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EDITORIAL

Vascular prevention and dementia

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The prevalence of dementia, including Alzheimer's disease, is expected to increase several-fold in the coming decades to an expected global prevalence of more than 100 million by 2050. This anticipated rise in prevalence can largely be attributed to increasing longevity and ageing of the baby boomer generation. The incidence of all-cause dementia nearly doubles every five years of age.

Given the expected dramatic increase in the incidence and prevalence of dementia, the identification of successful prevention and treatment strategies is critical. Current pharmaceutical options only modestly improve symptoms and cannot cure or prevent dementia. As a result, prevention of dementia through risk factor identification and modification is of the utmost importance until disease-modifying agents prove efficacious.

Although Alzheimer's disease and vascular dementia have traditionally been viewed as distinct disorders, it is now generally agreed that the two rarely occur in isolation. Both types of dementia share risk factors and histopathological features with atherosclerosis.[1] In addition, the presence and severity of cerebrovascular disease parallels the presence and severity of Alzheimer's disease. Thus, it is intuitively tempting to speculate that modification of vascular risk might reduce the risk of dementia.

In this issue, Richard *et al.*[2] discuss the current knowledge of the relation between vascular risk factors and dementia, and address the effect of treatment of vascular risk factors on incident dementia. Over the last decades epidemiological evidence has accumulated that increased vascular risk, especially during midlife, increases the risk of incident dementia. However, as the authors appropriately discuss, results of randomised clinical trials aiming at treatment of a single vascular risk factor in preventing cognitive decline or dementia are inconsistent. This lack of convincing trial results is only partly due to the relative lack of studies. Rather, it is the design of most cardiovascular studies that precludes firm conclusions on dementia prevention. Firstly, a long follow-up may be needed to assess the effects on dementia, which is both by nature and by definition a slowly evolving disease. In addition, a secure diagnosis of dementia is not as easy as diagnosing for example a heart attack, and usually requires cognitive decline of some magnitude before satisfying diagnostic criteria. Consequentially, a problem in the design of cardiovascular dementia prevention studies is that effects of risk modification on cardiovascular endpoints will often precede effects on clearly identifiable dementia. Any study thus runs the risk of early termination by data safety monitoring boards before the effect on dementia incidence can be firmly established. In terms of dementia prevention, it is thus possible that we will remain dependent on indirect evidence from suboptimally designed trials. Hopefully, ongoing studies particularly aimed at dementia prevention will provide a further basis for clinical recommendations.

Another important issue is cardiovascular risk modification in those already affected by dementia or earlier degrees of cognitive impairment. Conceivably, this may not be an issue for lipid lowering or platelet inhibition. For blood pressure lowering, however, the dilemma is complex.[3] In elderly individuals, in particular those with cerebrovascular pathology, cerebral autoregulation may be impaired, leaving vital brain tissue unprotected against the potentially harmful effect of lower perfusion pressure.[4] Low or even normal systemic blood pressure levels may be inadequate for optimal cerebral perfusion, causing a decline in brain function. Since many elderly subjects with dementia suffer from cerebrovascular pathology, higher blood pressure levels may be required in these patients to prevent further cognitive decline.[5] Preliminary evidence indeed suggests that lower blood pressure in combination with cerebrovascular pathology may have detrimental effects on the brain and consequently may aggravate existing cognitive impairment.[6,7] However, the level of blood pressure associated with such detrimental effects, as well as the determinants of inter-individual differences in this level, are unknown.

Meanwhile, the clinician is faced with the dilemma how to treat older patients with increased vascular risk. We believe it is prudent to start primary cardiovascular

prevention based on the conventional criteria, which include global cardiovascular event risk, comorbidity, and patient preference. Such a policy is supported by strong epidemiological evidence, but requires good clinical judgment just the same. In our view, fear of dementia, however justified, should not guide pharmacological cardiovascular risk factor management. In those who already have dementia or milder forms of cognitive impairment, clinicians should be careful. Antihypertensive therapy, if it is decided to be appropriate, should include proper follow-up with attention given to the hazard of cognitive deterioration, particularly if blood pressure drops significantly or reaches levels considered 'normal' by usual standards.

Many questions remain regarding the use of cardiovascular prevention regimens with advancing age especially in the older patient with complex diseases. Competing risks, comorbid conditions, polypharmacy and drug interactions, tolerability, and safety may alter the benefit/harm balance in older patients. In addition, applying these prevention regimens to older patients with multiple chronic diseases, a group that includes half of the population older than 65 years, may present the patient with an unsustainable treatment burden, making independent self-management and adherence difficult. Applying pharmacological cardiovascular prevention regimens in patients late in life should therefore build on principles of appropriate prescribing and includes a consideration of remaining life expectancy, goals of care, and potential benefits of medication.[8]

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REVIEW

Vascular risk factors and dementia – towards prevention strategies

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ABSTRACT

Several cohort studies have shown that vascular risk factors including hypertension, hypercholesterolaemia, diabetes mellitus, smoking, obesity and lack of physical exercise in midlife and to a lesser extent in late life, are associated with an increased risk of dementia.

The results from randomised controlled clinical trials on treatment of these risk factors are not conclusive for the effect on cognitive decline and dementia. Studies investigating the effect of a multi-component intervention aimed at vascular risk factors to prevent or slow down cognitive decline and dementia will hopefully give the answer as to whether such an intervention is efficacious. This requires large clinical trials in an elderly population with long follow-up and several competing risks, making it difficult from an organisational and methodological point of view. Major challenges for future studies are to select the optimal population, set the optimal treatment targets and select clinically relevant outcome parameters.

KEYWORDS

Dementia, cognitive decline, vascular risk factors, prevention, trial design

INTRODUCTION

Age is the strongest risk factor for dementia and with the increasing life expectancy the number of patients living with dementia worldwide is estimated to rise from 24 million currently to over 80 million by the year 2040.[1] The most common cause of dementia is Alzheimer's disease followed by vascular dementia and dementia with Lewy bodies.[2] These nosological entities in themselves are probably relatively rare and the majority of patients with dementia suffer from 'mixed dementia' characterised by multiple cerebral pathologies with prominent vascular involvement. This is especially the case in older dementia patients (over 80 years), which is the age group responsible for the increase in prevalence in the near future.[3,4] In several cohorts the occurrence of multiple cerebral pathologies has been confirmed.[5-8] Silent cerebral infarcts and white matter lesions increase the risk of future dementia and cerebrovascular lesions contribute to dementia severity in patients with Alzheimer's disease.[9-11] The attributable risk of vascular lesions for the occurrence of dementia in a model including age, Alzheimer changes, vascular lesions, Lewy bodies and atrophy is as high as 21%, which is higher than the attributable risk of cortical amyloid-beta plaques and neurofibrillary tangles together,[6] confirming the importance of the vascular component of dementia.

Currently no therapeutic options are available to target the neurodegenerative component of dementia, but the vascular component might offer opportunities for treatment and prevention strategies.[12,13] In this review the current knowledge of the relation between vascular risk factors and dementia, the effect of treatment aimed at vascular risk factors on incident dementia, and current ongoing randomised controlled trials (RCTs) evaluating the effect of treatment aimed at vascular risk factors are discussed. Finally, the design of future dementia prevention trials aiming at vascular risk modification to prevent dementia is discussed.

EPIDEMIOLOGICAL EVIDENCE

Results from several large prospective cohort studies have shown that vascular risk factors including hypertension, hypercholesterolaemia, diabetes mellitus (DM), obesity

and lack of physical exercise are all associated with an increased risk of dementia. Several systematic reviews on these associations have been published in recent years.[14-17] Most prospective cohort studies report on associations between midlife vascular risk factors and late life dementia and the associations are strongest for risk factors present in midlife. The associations in late life are less robust and some of these factors in late life, including hypertension and overweight, might even be associated with a decreased risk of dementia.[16,18,19]

Treatment aimed at several vascular risk factors has been associated with a decreased risk of incident dementia later in life from several of the same cohort studies. However, selection of study participants, lack of randomisation and confounding by indication are important sources of bias limiting the interpretation of treatment effects in observational studies.[20]

The relation between dementia and most relevant vascular risk factors is briefly discussed below.

Hypertension

On the subject of vascular risk factors and dementia most studies have focused on hypertension and the risk of dementia. Several cohort studies with long follow-up have consistently associated hypertension in middle-aged subjects with an increased risk of dementia and cognitive decline later in life.[21-24] This association is less clear for hypertension in late life and the relation appears to be age-dependent.[25,26] In fact, some longitudinal studies have shown that *low* blood pressure in later life (above 75 years of age) is associated with an increased risk of future dementia.[27-29] In addition, several cross-sectional studies in late life have shown an association between high blood pressure and better cognitive functioning and between low blood pressure and prevalent dementia.[30,31] Medical treatment of hypertension has been associated with a decreased incidence of dementia in several longitudinal studies, but results are difficult to interpret due to the mentioned sources of bias inherent to such analyses of associations.[32-34]

Cholesterol

Hypercholesterolaemia in midlife has consistently been associated with an increased risk of future dementia.[35,36] Hypercholesterolaemia in late life, however, has been associated with a decreased risk of dementia in some longitudinal studies.[19,37] No association between statin use and incident dementia has been observed in several large prospective cohort studies. [38-41]

Diabetes mellitus

Reports about the association between DM and incident dementia are fairly consistent.[17] DM is the only vascular risk factor which is associated with an increased dementia risk independent of age. This was shown in several longitudinal cohort studies on both midlife DM[36,42] and late life DM[43-46] and the risk of incident dementia. In addition, the occurrence of severe hypoglycaemia in patients with type 2 DM has been associated with an increased risk of future dementia; this risk increases with the number of hypoglycaemic episodes.[47]

Obesity and physical exercise

Midlife obesity has been associated with an increased risk of future dementia in several cohort studies with a very long follow-up.[48,49] As with hypertension, this relationship seems to be modified by age, as in late life a higher BMI is associated with a decreased risk of dementia, whereas a low BMI is associated with an increased risk of dementia.[18,50] Physical activity both in midlife and in late life has repeatedly been reported to be associated with a decreased risk of dementia.[51-53]

The presence of several risk factors in one subject probably has an additive effect and the constellation of vascular risk factors defined as the 'metabolic syndrome' has been associated to an increased dementia risk as well.[54,55]

Interpretation and comparison of the results from different prospective cohort studies is difficult for several reasons. In the first place the operationalisation of risk factors differs across the studies. There is no uniform definition of hypertension, hypercholesterolaemia, obesity or lack of physical exercise and different values or algorithms have been used in different studies. In addition, different outcome parameters have been used. Some studies used Alzheimer's disease as an outcome, some studies vascular dementia and some studies dementia in general. Considering the increasing awareness that the strict division between Alzheimer's disease and vascular dementia is no longer tenable and the probably high percentage of cases suffering from mixed dementia in most of the studies mentioned, all incident dementia outcomes are included in this review.

TREATMENT AND PREVENTION OF DEMENTIA

Currently available medical treatment

The currently available medical treatment of dementia is only symptomatic and limited to the use of acetylcholinesterase inhibitors (AChEI) in mild to moderate Alzheimer's disease and memantine, an NMDA receptor antagonist, in moderate to severe dementia. Gradually the indication for these drugs is being broadened to include vascular dementia and dementia with Lewy bodies (DLB). Most benefit from AChEI can probably be expected in DLB patients, especially when certain symptoms including hallucinations and attention deficit are prominent.[56] In spite of major efforts, including randomised controlled trials, to develop drugs that interfere with amyloid

metabolism, the neurodegenerative component of the disease is currently not amenable to treatment and no disease-modifying drugs are as yet available.[57,58]

Interventions aimed at vascular risk factors

While the neurodegenerative component of dementia is not yet amenable to treatment, the vascular component might offer a potential target for treatment or even prevention, as was already recognised almost 20 years ago.[12,13,59]

Most available evidence from randomised controlled clinical trials comes from cardiovascular studies using stroke, coronary heart disease or mortality as primary outcome measures, and assessing cognitive function or incident dementia as a secondary endpoint. This was recently systematically reviewed, and will be briefly discussed here.[20]

The strongest evidence for an effect on incident dementia is available for treatment of hypertension. Nevertheless, results from RCTs with different primary endpoints and different populations at baseline are inconsistent on the effect of antihypertensive treatment on cognitive decline or dementia.[60-65] So far only the Syst-Eur study has reported on a convincing effect of blood pressure (BP)-lowering therapy in hypertensive subjects over 60 years of age.[61] Treatment resulted in a 55% absolute risk reduction of incident dementia in the treatment arm of the study with an average follow-up of 3.9 years. A recent study in subjects over 80 years of age had to be terminated prematurely because of a favourable effect on several cardiovascular endpoints, and the follow-up of 2.2 years was too short to find an effect on cognition.[63] A meta-analysis including four RCTs investigating BP-lowering therapy revealed a hazard ratio of 0.87 (95% CI 0.76 to 1.00) for incident dementia.[63] Only two RCTs evaluated the effect of cholesterol-lowering therapy with a statin on cognition.[66,67] No effect on cognition was reported, and a Cochrane review confirmed that currently there is no evidence for an effect of statin treatment on cognitive decline.[68]

In spite of the repeatedly reported association between obesity and cognitive decline and incident dementia, no RCTs on this subject using cognitive decline or incident dementia have been performed.

One RCT evaluating the effect of intensive glucose control versus standard glucose control evaluated cognitive decline and incident dementia in diabetic subjects with a history of micro- or macro-vascular disease or at least one other cardiovascular risk factor. Although a significant effect on glycated haemoglobin level was achieved, this did not result in an effect on cognitive decline or incident dementia during a median follow-up of five years.[69]

The effects of multi-component interventions aimed at vascular risk factors are largely unknown in elderly populations. One small RCT among 400 patients at high cardiovascular risk assessed the effect of optimising pharmacological and non-pharmacological treatment of vascular risk factors on cognitive decline, but no effect was observed in this study, which may have been underpowered.[70] One RCT evaluating the effect of an intervention aimed at vascular risk factors compared a multi-component intervention to regular care in early Alzheimer's patients with cerebrovascular lesions on MRI.[71] After two years of follow-up no effect on cognitive decline was found, but the progression of white matter lesions was slightly less in the intervention arm.[72] This study is not a dementia primary prevention study, but a secondary prevention study, considering the diagnosis of early dementia. Caution is warranted when interpreting the results of these RCTs, and results cannot easily be translated to the whole population of elderly subjects.

DESIGN OF DEMENTIA PREVENTION TRIALS

The optimal design for an RCT to investigate the effect of interventions aimed at vascular risk factors is dependent on many variables, and the best design is subject of debate.

Population under study

The first major question is which population should be studied. As mentioned above, most epidemiological data confirm the relationship between midlife risk factors and late-life dementia, but the associations with risk factors in late life are less robust. The incidence of dementia in midlife is too low to find an effect of an intervention, unless a very long follow-up (i.e. ten years or longer) or large sample size (i.e. 10,000 subjects or more) is achieved. The realisation of such a study would be very complicated from a methodological point of view. The incidence of dementia in the population under study needs to be sufficiently high to find an effect of the intervention within a reasonable duration of follow-up. If the population under study is too old, the incidence of dementia might be high, but attrition due to death of other causes will seriously influence the results and these competing risks complicate the interpretation of such a study.

Therefore it seems reasonable to search for a practicable compromise between the downsides of an intervention in subjects who are either too young or too old, and aim for a population somewhere between 65 and 75 years of age at baseline.

Selecting subjects at increased risk of dementia could result in higher incidence during follow-up and limit the number of subjects needed in an intervention trial. Subjects could be selected based on previously developed dementia risk scores which take vascular risk factors into account.[73,74] By using such an 'enrichment strategy' the participation of large groups of subjects at very low risk of the primary

outcome, whose chance of benefiting from the intervention is very low, can be avoided. Whether or not subjects with a low dementia risk score or vascular risk score actually benefit from such an intervention is, however, unknown. Subgroup analyses of currently ongoing trials might offer an opportunity to evaluate this in the near future.

Another enrichment strategy commonly proposed is enrolling subjects who already have slight cognitive deficits, but who do not fulfil criteria for dementia yet, so-called mild cognitive impairment (MCI). Since MCI is considered a pre-dementia stage, and on average about 10% of the patients progress to dementia every year, such a study should not be considered as primary prevention, but rather as secondary prevention of dementia. As such this strategy could be valuable in addressing the same general research question: can modification of vascular risk factors prevent cognitive decline or dementia?

Risk factors under study

It is important to realise that even a very modest effect of treatment aimed at a specific risk factor can have a major impact at population level if the risk factor is highly prevalent, the so called prevention paradox. Effects of antihypertensive therapy may serve as an example. Hypertension is highly prevalent among elderly non-demented subjects, as was shown in several population-based cohorts and the baseline data of one of the ongoing intervention trials.[75] If this risk factor can be modified in a large proportion of the subjects, the effect on the overall incidence of dementia can be substantial, even if individual effects are negligible. In this context it is important to know the population attributable risk (PAR) of each risk factor to determine the potential effect of an intervention aimed at the prevention of dementia. If the PAR is high, i.e. a large proportion of incident dementia cases can be attributed to the presence of hypertension, it is more likely that this intervention will be effective at population level. In the case of hypertension, the PAR has been estimated to be as high as 27%, making it a very suitable treatment target for the prevention of dementia.[76]

When assessing the effect of a primary prevention intervention, the intended intervention should be affordable, acceptable to the patient, easy to implement on a large scale, have few serious adverse events and should not impose too heavy a burden on the health care system as a whole. Large groups of subjects would be exposed to the intervention. With the predicted increase of life expectancy and the resulting increase of older subjects in Western societies this issue will become even more relevant.

