Markers and outcome measures in chronic immune mediated arthritis: from association studies to prediction models

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List of abbreviations

ACPA	anti-citrullinated protein/peptide antibodies
ACR	American College of Rheumatology
AhFibA	anti-human fibrinogen antibodies
AKA	anti-keratin antibodies
ANCA	anti-neutrophyl cytoplasmic antibodies
APF	anti-perinuclear factor
AS	ankylosing spondylitis
ASCA	anti-*Saccharomyces cerevisiae* antibodies
AUC	area under the curve
CARD	caspase activation and recruitment domain
CCP	cyclic citrullinated peptide
CD	Crohn's disease
COR	common odds ratio
DAS	disease activity score
DMARD	disease modifying anti-rheumatic drug
ELISA	enzyme linked immunoassay
ESSG	European Spondyloarthropathy Study Group
HAQ	health assessment questionnaire
HLA-SE	HLA-shared epitope
IBD	inflammatory bowel dieseas
IIF	indirect immunofluorescence
LIA	line immunoassay
MLP	multilayered perceptrons
MDP	muramyl dipeptide
NOD	Nuclear oligomerization domain
NPV	negative predictive value
NSAID	non steroidal anti inflammatory drug
OR	odds ratio
pepA	peptide A
pepB	peptide B
PPV	positive predictive value
PsA	psoriatic arthritis
RA	rheumatoid arthritis
ReA	reactive arthritis
RF	rheumatoid factor
ROC	receiver operating characteristics
SNP	Single nucleotide polymorphism
SpA	spondyloarthropathy
TNF	tumor necrosis factor
UC	ulcerative colitis
uSpA	undifferentiated spondyloarthropathy

INTRODUCTION

General introduction

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General Introduction

When a rheumatologist is consulted by a patient with joint complaints, the physician attempts to find answers to the following questions: 1) What is the diagnosis? 2) What is the present disease activity? 3) What is the prognosis? 4) What is the best therapy to lower the present disease activity and to ameliorate the prognosis? The answers to those questions are formed by a complex composite picture, which is based on clinical findings and technical investigations.

The clinical findings are very important and include the personal and familial history, and the clinical examination. Generalisation of these findings can be obtained by specific questionnaires and standardized clinical measures. Blood tests are available to measure systemic inflammation or to search for diagnostic markers, present in different rheumatic diseases. Also some genetic tests may be helpful. Imaging techniques such as standard X-rays, especially useful to evaluate bone damage, provide other important tools. All those measures and markers can be seen as variables that, similarly to the hundreds of single brushstrokes of a master's painting, are combined in the physician's mind in order to find the answers to the mentioned questions. Picasso reduced a complex drawing of a bull in only 4 lines (front-page). In the present work, we will try to find out how a limited number of variables can be combined in models for clinical decision-making.

1. Description of the different rheumatic diseases

One of the most important activities of a rheumatologist is to treat and follow-up patients with chronic immune mediated arthritis. These encompass rheumatoid arthritis (RA), spondyloarthopathy (SpA), psoriatic arthritis (PsA) and arthritis associated with connective tissue diseases such as systemic lupus erythematosus (SLE) and systemic sclerosis.

In the present study we will limit the discussion to the most prevalent diseases: RA, SpA and PsA.

1.1. Rheumatoid arthritis (RA)

RA is the best-known immune mediated inflammatory joint disorder and affects about 0.3% to 1% of the general population, predominantly women [Lawrence 1998, Gabriel 1999, Guillemin 2005]. The disease is defined by a symmetric chronic polyarthritis characterised by inflammatory pain, morning stiffness and joint swelling. It typically affects the small joints of hands and feet (excluding the distal interphalangeal joints, more typically affected in PsA). The chronic joint inflammation may lead to destruction of cartilage and bone, which is radiologically seen by joint space narrowing and marginal erosions. Further joint destruction may occur by additional alterations of the

tendons and the joint capsule, resulting in deformations of hands and feet (Figure 1.1).

General symptoms such as fatigue and extra-articular manifestations may accompany the articular symptoms. Common extra-articular manifestations encompass rheumatoid nodules, serositis, hematologic manifestations (such as Felty's syndrome), vasculitis, secondary Sjögren's syndrome and amyloidosis. An important extra-articular manifestation, especially useful for diagnostic purposes is the occurrence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA). A combination of clinical and radiological symptoms and the presence of the rheumatoid factor resulted in classification criteria for RA, which are especially useful for study purposes (Table 1.1) [Arnett 1988].

In order to relieve pain and to avoid joint destruction, adequate therapy is needed. The therapeutic arsenal for RA encompasses non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs) and corticosteroids. NSAIDs control the symptoms of pain or stiffness, but they have no effect on the disease progression. Therefore they should be combined with DMARDs in order to suppress the local joint inflammation and to avoid joint destruction on the long term. Among the classical DMARDs, the historically used gold salts and anti-malarials have been replaced by sulfasalazine, methotrexate, and more recently leflunomide. The most frequently used biologicals block tumour necrosis factor-α (TNF). TNF blocking can be obtained by the use of a soluble TNF-receptor fusion protein (etanercept), a chimeric IgG anti-TNF-α monoclonal antibody (infliximab) or a recombinant humanized IgG monoclonal antibody (adalimumab). Other biologicals block interleukin-1 (Anakinra), CD20 (rituximab) or the T-cell co-stimulatory pathway (CTLA4-Ig). Finally, corticosteroids are a potent suppressor of the inflammatory response with a quick effect on pain and stiffness. Their well-known side effects limit their use [O'dell 2004, Olsen 2004, Haque 2005].

Figure 1.1: erosions and deformations caused by RA
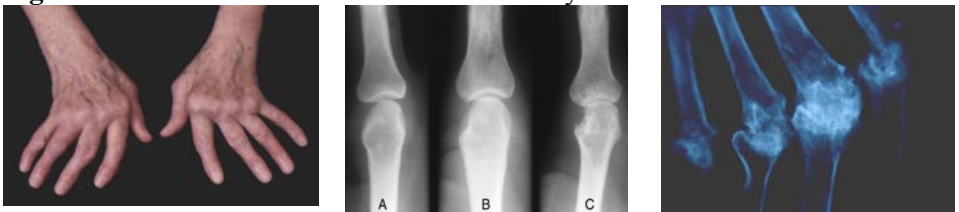
Table 1.1: ACR revised criteria for the classification of RA (1988)

1.	Morning stiffness in and around the joints, lasting at least one hour before maximal improvement
2.	Arthritis of three or more joint areas (soft tissue swelling or fluid, observed by a physician)
3.	Arthritis of hand joints (proximal interphalangeal, metacarpophalangeal, or wrist joints)
4.	Symmetric arthritis
5.	Rheumatoid nodules (observed by a physician)
6.	Serum rheumatoid factor (demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects).
7.	Radiographic changes on hand or wrist joints (erosions or bony decalcification)

A patient can be classified as having rheumatoid arthritis if at least 4 of these criteria are satisfied.

Item 1 through 4 must be present for at least 6 weeks

1.2. The spondyloarthropathy concept and its relation with inflammatory bowel disease

1.2.1 Spondyloarthropathy

Spondyloarthropathy (SpA) is a concept that includes different diseases with common characteristics such as peripheral arthritis (asymmetrical, pauci-articular, and mainly involving the lower limb), spinal inflammation (inflammatory back pain, sacroiliitis, spondylitis), enthesopathy, extra-articular manifestations (anterior uveitis, gut inflammation, skin symptoms), familial aggregation and the association with HLA-B27. The disease entities of SpA encompass ankylosing spondylitis (AS), reactive arthritis (ReA), arthritis associated with inflammatory bowel disease (IBD), some forms of PsA, some forms of juvenile chronic arthritis and undifferentiated SpA. Different classification criteria for the SpA subtypes and the SpA concept have been described in order to facilitate classification (e.g. for clinical studies).

For the SpA concept, a set of criteria proposed by the European Spondyloarthropathy Study Group (ESSG) [Dougados 1991] and the Amor criteria are available [Amor 1991] (Table 1.2.1.a and b). For reasons of simplicity, the ESSG criteria are more widely used. For AS the Rome [Kellgren 1962] and New York criteria [Bennett 1967] have been revised by van der Linden (Modified New York criteria) [van der Linden 1984] (Table 1.2.1.c).

The presence of sacroiliitis is an important criterion in all classification sets. Sacroiliitis can be observed on plain radiographs and is generally scored using the New York grading system: 0 – normal; 1 – suspicious; 2

– localized sclerosis, erosion, joint widening; 3 – diffuse sclerosis, erosion, widening; 4 – ankylosis [van der Linden 1984] (Figure1.2.1.a) . The prevalence of SpA can be estimated between 0.2% and 0.9% [Saraux 2005, Braun 1998, Lawrence 1998]. The therapeutic strategies for SpA were until recently limited to the use of NSAID, physiotherapy and sulfasalazine (for patients with peripheral arthritis). In analogy with RA, treatment with biologicals, such as TNF-α blockers proved also to be very effective [Van den Bosch 2002, Anadarajah 2005].

Table 1.2.1.a: ESSG criteria for spondyloarthropathy (1991)

Inflammatory spinal pain
OR
Synovitis Predominantly of the lower limbs Asymmetric
AND
One or more of the following:
- Positive family history (presence in first-degree or second-degree relatives of any of the following: AS, psoriasis, acute uveitis, reactive arthritis, inflammatory bowel disease)
- Psoriasis
- Inflammatory bowel disease
- Urethritis, cervicitis, or acute diarrhea within 1 month before arthritis
- Buttock pain alternating between right and left gluteal areas
- Enthesopathy
- Sacroiliitis

Table 1.2.1.b: Amor criteria for spondyloarthropathy (1991)

Clinical symptoms or past history of:	
1. Lumbar dorsal pain at night and/or morning stiffness of lumbar or dorsal area	1
2. Asymmetric oliogoarthritis	2
3. Buttock pain (if alternate buttock pain)	1(2)
4. Dactylitis	2
5. Heel pain or other well defined enthesopathy	2
6. Acute anterior uveitis	2
7. Non-gonococcal urethritis or cervicitis within 1 month before onset of arthritis	1
8. Acute diarrhea within 1 month before the onset of the arthritis	1
9. Psoriasis or balanitis or inflammatory bowel disease	2

10. Sacroiliitis ≥ grade 2 bilateral , ≥grade 3 if unilateral	2
11. Presence of HLA-B27 antigen and/or family history of ankylosing spondylitis, reactive arthritis, uveitis or inflammatory bowel disease	2
12. Clear-cut improvement within 48 h after taking NSAID or rapid relapse of pain after discontinuation	2
Number of points required for the diagnosis of SpA: 6 points	

Table 1.2.1.c: Modified New York criteria for AS (1984)

Diagnosis
Clinical criteria
- Low back pain and stiffness for more than 3 months which improves with exercise, and is not relieved by rest.
- Limitation of motion of the lumbar spine in both the sagittal and frontal planes.
- Limitation of chest expansion relative to normal values corrected for age and sex

Radiological criterion
- Sacroiliitis grade ≥2 bilaterally or sacroiliitis grade 3-4 unilaterally

Grading
Definite ankylosing spondylitis if the radiological criterion is associated with at least 1 clinical criterion
Probable ankylosing spondylitis if
1. Three clinical criteria are present
2. The radiological criterion is present without any signs or symptoms satisfying the clinical criteria (other causes of sacroiliitis should by considered)

Figure 1.2.1: Radiographic changes of sacrolliliits

1.2.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) represents a whole spectrum of disorders which affect the gastrointestinal tract and includes two major entities, namely Crohn's disease (CD) and ulcerative colitis (UC). CD is a transmural inflammatory disease that may involve any part of the alimentary tract, mainly the ileocolonic area and the perianal region, whereas UC is a mucosal disease with almost exclusive colonic involvement. This gut inflammation leads to symptoms of abdominal pain and (bloody) diarrhoea, and may result in different complications for which resective bowel surgery may be needed. The incidence of IBD is still increasing and responds to a lifetime risk for either one of the two diseases of about 0.5-1%. Worldwide, the incidence of UC seems to be higher than the incidence of CD, but in Belgium and France, CD seems to be more common than UC [Ekbom 2004]. Different treatment options are available, but interestingly, sulfasalazine and some TNF-α blockers are, similarly to SpA, also effective in CD [Panaccione 2004].

1.2.3. Shared pathogenic pathways in SpA and IBD.

The pathogenesis of SpA and IBD is still unknown, but there is growing evidence that a disturbed handling of bacterial antigens might be an important pathway in both diseases. In CD, it has been hypothesized that different bacteria such as *Yersinia*, *Listeria* and *Escherichia coli*, may have an important pathogenetic role [Hugot 2003]. Especially strains that are able to invade, survive, and replicate in host cells without inducing cell death, such as the adherent invasive *Escherichia coli* (AIEC) strains, might be important to induce persistent intestinal inflammation, breaching the intestinal barrier and, activating macrophages [Glasser 2001]. It can be hypothesized that this chronic intestinal inflammation may be a trigger for the occurrence of joint manifestations. Similarly, also in patients who primarily present with joint symptoms in the context of AS, ReA or uSpA, subclinical intestinal inflammation can be demonstrated [Mielants 1988, Mielants 1993, De Vos 1989, De Keyser 1998]. Also immunological similarities between IBD and SpA could be demonstrated. The αEβ7 integrin is up-regulated on gut mucosal T cells from patients with inflammatory bowel disease and on gut mucosal T cell lined from AS patients [Elewaut 1998, Van Damme 2001a]. An impaired Th1 cytokine profile seems to have an important role in the pathogenesis of both SpA and CD [Van Damme 2001b, Van Damme 2001c]. A similar increase in expression of the E-Cadherin/catenin complex has been demonstrated in active subclinical gut inflammation in SpA and CD patients [Demetter 2000a, Demetter 2000b]. The colon mucosa of patients with SpA and CD is enriched with macrophages expressing the scavenger receptor CD163, which can also be found in the synovial membrane of SpA patients [Baeten 2002, Demetter 2005]. On the other

hand, the similarities between CD and SpA do not explain the full spectrum of SpA or CD. For example HLA-B27 is a major genetic susceptibility factor for AS and ReA, but is less frequently seen in patients with IBD-related SpA [Mielants 1993].

1.3. Psoriatic arthritis

PsA shares features from RA and SpA. Moll and Wright initially described PsA as a (seronegative) inflammatory arthritis with psoriasis and suggested a classification system into 5 groups (Table 1.3a) [Moll 1973]. Since that time, a plea of other definitions and classification systems has been described. Moll and Wright proposed to consider patients with a positive RF as RA patients with concomitant cutaneous psoriasis. However, they indicated already that a positive RF may be due to false positivity (which can be found in about 5% of a healthy control population). Therefore, a working definition based on the presence of some features seemed to be helpful in distinguishing PsA from RA: a clinical inflammatory or radiographic enthesopathy, distal interphalangeal joint involvement, sacroilliitis or spinal inflammation, uncommon arthropathies, dactylitis and monoarthritis or oligoarthritis [McGonagle 1999]. Also the initial classification into 5 subtypes (Table) was not easily applicable in clinical practice since the pattern of the disease may evolve during follow-up. Especially the polyarticular disease will become more prevalent during follow-up by evolution from oligoarticular to polyarticular disease [Marsal 1999, Kane 2003]. In order to resolve those classification problems, an international study is underway to define the optimal classification criteria for PsA by the CASPAR study group [Taylor 2005].

The treatment of PsA is very similar to the treatment of RA and consists of the use of NSAIDs and DMARDs [Mease 2004]. Also biological agents such as the TNFα-blockers are highly effective [Mease 2005].

Table 1.3: Moll and Wright classification of psoriatic arthritis (1979)

Skin psoriasis with spondylitis or arthritis
1. Arthritis of the distal interphalangeal joints of the hands and feet
2. Arthritis mutilans with sacroiliitis
3. Symmetric arthritis indistinguishable from RA, but with negative RF
4. Asymmetric pauciarticular arthritis with small joint involvement
5. Ankylosing spondylitis with of without peripheral arthritis

2. Data analysis

The markers, obtained by technical investigations and clinical measures can be used in the clinical decision-making. This section will show that markers should not only be statistically significant, but also clinically significant. We will discuss some selected methods to evaluate the clinical importance of a marker or a clinical measure and how they can be combined in prediction models and composite indices.

2.1 The single variable

The validation of a single marker or clinical measure can go through different steps. The available methods to validate a single variable highly depend on the characteristics of the variable. A variable is called continuous, when the variable is a scale, such as height, age, temperature… In contrast, a variable is categorical when it is described by a limited number of categories. The simplest categorical variable is a dichotomous variable with only two categories, e.g. sex, yes/no, present/absent. When more than two categories exist, the variable can be described as multinomial or ordinal. A variable is ordinal when there is a certain hierarchy (e.g. good/better/best), in the other case it is called multinomial (e.g. 4 seasons).

2.1.1 How to measure association?

The exploration of a marker generally starts with the discovery that a certain marker is associated with a given disease. If the marker is continuous, an association will be reported if the height of the marker's level differs between the two investigated populations. If the marker is dichotomous, an association will be reported if the frequencies of a positive test differ between the two investigated populations. A frequently reported measure for an association between dichotomous variables is the odds ratio (OR) [Bland 2000, Glas 2003]. The formula to calculate the OR is explained in Table 2.1.1.a. An OR of 1 indicates that there is no association, an OR > 1 indicates a positive association and an OR <1 indicates a negative association. An OR of > 2 or 3 is important and means that the odds for disease is 2 or 3 times higher when the test is positive. When 2 different markers are both associated with a certain disease, one should always look whether there is also an association between those 2 different markers. If this is the case, one should be aware that the Simpson's paradox may occur. This paradox is described in Table 2.1.1.b [Simpson 1951] and proves that a reported association can disappear or invert after controlling for confounding variables. This can be seen both with dichotomous and continuous data and can be discovered by constructing 2x2x2 tables as displayed in Table 2.1.1.b [Agresti 1996]. More complex problems can be explored with logistic regression and general linear models which will be discussed later.

Generally, means, differences in mean, ORs… are calculated in a sample of the whole population and thus are in fact estimates of the real values of the whole population. This means that there may be a difference between this estimate and the real value. This uncertainty can be expressed by the 95% confidence intervals which indicate that the true value has a probability of 95% of lying in this range [Henderson 1993].

An alternative way to express this uncertainty is by means of p-values. This p indicates the probability that the null-hypothesis is true. In our example, we want to test whether the OR differs from 1. We thus can formulate a null-hypothesis that the OR is 1 and an alternative hypothesis that the OR differs from 1. When the 95% confidence interval for the OR does include 1, one must retain the null hypothesis and p will be more than 0.05. In the other case, when the 95% confidence interval for the OR does not include 1, we have less than 5% chance that the true OR is 1. In that case, the p-value corresponds to <0.05.

Table 2.1.1.a: Calculation of an odds ratio

		Disease	
		Present	Absent (healthy)
Test	Positive	a number of diseased patients with positive test	b number of healthy controls with a positive test
	Negative	c number of diseased patients with negative test	d number of healthy controls with a negative test

$$OR = \frac{a}{b} \bigg/ \frac{c}{d} = \frac{a*d}{b*c}$$

Table 2.1.1.b: The example of the Simpson's paradox as described by Simpson

Condition 1: Victim's Race	Condition 2: Defendant's Race	Death Penalty Yes	No	% Yes
White	White	53	414	11.3
	Black	11	37	22.9
Black	White	0	16	0
	Black	4	139	2.8
Total	White (483)	53	430	11.0
	Black (191)	15	176	7.9

Legend :

Simpson investigated whether there is a difference between whites and blacks to get the death penalty. This table may suggest that the white defendants have a greater chance to get the death penalty than blacks (11% whites vs. 7.9% blacks).

However, after controlling for the victim's race, it is clear that blacks get more death penalties: for killing a white victim 22.9% blacks vs. 11.3% whites get a death penalty; for killing a black victim, 2.8% blacks vs. no whites get the death penalty

This paradox can be explained by the association between death penalty and defendant's race, and by the association between victim's race and defendant's race. Blacks generally kill blacks and whites generally kill whites. Knowing that you have a greater change for a death penalty when you kill a white explains the initial higher number of death penalties for whites.

2.1.2 Clinical value of a marker or measure

An association should not only be statistically significant, but also clinically significant. This clinical significance highly depends on the application in which this marker will be used. In clinical practice, diagnosis and prediction are important aims. Diagnosis and prediction imply classification into categories. Methods to explore classification results will be discussed in the present chapter.

2.1.2.1 Dichotomous data

We reported already that the OR is an important measure to report association and that an OR of 2-3 may be clinically important. A lot of clinicians however don't like this OR since it is not easy to understand what really happens. Therefore many other measures have been invented and are highlighted in Tables 2.1.2.1.a and 2.1.2.1.c.

Table 2.1.2.1.a demonstrates that, applying a diagnostic test to a population of consecutive subjects with and without a certain disease will result in 4 possibilities. The number of true positives (TP) is the number of subjects that have the disease and a positive test, the number of true negatives (TN) is the number of subjects that do not have the disease and have a negative test. The two conditions that we want to avoid are: false negatives (FN= number of diseased subjects, but with a negative test) and the false positives (FP = number of non-diseased subjects with a positive test). Using those four possibilities, different ratios can be calculated to report the diagnostic properties of a test.

A very intuitive ratio is the predictive value: the positive predictive value (PPV) of a test is the probability for a disease, given the test is positive and is the number of TP divided by the total number of subjects tested positive. The negative predictive value (NPV) is the probability of absence of disease given that a test result is negative and can be calculated by dividing the number of TN by the total number of subjects tested negative. The problem of reporting the PPV and NPV is that these ratios are only valid in a consecutive cohort of patients and depend on the prevalence of disease in that specific cohort, or in Baysian terms, PPV and NPV depend on the a priori chance of disease. Therefore, sensitivity and specificity are more frequently used to describe the diagnostic properties of a test.

The sensitivity (sens) is the proportion of subjects with a positive test among the number of diseased subjects, and can be calculated by dividing the number of TP by the total number of diseased subjects. The specificity (spec) is the proportion of healthy subjects with a negative test result and can be calculated by dividing the number of TN by the total number of healthy subjects. Uncertainty of the estimates of sensitivity and specificity can be easily explored by the calculation of 95% confidence intervals [Harper 1999].

Another important ratio, especially for pure classification purposes is the accuracy. This is the number of TP and the number of TN divided by the total number of subjects. Again, this ratio depends also on the *a priori* chance of disease. The effect of the *a priori* chance on predictive value and accuracy can be calculated, applying Bayes' theorem. This theorem says that the *a priori* chance can be changed into a posterior chance, adapted by new data and results. The mathematical formula and the application of this formula to calculate PPV in terms of sensitivity, specificity, and *a priori* chance (or prevalence of the disease), are explained in Table 2.1.2.1.b. Although sensitivity and specificity are independent of the a priori chance or the prevalence of disease, they may vary between study populations. The sensitivity of a marker may be less in patients with early disease. Also, false positive tests may be more frequent in certain subpopulations (such as elderly, patients with another disease) leading to a decrease of the specificity of the test.

Table 2.1.2.1a: ratios to express association or diagnostic properties.

		Disease	
		Present	Absent (healthy)
Test	Positive	TP = True Positives	FP = False Positives
	Negative	FN = False Negatives	TN = True Negatives

Odds ratio
$$OR = \frac{TP}{FN} \bigg/ \frac{FP}{TN}$$

Relative Risk
$$RR = \frac{TP}{TP + FP} \bigg/ \frac{FN}{TN + FN}$$

Positive predictive value
$$PPV = TP/(TP + FP)$$

Negative predictive value
$$NPV = TN/(TN + FN)$$

Specificity
$$Spec = TN/(TN + FP)$$

Sensitivity
$$Sens = TP/(TP + FN)$$

Accuracy
$$Acc = (TP + TN)/(TP + FP + TN + FN)$$

Table 2.1.2.1.b: Bayes' theorem and deduction of formula for PPV

The Mathematical formula of Bayes' theorem: $P(H|D) = P(D|H) \cdot \dfrac{P(H)}{P(D)}$

(P = probability, H = hypothesis, D = data)

Or in words:

The probability of the hypothesis given the data [P(H|D)], is equal to the probability of the data, given that the hypothesis is correct [P(D|H)], multiplied by the probability of the hypothesis before obtaining the data [P(H)] divided by the averaged probability of the data [P(D)][Malakoff, 1999].

Applied to diagnostic testing we can substitute the constituents of the formula:

P(H|D) is the probability of disease if the test is positive (= PPV).

P(D|H) is the probability of a positive test if disease is present (=sensitivity);

P(H) is the a priori chance or prevalence for disease.

P(D) is the probability of a positive test result in the total population under study (both diseased and non-diseased) [= number FP/ tot number of subjects = (1-spec)*(1-prev)].

The formula thus becomes:

PPV= $\dfrac{\text{a priori chance * sensitivity}}{\text{Prevalence of a positive test in the total test population}}$

2.1.2.2 Dichotomizing continuous data: ROC curve analysis

A lot of markers have continuous properties, for which a certain cut-off can be chosen above which a test is considered positive. This is called dichotomizing the data and makes it possible to calculate sensitivities, specificities, but also PPV, NPV, accuracy… Generally, the definition of a high cut-off will reflect a high specificity with an impaired sensitivity. The definition of a lower cut-off will reflect a high sensitivity with an impaired specificity. How much the sensitivity decreases with increasing the specificity can be examined by the analysis of a receiver operating characteristics (ROC) curve. This plots the sensitivity (as a measure of the true positivity rate) in function of the 1-specificity (as a measure of the false positivity rate) (Figures 2.1.2.2 a-b). The optimal diagnostic test will show no or only a small decrease in sensitivity, when a cut-off with higher specificity is chosen, and vice versa. A way to express this in an objective measure is the calculation of the area under the curve (AUC). A maximal AUC of 1 indicates the most optimal test. An AUC of 0.5 indicates that the test has no diagnostic value. An accompanying table lists cut-offs, sensitivities and specificities (Figure 2.1.2.2.b) [Zweig 1993, Greiner 2000]. AUC of ROC analysis can be used to compare different diagnostic tests: the best test is the test with the highest AUC. Differences between AUC can be evaluated statistically by means of the Hanley test [Hanley 1983].

The calculation of the AUC is one way to compare different tests: it may occur that the AUC of 2 tests may be identical, but that the curves are crossing [Hoffman 2005, Pepe 2003] (Figure 3.2.2.2). Therefore, additional comparison of sensitivities at equal specificity levels (or vice versa) may be useful.

Figure 2.1.2.2.a: Example of a continuous test

Test result	Number of Controls	Number of Diseased patients	Total
1	5	0	5
2	10	0	10
3	45	0	45
4	78	0	78
5	92	1	93
6	93	2	95
7	92	4	96
8	78	5	83
9	45	7	52
10	15	9	24
11	5	11	16
12	0	12	12
13	0	13	13
14	0	14	14
15	0	13	13
16	0	12	12
17	0	11	11
18	0	9	9
19	0	7	7
20	0	5	5
21	0	3	3
22	0	1	1
Total	558	139	697

Legend:

We consider a continuous test that displays higher test result in diseased than in controls. This example will be used to explain ROC curve analysis, PPV, probabilities and logistic regression.

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Figure 2.1.2.2.b: Example of a ROC curve

Coordinates of the curve

Test result ≥	Sensitivity	1 – Specificity
0	1.000	1.000
1	1.000	.991
2	1.000	.973
3	1.000	.892
4	1.000	.753
5	.993	.588
6	.978	.421
7	.950	.256
8	.914	.116
9	.863	.036
10	.799	.009
11	.719	.000
12	.633	.000
13	.540	.000
14	.439	.000
15	.345	.000
16	.259	.000
17	.180	.000
18	.115	.000
19	.065	.000
20	.029	.000
21	.007	.000
22	.000	.000

AUC = 0.966

Legend:
Based on the example of fig 2.1.2.2.a, we calculated sensitivities and 1-specificities at each possible cut-off (Table). Plotting the sensitivities (y-axis) in function of the 1-specificities (x-axis) results in the ROC curve (Figure) with an area under the curve (AUC) of 0.966.

2.1.2.3 Using probabilities to handle a continuous test as such
An important drawback of defining a cut-off is that information may get lost. The use of PPV requires the definition of a cut-off (in our example, a cut-off of 10 [greater than or equal to 10] has a specificity of 99% and PPV = 96%), which makes that the information gets lost that a patient who displays a result of 10 still has less chance to have the disease (38%) than a patient that displays a result of 12 (100%) (Figure 2.1.2.2.a and Figure 2.1.2.3.). This explains why the use of PPV at a certain cut-off may overestimate the chance.

Figure 2.1.2.3: Comparison between probabilities and PPV

Test result	Calculated probability	PPV
1	0	0.21
2	0	0.21
3	0	0.22
4	0	0.25
5	0.01	0.3
6	0.02	0.37
7	0.04	0.49
8	0.06	0.67
9	0.13	0.86
10	0.38	0.96
11	0.69	1
12	1	1
13	1	1
14	1	1
15	1	1
16	1	1
17	1	1
18	1	1
19	1	1
20	1	1
21	1	1
22	1	1

Legend:
The dotted line shows the PPV of a positive test for each possible cut-off. The continuous line shows the probability for disease for each test result. When the cut-off is defined at more than or equal at 9, the test has a PPV of 86%. However, the probability for disease when a result of 9 is present is only 13%.
The figure and table is based on the case of Table 2.1.2.2.a

2.1.2.4 Introduction to logistic regression

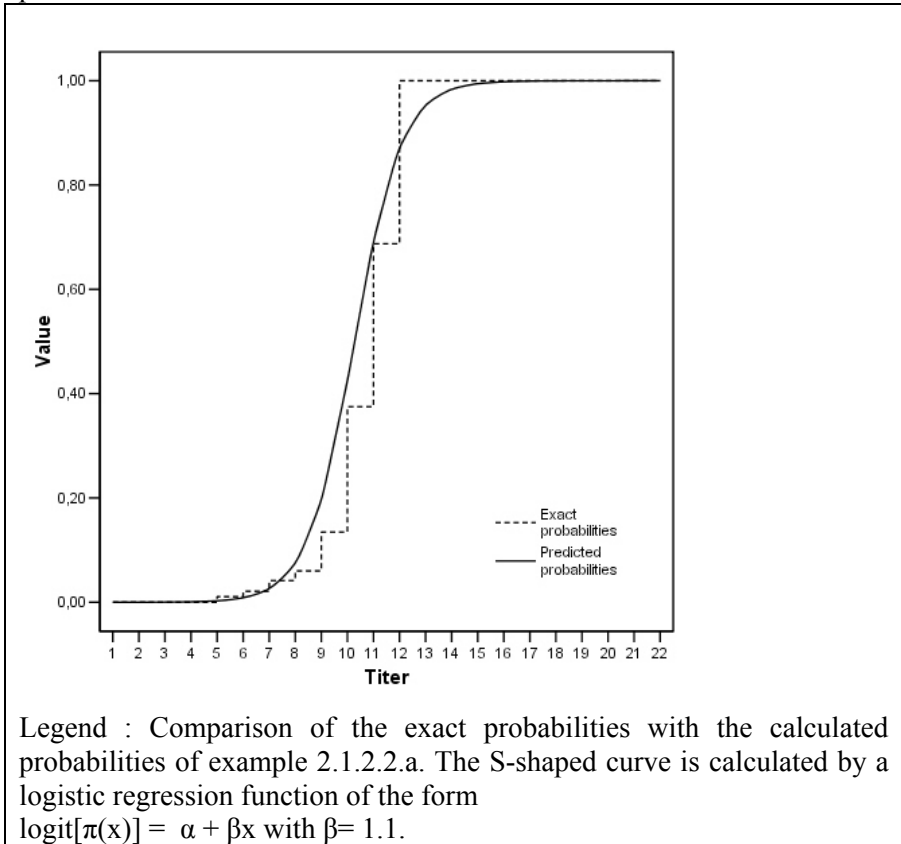In the presented example (Fig 2.1.2.2a) the test could have 22 results and there were 697 subjects included. This made it possible to easily calculate the exact probabilities for disease given a certain test result (Figure 2.1.2.4. However, a continuous test may have infinite possible results. In that case, it is impossible to calculate exact probabilities at each possible result. Therefore, a regression technique is needed which extrapolates between the observations. A probability (π) range from 0 to 1, therefore, the fitted regression curve should be S-shaped with a minimum of 0 and a maximum of 1. Such a S-shaped curve can be obtained by transforming the function of a straight line ($y = \alpha + \beta x$) into $\pi(x) = \exp(\alpha + \beta x)/(1 + \exp(\alpha + \beta x))$. This can be alternatively written as $\log[\pi(x)/(1-\pi(x))] = \alpha + \beta x$. The $\log[\pi(x)/(1-\pi(x))]$ is called "logit" which gave its name to this type of regression: logistic regression. The parameter β is the most important parameter and determines the rate of increase or decrease of the S-shaped curve. If β is zero, the curve becomes a horizontal straight line. Thus, if $\beta=0$, then the presence of disease is independent from the outcome of the test. If $\beta \neq 0$, the test can be used to predict disease or health. The parameters α and β and their confidence intervals are estimated through an iterative method. Figure 2.1.2.4 displays the predicted probabilities (as calculated by logistic regression) of the test of Table 2.1.2.2.a. compared to the exact probabilities for disease.

In its more complex form, different tests or variables ($x_1, x_2, \ldots x_i$) can be combined resulting in the following formula : $\text{logit}[\pi(x)] = \alpha + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_i x_i$. If a variable x displays a parameter β that is not significantly different from 0, this means that this variable has no additional value in a model to predict the probability for disease. The variables ($x_1, x_2, \ldots x_i$) may be either continuous or dichotomous. In the case that the variable is dichotomous, the parameter β has another nice interpretation. The exponent of β [$\exp(\beta) = e^{\beta}$] corresponds with the OR. If a model is fitted with only 1 variable, than $\exp(\beta)$ equals the OR calculated as described previously. In contrast, when a model is fitted with different explanatory variables, $\exp(\beta)$ can be interpreted as an "adjusted" OR. This may have important implications: as described by the Simpson's paradox, an association can disappear, or even invert if adjusted for other variables when those variables are associated.

Another difficulty that may arise is interaction. Interaction is the "statistical" term for what is better known as synergism in medical terms. For example, it can be assumed that a smoker has a 5% risk for a cardiovascular disease and a patient with diabetes has 2% risk for a cardiovascular disease. If a patient has both diabetes and smokes, those risks should be added. However when interaction occurs, the total risk for disease will be higher (or lower) than the sum of both risks.

Figure 2.1.2.4: comparison between the exact and the predicted probabilities

Legend : Comparison of the exact probabilities with the calculated probabilities of example 2.1.2.2.a. The S-shaped curve is calculated by a logistic regression function of the form
$\text{logit}[\pi(x)] = \alpha + \beta x$ with $\beta = 1.1$.

2.2 Combination of variables

2.2.1 The role of combining variables in composite indices and prediction models.

Generally, one single marker or one single clinical measure is not sufficient to classify, to make a diagnosis, or for predicting prognosis. We showed already that logistic regression can provide a helpful tool to calculate probabilities for a certain diagnosis or to predict a prognosis. Also disease activity can be described by combining different measures. This is especially important for rheumatic diseases, where it is impossible, due to their multifaceted aspects, to define disease activity or outcome by one single variable. In order to find the optimal prediction model, 2 cyclic steps should be taken: 1) feature selection, and 2) construction of the model. Different formal methods have been described, but it should be mentioned that a lot of composite indices have been constructed based on "gut feeling" and domain knowledge. We will describe only the formal methods hereafter.

2.2.2 Feature selection methods
2.2.2.1 Univariate
A simple method to select variables is by selecting them based on the "height of association" with the outcome. This can be measured by p-values, ORs, AUC of ROC curve analyses… The variables can thus be ranked based on those measures, and the highest ranked variables can be included in the model [Pepe 2003].

2.2.2.2 Multivariate
Feature selection methods that select a set of variables that have the best properties in the model is sometimes called multivariate. It may be possible that a single variable has good discriminative properties when it is tested by a univariate method, but that it performs badly in combination with others. Selecting a set of variables in a multivariate model can be done by the so-called forward or backward stepwise elimination. A forward stepwise method starts with one variable and scans all explanatory variables that can best be added. A backward method starts with all explanatory variables and scans which variables can best be eliminated without affecting the properties of the model.

2.2.3 Classification methods
2.2.3.1 Methods that require categorization of continuous variables
A commonly used method to explore combinations of two (or more) variables is by defining a cut-off and exploring the diagnostic properties of "AND" and "OR" combinations, for which an "AND" combination means double positivity for the two markers and an "OR" combination means positivity for one of the two markers. Another technique which

requires categorisation of the data is the construction of decision trees. The use of decision trees allows a very comprehensive way of classification since it visualises different steps that should be taken to obtain the conclusion. They are quite easy to construct manually for simple datasets but require more sophisticated software for more complex datasets. Multiple classes can easily be modelled, but both decision trees and AND/OR combinations have the major drawback that continuous data should be categorised which may result in a loss of information.

2.2.3.2 Methods that can handle continuous covariates as such: logistic regression and discriminant analysis.
In contrast to decision trees, for which the output is categorical, the output of logistic regression and discriminant analysis is continuous which allows choosing an optimal cut-off in function of the purpose.

Logistic regression has been explained previously. Classification into categories can be obtained when a cut-off is defined for the obtained predicted probabilities.

The results of discriminant analyses are mostly quite similar to the results obtained by logistic regression, but the theory behind the method is somewhat different. Discriminant analysis attempts to find linear combinations of independent variables that separate the groups of cases optimally. These combinations are called discriminant functions and have the form displayed in the equation: $d = b_0 + b_1 x_1 + \ldots + b_i x_i$ where d is the value of the discriminant function, i is the number of (predictor) variables, b is the value of the coefficient and x_i is the value of the case of the ith (predictor) variable. In other words, the method will try to find one (or more) functions that are linear combinations of the different variables (x_1 to x_i) by searching the coefficients (b).

The procedure run with SPSS (Chicago, Illinois) automatically chooses a first function that will separate the groups as much as possible. It then chooses a second function that is both uncorrelated with the first function and provides as much further separation as possible. The procedure continues adding functions in this way until reaching the maximum number of functions as determined by the number of predictors and the numbers of categories in classification variable. The scores of the different discriminant functions can be used to designate the different categories. For simple classification problems, only 1 discriminant function is needed.

Figure 2.2.3.2: Example of a discriminant analysis
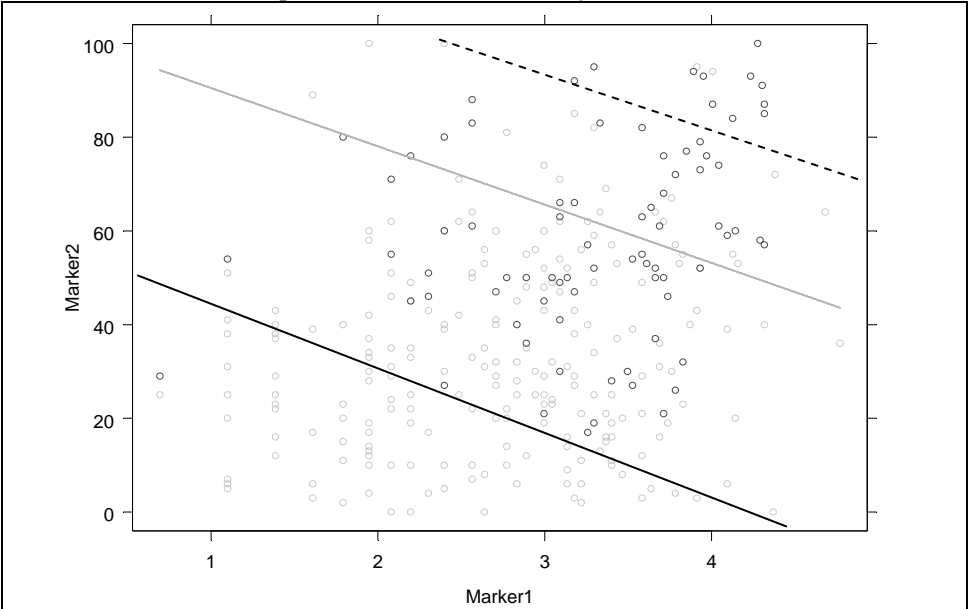
In this Figure, 2 markers were tested in 294 patients. We plotted the results of marker1 against the results of marker2 and represented each patient by 1 dot. The black dots are the diseased patients, the grey dot are the healthy controls. We performed discriminant analyses and the computer gave us the following discriminant function:

*D= -2.995 + 0.039 * Marker2 + 0.491 *Marker1*

Which can also be written as :

$$Marker2 = \left(\frac{2.995 + D}{0.039} \right) - \left(\frac{0.491}{0.039} \right) *Marker1$$

This formula has the form of y=a-bx and thus represents a straight line with a negative (or descending) slope. A cut-off for D can be defined according to the classification results that are desired. For example: the dotted line represents a cut-off for D of 1.2 and will be able to discriminate especially diseased patients in the right upper corner. The black line is calculated with a cut-off for D of -0.9 and will separate healthy controls in the left corner. The grey line is an intermediate line with a cut-off for D of 0.8.

2.2.3.3 Computer intensive methods including neural networks
More sophisticated classification methods are available including: support vector machines (SVM), Kohonen self-organising maps, and multilayered perceptrons (MLP) (which are also called neural networks). They are promoted for more complex classification problems, such as non-linear classification problems and datasets with missing values. They are very flexible, and therefore, a defined classification model, obtained in the so-called training set, should always be confirmed in a test-set. This implies that those methods should be applied only to datasets that are big enough to be split in a train and test file [Cortes 1995, Kohonen 1997, Haykin 1994, Ergun 2004].

