Immunoblot as a Diagnostic Tool in Neurosyphilis

Immunoblot zur Diagnostik der Neurosyphilis

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Summary: The sensitivity of the TPHA-test and the immunoblot regarding the detection of intrathecally produced *Treponema pallidum* (T.P.) specific antibodies was compared in 20 patients with and without neurological symptoms and positive TPHA screening in the serum. By immunoblot analysis we were able to detect a local immune response directed against the highly specific 14-, 17 kd antigens of T.P. in 16 of 20 cases. In contrast the iTPA-index (intrathecally produced *Treponema pallidum* antibody index) was positive (> 2) in only nine of these 16 cases. Equivocal results were obtained in a further two cases. Four patients proved to be negative after using both methods. None of the 20 control CSF and serum samples obtained from patients with negative TPHA and VDRL test results showed a staining of the 14-, 17 kd antigen. Under consideration of several activity parameters, ten neurosyphilis cases were classified as active disease (six patients with a positive iTPA-index and immunoblot, four with a positive immunoblot solely). Therefore, the immunoblot is recommended as a diagnostic tool in suspected neurosyphilis with a negative or borderline iTPA index.

Keywords: Neurosyphilis/diagnosis; Treponema Pallidum; Blotting, Western.

Zusammenfassung: Die Sensitivität von Immunoblot und TPHA-Test wurde verglichen bezüglich des Nachweises einer treponemenspezifischen intrathekalen Antikörpersynthese. Untersucht wurden 20 Patienten mit einem positiven TPHA-Test im Serum sowie der Verdachtsdiagnose Neurosyphilis. Mit dem Immunoblot gelang bei 16 von 20 Patienten der Nachweis einer lokalen Antikörpersynthese gegen die hochspezifischen 14 kd- und 17 kd-Antigene von *Treponema pallidum*. Im Gegensatz dazu war der iTPA-Index (intrathekal produzierte *Treponema pallidum*-Antikörper) nur in 9 dieser 16 Fälle positiv (über 2). Grenzwertige Resultate lagen bei zwei weiteren Fällen vor. Vier Patienten waren mit beiden Methoden negativ. Bei keinem Patienten der Kontrollgruppe mit negativem TPHA- und VRDL-Test konnten im Liquor oder Serum Antikörper gegen das 14- und 17 kd-Antigen nachgewiesen werden. Unter Berücksichtigung verschiedener Aktivitätsparameter lag eine behandlungsbedürftige Neurosyphilis bei 10 dieser 16 Patienten vor. Ohne Einsatz des Immunoblots hätte in vier dieser Fälle die Diagnose Neurosyphilis serologisch nicht gesichert werden können. Zusammenfassend erscheint die zusätzliche Durchführung des Immunoblots empfehlenswert bei der Verdachtsdiagnose Neurosyphilis und Vorliegen eines negativen bzw. grenzwertigen iTPA-Index.

Schlüsselwörter: Neurosyphilis/Diagnostik; Treponema pallidum; Western Blot.

Neurosyphilis remains an important and frequently encountered entity in industrialized countries with an incidence of one to two cases per 100 000 inhabitants [1]. Since the introduction of antibiotics the clinical pattern has changed and more patients are presenting with atypical forms [2].

The diagnosis of "possible neurosyphilis" can be made if two of the following three criteria are found in a patient with a positive serological finding in serum (*Treponema pallidum* hemagglutination assay (TPHA) or fluorescent treponemial antibody absorption test (FTA-Abs))

(1) acute or chronic neurological disease of unknown origin,

(2) pathological cerebrospinal fluid (CSF) findings,

(3) clinical improvement or CSF normalization after antibiotic therapy.

However, the only way to establish the diagnosis and to detect asymptomatic cases is the detection of intrathecally produced *Treponema pallidum*-specific antibodies usually measured by the TPHA-test and the calculated iTPA-index [3]. Recently several reports [4,5,6,7] have indicated that the *Treponema pallidum* immunoblot might be more sensitive and specific than the TPHA-test.

