Development and validation of serological immunoassays in laminin γ-1 pemphigoid and epidermolysis bullosa acquisita

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A tutto quello che mi mantiene vivo
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1. Introduction

1.1 Pemphigoid diseases of the skin

Pemphigoid diseases of the skin are a group of diverse diseases characterized by the presence of IgG autoantibodies (auto-ab) against different proteins of hemidesmosomes in the dermal epidermal junction (DEJ) of the skin and mucosa. Targeted proteins play a major role in the structural integrity of the skin and mucosa, so that targeting of these proteins by auto-ab leads to alteration of the basal membrane with the formation of blisters, erosions, papules, often supported by concomitant inflammatory skin infiltrates. The pemphigoids encompass distinct subtypes, bullous pemphigoid (BP) is the most common clinical subtype and is characterized by the presence of IgG auto-ab against the hemidesmosomal adhesion molecules, BP180 and BP230. Other subtypes include pemphigoid gestationis, linear IgA disease (LAD), mucous membrane pemphigoid (MMP), laminin gamma-1 (Lam γ-1) pemphigoid and epidermolysis bullosa acquisita (EBA). These diseases may be clinically hard to differentiate, since they often present clinical similarity, nonetheless the starting point for the discrimination between these different diseases is the correct diagnosis. It is crucial to differentiate between the different entities since different subtypes present also different target antigens, different prognosis and may require different treatment. For the proper diagnosis of the different clinical manifestations of pemphigoid diseases, it is critical to establish reliable serological tools for the detection of serum auto-ab against components of the DEJ.

1.2 Laminin γ-1 pemphigoid

Lam γ-1 pemphigoid is a rare variant of the pemphigoids which have been recently described as an autoimmune response against the γ chain of laminins (Dainichi, Kurono et al. 2009, Dainichi, Koga et al. 2010). The disease was independently described in 1996 by Zillikens et al. and Chen et al. as a new uncharacterized type of pemphigoid which was characterized by the presence of circulating reactive IgG auto-ab against a 200-kDa component of the DEJ. Therefore, the disease was firstly named anti p-200 pemphigoid
Serologically, the disease was characterized by the presence of linear IgG and C3 deposits by direct immunofluorescence (DIF) along the DEJ, while indirect immunofluorescence (IIF) showed dermal binding of serum IgG auto-ab on saline split human skin (SSS) (Meijer, Diercks et al. 2016). The latter finding differentiated this entity from BP, associating the disorder to EBA and MMP, since both show a similar staining pattern by IIF (Meijer, Diercks et al. 2016). Further investigations eventually showed that the non-collagenous p-200 was different from other known autoantigens and was identified as the γ-1 chain of laminins, leading to the final denomination of lam γ-1 pemphigoid by some groups (Solimani, Pollmann et al. 2018). Clinically, the disease has a variable appearance, it may resemble BP since it is associated with strong pruritus, presence of tense blisters which are mostly located on the extremities, papules, vesicles and urticarial plaques are frequently observed (Goletz, Hashimoto et al. 2014, Amber, Murrell et al. 2018). Nonetheless, Lam γ-1 pemphigoid may mimic other related bullous diseases such as dermatitis herpetiformis Duhring or in rare cases, the inflammatory type of EBA. Therefore a reliable diagnostic method is crucial to establish the correct diagnosis (Meijer, Diercks et al. 2016, Amber, Murrell et al. 2018, Solimani, Pollmann et al. 2018). Given the rarity of the disease and the difficulty to correctly diagnose this entity, there is a lack of good epidemiological data. So far around 100 cases of patients affected by Lam γ-1 pemphigoid cases have been published. The disease seems to have a more benign clinical course as BP. On the other hand, patients with Lam γ-1 pemphigoid are younger than BP patients (Dilling, Rose et al. 2007, Goletz, Hashimoto et al. 2014). Furthermore, Lam γ-1 pemphigoid is associated with psoriasis vulgaris (Chen, Shimizu et al. 1996, McFadden, Powles et al. 2013, Ohata, Ishii et al. 2015). A pathophysiological link between the two diseases has not yet been found (McFadden, Powles et al. 2013). Lastly, it seems that only a minority of patients affected by Lam γ-1 pemphigoid present concomitant involvement of the mucous membranes (Goletz, Hashimoto et al. 2014). Histologically there is a subepidermal loss of adhesion with a superficial inflammatory infiltrate consisting of eosinophils and/or neutrophils which resembles BP or LAD (Dilling, Rose et al. 2007). Although the pathogenicity of Lam γ-1-specific IgG has not yet been shown neither in vivo nor in vitro recombinants of the Lam γ-1 chain show prognosis as a reliable
target to serologically diagnose this rave variant (Koga, Ishii et al. 2013, Florea, Bernards et al. 2014).

1.3 Epidermolysis Bullosa Aquisita

EBA is a severe autoimmune disease with a chronic recalcitrant course which affects approximately 1-5 cases per million people. The disease has been firstly described by Eliott in 1895 (Kim and Kim 2013). Serologically, EBA is characterized by the presence of IgG auto-ab against human type VII collagen (ColVII) and by the detection of sera IgG binding to the dermal side of SSS. ColVII is the major component of anchoring fibrils, proteins which play a crucial role for the integrity of the skin (Stanley, Woodley et al. 1984). The binding of anti ColVII IgG leads to a destabilization of the anchoring fibrils and consecutively to a pronounced skin fragility and dermal loss of adhesion. ColVII is a collagenous protein with three trimers all consisting of a NH2-terminal (NC1) and a COOH-terminal (NC2) domain (Bentz, Morris et al. 1983). Although most of the patients show circulating IgG autoantibodies against the NC1 domain (Lapiere, Woodley et al. 1993), the NC2 domain is also targeted by EBA sera (Chen, Remmler et al. 1993, Fukumoto, Umekawa et al. 2004, Ishii, Yoshida et al. 2004). EBA has a mean onset of 50 years and two major clinical variants have been described: 1) a mechanobullous phenotype which resembles dystrophic EB with acral blistering and presence of scarring and milia and of hyper- and/or hypopigmentation and 2) an inflammatory type, which is characterized by a BP-like inflammatory phenotype, associated with complement activation and dermal neutrophil recruitment (Kim and Kim 2013). Treatment of EBA is often challenging due to its recalcitrant clinical course and prolonged skin fragility. Treatment is based mainly on systemic corticosteroids, frequently combined with immunosuppressive adjuvants such as mycophenolate mofetil, azathioprine or methotrexate (Gurcan and Ahmed 2011, Kim, Kim et al. 2011). Recently, treatment with high dose intravenous immunoglobulins, immunoadsorption and the anti-CD20 monoclonal antibody, rituximab, have been tried with variable success (Schmidt, Benoit et al. 2006, Schmidt and Zillikens 2010, Kim, Lee et al. 2012). Generally, the inflammatory type of EBA shows a better response to immunosuppressive agents.
However, the clinical presentation per se is not sufficient to correctly diagnose inflammatory EBA. This rare entity may mimic EB, BP, LAD and pemphigus vulgaris (PV). Therefore, serological detection of IgG against ColVII is crucial to correctly establish the diagnosis of EBA (Schmidt and Zillikens 2010).
2 Summary of published results

2.1 Study I: Diagnosis of anti-laminin γ -1 pemphigoid by immunoblot analysis

Impact factor (I.F): 4.287, own contribution 80%

F. S wrote the manuscript, performed the experiments, analysed data, designed figures, and recruited the patients. R. E. and M. H designed and supervised the study. T. H and N. I provided sera from their respective centres. R. P, T. S, R. E and M. H analysed data and revised the study. All authors revised and finally approved the manuscript.

_A part of the experiments (western blots analysis) was performed by Elke Herrmann, a medical technician in the Department of Dermatology and Allergology at the University of Marburg._

Given the lack of easily reproducible methods for the detection of IgG auto-ab against Lam γ-1, the scope of this study was to develop an easy-to-perform and reliable immune serological assay based on commercially available recombinant of Lam γ-1. In the present study, we developed a novel viable method for the serological detection of auto-ab against Lam γ-1d. To verify our diagnostic assay, we tested sera of Lam γ-1 pemphigoid patients by immunoblot (IB) analysis with three different proteins: two recombinant heterotrimers, namely Lam-111 and Lam 421, as well as with a recombinant Lam γ-1 chain monomer. To assess the specificity and sensitivity of IB analysis, we tested sera of 55 patients with Lam γ-1 pemphigoid and of 41 control sera, specifically 14 sera of patients with psoriasis vulgaris (PsO V.), 15 EBA sera and 12 healthy controls (HC) sera. The results of our IB analysis showed that 84% of the Lam γ-1 pemphigoid patients showed reactivity with Lam-111 and/or Lam-421 (46/55 patients). In a second step, the 9 non-reactive sera were tested with the Lam γ-1 monomer, in total 8/9 sera reacted with the Lam γ-1 monomer leading to an overall sensitivity of the combined IB assays of...
98.2%. Control sera were also tested with the three recombinant Lam γ-1 proteins showing a specificity of 88%. Specifically, 3/15 EBA sera, 1/14 Pso V sera and 1/12 HC sera reacted with the Lam γ-1 monomer, the three EBA sera reacted also against the Lam-421 heterotrimer. In summary, we established a sensitive and specific IB assay for the detection of serum IgG auto-ab against the putative major autoantigen of Lam γ-pemphigoid. This study is of relevance in the field of serological diagnostics for autoimmune blistering diseases. In light of the rarity of the disorder and the lack of viable diagnostic assays, the study may prevent incorrect disease diagnosis and evolving mistreatment. Explicitly, Lam γ- 1 pemphigoid is clinically characterized by blisters which are normally located on the extremities and by urticated itchy plaques; this clinical appearance is however typical also for other diseases like BP or DH so that a reliable serological assay is highly needed to correctly differentiate this entity. Already established assays for the detection of Lam γ-1 auto-ab are not available for most centers since they are based on enzyme linked immunosorbent assays (ELISA) with purified Lam γ-1 proteins (Groth et al., 2011) or based on dermal extract IB (Zillikens et al., 1996). The advantage of the present IB is that a) it is based on commercially available recombinant proteins and b) IB is a basic diagnostic methodic which is established in every laboratory with routine diagnostic for autoimmune diseases. This novel IB-assay should facilitate the diagnosis of Lam γ-1 pemphigoid.