Outcome measures

The optimal outcome measure for dementia prevention trials is subject of debate. In dementia research it is common to use deterioration on either a neuropsychological test battery or an extensive screening instrument as primary outcome measure. Small differences between groups can be detected this way, but clinical relevance of such differences is often unclear. Incident dementia in our opinion is a more clinically relevant outcome measure which is easy to interpret and with clear clinical relevance.

In addition to cognitive endpoints, other clinically relevant outcomes to be used are mortality, institutionalisation and disability or handicap. It is expected that interventions targeting vascular risk factors will sort an effect on vascular endpoints including stroke, myocardial infarction and peripheral vascular disease as well. Therefore outcome measures evaluating the effect on both cognitive decline and cardiovascular disease would be preferable. The Amsterdam Linear Disability Scale (ALDS) is a handicap scale which possesses these clinimetric properties; it assesses both basic and instrumental activities of daily living and is generic (i.e. not disease specific) and linear, as opposed to most handicap or disability scales which are disease-specific and ordinal.[77] Due to its test characteristics, both cognitive decline and handicap as a result of cardiovascular disease (e.g. stroke or myocardial infarction), will translate into deterioration on the ALDS.

ONGOING DEMENTIA PREVENTION INITIATIVES

Currently three RCTs investigating the effect of a multi-component intervention including treatment aimed at vascular risk factors are ongoing. All three studies aim at preventing cognitive decline or incident dementia, but the interventions and the selection of subjects are different.

The 'Prevention of Dementia by Intensive Vascular Care' trial (preDIVA) is a cluster-randomised trial among 3534 elderly non-demented subjects aged 70 to 78 years to evaluate the effect of a multi-component vascular intervention with a follow-up of six years.[75] In the intervention group subjects visit a practice nurse every four months, who assesses the presence of vascular risk factors including blood pressure, cholesterol level, diabetes mellitus, smoking, body mass index (BMI) and level of physical exercise.

Vascular risk factors are treated according to a standardised protocol in line with existing guidelines for cardiovascular risk management. Primary outcomes are incident dementia and disability as measured with the ALDS.[77] Secondary outcomes are all-cause mortality, cardiovascular disease (including stroke, myocardial infarction and peripheral vascular disease), death, cognitive decline and depression. The control group receives usual care.

The 'Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability' (FINGER) is a

randomised controlled trial among 1200 subjects aged 60 to 75 years with a high dementia risk score and with mild memory impairment evaluating the effect of a multi-domain intervention to delay cognitive impairment with a two-year follow-up with a planned extension.[78] In addition to management of vascular risk factors, the intervention comprises nutritional guidance, advice on physical activity and cognitive training. Primary outcomes are cognitive impairment, dementia and disability; secondary outcomes include depression, vascular disorders and quality of life. The control group attends regular health advice groups.

The Multidomain Alzheimer Prevention Trial (MAPT) is a randomised controlled clinical trial among 1200 'frail' elderly subjects over 70 years of age randomised to receive omega 3 capsules, a multi-domain intervention, both, or placebo with a three year follow-up.[79] The multi-domain intervention comprises cognitive training, physical training, nutritional counselling and preventive consultation including vascular risk factors assessment. The primary outcome is change in cognitive function as measured by a short memory test.

Considering the different populations under study and the different types of interventions, it is expected that the results from these three trials will result in valuable knowledge about effective intervention strategies and the type of population that will benefit most from it.

DISCUSSION

The mechanisms through which the vascular risk factors contribute to an increased dementia risk have not been fully elucidated yet. The multi-factorial aetiology of dementia in old age and the interplay between vascular changes and neurodegenerative changes in the brain prevents conclusions about the exact mechanistic processes underlying the clinical syndrome of cognitive decline. In addition to the contribution of (clinically silent) stroke to cognitive decline, blood-brain barrier (BBB) changes have been implicated to play an important role in the pathophysiology of the relationship between vascular risk factors and dementia and the interaction with neurodegenerative changes.[80,81]

In spite of the overwhelming evidence of associations between vascular risk factors and dementia and the reported associations between treatment of some vascular risk factors and dementia risk from prospective cohort studies, RCTs that confirm the exact effects of such interventions are lacking. Since treatment aimed at vascular risk factors to prevent coronary heart disease, stroke and peripheral vascular disease is undisputed, certainly as secondary prevention, this leads to the inevitable question whether new RCTs investigating the effect of intensive vascular care are ethical. In the first place it is not known whether such interventions can contribute to dementia prevention and therefore the research question is relevant. In the second place it is unknown whether there might be potential negative effects of such interventions in old age, such as potential side effects of a relatively low blood pressure and low cholesterol, illustrating the necessity of such studies. Both low BMI and decreasing blood pressure have been implicated as symptoms of early Alzheimer's disease, and the reported associations might therefore not represent a causal relationship, but a consequence of the disease. Finally, all subjects in the control arms of the ongoing intervention studies receive at least care as usual according to current guidelines, so no therapy with proven efficacy is withheld from any participant. This is a prerequisite for any future trial to be designed as well. In this way, future studies may either confirm or falsify the general claim that more intensive vascular care reduces the incidence of dementia.

The results of the observational studies and the limited evidence from RCTs on the effect of antihypertensive medication are encouraging. Due to the high incidence of dementia in the elderly population and the high prevalence of hypertension, a small treatment effect can result in a large effect at the population level. The treatment targets (e.g. BP level, cholesterol level) of vascular care in elderly populations are still to be determined, and might be different for primary and secondary prevention groups.

Results from RCTs as described above will hopefully answer the question whether a multi-component intervention aimed at vascular risk factors can lead to the prevention of dementia, and which factor of such an intervention has most effect.

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REVIEW

Gene expression profiles of the oestrogen receptor in breast cancer

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ABSTRACT

Breast cancer is a heterogeneous disease and existing clinicopathological classifications do not fully capture the diversity in clinical disease course. Since the oestrogen receptor (ER) plays a central role in the crosstalk between different signalling pathways in breast cancer, the expression of this receptor is important for the behaviour of breast cancer cells and is reflected in gene expression patterns of breast tumours. High throughput analysis of gene expression of breast cancer has increased the insights into ER signalling, including its relation with disease outcome and therapy response. Expression of ER and its numerous downstream targets are driving patterns of gene expression and dominate unsupervised analyses in the breast cancer specimens studied to date, regardless of microarray platform or statistical approach. This paper reviews gene expression studies either attempting to unravel the functional effect of ER or describing the gene expression profiles driven by ER in breast tumours. In addition, the development of molecular signatures predicting response to endocrine treatment will be discussed.

KEYWORDS

Breast cancer, oestrogen receptor, endocrine treatment, gene expression profiling, micro-array

INTRODUCTION

Hormones have been associated with breast cancer since Beatson showed that oophorectomy resulted in tumour regression in 1896.[1] Oestrogens play a predominant role in the growth of breast cancer. The identification of the oestrogen receptor (ER) by Jensen in 1960 shifted the paradigm of steroid hormone action from an enzymatic one to a model whereby steroids interact with a receptor to elicit defined biological responses.[2,3] Oestrogens bind to the ER, leading to dimerisation, conformational change and binding to oestrogen response elements (EREs) upstream of oestrogen-responsive genes including those responsible for proliferation of the tumour cells. Approximately 75% of breast tumours express the ER.[4] Patients with an ER-positive breast tumour and who have a likelihood to develop a relapse of disease will receive adjuvant endocrine treatment. The use of endocrine manipulation covers the spectrum of metastatic disease, adjuvant and neo-adjuvant therapy. Adjuvant endocrine therapy is a major contributor to the substantial decline in breast cancer mortality.

ENDOCRINE RESISTANCE

Tamoxifen has been the mainstay of treatment for ER-positive breast cancer for more than 30 years.[5,6] Tamoxifen is a selective ER modulator (SERM) that competes with oestrogens for ER binding. An alternative strategy includes the inhibition of aromatase using aromatase inhibitors (AIs) that result in a block in the production of oestrogen.[7,8] In addition, selective ER down-regulators (SERDs), such as fulvestrant, are used in the treatment of metastatic breast cancer patients.[9]

In patients with operable ER-positive tumours, tamoxifen reduces the risk of recurrence by 41% on average.[10] With that, tamoxifen has changed the clinical management of breast cancer dramatically. However, approximately 30% of the ER-positive breast cancer patients will develop a recurrence of their disease despite five years of adjuvant tamoxifen treatment.[10] Moreover, in the metastatic disease setting, half of the ER-positive breast cancer patients will not benefit from tamoxifen.[11] Endocrine resistance

is a major problem in the clinical management of breast cancer. *Figure 1* illustrates the impact of endocrine resistance in the Netherlands.

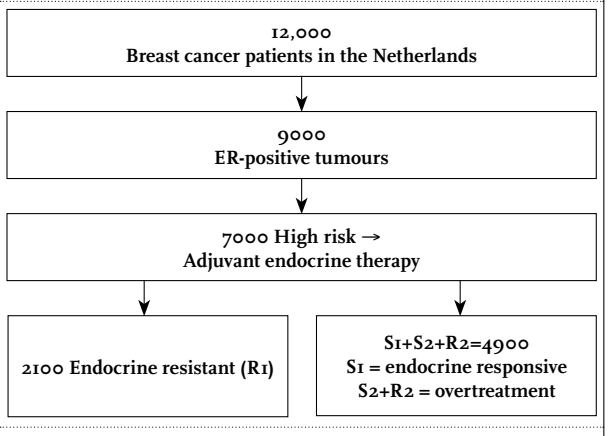
Several mechanisms may contribute to tamoxifen resistance.[8,12-15] First, genetic variations in genes coding for enzymes (cytochrome p450, CYP) that convert tamoxifen to its active metabolites can influence the effectiveness of tamoxifen. Patients with variant CYP2D6 alleles may have a higher risk of recurrence after adjuvant tamoxifen.[16,17] Secondly, a proportion of ER-positive tumours are intrinsically resistant to tamoxifen, for example due to high levels of growth factor receptors (GFRs) that may result in activation of signalling pathways in the tumour cells.[18-20] Mitogen-activated protein kinases (MAPK), protein kinase A (PKA) and p21-activated kinase 1 (PAK1) are well-characterised components of pathways that may be involved in tamoxifen resistance.[21-25] A crosstalk between the GFRs and ER has been described.[26] In addition, epigenetic and post-translational regulation of the ER may result in tamoxifen insensitivity via enhanced transcriptional activity.[27]

Thirdly, tumour growth can be stimulated by tamoxifen resulting in acquired resistance. Patients will eventually relapse despite an initial response.

Since AIs were introduced recently, it is largely unknown whether the resistance mechanisms known to be involved in tamoxifen resistance contribute to resistance to an AI as well.[8] In postmenopausal women, the only source of oestradiol (E2) is from the aromatisation of adrenal androgens. While peripheral conversion in adipose tissue contributes to detectable levels of circulating E2, local production via tumoural aromatase action results in 10- to 20-fold higher E2 concentrations in the tumour than in the serum.[28] Variations in tumour aromatase levels could therefore contribute to responsiveness to AIs. A small study suggested that the level of intratumoural aromatase activity could predict the response to the first-line AI, aminoglutethimide.[29]

Premenopausal patients who responded and relapsed after E2 withdrawal by ovarian suppression could respond to further suppression of E2 by the addition of an AI at the time of relapse.[30] This suggested that the initial resistance was due to the acquisition of an increased sensitivity to residual postmenopausal levels of E2, which could then be overcome by further reducing circulating levels of E2. Preclinical data from several laboratories support this hypersensitivity concept as a means of escape from E2 deprivation.[31,32] In part, this is caused by an adaptive increase in ER expression and function.

Figure 1. *Breast cancer patients in the Netherlands*



Proportion treated with adjuvant endocrine therapy and proportion of endocrine resistant ER-positive tumours. Numbers reflect estimates of patients per year.[4,10,117] R1 = Not cured by local treatment/Endocrine resistant (Recurrence); R2 = Cured by local treatment/Endocrine resistant (Overtreatment); S1 = Not cured by local treatment/Endocrine sensitive (Saved); S2=Cured by local treatment/Endocrine sensitive (Overtreatment).

CURRENT CLINICAL PICTURE

For premenopausal patients, tamoxifen is considered the standard adjuvant endocrine treatment. In addition, suppression of the ovarian function by means of oophorectomy or a luteinising hormone-releasing hormone (LHRH) analogue is effective, especially in women younger than 40 years.[33,34]

With regard to postmenopausal patients, recent randomised controlled trials showed that AIs are superior to tamoxifen in terms of disease-free survival (4.8% absolute difference at nine years), but failed to demonstrate a significant difference in overall survival.[35,36] Sequential tamoxifen for two or three years followed by an AI for two to three years resulted in a reduction in the risk of breast cancer recurrence and death.[37,38] The best sequence and timing for tamoxifen and AIs is still unclear.[39,40]

Almost all trials reported an increased risk of arthralgia and myalgia, as well as osteoporosis and subsequent fractures, when AIs were compared with tamoxifen.[35,36] In contrast, tamoxifen has been associated with an increased risk of endometrial cancer, especially in postmenopausal women.[37] The cardiovascular risk profile also differs between AIs and tamoxifen: thromboembolic events are more frequently seen with tamoxifen, and cardiac events are more common with AIs. Vasomotor and certain gynaecological symptoms are more frequent with tamoxifen than with AIs but quality of life, on average, appears to be similar.[35-38]

Clinicians decide whether a patient is likely to respond to endocrine treatment based on the presence of the ER and/or the progesterone receptor (PR) expression.[41] Although the predictive capacity of ER is indisputable, data on the predictive value of PR are conflicting and it could well be that PR is a prognostic as well as a predictive marker,

Box 1. *Definitions prognosis and prediction*

Prognostic marker
Any measurement available at time of diagnosis that is associated with disease-free or overall survival in the absence of adjuvant systemic therapy.

Predictive marker
Any measurement associated with response or lack of a response to a particular systemic therapy.

From: Disease of the Breast, edited by Jay Harris, © 2000.

just as the ER (definitions in *Box 1*).[10,42-44] Up till now meta-analyses have used cut-offs for ER and PR at 1% or 10% positive tumour cells. So, meta-analyses of endocrine treatment benefit have not provided the unambiguous cut-off for the percentages of ER and PR with regard to the predictive value of these markers, which is likely in a much higher range, between 50 to 100% positive tumour cells.[43,45]

Despite years of research on endocrine resistance, there are no other molecular markers, besides ER and PR, used in daily clinical practice to predict the likelihood of response to tamoxifen.[46] At present, no markers can be used to predict differential benefit from tamoxifen as opposed to AIs.

GENE EXPRESSION PROFILING TECHNOLOGY

Gene expression is a general term used to describe the transcription of information encoded within the DNA into messenger RNA (mRNA). It is assumed that for many genes there is a linear relation between the number of mRNA transcripts and functional proteins expressed in a cell. Gene expression profiling, in turn, is defined as the simultaneous measurement of the expression of a large number of genes. With gene expression profiling it has been possible to group gene transcripts of human tumours to create 'molecular signatures' that give more insight into the biology of cancer and consequently may predict clinical outcome. *Table 1* summarises the current applications of gene expression profiling. There are three techniques commonly used for gene expression profiling in clinical specimens.[47] These include gene expression profiling using two different microarray platforms (complementary DNA (cDNA) and oligonucleotide arrays) and multiplex quantitative reverse transcriptase polymerase chain reactions (qRT-PCR). On the cDNA microarray, double-stranded PCR products amplified from expressed sequences tag (EST) clones (length 300 to 1000 nucleotides) are spotted. Several ten thousands of different cDNA clones can be spotted onto the surface of a glass slide to produce a high-density cDNA array. The affixed DNA segments are known as probes. The drawback of studying gene expression using cDNA arrays is the frequent cross-hybridisation amongst homologous genes, alternative splice variants and antisense RNA. These problems have been overcome by oligonucleotide arrays, which use shorter probes of uniform length, usually 20 to 80 nucleotides. By constructing oligonucleotide arrays, complete control of the sequence is guaranteed; several different probes per gene can be spotted and many control spots provide information on contamination and hybridisation kinetics. Currently, there are four approaches for the production of oligonucleotide arrays. First, the oligonucleotides can be synthesised, purified and then printed by a robot or inkjet process onto glass slides (Agilent). Second, microarrays can be produced by *in situ* synthesis of oligonucleotides directly onto a solid surface using photolithographic technology (Affymetrix). Recently, a third technology was introduced based on bead-based arrays where the oligonucleotides are attached to microbeads that are then put onto microarrays (Illumina).[48] Finally, the fourth technique to measure gene expression in a high throughput fashion is real-time qRT-PCR, which is based on the quantification of mRNA after each round of amplification by PCR using a fluorescent reporter.[49] Current qRT-PCR assays can determine the expression of up to a few hundred genes simultaneously and may have an increased sensitivity compared with the array-based technology.

Table 1. *Gene expression profiling technologies*

	cDNA arrays	**Oligoarrays**			**Multiplex RT-PCR**
Manufacture	Clontech, academic microarray facilities	Agilent, academic microarray facilities	Affymetrix	Illumina	Taqman, Molecular Beacons, Scorpions
Probe	300-1000 nucleotide cDNA clone	60 mer oligonucleotides	20 mer oligonucleotides	50 mer oligonucleotides	~20b PCR primers
Probes per array	44,000	44,000	500,000	48,000	Up to 400
General information	Use is decreasing	Dual-channel system: expression values relative to reference	Single channel system: absolute expression values	Dual-channel system: oligos attached to beads	Most sensitive detection of mRNA levels

For the analysis and interpretation of microarray data, a range of computational tools is available. The two basic approaches are unsupervised hierarchical clustering analysis and supervised analysis.[50,51] Unsupervised hierarchical clustering analysis (or hierarchical clustering) orders both samples and genes on the basis of their similarity of gene expression. The object is to group together samples or genes that are 'close' to one another. A key component of the analysis is repeated calculation of distance measures between samples, and between clusters once samples begin to be grouped into clusters. The outcome is represented graphically as a dendogram. For example, gene expression studies using breast tumours are dominated by two main clusters: ER-negative *vs* ER-positive tumours.[52,53] In contrast, supervised analysis identifies gene expression patterns that discriminate samples on the basis of predefined clinical information such as tumour grade, disease outcome or endocrine responsiveness. Statistical analysis of expression data is complex and prone to false discoveries, e.g., identifying genes of interest just by chance. Therefore, it is crucial to validate molecular signatures in large independent series of patients before clinical application.

