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EDITORIAL

Enhanced extracorporeal elimination of valproic acid in overdose

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The treatment of the poisoned patient has been based on three main approaches: use of supportive nonspecific therapy, if available administration of antidotes and removal of the offending drug from the body. Gastric lavage and binding of nonabsorbed drug by activated charcoal are often used in an attempt to eliminate the intoxicating agent from the body. In addition, elimination of already absorbed drug can sometimes be enhanced by the induction of brisk diuresis coupled to manipulation of urine pH (e.g. alkalinise for salicylates) or applying extracorporeal techniques such as haemodialysis, haemofiltration or haemoperfusion.[1] In this issue of the journal Meek *et al*.[7] describe the application of haemodialysis in a patient with severe valproic acid (VPA) overdose and demonstrate an increased elimination after the start of haemodialysis.

It is likely that valproic acid overdose will become an increasing problem due to the extended therapeutic application of this drug in psychiatric patients. Valproic acid is currently not only used in epilepsy treatment but also in the treatment of bipolar disorders and migraine prophylaxis.[2] Most cases of overdose can be managed by supportive care with the use of single- and multiple-dose activated charcoal. Since absorption from the gastrointestinal tract is rapid and almost complete, a single-dose activated charcoal is expected to be sufficient for VPA overdose. However, absorption can be delayed after overdose, especially when enteric-coated formulations are ingested (as with Depakine® chrono in both presented cases) and in these cases multiple-dose activated charcoal is recommended.[3] Prospective studies in the management of the poisoned patient are lacking and available data are mainly from case reports or small retrospective studies using elimination kinetics as effect parameters (e.g. rapid decrease in serum concentrations). However, with a few exceptions (acetaminophen, ethylene glycol and theophylline) serum drug levels do not correlate well with the degree of toxicity or prognosis, probably reflecting the poor correlation with tissue concentrations at the receptor site or the individual differences in drug sensitivity.[1,4] Given the fact that most complications of intoxication occur in the initial hours and the inevitable delay in starting extracorporeal techniques, it is unclear whether these techniques are able to influence outcome at all. Therefore, no guidance based on evidence can be given in when to use extracorporeal elimination in drug overdose. Certain pharmacokinetic parameters of the ingested drug are nonetheless a prerequisite.[1] To be effectively eliminated by haemodialysis a substantial amount of the drug present in the body has to be available for extraction from the plasma compartment. This is favoured by a small volume of distribution (<1 l/kg), a low degree of protein binding and a low molecular weight (<500 daltons) which enables the drug to rapidly cross the dialysis membrane by diffusion. Furthermore, to add a clinically notable effect the drug clearance by dialysis has to be high, relative to the endogenous clearance of the drug. Gwilt and Perrier suggested that the amount of drug removed by haemodialysis can be estimated by dividing the percentage of free drug in plasma by the apparent volume of distribution (litre per kg of body weight).[5] When this fraction is greater than 80, six hours of dialysis should remove a significant amount (20 to 50%) of drug, when less than 20 only an insignificant amount (<10%) will be removed after six hours of dialysis. Except for its high protein binding (which is over 90%) VPA fulfils these criteria.[6] As discussed by Meek *et al*.[7]

the high protein binding of VPA results in free drug concentrations that are too small to use haemodialysis effectively under normal circumstances. Indeed, a negligible effect of haemodialysis on serum VPA concentrations has been demonstrated at therapeutic concentrations.[8] However, at increasing drug concentrations of VPA, protein binding becomes saturated thereby increasing the amount of drug available for diffusion, making dialysis a feasible option.[9] Elimination of highly protein-bound drugs can be enhanced by the use of haemoperfusion, which enables direct contact between blood and an absorbent, mostly charcoal. Special charcoal cartridges have to be available and unlike haemodialysis no correction of electrolytes and acid-base disorders are possible. Early haemoperfusion devices produced significant side effects, such as pyrogenic reactions, haemolysis, thrombocytopenia, and reduced fibrinogen concentrations.[1] These adverse effects have been largely overcome with modern preparatory methods.

Continuous haemofiltration techniques (arteriovenous or venovenous) have been advocated for drugs with a high tissue binding and hence volume of distribution.[10] After clearance of the plasma compartment these drugs tend to give a rebound effect by rapid diffusion from the tissue compartment into plasma. These techniques could therefore be an advantage for overdose of drugs with strong tissue binding (e.g. digoxin and tricyclic antidepressants). Since only limited data are available about the value of these continuous techniques and no comparison with repeated haemodialysis exists, no definitive conclusions can be drawn. The notion that extracting the responsible toxin from the plasma by extracorporeal techniques should improve the prognosis of the patient is intrinsically appealing. However, the lack of solid data on outcome means that the decision to use extracorporeal elimination techniques should be guided by whether removal of a substantial fraction of the drug from the body is possible and be carefully balanced with the risks of applying extracorporeal techniques (e.g. bleeding, infection). In this context the article by Meek *et al.*[7] provides favourable evidence for the use of haemodialysis in VPA intoxication.

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REVIEW

The serotonin syndrome

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ABSTRACT

The serotonin syndrome is a complex of symptoms that are thought to be largely attributable to changes in sensitivity in the serotonin receptor systems in the brainstem and the spinal cord due to drugs. Severe cases are almost always caused by a combination of two or more 'serotonergic' drugs, of which at least one is a selective serotonin reuptake inhibitor or a monoamine oxidase inhibitor. Usually, the syndrome heals spontaneously after withdrawal of the medication. Cessation of 'serotonergic' medication is the preferred treatment as well as supportive care.

INTRODUCTION

Case report[1]

A 50-year-old man was admitted to hospital with hyperhydrosis (diaphoresis), nausea, vomiting and diarrhoea. He was on fluoxetine (120 mg/day) for major depression, meprobamate (400 mg/day) for comorbid anxiety disorder and prometazine (13.55 mg/day) for insomnia ('off-label' use). The dose of fluoxetine had just been increased because it was insufficiently effective. The patient was agitated and confused and had insomnia. Hyperreflexia was present, but there were no focal neurological findings. His blood pressure was 155/80 mm Hg, heart rate 96 beats/min and regular, respiratory rate 20 breaths/min and temperature 37.2 °C. The findings of the complete blood count, blood potassium, blood glucose, liver and kidney function tests, and erythrocyte sedimentation rate were normal. A blood alcohol test was negative. ECG, chest radiograph, arterial blood gas measurements and a brain CT scan showed no anomalies.

A diagnosis of serotonin syndrome was made. The patient's medication was discontinued. Electrolyte solution and metoclopramide (10 mg every 8 hours) were administered intravenously. Clorazepate (20 mg orally every 12 hours) was also given. Nausea, vomiting, diaphoresis and diarrhoea disappeared within 72 hours. The patient's anxiety gradually subsided, and he was discharged five days later.

The treatment of depression has evolved greatly over the last two decades. The use of tricyclic antidepressants (TCAs) is decreasing, while the use of selective serotonin reuptake inhibitors (SSRIs) is increasing.[2] In 2001, prescriptions for SSRIs in Australia outnumbered those for tricyclics by two to one.[3] These figures in general compare with data from the Netherlands in the period 1999 and 2000.[2,4] Other new antidepressants with serotonergic properties are also being introduced. Although SSRIs and the other 'atypical' antidepressants (trazodone, venlafaxine and nefazodone, which was recently withdrawn from the market) are generally regarded as having lower toxicity than tricyclics, minor toxic effects are common, and serious toxicity can occur. Furthermore, it was recently concluded that the clinical relevance of differences in side-effect profile between TCAs and SSRIs is not great.[5] Apart from that, the efficacy of TCAs in general is somewhat greater than that of SSRIs.

Serotonin syndrome refers to a drug-induced syndrome that is characterised by mental, autonomic and neuromuscular changes.[6] It is not an idiosyncratic adverse reaction, but a complex of symptoms that are largely attributable to a changed serotonin sensitivity in the brainstem and spinal

cord. Serotonin syndrome was first described in 1955, but during the 1990s reports became increasingly common, as the signs, symptoms, and precipitants became more widely recognised and antidepressants were prescribed more often.[7-9] In the Netherlands up until now, a dozen adverse reactions have been reported to the Netherlands Pharmacovigilance Foundation (Lareb). Although severe cases have been reported with an overdose of a single drug, they usually only occur with a combination of two or more 'serotonergic' drugs, even when each is given at a therapeutic dose.

This article focuses on the pathophysiology, clinical features and diagnosis, incidence and the offending drugs, and treatment. Finally, a conclusion is drawn.

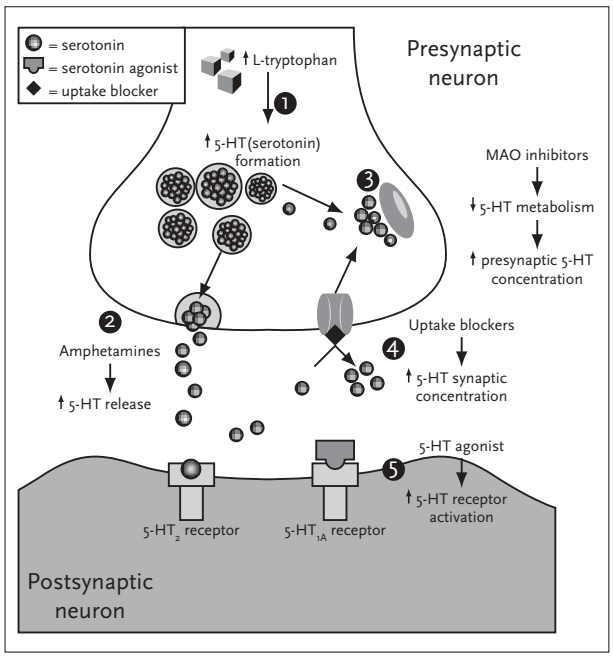

Figure 1

Mechanisms of serotonin syndrome

(1) Increased doses of L-tryptophan will proportionally increase 5-hydroxytryptamine (5-HT or serotonin) formation. (2) Amphetamines and other drugs increase the release of stored serotonin. (3) Inhibition of serotonin metabolism by monoamine oxidase (MAO) inhibitors will increase presynaptic 5-HT concentration. (4) Impairment of 5-HT transport into the presynaptic neuron by uptake blockers (e.g. selective serotonin reuptake inhibitors, tricyclic antidepressants) increases synaptic 5-HT concentration. (5) Direct serotonin agonists can stimulate postsynaptic 5-HT receptors.

PATHOPHYSIOLOGY

Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is synthesised from the amino acid tryptophan (*figure 1*). It has central and peripheral effects and there are at least seven different types of serotonin receptors.[10,11] Centrally, serotonin acts as a neurotransmitter with influences on mood, sleep, vomiting and pain perception. Depression is often associated with low concentrations of serotonin, but the functional meaning of this in the individual is not known. Peripherally, the primary effect of serotonin is on muscles and nerves. The majority of serotonin is synthesised and stored in the enterochromaffin cells of the gut where it causes contraction of gastrointestinal smooth muscle. Serotonin is also stored in platelets and promotes platelet aggregation. It also acts as an inflammatory mediator.

Serotonin syndrome

The pathophysiology of the serotonin syndrome remains poorly understood. It is thought to result from stimulation of the 5-HT_{1a} and 5-HT_2 receptors, and the drug classes implicated in serotonin syndrome reflect this theory. These include serotonin precursors, serotonin agonists, serotonin releasers, serotonin reuptake inhibitors, monoamine oxidase inhibitors (MAOIs), lithium and some herbal medicines (*table 1*).

Table 1

Mechanisms of serotonergic drugs implicated in serotonin syndrome[*12,13]*

Increase in serotonin production	L-tryptophan
Inhibition of metabolism of serotonin	MAO inhibitors, such as tranylcypromine MAO-A inhibitors, such as moclobemide MAO-B inhibitors, such as selegiline
Increase of serotonin release	Amphetamines, mirtazapine, anorectics
Serotonin reuptake inhibition	SSRIs: citalopram, fluvoxamine, fluoxetine, paroxetine, sertraline, venlafaxine in low dosage Nonspecific serotonin reuptake inhibitors: venlafaxine in high dosage, trazodone Tramadol Sibutramine TCAs: amitriptyline, clomipramine, doxepin, imipramine
Stimulation of serotonin receptors	Buspirone, pethidine, LSD, lithium

**Note: Interactions are more severe between drugs with different mechanisms of increasing serotonin. MAO = monoamine oxidase, TCA = tricyclic antidepressants.*

CLINICAL FEATURES AND DIAGNOSIS

General

The diagnosis of serotonin syndrome is purely clinical. It is based upon the recognition of a varied combination of signs and symptoms in the presence of selected 'serotonergic' medications. The diagnosis should not be made without identifying a cause. Serotonin syndrome most commonly occurs after a dose increase (or overdose) of a potent serotonergic drug or shortly after a second drug is added. Some of the drugs involved have very long half-lives (e.g. fluoxetine) and may have been stopped weeks before. There may be a history of recent overdose or use of illicit drugs, particularly ecstasy, amphetamines or cocaine. Herbal medicines may be implicated (St John's wort, ginseng, extracts from soya, or the food supplement S-adenosylmethionine).

Clinical features

The clinical features of serotonin syndrome are highly variable, reflecting the spectrum of toxicity (*table 2*). A distinction is usually made between major and minor symptoms. Diagnosis is made in the presence of three major symptoms and two minor symptoms.
The onset can be dramatic or insidious. The most useful features in the diagnosis of serotonin syndrome are hypereflexia and clonus (inducible/spontaneous/ocular). However, many patients taking SSRIs may display one or more of the clinical features without gross toxicity. Investigations are generally unhelpful in the diagnosis of serotonin syndrome, but may assist in treatment and in ruling out a differential diagnosis. The white cell count is often mildly raised and elevations in creatine kinase levels may occur, but both are nonspecific signs.

