Supplementary Online Content

Meijer JM, Diercks GFH, de Lang EWG, Pas HH, Jonkman MF. Assessment of diagnostic strategy for early recognition of bullous and nonbullous variants of pemphigoid. *JAMA Dermatol.* Published online January 9, 2019. doi: 10.1001/jamadermatol.2018.4390

This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. STARD 2015 checklist

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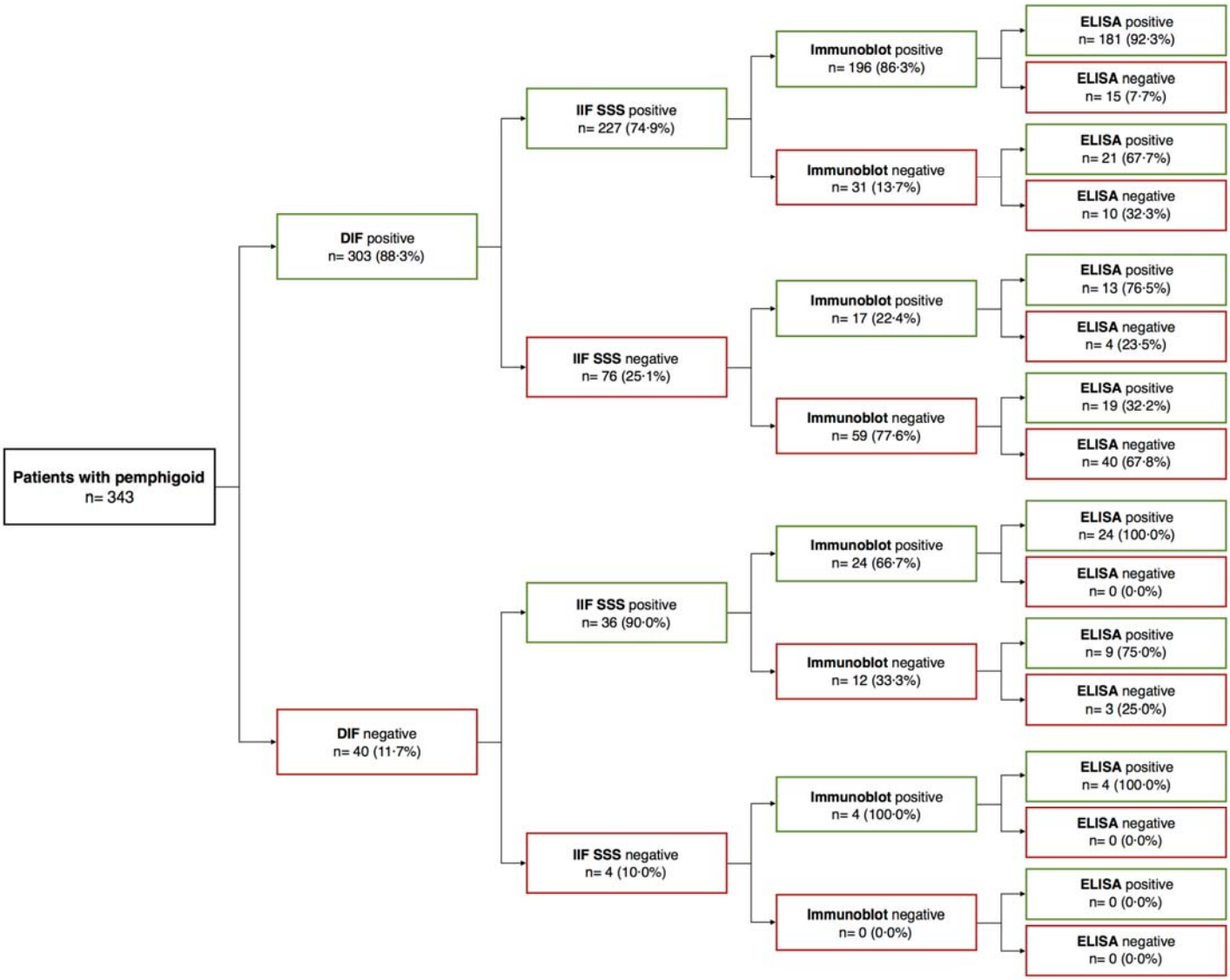
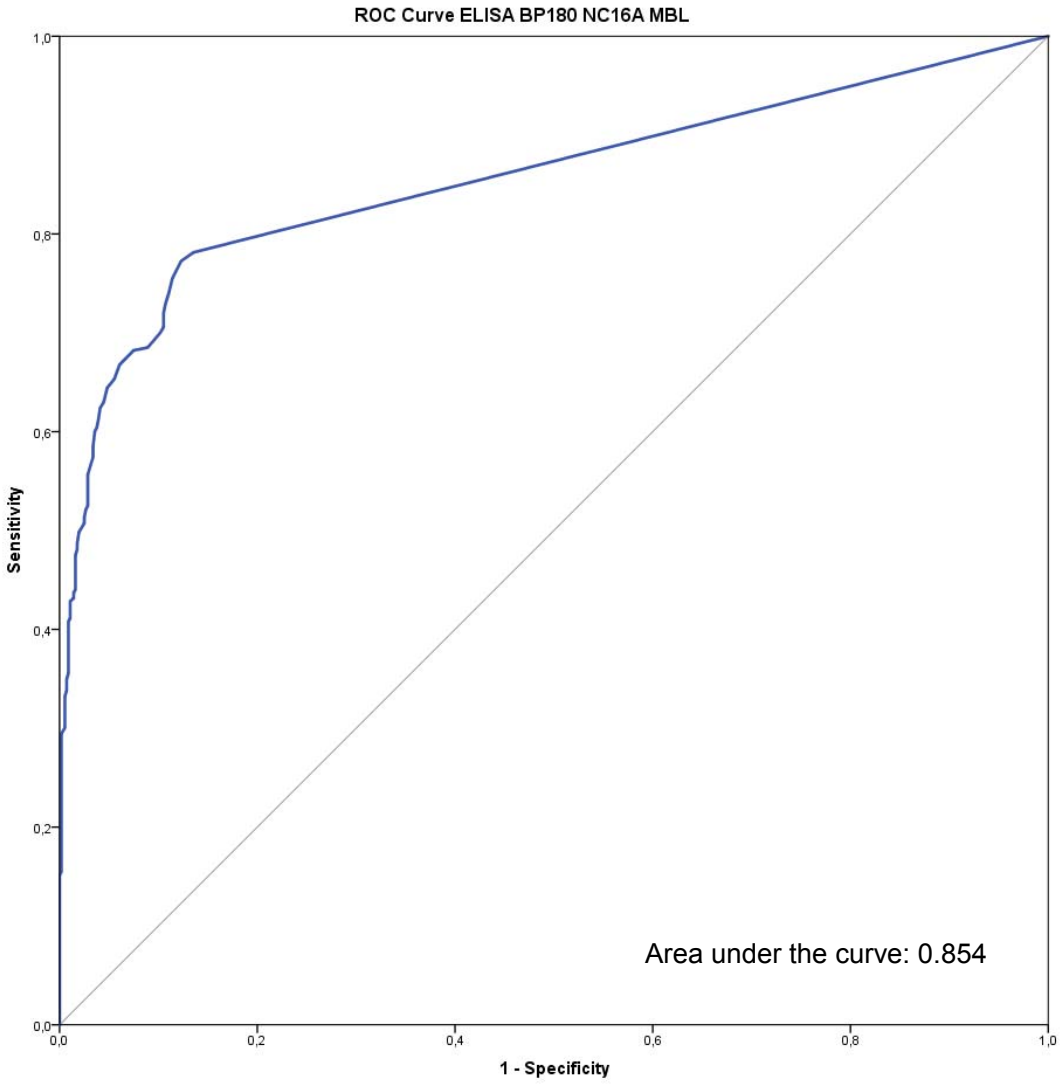
eFigure 1. Flow chart complementary to Figure 2

