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# Netherlands

# The Journal of Medicine

PUBLISHED IN COLLABORATION WITH THE NETHERLANDS ASSOCIATION OF INTERNAL MEDICINE

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The annual subscription fee within Europe is € 650,00, for the USA € 665,00 and for the rest of the world € 675,00. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis.

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# Ehlers-Danlos syndrome

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In 2001, Schalkwijk *et al.* described, in eight patients from five families, a new autosomal recessive type of Ehlers-Danlos syndrome (EDS), caused by mutations in the *tenascin-X (TNXB)* gene and leading to a total deficiency of tenascin-X.<sup>1</sup> Since then no other patients with tenascin-X deficiency have been published; apparently it is a rare type of EDS. This new type of EDS can be differentiated from the classic type, which it resembles most, by its mode of inheritance (autosomal recessive *vs* autosomal dominant) and the absence of abnormal scarring, which is one of the key features in the classic type of EDS.

Remarkably, this new type is associated with congenital adrenal hyperplasia when it is due to a deletion encompassing the *CYP21* gene.<sup>1,2</sup>

EDS consists of a group of inherited connective tissue disorders, mainly characterised by generalised joint hypermobility (= Beighton hypermobility score of 5 or more), skin hyperextensibility (measured at the volar side of the

underarm and/or at the flexed elbow), easy bruising and skin fragility (ranging from easy tearing after minor trauma to thin, broad scars and soft and velvety skin). It exhibits an enormous clinical and genetic heterogeneity. The monography by Beighton was the first comprehensive study of the syndrome and is still worth reading.<sup>3</sup> In the latest classification six types are recognised (see *table 1*).<sup>4</sup> EDS is not rare with an estimated prevalence of 1/5000. More than 90% of patients have either the classic or the hypermobility type.<sup>5</sup>

The classic type of EDS is characterised by all the main EDS features, namely hyperextensibility of the skin, broad and thin scarring ('cigarette-paper' scars), generalised joint hypermobility and easy bruising. Aortic dilatation and dissection is a rare complication of this EDS type. Well-known complications are recurrent (sub)luxations with chronic joint pain and early osteoarthritis, hiatus hernia, anal

**Table 1**  
*Villefranche classification of Ehlers-Danlos syndrome*

NEW NAME	OLD NAME	INHERITANCE	MUTATED GENES
Classic type	Gravis = type I Mitis = type II	AD	<i>COL5A1, COL5A2</i>
Hypermobility type	Hypermobile = type III	AD	<i>Unknown; ?TNXB?</i>
Vascular type	Arterial-ecchymotic = type IV	AD	<i>COL3A1</i>
Kyphoscoliosis type	Ocular-scoliotic = type VI	AR	<i>Lysyl-hydroxylase</i>
Arthrochalasia type	Arthrochalasia multiplex congenita = type VIIA and type VIIB	AD	<i>COL1A1, COL1A2</i>
Dermatosparaxis type	Dermatosparaxis = type VIIC	AR	<i>Procollagen, N-peptidase</i>

AD = autosomal dominant, AR = autosomal recessive.

prolapse, cervix uteri insufficiency with premature labour as a result and cicatricial hernia. There is a large interfamilial and intrafamilial clinical variability. The male-to-female ratio is almost equal to one. In about 50% of cases a mutation can be detected in cultured fibroblasts in the *COL5A1* or *COL5A2* genes.

The hypermobility type of EDS has generalised joint hypermobility and mildly hyperextensible and/or soft and velvety skin as its main features. The main problem is the joint hypermobility with its sequelae, recurrent (sub)luxations of mainly shoulder, patella and jaw, chronic joint pain which can be severe and invalidating, and early arthrosis. Complications related to internal organs are extremely rare. It should be distinguished from the benign joint hypermobility syndrome (BJHS), which lacks the skin symptoms of EDS. However, there is much debate whether BJHS and the hypermobility type of EDS are not one and the same disorder.<sup>6</sup> Personally, I adhere to the strict definition of BJHS, namely generalised joint hypermobility with Beighton scores >5, joint pain during a longer period in several joints and absence of skin features. Recently, Zweers *et al.* reported their tenascin-X findings in two groups of patients.<sup>7</sup> First, the haploinsufficient family members (n=20, mainly siblings, children, parents) of their patients with absent tenascin-X due to homozygous or compound heterozygous *TNXB* mutations: in this group, nine out of 14 women – and none of the six males – showed generalised joint hypermobility and four of these nine women also had a velvety skin. The second group consisted of 80 hypermobility type EDS patients, diagnosed by a medical specialist and recruited through the Dutch EDS patient organisation. In six (7.5%; all females) of these, tenascin-X haploinsufficiency was detected. Four of these six patients were examined: two had Beighton scores <5 and no abnormal skin, while of the two with Beighton scores >5, one had normal skin. What does this tell us? Firstly, that probably quite a few of the 80 hypermobility-type EDS patients do not have the hypermobility type of EDS nor the strictly defined BJHS, secondly, that a better controlled study is needed to clarify the role of tenascin-X in hypermobility disorders, and thirdly that most likely skin extensibility and joint mobility is the end result of the involvement of many genes and exogeneous factors ('multifactorial'). This is illustrated in among other ways by the fact that haploinsufficiency for tenascin-X does exist without phenotype, for a presumed autosomal dominant disorder far too many patients are sporadic (= not familial) and almost all patients with the hypermobility type of EDS and BJHS are female.

The vascular type of EDS is more rare than the two previous ones but it is the most serious and life-threatening of all. Thin skin due to lack of subcutaneous fat with prominent venous pattern and a peculiar face and easy bruising are

hallmarks as well the easy rupturing of internal organs as the large arteries, large intestines and uterus. Pregnant women with the vascular type of EDS are at an increased risk for severe complications. It is caused by mutations in *COL3A1* gene (cultured fibroblasts), which are detectable in nearly all patients.

The other three types are even rarer and will not be discussed in detail. Apart from these six EDS types and the new tenascin-X deficient type, there are more poorly defined EDS types and single family 'types'.<sup>4</sup> From clinical practice it is known that not every patient with an EDS phenotype can be classified in one of the well-defined types.

EDS is diagnosed and classified on the basis of history, family history and physical examination with laboratory confirmation when possible and indicated, at the protein level (tenascin-X in serum, collagen 1, 3 and 5 in fibroblasts) and/or at the molecular level (see text and *table 1*).

Interestingly, some often heard complaints are not readily found in textbooks on EDS: easy fatigue is one of these as is the ineffectiveness of local anaesthesia; the former is particularly clinically important and often distressing. Single reports on EDS patients with various clotting abnormalities (for example von Willebrand disease, factor IX deficiency, factor XIII deficiency, platelet release defect, fibronectin deficiency) have been published, but the cause of the easy bruising is much more likely to be due to vessel wall fragility than to a coagulation defect.<sup>5</sup>

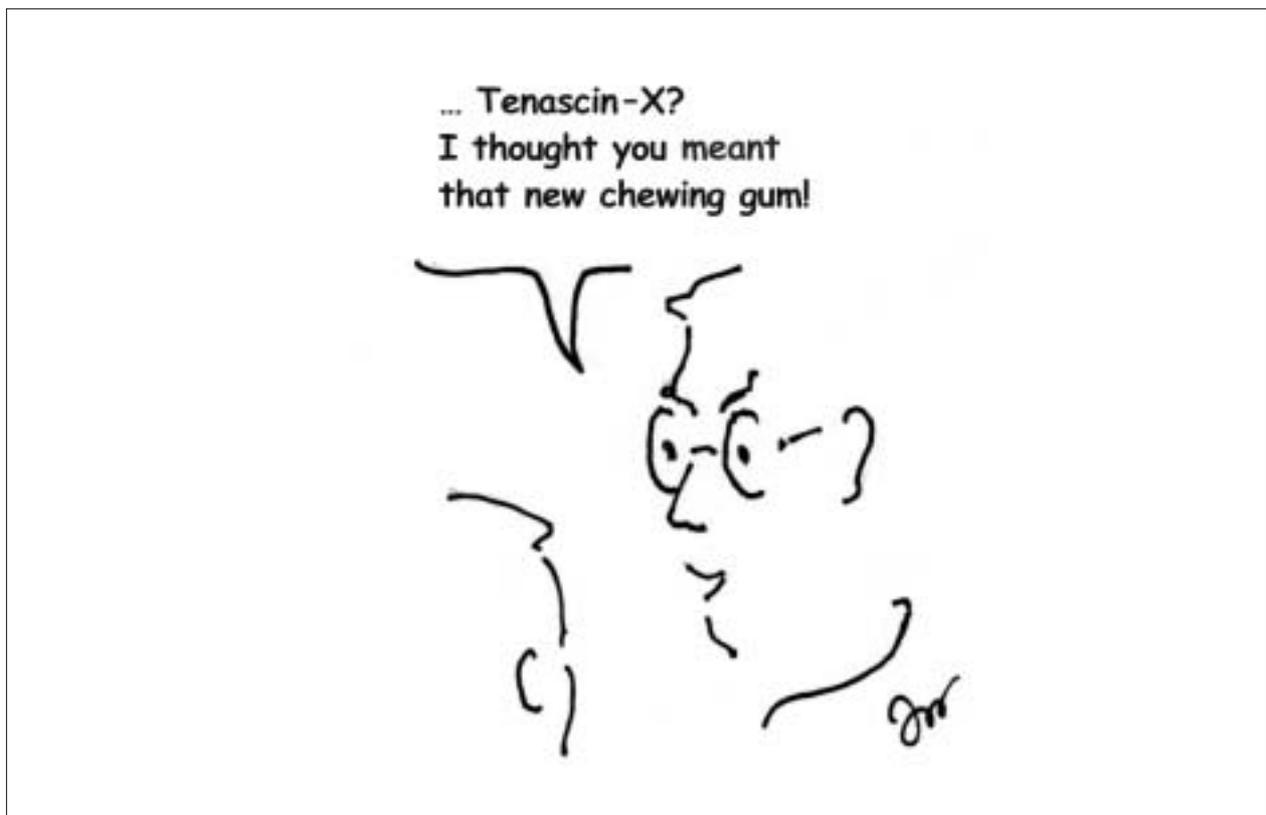
Management of EDS is largely supportive (e.g. physiotherapy, ergotherapy, rehabilitation) and preventive (e.g. surgical precautions, avoiding certain professions and sports, avoiding overweight); it is rarely surgical (e.g. arthrodesis). Genetic counselling is part of the management. Differential diagnosis of EDS includes other connective tissue disorders such as Marfan's syndrome, cutis laxa and osteogenesis imperfecta, but also a host of other diagnoses depending on the presenting complaint/symptom. Since it is also of great importance for the internist to recognise EDS in order to start timely management and to avoid unnecessary investigations, it is therefore wise of the editors of the Netherlands Journal of Medicine to publish the paper by Peeters *et al.*,<sup>8</sup> not so much to draw attention to this rare type of EDS, but more to EDS in general.

#### NOTE

There is an active Dutch patient organisation (Vereniging van Ehlers-Danlos patienten, [www.ehlers-danlos.nl](http://www.ehlers-danlos.nl)). In the Netherlands, the molecular and protein diagnostics of EDS (for types see *table 1*) are performed at the VU University Medical Center, Department of Clinical and Human Genetics, Laboratory for DNA and Protein Diagnostics, Amsterdam, head Dr G.Pals.

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# Hypothesis: normalisation of cytokine dysbalance explains the favourable effects of strict glucose regulation in the critically ill

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## ABSTRACT

Recent trials investigating the effects of strict glucose regulation in critically ill patients have shown impressive reductions in morbidity and mortality. Although the literature focuses on the possible toxic effects of high blood glucose levels, the underlying mechanism for this improvement is unclear. We hypothesise that strict glucose regulation results in modulation of cytokine production, leading to a shift towards a more anti-inflammatory pattern. This shift in the cytokine balance accounts for the reduction in morbidity and mortality.

To support our hypothesis, effects of glucose and insulin on cytokine release and effects of glucose, insulin, and cytokines on host defence, cardiac function and coagulation will be reviewed.

## INTRODUCTION

Diabetic patients are at risk for cardiovascular and various infectious diseases while infections will often run a complicated course.<sup>1</sup> The reason for this increased susceptibility to infections may be an impaired immune defence, microvascular and macrovascular comorbidity, the relative high number of medical interventions or – viewed from the microbial site – an altered adherence of micro-organisms to human cells under hyperglycaemic conditions.<sup>2</sup>

Reportedly, postoperative hyperglycaemia is an independent risk factor for the development of infectious complications.<sup>4</sup> A large recent study by Van den Berghe *et al.*<sup>4</sup> in critically ill patients showed that maintenance of blood glucose levels between 4.4 and 6.1 mmol/l results in a 42% reduction in mortality compared with conventional treatment aimed at blood glucose levels between 9.9 and 11.1 mmol/l. Patients with multiple-organ failure and a proven septic focus demonstrated the greatest improvement with intensive insulin therapy. In addition, in patients with acute myocardial infarction maintenance of blood glucose levels below 11.9 mmol/l has been shown to increase the success rate of thrombolysis,<sup>5</sup> preserves myocardial function,<sup>6</sup> and improves long-term outcome.<sup>7,8</sup>

How the strict control of blood glucose reduces morbidity and mortality is not known, but it is thought that the mechanism may be related either to a direct effect of normalisation of hyperglycaemia or to the concomitant higher insulin levels.

There is a striking similarity between the effects of high glucose levels on host defence, cardiac function and coagulation and the observation of elevated systemic levels of proinflammatory cytokines during critical illness. Cytokines, low-molecular-weight proteins produced by various immune-competent cells, have important modulating effects on the immune response. Cytokines have autocrine, paracrine and endocrine effects.<sup>9</sup> Whereas the local autocrine and paracrine effects may be beneficial in

containing the infection or tissue damage,<sup>10-13</sup> the systemic endocrine effects of the so-called proinflammatory cytokines, such as tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-1 (IL-1), are considered to be deleterious. During the normal response to infections or to noninfectious stressors, the tailored and balanced production of proinflammatory cytokines, aiming at stimulation of the innate immune response, and anti-inflammatory cytokines, curtailing the potential deleterious effects of inflammation, ensures a favourable outcome. However, when this balance is distorted towards an insufficient control of the infection or towards a dominant systemic proinflammatory status designated as 'lethal cytokinaemia',<sup>14</sup> the condition of the patient deteriorates and shock or death may develop. There are indications that glucose levels influence the type and quantity of cytokine release.

In the present article, we hypothesise that a better control of hyperglycaemia shifts the systemic cytokine profile towards a more anti-inflammatory balance resulting in a decreased morbidity and mortality of critically ill patients. This hypothesis is based on and derived from various *in vitro* and *in vivo* studies on the effect of glucose and insulin on immune functions, cardiac performance and coagulation.