Table 2
Clinical features of serotonin syndrome[14-16]

DOMAIN	MAJOR SYMPTOMS	MINOR SYMPTOMS
Psychic (cognitive and behavioural)	Confusion (semi)coma	Hyperactivity Agitation Insomnia Restlessness
Autonomic	Fever or hyperthermia Hypertranspiration (diaphoresis)	Tachycardia Tachypnoea Dyspnoea Hypotension or hypertension Flushing Diarrhoea
Neuromuscular	(Myo)clonus* (spontaneous /inducible/ocular) Hypertonia* Tremor Shivering Hyperreflexia	Incoordination Mydriasis Acathisia Ataxia

** Hypertonia and clonus are always symmetrical and are often much more dramatic in the lower limbs.*

Differential diagnosis

The differential diagnosis includes neuroleptic malignant syndrome, carcinoid syndrome, dystonic reactions, encephalitis, tetanus, thyroid storm and sepsis, as well as poisoning by anticholinergic drugs, cocaine, ecstasy, lithium, MAOIs, salicylates and strychnine. The serotonin syndrome and the other agitated deliriums share many clinical features, but clonus, hyperreflexia and flushing are the most specific signs. *Table 3* shows the characteristics of the other two most important conditions that should be involved in the differential diagnosis. Serotonin syndrome can also be confused with withdrawal of antidepressant treatment.[17] Stopping SSRIs can give rise to symptoms as fear, dizziness, lethargy, paraesthesiae nausea, vivid dreams, insomnia, being irritated quickly and depression.[18,19] These symptoms almost always appear, even if the dosage is reduced slowly. They especially occur with drugs that have a short half-life, such as paroxetine.[18,19] Of all the SSRIs, more than one case of withdrawal symptoms has been published.[19,20]

Table 3
*Differential diagnostic characteristics of the serotonin syndrome**

	DELIRIUM	NEUROLEPTIC MALIGNANT SYNDROME	SEROTONIN SYNDROME
Change in consciousness	+	+	+
Tremors	+	+	+
Tachycardia	+	+	+
Hypertension	+	+	+
Profuse transpiration	+	+	+
Repetitive movements	+	-	-
Disorders of perception and thinking	+	-	-
Acute onset	+	-	-
Fluctuating time course	+	-	-
Changes in sleep-wakefulness cycle	+	-	-
Good reaction on antipsychotics	+	-	-
Muscle rigidity	-	+	+
Hyperthermia	-	+	+
Nonspecific blood changes	-	+	+
Confusion	+	-	+
Restlessness and agitation	+	-	+
Disorders of coordination	+	-	+
Hyperreflexia	-	-	+
Myoclonus	-	-	+
Shivering	-	-	+

** Adapted with permission from Verhoeven WMA, et al. Het serotoninesyndroom; een miskende complicatie van antidepressiva. Ned Tijdschr Geneeskd 1995;139:2073-5.*[7]

INCIDENCE, OFFENDING DRUGS AND TIME COURSE

Incidence

Almost all data on the incidence of serotonin syndrome consist of case reports or small series of patients. In the UK, GPs of patients who were taking nefazodone were sent a questionnaire in which, among other things, information was requested on symptoms that were regarded as characteristic for the serotonin syndrome.[21] The diagnosis was made retrospectively when there were three or more major symptoms. The results showed that in 53 of 11,834 users, two or more major symptoms had occurred. In 19 patients a serotonin syndrome had occurred which equals to an incidence of 0.4 per 1000 patient-months of treatment with nefazodone. Eight patients suffered symptoms while not taking comedication. In users of other antidepressants (fluoxetine, moclobemide, paroxetine, sertraline, venlafaxine) serotonergic symptoms arose in equal amounts. Of the GPs who were interviewed, 85% were not familiar with the serotonin syndrome.

Offending drugs

In *table 4* drugs and drug groups are listed in which a severe serotonin syndrome has been described in the literature.

Most cases will involve either an SSRI or an MAO inhibitor and at least one other medication. Generally, drugs with two different mechanisms of action on serotonin must be present for a severe serotonin syndrome to develop. Yet, serotonin syndrome has also been described in patients taking only one drug, such as clomipramine or paroxetine.[8,21] Serotonin syndrome has also been described in patients using the popular hard drug ecstacy.[26]

Table 4
Combinations of drugs and individual drugs implicated in severe serotonin syndrome[8,12,22-25]

COMBINATIONS OF DRUGS	INDIVIDUAL DRUGS
Trazodone and buspirone	Almotriptan
Fluoxetine and sertraline	Eletriptan
Fluoxetine and tramadol	Naratriptan
Clomipramine and MAO inhibitor as	Rizatriptan
tranylcypromine, fenelzine and benzatropine	Sumatriptan
Clomipramine and trazodone	Zolmitriptan
Clomipramine and moclobemide	Dihydroergotamine
All SSRIs in combination with each other	Clomipramine
Venlafaxine and lithium,	Paroxetine
Venlafaxine and moclobemide	Ecstasy
Dextromethorphan and paroxetine	
Dextromethorphan and moclobemide	
Venlafaxine and fluoxetine	
Venlafaxine and mirtazapine	
Bromocriptine and levodopa and carbidopa	

Time course

In most cases, serotonin syndrome is a self-limiting condition and will improve on cessation of the offending drugs. Mild to moderate cases usually resolve in 24 to 72 hours. In severe cases patients require intensive care as the syndrome may be complicated by severe hyperthermia, rhabdomyolysis, disseminated intravascular coagulation and/or adult respiratory distress syndrome.

TREATMENT AND PREVENTION

Patients with moderate to severe serotonergic symptoms should be admitted to hospital. Those with hyperthermia should be admitted to an intensive care unit. All serotonergic medications should be ceased, and care taken that other precipitants are not inadvertently administered. Benzodiazepines may be used to control seizures and muscle hyperactivity. Specific treatment of hypertension is usually not required.

No randomised clinical studies have been published on the treatment of serotonin syndrome. Serotonin antagonists have been used in the management of moderate to severe serotonin syndrome. Some experience has been gained with cyproheptadine.[27] The initial dose is 4 to 8 mg orally. This may be repeated in two hours. If no response is seen after 16 mg it should be discontinued. If there is a response, then it may be continued in divided doses up to 32 mg/day (e.g. up to 8 mg four times daily).

Furthermore, case reports have been published regarding treatment of serotonin syndrome with mirtazapine.[28] Other drugs that have been suggested include chlorpromazine and propranolol, but these have more contraindications and adverse effects that limit their use.

After the patient has recovered, the ongoing treatment of the condition for which the serotonergic drug was prescribed should be reconsidered.

The prevention of serotonin syndrome involves awareness of the toxic potential of serotonergic drugs. The manufacturer's advice about washout periods should be carefully considered when switching antidepressants and patients should also be educated about possible drug interactions.

CONCLUSION

It is assumed that the serotonin syndrome results from a change in sensitivity in the serotonin receptor systems in the brainstem and spinal cord. Severe cases are almost always caused by a combination of two or more 'serotonergic' drugs, of which at least one is a selective serotonin reuptake inhibitor or a monoamine oxidase inhibitor. Yet, there are reports of serotonin syndrome resulting from single drug use.

The incidence of serotonin syndrome is not known. It is often not properly recognised because doctors are not familiar with it.
The syndrome usually heals spontaneously after withdrawal of the medication but the course can be so severe that the patient dies. Cessation of 'serotonergic' medication is the preferred treatment as well as supportive care.

NOTE

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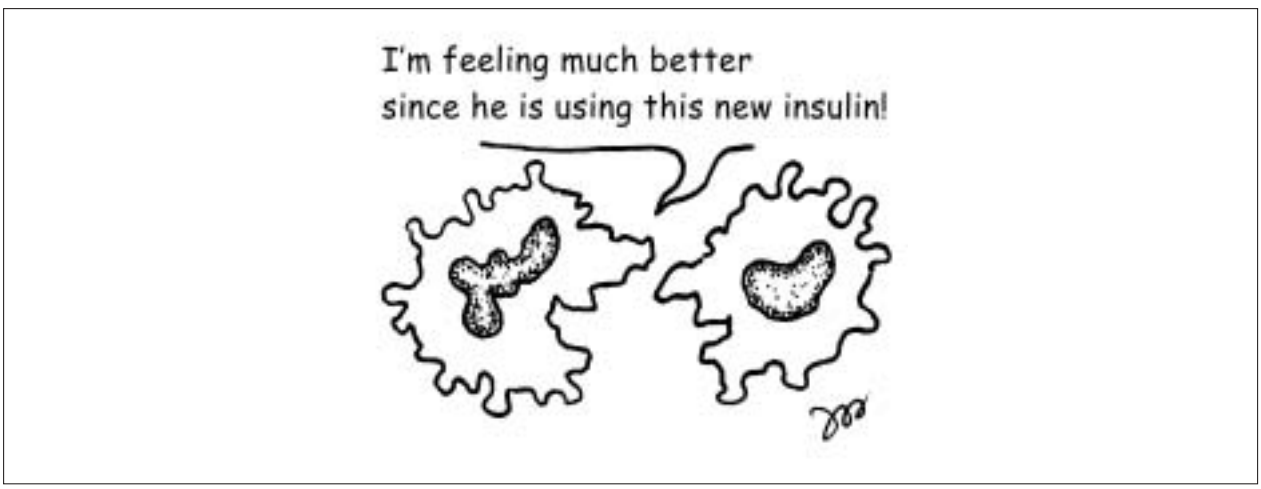

Bijl. The serotonin syndrome.

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Netherlands
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ORIGINAL ARTICLE

A phase I dose-escalating study of docetaxel plus folinic acid and 5-fluorouracil in anthracycline-pretreated patients with metastatic breast cancer

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ABSTRACT

Background: Since the need for nonanthracycline-containing chemotherapy regimens increases with the increased use of anthracyclines in earlier stages of breast cancer, we investigated the feasibility of the combination of docetaxel and 5-fluorouracil (5-FU) with folinic acid (FA). Patients and methods: Anthracycline-pretreated patients with metastatic breast cancer were eligible. Docetaxel was administered as a one-hour infusion every three weeks on day 1, FA 500 mg/m² (fixed dose) as a two-hour infusion on days 1 and 15 and 5-FU as a 24-hour infusion on days 1 and 15. The dose levels tested were (docetaxel/5FU in mg/m²): 60 /1800, 75/1800, 85/1800, 100/1800, and 100/2100. Results: Altogether 28 patients were accrued and treated in this multicentre open-label study. Dose-limiting toxicities (DLTs) were not observed at dose level 1, and in two patients in each of the higher dose levels. DLTs observed were grade III/IV infection (n=4), febrile neutropenia (n=2), diarrhoea (n=1) and erythema (n=1). Partial responses were observed in 10 out of 24 evaluable patients (42%, 95% confidence interval 22.1 to 63.4%). Dose escalation beyond the highest dose level (100/2100) was deemed inappropriate, because these dose levels correspond to recommended dose levels for each drug as a single agent. Conclusion: Combination of docetaxel (100 mg/m², one-hour infusion q3 weeks on day 1), FA (500 mg/m², two-hour infusion on days 1 and 15) and 5-FU (2100 mg/m², 24-hour infusion on days 1 and 15) is a feasible regimen with encouraging activity in anthracycline-pretreated patients.

INTRODUCTION

Breast cancer is the most common malignancy in women contributing to approximately 25% of malignant tumours and 20% of cancer deaths in female patients.[1] Women with metastatic breast cancer (MBC) have a median survival of between two and three years after documentation of metastasis.[2] Many cytotoxic agents have shown activity in MBC. The most active and most commonly used agents are cyclophosphamide, 5-fluorouracil (5-FU), doxorubicin, methotrexate and more recently the taxanes paclitaxel and docetaxel. Response rates (RR) for single agents in MBC vary between 20 to 70% with anthracyclines and taxanes being the most active single agents.

Among the newly introduced taxanes, differences exist between docetaxel and paclitaxel. Recently a head-to-head comparison of docetaxel and paclitaxel in MBC was presented. Docetaxel appeared more effective with higher RR (32 *vs* 25%), longer median time to progression (3.8 *vs* 5.7 months) and longer median survival (15.4 *vs* 12.7 months), but also the toxicity differed for the two drugs, with more pronounced myelosuppression for docetaxel as compared with paclitaxel.[3] Data on the use of docetaxel as a single agent indicate high RR ranging from 40 to 68%, even when used in second-line treatment (40 to 58%).[4-7] RR in a number of phase III studies indicate that docetaxel 100 mg/m², given every three weeks as second-line treatment for MBC, is superior to either the combination regimen of mitomycin/vinblastine (12 mg/m² iv every six weeks plus vinblastine 6 mg/m² iv every three weeks) or to adriamycin

75 mg/m² given every three weeks.[4,5] Superior RR were also confirmed in patients with a poor prognosis, such as metastasis in more than three organs or predominantly visceral metastases.[4-6] Considering combination chemotherapy, anthracycline and alkylating agent-based regimens are routinely used as first-line and sometimes second-line treatment for MBC. In the first line these combinations achieve approximately a 50 to 70% RR with a median duration of 8 to 16 months.[2,8-10] However, because anthracycline-based chemotherapy in the adjuvant setting is replacing the cyclophosphamide/methotrexate/5-fluorouracil (CMF)-type regimens, patients with MBC are increasingly being exposed to high cumulative doses of anthracyclines and therefore at risk of developing anthracycline resistance and cardiotoxicity. This has prompted the search for effective nonanthracycline-containing combination regimens in metastatic disease.

5-FU has been available since the 1950s[11] and still holds a position within the treatment of breast cancer. As a single agent it has only modest activity and much research has been focused on identifying agents that might modulate its cytotoxic effects.[12] In the clinical setting, 5-FU is most often incorporated as part of a combination regimen. Docetaxel in combination with 5-FU has been evaluated in nude mice against subcutaneously implanted advanced colon adenocarcinoma[13] and mammary adenocarcinoma MA 13/C.[14] Interestingly, in a mouse model of colon adenocarcinoma, a combination of docetaxel/5-FU was the most synergistic of all combinations tested, and no additional toxicity was observed when approximately 70% of the full dose of each agent was administered. Because of the promising synergistic activity in preclinical models of breast cancer, the combination docetaxel and 5-FU was studied in patients with MBC. We reasoned that in taxane-naive, anthracycline-pretreated patients with MBC, combining docetaxel with an intensified protracted infusion of FA/5-FU might further improve efficacy and prove useful as an alternative nonanthracycline-containing second-line treatment. Therefore, the primary objectives of this dose-finding study were to determine the maximum tolerated dose (MTD), dose-limiting toxicity (DLT), and recommended dose for phase II studies of the combination of docetaxel and FA/5-FU, with the latter being administered by protracted infusions on day 1 and 15, in anthracycline-pretreated, taxane-naive patients with MBC. Secondary objectives were to characterise the safety and to obtain preliminary evidence of the antitumour activity.

