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DEPARTMENT OF DERMATOLOGY AND ALLERGOLOGY
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STUDIES ON THE ROLE OF THE SKIN MICROBIOME IN HEALTHY SKIN AND UNDER PATHOGENIC CONDITIONS

Ph.D. thesis

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Publications related to the subject of the thesis

I. Tax G, Urbán E, Palotás Z, Puskás R, Kónya Z, Bíró T, Kemény L, Szabó K. **Propionic Acid Produced by *Propionibacterium acnes* Strains Contributes to Their Pathogenicity.** Acta Derm Venereol. 2015 Jun 3. doi: 10.2340/00015555-2154. [Epub ahead of print] IF: 3,025 (2014)

II. Szabó K, Tax G, Teodorescu-Brinzeu D, Koreck A, Kemény L. **TNFα gene polymorphisms in the pathogenesis of acne vulgaris.** Arch Dermatol Res. 2011 Jan;303(1):19-27. doi: 10.1007/s00403-010-1050-7. Epub 2010 Apr 13. IF: 2.279, Citation: 27 (24/3)

III. Szabó K, Tax G, Kis K, Szegedi K, Teodorescu-Brinzeu DG, Diószegi C, Koreck A, Széll M, Kemény L. **Interleukin-1A +4845(G> T) polymorphism is a factor predisposing to acne vulgaris.** Tissue Antigens. 2010 Nov;76(5):411-5. doi: 10.1111/j.1399-0039.2010.01530.x. Epub 2010 Aug 19. IF: 3.024, Citation: 15 (14/1)

Other publications

IV. Törőcsik D, Kovács D, Camera E, Lovászi M, Cseri K, Nagy GG, Molinaro R, Rühl R, Tax G, Szabó K, Picardo M, Kemény L, Zouboulis CC, Remenyik É. **Leptin promotes a proinflammatory lipid profile and induces inflammatory pathways in human SZ95 sebocytes.** Br J Dermatol. 2014 Dec;171(6):1326-35. doi: 10.1111/bjd.13229. Epub 2014 Nov 20. IF: 4.275 Citation: 1 (1/0)

V. Fazekas B, Polyánka H, Bebes A, Tax G, Szabó K, Farkas K, Kinyó A, Nagy F, Kemény L, Széll M, Ádám É. **UVB-dependent changes in the expression of fast-responding early genes is modulated by huCOP1 in keratinocytes.** J Photochem Photobiol B. 2014 Nov; 140:215-22. doi: 10.1016/j.jphotobiol.2014.08.002. Epub 2014 Aug 9. IF: 2.960

VI. Agodi A, Barchitta M, Valenti G, Quattrocchi A, Pettinato M, Tax G, Szabò K, Szell M. **Role of the TNFA -308G > A polymorphism in the genetic susceptibility to acne vulgaris in a Sicilian population.** Ann Ig. 2012 Sep-Oct;24(5):351-7. IF: -

Abbreviations

3'UTR	3' untranslated region
AA	acetic acid
AB/AM solution	antibiotic/antimycotic solution
APS	antimicrobial peptides
BA	butyric acid
BHI	brain heart infusion
BPE	bovine pituitary extract
BSA	bovine serum albumin
CAMP	Christie-Atkins-Munch-Peterson factor
CD	Crohn's disease
cfu	colony forming unit
Ci/nCi	cell index/normalized cell index
CMV	cytomegalovirus
DH5α	Dougles Hanahan 5 alpha
DMEM-HG	Dulbecco's Eagle Medium with high glucose
E. coli	*Escherichia coli*
EGF	epidermal growth factor
gDNA	genomic dezoxyribonucleic acid
GI	glycaemic index
HgB	hemoglobin
HPV	human papillomavirus
HPV-KER	immortalized human keratinocyte cell line
hRluc	*Renilla reniformis* luciferase gene
IBD	inflammatory bowel disease
IL-1A/IL-1α	interleukin-1 alpha gene/protein
IL-1ra	interleukin-1 receptor antagonist protein
IL1RN	interleukin-1 receptor antagonist coding gene
INFγ	interferon gamma
IRS	inner root sheath
KC-SFM	keratinocyte serum free medium

LDH	lactate dehydrogenase
MHC III	major histocompatibility complex III
MLST	multilocus sequence typing
MOI	multiplicity of infection
MS	mass spectrophotometry
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEK	normal human epidermal keratinocyte
NLS	nuclear localization signal sequence
OCT-1	octamer transcription factor-1
OD	optical density
ORS	outer root sheath
P. acnes	*Propionibacterium acnes*
PA	propionic acid
PBS	phosphate buffered saline
PBS-EDTA	phosphate buffered saline with ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
PFA	paraformaldehyde
RFLP	restriction fragment length polymorphism
RPMI	Roswell Park Medical Institute media
RT	room temperature
SCFA	short chain fatty acid
SEM	standard error of the mean
SNP	single nucleotide polymorphism
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TLR	Toll-like receptor
TNFA/TNFα	tumor necrosis factor alpha gene/protein
VNTR	variable number of tandem repeats

1. Introduction

1.1 The skin

The skin is the outer layer of our body with a surface area of about 1.8 m^2 and approximately 15 % of body weight in adults. It has a very complex structure composed of different cell types and tissues with ectodermal and mesodermal origin. Its major function is to provide a barrier between our body and the external environment, and to protect against the harmful impact of different physical, chemical and mechanical agents. Apart from that it also plays a major role in the regulation of body temperature, heat and cold sensation, or the control of evaporation (Jean L Bolognia Dermatology, Kanitakis J. 2002, Grice EA. 2011).

1.2 Anatomical structure of the human skin

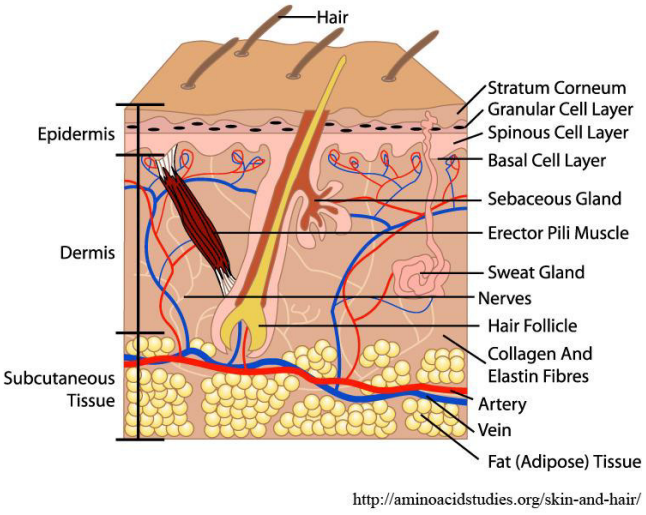

Figure 1. The anatomical structure of the human skin.

The epidermis is the outer layer of the skin, mainly composed of multiple layers of keratinocytes, also containing other cell types such as corneocytes, melanocytes, Langerhans- and Merkel cells. Keratinocytes are continuously generated by the division of epidermal stem cells located in the lower part of the epidermis, called *stratum basale*. They enter a characteristic differentiation process, during which they move suprabasally through the increasingly differentiated *stratum spinosum*, *granulosum*, and *corneum* (Figure 1). Underneath the epidermis locates the dermis, which is the middle layer of the skin. It is made up of collagen and elastin producing fibroblast, and provides flexibility and also strength for the organ. The deepest skin layer is the subcutaneous layer that is made up of fat and connective tissues. It is used mainly for fat storage and acts as padding and energy reserve (Bőrgógyászat és venerológia 2013, Kanitakis J. 2002, Braun-Falco, Jean L Bolognia Dermatology).

The skin also contains different skin accessory organs including the pilosebaceous unit (PSU), sweat glands, nails, various nerve endings and blood vessels. Among these the PSU

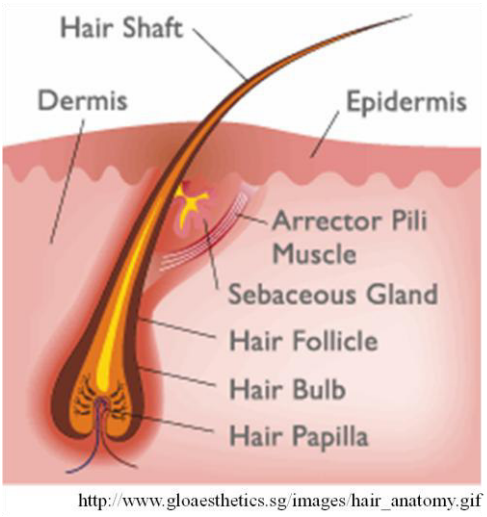

Figure 2. The anatomical structure of the pilosebaceous unit.

consists of the hair follicle, the hair shaft, the *arrector pili* muscle and the sebaceous gland (Figure 2).

The follicle has three major parts, the bulb, the isthmus and the infundibulum. The bulb consists of the matrix keratinocytes and the fibroblast-containing dermal papilla. This region is responsible for the growth of the hair shaft and the inner root sheath (IRS). The IRS surrounds the hair shaft and the duct of the sebaceous gland also fall into this region (Jean L Bolognia Dermatology). The sebaceous gland is an exocrine gland secreting a waxy substance, called sebum. It is produced in a holocrine way by the disintegration of glandular cells and composed of different lipids, such as triglycerides, esters of glycerol, squalene, wax and cholesterol. After its production sebum is emptied into the follicle and subsequently onto the skin surface where it exerts numerous functions, such as photoprotection, pro- and anti-inflammatory activity, transportation of several fat-soluble antioxidants and exhibiting an antimicrobial effect (Picardo M 2009).

1.3 The microbiome of the healthy human skin

From our birth we are exposed to a wide range of microorganisms, including bacteria, fungi and viruses (Grice EA 2011). Some of them are capable of inhabiting our skin and together with the various human cells forming a complex ecosystem. Major constituents of this community are the different bacterial species; approximately 1000 species belonging to 19 phyla has been detected in our skin (Grice EA 2009). They are mostly found in the epidermis and within the PSU (Grice EA 2008). The four dominant bacterium phyla and their most common representatives that colonize the human skin are the *Actinobacteria (Propionibacterium* and *Corynebacterium species)*, *Proteobacteria*, *Firmicutes (Staphylococcus species)* and *Bacteroidetes*. Apart from individual differences of the exact composition, regional variations are also exists in every individual because of alterations of environmental parameters including pH, temperature, moisture and the fine anatomic structure of the skin (Grice EA. 2011, 2014).

The exact function of the microbiome in the healthy skin and during pathogenic conditions is currently not clear (Gallo RL 2011, Littman DR 2011). Recent studies revealed

that the normal skin microbiome can protect us from pathogenic or harmful invaders by inhibiting their colonization using the available food sources and also by actively producing molecules with antimicrobial properties (antimicrobial peptides, APS) (Gallo RL 2011, Iwase T 2010). Apart from these functions they may also beneficially modulate our immune system (Gallo RL 2011, Lai Y 2009). These results suggest the importance of this complex ecosystem in the maintenance of skin homeostasis (Zeeuwen PL 2013).

1.4 The role of the microbiome in the pathogenesis of skin diseases

Under certain conditions that are currently not well defined, dysbiosis of the microbiome may lead to the pathogenesis of skin diseases. This can happen e.g. because of changes in the properties of the skin microenvironment, or as a result of local or systematic antibiotic treatments. In response to these events commensals, as well as pathogenic species may start an extensive growth and lead to the pathogenesis of several infectious and inflammatory diseases; including seborrheic dermatitis (*Malassezia spp*), atopic dermatitis (*Staphylococcus aureus*), post-operative infections (*Staphylococcus epidermidis*) or acne vulgaris (*Propionibacterum acnes*) (Bojar RA 2004, Grice EA 2011, Gallo RL 2011).

1.5 Acne vulgaris

Acne is currently described as the most common inflammatory skin disease of the pilosebaceous follicles, in which the dysbiosis of the skin microbiome, especially the extensive growth of the *Propionibacterium acnes* (*P. acnes*) bacterium is one of the important contributing factors. (Toyoda M 2001). It is mostly prevalent in puberty and estimated to affect more than 80-90% of the general adolescent population (Saitta P 2011).

1.5.1 Acne lesion formation and the types of lesions

Microscopically acne lesion formation starts with an abnormal keratinization and hyperkeratosis in the follicular infundibulum of the PSU.

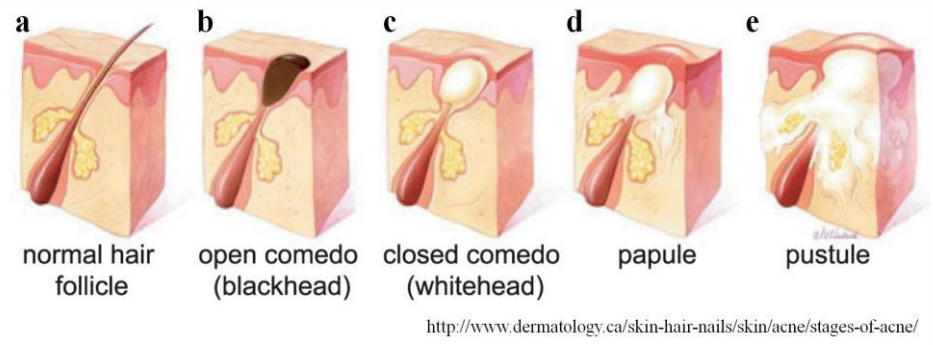

Figure 3. Pathogenesis of acne vulgaris. (Detailed description is present in the corresponding text.)

These events will lead to the generation of a plug and the formation of non-inflammatory open and closed comedones (Figure 3b and c). As a result, the constantly produced sebum will be trapped underneath the plug and cannot be emptied from the follicles to the skin surface. The increasingly anaerobic environment also supports the growth of the anaerobic microbes that resides in the PSU, among them the *P. acnes* bacterium. The constantly increasing pressure can cause a rupture of the follicle wall and the contents of comedo get out into the surrounding skin tissues (Figure 3d). These events may induce the activation of innate immune system and parallel to that inflammatory reaction in the affected skin areas. These will subsequently contribute to the generation of more severely acne lesions, including papules, pustules and in the most severe cases even nodules and cysts (Figure 3d and e) (Jean L Bolognia Dermatology).

1.5.2 The clinical characteristics of acne

Acne mostly affects the face, neck, back and chest, where the greatest density of PSUs

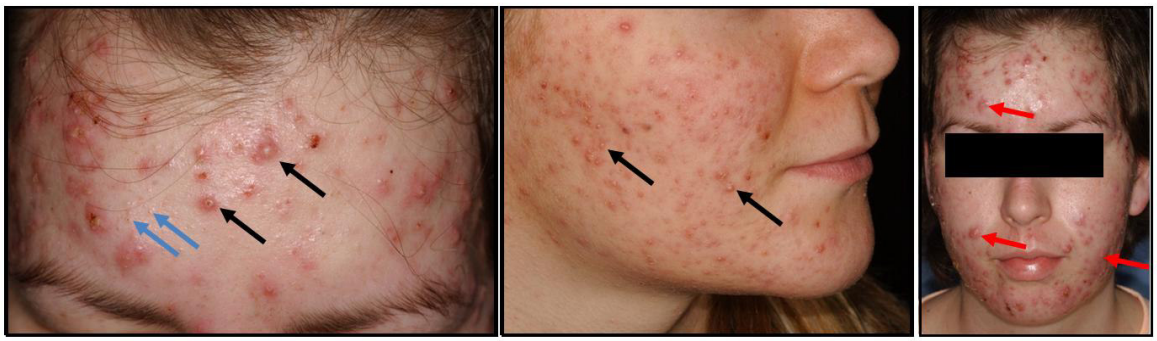

Figure 4. Clinical picture of acne vulgaris. The non-inflammatory comedones are marked by blue arrows. Black arrows mark the papules and pustules, whereas red arrows point to the most severe inflammatory form of acne lesions (nodules and cysts). (The images were selected from the collection of the Department of Dermatology and Allergology, University of Szeged.)

can be found (Dawson AL 2013, Jean L Bolognia Dermatology). Based on the severity of the symptoms, acne can be characterized into mild, moderate and severe forms. Mild acne typically affects the face with the presence of non-inflammatory and a few inflammatory lesions (*acne comedonica*). Moderate form can be characterized by the increased number of inflammatory papules and pustules (*acne papulo-pustulosa*) and in case of the severe form nodules and cysts are also present (*nodulo-cystic acne*) (Figure 4) (Dawson AL 2013).

1.5.3 Factors contributing to acne pathogenesis

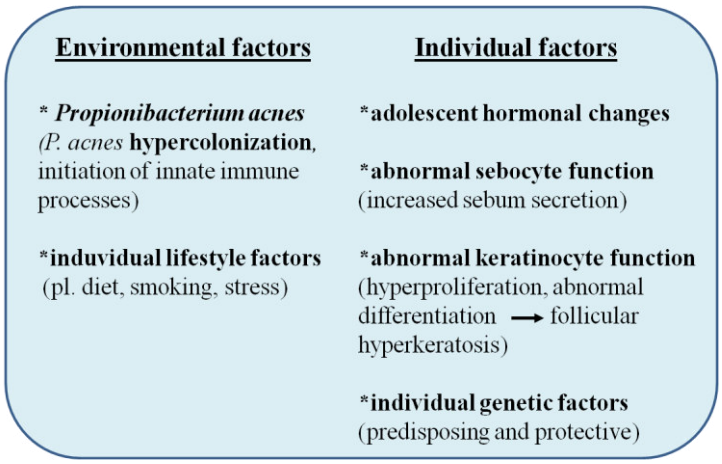

Figure 5. Different factors contributing to the pathogenesis of acne.

Acne is a typical mutifactorial disease, which means that several environmental and individual factors influence its pathogenesis (Figure 5). Among them production of hormones, especially androgen access, proliferation and hyperkeratinisation of the epidermal keratinocytes, extensive growth of the *P. acnes* and genetic predisposition considered to be the most important aspects (Dawson AL 2013, Koreck A 2003, Plewig G 1971, Pochi PE 1969, Ando I 1998, Bataille V 2002, Goulden V).

1.5.3.1 Hormonal changes and abnormal sebocyte function

The pathogenesis of acne is strongly linked to sebum production, which is controlled by hormonal stimulation. During puberty the endocrine changes stimulate the production of sex hormones. These lead to size increase in the sebaceous glands and subsequent elevation of sebum secretion, which is a food source of the *P. acnes* bacterium (Braun-Falco O 2010, Bergler-Czop B 2013).

1.5.3.2 The role of the skin microbiome in the pathogenesis of acne

P. acnes is one of the most common and important member of the skin microbiome in the adolescent and postadolescent skin (Oh J 2012). Its first colonization and growth coincides with the above described endocrine and hormonal changes. It is a Gram-positive, rod-shaped

anaerobic, but aerotolerant and lipophilic bacteria, which colonize the upper regions of the skin, as well as the PSU (Grice EA 2011). Under anaerobic conditions *Propionibacterium* species can hydrolyze various sebum components (triglycerides and fatty acids) and use the generated short chain fatty acids (SCFA), such as acetic acid (AA), propionic acid (PA) and butyric acid (BA) for their metabolism (Figure 6) (Allaker RP 1985).

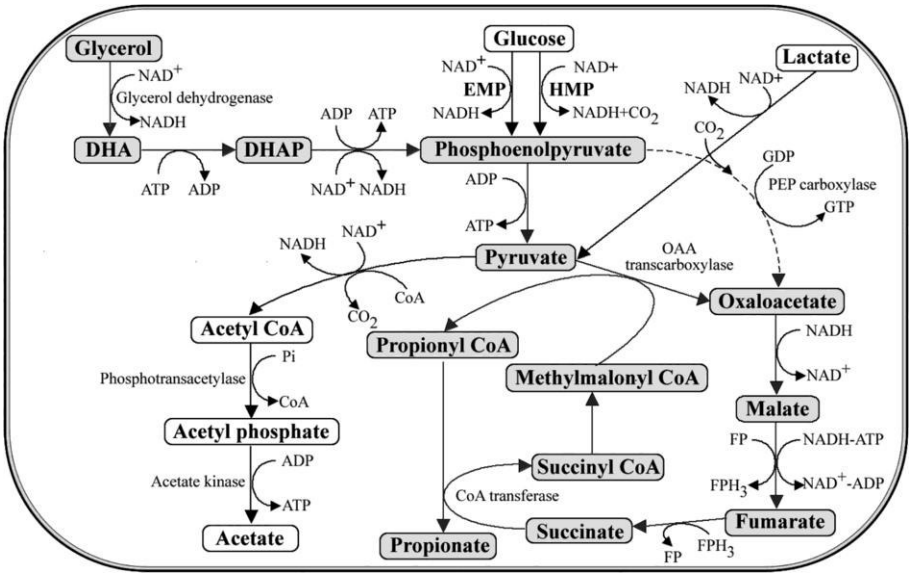

Figure 6. SCFA synthesis of *Propionibacterium* species (Zhuge X 2013)**.**

The *P. acnes* species is phylogenetically not homogenous. According to recent data obtained based on sequence analysis of bacterial *recA* and *tly* genes and the results of multilocus sequence typing (MLST) experiments, at least six distinct phylogenetic groups within the *P. acnes* species have been described (Figure 7) (McDowell A 2005, 2013).

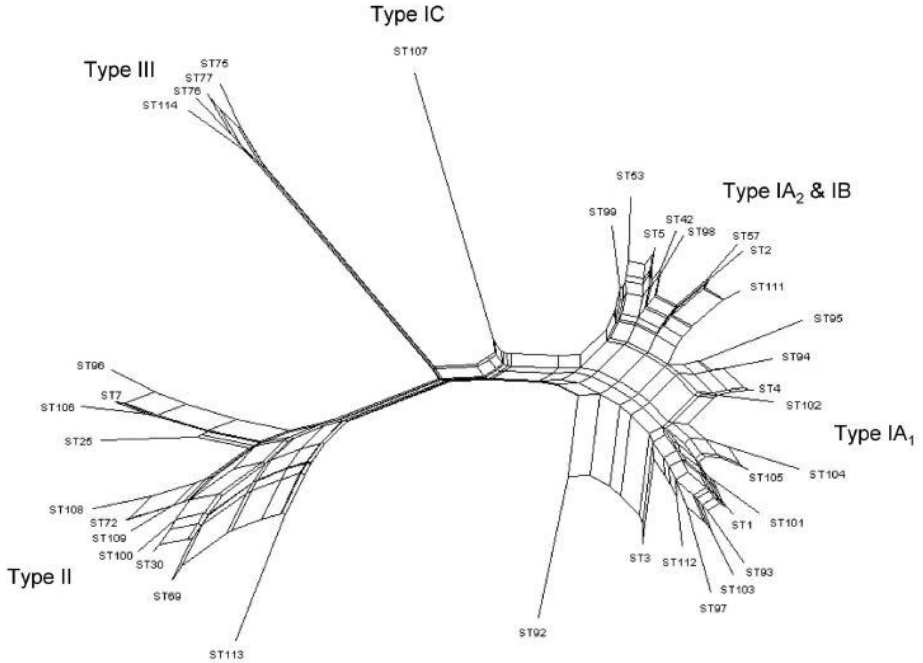

Figure 7. Phylogenetic tree of the different *P. acnes* isolates based on the results of MLST analysis Six different phylogenetic subgroups were identified within the *P. acnes* species. (McDowell A 2013).

Strains belonging to these subtypes may exhibit different virulence properties and has been suggested to differentially affect the cellular and molecular properties of human keratinocytes (Csukás Z 2004, Nagy I 2005).

1.5.3.3 Abnormal keratinocyte functions in acne pathogenesis

Abnormal cellular properties of the epidermal keratinocytes have been described during acne lesion formation, especially in the ductal region of the PSU. Cells become hyperproliferative and exhibit abnormal differentiation leading to the hyperkeratinization of the epidermal keratinocytes (follicular hyperkeratosis) (Plewig G, 1971). During these events molecular changes also happen in these cells. Results from our and other laboratories suggest that the keratinocytes recognize the presence of the *P. acnes* bacterium which in turn can activate pathogen recognition receptors, such as Toll-like receptor 2 and 4 (TLR 2, TLR4).

This activation initiates signaling events in the cytoplasm of the affected cells and consequently causes the activation and translocation of the NF-κB transcriptional factor and the subsequent regulation of genes playing an important role in the initiated innate immune and inflammatory events (Figure 8) (Nagy I. 2005, Nagy I. 2006, Kim J. 2005). These include e.g. different cytokines, chemokines and antimicrobial peptides (Nagy, 2005, Pomerantz JL 2002, Takeuchi O 1999).

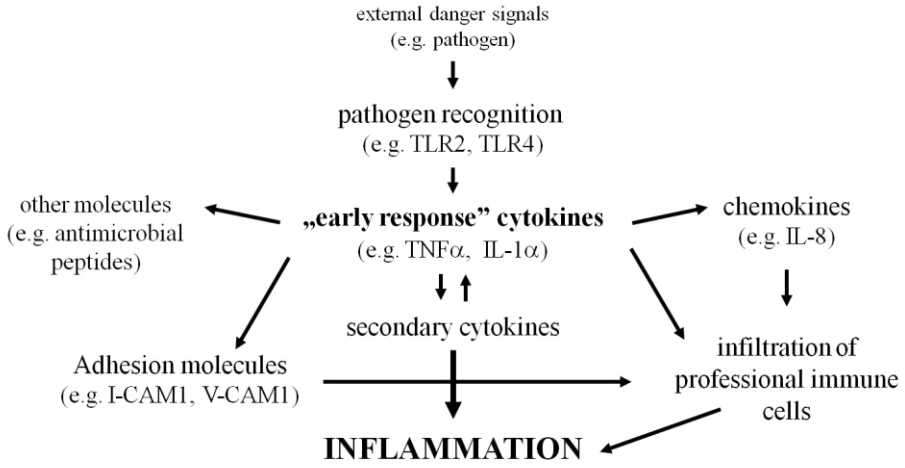

Figure 8. *P. acnes* induced signaling changes of keratinocytes. The bacterium activates TLR receptors and initiates signaling cascades, which subsequently lead to the activation of NF-κB transcription factor. These events regulate numerous cytokine, chemokine and antimicrobial peptide-coding genes (Szabó K. 2011).

