolische data analyseren is of de effecten waargenomen gedurende TNF blokkade indirect bepaald worden door veranderingen in inflammatoire status van deze patienten Dit zou impliceren dat elk ander anti-reumatisch medicijn dat een belangrijk anti-inflammatoir effect laat zien, vergelijkbare effecten op lipiden zou kunnen hebben als anti-TNF middelen Dit is een belangrijke vraag welke onderzocht zou moeten worden in toekomstige studies waarin RA patienten behandeld met anti-TNF en behandeld met andere therapeutische middelen betrokken worden Een positief antwoord op die

vraag zou belangrijke implicaties hebben niet alleen voor RA maar ook voor patienten met cardiovasculaire ziekten in het algemeen, met name het belichten van de cruciale rol van inflammatoire processen in de pathogenese van acute cardiovasculaire afwijkingen Daarnaast zou het interessant zijn om te bestuderen of de effecten waargenomen door ons mogelijk gegeneraliseerd kunnen worden naar alle TNF-blokkers De voordelige effecten van TNF blokkade op insuline sensitiviteit waargenomen bij patienten met RA zijn niet gelijk aan dat gerapporteerd bij diabetespatienten De mechanismen verantwoordelijk voor dit verschil zijn onbekend, echter verschillen tussen de weefselbron van TNF bij diabetes versus RA kan een oorzaak zijn geweest, en kan mogelijk onderwerp zijn voor toekomstig onderzoek Onze data met betrekking tot leptine zaait twijfels over de proinflammatoire en pathogene rol van leptine in RA, omdat chronische inflammatie inhiberende effecten op leptine concentraties liet zien Met betrekking tot adiponectine blijven sommige controversies nog bestaan en zullen moeten worden meegenomen in toekomstige studies, met name gerelateerd aan de significantie van een hogere of lagere plasmaconcentratie en de vertaling hiervan in het cardiovasculaire risico en/of pathogenetisch proces karakteristiek voor RA

Het is waarschijnlijk dat een verlaagde cytokine productiecapaciteit van immuuncellen een belangrijke bijdrage levert aan de vatbaarheid voor infecties bij RA patienten Echter, er is weinig bekend over de verdeling onder verscheidene typen van immuuncellen van anti-TNF effecten op cytokine productiecapaciteit Het is belangrijk voor het verdere begrip van de mechanismen betrokken in de ontwikkeling van verscheiden infectieuze bijwerkingen bij deze patienten, en zou daarom moeten worden beschouwd voor onderzoek in verdere studies Ten slotte zou dit ons begrip van de verschillen in therapeutisch spectrum tussen anti-TNF antilichamen en TNF oplosbare receptoren kunnen vergroten

Rezumat şi concluzii

În ultimul deceniu, agenţii care blochează TNF au fost consideraţi ca fiind un imens pas înainte în ceea ce priveşte posibilităţile terapeutice din bolile inflamatorii cronice, cum este şi poliartrita reumatoidă (PAR) Numeroase studii au arătat că anticorpii monoclonali anti-TNF precum şi receptorii solubili ai TNF sunt la fel de eficienţi în a reduce activitatea bolii şi a prelungii durata de viaţă a pacienţilor cu PAR Totuşi, deoarece TNF este o citochină pleiotropică, nu a fost greu de presupus că medicamentele anti-TNF vor influenţa în aceşti pacienţi şi alte procese fiziopatologice controlate de TNF, în afara celor legate de inflamaţia articulară Într-adevăr, în aceşti pacienţi au fost înregistrate un număr mai ridicat de infecţii grave comparativ cu pacienţii ce nu au primit această medicaţie, subliniind faptul că răspunsul imun este alterat de către aceste medicamente Înţelegerea mecanismelor responsabile de acest efect advers major este un lucru esenţial pentru a putea reduce frecventa infecţiilor grave la aceşti pacienţi În acest sens, capacitatea celulelor imune de a produce citochine ar putea deveni de o importanţă crucială