PATIENTS AND METHODS

Patient selection

In a period of two years, 28 patients with histologically confirmed breast cancer at first diagnosis were enrolled by four hospitals. Histological or cytological proof of metastasis was not required. Main inclusion criteria were: ≥1 chemotherapeutic regimen with anthracyclines for either an adjuvant setting or metastatic disease, ≤1 previous chemotherapy for metastatic disease and not suitable for endocrine therapy (prior 5-FU-containing chemotherapy was allowed provided it had been administered only as an iv bolus); measurable and/or evaluable disease; adequate haematopoietic reserve (white blood cells ≥3 x 10^9/l and thrombocytes ≥100 x 10^9/l); adequate liver function with bilirubin ≤ the upper-normal limit (UNL), ALAT and ASAT ≤2.5 the UNL, alkaline phosphatase ≤5 x UNL unless bone metastases were present in the absence of any liver disorder. Prior radiotherapy should have concluded at least four weeks before entering the study. Written informed consent was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committees.

Study design and treatment

To define the MTD of combined docetaxel, FA and 5-FU, the initial approach was to add escalating doses of docetaxel to a fixed dose of FA and 5-FU. In a later stage, the dose of 5-FU was escalated. Patients were treated on an outpatient basis with cycles every three weeks. Docetaxel was administered as a one-hour infusion every three weeks on day 1, FA 500 mg/m² as a two-hour infusion on days 1 and 15 and 5-FU as a 24-hour infusion on days 1 and 15. Efficacy for docetaxel at a dose of 60 mg/m² as a single agent has been observed. For 5-FU a wide range of routes and schedules of administration are available, and the starting dose of 1800 mg/m² is well within the acceptable range. With this information, the following dose levels were defined (docetaxel/5-FU in mg/m²): 60 /1800, 75/1800, 85/1800, 100/1800, and 100/2100. To enable the 24-hour infusion, all patients received an intravenous port system and portable pump. At least three patients were treated before a subsequent dose level was started. For toxicity evaluation, patients were required to receive at least two cycles of chemotherapy. Observed toxicity during these two complete cycles guided the final decision on the MTD. Doses were not escalated in individual patients. If DLT occurred in one of the three patients, an additional three patients were included at the same level. The MTD was reached if DLT was seen in two of the first three patients or in three out of six patients in one dose level.

Safety

Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria (NCI-CTC).[15] DLT was defined as follows: (1) a white blood cell count (WBC) ≤1 x 10^9/l or absolute neutrophil count (ANC) ≤0.5 x 10^9/l for more than seven days, (2) febrile neutropenia, defined as WBC

≤1 x 10^9/l or ANC ≤0.5 x 10^9/l and fever defined as either three oral temperature elevations above 38 °C during a 24-hour period or a single oral temperature above 38.5 °C, (3) severe infections requiring hospitalisation, i.e. pneumonia, sepsis, septic shock, in combination with WBC ≤1.9 x 10^9/l or ANC ≤0.9 x 10^9/l, (4) platelets ≤25 x 10^9/l, and (5) any grade III or IV nonhaematological toxicity. In case of grade ≥3 toxicity, treatment was discontinued until recovery to grade ≤1 and subsequent cycles were reduced to one dose level below and/or according to the specific toxicity. Prophylactic antiemetic treatment was allowed. Patients were treated until progression or unacceptable toxicity or patient's refusal or stable disease without symptomatic improvement after four cycles, whichever occurred first.

Table 1
Patient characteristics

Number enrolled	28
Number assessable	
for toxicity	28
for tumour response	24
Age in years	51 (34-72)
WHO performance status	
0	7
1	16
2	5
Prior chemotherapy	
• Adjuvant CMF	6
• 5-FU injections	28
• Chemotherapy for advanced disease	22

Study treatment
Premedication with dexamethasone was given to all patients to prevent the onset of hypersensitivity reactions and reduce the incidence and severity of fluid retention. Dexamethasone 8 mg (oral) was given twice a day on days -1 to 4 (day 1 is the day of docetaxel administration). Docetaxel was administered before FA with an interval of one hour between the end of the one-hour infusion of docetaxel and the start of the two-hour infusion of FA. The FA administration was immediately followed by the 24-hour 5-FU infusion.

Study assessments
Pretreatment evaluation included a complete history and physical examination, complete blood count, biochemistry assessment, urinalysis, chest X-ray and computerised tomography of chest or abdomen as required to evaluate measurable lesions. During treatment, physical examination, WHO performance status, full blood count, blood chemistry and toxicity assessment were obtained weekly or more frequently if clinically indicated. Tumour assessment was performed after every 3rd cycle of therapy. Standard WHO response criteria were used.

Statistical analysis
The analysis of this phase I study is primarily descriptive. Values are presented as median with ranges unless stated otherwise.

RESULTS

Patient population
Twenty-eight patients were enrolled in the study. The patient characteristics are summarised in *table 1*. All patients had received anthracycline-containing chemotherapy, mostly as epirubicin. Twenty-two patients received chemotherapy for metastatic disease.

Drug delivery
Twenty-eight patients received a total of 144 cycles of treatment (median 6, range 2 to 13), 17 received six or more treatment cycles. A total of five dose levels were tested. At the first dose level three patients were tested, at the third dose level seven patients because of logistics and at the other dose levels six patients were tested. All patients were evaluable for toxicity. Chemotherapy on day 1 was postponed by one week in 3/144 cycles because of haematological toxicity grade III/IV. In one patient on level 5 the dose of docetaxel was reduced for the next three cycles after the occurrence of febrile neutropenia. The dose of 5-FU on day 15 was omitted in the last cycle in four patients because of haematological toxicity grade III, because of progression in five patients and because of refusal in three patients; the dose of 5-FU on day 15 was omitted in any cycle in three patients because of haematological toxicity grade III.

Safety: haematological and gastrointestinal toxicity
Table 2 shows any grade III or IV haematological and gastrointestinal toxicity during treatment. Grade III or IV neutropenia occurred on day 1 and 14 of treatment during 10/144 cycles (grade III 8, grade IV 2). After a one-week delay, the WBC counts rose to levels above 3.0 g/l. In two patients treatment was stopped because of a DLT. At the different dose levels 1 to 5, the nadirs for platelets/WBC (g/l) were 136/1.1, 71/0.8, 20/0.4, 45/0.2 and 54/0.7, respectively. No grade III/IV mucositis or hand-foot syndrome or grade III/IV oedema or neurotoxicity was observed.

Dose-limiting toxicity
At level 1, no DLT events were seen (*table 3*). At level 2 (n=6) one serious infection related to the continuous infusion system and one case of diarrhoea NCI-CTC grade IV occurred. At level 3 (n=7) there was one serious infection due to staphylococcal pneumonia and one patient with leucopenic fever/febrile neutropenia. At level 4 (n=6)

Table 2
Haematological toxicity per dose level*

DOSE LEVEL	DOCETAXEL/5-FLUOROURACIL (MG/M²)	NO. OF PTS/ CYCLES	NEUTROPENIA		IDEM ON DAY TREATMENT		PLATELETS	
			III	IV	III	IV	I	II
1	60/1800	3/16	1/4	0/0	0/0	0/0	0/0	0/0
2	75/1800	6/22	4/5	1/1	0/0	0/0	0/0	0/0
3	85/1800	7/42	6/18	5/14	1/2	0/0	4/10	1/1
4	100/1800	6/36	3/6	1/3	1/1	1/1	0/0	1/2
5	100/2100	6/28	4/12	2/3	1/5	1/1	0/0	1/1
Total		28/144	18/45	9/21	3/8	2/2	4/10	3/4

** Worst NCI-CTC grade during entire treatment, per patient/total number of cycles per involved patients.*

Table 3
Gastrointestinal toxicity per dose level*

DOSE LEVEL	DOCETAXEL/5-FLUOROURACIL (MG/M²)	NO. OF PTS/ CYCLES	NAUSEA		VOMITING	
			III	IV	III	IV
1	60/1800	3/16	2/2	2/2	2/4	1/1
2	75/1800	6/22	2/2	1/1	2/2	1/1
3	85/1800	7/42	2/2	1/2	1/1	3/4
4	100/1800	6/36	1/1	1/4	1/5	0/0
5	100/2100	6/28	0/0	0/0	0/0	0/0
Total		28/144	7/7	5/9	5/12	5/6

** Worst NCI-CTC grade during entire treatment, per patient/total number of cycles per involved patients.*

there was one case of febrile neutropenia grade IV and one of staphylococcal sepsis. At level 5 (n=6) one serious infection related to the continuous infusion system and one erythema grade III/IV (possibly allergic) were observed. Because the maximum dose levels as applied for the single agents were reached at dose level 5, further dose escalation was not carried out, although formally the MTD was not reached. A third infusion with 5-FU/FA per cycle on day 8 was not considered feasible, as the nadir for docetaxel is on day 7 and the mean duration is seven days.[16]

Efficacy

Twenty-four patients were evaluable for response (*table 4*). Four were not evaluable. There were no complete responses. There were ten partial responses leading to an overall RR of 42% (95% confidence interval 22.1 to 63.%).

Table 4
Dose-limiting toxicities (DLT) per dose level

DOSE LEVEL	DOCETAXEL/5-FLUOROURACIL (MG/M²)	NO. OF PTS/ CYCLES	NO DLT	GRADE III/IV INFECTION	DIARRHOEA	FEBRILE NEUTROPENIA	ERYTHEMA
1	60/1800	3/16	3				
2	75/1800	6/22	4	1	1		
3	85/1800	7/42	5	1		1	
4	100/1800	6/36	4	1		1	
5	100/2100	6/28	4	1			1

Table 5
Dose reductions and relative dose intensity per dose level

DOSE LEVEL	DOCETAXEL/5-FLUOROURACIL (MG/M²)	CYCLES WITH DOSE REDUCTIONS /TOTAL CYCLES	NUMBER OF PATIENTS INVOLVED
1	60/1800	0/16	0/3
2	75/1800	0/22	0/6
3	85/1800	0/42	0/7
4	100/1800	0/36	0/6
5	100/2100	3/28	1/6
Total		3/144	1/28

Table 6
Response rates

DOSE LEVEL	DOCETAXEL/5-FLUOROURACIL (MG/M²)	NO. OF PATIENTS	NE	PR	SD	PD
1	60 /1800	3	0	1	2	0
2	75 /1800	6	1	2	1	2
3	85 /1800	7	0	3	2	2
4	100 /1800	6	0	4	2	0
5	100 / 2100	6	3	0	3	0
Total		28	4/28	10/24	10/24	4/24

DISCUSSION

Our study demonstrates that the combination of docetaxel, FA and 5-FU continuous infusion is effective and feasible at doses 100/500/2100 mg/m². The predominant dose-limiting toxicities were febrile neutropenia, grade III or IV infection, diarrhoea and erythema grade III/IV. In two patients the grade III/IV infection was related to the intravenous port system. Our study is thus in line with other phase I studies which show that concomitant administration is feasible and effective. Haematological toxicity was the predominant DLT in our as well as in other reported phase I studies. This haematological toxicity, however, did not lead to toxic deaths in any of these studies and thus appears to be manageable.[17-19] In one study 5-FU was given as bolus injections for three to five days with longer intervals of three to four weeks or as continuous infusions for five days every three to four weeks.[17,19] Further dose escalation beyond docetaxel 60 mg/m² was not feasible in these studies. The fact that in our study we were able to reach a dose of docetaxel 100 mg/m² is probably due to increased tolerability of prolonged infusions of 5-FU as compared with bolus injections, and to spreading the 5-FU infusions over two days in a three-week cycle. Meanwhile, phase II trials have demonstrated the efficacy of 5-FU/FA by bolus injection in patients pretreated with anthracyclines.[20] Experimental and clinical data indicate a far higher activity of 5-FU if it is given as a protracted infusion, and especially at higher dose intensity.[21-26] Although response rate was not the primary endpoint of the study, the observed antitumour activity of 42% of patients treated with this combination is in accordance with response rates observed in phase II studies in which docetaxel was combined with protracted 5-FU infusions.[20] The schedule presented in our study thus provides an alternative schedule of protracted infusions.

The oral fluoropyrimidine capecitabine has recently become available. Capecitabine was designed to generate 5-FU preferentially in tumour tissue, thus giving a more targeted approach. In a randomised phase III study in anthracycline-pretreated patients with MBC, combination of capecitabine and docetaxel led to improved RR (42 *vs* 30%, p=0.006), time to progression (6.1 *vs* 4.2 months, p=0.0001) and overall survival (14.5 *vs* 11.5 months, p=0.0126) when compared with docetaxel monotherapy.[27] These improvements in efficacy came at the cost of a somewhat higher toxicity, as expressed as a higher incidence of hand-foot syndrome and gastrointestinal side effects. When comparing the feasibility of capecitabine and the continuous 5-FU infusions described in this study, it is obvious that oral administration increases feasibility and patient comfort and precludes problems related to continuous infusion regimens observed in two patients in our study. With intermittent continuous 5-FU infusion we observed no hand-foot syndrome, which occurs in up to 25% of patients treated with capecitabine, and no mucositis. Formally the MTD was not reached in our study, but at dose level 5 the maximum dose levels were reached as applied for single agents and further dose escalation was not considered appropriate.

In conclusion the combination of docetaxel (100 mg/m², one-hour infusion q3 weeks on day 1), FA (500 mg/m², two-hour infusion on days 1 and 15) and 5-FU (2100 mg/m², 24-hour infusion on days 1 and 15) is a feasible regimen with encouraging activity in anthracycline-pretreated patients. A disadvantage of this regimen is the need for an indwelling central venous catheter with the associated increased risks of infection and thrombosis, and extra costs. Currently available oral formulations such as capecitabine have overcome these practical hurdles and have proven to prolong survival when given in combination with docetaxel.[27] For patients unable to receive anthracyclines, the encouraging activity of docetaxel combined with 5-FU/FA provides a valuable alternative.

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ORIGINAL ARTICLE

Increased expression of activation markers on monocytes and neutrophils in type 2 diabetes

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ABSTRACT

Background: Activation of leukocytes is obligatory for adherence to the endothelium and atherogenesis. Since leukocyte activation by triglycerides (TG) and glucose has been described *in vitro*, we hypothesised higher leukocyte activation in patients with type 2 diabetes.