Therefore we compared the sensitivity of the TPHA-test and the immunoblot regarding the detection of intrathecally produced *Treponema pallidum* specific antibodies. In addition we compared the classical activity parameters, i.e. CSF pleocytosis and the results of the venereal disease research laboratory (VDRL) test with the detection of IgM antibodies in serum and CSF by the immunoblot. As a study group we have chosen patients with and without neurological

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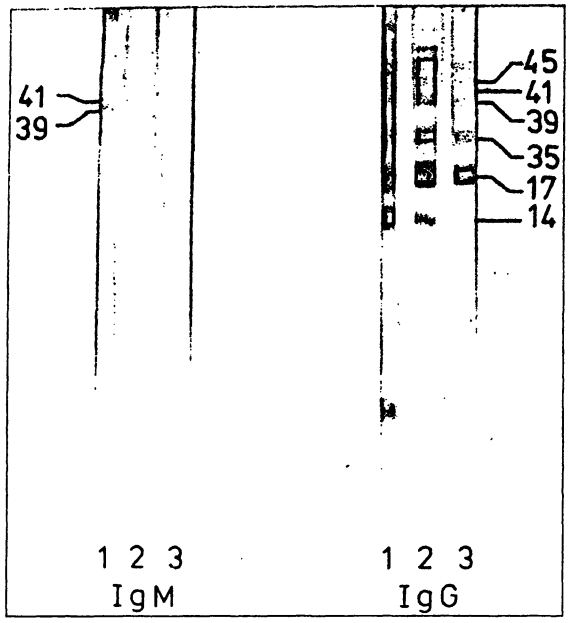

Figure 1 *Treponema pallidum* immunoblot of a patient with general paresis (case 4). Neurosyphilis is proven by a specific intrathecal IgG immune response. No highly specific IgM antibodies are detected despite an acute symptomatology, inflammed CSF and a positive VDRL-index.
Left side: Lane 1: IgM antibodies directed solely against the less specific 39 and 41 kd antigen in serum. Lane 2: (CSF) and lane 3 (diluted serum) showed no staining. All samples were diluted 1:100.
Right side: Strong IgG antibody bands against specific (14,17 kd) *Treponema pallidum* antigens in serum (lane 1), CSF (lane 2) and diluted serum (lane 3). The IgG bands of CSF are more intensive than the bands of diluted serum proving an intrathecal antibody synthesis. All samples were diluted 1:100. The IgG content in the paired CSF (lane 2) and diluted serum (lane 3) was 0.154 mg/dl.

symptoms and a positive TPHA screening in the serum.

Patients and methods

Between 1990 and 1993 a CSF analysis was performed in 20 TPHA seropositive (TPHA titres: 1:327680-1:320) patients (13 male, 7 female). Clinical and laboratory features are presented in Table 1 and 2. The age ranged from 15-76 years (median: 53 years). In all symptomatic patients extensive neurological, neuroradiological and neurophysiological diagnostic procedures were performed to classify the clinical picture. In case of a proven intrathecal-specific immune response the patients were further classified into different neurosyphilitic syndromes (e.g. general paresis) following the recommendations of *Prange* [3,8]. Routine laboratory investigation of CSF included cell count, total protein, lactate, albumin and IgG (nephelometric determination, Behringwere AG, Marburg, FRG). For the detection of CSF-restricted oligoclonal bands the isoelectric focusing method (Ampholine® PAGplate, Pharmacia, FRG) with protein staining was used. Serum and CSF was examined simultaneously. In all patients a *Borrelia burgdorferi* (B. b.) immunoblot and a ELISA (Sigma, Deisenhofen, FRG) was performed as described previously [9] to exclude a Borreliosis.

The TPHA-titres were carried out by routine procedures.

The differentiation between passive IgG transfer from serum and local synthesis of *Treponema pallidum*-specific IgG in the CNS was performed by calculating the iTPA-index (intrathecally *Treponema pallidum* antibody) as described by *Prange* [3]

$$\frac{\text{TPHA - titre (CSF) x total IgG (serum)}}{\text{total IgG (CSF) x TPHA - titre (serum)}}$$

A similar index was calculated for the VDRL test. An iTPA and VDRL index > 2 was regarded as positive.

As a control group, 20 patients (9 female, 11 men) with other neurological diseases and a negative *Treponema pallidum* (TPHA, VDRL) and *Borrelia burgdorferi* (ELISA, IFT-abs) serology were chosen.

The immunoblotting technique (Fig. 1) was performed as previously described by *Hensel* [7].