Among the first responders of bacterial recognition we find early response cytokines, including the tumor necrosis factor alpha (TNFα) and the interleukin 1 alpha (IL-1α) cytokines, playing a key role in the initiation and orchestration of the downstream innate

immune and inflammatory events (Figure 8). It has been shown that these cytokines exhibit increased expression level when abnormal hyperproliferation and differentiation occur during comedogenesis and also in later stages during the inflammatory lesion formation (Cunliffe WJ 2000, Ingham E 1992).

1.5.3.4 Individual genetic factors that modify the risk of acne

Classical genetic studies (identical twin, community- and family-based studies) have long been suggested the importance of inherited factors in the pathogenesis of acne, but their identification started only in the 1990s. (Szabó K. 2011). Inherited susceptibility to complex diseases is frequently a result of a combination of commonly occurring polymorphisms that may affect the expression, structure and/or function of various genes. (Hoogendoorn B, 2003). Based on all these it is feasible to imagine that such genetic factors of the above described early response cytokines can modify the reactivity of carrier individuals' to environmental attacks, e.g. may regulate the keratinocytes' response to the different members of the skin microbiome. If these are overly extensive or inappropriate, they may even result destructive, chronic inflammatory events. Because of these reasons the role of different polymorphisms of genes belonging to the TNF and IL-1 families has long been investigated in the genetic predisposition to various chronic inflammatory diseases (Fidder HH 2006, Waldron-Lynch F 2001, Pomerantz JL 2002, Takeuchi O 1999, Ingham E 1992, Jouvenne P 1999, Kawaguchi Y 2007).

The TNFA gene is located within the highly polymorphic major histocompatibility complex III (MHC III) region on chromosome 6p21.3. There are many single nucleotide polymorphisms (SNPs) within this gene, especially in its 5' regulatory region, whereas the coding- and the 3'-regions show a much higher degree of conservation (Waldron-Lynch F 1999). The most frequent promoter SNPs in Caucasian populations are situated at positions -238 (Waldron-Lynch F 1999, D'Alfonso S 1994), -308 (Wilson AG 1997), -857 (Herrmann SM 1998), -863 and -1031 (Higuchi T 1998) in relation to the transcription start site. Several reports indicate that these SNPs might affect the regulation of gene expression and have a role in the regulation of innate immune response.

The IL-1 family contains 11 cytokines, also important factors of immune regulation. Among them the interleukin-1α (IL-1α) is the most studied member of this family, and this is one of the best-known cytokines implicated in the pathogenesis of acne vulgaris (Aldana OL 1998, Guy R 2006, Guy R 1998), The expression level and function of both the IL1A gene

(chromosome 2q14) and the encoded IL-1α protein are tightly regulated at many levels, including the regulation of gene and protein expression or secretion (Barksby HE 2007). These functions may be disturbed as a result of functional polymorphisms located in the IL-1A locus.

The biologic function of the IL-1α cytokine is also regulated by a naturally occurring receptor antagonist protein, IL-1ra, encoded by the IL1RN gene. Genetic polymorphisms of this gene have frequently been implicated in the pathogenesis of various chronic inflammatory diseases (Kawaguchi Y 2007, Jouvenne P 1999, Clay FE 1994, Tarlow JK 1994).

1.5.3.5 Individual life-style factors in the pathogenesis of acne

Individual life-style conditions such as diet, stress, smoking or obesity may also contribute to the pathogenesis of acne, but there are several controversies about their precise involvement. Although there are many anecdotal facts, because of the lack of well-designed clinical studies, the real contribution of these factors are questionable and currently under a heavy scientific debate (Halverson JA 2009, Berra B 2009, Cordain L 2002, 2003, Schäfer T 2001, Mills CM 1993).

2. Aims

In the course of our studies we were interested in studying the exact role and properties of the skin microbiome in the healthy skin and during pathogenic conditions, with a special focus on the most common inflammatory skin disease, acne vulgaris.

In the first part of our studies we analyzed the interaction of the skin colonizing *P. acnes* bacterium and the epidermal keratinocytes, to find out if and how this commensal bacterium affects the cellular properties of the human cells. For that, we aimed to investigate:

-whether there is a difference in the effect of selected *P. acnes* strains belonging to different phylogenetic groups within the species (*889*: 1A, *6609*: 1B, *ATCC 11828*: II) on the cellular responses of an *in vitro* cultured immortalized human keratinocyte cell line, HPV-KER,

-whether the keratinocyte responses are dose-dependent,

-the nature of the bacterially-derived factors that are responsible for the induction of cellular responses in keratinocytes.

In the second part we aimed to identify and analyze genetic susceptibility or protective factors that may modify the carrier individuals' response to the presence of the skin microbiome, potentially leading to inappropriate and/or overly extensive reactions and as a result, the pathogenesis of acne vulgaris. Thus, in retrospective case-control studies we analyzed the role of:

-five different TNFA promoter polymorphism (-238G>A, -308G>A, -857C>T, -863C>A, -1031T>C),

-the +4845G>T SNP of the IL1A gene,

-and the IL1RN VNTR polymorphism in acne pathogenesis.

3. Materials and Method

3.1. *P. acnes* strains and culture conditions

P. acnes bacterial strains were cultured and stored as previously described in detail (Nagy I 2005) (Table 1). Briefly, *P. acnes* clinical isolates *889* (subtype IA) and *6609* (subtype IB) and the reference strain *ATCC 11828* (subtype II) were cultured on pre-reduced Columbia agar base supplemented with 5% (v/v) bovine blood, vitamin K1 and haemin (Oxoid, UK).

Name	Origin	Type
889	endocarditis	IA.
6609	healthy skin surface	IB.
ATCC 11828	cyst	II.

Table 1. Properties of the applied *P. acnes* strains

Bacteria were grown under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, Oregon, USA) at 37°C. Colonies initiated with single *P. acnes* cells were inoculated in brain heart infusion (BHI, pH 7.4; Oxoid) broth cultures, and the samples were incubated at 37°C for 48 hours. Bacterial suspensions were then pelleted by centrifugation at 2600 × g for 10 minutes, and the supernatant was discarded. After three washes in PBS-EDTA for 10 minutes each, the cells were harvested in 5 ml PBS-EDTA, and the cell number was estimated by measuring the optical density (OD) at 600 nm of the solution with a spectrophotometer. According to our previous observations, OD_{600}= 2 was equivalent to 1.5 x 10^9 cfu/ml. The number of bacterial cells was adjusted with PBS-EDTA to 1 × 10^9 cfu/ml, aliquoted and stored at −80°C until further use.

3.2 Immortalized human keratinocyte culture and treatment

For the generation of an immortalized keratinocyte cell line, normal human adult keratinocytes (NHEK) were obtained from a healthy individual undergoing routine plastic surgery at our department and transfected with the pCMV vector containing the HPV16/E6 oncogene. A stable cell line was established by continuous culturing over 70 passages before the start of our experiments (Polyánka and Szabó, submitted for publication).

The HPV-KER immortalized human keratinocyte cell line was cultured using a keratinocyte serum-free medium (KC-SFM, Life technologies, Carlsbad, USA) supplemented with 1% of an antibiotic/antimycotic (AB/AM) solution (Sigma-Aldrich, St. Louis, USA) for most of the experiments. The cells were kept under standard conditions (37°C in a humidified atmosphere containing 5% (v/v) CO_2) at all times.

For the bacterial treatments, HPV-KER cells were plated in AB/AM-free KC-SFM culture medium and incubated for 24 hours, before co-culturing with live *P. acnes* bacterium using different human cell:bacterium ratios (multiplicity of infection, MOI, refers to the number of bacteria that were added to the cell cultures during infection).

For the visualization of *P. acnes*-induced pH shifts, HPV-KER cells were plated in serum-free DMEM-HG supplemented with prequalified human recombinant Epidermal Growth Factor 1-53 (EGF 1-53) and Bovine Pituitary Extract (BPE). Bacterial treatments were performed as described above.

3.3 Real-time, label-free analysis of the interaction between HPV-KER cells and *P. acnes*

Cellular properties of the control and bacterium-treated HPV-KER and NHEK cells were followed in real-time using the xCELLigence system (ACEA Biosciences, San Diego, USA), allowing the label-free monitoring of various cellular events using impedance-based measurement. HPV-KER cells were plated at a density of 10,000 cells/well in fibronectin coated 96-well E-plates. Twenty-four hours after plating, they were treated with *P. acnes* strains at different doses (MOI = 25–300). HPV-KER treatments were performed in five and NHEK treatments were performed in three technical replicates. Impedance (Z) values were measured in every 60 minutes for 90 hours, from which a dimension free cell index (Ci) was calculated. Ci tracings (average of the technical replicates) were normalized to values recorded at the addition of the bacterium to the cultures, and the resulting nCi values were plotted. Each data point represents the mean ± the standard error of the mean (SEM).

3.4 Trypan Blue exclusion assay

HPV-KER cells were cultured in 12-well plates at a starting density of 125,000 cells/well. After 48 hours, the cells were treated with *P. acnes* strains at different MOIs (100 and 300) in triplicate. Samples were collected by trypsinization at 0, 6, 9, 12, 24, 36 and 48 hours post treatment, washed with PBS and stained with Trypan Blue dye (Sigma-Aldrich). Viable cells were counted using a hemocytometer.

3.5 Fluorescence microscopic analysis of the *P. acnes* and PA treated HPV-KER cultures

HPV-KER cells were cultured on the surface of glass coverslips (18x18 mm) in 6-well plates at a density of 300.000 cells/well. Forty-eight hours later they were treated with the

different *P. acnes* strains using high bacterial dose (MOI = 300) or with 2mM propionic acid (PA). Samples were collected at 48 hours post-treatment, by washing the cells with PBS and then fixed in 2% paraformaldehyde (PFA) solution for 10 minutes at room temperature. After washing two times in PBS, the cell membranes were permeabilized by 0,1 % Triton x-100 detergent (3 to 5 minutes at RT). The slides were washed again, and the filamentous actin (F-actin) was stained by Alexa Fluor 488® phalloidin (Life Technologies, Carlsbad, USA), in a 1:100 dilutions prepared in PBS, also containing 1% BSA. The slides were incubated for 30 minutes at RT. After the final PBS washes, the coverslips were mounted in Fluoromount-G mounting media (SouthernBiotech, Birmingham, USA).

3.6 Spectrophotometric hemoglobin and lactate dehydrogenase assays

Peripheral blood samples were taken with the subjects' written informed consent and the approval of the Scientific and Research Ethics Committee of the Medical Research Council, Hungary (protocol number: ACN-GENET-001). The studies were performed in accordance with the Declaration of Helsinki guidelines and its later revision. Erythrocytes were isolated from 5 ml peripheral blood samples obtained from each individual using a ficoll gradient separation method and subsequently resuspended in 10 ml RPMI 1640 media lacking phenol red dye (LONZA, Basel, Switzerland). Erythrocyte suspensions (500 µl) were treated with *P. acnes* strains (MOI = ~300) or with *P. acnes 6609* strain (MOI = 300) in the presence of 1, 2 and 5 mM PA. The supernatants were collected at 72 hours post-treatment, and the quantity of free hemoglobin was estimated by spectrophotometric analysis of the collected supernatant samples (OD at 540 nm).

The cytolytic effect of the *P. acnes* strains on HPV-KER cells was also measured by the colorimetric lactate dehydrogenase (LDH) assay using the Cytotoxicity Detection Kit^{PLUS} (Roche, Basel, Switzerland), according to the instruction of the manufacturer. Briefly, HPV-KER cells were plated in a 96-well plate at a density of 10,000 cells/well. After 24 hours incubation, the cells were treated with the *P. acnes* strains (MOI=100, 300). Released LDH was quantified after 72 hours by spectrophotometry (OD at 492 nm). The measured values were corrected with the background values of the culture medium, and the *P. acnes*-treated samples were also normalized to the values measured in corresponding *P. acnes*-treated cell-free controls.

3.7 Analysis of the pH changes of *P. acnes*-treated HPV-KER cultures

For the visualization of pH changes of the bacterial-treated HPV-KER culture supernatants color changes of a culturing media supplemented with a pH sensitive phenol-red dye was used. The color of this pH indicator molecule exhibits a gradual transition from yellow to red over the pH range 6.8 to 8.2. Thus, the HPV-KER cells were plated in serum-free DMEM-HG supplemented with prequalified human recombinant Epidermal Growth Factor 1-53 (EGF) and Bovine Pituitary Extract (BPE). Bacterial treatments were performed as described above. pH changes were monitored visually by observing the gradual color changes of the culturing media. For a more precise approximation we also used a pH test strip capable of the detection of the pH conditions between pH 6–7.5.

3.8 Mass spectrometry

All measurements were conducted on a Shimadzu GCMS-QP2010 SE device with a ZB-WaxPlus column of 30 m length. The column was heated at a constant rate (20°C/min) during all measurements starting at 50°C up until 230°C. The head pressure was 117.6 kPa and a 2 ml/min column flow was chosen. The mass spectrometer was operated in scan mode, where all m/z values between 10 and 500 were acquired after the 3.5 minutes (solvent elution) to protect the filament. For calibration serial dilutions of acetic (AA) and propionic acids (PA) were used. Each treatment was performed in triplicates. The samples were measured by the Department of Applied and Environmental Chemistry, University of Szeged.

3.9 Growth curve analysis of the different *P. acnes* strains

Bacteria were grown in brain heart infusion (BHI, pH 7.4; Oxoid) broth cultures, and the samples were incubated at 37°C for 48 hours under anaerobic conditions (anaerobic chamber; Bactron Sheldon Man, Oregon, USA). The cell numbers of the bacterial suspensions were estimated by measuring the optical density at 600 nm with a spectrophotometer. The OD_{600} values were adjusted with BHI to 0.02. Bacterial suspensions were incubated at 37°C under anaerobic conditions or under standard cell culturing conditions (at 37°C in a humidified atmosphere containing 5% (v/v) CO_2) and the OD_{600} values of the samples were measured at 24, 36, 48, 60 and 72 hours.

3.10 Study population and ethics of the genetic studies

For the genetic analyses, control individuals and acne patients were recruited at the University of Szeged, Department of Dermatology and Allergology and at the Department of Dermatology, Victor Babes University in Timisoara, and retrospective case-control studies were conducted. For that, peripheral blood samples were obtained from Caucasian individuals. Control subjects were recruited from university students, co-workers, and relatives of the participants at both locations. Acne vulgaris diagnosis was defined by trained dermatologists on the basis of physical examinations, patient records and questionnaires. Distribution of the study cohort is summarized in Table 2.

	subgroups based on the severity of acne	sample collection	distribution of females/males	mean age (year)
Control				29.5
group 0:	-	126	91/35	
Acne vulgaris		229	137/92	32.4
group 1:	*acne comedonica*	29	24/5	
group 2:	*acne papulo-pustulosa*	157	98/59	
group 3:	*nodulo-cystic acne*	43	15/28	

Table 2. Clinical characteristics of the study population.

Altogether our genomic collection consisted of samples from 126 healthy controls with no or only a few, mostly non-inflammatory lesions and 229 acne patients samples suffering from acne of various severity. The mean age of the control group was 29.5 years, compared to 32.4 of the patient group (Table 2). In both locations the same dermatologist (Andrea Koreck) supervised the sample collection to make sure that the categorization of the different acne patients and control was performed uniformly.

The study was approved by the Hungarian Research Ethics Committee and the Ethics Committee of the 'Victor Babes' University of Medicine and Pharmacy Timisoara. All subjects gave written consent before blood collection. The study was performed in accordance with the principles stated in the Declaration of Helsinki and its later revision.

3.11 Genomic DNA isolation

Genomic DNA was obtained from acne patients' and control subjects' peripheral blood leukocytes by a standard proteinase K digestion method, using the QIAamp Blood DNA Mini

Kit (QIAGEN, Hilden, Germany). The concentration (OD=260 nm measurement) and purity (OD260/280 nm ratio) of the isolated gDNA samples were measured by spectrophotometry, and they were diluted to a 20 ng/µl final concentration.

3.12 Restriction fragment length polymorphism (RFLP) analysis

Acne patients and controls were genotyped for five different promoter polymorphisms in the TNFA gene (-238G>A, rs361525; -308G>A, rs1800629; -857C>T, rs1799724; -863C>A, rs1800630; -1031T>C, rs1799964) and the +4845G>T (rs17561) SNP located at the coding region of the IL-1A gene, using a common PCR-based technique, called restriction fragment length polymorphism (RFLP) method. The list of the studied polymorphisms and the sequence of the used primers are listed in Table 3.

Polymorphisms	Amplification primers and restricion enzymes		
TNFA -1031T>C rs1799964	**Amplification primers**	**Restriction enzyme**	**Reference**
	F: 5' CAA GGC TGA CCA AGA GAG AA 3' R: 5' GTC CCC ATA CTC GAC TTT CAT 3'	BpiI	Soga Y. 2003
TNFA -863C>A rs1800630	**Amplification primers**	**Restriction enzyme**	**Reference**
	F: 5' GGC TCT GAG GAA TGG GTT AC 3' R: 5' CTA CAT GGC CCT GTC TTC GTT ACG 3'	TaiI	Skoog T. 1999
TNFA -857C>T rs1799724	**Amplification primers**	**Restriction enzyme**	**Reference**
	F: 5' AAG TCG AGT ATG GGG ACC CCC CGT TAA 3' R: 5' CCC CAG TGT GTG GCC ATA TCT TCT T 3'	HincIII	Kato T. 1999
TNFA -308G>A rs1800629	**Amplification primers**	**Restriction enzyme**	**Reference**
	F: 5' AGG CAA TAG GTT TTG AGG GCC AT 3' R: 5' TCC TCC CTG CTC CGA TTC CG 3'	NcoI	Wilson AG. 1992
TNFA -238G>A rs361525	**Amplification primers**	**Restriction enzyme**	**Reference**
	F: 5' AGA AGA CCC CCC TCG GAA CC 3' R: 5' ATC TGG AGG AAG CGG TAG TG 3'	MspI	Wilschanski M. 2007
IL1A +4845G>T rs17561	**Amplification primers**	**Restriction enzyme**	**Reference**
	F: 5'ATG GTT TTA GAA ATC ATC AAG CCT AGG GCA 3' R: 5' AAT GAA AGG AGG GGA GGA TGA CAG AAA TGT 3'	SatI	Agrawal AA. 2006

Table 3. Primer sequences and restriction enzymes used for the PCR–RFLP analyses.

Following PCR amplification the resulting fragments were digested and analyzed on a 5% NuSieve agarose gel (Lonza, ME, USA), where the DNA fragments were visualized using a GelRed (Biotium, Inc, Hayward CA, USA) stain.

3.13 Variable number of tandem repeats (VNTR) polymorphism analysis

Control and acne vulgaris patients were also genotyped for the IL1RN VNTR polymorphism located in intron 2 of the gene using a PCR-based method, similarly as it was reported by Tarlow *et al*. (Tarlow JK 1993). This polymorphism is a variable number (1–6 repetitions) of tandem repeats of an 86 bp motif. To determine the number of the carried repeats a gene region including the polymorphic sequences was amplified (Table 4).

Polymorphism	Amplification primers (Tarlow JK. 1993)
IL1RN VNTR	F: 5' CTC AGC AAC ACT CCT AT 3' R: 5' TCC TGG TCT GCA GGT AA 3'

Table 4. PCR amplification primers used to genotype the IL1RN VNTR polymorphism.

Following the amplification step the size of the resulting PCR fragments were determined based on an analysis using a 2 % agarose gel (Lonza, ME, USA), where the fragments were visualized with a GelRed stain (Biotium, Inc, Hayward CA, USA).

3.14 Generation of the TNFA luciferase reporter constructs

A piece of the proximal TNFA promoter was cloned into the pGL4.20 luciferase reporter plasmid (Promega, Madison, USA) similarly as it was described by Lv et al. (Lv K 2006). For that a 1012 base pair (bp) piece of the TNFA regulatory region was amplified using the following primers:

Forward primer: 5' CCG **CTC GAG** CCA CAG CAA TGG GTA 3'
Reverse primer: 5' CCC **AAG CTT** CGT CTG AGG GTT GTT TTC AGG 3'

The forward primer contained an extra XhoI, while the reverse a HindIII restriction enzyme recognition site (marked with bold letters) allowing the easy cloning of the resulting fragments to the chosen vector. Two construct were generated; one included the common (C) allele, whereas the other harbored the rare (T) allele at the -857th nucleotide position, corresponding to the two alleles of the rs1799724 (-857C>T) SNP. This was achieved by using gDNA samples of donors who were previously genotyped for this polymorphism. The resulting constructs were named as pGL4.20-TNFA-857C and pGL4.20-TNFA-857T, respectively, and the carried nucleotides at the polymorphic position were verified using the PCR-RFLP method.

Furthermore, the proximal part of the 3' untranslated region (3' UTR) of the TNFA gene was also cloned into both constructs, similarly as it was described by Denys et al (Denys A 2002). For that, a 1035 bp fragment was amplified using the following primers:

Forward primer: 5' AAT **TCT AGA** GGA GGA CGA ACA TCC AAC 3'
Reverse primer: 5' CG**G AAT TC**C CAG AGT TGG AAA TTC CCA TG 3'

In this case the forward primer contained an extra XbaI, while the reverse an EcoRI restriction enzyme recognition site (marked with bold letters) allowing the cloning of the resulting fragments to a pBS vector. From this the TNFA 3' UTR fragment was released by digesting with the XbaI enzyme, and used to replace a cassette including the SV40 late polyA signal, and the major part of the Puromycin resistance gene driven by an SV40 promoter the pGL4.20-TNFA-857C and pGL4.20-TNFA-857T constructs. Because of the bi-directional nature of this cloning step, clones in which the TNFA 3'UTR was inserted in a proper orientation was chosen after restriction mapping.

The generated vectors (pGL4.20-TNFA-857C-3' and pGL4.20-TNFA-857T-3') were transformed into chemically competent DH5α *Escherichia coli* (*E. coli*) bacterial cells. Transfection-grade plasmid DNA was isolated with the Endo Free Plasmid Maxi Kit (OMEGA Bio-Tek, Norcross, USA).

3.15 Transient transfection of the HPV-KER cells and luciferase reporter assay

HPV-KER cells were plated in 6-well plates at a density of 300.000 cells/well. Forty-eight hours later the cells were co-transfected with 1 μg of one of the two TNFA luciferase reporter plasmids (described in section 3.13). As normalization control 5 ng of the pGL4.75 [hRluc/CMV] constitutive Renilla luciferase vector (Promega, Madison, USA) was also added in case of each well.

The transfection reaction was performed using the X-tremeGENE 9 DNA transfestion reagent (Roche, Basel, Switzerland), according to the instructions of the manufacturer. Twenty-four hours after the addition of the transfection complex, the cell culture medium was replaced and the cells were treated with live *P. acnes 889* bacterium (MOI=300). Cells were then collected in 200 μl 1x Passive lysis buffer (Promega, Madison, USA) at 6 hours post-treatment, and they were centrifuged at 12,000g for 3 minutes at 4 °C. The cleared supernatants were kept at -80 °C until further processing.

Luciferase assay was performed using the Firefly & Renilla Dual Luciferase Assay Kit (Biotium, California, USA) according to the instruction of the manufacturer, and luciferase activities of the lysates were measured in a luminometer (Thermo Fisher Scientific, Waltham, USA). Each treatment was performed in three technical replicates and the measured firefly luciferase values were normalized to the Renilla luciferase values. Data represents the average of two parallel experiments.

3.16 Statistical analysis

In case of the molecular genetic studies statistical analysis was carried out on the various groups of patients and controls according to the rules of case–control allelic association study designs. First, genotype and allele frequencies for each polymorphism were calculated by determining the percentage of individuals carrying the different genotypes and alleles in each group. For the statistical analyses, the three most common genetic models (dominant, co-dominant and recessive) were evaluated for all the studied SNPs. The model that described the best the mode of inheritance (resulting the smallest p value) was chosen based on the obtained results Statistical significance was calculated using the Chi2 or the Fischer's exact test, depending on the characteristics of the analyzed data. Odds ratios (ORs) and their 95% confidence intervals (CIs) were also determined.

Chi-squared test for a linear trend were calculated to assess the relationship between the severity of acne symptoms and the different genotypes. For that, acne vulgaris patients were stratified into three subgroups according to the severity of their skin lesions (as described, in section 3.9). All the described analyses were done using the SPSS software (Version 17, SPSS, Chicago IL). Statistical significance was established at a $p<0.05$.

For all the other experiments data were compared using one-way analysis of variance (ANOVA) followed by Dunnett or Tukey's post hoc tests to determine statistical differences after multiple comparisons (SPSS, SPSS Inc., Chicago, IL). In all cases a probability value of less than 0.05 was considered significant. Unless otherwise noted, data were presented as mean ±SEM for three parallel experiments, where the different treatments were performed at least in triplicates.

4. Results

4. 1 *In vitro* monitoring of the interaction of the *P. acnes* bacterium and the epidermal keratinocytes

In order to gain a better understanding on the properties of the interaction that exists between the skin colonizing *P. acnes* bacterium and the epidermal keratinocytes, we set up an *in vitro* model system. For that, we used a human immortalized keratinocyte cell line (HPV-KER), previously established and characterized in our laboratory. In preliminary experiments we showed that these cells react similarly to the presence of the bacterium than that of cultured normal human keratinocytes; they grow in monolayer, exhibit keratinocyte-like morphologies and respond to the presence of *P. acnes* with similar gene expression changes than that of NHEK cells (Polyánka, Szabó *et al*. submitted for publication; Tax, 2015). Next, we aimed to determine if and how the bacterium affects the different cellular properties (e.g. growth and viability) of the HPV-KER cells when they are co-cultured.

