

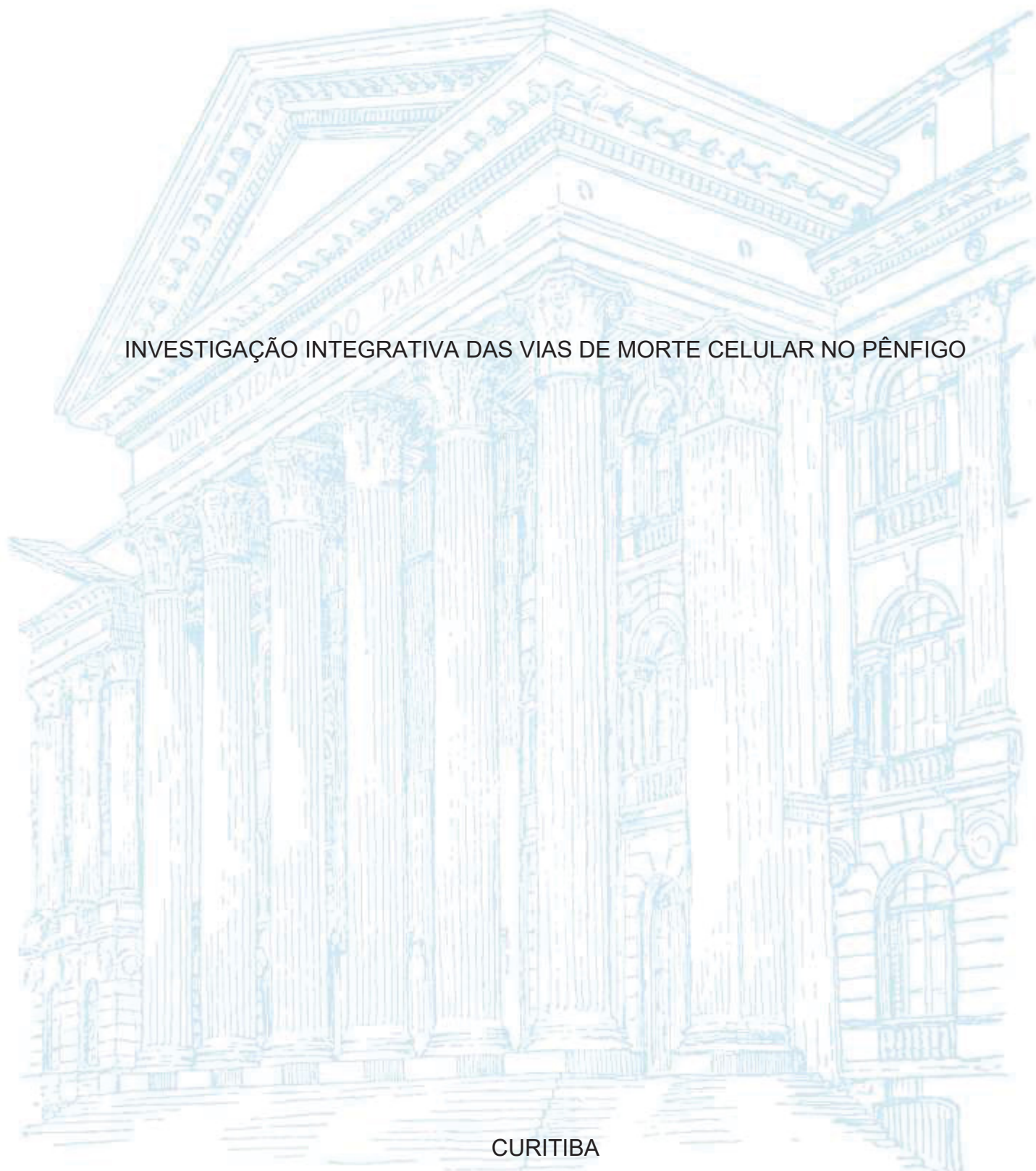
UNIVERSIDADE FEDERAL DO PARANÁ

VALÉRIA BUMILLER BINI HOCH

INVESTIGAÇÃO INTEGRATIVA DAS VIAS DE MORTE CELULAR NO PÊNFIGO

CURITIBA

2022



VALÉRIA BUMILLER BINI HOCH

INVESTIGAÇÃO INTEGRATIVA DAS VIAS DE MORTE CELULAR NO PÊNFIGO

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Orientadora: Profa. Dra. Angelica Beate Winter Boldt

Coorientadora: Profa. Dra. Jennifer Elisabeth Hundt

Coorientador: Prof. Dr. Gabriel Adelman Cippola

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*“Agora permanecem esses três: a fé, a esperança e o amor, mas o maior
deles é o Amor” 1 Coríntios 13:13*

RESUMO

Pênfigo é um grupo de doenças autoimunes bolhosas que causam dolorosas lesões cutâneas. As doenças pertencentes a este grupo são caracterizadas pela acantólise e pela produção de autoanticorpos contra proteínas de adesão celular. Apesar da relevância patogênica dos autoanticorpos IgG no processo acantolítico, os mecanismos exatos que levam os queratinócitos à morte permanecem desconhecidos. A apoptose tem sido sugerida como uma via que apresenta um papel importante em algumas dermatoses como no pênfigo foliáceo (PF) e no pênfigo vulgar (PV), antes ou após a perda de adesão celular. Dados os mecanismos subjacentes pouco compreendidos que levam à morte de queratinócitos no pênfigo, revisamos a literatura para moléculas e/ou características envolvidas nas 12 vias de morte celular descritas pelo Comitê de Nomenclatura em Morte Celular, ocorrendo em pacientes com pênfigo, linhagens celulares ou em miniórgãos de pele tratados com soros ou IgG de pacientes com pênfigo ou em modelo animal de pênfigo. Encontramos 61 estudos mencionando 97 moléculas envolvidas em vias de morte celular no pênfigo. A maioria dessas investigações se concentrou apenas na apoptose, mas quatro estudos recentes, utilizando ensaios de TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) e/ou microscopia eletrônica, desqualificaram essa via como um evento prévio de acantólise. Em seguida, investigamos em PF endêmico, variantes genéticas de genes cujos produtos estão envolvidos nas diferentes vias de morte celular. Neste estudo, encontramos 10 variantes associadas ($p < 0.005$) pertencentes a seis vias de morte celular: apoptose; morte celular imunogênica; necroptose; necrose; partanatos e piroptose. Também investigamos em PV e nas formas endêmica e esporádica de PF, variantes genéticas em genes nucleares cujos produtos codificam subunidades da cadeia respiratória mitocondrial ou seus fatores de montagem. Nesta análise, encontramos associação de uma variante do gene do componente essencial do complexo da cadeia respiratória I (subunidade A1 da ubiquinona oxidoredutase) e outra do complexo IV (fator de montagem do citocromo c oxidase heme A) exclusivamente com PF endêmico ($p < 0.005$). Além disso, realizamos uma caracterização ultraestrutural do miniórgão de pele humano utilizado para avaliar as proteínas candidatas, seguida de sua identificação nas biópsias de PF endêmico e esporádico. No miniórgão de pele humana, encontramos o receptor 1 do componente C5a (C5a) do complemento (C5aR1) aumentado ($p < 0.0001$), assim como em biópsias de PF endêmico e esporádico ($p < 0.0001$). Ainda, encontramos C5a aumentado no soro de pacientes de PF e PV ($p < 0.0001$). Para avaliar uma das vias de morte celular, investigamos a presença de armadilhas extracelulares de neutrófilos (NETs) no soro de pacientes de PF e PV, mas não observamos diferenças significativas. Uma validação funcional dos resultados encontrados nesta tese pode contribuir para o entendimento da etiologia do pênfigo e o desenvolvimento de novas drogas e alvos terapêuticos para a doença. Novas investigações relevantes sobre o pênfigo e as vias de morte celular, além da apoptose, devem ser feitas para superar os desafios de compreensão da etiopatogênese do pênfigo.

Palavras-chave: Pênfigo. Vias de morte celular. Cultura de miniórgão de pele. Sistema complemento. Subunidades da cadeia respiratória mitocondrial.

ABSTRACT

Pemphigus is a group of bullous autoimmune diseases that cause painful skin lesions. The diseases belonging to this group are characterized by acantholysis and the production of autoantibodies against cell adhesion proteins. Despite the pathogenic relevance of IgG autoantibodies in the acantholytic process, the exact mechanisms that lead keratinocytes to death remain unknown. Apoptosis has been suggested as a pathway playing an important role in some dermatoses such as pemphigus foliaceus (PF) and pemphigus vulgaris (PV), before or after loss of cell adhesion. Given the poorly understood underlying mechanisms that lead to keratinocyte death in pemphigus, we reviewed the literature for molecules and/or features involved in the 12 cell death pathways described by the Nomenclature Committee on Cell Death, occurring in patients with pemphigus, in cell lines, or in skin mini-organs treated with sera or IgG from patients with pemphigus or in an animal model of pemphigus. We found 61 studies mentioning 97 molecules involved in cell death pathways in pemphigus. Most of these investigations focused only on apoptosis, but four recent studies using TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assays and/or electron microscopy disqualified this pathway as an event prior to acantholysis. Next, we investigated in endemic PF, variants of genes whose products are involved in different cell death pathways. In this study, we found 10 associated variants ($p < 0.005$) belonging to six cell death pathways: apoptosis; immunogenic cell death; necroptosis; necrosis; pyroptosis and pyroptosis. We also investigated, in PV and in the endemic and sporadic forms of PF, variants in nuclear genes whose products encode subunits of the mitochondrial respiratory chain or their assembly factors. In this analysis, we found a variant in the gene of an essential component of respiratory chain complex I (ubiquinone oxidoreductase A1 subunit) and another of complex IV (cytochrome c oxidase heme assembly factor A) exclusively associated with endemic PF ($p < 0.005$). In addition, we performed an ultrastructural characterization of the human skin mini-organ used to evaluate candidate proteins, followed by their identification in biopsies of endemic and sporadic PF. We found the complement C5a receptor 1 (C5aR1) increased ($p < 0.0001$) in human skin mini-organ, as well as in biopsies of endemic and sporadic PF ($p < 0.0001$). Furthermore, the C5a component of the complement (C5a) was increased in serum of PF and PV patients ($p < 0.0001$). To assess one of the cell death pathways, we investigated the presence of neutrophil extracellular traps (NETs) in serum of PF and PV patients, but no significant differences were observed. A functional validation of the results found in this thesis may contribute to the understanding of the etiology of pemphigus and the development of new drugs and therapeutic targets for the disease. Further relevant investigations regarding pemphigus and cell death pathways beyond apoptosis must be conducted to overcome the challenges of understanding the etiopathogenesis of pemphigus.

Keywords: Pemphigus. Cell death pathways. Human skin organ culture. Complement system. Mitochondrial Respiratory Chain Subunits.

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1 INTRODUÇÃO

Pênfigo é um grupo de doenças autoimunes que atinge a epiderme, causando lesões bolhosas provocadas pela manifestação de autoimunidade e acompanhadas pelas sensações de ardor e calor nos pacientes (AMAGAI e STANLEY, 2012). Caracteriza-se pela formação de autoanticorpos principalmente contra glicoproteínas que compõem o desmossomo (JONES et al., 1986) e pelo desprendimento dos queratinócitos, processo denominado acantólise. Há dois tipos principais de pênfigo, o pênfigo foliáceo (PF) e o pênfigo vulgar (PV). No PF, a acantólise ocorre na camada granulosa subcórnea da epiderme e atinge a pele. No PV, a acantólise ocorre no estrato espinhoso suprabasal da epiderme; conseqüentemente as lesões são mais profundas e podem atingir a pele e as mucosas (SPINDLER et al., 2007).

No Brasil, o PF, também conhecido como fogo selvagem, é endêmico (CASTRO et al., 1983), assim como em outros países da América do Sul e na Tunísia (CASTRO et al., 1983; BASTUJI-GARIN et al., 1995). A maior prevalência da doença já observada foi encontrada nos anos 90 em duas comunidades indígenas localizadas no centro-oeste do Brasil (FRIEDMAN et al., 1995; HANS-FILHO et al., 1996). PF é a única doença autoimune e endêmica conhecida (CASTRO et al., 1983; BASTUJI-GARIN et al., 1995). Mundialmente, o PF e o PV ocorrem esporadicamente (MEYER e MISERY, 2010; ALPSOY et al., 2015).

O pênfigo apresenta modo de herança poligênico e etiologia multifatorial (DIAZ et al., 1989; RUOCCO et al., 2013; SCHMIDT et al., 2019; PETZL-ERLER et al., 2020). Diversos estudos já foram realizados para identificar componentes genéticos associados ao pênfigo (SCHMIDT et al., 2019; PETZL-ERLER et al., 2020). Dentre eles, alelos HLA foram associados ao aumento da propensão ao PF (PETZL-ERLER e SANTAMARIA, 1989; MORAES et al., 1997; PAVONI et al., 2003; BROCHADO et al., 2016), assim como genótipos dos genes *IL4* (PEREIRA et al., 2004) e *IL6* (PEREIRA et al., 2004), além de alelos do gene *TNF* tanto em PF como em PV (ROXO et al., 2013; TORZECKA et al., 2003; JAVOR et al., 2010; MOSAAD et al., 2012). Também, alelos dos genes *C3*, *C8A* e *C9* já foram associados à suscetibilidade ao PF (BUMILLER-BINI et al., 2018). Esses genes codificam componentes do sistema complemento que levam à morte celular pelo complexo de ataque à membrana. Além disso, já foi observada a expressão diferencial em células T CD4⁺ de nove genes envolvidos na morte celular programada entre pacientes com

PF e controles (MALHEIROS et al., 2014). Foi também observada expressão diferencial dos genes *CASP3*, *TNF* e *FAS* em pacientes com PV (FELICIANI et al., 2000; PACHECO-TOVAR et al., 2009).

O reconhecimento de glicoproteínas dos desmossomos pelos autoanticorpos IgG1 e IgG4 conduzem à acantólise (AMAIKAI e STANLEY, 2012). No entanto, o mecanismo subjacente à morte dos queratinócitos é desconhecido e debatido na comunidade científica. Apesar da relevância patogênica dos autoanticorpos IgG anti-desmogleína (DSG), o(s) exato(s) mecanismo(s) de perda da adesão celular e de morte celular, seja(m) como causa ou consequência destas, permanece(m) indecifrado(s). A apoptose tem sido sugerida como um evento que antecede a acantólise, mas alternativamente também como um evento posterior à perda de adesão celular (JANSE et al., 2014; SCHMIDT e WASCHE, 2009; SCHMIDT et al., 2009; LEE et al., 2009). Alguns autores afirmam que a morte celular no pênfigo ocorre pela apoptose (GNIADECKI et al., 1998; RODRIGUES et al., 2009) e outros, que não existem evidências da ocorrência desta (SCHMIDT et al., 2009; LEE et al., 2009; JANSE et al., 2014; SOKOL et al., 2015).

Sabendo que existem diversas outras vias que levam à morte celular (GALLUZZI et al., 2018) e que o mecanismo de morte celular no PF é desconhecido, assim como é a etiologia da doença, pretendeu-se investigar a associação de polimorfismos genéticos em genes envolvidos nas 12 diferentes vias de morte celular (apoptose intrínseca e extrínseca, necrose, necroptose, ferroptose, piroptose, partanatos, entose, netose, lisossomal, autofágica e imunogênica) com o PF endêmico. Após a identificação dos possíveis mecanismos de morte celular envolvidos no PF através do estudo de associação genética, realizaram-se ensaios para identificar a ocorrência de expressão proteica em um modelo de miniórgão de pele humana que mimetiza o fenótipo de pênfigo e em biopsias de pacientes de PF endêmico e esporádico. Foram também quantificadas, no soro de pacientes de PF endêmico e PV, proteínas séricas envolvidas nestes processos. Além disso, avaliou-se a expressão dos genes envolvidos nas vias de morte celular no modelo de miniórgão de pele humana. A identificação de uma (ou mais) via(s) de morte celular em pacientes com pênfigo fornecerá importantes informações para a compreensão da doença e indicação de novos alvos terapêuticos candidatos.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a possibilidade de associação genética e funcional de moléculas que participam de diferentes vias de morte celular com o pênfigo foliáceo e vulgar.

2.2 OBJETIVOS ESPECÍFICOS

- I. Revisar a literatura e identificar moléculas e características envolvidas nas diferentes vias de morte celular em pacientes com pênfigo foliáceo e pênfigo vulgar, linhagens celulares ou culturas de miniórgão de pele humana tratadas com soro ou imunoglobulina G de pacientes com pênfigo ou em modelo animal de pênfigo.
- II. Identificar possíveis associações de variantes de genes cujos produtos participam nas diferentes vias de morte celular com o PF endêmico.
- III. Identificar possíveis associações de variantes em genes nucleares cujos produtos codificam subunidades da cadeia respiratória mitocondrial ou seus fatores de montagem com três grupos: (i) PF endêmico, (ii) PF esporádico e (iii) PV.
- IV. Caracterizar de forma ultraestrutural, o modelo de miniórgão de pele humana que mimetiza PF e PV.
- V. Avaliar se há expressão diferencial de proteínas envolvidas nas vias de morte celular em um modelo de miniórgão de pele humana que mimetiza pênfigo, no soro e em biópsias de pacientes com PF esporádico e endêmico.

3 DESCRIÇÃO DOS CAPÍTULOS

Esta tese segue as normas da UFPR e se encontra em formato de capítulos correspondendo a manuscritos ou artigos, como permitido pelo Programa de Pós-Graduação em Genética dessa instituição. Dessa forma, está dividida em seis capítulos, cada qual abrangendo um dos manuscritos ou artigos produzidos ao longo do doutorado.

O primeiro capítulo corresponde ao artigo de revisão sobre pênfigo foliáceo e pênfigo vulgar e moléculas envolvidas nas diferentes vias de morte celular. Foi publicado neste ano no periódico *Life* com o seguinte título: “*Marked to Die-Cell Death Mechanisms for Keratinocyte Acantholysis in Pemphigus Diseases*”.

No segundo capítulo, apresentamos o artigo “*Condemned or Not to Die? Gene Polymorphisms Associated with Cell Death in Pemphigus Foliaceus*”, publicado no periódico *Frontiers in Immunology*, em 2019. Consiste em um estudo de associação genética com um grupo de pacientes com pênfigo foliáceo endêmico e controles, no qual as variantes genéticas estudadas são polimorfismos de nucleotídeo único (SNP - do inglês *single nucleotide polymorphisms*) localizados em genes cujos produtos são envolvidos nas diferentes vias de morte celular descritas. Os dados utilizados neste estudo são provenientes de uma genotipagem em larga escala realizada pelo grupo do LGMH, a partir da qual um estudo “GWAS”, do inglês, *Genome Wide Association Study*, foi realizado e publicado em 2021: “*Unsuspected Associations of Variants within the Genes NOTCH4 and STEAP2-AS1 Uncovered by a GWAS in Endemic Pemphigus Foliaceus*”, disponível em: [https://www.jidonline.org/article/S0022-202X\(21\)01228-8/fulltext](https://www.jidonline.org/article/S0022-202X(21)01228-8/fulltext).

O terceiro capítulo refere-se ao manuscrito “*DNA Polymorphisms in Mitochondrial Respiratory Chain Subunits are Associated with Endemic Pemphigus Foliaceus*”. Trata-se de uma comunicação concisa sobre um estudo de associação genética com um grupo de pacientes com PF endêmico, um grupo de pacientes com PF esporádico e um grupo de pacientes com PV. Nos grupos de PF endêmico e PV analisamos os SNPs localizados em genes previamente associados com doenças mitocondriais ou cujos produtos estão envolvidos na montagem das subunidades da cadeia respiratória. Para isso, utilizamos os mesmos dados provenientes de uma genotipagem de larga escala mencionado no capítulo anterior. Ainda, utilizamos

dados de genotipagem em larga escala gerados por nossos colaboradores do instituto de dermatologia de Lübeck (LIED), Alemanha.

No quarto capítulo, encontra-se o manuscrito “*Electron Microscopy of Desmosomal Structures in the Pemphigus Human Skin Organ Culture Model*”. Corresponde à investigação ultraestrutural dos desmossomos de um estabelecido modelo de miniórgão de pele. Este modelo de pele está descrito detalhadamente no seguinte artigo: *Translational Use of a Standardized Full Human Skin Organ Culture Model in Autoimmune Blistering Diseases*, disponível em: <https://pubmed.ncbi.nlm.nih.gov/30848563/>. A possibilidade de realizar o experimento descrito no manuscrito foi graças ao período do doutorado sanduíche realizado na Universidade de Lübeck, sob orientação da Profa. Dra. Jennifer Hundt. Da mesma maneira, os experimentos realizados nos manuscritos dos capítulos cinco e seis foram realizados na Universidade de Lübeck.

O quinto capítulo consiste no manuscrito “*C5a/C5aR1 Complement Expression in Pemphigus*”. Avaliamos um SNP previamente associado ao PF endêmico em um grupo de PF esporádico e um grupo de PV. Em PF esporádico, a associação foi confirmada. Seguimos com a investigação da expressão gênica e proteica do receptor 1 do componente C5a do complemento (Ca5R1) e seu ligante, componente 5a do complemento (C5a) no modelo de miniórgão de pele. Também quantificamos C5a em soro de PF endêmico e PV, assim como avaliamos a presença de armadilhas extracelulares de neutrófilos. Ainda, detectamos pela primeira vez células expressando C5aR1 em biópsias de pacientes com PF endêmico e esporádico.

Por fim, no sexto capítulo encontra-se o manuscrito “*Beyond Apoptosis: Starting the Investigation of Other Cell Death Pathways in Pemphigus*” no qual, pela primeira vez, a expressão de moléculas envolvidas em vias de morte celular, além da apoptose, foram investigadas utilizando um modelo de miniórgão de pele.

4 CAPÍTULO 1 – VIAS DE MORTE CELULAR NA DOENÇA DE PÊNFIGO

REVISÃO – “*Marked to Die-Cell Death Mechanisms for Keratinocyte Acantholysis in Pemphigus Diseases*”. Artigo publicado no dia 22 de fevereiro de 2022, no periódico “*Life*”, na seção “*Medical Research*”, na edição especial “*Mechanisms Underlying Skin Pathologies*”. Link para visualização online do artigo: <https://www.mdpi.com/2075-1729/12/3/329>.



Review

Marked to Die-Cell Death Mechanisms for Keratinocyte Acantholysis in Pemphigus Diseases

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Abstract: Pemphigus is a group of blistering autoimmune diseases causing painful skin lesions, characterized by acantholysis and by the production of autoantibodies against, mainly, adhesion proteins. We reviewed the literature for molecules and/or features involved in the 12 cell death pathways described by Nomenclature Committee on Cell Death, taking place in pemphigus patients, cell lines, or human skin organ cultures treated with sera or IgG from pemphigus patients or in pemphigus mouse models, and found 61 studies mentioning 97 molecules involved in cell death pathways. Among the molecules, most investigated were pleiotropic molecules such as TNF and CASP3, followed by FASL and CASP8, and then by FAS, BAX, BCL2, and TP53, all involved in more than one pathway but interpreted to function only within apoptosis. Most of these previous investigations focused only on apoptosis, but four recent studies, using TUNEL assays and/or electron microscopy, disqualified this pathway as a previous event of acantholysis. For PV, apoptolysis was suggested as a cell death mechanism based on pathogenic autoantibodies diversity, mitochondrial dysfunction, and p38 MAPK signaling. To answer those many questions that remain on cell death and pemphigus, we propose well-controlled, statistically relevant investigations on pemphigus and cell death pathways besides apoptosis, to overcome the challenges of understanding the etiopathology of pemphigus diseases.

Keywords: pemphigus; cell death pathways; apoptosis; apoptolysis



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1. Introduction

Pemphigus is an autoimmune blistering disease affecting the skin with painful and potentially lethal bullous lesions (reviewed by [1]). Based on the autoantibody reactivity, two major forms of pemphigus are differentiated. Pemphigus foliaceus (PF) is characterized by autoantibodies against desmoglein (DSG) 1, while DSG3 autoantibodies are characteristic of mucosal pemphigus vulgaris (PV), and reactivity against both autoantigens is seen in mucocutaneous PV [1]. Desmogleins are calcium-dependent adhesion molecules in the desmosomes that connect keratinocytes [2]. The binding of the autoantibodies to keratinocyte antigens leads to blister formation in the epidermis, caused by cell-cell detachment, a process called acantholysis, histologically hallmarked by intraepidermal split formation [1,3,4]. In PF, it takes place in the subcorneal layer of the epidermis but in PV, acantholysis occurs in the suprabasal layer [4]. In agreement with the DSG compensation hypothesis, the blistering sites differ between PF and PV due to the different expression sites of the DSG molecules in the epidermis: while DSG1 is mainly expressed in the upper

layers, DSG3 is expressed in the lower layers of the epidermis [5]. However, the misconception of this hypothesis is that there are more desmosomal cadherins promoting the integrity of the epidermis, beyond the DSGs (reviewed by [6]).

The pathogenicity of IgG from pemphigus patients has been demonstrated through both in vivo and in vitro experiments [7–9]. However, the precise mechanism by which pemphigus autoantibodies induce blistering is not fully elucidated. One hypothesis to explain this is the direct inhibition of DSG transinteraction, where the binding of autoantibodies causes steric hindrance and therefore loss of cell adhesion, which leads to acantholysis [10–13]. However, this seems to be more applicable to PV than to PF [14]. Beyond steric hindrance, signaling pathways induced by PF or PV-IgG binding, such as phospholipase C [15], p38 mitogen-activated protein kinase (p38 MAPK) [16,17]; c-Myc [18], epidermal growth factor receptor (EGFR), Map kinase (ERK), transcription factor c-Jun [19] and phosphorylation of DSG3 [20], among others [21], have been proposed to trigger acantholysis by launching intracellular events that disrupt desmosome homeostasis. Indeed, inhibition of the MAPKp38 signaling pathways blocks keratinocyte dissociation [16,17,21].

Apart from DSG, a wide range of other proteins are targeted by pemphigus autoantibodies. Among these are adhesion molecules, cell membrane receptors, and mitochondrial antigens [6,22]. These complex pathological mechanisms hinder the development of new treatment options, which remain based on oral corticosteroids [23], besides intravenous immunoglobulins (IVIg) [1,24]. Although the mechanistic reasons for IVIg treatment success are not fully understood, it protects target cells from apoptosis by interfering with signaling pathways, increasing the sensitivity to corticosteroids [24]. Recently, the anti-CD20 antibody rituximab, combined with corticosteroids, was found to induce complete remission off-therapy in almost 90% of patients [1,25].

Pemphigus occurs sporadically around the world. PF incidence is 0.75–5 cases/million per year [26] and PV incidence has a range to 0.6 in Switzerland to 32 in Israel (reviewed by [1]). Despite its worldwide sparing distribution, PF is endemic in South America and Tunisia [27–30]. In Brazil, endemic PF (EPF) is commonly known as fogo selvagem, “wild fire” in Portuguese, due to a popular belief that the blisters and burning sensation resulted from a curse or tribal rituals [31]. It is called “El Bagre” in Colombia, named after the gold-mining endemic region [32]. Endemic PF in Brazil reported the highest prevalence in 1996 (3.04%), in Limão Verde (Mato Grosso do Sul) [33].

Pemphigus is a multifactorial disease, resulting from a combination of genetic, epigenetic, and environmental factors [1,23,34–36]. The strongest genetic risk signature of pemphigus disease is the association with HLA class II alleles in several populations around the world (reviewed by [36]). Besides that, alleles from genes involved in many other mechanisms of the innate and adaptive immune system, including cell death pathways, were associated with pemphigus (reviewed by [36]); [37].

Among Brazilian PF environmental factors are bites of the sand (Phlebotominae) and black (Simuliidae) flies [38,39]. It is hypothesized that salivary antigens from these flies induce a cross-reaction leading to autoantibody production against DSG1 [40]. However, those flies also occur in Northern regions, with much lower, although growing, the incidence of the disease [41]. The possibility of a virus or other microorganism as triggers of the disease was also suggested [30,31]. Furthermore, the epidemiological landscape seems to follow the deforestation border, creating geographical clusters [39]. It was also observed that thiol, other calcium-sequestering components [31], and UVB radiation due to sunlight exposure might have an impact on the susceptibility of PF [42]. Epidemiological differences are not followed by any histopathological features that would distinguish endemic, from non-endemic PF. They are identical [43], except for higher anti-DSG1 IgM and IgE serum levels in endemic PF [44–46]. In contrast to PV, association with other autoimmune diseases and longitudinal mother-child transmission are very rare [30,47–54]. If affected, newborns soon recover [47]. Ruocco et al., 2013 listed several environmental factors associated with PV, such as thiol and phenol drugs, previous viral infections, sunburns, ultraviolet and ionizing radiation, surgical and cosmetic procedures, dietary factors, and emotional stress,

all of them supposed to facilitate the development of pemphigus [34]. Yet, low levels of 25-hydroxyvitamin D (25OHD) were reported in the serum of 34 PV patients compared to the healthy controls [55].

Here, we reviewed the connections between pemphigus and cell death, an issue that has been repeatedly studied (reviewed by [56–58]). Cell death is an essential mechanism to tissue homeostasis and protection against diseases such as cancer, infection, and autoimmune disorders [59]. According to the Nomenclature Committee on Cell Death (NCCD), there are 12 pathways orchestrating the cell death [60]. In a recent genetic association study of our group with EPF, we identified an association with polymorphisms of genes directly involved in apoptosis resistance, as well as gene variants whose products induce and regulate pyroptosis, necroptosis, necrosis, parthanatos, and immunogenic cell death pathways [37]. To our knowledge, only apoptosis had been investigated before in pemphigus [17,61–66], and some of the studies disqualified apoptosis as the major cell death mechanism [17,63,64,66].

Apoptosis and necrosis are the best-known ways of cell death. Apoptosis is a regulated cell death pathway that does not entail the activation of inflammation [67]. There are two ways of activating apoptosis by cysteine aspartate proteases, called caspases. Intrinsic apoptosis is the result of cell damage like DNA degeneration and extrinsic apoptosis is mediated by signaling molecules like TNF (reviewed by [60]). Caspases can also be activated independently of apoptotic signaling (reviewed by [68]) and cleave several adhesion molecules like DSG [69,70]. Apoptosis leads to the formation of apoptotic bodies that are removed by phagocytic cells (reviewed by [60]). In contrast, necrosis is the uncontrolled response of cells to tissue damage that results in cell death. The dying cells release their cell content into the environment so that necrosis is followed by an immune response (Reviewed by [71]).

Until some years ago, cell death pathways were summarized as apoptosis and necrosis. In this review, we aimed to open new views for the investigation in pemphigus considering other cell death pathways described by NCCD [60]. These include reactions of cells to different stress situations like overproduction of ROS (ferroptosis) or the accumulation of different proteins (parthanatos) or infection with a pathogen (necroptosis, pyroptosis). They differ from each other by the way they activate or regulate inflammation (passive or active) (reviewed by [72]). We laboriously reviewed the literature for molecules and/or features involved in cell death pathways described by NCCD, taking place in the pemphigus patient, cell lines or human skin organ cultures (HSOC) treated with sera or IgG from pemphigus patients, or pemphigus mouse models, providing an updated overview of this subject and perspectives for the future (the strategy search is described at the Supplementary Materials, Supplementary Figure S1). We compiled 61 studies for this review (Table 1), where 97 molecules, to the best of our knowledge, involved in cell death pathways were investigated in pemphigus (Figure 1).

Table 1. Studies included in this review-investigating cell death molecules in pemphigus.

References	Target(s) Investigated	Study Design			
		Patients	Human Skin Organ Culture	Cell Lines	Mouse Model
[73]	TNF	✓			
[74]	TNF	✓			
[24]	CASP3 *, CASP8 *, FASL *, FLIP1 *, CAPN1, DNA fragmentation, membrane cell permeability			✓	✓

Table 1. Cont.

References	Target(s) Investigated	Study Design			
[75]	TNF	✓			
[76]	iNOS *, TP53 *, BCL2, DNA fragmentation			✓	
[37]	TNF, TRAF2, CD36, SIRPA, CD47, HK1, PAK2, EIF2AK3, RAPGEF3, PRKN	✓			
[77]	CYCS			✓	
[78]	CYCS			✓	
[79]	CASP9, CYCS, SRC, p38 MAPK			✓	
[80]	CASP3, TNF, DNA fragmentation	✓			
[81]	TNF	✓			
[82]	DNA fragmentation	✓			
[83]	TNF	✓			
[84]	BAX, DNA fragmentation	✓			
[85]	BAG4, BCL2L11, CSF2RA, CSF2RB, GNAQ, IL15, LEPROT, MAP3K1, MAP3K4, MAP3K5, MAPK9, NSMAF, PPP2CA, SMAD2, SMAD4, ERAP1, ERBB4, BDNF, IRAK1, TNFSF13	✓			
[86]	TNF	✓			
[87]	TNF			✓	
[88]	TNF *	✓		✓	✓
[89]	CASP3, CASP8, membrane cell permeability, degraded cells and condensed chromatin	✓	✓	✓	
[19]	CASP3, CASP8, EGFR, ERK1/2, FASL, JUN, membrane cell permeability	✓	✓	✓	
[90]	BAX, BCL2, CASP3, CASP9, EGFR, MTOR, SRC, FAK				✓
[62]	DNA fragmentation	✓			
[63]	CASP3, CASP8, Fractin, PARP, DNA fragmentation	✓	✓		

Table 1. Cont.

References	Target(s) Investigated	Study Design		
[91]	TNF	✓		
[22]	mitochondrial damage		✓	
[92]	TNF	✓		
[93]	TP53, BAX	✓		
[94]	EIF2 α , PERK		✓	
[17]	CASP3, p38 MAPK, PARP, DNA fragmentation		✓	✓
[95]	BAX, BCLXL, CASP3, CASP6, DNA fragmentation			✓
[96]	BAX, BCL2, CASP3, NOD2, RIPK2, NF-kB p-p65, ROS, membrane cell permeability		✓	
[97]	TNF	✓		
[61]	CASP8, FAS, FASL, DNA fragmentation	✓	✓	✓
[68]	CASP3, PARP, DNA fragmentation	✓	✓	✓
[98]	ANXA4, BCL2A1, COP, GZMA, GZMH, LGALS1, LGALS12, NALP12, P2RX1, PIK3R1, PRKCA, SIAH1, TLR2, TNFRSF10A, TNFRSF18, TNFSF9, TNFSF10, TNFSF13	✓		
[99]	CASP3, CASP8, CASP9, CYCS, EGFR, FASL, JNK, p38 MAPK, SRC		✓	
[100]	FASL	✓		
[101]	TNF	✓		
[102]	TNF	✓		
[103]	BAX, CCND2, GADD34, PEA15, TSSC3, WAF1		✓	
[104]	TNF, FASL, membrane cell permeability		✓	
[105]	CASP3 *, FAZ *, FASL *, DNA fragmentation	✓		

Table 1. Cont.

References	Target(s) Investigated	Study Design		
[106]	AKT, BTC, EGF, EGFR, HER2, HER3, P-SCR, TNF, MTOR, DNA fragmentation			✓
[107]	FASL, CASP8, DNA fragmentation	✓		✓
[108]	TNF	✓		
[109]	BAX, CASP3, TP53	✓		✓
[110]	IL1B			✓
[65]	BCL2, FAS, IFNG, IL1-B, iNOS, TNF, DNA fragmentation	✓		
[111]	TNF	✓		
[112]	ST18	✓		
[113]	FAS	✓		
[64]	CASP3, FLIP, DNA fragmentation	✓		✓
[114]	membrane cell permeability			✓
[115]	NLRP3	✓		
[116]	membrane cell permeability		✓	
[117]	CXCL10, IFNG, TNF	✓		
[118]	LIVIN, XIAP	✓		
[119]	TNF, DNA fragmentation	✓		
[120]	ANXA5, BAX, BCL2, CASP1, CASP3, CASP8, FAS, FASL, FASR, TP53, DNA fragmentation, membrane cell permeability	✓	✓	✓
[121]	BCL2, CASP1, CASP3, CASP8, FASL, TP53,			✓
[122]	DNA fragmentation	✓		

* molecule investigated both at the gene and protein level. ✓investigated in ... (see header). AKT, AKT serine/threonine kinase 1; ANXA4, annexin A4; ANXA5, annexin A5; BAG4, BAG cochaperone 4; BAX, BCL2 associated X apoptosis regulator; BCL2, BCL2 apoptosis regulator; BCL2A1, BCL2 related protein A1; BCL2L1, BCL2 like 1; BDNF, brain derived neurotrophic factor; BCLXL, BCL2-like 1; BTC, betacellulin; CAPN1, calpain 1; CASP1, caspase1; CASP3, caspase 3; CASP6, caspase 6; CASP7, caspase 7; CASP8, caspase 8; CASP9, caspase 9; CCND2, cyclin D2; CD36, CD36 molecule; CD47, CD47 molecule; COP, caspase recruitment domain-containing protein 16; CSF2RA, colony stimulating factor 2 receptor subunit alpha; CSF2RB, colony stimulating factor 2 receptor subunit beta; CXCL10, C-X-C motif chemokine ligand 10; CYCS, cytochrome c; DSC3, desmocollin 3; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EIF2α, eukaryotic translation initiation factor 2 subunit alpha; EIF2AK3, eukaryotic translation initiation factor 2 alpha kinase 3; ERAP1, endoplasmic reticulum aminopeptidase 1; ERBB4, erb-b2 receptor tyrosine kinase 4; ERK1/2, mitogen-activated protein kinase; FAK, focal adhesion kinase; FAS, Fas cell surface death receptor; FASL, Fas ligand; FLIP1, TNFAIP interacting protein 2; FLIPL, CASP8 and FADD like apoptosis regulator; FLIPS; GADD34, protein phosphatase 1 regulatory subunit 15A; GNAQ, G protein subunit alpha q; GZMA, granzyme A; GZMH, granzyme H; HER, human epidermal growth factor receptor related; HER2, erb-b2 receptor tyrosine kinase 2; HER3, erb-b2 receptor tyrosine kinase 3; HK1,

hexokinase 1; IFNG, interferon gamma; IL1, interleukin 1; IL1B, interleukin 1 beta; *IL15*, interleukin 15; iNOS, inducible nitric oxide synthase; *IRAK1*, interleukin 1 receptor associated kinase 1; JNK, c-Jun NH2-terminal kinase; JUN, AP-1 transcription factor subunit; *LEPROT*, leptin receptor overlapping transcript; *LGALS1*, galectin 1; *LGALS12*, galectin 12; LIVIN, baculoviral IAP repeat containing 7; M3AR, M3 muscarinic acetylcholine receptor; *MAP3K1*, mitogen-activated protein kinase kinase 1; *MAP3K4*, mitogen-activated protein kinase kinase 4; *MAP3K5*, mitogen-activated protein kinase kinase 5; *MAPK9*, mitogen-activated protein kinase kinase 9; MTOR, mechanistic target of rapamycin kinase; *NALP12*, NLR family pyrin domain containing 12; NF-kB p-p65, nuclear factor kappa B p-p65; *NLRP3*, NLR family pyrin domain containing 3; NOD2, nucleotide binding oligomerization domain containing 2; *NSMAF*, neutral sphingomyelinase activation associated factor; *P2RX1*, purinergic receptor P2X 1; p38 MAPK, p38 mitogen-activated protein kinase; PAK2, p21 (RAC1) activated kinase 2; PARP, poly-(ADP-ribose) polymerase; *PEA15*, proliferation and apoptosis adaptor protein 15; PERK; *PIK3R1*, phosphoinositide-3-kinase regulatory subunit 1; *PPP2CA*, protein phosphatase 2 catalytic subunit alpha; *PRKCA*, protein kinase C alpha; PRKN, parkin RBR E3 ubiquitin protein ligase; RAPGEF3, Rap guanine nucleotide exchange factor 3; RIPK2, receptor interacting serine/threonine kinase 2; ROS, reactive oxygen species; *SIAH1*, siah E3 ubiquitin protein ligase 1; SIRPA, signal regulatory protein alpha; *SMAD2*, SMAD family member 2; *SMAD4*, SMAD family member 4; SPCA1, secretory pathway Ca^{2+} / Mn^{2+} -ATPase isoform 1; SRC, proto-oncogene tyrosine-protein kinase Src; ST18, ST18 C2H2C-type zinc finger transcription factor; *TLR2*, toll-like receptor 2; TNF, tumor necrosis factor; *TNFRSF10A*, TNF receptor superfamily member 10a; *TNFRSF18*, TNF receptor superfamily member 18; *TNFSF9*, TNF superfamily member 9; *TNFSF10*, TNF superfamily member 10; *TNFSF13*, TNF superfamily member 13; TP53, tumor protein p53; TRAF2, TNF receptor associated factor 2; *TSSC3*, pleckstrin homology like domain family A member 2; XIAP, X-linked inhibitor of apoptosis; *WAF1*, cyclin dependent kinase inhibitor 1A.

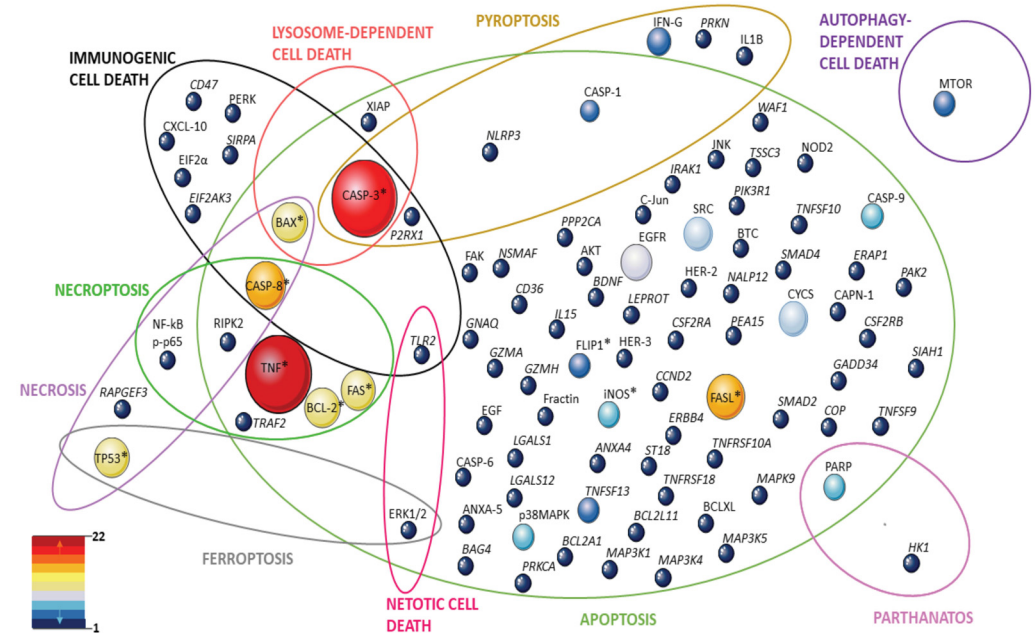


Figure 1. Representativeness of molecules in studies on cell death mechanisms and pemphigus. Ellipses represent cell death pathways and the intersections between them. The molecules investigated at the genetic, expression, and protein levels are shown by circles whose size corresponds to the number of articles in which they were investigated. They are distributed according to the pathways they are part of. The colors reinforce the representation of these molecules in the reviewed articles (dark red—most investigated, dark blue poorly represented). * molecule investigated both at the gene and protein level.

