Hanna-Leena Kelhälä

THE EFFECT OF SYSTEMIC TREATMENT ON IMMUNE RESPONSES AND SKIN MICROBIOTA IN ACNE
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 8 of Oulu University Hospital (Kajaanintie 50), on 7 October 2016, at 12 noon

UNIVERSITY OF OULU, OULU 2016
Abstract

Acne is a common skin disease that affects nearly every teenager but in some cases it persists into adulthood as a chronic disease. Acne severity ranges from mild comedonal to severe cystic and scarring disease. The pathogenesis of acne is multifactorial: increased sebum production, hormonal influences, the hypercornification of pilosebaceous ducts, overgrowth of Propionibacterium acnes (P. acnes) and inflammation around pilosebaceous follicles are considered to be the main pathogenetic factors but few further details are known. The role of innate immunity in the pathogenesis of acne has been studied quite extensively, but the contribution of adaptive immune responses is not well characterized. Although isotretinoin has been the most potent medication for acne over 30 years, its mode of action is partially unknown. Furthermore, modern 16S ribosomal RNA (rRNA) gene amplicon sequencing based methods allow quantification of the abundance of skin bacteria, but these culture-independent techniques have not yet been used to assess the effect of systemic treatments, isotretinoin and tetracycline antibiotics on acne skin microbiota.

In this study the innate and adaptive immune responses in the skin of acne patients were investigated. Skin biopsies were taken from early-stage acne lesions and from uninvolved skin of acne patients, and the samples were examined for the expression of cytokines, chemokines, transcription factors and antimicrobial peptides, using real-time polymerase chain reaction (PCR). In addition, the skin biopsies were examined by immunohistochemistry and Luminex technology, which was also used in the determination of cytokine levels in acne patients’ serum samples. The skin microbiota of swab samples was investigated using 16S rRNA gene amplicon sequencing.

We found that innate and adaptive immune responses, in particular the activation of the IL-17/Th17 axis, are involved in early-stage acne lesions. Furthermore, the expression of effector cytokine TGF-β and transcription factor of regulatory T cells (Tregs) FoxP3 mRNA was reduced in acne patients’ uninvolved skin compared to normal skin of healthy controls. Isotretinoin has no direct effect on innate and adaptive immune responses in newly formed acne lesions. It did, however, modify some innate immune responses: the expression of IL-1β and toll-like receptor 2 (TLR2) mRNA was reduced in uninvolved skin of acne patients, and the number of CD68+ macrophages increased in acne patients’ skin with isotretinoin treatment. After six weeks’ treatment, the abundance of P. acnes was similarly reduced with either isotretinoin or lymecycline treatment. However, previous studies and clinical experience show that isotretinoin is more clinically effective against acne than tetracyclines, probably because of the effect of isotretinoin on sebaceous glands and sebum excretion.

Acne is a disease, which at its worst results in significant scarring. Therefore it is important to understand the immunological factors behind its pathogenesis. Our findings show that both adaptive and innate immunity play an important role. Isotretinoin, although currently the most potent anti-acne drug, does not directly affect the adaptive immune response and therefore new medications are required to control the relevant immune responses, especially in severe cystic or fulminant acne.

Keywords: acne, adaptive immunity, innate immunity, isotretinoin, microbiota, Propionibacterium acnes, tetracycline
Tiivistelmä


Asiasanat: akne, hankittu immuuniteetti, isotretoniini, mikrobiomi, propionibakteeri, synnynnäinen immuniteetti, tetrasykliiri
To Kikka
Acknowledgements

This study was carried out at the Department of Dermatology, University of Oulu. The work was financially supported by MRC Oulu Doctoral Program, Oulu University Hospital VTR/EVO funding, the Finnish Dermatological Society and Oulun Lääketieteellinen Tutkimussäätiö.

I am deeply grateful to my principal supervisor Professor Antti Lauerma, MD, PhD. This work would not have been possible without your interest in the research topic and your financial support. I warmly thank my supervisor Docent Riitta Palatsi, MD, PhD, for the research ideas and bringing me to the complicated world of immunology. Your friendship and support throughout the project have been valuable. I would also like to express my deep gratitude to my third supervisor Professor Kaisa Tasanen-Määttä, the Head of Department of Dermatology, MD, PhD, for all the practical advice you have provided. Your guidance, positive attitude and encouragement have been invaluable.

I would like to extend my gratitude to the pre-examiners of this thesis, Professor Gregor Jemec, MD, PhD, and Docent Petteri Arstila, MD, PhD. I appreciate your valuable comments, which helped to improve the final version.

I also thank Steve Smith for revising the English language of this thesis.

I would also like to acknowledge my follow-up group members, Docent Petri Kulmala, MD, PhD, Päivi Hägg, MD, PhD, and Nina Kokkonen, PhD, for sharing your time and taking an interest in my thesis.

I owe my deepest gratitude to all patients and other volunteers for their contribution to this study.

I wish to thank warmly all collaborators and co-authors of the thesis project in Helsinki, Berlin, Sophia Antipolis and Oulu. I would like to give my special thanks to Docent Nanna Fyhrquist, PhD, for the great knowledge of immunology, to Velma Aho, MSc, for the understanding of bioinformatics, and to Johannes Voegel, MD, PhD, for the valuable co-operation. I also thank Professor Harri Alenius, PhD, and Docent Petri Auvinen, PhD, for your interest and positive attitude towards this project. I am also grateful to Docent Matti Kallioinen, MD, PhD, Juha Väyrynen, MD, PhD, and Riitta Vuento for collaboration.

I want to express my gratitude to Minna Kubin, MD, for your help with recruiting patients and collecting samples especially to the microbiome project during my maternity leave. I also thank other PhD students Suvi-Päivikki Sinikumpu, MD, and Anna-Kaisa Försti, MD, for providing important peer support
during PhD-project, and the excellent researcher Laura Huilaja, MD, PhD, for your friendly assistance.

I also wish to thank Professor Emeritus Aarne Oikarinen, the former Head of Department of Dermatology, MD, PhD, for providing me an opportunity to specialize in dermatology and work at the clinic.

I thank the former Deputy Chief Timo Järvinen, MD, and the Deputy Chief Päivi Hägg, MD, PhD, for organizing time for research work.

My thanks are owed to all former and present colleagues, nurses and secretaries at the Department of Dermatology, Oulu University Hospital. It has been a privilege to work with you and I am grateful for the support during the thesis project.

I also thank Mirja Kouvala for secretarial assistance and Seija Leskelä for assistance in the illustration of this thesis.

Outi, Tarja, Päivi, Maarit and Helena are thanked for the stimulating, usually non-scientific, discussions during coffee breaks.

I am privileged to have so many good friends around me to share the joys and sorrows of life. Thank you all, Pauliina, Nina, Tanja, Eija, Anna-Stiina and Anu, for your friendship.

I express my warmest thanks to my parents, Ritva and Leo, for your unfailing love and support during my life. You have always believed in me. I am also grateful to my brothers Antti and Matti, and the family members Miia, Anna, Niilo, Enni, Noelia and Martin, for your support and all happy moments.

I also thank my parents-in-law, Anna-Maija and Veikko, for your help with Emma and Rämä.

Finally, Dear Petri and Emma, thank you for your unconditional love and support during this project, in good days and bad days. Petri has solved many technical difficulties and should have deserved acknowledgements in all my papers. And Emma, Mummy’s diamond, you are the joy of my life. I love you!

Oulu, August 25, 2016

Hanna-Leena Kelhälä
### Abbreviations

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<tbody>
<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>CCL</td>
<td>chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FoxP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HBD</td>
<td>human β-defensin</td>
</tr>
<tr>
<td>hCAP-18</td>
<td>human cathelicidin</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>K6</td>
<td>keratin 6</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cell</td>
</tr>
<tr>
<td>LCN2</td>
<td>lipocalin 2</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Nod-like receptor P3</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
</tr>
<tr>
<td>P. acnes</td>
<td>Propionibacterium acnes</td>
</tr>
<tr>
<td>PAR-2</td>
<td>protease-activated receptor-2</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>Tc</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
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List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


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1 Introduction

Acne is a disease that affects almost every teenager, but it can also continue as a chronic disease through adulthood. As well as being one of the most common skin diseases, acne is also the eighth most prevalent disease worldwide (Tan & Bhate 2015). The clinical picture of acne varies from a mild manifestation to a severe fulminant condition with systemic symptoms. It can cause both visible and also mental scarring during teenage, a vulnerable stage of life. In addition to its psychosocial impact, treatment of acne can also pose a significant financial burden on the affected individual.

The pathogenesis of acne is multifactorial: increased sebum production, hormonal influences, the hypercornification of pilosebaceous ducts, overgrowth of Propionibacterium acnes (P. acnes) and inflammation around pilosebaceous follicles are considered to be the main pathogenetic factors but few further details are known (Williams et al. 2012). P. acnes is a normal skin commensal, especially common in sebaceous areas (Grice et al. 2009) and its role as a factor in acne pathogenesis is debated (Shaheen & Gonzalez 2011).

Acne is a human disease and the lack of animal model has challenged its research. Many acne studies are performed in vitro by stimulating sebocytes, keratinocytes or monocytes with P. acnes, but these approaches are far from representative of real life. There are an estimated $1 \times 10^6$ T cells in every cm$^2$ of normal skin and an estimated 20 billion ($2 \times 10^{10}$) T cells in one adult’s entire skin, twice the number found in the blood (Clark et al. 2006). This fact underlines the importance of studying human skin samples to understand the immunopathogenetic mechanisms of cutaneous diseases.

Immunological studies of acne have mainly focused on innate immune responses, and the impact of systemic treatments on acne skin are not completely understood. For example, isotretinoin is currently the most potent medication for acne but its mechanism of action is not completely understood. Furthermore, the effects of systemic treatments on acne skin microbiota have yet to be studied using modern 16S ribosomal RNA (rRNA) gene amplicon sequencing based methods.

The present study was designed to examine the immune responses in acne patients’ skin (I), the effects of isotretinoin treatment in acne skin (II), and the impact of systemic acne medications on microbiota (III).
2 Review of the literature

2.1 General background of acne

2.1.1 Clinical picture of acne

Acne is a multifactorial, complex human disease, affecting areas rich in pilosebaceous units, such as face, chest, shoulders and back. The clinical characterization of acne ranges from a mild comedonal condition to one that can be severe nodulocystic, scarring and even fulminant and systemic. Patients suffering acne typically have seborrhea and non-inflammatory (open and closed comedones) and inflammatory (papules and pustules) lesions and various degrees of scarring. In addition, in severe forms of acne nodules and cysts are seen (Zaenglein & Thiboutot 2012). Fig. 1 shows examples of different types of acne. Acne can also be present in newborns (neonatal acne) and infants (Zaenglein & Thiboutot 2012).

Acne is a common feature of many systemic endocrine disorders diseases like congenital adrenal hyperplasia (CAH) and polycystic ovary syndrome (PCOS), or nonendocrine disorders such as synovitis-acne-pustulosis-hyperostosis-osteitis (SAPHO), pyogenic arthritis-pyoderma gangrenosum-acne (PAPA), pyoderma gangrenosum-acne-suppurative hidradenitis (PASH), pyoderma gangrenosum-acne-suppurative hidradenitis-axial spondylarthritis (PASS) and pyogenic arthritis-pyoderma gangrenosum-acne-suppurative hidradenitis (PAPASH) syndromes (Braun-Falco et al. 2012, Bruzzese 2012, Chen et al. 2011, Marzano et al. 2013). The cause of acne can also be iatrogenic e.g. resulting from medication, especially glucocorticoids.

Acne grading

There are several systems for grading acne. At least 31 outcome measures are used in clinical trials (Barratt et al. 2009). The Leeds revised acne grading system used in this study is based on photographic images comprising 12 facial, 8 chest and 8 back severity categories, and 3 facial grades for noninflamed acne (O’Brien et al. 1998). Another grading system used in the study was Global Evaluation Acne Scale (Dreno et al. 2011), which is a 6-grade scale based on text descriptions.
Fig. 1. Clinical images showing examples of different types of acne. In comedonal acne (a) mainly macrocomedones are present. In papulopustular acne (b-d) papules and pustules predominate and scarring usually occurs. Nodules and cysts are the characteristics lesions in severe acne (e-f) and scarring is evident.
2.1.2 Prevalence, comorbidities and psychosocial impact of acne

Prevalence

Acne is the eighth most prevalent disease worldwide, affecting an estimated 9.4% of the global population, and is among the top three most prevalent skin conditions (Vos et al. 2012). Approximately 85% of teenagers suffers from acne, but acne is often a chronic disease that can persist into adulthood for unknown reasons (Bhate & Williams 2013). The severity of acne seems to increase with pubertal maturation in that comedonal acne predominates in preteens and inflammatory acne is likely to develop during the teenage years (Tan & Bhate 2015).

Comorbidities

Severe acne among adolescents is associated with sinopulmonary and upper gastrointestinal comorbidities (Silverberg & Silverberg 2014). A recent study suggests that there is correlation between acne and inflammatory bowel disease (IBD) (Alhusayen et al. 2013). Women with PCOS have increased risk of insulin resistance and metabolic syndrome (Housman & Reynolds 2014). Interestingly, postadolescent male acne patients more commonly have insulin resistance compared with controls without acne (Nagpal et al. 2016).

Psychosocial impacts of acne

Acne may have many psychosocial implications including dissatisfaction with personal appearance, social impairment, poor self-esteem and overall impaired quality of life. Anxiety, depression and even suicidal ideation have all been documented in patients with acne. Acne has a significant socioeconomic impact resulting from its psychosocial effects (Bhate & Williams 2013, Halvorsen et al. 2011, Ramrakha et al. 2015).

2.1.3 Genetic factors in acne

Epidemiological data indicates 78% heritability of acne in first-degree relatives, and more severe forms of acne and earlier occurrence of acne are associated with a family history of the condition (Ballanger et al. 2006, Ghodsi et al. 2009, Wei et al. 2010). In addition, several twin studies have suggested a substantial genetic basis
to acne (Bhate & Williams 2013). Studies suggest polygenic background in acne pathogenesis.

Interestingly, the increased frequency of the human leukocyte antigen (HLA)-Cw6 allele, which is strongly associated with psoriasis (Perera et al. 2012, Tilikainen et al. 1980) is also found in patients with severe acne (Karvonen et al. 1995).