4.1.1 Real-time monitoring of the growth properties of HPV-KER cultures

To monitor the cell growth properties, HPV-KER cells were seeded in 96-well plates at different cell densities (5,000, 10,000, 15,000 cells/well) and impedance-based RTCA analysis was performed using the xCELLigence system. Ci values were measured every 60 minutes for 72 hours and plotted as a function of time (Figure 9A).

According to the observed results the cells started to attach to the surface of the wells within 3 hours, indicated by a rapid increase of Ci values, and following a short lag period, they entered a growth phase. Plates seeded with 15000 cells/well reached confluence 36 hours after plating, whereas plates seeded with other cell densities remained in the growth phase during the entire experiment. Plating densities of 10,000 cells/well were thus used in subsequent experiments to detect changes in HPV-KER proliferation in response to *P. acnes* treatment.

4.1.2 *P. acnes* affects the cellular properties of HPV-KER cells in a strain-specific and dose-dependent manner

Next, we wanted to know whether the *P. acnes* bacterium affects the cellular properties of HPV-KER cells. To determine if there were any strain-specific differences we chose to study three bacterial strains belonging to different phylogenetic groups within the species (*P. acnes 889*: IA, *6609*: IB, *ATCC 11828*: II) (Nagy I 2005). We applied the bacteria using different MOIs to find out if the effects depended on the *P. acnes* dose. For the experiments, HPV-KER cells were treated with these strains 24 hours after plating, and cellular changes were monitored using the xCELLIgence system (Figure 9B–D).

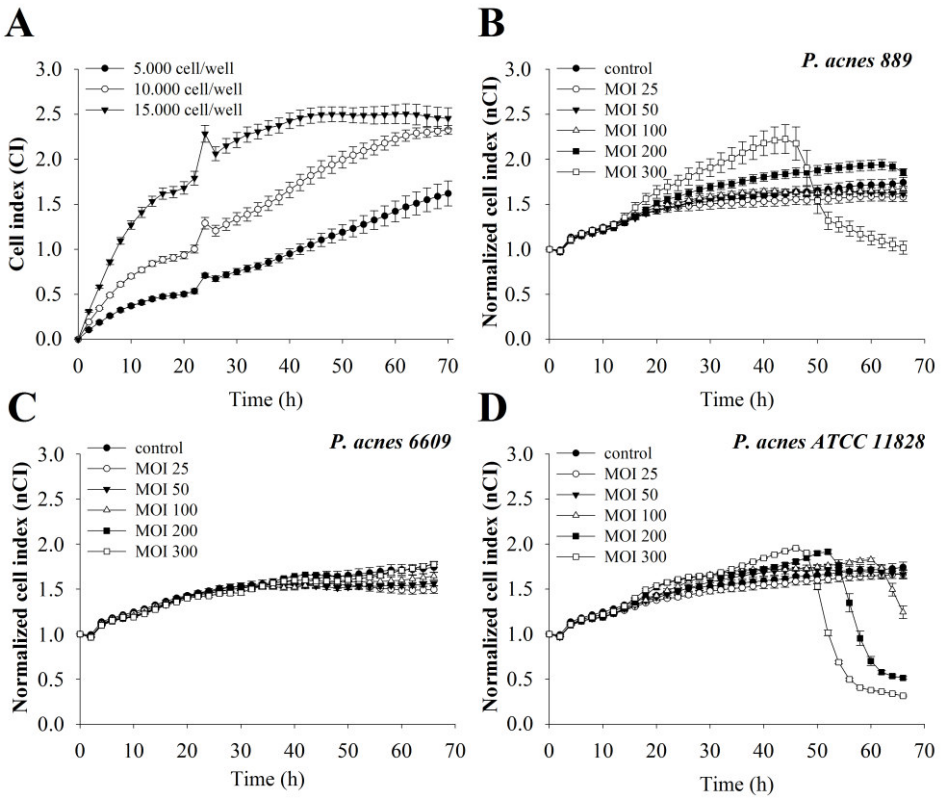

Figure 9. Strain-specific effects of *P. acnes* on cellular properties of HPV-KER cells. (A) HPV-KER cells were plated in a 96-well E-plate at densities of 5,000, 10,000 and 15,000 cells/well. The Ci values were plotted as a function of time. HPV-KER cultures (10,000 cells/well) were treated with *P. acnes 889* (B), *6609* (C) and *ATCC 11828* (D) at MOIs of 25–300, and nCi values were determined. (Representative image of 3 parallel experiments.)

Our results suggest that, when using low bacterial doses (MOI = 25, 50), no *P. acnes* strain had a significant effect on the properties of HPV-KER cells compared to untreated

controls. The cells continued to proliferate in the presence of the bacteria until they reached confluence, and subsequently entered to a stationary phase, marked by steady nCi values. In contrast, when the *P. acnes 889* strain was applied in a high dose (MOI = 300), a rapid elevation of the nCi values was detected at about 15 hours and continued to grow until 45 hours (Figure 9B). However, when higher bacterial loads (MOIs of 200 or 300) of the *889* and *ATCC 11828* strains were applied, a small increase followed by a sharp decrease was noted (Figure 9B and D, respectively). Similar changes were not observed for treatment with *P. acnes 6609* strain (Figure 9C).

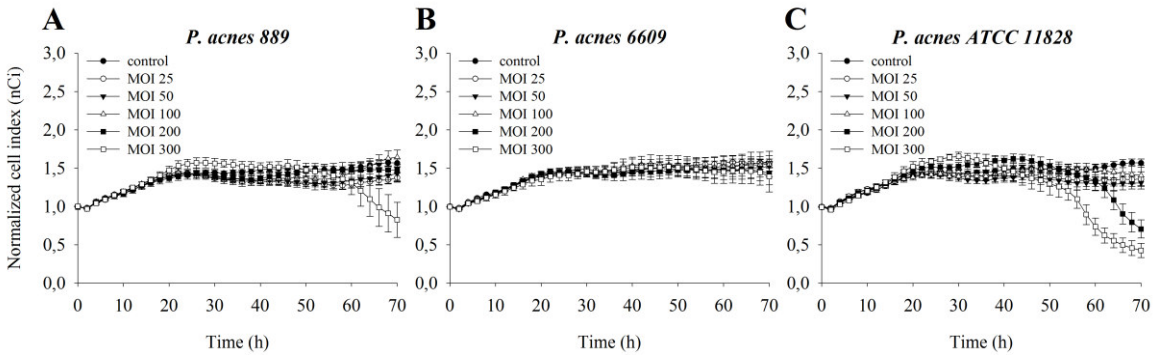

Figure 10. Strain-specific effects of *P. acnes* on cellular properties of NHEK cells. HPV-KER cultures (10,000 cells/well) were treated with *P. acnes 889* (A), *6609* (B) and *ATCC 11828* (C) at MOIs of 25–300, and nCi values were determined.

In order to prove that the above described effects were not HPV-KER specific, we also repeated the above experiment, but this time we treated NHEK cells using the same conditions. Our results indicate, that NHEK cells responded to the presence of the *P. acnes* strains and exhibited nCi changes similar to what we observed in case of the HPV-KER cells (Figure 10).

4.1.3 The *P. acnes 889* and *ATCC 11828* strains affect changes in HPV-KER cell number

Ci changes can reflect differences in the number or in the specific dimensions of cells attached to the electrodes. To determine the exact nature of the *P. acnes*-induced cellular events that corresponds to the observed nCi differences, we monitored the effect of the different *P. acnes* strains on the number of cells in the HPV-KER cell cultures by a Trypan Blue dye exclusion assay. High dose (MOI = 300) of the *P. acnes 889* strain resulted in increased cell numbers compared to untreated and low-dose treated samples (Figure 11A).

None of the other treatments resulted in an increase in cell number. However, the rate of increase in the cell number decreased 12 hours after high-dose treatment (MOI = 300) with the *ATCC 11828* strain (Figure 11C).

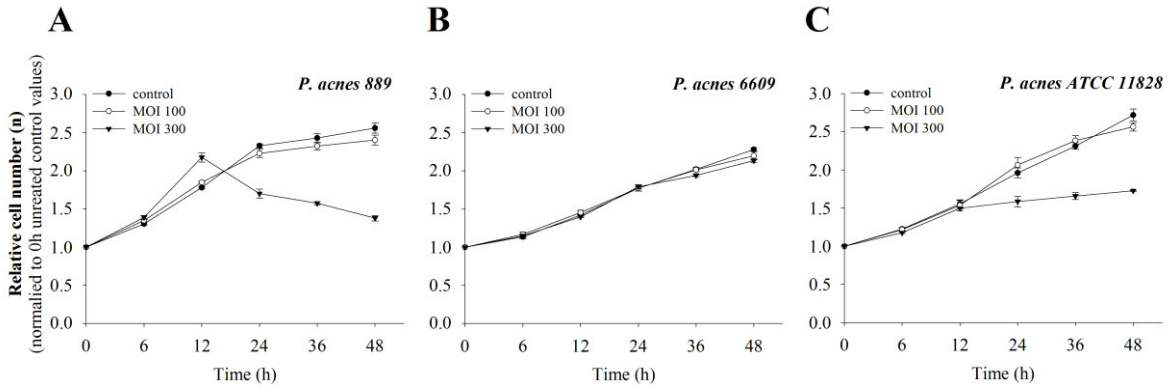

Figure 11. The *P. acnes 889* and *ATCC 11828* strains affect changes in HPV-KER cell number. To identify the cellular events corresponding to the detected nCI changes HPV-KER cells were plated in 12-well plates (125,000 cells/well) and treated with the *P. acnes* strains at MOIs of 100 and 300. The number of cells present in the cultures was determined by a Trypan Blue dye exclusion assay at different time points. (Average of 2 parallel experiments.)

4.1.4 High-dose treatment of the *P. acnes 889* and *ATCC 11828* strains induces microscopic changes in HPV-KER cells

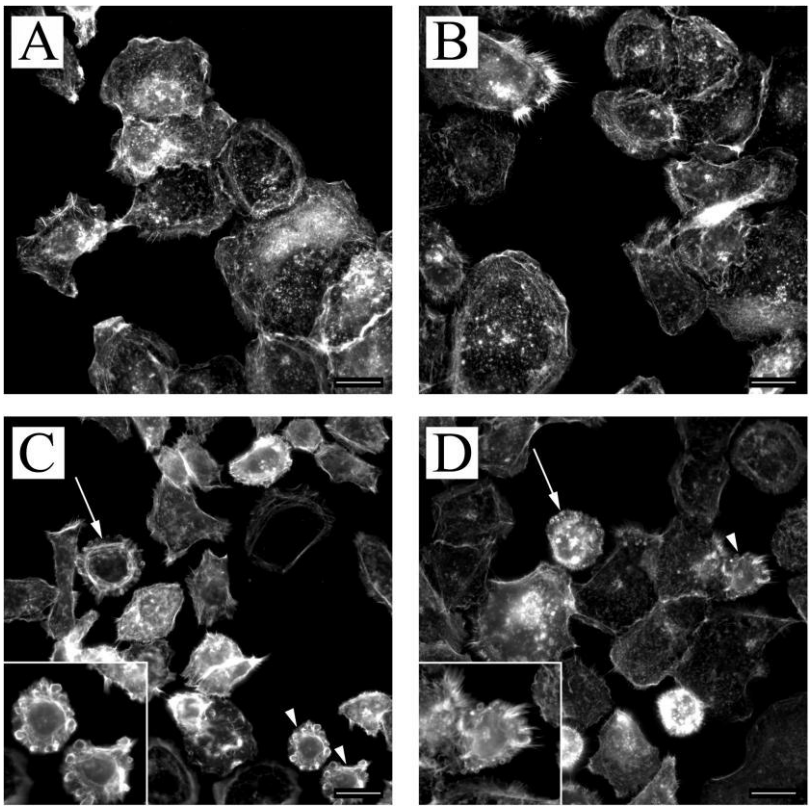

Figure 12. Microscopic analysis of *P. acnes*-treated HPV-KER cells. Immortalized keratinocytes were stained with rhodamine-labeled phalloidin to visualize cytoskeletal F-actin bundles for an overall evaluation of cellular morphology. The presence of abnormally shaped, round cells was detected in the cultures treated with (C) *P. acnes 889* and (D) *ATCC 11828* (MOI = 300), exhibiting a high degree of membrane irregularity (marked with white arrows and arrowheads), which was not apparent in (A) control and (B) *P. acnes 6609*-treated cultures. (Arrowheads point to cells presented in high magnification in the lower left corner. Scale bars: 10 μm)

To visualize cellular changes induced by *P. acnes* strains, we stained untreated and treated (MOI = 300) HPV-KER cells with rhodamine-labeled phalloidin and performed a fluorescent microscopic analysis. We noted the presence of abnormally shaped rounded cells exhibiting irregular membrane morphology 48 hours after treatment with the *P. acnes 889* and *ATCC 11828* strains (white arrows). This effect was not apparent in cells treated with the *P. acnes 6609* strain (Figure 12).

4.1.5 *P. acnes*-induced cytotoxicity is strain- and dose-dependent

To determine whether *P. acnes*-induced cytotoxicity was due to damage of the keratinocyte membrane caused by the bacterium or by bacterially derived toxins, the amount of free LDH enzyme released to the supernatant of the damaged cells was determined using an LDH assay of keratinocytes treated with different doses (MOIs of 100 and 300) of the *P. acnes* strains.

Higher LDH levels were measured in cells treated with the *889* and *ATCC 11828* strains. This effect appeared to be dose-dependent at 72 hours post-treatment. In contrast, no differences were detected in LDH levels in cells treated with the *P. acnes 6609* strain (Figure 13).

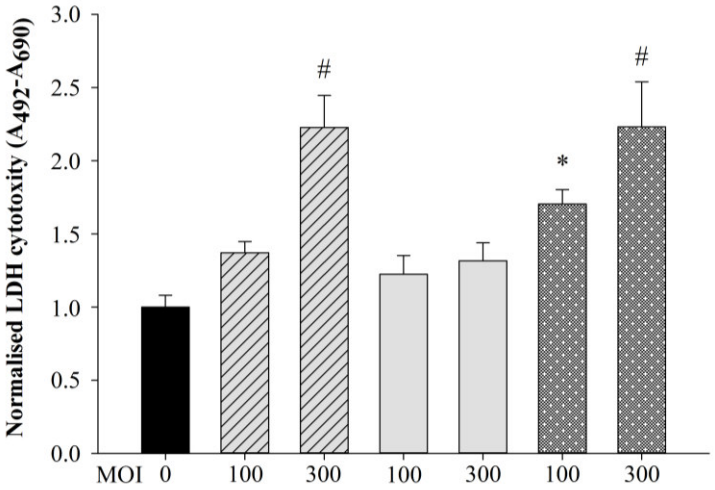

Figure 13. Cytotoxicity of *P. acnes* strains. HPV-KER cells were treated with *P. acnes* strains. After 72 hours, released LDH and was measured from cleared supernatants. High-dose (MOI = 300) treatment with the *P. acnes 889* and *ATCC 11828* strains lead to increased level of free LDH, which is indicative of membrane damage and subsequent cytotoxicity. (Statistical analysis: one-way ANOVA, Tuckey's post hoc test. * $p<0.05$, # $p<0.01$, compared to untreated control values. Results of *P. acnes 889*, *6609* and *ATCC 11828*-treated cleared supernatant samples are represented by striped, solid grey and dotted bars, respectively. Average of 3 parallel experiments.)

4.1.6 *P. acnes* exhibits a strain-specific hemolytic effect on human erythrocytes

To test whether the observed cytotoxic events caused by the selected *P. acnes* strains was specific to keratinocytes, we also treated washed human erythrocytes with the bacterium (MOI = 300). Spectroscopic analysis was performed to quantify the amount of free hemoglobin (HgB) released to the supernatant of the cell cultures as a result of possible membrane damage.

The free HgB results were similar of the results from the LDH assay: treatment with the *P. acnes 889* and *ATCC 11828* strains increased the concentration of free HgB in the culture supernatants at 72 hours post-treatment, whereas treatment with the *P. acnes 6609* strain had no such effect (Figure 14).

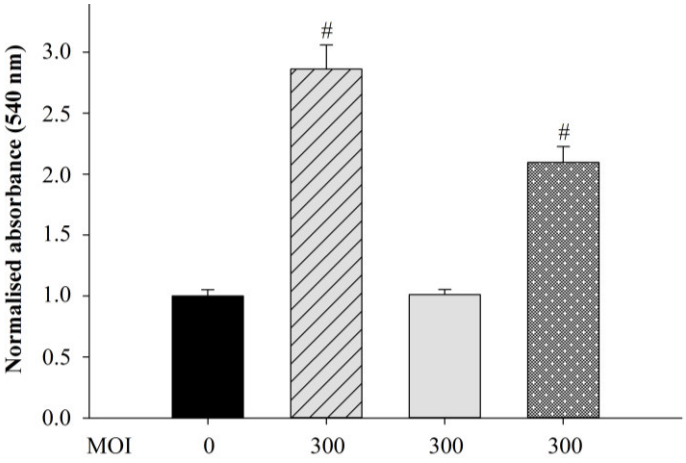

Figure 14. Hemolytic effect of *P. acnes* strains. Washed human erythrocytes were treated with *P. acnes* strains. After 72 hours, released HgB was measured from cleared supernatants. High-dose (MOI = 300) treatment with the *P. acnes 889* and *ATCC 11828* strains lead to increased level of free HgB, which is indicative of membrane damage and subsequent hemolytic effect. (Statistical analysis: one-way ANOVA, Tuckey's post hoc test. # $p<0.01$, compared to untreated control values. Results of *P. acnes 889*, *6609* and *ATCC 11828*-treated cleared supernatant samples are represented by striped, solid grey and dotted bars, respectively. Average of 3 parallel experiments.)

4.1.7 Some *P. acnes* strains decrease the pH of HPV-KER cell cultures

Metabolic activity of bacterial and human cells can modify the composition and chemical properties of the culturing media. To determine pH changes induced by *P. acnes*, HPV-KER cells were plated in serum-free DMEM-HG media, which contains a pH sensitive phenol-red dye. Cells were treated with live *P. acnes* bacterium (MOI = 25-300) as well as with heat inactivated ones (MOI = 300). We observed enhanced acidification of cultures treated with the *P. acnes 889* and *ATCC 11828* strains by visual inspection. We noted enhanced

acidification of the cultures treated with the *P. acnes 889* and *ATCC 11828* strains. The extent of pH changes was dose-dependent and strictly required the presence of live bacterium (Figure 15).

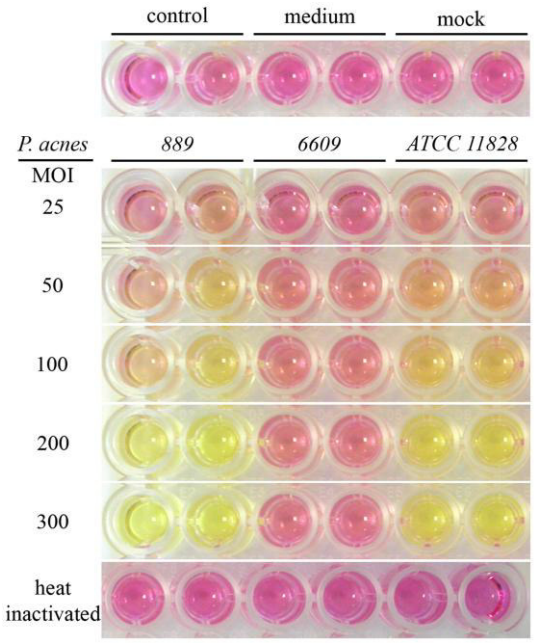

Figure 15. Dose-dependent pH changes induced by *P. acnes* strains. HPV-KER cells were treated with live and heat-killed bacteria at various MOIs in a DMEM-HG media, which contains a pH sensitive phenol-red dye. Culture pH was determined visually by inspecting the color changes of the phenol-red in the culturing medium. Gradual acidification was detected with increasing MOI in the live *P. acnes 889* and *ATCC 11828*-treated cultures. (Representative image of 3 parallel experiments.)

4.1.8 *P. acnes* production of PA may contribute to media acidification and cellular changes in the HPV-KER cultures

Next, we tried to determine the nature of the factors responsible for the observed pH and morphological changes, as well as keratinocyte membrane damage. Earlier reports suggested that *P. acnes* bacterium is able to synthesize SCFAs including PA, which is a unique product of its metabolism. To determine whether *P. acnes*-produced SCFAs contribute to the previously observed cellular changes, we treated the HPV-KER

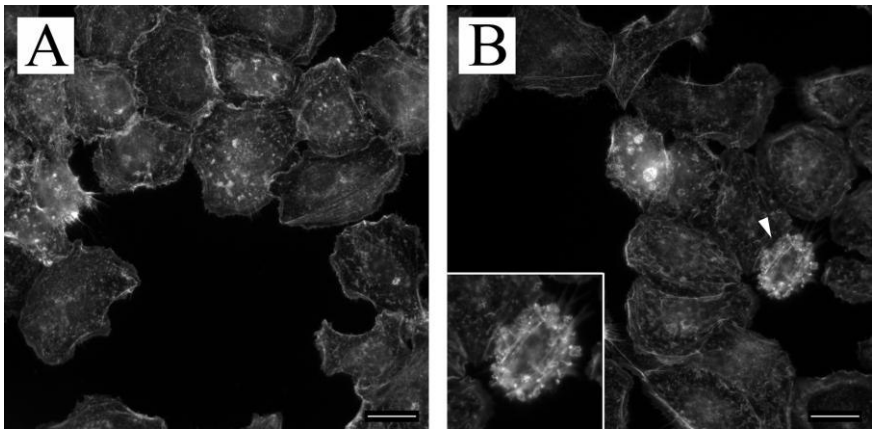

Figure 16. Microscopic analysis of PA-treated HPV-KER cells. (A) Control and (B) 48h PA-treated HPV-KER cells were stained with rhodamine-labeled phalloidin and subjected to microscopic analysis. Cells exhibiting irregular membrane morphology were detected in the PA-treated cultures. (White arrowhead points to a cell that is present in higher magnification in the lower left corner. Representative image of 3 parallel experiments. Scale bars: 10 μm)

cells with PA. Microscopic analysis of cells treated with PA and rhodamine-labeled phalloidin revealed similar irregular membrane morphologies observed earlier for *P. acnes* treatment (Figure 16). This result suggests that PA may be an important factor leading to the observed keratinocyte morphological changes.

4.1.9 PA secretion of *P. acnes* is strain- and dose-dependent

The results of our previous experiments provided only indirect evidence to the hypothesis that strain- and dose dependent differences are present in the amount of secreted SCFAs by the different *P. acnes* strains. To prove this, we treated HPV-KER cells with the bacterium (MOI = 100, 300), and determined the amount of secreted AA, PA and BA in the supernatant samples by mass spectrometric analysis.

The presence of AA and PA were detected in all samples. While the AA levels were similar in all cases, the amount of PA varied in a strains-, and dose-dependent manner at 72 hours post-treatment; higher levels were detected when higher bacterial loads (MOI = 300) of the *889* and *ATCC 11828* strains were applied (Figure 17D and H, respectively). Low levels of BA was also detected in the high dose (MOI = 300) *P. acnes 889*-treated HPV-KER culture supernatants (Figure 17D).

In order to prove that the detected SCFAs are bacterially-derived, we repeated the experiments, but this time in the absence of HPV-KER cells. While some AA was already present in the culturing media, bacterial fermentation clearly resulted in the release of additional amounts, while PA and BA were only detected in the bacterial-treated samples (Figure S1).

