Increased Levels of the Bullous Pemphigoid BP180 Autoantibody Are Associated with More Severe Dementia in Alzheimer’s Disease

Nina Kokkonen¹, Sanna-Kaisa Herukka², Laura Huilaja¹, Merja Kokki³, Anne M. Koivisto², Päivi Hartikainen², Anne M. Remes² and Kaisa Tasanen¹

Bullous pemphigoid (BP) is a subepidermal blistering skin disease, which has shown a strong association with neurological diseases in epidemiological studies. The BP autoantigens BP180 and BP230 are expressed in the cutaneous basement membrane and the central nervous system. Using BP180 and BP230 ELISA assays and immunoblotting against BP180, we analyzed the IgG reactivity in the sera of 115 patients with Alzheimer’s disease (AD) and 40 neurologically healthy controls. BP180 autoantibodies were found in 18% of patients with AD, whereas only 3% of controls had positive results ($P = 0.019$). BP230 values were higher and more often elevated in patients with AD than controls, but not significantly. None of the positive AD sera that recognized the full-length human BP180 in immunoblotting reacted with the cutaneous basement membrane in indirect immunofluorescence analysis. Moreover, a retrospective evaluation of the hospital records of the patients with AD revealed neither BP diagnosis nor BP-like symptoms. Interestingly, increased BP180-NC16A autoantibody values correlated with cognitive decline measured by mini-mental state examination scores, but not with the concentration of AD biomarkers in cerebrospinal fluid. Our findings further the understanding of the role of BP180 as a shared autoantigen in neurodermatological interactions and the association between BP and neurodegenerative diseases.

INTRODUCTION

Bullous pemphigoid (BP) is the most common subepidermal autoimmune blistering disease with increasing incidence among elderly people (Fürst et al., 2014; Joly et al., 2012; Langan et al., 2008; Schmidt and Zillikens, 2013). Typically, patients with BP have severe pruritus and blistering skin lesions, but in up to 20% of patients blisters are absent and only excoriations, eczematous, or urticated lesions occur (della Torre et al., 2012; Di Zenzo et al., 2012). At the molecular level, BP autoantibodies target two cutaneous basement membrane proteins, BP180 and BP230 (Nishie, 2014). The main antigen BP180, also known as collagen XVII or BP1A2, is a transmembrane protein of hemidesmosomes linking basal keratinocytes to the underlying dermis (Franzke et al., 2005; Van den Bergh and Giudice, 2003). IgG antibodies of patients with BP target the immunodominant non-collagenous 16A (NC16A) domain, which is located extracellularly close to the transmembrane domain of BP180 (Nishie, 2014; Schmidt and Zillikens, 2013). As well as in the skin, BP180 is also expressed in various other tissues (Claudepierre et al., 2005; Huilaja et al., 2008; Hurskainen et al., 2012) including the brain (Seppänen et al., 2006, 2007; Seppänen, 2013). The other autoantigen, BP230 (bullous pemphigoid antigen 1, BPAG1e), is an intracellular component of hemidesmosomes and a ligand of BP180. It is the epithelial isoform of dystonin, and its variants BPAG1a1 and BPAG1a2 are expressed in both the central and the peripheral nervous system (Kunzli et al., 2016; Powell et al., 2005). BP230 is targeted by autoantibodies in approximately 60% of patients with BP (Kasperkiewicz et al., 2012), but it seems that autoantibodies against BP230 result from intermolecular epitope spreading and never proceed to anti-BP180 autoantibodies (Di Zenzo et al., 2011, 2012).