Flow chart complementary to Figure 2 indicating ratios of test positivity and negativity.



eFigure 2. ROC curve, AUC and cross tabulation of BP180 NC16A ELISA

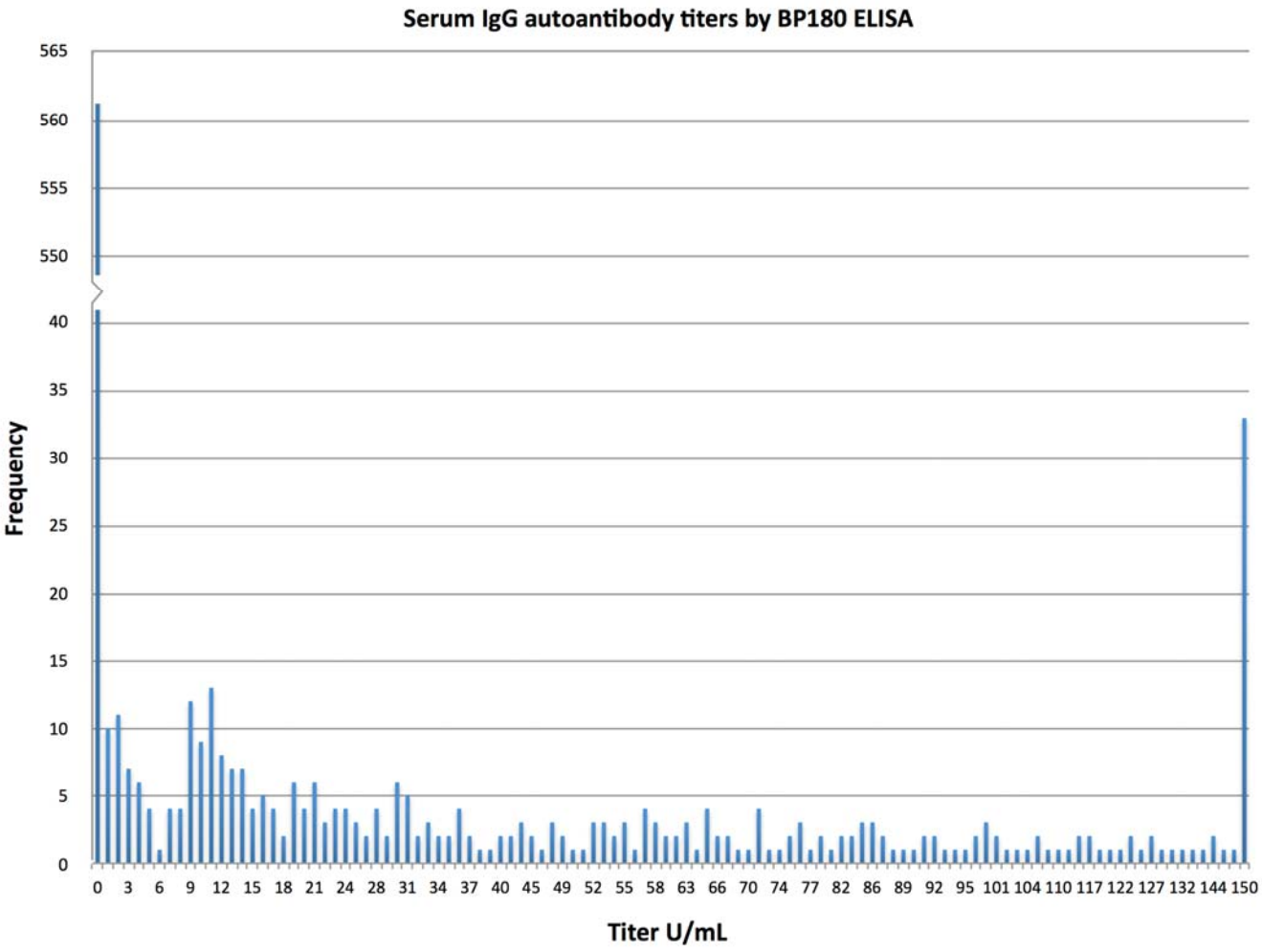
ROC curve, area under the curve (AUC) and cross tabulation of BP180 NC16A ELISA (MBL) for diagnosis of pemphigoid, based on manufacturers positivity cut-off 9 U/mL (MBL) and the reference standard.