## THE HYPOTHESIS

Normalisation of hyperglycaemia in acute and critically ill patients will shift the cytokine profile from a systemic proinflammatory status to a more balanced anti-inflammatory condition. By this mechanism, normoglycaemia improves and restores host defence, haemodynamics and coagulation abnormalities and decreases morbidity and mortality.

## EFFECTS OF GLUCOSE AND INSULIN ON HOST DEFENCE, ROLE OF CYTOKINES

While glucose is freely permeable across the cell membrane of leucocytes in diabetic patients, these cells display a decreased rate of glycolysis<sup>15</sup> and an increased glucose consumption after the addition of small amounts of insulin.<sup>16</sup> In diabetic patients, a variety of abnormalities in neutrophil function have been observed such as decreased chemotaxis, increased adherence to endothelium, decreased phagocytosis, decreased bacterial killing and overproduction of free radicals. These defects may result from short-term metabolic effects of hyperglycaemia and (relative) insulin deficiency or from long-term effects such as increased glycation. It has been shown *in vitro* that D-glucose and insulin,

alone or in combination, improve chemotaxis of neutrophils from healthy humans.<sup>17,18</sup> D-glucose alone is able to increase neutrophil locomotion in a dose-dependent fashion. However, as studied *in vivo*, N-formyl-methionyl-leucyl-phenylalanine (fMLP) and complement-induced chemotaxis is significantly lower in diabetic patients than in healthy controls, probably because of the increased expression of the adhesion promoting  $\beta$ 2-integrins CD11b and CD11c.<sup>19</sup> Because in addition, glucose increases the expression *in vitro* of adhesion molecules on endothelium such as E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1),<sup>20</sup> hyperglycaemia will promote the binding of leucocytes to endothelium.<sup>21</sup> Recently, it was demonstrated that K<sub>ATP</sub> channel blockade with glibenclamide inhibited fMLP and lipopolysaccharide-induced neutrophil activation and migration *in vitro* and *in vivo*,<sup>22</sup> suggesting that, independent of glucose and insulin, treatment of type 2 diabetic patients with glibenclamide could be detrimental to immune function. In type 2 diabetic patients circulating levels of cytokines and adhesion molecules are increased following a meal rich in carbohydrates.<sup>23</sup> This effect was inhibited by pretreatment with high doses of vitamin C and E, indicating that an oxidative mechanism mediates this effect.

Phagocytosis is impaired in diabetes.<sup>24</sup> This impairment is at least partly mediated by elevated levels of cytosolic calcium. In a prospective cohort study of patients with type 2 diabetes, higher fasting glucose levels were correlated with higher cytosolic calcium levels in neutrophils and with decreased phagocytic activity.<sup>25</sup> In type 2 diabetes mellitus this impaired phagocytic activity can be restored by glibenclamide therapy, either as a result of the glibenclamide-stimulated insulin production or decreased serum glucose levels. In diabetes, ingested bacteria seem to be protected from killing by intracellular bactericidal mechanisms.<sup>26</sup> Under hyperglycaemic conditions, proteins may be glycated resulting in the formation of so-called advanced glycation end products (AGEs). Recent studies have shown that these AGEs can bind to the corresponding receptor on neutrophils and impair intracellular bacterial killing.<sup>18</sup> Delamaire *et al.* reported that, although elevated in the unstimulated state, bactericidal activity of stimulated neutrophils was significantly depressed in diabetic patients.<sup>19</sup>

Neutrophils from diabetic patients show increased levels of basal free radical release as measured by chemoluminescence. This increased free radical production, in combination with increased adhesion, may induce local lesions in the vascular endothelium.<sup>19</sup>

Although all these defects in leucocyte function have been reported in diabetic patients, the exact mechanism and the effects of short-term glucose control are still not clear. We speculate that high glucose concentrations influence host defence by the modulation of cytokine synthesis.

This mechanism is sustained by a recent report by Morohoshi *et al.* that showed a glucose-dependent increase in basal TNF $\alpha$  and IL-6 production in human monocytes *in vitro*.<sup>27</sup> Similarly, studies by our group demonstrated a glucose-dependent increased production of TNF $\alpha$  by peripheral blood cells *in vitro* after stimulation with lipopolysaccharide, whereas glucose does not influence the production of the anti-inflammatory cytokine IL-10.<sup>28</sup> We also demonstrated that *in vivo* induced hypoglycaemia in hypoglycaemic human clamp models (achieved glucose levels 5.0, 3.5, and 2.5 mmol/l) resulted in a down-modulation of LPS-induced TNF $\alpha$  synthesis.<sup>29</sup> Together, these studies strongly suggest that glucose may stimulate the production of proinflammatory cytokines such as TNF $\alpha$  and IL-6, with no effect on the anti-inflammatory cytokine IL-10. Therefore, normalisation of hyperglycaemia may result in a shift of cytokine production towards a more balanced anti-inflammatory profile.

In addition to the effect of glucose on the production of pro- and anti-inflammatory cytokines, several effects of insulin on cytokine production have been shown.<sup>30</sup> Satomi *et al.* demonstrated that insulin blocks TNF $\alpha$  production by peritoneal macrophages.<sup>31</sup> Insulin has been shown to have a potential anti-inflammatory activity, since it inhibits the expression of the proinflammatory adhesion molecule ICAM-1 by endothelial cells<sup>32</sup> and increases the expression of the mRNA for macrophage migration inhibitory factor.<sup>33</sup> Rats treated with recombinant TNF $\alpha$  have a significant decrease in food intake, nitrogen balance and body weight compared with saline-treated control rats.<sup>34</sup> Concurrent insulin administration reversed all these changes. Five days of TNF $\alpha$  treatment caused severe interstitial pneumonitis, periportal hepatitis and increased wet organ weight of heart, lung, kidney and spleen. Concurrent insulin treatment led to near total reversal of these pathological changes. However, as systemic insulin administration results in lower glucose concentrations, the observed effects cannot be attributed to insulin *per se*.

Of the several questions that can be addressed, perhaps the most intriguing is *how* glucose mediates the TNF $\alpha$  production. A possible explanation involves nuclear factor kappa-B (NF $\kappa$ B). The inducible transcription factor NF $\kappa$ B was discovered in 1986<sup>35</sup> and is present in the cytoplasm of many different cell types, such as leucocytes, endothelial and epithelial cells. Activation of this protein initiates its translocation into the nucleus where it binds to specific sequences in the promoter regions of target genes. This will result in an increased rate of transcription of the gene, more messenger RNA (mRNA) and more protein. NF $\kappa$ B plays a pivotal role in the regulation of the synthesis of various proinflammatory proteins, including cytokines, cell-adhesion molecules and inducible nitric oxide synthase. In sepsis, NF $\kappa$ B expression is upregulated and a persistently increased expression correlates with nonsurvival.<sup>36</sup> The production

of NF $\kappa$ B is stimulated by lipopolysaccharide (LPS), TNF $\alpha$ , IL-1, and phorbol 12-myristate 13-acetate. The anti-inflammatory cytokine IL-10 inhibits NF $\kappa$ B formation. In type 2 diabetes it has been shown that IL-1 $\beta$  inhibits pancreatic  $\beta$  cell function and promotes Fas-triggered apoptosis in part by activating NF $\kappa$ B.<sup>37</sup> This suggests that an inflammatory process is involved in the pathogenesis of glucotoxicity in type 2 diabetes, which is mediated by NF $\kappa$ B.

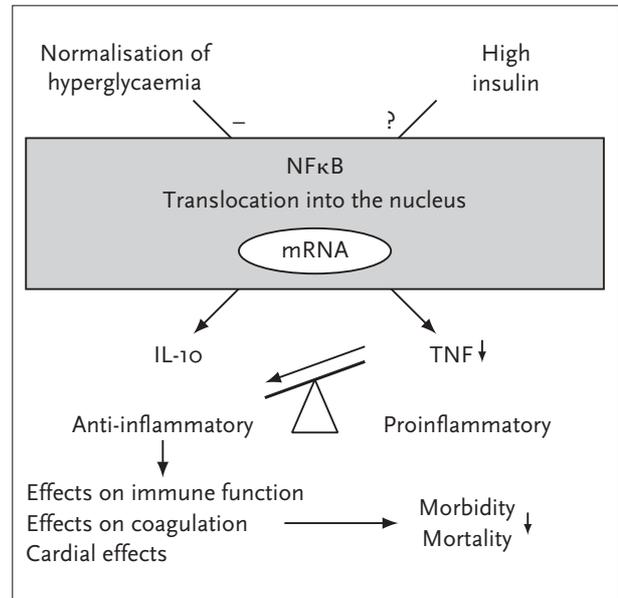
In Gram-negative sepsis, LPS or endotoxin binds to several receptors of which CD14 is one of the most extensively studied. After binding to LPS, this receptor forms a complex with the toll-like receptor 4 (TLR4).<sup>38</sup> Activation of TLR4 results in the activation of the NF $\kappa$ B signalling pathway and subsequently to the production of cytokines such as TNF $\alpha$ , IL-1, IL-2, IL-6, IL-8, and IL-12, and interferon- $\beta$  (IFN $\beta$ ). TNF $\alpha$ , in a positive regulatory loop, can lead to further excessive activation of NF $\kappa$ B and to cytokine-mediated effects such as endothelial cell injury, disseminated intravascular coagulation, septic shock or death.

Hyperglycaemia rapidly induces activation of NF $\kappa$ B within hours of its onset.<sup>39</sup> NF $\kappa$ B activation induced by elevated concentrations of carbohydrates is associated with increased monocyte adhesion to endothelial cells and the generation of oxygen radicals.<sup>40</sup> Adherence of leucocytes to human umbilical vein endothelial cells was strongly augmented by elevation of the glucose concentration in the perfusate from 5 mmol/l to 30 mmol/l. In the same experiment it was shown that this effect was paralleled by an intense NF $\kappa$ B activation.<sup>20</sup> So, direct effects of glucose on NF $\kappa$ B activation may well be the initiator of the deleterious effects of increased systemic cytokine production. However, as NF $\kappa$ B is also involved in the stimulation of some anti-inflammatory cytokines, more subtle stimulation of intracellular pathways is likely to be involved. Oxidative stress-mediated cellular damage may be involved in hyperglycaemia-induced activation (reviewed in),<sup>41</sup> as was shown by treating diabetic patients with antioxidants that significantly suppressed NF $\kappa$ B activation. By inhibiting NF $\kappa$ B activation, transcription of genes under NF $\kappa$ B control, such as adhesion molecules and cytokines, is prevented. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory chemokine produced primarily by the anterior pituitary gland.<sup>42</sup> LPS-induced secretion of MIF from the pituitary gland and from circulating/tissue resident monocyte/macrophages is thought to play an important role in the pathogenesis of endotoxaemia and sepsis and might be an integral part of the host's systemic stress response. In mice that received endotoxin, there was a dramatic fall in the pituitary content of MIF, associated with a concomitant increase in plasma levels of MIF.<sup>43,44</sup> *In vivo* studies in rats have shown that MIF protein is released from the pituitary, adrenal gland, liver, spleen, lung, kidney and skin within six hours of LPS injection.<sup>45</sup> Upon release, MIF is directly proinflammatory by activating

or promoting cytokine expression (TNF $\alpha$ ;<sup>46,47</sup> IL-1 $\beta$ , IL-2;<sup>48</sup> IL-6;<sup>46,49</sup> IL-8;<sup>50</sup> and interferon- $\gamma$ <sup>48,51</sup>). From this data it is stated that 'MIF's position within the cytokine cascade is to act in concert with glucocorticoids to control the set point of the immune and inflammatory response'.<sup>42</sup> Similarly to TNF $\alpha$ , high MIF levels are closely linked with poor outcome in patients with systemic inflammatory response syndrome (SIRS).<sup>52</sup> Again, there is a link with glucose metabolism: the insulin-secreting  $\beta$  cells in the pancreas express MIF, and both MIF and insulin colocalise by immuno-cytochemistry within the secretory granules.<sup>53</sup> MIF is secreted in response to glucose stimulation in a time- and concentration-dependent fashion. In turn, exogenously administered MIF potentiates insulin release. MIF thus plays an important regulatory role in carbohydrate metabolism and its secretion is regulated by glucose. Through effects on MIF, strict glucose regulation could influence the pro-/anti-inflammatory cytokine balance.

#### EFFECTS OF GLUCOSE AND INSULIN ON CARDIAC FUNCTION, ROLE OF CYTOKINES

Inflammation plays a pathogenic role in the development of acute coronary syndromes.<sup>54,55</sup> Increased levels of TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and MIF have been observed after acute myocardial infarction<sup>56,57</sup> and postinfarction IL-6 levels correlate significantly with the extent of myocardial tissue damage.<sup>58</sup> TNF $\alpha$  production forms part of an important intrinsic myocardial stress response system to injury. Within the myocardium TNF $\alpha$  is not only confined to the infarct and peri-infarct zone, but is also expressed in cardiac myocytes of the contralateral myocardium.<sup>59</sup> As can be concluded from a study in mice, TNF $\alpha$  has, apart from its pathogenic role in the acute inflammatory response during the acute ischaemia, also a protective role in limiting the ischaemia-induced apoptosis.<sup>60</sup> Several studies have suggested that treatment of acute myocardial infarction with infusions of glucose, insulin and potassium is beneficial.<sup>61-63</sup> It remains unclear whether normalisation of hyperglycaemia or the higher insulin levels account for these beneficial effects. Insulin has been shown to attenuate infarct size in the isolated rat heart, independent of the presence of glucose,<sup>64</sup> and it was also demonstrated that infarct size was linearly related to blood glucose concentration during acute hyperglycaemia independent of insulin concentration in dogs.<sup>65</sup> The DIGAMI study was initiated to test the hypothesis that rapid improvement of metabolic control in diabetic patients with acute myocardial infarction improves short- and long-term outcome.<sup>7,8</sup> Patients were randomised to insulin-glucose infusion followed by multidose subcutaneous insulin therapy or conventional therapy. In the



**Figure 1**  
*Representation of the hypothesis. Correction of high glucose concentration results in less NFκB activation and down-modulation of TNF $\alpha$  production, resulting in less spill-over to the systemic circulation. The production of the anti-inflammatory cytokine IL-10 is not influenced by glucose. This leads to a new pro- and anti-inflammatory cytokine balance with favourable effects on host defence, myocardial function, and coagulation. In the presence of little experimental evidence, the possible role of insulin remains questionable.*

intensive therapy group mortality rate was reduced by 30% after one year. This effect is most likely the result of better local metabolic control in the myocardium or, in accordance to the presently presented hypothesis, to an altered cytokine-mediated inflammatory response.