The diagnosis of BP is established by typical clinical presentation and the finding of linear IgG or complement 3 along the basement membrane zone in a direct immunofluorescence (IF) analysis of a perilesional biopsy (Di Zenzo et al., 2012; Schmidt and Zillikens, 2013). In addition, the detection of circulating autoantibodies by BP180-NC16A ELISA has high specificity and moderate sensitivity in diagnosing BP (Tampoia et al., 2012).
Several epidemiological studies have revealed an association of BP with a range of neurological diseases including dementia, cerebral stroke, Parkinson’s disease, multiple sclerosis, and motor neuron disease (Bastuji-Garin et al., 2011; Brick et al., 2014; Cordel et al., 2007; Langan et al., 2011; Ong et al., 2013; Stinco et al., 2005). An existing neurological disorder appears to increase the subsequent risk for BP (Brick et al., 2014; Langan et al., 2011), but conversely, increased risk for developing neurological diseases has also been reported after BP diagnosis (Brick et al., 2014; Yang et al., 2011).

The expression of BP autoantigens in the central nervous system and the association between BP and various neurodegenerative disorders suggest that BP180 and BP230 could act as shared antigens in cutaneous and neurological diseases (Kunzli et al., 2016; Seppänen, 2013). To test this hypothesis, we measured BP autoantibodies in well-characterized patients with Alzheimer’s disease (AD). We also investigated postulated associations between BP autoantibodies and age, gender, and cognitive decline as well as with the presence of cerebrospinal fluid (CSF) markers of AD pathology.

RESULTS
BP180 and BP230 autoantibodies in patients with Alzheimer’s disease
Sera from patients with AD (n = 115) and neurologically healthy control subjects (n = 40) were tested for BP180 and BP230 IgG autoantibodies using a well-established, commercially available ELISA analysis, and all anti-BP180 positive sera (24 AD samples and 3 controls) were further tested by immunoblotting against human recombinant BP180. A significantly higher proportion of AD samples (20%; 18%) than control samples (1%; 3%) were positive for both IgG autoantibodies against BP180-NC16A (based on a cutoff value >9 U/ml; Table 1, Figure 1) and the full-length BP180 (Figure 2). Accordingly, BP180 autoantibody values were significantly higher in the AD group (2.4 U/ml, range 0–72.0 U/ml) compared with those of the control group (2.0 U/ml, range 0.5–12.3 U/ml; P = 0.05; Table 1, Figure 1). We also tested 18 of the anti-BP180 positive AD samples and the one positive control sample using indirect IF analysis. Under the same conditions in which the sera from BP patients demonstrated a clear linear immunostaining on the cutaneous basement membrane zone, none of the tested AD sera nor the positive control sample showed any specific immunostaining either in normal or salt-split human skin (Supplementary Figure S1 online).

In the ELISA assay, IgG autoantibodies against BP230 were also found in a higher proportion of patients with AD than controls and BP230 autoantibody values were higher in the AD group than those of the controls, but neither of these differences was statistically significant (Table 1, Figure 1). Autoantibodies against only BP180 were detected in 16 patients with AD, 6 patients with AD had anti-BP230 positivity without having reactivity against BP180, and 8 patients with AD had autoantibodies against both BP180 and BP230.

A retrospective evaluation of the hospital records of patients with AD with increased anti-BP180 or -BP230 values revealed neither diagnosis nor clinical symptoms of BP in any patient.

The association of BP180 and BP230 autoantibodies with age, gender, cognitive impairment, and Alzheimer’s disease biomarkers
BP180 and BP230 ELISA values did not correlate with age in either the whole study group or the group of patients with AD. However, BP180 and BP230 ELISA values showed a statistically significant correlation to each other in the whole study group (r = 0.329, P < 0.001). This overall correlation was driven by the correlation in the patients with AD (r = 0.367, P < 0.001), as there was no such correlation when the control group was considered separately. In the AD group, autoantibodies against BP180-NC16A were significantly more common in females than males (P = 0.029) and the median BP180 ELISA value was significantly higher in females than males (P = 0.037). In contrast, there was no significant difference between the genders for positivity or values of BP230 ELISA.