2. Results and Discussion

2.1. Signaling Pathways: TNF, FAS, and Other Proteins

We found nine studies that investigated the association of pemphigus with polymorphisms of genes whose products are involved in cell death pathways (Supplementary Table S1). One of them investigated the association of polymorphisms of genes of all 12 cell death pathways (intrinsic apoptosis, extrinsic apoptosis, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic, NETotic, lysosome-dependent, autophagy-dependent and immunogenic pathways, listed by the NCCD [60] in a

case-control study of Brazilian EPF. There was association evidence for genes whose products are directly involved in apoptosis (*TNF rs1800630*A*, *TRAF2 rs10781522*G*, *CD36 rs4112274*T*, *PAK2 rs9325377*A*), as well as that induce and regulate pyroptosis (*PRKN rs9355950*C*), necroptosis (*TNF rs1800630*A*, *TRAF2 rs10781522*G*), necrosis (*RAPGEF3 rs10747521*A*), parthanatos (*HK1 rs7072268*T*), and immunogenic cell death (*EIF2AK3 rs10167879*T*, *SIRPA rs6075340*A*, *CD47_rs12695175_G*) pathways. This last pathway can be activated by environmental factors associated with PF susceptibility, such as UV irradiation, calcium-sequestering components, and components of fly saliva [37]. In a genome-wide association study with three PV populations, a variant *rs23043658*A* at gene C2H2C-type zinc finger transcription factor (*ST18*) which encodes a pro-apoptotic molecule, was associated in Jew and Egyptians. Also, its expression was upregulated in 8 skin biopsies of PV patients [112]. Seven of the association studies, done with PF and/or PV, focused on genetic polymorphisms of tumor necrosis factor (*TNF*) [37,73,86,91,101,111,119] (Supplementary Table S1). *TNF*-alpha is a proinflammatory cytokine involved in both apoptosis and necroptosis, mainly associated with the severity of different immune-regulated diseases [123], such as pemphigus [124]. Its encoding gene is located in 6p21.33 in the Major Histocompatibility Complex (MHC) class III region and is produced by macrophages, monocytes, neutrophils, T cells, and NK cells [125]. *TNF*-alpha binds to *TNFR1* and *TNFR2* receptors and ultimately regulates cell proliferation and differentiation, and the immune response [126]. Three polymorphisms may modulate the susceptibility to PF: *rs1800630*, *rs1800629*, and *rs361525*. *TNF rs1800630*A*, associated with lower *TNF* gene expression and protein levels [127], occurs in absolute linkage disequilibrium with the major *rs1800629*G* and *rs361525*G* alleles and was associated with the Brazilian EPF susceptibility [37]. Both the minor *rs361525*A* allele and the *rs1800629*G_rs361525*A* (GA) haplotype were associated with protection against the Brazilian EPF [111]. This was expected, since in contrast with the *rs1800630*A* allele, the *rs361525*A* variant is associated with a higher *TNF* gene expression [128,129]. The *rs1800629*GA+AA* was associated with a higher risk of sporadic pemphigus disease in Egypt for PV [101] and *rs1800629*A* in a Polish population with PF [119]. In contrast, the most common *rs1800629*G_rs361525*G* (GG) haplotype was associated with PV susceptibility in the Slovak population [91]. It was not associated in a small Argentinian study of 20 pemphigus patients [86]. *TNF* microsatellite loci (STR)*2 and *5 were associated with EPF susceptibility in Tunisia, while *TNF*6* with protection. Importantly, the susceptibility alleles are not in significant linkage disequilibrium with the main alleles associated with PF worldwide: *DRB1*04*, as well as the most associated in the Tunisian population, *DRB1*03* [73].

Higher *TNF* expression has been repeatedly associated with PV and PF [81,83,92,108] (Table 2 and Supplementary Table S2). However, *TNF* serum levels did not differ between 20 PV patients [117], as well as in 19 PV patients in the active stage and 24 patients in clinical remission compared to controls [102]. *TNF* serum levels were positively correlated with autoantibody titers, disease duration, and lesion numbers in the PV patients [74]. They were also higher in 25 PV Iranian, 10 PV Egyptian, and 25 PV Italian patients and a PV Japanese case report [75,92,108]. In the Italian PV patients, *TNF* and interleukin 6 (IL-6) levels were high at first and decreased after one month of therapy [83]. In agreement, *TNF* gene expression was evident in the epidermis of 19 PV patients [88]. This occurs especially near the blister, while it was almost not found in nonlesional skin of 6/7 PF and 5/6 PV patients [97]. *TNF* protein was also increased in the lesional and perilesional epidermis in 2/5 PV patients and lesional and perilesional dermis of all PV patients [80]. In addition, *TNF* was detected in the inflammatory exudate of more than 70% of 13 investigated EPF patients [65] and in the supernatants from peripheral blood molecular cells (PBMC) cultivated from 12 Brazilian EPF and 7 PV patients [81]. Pretreatment with anti-*TNF* strongly inhibited complement C3 gene expression and about 80% of cell detachment in normal human epidermal keratinocytes (NHuK) incubated with PV-IgG (IgG immunoglobulin obtained from PV patients). With the addition of anti-IL-1 α , acantholysis inhibition reached 84% [87,88]. Higher levels of IL-1B were observed in supernatants from Brazilian EPF PBMC even during the glucocorticosteroid therapy [110]. A lower incidence of PV-similar

blisters also occurred in TNF receptor knockout mice (*TNFR1R2*^{-/-}) after PV patient serum injection, compared with control wild-type mice (54 vs. 87%, $p < 0.05$) ([88]). Chiapa-Labastida [80] suggested that the accumulation of apoptotic cells in PV patients might enhance TNF production, which in turn activates apoptosis in more keratinocytes.

Table 2. Reported alterations in protein levels after blister formation in pemphigus patients.

Protein	Detected or Increased			Absent, Low or Decreased		
	PV	PF	PV and PF	PV	PF	PV and PF
BAX	[120] *			[84]		
BCL2		[65] *		[120] *		
CASP-1	[120] *					
CASP-3	[105] *					
	[109]			[64]		
	[80]			[89] *		[63] *
	[120] *					
CASP-8	[120] *					
	[89] *					[63] *
CXCL-10				[117]		
EGFR	[19] *					
FAS	[105] *					
	[61]					
	[113]	[65] *				
	[120] *					
FAS-L	[100] *					
	[105] *		[107]			[107] +
	[120] *					
Fractin						[63] *
IFN-G	[117]	[65] *				
IL-1B		[65] *				
iNOS		[65] *				
LIVIN	[118] +					
ST18	[112]					
PARP						[63] *
TNF	[92]					
	[83]			[75] #, +		
	[74]			[117]		
	[108]	[65] *	[81]	[102]		
	[80]			[83] +		
	[75] #					
TP53	[109]					
	[120] *					
XIAP	[118] +					

BAX, BCL2 associated X apoptosis regulator; BCL2, BCL2 apoptosis regulator; CASP1, caspase1; CASP3, caspase 3; CASP8, caspase 8; CXCL10, C-X-C motif chemokine ligand 10; FAS, Fas cell surface death receptor; FASL, Fas ligand; IFNG, interferon-gamma; iNOS, inducible nitric oxide synthase; LIVIN, baculoviral IAP repeat-containing 7; ST18, ST18 C2H2C-type zinc finger transcription factor; PARP, poly-(ADP-ribose) polymerase; TNF, tumor necrosis factor; TP53, tumor protein p53; XIAP, X-linked inhibitor of apoptosis; PF, pemphigus foliaceus; PV, pemphigus vulgaris. * Statistical significance was not mentioned in the article. + After therapeutic intervention. # Case report.

Another relevant and highly investigated protein in pemphigus is Fas [24,61,65,100, 105,107,113,120] (Figure 2, Table 2 and Supplementary Table S2). The Fas protein is a

membrane receptor of the TNF receptor family. Upon binding with Fas ligand (FasL), it triggers apoptosis, having fundamental importance in the maturation process of T and B lymphocytes and the homeostasis of the immune system [130]. Thus, mutations or unwanted activation of Fas may have very negative repercussions. Epidermal PV lesions expressed Fas and FasL proteins, as well as perilesional PV skin for Fas [61,105,120]. Fas was also expressed on keratinocytes of the lesional epidermis of 2/5 PV patients [113], and in the epithelium and the cells of the inflammatory exudate of 3 and 10 of 13 EPF patients, respectively [65]. Furthermore, NHuK treated with PV-IgG from two different PV patients presented higher *FASL* gene and FasL expression. After IVIg treatment, the PV-IgG of these patients did not induce *FASL* expression [24]. Interestingly, NHuK and HSOC treated with PV-IgG and/or FasL and/or TNF presented increased cell death and acantholysis [104]. FasL levels also increased in the supernatant of cultivated NHuK and HSOC treated/injected with PV-IgG, respectively. Moreover, the perilesional area of NHuK cultivated with PV-IgG presented enhanced expression of both Fas and FasL. Soluble FasL (sFasL) levels increased before and after blister formation, but membrane FasL numbers increased after blister formation [120]. Patients with mucosal PV had the highest mean value of sFasL (2775 pg/mL-2 patients), followed by the mucocutaneous group (1820 pg/mL-5 patients) and patients with only cutaneous involvement (1130 pg/mL-3 patients) [100]. In addition, sera from untreated pemphigus patients presented higher FasL levels than sera from corticosteroid treated patients (23 PV and 13 PF patients) and induced NHuK apoptosis [107]. FasL may induce keratinocyte apoptosis through caspase (CASP)-8 activation, based on the evidence that (1) FasL expression increased, followed by CASP-8 activation and by TUNEL-positive epidermal cells in NHuK treated with PV-IgG before acantholysis [61], (2) anti-FasL or CASP-8 inhibitor decreases cell death [107] and (3) FasL silencing through siRNA reduces PV-IgG-induced CASP-8 activation and Dsg3 cleavage in NHuK treated with patient IgG [61]. In a mouse model, anti-FasL injection greatly reduces blister formation after PV-IgG injection. Mutant mice without sFasL also had reduced acantholytic area, whereas mice with sFas, but lacking the membrane form of FasL, presented blisters after PV-IgG injection [61]. Interestingly, NHuK and HSOC treated with PV-IgG and/or Fas-L and TNF presented increased cell death and acantholysis [104]. Finally, serum levels of the apoptosis inhibitors livin and X-linked inhibitor of apoptotic proteins (XIAP) increased in all seven investigated pemphigus patients (5 PV and 2 PF) after IVIg treatment [118]. Interestingly, pathogenic anti-Dsg3 antibodies activate apoptosis in a FasL-independent way in cultured keratinocytes, increasing combined Annexin V and propidium iodide (PI) positivity at 4 h, with a peak at 12 h post-treatment. This leads to the “2-hit hypothesis”, where the first hit of both pathogenic and non-pathogenic antibodies cause structural cell membrane changes (cell stiffness), and the second hit is only mediated by pathogenic antibodies and induces FasL-independent apoptotic processes [114].

PV perilesional skin also expresses EGFR ([19]). This receptor may induce apoptosis if overexpressed [131]. PV-IgG injection did not change *EGFR* expression but increased the levels of autophosphorylated EGFR in NHuK and HSOC, 30 min after PV-IgG treatment. The numbers of phosphorylated extracellular signal-regulated kinases ERK1/2 did also increase and were detectable after 15 min, lasting up to 10 h. The plausible substrate of ERK, c-Jun, was phosphorylated after 4 h of treatment. Treatment with EGFR inhibitor (AG1478) or MAP (mitogen-activated protein) kinase (ERK) inhibitor (PD98059) reduced the numbers of apoptotic cells and CASP-3 activity in NHuK incubated with PV-IgG. The EGFR inhibitor also reduced Fas-L levels, whereas the MAPk inhibitor reduced the numbers of phosphorylated ERK1/2 and c-Jun. In conclusion, PV-IgG binding to membrane receptors activates EGFR and leads to dissociation of plakoglobin (cytoplasmic desmosomal component), phosphorylation of ERK and c-Jun, followed by an increase in sFasL secretion and organization of death-induced signaling complexes (DISC, composed by Fas, FADD, or Fas-associated protein with death domain and CASP-8) [19].

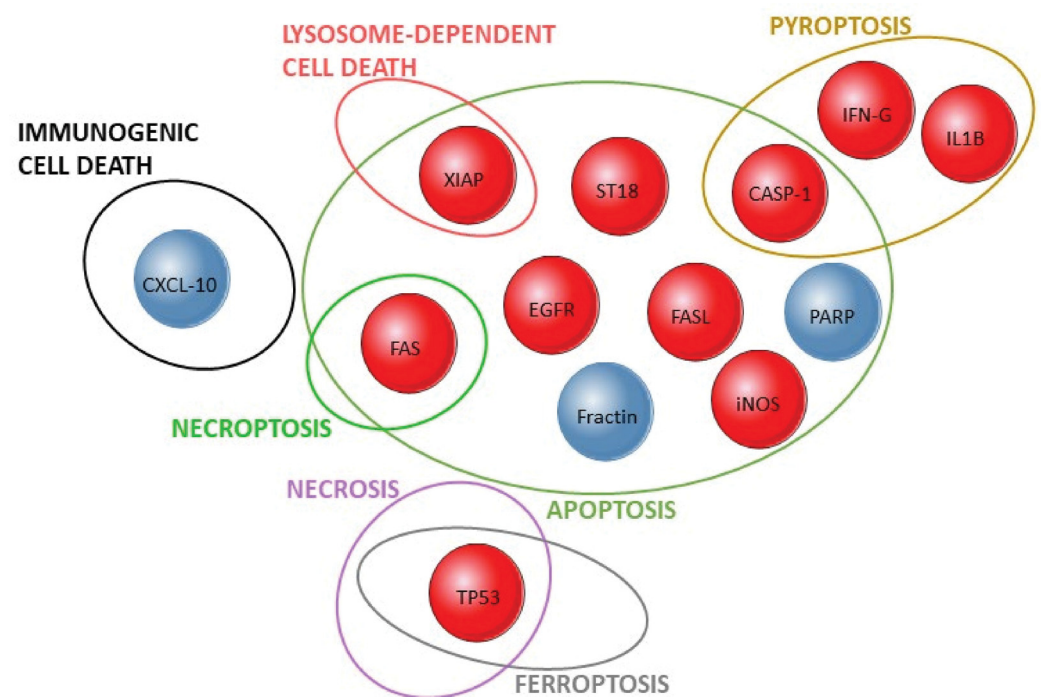


Figure 2. Validated results in human studies on cell death mechanisms and pemphigus. Ellipses represent cell death pathways and the intersections between them. The investigated proteins are shown by circles whose red color corresponds to their detection or increased expression in pemphigus patients, whereas blue corresponds to absent, low, or decreased expression. Discrepant results in the literature were excluded.

Global peripheral blood gene expression in PV patients and controls revealed many differentially expressed genes. A highlighted functional pathway was apoptosis (Supplementary Table S2). Several pro-apoptotic genes were upregulated in patients with active clinical PV [85]. Genome-wide gene expression profiles of peripheral CD4⁺ T cells comparing (1) Brazilian EPF patients vs. controls, (2) Brazilian patients with generalized vs. localized forms of EPF, and (3) untreated vs. treated Brazilian EPF patients, revealed many differentially expressed genes of programmed cell death (GO:0012501) (Supplementary Table S2) [98]. Among the genes encoding anti-apoptotic proteins and with decreased expression in T lymphocytes of patients undergoing corticosteroid treatment, *BCL2A1* (B-cell lymphoma 2-related protein A) stands out. Its product sequesters pro-apoptotic proteins B-cell lymphoma 2 (BCL) family, reduces the release of pro-apoptotic cytochrome c (CytC) from mitochondria, and inhibits caspase activation, promoting lymphocyte activation and survival [132]. Eleven and 4 of 13 Brazilian EPF patients expressed the antiapoptotic BCL-2 protein in the cells of the inflammatory exudate and the epithelium, respectively [65]. In agreement, NHuK or HSOC reduced BCL2 apoptosis regulator (*BCL2*) gene and protein expression after incubation with PV serum or PV-IgG [76,120]. BCL-2 plays a role in maintaining epithelial cell integrity and may inhibit the Th17 activation [133]. The Th1/Th17 immune responses play an important role in the pathogenesis of PV, with increased proapoptotic interferon-gamma (IFN- γ) and reduced chemokine interferon gamma-induced protein 10 (CXCL-10) levels in 20 investigated PV patients [117]. Most of the 13 investigated EPF patients mentioned before (11/13) also presented proinflammatory inducible nitric oxide synthase (iNOS), interleukin 1 (IL-1), and IFN γ in the inflammatory exudate [65]. NHuK incubated with PV serum also overexpressed *iNOS* and its product [76] creating high levels of reactive oxygen species (ROS). NHuK cultivated with sera from PV patients also increased levels of phosphorylated (p) pPERK (RNA-dependent protein kinase (PKR)-like ER kinase) and pEIF2 α , a translation initiation factor that acts as a central hub

of the stress response [94]. PERK is required at the contact sites between the endoplasmic reticulum (ER) and mitochondria to elicit apoptosis after ER stress-mediated by ROS [134].

Nguyen et al. looked into the antagonizing effects of PV-IgG and methylprednisolone (MP) in human keratinocytes on a genomic level. Their results showed that PV-IgG leads to a decrease in the transcription of 198 different genes and an enhanced transcription of 31 genes, whereas some of these genes are regulated reciprocally by MP. In keratinocytes treated with PV-IgG, the transcription of apoptosis-related genes like p53-activated fragment-1 (*WAF1*), IPL protein (*TSSC3*), apoptosis-associated protein (*GADD34*), phosphoprotein PEA-15, and cyclin D2 was down-regulated compared to the controls (Supplementary Table S3). On the other hand, the gene encoding proapoptotic *BAX-δ* is up-regulated, when comparing MP + PV-IgG vs. PV-IgG [103].

PV lesional skin also expressed the pro-apoptotic Bcl-2-associated X protein (BAX), as well as its regulator, tumor suppressor protein Tp53 [120] (Supplementary Table S4). BAX expression seemed at first unimportant in PV patients [84] but was later found increased in NHuK cultivated with PV sera [109] (Supplementary Table S4). The same treatment increased the *TP53* gene and protein expression [76,109]. In accordance, TP53 expression was higher in 48% of lesional and non-lesional skin biopsies of 25 PV patients. This seems to be a direct consequence of desmosomal disruption since intact DSG3 was found to inhibit TP53 expression and activity [109]. However, susceptibility to Brazilian EPF was not associated with two common polymorphisms of the corresponding genes, namely *BAX*-248 G > A (may alter RNA splicing, rs4645878) and *TP53* 12139 G > C (p.Arg72Pro, altering structure and function of the mature protein, rs1042522) [93]. The expression of the NLR family pyrin domain containing 3 (*NLRP3*) gene, whose product is a central player in the innate immunity [135], did not differ in 43 oral PV patients compared with 40 health controls [115].

Apoptosis progression is regulated in an orderly way by a series of signal cascades, coordinated by 14 proteases of the interleukin-1 β -converting enzyme family that play important roles in inflammation (caspases-1, -4, -5, and -11), in the cell cycle (CASP-2), in cell differentiation (CASP-14) and apoptosis. The last function divides these molecules into initiator (caspases-8, -9 and -10) and effector (caspases-3, -6 and -7) caspases [136]. Acantholytic cells of 17 PV biopsies presented *CASP3* gene and protein expression [105], and both lesional and perilesional epidermis and dermis from PV patients presented an increased number of cells with activated CASP-3 [80,109]. Higher CASP-1 and/ or CASP-3 expression was detected in NhuK and HSOC treated/ injected with PV-IgG [19,24,118,119], although the opposite for CASP-3 was true in another study [68]. After treatment of NhuK with PV-IgG, activated CASP-3 increased concomitantly with cell death (evaluated by methylene blue) and acantholysis [89].

As already mentioned before, CASP-8 is activated by the binding of FasL to the Fas receptor in the apoptosis extrinsic pathway. Although apoptosis was proposed to precede acantholytic detachment because CASP-8 activation preceded disruption of cell-cell contacts, this issue seems far from being resolved. Activated CASP-8 was detected in PV skin and higher CASP-8 levels were observed after [120] and before [89] blister formation in NhuK treated with PV-IgG. Interestingly, CASP-1 inhibitor (YVAD-CHO) decreased cell death and acantholysis in NhuK cultivated with PV-IgG (evaluated by trypan blue dye-TBD staining) and decreased acantholysis in HSOC injected with PV-IgG [120]. In accordance, higher *CASP3* and *CASP8* gene and protein expression were observed in NhuK treated with PV-IgG from two patients. After IVIg treatment, the PV-IgG lost its association with the expression of these genes, as well as with *FASL*, induced higher expression of the anti-apoptotic FLIP-I (FLICE-inhibitory protein) and reduced cell detachment and numbers of cells with double-strand DNA breaks (detected with the TUNEL assay terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) positive cells. Similar consequences were observed in the murine epidermis [24].

Apoptotic cells attract mononuclear phagocytes like CD1a+ and CD14+. CD1a+ phagocyte numbers increased not only in the perilesional epidermis but also in the lesional and

perilesional dermis of 5 active untreated PV patients. In the lesional and perilesional dermis, CD14+ cells also appeared in higher numbers. The authors of this study hypothesized that the mononuclear phagocytes may not easily reach the epidermis due to the high number of apoptotic cells in the dermis, which may explain their absence in the lesional epidermis [80].

2.2. Cell Death Controversy

2.2.1. TUNEL Assays—Are the Cells Dead or Alive?

The TUNEL assay allows the identification of endonuclease-driven DNA fragmentation (causing 3'-OH DNA termini), associated with different mechanisms of cell death, including but not restricted to apoptosis [137]. TUNEL+ cells were reported in 17 PV biopsies [105], in perilesional biopsies of 23 PV and 13 PF patients [107], in the lesional and perilesional epidermis and dermis of 5 PV patients [80], as well as in the epithelium (12/13) and inflammatory exudate (11/13) in Brazilian EPF patients [65] (Table 3). The PV blister roof was positive, lending support to the hypothesis that cell death precedes acantholysis [120]. Biopsies of 4 PF and 4 PV were positive for multiple pycnotics and condensed nuclei stained with TUNEL, extending beyond the region of acantholysis, even reaching the normal epidermis. PF biopsies presented abundant TUNEL+ cells clustered in the subcorneal zone and only single TUNEL+ keratinocytes in the upper spinous layer where the epidermis was cohesive. This may suggest an early onset of cell death in pemphigus, meaning that DNA fragmentation occurs before the development of frank acantholysis [62]. In 72% of 25 PV mucosa biopsies, the frequency of TUNEL+ cells was higher than 75% in the basal layer of perilesional tissue, while it was 100% in the tombstone region ($p = 0.038$). TUNEL positivity was also abundant in the parabasal region and the roof of the vesicles. Positivity was higher in the basal than in the parabasal layer, as in the tombstone area compared to the roof of the blister ($p = 0.0005$ and $p = 0.038$, respectively) [84]. In agreement, 14 of 15 PV patients showed TUNEL positivity in the basal layer, 13 in the blister roof, and 12 presented TUNEL+ acantholytic cells. Twelve of 15 controls presented TUNEL+ cells, but only in the granular layer [82]. NhuK incubated with PV serum or PV-IgG also presented TUNEL+ cells [76], as well as TBD+ cells [24] with TBD+ lesion edges in not detached cells and in perilesional area, indicating cell death [120]. TBD staining also indicated cell death in NhuK and HSOC treated with PV-IgG and/or Fas-L and TNF, along with increased acantholysis [104]. Cell viability, measured by TBD, decreased to 10% in HSOC treated with plasma from PV, while the viability was 60% in the controls [116]. In agreement, after acantholysis, TBD vital staining and annexin V-Cy3 showed dead cells at lesional and non-lesional areas in NhuK treated with PV-IgG. In HSOC injected with PV-IgG, through MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, cell viability decreased with increasing time of exposure and PV-IgG concentration [19]. In a PF mouse model, TUNEL+ cells were detected 8 h after PF IgG injection, whereas initial histological blisters were revealed after 12 h PF IgG injection. In addition, up-regulation of the expression of (pro-apoptotic) Bax at 2 and 4 h after PF IgG injection was reported [95], detection of cleaved CASP-3 and activated CASP-6 after PF IgG injection [95], as well as of down-regulated expression of (anti-apoptotic) Bcl-xl at 6, 8 and 20 h after PF IgG injection [95]. Lastly, the administration of caspase inhibitors blocked PF blistering, and TUNEL was negative [95] (Supplementary Table S5).

Table 3. Reported alterations in DNA degradation by Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick end Labeling Assay (TUNEL) or in cell membrane integrity by Trypan Blue or Annexin 5.

Type	Time	Detected or Increased			Absent, without Difference		
		PV	PF	PV and PF	PV	PF	PV and PF
HUMANS	After blister formation in patients	[105] * [120] * [80] [84] [82]	[65] *	[107] [62] *	[68] [64]	[122] *	[63] *
	After treatment with PF/PV IgG (before keratinocytes dissociation)	[120] * [17]			[68]		
CELL LINES	After keratinocytes dissociation	[76] [24] [120] * [104] [104] + [89] * [19] * [19] +, * [121] * [96] [114]		[107]	[68] [64]		
	After some therapeutic intervention in cell lines			[17] [107]	[19] * [24] [120] * [96]		[107]
	After injection with PF/PV IgG (before blistering formation)	[61] [106]	[95]		[68]		
MOUSE MODELS	After blistering formation	[24]	[17]		[68]		
	After some therapeutic intervention				[24] [106]	[95]	

PF, pemphigus foliaceus; PV, pemphigus vulgaris; IgG, immunoglobulin G. +: tissue culture or human skin organ culture. * Statistical significance was not mentioned in the article.

In contrast, 10 Brazilian EPF showed no difference regarding TUNEL detection between injured PF skin and healthy skin. Few cells were positive in both groups. On the other hand, a stem marker protein, P63, was highly expressed in both tissues [122]. Schmidt et al. [64] did not detect hallmarks of apoptosis in perilesional skin sections from two PV patients. TUNEL+ cells were found solely in the blister roof or around the intraepidermal split from 2 of 4 biopsies of one PV patient. In the other PV patient, in areas with intraepidermal splits, 1 of 4 biopsies presented TUNEL+ cells [64]. Positive cells for cleaved caspases could not be observed. HaCaT and NhuK cells were incubated with PV-IgG from 5 PV patients but no apoptotic characteristics were visible. They neither observed changes in the nuclear morphology nor increased TUNEL positivity although acantholysis was induced (visible through fragmented Dsg 3 immunostaining, cytokeratin retraction, and cell dissociation). Inhibition of caspases (z-VAD-fmk) or overexpression of FLIP did not prevent acantholysis in cultured cells, supporting the conclusion that apoptosis is not a necessary condition for PV acantholysis [64]. In agreement, Janse et al. found few TUNEL+ cells in 11 mucocutaneous PV biopsies (3 lesional, 3 perilesional, and 5 healthy skin), and 11 PF biopsies (5 lesional, 2 perilesional, and 4 healthy skin). They found no CASP-3, CASP-8, fractin and nuclear PARP (Poly [ADP-ribose] polymerase) in the biopsies, as well as in PV

and PF HSOC models [63]. Accordingly, human HaCaT keratinocytes and epidermis of adult and neonatal mice presented no fragmented nuclei after PV-IgG treatment, neither during the formation of the lesion nor after acantholysis. Similar to Janse's study, the analysis of six skin biopsies from PV patients didn't show TUNEL positive cells in non-lesional, perilesional, or most lesional tissue [68]. Moreover, maximal 7.5% and 3.5% of the cells in HSOCs injected with PV-IgG or with PF-IgG, respectively, were TUNEL+, depending on the extent of incubation time. There was no difference between healthy and lesioned skin fields [63].

2.2.2. Caspase Activity—Are the Cells Apoptotic or Not?

Apoptosis requires that CASP-3 and other caspases undertake poly ADP-ribose polymerase (PARP) cleavage [138]. However, neither a global actin collapse nor reduced full-length PARP nor increased cleaved PARP was observed in human and mouse keratinocytes and mouse epidermis after treatment with an experimental Dsg3 monospecific antibody (AK23) or PV-IgG [68], in agreement with the results obtained by others with 11 mucocutaneous PV and 11 PF biopsies [63]. Instead, there was an early transient, low-level activation of CASP-3 in mouse keratinocytes and AK23-treated adult mice one hour after AK23 or PV-IgG treatment, just before blistering. Furthermore, CASP-3 inhibition reduced acantholysis in cultured keratinocytes, neonatal and adult mice but inhibiting other caspases failed to prevent loss of cell adhesion. Thus, CASP-3 activation seems independent of the apoptotic signaling cascade, and apoptosis itself seems not needed for acantholysis in these PV models [68].

2.3. Morphology

Wang et al. (2004) investigated the different effects of PV-IgG on young (1–2 passages) and old (4–5 passages) cultured keratinocytes. Detachment of the cells occurred to a greater extent in aged cells as well as the number of dead cells whose death was induced with PV-IgG [121]. Higher FasL (m- and s-FasL) concentrations, DNA fragmentation, blebbing membranes, and apoptotic bodies confirmed these results. Activities of CASP-1, -3, and -8 also increased in aged cells, being consistent with down-regulated BCL-2 and increased p53. All in all, PV-IgG-induced apoptosis was evident in senescent cells, which might explain the higher severity and frequency of PV in older people [121]. Apoptotic cells (degraded cells and nuclei with condensed chromatin) were also reported in histological sections of lesional PV patients' skin stained by hematoxylin and eosin [89].

However, using electron microscopy, considered the gold standard for the identification of apoptotic cells [63,139]), no apoptotic features were observed in acantholytic, lesional PV, and PF skin [63]. In agreement, no nuclear changes were observed in perilesional skin biopsies of 2 PV patients and HaCaT culture incubated with PV-IgG [64].

2.4. Apoptolysis and Mitochondria

Grando et al. (2009) proposed that apoptosis followed acantholysis in the apoptolysis pathway, by the means of: (1) binding of three classes of autoantibodies against desmosomal autoantigens, mitochondrial autoantigens as acetylcholine receptors, and other autoantigens on the plasma membrane of keratinocytes, acting synergistically with proapoptotic serum and tissue factors; (2) activation of EGF receptor, Src, mTOR, p38 MAPK, and other signaling elements downstream of ligated antigens, elevating intracellular calcium levels and launching cell death cascades; (3) basal cell shrinkage by caspases that disrupt cytoskeleton components and then phosphorylate and cleave the cytoplasmic tails of transmembrane cadherins, dissociating inter desmosomal adhesion complexes; (4) massive collapse of structural proteins and production of scavenging antibodies, accompanied by total desmosome separation; (5) apoptotic death of acantholytic cells resulting from irreversible damage to mitochondrial and nuclear proteins [140].

In agreement with mitochondrial injury, PV sera disrupted the electron transfer chain and the electrochemical gradient of KCs, increasing proton leakage and the production of

reactive oxygen species (ROS). The mRNA expression of *BAX*, *CASP3*, nucleotide-binding oligomerization domain containing 2 (*NOD2*), receptor-interacting serine/threonine kinase 2 (*RIPK2*) and nuclear factor kappa B p-p65 (*NFkB p65*) increased and of *BCL2* decreased in human KCs treated with PV serum (Supplementary Table S3). These effects, as well as ROS production, stimulated either by UVB or PV serum, reversed by antioxidant naringenin (pre)treatment, preventing cell death [96]. Furthermore, PV patients produce PV-IgG against different mitochondrial proteins and in different amounts [22,78,99]. The absorption of antimitochondrial antibodies blocked/reduced the capacity of PV-IgG to induce acantholysis in KCs and a PV mouse model, respectively. Pretreatment with a combination of all three mitochondria-protecting drugs minocycline, nicotinamide, and cyclosporine A almost completely abolished acantholysis in the mouse skin [22]. Specifically, the serum of five PV patients contained antibodies targeting subunits of mitochondrial nicotinic acetylcholine receptors (mt-nAChRs), which normally keep the mitochondrial permeability transition pore (mPTP) closed. Stimulation of these receptors prevented the release of CytC through the mPTP and therefore the formation of the apoptosome [78]. Another three non-desmoglein antibodies causing skin acantholysis after injection in neonatal mice were identified in the sera of 12 PV patients lacking antibodies against desmoglein 1 or 3. They targeted desmocollin 3 (DSC3), the muscarinic acetylcholine receptor subtype M3 (M3AR), or the secretory pathway calcium ATPase (SPCA1), leading to higher CytC release. Adsorption of these autoantibodies prevented blister formation and a positive Nikolskiy sign in mice. Furthermore, the effect of these autoantibodies on apoptolysis markers was studied in cultured human keratinocytes. All of the three autoantibodies lead to an increase in the activity of p38 MAPK and CASP-9, important steps in apoptolysis signaling. Anti-Dsc3 and anti-SPCA1 also activated the Src kinase. Inhibition of SPCA1 through the autoantibodies triggered apoptotic signaling due to accumulation of Ca²⁺ and Mn²⁺ [79].

PV-IgG binds to neonatal Fc Receptor (FcRn) for internalization and intracellular trafficking of PVIgG to mitochondria. Among these PVIgG, anti-mitochondrial antibodies (AMA) and anti-Dsg synergize to cause acantholysis in the mouse model, increasing CytC release and changing the membrane potential of mitochondria, which might trigger the intrinsic apoptotic pathway. All these processes and the shrinkage of KCs were abolished by blocking FcRn [77]. PV-IgG binding leads to the activation of EGFR, tyrosine kinase Src, p38 MAPK, and c-Jun N-terminal kinase JNK in KCs with different time patterns, according to each patient. Patient source of PV-IgG further seems to influence: (1) the number of TBD-positive cells in cell monolayers, (2) acantholytic activity in a mouse model, (3) the response to CASP-3 inhibitors (DEVD-CHO, Z-DEVD-FMK, and Z-DCB-MK) and calpain inhibitors (MDL-28170, PD-150606, and CSP) in cell culture. Thus, PV-IgG from different PV patients seems to induce acantholysis through cell shrinkage (apoptosis) or cell swelling (oncosis preceding necrosis) [24]. Inhibitors of executioner caspases did not change EGFR, Src, and the first peak of p38 MAPK, but decreased JNK, the second peak of p38 MAPK, and CytC in keratinocytes treated with PV-IgG [99] (Supplementary Table S4).

Thus, irreversible mitochondrial damage is probably the reason for elevated CytC in keratinocytes treated with PV sera or pure PV-IgG fractions [77,99]. Elevated CytC, CASP-3, 8, and 9 confirmed activation of the intrinsic and extrinsic apoptosis pathways in keratinocytes treated with PV sera or pure PVIgG fractions, being PV sera much more effective in activating FasL and subsequently CASP-3 and 8. Anti-FasL decreased CASP-8 activation in both groups, and CASP-3 in KCs treated with PV-sera. However, the addition of anti-FasL antibodies did not modify CASP-9 activity and CytC levels, both remained elevated [99].

2.5. p38 MAPK, and Other Signaling Molecules

Two peaks of p38 MAPK were observed in KCs cultivated with PV-IgG and in a mouse model injected with PF-IgG. The first one occurred before the acantholysis, 30 min after addition of PV-IgG, and 2–4 h after PF-IgG injection in KCs and a mouse model,

respectively. In KCs, cleaved PARP and positive TUNEL increased after 6 h of PV-IgG addition after the second peak of p38 MAPK. In the mouse model, the second peak of p38 MAPK occurred between 8–21 h after PF IgG injection and positive TUNEL, cleaved CASP-3 and cleaved PARP increased after acantholysis triggered by PF IgG injection. Interestingly, inhibition of the first peak of p38 MAPK blocked blistering. The inhibition of the p38 MAPK second peak did not block the blister formation. However, it blocked the activation of CASP-3 in mice that received the p38 MAPK inhibitor 4 h after injection of PF IgG [17] (Supplementary Table S5). Furthermore, inhibitors of executioner caspases (CASP-3 and 7) did not change EGFR, SRC, and the first peak of p38 MAPK, but decreased JNK, the second peak of p38 MAPK and CytC levels in keratinocytes treated with PV-IgG [99].

Mice treated with PV-IgG presented higher phosphorylation of the epidermal growth factor receptors HER1, HER2, HER3 and an upregulation of the HER ligands betacellulin, EGF, and transforming growth factor TGF- α as well as an increased expression of Src and mTOR. TUNEL positive cells increased, before blistering, mTOR mediates the apoptotic process and Akt promotes cell survival through regulation of pro-apoptotic molecules. PV-IgG injection up-regulated mTOR but down-regulated Akt, which might drive cells to apoptosis. In agreement, inhibition of HER, mTOR, pan-caspase, or Src decreases the number of apoptotic cells and acantholysis in PV neonatal mice. Inhibition of Src or HER resulted in a decrease of mTOR levels as well. Inhibition of HER resulted in P-HER and Src decreased [106].

The importance of focal adhesion kinase (FAK) in blister formation was shown by their absence in mice pretreated with FAK-inhibitor, before PV-IgG injection. Furthermore, the inhibition of mTOR, Src, and HER isoforms before the treatment with PV-IgG decreased the levels of phosphorylated FAK. Higher Bcl-2 levels and lower levels of Bax, CASP-3, and CASP-9 followed FAK inhibition compared to the injection of PV-IgG without any pretreatment [90] (Supplementary Table S5).

3. Conclusions and Perspectives

In conclusion, we point to some possibilities to explain the divergent results for apoptosis as an upstream or downstream event of acantholysis: (1) many investigations were only descriptive, without enough statistical power for a meaningful analysis; (2) several of them limited their findings to the detection a specific molecule and/or TUNEL positive cells, frequently without a control group, neither with positive and negative controls for immunohistochemistry / immunofluorescence [19,62,63,65,89,97,105,120–122]; (3) there is no follow-up investigation to ensure that acantholysis follows apoptosis in nonlesional areas presenting pro-apoptotic molecules, a common problem for human biopsies [80,107,120]; (4) the usage of only one or two IgG pools from pemphigus patients on cell lines and mouse models may bias the results due to the heterogeneity of their composition, including different anti-mitochondrial IgG, not purified for anti-DSG1/DSG3, which may trigger divergent cellular signaling and pro-apoptotic and/or pro-necrotic (oncotic) outcomes [6,24]; (5) PV serum was seldom incorporated in cell death investigations, but different outcomes result from the usage of PV-IgG or PV sera, revealing the importance of its components [94]; (6) the genetic regulation of cell death pathways was poorly investigated in pemphigus, despite the fact that patient genetic background may unveil the susceptibility to deregulated mechanisms that precede the production of anti-DSG1 and/or 3 IgG, before self-tolerance is broken. (7) most authors investigated only apoptosis, one of 12 cell death pathways, but the importance of other key cell death molecules and pathways, as those responsive to environmental factors for PF, cannot be underestimated; (8) a complex combination of genetic susceptibility background, pemphigus-IgG production and environmental factors as mosquito bites and calcium sequestrant components may ultimately trigger cell death in the disease, which must be controlled for and thoroughly investigated [37], (9) Lastly, multiple pleiotropic effects of the investigated molecules are important to account for (Supplementary Figure S2). Taken together, this review is intended to motivate future well-controlled, statistically relevant investigations on pemphigus and keratinocyte cell

death pathways besides apoptosis, given the importance of this knowledge to overcome the urgent challenges of preventive, personalized, participative, and predictive Medicine for these autoimmune diseases.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/life12030329/s1>, Figure S1: PRISMA study selection diagram flow [141,142]; Figure S2: Wikipathways of molecules involved in cell death pathways. Table S1: Genetic background in pemphigus vulgaris and pemphigus foliaceus patients; Table S2: Reported alterations in mRNA expression after blister formation in patients; Table S3: Reported alterations in mRNA expression after keratinocytes dissociation in cell lines; Table S4: Reported alterations in protein levels in cell lines; Table S5: Reported alterations in protein levels in a mouse model.

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SUPPLEMENTARY MATERIAL

Market to Die-Cell Death Mechanisms for Keratinocyte Acantholysis in Pemphigus Diseases

1. Materials and Methods

To investigate the relationships between cell death pathways and pemphigus, a PubMed literature search was performed. Articles according to the criteria listed below and published until April 20, 2021, were included.

1.1. Search strategy

All original research and review articles were searched in the electronic database PubMed using the term ("pemphigus"[MeshTerm]) combined, through the descriptor "AND", with ("cell death"[MeshTerm]), ("apoptosis"[MeshTerm]), ("necrosis"[MeshTerm]), ("necroptosis"[MeshTerm]), ("ferroptosis"[MeshTerm]), ("pyroptosis"[MeshTerm]), ("parthanatos"[MeshTerm]), ("entotic"[MeshTerm]), ("NETotic"[MeshTerm]), ("lysosome-dependent"[MeshTerm]), ("autophagy-dependent"[MeshTerm]), and ("immunogenic cell death"[MeshTerm]). Articles not related to the human species were excluded. 251 articles were selected, 32 being duplicates. Thus, 219 were kept for analysis.

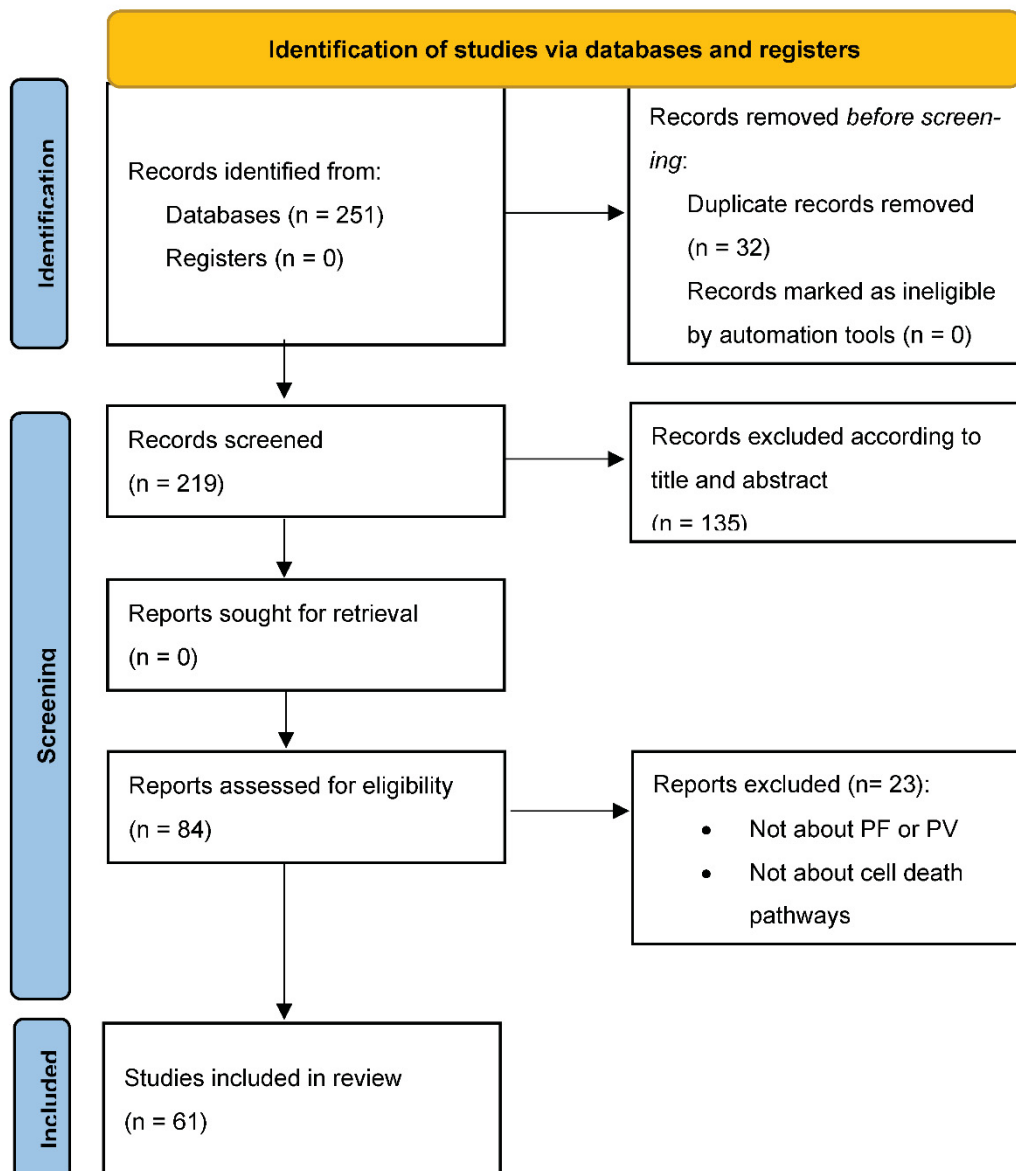
1.2. Inclusion criteria

All studies that met the following criteria were included: (1) the article refers to pemphigus vulgaris or foliaceus; and (2) address cell death pathways in these diseases. Exclusion criteria for language were not applied. After these steps, 84 articles were determined for full reading and final check-up. 61 articles were included in the review [141] (**Supp. Figure 1**).

1.3. Data extraction

The selection process was carried out using Rayyan software [142] by two researchers. Data extraction was performed by four independent reviewers and, in parallel, checked by another. All relevant information was classified in a spreadsheet, such as the molecules investigated in each study, variations in their expression, the chronology of interventions, as well as their respective results, in addition to the method by which they were obtained and the means (e.g. human, animal or cell lines).

Supplementary Figure S1. PRISMA study selection diagram flow.



2. Supplementary tables

Supplementary Table S1. Genetic background in pemphigus vulgaris and pemphigus foliaceus patients.

