# Publication of the Month 2002

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December 12/02:

# EliA™ dsDNA

The measurement of antibodies against double-stranded DNA (anti-dsDNA) is a useful tool for the diagnosis of systemic lupus erythematosus (SLE), and is one of the 11 diagnostic criteria for SLE according to the American College of Rheumatology (ACR). Additionally, titers of dsDNA antibodies have been reported to be dependent on the activity of disease. There are several established techniques for detection of dsDNA antibodies, such as Farr RIA, Crithidia luciliae indirect immunofluorescence (CLIFT) and ELISA. Recently (in April 2001), Pharmacia Diagnostics has launched an entirely new, fully automated fluorescent immunoassay for dsDNA antibodies detection: EliA dsDNA.

In the following current publication, EliA dsDNA was compared to Varelisa dsDNA, Farr RIA and CLIFT with respect to sensitivity, specificity as well as positive and negative predictive value:

*Hernando M, González C, Sánchez A et al.(2002)*
**Clinical Evaluation of a New Automated Anti-dsDNA Fluorescent Immunoassay**
Clin Chem Lab Med 40, 1056 - 1060

Anti-dsDNA antibodies have been measured in the sera of 179 patients derived from Salamanca University Hospital. 76 of them were sera of SLE patients (14 men, 62 women) and the remaining 103 sera constituted the control group consisting of patients with Sjögren's syndrome, rheumatoid arthritis, connective tissue diseases, hepatopathies, viral hepatitis, renal disease, diabetes, exanthema and hypertension.

The results achieved can be found in Table 2 and Table 3 (p. 1058). For patients with SLE, sensitivity of EliA was only somewhat lower (39.5 %) than that for Varelisa (44.7%) which displayed the highest value. In the group of patients with active SLE, EliA and Varelisa showed highest sensitivity (70.8 %). The specificity was higher for EliA (93.2 %) than for Varelisa (82.5 %) and nearly reached the value for Farr assay (96.1 %).

These results reveal that EliA displays very favourable performance characteristics compared to the other methods. Additionally, the big advantages of EliA with respect to handling, precision and flexibility should be kept in mind.

November 11/02:

# ANCA Testing Algorithm

In 1985 antineutrophil cytoplasmic antibodies (ANCA) were recognized as a sensitive and specific marker for Wegener's Granulomatosis (WG). The antigen of the specific cytoplasmic indirect immunofluorescence (IIF) staining pattern (C-ANCA) was identified as proteinase 3 (PR3). During the routine screening of sera for the presence of ANCA it became apparent that some sera produce a perinuclear to nuclear fluorescence pattern (P-ANCA) clearly different from the C-ANCA pattern. Many P-ANCA-positive sera were shown to be directed against myeloperoxidase (MPO). The presence of antibodies to MPO is associated with idiopathic or vasculitis-associated necrotizing crescentic glomerulonephritis.

Around 75% of all vasculitis cases are ANCA positive with MPO or PR3 specificities. ANCA patterns may also occur due to antibodies to other antigens, usually not related with vasculitides, but with inflammatory bowel diseases, liver diseases, rheumatoid arthritis or cystic fibrosis.

Alan Wiik from Denmark proposed a differentiation between ANCA (MPO, PR3) and neutrophil-specific antibodies.

Currently at most institutions, ANCA testing is performed with immunofluorescence and only for confirmation with ELISA. ANCA testing guidelines have recently been put forth, recommending dual testing on each sample with standard IIF and solid-phase ELISA. The following article calls the routinely used algorithm for ANCA testing into question.

*Russell KA, Wiegert E, Schroeder DR, Homburger HA, Specks U (2002)*
**Detection of Anti-Neutrophil Cytoplasmic Antibodies under Actual Clinical Testing Conditions**
Clin Immunol 103, 196-203

Russell et al. compared the performance of standard IIF, PR3-, and MPO-ANCA-specific direct ELISA, and a PR3-ANCA-specific capture ELISA used alone and in combination. The study was performed under routine use conditions of a high-volume reference laboratory, the laboratory of the Mayo Clinic, Rochester.

Russell et al. found, that screening each submitted sample by both ELISA and IIF may not be necessary. Neither sensitivity nor specificity was improved if the combination of ELISA and IIF testing was applied to every sample compared to using ELISA alone. Yet, the most efficient and diagnostically accurate pairing of assays for ANCA detection in patients suspected for WG/PA was the combination of screening all samples by PR3-ANCA and MPO-ANCA direct ELISA with subsequent confirmation of all positives with the matching fluorescence pattern by IIF.

Interestingly, under high-throughput routine conditions, the PR3-ANCA capture ELISA seems to offer no diagnostic advantages over the very sensitive direct ELISA it was compared to.

October 10/02:

# Thyroid Diseases

Thyroglobulin antibodies (Tg Ab), TSH receptor antibodies (TSHR Ab) and thyroid peroxidase antibodies (TPO Ab) are known to occur in association with thyroid diseases, such as Graves' disease, Hashimoto's thyroiditis, hyperthyroidism and thyroid tumors. Apart from these well-known facts, interesting observations around thyroid diseases have been reported recently:

*Huber G, Staub J-J, Meier C, Mitrache C, Guglielmetti M, Huber P, Bravermann LE (2002)*
**Prospective Study of the Spontaneous Course of Subclinical Hypothyroidism: Prognostic Value of Thyrotropin, Thyroid Reserve, and Thyroid Antibodies**
J Clin Endocrinol Metab 87, 3221 - 3226

Subclinical hypothyroidism is defined by elevated TSH (thyroid stimulating hormone) secretion by anterior pituitary in the presence of normal concentrations of circulating thyroid hormones. It has been associated with an increased risk for various disorders, such as coronary heart disease or peripheral arterial disease, but the major concern is the development of overt hypothyroidism. Due to the high prevalence of this syndrome and its potential cost implications for the health care system, it is very important to detect patients early who might progress to the overt stage of the disease.