The severity of dementia of patients with AD was evaluated using the mini-mental state examination (MMSE) (Table 1). MMSE data were available for 89 patients with AD. Interestingly, there was a negative correlation between the MMSE score and the BP180 ELISA value (r = −0.287, P = 0.007), that is, patients with lower MMSE, denoting more severe dementia, had higher serum anti-BP180 levels. As expected, the MMSE score showed negative correlation with age (r = −0.351, P = 0.001). Regarding AD biomarkers, there was no correlation between serum anti-BP180 values and beta-amyloid (Aβ1-42), total tau, or hyperphosphorylated tau (p-tau181) in the CSF samples of patients with AD.

DISCUSSION
Based on the association of several neurological diseases with BP and the central nervous system expression of BP

Table 1. Characteristics, bullous pemphigoid autoantibodies and Alzheimer’s disease (AD) biomarkers of neurologically healthy controls and patients with AD

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients with AD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>40</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>Sex M/F</td>
<td>14/26</td>
<td>41/74</td>
<td>ns</td>
</tr>
<tr>
<td>Age</td>
<td>66.8 ± 7.0</td>
<td>72.0 ± 7.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMSE</td>
<td>na</td>
<td>20 (8–29)</td>
<td></td>
</tr>
<tr>
<td>BP180 (U/ml)</td>
<td>2.0 (0.5–12.3)</td>
<td>2.4 (0–47.7)</td>
<td>0.05</td>
</tr>
<tr>
<td>BP180 –/+</td>
<td>37/1 (97.3%)</td>
<td>91/20 (82.18%)</td>
<td>0.019</td>
</tr>
<tr>
<td>BP230 (U/ml)</td>
<td>1.35 (0–24.1)</td>
<td>2.0 (0–35.8)</td>
<td>ns</td>
</tr>
<tr>
<td>BP230 –/+</td>
<td>37/3 (95.75%)</td>
<td>101/14 (88.12%)</td>
<td>ns</td>
</tr>
<tr>
<td>Aβ1-42 (pg/ml)</td>
<td>728 (263–1,078)</td>
<td>443 (139–1,294)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tau (pg/ml)</td>
<td>266 (90–582)</td>
<td>554 (48–1,994)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p-Tau181 (pg/ml)</td>
<td>51 ± 14</td>
<td>90 ± 35</td>
<td>&lt;0.001</td>
</tr>
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Data given as median (range) unless otherwise indicated.
Abbreviations: Aβ1-42, beta-amyloid; BP, bullous pemphigoid; MMSE, mini-mental state examination; na, not available; ns, not significant; p-Tau181, hyperphosphorylated tau; Tau, total tau.
1Data given as mean ± standard deviation.
2Data available for 89 patients with AD.
3Two controls and four patients with AD were excluded because of lack of reactivity against BP180 in immunoblotting.
4Because of a method change, the levels are 10% higher than in the AD group.
autoantigens, it has been suggested that neuroinflammation could lead to a cross-reactive immunoresponse between neural and cutaneous antigens (Kunzli et al., 2016; Langan et al., 2011; Seppänen, 2013). This theory is supported by our current study, which revealed the presence of increased IgG autoantibodies against BP180 in 18% of patients with AD. The prevalence of BP autoantibodies in our control samples was slightly higher than in previous studies, which report that IgG autoantibodies against BP180 and BP230 exist in 0–2.0% of the general population (see Prussmann et al., 2015). The association between BP autoantibodies and dementia has been previously shown in a French study in which the reactivity against BP180-NC16A was analyzed in 138 subjects aged over 69 years with no signs of BP (69 patients with dementia and 60 nondementia controls) (Foureur et al., 2006). That study found BP180 autoantibodies in 7% of subjects with dementia (MMSE ≤ 24), but not in controls (MMSE > 24). When compared with the previous study, the prevalence of increased BP180-NC16A autoantibodies in our study was clearly higher among patients with AD. Differing ELISA results were reported in a recent study in which only one of 26 patients with various dementia types had autoantibodies against BP180-NC16A (Messingham et al., 2016). However, six of the same patients showed IgG reactivity in immunoblotting against the entire intra- or extracellular domain of BP180 (Messingham et al., 2016), which closely resembles our current result. In addition, nine patients with Parkinson’s disease (n = 24) had antibodies to BP180, but not against the NC16A domain (Messingham et al., 2016). Another recent study failed to detect any autoantibodies against BP180 or BP230 in patients with Parkinson’s disease (n = 50) and multiple sclerosis (n = 50) despite the use of several antigens and diagnostic methods (Recke et al., 2016).

