THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Role of PPARγ in human sebaceous gland biology and pathology

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I. ABBREVIATIONS

AA: arachidonic acid (eicosatetraenoic acid, 20:4)

AC: adipocyte

ADRP: adipose differentiation related protein

AF-1:amino terminal activation function domain

AR: androgen receptor

AS: acne involved skin

CAH: congenital adrenal hyperplasia syndrome

CE: cholesterol esters

C/EBP:CAATT enhancer binding protein

CH : cholesterol

CER: ceramides

COX: cyclooxygenase

DBD: DNA binding domain

DG: diglycerides

DHT: dihidrotestosterone

ESI-MS/MS: direct flow injection electrospray ionization tandem mass spectrometry

FFA: free fatty acids

FGFR: Fibroblast growth factor

GAGS: Global Acne Grading System Score

GLP: glycerophospholipids

HAIR-AN: hyperandrogenism-insulin resistance acanthosis nigricans

HE: hematoxillyn-eosin stain

HexCer: hexosylceramides

HPLC-TOF-MS: high pressure liquid chromatography time of flight mass spectrometry

IF: immunofluorescence

IHC: immunohistochemistry

ISTD: internal standard

IGFR-1: insulin like growth factor receptor

KLF: Krüppel like factor

LBD: ligand binding domain

LCM: laser capture microdissection

LPC: lisophosphatidylcholine

LTB4: leukotriene B4

MC5R: Melanocortin 5 receptor

MMP: matrix metalloproteinases

NAS: non-acne involved skin

P. acnes: Propionibacterum acnes

PAPA: pyogenic arthritis-pyoderma gangrenosum-acne

PC: phosphatidylcholine

PCO: polycystic ovary syndrome

PE: phosphatidylethanolamine

PI: phosphatidylinositol

PG: prostaglandin

PGAR: PPARγ angioprotein related protein

PPAR: peroxisome proliferator-activated receptor

PS : phosphatidylserine

PG: phosphatidylglycerol

Plasm: PE-based plasmalogens

qPCR: quantitative polymerase chain reaction

RFU: relative fluorescence units

RT-qPCR: Real Time –quantitative PCR

RSG: rosiglitazone

RXR: retinoid X receptor

SAHA: seborrhoea-acne-hirsutism-androgenetic alopecia syndrome

SAPHO: synovitis-acne-pustulosis-hyperostosis-osteitis

SC: sebocyte

SG: sebaceous gland

SM: sphingomyelin

SREBP: sterol response element binding protein

SSD: signal sensing domain

SZ95: human immortalized sebocyte cell line

TF: transcription factor

TG: triglycerides

TLR:Toll like receptor

TZD:thiazolidione

WE: wax esters

5-KETE : 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid

15-KETE: 15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid

12-KETE: 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid

II. INTRODUCTION

Human skin is our largest organ, approximately 2 m^2 large. But, it is relatively thin, only 0,1 - 1mm. Still, our skin has a unique role in defending us and communicating with our environment. In order to serve these roles, all structures, cells that are part of this organ must be precisely coordinated and regulated. Skin has two layers, epidermis and dermis. The upper part of skin is epidermis, where keratinocytes (KC), immune cells, macrophages, the Langerhans cells, and also small protein and lipid molecules build up the skin barrier. On the surface, and between KCs of epidermis, a lipid layer protects the coherence of KCs. Also, on the surface of epidermis a complex microbial ecosystem in density of 1 million/cm^2 appears, which also takes part in homeostasis of our skin. Many dermal structures support epidermal cells. Vessels of the dermis transport nutrition, oxygen, immune cells, cytokines, hormones, chemokines, and also lipoprotein complexes. Neurons, and neuronal receptors are important in deep and surface sensation, and regulation of other dermal structures, like the appendages. Skin appendages, like apocrine sweat glands or hair follicles are important in thermoregulation, epidermal defense and ion balance. Furthermore, sebaceous glands (SG), had been termed as a living fossil for decades, was re-discovered 20 years ago, and found to be major center for lipid production for the epidermal surface. This SG is a tiny, less than a half millimeter sized gland, consists of thousands of lipid containing sebocytes (SC). SCs produce 150-300µg/cm^2 sebum. SG plays role in barrier function and

communication with our environment. SGs also produce and store specific hormones, cytokines and regulate immune cells. This way, SG plays a major role in homeostasis of whole skin. They are most similar to adipocytes (AC), but unique organs with pivotal position in the skin. However, to better understand the regulatory mechanisms, which control these unique cells, the best is to learn about AC, and adipogenesis.