**Personal involvement in the study**

The study was presented in its preliminary form and then its final version, respectively during the 43nd annual meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Vienna, Austria (2016) and the 45th Annual Meeting of the ADF, Zurich, Switzerland (2018). The study is published in the Journal of the European Academy of Dermatology and Venereology as an original research article.
2.2 Study II: Serological diagnostics in the detection of IgG autoantibodies against human collagen VII in epidermolysis bullosa acquisita: a multicenter analysis


I.F 6.129, own contribution: 20%

T. S wrote the manuscript, T. S, M. H, R. E and M. H designed and developed the study. T. S S.L-J, G. G and F. S performed the experiments, analyzed data, and prepared the figures. F. S recruited patients and provided clinical information. G. D-Z, A.V. M, M. G, E. C, J. S. K, C. S, I. L-J, M. S, S. U, H. J, R. G, M. K, N. I, T. H provided patients material from their respective centers. B. G provided statistical analysis. F. S, R. E, M. H revised the study. All authors revised and finally approved the manuscript.

EBA is a rare and life-threatening disease of the skin caused by the presence of circulating IgG auto-ab against human ColVII, a major compound of anchoring fibrils which are critical for the dermal-epidermal adhesion. Serologically, the clinical diagnosis of EBA is supported by the detection of IgG deposits along the DEJ by DIF, dermal binding on SSS of serum IgG and the detection of IgG auto-ab against ColVII by IB or ELISA. Nonetheless, it is often challenging to detect the IgG auto-ab against ColVII based on different methods with variable specificities and/or sensitivities. In an international retrospective multicenter study, we studied a large cohort of patients with EBA and evaluated the specificity and sensitivity of four different diagnostic assays for the detection of anti ColVII IgG auto-ab. Analyzed assays were two commercial ELISA systems, IIF on SSS and an in-house IB developed in our laboratory. The first ELISA kit (MBL, Nagoya, Japan) is based on human ColVII containing the NC1/NC2 domains
ELISA kit, the second ELISA (Euroimmun, Lübeck, Germany) is based on the human ColVII-NC1 domain. The in-house IB is based on baculovirus-derived recombinant overlapping fragments that span the NC1 subdomain of ColVII. To assess specificity and sensitivity of these four different methods, we analyzed a total of 95 EBA which were collected in 14 centers from seven countries (Italy, Germany, Croatia, Turkey, Czech Republic and Japan). The diagnosis of EBA was made in the different contributing centers based on clinical and serological criteria, all the patients showed positive DIF and were positive in at least one of the three serological tests employed in our study (IIF on SSS, WB, ELISA). In addition, 100 BP sera and 50 PV sera as well as 50 sera from HC were included in the study. Diagnosis of BP and PV was made based on clinical phenotype, positive DIF of the perilesional skin and serological detection of IgG auto-ab against the respective autoantigens. When comparing the different assays, the highest sensitivity was obtained with the NC1/NC2 ELISA (97.9%, i.e. 93/95 positive sera). The sensitivity of the NC1 ELISA was almost comparable (89.5%, 85/95 positive sera) while WB and IIF on SSS showed a lower sensitivity of 85.5% and 74.4%, respectively. The findings with the control sera revealed a high specificity for all four tested methods. NC1/NC2 and NC1 ELISA showed the highest specificity (99% and ~100%). In the NC1/NC2 ELISA, 1 BP serum reacted against human ColVII while 1 BP and 1 PV sera were reactive in the NC1 ELISA. WB was less specific than IIF on SSS (94% versus 99%). Furthermore, we tried to correlate anti-ColVII IgG auto-ab with the clinical course of 3 selected patients with EBA. In 2 of the 3 followed patients, reduction of IgG auto-ab in ELISA (NC1/NC2 and NC1 ELISA) correlated with clinical improvement, while there was no more correlation in the third EBA patient. This study is so far, regarding EBA studies and serological detection of anti IgG ColVII auto-ab, the international retrospective multicenter study with the largest cohort of EBA patients. We here show that the ColVII NC1/NC2 ELISA shows the highest sensitivity among the 4 different studied methodic and that the NC1 ELISA also shows high levels of sensitivity. Both kits are superior to IIF on SSS and to WB. The slight difference in sensitivity between the two ELISA assays may be explained by the fact that the NC1/NC2 ELISA covers both, NH2- and COOH-terminus of the ColVII heterotrimer. As shown in different studies, the majority of EBA patients have IgG auto-ab which target the ColVII-NC1 domain while a minority of EBA patients have circulating IgG auto-ab against the ColVII-NC2 domain. In our cohort, 2% of the tested
EBA sera had serum IgG auto-ab that were exclusively directed against the NC2 domain of ColVII.
3. Discussion

3.1 Study I: Development of a new immunoassay for the detection of autoantibodies against Laminin γ-1

Lam γ-1 pemphigoid is a recently described blistering disease of the skin which was firstly described almost simultaneously by Zillikens et al. and Chen et al. twenty years ago (Chen, Shimizu et al. 1996, Zillikens, Kawahara et al. 1996). Initially, the disease was called anti p-200 pemphigoid, since patients’ sera showed serum IgG reactivity against a 200 kDa protein of dermal extracts. Furthermore, DIF showed the presence of linear IgG deposits along the DEJ and IIF presented decoration of the DEJ by SSS, thus differentiating this blistering entity from BP but not from EBA or MMP (Dilling, Rose et al. 2007). Clinically, the disease was characterized by blisters, papules and erosions which often precipitate on the extremities (Meijer, Diercks et al. 2016). Interestingly, especially among the Japanese patients, Lam γ-1 pemphigoid is associated with Pso V. (Dilling, Rose et al. 2007). However, a common pathogenic link for this concomitance has not yet been not found. Further studies showed that the putative major autoantigen is the γ-1 chain of laminin heterotrimers present in the skin. This finding led to the present denomination of the disease, Lam γ-1 pemphigoid (Dainichi, Koga et al. 2010). However, in vivo and ex vivo studies failed to demonstrate that IgG against Lam γ-1 is pathogenic, nonetheless the detection of these IgG auto-ab has been widely accepted as reliable serological marker for the diagnosis of Lam γ-1 pemphigoid (Vafia, Groth et al. 2012). Despite the availability of IB analysis with dermal extracts, and IIF on SSS, a major limitation is the lack of commercially available and easily reproducible assays for the serological detection of anti-Lam γ-1 IgG auto-ab. This limitation strongly hampered the acquisition of clinical, therapeutically and epidemiological information, with the consequence that many cases of Lam γ-1 pemphigoid, based on IIF on SSS only, were misdiagnosed as EBA. Different groups attempted to develop reproducible methods to facilitate the diagnosis of Lam γ-1 pemphigoid. Groth et al. developed an ELISA based on a non-commercial recombinant Lam γ-1 protein fragment, which although highly sensitive was only available in few specialized centers (Groth, Recke et al. 2011). Zillikens et al. performed IIF SSS analysis on Lam-332 and Human ColVII Knockout...
skin models which helps to distinguish Lam $\gamma$-1 pemphigoid from EBA and MMP (Zillikens, Ishiko et al. 2000). This methods was conceptually based on the fact that all these three diseases show dermal IgG deposits by IIF on SSS of the patients sera. However this is a) an indirect model for detection of auto-ab b) most centers in the world do not dispose of knockout skin samples. Lastly, the group of Meijer et al. showed that DIF from perilesional skin of Lam $\gamma$-1 pemphigoid patients showed a peculiar serrated pattern (n-serrated) due to the IgG deposits at the DEJ, which helps to differentiate it from EBA (u-serrated IgG deposits) (Meijer, Diercks et al. 2016, Meijer, Atefi et al. 2017). Nonetheless also these methods are not widely available and need intensive training of the users beforehand. In our study, we aimed to develop an easy-to-reproduce immunoblot which can be performed in every laboratory with basic lab skills. This IB is based on two laminin heterotrimers, Lam-421 and Lam-111, and on a recombinant, purified, isolated Lam $\gamma$-1 chain. All these three proteins are commercially available. This IB also showed to be highly specific: Based on a total of 41 control era, the overall observed specificity was of 87.8%. In conclusion, blistering diseases crucially rely on the development and presence of specific and sensitive immunoassays for the detection of circulating auto-ab. In Summary we developed an easy-to-perform and reliable immunoserological assay which could be theoretically implemented worldwide to establish the diagnosis of Lam $\gamma$-1 pemphigoid.