Recently several large genome-wide association studies (GWAS) have been conducted on acne patients with severe disease. A GWAS performed in United Kingdom identified three susceptibility loci: 11q13.1, 5q11.2 and 1q41, which contain genes linked to the transforming growth factor-β (TGF-β) cell signalling pathway (Navarini et al. 2014). In a Chinese population susceptibility loci at 11p11.2 and 1q24.2 were identified which involve genes related to androgen metabolism, inflammation processes and scar formation (He et al. 2014). In addition, the chromosomal locus 8q24, which has been previously associated with several types of cancer, was found among European-American patients with acne (Zhang et al. 2014).

Furthermore several candidate genes, which are known to regulate hormone metabolism and innate immune functions, have been implicated in acne pathogenesis, including those that code for tumor necrosis factor (TNF), tumor necrosis factor receptor 2 (TNFR2), interleukin 1A (IL1A), toll-like receptor 2 (TLR2), androgen receptor (AR), insulin-like growth factor 1 (IGF-1), resistin, cytochrome P450 family 1 subfamily A polypeptide 1 (CYP1A1), cytochrome P450 family 17 subfamily A polypeptide 1 (CYP17A1) and cytochrome P450 family 21 subfamily A polypeptide 2 (CYP21A2) (He et al. 2006, Ostlere et al. 1998, Paraskevaidis et al. 1998, Tasli et al. 2013, Tian et al. 2010, Yang et al. 2014, Yang et al. 2009, Younis & Javed 2014).

### 2.1.4 Dietary factors in acne

Conflicting data have been published concerning a relationship between diet and acne (Bhate & Williams 2013). The absence of acne in non-Westernized populations in Papua New Guinea and Paraguay, together with development of acne in Inuit, Japanese and Chinese populations, following changes to nutrition habits, suggest that high glycaemic load of the Western diet could have a role in the pathogenesis of acne (Cordain et al. 2002, Zouboulis et al. 2005). In recent years studies have been published concerning the role of a low glycaemic index (GI) diet in attenuating acne (Kwon et al. 2012, Smith et al. 2007). A recent study showed
that acne patients had higher GI and glycaemic load levels than controls (Cerman et al. 2016).

Possible mechanisms behind the association between acne and diet have been proposed in several recent papers by Melnik and co-workers (Melnik 2012, Melnik & Zouboulis 2013). One such proposed mechanism is enhanced mechanistic target of rapamycin complex (mTORC) signalling, resulting from increased insulin/insulin-like growth factor 1 (IGF-1) signalling promoted by a diet containing hyperglycaemic carbohydrates and insulintropic/dairy products (Melnik 2016). Recently mTOR expression and mTORC-S6K1 signalling have been shown to be up-regulated in acne patients’ skin (Agamia et al. 2016, Monfrecola et al. 2016).

2.2 Pathogenetic factors in acne

The main factors contributing to pathophysiology of acne are increased and/or altered sebum production (supposed largely androgen-hormone mediated), hypercornification of the pilosebaceous duct, follicular colonization with \( P. \text{acnes} \) and inflammatory processes around pilosebaceous units (Fig. 2) (Williams et al. 2012).

![Histologic pictures of normal pilosebaceous follicle (a), comedone (b) and inflammatory acne lesion with rupture of the follicular wall (c) (modified from Williams et al. 2012).](image)
2.2.1 The effect of hormones in acne

Acne does not develop without hormonal stimulation. Pilosebaceous units are hormonally regulated. Several hormones that regulate sebum secretion are also linked to acne e.g. androgens, estrogens, growth hormone (GH), insulin, IGF-1, corticotropin-releasing hormone (CRH), melanocortins such as melanocyte-stimulating hormone (MSH), and glucocorticoids (GCs) (Lolis et al. 2009).

Androgens, which are produced in the adrenal glands, gonads and skin, are the most important regulators of sebaceous glands affecting sebaceous gland growth and development. Androgens increase the size of sebaceous glands and stimulate sebum production (Pochi & Strauss 1969). Testosterone and dihydrotestosterone (DHT) are physiologically active hormones in skin. Androgen receptors (ARs) have been localized to the sebaceous glands but the results of AR immunoreactivity in the outer root sheath keratinocytes of hair follicles are inconsistent (Kariya et al. 2005, Liang et al. 1993). Several possible roles of androgens in acne development have been proposed: a permissive role of androgens in acne initiation, localized overproduction of androgens in acne sites, or elevated expression and responsiveness of androgen receptors may each play a part (Kurokawa et al. 2009).

Elevated serum levels of adrenal dehydroepiandrosterone sulphate (DHEAS), which is a precursor of testosterone, correlate with sebum production in both sexes and with the presence of acne vulgaris in girls during the prepubertal period (Lucky et al. 1994, Stewart et al. 1992). Most patients with longstanding severe therapy-resistant cystic acne have increased serum androgen levels (Marynick et al. 1983). Female acne vulgaris patients usually have circulating androgen levels within the normal range (Levell et al. 1989) but some patients with excessive androgen levels e.g. those with congenital adrenal hyperplasia or women with PCOS, suffer from acne (Housman & Reynolds 2014, Thiboutot et al. 1999). In addition, the intake of androgenic-anabolic steroids leads often to so-called ‘bodybuilding acne’ (Melnik et al. 2007). Conversely, men who have non-functional androgen receptors do not produce sebum and do not develop acne (Imperato-McGinley et al. 1993). Furthermore, androgens locally produced in the skin by increased activity of steroid metabolizing enzymes, especially 5α-reductase and 17β-hydroxysteroid dehydrogenase, may contribute to androgen effects in acne patients (Thiboutot et al. 1999).

In addition to androgens, GH and IGF-1, are hormones also considered to be important in the onset of acne. GH is maximally secreted and serum levels of IGF-1 are highest in adolescents during the time when acne is most prevalent. Moreover,
conditions of GH excess, such as acromegaly, are associated with seborrhea and development of acne. (Thiboutot 2004). Patients with Laron syndrome, which is characterized by primary growth hormone insensitivity and inadequate production of IGF-1 are devoid of acne (Klinger et al. 1998).

The complete CRH signalling pathway is active in acne-involved skin especially in the sebaceous glands. CRH may activate lipid pathways leading to the development and stress-induced exacerbation of acne by affecting immune and inflammatory processes (Ganceviciene et al. 2009).

Treatment with GCs may cause acneiform eruptions and exacerbate pre-existing acne. GCs increase toll-like receptor 2 (TLR2) expression in keratinocytes and sebocytes (Lee et al. 2013, Shibata et al. 2009). The bioavailability of GCs is regulated by isoenzymes of 11β-hydroxysteroid dehydrogenase type 1 (HSD11β1), which catalyses the conversion of cortisone to active cortisol. HSD11β1 is expressed in human skin in keratinocytes, fibroblasts and sebocytes and it is up-regulated in acne lesions (Lee et al. 2013). HSD11β1 regulates GC-induced lipid synthesis and TLR2 expression in sebocytes in vitro, and may play a key role in the modulation of the action of GCs on sebocytes. This may partly explain the pathogenesis of stress- and steroid-related acne (Lee et al. 2013).

Increased fibroblast growth factor receptor-2 (FGFR2) - signalling caused by FGFR2-gain-of-function mutations is associated with acne in Apert syndrome. This indicates that FGFR2-signaling also has a role in the pathogenesis of acne (Chen et al. 2011).

### 2.2.2 Sebum production and changes in lipid composition

It is generally accepted that increased sebum production by androgens is a major pathogenic factor promoting acne. Many acne patients have increased levels of sebum secretion, but some have normal sebum efflux and conversely, hyperseborrhea does not always lead to the development of acne (Youn et al. 2005). Interestingly, one study found that acne patients not responding to the antibiotic treatment had a significantly higher mean sebum excretion rate (SER) than that of matched untreated acne patients (Eady et al. 1988). Several studies have indicated that sebum composition may be more important than its quantity. Human sebum contains triglycerides, free fatty acids (FFAs), wax esters, squalene, cholesterol esters and cholesterol. *P. acnes*-produced lipases are known to release FFAs from triglycerides produced in sebaceous glands (Marples et al. 1971). Several alterations in sebum composition have been described in acne patients. Those
considered most important are decreased levels of linoleic acid, an altered ratio of saturated to unsaturated fatty acids, and squalene peroxidation products. These alterations are suggested to be involved in the formation of, and inflammatory changes in, comedones (Ottaviani et al. 2010). For example unsaturated FFAs – oleic acid and palmitoleic acid – in sebum are shown to alter the calcium dynamics in epidermal keratinocytes and to induce abnormal follicular keratinization (Katsuta et al. 2005). In addition, oleic acid has been shown to be comedogenic in a rabbit ear model (Choi et al. 1997).

Peroxisome proliferator-activated receptors (PPARs) are nuclear transcription factors involved in the control of lipid metabolism, cell proliferation, differentiation and inflammatory responses that modulate skin homeostasis and pilosebaceous unit function. PPARs are activated by endogenous ligands that are derived from the metabolism of fatty acids, and by exogenous ligands developed as drugs for the treatment of metabolic or inflammatory diseases. PPARs are widely expressed in the epidermis, sebaceous glands and hair follicles (Trivedi et al. 2006a). PPAR modulators are considered to be possibly beneficial in acne, particularly because of their anti-inflammatory and lipogenesis-regulating properties (Ramot et al. 2015). A recent study showed that PPARγ levels and signalling activity are decreased in sebocytes from acne patients (Dozsa et al. 2016).

Nerve fibres expressing substance P (SP) are present around sebaceous glands in acne patients’ skin. SP promotes both proliferation and differentiation of sebaceous glands in vitro and induces its degrading enzymes, neutral endopeptidases, implicating the pathologic significance of neurogenic and psychogenic aspects in the disease process (Toyoda et al. 2002).

2.2.3 Hypercornification of the pilosebaceous duct and comedogenesis

Hyperproliferation of keratinocytes in the pilosebaceous follicle have been demonstrated by immunohistochemical analysis of comedones. Increased immunoreactivity of Ki-67, a cellular marker for proliferation and indicator of active DNA synthesis, has been demonstrated in the intrafollicular and interfollicular epidermis of acne patients (Knaggs et al. 1994).

Keratins K6, K16 and K17 are stress-inducible, and rapidly switched on e.g. after injury and UV-irradiation. They are present also in inflammation and in hyperproliferative disorders like in psoriasis (Moll et al. 2008). In normal human skin K6 and K16 are expressed suprabasally in the outer root sheath below the
opening of the sebaceous duct. In addition, K6 expression is detected in cells of the sebaceous gland and duct. K17 is expressed suprabasally in the infrainfundibulum, sebaceous duct, the outer root sheath below the opening of the sebaceous ducts and in the companion layer (Kurokawa et al. 2011). The expression of K6, K16 suprabasally and K17 panepithelially is increased in comedones compared with normal skin (Hughes et al. 1996). In psoriasis, the high levels of expression of K17 in psoriatic lesions contribute to local abnormal immune reactions by forming a K17/T-cell/cytokine autoimmune positive feedback loop (Jin & Wang 2014).

As are keratins, filaggrin, is a differentiation-specific marker for the epidermal keratinization process. Filaggrin aggregates keratin filaments into macrofibrils (Dale et al. 1985). Compared with normal skin, the expression of filaggrin is more pronounced in acne lesions in the lower parts of the sebaceous duct and the infundibulum, indicating a disorder of the terminal phase of keratinocytic differentiation in these areas (Kurokawa et al. 1988). There is also increased expression of α2, α3 and α6 integrins in comedones and on basal keratinocytes of the epidermis and follicle wall in acne patients’ uninvolved skin and in early lesions (Jeremy et al. 2003). Integrins regulate the balance between proliferation and differentiation (Watt 2002).

Akaza and colleagues suggested that P. acnes relates to acne pathogenesis by influencing the differentiation of keratinocytes that may be dependent on the type of bacteria (Akaza et al. 2009). All P. acnes strains used in their study increased transglutaminase (TGase), K17 and decreased K1 and K10 mRNA expression levels in normal human epidermal keratinocytes (NHEK) in vitro. Some P. acnes strains increased involucrin and K6 and decreased filaggrin, K6 and K16 expression levels.

Finally, the cytokine interleukin-1 (IL-1) is considered to have a central role in hyperkeratosis of the pilosebaceous duct; this will be discussed later in chapter 2.4.2.

### 2.2.4 Bacterial colonization in acne

**Skin microbiota**

The fetal skin is sterile in utero but rapidly after birth, environmental microbes start to colonize the skin forming a complex microbial ecosystem that is in homeostasis with its host (Zeeuwen et al. 2013). An estimated 1 million bacteria reside per
square centimeter of skin for a total of over $10^{10}$ bacterial cells covering a human (Grice et al. 2008). Modern molecular approaches including 16S rRNA gene sequencing methods have revealed a greater diversity of organisms colonizing the skin than previously estimated using earlier culture based methods. Most bacteria of the skin, the gastrointestinal tract and the oral cavity bacteria belong to four phyla: Actinobacteria, Firmicutes, Bacteroides and Proteobacteria but the proportions of each differ widely depending on site (Grice & Segre 2011). The skin microbiota is dependent on the physiology of the skin site in such a way that specific bacteria are associated with moist, dry and sebaceous microenvironments (Fig. 3) (Grice et al. 2009). Bacterial genomic sequence data have showed that Propionibacteria and Staphylococci species are the dominant bacteria in sebaceous areas of adult skin (Grice et al. 2009, Staudinger et al. 2011). There are significant differences in microbiota between children and adults. The shifts in the skin and nares microbiota are puberty-dependent: The prevalence of Propionibacterium, Corynebacterium and other lipophilic bacteria increases at puberty, when the sebaceous glands mature and sebum secretion increases (Leyden et al. 1975a, Oh et al. 2012).