Gene	Genetic variant	Associated		Not Associated	
		PV	PF	PV	PF
<i>BAX</i>	<i>rs4645878 G>A (-248)</i>				[93]
<i>CD36</i>	<i>rs4112274*T</i>		[37]		
<i>CD47</i>	<i>rs12695175*G</i>		[37]		
<i>EIF2AK3</i>	<i>rs10167879*T</i>		[37]		
<i>HK1</i>	<i>rs7072268*T</i>		[37]		
<i>PAK2</i>	<i>rs9325377*A</i>		[37]		
<i>PRKN</i>	<i>rs9355950*C</i>		[37]		
<i>RAPGEF3</i>	<i>rs10747521*A</i>		[37]		
<i>SIRPA</i>	<i>rs6075340*A</i>		[37]		
<i>ST18</i>	<i>rs2304365*A/A</i>	[112]			
<i>ST18</i>	<i>rs4074067*T</i>	[112]			
<i>ST18</i>	<i>rs10504140*A</i>	[112]			
<i>TNF</i>	<i>rs1800630*A (-863)</i>		[37]		[111]
<i>TNF</i>	<i>rs1800629*A (308*A)</i>		[119]	[101] [86] [91]	[101] [111] [86]
<i>TNF</i>	<i>rs1800629*A/rs361525*G (308*G/238*G)</i>	[91]			
<i>TNF</i>	<i>rs1800629 GA+AA (308)</i>	[101]			[101]
<i>TNF</i>	<i>rs361525*A (238*A)</i>		[111]		
<i>TNF</i>	<i>rs1800629*G / rs361525*A (308*G/238*A)</i>		[111]		
<i>TNF</i>	<i>STR *2, *5, *6</i>		[73]		
<i>TNF</i>	<i>STR *a2 / *a5</i>		[73]		
<i>TP53</i>	<i>rs1042522 C>G (12139)</i>				[93]
<i>TRAF2</i>	<i>rs10781522*G</i>		[37]		

[73]: Tunisian EPF, [37]: Brazilian EPF, [93]: Brazilian EPF, [86]: Argentinian population. STR: microsatellite. *BAX*, BCL2 associated X apoptosis regulator; *CD36*, CD36 molecule; *CD47*, CD47 molecule; *EIF2AK3*, eukaryotic translation initiation factor 2 alpha kinase 3; *HK1*, hexokinase 1; *PAK2*, p21 (RAC1) activated kinase 2; *PRKN*, parkin RBR E3 ubiquitin protein ligase; *RAPGEF3*, Rap guanine nucleotide exchange factor 3; *SIRPA*, signal regulatory protein alpha; *ST18*, ST18 C2H2C-type zinc finger transcription factor; *TNF*, tumor necrosis factor; *TP53*, tumor protein p53; *TRAF2*, TNF receptor associated factor 2; PF, pemphigus foliaceus; PV, pemphigus vulgaris.

Supplementary Table S2. Reported alterations in mRNA expression after blister formation in patients.

mRNA	Increased		Decreased	
	PV	PF	PV	PF
<i>ANXA4</i>		[98]		
<i>BAG4</i>	[85]			
<i>BCL2A1</i>		[98]+		
<i>BCL2L11</i>	[85]			
<i>BDNF</i>			[85]	
<i>CASP3</i>	[105]*			
<i>COP</i>		[98]		
<i>CSF2RA</i>	[85]			
<i>CSF2RB</i>	[85]			
<i>ERAP1</i>	[85]			
<i>ERBB4</i>			[85]	
<i>FAS</i>	[105]*			
<i>FASL</i>	[105]*			
<i>GNAQ</i>	[85]			
<i>GZMA</i>		[98]		
<i>GZMH</i>		[98]		
<i>IL15</i>	[85]			
<i>IRAK1</i>	[85]			
<i>LEPROT</i>	[85]			
<i>LGALS1</i>		[98]		
		[98]+		
<i>LGALS12</i>		[98]		
		[98]+		
<i>MAP3K1</i>	[85]			
<i>MAP3K4</i>	[85]			
<i>MAP3K5</i>	[85]			
<i>MAPK9</i>	[85]			
<i>NALP12</i>		[98]		
<i>NLRP3</i>				
<i>NSMAF</i>	[85]			
<i>P2RX1</i>		[98]+		
<i>PIK3R1</i>				[98]+
<i>PPP2CA</i>	[85]			
<i>PRKCA</i>				[98]
<i>SIAH1</i>				[98]
<i>SMAD2</i>	[85]			
<i>SMAD4</i>	[85]			
<i>TLR2</i>		[98]		
<i>TNF</i>	[88]	[97]*		

	[97]*		
<i>TNFRSF10A</i>			[98]+
<i>TNFRSF18</i>		[98]	
<i>TNFSF9</i>		[98]	
<i>TNFSF10</i>		[98]	
<i>TNFSF13</i>	[85]		
<i>TNFSF13B</i>		[98]	
		[98]+	

ANXA4, annexin A4; *BAG4*, BAG cochaperone 4; *BCL2A1*, BCL2 related protein A1; *BCL2L11*, BCL2 like 11; *BDNF*, brain derived neurotrophic factor; *CASP3*, caspase 3; *COP*, caspase recruitment domain-containing protein 16; *CSF2RA*, colony stimulating factor 2 receptor subunit alpha; *CSF2RB*, colony stimulating factor 2 receptor subunit beta; *ERAP1*, endoplasmic reticulum aminopeptidase 1; *ERBB4*, erb-b2 receptor tyrosine kinase 4; *FAS*, Fas cell surface death receptor; *FASL*, Fas ligand; *GNAQ*, G protein subunit alpha q; *GZMA*, granzyme A; *GZMH*, granzyme H; *IL15*, interleukin 15; *IRAK1*, interleukin 1 receptor associated kinase 1; *LEPROT*, leptin receptor overlapping transcript; *LGALS1*, galectin 1; *LGALS12*, galectin 12; *MAP3K1*, mitogen-activated protein kinase kinase kinase 1; *MAP3K4*, mitogen-activated protein kinase kinase kinase 4; *MAP3K5*, mitogen-activated protein kinase kinase kinase 5; *MAPK9*, mitogen-activated protein kinase kinase kinase 9; *NALP12*, NLR family pyrin domain containing 12; *NLRP3*, NLR family pyrin domain containing 3; *NSMAF*, neutral sphingomyelinase activation associated factor; *P2RX1*, purinergic receptor P2X 1; *PIK3R1*, phosphoinositide-3-kinase regulatory subunit 1; *PPP2CA*, protein phosphatase 2 catalytic subunit alpha; *PRKCA*, protein kinase C alpha; *SIAH1*, siah E3 ubiquitin protein ligase 1; *SMAD2*, SMAD family member 2; *SMAD4*, SMAD family member 4; *TLR2*, toll-like receptor 2; *TNF*, tumor necrosis factor; *TNFRSF10A*, TNF receptor superfamily member 10a; *TNFRSF18*, TNF receptor superfamily member 18; *TNFSF9*, TNF superfamily member 9; *TNFSF10*, TNF superfamily member 10; *TNFSF13*, TNF superfamily member 13. PF, pemphigus foliaceus; PV, pemphigus vulgaris. +After therapeutic intervention. *Statistical significance was not mentioned in the article.

Supplementary Table S3. Reported alterations in mRNA expression after keratinocytes dissociation in cell lines.

mRNA	Increased		Decreased or without difference	
	IgG-PV	After therapeutic intervention	IgG-PV	After therapeutic intervention
<i>BAX</i>	[96]	[103]		[96]
<i>BCL2</i>			[96] [76]	
<i>CASP3</i>	[96] [24]			[24] [96]
<i>CASP8</i>	[24]			[24]
<i>CCND2</i>			[103]	
<i>FASL</i>	[24]			[24]
<i>FLIP1</i>	[24]	[24]		
<i>GADD34</i>			[103]	
<i>iNOS</i>	[76]			
<i>PEA15</i>			[103]	
<i>TNF</i>	[88]#			
<i>TP53</i>	[76]			
<i>TSSC3</i>			[103]	
<i>WAF1</i>			[103]	

BAX, BCL2 associated X apoptosis regulator; *BCL2*, BCL2 apoptosis regulator; *CASP3*, caspase 3; *CASP8*, caspase 8; *CCND2*, cyclin D2; *FASL*, Fas ligand; *FLIP1*, TNFAIP interacting protein 2; *GADD34*, protein phosphatase 1 regulatory subunit 15A; *iNOS*, inducible nitric oxide synthase; *PEA15*, proliferation and apoptosis adaptor protein 15; *TNF*, tumor necrosis factor; *TP53*, tumor protein p53; *TSSC3*, pleckstrin homology like domain family A member 2; *WAF1*, cyclin dependent kinase inhibitor 1A. PF, pemphigus foliaceus; PV, pemphigus vulgaris. #Before keratinocytes dissociation. *Statistical significance was not mentioned in the article.

Supplementary Table S4. Reported alterations in protein levels in cell lines.

Protein	Detected or increased		Absent, low or decreased	
	PV	After therapeutic intervention	PV	After therapeutic intervention
ANXA-5	[120]*#			
BAX	[109]			
BCL2		[96]	[120]* [121]*	
CAPN-1	[24]			
CASP-1	[120]*# [121]* [120]*# [121]*			
CASP-3	[24] [89]* [19]* [99]# [120]* [121]*		[68]# [68]	[19]* [99]
CASP-8	[24] [89]*# [99]# [99]#			[61] [99]
CASP-9	[99] [79]			[99]
c-Jun	[19]*# [99]#			[19]*
CYCS	[99] [79] [77]#			[79] [99]
EGFR	[19]*# [99]#			
p-ERK1/2	[19]*#			[19]*
FAS	[120]*# [120]*			
FASL	[120]*# [121]*# [24]			[19]*
FLIP1	[24]			
IL1B		[110]		
iNOS	[76]			
JNK	[99]#			[99]
NF-kB p-p65	[96]			[96]
NOD2	[96]			[96]
RIPK2	[96]			[96]
SRC	[99]# [79] [99]#			
p38 MAPK	[99] [17]# [79]			[99]

PARP	[17]#	[68]
p-EIF2 α	[94]#	
p-PERK	[94]#	
TNF	[120]*	
	[76]	
TP53	[109]	
	[120]*	

ANXA5, annexin A5; BAX, BCL2 associated X apoptosis regulator; BCL2, BCL2 apoptosis regulator; CAPN1, calpain 1; CASP1, caspase1; CASP3, caspase 3; CASP8, caspase 8; CASP9, caspase 9; CYCS, cytochrome c; EGFR, epidermal growth factor receptor; ERK1/2, mitogen-activated protein kinase; FAS, Fas cell surface death receptor; FASL, Fas ligand; FLIP1, TNFAIP interacting protein 2; GADD34, protein phosphatase 1 regulatory subunit 15A; IL1B, interleukin 1 beta; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH2-terminal kinase; JUN, AP-1 transcription factor subunit; NF-kB p-p65, nuclear factor kappa B p-p65; NOD2, nucleotide binding oligomerization domain containing 2; RIPK2, receptor interacting serine/threonine kinase 2; SRC, proto-oncogene tyrosine-protein kinase Src; p38MAPK, p38 mitogen-activated protein kinase; PARP, poly-(ADP-ribose) polymerase; p-EIF2 α , eukaryotic translation initiation factor 2 subunit alpha; PERK, eukaryotic translation initiation factor 2 alpha kinase 3; TNF, tumor necrosis factor; TP53, tumor protein p53. PF, pemphigus foliaceus; PV, pemphigus vulgaris. #Before keratinocytes dissociation. *Statistical significance was not mentioned in the article.

Supplementary Table S5. Reported alterations in protein levels in mouse model.

Protein	Detected or increased		Absent, low or decreased	
	PV	PF	PV	PF
AKT			[106]	
BAX	[90]	[95]#	[90]+	
BCL2	[90]+		[90]	
BCL-XL				[95]# [95]
BTC	[106]			
CASP3	[90]	[95]# [17]	[68]# [68] [90]+	[17]+
CASP6		[95]#		
CASP9	[90]		[90]+	
CYCS	[77]			
EGF	[106]		[106]+	
EGFR	[106]			
FAK			[90]+	
FASL	[61]#			
HER2	[106]			
HER3	[106]			
MTOR	[106]		[106]+	
p38 MAPK		[17]# [17]		
PARP		[17]	[68]	
SRC	[106]		[106]+	
TNF	[106]			

AKT, AKT serine/threonine kinase 1; BAX, BCL2 associated X apoptosis regulator; BCL2, BCL2 apoptosis regulator; BCLXL, BCL2-like 1; BTC, betacellulin; CASP3, caspase 3; CASP6, caspase 6; CASP9, caspase 9; CYCS, cytochrome c; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; FASL, Fas ligand; HER2, erb-b2 receptor tyrosine kinase 2; HER3, erb-b2 receptor tyrosine kinase 3; MTOR, mechanistic target of rapamycin kinase; p38MAPK, p38 mitogen-activated protein kinase; PARP, poly-(ADP-ribose) polymerase; SRC, proto-oncogene tyrosine-protein kinase Src; TNF, tumor necrosis factor; PF, pemphigus foliaceus; PV, pemphigus vulgaris. +After therapeutic intervention. #Before blistering formation. *Statistical significance was not mentioned in the article.

Supplementary Figure S2. Wikipathways of molecules involved in cell death pathways. Representative for the pleiotropic effect of the investigated molecules. Search: <https://fuma.ctglab.nl/>.

5 CAPÍTULO 2 – ESTUDO DE ASSOCIAÇÃO GENÉTICA NO PÊNFIGO FOLIÁCEO

PESQUISA ORIGINAL – “*Condemned or Not to Die? Gene Polymorphisms Associated with Cell Death in Pemphigus Foliaceus*”. Artigo publicado no dia 18 de outubro de 2019, no periódico “*Frontiers in Immunology*”, no tópico de pesquisa “*Skin Autoimmunity*”. Link para visualização online do artigo: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.02416/full>.



Condemned or Not to Die? Gene Polymorphisms Associated With Cell Death in Pemphigus Foliaceus

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Pemphigus foliaceus (PF) is an autoimmune blistering skin disease that occurs sporadically across the globe and is endemic in Brazil. Keratinocyte adhesion loss (acantholysis) is associated with high levels of anti-desmoglein 1 IgG autoantibodies, but the role of cell death is poorly understood in PF. Current evidence disqualifies apoptosis as the major cell death mechanism and no other process has yet been investigated. To approach the role of variation in genes responsible for cell death pathways in pemphigus susceptibility, we systematically investigated the frequencies of 1,167 polymorphisms from genes encoding products of all 12 well-established cell death cascades (intrinsic and extrinsic apoptosis, necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic, NETotic, lysosome-dependent, autophagy-dependent, and immunogenic). By multivariate logistic regression, we compared allelic and genotypic frequencies of 227 PF patients and 194 controls obtained by microarray hybridization. We found 10 variants associated with PF ($p < 0.005$), belonging to six cell death pathways: apoptosis (*TNF*, *TRAF2*, *CD36*, and *PAK2*), immunogenic cell death (*EIF2AK3*, *CD47*, and *SIRPA*), necroptosis (*TNF* and *TRAF2*), necrosis (*RAPGEF3*), parthanatos (*HK1*), and pyroptosis (*PRKN*). Five polymorphisms were associated with susceptibility: *TNF* rs1800630*A ($OR = 1.9$, $p = 0.0003$), *CD36* rs4112274*T ($OR = 2.14$, $p = 0.0015$), *CD47* rs12695175*G ($OR = 1.77$, $p = 0.0043$), *SIRPA* rs6075340*A/A ($OR = 2.75$, $p = 0.0009$), and *HK1* rs7072268*T ($OR = 1.48$, $p = 0.0045$). Other five variants were associated with protection: *TRAF2* rs10781522*G ($OR = 0.64$, $p = 0.0014$), *PAK2* rs9325377*A/A ($OR = 0.48$, $p = 0.0023$), *EIF2AK3* rs10167879*T ($OR = 0.48$, $p = 0.0007$), *RAPGEF3* rs10747521*A/A ($OR = 0.42$, $p = 0.0040$), and *PRKN* rs9355950*C ($OR = 0.57$, $p = 0.0004$). Through functional annotation, we found that all associated alleles, with the exception of *PRKN* rs9355950*C, were previously associated with differential gene expression levels in healthy individuals (mostly in skin and peripheral blood). Further functional validation of these genetic associations may contribute to the understanding of PF etiology and to the development of new drugs and therapeutic regimens for the disease.

Keywords: pemphigus, cell death, skin disease, autoimmune disease, apoptosis, pyroptosis, necroptosis, immunogenic cell death

INTRODUCTION

Pemphigus foliaceus (PF) is an autoimmune bullous disease of the skin, characterized by the production of autoantibodies that recognize the desmosome protein desmoglein 1 (DSG1) (1, 2). The binding of antibodies to this cell adhesion molecule is accompanied by acantholysis (keratinocyte detachment) and lesions in the superficial granular layer of the epidermis. In contrast, a different form of pemphigus called pemphigus vulgaris (PV) is characterized by anti-desmoglein 3 antibodies, which bind in deeper layers of the skin and cause blisters in the skin and mucosa (3). PF occurs sporadically around the world—0.75–5 cases/million per year (4). In Brazil, PF is endemic and commonly known as *fogo selvagem* (“wild fire” in Portuguese). The prevalence of PF in Limão Verde, located in the Brazilian state of Mato Grosso do Sul, is of 3.04%, one of the highest prevalences ever reported for autoimmune diseases (5). PF pathogenesis is multifactorial, resulting from poorly understood interactions between multiple environmental and genetic factors (6, 7).

Cell Death in Pemphigus: An Unsolved Issue

Despite the pathogenic relevance of IgG autoantibodies in the acantholytic process, the exact mechanisms that lead keratinocytes to death remain unknown (8). Apoptosis has been suggested to play an important role in some dermatoses with positive Nikolsky's sign (skin detachment after slight rubbing) as in PV and PF (9–12), either prior (10, 13–15), or after the loss of cell adhesion (8, 16–18). As early as 1998, Gniadecki et al. reported many apoptotic keratinocytes in acantholytic tissue and in the cohesive epidermis just under the blisters of sporadic PF and PV lesional skin biopsies, as judged by positive TUNEL signs (terminal deoxynucleotidyl transferase dUTP nick end labeling) (9). Rodrigues et al. (19) also found TUNEL-positive keratinocytes in 12/13 biopsies of perilesional skin of endemic PF lesions. Among them, 10/13 presented keratinocytes with intense expression of proapoptotic inducible nitric oxide synthase (iNOS) and 3/13, with rather discrete-moderate expression of proapoptotic FAS protein. Anti-apoptotic Bcl-2 occurred in 4/13 biopsies only, being much more abundant in the inflammatory infiltrate, which also had discrete-moderate expression of interleukin 1, interferon gamma, and tumor necrosis factor alpha (TNF- α) proinflammatory cytokines (11/13) (19). After the passive transfer of sporadic PF-antibodies in the experimental neonatal mouse model, keratinocytes expressed the proapoptotic Bax factor, followed by activation of caspases (CASP) 3 and 6, and down-regulation of the anti-apoptotic Bcl-x(L) factor. In this model, apoptotic inhibitors abrogated the acantholytic process (14). Nevertheless, p38MAPK signaling occurred in this same model in two phases, and the first peak of activation coincided with acantholysis, prior to the second peak that induced activation of CASP-3 (18). Taking into account ultra-violet (UV) light exposure as a known risk factor for endemic PF, it is interesting that caspases-3 and -7 cleave desmoglein-1 intracellularly and that knock-down

of desmoglein-1 protects cells from UV induced apoptosis in irradiated keratinocytes (20).

On the other hand, only few apoptotic cells were detected in skin biopsies of endemic PF patients, whereas p63 marked many undifferentiated cells distributed over the whole epidermis, both in injured and non-injured skin (21). Electron microscopy did not reveal any morphological signs of apoptosis—retraction of pseudopods, pyknosis, karyorrhexis, and plasma membrane blebbing—in acantholytic tissue of PV and PF patients (8, 22). There were, as well, no signs for activation of caspases (as cleaved CASP3 and CASP8, fractin, or nuclear poly (ADP-ribose) polymerase—PARP) in PV and PF biopsies, nor in PV or PF IgG-incubated healthy breast reduction skin biopsies (8). A possibility suggested by Schmidt and Waschke (16) is that caspase signaling adds in desmosome destabilization, but that apoptosis itself is not responsible nor needed for acantholysis to occur.

Thus, whereas some authors state that apoptosis causes cell death in PF (9, 19, 23), others found no clear evidence of such event (8, 17, 18, 22). The uncertainty about how cell death takes place in PF, as well as the scarcity of genetic association studies on this topic, prompted the current investigation encompassing genes of all known cell death routes. In fact, there are several pathways orchestrating cell death, classified following morphological, biochemical, and functional features. In 2018, the Nomenclature Committee on Cell Death (NCCD) proposed 12 pathways orchestrating cell death, supported by genetic, biochemical, and functional results: intrinsic apoptosis, extrinsic apoptosis, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic, NETotic, lysosome-dependent, autophagy-dependent, and immunogenic pathways (24). All of them are classified as regulated cell death (RCD) routes. All routes depend on the molecular machinery (causing the activation of one or more signal transduction pathways), which can be pharmacologically and/or genetically modulated. RCD begins with excessive cellular stress, caused by non-coped perturbations of the intra- and extracellular environment (24, 25). Given the poorly understood underlying mechanisms leading to keratinocyte death in PF, we intended to identify genetic variants associated with PF analyzing variants from genes whose products are known to be directly involved in RCD routes.

MATERIALS AND METHODS

Population Sample

A total of 227 PF patients and 194 unrelated controls with no diagnosis or familial history of autoimmune illnesses were analyzed in this study. Patients received clinical and/or immunohistological diagnosis. Patients and controls were recruited from 1984 to 2015 in South/Southeastern/Central-Western Brazilian hospitals: Hospital Adventista do Pênfigo (Campo Grande, Mato Grosso do Sul), Lar da Caridade—Hospital do Fogo Selvagem (Uberaba, Minas Gerais), Hospital das Clínicas—University of São Paulo (Ribeirão Preto, São Paulo), Hospital de Clínicas—Federal University of Paraná, Hospital de Dermatologia Sanitária São Roque, and Hospital Santa Casa de Misericórdia (Curitiba, Paraná). All individuals

enrolled in this study were unrelated, predominantly of European ancestry and living in rural endemic areas. No history of other autoimmune diseases was reported for patients, as well as no history of any autoimmune disease for the controls. The median age was 40.9 years for patients (minimum 6, maximum 83) and 44.8 years for controls (minimum 11, maximum 86). In both groups, 52% were women. Peripheral blood was collected, from which DNA was extracted by the phenol-chloroform-isoamyl alcohol protocol (26). Patients and controls voluntarily agreed to participate in the study and gave their informed consent. This study was carried out according to the Declaration of Helsinki, with the approval from the Brazilian National Ethics Committee (CONEP) under the protocol number CAAE 02727412.4.0000.0096 and approval number 505.988.

Selection of Candidate Genes and Genotyping

In agreement with NCCD, we selected genes encoding proteins involved in essential aspects of at least one of the following cell death cascade processes: intrinsic apoptosis, extrinsic apoptosis, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic, NETotic, lysosome-dependent, autophagy-dependent, and immunogenic pathways (24). We identified the genomic positions of each gene, considering regulatory regions of one thousand base pairs upstream and downstream from the transcription start and end sites, respectively, of the longest transcript, according to the GRCh37/hg19 human genome version (available at: http://www.lgmh.ufpr.br/data/Supplementary_material_1_Bumiller-Bini_2019.xlsx).

Genotyping was performed using microarray hybridization (CoreExome-24 v1.1 Illumina®) in 194 and 227 DNA samples from controls and endemic PF patients, respectively. A total of 3,798 SNPs were extracted from DNA microarray data, filtered based on genotyping quality, and on population genetics criteria: excluding those SNPs with minor allele frequencies <1%, with genotypic distributions deviating from those expected by Hardy-Weinberg equilibrium in controls ($p < 0.05$) and/or with high linkage disequilibrium ($r^2 \geq 0.8$). After filtering, a total of 1,167 SNPs remained for subsequent analyses (Figure 1, set available at: http://www.lgmh.ufpr.br/data/Supplementary_material_2_Bumiller-Bini_2019.xlsx).

Association Analysis

Association analysis was carried out by binary logistic regression, using sex and two principal components (PCA) as co-variables. The PCA eliminates spurious associations due to possible population stratification. The significance level was set to $p < 0.005$ (27). The analyses were performed using PLINK software version 1.1.9 (28). We applied Fisher's exact test to perform the stratified association analysis (29), in this case, with a significance level set to $p < 0.05$.

In silico Analysis

We performed *in silico* analysis to explore the genetic associations identified in this study. Linkage disequilibrium within the Iberian population (major ancestral of the Euro-Brazilian population) was evaluated with LDlink (30). The predicted regulatory

impact of the genetic variants was verified using ENSEMBL (31), GTEx portal (32), UCSC (<https://genome.ucsc.edu/cgi-bin/hgGateway>) (33), HaploReg (<https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php>) (34), Innatedb (<https://www.innatedb.com>) (35), and Blood eQTL (expression quantitative trait loci) browser (36), which assemble public datasets, and published data. GTEx portal and the Blood eQTL browser inform whether a SNP is an expression quantitative trait loci (eQTL). Innatedb informs whether there is a physical interaction between proteins.

RESULTS

Association Analysis

We found 10 SNPs associated with PF ($p < 0.005$) located in 10 different genes participating in six RCD routes: apoptosis (*CD36*, *PAK2*, *TNF*, and *TRAF2*), immunogenic cell death (*CD47*, *EIF2AK3*, and *SIRPA*), necroptosis (*TNF* and *TRAF2*), necrosis (*RAPGEF3*), parthanatos (*HK1*), and pyroptosis (*PRKN*) (Table 1). Altogether, the less frequent alleles of five SNPs were associated with PF susceptibility, while the less frequent alleles from the other five SNPs, with protection. All associated variants were located within non-protein coding regions.

Functional Annotation in silico Analysis

To explore the potential effects of all 10 genetic variants associated with PF, we used functional annotation available in reference public databases. As outlined below, most of the associated variants are associated with gene expression in different tissues (Table 2). Nevertheless, the association of these variants (with disease and with gene expression) may also be explained by strong linkage disequilibrium with other causal variants (hitch-hiking effect).

In accordance with functional annotation, *TNF_rs1800630*, *TRAF2_rs10781522*, *PAK2_rs9325377*, *EIF2AK3_rs10167879*, *SIRPA_rs6075340*, *PRKN_rs9355950*, and *HK1_rs7072268* SNPs are located within predicted transcription factor binding sites. On the other hand, the location of *TNF_rs1800630*, *TRAF2_rs10781522*, *PAK2_rs9325377*, *SIRPA_rs6075340*, *CD47_rs12695175*, *CD36_rs4112274*, *HK1_rs7072268*, and *PRKN_rs9355950* SNPs overlap with enhancers and/or promoters that are important in several tissues, including skin, T and B cells from blood (enriched in H3K27Ac) (33, 34). Despite being located downstream of the last exon of *LTA* and far from the transcription start site of *TNF*, *TNF_rs1800630* is located in a DNase hypersensitive region bound by RNA polymerase II subunit A, providing strong evidence for active transcription of this region in B lymphocytes (33, 34).

Furthermore, the *SIRPA* protein interacts physically with *CD47*, suggesting a possible gene interaction/synergistic effect of the associated polymorphisms of both genes on disease susceptibility (35). In fact, carriers of *SIRPA_rs6075340*A* and *CD47_rs12695175*G* alleles were more frequent among patients than controls ($OR = 2.02$ [95%CI = 1.08–3.81], $p = 0.0202$). *TNF* also interacts physically with the *TNF* receptor *TRAF2*, as shown in a cervical cancer cell line (35). Thus, an additive susceptibility

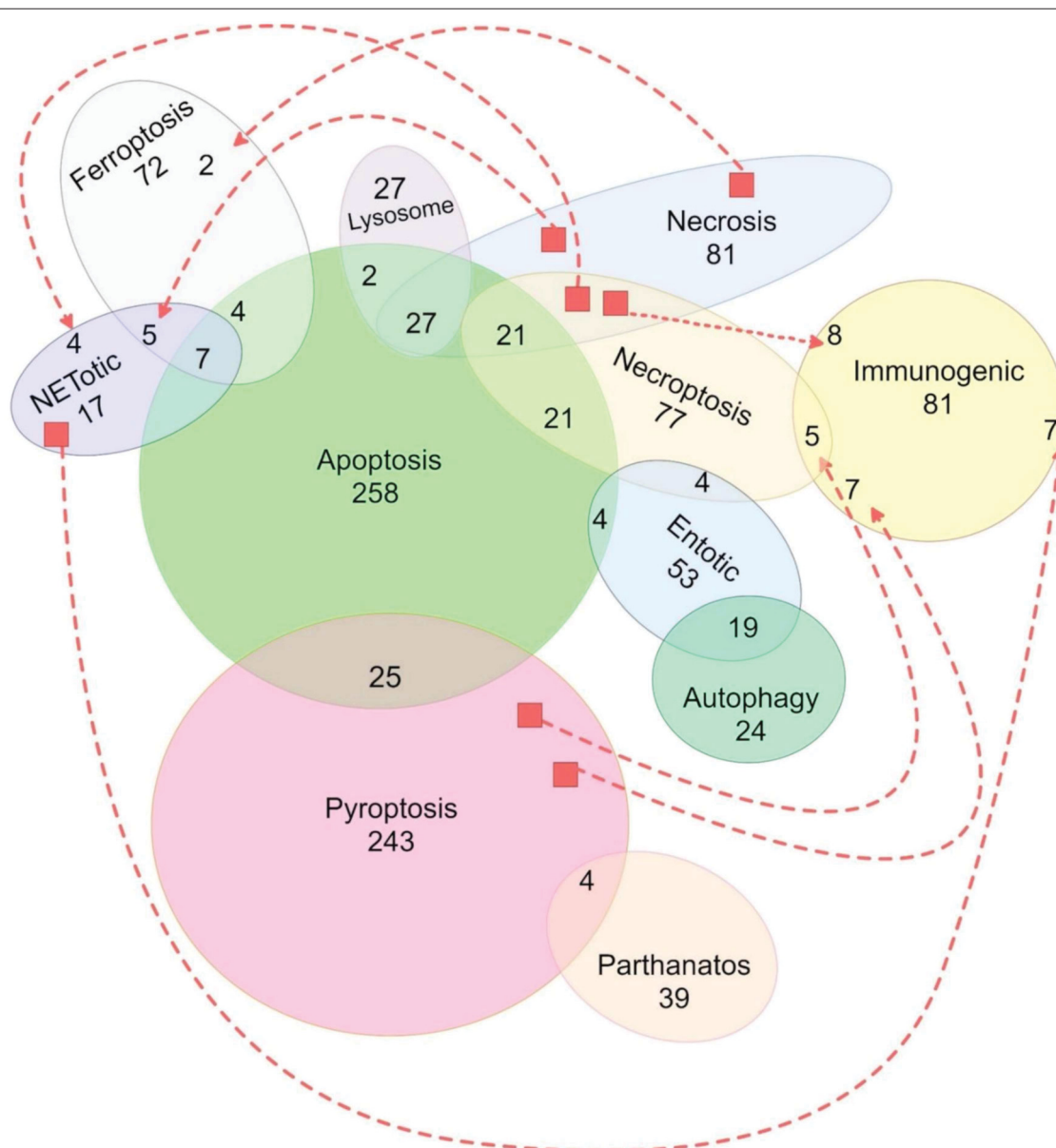


FIGURE 1 | Representation of the distribution of all 1,167 SNPs used in this study according to their related cell death pathway(s). Traced lines were used to represent additional shared SNPs (unions) between two pathways that were not otherwise graphically united. Genes from the intrinsic and extrinsic apoptosis pathways were represented together. Unrepresented SNPs, shared among pathways: one between apoptosis, immunogenic, lysosome, and necrosis pathways; two between apoptosis, immunogenic, lysosome, and pyroptosis pathways; two between necroptosis, necrosis, pyroptosis, NETotic, and immunogenic pathways; three between pyroptosis and necroptosis; three between apoptosis, necroptosis, necrosis, and NETotic pathways; four between apoptosis, necrosis, and immunogenic pathways; and four between apoptosis, entotic, and immunogenic pathways).

effect was evident in carriers of *TNF_rs1800630*A* and *TRAF2_rs10781522*A* ($OR = 4.78$ [95%CI = 2.18–10.94], $p < 0.0001$) (Supplementary Tables 1, 2).

DISCUSSION

Although the underlying molecular mechanisms of RCDs overlap considerably, our approach allowed us to suggest the possible

role of non-apoptotic RCDs and raise hypotheses to explain the genetic associations that we observed.

TNF, *TRAF2*, and *PAK2*: Apoptosis and Necroptosis

The genetic associations of *TNF_rs1800630*A* (also known as $-863*A$, $OR = 1.9$, $p = 0.0003$) and *TRAF2_rs10781522*G* ($OR = 0.64$, $p = 0.0014$) with PF point to a specific role

TABLE 1 | Cell death-related gene variants associated with PF.

Gene	SNP	MAF (%)		Model	CONTR	CASE	OR	95%CI	p
		Contr	Case						
APOPTOSIS AND NECROPTOSIS									
TNF 6p21.33	rs1800630	15.7	26.2	add	61/327	117/329	1.90	[1.34–2.70]	0.0003
	C>a			rec	7/187	14/209	1.79	[0.70–4.54]	0.2214
	Promoter			dom	54/140	103/120	2.24	[1.49–3.38]	0.0001
TRAF2 9q34.3	rs10781522	49.2	38	add	191/197	171/279	0.64	[0.48–0.84]	0.0014
	g>A			rec	47/147	34/191	0.58	[0.35–0.95]	0.0305
	Intron 9			dom	144/50	137/88	0.52	[0.34–0.79]	0.0024
APOPTOSIS									
PAK2 3q29	rs9325377	49.5	42	add	196/192	190/162	0.72	[0.54–0.94]	0.0173
	a>G			rec	55/139	36/190	0.47	[0.29–0.77]	0.0023
	Intron 1			dom	141/53	154/72	0.81	[0.53–1.25]	0.3449
CD36 7q21.11	rs4112274	7.51	15.27	add	29/357	69/383	2.14	[1.33–3.44]	0.0015
	C>t			rec	1/192	5/221	3.92	[0.45–34.2]	0.2169
	Intron 3			dom	28/165	64/162	2.23	[1.34–3.68]	0.0018
IMMUNOGENIC									
EIF2AK3 2p11.2	rs10167879	17.6	9.9	add	67/313	43/393	0.48	[0.31–0.73]	0.0007
	C>t			rec	5/185	4/214	0.62	[0.16–2.40]	0.4940
	Intron 14			dom	62/128	39/179	0.41	[0.26–0.66]	0.0002
SIRPA 20p13	rs6075340	33.5	42.1	add	130/258	191/263	1.50	[1.13–2.00]	0.0055
	a>G			rec	17/177	47/180	2.75	[1.51–4.98]	0.0009
	Intron 2			dom	113/81	144/83	1.35	[0.90–2.02]	0.1449
CD47 3q13.12	rs12695175	11.4	17.9	add	44/342	81/371	1.77	[1.20–2.63]	0.0043
	T>g			rec	4/189	10/216	2.23	[0.68–7.30]	0.1850
	Intron 6			dom	40/153	71/155	1.95	[1.23–3.07]	0.0041
NECROSIS									
RAPGEF3 12q13.11	rs10747521	40.1	36.7	add	155/231	165/285	0.81	[0.61–1.1]	0.1676
	a>G			rec	36/157	23/202	0.42	[0.23–0.76]	0.0040
	Intron 1			dom	155/231	165/285	1.03	[0.20–0.69]	0.8837
PARTHANATOS									
HK1 10q22.1	rs7072268	43.0	53.1	add	167/221	241/213	1.48	[1.13–1.94]	0.0045
	t>C			rec	37/157	70/157	1.57	[1.02–2.42]	0.0412
	Intron 5			dom	130/64	171/56	1.87	[1.18–2.95]	0.0076
PYROPTOSIS									
PRKN 6q26	rs9355950	34.3	22.9	add	133/255	104/350	0.57	[0.42–0.78]	0.0004
	T>c			rec	25/169	11/216	0.35	[0.16–0.73]	0.00502
	Intron 4			dom	108/86	93/134	0.55	[0.37–0.81]	0.0024

Logistic regression association tests were done with allele frequencies ("add"), frequency of homozygotes for the minor allele ("rec"- recessive model), and summed frequencies of heterozygotes and homozygotes for the minor allele ("dom"—dominant model). The minor alleles in our sample are given in lowercase; they are the reference for the associations. In bold, significant associations ($p < 0.005$). SNP, single nucleotide polymorphism; MAF, minor allele frequency; CONTR, controls; CASE, cases; Model, association tests; OR, odds ratio; CI, confidence interval; TNF, tumor necrosis factor alpha; TRAF2, TNF receptor associated factor 2; PAK2, p21 (RAC1) activated kinase 2; CD36, CD36 molecule; EIF2AK3, eukaryotic translation initiation factor 2 alpha kinase 3 (also known as PERK); SIRPA, signal regulatory protein alpha; CD47, CD47 molecule; RAPGEF3, Rap guanine nucleotide exchange factor 3 (also known as EPAC); HK1, hexokinase 1; PRKN, parkin RBR E3 ubiquitin protein ligase (also known as PARK2).

of the death receptor pathway in the disease. As the major proinflammatory cytokine mediating apoptosis and necroptosis, TNF binds TNF type I receptor (TNFRI), activating NF- κ B through TRADD (TNFR-associated death domain), and TRAF2 recruitment. This sequence of events culminates in cell survival and inflammation (37, 38). In the absence of TRAF2, TNF binding to TNFRI builds "death-inducing signaling complexes" that can activate either necroptosis or caspase-dependent cell

death. In the latter case, this leads to the activation of CASP8 and of the apoptotic cascade (37, 39). On the other hand, TRAF2 was recently reported to positively regulate caspase-2 activation, which initiates apoptosis and is a negative regulator of necroptosis (40, 41). For necroptosis to ensue, caspase inactivity or absence must prevail [thus, in the absence of TRAF2 (38)] (42). Interestingly, although *TNF rs1800630**A carriers present reduced *TNF* expression, *TRAF2 rs10781522**G is associated

TABLE 2 | Cell death-related gene variants associated with PF.

Variant	Allele associated with PF	eQTL effect (25, 29)	Tissues
<i>TNF_</i> <i>rs1800630</i>	A risk	low expression: <i>TNF</i> ($p = 3.71 \times 10^{-21}$) and <i>LTA</i> ($p = 2.88 \times 10^{-24}$) (36). high expression: <i>DDX39B</i> ($p = 2.6 \times 10^{-4}$), <i>CSNK2B</i> ($p = 3.14 \times 10^{-11}$), <i>HCP5</i> ($p = 2.5 \times 10^{-3}$) and <i>MICB</i> ($p = 2.0 \times 10^{-15}$) (36).	Peripheral blood.
<i>TRAF2_</i> <i>rs10781522</i>	G prot	low expression: <i>C8G</i> ($p = 2.6 \times 10^{-5}$), <i>AGPAT2</i> ($p = 9.1 \times 10^{-4}$), and <i>CLIC3</i> ($p = 4.79 \times 10^{-17}$) (32, 36). high expression: <i>TRAF2</i> ($p = 2.73 \times 10^{-4}$), <i>PHPT1</i> ($p = 1.2 \times 10^{-6}$)	Skin, peripheral blood, muscle.
<i>PAK2_</i> <i>rs9325377</i>	A prot	low expression: <i>PAK2</i> ($p = 1.2 \times 10^{-6}$) (32). high expression: <i>PIGX</i> ($p = 9.7 \times 10^{-7}$). <i>PIGX</i> maps just upstream of <i>PAK2</i> , on the same strand of chromosome region 3q29 (32).	Skin, spleen.
<i>CD36_</i> <i>rs4112274</i>	T risk	low expression: <i>CD36</i> ($p = 2.37 \times 10^{-29}$) (36).	Peripheral blood.
<i>EIF2AK3_</i> <i>rs10167879</i>	T prot	low expression: <i>AC062029.1</i> ($p = 3.0 \times 10^{-4}$) (32) high expression: <i>EIF2AK3</i> ($p = 3.5 \times 10^{-4}$), <i>ANKRD36BP2</i> ($p = 1.6 \times 10^{-6}$) (32).	Skin, whole blood.
<i>SIRPA_</i> <i>rs6075340</i>	A risk	low expression: <i>SIRPA</i> ($p = 4.1 \times 10^{-7}$) (32). high expression: <i>SIRPB1</i> ($p = 6.6 \times 10^{-98}$) (32).	Esophagus, skin.
<i>CD47_</i> <i>rs12695175</i>	G risk	low expression: <i>CD47</i> ($p = 3.37 \times 10^{-4}$) (36). high expression: <i>MYH15</i> ($p = 1.3 \times 10^{-8}$) (32).	Peripheral blood, skin.
<i>RAPGEF3_</i> <i>rs10747521</i>	A prot	low expression: <i>RAPGEF3</i> ($p = 2.0 \times 10^{-8}$) (32). high expression: <i>PCED1B-AS1</i> (32).	Lung, heart.
<i>HK1_</i> <i>rs7072268</i>	T risk	high expression: <i>HK1</i> ($p = 2.3 \times 10^{-6}$) (32).	Tibial nerve.

PRKN_rs9355950 is not an eQTL.

eQTL, expression quantitative trait loci; *LTA*, lymphotoxin alpha; *DDX39B*, dead box polypeptide 39 B; *CSNK2B*, casein kinase II beta; *HCP5*, HLA complex P5; *MICB*, major histocompatibility complex class I-chain related gene B; *PHPT1*, phosphohistidine phosphatase 1; *C8G*, complement C8 gamma chain; *AGPAT2*, 1-acylglycerol-3-phosphate O-acyltransferase 2; *CLIC3*, chloride intracellular channel 3; *PIGX*, phosphatidylinositol glycan anchor biosynthesis class X; *ANKRD36BP2*, ankyrin repeat domain 36B pseudogene 2; *SIRPB1*, signal regulatory protein beta 1; *MYH15*, myosin heavy chain 15.

with higher *TRAF2* gene expression in peripheral blood (36). In contrast with *TNF*, the allele from *TRAF2* was associated with PF protection. Since both molecules are known to interact physically (35), certain allelic combinations of these genes present an additive effect toward susceptibility to the disease.

Moreover, many associations of *TNF_rs1800630*A* with diseases have been reported. Those of enhanced susceptibility to cancer seem to indicate that reduced TNF levels increase the chance of inappropriate cell proliferation, due to insufficient signaling for apoptosis/necroptosis [e.g., hepatocellular carcinoma (43), non-Hodgkin lymphoma (44, 45), gastric cancer (46), and colon cancer (47)]. On the other hand, the same allele was associated with progression to and severity of infections, as for severe malaria, including cerebral malaria (48, 49), HPV-associated oral squamous carcinoma (50), HBV chronification (51), as well as with chronic disorders, which may rely on insufficient immunological response to different kinds of aggressors [steroid-induced osteonecrosis of the femoral head (52), progression of acute pancreatitis to systemic inflammation and multi-organ dysfunction syndrome (53), chronic periodontitis (54), and cardiovascular heart disease (55)].

The *TNF_rs1800630*A* was associated with predisposition to autoimmune disorders affecting the skin and mucosal tissue, as vitiligo (56), systemic lupus erythematosus (SLE) (57), and Crohn's disease (58). As in these autoimmune skin disorders, the *rs1800630*A* was associated with susceptibility

to endemic PF, in the Brazilian population ($OR = 1.9$, $p = 0.0003$). Interestingly, *TNF* microsatellite polymorphisms were associated with susceptibility to PF in Tunisia, where the disease is also endemic (59). The *rs1800630*A* causes decreased *TNF* transcription and lower serum TNF levels (51, 60–62). Furthermore, carriers of *rs1800629*A* (also known as $-308*A$) presented higher susceptibility to pemphigus (both PV and PF) (63). This allele is associated with TNF expression levels in whole blood, similar as observed for *rs1800630*A*. Thus, although both occur in different haplotypes (*rs1800630_rs1800629*CA* and *AG*, with *CG* representing more than 70% of the allelic combinations in the Iberian population), both present the same effect on reducing gene expression and increasing PF susceptibility.

In addition, the *TRAF2* PF protective allele is associated with altered expression of at least five genes implicated in cell survival or death (*PHPT1*, *PTGDS*, *LCNL1*, *C8G*, *CLIC3*). It is also associated with the expression of one gene related to size and inflammatory itching reaction, after mosquito bites (*AGPAT2*) (64–70). The *AGPAT2* association is particularly interesting, given the epidemiological association of endemic PF with massive and continued exposure to mosquito bites (71, 72).