#### EFFECTS OF GLUCOSE AND INSULIN ON COAGULATION, ROLE OF CYTOKINES

In critically ill patients, derangement of the coagulation system comprises enhanced activation of coagulation, depression of inhibitory mechanisms of coagulation, and inhibition of fibrinolysis. An antifibrinolytic endothelium phenotype is associated with multiorgan failure and mortality.<sup>66</sup> Derangement of the coagulation system appears to be mediated by various cytokines and may contribute to the pathogenesis of multiple organ failure. In human umbilical vein endothelial cells, TNF $\alpha$  alters the coagulant activity by stimulation of the production and surface expression of tissue factor and by inhibition

of anticoagulant mechanism, particularly the activation of the protein C system.<sup>67,68</sup> In healthy human volunteers, infusion of TNF $\alpha$  activates the coagulation system identically to what is seen in septic patients or in volunteers after endotoxin infusion,<sup>69,70</sup> and this activation of the coagulation seems to be, at least initially, dependent on activation of the extrinsic pathway of blood coagulation.<sup>71</sup> In septic patients, the fibrinolytic system becomes initially activated and subsequently inhibited.<sup>72</sup> Blockade of TNF $\alpha$  with monoclonal antibodies inhibits the fibrinolytic response upon the administration of endotoxin.<sup>73,75</sup> The mechanisms underlying the effects of TNF $\alpha$  on the fibrinolytic system are not completely clear. It is suggested that the rapid effects are not due to enhanced gene expression observed *in vitro*,<sup>76,77</sup> but to release of stored plasminogen activators probably from the vascular endothelium and endotoxin-activated platelets.

The level of plasminogen activator inhibitor type I (PAI), an inhibitor of fibrinolysis, is higher in diabetic patients as compared with nondiabetics, and associated with the occurrence of myocardial infarction.<sup>78</sup> Systemic insulin treatment decreases the plasma activity of PAI in type 2 diabetic patients.<sup>79</sup> Human administration of recombinant IL-6, another proinflammatory cytokine, resulted in an increased thrombin generation<sup>80</sup> and antibodies directed against IL-6 are able to completely abolish the endotoxin-induced activation of coagulation in chimpanzees.<sup>75</sup> Also, diabetes has been associated with platelet hyperactivity to agonists *in vitro*, and alterations in a number of

mechanisms involved in platelet activation occur in diabetic platelets. These alterations include increased presence of glycoprotein receptors for agonists and adhesive proteins on the platelet surface, increased fibrinogen binding, decreased membrane fluidity, enhanced arachidonate pathway activation with increased thromboxane A<sub>2</sub> formation, and increased phosphoinositide turnover leading to increased inositol triphosphate production, calcium mobilisation, and protein phosphorylation.<sup>81</sup> Tight glycaemic control with insulin therapy reduces the excretion levels of a thromboxane metabolite by approximately 50%.<sup>82</sup> Again, the mechanism responsible for this effect is unclear.<sup>83</sup> In accordance with our hypothesis, the effect of systemic insulin treatment could be explained by better glucose control, subsequent inhibition of proinflammatory cytokines, resulting in a reduced cytokine-induced procoagulatory state.

Recently, it was shown that supplementation of the anticoagulatory protein recombinant activated protein C (aPC) reduces the mortality in critically ill patients.<sup>84</sup> Apart from the insulin study by Van de Berghe *et al.*,<sup>4</sup> this is the only immunomodulatory strategy so far that has proven to be efficacious. The effects of aPC, however, appear to involve more than its antithrombotic activity. Similar to one of the effects of strict glucose control, aPC influences translocation of NF $\kappa$ B and cytokine production in an anti-inflammatory way.<sup>85</sup> Normalisation of hyperglycaemia may therefore be beneficial either directly or by a restoration of the cytokine balance, through a positive effect on the immune function, through the improvement of haemodynamics or through a decrease of the undesirable procoagulatory state.

**Table 1**

*Summary of the effects of hyperglycaemia (compared with normoglycaemia)*

EFFECTS ON HOST DEFENCE	
Nf $\kappa$ B induction	↑
Production of proinflammatory cytokines, MIF	↑
Production of anti-inflammatory cytokines	=
Expression of adhesion molecules	↑
Binding of leucocytes to endothelium	↑
Phagocytic activation	↓
Intracellular bacterial killing	↓
EFFECTS ON CARDIAC FUNCTION	
Infarct size	↑
Postinfarction mortality	↑
EFFECTS ON COAGULATION	
PA-I concentration	↑
Thromboxane concentration	↑
Platelet activity	↑

## CONCLUSION AND FUTURE DIRECTIONS

Strict control of plasma glucose in acute and critically ill diabetic and nondiabetic patients has been shown to improve outcome in several clinical settings. The mechanism of action of these beneficial effects is not known, although in critically ill patients, the control of glucose levels rather than the absolute levels of insulin appear to account for the positive effects of strict regulation.<sup>86</sup> From the literature and our own studies it can be concluded that hyperglycaemia augments the production of proinflammatory cytokines. There is a striking similarity between the effects of glucose on host defence, cardiac performance and coagulation and the proinflammatory cytokine status associated with critical illness. Although in acute or critically ill patients various mutually dependent overlapping and redundant pathophysiological mechanisms are known to influence the clinical course in a complex fashion, we claim that the modulation of cytokines explains the beneficial effect of a more strict glycaemic control. Cognisant the

beneficial local autocrine and paracrine action and the potentially deleterious systemic endocrine effect of pro-inflammatory cytokines, we suggest that future studies to test this hypothesis encompass local and systemic cytokine production. Further understanding of the mechanism by which intensive therapy with insulin reduces morbidity and mortality in acute and critically ill patients will probably take place at the crossroads of metabolic control, immune response, cardiac performance and coagulation.

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## Advertentie Thyrax

# Baroreflex failure: a neglected type of secondary hypertension

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## ABSTRACT

The arterial baroreflex buffers abrupt transients of blood pressure and prevents pressure from rising or falling excessively. In experimental animals, baroreceptor denervation results in temporary or permanent increases in blood pressure level and variability, depending on the extent of denervation. In humans, the clinical syndrome of baroreflex failure may arise from denervation of carotid baroreceptors following carotid body tumour resection, carotid artery surgery, neck irradiation and neck trauma. The syndrome is characterised by acute malignant hypertension and tachycardia followed by labile hypertension and hypotension. Baroreflex failure can be a cause of hypertension and should also be considered in the differential diagnosis of pheochromocytoma. Patients with suspected baroreflex failure should be referred to specialised centres for diagnostic testing and treatment.

## BARORECEPTORS

In the late 1920s, Hering and Koch were the first to recognise the reflex nature of changes in heart rate and blood pressure evoked by external massage of the neck. The afferents were tracked as nerve endings at the carotid bifurcation.<sup>1,2</sup> The arterial baroreflex buffers abrupt transients of blood pressure and originates from stretch sensitive receptors in the arterial wall of the carotid sinus and the aortic arch and large vessels of the thorax.<sup>3,4</sup> Afferent fibres from carotid sinus baroreceptors join the glossopharyngeal nerve (ninth cranial nerve) and project to the nucleus tractus solitarius in the dorsal medulla, which in turn projects

to efferent cardiovascular neurones in the medulla. In addition to carotid baroreceptors, stretch-sensitive baroreceptors are also located in the aortic arch, heart and large pulmonary vessels. The extra-carotid baroreceptors transmit their afferent information along with the vagal nerves to the same brain stem nuclei. The efferent limbs of the baroreflex loop consist of sympathetic and parasympathetic fibres to the heart as well as to blood vessels.

## EXPERIMENTAL AND IATROGENIC DENERVATION OF BARORECEPTORS

Arterial baroreceptors provide a tonic inhibitory influence on sympathetic tone, thus controlling peripheral vasoconstriction and cardiac output.<sup>1,2</sup> Therefore, baroreceptor denervation would be expected to result in a sustained increase in sympathetic tone and, as a consequence, a sustained increase in blood pressure. Indeed, experimental denervation of carotid, aortic and cardiopulmonary baroreceptors in dogs produces a persistent increase in blood pressure level and variability.<sup>5</sup> Following selective carotid and/or aortic baroreceptor denervation, the increase in blood pressure and heart rate is usually temporary.<sup>5-8</sup> The first report on baroreceptor denervation in humans appeared in the 1930s.<sup>9</sup> Unilateral section of the glossopharyngeal nerve in five patients with glossopharyngeal neuralgia produced a prompt and pronounced rise in blood pressure, which lasted 5 to 12 days. In 1956, a patient died from a fatal hypertensive crisis following unilateral carotid sinus denervation, which had been performed for the relief of recurrent syncope due to a

hypersensitive carotid sinus syndrome.<sup>10</sup> In 1985, Fagius and Wallin reported the effects of experimental chemical blockade of carotid baroreceptors in humans.<sup>11</sup> These authors performed bilateral anaesthetic blockade of vagal and glossopharyngeal nerves upon each other, which resulted in an elevation of blood pressure and tachycardia, accompanied by a strong increase in muscle sympathetic nerve activity.

Apart from these experimental studies, inadvertent baroreceptor denervation may occur as a complication of bilateral carotid body tumour resection,<sup>12-14</sup> radiotherapy and surgery for laryngeal/pharyngeal carcinoma,<sup>12,15,16</sup> bilateral<sup>17,18</sup> and unilateral<sup>19,20</sup> carotid endarterectomy and trauma of the neck.<sup>12</sup> Disruption of the baroreflex has also been reported in the event of ischaemic or neurodegenerative lesion of the nucleus tractus solitarii.<sup>21</sup> The clinical syndrome of baroreflex failure has now been characterised as a separate clinical entity.<sup>12,22</sup>

## BAROREFLEX FAILURE SYNDROME

The acute form of baroreflex failure is encountered following loss of glossopharyngeal or carotid sinus nerve function due to surgical intervention or accidental injury.<sup>17-20</sup> It is characterised by severe, unremitting hypertension, tachycardia, and headache (*table 1*). The systolic blood pressure typically exceeds 250 mmHg,<sup>22</sup> which may lead to hypertensive encephalopathy<sup>17,19</sup> and (fatal) cerebral haemorrhage.<sup>10,20</sup> Hypertensive crisis may evolve over days and weeks into the more chronic volatile hypertension phase.<sup>12,14,18,23,24</sup> In addition, volatile hypertension may result from a gradual decline in baroreflex function due to neck irradiation.<sup>12,16</sup> Irradiation may affect the stretch-induced afferent baroreceptor activity due to direct trauma of baroreceptors or by inducing atherosclerosis and fibrosis of the carotid sinus arterial wall.<sup>25-27</sup> Volatile hypertension due to baroreflex failure is characterised by paroxysms of abrupt sympathetic activation, including excessive increments in plasma catecholamine levels.<sup>12,22</sup> Surges of blood pressure and tachycardia may occur spontaneously or are elicited by mental stress or physical stimuli such as exercise, cold and sexual arousal.<sup>28</sup> These bouts of sympathetic activation may be accompanied by severe headache, palpitations, diaphoresis, light-headedness and anxiety.<sup>12</sup> Intraocular pressure may be increased.<sup>29</sup> In addition, emotional instability appears to be a prominent feature in this phase of baroreflex failure. Apart from hypertensive surges, hypotensive valleys may occur during sleep.<sup>16</sup> In rare cases, inadequate baroreflex buffering of cardiovagal efference is the most prominent feature, resulting in malignant vagotonia with hypotension, bradycardia, and asystole.<sup>30</sup> Accompanying symptoms of this so-called 'selective baroreflex failure' include fatigue and dizziness, with possible progression to frank syncope.

**Table 1**  
*Symptoms and signs of baroreflex failure*

ACUTE PHASE FOLLOWING BARORECEPTOR DENERVATION (DAYS-WEEKS)	
Severe sustained elevation of blood pressure (systolic pressure typically >250 mmHg)	
Tachycardia	
Elevation of plasma catecholamines	
Headache	
Complications of hypertension	Encephalopathy
	Cerebral haemorrhage
CHRONIC PHASE (WEEKS-YEARS)	
Common	
Volatile hypertension and tachycardia	
Paroxysm of	Palpitations
	Headache
	Diaphoresis
	Light headedness
	Anxiety
	Emotional instability
	Increased intraocular pressure
Rare	
Hypotension (during sleep)	
Bradycardia, asystole	
Fatigue	
(Orthostatic) dizziness	
Syncope	

## WHEN SHOULD BAROREFLEX FAILURE BE CONSIDERED?

A history of prior (iatrogenic) trauma of the neck is the most important clue in suspecting the diagnosis of baroreflex failure. The diagnosis of baroreflex failure should be considered in patients with a negative work-up for pheochromocytoma, since this carries a strong clinical resemblance to baroreflex failure. Apart from pheochromocytoma, which should be ruled out by the proper biochemical and radiographic investigations, the differential diagnosis of baroreflex failure includes paroxysmal tachycardia, migraine, hyperthyroidism, renovascular hypertension, alcohol withdrawal, drug use (e.g. amphetamines or cocaine), mastocytosis, carcinoid syndrome, intracranial lesions and psychological disorders (panic attack, generalised anxiety disorder).<sup>22</sup>