GENOME-WIDE ANALYSIS OF OESTROGEN RECEPTOR FUNCTION

Oestrogens are known to regulate the proliferation of breast cancer cells and to alter phenotypical properties. However, the mechanisms and pathways by which oestrogens regulate these events are only partially understood. With the sequencing of the human genome as well as the advent of microarray technology, it is now possible to investigate the complexities of ER-mediated gene transcription on a more global scale rather than studying one oestrogen-responsive target at a time. Many gene expression profiling studies have been conducted identifying E2-responsive genes, the number ranged from 100 to 1000.[54-59] The large quantitative and qualitative differences are most probably due to the use of different cell lines, treatments, microarray platforms and statistics. Collectively, expression profiles show that E2 influences a large variety of targets including genes involved in cell cycle and proliferation, apoptosis and transcriptional regulation.

Using gene expression profiling, researchers identified patterns of genes that are either stimulated or inhibited by E2 in ER-positive MCF-7 human breast cancer cells.[56,60,61] In addition they show that numerous cell cycle-associated genes as well as expression of novel transcription factors, receptors and signalling pathways are modulated by E2, many of which could play roles in mediating the effects of E2 on breast cancer proliferation. Subsequently, to better understand the actions of endocrine treatment, microarray analysis was performed after exposure of breast cancer cells to different ER-targeted drugs.[62,63] The gene expression changes induced as a response to SERMs such as tamoxifen and raloxifene or the anti-oestrogen fulvestrant indicated the agonistic and/or antagonistic actions on a large set of E2-regulated genes. Although the regulation of the majority of E2-regulated genes is either partially or fully reversed by SERMs and fulvestrant, differences can be observed among these ligands in their balance of agonistic, partial antagonistic or fully antagonistic activities on E2-regulated genes. In addition, in 2006 Oh and colleagues used this strategy to classify ER or PR-positive breast carcinomas, applying supervised analysis (significant analysis of microarray data 'SAM', software for expression data mining) on gene expression data of ER-positive MCF-7 cells treated with E2.[64,65] Using this approach, they identified 822 genes that were shown to be E2 regulated. These genes were used to develop an outcome predictor, which was then validated on independently published breast cancer datasets. Also, Musgrove *et al*. used their E2-induced gene signatures to predict survival in tamoxifen-treated patients.[60]

Translational research performed at the Netherlands Cancer Institute, the Netherlands, showed that combining *in vitro* experiments with gene expression analyses of clinical breast cancer samples can improve the understanding of ER function in cancer patients. Using fluorescence resonance energy transfer (FRET) that detects changes in the conformation of ER, the efficacy of anti-oestrogens to inactivate ER was studied.[24] Phosphorylation of serine 305 in the hinge region of ER by PKA induced resistance to tamoxifen. In clinical samples, the downregulation of a negative regulator of PKA, PKA-RIα, was associated with tamoxifen resistance. Activation of PKA by downregulation of PKA-RIα converted tamoxifen from an ER inhibitor into a growth stimulator. To further test whether ERαS305-P is indeed associated with PKA in human breast tumours, Michalides and colleagues evaluated gene expression of tumours known to have a phosphorylated ERα at serine 305. Nineteen pathways were differentially expressed in ERαS305-P-positive tumours and these pathways were enriched for pathways that include one or more PKA subunits.[25]

Whereas oestrogens exert their effects by binding to nuclear ER and directly altering transcription, they can also initiate extranuclear signalling through activation of kinase cascades. Madak *et al*. investigated the impact of E2-mediated extranuclear-initiated pathways on global gene expression.[66] Their findings document that E2 action initiated outside the nucleus stimulates the transcription and expression of a significant (~25%) portion of the total number of E2-regulated genes.

ER-mediated transcription has been intensively studied on a small number of endogenous target promoters.[67,68] Recently, ER-binding sites were mapped in a less-biased way that did not depend on pre-existing concepts of classic promoter domains and subsequently several new features of ER-mediated transcription were identified, such as the facilitation of ER binding to chromatin leading to gene transcription.[69] A number of proteins have been identified as ER co-factors using chromatin immunoprecipitation (ChIP), which has revolutionised our understanding of ER action.[70] Unbiased ChIP-microarray (ChIP-chip) work identified a total of 3665 ER binding sites throughout the entire genome.[69,71] A similar genome-wide approach mapped 1234 ER binding sites across the genome.[72] Combining this unique resource with gene expression data from breast cancer patients, it correctly predicted that the genes co-expressed with the ER and thereby identified important and previously unexplored regions of the genome that could be the critical regulators of the oestrogen dependence of breast cancer.

GENE EXPRESSION PROFILES DRIVEN BY OESTROGEN RECEPTOR

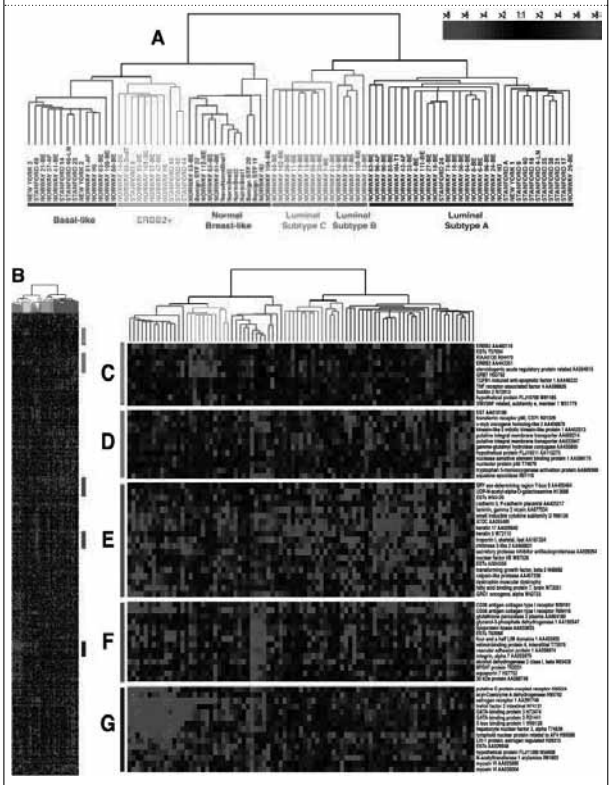
The first large-scale study of gene expression profiling in breast cancer was performed by Perou and colleagues who showed that based on overall gene expression profiles, breast carcinomas can be subdivided into five molecular subtypes (*figure 2*).[52] Three biologically distinct subgroups of ER-negative breast tumours have been identified: the 'basal-like' group, which expresses cytokeratin-5 and cytokeratin-17; the 'HER2-positive' group, expressing several genes located in the human epidermal growth factor receptor 2 (HER2) amplicon including HER2 and the gene encoding for growth factor receptor-bound protein 7 (GRB7); and the 'normal-breast-like' group, which expresses genes usually expressed in normal breast. The ER-positive tumours that were originally found to be a single group have in subsequent studies been separated into at least two distinct groups: the 'luminal A' subtype, which expresses high levels of cytokeratin-8 and cytokeratin-18 and other breast luminal genes, and the 'luminal B' subtype, expressing low levels of these genes.[73] Importantly, these five subtypes also represent clinically distinct subgroups of patients. For example, the ER-negative 'basal-like' and 'HER2-positive' subtypes are associated with a shorter overall and disease-free survival, whereas the ER-positive 'luminal A' tumours have the best outcome. These findings have been confirmed in independent datasets.[74,75] It has to be realised that classifications generated by hierarchical clustering may be unstable. For example, adding more breast cancer samples resulted in a changed dendrogram, as demonstrated by the disappearance of the luminal C subtype.[74] Furthermore, it can be argued that these analyses do not provide more information than currently given by histological grade and immunohistochemistry (IHC) for ER and HER2 of the tumour. For example, recently Cheang *et al*. showed that expression of ER, PR, HER2 and Ki67 determined by IHC appear to distinguish luminal A from luminal B breast cancer subtypes.[76]

The gene expression grade index (GGI), which defines histological grade based on gene expression profiles, could also define two ER-positive molecular subgroups (high and low genomic grade).[77,78] Despite tracking a single biological pathway, these subgroups were highly concordant with the previously described luminal A and B classifications.

Subsequent studies confirmed that there are large-scale gene expression differences between ER-positive (most 'luminal-like') and ER-negative (most 'basal-like') cancers. *Table 2* summarises different studies describing the dominant gene expression pattern in breast carcinomas driven by ER. To study the characteristics of ER-positive and ER-negative breast tumours in more detail, Gruvberger and colleagues profiled a homogeneous group of lymph node-negative breast cancers.[79] They reported that ER-positive and ER-negative tumours display remarkably different molecular phenotypes. To gain insight into the genes of this dominant expression signature, Van 't Veer *et al*. associated gene expression data with ER expression as determined by IHC.[53] Out of 39 tumours stained negative for ER by IHC, 34 clustered together. By this unsupervised approach, known ER target genes formed a cluster with the ER gene (ESR1). Supervised classification showed that 550 genes optimally reported the dominant pattern associated with ER status; reporter genes included cytokeratin-18, bcl-2, HER3 and HER4. Twenty-one out of the 50 ER reporter genes as determined by Gruvberger *et al*. were also present in the 550 gene list.[79]

Since the introduction of high throughput analysis of gene expression, several molecular signatures predicting prognosis in breast cancer patients have been developed.[80-83] All classifiers have been developed using different microarray platforms and approaches to select genes. Consequently, a direct comparison between the various gene lists generated is difficult. However, these different gene sets show significant agreement in the outcome predictions for individual patients and are probably tracking a common set of biological phenotypes.[75] In addition to the degree of proliferation and histological grading, information on ER signalling is present in all prognostic signatures. Wang and colleagues included this information in the development of their prognostic test.[83] Tumours used for their discovery study were allocated to one of two subgroups stratified by ER status. Markers selected from each subgroup (60 genes for ER-positive tumours and 16 for ER-negative tumours) were combined to form a single signature to predict

Figure 2. *Molecular subtypes of breast cancer*



Gene expression patterns of 85 experimental samples representing 78 carcinomas, three benign tumours and four normal tissues analysed by hierarchical clustering of cDNA clones. a Tumour specimens were divided into five (or six) subtypes based on differences in gene expression: luminal A, luminal B, luminal C, normal breast-like, basal-like and HER2+. b Full cluster diagram scaled down, bars on the right represent the inserts present in c–g. c HER2 amplicon. d Unknown cluster. e Basal epithelial cell-enriched cluster. f Normal breast-like cluster. g Luminal epithelial gene cluster containing ER.

tumour metastasis in a subsequent independent validation consisting of both ER-positive and ER-negative tumours. This result supports the idea that the extent of heterogeneity and the underlying mechanisms for disease progression could differ for the two ER-based subgroups of breast cancer patients. In addition, Dai *et al*. showed within a subset of young patients (<55 years) characterised by relatively high ER expression for their age (i.e., the ER/age high group) that the occurrence of metastases is strongly predicted by a homogeneous gene expression pattern almost entirely consisting of cell cycle genes.[84] By combining information on expression of ER with clinical variables such as age at diagnosis, a subgroup of patients was identified in which expression of proliferation-associated genes is a very strong predictor of outcome. All the above findings describe the marked influence of ER and its numerous targets on gene expression in breast cancer. Expression of ER drives patterns of gene expression and dominates unsupervised analyses in the breast cancer specimens studied to date, regardless of microarray platform or statistical approach. mRNA levels of ER (gene name ESR1) show strong correlation with protein expression.[52,53,85] Although there is preliminary evidence that quantitative mRNA levels of ESR1 and gene lists containing ER target genes could be predictive for outcome after endocrine treatment, clinical application of these tests requires further investigation.

In 2010, Dunbier and colleagues were the first to show a relationship between the expression of E2-dependent genes in ER-positive breast carcinoma and basal levels of E2 in plasma.[86] Their data challenge the view and strongly suggest that differences in plasma E2 levels between patients have a significant influence on the behaviour of breast tumours.

Table 2. *Gene expression profiling using human breast tumours to identify genes related to oestrogen receptor (ER)*

Microarray type	Samples	ER-related genes	Identified by	Prediction results	Reference
Oligonucleotide 25k, Agilent	98 Breast tumours	550	Unsupervised clustering	95% of ER status (IHC) predicted correctly (training only)	53
cDNA array 10k ESTs	38 Breast tumours	105	Supervised analysis	16/20 ER status (IHC) predicted (validation)	85
cDNA array 4.5k ESTs	38 Breast tumours	98	Median difference per gene in ER+ vs ER- tumours	46 genes more expressed in ER+, 52 genes more expressed in ER-	109
cDNA array 6,728 clones	58 Breast tumours	Top 100	Artificial neural networks models	100% of ER status (LBA) predicted correctly (validation)	79
cDNA array 8,102 clones	85 Breast tumours and normal tissue	427	Differentially expressed between subtypes of breast tumours	Discrimination of ER+ (luminal) vs ER- tumours (basal, HER2, normal-like subtypes)	52,73
Oligonucleotide Hu6800 Affymetrix	49 Breast tumours	Top 100	Correlation coefficient per gene with ER+ and ER- tumours	8/9 ER status (IHC) predicted correctly (validation)	110
Oligonucleotide 44k Agilent	65 Breast tumours and MCF7 cell line	822	Stimulation of MCF7 cells with oestradiol	Good discrimination of relapse-free survival	64

IHC = immunohistochemistry.

While most gene expression studies have focused on the presence or absence of ER, Creighton *et al*. examined RNA expression of ER-positive breast cancers in relation to the presence of PR.[87] ER+/PR- breast cancer defined by gene expression profiling (i.e., tumours neither truly ER+/PR+ nor ER-/PR- but sharing expression patterns with both) tended to have a poor outcome and this was not observed when using the IHC assays to determine ER and PR status. This shows that gene expression profiles may provide a clinically relevant tool to assess PR levels for diagnostic or therapeutic purposes.

MOLECULAR SIGNATURES PREDICTING RESPONSE TO ENDOCRINE TREATMENT

Adjuvant tamoxifen treatment reduces the breast cancer death rate by 31% in patients with ER-positive disease.[10] Gene expression studies have consistently confirmed the heterogeneity of ER-positive breast cancer and may provide insights into the mechanisms of response to endocrine treatment.

Current research efforts are focusing on the discovery of molecular signatures that might identify those patients most responsive to tamoxifen. The expression of ER does not guarantee functional activity and other molecular events unrelated to ER signalling can also influence sensitivity to endocrine treatment regimens. A multigene assay calculating a recurrence score (Oncotype DXTM) represents an important conceptual evolvement in the diagnosis of ER-positive breast cancer.[81] This RT-PCR-based assay was derived from 250 candidate genes selected by a literature search of the most important microarray studies in breast cancer. For the recurrence score, out of these 250, 16 genes were selected as well as five control genes. This assay measures ER mRNA levels in a quantitative and reproducible manner and also measures expression of several downstream ER-regulated genes (PR, bcl2 and SCUBE2) that probably contain information on the functionality of ER. The same assay also quantifies HER2 expression and proliferation-associated genes (Ki67, cyclin B1 and survivin). This RT-PCR-based test has been optimised for paraffin-embedded material and has been shown to accurately identify a group of patients with excellent prognosis when treated with adjuvant tamoxifen.[81,88] A disadvantage included the pre-selection of genes and a subsequent algorithm that may not encompass more than quantitative ER and PR levels, proliferation and HER2 expression, all currently easy to test and hence may provide no new biological insights into tamoxifen response. Another study, conducted in 60 ER-positive breast carcinomas treated with adjuvant tamoxifen, suggested the utility of a two-gene index of HOXB13 and IL17BR in identifying a subset of patients who are at risk for relapse of disease.[80] In an independent dataset of patients receiving tamoxifen, Reid *et al*. reported that the two-gene index failed to detect differences in outcome.[89] Taking into account that Fan and colleagues calculated the two-gene index using microarray data, again no association with outcome was seen.[75] However, in three other large cohorts the two-gene index showed a relation with tumour aggressiveness and response to first-line tamoxifen monotherapy for relapse of disease.[90-92] In studies of relatively small sample size, a model based on analysis of only two genes is much more likely to be sensitive to technical differences or patient selection. Further, in a substantial proportion of ER-positive tumours HOXB13 expression was below the detection level.[92] Rodriguez *et al*. showed by functional experiments that HOXB13 is an ER target gene and that its repression is mediated by DNA methylation in ER-positive tumours.[93] The observation by Wang *et al*. that HOXB13 and IL17BR expression strongly correlates with the expression of ER, PR and HER2 as determined by the routinely used IHC supports this regulation mechanism.[94] Independent studies will reveal whether HOXB13 and IL17BR might be useful predictive markers when used instead of IHC or add information to the standard markers.

In addition, using Affymetrix Gene Chip arrays, investigators from the Jules Bordet Institute, Belgium, selected 181 genes by Cox proportional regression analysis to predict patients having an early relapse after adjuvant tamoxifen treatment.[95]

While the recurrence score and two-gene index might be very helpful in predicting the likelihood of relapse of disease, a major limitation of these tests is that tamoxifen is prescribed as adjuvant treatment. A disadvantage of assessing response in the adjuvant setting is that both the response of tumour cells to tamoxifen and intrinsic aggressiveness of the malignancy are measured. Furthermore, some resistant tumours will not recur because they were already cured by surgery and radiation. The proportion of this group of patients is unknown.