Therefore, the aim of this study was to identify risk factors for occurrence of overt hypothyroidism among 82 patients with the subclinical form over a mean period of 9.2 years. During this period, 28 % of the patient cohort developed overt hypothyroidism. For this collective, basal serum TSH was identified as strongest predictive factor for disease progression, followed by positive microsomal (TPO) antibodies and impaired thyroid reserve (see Table 2, p. 3224). Particularly in the group of patients with grade II dysfunction (TSH 6 – 12 mU/l), the risk for overt hypothyroidism can be assessed by measurements of microsomal or TPO antibodies and thyroid reserve as prognostic factors.

*Chung J-K, Park YJ, Kim TY, So Y, Kim S-K, Park DJ, Lee DS, Lee MC, Cho BY (2002)*
**Clinical significance of elevated level of serum antithyroglobulin antibody in patients with differentiated thyroid cancer after thyroid ablation**
Clin Endocrinol 57, 215 – 221

In this study, 226 patients with differentiated thyroid carcinoma (DTC) after thyroid ablation have been investigated with respect to serum thyroglobulin antibodies and disease status. All of these patients had undetectable thyroglobulin. Serum thyroglobulin is used as indicator for recurrence of DTC, as thyroid cancer cells secret this protein.

22.6 % of the patient collective (n = 51) were found to be positive for Tg Ab. In this group, 49.0 % were confirmed with recurrence of disease, in contrast to only 3 – 4 % in the Tg Ab negative group. Evidently, in Tg Ab positive patients these antibodies interfere with measurement of thyroglobulin so that low levels of thyroglobulin are found although thyroglobulin producing cancer cells are present.

The authors conclude that persistently elevated Tg Ab levels appear to serve as a useful indicator for recurrent or persistent DTC in patients with undetectable serum thyroglobulin. In such patient populations, the routine measurement of Tg Ab may be indicated.

*Wada N, Mukai M, Kohno M, Notoya A, Ito T, Yoshika N (2002)*
**Prevalence of Serum Anti-Myeloperoxidase Antineutrophil Cytoplasmic Antibodies (MPO-ANCA) in Patients with Graves' Disease Treated with Propylthiouracil and Thiamazole**
Endocr J 49, 329 – 334

Graves' disease is an autoimmune thyroid disorder characterized by hyperthyroidism. This dysfunction is caused by stimulatory antibodies directed against the TSH receptor. Additionally, in most cases TPO Ab (and Tg Ab) are present. One possibility to treat Graves' disease is the application of anti-thyroid drugs, like propylthiouracil or thiamazole. Their mechanism of action includes the inhibition of thyroid peroxidase (thereby reducing the generation of iodinated thyroglobulin and finally thyroid hormones) and a long-term immunosuppressive effect often leading to reduced TSHR Ab titers. Propylthiouracil has been reported to induce ANCA-associated vasculitis as side effect in few Graves' disease patients.

In this study, 61 patients with Graves' disease were included, 32 of them being treated with propylthiouracil and 29 being treated with thiamazole. For all patients, MPO-ANCA was determined. 25 % of the patients treated with propylthiouracil were found to be positive for MPO-ANCA whereas in the group of patients treated with thiamazole the corresponding value amounted to only 3.4 %. None of the MPO-ANCA positive patients were diagnosed as having classical ANCA-associated vasculitis. According to the authors, this may be due to either low titers of MPO-ANCA or short duration of antibody persistence.

Anyway, these results confirm MPO-ANCA induction by the anti-thyroid drug propylthiouracil in patients with Graves' disease.

September 09/02

# Best performance of Celikey

Due to the large number of recent publications dealing with the performance of commercial anti-tissue transglutaminase antibody ELISAs (including Celikey [R]), this Publication of the Month again refers to this topic:

IgA anti-endomysial antibodies (EMA), detected by indirect immunofluorescence (IIF), are the classical serological marker for celiac disease (CD) and have enabled the development of non-invasive screening tests for this condition. The sensitivity and specificity of this method lie in the range of 84 - 100 % and 94 - 100 %, respectively. Since Dieterich et al. described the ubiquitous enzyme tissue transglutaminase (tTG) as major autoantigenic target for IgA EMA in 1997, a wide variety of in-house and commercial IgA anti-tTG antibody ELISAs has been developed and studied. Most studies used guinea pig tTG as antigen, but purified erythrocyte and recombinant human tTG were also employed. Although many studies have concluded that the IgA tTG assay has a comparable performance to the IgA EMA IIF technique, several discrepant findings remain.

The following comprehensive study compares 13 commercial IgA anti-tTG antibody ELISAs with respect to sensitivity and specificity (7 guinea pig tTG based ELISAs and 6 ELISAs with human tTG, in 4 cases being recombinant):

*Wong RCW, Wilson RJ, Steele RH, Radford-Smith G, Adelstein S (2002)*
**A comparison of 13 guinea pig and human anti-tissue transglutaminase antibody kits**
J Clin Pathol 55, 488 - 494

The following commercial assays were included in the study:

| | **Company (Assay)** | **Antigen** |
|---|---|---|
| 1 | Aesku.Lab (AESKULISA) | human, recombinant |
| 2 | The Binding Site | guinea pig, liver |
| 3 | | human, recombinant |
| 4 | Eurospital | guinea pig, liver |
| 5 | | human, recombinant |
| 6 | Genesis Diagnostics | guinea pig, liver |
| 7 | Immco Diagnostics (ImmuLisa) | guinea pig, liver |
| 8 | Immunopharmacology Research Diagnostics | guinea pig, liver |
| 9 | Inova (QUANTA Lite) | guinea pig, liver |
| 10 | | human, erythrocyte |
| 11 | Medipan Diagnostics (Medizyme) | guinea pig, liver |

| | | |
|---|---|---|
| 12 | Orgentec Diagnostika | human, purified |
| 13 | Pharmacia Diagnostics (Varelisa CelikeyR) | human, recombinant (baculovirus) |

In total, 49 EMA positive adults with histologically proven CD and 64 adult disease controls were analyzed. Results (IgA tTG antibody values for CD patients and non-CD controls plus ROC plots) are presented for guinea pig-based assays in Fig. 1 and for assays using human antigen in Fig. 2. Sensitivity and specificity at manufacturers' Cut-offs and according to ROC analysis can be found for both groups in Table 1 and Table 2, respectively.