Methods: Using flow cytometry, we studied the expression of the leukocyte activation markers CD11A, CD11B, CD62L and CD66B in 15 patients with type 2 diabetes without clinical evidence of atherosclerosis (55±7 years) and in 15 healthy controls (53±2 years). All patients were on oral antidiabetic treatment (glyHb 6.3±0.9%) and not taking statins or anti-inflammatory drugs.

Results: In comparison with controls, the patients had a higher waist circumference (1.08±0.09 *vs* 0.94±0.11 m, p<0.005) and higher fasting glucose (8.4±2.3 *vs* 5.3±0.7 mM, p<0.005), whereas fasting plasma lipids were not statistically different. The leukocyte count was higher in the patients (6.55±1.55 *vs* 5.07±1.10 x 10^9cells/l, p<0.005) due to higher neutrophils and lymphocytes (+34% and +24%, p<0.05 for each). CD11B on monocytes and CD11B and CD66B on neutrophils were higher in the patients (+30%, +52% and +43%, p<0.05 for each). Fasting glucose, waist circumference, body mass index and systolic blood pressure were positively associated with the leukocyte and neutrophil count. The expressions of CD11B and CD66B on monocytes and neutrophils were strongly positively interrelated, but unrelated to TG and glucose.

Conclusion: In patients with type 2 diabetes, the expression of activation markers on monocytes and neutrophils is enhanced and not correlated to fasting glucose or TG. These results suggest a proinflammatory situation in type 2 diabetes and most likely represent increased adhesive capacity of neutrophils and monocytes to the endothelium.

INTRODUCTION

It is generally accepted that atherosclerosis is a low-grade, chronic inflammatory disease.[1,2] In response to endothelial injury, atherogenesis is initiated by resident and recruited leukocytes, eventually resulting in release of various inflammatory mediators.[2] A relationship between leukocyte count and the incidence of coronary heart disease (CHD) and mortality has been described.[3] In addition, increased leukocyte counts are positively associated with several traditional CHD risk factors such as smoking, hyperlipidaemia and insulin resistance.[4,5]

A prerequisite for adherence of leukocytes to the endothelium is that both cell types are activated.[6] Studies with animal models have established the importance of the interaction between leukocytes and endothelial cells with regard to atherogenesis, since blocking of specific selectins reduced plaque formation[7,8] and prevented endothelial dysfunction.[9] A plausible explanation for a proinflammatory state in various metabolic disorders could be that triglycerides (TG) and glucose are able to induce activation of leukocytes and endothelial cells. Activation of endothelial cells has been described *in vitro*.[10-12] In contrast, the effects of glucose and TG on the activation of leukocytes have not been studied in detail. Activation of monocytes in particular, but also neutrophils, has been described *in vitro*. In these studies cell activation was established by gene expression,[13,14] cytokine production, nuclear factor kappa B (NF-κB) activation[14] and intracellular signalling.[15] The effects of TG were studied using artificial TG emulsions.[15] A more appropriate way of measuring would be flow cytometric quantification of the expression of leukocyte activation markers that are involved in the interaction with the endothelium.[16]

Increased expression of these markers in ischaemic heart disease has been described earlier,[17,18] but there are only limited data available on patients at risk for CHD without clinical atherosclerosis. Since *in vitro* data are suggestive of activation of monocytes and also neutrophils, in particular in conditions associated to the metabolic syndrome, we carried out the present observational study to determine the expression of leukocyte activation markers in a group of patients with type 2 diabetes and in healthy controls.

METHODS

Subjects and study design

Diabetic patients and healthy controls aged 45 to 65 years were recruited by advertisement. Exclusion criteria for the patients were smoking, alcohol intake >2 units/day, glycated haemoglobin (glyHb) >8.5 %, presence of renal disease or a liver disorder, clinically established CHD and use of insulin. Due to a possible anti-inflammatory effect of statins with regard to leukocyte-endothelium adherence,[19-22] patients on statins were also excluded. Exclusion criteria for the controls were fasting hyperlipidaemia (plasma cholesterol >6.5 mM, plasma TG >2.0 mM), fasting plasma glucose >6.5 mM, body mass index (BMI) >30 kg/m^2, smoking, alcohol intake >2 units/day, presence of clinically established CHD or renal and liver diseases, a family history of premature CHD or type 2 diabetes mellitus and use of any drugs known to affect lipid metabolism. None of the patients and controls were on special diets, antioxidants, antihypertensive or anti-inflammatory drugs. For both groups recent infection suggested by a positive medical history was an exclusion criterion. All subjects gave written informed consent. The study was approved by the Independent Ethics Committee of Institutional Review Board of the University Medical Centre, Utrecht. Subjects visited the hospital after an overnight fast for at least ten hours and they were asked not to drink alcohol on the day before the test. Blood pressure and waist circumference were measured and the BMI was calculated. Venous blood samples were obtained in sodium EDTA (2 mg/ml).

Analytical methods

Blood cell counts and differentials were determined automatically using a Celldyn-3500 (Abbott, Abbott Park, IL, USA). One blood sample was stored at 4 °C to determine expression of leukocyte activation markers. All other blood samples were chilled and centrifuged immediately for 15 minutes at 800 g at 4 °C, after which plasma was stored at -80 °C. Total cholesterol, HDL cholesterol obtained after precipitation with heparin/MnCl2 and TG were measured in duplicate by colorimetric assay with the CHOD-PAP and GPO-PAP kits, respectively (Roche diagnostics, Germany). LDL cholesterol was calculated using the Friedewald formula. Glucose was measured by glucose oxidase dry chemistry (YSI, USA) and GlyHb was measured photometrically (Hitachi 911, Roche, Germany) in the diabetic patients only.

Leukocyte activation markers

Using fluorescent labelled monoclonal antibodies (MoAbs), the cell surface expression of two pairs of leukocyte activation markers was detected by direct immunofluorescence in duplo and evaluated by flow cytometry: a combination of fluorescein isothiocynate (FITC) conjugated CD66B (CLB, the Netherlands) and phycoerythrin (PE) conjugated CD11B (DAKO, Denmark) and a combination of FITC conjugated CD62L (Cymbus Biotechnology, UK) and PE conjugated CD11A (Diaclone, France). CD11A (also termed LFA-1), CD11B (also termed MAC-1 or CR3) and CD62L (also termed leukocyte selectin) are the most important markers for the early adhesion of leukocytes to the endothelium.[23,24] CD66B (also termed CEACAM8) is a degranulation marker of neutrophils and is not expressed on lymphocytes and monocytes.[25] Using isotype matched MoAbs, nonspecific binding of each label was ruled out in five subjects (data not shown). To avoid *in vitro* activation, the leukocytes were incubated with MoAbs in whole blood at a saturating concentration of 1:10 for 30 minutes in the dark on ice. Erythrocytes were lysed by adding ice-cold isotonic erythrocyte lysing solution (NH_4Cl 0.19 M; $KHCO_3$ 0.01 M; $Na_2EDTA{\bullet}2H_2O$ 0.12 M, pH 7.2) for approximately 15 minutes and centrifuged at 500 x g for five minutes at 4 °C. The remaining leukocyte suspension was washed twice in ice-cold PBS supplemented with bovine serum albumin (BSA 0.2%). Within one hour a total of 5000 cells/sample was analysed by flow cytometry using a fluorescence activated cell counter (FACS, Becton-Dickinson) and CellQuest software. Neutrophils, lymphocytes and monocytes were identified by their characteristic forward and side scattering properties. Fluorescence intensity of each cell was expressed as the average mean fluorescence intensity (MFI) of the duplo, given in arbitrary units (AU).

Statistics

Data are given as mean ± SD. Differences were tested using a Chi-square or Student's t-test. For TG and leukocyte counts, calculations were performed after logarithmic transformation. Bivariate correlations were calculated using Spearman's correlation coefficients. All significantly correlated variables were used as independent variables in stepwise multiple regression analysis with the average MFI of the leukocyte activation markers as dependent variables. For statistical analysis SPSS version 10.0 was used. P values <0.05 (two-tailed) were considered statistically significant.

RESULTS

Subject characteristics

All patients were on unchanged oral antidiabetic treatment with either a sulphonylurea derivative (n=6), metformin (n=5) or a combination of both (n=4) for more than three months. In comparison with controls, the patients with type 2 diabetes showed a higher frequency of characteristics of the metabolic syndrome, e.g. central obesity, a higher blood pressure and higher fasting glucose (*table 1*). None of the lipid parameters from *table 1* showed a significant difference between patients and controls. Furthermore, when compared with controls, the patients showed a higher leukocyte count (6.55±1.55 *vs* 5.07±1.10 x 10^9 cells/l, p<0.005) due to higher numbers of neutrophils and lymphocytes (+34% and +24% respectively, p<0.05 for each, *figure 1*).

Table 1

Baseline characteristics (mean ± SD) of the study group consisting of healthy controls and patients with type 2 diabetes

	CONTROLS (N=15)	DM TYPE 2 (N=15)
Gender (M/F)	13/2	10/5
Age (years)	53 ± 2	55 ± 5
BMI (kg/m²)	25.7 ± 2.7	31.6 ± 4.8 **
Waist (m)	0.94 ± 0.11	1.08 ± 0.09 **
BP systolic (mmHg)	128 ± 12	148 ± 11 **
BP diastolic (mmHg)	86 ± 5	89 ± 8
Glucose (mM)	5.3 ± 0.7	8.4 ± 2.3 **
GlyHb (%)	ND	6.3 ± 0.9
Triglycerides (mM)	1.69 ± 0.67	2.50 ± 1.45
Cholesterol (mM)	5.8 ± 0.9	6.3 ± 1.5
LDL-c (mM)	3.8 ± 0.7	3.9 ± 1.5
HDL-c (mM)	1.21 ± 0.30	1.27 ± 0.57
Cholesterol/HDL-c	5.0 ± 1.2	5.6 ± 2.1

*ND not determined, Student's t-test: * p<0.05, ** p<0.005 patients vs controls.*

Leukocyte activation

In comparison with controls, the diabetic patients showed a higher expression of CD11B on monocytes (253±89 *vs* 194±45 AU, p<0.05) and of CD11B and CD66B on neutrophils (307±175 *vs* 202±66 and 10.7±5.0 *vs* 7.5±2.5 AU, respectively, p<0.05 for both comparisons, *table 2* and *figure 2*). The expression of CD11A or CD62L in any of the cell types was not statistically different between patients and controls (*table 2*). Subanalysis of the diabetic patients according to the type of oral antidiabetic treatment did not show differences in parameters from *table 1* or *figures 1* and *2*. When patients and controls were analysed together, BMI, waist circumference, fasting glucose and systolic blood pressure were positively related to the leukocyte count (R=0.54, R=0.48, R=0.59 and R=0.43, respectively, p<0.05 for each) and neutrophil count (R=0.53, R=0.50, R=0.53 and R=0.46, respectively,

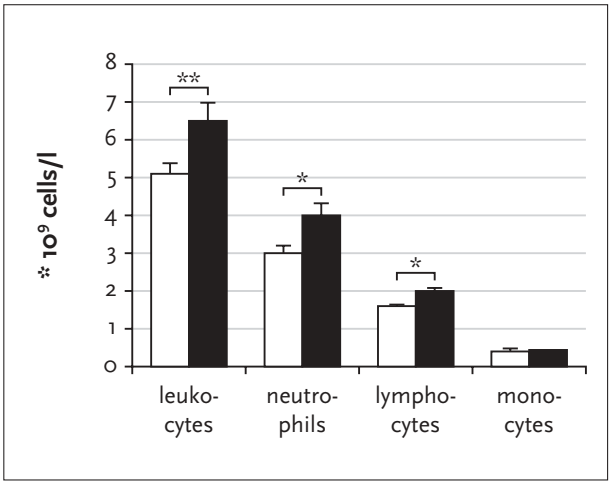

Figure 1

*Leukocyte count and differentiation into monocytes, lymphocytes and neutrophils in healthy controls (n=15, open bars) and in patients with type 2 diabetes (n=15, closed bars) Student's t-test: * p<0.05, ** p<0.005 patients vs controls.*

Table 2

Expression of leukocyte activation markers in healthy controls and in patients with type 2 diabetes

	CONTROLS (N=15)	DM TYPE 2 (N=15)
CD11A		
Monocytes	80 ± 18	80 ± 20
Lymphocytes	57 ± 12	60 ± 13
Neutrophils	34.1 ± 7.7	35.2 ± 8.9
CD11B		
Monocytes	194 ± 46	253 ± 89 *
Lymphocytes	31.3 ± 7.2	30.1 ± 10.5
Neutrophils	202 ± 66	307 ± 175 *
CD62L		
Monocytes	96 ± 22	94 ± 22
Lymphocytes	51 ± 8	53 ± 11
Neutrophils	81 ± 25	85 ± 18
CD66B		
Neutrophils	7.5 ± 2.5	10.7 ± 5.0 *

*Data are given as mean fluorescence intensity per cell (MFI, in arbitrary units) ± SD. Student's t-test: * p<0.05 patients vs controls. Note that CD66B is not expressed on monocytes and lymphocytes.*

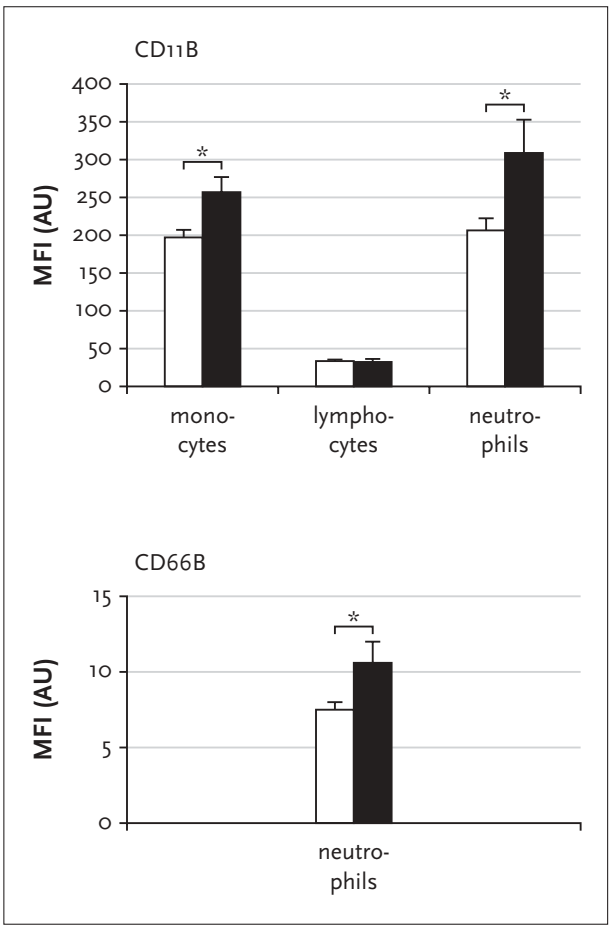

Figure 2

Expression of leukocyte activation markers CD11B on neutrophils, lymphocytes and monocytes (upper panel) and of CD66B on neutrophils (lower panel) in healthy controls (n=15, open bars) and in patients with type 2 diabetes (n=15, closed bars)
*Data are given as mean fluorescence intensity per cell (MFI, in arbitrary units). Student's t-test: * p<0.05 patients vs controls. Note: the different Y-axis scale in each panel.*

p<0.05 for each). From all parameters in *table 1*, age was significantly related to the expression of CD11B on monocytes and of CD11B and CD66B on neutrophils (R=0.68, R=0.65 and R=0.47, p<0.01 for all). Furthermore, CD66B expression on neutrophils was significantly related to systolic and diastolic blood pressure (R=0.48 and R=0.42, p<0.05 for each). The expression of these markers was strongly interrelated (monocyte and neutrophil CD11B: R=0.83, neutrophil CD66B and CD11B: R=0.57 and neutrophil CD66B and monocyte CD11B: R=0.57, p<0.001 for all comparisons). Stepwise linear regression showed that the models to explain the variation in the expression of these markers did not improve by adding age and or blood pressure.