In contrast, Jansen and colleagues discovered, using cDNA microarrays, an 81-gene signature in tumours of breast cancer patients treated with tamoxifen for their metastases.[96] In this palliative setting, tumour response can be visualised. Subsequently, this response profile was tested on 66 independent cases and could select patients who had a short time to tumour progression (TTP). The genes were involved in oestrogen action, apoptosis, extracellular matrix formation and immune response. Recently, these 81 genes were validated in tumour samples from another hospital using a more advanced microarray platform.[97] It is provocative to speculate on the predictive value of this tool if used for adjuvant treatment decisions. Identification of a subset of patients who might have more

chance to be cured by tamoxifen instead of an AI may open the door to more individualised medicine.

While adjuvant tamoxifen treatment reduces the risk of breast cancer death by 31%, AIs slightly improve disease-free survival compared with tamoxifen.[98] In addition, a survival benefit has been shown for sequential tamoxifen and an AI.[37,99] A molecular test helping clinicians to make a choice between starting with tamoxifen, an AI or rather with chemotherapy would have enormous potential for tailoring treatment. Mackay *et al.* conducted gene expression profiling on pre-treatment and post-treatment biopsies of breast cancer patients who received an AI for two weeks before surgery.[100] Profound changes in gene expression were seen after treatment, including many classical E2-dependent genes (TFF1, CCND1, PDZK1 and AGR2) as well as a prominent decrease in the expression of proliferation-related genes. Using a similar approach, Miller and colleagues identified letrozole-induced changes in gene expression associated with cell cycle progression, organ development, extracellular matrix regulation and inflammatory response.[101-103] With regard to the steroidal anti-oestrogen AI exemestane, Harvell and colleagues identified 50 genes that can predict response or intrinsic resistance to neoadjuvant exemestane treatment.[104] This study showed upregulation of a lipogenic pathway in non-responsive tumours that may serve as a marker for intrinsic resistance. Subsequently Harvell *et al.* demonstrated that an AI alone alters gene expression five times more than an AI in combination with tamoxifen, and is 11 times more effective in modifying expression of E2-regulated genes.[105] Moreover, *in vitro* studies suggest that gene profiles unique to AI resistance are inherently different from tamoxifen resistance profiles.[106] Larger datasets and samples derived from a randomised trial are necessary to enable the identification of markers or gene signatures specifically associated with AI response.

FUTURE PERSPECTIVE

The published literature is awash with examples of biomarkers promising to predict responses to endocrine therapy in breast cancer. However, only two molecular markers, ER and PR, have become standard measurements in the management of breast cancer patients with regard to assessment of endocrine sensitivity. Moreover, even their exact predictive value, e.g. sensitivity and specificity at a well-optimised cut-off value, is largely unknown regarding the important clinical question: has an individual patient more benefit from tamoxifen or an AI? Apparently the discovery of a biomarker related to endocrine responsiveness is relatively easy. However, translation of the findings into clinical practice seems extremely difficult. In the majority of clinics the endocrine dependence of a breast carcinoma is simply rated as ER-positive or ER-negative. Around the world several cut-offs are used to determine whether a tumour is ER-positive. Meta-analyses have never showed an analysis that addressed at which particular cut-off the ER was best at predicting tamoxifen benefit. Although the presence or absence of ER is widely used to guide therapy, less attention has been paid to the quantitative aspects of ER. Thirty years ago, McGuire and colleagues observed that the response of metastatic disease to endocrine treatment was directly related to the level of ER expression.[107] The Oxford overview analysis has extended this to primary disease showing a greater proportional reduction in recurrence rate with tamoxifen treatment in high *vs* low ER-positive tumours.[108] However, a quantitative measurement of ER is still not used in the clinic. Besides ESR1 mRNA levels, tumour profiling using genes that incorporate an ERE in their promoter could be informative with regard to the assessment of endocrine sensitivity. Future research should focus on how exactly ER activity has to be quantified.

In a short period of time, analysis of gene expression in breast cancer has increased the understanding of ER signalling and the diversity of ER-positive and -negative breast cancer subtypes. However, there are still many questions remaining that could be answered by continuing research using gene expression profiling of human tumour samples. The advantage of microarray technology is that thousands of genes can be studied at the same time instead of focusing on a single gene of interest. Regarding the genes responding to activation of ER, several lists of either putative ER targets or genes correlating with ER expression have been published.[53,64,71,80,109,110] However, currently there is no consensus on the comprehensiveness of these gene sets. A complete overview of genes also including processes in which ER is influencing gene expressing by functioning as a transcriptional co-factor or driving other co-factors, is still lacking. Furthermore, gene expression profiling is not suitable to pinpoint post-translational modifications of ER or epigenetic regulation by ER by binding to chromatin.

While the description of breast cancer phenotypes in distinct molecular subtypes, as first portrayed by Perou and colleagues, has been exciting, further refinement of subdivision of ER-positive breast cancer is needed.[52,111] How to define the group of patients with a very good outcome for which systemic treatment can be safely omitted? And since some ER-positive tumours show a moderate response to chemotherapy, it will be very interesting to screen this subgroup for specific drug targets.[108,112-114] If these can be identified, clinicians can offer endocrine treatment combined with targeted therapy.

Although the high throughput analysis of gene expression of breast cancer cells has increased the insights into the behaviour of the disease, the relation with outcome and

therapy response, accurate and robust validation of the candidate response profiles is necessary before clinical application. Standardisation of technology and properly designed clinical trials performed on a large scale will be essential. Moreover, the discrimination of the prognostic value of a set of genes, e.g., aggressiveness of tumour cells regardless of systemic treatment versus the capacity to predict response to a specific drug needs more detailed investigation.

Currently, whole genome analyses require frozen material. The isolation of sufficient and high-quality mRNA from formalin-fixed paraffin-embedded (FFPE) material will allow the analysis of the complete genome from archived material. Besides, it saves the complex logistics of the storage of frozen material. Important challenges for the future include the implementation of a technically robust gene expression technology in daily clinical practice, and to combine multiple separate predictive tests into a single assay to improve cost-effectiveness. In an ideal world, a breast tumour will be profiled using a single microarray resulting in information on prognosis, endocrine resistance, chemo-sensitivity, expression of drug targets and genetic variation in drug metabolising enzymes.

Series of prospectively designed clinical studies enrolling patients whose clinical characteristics match the intended use of the test are needed. Since endocrine treatment has an undisputed efficacy, a trial incorporating a study arm that withholds adjuvant endocrine treatment for intermediate-high risk ER-positive breast cancer patients is impossible to conduct. However, collecting material from patients randomised between tamoxifen and an AI may enable the discovery of gene profiles that predict the response to either tamoxifen or an AI. In the MINDACT trial novel gene expression signatures predicting clinical response in patients treated with sequential tamoxifen-letrozole *vs* letrozole alone will be compared.[115] In addition, the trans-ATAC has been set up as a follow-up of the ATAC trial to try to identify the molecular characteristics of tumours of patients that benefit more from anastrozole than tamoxifen and pinpoint the resistance mechanisms that still allow many patients to relapse.[116]

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REVIEW

Pathophysiology and prevention of diverticulitis and perforation

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ABSTRACT

Objective: This article gives an overview of the current evidence and theories in the pathophysiology of diverticulosis, diverticulitis and perforation and discusses its prevention.
Background: Diverticular disease is one of the most common diseases related to the gastrointestinal tract in Western countries. The pathogenesis of this disease process is probably multifactorial, but remains poorly understood and inadequately investigated.
Methods: A literature search was performed in order to give an overview of the current evidence and theories in the pathophysiology of diverticula formation and the factors related to progression towards inflammation and even perforation. Strategies for prevention of (perforated) diverticulitis are also discussed.
Results/conclusion: The pathogenesis of diverticular disease and its complications seems to be a result of a complex interaction between exposure to a low-fibre diet, possible genetic influences, the coexistence of other bowel diseases and the impact of medicine use. This eventually leads to alterations in colonic pressures and motility and structural changes of the colon wall. Unfortunately the evidence is frequently conflicting in the present literature or lacking altogether.

KEYWORDS

Pathophysiology diverticulitis, diverticular disease, prevention, perforation

INTRODUCTION

The prevalence of diverticulosis is estimated at 5% by the age of 40 years, up to 65% at 80 years of age.[1,2] Its exact prevalence is difficult to assess because most people remain asymptomatic.[2] Only 10 to 25% of patients with diverticulosis will manifest any related clinical symptoms.[2,3]
The pathogenesis of this disease process is probably multifactorial involving dietary habits, changes in colonic pressure, motility and wall structure associated with ageing.[4] The reason why a subgroup of individuals progresses from asymptomatic to symptomatic or even complicated diverticular disease remains poorly understood. This article gives an overview of the current evidence and theories in the pathophysiology of diverticulosis, diverticulitis and perforation and discusses its prevention.

PATHOPHYSIOLOGY DIVERTICULA OF THE COLON

In Western nations diverticula are most common in the left colon. This is in contrast to Asian nations where they occur primarily in the right colon.[5] This difference suggests a role for genetic, environmental or lifestyle factors in the aetiology of diverticular disease.[6]
Diverticula are most notable in the left colon, with up to 99% having some degree of sigmoid involvement.[7] They protrude most commonly in four rows between the antimesenteric and mesenteric taenia.[8] The majority of diverticula pass through the bowel at weak points in the circular muscle layer where the blood vessels penetrate it to supply the mucosa.[8,9] This suggests that intraluminal pressure might play a role in their formation. These pulsion diverticula are in fact 'false' diverticula as not all layers of the bowel wall are involved.[4]
The maintenance of the colonic wall is provided by extracellular matrix, with components such as collagen and elastin.[10] The mechanical characteristics of the bowel

are maintained via circular and longitudinal muscle layers. The circular muscle thickens in regular bands of contraction (plicae circulares) which control peristalsis. The longitudinal muscle also condenses in thick bands (taeniae coli) which serve to pull the colon to a relatively short functional length. Thickening of the muscular layer is one of the most consistent features of diverticulosis.[7]

Accumulation and aberrant deposition of connective tissue fibres (elastin and collagen) underlie the altered muscle morphology.[11] The muscle cells themselves do not change, but the taeniae become thickened secondary to elastin depositions, which leads to contraction in this layer and thickening of the circular muscle layer.[11-13] This narrows the lumen. In addition, systematic contractions of the circular muscle divide the bowel into a series of compartments. Altogether these colonic wall changes lead to an increase in intracolonic pressures.[14-16]

Elastin depositions and cross-linking of collagen continue throughout life in all layers of the colonic wall.[17] Increased elastin deposition may result from intermittently increased colonic pressure, which in turn is due to reduced faecal load produced by a Western low-fibre diet. Together with a decrease in tensile strength of the colonic wall, caused by an increase in cross-linking of collagen fibres with age and caused by a low-fibre diet as well, these changes in muscle morphology will result in weakening of wall resistance.[13,18-20] The increased depositions of these two connective tissue fibres (elastin and collagen III) are observed to be more pronounced in diverticular disease.[21,22]

It is thought that a disruption of the balance between matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) may be involved in the pathogenesis of diverticular disease, through remodelling of the colonic extracellular matrix, particularly collagen.[10] An overexpression of TIMPs in the muscular layer affects the turnover of extracellular matrix, resulting in the formation of diverticula and their complications.[7,10,22] An increased synthesis of type III collagen is observed in diverticulosis, but its significance remains to be elucidated.[21] The disturbance of the collagen texture (lower ratio of mature collagen type I and immature collagen type III) is thought to weaken the bowel wall, hence leading to the onset of diverticula.

Besides colonic wall changes, disordered motility has also been suggested as a cause of increased intraluminal pressure and hence as a pathogenetic factor in diverticulosis.[23] Colonic motility is influenced by the ageing process of its smooth muscle, causing an increase in segmental contractile activity.[24] Patients with symptomatic diverticular disease have shown to have higher motility indices than asymptomatic patients or healthy persons.[23] Nevertheless, absolute evidence is still lacking, since most studies on colonic motility and myoelectrical activity were biased by poor patient selection, heterogeneity of clinical conditions, recording techniques, and duration of the recording and mostly based on small numbers of patients.[23]

Neurophysiopathological data to support the increased colonic motility are sparse in the present literature. The high intracolonic pressure might be related to an imbalance in usual excitatory and inhibitory neural influences (increased cholinergic stimulation). Cholinergic nerves were dominantly present in the diverticular colon compared with controls.[25,26] Moreover, patients with diverticular disease have shown substantial structural alterations of the enteric nervous system mainly characterised by a significant lower number of glial cells and a lower number of interstitial cells of Cajal in the mesenteric plexus and within the muscle.[27] These cells are emerging as potential colonic pacemaker cells, and their loss might explain intestinal motor abnormalities reported in diverticular disease.

The influence of Western diet habits (red meat, low fibre) on the evolution of diverticular disease has been well established.[1,28,29] These dietary factors lead to increased colonic transit times, smaller stool volumes and subsequently to raised intracolonic pressures, all of which may contribute to the development of diverticulosis.[30] An increase in diverticular disease in developing countries has been documented, concurrent with the adoption of Westernised dietary habits.[4] An unexplained curiosity in the increase in diverticular disease in Asia is that it is mostly right sided, which suggests a genetic component in the development of diverticulosis.[31]

Some genetic disorders have been associated with a strong predisposition towards diverticula formation. Most of these syndromes are associated with a connective tissue disorder (Ehler-Danlos, polycystic kidney disease).[6] But literature is conflicting about this matter.[32] The same is thought about Saint's triad (the aggregation of gallstones, diverticulosis of the colon and hiatus hernia in elderly people). Connective tissue abnormalities causing herniosis might be the causing factor in this triad, although fibre-depleted diets may also be causatively related to Saint's triad.[33]

Recent studies show increasing mitochondrial dysfunction in the ageing colonic epithelia and this correlates well with diverticular disease prevalence.[34] It remains unclear whether these findings play a role in pathogenesis or are simply related to ageing.

It has been suggested that the irritable bowel syndrome may be an early stage in the development of diverticulosis.[35,36] Although a lack of dietary fibre and higher colonic motility activities caused by changes in the enteric nervous system have been implicated as aetiological factors in both conditions, available evidence supporting this theory is conflicting in the present literature. As both conditions are relatively common, the likelihood of coincidental occurrence in the same individual is

quite high.[37] It is therefore almost impossible to predict which patients are symptomatic as a direct result of their diverticulosis. In the same manner, persistence of symptoms after surgical resection for symptomatic diverticular disease can be explained.[37]
In conclusion, the evidence from studies in man suggests a relationship between diet/lifestyle and diverticular disease, but there remains a lack of robust definitive evidence.

PATHOPHYSIOLOGY OF DIVERTICULITIS

It is estimated that 10 to 25% of patients with diverticulosis will experience inflammation at some point during their lives.[38] Like the pathophysiology of diverticula, the aetiology of diverticular inflammation is also speculative. Development of diverticulitis has been described similarly to that of appendicitis. Diverticula may become acutely inflamed through impacted faeces, leading to an obstruction of the lumen, raising intradiverticular pressure by continuing mucus formation and ultimately causing ulceration within the diverticular mucosa.[39] This event then allows for proliferation of bacteria, diverticula distension, and localised ischaemia. Eventually, perforation of variable extent may result, accounting for a range of symptoms.[40,41] It is possible that the increased colonic pressure in diverticular disease is also responsible for pushing fecaliths into the diverticula.
Dietary shifts during the past century have likely not only influenced colonic motility, but also altered colonic flora.[42] The colonic environment has likely undergone radical changes in the past century due to decreases in both soluble and insoluble fibre. Higher levels of *Bacteroides* and lower levels of *Bifidobacteria* have been found in studies comparing gut flora between Westernised and rural populations. This change in colonic microbial environment may be an important element in the transformation of asymptomatic diverticular disease into diverticulitis, but its exact role has not been adequately defined.[43]
In addition to the 'typical' form of diverticulitis, it is increasingly recognised that luminal mucosal inflammation may coexist with diverticula.[44,45] This low-grade inflammation shares histological features with inflammatory bowel disease. The pathogenesis of this so-called diverticular colitis, sigmoiditis, or segmental colitis is unknown, as is its relationship with inflammatory bowel disease.[45,46] Nevertheless, low-grade diverticular colitis might be the reason why some patients are chronically symptomatic. This phenomenon has been described before in inflammatory bowel disease, where colonic symptoms may persist after resolution of inflammation.[47] The acute diverticular inflammation may have provoked an alteration in colonic neuromuscular function and may be responsible for chronic symptoms, even in the absence of inflammation.