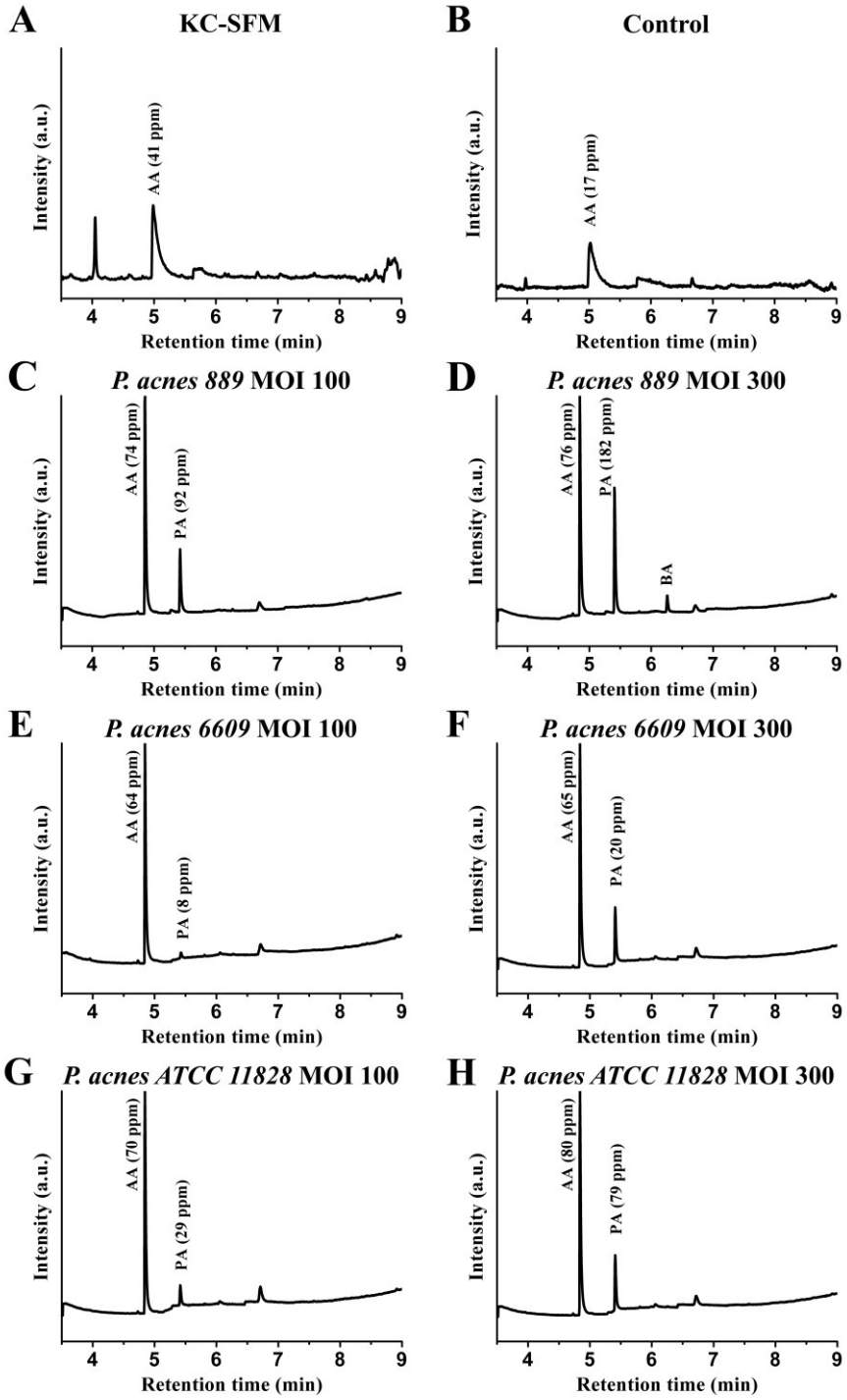

Figure 17. Identification of SCFAs in HPV-KER culture supernatant samples. HPV-KER cells were treated with *P. acnes* strains. After 72 hours, released SCFAs were measured by MS. While the level of AA was relatively stable in the samples, the PA levels exhibited strain- and dose dependent differences; higher amounts detected in the high dose (MOI = 300) *P. acnes 889* and *ATCC 11828*-treated cultures. In the (A) control medium (KC-SFM) and (B) the untreated HPV-KER supernatant samples the presence of PA and BA was not detected.

4.1.10. Comparison of the growth properties of the different *P. acnes* strains

One of the studied strains, *P. acnes 6609* appeared to be the least effective in our *in vitro* assays, and MS analysis of the bacterial treated HPV-KER supernatants showed that the PA levels were the smallest in these samples. Variations in the amount of secreted SCFAs may reflect differences in the growth and/or the metabolic properties of certain *P. acnes* strains. To test this hypothesis we analyzed the growth properties of our three strains by spectrophotometric growth curve analysis. We found that under both anaerobic and aerobic conditions *P. acnes 6609* was the slowest-growing strain, suggesting that within a given time frame these *P. acnes 889* and *ATCC11828* outnumber the *6609*, and as a consequence fewer bacterial cells might produce less SCFAs. It has to be noted that based on these results we cannot rule out the possibility of differences in the metabolic properties of the used strains, either (Figure 18).

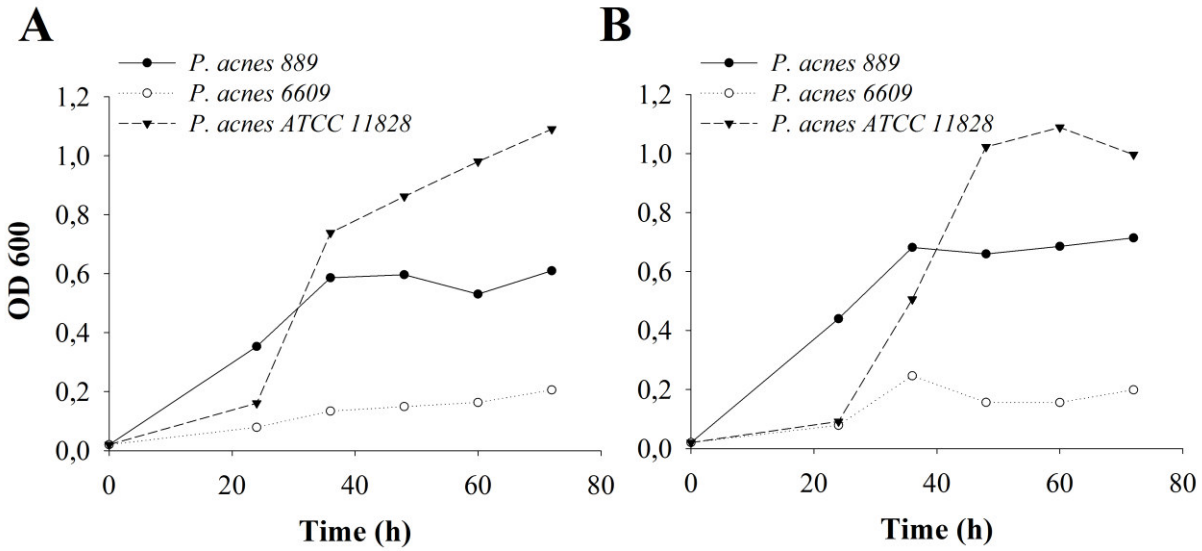

Figure 18. Growth properties of the different *P. acnes* strains. *P. acnes* strains (*889*, *6609*, *ATCC 11828*) were cultured in BHI medium at (A) 37°C under anaerobic conditions or (B) at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. OD_{600} values were measured up to 72 hours. The three *P. acnes* isolates exhibited differences in the growth properties; the *6609* strain was the slowest growing among the analyzed ones.

4.1.11 Combined treatment of the *P. acnes 6609* strain and PA induces cytotoxicity

The results of our previous studies strongly suggest that PA may be a factor responsible of the *P. acnes*-induced keratinocyte death, and that this SCFA is secreted at a different level by the studied strains. As our gathered data consistently indicated that *P. acnes 6609* was the

least effective strain in all our investigations, we concluded that this may be due to the impaired production of SCFAs, among them PA by this strain. To prove this hypothesis, we treated washed human erythrocytes with the *P. acnes 6609* (MOI = 300) in the presence of 1, 2 and 5 mM PA (Figure 19) and analyzed the level of induced cytotoxicity by measuring the level of free HgB in the supernatant samples

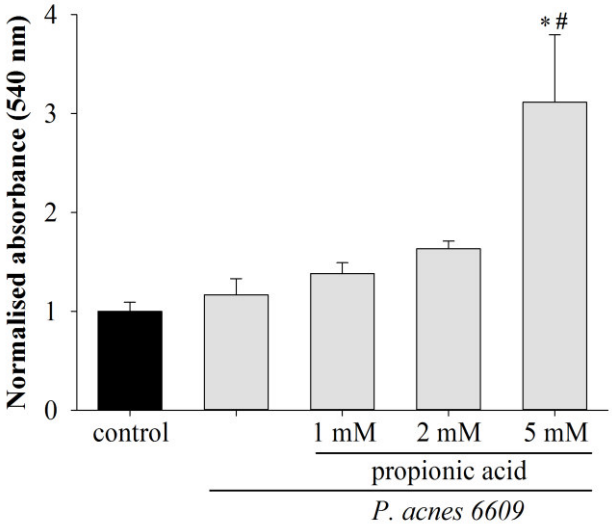

Figure 19. Cytotoxicity of PA in the presence of the *P. acnes 6609* strain. Washed human erythrocytes were treated with PA and/or a high dose of the *P. acnes 6609* strain (MOI = 300) and the amount of free hemoglobin was measured in a spectrophotometric assay. The greatest absorbance, indicative of membrane damage and subsequent cytotoxicity, were observed for the double treated cells. (Average of three parallel experiments. Statistical analysis: ANOVA, post hock Dunnett test, statistical significance established at $p<0.05$. Marks represent comparisons to untreated control (*) *or P. acnes 6609* MOI 300 (#) treated samples.

While this strain did not induce cytotoxicity alone, it led to the appearance of increasing free hemoglobin levels parallel with the increasing PA concentrations (as described in section 4.1.6). Our results thus suggest that while PA treatment clearly induces cytotoxicity, other bacterially derived factors may also contribute to this effect.

4.2 Identification and molecular characterization of inherited factors contributing to acne pathogenesis

During the course of our studies we aimed to characterize genetic susceptibility or protective factors that may modify the *P. acnes*-induced innate immune- and inflammatory events.

Innate immune activation and inflammation plays an important role in acne pathogenesis, and pro-inflammatory cytokines, especially TNFα and IL-1α, are known to be key factors in these events (Pomerantz JL 2002, Takeuchi O 1999, Ingham E 1992, Cunliffe WJ 2000). Based on this we hypothesized that common genetic factors affecting either the regulation of

the coding genes, or alternatively, the structure and/or function of the encoded proteins may modify the carriers risk to develop acne. In order to test this hypothesis, we reviewed the available literature data and selected known polymorphisms of the TNFA, IL-1A and IL1RN genes and tested them in retrospective case-control studies.

4.2.1 Studying the role of different TNFA promoter SNPs in the genetic predisposition to acne

While the coding part of the TNFA gene in relatively conserved, the promoter region exhibits a high level of genetic polymorphism (Waldron-Lynch F 1999). SNPs in this region may affect the regulation of the gene, and several of them have already been associated with an increased risk to develop different chronic inflammatory diseases (Fidder HH 2006, Waldron-Lynch F 2001). In order to investigate the role of such regulatory TNFA promoter polymorphims in the pathogenesis of acne, we selected five different ones (-238G>A, -308G>A, -857C>T, -863C>A, -1031T>C) and analyzed them in the available control and acne patient samples in retrospective case-control studies using the PCR-RFLP method. Clinical characteristics of the study group are present in the Materials and Methods section (Table 2).

4.2.1.1 The -1031T>C, -863C>A and -238G>A TNFA promoter polymorphisms are not associated with acne pathogenesis

In our studies we compared the genotype and allele frequencies in the control and patient cohort. In case of the above SNPs located at the -1031st, -863rd and -238th position (counted from the transcription start site) the distribution of the various genotype and allele frequencies in the control individuals was similar to that of acne patients (Figure 20 and Table 5).

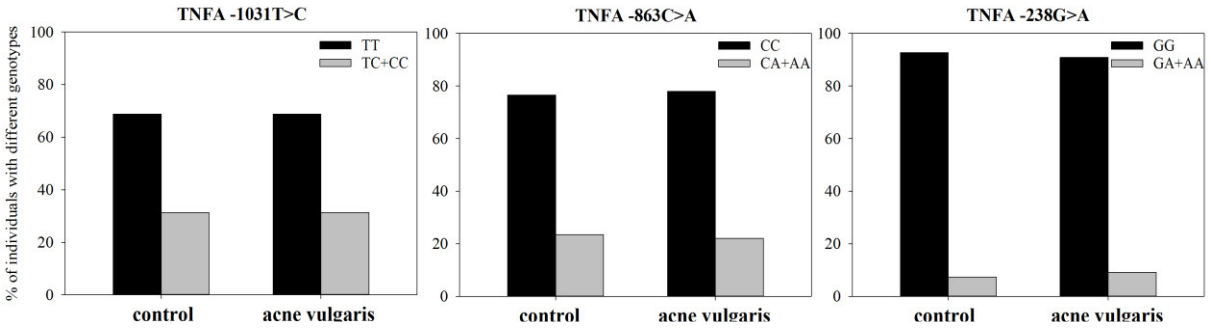

Figure 20. Distribution of various genotypes in the control and acne group. In case of the -1031T>C, -863C>A and -238G>A TNFA promoter polymorphisms, we could not detect differences between the control and acne groups.

In order to uncover potential gender specific differences, next we reanalyzed our data on the groups of males and females separately. Again, no statistically significant differences were detected (Table 6).

Finally, we also divided the patient cohort into groups based on the severity of individual lesions, and the clinical type of acne presented. We did not find any differences as a result of this analysis, either (Table 6). Overall these results indicate that the -1031T>C, -863C>A and -238G>A TNFA promoter polymorphisms are not associated with the pathogenesis of acne in our study population.

Polymorphism	Control n (%)	Acne vulgaris n (%)	Chi2 (P value)*	Odds ratio#	95% CI
TNFA -1031T>C	112	224			
Genotype frequency			1	1	0.61-1.63
TT	77 (68.7)	154 (68.8)			
TC	32 (28.6)	57 (25.4)			
CC	3 (2.7)	13 (5.8)			
Allele frequency			0.619		
T	186 (83.1)	365 (81.5)			
C	38 (16.9)	83 (18.5)			
TNFA -863C>A	111	222			
Genotype frequency			0.781	0.93	0.54-1.59
CC	85 (76.6)	173 (77.9)			
CA	25 (22.5)	43 (19.4)			
AA	1 (0.9)	6 (2.7)			
Allele frequency			0,933		
C	195 (87.8)	389 (87.6)			
A	27 (12.2)	55 (12.4)			
TNFA -857C>T	124	221			
Genotype frequency			**0.010**	1.79	1.14-2.81
CC	66 (53.2)	151 (68.3)			
CT	54 (43.6)	64 (29.0)			
TT	4 (3.2)	6 (2.7)			
Allele frequency			**0.023**		
C	186 (75.0)	366 (82.8)			
T	62 (25.0)	76 (17.2)			
TNFA -308G>A	126	229			
Genotype frequency			0,092	1.52	0.93-2.48
GG	95 (75.4)	153 (66.8)			
GA	30 (23.8)	72 (31.4)			
AA	1 (0.8)	4 (1.8)			
Allele frequency			0.095		
G	220 (87.3)	378 (82.5)			
A	32 (12.6)	80 (17.5)			
TNFA -238G>A	124	229			
Genotype frequency			0.539	1.29	0.57-2.91
GG	115 (92.7)	208 (90.8			
GA	9 (7.3)	20 (8.7)			
AA	0 (0)	1 (0.5)			
Allele frequency			0.467		
G	239 (96.3)	436 (95.2)			
A	9 (3.6)	22 (4.8)			

* **Statistical analysis using $\chi 2$ analysis with Pearson's correction**

Odds ratio of homozygote and heterozygote carriers of the minor alleles was determined together against homozygotes of the common alleles

Table 5. Genotype and allele frequencies of the studied TNFA promoter polymorphisms.

Polymorphism	Genotypes, n (%)								
	Combined			Female			Male		
TNFA -1031T>C	TT	TC + CC	*P* value*	TT	TC + CC	*P* value*	TT	TC + CC	*P* value*
Control (n=112)									
0	77 (33.3)	35 (33.3)	0.534	55 (38.7)	28 (37.8)	0.483	22 (24.7)	7 (22.6)	0.590
Acne (n=224)									
1	14 (6.1)	11 (10.5)		11 (7.7)	10 (13.5)		3 (3.4)	1 (3.2)	
2	108 (46.8)	48 (45.7)		63 (44.4)	34 (45.9)		45 (50.6)	14 (45.2)	
3	32 (13.9)	11 (10.5)		13 (9.2)	2 (2.7)		19 (21.3)	9 (29.0)	
TNFA -863C>A	CC	CA + AA	*P* value*	CC	CA + AA	*P* value*	CC	CA + AA	*P* value*
Control (n=111)									
0	85 (32.9)	26 (34.7)	0.951	61 (38.1)	18 (36.0)	0.870	24 (24.5)	8 (32.0)	0.955
Acne (n=222)									
1	19 (7.4)	5 (6.7)		17 (10.6)	4 (8.0)		2 (2.0)	1 (4.0)	
2	121 (46.9)	33 (44.0)		69 (43.1	26 (52.0)		52 (53.1)	7 (28.0)	
3	33 (12.8)	11 (14.7)		13 (8.1)	2 (4.0)		20 (20.4)	9 (36.0)	
TNFA -857C>T	CC	CT + TT	*P* value*	CC	CT + TT	*P* value*	CC	CT + TT	*P* value*
Control (n=124)									
0	66 (30.4)	58 (45.3)	0.001	50 (32.2)	41 (48.8)	0.012	16 (21.3)	17 (37.8)	0.029
Acne (n=221)									
1	14 (6.5)	12 (9.4)		13 ((9.2)	11 (13.1)		1 (1.3)	2 (4.4)	
2	104 (47.9)	47 (36.7)		67 (47.2)	29 (34.5)		37 (49.3)	18 (40.0)	
3	33 (15.2)	11 (8.6)		12 (8.5)	3 (3.6)		21 (28.0)	8 (17.8)	
TNFA -308G>A	GG	GA + AA	*P* value*	GG	GA + AA	*P* value*	GG	GA + AA	*P* value*
Control (n=126)									
0	95 (38.3)	31 (29.0)	0.101	69 (44.5)	22 (30.6)	0.022	26 (28.0)	9 (25.7)	0.918
Acne (n=229)									
1	20 (8.1)	9 (8.4)		16 (10.3)	8 (11.1)		4 (4.3)	1 (2.9)	
2	104 (41.9)	52 (48.6)		63 (40.6)	34 (47.2)		41 (44.1)	18 (51.4)	
3	29 (11.7)	15 (14.0)		7 (4.5)	8 (11.1)		22 (23.7)	7 (20.0)	
TNFA -238G>A	GG	GA + AA	*P* value*	GG	GA + AA	*P* value*	GG	GA + AA	*P* value*
Control (n=124)									
0	115 (35.6)	9 (30.0)	0.984	80 (39.2)	9 (40.9)	0.544	35 (29.4)	0 (0)	0.193
Acne (n=229)									
1	25 (7.7)	4 (13.3)		21 (10.3)	3 (13.6)		4 (3.4)	1 (12.5)	
2	142 (44.0)	15 (50.0)		88 (43.1)	10 (45.5)		54 (45.4)	5 (62.5)	
3	41 (12.7)	2 (6.7)		15 (7.4)	0 (0)		26 (21.8)	2 (25.0)	

*Statistical analysis using the Chi2 for linear trend probe

Goup 0: control patients, Group 1: acen comedonica subgroup of patients, Group 2: acne papulo-pustulosa subgroup of patients, Group 3: nodulo-cystec acne subgroup of patients

Table 6. Gender-specific genotype and allele frequencies of various promoter SNPs of the TNFA gene in control individuals and in subgroups of acne patients.

4.2.1.2 The -308G>A TNFA polymorphism may have a role in the acne pathogenesis in female patients

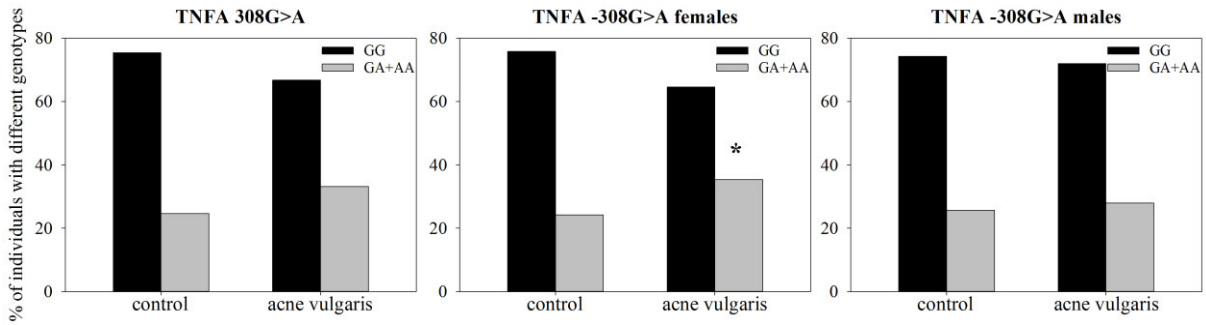

Figure 21. Gender-specific genotype frequencies of the TNFA -308G>A SNP in control individuals and acne patients. In case of the -308G>A TNFA promoter polymorphisms, we found positive association between the minor A allele and acne patients in females (Pearson Chi2 test p = 0.0458).

Next, we analyzed the TNFA -308G>A SNP and found a tendency for a more frequent occurrence of the rare A allele-containing genotypes in the patient group (Figure 21), but this difference did not reach a statistical significance in the overall dataset (Table 5).

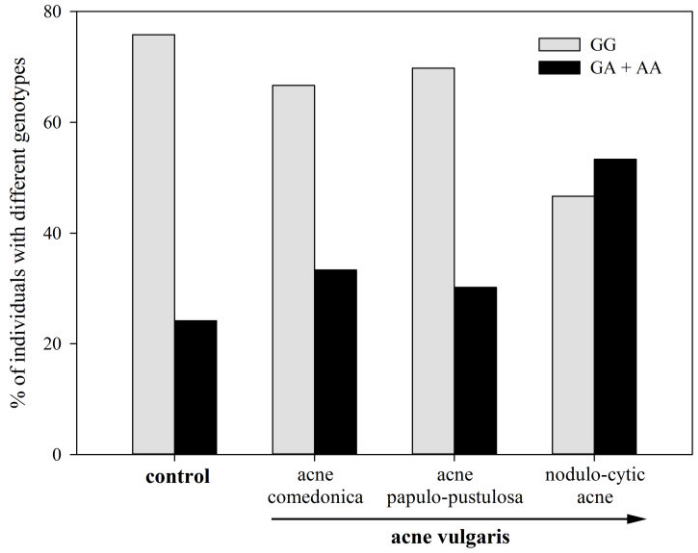

Figure 22. Distribution of various genotypes in the control and acne subgroups in females. In case of the -308G>A SNP the ratio of combined heterozygote and homozygote mutant (GA + AA) female patients are increasing parallel to the severity of inflammatory symptoms in the different acne subgroups, suggesting that the minor A allele might have a predisposing effect in female patients of

However, after the stratification of the two genders, a positive association was detected between the minor A allele and acne in female patients using the dominant inheritance model, whereas this was not the case in males (Figure 21).

We also analyzed the association between the severity of acne and the distribution of TNFA genotypes (Figure 22) in the various acne groups in both genders, and found an increasing frequency of the rare allele-containing GA+AA genotypes parallel in the different acne groups in female patients (Table 6). Overall, these data suggest that the rare A allele of the -308 TNFA polymorphism may have a role in the genetic predisposition to acne in the female patients of our study population.

4.2.1.3 The -857C>T TNFA promoter polymorphism has a protective role in the pathogenesis of acne.

Next, we genotyped our study population for the -857C>T TNFA polymorphism, and found a statistically significant difference in the distribution of the genotype and allele frequencies using the dominant genetic model (Table 5). Interestingly, the carrier frequency of the major C allele was higher in case of the acnes patient group, suggesting that the major C allele of this SNP is the one that is positively associated with acne.

Next, we analyzed the genders separately, but did not observe any gender specific differences (Table 6).

Last, but not least we also looked if the distribution of the various genotype and allele frequencies showed a correlation with the severity of the disease in the different patient groups (Table 6, Figure 23). We found that the frequency of the minor allele-containing genotypes decreased parallel to the severity of inflammatory acne symptoms.

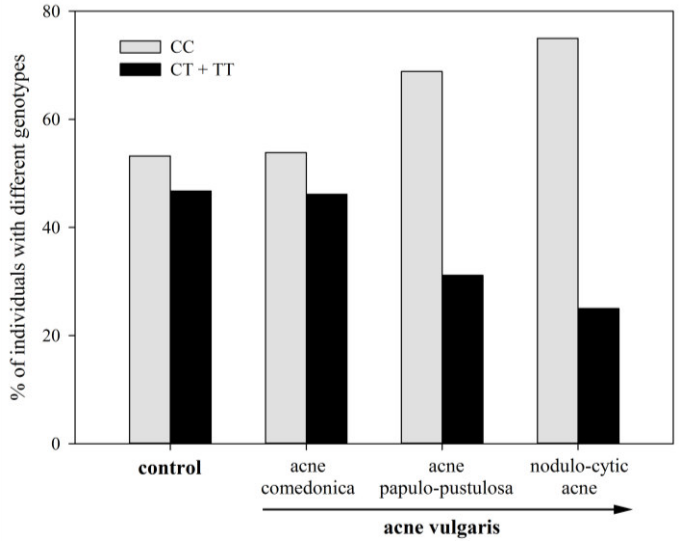

Figure 23. Distribution of various genotypes in the overall control and acne patient groups. In case of the-857C>T SNP the percentage of the homozygote wild type (C/C) individuals in the various groups is increasing parallel to the increase of the severity of inflammatory symptoms, suggesting that the major C allele is associated with acne vulgaris in our study population. The arrow indicates increasing severity of the inflammatory symptoms in the acne subgroups (Chi2 test for linear trend, p = 0.001).

4.2.1.4 Studying the effect of the TNFA -857C>T polymorphism on the promoter activity of the TNFA gene by a luciferase reporter assay

The TNFA -857C>T SNP was previously identified as a functional polymorphism in the promoter region of the TNFA gene (Hohjoh H 2001). In order to investigate the effect of the SNP on the regulation of the TNFA promoter activity in keratinocytes, we generated luciferase reporter constructs carrying the minor T or the major C allele at the -857th nucleotide position in the TNFA promoter fragment (pGL4.20-TNFA-857C-3' and pGL4.20-TNFA-857T-3', respectively). The two constructs were transiently transfected to HPV-KER cells and 24 hours later the cells were treated with the *P. acnes 889* strain. We measured luciferase activities 6 hours post-treatment.

In case of the construct containing the rare T allele (pGL4.20-TNFA-857T-3') we measured significantly lower basal luciferase activities and this difference appeared to be statistically significant (Figure 24). *P. acnes* treatments lead to increased promoter activities in case of both constructs, but the induced luciferase levels were also lower when the pGL4.20-TNFA-857T-3' plasmid was used (Figure 24).