Another PF susceptibility allele, *CD36_rs4112274*T*, is associated with decreased *CD36* gene expression in blood. This receptor is a mediator of both endoplasmic reticulum stress and the generation of reactive oxygen species in the intrinsic apoptosis pathway (73). Its predicted down-regulation in most PF patients is an additional argument favoring a less prominent

role (if any) for apoptosis in the disease. Its expression in keratinocytes correlates with early wound healing (74). Thus, lower expression is also predicted to increase the extent of PF epidermal lesions.

The *PAK2 rs9325377* A/A* was associated with PF protection ($OR = 0.48$, $p = 0.0023$). The *PAK2* product is activated through proteolytic cleavage, by caspase-mediated apoptosis. Cleavage of *PAK2* regulates morphological changes in apoptotic cells and always correlates with apoptotic cell death (75). The variant *PAK2 rs9325377* A* occurs in strong linkage disequilibrium with the *PAK2 rs6583176* A* in the Iberian population ($D' = 0.93$), and both alleles were associated with increased susceptibility to gastric cancer (76). Thus, it is conceivable that they are associated with apoptosis failure, through yet unknown mechanisms. The association of the *PAK2 rs9325377* A* with higher *PIGX* expression further reinforces this possibility, since *PIGX* knockdown may inhibit breast cancer cell growth (77).

Taken together, decreased *TNF* gene expression (suggested by *TNF rs1800630* A* association) increases PF susceptibility, whereas higher *TRAF2* gene expression (suggested by *TRAF2 rs10781522* G* association) seems to protect against the disease, as well as *PAK2 rs9325377* A/A*. Moreover, *TRAF2 rs10781522* G* is associated with lower expression of C8G in the skin, expected to decrease the production of a complement component protecting keratinocytes against apoptosis and necroptosis (32). Higher *TNF* and *TRAF2* levels are thus predicted to be protective against the disease, both preferentially leading to cell survival and inflammation. The fact that we did not find a clear association between any of these gene polymorphisms and apoptosis or necroptosis is in agreement with previous findings using electron microscopy (8, 22).

EIF2AK3, CD47, and SIRPA: Immunogenic Cell Death

We identified genetic associations of *EIF2AK3 rs10167879* T* with PF protection, and of *SIRPA rs6075340* A/A* and *CD47 rs12695175* G* with susceptibility to PF. These genes encode products that participate in the immunogenic activation by CALR (calreticulin) (78), highlighting CALR as a molecule likely associated with damage-associated molecular patterns (DAMP) that may follow or precede the immunogenic pathway in PF. This cascade can be activated by a relatively limited set of stimuli, that may also include environmental factors associated with PF susceptibility, as UVB (79), thiol and other calcium-sequestering components (80), and components of fly saliva (71, 72) (Figure 2).

Besides its association with PF protection, *EIF2AK3 rs10167879* T* is also associated with higher *EIF2AK3* and lower *EIF2AK3-DT* (its antisense lncRNA) expression (32). This is the first documented association between this allele and any disease. The association is supported by the fact that deregulation of *EIF2AK3* (also known as PERK) has been reported as one of the earliest pathogenic events in PV independently of IgG. Also, *EIF2AK3* is activated in keratinocytes exposed to PV serum, as demonstrated by an increase in its phosphorylated levels and in phosphorylation

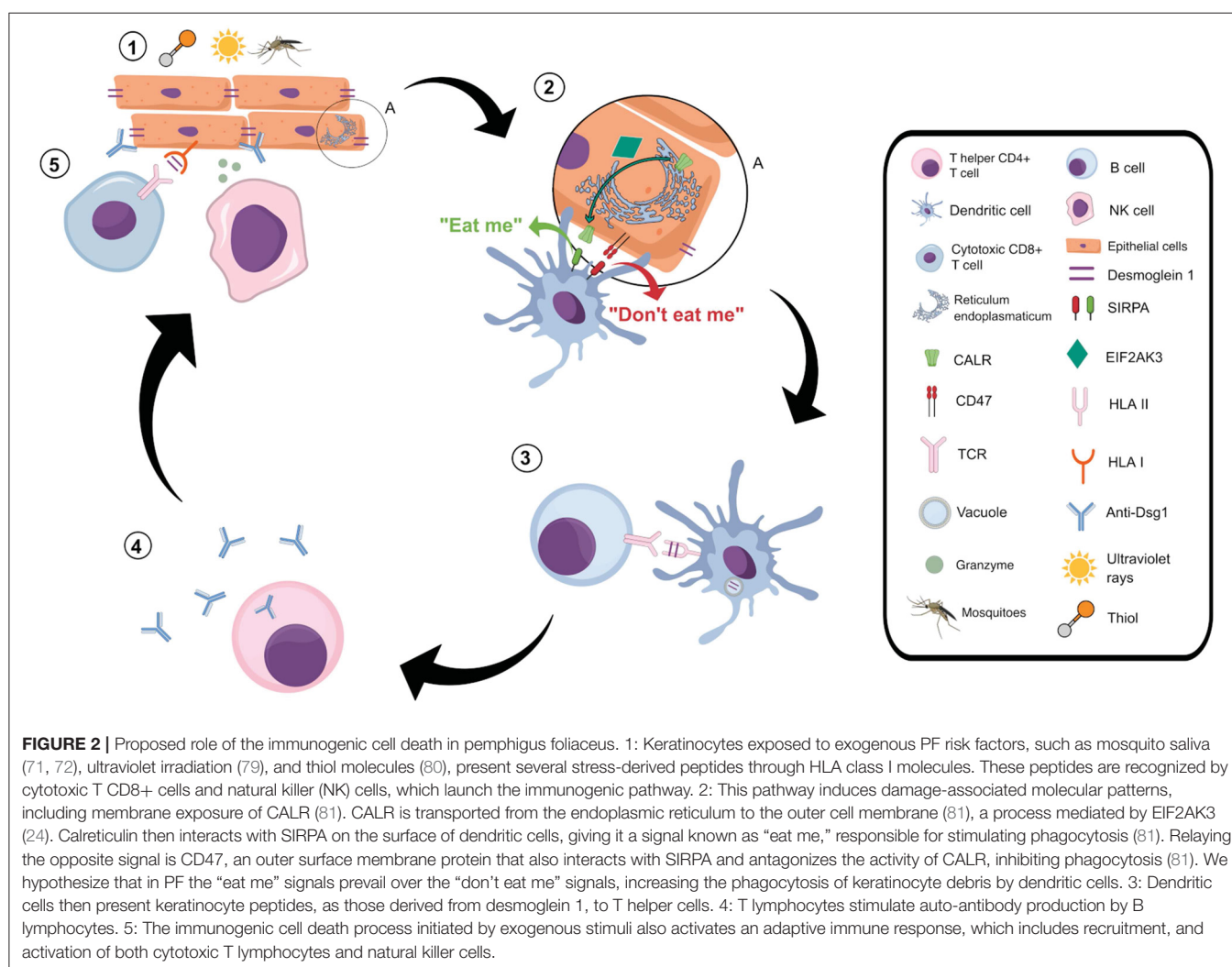
of its target, eIF2 α . Decreased *EIF2AK3* expression, mediated by small interfering RNA, reduced the effects of PV serum on cell cycle and keratinocyte viability, two PV hallmarks (82). In agreement with this, Cipolla et al. (83) formerly proposed that PF IgG and/or non-IgG extracellular factors may lead to endoplasmic reticulum (ER) stress, resulting in C/EBP-homologous protein (CHOP) induction via *EIF2AK3* (PERK), and activation of transcription factor 6 (ATF6). The association with *EIF2AK3 rs10167879* T* is puzzling, since it would imply that high *EIF2AK3* levels are protecting against PF. Nevertheless, it may be explained in the light of its pleiotropy and a possible effect on differential transcription of its two poorly characterized isoforms (31). The *SIRPA rs6075340* A* and *CD47 rs12695175* G* alleles are associated with lower expression of their respective genes (36), in addition to their synergistic association with PF susceptibility. The interaction between *CD47* and *SIRPA* is capable of antagonizing the activity of surface-exposed CALR, responsible for emitting “eat me” signals for phagocytosis (81). The lower expression of those two physically interacting molecules is expected to lead to excessive internalization of cell debris and antigen presentation, increasing PF autoantibody production (Figure 2). This hypothesis agrees with our prior results on complement receptor polymorphisms, which would protect against PF development by modulating scavenging efficiency of acantholytic cell debris (7). A further argument in favor of the importance of the immunogenic cell death pathway in PF is the up-regulated expression of immunogenic deadly granzyme *GZMA* and *GZMB* genes in T lymphocytes of untreated patients with generalized lesions (84).

PRKN, HK1, and RAPGEF3: Pyroptosis, Parthanatos and Necrosis

PRKN rs9355950 C* was associated with PF protection ($OR = 0.57$, $p = 0.0004$). This gene encodes the mitophagy-regulating Parkin protein, which prevents cell death by causing ubiquitination of mitochondrial proteins presented by damaged mitochondria. Pyroptosis amplifies mitochondrial damage through caspase 1-driven cleavage and inactivation of *PRKN* (85). Since the associated effects of this variant on gene expression are unknown, the importance of pyroptosis on PF susceptibility remains elusive.

HK1 rs7072268 A* was associated with increased susceptibility to PF ($OR = 1.48$, $p = 0.0045$) and also with higher gene expression levels in nervous tissue (32). Furthermore, this variant is associated with glycated hemoglobin levels (86). It is conceivable that it may be actually associated with enhanced poly(ADP-ribosylation) of hexokinase 1, occurring through the activation of PARP [poly(ADP-ribose) polymerase], with consequent glycolysis inhibition and induction of parthanatos-induced cell death (87).

Finally, the *RAPGEF3 rs10747521* A/A* genotype of the necrosis pathway, associated with protection against the disease ($OR = 0.42$, $p = 0.004$), is also associated with lower *RAPGEF3* gene expression, as well as of its neighboring gene, *SLC18A1*. This protein has a role in inhibiting the p38MAPK pathway (88), activated in PF (83). Higher *PCED1B-AS1* lncRNA



expression is also associated with the same variant, predicted to enhance monocyte apoptosis and reduce autophagy (89). The reasons subjacent to this association await the results of future functional studies.

CONCLUSION

For the first time, SNPs located within genes involved in all known cell death cascades were systematically investigated in a single disease. The genetic association profile with *TNF*, *TRAF2*, *CD36*, and *PAK2* variants favors cell survival and inflammation, instead of apoptosis/necroptosis, to explain resistance against the disease. On the other hand, susceptibility is conferred by variants of *CD47* and *SIRPA* of the immunogenic cell death pathway, proposed to lead to excessive internalization of cell debris, and antigen presentation, which may increase PF autoantibody production. The importance of other pathways as pyroptosis, parthanatos, and necrosis, represented by one association each in our setting, cannot be disclosed and shall be

further investigated. Functional validation of these associations, especially of genes encoding common isoforms, as *EIF2AK3*, will provide a better understanding of PF pathogenesis and contribute to the development of new drugs and to therapeutic improvement for the disease.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the LGMH repository: http://www.lgmh.ufpr.br/data/Supplementary_material_1_Bumiller-Bini_2019.xlsx; http://www.lgmh.ufpr.br/data/Supplementary_material_2_Bumiller-Bini_2019.xlsx.

ETHICS STATEMENT

This study was carried out in accordance with the guidelines of the Conselho Nacional de Ética em Pesquisa (CONEP) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the

Declaration of Helsinki. The protocol was approved by CONEP (No. 505.988).

AUTHOR CONTRIBUTIONS

MP-E, AB, VB-B, GC, MS, DA, and MB contributed to conception of the work. AB and VB-B designed the study. AB, GC, and MB supervised the study. MP-E provided the samples. MP-E and AB provided resources. DA performed microarray hybridization. VB-B and MS did the statistical analysis and drafted the manuscript. AB, MP-E, GC, DA, and MB edited it. All authors revised the work critically for intellectual content and approved the final version of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.02416/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Table 1: Stratified analyses between rs6075340 (*A/G*) of *SIRPA* and rs12695175 (*T/G*) of *CD47*.

	<i>SIRPA</i>	<i>CD47</i>	Cases	Controls	OR	[95%CI]	p
	rs6075340	rs12695175					
A)			n = 88	n = 47			
	<i>A/A</i>	<i>T/T</i>	32	12	1.66	[0.71 – 4.03]	0.2489
	<i>G+</i>	<i>G+</i>	56	35	0.60	[0.24 – 1.39]	
B)			n = 138	n = 146			
	<i>A/A</i>	<i>G+</i>	15	5	3.42	[1.14 – 12.39]	0.0190
	<i>G+</i>	<i>T/T</i>	123	141	0.29	[0.08 – 0.87]	
C)			n = 173	n = 172			
	<i>A/A</i>	<i>G/G</i>	2	0	-	-	0.4986
	<i>G+</i>	<i>T+</i>	171	172	-	-	
D)			n = 53	n = 21			
	<i>A/A</i>	<i>T+</i>	45	17	1.31	[0.25 – 5.74]	0.7314
	<i>G+</i>	<i>G/G</i>	8	4	0.75	[0.17 – 3.89]	
E)			n = 117	n = 99			
	<i>A+</i>	<i>T/T</i>	95	86	0.65	[0.28 – 1.45]	0.2734
	<i>G/G</i>	<i>G+</i>	22	13	1.52	[0.68 – 3.52]	
F)			n = 109	n = 94			
	<i>A+</i>	<i>G+</i>	49	27	2.02	[1.08 – 3.81]	0.0202
	<i>G/G</i>	<i>T/T</i>	60	67	0.49	[0.26 – 0.92]	
G)			n = 88	n = 82			
	<i>A+</i>	<i>G/G</i>	8	3	2.61	[0.60 – 15.88]	0.2144
	<i>G/G</i>	<i>T+</i>	80	79	0.38	[0.06 – 1.66]	
H)			n = 138	n = 111			
	<i>A+</i>	<i>T+</i>	136	110	0.61	[0.01 – 12.04]	1.0
	<i>G/G</i>	<i>G/G</i>	2	1	1.61	[0.08 – 96.23]	

In bold: significant (Fisher exact test). Fisher exact test was performed using the online web statistical calculator Astasa [www.astatsa.com]. OR: Odds Ratio. CI: Confidence interval. SNP: single nucleotide polymorphisms.

Table 2: Stratified analyses between 10781522 (*A/G*) of *TRAF2* and rs1800630 (*A/C*) of *TNF*.

	<i>TRAF2</i>	<i>TNF</i>	Cases	Controls	OR	[95%CI]	p
	rs10781522	rs1800630					
A)	<i>A/A</i> <i>G+</i>	<i>A/A</i> <i>C+</i>	n = 129 4 125	n = 145 4 141	1.12 0.88	[0.20 – 6.18] [0.16 – 4.86]	1.0
B)	<i>A/A</i> <i>G+</i>	<i>C+</i> <i>A/A</i>	n = 94 84 10	n = 49 46 3	0.54 1.81	[0.09 – 2.28] [0.43 – 10.79]	0.5428
C)	<i>A/A</i> <i>G+</i>	<i>C/C</i> <i>A+</i>	n = 115 50 65	n = 80 38 42	0.85 1.17	[0.46 – 1.57] [0.63 – 2.17]	0.6610
D)	<i>A/A</i> <i>G+</i>	<i>A+</i> <i>C/C</i>	n = 108 38 70	n = 114 12 102	4.58 0.21	[2.16 – 10.34] [0.09 – 0.46]	<0.0001
E)	<i>A+</i> <i>G/G</i>	<i>A/A</i> <i>C+</i>	n = 42 11 31	n = 52 6 46	2.69 0.37	[0.81 – 9.84] [0.10 – 1.23]	0.1044
F)	<i>A+</i> <i>G/G</i>	<i>C+</i> <i>A/A</i>	n = 181 178 3	n = 142 141 1	0.42 2.37	[0.01 – 5.31] [0.18 – 125.54]	0.6336
G)	<i>A+</i> <i>G/G</i>	<i>C/C</i> <i>A+</i>	n = 126 106 20	n = 125 109 16	0.77 1.28	[0.35 – 1.67] [0.59 – 2.80]	0.5896
H)	<i>A+</i> <i>G/G</i>	<i>A+</i> <i>C/C</i>	n = 97 83 14	n = 69 38 31	4.78 0.20	[2.18 – 10.94] [0.09 – 0.45]	<0.0001

In bold: significant (Fisher exact test). Fisher exact test was performed using the online web statistical calculator Astasa [www.astatsa.com]. OR: Odds Ratio. CI: Confidence interval. SNP: single nucleotide polymorphisms.

6 CAPÍTULO 3 – ESTUDO DE ASSOCIAÇÃO GENÉTICA NO PÊNFIGO FOLIÁCEO E PÊNFIGO VULGAR

PESQUISA ORIGINAL – *Concise Communication* – “*DNA Polymorphisms in Mitochondrial Respiratory Chain Subunits Are Associated with Endemic Pemphigus Foliaceus*”. Manuscrito que será submetido no periódico “*Experimental Dermatology*” - <https://onlinelibrary.wiley.com/journal/16000625>.

DNA POLYMORPHISMS IN MITOCHONDRIAL RESPIRATORY CHAIN SUBUNITS ARE ASSOCIATED WITH ENDEMIC PEMPHIGUS FOLIACEUS

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DNA POLYMORPHISMS IN MITOCHONDRIAL RESPIRATORY CHAIN SUBUNITS ARE ASSOCIATED WITH ENDEMIC PEMPHIGUS FOLIACEUS

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6.1 ABSTRACT

Mitochondrial reactive oxygen species (ROS) may result from skin ultraviolet exposure, a known risk factor for pemphigus foliaceus (PF) and vulgaris (PV). We evaluated previously obtained genotypes of 107 and 151 single nucleotide polymorphisms (SNPs) from nuclear genes whose products encode mitochondrial respiratory chain subunits or its assembly factors in 227 endemic PF patients (EPF) and 194 controls, and in 241 PV patients and 1188 controls, respectively. The intronic *rs5910697*G* variant of *NDUFA1* (ubiquinone oxidoreductase subunit A1), associated with lower mRNA levels in endothelial cells, was also associated with EPF protection (OR=0.40; [95%CI=0.23-0.72]; p=0.00198). It encodes an essential component of the respiratory chain complex I, transferring electrons from NADH to ubiquinone. The *rs2159132*G* variant of *COX10* (cytochrome c oxidase assembly factor heme A, catalyzing the electron transfer from reduced cytochrome c to oxygen) was associated with EPF susceptibility (OR=2.01; [95%CI=1.30-3.08]; p=0.00143). This variant causes the loss of a stop codon and is associated with increased levels of an uncommon transcript lacking exon 3 in different tissues, subjected to nonsense mediated decay. No associations were found with sporadic PF or PV. Thus, genes encoding mitochondrial respiratory chain subunits may have relevance for EPF development, and possible reasons for the genetic associations shall be the focus of further functional studies.

KEYWORDS: pemphigus foliaceus; pemphigus vulgaris; mitochondrial Respiratory chain subunits; cell death; autoimmune diseases.

6.2 BACKGROUND

The etiology of pemphigus, a group of autoimmune blistering diseases, is not fully understood. Desmogleins (DSG) are their hallmark autoantigens: DSG1 for pemphigus foliaceus (PF), whose blisters occur in the granular layer of the epidermis, and DSG3 for pemphigus vulgaris (PV), whose lesions occur in the suprabasal layer (Eyre and Stanley 1987; Spindler et al. 2007; Schmidt et al. 2019). Mitochondrial autoantigens have also been described (Marchenko et al. 2010; Grando 2012; Chen et al. 2015; Chernyavsky et al. 2015). PV patients release increased levels of cytochrome c, indicating mitochondrial damage. They also presented autoantibody combinations directed against mitochondrial components (Marchenko et al. 2010), including NDFUS1, an important component of the first complex of the respiratory chain (Kalantari-Dehaghi et al. 2013). In contrast, only two out of 28 Brazilian patients with endemic PF (EPF) presented anti-mitochondrial antibodies (Mendes and Castro 1976), and none was detected in 120 EPF patients, of which 63% presented anti-DSG1 (Nisihara et al. 2003).

Environmental risk factors interacting with gene variants and deregulating gene expression (Malheiros et al. 2014, Petzl-Erler 2020) modulate PF susceptibility and clinical presentation, affecting antibodies (Calonga-Solís et al. 2020), antigen-presenting molecules (Petzl-Erler and Santamaria 1989), complement system (Bumiller-Bini et al. 2018), and cell death pathways (Bumiller-Bini et al. 2019). Besides adenosine triphosphate (ATP) production by the oxidative phosphorylation process, mitochondria also have a central role in cell death, mainly in intrinsic apoptosis (Bock and Tait 2020). Primarily, mitochondrial outer membrane permeabilization leads to cell death through cytochrome c release and caspase activation (Bock and Tait 2020). Further on, deregulated mitochondrial functions may have a devastating outcome by increasing the production of reactive oxygen species (ROS), therefore fostering cell death mechanisms (Lu et al. 2009; Feichtinger et al. 2014; Bock and Tait 2020).

Interestingly, skin ultraviolet exposure, a known risk factor for PF (Reis et al. 2000), increases ROS formation. Although the status and the role of oxidative stress remain to be explored in Brazilian PF, it has been recently reported that patients with the EPF in Peru have higher serum levels of malondialdehyde, a marker of oxidative stress (Gutierrez et al. 2018). Consonantly, this marker is significantly increased in

EPF and PV patients from Tunisia (Abida et al. 2012). PV patients also have reduced total antioxidant capacity (Javanbakht et al. 2015; Shah et al. 2016) or increased levels of oxidative stress markers (Yesilova et al. 2013). Dietetic supplementation of L-carnitine, which has antioxidant properties, resulted in better profiles of oxidative stress parameters in PV patients (Mohammadi et al. 2018).

Among the causes of mitochondrial deregulation are mutations in genes that compose respiratory chain subunits (Brealey et al. 2002; Fernandez-Vizarra and Zeviani 2021). Interestingly, *MT-ATP8* (mitochondrially encoded ATP synthase 8) single nucleotide polymorphisms (SNPs) were associated with susceptibility to bullous pemphigoid (BP), another autoimmune skin blistering disease (Grando 2015; Hirose et al. 2015). However, polymorphisms of mitochondrial protein-encoding genes have not been investigated in pemphigus disease.

6.3 QUESTIONS ADDRESSED

Considering the detection of mitochondrial autoantibodies in autoimmune skin blistering diseases and the importance of mitochondria signaling for oxidative stress and cell death, we investigated a possible association between variants of nuclear genes whose products have been previously implicated in mitochondrial illnesses (Ylikallio and Suomalainen 2012) and that encode mitochondrial respiratory chain subunits or their assembly factors with the susceptibility to the Brazilian endemic form of PF (EPF), sporadic PF (SPF) and PV.

6.4 EXPERIMENTAL DESIGN

6.4.1 Endemic Brazilian PF samples

We compared 227 Brazilian EPF patients and 194 controls. Patients received clinical and/or immunohistological diagnosis. No diagnosis of any autoimmune disease for controls was reported, as well as no other autoimmune disease for patients. Both groups had equivalent gender distributions. The median age was 40.9 years for patients and 44.8 years for controls (Bumiller-Bini et al. 2019). Patients and controls were genetically unrelated, of predominant European ancestry (Calonga-Solís et al. 2020) and living in rural endemic areas. They were contacted from 1984 to 2015 at reference hospitals in Brazil, as previously described (Bumiller-Bini et al.

2019). Peripheral blood was collected from which DNA was extracted by the phenol-chloroform-misoamyl alcohol protocol (Sambrook et al., 2001). All individuals voluntarily agreed to take part in the study and gave their informed consent. This investigation is in agreement with the Declaration of Helsinki and was approved by the Brazilian National Ethics Committee (CONEP) under the CAAE 02727412.4.0000.0096 protocol number and 505.988 approval number.

6.4.2 Sporadic PF and PV samples

For SPF, we compared 76 PF patients (46% female) and 150 controls (50% female), with predominant German ancestry. The mean age was 60.1 (25-88) years for patients and 60.3 (21-77) years for controls. The PV group included 241 PV patients and 1188 controls, all of German origin, unrelated and with no clinical history of other autoimmune diseases. In agreement with the guideline of the German Society of Dermatology, patients were diagnosed based on their clinical phenotype and a positive direct immunofluorescence microscopy of a perilesional skin biopsy and/or detection of serum autoantibodies against DSG1 (for PF) and DSG3 (for PV) by ELISA (Euroimmun) (Joly et al. 2020). The blood samples from SPF and PV patients were collected by the German Autoimmune Bullous Diseases Genetic Study Group in German hospitals. The DNA from SPF patients was isolated from whole blood samples using the QIAamp DNA Maxi Blood Kit (Qiagen, Hilden, Germany). For PV patients, DNA was isolated from total blood samples using the Smart Blood DNA midi prep (Analytik Jena, Germany). This investigation is in agreement with the Declaration of Helsinki and was approved by the Ethics Committee of the University of Lübeck (08–156,12–178).

6.4.3 Genotyping

The genotyping assays were performed using the Human Infinium® CoreExome-24 Beadchip (Illumina Inc., San Diego, CA, USA) for the EPF group, and the Global Screening Array (Illumina Inc., San Diego, CA, USA) for the PV group, all according to the manufacturer's instructions. For the sporadic PF group, the iPLEX platform of the MassARRAY system (Agena Bioscience, San Diego, CA, USA) was used. The primer sequences are available in **Supp. Table 1**.

6.4.4 Selection of candidate genes and microarray data quality control

We extracted the genotyping data for 40 genes (**Supp. Table 2**) mapping in nuclear DNA and encoding mitochondrial respiratory chain subunits or their assembly factors which have been previously implicated in other illnesses (Ylikallio and Suomalainen 2012). A total of 483 single nucleotide variants (SNVs) and 183 SNVs were extracted for EPF and PV groups, respectively. Those with minor allele frequencies (MAF) < 1% and with genotypic distribution deviating from those expected by Hardy-Weinberg equilibrium in controls ($p < 5\%$) were excluded from the downstream analyses. After this filtering step, for EPF, 107 single nucleotide polymorphisms (SNPs) remained and were evaluated through binary logistic regression, correcting the association for two principal components (PCA). For PV, 151 SNPs were evaluated through binary logistic regression. The significance level was set to $p < 0.005$ (Benjamin et al., 2018). The analyses were performed using PLINK software version 1.1.9 (Purcell et al. 2007). Subsequently, the associated SNPs in the EPF cohort were evaluated in the SPF cohort through binary logistic regression. The significance level was set to $p < 0.05$.

6.5 RESULTS

We found two associated variants with EPF (**Supp. Table 3**). The intronic *rs5910697*G* variant located in the *NDUFA1* (ubiquinone oxidoreductase subunit A1) gene, which encodes a component of complex I, was associated with EPF protection (OR=0.40; [95% CI=0.23-0.72]; $p=0.00198$). We also identified one nonsense variant (*rs2159132*G*) associated with susceptibility to EPF (OR=2.009 [95% CI=1.30-3.08]; $p=0.00143$) in the *COX10* gene which encodes a subunit of complex IV (**Table 1**). However, no association was found for SPF (**Table 1**), neither for PV (**Supp. Table 4**).

The *rs5910697*G* occurs in the first intron of the *NDUFA1* gene in the X chromosome (Howe et al. 2021). *NDUFA1* is a structural subunit of complex I of the respiratory chain. Although most likely non-catalytic (Stroud et al. 2016), its deficiency has been causally implicated in Leigh syndrome and mitochondrial encephalopathy (Au et al. 1999; Fernandez-Moreira et al. 2007; Rahman et al. 2015). Its polymorphisms have also been associated with Alzheimer's disease and basal cell carcinoma and its low expression, with skin pigmentation and aging (Manners et al. 2018; Mamelak et al. 2005; Nakama et al. 2012).

To the best of our knowledge, the association of the *rs5910697*G* variant with protection against PF is the first one reported between this intronic variant and an illness. This SNP overlaps with a regulatory region (Howe et al. 2021). The associated allele is inserted within an expression quantitative trait loci (eQTL) haplotype with 24 SNPs, covering more than 85,500 bp and with cc. 5% frequency in non-Finnish Europeans, whose most associated allele overlaps a region recognized by at least 45 different regulatory proteins, within an active promoter region in human vein endothelial umbilicary cells (HUVEC, Encode data available in the UCSC Genome browser). It is associated with reduced expression of the *NDUFA1* gene in tibial arteries (endothelial cells) and of the *UPF3B* gene in sun-exposed skin (Gtex Consortium 2020). We suggest that its lower expression increases extravasation of EPF autoantibodies in the skin, by dynamin-dependent endocytic vesicle formation in blood endothelial cells (Ono et al. 2019).

UPF3B is in the X chromosome and encodes a subunit of the exon-junction complex (EJC), a multiprotein complex responsible for binding to mRNAs with premature stop codons and signaling to several different proteins. This signaling may affect different molecular processes, such as pre-mRNA splicing, nonsense-mediated mRNA decay, mRNA transport to the cytoplasm and translation, which is then enhanced (Le Hir et al. 2015; Gao and Wilkinson 2017; Jolly et al. 2013). The EJC is crucial for germ cell formation, cell differentiation, and cell division, and mutations in this gene have been linked to intellectual disability, autism, ADHD, and childhood onset schizophrenia (Le Hir et al. 2015; Howe et al. 2021). Using small hairpin RNAs (shRNAs) to knock-down *UPF3B*, Jolly and collaborators demonstrated that reduced *UPF3B* expression in neuroprogenital cells increased the number of proliferative cells and shortened the division cycle which, in turn, resulted in increased total cells and percentage of undifferentiated cells (Jolly et al. 2013). It is possible that a lower expression of *UPF3B* caused by the presence of the *rs5910697*G* variant has a similar effect in the skin, increasing the number of undifferentiated cells, usually located in the basal layer. We speculate that this could be protective against PF onset by rapidly restoring the skin after insults such as sun exposure, conceivably reducing antigen presentation and autoantibody formation. Further studies are necessary not only to understand the association found here, but also the role of mRNA surveillance mechanisms in PF.

In addition, we found *COX10* rs2159132*G associated with susceptibility to EPF. This variant causes the loss of a stop codon and is associated with increased levels of an uncommon transcript lacking exon 3 (ENST00000580561.1) in different tissues. The ancestral allele (rs2159132*A), found in most primates, and with almost 50% frequency worldwide, creates a stop codon ([ENSP00000462190.1:p.Trp61Ter](#)). Thus, the associated G variant is predicted to increase the levels of a *COX10* mRNA variant, lacking 106 amino acids normally encoded by the third exon of the gene. *COX10* is necessary for the formation of complex IV. The protein is responsible for adding a farnesyl group to a protoheme, an essential step in creating heme A, then added to the *MTCO1* subunit of complex IV to facilitate electron transfer (Taanman 1997; Moraes et al. 2004). Mutations in *COX10* have been identified in patients presenting with multisystem mitochondrial syndromes of generally early onset, severe symptoms, and dire prognosis (Valnot et al. 2000; Pitceathly et al. 2013; Antonicka et al.) *COX10* alterations have also been associated with susceptibility to sudden cardiac death, azoospermia, and atherosclerosis (Lian et al. 2009; Holvoet et al. 2019; Yang et al. 2021). We suggest that the protein isoform encoded by this mRNA variant transcript dysregulates mitochondrial function, and that this dysregulation enhances EPF susceptibility.

6.6 CONCLUSIONS & PERSPECTIVES

In this study, we aimed to provide a first glimpse into the influence of genetic mitochondrial alterations in pemphigus diseases. Our results revealed two variants associated with EPF, but not with SPF or PV. Many factors could be involved in this disparity. Although related, PF and PV are different diseases with different clinical presentations, triggers and pathogenesis (Hammers and Stanley 2016; Petzl-Erler 2020). Additionally, the populations analyzed had different geographic origins. While the SPF and PV cohorts were mostly German, the EPF group was composed of Brazilian patients of general European ancestry. Remarkably, the frequency of the rs5910697*G polymorphism, which is associated with protection against PF, can vary from 6% amongst an European population, or 8% in Latin American populations, to 18% in African populations (Clarke et al. 2017). Furthermore, the microarrays used for genotyping the Brazilian and German participants were different, detecting different polymorphisms. SNPs in the genes whose products encode mitochondrial respiratory chain subunits may alter the susceptibility to the disease and be relevant

for PF development. Possible reasons for the observed associations should be explored by further functional studies. Furthermore, a multidrug treatment including intravenous immunoglobulin and two mitochondrion-protecting drugs, minocycline and niacinamide, rapidly controlled and maintained PV remission (Grando 2019). Minocycline also contributed to reduced BP disease activity and more rapid corticosteroid dose reduction (Loo et al. 2001). Based on these results and the mentioned findings of others regarding the oxidative stress status in different forms of pemphigus, we suggest that antioxidative diets may add to the therapeutic management of Brazilian PF.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

VBB, ABWB, GAC, MLPE, and MBS contributed to the conception of the work. VBB, ABWB and MBS designed the study. MLPE provided the samples. DGA performed microarray hybridization. VBB did the statistical analysis. VBB, MBS and GAC drafted the manuscript. All authors revised the work critically for intellectual content and approved the final version of the work.

Table 1. Genetic variants associated with endemic pemphigus foliaceus and sporadic pemphigus foliaceus.

		ENDEMIC PEMPHIGUS FOLIACEUS							SPORADIC PEMPHIGUS FOLIACEUS								
GENE	SNP	MAF (%) Contr	MAF (%) Pat	MODEL	CONTR	PAT	OR	95%CI	p	MAF (%) Contr	MAF (%) Pat	MODEL	CONTR	PAT	OR	95%CI	p
NDUFA1 Xq24	rs5910697	13.45	6.31														
	A>g			add	29/171	13/225	0.40	[0.23-0.72]	0.0019	5.32	10.67	add	15/267	16/134	1.54	[0.88-2.68]	0.1302
	Intron 1			dom	NA	NA	NA	NA	NA			dom	NA	NA	NA	NA	NA
				rec	NA	NA	NA	NA	NA			rec	NA	NA	NA	NA	NA
COX10 17p12	rs2159132	40.72	50.22	add	158/230	228/226	1.45	[1.10-1.92]	0.0088	41.38	42.57	add	120/170	63/85	1.05	[0.70-1.56]	0.8128
	g>A			dom	122/72	176/51	2.00	[1.31-3.08]	0.0014			dom	93/52	51/23	1.24	[0.68-2.25]	0.481
	Exon 4			rec	36/158	52/175	1.26	[0.78-2.04]	0.3426			rec	27/118	12/62	0.84	[0.40-1.78]	0.6602

Logistic regression association tests were done with allele frequencies ("add"), frequency of homozygotes for the minor allele ("rec"—recessive model), and summed frequency of heterozygotes and homozygotes for the minor allele ("dom"—dominant model). The minor alleles in our population are given in lowercase, they are the reference for the association analyses. In bold: significant associations ($p < 0.005$); SNP, single nucleotide polymorphism; MAF, minor allele frequency; CONTR, controls; PAT, patients, Model, association tests; OR, odds ratio; CI, confidence interval; PF, pemphigus foliaceus. *NDUFA1*, ubiquinone oxidoreductase subunit A1; *COX10*, cytochrome c oxidase assembly factor heme A:farnesyltransferase COX10.

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6.8 SUPPLEMENTARY MATERIAL

The supplementary material is available in:

<https://docs.google.com/spreadsheets/d/1vuxGGKgv8RdsLJip263Vg0HpeScbOUmt/edit?usp=sharing&ouid=115289756184035320172&rtpof=true&sd=true>

7 CAPÍTULO 4 – INVESTIGAÇÃO ULTRAESTRUTURAL DOS DESMOSSOMOS DE UM MODELO DE MINIÓRGÃO DE PELE

PESQUISA ORIGINAL – Letter to the editor – *Electron Microscopy of Desmosomal Structures in the Pemphigus Human Skin Organ Culture Model*. Manuscrito submetido no periódico *Journal Investigative in Dermatology* - <https://www.jidonline.org/>.

ELECTRON MICROSCOPY OF DESMOSOMAL STRUCTURES IN THE PEMPHIGUS HUMAN SKIN ORGAN CULTURE MODEL

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SHORT TITLE: Electron microscopy in pemphigus HSOC

ABBREVIATIONS

DSG – Desmoglein

ETA - *Staphylococcus aureus* exfoliative toxin

HSOC - Human skin organ culture

IVIG - Intravenous Immunoglobulin

PF - Pemphigus foliaceus

PV - Pemphigus vulgaris

scFv - Single-chain variable fragment

TEM - Transmission electron microscopy

7.1 TO THE EDITOR

Pemphigus are rare, potentially life-threatening, chronic autoimmune skin blistering diseases, with pathogenic autoantibodies mainly directed against the structural desmosomal proteins desmoglein 1 (DSG1) and/or DSG3 (Schmidt et al., 2019). Pemphigus foliaceus (PF) presents autoantibodies against DSG1, and mucosal pemphigus vulgaris (PV), autoantibodies against DSG3. Mucocutaneous pemphigus presents reactivity against both autoantigens (Schmidt et al., 2019). DSGs are cadherin-type Ca^{2+} -dependent transmembrane adhesion molecules (Schmidt et al., 2019). Anti-DSG1/3 IgG binding causes acantholysis with desmosomal splitting and keratinocyte separation, hallmarked by intraepidermal split formation, clinically flaccid blisters and secondary erosions (Schmidt et al., 2019). Following DSG1/DSG3 distribution, desmosomal splits occur in the mucosa and skin suprabasal stratum in PV, but solely in the skin stratum granulosum in PF (Schmidt et al., 2019). Corticosteroids are the standard pemphigus treatment (Schmidt et al., 2019). The anti-CD20 antibody rituximab, combined with corticosteroids, further induces complete remission off-therapy within 24 months in 89% of patients (Joly et al. 2017). Due to the extended time to achieve remission, the necessity of new therapeutic options remains. Model systems allow the identification and testing of new therapeutic targets. In pemphigus, *in vitro* models (Van Der Wier et al., 2010) and mouse models (Amagai et al. 2000; Schulze et al. 2012) can be used to that end. While the aforementioned *in vitro* models duplicate certain aspects of pemphigus pathogenesis, mouse models are better suited to assess the impact of an *in vivo* intervention. Mouse models of pemphigus, however, are hampered by the relatively complex experimental procedures (Amagai et al. 2000; Schulze et al. 2012) or by the differences in DSG expression patterns between mice and men (Egu et al. 2017). To overcome these limitations and to implement the 3R principles of animal research, including to replace animal experiments by appropriate alternatives, we recently developed a highly standardized human skin organ culture (HSOC) model of pemphigus (Burmester et al. 2019). Here, a bi-specific anti-DSG1/DSG3 single-

chain antibody variable fragment (scFv) binding to both DSG1 and 3, termed Px4-3 (Hammers et al. 2015), is injected into human skin. This consistently induces intraepidermal splits and the model is amendable for therapeutic interventions (Burmester et al. 2020). To obtain additional insights into the mechanisms of how Px4-3 induces split formation, we evaluated the ultrastructural morphology of desmosomal dissociation in the HSOC model. As a reference and positive control, *Staphylococcus aureus* exfoliative toxin (ETA), mimicking DSG1 autoantibodies-mediated effects (Amagai et al. 2000), was injected into the human skin. Next, we investigated the alterations in interdesmosomal widening, desmosome number and length induced by either Px-4-3 or ETA.

The local ethics committee approved this study (06-109), realized according to the Declaration of Helsinki. We performed the HSOC following established protocols (Burmester et al. 2019). In brief, human skin samples were obtained from donors without a history of skin diseases and were injected intradermally with either 50 μ L of Px4-3 (60 ng) or ETA (100 ng). Normal human IgG was used as a control. Each experimental condition was evaluated in abdominal skin biopsies from five different donors. After 24 hours of incubation, we processed the samples for histological and ultrastructural electron microscopy analyses, as described (Burmester et al. 2019; Zimmer et al. 2000). To demonstrate the Px4-3 binding at the desmosomes and to confirm epidermal split formation, we performed immunogold-labeling (Figure 1) and hematoxylin-eosin staining (Figure 2). For transmission electron microscopy (TEM) studies, fixation was performed using paraformaldehyde/piperazine-N-N' bis (20-ethanol sulfonic acid) 5 %, followed by Monti Graziadei or polyvinylpyrrolidone-saccharose. After slide processing, we took 10 to 13 TEM pictures magnified 80,000-fold for each condition and skin. We used the “iTEM” software to count the desmosomes, measure their length and interdesmosomal widening. For Gaussian-distributed data, we used one-way ANOVA and Bonferroni post-test; for non-Gaussian-distributed data, Kruskal-Wallis and

Dunn's post-test. P-values lower than 0.05 were considered significant. Supplementary Material presents more detailed information.

Immunogold-labeling demonstrated Px4-3 binding to DSG1 and DSG3 in the PV model (Figure 1c). As expected, Px4-3 or ETA-injected skin specimens developed split formation at the corresponding intraepidermal layers (Figure 2a). Px4-3 but not ETA injection reduced the desmosome number compared to control skin (Figure 2b). Interdesmosomal widening was seen in both Px4-3 or ETA injected skin and were significantly higher in both groups compared to control skin (Figure 2d). The length of the desmosomes does not differ between Px4-3 and ETA injected and controls (Figure 2c).

Consistent with our findings, Sokol et al. (2015) reported a reduced number of desmosomes in skin biopsies of PV patients. Egu et al. (2017) observed the same in human skin injected with IgG from patients with mucocutaneous PV (DSG1 and DSG3 autoantibodies) and with pathogenic monoclonal anti-DSG3 antibodies from a pemphigus mouse model, as well as higher interdesmosomal widening. Both studies, in contrast to the HSOC model, reported reduced desmosomal size. Van der Wier et al. (2012) reported a reduced number of desmosomes in Nikolsky-positive PF biopsies but no difference in Nikolsky-negative PF biopsies compared to normal skin (van der Wier et al. 2012; van der Wier et al. 2014). In line with our findings, they also did not find changes in the desmosomal sizes in mucosal-dominant PV and Nikolsky-negative mucocutaneous PV biopsies compared to controls (van der Wier et al. 2014). Thus, the ultrastructural morphological features of desmosomes in the pemphigus HSOC model are similar to patient lesions (Sokol et al. 2015). Taken together, although desmosomal sizes differ between our model and PV/PF biopsies and other pemphigus models (Egu et al. 2017; Sokol et al. 2015; Stahley et al. 2016), the lower number of desmosomes (Egu et al. 2017; Sokol et al. 2015) and the higher interdesmosomal widening are observed across our model, as well as in patient skin biopsies (Egu et al. 2017).

We conclude that ultrastructural hallmarks of desmosome morphology following Px4-3 binding are reproduced in our HSOC model, reflecting the lesional skin of pemphigus patients. The reproducibility of the HSOC pemphigus model makes it an attractive tool to unravel novel therapeutic targets and evaluate new treatments targeting pemphigus pathology.

DATA AVAILABILITY STATEMENT

The data used in this paper is available in the Supplementary Table 1.

CONFLICT OF INTEREST STATEMENT

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS STATEMENT

J.E.H. and M.K. contributed to the conception of the work. J.E.H., U.K.R., V.B-B.H. designed the study. J.E.H., C.M.H., R.J.L., D.Z. provided the infrastructure and material for the HSOC. M.K. made available the infrastructure and material for the electron microscopy. U.K.R., V.B-B.H. performed the HSOC. U.K.R. collected the data. V.B-B.H. did the statistical analysis. V.B-B.H., U.K.R., J.E.H., A.B.W.B. drafted the manuscript. All authors critically evaluated the data, revised the work for intellectual content, revised the manuscript, and approved its final version.