III. ADIPOGENESIS

1. Mechanism of adipogenesis

Former studies showed, that the process of adipogenesis in ACs and in SCs have many common mechanisms (Rosen, Eguchi, & Xu, 2009). In adipogenesis or in the formation of new fat cells key adipogenic transcription factors play important roles (Farmer, 2006). Key signaling pathways (Figure 1.) regulate AC differentiation: pro-adipogenic extracellular signals like insulin and FGF, and anti-adipogenic ones, like the Wnt and hedgehog pathways. Other key molecules, PPARγ, C/EBPα, KLFs, and other pro- and anti-adipogenic transcription factors (discussed below) are required for initiating and regulating the transcriptional cascade that regulates the development of new fat cells, the expression of key metabolic enzymes, signaling components, and adipocytokines in the mature fat cell (Rosen & MacDougald, 2006).

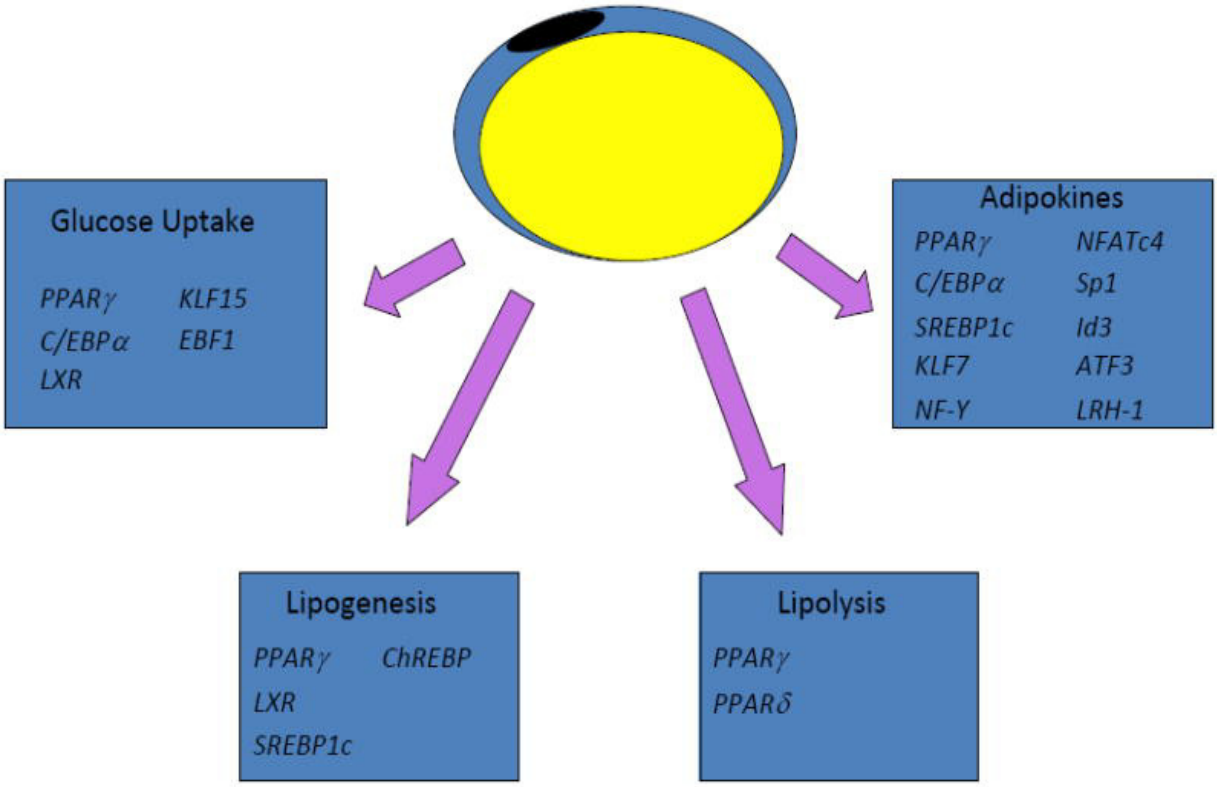


Figure 1. ACs and its regulators. AC perform a variety of functions controlled by distinct yet overlapping sets of factors. Adipokine secretion by fat cells is under the control of a large number of transcriptional pathways (Rosen et al., 2009).