2.3 How to handle the dimension time?
In statistics, time is a very important and powerful variable. However, to analyse time, specific statistical models are needed. In this paragraph, we will discuss methods to handle repeated measurements over time and survival analysis.
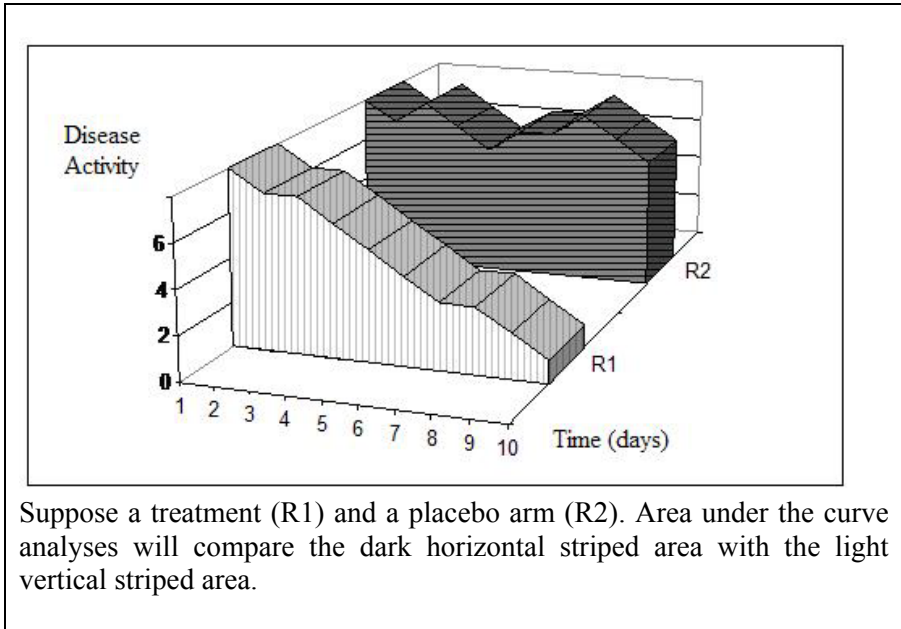
2.3.1 Repeated measurements over time
A common design of longitudinal studies is to measure a single outcome variable, for example swollen joint count, repeated at different time points.

2.3.1.1 Classical methods
Classically, differences between two different time points are measured by a paired sample test such as the paired t-test or the Wilcoxon Rank test. Those tests evaluate e.g. whether there is a difference between the baseline-state and the state after treatment. In order to control for a potential placebo effect, a control group must be included and should show no decrease or a significantly smaller decrease over time than the treatment group. Such methods, however can handle only 2 different time points. Therefore, it might be useful to calculate summary measures, such as an area under the curve (AUC) (Figure 2.3.1.1). AUC analysis can handle different measures in between the baseline measures and the last evaluation.
When disease activity is compared between two treatment groups e.g. in a placebo-controlled trial, it is important to mention that not only the (mean) evolution of the disease activity over time should be evaluated, but also the number of patients that are finally in "remission" or present a pre-defined minimal disease activity [Wells 2005, Cook 2004, Schiff 2002, Liu 1998].

Figure 2.3.1.1 Area under the curve analysis applied for longitudinal data.

Suppose a treatment (R1) and a placebo arm (R2). Area under the curve analyses will compare the dark horizontal striped area with the light vertical striped area.

2.3.1.2 General linear mixed models

General linear mixed models (GLMM) are, similar to logistic regression, an extension of classical (linear) regression. For GLMM, the equation remains $y=\alpha+\beta x$, but it allows a correction for repeated measurements within one individual. If you want to analyse the evolution of the weight over time in 5-year old children, one can take 100 children and measure them every 3 months in that year, thus obtaining 400 paired observations of height and weight from 100 children. To estimate the coefficients of the function "height= $\alpha + \beta*$time", those 400 observations should not be used as such, as every child has been measured 4 times. It can be assumed that there is a correlation between heights measured within each child. Therefore, a method should be used that can correct for the within-child correlation of height. The GLMM's provide different methods to handle such "within-child" correlations. Suppose 50 of those children are treated with a low caloric diet and 50 with a high caloric diet, and you are interested in the effect of the diet on the weight. In that case, the interaction or synergism between time and diet is of interest. This interaction term shows how the diet affects the weight over time. We thus will fit a model "weight=$\alpha+\beta_1$time + $\beta_2$diet + β_3 diet*time". In both models, the final estimate of the effect of the diet will be given by the "diet*time" effect. The notation diet*time is used to express interaction.

What are the advantages of such types of analyses? GLMM has the advantage that it is very flexible. In contrast to AUC analysis, GLMM

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can not only handle measurements of more than 2 time points, but it can also handle unbalanced data (that time points may differ between patients). Not only one effect, but also different covariates can be included in the model, for example sex. Also missing values can be handled by GLMM upon the condition that the reasons for missingness can be retrieved or modelled by the data (for example, when missing values occur more frequently in patients with a high disease activity, and high disease activity is monitored in the dataset) [Fitzmaurice 2004, Renard 2002].

2.3.2. Survival analysis
A special case of modelling time is represented by survival analysis. This method evaluates at each time point the number of patients that are still alive (or that are still under treatment) compared to the number of patients that are already death (or stopped treatment). Differences between (treatment) groups can be compared by the so-called log-rank test (or alternative methods), but another extension of linear regression models, called Cox regression is more flexible. In analogy with logistic regression which gives an estimate of the OR, a Cox regression model provides estimates of the hazard ratio. The hazard ratio is a measure of the momentary risk to die (or to stop treatment).
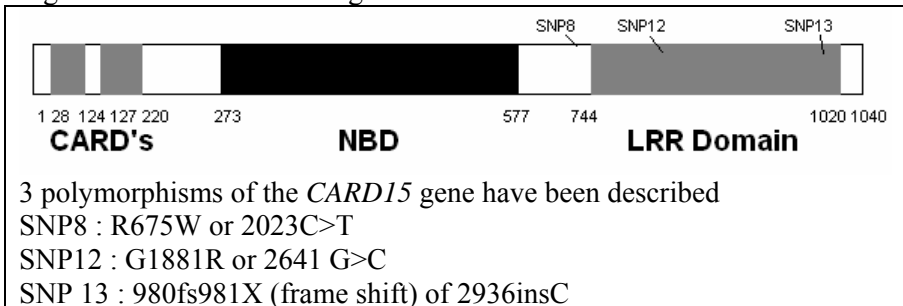
3. Selected biomarkers and clinical measures
3.1. Genetic markers
3.1.1 CARD15

In 2001, 3 polymorphisms in the *CARD15* (or *NOD2*) gene have been associated with CD by 2 independent researchers [Hugot 2001, Ogura 2001]. Carriage of at least one polymorphism can be found in about 38.5% - 57% of CD patients and in about 15% of controls [Hugot 2001, Ahmad 2002]. Different disease phenotypes have been associated with carriage of CARD15 polymorphisms, of which ileal disease seems to be the most strongly associated phenotype [Vermeire 2004]. The CARD15 gene encodes for the NOD2 protein, which is constitutively expressed in myeloid cells as well as in Paneth cells. NOD2 expression can be induced under inflammatory conditions. The NOD2 protein is believed to serve as a microbial sensor by recognition of muramyl dipeptide (MDP), which is a peptidoglycan motif present in the cell wall of bacteria. The effect of the CARD15 polymorphisms on the activity of the receptor and the occurrence of disease remains to be further investigated [Eckmann 2005].

Figure 3.1.1: The CARD15 gene

3 polymorphisms of the *CARD15* gene have been described
SNP8 : R675W or 2023C>T
SNP12 : G1881R or 2641 G>C
SNP 13 : 980fs981X (frame shift) of 2936insC

3.1.2 The HLA shared epitope

The best-known genetic marker associated with RA is the HLA shared epitope (HLA-SE). This HLA-SE consists of one of the following motifs of amino acids QRRAA, QKRAA or RRRAA at positions 70-74 at the antigen binding groove of the DRbeta chain, shared respectively by HLA-DRB1*01 (DBR1*0101, DRB1*0102), DRB1*04 (DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0408), DRB1*1001 and others. Several studies found that one or two copies of the HLA-SE can be found more frequently in patients with RA (+/- 65%) compared to controls (+/- 45%), and resulted in different hypotheses of its pathogenetic importance [Winchester 2004, Winchester 1999, Fries 2002]. Evidence has been given that carriage of one or two copies of the HLA-SE predisposes to more erosive disease, however large differences between ethnic groups exist [Moxley 2002, Gorman 2004].

3.2 Serological markers

3.2.1 ASCA

Anti-*Saccharomyces cerevisiae* antibodies (ASCA) are directed against the cell wall mannan of *Saccharomyces cerevisiae,* commonly known as baker or brewer's yeast [Sendid 1996]. The sensitivity and specificity of ASCA for CD range from 39% to 65% and from 80% to 97.5% [McKenzie 1990, Quinton 1998, Vermeire 2001, Sandborn 2001]. Combinations of ASCA with other serological markers such as pANCA or antibodies against bacterial antigens are under investigation to obtain a better serological tool for IBD [Mow 2004]. In CD patients, ASCA have been linked with earlier onset of disease, ileal involvement, penetrating and stricturing disease and need for resective bowel surgery [Quinton 1998, Mow 2004, Vermeire 2001(2), Peeters 2001, Vasiliauskas 2000]. The pathophysiological role of ASCA is not yet clear [Main 1988, Oshitani 2004]. A genetic influence on ASCA formation has been suggested by family and twin studies: unaffected twins and unaffected relatives have higher ASCA titres compared to healthy controls [Seibold 2001, Glas 2002, Lindberg 1992, Sutton 2000]. Interestingly, ASCA, only in its IgA isotype, can also be found in patients with SpA [Hoffman 2003, Riente 2004, Torok 2004].

3.2.2. RA-associated antibodies: Anti-citrullinated protein/peptide antibodies (ACPA) and rheumatoid factor (RF)

The occurrence of RF in RA patients is well known since many years and constitutes one of the ACR criteria for RA [Arnett 1988]. RF is an immunoglobulin directed to the Fc part of IgG. Although different isotypes may be present, it is typically the IgM RF that is detected by the widely used agglutination assays such as the Waaler Rose test or the latex fixation test and by nephelometry. More recently, a group of antibodies that recognise citrullinated proteins or peptides have been described (anti-citrullinated protein/peptide antibodies: ACPA). Substrates for such tests include citrullinated proteins (filaggrin, fibrinogen) or synthetic peptides. The history of ACPA has been reviewed by Peene et al [Peene 2004]. Shortly after the description of the anti-perinuclear factor (APF, detected by indirect immunofluorescence and directed against components in the perinuclear granules of human buccal mucosa cells) [Nienhuis 1964], also anti-keratin-antibodies (AKA, directed against epithelium of rat oesophagus) were reported to be associated with RA [Young 1979]. Moreover, it was shown that the epitopes recognised by both APF and AKA contain citrullinated residues, converted from arginine into citrulline by the enzyme peptidylarginine deiminase (PAD) [Sebbag 1995, Girbal-Neuhauser 1999] (Figure 3.2.2.a).

This knowledge of the epitope-specificity made it possible to develop more standardized and user-friendly tests. Substrates for such tests

include deiminated proteins (filaggrin or fibrinogen) or peptides. The most widely available synthetic citrullinated peptide is known as CCP (cyclic citrullinated peptide). This substrate is available in an enzyme linked immuno sorbant assay (ELISA)-format [Schellekens 2000]. The first available CCP assay (anti-CCP1) was further optimized by screening of dedicated peptide libraries (anti-CCP2) [Vossenaar 2002]. Alternative peptides include pepA and pepB, which are detected by line immune assay (LIA) [Union 2002]. A recently described alternative citrullinated substrate is deiminated human antifibrinogen (AhFibA) [Nogueira 2002]. The reported sensitivity and specificity of the different tests may differ between studies. As mentioned in the statistics section, comparison between 2 diagnostic tests should be done on the same population using ROC curve analysis. Side-by-side comparison of ACPA and RF has been performed in different populations, indicating that ACPA has better diagnostic properties than RF testing [De Rycke 2004, Hoffman 2005, Nielen 2005]

Fig 3.2.2 Citrullination

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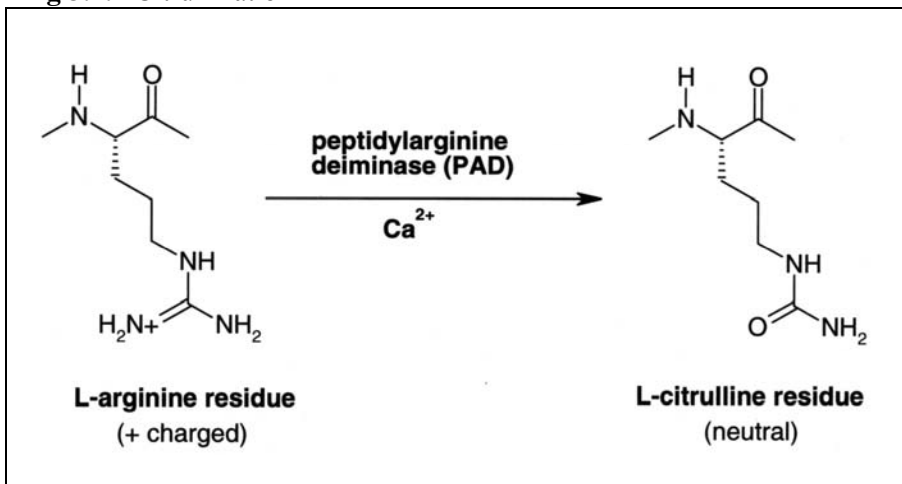
3.3. Single clinical measures and composite indices to evaluate disease activity in chronic arthritis.

Multiple measures exist to evaluate disease activity and response to treatment. Amongst many others, they may include a measure for the number of swollen joints (swollen joint count), a measure for the number of tender joints (tender joint count, Ritchie articular index), a measure to evaluate systemic inflammation (ESR, CRP), functional measurements (morning stiffness, grip strength) and patient's and physician's questionnaires [Anderson 1989, Felson 1993]. An important tool in questionnaires is the visual analogue scale (VAS). The examinee has to evaluate for example his pain by placing a vertical line on a 100 mm line. Scores that are measured by VAS are the physician's assessment of global disease activity, the patient's assessment of global disease activity or the patient's assessment of pain.

A commonly used questionnaire is the health assessment questionnaire (HAQ) [Fries 1980], which measures disability. Another important questionnaire is the SF-36 [Ware 1992] and measures eight multi-item dimensions: physical functioning, social functioning, role of limitations due to physical problems, role of limitations due to emotional problems, mental health energy/vitality, pain and general health perception.

In the early 90's, it was common practice to use different of those single outcome measures to evaluate disease activity or response to therapy in placebo controlled trials. However, this imposed the problem of multiple comparisons [Bland 1995, Feise 2002, Curran-Everett 2002, Rothman 1990]. This problem of multiple comparisons might be overcome by some specific statistical methods, which are in favour of the power, but at the expense of standardisation [Anderson 2003]. Therefore, composite indices were constructed, so that a single outcome measure could be used as the primary outcome, and thus corrections for multiple comparisons were no longer needed. Moreover, in the ideal situation, the new composite index should be more efficient than its single components to discriminate responders from non-responders. This leads to the construction of several composite indices, of which the best knowns are: the ACR response criteria [Felson 1995] (Table 3.3.a) and the disease activity score (DAS) [van der Heijde 1993]. The initial DAS has been later on transformed into the DAS28 [Prevoo 1995]. Also alternative disease activity scores have been proposed such as simplified disease activity index (SDAI) and clinical disease activity index (CDAI) [Smolen 2003, Aletaha 2004] (Table 3.3b). For clinical trials, EULAR response criteria based on the DAS score have been developed also for the use in clinical trials [Van Riel 1996]

Table 3.3a: ACR Response criteria (Felson, 1995)

20, 50 or 70% improvement in 5 of 7 set variables Improvement in the first two is required
Tender joint count Swollen joint count Acute phase reactant Patient's pain Patient's global assessment of disease activity Observer's global assessment of disease activity Physical disability (HAQ)

Table 3.3b: Formulas to calculate DAS and DAS28 based on three or four measurements

Score		Formula
DAS	DAS based on RAI and 44 swollen joint count	$0.54*sqrt(RAI) + 0.065*sqrt(SJC44) + 0.330*ln(ESR) + 0.0072*pt$ global VAS
DAS-3	DAS without pt global VAS	$0.54*sqrt(RAI) + 0.065*sqrt(SJC44) + 0.330*ln(ESR) + 0.22$
DAS28	DAS based on 28 swollen and tender joint counts	$0.56*sqrt(TJC28) + 0.28*sqrt(SJC28) + 0.70*ln(ESR) + 0.014*pt$ global VAS
DAS28-3	DAS28 without pt global VAS	$[0.56*sqrt(TJC28) + 0.28*sqrt(SJC28) + 0.70*ln(ESR)]*1.08 + 0.16$
DAS28-CRP	DAS28 calculated with CRP in stead of ESR	$0.56*sqrt(TJC28) + 0.28*sqrt(SJC28) + 0.36*ln(CRP+1) + 0.014*$ pt global VAS $+ 0.96$
DAS28-CRP-3	DAS28-CRP without pt global VAS	$[0.56*sqrt(TJC28) + 0.28*sqrt(SJC28) + 0.36*ln(CRP+1)] * 1.10 + 1.15$
SDAI	Simplified disease activity score as a simple sum	TJC28+SJC28+pt global VAS/10+phys global VAS/10+ CRP(mg/dl)
CDAI	Clinical disease activity score without CRP	SJC28+TJS28+pt global VAS/10+phys global VAS/10

SJC44/28 = 44/28 swollen joint count; TJC28 = 28 tender joint count; RAI =Ritchie articular index (53 joints in 26 units, graded for tenderness); ESR = Erythrocyte sedimentation rate

Sqrt= squared root; Ln= natural logarithm; Conversion formula to calculate the DAS28 from the original DAS: DAS28 = (1.072)*DAS) + 0.938

Table 3.3c: Definition of the DAS response criteria

DAS28 at Endpoint	Improvement in DAS 28 from Baseline		
	>1.2	>0.6 and ≤ 1.2	≤1.6
≤ 3.2	*Good*	*Moderate*	*None*
>3.2 and ≤3.7	*Moderate*	*Moderate*	*None*
>5.1	*Moderate*	*None*	*None*

4. Research objectives

The aim of the present work was to explore the characteristics of different markers and measures in chronic arthritis.

Before a marker can be used in clinical practice, this marker should be associated with the disease. Associations with the carriage of CARD15 polymorphisms were investigated in the first part of this work. In Chapters 1 and 2, we investigated whether carriage of CARD15 polymorphisms are associated with SpA-like features in Crohn's disease patients and with Crohn-like features in SpA patients. In Chapter 3, we explored whether carriage of CARD15 polymorphisms is associated with the occurrence of ASCA.

In the second part, we explored how a marker can be used in daily clinical practice by the exploration of the diagnostic characteristics of ACPA testing. In Chapter 4 we reviewed some association studies and the diagnostic properties of ACPA and RF. In Chapter 5, we investigated whether ACPA positivity, as a highly specific marker for RA, might occur in PsA. We investigated the diagnostic properties of different combinations of ACPA, SE and RF by means of calculations of probabilities in Chapter 6. In Chapter 7, we finally compared the diagnostic properties of tests and the agreement between different ACPA assays.

Part 3 of this work aimed to evaluate different clinical measures and composite indices to measure response to treatment in RA and PsA. In Chapter 8, we investigated 511 RA patients under infliximab therapy that where enrolled in a compassionate use program. After a loading regimen at week 0, 2, 6 and14, the treating rheumatologist could decide to give a dose increase. This decision to give a dose increase can be considered as a measure of insufficient response. In the analysis presented, we investigated which single measures and composite indices could best discriminate this decision and whether they can be combined in a composite index. In Chapter 9, we evaluated the 4 years effect of infliximab therapy and how the DAS28 can be used to measure sustained response to therapy and which measure or composite index can be best used to predict the survival of the therapy. Finally, we evaluated in Chapter 10 whether the measurement of DAS28 can also be used in PsA patients.

In the last part (Chapter 11), we evaluated different methods to construct prediction models by computer intensive methods. Discriminant analysis and logistic regression may be less performing in real life datasets with missing values and high dimensionality. Therefore, we evaluated whether

different computer intensive methods can handle those problems more effectively.

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CHAPTER 1
Radiological sacroiliitis, a hallmark of spondylitis, is linked with
CARD15 gene polymorphisms in patients with Crohn's disease.

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Background: Sacroiliitis is a common extraintestinal manifestation of Crohn's disease but its association with the HLA-B27 phenotype is less evident. Polymorphisms in the CARD15 gene have been linked to higher susceptibility for Crohn's disease. In particular, associations have been found with ileal and fibrostenosing disease, young age at onset of disease, and familial cases.

Objectives: To investigate whether the presence of sacroiliitis in patients with Crohn's disease is linked to the carriage of CARD15 polymorphisms.

Methods: 102 consecutive patients with Crohn's disease were clinically evaluated by a rheumatologist. Radiographs of the sacroiliac joints were taken and assessed blindly by two investigators. The RFLP-PCR technique was used to genotype all patients for three single nucleotide polymorphisms (SNP) in the CARD15 gene. Every SNP was verified by direct sequencing. The HLA-B27 phenotype was determined.

Results: Radiological evidence of sacroiliitis with or without ankylosing spondylitis was found in 23 patients (23%), of whom only three were HLA-B27 positive. In contrast, 78% of patients with sacroiliitis carried a CARD15 variant v 48% of those without sacroiliitis (p=0.01; odds ratio 3.8 (95% confidence interval, 1.3 to 11.5)). Multivariate analysis (logistic regression) showed that the association between sacroiliitis and CARD15 polymorphisms was independent of other CARD15 related phenotypes (ileal and fibrostenosing disease, young age at onset of disease, familial Crohn's disease) (p=0.039).

Conclusions: CARD15 variants were identified as genetic predictors of Crohn's disease related sacroiliitis. An association was demonstrated between these polymorphisms and an extraintestinal manifestation of Crohn's disease.

There is increasing evidence that Crohn's disease is a multigenic disorder resulting in different phenotypes.[1] Although several susceptibility loci have been described, only one gene has been clearly identified as being associated with an increased susceptibility to Crohn's disease. Three single nucleotide polymorphisms (SNP) in the CARD15 gene (R702W, G908R, and 1007fs) increase the risk for Crohn's disease by a factor of 3 for heterozygotes and by about a factor of 40 for homozygotes or compound heterozygotes.[2–4]

The identification of associated phenotypes remains unclear and controversial but suggests an association with ileal and fibrostenosing disease, familial predisposition, and earlier age at onset.[5–8]

No association has been reported between CARD15 polymorphisms and extraintestinal manifestations of Crohn's disease. However, data on articular involvement are scarce and limited to two studies.[6 7] Lesage et al reported articular involvement in only 6% of the patients, but without a clear definition and without separation between peripheral and axial disease.[6] Ahmad et al recorded pauciarticular arthritis in 13% of the patients, also with no information about axial disease.[7] The data in these studies were gathered through questionnaires and the patients' clinical records.

Besides Crohn's disease, CARD15 variants have also been linked to Blau syndrome and psoriatic arthritis.[9 10] Current data show no association between CARD15 and idiopathic ankylosing spondylitis (AS)[11–14]; however, in the Crohn's disease variant the HLA-B27 association is weaker than in idiopathic AS, pointing to another genetic link.[15–17] Indeed, Van der Paardt et al have already suggested that it would be

interesting to determine whether there is an association between CARD15 and the subgroup of patients with Crohn's disease and AS.[12]

Our aim in this cross sectional clinical and radiological study was to evaluate, in a cohort of Crohn's disease patients, the possible association between CARD15 polymorphisms and the presence of radiological sacroiliitis, a hallmark of spondylitis and the most frequent objective rheumatic manifestation of Crohn's disease.[18]

METHODS

Study population

One hundred and two white patients with Crohn's disease, attending the gastroenterology department of the Ghent University Hospital, agreed to participate in this cross sectional study and were included consecutively over a period of 13 months. The diagnosis of Crohn's disease was based on clinical, endoscopic, histological, and radiological findings. All patients were seen by both a gastroenterologist and a rheumatologist. The localisation of the disease was assessed as ileal, ileocolonic, or colonic. Types and amount of surgical interventions were recorded. The need for resective small bowel surgery was used as an index of fibrostenosing disease. A positive family history was defined as the presence of at least one first, second, or third degree relative with

proven Crohn's disease. The age at diagnosis of the disease was also recorded.

For controls, we used a group of 54 patients without any symptoms or clinical evidence of Crohn's disease, spondyloarthropathies, or sclerosing cholangitis, who were attending the department of hepatology.

The study was approved by the regional ethics committee and all patients gave their signed informed consent.

Assessment of articular involvement

All patients were assessed for the presence of inflammatory low back pain, had measurements of the Schöber index and chest expansion, and were examined for peripheral arthritis and enthesopathy (fasciitis plantaris or Achilles tendon tendinitis).

A history of peripheral arthritis or enthesopathy was recorded if observed, confirmed by a physician, and noted in the patient's medical record. Inflammatory low back pain was defined by the history or presence of spinal pain in the neck, dorsal, or cervical region with at least four of the five following criteria: onset before the age of 45 years, insidious onset, improvement with exercise, association with morning stiffness, and duration of at least three months.[19] The modified New York criteria were used to determine the diagnosis of AS.[20]

Radiological classification

Radiographs of the sacroiliac joints were made and assessed blindly by two rheumatologists. They were scored using the New York grading system: 0, normal; 1, suspicious; 2, localised sclerosis, erosion, joint widening; 3, diffuse sclerosis, erosion, widening; 4, ankylosis.[20] Radiological sacroiliitis was diagnosed by the presence of at least unilateral sacroiliitis grade 2, and only when agreed on by both radiological assessors.[18 21]

CARD15 and HLA-B27 typing

Genomic DNA was extracted from whole blood using a Qiagen blood and cell culture DNA kit (Qiagen, Valencia, California, USA).

All patients were genotyped for R702W (SNP8), G908R (SNP12), and 1007fs (SNP13) using a restriction fragment length polymorphism polymerase chain reaction technique (RFLP-PCR), followed by separation on 2.5% agarose gel. The missense mutation R702W (GenBank accession number G67950) abolishes the restriction site for $MspI$ (5'-CAGCCC TGATGACATTTCTCTT-3' and 5'-AGCCGCTCCTCCTGCATCTC GTA-3'), resulting in an intact 130 base pair (bp) band for mutant alleles, compared with two bands of 54 and 76 bp for wild type alleles. The missense mutation G908R (GenBank accession number G67951) creates a restriction site for $HinP1I$. The frameshift mutation 1007fs (GenBank accession number G67955) creates a restriction site for $NlaIV$ (5'-CTGAGCCTTTGTTGATGAGC-3' and 5'-TCTTCCAACCACAT CCCCATT-3'). The presence of a mutant allele results in two bands of 219 and 41 bp, while the wild type allele produces a single 260 bp product. We verified every SNP by direct sequencing of the PCR product in an ABI 377 analyser (Applied Biosystems, Foster City, California, USA).

HLA-B27 typing was undertaken using Dynal Classic SSP (Dynal Biotech, Bromborough, Cheshire, UK).

Statistical analysis

Odds ratios were calculated with Pearson's χ^2 test or Fisher's exact test when the expected count was less than 5 in at least one cell. A model for multivariate analysis (logistic regression) was constructed, with sacroiliitis as the dependent variable. As covariates we included the CARD15 genotype, HLA-B27, and CARD15 related phenotypes. A probability (p)

value of less than 0.05 was considered to indicate statistical significance. Statistical analyses was carried out using SPSS software (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Patient characteristics

In all, 102 patients with Crohn's disease were included (34 men, 68 women), mean age 41.6 years (range 18 to 71). The mean age at diagnosis was 28.5 years (range 9 to 57). Twenty two of the patients (21.6%) had ileal disease, 26 (25.5%) had colonic disease, and 54 (52.9%) had ileocolonic involvement. Fifty two patients (51.0%) had needed resection of a small bowel segment. Fifteen patients (14.7%) had a family history of Crohn's disease.

Radiological sacroiliitis and other rheumatic manifestations

Radiological sacroiliitis was found in 23 patients: eight had unilateral sacroiliitis grade 2, 14 had bilateral sacroiliitis grade 2, and one had unilateral sacroiliitis grade 3. Nine patients fulfilled the criteria for AS. Among the 14 remaining patients, three had inflammatory low back pain without fulfilling the AS criteria, and 11 were clinically asymptomatic.

The history or presence of peripheral arthritis was recorded in 17 patients, of whom five actually had arthritis at the time of study assessment. Three patients had monoarticular arthritis and 14 had oligoarticular involvement. A history of enthesopathy was present in 11 patients, of whom four had tendinitis at the time of the study.

CARD15 status

The overall prevalence of CARD15 polymorphisms in the Crohn's disease group was significantly higher than in the control group, at 56/102 (54.9%) v 8/54 (15%), p<0.001; odds ratio (OR) 7.00 (95% confidence interval (CI), 3.00 to 16.31)). Eighteen of 23 patients with radiological sacroiliitis (78%) carried at least one CARD15 variant, compared with 38 of 79 patients (48%) without sacroiliitis (p = 0.01; OR 3.88 (95% CI, 1.31 to 11.49)) (table 1). There were two compound heterozygotes and no homozygotes in the group of patients with sacroiliitis (table 1). Seven of nine patients with AS carried a CARD15 variant (table 2).

In another perspective, among all carriers of CARD15 variants, 32% of the patients had sacroiliitis compared with 11% in patients without these variants.

Table 1 Carrier frequency of CARD15 variants in patients with Crohn's disease

	R702W	G908R	1007fs	Overall*
Crohn's disease (n = 102)				
CARD15+/−	33	6	21	56
CARD15+/+	2	1	0	
Crohn's disease with no SI (n = 79)				
CARD15+/−	21	6	13	38
CARD15+/+	2	1	0	
Crohn's disease with SI (n = 23)				
CARD15+/−	12	0	8	18
CARD15+/+	0	0	0	

Numbers of patients carrying R702W, G908R, or 1007fs variants.
*Total number of patients in the group carrying at least one variant. The sum of all allelic CARD15 variants is greater than the overall number of patients carrying at least one variant, as some patients carry two different single nucleotide polymorphisms (compound heterozygotes). CARD15+/−, heterozygous for CARD15 gene; CARD15+/+, homozygous for CARD15 gene; SI, sacroiliitis.

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Table 2 Carrier frequency of CARD15 variants and HLA-B27 in patients with Crohn's disease

	CARD15 +/ or +/+	HLA-B27+
Total (n = 102)	56 (55%)	6 (6%)
No sacroiliitis (n = 79)	38 (48%)	3 (4%)
Sacroiliitis (n = 23)	18 (78%)	3 (13%)
AS (n = 9)	7	3
Non-AS (n = 14)	11	0

CARD15 +/ or +/+: carriage of at least 1 CARD15 variant
HLA-B27+: carriage of HLA-B27
AS, Crohn's disease patients with sacroiliitis fulfilling the ankylosing spondylitis (AS) criteria; non-AS, Crohn's disease patients with sacroiliitis not fulfilling the AS criteria.

Table 3 Multivariate analysis (logistic regression) with "sacroiliitis" as the dependent variable

Covariate	p Value	OR	95% CI
CARD15 variants	0.039	3.38	1.06 to 10.75
HLA-B27	0.201	3.48	0.51 to 23.81
Ileal involvement	0.517	1.84	0.29 to 11.49
History of small bowel resection	0.145	2.56	0.72 to 9.09
Family history of Crohn's disease	0.857	1.14	0.28 to 4.65
Age at diagnosis	0.706	0.99	0.94 to 1.04

Logistic regression shows that the association between sacroiliitis and CARD15 variants is independent of known CARD15 related phenotypes or HLA-B27.
CI, confidence interval; OR, odds ratio.

There was no significant association between the presence of CARD15 polymorphisms and peripheral arthritis (p = 0.37; OR 1.6 (95% CI, 0.55 to 4.81)) or enthesopathy (p = 0.34; OR 0.5 (0.14 to 1.94)).

HLA-B27 status

Overall, six of the 102 patients with Crohn's disease (6%) carried HLA-B27. HLA-B27 positivity was present in three of the 23 patients (13%) with sacroiliitis. Those three patients were also carriers of CARD15 variants and fulfilled the modified New York criteria for AS (table 2).

Multivariate analysis

Logistic regression showed the presence of CARD15 variants as the only significant predictor of sacroiliitis (p = 0.039), independent of HLA-B27, ileal involvement, need for resective small bowel surgery, familial Crohn's disease, or age at onset (table 3).

DISCUSSION

Our data identify CARD15 variants as a possible genetic predictor of sacroiliitis in Crohn's disease. Sacroiliitis is a distinct extraintestinal manifestation of Crohn's disease. In contrast to the clinically evident peripheral arthritis, the prevalence of axial involvement in Crohn's disease is probably underestimated.[18 22] Previous studies showed that 10–32% of patients with inflammatory bowel disease (IBD) have evidence of sacroiliitis, a hallmark of spondylitis, on conventional radiography.[15 17 18 21 23–25] With technetium scintigraphy, tracer uptake in the sacroiliac joints can be seen in up to 52% of patients.[26] Many patients with sacroiliitis remain asymptomatic.[15 17 18 23 27] Only about one quarter of the patients with radiological sacroiliitis also fulfil the criteria for AS.[18] In contrast to idiopathic AS, involvement of the sacroiliac joints is asymmetrical in about 25% of the patients with Crohn's disease.[18 21 22]

In our cohort, 23% of patients had evidence of sacroiliitis of at least grade 2 unilaterally on conventional radiographs. Diagnosis of AS was retained in 39% of these patients with sacroiliitis.

In contrast to other forms of spondyloarthropathy, the association between axial involvement and HLA-B27 is weak in IBD patients. Whereas HLA-B27 is present in more than 90% of patients with idiopathic AS, the prevalence decreases to 25–75% in patients with AS associated with IBD, and to low or even normal prevalences in patients with asymptomatic IBD-associated radiological sacroiliitis.[15–17 24]

Recently, the possibility of an as yet undefined common genetic link between intestinal inflammation and sacroiliac changes was again suggested in a study assessing first degree relatives of patients with AS.[28] This study showed an association between asymptomatic intestinal inflammation, recorded in 41% of these relatives, and sacroiliac changes

suggestive of early AS. The presence of intestinal inflammation and sacroiliac changes did not relate to the HLA-B27 status of these subjects.

Our data suggest that CARD15 polymorphisms may predispose to sacroiliac involvement in Crohn's disease: 78% of Crohn's patients with sacroiliitis carried at least one variant, versus 48% of patients without sacroiliitis. Multivariate analysis showed that the presence of CARD15 variants was the only significant predictor of sacroiliitis, independent of other known CARD15 related phenotypes such as ileal involvement, fibrostenosing disease, family history, and age of onset, and independent of HLA-B27.

Interestingly, the three patients with sacroiliitis who carried HLA-B27 also carried a CARD15 variant, and all had clinical AS. In contrast none of the patients with isolated sacroiliitis carried HLA-B27. Whether or not the presence of both genetic markers in Crohn's patients with sacroiliitis predisposes to evolution to AS cannot be concluded from this study. It seems interesting, however, to explore this question with larger studies.

The percentage of Crohn's disease patients with CARD15 variants was higher in our study (54.9%) than generally reported. A variation in the methodology was excluded as the prevalence in our control group was similar to values in previous reports. Moreover, the prevalence of CARD15 variants in our group was not significantly greater than in another Flemish population studied by Vermeire et al (46.1%).[29]

To the best of our knowledge, no studies are available about a possible relation between the CARD15 genotype and sacroiliitis in Crohn's disease. Until now, studies in idiopathic AS have not been able to demonstrate a possible association with CARD15 variants.[11–14] However, these studies provided no information on the possible presence and relative number of patients with Crohn's disease who were included. Only one study included cases of AS with Crohn's disease and ulcerative colitis.[30] The investigators could not demonstrate a higher prevalence of CARD15 variants in patients with AS plus Crohn's disease compared with idiopathic AS, AS plus ulcerative colitis, or healthy controls. The low prevalence of CARD15 variants in their Crohn's population with AS was not compared with the prevalence in a general Crohn's disease population. Moreover, they unexpectedly showed a possible association between the G908R CARD15 variant and AS with ulcerative colitis.

In contrast to that study, we found CARD15 variants in seven of nine Crohn's patients with AS. Although it only represents a small group of patients in our study, a difference in prevalence of CARD15 variants between Crohn's disease related AS and idiopathic AS (and also for HLA-B27) could reflect a different aetiopathogenetic mechanism.

The CARD15 gene encodes for an intracellular protein that is found in monocytes, macrophages, epithelial cells, granulocytes, and dendritic cells. The gene product acts as a

receptor for bacterial cell wall components like muramyldipeptides derived from peptidoglycan and lipopolysaccharides, resulting in the activation of NF-κB. The polymorphisms described could cause a disturbed cellular response to bacterial components, leading to intracellular persistence of pathogens.[31] Previous studies have suggested that recirculation of inflammatory cells between the gut and peripheral joints might contribute to inflammation at a distance.[32–36]

Conclusions

In Crohn's disease, CARD15 variants could play a role in the development of sacroiliitis, as an extraintestinal manifestation of the disease. However, the exact mechanism of the link between gut and axial joints remains to be elucidated.

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CHAPTER 2
CARD15 gene polymorphisms in patients with spondyloarthropathies
identify a specific phenotype previously related to Crohn's disease.

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EXTENDED REPORT

CARD15 gene polymorphisms in patients with spondyloarthropathies identify a specific phenotype previously related to Crohn's disease

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Background: The association between spondyloarthropathy and Crohn's disease is well known. A risk for evolution to Crohn's disease has already been shown in the subgroup of patients with spondyloarthropathy associated with chronic gut inflammation.
Objective: To investigate whether the reported polymorphisms in the CARD15 gene, a susceptibility gene for Crohn's disease, are associated with the presence of preclinical intestinal inflammation observed in spondyloarthropathies.
Methods: 104 patients with spondyloarthropathies were studied. All underwent ileocolonoscopy with biopsies between 1983 and 2004. The prevalence of three single nucleotide polymorphisms in the CARD15 gene (R702W, G908R, and 1007fs) was assessed using restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR); the patients were compared with an ethnically matched Crohn's disease population and a control population.
Results: The carrier frequency of R702W, G908R, or 1007fs variants in the spondyloarthropathy populations (20%) was similar to the control population (17%), but increased to 38% in the spondyloarthropathy subgroup with chronic gut inflammation. This frequency was significantly higher than in the other spondyloarthropathy subgroups (p=0.001) or the control group (p=0.006), but not different from the Crohn's disease group (49%) (NS). This indicates that CARD15 polymorphisms are associated with a higher risk for development of chronic gut inflammation.
Conclusions: CARD15 gene polymorphisms clearly identify a subgroup of patients with spondyloarthropathies associated with chronic intestinal inflammation.

The spondyloarthropathies are a group of interrelated inflammatory diseases characterised by a pauciarticular, peripheral, asymmetrical arthritis with or without axial involvement, with ankylosing spondylitis as the prototype.[1] Reported prevalences of spondyloarthropathy vary between 0.2% and 1.9%.[2][3] Although an association with HLA-B27 is strong, recent genetic studies suggest a polygenic model of susceptibility.[4–7]

In up to 60% of spondyloarthropathy patients, articular involvement is associated with subclinical histological evidence of chronic or acute gut inflammation in the ileum or colon.[8–10] We described a long term evolution to overt Crohn's disease in 13% of patients with initial chronic gut inflammation.[11][12] The presence of chronic intestinal inflammation was not related to HLA-B27, but a weak association with HLA-B62 was found.[11]

The observed immunological similarities between spondyloarthropathy with gut inflammation and Crohn's disease support the concept that this subgroup of spondyloarthropathy patients can be considered a model for early immune alterations related to Crohn's disease. An enrichment of gut mucosal T cell lines with αEβ7 integrin and an increased expression of its ligand, E-cadherin, is found in the intestine in Crohn's disease as well as in spondyloarthropathy patients.[13][15] Recirculation of gut primed T cells to synovial tissue is one potential mechanism whereby gut and synovial inflammation could be linked. This hypothesis is supported by an altered expression of β7 integrins, which are highly expressed within the gut, on synovial T cells from patients

with spondyloarthropathies compared with rheumatoid arthritis.[16] Another potential mechanism includes trafficking of antigen presenting cells between gut and joints. Consistent with this was the augmented infiltration of gut mucosa and synovium with CD163 positive macrophages (producing interleukin 1 (IL1) and tumour necrosis factor α (TNFα) in both Crohn's disease and spondyloarthropathy.[17][18] Finally, a comparable beneficial clinical effect of infliximab, a monoclonal antibody to TNFα, suggests a key role for this cytokine in both diseases.[19][20]

In 2001, a correlation was reported between polymorphisms in the CARD15 gene and an increased susceptibility to Crohn's disease.[21][23] Three independent single nucleotide polymorphisms (SNPs) in CARD15 are associated with Crohn's disease in around 30–46% of patients (one frame shift mutation (1007fs (SNP13)) and two missense mutations (R702W (SNP8) and G908R (SNP12)).[22][24] These variants increase the risk for Crohn's disease by a factor of 3 for heterozygotes and by a factor of 38 or 44 for, respectively, homozygous or compound heterozygous individuals.[22] Lower prevalences have been described in Crohn's disease patients in Scotland, Ireland, and northern Europe, whereas no association could be found in Japan.[25][26]

The CARD15 gene encodes for an intracellular protein, which is expressed in monocytes, granulocytes, and dendritic, epithelial, and Paneth cells, and has binding affinity for

Abbreviations: CARD, caspase recruitment domain; ESSG, European Spondylarthropathy Study Group; SNP, single nucleotide polymorphism

bacterial cell wall components such as muramyldipeptides.[29] The *CARD15* protein is involved in NFκB activation and in apoptosis by two N-terminal caspase recruitment domains (hence the term CARD), although its precise pathogenic role in Crohn's disease remains to be determined.[29–31]

CARD15 gene polymorphisms have also been linked to another related disorder, Blau's syndrome, characterised by granulomatous inflammation of the uvea, skin, and joints.[32]

Several studies have been carried out to investigate the role of *CARD15* polymorphisms in spondyloarthropathies. These studies did not show an association with spondyloarthropathies or ankylosing spondylitis in particular.[33–37] An increased prevalence of *CARD15* polymorphisms was found in psoriatic arthritis though not in psoriatic skin disease.[38–40] However, a recent Italian study could not confirm this association.[41] Nevertheless, this finding could emphasise the importance of investigating the possible role of these genetic variants in specific clinical subpopulations of patients. In Crohn's disease, *CARD15* polymorphisms also seem to be related to certain clinical phenotypes.[42–46]

In view of the apparent correlation between gut inflammation in spondyloarthropathies and their clinical evolution to Crohn's disease, we investigated whether the presence of polymorphisms in this susceptibility gene for Crohn's disease is associated with gut inflammation in spondyloarthropathy.