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FIGURE LEGENDS

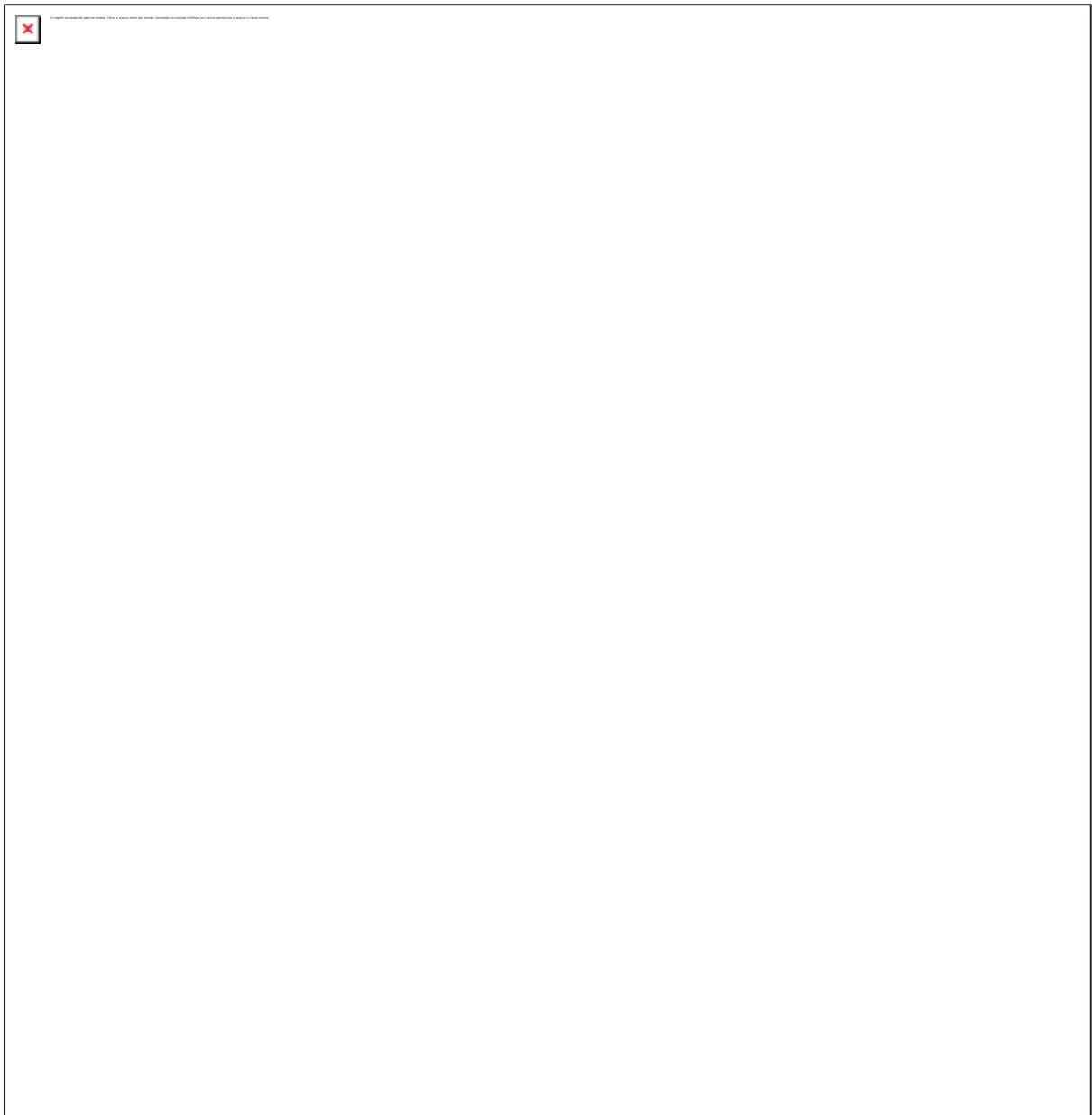


Figure 1. Ultrastructural and immunopathological characterization of the human skin organ culture model for pemphigus vulgaris.

(a. I) Electron micrograph showing desmosomes. One of the desmosomes is still intact (red oval circle), others are disrupted (red stars). The big gold particles detect DSG1. **(a. II)**

Picture showing indirect immunofluorescence microscopy staining for antibodies against DSG1. **(b. I)** Electron micrograph showing disrupted desmosomes. The gold particles (red arrows) mark DSG3 **(b. II)** Indirect immunofluorescence microscopy with intercellular staining by antibodies to DSG3. **(c. I)** Electron micrograph showing a stretched desmosome. Gold particles (red arrows) detect Px4-3 (anti-DSG1/3 scFv) within the desmosome. **(c. II)** Direct immunofluorescence microscopy against Px4-3. Left column: Electron micrographs (100,000-fold; scale bars 0.2 μm), right column: Micrographs (200-fold; scale bars 100 μm).

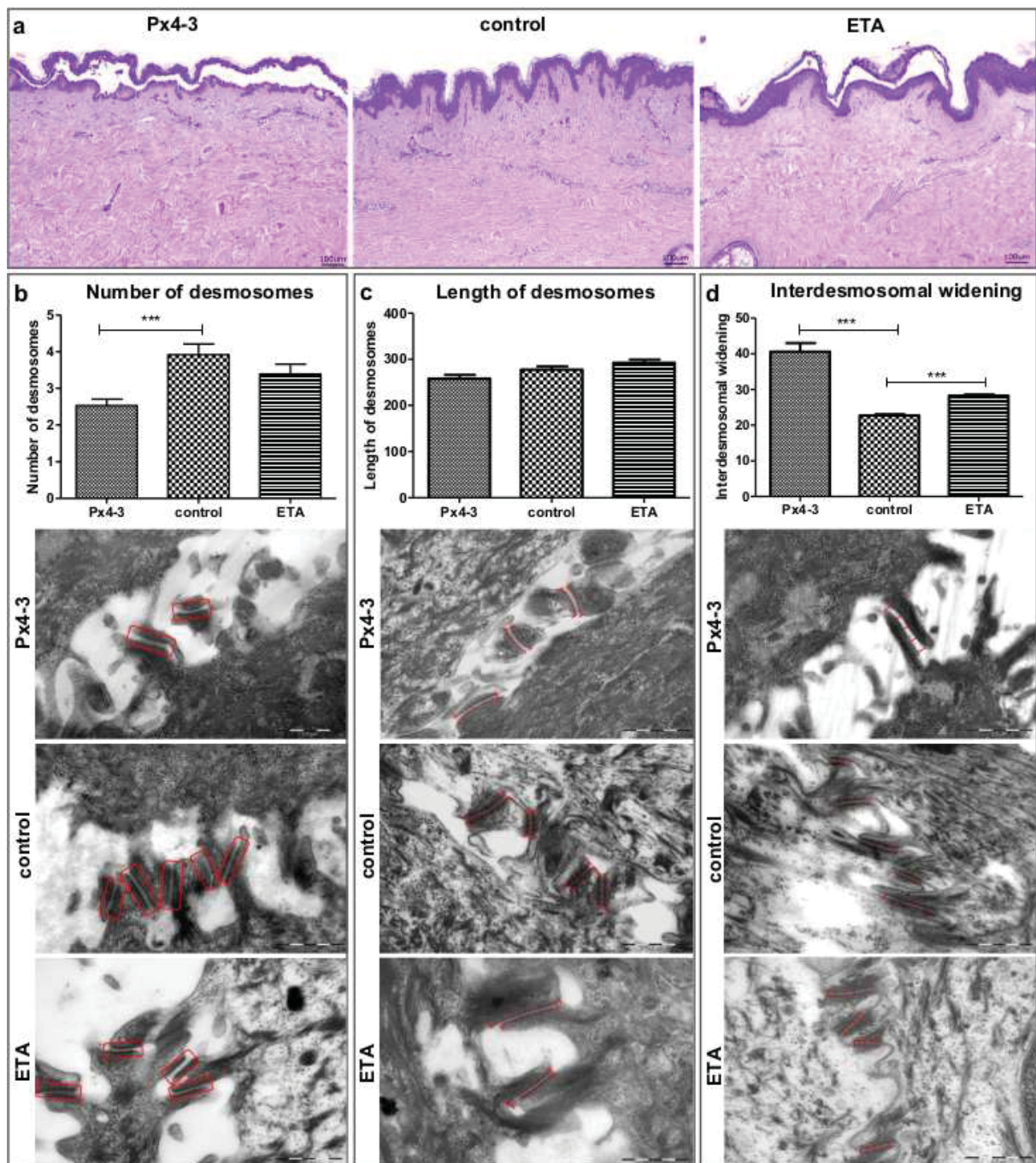


Figure 2. Desmosomal number, length and interdesmosomal widening in the human skin organ culture model for pemphigus.

(a) Light micrographs of hematoxylin & eosin stainings (100-fold, scale bar 0.5 μ m, N = 5) of human skin organ culture specimens after injection of Px4-3, normal human IgG (negative control) or ETA (positive control and reference). (b) Electron micrographs showing the number of desmosomes in the three different conditions. Px4-3 injected has a smaller number of desmosomes compared to the control. (c) Electron micrographs showing the length of

desmosomes. Px4-3 and ETA injected do not differ compared to the control. **(d)** Electron micrographs showing interdesmosomal widening. Px4-3 injected and ETA injected show larger interdesmosomal space than the control (80,000-fold, scale bar 0.5 μm , N = 5).

7.3 SUPPLEMENTARY MATERIALS

Tissue collection

Skin donors of elective surgery from the practice of Dr. Valina in Lübeck, of Dr. Schlichter in Bremen and from the surgery of the University Clinic Schleswig-Holstein, Campus Lübeck were informed about the opportunity to donate skin for experimental work approved by the ethics committee of the University of Lübeck (06-109). Five skin donors voluntarily agreed to participate in the study and gave their informed consent.

Human skin organ culture

From five different donors, for each experimental condition, we sampled two pieces of skin. We injected 50 μ L of anti-desmoglein (DSG) 1/3 single-chain variable fragment (scFv). The scFv is a monoclonal antibody directed against DSG1 and 3, termed Px4-3, able to induce acantholysis. As a reference and positive control, we injected 50 μ L of exfoliative toxin A (ETA), a toxin of the bacterium *Staphylococcus aureus*. As a negative control, we administrated 50 μ L of normal human IgG. Intravenous immunoglobulin G (IVIG) was used as the source of normal human IgG. The skin specimens were harvested after incubation at 37 °C and 5 % CO₂. We used the first skin sample for hematoxylin-eosin (HE) staining and three different immunofluorescence stainings directed against DSG1, DSG3 and Px4-3. We used the second skin sample from each experimental condition for four 2 mm biopsy punches. After punching, the biopsies were transferred directly into 5 % paraformaldehyde (PFA)/piperazine-N,N'-bis(2-ethanesulfonic acid)(PIPES) for fixation. They remained there for two to four hours (hrs). After lying in PFA/PIPES, we washed the punches in 0.14 M phosphate buffered saline (PBS). We stored two of the four punches in glutaraldehyde in cacodylate buffer for later araldit-embedding for standard transmission electron microscope (TEM)

pictures. We put the other two punches in polyvinylpyrrolidone (PVP)-saccharose for later immunogold-labeling.

Sample preparation for electron microscopy

The two skin punches that we stored in PVP-saccharose were used for the immunogold-labeling. The other two punches of each sample we put into glutaraldehyde in cacodylate buffer (instead of PVP-saccharose). After the storage in glutaraldehyde in cacodylate buffer, the skin punches were embedded in araldit.

Araldit-embedding for standard electron microscopy

For the araldit-embedding, the biopsies first had to be fixed in glutaraldehyde 2.5 % for a minimum of 2 h. We washed the skin three times, every 5 min, in cacodylate buffer (pH = 7.2 – 7.4; 0.1 M). After that, we performed osmium fixation in 1 – 2 % OsO₄ (in cacodylate buffer) for 2 h, followed by another three washing steps each for 10 min in cacodylate buffer. Dehydration is accomplished with an ethanol-series with increasing concentrations - twice 10 min starting with 30 up to 100 % ethanol, followed by incubation in 70 % ethanol with 1 % uranylacetate for a minimum of 1 h and protected from UV-light, concluding finally with 70 % ethanol overnight at 4 °C. After the dehydration, the punches were plunged twice into propylenoxid for 30 min and then transferred to propylenoxid-resin mixtures and a pure resin-mixture. After embedding in araldit, we stored the samples at room temperature (RT) until further usage.

We cut the samples with an ultramicrotome (Ultracut E, Reichert-Jung, Leica Mikrosysteme Vertrieb GmbH®, Wetzlar, Germany). After that, we included the sliced samples into Leica EM AC20 Automatic Contrasting Instrument (Leica Mikrosysteme Vertrieb GmbH®, Wetzlar, Germany). We took 10 to 13 pictures for each experimental model at the TEM

(JEOL JEM-1011, JEOL®, Tokyo, Japan). These pictures were used for our analyses of the desmosomes.

For this, we used the program iTEM (www.soft-imaging.net). First of all, we measured the area of the TEM pictures. We took all of the micrographs for the analyses of the desmosomes in 80,000 magnification. First, we counted the desmosomes in every micrograph. For each of these counted desmosomes, we measured desmosomal length and interdesmosomal widening. For determining the length of the desmosomes, we measured both desmosomal plaques of each desmosome, using the feature "polyline". After that, we calculated the average of the two values for each desmosome. We measured the distance between the desmosomal plaques by choosing five points. Finally, we calculated the average of the five values for each desmosomal distance.

Immunogold-labeling in electron microscopy

We included the punches from the PVP-saccharose into a medium for thawing onto pins (Leica Mikrosysteme Vertrieb GmbH®, Wetzlar, Germany). We cut the skin biopsies and put the pins underneath the stereo magnifier using a clamp. Using a loop, the slide got wetted homogeneously with a thawing medium. The skin biopsies were freed from PVP-saccharose with filter paper. After that, we placed them onto the pins. Finally, we stored the pins with the skin punches in liquid nitrogen until further usage. We cut them with the cryo-ultramicrotome (Leica EM FCS, Leica Mikrosysteme Vertrieb GmbH®, Wetzlar, Germany) to produce 60 to 80 nm thick sections. For cutting, we used three different temperatures within the ultra-cryo. The block temperature was set between -110 °C to -120 °C, the temperature of the diamond knife, between -100 °C to -115 °C and the temperature of the chamber, -130 °C. We gently put the sections onto grids (Formvar covered, mesh = 100).

The immunogold-labeling followed the cutting process. First, we incubated the sections for 15 mins with 10 % fetal calf serum (FCS) (1 drop to block the unspecific antigens), then 45 min with the primary antibody [for DSG1: mouse anti-human desmoglein-1 (Origene®, Rockville, United States of America, # AM26377PU-N), for DSG3: mouse anti-human desmoglein-3 (Origene®, Rockville, United States of America, # SM2037PS), for Px4-3: rat anti-HA mAb high affinity 0,1 µg/µL (Roche®, Basel, Switzerland); each of the antibodies was diluted in 5 % FCS]. After this, 15 min of washing with 0.14 M PBS (5 drops) followed. Then, 45 min incubation with the secondary antibody [6 nm Colloidal Gold-AffiniPure Goat Anti-Rat IgG (Jackson Immuno Research®, West Grove United States of America, # 112-195-167), diluted in 5 % FCS/ 12 nm Colloidal Gold-AffiniPure Goat Anti-Mouse IgG (Jackson Immuno Research®, West Grove United States of America, # 115-205-146), diluted in 5 % FCS]. After the incubation, 30 min of washing was done in 0.14 M PBS (6 drops) and 5 min of washing with ddH₂O (3 drops). Then, we put the sections onto ice with 100 µL uranylacetate (4 %) and we poured 900 µL of methylcellulose (2 %) on top. Sections were dried afterwards. After immunogold-labeling, we took the pictures with the routine transmission electron microscopy (JEOL JEM-1011, JEOL, Tokyo, Japan).

Hematoxylin and eosin staining

After embedding into paraffin, we cut 4 µm thick sections from each paraffin block. We deparaffinized the dried sections in a xylene bath and dehydrated them in an increasing ethanol concentration series. Afterward, we dipped the slides into distilled water, then into papanicoulos-hematoxylin for 5 min. After the hematoxylin, we rinsed the slides in water, put them into ammonia water, rinsed them in the water again and then dipped them into distilled water. The eosin bath followed and another increasing ethanol concentration series as well, as xylene baths. In the end, the coverslipping machine coverslipped the sections.

Immunofluorescences staining for desmogleins

We cut the skin into 6 μm sections. These were used for immunofluorescence stainings directed against DSG1 and 3. The sections first thawed for 10 min. After fixation in acetone for 20 min, we encircled each section using a fat pen (DAKO®, Hamburg, Germany, # S2002). Two washing steps in PBS followed, each for 5 min. Preincubation with 10 % goat normal serum (GNS) for 20 min followed. The primary antibody of each staining [for DSG1: (Origene®, Rockville, United States of America, # AM26377PU-N) mouse anti-human DSG1, for DSG3: (Origene®, Rockville, United States of America, # SM2037PS) staining the primary antibody mouse anti-human DSG 3) and 2 % GNS was added diluted in PBS (1:100).] We stored the humid chamber including the sections overnight in the fridge at 4 °C. Three washing steps in PBS followed, each for 5 min. Then, we performed the incubation with the secondary antibody [for DSG1: CyTM 3-conjugated AffiniPure goat anti-mouse IgG (Jackson Immuno Research®, West Grove United States of America, # 115-165-146), for DSG3: Goat Anti-Mouse IgG (H + L) Alexa Fluor 488 (Jackson Immuno Research®, West Grove United States of America, # A11029); dilution: 1:200 in PBS] for 45 min. We washed the sections three times in PBS, each for 5 min. We covered the slides with 4,6-diamidine-2-phenylindole (DAPI)-fluoromount-G and coverslips, dried for one hour and stored them at -20° C.

Immunofluorescence staining for Px4-3

We prepared washing and blocking buffers. For the washing buffer, we added 100 mL TBS, 500 μL 2 M CaCl_2 , 900 mL NanoPure ddH₂O and 500 μL of Tween 20. The blocking buffer consists 2.5 g of bovine albumin (BSA), which was filled into a falcon tube and filled up with washing buffer to a volume of 250 mL. We filtered the mixture before storing it at 4 °C. The sections were thawed for 3 to 5 min. We encircled each section using a fat pen. We washed the sections twice with washing buffer for 5 min, then blocked them with the blocking buffer

for 15 min and afterwards incubated them with the secondary antibody [Rat Anti- HA High-Affinity Antibody monoclonal (Roche®, Basel, Switzerland, # 11867423001), dilution 1:100 in blocking buffer] for 1 h. After three times washing, we added the tertiary antibody [Goat Alexa Fluor 594 Anti-Rat IgG (Life Technologies®, Carlsbad, United States of America, # A11007), dilution 1:200 in blocking buffer] for 1 h. Afterwards, we washed the sections three times in washing buffer, each for 5 min. We covered the slides with DAPI-fluoromount-G and coverslips, dried them for one hour and stored them at - 20 °C.

Microscopical analyses

While using the Keyence BZ 9000 (Keyence®, Osaka, Japan) the HE-stained slides were observed with 100 magnification. We took pictures along the whole epidermis.

Semi-quantitative histomorphometry

Every picture taken described a visual field of the skin. For every visual field, we measured the length of the epidermis using the program ImageJ (<https://fiji.sc>). We measured the whole epidermis length as well as the split formation within the visual fields. Two hundred µm of both skin edges were taken out of the evaluation to avoid cutting artifacts.

Supplementary Table 1. Number of desmosomes, length of desmosomes and interdesmosomal widening in five Px4-3-injected HSOcCs, five controls and five ETA-injected HSOcCs.

Picture number	Number of desm.	Desm. number	Length	Int. wid.	Picture number	Number of desm.	Desm. number	Length	Int. wid.	Picture number	Number of desm.	Desm. number	Length	Int. wid.
Px4-3 HSOcC 1					Control HSOcC 1					ETA HSOcC 1				
1	2	1 2	338.89 328.74	22.83 26.06	1	9	1 2 3 4 5 6 7 8 9	307.28 259.55 146.98 256.44 247.76 279.58 317.99 352.24 237.93	14.76 15.24 22.05 17 16.75 24.36 21.76 24.94 25.84	1	2	1 2	203.03 290.13	25.99 24.12
2	4	1 2 3 4	188.6 164.96 292.36 281.18	30.26 24.77 30.09 33.02	2	6	1 2 3 4 5 6	252.2 328.41 152.14 231.48 296.65 214.12	25.09 21.53 24.64 13.9 23.24 29.95	2	3	1 2 3	327.45 470 275.28	28.85 26.37 23.5
3	5	1 2 3 4 5	313.98 104.66 210.98 396.1 223.49	26.13 31.7 18.82 116.4 31.32	3	2	1 2	385.67 355.52	19.3 24.21	3	3	1 2 3	228.38 237.11 333.23	23.57 21.26 25.42
4	3	1 2 3	176.42 103.7 174.04	31.08 27.27 50.41	4	3	1 2 3	309.43 111.52 441.45	21.55 23.68 23.76	4	3	1 2 3	283.79 263.86 498.62	24.08 23.56 25.09
5	2	1 2	346.47 119.63	23.11 22.78	5	3	1 2 3	328.72 213.6 378.23	22.46 24.74 22.3	5	6	1 2 3 4 5 6	205.29 224.62 148.88 195.98 217.2 120.96	24.41 25.86 21.26 29.74 26.39 31.36
6	3	1 2 3	68 113.38 212.66	26.75 27.53 27.96	6	2	1 2	593.44 378.04	23.22 22.03	6	4	1 2 3 4	431.46 309.87 236.2 293.83	25.45 25.3 22.87 24.33
7	1	1	218.14	35.19	7	2	1 2	196.25 320.91	23.03 22.03	7	2	1 2	254.28 152.19	29.47 26.7
8	1	1	206.79	29.34	8	8	1 2 3 4 5	331.52 362.73 255.43 173.91 251.58	24.14 18.43 22.93 20.54 23.2	8	3	1 2 3	263.49 266.02 200.08	22.41 23.73 17.45

[illegible]

6	2	1	378.83	38.67	6	1	1	312.86	13.38	6	1	1	640.54	34.56
7	1	1	217.28	29.86	7	10	1	181.95	20.63	7	3	1	459.82	35.64
			372.7	30.92			2	279.6	22.36			2	481.09	21.16
			.	.			3	336.1	20.79			3	293.1	22.17
			.	.			4	301.53	23.32				.	.
			.	.			5	275.9	19.75				.	.
			.	.			6	279.46	20.28				.	.
			.	.			7	266.74	19.8				.	.
			.	.			8	252.67	19.44				.	.
			.	.			9	271.25	24.44				.	.
			.	.			10	266.74	20.33				.	.
8	3	1	167.53	24.6	8	4	1	252.67	27.57	8	2	1	232.75	32.67
		2	331.1	31.27			2	271.25	17.04			2	230.74	28.53
		3	160.22	28.43			3	125.39	17.31				.	.
			.	.			4	223.38	22.28				.	.
9	3	1	235.44	35.85	9	2	1	138.1	21.86	9	5	1	275.9	26.83
		2	223.23	42.84			2	304.91	21.94			2	266.18	23.39
		3	107.38	28.07			.	.	.			3	215.54	21.9
			.	.			.	.	.			4	134.91	21.43
			.	.			.	.	.			5	199.14	25.32
10	3	1	165.44	29.37	10	2	1	160.26	21.75	10	2	1	371.99	30.63
		2	266.66	29			2	356.96	18.33			2	235.07	28.49
		3	176.52	32.99			.	.	.				.	.
11	1	1	188.15	30.39	11	2	1	117.32	21.16	11	4	1	309.38	26.1
			.	.			2	222.76	24.78			2	85.43	18.66
			.	.			.	.	.			3	354.38	23.99
			.	.			.	.	.			4	204.54	20.17
			.	.			5	209.5	30.64				.	.
12		5			12		1	296.13	25.83	12	3	1	220.57	25.21
							2	240.62	32.86			2	306.09	25.72
							3	264.39	23.5			3	149.54	33.76
							4	275.5	25.57				.	.
							5	209.5	30.64				.	.
13		3			13		1	249.49	22.76	13	3	1	179.61	31.26
							2	428.62	20.75			2	425.15	27.53
							3	295.36	20.65			3	178.44	33.58
										14	1	1	298.94	25.3
Px4-3					Control					ETA				
HSOC 3					HSOC 3					HSOC 3				
1	3	1	153.76	43.4	1	5	1	123.54	32.39	1	1	1	228.31	31.04
		2	165.91	25.81			2	320.87	28.07				.	.
		3	233.96	240.9			3	276.3	25.59				.	.
			.	.			4	280.73	17.06				.	.
			.	.			5	188	20.47				.	.
2	1	1	261.95	30.5	2	1	1	333.79	24.98	2	5	1	247.05	28.75
			.	.			.	.	.			2	524.96	26.65
			.	.			.	.	.			3	280.07	31.81
			.	.			.	.	.			4	303.47	34.1

3	1	1	1	309.6	61.85	.	3	2	1	1	325.55	26.3	.	3	2	1	5	460.07	25.3
				.	.	.			2	2	383.8	16.36	.					313.46	26.76
4	1	1	1	358.39	36.54	.	4	3	1	1	184.09	22.77	.	4	3	1		277.45	24.27
				.	.	.			2	2	291.29	22.82	.			2		231.48	22.25
5	3	1	1	283.09	36.7	.	5	2	1	3	239.88	20.81	.	5	2	1	3	284.52	38.49
		2	2	511.78	31.4	.			1	1	164.9	23.37	.			1		286.8	31.31
		3	3	266.63	35.72	.			2	2	259.2	26.44	.			2		331.74	31.15
6	1	1	1	273.9	23.8	.	6	6	1	1	421.48	29.33	.	6	6	1		264.63	31.68
				.	.	.			2	2	403.33	18.32	.			2		178	23.67
				.	.	.			3	3	173.89	14.48	.			3		280.73	41.28
				.	.	.			4	4	296.77	18.62	.			4		222.73	29.82
				.	.	.			5	5	280.68	18.41	.			5		240.63	28.16
				.	.	.			6	6	244.53	18.13	.			6		218.29	23.95
7	2	1	1	362.54	37.54	.	7	7	1	1	238.95	25.64	.	7	4	1		224.59	26.6
		2	2	197.65	31.43	.			2	2	200.91	20.54	.			2		212.77	18.1
				.	.	.			3	3	179.36	17.25	.			3		268.23	22.05
				.	.	.			4	4	229.29	17.65	.			4		236.44	34.55
				.	.	.			5	5	254	20.18	.					.	.
				.	.	.			6	6	140.1	23.24	.					.	.
				.	.	.			7	7	268.72	10.44	.					.	.
8	2	1	1	397.98	30.48	.	8	8	1	1	274.75	16.03	.	8	4	1		211.44	20.66
		2	2	276.47	26.31	.			2	2	150.84	14.89	.			2		250.91	30.17
				.	.	.			3	3	222.19	12.45	.			3		364.75	18.74
				.	.	.			4	4	181.7	27.66	.			4		338.42	37.18
				.	.	.			5	5	255.31	16.57	.					.	.
				.	.	.			6	6	206.57	17.42	.					.	.
				.	.	.			7	7	340.05	20.15	.					.	.
				.	.	.			8	8	230.77	19.08	.					.	.
9	1	1	1	309.5	31.81	.	9	5	1	1	170.6	20.18	.	9	2	1		354.8	31.13
				.	.	.			2	2	287.65	20.06	.			2		404.69	26.82
				.	.	.			3	3	333.66	28.42	.					.	.
				.	.	.			4	4	247.11	21.45	.					.	.
				.	.	.			5	5	560.01	21.25	.					.	.
10	2	1	1	226.4	26.13	.	10	3	1	1	249.23	23.82	.	10	3	1		226.45	23.05
		2	2	431.94	26.27	.			2	2	308.64	22.44	.			2		530.34	26.6
				.	.	.			3	3	229.63	17.23	.			3		336.54	33.88
11	3	1	1	245.01	28.68	.	11	4	1	1	253.46	20.99	.	11	11	1		357.26	34.62
		2	2	198.95	23.4	.			2	2	278.1	17.61	.			2		331.83	27.18
		3	3	233.52	85.38	.			3	3	318.93	22.26	.			3		311.11	30.59
				.	.	.			4	4	470.84	23.48	.			4		278.91	28.25
				.	.	.					.	.	.			5		220.13	25.36
				.	.	.					.	.	.			6		226.93	28.63
				.	.	.					.	.	.			7		295.09	21.88
				.	.	.					.	.	.			8		325.43	28.48
				.	.	.					.	.	.			9		350.02	22.1
				.	.	.					.	.	.			10		262.25	28.55

12	2	1	470.51	135	.	12	4	1	276.01	18.03	.	11	3	1	323.97	220.28	25.26
	2	2	235.11	33.47	.		2	2	442.61	22.16	.		2	2	580.74	23.77	28.94
13	6	1	516.26	25.05	.	12	4	3	490.19	24.45	.	13	5	3	350.94	24.38	.
		2	227.86	24.8	.		4	4	442.78	23.26	.						.
14	8	1	187.31	37.58	.	12	4	1	276.01	18.03	.	13	5	1	194.02	25.88	.
		2	139.85	31.05	.		2	2	442.61	22.16	.		2	2	290.77	21.6	.
15	1	1	296.87	26.93	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		2	258.92	24.46	.		4	4	442.78	23.26	.		4	4	281.95	28.79	.
Px4-3 HSOC 4	2	1	179.12	82.91	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	266	23.85	.			6	259.16	29.94	.						.
1	2	1	229.62	34.74	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	138.41	35.74	.			2	442.61	22.16	.		2	2	290.77	21.6	.
2	3	1	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		2	374.88	30.68	.			4	442.78	23.26	.		4	4	281.95	28.79	.
3	3	3	416.95	33.91	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		3	.	.	.			6	259.16	29.94	.						.
3	3	1	239.46	35.06	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	260.32	31.97	.			2	442.61	22.16	.		2	2	290.77	21.6	.
4	3	3	360.28	133.4	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	.	.	.			4	442.78	23.26	.		4	4	281.95	28.79	.
5	3	1	179.12	82.91	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	266	23.85	.			6	259.16	29.94	.						.
6	3	1	229.62	34.74	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	138.41	35.74	.			2	442.61	22.16	.		2	2	290.77	21.6	.
7	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
8	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
9	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
10	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
11	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
12	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
13	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
14	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
15	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
16	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
17	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
18	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
19	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
20	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
21	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
22	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
23	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
24	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
25	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
26	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
27	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
28	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
29	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
30	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
31	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
32	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
33	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
34	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
35	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
36	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
37	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
38	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
39	3	1	179.12	82.91	.	12	4	1	187.31	37.58	.	13	5	1	194.02	25.88	.
		2	266	23.85	.			2	442.61	22.16	.		2	2	290.77	21.6	.
40	3	3	485.03	28.15	.	12	4	3	490.19	24.45	.	13	5	3	232.07	28.03	.
		3	416.95	33.91	.			4	442.78	23.26	.		4	4	281.95	28.79	.
41	3	1	239.46	35.06	.	12	4	5	131.17	32.88	.	13	5	5	197.26	25.88	.
		2	260.32	31.97	.			6	259.16	29.94	.						.
42	3	1	179.12	82.91	.												

4	2	5	154.67	36.7	4	2	1	255.95	22.95	4	2	1	379.77	29.05
		1	206.62	34.04			2	220.48	27.71			2	254.4	34.52
5	2	2	271.91	34.36	5	2	1	630.57	32.77	5	9	1	263.15	34.55
		2	249.79	36.75			2	227.72	30.87			2	237.99	37.81
			.	.				.	.			3	370.68	36.82
			.	.				.	.			4	229.16	33.39
			.	.				.	.			5	367.05	31.64
			.	.				.	.			6	203.93	36.89
			.	.				.	.			7	530.21	33.82
			.	.				.	.			8	245.68	35
			.	.				.	.			9	292.01	37.85
6	1	1	440.75	54.69	6	2	1	221.38	26.84	6	8	1	230.96	32.79
			.	.			2	316.11	30.29			2	316.67	32.68
			.	.				.	.			3	263.97	26.62
			.	.				.	.			4	317.52	33.13
			.	.				.	.			5	212.13	26.98
			.	.				.	.			6	220.26	28.2
			.	.				.	.			7	195.54	36.99
			.	.				.	.			8	162.73	35.49
7	1	1	241.07	35.72	7	2	1	228.26	26.53	7	2	1	457.58	35.44
			.	.			2	368.16	28.89			2	214.21	36.5
8	1	1	306.78	42.99	8	2	1	714.39	29.85	8	3	1	291.08	29.53
			.	.			2	198.61	25.64			2	362.73	26.89
			.	.				.	.			3	235.19	29.14
9	3	1	150.37	35.1	9	2	1	423.29	20.1	9	10	1	366.47	29.01
		2	172.15	32.28			2	305.92	16.68			2	327.84	32.58
		3	265.92	45.3				.	.			3	189.17	27.72
			.	.				.	.			4	192.59	27.08
			.	.				.	.			5	353.24	32.02
			.	.				.	.			6	269.45	28.7
			.	.				.	.			7	382.32	29.5
			.	.				.	.			8	246.24	29.04
			.	.				.	.			9	253.21	30.71
			.	.				.	.			10	280.75	30.79
10	2	1	202.79	37.01	10	5	1	349.74	25.66	10	1	1	492.52	21.31
		2	245.18	33.81			2	245.14	22.82				.	.
			.	.			3	276.42	22.41				.	.
			.	.			4	353.83	21				.	.
			.	.			5	233.81	20.61				.	.
11	1	1	318.44	40.84	11	4	1	195.2	24.73	11	3	1	296.88	39.03
			.	.			2	197.68	21.85			2	247.76	29.11
			.	.			3	216.1	22.13			3	259.73	30.05
			.	.			4	285.36	23.85				.	.
12	2	1	290.59	30.24	12	5	1	414.2	23.19	12	5	1	253.63	20.69
		2	200.93	47.4			2	495.76	18.21			2	221.31	34.39
			.	.			3	150.98	22.57			3	187.11	27.46
			.	.			4	374.97	22.21			4	225.93	26.75

13	4	1	289.62	.	.	13	3	5	294.91	18.97	13	9	5	171.19	28.06
		2	194.4	45.48	.										
		3	137.16	51.28	.										
		4	141.85	53.05	.										
				43.9	.										
			.	.	.										
			.	.	.										
			.	.	.										
			.	.	.										
			.	.	.										
14	1	1	251.77	39.78	.	14	5	1	117.09	5.098	14	6	1	145.19	22.34
			.	.	.										
			.	.	.										
			.	.	.										
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			.	.	.										

Desm. Desmosome; Int. Wid. Interdesmosomal widening.

8 CAPÍTULO 5 – EXPRESSÃO DE C5A/C5AR1 DO COMPLEMENTO NO PÊNFIGO

PESQUISA ORIGINAL – *C5a/C5aR1 Complement Expression in Pemphigus*.

Manuscrito que será submetido no periódico *Immunology* -
<https://onlinelibrary.wiley.com/journal/13652567>.

ORIGINAL ARTICLE

C5a/C5aR1 COMPLEMENT EXPRESSION IN PEMPHIGUS**Short title:** C5a/C5aR1 in pemphigus

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8.1 ABSTRACT

The binding of complement 5a (C5a) to C5a receptors (C5aR) is a key factor in autoimmune blistering diseases such as epidermolysis bullosa acquisita and bullous pemphigoid. In pemphigus, though, the role of this event is poorly known. Pemphigus foliaceus (PF) presents autoantibodies mainly against desmoglein 1 (DSG1), while pemphigus vulgaris (PV), against DSG3. Both are rare, sporadic diseases; PF, however, may also have endemic forms (EPF). In this work, we confirmed that the EPF-associated *C5AR1 rs10404456*C* is also associated with sporadic PF (SPF OR=2.41; [95% CI=1.45-3.99]; $p<0.001$), but not with PV. This allele is one of 18 variants within a haplotype associated with lower *C5AR1* expression in sun-exposed skin. Keratinocytes are among the cells with lowest basal *C5AR1* expression, whose up-regulation favors proliferation of epithelial cells. Even so, C5aR1 detection was conspicuous in keratinocytes of the investigated biopsies and human skin organ cultures (HSOC). We propose that higher *C5AR1* keratinocyte expression is needed to counteract PF blister formation. In contrast, high *C5AR1* (but not C5) mRNA expression and C5aR1 (but not C5a)-positive cell counts occurred in the dermis of Px4-3-treated PV-mimicking HSOC, the last being lower in HSOCs treated with PF-mimicking exfoliative toxin A ($P<0.001$). Higher numbers of C5aR1 protein expressing cells also occurred in epidermis of EPF ($P<0.0001$), as well as in dermis of SPF ($P<0.001$) and EPF ($P<0.01$). This expression is probably given by Langerhans cells and fibroblasts in the Px4-3-treated HSOCs and by infiltrating neutrophils attracted by higher C5a serum levels in PF and PV ($P<0.001$). However, serum levels of neutrophil extracellular traps, induced by C5a and examined in EPF and PV patients, did not differ. These last results highlight the proinflammatory role of the C5aR1-C5a axis and, ultimately, of the complement cascade in PF pathogenesis, regardless of its sporadic or endemic etiology, and possibly also in PV. They also lead us to suggest a shared role of the complement system in different pemphigus forms and a beneficial effect from C5a therapeutic inhibition.

Keywords: Pemphigus foliaceus, Pemphigus vulgaris, Desmoglein, Inflammation, C5a, C5aR1, organ culture, neutrophil extracellular traps, autoimmune disease

Abbreviations:

AP- alternative pathway; **C5a**- complement component 5a; **C5aR1**- complement C5a receptor 1 ; **C5aR2**- complement C5a receptor 2; **CP**- classical pathway; **CS**- complement system; **EBA** - epidermolysis bullosa acquisita; **EPF** - endemic pemphigus foliaceus; **ETA** - exfoliative toxin A; **HSOC** - human skin organ culture; **IVIG** - intravenous immunoglobulin; **LP**- lectin pathway; **MV**- microvesicles; **NHEK** - Normal Human Epidermal Keratinocytes; **NETs** - neutrophil extracellular traps; **PV** - pemphigus vulgaris; **Px4-3** - single-chain variable fragment (scFv) of an antibody directed against DSG1 and DSG3; **scFv** - single chain variable fragment; **RT** - room temperature; **SNP** - single nucleotide polymorphism; **SPF**- sporadic pemphigus foliaceus;

8.2 INTRODUCTION

Dysregulated activation of the complement system (CS) generates an excess of anaphylatoxins like C5a, whose recognition by C5a receptors (C5aR) attracts inflammatory cells that further enhance tissue injury. The involvement of the C5a/C5aR axis has already been observed in autoimmune diseases, such as psoriasis (1,2), anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (3), rheumatoid arthritis (4–6), and pemphigoid diseases (6–10). In bullous pemphigoid, the C5a/C5aR1 axis causes blisters through activation of the p38 mitogen-activated protein kinase (MAPK) pathway, and subsequent mast cell degranulation (7). In the incipient epidermolysis bullosa acquisita (EBA) mouse model, treatment with anti-C5 significantly ameliorates the disease (8). Despite its pathogenic importance in these two blistering autoimmune diseases, C5a/C5aR role in pemphigus is still unclear.

Pemphigus incidence varies considerably in the worldwide population (11), being higher for pemphigus vulgaris (PV) than for pemphigus foliaceus (PF) (reviewed by 12). However, although PF occurs sporadically (SPF) around the world, it is uniquely endemic (EPF) in some South American countries and in Tunisia, contrasting with any other autoimmune disease (13–16). Loss of keratinocyte adhesion (acantholysis) in PF and PV occurs in the granular layer of the skin and in the suprabasal layer of the epidermis (affecting skin and mucosa), respectively (17,18). The hallmark autoantibodies are directed against desmoglein (DSG) adhesion molecules: DSG1 for PF; DSG3 or DSG1 and DSG3 for PV (17). However, pemphigus autoantibodies also target other adhesion molecules, mitochondrial antigens, and cell membrane receptors; recently, anti-C5aR1 stood out as part of a specific PV DSG3-independent biomarker combination (reviewed by 10,19). Furthermore, C5a levels were found to be higher in PV patients, correlating with gut microbiota composition (20). Pemphigus diagnosis may rely on the detection of anti-DSG1 and/or anti-DSG3 in serum, and/or of complement component C3 in the keratinocytes of perilesional biopsies (11).

C3 cleavage is a previous central step of the CS cascade by generating the C5 convertase, which cleaves C5 and generates C5a, as well as the membrane attack complex (C5b-9 or MAC). C5a is a potent anaphylatoxin that exerts a predominant pro-inflammatory activity through interactions with the classical G protein-coupled receptor C5aR1 (CD88) and with the non-G protein-coupled receptor C5aR2 (GPR77), both expressed in various immune and non-immune cells (21). It also

recruits neutrophils that may release extracellular traps (NETs), featuring NETotic cell death (22,23). NETs are involved in host tissue injury and inflammation and were already associated with autoimmune diseases, acute injuries, atherosclerosis, vasculitis, Alzheimer's disease, and cancer (24,25), but have not so far been investigated in pemphigus.

Despite previous results on C5-deficient and complement-depleted mice showing that CS activation seems not to be required for pemphigus lesion formation (26), there are several issues that forbid a perfect transposition of these results to humans (for a discussion, see Bumiller-Bini et al. 2018). PF patients present increased serum levels of C3 and C-reactive protein (opsonins), Ba from the alternative and C4d from the classical/lectin pathways of the cascade (27–30). Mannose-binding lectin and ficolin 2 (pattern-recognition receptors that initiate the lectin pathway), besides C5b-9, were detected in PV biopsies (31). Furthermore, genetic variants of several complement components, including *C5AR1*, have been associated with EPF. We actually found an association of *rs10404456**C located in the *C5AR1* gene with susceptibility to EPF (32). Here, we aimed to evaluate this variant in a SPF group and the C5aR1 and C5a expression status in a human skin organ culture (HSOC) model mimicking pemphigus as well as in pemphigus skin biopsies and serum. We found *rs10404456**C also associated with SPF, in addition to: (i) high *C5AR1* mRNA and protein expression in a Px4-3-treated HSOC, PV-mimicking model; (ii) high C5a levels in EPF and PV serum; and (iii) high counts of C5aR1-expressing cells in the epidermis of EPF and in the dermis of EPF and SPF biopsies. Altogether, these results further advance our knowledge on the possible role of these molecules in pemphigus pathophysiology.

8.3 MATERIALS AND METHODS

8.3.1 Ethics Committee

This study was performed according to the Declaration of Helsinki, approved by the Brazilian National Ethics Committee (CONEP) under the protocol number CAAE 02727412.4.0000.0096 (approval number 2.045.739) and by the Ethics Committee of the University of Lübeck (Biobank PopGen, number 2017-018; 06-109, 08-156 and 12-178).

8.3.2 Samples

8.3.2.1 Case-control study association

The blood samples from SPF and PV patients were collected by the German Autoimmune Bullous Diseases Genetic Study Group in German hospitals. SPF comprised 76 PF patients (46% female) and 150 controls (50% female), with predominantly German ancestry. The mean age was 60.1 years old for patients (minimum 25, maximum 88) and 60.3 years old for controls (minimum 21, maximum 77). The DNA was isolated from whole blood samples using the QIAamp DNA Maxi Blood Kit (Qiagen, Hilden, Germany). The PV group is composed of 241 PV patients and 1,188 controls, being all subjects of German origin. All subjects were unrelated and with no clinical history of other autoimmune diseases. DNA was isolated from total blood samples of patients with the use of Smart Blood DNA midi prep (Analytik Jena, Germany). In agreement with the guideline of the German Society of Dermatology, patients were diagnosed based on their clinical phenotype and a positive direct immunofluorescence microscopy of a perilesional skin biopsy and/or detection of serum autoantibodies against DSG1 (for PF) and DSG3 (for PV) as detected by ELISA (Euroimmun) (33).

8.3.2.2 Human skin organ culture

Eight skin donors obtained from plastic surgeries from Bremen (Aestheticum Praxisklinik) and Lübeck (Praxis und Klinik für Plastische Chirurgie), Germany, voluntarily agreed to participate in the study and donated skin for experimental work. Human breast or belly skin of eight female patients were used for HSOC.

8.3.2.3 C5a and NET detection in serum

Serum from 23 PV and 95 EPF patients were selected for NET detection, and among these, 18 PV and 35 EPF patients were used for C5a level detection. A total of 97 unrelated controls with no diagnosis or familial history of autoimmune illnesses were analyzed for NET detection, and 35 of them for C5a level detection. Patients received clinical and/or immunohistological diagnosis. Patients and controls were recruited from 1984 to 2015 in South/ Southeastern/ CentralWestern Brazilian hospitals:

Hospital Adventista do Pênfigo (Campo Grande, Mato Grosso do Sul), Lar da Caridade - Hospital do Fogo Selvagem (Uberaba, Minas Gerais), Hospital das Clínicas - University of São Paulo (Ribeirão Preto, São Paulo), Hospital de Clínicas - Federal University of Paraná, Hospital de Dermatologia Sanitária São Roque (Piraquara, Paraná), and Hospital Santa Casa de Misericórdia (Curitiba, Paraná). All individuals described here were unrelated, predominantly of European ancestry and living in rural endemic areas. No history of other autoimmune diseases was reported for patients, as well as no history of any autoimmune disease, for controls. The description of the groups is available in **Supp. Table 1**.

8.3.2.4 Pemphigus biopsies

Three biopsies from SPF were obtained by the German Autoimmune Bullous Diseases Genetic Study Group in German hospitals, two from women born in 1948 and in 1980 and one from a man born in 1937. Eight biopsies from different EPF patients were collected at the Hospital das Clínicas - University of São Paulo (Ribeirão Preto, São Paulo) during the years of 2007-2008 and 2014-2015. Patients were diagnosed based on their clinical phenotype and a positive direct immunofluorescence microscopy of a perilesional skin biopsy against DSG1 (33). Besides, nine “control” biopsies were obtained from donors of elective surgery.