În timpul răspunsului inflamator, TNF se secretă în mod abundent şi atinge concentraţii crescute atât în sânge cât şi în ţesutul respectiv Acestea vor influenţa metabolismul intermediar, având ca rezultat modificări ale profilului lipidelor precum şi ale homeostaziei glucozei/insulinei Deşi aceste modificări sunt menite să favorizeze răspunsul imun acut, ele vor avea efecte negative pentru organism în cazul în care vor persista pe termen îndelungat, cum ar fi cazul pacientilor cu PAR Într-adevăr, studii recente au arătat că pacientii cu PAR au un profil lipidic modificat, o scădere a toleranţei la glucoză şi o accelerare a procesului de ateroscleroză, rezultând într-o creştere al riscului pentru boli cardiovasculare comparativ cu cel din populaţia generală De aceea, am presupus iniţial că blocarea terapeutică a TNF ar putea avea şi o serie de "efecte adverse favorabile", îmbunătăţind profilul lipidelor, toleranţa la glucoză şi riscul de boli cardiovasculare la aceşti pacienţi Scopul acestei teze a fost de a cunoaşte mai bine modul în care medicamentele anti-TNF modulează atât metabolismul intermediar cât şi răspunsul imun la pacientii cu PAR

Prima parte a acestei teze analizează capacitatea anticorpilor anti-TNF de a modifica concentratia sangvină a lipidelor În Capitolul 2 au fost investigate efectele pe termen scurt ale terapiei cu medicamente ce blochează TNF asupra concentraţiei plasmatice a lipoproteinelor Studii anterioare au arătat că TNF este capabil să crească concentratia trigliceridelor şi să o scadă pe cea a HDL-colesterolului La două săptămâni de la administrarea de *adalimumab*, un anticorp monoclonal integral-uman împotriva TNF, am observat o creştere semnificativă a concentraţiei de HDL-colesterol împreună cu o scădere a raportului colesterol total HDL Din contră, nici o

modificare nu a putut fi observată în grupul tratat cu placebo De aceea am concluzionat că la scurt timp după ce a fost inițiată, terapia cu blocanți de TNF la pacienții cu PAR este însoțită de un efect anti-aterogenic datorat atât unei scăderi al statusului inflamator cât și unei îmbunătățiri al profilului lipidelor la acești pacienți Totuși, pentru a avea semnificație clinică, aceste rezultate ar fi trebuit confirmate în cazul pacienților tratați pe perioade mai lungi de timp cu acești agenți terapeutici

Aceast fapt a fost adresat în Capitolul 3 al tezei, în care ne-am întrebat dacă efectele observate la scurt timp de la inițierea terapiei cu anti-TNF sunt susținute de-a lungul unei perioade mai îndelungate de tratament În acest sens, un grup de pacienți cu PAR ce urmau să înceapă terapia cu infliximab, un anticorp monoclonal chimeric împotriva TNF În acest studiu am reușit să confirmăm efectele pe timp scurt ale terapiei cu anti-TNF asupra profilului lipidic pe care le observasem anterior Totuși, am observat o creștere a colesterolului total și a raportului colesterol total HDL la 6 luni și chiar la 12 luni de la începerea terapiei, ceea ce sugerează o înrăutățire a profilului lipidic Am concluzionat așadar că blocada terapeutică de lungă durată a TNF înrăutățește profilul lipidic în PAR Totuși, impactul acestei modificări asupra riscului global de boli cardiovasculare este dificil de estimat, datorită efectelor benefice ale medicamentelor anti-TNF, independente de lipide, și anume scăderea concentrației de homocisteină, îmbunătățirea sensibilității la insulină și reducerea statusului inflamator

În lumina noilor date din literatură ce sugerează că inflamația poate modula mecanismele anti-aterogenice ale HDL, am investigat în Capitolul 4 al tezei dacă și în ce mod blocarea TNF interferă cu acest proces Capacitatea antioxidantă a HDL a fost evaluată prin măsurarea activității paraoxonazei (PON)-1, principala enzimă responsabilă de capacitatea antioxidantă a HDL În același scop, am dezvoltat pe lângă cele amintite mai sus un test in-vitro pentru a evalua capacitatea globală a HDL de a neutraliza direct oxLDL Deși concentrația plasmatică a HDL nu s-a schimbat la 6 luni după inițierea terapiei cu anti-TNF, capacitatea antioxidantă a HDL s-a îmbunătățit semnificativ în același interval de timp Rezultatele noastre sugerează că efectele generale ale terapiei cu agenți anti-TNF asupra lipidelor circulante sunt totuși benefice și ar avea relevantă clinică, de vreme ce tratamentul cu anti-TNF poate scădea incidența infarctului miocardic la pacienții cu PAR ce răspund la această terapie