3.2 Study II: Analysis of serological diagnostic methods for the detection of IgG autoantibodies against human collagen VII in epidermolysis bullosa acquisita

EBA is a severe, rare autoimmune bullous disease of the skin and is serologically characterized by the presence of IgG auto-ab directed against ColVII, the major component of anchoring fibrils (Kim and Kim 2013). By IIF the diagnosis of EBA is hard to establish as EBA sera, similarly to MMP and Lam $\gamma$-1 pemphigoid sera, show I IIF reactivity with the dermal side of SSS. Clinically, EBA presents with a wide spectrum of skin manifestations and may be hard to be distinguished from BP, dystrophic EB and LAD. Nonetheless, two different types of EBA have been described: a mechanobullous type with extensive blistzering and scarring at the acral sides and mucous membranes and
an inflammatory type which is associated with local inflammation, complement activation and neutrophil recruitment (Kim and Kim 2013). In general, EBA patients present severe and prolonged skin fragility which is reflected by the presence of tense blisters, typically prominent on mechanically stressed skin areas and mucous membranes, with oral, conjunctival or genital lesions. Blisters often present with secondary milia formation and heal with scarring (Kim and Kim 2013). EBA is a potentially lethal disease and often shows a chronic relapsing course. Present therapeutic options are widely based on corticosteroids, often combined with adjuvant immunosuppressive drugs such as azathioprine, mycophenolate mofetil or cyclophosphamide (Gurcan and Ahmed 2011). In addition, immunoadsorption, intravenous immunoglobulins or anti-CD20 monoclonal antibody (rituximab) have shown positive effects in refractory cases (Schmidt, Benoit et al. 2006, Kim, Lee et al. 2012). Given the severity of EBA, it is crucial to develop highly specific and sensitive tools to establish the diagnosis based of EBA based on serological analysis. In this international multicentric retrospective study, we investigated a large cohort of sera coming from different European countries and Japan. The scope of the study was to evaluate the diagnostic power of four different methods for the detection of anti ColVII IgG auto-ab. The study included 95 EBA sera which have been tested by DIF, IIF, WB and with two different ELISA kits, respectively a ColVII NC1/NC2 ELISA (MBL, Nagoya, Japan) and a ColVII-NC1 ELISA (Euroimmun, Lübeck, Germany). Furthermore, 200 control sera were included in this study, 100 sera from BP patients, 50 PV and 50 HC. Our results show that the highest sensitivity was obtained with the NC1/NC2 ELISA (97.9%), followed by the NC1 ELISA (89.5%). WB analysis had a sensitivity of 85.3%, while IIF analysis showed the lowest sensitivity among these four techniques (85.3%). Similarly, both ELISA were highly specific (~100), IIF analysis also delivered high specificity (99%) while WB analysis showed the lowest specificity (94%) Of note, sensitivity of the NC1/NC2 ELISA was importantly higher than the ELISA against only the NC1 epitope. This finding confirms that, although most of the tested EBA sera contained IgG auto-ab against the NC1 epitope (Lapiere, Woodley et al. 1993), a significant number of sera also reacted with the less frequently targeted NC2 subdomain of ColVII (Fukumoto, Umekawa et al. 2004, Ishii, Yoshida et al. 2004). In conclusion, we here show that two commercially available ELISA assays (ColVII-NC1/NC2 ELISA
and ColVII-NC1 ELISA) provide the highest specificity and sensitivity and should be used in first place for making the diagnosis of EBA.
4. References


5. Summary

Pemphigoid diseases encompass a heterogeneous group of subepidermal autoimmune bullous diseases of the skin and mucosa. These disorders are characterized by autoantibodies that target distinct structural proteins of the basement membrane zone which are crucial for the integrity of the skin. Targeting of these proteins leads to skin fragility, detachment of the epidermis and eventually formation of blisters and skin inflammation. The clinical presentation of the pemphigoids is heterogeneous, the subtypes are divided by clinical symptoms, target antigens and response to treatment. A crucial step for the proper classification is the serological detection of the autoantigens directed against distinct components of the dermal epidermal junction. Thus, it is crucial to develop reliable and reproducible diagnostic methods which help clinicians to differentiate between different entities and therefore to select the best treatment. This thesis is focused on Laminin γ-1 pemphigoid and epidermolysis bullosa acquisita, the study of their clinical appearance and the development and validation of serological assays to correctly diagnose these two entities. Both autoimmune blistering diseases are rare and often hard to diagnose with standard serological assays. In the first study presented in this thesis, I discuss the development of an immunoblot assay for the detection of autoantibodies against Laminin γ-1. Laminin γ-1 pemphigoid is a novel subepidermal blistering disease which has been first described in 1996. Since then, different immune serological methods for the detection of circulating autoantibodies have been described, however these are often not reproducible for most of the centers worldwide, since often advanced lab skills are required. In our study, we present a novel immunoblot assay based on three commercially available Laminin γ-1 recombinants, which can be reproduced in almost every laboratory with basic skills. In the second study, we thought to serologically identify for the best immunoassay for the detection of serum IgG autoantibodies against anti-human collagen VII in patients with epidermolysis bullosa acquisita. Epidermolysis bullosa acquisita is a severe blistering diseases characterized by circulating IgG autoantibodies targeting human collagen VII. In this study, four different diagnostic assays (immunoblot, indirect immunofluorescence with saline split human skin and 2 enzyme linked immunosorbent assays) have been tested and compared with regard to their sensitivity and specificity in a large cohort of epidermolysis...
In summary, our study showed that enzyme linked immunosorbent assays based on recombinant human collagen VII NC1-NC2 or collagen VII-NC1 proteins provide the highest sensitivity and specificity for the detection of anti-collagen VII IgG and should be preferred to immunoblot analysis or indirect immunofluorescence on saline split human skin in making the diagnosis of epidermolysis bullosa acquisita.
6. Zusammenfassung

7. Published results

7.1 Study I


Diagnosis of anti-laminin γ-1 pemphigoid by immunoblot analysis

Diagnosis of anti-laminin γ-1 pemphigoid by immunoblot analysis

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Abstract

Background Anti-laminin-γ1 (lam-γ1) pemphigoid, a recently described immunobullous disorder sharing immune serological features of bullous pemphigoid and epidermolysis bullosa acquisita (EBA), is characterized by the detection of serum IgG autoantibodies against the lam-γ1 chain, a 200 kDa heterotrimeric component of the dermal-epidermal junction (DEJ).

Objective The aim of the study was to develop an easy-to-perform and reliable assay for the serological detection of anti-lam-γ1 IgG autoantibodies. The clinical appearance alone is not sufficient to establish diagnosis of anti-lam-γ1 pemphigoid and rather requires immune serological evidence of (i) IgG reactivity against the dermal portion of salt-split human skin; (ii) exclusion of IgG against other components of the DEJ; and (iii) IgG reactivity with a 200 kDa protein of dermal extracts by immunoblot analysis (IB).

Methods The sera of 55 patients with anti-lam-γ1 pemphigoid were tested by IB with two recombinant heterotrimers, laminin 111 (lam-111) and laminin 421 (lam-421), as well as with a recombinant lam-γ1 chain monomer. Additionally, a total of 41 control sera from patients with EBA (n = 15), psoriasis vulgaris (PV; n = 14), and healthy controls (HC; n = 12) were tested.

Results Immunoblot analysis revealed a positive reactivity with lam-111 and/or lam-421 in 46/55 (84%) of anti-lam-γ1 pemphigoid sera. Moreover, 8/9 of the initially non-reactive sera were positive with the lam-γ1 monomer, leading to an overall sensitivity of 98.2%. Analyses of 41 control sera with the three lam-γ1 recombinants led to a specificity of 88%. Specifically, 3/15 EBA sera, 1/14 PV serum and 1/12 HC serum reacted with the lam-γ1 monomer while only the 3 EBA sera reacted with lam-421.

Conclusions Here we show a novel two-step IB assay using the two recombinant laminin trimers and lam-γ1 chain monomer for the detection of anti-lam-γ1 serum IgG with high sensitivity and specificity. This assay will facilitate the diagnosis and further characterization of this disease.

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Conflicts of interest None declared.

Funding source None declared.