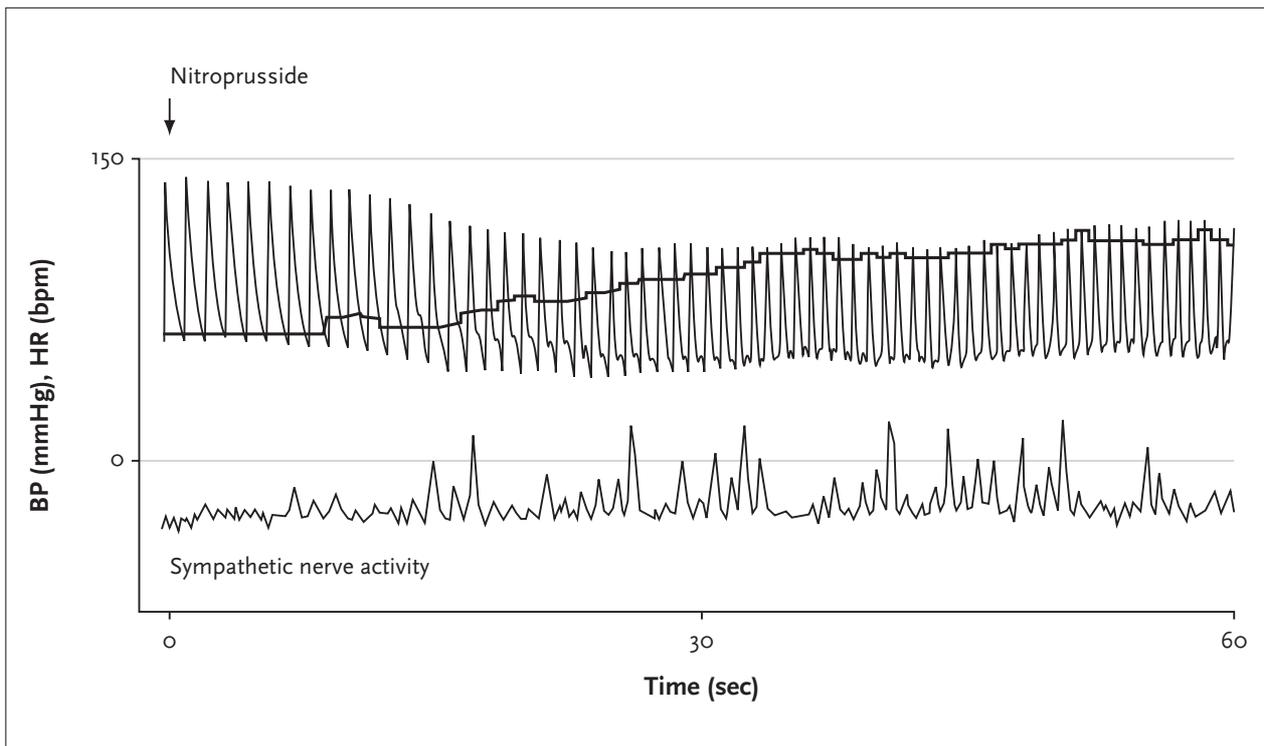
Labile hypertension and hypotension can be demonstrated by a 24-hour ambulatory blood pressure recording.<sup>17</sup> Patients with a suspicion of baroreflex failure should be referred

to a specialised centre for the evaluation of autonomic cardiovascular function. Disruption of the baroreflex arch is demonstrated by absence of reflex bradycardia and tachycardia in response to intravenous injection of pressor drugs such as phenylephrine and depressor drugs as nitroprusside, respectively.<sup>12,31</sup> The baroreflex modulation of muscle sympathetic nerve activity can be assessed by micro-neurography of sympathetic fibres within the peroneal nerve (*figure 1*).<sup>32,33</sup> Additional cardiovascular reflex tests such as Valsalva's manoeuvre, standing up, forced breathing, cold face test, cold pressor test and mental arithmetic<sup>34</sup> can be used to tease out the localisation of the baroreflex lesion.<sup>34</sup> Baroreflex failure is essentially different from autonomic neuropathy, which is either primary (pure autonomic failure, multiple system atrophy) or secondary (e.g. diabetes mellitus). In contrast to baroreflex failure which is caused by lesions of the afferent innervation of baroreceptors, autonomic neuropathy is characterised by abnormal efferent innervation to the heart and resistance vessels. The key feature of autonomic neuropathy is (severe) orthostatic hypotension.<sup>35</sup> Syncope due to the hypersensitive carotid sinus syndrome is caused by excessive afferent nerve impulses towards the brainstem, causing cardioinhibition and/ or vasodepression resulting in syncope.<sup>36</sup>

## TREATMENT AND PROGNOSIS

Information on the treatment of acute, postsurgical baroreflex failure is scarce and relies on observational case studies. As in any form of hypertensive crisis, antihypertensive treatment in baroreflex failure is aimed at the prevention of hypertensive encephalopathy, (cerebral) haemorrhage, myocardial infarction, heart failure and hypertensive retinopathy. Haemodynamic monitoring of these patients in a medium or intensive care unit is warranted in the acute phase. Theoretically, intravenous administration of drugs with a short half-life is preferred in view of the strong blood pressure lability. Drugs that have been used in this setting include nitroprusside, phentolamine and labetalol.<sup>37,38</sup> Apart from antihypertensive treatment, adequate analgesic and sedative therapy for relief of postsurgical discomfort and baroreflex failure related symptoms such as headache and palpitations are indicated.

In the phase of labile hypertension, the primary goal of therapy is to reduce the frequency and magnitude of surges in blood pressure and heart rate. Clonidine, a centrally and peripherally acting  $\alpha$ -adrenoreceptor and imidazoline



**Figure 1**  
Assessment of baroreflex control of heart rate (upper panel) and muscle sympathetic nerve activity (lower panel) in a normal subject. Nitroprusside-induced hypotension elicits a baroreflex mediated increase in heart rate and muscle sympathetic nerve activity. BP = blood pressure, HR = heart rate.

agonist, has been shown to reduce both frequency and severity of pressor surges.<sup>12,20-22</sup> Both central inhibition of noradrenergic neurotransmission and sedative effects may contribute to the beneficial effect of clonidine in baroreflex failure. If tolerated by the patient, daily doses as high as 1.2 to 2.4 mg may be required. The  $\alpha$ -adreno-receptor blocker phenoxybenzamine has also been shown to reduce the magnitude of blood pressure surges.<sup>12</sup> In patients who have been well controlled for months to years, clonidine may be tapered off and replaced by high doses of benzodiazepines, such as diazepam.<sup>22</sup> Apart from these agents, experimental treatment of baroreflex failure includes (non-registered) inhibitors of norepinephrine release. Agents that increase synaptic norepinephrine concentrations and may thereby elicit profound pressor responses are probably better avoided in baroreflex failure.<sup>22</sup> These include tricyclic antidepressants, amphetamines, mono amino oxidase A inhibitors, cocaine, and tyramine-containing food and beverages. Additional nonpharmacological strategies include avoidance of individual factors that evoke sympathetic surges and (relaxation) biofeedback training.<sup>39-41</sup> In the rare patients with malignant vagotonia due to selective baroreflex failure, insertion of a pacemaker may be necessary.<sup>39</sup> In the rare patients with predominant hypotension, low doses of fludrocortisone and increased dietary salt may be indicated.<sup>22</sup> Regarding the polar shifts in blood pressure, treatment of baroreflex failure can be challenging and frequent follow-up of patients is warranted. The clinical expression of baroreflex failure varies considerably among patients, probably depending on the extent of denervation of baroreceptors. In addition, the natural cause of baroreflex failure is unpredictable. In most cases, the pressor surges attenuate over time, but in some cases, volatile hypertension is permanent.<sup>12</sup>

## CONCLUSION

Baroreceptors are essential for buffering acute changes in blood pressure and prevent it from rising or declining excessively. The syndrome of baroreflex failure results from denervation of arterial and/or cardiopulmonary baroreceptors and is characterised by labile hypertension and rarely hypotension. Baroreflex failure is particularly likely to develop following carotid body tumour surgery, carotid artery surgery, neck irradiation and injury. Baroreflex failure should be considered in the differential diagnosis both of the cases with (severe) hypertension with a striking blood pressure variability and in patients with suspected pheochromocytoma. Referral to a specialised centre for correct diagnosis and specific therapy is warranted.

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# Testing families with HFE-related hereditary haemochromatosis

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## ABSTRACT

HFE-related hereditary haemochromatosis is the most common autosomal recessive disorder in the Caucasian population. In 1996 the responsible gene (called HFE) was identified. Two mutations (C282Y and H63D) are considered most important and occur frequently in the Caucasian population.

We describe a family of an affected proband in which first- and second-degree relatives were tested phenotypically and genotypically. In second-degree relatives both C282Y homozygosity as well as compound heterozygosity were found. Family testing can be useful to detect persons who will possibly develop iron overload. We must be aware that testing first-degree relatives only carries a 2.5% chance that persons at risk of developing iron loading will not be detected. Cascade screening of second-degree relatives might be cost-effective.

## INTRODUCTION

HFE-related hereditary haemochromatosis (HFErHH) is the most common autosomal recessive disorder in the Caucasian population,<sup>1</sup> the prevalence ranging from 0.25 to 0.5%.<sup>2-4</sup> A common trait of the disease is iron loading in tissues and organs such as liver, heart, joints, pancreas and pituitary gland. Ultimately this can lead to liver cirrhosis, cardiomyopathy, arthropathy, diabetes mellitus and hypogonadism.

In 1996 the gene responsible for HFE-related hereditary haemochromatosis (called HFE gene, coding for the HFE protein) was identified.<sup>5</sup> The HFE protein is involved in

the regulation of iron absorption.<sup>6</sup> Multiple mutations in the HFE gene have been described.<sup>7</sup> The most important one is the C282Y mutation, which means that at amino-acid 282 cysteine is substituted by tyrosine. A second (H63D, aspartate-to-histidine substitution at amino-acid 63) is also considered of importance.

The C282Y and H63D mutations occur frequently in the Caucasian population (see *table 1*).<sup>8</sup> Among all Caucasian HFErHH patients 80 to 90% are homozygous for the C282Y mutation, 1% is homozygous for the H63D mutation, 5% are compound heterozygous (which means heterozygous for both the C282Y as well as the H63D mutation), and 3 to 10% are heterozygous for either C282Y or H63D mutation, possibly with other mutations.<sup>9</sup>

Since the discovery of the HFE gene, it is possible to detect persons at risk of developing haemochromatosis in a presymptomatic stage. The Dutch Haemochromatosis Association advises testing of first-degree relatives of affected probands.<sup>10</sup> In this original article, we will describe a family in which C282Y homozygosity and compound heterozygosity were found in second-degree relatives. The role and extent of genetic testing of families of affected probands will be discussed.

## CASE REPORT

Patient A, 60 years old and diagnosed with diabetes mellitus nine years ago, presented with complaints of chronic fatigue. Physical examination did not reveal any abnormalities. Blood examination had the following results (reference range between brackets): haemoglobin 10.3 mmol/l (8.5-10.9), mean corpuscular volume 94 fl (80-100), aspartate aminotransferase 76 U/l (<40), alanine

**Table 1**  
Distribution of genotype in the Caucasian population and in HH patients

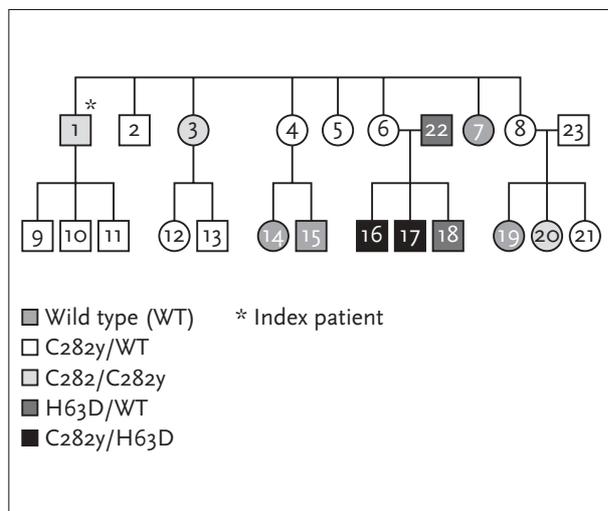
	CAUCASIAN POPULATION	HFE-RELATED HEREDITARY HAEMOCHROMATOSIS PATIENTS
C282Y/C282Y (homozygosity)	0.5%	80-90%
H63D/H63D (homozygosity)	2%	1%
C282Y/H63D (compound heterozygosity)	2%	5%
C282Y/WT (heterozygosity)	10%	Rest
H63D/WT (heterozygosity)	20%	Rest

WT = wildtype = unmutated gene.

aminotransferase 79 U/l (<45), lactic dehydrogenase 976 U/l (<450),  $\gamma$ -glutamyl transpeptidase 216 U/l (<50), alkaline phosphatase 154 U/l (40-120), ferritin 1318  $\mu$ g/l (30-300), iron 39  $\mu$ g/l (14-28), iron-binding capacity 55  $\mu$ g/l (45-77) and transferrin saturation (iron divided by iron-binding capacity) 71% (<45%). Ultrasound examination of the liver showed no abnormalities. Serological testing for infection with hepatitis A, B and C, Epstein-Barr virus and cytomegaly virus was negative. Liver biopsy showed iron loading with mild periportal fibrosis. The patient turned out to be C282Y homozygous.

#### Testing of family members

All first- and second-degree relatives of the patient were tested both phenotypically (ferritin and transferrin saturation) and genotypically. The spouses of some of the first-degree relatives were tested as well. The results of this screening are shown in the family tree (figure 1) and table 2. Among the ten first-degree relatives one C282Y homozygote was found. Among ten second-degree relatives two compound heterozygotes and one C282Y homozygote were found.



**Figure 1**

#### DISCUSSION

HFE-related hereditary haemochromatosis seems to be a favourable disorder for screening. The disease has a long presymptomatic phase, an economical and simple treatment is available and – if treated in time – patients have the same life-expectancy as healthy persons.<sup>11</sup>

Two modalities exist for screening haemochromatosis: phenotypic testing (ferritin level and transferrin saturation) and genotypic testing (DNA examination).

Phenotypic testing detects persons with elevated iron stores. However, phenotypic testing is a one-time measurement, so a normal test result does not exclude future iron loading. Genotypic testing detects persons who are at risk of developing iron overload, but not all detected persons will develop iron loading, indicating that HFE-related hereditary haemochromatosis is not a monogenetic disorder.

Therefore, genotypic population screening for HFE-related hereditary haemochromatosis is still a matter of debate, because the clinical penetrance of the disorder (that means the percentage of persons with an ‘at risk’ genotype who will develop symptomatic iron loading) seems to be much lower than previously thought. Although C282Y homozygosity has a phenotypic expression (elevated ferritin and transferrin saturation) of 50 to 90%,<sup>12,13</sup> the importance with respect to morbidity and mortality seems to be low. Beutler *et al.* found 152 C282Y homozygotes in a group of 41,038 healthy volunteers.<sup>13</sup> Complaints and symptoms that could be ascribed to haemochromatosis were not more prevalent in these C282Y homozygotes than in a control group, so that the clinical penetrance in this study was only 1%.