8.3.3 Association study in SPF

We genotyped the *rs10404456 C>T* SNP in 76 SPF patients and 150 controls with the iPLEX platform of the MassARRAY system (Agena Bioscience, San Diego, USA). The primer and extension sequences are listed in **Supp. Table 2**. All the methods applied in this study are summarized in **Figure 1**.

8.3.4 Genotyping, imputation and PV genome-wide association study

After all quality control (QC) steps, described in **Supplementary materials**, 486,084 variants for 241 cases and 1,188 controls (680 female and 761 male) remained. For imputation, the Sanger imputation server at <https://imputation.sanger.ac.uk/> (34) was used with 1000 Genomes Phase 3 as a reference panel for EAGLE 2/Impute. As

post-imputation QC, variants with INFO score less than 0.8, multiallelic sites and indels were removed. This resulted in 5,381,437 variants for which association was tested using SNPTTEST version 2 (35) using sex and principal components 1 to 5 as covariates.

Within the GWAS, 5,073 SNPs have been assessed within a 1 Mb window from rs10404456, with 78 having a p-value under 0.01 (see **Suppl. Table 3**; lowest p-value is 0.000144021). Proxy variants for rs10404456 were computed using LDLINK (<https://ldlink.nci.nih.gov>) (36) with European 1000 Genomes populations; 837 proxy variants were reported (down to a R^2 linkage disequilibrium of 0.01).

8.3.5 Human skin organ culture (HSOC)

We performed the HSOC following established protocols (37). Briefly, the eight skins obtained from plastic surgeries were stored in William's E medium (Bio & Sell GmbH; Feucht, Germany) at 4°C. Within 24 hours after surgery, each skin was prepared for culturing, fat trimming, and cutting into 1 cm x 1 cm pieces, preserving all layers of the skin. The skin was cultured in 1 mL of William's E medium per well in six-well TC plates (Sarstedt AG & Co.; Nümbrecht, Germany) in TC inserts (Sarstedt AG & Co.; Nümbrecht, Germany) with a permeable membrane, with 4 µm pores, on the bottom in order to only supply the lowest layer of the skin with nutrients. Insulin syringes (Becton Dickinson GmbH; Heidelberg, Germany) were used to inject 50 µL solutions of (i) single-chain variable fragment (scFv) of an antibody directed against DSG1 and DSG3 (Px4-3; 60 ng), (ii) intravenous immunoglobulin (IVIG; 7.5 µg; Biotest AG; Dreieich, Germany), or (iii) exfoliative toxin A (ETA; 100 ng) between the epidermis and dermis of two skin pieces per well plate. Each experimental condition was evaluated in skin biopsies from eight different donors, excepting ETA, for which seven skins were used. Px4-3 was used as a model mimicking the PV phenotype, IVIG was used as a negative control, and ETA was used as a positive control and mimicking PF phenotype. Incubation followed at 37°C and 5% CO₂ for 24 hours. Next, one skin piece per condition was frozen in RNase-free tubes in liquid nitrogen and stored at -80°C. One-half of the second pieces was put into Tissue Tek Intermediate (15x15x5 mm) cryomolds (Sakura Finetek Europe B.V.; Alphen aan den Rijn, The Netherlands) with the cutting side facing downwards, covered with TissueTek optimum cutting temperature (O.C.T.) Compound (Thermo Fisher

Scientific; Waltham, USA) and then frozen at -20°C. Long-term storage took place at -80°C. The other half was put into paraffin molds (MEDITE Medical GmbH; Burgdorf, Germany) and stored in 4% Roti-Histofix (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) at room temperature.

8.3.6 Expression levels of *C5* and *C5AR1* mRNA in HSOC

RNA was extracted from frozen skin samples using mirVana™ miRNA Isolation Kit (Ambion, Life Technologies Corporation; Carlsbad, CA, USA), according to the manufacturer's instructions for large RNA fraction enrichment. After removal of contaminant DNA with TURBO DNA-free™ Kit (Invitrogen AG; Carlsbad, CA, USA), RNA samples were submitted to reverse transcription, using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Waltham, MA, USA), according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (RT-qPCR) was used to measure the expression levels of *C5AR1* and *C5* genes, which were normalized to the expression of *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene. All qPCR reactions were performed in triplicates using TaqMan® Fast Advanced Master Mix (Applied Biosystems; Waltham, MA, USA) and specific probes (*C5AR1* - Hs00383718_m1; *C5* - Hs01004342_m1; *GAPDH* - Hs99999905_m1; Thermo Fisher, Waltham, MA, USA) using 20 ng of cDNA, according to the manufacturer's protocol. All qPCR amplifications were performed using StepOne Real-Time PCR Systems (Applied Biosystems, Waltham, MA, USA).

8.3.7 Staining

We performed: Haematoxylin and eosin staining; Desmoglein 1 and 3 staining with anti-desmoglein 1 and 3 single chain variable fragments; human IgG, C5a and C5aR1 immunofluorescence. The methods are detailed in the **Supplementary materials**.

8.3.8 Investigation of the C5a levels in serum

To analyze C5a serum levels of patients with EPF (N = 35), PV (N = 18), and controls (N = 35), we performed an enzyme-linked immunosorbent assay (ELISA) using the MicroVue C5a EIA kit (Quidel Corporation, cat. A025), following the protocol recommended by the manufacturer.

8.3.9 Neutrophil extracellular traps

We investigated whether NETs were present in serum of EPF (N = 91), PV (N = 26), and controls (N = 97). For this, we used an adapted ELISA test with immunofluorescence (38,39). For more details about the protocol, consult (25).

8.3.10 Statistics

After testing for Hardy-Weinberg equilibrium using PLINK software version 1.1.9 (40), the distribution of rs10404456 genotypes was analyzed by binary logistic regression according to the additive, dominant and recessive models, using the PLINK software version 1.1.9 (40). The limit of significance adopted for all analysis was $p < 0.05$. The Genehopper-Qtlizer tool was further used to identify SNPs in linkage disequilibrium (LD) with rs10404456 (with a correlation of $r^2 > 0.8$ between alleles), which were also simultaneously associated as eQTLs (expression quantitative trait loci) with *C5AR1* gene expression in sun-exposed skin (41,42). Global frequency of the resulting eQTL haplotype was determined with the LDHap tool, using data from the 1000 Genomes Project (43). Regulatory proteins identified by chromatin immunoprecipitation, methylation states of 5'CpG3' dinucleotides by Methyl 450K Bead Arrays from ENCODE/HAIB and chromatin segmentation states revealed by epigenomic data modeled with a multivariate hidden Markov chain were found using the GRCh37 human genome version in the UCSC Genome Browser and searching for sequences occupied by the haplotype eQTLs in epithelial cell lines (as A549) and normal human epidermal keratinocytes (NHEK) (44,45). Protein Atlas was used to localize the protein expression in the tissues (46). To check the normality distribution of the data, D'Agostino & Pearson omnibus normality test and Shapiro-Wilk normality test were used.

Split measurement was performed on pictures taken of the HE staining, the details are in **Supplementary materials**. Kruskal-Wallis tests with Dunnett's post test were

8.4 RESULTS

8.4.1 The *C5AR1* rs10404456*C allele is also associated with susceptibility to sporadic PF

The distribution of the rs10404456 genotypes were in agreement with those expected under Hardy-Weinberg equilibrium. The *rs10404456**C allele of *C5AR1* gene, previously associated with susceptibility to endemic PF (32), was also associated with the sporadic form of PF ($p < 0.001$) (**Table 1**).

Table 1. Association of *rs10404456**C from the *C5AR1* gene with endemic and sporadic pemphigus foliaceus.

	MAF (%)		MODEL	CONTR	PAT	OR	95% CI	<i>p</i>
ENDEMIC PEMPHIGUS FOLIACEUS	Contr	Pat	add	123/263	183/267	1.43	[1.07-1.90]	0.0155
	N = 193	N = 225	rec	20/173	38/187	1.67	[0.93-3.00]	0.0836
	31.87	40.67	dom	103/90	145/80	1.65	[1.04-2.30]	0.0299
SPORADIC PEMPHIGUS FOLIACEUS	N = 140	N = 59	add	58/222	44/74	2.41	[1.45 – 4.00]	0.0006
			rec	7/133	5/54	1.76	[0.53-5.75]	0.3523
	20.71	37.29	dom	51/89	39/20	3.40	[1.79-6.45]	0.0002

Logistic regression association tests were done with allele frequencies (“add”), frequencies of homozygotes for the minor allele (“rec”—recessive model), and summed frequencies of heterozygotes and homozygotes for the minor allele (“dom”—dominant model). In bold, significant associations ($p < 0.05$); SNP, single nucleotide polymorphism; MAF, minor allele frequency; CONTR, controls; PAT, patients; Model, association tests; OR, odds ratio; CI, confidence interval; PF, pemphigus foliaceus. Endemic pemphigus foliaceus data from Bumiller-Bini et al., 2018.

The rs10404456 variant has been removed during post-imputation QC of PV GWAS data because its INFO-score is 0.75 (in both cases and controls), which is less than the minimum INFO score of 0.8 used as the threshold for SNPs to be included in the GWAS. Imputed rs10404456 has an allele frequency of 72.8% in cases ($N = 241$ individuals) and of 74.7% in controls ($n = 1,188$ individuals). The Chi-squared test p -value is 0.4198, i.e. not significant (C in cases: 131. C in controls: 601. T in cases: 351. T in controls: 1775). There is no overlapping between the 78 GWAS variants with p -values under 0.01 (cf. **Suppl. Table 3**) and these proxy variants. Based on the GWAS, rs10404456 is thus not associated with PV.

The rs10404456 polymorphism is located in the 5' untranslated region of the first exon among the only two exons of *C5AR1* (Chr19:47,290,023-47,322,066, GRCh38). The PF-associated *rs10404456**C minor allele creates a 5'CpG3' site (UCSC Genome Browser). This allele is in fact associated with lower *C5AR1* expression in sun-exposed skin (but not in blood) (GTEx $p = 3.6e-7$). We further identified another 17 eQTLs in LD with rs10404456 ($r^2 > 0.8$), all of them with minor alleles associated with lower *C5AR1* gene expression in sun-exposed skin (GeneHopper). Interestingly, the same haplotype has, in the same tissue (GTEx), the opposite effect on the

neighboring *DHX34* (DExH-Box Helicase 34) gene, located on the same strand. The 18 SNP haplotype spans 8200 bp. Seven of the 18 SNPs create while only two disrupt potentially methylated 5'CpG3' dinucleotides. Three neighboring SNPs (rs11670789, rs11671717, and rs7253663) occur in a region recognized by 46 regulatory proteins, 12 of which were identified in the A549 epithelial cell line (**Supp. Table 4**). The CCCTC-binding factor (CTCF) stands out among them, being repeatedly found in NHEK. In these cells, rs11670789 locates exactly at a chromatin segmentation spot dividing a weak enhancer and a neighboring weak promoter (data from the ENCODE database at the UCSC Genome Browser).

8.4.2 C5aR1 mRNA and protein expression are increased in the PV HSOC model

As expected, Px4-3 or ETA-injected skin specimens developed splits at the corresponding intraepidermal layers: in the suprabasal layer after Px4-3 injection, mimicking the PV phenotype ($p < 0.0001$); and in the granular layer after ETA injection, mimicking the PF phenotype ($p < 0.0001$) (**Figures 2.A** and **2.B**). In order to characterize our model, we verified the expression of DSG1 (**Figure 2.C**) and DSG3 (**Figure 2.D**) in the epidermis. We also demonstrated Px4-3 binding the desmosomes (**Figure 2.E**).

The expression of *C5* and its product did not differ in any treatment (**Figures 3.A** and **3.B**). On the other hand, the expression of *C5AR1* mRNA ($p < 0.05$, FC= 2.71) and of its protein in the epidermal and dermal layers are higher in skins injected with Px4-3, mimicking the PV phenotype ($p < 0.05$) (**Figures 3.C** and **3.D**). Surprisingly, skin injected with ETA presented lower C5aR1 protein expression compared with controls ($p < 0.05$) (**Figure 3.D**).

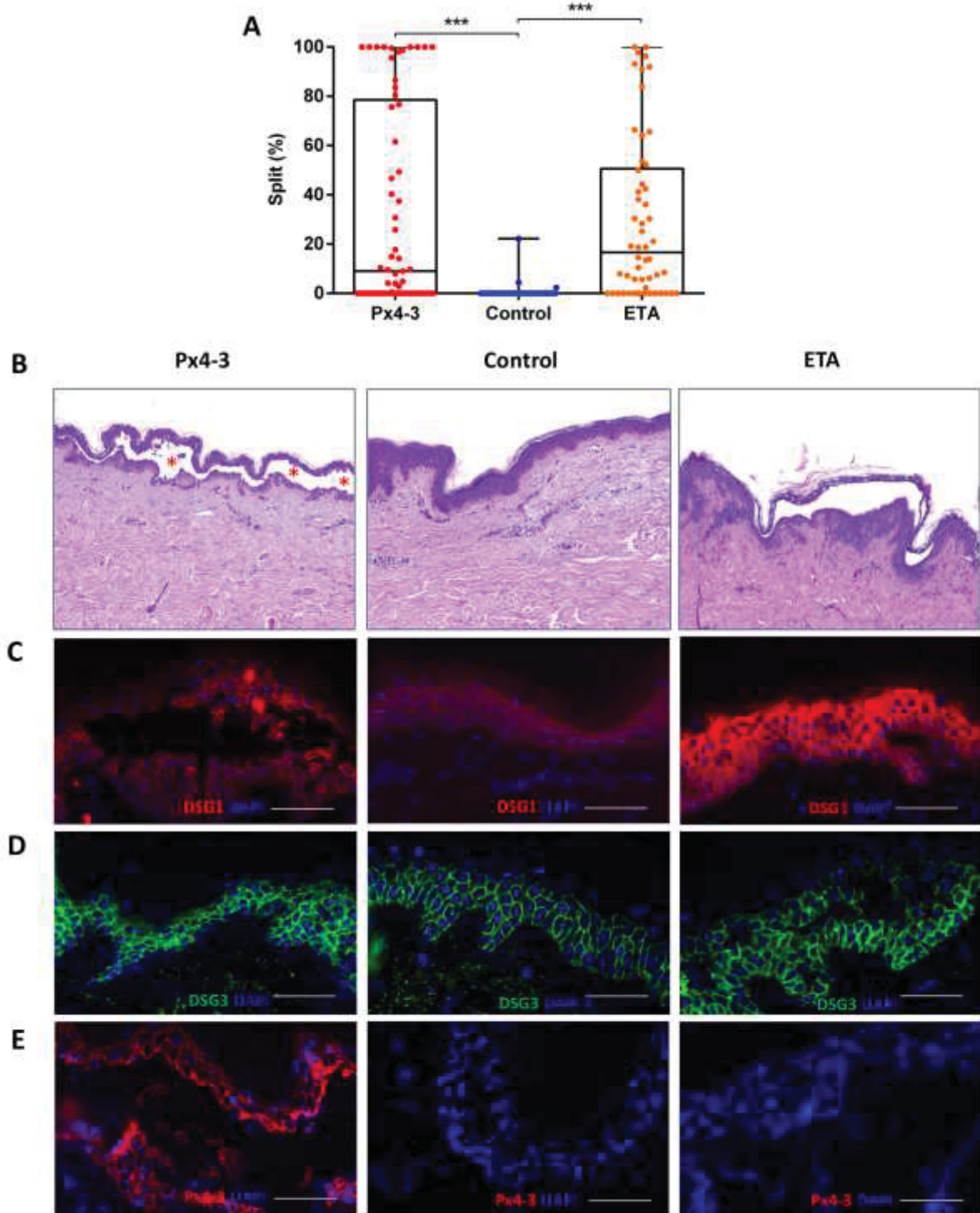


Figure 2. Characterization of the human skin organ culture (HSOC) model. **A.** Split formation in each HSOC treatment. **B.** Light micrographs of hematoxylin and eosin stainings (100-fold, N = 8) of HSOC specimens after injection of Px4-3, normal human IgG (negative control) or ETA (positive control and reference) **C.** Immunofluorescence microscopy against DSG1. **D.** Immunofluorescence microscopy against DSG3. **E.** Direct immunofluorescence microscopy against Px4-3. (600-fold, scale bar = 50 μ m, N = 8). *** p < 0.0001.

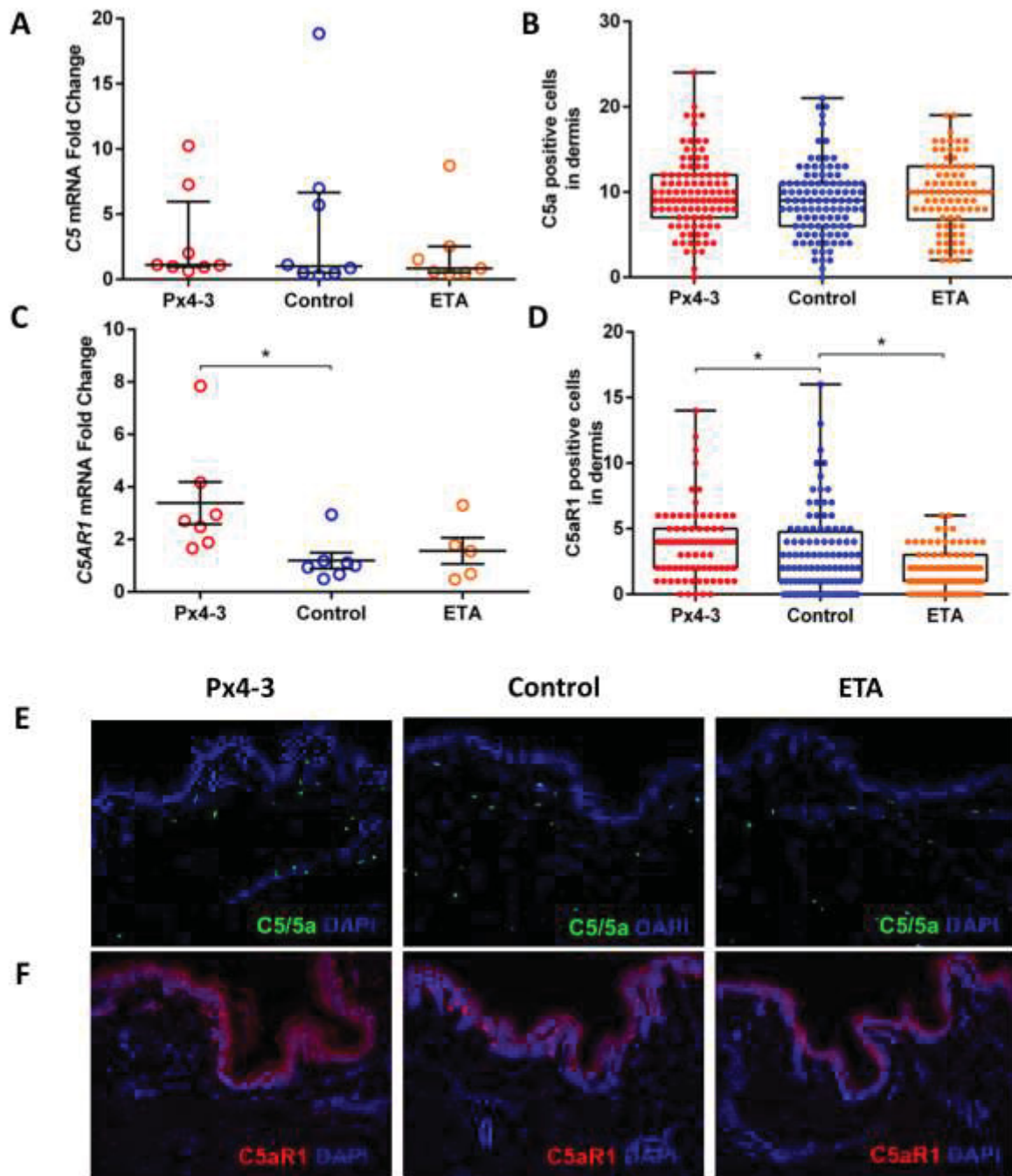


Figure 3. Evaluation of C5a and C5aR1 in the human skin organ culture (HSOC) model. **A.** C5 mRNA expression in HSOC treated with Px4-3, IVIG (control) and ETA. **B.** C5a-positive cells in the dermis of Px4-3, IVIG (control) and ETA treatments. **C.** C5aR1 mRNA expression in HSOC treated with Px4-3, IVIG (control) and ETA. **D.** C5aR1-positive cells in the dermis of Px4-3, IVIG (control) and ETA treatments. **E.** Immunofluorescence microscopy against C5a (200-fold, N = 8). **F.** Immunofluorescence microscopy against C5aR1 (200-fold, N = 8). * $p < 0.05$.

8.4.3 Endemic PF and PV patients have higher levels of C5a, but not of NETs in serum

C5a Serum levels were higher in both PV and EPF patients compared with controls ($p < 0.001$ for both comparisons) (**Figure 4.A**). They did not correlate with NET levels in serum of pemphigus patients, investigated for the first time in this study, nor did NET levels differ from controls (**Figures 4.B** and **4.C**).

8.4.4 Sporadic PF and Endemic PF patients have increased numbers of C5aR1-expressing cells in the dermis

The biopsies were characterized by HE staining as well as by DSG1 and DSG3 expression in the epidermis (**Supp. Figures 1.A and 1.B**). For the first time, C5a and C5aR1 were detected in biopsies from SPF patients (**Figure 4.D** and **4.F**). C5a-positive cells were increased in the epidermis of only one out of three SPF biopsies (**Figure 4.D**). In the other two, they occurred exclusively in the dermis. Therefore, C5a-bound cells were not associated with SPF, but cell-free C5a deposits were not quantified (**Figure 4.E**). Interestingly, we found an increased count of C5aR1-expressing cells in the dermis (but not in the epidermis) of SPF biopsies ($p < 0.0001$) (**Figures 4.F** and **4.G**). On the other hand, C5aR1-positive cells were increased in both the epidermis ($p < 0.0001$) and the dermis ($p < 0.01$) of EPF biopsies (**Figures 4.H, 4.I** and **4.J**).

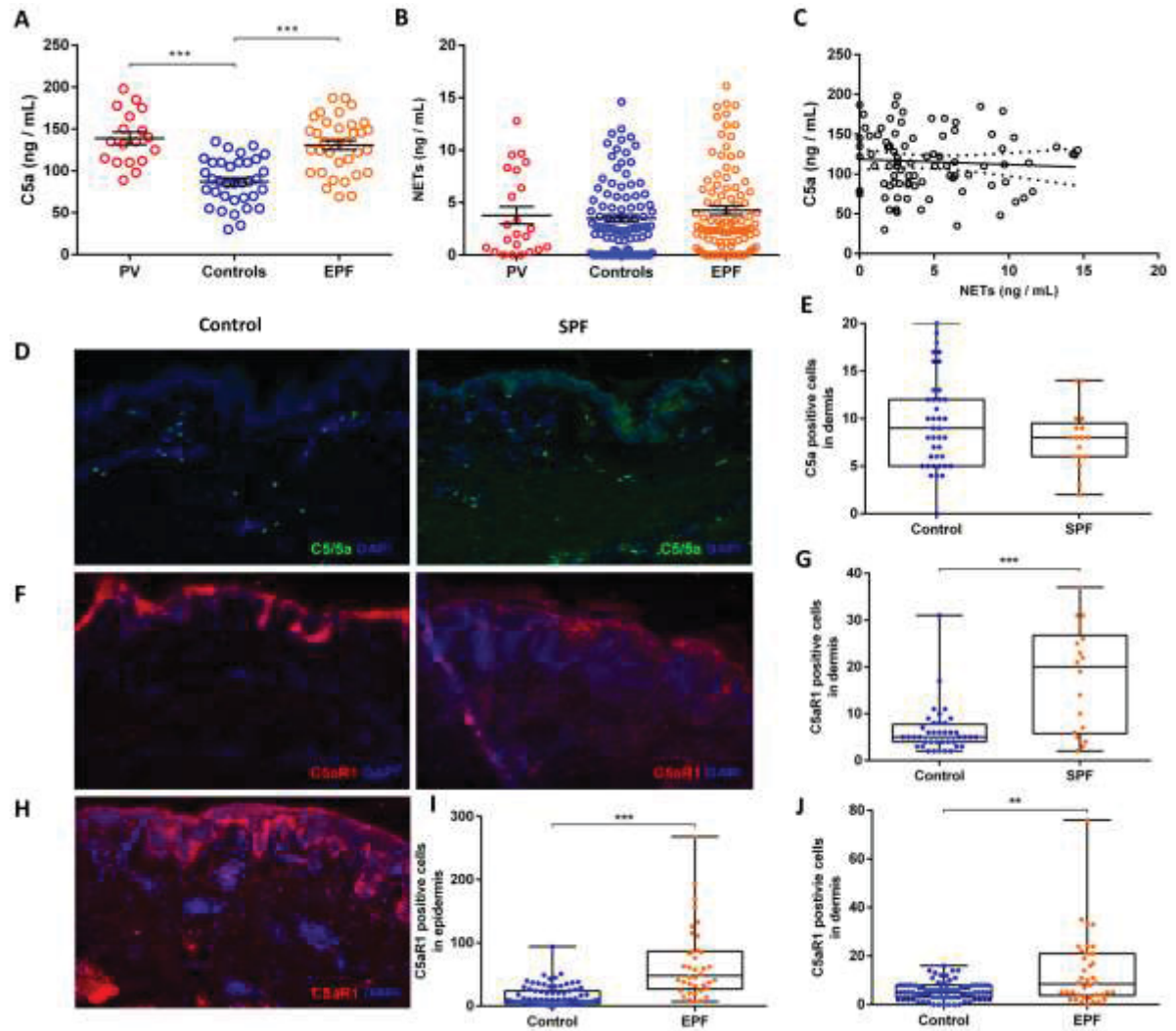


Figure 4. Evaluation of C5a/C5aR1 in the PV, SPF and EPF patients. **A.** Quantification in patients serum of C5a: PV, mean= 138.9 [89 - 198] ng/mL; controls, mean= 87.2 [30 - 135] ng/mL; PF, mean= 130.7 [69 -187] ng/mL. **B.** Quantification in patients serum of NETs: PV, median = 2.014 [0 - 12.79] ng/mL; controls, median = 2.540 [0 - 14.61] ng/mL; PF, median = 2.798 [0 - 16.13] ng / mL. **C.** Absence of correlation between C5a and NET levels ($r = -0.1027$, $p = 0.34$). **D.** Immunofluorescence microscopy for C5a (200-fold, N= 3). **E.** C5a-positive cells in the dermis of controls and SPF patients (200-fold, N= 3). **F.** Immunofluorescence microscopy for C5aR1 (200-fold, N= 3). **G.** C5aR1-positive cells in the dermis of controls and SPF patients. **H.** Immunofluorescence microscopy for C5aR1 in EPF biopsies (200-fold, N= 8). **I.** C5aR1-positive cells in the epidermis of controls and EPF patients. **J.** C5aR1-positive cells in the dermis of controls and EPF patients. *** $p < 0.0001$.

8.5 DISCUSSION

To further advance our understanding of the possible role of the C5a/C5aR1 complement axis in pemphigus pathophysiology, we (i) evaluated if *rs10404456**C is also associated with susceptibility to SPF and PV; (ii) measured the expression of *C5/C5a* and *C5AR1/C5aR1* at the mRNA/protein levels in HSOC models; (iii) measured C5a and NET levels in PV, SPF and EPF patient serum; and (iv) investigated C5a deposition and C5aR1 expression in SPF and EPF patient biopsies. First, we also found an association of the *C5AR1 rs10404456**C allele with susceptibility to SPF, but not with PV. The associated variant is embedded within a haplotype with a 36% global frequency and spanning the promoter, exon 1 and the first intron of the *C5AR1* gene (LD Link data from the 1000 Genomes project). This haplotype harbors 18 eQTLs whose minor alleles are associated with lower *C5AR1* and higher *DHX34* expression (a *C5AR1* neighbor gene, located on the same DNA strand) in sun-exposed skin. *DHX34* encodes an RNA helicase involved in nonsense-mediated mRNA decay (NMD). Polymorphisms of this gene were associated with polyautoimmunity, although the role of this protein in pemphigus diseases remains to be clarified (47).

Moreover, since the investigated groups are unrelated and from different geographical localizations (EPF from Brazil and SPF from Germany), the environmental PF triggers are expected to be distinct. Thus, genetically predicted lower *C5AR1* expression in sun-exposed skin seems to be intrinsically associated with PF pathogenesis, prompting further functional investigation of C5aR1 in pemphigus.

Interestingly, only 0.83% of 1,320 basal keratinocytes investigated by single-cell RNA sequencing (scRNA-Seq) in healthy, generally sun-exposed skin (lower leg) expressed the *C5AR1* gene (GTEx portal). This is in agreement with the condensed chromatin structure adopted by the promoter region in NHEK, including the region occupied by the PF-associated *rs10404456* polymorphism. Moreover, three upward SNPs (*rs11670789*, *rs11671717* and *rs7253663*) of the eQTL haplotype occur in a highly regulatory region occupied by CTCF and another 11 regulatory proteins in an epithelial cell line (A549). One of these SNPs (*rs11670789*) occupies a chromatin structure division spot, separating a weak enhancer from a weak promoter in NHEK (ENCODE, data available in the UCSC Genome Browser). We hypothesize that the low *C5AR1*-expressing haplotype modulates the binding of these regulatory proteins,

increasing condensation, which is probably reinforced by DNA methylation on 5'CpG3' dinucleotides created by seven of the 18 alleles of the eQTL haplotype.

In conclusion, maximized down-regulation of this gene in sun-exposed skin keratinocytes increases the odds of PF development in individuals carrying the *C5AR1* susceptibility haplotype. This result apparently challenges the paradigm of C5aR1 playing a proinflammatory role in autoimmune blistering diseases. Nevertheless, we have to take the following into account: (i) C5aR1 expression favors cell survival and carcinogenesis through the PI3K/AKT pathway (48–52); (ii) epigenetic mechanisms favoring keratinocyte proliferation are predicted to protect against PF lesions (53), in contrast to psoriasis (1,2); and (iii) ultraviolet irradiation of skin is a well-known PF risk factor (54,55) and the associated haplotype displays an eQTL effect specifically on sun-exposed skin (and not in blood cells of the immune response, which commonly express high C5aR1 levels, nor in cells of sun-protected skin - GTEx portal). As a limitation, we did not check for the *C5AR1* genotype of the skin donors for the HSOC models, nor of the skin biopsy donors; in addition, skin biopsies used for HSOCs were from generally sun-protected regions (breast and belly).

Despite low constitutive *C5AR1* gene expression, keratinocytes of both normal and treated HSOC skin displayed conspicuous C5aR1 intracellular protein levels, due to high mRNA or protein stability. However, the numbers of C5aR1 protein-expressing keratinocytes did not differ, even after challenging them with different blister triggers. This contrasts with patient EPF, but not SPF skin, which displayed higher numbers of C5aR1-positive keratinocytes. Although SPF patients did not follow the same expression pattern, sample size at our disposal was smaller. Higher counts of C5aR1-expressing keratinocytes in EPF epidermis agree with the genetically predicted benefit of C5aR1 protein to counteract blister formation in the upper skin.

In contrast to the epidermis, C5aR1-positive cell counts in the dermis clearly differed between normal and treated HSOC. They were lower in ETA-treated HSOCs, independent of mRNA expression, and thus independent of *C5AR1* and also of *C5* gene regulation. ETA-induced reduction of C5aR1-expressing cells within the dermis (as Langerhans cells and fibroblasts) seems most probably to be a product of intracellular protein consumption by inhibiting C5aR1 recycling in the Golgi complex. Quite the opposite occurred in PF biopsies, calling attention to the fact that ETA-induced blisters, despite being phenotypically similar to those in PF, do not share the

same etiological mechanisms. This actually reinforces the pathological role played by anti-DSG1 antibodies in the disease (17).

In contrast, *C5AR1* mRNA levels and counts of C5aR1-positive cells increased in the HSOC dermis after Px4-3, PV-mimicking treatment. These contrasting results may reflect the use of different blister triggers in the HSOC models (exfoliative toxin A for PF vs. single-chain variable fragment directed against DSG1 and DSG3 (Px4-3) for PV) and the up-regulating effect of specific PV antibody fragments on *C5AR1* gene expression in cells populating the dermis of HSOC models, such as resident Langerhans macrophages and fibroblasts (GTEx portal).

Despite the advantages of HSOCs in relation to animal models regarding DSG histological distribution, gene regulation and the reproduction of the architecture of human skin (37), HSOCs are disconnected from normal blood flow and thus from systemic far-reaching immune responses. In fact, immunofluorescence dermal profiles differ due to the nature of C5aR1 protein-expressing cells in HSOCs and patient biopsies. Based on scRNA-Seq of sun-exposed skin cells, we predict that Langerhans macrophages and neutrophils are the highest C5aR1-expressing cells among those residing in and migrating to the dermis, respectively, being the latter probably absent in HSOCs (GTEx portal data, Protein atlas). Increased counts of C5aR1-expressing cells in the dermis of patient biopsies is in accordance with neutrophil infiltration in the dermis of lesional and perilesional PF and PV skin (56–58) (**Figure 5**).

The *C5* gene is constitutively expressed at low levels in sun-exposed skin cells, but *C5* gene regulation and C5a-positive cell counts did not differ from those of HSOC treatments. This leads us to suggest that blood serum is the actual source of C5 complement-mediated cleavage and cell-free C5a deposits found in the dermis of PF patients (not quantified). Higher C5a levels were actually found in the serum of PV and EPF patients in this study and also by others (20,59). C5a is a potent neutrophil chemokine, and neutrophils stand out among the immune cells with highest basal *C5AR1* expression in different RNA-Seq databases (60) and HPA, available in the Protein Atlas). C5a activates the neutrophil response, inducing significant morphological changes, and enhancing neutrophil adhesion to endothelial and epithelial cells, phagocytosis, and NET production (6,22,61). However, we did not find any association between EPF or PV and NET release, possibly ruling out this pathway in pemphigus pathophysiology. C5a also induces the shedding of

microvesicles containing C5aR1 receptors from neutrophils (**Figure 5**), reducing the expression of these molecules in neutrophils and leading to profound pro-inflammatory signals (61). To be confirmed, this possible effect needs further investigation.

Pemphigus etiology is still not fully understood. Primary treatments are systemic corticosteroids, often accompanied by side effects (reviewed by (12)). Other options such as plasmapheresis and intravenous immunoglobulin are available, as well as a monoclonal antibody against CD20+ B cells (Rituximab) (18). Anti-C5a and anti-C5aR1 can be a potential therapeutic target in pemphigus disease (62), but more functional assays are necessary to elucidate the role of the C5a/C5aR1 axis in pemphigus disease. Although the C5a/C5aR1 axis is commonly associated with harmful effects on tissue integrity, exacerbating inflammation (1,2), genetic evidence suggests that a higher basal expression of the *C5AR1* gene in sun-exposed keratinocytes actually protects against PF. In fact, others have found its expression to be crucial in the defense against *Toxoplasma* infection (63). The results described in this work contrast the protective role of C5aR1 in keratinocytes against PF development, regardless of its sporadic or endemic epidemiology, and the proinflammatory role of the C5a/C5aR1 axis and ultimately of the complement cascade in PF and PV pathogenesis. They also lead us to suggest a shared role of the complement system in different pemphigus forms and a potential beneficial effect of its therapeutic inhibition in immune cells, especially in neutrophils.

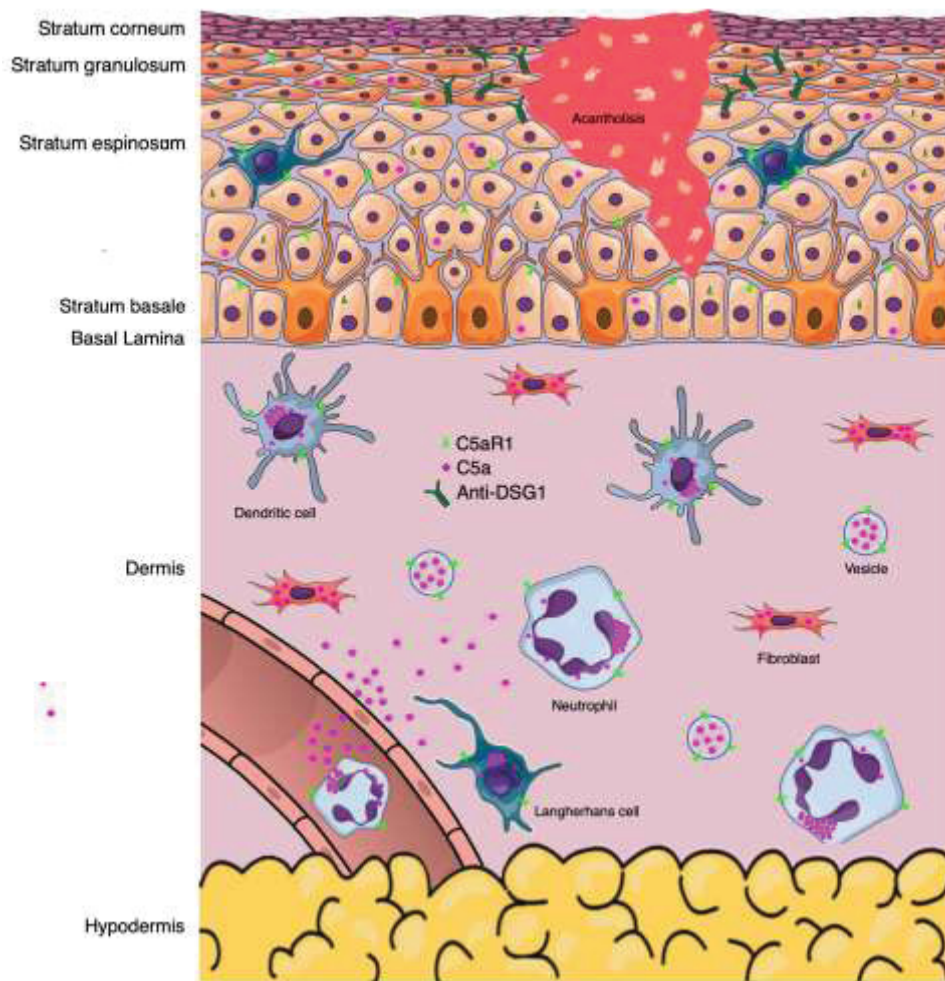


Figure 5. Hypothesis explaining increased counts of C5aR1-expressing cells in patients with PF. The *C5aR1* gene is up-regulated in dermal Langerhans, dendritic cells and fibroblasts, as well as in circulating cells such as neutrophils, which are chemoattracted to inflammation sites through higher local C5a levels. After C5a binding, C5aR1 is phosphorylated, rapidly internalized and transported to the Golgi apparatus for protein recycling. It may also be found in extracellular vesicles in the dermis.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTIONS

VB-BH, ABWB and JEH designed the study; MLPE, ES, AMFR, GAC, AMR, NB, DZ, CP, MWo, MS provided the samples; VB-BH, EL, AP, RMN, YRZ, KSTY, TDJF MWi and AF performed the experiments; VB-BH analyzed the data; VB-BH, GCK, ABWB, AP and EL drafted the manuscript. JEH, ES and HB financed the research. All authors critically reviewed this manuscript.

DATA AVAILABILITY STATEMENT

https://docs.google.com/spreadsheets/d/1PypoBV0SKHQ1_cqdzJVTr3woZw1lbQBk/edit?usp=sharing&ouid=115289756184035320172&rtpof=true&sd=true

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8.7 SUPPLEMENTARY MATERIALS

Supplementary methods

Genotyping, imputation and PV genome-wide association study

Genotyping for 306 PV cases has been performed using Genome Sequence Archive for Human (GSA) version 1, and for 76 PV cases using GSA version 2. As control, genotype data of 1,219 ancestry-matched individuals obtained using GSA version 1 have been used. To address potential genotyping batch effects between GSA version 1 and version 2, N=1,009 variants with association p-value lower 0.001 in a comparison of GSA version 1 versus GSA version 2 genotyped patients were removed. Further SNP quality control (QC) was the removal of variants with (i) ambiguous allele pairs (A/T and C/G), (ii) a call rate of less than 0.98, (iii) minor allele count less than 5 and (iv) a rejection of Hardy-Weinberg Equilibrium at a significance level of 0.00001 in cases and 0.001 in controls. In individual-level QC, samples with (i) a call rate of less than 95% and (ii) an inbreeding coefficient less than -0.2 or larger 0.2 were removed. Cryptic relatedness was assessed using KING (Manichaikul et al., 2010) within PLINK 2.0 and we removed one individual for each pair of individuals with a relationship of second degree or closer, i.e., using a cutoff of 0.0884. Lastly, we performed a principal component analysis to check for outliers. Using 8 iterations we repeatedly removed individuals that deviated more than 4 standard deviations from the mean in at least one of the first 5 principal components.

Staining

Haematoxylin and eosin (HE)

Paraffin slides were incubated in one xylene bath (Merck; Darmstadt, Germany) for 20 minutes and swiveled back and forth in two others. Subsequently, the slides were swiveled in three ethanol (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) baths with descending concentrations (99,8%, 96%, 70%) and finally in distilled water (Klinisch Experimentelle Forschungseinrichtung; Universität zu Lübeck, Germany). Incubation for 5 minutes in haematoxylin solution according to Papanicolaou (Merck; Darmstadt, Germany) followed as well as a washing step with tap water. Next, the slides were revolved in an acetic acid alcohol solution (Th. Geyer GmbH & Co. KG (Chemsolute); Renningen, Germany), washed and swiveled in an ammonia solution

(Merck; Darmstadt, Germany). The staining for eosin (Waldeck GmbH & Co. KG; Münster, Germany) followed after another washing step for 20 seconds. Ensuingly, the slides were dehydrated using an ascending ethanol row (reverse from before including xylene at last) and covered with a cover slide (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) using the Leica CM-3050S automatic cover dispenser (Leica Mikrosysteme Vertrieb GmbH; Wetzlar, Germany).

Desmoglein 1 and 3

Three different stainings were performed, one to visualize DSG1, the second for DSG3 and the last for both. Cryo slides were used for all. First, the slides were dried at room temperature (RT) for 10 minutes and then fixed in acetone (Thermo Fisher Scientific; Waltham, MA, USA) at -20 °C. Afterwards, the sections were circled using a fat pen (DAKO Pen S2002; Agilent; Santa Clara, CA, USA) without touching the section, followed by three washing steps in PBS for 10 minutes each using a humid chamber (abArt UG und Co. KG; Waldbrüttelbrunn, Germany). For preincubation, 10% normal goat serum (DAKO GmbH; Jena, Germany) in PBS was used for 20 minutes. Next, incubation with the primary antibody diluted in 2% normal goat serum in PBS (mouse anti-human desmoglein 1, 1:100; mouse anti-human desmoglein 3, 1:50; both from OriGene Technologies, Inc.; Rockville, MD, USA) overnight at 4°C took place. The following day, the slides were washed three times in PBS for 5 minutes and incubated with the secondary antibody (DSG1: CyTM3-conjugated AffiniPure goat anti-mouse, Clone: polyclonal, Jackson ImmunoResearch; DSG3: goat anti-mouse IgG (H+L) Alexa Fluor 488, A11029 Clone: polyclonal, Thermo Fisher Scientific; Waltham, MA, USA) 1:200 in 2% normal goat serum in PBS for 45 minutes at RT. In the staining of both DSG1 and DSG3, the incubations of primary and secondary antibodies of DSG3 were done after the incubations of primary and secondary antibodies of DSG1. Finally, another three-part washing step was conducted and the slides were covered with 4,6-diamidino-2-phenylindole DAPI Fluoromount-G (Southern Biotech; Birmingham, AL, USA). After a drying time of 1 hour protected from light, the slides were stored at -20°C.

Anti-desmoglein 1 and 3 single chain variable fragment staining

This staining was performed to evaluate whether the injected single chain variable fragment (scFv) was bound to the DSGs and was responsible for blister formation.