Introduction

Twenty years ago, anti-laminin γ1 (lam-γ1) pemphigoid which is also known as anti-p200 pemphigoid, was independently described by Zillikens et al. and by Chen et al. as a novel autoimmune bullous skin disorder with IgG autoantibodies against the dermal-epidermal junction (DEJ) which did not target any of the known autoantigens of the DEJ including bullous pemphigoid (BP) 180, BP230, laminin-332 (lam-332), and type VII collagen (ColVII). 1,2 The disorder was characterized clinically by BP-like inflammatory phenotype and, immune serologically, by serum IgG against a 200 kDa protein of dermal extracts leading to the initial denomination of anti-p-200 pemphigoid. 3 Further studies showed that the non-collagenous p-200 protein was a new autoantigen different from other known autoantigens and was synthesized by keratinocytes and fibroblasts. 4–6 Finally, the p-200 protein was identified as the γ-1 chain of laminin leading to the later denomination as anti-lam-γ1 pemphigoid. 7,8 Lam-γ-1 is a ubiquitous protein present in almost every tissue and a component of multiple laminin heterotrimers. 9 In the
skin, the protein is located in the DEJ within the lower lamina lucida.\textsuperscript{10,11}

Clinically, anti-lam-γ1 pemphigoid mostly resembles BP with tense blisters, urticated papules and plaques and pronounced pruritus. The blisters often exhibit an acral distribution (Fig. 1). In general, patients respond well to topical and systemic immunosuppressive treatment with a response pattern similar to BP.\textsuperscript{3,8} As sera of anti-lam-γ1 pemphigoid patients show a reactivity pattern by IIF similarly to EBA, i.e. IgG reactivity within the dermal side of salt-split human skin, these patients were occasionally misdiagnosed as inflammatory type of EBA.\textsuperscript{2,13} However, the etiopathogenetic links between the two disorders are not yet well-understood.\textsuperscript{14,15} Furthermore, laminins are also known autoantigens in other inflammatory diseases such as lupus erythematosus and vasculitis.\textsuperscript{16,17}

Almost 100 cases of anti-lam-γ1 pemphigoid have been published so far,\textsuperscript{16–20} and, except one all reported cases were IgG mediated.\textsuperscript{21} Due to its rarity and limited availability of specific diagnostic methods, anti-lam-γ1 pemphigoid is largely underdiagnosed and mistreated as the inflammatory type of EBA.\textsuperscript{2} At present, the diagnosis of anti-lam-γ1 pemphigoid is based on: (i) direct immunofluorescence (DIF) of perilesional skin; (ii) indirect immunofluorescence microscopy (IIF) with salt-split human skin (SSS), and (iii) immunoblot analysis (IB) with dermal extracts (Fig. 3).\textsuperscript{12} Histopathology of the skin reveals subepidermal loss of adhesion with a superficial inflammatory infiltrate containing eosinophils and/or neutrophils which resembles BP or linear IgA bullous dermatosis.\textsuperscript{22} Immune serologically, DIF shows linear deposits of IgG and C3 along the DEJ while IIF shows dermal IgG binding to SSS which discriminates this disorder from BP. Further biochemical and genetic approaches revealed that the autoantigen is a non-collagenous glycoprotein which is synthesized by keratinocytes and dermal fibroblasts.\textsuperscript{2} Recently, Dainichi et al.\textsuperscript{23} identified the lam-γ1 chain as the target antigen of the majority of the patients’ sera.

Even though \textit{ex vivo} and \textit{in vivo} studies have failed to show direct pathogenicity of anti-lam-γ1 IgG autoantibodies,\textsuperscript{24–26} the serological detection of anti-lam-γ1 IgG still remains the gold standard for establishing the diagnosis of anti-lam-γ1 pemphigoid. At present, IB analysis with purified lam-γ1 or dermal extract represents the most reliable method for the serological detection of lam-γ1 IgG autoantibodies. This method is, however, only performed in a few specialized centres and largely depends on the quality of the employed protein preparations.

In this study, we present an easy-to-perform and highly sensitive IB assay for the serological detection of anti-lam-γ1 IgG, which is based on three commercially available recombinant proteins containing the lam-γ1 chain. This novel IB assay should enable many laboratories to correctly diagnose and subsequently properly treat anti-lam-γ1 pemphigoid.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Clinical, histopathological and immunofluorescence microscopic findings in anti-laminin γ1 pemphigoid. (a–d) Excoriated pruritic papules (a), tense blisters (b), urticated papules and plaques (c), and concomitant plantar psoriasis (d) in a patient with anti-laminin γ1 pemphigoid. (e) Subepidermal loss of adhesion with superficial dermal inflammatory infiltrate (e). (f,g) IgG deposits along the dermal-epidermal junction by direct immunofluorescence (f), and serum IgG autoantibodies labelling the dermal side of salt-split human skin (g).}
\end{figure}
Materials and methods

**Patients**
The study included a total of 49 Japanese and 6 European patients with anti-lam-γ1 pemphigoid who fulfilled the following criteria: (i) presence of blisters, urticarial lesions and pruritic, excoriated papules; (ii) linear IgG deposits along the DEJ by DIF of perilesional skin; (iii) serum IgG reactivity with the dermal side of human SSS by IIF; and (iv) absence of serum IgG against other components of the DEJ (BP180, BP230, ColVII and Lam-332). In addition, all the 49 Japanese sera showed IgG reactivity against a 200 kDa protein of dermal extracts by IB. The 55 anti-lam-γ1 pemphigoid patients include 33 were male and 13 female, while the genders for 9 patients were retrospectively not identifiable gender. The mean age of the patients at disease onset was 63.6 years. Sera from patients with psoriasis vulgaris (n = 14), EBA (n = 15), and healthy individuals (n = 12) served as controls. The protocol of the present study was approved by the Ethics Committee of the Medical Faculty of Philipps University Marburg, Germany.

**Indirect immunofluorescence microscopy with salt-split normal human skin**
Preparation and evaluation of immunofluorescence microscopy on salt-split human skin was performed as previously described.27

**Exclusion of other circulating autoantibodies**
IgG serum autoantibodies against BP180, BP230 and ColVII were excluded using commercially available ELISA (Euroimmun, Lübeck, Germany); according to the manufacturers’ protocol.27 Presence of autoantibodies against laminin 332 were excluded by ELISA using native human laminin 332 as previously described.28

**Immunoblot analysis with recombinant laminin proteins**
Recombinant lam-111 and recombinant lam-421 were purchased from Biolamina, Sundbyberg, Sweden. Purified lam-γ1 and rabbit polyclonal anti-lam-γ1 antibody were purchased from OriGene, Rockville, MD, USA. SDS/PAGE and IB was performed as follows: according to their molecular weight, proteins were separated by SDS-Page in a discontinuous buffer system using the Multigel system (Biometra, Gottingen, Germany). The polyacrylamide gel consists of a lower separation gel at a concentration of 7.5% and the upper stacking gel containing 30% acrylamide. The proteins were denatured by boiling (for 5 min at 95°C) and 8 μg of lam-111, lam-421 and 2.5 μg of lam-γ1-chain was applied on the chamber. After separation, the proteins were transferred to a nitrocellulose membrane (Peplab Biotechnologie, Erlangen, Germany) at 300 mA, 150 V for 1.5 h. Thereafter, the membrane was washed with TBS and blocked with TBS/10% milk powder. Afterwards, the membranes were incubated with patients’ sera diluted at 1 : 200 in PBST (1 × PBS + 0.1% Tween-20) overnight and incubated by 4°C under shaking conditions. Positive controls included a lam-γ1-reactive human serum (1 : 200) and a rabbit anti-lam-γ1 primary antibody (OriGene), healthy control (HC) sera served as a negative control. IgG binding was visualized by a digital chemiluminescence reader (PEQLAB, Erlangen, Germany).

**Sensitivity of immunoblot analysis with recombinants laminins**
The studied cohort of anti-lam-γ1 pemphigoid patients’ sera was characterized by IgG reactivity with the dermal portion of human SSS, and a lack of IgG reactivity with well-known autoantigens of the DEJ including BP180, BP230, lam-332, and ColVII. Moreover, 49/55 tested sera were previously shown to display IgG reactivity against a 200 kDa protein of human dermal extracts. In a first step, all the sera were tested with two heterotrimers containing the lam-γ1 chain, i.e. lam-111 and lam-421. As shown in Fig. 2 Table 1, 46/55 sera showed IgG reactivity to either lam-111 and/or lam-421 resulting in a sensitivity of 83.6%. In a second step, all the nine sera which were neither reactive with lam-111 nor with lam-421 were tested by IB with purified lam-γ1 monomer. A total of 8/9 sera were IgG reactive with the lam-γ1 chain (Fig. 2 and Table 1).

To prove that the portion of lam-421 and lam-111 which reacted with the lam-γ1 sera by IB analysis was indeed the γ1 chain of laminin, we tested 10 sera reactive with both, lam-111 and lam-421 with lam-γ1 monomer. In fact, all these sera showed IgG reactivity against the 200 kDa protein (data not shown). Only one single patient with serum IgG reactivity against the dermal side of human SSS (anti-collagen VII IgG negative) did not react with any of the laminins and this serum was eventually considered as negative (1.8%; Table 1). Overall, combining IB analysis with the heterotrimers, lam-111 and lam-421, and the monomeric lam-γ1 chain (step 1 and 2), resulted in a final sensitivity of 98.2% (54/55 sera positive).

Remarkably, the recombinant lam-γ1 of the present IB had a molecular weight close to 250 kDa (Fig. 2). This largely depends on differential NH2-linked glycosylation leading to a variable molecular weight of lam-γ1 ranging from 200 to 250 kDa.29

**Specificity of immunoblot with recombinants laminins**
This study included a total of 41 control sera, i.e. 12 HC, 14 sera from patients with psoriasis (as anti-lam-γ1 pemphigoid is clinically associated with psoriasis) and 15 EBA sera (as EBA sera also show IgG reactivity with the dermal part of human SSS; Fig. 2 and Table 2). Upon IB analysis with lam-111 and lam-421, 3 EBA sera showed positive IgG reactivity. Of note, 5 control sera showed IgG reactivity against the lam-γ1 monomer, i.e.1 HC
serum, 1 PV serum and 3 EBA sera. Three EBA sera showed additional IgG reactivity against lam-421. Overall, 5/41 control sera showed IgG reactivity against at least one of the three laminins leading to a specificity of 87.8% (Table 2).