METHODS

Study population
This study included 104 white patients with spondyloarthropathies (according to the ESSG criteria[47]), who underwent an ileocolonoscopy with concomitant ileal and colonic biopsies between 1983 and 2004. This population consisted of 74 male and 30 female patients with a mean age of 46 years (range 21 to 77). Spondyloarthropathy patients were systematically referred by the rheumatologist for an ileocolonoscopy with biopsies, independent of the presence of gastrointestinal symptoms.

Patients with a diagnosis of clinical Crohn's disease or psoriasis before the diagnosis of spondyloarthropathy were excluded from the study.

A subgroup of 54 patients with long term follow up since the time of their diagnosis of spondyloarthropathy (ranging from 17 to 49 years) was recently clinically reassessed. New follow up colonoscopies were not carried out.

The total spondyloarthropathy population consisted of 75 patients with ankylosing spondylitis according to the modified New York criteria[48] and 29 with an undifferentiated

form of spondyloarthropathy. Eighteen patients with ankylosing spondylitis had only axial involvement, while 57 also had peripheral disease (defined as the history or presence of peripheral arthritis, enthesitis, or both). Twenty five patients with undifferentiated spondyloarthropathy had peripheral disease and four only had axial involvement. These four patients had inflammatory low back pain and fulfilled the ESSG criteria but not the modified New York criteria for ankylosing spondylitis.

HLA-B27 status was known in 81 patients. In 53 patients both HLA-B27 and HLA-B62 status was known.

A population of 156 consecutive patients with Crohn's disease proven on clinical, endoscopic, and histological grounds was also included. This cohort included 57 male and 99 female patients with a mean age of 38 years (range 18 to 80). Prevalences were also compared with those in a control population of 140 individuals.

The study was approved by the local ethics committee. All patients signed their informed consent.

Histological classification
A classification of histological lesions was used as reported in previous studies.[8 11 12 49 50] Three subgroups were distinguished: patients with normal gut histology, and those with acute and chronic inflammation.[51]

In acute inflammatory lesions normal architecture was well preserved. A mucosal and epithelial infiltrate of neutrophils and eosinophils was present, without a significant increase in lymphocytes. Small superficial ulcers covered with fibrin and neutrophils overlying hyperplastic lymphoid follicles were occasionally observed. The lamina propria was oedematous and haemorrhagic and contained mainly polymorphonuclear cells. The pattern of inflammation was similar to that seen in acute self limiting bacterial enterocolitis.

The principal features of chronic inflammatory lesions were mucosal architectural alterations with crypt distortion and atrophy in the colon, and villous blunting and fusion in ileal mucosa. In both ileum and colon there was an increased mixed cellularity and formation of basal lymphoid aggregates in the lamina propria. Whenever one of several biopsies featured chronic lesions, regardless of acute or active inflammation in other fragments, a diagnosis of chronic inflammation was made.

Although non-steroidal anti-inflammatory drugs may induce intestinal disorders, we and others excluded these drugs as factors in the aetiology of reported chronic inflammation.[8 10 52]

CARD15 genotyping (R702W, G908R, and 1007fs), and HLA-B27 and HLA-B62 typing
Genomic DNA was extracted from whole blood using the Qiagen blood and cell culture DNA kit (Westburg BV, Leusden, Netherlands). All patients were genotyped for R702W, G908R, and 1007fs using restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR), followed by separation of the DNA fragments on a 2.5% agarose gel. The missense mutation R702W (GenBank accession number G67950) abolishes the restriction site for *MspI*, resulting in an intact 130 base pair (bp) band for mutant alleles compared with two bands of 54 and 76 bp for wild type alleles (forward primer: 5'-CAGCCCTGATGACATTT CTCTT-3'; reverse primer: 5'-AGCCGCTCCTCCTGCATCTCG TA-3'). The missense mutation G908R (GenBank accession number G67951) creates a restriction site for *HinPII*. The frameshift mutation 1007fs (GenBank accession number G67955) creates a restriction site for *NlaIV*. The presence of a mutant allele results in two bands of 219 and 41 bp, while the wild type allele produces a single 260 bp product (forward

Table 1 Prevalence of *CARD15* variants in the populations according to subtypes defined at baseline

Classification	n	Carriers of CARD15 variant(s)
Controls	140	24 (17%)
Crohn's disease	156	77 (49%)*
Spondyloarthropathy	104	21 (20%)†
Ankylosing spondylitis	75	16 (21%)
Undifferentiated spondyloarthropathies	29	5 (17%)
Gut histology in the spondyloarthropathy population		
Chronic inflammation	40 (38%)	15 (38%)‡
Acute inflammation	24 (23%)	0 (0%)
Normal histology	40 (38%)	6 (15%)

*χ²: p<0.001 (carrier frequency in Crohn's disease v control population).
†χ²: p=0.5 (carrier frequency in general spondyloarthropathies v control population).
‡χ²: p=0.001 (chronic inflammation in patients with *CARD15* variant v chronic inflammation in patients without *CARD15* polymorphism).

Table 2 Carrier frequency of CARD15 variants in patients with spondyloarthropathies, Crohn's disease, and controls (%)

	Spondyloarthropathy (n = 104)				Crohn's disease (n = 156)				Controls (n = 140)			
	R702W	G908R	1007fs	Overall*	R702W	G908R	1007fs	Overall*	R702W	G908R	1007fs	Overall*
CARD15 $^{/+}$	12 (12)	4 (4)	4 (4)	21 (20)	39 (25)	11 (7)	27 (17)	77 (49)	18 (13)	1 (1)	6 (4)	24 (17)
CARD15 $^{/}$	0 (0)	0 (0)	1 (1)		4 (3)	3 (2)	0 (0)		0 (0)	0 (0)	0 (0)	

Data are numbers of patients carrying R702W, G908R or 1007fs variants.
CARD15 $^{/+}$, heterozygous; CARD15 $^{/}$, homozygous.
Overall, total number of patients in the group carrying at least one variant.
*The sum of all allelic CARD15 variants is greater than the overall number of patients carrying at least one variant, as some patients carry two different single nucleotide polymorphisms, thus displaying compound heterozygous status.

primer: 5'-CTGAGCCTTTGTTGATGAGC-3'; reverse primer: 5'-TCTTCCAACCACATCCCCATT-3').

In the patients with known HLA-B27 and HLA-B62 status, typing of these markers was done using the microlymphocytotoxity test, according to Terasaki and McClelland.[53]

Statistical analysis

Statistical significance was determined by the χ^2 test and odds ratios using SPSS (SPSS Inc, Chicago, Illinois, USA). Multivariate analysis (logistic regression) was carried out to investigate whether an association found in univariate analysis was independent of other genetic markers. Probability (p) values of less than 0.05 were considered significant.

RESULTS

We subdivided our cohort into three groups according to gut histology. Forty patients (38%) had normal histology, 24 (23%) had acute gut inflammation, and 40 (38%) had chronic gut inflammation (table 1).

Univariate analysis
Prevalence of CARD15 polymorphisms in the various populations

The prevalences of CARD15 polymorphisms in the total spondyloarthropathy (20%), specific ankylosing spondylitis (21%), and undifferentiated spondyloarthropathy (17%) populations did not differ significantly (table 1). All except one (homozygous for the 1007fs allelic variant) were heterozygous for at least one mutation. The prevalences of R702W, G908R, and 1007fs allelic variants in these spondyloarthropathy populations were 12%, 4%, and 5% respectively

Table 3 Prevalences of CARD15 polymorphisms according to the presence of mainly axial or peripheral involvement in the total spondyloarthropathy group (n = 104), the ankylosing spondylitis group (n = 75), and the undifferentiated spondyloarthropathy group (n = 29)

	CARD15			
	Wild type	Variant	Total	
Total spondyloarthropathy group				
Axial	22	0	22	p = 0.006
Peripheral	61	21	82	
Total	83	21	104	
Ankylosing spondylitis group				
Axial	18	0	18	p = 0.006
Peripheral	41	16	57	
Total	59	16	75	
Undifferentiated spondyloarthropathy group				
Axial	4	0	4	p = 1.0
Peripheral	20	5	25	
Total	24	5	29	

(table 2). No compound heterozygosity was found. All carriers of CARD15 polymorphisms in the spondyloarthropathy group had (a history of) peripheral disease (table 3). There were no significant differences in disease duration or the duration of the follow up period between the spondyloarthropathy patients carrying CARD15 polymorphisms and the patients without these polymorphisms (data not shown).

In the Crohn's disease population, a carrier frequency of 49% (77 of 156 patients) was observed (table 1). Forty three Crohn's disease patients carried at least one R702W polymorphism, 14 carried at least one G908R polymorphism, and 27 carried at least one 1007fs polymorphism. Fourteen patients carried two polymorphisms, of which seven were homozygous and seven were compound heterozygous (table 2).

In the control group, 24 individuals (17%) carried CARD15 polymorphisms (table 1). All except one (compound heterozygous for the R702W and 1007fs variant) were single heterozygotes (table 2).

The prevalence of polymorphisms in the spondyloarthropathy cohort (20%) was not different from that in the control group (17%) (p = 0.5; odds ratio (OR) = 1.22 (95% confidence interval (CI), 0.64 to 2.34)) and significantly lower than in our Crohn's disease population (49%) (p < 0.001; OR = 3.85 (95% CI, 2.17 to 6.83)).

Association between CARD15 polymorphisms and intestinal inflammation in spondyloarthropathy patients

The carrier frequency in the subpopulation of spondyloarthropathy patients with chronic gut inflammation was 38% (15 of 40 patients) which was significantly higher than in the control population (p = 0.006; OR = 2.9 (95% CI, 1.33 to 6.30)) or the other spondyloarthropathy populations (p = 0.001; OR = 5.80 (2.02 to 16.68)) and not statistically different from that in our Crohn's disease population (49%, p = 0.2; OR = 1.62 (0.80 to 3.31)) (table 1).

Of all spondyloarthropathy patients carrying CARD15 polymorphisms, 71% (15 of 21 patients) had chronic gut inflammation, none had acute inflammation, and 29% had normal histology (table 1). The only spondyloarthropathy patient carrying two CARD15 variants also had chronic gut inflammation. In contrast, only 25 of 83 patients with a wild type genotype (30%) had chronic gut inflammation, 29% had acute inflammation, and 41% had normal histology. Consequently, the presence of CARD15 polymorphisms was associated with a higher risk of the development of chronic gut inflammation.

There were no significant differences between the ankylosing spondylitis and the undifferentiated spondyloarthropathy groups for the prevalence of CARD15 polymorphisms in patients with normal gut (3/29 v 3/11, respectively; p = 0.3; OR = 3.3 (95% CI, 0.5 to 19.4)), acute gut inflammation (0/13 v 0/11), or chronic gut inflammation (13/33 v 2/7; p = 0.7; OR = 1.6 (95% CI, 0.3 to 9.7)).

In the subgroup of 54 patients who were clinically reassessed, four had evolved from histological chronic gut inflammation towards clinically overt Crohn's disease. Two of these four patients carried CARD15 polymorphisms. The other 22 patients with chronic gut inflammation in this group did not develop clinical Crohn's disease.

Association between CARD15 polymorphisms and HLA-B27 in spondyloarthropathy patients

There was no significant association between the presence of these two genetic markers. Six of 34 HLA-B27 negative patients carried CARD15 polymorphisms v 13 of 47 HLA-B27 positive patients (p = 0.3; OR = 1.8 (95% CI, 0.6 to 5.3)).

Multivariate analysis

In the subgroup of 53 spondyloarthropathy patients in whom both HLA-B27 and HLA-B62 status was known, logistic regression was undertaken (with the presence of chronic gut inflammation as the dependent variable). This showed that the association between chronic gut inflammation and CARD15 polymorphisms (p = 0.01; OR = 17.3 (95% CI, 2.0 to 152.3)) was independent of HLA-B27 (p = 0.42; OR = 1.7 (0.5 to 6.0)) and HLA-B62 (p = 0.28; OR = 2.5 (0.5 to 13.0)).

DISCUSSION

In this study we describe a novel and remarkably strong association between variants in a host defence gene located on chromosome 16 (CARD15) and a distinct form of gut inflammation in patients with spondyloarthropathies. The prevalence of CARD15 polymorphisms in this subgroup of spondyloarthropathy patients was not significantly different from that seen in patients with Crohn's disease.

Three single nucleotide polymorphisms have been associated with Crohn's disease.[21–23] One variant (1007fs) encodes a truncated protein which results in altered activation of NFκB in response to bacterial stimuli.[29–31] The two other single nucleotide polymorphisms (R702W and G908R) result in an amino acid substitution.

More recently, several groups assessed the linkage of CARD15 variants in Crohn's disease to particular clinical phenotypes, but the results of these retrospective studies are disparate. The presence of two mutations has been linked to younger age at onset and preferential involvement of small bowel.[43] Preference for ileal involvement was also reported by Cuthbert et al[42] and by Ahmad et al.[44] Fibrostenosing disease was the dominant type in a study by Abreu et al.[45] In these studies, no association of CARD15 variants with extraintestinal involvement could be shown.

Our study shows a new association between these three Crohn's disease associated variants in the leucine-rich region of the CARD15 gene and a distinct subpopulation of patients with spondyloarthropathies. As in previous reports, the overall prevalence of mutations in spondyloarthropathy patients was not statistically different from the prevalence in our control population.[35–37] However, unlike previous studies, we identified a distinct clinical subgroup—characterised by the presence of chronic inflammatory gut lesions— with a high prevalence of CARD15 polymorphisms similar to the Crohn's disease population and significantly higher than in the control population or the other spondyloarthropathy patients. Previous studies from our group showed that these patients with chronic gut inflammation were at particular risk for progression to Crohn's disease.[11 12]

Strikingly, in the present study none of the spondyloarthropathy patients with only axial disease carried CARD15 polymorphisms. Carriers of these polymorphisms all had (a history of) peripheral disease. This is in accord with previous studies of patients with ankylosing spondylitis where more

chronic gut inflammation could be found in those with peripheral disease than in those with strict axial disease.[11]

One previous study investigated CARD15 polymorphisms in patients with ankylosing spondylitis, Crohn's disease, and ulcerative colitis.[34] It did not show a higher prevalence of CARD15 variants in patients with ankylosing spondylitis plus Crohn's disease compared with idiopathic ankylosing spondylitis, ankylosing spondylitis plus ulcerative colitis, or healthy controls. However, the low prevalence of CARD15 variants in the Crohn's disease population with ankylosing spondylitis was not compared with the prevalence in a general Crohn's disease population and it unexpectedly revealed a possible association between the G908R CARD15 variant and ankylosing spondylitis plus ulcerative colitis.

Moreover, in a recent study we found an association between CARD15 polymorphisms and the presence of radiological sacroiliitis in Crohn's disease patients, unrelated to the HLA-B27 status of these subjects. These data already suggested a role for the CARD15 gene in the link between gut and joint inflammation.[46]

Our findings confirm the previous reported clinical, therapeutic, and immunological links between spondyloarthropathies and Crohn's disease and provide clear genetic proof for the association between these two disorders. As chronic gut inflammation in the majority of spondyloarthropathy patients remains asymptomatic, this might suggest that CARD15 polymorphisms could be linked to the development of (subclinical) chronic gut inflammation rather than to Crohn's disease as such.

The underlying pathogenic mechanisms that could explain the phenotypic expression of CARD15 mutations in spondyloarthropathies need to be investigated. CARD15 encodes a cytosolic protein that could play a role in spondyloarthropathies by interference with transport of antigens by macrophages from mucosal surfaces to the joints.[55] CARD15 seems to function as an intracellular receptor for bacterial components, where the C-terminal leucine-rich repeat domain (LRR domain) is crucial for responsiveness. The cellular response to bacterial products has been shown to be altered in HEK293T cells transfected with expression plasmids containing any of the three SNPs.[29 31] Moreover, expression of CARD15 in myeloblastic and epithelial cells is enhanced by proinflammatory cytokines and bacterial components, through NFκB.[30 31 56] This response is likely to mediate cytokine production including TNFα, suggesting that upregulation of CARD15 may be part of a positive regulatory loop and facilitate the response of the host to pathogens. A genetically determined disturbance of handling of bacterial products in the intestinal tract, leading to altered transport of antigens by macrophages to synovial tissue, is an interesting hypothesis that should be investigated in spondyloarthropathy. A further identification and characterisation of inflammatory cells involved in gut and joint inflammation may also lead to new therapeutic targets.

Conclusions

A distinct phenotype associated with the three main Crohn's disease associated CARD15 variants is reported in patients with spondyloarthropathies. Our data show that the presence of CARD15 variants in spondyloarthropathy patients strongly predisposes to chronic intestinal inflammation, defining a population at risk for evolution to Crohn's disease. However, the persistence of the subclinical character of the inflammation in a large proportion of patients may reflect the fact that Crohn's disease is a multigeneic disease or alternatively that the heterozygous carriage of CARD15 polymorphisms predisposes only to subclinical inflammation.

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CHAPTER 3

CARD15 polymorphisms are associated with anti-Saccharomyces
cerevisiae antibodies in Caucasian Crohn's disease patients.

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CARD15 polymorphisms are associated with anti-*Saccharomyces cerevisiae* antibodies in caucasian Crohn's disease patients

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Summary

Carriage of *CARD15* gene polymorphisms and the serological marker anti-*Saccharomyces cerevisiae* antibodies (ASCA) are two markers for Crohn's disease (CD). Similar phenotypes have been associated with both markers. In the present study we analysed whether both markers were associated with each other and, if so, whether this association could be explained by a direct link or by an indirect association with those phenotypes. Therefore, we included 156 consecutive Caucasian CD patients and assessed the prevalence of the three common single nucleotide polymorphisms in the *CARD15* gene. Serum samples were analysed for IgA and IgG ASCA by ELISA. CD patients with *CARD15* polymorphisms were more frequently ASCA positive (OR 2·7 (1·4–5·2); $P = 0·002$) and had higher titres for ASCA IgA ($P = 0·005$) and ASCA IgG ($P < 0·001$) compared to patients carrying the wild type polymorphisms. Multivariate analysis demonstrated that this association was independent from ileal disease, penetrating disease and stricturing disease, the need for resective bowel surgery, familial cases, smoking habits and early age at onset. Homozygotes or compound heterozygotes for *CARD15* polymorphisms had significantly more frequent ASCA positivity compared to single heterozygotes (OR 9·1 (1·1–74·2), P_c (corrected P-value) = 0·030). These data indicate that there is a significant association between the carriage of *CARD15* polymorphisms and ASCA, independent of the described phenotypes. Moreover, ASCA positivity is more frequent in CD patients carrying 2 *CARD15* polymorphisms compared to single heterozygotes.

Keywords: ASCA, CARD15, Crohn's disease, caucasian, antibodies

Introduction

Although the exact pathogenesis of Crohn's disease (CD) remains unclear, it is well accepted that an impaired microbial immune response, triggered by environmental and genetic factors, is important [1–4]. Recently, 3 polymorphisms in the *CARD15* gene (two missense mutations (R702W and G908R) and one frame shift mutation (1007fs)), were independently associated with CD [5,6]. It has been estimated that heterozygotes have a 3-fold risk to develop CD and homozygotes or compound heterozygotes a 40-fold risk to develop the disease [5,6]. Genotype-phenotype studies showed different possible associations of these *CARD15* polymorphisms with ileal and stricturing disease [2,7–10], familial cases [11] and early onset of disease [12].

Anti-*Saccharomyces cerevisiae* antibodies (ASCA) are directed against the cell wall mannan of *Saccharomyces cerevisiae*, commonly known as baker's or brewer's yeast [13]. ASCA are considered as a serological marker for CD. However, their pathophysiological role is not yet clear [14]. Sensitivity and specificity of ASCA for CD range from 39% to 65% and 80% to 97·5% [15–18], respectively. Combinations of ASCA with other serological markers as pANCA, I2 and OmpC antibodies are under investigation to obtain a better

serological diagnostic tool for IBD [19]. In CD patients, ASCA are linked with earlier onset of disease [16], ileal involvement, penetrating and stricturing disease and need for resective bowel surgery [19–22]. One study also pointed at a possible negative association with smoking behaviour [23]. Previous family and twin studies already suggested a genetic influence on ASCA formation. Indeed, family and twin studies revealed that unaffected twins and unaffected relatives have higher ASCA titres compared to healthy controls [20,24–27]. The aim of the present study was to analyse whether CARD15 and ASCA are related and if so, whether this association can be explained by a direct link or an association by phenotypes.

Materials and methods

Study population and assessment of clinical characteristics

The study population consisted of 156 consecutive, Caucasian CD patients (57 male, 99 female). The diagnosis of CD was based on clinical, endoscopical, histological and/or radiological findings. All patients were seen by a gastroenterologist. Family and personal medical history, onset of disease, localizations of inflammation, amount and types of surgical interventions, history or current presence of fistulae, past and present intake of medication and smoking habits were recorded. Familial Crohn's disease was considered when at least one first, second or third degree relative also had a proven Crohn's disease. The specific anatomical localization was based on documented areas of macroscopical and/or microscopical inflammation and was subdivided into 3 categories: ileal, ileocolonic or colonic disease. Penetrating disease was defined by the history or actual presence of fistulae, abscesses or perforations. Fistulae secondary to surgery were excluded. Stricturing disease was considered in those patients without fistulae, who had radiological or surgical evidence for stenosis.

Additionally, we tested ASCA in 188 controls including 24 osteoarthritis patients, 56 rheumatoid arthritis patients and 108 blood donors of whom 87 could also be typed for *CARD15* variants.

Detection of ASCA by ELISA

The Medizym ASCA kits (Medipan Diagnostica, Germany) were used. Tests were performed according to instructions from the manufacturer, including the use of cut-offs that were determined at 20 U/ml for both IgA and IgG ASCA. Briefly, serum was diluted 1 : 50 and applied to the microtitre plates (100 µl/well), coated with cell wall mannan from a mixture of different *Saccharomyces* strains. The plates were incubated for one hour at 37°C. To remove unbound serum components, plates were washed five times. Consequently 100 µl of conjugate, specific for either IgG of IgA, coupled

with horseradish peroxidase was added, followed by an incubation period of 30 min at 37°C. The plates were washed again five times, after which substrate was added (3,3′,5,5′-tetramethylbenzidine in citrate buffer containing hydrogen peroxide). Plates were incubated in the dark at room temperature for 10 min. The reaction was stopped using a stop solution containing sulphuric acid, turning the colour of the solution from blue to yellow. Plates were read at a wavelength of 450 nm.

ASCA IgG and IgA levels were determined using a standard curve, for which the manufacturer supplied calibrators. Study personnel were blinded for diagnosis during these assays. Each sample was tested in duplo and 2 positive control samples were run on each plate. The mean values for the IgG-samples were 60 U/ml and 29 U/ml with a coefficient of variation (CV%) of 2% and 4·5%, respectively. The mean values for the ASCA IgA samples were 18 U/ml and 28 U/ml with a CV% of 6·9% and 5·6%, respectively. The mean CV% between duplo's of all samples was 3·15% for ASCA IgA and 4·23% for ASCA IgG. Unless otherwise specified, ASCA were considered positive when either ASCA IgA or IgG was positive.

Genotyping of R702W, G908R and 1007fs and sequencing

Genomic DNA was extracted from whole blood using Qiagen blood and cell culture DNA kit (Qiagen, Germany). All patients were genotyped for R702W, G908R and 1007fs using a RFLP-PCR technique, followed by separation on 2·5% agarose gel. The missense mutation R702W (GenBank accession number G67950) abolishes the restriction site for *MspI* (5′-CAGCCCTGATGACATTTCTCTT-3′ and 5′-AGC CGCTCCTCCTGCATCTCGTA-3′), resulting in an intact 130-bp band for mutant alleles compared to two bands of 54- and 76-bp for wild type alleles. The missense mutation G908R (GenBank accession number G67951) creates a restriction site for *HinPII*. The frame shift mutation 1007fs (GenBank accession number G67955) creates a restriction site for *NlaIV* (5′-CTGAGCCTTTGTTGATGAGC-3′ and 5′-TCTTCCAACCACATCCCCATT-3′). The presence of a mutant allele results in two bands of 219 and 41 base pairs, while the wild type allele produces a single 260-bp product.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS inc., Chicago, Illinois, USA). Groups were compared with Mann–Whitney U-test because normality was not achieved. Dichotomous data were analysed using Pearson's χ^2 test or with Fisher's exact test when the expected count was less than five in at least one cell. Odds ratios were calculated with their corresponding 95% confidence interval (CI). When indicated, a corrected P-value (P_c) was calculated using Bonferroni's correction. We also calculated conditional odds ratios

and their corresponding 95% CI by binary logistic regression.

Ethics

The study was approved by the local ethics committee. All patients gave written informed consent.

Results

Patients characteristics

Thirty-eight patients had ileal disease, 79 patients had ileo-colonic disease and 39 patients had only colonic involvement. Seventy-three patients needed resective small bowel surgery, 92 patients had penetrating disease and 33 patients had stricturing disease. Thirty patients had at least one affected relative. Forty patients were ex-smokers and 44 patients smoked at the time of evaluation. Mean age was 38 years (range 18–80 years) and mean age at diagnosis was 27 years (range 9–66 years). In 48 patients, onset of disease was before the age of 20.

Prevalence of *CARD15* mutations

Seventy-seven (49·3%) of 156 CD patients carried *CARD15* polymorphisms. Forty-three CD patients carried at least one R702W polymorphism, 14 patients carried at least one G908R polymorphism and 27 patients carried at least one 1007fs polymorphism. Fourteen patients carried two polymorphisms of which 7 patients were homozygous and 7 patients compound heterozygous (Table 1). In a local control population, 19/87 (21·8%) controls carried at least one *CARD15* variant.

Prevalence of ASCA positivity

Eighty-two (52·6%) CD patients were ASCA positive, 71 (45·5%) patients had ASCA IgA and 57 (36·5%) patients ASCA IgG. In a control population, 5/188 (2·6%) tested positive for ASCA (3/24 OA patients, 0/56 RA patients and 2/108 blood donors), confriming the high specificity of the test. Only 1/87 controls who were typed for *CARD15* polymor-

Table 1. Number of patients carrying R702W, G908R or 1007fs polymorphisms.

	CD patients ($n = 156$) n (%)
R702W heterozygotes	33 (21·3%)
G908R heterozygotes	9 (5·8%)
1007fs heterozygotes	21 (13·5%)
R702W homozygotes	4 (2·6%)
G908R homozygotes	3 (1·9%)
R702W + G908R compound heterozygotes	1 (0·6%)
G908R + 1007fs compound heterozygotes	1 (0·6%)
R702W + 1007fs compound heterozygotes	5 (3·2%)

Number of CD patients carrying the different *CARD15* polymorphisms. There were no patients homozygous for 1007fs.

phisms had ASCA. This control carried the 1007fs *CARD15* polymorphism.

Univariate analysis of association between *CARD15* genotype and ASCA positivity

Fifty of 77 (64·9%) carriers of *CARD15* polymorphisms had positive ASCA IgA or IgG, in contrast to 32 (40·5%) of 79 wild type patients (OR 2·7; 95% CI (1·4–5·2), $P = 0·002$).

Forty-four (57·1%) of 77 carriers of CARD15 polymorphisms were positive for ASCA IgA *versus* 27 of (34·2%) 79 wild type patients (OR 2·87; 95% CI (1·49–5·50), $P = 0·004$) and 37 (48·1%) of 77 carriers tested positive for ASCA IgG *versus* 20 (25·3%) of 79 wild type patients (OR 2·7; 95% CI (1·4–5·4), $P = 0·003$) (Table 2).

Carriage of 1 *versus* 2 *CARD15* polymorphisms and ASCA positivity

Carriers of 2 *CARD15* polymorphisms were more frequently ASCA positive compared to patients who carried only 1 polymorphism: 13 (93%) of 14 patients who carried 2 *CARD15* variants were ASCA positive compared to 37 (58·7%) of 63 carriers of 1 polymorphism (OR 9·135, 95% CI (1·1–74·2), $P = 0·015$, $P_c = 0·030$). When looking at ASCA IgA and ASCA IgG separately, we could again find higher prevalences of ASCA IgA or ASCA IgG in those patients who

Table 2. ASCA status in relation to the carriage of *CARD15* polymorphisms.

	ASCA positivity (IgA or IgG)		ASCA IgA positive		ASCA IgG positive	
Carriage of *CARD15* polymorphisms						
No	32 (40·5%)	$P = 0·002$	27 (34·2%)	$P = 0·004$	20 (25·3%)	$P = 0·003$
Yes	50 (64·9%)		44 (57·1%)		37 (48·1%)	
Total	82 (52·6%)		71 (45·5%)		57 (36·5%)	

ASCA positivity for ASCA IgA or IgG, ASCA positivity for ASCA IgA and ASCA positivity for ASCA IgG in relation to the carriage of *CARD15* polymorphisms.

carried 2 polymorphisms, but it did not reach significance for ASCA IgG (IgA: OR 5·81, 95% CI (1·0–28·1), $P = 0·017$, $P_c = 0·034$; IgG: OR 3·33, 95% CI (0·9–77·8) $P = 0·053$, $P_c = 0·106$).

Association between carriage of CARD15 polymorphisms and ASCA titres

When considering the ASCA titres as continuous data, they were significantly higher in patients who carried a common CD associated CARD15 polymorphism compared to those who carried only wild type polymorphisms (ASCA IgA: median 23·5 U/ml versus 14·4 U/ml, $P = 0·005$; ASCA IgG: median 19·4 U/ml versus 7·2 U/ml, $P < 0·001$).

Carriage of 1 versus 2 CARD15 polymorphism and ASCA titres

Carriers of 2 CARD15 variants had higher titres compared to patients who carried only 1 variant for ASCA IgA (Median 46·3 U/ml versus 20·5 U/ml, $P = 0·033$, $P_c = 0·066$) and ASCA IgG (Median 31·6 U/ml versus 17·5 U/ml, $P = 0·187$, $P_c = 0·37$), but this was not statistically significant.

Association between carriage of different CARD15 polymorphisms and ASCA positivity

The data on ASCA positivity in relation to the different CARD15 polymorphisms are shown in Table 3. These results provide no arguments for a higher association between one specific polymorphism and ASCA positivity.

Multivariate analysis by logistic regression

Multivariate analysis was performed with a logistic regression model, using ASCA as dependent variable and familial CD, onset before the age of 20 years, ileal, penetrating and stricturing disease, need of resective bowel surgery, present smoking and carriage of CARD15 polymorphisms as covariates (Table 4). In this model, carriage of CARD15 polymorphisms and ileal disease were significant and independent predictors for ASCA positivity.

Table 3. Carriage of different CARD15 polymorphisms in function of ASCA positivity.

	ASCA positivity		
	No	Yes	
Carriage of at least 1 R702W polymorphism	15	28	$P = 0·575$
Carriage of at least 1 G908R polymorphism	3	11	
Carriage of at least 11007fs polymorphism	10	17	

Pearson's χ^2 test shows that ASCA positivity is not significantly different between the 3 CARD15 polymorphisms.

Discussion

This study describes the association between a genetic predictor of CD (carriage of a CARD15 polymorphism) and a serological marker for CD (ASCA).

So far, data on the association between CARD15 and ASCA are very disparate. Data from Abreu et al. [7] suggested an association between the 1007fs CARD15 polymorphism and ASCA positivity, but the authors eventually rejected that hypothesis since this association could not be confirmed in a second, smaller test population (in which however, the prevalence of ASCA positivity was significantly different from the prevalence in the first test population) [7]. In a Scottish population, where the prevalence of CARD15 polymorphisms is much lower, no significant association between CARD15 polymorphisms and ASCA could be found, although there was a trend towards an association between the carriage of 2 copies and ASCA positivity [28]. Another recent study in a Dutch population, described an association between ASCA positivity or atypical pANCA positivity and carriage of the 1007fs or G908R polymorphism [29].

In our present study, we show that there is an association between the carriage of at least one of the three common CARD15 polymorphisms (R702W, G908R and 1007fs) and ASCA. We also found that patients, carrying 2 CARD15 polymorphisms were more frequently ASCA positive compared to patients carrying 1 polymorphism. Multivariate analysis, showed that the presence of CARD15 polymorphisms is a significant predictor of ASCA positivity, conditionally independent from the related phenotypes. This indicates that this association between the carriage of CARD15 polymorphisms and ASCA can not be explained only by an indirect link with the common associated, clinical phenotypes. In the same analysis, ileal disease was also a significant, independent predictor of ASCA. This might suggest that other (environmental?) factors might also contribute to the formation of

Table 4. Multivariate analysis with ASCA positivity as dependent variable.

Covariate	P-value	OR	95% CI
Carriage of CARD15 polymorphisms	0·028	2·26	1·09–4·67
Familial CD	0·218	1·76	0·71–4·35
Age of onset before 20	0·608	1·23	0·56–2·71
Ileal disease	0·002	4·48	1·72–11·62
Need of resective surgery	0·143	1·91	0·82–4·56
Present smoking	0·060	0·46	0·20–1·03
Penetrating disease	0·170	2·02	0·74–5·55
Stricturing disease	0·722	1·38	0·23–2·75

Logistic regression model with ASCA positivity as dependent variable and carriage of CARD15 polymorphisms and related phenotypes as covariates. The calculated odds ratio's (OR) with their 95% confidence intervals (95% CI) are shown.

ASCA. The fact that data on a possible association between carriage of *CARD15* polymorphisms and ASCA are so disparate might be explained by differences in environmental factors and different genetic backgrounds. Associations between genetic markers and serologic markers have previously been described for several diseases. In insulin dependent diabetes, disease associated autoantibodies are associated with susceptibility HLA class II alleles [30]. Coeliac associated antibodies can identify healthy first-degree relatives who express coeliac associated HLA haplotypes [31]. In rheumatoid arthritis, rheumatoid factor is associated with the HLA shared epitope and the −2849 IL-10 promoter polymorphism [32,33]. Carriage of different HLA class II alleles is associated with a specific antibody response to nuclear antigens, in particular anti-SSA/Ro52, both in primary Sjögren's syndrome and lupus [34,35] and may be more strongly associated with the antibody subsets than with the disease status itself [36]. Other nuclear antibodies (anti-SSB/La), associated with HLA class II genes, have also recently been linked with carriage of polymorphisms in the genes for transforming growth factor β and tumour necrosis factor α in patients with primary Sjögren's syndrome [37]. Different hypotheses can be generated to explain how the carriage of different HLA types or different gene polymorphisms on the promoters of cytokines, might influence antibody responses [33,35]. How the different polymorphisms on the *CARD15* gene are involved in the antibody response against mannan from *Saccharomyces cerevisiae* is still unclear. The *CARD15* gene encodes for the Nod2/CARD15 protein, a member of the Apaf-1/Ced-4 family of apoptosis regulators and an intracellular protein expressed in monocytes, macrophages, epithelial cells, granulocytes and dendritic cells. Nod2 activates the NF-κB pathway after stimulation by bacterial products. Initial reports suggested lipopolysaccharide as a possible ligand for Nod2, but recently, 2 independent groups highlighted muramyl dipeptide as the major activator of the Nod2-receptor [6,38–41]. The activation of the NF-κB–pathway is followed by an enhanced production and secretion of proinflammatory cytokines [42].

It has been suggested that the described polymorphisms in the *CARD15* gene give rise to an impaired NF-κB activation by a deficient recognition of microbial antigens. This might result in an impaired killing of intracellular microbes, which leads to inflammation [38,40]. Whether ASCA is just a side-effect of this inflammation or whether ASCA have themselves a pathological role by cross-reactivity with self-antigens in human gut or other tissues is still unknown [43].

In conclusion, our data suggest that there is an association between the presence of *CARD15* gene polymorphisms and the serological marker ASCA in a West-European CD population. This could be interpreted in a double way. *CARD15*, as an immune response gene, may modulate in some way humoral immunity predisposing to the generation of ASCA. Or, carriage of *CARD15* polymorphisms and ASCA may be both associated with a particular, as yet undefined, phenotypical subset of CD. However, we proved in the present study that the association between carriage of *CARD15* polymorphisms and ASCA could not be explained by an indirect link with the common *CARD15* and ASCA related phenotypes.

Acknowledgements

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CHAPTER 4

Anti-citrullinated protein/peptide antibodies (ACPA) in
rheumatoid arthritis: specificity and relation with rheumatoid
factor.

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Anti-citrullinated protein/peptide antibodies (ACPA) in rheumatoid arthritis: Specificity and relation with rheumatoid factor

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Abstract

Anti-citrullinated protein/peptide antibodies (ACPA) are highly specific and sensitive markers for rheumatoid arthritis (RA). For instance, for the anti-CCP2 assay, sensitivities ranging from 55% to 80% and specificities ranging from 90% to 98% have been reported. Despite their high specificity, recent reports have suggested that ACPA may be found in some patients with other rheumatic autoimmune diseases, including psoriatic arthritis, systemic lupus erythematosus and Sjögren's syndrome. Also, the differences between the classical rheumatoid factor (RF) and ACPA, as well as the complementarity between both tests have recently been demonstrated more clearly. Indeed, both antibody systems have a different association with specific RA features like extra-articular manifestations, a different association with the HLA shared epitope and, behave differently following anti-TNF therapy.
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Keywords: Anti-citrullinated peptide/protein antibodies (ACPA); Rheumatoid factor; Rheumatoid arthritis; Specificity

Contents

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1. Introduction and short history of ACPA

Autoantibody formation is a common manifestation of rheumatoid arthritis (RA). The oldest and best-known antibody is the rheumatoid factor (RF), which constitutes one of the revised American College of Rheumatology (ACR) criteria for RA. In 1964, a new antibody was described: the antiperinuclear factor (APF). This factor was the first of a new group of antibodies, which we currently know to be directed against citrullinated residues and known as anti-citrullinated protein/peptide antibodies (ACPA). After the description of the APF (detected by indirect immunofluorescence and directed against components in the perinuclear granules of human buccal mucosa cells) anti-keratin antibodies (AKA; directed against epithelium of rat oesophagus) were reported to be associated with RA. Moreover, it was shown that the epitopes recognised by both APF and AKA contain citrullinated residues, converted from arginine into citrulline by the enzyme peptidylarginine deiminase (PAD) [1]. Since the epitope-specificity of ACPA was elucidated; and, in order to optimize stan-

dardization of ACPA detection, more user-friendly tests were developed. Substrates for such tests include deiminated proteins (filaggrin or fibrinogen) or peptides. The most widely available synthetic peptide is known as CCP (cyclic citrullinated peptide). The first available CCP assay (anti-CCP1) was further optimized by screening of dedicated peptide libraries (anti-CCP2). Alternative peptides include pepA and pepB [2]. The characteristics of those different assays have recently been reviewed by Peene et al. [3].

2. ACPA as a highly specific marker for rheumatoid arthritis

In general, all recent ACPA assays have a good diagnostic performance for the diagnosis of RA. Anti-CCP2 assays have a sensitivity of 55% to 80% and a specificity of 90% to 98% for established RA [4–6]. Sensitivities and specificities can differ between studies, due to the use of different patient populations, different control populations [7], different assays, and

Table 1
Comparison of the sensitivity and specificity of RF and ACPA at a preset specificity level of ≥ 98% specificity

			Sens at ≥ 98% spec (%)	AUC (95% CI)
Established disease	Nielen, 2005 [27]	IgM-RF	45.2	0.73 (0.68–0.77)
		Anti-CCP2	71.1	0.90 (0.87–0.93)
		ACF	67.6	0.89 (0.86–0.93)
	De Rycke, 2004 [12]	IgM-RF	12.8	0.83 (0.78–0.88)
		Anti-CCP2	73.7	0.89 (0.84–0.93)
		Anti-PepA	63.6	NA
		Anti-PepB	54.2	NA
Undifferentiated joint disease	Hoffman, 2005 [14]	IgM-RF LF	41.7	0.84 (0.80–0.88)
		IgM-RF WR	32.6	0.82 (0.77–0.87)
		Anti-PepA	58.7	0.78 (0.73–0.84)
		Anti-PepB	48.3	0.79 (0.74–0.84)

ACF=Anti citrullinated fibrinogen antibodies, NA=not applicable, CI=confidence interval.

the use of different cut-offs. In general, tests are validated in established disease. From a clinical point of view, tests should not only be evaluated in established disease, but also in patients with early disease and in patients with undifferentiated inflammatory joint disease. Depending on the different cohorts of patients and the different assays, the sensitivities for the anti-CCP2 assay in these groups ranged from 43% to 64% with specificities of 96% to 98% [8–10]. In general, ACPA perform better than RF for the diagnosis of RA. Comparing both tests should be done by the comparison of the areas under the curve (AUC) of a receiver operating characteristic (ROC) curve or by comparison of sensitivities at equal specificity levels [11]. This exercise has been done for anti-CCP antibodies [12,13], anti-citrullinated fibrinogen antibodies (ACF) [13] and anti-pepA/pepB antibodies [12,14] (Table 1). Those ROC curve analyses indicate that, using a high cut-off for RF, an equal specificity as ACPA testing can be obtained, in disadvantage of the sensitivity of RF.