The used cryo slides were dried for 5 minutes at RT and the sections encircled with the fat pen as described above. Two washing steps with the washing buffer [100 mL 10x TBS (61 g Trizma-Base + 88 g sodium chloride + distilled water for 1 L) + 500 µL 2 M calcium chloride + 900 mL distilled water + 500 µL Tween20 (Euroimmun, Medizinische Labordiagnostika AG; Lübeck, Germany)] followed for 3 minutes each. Afterwards, blocking of nonspecific binding sites was performed using a blocking buffer with 1% bovine serum albumin (BSA) [0.5 g BSA (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) + 50 mL washing buffer; sterile filtered] with an incubation time of 30 minutes. Subsequently, the blocking buffer was discarded and the sections were incubated with the secondary antibody rat anti-hemagglutinin monoclonal antibody high affinity (Roche Deutschland Holding GmbH; Grenzach-Wyhlen, Germany) in a dilution of 1:100 in blocking buffer for 1 hour. Another washing step followed, 3 times for 3 minutes using the washing buffer. Incubation with the tertiary antibody goat anti-rat IgG (H+L) Alexa Fluor 594 (Invitrogen AG; Carlsbad, CA, USA), 1:200 in blocking buffer, was performed for 30 minutes as well as a last washing step. These slides were also covered with DAPI Fluoromount-G and stored at -20 °C after 1 hour of drying in a light protected place.

C5a

Paraffin slides were used to stain for C5/5a by deparaffinising. Firstly, the slides were put subsequently into two baths containing Roti Clear (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) for 15 minutes each. Afterwards, a descending alcohol row followed (99.8%, 96%, 70% and 50% ethanol; Carl Roth GmbH + Co. KG; Karlsruhe, Germany) for 5 minutes each, as well as a bath with distilled water for 5 minutes. The sections on the slides were then encircled using a fat pen (DAKO Pen S2002; Agilent; Santa Clara, CA, USA) and washed with phosphate-buffered saline (PBS; Thermo Fisher Scientific Gibco; Waltham, MA, USA) mixed with Tween20 (Euroimmun, Medizinische Labordiagnostika AG; Lübeck, Germany; 1 L PBS + 500 µL Tween20) three times for 5 minutes. Ready-to-use pepsin (Thermo Fisher Scientific Gibco; Waltham, MA, USA) was used for retrieval of the antigens by incubating the sections for 10 minutes at 37°C followed by another washing step with PBS and Tween20. Furthermore, unspecific binding sites were blocked by incubation of the sections with 3% bovine serum albumin (BSA; Carl Roth GmbH + Co. KG; Karlsruhe, Germany) diluted in the washing buffer for 1 hour at room temperature

(RT). In the next step, the sections were covered with the primary antibody mouse anti-human C5/5a des-Arg diluted 1:40 in 3% BSA in the washing buffer. Incubation overnight at 4°C followed. The day after, a washing step was conducted before incubation with the secondary antibody. Donkey anti-mouse IgG (H+L) diluted 1:100 in 3% BSA in the washing buffer was used for this step. The duration of the incubation was 1 hour at RT. Another washing step was performed and the slides were covered with 4,6-diamidino-2-phenylindole (DAPI) Fluoromount-G (Southern Biotech; Birmingham, AL, USA), dried at RT for 1 hour and finally stored at -20°C. C5a was evaluated in the dermis of the HSOC treatments and patients.

C5aR1

As paraffin slides are used for this staining as well, the procedure is similar, but different reagents and antibodies were used. In the same way, the slides were deparaffinized, washed, and encircled. For the antigen retrieval, a proteinase K solution (20 µg/mL in TE buffer; QIAGEN N.V.; Hilden, Germany) was used for 15 minutes at 37°C on the sections. After a three-part washing step, blocking was performed using 5% normal donkey serum diluted in PBS for 1 hour at RT. Subsequently, the slides were incubated with the primary antibody rabbit anti-human C5aR1 (Thermo Fisher Scientific Gibco; Waltham, MA, USA) in a dilution of 1:100 in 5% normal donkey serum in PBS at 4°C overnight. The next day, a washing step was conducted and incubation with the secondary antibody donkey anti-rabbit IgG (Jackson ImmunoResearch Europe Ltd.; Ely, UK). It was diluted 1:200 in 5% normal donkey serum in PBS and put on the sections for 1 hour at RT. Lastly, another washing step followed and the slides were embedded with DAPI Fluoromount-G, dried and stored at -20 °C. C5aR1 was evaluated in the epidermis and in the dermis of the HSOC treatments and patients.

Split measurement

Split measurement was performed on pictures taken of the HE staining with the Keyence fluorescence microscope (BZ9000 BIOREVO, Keyence; Osaka, Japan). Pictures of each section per condition were taken on a 100 magnification in the manner that pictures were not overlapping regarding the epidermis. Measurement of the length of the epidermis and the length of the split if one occurred, was conducted

using ImageJ (Wayne Rasband) and the percentage of splits in the whole section was calculated.

Supplementary tables

Supp. Table 1. Subject description Clinical and demographic aspects of subjects enrolled in the measurements of NETs and C5a.

Subjects used for NETs detection	PV (N = 23)	Control (N = 97)	EPF (N = 95)
Median age (min, max)	45 (16, 80)	51 (19, 86)	39 (12, 76)
Sex (women)	65 %	58 %	57%
Subjects used for C5a detection	PV (N = 18)	Control (N = 35)	EPF (N = 35)
Median age (min, max)	45 (16, 80)	53 (20, 73)	37 (12, 74)
Sex (women)	72 %	46 %	60 %

Min, minimum; max, maximum; PV, pemphigus vulgaris; EPF, endemic pemphigus foliaceus

Supp. Table 2. Primer sequences for genotyping the SNP rs10404456 from gene C5aR1 with the massARRAY iPLEX Platform.

Forward Primer Sequence	ACGTTGGATGAAGAGATGGCCCCAAATAGG
Reverse Primer Sequence	ACGTTGGATGAAACCGAGGAAGCTCCTGTG
Extended Primer Sequence	taatCTCCTGTGCAGGCTC

Supp. Table 3. SNPs within a 1 Mb window from rs10404456 associated with pemphigus vulgaris

CHR	position	Allele A	Allele B	MAF in cases	MAF in controls	OR	P value
19	48265232	C	A	0,104876	0,062016	1,77208	0,000144021
19	47244530	C	A	0,259025	0,330135	0,709307	0,000152327
19	47253284	T	C	0,259336	0,331524	0,706014	0,000165717
19	47247786	G	A	0,259232	0,33064	0,708455	0,000181028
19	47241659	C	T	0,258921	0,329882	0,709734	0,000184611
19	47253218	C	T	0,258921	0,330577	0,707509	0,000194364
19	48264879	A	G	0,104876	0,062521	1,75681	0,000195183
19	47239919	G	C	0,258817	0,328767	0,712941	0,000213157
19	47238659	G	A	0,258402	0,328809	0,711264	0,000213451
19	47248979	C	G	0,260685	0,33104	0,712535	0,00022057
19	47236268	G	C	0,25861	0,327567	0,716056	0,000230591
19	47235715	C	T	0,251141	0,321002	0,70938	0,000275559
19	47215974	G	A	0,258402	0,32763	0,715076	0,000311128

19	47199156	C	T	0,263382	0,329819	0,72654	0,000376473
19	47265153	C	T	0,403527	0,469676	0,763879	0,000450785
19	47268978	T	A	0,402075	0,469255	0,760566	0,000482173
19	48272855	T	C	0,0760373	0,0424242	1,85751	0,000636559
19	48275274	C	T	0,075	0,0418561	1,85606	0,000686817
19	48269847	C	T	0,075	0,0419613	1,8512	0,000712255
19	48285809	T	C	0,0752075	0,0422138	1,84515	0,000802794
19	48279352	C	G	0,0743776	0,0417719	1,84329	0,000929006
19	47210395	C	T	0,0752075	0,111237	0,649758	0,00103693
19	47291561	A	G	0,306432	0,239373	1,40392	0,00114574
19	47284403	C	G	0,142324	0,193077	0,693516	0,0011492
19	48268184	C	G	0,0787344	0,0454545	1,79473	0,00138185
19	48267703	C	T	0,0791494	0,0456019	1,79889	0,00139398
19	48267819	C	T	0,0791494	0,0456019	1,79889	0,00139398
19	48268194	C	T	0,0791494	0,0456019	1,79889	0,00139398
19	48266776	C	T	0,0787344	0,0454966	1,79299	0,00140571
19	47273595	C	G	0,131432	0,182218	0,679113	0,00144635
19	48264923	A	G	0,0784232	0,0452652	1,79487	0,0014794
19	48264989	G	T	0,0784232	0,0452652	1,79487	0,0014794
19	48265818	T	C	0,0784232	0,0452652	1,79487	0,0014794
19	48265972	G	A	0,0784232	0,0452652	1,79487	0,0014794
19	48266071	G	A	0,0786307	0,0454125	1,7939	0,00149107
19	47266630	T	C	0,140664	0,188889	0,7029	0,00151888
19	47275221	C	T	0,131846	0,182344	0,681005	0,00153547
19	47277712	C	T	0,132573	0,182807	0,683206	0,00154456
19	47265960	G	A	0,140664	0,188742	0,703576	0,00156388
19	47276835	G	C	0,13278	0,182934	0,683861	0,00166626
19	47174205	A	G	0,160996	0,203683	0,75021	0,00178186
19	47282162	G	A	0,156846	0,206524	0,714714	0,00187001
19	47254540	G	C	0,352905	0,288699	0,744225	0,00219112
19	47273017	G	A	0,355187	0,289331	0,739102	0,00252687
19	47269370	A	G	0,357054	0,29154	0,741011	0,00263471
19	47272725	C	G	0,355394	0,289268	0,738206	0,00275891
19	47274696	G	A	0,353423	0,288931	0,743374	0,00282085
19	47269665	T	C	0,355394	0,289226	0,738055	0,00283578
19	47272083	G	A	0,355498	0,28952	0,738779	0,0028504
19	47274172	G	A	0,353423	0,289099	0,743983	0,00286496
19	47274659	A	G	0,353423	0,289099	0,743983	0,00286496
19	47275107	G	A	0,353423	0,289205	0,744364	0,00287599
19	47275163	A	G	0,352697	0,288678	0,744825	0,00300977
19	47274071	C	T	0,350311	0,288047	0,75035	0,00304628
19	47275804	C	A	0,352697	0,288931	0,745741	0,0030634
19	48259220	C	T	0,0715768	0,0414773	1,78163	0,00306699
19	47241217	G	A	0,350311	0,291077	0,761485	0,00397556
19	47232223	C	T	0,347303	0,286258	0,753738	0,00403342
19	48384449	C	A	0,176452	0,143013	1,28391	0,00446044
19	47270442	G	A	0,35612	0,293392	0,75072	0,0045349
19	47615306	G	A	0,0456432	0,0238215	1,95986	0,00503523
19	48464766	C	G	0,134025	0,103998	1,33341	0,00647471
19	47245792	T	C	0,485373	0,422875	0,776886	0,00730494
19	48466456	C	T	0,150207	0,115173	1,35796	0,00749475
19	48379260	A	T	0,13195	0,102041	1,33766	0,00767742
19	48380622	A	G	0,13195	0,102041	1,33766	0,00767742
19	47230113	C	T	0,487448	0,424853	0,776728	0,00788569
19	47257668	T	C	0,533921	0,481587	0,810925	0,00794101

19	48455853	G	C	0,0825726	0,0637837	1,32109	0,00809762
19	48455856	C	G	0,0825726	0,0637837	1,32109	0,00809762
19	47082454	T	C	0,280083	0,325589	0,805858	0,00810207
19	48382516	C	T	0,129046	0,100421	1,32728	0,00839789
19	46926178	C	T	0,123963	0,0991162	1,28615	0,0084721
19	48174216	A	G	0,244917	0,197096	1,32133	0,00902809
19	48236665	T	C	0,0664938	0,0442971	1,53678	0,00944277
19	47227327	C	A	0,487759	0,426873	0,782197	0,00953466
19	48397380	G	A	0,451971	0,476557	1,10392	0,00975621
19	47227295	C	A	0,487759	0,427252	0,783409	0,00987605

CHR, chromosome; position (GRCh37); allele A, minor allele frequency; allele B. the other allele; MAF, minor allele frequency; OR, odds ratio.

Supp. Table 4. SNPs with high disequilibrium linkage with the rs10404456.

SNP	Position	Alleles	r ²	Beta	P value	Original	Mutated
rs73063338	chr19:47807516	A=0.295	0.98	-0,18	8.4e-7	CpA	CpG
rs11670768	chr19:47808431	A=0.257	0.86	-0,23	4.8e-10	CpG	CpA
rs11670789	chr19:47808762	G=0.261	0.87	-0.22	5.1e-9	CpA	CpG
rs11671717	chr19:47808798	C=0.261	0.88	-0.22	5.1e-9	No CpG	
rs7253663	chr19:47808828	G=0.261	0.87	-0.22	5.1e-9	CpT	CpG
rs4287683	chr19:47809671	A=0.262	0.88	-0.22	3.6e-9	No CpG	
rs4538111	chr19:47809689	T=0.262	0.88	-0.22	3.6e-9	No CpG	
rs4508517	chr19:47809714	T=0.262	0.88	-0.22	3.6e-9	No CpG	
rs62128435	chr19:47810085	C=0.261	0.88	-0.22	1.7e-9	No CpG	
rs67834691	chr19:47810358	A=0.261	0.88	-0.22	3.6e-9	CpG	CpA
rs4595896	chr19:47810900	G=0.295	0.99	-0,19	3.0e-6	CpA	CpG
rs60925053	chr19:47812689	A=0.261	0.88	-0.22	3.6e-9	No CpG	
rs59259352 *	chr19:47812757	G=0.261	0.88	-0.22	3.6e-9	CpA	CpG
rs10404456	chr19:47812900	C=0.292		-0,19	3.6e-7	TpG	CpG
#							
rs11670330 *	chr19:47813165	A=0.261	0.88	-0.22	3.6e-9	CpG	CpA
rs11668733	chr19:47815156	C=0.261	0.88	-0.22	2.4e-9	TpG	CpG
rs11668768	chr19:47815192	C=0.261	0.88	-0.22	2.4e-9	No CpG	
rs4427917	chr19:47815715	C=0.261	0.88	-0.22	2.4e-9	No CpG	

SNP, single nucleotide polymorphism. # associated SNP. In bold: SNPs located within a region recognized by at least one of 46 regulatory proteins in different cell lines. Twelve of them were identified by chromatin immunoprecipitation in epithelial cells: MYC (MYC proto-oncogene, bHLH transcription factor) in MCF10A-Er-Src non-tumorigenic epithelial cell line from mammary gland; BCL3 (BCL3 transcription coactivator), CTCF (CCCTC-binding factor), CREB1 (cAMP responsive element binding protein 1), ELF1 (E74 like ETS transcription factor 1); FOSL2 (FOS like 2, AP-1 transcription factor subunit), FOXA1 (forkhead box A1), MAX (MYC associated factor X), NR3C1 (nuclear receptor subfamily 3 group C member 1, also known as glucocorticoid receptor), RAD21 (RAD21 cohesin complex component), TCF12 (transcription factor 12) and USF1 (upstream transcription factor 1) in A549, an epithelial cell line derived from a lung carcinoma tissue. CTCF was also repeatedly observed in normal human keratinocytes (NHEK) of 5 different sources. NHEK were not investigated for the other mentioned proteins. Available in: <https://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&lastVirtModeType=default&lastVirtModeExtraState=&virtMo>

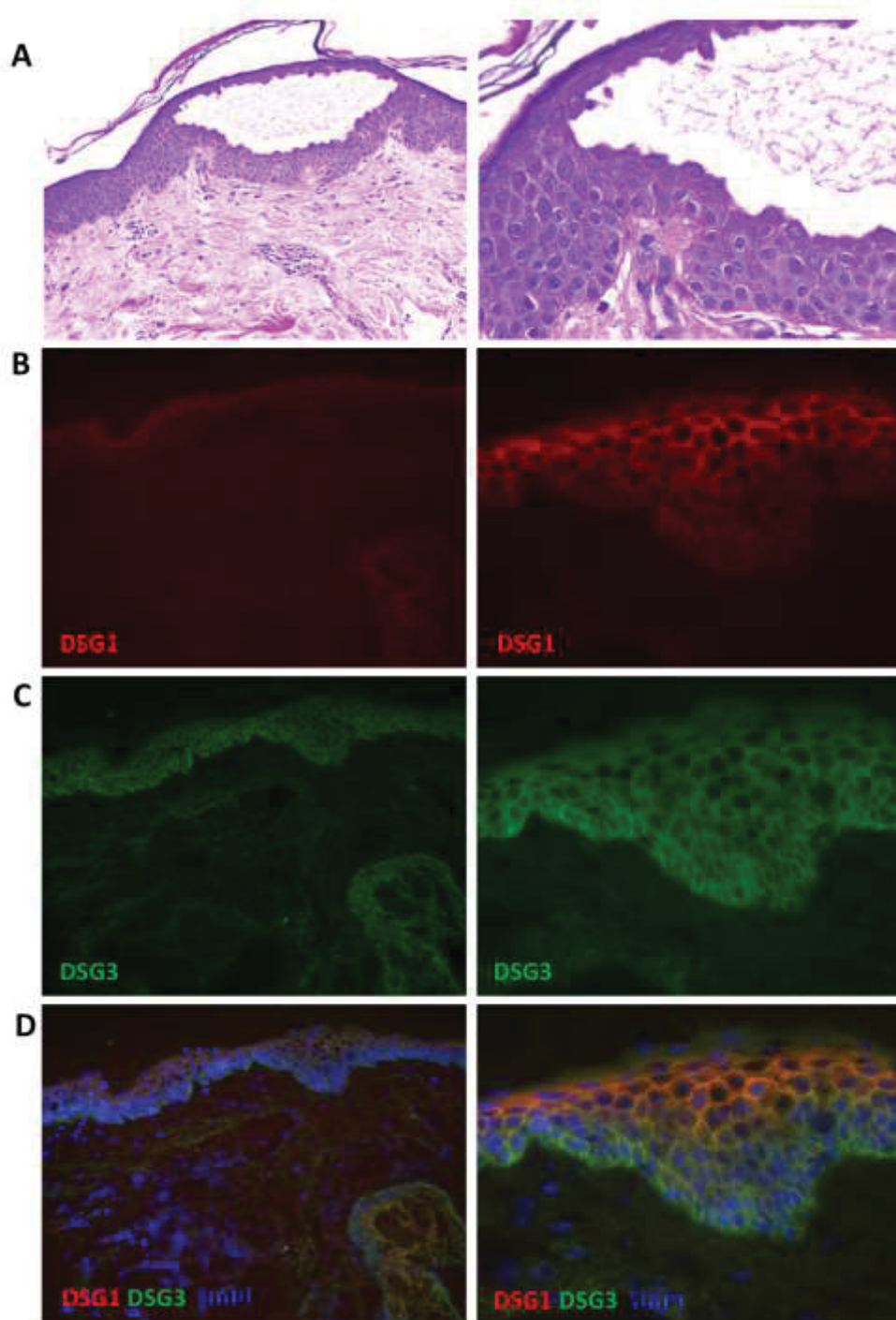
deType=default&virtMode=0&nonVirtPosition=&position=chr19%3A47804239%2D47824338&hgid=1289357561_sUKZZVsrPaSd9MaEkWJ8xj2ERITO

* rs59259352 and rs11670330 occur next to cg23058911 (chr19:47812780) and cg10224107 (chr19:47813131), being partially methylated and unmethylated, respectively, in GM12878, K562 and HepG2 cell lines. Despite being recognized by CTCF in A549 cell lines, CTCF did not bind the region harboring rs11670330 in NHEK. This region actually appears condensed in NHEK.

Available in: [https://genome.ucsc.edu/cgi-](https://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr19%3A47804239%2D47824338&hgid=1289357561_sUKZZVsrPaSd9MaEkWJ8xj2ERITO)

[bin/hgTracks?db=hg19&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr19%3A47804239%2D47824338&hgid=1289357561_sUKZZVsrPaSd9MaEkWJ8xj2ERITO](https://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr19%3A47804239%2D47824338&hgid=1289357561_sUKZZVsrPaSd9MaEkWJ8xj2ERITO)

Supplementary figure



Supp. Figure 1. Characterization of SPF biopsies **A.** Light micrographs of hematoxylin & eosin staining of sporadic pemphigus foliaceus. **B.** Immunofluorescence microscopy against DSG1. **C.** Immunofluorescence microscopy against DSG3. **D.** Immunofluorescence microscopy against DSG1 and DSG3. On the left the magnification is 200-fold. On the right, it is 600-fold.

**9 CAPÍTULO 6 – MARCADORES DE VIAS DE MORTE CELULAR INVESTIGADOS
EM UM MODELO DE MINIÓRGÃO DE PELE QUE MIMETIZA PÊNFIGO**

PESQUISA ORIGINAL – *Beyond Apoptosis: Starting the Investigation of Other Cell Death Pathways in Pemphigus*. Manuscrito que será submetido no periódico Cell death and diseases- <https://www.nature.com/cddis/>.

ORIGINAL ARTICLE

BEYOND APOPTOSIS: STARTING THE INVESTIGATION OF OTHER CELL DEATH PATHWAYS IN PEMPHIGUS**Short title:** Cell death pathways in pemphigus

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9.1 ABSTRACT

Pemphigus foliaceus (PF) and pemphigus vulgaris (PV) are autoimmune blistering diseases, characterized by the presence of autoantibodies mainly against desmoglein (DSG) 1 and DSG3. The role of cell death in this group of diseases is still poorly understood. Here, we investigated supernatants and biopsies of human skin organ culture (HSOC) treated with: (i) either exfoliative toxin of *Staphylococcus aureus* (ETA) (N = 7) or single chain variable fragment (scFv) of an antibody directed against DSG1 and DSG3 (Px4-3) (N = 8) to mimic PF or PV phenotypes, respectively, or intravenous immunoglobulin (IVIG) as a control (N = 8); (ii) serum from PF and PV patients (N = 5). As expected, the HSOC models injected with ETA and Px4-3 presented blisters in the granular and deep layers of the epidermis, respectively; (iii) IgG isolated from endemic controls (negative or positive for anti-DSGs), from active or remitting endemic PF patients, or from active PV patients (N = 5). Treatment with patient serum did not induce any split formation, whereas concentrated IgG of any source, including of controls, caused skin blisters. There was no difference in gene expression of Px4-3- or ETA-treated HSOCs for: *CD47*, *SIRPA* and *EIF2AK3* (immunogenic cell death - ICD); *TNF* and *TRAF2* (apoptosis and necroptosis); *IL1B*, *IL18* and *IL1A* (pyroptosis and inflammatory response). There was also no difference in these treatments for cell proliferation (Ki67), death (TUNEL) and CALR (ICD marker) expression. Nonetheless, RIPK1 (necroptosis) and cleaved CASP3 (apoptosis) abundances decreased in the Px4-3-treated HSOC model ($p < 0.005$). The ETA-treated HSOC presented higher supernatant lactate dehydrogenase (LDH) levels ($p < 0.001$), followed by a trend in the PX4-3 PV model ($p = 0.09$), confirmed in PV serum-treated HSOCs supernatants ($p < 0.001$). Surprisingly, IgG isolated from PF and PV patients decreased LDH supernatant levels. Our results highlight advantages and limitations of the HSOC model in investigating pemphigus acantholysis and cell death. In this work, we found evidence against apoptosis and necroptosis as relevant cell death routes for PV and, to some extent, for EPF. Despite these clues, the investigation of other cell death markers was not conclusive, and other functional essays are needed to understand their relative importance in pemphigus diseases.

Keywords: pemphigus foliaceus, pemphigus vulgaris, human skin organ culture, cell death, LDH, apoptosis.

9.2 INTRODUCTION

Pemphigus is a group of blistering autoimmune diseases causing painful bullous lesions in the skin^{1,2}. It is characterized by acantholysis, the loss of epidermal cell cohesion, and by the production of autoantibodies against adhesion proteins that connect keratinocytes: desmoglein (DSG) 1 for pemphigus foliaceus (PF) and DSG3 for pemphigus vulgaris (PV)^{1,2}. Despite the established importance of these autoantibodies in acantholysis, the role played by cell death in pemphigus is far from being unveiled^{3,4}.

Both diseases occur sporadically around the world, being the incidence of PF around 0.75-5 cases/million per year⁵ and of PV in a range of 0.6 (in Switzerland) to 32 cases/million per year (in Israel)². Despite being rare, PF is endemic (EPF) in South America and Tunisia⁶⁻⁹. In Brazil, EPF is commonly known as “*fogo selvagem*”, meaning “wild fire” in Portuguese (in literal translation), with the highest prevalence reported in 1996 (3.04%), in Limão Verde (Mato Grosso do Sul state)¹⁰. Genetic, epigenetic and environmental factors have been associated with pemphigus diseases^{2,11-13}. In a recent genetic association study of EPF, we identified associations with polymorphisms of genes involved in six out of 12 pathways orchestrating cell death¹⁴, namely resistance against apoptosis, as well as induction and regulation of pyroptosis, necroptosis, necrosis, parthanatos, and immunogenic cell death⁴. Before this study, apoptosis had been the exclusive subject of investigation on this matter in pemphigus^{3,15-20}. Even so, most of the former studies disqualified apoptosis as the major cell death mechanism for PV and PF^{3,16-18,21}. Here, we aimed to investigate the RNA and protein levels from genes involved in cell death pathways previously associated with EPF, in human skin organ culture (HSOC) challenged with triggers of PF- or PV-like skin blisters, or IgG or serum from PV and PF patients.

9.3 METHODS

9.3.1 Ethics Committee

This study was performed according to the Declaration of Helsinki, and approved by the Ethics Committee of the University of Lübeck (approval number 06-109) and by

the Brazilian National Ethics Committee (CONEP) under the protocol number CAAE 02727412.4.0000.0096 (approval number 2.045.739).,

9.3.2 Samples

9.3.2.1 Samples for human skin organ culture (HSOC)

Eight skin donors from plastic surgeries from Bremen (Aestheticum Praxisklinik) and Lübeck (Praxis und Klinik für Plastische Chirurgie), Germany, voluntarily agreed to participate in the study and donated skin for experimental work. Human breast or belly skin of eight female patients were used for the HSOCs.

9.3.2.2 Serum samples injected in HSOCs

We used five sera from EPF and five from PV patients, five sera from controls living in endemic areas (EC) and five from controls (SC). These serum samples were different from those used for IgG isolation (see below).

9.3.2.3 Samples for IgG isolation

Serum or plasma from six EPF and two PV patients, none of them under systemic treatment, as well as from four EC (**Table 1**) were pooled in equal amounts (approximately 1 mL from each donor, excepting G4) and used to isolate IgG with the Pierce™ Protein A IgG Purification Kit (Thermo Scientific) following the manufacturer's instructions. The samples were arranged in five different groups as follows: (G1) anti-DSG1/DSG3-negative ECs; (G2) anti-DSG1-positive EPF with lesions; (G3) anti-DSG1/DSG3-positive PV with mucocutaneous lesions; (G4) anti-DSG1/DSG3-positive EC; (G5) anti-DSG1-negative EPF without lesions (full remission).

Table 1. Sample groups (plasma/serum pooled when N > 1).

(G1) anti-DSG1/DSG3-negative EC		
Sample	Age (years)	Gender
C630	74	F
C635	68	M
C692	66	M
(G2) anti-DSG1-positive EPF with lesions		
Sample	Age (years)	Gender
P414	28	F
P501	21	F
P507	51	F

P521	41	F
(G3) anti-DSG1/DSG3-positive PV with mucocutaneous lesions		
Sample	Age (years)	Gender
P504	52	M
P519	42	M
(G4) anti-DSG1/DSG3-positive EC		
Sample	Age (years)	Gender
C690	51	F
(G5) anti-DSG1-negative EPF without lesions (full remission)		
Sample	Age (years)	Gender
P242	62	F
P307	31	F

EPF, endemic pemphigus foliaceus; PV, pemphigus vulgaris; EC, endemic controls; DSG1, levels of anti-DSG1 (U/mL); DSG3, levels of anti-DSG3 (U/mL); HAP, Hospital Adventista do Pênfigo; FMRP, Hospital de Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo; F, female; M, male.

9.3.2.4 Samples for granzyme measurement

Serum from 18 PV and 35 EPF patients were used for measuring granzymes A and B levels. A total of 35 unrelated controls (both of endemic and nonendemic regions) with no diagnosis or familial history of autoimmune illnesses were analyzed. Patients received clinical and/or immunohistological diagnosis. Patients and controls were recruited from 1984 to 2015 in South/ Southeastern/ CentralWestern Brazilian hospitals: Hospital Adventista do Pênfigo (Campo Grande, Mato Grosso do Sul); Lar da Caridade - Hospital do Fogo Selvagem (Uberaba, Minas Gerais); Hospital das Clínicas - University of São Paulo (Ribeirão Preto, São Paulo); Hospital de Clínicas - Federal University of Paraná (Curitiba, Paraná); Hospital de Dermatologia Sanitária São Roque (Piraquara, Paraná); and Hospital Santa Casa de Misericórdia (Curitiba, Paraná). All individuals described here were unrelated, predominantly of European ancestry and living in rural endemic areas. No history of other autoimmune diseases was reported for patients, as well as no history of any autoimmune disease, for controls. The description of the groups is available in **Supp. Table 1**.

9.3.2.5 Pemphigus biopsies samples

We used one EPF and one PV biopsies to perform RIPK1 staining. Both biopsies were collected at the Hospital das Clínicas - University of São Paulo (Ribeirão Preto, São Paulo) during the years of 2007-2008 and 2014-2015. Patients were diagnosed based on their clinical phenotype and a positive direct immunofluorescence microscopy of a perilesional skin biopsy against DSG1 for PF and DSG3 for PV.

9.3.3 HSOC

We performed the HSOC following established protocols²². Briefly, the eight skins obtained from plastic surgeries within 24 hours of surgery were prepared for culturing preserving all layers of the skin. The skin was cultured in 1 mL William's E medium per well in six-well TC plates (Sarstedt AG & Co.; Nümbrecht, Germany). Insulin syringes were used to inject: (i) 50 μ L solutions of intravenous immunoglobulin (IVIG; 7.5 μ g; Biotest AG, Dreieich, Germany) (N = 8), or of a single chain variable fragment (scFv) of an antibody directed against DSG1 and DSG3 (Px4-3; 60 ng) (N = 8) or of the exfoliative toxin A of *Staphylococcus aureus* (ETA; 100 ng; # ST101, Toxintechonology)^{23,24} (N = 7); (ii) 150 μ L (50 μ L at 0, 2 h and 4 h) of serum from PF, PV patients or controls (N = 5); (iii) 150 μ L (= 3mg) (1mg/50 μ L at 0, 2 h and 4 h) of IgG isolated from PF or PV patients or controls (N = 5) (**Table 1**) between epidermis and dermis of two skin pieces per well plate. Incubation followed at 37°C and 5% CO₂ for 24 h. Next, one skin piece per condition was frozen in RNase-free tubes in liquid nitrogen and stored at -80°C. One half of the second pieces were put into TissueTek Intermediate cryomolds with the cutting side facing downwards, covered with TissueTek optimum cutting temperature Compound (Thermo Fisher Scientific; Waltham, USA) and frozen at -20°C. The other half was put into paraffin molds and stored in 4% Roti-Histofix (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) at room temperature (RT). Later, they were embedded in paraffin with the cutting side facing downwards. The supernatant was transferred into SafeSeal reaction tubes and stored at -80°C.

9.3.4 mRNA expression

RNA was extracted from frozen samples of the first (IVIG, Px4-3 or ETA) HSOC treatment using mirVana™ miRNA Isolation Kit (Ambion, Life Technologies Corporation; Carlsbad, CA, USA), according to the manufacturer's instructions for large RNA fraction enrichment. After removal of contaminant DNA with the TURBO DNA-free™ Kit (Invitrogen AG; Carlsbad, CA, USA), the RNA samples were submitted to reverse transcription with the use of the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Waltham, MA, USA), according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qPCR) was used to measure gene expression levels of cluster differentiation *CD47*, eukaryotic translation initiation factor 2 alpha kinase 3 *EIF2AK3*, interleukins *IL1A*, *IL1B*, *IL18*, signal regulatory protein alpha *SIRPA*, tumor necrosis factor *TNF* and TNF receptor associated factor 2 *TRAF2*, which were normalized to the expression of the ribosomal protein L13 housekeeping gene *RPL13A*. The qPCR reactions were performed in triplicates using TaqMan® Fast Advanced Master Mix (Applied Biosystems; Waltham, MA, USA), 20 ng of cDNA and the following specific probes: *CD47* - Hs00179953_m1; *EIF2AK3* - Hs00984003_m1; *IL1A* - Hs00174092_m1; *IL1B* - Hs01555410_m1; *SIRPA* - Hs00757426_s1; *TNF* - Hs00174128_m1; *TRAF2* - Hs00184192_m1; *RPL13A* - Hs04194366_g1 (Thermo Fisher; Waltham, MA, USA), according to the manufacturer's protocol. The qPCR amplifications were performed using StepOne Real-Time PCR Systems (Applied Biosystems; Waltham, MA, USA).

9.3.5 Stainings

9.3.5.1 Haematoxylin and eosin (HE)

HE staining was performed with paraffin slides that were first incubated in a xylene bath (Merck; Darmstadt, Germany) for 20 min and then swiveled back and forth in two other baths. Subsequently, swiveling in three ethanol baths (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) with descending concentrations (99.8%, 96%, 70%) and finally in distilled water (Klinisch Experimentelle Forschungseinrichtung; Universität zu Lübeck, Germany) followed. Next, the slides were incubated for 5 min in a haematoxylin solution according to Papanicolaou (Merck; Darmstadt, Germany) and washed with tap water. Afterwards, the slides were slued in an acetic acid alcohol solution (Th. Geyer GmbH & Co. KG; Renningen, Germany), washed again and swiveled in an ammonia solution (Merck; Darmstadt, Germany). The staining for eosin (Waldeck GmbH & Co. KG; Münster, Germany) followed after another washing step for 20 sec. Dehydration of the section was obtained using an ascending ethanol row (reverse from before including xylene as a last step) and covered with a cover slide (Carl Roth GmbH + Co. KG; Karlsruhe, Germany), using the Leica CM-3050S automatic cover dispenser (Leica Mikrosysteme Vertrieb GmbH; Wetzlar, Germany).

9.3.5.2 Desmoglein (DSG) 1 and 3

Three different stainings were performed on cryo slides to visualize DSG1, DSG3 or both together. The slides were dried at RT for 10 min and then fixed in acetone (Thermo Fisher Scientific; Waltham, MA, USA) at -20°C for 10 min as well. Chosen sections were encircled with a fat pen (DAKO Pen S2002; Agilent; Santa Clara, CA, USA), without contact. Then, slides were washed three times in PBS for 10 min, each time in a humid chamber (abArt UG und Co. KG; Waldbrüttelbrunn, Germany). The sections were pre-incubated with 10% normal goat serum (DAKO GmbH; Jena, Germany) in PBS, for 20 min. Next, goat serum was discarded and the primary antibody diluted in 2% normal goat serum in PBS (mouse anti-human DSG1, 1:100; mouse anti-human DSG3, 1:50; both from OriGene Technologies, Inc.; Rockville, MD, USA) was added for overnight incubation at 4°C. The following day, another washing step was performed and the slides were incubated with 1:200 of the secondary antibody (for DSG1, CyTM3-conjugated AffiniPure goat anti-mouse, polyclonal, Jackson ImmunoResearch; for DSG3, goat anti-mouse IgG (H+L) Alexa Fluor 488, A11029, polyclonal, Thermo Fisher Scientific; Waltham, MA, USA) in 2% normal goat serum in PBS for 45 min at RT. For the double staining of both DSG1 and DSG3, the incubations of primary and secondary antibodies of DSG3 were done after the incubation of primary and secondary antibodies of DSG1. Finally, another three-step wash was conducted and the slides were covered with 4,6-diamidino-2-phenylindole DAPI Fluoromount-G (Southern Biotech; Birmingham, AL, USA). Protected from light, the slides were dried for 1 h and then stored at -20°C.

9.3.5.3 Anti-desmoglein 1 and 3 single chain variable fragment (anti-DSG1/3 scFv)

Anti-DSG1/3 scFv staining was used to validate their binding in the blisters. The staining was conducted on cryo slides that were dried, fixed in acetone and encircled in the same manner as described above. This was followed by two-times washing for 3 min using washing buffer [100 mL 10x TBS (61 g Trizma-Base + 88 g sodium chloride + distilled water for 1 L) + 500 µL 2 M calcium chloride + 900 mL distilled water + 500 µL Tween20 (Euroimmun, Medizinische Labordiagnostika AG; Lübeck, Germany)]. Blocking nonspecific binding sites was subsequently performed by incubation for 30 min with a blocking buffer containing 1% bovine serum albumin (BSA) [0.5 g BSA (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) + 50 mL washing buffer; sterile filtered]. After discarding the blocking buffer, incubation for 1 h

with the secondary antibody (rat anti-hemagglutinin monoclonal antibody high affinity, 1:100; Roche Deutschland Holding GmbH; Grenzach-Wyhlen, Germany) followed. Another three-times washing step for 3 min was performed before incubating the sections with the tertiary antibody (goat anti-rat IgG (H+L) Alexa Fluor 594, 1:200; Invitrogen AG; Carlsbad, CA, USA) for 30 min. A final washing step was conducted as before and the slides were covered with DAPI Fluoromount-G, dried for 1 h protected from light and stored at -20°C.

9.3.5.4 Human immunoglobulin G (IgG)

Staining against human IgG was conducted for qualitative analysis of the injected antibodies in the HSOC. The washing and blocking buffers were the same as described for anti-DSG1/3 scFV staining. Cryo slides were dried, followed by fixation as previously described. Afterwards, the slides were washed with the washing buffer 3 times for 5 min and encircled with the fat pen. In the next step, the blocking buffer was used for blocking nonspecific binding sites at RT for 30 min. Subsequently, the sections were incubated with the secondary antibody (F(ab)₂ anti-human IgG FITC, 1:100; Bio-Rad Laboratories, Inc.; Hercules, CA, USA) for 1 h at RT, as no primary antibody is needed. Finally, another washing step was performed and the slides were covered with DAPI Fluoromount-G, after drying for 1 h at RT. Afterwards, the sections were stored at -20°C.

9.3.5.5 Nuclear Ki67 protein and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (Ki67-TUNEL)

This double staining was performed on cryo slides with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Merck; Darmstadt, Germany). The slides were dried at RT for 5 min. A 1% formaldehyde solution in PBS (2.7 mL formaldehyde + 97.3 mL PBS) was used for subsequent fixation at RT for 10 min. The slides were washed 3 times in PBS, for 5 min. A second fixation followed for 5 min at -20°C in acetic acid mixed with ethanol (1:2). Next, the sections were encircled as previously described and incubated for 5 min in the equilibrium buffer. The enzyme reaction with the terminal deoxynucleotidyl transferase was performed on half of the sections, the other half was used as the negative control (reaction buffer without enzyme). The sections were incubated with the enzyme solution (or only with the reaction buffer) for 1 h at 37°C, in the incubator. Afterwards, the stop buffer at a temperature of 37°C

was applied and also incubated at 37°C for 10 min. Before the preincubation with 10% normal goat serum at RT for 20 min, a three-step wash in PBS was performed. Next, the primary antibody (FLEX mouse anti-human Ki67 antigen; DAKO GmbH; Jena, Germany) was applied overnight at 4°C. The next day, the slides were washed again with PBS and incubated with the first fluorescein-labeled anti-digoxigenin secondary antibody for 30 min at RT to visualize the enzyme. Another wash step was performed, followed by the incubation of the second secondary antibody (goat anti-mouse IgG rhodamine red) directed against Ki67, for 45 min. The sections were washed, covered with DAPI Fluoromount-G, dried and stored at -20°C.

9.3.5.6 Cleaved caspase 3 (cCASP3)

Cryo slides were dried at RT for 10 min. Subsequent fixation took place at -20°C in acetone for 10 min. The slides were washed with a PBS buffer 3 times for 5 min, and sections were encircled with the fat pen. Blocking with 5% normal goat serum in PBS followed for 1 h. Afterwards, the sections were incubated with the primary antibody (rabbit anti-human cleaved CASP-, 1:400; Cell Signaling Technology, Inc.; Danvers, MA, USA) diluted in 0.3% Triton X-100 (Sigma Aldrich; St. Louis, MO, USA) in PBS overnight at 4°C. The next day, another washing step with PBS followed three times for 5 min. Thereupon, incubation with the secondary antibody (goat anti-rabbit Alexa Fluor 488, polyclonal, 1:800; Jackson ImmunoResearch Europe Ltd.; Ely, UK) diluted in 0.3% Triton X-100 and in 2% normal goat serum in PBS followed for 90 min at RT. Lastly, the slides were washed once more, covered with DAPI Fluoromount-G, dried and stored at -20°C.

9.3.5.7 Calreticulin (CALR) and Receptor-interacting protein kinase 1 (RIPK1)

These stainings were performed using paraffin slides that were rehydrated in a descending ethanol row starting with two xylene baths for 15 min each. This was followed by baths with 99.8%, 96%, 75% and 50% ethanol and finally with distilled water, for 5 min each. Then the antigen retrieval was conducted by cooking the slides in citrate buffer [2.9 g sodium citrate dihydrate (Sigma-Aldrich; St. Louis, MO, USA) + 0.5 mL Tween20 + 1 L distilled water, pH = 6] in a pressure cooker for 10 min at 310°C. After cooling down, the sections were encircled and washed 3 times with PBS for 5 min. Subsequently, unspecific binding sites were blocked with 10% normal goat serum in PBS for 20 min. For CARL staining, the sections were incubated overnight

with anti-CALR primary antibody (polyclonal; Abcam plc.; Cambridge, UK) diluted 1:100 in 2% normal goat serum in PBS at 4°C. Anti-RIPK1 (monoclonal; Abcam plc.; Cambridge, UK) served as the primary antibody for RIPK1 staining. Incubation with the secondary antibody goat anti-rabbit IgG (Jackson ImmunoResearch Europe Ltd.; Ely, UK), for CALR staining, or goat anti-mouse IgG (H+L) Alexa Fluor 488 (Life Technologies; Carlsbad, CA, USA), for RIPK1, were diluted 1:200 in 2% normal goat serum in PBS for 45 min at RT followed by a washing step of three times for 5 min. Another washing step was conducted and the slides were covered with DAPI Fluoromount-G, dried and stored at -20°C.

9.3.6 Quantification of soluble proteins

9.3.6.1 Lactate dehydrogenase (LDH) levels in HSOC supernatants

LDH assay was performed using the Cytotoxicity Detection Kit (F. Hoffmann-La Roche AG; Basel, Switzerland) and the supernatants of the HSOCs as samples. Before use, the components and samples were thawed. The measurements were performed in triplicate. William's E medium (used for culture) was used as the blank. Furthermore, 100 µL of the reaction solution with 1 catalyst:46 dye ratio was added to every well except the blank, using a multichannel pipette. An incubation of 30 min at RT in the dark followed. Afterwards, fluorescence at 490 nm was measured using a microplate reader (Tecan Group AG; Männedorf, Switzerland).

9.3.6.2 Granzymes A and B (GZMA and GZMB) levels in serum

An enzyme-linked immunosorbent assay (ELISA) was performed to measure GZMA and GZMB serum levels of patients with EPF (N = 35), PV (N = 18), and controls (N = 35), using the Human Granzyme A and B ELISA Kits (Abcam, cat. ab255728 and ab46142), following the protocol recommended by the manufacturer.

9.3.7 Statistics

Split measurement was performed on pictures of the HE staining with the Keyence fluorescence microscope (BZ9000 BIOREVO, Keyence; Osaka, Japan). Pictures were taken with 100x magnification without overlapping. Length measurement of the epidermis and of the split (if one occurred), was conducted with ImageJ (Wayne

Rasband). Split ratio (lesion length/ epidermis length) was calculated in the whole section. Data distribution agreement with expected normality was tested with D'Agostino & Pearson omnibus normality test and Shapiro-Wilk normality test. In accordance with the results, Kruskal-Wallis tests with Dunnett's post test were used for comparing split measurements and numbers of cells positive for Ki67 and TUNEL (indicative of cell proliferation and cell death, respectively), RIPK1 (a necroptosis marker), CALR (an ICD marker) and cCASP3 (an apoptosis marker). On the other hand, LDH levels were compared through one-way ANOVA with Dunnett's post test. For gene expression analyses, fold change (FC) values were calculated by the $2^{-\Delta\Delta Cq}$ comparative quantification cycle (Cq) method and compared using Kruskal-Wallis tests with Dunnett's post test. All tests were performed, using GraphPad Prism v.5 software (GraphPad Software Inc.; San Diego, CA, USA). The limit of significance adopted for all analysis was $p < 0.05$.