However, the bacterial flora of acne patients does not seem to differ from that of unaffected persons. Earlier culture based methods have revealed that the microflora in areas rich in sebaceous glands of both healthy adults and acne patients consists mainly of the bacterial genera Propionibacterium, Staphylococcus and the yeast Malassezia, and in lesser density Microsporium, aerobic coryneforms and gram-negative bacteria (Marple et al. 1974, Numata et al. 2014, Till et al. 2000). A recent study using 16S rRNA gene amplicon sequencing demonstrated a similar relative abundance of P. acnes, Staphylococcus epidermis, Propionibacterium humerusii and Propionibacterium granulosum in the nose follicular casts from acne patients and those from healthy individuals (Fitz-Gibbon et al. 2013).
Fig. 3. Characteristics of the adult skin and microbiota (modified from Grice et al. 2009, Grice & Segre 2011).
Propionibacterium acnes

This short pleomorphic bacillus was detected in comedones as early as 1896 by Unna, was later named as *P. acnes* (previously classified as *Corynebacterium parvum*) and is considered to be important in the pathogenesis of acne (Pochi & Strauss 1961). *P. acnes* is gram-positive, nonmotile aerotolerant anaerobe which is a normal inhabitant of human pilosebaceous units (Bojar & Holland 2004). There are strong *in vivo* and *in vitro* data pointing to a prominent role of *P. acnes* in the pathogenesis of acne. However, the role of *P. acnes* as a causal agent of acne is still unclear (Shaheen & Gonzalez 2011), as is its correlation with acne severity. Leeming and co-workers (1998) found that the count of viable bacteria within follicles does not correlate with the severity of inflammation and that not all inflamed lesions contain viable *P. acnes* (Leeming *et al.* 1988). In a recent study, facial samples of acne patients presented a higher prevalence of follicular *P. acnes* colonization, and extensive *P. acnes* macrocolonies were more common than in control samples (Jahns *et al.* 2012).

Acne is not considered a classic infectious disease but immunologic reactivity against *P. acnes* may contribute to the inflammation in acne. Decoding of the *P. acnes* genome has revealed the pathogenic potential of the bacterium including factors involved in degrading host molecules (e.g. sialidases, neuraminidases, endoglycoceramidases, lipases, and pore-forming factors), and in conferring cell adhesion and/or mediating inflammation (e.g. heat shock proteins) (Bruggemann *et al.* 2004).

Recent studies have shown that certain strains of *P. acnes* were highly associated with acne and other strains were enriched in healthy skin (Fitz-Gibbon *et al.* 2013). In addition to acne, *P. acnes* has been linked with other conditions e.g. SAPHO, sarcoidosis and primary biliary cirrhosis. It can also cause infections such as ocular and dental infections, endocarditis, prosthetic joint and orthopaedic device-related infections (Perry & Lambert 2011). Acne has been associated with *P. acnes* phylotype IA, ophthalmic infections IA1 and IA2, and soft tissue and medical implant infections types IB, II and III (McDowell *et al.* 2012). The virulent genes carried in disease-associated strains may explain the association of certain strains with acne (Tomida *et al.* 2013). Moreover, different strains of *P. acnes* have different inflammatory potential to modulate cutaneous innate immunity (Jasson *et al.* 2013).

The presence of *P. acnes* in macrocolonies or biofilms in sebaceous follicles is more common in acne patients than in controls (Jahns *et al.* 2012). Biofilm
formation of *P. acnes* is associated with increased production of virulence factors and increased resistance to antimicrobial agents (Coenye *et al.* 2007).

*P. acnes* bacteriophages have also been identified. They are highly homogenous and show limited genetic diversity. Furthermore, these bacteriophages have a broad killing activity against clinical isolates of *P. acnes* that is perhaps related to the lipid-rich anaerobic environment where the phages’ bacterial hosts reside (Marinelli *et al.* 2012). Interestingly, *P. acnes* phages may be candidates for the development of a phage-based therapy for acne.

### 2.3 Histopathology of acne

The obstruction of pilosebaceous follicles causes microcomedones, subclinical acne lesions, which are observed only under microscopic examination. Keratinocytes accumulate particularly in the infrainfundibular region of the pilosebaceous duct. The infundibulum is continuous with the epidermis but the infrainfundibulum part differs from the epidermis, especially in that its granular layer is inconspicuous and the horny cells it produces are fragile. The ducts of sebaceous glands are similar to the infrainfundibulum. The microcomedone can develop into either a non-inflammatory comedo or an inflammatory lesion, papule, pustule, nodule or cyst (Kligman 1974).

In comedo formation the granular layer becomes more prominent and the cells of the horny layer more distinct. The sebaceous ducts begin to go through similar hyperkeratotic changes (Kligman 1974). Electron microscope examinations have showed that the most striking difference between comedones and normal follicles is the presence of intracellular lipid inclusions within keratinized and granular cells. Lipid inclusions are not apparent in the normal mammalian epidermis but they have been observed e.g. in parakeratotic psoriatic scales and in parakeratotic layers of eczema (Knutson 1974). Increased numbers of desmosomes and tonofilaments are seen in the follicular keratinocytes in comedones (Toyoda & Morohashi 2001).

As more keratinous material accumulates, the follicular wall continues to distend and become thinner. Simultaneously sebaceous glands undergo progressive atrophy and appear to dedifferentiate resulting in a fully developed comedo which contains hardly any sebaceous cells. The lipid-forming lobules of the sebaceous gland are shrunken while the undifferentiated cell pool becomes relatively larger. However, small clusters of sebum-producing cells are always found. During comedo formation the tiny hair follicle seems not undergo fundamental changes (Kligman 1974). The follicular infundibulum is dilated and the attenuated wall of
the comedo is plugged with keratinized cells, sebum, hair and focal masses of bacteria (Knutson 1974). If the follicular orifice remains normal in size, the comedo closes but when it widens, an open comedo is formed. Comedones are associated with mild mononuclear inflammation around vessels in the adjacent papillary dermis (Ioffreda 2009).

In papules and pustules the inflammatory reaction consists of neutrophils and macrophages, which localize intradermally in papules and more superficially within the epidermis in pustules. Under electron microscope examination the keratinized cells, macrophages and neutrophils contain lipid inclusions in the inflammatory infiltrates (Knutson 1974).

In papules and nodules the neutrophilic inflammation is massive and the remaining sebaceous lobules, pilary unit and epithelium may be totally destroyed. The horny core containing the comedo, along with lips, bacteria and hairs in the dermis is removed by macrophages. Approximately 7–10 days after follicular rupture the neutrophils are replaced by lymphocytes, histiocytes and foreign-body giant cells, creating granulation tissue and eventually leading to scar formation (Kligman 1974).

2.4 Immunopathogenetic factors in acne

The immune system protects the skin against harmful microbial, chemical and physical insults, and the activation of innate immunity provides the first rapid but non-specific response against those harmful attacks. The activation of the adaptive immune system is more specific due to immunologic memory (Schwarz 2012). However, the activation of both innate and adaptive immunity are very early events in the formation of acne lesions (Jeremy et al. 2003, Layton et al. 1998). Compared with adaptive immune reactions, innate immune responses in acne pathogenesis have been studied quite extensively.

2.4.1 Innate immune reactions in acne

Innate immune responses in acne are mediated by a variety of different cell types including monocytes, macrophages, neutrophils and dendritic cells (DCs) as well as nonimmune cells, such as keratinocytes and sebocytes. The possible contribution of *P. acnes* in particular, in the activation of innate immune reactions has been demonstrated by several *in vitro* studies in keratinocytes, sebocytes and in peripheral blood monocytes (Graham et al. 2004, Jugeau et al. 2005, Kim et al.
However the clinical relevance of in vitro data is partly unknown.

One of the earliest signs of skin inflammation in acne is the accumulation of CD68+ macrophages in the uninvolved skin of acne patients and in early-stage (less than 6 hours old) lesions together with CD3+, CD4+ T cells resembling a type IV delayed hypersensitivity response (Jeremy et al. 2003). As the lesion matures, the numbers of neutrophils and CD8+ cells increase (Layton et al. 1998, Norris & Cunliffe 1988). After rupture of the follicular wall, the inflammatory response is mediated mainly by neutrophils and later by macrophages and foreign-body giant cells (Ioffreda 2009). It is possible but unusual for CD4+ T cells to accumulate in the inflammation site before neutrophils.

Professional antigen-presenting cells (APCs) have not been extensively studied in acne. It has been noted that CD1a+ cells have reduced density in the epidermis and dermis around uninvolved follicles of acne patients (Jeremy et al. 2003). However, in acne lesions aged 6 hours and more, CD1a+ cells are found within the epidermis and follicle wall as well as in the periductal dermal infiltrates (Holland et al. 2004). CD1a is the major human CD1 protein expressed especially on Langerhans cells (LCs). FFAs, squalene and wax esters have recently been identified as autoantigens presented by CD1a and recognized by autoreactive T cells (de Jong et al. 2014). Moreover, HLA-DR expression is not increased within cellular infiltrates in uninvolved follicles of acne patients but in older lesions (6-72 h) HLA-DR expression is increased in perivascular and periductal infiltrates including some basal keratinocytes (Jeremy et al. 2003, Layton et al. 1998). HLA-DR is one of the major histocompatibility complex (MHC) class II molecules, where antigenic peptide fragments are bound on APCs and presented to CD4+ T cells. DCs, including LCs and dermal DCs, B cells and macrophages are the major MHC II expressing APCs.

Complement activation

Complement is a key component of innate immunity but its role in the pathogenesis of acne is not very well understood. In addition to its important role in cutaneous defence against microbial infection, complement also mediates inflammation and tissue injury (Panelius & Meri 2015).

Comedonal contents and P. acnes have been shown to activate complement via both the classic and the alternative pathways (Webster et al. 1979). Complement-3 (C3) immunoreactivity has been detected in early-stage inflammatory acne lesions.
in the walls of small dermal blood vessels and at the dermo-epidermal junction. In late-stage inflammatory lesions C3 deposition is much less prominent (Dahl & McGibbon 1979, Scott et al. 1979).

**Antimicrobial peptides**

Antimicrobial peptides (AMPs) are evolutionarily conserved molecules, which protect the skin and other epithelia against pathogens and form a fast-acting chemical barrier that also regulates the normal flora of the skin and mucosa. Several AMPs are induced in acne, which suggests that they influence acne pathogenesis. However, it is not clear whether AMPs have a beneficial or detrimental antimicrobial effect by promoting inflammation (Harder et al. 2013).

The AMPs human β-defensin 1 and 2 (HBD 1 and 2), cathelicidin (hCAP18), psoriasin (S100A7), koebnerisin (S100A15), lactoferrin, lysozyme, RNase7 granulysin, human neutrophil proteins 1-3 (HNP1-3) and neutrophile gelatinase-associated lipocalin (NGAL; the product of the lipocalin 2 [LCN2] gene), are up-regulated in acne vulgaris lesions (Borovaya et al. 2014, Chronnell et al. 2001, Ganceviciene et al. 2006, Lumsden et al. 2011, Trivedi et al. 2006b). Gene expression analysis of noninflamed skin of acne patients have shown that S100A7 and S100A9 are up-regulated during isotretinoin treatment (Nelson et al, 2008).

In addition, *P. acnes* is shown to induce HBD2 and hCAP18 in sebocytes and keratinocytes *in vitro* (Lee et al. 2008, Nagy et al. 2005, Nagy et al. 2006).

**Toll-like receptors**

Pathogens or danger signals are recognized by the innate immune system via pattern recognition receptors (PRRs) of which the most prominent group is the TLRs (Bangert et al. 2011). The importance of the TLR-mediated immune response in acne is supported by the presence of TLR2-expressing cells in acne lesions. TLR2 is expressed in macrophages around pilosebaceous follicles and its expression is increased during the evolution of the disease (Kim et al. 2002). The expression of TLR2 and TLR4 is also increased in epidermal keratinocytes in biopsies of inflammatory acne lesions (Jugeau et al. 2005).

*P. acnes* has been shown to trigger both TLR2 and TLR4 in monocytes, keratinocytes or sebocytes *in vitro* (Jugeau et al. 2005, Kim et al. 2002, Nagy et al. 2005). In addition, the bacterial components of *P. acnes*, peptidoglycans and
lipoteichoic acid, are considered to act as possible activators of TLR2 and TLR4 in acne (Jugeau et al. 2005).

2.4.2 Cytokines in acne

Soluble mediators of immunity and inflammation are actively secreted by immune and nonimmune cells and they initiate, mediate and perpetuate inflammation and tissue damage (Schwarz 2012). Several cytokines are involved in acne.

Cytokine IL-1 and inflammasome activation

IL-1 is the most common initiator of keratinocyte activation. Activated keratinocytes are hyperproliferative and produce paracrine signals to alert fibroblasts, endothelial cells, melanocytes and lymphocytes, as well as autocrine signals to adjacent keratinocytes, thus maintaining the activated state (Freedberg et al. 2001). The role of IL-1 in the initiation of acne lesions is important. Comedones in vivo are rich in IL-1α-like bioactivity and IL-1α immunoreactivity is shown in early phase (<6 hours) papules and uninvolved acne skin (Ingham et al. 1992, Jeremy et al. 2003). In the uninvolved skin of acne patients, increased IL-1α labelling is detected in all layers of both the interfollicular and down the follicle wall compared to controls. In the epidermis of inflamed lesions, significant increases in IL-1α labelling is also observed compared with uninvolved skin but down the follicular wall increased IL-1α labelling is only present in infrainfundibular basal cells (Jeremy et al. 2003). Furthermore, exposure to IL-1α causes hypercornification of the infundibulum similar to that seen in comedones in isolated, cultured human infundibulum in vitro (Guy et al. 1996).

Inflammasomes are cytosolic protein complexes that recognize a diverse range of inflammation-inducing stimuli including both exogenous and endogenous signals e.g. microbial, stress and damage signals, and respond by activating caspase-1 and producing proinflammatory cytokines IL-1β and IL-18 (Strowig et al. 2012). Recent reports support Nod-like receptor P3 (NLRP3)-inflammasome activation in acne (Kistowska et al. 2014, Li et al. 2014, Qin et al. 2014). Inflammasome proteins NLRP3 and caspase-1 are expressed in CD68+ macrophages and sebaceous glands in acne lesions in vivo, and P. acnes triggers NLRP3- inflammasome activation in vitro resulting in IL-1β release from sebocytes and monocytic cells but not from keratinocytes (Kistowska et al. 2014, Li et al. 2014, Qin et al. 2014). Furthermore, mature-IL1β is expressed at the site
of cutaneous inflammation, in the presence of macrophages surrounding the pilosebaceous unit and in sebaceous glands in acne lesions (Kistowska et al. 2014, Li et al. 2014). *P. acnes*-induced inflammasome activation depends on the internalization of the bacteria by antigen-presenting cells, lysosomal maturation, activation of cathepsin B, generation of reactive oxygen species (ROS), and potassium efflux (Kistowska et al. 2014).