Human tTG- based assays generally demonstrated superior performance (particularly with respect to specificity) compared to guinea pig based assays. However, they also resulted in several false positive and false negative results.

The two kits (The Binding Site, Genesis Diagnostics) using Ca++ activated tTG as antigen did not show a particular advantage with respect to performance, although it had been suggested some years ago that Ca++ ions might improve the differentiation between CD patients and non-CD controls. This result is in line with recent findings that Ca++ increases both signal and background values.

The assay with the clear-cut best performance was CelikeyR, presenting with 100 % sensitivity and 100 % specificity. This result is even more impressive, as it was obtained using the Cut-off value recommended by Pharmacia Diagnostics, whereas for the majority of the other assays the Cut-off values had to be optimized by ROC analysis.

August 08/02

# Antiphospholipid Syndrome

The antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by a combination of arterial and/or venous thrombosis, recurrent fetal loss, often accompanied by a mild-to-moderate thrombocytopenia, and elevated titres of antiphospholipid antibodies (aPL).

The criteria for diagnosis of APS are continuously being discussed and the most recent outcome are the preliminary classification criteria for definite antiphospholipid syndrome published in 1999 after a workshop in Sapporo (see Publication of the Month May 05/00).

Although a great variety of clinical features have been described in patients with APS, the real prevalence of most of these manifestations is unknown. The aim of a large multicenter study, organized by a European consortium, the Euro-Phospholipid Project Group, was to analyze prevalence and characteristics of the main clinical and immunological manifestations of APS at disease onset and during its evolution in a cohort of 1,000 APS patients (which will be followed up during the next 10 years). The results of this study are presented in the following publication:

*Cervera R, Piette J-C, Font J, Khamashta MA, Shoenfeld Y, Camps MT, Jacobsen S et al (2002)*
**Antiphospholipid Syndrome. Clinical and Immunological Manifestations and Patterns of Disease Expression in a Cohort of 1,000 Patients**
Arthritis Rheum 46, 1019 - 1027

Only patients with definite APS, as recommended by the international consensus statement at Sapporo, were included in the cohort. The collective consisted of 82 % female and 18 % male patients. 53.1 % suffered from primary APS, which is independent of any underlying disease, and 36.2 % had APS associated with SLE. 87.9 % of all patients were positive for anticardiolipin antibodies and 53.6 % displayed LA (lupus anticoagulant) activity.

With respect to cumulative clinical manifestations, deep vein thrombosis was the most common one (38.9 %), followed by thrombocytopenia (29.6 %), livedo reticularis (24.1 %), migraine (20.2 %), stroke (19.8 %), early fetal loss (35.4 % of pregnancies), late fetal loss (16.9 % of pregnancies), transient ischaemic attack (11.1 %), hemolytic anaemia (9.7 %) and some further more. Surprisingly, some of these rather frequent symptoms, such as thrombocytopenia or livedo reticularis, had been considered "minor" in the Sapporo criteria. Another, yet positive surprise was that 74 % of the women who became pregnant succeeded in having at least one live birth. This is most probably due to the considerable progress that was made in the last decade with respect to follow-up and therapy. Additionally, some differences with respect to the frequency of certain clinical manifestations were found for primary vs. secondary APS, female vs. male patients and dependent on the age of disease onset. This finding might render it possible to define specific, more homogeneous subsets of APS.

July 07/02

# tTG IgA Antibodies: Excellent Performance of Celikey

Celiac disease (CD) is an autoimmune disease triggered by ingestion of gluten in genetically susceptible individuals. It leads to characteristic morphological damage in the small intestine (villous atrophy). CD is often manifested by a variety of symptoms, ranging from overt enteropathy to non-specific and silent forms. Although, due to this heterogeneity of manifestations, early diagnosis is not easy to perform, it is very important for an initiation of treatment on time in order to reduce the risks associated with prolonged gluten exposure.

In 1969, intestinal biopsy was introduced as the gold standard in diagnosing CD. Autoantibodies associated with CD are known since 1958, when antibodies to gliadin (AGA) were described. Endomysium antibodies (EMA) were detected in 1978. The combination of IgG-AGA, IgA-AGA and IgA-EMA has since then proved to be an excellent tool for diagnosing CD. Detection of EMA, however, is performed by indirect immunofluorescence on monkey oesophagus. Hence it has all technical disadvantages of this subjective and elaborate method.

In 1997, Dieterich et al. identified the enzyme tissue transglutaminase (tTG) as the main endomysial autoantigen. Subsequently, ELISAs have been established using first guinea pig tTG (gp-tTG-ELISA) and then human enzyme as antigen (h-tTG-ELISA). Celikey at present represents the only commercially available ELISA employing human tTG expressed in the eucaryotic baculovirus system. This assay is currently being evaluated by numerous centers. Three of these studies are presented here:

(1) *Clemente MG, Musu MP, Frau F, Lucia C, De Virgiliis S (2002)*
**Antitissue Transglutaminase Antibodies Outside Celiac Disease**
J Pediatr Gastroenterol Nutr 34, 31 - 34

(2) *Wolters V, Vooijs-Moulaert A-F, Burger H, Brooimans R, De Schryver J, Rijkers G, Houwen R (2002)*
**Human tissue transglutaminase enzyme linked immunosorbent assay outperforms both the guinea pig based tissue transglutaminase assay and anti-endomysium antibodies when screening for coeliac disease**
Eur J Pediatr 161, 284 – 287

(3) *Bürgin-Wolff A, Dahlbom I, Hadziselimovic F, Petersson CJ (2002)*
**Antibodies Against Human Tissue Transglutaminase and Endomysium in Diagnosing and Monitoring Coeliac Disease**
Scand J Gastroenterol 37, 685 – 691

The common outcome of these three evaluations is an excellent performance of Celikey, with only minor discordances compared to EMA.