PATHOPHYSIOLOGY OF PERFORATED DIVERTICULITIS

Although diverticulosis is common, complications requiring surgery occur in only approximately 1% of patients with the disease.[48] The incidence of diverticular perforation has been estimated at 4/100,000 population per annum.[49] About 80% of patients presenting with perforated diverticulitis do not have a previous history of diverticular disease.[49]
The natural history of complicated diverticulitis remains poorly understood, probably because consultant surgeons see only two to three cases a year and almost a third of patients die from unrelated causes during follow-up.[50] In case of perforated diverticulitis this percentage might be even higher, up to 50% within five years.[51]
The aetiology of perforation remains unknown, but as stated before, it is thought to be a result of excessive rise in intradiverticular pressure and focal necrosis.[52] This local perforation may form pericolic phlegmones and pus collections (Hinchey I).[53] If this process progresses, localised abscesses may be formed between loops of small bowel or in the pelvic peritoneum (Hinchey II). If the pus cannot be contained, the abdominal peritoneum gets contaminated producing generalised purulent peritonitis (Hinchey III). The same is found when a large intraperitoneal diverticular abscess ruptures into the abdominal cavity.[54] If the initial perforation is large, faecal contamination of the abdominal cavity can occur (Hinchey IV).[53]
Patients with diverticular disease in general show raised intracolonic pressures, especially in the sigmoid colon.[14] As almost all diverticular perforations occur in the sigmoid colon, these pressure changes must be an important aetiological factor. Besides that, the properties of the colonic wall are likely important, because diverticula consist predominantly of mucosa, lacking a smooth muscle layer. The mucosal barrier is vulnerable and may be impaired by various exogenous factors.[55]
NSAIDs have been implicated as a risk factor for perforation in diverticulitis.[49,56-58] NSAIDs inhibit the cyclo-oxygenase enzyme and cause topical mucosal damage, increasing colonic permeability. Besides, they reduce prostaglandin synthesis, which is important in maintaining an effective mucosal barrier.[59]
Corticosteroids and opiate analgesics are also related to an increased perforation rate.[60,61] Corticosteroids have strong immunosuppressive and anti-inflammatory effects, which may result in an impaired ability to contain the perforation initially.[57] This will lead to more severe

inflammatory complications. Besides, symptoms and signs in the immunosuppressed patient may well be masked, often delaying and underestimating diagnosis and its severity.[62,63] The prevalence of diverticulosis in immunosuppressed patients may not differ from that in the rest of the population, but there is undoubtedly a much higher incidence of complicated diverticulitis in such patients.[63] Opiates slow intestinal transit and raise intracolonic pressures.[61] By slowing transit time, the diverticular mucosa may have a prolonged exposure to potentially damaging pathogens, such as bacteria.

Unfortunately the causal relationship between these drugs and perforated diverticulitis is mainly based on (small) case series or case-control studies. Hard evidence is lacking in the present literature. Besides, if these drugs are a true risk factor for perforation, they would account for less than 20% of cases: other risk factors must be important.[54]

The role of smoking and alcohol intake in perforated diverticulitis is also unknown. Nicotine might predispose to diverticular inflammatory complication by reducing mucosal immunity,[64,65] but hard evidence is lacking in the present literature.[66]

Since the incidence of diverticulosis increases with age, the majority of patients presenting with symptoms are the elderly. Complicated diverticulitis is also observed predominantly in older patients. This problem is caused by an unusual presentation of diverticular complications in the elderly patient, with consequent delay in diagnosis. Polypharmacy may further exacerbate this problem and may even increase the risk of developing complications (NSAIDs, corticosteroids).[67] The relatively high incidence of comorbidities in the elderly and the unusual presentation of the disease will lead to a very high morbidity and mortality rate for this group of patients.[51]

On the other hand, complicated (perforated) diverticulitis is relatively frequently seen in younger (male) patients.[68] Although diverticulitis is uncommon in patients less than 40 years old, accounting for only 5% of all patients admitted for diverticulitis, it has been thought to be a more virulent condition in this age group.[69,70] But again the present literature is conflicting. Several recent publications have suggested that the disease is not more virulent in the younger patients.[71-73] The high rate of complications and perforations may be attributed to a high misdiagnosis rate because diverticulitis may not be suspected in younger patients with abdominal complaints.[74,75]

PREVENTION OF DIVERTICULITIS AND PERFORATION

The possible role of diet and lifestyle offers strategies for prevention. Large prospective studies have identified a preventive effect of both vegetable and high fibre intake and physical exercise in the development of diverticular disease, as well as diverticulitis.[76-78] The protective action of dietary fibre would make the stools bulkier, thereby increasing the colon size and decreasing intraluminal pressures, and reducing colonic transit time.[79,80] Fibre as a dietary supplement may be beneficial in prevention. It is nevertheless remarkable that the incidence of diverticular disease has not been found to be reduced, while several studies have shown an increased intake of fibres in Western populations over the last three decades.[81] The exact role of fibres in the pathophysiology of diverticulosis and its prevention remains unclear. And when symptoms have developed, evidence of a benefit of fibre is even less convincing.[81]

A reduction in transit time was the consistent finding in most of the studies that addressed the effect of physical exercise on colonic function. An increase in colonic motor activity has been postulated; however, the exact mechanism of this effect is still not clear.[78]

As mentioned above, patients with symptomatic diverticular disease have shown to have higher motility indices than asymptomatic patients or healthy persons.[82] This suggests that anticholinergic or antispasmodic drugs might improve symptoms by diminishing muscular contractions. Nonetheless, there is no evidence to support this in the present literature.[40]

One of the latest therapies for the prevention of recurrent diverticulitis is the use of mesalazine, rifaximin or a combination of both.[83,84] The rationale for this is that mesalazine inhibits some key factors of the inflammatory cascade.[85] The protective role of mesalazine in the recurrence of symptomatic diverticular disease is thought to be similar to that for the use in chronic inflammatory bowel disease.[85,86]

Another very recent therapy is the use of probiotics.[87] Probiotics diminish changes in the spectrum of intestinal microflora and the adherence and translocation of pathogens. They also regulate production of antimicrobials and interact as competitive metabolites with pro-inflammatory organisms. Especially the combination of *Lactobacilli* spp. with rifaximin seems effective in reducing severe forms of diverticulitis and the prevention of recurrences, hence reducing surgical treatment significantly.[88,89]

The role of surgery in the prevention of (complicated) diverticular disease is unclear. Formally, elective sigmoid resection was recommended after two episodes of uncomplicated diverticulitis to prevent serious complications of recurrent colonic diverticulitis.[90] This guideline was based on the assumption that recurrent episodes of diverticulitis will lead to more complications and higher mortality. The data to support this assumption are based on small and older studies. Advances in diagnostic modalities, medical therapy, and surgical techniques over the past two decades have changed both the management and outcomes of diverticulitis.[91]

Patients treated nonoperatively would be expected to do well without elective colectomy, since most patients will not have further episodes of diverticulitis.[92,93] Recurrent episodes of diverticulitis do not lead to more complications and more conservative treatment failure.[94,95] At present it is thought that elective resection for uncomplicated diverticulitis does not alter outcome, nor does it decrease mortality or prevent severe complications of the disease such as perforation.[96,97] For approximately 80% of the patients perforation is the first manifestation of diverticular disease.[49]

Finally an association between the use of calcium channel antagonists and perforated colonic diverticular disease was demonstrated.[98] Calcium channel antagonists, which reduce colonic contractility and tone, protected against perforation. Further studies are required to confirm this association, but it may represent a potentially useful preventive therapy.

CONCLUSION

Although diverticular disease is one of the most common diseases related to the gastrointestinal tract in Western countries its pathophysiology remains poorly understood and inadequately investigated. Much of the evidence suggests that the pathogenesis of diverticular disease is a result from a lifelong exposure to a low-fibre diet, leading to alterations in colonic pressures and motility and colon wall structural changes. Unfortunately the 'evidence' is frequently conflicting in the present literature or lacking altogether. This complex interaction between colonic structure, motility and diet, the possible genetic influences, the coexistence of other bowel diseases and the impact of medicine use, makes it difficult to investigate. It may even be so that clinical subtypes of diverticular disease exist in terms of pathophysiology and symptomatology requiring different treatment strategies. Further basic and clinical investigations need to be done to fill up the several gaps in the knowledge of pathophysiology of diverticulosis and diverticulitis and its treatment and prevention. For the same reason, there is a need for further good quality epidemiological research to identify risk factors in diverticular perforation. Whether new insights into the aetiology will lead to new surgical strategies for prevention and treatment of perforated diverticulitis remains to be seen.

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ORIGINAL ARTICLE

Chronic yersiniosis due to defects in the TLR5 and NOD2 recognition pathways

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ABSTRACT

Infection with *Yersinia enterocolitica* leads to a self-limiting disease, but in a small number of cases a protracted course can develop. The host genetic factors contributing to the advancement of the disease to the chronic phase are not known. We describe a patient suffering from an abdominal inflammatory mass due to chronic yersiniosis. Functional assays revealed defects in the recognition of flagellin by Toll-like receptor 5 (TLR5) and of muramyl dipeptide by NOD2, leading to a defective inflammatory response to *Yersinia enterocolitica*. Genetic sequencing showed that the patient was compound heterozygous for five different mutations in TLR5, while being homozygous for the 3020insC NOD2 mutation. In conclusion, we describe a patient in whom specific defects in the TLR5 and NOD2 recognition pathways led to chronic yersiniosis.

KEYWORDS

Cytokine, Crohn's disease, NOD2, TLR5, *Yersinia*

INTRODUCTION

Yersinia enterocolitica is a flagellated Gram-negative bacterium that is pathogenic for humans. Infection occurs through consumption of contaminated water or food, and when healthy individuals are infected, generally a self-limiting intestinal inflammation ensues. Occasionally, more serious protracted disease develops.[1] Chronic mesenteric lymphadenitis, ileitis and hepatitis are major manifestations of more severe yersiniosis, as are extra-intestinal presentations such as reactive arthritis and erythema nodosum.[2]

Y. enterocolitica infects the Peyer's patches and other elements of the mucosa-associated lymphoid tissue, and here the organism may persist for long periods of time. In recent years, many of the virulence factors of *Y. enterocolitica* have been discovered; among these the *Yersinia* outer proteins (Yop) and LcrV are the most prominent.[3] Delivery of Yop into the cytoplasm of host cells through a type III secretion/translocation system is crucial in the pathogenesis of the infection.[4] LcrV is a released multifunctional molecule that acts as an immunomodulatory molecule by interaction with CD14 and Toll-like receptor (TLR) 2 and subsequent induction of interleukin-10 (IL-10).[5] In addition to recognition by TLR2, other pattern recognition receptors of the innate immune system are likely to play a role in the interaction of *Y. enterocolitica* with the host. As *Yersinia* spp are flagellated bacteria, recognition of flagellin by TLR5 also contributes to the recognition by the host,[6] while TLR4 is important for induction of apoptosis.[7] NOD2, the intracellular recognition receptor for peptidoglycans and muramyl dipeptide (MDP)[8,9] could also be expected to play a role in the interaction between *Y. enterocolitica* and host cells.

From experiments in murine models it is known that interferon γ (IFNγ) and the cytokines responsible for its induction, IL-12 and IL-18, are essential for protective immunity against *Y. enterocolitica*.[10-12] In addition, other proinflammatory cytokines such as IL-6 are also involved in the protective anti-*Yersinia* mechanisms,[13] while triggering IL-10 through an LcrV/TLR2-dependent mechanism is essential for virulence.[14]

In this paper we report the history of a patient with a severe chronic yersiniosis. The persistence of *Yersinia* was accompanied by a defective cytokine response to the micro-organisms by leucocytes isolated from the patient,

which was very likely responsible for the protracted course of the disease. We also pinpoint the defective cytokine response to defects in TLR5- and NOD2-mediated recognition of *Y. enterocolitica* in this patient.

PATIENTS AND METHODS

Case report

A 28-year-old male was referred to the outpatient clinic for infectious diseases at the Radboud University Nijmegen Medical Centre. In February 2003, the patient developed right-sided abdominal pain followed by chills and high fever. He was admitted 14 days after the start of the illness. A CT scan revealed multiple enlarged mesenterial lymph nodes close to the aortic bifurcation. The immunoblot for the *Y. enterocolitica* antibodies was positive, showing specific IgG and IgA reactivity against Yop1, 3, 3A, 4 and 5. A presumptive diagnosis of *Y. enterocolitica* mesenterial adenitis was made, and subsequently the patient was treated with ciprofloxacin for a total of six months. Despite this treatment, the patient's fever, malaise and elevated C-reactive protein persisted. An extensive work-up search for alternative diagnoses such as Whipple's disease was fruitless. Crohn's disease was unlikely as a small bowel X-ray series and colonoscopy revealed no abnormalities. A biopsy taken from a mesenterial lymph node mass yielded granulomatous inflammation with epitheloid cells and perilymphadenitis. No micro-organisms were seen and further microbiological investigations remained negative. As the immunoblot against *Yersinia* epitopes remained positive and his abdominal complaints persisted, we decided to perform positron emission tomography using 18F-fluorodeoxyglucose (FDG-PET), which revealed an FDG accumulation in the lower abdomen consistent with a mass with a diameter of 7 cm.

The patient started treatment with minocyclin (100 mg/day) for additional six months. During this treatment the fever and complaints subsided and a subsequent FDG-PET showed a decrease of abdominal mass. No side effects of minocyclin treatment were reported.

Materials

Muramyl dipeptide, poly I:C and flagellin were purchased from Sigma Chemical Co (St. Louis, MO), and synthetic Pam3Cys from EMC Microcollections (Tübingen, Germany). LPS (*Escherichia coli* 055:B5) was purchased from Sigma, and repurifed as previously described.[15] LcrV was kindly provided by Prof. J. Heesemann (Munchen, Germany).

Isolation of peripheral blood mononuclear cells and cytokine stimulation

After informed consent, venous blood was drawn from the cubital vein of the patient (collected after minocyclin treatment, during a stable phase of the disease, without acute signs of inflammation), healthy family members and five healthy volunteers (all male, age 22-35 years old) into three 10 ml EDTA tubes (Monoject, s-Hertogenbosch, the Netherlands). In addition, cells isolated from four patients with Crohn's disease homozygous for 3020insC NOD2 mutation were also used as an additional control group. Isolation of mononuclear cells (MNC) was performed as described elsewhere,[16] with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Cells were washed twice in saline and suspended in culture medium (RPMI 1640 DM) supplemented with gentamicin 10 µg/ml, L-glutamine 10 mm and pyruvate 10 mm. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, the Netherlands) and the number was adjusted to 5×10^6 cells/ml.

5×10^5 MNC in a 100 µl volume were added to round-bottom 96-wells plates (Greiner, Alphen a/d Rijn, the Netherlands) and incubated with either 100 µl of culture medium (negative control), various concentrations of heat-killed *Y. enterocolitica*, or LcrV (5 µg/ml). In addition, stimulation experiments with various TLR and NOD2 stimuli were performed in separate wells: MDP (10 µg/ml), Pam3Cys lipopeptides (1 µg/ml), poly I:C (50 µg/ml), purified *E. coli* LPS (1 ng/ml), flagellin (10 ng/ml). After 24 hours incubating at 37°C, the supernatants were stored at -80°C. Cytokine concentrations were measured by specific commercial ELISA kits from R&D Systems (Minneapolis, MN).

Genotyping of TLR5 gene

Amplification of the six exons of the TLR5 gene was performed using 11 polymerase chain reactions (PCR**).** After DNA purification, the sequencing was performed at the Clinical Genetic Centre Nijmegen, Radboud University Nijmegen Medical Centre, using the Big Dye terminator version 2 Chemic (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The sequence was checked using Biology Workbench 3.2.

Genotyping of 3020insC NOD2 gene variant

Blood was collected from the patient and his family (mother, father, and three sisters). PCR amplification of NOD2 gene fragments containing the polymorphic site 3020insC was performed in 50 µl reaction volumes containing 100 to 200 ng genomic DNA, as previously described.[17] The 3020insC polymorphism was analysed by Genescan analysis on an ABI Prism 3100 Genetic Analyser according to the protocol of the manufacturer (Applied Biosystems).

Patient consent

Written consent was given by the patient and family. The analysis performed in the study took place within the diagnostic work-up of the disease, and therefore no ethics commission approval was necessary.

RESULTS

Cytokine production induced by Y. enterocolitica and LcrV in PBMC of healthy volunteers

In a first series of experiments we measured the cytokine responses of peripheral blood mononuclear cells (PBMC) of five human volunteers exposed to *Y. enterocolitica*. With 5 x 10^3 and 5 x 10^4 cfu/ml *Y. enterocolitica*, a sizeable cytokine response was seen, the IL-6 response being the most accentuated (*figure 1A*). Strong stimulation of IL-10 (1444±383 pg/ml) and tumour necrosis factor (714±215 pg/ml) by 5 x 10^4 cfu/ml *Y. enterocolitica* was also measured.

The response to LcrV in a dose range between 1.25 and 5 μg/ml was lower for all cytokines: low amounts of IL-10 and TNF, but a clear dose response for IL-6 (*figure 1B*).

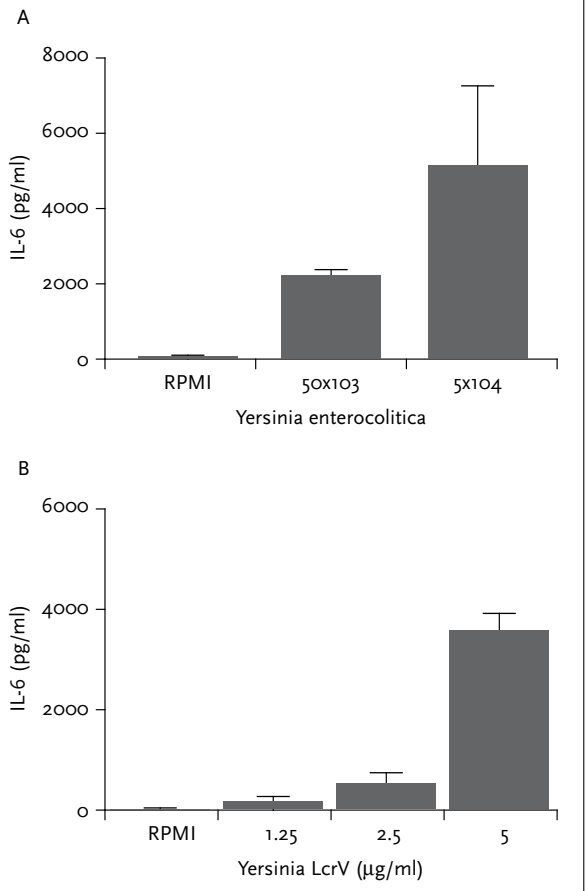
PMBC responses to Y. enterocolitica and TLR/NOD2 ligands

Interestingly, when PBMC of the patient with chronic yersiniosis were exposed to whole *Yersinia* micro-organisms, a strongly blunted IL-6 response was observed. The IL-6 response to LcrV in the patient was in the normal range (*figure 2A*). The IL-10 response to LcrV was low and also did not differ between patient and controls (not shown).