3. Reports on appearance of ACPA in non-RA rheumatic diseases

Despite the high specificity of ACPA for RA, there are several reports about the occurrence of ACPA in non-RA rheumatic diseases.

3.1. Systemic lupus erythematosus (SLE)

In a recent study of 201 consecutive SLE patients (diagnosed as SLE and fulfilling the ACR criteria for SLE), we found anti-CCP2 antibodies in 11 patients (5.5%) patients of whom 4/7 evaluable patients had no erosive disease [15]. Mediwake et al. reported anti-CCP1 antibodies in 2/10 (20%) of SLE patients with erosive arthritis and in 1/50 (2%) of SLE patients without erosive disease and noted that SLE patients were less frequently positive for anti-CCP1 antibodies than for RF or anti-RA33 antibodies [16].

3.2. Primary Sjögren's syndrome

Gottenberg et al. studied 143 patients with primary Sjögren's syndrome, excluding patients with erosive disease [17]. In 13.3% patients, anti-CCP2 antibodies were present. It should be noted that 11 of these

patients had synovitis and 9 fulfilled the ACR classification criteria for RA. The mean disease duration in those non-erosive ACPA positive patients was 10 years. However, future evolution towards erosive disease could not be ruled out.

3.3. Psoriatic arthritis (PsA)

We recently investigated sera of 192 PsA patients. Eighteen of them (= 9.4%) had anti-CCP2 antibodies (some of them at high titers), which was significantly higher than 1.5% expected by the specificity level of 98.5% in a random rheumatic control population. Thirteen of them were also positive in an alternative ACPA assay (anti-pepA/pepB assay) [18]. Another report found anti-CCP antibodies in 16 of 102 PsA patients (15.7%) and described association with RF positivity and more involved and erosive joints [19].

3.4. Juvenile idiopathic arthritis (JIA)

Since some subgroups of patients with JIA will develop a persistent erosive disease, similarly to RA, it might be questioned whether ACPA are associated with this disease. Two studies found a general prevalence of anti-CCP antibodies in 2–5% of JIA patients [20,21], mainly in polyarticular and RF positive patients. The prevalence of anti-CCP antibodies may reach 13% in patients with a polyarticular onset of the disease [22].

3.5. Hepatitis C

RF can be found in about 30% to 70 % of patients with a hepatitis C infection [23,24]. In contrast, false positivity of anti-CCP antibodies in this disease context has been reported to be significantly lower (0–7%)[23,24]. This is of particular importance given the fact that patients with hepatitis C infection may present with arthralgia or synovitis, in which situation the differential diagnosis with RA is often difficult.

3.6. Explanations for ACPA false positivity

Different hypotheses can be constructed to explain the ACPA positivity in patients with SLE, Sjögren's syndrome or PsA. In a cohort of patients with non-RA

rheumatic diseases, co-incidence of a non-RA rheumatic disease and (latent) RA is theoretically possible in about 1%. It remains very difficult to pick up the single patient with a non-RA rheumatic disease and concomitant (latent) RA. More patients might display ACPA due to false positivity (which is at least 1.5%) than there are patients with (latent) RA (estimated on 1%). It would be wise to follow-up carefully those ACPA positive patients with a non-RA rheumatic disease in order not to miss an eventual evolution to RA. Indeed, several studies indicated that ACPA positive patients are more susceptible to develop persistent erosive disease [10,25] and that ACPA may occur years before RA [26,27]. Another hypothesis to explain ACPA positivity in those patients with non-RA rheumatic diseases is that reactivity with citrullinated proteins or peptides could be false positive, due to non-specific binding with the substrate. This phenomenon has been observed in animal models [28]. Therefore, it might be useful to confirm ACPA reactivity with different citrullinated substrates [15,17,18] or by a control assay with the non-deiminated control substrate. However, in clinical practice, ACPA are generally tested by only one assay, leaving the clinician unable to make a distinction between true reactivity against citrullinated peptides and reactivity against non-specific residues.

4. Phenotypic correlations with RF and ACPA

It is generally accepted that patients with extra-articular manifestations such as rheumatic nodules are more frequently RF positive. This is in contrast with ACPA. Indeed, RF positivity and not ACPA positivity seemed to be more frequent in patients with compared to patients without such extra-articular manifestations [12]. Similar results were previously found with AFA [29]. Both RF and ACPA have been associated with more severe and more erosive disease [7,25,30].

5. Associations of RF and ACPA with the HLA shared epitope

In RA patients, there is an association between ACPA and the HLA shared epitope. This is in contrast to RF, which seems to be less associated with the HLA

shared epitope. This is reflected by higher odds ratios (OR) for the association between ACPA and the HLA shared epitope than for the association between RF and the HLA shared epitope [31,32]. Three studies directly compared these associations. In a study with long-standing RA patients, ACPA, and not RF, were significantly more frequent among those who had one or two copies of the HLA shared epitope, versus those who had no HLA shared epitope [12]. Goldbach-Mansky found that different ACPA had a higher OR for the association with the HLA shared epitope than RF in patients with synovitis of recent onset and early RA [33]. Similar results were found in a study with patients with undifferentiated joint disease [34]. The pathophysiological significance of the T-cell response to citrullinated epitopes (and its relation with the HLA shared epitope) has been reviewed by Sebbag et al. [1].

6. Evolutions of RF and ACPA concentrations following biological therapy

Four studies evaluated the effect of ACPA and RF concentrations after infliximab treatment. All studies found a significant decrease in RF titers. Two of them found no decrease in ACPA concentrations [35,36] after 22 and 30 weeks; another study described a decrease of ACPA levels at week 24 [37]; the fourth study found a decrease of ACPA levels at week 30; however at week 52 and week 78 there was an evolution towards baseline levels [38]. The discrepant behavior between ACPA and RF following biological therapy has also been demonstrated in a study with diverse non-biological treatments in which a significant decrease of both RF and ACPA was seen. The decrease of RF titers was associated with effective treatment. In contrast, a decrease of ACPA titers was associated with shorter disease duration [39]. After rituximab therapy, which induces B-cell depletion, a significant decrease of ACPA or RF could only be observed in patients with response to treatment and original titers were regained after relapse [40].

7. Future use of RF and ACPA

In general, ACPA are superior to RF for diagnosing RA. In view of the reduced costs of RF

versus ACPA, it is relevant to identify situations where the diagnostic performance of RF equals that of ACPA. This is the case if RF reaches very high levels or if intermediately high RF levels occur in patients with a high pretest probability for RA [14]. A guideline for clinicians might thus be a two-step testing model. One can start with RF: when there is a high pretest probability for RA (based on the clinical symptoms), or when there are very high RF titers, RF testing may be sufficient. In case of lower pretest probability or when RF is negative or intermediately high, additional ACPA testing is advised.

In prediction models to forecast persistence, disability or erosive disease both ACPA and RF are important. In most models both RF and ACPA are significant covariates, indicating that they are complementary [7,25,30].

8. Conclusions

ACPA and RF are distinct antibody systems yielding different information. They also differ in their association with the HLA shared epitope and with extra-articular manifestations, and behave differently following therapy. It can be stated that ACPA have better diagnostic properties than RF, which is reflected by a higher sensitivity at a preset high specificity level. However, the presence of ACPA does not exclude other rheumatic diseases and should always be interpreted in the full clinical context. Since ACPA assays are more expensive than RF assays, one may consider a two-step testing model with RF testing in a screening step and additional ACPA testing under specific conditions. On the other hand, in cases where ACPA testing is more widely available and used as a screening test, additional RF testing can still be quite useful, amongst others because of its association with extra-articular manifestations.

Acknowledgments

Take-home messages

■ ACPA are highly specific markers for rheumatoid arthritis; yet, they do not exclude the diagnosis of other autoimmune diseases including psoriatic arthritis, systemic lupus and Sjögren's syndrome.
■ RF and ACPA are distinct and complementary antibody systems.

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CHAPTER 5

Anti-citrullinated peptide antibodies may occur in patients with psoriatic
arthritis.

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Anti-citrullinated peptide antibodies may occur in patients with psoriatic arthritis

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Background: Anti-cyclic citrullinated peptide (anti-CCP) antibodies are considered highly specific markers of rheumatoid arthritis. Despite the high specificity of the test, anti-CCP antibodies have also been observed in psoriatic arthritis.

Objective: To determine the frequency of anti-CCP antibodies in psoriatic arthritis and to describe the clinical characteristics of such patients.

Methods: Serum samples from 192 patients with psoriatic arthritis were analysed for anti-CCP antibodies. A previously defined cut off point was applied at a specificity level of $\geqslant$98.5% (42 U/ml). Antibodies against pepA and pepB (two synthetic citrullinated peptides) were determined on samples containing anti-CCP antibodies by line immune assay. The swollen joint count and the numbers of affected joints (present or past) were recorded. Clinical features were noted and if available radiographs of hands and feet were scored for erosions. Rheumatoid factor was determined in all samples.

Results: Anti-CCP antibodies were found in 15 patients (7.8%); 13 of 15 anti-CCP2 positive samples were also positive for anti-pepA or pepB antibodies. The prevalence of anti-CCP antibodies was higher than expected in view of the highly specific cut off applied in the test. Detailed analysis of the clinical and radiological features makes it improbable that the high prevalence of anti-CCP antibodies resulted solely from concomitant psoriasis and rheumatoid arthritis or from misclassification.

Conclusions: Anti-CCP antibodies may be present in patients with psoriatic arthritis. Although some of the present cohort could have had psoriasis with concomitant rheumatoid arthritis, a proportion at least had the typical characteristics of psoriatic arthritis as the primary diagnosis.

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Psoriatic arthritis is a type of inflammatory joint disease in which axial or peripheral arthritis is associated with psoriasis. The picture of psoriatic arthritis is broad and comprises oligoarticular or polyarticular peripheral arthritis and axial involvement.[1] Psoriatic arthritis shares features of spondyloarthropathies and rheumatoid arthritis. Evolution from oligoarticular disease to polyarticular disease has been described.[2 3] McGonagle *et al* suggested features of psoriatic arthritis that are helpful in distinguishing it from rheumatoid arthritis.[4] These comprise the following: asymmetrical oligoarticular disease predominantly of the lower limbs, distal interphalangeal joint (DIP) involvement, enthesitis, dactylitis, typical radiological features (pencil-in-cup phenomenon, ankylosis of small hand or feet joints, arthritis mutilans (fig 1)), radiological sacroiliitis, and inflammatory low back pain. The broad spectrum of the disease makes it difficult to develop good criteria for diagnosis and classification. Although different criteria have been suggested, none are widely accepted.[2 4 5 9] At present an international multicentre validation of diagnostic criteria is under way.[6]

Anti-cyclic citrullinated peptide (CCP) antibodies are antibodies against synthetic citrullinated peptides and are specific markers of rheumatoid arthritis. They belong to a group of anti-citrullinated protein/peptide antibodies (ACPA). Rheumatoid sera were found to contain antibodies that were detected by indirect immunofluorescence in rat oesophagus (the so called anti-keratin antibodies)[10] or in human buccal mucosal cells (the so called anti-perinuclear factor), and on western blotting of human epidermis (anti-filaggrin antibodies).[11] This reactivity was found to be critically dependent on the presence of epitopes containing the amino acid citrulline[11] Based on this knowledge, other assays were developed using synthetic citrullinated peptides such as CCP,[12 13] peptide A (pepA), and peptide B (pepB).[14 15] The anti-CCP enzyme linked immunosorbent assay (ELISA) is a more widely available test, based on citrulline containing peptides derived from filaggrin and later improved by screening dedicated peptide libraries.[13 15 20]

Despite the described high specificity of the anti-CCP test, we and others[19] have identified patients with psoriatic arthritis who are positive for anti-CCP antibodies. Based on these observations, we analysed a large group of patients diagnosed by their treating rheumatologist as having psoriatic arthritis, based on the presence of psoriasis with arthritis. We have assessed the prevalence of anti-CCP antibodies in these cases and described in detail their clinical and radiological characteristics in order to determine whether they can be considered genuine cases of psoriatic arthritis.

METHODS

Patients and characteristics

We included patients with skin or nail psoriasis who also had spondylitis or peripheral arthritis, and in whom serum samples had been sent to the laboratory of the department of rheumatology for routine work up. Spare serum was available from 192 patients. The study was approved by the local ethics committee. Patient and disease characteristics of all 192 patients included in the study are given in table 1.

Figure 1 Radiographs of the hands of a patient with positive anti-cyclic citrullinated peptide antibodies (male, aged 68 years), showing ankylosis of different finger interphalangeal joints.

Table 1 Patients and disease characteristics

Demographic features	
Sex (male/female ratio)	111/81
Mean age (years) (range)	47 (16 to 78)
Mean disease duration (years) (range)	10 (0 to 47)
Familial psoriasis	77/192 (40%)
Familial IBD, PsA, or AS	27/192 (14%)
Actual use of DMARDs	163/192
Clinical features	
Skin involvement	172/192 (90%)
Nail involvement	105/192 (55%)
Inflammatory low back pain	76/192 (40%)
DIP involvement	75/192 (39 %)
Enthesitis	84/192 (44%)
Dactylitis	78/192 (41%)
Peripheral arthritis	187/192 (97%)
Persistent asymmetrical mono- or oligoarticular disease, predominantly of the lower limbs	43/192 (22%)
Radiological features	
Erosive disease	64/139 (46%)
Typical radiographic features	23/139 (16%)
Sacroiliitis	42/149 (28%)
Number of patients with at least one feature helpful in discriminating psoriatic arthritis from rheumatoid arthritis.[1]	177/192 (92%)

AS, ankylosing spondylitis; DIP, distal interphalangeal joint; DMARD, disease modifying anti-rheumatic drug; IBD, inflammatory bowel disease; PsA, psoriatic arthritis.

The clinical and radiological characteristics of the patients were obtained by retrospective analysis of the clinical records and review of the radiographs. A swollen joint count (SJC) was calculated at the sampling date; 74 joints were taken into account and included shoulders, elbows, wrists, metacarpophalangeal joints, temporomandibular joints, acromioclavicular joints, sternoclavicular joints, knees, ankles, tarsus, metatarsophalangeal joints, and all interphalangeal joints, counted as distinct units. Based on radiographs, the number of erosive or destructed hand or foot joints was calculated, taking into account the wrist, all metacarpophalangeal joints, the tarsus, all metatarsophalangeal joints, and all interphalangeal joints as distinct units (radiological joint count (RXJC), maximum 60). Enthesopathy, inflammatory back pain, and radiological sacroiliitis were defined according to the ESSG criteria for spondyloarthropathy,[21] briefly summarised as follows:

- *enthesopathy*: past or present spontaneous pain or tenderness on examination of the site of the insertion of the Achilles tendon or plantar fascia;
- *inflammatory back pain*: history of or present symptoms of spinal pain in the back or neck meeting at least four of the following criteria: onset before age 45, insidious onset, improvement with exercise, association with morning stiffness, and of at least three months' duration;
- *radiological sacroiliitis*: bilateral grade 2 or more or unilateral grade 3 or more.

Dactylitis was defined according to the definitions by Moll and Wright[1] as follows: interphalangeal involvement with flexor tendon sheath effusion; pencil-in-cup phenomenon; arthritis mutilans. Ankylosis of small hand or foot joints was considered a typical radiological feature of psoriatic arthritis.[4]

Anti-CCP antibodies

Anti-CCP antibodies were detected by a commercially available ELISA containing synthetic peptides (Immunoscan rheumatoid arthritis, mark 2, Eurodiagnostica, Arnhem, Netherlands). The ELISA was carried out according to the manufacturer's instructions. Briefly, serum samples were diluted 1/50 with dilution buffer and incubated for one hour at 37°C. After removing the liquid and washing three times with rinsing buffer, the conjugate solution (peroxidase conjugated anti-human IgG antibodies) was added into each well and incubated for one hour at 37°C. After three washing steps with rinsing buffer, the substrate solution (tetramethyl

benzidine) was added and incubated for 30 minutes at room temperature. The stop solution (sulphuric acid, 0.5 mol/l) was added and the absorbance values were read immediately at 450 nm.

Rheumatoid factor (RF)

RF was determined by the latex fixation method. A suspension of uniform polystyrene particles sensitised in glycine buffer with heat altered human IgG (BD Diagnostic Systems, Sparks, Maryland, USA) was incubated with progressive dilutions of human sera in microtitre wells. After incubation, the plates were inspected for observable agglutination. The dilution titre present in the last well showing agglutination was recorded.

Calculation of cut off points

In an independent cohort of patients with inflammatory joint symptoms (containing only four patients with psoriatic arthritis), we previously defined cut off points for anti-CCP antibodies and RF as a function of a preset specificity level.[18] For anti-CCP antibodies a cut off of $\geqslant 42$ U/ml corresponded to a specificity of $\geqslant 98.5\%$ (98.6%; 95% confidence interval (CI), 95.8 to 99.8). For RF, a cut off of $\geqslant 160$ corresponded to a specificity of $\geqslant 95\%$ (95.5%; 95% CI, 91.7 to 99.3). An RF titre of $\geqslant 1280$ corresponded to a specificity of $\geqslant 98.5\%$ (98.6%; 95% CI, 95.8 to 99.8), comparable with the specificity of the anti-CCP test.[18]

Anti-pepA and anti-pepB antibodies

To confirm ACPA reactivity, anti-pepA and pepB antibodies were determined on all samples showing anti-CCP reactivity by a line immune assay (prototype of INNO-LIA™ rheumatoid arthritis, Innogenetics, Gent, Belgium), as described previously.[14] This test detects antibodies against two synthetic citrullinated peptides, pepA and pepB. Previous studies indicated that the sensitivity and specificity of anti-pepA antibodies were, respectively, 63.4% and >98.5% (100; 95% CI, 98.0 to 100), and the sensitivity and specificity of anti-pepB antibodies were 54% and >98.5% (99.3; 95% CI, 96.8 to 100).[14 18] Briefly, serum samples were diluted 1/100 and incubated with the strip for one hour at room temperature.

Table 2 Clinical features of patients with anti-CCP antibodies

Sex, age (y)	Dis dur	Anti-CCP (U/ml)	Anti-pepA or pepB reactivity	RF (titre)	SJC	RXJC	Asym oligo dis	Features helpful in distinguishing PsA from RA	Family history	DMARD*	Psoriasis subtype
F, 39	15	54	PepA+PepB	160	6	19	No(1)	Enthesitis, dactylitis, inflammatory low back pain, DIP involvement		Infliximab + MTX + SASP	Nail involvement
M, 24	5	54	Neg	320	10	17	No(1)	DIP involvement	Psoriasis, IBD	CyA-CS + MTX +Dpen	Nail involvement
M, 60	5	72	PepA+PepB	1280	4	3	No(1)			MTX	Vulgaris
M, 68	17	93	Neg	640	10	34	No(1)	DIP involvement, enthesitis, dactylitis, sacroiliitis, ankylosis		MTX+CS	Vulgaris and nail involvement
M, 67	20	353	PepA	2560	2	38	No(1)	DIP involvement, enthesitis, dactylitis		MTX+CS	Vulgaris and nail involvement
F, 50	5	500	PepA+PepB	80	0	NA	No(1)	Enthesitis, dactylitis, sacroiliitis, inflammatory low back pain	Psoriasis	Infliximab+MTX	Vulgaris
M, 37	6	1100	PepA+PepB	160	6	1	No(1)		Psoriasis, PsA	MTX+SASP	Vulgaris and nail involvement
F, 54	6	1113	PepA+PepB	80	4	0	No(1)	Ankylosis	Psoriasis	MTX+SASP	Nail involvement
F, 68	6	1375	PepA+PepB	160	2	14	No(1)	Enthesitis	Psoriasis	Infliximab +MTX	Vulgaris
F, 48	9	1456	Pep B	320	5	0	No(1)			Etanercept +CyA+MTX	Vulgaris and nail involvement
F, 31	1	>1600	PepA+PepB	640			No(1)			SASP	Vulgaris and nail involvement
F, 51	8	>1600	PepA+PepB	1280	1	0	No(1)	Dactylitis, enthesitis	Psoriasis	Absent	Vulgaris and nail involvement
M, 46	1	>1600	PepA+PepB	320	0	0	Yes	Enthesitis	Psoriasis	SASP	Vulgaris and nail involvement
M, 63	5	>1600	PepA+PepB	1280	9	7	No(1)	DIP involvement, enthesitis, dactylitis		MTX	Vulgaris
M, 69	6	>1600	PepA+PepB	160	0	NA	No(2)			MTX+gold	Vulgaris

*Current treatment in bold

asym oligo dis, asymmetrical oligoarticular disease (No(1); polyarticular disease; No(2), symmetrical oligoarticular disease); CCP, cyclic citrullinated peptide; CS, corticosteroids; CyA, ciclosporine; DIP, distal interphalangeal joint; Dis dur, disease duration; Dpen, D-penicillamine; IBD, inflammatory bowel disease; MTX, methotrexate; NA, no radiographs available; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; RXJC, radiological erosive or destructive joint count; SASP, salazopyrine; SJC, number of swollen joints; y, years.

After aspirating the liquid and washing three times, the substrate solution NBT/BCIP (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) was added. The reaction was stopped in 0.1 M sulphuric acid and strips were dried. During each test, a reference cut off control strip was included, providing a cut off intensity for each antigen line. Tests were read visually by comparing the intensity of the test strip with the intensity obtained with the reference sample.

Statistical analysis

The Mann–Whitney U test was used to evaluate differences between groups,. Exact χ^2 tests were computed for analysis of categorical data. We determined 95% confidence intervals (CI) around specificities as described by Harper and Reeves.[22] Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS v12.0, Chicago, Illinois, USA).

RESULTS

Anti-citrullinated peptide antibodies and rheumatoid factor

Fifteen patients with psoriatic arthritis of the total cohort of 192 (7.8%) had anti-CCP antibodies. In these 15 patients, ACPA reactivity was further confirmed with an independent citrullinated peptide substrate (pepA and pepB). Thirteen of 15 patients with anti-CCP reactivity had anti-pepA or anti-pepB reactivity (table 2).

Sixteen of 192 patients (8.3%) were positive for RF at the cut off corresponding to the 95% specificity level. Three of these had no anti-CCP antibodies. At the 98.5% specificity level, we found that four patients (2%) were positive for RF, and all of these had anti-CCP antibodies.

Clinical features of anti-CCP positive patients

The clinical features of the patients with anti-CCP antibodies are summarised in table 2. Differences between patients with and without anti-CCP antibodies are indicated in table 3. There were no major differences between the two patient groups for median disease duration or treatment. Patients with anti-CCP antibodies had a larger number of eroded joints (median 3 v 0, p = 0.032) but no significant difference in the swollen joint count at the date of sampling. There was no difference in the occurrence of typical radiological features or sacroiliitis. Of all patients with fewer than five affected joints (present or past and based on radiographs), 43 had predominantly lower limb involvement. Ten patients with anti-CCP antibodies had at least one feature suggested by McGonagle as being helpful in discriminating psoriatic arthritis from rheumatoid arthritis.[4] These features comprise: asymmetrical oligoarticular disease, predominantly of the lower limbs; distal interphalangeal joint (DIP) involvement; enthesitis; dactylitis; typical radiological features (pencil-in-cup phenomenon, ankylosis of small hand or foot joints, arthritis mutilans); radiological sacroiliitis; and inflammatory low back pain.

Table 3 Comparison between CCP negative and CCP positive patients

	CCP negative	CCP positive	p Value
Demographic features			
Median disease duration (range)	7 years (0 to 47)	6 years (1 to 20)	NS
Use of DMARD or biological agent	149/177	14/15	NS
Clinical features			
SJC	2 (0 to 21)	4 (0 to 10)	NS
RXJC	0 (0 to 44)	3 (0 to 38)	p = 0.044
Typical radiographic features*	20/126 (16%)	3/13 (23%)	NS
Sacroiliitis	40/136 (29%)	2/13 (15%)	NS
RF positive at 98.5% specific level	0/177	4/15 (27%)	p < 0.001
Persistent oligoarticular disease predominantly of the lower limbs	42/177 (24%)	1/15 (7%)	NS

Values are mean (range) or n (%) unless stated otherwise.
*Pencil-in-cup phenomenon, arthritis mutilans, ankylosis of small hand or foot joints.
CCP, cyclic citrullinated peptide; DMARD, disease modifying antirheumatic drug; RXJC, radiological joint count (number of erosive or destructive joints); SJC, swollen joint count.

Observed number of patients with anti-CCP antibodies v expected number given the specificity of the test

In a separate study, we calculated cut off points at a preset specificity level of at least 98.5% (a cut off of 42 U/ml for anti-CCP antibodies). The corresponding specificity and 95% CI were 98.6% (95.8% to 99.8%).[18]

In this cohort of 192 patients with psoriatic arthritis, we found 15 (7.8%) with anti-CCP antibodies. This is more than expected in view of the specificity and 95% CI obtained previously. Considering only those 10 patients with at least one feature suggested as helpful in distinguishing psoriatic arthritis from rheumatoid arthritis,[4] there were still more patients with anti-CCP antibodies than expected (10 of 187; 5.3%).

DISCUSSION

In this study, we found that 15 of 192 patients (7.8%) with psoriasis (skin or nail or both) and peripheral or axial arthritis had anti-CCP antibodies. This is more than would be expected in view of the high specificity of the test. PepA or pepB reactivity was confirmed in 13 of these 15 patients, pointing to specific anti-citrullinated peptide reactivity. Although patients with anti-CCP antibodies had a similar disease duration and similar treatment, they tended to have a higher radiological joint count than patients without anti-CCP antibodies. Some also had rheumatoid factor. These data raise the question of whether positivity for anti-CCP antibodies in such patients is caused by the co-occurrence of rheumatoid arthritis and psoriasis. When we excluded the patients who did not have at least one feature helpful in discriminating between psoriatic arthritis and rheumatoid arthritis,[4] and looked at differences in joint involvement, the patient subgroups were too small to reach significance (data not shown). It has been shown that patients who have anti-CCP antibodies and who are also positive for the rheumatoid arthritis associated HLA shared epitope are at increased risk of developing rheumatoid arthritis (odds ratio = 66.8).[23] However, HLA data were not available in the present study.

The differential diagnosis between rheumatoid arthritis (in patients with psoriasis) and psoriatic arthritis may be difficult.[24 25] Moll and Wright proposed that patients who are positive for rheumatoid factor should be considered to have rheumatoid arthritis, though they admitted that false positivity may occur.[1] As RF is false positive in around 5% of healthy controls and in a larger proportion of patients with chronic diseases,[26] we did not exclude patients with a positive RF test at the standard cut off. Using a cut off at a higher specificity would reduce the number of false positive results. However, in the present study we found that one patient of the four with RF at the 98.5% specific cut off point had a disease picture that was not fully compatible with rheumatoid arthritis, while of the remaining three patients two had dactylitis, enthesitis, and DIP involvement—features that are typically attributed to psoriatic arthritis or spondyloarthropathy.[1 21] Excluding patients who fulfil the ACR criteria for rheumatoid arthritis was not helpful either, as many patients with psoriatic arthritis fulfil those criteria.[26 27] Applying the ESSG criteria for spondyloarthropathy[21] does not include the patients with a symmetrical pattern of arthritis that is indistinguishable from rheumatoid arthritis. A French group suggested a classification system based on weighted clinical, radiological, and HLA criteria.[7] The major drawback of this classification system is the inclusion of HLA data.[29] Also, in our present cohort, some psoriasis patients with dactylitis—which is considered to be one of the most typical manifestations of psoriatic arthritis[1]—did not fulfil those criteria. McGonagle *et al* suggested that one should consider all patients with arthritis and psoriasis or a familial history of psoriasis in combination with clinical or radiographic enthesitis, DIP disease, radiological sacroiliitis, uncommon arthropathies, dactylitis, monoarthritis, or asymmetrical oligoarthritis as having psoriatic arthritis.[4] Patients with polyarticular disease without any of those features and without evidence of enthesitis on magnetic resonance imaging were considered to have rheumatoid arthritis.[4] Using this algorithm, which needs to be validated further, we found that 10 patients showed at least one feature suggested by McGonagle to be helpful in discriminating psoriatic arthritis from rheumatoid arthritis. The number we thus obtain (5.3%) is still higher than the 1.5% expected by the high specificity of the assay, obtained in an independent cohort.[18]

It is possible that some patients with typical psoriatic arthritis and positive anti-CCP or RF antibodies may have psoriatic arthritis patients with concomitant rheumatoid arthritis.[4] This would presuppose a higher prevalence of rheumatoid arthritis in a psoriatic arthritis population than in the general population, in which it is estimated to be 1%. Another possibility is that several psoriatic arthritis patients with anti-CCP antibodies had a disease picture that was not compatible with rheumatoid arthritis.

Our findings show that anti-CCP antibodies are more frequently present in a psoriatic arthritis population than in the populations generally used to assess the specificity of the test, such as healthy controls, patients with different rheumatic diseases, or patients with variant rheumatic complaints. Similarly, the presence of anti-CCP antibodies has also been described in patients with systemic lupus erythematosus[28 29] and primary Sjögren's syndrome.[30] These

findings raise other questions. Thus it could be hypothesised that reactivity for CCP could be a false positive, caused by reactivities to non-specific epitopes in the citrullinated peptide substrate. This phenomenon has been observed in animal models.[31] However, we confirmed real ACPA reactivity with two independent synthetic citrullinated substrates (pepA and pepB), showing the presence of anti-pepA or pepB antibodies in 13 of the 15 psoriatic arthritis patients with anti-CCP antibodies. This supports the view that the observed CCP positivity was caused by genuine anti-citrullinated peptide antibodies.

Another hypothesis could be that those patients with anti-CCP antibodies suffer from an overlap with a preclinical form of rheumatoid arthritis. Indeed, anti-CCP antibodies may be present years before the clinical manifestation of rheumatoid arthritis.[32 33] Additionally, current DMARD treatment in those patients might prevent the full expression of rheumatoid arthritis. Thus, as suggested before, patients with anti-CCP antibodies require a cautious clinical and radiographic follow up to confirm the absence of future evolution to rheumatoid arthritis.[30]

Conclusions

Our data suggest that anti-CCP antibodies may be present in patients with psoriatic arthritis Although it is possible that some of these patients have rheumatoid arthritis with concomitant psoriasis, in a proportion of them at least there are typical characteristics of psoriatic arthritis. The number of patients with psoriatic arthritis who have anti-CCP antibodies is greater than would be expected in view of the high specificity of the test.

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CHAPTER 6

Effect of the association between the HLA shared epitope and anti-citrullinated protein/peptide antibodies on modeling the probability for rheumatoid arthritis during diagnostic work-up.

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Abstract

Objectives: In the present analysis, our objective was to calculate the probabilities for rheumatoid arthritis (RA) in a consecutive cohort of patients during diagnostic work-up. Therefore, we fitted different logistic regression models evaluating the value of HLA-shared epitope (SE) determination and testing for rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA).

Methods: The study included 1003 consecutive patients, presenting a new diagnostic problem for which RA was included in the differential diagnosis. All patients were tested for ACPA, RF and HLA-SE.

Results: After 1 year, diagnoses were established: 153 patients had definite RA and 629 patients had RA excluded. RF, used as a continuous marker is useful to evaluate the probability for RA. Combined RF and SE testing may provide additional predictive information, but combined ACPA and RF testing is superior. The redundancy of SE testing in a model that includes ACPA testing can be explained by the high association between ACPA and SE both in RA and non-RA patients. The value of RF testing increased if patients presented with at least one swollen joint at baseline.

Conclusion: In the present study, we calculated valid probabilities for RA during routine diagnostic work-up and showed that the potential additional value of SE testing disappears when ACPA testing is available. Combined RF and ACPA testing is useful, especially when RF is considered as a continuous parameter reflecting an increasing probability for RA at higher RF titers. The value of (continuous) RF testing increases when the *a priori* chance is higher.

INTRODUCTION

Diagnosis and intensive treatment at an early stage in case of rheumatoid arthritis (RA) is an important factor in slowing the radiological progression [1]. Although the diagnosis of RA is mainly based on clinical features, these might be insufficient in an early stage of the disease, hampering clinical diagnosis. Therefore, additional serological and genetic tests may be useful. The oldest and best-known serological antibody (Ab) test is the rheumatoid factor (RF), which is part of the revised American College of Rheumatology (ACR) criteria for RA [2]. More recently, anti-citrullinated protein/peptide antibodies (ACPA) have been described. These are highly specific markers for RA and combine a good sensitivity (45 to 80%) with a very high specificity (89 to100%) [3,4,5]. Detection of ACPA can be achieved using the antigenic substrates citrullinated peptide A (pepA) and citrullinated peptide B (pepB) incorporated in a line immunoassay (LIA®) [6], or by ELISA tests that are available for cyclic citrullinated peptides (CCP) [7] and

deiminated fibrinogen [8]. All those last generation assays display comparable sensitivities and specificities [4,9]. Genetic markers <u>might</u> also have a role in RA diagnosis; much attention has been given to the HLA shared epitope (SE), which is found more often in RA patients than controls [10,11]. Since RF was the only available serologic marker until relatively recently, although not recommended, an additional assessment for the presence of the SE was sometimes performed.

Combinations of RF, SE, and ACPA have been used as such, or with other clinical or radiological measures, in models to predict RA or radiological progression [12,13,14]. The predictive value of these models depends on 1) the characteristics of the investigated population and 2) the prevalence (or *a priori* chance) of RA or persistent erosive disease, varying from 24.2% to 68% between different early arthritis cohorts [12,15].

Although most of these models have been applied in early arthritis cohorts, few data are available about the combined value of RF, SE and ACPA testing for RA diagnosis in a routine clinical diagnostic set up. The aims of the study were threefold: to test the value of RF, ACPA and SE profiles in different models to evaluate the probability for RA; to assess the added value of SE and RF testing now that ACPA testing is widely available; and to investigate the optimal combination of these three parameters.

PATIENTS AND METHODS
Patients
The present analysis is based on a prospective study in which 1003 consecutive patients from three academic and nonacademic centers were enrolled [16]: the Department of Rheumatology, Ghent University Hospital (Ghent, Belgium); the Locomotor Center, Elisabeth Hospital (Sijsele-Damme, Belgium); and the Department of Rheumatology, St-Augustinus Hospital (Wilrijk, Belgium). The local ethics committees approved this study October 1997, and informed consent was obtained from all patients. Patients were included from november 1997 to December 1999.

Patients were seen by one of the participating rheumatologists and entered consecutively the study if they presented with a new diagnostic problem for which RA was included in the differential diagnosis. This setting typically reflects the case where a rheumatologist would request RF or ACPA testing. Blood was taken at inclusion; serum samples obtained were aliquoted and frozen at –20°C, and whole blood was stored at -80° C. Participating rheumatologists were asked to fill in a file at baseline and after one year of follow-up asking for the clinical diagnosis established by the treating rheumatologist by ticking a box containing one of the following diagnoses (in ascending probability for RA): definite

non-RA, potential RA, proable RA and non-RA. To improve the comparability of our results, further classification of all RA patients was performed after one year by systematically checking the (cumulative) RA classification criteria by an independent investigator [2]. Patients fulfilling both the clinical diagnosis for definite RA and the classification criteria were further taken into account as RA patients. Patients in which RA was excluded were taken as the non-RA control group.

Rheumatoid factor
RF was determined by the latex fixation test. A suspension of uniform polystyrene particles sensitized in glycine buffer with heat-altered human IgG (Difco Laboratories, Detroit, MI) was diluted 1/20 and incubated with progressive dilutions of human sera in microtiter wells. The reagents were mixed and incubated at 37°C for 2 hours. The plates were then shaken gently and inspected for observable agglutination. Titers were converted to U/mL using a reference serum, to correct for inter-assay variation. A low (>95% specificity level, 25 U/ml) and a high (>98% specificity level, 100U/ml) cut-off was defined based on a previously described cohort [4]

Detection of anti-pepA antibodies by line immunoassay (LIA®).
Anti-pepA Ab were detected by a research LIA® containing the synthetic citrullinated peptide referred to as pepA (INNO-LIA™ RA - for research use only - Innogenetics, Ghent, Belgium) [4,6]. Air-dried strips were scanned using a HP Scanjet 5P scanner. A reference sample was included in each test run. In order to minimize test-to-test variability, the scan values of individual samples were corrected by dividing them by the scan values of the cut-off sample. Anti-pepA positivity was defined as a corrected scan value of ≥ 1, at which the test had a specificity of $\geq 98.5\%$ and a sensitivity of 63.6% in an independent cohort of patients [4].

HLA typing by INNO-LiPA (line probe assay) technology
DNA was extracted from whole blood samples and amplified using the INNO-LiPA HLA-DRB1 or –DRB decoder amplification kits (Innogenetics, Ghent, Belgium) as instructed by the manufacturer. HLA typing was performed with the INNO-LiPA HLA-DRB1 or –DRB decoder kits (Innogenetics, Ghent, Belgium), that are based on the reverse hybridization principle; specific oligonucleotide probes are immobilized as parallel lines on membrane-based strips. The amino acid sequences QRRAA, QKRAA, and RRRAA at positions 70-74 constitute the RA SE sequence. Patients were classified into two groups according to the inheritance of zero versus one or two copies of the SE.

Statistical methods

The dataset, previously described [16], is here analyzed by logistic regression techniques. Different models with all possible combinations of anti-pepA Ab, SE and RF-results as explanatory variables and RA diagnosis as explained variable were fitted with logistic regression. The validity of the models was confirmed by fitting a full model and performing backwards elimination of the interaction terms. Logistic regression models are an extension of the general linear regression and fit S-shaped curves by modeling the logit [$= \log(x/(1/x))$] of the probabilities for a dichotomous outcome, in this case, the probability for RA ($= \pi$), using the formula $\text{logit}(\pi) = \log(\pi/(1-\pi)) = \alpha + \beta_1 x_1 + \beta_2 x_2 + \dots$. The predicted probabilities thus obtained can be plotted against the different explanatory variables x [17]. This method allows to evaluate the variables in a continuous way and to visualize the models so that they are easier to interpret.

We calculated odds ratios and their 95% confidence intervals, as a measure of the correlation between dichotomous variables. Homogeneity between marginal odds ratios were calculated with the Breslow-Day statistic. Common odds ratios were calculated by means of the Mantel-Haenszel test [17].

The analyses were performed using 2 classical statistical packages: SPSS 12.0, Chicago, Il, USA and S-Plus 6.1, Insightful Corporation, Seattle, USA.

RESULTS

Patient characteristics

After one year of follow-up, the treating rheumatologist diagnosed each patient according to the following categories: definite RA (n = 153), probable RA (n = 72), potential RA (n = 75), non-RA (n = 629), or lost to follow-up (n=74). Only patients diagnosed by their treating rheumatologist as definite RA and fulfilling the revised ACR criteria for RA [2] were further considered in the RA-group (n = 144). The non-RA control population (n=629) had the following diagnoses: osteoarthritis (38%), abarticular rheumatic symptoms (including peri-arthritis scapulohumeralis, non-rheumatic tendinopathies,…) (14%), spondyloarthropathy (10%), connective tissue diseases including polymyalgia rheumatica (15%), psoriatic arthritis (7%), crystal induced arthritis (1%), fibromyalgia and aspecific arthralgias (5%), other and undifferentiated diseases including infections, malignancies and neurological disorders (10%). We finally lost 9 non-RA patients and 3 RA patients, due to lack of serum or DNA samples of good quality. The mean age of the RA patients was 58 year and 51 year for the non-RA patients. In both populations, 66% of patients were female. The mean duration of symptoms was 19.3 months in the RA group and 15.9 months in the non-RA group. We further identified a subpopulation of 498

patients with at least 1 swollen joint at baseline including 134 RA and 230 non-RA patients.

Test results
Six hundred twenty non-RA and 141 RA patients were tested for anti-pepA Ab, RF and SE. A positive anti-pepA result was observed in 78 (55.3%) RA and 13 (2.1%) non-RA patients. Median pepA scan values were 2.15 (range 0-9.62) for RA patients and 0.04 (range 0.00-6.07) for non-RA patients. Ninety (63.8%) RA and 264 (42.6%) non-RA patients carried at least one copy of the SE. Two copies were found in 32/141 (21.1%) of the RA patients and in 30/620 (5.1%) of the non-RA patients. The median RF titers were 50 U/ml (range 0-1600) for the RA and 0 U/ml (range 0-1600) for the non-RA patients.

Comparison between PPV and predicted probabilities.
Plots of the positive predictive values and predicted probabilities in function of RF titers are shown in Figure 1. Positive predictive values (PPV) were calculated by defining different cut-offs at different titer steps and plotted against RF or anti-pepA Ab titers (Figure 1a). Predicted probabilities, calculated by logistic regression, are shown in Figure 1b.

Logistic regression models in the global population
Different logistic regression models were fit with different combinations of anti-pepA, RF and SE-testing. These analyses revealed that ACPA testing in combination with SE has no additional value. This resulted in 2 final models: 1) a model with combined RF and SE testing, and 2) a model with combined RF and ACPA testing. The results of these 2 models are visualized in the predicted probability plots in Figure 2 and Figure 3, showing that the added value of combined RF and SE testing is limited compared to combined RF and ACPA testing.

Logistic regression models in the subpopulation of patients with at least 1 swollen joint at baseline.
Similarly as observed in the global population, (continuous) RF testing has additional value to ACPA testing alone. Moreover, in this sub-population with at least 1 swollen joint, additional (continuous) RF testing seems to add more value than in the global population: lower RF titers become more relevant. The predicted probability curves are shifted up and have steeper slopes (Figure 4).

Correlation between SE and anti-pepA Ab positivity
When the dataset was split into RA and non-RA patient groups, we obtained the following marginal odds ratios for the association of anti-pepA Ab positivity with SE-positivity: 4.63 (95% CI 1.26-17.00, p=

0.011) for non-RA patients and 4.21 (95% CI 2.03-8.74, p<0.001) for RA patients. Application of the Breslow-Day test indicated that the ORs for the association between SE and anti-pepA Ab positivity were not significantly different (which also means that there is no interaction between SE and anti-pepA Ab positivity). This allowed us to calculate an overall common odds ratio for the relation between anti-pepA Ab positivity and SE positivity, which was 4.31(95% CI 2.28-8.17, p<0.001).