Adult male rabbits were used for the passage of *Treponema pallidum.* When orchitis developed, rabbits were sacrificed and the testes removed aseptically, sliced and washed repeatedly with 10 ml of RPMI 1640 medium. Cellular debris was removed by centrifugation at 1400 *g* for 30 minutes at 4 °C. The pellets containing the treponemes were washed three times with 0.2 mol/l phosphate-buffered saline (pH 7.4) containing 0.001mol/l dithioerythrit (DTE). The treponemes were finally concentrated c. 100-fold to the primary volume by centrifugation. 1 ml of the samples was dissolved in 150 µl solubilizing buffer (20% sodium dodecyl sulfate [SDS], 1.5 mol/l DTE, 0.1 mol/l Phenylmethylsulfonylfluorid [PMSF]), boiled for 10 minutes and subjected to slab SDS polyacrylamide gel (15%) (90 mm long, 1 mm thick) and ran at 30-40 mA for 16-18 hours. Proteins were transferred onto polyvinylidene difluoride sheet (Immobilon®, Millipore, USA). Transfer efficiency was controlled by staining the paper blots with amido black. After protein transfer, the blots were incubated for 1 hour in blocking buffer (0.02 mol/l Tris, 0.5 mol/l NaCl, 0.002 mol/l E-Aminocapronacid, 0.0001 mmol/l PMSF, 0.001% gelatine, 1% fetal calf serum, 0.1 mg/ml Gentamycin) and stored dry at -70 °C until use.
The blotted membranes were probed with serum and CSF samples, both diluted 1:100 in blocking buffer. In addition serum specimens diluted to the same IgG con-

Non standard abbreviations: CSF, cerebrospinal fluid; DTE, dithioerythrit; ELISA, enzyme-linked immmunosorbent assay; FTA-Abs, fluorescent treponemal antibody absorption (test); iTPA, intrathecally produced *Treponema pallidum* antibody; PMSF, Phenylmethylsulfanylfluorid; T.P., *Treponema pallidum;* TPHA, *Treponema pallidum* hemagglutination assay;VDRL,Venereal Disease Research Laboratories.

Table 1 Clinical findings in 20 patients with different clinically based syphilitic syndromes***

case no.	age (year) sex*	symptomatology	symptoms** duration	progressive	therapy past	therapy now	syphilitic syndrome	additional diagnosis
1	35 m	psychosis	4 weeks	yes	no	yes	general paresis	Ø
2	43 m	stroke	1 week	yes	no	yes	meningovasc.neurosyph.	Ø
3	32 m	none	Ø	Ø	no	yes	asymptomatic neurosyph.	Ø
4	55 f	dementia	8 weeks	yes	no	yes	general paresis	Ø
5	54 m	dementia	20 years	no	yes	yes	general paresis	Ø
6	68 m	dementia	8 years	no	yes	no	general paresis	Ø
7	81 m	personality changes	1 year	yes	no	yes	unclassificable neurosyph.	Ø
8	38 m	none	Ø	Ø	yes	no	inactive neurosyph.	Ø
9	68 m	psychosis	3 days	yes	no	no	inactive neurosyph.	alcoholic delirium
10	52 m	psychosis	6 weeks	yes	no	yes	general paresis	Ø
11	73 f	tetraparesis	2 weeks	yes	no	no	inactive neurosyph.	chronic polyradiculoneuritis
12	15 f	headache	2 weeks	yes	no	yes	meningovasc. neurosyph.	Ø
13	53 m	personality changes	2 weeks	yes	no	yes	unclassificable neurosyph.	Ø
14	65 m	personality changes	6 years	yes	no	yes	unclassificable neurosyph.	Ø
15	55 f	depression	3 years	yes	yes	no	inactive neurosyph.	neurotic depression
16	76 f	stroke	4 weeks	no	no	no	inactive neurosyph.	intracerebral hemorrhage
17	27 m	skin lesion	8 weeks	yes	no	yes	secondary syphilis	Ø
18	36 f	none	Ø	Ø	no	no	latent syphilis	Ø
19	68 f	facial palsy	2 days	yes	no	no	latent syphilis	idiopathic facial palsy
20	60 m	paraparesis	3 weeks	yes	no	no	latent syphilis	meningeal carcinomatosis

* m = male; f = female
** prior to lumbar puncture
*** on the basis of clinical judgement and serological results, patients 19 had a positive iTPA and an immunoblot indicating an intrathecal antibody synthesis, 10–16 a positive immunoblot only, and 17–20 a normal CSF with no detectable intrathecal antibody synthesis (see Table 2)

Table 2 CSF analysis and serological findings in 20 patients with different clinically based syphilitic syndromes