The differing results of these studies may be explained by methodological differences or differences between the study populations. The patients with dementia in our study were diagnosed according to internationally accepted diagnostic criteria for AD including clinical and neuropsychological examination, brain imaging and testing for CSF AD biomarkers (McKhann et al., 1984), whereas the other studies do not describe in detail the criteria by which their dementia diagnoses were made (Foureur et al., 2006; Messingham et al., 2016).

Our current study shows that BP180 autoantibodies had a significant association with cognitive decline: the lower the MMSE score, the higher the value of the BP180 ELISA. This is in line with an epidemiological study, which showed that more severe dementia (MMSE ≤ 17) is associated with a twofold increase in the risk of BP (Bastuji-Garin et al., 2011). In contrast, there was no correlation between the level of BP autoantibodies and the concentration of AD biomarkers in CSF samples. Aβ1-42 is a marker for the deposition of Aβ, and total tau and p-tau181 can be used to quantify neurofibrillary tangles and neuronal loss in the brain (Tapiola et al., 2009).
Among these three markers, only a decrease in the concentration of p-tau\textsubscript{181} in CSF correlates weakly with cognitive decline measured by the MMSE (Seppäla\textsubscript{e} et al., 2011). The negative correlation between the level of BP180-NC16A autoantibodies and MMSE values demonstrates the need for further studies to analyze whether BP180 ELISA could be used as a predictive serum marker of AD onset or prognosis. Surprisingly, we found that IgG antibodies against BP180 were significantly more common in women than men with AD. Although the incidence of BP is higher in women than in men (Joly et al., 2012; Langan et al., 2008), dementia seems to be equally associated with BP in both genders (Chen et al., 2011; Langan et al., 2011).

The expression of the neural isoform of BP230 in the brain has often been suggested to explain the association between BP and neurological disorders (Kunzli et al., 2016; Langan et al., 2011; Seppänen, 2013), but so far attempts to demonstrate a cross-reactive immune reaction between the central nervous system and skin variants have failed (Kunzli et al., 2016). The neural isoforms of BP230 are widely expressed in both the central nervous system and the peripheral nervous system (Kunzli et al., 2016), whereas BP180 is expressed in different anatomical regions of the human brain (Seppänen, 2013). Of note, particularly strong BP180 expression has been detected in pyramidal cells of the hippocampus and the ganglionic layer of the cortex, regions that are well-recognized predilection areas for AD-related lesions (Seppänen et al., 2006, 2007; Seppänen, 2013). Our current data suggest that an autoimmune reaction against BP180, rather than BP230, seems to be associated with neurodegenerative disorders. In fact, reactivity against BP180 is more relevant for the development of clinical BP, because IgG recognition of the BP180 ectodomain is an early and crucial event in the pathogenesis of BP, which is followed by IgG reactivity against BP230 through epitope spreading events (Di Zenzo et al., 2011).