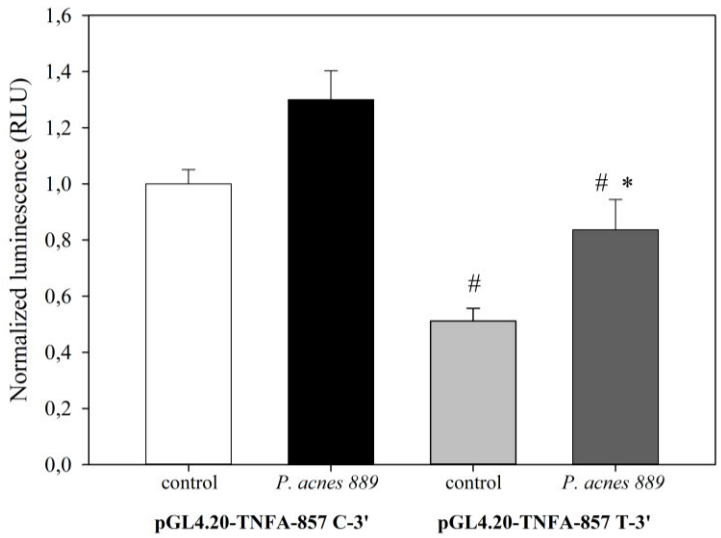

Figure 24. TNFA luciferase reporter assay. HPV-KER cells were treated with high dose of the *P. acnes 889* (MOI = 300) for 6 hours. In the presence of the minor T allele-containing construct even though the fold changes were comparable, both the basal as well as the induced promoter activities were lower in case of the rate T-allele containing construct. (All data were normalized to the basal activities measured in case of the common C-allele containing construct, without *P. acnes* treatment. Error bars: mean± SEM. Statistical analysis: one-way ANOVA, Tuckey's post hoc test. (# comparison to the pGL4.20-TNFA-857C-3' transfected, untreated control values ($p<0.01$), *comparison to comparison to the pGL4.20-TNFA-857T-3' transfected, untreated control values ($p<0.05$).

4.2.2 Studying the role of selected polymorphisms of IL-1 family members in the genetic predisposition to acne

The IL-1 family plays an important role in immune regulation. Among them the interleukin-1α (IL-1α) is the most studied member of this family and this is one of the best-known cytokines implicated in the pathogenesis of acne vulgaris (Aldana OL 1998, Guy R 2006, Guy R 1998). IL-1ra is a naturally occurring receptor antagonist protein encoded by the IL1RN gene. Genetic polymorphisms of these two genes have frequently been implicated in the pathogenesis of various chronic inflammatory diseases (Kawaguchi Y 2007, Jouvenne P 1999, Clay FE 1994, Tarlow JK 1994).

In order to investigate the exact role of the IL-1A and IL1RN polymorphisms in the acne pathogenesis, we chose to study the IL-1A +4845G>T SNP and the IL1RN VNTR polymorphism. We analyzed them in the available control and acne patient samples also in retrospective case-control studies.

4.2.2.1 The rare T allele of the IL-1A +4845G>T SNP may be a genetic predisposing factor

We determined the frequency of various genotypes and alleles of the IL-1A +4845 SNP in controls and acne patients (Table 7) and we found statistically significant differences using the co-dominant mode of inheritance (Pearson's $\chi 2$ test, $p = 0.03$ in both cases).

Polymorphism	Genotype frequency n (%)			Allele frequency n (%)		
IL-1A +4845G>T	GG	GT + TT	*P* value*	G	T	*P* value*
Control (127)	67 (52.76)	60 (47.24)	0,03	186 (73.23)	68 (26.77)	0,03
Acne vulgaris (217)	89 (41.01)	128 (58.99)		282 (64.98)	152 (35.02)	

Chi2 analysis 2 x 2 table

Table 7. Genotype and allele frequencies of IL-1A +4845G>T SNP in control individuals and acne patients. The minor T allele is positively associated with acne.

The analysis of the distribution of the genotype frequencies in the three acne subgroups showed that the frequency of the IL-1A +4845 genotypes positively correlated with the severity of acne symptoms (Figure 25). We also analyzed the genders separately in the three acne subgroups, but did not observe marked gender specific differences (data not shown).

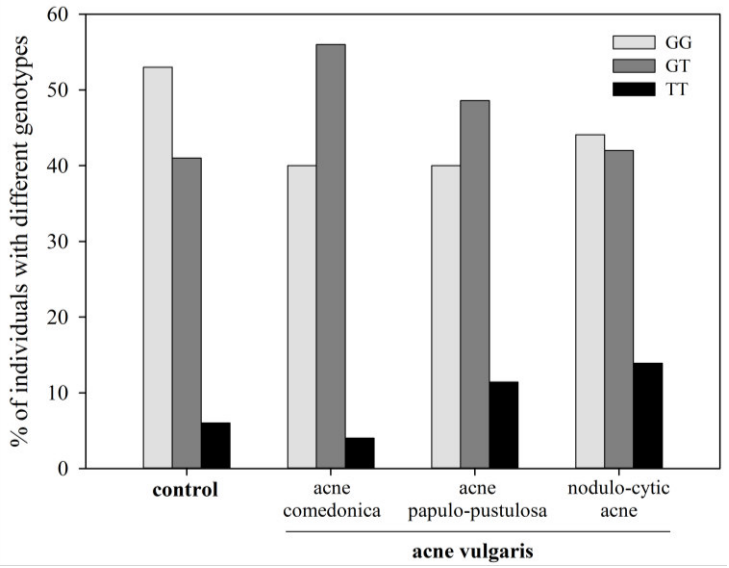

Figure 25. Distribution of various genotypes in the control and acne subgroups. In case of the IL-1A +4845G>T polymorphism the percentage of individuals carrying the minor T allele in a homozygote form correlates with the severity of acne symptoms, suggesting that the minor T allele is positively associated with acne. (Chi2 for linear trend analysis, $p = 0.03$)

4.2.2.2 The IL1RN VNTR polymorphism does not associate with the acne pathogenesis

Finally we also analyzed the VNTR polymorphism of the IL1RN gene in controls and acne patients, frequently associated with the pathogenesis of different chronic inflammatory diseases (Clay FE 1994, Tarlow JK 1994) by PCR amplification and subsequent fragment size determination using an agarose gel electrophoresis.

Polymorphism	Genotype frequency n (%)						Allele frequency n (%)			
IL1RN VNTR	1/1	1/2	1/3	2/2	2/3	*P* value*	1	2	3	*P* value**
Control (118)	56 (47.46)	47 (39.83)	5 (4.27)	8 (6.78)	2 (1.69)	0.96	164 (69.49)	65 (27.54)	7 (2.97)	0.83
Acne vulgaris (226)	111 (49.11)	88 (38.94)	8 (3.54)	17 (7.53)	2 (0.88)		318 (70.35)	124 (27.43)	10 (2.21)	

* Chi2 analysis 2 x 5 table; ** Chi2 analysis 2 x 3 table

Table 8. Genotype and allele frequencies of IL1RN VNTR polymorphism in control individuals and acne patients. In case of the IL1RN VNTR polymorphisms, we could not detect differences between the control and acne groups.

We detected three different alleles in our study population: the most frequent, four-repeat-containing allele 1 $(86\ bp)_4$, the two-repeat-containing allele 2 $(86\ bp)_2$, and allele 3, including five tandem repeats $(86\ bp)_5$. In the statistical analysis we did not observe any differences in the distribution of various genotype frequencies between the control and patients group and similar results were also found when the allele frequencies were compared. These results suggest that this polymorphism did not contribute to acne pathogenesis in our study population (Table 8, Figure 26).

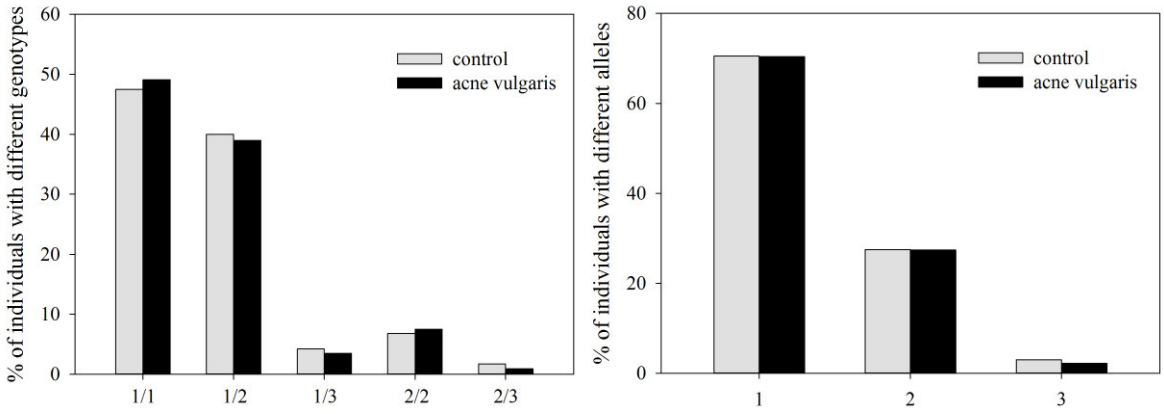

Figure 26. Distribution of various genotypes and alleles in the control and acne group. In case of the ILRN VNTR polymorphism, we could not detect differences in the genotype and allele frequencies between the control and acne groups.

5. Discussion

The skin is a complex tissue composed of many different cell types, but not all of them of human origin. According our current view the specialized microbial flora that is located mostly on the surface of the skin and within the PSU is an important and integral component, and together they form a complex ecosystem (Grice EA 2008). The exact function of this microbial community is not yet fully understood, but it has been suggested that the balanced interaction of the microbial and the human cells is important for the maintenance and promotion of healthy skin functions (Zeeuwen PL 2013).

The composition of skin microflora differs on various body parts, and although *P. acnes* is a major constituent, it is especially abundant in the sebum-rich regions from the start of the teenage years (Trivedi B 2012). This Gram-positive *Actinobacteria* preferentially resides on the face, shoulders, upper chest and back, which are also the regions that are most affected by acne vulgaris during puberty. Even though these data suggested the possible role of this commensal bacterium in acne pathogenesis for a long time, nowadays there is still a heavy scientific debate about its exact contribution (Dessinioti 2010, Shaheen 2011, Williams, 2012, Christensen GJ 2013). It is still not clear how and why an otherwise harmless commensal, such as the *P. acnes* bacterium, which is also a prominent member of the skin microbiome would turn to pathogenic. One of the possible explanations is that the *P. acnes* species is not homogenous, and the different phylogenetically distinct subgroups (phylogroups) that have recently been identified may also differ in their pathogenic properties, and thus can differentially affect various cellular and molecular properties of certain human cell types (McDowell A2013, Nagy I 2005). To analyze this we systematically investigated the effect of selected *P. acnes* strains (*889, 6609* and *ATCC 11828*) representing different phylogroups on HPV-KER cells. Using a detailed, real-time, label-free, impedance measurement-based approach, we found clear strain and dose-specific differences. High-dose treatment with *P. acnes 889* lead to a transient increase in the number of HPV-KER cells. This presumed increase in proliferation is very intriguing, as one of the initial and characteristic cellular changes that take place *in vivo* during acne lesion development is ductal hyperproliferation and hypercornification, the abnormal growth and differentiation of ductal keratinocytes of the PSU (Ganceviciene R 2006, Jeremy AH 2003). Our results suggest that selected *P. acnes*

strains may contribute to these early cellular changes in areas where the local presence of the bacterium reaches a certain threshold cell density.

High dose-treatment of *P. acnes 889* and *ATCC 11828* also caused the appearance of morphologically distinct, shrunken cells exhibiting extensive membrane irregularities. Recently, it was shown that these types of membrane reorganizations often occur in response to cellular damage caused, for example, by mechanical cell injury or the presence of various pore-forming toxins. Injured cells form blebs to localize and delimit the damaged area in an attempt to limit the loss of cellular constituents as well as to allow repair (Babiychuk EB 2011). In case of severe, irreparable damage these events may even lead to cytotoxicity. Currently we hypothesize that such events may be responsible for the cellular changes we observed in the *P. acnes*-treated HPV-KER cultures, as we could not prove the presence of apoptotic or necrotic events using classical methods such as DNA laddering or flow cytometry (data not shown). The observed cytotoxic events did not appear to be the result of a cell-type-specific interaction: similar effects were also detected in washed human erythrocytes and other cell types treated with *P. acnes* (Csukás Z 2004, Nakatsuji T 2011).

The production of bacterium-derived secreted pore-forming exotoxins may be responsible for the observed changes. Once such molecule, the *P. acnes* CAMP factor has been shown to be secreted at different rates by various strains (Nakatsuji T 2011, Valanne S 2005). However, the observation that the cellular changes correlated with marked pH changes in the cultures led us to hypothesize that these effects are due, at least partly to PA, a *P. acnes* metabolic product resulting from bacterial fermentation (Allaker RP 1987). This hypothesis is supported by reports that PA causes cytotoxicity in various cell types (Allaker RP 1987, Csukás Z 2004). We chose to test this hypothesis with *in vitro* experiments. Our MS analysis revealed the presence of AA and PA in the supernatant of *P. acnes*-treated HPV-KER cells. While the amount of AA was relatively invariant, strain- and dose dependent differences were measured in the PA levels, whereas BA was only detected in the high-dose *P. acnes 889*-treated cells. Next, we showed that the detected PA, BA and the part of AA is of bacterial origin. Taken all these together we hypothesized that PA may contribute to the biologic effect of *P. acnes* in keratinocytes, and we tested this idea in further *in vitro* experiments (Figure S1).

Currently, the available data on the concentration of PA in the PSU is limited. However, measurements in other, more easily accessible organs (e.g., the colon), where fermenting

bacterial species are present suggests that PA levels can be as high as 20–140 mM (Garland SH 2001). These concentrations are sufficient to control pathogenic microbes: minimal bactericidal or fungicidal PA concentrations for different species were found to be 10–25 mM (Wang Y 2014). Based on these data, we chose PA concentrations of 1–10mM for our *in vitro* experiments that was in a range often used by other investigators (Allaker RP 1985). Our results indicated that these treatments were sufficient to induce cellular changes in HPV-KER cells.

The function of PA in the skin is expected to be complex. PA can contribute to skin acidification, which inhibits the colonization and growth of harmful invaders (Elias PM 2007). In addition, PA exhibits antimicrobial properties that may be independent of its acidic nature (Wang Y 2014). High PA concentrations, however, may also have deleterious effects, leading to cellular damage and, thus, compromising the integrity of the epidermal barrier.

Our observations suggest that the cellular events that are initiated by the bacterium are strongly dose-dependent. This challenges the generally accepted view that there is no correlation between the bacterial load and the occurrence and severity of acne (Leeming JP 1988). However, our findings support novel studies suggesting that *P. acnes* is detected more frequently in acne vulgaris than in control skin samples (Jahns AC 2012). The differences between earlier and current results may reflect variations in the sampling techniques used, as the bacterium is often present in follicles as a biofilm, making quantitative studies more challenging (Jahns AC 2012, Alexeyev OA 2012).

Various *P. acnes* strains exhibited differences in our *in vitro* analyses. One of the studied strains, *P. acnes 6609* appeared to be the least effective in our assays. MS analysis of the bacterial treated HPV-KER supernatants showed that the PA levels were the lowest in these samples, which effect may be a direct consequence of the impaired growth properties of *P .acnes 6609*, compared to the other two studied strains (Figure 18). We propose that such differences may contribute to the determination of individual acne lesion severity in the affected individuals.

Overall, our results suggest that members of the microbiome modify the behavior of the human cells. Some of these cellular events are similar to what we can observe during the pathogenesis of skin diseases, such as in the case of acne. The exact outcome of the interaction of our microbiome and the human cells, however, are clearly dependent on the

exact microbiological properties of the bacterial cells, as well as on their abundance in the environment.

Apart from the above described bacterial properties, the response of different individuals' to the presence of the skin microbiome may also differ, and inherited factors may modify the outcome and extent of these reactions. To study these effects in more detail, we set out to identify and analyze genetic susceptibility or protective factors that may modify the risk of developing various clinical forms of acne vulgaris. Based on the available own and literature data we selected different genes playing an important role in the initiation and/or maintenance of *P. acnes*-induced innate immune and inflammatory events. Such molecules are the TNFα and IL-1α pro-inflammatory cytokines which are overexpressed in response to the recognition of the *P. acnes* bacterium following TLR 2 and 4 activation of the keratinocytes (Nagy I 2005, Kim J 2005).

The TNFA gene is located on the short arm of chromosome 6 (6p21.3) in the major histocompatibility class III region, where a high degree of genetic polymorphism is a characteristic feature. Different types of polymorphisms exist in the TNFA promoter, including SNPs and microsatellites, whereas the coding region is much highly conserved (Waldron-Lynch F 2001). The cytokine TNFα is a key molecule in various biologic processes, and its misregulation can have deleterious effects for the host organism. The SNPs in the promoter region can play a role in the allele-specific regulation of gene expression and are often reported to act as protective or disease-predisposing factors in the development of various inflammatory and infectious diseases and certain types of cancers (Knight JC 2005, Smith AJ 2008).

In classical retrospective case-control studies we investigated the role of 5 different SNPs located at the regulatory region of the TNFA locus. Among them no correlation has been found in case of the −1031T>C, −863C>A, -238G>ASNPs and acne. In case of the -308G>A polymorphism no association was detected in our overall dataset, but a significant association was discovered between the minor A allele and acne in female patients. These results differ in some aspect from the results obtained in other ethnic groups. The report by Baz et al. (Baz K 2008) demonstrates a strong association between the minor A allele of this SNP and acne vulgaris in a Turkish population, but no gender-specific differences were detected. In contrast to that, Sobjanek *et al*. reported similar results on a Polish population, with no association

between their overall dataset and acne, but they did not investigate gender-specific differences (Sobjanek M 2009). The reason for these differences is currently not clear, but it is interesting that the frequency of the A allele exhibits large geographic difference from east to west and the regional variations might be explained by differences in the linkage groups associated with the TNFα -308 SNP in the studied, geographically distant populations (Baz K 2008, Szabó K 2011).

Our analysis of the −857C>T SNP revealed that the frequent C allele exhibited a positive association with acne, whereas the minor T allele seemed to have a protective effect (Szabó K 2011). *In silico* sequence analysis revealed that in the presence of the minor T allele, a novel transcription factor binding site (OCT-1) is generated at the promoter region of the TNFA gene, immediately next to a pre-existing NF-κB binding site. As a result, an altered regulation of the gene might be generated in response to various stimuli, which involves the activation of NF-κB (van Heel DA 2002). To functionally test this hypothesis we generated allele specific reporter constructs, in which the firefly luciferase gene was placed under the regulation of the proximal TNFA promoter region. Two construct were generated; one included the common (C) allele, whereas the other harbored the rare (T) allele at the site corresponding to the -857th nucleotide position (Lv K 2006). Furthermore, the proximal part of the 3' untranslated region (3' UTR) of the TNFA gene was also cloned into both constructs, similarly as it was described by Denys *et al.* (Denys A 2002). The reason for that was that this region is known to contain specific sequences required for the destabilization of the TNFA mRNA and as a result, the mRNA has a rapid turnover and a short half life in the cells. By this modification we wanted to mimic these properties of the endogenous TNFA mRNA to avoid the artificial accumulation of our synthetic constructs that can potentially mask subtle expression differences resulting from the effects of the -857 TNFA alleles. Our reporter analysis showed differences in the level of basal, as well as in the *P. acnes* induced luciferase levels, with significantly lower activities measured in case of the rare TNFA -857T allele. This suggests that NF-κB-driven signaling events may be compromised in case of this allele leading to a protection in the carrier individuals from the over activation of immune and inflammatory events in response to various external stimuli.

Further case–control studies also revealed a positive association between the rare T allele of the +4,845(G>T) SNP of the IL-1A gene and acne, as well as a correlation between the

severity of acne symptoms and the frequency of the minor allele–containing genotypes (Szabó K. 2010). IL-1α protein is synthesized as pre-IL-1α and processed into the mature form by the enzymatic cut between amino acids 117 and 118 (Kobayashi Y 1990). Both isoforms are biologically active, with different subcellular localizations and hence biologic functions (Mosley B 1987, Lee S 2008). Pre-IL-1α has a predominantly nuclear localization, whereas the mature protein exhibits a cytoplasmic localization and can be secreted in response to the appropriate signals (Kobayashi Y 1990). The SNP at position +4,845 of the gene causes an

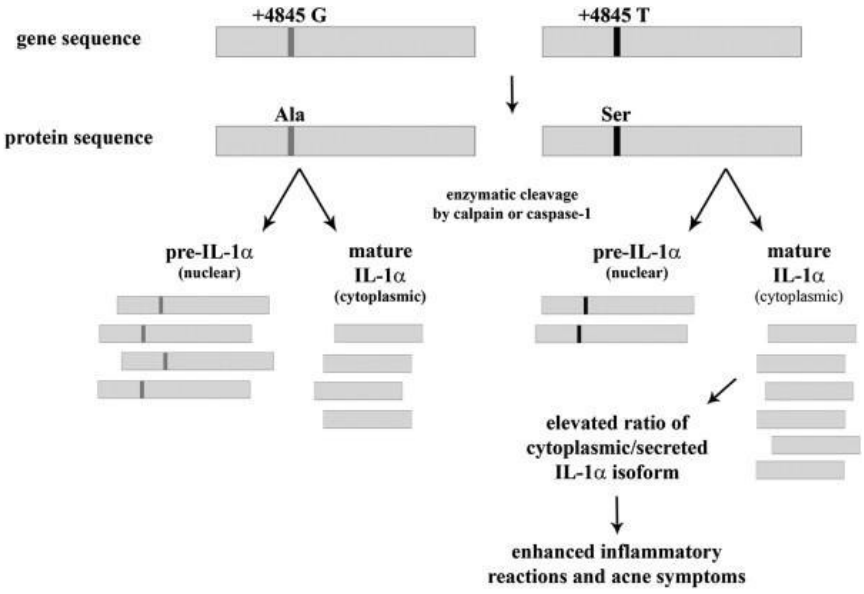

Figure 27. The IL-1A +4,845 (G>T) SNP causes an alanine to serine substitution close to the proteolytic cleavage site and might lead to enhanced cleavage when the rare T allele is present. This results in a shift in the ratio of nuclear versus secreted IL-1α isoforms. Excess secretion of mature IL-1α can lead to an activation cycle of the same and surrounding cells in an autocrine and paracrine manner, leading to uncontrolled inflammatory reactions (Szabó K 2011).

alanine to serine substitution close to the proteolytic cleavage site and might result in enhanced calpain-mediated cleavage when the rare T allele is present (Lee S 2008, Kawaguchi Y 2007). This leads to different ratios of nuclear versus secreted IL-1α isoforms and subsequently can have a potent effect on the regulation of epidermal homeostasis; carriers of the rare T allele may therefore be at higher risk of more severe acne symptoms (Figure 27) (Szabó K. 2010).

Lastly, we have also investigated the role of the VNTR polymorphism of the IL1RN gene, a naturally existing IL-1 receptor antagonist. Although it has been demonstrated in many cases that various alleles of this polymorphism often act as predisposing factors in the development of chronic inflammatory diseases, no association with acne was reported (Szabó K 2010).

6. Conclusion

Although the colonization of the human skin by the different commensal and symbiotic microbes starts immediately after birth, the *P. acnes* bacterium only becomes dominant in the PSU-rich skin regions during adolescence, when hormonal changes lead to follicular hyperplasia and increased sebum secretion. The bacterium can activate the pattern-recognition receptors and initiate cellular and molecular changes in the surrounding keratinocytes (Nagy I 2005). These events may be responsible for the initiation of an alert state of the cells within the follicles. Further increasing sebum secretion due to hormonal changes will lead to enhanced *P. acnes* growth that may result increased cell proliferation and abnormal differentiation of ductal keratinocytes. Dead corneocytes together with the waxy sebum may generate a thick substance forming a plug, which closes the ductal region of the PSU and the formation of comedones. This plug prevents sebum and bacterially-derived metabolic products from being emptied from the follicles to the skin surface. The changing microenvironment and the increasing anaerobic conditions may induce a modulation in the bacterial metabolism, and fermentation of the sebum components will lead to the release of SCFAs by *P. acnes* (Greenman J. 1981). The resulting increased acidification and elevated levels of molecules such as PA, may lead to keratinocyte cytotoxicity and increased vulnerability and subsequent rupture of the PSU wall. Bacterial metabolites and sebum components now come in close contact with the deeper skin tissues leading to the formation of inflammatory skin lesions. The severity of these reactions, however, is individually different and may depend on the combinations of carried genetic predisposing and protective factors of key genes.

All the above described events possibly take place in the adolescents, during a transition period in which adaptive immune events have not been established, or they are not accustomed to the presence of the *P. acnes* bacterium in our skin. When they are fully established, they may provide another, even higher level of control and regulation and thus contribute to the subsequent healing of acne symptoms after puberty.

Our data proves that a complex interplay of many host- and bacterial factors are important for the maintenance of healthy skin functions. A shift of this balance, called dysbiosis, can lead to various diseases, such as the most common inflammatory skin disease, acne vulgaris.