Based on these findings we assessed the response to well-defined TLR ligands.

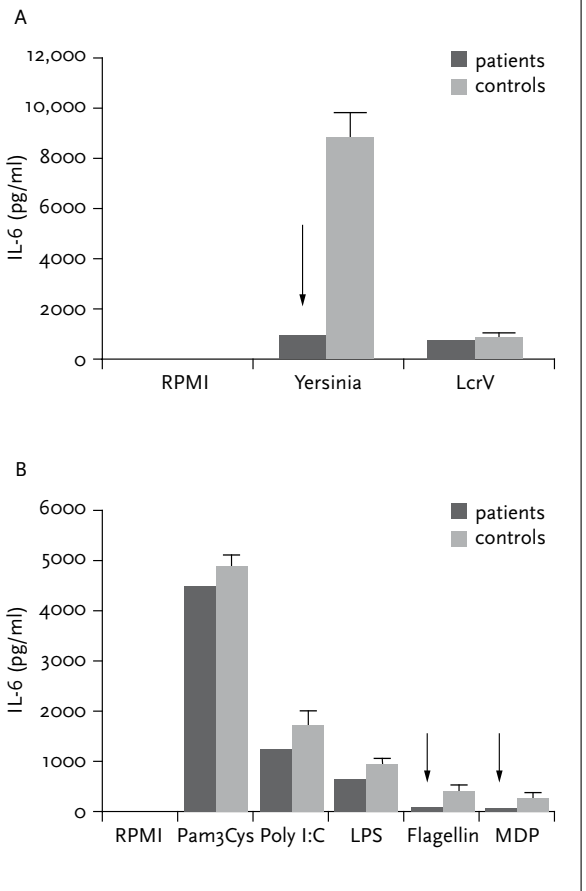
In these experiments we found virtually no cytokine response to flagellin, the ligand for TLR5, and MDP, the ligand for NOD2, and normal responses to ligands for TLR2, TLR3 and TLR4 (*figure 2B*). We repeated these studies four times, always with the same results.

Figure 1. *Cytokine production induced by Y. enterocolitica and LcrV*



A. Freshly isolated peripheral blood mononuclear cells from human volunteers were stimulated with Y. enterocolitica (5 x 10^3 and 5 x 10^4 cfu/ml) (panel A) or LcrV in various concentrations (panel B), and IL-6 was measured by ELISA 24 hours later (n=5, mean ± SD).

Figure 2. *Cytokine responses to Y. enterocolitica, TLR and NOD2 ligands*



A. Peripheral blood mononuclear cells of the patient with chronic yersiniosis and of five control volunteers were exposed to whole *Yersinia* micro-organisms (5 x 10^4 cfu/ml) or LcrV (5 μg/ml). IL-6 concentrations were measured after 24-hour incubation. B. Cytokine responses after stimulation with various TLR and NOD2 ligands: Pam3Cys (TLR2, 1 μg/ml), poly I:C (TLR3 50 μg/ml), LPS (TLR4, 1 ng/ml), flagellin (10 ng/ml) and MDP (10 μg/ml). IL-6 concentrations were measured after 24 hours of incubation.

Genetic studies

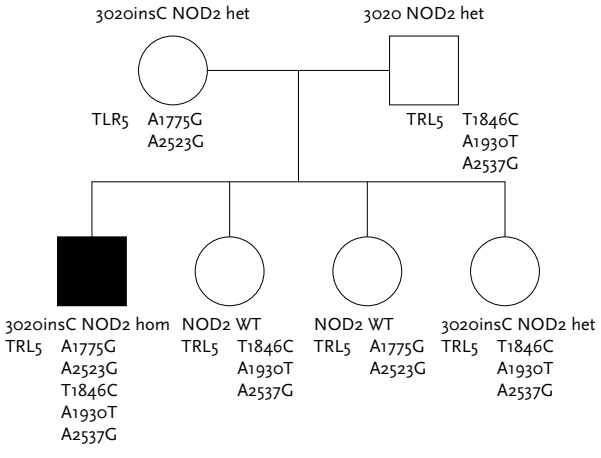
To explain the strong defects in specific stimulation with flagellin and MDP, we hypothesised that these are due to genetic defects in their receptors TLR5 and NOD2. TLR5 gene was sequenced in both the patient, his three sisters and parents. The three most common NOD2 mutations known to be associated with a decreased recognition of MDP were also determined in the family.

Sequencing showed that the patient possessed TLR5 as well as NOD2 variants. The TLR5 gene of the patient contained five single nucleotide polymorphisms, three of which had already been described, namely A2523G (lys-lys), T1846C (phe-leu) and A1775G (asp-ser).[18] Two new single nucleotide polymorphisms were discovered: A1930T (ile-phe) and A2357G (asp-gly). Comparison of the human DNA sequence with that of other species (mouse, rat, chimpanzee) shows that the A in the 1930 position is highly conserved among different species, suggesting this is an important residue. The position 2357 is more variable (G in other species and A in human).

The mutations are part of two haplotypes which were inherited from his mother (A1930T, A2357G and T1846C) and his father (A1775G and A2523G) (*figure 3*), leading to compound heterozygosity for TLR5 in the patient. As both chromosomes inherited from his parents contained non-synonymous mutations, very likely leading to defects in the function of the molecule, it is highly probable that the complete incapacity of the cells of the patient to respond to flagellin was due to these mutations in the TLR5 gene. Interestingly, one sister was also compound heterozygous with severely impaired flagellin responses (*figure 3*), whereas the other two sisters were heterozygous for mutations inherited either from their mother or their father (*figure 3*). Their cytokine responses, as well as those of their parents, were low compared with control volunteers, but significantly higher than those of their compound heterozygous brother and sister (*figure 3*).

With regard to the NOD2 mutation, the patient was found to be homozygous for the NOD2 3020insC mutation, while his parents are heterozygous. One sister was also found to be heterozygous for the mutation, whereas the other two sisters were homozygous for the wild-type allele (*figure 3*). The 3020insC NOD2 mutation is a well-known mutation resulting in a total loss of MDP-recognition capacity.[19,20]

Figure 3. *Genetic studies in the family of the patient*



TLR5 gene was sequenced in both the patient, his three sisters and parents. The three most common NOD2 mutations known to be associated with a decreased recognition of muramyl dipeptide were also determined in the family. The mutation analysis identified by sequencing is presented for both TLR5 and NOD2.

Pro- and anti-inflammatory cytokine responses in patient, family and patients with Crohn's disease and defective NOD2

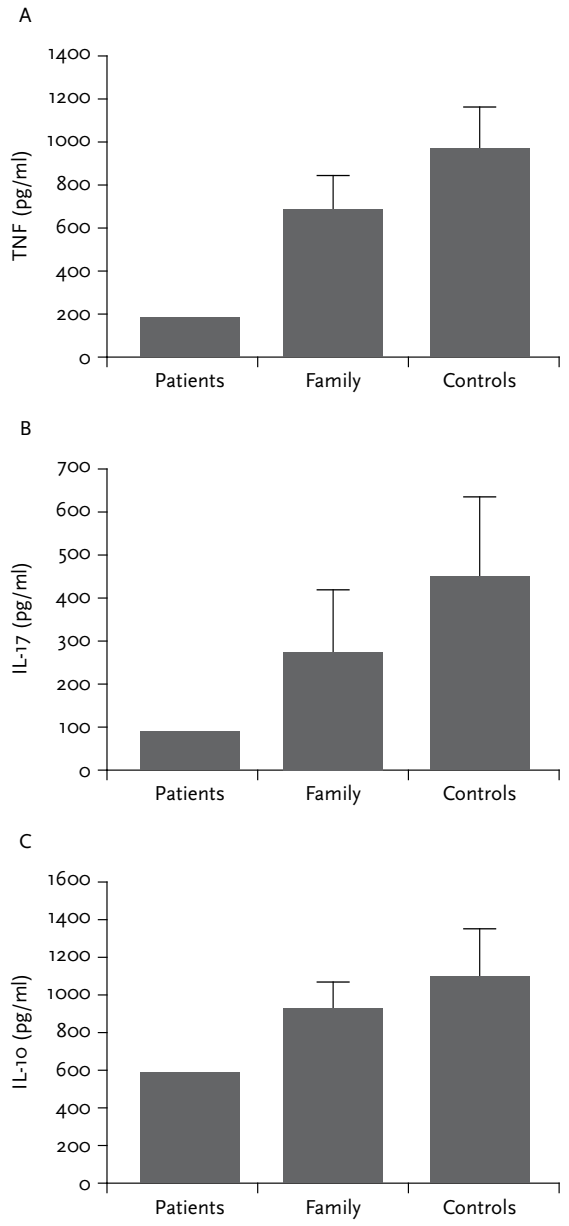
Next we investigated production of the pro-inflammatory cytokines TNF, IL-17 and the anti-inflammatory cytokine IL-10 by PBMC of the patient and the family, when the cells were exposed to *Y. enterocolitica*. As can be seen in *figure 4*, the production of TNF and IL-17 by cells of the patient is considerably lower than that of family and Crohn's disease patients, whereas IL-10 production is completely normal.

DISCUSSION

In this report we present a patient with presumed chronic yersiniosis, who was found to have a virtually absent pro-inflammatory cytokine response when his cells were exposed specifically to *Y. enterocolitica*. This defect was due to the loss of recognition of flagellin by TLR5, and the failure to induce a response through NOD2. Genetic studies revealed that the patient was compound heterozygous for several mutations in the gene coding TLR5 (two of which not reported before) and that he was homozygous for the 3020insC NOD2 mutation.

It is most likely that the genetic changes in TLR5 led to the poor cytokine response to flagellin and strongly contributed to the defective response to whole *Yersinia*. Support for this hypothesis is provided by the studies that reported an increased susceptibility to another facultative intracellular pathogen, *Legionella pneumophila*, in individuals bearing TLR5 mutations that led to defective responses to flagellin.[18] The contribution of the NOD2 3020insC mutation to the lack of response to whole *Yersinia* is unclear. Although we have not found an abnormal cytokine response after *Y. enterocolitica* stimulation of cells isolated from Crohn's disease patients homozygous for the 3020insC NOD2 gene mutation (*figure 4*), one could assume synergistic effects between NOD2 and TLR5 deficiencies. The synergy between TLR5 and NOD2, however, is an area of some controversy

Figure 4. *Pro- and anti-inflammatory cytokine responses in patient, family and patients with Crohn's disease and defective NOD2*



The production of the pro-inflammatory cytokines TNF, IL-17 and the anti-inflammatory cytokine IL-10 was assessed by stimulating peripheral blood mononuclear cells of the patient and family with *Y. enterocolitica* micro-organisms (5x10⁴ cfu/ml). In addition to the patient, cells from the five family members, and four patients with Crohn's disease homozygous for the 3020insC mutation were tested.

in the literature. In previous studies, we were unable to demonstrate significant synergy between these two receptor pathways,[19] while Van Heel *et al.* found evidence of synergy, albeit to a much lower degree than between TLR4 and NOD2.[20] Therefore, the data presented here point to a more important role of TLR5 for recognition of *Y. enterocolitica* and the disease process.

The finding that our patient was homozygous for the 3020insC NOD2 mutation, while not having Crohn's disease, is remarkable, since the relative risk for Crohn's disease in individuals bearing this NOD2 genotype is up to 42.[21] Thus, the first question to be addressed is whether the pathology observed is an infiltrate of Crohn's disease. However, the histological picture of the biopsy taken after colonoscopy does not support this diagnosis. Moreover, the patient had normal stools, did not display any other symptoms related to Crohn's disease, and none of the additional investigations have revealed intestinal abnormalities. A second possibility would be that the patient does not have Crohn's disease yet, but will still develop the disease. Although this cannot be ruled out, it is of interest to note that the mean age of diagnosis in 'genetic' Crohn's disease is lower than that of common Crohn's disease.[22]

Yet another more interesting possibility could be that the patient is protected from Crohn's disease because of the concomitant TLR5 defect. Indeed, it has been reported that TLR5 mutations are less frequent in Crohn's disease,[23] in agreement with the observation that flagellin is a dominant antigen in this disease.[24] The defective production of pro-inflammatory cytokines in our patient, albeit most prominent with *Yersinia* as a stimulant, would fit with this observation. Elsewhere, we have defended the thesis that a net pro-inflammatory cytokine status (with reduced anti-inflammatory response) contributes to the development of Crohn's disease in individuals with defective NOD2.[17] The recent finding that IL-23 receptor polymorphisms protect against Crohn's disease is in line with this concept.[25] In addition, recent studies point to IL-17 as a key mediator in Crohn's disease[26,27] and in this respect our finding of a strongly diminished IL-17 response in the patient is of great interest.

In many preclinical studies on chronic yersiniosis, a major role for IL-10 has been found, probably due to the inhibitory effects of IL-10 on an adequate antibacterial effector mechanism mediated through the proinflammatory cytokines TNFα, IL-6 and interferon-γ.[3] The cytokine production patterns observed in our patient closely resemble this situation: *Yersinia*-induced production of proinflammatory cytokines was low, while the IL-10 production was normal. The production of the latter cytokine apparently is not TLR5 and NOD2 dependent. In the literature, the virulence factor LcrV acting through TLR2 is considered a major stimulant for IL-10 production in mice,[14] although this is a somewhat controversial issue.[28] In the stimulation assay used in our study LcrV did not induce significant amounts of IL-10, neither in our patient nor in the controls. Possibly other *Yersinia* components, such as YopH and YopJ/YopP, could also induce IL-10 induction in human monocytes.[28]

A potential limitation is that the yersiniosis has merely been diagnosed by serology, and therefore the diagnosis

is still presumptive. However, recovering *Y. enterocolitica* from chronic extraintestinal sites has proven extremely difficult. It is not unusual in such cases to rely on serology,[1] although even the Western blot using the various *Yersinia* outer membrane proteins meets with some cross-reactivity and false-positives.[29] The onset of the disease, the development of the abdominal mass histologically compatible with a granulomatous infection and the gradual response to antibiotics, are strong arguments in favour of chronic yersiniosis.

CONCLUSION

In conclusion, the patient reported here suffered from a chronic inflammatory mass due to chronic yersiniosis. The finding of mutated genes for TLR5 leading to an almost complete loss of response to flagellin puts this patient in the category of selective immunodeficiencies.[30] This TLR5 defect also contributes to the lack of proinflammatory response to whole *Yersinia*, while the contributing role of the loss of NOD2-induced signals is unclear. In addition, it is attractive to speculate that the loss-of-function of TLR5 is protective against the development of Crohn's disease in this patient homozygous for NOD2 mutations.

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CASE REPORT

Central nervous system involvement in a rare genetic iron overload disorder

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ABSTRACT

In most genetic iron overload disorders the diagnosis can be rejected when transferrin saturation is low. We describe a patient and her family with hyperferritinaemia and low transferrin saturation with iron accumulation in the central nervous system (CNS) and liver due to hereditary aceruloplasminaemia. In this rare genetic iron overload disorder oxidation of iron is disturbed, resulting in storage of iron in the CNS and visceral organs.

KEYWORDS

Iron overload disorder, hereditary aceruloplasminaemia, hereditary haemochromatosis

What was known on this topic?
The diagnostic approach in patients suspected for hereditary haemochromatosis is focused on hyperferritinaemia and a high transferrin saturation due to the high frequency of HFE-related haemochromatosis.

What does this add?
Hyperferritinaemia in combination with a low transferrin saturation does not always exclude an iron overload disorder. Particularly when neurological symptoms occur in the presence of persistent hyperferritinaemia, hereditary aceruloplasminaemia needs to be excluded.

CASE REPORT

A 59-year-old woman presented at the neurology department with ataxia, involuntary movements and mild cognitive impairment. Her medical history was notable for chronic obstructive pulmonary disease and long-lasting microcytic anaemia, intermittently treated with iron supplements. Brain magnetic resonance imaging (MRI) revealed global cortical atrophy and an abnormal signal of the basal ganglia suggestive for storage disease (*figure 1*). Laboratory results showed a haemoglobin of 7.1 mmol/l (normal 7.2 to 9.8 mmol/l), MCV 74 fl (normal 81 to 96 fl), ferritin 1320 µg/l (normal 14 to 150 µg/l) combined with low serum iron (3.0 µmol/l; normal 10 to 30 µmol/l) and decreased transferrin saturation (6.5%; normal 15 to 50%). Serum copper was low (<8 µmol/l; normal 13 to 24 µmol/l) with a normal urine excretion of copper. Retinal degeneration and Kayser-Fleischer rings were not present. Genetic analysis for Huntington's disease was negative. Iron accumulation in the liver was proven with MRI, showing an iron concentration of 350 µmol/g (normal <36 µmol/g) according to the protocol of Gandon (University of Rennes, France) and liver biopsy (grade 4 iron accumulation). In conclusion, an iron overload disease in combination with low transferrin saturation was observed. Differential diagnostic considerations were dysmetabolic hyperferritinaemia, ferroportin disease or hereditary aceruloplasminaemia (*figure 2*). Ceruloplasmin concentration was markedly decreased (31 mg/l, normal 200 to 350 mg/l) and the diagnosis of hereditary aceruloplasminaemia was made. Additional genetic analysis showed a homozygote mutation (Gly650Arg) in the ceruloplasmin gene on chromosome 3. This mutation has not been previously reported in the literature. Family screening revealed that both children with a slightly decreased ceruloplasmin concentration (128 mg/l and 139 mg/l respectively) and normal serum ferritin were heterozygous for the mutation.