In contrast to population screening, family testing is an accepted screening strategy to detect asymptomatic persons with (an elevated risk for) iron loading. Detecting a C282Y homozygote with elevated iron stores implies phlebotomy therapy, whereas C282Y homozygotes without phenotypic expression should be re-tested phenotypically within several years. In contrast to C282Y homozygosity, the relevance of compound heterozygosity appears to be much lower

**Table 2**  
*Phenotype of the tested family members*

Family member	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Iron	39	5	*	17	18	15	8	19	5	28	15	23	37	14	22	26	24	63	4	11	16	12	22
TIBC	55	52	*	63	75	72	77	89	74	67	70	98	68	87	67	84	76	88	115	57	83	76	74
Tf-saturation	71	10	*	27	24	20	11	21	7	41	21	23	54	16	33	31	31	71	4	19	19	16	30
Ferritin	1318	355	580	40	42	37	159	77	10	109	397	44	182	37	91	148	54	42	122	213	41	145	242
Age	60	57	55	54	53	49	46	44	36	34	31	29	26	30	27	24	22	18	21	18	13	52	49

TIBC = total iron-binding capacity, Tf = transferritin, \* not available.

since only 1 to 2% with this genotype develop a mild iron loading.<sup>14</sup> As can be seen from figure 1 and table 2, genotype and phenotype often correlate poorly. However, the C282Y homozygote in the second-degree relatives (no. 20) was still young and it is possible that she will develop iron loading later in life. Furthermore, elevated iron parameters can be caused by conditions other than HFE-related hereditary haemochromatosis, as was recently discussed by Jacobs *et al.*<sup>15</sup>

The Dutch Haemochromatosis Association advises testing of first-degree relatives (siblings, children, parents) of C282Y homozygous patients both phenotypically and genotypically. In our patient second-degree relatives were tested as well. Among these second-degree relatives both C282Y homozygosity and compound heterozygosity were found, which means that spouses of first-degree relatives had introduced a C282Y and a H63D mutation into the family.

As is the case in the general population, relatives of patients with HFE-related hereditary haemochromatosis with an 'at risk' genotype do not inevitably develop iron loading. However, in families the clinical penetrance of C282Y homozygosity appears to be higher than in the general population and is reported to be 40 to 67%.<sup>16</sup> Bulaj *et al.* examined the penetrance in 214 C282Y homozygotes, including second-degree relatives, who were detected by family testing.<sup>17</sup> From all men, 85% had an increased iron supply, 38% had signs and/or symptoms that could be ascribed to iron loading, including liver cirrhosis in 12%. However, a control group was not included in this study. Because of this higher clinical penetrance it can be questioned whether family testing for HFE-related hereditary haemochromatosis should include second-degree relatives. Extended family testing (first to third degree) has been advocated as an alternative to population screening, leading to detection of 40% of C282Y homozygotes.<sup>18</sup> It is thought that the higher penetrance in families is caused by additional genetic and environmental factors that promote penetrance, although a search for such genetic factors was unrewarding.<sup>19</sup> In second-degree relatives a dilution of these penetrance-promoting factors is expected. However, the magnitude and effect of this expected dilution is unknown so the

penetrance in second-degree relatives is difficult to predict. A current two-year multicentre study of the clinical penetrance in first-degree relatives of Dutch HFE-related hereditary haemochromatosis patients will hopefully shed some light on this issue ([www.zonmw.nl](http://www.zonmw.nl)). If penetrance in these first-degree relatives turns out to be low, screening second-degree relatives will probably not be cost-effective. However, if the penetrance is in the reported range of 50%, a study of penetrance in second-degree relatives is needed. The chance of C282Y homozygosity in second-degree relatives of a C282Y homozygous index patient can be calculated as follows:

$$\begin{aligned}
 &0.5 \text{ (= the chance of having a heterozygous sibling)} \times 0.1 \\
 &\text{(= the chance of a heterozygous spouse)} \times 0.25 \text{ (= the} \\
 &\text{chance that both parents pass the mutation to their off-} \\
 &\text{spring)} = 1.25\% \\
 &+ \\
 &0.25 \text{ (= the chance of having a homozygous sibling)} \times 0.1 \\
 &\text{(= the chance of a heterozygous spouse)} \times 0.5 \text{ (= the chance} \\
 &\text{that the spouse passes the mutation)} = 1.25\% \\
 &\text{Total} = 2.5\%^*
 \end{aligned}$$

\*In this calculation C282Y heterozygosity of the parents of the index patient is assumed and the chance of a homozygous spouse is neglected.

At first sight this chance seems rather small. However, the yield of genetic testing in second-degree relatives can be enhanced by first testing the spouse of the first-degree relative (so-called cascade screening). If the sibling is C282Y heterozygous or homozygous, the spouse should be tested. The initial increase in costs and number of persons that need to be tested will be 75%. If the spouse carries no mutation, testing the children can be omitted. However, if the spouse is C282Y heterozygous (10% chance) there is at least a 25% chance of C282Y homozygosity in their offspring, which is the same as for siblings of the proband. The above-described approach proved to be cost-effective in screening children of affected probands.<sup>20</sup> The difference in chance of C282Y homozygosity between children (5%)

and second-degree relatives (2.5%) is rather small. However, in the aforementioned study the clinical penetrance in children was assumed to be 40%, whereas the penetrance in second-degree relatives is unknown. The findings of this study cannot thus be automatically extrapolated to screening second-degree relatives. It has been argued that detecting asymptomatic C282Y homozygotes would impose a psychological and economical burden upon these persons. Insurance denial has indeed been reported. However, a recent study showed that the quality of life and psychological well-being in asymptomatic C282Y homozygotes detected by screening is not different from unaffected persons. In conclusion, testing second-degree relatives of patients with HFE-related haemochromatosis can be helpful in detecting persons who will possibly develop iron loading in the future. Testing first-degree relatives only implies a 2.5% chance that persons who are at risk for developing iron loading will not be detected. Testing second-degree relatives by means of cascade screening might be cost-effective. However, before recommendations on screening second-degree relatives can be made, studies to cost-effectiveness and penetrance are needed.

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# A clinical and cardiovascular survey of Ehlers-Danlos syndrome patients with complete deficiency of tenascin-X

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## ABSTRACT

**Background:** We recently described a new autosomal recessive type of Ehlers-Danlos syndrome (EDS) based on a deficiency of the extracellular matrix protein tenascin-X (TNX). TNX-deficient patients have hypermobile joints, hyperextensible skin and show easy bruising. Because of the reported cardiovascular abnormalities in other EDS types and the excessive haematoma formation after mild trauma in TNX-deficient individuals, we investigated whether cardiovascular or coagulation abnormalities occur in these patients.

**Methods:** We examined seven TNX-deficient patients. One of them had a mitral valve prolapse and died postoperatively after valve replacement, before the study was completed.

**Results:** Bleeding time and coagulation factors (INR, APTT, PT and fibrinogen) were all within the normal range. Ultrasonographic examination of the carotid and femoral arteries showed normal vessel wall compliance and distensibility. Echocardiography showed a slight billowing of the mitral valve in two patients from one family. All patients had normal diameters of aortic root and ascending aorta.

**Conclusion:** Although the patient group is small, there are no indications of generalised cardiovascular abnormalities in this type of EDS. We would recommend echocardiography for all these patients at the first evaluation and when a cardiac murmur appears.

## INTRODUCTION

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of inherited connective tissue disorders characterised by hyperextensibility of the skin, hypermobility of joints and tissue fragility which results in easy bruising and (in the classical type) atrophic scars following superficial injury. Six well-defined types of EDS have been described<sup>1</sup> and the syndrome is generally regarded as a disorder of fibrillar collagen. Recently, we showed that deficiency of the extracellular matrix protein tenascin-X (TNX), encoded by the TNXB gene, causes a new type of EDS with autosomal recessive inheritance.<sup>2</sup> Patients with a complete deficiency of TNX showed marked joint hypermobility, skin hyperextensibility and easy bruising, resembling the classical type of EDS. The six patients studied showed a variety of other clinical manifestations (such as spina bifida occulta, IgA deficiency, gastrointestinal bleeding, arteriosclerosis, goiter) although it is not clear whether these are causally related to TNX deficiency.

TNX deficiency is associated with abnormalities in collagen and elastic fibres,<sup>3,4</sup> which are principal components of heart valves and large vessels. Cardiac abnormalities such as mitral valve prolapse and aortic dilatation are reported to be common features of certain EDS types,<sup>4,5</sup> although this has been questioned in a more recent paper.<sup>6</sup> Here we undertook an echocardiographic examination of six patients who were available for study and we investigated vessel wall properties of carotid and femoral arteries by ultrasonography. All these patients had excessive haematomas after relatively mild trauma or spontaneous haematomas. Although the most likely explanation for this phenomenon is vascular fragility due to connective tissue abnormalities, we wanted to exclude the possibility that the absence of serum TNX

caused abnormalities in coagulation. We therefore tested platelet function and performed screening coagulation tests.

## MATERIALS AND METHODS

### Patients

Six TNX-deficient patients were seen in our outpatient clinic. Three of them were identified through analysis of serum TNX levels in 151 Ehlers-Danlos patients. The other three were siblings. Initially we included seven patients; one of these patients died following cardiac surgery because of sepsis after a mitral valve replacement. These patients were described earlier.<sup>2</sup>

### Clinical features

From the six patients medical history was recorded and physical examination was carried out with an emphasis on joint hypermobility, which was scored on a nine-point scale according to Beighton.<sup>1</sup> An electrocardiogram was performed in all patients.

### Laboratory measurements

Blood was taken for measurement of screening coagulation tests (INR, APTT, PT and fibrinogen). Platelet function was tested by means of Ivy, simplate and aggregation tests. Von Willebrand factor was measured as a marker for endothelial function

### Echocardiography

All patients underwent echocardiography, which was performed with standard equipment (GE ultrasound systems, Vingmed V, Horten, Norway, with second harmonic imaging (octave mode: transmit frequency of 1.7-1.9 MHz, receive frequency of 3.4-3.8 MHz)). Digitised measurements of the aortic root were taken in two-dimensional parasternal long-axis views at end diastole using the leading-edge technique at the four aortic levels described by Roman *et al.*:<sup>7</sup> at the annulus, sinuses of Valsalva, supra-aortic ridge and proximal ascending aorta. Echocardiographic evidence of mitral valve prolapse consisted of severe bowing of the anterior and/or posterior mitral valve leaflet(s) into the left atrium and with the coaptation point of the leaflets on the atrial side of the mitral annulus.<sup>8</sup> If the mitral valve leaflets showed bowing but the coaptation point was still on the ventricular side of the annulus or at the level of the annulus it was defined as 'billowing'.

### Vessel wall compliance

Cross-sectional compliance (CC) and distensibility coefficients (DC) are defined as the absolute and relative change in volume for a given change in pressure, respectively. Since arterial volume cannot be measured directly, simplified models have been used which assume that arteries have a circular shape and that the arterial length does not change

during the cardiac cycle. With this simplification, CC and DC of separate arteries can be assessed by measuring diameters and diameter changes ultrasonographically using a vessel wall movement detector (Wall Track System, Maastricht, the Netherlands) as described by the group of Hoeks and Reneman.<sup>9,10</sup> Measurements were performed by one person according to a protocol described by Van den Berkmortel *et al.*<sup>11</sup> Traces were recorded at the following sites: 1) the right and left common carotid arteries (2 cm proximal of the bulb); 2) the right common femoral artery (at least 1 cm proximal of the bifurcation into the deep and superficial femoral artery).

## RESULTS

All TNX-deficient patients had hyperextensible skin, hypermobile joints and easy bruising, as reported earlier. There were no signs of atrophic scarring. One of the patients had coexisting congenital adrenal hyperplasia. Three patients complained of recurrent (sub)luxation/dislocation of joints and two patients had chronic joint pain.

One patient had a mild systolic murmur at the apex; the other patients had normal heart sounds. The electrocardiogram showed no abnormalities. Echocardiographic analysis showed no evident mitral valve prolapse, but two patients of the same family had billowing of the mitral valve. The diameter of the ascending aorta and aortic root was normal in all six patients available for study. Initially we included seven patients in this study. This seventh patient had a mitral valve prolapse that was complicated by a *Staphylococcus aureus* endocarditis in 1990. Mitral valve replacement was performed in July 2002. During surgery, the tissues of the patient appeared fragile and bled easily. Unfortunately, the patient died 16 days postoperatively due to sepsis. The mitral valve showed excessive tissue and a chorda rupture of the anterior mitral valve leaflet; microscopically there were myxoid degenerative changes. We did not observe fragmentation and clumping of elastic fibres in this mitral valve as we recently found for the dermal elastic fibres in these patients.<sup>4</sup> There were no signs of neovascularisation or inflammation. As large arteries are also rich in elastic fibres we examined the vessel wall compliance and distensibility of these arteries; these results were comparable with those of earlier measurements from healthy controls.<sup>12</sup> Screening coagulation tests and platelet function tests were also normal.

## DISCUSSION

There are several reports showing a high prevalence of cardiovascular abnormalities in EDS patients. Aortic rupture as seen in the vascular type of EDS is the most serious complication but also in the classical type of EDS cardiovascular complications were observed (Leier *et al.*)<sup>5</sup> In

hypermobility syndrome patients, Grahame *et al.* found mitral valve prolapse to be more frequent in patients with a high hypermobility score, but echocardiography was only performed with the M-mode technique.<sup>13</sup> A more recent study by Dolan *et al.* showed no evidence of increased frequency of mitral valve prolapse in patients with EDS.<sup>6</sup> This apparent discrepancy can be explained by a possible bias in the study by Leier *et al.* because a substantial number of the reported patients in that study initially presented to a cardiologist.<sup>5</sup> In our study, two patients of the same family had billowing of the mitral valve and one unrelated patient had a mitral valve prolapse. Because of the small patient group it is too preliminary to conclude that there is an association between TNX-deficient type EDS and cardiac abnormalities. In addition, billowing and prolapse of the mitral valve are not uncommon in the general population. Studies show a prevalence of 1 to 2%.<sup>14,15</sup>

At this point, no general recommendation can be made as to whether all EDS patients should be subjected to echocardiography. Former studies show that cardiac abnormalities and aortic root dilatation occur in all subtypes of EDS<sup>3,16,17</sup> but studies on the prevalence of cardiac abnormalities within the specific EDS subtypes and studies concerning long-term outcome of the cardiovascular abnormalities have not been carried out. The EDS type based on TNX deficiency described here can easily be discriminated from the other types, because of the absence of atrophic scarring and family history. We would recommend echocardiography for these patients at the first evaluation and when a cardiac murmur appears. If there is a mitral valve prolapse with coexisting mitral insufficiency patients should be followed yearly or every other year according to the severity of the disorder. In case of interventions patients should be treated according to the endocarditis prophylaxis.

We recently found that dermal elastic fibre structure is disturbed in TNX-deficient patients.<sup>3,4</sup> Whether these patients suffer from a more generalised elastinopathy remains to be investigated. Our measurements of arterial vessel wall mechanical properties showed no evident abnormalities nor did we observe abnormal elastic fibres in the mitral valve that was available for study. In large blood vessels there is co-expression of TNX and tenascin-C (TNC), which is another member of the tenascin gene family.<sup>18</sup> TNC could possibly compensate for the absence of TNX leading to normal vessel wall properties.