ELISA BP180 NC16A	Pemphigoid present		Pemphigoid not present		Total		Predictive values	
Test positive	True-positive, TP	240	False-postive FP	57	TP+FP	297	PPV = TP/(TP+FP)	80,81%
Test negative	False-negative, FN	103	True-negative TN	504	TN+FN	607	NPV = TN/(TN+FN)	83,03%
Total	TP+FN	343	FP+TN	561		904		
	Sensitivity = TP/(TP+FN)	69,97%	Specificity = TN/(FP+TN)	89,84%				

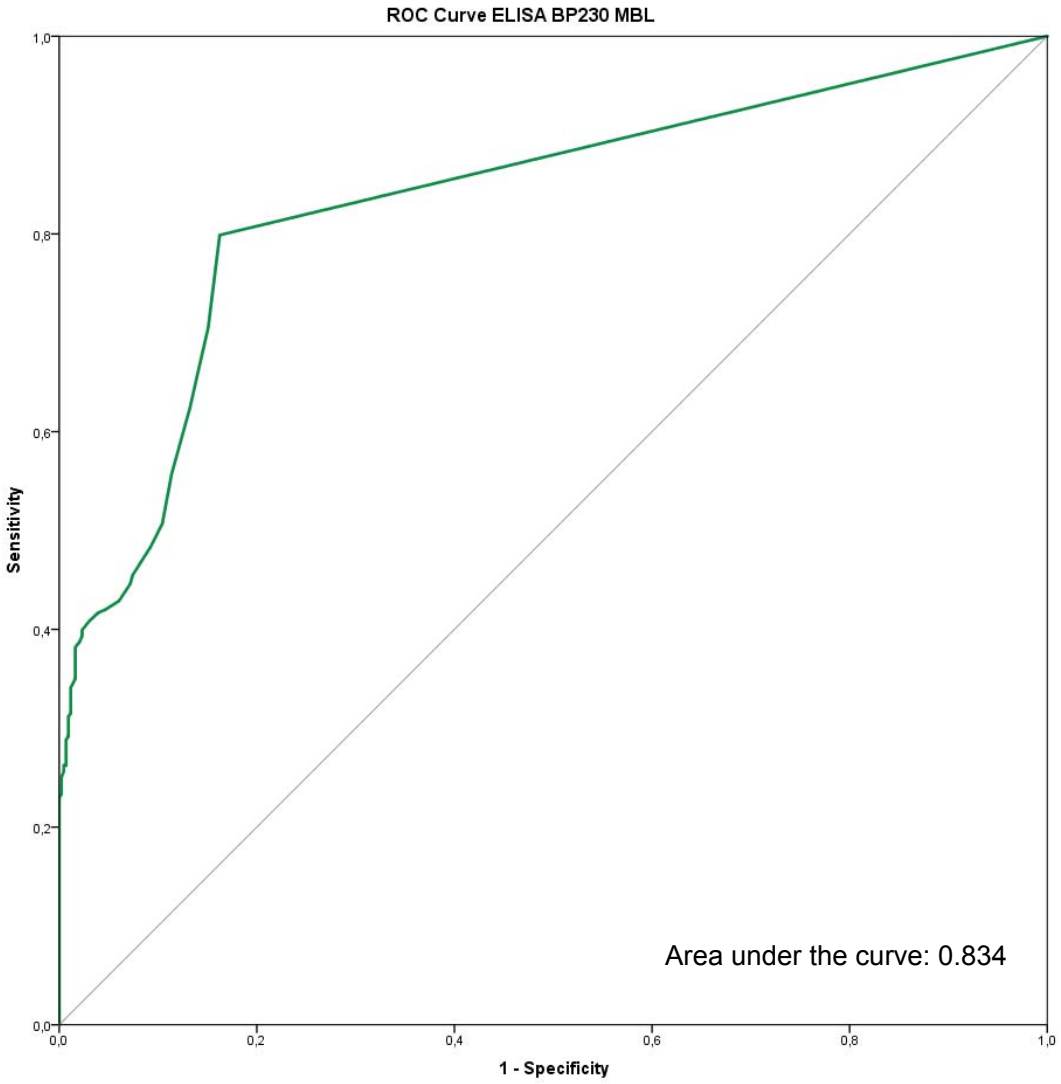
eFigure 3. Distribution of test results of BP180 NC16A ELISA

Distribution of IgG autoantibody serum concentrations of BP180 NC16A ELISA.