2. Transcription and transcription factors in adipogenesis

The expression of human genes is regulated by several regulator proteins. Starting with tanscription, the reading of DNA, and transcribing to RNA. The key enzyme in this process is the RNA polymerase II is regulated by multiple, precisely regulated molecules, the transcription factors (TF).

2.1 Structure of transcription factors

Structure of transcription factors contain one or more DNA-binding domains (DBDs), which attach to specific sequences of DNA adjacent to the genes that they regulate. TF also has a trans-activating domain , which contains binding sites for other proteins such as transcription coregulators, like p53, NFKB, etc. The binding sites of trans-activating domain are frequently referred to as activation functions (AFs). TF might have an optional signal sensing domain (SSD) (e.g., a ligand binding domain (LBD)), which senses external signals and, in response, transmits these signals to the rest of the transcription complex, resulting in up- or down-regulation of gene expression. Also, the DBD and signal-sensing domains may reside on separate proteins that associate within the transcription complex to regulate gene expression. As these molecules are responsible for regulation, if these molecules are damaged, ie. mutated, the process of gene expression might be defected, disregulated, leading to specific defects of the cell, for example mutation of HOX family might result in cancer (Shah & Sukumar, 2010).

2.2 Classification of transcription factors

TF's can be classified based on their mechanistical regulatory or functional characteristics. Mechanistic classification of TF's include general and upstream/specific TF's groups. General TF's interact with the core promoter region surrounding the transcription start site(s), these TFs are the same

structures in all cell types. Upstream/specific TF's vary depending on recognition sequences they bind to, and they are cell-type specific.

Functional classification consists two groups, focusing on active period of the TF's during cell life time. A constitutively active group (for example: general transcription factors, Sp1, NF1, CCAAT) and conditionally active group. TFs in conditionally active group can be active during specific developmental period of the cell. For example GATA transcription factors (TF which are able to bind DNA sequence GATA) and Hox gene product TFs (a subset of homeotic genes, which control the body plan of an embryo along the cranio-caudal axis). Also, conditionally active TF's can be activated by a signal molecule. These signals molecules might be extra- or intracellular, or cell membrane receptor dependent. These latters are called nuclear factors. Nuclear factors can be resident nuclear factors, which localized in the nucleus, like cAMP response element-binding protein(CREB), activator protein-1(AP-1). Nuclear factors can be latent cytoplasmic factors, which localized in cytoplasm, and upon ligand activation translocate to the nucleus signal transducer and activator of transcription proteins (STAT) or nuclear factor kappa-light-chain-enhancer of activated B cells protein (NF-κB).

2.3 Key transcription factors in adipogenesis

2.3.1 CCAAT-enhancer-binding proteins (C/EBPs)

C/EBPs are TFs which contain highly conserved, basic-leucine zipper domain at the C-terminus that is involved in dimerization and DNA binding. Multiple members of the receptor family are involved in adipogenesis. The pro adipogenic factor C/EBPα and PPARγ have an important synergistic relationship. However, PPARγ can promote differentiation in the absence of C/EBPα (Dipak & Pelagia, 2002).

2.3.2 Krüppel-like factors (KLFs)

The Krüppel-like factors (KLFs) are a large family of Cys2/His2 zinc finger DNA-binding proteins related to the Drosophila melanogaster segmentation gene product, Krüppel. KLFs have been shown to play an active role in adipogenesis, KLF5 activates PPARγ coding DNA promoter region, and induces PPARγ expression. (Banerjee et al., 2003; Birsoy, Chen & Friedman, 2008; Kawamura et al., 2006; Oishi et al., 2005; Sue et al., 2008; Li et al., 2005; Mori et al., 2005).