		CSF analysis							T. pallidum immunoblot			
case no.	syphilitic* syndrome	cell count (mm^3)	protein (g/l)	oligoclonal bands	iTPA Index	serum VDRL	CSF VDRL	VDRL Index	i.a.s.** IgM	i.a.s.** IgG	serum IgM	serum IgG
1	GP	18	2.18	+	23.0	1:128	1: 8	5.8	+***	+	+	+
2	MV	14	0.82	–	8.5	1: 32	1: 1	4.2	–****	+	–	+
3	ASY	12	0.74	+	62.2	1: 64	1: 8	15.5	–	+	–	+
4	GP	8	0.67	+	4.9	1: 32	1: 1	12.2	–	+	–	+
5	GP	0	0.43	–	19.0	1: 16	–	–	–	+	–	+
6	GP	2	0.23	+	46.6	1: 2	–	–	–	+	–	+
7	UNCL	8	1.49	–	20.0	1: 2	–	–	–	+	+	+
8	NSY	0	0.71	+	12.2	1: 2	–	–	–	+	–	+
9	NSY	2	0.40	+	8.2	1: 8	–	–	–	+	–	+
10	GP	1	0.92	+	2.0	1:256	1:16	4.8	–	+	+	+
11	NSY	0	3.12	–	2.0	1: 4	–	–	–	+	–	+
12	MV	166	0.71	–	–	1: 2	–	–	–	+	–	+
13	UNCL	2	0.26	–	–	1: 16	–	–	–	+	+	+
14	UNCL	1	0.23	–	–	1: 2	–	–	–	+	+	+
15	NSY	1	0.32	–	–	–	–	–	–	+	–	+
16	NSY	0	0.36	–	–	1: 4	–	–	–	+	–	+
17	sec.syphilis	3	0.37	–	–	1: 4	–	–	–	–	+	+
18	lat.syphilis	1	0.40	–	–	1: 4	–	–	–	–	–	+
19	lat.syphilis	1	0.26	–	–	–	–	–	–	–	–	+
20	lat.syphilis	1	1.06	–	–	–	–	–	–	–	–	+

GP = general paresis, MV = meningovascular neurosyphilis, ASY = asymptomatic neurosyphilis, UNCL = unclassificable neurosyphilis, NSY = neurosyphilis, inactive
** i.a.s = intrathecal antibody synthesis (14-, 17 kd antigen)
*** = prescence of reactivity (+)
**** = absence of reactivity (–)

tent as the CSF were used to prove the intrathecal synthesis of *Treponema pallidum* specific antibodies. After incubation overnight at room temperature the blots were washed twice with washing buffer (20 mmol/l Tris, 500 mmol/l NaCl, pH 7.5) and incubated for 90 minutes with the appropriate peroxidase labelled second antibody (rabbit antihuman IgG, IgM) (Dako Immunglobulins, Copenhagen, Denmark) in a dilution of 1:1000. The colour was developed by incubating the blots in substrate solution (0.003 mol/l Carbazol, 0.18 mmol/l Dimethylformamid, 0.08 mol/l Natriumacetat, 0.02% H_2O_2) for 20 minutes.

The same luetic sera with a broad reactivity pattern was integrated in triplicate in all assays to ensure reproducibility of electrophoretic transfer. The molecular weights of the *Treponema pallidum*-specific proteins were calibrated with a low molecular weight marker kit (Pharmacia, Uppsala, Sweden). IgG and IgM antibody bands were evaluated qualitatively (eye-scanning) against several antigens of *Treponema pallidum* (Fig. 1). However, to favour specificity only IgM or IgG antibody bands against the highly specific 14- and 17 kd antigens were evaluated for diagnostic purposes [4,5,6,7,10].

An intrathecal synthesis was assumed if antibodies in the CSF were solely or more strongly detected than in the matched serum diluted to the same IgG concentration [11].

Results

By the immunoblot analysis (Fig. 1) we were able to detect an intrathecal IgG immune response against the *Treponema pallidum*-specific antigens (14 kd alone [n = 1] 17 kd alone [n = 4], 14- and 17 kd [n = 11]) in 16 of 20 cases (Tab. 2). In contrast the iTPA-index proved a local immune response in only nine of these 16 cases. Equivocal results were obtained in two cases by iTPA-index. Four cases proved to be negative by both methods. IgM antibodies were frequently detected by the immunoblot. However, antibodies against the highly specific 14- and 17 kd antigen showed only one patient in the CSF and a further five in the serum. In all cases a *Borrelia burgdorferi* infection could be excluded by serological methods.