Publication 1 clearly shows that Celikey is superior to gp-tTG-ELISA, particularly with respect to specificity. The authors have analyzed not only untreated patients with CD and healthy controls but also patients with other autoimmune diseases. Among these patients, 50 % of those with autoimmune liver disease (n = 9/18 for autoimmune hepatitis, n = 5/10 for primary biliary cirrhosis) and 6.5 % of those with insulin-

dependent diabetes mellitus (n = 3/46) tested positive with gp-tTG-ELISA, whereas applying Celikey only one patient with autoimmune hepatitis and one patient with diabetes were found positive (Table 1, p. 32). By intestinal biopsy, both of them displayed histological features of CD and they successfully started a gluten-free diet. Most interestingly, this reduced specificity of the gp-tTG-ELISA is due to impurities in the antigen. As shown by immunoblot, none of the EMA-negative sera that reacted positively in the gp-tTG-ELISA recognized the tTG protein band at 78 kDa, whereas EMA-positive sera from CD patients did (Figure 2, p. 33). This result clearly shows that the reduced specificity of gp-tTG-ELISAs that has also been reported previously, is not due to the presence of latent CD in so far asymptomatic patients (as has been hypothesized before) but is related to contaminants in the antigen. In addition to the low specificity, the gp-tTG-ELISA displayed a reduced sensitivity compared to Celikey (94 % vs. 100%).
The authors conclude that, due to its high rate of false-positive and false-negative results, gp-tTG-ELISA is unsuitable for screening CD. Celikey, however, is clearly superior in both respects. And, according to the authors, it has to be assessed by further sudies, if h-tTG-ELISA could replace traditional EMA testing or if a combination of both methods might be the optimal tool for diagnosis of CD.

In publication 2, Celikey was compared with the other currently available serological methods for diagnosing CD (gp-tTG-ELISA, EMA, AGA). 101 Sera from patients with unspecific gastrointestinal complaints (52 with CD, 49 without CD) were analyzed. The outcome of the study was highest sensitivity (96 %) and specificity (100 %) for Celikey, with considerably lower values for the other assays (Table 1, p. 286). Based on these data, the authors conclude that an ELISA employing human tTG as antigen is currently the serological method of choice for identifying patients with CD in the absence of IgA deficiency.

In publication 3 Celikey was evaluated in comparison with traditional EMA testing. The study population consisted of 208 CD patients and 157 controls. For Celikey, a sensitivity and specificity of 96 % and 99 %, respectively were determined (area under the ROC curve: 98.3 %). Only 4 out of 365 patients (1 %) displayed discordant results for Celikey vs. EMA testing. Both antibody levels measured by Celikey and EMA titers were closely correlated to the duration of gluten-free diet and gluten challenge.
From these observations the authors conclude that IgA tTG ELISA can be used as an accurate observer-independent alternative to EMA testing in diagnosing and monitoring CD.

It can be summarized that Celikey presents with an excellent performance clearly surmounting that of gp-tTG-ELISA. Due to only minor discrepancies compared to EMA testing it may be suggested as technically feasible alternative or complement to EMA testing by indirect immunofluorescence, particularly in large screening programs for CD.

June 06/02

# Antiphospholipid Antibodies

Antiphospholipid (aPL) antibodies are a family of autoantibodies that have been described to occur associated with thromboses (venous or arterial) or recurrent fetal losses. The corresponding clinical picture is referred to as the antiphospholipid syndrome (APS), either as isolated form (primary APS or PAPS) or as secondary variant (in conjunction with a connective tissue disease). aPL Antibodies are particularly frequent in the sera of patients with systemic lupus erythematosus (SLE) and other autoimmune diseases. However, it should be emphasized that there are also many patients that have laboratory evidence of aPL antibodies without known clinical consequences. Among young, apparently healthy control subjects aPL antibodies are found at a prevalence of 1 to 5 percent.

The antigen target of aPL antibodies is still a matter of debate. Since the early 1990s it is known that some anticardiolipin antibodies require the presence of the plasma phospholipid binding protein ß2-glycoprotein I in order to bind cardiolipin. This requirement is typical for aPL antibodies from patients with the antiphospholipid syndrome, whereas patients with syphilis or other infectious diseases display aPL antibodies that react directly with cardiolipin.

In the following recent publication a current overview is given comprising relevant aspects of aPL antibodies and of the antiphospholipid syndrome:

*Levine JS, Branch DW, Rauch J (2002)*
**The Antiphospholipid Syndrome**
N Engl J Med 346, 752 – 763

Treated items are, besides a short historical summary, detection of aPL antibodies by different methods (lupus anticoagulant vs. ELISA), criteria for classification and diagnosis of APS, epidemiology, clinical features, pathological features and the catastrophic antiphospholipid syndrome. With regard to the pathogenesis, four (so far unproven) possibilities are discussed, one of which, the interference of aPL antibodies with binding of annexin-V to phospholipids, is exemplified in more detail in the following publication:

*Rand JH (2002)*
**Molecular Pathogenesis of the Antiphospholipid Syndrome**
Circ Res 90, 29 - 37

Annexin-V has potent anticoagulant activity in vitro, which is based on its high affinity for anionic phospholipids and its capacity to displace coagulation factors from phospholipid surfaces. Since both aPL antibodies and annexin-V have affinity for anionic phospholipids, it is hypothesized that these autoantibodies might interfere with the formation of the anti-thrombotic annexin-V shield over phospholipids. Details on this possible pathogenic mechanism including molecular features of annexin-V and an attractive explanation for the paradox of the LA effect (aPL antibodies induce prolongation of the coagulation time in vitro) are found in this publication.