Efectele terapiei cu anti-TNF asupra sensibilității la insulină au fost investigate în Capitolul 5 Folosind ca metodă "standardul de aur" pentru evaluarea sensibilității la insulină, și anume testul

hiperinsulinemic-euglicemic, am observat că blocarea terapeutică de scurtă durată a TNF poate îmbunătăţii sensibilitatea la insulină. Aceasta a fost asociată cu scăderea statusului inflamator al acestor pacienţi. Datele noastre sunt în acord cu cele din studii anterioare ce arătau că terapia cu anti-TNF este în măsură să corecteze tulburările din metabolismul glucozei la pacienţii cu PAR şi sprijină în acelaşi timp ideea că aceste medicamente au un efect benefic asupra riscului de boli cardiovasculare la aceşti pacienţi. Interesant, în ciuda datelor încurajatoare obţinute în urma studiilor pe animale de laborator precum şi în cazul pacienţilor cu boli inflamatorii cronice, efectele benefice anterior amintite ale blocării în scop terapeutic a TNF nu au putut fi încă demonstrate la pacienţii cu diabet zaharat. Doza sau poate chiar medicamentul în sine ar putea constitui o explicaţie a acestui fapt.

Influenţa tratamentului cu anti-TNF asupra concentraţiilor sangvine ale leptinei şi adiponectinei a fost investigată în Capitolele 6 şi 7. Am observat că inflamaţia cronică caracterisitcă PAR şi suprimarea ei din timpul tratamentului cu anti-TNF au o influenţă minoră asupra concentraţiei plasmatice de leptină, în schimb pot modifica considerabil concentraţia adiponectinei circulante. Pacienţii cu PAR au o concentraţie mai ridicată de adiponectină comparativ cu subiecţii sănătoşi, şi aceasta a fost redusă în urma tratamentului cu anti-TNF. Indicele de masă corporală (IMC) şi deci cel mai probabil ţesutul adipos, a rămas principalul determinant al concentraţiei plasmatice a leptinei, ceea ce este în acord cu studii anterioare efectuate la pacienţi cu PAR dar şi cu alte boli inflamatorii cronice. Rezultatele studiilor noastre ar putea deci să aştearnă o oarecare notă de dubiu asupra rolului şi deci contribuţiei leptinei circulante la apariţia poliartritei reumatoide. Efectele supresive asupra concentraţiei plasmatice a adiponectinei observate în cazul terapiei concomitente cu anti-TNF şi glucocorticoizi oral nu au mai fost descrise anterior şi le vom investiga mai departe în studii viitoare.

Un al doilea efect major al terapiei cu anti-TNF este cel asupra mecanismelor de apărare ale sistemului imun. Efectele blocării terapeutice a TNF asupra răspunsului imun la diferiţi agenţi microbieni au fost investigate în Capitolele 9 şi 10. Iniţial am observat la pacienţii cu PAR mai ales o scădere a capacităţii celulelor imune de a produce interferon-gamma (IFNγ) ca răspuns la stimuli bacterieni, comparativ cu cea de la subiecţii sănătoşi. De remarcat faptul că tratamentul anti-TNF cu infliximab a fost iniţial sugerat ca fiind principalul factor responsabil pentru aceasta, de vreme ce capacitatea celulelor de a produce IFN γ a revenit la normal după 3 săptămâni de la întreruperea terapiei. În schimb, un efect inhibitor modest şi reproductibil al adalimumabului asupra producţiei de IFNγ a fost observat la 3 luni de la startul terapiei, în timp ce tratamentul cu

etanercept nu a influențat producerea de IFNγ. Şi totuşi, amploarea acestei scăderi observate în cazul adalimumabului face improbabil faptul ca această inhibare a producției de IFNγ să aibă repercursiuni majore asupra susceptibilității la infecții, doar dacă acest efect e mai puternic la nivel de țesut. Tratamentul cu oricare dintre cele două medicamente nu a afectat producția de IL-1β, în timp ce singura diferență în ceea ce privește eliberarea de IL-6 din celule a fost observată doar în cazul pacienților tratați cu etanercept. Deci, atunci când sunt stimulate cu produşi bacterieni celulele albe sangvine răspund în mod similar în ceea ce privește producția de citochine pro-inflamatorii pe tot parcursul tratamentului cu etanercept şi doze mici de adalimumab. Acest fapt ar putea explica incidența mai scăzută a infecțiilor la pacienții tratați cu aceste medicamente comparativ cu cei ce primesc infliximab.