The detection of BP180 autoantibodies has been shown to have high sensitivity and moderate specificity for diagnosing BP and it has even suggested to be used in the diagnostic screening for BP (Sakuma-Oyama et al., 2004; Tampoia et al., 2012). Using immunoblotting we confirmed that the IgG autoantibodies of patients with AD recognized a 180-kDa protein that was sensitive to collagenase digestion, and could therefore be identified as BP180. Therefore, it is intriguing that sera from our patients with AD showed no IgG reactivity against the cutaneous basement membrane zone in indirect IF analysis. However, our negative IF results reflect those of the aforementioned work of Messingham et al. They reported that anti-BP180 positive sera from four patients with Parkinson’s disease lacked reactivity with skin in indirect IF, but instead recognized tyrosine-hydroxylase positive neurons in human and rat substantia nigra (Messingham et al., 2016). Viewed with our current data, these results suggest that autoantibodies found in patients with neurological diseases are not identical to those in patients with BP. Perhaps the patients with AD or other neurological diseases who have positive BP180 antibodies represent a subgroup of “predisease state” BP patients. These patients first have neuronal immunoreactivity against BP180 and later on, perhaps due to some additional triggers, some also develop cutaneous autoimmunity. The time from initial symptoms to clinically typical BP may range from several weeks to more than 10 years (Schmidt et al., 2014), but currently the typical latency between the detection of IgG reactivity against BP180 and the appearance of clinical BP symptoms is unknown. After a diagnosis of dementia, people aged 65 or older survive a median of 3–8 years, but some live for as long as 20 years (see Winblad et al., 2016). Thus it is possible that the majority of patients with AD and other dementia with BP180 autoantibodies die before they develop reactivity against the skin. This hypothesis is supported by epidemiological data: the prevalence of dementia is 5–7% in most regions of the world and AD is estimated to account for 50–70% of all dementia cases (see Winblad et al., 2016). Because the incidence of BP varies between 0.25 and 4.28/100,000 per year (Förstl et al., 2014; Joly et al., 2012; Langan et al., 2008; Schmidt and Zillikens, 2013), a crude estimate would indicate that indeed only a very small subgroup of anti-BP180 positive AD patients finally develop clinical BP. On the other hand, in our study, increased anti-BP180 values correlated with more severe dementia. If BP manifests as pruritus and nonspecific symptoms such as erosions, excoriations, eczema, or papules (Di Zenzo et al., 2012; Schmidt and Zillikens, 2013), its diagnosis can be easily missed, especially in patients with severe AD whose ability to communicate is impaired and who are usually treated in geriatric units or nursing homes rather than hospitals.

We conclude that levels of IgG antibodies against BP180 are detected in almost 20% of patients with AD, and that BP180-NC16A ELISA values are significantly associated with the severity of dementia. Future studies are required to assess the exact epitopes and IgG subtypes of BP180 antibodies in neurological patients and measure BP autoantibodies in CSF samples of patients with AD, and thereby clarify the molecular mechanisms leading to failure of immunological tolerance against BP autoantigens in the human brain. The key question to be answered is why the BP180 autoantibodies of patients with AD do not bind to the cutaneous basement membrane. This could be explored, for example, by comparing the immunoreaction in the human brain between specific BP180 antibodies and anti-BP180 positive samples from patients with various neurological diseases as well as by analyzing whether autoantibodies from patients with BP bind to neural tissue. Finally, large prospective studies with well-characterized study populations and long enough follow-up periods will help us better understand predisposing factors and additional triggers of the onset of BP and its association with AD and other neurological diseases.

**MATERIALS AND METHODS**

**Patient samples**

The study was performed according to the principles of the Declaration of Helsinki. The research ethics committee of Kuopio University Hospital approved the collection of human AD and control samples. The collection of BP sera samples and control skin biopsies for indirect IF analysis was approved by the Ethical Committee of the Northern Ostrobothnia Hospital District. All samples were taken after written informed consent.