Discussion

Anti-lam-γ1 (anti-p200) pemphigoid is a peculiar pemphigoid variant; i.e., most patients with this disease present clinically

Table 1  Sensitivity of immunoblot analysis with three recombinant laminins containing the lam-γ1 chain

<table>
<thead>
<tr>
<th>Sera from patients with anti-lam-γ1 pemphigoid</th>
<th>lam-111 (%)</th>
<th>lam-421 (%)</th>
<th>lam-γ1 (%)</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 1 – lam-111 and lam-421 heterotrimers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36/55 positive</td>
<td></td>
<td></td>
<td></td>
<td>65.4</td>
</tr>
<tr>
<td>44/55 positive</td>
<td></td>
<td></td>
<td></td>
<td>80.0</td>
</tr>
<tr>
<td>46/55 positive</td>
<td></td>
<td></td>
<td></td>
<td>83.6</td>
</tr>
<tr>
<td><strong>Step 2 – lam-γ1 monomer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/9+</td>
<td></td>
<td></td>
<td></td>
<td>88.8</td>
</tr>
<tr>
<td><strong>Total 54/55 positive</strong></td>
<td></td>
<td></td>
<td></td>
<td>98.2</td>
</tr>
</tbody>
</table>
with a BP-like inflammatory phenotype but shows serologically EBA-like reactivity by IIF using SSS.\(^1\) Due to its rarity and the lack of routinely used diagnostic procedures, the disorder is frequently under-diagnosed.\(^3\) Clinically, anti-lam-\(c\)-1 pemphigoid is defined by urticated plaques and tense blisters which are characteristically located on the extremities and do not present signs of scarring or milia formation. However, the clinical features may show a wide variability including those of BP, EBA, and dermatitis herpetiformis.\(^2\) The recent identification of the lam-\(c\)-1 chain as target autoantigen of the majority of anti-lam-\(c\)-1 (anti-p200) pemphigoid sera has opened an avenue to more precisely diagnose this rare pemphigoid variant.\(^2\)

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### Table 2: Specificity of immunoblot analysis with three recombinant laminins containing the laminin-\(\gamma\)-1 chain

<table>
<thead>
<tr>
<th>Control group</th>
<th>lam-111</th>
<th>lam-421</th>
<th>lam-(\gamma)-1</th>
<th>Total</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>0/12+</td>
<td>0/12+</td>
<td>1/10</td>
<td>1/12+</td>
<td>91.7</td>
</tr>
<tr>
<td>Psoriasis vulgaris</td>
<td>0/14+</td>
<td>0/14+</td>
<td>1/14+</td>
<td>1/14+</td>
<td>92.9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3/41+</td>
<td>3/41+</td>
<td>5/39+</td>
<td>5/41+</td>
<td>87.8</td>
</tr>
</tbody>
</table>

---

**Figure 3** Diagnostic algorithm for establishing the diagnosis of anti-laminin-\(\gamma\)-1 pemphigoid. Shown is an algorithm to distinguish subepidermal autoimmune blistering diseases. The clinical appearance and presence of IgG deposits along the dermal–epidermal junction (DEJ) or IgG reactivity with the dermal side of the salt-split skin (SSS) is not sufficient to establish proper diagnosis of a distinct subepidermal blistering dermatosis. Detection of serum IgG autoantibodies is needed to distinguish between anti-lam-\(\gamma\)-1 pemphigoid, epidermolysis bullosa acquisita (EBA) and mucous membrane pemphigoid (MMP). Immunoblot analysis (IB) with commercially available laminin (lam)-111 and lam-421 strongly facilitates the detection of serum IgG reactivity with the lam-\(\gamma\)-1 heterotrimers, IB can be performed with the lam-\(\gamma\)-1 monomer. DIF, direct immunofluorescence; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; IB, immunoblot, IIF, indirect immunofluorescence.
We here established a highly sensitive and specific IB assay for the detection of serum IgG autoantibodies against lam-\(\gamma\)-1, the putative major autoantigen of anti-lam-\(\gamma\)-1/anti-p200 pemphigoid. The present IB assay is based on three recombinant proteins, i.e. the heterotrimers, lam-111 and lam-421, and the lam-\(\gamma\)-1 monomer. The great advantage of the newly developed immunoassay is the accessibility of the employed lam-\(\gamma\)-1 recombinants which are commercially available to many laboratories worldwide. The proposed two-step IB analysis with two lam-\(\gamma\)-1 heterotrimers, i.e. lam-111 and lam-421, and a lam-\(\gamma\)-1 monomer in case of no reactivity to the heterotrimers, allows the in vitro detection of lam-\(\gamma\)-1-specific IgG autoantibodies at a high sensitivity and specificity. Using the proposed two-step IB assay, a total of 54/55 sera from anti-lam-\(\gamma\)-1 pemphigoid patients were found to be positive (98.2%), while 46 sera were already found to be lam-\(\gamma\)-1-reactive at the first step IB with the two lam-\(\gamma\)-1 heterotrimers (83.6%; Table 1). Using 41 control sera, we observed a specificity of 87.8% of this two-step IB assay (Table 2). In detail, 3 EBA sera reacted by IB with all the lam-\(\gamma\)-1 recombinant proteins while 1 HC and 1 PV serum reacted with the lam-\(\gamma\)-1 monomer only. In the case of the 3 EBA sera, chronic skin inflammation at the DEJ may have led, e.g. through epiblast spreading, to the development of autoantibodies against lam-\(\gamma\)-1. However, this assumption needs to be verified prospectively in a cohort of EBA patients.

At present, the diagnosis of anti-lam-\(\gamma\)-1 pemphigoid is based on a mix of clinical, histopathological and immune serological findings.\(^2\) Although the significance of lam-\(\gamma\)-1-specific IgG autoantibodies is still unresolved, as \textit{ex vivo} and \textit{in vivo} models failed to show pathogenic properties of these autoantibodies,\(^4\) their detection in the patients’ sera is a diagnostic hallmark in establishing diagnosis of anti-lam-\(\gamma\)-1 pemphigoid. Previously, detection of IgG serum autoantibodies against a 200 kDa protein of dermal extracts was the standard immune serological procedure which is highly reliable but only available in a few specialized centres.\(^5\) Recently, Groth et al.\(^6\) developed an ELISA assay utilizing a recombinant lam-\(\gamma\)-1 fragment, which is also restricted to a few centres with the capacity to produce the recombinant protein. Zillikens et al.\(^7\) developed an assay based on IIF knockout analysis which allowed exclusion of EBA and anti-lam332-type mucous membrane pemphigoid. Lastly, Meijer et al.\(^8\) analyzed the serrated pattern profile of perilesional skin of anti-lam-\(\gamma\)-1 pemphigoid patients and found that, in contrast to the u-serrated pattern for EBA sera, anti-lam-\(\gamma\)-1 pemphigoid presented with a n-serrated pattern, characteristic of BP. The lack of easily reproducible techniques strongly limits the diagnosis of anti-lam-\(\gamma\)-1 pemphigoid and leads to an under-recognition of this pemphigoid variant.

Epidemiological data are poor and based on small cohorts.\(^9,10\) In the reported studies, patients with anti-lam-\(\gamma\)-1 pemphigoid seem to be younger than BP patients with an age of disease onset varying from 60 to 65 years.\(^11\) In our cohort, the mean age of onset was 63.6 years, which confirms previous data suggesting a slight earlier onset of the disease. Furthermore, our study cohort showed male predominance.

In conclusion, the lack of easily reproducible assays for the detection of IgG autoantibodies against lam-\(\gamma\)-1 represents a major restriction for establishing the diagnosis of anti-lam-\(\gamma\)-1 pemphigoid, a rare but significant pemphigoid variant. We here developed a reliable serological IB assay for the detection of serum autoantibodies based on the use of commercially available recombinant proteins, i.e., heterotrimeric lam-111 and lam-421 proteins and a monomeric lam-\(\gamma\)-1 chain. This novel, highly sensitive and widely accessible two-step IB assay will help to better diagnose anti-lam-\(\gamma\)-1 pemphigoid, a currently under recognized pemphigoid variant.

References

Diagnosis of anti-laminin c-1 pemphigoid by immunoblot analysis

7.2 Study II


Serological diagnostics in the detection of IgG autoantibodies against human collagen VII in epidermolysis bullosa acquisita: a multicenter analysis

Serological diagnostics in the detection of IgG autoantibodies against human collagen VII in epidermolysis bullosa acquisita: a multicentre analysis

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 Conflicts of interest
M. Hertl received an unrestricted grant from Medical and Biological Laboratories (MBL, Nagoya, Japan). T.H. has worked on previous collaborations with MBL. M.S. received a registration fee and acted as a scientific advisor for MBL, but did not have direct influence on the marketing or production activity.

T.S., M. Hoch, T.H. and M. Hertl contributed equally.

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Summary

Background Epidermolysis bullosa acquisita (EBA) is a rare, potentially devastating autoimmune disease of the skin. IgG autoantibodies directed against type VII collagen (Col7), the major component of anchoring fibrils, induce skin fragility leading to cutaneous and mucocutaneous blister formation, which is mostly of a scarring phenotype. Thus, powerful and reproducible diagnostic assays are critical to establish the diagnosis of EBA early to avoid irreversible sequelae.

Objectives The present international, retrospective multicentre study included a large cohort of patients with EBA and evaluated the diagnostic power of four different diagnostic assays for the detection of anti-Col7 IgG autoantibodies.