Concluzii şi perspective în viitor

În această teză au fost investigate pe de o parte efectele terapiei cu medicamente ce blochează TNF asupra metabolismului intermediar iar pe de altă parte efectele aceleiaşi terapii asupra mecanismelor de apărare imună. Datele prezentate în capitolele 2-4 demonstrează clar că medicamentele anti-TNF pot modula profilul lipidelor la pacienții cu PAR. Aceste efecte se reflectă nu doar la nivelul concentrațiilor plasmatice (LDL, HDL şi trigliceride), dar şi la nivelul funcțional (capacitatea antioxidantă a HDL). Una dintre concluziile principale ce rezultă din aceste studii este deci aceea că terapia anti-TNF moduleaza profilul plasmatic al lipoproteinelor şi îmbunătățește în acelaş timp capacitatea antioxidantă a HDL. O altă concluzie ce rezultă din capitolul 5 al tezei este aceea că medicația anti-TNF poate ameliora sensibilitatea la insulină la pacienții cu PAR. Din datele prezentate în capitolele 6 şi 7 putem trage concluzia că inflamația şi supresia ei din timpul terapiei cu anti-TNF au un impact important mai ales asupra concentrației plasmatice de adiponectină a pacienților cu PAR. În fine, evaluarea capacității imunomodulatorii a medicamentelor anti-TNF prezentată în capitolele 9 şi 10 a dus la concluzia că această terapie este în stare să influențeze răspunsul imun la diferite microorganisme.

Un punct important pentru discuții, mai ales dacă facem referire la datele noastre "metabolice", ar fi dacă efectele observate în timpul tratamentului cu anti-TNF sunt indirect determinate de modificarea statusului inflamator al acestor pacienți. Acest lucru ar implica faptul că efectele asupra lipidelor ale oricărui alt medicament anti-reumatic cu o importantă componentă anti-inflamatorie ar trebui să fie similare cu cele ale agenților anti-TNF. Aceasta este o întrebare importantă care merită să fie investigată în studii viitoare ce vor înrola atât pacienți cu PAR

tratați cu anti-TNF cât și pe cei tratați cu alte regimuri medicamentoase Un răspuns pozitiv la această întrebare ar putea avea implicații deosebit de importante nu doar pentru pacienții cu PAR dar și pentru pacienții cu boli cardiovasculare în general, subliniind încă o dată rolul crucial al proceselor inflamatorii în patogeneza evenimentelor cardiovasculare acute În plus, ar fi interesant de studiat dacă efectele observate de noi ar putea fi generalizate la toate medicamentele aparținând grupei blocanților TNF Efectele benefice ale terapiei anti-TNF asupra sensibilității la insulină observate la pacienții cu PAR diferă de cele raportate la pacienții cu diabet zaharat Mecanismele responsabile pentru această discrepanță nu sunt încă cunoscute Posibila diferență între diabetul zaharat și poliartrita reumatoidă în ceea ce privește țesutul sursă al TNF ar putea reprezenta o cauză, și deci ar merita să fie supusă unei investigații mai ample într-un studiu viitor Datele noastre privitoare la leptină pun sub semnul întrebării rolul pro-inflamator al leptinei circulatorii în patogeneze PAR, deoarece inflamația cronică a arătat că are efecte inhibitorii asupra nivelului circulant de leptină În ceea ce privește adiponectina, rămân încă câteva controverse ce vor trebui sa fie investigate în studii viitoare, în mod special cele legate de semnificația funcțională a unei concentrații plasmatice crescute sau scăzute și impactul acesteia asupra riscului de boli cardiovasculare și/sau asupra proceselor patogenetice caracteristice PAR Se pare că o diminuare a capacității celulelor imune de a produce citochine are o contribuție importantă la creșterea susceptibilității la infecții a pacienților cu PAR Totuși, se cunoaște încă puțin despre tipul anume de celule responsabil pentru aceasta și efectele terapiei cu anti-TNF asupra lor Acest lucru este important pentru a înțelege mai departe mecanismele implicate în apariția infecțiilor severe din timpul tratamentului cu anti-TNF și deci vor trebui luate în considerare în cadrul unor studii viitoare Acesta ar putea spori în plus și capacitatea noastră de a înțelege diferențele privind spectrul terapeutic al medicației bazate pe anticorpi anti-TNF comparativ cu agenții ce au la bază receptori solubili ai TNF