**Other cytokines**

Analysis of skin biopsies from acne lesions using RT-PCR, gene array and immunohistochemical staining techniques have demonstrated an increased expression of the proinflammatory cytokines tumor necrosis factor-α (TNF-α), IL-6, IL-8, IL-17A, interferon-γ (IFN-γ), IL-21 and the anti-inflammatory cytokine IL-10 (Agak et al. 2014, Alestas et al. 2006, Kang et al. 2005, Kistowska et al. 2015, Trivedi et al. 2006b). The concentration of IL-17, IL-4, IFN-γ and TNF-α is elevated in serum samples of patients with moderate-to-severe nodulocystic acne (Karadag et al. 2012).


Based on data obtained from *in vitro* studies, it has been hypothesized that the cytokine milieu simultaneously induces comedogenesis, and de-differentiation of sebocytes into a keratinocyte-like phenotype and sebaceous gland atrophy (Downie et al. 2002). Proinflammatory cytokines also induce adhesion molecules on endothelial cells to facilitate recruitment of inflammatory cells into the skin. Subsequent to the proinflammatory stimulus by cytokines, expression levels of intercellular adhesion molecule 1 (ICAM-1), E-selectin and vascular cell adhesion molecule 1 (VCAM-1) are increased in early-stage inflammatory acne lesions (Jeremy et al. 2003, Layton et al. 1998)

**2.4.3 Adaptive immune reactions in acne**

Antigen-specific T and B lymphocytes mediate adaptive immune responses. An adaptive immune response corresponds to type and strength of the innate response...
Activated B cells secrete immunoglobulins and are involved in humoral immunity, whereas T cells are involved in cell-mediated immune responses.

**T cells**

T cells are divided into two main groups, CD4+ helper T cells (Th) and CD8+ cytotoxic T cells (Tc), and they identify antigen-derived peptides by their antigen receptor. Th cells recognize antigens bound to the MHC class II molecules expressed on APCs. Most Tc cells recognize the endogenous antigens presented by class I MHC molecules, which are expressed normally on all nucleated cells. Recognition of foreign antigen-derived peptides by naïve T cells leads to a process that includes massive proliferation and differentiation into distinct T cell subsets according to the corresponding cytokine profile (Bangert et al. 2011, Zhu & Paul 2010). Th cells help B cells to produce antibodies, activate Tc cells, and recruit and activate other immune cells. Tc cells act as killer cells, but like CD4+ cells, they can also exert regulatory functions and produce different cytokines (Zhu & Paul 2010).

Th cells are the central players in the adaptive immune responses. Many types of specialized Th cells have been identified. The main Th cell lineages are Th1, Th2, Th17 and regulatory T (Treg) cells (Fig. 4). Th1 cells promote cell-mediated immunity by activating macrophages and CD8+ T cells to kill viruses and other intracellular pathogens. Th1 cells also contribute to the pathogenesis of autoimmune diseases. Th2 responses are critical for IgE production and activation of eosinophils, mast cells and basophils. Th2 are important for the elimination of helminthic parasites but nowadays they are known better for their role in the pathogenesis of asthma and other related allergic conditions (Schwarz 2012). Th17 cells recruit and activate neutrophils and contribute to the host defence against extracellular bacteria and fungi. They contribute to chronic inflammation associated with many inflammatory and autoimmune disorders (Beringer et al. 2016). Treg cells are immunosuppressive and maintain self-tolerance, prevent autoimmunity and control immune responses during infection and cancer (Schwarz 2012). In addition to the four major lineages, other potential new Th lineages have been proposed including Th3, Th9, Th22, follicular Th (Tfh) cells and type 1 regulatory (Tr1) cells. Since the signature cytokines produced by these Th cells are also the products of Th1/Th2/Th17/Treg cells and the transcription factors they
express are not unique, it is possible that these cells represent subsets of the four main lineages (Zhu & Paul 2010).

In addition to heterogeneity between different Th lineages, individual cells within same lineage may display different patterns of cytokine production and express transcription factors differentially. Moreover, Th cells are capable to switch from one lineage to another or to a mixed phenotype suggesting that Th cells are plastic. At early stage of Th differentiation, each Th lineage can be plastic but at later stages, only the majority of Th17 and Treg cells may alter their cytokine production profile (Zhu & Paul 2010).

The prevalence of T-cells and macrophages in early-stage acne lesions indicate their key role in inflammation of acne. CD3+CD4+ T-cells are the major leukocytes in the inflammatory infiltrates of early-stage (6–72h) acne lesions and the number of CD8+ T-cells increases with the age of the lesion (Norris and Cunliffe, 1988; Layton et al, 1998). Additionally, the numbers of CD4+ T-cell (most of which are CD45RO+ memory/effector cells) and CD68+ macrophages are elevated in the uninvolved follicles of acne patients, and in acne lesions less than 6 hours old compared with healthy skin controls, indicating that adaptive immune reactions in acne begin even before microcomedo formation. (Jeremy et al. 2003).

Karvonen and co-workers (1994) have shown that specific cell-mediated immunity to *P. acnes* increases during the course of severe inflammatory acne (Karvonen et al. 1994). *P. acnes* has been shown to induce T-cell proliferation (Jappe et al. 2002). In addition, *P. acnes*-reactive CD4+ T cells with an IFN-γ cytokine profile have been found in early-stage papular inflammatory lesions (Mouser et al. 2003) suggesting the presence of T helper 1 (Th1) cells in acne. Recently cells with Th1 and Th17 profile have been found in inflammatory acne lesions (Kistowska et al. 2015). Moreover, *P. acnes* has been shown to trigger Th17, Th1 and mixed Th17/Th1 responses in peripheral blood mononuclear cells (PBMCs) in vitro (Agak et al. 2014, Furusawa et al. 2012, Kistowska et al. 2015, Sugisaki et al. 2009).
Fig. 4. The differentiation of T helper cells. The interaction of antigen presenting cell (APC) with naïve CD4+ T cells in the various cytokine milieus induces certain transcription factors in the T cells (Tbet, GATA3, RORγt, FoxP3), which direct differentiation into Th1, Th2, Th17 and Treg cells.

**Humoral immunity**

As well as an increase in the number of Th cells, the number of B cells also seemed to correlate with increased severity of acne in the peripheral blood (Holland et al. 1983). The antibody on the B cell recognizes the specific antigen and eventually with the assistance of helper T cells, the B cell differentiates into antibody producing plasma cells. Secreted immunoglobulins (Ig) bind to antigens and then neutralize them or facilitate phagocytosis or complement activation (Bangert et al. 2011). Puhvel et al. (1966) found that complement-fixing antibody titres to *P. acnes* are in the levels found for most adults with mild acne but titres increase with the severity of inflammation (Puhvel et al. 1966). Increased total IgG has been observed in patients with severe acne (Holland et al. 1986, Ingham et al. 1987) and antibody titers to other skin organisms such as *Staphylococcus epidermidis* have not been noticed (Ashbee et al. 1997). In severe acne the elevated antibody
response is directed against carbohydrate structures in the *P. acnes* cell wall (Webster *et al.* 1985). Unfortunately, the studies about humoral immunity in acne have not been published recently and therefore it is difficult to evaluate the role humoral immunity in the pathogenesis of acne.

### 2.4.4 Other factors contributing to the immunopathogenesis of acne

**PAR-2 and proteases**

Protease-activated receptor-2 (PAR-2) is expressed by almost all cell types in the skin, including keratinocytes, fibroblasts, sebocytes, endothelial cells, sensory neurons and inflammatory cells, and is activated by endogenous or exogenous ligands (Rattenholl & Steinhoff 2008). In the human skin PAR-2 is thought to regulate the homeostasis of the permeability barrier, keratinocyte cornification/differentiation, inflammation, pruritus, pigmentation and wound healing (Lee *et al.* 2015). In inflammatory acne lesions PAR-2 expression is increased in sebaceous glands (Lee *et al.* 2015) and in the suprabasal layers of the follicular epithelium lining of the comedone (Lee *et al.* 2010). PAR-2 activation has been shown to mediate sebocyte differentiation and to induce lipogenesis and the proinflammatory cytokines, HBD-2 and LL-37 in sebocytes and keratinocytes (Lee *et al.* 2010, Lee *et al.* 2015).

*P. acnes* produces proteases (Ingram *et al.* 1983) and via PAR-2 triggering *P. acnes* has been shown to stimulate the expression of IL-1α, IL-8, TNF-α, HBD-2, LL-37 and several matrix metalloproteinases (MMPs), including MMP-1, -2, -3, -9 and -13, in keratinocytes and sebocytes *in vitro* (Lee *et al.* 2010, Lee *et al.* 2015). In cultured human dermal fibroblasts *P. acnes* increases the expression of pro-MMP-2 through TNF-α (Choi *et al.* 2008).

MMPs are a family of zinc-dependent endopeptidases, which degrade extracellular matrix components under both normal and inflammatory conditions (Kähäri & Saarialho-Kere 1997) and several MMPs including MMP-1, -2, -3 and -9 are up-regulated in acne lesions (Kang *et al.* 2005, Lee *et al.* 2010, Trivedi *et al.* 2006b). Facial sebum of acne patients also contains MMP-1, MMP-13 and tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 which are supposed to originate from keratinocytes and sebocytes (Papakonstantinou *et al.* 2005). The up-regulated MMPs may contribute to acne pathogenesis by inducing inflammation and tissue destruction (Lee *et al.* 2010). Increased MMP activity produces greater
 amounts of degraded collagen in lesional acne skin (Kang et al. 2005). Matrix breakdown is followed by its imperfect repair, which is thought to result in acne scarring.

The roles of other endogenous proteases in acne have not been extensively studied. Granzyme B is strongly up-regulated in inflammatory acne lesions (Trivedi et al. 2006b). Tcs and natural killer cells (NKs) have granzyme B in their granules that they use to destroy target cells (Voskoboinik et al. 2015). Tregs can also secrete granzyme A and B (Zhou et al. 2015). Protease inhibitor 3 or skin-derived antileukoprotease (SKALP), which is an elastase-specific protease inhibitor, is also up-regulated in acne patients’ skin (Trivedi et al. 2006b) as well as in the epidermis of psoriatic skin and in Netherton syndrome (Raghunath et al. 2004).

The serine proteases pro-kallikrein 5 and kallikrein 7 are expressed in the interfollicular epidermis, the pilary canal and secondary sebaceous ducts (Ekholm et al. 2000). Their expression in acne is largely unknown in contrast to rosacea, in which the activity of kallikrein 5 is increased (Yamasaki et al. 2007). Both kallikrein 5 and 7 control the activation of hCAP18 (Yamasaki et al. 2006), the expression of which is elevated in acne lesions (Borovaya et al. 2014).

2.5 Systemic medications for acne vulgaris

Isotretinoin and systemic antibiotics, especially tetracyclines combined with topical treatments (benzoyl peroxide, retinoids, azelaic acid), are recommended for severe acne, and for milder forms of acne that are treatment-resistant or relapsing (Nast et al. 2012, Zaenglein et al. 2016). The acne medications currently available in Finland are presented in Table 1. There are global concerns about increasing antibiotic resistance. Antibiotic resistance in acne may lead to treatment failures. The resistance can also occur in more pathogenic organisms than P. acnes (Walsh et al. 2016). The use of oral erythromycin and other macrolides to treat acne is recommended to be limited only to those acne patients who cannot use tetracyclines. Antibiotics are not recommended for use as monotherapy and oral antibiotic use should ideally last no longer than three months (Nast et al. 2012, Zaenglein et al. 2016). Moreover, combined oral contraceptives or spironolactone may be used in the treatment of inflammatory acne in females.
Table 1. Acne medications available in Finland.

<table>
<thead>
<tr>
<th>Topical</th>
<th>Systemic</th>
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<tbody>
<tr>
<td>Benzoyl peroxide (BPO) 5% gel</td>
<td>Tetracycline (500-1000 mg)</td>
</tr>
<tr>
<td>Adapalene 0.1% gel</td>
<td>Doxycycline (100-200 mg)</td>
</tr>
<tr>
<td>Tretinoin 0.025% and 0.05% cream</td>
<td>Lymecycline (300-600 mg)</td>
</tr>
<tr>
<td>Azelaic acid 20% cream, 15% gel</td>
<td>Erythromycin (500-1000 mg)</td>
</tr>
<tr>
<td>Clindamycin 1% solution, emulsion</td>
<td>Combined oral contraceptives</td>
</tr>
<tr>
<td>BPO 5% + Clindamycin 1% gel</td>
<td>Spironolactone (50-200 mg)</td>
</tr>
<tr>
<td>Clindamycin 1% + Tretinoin 0.025% gel</td>
<td>Isotretinoin (0.5-1.0 mg/kg)</td>
</tr>
<tr>
<td>Adapalene 0.1% + BPO 2.5% gel</td>
<td>Corticosteroids</td>
</tr>
</tbody>
</table>

1 Fixed combinations. 2 Daily dosage.

2.5.1 Isotretinoin

Although the anti-inflammatory drug isotretinoin (13-cis-retinoic acid, 13-cis RA) has proven highly efficient in the treatment of acne over 30 years but its mode of action is not completely understood. Isotretinoin is regarded as a prodrug, because 13-cis RA itself does not activate retinoic acid receptors (RARs) or retinoid X receptors (RXRs). These receptors are activated by isotretinoin’s active metabolites, including all-trans retinoic acid (ATRA) (On & Zeichner 2013). Isotretinoin influences cell-cycle progression, cellular differentiation, cell survival and apoptosis, resulting in reduced sebum production and alteration of the composition of skin surface lipids (Landthaler et al. 1980, Nelson et al. 2006, Strauss et al. 1987) and attenuated growth and proliferation of infrainfundibular keratinocytes (Ganceviciene & Zouboulis 2010). Isotretinoin has no direct antimicrobial effects, but causes the reduction of surface and ductal P. acnes (King et al. 1982) as a result of the altered microenvironment due to reduced sebum production and smaller sebaceous glands. The anti-inflammatory effects of isotretinoin are also considered important. Isotretinoin and other retinoids down-regulate TLR2 expression on monocytes (Dispenza et al. 2012, Liu et al. 2005). Furthermore, retinoids induce Treg differentiation and down-regulate Th17 differentiation and neutrophil migration (Elias et al. 2008, Wozel et al. 1991, Xiao et al. 2008) but it is unknown whether these mechanisms are modulated by isotretinoin in acne. Isotretinoin also down-regulates the expression of MMPs -9 and -13, which play a central role in inflammatory matrix remodelling (Papakonstantinou et al. 2005). Finally, retinoids stimulate the release of GM-CSF, monocyte chemotactic protein-1 (MCP-1) and IL-10 (Wojtal et al. 2013) in cultured monocytes and macrophages.
2.5.2 Tetracyclines

Clinical use of oral antibiotics for acne seems to be based primarily on empirical evidence, and clinical trials demonstrating dose-responses and comparative efficacy are widely lacking (Larsen & Jemec 2003, Simonart et al. 2008). Antibiotics reduce the number of inflammatory lesions but none clear acne completely (Williams et al. 2012). Tetracycline, lymecycline and doxycycline are the antibiotics of the tetracycline group used in Finland.