None of the 20 examined control CSF and serum samples showed a staining of the 14- and 17 kd antigens. However slight reactions in the 41-, 45 kd range could be depicted in several control sera. In contrast the 20 sera from syphilitic patients recognized beside the evaluated antigens (14-, 17 kd) a broad variety of other *Treponema pallidum* proteins most commonly in the 60-, 47-, 45-, 41-, 39-, 35-, 30 kd range.

In nine patients (Tab. 1 and 2, case 1-9) with an equally diagnostic iTPA-index and immunoblot the activity parameters were analysed. In three untreated patients [(meningovascular neurosyphilis, basilar artery thrombosis, case 2), (asymptomatic neurosyphilis, case 3) and (general paresis, case 4)] with pleocytosis no specific IgM antibodies in the serum and CSF were found, but a positive VDRL index indicated an intrathecal synthesis of cardiolipin-specific antibodies (Table 2). In contrast patient No. 7 presenting with personality changes had IgM serum antibodies but only a low VDRL titre. No IgM antibodies or pleocytosis were depicted in two patients with a general paresis lasting for a decade and treated several times with intravenous penicillin. Case 8 and 9 had an inactive neurosyphilis with low to medium VDRL-titres only.

In a second group (Table 1 and 2, case 10-16) with negative or equivocal iTPA indices several patients showed active neurosyphilis. Case 10 with a borderline index had the characteristic clinical feature of a general paresis with the classical grandiose delusional state and case 12 a meningovascular neurosyphilis presenting with a meningitis. The patients No. 13 and 14 developed over years personality changes and depression. These patients had either *Treponema pallidum*-specific IgM serum antibodies (case 10, 13, 14) or/and a high VDRL titre (> 1:8) (case 10, 13) or/and a pleocytosis (case 12) indicating active disease. A inactive neurosyphilis was found in cases 11, 15 and 16 with the clinical diagnosis of chronic polyradiculoneuritis, neurotic depression and intracerebral hemorrhage. All activity parameters were negative in these cases.

In four cases (Table 1 and 2, case 17-20) neither the immunoblot nor the iTPA index indicated *Treponema pallidum*-specific antibody synthesis in the CSF. One of these patients (case 17) had an active syphilis with pink macules on the trunk and specific IgM antibodies in serum.

A clear correlation between non-specific CSF parameters and the clinical picture were infrequently found in cases of neurosyphilis: - e.g. case 11 had an elevated total protein and a polyradiculitis, case 3 had no symptoms but a typical inflammed CSF and in contrast case 10 (general paresis) lacked a pleocytosis.

Discussion

The *Treponema pallidum* Western immunoblot assay was introduced in 1985 by *Hensel* [7] as a potential confirmatory test for syphilis. Its superiority to other tests was confirmed by various other authors. *Byrne* [5] found a specificity and sensitivity of the immunoblot of 100% and 93.8% vs. the FTA-abs of 91.7% and 92.0% in clinical defined samples. *Hensel* [7] reported immunoblot positivity in 60% of sera with a borderline TPHA-test. *Dettori* [6] followed eight patients over four years who passed from a positive to a negative TPHA-test in the serum, but were still reactive in the immunoblot. For the immunoblot the endpoint of reactivity was at least 3 to 4 serial dilutions greater than that for the FTA-abs test.

In addition by immunoblot analysis not only the immunoglobulin class but also the specificity of the single antibody can be determined. This is important because *Treponema pallidum* shares common epitopes with other bacteria. Therefore the presence of a single antibody population that is cross-reactive with *Treponema pallidum* can be sufficient to produce a positive TPHA-test. The 14- and 17 kd antigens are generally considered specific for *Treponema pallidum* infections [4,5,6,7,10] and were observed in all our patient sera but not in the control group. Beside these antigens *Treponema pallidum* sera recognized a wide variety of other *Treponema pallidum* proteins (e.g. 60, 47-, 45-, 41-, 39-, 35- and 30 kd). However the

occurrence of antibodies against these antigens varies and cross-reactivity occurs.

The most important serological pitfall can occur in borreliosis, therefore it is necessary to exclude in all patients a Borrelia infection. This is possible with a B. b. immunoblot. Patients with a B. b. infection have a different band pattern as in syphilis and usually show no reactivity in the 14, -17 kd range. [Wellensiek, H. J., personal communication].