Methods Overall, 95 EBA sera and 200 control sera consisting of 100 bullous pemphigoid sera, 50 pemphigus vulgaris sera and 50 sera of healthy controls were tested for anti-Col7 IgG autoantibodies using indirect immunofluorescence (IIF), two commercial enzyme-linked immunosorbent assay (ELISA) systems and Western blot (WB) analysis. EBA sera were taken from patients with positive direct immunofluorescence and IgG reactivity in at least one of the immunoserological assays (IIF, ELISA, WB).

Results A Col7-NC1/NC2 ELISA (MBL, Nagoya, Japan) showed the highest sensitivity (97.9%), followed by a Col7-NC1 ELISA (Euroimmun, Lübeck, Germany) (89.5%), WB with Col7-NC1 (85.3%), and IIF on saline-split human skin (74.7%). The specificities of both ELISA systems were comparable (NC1 98.7%, NC1/NC2 99.3%). Furthermore, WB was more sensitive than IIF, which was more specific.

Conclusions The two commercially available ELISA systems allow for a highly sensitive and specific diagnosis of EBA. The sensitivity of the Col7-NC1/NC2 ELISA is...
significantly higher compared with the ELISA based on the Col7-NC1 domain only.

Epidermolysis bullosa acquisita (EBA) is a severe, acquired autoimmune bullous disease with a prevalence of approximately one to five cases per million people. It is characterized by the presence of IgG autoantibodies directed against type VII collagen (Col7), a major component of anchoring fibrils, which are responsible for the anchorage of the epidermal basal membrane zone (BMZ) to the dermis. Binding of anti-Col7 IgG leads to a destabilization of the BMZ resulting in severe skin fragility and subsequent subepidermal loss of adhesion. Thereby, blisters are induced by a direct antibody impact (mechanobullous type) or by an antibody-induced local inflammation cascade including complement activation and neutrophil recruitment (inflammatory type).

Epidermolysis bullosa acquisita was first described by Elliot in 1895 and occurs independently of age, sex or ethnic group. It is genetically associated with the human leucocyte antigen (HLA) major histocompatibility class (MHC) II allele HLA-DR2, which is also associated with other autoimmune diseases. Clinically, EBA presents with cutaneous and mucocutaneous blisters and erosions, which often lead to a scarring phenotype with miliaria formation and nail loss.

Owing to the wide spectrum of clinical manifestations of EBA, various differential diagnoses including dystrophic epidermolysis bullosa, bullous pemphigoid (BP), linear IgA dermatosis and pemphigus vulgaris (PV), have to be considered (Fig. 1). The diagnosis of EBA is based on the clinical phenotype in combination with direct immunofluorescence (DIF), indirect immunofluorescence (IIF) on saline-split skin (SSS) and the serological detection of anti-Col7 IgG autoantibodies. The present international retrospective multicentre study compared the specificity and sensitivity of four different standardized serological assays for the detection of anti-Col7 IgG, including two commercial enzyme-linked immunosorbent assay (ELISA) systems, IIIF on SSS and Western blotting (WB). The results demonstrate that the ELISA systems delivered the highest sensitivity, followed by IIIF and WB. Of note, a Col7-NC1/NC2 ELISA showed a significantly higher sensitivity than a Col7-NC1 ELISA, even though their specificities were comparable. Furthermore, WB was more sensitive than IIIF while IIIF showed a higher level of specificity than WB.

Materials and methods

Patient and control sera

A total of 95 EBA sera were analysed in this retrospective multicentre study. For each serum the centres filled out a study form that included information on the methods used for the immunoserological assessment of EBA (Table S1; see Supporting Information), clinical phenotype and treatment. The spectrum of diagnostics included clinical appearance, histology, DIF of perilesional skin, IIIF (SSS–IgG reactivity with the dermal portion), ELISA and WB analysis. All of the sera were from patients with EBA with positive DIF and were positive in at least one serological test (IIIF/ELISA/WB). A total of 98% of the tested EBA sera were initially tested by IIIF (SSS and/or monkey oesophagus) in the contributing centres.

Overall, 3% of the sera were tested by ELISA only. Owing to the limited relevance of monkey oesophagus analysis, the monkey oesophagus data were deleted. Thus, 91 of 95 EBA
sera (96%) were initially tested by SSS in the contributing centres. Of these 91 sera, three (3%) sera tested negative. As shown in Table S1 (see Supporting Information), 97% of the SSS-tested EBA sera were initially positive, whereas only 75% were positive upon reanalysis in the present study (including four samples that were not initially tested in the respective centres). The observed difference in the sensitivity of SSS analysis of the EBA sera may be related to factors dependent on the time period between initial analysis at the contributing centre and our analysis, such as storage of the sera. This became apparent when the study showed that SSS was the method of lowest sensitivity compared with WB and ELISA. In contrast, all the EBA sera that initially tested positive by ELISA in the contributing centres remained positive upon reanalysis in our laboratory. It is noteworthy that all of the six EBA sera that were initially tested by SSS in only the contributing centres were also positive by SSS upon reanalysis in our laboratory.

Overall, 41% of the sera were collected from European patients with EBA (Italy, Germany, Croatia, Turkey, Czech Republic) and 51% from Japanese patients with EBA. A total of 16 of the 48 Japanese EBA sera had already been included in a previous study by Komorowski et al. The controls included 100 sera from patients with BP, 50 sera from patients with PV and 50 sera from healthy controls. BP and PV were diagnosed by clinical phenotype, DIF of perilesional skin and the serological detection of IgG against BP antigen 180 (BP180)/BP230 or desmoglein (Dsg) (Dsg1 and Dsg3, respectively). This study was conducted in accordance with the Declaration of Helsinki guidelines and approved by the local ethics committee of the medical faculty of the University of Marburg, Marburg, Germany.

**Immunofluorescence microscopy on saline-split human skin**

Healthy human skin was incubated in a 1 mol L⁻¹ NaCl solution for 48–72 h. The skin specimens were then washed three times in phosphate-buffered saline (PBS) for 15 min, embedded in optimum cutting temperature Tissue Tec (Sakura Fine-tek, Staufen, Germany) and stored at −80 °C. After the preparation of microscope slides (Superfrost Plus, Langenbrink, Emmendingen, Germany) with cryo sections (Microtom Micron HM-560), EBA and control sera were incubated

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**Fig 1.** Clinical differential diagnosis of autoimmune bullous skin disorders. (a) Epidermolysis bullosa acquisita (skin); (b) bullous pemphigoid; (c) laminin-γ1 pemphigoid; (d) epidermolysis bullosa acquisita (oral mucosa); and (e) pemphigus vulgaris (oral mucosa).

**Fig 2.** Synopsis of diagnostic assays utilizing distinct subdomains of collagen VII (Col7). WB, Western blot; ELISA, enzyme-linked immunosorbent assay.
Serological diagnostics for anti-Col7 IgG autoantibodies in EBA, T. Schmidt et al.

Sodium dodecyl sulfate gel electrophoresis and Western blotting

The recombinant fragments of human Col7 NC1(1) (aa17-aa610), NC1(2) (aa273-857) and NC1(3) (aa611-1253) (Fig. 2) were produced in a baculovirus-based eukaryotic expression system as previously described. For gel electrophoresis, equal amounts of Col7-NC1 proteins were denaturated by Laemmli buffer and separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. The WB procedure was performed as previously described. Patient sera were diluted 1 : 200 in PBST (1× PBS + 0.1% Tween-20) and incubated overnight at 4°C under shaking conditions. An EBA serum that tested positive (1 : 200) and a rabbit anti-E-Tag primary antibody (Abcam, Cambridge, U.K.) served as a positive control and a serum from a healthy donor was used as a negative control. Analysis was performed using a digital chemoluminescence reader (PEQLAB, Erlangen, Germany).

Collagen VII enzyme-linked immunosorbent assay

The NC1 ELISA [Anti-Type VII Collagen-ELISA (IgG), Euroimmun, Lübeck, Germany] and the NC1/NC2 ELISA (MESCUCP Anti-Type VII Collagen Test, MBL, Nagoya, Japan) were performed according to manufacturers’ protocol. Optical densities were measured at a wavelength of 450 nm and a reference of 620 nm by photometer Sunrise®-Basic (Tecan, Göding, Austria). Calculation of RE mL⁻¹ (Euroimmun ELISA) and U mL⁻¹ (MBL ELISA) was performed according to the manufacturers’ instructions. Samples showing values greater or equal to 20 RE mL⁻¹ (Euroimmun ELISA) or 6 U mL⁻¹ (MBL ELISA) were considered to be positive.