eFigure 4. ROC curve, AUC and cross tabulation of BP230 ELISA

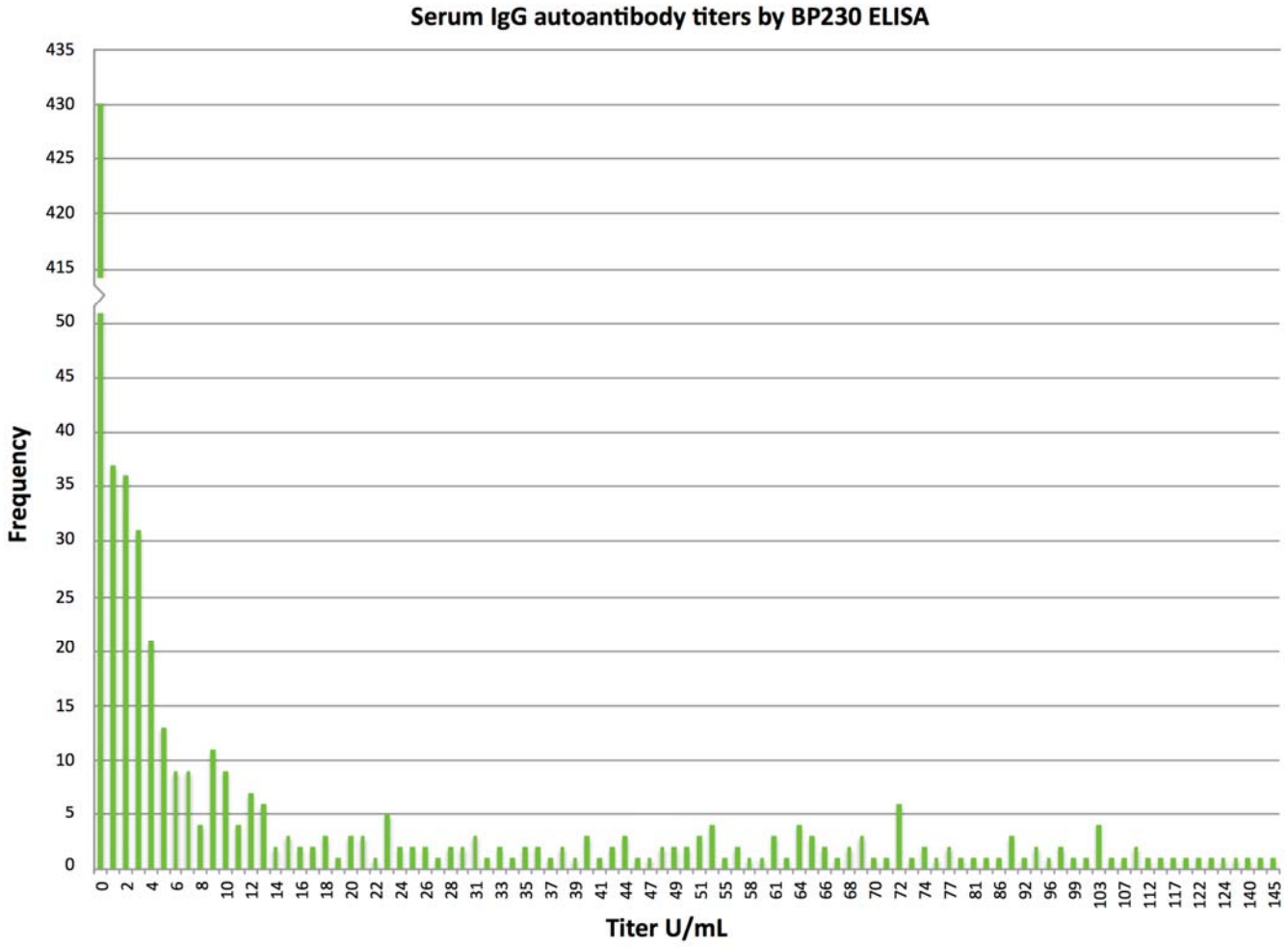
ROC curve, area under the curve (AUC) and cross tabulation of BP230 ELISA (MBL) for diagnosis of pemphigoid, based on manufacturers (MBL) positivity cut-off 9 U/mL and the reference standard.



ELISA BP230	Pemphigoid present		Pemphigoid not present		Total		Predictive values	
Test positive	True-positive, TP	153	False-postive FP	31	TP+FP	184	PPV = TP/(TP+FP)	83,15%
Test negative	False-negative, FN	190	True-negative TN	400	TN+FN	590	NPV = TN/(TN+FN)	67,80%
Total	TP+FN	343	FP+TN	431		774		
	Sensitivity = TP/(TP+FN)	44,61%	Specificity = TN/(FP+TN)	92,81%				

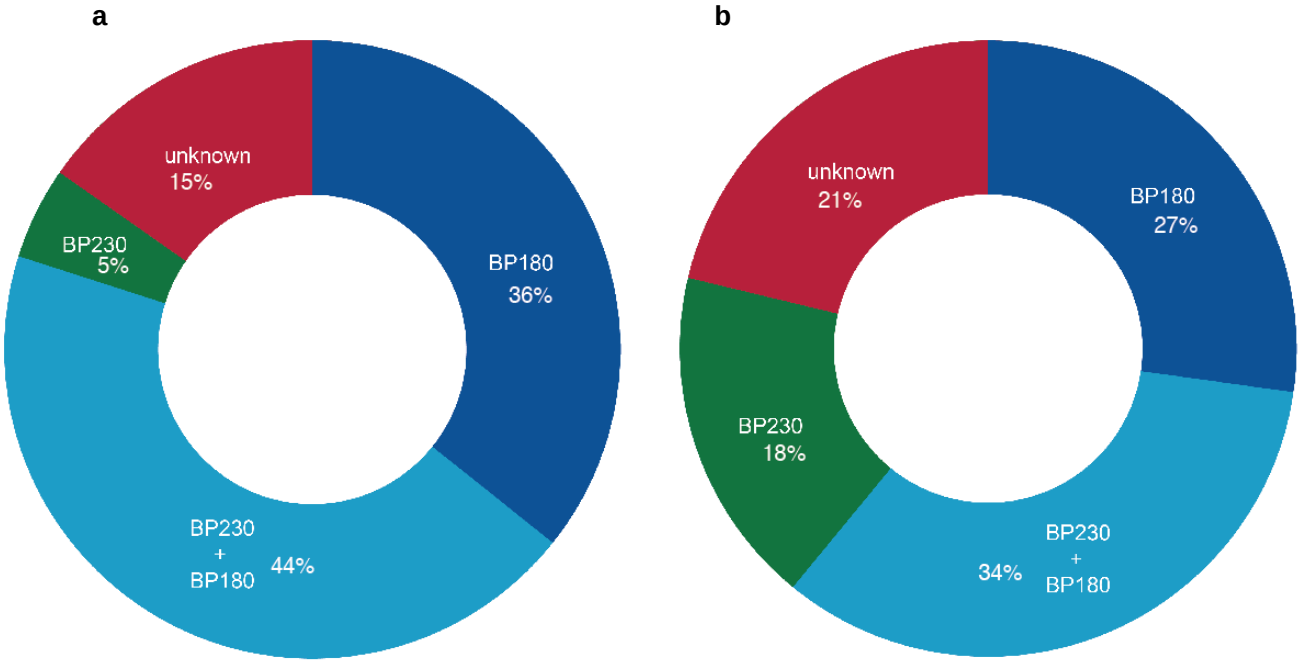
eFigure 5. Distribution of test results of BP230 ELISA

Distribution of IgG autoantibody serum concentrations of BP230 ELISA.



eFigure 6. Distribution of target autoantigens BP180 and BP230 in patients with pemphigoid

Targeted autoantigens in patients with bullous pemphigoid (a; n=239) and nonbullous pemphigoid (b; n=74). Significantly more often solely BP230 as target autoantigen (b; 18% in green), and absence of detectable circulating anti-BP180 autoantibodies (dark blue) in patients with nonbullous pemphigoid.



eTable1.