Correlation between the SE and RF positivity
The odds ratios for RF positivity with SE positivity at the low cut-off were 0.72 (95% CI 0.42-1.24, p = NS) for the non-RA patients and 3.1 (95% CI 1.5-6.5, p= 0.002) for the RA patients. Using the high RF cut-off, the ORs were 0.5 (95% CI 0.165-1.716, p=NS) for the non-RA patients and 2.8 (95% CI 1.3-5.8, p=0.008) for the RA patients. However, when corrected for anti-pepA Ab positivity, the significant association between RF and SE, initially observed in the RA population, disappeared. The ORs thus obtained were 1.7 (95% CI 0.5-3.1, p = NS) at the low RF cut-off and 1.7 (95% CI 0.7-3.8, p= NS) using the high RF cut-off.

DISCUSSION
The set-up of the present study represents real-life clinical practice by the inclusion of consecutive patients seen by a rheumatologist (2 non-academic centres, 1 university centre), for a new diagnostic problem in which RA was included in the differential diagnosis. After 1 year, diagnoses were established as RA, non-RA and a group of patients with persistent undifferentiated disease. In order to avoid misclassifications, the patients with persistent undifferentiated disease were further excluded from the present analysis. Such a study design allows the calculation of representative predictive values and the estimation of representative probabilities for RA. This contrasts with other types of case-control studies where the calculated predictive values should not be extrapolated since predictive values depend on the prevalence (or the *a priori* chance) of the disease [16,18,19]. In contrast to the classical exploration by means of sensitivity, specificity, and predictive value, we evaluated the (predicted) probabilities for disease (figures 1b, 2b, 3 to 6). The use of probabilities is especially helpful when a continuous marker that is known to express higher titers in diseased than in non-diseased patients is considered (which is the case for both RF testing). The use of positive predictive values (PPV) requires the definition of a cut-off point (for example, an RF cut-off at 25 U/ml, which corresponds with a PPV of 61.5% in the present study). This cut-off has the disadvantage that the information gets lost that a patient who displays a titer of 100 U/ml still has a greater chance to have RA than a patient who displays only 25 U/ml. Therefore, the use of PPV may overestimate the probability for

disease in patients displaying low positive titers (figures 1a and 2a compared with figure 1b and 2b). In the present analysis, we used logistic regression to calculate those probabilities. Although logistic regression is a parametric regression technique and therefore provides only an estimate for the probabilities by extrapolation, it has the great advantage that it easily allows the exploration of combinations of different markers in a continuous and categorized manner. This is in contrast to the commonly used "and/or combinations" at pre-set sensitivity or specificity levels for which dichotomization by defining a cut-off is needed [15, 16, 20].

Plotting (predicted) probabilities by means of logistic regression, as performed in the present analyses should not be confused with the cumulative probability plots, described by Landewé [21].

We thus calculated (predicted) probabilities for RA by means of logistic regression, using different combinations of RF, the presence of SE and anti-pepA Ab testing. These analyses demonstrated that SE testing did not significantly contribute to a model where anti-pepA Ab testing is already present. In contrast, SE testing contributed significantly when only RF testing was performed (Figure 2). The redundancy of SE testing when ACPA testing is available was also demonstrated in a model for prediction of radiological progression and persisting erosive disease [12, 14]. This redundancy of SE testing when ACPA testing is available can be explained by the high association (without interaction for diagnosis of RA) between SE and ACPA in RA and non-RA patients (OR = 4.3). It is important to highlight that this association between ACPA and SE is also observed in non-RA patients. The association between ACPA and SE has also been described in patients with RA and undifferentiated arthritis and has led to hypotheses about the induction of ACPA [3,22-30]. Interestingly, this association between ACPA and SE might also be the reason for the observed association between RF and SE, which disappears after correction for ACPA positivity. Berglin et al showed that combined testing for ACPA and SE had an additional value for predicting RA in healthy blood donors [27]. This can be explained by the fact that some SE-positive blood donors who later developed RA had not(yet) detectable ACPA at the time of blood sampling: only 16/34 (47%) of the SE-positive patients who later developed RA were ACPA positive (in contrast to 78% in the present study).

In contrast to the clear association between ACPA and the SE, there seems to be no (or only a weak) correlation between RF and the SE [4,22,23]. This explains why combined SE and RF testing displayed a statistically significant additional value to RF testing alone, as displayed in Figure 2. However, this additional value is limited when compared to to the important additional value provided by the combination of ACPA and RF testing (described in Figures 3).

Interestingly, these models show also that the probability for RA, given a positive ACPA test, is less than 80% when RF is negative, but increases when higher RF titers are present. At very high RF titers, the value of additional ACPA testing appears to be reduced (Figure 3). In contrast, ACPA has more added value when only intermediate positive RF titers are displayed. The models from Figures 1-3 have been calculated in a population of patients in whom the treating rheumatologist would routinely ask for a rheumatoid factor test. We also evaluated the value of RF and ACPA testing in the subgroup of patients with at least one swollen joint at baseline (Figure 4). These figures show that intermediate RF titers become more relevant when the *a priori* chance for RA is higher.

These findings might be used to propose multi-step testing models in which different RF cut-offs are defined. In such models, additional ACPA testing might not be required in patients with a high positive RF test or in patients with an intermediate high positive RF but with a high clinical probability for RA [13]. In contrast, additional ACPA testing seems to add much value in cases with low positive RF. The value of the combined testing of RF and ACPA has also been demonstrated in models of early or undifferentiated arthritis to predict RA or erosive disease [12,32,33]. Logistic regression can be useful to evaluate the diagnostic value of different lab tests by different models and plots. The conditions for the generalization of those models and plots are similar to the conditions to generalize predictive values (PPV, NPV): populations and *a priori* chance for disease should be similar [34]. Logistic regression can also be used to construct prediction models including variables from medical history and physical examination, but generalization of such models may be more difficult when more variables are used [12, 35, 36]. To conclude, this study provides unbiased models to calculate the probabilities for the development of RA in a diagnostic set-up. Although not recommended in daily clinical practice [37], we showed that, in case ACPA results are not available, SE-testing may add some diagnostic information in addition to RF results. However, since ACPA testing has become available, additional SE testing seems no longer appropriate in a diagnostic work-up, due to the high correlation between ACPA and the SE. Finally, it would appear that a diagnostic strategy that combines ACPA testing and RF-testing in a single or multi-step method, is superior to single RF or single ACPA testing alone. RF testing is especially useful when it is considered as a continuous parameter that reflects a higher probability for RA when higher titers are displayed.

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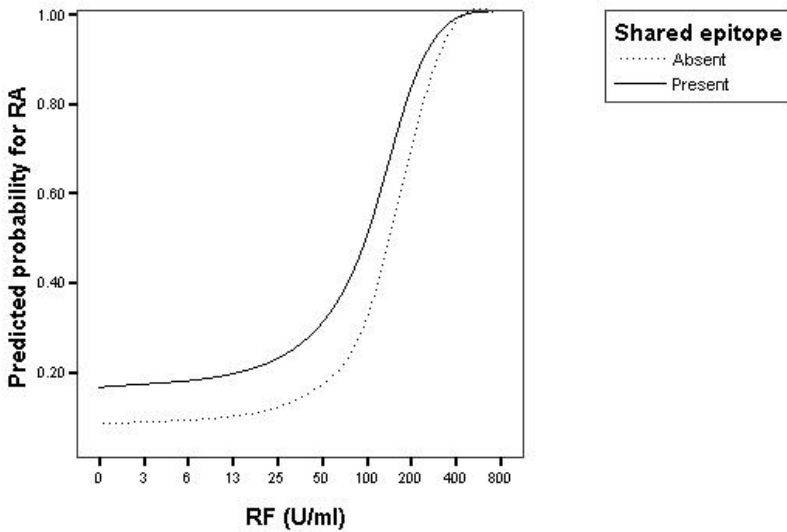
FIGURES

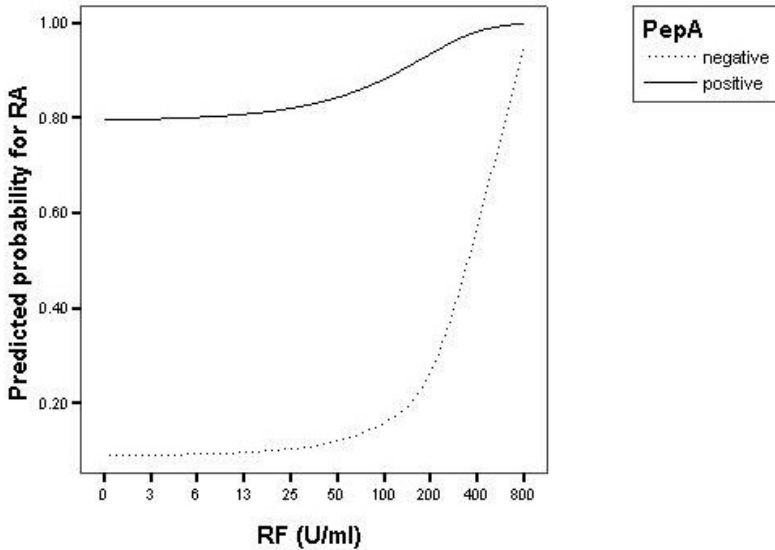**Figure 1: Plots of PPV and predicted probability of RF.**
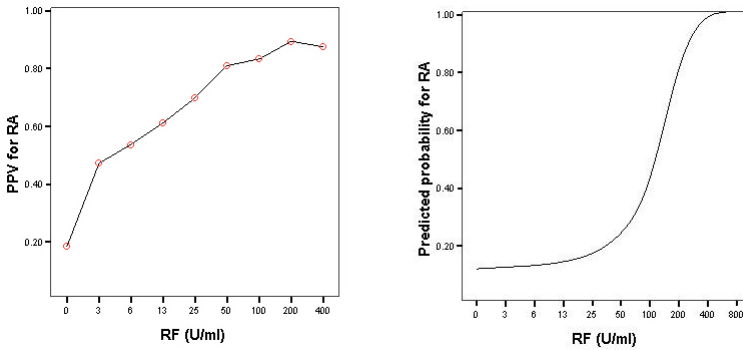
(a) PPV were calculated by defining different cut-offs at different titers. (b)Predicted probabilities were calculated by logistic regression.

**Figure 2: Plot of the predicted probabilities in function of RF titers and SE testing.**

This figure illustrates that SE testing has a small additional value to RF testing for the diagnosis of RA.

Figure 3: Plots of the predicted probabilities in function of RF titers and ACPA testing

This figure illustrates that ACPA testing has a big additional value to RF testing, especially in the low RF range.

Figure 4: Plots of the predicted probabilities in function of RF titers in patients with at least one swollen joint at baseline.
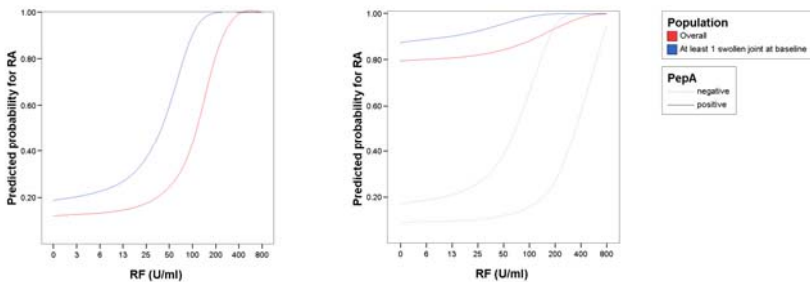
In this figure, we evaluated the effect of the baseline presence of at least one swollen joint on the interpretation of RF results. In figure 4a, we evaluated single continuous RF testing in patients with at least one swollen compared with the model from Figure 3 (red). In

figure 4b, we evaluated a model of combined RF – ACPA testing calculated in the global population (red, Figure 3) compared with a model calculated in the subgroup of patients with at least 1 swollen joint at baseline (blue).

ADDITIONAL TABLES
Table : Different logistic regression models to predict RA

	Beta	OR	95%CI		p-value
Model 1: pepA-RF-SE					
Intercept	-2.445				
PepA scan value	1.065	2.90*	2.14	3.92	<0.001
RF titer	0.006	1.01*	1.00	1.02	0.002
SE	0.219	1.25	0.76	2.08	NS
Model 2: pepA-SE					
Intercept	-2.291				
PepA scan value	1.287	3.62*	2.67	4.91	<0.001
SE	0.113	1.12	0.69	1.81	NS
Model 3: RF-SE					
Intercept	-2.380				
SE	0.769	2.15	1.40	3.30	<0.001
RF titer	0.016	1.03*	1.00	1.02	<0.001
Model 4: pepA continuous-RF trichotomous					
Intercept	-2.535				
PepA scan value	0.925	2.52*	1.81	4.08	<0.001
RF low positive	1.404	4.073	1.74	9.52	0.001
RF high positive	1.191	3.292	1.16	9.32	0.025
Model 5: pepA positive-RF continuous					
Intercept	-2.325				
Anti-pepA positivity	3.676	39.5	18.6	83.7	<0.001
RFtiter	0.007	1.01*	1.00	1.02	<0.001
Model 6: pepA positive-RF continuous in patients with at least one swollen joint at baseline					
Intercept	-1.608				
Anti-pepA positivity	3.537	34.359	7.739	152.534	<0.001
RFtiter	.023	1.023	1.013	1.033	<0.001

* The OR for the continuous parameter RF or anti-pepA Ab should be interpreted differently from the OR for the dichotomous anti-pepA Ab and SE testing [34].

Table: Results of the test in the different subpopulations of the cohort.

Diagnosis	n (included in study)	n (complete case)	RF LF low	RF LF high	pepA	HLA-SE positive
Lost to follow-up	74	73	11%	4%	7%	49%
Definite RA, ACR criteria fulfilled	144	141	69%	42%	55%	64%
Definite RA, ACR criteria not fulfilled	9	9	40%	20%	30%	67%
Probable RA, ACR criteria fulfilled	37	36	35%	24%	22%	65%
Probable RA, ACR criteria not fulfilled	35	34	29%	17%	18%	45%
Potential RA	75	75	13%	5%	7%	44%
Non RA	629	620	10%	2%	2%	43%
Overall	1003	988	16%	10%	11%	47%

RFLF low $>/= 25$ U/ml
RFLF high $>/= 100$ U/ml
pepA $>/= 1$

CHAPTER 7

Diagnostic value of anti-human citrullinated fibrinogen ELISA and comparison with four other anti-citrullinated protein assays.

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* Equal contribution

Research article

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Diagnostic value of anti-human citrullinated fibrinogen ELISA and comparison with four other anti-citrullinated protein assays

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Abstract

We studied the diagnostic performance of the anti-human citrullinated fibrinogen antibody (AhFibA) ELISA for rheumatoid arthritis (RA) in a consecutive cohort (population 1) and evaluated the agreement between the AhFibA ELISA and four other assays for anti-citrullinated protein/peptide antibodies (ACPAs) as well as rheumatoid factor in patients with longstanding RA (population 2). Population 1 consisted of 1024 patients with rheumatic symptoms; serum samples from these patients were sent to our laboratory for ACPA testing within the context of a diagnostic investigation for RA. Ninety-two of these patients were classified as having RA according to the American College of Rheumatology criteria and 463 were classified as non-RA patients. Population 2 consisted of 180 patients with longstanding RA and was used to assess agreement and correlations between five ACPA assays: anti-cyclic citrullinated peptide (CCP)1 and anti-CCP2 antibodies were detected using a commercially available ELISA, AhFibA using ELISA, and anti-PepA and anti-PepB antibodies using line immunoassay. Applying previously proposed cut-offs for AhFibA, we obtained a sensitivity of 60.9% and a specificity of 98.7% in population 1. Receiver operating characteristic curve analysis could not detect a significant difference in diagnostic performance between the AhFibA ELISA and anti-CCP2 assay. Performing a hierarchical nearest neighborhood cluster analysis of the five different ACPA assays in population 2, we identified two clusters: a cluster of anti-pepA, anti-pepB and anti-CCP1, and a cluster of AhFibA and anti-CCP2. In conclusion, we found that AhFibA and anti-CCP2 antibodies had similar diagnostic performance. However, disagreement between ACPA tests may occur.

Introduction

Clinical indicators of rheumatoid arthritis (RA) are pain and swelling of the proximal interphalangeal and metacarpophalangeal joints. Larger joints such as knee, elbow and ankle joints may also be affected. Synovial inflammation and joint destruction together with the extra-articular manifestations of the disease are responsible for a severe decline in the RA patient's quality of life. It is important to identify RA early. Joint erosions, which are irreversible, occur early in the disease process and intervention with aggressive therapy is most successful if it is applied early in the disease course [1]. A sensitive and specific serological test is needed for application in this window in the disease course, when often not all clinical manifestations are apparent.

Several autoantibody systems have been described in this autoimmune disease [2]. The presence of the rheumatoid factor (RF), directed against the Fc part of an IgG molecule, is one of the American College of Rheumatology (ACR) criteria for RA [3]. This antibody is present in about 65–75% of RA

ACPA = anti-citrullinated protein/peptide antibody; ACR = American College of Rheumatology; AhFibA = anti-human fibrinogen (auto)antibodies; CCP = cyclic citrullinated peptide; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; OD = optical density; PBS = phosphate-buffered saline; RA = rheumatoid arthritis; RF = rheumatoid factor; ROC = receiver operating curve.

patients. However, because it is also found in patients with other autoimmune diseases or infectious diseases, and even in the healthy elderly, it has limited specificity. The presence of anti-citrullinated protein/peptide antibodies (ACPAs), on the other hand, is significantly more specific for RA. ACPAs are directed against various proteins that have one trait in common; some of their arginines have been converted to citrulline by post-translational modification, catalyzed by peptidy-larginine deiminase enzymes [4,5].

Depending on the substrate, various assays for detection of ACPAs have been developed. Human buccal mucosa cells and rat oesophagus provide the antigenic substrate for anti-perinuclear factor and anti-keratin antibodies [6,7]. The difficulty in standardizing these natural substrates, together with arbitrary interpretation of the indirect immunofluorescence pattern, has hampered the widespread use of these tests. Because it was shown that both anti-perinuclear factor and anti-keratin antibodies reacted against citrullinated filaggrin related proteins [8], the latter was used for detection of ACPAs in immunoblot assays and in an ELISA, resulting in an assay with 52% sensitivity at a specificity of 95% in a cohort of patients with established disease [9,10]. An ELISA using a cyclic citrullinated peptide (CCP) derived from filaggrin was commercialized (anti-CCP1) [11]. Numerous studies were reported in which sensitivities ranged from 41% (with a corresponding specificity of 97.8% [12]) to 68% (with a corresponding specificity of 98% [11]) in established RA. The line immunoassay format was used with two filaggrin based peptides (pepA and pepB), obtained from the results of epitope mapping as well as molecular modelling and computational chemistry [13]. The sensitivity of this assay was 63.6% for pepA and 54.2 % for pepB at a specificity of 98.5% in established RA [14]. With the development of the second generation anti-CCP2 ELISA, sensitivities ranging from 65% [15] to 80% [16] at a high level of specificity have been reported in established RA. Recently, the presence of citrullinated fibrin in the synovial membrane of RA patients [17] and the use of cit-rullinated fibrinogen to assay the serum antibodies to deiminated fibrinogen (anti-human citrullinated fibrinogen antibody [AhFibA]) was described [18,19].

The aim of the present study was to assess the diagnostic performance of the AhFibA ELISA for RA in a consecutive population of patients of whom serum was sent to our laboratory for RA serological testing. We also studied the agreement between five different ACPA assays and RF in a cohort of patients with established disease.

Materials and methods
Study population 1
Study population 1 was established to evaluate the diagnostic performance of the AhFibA assay and to compare the diagnostic performance with an anti-CCP2 antibody assay by means of receiver operator characteristic (ROC) curve analysis. This

cohort consisted of 1024 patients with rheumatic symptoms, from whom serum samples were consecutively sent to our laboratory for ACPA determination within the context of a diagnostic investigation. Patients were diagnosed by a clinician by reviewing of files and the patients were classified in accordance with the ACR classification criteria for RA [3]. Eighty-one patients were lost to follow up. We thus diagnosed 92 individuals as having RA, and all of these patients met the ACR criteria for RA. In 463 patients the diagnosis of RA could be excluded. A further 388 patients had undifferentiated arthritis and were further withdrawn from the analysis. The most frequent diseases diagnosed in the non-RA patients were osteoarthritis (31%), soft tissue mechanical complaints 20% (including peri-arthritis scapulohumeralis and tendinopathies), spondyloarthropathy (13%), systemic lupus erythematosus (9%), vasculitis (6%), polymyalgia rheumatica (5%), other connective tissue diseases (including scleroderma and Sjögren's syndrome; 2%), adult patients with juvenile idiopathic arthritis (1%), psoriatic arthritis (5%), crystal arthritis (3%) and other diseases including infections, malignancies and neurological disorders (5%).

Of the RA patients, 65.2% were female, the median age was 55 years (range 22–85 years) and the median disease duration was 3 years (range 0–40 years). In the non-RA patients, 66.2% were female and the median age was 51 years (range 11–83 years). Of the RA patients 64% were receiving disease-modifying antirheumatic drug therapy, predominantly methotrexate ($n = 34$), sulphasalazine ($n = 11$) and leflunomide ($n = 5$). Combination therapies were administered to six patients. None of the patients were being treated with anti-tumour necrosis factor therapy at the time of sampling. Corticosteroids were being received by 30% of patients.

Study population 2
Study population 2 consisted of 180 consecutive RA patients with longstanding disease of at least 4 years (median disease duration 9 years; range 4–39 years) [14]. In this cohort we compared the AhFibA assay with four other ACPA assays. All patients were treated with classic disease-modifying antirheumatic drug therapy (methotrexate, gold salts, or sulphasalazine). None of the patients received leflunomide, anti-tumour necrosis factor therapy, or other biologicals. Concomitant corticosteroids were used in one-third of the patients.

Rheumatoid factor assay
RF was determined using the latex fixation test. A suspension of uniform polystyrene particles sensitized in glycine buffer with heat-altered human IgG (Difco Laboratories, Detroit, MI, USA) was diluted 1/20 and incubated with progressive dilutions of human sera in microtitre wells. The reagents were mixed and incubated at 37°C for 2 hours. The plates were then shaken gently and inspected for observable agglutination. The dilution titre present in the last well showing agglutination was recorded.

Detection of anti-pepA and anti-pepB antibodies by line immunoassay

Anti-pepA and anti-pepB Abs were detected by a research line immunoassay containing two citrulline-containing peptides, as described previously (INNO-LIA™ RA [for research use only]; Innogenetics, Ghent, Belgium) [13,14]. The cut-off defined for anti-pepA and anti-pepB antibodies corresponds with a specificity of 100% and 99.3% and a sensitivity of 63.6% and 54.2%, respectively [14].

Detection of anti-CCP1 and anti-CCP2 antibodies by ELISA

Anti-CCP1 and anti-CCP2 antibodies were detected using a commercially available ELISA containing synthetic CCPs (Immunoscan RA, mark 1 and mark 2; Eurodiagnostica, Arnhem, The Netherlands). The ELISA was performed in accordance with the manufacturer's instructions. Briefly, serum samples were diluted 1/50 with dilution buffer and incubated for 1 hour at 37°C. After removing the liquid and washing three times with rinsing buffer, the conjugate solution (peroxidase conjugated anti-human IgG antibodies) was added into each well and incubated for 1 hour at 37°C. After three washing steps with rinsing buffer, the substrate solution (tetramethyl benzidine) was added and incubated for 30 min at room temperature. The stop solution (sulfuric acid; 0.5 mol/l) was added and the absorbance values were read immediately at 450 nm. A 98.5% specificity cut-off has previously been set at 42 U/ml (sensitivity 75.4%) for the anti-CCP2 assay [14] and a 98% specific cut-off has been set at 92 U/ml for the anti-CCP1 assay [11].

Detection of AhFibA antibodies by ELISA

The AhFibA-ELISA was developed previously [18,20]. Briefly, plasminogen depleted human fibrinogen (Calbiochem, Meudon, France) was further affinity purified on a protein G column (HiTrap protein G; Amersham Biosciences, Orsay, France). Deimination was performed for 2 hours at 37°C with 7 units rabbit skeletal muscle peptidylarginine deiminase per milligram fibrinogen (Sigma, Lyon, France) in deimination buffer (0.1 mol/l Tris-Hcl [pH 7.4], 10 mmol/l CaCl$_2$, 5 mmol/l DTT). Microtitration plates (MaxiSorp, Nunc, Denmark) were coated overnight with human deiminated fibrinogen (5 µg/ml) diluted in phosphate-buffered saline (PBS; pH 7.4). The plates were blocked with PBS containing 2% bovine serum albumin. A volume of 100 µl sera, diluted to 1:50 in 2 mol/l NaCl PBS, was applied and plates were incubated for 1 hour. After washing, plates were incubated with horseradish peroxidase labelled goat anti-human IgG antibodies (γ-chain specific) for 1 hour and washed again. All incubations and washing steps were performed at 4°C. Bound antibodies were detected with ortho-phenylene diamine dihydrochloride (Sigma, St. Louis, MO, USA). The reaction was stopped with 50 µm of 3 mol/l sulphuric acid.

Plates were read using a Multiskan plate reader (Thermo Labsystem, Cergy-Pontoise, France). Serum samples were tested twice and results were averaged. A serum was considered positive for AhFibA above a previously defined cut-off corresponding with the 98.5% specificity level (optical densitiy (OD) ≥ 0.12 nm) [18].

Statistical analysis

ROC curve analyses were performed. Differences between areas under the curve were evaluated, as proposed by Hanley [21]. Agreement between dichotomized variables was measured by the (weighted) κ statistic. Proportions of matched-pair data were compared by means of the McNemar test. A hierarchical nearest neighbourhood cluster analysis of variables was performed based on squared Euclidian distances. All analyses were performed using the commercial available statistical package SPSS 11.0 (SPSS Institute Inc., Chicago, Il, USA).

Results

Diagnostic performance of the AhFibA assay for RA and comparison with the anti-CCP2 and RF assay in population 1

The previously proposed AhFibA cut-off of OD ≥ 0.12 corresponded to a specificity level of 98.5% [18]. When we applied this cut-off to population 1, we obtained a sensitivity of 60.9% and a specificity of 98.7% for the AhFibA assay. Seven non-RA patients tested false positive for AhFibA: one patient with systemic lupus erythematosus, one with psoriatic arthritis, one with polymyositis, one with polymyalgia rheumatica and three with osteoarthritis.

The ROC curve comparing the diagnostic performance of AhFibA ELISA, anti-CCP2 assay, and RF in population 1 is shown in Figure 1a, with detail of the curve in the high specificity region shown in Figure 1b. There were no significant differences in the area under the ROC curve analyses of the AhFibA assay compared with the anti-CCP2 assay (0.824 versus 0.854; P = NS) [21]. The sensitivities at cut-offs defining comparable specificity levels were similar for the AhFibA ELISA and the anti-CCP2 assay, but they were significantly higher than the sensitivities of the RF test (Table 1). Applying the McNemar test, there were no significant differences between the two ACPA tests after dichotomization at the cut-offs presented in Table 1.

Agreement between the AhFibA assay and anti-CCP2 in population 1

In Figure 2 the results of the anti-CCP2 assay are plotted against the results of the AhFibA assay. Table 2 shows the cross-tabulation of the results of the AhFibA and anti-CCP2 ELISAs after dichotomization at the 98.5 % specificity level both for the RA and the non-RA patients. The κ statistic, as a measure of agreement between AhFibA and anti-CCP2 ELISA, calculated on the global population, was 0.845. After splitting the population into RA and non-RA patients, we

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Figure 1
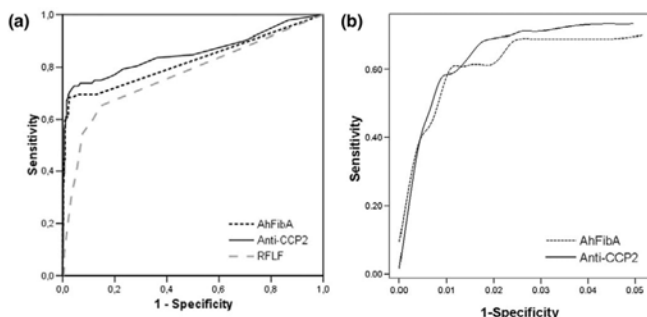
ROC curve analyses of the RF, AhFibA and anti-CCP2 assay in population 1. **(a)** The whole curve is shown, and **(b)** with a focus on detail of the ROC curve at the 98% specificity level for the AhFibA and anti-CCP2 assay in population 1. AhFibA, anti-human fibrinogen (auto)antibodies; CCP, cyclic citrullinated peptide; RF, rheumatoid factor; ROC, receiver operating curve.

obtained a κ of 0.765 for the RA patients and κ of 0.420 for the non-RA patients. Hence, agreement of both assays is especially impaired in non-RA patients; only 11 non-RA patients exhibited any ACPA reactivity, of which only three were positive for both AhFibA and anti-CCP2 ELISAs. Those three patients had the following diagnoses: osteoarthritis, psoriatic arthritis and polymyositis. We calculated that the specificity in case of double ACPA positivity is 99.4%, with a sensitivity of 58.7%.

Agreement between the five ACPA assays and RF in population 2

In this cohort of longstanding RA patients, the agreement between the AhFibA assay and the anti-CCP2 assay corresponded with the agreement observed in the RA patients of population 1. After dichotomization at a >98% specificity level, as defined in Materials and methods (see above), we calculated the sensitivities listed in Table 3; sensitivities, especially for the AhFibA assay, observed in population 2 were higher than those in population 1. The results of the κ statistic as a measure of agreement between dichotomized tests are listed in Table 4, confirming the moderate agreement between the different ACPA tests. A hierarchical nearest neighborhood cluster analysis of variables was performed with the results of RF and five ACPA assays: anti-CCP1, anti-CCP2, anti-pepA, anti-pepB and AhFibA (Figure 3).

Table 1

Sensitivities and specificities of AhFibA and anti-CCP2 assay in the two populations

	Cut-off	Population 1		Population 2
		Specificity	Sensitivity	Sensitivity
AhFibA antibody (OD)	0.027	0.945	0.685	0.800
	0.11	0.985	0.609	0.711
	0.12	0.987	0.609	0.700
	0.19	0.990	0.598	0.678
anti-CCP2 antibody (U/ml)	14.5	0.950	0.728	0.744
	37	0.985	0.674	0.680
	42	0.985	0.641	0.650
	135	0.990	0.565	0.551
RF	160	0.948	0.391	0.556
	640	0.985	0.163	0.244
	1280	0.993	0.087	0.122

AhFibA, anti-human fibrinogen (auto)antibodies; CCP, cyclic citrullinated peptide; OD, optical density; RF, rheumatoid factor.

Figure 2
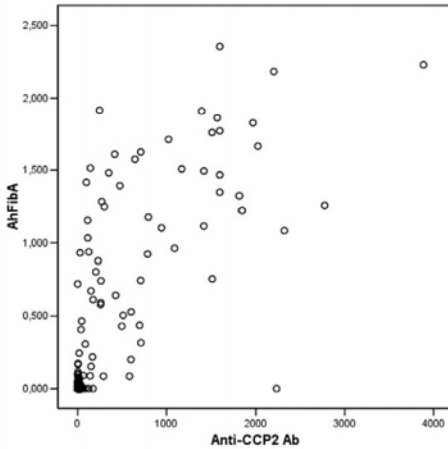
Scatter plot: AhFibA assay versus anti-CCP2 assay in population 1. Ab, antibody; AhFibA = anti-human fibrinogen (auto)antibodies; CCP = cyclic citrullinated peptide.

This analysis identified the ACPAs apart from the RF. Within ACPAs, we identified different clusters: a cluster of pepA, pepB and anti-CCP1, and a cluster of AhFibA and anti-CCP2.

Discussion

In the present study, we describe the diagnostic performance of an assay, based on the detection of AhFibA. We compared the diagnostic value of the AhFibA ELISA and the anti-CCP2 ELISA, and conclude that both assays perform equally well, which is reflected by similar ROC curves and similar sensitivities and specificities. There were some non-significant differ-

Table 2

Agreement between AhFibA assay and anti-CCP2 assay at the 98.5% specificity level in population 1

			Anti-CCP2		Total	κ	
			Neg	Pos			
Non-RA	AhFibA	Neg	452	4	456	0.420	0.845
		Pos	4	3	7		
	Total		456	7	463		
RA	AhFibA	Neg	28	8	36	0.765	
		Pos	2	54	56		
	Total		30	62	92		

AhFibA, anti-human fibrinogen (auto)antibodies; CCP, cyclic citrullinated peptide; Neg, negative; Pos, positive; RA, rheumatoid arthritis.

Table 3

Sensitivities of the different ACPA assays in population 2 after dichotomization at specificity level ≥ 98%

	Sensitivity	Cut-off (at 98% specificity)	Ref.
AhFibA	70%	0.12 OD	[18]
Anti-CCP1	51%	92 U/ml	[11]
Anti-CCP2	65%	42 U/ml	[14]
Anti-PepA	57%	1	[13,14]
Anti-PepB	55%	1	[13,14]

ACPA, anti-citrullinated protein/peptide antibody; AhFibA, anti-human fibrinogen (auto)antibodies; CCP, cyclic citrullinated peptide; OD, optical density.

ences in sensitivities of the AhFibA and anti-CCP2 assay between populations 1 and 2.

In contrast to the comparable diagnostic performance of the AhFibA and anti-CCP2 antibodies, the agreement between the two assays in population 1 was only moderate, and was especially impaired in the non-RA patients. Indeed, at the 98.5% specificity level only 11 non-RA patients exhibited any ACPA reactivity, of which only three were positive for both AhFibA and anti-CCP2 antibodies (Table 2). Double ACPA positivity thus resulted in a specificity of 99.4% with a sensitivity of 58.7%.

In population 2, we also evaluated the agreement between the AhFibA ELISA and four other ACPA tests. This confirmed the moderate agreement between the different ACPA assays. Agreement between the different ACPA assays may be important for the implementation of prediction models. Different prediction models for diagnosis of (persistent) erosive disease have been described by means of different ACPA assays [22,23]. Taking into account the similarities between the different ACPA tests, we performed a cluster analysis. Separated from RF, we found a clustering of anti-pepA, anti-pepB and anti-CCP1 assays on one side and anti-CCP2 and AhFibA assays on the other. The clustering of RF at a long distance from ACPAs illustrates the different nature of the antibody systems [24]. Two different explanations can be hypothesized to account for the two clusters within the ACPA tests. First, the anti-pepA, anti-pepB and anti-CCP1 assay use a citrullinated epitope derived from filaggrin. Filaggrin is not the natural autoantigen for ACPA because it is only expressed in epidermis. The substrate of the anti-CCP2 ELISA comprises cyclic peptides selected from libraries containing citrullinated peptides screened with RA sera; these peptides could have a lower degree of homology with filaggrin [25]. The second potential explanation is that both the AhFibA and the anti-CCP2 ELISA use multiple citrullinated epitopes for the detection of ACPAs. Because it was demonstrated that individual

Table 4

Value of κ statistic between the different ACPA assays in population 2

	AhFibA	Anti-CCP1	κ Anti-CCP2	Anti-PepA	Anti-PepB
AhFibA	-				
Anti-CCP1	0.552	-			
Anti-CCP2	0.710	0.609	-		
Anti-PepA	0.540	0.766	0.605	-	
Anti-PepB	0.618	0.811	0.679	0.842	-

Values for κ statistic were calculated after dichotomization with previously defined >98% specific cut-offs [5,14,18]. ACPA, anti-citrullinated protein/peptide antibody; AhFibA, anti-human fibrinogen (auto)antibodies; CCP, cyclic citrullinated peptide.

Figure 3
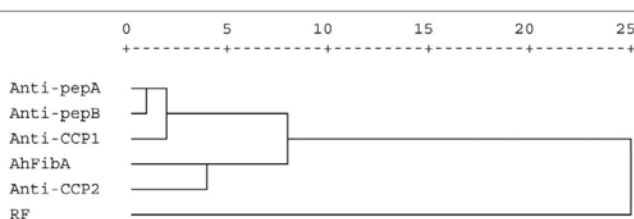
Dendrogram of the cluster analysis of the different ACPA assays in population 2. ACPA, anti-citrullinated protein/peptide antibody; AhFibA, anti-human fibrinogen (auto)antibodies; CCP, cyclic citrullinated peptide; RF, rheumatoid factor.

RA patients reacted with different citrullinated epitopes [5], the sensitivity of an ACPA test is expected to increase when more than one citrullinated epitope is used.

Increasing the sensitivity at a high specificity level for ACPA detection appears difficult to achieve. Further characterization of the synovial citrullinated proteins apart from fibrinogen may provide new substrates for detection of ACPAs, which might increase the sensitivity and specificity of the future ACPA assays [26]. However, it can be hypothesized that there may be a limit to the sensitivity of ACPA assays for RA. It could be argued that there are two subpopulations within RA patients [27,28]: a population with ACPAs can be detected, which has an increased prevalence of the HLA shared epitope and with a worse functional and radiological outcome; and a population without ACPAs but with reactivities against several human cartilage gp39 peptides and type II collagen, with no increased prevalence of the HLA shared epitope and with a better radiological and functional prognosis. Also, ACPA positivity, if observed in non-RA patients, can preferentially be observed in patients who carry the HLA shared epitope, suggesting an important association between ACPA and the HLA shared epitope [29].

Conclusion
Detection of autoantibodies against human citrullinated fibrinogen performs as well as the anti-CCP2 ELISA, because it has similar diagnostic characteristics. Despite the similar diagnostic characteristics of the different ACPA tests, we found that the agreement between the different available assays is only moderate, especially in non-RA patients.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
BVC and TC drafted the manuscript. BVC performed the statistical analysis. BVC, TC, LDR and AD constructed the datasets. BVC, TC, DD, DE, GS and FDK participated in the study design. LN, CC, MS, CV and GS participated in the development of the AhFibA ELISA. All authors read and approved the final manuscript.

Acknowledgements

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CHAPTER 8

A.
DAS28 best reflects the physician's clinical judgment of response to infliximab therapy in rheumatoid arthritis patients: validation of the DAS28 score in patients under infliximab treatment.

Vander Cruyssen B*, Van Looy S*, Wyns B, Westhovens R, Durez P, Van den Bosch F, Veys EM, Mielants H, De Clerck L, Peretz A, Malaise M, Verbruggen L, Vastesaeger N, Geldhof A, Boullart L, De Keyser F.

B.
DAS28: a useful instrument to monitor infliximab treatment in patients with
rheumatoid arthritis.

van Riel PL, Fransen J.

Research article

DAS28 best reflects the physician's clinical judgment of response to infliximab therapy in rheumatoid arthritis patients: validation of the DAS28 score in patients under infliximab treatment

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Abstract

This study is based on an expanded access program in which 511 patients suffering from active refractory rheumatoid arthritis (RA) were treated with intravenous infusions of infliximab (3 mg/kg+methotrexate (MTX)) at weeks 0, 2, 6 and every 8 weeks thereafter. At week 22, 474 patients were still in follow-up, of whom 102 (21.5%), who were not optimally responding to treatment, received a dose increase from week 30 onward. We aimed to build a model to discriminate the decision to give a dose increase. This decision was based on the treating rheumatologist's clinical judgment and therefore can be considered as a clinical measure of insufficient response. Different single and composite measures at weeks 0, 6, 14 and 22, and their differences over time were taken into account for the model building. Ranking of the continuous variables based on areas under the curve of receiver-operating characteristic (ROC) curve analysis, displayed the momentary DAS28 (Disease Activity Score including a 28-joint count) as the most important discriminating variable. Subsequently, we proved that the response scores and the changes over time were less important than the momentary evaluations to discriminate the physician's decision. The final model we thus obtained was a model with only slightly better discriminative characteristics than the DAS28. Finally, we fitted a discriminant function using the single variables of the DAS28. This displayed similar scores and coefficients as the DAS28. In conclusion, we evaluated different variables and models to discriminate the treating rheumatologist's decision to increase the dose of infliximab (+MTX), which indicates an insufficient response to infliximab at 3 mg/kg in patients with RA. We proved that the momentary DAS28 score correlates best with this decision and demonstrated the robustness of the score and the coefficients of the DAS28 in a cohort of RA patients under infliximab therapy.

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Introduction

Rheumatoid arthritis (RA) is a complex disease with a broad spectrum of manifestations that requires an early intensive therapy in order to avoid joint destruction and physical disability. In order to measure the effect of therapy in daily practice and in clinical trials, many variables have been recorded and different composite indices have been proposed to measure the remaining disease activity or the response to treatment. Those variables may cover items such as patient self-reported questionnaires, physician's scores including different joint scores, and serum markers of systemic inflammation.

Infliximab, in combination with methotrexate (MTX), is a highly effective therapy for a majority of RA patients. After an induction scheme at weeks 0, 2 and 6, the indicated dose of this therapy is 3 mg/kg every 8 weeks, although the ATTRACT trial suggested that a higher dose of 10 mg/kg every 8 weeks or a shorter perfusion interval may add benefit [1-3].

The present study is based on an expanded-access program in which patients suffering from active refractory RA were treated with intravenous infusions of infliximab (3 mg/kg + MTX) at weeks 0, 2, 6 and every 8 weeks thereafter. At week 22, patients not optimally responding to treatment could receive a dose increase of 100 mg (1 vial) per infusion from week 30 onwards [4]. The effect of dose escalation for the patients of this cohort has been discussed previously [4]. The decision to increase the dose was based on the treating rheumatologist's clinical judgment and can be considered as a measure of insufficient response to infliximab. It might be questioned which variables can be measured to best evaluate the effect of therapy and remaining disease activity in daily practice (and in clinical trials). The aim of the present analyses was to evaluate whether the decision to increase the dose could be reflected by using single variables or composite indices, alone or together in a model. We also wanted to evaluate whether this decision was mainly based on differences over time or on momentary disease activity.