Tetracyclines are broad-spectrum antibiotics, effective against various pathogens including rickettsiae, Gram-positive, and Gram-negative bacteria (Griffin et al. 2010). The antimicrobial properties of tetracyclines are known to reduce amount of propionibacteria and staphylococci on acne patients’ skin (Eady et al. 1990, Goltz & Kjartansson 1966, Mills et al. 1972, Oprica et al. 2007). In vitro studies have shown that tetracyclines also have anti-inflammatory effects that could have an important role in acne. They inhibit MMPs, scavenge ROS and have anti-apoptotic effects (Griffin et al. 2010). Tetracyclines may also down-regulate proinflammatory cytokines such as IL-1β, TNF-α, IL-6 and the chemokines IL-8, MCP-1, Chemokine (C-C motif) ligand 3 (CCL3) and CCL4 (Bender et al. 2008, Krakauer & Buckley 2003) and inhibit neutrophil migration and degranulation (Gabler & Creamer 1991).
3 Aims of the study

In this study, our aim was to clarify innate and adaptive immune responses in clinically early-stage inflamed acne lesions and to examine the effects of isotretinoin treatment on acne patients’ skin. The study was also designed to explore the differences between the skin microbiota of normal and acne skin, and especially the effects of systemic medications on acne patients’ skin microflora. The specific aims were:

1. To investigate adaptive immune responses and identify the types of CD4+ T cells in evolving acne lesions (I).
2. To explore how isotretinoin treatment affects innate immune reactions and T helper cells in acne patients’ skin (II).
3. To characterize the microbiota at different acne skin sites (cheek and back), and how it is affected by treatment with isotretinoin or lymecycline (III).
4 Materials and methods

4.1 Study subjects (I-III)

A total of 79 acne vulgaris patients (30 female and 49 male; age range 15-41 years (mean 23.5 ± 5.4) were included in the study. We recruited patients with acne vulgaris who attended the outpatient department of Oulu University Hospital in Finland (I-III) or Charite Universitätsmedizin Berlin in Germany (I) for treatment. Because study I was performed at two separate clinical centers – Oulu and Berlin – at which the patient cohorts were examined using partially different methods, the terms “Finnish cohort” and “German cohort” are used to define the patient groups. Severity of acne was assessed using the Leeds revised acne grading system (O’Brien et al. 1998) in the Finnish cohort (I-III) and using the Global Evaluation Acne Scale (Dreno et al. 2011) in the German cohort (I).

Patients were treated at their physicians’ discretion with isotretinoin or lymecycline (II, III), and the treatment was allocated to each patient based on their severity of acne and their previous medical history and treatment responses.

4.1.1 Skin biopsy samples (I, II)

Two punch biopsies were taken from the back or chest skin of each acne patient under local anesthetic. The samples were taken from areas clinically identified as early-stage inflammatory lesions (comedones with erythematous flare or small papules). The 3 mm-diameter (German cohort) or 4 mm-diameter (Finnish cohort) punch biopsies involved both the acne lesion at the centre, and surrounding non-inflamed skin. One sample from each patient was embedded in paraffin for immunohistochemistry analysis and the other was flash frozen in liquid nitrogen. Biopsies from German cohort for the microarray analysis were stored in RNA Stabilization Reagent. One six-millimeter biopsy was taken from each patient, from an area of normal appearing skin at least 1 cm away from any acne lesion and the sample was halved. One half was put in paraffin and another half in liquid nitrogen. In addition, further skin biopsies were taken from each of the acne subjects participating in study II approximately six weeks after the initiation of isotretinoin treatment at a dose 0.4–0.6 mg/kg/day. A third set of samples were taken from four of these patients on average 40 weeks after completing isotretinoin therapy. The control skin biopsies were taken from the back of psoriasis patients’ lesional and
non-lesional skin (used in study I) and from healthy volunteers’ back skin (used in study I, II), and put in liquid nitrogen. All samples put in liquid nitrogen were stored at -80 °C until total RNA isolation.

4.1.2 Serum samples (II)

Blood samples were taken before the onset of isotretinoin treatment, and 4–7 weeks later. Sera collected were stored at -20 °C until analysis.

4.1.3 Swab samples (III)

Skin washing, make-up, skin creams and deodorants were avoided for 24 hours prior to sampling. Samples were taken from the cheek, back and armpit using sterile cotton swabs and the sampler wore sterile gloves. A 4 cm² skin area was rubbed 20 times with a cotton stick (10 times in one direction and 10 times perpendicular to this direction). The swabs were put in phosphate-buffered saline solution and stored at -20 °C until DNA extraction. Swab samples were taken before isotretinoin or lymecycline therapy and approximately six weeks after the initiation of those treatments.

4.2 Ethical considerations

All samples from study subjects were taken with informed and written consent. The study plan considering this work was accepted by the Ethical committee of the Northern Ostrobothnia Hospital District (I-III) and the Ethical committee of Oulu University Hospital in Finland and the Charite Universitätsmedizin Berlin in Germany (I). All clinical investigations were conducted in accordance with the principles of the Declaration of Helsinki.

4.3 DNA microarray analysis (I)

Affymetrix was conducted at the Galderma Research and Development (R&D) Center in the Sophia Antipolis technology park, France. For RNA extraction the samples were homogenized in a Qiagen lysis buffer using a Potter-Elvehjem homogenizer (Dominique Dutscher S.A, Brumath, France). Total RNA was extracted using miRNeasy extraction kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA quantity was measured using a NanoDrop
8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA quality was controlled using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany). Probes were synthesised and then hybridized on Affymetrix U133 Plus 2.0 chips (Affymetrix, Santa Clara, CA, USA). All chips were normalized using a Robust Multichip Average (RMA) method (Bolstad et al. 2003). Only Affymetrix identifiers (IDs) with expression ≥ 2exp6 in at least one condition were selected. Data analysis was performed on Array Studio software (OmicSoft, Cary, NC, USA). Mean expression levels were obtained by calculating the geometrical means of the RMA-normalized data for involved and non-involved sample groups, respectively.

### 4.4 RNA isolation and real-time PCR analysis (I, II)

The analyses were conducted in the Unit of Systems Toxicology of the Finnish Institute of Occupational Health in Helsinki, Finland (I-II) and in the Galderma R&D Center (I). Snap frozen skin biopsies were homogenized in TRIsure reagent (Bioline, London, UK) and total RNA was extracted according to the protocol provided by the manufacturer and used as a template for cDNA synthesized by standard methods. Quantitative real-time PCR analysis was performed using an Applied Biosystems 7500 Fast Real Time PCR-system (Applied Biosystems, Foster City, CA, USA) in Finland and an Applied Biosystems 7900HT machine in France using commercial primers and probes from Applied Biosystems. 18S RNA was used as an endogenous control to ensure equal amount of RNA in each sample. The relative unit (RU) for each sample was calculated as follows: the cycle threshold value (CT) of a sample was determined according to the manufacturer’s instructions (Applied Biosystems, CA, USA). Then the CT value of the 18S rRNA sample was subtracted from that of the corresponding target cytokine to obtain the ΔCT. Next, the average of 18S CTs of each sample was subtracted from the calibrator CT value obtained from the "no template control" (NTC) to get the calibrator ΔCT. The calibrator ΔCT was then subtracted from the ΔCT of each experimental sample to obtain the ΔΔCT. Finally, the RU of the target was calculated using the equation 2– ΔΔCT.

### 4.5 Cytokine profiling by Luminex technology (I)

Luminex assays were performed in the Galderma R&D Center. Cytokines were extracted from the biopsies taken from German patients’ non-lesional and lesional
skin, which were crushed manually for 4 minutes with tissue grinder Potter-Elvehjem (Dominique Dutscher S.A, Brumath, France) in 200 µL ice-cold phosphate buffer saline (PBS) containing Triton X100 0.2% and protease inhibitors (cOmplete, Mini, EDTA-free; Roche Applied Science, Mannheim, Germany). Samples were stirred for 30 minutes at 1400 RPM and then centrifuged for 5 minutes at 10 000 RPM at 4 °C. Supernatants were harvested and the concentration of proteins was determined using a colorimetric method (Bio-Rad DC Kit; Bio-Rad Laboratories, Hercules, CA, USA). Cytokines were quantified in duplicate using the following Luminex assays from Life Technologies, Carlsbad, CA, USA: Human Cytokine, Premixed 23 Plex, Immunoassay Procarta kit and Milliplex Human Cytokine, as well as the Premixed 42 Plex, Immunoassay kit (Merck Millipore, Billerica, MA). Cytokine quantities were normalized to the total concentration of protein.

4.6 Immunohistochemistry (I, II)

Immunohistochemical analysis were performed in the Department of Pathology, University of Oulu and Oulu University Hospital (I, II) and in the Galderma R&D Center (I). For the immunohistochemistry of Finnish patient samples, paraffin-embedded 3.5 µm sections were deparaffinized and rehydrated. Heat-induced antigen retrieval was performed in a microwave oven. Endogenous peroxidase activity was neutralized. The sections were incubated with monoclonal mouse anti-human antibodies to CD4 (1:50, Novocastra, Newcastle, UK), CD8 (1:200, Novocastra), CD68 (1:10000, Dako, Copenhagen, Denmark), CD83 (1:25, Dako), FoxP3 (1:100, Abcam Ltd., Cambridge, UK), Tbet (1:200, Abcam Ltd.) and goat polyclonal anti-human antibody to IL17 (1:100, R&D systems, Minneapolis, MN, USA). Bound antibodies were detected using the NovoLink Polymer detection system (Leica Biosystems, Newcastle, UK), the EnVision system (Dako) or Goat-HPR-Polymer kit (Biocare Medical, Concord, CA, USA). 3, 3'-Deaminobenzidine (DAB) was used as the chromogen and hematoxylin as the counterstain. Histological images were obtained with an Olympus DP25 camera (Olympus, Center Valley, PA, USA) attached to a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) using x 20 objective. The positive cells were counted using ImageJ software, as described previously (Väyrynen et al. 2012).

For the immunohistochemistry of German patient samples, deparaffinised sections were treated for antigen retrieval in 10 mM citrate buffer, pH 6.0 at 98°C for 20 minutes. Mouse antihuman CD3 (clone Ab-9, Thermo Scientific, Fremond,
CA) was used to detect T-lymphocytes. IL-17A was detected on the same section with a goat polyclonal antihuman IL-17A antibody (R&D systems, Minneapolis, MN). CD3 was detected with Alexa fluor 594 conjugated antibody and IL-17A was revealed after amplification (Biotin Streptavidin FITC, Thermo Fisher Scientific, Waltham, MA, USA). Positive cells were counted visually on slides scanned with a NanoZoomer slide imaging system (Hamamatsu, Japan).

4.7 Serum cytokine measurements (II)

A human Th17 Magnetic Bead Panel (Merck Millipore, St. Charles, MO, USA) was used for the detection of cytokines by Luminex technology following the manufacturer’s instructions. Measurements were performed in the Unit of Systems Toxicology at the Finnish Institute of Occupational Health in Helsinki.

4.8 DNA extraction, sequencing and sequence data analysis (III)

4.8.1 DNA extraction, PCR amplification and 16S rRNA gene sequencing

The analyses were carried out at the DNA Sequencing and Genomics Laboratory of the Institute of Biotechnology, University of Helsinki. Total DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions.

PCR amplification was conducted with an Arktik Thermal Cycler (Finnzymes Diagnostics/Thermo Scientific, Vantaa, Finland) in two steps. The V1-V3 region of the 16S rRNA gene was amplified using the universal bacterial primers pA (AGAGTTTGATCMTGGCTCAG) (Lane 1991) and pD' (GTATTACCGCGGTGTGCTG) (Edwards et al. 1989), with partial Illumina TruSeq adapters (Illumina, San Diego, CA, USA) sequences added to the 5’ ends of the primers, (ATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT and GTGACTGGA GTTCAGACGTGTGCTCTTCCGATCT, respectively). Two 25 µl technical replicates of each sample were run and subsequently pooled. The cycling conditions were as follows: initial DNA denaturation at 98 °C, followed by 15 cycles at 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 10 s, and a final extension for 5 min at 72 °C. Between 11 and 59 ng of template DNA was used in the reaction. Each PCR run included a PCR blank with no template DNA. The PCR products
were purified with Exonuclease I (Thermo Scientific) and Thermosensitive Alkaline Phosphatase (FastAP; Thermo Scientific). A second PCR was performed with full-length TruSeq P5 and Index containing P7 adapters and 1–5 ml from the first PCR as a template. The cycling conditions were identical to the previous ones except that 18 cycles were run.

The final PCR products were purified with Agencourt® AMPure® XP magnetic beads (Agencourt Bioscience), pooled, and sequenced in two separate runs on a MiSeq Sequencer (Illumina) using a v2 600 cycle kit paired-end (325 bp + 285 bp).

4.8.2 Sequence data analysis and data trimming

Preliminary quality control and primer removal were performed with cutadapt software (Martin 2011). Due to the differing quality profiles of the two sequencing runs, different parameters were used: -q 30 and -m 150 for the first run, -q 28, -m 100 and –e 0.2 for the second run. The sequence data were analyzed further using mothur software (Schloss et al. 2009), as described in the MiSeq SOP http://www.mothur.org/wiki/MiSeq_SOP (accessed 20 June 2015). The reads were paired; all ambiguous nucleotides, reads over 550 bases in length and chimeras were removed; the sequences were clustered into Operational Taxonomic Units (OTUs), and taxonomical classifications were assigned. All singleton OTUs were trimmed before further analysis.