In addition to the classical and well-characterized association of aPL antibodies with APS, some other diseases, particularly neurological ones, have been described to be

possibly linked to aPL antibodies. One recent example is found in the following publication that describes a high prevalence of anti-cardiolipin, anti-ß2 glycoprotein I and anti-prothrombin antibodies in young patients with epilepsy:

*Cimaz R, Romeo A, Scarano A, Avcin T, Viri M, Veggiotti P, Gatti A, Lodi M, Catelli L, Panzeri P, Cechini G, Meroni PL (2002)*
**Prevalence of Anti-cardiolipin, Anti-ß2 Glycoprotein I, and Anti-prothrombin Antibodies in Young Patients with Epilepsy**
Epilepsia 43, 52 - 59

For sera from 142 consecutive patients with epilepsy an overall positivity of 28.8 % (n=41) was found, 15 patients being positive for anti-cardiolipin antibodies, 25 for anti-ß2-glycoprotein I antibodies and 18 (out of 90 tested) for anti-prothrombin antibodies. An elevated percentage of IgG anticardiolipin-positive patients in a cohort of unselected epilepsy patients compared to control sera has been reported previously. Although being still rather hypothetical, according to the authors, a pathogenic role for these antibodies cannot be excluded. Possible mechanisms might be microinfarcts secondary to ischemic events or immune-mediated processes directed against endothelial or neuronal cells.

May 05/02

# ANCA: Comparison of capture assay vs. conventional ELISA

Anti-neutrophil cytoplasmic antibodies (ANCA) are often found in systemic vasculitides, a group of diseases of unknown etiology that are characterized by widespread inflammation in small blood vessels. ANCA directed against the lysosomal enzyme proteinase 3 (PR3-ANCA) are of great interest because they have been reported to be associated with active Wegener's granulomatosis (WG). In WG, PR3-ANCA titers are regarded as very helpful in monitoring treatment and are discussed as possible indicators for relapses.

The current standard technique for ANCA screening is indirect immunofluorescence (IIF) using ethanol fixed human neutrophils as substrate. One of the resulting fluorescence patterns, the C-ANCA pattern (cytoplasmic ANCA), is in most cases caused by PR3 antibodies. Positive ANCA results obtained by IIF should always be confirmed by ELISA. Discrepancies between these methods may be due to antibodies against so far unknown proteins or to conformational changes of the antigen occurring during the purification and/or coating process in ELISA.

In order to overcome the latter problems, some years ago an indirect technique (capture assay) was developed for PR3 coating instead of direct antigen binding to the microtiter plate.

In the following recent publication, capture assay and conventional ELISA for PR3-ANCA detection were compared with respect to sensitivity and specificity for detection of relapses of systemic vasculitides by analysis of titer increases:

*Gisslen K, Wieslander J, Westberg G, Herlitz H (2002)*
**Relationship between anti-neutrophil cytoplasmic antibody determined with conventional binding and the capture assay, and long term clinical course in vasculitis**
J Intern Med 251, 129 - 135

A total of 245 serum samples were analysed from 10 patients with systemic vasculitides (9 with WG, 1 with MPA). These samples had been collected during regular patients' visits 3 – 4 times a year over a 15-year period (1984 – 1999) at the Department of Nephrology, Sahlgrenska University Hospital, Göteborg. Periods of active disease were defined retrospectively by appearance of clinical symptoms and rising laboratory indices (ESR, CRP, haematuria, serum creatinine), based on the hospital charts.

For both conventional ELISA and capture assay, in-house techniques were used. In case of the capture assay coating was performed using a monoclonal antibody that in a second step was allowed to capture purified PR3.

In total, 29 episodes of active vasculitis were found for the analysed 10 patients. A PR3-ANCA rise was defined as (at least) doubling of the titer compared with the preceding period of remission.

Overall, the capture assay displayed a higher sensitivity for detection of relapses by rises of titer than the conventional ELISA (100 % vs. 79 %, see Table 2). Thus, 6 relapses were only preceded by significant increases in the titers measured by the capture PR3-ANCA assay.

On the other hand, the specificity of rises in titer for prediction of relapses was higher for conventional ELISA than for the capture assay (increase in titer not followed by relapses: *conventional ELISA*: 2 for 29 relapses; *capture ELISA*: 6 for 29 relapses).

Additionally, the PR3 titer determined by the capture assay exceeded the conventionally measured PR3-ANCA titer by one to four times in all but one patient. 8 out of 10 patients displayed significant ANCA titers between relapses when measured with the capture assay.

Increased sensitivity of the capture assay compared to conventional ELISA has been reported before and may be due to a better conservation of native conformational epitopes on the PR3 molecule. This study, however, clearly reveals that the specificity of the capture assay is limited and considerably lower than for conventional ELISA. This becomes evident from persistently high ANCA titers during periods of remission and a considerably higher rate of "false-positive" ANCA rises.

This observation is of great significance as the initiation of treatment based on increases of PR3-ANCA titers is still a matter of debate and becomes even more prone to discussions in the case of results obtained with the capture assay.

April 04/02

# Unusual ANA/ENA Antibody Associations

Autoantibodies directed against antinuclear antigens (ANA) or extractable nuclear antigens (ENA) are mainly found in patients with connective tissue diseases. The different antigen specificities of this group of autoantibodies, such as anti-dsDNA or anti-Sm, have been reported to occur in association with special autoimmune diseases and in defined combination with other ANA/ENA antibodies. However, apart from these well known disease associations and antibody coexistences, unusual coincidences of ANA/ENA antibodies have been described. This Publication of the Month presents three articles exemplifying this issue:

*Dick T, Mierau R, Bartz-Bazzanella P, Alavi M, Stoyanova-Scholz M, Kindler J, Genth E (2002)*
**Coexistence of antitopoisomerase I and anticentromere antibodies in patients with systemic sclerosis**
Ann Rheum Dis 61, 121 – 127

Systemic sclerosis (SSc) is characterized by antitopoisomerase I antibodies (Scl-70) as well as by anticentromere antibodies. In large series of patients these antibodies have been described to be mutually exclusive, Scl-70 antibodies being associated with diffuse scleroderma and anticentromere antibodies occurring in patients with CREST syndrome or limited cutaneous SSc. In the study presented, however, three cases of coexistence of these two antibodies are reported – a phenomenon which occurs in < 1% of patients with SSc.