eTable 1. Distribution of age groups and predictive value for diagnosis of pemphigoid.				
Age group (years)	**n**		**Odds ratio**	**p-value**
<49	271			
50-54	76		1.16	.68
55-59	83		0.86	.67
60-64	85		1.96	.03
65-69	90		2.53	.001
70-74	121		2.28	.002
75-79	133		4.21	<.001
80-84	130		4.52	<.001
85-89	87		6.59	<.001
>90	49		9.66	<.001

RESEARCH PROTOCOL

‘Diagnostic accuracy of direct immunofluorescence and immunoserology in diagnosis of pemphigoid: towards minimal diagnostic criteria’

Retrospective Study

May 2015

PROTOCOL TITLE 'Diagnostic accuracy of direct immunofluorescence and immunoserology in diagnosis of pemphigoid: towards minimal diagnostic criteria'

Protocol ID	**201500760**
Short title	**Minimal criteria of pemphigoid**
EudraCT number	***Not applicable***
Version	**1.3**
Date	**1-05-2015**
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Sponsor	***University Medical Center Groningen***
Subsidising party	***University Medical Center Groningen***
Laboratory sites	***University Medical Center Groningen*** ***Immunodermatology Laboratory***
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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

BMZ	**Basement membrane zone / dermal epidermal junction**
BP	**Bullous pemphigoid**
C3c	**Complement C3**
CIs	**Confidence Intervals**
DIF	**Direct immunofluorescence**
ELISA	**Enzyme-Linked Immuno Sorbent Assay**
IB	**Immunoblot**
IIF	**Indirect immunofluorescence**
IgA	**Immunoglobulin A**
IgG	**Immunoglobulin G**
IgM	**Immunoglobulin M**
METC	**Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)**
MMP	**Mucous membrane pemphigoid**
MO	**Monkey oesophagus *substrate***
SSS	**Salt-split skin *substrate***
UMCG	**University Medical Center Groningen**
Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale: Pemphigoid is the most common subepidermal autoimmune blistering skin disease, which has a typical clinical presentation with tense blisters and pruritus (bullous pemphigoid). Pemphigoid mostly affects elderly: the mean age at time of diagnosis is over seventy years old. The atypical nonbullous clinical phenotype is characterized by pruritus and urticarial plaques or eczematous eruptions. This nonbullous variant contributes up to 20% of all pemhigoid patients. A diagnostic delay often occurs in the nonbullous presentations of pemphigoid, because it is often not recognized. Direct immunofluorescence microscopy (DIF) on a perilesional skin biopsy is the current reference standard for diagnosis of pemphigoid. Several immunoserological tests are used to confirm and subtype the diagnosis of pemphigoid and measure disease activity.

Objectives: to assess the diagnostic accuracy of available routine diagnostic tests for diagnosis of pemphigoid, including DIF on a skin biopsy and various immunoserological tests. More specific, to determine the additional value of indirect immunofluorescence on salt-split skin (IIF SSS) for diagnosis of pemphigoid. Secondary objectives are to compare various biopsy sites for DIF microscopy, to determine the optimal diagnostic procedure for pemphigoid and establish minimal diagnostic criteria.

Study design: retrospective, single-centre, multivariable diagnostic accuracy study. Subjects suspected for pemphigoid will be enrolled of whom between 2002 and May 2015 at least one skin biopsy for DIF and serum for IIF SSS and at least one other serological test (IIF on monkey esophagus, immunoblot BP180/BP230, ELISA BP180 NC16A/BP230) is performed. Paired skin biopsies and serum samples will be used from our UMCG biobank and registry database for collection of clinical data.

Intervention: not applicable.

Main study parameters/endpoints: primary study parameters of diagnostic accuracy are the binary classified outcomes of index tests DIF microscopy, IIF SSS, IIF MO and of immunoblot, and the continuous titers of ELISA assays with a positivity cut-off of ≥9 U/ml. Secondary study parameters are standardized biopsy sites for DIF biopsies, and area under the curve of ROC curve analysis of multivariate models with 95% CIs.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: not applicable in this retrospective cross-sectional study with biobank materials and registry database.

INTRODUCTION AND RATIONALE

Pemphigoid is the most frequent autoimmune bullous disease and mainly affects elderly above 70 years old, the disease is associated with a significant morbidity and a one-year mortality up to 6 times higher than age-matched controls.(1,2) Diagnosis of pemphigoid is based on detection of in-vivo and circulation auto-antibodies, targeted against hemidesmodomal structural proteins BP180 and BP230 of the skin and mucosa.(1) The annual incidence of pemphigoid in Europe has increased significantly in the last decades, which might be attributed to an increasing aging population, better laboratory diagnostic techniques, and the recognition of atypical clinical presentations of pemphigoid.(2,3) Typically, patients present with tense blisters and urticarial plaques on the skin, accompanied by intense pruritus. In recent years recognition and reporting of atypical nonbullous clinical presentations has increased. Nonbullous clinical features in up to 20% of patients with pemphigoid may consist of eczematous lesions, prurigo-like or urticarial lesions, or only pruritus and secondary excoriations.(4-6) These insights make clear pemphigoid of the skin is not always bullous, but a polymorphic disease with a spectrum of clinical presentations. The possibly difficult recognition of patients with pemphigoid with nonbullous clinical features emphasizes the need for an effective diagnostic strategy. A consensus of diagnostic criteria such as defined in mucous membrane pemphigoid is lacking.(7) Currently, the diagnosis of pemphigoid is based on several combined criteria of clinical features and clinical criteria, histopathology, DIF microscopy and immunoserology.(8,9) However, the clinical criteria are based on patients with typical bullous clinical features and each study may define patients with pemphigoid differently. The lack of minimal diagnostic criteria and a consistent reference standard is an obstacle for both diagnostic accuracy studies and clinical trials.(10) According to the 2015 European Guideline on diagnosis and management of pemphigoid, a positive perilesional biopsy for DIF is a necessity for diagnosis of pemphigoid.(11) In addition, the search for specific circulating IgG autoantibodies by immunoserological test, such as IIF, IB and ELISA are recommended for proper diagnosis and classification of pemphigoid subtypes, but only confirmative for pemphigoid together with positive DIF studies.

In this study, we aim to investigate the diagnostic accuracy of various available immunopathological and serological laboratory test for diagnosis of pemphigoid, in a large cohort with patients with both bullous and nonbullous clinical features. Furthermore, this study aims to assess the additional diagnostic value of indirect IF on salt-split skin, the optimal diagnostic strategy and minimal diagnostic criteria for pemphigoid.