2.3.3 Early B cell factors (EBFs)

Early B cell factors (EBFs) are atypical helix-loop-helix factors important for the control of B lymphocyte specific genes and for the transcriptional regulation of genes in olfactory receptor neurons. Knockdown experiments showed that EBF1 and EBF2 are required for adipogenesis. C/EBPα, SREBP-1 and PPARγ have been showed to be indirect EBF targets (Akerblad et al., 2002).

2.3.4 GATA factors, Krox20, STAT5, CREB, SREBP-1

GATA transcriptional factors play important roles in a variety of developmental processes. These effects are mediated through the direct suppression of PPARγ expression (Farmer, 2006).

Investigators have identified Krox20 as a factor that acts early in the adipogenic program and appears to contribute to induction of C/EBPα expression. It is likely that additional factors of parallel pathways are induced early in the adipogenic program and converge on PPARα at a stage downstream of C/EBPα and C/EBPα, such as the helix-loop-helix (HLH) transcription factor sterol response element binding protein (SREBP1c/ADD-1). A potential role for SREBP1c in regulating adipogenesis derives from studies showing that its expression is significantly enhanced in 3T3-L1 adipocytes in response to insulin (Kim et al., 1998). Additional studies suggest that SREBP1c contributes to the production of PPARα ligands, thereby facilitating the action of PPARα (Kim et al., 1998).

IV. NUCLEAR HORMONE RECEPTOR SUPERFAMILY

1. Grouping of nuclear hormone receptors

As described above, NRs are transcriptional factors located in either residently in the nucleus or only transiently, upon a ligand activation, when the ligand-receptor complex translocates to the nucleus. In the nucleus, NRs attach to their target genes on the hormone response element (HRE) sequence in the DNA

and form monomer, homodimer or heterodimer (Figure 2.). 49 various NR's are identified to date. The first molecules were described 30 years ago. There are several classifications exist, the first was based on the receptors ligand binding capacity. There were 'classical' nuclear receptors, with known ligands, and 'orphan' receptors with not known ligands. Receptors form heterodimer or homodimers. This gives a possibility that multiple ligands might activate the same receptor complex synchronously. Novel classification is based on a phylogenetic location of the molecule on the phylogenic tree. Although it is more accurate, the former names of molecules are still more widely used, ie. PPARα, PPARβ/δ and PPARγ were renamed as NR1C1, NR1C2 and NR1C3.