*Yamane K, Ihn H, Kubo M, Kuwana M, Asano Y, Yazawa N, Tamaki K (2001)*
**Anti-U1RNP antibodies in patients with localized scleroderma**
Arch Dermatol Res 293, 455 – 459

The authors describe the presence of anti-U1RNP antibodies (reactivity with 70 kD protein) in patients with localized scleroderma (frequency 3%). This finding has not been reported previously. U1RNP antibodies are known to be a serological marker for MCTD, but may also be detected in patients with definite systemic sclerosis or systemic lupus erythematosus (SLE).

*Gussin HAE, Ignat GP, Varga J, Teodorescu M (2001)*
**Anti-topoisomerase I (Anti-Scl-70) antibodies in patients with systemic lupus erythematosus**
Arthritis Rheum 44, 376 - 383

As also reported in earlier publications, this study detects Scl-70 antibodies by ELISA in 25% of SLE patients. These were not classified as having also systemic sclerosis. This unusual observation was confirmed by immunoblotting, immunoabsorption and further methods. Significant correlations of Scl-70 antibodies were found for disease activity and for dsDNA abs.

March 03/02

# Evaluation of three commercial dsDNA-ELISAs

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with a prevalence of approximately 40/100,000 and an incidence between 1.8 and 7.6 cases/100,000 patient years. As the early symptoms in many cases are not disease-specific (such as arthritis, febrile temperatures or increased light sensitivity), clinical diagnosis is frequently difficult. Serological diagnosis therefore is an important aid in the primary diagnosis of SLE. Anti-nuclear antibodies are the most prominent ones out of a whole range of autoantibodies. Merely 5 % of patients with SLE do not display anti-nuclear antibodies during the course of their disease.

Only few of these autoantibodies, however, are specific for SLE, such as anti-dsDNA, anti-Sm, anti-PCNA and anti-ribosomal P-protein antibodies. Of these, only anti-dsDNA antibodies are frequent enough to be useful for diagnostic purposes. Currently, mainly three different assay methods are used to determine dsDNA antibodies: Crithidia luciliae immunofluorescence test (CLIFT), radioimmunoassay (FARR assay) and ELISA. The immunofluorescence technique is considered highly specific but suffers from poor sensitivity. The FARR assay is assumed to detect only disease-relevant antibodies with high affinity and is characterized by a high positive predictive value and specificity. ELISAs, on the other hand, have been described to be more sensitive but of lower specificity.

Comparative evaluations employing current commercial tests of these assay types are of immense importance to gain a notion about the concrete strengths and disadvantages of the various assays in practice. In the following recent publication, the authors have comparatively evaluated three commercial dsDNA antibody ELISAs (including Varelisa dsDNA Antibodies from Pharmacia) in relation to CLIFT:

*Linnemann M, Geisen C, Menn S, Herzberg C, Dinser R, Wielckens K (2002)*
**Comparison of Three dsDNA-ELISAs with Regard to their Efficiency in the Diagnosis of Systemic Lupus Erythematosus**
Clin Lab 48, 45-52

The comparative evaluation comprised the following assays: ELISA from Euroimmun (using dsDNA from salmon), ELISA from Binding Site (using dsDNA from calf thymus), Varelisa from Pharmacia (using plasmid dsDNA) and CLIFT from Euroimmun. Measurement ranges for the ELISAs as indicated by the manufacturers were 10 – 800 IU/ml for the Euroimmun assay, 12.3 – 1000 IU/ml for the Binding Site assay and 1 – 200 IU/ml for Varelisa.

Sera from 18 patients with SLE (16 of them in partial or complete remission) and 64 sera from patients with other, partly autoimmune, diseases (such as rheumatoid arthritis, systemic sclerosis, psoriatic arthritis, hepatitis C and malignancy) were employed for the determination of diagnostic sensitivity, specificity and efficiency of the different test methods. Sera from 57 healthy controls were used to assess the respective cut-off values. Additionally, various test characteristics were determined, such as variability, detection limit, linearity and rate of retrieval.

With respect to precision, the results for the three different ELISA assays were comparable, with both intra- and interassay variation being below 10 % for most conditions tested (see Table 1a and 1b). In the lowest measurement range below the cut-off (Varelisa mean: 7.91 IU/ml), the intra-assay variability of Varelisa was higher

compared to that of the other two assays (9.93 % vs. 7.43 % and 2.98 %), for the more relevant intermediate (Varelisa mean: 62.09 IU/ml) and high (Varelisa mean: 183.40 IU/ml) values the outcome was inverse (3.48 % for Varelisa vs. 10.18 % and 6.08 %; 3.93 % for Varelisa vs. 5.87 % and 5.83 %), which emphasizes an advantage of Varelisa.

The detection limit was calculated for each test by determining the arithmetic mean of 15 negative samples plus three standard deviations. For the assay of Binding Site, the measured detection limit was identical to that indicated by the manufacturer, for the assay from Euroimmun and for Varelisa it was higher (see Table 2). Of note, however, the detection limits indicated by Euroimmun and Binding Site were outside the lower limit of the measurement range, which means that no valid standard curve exists for this range. According to the authors, manufacturers should include the method used to determine the detection limit in the test description.

The linearity of each ELISA assay was assessed by testing consecutive dilutions (1:1 to 1:32), using dilution buffers provided by the manufacturer. All assays tested displayed considerable deviations from an ideal linear performance (positive variation of all assays: roughly 20 %, up to 60 %) (see Figure 1). For the FARR assay from Amersham and other tests, similar results have been reported (McMillan SA, Fay AC (1988); J Clin Pathol 41,1223 – 1228).