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9. Supplementary figure

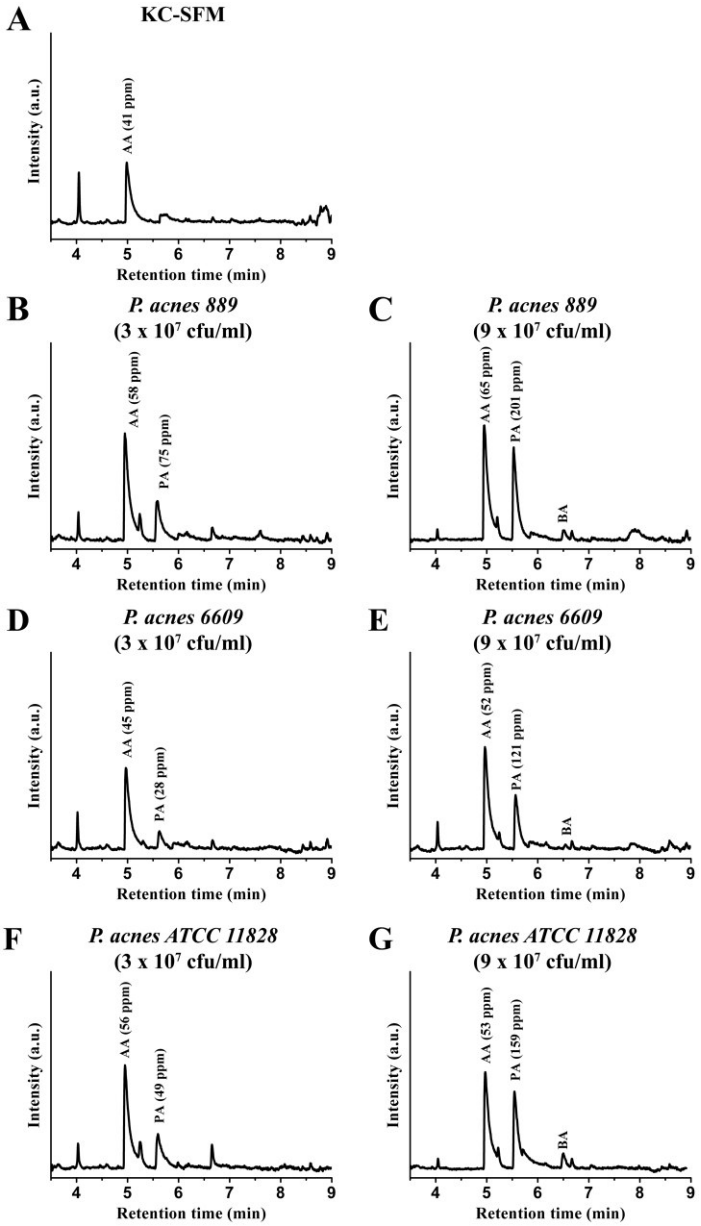

Figure S1. Identification of SCFAs in *P. acnes* culture supernatant samples. In order to prove that secreted SCFAs are by the different *P. acnes* strains, AB/AM-free KC-SFM culture medium was inoculated with live *P. acnes* bacterium alone using different starting concentrations (3 x 10^7 and 9 x 10^7 cfu/ml). After 72 hours, released SCFAs were measured in the culture supernatants by MS. While the level of acetic acid (AA) was relatively stable in the samples, the propionic acid (PA) and butyric acid (BA) levels exhibited strain- and dose dependent differences; higher amounts detected in the high dose (1 x 10^9 cfu/ml) *P. acnes 889* and *ATCC 11828*-treated cultures.

I.

INVESTIGATIVE REPORT

Propionic Acid Produced by *Propionibacterium acnes* Strains Contributes to Their Pathogenicity

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***Propionibacterium acnes* is an important member of the skin microbiome. The bacterium can initiate signalling events and changes in cellular properties in keratinocytes. The aim of this study was to analyse the effect of the bacterium on an immortalized human keratinocyte cell line. The results show that various *P. acnes* strains affect the cell-growth properties of these cells differentially, inducing cytotoxicity in a strain-specific and dose-dependent manner. We propose that bacterially secreted propionic acid may contribute to the cytotoxic effect. This acid has a role in maintaining skin pH and exhibits antimicrobial properties, but may also have deleterious effects when the local concentration rises due to excessive bacterial growth and metabolism. These results, together with available data from the literature, may provide insight into the dual role of *P. acnes* in healthy skin and during pathogenic conditions, as well as the key molecules involved in these functions. *Key words: immortalized keratinocyte cell line (HPV-KER);* Propionibacterium acnes*; acne vulgaris; short-chain fatty acid; propionic acid*.**

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Human skin harbours a specialized microbiota. Although the existence of this microbial community has been known for a long time, its exact function and contribution to healthy skin functions, and its role in the initiation of skin diseases during pathogenic conditions, has not been clearly established (1, 2). A complex and dynamic interaction has been recognized between the microbes and skin cells, and an increasing volume of data indicates their importance for the development and maintenance of healthy skin (3). Under certain conditions that are currently not well defined, dysbiosis may lead to the pathogenesis of different skin diseases, including the most common and well-known multifactorial, chronic inflammatory skin disease, acne vulgaris (4).

According to our current understanding, acne lesions start to develop around puberty as a result of hormonal changes that include an androgen excess, leading to enhanced sebum secretion and the subsequent hypercolonization of the otherwise commensal *P. acnes* bacterium (5). In addition, abnormal keratinocyte and sebocyte function, as well as innate immune and inflammatory events, have been shown to play a major role during lesion formation (6). However, the exact cause and sequence of these events, and whether *P. acnes* plays role in the initiation of the unusual keratinocyte and sebocyte behaviour, are not known (7).

P. acnes exhibits cytotoxic and haemolytic activities (8) and can activate the innate immune system via different pathogen recognition receptors, such as the Toll-like receptors (TLR); TLR2 and TLR4 in particular have been implicated in these processes (9, 10). As a result of this activation, a transcriptional program is initiated that is responsible for the activation and subsequent nuclear translocation of the nuclear factor (NF)-κB transcription factor (11). These activities result in changes in the expression of several genes, including those for proinflammatory cytokines, chemokines and antimicrobial peptides, subsequently leading to innate immune and inflammatory events in the affected cells (9, 10).

Based on sequence differences in bacterial *recA* and *tly* genes and the results of multi-locus sequence typing (MLST) experiments, 6 phylogenetic groups within the *P. acnes* species have been described (IA_1, IA_2, IB, IC, II, III) (12, 13). It has been suggested that the strains belonging to these phylogenetic groups might exhibit different virulence properties as well as differentially affect the cellular and molecular properties of human keratinocytes (10, 14).

To systematically analyse this phenomenon, we investigated the effect of 3 selected *P. acnes* strains on various cellular responses of an *in vitro* cultured immortalized human keratinocyte cell line, HPV-KER (Polyánka & Szabó, in preparation). Results from different strains were compared in order to determine whether they exerted differential effects.

Our results show that the selected *P. acnes* strains have differential effects on the cellular properties (growth

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and viability) of HPV-KER cells. The observed effects are dose-dependent and correlate well with the bacterial load that is present in the environment of the human cells. Previous studies suggest that pattern-recognition receptor activation (e.g. mediated by TLR2 and TLR4) by conserved bacterial pathogen-associated molecular patterns and the subsequent initiation of the NF-κB-dependent transcriptional program may be responsible for the activation of an alternative keratinocyte program (10). Our results indicate that, apart from this signal transduction, strain-specific differences in the concentration of acidic metabolites secreted by *P. acnes*, including the short-chain fatty acid (SCFA) propionic acid (PA), may also contribute to the observed effects.

MATERIALS AND METHODS (See Appendix S1[1])

RESULTS

Strain-specific effects of P. acnes *on cellular properties of HPV-KER cells*

In order to systematically analyse the effect of different *P. acnes* strains on the cellular properties of human *in vitro* cultured immortalized keratinocytes a novel cell line, HPV-KER, was used. These were shown to grow in monolayer, exhibit keratinocyte-like morphologies and respond to the presence of *P. acnes* with similar gene expression changes to those of normal human adult keratinocytes (NHEK) cells (Fig. S1[1]).

To monitor growth properties, HPV-KER cells were seeded in 96-well plates at different cell densities (5000, 10,000, 15,000 cells/well) and impedance-based analysis was performed using the xCELLigence system. Cell index (Ci) values were measured every 60 min for 72 h and plotted as a function of time (Fig. S2[1]).

The cells started to attach to the surface of the wells within 3 h, indicated by a rapid increase in Ci values, and, after a short lag period, they entered a growth phase. Plates seeded with 15,000 cells/well reached confluence 36 h after plating, whereas plates seeded with other cell densities remained in the growth phase during the entire experiment. Plating densities of 10,000 cells/well were used in experiments to detect changes in HPV-KER proliferation in response to *P. acnes* treatment.

For determining the strain-specific effects of *P. acnes* treatment, the experiment was repeated with the addition of the *P. acnes* strains 24 h after plating (Fig. 1).

Our results suggest that, when using low multiplicity of infections (MOIs) (25 and 50), no *P. acnes* strain had a significant effect on the properties of HPV-KER cells compared with untreated controls. The cells continued to proliferate in the presence of the bacteria until they reached confluence, and subsequently entered a stationary phase, marked by steady normalized cell index (nCi) values. In contrast, when the *P. acnes 889* strain was applied at a high dose (MOI=300), a rapid elevation of the nCi values was detected at approximately 15 h and continued until 45 h (Fig. 1A). However, when higher bacterial loads (MOIs of 200 or 300) of the *889* and *ATCC 11828* strains were applied, a small increase followed by a sharp decrease were noted (Fig. 1A and C, respectively). Similar changes were not observed for treatment with *P. acnes 6609* strain (Fig. 1B).

The above experiment was repeated, but this time instead of the HPV-KER cells, we treated NHEK ones using the same conditions. Our results indicate that NHEK cells responded to the presence of the *P. acnes* strains and exhibited nCi changes similar to those observed in the HPV-KER cells (Fig. S3[1]).

P. acnes 889 *and* ATCC 11828 *strains affect HPV-KER cell numbers*

Ci changes can reflect differences in the number or the specific dimensions of cells attached to the electrodes. To determine the exact nature of the *P. acnes*-induced cellular events that corresponds to the observed nCi

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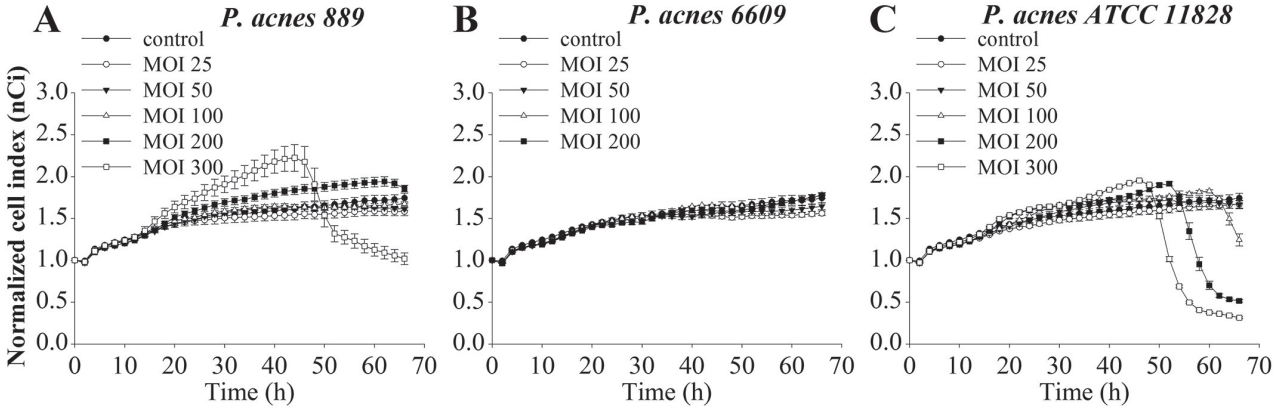

Fig. 1. Strain-specific effects of *P. acnes* on cellular properties of HPV-KER cells. HPV-KER cultures (10,000 cells/well) were treated with: (A) *P. acnes 889*, (B) *6609*, and (C) *ATCC 11828* at multiplicity of infections of 25–300, and normalized cell index (nCi) values were determined. The cell index (Ci) values were plotted as a function of time.

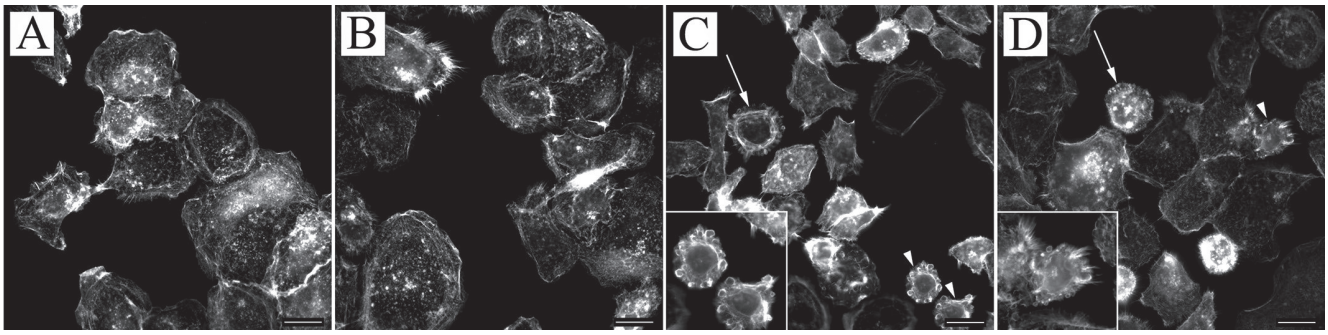

Fig. 2. Microscopic analysis of *P. acnes*-treated HPV-KER cells. Immortalized keratinocytes were stained with rhodamine-labelled phalloidin to visualize cytoskeletal F-actin bundles for an overall evaluation of cellular morphology. The presence of abnormally shaped, round cells was detected in the cultures treated with: (C) *P. acnes 889* and (D) *ATCC 11828* (multiplicity of infections 300), exhibiting a high degree of membrane irregularity (marked with white arrows and arrowheads), which was not apparent in: (A) control and (B) *P. acnes 6609*-treated cultures. (*Arrowheads* indicate cells depicted at high magnification in the lower left corner. Scale bars: 10 μm).

differences, we monitored the effect of the different *P. acnes* strains on the number of cells in the HPV-KER cell cultures.

High dose (MOI=300) of the *P. acnes 889* strain resulted in increased cell numbers compared with untreated and low-dose treated samples (Fig. S4A[1]). None of the other treatments resulted in an increase in cell number. However, the rate of increase in the cell number decreased 12 h after high-dose treatment (MOI=300) with the *ATCC 11828* strain.

High-dose treatment of the P. acnes 889 *and* ATCC 11828 *strains induces microscopic changes in HPV-KER cells*

To visualize cellular changes induced by *P. acnes* strains, we stained untreated and treated (MOI=300) HPV-KER cells with rhodamine-labelled phalloidin and performed a fluorescent microscopic analysis. We noted the presence of abnormally shaped rounded cells exhibiting irregular membrane morphology 48 h after treatment with the *P. acnes 889* and *ATCC 11828* strains (white arrows). This effect was not apparent in cells treated with the *P. acnes 6609* strain (Fig. 2).

P. acnes-*induced cytotoxicity is strain- and dose-dependent*

To determine whether *P. acnes*-induced cytotoxicity was due to damage of the keratinocyte membrane caused by the bacterium or by bacterially derived toxins, the amount of free lactate dehydrogenase (LDH) enzyme released to the supernatant of the damaged cells was determined using an LDH assay of keratinocytes treated with different doses (MOIs of 100 and 300) of the *P. acnes* strains.

Higher LDH levels were measured in cells treated with the *889* and *ATCC 11828* strains. This effect appeared to be dose-dependent at 72 h post-treatment. In contrast, no differences were detected in LDH levels in cells treated with the *P. acnes 6609* strain (Fig. 3A).

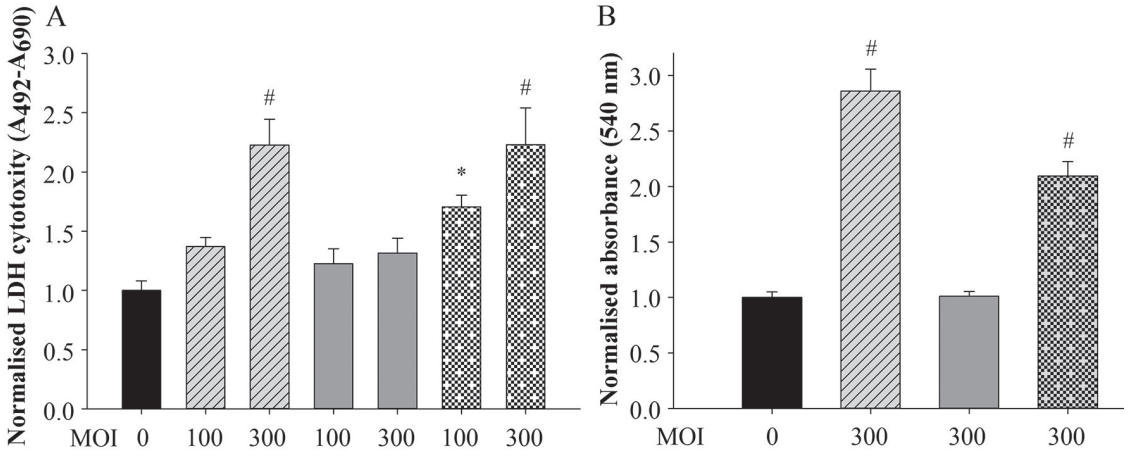

Fig. 3. Cytotoxicity of *P. acnes* strains. HPV-KER cells and washed human erythrocytes were treated with *P. acnes* strains. After 72 h, released (A) lactate dehydrogenase (LDH) from HPV-KER cells, and (B) haemoglobin from erythrocytes, were measured from cleared supernatants. High-dose (multiplicity of infection =300) treatment with the *P. acnes 889* and *ATCC 11828* strains led to increased levels of free LDH and haemoglobin, which is indicative of membrane damage and subsequent cytotoxicity. (Mean of 3 parallel experiments. Statistical analysis: one-way analysis of variance (ANOVA), Tuckey's *post hoc* test. $^{*}p<0.05$, $^{\#}p<0.01$, compared with untreated control values, marked with solid black bars. Results of *P. acnes 889 6609* and *ATCC 11828*-treated cleared supernatant samples are represented by striped, solid grey and dotted bars, respectively).

P. acnes *exhibits a strain-specific haemolytic effect on human erythrocytes*

To test whether the cytotoxic events caused by the selected *P. acnes* strains was specific to keratinocytes, we also treated washed human erythrocytes with the bacterium (MOI=300). Spectroscopic analysis was performed to quantify the amount of free haemoglobin released to the supernatant of the cell cultures as a result of possible membrane damage.

The free haemoglobin results were similar to the results of the LDH assay: treatment with the *P. acnes 889* and *ATCC 11828* strains increased the concentration of free haemoglobin in the culture supernatants at 72 h post-treatment, whereas treatment with the *P. acnes 6609* strain had no such effect (Fig. 3B).

Some P. acnes *strains increase the pH of HPV-KER cell cultures*

To determine pH changes induced by *P. acnes*, HPV-KER cells were plated in serum-free Dulbecco's Modified Eagle's Medium, high glucose (DMEM-HG) media, which contains a pH-sensitive phenol-red dye. We observed enhanced acidification of cultures treated with the *P. acnes 889* and *ATCC 11828* strains by visual inspection. The extent of acidification was dose-dependent (Fig. S5[1]).

P. acnes *production of PA may contribute to media acidification and cellular changes in the HPV-KER cultures*

The *P. acnes* bacterium generates SCFAs during metabolism. To determine whether the presence of SCFAs contributes to the observed cellular changes, we treated the HPV-KER cells with PA. Microscopic analysis of cells treated with SCFAs and rhodamine-labelled phalloidin revealed similar irregular membrane morphologies observed for *P. acnes* treatment. This result suggests that PA may be a factor leading to the observed keratinocyte morphological changes (Fig. 4).

PA secretion of P. acnes *is strain- and dose-dependent*

To determine whether there are differences in the amount of *P. acnes*-secreted SCFAs, we treated HPV-KER cells with the bacterium (MOI 0=100, 300), and the supernatant samples were subjected to mass spectrometry (MS) analysis.

The presence of acetic acid (AA) and PA were detected in all samples. While the AA levels were similar in all cases, the amount of PA varied in a strain-, and dose-dependent manner at 72 h post-treatment (Fig. 5); higher levels were detected when higher bacterial loads (MOI =300) of the *889* and *ATCC 11828* strains were applied (Fig. 5D and H, respectively). Low levels of butyric acid (BA) were also detected in the high-dose (MOI=300) *P. acnes 889*-treated HPV-KER culture supernatants (Fig. 5D).

In order to prove that the detected SCFAs are bacterially-derived, we repeated the experiments, but this time in the absence of HPV-KER cells. While some AA was already present in the culturing media, bacterial fermentation clearly resulted in the release of additional amounts. PA and BA were only detected in the bacterial-treated samples under certain conditions (Fig. S6[1]).

Combined treatment with the P. acnes 6609 *strain and PA induces cytotoxicity*

To determine whether PA plays a role in the bacterium-induced cytotoxic effects, we treated washed human erythrocytes with the *P. acnes 6609* strain (MOI=300) in the presence of 1, 2 and 5 mM PA (Fig. S7[1]). While this strain did not induce cytotoxicity alone, it led to the appearance of increasing levels of free haemoglobin parallel with the increasing concentrations of PA (Fig. S6[1]).

DISCUSSION

Human skin harbours a specialized microbial flora that is located mostly on the surface of the skin and within

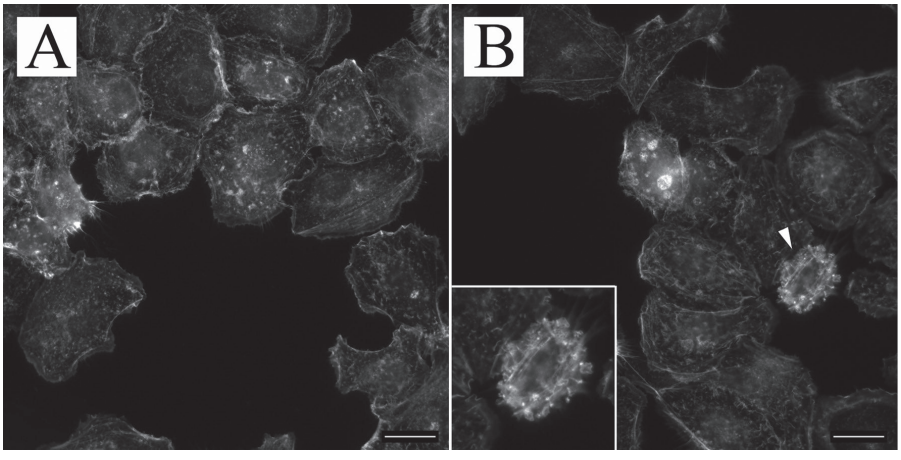

Fig. 4. Microscopic analysis of propionic acid (PA)-treated HPV-KER cells. (A) Control and (B) 48 h PA-treated HPV-KER cells were stained with rhodamine-labelled phalloidin and subjected to microscopic analysis. Cells exhibiting irregular membrane morphology were detected in the PA-treated cultures. (Arrowheads indicate cells depicted at high magnification in the lower left corner. Scale bars: 10 µm).

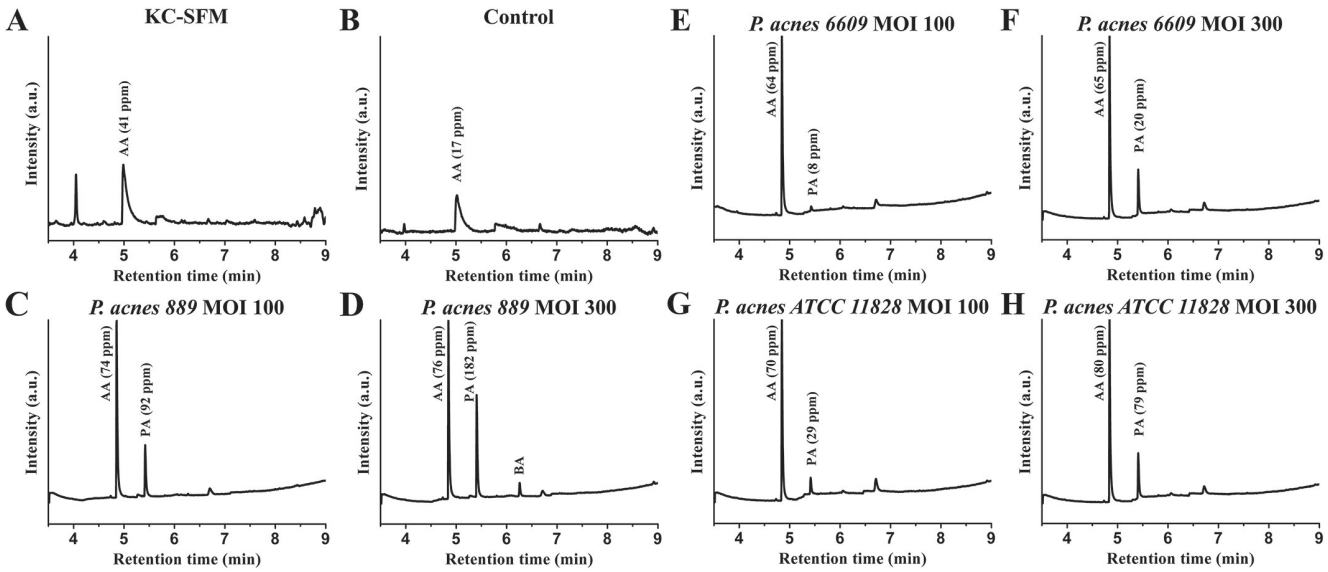

Fig. 5. Identification of short-chain fatty acids (SCFAs) in HPV-KER culture supernatant samples. (C–H) HPV-KER cells were treated with *P. acnes* strains. After 72 h, released SCFAs were measured by mass spectrometry (MS). While the level of acetic acid (AA) was relatively stable in the samples, the propionic acid (PA) levels exhibited strain- and dose-dependent differences; higher amounts detected in the high-dose (MOI=300) *P. acnes 889* and *ATCC 11828*-treated cultures. In (A) the control medium (keratinocyte serum-free medium (KC-SFM), and (B) the untreated HPV-KER supernatant samples the presence of PA and butyric acid (BA) were not detected.

the pilosebaceous unit (15). The exact function of this microbial community is not yet fully understood; however, it has been suggested that the interaction of the skin flora with skin cells is important for the maintenance and promotion of healthy skin functions (3). The composition of skin microbiota differs on various body parts, and although *P. acnes* is a major constituent, it is especially abundant in the sebum-rich regions (16). This Gram-positive *Actinobacteria* resides preferentially on the face, shoulders, upper chest and back, which are also the regions that are most affected by acne vulgaris during puberty. Thus, *P. acnes* has long been believed to play a role in the pathogenesis of this skin disease (4).