Serum samples were collected at the Kuopio University Hospital from patients with dementia and from neurologically healthy control
subjects. All patients with dementia were diagnosed with probable AD according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria (McKhann et al., 1984). The diagnosis was based on clinical and neuropsychological examination, brain imaging, and CSF AD biomarkers. All control samples were taken from patients attending the hospital for knee replacement operations. The mean age of the patients with AD was slightly higher than that of controls. In both control and AD groups, there were equal female predominance (41 of 74 in the study group and 14 of 26 in the control group) corresponding to that in patients with AD in general. The MMSE score at the time of the clinical examinations was obtained from patient records. We obtained CSF samples from both the patients with dementia and the controls by lumbar puncture in the L3-4 or L4-5 interspace and stored at −80 °C until measurement. The CSF concentrations of AD markers (p-tau181, p-tau143, and total tau) were measured as previously described in detail (Herukka et al., 2005). The patient records of all patients with BP180 or BP230 ELISA positivity were checked for skin diseases. A dermatologist did not check the cutaneous status of patients with AD or controls.

BP180 and BP230 ELISA
Circulating antibodies against BP180 and BP230 were analyzed from serum samples stored at −80 °C until measurement with commercially available kits (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer’s instructions. A cutoff ELISA value of 9 U/ml was used, with a value of >9 U/ml being a positive result. BP180 ELISA was performed twice for each sample and the mean value was used for further analysis.

Immunoblotting
COS-7 cells were transiently transfected with a plasmid containing human Col17A1 cDNA (Franzke et al., 2004) and Lipofectamine 3000 (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. After a 24-hour transfection period, the culture medium was changed and 50 µg/ml ascorbic acid added to the cells. Then, 24 hours later the cells were lysed in a RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO], 2 mM EDTA) and aliquots of cell extracts were treated with 40 U/ml highly purified bacterial collagenase (type III, Sigma-Aldrich) for 2 hours at 37 °C. Proteins were size-separated under denaturing conditions, electrophoresed onto a nitrocellulose membrane, and detected with human serum samples as previously described (Hurskainen et al., 2015). Shortly, the study serum samples were used as 1:50 diluted purified bacterial collagenase (type III, Sigma-Aldrich, Manassas, VA) as a secondary antibody. ECL Prime substrates (GE Healthcare, Buckinghamshire, UK) with an LAS-3000 Image analyzer (Fuji, Tokyo, Japan) were used for visualization. Polyclonal rabbit anti-human NC16A (Schumann et al., 2000) and goat anti-rabbit IgG-peroxidase (Sigma-Aldrich) antibodies were used to detect BP180 protein in immunoblots.

Indirect immunofluorescence (IF)
Indirect IF analysis was performed as previously described (Hurskainen et al., 2015). Briefly, 5-µm frozen nonfixed sections of human skin (treated or not with 1 M NaCl) were blocked (1% BSA in phosphate buffered saline) and human sera in 1:4 to 1:200 dilutions were used as primary antibodies. Rabbit anti-human IgG-FITC (DAKO, Glostrup, Denmark) was used as a secondary antibody. ImmunoQuant-embedded sections (Shandon, Thermo Scientific, Mid-dletown, VA) were photographed with an Olympus FluoView FV1000 confocal microscope using a ×60 oil objective. Identical settings and exposure times were used to photograph all sections.

Statistical analysis
Data were entered and statistical analyses were conducted using the IBM SPSS Statistics version 21.0 software for Windows (IBM, Chicago, IL). The Kolmogorov-Smirnov test was used to test the normality of distribution of the variables. The data for continuous variables are presented as mean (standard deviation) of median (range) when the assumption for normality was not met. Differences between the groups were analyzed using either analysis of variance for normally distributed data or the Mann-Whitney U test when the assumptions were not met. Pearson correlation or Spearman’s rho test was used to analyze correlations between continuous variables. For the nominal variables, the results are presented as the number (percentage) of subjects when appropriate and the χ² test was used for testing the statistical difference between groups. A two-sided P value of 0.05 was considered as the limit of statistical significance.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
We thank Anja Mattila and Riitta Vuento for their expert technical assistance. This study was supported by the Academy of Finland grant to NK, Oulu University Hospital to KT, and Kuopio University Hospital to S-KH and AMR.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.09.010.

REFERENCES