Results

Sensitivity of diagnostic assays

Four diagnostic assays were employed to detect anti-Col7 IgG in the sera of patients with EBA. These included IIF with the standard substrate SSS, an in-house WB with the recombinant human CO7-NC1 domain and two commercial ELISA systems with the human Col7-NC1 and Col7-NC1/NC2 domains, respectively (Fig. 2). A total of 95 EBA sera were tested. A total of 100 BP sera, 50 PV sera and 50 healthy control sera were also tested. The highest sensitivity was obtained with the NC1 ELISA (89.5%) and the NC1/NC2 ELISA (97.9%) followed by WB (85.3%) and IIF with SSS (74.7%). Of note, the NC1/NC2 ELISA exhibited a significantly higher sensitivity compared with the NC1 ELISA (P = 0.005) (Table 1). Specifically, in the analysis of the cohort of 95 patients with EBA, the NC1/NC2 ELISA delivered 93 positive results and the NC1 ELISA delivered 85 positive results. WB with human Col7-NC1 revealed a significantly higher sensitivity rate than IIF on SSS (P = 0.033), whereas the NC1 ELISA and WB results showed no statistical difference regarding sensitivity (P = 0.346) (Table 1). Two sera tested positive using WB or

Table 1 Sensitivity of diagnostic assays in epidermolysis bullosa acquisita

<table>
<thead>
<tr>
<th>F-values (95% CI)</th>
<th>SSS</th>
<th>WB</th>
<th>NC1 ELISA</th>
<th>NC1/NC2 ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSS</td>
<td>0.033 (−0.0205 to −0.009)</td>
<td>0.007 (0.061 to 0.243)</td>
<td>0.000 (0.148 to 0.329)</td>
<td></td>
</tr>
<tr>
<td>WB</td>
<td>0.013 (−0.205 to −0.009)</td>
<td>0.346 (−0.049 to 0.136)</td>
<td>0.001 (0.059 to 0.212)</td>
<td></td>
</tr>
<tr>
<td>NC1 ELISA</td>
<td>0.002 (0.061 to 0.243)</td>
<td>0.346 (−0.049 to 0.136)</td>
<td>0.005 (0.042 to 0.157)</td>
<td></td>
</tr>
<tr>
<td>NC1/NC2 ELISA</td>
<td>0.000 (0.148 to 0.329)</td>
<td>0.001 (0.059 to 0.212)</td>
<td>0.005 (0.042 to 0.157)</td>
<td></td>
</tr>
</tbody>
</table>

CI, confidence interval; SSS, saline-split skin; WB, Western blot; ELISA, enzyme-linked immunosorbent assay.
IIF only. Surprisingly, all of the six additionally positive test results of the NC1/NC2 ELISA originated from European EBA sera (Italy n = 3, Germany n = 2, Czech Republic n = 1), which remained below the cut-off value of the NC1 ELISA (cut-off level 20 RE mL\(^{-1}\)).

**Specificity of diagnostic assays**

The specificity of the diagnostic assays was calculated based on the analysis of 50 healthy control sera, 50 PV and 100 BP sera. Overall, the specificity was high (i.e. > 90%) for all methods tested (Fig. 3). The WB delivered a specificity of 94%, IIF with SSS delivered a specificity of 99%, and the two ELISA systems delivered a specificity of 99–100%. The NC1 ELISA showed false-positive results for one patient with PV and one patient with BP (Fig. 4a), while the NC1/NC2 ELISA was false-positive in one patient with BP (Fig. 4c). Using WB, three healthy control sera, six BP sera and three PV sera were considered to be positive. Hence, the healthy control sera showed the lowest number of false-positive results (n = 3) and the WB revealed a significantly lower overall specificity compared with ELISA and IIF (NC1 ELISA, P = 0.004; NC1/NC2 ELISA, P = 0.002; IIF, P = 0.001) (Table 2).

**Diagnostic and predictive power of collagen VII enzyme-linked immunosorbent assays**

ROC analysis [area under the curve (AUC)] revealed no statistical differences between the NC1 ELISA (AUC healthy controls 0.983; AUC BP + PV 0.987) and the NC1/NC2 ELISA (AUC healthy controls 0.995; AUC BP + PV 0.995) (Fig. 4a, d). We also tried to correlate anti-Col7 IgG autoantibodies with the clinical course of selected patients with EBA. In patient 1, as expected, immune adsorption led to a strong decrease of anti-Col7 serum IgG levels (T2) but in the course of disease, anti-Col7 serum IgG increased again (T3) and was associated with a worsening of the clinical status. As illustrated by Autoimmune Bullous Skin Disorder Intensity Score and clinical pictures, anti-Col7 IgG correlated well with disease activity (Fig. 5a). In contrast, in patient 2, an increase of anti-Col7 IgG was associated with a slight improvement of skin involvement (TPI > TP2). In was noteworthy that in this patient antibody titres were very consistent on a high level over time (TP1 > TP6) (Fig. 5b). In patient 3, complete clinical remission was accompanied by a strong decrease of anti-Col7 serum IgG (Fig. 5c). In all three patients with EBA, changes in anti-Col7 serum IgG concentrations measured by optical density were comparable between the Col7-NC1 and Col7-NC1/NC2 ELISAs but ran at different levels (Fig. 5a–c). Overall, anti-Col7 IgG serum concentrations were largely correlated with the clinical disease activity (Fig. 5a–c).

**Discussion**

In this international retrospective multcentre study, we investigated the largest cohort to date of European and Japanese patients with EBA in order to validate the diagnostic power of serological assays designed to detect IgG autoantibodies against Col7, the autoantigen of EBA. Here we demonstrate that two commercially available human Col7-NC1 ELISAs showed the highest sensitivity among the in vitro diagnostic assays tested and were superior to IIF with human SSS and to a WB with overlapping fragments that span the NC1 subdomain of Col7. Moreover, one of the two Col7 ELISAs, which additionally contained the NC2 domain of Col7, showed the highest sensitivity and was thus superior to the Col7-NC1 ELISA.

Epidermolysis bullosa acquisita is a potentially devastating, chronic relapsing autoimmune bullous disorder of the skin and mucous membranes that is characterized by a pronounced and persistent fragility of the skin leading to blisters and erosions with a scarring phenotype. Novel anti-inflammatory therapies like high-dose intravenous immunoglobulins, the anti-CD20 monoclonal antibody, rituximab and immunoabsorption have been employed in EBA treatment with variable success. The inflammatory type of EBA that clinically resembles BP responds well to immunosuppressive drugs. In contrast, mechanobullous EBA is largely...
Fig 4. Performance of two enzyme-linked immunosorbent assay (ELISA) systems in epidermolysis bullosa acquisita (EBA). (a) Combined box-and-whisker plots showing collagen VII (Col7) NC1 ELISA results. (b) Receiver operating characteristic (ROC) analysis of Col7-NC1 ELISA results. (c) Combined box-and-whisker plots showing Col7-NC1/NC2 ELISA results. (d) ROC analysis of Col7-NC1/NC2 ELISA results. BP, bullous pemphigoid; PV, pemphigus vulgaris; HC, healthy controls; AUC, area under the curve.

Table 2 Specificity of diagnostic assays in epidermolysis bullosa acquisita

<table>
<thead>
<tr>
<th>P-values (95% CI)</th>
<th>SSS</th>
<th>WB</th>
<th>NC1 ELISA</th>
<th>NC1/NC2 ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSS</td>
<td></td>
<td>0.001 (0.035–0.102)</td>
<td>0.157 (–0.036 to 0.009)</td>
<td>0.317 (–0.028 to 0.014)</td>
</tr>
<tr>
<td>WB</td>
<td>0.001 (0.035–0.102)</td>
<td></td>
<td>0.004 (0.02–0.092)</td>
<td>0.002 (0.024–0.098)</td>
</tr>
<tr>
<td>NC1 ELISA</td>
<td>0.157 (–0.036 to 0.009)</td>
<td>0.004 (0.02–0.092)</td>
<td></td>
<td>0.564 (–0.019 to 0.031)</td>
</tr>
<tr>
<td>NC1/NC2 ELISA</td>
<td>0.317 (–0.028 to 0.014)</td>
<td>0.002 (0.024–0.098)</td>
<td>0.564 (–0.019 to 0.031)</td>
<td></td>
</tr>
</tbody>
</table>

CI, confidence interval; SSS, saline-split skin; WB, Western blot; ELISA, enzyme-linked immunosorbent assay.
refractory to immunosuppressive treatment. In addition, chronic immunosuppressive treatment is associated with major adverse effects.\textsuperscript{15,19} Thus, early diagnosis is essential for the optimal therapeutic and functional management of EBA. The detection of IgG autoantibodies directed against Col7, which play an essential pathogenic role (Table 3), is central to the proper diagnosis of the different clinical EBA variants.\textsuperscript{4,10,11,16,20–29} In general, anti-Col7 IgG serum concentrations correlate with the disease status,\textsuperscript{30,31} which is also reflected by the longitudinal analyses of three selected patients with EBA in the present study (Fig. 5).

Currently, in addition to the clinical appearance (which may be quite heterogeneous), the diagnosis of EBA is established based on the detection of tissue-bound (by DIF) and serum anti-Col7 IgG antibodies (by IIF, WB or ELISA). It has been estimated that 50–80% of patients with EBA exhibit both, tissue-bound and circulating anti-Col7 autoantibodies.\textsuperscript{32} The gold-standard technique for the detection of tissue-bound IgG is immunoelectron microscopy (IEM), which provides the highest sensitivity.\textsuperscript{33} Recently, Terra et al.\textsuperscript{29} developed a more refined and highly sensitive DIF analysis of EBA that is based on the detection of a u-serrated IgG deposition pattern at the dermoepidermal BMZ. However, at present, the overall significance of both IEM and DIF serration pattern analysis is limited as only a few laboratories worldwide provide this diagnostic technique for EBA. Thus, in current clinical practice, DIF has proven to be a highly reproducible and robust technique and is considered to be a reference method for detection of tissue-bound IgG autoantibodies in EBA.\textsuperscript{34–36} Consequently, all of the published studies on the sensitivity and specificity of immunoserological assays in EBA refer to DIF as the gold standard. However, its diagnostic power may be overestimated as DIF is less sensitive than IEM and DIF serration pattern analysis.\textsuperscript{33} This is reflected in studies by Seta et al.\textsuperscript{28} and Terra et al.\textsuperscript{29} where a positive IEM or serration pattern result served as an inclusion criterion for the assessment of the diagnosis of EBA. In these studies, the sensitivity of the NC1/NC2 ELISA was much lower (30% and 54%) compared with serological studies that defined classical DIF as the gold standard.