Detailed microbiological and, later, sequence-based phylogenetic analyses of various clinical isolates have indicated that this bacterial species is not homogenous, and different phylogenetically distinct subgroups (phylogroups) have been identified (13). It has been suggested that different strains belonging to these phylogroups may differentially affect various cellular and molecular properties of certain human cell types (10, 17).

In order to gain a better understanding of the properties of the interaction between the skin-colonizing *P. acnes* bacterium and the epidermal keratinocytes, we set up and systematically analysed an *in vitro* model system, using HPV-KER, a human immortalized keratinocyte cell line, previously established and characterized in our laboratory. We wanted to use a cell line that is easy to maintain, the availability of which is not limited, but which responds to the presence of the bacterium *in vitro* similarly to NHEK cells. In preliminary experiments we evaluated the well-known and widely used HaCaT cells. However, possibly because of their abnormally high NF-κB transcription factor levels (18), which is a known mediator of the TLR-mediated signalling events, HaCaT cells responded differently compared with NHEKs. In contrast, the newly established immortalized keratinocyte cell line HPV-KER reacted similarly to the presence of the bacterium (Figs S1–S3[1]).

Next, we systematically analysed the effect of selected *P. acnes* strains (*889*, *6609* and *ATCC 11828*) representing different phylogroups on HPV-KER cells. Using a detailed, real-time, label-free, impedance-based approach, we found clear strain- and dose-specific differences.

High-dose treatment with *P. acnes 889* leads to a transient increase in the number of HPV-KER cells up to 12 h after treatment. This presumed increase in proliferation is very intriguing, as one of the initial and characteristic cellular changes that take place *in vivo* during acne lesion development is ductal hypercornification. This hyperproliferation and abnormal differentiation of keratinocytes is particularly notable for keratinocytes lining the ductal region of the pilosebaceous units (19, 20). In the follicles, *P. acnes* mostly comes into direct contact with differentiated keratinocytes, whereas proliferating basal cells, that are modelled in our system reside in deeper tissue compartments. Our results suggest that selected *P. acnes* strains may contribute to these early changes in areas where the local presence of the bacterium reaches a certain threshold cell density through the presence of keratinocyte and/or bacterially-derived secreted factors that may reach these cells.

A drop in nCi values was noted in cultures treated with high doses of *P. acnes 889* and *ATCC 11828*.

Microscopic analysis of these cultures revealed the presence of morphologically distinct, shrunken cells exhibiting extensive membrane irregularities. Recently, it was shown that these types of membrane reorganizations often occur in response to cellular damage caused, for example, by mechanical cell injury or the presence of various pore-forming toxins. Injured cells form blebs to localize and delimit the damaged area in an attempt to limit the loss of cellular constituents and to allow repair (21). We hypothesize that the observed morphological changes and, in the case of severe, irreparable damage, cell morphological changes and death, are responsible for the observed drop in nCi values detected in our experiments. These events did not appear to be the result of a cell-type-specific interaction: similar effects were also detected in washed human erythrocytes and other cell types treated with *P. acnes* (8, 14).

The production of bacterium-derived secreted pore-forming exotoxins may be responsible for the observed changes. One such molecule, the *P. acnes* Christie, Atkins, Munch-Peterson (*CAMP*) factor, has been shown to be secreted at a different rates by various strains (8, 22). However, the observation that the cellular changes correlated with marked pH changes in the cultures led us to hypothesize that these effects are due to PA, a *P. acnes* metabolic product resulting from bacterial fermentation (23). This hypothesis is supported by reports that PA causes cytotoxicity in various cell types (14, 23). We chose to test this hypothesis with *in vitro* experiments. Our MS analysis revealed the presence of AA and PA in the supernatant of *P. acnes*-treated HPV-KER cells, and strain- and dose-dependent differences were measured in the PA levels. We also measured BA, but only in the high-dose *P. acnes 889*-treated cells. Next, we proved that the detected PA, BA and the majority of AA is of bacterial origin. Based on these results, we hypothesized that PA may be responsible for the previously observed effects, and we tested this idea in further *in vitro* experiments (Fig. S6[1]).

Available data on the concentration of PA in the pilosebaceous unit are limited. However, measurements in other, more easily accessible, organs (e.g. the colon), where fermenting bacterial species are present suggests that PA levels can be as high as 20–140 mM (24). These concentrations are sufficient to control pathogenic microbes: minimal bactericidal or fungicidal PA concentrations for different species were found to be 10–25 mM (25). Based on these data, we chose PA concentrations of 1–10 mM for our *in vitro* experiments. Our results indicate that these treatments were sufficient to induce cellular changes in HPV-KER cells.

The function of PA in the skin is expected to be complex. PA contributes to skin acidification, which inhibits the colonization and growth of harmful invaders (26). In addition, PA exhibits antimicrobial properties that may be independent of its acidic nature (25). High PA concentrations, however, together with other bacterial factors, may also have deleterious effects, leading to cellular damage and, thus, compromising the integrity of the epidermal barrier.

Our observations suggest that the cellular events that are initiated by the bacterium are strongly dose-dependent. This challenges the generally accepted view that there is no correlation between the bacterial load and the presence of acne (27). However, our findings support novel studies suggesting that *P. acnes* is detected more frequently in acne vulgaris than in control skin samples (28). The differences between earlier and current results may reflect variations in the sampling techniques used. The bacterium is present in follicles as a biofilm, making quantitative studies more challenging (28, 29).

One of the studied strains, *P. acnes 6609* appeared to be the least effective in our *in vitro* assays. MS analysis of the bacterial treated HPV-KER supernatants showed that the PA levels were the smallest in these samples. This appears to be the direct consequence of the impaired growth properties of *P. acnes 6609*, compared with the other 2 studied strains (Fig. S8[1]). Such differences may contribute to the determination of individual acne lesion severity in the carriers.

Similar to other members of the skin microbiome, various *P. acnes* strains activate pattern-recognition receptors to initiate signalling events in the cytoplasm of the affected cells (10). Thus, the presence of the bacterium may be responsible for the initiation of an alert state in the surrounding cells within the follicles. According to our current model, a plug closes the ductal region of the pilosebaceous unit during comedo formation. The environment within the follicle (increasingly anaerobic conditions, constant temperature and moisture, continuously generated sebum) aids the growth of *P. acnes*. The plug prevents sebum and bacterially-derived metabolic products from being emptied from the follicles to the skin surface, generating a gradual change in the microenvironment. *P. acnes* is an anaerobic, aerotolerant species, and as a result has the ability to grow under both anaerobic and microaerobic conditions. Thus, in the healthy follicles it is capable of oxidative phosphorylation under microaerophilic conditions. During comedo formation, the increasingly anaerobic conditions may induce a switch in the bacterial metabolism to fermentation of the sebum components and the subsequent release of SCFAs (30). As a result, excess acidification and increased concentration of certain molecules, such as PA, may lead to keratinocyte cytotoxicity, compromising the comedo wall and making it more vulnerable. This vulnerability, together with the increasing concentrations of molecules that promote innate immune and inflammatory events, and the severity of lesions, increases. The effect of PA is strikingly similar in the pathogenesis of periodontitis, another bacterially-mediated human inflammatory disease (31).

Together with available data from the literature our results may enhance the understanding of the dual role of *P. acnes* in healthy skin and during pathogenic conditions.

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The authors have no conflicts of interest to declare.

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ORIGINAL PAPER

TNFα gene polymorphisms in the pathogenesis of acne vulgaris

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Abstract Inflammation plays an important role in acne pathogenesis, and pro-inflammatory cytokines are key factors in these events. Tumor necrosis factor alpha (TNFα) is a central molecule coded by a gene that shows high level of genetic polymorphisms especially in its promoter region. Single nucleotide polymorphisms (SNPs) of the TNFα gene have been shown to be associated with an increased risk to develop chronic inflammatory diseases. In order to find out if known TNFα regulatory SNPs (−1031T>C, −857C>T, −863C>A, −308G>A, −238G>A) have a role in the development of the inflammatory reactions in acne vulgaris, we analyzed our genomic collection in a retrospective case–control study using the PCR–RFLP method, and we compared the resulting genotype and allele frequencies. There were no significant differences in the observed genotype or allele frequencies between the control and acne group in case of the −1031, −863, −238 SNPs; however, the TNFα −857 minor T allele was found to act as a protective factor in our study population in acne, and a higher occurrence of the minor −308 A allele in female acne patients was also noted. Genetic variants of the TNFα gene may affect the risk of acne vulgaris. Our results can help to elucidate the molecular events leading to acne development.

Keywords Acne vulgaris · Tumor necrosis factor alpha (TNFα) · Single nucleotide polymorphism · −857C>T · −308G>A

Introduction

The pathogenesis of acne vulgaris is a complex process in which several factors have been implicated [24] including excessive androgenic stimulation and sebum secretion [32], abnormal differentiation, proliferation and hyperkeratinisation of the epidermal keratinocytes lining especially the duct region of the sebaceous unit [31]. These processes lead to occlusion of the follicular orifice, and subsequent hypercolonisation by *Propionibacterium acnes* (*P. acnes*) bacterium and inflammation. The affected persons' individual genetic background may also play an important role. Despite the difficulties associated with the study of polygenic diseases such as acne vulgaris, genetic studies clearly demonstrate the importance of hereditable genetic factors in acne predisposition [2, 4, 14, 16, 30].

Epidermal keratinocytes are important regulators of the immune responses of healthy human skin in response to various external stimuli, as they are able to produce

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cytokines, chemokines and antimicrobial peptides in response to pathogenic attacks [6, 15, 28]. Toll-like receptor 2 and 4 (TLR2, TLR4) are key molecules for the recognition of *P. acnes* bacterium [23, 28]. The activation of these receptors initiates a signaling cascade in the cytoplasm of these cells and this subsequently causes the activation of the NF-κB transcription factor which, in turn, regulates the expression of a number of genes, among them the genes coding the cytokines tumor necrosis factor alpha and interleukin-1 alpha (TNFα and IL-1α) [33, 39]. Both of the above-mentioned cytokines have been implicated in the initiation and maintenance of inflammatory and immune responses in acne lesions. They have been shown to exhibit elevated expression already at the early stage when keratinocyte hyperproliferation and abnormal differentiation occur during comedo formation and also in later stages when extensive inflammation takes place [7, 20].

Simple monogenic genetic disorders are often caused by various missense and nonsense mutations that happen in the protein coding part of disease susceptibility genes. It has been proposed that the genetic susceptibility to complex diseases, however, is frequently a result of a different set of mutations, namely polymorphisms that affect the expression rather than the structure and function of various genes [19]. The TNFα locus is located within the highly polymorphic major histocompatibility III (MHC III) region on chromosome 6p21.3. There are many single nucleotide polymorphisms (SNPs) within this gene, especially in its 5′ regulatory region, whereas the coding- and the 3′-regions show a much higher degree of conservation [41]. The most frequent promoter SNPs in Caucasian populations are situated at positions −238 [8, 41], −308 [47], −857 [17], −863 and −1031 [18] in relation to the transcription start site. Several reports indicate that these SNPs might affect the regulation of gene expression by, e.g., interfering with transcription factor binding sites and other regulatory elements leading to allele-specific variations in the gene expression levels.

In order to investigate whether known and relatively frequent TNFα promoter polymorphisms predispose the carrier individuals to develop acne, we studied five SNPs (−238G>A, −308G>A, −857C>T, −863C>A, and −1031T>C) and performed PCR–RFLP analyses to compare the frequency of various genotypes in control individuals and acne patients. In case of the −857C>T SNP we found a statistically significant association between the major C allele and acne, and higher occurrence of the minor −308 A allele in female acne patients was also noted, although its significance in acne vulgaris needs more detailed analysis.

Results

TNFα −1031T>C, −863C>A, −238G>A polymorphisms are not associated with acne vulgaris

The number of patient and control samples that was genotyped for the TNFα −1031T>C, −863C>A, −238G>A polymorphisms is shown in Table 1. No association has been detected between any alleles of the −1031T>C, −863C>A, −238G>A polymorphisms and acne vulgaris, as the distribution of the various genotype and allele frequencies in control individuals was similar to that of acne patients (−1031: T/T = 68.7%, T/C + TT = 31.3 in controls vs. T/T = 68.8%, T/C + TT = 31.2% in acne patients, Pearson's χ^2 test 2 × 2 table, $P = 1$; −863: C/C = 76.6%, C/A + AA = 23.4% in controls vs. C/C = 77.9%, C/A + AA = 22.1% in acne patients, Pearson's χ^2 test 2 × 2 table, $P = 0.781$; −238: G/G = 92.7%, G/A + AA = 7.3% in controls vs. G/G = 90.8%, G/A + A/A = 9.2% in acne patients, Pearson's χ^2 test 2 × 2 table, $P = 0.539$).

We have not detected any association between the severity of acne and the different genotypes either (χ^2 for linear trend analysis, $P = 0.534$, 0.951, and 0.984, respectively), and this was also the case when we subdivided both the control and the acne group into females and males to detect potential gender-specific differences (Table 2). Our data suggest that these polymorphisms are not involved in acne predisposition in our study population.

TNFα −857C>T polymorphism is associated with acne vulgaris

Altogether 124 control and 221 acne patients were genotyped for the TNFα −857C>T SNP. The observed genotype and allele frequencies for acne patients and controls are presented in Table 1. In case of this SNP the genotype distribution of control individuals (C/C: 53.2%, C/T + T/T: 46.8%) showed a statistically significant difference when compared to acne patients (C/C: 68.3%, C/T + T/T: 31.7%), (Pearson's χ^2 test 2 × 2 table, $P = 0.010$). Interestingly, our data showed that in case of the −857C>T SNP the major C allele was positively associated with acne, as the odds of developing acne vulgaris were increased for the homozygote wild type (C/C) individuals (OR = 1.793, at 95% CI 1.14–2.81).

The severity of the acne was also in association with the TNFα genotypes (χ^2 test for linear trend, $P = 0.001$). Interestingly, the percentage of C/C versus combined C/T + T/T individuals was similar in controls compared to Group 1 of acne patients, containing individuals exhibiting

Table 1 Genotype and allele frequencies of various promoter SNPs of the TNFα gene in control individuals and acne patients

Polymorphism	Control *n* (%)	Acne vulgaris *n* (%)	χ^2 (*P* value)[a]	Odds ratio[b]	95% CI
TNFα −1031T>C	112	224			
Genotype frequency			1	1	0.61–1.63
T/T	77 (68.7)	154 (68.8)			
T/C	32 (28.6)	57 (25.4)			
C/C	3 (2.7)	13 (5.8)			
Allele frequency			0.619		
T	186 (83.1)	365 (81.5)			
C	38 (16.9)	83 (18.5)			
TNFα −863C>A	111	222			
Genotype frequency			0.781	0.93	0.54–1.59
C/C	85 (76.6)	173 (77.9)			
C/A	25 (22.5)	43 (19.4)			
A/A	1 (0.9)	6 (2.7)			
Allele frequency			0.933		
C	195 (87.8)	389 (87.6)			
A	27 (12.2)	55 (12.4)			
TNFα −857C>T	124	221			
Genotype frequency			0.010	1.79	1.14–2.81
C/C	66 (53.2)	151 (68.3)			
C/T	54 (43.6)	64 (29.0)			
T/T	4 (3.2)	6 (2.7)			
Allele frequency			0.023		
C	186 (75.0)	366 (82.8)			
T	62 (25.0)	76 (17.2)			
TNFα −308G>A	126	229			
Genotype frequency			0.092	1.52	0.93–2.48
G/G	95 (75.4)	153 (66.8)			
G/A	30 (23.8)	72 (31.4)			
A/A	1 (0.8)	4 (1.8)			
Allele frequency			0.095		
G	220 (87.3)	378 (82.5)			
A	32 (12.6)	80 (17.5)			
TNFα −238G>A	124	229			
Genotype frequency			0.539	1.29	0.57–2.91
G/G	115 (92.7)	208 (90.8)			
G/A	9 (7.3)	20 (8.7)			
A/A	0 (0)	1 (0.5)			
Allele frequency			0.467		
G	239 (96.3)	436 (95.2)			
A	9 (3.6)	22 (4.8)			

[a] χ^2 analysis with Pearson's correction

[b] Odds ratio of homozygote and heterozygote carriers of the minor alleles was determined together against homozygotes of the common alleles

a non-inflammatory form of acne. In contrast, decreased minor allele frequencies were detected in patients exhibiting more and more severe symptoms (Fig. 1a). Similar results were obtained when we looked at males and females separately, suggesting that in case of the −857C>T SNP the observed association could be observed in both genders.

The TNFα −308G>A polymorphism and acne vulgaris

126 control individuals and 229 acne patients were genotyped for the TNFα −308G>A. The genotype and allele frequencies found in acne patients and controls are presented in Table 1. In case of this SNP the genotype distribution of controls (G/G: 75.4%, G/A + A/A: 24.6%)

Table 2 Gender-specific genotype and allele frequencies of various promoter SNPs of the TNFα gene in control individuals and in subgroups of acne patients

Polymorphism	Genotypes, *n* (%)								
	Combined			Female			Male		
−1031T>C	TT	TC + CC	*P* value*	TT	TC + CC	*P* value*	TT	TC + CC	*P* value*
Control ($n = 112$)									
0	77 (33.3)	35 (33.3)	0.534	55 (38.7)	28 (37.8)	0.483	22 (24.7)	7 (22.6)	0.590
Acne ($n = 224$)									
1	14 (6.1)	11 (10.5)		11 (7.7)	10 (13.5)		3 (3.4)	1 (3.2)	
2	108 (46.8)	48 (45.7)		63 (44.4)	34 (45.9)		45 (50.6)	14 (45.2)	
3	32 (13.9)	11 (10.5)		13 (9.2)	2 (2.7)		19 (21.3)	9 (29.0)	
−863C>A	CC	CA + AA	*P* value*	CC	CA + AA	*P* value*	CC	CA + AA	*P* value*
Control ($n = 111$)									
0	85 (32.9)	26 (34.7)	0.951	61 (38.1)	18 (36.0)	0.870	24 (24.5)	8 (32.0)	0.955
Acne ($n = 222$)									
1	19 (7.4)	5 (6.7)		17 (10.6)	4 (8.0)		2 (2.0)	1 (4.0)	
2	121 (46.9)	33 (44.0)		69 (43.1)	26 (52.0)		52 (53.1)	7 (28.0)	
3	33 (12.8)	11 (14.7)		13 (8.1)	2 (4.0)		20 (20.4)	9 (36.0)	
−857C>T	CC	CT + TT	*P* value*	CC	CT + TT	*P* value*	CC	CT + TT	*P* value*
Control ($n = 124$)									
0	66 (30.4)	58 (45.3)	0.001	50 (35.2)	41 (48.8)	0.012	16 (21.3)	17 (37.8)	0.029
Acne ($n = 221$)									
1	14 (6.5)	12 (9.4)		13 (9.2)	11 (13.1)		1 (1.3)	2 (4.4)	
2	104 (47.9)	47 (36.7)		67 (47.2)	29 (34.5)		37 (49.3)	18 (40.0)	
3	33 (15.2)	11 (8.6)		12 (8.5)	3 (3.6)		21 (28.0)	8 (17.8)	
−308G>A	GG	GA + AA	*P* value*	GG	GA + AA	*P* value*	GG	GA + AA	*P* value*
Control ($n = 126$)									
0	95 (38.3)	31 (29.0)	0.101	69 (44.5)	22 (30.6)	0.022	26 (28.0)	9 (25.7)	0.918
Acne ($n = 229$)									
1	20 (8.1)	9 (8.4)		16 (10.3)	8 (11.1)		4 (4.3)	1 (2.9)	
2	104 (41.9)	52 (48.6)		63 (40.6)	34 (47.2)		41 (44.1)	18 (51.4)	
3	29 (11.7)	15 (14.0)		7 (4.5)	8 (11.1)		22 (23.7)	7 (20.0)	
−238G>A	GG	GA + AA	*P* value*	GG	GA + AA	*P* value*	GG	GA + AA	*P* value*
Control ($n = 124$)									
0	115 (35.6)	9 (30.0)	0.984	80 (39.2)	9 (40.9)	0.544	35 (29.4)	0 (0)	0.193
Acne ($n = 229$)									
1	25 (7.7)	4 (13.3)		21 (10.3)	3 (13.6)		4 (3.4)	1 (12.5)	
2	142 (44.0)	15 (50.0)		88 (43.1)	10 (45.5)		54 (45.4)	5 (62.5)	
3	41 (12.7)	2 (6.7)		15 (7.4)	0 (0)		26 (21.8)	2 (25.0)	

* Statistical analysis using the χ^2 for linear trend probe

Group 0: control patients, Group 1: acne comedonica subgroup of patients, Group 2: acne papulo-pustulosa subgroup of patients, Group 3: nodulo-cystic subgroup of patients

showed a difference when compared to acne patients (G/G: 66.8%, G/A + A/A: 33.2%), but this difference was not statistically significant (Pearson's χ^2 test, 2×2 table, $P = 0.092$).

Also, no association was detected between the severity of acne and the distribution of TNFα genotypes in the various subgroups (χ^2 test for linear trend, $P = 0.101$). The structured data showed that there was a positive association

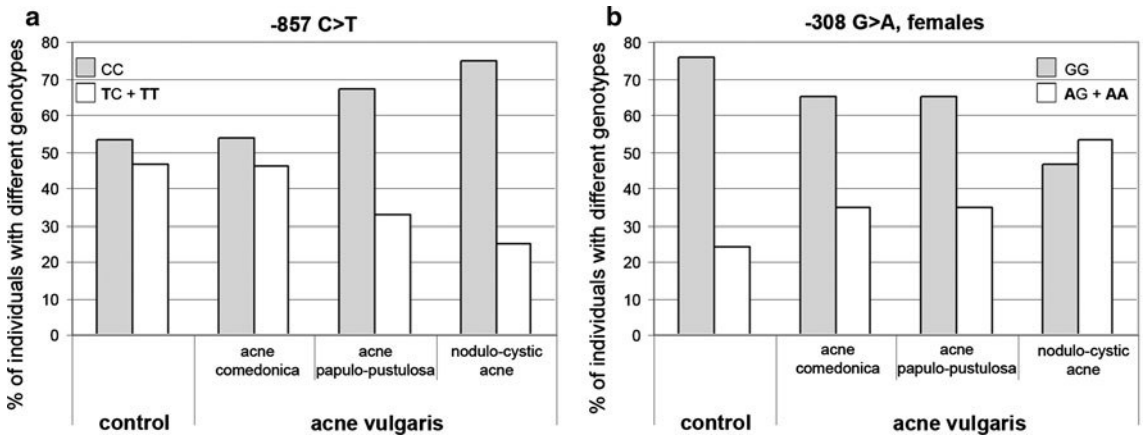

Fig. 1 Distribution of various genotypes in the control and acne subgroups. *Gray bars* represent the percentage of homozygote wild type individuals, whereas *white bars* show the percentage of individuals carrying one or two copies of the minor alleles (heterozygotes, or homozygote mutants) in the different groups. **a** In case of the −857C>T SNP the percentage of the homozygote wild type (C/C) individuals in the various groups is increasing parallel to the increase of the severity of inflammatory symptoms, suggesting that it is the major C allele that is associated with acne vulgaris in the studied population. **b** In case of the −308G>A SNP the ratio of combined heterozygote and homozygote mutant (GA + AA) female patients are increasing parallel to the severity of inflammatory symptoms in the various acne subgroups, suggesting that the minor −308 A allele might have a role in acne pathogenesis in the female patients of the studied population

between the minor −308 A allele and acne in females (χ^2 test for linear trend, $P = 0.022$), whereas this was not the case in males (χ^2 test for linear trend, $P = 0.918$) (Table 2).

Geographic differences of the observed allele frequencies of TNF −308G>A SNP

We have also compared the observed allele frequencies of the TNF −308G>A SNP with the two other published dataset investigating the association of this polymorphism and acne vulgaris [5, 37]. We found that the frequency of the minor allele containing G/A + A/A genotypes were different in the three studies (Turkish population: 13.2%; Polish population: 34.6%, our results: 24.6%), and our data lie in between the other two observed ones (Table 3).

Table 3 Observed TNFa −308G>A genotype frequencies of control individuals in various populations (acne studies)

	GG, *n* (%)	GA, *n* (%)	AA, *n* (%)
Turkish population[a]	99 (86.8)	15 (13.2)	0 (0)
Polish population[b]	49 (65.4)	25 (33.3)	1 (1.3)
Present study	95 (75.4)	30 (23.8)	1 (0.8)

[a] Baz et al. [5]

[b] Sobjanek et al. [37]

Discussion

In the healthy epidermis the keratinocytes slowly differentiate from the basal epidermal layer through the squamous, granular, and cornified layers. According to the keratinocyte activation hypothesis by Freedberg et al. [12] these natural differentiation events are often disturbed by various external stimuli that lead to the activation of epidermal cells. In this state alternative pathways are being opened for the affected cells that will subsequently lead to disturbed cell proliferation and differentiation. This special state is also marked by changes in the cellular cytoskeletal structures, and altered expression of various genes, e.g., cell surface receptors, keratins, adhesion molecules, cytokines. Key molecules that have been shown to play an important role in these events include TNFα, transforming growth factor alpha (TGFα), transforming growth factor beta (TGFβ), interferon gamma (INFγ), and interleukin 1 (IL-1) [3, 12, 27, 29]. It has been proposed in the literature that keratinocyte activation might play an important role in acne pathogenesis [7], but further studies are required to decipher the primary initiator of the events, the exact nature of this activated state, and the sequence of steps that take place at the molecular level.