One of the clinical features of the TNX-deficient patients is easy bruising. This could be caused by mechanical fragility of the vascular connective tissue. Since a 140 kDa TNX fragment is present in plasma, this could theoretically affect the intrinsic coagulation properties. We found, however, no impaired coagulation in these patients.

In conclusion, we would recommend echocardiography for patients with EDS due to TNX deficiency, at the first evaluation and when a cardiac murmur appears.

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# A 72-year-old patient with diarrhoea and abdominal pain

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## CASE REPORT

A 72-year-old diabetic male was referred by his general practitioner because of gradually increasing diarrhoea and abdominal pain over several days. On examination, he had mild acidotic breathing, and he appeared slightly dehydrated. The abdomen was distended and tender on palpation. Blood chemistry revealed slight derangement of his diabetes, and a neutrophil count of  $12 \times 10^9/l$ , liver enzymes were slightly elevated (ASAT 60, ALAT 70 U/l). During clinical observation, symptoms worsened, and a plain abdominal radiograph was performed (*figure 1*).

## WHAT IS YOUR DIAGNOSIS?

See page 175 for the answer to this photo quiz.



**Figure 1**  
*Plain abdominal (antero-posterior) radiograph in supine position*

# Extreme leucocytosis and splenomegaly in metastasised melanoma

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## ABSTRACT

A 63-year-old woman presented to the internist with fatigue, cough, low-grade fever, splenomegaly and leucocytosis up to  $130 \times 10^9/l$ . Although a diagnosis of chronic myelogenous leukaemia was initially entertained, she turned out to have a metastasised melanoma.

The differential diagnosis and workup is discussed, as well as potential mechanisms by which the tumour could have induced the leucocytosis, such as the production of G-CSF or similar mediators, and the prognostic significance of this phenomenon.

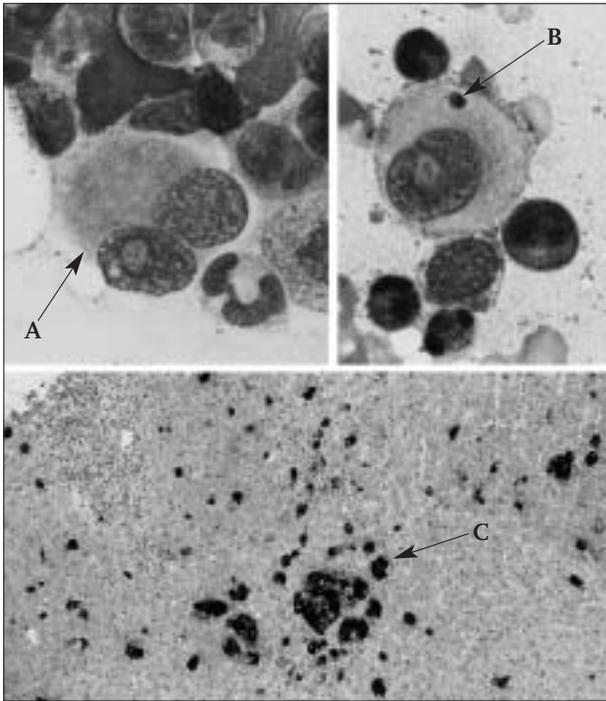
## CASE REPORT

A 63-year-old woman was referred to the internist because of progressive fatigue and breathlessness for several months. She even got breathless from talking. She had lost 15 kg of weight and had intermittent fevers up to  $38.6^\circ\text{C}$ . There were no night sweats. Several courses of oral broad-spectrum antibiotics had not had any effect on her symptoms. The previous medical history was unremarkable apart from an unspecified lung operation at the age of 13 and chronic obstructive pulmonary disease. Ten months earlier a melanoma had been removed from her left ankle, histologically Breslow depth 1.5 mm and Clarke level III. Her medication consisted of inhaled bronchodilators, steroids and acetylcysteine. The family history included a brother with multiple sclerosis and a brother with colonic carcinoma. On physical examination a pale and breathless woman was seen. She was 1.65 m tall and her weight was 60.3 kg. The pulse was 100 beats/min, blood pressure 120/70

mmHg and rectal temperature  $39.0^\circ\text{C}$ . There were no enlarged lymph nodes and no pathological findings of heart and lungs. No breast lumps were palpable. Her liver was palpable under the right costal margin and the spleen was palpable 10 cm under the left costal margin. There was no peripheral oedema and no other findings at the extremities.

Laboratory findings were: Hb 5.5 mmol/l (8.91 mg/dl), thrombocyte count  $377 \times 10^9/l$ , leucocyte count  $62.4 \times 10^9/l$  (differential: neutrophils 73%, eosinophils 11%, basophils 0%, lymphocytes 11%, monocytes 4%, some metamyelocytes and myelocytes; erythroblasts were not observed). The electrolytes and renal function were normal. Liverfunction tests: ASAT 21 U/l, ALAT 12 U/l, LDH 527 U/l, alkaline phosphatase 295 U/l,  $\gamma\text{GT}$  196 U/l, ferritin 379  $\mu\text{g/l}$  and serum iron 3.1  $\mu\text{mol/CRP}$  was elevated at 101 mg/l.

Bone marrow biopsy showed hypercellular bone marrow with all cell lines represented, though a strong increase in granulopoiesis was seen. Occasionally some very atypical cells were seen with polymorphic hyperchromatic nuclei, a coarse chromatin pattern and enlarged nucleoli. Bone marrow cytology showed an increased granulopoiesis (70%) with moderate left shift, 2% blasts, increase of eosinophilic elements (14%) with multiple precursors. There was decreased erythropoiesis and megakaryopoiesis. Scattered through the marrow there were large, very atypical cells with an epitheloid aspect, one or more round to oval nuclei, abundant cytoplasm which sporadically contained blue-grey granula (see figure 1). These cells were identified as (amelanotic) melanoma cells.



**Figure 1**

*Microscopy*

Top: Bone marrow aspirate with large, very atypical cells with an epithelioid aspect, one or more round to oval nuclei (A), abundant cytoplasm containing sporadic blue-grey granula (B).

Bottom: Liver biopsy (HMB-45 and S100 staining) with agglomerates (C) of melanoma cells and marked hypergranulocytosis of the sinuses.

Cytogenetics showed 20 cells with a normal female karyotype and no *bcr-abl* fusion on fluorescent *in situ* hybridisation (FISH) in ten metaphases.

Blood cultures (two separate 3-10 ml aliquots containing aerobic and anaerobic media) were negative. Mid-stream urine examination showed several leucocytes per high-power field and upon culture contained mixed bacterial flora. Sputum was negative for auramine and Ziehl-Neelsen staining but on culture showed *Haemophilus parainfluenzae*.

Ultrasound examination of the abdomen showed an enlarged liver with multiple lucencies, a very large spleen of 20 cm with one lucency possibly due to metastasis, some ascites and no enlarged lymph nodes.

Computed tomography (CT) of chest and abdomen showed no signs of a primary tumour, signs of infiltration in the left lower pulmonary lobe, many metastases in the liver and a solitary one in the spleen, one pathological lymph node around the abdominal aorta and some ascites.

Ultrasound-guided biopsy of one of the liver lesions showed disseminated melanoma cells in small clusters and

marked granulocytosis of the sinusoids. Immunohistochemical staining was positive for melanoma (HMB-45, S100, Schmorl's stain weak).

During the stay in hospital the leucocytosis increased to  $130.3 \times 10^9/l$  despite antibiotic therapy and the patient's condition worsened with night sweats and progressive fatigue. She declined chemotherapy, and was released with terminal home care; she died at home one day after discharge. A postmortem examination was not performed.

**DISCUSSION**

Extreme leucocytosis can be caused by infection, malignancy and several other, less common causes. When leucocyte counts exceed  $50 \times 10^9/l$  it is referred to as 'leukaemoid reaction' (see table 1). Reding *et al.* studied 100 patients presenting with leucocytosis exceeding  $25 \times 10^9/l$  and found infection as a cause in only 48%.<sup>1</sup> In 15% malignancy was diagnosed and in the remaining 37% other, less common secondary causes were found (such as haemorrhage, glucocorticoid therapy, rhG-CSF therapy).

**Table 1**

*Causes of neutrophilic leukaemoid reaction (leucocyte count  $>50 \times 10^9/l$ )<sup>1,15</sup>*

<b>INFECTIONS</b>
Pneumonia
Meningitis
Diphtheria
Tuberculosis
Shigellosis
<b>INTOXICATIONS/MEDICATION</b>
Mercury poisoning
Steroids
Adrenergic agents
All-trans retinoic acid (ATRA)
G-CSF
<b>PHYSICAL CAUSES</b>
Serious burns
<b>MALIGNANCY</b>
Bone metastasis
Multiple myeloma
Myelofibrosis
Hodgkin's disease
Other malignancy
<b>SERIOUS HAEMORRHAGE</b>
<b>ACUTE HAEMOLYSIS</b>
<b>ECLAMPSIA</b>
<b>PREMATURITY/NEONATES</b>
<b>HEREDITARY/CONGENITAL</b>

The leucocyte count was not predictive of the cause. The 15 patients with malignancy mostly had primary haematological disorders, but metastatic carcinoma was diagnosed in six of them.

Fatigue, splenomegaly and leucocytosis as presenting signs in a middle-aged patient all point at the possibility of myeloproliferative syndrome (MPS). However, careful review of the history, physical examination, infectious parameters (such as blood culture, chest X-ray and mid-stream urine specimen), blood film and bone marrow aspirate and trephine can give clues as to a secondary cause.

After excluding infectious disease and drug-related causes of leucocytosis, the diagnostic effort should concentrate on the possibility of malignancy. To differentiate between primary haematological and secondary causes, normal to low basophil granulocytes on the blood film, as well as negative cytogenetics (no t(9;22)(q34;q11) translocation causing *bcr-abl* fusion) make myeloproliferative disease very unlikely.

There are roughly two ways in which nonhaematological malignant disease can lead to leucocytosis. Firstly, bone marrow invasion by metastases can cause a leucoerythroblastic picture. Secondly, an oft-reported form of leucocytosis in malignancy is a leukaemoid reaction due to production of cytokines or other mediators by tumour cells.

In our patient, infectious causes were considered unlikely, with blood cultures remaining sterile all through the patient's stay in hospital. She was not taking any oral medication associated with a raised leucocyte blood count. The absence of *bcr-abl* fusion on cytogenetics and the normal basophil count made the diagnosis of chronic myelogenous leukaemia (CML) very unlikely (<5% of CML patients are *bcr-abl* negative).<sup>2</sup>

The finding of metastatic lesions of the primary melanoma in the liver biopsy, as well as malignant cells in the bone marrow, confirmed the suspected diagnosis of tumour-induced leucocytosis. Bone marrow infiltration as such was not perceived to be an explanation for the symptoms, given the absence of leucoerythroblastosis in the blood. Therefore, the diagnosis was leukaemoid reaction due to the production of a humoral factor by a primary melanoma.

Virtually every type of malignant tumour has been reported to induce leucocytosis, for example glioblastoma multiforme,<sup>3</sup> head and neck carcinoma<sup>4</sup> and malignant mesothelioma.<sup>5</sup> Generally the secretion of G-CSF or GM-CSF, most commonly the former,<sup>6</sup> by the tumour cells seems to be the predominant mechanism of the leukaemoid reaction in malignancy.<sup>7</sup>

Malignant melanoma has been reported to secrete G-CSF or GM-CSF,<sup>8-11</sup> and this feature appears to be a marker of

poor prognosis.<sup>10</sup> In the patient reported by Carey and Kunz<sup>11</sup> the leucocytosis resolved when the adrenal metastases were removed through bilateral adrenalectomy. Eosinophilic leukaemoid reaction has been reported in a case of melanoma secreting extremely high quantities of IL-5.<sup>12</sup> Concerning the contribution of G-CSF production to a tumour's malignant potential, Safarians *et al.* found that G-CSF-secreting melanoma cells do not express G-CSFR, thereby ruling out an autocrine mechanism.<sup>10</sup> Otherwise, G-CSF can stimulate endothelial proliferation and migration, thereby assuring the tumour of a steady blood supply through angiogenesis.<sup>13</sup> Moreover, the granulocytes themselves can benefit the tumour by inducing further malignant transformation.<sup>14</sup> Finally, Safarians *et al.* suggest that the expression of G-CSF could be a side effect of the activation of other oncogenes, which increase the tumour's capability of malignant transformation, growth and dissemination.<sup>10</sup>

## CONCLUSION

The diagnosis in this patient turned out to be metastasised melanoma with extreme granulocytosis, possibly due to G-CSF production by the tumour cells.

## ACKNOWLEDGEMENT

Dr M.C.B. Gorsira, Diaconessenhuis Leiden, for providing the liver biopsy slide.

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## Bijsluiter A

# Symmetrical, painful ulceration of the lower limbs in a vascular surgery ward: a diagnostic challenge

## Pyoderma gangrenosum associated with IgG- $\kappa$ paraproteinaemia

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### ABSTRACT

We describe a 61-year-old patient who had been suffering from chronic ulcers of both legs for 18 months. Initially, his condition was diagnosed as ischaemic because of an ankle-brachial index of 0.6, as confirmed by additional angiography.

A successful femoro-infragenuous bypass procedure was performed, but the ulcers increased in size and number. He was then extensively analysed for a possible (macro)vascular origin of his symptoms. Angiographic analysis of both legs showed no arterial stenosis or occlusion. Despite the extensive experience of the vascular surgeons with leg ulcers, consultations by internal medicine, vascular medicine and dermatology, and tissue examination by our pathologists, pyoderma gangrenosum was not recognised. During a multidisciplinary meeting one of the specialists, to whom the lesions were shown, immediately considered the diagnosis on clinical grounds. The additional finding of IgG- $\kappa$  paraproteinaemia and improvement of the ulcers on treatment with corticosteroids were consistent with the diagnosis. Although the majority of patients on the vascular surgery ward have ulcers caused by ischaemia or a combined arterial/venous origin, another (rare) cause, namely pyoderma gangrenosum in association with IgG- $\kappa$  paraproteinaemia without the presence of multiple myeloma, should be taken into account.