OBJECTIVES

Primary Objective: to assess the diagnostic accuracy of routine diagnostic tests DIF microscopy, indirect immunofluorescence, immunoblot and ELISA, and to determine the additional value of indirect immunofluorescence on salt-split skin for diagnosis of pemphigoid.

Secondary Objective(s):

- To assess differences in biopsy sites for diagnostic accuracy of DIF microscopy.
- To determine the diagnostic accuracy of DIF microscopy in subjects with nonbullous clinical features.
- To determine the optimal diagnostic strategy for diagnosis of pemphigoid.
- To compose minimal diagnostic criteria of pemphigoid.

STUDY DESIGN

This study is designed as a retrospective, single-center cross-sectional study at the national referral centre for Autoimmune Bullous Diseases in The Netherlands (Department of Dermatology, University Medical Center Groningen). Assessment of all laboratory tests was conducted at the Immunodermatology Laboratory of the department of Dermatology.

Reference standard and study criteria

This study is in accordance with the Standards for Reporting Diagnostic Accuracy.(12) A consensus reference standard for diagnosis of pemphigoid is not available. Combined clinical and laboratory criteria are defined differently in past studies. DIF microscopy is considered as criterion standard for diagnosis of pemphigoid, whether or not combined with IIF microscopy and/or ELISAs.(11) In order to test the diagnostic accuracy of the criterion standard DIF microscopy, a composite reference standard is used for diagnosis of pemphigoid making use of all combined available clinical, immunopathological and serological findings.

Study criteria for diagnosis of pemphigoid are defined as:

1) compatible clinical features of bullous or nonbullous pemphigoid (5,11)

and

2) compatible findings by DIF microscopy consisting of linear depositions of IgG and/or C3c along the basement membrane zone(11)

and/or

3) compatible positive immunoserology defined as positive epidermal side staining by IIF SSS or positive findings by ELISA BP180 NC16A, and positivity in at least one other serological assay (immunoblot to BP180 or BP230, IIF MO or ELISA BP230).

To identify possible false-positive test results, of all subjects with single positive test results or with indeterminate test results the medical file, clinical features and histopathology will be analyzed (when available). Paired data of light microscopy studies are not available in all patients and may be non-specific in patients with nonbullous pemphigoid and therefore not included in the study criteria and only analyzed in above-mentioned subgroups.

STUDY POPULATION

Population (base)

The study population consists of subjects with suspected pemphigoid from the UMCG registry database for the period 1st January 2002 – 1st May 2015 who are evaluated at the UMCG Center for Blistering Diseases, or of whom tissue samples (biopsies and serum) were referred from secondary care hospitals to the UMCG Immunodermatology Laboratory. Non-pemphigoid control subjects can have various other skin disorders in which diagnosis of pemphigoid can be clearly excluded.

Inclusion criteria

- Subjects suspected of having pemphigoid
- Minimum performed laboratory tests of samples taken at time of first diagnosis, before introduction of immunosuppressive therapy and within inclusion window of 4 weeks:
    - skin biopsy for DIF microscopy
    - indirect immunofluorescence on 1M salt-split human skin substrate
    - ≥1 routine serological assay performed: indirect immunofluorescence on monkey esophagus, immunoblot, ELISA BP180 NC16A and/or ELISA BP230.

Exclusion criteria

- Subjects with only mucosal biopsies
- Subjects with diagnosis of other AIBD based on reported clinical features, DIF microscopy and/or immunoserology, including:
    - Mucous membrane pemphigoid
    - Epidermolysis bullosa acquisita (linear u-serrated IgG along BMZ)
    - Linear IgA disease (IgA in DIF and/or IIF SSS in absence of IgG)
    - Anti-p200/anti-laminin γ1 pemphigoid (dermal side staining IIF SSS)
    - (paraneoplastic) pemphigus vulgaris or pemphigus folicaceus (DIF anti-ECS deposition)
- Immunosuppresive therapy

Sample size calculation

Sample size calculation is based on the main study parameter DIF microscopy with a sensitivity of 90.8% based on the study of Sárdy et al.(13) A power of 80% (0.8) and confidence interval of 95% calculates a sample size of 129 subjects with positive DIF.
$N=(1.96)^2*0.908(1-0.908)/(0.05)^2 = 129$

METHODS

Main study parameters/endpoints

Index test: direct immunofluorescence microscopy

Direct immunofluorescence microscopy is performed as described before.(14,15) Immunopathological examination includes DIF microscopy with detection of deposits of IgG, IgA, IgM and C3 complement (C3c). All secondary antibodies are labeled with fluorescein isothiocyanate (Protos Immunoresearch, Burlingame CA, USA; IgG: Cat.311, IgA: Cat 312, IgM: Cat313 and C3c:F201 Dako, Glostrup, Denmark). For DIF microscopy detection of linear and/or n-serrated deposits of IgG and/or C3c along the epidermal basement membrane zone are considered positive. Biopsies are routinely transported and stored overnight in physiologic saline solution (0.9% NaCl), although liquid nitrogen and Michel's medium could also be used for a small number of samples from referral hospitals. For microscopic examination and routine serration pattern analysis (≥2009) either a Leica DM2000 with a Leica HCX PL Fluotar 40x/0.75 dry lens and 10x objective or DMRA microscope with a Leica PL Apo 40x/0.85 dry lens and 10x objective is used (Leica Microsystems, Wetzlar, Germany. Total 400x magnification).

Index test: indirect immunofluorescence microscopy on salt-split human skin

Human salt-split skin is prepared from donated human skin tissue obtained from routine reduction mammoplasty and abdominoplasty surgery, with signed informed consent. Six millimeter diameter skin biopsies skin are incubated in 0.9% NaCl (1 mol/L) solution for approximately 20 hours at room temperature, during the process random sample biopsies are washed with phosphate buffered saline (PBS) for 25-35 minutes, snap frozen in liquid nitrogen and 4µm sections cut for quality control of the artificial split by light microscopy. After required quality and size of artificial split are obtained, biopsies are stored in aluminium cans, snap frozen in liquid nitrogen and stored at -80°C. New SSS substrates are validated with positive and negative controls. Sera are incubated in 1:8 dilution in PBS and bound IgG visualized with FITC-labeled secondary antibodies ((Protos Immunoresearch, Burlingame CA, USA; IgG: Cat.311, IgA: Cat 312).