Methods

Study population

A total of 511 patients, suffering from active refractory RA [5], were treated with intravenous infusions of infliximab (3 mg/kg) at weeks 0, 2, 6 and every 8 weeks thereafter in combination with MTX (a minimal dose of 15 mg/kg was recommended). Between week 0 and week 22, 37 patients dropped out for the following reasons: 16 patients stopped due to side effects (four infusion reactions, five infections, one malignancy, one pancytopenia, five disease-related complications), 12 patients stopped for withdrawal of consent and 9 patients stopped for protocol violation. Of the remaining 474 patients, 102 (22%) patients, who were not optimally responding to treatment according to the treating rheumatologist's opinion, received a dose increase of 100 mg (1 vial) per infusion from week 30 on. Throughout the first 22 weeks, dosage of MTX, steroids and non-steroidal anti-inflammatory drugs remained unchanged.

Evaluated variables

When designing the model, we took the following single variables into account at weeks 0, 6, 14 and 22: 28 and 66/68 swollen/tender joint counts, erythrocyte sedimentation rate (ESR; mm/h), C-reactive protein (CRP; mg/l), Health Assessment Questionnaire (HAQ; 0–3), physician's global assessment of disease activity (visual analogue scale (VAS); 0–100 mm), patient's global assessment of disease activity (VAS 0–100 mm), patient's assessment of pain (VAS 0–100 mm), patient's assessment of fatigue (VAS 0–100 mm) and all subscales of the SF-36 questionnaire (0–100 points) [6]. DAS28 (Disease Activity Score including a 28-joint count) [7] and other composite scores such as simplified disease activity index (SDAI), clinical disease activity index (CDAI) [8,9] and the alternative DAS28 scores [10,11] (Table 1) were calculated after data collection so that the treating rheumatologist was unaware of the exact values of those composite scores. Also, differences over time and the DAS28 response (no, moderate or good) and the ACR (American College of Rheumatology) response (no/20/50) were computed [12,13].

Table 1

Formulae to calculate the different DAS and SDAI score

Score	Formula	AUC (95% CI)	Sens at 95% spec, % (95% CI)
DAS28	$0.56 \cdot \mathrm{sqrt}(28TJC) + 0.28 \cdot \mathrm{sqrt}(28SJC) + 0.70 \cdot \ln(ESR) + 0.014 \cdot \mathrm{pt\ global\ VAS}$	0.840 (0.791–0.889)	42.5 (36.9–48.1)
DAS28-3	$[0.56 \cdot \mathrm{sqrt}(28TJC) + 0.28 \cdot \mathrm{sqrt}(28SJC) + 0.70 \cdot \ln(ESR)] \cdot 1.08 + 0.16$	0.815 (0.763–0.868)	37.8 (32.3–43.3)
DAS28-CRP	$0.56 \cdot \mathrm{sqrt}(28TJC) + 0.28 \cdot \mathrm{sqrt}(28SJC) + 0.36 \cdot \ln(CRP+1) + 0.014 \cdot \mathrm{pt\ global\ VAS} + 0.96$	0.829 (0.782–0.876)	35.8 (30.4–41.2)
DAS28-CRP-3	$[0.56 \cdot \mathrm{sqrt}(28TJC) + 0.28 \cdot \mathrm{sqrt}(28SJC) + 0.36 \cdot \ln(CRP+1)] \cdot 1.10 + 1.15$	0.806 (0.755–0.858)	28.9 (23.8–33.9)
SDAI	$28TJC + 28SJC + CRP/10 + \mathrm{pt\ global\ VAS}/10 + \mathrm{phys\ global\ VAS}/10$	0.824 (0.776–0.873)	40.7 (35.1–46.2)
CDAI	$28TJC + 28SJC + \mathrm{pt\ global\ VAS}/10 + \mathrm{phys\ global\ VAS}/10$	0.821 (0.772–0.870)	37.8 (32.3–43.2)

DAS28-3 and DAS28-CRP-3 are the DAS28 and DAS28-CRP scores calculated without the patient's global disease activity VAS. AUC, area under the curve; CDAI, clinical disease activity score; CRP, C-reactive protein; DAS, disease activity score; ESR, erythrocyte sedimentation rate; phys, physician; pt, patient; SDAI, simplified disease activity score; SJC28, 28 swollen joint count; TJC28, 28 tender joint count; VAS, Visual Analogue Scale.

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Statistics

We opted to use only statistical methods that are available in a classical statistical package (SPSS 12.0; SPSS, Inc, Chicago, IL, USA) or could be computed manually. When needed, the continuous variables were normalized (by taking the square root of the joint counts and the natural logarithm of CRP and ESR). Robustness of the discriminant analyses and logistic regressions was confirmed by the use of a random train and test set. Missing values were handled by pairwise complete case analysis. This means that a case with no missing values for a group of variables is included in the analysis of that group of variables. The case may have missing values for variables used in other analyses. Confidence intervals (95% CI) for sensitivity or specificity were calculated based on the method proposed by Harper [14]. The areas under the curves (AUCs) of receiver operating characteristic (ROC) curves were calculated. A higher AUC indicates that a single variable has better discriminative characteristics. A statistical test to compare AUCs of two variables tested on the same population has been described by Hanley [15]. Continuous and categorical variables were compared by adapting the cut-off of the continuous variables to the same specificity level as the categorical variable so that sensitivities could be evaluated and compared [16]. The selection and comparison of variables by curve analysis was performed since this method gives a valid ranking of variables and does not (in contrast to ranking methods based on p values) depend on the number of subjects available for that specific variable [17]. In order to find the true maximal model and to avoid sticking at a local maximal model, we used different strategies for the construction of the final model: binary logistic regressions and discriminant analyses were performed with the default options of SPSS 12.0 and stepwise construction of models was performed by conditional forward and backward elimination for logistic regression and by Wilk's lambda for discriminant analysis using the strategy described by Hosmer and Lemeshow [18].

Ethics

All patients signed informed consent. This study was approved by the local ethics committees.

Results

Ranking of continuous variables

In order to select the most important variables that correlate with the decision to give a dose increase at week 22, we calculated the AUC of ROC curve analysis for all continuous variables and ranked them based on this AUC [17]. Since crossing over of ROC curves may affect the diagnostic properties of a variable without changing the AUC, we also ranked the variables based on sensitivity levels by adapting the cut-off to a given preset specificity level of 95% [16].

Both ranking methods displayed that the DAS28 score at week 22 had the highest ability to discriminate the physician's decision to give a dose increase. Table 2 displays the 10 most

important variables ranked by AUC of ROC curve analysis and by the sensitivity at the 95% specificity level. Using the method described by Hanley [15], we found that there was a significant difference in AUC between the two first ranked parameters: DAS28 at week 22 and the 28 tender joint count at week 22 (AUC = 0.840 versus 0.797, $p = 0.02$). Additionally, most variables were ranked in such a way that each variable was represented first by its measure at week 22 before it was represented by a measure at another week.

Evaluation of the response scores

To evaluate categorical scores, we adapted the cut-off of the variable with the highest ranking (DAS28 at week 22) to the specificity of the categorical score and compared the sensitivities [16]. For the decision to give a dose increase, ACR response not reaching the ACR20 criterion ('no ACR response') had a sensitivity of 69.6% (95% CI: 65.2–74.0) and a specificity of 64.2% (95% CI: 59.6–68.8). When we adapted the cut-off of the DAS28 at week 22 to a specificity of 64.2% (DAS28 = 4.01), we obtained a sensitivity of 80.0% (95% CI: 75.2–84.7). 'No DAS28 response' had a sensitivity of 46.7% (95% CI: 40.8–52.6) and a specificity of 83.3% (95% CI: 78.9–87.7). When we adapted the cut-off of the DAS28 to a specificity of 83.3% (DAS28 = 4.77), we obtained a sensitivity of 67.5% (95% CI: 61.9–73.1). Similar results were obtained when looking at the ACR50 and the good DAS28 response criterion (Table 3).

Additionally, we fitted a logistic regression model with the decision to give a dose increase as a dependent variable and DAS28 at week 22, DAS28 response and ACR response as categorical covariates. These analyses retained DAS28 at week 22 as the only significant covariate in the model (data not shown).

Effects of change of scores over time on the physician's decision

To evaluate the effect of differences over time, we plotted the means of the most important normalized continuous variables over time (Fig. 1). The plot of the variable with the highest ranking (DAS28) shows that patients who get a dose increase have a (significantly) higher disease activity at baseline and, after an initial decrease of disease activity, regain disease activity from week 6 on. To evaluate this, we calculated differences in DAS28 scores between baseline and week 22 (delta DAS28 0–22), and between week 6 and week 22 (delta DAS28 6–22). Indeed, patients who get a dose increase regain some disease activity between week 6 and week 22 (mean delta DAS28 6–22: -0.4 versus +0.4, $p < 0.001$), which is reflected in a smaller decrease of disease activity between baseline, and week 22 (mean delta DAS28 0-22: -2 versus -1, $p < 0.001$). However, the AUC of the ROC curve of delta DAS28 0–22 was 0.725 (95% CI: 0.659–0.790) and the AUC for delta DAS28 6–22 was 0.672 (95% CI: 0.590–0.754), which is much lower than the AUC of the momentary

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Table 2

Variables with the highest ranking based on ROC curve AUC and sensitivities at 95% specificity

	AUC	95% CI of AUC
DAS28 w22	0.840	0.791–0.889
28 TJC w22	0.797	0.744–0.850
Physician global VAS w22	0.786	0.736–0.836
Patient pain VAS w22	0.764	0.71–0.814
DAS28 w14	0.750	0.685–0.815
Patient disease activity VAS w22	0.750	0.689–0.802
66TJC w22	0.740	0.689–0.791
28TJC w14	0.721	0.662–0.780
66SJC w22	0.717	0.660–0.774
ESR w22	0.716	0.654–0.779
	Sensitivity (%) at 95% specificity level	95% CI of sensitivity
DAS28 w22	42.5	36.9–48.1
Physician global VAS w22	32.7	28.4–37.0
28SJC w22	29.8	24.7–34.9
Patient pain VAS w22	26.8	22.7–30.9
66SJC w22	24.5	20.6–28.4
ESR w22	24.1	19.7–28.5
66SJC w14	23.0	18.6–27.4
CRP w22	21.3	17.3–25.3
Patient disease activity VAS w22	20.4	16.6-4.2
DAS28 w14	20.3	15.3–25.3

AUC, area under the curve; TJC, tender joint count; SJC, swollen joint count; ESR, erythrocyte sedimentation rate; CI, confidence interval; CRP, C-reactive protein; w, week.

Table 3

Sensitivity and specificity of the response scores compared with DAS28 set at equal specificity

	Sensitivity and specificity of the different response scores		Sensitivity of DAS28 at the same specificity level	
	Specificity (%)	Sensitivity (%)	Sensitivity (%)	According DAS28 score
No moderate DAS response	83.3	46.7	67.5	4.8
No good DAS response	42.4	96.3	97.3	3.1
No ACR20 response	64.2	69.6	80.0	4.0
No ACR50 response	33.6	85.9	97.5	2.9

DAS, disease activity score.

DAS28 (0.840) at week 22. Additionally, when we fitted a logistic regression model with the decision to give a dose increase as a dependent variable and DAS28 at week 22, delta DAS28 0–22 and delta DAS28 6–22 as covariates, only DAS28 at week 22 was a significant variable in the model.

Similar analyses were performed for the other variables. The AUC of the differences between weeks 0–22, weeks 6–22 and weeks 14–22 of the other variables were all less than 0.700 (data not shown). These analyses indicate that, although the differences in disease activity over time are statistically significant, those differences over time are not impor-

Figure 1
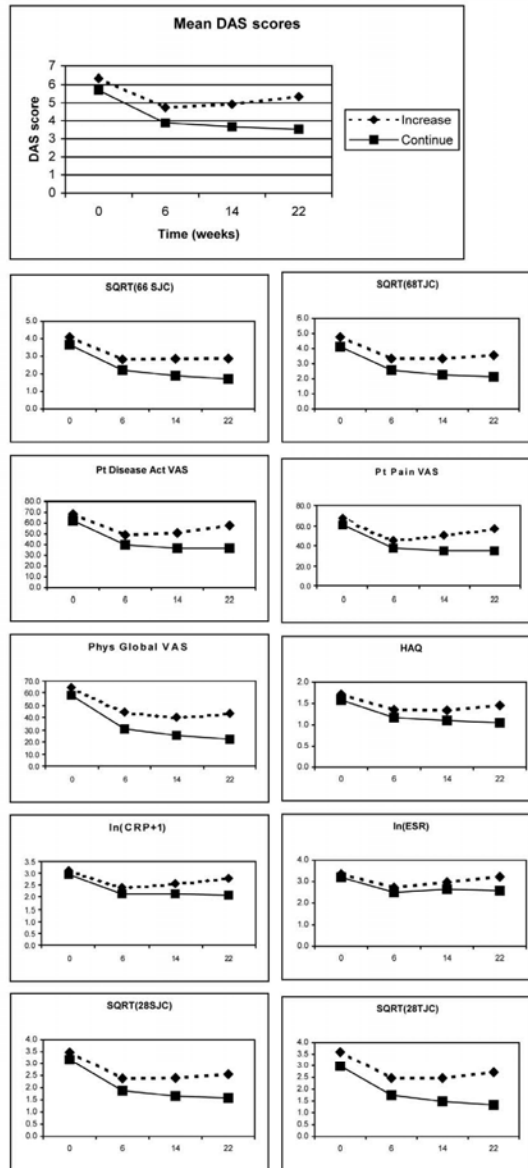
Plot of the mean scores over time. Act, activity; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; SJC, swollen joint count; TJC, tender joint count; pt, patient; Phys, physician; SQRT, variable normalized by taking the squared root; ln, variable normalized by taking the natural logarithm; VAS, visual analogue scale.

tant enough to incorporate in a model to discriminate the physician's decision.

Building a model to discriminate the physician's decision to give a dose increase

The first three analyses (ranking of continuous variables, evaluation of the response scores and effects of change of scores over time on the physician's decision) allowed us to narrow the selection of variables for the model by eliminating variables that are already incorporated into the DAS28 (or are highly related to them such as CRP and 68 tender joint and 66 swollen joint count) and taking into account only those variables at week 22. This resulted in the following list: DAS28, HAQ, physician global VAS, patient pain VAS, patient fatigue VAS and the scores of the SF36 questionnaire at week 22. We screened those variables using forward and backward elimination in a logistic regression model and by the stepwise Wilk's lambda method. The probability scores of the logistic regression and discriminant scores we thus obtained were compared using ROC curve analysis. The model with the highest AUC was a model from discriminant analysis with the following variables (and standardized canonical discriminant function coefficients): DAS28 week 22 (0.863), physician global VAS (0.796), patient pain VAS (0.735), and physical functioning (-0.227). The discriminant score of this model had an AUC of 0.870 (95% CI: 0.828–0.912) with a sensitivity at the 95% specificity level of 45.5% (95% CI: 38.7–50.3).

Figure 2
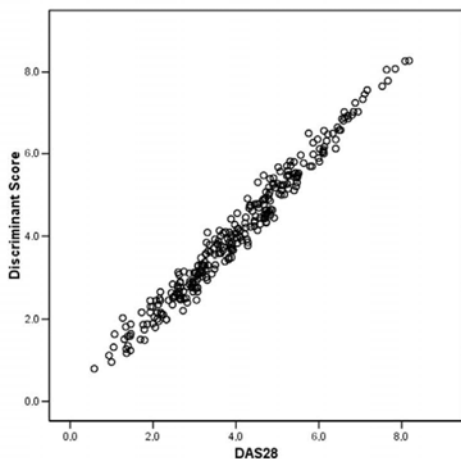
Validation of the DAS28 score and coefficients (see text). ESR, erythrocyte sedimentation rate; VAS, visual analogue scale.

Evaluation of the discriminant score of the variables of DAS28

To validate the score and coefficients of the DAS28, we calculated a discriminant function using the (normalized) variables of the DAS28 score: 28 tender and swollen joint count, ESR and patient global VAS. After rescaling, we obtained the following discriminant coefficients: 0.52 for 28 tender joint count (28TJC), 0.28 for 28 swollen joint count (28SJC), 0.56 for ESR and 0.025 for patient disease activity. This discriminant score had an AUC of 0.844 (0.797–0.891) and a sensitivity at the 95% specificity level of 43.8% (95% CI: 38.1–49.2), which is equal to the DAS28 at week 22. The Pearson's correlation coefficient between this discriminant score and the DAS28 was 0.986 (Fig. 2). We also performed logistic regression with similar results (data not shown).

Comparison with the other DAS scores and SDAI/CDAI

Since different alternative methods are available to calculate the DAS scores (Table 1), we additionally evaluated the properties of those alternative scores. We also evaluated the SDAI and CDAI [8,9], after normalization, by taking the squared root. The Pearson's correlation coefficient of those alternative scores with the DAS28 at week 22 was 0.982 for the DAS28-3, 0.952 for the DAS28-CRP, 0.928 for the DAS28-CRP-3, 0.914 for the SDAI and 0.893 for the CDAI. The AUC and sensitivity at the 95% specificity level are shown in Table 1 and indicate that all those alternative scores perform similarly or slightly worse than the original DAS28.

Detailed ROC curve analysis of the DAS28

We plotted the ROC curve of the DAS28 in Fig. 3 and listed sensitivities and specificities in Table 4. Also, predictive values and the accuracies of classification in function of the different DAS28 cut-offs are shown in Table 4. Beneath a cut-off of 3.2, we found a high predictive value for continuing the current dose as a measure of good response. The maximal accuracy of 84% could be found at a cut-off of 5.5.

Discussion

The aim of the present analyses was to evaluate which single or composite variables, combined in a model, could discriminate the treating rheumatologist's decision to give a dose increase of infliximab to RA patients not optimally responding to an indicated dose of 3 mg infliximab every 8 weeks. Since different variables on different time points were available, we started to rank the continuous variables based on the AUC of ROC curves and sensitivities at the 95% specificity level. This strategy has previously been proposed for microarray data [17]. The calculation of sensitivities at the 95% specificity level is important in order not to overlook some variables with a relative small AUC but with a high specificity [16]. So, both methods ranked the DAS28 at week 22 as the variable which best discriminates the decision to give a dose increase. In a second and third analysis, we looked at whether response scores and differences in disease activity over time

Table 4

Performance at different cut-offs of DAS28 at week 22 for dose increase

DAS cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy
2.0	0.99	0.13	0.23	0.98	0.31
2.5	0.99	0.22	0.25	0.99	0.38
2.6	0.99	0.26	0.26	0.99	0.41
3.0	0.98	0.38	0.29	0.98	0.50
3.2	0.96	0.46	0.32	0.98	0.57
4.0	0.79	0.66	0.38	0.92	0.68
4.5	0.76	0.77	0.47	0.92	0.77
5.0	0.58	0.87	0.55	0.89	0.81
5.1	0.53	0.88	0.55	0.88	0.81
5.5	0.43	0.95	0.69	0.86	0.84
6.0	0.34	0.97	0.73	0.85	0.83
6.5	0.19	0.98	0.72	0.82	0.81

DAS, disease activity score; PPV, positive predictive value (predictive value to give a dose increase as a measure of insufficient response); NPV, negative predictive value (predictive value to continue on the current dose as a measure of good response); PPV, NPV and accuracy were calculated using the following formulae:

a) $PPV = \dfrac{sensitivity * a\_priori\_chance}{sensitivity * a\_priori\_chance + (1 - specificity) * (1 - a\_priori\_chance)}$

b) $NPV = \dfrac{specificity * (1 - a\_priori\_chance)}{specificity * (1 - a\_priori\_chance) + (1 - sensitivity) * a\_priori\_chance}$

c) $Accuracy = sensitivity * a\_priori\_chance + specificity * (1 - a\_priori\_chance)$

The a priori chance is given by the percentage of patients that need a dose increase as a measure of insufficient response.

could give additional information to discriminate the rheumatologist's decision. Those analyses indicated that variables, including differences over time, seem to be less important than the momentary remaining disease activity at week 22, to discriminate the rheumatologist's decision.

After the prior selection of variables, based on the findings of the previous steps, we built the final model to discriminate the rheumatologist's decision, which was only slightly better than the DAS28. We think that the small gain in discriminative properties in comparison with the DAS28 is not enough to accept the increased complexity of this model. Moreover, in contrast to the DAS28, this model included the physician's global assessment of disease activity (VAS), which is investigator-dependent and has the draw-back that it cannot be calculated by a study nurse. All four analyses together indicated that the DAS28 is an important variable for evaluating insufficient response to infliximab therapy (especially in daily practice) and that this variable can only slightly be improved by adding supplemental variables.

DAS was developed in the early 1990s [19,20] and later on, it was transformed into the DAS28 [7] in an era when therapy with biologicals was not yet available. In those initial studies, patients were scored by the same two independent nurses

and the decision to change disease-modifying antirheumatic drug (DMARD) therapy during a follow-up period of up to 3 years was considered as a measure of insufficient response [20]. The present study is a multi-center study where patients were scored by the treating physician and the decision to give a dose increase of infliximab could happen only at one time point. This difference in study design and therapy may explain why in the present study the AUC of DAS28 is smaller than in other studies (AUC = 0.840 versus 0.933) [21]. Therefore, it is remarkable that despite those differences in study design, we could calculate a discriminant function (in the fifth analysis) that correlated so well with the DAS28 by using the 28SJC, 28TJC, ESR and patient disease activity VAS as independent variables and the physician's decision as a grouping variable. Not only the discriminant scores, but also the coefficients of this discriminant function were quite similar to the coefficients of the DAS28, indicating the robustness of the scores and coefficients of the DAS28 score.

In another, final analysis, we evaluated the alternative DAS scores and the squared root transformed SDAI and CDAI. All those alternative scores have a slightly worse AUC than the original DAS28, but seem good enough to be useful when some other variables are not available. We think the use of the DAS28 is feasible and time-effective using a preprogrammed

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Figure 3
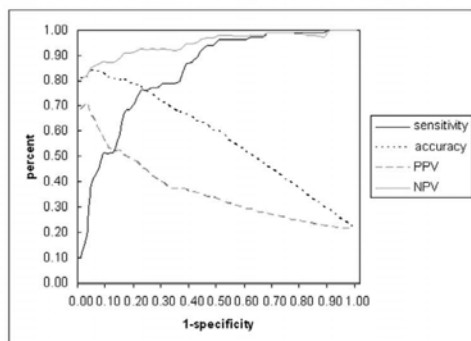
ROC curve analysis of the DAS28 at week 22 (plotting the 1-specificity versus the sensitivity). Also the accuracy, PPV and NPV are plotted. PPV, positive predictive value (predictive value to give a dose increase as a measure of insufficient response); NPV, negative predictive value (predictive value to continue on the current dose as a measure of good response).

calculator, spreadsheet or web-based calculator [11]. The unique characteristics of the DAS score make it a useful measure in a lot of applications. DAS28 as a continuous variable is a sensitive tool for measuring response to treatment in randomized controlled trials and facilitates the use of more complex statistical methods that can handle repeated measures over different time points [22-24].

Other studies demonstrated that a low DAS is an important prognostic factor of persistent remission and that DAS correlates with radiological progression [25,26]. DAS may also be a useful parameter in daily clinical practice as a treatment goal and to evaluate the actual disease activity (which cannot be assessed by the categorical response scores) [27-31]. Our findings that the physician's decision to give a dose increase can best be modeled by a combination of measurements of remaining/momentary disease activity, represented by the DAS28 does not reduce the value of the response scores such as ACR response or DAS response scores. Indeed, those scores are important for measuring differences over time as a measure of global treatment effects in clinical trials [12,13] but, as demonstrated by the present study, are not useful for evaluating the momentary disease activity in a single patient, which is important in daily practice. The continuous properties of the DAS28 score provide the additional opportunity for a cut-off, which can be chosen as a function of the purpose. Interestingly, we found a high predictive value for continuing the current dose as a measure of good response below a cut-off of 3.2. It is noteworthy that a DAS score of 3.2 is an important threshold for a good DAS response according to the EULAR criteria [12]. In contrast, for classification purpose, a higher cut-off (5.5) is more appropriate since this level

displayed the highest accuracy. One should be aware that the displayed predictive values and accuracies may be highly influenced by the prevalence of insufficient response, reflected by the need for a dose increase, which was 21.5% in the present study. A lower *a priori* chance of the need for a dose increase may increase the accuracy of DAS (given the fixed cut-off of 5.5) and vice versa. Indeed, at a cut-off with a high specificity, the accuracy will increase when the *a priori* chance decreases (applying formula c given in the legend to Table 4).

Conclusion
The results of the present analyses indicate that the momentary DAS28 as a continuous composite index correlates best with the decision to give a dose increase of infliximab, which is a measure of insufficient response. The discriminative characteristics of the DAS could be slightly improved by the use of supplemental variables, although this results in the disadvantage of a more complex model and calculations. This study also demonstrates the robustness of the scores and coefficients of the DAS28 in a cohort of RA patients under infliximab therapy and therefore validates the DAS28 as a measure of disease activity in patients under treatment with biologicals.

Competing interests
AG is an employee of Centocor, Leiden, The Netherlands; NV is an employee of Schering-Plough, Brussels, Belgium; and PD and RW were consultants for Schering-Plough Belgium during the clinical study.

Authors' contributions
BVC and SVL performed the statistical analysis, constructed the datasets and drafted the manuscript under the direct supervision of LB and FDK. RW, PD, FVdB, EV, HM, LDC, AP, MM, LV and FDK recruited and followed-up the arthritis patients. BVC, SVL, BW, NV, AG, LB, RW, PD and FDK participated in the study design. RW and PD were the initial investigators of the Belgian infliximab expanded-access program, in which the patients were enrolled. All authors have read and approved the final manuscript

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Commentary

DAS28: a useful instrument to monitor infliximab treatment in patients with rheumatoid arthritis

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Abstract

The Disease Activity Score using 28 joint counts (DAS28) has been developed in a cohort of patients with rheumatoid arthritis in which only conventional anti-rheumatic treatments were used. It has extensively been validated to monitor disease activity in daily clinical practice as well as in clinical trials. The study of Vander Cruyssen and colleagues showed that the DAS28 correlated best with the decisions of rheumatologists to increase the infliximab dose because of insufficient response. This result once more confirms the validity of the DAS28 to monitor disease activity in patients with rheumatoid arthritis and to titrate treatment with biologicals.

In daily clinical practice, the Disease Activity Score using 28 joint counts (DAS28) is used to monitor the disease activity of rheumatoid arthritis patients treated with disease-modifying anti-rheumatic drugs (DMARDs) and biological agents. This is useful to inform the rheumatologist about whether the treatment is producing the expected effects in an appropriate period of time or whether the treatment should be more intensified.

In an article in the present issue, Vander Cruyssen and colleagues investigated which variables can best be measured to evaluate the effect of therapy and the remaining disease activity in daily clinical practice [1]. This study was based on a cohort of 511 patients with active refractory rheumatoid arthritis who were treated with infliximab [2]. Patients who were judged by their physicians to have an insufficient response at week 22 received a dose increase at week 30. According to the authors, the decision to increase the dose was based on clinical judgement, without knowledge of outcome measures such as the DAS28. In their study, the authors found that the DAS28 as a continuous composite index correlated best with the decision to give a dose increase of infliximab, which was used as a surrogate measure of insufficient response. The discriminative capacity

of the DAS28 could only slightly be improved by the inclusion of supplemental variables in the regression model. Recalculation of the DAS28 coefficients in a discriminative function obtained similar coefficients and the same discriminative capacity as the original DAS28. For a better understanding of these results, it is informative to know how the Disease Activity Score and the DAS28 were developed back in the 1990s.

The DAS28 was developed in a similar way to the Disease Activity Score, but the DAS28 contains reduced, ungraded, joint counts and has different weights [3,4]. The DAS28 was developed in a cohort from an outpatient clinic, using the data from 227 early rheumatoid arthritis patients that were followed-up for 9 years between 1985 and 1994. Because no gold standard for disease activity is available, decisions on DMARD therapy were used as an external standard of 'high' and 'low' disease activity in the development of the DAS28. The DAS28 formula optimally discriminated between these two clinically relevant states. The validity of the DAS28 was tested using a similar cohort from another clinic. Since their development, the Disease Activity Score and the DAS28 have extensively been validated [5].

An interesting finding from the study of Vander Cruyssen and colleagues is that they also used decisions to change (infliximab) treatment as a proxy for the underlying disease activity, and produced the same DAS28 as found 20 years earlier in a cohort in which only conventional DMARDs were used, without a need to change its content or form. This means that the DAS28 is able to discriminate between clinically relevant states of disease activity, rather than discriminating a 'readiness' to change treatment (from physicians and patients) to start, to stop or to continue DMARD treatment. This enforces the validity and generalisability of the DAS28.

DAS28 = Disease Activity Score using 28 joint counts; DMARD = disease-modifying anti-rheumatic drug.

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The authors reached their conclusion based on a series of analyses comparing the performance of multiple measures in several ways. The authors used receiver-operating characteristic curves and sensitivity, specificity and predictive values to rank the measures in order of their performance. As the authors state, these statistics for diagnostics may be used to rank measures in a study, but it is difficult to generalise the values for sensitivity, specificity, and so on, beyond the study. This difficulty occurs because all values for these statistics heavily depend on the distributions found in the study (see Figure 1 in [1]). Moreover, the use of sensitivity, specificity, and so on, does not reflect the way the DAS28 is used, as one would not use the DAS28 to 'diagnose' physician opinion on whether or not to increase the infliximab dose.

However, the results of Vander Cruyssen and colleagues can best be understood when looking at Figure 1 in their article [1], depicting the differences in disease activity measures between both groups of patients. Two lessons can be learned from this figure.

First, higher scores of the DAS28 and the other disease activity measures are found in patients in which a decision was made to increase the dose of infliximab. Only a few other studies used external criteria for high and low disease activity to study the validity of the Disease Activity Score and the DAS28. In a study performed in Italy in the late 1990s, it was found that the Disease Activity Score was the best measure to discriminate between predefined states of low and high disease activity, in a sample of 202 patients [6]. A recent study used a different, opinion-based, approach, with expert rating ($n = 35$) of a sample of clinical profiles that were categorised into remission, low disease activity, moderate disease activity and high disease activity [7]. Interestingly, the cut-off criteria for the DAS28 that were found in this way were only slightly different from the established cut-off points for the DAS28, which can therefore be regarded as confirmation.

The second interesting finding from Vander Cruyssen and colleagues' study, which was not highlighted in the article, is that more than 50% of the patients in which the infliximab dose was not increased had DAS28 >3.2, which means 'moderate' or 'high' disease activity. One may ask whether a dose increase would also have been indicated in these patients, as the aim is to reach low disease activity or even remission. This illustrates that the target of anti-rheumatic treatment is moving in time. It is therefore an extra advantage to use a continuous measure with absolute values to measure disease activity in daily clinical practice and clinical trials.

Conclusion

The study of Vander Cruyssen and colleagues confirms that the DAS28 is a valid measure to monitor disease activity and to titrate treatment with biologicals [8].

Competing interests

The author(s) declare that they have no competing interests.

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CHAPTER 9

Four year follow-up of infliximab therapy in rheumatoid arthritis patients
with longstanding refractory disease: attrition and long-term evolution of
disease activity.

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Research article

Four-year follow-up of infliximab therapy in rheumatoid arthritis patients with long-standing refractory disease: attrition and long-term evolution of disease activity

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Abstract

Although there is strong evidence supporting the short-term efficacy and safety of anti-tumour necrosis factor-α agents, few studies have examined the long-term effects. We evaluated 511 patients with long-standing refractory rheumatoid arthritis treated with intravenous infusions of infliximab 3 mg/kg at weeks 0, 2, 6, and 14 and every 8 weeks thereafter for 4 years. Among the initial 511 patients included in the study, 479 could be evaluated; of these, 295 (61.6%) were still receiving infliximab treatment at year 4 of follow-up. The most common reasons for treatment discontinuation were lack of efficacy (65 patients, 13.6%), safety (81 patients, 16.9%), and elective change (38 patients, 7.9%). Analysis of disease activity scores (DAS28 [disease activity score based on the 28-joint count]) over time showed that, after the initial rapid improvement during the first 6 to 22 weeks of therapy, a further decrease in disease activity of 0.2 units in the DAS28 score per year was observed. DAS28 scores, measured at week 14 or 22, were found to predict subsequent discontinuation due to lack of efficacy. In conclusion, long-term maintenance therapy with infliximab 3 mg/kg is effective in producing further reductions in disease activity. Disease activity measured by the DAS28 at week 14 or 22 of infliximab therapy was the best predictor of long-term attrition.

Introduction

After demonstration of effectiveness of anti-tumour necrosis factor (TNF)-α agents in patients with rheumatoid arthritis (RA) [1-3], their use has become common practice in treating patients with RA not responding to classical disease modifying anti-rheumatic drugs (DMARDs). Although there is strong evidence in support of the short-term efficacy and safety of these agents, data are still insufficient with regard to the long-term effects.

Long-term treatment continuation rates reflect safety, efficacy, and compliance to therapy and may vary between data from clinical trial extensions and treatment registries. Infliximab, primarily used in combination with methotrexate (MTX), is a highly

ACR = American College of Rheumatology; AUC = area under the curve; CI = confidence interval; CRP = C-reactive protein; DAS28 = disease activity score based on the 28-joint count; DMARD = disease-modifying anti-rheumatic drug; ESR = erythrocyte sedimentation rate; HAQ = health assessment questionnaire; MTX = methotrexate; RA = rheumatoid arthritis; ROC = receiver operating characteristic; SD = standard deviation; SE = standard error; SF = short form; TNF = anti-tumour necrosis factor; VAS = visual analogue scale.

effective therapy for the majority of patients with RA [4]. After an induction scheme with intravenous infliximab infusions given at weeks 0, 2, and 6, infliximab is typically administered at a dosage of 3 mg/kg every 8 weeks in combination with MTX. However, results of the ATTRACT (Anti-TNF Trial in Rheumatoid Arthritis with Concomitant Therapy) trial suggested that a higher dosage (10 mg/kg every 8 weeks) or a shorter perfusion interval may add benefit, which is reflected by the use of dosage increases in some studies [5,6].

In most countries, anti-TNF-α therapy is reserved for patients who are refractory to classical DMARD therapy. These patients may require TNF-α blockade for an extended time. We analysed data from patients who entered the Belgium expanded access program and received infliximab 3 mg/kg in combination with MTX. Patients in this program could receive infliximab therapy (provided by Schering-Plough, Brussels, Belgium) until the product became reimbursed. We aimed to (a) evaluate attrition of infliximab therapy in patients with long-standing refractory RA over a 4-year period, (b) document the reasons for discontinuation, (c) describe the long-term course of disease activity, and (d)evaluate early predictors of long-term continuation of the therapy.

Materials and methods
Study population
Five hundred eleven patients with RA entered the Belgium expanded access program between February 2000 and September 2001. These were the first Belgian patients to be treated with TNF blockade outside of the clinical trial setting after EMEA (European Medicines Evaluation Agency) approval of infliximab for the treatment of patients with RA and to receive infliximab from Schering-Plough for free as part of a Medical Need Program (the Belgian expanded access program) until the product became reimbursable.

Patients were observed at seven Belgian university centres. Clinical evaluations performed with each infliximab infusion included the 28 and 66/68 swollen and tender joint counts, erythrocyte sedimentation rate (ESR) (mm/hour), C-reactive protein (CRP) (mg/l), health assessment questionnaire ([HAQ] on a scale of 0–3) [7], physician's global assessment of disease activity using a visual analogue scale ([VAS] 0–100 mm), patient's global assessment of disease activity (VAS 0–100 mm), patient's assessment of pain (VAS 0–100 mm), patient's assessment of fatigue (VAS 0–100 mm), and all subscales of the short form (SF)-36 questionnaire (0–100 points) [8].

Along with the clinical evaluations performed on the day of each infusion, all physicians completed an evaluation of the 4-year experience. The evaluation provided an assessment of the actual therapy patients were receiving by year 4. If patients were withdrawn from infliximab therapy, the following information was collected: reasons for withdrawal (inefficacy, safety,

death, or lost to follow-up); DAS28 [9,10]; physician's global VAS, CRP, and HAQ scores prior to infliximab withdrawal; and actual therapy at year 4.

All patients had long-standing, active, refractory RA. After an induction regimen of 3 mg/kg at weeks 0, 2, and 6, all patients received maintenance therapy every 8 weeks. At week 22, the treating rheumatologist had the option of increasing the dosage by 100 mg [11,12]. The standard infliximab dosage of 3 mg/kg every 8 weeks was reinstituted in a majority of patients beginning in June 2002, the time at which infliximab became a reimbursable medicine in Belgium. During the first 6 months, steroid and MTX dosages were kept stable; dosages could be adjusted from month 6 onward.

All patients gave written informed consent.

Variables evaluated in the prediction of infliximab continuation
To predict long-term continuation of infliximab therapy early in the treatment course, we assessed the relationship between infliximab discontinuation and the following single variables at each of weeks 0, 6, 14, and 22: 28 and 66/68 swollen/tender joint counts, ESR, CRP, HAQ, physician's global assessment of disease activity, patient's global assessment of disease activity, patient's assessment of pain (VAS 0–100 mm), patient's assessment of fatigue, and all subscales of the SF-36 questionnaire. The patients' DAS28 scores and response (no, moderate, or good) and the American College of Rheumatology (ACR) response (no, 20, 50, or 70) were calculated after data collection so that the treating rheumatologist was unaware of the exact values of those composite scores [9,10,13].

Statistical analysis
Statistical methods available in a classical statistical package (SPSS 12.0; SPSS Inc., Chicago, IL, USA) were employed in all analyses. The estimation of the slope of the course of disease activity from week 22 forward was estimated by means of linear mixed model analysis with an unstructured covariance matrix with random intercept and random slope [14]. Measurements from weeks 0, 6, 14, and 22 and the last clinical evaluation were included, as were available measures from other time points. When necessary, the continuous variables were normalised by taking the square root of the joint counts and the natural logarithm of CRP and ESR. Areas under the curve (AUCs) of receiver operating characteristic (ROC) curves were calculated. A higher AUC indicates that a single variable has better discriminative characteristics. The cutoff of the continuous variables was adapted to the same specificity level as the categorical variable so that sensitivities could be evaluated and compared between continuous and categorical variables [15]. The selection and comparison of variables by ROC-curve analysis was performed because this method gives a valid ranking of variables and does not depend on the number of

Figure 1
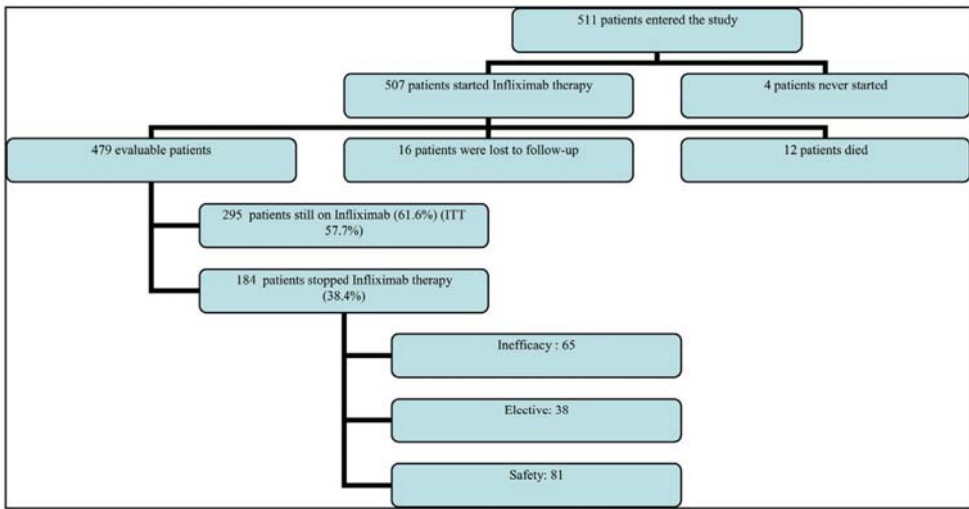
Flow chart of the patient population. ITT= intention to treat.

Figure 2
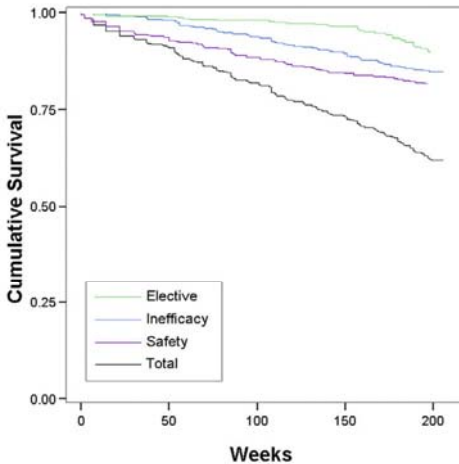
Kaplan-Meier plot and cumulative discontinuation due to the different stop reasons.

patients available for that specific variable (in contrast to ranking methods based on p values) [16].

Survival analysis and predictors of continuation of infliximab therapy were analysed by means of Kaplan-Meier analysis and Cox regression. Cox regression analysis was performed with the default options of SPSS 12.0, and stepwise construction of models was performed by conditional forward and backward elimination using the strategy described by Hosmer and Lemeshow [17]. Survival data were calculated after censoring at 4 years.