DISCUSSION

When compared with controls, in type 2 diabetic patients, leukocyte cell counts were increased and neutrophils and monocytes showed a higher expression of activation markers. An increased leukocyte count in patients with type 2 diabetes was observed earlier in the ARIC study.[5] In that study it was hypothesised that the leukocyte count reflects the pathogenesis of type 2 diabetes. In line with this, three traditional CHD risk factors in diabetic patients, glucose, obesity and blood pressure, were positively related to the leukocyte and neutrophil count in the present study and in earlier reports.[4,5]

Leukocyte adherence starts with rolling along the vessel wall, largely mediated via leukocyte selectin (L-selectin or CD62L).[6] Upon *in vitro* stimulation, L-selectin is rapidly shed from the leukocytes, in order to improve a more tight adherence via β2 integrins (in particular CD11B, but also CD11A).[23,26] Since we hypothesised a higher degree of activation in the diabetic patients, we expected to find a lower expression of CD62L per cell in these subjects. However, in the present study, which is to the best of our knowledge the first to compare cellular CD62L in diabetic patients with healthy controls, we did not observe differential expression of CD62L. On the other hand, the most important marker involved in tight leukocyte adherence, CD11B, showed a higher expression on monocytes and neutrophils of the diabetic patients. This may suggest that these cells have increased adhesive properties thereby potentially enhancing the risk of vascular complications. Previous reports of CD11B expression in patients with type 2 diabetes are contradictory. Increased neutrophil CD11B expression in type 2 diabetes has been reported,[27] while another study did not show different expression of CD11A and CD11B on monocytes and neutrophils in diabetic patients *vs* controls.[28] Furthermore, in comparison with controls, enhanced neutrophil CD11B expression after *ex vivo* stimulation has been described in patients with type 2 diabetes.[29] This finding may suggest increased responsiveness of these cells.

CD66B is a marker of release of substances such as collagenase and gelatinase from specific granules.[30,31] Upon *ex vivo* stimulation, expression of CD66B is rapidly upregulated.[25] CD66B has not been associated with atherosclerosis before, but under proinflammatory conditions activated neutrophils secreting these enzymes in the vicinity of the endothelium, could negatively affect endothelium function and plaque stability. We found a positive association between neutrophil CD66B expression and diastolic and systolic blood pressure. In diabetic patients, besides increased *ex vivo* neutrophil responsiveness, also impaired *ex vivo* degranulation has been shown and these effects where most pronounced in the patients with hypertension and microalbuminuria.[29] We have not

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stimulated leukocytes *ex vivo*, but higher *in vivo* CD66B expression does not exclude degranulation dysfunction in response to acute activating stimuli. In the present study, the higher degree of CD66B expression in the patients is in line with increased expression of CD11B and the strong correlations between the expressions of these markers are suggestive of a common underlying mechanism. Supportive for this is that in resting neutrophils 75% of CD11B is colocalised with CD66B in secretory granules.[32] It was remarkable that the expressions of CD11B and CD66B were best predicted by each other whereas age and blood pressure, which were also correlated by univariate analysis, did not improve the models. This may also be a result of a common mechanism of expression of these markers resulting in amplification of the effect of age and blood pressure. BMI or gender were not correlated in the whole group correlation analysis, so we do not expect gender and BMI to explain the differences in leukocyte activation between the patients and controls.

On lymphocytes the expression of the studied markers was low and not different between the patients and controls. The selected markers are most certainly not the most appropriate to reflect lymphocyte activation. Indeed, upon *in vitro* stimulation the change in expression of these markers was markedly lower on lymphocytes than on monocytes and neutrophils (data on file).

While the leukocyte count was positively related to fasting glucose, a similar relationship with TG, another important characteristic of the metabolic syndrome, was not observed. In addition, both TG and glucose were unrelated to the expression of activation markers on leukocytes, which is in agreement with other studies.[27,33] From many *in vitro* studies it is known that both TG and glucose can activate leukocytes.[13-15,34-36] and that under hyper-TG or hyperglycaemic conditions adherence of leukocytes to the endothelium is increased due to activation of endothelial cells.[12,20,37] Monocytes from diabetic patients showed increased superoxide production upon *ex vivo* stimulation, suggestive of increased responsiveness of these cells. However, this activation was related to plasma TG only and not to elevated glucose or glyHb.[36] The *in vivo* situation seems even more complex, since in diabetic patients and controls after a glucose load, monocyte but not neutrophil CD11B expression increased, unrelated to the glucose rise.[28] Furthermore, we have recently reported acute increments of neutrophil counts during the postprandial phase after ingestion of a glucose and fat load, suggesting a proinflammatory effect of glucose and TG-rich lipoproteins on leukocytes.[38,39] In another study in healthy subjects we have observed increased expression of monocyte and neutrophil CD11B, CD62L and CD66B after an oral fat load, while only the CD11B increments were related to the TG increment (data on file). A possible explanation for the weak *in vivo* relationship could be that advanced glycaemic end products (AGE) or the type of fatty acids or lipoproteins are more important than glucose or TG.[16,40] Regarding AGEs, in the present study we could not find a relationship between glyHb and the activation of leukocytes. This may be due to the relative tight glucose control of the diabetic patients we studied. In addition, leukocytes could become activated indirectly via triggering of endothelial cells by TG or glucose,[12,20,37] by unstable leukocyte-enriched atherosclerotic plaques,[41] or by endothelial injury in general, since coronary angioplasty has been shown to induce leukocyte activation.[42] We also have to underline that, as has been shown by others, the leukocytes we obtained by peripheral sampling are most certainly less activated than the cells contributing to local inflammatory processes, since activated leukocytes will probably adhere to the endothelium *in vivo*.[41]

In conclusion, the expression of activation markers on monocytes and neutrophils is enhanced in patients with type 2 diabetes, independent of fasting glucose and TG. These results suggest a proinflammatory situation in type 2 diabetes and most likely represent increased adhesive capacity of neutrophils and monocytes to the endothelium. The clinical relevance of leukocyte activation and the exact mechanisms whereby type 2 diabetes leads to this activation need to be studied.

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A pilot study exploring the role of glucocorticoid receptor variants in primary biliary cirrhosis and primary sclerosing cholangitis

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ABSTRACT

Background: In primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) significant therapeutic effects of glucocorticoids have not been documented. The most important clinical problem in patients with these diseases is fatigue, which is occasionally invalidating. Abnormalities in the hypothalamo-pituitary-adrenal axis have been suggested as a cause of fatigue. Most effects of glucocorticoids are mediated by the glucocorticoid receptor (hGR α). Recently a causative role for a splicing variant of the glucocorticoid receptor (hGR β) has been proposed in glucocorticoid resistance in asthma and ulcerative colitis, whereas another splicing variant (hGR P) might be associated with glucocorticoid-resistant haematological malignancies. The aims of the present pilot study were to assess abnormalities in glucocorticoid receptor expression and to relate these abnormalities to the development of fatigue and to disease activity and severity in autoimmune cholestatic liver disease.

Methods: Five fatigued and five nonfatigued patients with PBC or PSC were included, and the results were compared with healthy controls.

Results: The expression of hGR P was not different from controls, but hGR β mRNA was significantly increased (p=0.02) and hGR α mRNA decreased (p=0.015). There were no significant differences between fatigued and nonfatigued patients. A significant negative correlation between the serum activity of alkaline phosphatase and hGR α and hGR P mRNA was found.

Conclusion: Although there was no relation with fatigue, abnormalities in hGR expression appear to occur in patients with these diseases, and may play a role in its pathophysiology and the poor response to glucocorticoid treatment.

INTRODUCTION

Primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are chronic cholestatic liver diseases with a relatively favourable prognosis for most patients.[1,2] Clinically, the most frequent and occasionally invalidating symptom is fatigue. In PBC, the prevalence of fatigue of any severity is around 85%. Although fatigue has not been studied as extensively in PSC, it appears to occur with comparable frequency in patients with this disease.[3-5] There is no correlation with the biochemical or histological severity of the disease.[6] Although several studies have attempted to elucidate the pathophysiological mechanisms causing fatigue in cholestatic liver diseases, these have so far remained unknown.[7] In addition, there have been no reports of drugs or other treatment modalities with a beneficial effect on fatigue. Although the widely used drug ursodeoxycholic acid improves the biochemical abnormalities in these diseases, it usually has no effect on fatigue.[6,8]

Since fatigue is the most important and still an untreatable problem in many patients with these diseases, attempting to elucidate the mechanisms leading to fatigue may be an important step in finding an effective treatment. One of the possible mechanisms is dysfunction of the hypothalamo-pituitary-adrenal axis, a role of which has been implicated in the pathophysiology of chronic fatigue.[9] Since the actions of glucocorticoids are mediated by the intracellular glucocorticoid receptor, this receptor has been studied for defects associated with abnormalities in glucocorticoid function.[10] Previously, three splicing variants of the glucocorticoid receptor have been described. The hGR α is the active form of the hGR, while hGR β and hGR P are derived by alternative splicing of the original transcript.[11,12]

The hGR P has been reported to increase the activity of hGR α in several cell lines, and it has been suggested that it may be related to glucocorticoid resistance in haematological malignancies.[12,13] Increased expression of the β-variant of the glucocorticoid receptor (hGR), which is formed by alternative splicing of the hGR gene-transcript and is present in normal human tissues, was associated with glucocorticoid resistance in asthma and ulcerative colitis.[14-19] Although the mechanism causing the increased expression of hGR β is partially unclear, it has been repeatedly found that induction by proinflammatory cytokines may be involved.[20-22] This finding led to the hypothesis that glucocorticoid resistance might be the result of an abnormal inflammatory response.[23] Since an increased production of inflammatory cytokines has also been observed in cholestatic liver diseases, the expression of hGR β might be increased in these diseases.[24-27] In addition, glucocorticoid treatment is not recommended in these diseases since studies assessing the efficacy of glucocorticoids found only modest effects, suggesting that relative glucocorticoid resistance might exist.[28,29] No studies attempting to find a relation between expression of hGR β in chronic inflammatory diseases and fatigue have been reported. We hypothesised that increased expression of hGR β might not only be present in these diseases, but that it might also be associated with fatigue. The present study was performed to determine whether levels of the variants of the hGR in peripheral blood mononuclear leukocytes are different from controls in these cholestatic liver diseases, as well as to assess the relation between hGR expression and fatigue.

PATIENTS AND METHODS

In the present pilot study five patients with a diagnosis of PBC or PSC without fatigue and five patients with chronic and significant fatigue were included. Sex, age and dose of ursodeoxycholic acid were recorded. Serum activity of aspartate aminotransferase, alkaline phosphatase, total serum bilirubin and total immunoglobulin M were measured as markers of disease severity and activity. Fatigue severity was quantified using a visual analogue scale (VAS) and the Fisk fatigue severity scale (FFSS).[30] The FFSS includes social, cognitive and physical domains, in which these aspects of fatigue are quantified. It has been validated for use in primary biliary cirrhosis.[31] A visual analogue scale was used in order to quantify pruritus. Informed consent was obtained from each patient and the study was approved by the institutional review committee.

Laboratory techniques

Blood samples were obtained from a group of 12 healthy controls and the 10 patients. To isolate peripheral blood mononuclear leukocytes, the samples were diluted twofold with saline and layered over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden).

Density gradient centrifugation was performed at 1410 rpm for 30 minutes at room temperature. The peripheral blood mononuclear leukocytes enriched interphase was isolated and washed twice with saline and the final pellet was suspended with saline. RNA was immediately isolated using a high-resolution RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). After RNA elution in 55 μl elution buffer the concentration of isolated RNA was measured using a Ribo-Green RNA Quantitation Reagent and Kit (Brunschwig Chemie, Amsterdam, the Netherlands). Using 5 μM Random Hexamers and 200 nM Oligo-dt-primers in a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, USA), 800 ng of total RNA was used for reverse transcription reaction of 50 μl. Reactions lacking reverse transcriptase were also run to generate controls for assessment of genomic DNA contamination. For the different hGR splice variants 2 μl of the resulting cDNA were amplified in real-time PCR assays on the ABI Prism 7700 (ABS, Nieuwerkerk a/d IJssel, the Netherlands) in a total volume of 25 μl containing 300 pmol of each primer and 200 pmol probe in a qPCR-core kit (Eurogentec, Liege, Belgium). After an initial denaturation at 95 °C for ten minutes, PCR was performed for 42 cycles of denaturation for 15 seconds and annealing for one minute at 60 °C.