9.4 RESULTS

9.4.1 Blisters were induced in HSOCs treated with (1) Px4-3 and ETA, but not with IVIG, (2) pooled IgG isolated from PF or PV patients, but not with (3) serum from PF and PV patients

Confirming this approach as suitable for mimicking pemphigus, injection of Px4-3^{22,25} or ETA^{23,24} in HSOC specimens induced split formation at the expected intraepidermal layers: in the suprabasal layer after Px4-3 injection, mimicking the PV phenotype ($p < 0.0001$); and in the granular layer after ETA injection, mimicking the PF phenotype ($p < 0.0001$) (**Figures 1A and 1B**). Px4-3 immunofluorescence showed its binding to DSG1 and DSG3. Membranes of the keratinocytes were stained in red, presenting a honeycomb pattern which is typical for stained DSG (**Figure 1C**). Qualitative stainings for DSG1 and DSG3 displayed the typical honeycomb pattern with different intensities throughout the epidermis (**Figure 1D**). Therefore, DSG1 is mainly expressed in the superficial layers of the epidermis, whereas DSG3 shows greater abundance in the basal layers. These results indicated that culturing the skin for 24 h under these conditions does not impair the function nor the structure of DSG1 and DSG3.

Pooled serum from EPF and PV patients was not able to induce blisters in the HSOCs. However, anti-DSG IgGs bound keratinocytes at the corresponding skin layers (**Supp. Figure 1**). In sharp contrast to these results, concentrated IgGs, regardless of their source, surprisingly elicited blisters (**Supp. Figure 2**). However, only IgGs from PF and PV patients (G2 and G3) bound keratinocytes (**Supp. Figure 3**). On the other hand, neither PV nor EPF serum-injected HSOCs formed any blisters. As the results were controversial, we did not perform subsequent staining and qPCR with these HSOC treatments. We only used the supernatants of these HSOCs to measure LDH levels.

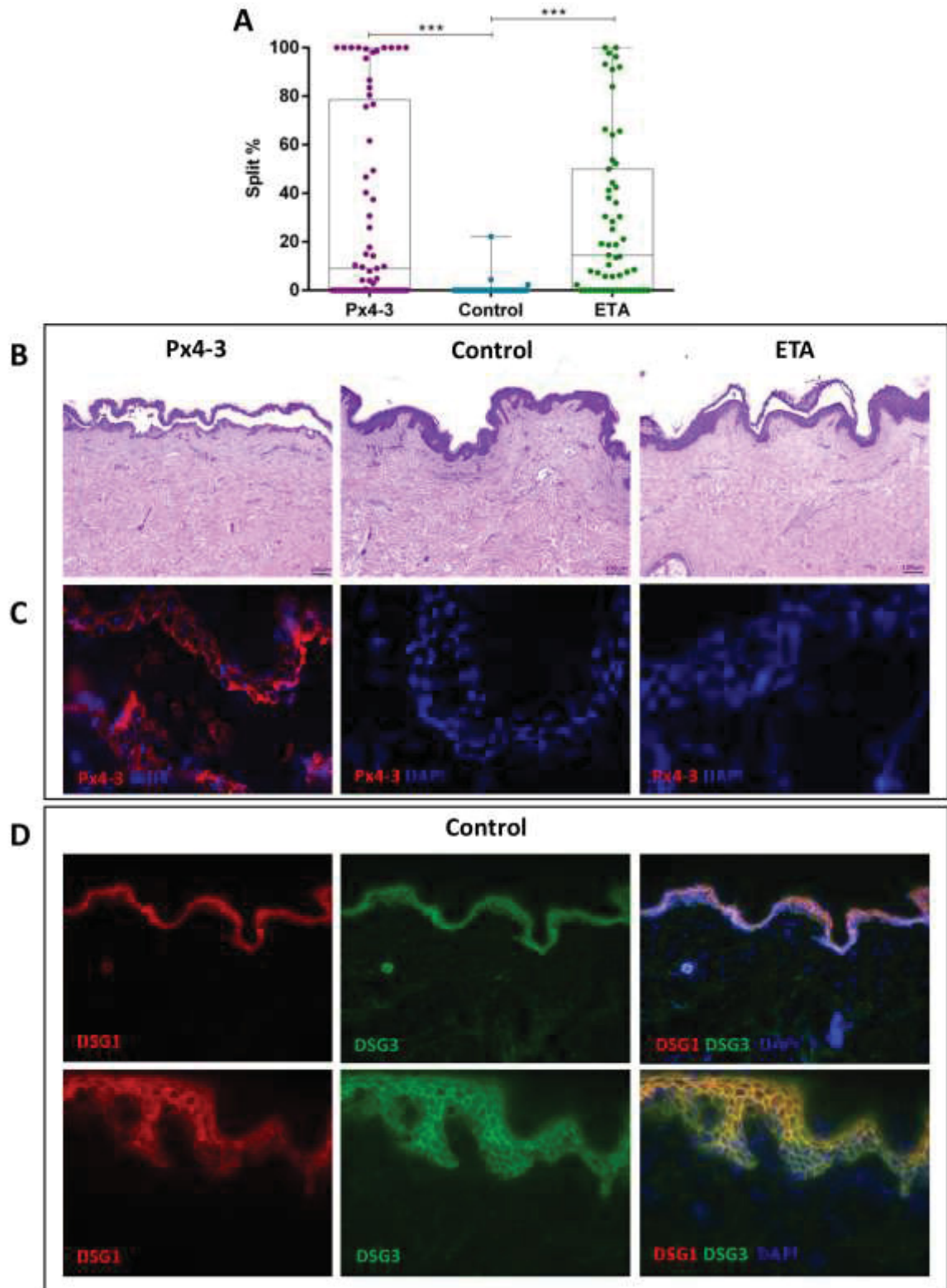


Figure 1. HSOC characterization. **A.** Split formation in each treatment. Data is given by split ratio. **B.** Light micrographs of HE stained HSOC specimens (100-fold, N = 8) after injection of Px4-3 (PV-like), IVIG (negative control) or ETA (PF-like, as a positive control and reference). **C.** Direct immunofluorescence microscopy for Px4-3. **D.** Immunofluorescence

microscopy for DSG1, DSG3 and DSG1/DSG3 in IVIG-injected control HSOCs (upper figures, 100-fold; lower figures, 600-fold). *** $p < 0.0001$.

9.4.2 Gene expression levels and positive cell counts for Ki67 and TUNEL did not differ from controls

The *CD47*, *EIF2AK3*, *IL1A*, *IL1B*, *IL18*, *SIRPA*, *TNF* and *TRAF2* genes were not differentially expressed between HSOCs with different treatments (Px4-3, control and ETA) ($p > 0.05$) (**Supp. Figure 4**). Also, cell counts positive for Ki67 and TUNEL in the epidermis of HSOCs treated with ETA or Px4-3 did not differ from those for controls (HSOCs injected with IVIG) (**Figure 2**). Yet, GZMA and GZMB were not detected in sera from pemphigus patients and controls, probably due to the sera used being old.

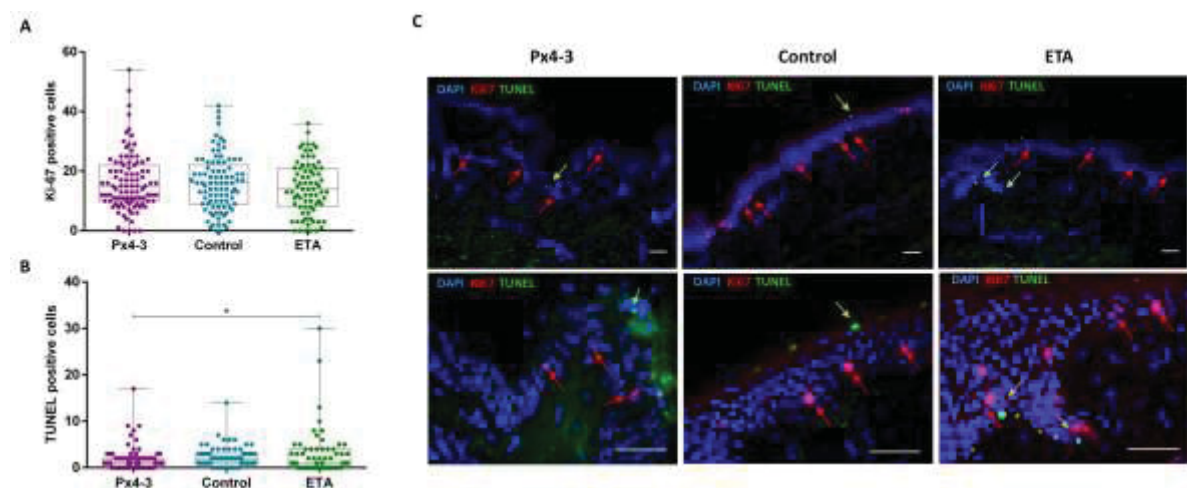


Figure 2. Evaluation of Ki-67-TUNEL in HSOCs. **A.** Ki-67-positive cells in the epidermis after Px4-3 (PV-like), IVIG (negative control) and ETA (PF-like) HSOC treatments (200-fold, N = 8). **B.** TUNEL-positive cells in the epidermis of Px4-3 (PV-like), IVIG (control) and ETA (PF-like) treatments (200-fold, N = 8). **C.** Immunofluorescence microscopy for Ki67 (red) and TUNEL (green) in the epidermis of Px4-3 (PV-like), IVIG (controls) and ETA (PF-like) treatments (200-fold, N = 8)

9.4.3 Px4-3-treated HSOCs (PV-like) present fewer positive cells for cCASP3 and lower RIPK1, but not CALR, protein expression

Total counts of cells containing cCASP3 were lower in HSOCs injected with Px4-3 (PV-like) compared with ETA-injected (PF-like) and IVIG-injected (controls) HSOCs

($p < 0.0001$) (**Figures 3A and 3B**). There was no difference for CALR protein expression (**Figures 3C and 3D**), but reduced RIPK1 protein levels were detected in the PV-like model compared to the PF-like model and IVIG-injected control (**Figures 3E and 3F**). For the first time, the expression of RIPK1 was observed in PV and PF biopsies of patients (**Figure 4**).

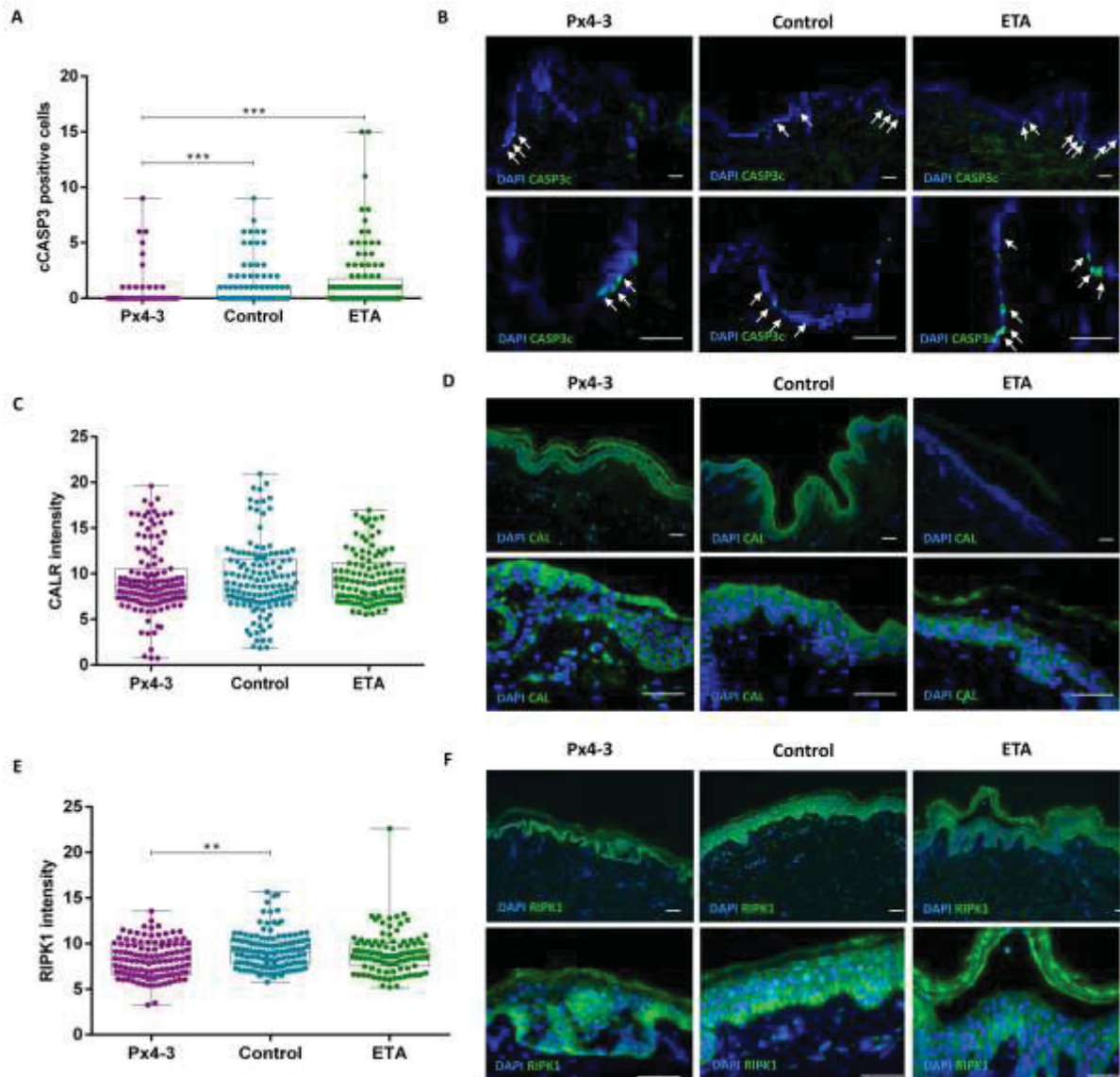


Figure 3. Evaluation of cASP3, CALR and RIPK1 in HSOCs. **A.** cASP3-positive cells in the epidermis of Px4-3 (PV-like), IVIG (negative control) and ETA (PF-like) HSOC treatments. **B.** Immunofluorescence microscopy against cASP3 (200-fold, N = 8). **C.** CALR intensity in the epidermis of Px4-3, IVIG and ETA HSOC treatments. **D.** Immunofluorescence microscopy against CALR (200-fold, N = 8). **E.** RIPK1 intensity in the epidermis of Px4-3, IVIG and ETA HSOC treatments. **F.** Immunofluorescence microscopy against RIPK1 (200-fold, N = 8). ($p < 0.005$).

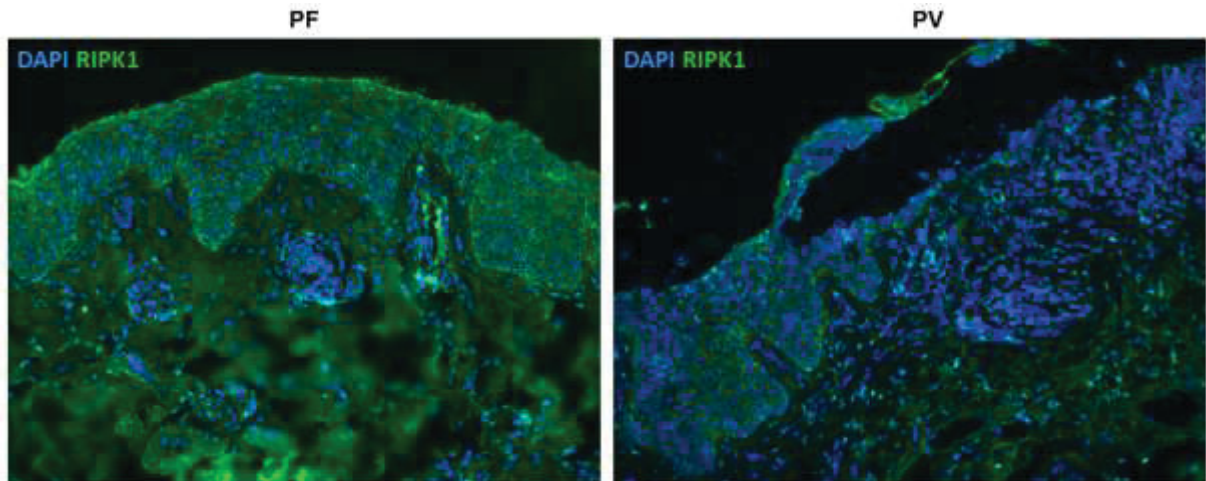


Figure 4. Immunofluorescence microscopy against RIPK1 in a PF perilesional biopsy and PV lesional biopsy (200-fold).

9.4.4 ETA and IgG from EPF patients present opposite effects on LDH levels

Compared to controls (IVIg), high LDH levels were measured in supernatants from the ETA-treated model ($p < 0.001$). For the Px4-3 treated model, a trend was found in the same direction ($p = 0.09$) (**Figure 5A**). Surprisingly, the injection of IgG from EPF or PV patients decreased LDH levels (**Figure 5B**). Furthermore, LDH levels were higher in supernatants of HSOCs treated with PV serum, but not in those treated with EPF serum ($p < 0.001$) (**Figure 5C**).

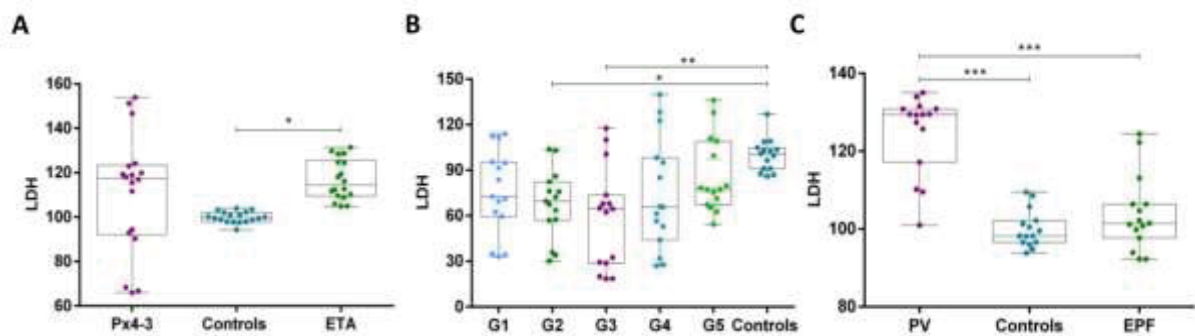


Figure 5. LDH levels in supernatants of HSOCs injected with **(A)** Px4-3 (PV-like), IVIg (negative control) and ETA (PF-like) HSOC treatments, **(B)** IgG isolated from EC subjects or EPF or PV patients, as well as from “controls”, **(C)** serum from PV patients, controls or EPF patients. PV, pemphigus vulgaris; EPF, pemphigus foliaceus; Px4-3, single chain variable fragment (scFv) of an antibody directed against DSG1 and DSG3; ETA, exfoliative toxin of *Staphylococcus aureus*; G1, anti-DSG1/DSG3-negative ECs; G2, anti-DSG1-positive EPF with lesions; G3, anti-DSG1/DSG3-positive PV with mucocutaneous lesions; G4, anti-DSG1/DSG3-positive EC; G5, anti-DSG1-negative EPF without lesions (full remission).

9.5 DISCUSSION

In this work, we started the evaluation of cell death routes that may be held responsible for keratinocyte acantholysis and death in pemphigus, using cultured human skin. While keratinocyte cell cultures²⁶ may mimic certain aspects of pemphigus pathogenesis, they do not reproduce skin cell structure and complexity. Mouse models of pemphigus, however, are hampered by the relatively complex experimental procedures^{23,27}. Moreover, their skin does not follow human skin desmoglein distribution and their skin cell (epi)genomes differ from humans, and thus respond differently to pemphigus triggers^{28,29}. To overcome these limitations, to implement the 3R principle and to more specifically replace animal experiments, we used HSOC models of pemphigus²². HSOC, however, also has some limitations, such as its disconnection from innervation and blood circulation.

Thus, despite the fact that Px4-3 and ETA induced PV- and PF-similar blisters in the HSOCs, respectively, in contrast with IVIG (control), results with these triggers may not recapitulate pemphigus pathogenic processes. Moreover, patient serum containing actual anti-DSG antibodies were not able to induce skin blisters, despite recognizing keratinocyte desmosomes. This was not a mere consequence of IgG abundance, since concentrated IgGs, regardless of their source, were able to induce skin lesions. Thus, it seems clear that IgG antibodies and serum components are not sufficient to induce pemphigus-like skin blisters when directly injected in the skin. Furthermore, blisters may be induced simply by an IgG excess, regardless of antibody specificity. These findings inspire caution when interpreting results in pemphigus models, achieved with supraphysiological IgG concentrations.

HSOCs disconnection from circulating blood cells and other missing elements may explain why the genes whose products are considered cell death markers^{4,14} did not change gene expression after Px4-3 and ETA treatments, despite the occurrence of acantholysis. These include markers associated with ICD (*SIRPA*, *CD47*, *EIF2AK3*), pyroptosis (*IL1B*, *IL1A*, *IL18*), necroptosis and apoptosis (*TNF* and *TRAF2*). We used a specific keratinocyte housekeeping gene, *RLP13A*, to normalize gene expression levels^{30–33}. Thus, absence of differential gene expression in these models seems trustable for the most abundant cell type within the HSOCs, although single cell RNA sequencing may reveal additional subtle differences among keratinocyte layers, as well as in other resident cells types, as melanocytes and Langerhans macrophages.

We also did not identify cell proliferation and cell death differences among these same treatments, using Ki67-TUNEL stainings. No difference was also found for CALR, an ICD marker¹⁴. The absence of some differences for ICD-related genes may derive from the relatively low abundance of ICD marker-presenting phagocytic cells in the HSOCs (which commonly present SIRPA)⁴. However, cCASP3-positive cells and RIPK1 protein levels, markers of apoptosis and necroptosis¹⁴, respectively, decreased after Px4-3 injection (PV-like model). RIPK1 was also poorly expressed in PV lesions, compared with EPF perilesional epidermis. These cell death routes seem to be less activated in the Px4-3-treated, PV-like model, compared with controls. There were no differences regarding the ETA-treated, PF-like model. These results reinforce our conclusions and those of others, who demonstrated that apoptosis is not a causal route for keratinocyte cell death in pemphigus²¹. They also are the first evidence that the same conclusion may apply for necroptosis as well, at least for PV. LDH release is a widely used marker in cytotoxicity assays. It is a soluble, stable cytosolic enzyme that is rapidly released into the cell culture medium after disruption of the cell plasma membrane³⁴. LDH increased after ETA treatment of HSOC, probably because of DSG1 degradation, but decreased after injections with concentrated IgGs from PF and PV patients. It also presented a trend for higher levels after Px4-3 treatment of HSOC, confirmed by higher LDH levels after HSOC injection with PV sera. These results seem to reinforce Px4-3 treatment of HSOC as a suitable PV-mimicking model, whereas ETA, despite its reported attack on desmosomal DSG1, probably causes the PF-similar blisters by more distantly related mechanisms²³. Higher LDH levels were also found by others in the basal and prickle epidermal layers, as well as in Langerhans cells of PV patient biopsies³⁵. Our results highlighted advantages and limitations of the HSOC model in the investigation of cell death routes, using blister triggers that mimic PF and PV lesions, as well as patient serum and IgGs. For PV and to some extent for EPF, we found evidence against apoptosis and necroptosis as relevant cell death routes. However, the investigations of other cell death markers were not conclusive, and other functional essays are needed to describe their relative importance in pemphigus diseases.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTIONS

VB-BH, ABWB and JEH designed the study; MLPE, ES, GAC, NB provided the samples; VB-BH, EL, AP performed the experiments; VB-BH analyzed the data; VB-BH, AP and ABWB drafted the manuscript. JEH financed the research. All authors critically reviewed this manuscript.

DATA AVAILABILITY STATEMENT

[Data - cell death pathways manuscript.xlsx](#)

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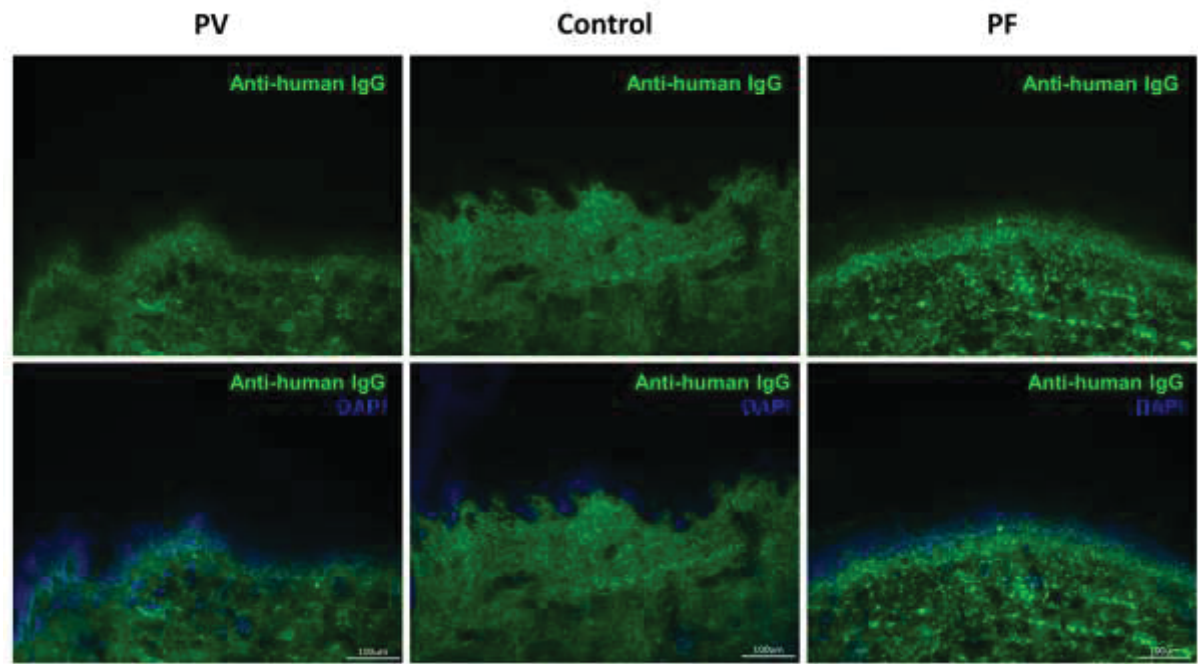
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9.7 SUPPLEMENTARY MATERIAL

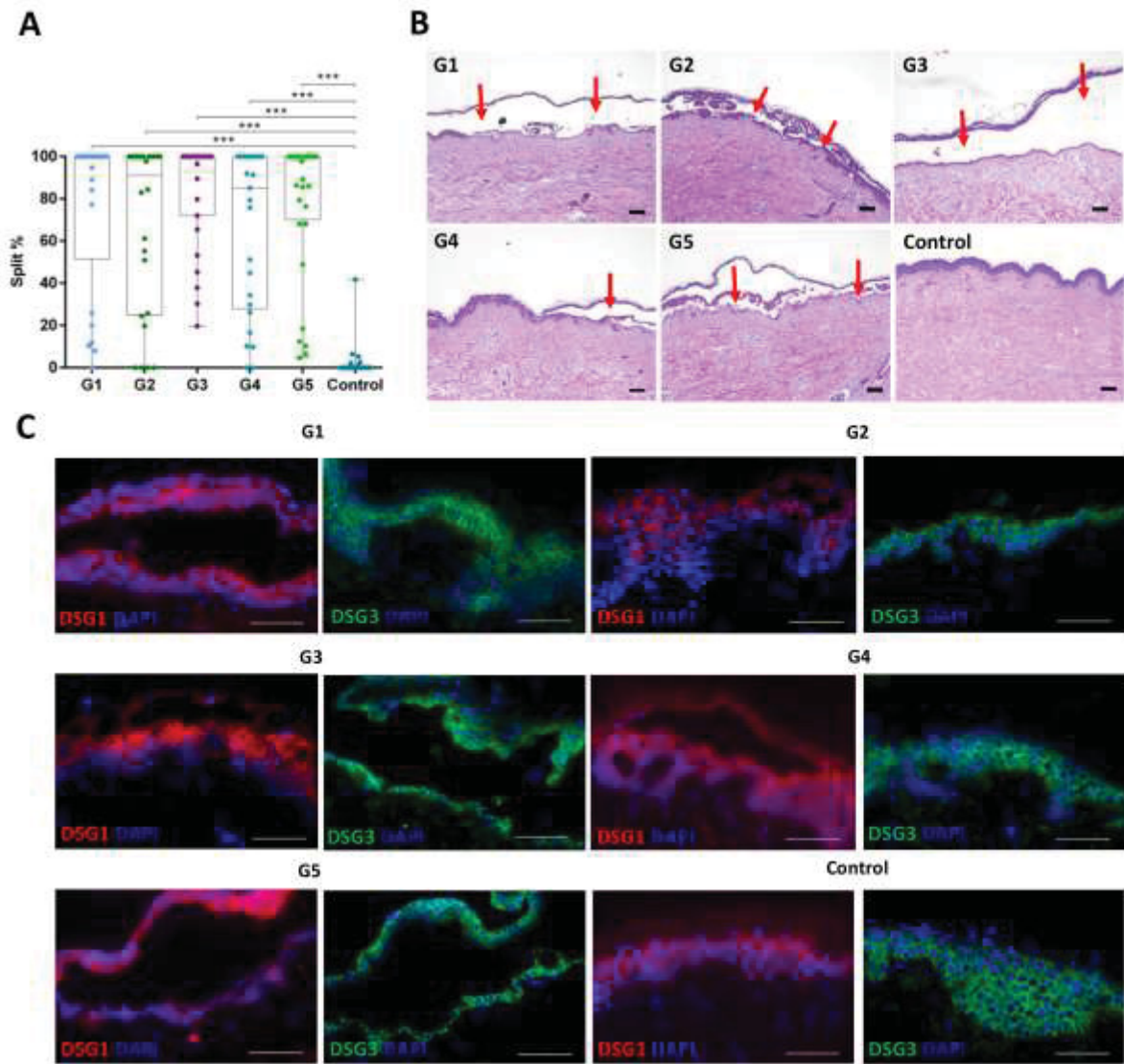
Supp. Table 1. Subject description Clinical and demographic aspects of subjects enrolled in the measurements of granzymes A and B.

Subjects	PV (N = 18)	Control (N = 35)	EPF (N = 35)
Median age (min, max)	45 (16, 80)	53 (20, 73)	37 (12, 74)
Sex (women)	72 %	46 %	60 %

Min, minimum; max, maximum; PV, pemphigus vulgaris; EPF, endemic pemphigus foliaceus

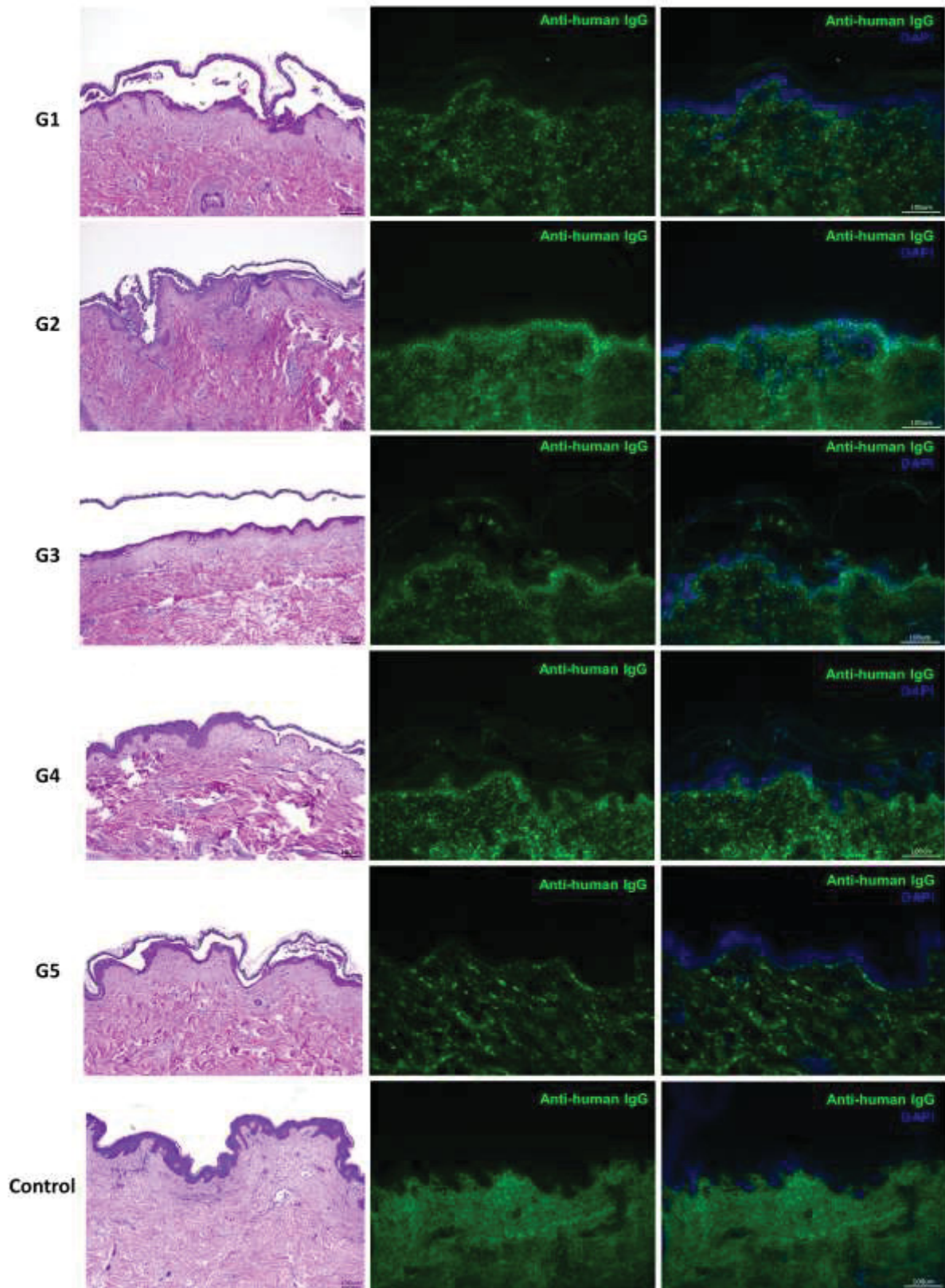


Supp. Figure 1. Immunofluorescence against the human IgG binding in the keratinocytes of the human skin organ culture (HSOC) treated with serum from pemphigus patients. In the top, human skin organ culture specimens after injection of serum from: pemphigus foliaceus patients, controls and pemphigus vulgaris patients, respectively. No split was formed in this treatment. PV, pemphigus vulgaris; PF, pemphigus foliaceus.



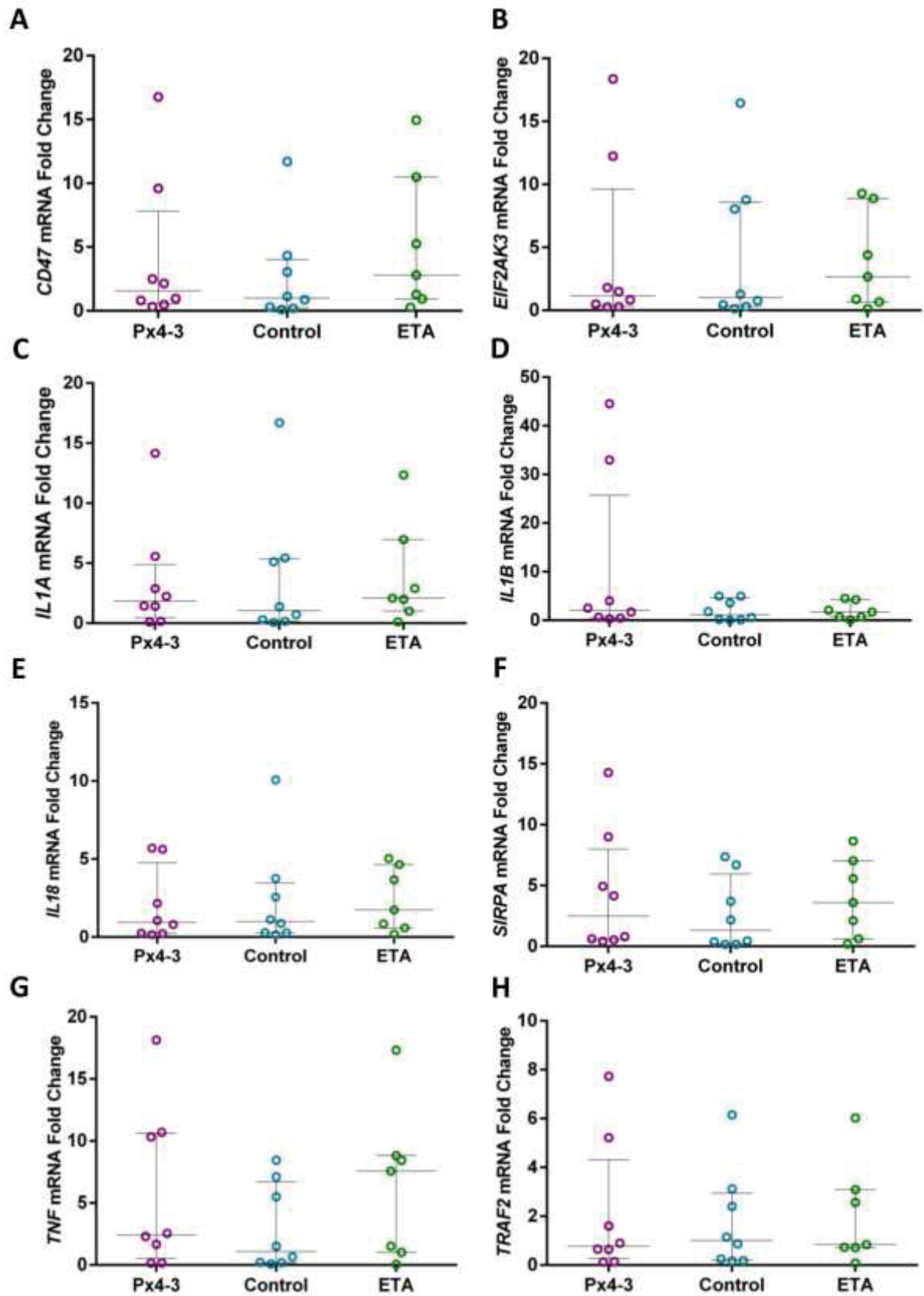
Supp. Figure 2. Characterization of the human skin organ culture (HSOC) treated with IgG .

A. Split formation in each treatment. **B.** Light micrographs of hematoxylin & eosin stainings (100-fold, N = 5) of human skin organ culture specimens after injection of IgG from: G1) Controls from endemic areas without anti-Dsg1 and anti-Dsg3; 2) PF with lesions and not under systemic treatment and with only anti-Dsg1; 3) PV with mucocutaneous lesions and not under systemic treatment and with anti-Dsg1 and anti-Dsg3; 4) Controls from endemic areas with anti-Dsg1 and anti-Dsg3; 5) PF without lesions and not under systemic treatment and without anti-Dsg1 and anti-Dsg3 (full remission). **C.** Immunofluorescence microscopy against DSG1, in red. Immunofluorescence microscopy against DSG3, in green (600-fold, scale bar 50 μ m, N = 5). *** $p < 0.0001$. (G1) anti-DSG1/DSG3 negative ECs; (G2) anti-DSG1 positive EPF with lesions; (G3) anti-DSG1/DSG3 positive PV with mucocutaneous lesions; (G4) anti-DSG1/DSG3 positive EC; (G5) anti-DSG1 negative EPF without lesions (full remission).



Supp. Figure 3. The human IgG binding in the keratinocytes of the human skin organ culture (HSOC) treated with IgG . In the left, light micrographs of hematoxylin & eosin stainings (100-fold, N = 5) of human skin organ culture specimens after injection of IgG from: (G1) anti-DSG1/DSG3 negative ECs; (G2) anti-DSG1 positive EPF with lesions; (G3) anti-

DSG1/DSG3 positive PV with mucocutaneous lesions; (G4) anti-DSG1/DSG3 positive EC; (G5) anti-DSG1 negative EPF without lesions (full remission). In the middle and in the right, immunofluorescence against human IgG. Split were formed in all treatments.



Supp. Figure 4. Gene expression results in HSOC treated with Px4-3, IVIG and ETA. No association was found. Px4-3, single chain variable fragment (scFv) of an antibody directed against DSG1 and DSG3; ETA, exfoliative toxin of *Staphylococcus aureus*. Px4-3, single

chain variable fragment (scFv) of an antibody directed against DSG1 and DSG3; ETA, exfoliative toxin of *Staphylococcus aureus*. *CD47*, cluster differentiation *CD47*; *EIF2AK3*, eukaryotic translation initiation factor 2 alpha kinase 3; *IL1A*, interleukins 1 A; *IL1B*, interleukins 1 B; *IL18*, interleukins 18; *SIRPA*, signal regulatory protein alpha; *TNF*, tumor necrosis factor; *TRAF2*, TNF receptor associated factor 2 *TRAF2*.

10 CONSIDERAÇÕES FINAIS

Nosso trabalho contribuiu para esclarecer parte da etiologia e mecanismos patológicos do pênfigo, as quais ajudarão no entendimento da doença e oferecem novas perspectivas de investigação.

Escrevemos uma revisão atualizada sobre moléculas e características envolvidas em vias de morte celular no pênfigo foliáceo e pênfigo vulgar. Analisamos os resultados de forma sistemática e as divergências entre os diferentes trabalhos foram destacadas, assim como as possíveis falhas que devem ser evitadas em futuros trabalhos, especialmente a carência de estudos com outras vias de morte celular além da apoptose.

Nossos estudos de associação genética, tanto relacionados às vias de morte celular como às subunidades da cadeia respiratória mitocondrial, foram pioneiros na investigação do pênfigo. A validação funcional das associações encontradas também proporcionará uma melhor compreensão da patogênese do pênfigo.

Pela primeira vez, SNPs localizados em genes envolvidos em todas as cascatas de morte celular conhecidas foram sistematicamente investigados em uma única doença. Associações genéticas foram encontradas com genes cujos produtos são envolvidos na apoptose, necrose, necroptose, partanatos, piroptose e via de morte celular imunogênica. O perfil de associação genética com variantes de *TNF*, *TRAF2*, *CD36* e *PAK2* favorece a sobrevivência celular e a inflamação. Por outro lado, a suscetibilidade é conferida por variantes de *CD47* e *SIRPA* da via de morte celular imunogênica, que levaria à internalização excessiva de *debris* celulares e apresentação de antígenos, o que pode aumentar a produção de autoanticorpos de PF endêmico.

Ainda, de forma inédita, considerando as subunidades da cadeia respiratória mitocondrial, encontramos associação de uma variante do gene do componente essencial do complexo da cadeia respiratória I e outra do complexo IV com PF endêmico. Neste trabalho foi possível realizar uma abordagem que evidenciou diferenças entre PF endêmico, PF esporádico e PV.

Nós caracterizamos de forma ultraestrutural os desmossomos do modelo de miniórgão de pele humana que mimetiza o fenótipo final de pênfigo. Este modelo já foi utilizado para testar possíveis fármacos que combatem o pênfigo.

Com a utilização do miniórgão de pele, foi possível avaliar a expressão de C5aR1 e C5a. Interessantemente, a expressão de C5aR1 foi elevada tanto ao nível do número de células que expressam o receptor na derme, como ao nível de mRNA para o modelo de PV (e não para o modelo de PF, onde as células positivas para C5aR1 ocorreram em menor número). Surpreendentemente, células expressando C5aR1 também ocorreram em maior número na derme, tanto em pacientes com PF endêmico (nestes também na epiderme), quanto naqueles com PF esporádico. Estes resultados ressaltam a importância da expressão de C5a/C5aR1 no pênfigo. Pela primeira vez, C5a foi encontrado aumentado em soro de pacientes com PF endêmico. Também, os níveis de C5a foram elevados em PV, corroborando estudos anteriores. Pela primeira vez, os níveis de NETs foram mensurados em soro de pacientes com PF endêmico e PV, porém, nenhuma associação foi encontrada.

Nossos resultados no miniórgão de pele destacaram vantagens e limitações na investigação de vias de morte celular. Para PV e até certo ponto para EPF, encontramos evidências contra apoptose e necroptose como vias de morte celular relevantes. No entanto, as investigações de outros marcadores de morte celular não foram conclusivas, sendo necessários outros ensaios funcionais para descrever sua importância relativa nas doenças do pênfigo.

Novos estudos investigando as vias de morte celular devem ser realizados no pênfigo. Validações funcionais dos resultados encontrados nesta tese podem contribuir para o entendimento da etiologia do pênfigo e o desenvolvimento de novas drogas e alvos terapêuticos para a doença.

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