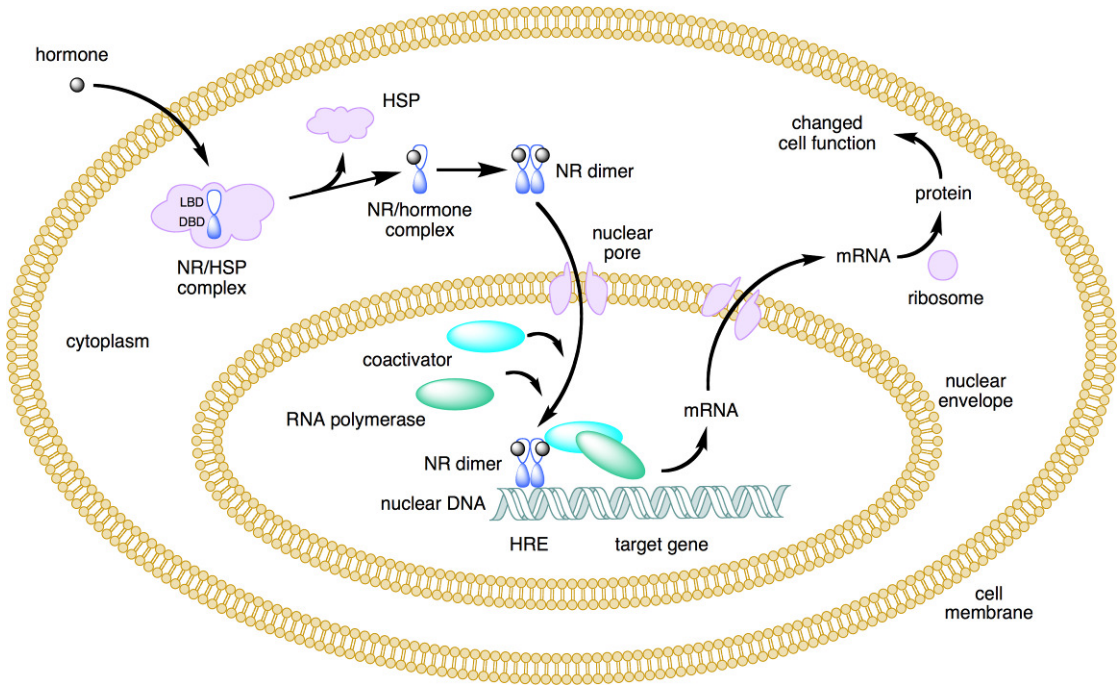


Figure 2. Nuclear receptor: mode of activation. Source:en.wikipaedia.org

Classification of nuclear receptors depends on ligand binding and dimerization activity. Members of each group are listed in table 1.

Class of nuclear receptor	Members (for example)
steroid receptors (SRs) or Type I	estrogen receptor (ER) progesteron receptor (PG) glucocorticoid receptor (GR) androgen receptor (AR) mineralocorticoid receptor (MR)
retinoid X receptors (RXR) or Type II	retinoid X receptor (RXR) thyroid hormone (TR) vitamin D (VDR) all-trans retinoic acid receptors (RARs) **peroxisome proliferation activated receptors (PPARs)**
dimeric orphan receptors	estrogen related receptors (ERRs) testis receptors (TR2 and TR4) chicken ovalbumin promoter transcription factor (COUP-TF)
monomeric orphan receptors	nerve growth factor-induced-B (NGFI-B)

Table 1. Classification of nuclear receptors

Structure of NRs (except a few, ie.Dax-1, Shp-1) is common in all vertebrates (Figure 3.): the receptor contains an amino-terminal activation function domain (AF-1), a DNA- binding domain (DBD), a hinge region, and a carboxy-terminal ligand binding domain (LBD). LBD binds ligands, contain an activation function-2 (AF-2) domain, which conformation is changing depending on the bound ligand (Thompson & Kumar, 2003).

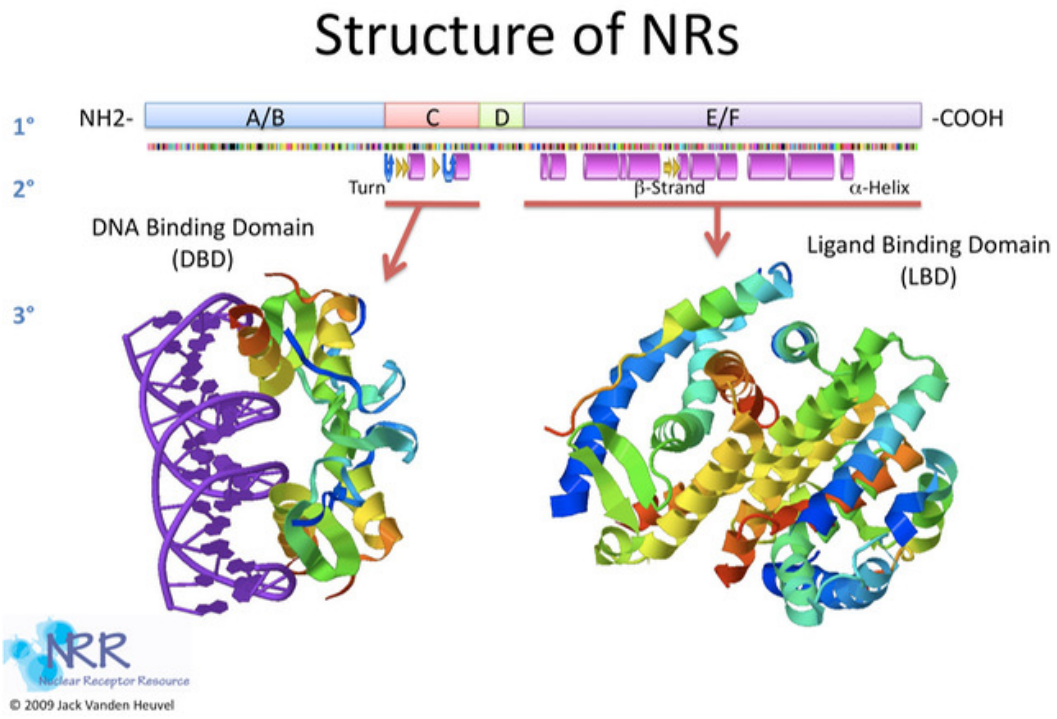


Figure 3. Structural organization of nuclear receptors. Source: http://nrresource.org

Then, after a ligand binds to the receptor, the receptor attaches to the DNA with its DBD, recognizes promoter region of various target genes (Khorasanizadeh & Rastinejad, 2001).