Rarely the immunoblot technique was applied to the CSF [6,7] and to our best knowledge, only in one study [4] CSF and serum samples diluted to the same total IgG content were examined. This is important to differentiate locally produced antibodies from antibodies which are derived from the serum [11,19]. In our study we were able to detect with the immunoblot in 16 of 20 cases with a positive TPHA-titre in serum a local *Treponema pallidum*-specific immune response directed against the 14- or/and 17 kd antigen. With the reference method (iTPA index) in intrathecal production was proven in only nine cases. In a further two cases equivocal results were obtained. These findings are supported by the observation of *Prange* [3] who, with the iTPA-index found, a local synthesis of specific antibodies in only 82% of 261 patients with clinical symptoms of neurosyphilis. However, *Bollensen* [4] reported different experiences with a immunoblot positivity in only three of eleven patients with various syphilitic conditions. In his patients the iTPA-index was strongly positive in five cases, further three had indices of 2.2, 2.1 and 1.7 (values between 1.5 and 2 were regarded as equivocal). The lower sensitivity of the immunoblot in this study might be due to technical reasons (e.g. antigen preparation, blotting conditions).

A local specific IgM production was detected in only one patient (case 1, immunoblot not shown), further five patients showed IgM antibodies solely in the serum (14-, 17 kd antigen). No good correlation was seen with the VDRL-titre, the traditional activity parameter. However recent studies have shown that false-negative reactions by VDRL-titre occur in 30% of patients with neurosyphilis [12,13,14,15]. False positives were reported in a wide variety of conditions such as pregnancy, chronic and acute infections. Usually such cases produce titres less than 1:8 [1]. Generally the detection of *Treponema pallidum*-specific IgM antibodies is regarded as the best activity parameter. However, in late stages of active neurosyphilis the immune response might already have switched to a predominating IgG response. In addition to favour specificity we evaluated only IgG and IgM antibodies against the highly specific 14-, 17 kd antigens. The high dilution of CSF and serum (1:100) in this study might be a further explanation for the lack of correlation between the occurence of IgM antibodies and a positive VDRL-titre as seen in all patients.

The cell count is generally a good activity parameter. However, the cell count was normal in three neurosyphilitic patients with specific IgM antibodies in the serum or a positive VDRL-index indicating active disease. Similar experiences made several other authors [8,15,16]. In untreated neurosyphilis a pleocytosis was reported in only 50-80%.

With the immunoblot a neurosyphilis was proven in 16/20 cases by the detection of a *Treponema pallidum*-specific intrathecal antibody synthesis. Using solely the iTPA-index the serological confirmation of a secondary CNS-invasion of *Treponema pallidum* would have been missed in seven cases. In four of these 16 patients the current clinical symptomatology was most likely due to other diseases (Table 1). Therefore they were classified additionally to the cases 3 and 8 as inactive neurosyphilis. The clinical pictures of the remaining ten patients were compatible with a general paresis, meningovascular or unclassificable neurosyphilis.

No single parameter was sufficient for itself as a activity criteria in our patient group. Therefore, we propose in accordance with other authors [8,12,13] to treat patients with neurosyphilis, if they have a pleocytosis or/and a positive VDRL index, respectively a VDRL-titre > 1:8 in the serum or/and specific IgM antibodies in serum or CSF.

Ten patients with neurosyphilis met these criteria and were subjected to an intravenous high-dose penicillin therapy. Four had a general paresis (case 1, 4, 5, 10), two a meningovascular neurosyphilis (case 2, 12), three an unclassificable neurosyphilis (case 7, 13, 14) and one an asymptomatic neurosyphilis (case 3). Without the immunoblot four patients would have been not correctly classified and probably treated only with intramuscular penicillin which is insufficient in neurosyphilis.

In the remaining six patients (case 6,8,9,11,15,16) the negativity of the activity parameters correlated with the clinical picture. Five cases were asymptomatic or had other diseases and one had a treated general paresis, clinically unchanged for years. The positive oligoclonal bands in three of these patients are most likely consistent with an immunological scar syndrome and are not due to a *Treponema pallidum* persistence [8,17,18]. A syphilitic CNS manifestation could be excluded serologically in case 17 to 20. In case 17 an intramuscular penicillin therapy was initiated after a syphilitic skin lesion and specific IgM antibodies were found.

In conclusion we strongly recommend the immunoblot as a diagnostic tool in suspected neurosyphilis cases with a negative or borderline iTPA index. However, clinical judgement is still essential in differentiating inactive from active neurosyphilis cases.

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