### INTRODUCTION

Pyoderma gangrenosum (PG) is an uncommon, idiopathic, chronic ulcerative inflammatory skin disease.<sup>1-5</sup> Four types of PG have been recognised: the ulcerative, pustular, bullous and vegetative types.<sup>4</sup> The first type is characterised by a severely painful ulceration surrounded by an erythematous halo. In the majority of the affected patients there is an underlying condition, usually inflammatory bowel diseases (such as ulcerative colitis, Crohn's disease and diverticular disease), arthritis (such as rheumatoid arthritis, spondylarthropathy), chronic hepatitis and haematological malignancy (including acute and chronic myeloid leukaemia, myelofibrosis, lymphoma) and solid tumours of the colon, bladder, prostate, breast, bronchus, ovary, and adrenocortical carcinoma. This type is commonly located on the lower limb, but location on the trunk, penis, head and neck, breast, and ocular sites have also been reported. The ulcerative form of PG typically affects people in the age range 25 to 55 years. The second type is also painful with pustules again surrounded by an erythematous halo. This condition is usually associated with inflammatory bowel disease (ulcerative colitis) and rarely with polycythaemia rubra vera, hepatobiliary disease and pyostomatitis vegetans. The third type (also designated as atypical PG) is characterised by painful vesicles, with a tendency to enlarge rapidly in waves with central necrosis and erosion with an erythema as a surrounding halo. This type is usually present in

combination with haematological dyscrasias. Confusion with Sweet's disease (acute febrile neutrophilic dermatosis) can easily occur. Sweet's disease is very uncommon and characterised by uncomfortable chronic erythematous plaques with sinus discharge. Other disorders associated with PG include HIV, hepatitis C, thyroid disease, diabetes mellitus, cryoglobulinaemia, lupus erythematosus, dermatomyositis, sarcoidosis, vasculitis (Wegener's granulomatosis, Takayasu's arteritis, Behçet's disease) and paroxysmal nocturnal haemoglobinuria (*table 1*).

The association of the ulcerative type of PG with benign monoclonal gammopathy, especially of immunoglobulin A (IgA), has also been described.<sup>6-9</sup> We report a patient suffering from the ulcerative type of PG of both legs, which was found to be associated with IgG-κ paraproteinaemia.

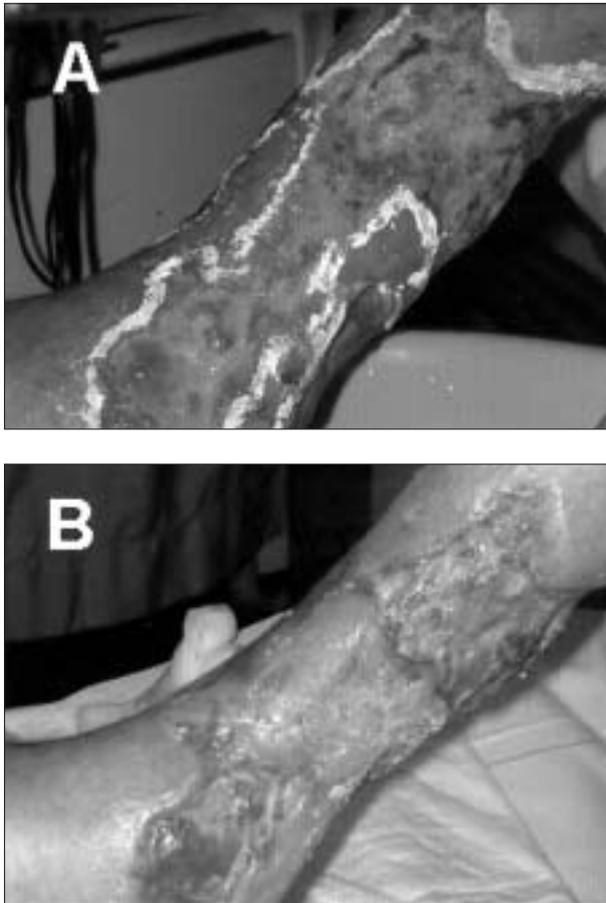
## CASE REPORT

A 61-year-old patient was admitted to the department of vascular surgery because of chronic ulcers of both legs (for 18 months). These ulcers were initially identified as ischaemic lesions (an ankle-brachial index of 0.6) and femoro-infragenual bypass surgery was performed. Soon after this operation, he developed more painful ulcers, despite normalisation of the ankle-brachial index. Moreover, subsequent angiographic analysis of the arteries of the legs showed an open bypass without any indication for (macro)vascular stenosis/occlusion. Duplex examination did not show venous occlusion either. The patient had been treated with several antibiotics, without any improvement in the clinical situation. Then he was sent to our university hospital. Cardiovascular risk factors, including hypertension and dyslipidaemia, were adequately controlled. He was not complaining of headache, fever or changes in bowel movement. Further medical history was unremarkable. Physical examination revealed a normal blood pressure, normal temperature, lack of tenderness of the temporal arteries, and large ulcerations involving the pretibial area of both legs, with blood and pus in the centre and a region of deep necrosis (*figure 1A*). Laboratory data disclosed an elevated erythrocyte sedimentation rate (100 mm/h), Hb 6.2 mmol/l, MCV 82 fl, leucocytosis  $11.9 \times 10^9/l$  with 90% neutrophils, normal platelet count, slightly elevated creatinine level 152 μmol/l, and a normal calcium. Thyroid function was normal. Tests for hepatitis B, C and lues were negative. Antinuclear antibodies, rheumatoid factor, anti-neutrophil cytoplasmic antibodies and antiphospholipid antibodies were absent. A chest radiogram and a computer tomography of the abdomen revealed no abnormalities. A skin biopsy revealed lymphocytic as well as neutrophilic inflammation. Moreover, the biopsy showed plasma cells, necrosis and re-epithelialisation. Vasculitis was absent in the biopsy.

**Table 1**

*Diseases associated with pyoderma gangrenosum*

<b>INFLAMMATORY BOWEL DISEASE</b>	
(Chronic) ulcerative colitis	
Crohn's disease	
Diverticular disease	
Regional enteritis	
<b>ARTHRITIS</b>	
Seronegative with inflammatory bowel disease	
Seronegative without inflammatory bowel disease	
Rheumatoid arthritis	
Spondylarthropathy	
<b>HAEMATOLOGICAL DISEASE</b>	
(Acute and chronic) myelocytic leukaemia	
Monoclonal gammopathy (IgA)	
Hairy cell leukaemia	
Myelofibrosis	
Polycythaemia rubra vera	
Lymphoma: Hodgkin's disease, non-Hodgkin's disease, cutaneous T cell	
<b>IMMUNE ABNORMALITIES</b>	
Humoral	Congenital and acquired hypogammaglobulinaemia
	Selective, complete and hyperimmunoglobulin E syndrome
	Streaking leucocyte factor
Cell-mediated	Defective neutrophil function: reduced chemotaxis and impaired phagocytosis and oxygen uptake, aberrant neutrophil trafficking
	Abnormal monocyte function
	Abnormal response to skin antigens
	Congenital deficiency in leucocyte-adherence glycoproteins
	Immunodeficiency/immunosuppression
<b>SOLID TUMOURS ASSOCIATED WITH PG</b>	
Colon, bladder, prostate, breast, bronchus, ovary, adenocortical carcinoma	
<b>DRUGS TRIGGERING PG</b>	
Alpha 2b-interferon	
<b>OTHERS</b>	
Chronic active hepatitis, cryoglobulinaemia and hepatitis C, thyroid disease, pulmonary disease, hidradenitis suppurativa, acne conglobata, sarcoidosis, atrophic gastritis, diabetes mellitus, systemic lupus erythematosus, Takayasu's arteritis, dermatomyositis, HIV, Wegener's granulomatosis, sensorineuronal deafness, paroxysmal nocturnal haemoglobinuria, peripheral ulcerative keratitis, lung injury, primary biliary cirrhosis	



**Figure 1**  
*Ulcerative pyoderma gangrenosum before (A) and after (B) treatment with steroids*

Initially a definitive diagnosis could not be made. During a multidisciplinary meeting, however, one of the specialists, to whom the lesions were shown, immediately considered the diagnosis pyoderma gangrenosum on clinical grounds. Further laboratory analysis, i.e. serum protein electrophoresis, showed an IgG- $\kappa$  paraproteinaemia. To see whether there was a systemic skeletal involvement additional radiograms were performed, without signs of multiple myeloma. Moreover, neither cytology nor histology of the bone marrow showed a localisation for multiple myeloma. The ulcers of both legs started to heal within a week after treatment with prednisolone 1 mg/kg was initiated (figure 1B). Altogether, the presence of the monoclonal gammopathy and the improvement of the lesions after treatment were consistent with PG.

## DISCUSSION

Pyoderma gangrenosum in association with IgG- $\kappa$  paraproteinaemia is uncommon.<sup>10-12</sup> Although the association with monoclonal IgA gammopathy is well recognised,

multiple myeloma is rarely found. Also in our patient, we could not find signs of systemic skeletal involvement. The conditions that may mimic ulcerative PG are systemic vasculitis, dermatitis artefacta, infection (mycobacterial, atypical mycobacterial, amebiasis, syphilis, herpes simplex, deep fungal infection), drug reactions (hydroxyurea), insect bite (spider), synergistic gangrene, antiphospholipid syndrome and cutaneous neoplasma (table 2). PG is a diagnosis of exclusion, so the above-mentioned conditions should be ruled out before the diagnosis of PG is made. Weenig *et al.* have described that substantial misdiagnosis of skin ulcers as PG can occur (approximately 10%).<sup>10</sup> Misdiagnosis of PG can expose patients to risks associated

**Table 2**  
*Differential diagnosis of pyoderma gangrenosum*

<b>ULCERATIVE PG</b>
Systemic vasculitis
Dermatitis artefacta
Infection: (atypical) mycobacterial, amebiasis, syphilis, herpes simplex (especially in HIV patients), deep fungal infections
Drug reactions (hydroxyurea)
Insect bite (spider)
Synergistic gangrene
Antiphospholipid syndrome
Cutaneous neoplasms
<b>PUSTULAR PG</b>
Infection: bacterial, viral, fungal
Gonococcal septicaemia
Folliculitis
Pustular vasculitis
Pustular drug eruptions
Bowel bypass syndrome
Pyostomatitis vegetans
<b>BULLOUS PG</b>
Atypical Sweet's syndrome
Insect/arthropod bite
Viral infection (in immunocompromised patients)
Acute cellulitis
Bullous mycosis fungoides
Bullous dermatoses (erythema multiforme, etc.)
<b>VEGETATIVE PG</b>
Pyoderma vegetans
Blastomycosis-like pyoderma
Deep fungal infection
(Atypical) mycobacterial infection
Dermatitis artefacta
Cutaneous neoplasm

with its treatment. In that study, 95 patients appeared to have skin ulcers caused by other diseases, although initially PG was suspected. These causative diseases were vascular occlusive or venous disease (such as antiphospholipid syndrome, venous stasis ulceration and type I cryoglobulinaemia), vasculitis (among them Wegener's granulomatosis, polyarteritis nodosa and cryoglobulinaemic (mixed) vasculitis), cutaneous involvement of malignant process (usually lymphoma), primary cutaneous infection (such as deep fungal infection, herpes simplex virus type 2, cutaneous tuberculosis), drug-induced or exogenous tissue injury (among them Münchhausen's syndrome/factitial disorder, hydroxyurea-induced ulceration). The authors thus stress the need to follow a thorough diagnostic evaluation, including collecting careful medical historical data, being aware of characteristic features on physical examination, performing a skin biopsy for not only histopathological purposes but also for tissue culture, and ordering laboratory investigations with the aim of ruling out diagnoses that mimic PG (such as erythrocyte sedimentation rate, complete blood count, blood chemistry, protein electrophoresis, chest radiography, antiphospholipid, antinuclear, and antineutrophil cytoplasmic antibodies). When PG is eventually established as the diagnosis, the patient should be evaluated thoroughly to rule out other diagnoses after long-term follow-up.

However, in our patient, the opposite of what Weenig *et al.* described in their study population happened. Namely, it took rather a long time before a definitive diagnosis of PG was made, due to the other possible causes of the ulceration. Skin biopsies in all forms of PG are characterised by a central necrosis accompanied by a massive peripheral neutrophilic infiltration and perivascular and intramural lymphocytic infiltrates.<sup>13</sup> A possible pathogenetic mechanism underlying the association between PG and monoclonal gammopathy could be the defective monocyte function, as was suggested by Norris *et al.*<sup>14</sup> and Jones *et al.*<sup>12</sup> Other possible mechanisms include abnormality of neutrophil and monocyte chemotaxis, phagocytosis (including defective leucocyte adhesion glycoproteins), mast cell activation, hypogammaglobulinaemia or association with hyperimmunoglobulinaemia E.<sup>5,13</sup> Crowson *et al.* mention in their review article that the pathogenetic mechanism and the initial trigger are dependent on the associated/underlying systemic disease, eventually leading to the endpoint of a neutrophilic dermatopathy with diverse destruction of the epidermis and the adnex.<sup>13</sup>

In conclusion, we report a patient with chronic ulcers of both legs with a history of peripheral bypass surgery and (cardio)vascular risk factors. Despite an extensive search for an ischaemic aetiology, a rare diagnosis, pyoderma gangrenosum associated with IgG- $\kappa$  paraproteinaemia, was found to be the cause of the lesions. This case illustrates that the diagnosis of pyoderma gangrenosum is not

easy: many specialists were involved before one of them coined the possibility of a PG. Since the disease is rare, recognition is difficult. Suspicion of PG should be raised when ulcers are present on the lower legs that are painful and symmetrical and for which there is no other obvious explanation. A thorough diagnostic effort must be made to reveal underlying disease when PG is diagnosed.

#### ACKNOWLEDGEMENT

We would like to thank dr. F. Stam for his help.

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# Recurrent acute pancreatitis after isoniazid

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## ABSTRACT

**Drug-induced acute pancreatitis should be in the differential diagnosis of acute abdomen occurring soon after initiation of tuberculosis treatment and chemoprophylaxis. Isoniazid-induced pancreatitis is potentially reversible; early recognition and drug withdrawal are warranted in the appropriate clinical setting. We present a case of reversible acute pancreatitis after isoniazid treatment of genitourinary tuberculosis, followed by recurrence of pancreatitis 12 years later when the patient received isoniazid again for pulmonary tuberculosis. Isoniazid-induced pancreatitis, if highly suspicious or confirmed with re-challenge test, mandates permanent avoidance of the drug.**

## INTRODUCTION

Isoniazid has been used for treatment of *Mycobacterium tuberculosis* for over 50 years. Although it was initially considered to be free of serious side effects, the potential adverse effect of isoniazid hepatotoxicity has now become evident. Furthermore, as our experience with isoniazid accumulates, there have been more reports of isoniazid-induced pancreatitis in post-marketing surveillance. This category of adverse effect, albeit less frequent than hepatotoxicity, should be promptly recognised because development of acute pancreatitis mandates drug withdrawal and permanent isoniazid avoidance as illustrated in our case.