To detect the expression of the hGR splice variants we used the same upstream primer: 5'-TGT TTT GCT CCT GAT CTG A-3', encoding part of exon 6, as well as the same taqman probe: 5'-FAM-TGA CTC TAC CCT GCA TGT ACG AC-TAMRA-3', encoding part of exon 7, for all isoforms. To discriminate hGR α, β and P from each other we used specific downstream primers. The sequences of these reverse primers are as follows: rev-α: 5'-TCG GGG AAT TCA ATA CTC A-3', encoding part of exon 9α, rev-β: 5'TGA GCG CCA AGA TTG T-3', encoding part of exon 9β, and rev-P: 5'-GTT TCT GCC ATA CCT ATT TG-3', encoding part of intron 7. The expression levels were determined relatively by using the expression of the HPRT housekeeping gene (hyoxantine phosphoribosyltransferase with the forward primer (500 pmol): 5'CAC TGG CAA AAC AAT GCA GAC T-3', the reverse primer (500 pmol): 5'-GTC TGG CTT ATA TCC AAC ACT TCG T-3', and the probe (200 pmol): 5'FAM-CAA GCT TGC GAC CTT GAC CAT CTT TGG A-TAMRA-3'.

Because of the supposed interactions between the hGR β and hGR P variants with the active hGR α variant, we calculated the hGR α/hGR β and the hGR α/hGR P ratios.

Statistical analysis

Differences in the expression of variants of hGR mRNA between patients and controls, and differences between fatigued and nonfatigued patients were tested using

Mann-Whitney's nonparametric test for independent samples. Correlations between the severity of fatigue and laboratory values and the hGR expression were tested using Pearson's correlation method. Logarithmic transformations of laboratory values were used. All statistical tests were performed using SPSS version 9.0.

RESULTS

Ten patients with cholestatic liver disease were included in the study, five of whom complained about fatigue. Seven patients had a diagnosis of PBC and three had been diagnosed with PSC. A summary of patient characteristics is shown in *table 1*.

To assess differences in hGR mRNA levels related to the presence of the disease, we compared the levels of the three variants of the hGR, as well as the hGR α/hGR P ratio and the hGR α/hGR β ratio with the levels in a group of healthy controls. These tests resulted in non-significant p values for hGR P and p values of 0.015 for the hGR α variant and 0.02 for the hGR β variant, with decreased numbers of hGR α and increased numbers of hGR β mRNA in patients *vs* controls. In addition, the hGR α/hGR β ratio was significantly decreased in patients (*table 2*).

Tests for differences between fatigued and nonfatigued patients were performed, and resulted in p values of 0.99 for hGR α, 0.65 for hGR β and 0.28 for hGR P. No significant correlations were found between GR mRNA levels and quantified fatigue (*table 3*).

Finally, correlation testing was performed to find associations between the GR variants and the markers of disease severity and activity. A significant, negative correlation was found between the serum alkaline phosphatase activity and hGR α and hGR P, as well as total hGR mRNA. For the levels of aspartate aminotransferase, bilirubin and immunoglobulin M, no significant correlations were found (*table 4*). *Figure 1* illustrates this relation between alkaline phosphatase and total hGR mRNA.

Table 2
Glucocorticoid receptor mRNA levels, number of copies in 2 µl cDNA obtained from a 50 µl reverse transcriptase reaction of 800 ng RNA

RELATIVE NUMBER OF COPIES	MEAN (PATIENTS)	MEAN (CONTROLS)	P VALUE
hGR α	41,887	56,025	0.02
hGR β	69	42	0.02
hGR P	8,889	11,922	0.09
hGR α/ hGR P	4.7	5.1	0.19
hGR α/ hGR β	679	1,523	0.001
Total	50,844	67,752	0.02

hGR = human glucocorticoid receptor.

DISCUSSION

In the present study we found increased levels of hGR β mRNA and decreased levels of hGR α mRNA in patients with cholestatic liver disease compared with healthy controls. As a result, the hGR α/hGR β ratio was significantly decreased. In addition, there was a significant inverse

Table 1
Patient characteristics

	ALL PATIENTS	FATIGUE +	FATIGUE -
Male/female	4 / 6	3 / 2	1 / 4
PSC/PBC	3 / 7	3 / 2	0 / 5
Age	58 (40-76)	53 (40-62)	64 (55-76)
UDCA dose (mg/day)	900 (0-1200)	900 (0-1200)	900 (600-1200)
Bilirubin (µmol/l)	15.5 (7-89)	15 (10-89)	16 (7-43)
Alkaline phosphatase (U/l)	171 (72-441)	188 (122-441)	154 (72-427)
Aspartate aminotransferase (U/l)	40 (27-241)	39 (27-241)	40 (30-65)
Immunoglobulin M (g/l)	1.8 (1.1-3.9)	2.8 (1.2-3.9)	1.6 (1.1-2.4)
Visual analogue score for pruritus (cm)	0.6 (0-3.3)	0.9 (0-2.5)	0 (0-3.3)
Visual analogue score for fatigue (cm)	3.15 (0-9.3)	6.2 (4.3-9.3)	0 (0-2.0)
FFSS physical domain	9 (1-32)	24 (10-32)	1 (1-8)
FFSS cognitive domain	4.5 (0-21)	7 (4-21)	2 (0-5)
FFSS social domain	7.5 (0-42)	31 (9-42)	3 (0-6)

Values are medium (range). PSC = primary sclerosing cholangitis, PBC = primary biliary cirrhosis, FFSS = Fisk fatigue severity scale.

Table 3
Correlation between hGR mRNA and fatigue

	HGR α		HGR β		HGR P		TOTAL	
	coeff.	**P**	**coeff.**	**P**	**coeff.**	**P**	**coeff.**	**p**
VAS	-0.089	0.81	-0.29	0.43	0.12	0.75	-0.02	0.96
FFSS physical domain	-0.26	0.47	-0.30	0.40	-0.07	0.86	-0.20	0.58
FFSS cognitive domain	-0.93	0.80	0.21	0.55	0.14	0.71	-0.01	0.97
FFSS social domain	-0.26	0.47	-0.10	0.79	0.15	0.69	-0.12	0.74

Coeff. = coefficient, hGR = human glucocorticoid receptor, VAS = visual analogue scale, FFSS = Fisk fatigue severity scale.

Table 4
Correlation between hGR mRNA and biochemical markers of disease activity and severity

	HGR α		HGR β		HGR P		TOTAL	
	coeff.	**P**	**coeff.**	**P**	**coeff.**	**P**	**coeff.**	**p**
Total serum bilirubin	-0.38	0.28	0.11	0.76	-0.063	0.86	-0.28	0.43
Alkaline phosphatase	-0.65	0.041	-0.28	0.44	-0.64	0.049	-0.68	0.03
Aspartate aminotransferase	-0.39	0.27	-0.20	0.58	-0.61	0.063	-0.48	0.16
Immunoglobulin M	-0.10	0.78	0.11	0.76	-0.31	0.39	-0.18	0.62

hGR = human glucocorticoid receptor, coeff. = coefficient.

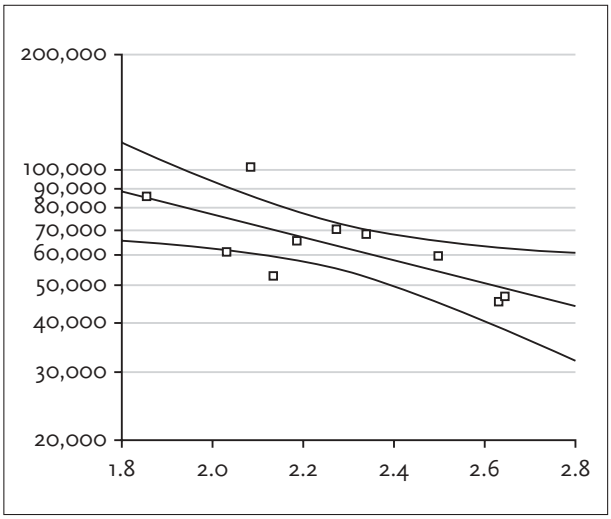

Figure 1
Relation between total GR mRNA and serum activity of alkaline phosphatase
[10]Log(alkaline phosphatase) is shown on the X-axis and total hGR mRNA is shown on the Y-axis.

relation between the hGR α and hGR P variants and the serum activity of alkaline phosphatase, a routinely used marker of disease activity in PBC and PSC. A correlation between the receptor variants and fatigue was not found. An association between increased levels of hGR β mRNA and glucocorticoid resistance in asthma and ulcerative colitis has been reported previously.[14-17,20,21] In addition, increased expression of this variant has been observed in patients with hormone resistant nephrotic syndrome, chronic lymphatic leukaemia and nasal polyps.[32-35] Several *in vitro* studies have shown that expression of hGR β can be induced by the inflammatory cytokines Il-2, Il-4, Il-7, Il-8 and TNF-α.[20-22] The increase of hGR β as a result of cytokine exposure correlated with a decrease in glucocorticoid sensitivity in one of these studies.[22] Further, the frequency of a polymorphism associated with increased stability of hGR β mRNA was increased in patients with rheumatoid arthritis. The authors suggested that this could be a cause of glucocorticoid resistance, which is a common problem in this condition.[36] However, despite a significant number of studies reporting it, controversy regarding the negative effects of this variant does still exist, since several other *in vivo* and *in vitro* studies found no effects of the hGR β variant, and the mechanisms responsible for the dominant negative effect are largely unknown.[13,19,37-39] In the present study, levels of hGR β mRNA were much lower than those of the other variants. This does not exclude a role for this variant in causing glucocorticoid resistance, since similar results have been obtained in previous studies reporting quantitative hGR mRNA levels, and it might have several explanations.[14,35] First, mRNA does not necessarily correspond with protein levels, and hGR β protein levels could better reflect the

mechanism leading to glucocorticoid resistance, although a previous paper reported very low or undetectable hGR β protein levels in the presence of similar mRNA levels as in the present study.[19] Second, in the present study blood samples were studied, whereas the disease occurs primarily in the liver. Thus, studying blood samples may have diluted the hypothetically higher intrahepatic hGR β levels. Third, significantly lower levels of hGR β compared with total hGR levels might be needed to induce glucocorticoid resistance, although the mechanism responsible for this presumed dominant negative effect of the hGR β variant is unclear.[23]

Thus, the increased levels of hGR β mRNA in patients in the present study compared with healthy controls may have been caused by the inflammatory nature of these liver diseases, and it can be hypothesised that the modest efficacy of glucocorticoid treatment in these diseases could be caused by an increased expression of hGR β.[28,29] Another explanation for the increased hGR β mRNA levels, in parallel to the hypothesis by Derijk *et al.* in rheumatoid arthritis, is that patients with increased hGR β expression are at increased risk of developing autoimmune diseases due to resistance to endogenous glucocorticoids.[36]

An inverse correlation between the levels of hGR α and hGR P, and therefore total hGR mRNA, and the serum activity of alkaline phosphatase was found, whereas we found no correlation with the other markers of disease activity or severity. Such a relation with disease activity has been reported previously in patients with systemic lupus erythematosus, where glucocorticoid sensitivity correlated with total hGR levels.[40] In patients with rheumatoid arthritis, hGR levels were decreased in patients compared with controls.[41,42] These studies suggest that, in addition to hGR β expression, hGR α and hGR P levels might also play a role in determining disease activity and glucocorticoid resistance.

The most important limitation of the present study is its small sample size, and therefore confirmation of the results of the present study in a subsequent larger study would be valuable. In addition, the present study design does not allow conclusions with regard to the cause of abnormalities in hGR expression.

In conclusion, we found increased expression of hGR β mRNA in patients with cholestatic liver diseases as compared with controls and an inverse relation between the hGR α and hGR P mRNA and the serum activity of alkaline phosphatase. This suggests that the glucocorticoid receptor might be involved in the pathogenesis of these diseases as well as in their relative glucocorticoid resistance. Since we found no correlation with fatigue, it seems unlikely that differential expression of hGR variants plays a major role in the aetiology of this distressing symptom of PBC and PSC.

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A remarkable ECG of a patient with swollen legs

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CASE REPORT

A 69-year-old male with an unremarkable medical history was admitted to the hospital because of predominantly right-sided heart failure. Despite initiation of furosemide two weeks before admission, his physical condition did not improve. Physical examination revealed oedematous legs and presacral oedema. His blood pressure was 100/60 mmHg with a pulse frequency of 88 beats/min. The laboratory results were as follows: normal peripheral blood cell count, urea 7.5 mmol/l, creatinine 80 µmol/l, ASAT 38U/l, ALAT 40 U/l, γ-glutamyltransferase 55 U/l, alkaline phosphatase 118 U/l and C-reactive protein 3 mg/l.
The ECG (*figure 1*) showed sinus rhythm with microvoltages in the frontal leads and slow R-progression in the precordial leads. Echocardiography revealed a concentric hypertrophic heart with moderate left systolic function, based on diffuse hypokinesia. Doppler showed a restrictive diastolic flow pattern.
Investigations showed hypoalbuminaemia (24 g/l) and absence of monoclonal gammopathy. Albuminuria of 2 g/day was documented.

WHAT IS YOUR DIAGNOSIS?

See page 340 for the answer to this photo quiz.

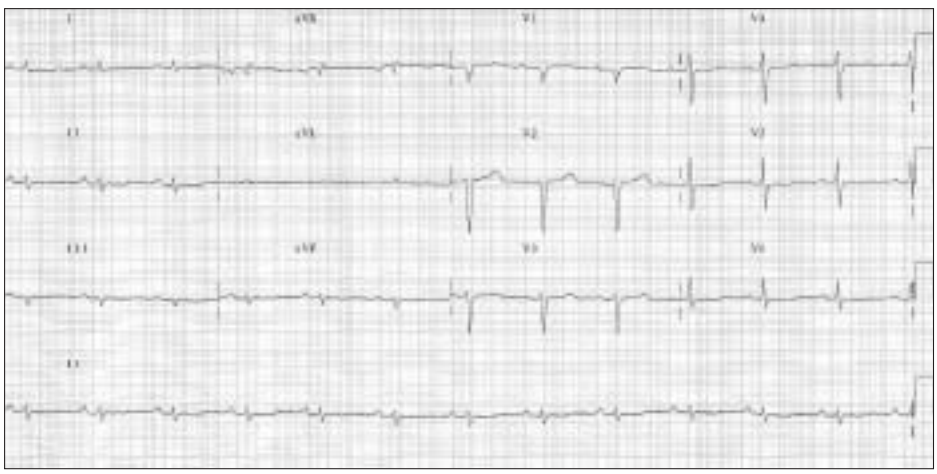

Figure 1
The ECG shows sinus rhythm with microvoltages in the frontal leads and slow R-progression in the precordial leads

A colour version of this figure is available on www.njmonline.nl

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CASE REPORT

Valproic acid intoxication: sense and non-sense of haemodialysis

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ABSTRACT

Introduction: Valproic acid is increasingly used in the treatment of epilepsy, and also prescribed for bipolar affective disorders, schizoaffective disorders, schizophrenia and migraine prophylaxis. We describe two case reports involving valproic acid intoxication with ingestion of ethanol.