Serological assays

The routine multi-step serological laboratory test procedure for subjects with suspected pemphigoid includes IIF microscopy on monkey oesophagus (MO) substrate, immunoblot and commercially available anti-BP180 NC16A and anti-BP230 ELISA tests (Medical and Biological Laboratories Co Ltd, Nagoya, Japan). IIF on monkey esophagus substrate is

performed on corresponding organs obtained from healthy animals used for other research purposes by the National Institute for Public Health and the Environment (RIVM) in the Netherlands for the period 2002 to 2012. Since 2012 commercially available IIF slides (Primate Esophagus Slide 2155, Immco Diagnostics, Buffalo NY, USA) are being used. Sera is incubated in 1:40 dilution and visualized with FITC-labeled IgG secondary antibody (Inova Diagnostics 504014, San Diego CA, USA). Detection of circulating anti-BMZ IgG autoantibodies is considered positive. Immunoblot with keratinocyte extract is performed as described before in detail.(16) Anti-BP180 NC16A and anti-BP230 ELISA tests are performed according to the manufacturer's protocol and defined positivity cut-off value ≧ 9 u/L. Routine performed ELISA testing is introduced in 2007, missing ELISA tests of patients with confirmed diagnosis of pemphigoid will be post-hoc performed.

Secondary study parameters/endpoints

DIF biopsy sites

Registration of biopsy site is routinely performed at the Department of Dermatology, University Medical Center Groningen. Biopsy sites are defined in advance as: 1) perilesional skin; erythematous nonbullous skin, within a 1-2cm radius when a bullae or erosion is present, 2) lesional skin; (every) nonbullous skin lesion, and 3) healthy skin; normal appearing noninflamed skin. Healthy skin biopsies are routinely taken from the medial side of the upper arm. For referring hospitals a perilesional biopsy is recommended by the Center for Blistering Diseases and registration of biopsy site is required.

Other study parameters

- Age
- Sex (male/female)
- Transport medium (saline, Michel's medium, liquid nitrogen)
- Duration in transport medium (24/48 hours)
- Clinical feature: pruritus (yes/no)
- Clinical feature: bullae (yes/no)
- DIF serrated pattern (linear/linear n-serrated)

STATISTICAL ANALYSIS

Primary study parameter(s)

Single test diagnostic accuracy of binary classified index tests DIF microscopy and IIF SSS and the serological assays is based on the composite reference standard. For each comparison 2x2 contingency tables will be used to present and calculate sensitivities, specificities, positive and negative predictive values, positive and negative likelihood ratios and diagnostic odds ratio, with 95% confidence intervals (95% CIs). Sensitivities and specificities of diagnostic test will be compared head-to-head using McNemar test. Diagnostic odds ratio represents the odds of each test to correctly detect presence or absence of the target condition and quantify the ability to predict diagnosis of the target condition.

The added diagnostic value of IIF SSS and the optimal diagnostic strategy for diagnosis of pemphigoid will be asses by receiver operator curve (ROC) analysis. The AUC and 95% CIs will be calculated for the multivariate basic model with clinical predictors, and for extended multivariate models with the most commonly used tests: I) ELISA NC16A, II) IIF SSS, III) IIF SSS and ELISA NC16A, IV) DIF, V) DIF and ELISA NC16A, VI) DIF and IIF SSS and VII) DIF and IIF SSS and ELISA NC16A.

Secondary study parameter(s)

To assess differences in biopsy sites for diagnostic accuracy of DIF microscopy, groups of lesional, perilesional and healthy skin biopsies will be compared using Chi-square test or Fisher’s Exact test when variables contain low numbers. To assess differences in biopsy sites for diagnostic accuracy of DIF microscopy in subjects with nonbullous clinical features, lesional, perilesional and healthy skin biopsies will be compared using Chi-square test or Fisher’s Exact test when variables contain low numbers.

Other study parameters

Demographic variables will be summarized using descriptive statistics. Mann-Whitney U test and Chi-square test (or Fisher’s exact test when variables contain low numbers) will be used to compare medians and proportions. All p-values are two-sided and a p-value <0.05 will be considered statistically significant. Statistical analysis will be performed using SPSS version 22 (SPSS Inc., Chicago, IL, USA).

ETHICAL CONSIDERATIONS

Regulation statement

This study will be conducted according to the principles of the Declaration of Helsinki (Seventh revision, 2013). According to national regulations in The Netherlands, this type of retrospective non-interventional study with a registry of laboratory data and samples from routine diagnostic purposes does not require approval from the local medical ethical committee.

ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

Handling and storage of data and documents

The UMCG ACLAB registry includes standardized data on clinical features, transport medium, duration of transport, standardized biopsy sites, differential diagnosis at first presentation, results of immunofluorescence and immunoserology, differential diagnosis based on immunofluorescence, dates and biobank codes. Biobank materials are coded and stored at -80 °C. The UMCG ACLAB registry is built in Microsoft Access and continuously quality checked by database managers. Query criteria are based on the study inclusion and exclusion criteria (4.2 and 4.3) and exported to SPSS and processed. Data will be handled confidentially. Data will be saved for 15 years after completion of this study. saved in accordance with the Dutch Personal Data Protection Act. This study is designed honouring the CCMO statement on publication policy. This is an investigator initiated study. The results of the study will be made public unreservedly; they will be offered for publication in a peer reviewed journal. In a publication all data will be handled anonymously.

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umcg	**Preparation human salt-split skin substrate**	Date: November 2017
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1.0 Title

Preparation human salt-split skin substrate

2.0 Aim

To prepare a human skin substrate for detection and localization of specific autoantibodies in patient serum against skin proteins along the epidermal basement membrane zone.

3.0 Rationale

Biopsies of healthy human skin will be incubated in saline solution until artificial splitting between dermis and epidermis is achieved. The artificial split will reproducibly occur in the lamina lucida. Some of the antigens will be located in the epidermal side (roof) of the artificial split, while other antigens will be located at the dermal side (floor) of the artificial split. The substrate can be used for detection of circulating autoantibodies in pemphigoid diseases and further subtyping of antigen specificity of autoantibodies.