The final tables with numbers of reads per taxon per sample were exported to the phyloseq R package (McMurdie & Holmes 2013), which was used to trim the data further. Several taxa deemed to be likely contaminants, based on their presence in PCR blanks or previous experience and published results on contamination (Salter et al. 2014), were removed. Samples in which more than 50% of sequence reads consisted of likely contaminants were deemed unreliable and were excluded from analyses. The numbers of the suspected contaminant taxa differed notably depending on the sample location (armpit, back and cheek), sample group (control, acne before and after treatment) and sequencing run, and NMDS ordination plots showed clustering according to run even after contaminant removal. Because of these differences, the data were further trimmed to include only those OTUs present in both sequencing runs.

A representative sequence for Otu00001 was compared with Basic Local Alignment Search Tool (BLAST) against the NCBI’s 16S rRNA collection and with
the Ribosomal Database Project’s (RDP) SeqMatch tool to determine whether Otu00001 represented *P. acnes*.

### 4.9 Statistical analysis (I-III)

For microarray data, a two sided paired T test was performed using Array Studio (OmicSoft Corporation, USA), to determine which genes were significantly differentially expressed between involved and non-involved groups, and Benjamini-Hochberg false discovery rate (FDR) multiple testing correction (Benjamini & Hochberg 1995) was applied (I). Statistical analyses of RNA expression levels were done with ANOVA, Student's t-test or, when variances were significantly different, with a nonparametric Mann-Whitney U test using GraphPad Prism software (GraphPad Software Inc., LaJolla, CA, USA) (I, II). For immunohistochemical data statistical analysis, a two-tailed Mann-Whitney U test was performed using IBM SPSS Statistics 19 (IBM, Chicago, IL) (I, II). For the microbiota study most statistical analysis was performed using R. Alpha diversity indices, which were calculated using the “estimate_richness” command of the phyloseq package (McMurdie & Holmes 2013), and compared using the Wilcoxon rank sum test (command pairwise.wilcox.test), with the multiple comparison correction method “holm”. DESeq2 was used to compare the relative abundance of bacterial taxa (Love et al. 2014) (III). For comparisons of acne grades, Wilcoxon’s two-tailed signed rank test was performed (II, III). For all statistical comparisons, p-values below 0.05 were considered statistically significant.
5 Results

5.1 IL-17A/Th17- axis in clinically early inflamed acne lesions (I)

In the German patient cohort a total of 904 genes were identified by the analysis of gene expression, 509 were up-regulated and 395 were down-regulated in acne lesions compared with uninvolved skin of acne patients. Interestingly, the bioinformatic analysis of differentially modulated genes showed that the Th17-derived cytokine network and the IL-17 signalling pathway were among the top-four in gene enrichment analysis (I, Tables 3-4, Figure 3).

The findings of the RT-PCR analysis confirmed those of the Affymetrix results. In acne lesions the main effector cytokine of Th17 cells, IL-17A mRNA was significantly elevated compared with uninvolved skin, as were the other Th17 cell products, IL-22, IL-26 and TNF-α. Moreover, the gene expression of the Th17 lineage inducing cytokines IL-1β, IL-6, transforming growth factor-β (TGF-β) and IL-23p19 (IL-23a) were all up-regulated. mRNA levels of chemokines CSF2 and CCL20, which both are induced by IL-17, were also elevated (I, Table 5, Figure 4).

At the protein level the increased expression of the markers and cytokines characterizing the Th17 cell subtype were demonstrated using Luminex technology in German samples (I, Table 6). Accordingly, immunohistochchemical analysis showed an increased number of IL-17A+ cells in the papillary dermis and around pilosebaceous follicles in acne lesions compared with uninvolved skin of acne patients. The IL-17A+ cells were scattered through the superficial and deep dermis both in acne lesions and in normal skin. The IL-17A+ cells were identified mainly as CD3+ lymphocytes but some of them were neutrophils and mast cells (I, Table 7, Figure 5).

5.2 Th1 and Treg cells in acne lesions (I)

Because CD4+ cells and CD68+ macrophages had previously been detected around pilosebaceous follicles in the early phase of acne lesions formation (Jeremy et al. 2003, Layton et al. 1998), our aim was to analyze CD4+ cells most closely. To investigate CD4+ cells other than Th17 cells we measured the expression of signature cytokines and transcription factors of different Th lineages on the mRNA level by quantitative RT-PCR (Finnish cohort) and by Affymetrix microarray technology (German cohort).
As well as the Th17-polarized immune response, we found induction of Th1-associated genes. The mRNA levels of effector cytokines of Th1 lineage, IFN-\(\gamma\) and IL-12p40, were significantly elevated in acne lesions, as were those of the transcription factor Tbet, the chemokine receptor CXCR3, and the chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10 and CXCL11 (I, Figure 8, Table 10).

In addition to the Th1/Th17 immune response, Tregs were induced in acne lesions in both cohorts according to the elevated mRNA levels of the transcription factor FOXP3 and signature cytokines TGF-\(\beta\) and IL-10 (I, Figure 8, Table 11). In contrast, in acne patients’ uninvolved skin the expression levels of TGF-\(\beta\) and FOXP3 mRNA were lower than those in the skin of healthy control subjects (I, Figure 8; II, Figure 1).

Regarding the effector cytokines of Th2 lineage, we detected IL-4 mRNA in low levels in acne lesions, but found increased expression of neither IL-13 nor transcription factor GATA3.

Finally, immunohistochemical stainings from Finnish patients’ samples were performed to detect subtypes of CD4+ cells in inflammatory infiltrates of acne lesions. T-bet+ and FoxP3+ cells were located in the papillary dermis and perifollicularly in the same areas as CD4+ cells. The numbers of T-bet+ and FoxP3+ cells were significantly higher in acne lesions compared with uninvolved skin in papillary dermis but around pilosebaceous follicles the Tbet+ cell count was elevated but not that of FoxP3+ cells. The presence of CD68+ macrophages, CD8+ cells and CD83+ DCs were also detected in acne lesions (I, Figure 5, Table 7).

5.3 The effect of isotretinoin treatment on immune responses in acne (II)

5.3.1 The effect of isotretinoin on Th17/Treg balance

The mRNA expression levels of Th17 or Treg markers in early-stage lesions were equally amplified in acne lesions before and during isotretinoin treatment (II, Figure 2). Lesion immunohistochemistry found similar numbers of CD4+, IL-17A+ and FoxP3+ cells in the upper dermis and around the sebaceous follicles before and during treatment (II, Supplementary Table S2).

Finally, we analyzed serum levels of signature cytokines (IL-17A, IFN-\(\gamma\), IL-10) of different Th lineages. We observed slightly (but not significantly) higher levels of IFN-\(\gamma\) and IL-17 in acne patients compared with healthy volunteers.
Isotretinoin treatment showed a trend of lowering the level of IL-17A and IFN-γ, but not IL-10 in the serum (II, Supplementary Figure S6).

5.3.2 The effect of isotretinoin on innate immune responses and apoptosis markers

We observed a significant increase in the number of CD68+ cells in dermal infiltrates of acne lesions (P=0.032) and in uninvolved skin of acne patients (P=0.037) during isotretinoin treatment, compared with untreated skin (II, Supplementary Table S2, Supplementary Figure S5).

Similar to the markers of adaptive immune responses, isotretinoin treatment did not affect the expression of innate immune signals in newly-formed acne lesions (II, Supplementary Figures S3 and S4). However, we observed that gene expression of IL-1β mRNA expression was slightly up-regulated in acne patients’ uninvolved skin compared with healthy volunteers, and isotretinoin reduced IL-1β expression with a long-lasting effect (II, Figure 1). Similarly, TLR2 mRNA was significantly decreased (P<0.001) in uninvolved skin during treatment (II, Figure 1). In contrast, psoriasin S100A7 and LCN mRNA levels were low in acne patients’ uninvolved skin at baseline but those levels returned to normal after isotretinoin treatment (II, Figure 1). Moreover, in acne lesions we observed close to significant (p=0.058) elevation of the expression of TNF-related apoptosis-inducing ligand (TRAIL) at the mRNA level during isotretinoin treatment, but the level of LCN2 mRNA was not modified by isotretinoin (II, Supplementary Figure S4).

5.4 Characterization of acne patients’ skin microbiota in typical acne areas and the correlation of bacterial composition with acne severity (III)

Four bacterial phyla dominated acne patients’ skin microbiota: Actinobacteria, Firmicutes, Proteobacteria and Bacteroides. The predominant bacteria at the genus level on the face and back were Propionibacterium, Staphylococcus, Corynebacterium, Streptococcus and Micrococcus. In the armpit, the main genera were Staphylococcus, Propionibacterium and Corynebacterium. The same dominating phyla and taxonomic groups were present in controls at each location (III, Figures 1 and 2a, Supplementary Table S4.)

Otu00001 (Propionibacterium) was the most abundant in both acne patient and controls in cheek and back samples. Sequence comparisons suggested that
Otu00001 corresponded to *P. acnes*, Otu00002 (*Staphylococcus*) and Otu00001 (*Propionibacterium*) dominated in armpit samples from both acne patients and controls (III, Supplementary Table S5).

Next, we analysed the differences in the abundance of specific bacteria between acne patients and controls. Five OTUs were significantly less abundant in the cheek samples of acne patients compared with those from controls: Otu00010 (*Streptococcus*), Otu00051 (*Fusobacterium*), Otu00046 (*Gemella*), Otu00080 (*Granulicatella*) and Otu00145 (*Neisseria*) (III, Figure 3b, Table 1). However, there were no differences in either back or armpit samples.

Then we studied if bacterial taxa correlated with the acne severity grades. We observed that *Propionibacteria* were significantly more abundant in cheek samples of acne patients who had greater acne scores. In contrast, *Corynebacteria* on the back was associated with lower grades (III, Supplementary Table S8).

### 5.5 The impact of isotretinoin and tetracycline treatments on acne patients’ microbiota and microbial diversity (III)

Isotretinoin and lymecycline treatments significantly decreased cheek and back acne severity, and both simultaneously seemed to decrease the relative abundance of *Propionibacteria* in samples taken from same areas (III, Supplementary Figure S1, Figure 1). In statistical comparisons both isotretinoin and lymecycline significantly reduced the abundance of Otu00001 (*Propionibacterium*) in cheek samples. In addition, the abundance of Otu00015 (*Propionibacterium*) was significantly lower, whereas Otu00010 (*Streptococcus*), Otu00085 (*Corynebacterium*) and Otu00207 (unclassified genus, family Pasteurellaceae) were significantly more abundant in cheek samples after isotretinoin treatment (III, Table 1, Figure 3b). However, in back samples *Propionibacteria* were less abundant among lymecycline-treated patients but not isotretinoin-treated patients (III, Table 1, Figure 3b). In armpit samples significant differences between untreated and treated acne patients were not detected.

We used the inverse Simpson and Shannon indices to compare alpha diversity between different sample groups and locations. Those indices take into account the number of taxa present and their abundance in the community. Alpha diversity did not significantly differ between controls and acne patients in separate body locations. However, after both treatments the microbiota in cheek and back samples of treated acne patients was more diverse than that of untreated patients. In contrast,
both treatments significantly decreased alpha diversity in the armpit samples (III, Figure 2b).
6 Discussion

6.1 The activation of IL-17A/Th17 axis in acne (I, II)

Study I was designed to determine the adaptive immune responses (in particular, T cell subsets), and the innate immune responses in the skin of acne vulgaris patients. The most important finding was the activation of the IL-17A/Th17 axis in clinically early-stage inflammatory acne lesions. This is currently an area of interest, as demonstrated by the reporting of the presence of IL-17/Th17 cells in acne by two other research groups (Agak et al. 2014, Kistowska et al. 2015) almost simultaneously with the publication of our findings.

IL-17 may contribute to the pathogenesis of acne in different ways depending on lesions age or type. The accumulation of IL-17 positive cells around typical closed comedone-type acne lesions examined by immunohistochemistry indicates that IL-17 might have role in early inflammatory events in acne (Agak et al. 2014). The lesions investigated in our study were clinically early-stage inflammatory acne lesions (comedones with erythematous flare or small papules) in which the neutrophils were in a minority. The most prominent function of IL-17 is neutrophil chemotaxis mediated by the production of granulocyte colony-stimulating factor (G-CSF) and chemokines such as IL-8/CXCL8. IL-17 also induces chemoattractants e.g. CCL20, GM-CSF for lymphocytes, DCs and monocytes, and modulates the production of AMPs. Importantly, IL-17 synergizes with other inflammatory cytokines in particular TNF-α, IL-1β, IL-22 and IFN-γ (Beringer et al. 2016).

In addition to its potential function to attract the neutrophils in early phases of acne inflammation, IL-17 may account for the increased expression of AMPs in acne lesions, which was also observed in our study. IL-17 together with IL-1 and TNF-α may induce the production of several MMPs, whose expression is also increased in acne (Agarwal et al. 2008, Kang et al. 2005). Acne pustules contain large numbers of neutrophils which could for their part secrete more IL-17 and intensify the inflammation. In more severe forms of acne with nodular or cystic lesions there could be very intensive IL-17 reaction, because of massive neutrophilic inflammation.

As well as by CD4+ cells and neutrophils, IL-17 can also be produced by several other cell types including Tc cells, group 3 innate lymphoid (ILC3) cells, γδT cells, NK cells, mast cells and macrophages (Isailovic et al. 2015).
Keratinocytes are the principal cellular target of IL-17A, but IL-17 receptors are constitutively expressed on fibroblasts, osteoblasts, chondrocytes, macrophages, DCs and endothelial cells (Maddur et al. 2012). In our study we noticed that IL-17 expressing cells were mainly lymphocytes with a minority of neutrophils and mast cells.

The activation of the IL-23/IL-17/Th17 axis is considered to be important in driving pathogenic inflammation in psoriasis (Di Cesare et al. 2009). Acne and psoriasis have some histological features in common, namely keratinocyte hyperproliferation that occurs in the pilosebaceous follicle in acne and in the epidermis in psoriasis. Both diseases demonstrate elevated expression of both TLR2 and AMPs, and neutrophilic inflammation. This is why we chose psoriasis patients as a control group for quantitative RT-PCR. Indeed, we found that several effector cytokines and markers of Th17, Th1 and Treg cells were induced both in acne and in psoriasis, however usually to a greater extent in psoriasis. Interestingly, the expression of cytokines IL-1β, TGF-β and IL-10 mRNA was higher in acne lesions than in psoriasis samples. TLR2 mRNA was up-regulated in both acne and psoriasis but the expression of TLR4 was only increased in acne. All examined AMPs (human β-defensin 2 and 3, S100A7, S100A9 and cathelicidin, lipocalin 2) were up-regulated to a significantly greater extent in psoriasis than acne except for cathelicidin, which was similarly expressed in both diseases. The stronger up-regulation of AMPs and many cytokines in psoriasis may be due to hyperplasia of the epidermis. Moreover, in the biopsies taken from acne lesions there is also healthy skin around the lesions whereas in psoriasis biopsies the whole skin is affected. It is unknown if cathelicidin has a specific role in the pathogenesis of acne.