Methods: One patient was treated by supportive care, one patient received haemodialysis.

Results: From analysis of plasma concentrations before and during haemodialysis (pre- and post-filter) it is shown that valproic acid can be effectively eliminated by haemodialysis when plasma levels are way above 100 μg/ml. In the literature, plasma protein binding is reported to be around 90% for levels within the therapeutic range. In our patient plasma protein binding was around 50% after treatment with haemodialysis.

Conclusion: These findings make haemodialysis in valproic acid intoxication a sensible therapeutic option with increasing efficiency when plasma concentration is high. Furthermore our findings suggest that lowering valproic acid concentrations to a therapeutic level by haemodialysis does not necessarily result in an immediate, simultaneous increase in plasma protein binding of valproic acid.

INTRODUCTION

Valproic acid (VPA) is increasingly used in the treatment of epilepsy, and is also prescribed for bipolar affective disorders, schizoaffective disorders, schizophrenia and migraine prophylaxis.[1,2] Patients with these diseases are prone to self-poisoning. Self-poisoning with VPA may lead to coma, respiratory failure, renal failure, acute pancreatitis, central nervous system depression, hepatotoxicity, leucopenia, thrombocytopenia, and drowsiness.[3-5] Although some side affects of VPA are not dose-dependent, toxic effects are associated with daily doses above 1800 mg[6] and blood levels above 100 μg/ml.[7] VPA has a low molecular weight of 144 daltons and a small distribution volume (0.1 to 0.5 l/kg). VPA is metabolised in the liver and excreted in the urine. At therapeutic levels (50 to 100 μg/ml), VPA is almost completely bound (90 to 95%) to plasma proteins with, as a consequence, a limited free drug fraction.[8] Thus, at the therapeutic serum level, drug removal obtained through haemodialysis (HD) is negligible. However, in case of VPA overdose, the free drug fraction increases as VPA protein binding sites become saturated, and plasma concentration of the drug increases significantly.[8-10] In this situation, HD may be a useful tool for eliminating VPA. We report two cases of VPA self-poisoning. We studied the natural course in one patient and the effect of HD in the other patient.

CASE 1

A 38-year-old man with a history of psychiatric disease was found naked and unconscious. Empty medication strips were found on the ground beside him. Sixty tablets of valproic acid (Depakine® chrono) were missing.
He arrived in the emergency room in coma, with a Glasgow

coma score of 3. He had a regular heart rate of 92 beats/min, blood pressure of 100/45 mmHg, temperature of 36 °C, and a respiratory rate of 20 breaths/min. Blood toxicology revealed ethanol values of 1.1%. The serum valproic acid (VPA) concentration on admission was 846 µg/ml (therapeutic range 50 to 100 µg/ml).
Initial treatment included enteral activated charcoal, nasal oxygen, and fluid therapy. The patient was admitted to the intensive care unit. He was rehydrated, and $MgSO_4$, and L-carnitine were administered intravenously.[11]
Haemodialysis (HD via a right femoral venous catheter) was initiated 3.5 hours after admission and was performed for 4.5 hours. During this HD session, the blood flow rate was 300 ml/min, and dialysate flow rate was 500 ml/min. Blood samples for determination of VPA and free VPA concentrations were drawn at one, two, three and four hours after dialysis initiation both pre- and post-filter. A fluoresence polarisation immunoassay (FPIA; Cobas Integra, Mannheim, Germany) was used for analysis of total VPA concentrations in plasma. After ultrafiltration, free VPA concentrations were determined by a specific and sensitive gas chromatography method with flame ionisation detection (GC-FID), with a limit of detection of 1.5 µg/ml. Percentage plasma protein binding of VPA was calculated as 100 x (total VPA concentration – free VPA concentration)/total VPA concentration.
After HD, the serum VPA concentration decreased from 723 to 313 µg/ml. Eighteen hours after the start of HD the VPA concentration had decreased to 86 µg/ml (*figure 1*). During dialysis, the patient showed stable haemodynamic parameters. He regained consciousness and no toxic effects, including hepatotoxicity, occurred. His subsequent hospital course was uncomplicated.

CASE 2

A 37-year-old man with a history of epilepsy (treated with Depakine® chrono) following a road traffic injury 14 years previously, multiple suicide attempts, and alcohol dependence, arrived in the emergency room with a Glasgow coma score of 6. He had a regular heart rate of 80 beats/min, blood pressure of 105/70 mmHg, temperature of 36.8 °C, and a respiratory rate of 15 breaths/min. Blood toxicology revealed an ethanol level of 2.8%. The serum valproic acid (VPA) concentration on admission was 260 µg/ml (therapeutic range, 50 to 100 µg/ml). Initial treatment included enteral activated charcoal, nasal oxygen and fluid therapy. The patient was admitted to the intensive care unit. He was treated with supportive care. After 24 hours, his serum VPA concentration decreased to 186.9 µg/ml; 48 hours thereafter the VPA concentration had decreased to 52.9 µg/ml (*figure 1*). It is shown in this case that the half-life of VPA is prolonged in supratherapeutic ranges (e.g. approximately 24 hours). The patient remained haemodynamically stable. His subsequent hospital course was uncomplicated.

DISCUSSION

Valproic acid (VPA) is used in the treatment of epilepsy and psychiatric disorders. We have reported two cases of VPA self-poisoning and have studied the natural course in one patient and the effect of HD in the other. The patient who was treated with HD showed a better elimination rate than the patient on supportive care. However, HD is not totally free from complications and is not widely or

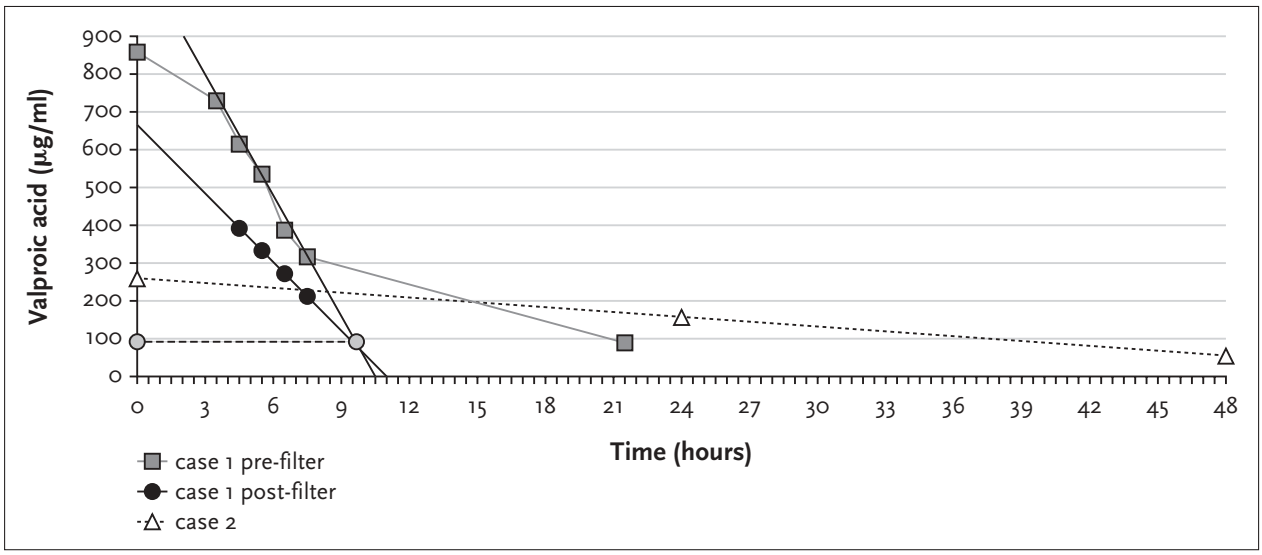

Figure 1
Valproic acid concentration vs time
In case 1 the pre- and post-haemodialysis filter VPA levels are shown. Case 2 represents serum levels. The line at 100 µg/ml is drawn to determine the level were the elimination fraction is zero (see text).

rapidly available in most hospitals. The most common complications during HD are hypotension (20 to 30%), cramps (5 to 20%), nausea and vomiting (5 to 15%), headache (5%), chest pain (2 to 5%), back pain (2 to 5%), itching (5%), and fever and chills (<1%).[12]

On the other hand, if HD is not applied in acute VPA self-poisoning, risks are taken. Self-poisoning with VPA may lead to coma, respiratory failure, renal failure, acute pancreatitis, central nervous system depression, hepatotoxicity, leucopenia, thrombocytopenia, and drowsiness.[3-5] Hepatotoxicity may be asymptomatic with elevated serum liver enzymes, or may present as fatal hepatic failure.[5,7] Therefore, at very high plasma levels the risks of HD may outweigh the risks of prolonged toxic VPA levels if supportive care is given without HD. However, VPA is poorly water-soluble, suggesting that it would be poorly dialysed from the blood into the dialysate. In contrast, the volume of distribution (0.1 to 0.5 l/kg) and low molecular weight suggest it would be effectively removed from circulation by dialysis. According to the current literature, at therapeutic levels VPA circulates almost completely protein bound.[8] The protein binding decreases to 70 and 35% when drug concentrations are >150 μg/ml, and >300 μg/ml, respectively.[10,13] The toxicokinetics, as derived from the literature on VPA, are summarised in *table 1*.[14-16]

Table 1
Toxicokinetics of VPA

Peak plasma level	50-100 μg/ml
Time to peak plasma level	1-4 hours
Volume of distribution	0.15-0.40 l/kg
Plasma protein binding	90%
Elimination half-life	7-15 hours
Excreted unchanged	1-3%

With supratherapeutic VPA concentrations, the free drug fraction increases as VPA protein binding sites become saturated, and HD may be an increasingly efficient tool to eliminate VPA and lower the free drug fraction. As shown in *figure 1*, the concentration differences between pre- and post-filter blood samples are greater in the high plasma concentration range than in the low plasma concentration range. This elimination fraction (EF) is shown in *table 2*. By extrapolating these results, we calculated that the EF will be zero with a plasma level of 100 μg/ml. Above this cut-off point of the VPA concentration, HD will contribute to VPA elimination. However, a low free drug fraction will decrease the effectiveness of HD.

Klotz and Antonin studied the pharmacokinetics and bioavailability of VPA.[10] At the therapeutic concentration of 80 μg/ml, the plasma protein binding of VPA was determined (the four-hour blood sample). They found that therapeutic concentrations of VPA revealed relatively strong plasma protein binding between 80 and 94% after a single iv bolus of 400 mg. The results were obtained from six healthy volunteers,[10] and used as a reference in numerous articles. In contrast, at a therapeutic level after intoxication, we found a lower protein bound fraction of around 50%. The reason for this finding is not completely clear.

We found an increase in protein binding with lowering of the total VPA concentration. This is in agreement with a case report by Franssen *et al.*[17] in which they describe a patient with a severe VPA intoxication successfully treated by HD and haemoperfusion. In that particular case, during two treatment sessions, protein binding of VPA rose from 32%, at the start, to 54%.[17] There may be two explanations for the fact that when lowering toxic total VPA concentrations to a therapeutic level by HD, protein binding does not immediately reach the normal level of around 90%. HD may increase free fatty concentrations, leading to an increase in free VPA concentrations by displacement of protein binding.[18] However, De Smet *et al.* reported that

Table 2
VPA concentrations, free fractions, and plasma protein binding in case 1

TIME (HOURS)	VPA SERUM CONCENTRATION PRE-FILTER (μG/ML)	FREE DRUG FRACTION (μG/ML) (% PLASMA PROTEIN BINDING)	VPA CONCENTRATION POST-FILTER (μG/ML)	FREE DRUG FRACTION POST-FILTER (μG/ML) (% PLASMA PROTEIN BINDING)	ELIMINATION FRACTION
0	846	398 (53%)			
3.5	723	425 (41%)	*		
4.5	616	358 (42%)	391	204 (48%)	36.5% ((616-391)/616)
5.5	535	313 (41%)	317	157 (50%)	40.7%
6.5	379	238 (37%)	265	112 (58%)	30.0%
7.5	313	165 (47%)	210 **	88 (58%)	32.9%
21.5	86	33 (59%)			

** Start of haemodialysis, ** end of haemodialysis.*

the use of heparin during dialysis may alter lipase activity in the blood sample collection tubes (the free fraction does not increase during HD when lipase activity is neutralised at the time of blood sampling), resulting in higher free fatty acid concentration competing with VPA for protein binding sites. Thus, during dialysis, measured free VPA concentrations may be higher than in reality, and previous reported increases are probably artefacts.[19]

It was shown earlier that the protein binding of VPA may alter due to age and concomitant medication.[20,21] However, neither of these factors were present in our patient. *Table 2* shows an increase in protein binding measurements and an ongoing decrease in VPA concentrations. It can be speculated that a further increase in protein binding would appear with decreasing VPA concentrations.

In conclusion, although it seems logical to use HD in VPA levels above 100 μg/ml, a decision will be made based upon the clinical situation and the possible disadvantages of HD. In practice, the cut-off point when to use HD will be higher than the 100 μg/ml as illustrated by our second case. In the first patient with a relatively high VPA level, HD was successfully applied, whereas in our second patient with a much lower VPA level, no forced elimination was performed. In addition, we have shown in our patient that the protein binding at high VPA levels, and after normalisation of the level by HD, differs from protein binding data at therapeutic levels under normal circumstances.