4.0 Definitions and abbreviations

SSS: salt-split human skin

BMZ: basement membrane zone

5.0 Laboratory equipment

5.1 Microscope

Phase-contrast microscopy *(Olympus BK40 CY)*

5.2 Tools

Silicone half sphere

Needles or pins

Biopsy punch 6mm *(Stiefel)*

Plastic biopsy transport tube 5mL with screw caps *(BIOzym)*

Rotary shaker

Aluminium vial *(Cryovial 15mL 29x30mm with screw cap)*

6.0 Stepwise protocol

- Check for scheduled routine reduction mammoplasty and abdominoplasty surgery.
- Ask permission and partaking by operating (plastic) surgeon.
- Ask informed consent of patient undergoing routine surgery.
- Pick up the residue skin after surgery.
- Tighten up the skin on a silicone half sphere with needles or pins
- Take skin punch biopsies of 6mm.
- Put the biopsies in a plastic transport tube filled with saline solution, maximum of 6 biopsies in 5mL tube.
- Rotate the tubes at room temperature with a rotary shaker.
- Take a sample to check for artificial splitting after approximately 20 hours; wash a biopsy 25-35 minutes in PBS en free in liquid nitrogen. Directly after freezing cut a 4µm cryosection and check under the microscope for artificial splitting between dermis and epidermis. When splitting did not occur of the artificial split does not have the required size yet, take a new sample at a later time until artificial splits have the required size (interval to be determined by technician). Wash all biopsies 25-35 minutes in PBS, place 4-5 biopsies in one aluminium vial, snap freeze in liquid nitrogen and store at -80°C until usage.

7.0 Quality check

New prepared human SSS substrate must be checked by staining with predetermined positive and negative control serum.

umcg	**Detection of circulating autoantibodies against skin proteins using immunofluorescence analysis on human salt-split skin substrate**	Date: November 2017
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1.0 Title

Detection of serum antibodies against skin proteins using immunofluorescence on human salt-split skin substrate.

2.0 Aim

To determine binding characteristics of antibodies in patient serum against skin proteins along the epidermal basement membrane zone to determine presence of auto-immune bullous diseases.

3.0 Rationale

Biopsies of healthy human skin will be incubated in saline solution until artificial splitting between dermis and epidermis is achieved. The artificial split will reproducibly occur in the lamina lucida. Some of the antigens will be located in the epidermal side (roof) of the artificial split, while other antigens will be located at the dermal side (floor) of the artificial split. The substrate can be used for detection of circulating autoantibodies in pemphigoid diseases and further subtyping of antigen specificity of autoantibodies. Salt-split human skin biopsies are deep frozen and 4 µm cryosections are cut and incubated with patient serum. Binding of antibodies is visualized with fluorescent labeled antibodies against human immunoglobulins.

4.0 Definitions and abbreviations

BMZ: basement membrane zone

BP: bullous pemphigoid pemphigoid

Cryosection: thin tissue section

FITC: fluorescein isothiocyanate

LAD: linear IgA disease

SSS: salt-split human skin substrate

5.0 Laboratory equipment

5.3 Microscope

Fluorescence microscope

5.4 Tools

Glass container (14x28x5cm)

Incubation container with grid and glass plate

Washing equipment and grid

Hairdryer with cold air setting

Coverslips (24x32mm)

Filter paper

6.0 Sample

Human serum >50 microliter, deep frozen.

7.0 Procedure

7.1 Preparation

Human serum defrosted to room temperature. The standard dilution is 1:8 in PBS.

7.2 Reagents and safety

FITC-labeled antibodies Goat F(ab)2 anti-human IgA/IgG/IgM: dilute in PBS/OVA until acquired concentration.

PBS/OVA: 1 gr OVA in 100mL PBS (=1%)

PBS/glycerin (1:1): 1 part PBS plus 1 part glycerin

7.3 Stepwise protocol staining

- General note: be sure not to let sections dry out
- Make a work list of tested serum samples
- Salt-split human skin substrate is prepared according to protocol “Preparation human salt-split skin substrate”
- 4µm cryosections are cut according to standard protocol and surrounded by a PAP ring on object glass circling the tissue section
- Sections will be dried with a hairdryer with cold air setting with a minimum of 15 minutes and maximum of 4 hours
- Immerse sections in a glass container with PBS with a minimum of 5 seconds
- Sections need to be run off after immersion

- Orientate sections horizontally in damp incubation container
- Apply as much diluted serum on each section until the applied PAP ring circling the tissue sample is completely filled
- Cover the incubation contained with a glass plate and darkening material/object
- Incubate for 30-40 minutes with room temperature
- Run off the incubation solution in the incubation container and put the objects glasses in an object holder
- Wash the sections with PBS
- Put the glass object holder in the washing machine
- Wash sections mechanically for 15-20 minutes with PBS
- Run off the sections
- Orientate sections horizontally in incubation container
- Apply as much FITC labeled antibody on each section until the applied PAP ring circling the tissue sample is completely filled. Of each tissue sample one section will be incubated with Goat(Fab)2-HuIgA/FITC and one with Goat(Fab)2-HuIgG/FITC. Incidentally sections will be incubated with Goat(Fab)2-HuIgM/FITC.
- Incubate for 30-40 minutes with room temperature
- Run off the incubation solution in the incubation container and put the sections in an object holder
- Wash the sections with PBS
- Put the glass object holder in the washing machine
- Wash sections mechanically for 15-20 minutes with PBS
- Dry the object glasses surrounding the tissue section with filter paper
- Enclose the section with 24 µL PBS/glycerin (1:1) and coverslip
- Put the object glasses in a map, add the work list and store cooled and dark
- Register tested serum samples in log
- Sections need to be examined within 1 month

8.0 Quality check

Each staining procedure must include predetermined positive control human sera of one patient with confirmed bullous pemphigoid, one patient with confirmed EBA and one patient with confirmed LAD. Negative control sera includes human one serum with predetermined negative results with Goat(Fab)2-HuIgA/FITC and one serum with negative result of Goat(Fab)2-HuIgG/FITC. All staining procedures need to be replicated when control sera do not meet the requirements.