On the skin surface inactive precursors of cathelicidin are cleaved from the inactive precursor to LL-37 by serine proteases of the kallikrein family. LL-37 and its peptide fragments may be important in pathogenesis of rosacea (Steinhoff et al. 2013). In psoriasis LL-37 has shown to be crucial mediator of plasmacytoid DCs (pDCs) activation. By formation of aggregates with self-DNA, LL-37 may initiate an autoinflammatory cascade in psoriasis (Batycka-Baran et al. 2014).

IL-17/Th17 cells’ primary function is first line host defense at epithelial and mucosal barriers against bacteria and fungi. In addition to psoriasis and psoriatic arthritis, IL-17 contributes to the chronic inflammation associated with many inflammatory and autoimmune disorders e.g. rheumatoid arthritis, ankylosing spondylitis, IBDs and multiple sclerosis. The immunological mechanisms in the other acne diseases, acne rosacea and hidradenitis suppurativa (HS), have recently been studied. Th1/Th17 polarized inflammation and macrophage infiltration were
the hallmark in all subtypes of rosacea (Buhl et al. 2015), and the IL-1β-IL-23/Th17/IL-17 pathway has also been implicated in the pathogenesis of HS (Lima et al. 2016, Schlapbach et al. 2011). Shared features of severe forms of acne, rosacea and HS are the formation of inflammatory lesions and chronicity, but the distinct roles of the microbiome may also contribute to the diseases. The formation of Th17 memory cells in chronic acne, rosacea and HS may drive the chronic relapsing-remitting nature of these diseases.

IL-1 is the key cytokine in the early phase of the inflammatory reaction in acne (Jeremy et al. 2003) and recent data indicate the activation of inflammasome in inflammatory acne (Kistowska et al. 2014, Li et al. 2014, Qin et al. 2014). Inflammasome activation and active IL-1β potentiate Th17 cell-dominant immune responses (Meng et al. 2009). In addition to Th17, active IL-1β together with other cytokines drives the generation of adaptive immunity including Th1 effector cells. It is probable that in severe forms of acne e.g. in cystic acne, the activation of inflammasome and subsequent production of Th17 and Th1 cells contributes to the powerful activation of adaptive immunity that is indicated by the increased levels of IL-17 and IFN-γ in serum samples of moderate to severe nodulocystic acne patients (Karadag et al. 2012). In our study (II) the lack of significance in the differences in the values of IL-17 and IFN-γ in serum could be due to very low levels of cytokines, which are challenging to measure accurately, and also due to the small number of study subjects. However, the most likely explanation for the low cytokine levels detected in our study, is that our patients did not suffer severe forms of acne vulgaris. In psoriasis, serum IL-17 concentrations are highest in pustular psoriasis and plaque psoriasis with PASI score>10 (Yilmaz et al. 2012). It is possible that cytokine levels correlate with the severity of disease and the area of skin inflammation also in acne.

There is strong in vitro evidence that P. acnes can induce Th17 and Th17/Th1 differentiation (Agak et al. 2014, Furusawa et al. 2012, Kistowska et al. 2015, Sugisaki et al. 2009). P. acnes come into close contact to dermal DCs and macrophages after the follicular wall of the pilosebaceous duct ruptures, initiating the inflammatory cascades. It is reasonable to believe that P. acnes in severe acne potentiates IL-17-mediated inflammation. The fact that patients with moderate and severe acne have significantly higher titres of IgG antibodies to P. acnes than patients with milder forms of acne (Ashbee et al. 1997, Holland et al. 1986) support this view. In milder acne there are no differences between acne patients and controls in the humoral response against P. acnes (Till et al. 2000).
P. acnes-induced Th17 and Th17/Th1 cell-mediated recall responses can be observed in all healthy donors suggesting that such a pool of memory cells is present in most healthy individuals (Kistowska et al. 2015). The reason for this finding could be that almost everybody has acne at some point in their life.

6.2 The effects of isotretinoin treatment in acne patients (II)

In the second study of the thesis we investigated the effect of isotretinoin in acne patients’ skin and serum samples. Isotretinoin is the most efficacious medication in the treatment of acne, but despite of its wide use, its mode of action remains incompletely understood. Since retinoids inhibit inflammatory Th17 responses, and promote regulatory T cell responses (Elias et al. 2008, Mucida et al. 2007, Xiao et al. 2008), our working hypothesis was that systemic isotretinoin treatment might influence the Th17/Treg balance in the skin. In contrast to our expectations, isotretinoin treatment had no effect on adaptive immune responses in newly-formed acne lesions. This indicates that when the inflammation is already initiated in an acne lesion, isotretinoin cannot attenuate it. However, there was a slight (but statistically non-significant) reduction of IL-17A and IFN-γ in the serum due to reduced inflammation. As seen in acne, the IL-17/IL-23 axis contributes to the pathogenesis of IBD (Catana et al. 2015). It has previously been suggested that isotretinoin treatment could induce colitis in acne patients but recent studies have demonstrated that the use of isotretinoin is not associated with increased risk of IBD (Coughlin 2015, Lee et al. 2016, Racine et al. 2014, Rashtak et al. 2014). In our study isotretinoin did not aggravate Th17 responses in acne patients, but at the moment the effect of isotretinoin on the level of IL-17 in IBD patients’ intestine is not known.

In acne lesions, the expression of both TLR2 and TLR4 is up-regulated (Jugeau et al. 2005, Kim et al. 2002). Accordingly, we also detected their increased expression in our studies (I, II). In acne lesions TLR2 is expressed mainly by keratinocytes and macrophages infiltrating around the pilosebaceous follicles (Jugeau et al. 2005, Kim et al. 2002). TLRs represent an ancient front-line defense system that enables the host to sense the presence of microbial components (pathogen-associated molecular patterns; PAMPs) or endogenous danger signals (danger-associated molecular patterns; DAMPs) within minutes, engaging intracellular downstream cellular pathways followed by the production of pro-inflammatory mediators (Schwarz 2012). The infiltration of TLR2+ cells is an early event in the evolution of acne lesions (Kim et al. 2002), so it is unlikely that P.
Acne triggers innate immune responses before rupture of pilosebaceous follicles. FFAs can act as possible DAMPs activating TLR2 and TLR4 (Lwin et al. 2014), and initiate inflammation and the formation of acne lesions. Given its role in the initiation of inflammation, the down-regulation of TLR2 expression might represent one of the mechanisms by which isotretinoin therapy exerts its effects in acne. Indeed, we observed that the expression of TLR2, but not TLR4, is significantly reduced during isotretinoin treatment in uninvolved skin in acne patients. This is in line with previous studies that demonstrated down-regulation of TLR2 expression and function in all-trans ATRA- or isotretinoin-treated monocytes in vitro (Liu et al. 2005) and in vivo (Dispenza et al. 2012).

One important, natural antimicrobial defense system is the production of AMPs, several of which are induced in acne. Some AMPs (e.g. hBD-2, hCAP-18/LL-37, RNAse7 and S100A7) efficiently limit the growth of *P. acnes*, but may at the same time contribute to the formation of comedones by stimulating keratinocyte migration and proliferation, and by activating immune cells (Harder et al. 2013). In our work the levels of AMPs in the acne lesions were not affected by isotretinoin treatment. Recently Borovaya and co-workers found that lesional expression of many AMPs, including HBD-2 and S100A7 was decreased after 2 months of isotretinoin treatment (Borovaya et al. 2014). The discrepancy between the results of that study and ours might be due to different sampling methods: in our study we took 4 mm punch biopsies but Borovaya and co-workers (2014) took shave biopsies. Moreover, our biopsies were taken after 6 weeks’ isotretinoin treatment, whereas in the other study they were taken after 2 months.

S100A7, one of the major skin AMPs, is expressed in keratinocytes and sebocytes, and is highly induced in inflammatory skin diseases like atopic dermatitis and psoriasis as well as wounds (Harder et al. 2010). The expression of S100A7 is known to be increased at the ductus seboglandularis in acne comedones (Ganceviciene et al. 2006). We found that the expression of S100A7 was lower in the uninvolved skin of acne patients compared with that of non-acne controls. Interestingly, systemic isotretinoin treatment resulted in the up-regulation of the expression of S100A7 in acne patients’ uninvolved skin, reaching similar levels as in healthy volunteers. We observed a similar trend for the expression of LCN2. A previous gene expression study also revealed that LCN2, S100A7 and S100A9 were among the most highly up-regulated genes in acne patients’ uninvolved skin after a week of isotretinoin treatment (Nelson et al. 2008), but the increase was no longer detectable after 8 weeks of treatment (Nelson et al. 2009). In line with the findings of the in vivo studies, it has been shown that LCN2 and S100A7 were up-
regulated by retinoic acid in cultured keratinocytes (Lee et al. 2009). LCN2 exerts its antimicrobial effects by sequestering iron, thus limiting bacterial growth (Yang et al. 2002), but it also has a function in isotretinoin-induced sebocytes apoptosis (Nelson et al. 2006). At the moment we cannot explain why the levels of S100A7 and LCN2 mRNA were slightly lower in uninvolved skin of our acne patients than in controls. Nelson and co-workers hypothesized that the up-regulation of S100 proteins by 13-cisRA at the beginning of the treatment course could relate to the “acne-flare” observed in some patients receiving retinoids (Nelson et al. 2009). Taken together, it is clear that more studies, both clinical and experimental, are required better to understand the effect of isotretinoin on the expression and function of AMPs in acne.

Isotretinoin reduces the size of human sebaceous glands and their levels of secretion, and induces many genes that in vitro studies demonstrate sebocyte apoptosis and cell cycle control, including LCN2 (encodes NGAL), TNFSF10 (which encodes TRAIL) and FAS (Nelson et al. 2006, Nelson et al. 2008). TRAIL is a type II transmembrane protein with a soluble form, and both the membrane and soluble forms induce apoptosis in a variety of cell types via the activation of apoptotic receptors on the cell surface receptors (Griffith et al. 1998). Recent data suggest that TRAIL plays a key role in the sebocyte-specific effects of isotretinoin, and that its expression is increased in acne patients’ skin acne after one week of isotretinoin therapy (Nelson et al. 2011). We observed that TRAIL expression remained slightly up-regulated in acne lesions after six weeks of isotretinoin therapy i.e. at the time point when clinical improvement usually begins.

The presence of CD68+ macrophages is detectable already in the very early stages of acne lesion development, even before microcomedo formation and breakage of the follicular wall, suggesting a prominent role for macrophages in the development of acne lesions (Jeremy et al. 2003). Isotretinoin therapy significantly increased the number of CD68+ macrophages in both lesional and uninvolved skin in our acne patients. All retinoids stimulate the release of IL-10, GM-CSF and MCP-1 in monocytes and macrophages in vitro (Wojtal et al 2013). This stimulation could explain the increased number of CD68+ macrophages in the acne skin in our study and could also partly explain the increased amount of IL-10 during isotretinoin treatment.
6.3  **Skin microbiota of acne vulgaris patients and its correlation with acne severity (III)**

In the third publication we analyzed the microbiota of acne patients and the effects of systemic treatments on it, using 16S rRNA gene amplicon sequencing. With this method a much wider variety of bacteria than just *P. acnes* can be investigated, but so far only a few studies have utilized this method in acne. Results from a recent exploration of the nose follicular casts from acne patients are in line with our data, in that no difference was detected between acne patients and healthy controls in the relative abundance of *P. acnes* (Fitz-Gibbon *et al.* 2013). In that study the other common bacteria were *Staphylococcus epidermidis*, *Propionibacterium humerusii* and *Propionibacterium granulosum* (Fitz-Gibbon *et al.* 2013). The researchers also used metagenomics shotgun sequencing, which allows for the identification of microbes at the strain level, as well as the analysis of their functional potential. In our study the taxonomical level used was OTU and only Otu00001 (*Propionibacterium*) was defined to correspond to *P. acnes*. In addition to *P. acnes*, other most abundant bacteria in our study were Otu00002 (*Staphylococcus*), Otu00010 (*Streptococcus*) and Otu00008 (*Corynebacterium*) in cheek samples of acne patients and controls. In another study the pilosebaceous follicles of healthy controls were exclusively colonized by *P. acnes*, whereas those of acne patients contained mainly *P. acnes*, *Staphylococcus epidermidis*, with minor proportions of other species (Bek-Thomsen *et al.* 2008). It is probable that a low number of study subjects (three controls and five acne patients) may have influenced the results of the latter study.

Our analyses showed that *P. acnes* dominates the sebaceous areas both in acne patients and controls. However, Otu00010 (*Streptococcus*), Otu00051 (*Fusobacterium*), Otu00046 (*Gemella*), Otu00080 (*Granulicatella*) and Otu00145 (*Neisseria*) were significantly less abundant in the cheek samples of untreated acne patients than in those of controls. These bacteria are facultatively or strictly aerobic, occurring in skin, oral and respiratory track flora (Cargill *et al.* 2012, Davis 1996) and may have lost to *P. acnes* the battle for the same ecological niche in acne patients’ skin.

Interestingly, our data indicate that among patients with higher acne scores *P. acnes* is more abundant on the face and *Corynebacteria* is less abundant on the back. Previous studies have not demonstrated the correlation between the amount of *P. acnes* and acne severity (Cove *et al.* 1980, Leyden *et al.* 1975b). By contrast, there was a trend for lower (but non-significantly lower) levels of *P. acnes* as the
severity of inflammation increased (Leyden et al. 1975b). Of note, 16S rRNA gene amplicon sequencing is used for bacterial identification and essentially it is the comparisons of relative abundances, but it does not provide information about the amount of bacterial biomass or the absolute number of bacteria in the samples.