ACKNOWLEDGMENTS

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CASE REPORT

Autoimmune haemolysis as an unusual cause of anaemia in von Recklinghausen's disease

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ABSTRACT

Von Recklinghausen's disease, now classified as neurofibromatosis type 1 (NF-1), is a relatively frequent autosomal dominant disorder and has clinical manifestations, such as cafe-au-lait spots, freckling, generalised cutaneus neurofibroma, Lisch nodules, short stature, optic glioma and central nervous system tumours. In adults, anaemia in the course of NF-1 is usually due to gastrointestinal tumour bleeding. Association of NF-1 and autoimmune haemolytic anaemia is unusual. Here, we report a 48-year-old woman with NF-1 presenting as autoimmune haemolytic anaemia. We also reviewed the literature about the association of NF-1 and autoimmune diseases.

INTRODUCTION

Neurofibromatosis type 1 (NF-1) is a common autosomal dominant neurocutaneus syndrome.[1] This syndrome is associated with a variety of benign and malignant tumours such as neurofibromas, neurofibrosarcomas, pheochromocytomas, central nervous system (CNS) tumours. In addition, children with NF-1 have increased risk for malignant myeloid disorders which may present with anaemia. However, in adults, there is insufficient data about the association of NF-1 and haematological malignancies. In adults, patients with NF-1 may present with anaemia due to bleeding from gastrointestinal tumours.[2-5] On the other hand, severe anaemia due to autoimmune haemolysis in NF-1 is unusual. We report a case of NF-1 presenting as autoimmune haemolytic anaemia with unexpected laboratory findings and dramatic response to oral prednisolone therapy.

CASE REPORT

The patient was a 48-year-old woman with NF-1 who had been taking oral iron preparations for anaemia for ten years. She was not taking any other medications. There was no information about previous laboratory tests since these tests were performed at another hospital ten years ago. Furthermore, the patient had not had any medical follow-up during the last ten years. She was referred to our hospital to examine the gastrointestinal tract as a source of iron loss. She had had no menstrual bleeding for four years. Medical history of the patient revealed no fever or weight loss suggesting lymphoma, no joint pain suggesting autoimmune disease, or no painful episodes suggesting sickle cell anaemia. Physical findings on admission were kyphosis and a short stature with a height of 140 cm and weight 43 kg. Blood pressure was 110/60 mmHg. There was no hepatosplenomegaly or lymphadenopathy. Cafe-au-lait spots, freckles and multiple soft skin tumours were observed over her entire body (*figure 1*). In addition, her daughter and three sisters had similar cutaneus physical findings.

The laboratory findings showed a reduction in red blood cells, haemoglobin, haematocrit, increase in reticulocytes, presence of normoblasts and normal mean corpuscular volume, lactate dehydrogenase (LDH), total bilirubin, unconjugated bilirubin (UB), iron, transferrin saturation and ferritin values (*table 1*). Liver and renal function tests were all within normal ranges. Levels of vitamin B12 and folic acid were in normal ranges. Haemoglobin electrophoresis was normal and no monoclonal gammapathy was detected in immunoelectrophoretic study. Direct Coombs' test was positive. Test for haptoglobin was not performed because of technical problems.

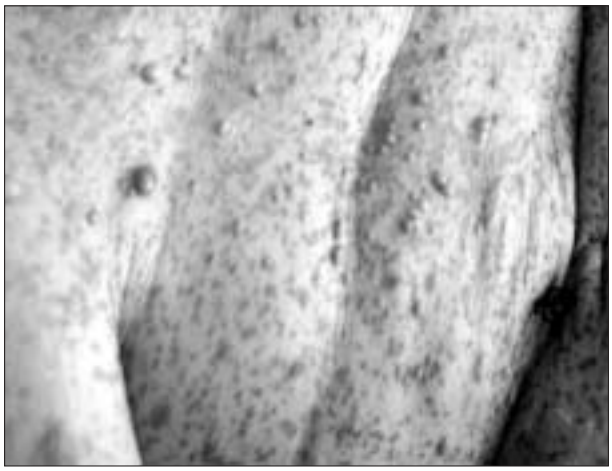

Figure 1
Cutaneus findings of abdominal region
Note the freckles and multiple soft skin tumours.

Table 1
Laboratory data on admission and 20th day of therapy

	ON ADMISSION	20TH DAY OF THERAPY
White blood cells	23.2 x 10^9/l	9.7 x 10^9/l
Red blood cells	1.96 x 10^{12}/l	4.38 x 10^{12}/l
Haemoglobin	2.5 mmol/l	7.6 mmol/l
Haematocrit	0.12 l/l	0.36 l/l
Mean corpuscular volume	81 fl	82 fl
Platelets	213 x 10^9/l	270 x 10^9/l
Iron (9-27 µmol/L)	11.7 µmol/l	14.0 mmol/l
Transferrin saturation	22%	25%
Ferritin (30-150 µg/L)	20 µg/l	28 µg/l
Reticulocytes	12%	2%
Corrected reticulocytes	4%	2%
Direct Coombs' test	positive	not performed
Normoblasts	6%	not seen
Peripheral blood smear	anisocytosis, polychromasia, spherocytosis, normoblasts	none
Lactate dehydrogenase	270 U/l (230-460 U/l)	310 U/l
Total bilirubin (5.1-17µmol/l)	4 µmol/l	3 µmol/l

Peripheral blood smear showed anisocytosis, polychromasia, normoblasts and spherocytosis. Infectious serologies including mycoplasma, hepatitis B, C, Cytomegalovirus, Ebstein-Barr virus and human immunodeficiency virus were negative. Tests for antinuclear antibody and anti-double-stranded DNA were also negative. Bone marrow examination was planned, but the patient refused it. Although laboratory findings were consistent with autoimmune haemolytic anaemia, normal values of LDH and UB were unexpected results. Furthermore, she had been anaemic for ten years. To exclude gastrointestinal bleeding, double-contrast barium studies of colon and small bowel, upper gastrointestinal endoscopy and rectosigmoidoscopy were performed and they revealed no gastrointestinal lesions. Angiography was not performed because of the absence of active gastrointestinal bleeding. Stool testing for occult blood was negative three times.

Abdominal ultrasonography revealed suspect hypoechogenic small lesions of the liver. Computed tomography as well as magnetic resonance imaging showed multiple, smaller than 1 cm, cystic lesions of the liver. We considered these lesions to be incidental since laparoscopy showed no liver surface lesions and biopsy revealed normal liver histology; these investigations were performed to exclude hepatic neurofibromatosis.

With the laboratory findings above, the diagnosis of autoimmune haemolytic anaemia was made and oral prednisolone was administered at a dose of 1 mg/kg. After 20 days, without any blood transfusions, all the haematological parameters were within the normal ranges (*table 1*).

DISCUSSION

NF-1, previously known as von Recklinhausen's disease, is a relatively common autosomal disease affecting one in 2190 to 7800 people.[6] The specific gene maps to chromosome 17q11.2.[7] Clinical manifestations of NF-1 include cafe-au-lait spots, freckling, generalised cutaneus neurofibroma, Lisch nodules, short stature, optic glioma and CNS tumours. NF-1 patients have increased risk of developing malignancies.[8] They have a high spontaneous mutation rate[1] and mutations in tumour suppressor genes play an important role in the development of tumours.[9] The most common cause of death is CNS tumours.[10]

The complication of gastrointestinal lesions has been reported in 25% of NF-1 patients.[11] When anaemia is seen in the course of NF-1, gastrointestinal system tumours have to be searched for carefully.[2-5] In some cases, only angiography was successful in identifying the focus of bleeding.[12,13] Bell *et al.*[14] reported an NF-1 patient with unexplained chronic anaemia for 21 years and finally diagnosed as retroperitoneal neurofibrosarcoma invading small intestine.

There are few reports of NF-1 patients associated with autoimmune diseases. Migita *et al.*[15] presented a case of

NF-1 associated with mixed connective tissue disease and treated with glucocorticoids. Scadding[16] reported the association of NF-1 with fibrosing alveolitis and autoimmune haemolysis. Toth *et al.*[17] presented an NF-1 patient with unexplained iron-deficient anaemia and secondary adrenal insufficiency who was successfully treated with glucocorticoids. The pathogenesis by which NF-1 is associated with autoimmune diseases is unknown. Gerosa *et al.*[18] reported that there were borderline levels of anti-DNA antibody and immune complex in some NF-1 patients. On the other hand, as with the present case, previously reported associations might be coincidental. There is insufficient evidence that autoimmune process plays a role in NF-1.

In our patient, although normal values of LDH and UB were inconsistent, positive direct Coombs' test and findings of peripheral blood smear brought us to the diagnosis of autoimmune haemolytic anaemia. The response to glucocorticoid administration was excellent without any blood transfusion which supported our diagnosis. An important point was that autoimmune haemolytic anaemia might be associated with haematological malignancies or autoimmune diseases. Although the patient was an adult, bone marrow examination was planned to exclude the diagnosis of myelodysplastic syndrome, but the patient refused it. Nevertheless, we considered that in addition to the age of the patient, dramatic response to glucocorticoid therapy was enough to exclude myelodysplastic syndrome. Lymphoma was excluded by the absence of fever, weight loss, organomegaly, or lympadenopathy. No monoclonal gammapathy was detected in immunoelectrophoretic study which strongly excluded multiple myeloma. An autoimmune disease was not considered since there were no clinical symptoms such as joint pain, weight loss or fever. In addition, antinuclear antibody testing was negative.

In conclusion, evaluation of anaemia in NF-1 patients seems to be difficult. While a careful search of the gastrointestinal tract is needed, it must also be borne in mind that NF-1 patients may present with autoimmune haemolytic anaemia. Furthermore, laboratory findings such as normal values of LDH and UB may not exclude autoimmune haemolysis.

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ANSWER TO PHOTO QUIZ (ON PAGE 332)

A REMARKABLE ECG OF A PATIENT WITH SWOLLEN LEGS

DIAGNOSIS

The combination of a hypertrophic myocardium with diffuse diminished left ventricular function and glomerular involvement (macroalbuminuria) was suspicious for a systemic disease. The ECG pattern was concordant with cardiac amyloidosis.

Invasive work-up confirmed restriction with square root sign, elevated pulmonary wedge pressure, and left ventricular end-diastolic pressure. Myocardial amyloidosis was histologically confirmed by endomyocardial biopsy.

This case demonstrates the two most common and diagnostically useful ECG patterns in primary amyloidosis: pseudoinfarction pattern (sensitivity 63 to 80%) and low QRS voltage (sensitivity 60 to 93%).[1,2]

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ABOUT THE COVER

‘Schuim der Zee’

Ellen Baptist

Ellen Baptist (1945), the artist of this month’s cover, was educated in Arnhem at the Academy of Design. She lives and works in Huissen. She paints, makes woodcarvings, dry-needle prints, dips paper and makes exclusive books for artists. In 2004 she developed a new technique: cast printing. These prints originate from casting coloured paper pulp on a wet pulp basis. Baptist also teaches at the Open Academy in Bemmel and gives workshops in different techniques in her studio and if required on location.

This print is part of a series of woodprints on self-dipped paper.
The series was given the title “Verlangen”. For this special occasion two prints from this series can be bought separately.
The whole series is available at a price of € 681, the price of the two separate prints will be € 150 each.
A limited edition of the original print can be ordered at Galerie Unita, Rijksstraatweg 109, 6573 CK Beek-Ubbergen, the Netherlands, e-mail: galerie-unita@planet.nl or on our website: www.galerie-unita.com.

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The Netherlands Journal of Medicine publishes papers in all relevant fields of internal medicine. In addition to reports of original clinical and experimental studies, reviews on topics of interest or importance, case reports, book reviews and letters to the Editor are welcomed.

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Manuscripts submitted to the Journal should report original research not previously published or being considered for publication elsewhere. Submission of a manuscript to this Journal gives the publisher the right to publish the paper if it is accepted. Manuscripts may be edited to improve clarity and expression.

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Language
The language of the Journal is English. English idiom and spelling is used in accordance with the Oxford dictionary. Thus: Centre and not Center, Tumour and not Tumor, Haematology and not Hematology.

Preparation of manuscripts
Type all pages with double spacing and wide margins on one side of the paper. To facilitate the reviewing process number the pages; also we would appreciate seeing the line numbers in the margin (Word: page set-up - margins - layout - line numbers). Divide the manuscript into the following sections: Title page, Abstract, Introduction, Materials and methods, Results, Discussion, Acknowledgements, References, Tables and Figures with Legends.

A *Covering letter* should accompany the manuscript, identifying the person (with the address, telephone and telex numbers, and e-mail address) responsible for negotiations concerning the manuscript: the letter should make it clear that the final manuscript has been seen and approved by all authors. Conflicts of interest, any commercial affiliations, consultations, stock or equity interests should be specified. In the letter 1-3 sentences should be dedicated to what this study adds. All authors should sign the letter.

The *Title page* should include authors' names, degrees, academic addresses, address for correspondence including telephone, fax and e-mail, and grant support. Also the contribution of each author should be specified.
The title should be informative and not exceed 90 characters, including spaces. Avoid use of extraneous words such as 'study', 'investigation' as well as priority claims (new, novel, first). Give a running title of less than 50 characters. If data from the manuscript have been presented at a meeting, list the name, date and location of the meeting and reference and previously published abstracts in the bibliography. Give a word count (including references, excluding tables and legends) at the bottom of this page.

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The *Abstract*, not exceeding 200 words, should be written in a structured manner and with particular care, since this will be the only part of the article studied by some readers. In original articles, the abstract should consist of four paragraphs, labelled Background, Methods, Results, and Conclusion. They should briefly describe the problem being addressed in the study, how the study was performed and which measurements were carried out, the most relevant results, and what the authors conclude from the results.

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The *Materials and methods* should be sufficiently detailed so that readers and reviewers can understand precisely what has been done without studying the references directly. The description may be abbreviated when well-accepted techniques are used.

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Examples:

[1.] Smilde TJ, Wissen S van, Wollersheim H, Kastelein JJP, Stalenhoef AFH. Genetic and metabolic factors predicting risk of cardiovascular disease in familial hypercholesterolemia. Neth J Med 2001;59:184-95.

[2.] Kaplan NM. Clinical Hypertension. 7th Edition. Baltimore: Williams & Wilkins; 1998.

[3.] Powell LW, Isselbacher KJ. Hemochromatosis. In: Harrison's Principles of Internal Medicine, 15th Edition, Braunwald E, Fauci AS, Kasper DL, et al. (eds). New York: McGraw-Hill; 2001. p. 2257-61.

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