Results
Continuation rates of infliximab therapy

Of the initial 511 patients enrolled in the study, 507 effectively started infliximab therapy. All patients had long-standing, active, refractory RA, which is reflected by a mean baseline failure of 3.9 DMARDs and a mean disease duration of 10 years at baseline. After 4 years, 12 (2%) patients treated with infliximab had died (3 patients due to infections, 5 due to cardiovascular disease or lung embolism, and 4 due to other reasons; no patients died due to tuberculosis or anaphylactic reactions), and 16 (3%) patients were lost to follow-up. Of the 479 remaining patients, 295 (61.6%) patients were still receiving infliximab treatment after 4 years of therapy. One

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Table 1

Infliximab attrition rates at different time points

| | Global continuation rates | | | Discontinuation rates | | | | | | | | |
| | | | | Inefficacy | | | Elective | | | Safety | | |
Year	Percentage	95% CI		Percentage	95% CI		Percentage	95% CI		Percentage	95% CI	
1	90.9	88.3	93.5	1.8	0.6	3.0	0.9	0.1	1.7	6.4	4.2	8.6
2	80.7	77.2	84.2	6.4	4.2	8.6	1.4	0.3	2.5	11.5	8.6	14.4
3	69.6	65.5	73.7	11.2	8.4	14.0	3.4	1.8	5.0	15.8	12.5	19.1
4	61.6	57.2	66.0	13.6	10.5	16.7	7.9	5.5	10.3	16.9	13.5	20.3

CI, confidence interval.

hundred eighty-four (38.4%) patients were withdrawn from treatment for the following reasons: 81 (16.9%) due to safety issues (including 28 infections, 18 immune-allergic reactions, and 9 malignancies), 65 (13.6%) due to inefficacy, and 38 (7.9%) for elective reasons (Figure 1). The main elective reason to stop infliximab treatment was the decision by the physician or the patient to switch to a subcutaneous TNF-α blocker. Those subcutaneous TNF-α blockers became available in February 2003 for etanercept and in May 2004 for adalimumab. Figure 2 provides the Kaplan-Meier plot of the attrition on infliximab therapy, illustrating the continuation rates after 1, 2, and 3 years; these data are also displayed in Table 1.

Evaluation of the actual therapy at year 4 in the patients withdrawn from infliximab therapy

Data on the current DMARD or newly started biological therapy could be obtained in 142 of the 184 patients who discontinued infliximab therapy. Fifty percent of patients were switched to another biological therapy (Table 2).

Evolution of the DAS28 score over time

Patients continuing with infliximab therapy had a mean (standard deviation [SD]) DAS28 score of 3.0 (SD 1.3) at the year 4 clinical evaluation. In comparison, the mean DAS scores

Table 2

Use of biologic agents after withdrawal of infliximab therapy

| New biologic therapy | Reason for withdrawal | | | Total |
	Lack of efficacy	Patient request	Safety issues	
None	24	3	44	71
Adalinumab	10	23	3	36
Etanercept	6	4	8	18
Rituximab	8	0	1	9
Abatacept	4	0	4	8
Total	52	30	60	142

before the stop of therapy were 5.4 (SD 1.5) among patients who stopped due to inefficacy, 3.5 (SD 1.3) in patients who stopped due to safety reasons, and 3.0 (SD 1.0) in the patients withdrawn from infliximab for elective reasons. DAS28 scores at the relevant time points are depicted in Figure 3. This figure also suggests that, after an initial rapid decrease in disease activity between weeks 0 and 6, there appears to be a further decrease in disease activity through year 4. This decrease, calculated in the total population (all patients, including those who withdrew from therapy due to inefficacy), was estimated as a mean of 0.2 (standard error of the mean 0.03, $p < 0.0001$) DAS units per year. The same results were obtained in models that corrected for infliximab dosage and/or concomitant corticosteroid, MTX, or leflunomide use by adding them as (non-significant) covariates to the mixed models analysis.

Among the patients still receiving infliximab at year 4, 62% had a low level of disease activity, as defined by a DAS28 score of less than 3.2. Nearly half (49.5%) of these patients had minimal disease activity (DAS28 less than 2.85 or no swollen joints, no tender joints, and ESR less than 10 [18]). Of note, 17.4% of the patients had no swollen joints, no tender joints, and an ESR less than 10.

Prediction of attrition of infliximab therapy

Ranking of all clinical evaluations conducted at weeks 0, 6, 14, and 22 showed that the DAS28 scores at weeks 14 and 22 are the most important measurements in predicting later withdrawal from infliximab therapy due to inefficacy, with ROC AUCs of 0.731 (standard error [SE] 0.06) and 0.706 (SE 0.06), respectively. None of the other parameters evaluated for prediction of treatment withdrawal had an AUC higher than 0.65, and they were therefore omitted from further evaluation. We combined in one Cox regression model the continuous DAS28 scores (at week 14 or week 22) with the response scores (ACR 20–50–70 response and no-moderate-good DAS response score). None of the models showed a significant additional value of those response scores to the continuous DAS28 alone. This indicates that the DAS28 at week 14

Figure 3
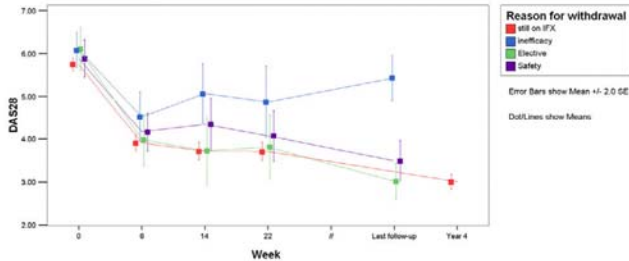
Evolution of the DAS28 (disease activity score based on the 28-joint count) scores and its components over time. DAS28 scores at the last clinical evaluation (evaluation at discontinuation of infliximab (IFX) treatment; median 119 weeks, interquartile range = 74 weeks) and the year 4 evaluation (median 205 weeks, interquartile range = 22 weeks). SE, standard error.

or 22 can predict long-term attrition better than DAS response or ACR response can.

The hazard ratio for withdrawal from infliximab therapy due to lack of efficacy was 1.9 (95% confidence interval [CI] 1.4–2.5) for high DAS scores at week 14 and was 1.7 (95% CI 1.3–2.1) for high DAS scores at week 22. These findings indicate that the likelihood of withdrawing from therapy increases by 70% to 90% when the DAS score increases by one unit.

To translate this hazard ratio in terms of sensitivity and specificity to predict withdrawal from therapy, we performed ROC curve analysis (Figure 4). At the 95% level of specificity, the

Figure 4

Receiver operating characteristic (ROC) analysis of DAS28 (disease activity score based on the 28-joint count) to predict withdrawal from treatment due to inefficacy.
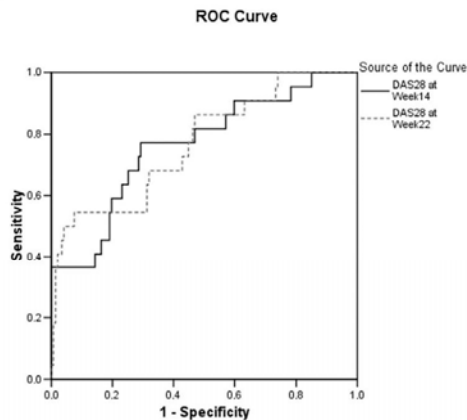
week 14 and week 22 DAS28 scores show 35% and 37% sensitivities, respectively, of predicting withdrawal of infliximab therapy due to inefficacy (Table 3). Taking into account the 13.6% a *priori* chance to withdraw therapy due to inefficacy, a DAS28 of at least 6 at week 14 or 22 increases the probability of withdrawing from therapy to more than 50% (Table 3, Figure 4).

Transiently increasing the infliximab dose at week 22 in a subgroup of those patients with persistently high disease activity did not lead to a better attrition rate (data not shown).

Discussion

In the present study, we prospectively evaluated the 4-year continuation rates and efficacy of infliximab in a large cohort of patients with long-standing refractory RA. Relatively few patients were lost to follow-up, which is important when estimating continuation rates and efficacy of therapy. After a 4-year study period, 61.6% of patients enrolled in the study were still receiving infliximab therapy. Over the same time period, 13.6% of patients discontinued infliximab therapy due to lack of efficacy, 16.9% due to safety issues, and 7.9% due to elective reasons. This is the first study that describes 4-year infliximab continuation rates in a large cohort of patients. Long-term continuation rates have also been reported for etanercept (25 mg) and adalimumab (40 mg) in open-label extensions of double-blind controlled trials [19,20]. In early RA and MTX-naïve patients who received etanercept, 63% of the 468 patients who entered the 3-year open-label extension were still receiving etanercept at the 5-year follow-up [19]. Similarly, 4 years after the initiation of the ARMADA (Anti-TNF Research Study Program of the Monoclonal Antibody D2E7 in Patients with RA) trial, 64% of the 271 enrolled patients were still receiving adalimumab therapy (mean duration of treatment = 3.4 years) [20].

Table 3

Sensitivity, PPV, and NPV of the DAS28 to predict infliximab discontinuation due to lack of efficacy

Specificity level	DAS28 at week 14				DAS28 at week 22			
	Cutoff	Sensitivity	PPV	NPV	Cutoff	Sensitivity	PPV	NPV
0.90	5.47	0.36	0.34	0.90	5.55	0.41	0.41	0.90
0.95	5.97	0.35	0.51	0.90	6.10	0.37	0.56	0.91
0.98	6.40	0.32	0.90	0.90	6.85	0.16	0.62	0.89

Sensitivities, PPVs, and NPVs are presented at different specificity levels of the DAS28 at week 14 or 22. DAS28, disease activity score based on the 28-joint count; NPV, negative predictive value; PPV, positive predictive value.

Results of some smaller studies showed similar or lower infliximab continuation rates after 3 years (Voulgari *et al.* [21], $n =$ 84, 59%), after 2 years (Geborek *et al.* [22], $n =$ 135 75%; Wendling *et al.* [23], $n =$ 41, 67%), and at 1 year (Flendrie *et al.* [24], $n =$ 120, 58%; Zink *et al.* [25], $n =$ 343, 65%; Chevillotte *et al.* [26], $n =$ 60, 64%). Differences between results may be explained by differences in study populations and different availability of treatment alternatives.

Another manner of accessing long-term data is through national registry databases, which are primarily maintained to evaluate effectiveness and safety issues [27,28]. Evaluation of the efficacy of long term therapy shows that, after 4 years of infliximab therapy, the majority of patients had a low level of disease activity and that approximately half of the patients met the criteria for minimal disease activity [18]. Moreover, we demonstrated that, after the initial rapid response to infliximab therapy between baseline and week 22, a further decrease in disease activity was observed over the remaining 3.5 years. This decrease in disease activity was observed in both the patients who continued with infliximab therapy and those who discontinued treatment later on due to safety or elective reasons. It is also important to mention that low levels of disease activity could be achieved and maintained with a standard infliximab dosage of 3 mg/kg and that dosage increases were transient in a majority of patients. The higher percentage of patients who need a dosage increase in some American studies might be explained by greater flexibility in the U.S. label or by a lower concomitant use of MTX in U.S. practice as compared with our study, in which 90% of the patients continue on infliximab + MTX combination therapy.

Also, transiently increasing the infliximab dose at week 22 in a subgroup of patients with a persistently high level of disease activity did not appear to affect the continuation rate.

We also assessed whether long-term response to infliximab therapy could be predicted early in the treatment protocol. Our findings suggest that persistently high levels of disease activity after an induction regimen of infliximab, as measured by the DAS28 score at week 14 or 22, was predictive of subsequent treatment discontinuation due to lack of efficacy. This observation corroborates the notion that a change in treatment strategy should be considered for patients with high levels of disease activity after 6 months of infliximab therapy. Switching to alternative therapies after 3 to 6 months if no therapeutic effect is observed is common in daily clinical practice and has been used in different studies to explore treatment options [29-31]. The optimal time point (that is, week 14 or 22) for determining whether a patient should continue therapy remains to be established and should take into account that predictive values may be highly influenced by the *a priori* chance to withdraw from treatment due to inefficacy, which was 13.6% in the present population. This *a priori* chance was modified to an *a posteriori* chance of 50% when the DAS28 at week 14 or 22 was higher than 6.

However, the data presented here clearly show that this decision is best made using the DAS28 score and not employing single measurements of swollen or tender joint count or response scores such as DAS28 response or ACR response score.

Conclusion

The results of this study highlight that infliximab therapy is safe and effective for long-term (that is, 4 years) treatment of refractory RA. After an initial rapid response to the therapy, patients receiving infliximab continue to experience less disease activity over time. Our findings also indicate that the decision to continue infliximab therapy is best made using the DAS28 score.

Competing interests

Authors' contributions

BVC and SVL performed the statistical analysis, constructed the datasets, and drafted the manuscript. RW, PD, FVdB, HM, LDC, AP, MM, LV, and FDK recruited and observed the patients with arthritis. BVC, SVL, BW, NV, AG, LB, RW, PD,

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CHAPTER 10

Comparison of different outcome measures for psoriatic arthritis patients treated with infliximab or placebo.

Vander Cruyssen B, De Keyser F, Kruithof E, Mielants H, Van den Bosch F.

LETTER TO THE EDITOR

Psoriatic arthritis (PsA) shares features with the spondyloarthropathy (SpA) concept and with rheumatoid arthritis (RA). Treatment of PsA should target the skin, the SpA-like features and the RA-like features of the disease. Effects of therapy can be measured by one index that covers different axes at once or by evaluating each axis separately [1]. In the present analysis, we aimed to evaluate different composite indices that have been validated in RA and compare with the Psoriatic Arthritis Response Criteria (PsARC) [2].

The study population consisted of eighteen PsA patients, previously enrolled in a randomized mono-center double-blind placebo-controlled study evaluating infliximab in SpA [3]. This study included 18 PsA patients of whom 9 received placebo. Patients were evaluated at baseline and weeks 1, 2, 6, 8 and 12 which included the evaluation of the single components included the PsARC, DAS28 [4] and DAS response [4]. A modified ACR (mACR) response was calculated by using the BASFI or the Dougados functional index in stead of the HAQ [5, 6, 7].

Although only 18 patients were included in the present study, this was enough to evaluate the value of the different outcome measures in psoriatic arthritis as reflected by the significant differences between groups after treatment. The results of the mACR response were identical when BASFI or Dougados functional index were used. PsARC response, mACR20 response and DAS28 response were similarly effective to discriminate the 2 treatment groups (Table 1). The mACR response and the DAS28 response can not only be used as a dichotomous (response yes or no) variable, but also as ordinal variables (0-20-50-70 response for the mACR response and no-moderate-good response for the DAS28). The evaluation of such ordinal categories by a special measure of association (such as the ordinal gamma statistic [8]), adds statistical power (Table1).

DAS28 as a continuous variable can be evaluated by measuring changes over time or by comparing the remaining disease activity at week 12 between the two treatment arms (Table 1). The effect size (ES) [10] and standardized response mean (SRM) [9] of DAS28 were respectively 1.9 and 1.7 in the infliximab treated group which is similar for PsA as for RA (personal observations). Profiles over time can be evaluated by the comparison of areas under the curve (Table1) or by linear mixed models analysis. Mixed models analysis can correct for small differences in baseline conditions between groups [10]. We therefore fitted a general linear mixed model with DAS28 as outcome variable resulting in a significant effect of the treatment over time, reflected by a significant treatment effect at week 2 (mean = -1.15 vs. baseline, p=0.0156), week 6 (mean = -2.12 vs baseline, p<0.0001) and week 12 (mean=-2.39 vs. baseline, p< 0.0001), resulting in a high significant global treatment effect (F-test: p<0.0001).

Not only DAS28, but also other variables, such as tender or swollen joint counts can be used as continuous outcome measures, evaluated at different time points. However, as shown in Figure 1, DAS28 can discriminate more efficiently the two treatment groups than each of its 4 components alone (less overlap of error bars) or the 66/68 joint counts. This also suggests that the reduction of the number of evaluable joints by the use of a 28 joint count is not a draw-back for the efficacy of the DAS28.

In conclusion, we evaluated different RA related outcome measures and statistical methods to measure effects of therapy in PsA patients. We showed that the different response scores are equally efficient. Also, the DAS28, as a measure of absolute disease activity, can be used as a powerful tool to evaluate effects of therapy in PsA patients.

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TABLES AND FIGURES

Table 1: Patients' baseline characteristics and comparison of the different outcome measures evaluating the effects of therapy.

	Placebo	Infliximab	Difference Between groups
Baseline characteristics			
Age (years) [$]	47(39-63)	48(30-65)	NS
male/female*	4/5	5/4	NS
Disease duration (years) [$]	5(3-26)	5(1-18)	NS
Patient global assessment VAS[$]	47(24-97)	66(42-84)	NS
Physician global assessment VAS[$]	65(51-77)	65(60-85)	NS
66 swollen joint count[$]	8 (1-17)	9(3-14)	NS
28 swollen joint count[$]	4 (1-7)	5 (1-9)	NS
28 tender joint count[$]	5 (1-10)	4 (1-8)	NS
ESR (mm/first hour) [$]	8 (4-43)	11 (1-26)	NS
CRP (mg/dl) [$]	0.8 (0.27-1.51)	0.96 (0-2.41)	NS
DAS28 §	3.78 (1.16)	4.32(1.21)	NS
HLA-B27 positive/negative *	3/6	2/7	NS
Effects of therapy			
mACR no/20/50/70 responders*	8/0/1/0	0/3/2/3	γ= 0.861 +/-0.142 p=0.002 [A]
mACR 20 responders (no/yes)*	8/1	1/8	OR= 28 (CI:2.1-379) p=0.015 [B]
No/moderate/good DAS28 response*	7/2/0	0/1/7	γ= 0.944 +/-0.067 p=0.001 [A]
Moderate DAS28 respons (no/yes)*	7/2	0/8	OR= 28 (CI:2.1-379) p=0.015 [B]
PsARC response : no-yes*	7/2	0/9	p=0.002 [B]
Remaining DAS28 score at week 12[$]	3.91(1.48)	2.06(0.84)	p=0.0065 [C]
Difference in DAS28 score between baseline and week 12[$]	+0.127(0.84) p=NS [D]	-2.26(1.33) p=0.0009 [D]	
Area under the curve	-0.95	-29.31	p=0.0056 [E]

Legend:
$ Values are the median (range), § Values are the mean (standard deviation), * Values are number of patients in each category
Statistical methods : A: Exact Gamma statistic, B: Fisher Exact, C: t-test, D: paired sample t-test, E: Mann-Whitney test,
Abbreviations: NS= not significant, VAS= visual analogue scale, ESR= erythrocyte sedimentation rate, CRP= c-reactive protein, CI= 95% confidence interval, γ= Gamma statistic [8], OR = odds ratio.

Figure 1: Plots of the DAS28 score and different single measures.
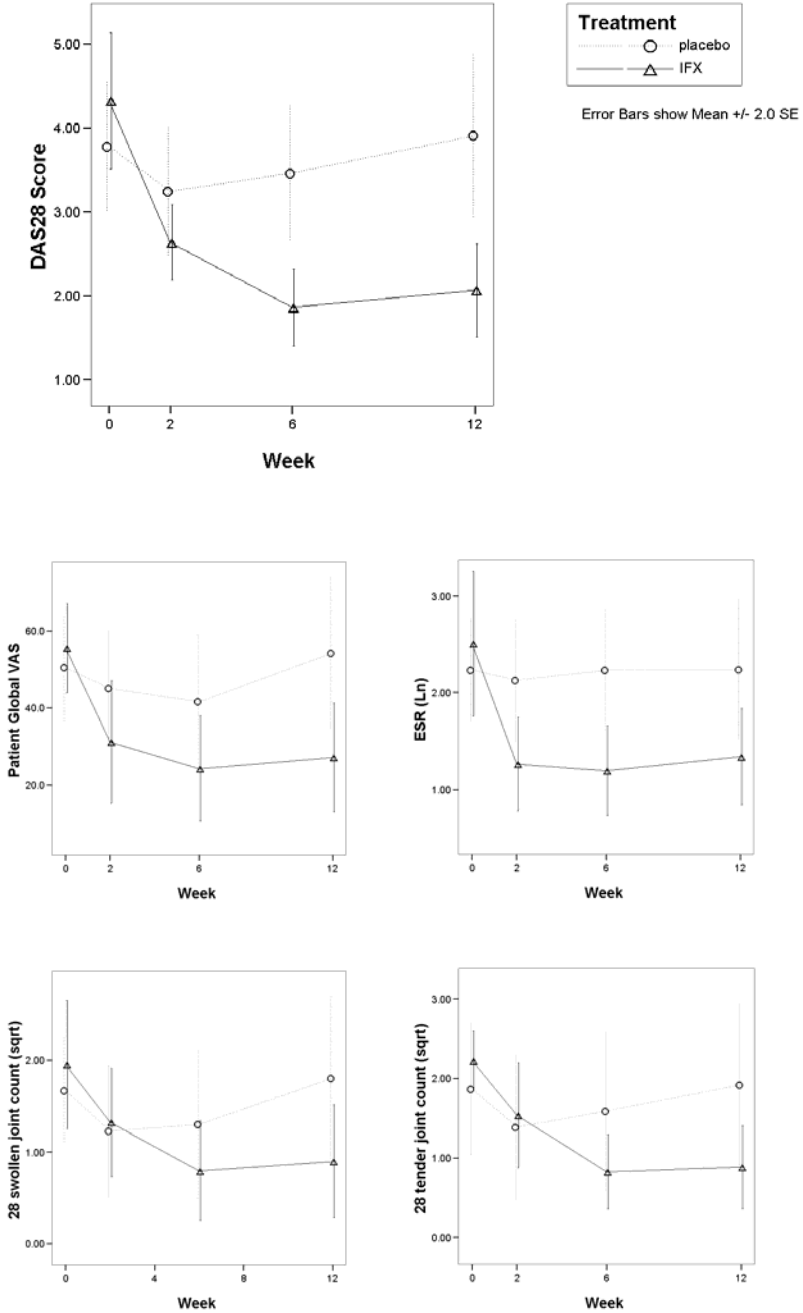
Legend:

Panel A: Evolution of the DAS28 over time. Panel B-E: Evolution of the components of the DAS28 over time.

Panel F-G: Evolution of 66 and 68 swollen en tender joint counts over time.

Those plots show that there is more overlap of the error bars of the individual components and the 66 and 68 swollen joint counts than the DAS28.

Abbreviations: Sqrt= squared root, ln= natural logarithm, VAS= visual analogue scale.

CHAPTER 11
Prediction of dose intensification for Rheumatoid Arthritis patients under
Infliximab treatment.

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* Equal contribution

Prediction of dose escalation for rheumatoid arthritis patients under infliximab treatment

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that leads to irreversible joint destruction. To prevent this, new biological therapies, such as infliximab, have been successfully developed. The present analysis is based on an expanded access program in which 511 RA patients with chronic refractory disease were treated with infliximab. They received a standard dose of 3 mg/kg on weeks 0, 2, 6, 14 and every 8 weeks thereafter. On week 22, the treating rheumatologist evaluated the situation of every patient and decided whether the current dose should be increased or not. This decision can be considered as a measure of insufficient response. In the present analysis, 3 machine-learning classification techniques—the self-organizing map (SOM), multilayered perceptron (MLP) and support vector machine (SVM)—are implemented to model the decision to give a dose increase. Their performance on increasingly multivariate real-life data will be studied and compared to classical statistics—linear discriminant analysis (LDA) and logistic regression (LR). Results show that the SOM is an excellent tool for data visualization but not for classification. All the remaining methods show good classification performance, if configured well. However, as the number of features increases, the performance decreases. The SVM suffers to a lesser degree from this curse of dimensionality. Expectation maximization (EM) comes out as a good method to cope with missing values in such real-life data.
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Keywords: Medical data analysis; Machine learning; Classification

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1. Introduction

Rheumatoid arthritis (RA) is one of the most common forms of chronic immune-mediated inflammatory arthritis. The disease, which affects about 0.5 1% of the population, finally leads to destruction of the joints, resulting in an important functional impairment and loss of quality of life.

Over the past years, a tremendous evolution in treatment strategies of RA made it possible to control the local joint inflammation, resulting in a decrease of pain, symptoms and joint destruction, finally leading to a better quality of life. The most important breakthrough in this treatment

strategy was the discovery of new biological drugs of which TNF-α blocking agents seem to be highly effective in targeting the joint inflammation and preventing joint destruction. Infliximab (Remicade®) is one such drug that provides the physicians a powerful tool in the treatment of different chronic inflammatory (joint) disorders, such as RA and spondyloarthropathies (Quinn et al., 2005; De Keyser et al., 2004).

The present study is based on an expanded access program in which 511 patients with refractory RA originating from 7 Belgian university hospitals were included. Infliximab was administered at 3 mg/kg by intravenous infusions at week 0, 2, 6 and every 8 weeks thereafter. Different studies suggest, however, that this standard regimen may not be sufficient in some subgroups of patients. Therefore, at week 22, it was decided that the treating rheumatologist could increase the dose based on his clinical judgment. This decision to give a dose increase can be considered as a subjective measure of insufficient response (Durez et al., 2005).

Although great efforts have been made to construct diagnostic criteria and criteria for the evaluation of response to treatment, these are not perfect, and the clinical judgement of the treating rheumatologist still adds to their performance. Since this clinical judgment is a subjective measure, the aim of the present analysis is to build a model to predict this decision based on 19 features that were measured over 4 points in time (76 features in total). These features consist of several clinical measurements and health scores reported by both the patient himself and the treating rheumatologist. To build this classification model, we will use machine-learning techniques as well as classical statistics. Their performance on this real-life data will be studied and compared to each other.

The remainder of this paper is organized as follows. Section 2 describes the composition of the data used in this study. In Section 3, we give an overview of the different classification methods used, followed by a description of the way we evaluated them in Section 4. Results will be presented in Section 5, comparing the efficiency of the different classifiers on the different data sets. These results are discussed in Section 6 and finally, the conclusions are summarized in Section 7.

2. The data

The following single variables were measured at weeks 0, 6 and every 8 weeks thereafter: (1) measurements of the number of affected joints: the 28 and 66 swollen and tender joint count (SJC, TJC); (2) objective serum measures of inflammation: erythrocyte sedimentation rate (ESR: mm/h) and C-reactive protein(CRP: mg/l) and (3) different patient and physician reported "subjective" scales: health assessment questionnaire (Fries et al., 1980) (0 3); physician's assessment of global disease activity visual analog scale (VAS: 0 100 mm); patient's assessment of global disease

activity VAS (0 100 mm); patient's assessment of pain VAS (0 100 mm); patient's assessment of fatigue VAS (0 100 mm) and the patient's evaluation of general functioning, measured by 8 items of the SF-36 questionnaire (Ware and Sherbourne, 1992).

In this study, we will take into account the data on weeks 0, 6, 14 and 22 to model the physician's decision at week 22 to give a dose increase from week 30 on. Nineteen features, measured at 4 time points result in 76 features.[2] Out of these data, 3 different data sets will be constructed, each with a different number of features:

- *Data set 1:* The input consists of all selected data, 76 features in total, which unfortunately contain a lot of missing values, which is a common phenomenon in real-life data sets.
- *Data set 2:* The input consists of all 19 features at week 22.
- *Data set 3:* The input consists of only 4 selected features at week 22 (28 tender and swollen joint counts, ESR and patient's global disease activity VAS), which give a reasonable indication of disease activity since those variables are considered as a core set of variables, employed in the so-called DAS disease activity score (van der Heijde et al., 1990).

A comparison of the classification results obtained by the different methods between these 3 data sets will give us an idea about the possibilities of the different methods to handle data sets with an increasing dimensionality, and how they can handle multicolinearity.

Since not only multiple dimensions and multicolinearity, but also missing values are common problems encountered in such clinical data sets, we considered 3 different methods to cope with missing values:

- *All cases (AC):* Some methods can intrinsically handle missing data, so that the data can be fed as it is. It could be argued that this is the most honest way to handle missing data.
- *Complete cases (CC):* Another way to handle missing data is simply to ignore them. In this case, all patients who have one or more features missing are left out. This method is only used on data sets 2 and 3, since data set 1 does not contain any complete case. This is the easiest way to handle missing data, but not the preferred way since the population is reduced and hence the results could be biased.
- *Expectation maximization (EM):* Missing data are imputed using the EM method. In general, data imputation should be avoided. However, since some of

[2]Since some of these features represent the same feature on different points in time and others represent the same physical entity from a different perspective—such as physician's and patient's assessment of global disease activity are both measures for disease activity—a few of these 76 features are correlated, which was reflected by their correlation coefficients.

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the discussed methods simply cannot handle missing data, EM is considered as a valid alternative (Dempster et al., 1977).

The combination of different features and different ways to handle missing values, leads to a total of 8 data sets: 76 features (fts) AC, 76 fts EM, 19 fts AC, 19 fts CC, 19 fts EM, 4 fts AC, 4 fts CC and 4 fts EM. Each of these sets is randomly split up into a training set, containing 2/3 of the data, and a testing set with the remaining 1/3 of the data. The training set will be used to construct classification models. The testing set acts as previously unseen data to evaluate and compare these models.

3. Classification methods

3.1. Self-organizing maps

The self-organizing neural network architecture as it is used in the underlying research was introduced by Kohonen (1997) and is organized as a 2-dimensional map with neurons (nodes), where each neuron is connected to each component of an N-dimensional input vector by a weighted connection.

The training algorithm of the Kohonen network employs both competitive and unsupervised methods. By iteratively presenting a set of training samples (vectors with N components) at the input, the weights at the connections toward the input vector of the node with the minimum distance with regard to the current input are adapted (Fig. 1). The net result from the training phase is that inside the 2-dimensional plane, clusters of similar nodes are spontaneously being formed (Wyns et al., 2004). The self-organizing map (SOM) algorithm is primarily designed for data clustering and visualization, not for classification.

Furthermore, batch training is used, where the effect of the training data on their winning nodes and corresponding
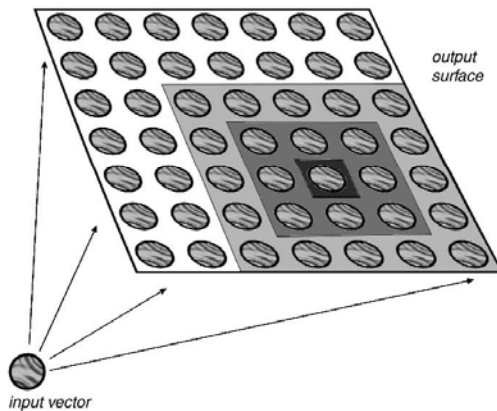

Fig. 1. The self-organizing map network structure.

neighboring nodes is calculated for all the training data at once. This avoids different results when using different orderings of the training data as is the case with sequential training. The number of training steps is determined by the starting neighborhood radius, which is chosen equal to the smallest lattice dimension. The algorithm is extended to cope with missing data as previously described (Samad and Harp, 1992). The following enhancements were made to speed up the training algorithm and to improve classification performance.

3.1.1. Number of nodes and lattice dimensions

Since the SOM algorithm is quite robust concerning over-fitting adding more nodes just results in more dead nodes after training the number of nodes can safely be chosen equal to the number of training vectors, or even more. The SOM is linearly initialized, i.e. in the plane defined by the eigenvectors corresponding to the largest eigenvalues. As a heuristic, the number of nodes in the SOM is chosen as n/a, where n is the number of training vectors and a is the proportion of the data variance explained by the direction of the eigenvectors corresponding to the 2 largest eigenvalues. The proportion of the SOM dimensions is chosen as described in (Kohonen, 1997): equal to that of the 2 largest eigenvalues.

3.1.2. Data quality

The SOM is quite vulnerable to class imbalance: 1 class being represented by more training data than the other. According to Batista et al. (2004), a good way to clean and balance data is to combine the removal of Tomek links (Tomek, 1976) and the over-sampling of the data using the Synthetic Minority Over-sampling Technique (SMOTE) (Chawla et al., 2002). This was also done in this study.

3.1.3. SOM data visualization

After training, the training data are split up according to the class labels. For each class a hit map is constructed; for each training vector the closest lattice node is searched and the hit counter corresponding to this node is increased. The values of these hit counters are then smoothed out over the neighboring lattice nodes by applying a Gaussian neighborhood function on them. In this way, the non-referenced nodes also are labeled with a "hit count" for each class and the influence of each hit is extended to the neighboring nodes. In the next step we create a probability map: for each node the hits for all the classes are summed up. The probability for each class in that node is then given by $hits_{class}/total\ hits$. Finally, a label map can be constructed by giving each node a label corresponding to the class with the highest probability in that node.

3.1.4. SOM classification

Using the described method for data visualization using the SOM, new previously unseen data vectors can easily be classified. This is done by looking up the closest lattice

node and assigning the corresponding label from the label map to the new data vector.

3.2. Multilayered perceptrons

Multilayer perceptrons (MLPs) are models for computational systems, which imitate the behavior of biological neurons the human brain by using a large number of structural interconnected artificial neurons. The MLP is trained using the back-propagation algorithm as described in Haykin (1994). Training was done during 1000 cycles in all experiments and the learning rate was initialized at 0.01. The mean squared network error was monitored during each cycle and when it was rising, the learning rate was decreased to prevent the algorithm from moving away from the optimum. Note that in each training cycle the training vectors are picked randomly out of the training set. This way, 2 different runs do not necessarily result in the same final neural network configuration.

To design the network structure, the training data were first visualized using the SOM. In this way, we obtain a 2-dimensional representation of the high-dimensional structure of the data. According to this observed structure, the
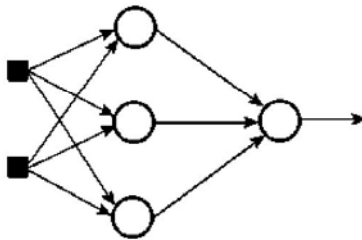

Fig. 2. A multilayered perceptron with (from left to right) 1 input layer, 1 hidden layer and 1 output layer.

MLP is designed; 2 hidden layers are needed when several clusters are visible and the number of nodes in the hidden layers is chosen according to the complexity of these clusters (Fig. 2).

MLPs are fault tolerant: they still produce a reasonable output when 1 or more nodes in the network malfunction. This principle of graceful degradation is exploited to adapt the algorithm toward coping with missing data. Missing inputs are expressed as zeros and therefore the network will behave as if the corresponding input node is faulty. By design, the network will still produce a reasonable output.

3.3. Support vector machines

First discussed by Cortes and Vapnik (1995), a support vector machine (SVM) is a hyperplane that separates data from 2 classes with maximal margin, meaning that the distance from the closest examples to the hyperplane is maximized (Fig. 3). The use of the maximum-margin hyperplane is motivated by the statistical learning theory, which provides probabilistic error bounds. The original SVM was a linear classifier. However, using the kernel trick with a non-linear kernel function (Aizerman et al., 1964) causes the algorithm to operate in a different space, allowing for non-linear decision functions.

The analysis is restricted to the linear kernel and the radial basis function (RBF) kernel. With the linear kernel, there is only 1 parameter that needs to be tuned: the parameter C. This parameter defines the cost of a single classification error and thus controls to a certain extent the generalization potential of the classifier. A higher C-value results in fewer classification errors on the training set, but the margin will be smaller. The linear kernel is obviously only suited for data that are approximately linearly separable. The RBF kernel on the other hand is a commonly used non-linear kernel and thus allows the
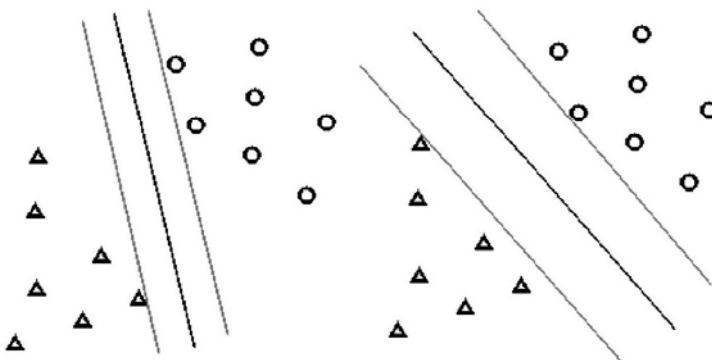

Fig. 3. A linear separation between 2 classes versus a maximal margin separation.

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classification of non-linear data. With this kernel, there is an extra parameter that needs to be tuned: the parameter γ. This parameter defines the width of the RBF kernel function. When γ is high, the classification model will be constructed with a high influence of individual data points; the model will have a high degree of non-linearity. On the other hand, when γ calculates to zero, the kernel function will behave like a linear kernel function, as stated in Keerthi and Lin (2003).

To tune the parameters only C for the linear kernel and C and γ for the RBF kernel a grid search using 5-fold cross-validation (Mosier, 1951) for the RBF kernel and leave-one-out cross-validation for the linear kernel was carried out . This way, for a number of (combinations of) parameter values, a performance estimate of the classifier is obtained. The optimal performance is pointed out and the corresponding parameter values are chosen. When more than 1 optimal combination was found, we chose the combination with the lowest value for C, for the sake of generalization.

To select the kernel function linear or RBF we observe a SOM data visualization. If this visualization reveals a nearly linear structure, the linear kernel is used, otherwise the RBF kernel is selected. In theory, the RBF kernel behaves like a linear one for $\gamma \to 0$. However, in practice, when the data behaves linear alike, the linear kernel outperforms the RBF one.

3.4. Linear discriminant analysis

Linear discriminant analysis (LDA) is also known as Fisher's linear discriminant, named after its inventor (Fisher, 1936). It is commonly used as a method to determine which features discriminate best between 2 or more occurring groups. In the 2-group case as in this study the groups are assigned a number, e.g. 0 for continued dose and 1 for dose increase. LDA then searches for a linear combination of the inputs:

$$d = a_0 + a_1 x_1 + a_2 x_2 + \cdots + a_n x_n,$$

where n is the number of inputs. The coefficients a_i are optimally chosen similar to multiple regression: the squared difference between the d's calculated by filling in the feature values of each training vector, and their real group number is minimized. Classification of previously unseen data is done by calculating the corresponding d value and defining a threshold. A data vector with a d above this threshold is classified as one group and one with a value below the threshold is classified as the other group. For this study, we used the LDA implemented in the SPSS statistical package.

3.5. Logistic regression

In the case of logistic regression (LR) (Hosmer and Lemeshow, 1989), the chance of a patient to get a dose increase is modeled as

$$p = \frac{e^{a_0 + a_1 x_1 + \cdots + a_n x_n}}{1 + e^{a_0 + a_1 x_1 + \cdots + a_n x_n}}, \tag{1}$$

where n is the number of input features. By applying the logit function this formula transforms into

$$\text{logit}(p) = \ln\left(\frac{p}{1-p}\right) = a_0 + a_1 x_1 + \cdots + a_n x_n,$$

which is a linear function in the x_i and allows the coefficients a_i to be estimated through a maximum likelihood method. The likelihood of the training set to occur can be written as a function of the a_i using (1). Then the a_i that maximizes this likelihood is found through an iterative algorithm.

Classification of previously unseen data is done by calculating the corresponding p and defining a threshold. A data vector with a p above this threshold is classified as one group and one with a value below the threshold is classified as the other group.

4. Evaluation method

Traditionally, a classifier aims at an output with an optimal global accuracy. However, in most real-life applications, and in particular in this medical application, the impact of a wrong classification depends strongly on the real class of the object. Therefore, a score indicating the probability of a patient belonging to a certain class is needed rather than a single "hard" classification result. For the SOM, such a score is obtained by looking at the winning node's probabilities rather than the assigned labels. In the case of MLPs, we look at the "raw" output of the output neuron, i.e. we omit the sign operator. A score can be obtained from SVMs by looking at the distance to the separating hyperplane. The sign of this distance determines the class of the object. With LDA a discriminant score is obtained and with LR we obtain a probability for dose increase for each patient. We can thus obtain a suitable score with every considered method.

On the obtained score, a threshold can be defined: every patient with a score above this threshold is labeled as "needs a dose increase" further on called positive and every other as "does not need a dose increase" further on called negative. By default, this threshold equals 0.5 for the SOM, 0 for the MLP and the SVM, for LDA this depends on the class coding and for LR it equals 0.5. By varying this value, a collection of classifiers is constructed out of a single classification model output. For each of these classifiers, the proportion of positive patients that is labeled as such out of all positive patients this is the sensitivity of the classifier as well as the proportion of negative patients that is labeled as negative out of all negative patients this is the specificity of the classifier can be calculated. A receiver operator characteristic (ROC) curve (Swets, 1988) plots the evolution of
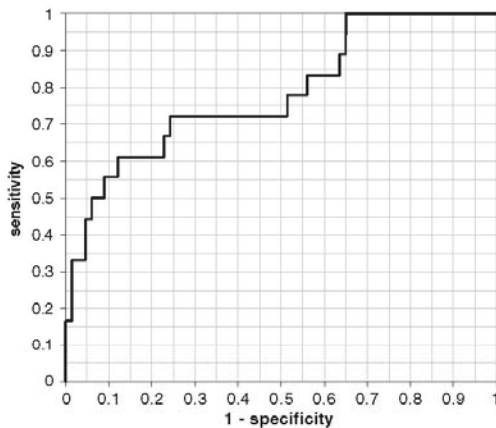
Fig. 4. A receiver operator characteristic (ROC) curve with area under the curve (AUC) = 0.784, sensitivity at specificity 0.95 = 0.44 and specificity at sensitivity 0.95 = 0.35.

Table 1
Results for the self-organizing map (SOM) and multilayered perceptron (MLP) classifiers on the all cases data sets for a decreasing number of features

	76 fts		19 fts		4 fts	
	SOM	MLP	SOM	MLP	SOM	MLP
AUC	0.691	0.758	0.656	0.749	0.745	0.772
Sens at Spec 0.95	0.235	0.324	0.176	0.324	0.206	0.265
Spec at Sens 0.95	0.132	0.215	0.091	0.240	0.084	0.402

Table 2
Results on the complete cases data set using all 19 features on week 22

	SOM	MLP	SVM	LDA	LR
AUC	0.759	0.793	0.784	0.772	0.742
Sens at Spec 0.95	0.278	0.500	0.444	0.500	0.444
Spec at Sens 0.95	0.045	0.205	0.348	0.121	0.212

Table 3
Results on the complete cases data set using the 4 selected features on week 22

	SOM	MLP	SVM	LDA	LR
AUC	0.776	0.854	0.863	0.876	0.878
Sens at Spec 0.95	0.423	0.462	0.308	0.385	0.385
Spec at Sens 0.95	0.205	0.548	0.507	0.575	0.575

sensitivity and specificity for a threshold varying from the maximum score value corresponding to a specificity of 1 and a sensitivity of 0 to the minimum score value corresponding to a specificity of 0 and a sensitivity of 1. The x-axis shows 1 specificity and the y-axis shows sensitivity. In this way, we obtain a monotonically raising curve (Fig. 4).