Figure 1. *MRI brain showing a hypointense signal of the basal ganglia*

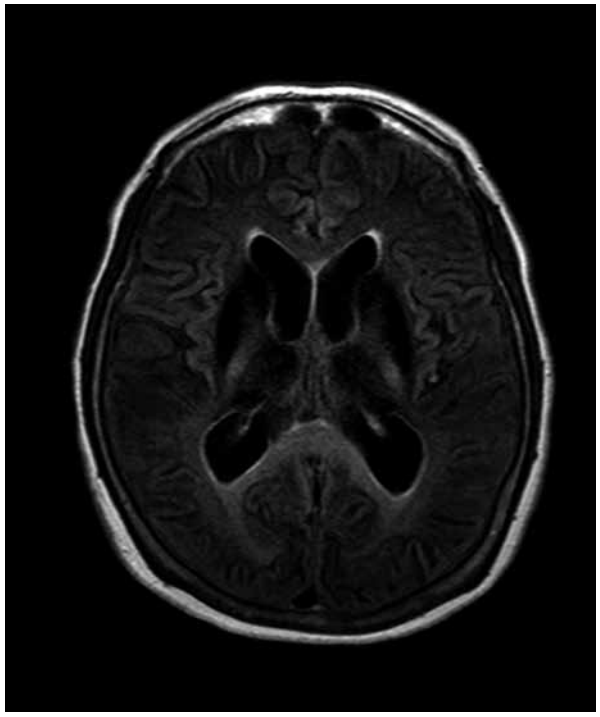
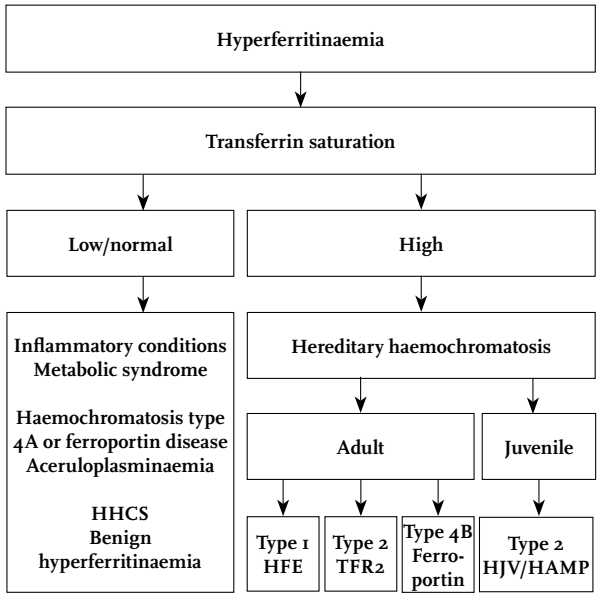


Figure 2. *Diagnostic diagram of hyperferritinaemia*



***Adapted from Camaschella, Haematologica 2009.[20] HHCS = hereditary hyperferritinaemia cataract syndrome; TFR2 = transferrin receptor 2; HJV = haemojuvelin; HAMP: = hepcidin antimicrobial peptide.**

The patient's sister was also diagnosed with hereditary aceruloplasminaemia because of an iron overload disorder in combination with low transferrin saturation (8.3%) and low ceruloplasmin concentration (6 mg/l). The patient's other sibling had normal laboratory results. In our patient treatment with phlebotomies was impossible because of the anaemia. Instead the patient received the oral iron chelator deferasirox, resulting in normalisation of the ferritin concentration without resolving the neurological symptoms. A few months later she developed diabetes mellitus.

DISCUSSION

Ninety percent of the cases with haemochromatosis are related to mutations of the HFE gene.[1] Furthermore, the prevalence of HFE gene mutation in the general population is high. Therefore, the diagnostic approach in patients suspected for haemochromatosis is focused on the classic HFE-related haemochromatosis. Hyperferritinaemia and increased transferrin saturation are used as discriminating tests. Normal or low transferrin saturation excludes the diagnosis of HFE-related haemochromatosis.[2] However, as illustrated in our case presentation, using these laboratory parameters, rare non-HFE-related genetic haemochromatosis such as hereditary aceruloplasminaemia can be missed.

Aceruloplasminaemia is a rare autosomal recessive iron overload disorder, due to mutations in the ceruloplasmin gene on chromosome 3q21-24.[3,4] The disease has mainly been described in the Japanese, but is also rarely seen in whites.[5,6] The incidence of aceruloplasminaemia in Japan is estimated to be approximately one per 2,000,000 in non-consanguineous marriages. There are no reliable data regarding incidence and prevalence in Western European countries.[6] Forty mutations of the ceruloplasmin gene have been described,[7,8] leading to failure to incorporate copper during synthesis, which causes secretion of an apoprotein without oxidase activity that diminishes rapidly.[9]

Ceruloplasmin plays a major role in iron mobilisation from the tissue stores. It catalyses oxidation of ferrous to ferric iron, which is essential for release to transferrin.[10,11] Deficiency of ceruloplasmin results in iron deposition in liver, pancreas, basal ganglia and other organs.[3] Aceruloplasminaemia is a lethal disease that typically presents in the fourth or fifth decade with neurological symptoms, retinal degeneration and diabetes mellitus. Brain involvement, particularly of the basal ganglia, thalamus and dentate nucleus, can present with various symptoms, such as cerebellar ataxia, involuntary movement, Parkinsonism, craniofacial dyskinesia and cognitive impairment.[12] Iron accumulation in the CNS is a unique finding among the classical iron overload syndromes.[9]

The diagnosis of aceruloplasminaemia can be made when there is an absence of serum ceruloplasmin, in

combination with low serum copper, low serum iron, high serum ferritin and increased hepatic iron concentration. Concomitantly a mild anaemia is also a constant finding in aceruloplasminaemia.[13,14] The diagnosis is supported by characteristic findings on MRI that are compatible with iron accumulation in liver and brain.

Early recognition and intervention is essential to alter the fatal course of this disease. Because of the anaemia, the role for phlebotomies is limited. Iron chelation therapy can be used for treatment of aceruloplasminaemia.[7,15-18] Normalisation of serum ferritin and decrease in hepatic iron overload have been described. However, its ability to remove iron from the CNS is doubted. Only one case described improvement of neurological symptoms while on the oral iron chelator deferasirox.[19] Therefore, one should focus on early awareness and treatment of this rare disease with iron chelators to prevent devastating neurological damage in patients with aceruloplasminaemia. In patients with high serum ferritin and low transferrin saturation in the absence of inflammation, alcoholism or dysmetabolic syndrome, MRI of the liver and serum ceruloplasmin should be considered. However, additional tests in patients with high ferritin level and normal transferrin saturation are not routinely recommended.

CONCLUSION

It is important to consider the possibility of a genetic iron overload syndrome in patients with neurological symptoms and hyperferritinaemia, even in the presence of low transferrin saturation. Otherwise the rare genetic iron overload disorder aceruloplasminaemia can be missed. Early treatment with oral iron chelators may prevent progression of neurological symptoms in patients with iron overload due to aceruloplasminaemia.

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PHOTO QUIZ

An abdominal mass: not a 'clear cut' case!

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CASE REPORT

A 52-year-old woman, previously healthy, was referred with progressive pain in the right inguinal region, after a fall two weeks before. She mentioned no other complaints. Physical examination was normal. Laboratory results showed: erythrocyte sedimentation rate 109 mm/h, C-reactive protein 239 mg/l and leucocytes 26.4 x 10^9/l with 83% granulocytes. Kidney and liver functions were normal. Ultrasound (*figure 1*) and CT scan (*figure 2*) revealed a large solid mass in the small pelvis with multiple abscesses extending into the right tubo-ovarian region and iliopsoas muscle, causing obstruction of the urether and hydronephrosis of the right kidney. In the cavum uteri a small hyperdense structure was visible, consistent with an intra-uterine contraceptive device (IUD). It turned out to have been in place for almost 30 years! The consulted gynaecologist proposed an exploratory laparotomy for diagnostic purposes as a malignancy was suspected.

Figure 1. *Ultrasound. Panel A shows hydronephrosis of the right kidney (arrow) with diffuse loss of cortex, suggesting the existence of a long-term obstruction (1. liver; 2. inferior vena cava; 3. aorta; 4. vertebra). Panel B shows a fluid collection in the right fossa iliaca (arrow) next to the right iliopsoas muscle (1) and above the ileum wing (2)*

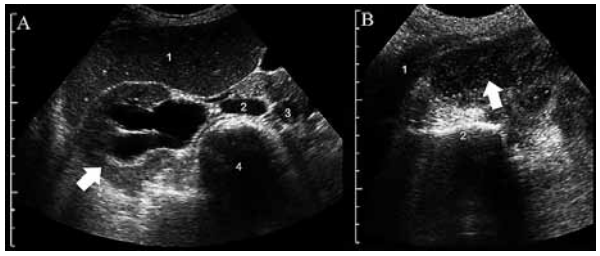
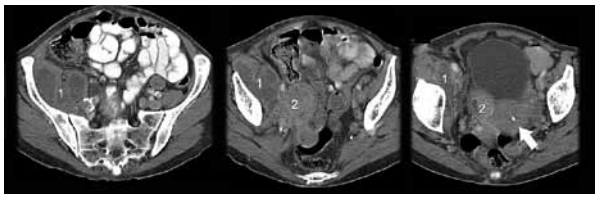


Figure 2. *CT scan after oral and intravenous contrast administration. Multiple abscesses (1), originating from a solid mass in the small pelvis (2), extending into the right iliopsoas muscle and tubo-ovarian region. In the cavum uteri a small hyperdense structure is visible, matching the outline of an IUD (arrow)*



WHAT IS YOUR DIAGNOSIS?

See page 320 for the answer to this photo quiz.

DIAGNOSIS

The presence of the IUD in combination with the elevated inflammatory markers gave rise to the suspicion of actinomycosis. To avoid unnecessary surgery we aspirated fluid from the psoas abscess. A Gram stain showed Gram-positive branching rods, suggestive of *Actinomyces* (*figure 3*). The IUD was removed, treatment with intravenous penicillin (6 million U/day) was started and a double-J-catheter was inserted to prevent deterioration of the right kidney function. Initially the patient developed a 'psoas sign' (*figure 4*), which improved after percutaneous drainage of the psoas abscess. Twenty days after admission the diagnosis was confirmed: *Actinomyces israelii* was cultured from the abscess fluid. After four weeks of intravenous penicillin she was discharged in a good clinical condition. Treatment was continued with oral feneticillin (4 dd 1 gram). Eight months after initiation of the antibiotic regimen, a CT scan no longer showed the extensive abnormalities previously observed. The feneticillin was stopped and the double-J-catheter was removed. There have been no signs of relapse.

Figure 3. *Gram stain showing the typical Gram-positive branching rod shaped bacteria, suggestive of Actinomyces*

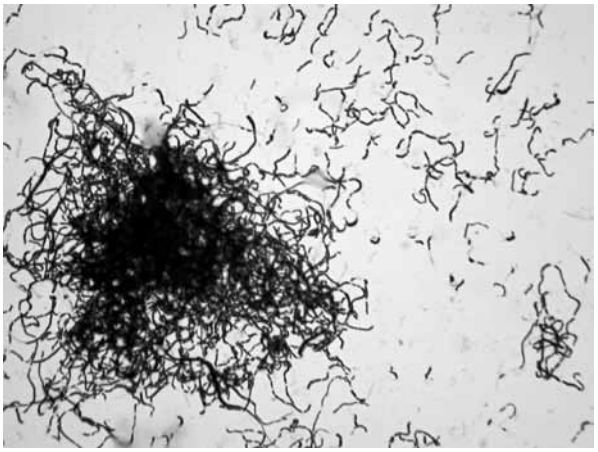


Figure 4. *Our patient with her right leg bent in a preferred position, suggestive of a 'psoas sign' (photograph taken with patient's permission)*

Infections with *Actinomyces* are rare, with an estimated yearly incidence of 1 per 100,000.[1] *Actinomyces* can be found as part of the commensal flora.[2] Actinomycosis can occur in immunocompetent individuals and most frequently involves the head/neck region and the pelvis. Actinomycosis of the pelvis originates from the female internal genital organs and is associated with presence of an IUD; the occurrence significantly increases with the use of copper-containing IUDs and the length of IUD use.[3-8] Actinomycosis is often mistaken for malignancies, due to chronic granulomatous disease with abscesses, fistulas and fibrotic masses.[9-13] This tumour-like behaviour frequently results in unnecessary surgery. To ensure early diagnosis a Gram stain should be performed, which shows typical Gram-positive branching rods (*figure 3*). Microscopic examination can reveal the characteristic yellow or brown 'sulphur granules', which consist of *Actinomyces* micro-organisms, tissue debris and calcium phosphate.[3,14] Because it concerns slow-growing anaerobic bacteria, cultures should be held for a long time and under strict anaerobic conditions. They should preferably be with abscess content.[9] For initial treatment high-dose intravenous penicillin is preferred, because the minimal inhibitory concentration for *Actinomyces* is high and antibiotics poorly penetrate the fibrotic masses and abscesses. After two to six weeks, intravenous penicillin can be switched to oral feneticillin, after an oral absorption assay is performed to ensure adequate feneticillin serum levels. Prolonged antibiotic treatment, varying from 6 up to 12 months, is necessary to prevent relapse.

This case illustrates the importance of including actinomycosis in the differential diagnosis of an abdominal mass, particularly in the presence of an IUD. Timely diagnosis with a Gram stain can prevent unnecessary surgery. Complete cure of the often extensive abnormalities can be achieved by antibiotic treatment and drainage of abscesses. Surgery is only justified in case of specific complications such as mechanical ileus.

ACKNOWLEDGMENTS

We would like to thank Dr. J.B.C.M. Puylaert (Department of Radiology, Medical Centre Haaglanden, The Hague) for providing us with the radiological images and descriptions.

We would like to thank M. Galli-Leslie (Department of Internal Medicine, Medical Centre Haaglanden, The Hague) for her advice on the English text.

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PHOTO QUIZ

Shoulder pain in two HIV-seropositive patients

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CASE 1

Patient K, born in 1967, started complaining about progressive pain in his left paretic shoulder in the summer of 2008. Both passive and active movements were painful. The X-ray is shown in *figure 1A*. The patient's medical history was remarkable for the diagnosis of *Pneumocystis jiroveci* pneumonia (PJP) in 2002, with a CD4 count of 1 x 10^6/l and a plasma HIV-RNA of 140,000 copies/ml. After treatment of the PJP, antiretroviral therapy consisting of stavudine, lamivudine and lopinavir/ritonavir was started; after one year lopinavir/ritonavir was substituted by nevirapine because of a cholesterol of 5.9 mmol/l. His HIV-RNA became undetectable (<40 copies/ml) and his CD4 count increased steadily to 520 x 10^6/l. In 2003 he suffered from a cerebrovascular accident. An MRI showed infarction in the area of the left medial cerebral artery, and multiple T2 hyperintense lesions of gray and white matter in the basal ganglia and peripheral parts of the brain. With the presumed diagnosis of vasculitis, prednisone was started and patient received a cumulative dose of 10.5 gram between 2003 and 2004. In this period alendronine acid was started.

CASE 2

Patient L, born in 1963, was diagnosed with HIV infection in 1999. At that time the CD4 count was 40 x 10^6/l, and toxoplasmosis cerebri was diagnosed. After treatment, antiretroviral therapy was started with stavudine, lamivudine and indinavir/ritonavir. Within a few months the plasma HIV-RNA declined below the detection level, and within one year the CD4 count increased to 500 x 10^6/l. In June 2005, she had reactivation of the toxoplasmosis, i.e. on a CT scan oedema surrounding the calcified old toxoplasmosis lesion. She was successfully treated with sulfadiazine/pyrimethamine and dexamethasone. Steroids were gradually tapered and stopped in the summer of 2006. In the summer of 2008 she developed pain in the upper left arm. The X-ray is shown in *figure 1B*.

Figure 1A. *X-ray of left humerus of patient K showing sclerotic changes centrally*

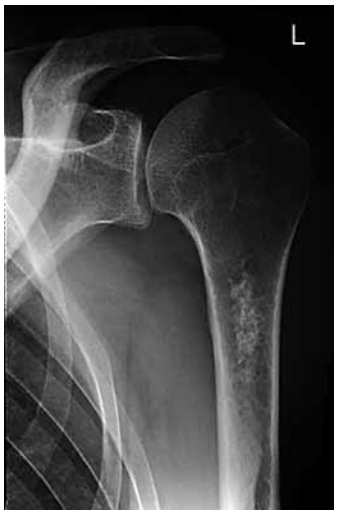
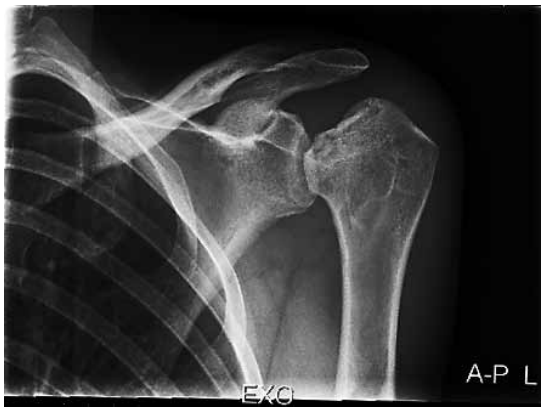


Figure 1B. *X-ray of left humerus of patient L showing subchondral collapse*



WHAT IS YOUR DIAGNOSIS?

See page 325 for the answer to this photo quiz.

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PHOTO QUIZ

An unusual urinary tract infection!

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CASE REPORT

A 30-year-old lady presented to the specialist parasitology diagnostics department in Karachi, Pakistan, with a complaint of having noticed 'eggs' in her urine. She had been diagnosed previously as having 'schistosomiasis' at a peripheral diagnostic laboratory. Past medical history was significant for a laparotomy for uterine perforation one year ago, and her husband also reported her to have presented previously to many physicians with multiple symptoms including abdominal pain and dysuria. 'Motile eggs' had often been seen in the patient's urine over the past six months, which appeared typically when she was dehydrated and passed less urine than normal. Her dysuria was intermittent. Several concentrated microscopy smears of the urine were negative for schistosoma eggs, and the patient was reassured. However, on the insistence of the patient's husband, the first submitted specimen was re-examined for 'motile worms'. Gross examination of the specimen showed a small (0.5 cm) segmented 'worm'. Microscopy revealed the diagnosis (*figure 1*). The patient was subsequently advised to undergo cystoscopy and bladder biopsy to confirm the unusual infestation.