## CASE REPORT

A 25-year-old Chinese patient with a history of systemic lupus erythematosus presented with malaise and progressive uraemic symptoms. There was no history of alcohol abuse. Plasma creatinine was 18.0 mg/dl (normal range, 0.6 to 1.4 mg/dl). Urinary sediment was unremarkable except for sterile pyuria. Advanced renal failure with quiescent lupus nephritis was diagnosed, followed by identification of acid-fast bacilli in the early morning urine. Intermittent peritoneal dialysis was commenced after Tenckhoff catheter insertion, together with implementation of quadruple tuberculosis treatment consisting of isoniazid 200 mg daily (5.5 mg/kg/day), rifampicin, pyrazinamide and ethambutol.

The patient developed acute severe pain in the epigastrium region three weeks after initiation of tuberculosis therapy, before the scheduled date for continuous ambulatory peritoneal dialysis training. Examination revealed diffuse abdominal tenderness. His serum and peritoneal dialysate amylase levels were 2071 U/l (normal range, 44-128 U/l) and 1478 U/l, respectively. An erect chest radiograph demonstrated right apical granuloma only. Abdominal sonography and computed tomographic imaging showed an oedematous pancreas, in the absence of biliary tract disease. The clinical picture of acute pancreatitis resolved with conservative management and withdrawal of isoniazid therapy. There were no other identifiable causes of acute pancreatitis.

Subsequent cadaveric kidney transplantation was complicated by progressive allograft dysfunction secondary to chronic rejection. Twelve years later, reactivation of tuber-

culosis occurred with pulmonary involvement. When isoniazid 200 mg daily, rifampicin and levofloxacin were started, his plasma creatinine was 6.7 mg/dl with an estimated glomerular filtration rate of 12 ml/min/1.73 m<sup>2</sup>.

After three weeks of tuberculosis therapy, the patient presented acutely with marked epigastric tenderness. The serum amylase level rose from 429 U/l to 2100 U/l within five days. He had been receiving prednisolone, cyclosporin and azathioprine for more than ten years, and the serum amylase levels were normal in between the two attacks. Ultrasound imaging confirmed the clinical impression of acute nonbiliary pancreatitis. Again, the pain resolved 72 hours after discontinuation of isoniazid, accompanied by biochemical and radiological evidence of improvement. No further bouts of pancreatitis were encountered thereafter.

## DISCUSSION

Drug-related acute pancreatitis is uncommon.<sup>1</sup> As always, causal relationship based on anecdotal case report(s) has been a matter of controversy. Theoretically, it should be clearly documented that pancreatitis developed during treatment with the implicated drug, was reversed by withdrawal of the drug, and recurred when the drug was reintroduced.<sup>2</sup>

We have previously reported another end-stage renal disease patient with isoniazid-induced pancreatitis, which was

supported by means of re-challenge test.<sup>1</sup> In case of tuberculosis therapy, controlled re-challenge with the drug suspected of causing pancreatitis is deemed necessary in view of the limited range of drugs available for *Mycobacterium tuberculosis* infection as well as the confounding drug culprit rifampicin.<sup>3</sup> Now considered the first-line essential antituberculous drug, isoniazid should be included in all tuberculosis treatment regimens unless contraindicated.

According to the case reports in literature,<sup>1,4-9</sup> acute pancreatitis associated with isoniazid develops within three weeks after drug administration and consistently recurs, with a much earlier onset, if re-challenged soon after resolution of pancreatitis (*table 1*). The role of concurrent rifampicin may not be directly contributory since this complication has been documented in several cases of isoniazid monotherapy (mostly chemoprophylaxis therapy).<sup>4,6,9</sup> It remains unclear whether the reaction of isoniazid-induced pancreatitis occurs in a dose-dependent manner or in the setting of a hypersensitivity syndrome to isoniazid.

Given the short time lapse (i.e. hours between re-challenge and occurrence of pancreatitis (*table 1*), the latter is most likely. The current case allows us to evaluate recurrence of pancreatitis as isoniazid was re-introduced 12 years after the initial episode. Irrespective of the mechanisms of isoniazid-induced pancreatitis,<sup>1</sup> the message here is clear: isoniazid should be permanently avoided once it has been documented to cause acute pancreatitis.

**Table 1**  
*Well-documented case reports of isoniazid-induced acute pancreatitis*

REFERENCE	SUBJECT SEX/AGE	ESRD	DAILY TREATMENT DOSE (MG)	DIAGNOSIS	AMYLASE IN SERUM (U/L)	ONSET (DAY(S)) OF PANCREATITIS AFTER START OF ISONIAZID	ONSET OF PANCREATITIS AFTER RE-CHALLENGE
4	F/43	-	300	Chemoprophylaxis, sarcoidosis	Not done	4	2 hours
5	M/31	-	300	Gastrointestinal tuberculosis	758	14	6 hours
6	M/68	-	300	Urinary bladder treatment	458	0.5	No re-challenge
1	M/31	+	300	Pulmonary tuberculosis, IgA nephropathy	1672	21	5 days
7	M/80	+	300	Tuberculous spondylitis, ischaemic nephropathy	782	2	No re-challenge
8	M/42	-	300	Tuberculous spondylitis	645	11	8 hours
9	F/28	-	300	Chemoprophylaxis	356	21	No re-challenge
Our case report	M/25	+	200	Genitourinary tuberculosis, systemic lupus erythematosus	2071	21	21 days

ESRD = end-stage renal disease, F = female, M = male.

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Bijsluiter B

ANSWER TO PHOTO QUIZ (ON PAGE 163)

A 72-YEAR-OLD PATIENT WITH DIARRHOEA AND ABDOMINAL PAIN

DIAGNOSIS

The plain abdominal radiograph reveals what was suspected during the repeat physical examination: a caecal blow-out. The radiograph shows gas around the pathologically distended caecal part of the colon, and gas collection anterior and medial of the caecum.

The computed tomography (figure 2) confirmed this diagnosis, and caecal perforation with early peritonitis was found during laparotomy. An ileocaecal resection was carried out. The postoperative course was complicated by multiple organ failure. The patient was transferred to our intensive care unit. Despite fluid resuscitation, mechanical ventilation, vasopressor and antimicrobial treatment, and renal replacement therapy, he died from refractory septic shock.

The cause of his diarrhoea was not established. In hospital populations, caecal perforation is a well-known complication of *Clostridium difficile* toxin-mediated diarrhoea.<sup>1</sup> Several infectious conditions have occasionally been associated with diarrhoea, toxic megacolon and caecal perforation. These infections include bacteria (*Salmonella* spp, tuberculosis), helminths (*Schistosoma mansoni*) and viruses (CMV). Besides, collagen-vascular diseases such as Wegeners' granulomatosis may occasionally cause a similar clinical pattern. Idiopathic colonic pseudo-obstruction (Ogilvie's syndrome) may also cause caecal blow-out.<sup>2</sup>

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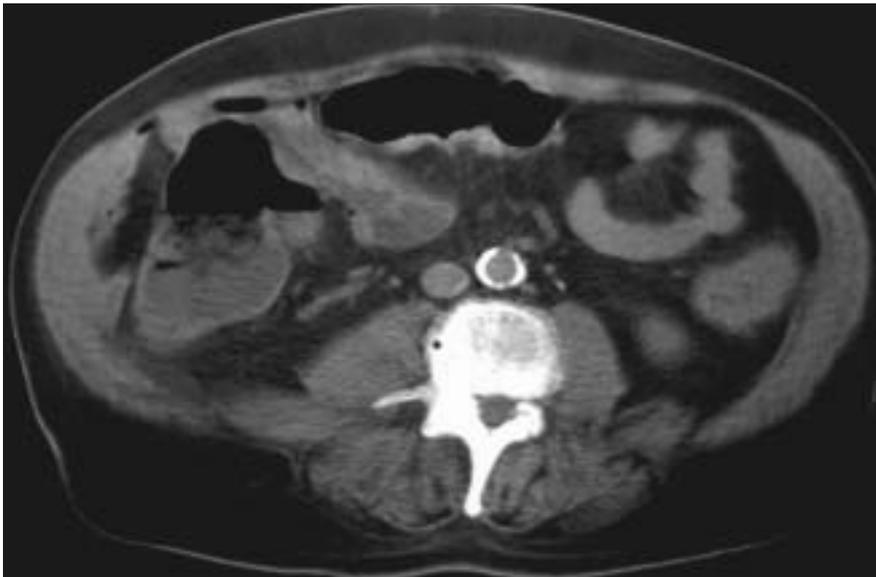


Figure 2  
Abdominal CT confirms the presence of gas outside the caecal lumen that is clearly distended

# The Netherlands Journal of Medicine Awards for best original article, case report and review

At the recent Convention for Internal Medicine (Internistendagen) in Maastricht on 22 and 23 April 2004, *the Netherlands Journal of Medicine* awarded the following prizes: the best original article, best case report and best review published in *the Netherlands Journal of Medicine* in 2003.

A jury consisting of Professor P.W. de Leeuw, Dr C. Gaillard and Dr W. Hart selected the following papers:

## BEST ORIGINAL ARTICLE

**Candida-specific interferon- $\gamma$  deficiency and toll-like receptor polymorphisms in patients with chronic mucocutaneous candidiasis**

C.A.A. van der Graaf, M.G. Netea, J.P.H. Drenth, *et al.* *Neth J Med* 2003;61(11):365-9.

## BEST CASE REPORT

**Rituximab in the treatment of relapsing idiopathic thrombocytopenic purpura**

N.P. Riksen, J.J. Keuning, G. Vreugdenhil. *Neth J Med* 2003;61(7):262-5.

## BEST REVIEW

**Adipose tissue as an endocrine organ: impact on insulin resistance**

I.M. Jazet, H. Pijl, A.E. Meinders. *Neth J Med* 2003;61(6):194-206.

The winner may choose one of the original prints of the graphic art published on the covers of this journal.



*Drs. C.A.A. van der Graaf, Drs. N.P. Riksen, Prof. dr. J.W.M. van der Meer, Drs. I.M. Jazet*

## 'Red tree'

Piet Warffemius



This month's cover is a silk-screen printing by Piet Warffemius.

Piet Warffemius lives and works in The Hague. His work can be described as mysterious, almost unreachable and sometimes bewitching. In recent prints, he often uses images of trees. What he wants to do in the first place is express feelings and

impressions in colour and shape.

The viewer is free to interpret the picture. An original print is available at a price of € 275. You can order the print at Galerie Unita, Rijksstraatweg 109, 6573 CK Beek-Ubbergen, the Netherlands, by e-mail: [galerie-unita@planet.nl](mailto:galerie-unita@planet.nl), or see the website: [www.galerie-unita.com](http://www.galerie-unita.com).

### Aims and scope

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.

### Manuscripts

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.

### Declaration

It is the author's responsibility to seek permission from the person or party concerned for the use of previously published material, such as tables and figures. In addition, persons who are recognisable on photographs must have given permission for the use of these.

### 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.

*Subheadings* should not exceed 55 characters, including spaces.

*Abbreviations:* Measurements should be abbreviated according to SI units. All other abbreviations or acronyms should be defined on the first appearance in the text. Use a capital letter for proprietary names of substances and materials. At first mention of a chemical substance, use the correct chemical designation as well as the generic name.

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.

The *Introduction* should be brief and set out the purposes for which the study has been performed.

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.

The *Results* should be presented precisely without discussion.

The *Discussion* should directly relate to the study being reported. Do not include a general review of the topic, but discuss the pertinent literature.

*Acknowledgement:* All finding sources should be credited here. Also a statement of conflicts of interest should be put here.

*References* should be numbered consecutively (in square brackets) as they appear in the text. Type the reference list with double spacing on a separate sheet. References should accord with the system used in Uniform requirements for manuscripts submitted to biomedical journals (N Engl J Med 1991;324:424-8).

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.

Please note that the first six authors should be listed; when seven or more, list only the first three and add *et al.* Do not include references to personal communications, unpublished data or manuscripts either 'in preparation' or 'submitted for publication'. If essential, such material may be incorporated into the appropriate place in the text. Recheck references in the text against reference list after your manuscript has been revised.

*Tables* should be typed with double spacing each on a separate sheet, numbered consecutively with Arabic numerals, and should contain only horizontal lines. Provide a short descriptive heading above each table with footnotes and/or explanation underneath.

*Figures* must be suitable for high-quality reproduction. Submit line drawings made in Word or other computer programmes but not in a PowerPoint file. India ink drawings or sharp, strongly contrasting photographic prints on glossy paper are also acceptable. Lettering should be complete, of professional quality, and of a size appropriate to that of the illustration of drawing, with the necessary reduction in size taken into account. Figures should be no larger than 12.5 x 18 cm. Submit half-tone illustrations as black-and-white prints on glossy paper, with as much contrast as possible. Identify each figure on the back with a typed label, which shows the number of the figure, the name of the leading author, the title of the manuscript and the topside of the figure. Colour figures are occasionally possible and will be charged to the authors.

*Legends for figures* should be typed, with double spacing, on a separate sheet.

#### **Brief reports**

Brief reports containing concise reports on original work will be considered for publication. Case reports which are

relevant for understanding the pathophysiology or clinical presentation of disease may also be accepted under this heading. Articles published in this section should be no longer than 1000 words, and be supplied with a summary of about 60 words, preferably no more than two figures and/or tables, and no more than 15 references.

#### **Letters to the editor**

Letters to the editor referring to articles previously published in the journal will be considered by the editors; letters should be no more than 500 words and sent both on disk or e-mail and in hard copy.

#### **Submission**

Manuscripts should be sent to the Editor in chief, Prof. J.W.M. van der Meer, University Medical Centre St Radboud, Department of General Internal Medicine, PO Box 9101, 6500 HB Nijmegen, the Netherlands, tel.: +31 (0)24-361 04 59, e-mail: g.derksen@aig.umcn.nl. They should be submitted in four complete copies, which include four sets of the figures; authors should retain one copy of the manuscript. Rejected manuscripts will not be returned to the author unless specially requested at the time of submission.

#### **Reviewing process**

After external and editorial review of the manuscript, the authors will be informed about acceptance, rejections or revision. Unless stated otherwise in our letter, we require revision within three months.

#### **Acceptance**

After acceptance we prefer electronic submission of text and figures, either by e-mail to g.derksen@aig.azn.nl or on floppy disk. A disk plus two final and exactly matching printed versions should be submitted together. It is important that the file saved is in the native format of 'Word' or any other computer programme used. Label the disk with the name of computer programme used, your name, and the name of the file on the disk.

#### **Proofs**

Proofs will be sent to the authors to be carefully checked for printer's errors. Changes or additions to the edited manuscript cannot be allowed at this stage. Corrected proofs should be returned to the publisher within two days of receipt.

#### **Offprints**

These are not available. The first author receives two sample copies of the journal with the published article.

#### **Books for reviewing**

Books, which are to be considered for review, should be sent to the Editor in chief.