6.4 Impact of systemic treatments on the acne skin microbiota (III)

Previous studies have demonstrated that isotretinoin reduces the number of surface and ductal *P. acnes* (King et al. 1982) and tetracycline treatments decrease the numbers of *P. acnes* and *Staphylococci* in acne patients’ skin (Eady et al. 1990, Goltz & Kjartansson 1966, Mills et al. 1972, Oprica et al. 2007). 16S rRNA gene amplicon sequencing has not previously been used to determine the effect of systemic acne treatments on the bacterial composition of acne patients’ skin. In accordance with previous studies (Eady et al. 1990, Goltz & Kjartansson 1966, King et al. 1982, Mills et al. 1972, Oprica et al. 2007), we found that six weeks’ treatment with isotretinoin or lymecycline significantly decreased the abundance of *P. acnes* in cheek samples while also reducing the clinical severity of acne. However, based on the findings of previous studies and clinical experience, isotretinoin is more effective than tetracyclines in the treatment of acne, which is probably due to the effect of isotretinoin on the sebaceous glands and sebum excretion.

Interestingly, after isotretinoin treatment several OTUs such as Otu00010 (*Streptococcus*), Otu00085 (*Corynebacterium*) and Otu00207 (unclassified genus, family Pasteurellaceae) were significantly more abundant in cheek samples than before treatment, likely because of drier skin caused by isotretinoin. Surprisingly, while lymecycline reduced the abundance of *P. acnes* in back skin samples, isotretinoin did not, although acne was clinically improved by both treatments in this area. However, the number of samples available for analysis from the back area was lower than that from the face, which may have affected these conflicting results.

We also examined the armpit, since moist areas are known to have different bacterial composition from sebaceous areas (Grice et al. 2009). *Staphylococcus*, *Propionibacterium* and *Corynebacterium* were the main genera in armpit samples from both acne patients and controls. This result is in line with previous work by Grice and coworkers (2009) who found that *Staphylococcus* and *Corynebacterium* spp. are the most abundant organisms colonizing moist areas (Grice et al. 2009). Systemic acne treatments are likely to influence the microbiota of non-acne skin regions, such as the armpit. However, we could not detect significant differences in
the abundance of specific bacteria in armpit samples after isotretinoin or lymecycline treatment. Thus our result is different to that of an older study, which after one month of isotretinoin treatment, detected significant reductions in \( P. \text{acnes} \) and gram negative bacteria counts in armpit samples, but an increase in that of \( S. \text{aureus} \) (Leyden et al. 1986).

6.5 Limitations of the study

In studies I and II the biopsied acne lesions were clinically early-stage lesions, meaning comedones with erythematous flare or small papules. Many previous studies (Holland et al. 2004, Jeremy et al. 2003, Kim et al. 2002, Layton et al. 1998) have used the mapping technique described by Norris and Cunliffe (1988) to select for biopsy lesions within accurately-determined specific age ranges (Norris & Cunliffe 1988). Therefore our results are not directly comparable to those of previous studies. However, the biopsied 6–72 h lesions in the study of Norris and Cunliffe (2008) had a morphology of small papules with a minimal surrounding erythematous flare. Among these, lymphoid cells outnumbered neutrophils (polymorphonuclear cells, PMNs) in the 6 h biopsies, with neutrophils becoming increasingly numerous in the 24–72 h lesions (Norris & Cunliffe 1988). The morphology of biopsied lesions in our study was similar: we performed histological analysis that showed that the biopsied lesions contained neutrophils, but follicular wall rupture was not usually seen. Further, we widely measured mRNA levels (I, II) but the fact that we did not delve into the protein expressions of many inflammatory markers might be considered limitation of this study. However, several cytokines, TLRs and antimicrobial peptides have already been studied in acne and are known to be expressed at the protein level (Harder et al. 2013, Jugeau et al. 2005, Kim et al. 2002, Kistowska et al. 2014, Kistowska et al. 2015, Li et al. 2014, Lumsden et al. 2011).

In study III, contaminants presented a challenge for the analysis. This is a well-known problem in skin microbiome studies, since bacteria exist everywhere and completely aseptic conditions are difficult to attain during sample collection, storage, preparation and processing. The problem is more obvious in so-called lower biomass samples e.g. skin swabs and bronchoalveolar lavage (BAL) fluids where fewer bacteria are present, compared with high biomass samples e.g. stool (Aho et al. 2015). Furthermore, skin samples from low-diversity and high-biomass sebaceous sites can offer greater sequence yields than those from dry or moist areas (Oh et al. 2014). This is probably the reason why the contaminant-problem was
more obvious in the samples from isotretinoin-treated acne patients, whose skin was drier because of treatment. Moreover, it has to be remembered that the 16S rRNA gene amplicon sequencing approach does not differentiate living cells from dead ones, and as mentioned earlier, it does not measure the absolute number of bacteria, only relative abundances.

Finally, a greater number of samples for analysis, in particular in studies II and III may have allowed us to confirm the results from serum samples and the effects of systemic treatments.

6.6 Future perspectives

There are several aspects to the multifactorial pathogenesis of acne that need to be clarified in future. Firstly, it would be interesting to know which cells contribute to the secretion of the cytokines IL-1α, IL-1β, IL-17 and IL-22 in different types of acne lesions. Another interesting question is whether the severity of disease correlates with the serum levels of certain cytokines, such as IL-17 and IL-1β. It has been shown that in patients with severe acne, requiring isotretinoin treatment, serum levels of IL-1 did not differ from those of healthy volunteers (Dispenza et al. 2012), but the situation could be different in other forms of acne, such as acne fulminans.

The pathogenesis of acne fulminans is unknown. It is a rare, severe ulcerative form of acne with an acute onset and systemic symptoms, such as fever, chills and musculoskeletal pain. Some patients also develop aseptic bone osteolysis and/or myositis (Zaba et al. 2011). It is tempting to speculate that in severe nodulocystic acne or acne fulminans there is powerful activation of inflammasome by P. acnes in genetically predisposed patients. Acne fulminans primarily affects 13–16 year-old males (Zaba et al. 2011), an age when levels of P. acnes and androgens increase (Leyden et al. 1975b). It is likely that IL-1β contributes to the osteolytic lesions seen in acne fulminans, because IL-1β can induce the differentiation of bone-resorbing osteoclasts (Schett et al. 2016). At the systemic level the main action of IL-1β is the induction of the fever response by the hypothalamus, so IL-1β may cause the fever present in acne fulminans (Sims & Smith 2010). Moreover, in the ulcerative/necrotic skin lesions typical of acne fulminans large amounts of IL-1α are probably liberated from dying cells. It would be interesting to investigate the serum cytokine levels of IL-1α, IL-1β and IL-17A and other proinflammatory cytokines in samples from acne fulminans patients and compare them to those found in samples from patients suffering cystic acne or milder forms of acne.
Because of the skin ulceration seen in acne fulminans there may also be differences between the bacterial microbiota of acne vulgaris and acne fulminans patients.

At the moment the function of Tregs in acne is largely unknown. Tregs are characterized by expression of transcription factor Foxp3, and they comprise a dynamic and heterogeneous population of cells, which controls immune responses, prevents autoimmunity and participates in the resolution of inflammation. Tregs exert their suppressive functions in several ways e.g. by releasing inhibitory cytokines (IL-10, TGF-β, IL-35) or immunosuppressive metabolites such as adenosine, or inducing apoptosis of target effector T cells via perforin or granzymes (Zhou et al. 2015). To the best of our knowledge, our study was the first to show the presence of FoxP3+ cells in acne lesions (I). Previously Dispenza and coworkers (2012) have shown that peripheral proportions of CD4+, CD25+ and FoxP3+ Tregs did not differ between acne patients and normal volunteers. Furthermore, they found that isotretinoin treatment had no influence on peripheral Tregs (Dispenza et al. 2012). Interestingly, we found decreased expression of TGF-β and FoxP3 in acne patients’ non-lesional skin (II). It is possible that in mild acne Tregs and macrophages works properly and demarcate acne lesions, but in severe forms there might be aberrant function of these cells. Interestingly, dysregulated TGF-β mediated signaling has been linked to the genetic susceptibility to severe acne (Navarini et al. 2014). TGF-β is needed for the induction of induced Tregs (iTregs) which develop from naïve T cells in peripheral tissues (Schwarz 2012). However, Tregs are very heterogeneous cell group and, like other T cells, have demonstrated plasticity that may complicate research.

The roles of macrophages and different DCs in acne could also be investigated in more detail. Here we reported the presence of CD83+ cells in inflammatory infiltrates of acne. CD83 is stably expressed on activated, mature DCs and studies have indicated roles of CD83 in the modulation of antigen presentation and CD4+ T cell generation (Cao et al. 2005). In early-stage acne lesions an increased number of CD1a+ cells, supposed to be Langerhans cells, has been detected mainly within the epidermis and follicle wall, but some are also present in the periductal infiltrate with isolated cells scattered through the papillary dermis (Holland et al. 2004). A recent interesting study showed that the natural ligands of CD1a – FFAs, squalene and wax esters – activated autoreactive T cells. This discovery of the antigenic properties of skin oils led the authors to speculate that alterations in lipid content might influence diseases like atopic dermatitis and psoriasis (de Jong et al. 2014). The altered amount and quality of sebum in acne is well known and Zouboulis et al (2013) among others have suggested that alterations in sebum composition may
initiate acne lesions (Zouboulis et al. 2013), but this view has not been validated. Actually human CD4+ T cells that produce IL-22 recognize CD1a-lipid complexes on Langerhans cells (Colonna 2010). We found increased IL-22 mRNA expression in acne lesions, whereas another group did not (Kistowska et al. 2015). The production of IL-22 by Th22 or Th17 cells mediates multiple effects on keratinocytes including hyperproliferation, differentiation, migration, and proinflammatory cytokine and AMP production (Sabat & Wolk 2011). In future, experiments designed to determine whether there are increased number of CD1a activated autoreactive T cells or pDCs in acne could help to clarify if there is any autoimmunity in acne.

Isotretinoin has been the most potent medication for acne over 30 years. However, it does not rapidly ameliorate the skin symptoms that often lead to scarring. This may be because, as revealed in our study, isotretinoin does not directly affect adaptive immune responses in acne patients’ skin (II). It is essential for acne medication to reduce sebum production and *P. acnes* simultaneously, thus it would be ideal to combine isotretinoin with a medication that prevents the adaptive immune responses. Indeed, immunosuppressive corticosteroids are indicated in severe cystic or fulminant acne to control the skin and systemic symptoms before starting isotretinoin therapy. In a recent Phase 2 study, administration of a human monoclonal IL-1α antibody to patients with moderate-to-severe acne vulgaris resulted in rapid improvement of skin lesions (Carrasco et al. 2015). There are also reports concerning beneficial effects of TNF-α blockade in severe acne (Campione et al. 2006, Colsman & Sticherling 2008, Rispo et al. 2016), although TNF-α blockade is also associated with acneiform eruptions and even cystic acne (Kashat et al. 2014, Steels et al. 2009). Further studies are also needed to determine whether IL-17 antibodies are effective in the treatment of severe forms of acne and whether it would be beneficial to combine low doses of isotretinoin with biologicals.

Furthermore, it would be interesting to study the long-term effects of systemic treatments on acne patients’ skin microbiota using 16S rRNA gene amplicon sequencing. Antibiotics act on the gut microbiota by decreasing its diversity and modifying its composition in a way that can last for months or even years (Becattini et al. 2016). Previous studies using culture-based methods showed that bacterial populations were not significantly altered in patients whose acne responded to a 3-month period of tetracycline treatment (Cove et al. 1980, Cunliffe et al. 1973). Oprica and co-workers (2007) also showed that initially decreased levels of *P. acnes* started to increase after a 3–4-month course of tetracycline treatment, and
returned to initial levels within two weeks of cessation of the antibiotic treatment (Oprica et al. 2007). It would be interesting to discover why the effect of tetracycline to the levels of *P. acnes* on sebaceous skin area is so temporary.

Finally, future research should focus on determining the differences between the microbial functions in healthy and acne skin. The use of metatranscriptomics and metaproteomics could provide more comprehensive functional information, and further define the role of *P. acnes* and other microbes in acne.
7 Conclusions

The work presented in this thesis represents new findings about inflammatory mechanisms in the skin of acne vulgaris patients, particularly regarding the activation of the IL-17/Th17 axis, which may together with IL-1, be an essential component in the immunopathogenesis of acne (I). It is probable that the magnitude of innate and adaptive immune responses define the severity of acne. Furthermore, our new findings obtained by analysing skin samples from isotretinoin-treated acne patients show that isotretinoin does not directly affect adaptive immune responses. The data also support the view that more effective treatments which influence adaptive immunity e.g. biologicals may be useful in combination with isotretinoin, in particular in severe acne (II). Moreover, our study is the first to investigate the effect of isotretinoin and lymecycline treatments on acne patients’ skin microbiota using 16S rRNA gene amplicon sequencing based methods (III). Based on our results, we draw the following conclusions:

1. The IL-17/Th17- axis is activated in clinically early-stage acne lesions. The inflammatory infiltrates contain the CD4+ cells that are Th1, Th17 and Tregs, and also CD68+ macrophages, mature CD83+ DCs cells and CD8+ T cells.
2. The expression levels of TGF-β and FOXP3 mRNA are lower in acne patients’ uninvolved skin than in healthy controls’ normal skin.
3. Several innate immune signals such as TLR2 and TLR4, proinflammatory cytokines and AMPs are activated simultaneously with adaptive immune responses.
4. Isotretinoin treatment does not directly affect either innate or adaptive immune responses in newly formed acne lesions except for macrophages, which are abundant in acne patients’ skin. This implies that once a single acne lesion is formed, isotretinoin does not effectively stop the inflammation.
5. Isotretinoin decreases the expression of TLR2, but not TLR4, seen in acne patients’ nonlesional skin.
6. IL-1β is present in uninvolved skin of acne patients and its presence is reduced by isotretinoin treatment.
7. Isotretinoin restores the low levels of S100A7 in uninvolved skin of acne patients to the levels of normal skin controls.
8. The skin microbiota does not differ between acne patients and healthy skin controls and the abundance of *P. acnes* correlates with greater acne scores.
9. Isotretinoin and lymecycline both diminish the relative abundance of \( P.\ acnes \) in acne areas and the decreased dominance of \( P.\ acnes \) increases the diversity of the microbiota.
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Original publications


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Original publications are not included in the electronic version of the dissertation.
1368. Kröökkki, Olga (2016) Multiple sclerosis in Northern Finland: epidemiological characteristics and comorbidities
1369. Mosorin, Matti-Aleksi (2016) Prognostic impact of preoperative and postoperative critical conditions on the outcome of coronary artery bypass surgery
1372. Helikala, Anne (2016) Ketoacidosis at diagnosis of type 1 diabetes in children under 15 years of age
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