To test the correctness of the method, the rate of retrieval was determined by addition of different defined quantities of analyte to patient or control samples. The ratio of measured value/expected value was calculated. It describes the rate of recovery. The results, as depicted in Table 3, reveal that the assay from Euroimmun showed a symmetrical deviation towards an underestimation of the measured values for the entire concentration range analyzed. The assays from Binding Site and particularly from Pharmacia performed well, with a non-systematic maximal deviation below 18 % and 13 %, respectively. The only exception was the lowest value (mean: 8.25 IU/ml) for Varelisa (Deviation: - 28.61 %). With respect to this result it has to be borne in mind that small deviations lead to high relative differences at low antibody levels.

The cut-off value of each assay was determined by testing control samples from 57 healthy individuals. In each case, the measured value was considerably lower than that indicated by the manufacturer (see Table 4), e.g. 19.3 IU/ml instead of 55 IU/ml for Varelisa. However, the results obtained from the control subjects were not distributed normally.

To determine the diagnostic efficiency at various cut-off values, ROC (Receiver Operator Characteristics) diagrams were generated by calculating the sensitivity (based on 18 SLE patients) and the specificity (based on 64 patients with other diseases) for each chosen cut-off and assay. Twelve different cut-off values were applied for each test, including that indicated by the manufacturer and that determined by the authors (see Figure 2). If two cut-offs yielded identical efficiencies, the cut-off equivalent to higher specificity was preferred. The (via ROC plot) optimized values for the diagnostic efficiency of the tested ELISAs were very high, amounting to 90.2 % to 92.7 % and were higher than that obtained for CLIFT (89 %; cut-off : titer 1:10). Most importantly, the cut-off value resulting in the highest diagnostic efficiency was identical to the upper limit of the grey zone indicated by Pharmacia for the Varelisa assay, whereas for the test from Euroimmun it was far below the cut-off value proposed by the manufacurer (66 IU/ml vs. 100 IU/ml) and for the assay from Binding Site it was similar to the lower limit of the grey zone (29 IU/ml vs. 30 IU/ml; upper limit: 75 IU/ml) (see Table 5). The sensitivity achieved by the tested ELISAs was not particularly high (55.6

– 66.7 % compared to 50 % for CLIFT). This is partly due to the approach to optimize diagnostic efficiency. Another important reason is that most of the SLE patients included in the study were currently in remission under immunosuppressive therapy.

In summary, the authors found a very high diagnostic efficiency for dsDNA antibody ELISAs from Euroimmun, Binding Site and Pharmacia. The performance of these assays was superior to CLIFT. However, for the assays from Euroimmun and Binding Site, the high performance values were obtained only after optimization of the cut-off value with the help of ROC diagrams (towards lower values), whereas for the Varelisa assay optimal output was achieved at the cut-off indicated by Pharmacia (upper limit of grey zone). With respect to the other test characteristics (variability, detection limit, linearity, rate of retrieval), all three ELISAs performed reasonably well, with Varelisa displaying advantages with respect to variability and retrieval rate. A critical point was the systematic negative deviation of the retrieval rates achieved by the ELISA from Euroimmun.

February 02/02

# New therapeutic approaches for autoimmune diseases

Systemic lupus erythematosus (SLE) represents the prototype of an autoimmune disease, characterized by flares and organ manifestations as well as by autoantibodies directed against nuclear antigens. Currently, the most widely used therapies of SLE comprise predominantly steroids and immunosuppressive or cytotoxic agents. However, this approach is limited by the occurrence of partly severe side effects.
As an alternative, plasmapheresis has been investigated with the assumption that the removal and replacement of plasma could have a beneficial effect in SLE. The outcome of different studies, however, was controversial.
As SLE is regarded as immune complex-mediated disease, another possibility is the use of C1q as ligand for a new immunoadsorption column. This technology may be promising due to the unique capability of C1q for multifunctional binding and removal from the circulation of pathogenic circulating immune complexes, anti-C1q antibodies and inflammatory proteins such as CRP, fibrinogen and fibronectin.

In the following recent publication, results from the treatment of SLE patients using this novel approach are presented:

*Pfueller B, Wolbart K, Bruns A, Burmester GR, Hiepe F (2001)*
**Successful treatment of patients with systemic lupus erythematosus by immunoadsorption with a C1q column: a pilot study**
Arthritis Rheum 44, 1962 - 1963

In a phase I/II clinical trial, 8 SLE patients fulfilling the ACR criteria and showing symptoms of a disease flare were treated by C1q immunoadsorption. Six immunoadsorptions were performed on a given patient every other day. During the treatment no severe side effects were observed. The clinical activity, as assessed by patient global assessment and the European Consensus Lupus Activity Measure (ECLAM), showed a statistically significant improvement in 7 of 8 patients. Significant amelioration was observed in joint, renal and cutaneous manifestations. The effects lasted for different periods, up to > 1 year in 2 cases. 5 of 8 patients showed a decrease in levels of circulating immune complex bound IgG after 6 immunoadsorptions.
Based on these promising results, a randomized, controlled, clinical trial with a larger group of patients is warranted to evaluate the clinical and immunological effects of this therapy in SLE.

Another possible new therapeutic approach for autoimmune diseases, which however is still in the research phase, is antigen-specific immunotherapy. This potential future perspective is discussed in the following publication:

*Peakman M, Dayan CM (2001)*
**Antigen-specific immunotherapy for autoimmune disease: fighting fire with fire?**
Immunology 104, 361 - 366

Antigen-specific immunotherapy is based on the observation some decades ago that prior systemic administration of a protein antigen could inhibit the subsequent generation of an immune response to the same antigen. In fact, this strategy has resulted in protection from disease in a large range of animal models. However, most

of these models were not spontaneous models of human autoimmune disease, but models induced by administration of antigen under immunizing conditions. One exception are non-obese diabetic (NOD) mice that spontaneously develop diabetes with islet infiltration and complete destruction of ß-cells.
In the presented review, several possible mechanisms for antigen-induced immunosuppression such as induction of anergy or regulatory T cells, and immune deviation are discussed. Additionally, advantages and disadvantageas of the application of peptides compared to whole protein antigens are presented.