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Curriculum vitae

Călin Popa, de schrijver van dit proefschrift, werd op 29 December 1976 te Sibiu, Roemenie, geboren In 1995 heeft hij zijn studies aan het "Gheorghe Lazăr" Wiskunde en Natuurkunde Lyceum te Sibiu, afgerond Tussen 1995 en 2001 heeft hij zijn medische opleiding aan het "Iuliu Haţieganu" Geneeskunde Faculteit te Cluj-Napoca, Roemenie, gedaan In 2000 kreeg hij een beurs van het Europese Commissie in het kader van het Erasmus Mundus programma en ging hij naar Universita Degli Studi di Milano, Italie Tijdens 3 manden, heeft hij als student onderzoek gedaan op Afdeling Vasculaire Geneeskunde, Ospedalle "Luigi Sacco" Milano, onder de begeleiding van Prof M Catalano Terug in zijn eigen land, was hij begonnen met het onderzoeken van het toepassingsheid van capilaroscopie in het diagnose van vasculaire aandoeningen In 2003 begint hij als AGIO tot internist in Roemenie Vanaf augustus 2004 t/m januari 2007 werkte hij als arts-onderzoeker op de Afdeling Reumatologie (hoofd Prof P L C M van Riel) en Algemene Interne Geneeskunde (hoofd Prof J W M van der Meer), UMC St Radboud Nijmegen In deze periode werd het in dit proefschrift beschreven onderzoek verricht Tijdens deze periode heeft hij meerdere beursen en prijzen gewonen, onder andere de Frederika Fischer Foundation Fellowship Award 2003 (names International Cytokine Society – ICS) en de EULAR/Abbott Young Investigator Award 2006 Tussen januari 2007 en juni 2008 was hij werkzaam als post-doc onderzoeker in de Laboratorium van Reumatologie van NCMLS (Prof W B van den Berg) In deze periode verdiepte hij zijn kennis over immunologie bij het onderzoeken van de rol van dendritische cellen in de pathogenese van systemisch sclerose, onder de begeleiding van Dr T Radstake Hij is vanaf september 2008 werkzaam in het Jeroen Bosch Ziekenhuis in Den Bosch, waar hij met zijn opleiding tot reumatoloog is begonnen Hij is gehuwd met Delia en heeft een jongetje, Andrei Teodor

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Stellingen

behorend tot deze proefschrift

1. The immune system through TNF is able to modulate many pathways of the intermediary metabolism; in acute situations the changes are beneficial for the host; if persisting, they might be harmful (this thesis).
2. The Yin-Yang principle applies also to anti-TNF drugs (this thesis).
3. The cardiovascular risk profile of RA patients improves during anti-TNF treatment; testing this effect in patients with acute coronary syndromes is still "a bridge too far" (this thesis).
4. It is doubtfull whether circulating leptin plays a pro-inflammatory and pathogenetic role in RA, as chronic inflammation showed inhibitory effects on leptin concentrations (this thesis).
5. Anti-TNF agents still retain an important capacity to increase the susceptibility to infections; therefore, prevention measures i.e. screening for T.B. might become crucial in order to prevent serious infections in patients receving TNF blockers (this thesis).
6. Pay attention to your wishes; they may one day come true!
7. Sometimes you need to have luck; quite surprising, your actions may contribute to your own luck.
8. You will always achieve more if you work together than if you do it on your own; nevertheless, this simple true phrase gets a lot more complicated if you are going to apply it in research.
9. The reachest person is not the one who has the most, but the one who needs the least.
10. Life is like a box of choclates. You never know what you're gonna get (Forest Gump)...