The area under this curve (AUC) is a general measure of the quality of a classification method. Optimally, the AUC equals 1. This occurs when the classification method succeeds in separating the 2 classes perfectly. Thus, the AUC is a more sophisticated quality measure than the obtained global accuracy.

The evolution at the beginning and the end of the ROC curve is very important. These regions correspond to classifiers that identify a large part of the positive group in the case of a high sensitivity or a large part of the negative group in the case of a high specificity. In medical applications, it is often important to select 95% of the positive group while selecting the least possible patients from the negative group and vice versa. Therefore, we shall consider the specificity at a sensitivity level of 95% and the sensitivity at a specificity level of 95% as a performance measure too.

5. Results

Since MLP training does not result in the same neural network configuration after each run, we ran the algorithm 100 times and reported the median over these runs for each evaluation score. The other classifiers produce the same result on each run, i.e. they are independent of the order of the training vectors in the training data set.

5.1. Results for the all cases data sets

Since these data sets contain missing values and the SVM, LDA and LR algorithms cannot cope with these, only the modified SOM and MLP algorithms are used on these data sets. These results are shown in Table 1.

5.2. Results for the complete cases data sets

Data set 1, which contains 76 features, does not have any complete case. Therefore, only the 2 other data sets could be used in this analysis. These results are shown in Tables 2 and 3.

5.3. Results for the expectation maximization data sets

Since missing data are now filled in using an EM method, all classification methods can be applied to all data sets. The results are shown in Tables 4 6.

6. Discussion

6.1. The SOM and classification

As already stated by Kohonen (1997), the SOM is primarily designed for data visualization and clustering.

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Table 4
Results on the expectation maximization data set using all data on the 4 selected weeks (76 features in total)

	SOM	MLP	SVM	LDA	LR
AUC	0.631	0.738	0.788	0.746	0.695
Sens at Spec 0.95	0.147	0.176	0.324	0.176	0.265
Spec at Sens 0.95	0.008	0.207	0.074	0.107	0.116

Table 5
Results on the expectation maximization data set using all 19 features on week 22

	SOM	MLP	SVM	LDA	LR
AUC	0.712	0.790	0.758	0.802	0.788
Sens at Spec 0.95	0.294	0.368	0.324	0.324	0.324
Spec at Sens 0.95	0.273	0.211	0.140	0.289	0.289

Table 6
Results on the expectation maximization data set using the 4 selected features on week 22

	SOM	MLP	SVM	LDA	LR
AUC	0.705	0.813	0.804	0.825	0.828
Sens at Spec 0.95	0.324	0.412	0.412	0.353	0.353
Spec at Sens 0.95	0.075	0.411	0.402	0.542	0.449

Although it can easily be modified towards classification, the performance will not be optimal through design. After all, the SOM algorithm is designed to preserve data topology as much as possible in the transformation from the original high-dimensional data space towards the 2-dimensional lattice space. With classification, however, the topology of the data is of lesser concern. It would be better to have a transformation that stresses out the distinction between the different classes instead. A second issue in this matter is that of generalization. The SOM algorithm has no concept at all of generalization, since it is not a classification algorithm. Therefore, this problem is moved from the original high dimensional data space towards the 2-dimensional lattice space. Coping with the generalization step in a space of lower dimensionality implies that it will be at best equally well compared to generalization in the original data space with higher dimensionality. Overall, generalization in a space with lower dimensionality will be worse, hence worse classification results expected.

This observation is clearly confirmed by the results presented in Tables 1 6. All this leads to the conclusion that the SOM is not suitable for classification problems. Nevertheless, the algorithm stays very useful in a first data analysis step to help the process of designing a classifier such as the MLP or the SVM.

6.2. Comparing classifier performance

Now that we have excluded the SOM from the classifiers in the previous paragraph, we can focus on the remaining classification models.

LDA is actually nothing more than a special formulation of a single-node perceptron with a linear activation function. Since such a perceptron is only 1 possible design of a MLP MLPs can have more layers and nodes, increasing their non-linear character the performance of LDA will be at best equal to that of an MLP. LR is a special formulation of a single-node perceptron with a sigmoid activation function. We can thus conclude that LR performance will also be at best equal to that of an MLP.

One of the main differences between MLPs and SVMs is that the non-linear transformation after which linear class separation is performed is quasi-fixed with SVMs, where it is adjusted toward optimal performance during training with MLPs. The only flexible part in the SVM case, are the kernel parameters, such as γ in the case of a RBF kernel. This is an argument in favor of the MLP.

Another difference between MLPs and SVMs lies within the final process of linear class separation. MLPs just find 1, amongst many other possible separating hyperplanes, while SVMs find the hyperplane that has the largest margin between the 2 classes. Therefore, the generalization capacity of a SVM is expected at least equal to that of an MLP. This is an argument in favor of the SVM.

On the basis of these theoretical considerations on MLPs vs. SVMs, it is hard to come to a conclusion concerning their classification performance. However, since MLPs do not produce the same output after each run and since they need a lot more careful design and configuration prior to the actual training, MLPs can be considered less stable and more susceptible to configuration flaws. Therefore, we expect the performance of SVMs to be at least equally good to that of MLPs. In exceptional cases, however, it is possible that MLPs outperform SVMs, e.g. when a suitable kernel function is not found.

These considerations lead to the following order in which we expect the classification performance of the different methods:

$$\begin{matrix} LDA \\ LR \end{matrix} \leqslant MLP \leqslant SVM. \tag{2}$$

This performance expectance is, however, not clearly seen in the results in Section 5. To achieve a more global view on the classifiers performance, we averaged the results out per performance measure per classification method. These average results are shown in Fig. 5. In this figure, it can be seen that there is no noticeable difference in performance between the considered methods.

The many exceptions to formula (2) in the individual results presented in Section 5 can be explained by noting that the used data are just 1 sample out of the population. There is always a chance that on this particular data set, a
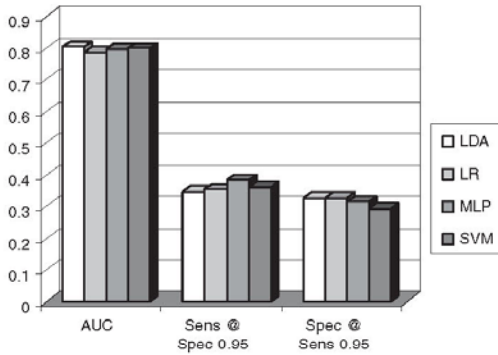
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Fig. 5. The average performance per classification method per performance measure.
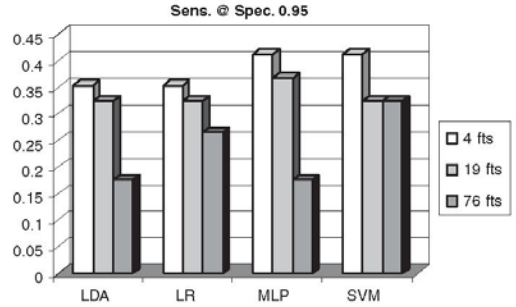

Fig. 6. The change in performance when the number of considered features increases.

Fig. 7. Performance of different methods for coping with missing data.

(say) LDA performs better than a (say) SVM although from a theoretical point of view this is impossible. Another important remark in this context is that a slight difference in design of, e.g. a MLP can have a large impact on the classification performance. Such configuration issues may also cause deviations to formula (2).

6.3. Curse of dimensionality

If the same data are considered, an increasing amount of dimensions means the data become distributed more sparsely into the data space. This implies that it becomes harder for a classifier to find an appropriate decision surface, hence worsening its performance. This decrease in performance when the dimensionality increases is called the curse of dimensionality. Since the same data are available with 76 features, 19 features and 4 features via the EM imputation method, we can look at the impact of an increasing number of features on the different classification methods used in this study. An overview of the performance measure "sensitivity at 0.95 specificity" is given in Fig. 6. An analog behavior is observed in the other 2 performance measures. It is clear that the SVM is less susceptible to this curse of dimensionality than the other methods used in this study.

6.4. Missing data

With the AC data sets, missing values are handled by the SOM and MLP as described in Sections 3.1 and 3.2. Since the real values are not known, it can be argued that this method of coping with missing values is the most honest. However, since the training algorithm of the SOM and MLP must proceed when a missing value is encountered, something has to be done. In the case of the SOM, the corresponding weight is simply left as it is. This corresponds to filling in the most expected value considering the network status. For MLPs a zero is inserted. Since all data
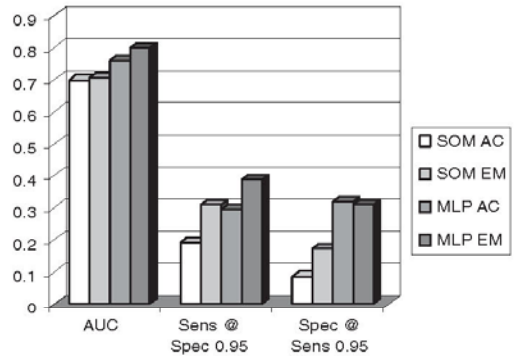
are standardized into the $[-1,1]$ interval during preprocessing, this method corresponds to filling in the mean value of the feature. From this point of view, the method used for the SOM algorithm is expected to perform at least equally well than the MLP one.

Although the SOM method fills in a most expected value, this estimate will be far from optimal in the beginning of the training, since the SOM is not yet fully trained. Therefore, we expect this method to perform at best equally well as the EM method.

Fig. 7 compares the methods used in the SOM algorithm and the MLP algorithm with the performance on EM imputed data. For the construction of this graph, the performance on the EM and AC data was averaged out over data sets 2 and 3. Data set 1 with all 76 features was left out since the MLP is highly susceptible to the curse of dimensionality as shown in the previous section.

As can be seen in Fig. 7, the previously mentioned considerations hold. The EM method clearly has a positive impact on the performance compared to the handling of missing data by the classification methods themselves. This impact is larger for MLPs. The SOM algorithm does not

benefit much from the EM method, indicating that it can manage missing data by itself very well.

6.5. Implications for medical decision making

In order to decide which patients can earn the maximum profit of new (expensive) therapies that are provided with the new biologicals, it becomes more and more important to increase the performance of diagnostic tools and to obtain adequate outcome measures. Increasing the performance of diagnostic tools can be done by the exploration of new diagnostic markers, such as the discovery of anti-citrullinated peptide/protein antibodies, which facilitates the early diagnosis of RA (Peene et al., 2004). However, it becomes more and more clear that combining markers in prediction models may provide another important tool to facilitate diagnoses and predict prognoses (Wyns et al., 2004; Visser et al., 2002). This question is even more prominent since new techniques, such as line immunoassays (Meheus et al., 1999), laser bead immuno-assays (Fulton et al., 1997), and microarrays (Southern, 2001) are available that report 10, 100 or even 10,000 markers at once. Similarly, combining different measures into adequate composite indices to measure disease activity and response to therapy is important as well (Fransen and van Riel, 2004).

The present analysis directly compares the different classification methods by the use of a unique real-life data set and thus reveals some pros and contras of different classification methods used for the construction of a composite indices or prediction models. An important message is that simple methods such as logistic regression and discriminant analysis perform very well when the number of variables is limited, but that their performance impair when more dimensioned data sets are used, and thus require a feature selection method in cases where many variables are available. In cases where no feature selection can be performed, more complex methods that suffer to a lesser degree from the curse of dimensionality such as SVM tend to be more relevant.

7. Conclusions

Summarizing, the SOM is an excellent tool for data visualization and clustering. Although it can easily be extended to a classifier, its performance is rather mediocre. LDA and LR are by far the most easy methods in usage: no configuration is required. But they only produce reasonable results when the data are quasi-linear. MLPs require a lot of configuration: the network structure must be designed and several parameters need to be set. This makes them very difficult to use, but nevertheless, when it is done properly, they produced very satisfying results compared to the other methods, which require less fine-tuning. SVMs do not have these many configuration parameters. In fact, they have only 1 or 2, once a proper kernel function is chosen or designed. Moreover, the SVM is less susceptible to the curse of dimensionality than the other considered methods. Concerning missing data: in this real-life data set, EM is an acceptable method to cope with those.

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DISCUSSION

1.
CARD15 and ASCA in SpA and Crohn: explorative association studies

2.
ACPA and RF as diagnostic markers for RA: validation of its diagnostic value and construction of (diagnostic) prediction models

3.
Evaluation of different measures and composite indices as a measure of (insufficient) response to treatment in RA (and PsA)

4.
Optimalization of the statistical methods to construct prediction models

1 CARD15 and ASCA in SpA and Crohn: explorative association studies

The first requirement of a new biomarker is that this marker should be associated with the disease or with a specific clinical subtype. Carriage of CARD15 polymorphisms has been associated with Crohn's disease (CD). The CARD15 gene encodes for the intracellular NOD receptor and a change in function of this receptor might lead to disturbed early inflammatory monocyte response to microbial agents finally resulting in gut inflammation [Peeters 2006]. As CD and gut inflammation are related with SpA, we investigated whether the carriage of CARD15 polymorphisms is associated with specific SpA related symptoms such as arthritis, inflammatory low back pain or sacroiliitis (Chapter 1). We found an association between the carriage of CARD15 mutations and the occurrence of sacroiliitis. This association was independent of different possible confounding factors including HLA-B27.

We investigated the opposite question in Chapter 2 by investigating SpA patients who had undergone a colonoscopy and found that SpA patients with chronic gut inflammation are more frequently positive for the CD associated CARD15 polymorphism.

In Chapter 3, we report that CD patients who carry CARD15 polymorphisms are more frequently positive for ASCA and have higher titres than patients carrying wild types. We observed also a gene dose effect: homozygotes or compound heterozygotes were more frequently ASCA positive than single heterozygotes, which are on their turn more frequently ASCA positive than wild types.

In the worldwide research for new genes for complex diseases such as SpA and CD disease, genetic association studies, as performed in these first 3 Chapters, are a powerful tool to explore the relationship between genes and the occurrence and clinical presentation of diseases. However, such associations should be interpreted with caution. It has been demonstrated that many reported association studies cannot be replicated or corroborated. This non-reproducibility may have different causes that are reviewed in Table 2.1. Some of these problems can be illustrated by the non-reproducibility of the association between CARD15 and ASCA reported in different analyses of a British cohort of CD patients [Arnott 2004, Walker 2004, Smith 2004]. In contrast to other, more recent studies that confirm the association between carriage of CARD15 mutations and ASCA [Amnese 2005, Riis 2005], this association could not be found in this British cohort of which several reports are published [Arnott 2004, Walker 2004, Smith 2004]. The non-reproducibility of the association between CARD15 and ASCA in this British cohort might be explained by a combination of heterogeneity and lack of power. First, the prevalence of carriage of CARD15 mutations in the CD patients enrolled

in that British cohort is only 24%, which is significantly lower than the 45.5% reported in our Belgian cohort. The low prevalence of carriage of CARD15 mutations in this cohort may lead on its turn to a decrease of power to detect a difference of ASCA titres between CARD15 carriers and wild type patients and resulting in the false conclusion that there is no association between carriage of CARD15 mutations and ASCA.

The reasons why the association between carriage of CARD15 polymorphisms and sacroiliitis in Crohn's disease patients could not be replicated in a Belgian multicentre study are more difficult to unravel [Peeters 2006]. We first observed significantly different frequencies of sacroiliitis between centers. Secondly, differences in disease duration might explain that some patients, prone to develop sacroiliitis had not yet developed sacroiliitis. Also differences in association between CARD15 and ileal disease between centers could be observed. Whether several of such confounding factors have caused this non-reproducibility or whether this non-reproducibility was caused due to random error remains uncertain.

The clinical relevance of most reported genetic associations for diagnosis and prognosis is generally low. Also in our studies, the sensitivities and specificities of the presence of CARD15 genes for the prediction of sacroiliitis or chronic gut lesions were low. Many genes discovered in complex multigenetic disorders are associated with the disease with ORs between 1.1 and 1.5. Although this is too low for clinical decision-making, such associations may be interesting from a pathological point of view [Ioannidis 2003]. Previous studies could not detect a significant association between carriage of CARD15 mutations and the susceptibility for SpA [Micelli-Richard 2002, D'Amato 2002, Ferreiros-Vidal 2003, van der Paardt 2003]. However, none of them was sufficiently powered to detect an OR of 1.5.

Also in Crohn's disease, the clinical relevance of CARD15 determination should be further established. Combinations of different serological tests have been explored to facilitate the diagnosis of Crohn's disease. In such models, CARD15 determination may have only limited additional value to serological testing [Vermeire 2004] which can be, at least partially, explained by its association with ASCA, which is more easy and cheaper to determine. Although for the moment not useful for clinical decision making, the reported association studies may be important for further understanding SpA and Crohn's disease and fine-tunning disease definitions and disease categories [Cardon 2001].

Table 2.1: Possible reasons for the non-reproducibility of genetic association studies.

1) <u>Statistical reasons</u>
    a. Lack of power (false negativity):
          Large sample sizes are needed to detect a statistical significant association. It has been estimated that most genetic associations between a polymorphic locus and disease yield an odds ratio between 1.1 and 1.5, requesting samples sizes of more than 1000 patients.
    b. Multiple comparison (false positivity – type I error):
          The more hypotheses that are tested, the more chance there is on false positive findings
    c. Interaction
          A disease may be caused by the interaction between two genes or by interaction between genes and the environment. Thus, when interaction terms are not investigated, the existence of a true association in a subgroup of patients can be overlooked.
    d. Stratification
          Population stratification occurs when genetic variants are studied in a mixture of genetically distinct populations. If the disease is more common in a particular ethnic group, any polymorphism that is associated with that ethnic group will appear to be associated with the disease.
    e. Random error
          When the random error is controlled at 5% ($p<0.05$), there is still a possibility of 5% that the study can not be replicated.
2) <u>Publication bias</u>
          It may happen that subsequent studies cannot replicate both initial positive or negative results. However, positive findings are more likely to be published and attract more subsequent studies.
3) <u>Heterogeneity</u>
    a. Locus and allelic heterogeneity
          This is a form of genetic expression in which distinct mutant alleles at the same or at different loci lead to the same disease phenotype.
    b. Definition of the phenotype
          Differences in the definition of the phenotype

may attribute patients to different phenotypes, resulting in differences in association.

  c. Definition of the genotype

    Differences in analysis on allele level or on haplotype level may cause differences in association.

4) <u>Linkage disequilibrium</u>

    This is the tendency that genes and other genetic markers located close to each other on the same chromosome inherit together.

Refs: Salanti 2005, Ioannidis 2001, Cardon 2001, Cardon 2003, Colhoun 2003, Gambaro 2000, Thomas 2004, Cappelleri 1996.

2 ACPA and RF as diagnostic markers for RA: validation of their diagnostic value and construction of a (diagnostic) prediction model.

After a marker proved to be associated with a disease, it is important to explore its diagnostic properties (including sensitivity and specificity) in diverse subpopulations. Different studies in RA patients and different control populations previously highlighted the excellent diagnostic properties of ACPA testing [Chapter 4, Schellekens 2000, Pinheiro 2003, Union 2002, Noguiera 2002]. In the next steps, it may be important to translate the results of sensitivities and specificities to the single patient in daily clinical practice. We first addressed the question whether ACPA may occur in PsA patients. We and others demonstrated that ACPA positivity can be seen in 5.6% to 15.6% of PsA patients [Chapter 5, Alenius 2005, Bogliolo 2005, Korendowych 2005]. Selected false positivity of ACPA testing has also been reported in patients with systemic lupus erythematosus (SLE) [Hoffman 2005, Mediwake 2001] and primary Sjögren's syndrome [Gottenberg 2005, Tobon 2005]. Also, PsA and SLE patients with a positive ACPA test have a higher risk to develop more erosive disease [Korendowych 2005, Boglioglio 2005, Inanc 2006].

During the process of the development of a new marker, it may occur that different methods are developed to test the same or a similar marker. We evaluated the diagnostic properties and the agreement between the different ACPA tests in Chapter 7. Although anti-CCP and AhFibA assays had similar sensitivities and specificities, the correlation/agreement between those different tests was only moderate. This impaired agreement was especially observed in the non-RA patients in whom a (false) positive ACPA test could be confirmed in only 3 of 11 cases. Some biological explanations for this observation have been formulated in the same chapter. Specific problems with the moderate agreement between the mentioned ACPA assays may occur when prediction models are described. Generalising prediction models becomes more difficult when the agreement between tests is only moderate.

Another way to translate sensitivities and specificities to the single patient in daily clinical practice is by calculating the positive predictive value of a test. However, in the introduction and in Chapter 6, we indicated that thinking in probabilities for disease may be better. Thinking in probabilities, modelled by logistic regression, is especially helpful when different diagnostic markers are combined in order to select the most appropriate combination of different diagnostic tests. We thus showed that HLA-SE testing is not useful in combination with ACPA testing. This can be explained by the high association between ACPA and HLA-SE in patients with established disease. We previously described another association between a genetic marker and a serological marker (Chapter 3). Different associations between genetic alterations and the

occurrence of (auto) antibodies have been described in many other diseases. In Type 1 diabetes, different disease associated antibodies are associated with susceptible HLA class II alleles defining different subtypes of Type 1 diabetes [Delamaire 1995, Sabbah 1999, Hermann 2004, Gorus 1994]. Carriage of other HLA class II alleles have been associated with a specific antibody response to nuclear antigens, in particular anti-SSA/Ro52, both in primary Sjögren's syndrome and lupus [Smolens 1987, Gottenberg 2003, Tsao 2002]. Other nuclear antibodies (anti-SSB/La), associated with HLA class II genes have also been linked with carriage of polymorphisms in the genes for transforming growth factor-β and tumor necrosis factor-α in patients with primary Sjögren's syndrome [Gottenberg 2005]. Different hypotheses can be generated to explain how the carriage of different HLA types or different gene polymorphism on the promoters of cytokines may influence antibody responses [Lard 2003, Gottenberg 2003].

By combining the knowledge of the genetic susceptibility of carriers of HLA-DQ2 or HLA-DQ8 genes with the discovery of the almost pathognomonic autoantibodies against tissue transglutaminase, important progress has been made in the understanding of the pathogenesis of celiac disease [Mowat 2003, Reif 2004].

Similarly, different hypotheses for the pathogenesis of RA have been generated. The carriage of the HLA-SE leading to genetic susceptibility for RA generated the hypothesis that specific proteins or peptides, relevant for the pathogenesis of RA, might have a high affinity for the HLA-SE. This focused some investigators on the human cartilage gp-39, which is a protein that can be retrieved in RA cartilage and synovium [Baeten 2004]. Citrullination of peptides might lead to a better interaction with the HLA-SE [Hill 2003] however citrullination is not mandtory for binding of fibrinogen to HLA-DR [Auger 2005].

Serological testing cannot only be useful for establishing a diagnosis, but also for estimating a prognosis. Different models to predict erosions have previously been suggested [Visser 2002, Nell 2005, Vencovsky 2003, Genevay 2002].

In some diseases, it has been proven that antibodies may occur before clinical symptoms are present, and thus can be used for early screening strategies and early therapeutic intervention. Such preventive therapeutic strategies are especially investigated for type I diabetes [Wilson 2001]. Similarly, it has been proposed to perform first a genetic testing and, if positive, to perform subsequent serial autoantibody testing in patients with a high risk to develop celiac disease [Liu 2005]. Whether such testing strategies might be useful for RA before any symptom has occurred remains to be proven [Berglin 2004].

3. Evaluation of different measures and composite indices as a measure of (insufficient) response to treatment in chronic arthritis.

In the Chapters 8-10, we focused on clinical measures and composite indices to evaluate RA disease activity. An important issue in this context is the question how results from clinical trials can be best translated to daily clinical practice.

The aim of a therapeutic strategy is to ameliorate the outcome. For RA, the therapy should target to reduce the symptoms of pain and swelling, to conserve the function and to avoid joint-destruction. In clinical trials however, "surrogate outcomes" are frequently defined [Piantadosi 1997]. Such surrogate outcomes are only valid if they proxy an outcome, in this case less destructive disease [Tugwell 1993]. For rheumatic diseases, the special study group OMERACT, has defined three conditions wchich should be fulfilled by an appropriate outcome measure: it should be true, discriminative and feasible [Boers 1998]. An outcome measure is true, when it measures what it intends to measure. Discriminative means that an outcome measure should also be able to discriminate between the situations of interest. This is important for classification or for the determination of a prognosis. Finally, an outcome measure should be feasible which means that the measure should be applied easily and should be easy to interpret [Boers 1998].

Statistical methods can be used to evaluate the discriminative properties of an outcome measure [Verhoeven 2000]. In Chapter 8, we defined the best standard for insufficient response to therapy as the physician's decision to give a dose increase of Infliximab therapy. In this study, we aimed to find the most appropriate single measure or composite index that could discriminate this decision. Therefore, we ranked all possible outcome measures based on their AUC of ROC curve analyses and the sensitivities at the 98% specificity level. This ranking showed that the DAS28, reflecting the disease activity at the moment of the decision, had the highest ranking. In the next steps, we tried to find a better model by combining different outcome measures, but we found that the final model had only slightly better discriminating properties compared to the DAS28. Finally, we recalculated the scores and coefficients by means of discriminant analysis and found similar scores and coefficients as the DAS28. We thus validated for the first time the DAS28 in a cohort of RA patients under biological therapy, more than 10 years after the initial development of the DAS. Differences between the initial study and the present study are highlighted in Table 1.3.

The evaluation of DAS is useful to evaluate the disease activity of RA patients in placebo-controlled trials [Fuchs 1993, Landewé 2002], and we suggested that it may also be used to evaluate the effect of therapy in PsA patients [Chapter 10]. The repeated measurement of DAS28 is a valid method to evaluate effects of therapy by applying area under the curve or

mixed models analysis. Interestingly, although PsA patients may show less swollen and tender joint counts than RA patients, we showed that this seems not to matter for the use of the DAS28. This can be at least partially explained by the squared root transformation applied to the SJC and TJC which leads to the phenomenon that a change in the low ranges has a higher impact on the DAS28 than the same change in the higher joint count ranges.

The ideal outcome measure should not only be applicable in clinical trials, but also in daily clinical practice so that results from trials can be easily translated into concepts for daily clinical practice. There is rising evidence that the DAS28 can be used both in clinical trials and in daily clinical practice. The response scores "ACR response" and "DAS response" measure differences over time and are thus not suited to evaluated momentary disease activity [Fransen 2004]. Compared to DAS, simplified disease activity index (SDAI) and clinical disease activity index (CDAI) are not normally distributed, which is a drawback for some statistical methods [Van der Heijde 1993, Smolen 2003].

Tight monitoring of the DAS with subsequent therapy adjustments, leads to a global improvement of the disease activity, a better functional outcome and a lower radiological progression rate [Grigor 2004, Welsing 2004, Landewé 2002, Gossec 2003, Drossaers-Bakkers 1999, Fansen 2005].

In Chapter 9, we showed that the DAS28, measured at week 14 or week 22 is a predictor of infliximab survival. If there is a remaining high disease activity at week 14 to 22 after start of infliximab, other therapeutic strategies should be considered.

Although we showed in Chapters 8 and 9, that DAS28 is the best composite index to model the physician's concept of disease activity, it is important to state that this is true on the population level, but that exceptions may occur on the individual level. This has been explored by different authors. First, studies evaluating remission criteria showed that tender joints and swollen joints may be present in the case that the classical DAS28 remission criterion (DAS28 < 2.6) [Prevoo 1996] is fulfilled, suggesting that some single patients with a low DAS28 may in fact still have some disease activity [Van der Heijde 2005, Mäkinen 2005]. Second, it may be important to keep in mind that toe joints are not evaluated by the DAS28. Also, there is no perfect fit between DAS28 and the physician's appreciation of disease activity VAS [Wolfe 2005, Chapter 8]. Taking into account the reasons for DAS28 to over or underestimate disease activity, DAS28 may be a useful tool to evaluate disease activity in RA patients in daily clinical practice. Thus, using DAS28 may be helpful so that results and treatment goals achieved in randomised clinical trials and studies of treatment strategies can be easily translated into daily clinical practice treatment goals.

Table 3: Comparison of the original construction of the DAS with the present validation study.

Initial studies*°	Present study§
113*/227° patients	511 patients
Recent onset RA (< 1 year)	Long-standing disease
DMARD-therapy	Infliximab (+ MTX)-therapy
Different time points	1 time point (week 22)
1 centre (6 rheumatologists)	Different centres
Scored by research nurses	Scored by a physician

* Van der Heijde 1993: DAS44
° Prevoo 1995: DAS28
§ Chapter 8: DAS28

4. Optimalization of statistical methods to construct prediction models

In Chapter 11 we evaluated the use of more sophisticated classification methods in multidimensional classification issues and compared them with discriminant analysis or logistic regression. We showed that, when a limited number of variables are considered, discriminant analysis and logistic regression perform rather well, but that their performance gets worse when more dimensional datasets are used requiring a feature selection method. In cases where no feature selection can be performed, more complex methods such as support vector machines (SVM) and multilayered perceptrons (MLP) seem to be more relevant. This is congruent with another study that compared MLP with logistic regression. Similar performance of MLP and logistic regression was observed for dichotomous outcomes. However, for ordinal outcomes, MLP performed better [Ergün 2004].

Those studies suggest that for simple classification problems, logistic regression and discriminant analysis perform rather well. Only for more dimensional datasets, or non-linear data, more complex methods should be considered. All methods however seem to suffer from the so-called "curse of multidimensionality" [Bellman 1957]. This means that when more variables are available, classification methods perform less well. Adding more variables for classification not only adds information, but also noise which may impair the classification task. This problem becomes more and more relevant with the introduction of new assays in which multiple markers can be tested simultaneously by means of micro-bead assays [Fulton 1997] or micro-array analysis [Southern 2001]. In Chapter 11 we showed that, when no feature selection method can be used, among the evaluated methods, SVM seem to be the best choice. Which feature selection method should be used, remains to be further established. We used a combination of formal methods such as ROC curve analysis and logistic regression with domain knowledge [Chapter 8, 11]. However, more difficulties will arise in more complex problems generated by micro-array analysis: sample sizes are generally smaller and more variables are available.

To conclude, high dimensional datasets remain a challenge for statistical analysis. New methods are under investigation for feature selection and classification.

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SUMMARY

This work aims to illustrate how single biomarkers and standardized clinical measures can be used in the daily clinical practice rheumatologist's decision making and how different markers and measures can be combined into prediction models and composite indices.

The first part focuses on association studies. First, a potential biomarker (or clinical measure) should be associated with the disease. Association studies provide a powerful tool to discover new markers but have the major draw-back that replication may be difficult. Three association studies of carriage of Crohn's disease associated CARD15 polymorphisms are shown. We first investigated the association between carriage of CARD15 polymorphisms and SpA related symptoms in Crohn's disease. Inversely, the association between gut inflammation in SpA and carriage of CARD15 polymorphisms was explored. These associations may suggest a genetic link between gut and joint inflammation. Also, an association between carriage of CARD15 polymorphisms and anti-*Saccharomyces cerevisiae* antibodies in Crohn's disease patients is demonstrated. Although the discovery of CARD15 polymorphisms may be important for the understanding of Crohn's disease and Spondyloarthropathy, their diagnostic value is weak and therefore, CARD15 determination is not recommended for daily clinical practice.

The diagnostic properties of a test are explored into more detail in part 2, illustrated by the RF and ACPA determination in RA. ACPA is a very specific test for RA, but the specificity of this test may differ between study populations. A higher ACPA false-positivity rate than expected (in a random control population) can be observed in PsA patients. Combinations of ACPA, RF and HLA-SE testing are explored by different (predicted) probabilities plots, based on logistic regression. They demonstrate that combined ACPA and continuous RF-testing is the most relevant combination. Finally, different ACPA tests are compared side-by-side showing that different ACPA tests may have similar diagnostic properties, but that disagreement between tests may occur.

In part 3, different single clinical measures and composite indices to measure response to treatment in RA (and PsA) are evaluated. 511 RA patients treated with infliximab are investigated. After a loading regimen at week 0, 2, 6 and 14, the treating rheumatologist could decide to give a dose increase at week 22. This decision to give a dose increase can be considered as a measure of insufficient response and could be best

modeled by the DAS28 at the moment of decision. The same patients were followed over 4 years and also DAS28, measured at week 14 or week 22, could best predict 4-year attrition to therapy. DAS28 as a continuous measure of disease activity in RA patients can be used in clinical trials and in daily clinical practice to make treatment decisions. Also, measuring of the DAS28 score in PsA may be useful to evaluate effects of therapy in placebo-controlled trials.

In the last part, different methods to construct prediction models are evaluated. Discriminant analysis and logistic regression may be less performing in real life datasets with missing values and high dimensionality. In such cases, computer intensive methods – such as support vector machines and multilayerd perceptrons - may be useful.

SAMENVATTING

In deze thesis wordt geïllustreerd hoe merkers uit bloedanalyses en gestandaardiseerde klinische metingen de reumatoloog kunnen helpen om beslissingen te nemen in de dagelijkse klinische praktijk en hoe verschillende merkers en metingen samengevoegd kunnen worden in modellen en indexen.

Het eerste deel van dit werk handelt over associatie studies. Een biomerker (of klinische meting) moet geassocieerd zijn met de ziekte. Associatie studies kunnen gemakkelijk nieuwe merkers aan het licht brengen, maar hebben het grote nadeel dat het vaak moeilijk is om de resultaten te reproduceren. Drie associatie studies worden getoond met betrekking tot polymorphismen in het CARD15 gen, die frequenter voorkomen bij patiënten met de ziekte van Crohn. De associatie tussen het dragen van een CARD15 polymorphisme en het voorkomen van SpA-gerelateerde symptomen in Crohn patiënten wordt nagegaan. Anderzijds wordt ook de relatie tussen het dragen van een CARD15 polymorphisme en darm ontsteking in SpA nagegaan. Deze associaties suggereren een genetische link tussen darm en gewricht. Verder wordt ook aangetoond dat patiënten met de ziekte van Crohn die een CARD15 polymorphisme dragen frequenter antistoffen aanmaken tegen *Saccharomyces cerevisiae* (of bakkersgist). Hoewel deze associatie studies ons mogelijk meer leren over het verband tussen de ziekte van Crohn en SpA, heeft het testen op de aanwezigheid van CARD15 polymorphismen weinig diagnostische waarde en wordt dit niet aanbevolen voor de dagelijkse praktijk.

In het tweede deel van dit werk wordt nagegaan aan welke eigenschappen een diagnostische test moet voldoen aan de hand van 2 serologische tests voor reumatoïde artritis (RA): reumafactor (RF) en de antistoffen tegen gecitrullineerde eiwitten (ACPA). Dergelijke ACPA tests zijn zeer specifiek voor RA maar de specificiteit kan verschillen tussen studiepopulaties. Zo komt een (vals) positieve ACPA test bij patiënten met psoriasis artritis frequenter voor dan kan verwacht worden, gegeven door de specificiteit van de test in willekeurige controle populaties.
Verschillende combinaties van ACPA, RF en de HLA-SE worden bestudeerd aan de hand van kansgrafieken die berekend werden met behulp van logistische regressie. Hiermee kan aangetoond worden dat een combinatie van een continue reumafactor test met een ACPA test de beste testbatterij is. Tot slot worden een aantal ACPA tests direct met elkaar vergeleken waarbij aangetoond wordt dat deze tests vergelijkbare diagnostische kenmerken hebben, maar dat discrepante resultaten kunnen voorkomen.

In deel 3 worden verschillende gestandaardiseerde klinische metingen en indexen bestudeerd die gebruikt worden om het effect van een behandeling te meten bij patiënten met reumatoïde (of psoriasis) artritis. Hiervoor worden 511 RA patiënten bestudeerd die behandeld werden met inflximab. Nadat de patiënten een oplaaddosis hadden gekregen op week 0, 2, 6 en 14 kon de arts op week 22 beslissen om een dosisverhoging te geven. Deze beslissing om een dosisverhoging te geven kan beschouwd worden als een maat van onvoldoende respons en kan het best gemodelleerd worden door het meten van de DAS28 op het moment van de beslissing zelf. Dezelfde patiënten werden ook over 4 jaar gevolgd waarbij aangetoond wordt dat de ziekte activiteit, gemeten op week 14 of week 22 met behulp van de DAS28 het best vroegtijdig kan voorspellen of patiënten ook nog na 4 jaar onder behandeling blijven. DAS28 als een continue maat van ziekteactiviteit bij RA patiënten kan gebruikt worden in klinische studies en in de dagelijkse kliinische praktijk om beslissingen te maken over therapie. Verder wordt ook aangetoond dat het meten van de DAS28 ook kan gebruikt worden om het effect van therapie te evalueren bij patiënten met psoriasis artritis.

In het laatste deel worden een aantal methoden geëvalueerd die gebruikt worden om voorspellingsmodellen en indexen te maken. Discriminant analyse en logistische regressie zijn vaak minder performant in datasets met ontbrekende waarden en hoog-dimensionele datasets. In dergelijke gevallen kan het nuttig zijn om computer intensieve methoden te gebruiken zoals support vector machines en multilayered perceptrons.

CURRICULUM VITAE

Bert Vander Cruyssen is born 21th November 1975 in Kortrijk, Belgium. He obtained the degree of medical doctor in 2000 maxima cum laude at the Catholic University of Louvain. He started his training in internal medicine in July 2000 for one year at the St-Lucas' Hospital in Bruges followed by one year at the Ghent University Hospital. From October 2002 to October 2006, he performed research at the department of Rheumatology, Ghent University, funded by a concerted action grant GOA 2001/12051501. From October 2006 to July 2008, he will finalize his training in rheumatology at the Ghent University Hospital. He is married with Katrien Vandewiele, anesthesiologist, and proud father of three sons (Thibault, Matisse and Henri).

List of Publications
A1 Publications

Peeters H*, Vander Cruyssen B*, Laukens D, Coucke P, Marichal D, Van Den Berghe M, Cuvelier C, Remaut E, Mielants H, De Keyser F, Vos MD. Radiological sacroiliitis, a hallmark of spondylitis, is linked with CARD15 gene polymorphisms in patients with Crohn's disease. Ann Rheum Dis. 2004;63:1131-4. (*equal contribution)

Laukens D, Peeters H, Marichal D, Vander Cruyssen B, Mielants H, Elewaut D, Demetter P, Cuvelier C, Van Den Berghe M, Rottiers P, Veys EM, Remaut E, Steidler L, De Keyser F, De Vos M. CARD15 gene polymorphisms in patients with spondyloarthropathies identify a specific phenotype previously related to Crohn's disease. Ann Rheum Dis. 2005;64:930-5.

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in rheumatoid arthritis: specificity and relation with rheumatoid factor. Autoimmun Rev. 2005;4:468-74. Review.

<u>Vander Cruyssen B*</u>, Van Looy S*, Wyns B, Westhovens R, Durez P, Van den Bosch F, Veys EM, Mielants H, De Clerck L, Peretz A, Malaise M, Verbruggen L, Vastesaeger N, Geldhof A, Boullart L, De Keyser F. DAS28 best reflects the physician's clinical judgment of response to infliximab therapy in rheumatoid arthritis patients: validation of the DAS28 score in patients under infliximab treatment. Arthritis Res Ther 2005;7:R1063-R1071.(* equal contribution)

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Van Looy S*, <u>Vander Cruyssen B*</u>, Meeus J, Wyns B, Boullart L, F De Keyser, Comparison of Machine Learning models for prediction of dose increase in patients with rheumatoid arthritis. Eng Appl Artif Intel. epub-ahead of print (*Equal contribution).

Peeters H, <u>Vander Cruyssen B</u>, Mielants H, Vlam K, Vermeire S, Louis E, Rutgeerts P, Belaiche J, De Vos M. Sacroiliitis in Crohn's disease: a multicentre association study. [Submitted to IBD].

<u>Vander Cruyssen B,</u> De Keyser F, Kruithof E, Mielants H, Van den Bosch F.
Comparison of different outcome measures for psoriatic arthritis in patients treated with infliximab or placebo [In press in Ann rheum dis].

<u>Vander Cruyssen B*</u>, Cantaert T*, Nogueira L, Clavel C, De Rycke L, Dendoven A, Sebag M, Deforce D, Vincent C, Elewaut D, Serre G, De Keyser F. Diagnostic value of anti-human citrullinated fibrinogen ELISA and comparison with four other anti-citrullinated protein assays. Arthritis Res Ther. 2006;8:R122 (* Equal contribution);

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A2 publications

Vander Cruyssen B, De Keyser F. Rheumatoid factor and anticitrullinated protein/peptide antibodies. In "The Year in Rheumatic Disorders 5" edited by Madhok, Capell and Luthra. Oxford: clinical publishing; 2006:49-64.

Vander Cruyssen B, De Keyser F. Natural history, prognosis, socioeconmic aspect and quality of life of reactive arthritis. In "Psoriatic and reactive arthritis" edited by Ritchlin and Fitzgerald. Philadelphia: Elsevier 2006. [In press].

A3 publications

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DANKWOORD

Wanneer een arts een beslissing neemt rond een diagnose of een therapie houdt hij rekening met enorm veel factoren, die kunnen vergeleken worden met een complex schilderij. Dit werk probeert te illustreren hoe verschillende penseeltrekken kunnen gecombineerd worden in een ruwe schets.
De kleinste penseeltrek, of dit nu een hoog technologische merker is, of een eenvoudig klinisch teken, is het resultaat van werk van verschillende mensen. Zo dus ook deze thesis.

Het is evident dat dit werk enkel tot stand kon komen dankzij de enorme wetenschappelijke bagage van de dienst en de coaching door mijn promotor prof F De Keyser.

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