Figure 1. *Photomicrograph (400 x).*

WHAT IS YOUR DIAGNOSIS?

See page 326 for the answer to this photo quiz.

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Large nocturnal eyes causing gastrointestinal bleeding in asymptomatic multiple myeloma

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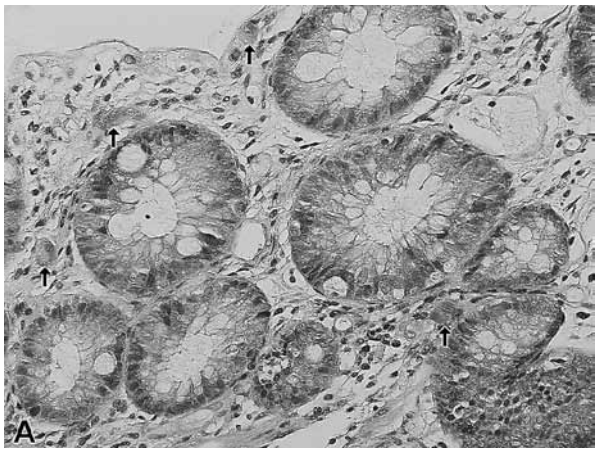
CASE REPORT

A 67-year-old male was transferred to our Intensive Care Unit because of recurrent severe gastrointestinal bleeding after treatment for *Enterococcus faecalis* urosepsis with amoxicillin, corticosteroids, fluid resuscitation and norepinephrine. About six months earlier, multiple myeloma (MM) IgG-lambda type had been diagnosed with 15% bone marrow infiltration. Since no evidence of related end-organ damage was present, fulfilling the criteria for asymptomatic myeloma, an expectative management was chosen.[1] At admission, laboratory examination showed elevated C-reactive protein (177 mg/l) with leucocytosis (16 x 10^9/l) and lymphopenia (0.5 x 10^9/l). Blood and stool cultures remained negative. Angiography revealed contrast extravasations in the upper gastrointestinal tract, but unsuccessful coil embolisation necessitated short bowel resection. Histologically, the resected specimen showed generalised ulcerations. T-cell counts were decreased (CD4 0.23/CD8 0.22 x 10^9/l) with ANA/ANCA, HIV and Epstein-Barr virus testing negative. Colonoscopy also showed a diffuse ulcerative mucosa with biopsies demonstrating reactive colonic mucosa with some inflammation (*figure 1*). A few microthrombi were found in submucosal vessels.

Figure 1. *Reactive colonic mucosa with viral inclusions, mainly in endothelial cells (arrows)*



WHAT IS YOUR DIAGNOSIS?

See page 327 for the answer to this photo quiz.

DIAGNOSIS

In case 1, the X-ray of the shoulder showed sclerotic changes central in the left humerus without cortical changes or osteolysis and was compatible with avascular necrosis. In case 2, the X-ray showed avascular necrosis of the left upper humerus, with subchondral collapse. Avascular necrosis (AVN) of the bone has been described in HIV-infected patients since 1990 and the incidence seems to be increasing.[1] An annual incidence as high as 1.19 per 1000 patients has been reported, which is 29-fold higher than the population-based incidence.[1] In HIV-negative patients, the majority of AVN is of the femoral head, but it has also been described in humerus, knee, ankle and smaller joints.[2] The explanation for the high percentage of AVN occurring in the femoral head is multifactorial, but mechanical stress probably plays an important role.[2] This was shown in rats that were forced to stand on their hind legs while being fed; 33% developed AVN.[2] AVN has been associated with more than ten different disease entities, trauma being the most frequently occurring aetiology.[2] Of non-traumatic causes, use of corticosteroids is most commonly reported, but also the consumption of alcohol, infections (among which HIV infection), hyperbaric events, storage disorders, marrow infiltrating diseases, coagulation defects and some autoimmune diseases have been described.[2] The final common pathway for the development of AVN is a compromise in the blood flow to the bone. In non-traumatic AVN, epidemiology suggests that pathogenesis is often multifactorial. The concept of cumulative cell stress stems from data that show a higher rate of steroid-induced AVN in systematically ill patients. Both patients described here fit this theory, since they had used high cumulative doses of steroids, were infected with HIV for a long period, and had hypercholesterolaemia (patient K). Whether there is a causal association with HIV infection or antiretroviral treatment has not been completely elucidated. Prior corticosteroid use has been reported to be a significant risk factor, although traditional risk factors such as alcoholism, radiation therapy, hypercholesterolaemia, and hypertriglyceridaemia were also described.[2] In a case-control study from France[3] describing 12 HIV-seropositive patients with AVN, in the multivariate analysis only prior steroid use and alcohol abuse were associated with AVN. CD4 count nadir, the use of highly active antiretroviral therapy (HAART), use of protease inhibitors, duration of antiretroviral therapy, or HIV load over 500 copies/ml were not statistically significantly associated with AVN, and neither were HAART-induced metabolic disorders such as fat wasting, fat accumulation, high cholesterol or hypertriglyceridaemia or diabetes mellitus.[3] In another case-control study of 26 patients with HIV and AVN, a lower CD4 count nadir and previous opportunistic infections were associated with AVN, whereas duration of therapy was not.[4]

To our knowledge, our patients are the first reported HIV-infected patients with AVN of the upper humerus. Early imaging of painful bones and joints in HIV patients with concurrent or prior steroid or alcohol use seems warranted.

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ANSWER TO PHOTO QUIZ (PAGE 323)

AN UNUSUAL URINARY TRACT INFECTION!

DIAGNOSIS

The photomicrograph reveals a distorted fly larva with several spines projecting from its anterior end. Infestation of human tissue by fly larvae is termed myiasis.[1] The larva was examined after a 48-hour delay and hence its morphology was not preserved. Genus determination based on chitinous plates at its posterior end was also not conclusive for the same reason.

Urinary infestation is a rare manifestation of myiasis[1] and only a few cases have been reported in literature. Most cases are due to poor personal hygiene.[2] Cases following surgery on the urogenital tract have also been reported.[3] This was also the likely predisposing factor in our patient. However, the bladder biopsy was not submitted to our centre and hence a confirmatory diagnosis of urinary myiasis could not be made. Cases of myiasis are usually diagnosed after prolonged antibiotic therapy of symptoms and due to the unusual features of the larvae, the diagnosis may be missed by physicians and microbiologists.[1,4] Awareness of such cases by lab technicians and physicians facilitates rapid diagnosis.

Treatment of myiasis consists of manual or surgical removal of the fly larvae.[1] This was explained to the patient and further management was deferred until definitive diagnosis by cystoscopy and biopsy.

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ANSWER TO PHOTO QUIZ (PAGE 324)

LARGE NOCTURNAL EYES CAUSING GASTROINTESTINAL BLEEDING IN ASYMPTOMATIC MULTIPLE MYELOMA

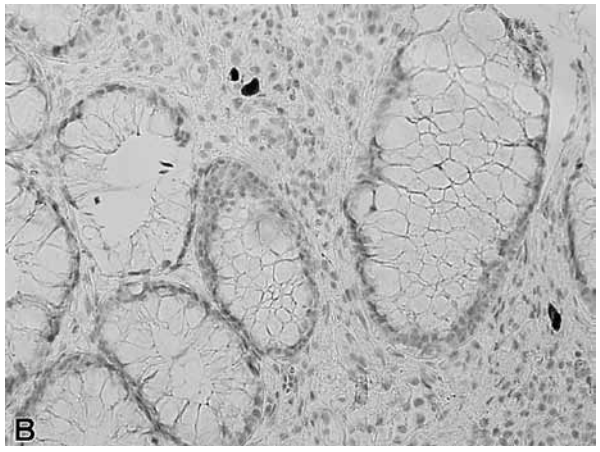
DIAGNOSIS

Our patient had positive cytomegalovirus (CMV) IgG and negative IgM serum antibodies with high levels of CMV viraemia (2.2 x 10^5c/ml) indicative of acute reactivated CMV infection. Immunohistochemical staining for CMV on colonic biopsies was positive (*figure 2*). CMV is an extremely common pathogen worldwide, with 50 to 80% of adults infected by the age of 40 years. Although most healthy people infected by CMV have no symptoms, CMV infection can be life-threatening in immunocompromised patients, affecting different organ systems including the gastrointestinal tract. The CMV enterocolitis of our patient was probably induced by multiple myeloma associated T-cell dysfunction and perhaps worsened by the corticosteroids used to treat his initial *E. faecalis* sepsis. Despite initiation of ganciclovir, our patient died as anastomotic leakage resulted in abdominal sepsis with multiple organ failure.

Multiple myeloma (MM), also known as Kahler's disease, is the second most prevalent haematological malignancy representing 2% of all cancer deaths. At diagnosis, before treatment, a significant number of MM patients exhibit impaired cellular and humoral immune responses.[2] In one study, about 10% of patients died early after MM diagnosis as a result of bacterial infections, in particular with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Escherichia coli*.[3] However, fatal CMV infection in untreated MM is rare, with to our knowledge only a few cases published.[4] Gastrointestinal CMV infection induces mucosal ulcerations resulting in abdominal pain, fever and bloody diarrhoea. CMV also promotes the formation of micro-thrombi, leading to secondary ischaemic damage. Diagnosis may be obtained by colonoscopy with biopsies. Upon direct visualisation by colonoscopy, inflammation with focal mucosal haemorrhage, oedematous folds, and polypoid lesions can be seen. Histological specimens typically show viral inclusions, referred to as owl's eyes (*arrows figure 1*).

In conclusion, asymptomatic and thus often untreated myeloma patients may display cellular and humoral immunodeficiencies which constitute an important predisposing factor for fatal opportunistic bacterial and viral infections.

Figure 2. *The immunohistochemical stain for CMV is positive in the nucleus of virus containing cells (black dots)*



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CASE REPORT

Semi-final masked hypertension

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ABSTRACT

Masked hypertension is normal blood pressures (BP) in a clinical setting and high BP during ambulatory monitoring.[1] Although these patients are at higher cardiovascular risk, there is still no clear consensus definition of masked hypertension.

KEYWORDS

Hypertension, ambulatory monitoring, cardiovascular events

CASE REPORT

A 48-year-old male with new-onset atrial fibrillation and a mean blood pressure (BP) of 110/70 during repetitive measurements at the outpatient clinic was scheduled for 24-hour ambulatory BP monitoring due to unexplained left ventricular hypertrophy. His BP was clearly high in the evening of the ambulatory monitoring as shown in *figure 1*. Reassessing the date and time of measurements revealed peeks of hypertensive episodes concomitant with the goals of the FIFA 2010 world cup semi-final match Uruguay vs. Netherlands (*figure 1*). This high susceptibility for external influences and triggered masked hypertension is a risk factor of developing cardiovascular events. Important sport events are known to provoke a sufficient level of stress to trigger symptomatic cardiovascular events.[2,3]

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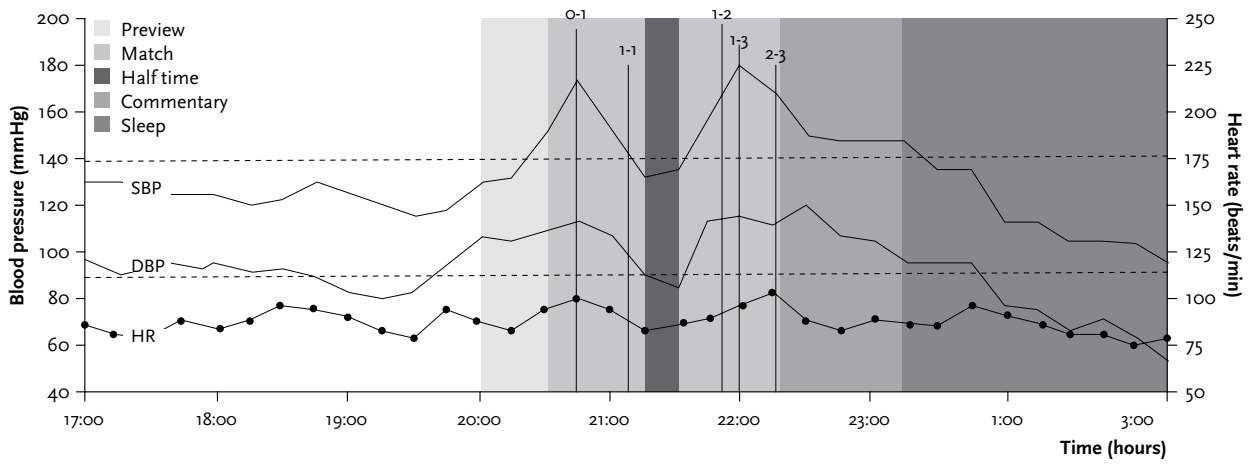
Figure 1. *Registration of 24-hour ambulatory blood pressure measurement*



SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate.

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LETTER TO THE EDITOR

Intermittent use of pantoprazole and famotidine in severe hypomagnesaemia due to omeprazole

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Dear Editor,

We read with interest the article 'Hypomagnesaemia due to use of proton pump inhibitors- a review', by Kuipers *et al.*[1] We present here a patient with severe hypomagnesaemia due to omeprazole. We emphasise that the intermittent use of pantoprazole and a histamine H2-receptor antagonist may be an appropriate therapeutic alternative for normalising serum magnesium concentrations, without recurrence of gastro-oesophageal symptoms.

The patient is a 67-year-old man who presented in April 2010 with paresthesia, numbness and weakness in his limbs. His medical history included hypertension, chronic obstructive airways disease and ischaemic heart disease. In October 2006 and December 2006 he was hospitalised because of tetany, with severe hypocalcaemia and hypomagnesaemia, which was attributed to alcoholism, without further studies. In 2006 he was on omeprazole therapy for gastroprotection. The patient was followed up in the outpatient clinic until July 2007, with serum magnesium at the lower limit of normal range or slightly reduced despite oral magnesium supplements. After that, he was lost to follow-up; he continued treatment with omeprazole until his current hospitalisation. The laboratory findings showed hypocalcaemia (1.55 mmol/l), hypomagnesaemia (0.14 mmol/l) and a level of 25-OH-vitamin D at 27.45 nmol/l (our laboratory range, 29.9 to 134.7). The complete blood count, and serum levels of albumin, glucose, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, sodium, potassium, and phosphorus were normal. He denied alcohol use, and other causes of hypomagnesaemia were absent, including family history of genetic electrolyte disorders, diarrhoea, and use of laxatives, or diuretics. The slightly low level of 25-OH vitamin D coincided with extraction at the beginning of spring. His medications were aspirin, losartan, diltiacem, fluticasone/salmeterol inhalers and omeprazole 20 mg daily. Omeprazole was stopped and famotidine (40 mg/12 h) initiated. Likewise, he began receiving intravenous magnesium sulphate and calcium gluconate. Then, oral replacement therapy with magnesium supplements (12 mEq/day), calcium carbonate and vitamin D was prescribed. Seven days after admission, the fractional excretion of magnesium was 1.05 (urinary magnesium of 0.49 mmol/l). Fourteen days after admission, the fractional excretion of magnesium was 9.33 (urinary magnesium of 8.27 mmol/l), with a serum magnesium of 0.86 mmol/l. Several authors have raised the possibility that genetic factors might result in increased susceptibility to PPI-induced hypomagnesaemia, as might be the case with heterozygous carriers of TRPM6 mutations.[1-3] In our patient, we searched for mutations by sequencing of all 39 exons of the TRPM6 gene and did not find mutations that can be seen in patients with familial hypomagnesaemia with secondary hypocalcaemia.[4] The laboratory reported the presence of single-nucleotide polymorphisms, rs7018994 (homozygous), rs7859201 (homozygous) and rs11144089 (heterozigous), and to our knowledge, at present, it is unknown whether the combination of these polymorphisms might have a negative impact on protein activity.[5]

After stopping omeprazole, the patient complained of reflux dyspepsia. A gastroscopy showed peptic oesophagitis and thickening of the gastric folds. A distal duodenal biopsy was normal and the IgA anti-tissue transglutaminase antibodies were negative. The gastroenterologists recommended to resume treatment with omeprazole 40 mg daily, and seven days after resuming omeprazole (and continuing with 12 mEq daily of oral magnesium supplements) the level of serum magnesium decreased from 0.86 mmol/l to 0.70 mmol/l, and the fractional excretion of magnesium

decreased to 1.13 (urinary magnesium decreased from 8.27 mmol/l to 0.41 mmol/l). Given the inadequate control of the gastrointestinal discomfort with famotidine in high dosage, and malabsorption of magnesium with omeprazole, we chose alternate-day therapy with omeprazole and famotidine, combined with domperidone and magnesium supplements (12 mEq daily). With this therapeutic regimen the patient was asymptomatic but the fractional excretion of magnesium was low, indicating magnesium deficiency.[6] Some researchers have postulated that pantoprazole is the least potent PPI at suppressing gastric acid secretion.[7] Thus, we replaced omeprazole by pantoprazole 40 mg/24 hour, three days a week (on Monday, Wednesday and Friday), and famotidine 40 mg/24 hour, four days a week (on Tuesday, Thursday, Saturday and Sunday), combined with oral domperidone and magnesium (12 mEq daily). With this last therapeutic regimen, which reduces the administration of PPIs an extra day per week, the patient is asymptomatic and 20 days later the fractional excretion of magnesium had increased to 5.02.

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2. Kaplan NM. Clinical Hypertension. 7th ed. Baltimore: Williams & Wilkins; 1998.
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