One critical aspect for the use of antigen-specific immunotherapy against human autoimmune diseases is the timing of therapy. Peptide immunotherapy in rodents has generally been administered at an early stage of disease (e.g. 4 – 6 weeks of age in NOD mice) or before priming in models in which disease is induced by immunization. At these stages it has been comparatively easy to achieve major effects on disease with a single peptide. Human autoimmune diseases with their insidious, asymptomatic onset, however, are usually diagnosed at more advanced stages, when organ infiltration and damage are already established. To achieve a detectable benefit under these conditions, peptide immunotherapy will probably need to be administered by the optimal protocol and target multiple epitopes of more than one antigen.
The most relevant prerequisite for the use of antigen or peptide immuntherapy in man is that it is safe. Possible severe side effects might include exacerbation of the developing disease or precipitation of new autoimmune diseases as well as induction of hypersensitivity.

All these aspects have to be studied thoroughly and have to be clarified, before it can be evaluated if antigen-specific immunotherapy with its great potential benefit may be successfully applied for the treatment of human disease.

January 01/02

# ANA Testing

One important tool for diagnosis of connective tissue disease (CTD) is detection of antinuclear antibodies (ANA) in patients' sera. The classically used screening technique for ANA is indirect immunofluorescence (IIF) on HEp-2 cells. Positive fluorescence staining indicates the presence of ANA but does not allow precise identification of these autoantibodies. For that purpose, additional testing is required, employing techniques such as ELISA, immunodiffusion, western blotting or, the more recently developed, line immunoassay (Innogenetics). Anyway, only defined fine specificities of ANA (and not merely their presence) are characteristic for certain CTDs and may provide valuable clues to the diagnosis.

It is interesting to find out, which is the probability of detecting ANA in a series of sera referred for routine testing, employing IIF on HEp-2 cells as initial screen, and how the various autoantibodies are distributed among ANA positive serum samples. A study aimed at providing data on these issues was recently performed at the Department of Rheumatology at the University Hospital of Ghent. It is described in the following publication:

*Peene I, Meheus L, Veys EM, DeKeyser F (2001)*
**Detection and identification of antinuclear antibodies (ANA) in a large and consecutive cohort of serum samples referred for ANA testing**
Ann Rheum Dis 60, 1131 - 1136

Serum samples from 10550 consecutive patients (1 sample/patient) that had been sent to the rheumatology laboratory at Ghent University Hospital during a three year period were screened for ANA positivity at a 1:40 dilution employing IIF on HEp-2 cells. In ANA positive samples, further identification of the fine reactivities of these autoantibodies was obtained with IIF on Crithidia luciliae (for detection of anti-dsDNA antibodies) and by immunodiffusion and line immunoassay (for detection of antibodies against extractable nuclear antigens (ENA)).

The prevalence of positive ANA testing by IIF on HEp-2 cells in the analysed population amounted to 23.5 %. In this context it is worthwile being mentioned that the outcome of IIF testing is known to be highly dependent on the chosen dilution of the sera, which in this case was set at 1:40 and therefore should permit highest sensitivity of the screening test. With respect to the low dilution of patients' sera, the observed percentage of positive samples is rather small, the more so since Tan et al. found 31.7 % positivity in healthy individuals at the same dilution (Tan EM, Feltkamp TEW, Smolen JS et al. (1997) Range of Antinuclear Antibodies in "Healthy" Individuals. Arthritis Rheum 40, 1601 – 1611). Although this discrepancy may be due to methodological differences, it should be emphasized that screening for ANA positivity only at a 1:40 dilution nowadays is not well accepted.

In the authors' study the most prevalent fluorescent pattern was speckled (42.5 %), followed by homogeneous (41.4 %), nucleolar (10.6 %) and centromere (3.9 %).

1986 of these ANA positive serum samples were further analysed using the described techniques. In 21.4 % of them at least one fine reactivity could be identified. This comparably small percentage may be explained by the use of the low sera dilution of 1:40 for the initial screening. The single specificities were distributed as follows: 3.2 % anti-dsDNA, 15.8 % anti-ENA (anti-SSA, anti-SSB, anti-RNP, anti-Sm, anti-Scl70, anti-

Jo-1), 0.5 % anti-rRNP and 4.0 % anti-CENP-B. In a substantial number of patient serum samples, multiple reactivities were identified (two reactivities in 6.6 % , three reactivities in 1.0 %, more than 3 reactivities in 0.3 %). The most common identified antinuclear autoreactivity was directed against SSA (10.5 %) and SSB (6.7 %). In any case, the stronger the fluorescence intensity in the initial ANA screening, the higher the detection rate of fine specificities. For the detection of single ENA reactivities, line immunoassay was more sensitive than immunodiffusion (15.4 % vs. 7.7 %). The higher sensitivity could mainly be attributed to the better detection by this assay of anti-Ro 52 and anti-SSB antibodies. On the other hand, the specificity of this recently developed dot blot variant was not analysed. It may be summarized that about 1/5 of consecutive serum samples sent for ANA testing to the Department of Rheumatology (University Hospital Ghent) were ANA positive when tested with IIF on HEp-2 cells (1:40 dilution). A positive ANA result, however, has only weak predictive value for diagnosing SLE or other CTD (even in serum samples specifically referred for ANA testing) so that additional tests for the identification of fine specificities are required that significantly increase the predictive diagnostic value up to a level that is of real diagnostic significance in specialist practice. By employing such additional tests, the authors found that about 1/5 of ANA positive patients displayed defined ANA reactivities related to CTD, thus being of practical relevance for diagnosis.

These results clearly show that initial screening for ANA employing IIF on HEp-2 cells as tool for diagnosis of CTD is a sensitive, but highly unspecific method. More specific techniques such as ELISA-based ANA screens with their defined, restricted and adaptable antigen panels may represent valuable alternatives and should be evaluated with emphasis.