After the initiation of lesions, in later stages of acne development, inflammation will dominate the pathogenic events. Severe inflammation can develop after the wall of the comedo brakes because of the increasing amount of stagnant sebum that comes into contact with the dermis. Free fatty acids in the sebum are cytotoxic, and *P. acnes* secreted lipases also have an irritative effect. As neutrophils arrive to the follicles they also release various inflammatory factors such as lysosomal enzymes and

reactive oxygen species that will damage the follicle wall even further [1, 26, 43], and thus exacerbate the inflammation.

Data gathered so far about the role of TNFα in the pathogenesis of acne vulgaris suggest that this cytokine might be an important molecule and has a dual role in acne pathogenesis. On one hand it seems to be involved in early stages in the initiation of lesion formation, in the regulation of innate immune events. On the other hand, TNFα is also an important player in later stages, in the development and control of inflammatory reactions, and has been suggested to be associated with excessive inflammation and thus the immunopathology of acne vulgaris. All these point out the importance of tight control of the TNFα level which is crucial for the maintenance of epidermal homeostasis, as both too little and too much protein can be deleterious for the affected host.

The level of TNFα is regulated at many different levels. These include transcriptional, RNA stability, and translational regulation, and important elements of the transcriptional regulation of the various polymorphisms (SNPs and microsatellites) that can be found at the 5′ regulatory (promoter) region of the gene. They are possibly playing a role by interfering with the various transcription factor binding sites that can be found nearby the polymorphism of interest, and thus lead to allele-specific differences in the TNFα mRNA level. All these will also affect the homeostasis of the tissue of interest, and subsequently will result in the progression of the diseases. In order to gain a deeper understanding of the role of TNFα in acne pathogenesis, we genotyped five known polymorphisms of this gene (−238G>A, −308G>A −857C>T, −863C>A, and −1031T>C) in control individuals and acne patients. We chose these particular SNPs because they have been associated with various inflammatory and immune-mediated diseases (e.g., inflammatory bowel disease (IBD) [11], COPD [21], rheumatoid arthritis [42], pemphigus [34], Graves disease [35], infectious diseases [9, 13]), and has been shown to be present in Caucasian individuals.

The data we have presented here strongly suggest that various alleles and of the TNFα gene are associated with acne vulgaris in our study population. Interestingly, in the case of the −857C>T polymorphism, the major C allele exhibited a positive association with acne whereas the minor T allele seems to have a protective effect. In case of this polymorphism a model has been suggested how this SNP can impact the pathogenic process. In the presence of the minor T allele a novel transcription factor binding site (OCT-1) is generated at the promoter region of the TNFα gene, right next to a preexisting NF-κB binding site. The OCT-1 (octamer transcription factor-1) protein has been shown to physically interact in vitro with the p65 subunit of NF-κB, thus leading to altered gene expression pattern in response to various stimuli that involves activation of NF-κB. Interestingly, the protective nature of the −857 T allele has also been suggested by van Heel et al. [40] who found very similar association between this allele and IBD in a British population. In another study from New Zealand [10] found that even though they could not detect an overall effect of this SNP in their IBD patient cohort, they did show decreased risk for specific types and locations of CD (ileocolonic Crohn's-disease and also decreased need for bowel resection) in their IBD patients.

In our data the percentage of C/C versus combined C/T + T/T individuals in case of the −857C>T SNP was similar in controls and patients with non-inflammatory acne (Group 1), whereas lower minor allele frequencies were detected in patients suffering from inflammation dominated forms of acne (Groups 2 and 3). Based on these results, we concluded that this SNP might play a prominent role in determining the severity of inflammatory events during later stages of acne development.

A positive association was also noted for the minor A allele of −308G>A in female acne patients. These associations were even more pronounced when we correlated the severity of acne with the observed genotypes (linear by linear association). In this case we detected higher minor A allele frequencies in the groups where patients exhibited more severe forms of inflammatory acne. There are numerous studies investigating the role of the TNFα −308G>A SNP in various diseases in several different populations, and many of them suggest that this polymorphism is important in determining the host's TNFα responses after various stimuli [25, 46, 47].

Recently, Baz et al. [5] reported a strong association between the minor A allele of this SNP and acne vulgaris in a Turkish population. In contrast to our result, however, they did not find an association between the severity of acne and the various genotypes, and also no gender-specific differences were detected. The reason for these dissimilarities is not clear, but might reflect differences in the linkage groups that are associated with the TNFα −308 SNP in the two studied population. The TNFα gene is closely linked to various HLA alleles [10], and this fact sometimes makes it difficult to distinguish between the effect of TNFα from that of other closely linked genes of the major histocompatibility complex. Our results, however, are more similar to the ones recently reported by Sobjanek et al. [37]. They did not find any association between the TNFα −308G>A and the −238G>A SNPs and acne vulgaris in the studied Polish population. Further in vitro and also genetic studies involving higher number of controls and patients in various populations would be advantageous to prove the effect of the −308 A allele in acne pathogenesis.

Baz et al. [5] also compared the reported genotype frequencies regarding the TNFα −308 SNP in control subjects from various studies performed in different ethnic groups. They included studies that investigated the association of this SNP and various diseases and found that the frequency of the GA heterozygote genotype is very low in various Asian ethnic groups (8–18%), whereas this value is much higher in Western European study cohorts (27–35.5%). So far there are data available from three different studies that are investigating exclusively the association of the TNFα −308 SNP and acne vulgaris [5, 37, present study]. Comparing the allele frequencies of the controls we find that the frequency of combined G/A + A/A genotypes we observed (24.6%) is in between the Turkish (Asian) and Polish (Western-European) data. This can be explained by the unique geographic position of Hungary and Romania, and also by historic influences (Osman subjection, Austro-Hungarian Monarchy).

Carrying various polymorphisms of different genes can predispose the carrier individuals to develop certain diseases by affecting the mRNA expression, or the stability and/or the structure of the encoded protein. In our study we have identified two SNPs in the regulatory region of the TNFα gene that showed association with acne vulgaris.

Materials and methods

Study population and ethics

Blood samples were obtained from 364 Caucasian individuals from Szeged collected by the University of Szeged Department of Dermatology and Allergology in Hungary and by the Department of Dermatology, Victor Babes University in Timisoara, Romania. Acne patients were collected from patients of these two Dermatology Departments. Control subjects were recruited from medical students studying at these two locations as well as from co-workers, and relatives of the participants.

A retrospective case–control study was conducted, and acne vulgaris diagnosis was defined as a dermatologists' diagnosis based on physical examination, patient records, and questionnaires. Acne patients were classified based on severity of the disease into three subgroups, as determined: Group 1 contained patients exhibiting non-inflammatory type of acne (acne comedonica), Group 2 comprised patients showing mild to moderate inflammatory (acne papulo-pustulosa) acne and Group 3 consisted of patients suffering from severe acne (nodulo-cystic acne). The control group comprised individuals showing none or only a very few mostly non-inflammatory types of lesions during their life. Altogether our genomic collection contained 126 controls (91 females and 35 males), and 229 acne patient samples (136 females and 93 males). All the participants were older than 20 years at the time of recruitment, and mostly co-workers and university students were recruited. Thus, the median age of the control group was 29.5 years, compared to 32.4 of the patient group.

In both locations the same dermatologist (Andrea Koreck) supervised the sample collection to make sure that the categorization of the different acne patients and control was performed uniformly at the two locations.

The study was approved by the Hungarian Research Ethics Committee and the Ethics Committee of "Victor Babes" University of Medicine and Pharmacy Timisoara. All subjects gave written consent before blood collection. The study was performed in accordance with the principles stated in the Declaration of Helsinki and its later revision.

Polymorphism analyses

Genomic DNA was obtained from peripheral blood leukocytes by a standard proteinase K digestion method, using the QIAamp Blood DNA Mini Kit (QIAGEN, Germany).

Acne patients and controls were genotyped for five promoter polymorphisms in the TNFα gene; −238G>A (rs361525), −308G>A (rs1800629), −857C>T (rs1799724), −863C>A (rs1800630), −1030T>C (rs1799964) using the PCR–RFLP method, as described previously (for references see Table 4).

Electrophoresis of the digested PCR products was performed on an 5% NuSieve agarose gel (Lonza, ME, USA). Gels were stained with GelRed (Biotium, Inc, Hayward, CA, USA) in order to visualize the DNA fragments. For representative gel pictures see Online Resource 1.

Selected samples were reamplified, purified, and subjected to sequencing analysis. These results showed a 100% concordance to the results we obtained by the PCR–RFLP analysis (Online Resources 2 and 3).

Statistical analysis

Statistical analysis was carried out on the groups of acne patients and controls according to the rules of case–control allelic association study design for all five SNPs. Genotype frequencies were calculated by determining the percentage of individuals carrying the different genotypes in both groups, and the statistical significance of the association was determined using the χ^2 test (2 × 2 table), using the SPSS software (Version 17, SPSS, Chicago, IL). In the analysis the number or homozygote wild type individuals was compared to the combined number of hetero-, and homozygote mutants, because the number of individuals with homozygote mutant genotype was too low ($n < 5$). Statistical significance was established at a P value of 0.05.

Table 4 Primer sequences and restriction enzymes used for the PCR–RFLP analyses

SNP	Amplification primers	Restriction enzyme	Reference
−238G>A (rs361525)	F: 5′-AGAAGACCCCCCTCGGAACC-3′	*Msp*I	Wilschanski [44]
	R: 5′-TGCACCTTCTGTCTCGGTTT- 3′		
−308G>A (rs1800629)	F: 5′-AGGCAATAGGTTTTGAGGGCCAT-3′	*Nco*I	Wilson [45]
	R: 5′-TCCTCCCTGCTCCGATTCCG-3′		
−857C>T (rs1799724)	F: 5′-AAGTCGAGTATGGGGACCCCCCGTTAA-3′	*Hinc*II	Kato [22]
	R: 5′-CCCCAGTGTGTGGCCATATCTTCTT-3′		
−863C>A (rs1800630)	F: 5′-GGCTCTGAGGAATGGGTTAC-3′	*Tai*I	Skoog [36]
	R:5′-CTACATGGCCCTGTCTTCGTTACG-3′[a]		
−1030T>C (rs1799964)	F: 5′-CAAGGCTGACCAAGAGAGAA-3′	*Bpi*I	Soga [38]
	R: 5′-GTCCCCATACTCGACTTTCAT-3′		

[a] Underlined nucleotides are mismatched nucleotide sequences

In case of the −857C>T SNP two proportions *Z* test was used to test the power of the detected association.

Odds ratios (ORs) for risk of acne and their 95% confidence intervals (CIs) were also calculated in a way that homozygote and heterozygote minor allele carriers were compared together with the homozygote wild type individuals for the various SNPs.

χ^2 tests for linear trend were calculated to assess the relationship between the severity of acne symptoms and the number of minor alleles the individual carries (homozygote wild type = 0, heterozygote = 1, homozygote mutant = 2). Again, statistical significance was established at a *P* value of 0.05.

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Conflict of interest statement The authors state no conflict of interest.

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III.

TISSUE ANTIGENS
IMMUNE RESPONSE GENETICS

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BRIEF COMMUNICATION

Interleukin-1A +4845(G>T) polymorphism is a factor predisposing to acne vulgaris

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Key words
acne vulgaris; IL1RN; interleukin-1 alpha; +4845 (G>T); single nucleotide polymorphism; VNTR

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Abstract

Acne vulgaris is a common chronic inflammatory skin disease of multifactorial origin. The aim of this study was to clarify whether known polymorphisms of the interleukin-1A (IL1A) and IL1RN genes play a role in the pathogenesis of acne vulgaris. A positive association was found between the minor T allele of the IL1A +4845(G>T) single nucleotide polymorphism (SNP) and acne, whereas no association was found with respect to any alleles of the variable number of tandem repeats (VNTR) polymorphism of the IL1RN gene. The severity of inflammatory acne symptoms correlated with the percentage of individuals carrying the homozygote T/T genotype. These results may help to elucidate the molecular events leading to the development of acne.

Acne vulgaris is a multifactorial inflammatory skin disease of the pilosebaceous follicle, caused by both host and environmental factors and microbial components. The pathogenesis of acne is thought to involve an interplay between a number of factors (1), including excess androgenic stimulation and sebum hypersecretion (2), abnormal differentiation, proliferation and hyperkeratinization of the epidermal keratinocytes, especially those lining the duct region of the sebaceous unit (3). All these processes lead to occlusion of the follicular orifice by a plug containing mostly a mixture of keratin and dead skin cells, together with sebum, hypercolonization by the bacterium *Propionibacterium acnes*, and the appearance of early skin symptoms called comedos. In later stages, the lesions and the surrounding tissues can become inflamed, and papules, pustules or, in more severe cases, nodules and cysts can develop. The severity of the inflammatory reaction and the subsequent acne symptoms vary considerably in the affected subjects, in part because of individual genetic susceptibility factors (4–9).

Interleukin-1α (IL-1α), one of the best-known cytokines implicated in the pathogenesis of acne vulgaris (10–12), is encoded by the gene interleukin-1A (IL1A). The IL-1α protein is a multifunctional pleiotropic cytokine that affects different cell types, with an important role in linking the innate and adaptive immune responses. It is also a central molecule in cutaneous inflammatory reactions: it is itself highly inflammatory, and has been shown to exert deleterious effects if its regulation and production are disturbed. For these reasons, the expression level and function of both the IL1A gene and the encoded IL-1α protein are tightly regulated at many levels, including the regulation of gene and protein expression and secretion (13), with additional regulation by the naturally existing receptor antagonist protein IL-1ra, encoded by the IL1RN gene.

In the pathogenesis of acne, IL-1α plays a role in the initiation of lesion formation: increased IL-1α immunoreactivity has been shown in the early steps of comedo formation (10). Moreover, external treatment with the IL-1α protein leads

to comedonal features, e.g. hyperproliferation and abnormal differentiation in isolated pilosebaceous units *in vitro* (11, 12).

All these data point to the importance of the tight regulation of the IL-1α level and bioactivity in the epidermal keratinocytes, and we therefore decided to study the effects of known genetic polymorphisms of the IL1A and IL1RN genes. The chosen polymorphisms were previously implicated as genetic susceptibility or protective factors in the pathogenesis of various chronic inflammatory diseases. Our results suggest that the variable number of tandem repeat (VNTR) polymorphism of the IL1RN gene does not show an association with acne, whereas the +4845(G>T) single nucleotide polymorphism (SNP) of the IL1A gene proved to be correlated with the disease in our study population.

Peripheral blood samples for the studies were obtained from 344 Caucasian individuals in Szeged, Hungary and Timisoara, Romania. A retrospective case-control study was conducted. Acne vulgaris was unambiguously diagnosed by the dermatologists on the basis of physical examinations, patient records and questionnaires. The same questionnaires were used at both universities. The distance between the two locations is only 120 km, and the populations at both locations can be regarded as typically Central-European. The acne patients were divided into three subgroups according to the severity of the disease: group 1 comprised patients exhibited non-inflammatory acne (acne comedonica), group 2 those with mild to moderate inflammatory acne (acne papulo-pustulosa), and group 3 those with severe acne symptoms (nodulo-cystic acne). The control group comprised individuals with none or only a few, mostly non-inflammatory lesions, who had never had severe acne symptoms. Overall, this genomic collection consisted of samples from 127 healthy controls (91 females and 36 males) and 229 acne patients samples (136 females and 93 males).

The study was approved by the Hungarian Research Ethics Committee and the Ethics Committee at Victor Babes University of Medicine and Pharmacy, Timisoara. All participating subjects gave their written consent before blood collection. The study was performed in complete accordance with the principles stated in the Declaration of Helsinki and its later revision.

Genomic DNA was obtained from peripheral blood leukocytes by a standard proteinase K digestion method, using the QIAamp Blood DNA Mini Kit (Qiagen, Hilden, Germany).

Acne patients and controls were genotyped for the +4845 (G>T) (rs17561) SNP at the IL1A gene using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method described by Agrawal et al. (14), whereas the IL1RN VNTR polymorphism was genotyped as reported by Tarlow et al. (15).

Statistical analysis was carried out on the groups of acne patients and controls in accordance with the rules of case-control allelic association study designs. Genotype frequencies were calculated by determining the percentages of individuals carrying the different genotypes in both groups, and the statistical significance of the association was determined by the chi-squared (χ^2) test, using the SPSS software (Version 17, SPSS, Chicago, IL). In the analysis of the IL1A +4845(G>T) SNP, the number of homozygote wild-type individuals was compared with the combined number of hetero- and homozygote mutants (GG *vs* TG + TT) in a 2 × 2 table. Statistical significance was established at a P-value of 0.05. Odds ratios (ORs) for the risk of acne and their 95% confidence intervals (CIs) were also calculated.

Among the cases of IL1RN VNTR polymorphism, five different genotype categories were identified, and a 2 × 5 table was used.

Chi-squared tests for a linear trend were calculated to assess the relationship between the severity of acne symptoms and the different genotypes. For this analysis, acne patients were stratified into three subgroups on the basis of disease severity, as described earlier, and analysed separately using the SPSS software. Statistical significance was again taken as $P < 0.05$.

One hundred and eighteen control individuals and 226 acne patients were genotyped for the VNTR polymorphism of the IL1RN gene, which is an 86-bp-long tandem repeat sequence in intron 2 of the IL1RN gene and which has been shown to be a susceptibility factor for various autoimmune, inflammatory and infectious diseases (16–18). Three different alleles were detected in our samples: the most frequent, four-repeat-containing allele 1 $(86\ bp)_4$, the two-repeat-containing allele 2 $(86\ bp)_2$, and allele 3, involving five tandem repeats $(86\ bp)_5$.

Comparison of the control and patients group did not show any difference in the distribution of various genotype frequencies. A similar result was obtained when the allele frequencies were compared (Table 1).

Table 1 Comparison of IL1RN VNTR polymorphism in control individuals and acne patients

VNTR	Genotype frequency *n* (%)						Allele frequency *n* (%)			
IL1RN	1/1	1/2	1/3	2/2	2/3	χ^2 *P*-value	1	2	3	χ^2 *P*-value
Controls	56 (47.5)	47 (40.0)	5 (4.2)	8 (6.8)	2 (1.7)	0.96[a]	164 (70.5)	65 (27.5)	7 (3.0)	0.83[b]
Patients	111 (49.1)	88 (39.0)	8 (3.5)	17 (7.5)	2 (0.9)		318 (70.4)	124 (27.4)	2 (2.2)	

VNTR, variable number of tandem repeat polymorphism.
[a] χ^2 analysis 2 × 5 table.
[b] χ^2 analysis 2 × 3 table.

Table 2 Comparison of IL1A +4845(G>T) polymorphism in control individuals and acne patients

SNP	Genotype frequency *n* (%)			Allele frequency *n* (%)		
+4845(G>T)	G/G	T/G + T/T	χ^2 *P*-value	G	T	χ^2 *P*-value
Controls	67 (52.8)	52 + 8 (47.2)	0.03	186 (73.2)	68 (26.8)	0.03
Patients	89 (41.0)	104 + 24 (59.0)		282 (65.0)	152 (35.0)	

SNP, single nucleotide polymorphism.

These led us to conclude that this polymorphism did not contribute to acne pathogenesis in our study population.

The observed genotype and allele frequencies for the 127 controls and 217 acne patients genotyped for the IL1A +4845(G>T) SNP are presented in Table 2. In the case of this SNP, the distributions of the various genotype and allele frequencies exhibited statistically significant differences between the control individuals and the acne patients (Pearsons χ^2 test 2×2 table, $P = 0.03$ in both cases). Our data also indicated that the rare T allele was positively associated with acne: the OR of acne vulgaris development was higher for individuals who carried the minor T allele in at least one copy (OR = 1.61 at 95% CI 1.03–2.5).

The results of the analysis of the distribution of the genotype frequencies in the three acne subgroups corroborated that the IL1A +4845(G>T) polymorphism is indeed an acne susceptibility factor, because the severity of the acne correlated with the observed IL1A genotypes (χ^2 for linear trend analysis, $P = 0.03$), as shown in Figure 1. The percentage of homozygote wild types (**G/G**) relative to individuals carrying at least one minor allele (**G/T + T/T**) was fairly constant within the subgroups, but the of the acne symptoms increased in parallel with the percentage of individuals carrying the minor allele in a homozygote form (**T/T**).

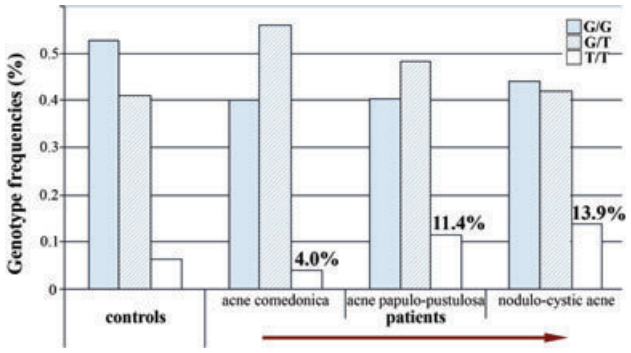

Figure 1 Observed genotype frequencies of the IL1A +4845(G>T) (rs17561) SNP in the control individuals and the subgroups of acne patients. The percentage of homozygote wild types (G/G) is fairly constant within the acne subgroups. In contrast, the percentage of individuals carrying the minor allele in a homozygote form (T/T) correlates with the severity of acne symptoms (acne comedonica: 4.0%, acne papulo-pustulosa: 11.6%, nodulo-cystic acne: 13.9%). The red arrow indicates increasing severity of the inflammatory symptoms in the acne subgroups.

Overall, these data led us to propose that the IL1A +4845(G>T) SNP acts as a genetic predisposing factor in the pathogenesis of acne vulgaris in our study population.

The present study related to the roles of the IL1A +4845(G>T) SNP and the VNTR polymorphism of the IL1RN gene in the pathogenesis of acne vulgaris. We chose to study these two polymorphisms because of the available data on the roles of IL-1α and related genes in the pathogenesis of this skin disease. The published data suggest the involvement of IL-1α both in the early stages of lesion formation in acne pathogenesis, at the initiation and regulation of the innate immune events, and also in the later stages, in the development and control of the inflammatory reactions (10–12). The evidence suggests that it is often not the exact level of the IL-1α protein that is important, but rather the ratio of the IL-1α protein and its antagonist IL-1RA. Any shift in this ratio will lead to an imbalance and hence the initiation of pathogenic events (19).

No differences were found in between the controls and the acne group in the distributions of the various genotypes and alleles in the cases of VNTR polymorphism of the IL1RN gene, suggesting that it probably did not play a role in the predisposition to acne in our population.

In contrast, a positive association was found for the +4845(G>T) SNP, suggesting that this SNP is indeed a genetic susceptibility factor. Furthermore, a linear association was shown between the severity of acne symptoms and the frequency of the minor allele-containing genotypes (Figure 1).

The IL-1α protein is synthesized as pre-IL-1α and processed into its mature form by the enzymatic cut between amino acids 117 and 118 (20). Both the premature and the mature IL-1α isoforms are biologically active, but their subcellular localizations and biological functions differ. Pre-IL-1α exhibits a predominantly nuclear localization, which is a result of a nuclear localization signal sequence (NLS) situated in the N-terminal pre-sequence. Upon maturation, the N-terminal is cleaved off by specific proteases (calpain, caspase-1) and the mature protein is generated. On elimination of the NLS signal, the resulting mature IL-1α takes up a cytoplasmic localization and this molecule can then be secreted in response to the appropriate signals (20).

The SNP at position +4845 of the gene causes an alanine to serine substitution of amino acid 114 of pre-IL-1α, which is immediately adjacent to the proteolytic cleavage site, and enhanced calpain-mediated cleavage can be observed when

the rare T allele is present at this position. The exact mechanism as to how this SNP modulates the cleavage requires further investigations (21, 22).

The IL1A +4845 SNP causes changes in the ratio of nuclear *vs* secreted IL-1α isoforms, which can affect epidermal homeostasis. Nuclear IL-1α has a role in transcriptional regulation (23), whereas the mature secreted form activates signalling cascades in an autocrine and a paracrine manner. It emerged from the present study that homozygote carriers of the rare T allele have a higher chance of suffering from more severe acne symptoms. The previously published *in vitro* data showed more extensive cleavage of pre-IL-1α and the ratio of nuclear *vs* secreted IL-1α is shifted towards higher amounts of the secreted isoform, which may subsequently lead to an altered IL1A/IL-1RA ratio.

Similar case-control studies of the effects of the +4845 G>T SNP have been conducted in a number of chronic inflammatory diseases, and a similar association has been reported in rheumatoid arthritis (21), chronic polyarthritis (24), polymyalgia rheumatica (25) and nasal polyposis (26), suggesting similarities in molecular pathogenesis of these diseases and acne vulgaris.

In summary, our data indicate that the rare allele of the IL1A +4845(G>T) SNP is associated with acne vulgaris in our study population. This, together with our previous findings (27) that a tumour necrosis factor-alpha (TNF-α) regulatory SNP also contributes to acne pathogenesis, suggests that genetic variations of primary pro-inflammatory cytokines causing alterations either in the fine regulation of these genes or in the function of the resulting proteins can result in imbalances in cellular homeostasis and hence a subsequent susceptibility to various immune mediated diseases such as acne vulgaris.

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