The present study clearly shows that WB using human Col7 and IIF with the standard substrate SSS, are less sensitive than the commercial Col7 ELISAs. Nevertheless, SSS is a critical technique for the immunoserological distinction between BP (epidermal IgG reactivity) and EBA, laminin-\gamma1 pemphigoid (all dermal IgG reactivity) and mucous membrane pemphigoid

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Serological diagnostics for anti-Col7 IgG autoantibodies in EBA, T. Schmidt et al. 1689

Fig 5. Longitudinal analysis of anticollagen VII (anti-Col7) IgG serum concentrations and clinical course of patients with epidermolysis bullosa acquisita (EBA). (a) Patient 1. Autoimmune bullous skin disorder (ABSIS) score, anti-Col7 IgG by enzyme-linked immunosorbent assay (ELISA) (Col7-NC1:RE mL\textsuperscript{−1}; Col7-NC1/NC2:U mL\textsuperscript{−1}) and illustration of clinical symptoms (T4, T5, T6 and T7). (b) Patient 2. Anti-Col7 IgG by ELISA (Col7-NC1:RE mL\textsuperscript{−1}; Col7-NC1/NC2:U mL\textsuperscript{−1}) and description of clinical symptoms. (c) Patient 3. Anti-Col7 IgG by ELISA (Col7-NC1:RE mL\textsuperscript{−1}; Col7-NC1/NC2:U mL\textsuperscript{−1}) and description of clinical phenotype. IA, immunoabsorption.
Table 3 Sensitivity and specificity of diagnostic assays in epidermolysis bullosa acquisita

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>EBA sera</th>
<th>Control sera</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>EBA sera</th>
<th>Control sera</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Recombinant protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chen et al.</td>
<td>1997</td>
<td>24</td>
<td>39</td>
<td>78</td>
<td>71.4</td>
<td>78</td>
<td>39</td>
<td>100</td>
<td>71.4</td>
<td>Col7-NC1</td>
</tr>
<tr>
<td>Chen et al.</td>
<td>2007</td>
<td>32</td>
<td>27</td>
<td>–</td>
<td>66.6</td>
<td>83.3</td>
<td>–</td>
<td>100</td>
<td>71.4</td>
<td>Col7-NC1 (aa1-227)</td>
</tr>
<tr>
<td>Pendaries et al.</td>
<td>2009</td>
<td>41</td>
<td>55</td>
<td>93.5</td>
<td>73</td>
<td>66.6</td>
<td>(combination of all fragments)</td>
<td>100</td>
<td>(combination of all fragments)</td>
<td>Col7-NC1 peptides (aa17-610, aa273-857, aa611-1253)</td>
</tr>
<tr>
<td>Mueller et al.</td>
<td>2010</td>
<td>15</td>
<td>50</td>
<td>93.5</td>
<td>73</td>
<td>66.6</td>
<td>(combination of all fragments)</td>
<td>100</td>
<td>(combination of all fragments)</td>
<td>Col7-NC1 peptides (aa17-610, aa273-857, aa611-1253)</td>
</tr>
<tr>
<td>Saleh et al.</td>
<td>2011</td>
<td>49</td>
<td>95</td>
<td>100</td>
<td>89.8</td>
<td>93.8</td>
<td>(combination of two fragments)</td>
<td>98.1</td>
<td>(combination of two fragments)</td>
<td>Col7-NC1</td>
</tr>
<tr>
<td>Marzano et al.</td>
<td>2013</td>
<td>14</td>
<td>143</td>
<td>100</td>
<td>93</td>
<td>85.7</td>
<td>(combination of two fragments)</td>
<td>98.1</td>
<td>(combination of two fragments)</td>
<td>Col7-NC1</td>
</tr>
<tr>
<td>Licarette et al.</td>
<td>2012</td>
<td>50</td>
<td>363</td>
<td>–</td>
<td>–</td>
<td>94</td>
<td>(combination of all fragments)</td>
<td>97.3</td>
<td>(combination of all fragments)</td>
<td>Col7-NC1</td>
</tr>
<tr>
<td>Kuo et al.</td>
<td>2013</td>
<td>28</td>
<td>46</td>
<td>57.1</td>
<td>54</td>
<td>97.8</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
<tr>
<td>Komorowski et al.</td>
<td>2012</td>
<td>73</td>
<td>389</td>
<td>99.8</td>
<td>91.8</td>
<td>91.8</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
<tr>
<td>Terra et al.</td>
<td>2013</td>
<td>24</td>
<td>85</td>
<td>83.3</td>
<td>91.7</td>
<td>79.2</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
<tr>
<td>Calabresi et al.</td>
<td>2014</td>
<td>24</td>
<td>85</td>
<td>83.3</td>
<td>91.7</td>
<td>79.2</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
<tr>
<td>Horvath et al.</td>
<td>2016</td>
<td>21</td>
<td>40</td>
<td>100</td>
<td>–</td>
<td>81</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
<tr>
<td>Seta et al.</td>
<td>2016</td>
<td>77</td>
<td>67</td>
<td>27</td>
<td>65 (30)</td>
<td>97 (96)</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
<tr>
<td>Marzano et al.</td>
<td>2016</td>
<td>6</td>
<td>11</td>
<td>100</td>
<td>–</td>
<td>100</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
<tr>
<td>Present study</td>
<td>2017</td>
<td>95</td>
<td>200</td>
<td>74-74</td>
<td>85-26</td>
<td>89.47</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
<tr>
<td>Present study</td>
<td>2017</td>
<td>95</td>
<td>200</td>
<td>74-74</td>
<td>85-26</td>
<td>97.87</td>
<td>–</td>
<td>98.1</td>
<td>Col7-NC1</td>
<td></td>
</tr>
</tbody>
</table>

EBA, epidermolysis bullosa acquisita; SSS, saline-split skin; WB, Western blot; ELISA, enzyme-linked immunosorbent assay. aManufacturer: MBL, Nagoya, Japan (Col7-NC1: aa21-1253, NC2: aa2783-2944). bManufacturer: Euroimmun, Lübeck, Germany (Col7-NC1: aa1-1253).
findings are in line with a previous study by Komorowski et al., who found similar rates of sensitivity (91/96) in their cohort of 73 patients with EBA (Col7-NC1 and WB with human Col7-NC1). Furthermore, our findings clearly show that supplementation of the Col7-NC1 ELISA with the human Col7-NC2 subdomain leads to a significant increase in the diagnostic power of the Col7 ELISA.

References


Serological diagnostics for anti-Col7 IgG autoantibodies in EBA, T. Schmidt et al.


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1: Serological diagnostics to establish the diagnosis of epidermolysis bullosa acquisita by the contributing clinical centres (n=95).
PERSONAL DATA
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Ruprecht-Karls-Universität, Medizinische Fakultät
Heidelberg, Germany
2007 - 2014
Medical University, Università Degli Studi Di Parma, Parma, Italy. Evaluation 105/110
Bachelor thesis: Comparison of Kindlin-1 expression in Primary keratinocytes and skin samples respecting the amount of UV-irradiation (Experiments and development of the thesis project from 8/2013 until 1/2014 at Klinik für Dermatologie und Allergologie, Albert-Ludwigs-Universität Freiburg, Supervisor: Prof. Dr. med Cristina Has)

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2001 - 2006
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PRIZES AWARDED
Translational Research Prize, project title: "Therapeutic inhibition of IL-17 leads to clinical remission of lichen planus" ADF 2018, Zurich

LIST OF PUBLICATIONS

Serological diagnostics in the detection of IgG autoantibodies against human collagen VII in epidermolysis bullosa acquisita: a multicentre analysis. Br J Dermatol 12, 2017 Dec; 177(6); 1683-1692

Amber KT, Lamberts A, Solimani F, Agnoletti AF, Didona D, Euverman I, Cozzani E, Yueh LH, Di Zenzo G, Leshem YA, Mimouni D, Hertl M, Horvath B
Determining the incidence of Pneumocystis Pneumonia in Patients With Autoimmune Blistering Diseases Not Receiving Routine Prophylaxis JAMA Dermatol. 2017 Nov 1;153(11):1137-1141

Solimani F, Juratli H, Hoch M, Wolf R, Pfützner W.

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9. Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren Damen und Herren

In Marburg: Eming, Hertl, Pfützner

In Freiburg: Has, Happle

In Parma: Amore, Bacciu Bettuzzi, Calderaro, Ceccarelli, Chetta, Cucurachi, De Angelis, Del Rio Fabrizi, Fanelli, Feliciani, Fiaccadori, Frusca, Gallese, Gandolfi, Gazzola, Giuliani, Meschi, McCharthy, Mori, Mutti, Raposio, Rizzolatti, Parmigiani Roncoroni, Rossi, Sansoni, Scandroglio, Silini, Vitale.
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Die Hinweise zur Erkennung von Plagiaten habe ich zur Kenntnis genommen.

Ort, Datum, Unterschrift

Doktorand