The function of NRs' is the regulation of major cellular signalling pathways, and the regulation differentiation processes.

2. Peroxisome proliferator-activated receptors (PPARs)

PPARs were originally described as target of xenobiotic compounds that triggered proliferation of peroxisomes in rodent liver cells (Dreyer et al., 1992). Later, 3 isoforms were recognized, PPARα, PPARβ/δ, PPARγ. Like many NRs, endogenous ligands of PPARs were not known (orphan receptors), and later it

was found that several ligands could bind to the receptor, then PPARs became 'adopted'. Like other NRs, the structure of PPARs consist of weak ligand binding potential N-terminal AF-1 domain, DBD (two zinc-finger domain), hinge region and an LBD. The structure of LBD is highly conserved, and its transcriptional activity is ligand-dependent. The PPARs act as members of transcriptional multiprotein complex, they can dimerize, and the LBD, DBD domains can act as co-repressors and co-activators. Stucture of the three subtypes are very similar, but they regulate different processes in the cells. Ligands for PPARs can be exogenous, xenobiotic compounds and endogeneous ligands, molecules which are produced by the human organ, summarized in Table 2. (Vamecq & Latruffe, 1999; Ramot et al., 2015).

PPAR subtypes	exogenous ligands	endogenous/natural ligands
PPARα	WY 14.643, gemfibrozil, clofibrate, fenofibrate	Unsaturated fatty acids, LTB4,8-HETE, arachidonic acid, palmitoylethanolamide
PPAR β/δ	GW501516	Unsaturated fatty acids, carbaprostacyclin, Prostaglandin A1 (PGA1), PGD2 and PGD1, 15-HETE and the toxic lipid 4-hydroxynonenol (4-HNE)
PPAR γ	pioglitazone, rosiglitazone,troglitazone, ciglitazone, S26948, Farglitazone	Unsaturated fatty acids, 15-HETE, 9- and 13-HETE, 15-deoxy-12,14-PGJ2

Table 2. Ligands for PPARs.

Drug induced PPARγ activation result in adipocyte differentiation, and foam cell formation, also has an inhibitory effect on tumor cell growth. Several non-

steoid antiinflammatory drugs also bind to PPARα and γ (indomethacin, fonoprofen, flufenamic acid, ibuprofen).

2.1 PPARs in various cell types

Cardiac muscle cells express PPARα, and also all cells which have gluconeogenesis: liver, intestine, renal cortex. PPARγ expression is predominantly in white adipose cells and immune cells, macrophages and dendritic cells (Szatmari et al., 2004; Szeles et al., 2007). Interestingly, there is only little difference in processes regulated by PPARγ between rodents and humans, however, PPARα has much. Lipid metabolism (PPARγ,/α), hepatocarcinogenesis (PPARα), inflammatory lipid-mediator metabolism (PPARα/ γ), adipocyte differentiation (PPARγ) are processes thes molecules regulate, as hormone –dependent receptors. Peroxisome proliferator-activated receptors (PPARs) also play roles in sebaceous lipogenesis, and differentiation (Alestas et al., 2006; Trivedi et al., 2006).

2.2 Peroxisome proliferator-activated receptor γ (PPARγ)

The nuclear hormone receptor PPARγ (NR1C3) was initially characterized as a master regulator of adipogenesis (Tontonoz et al., 1995), and its firstly identified target gene was FABP4/aP2 gene (Dreyer et al., 1992), later other target genes were found (Figure 4). However, later several studies revealed, that

PPARγ is present and active in many other cell types, for example immune cells, skeletal muscle cells, gastrointestinal epithel cells.

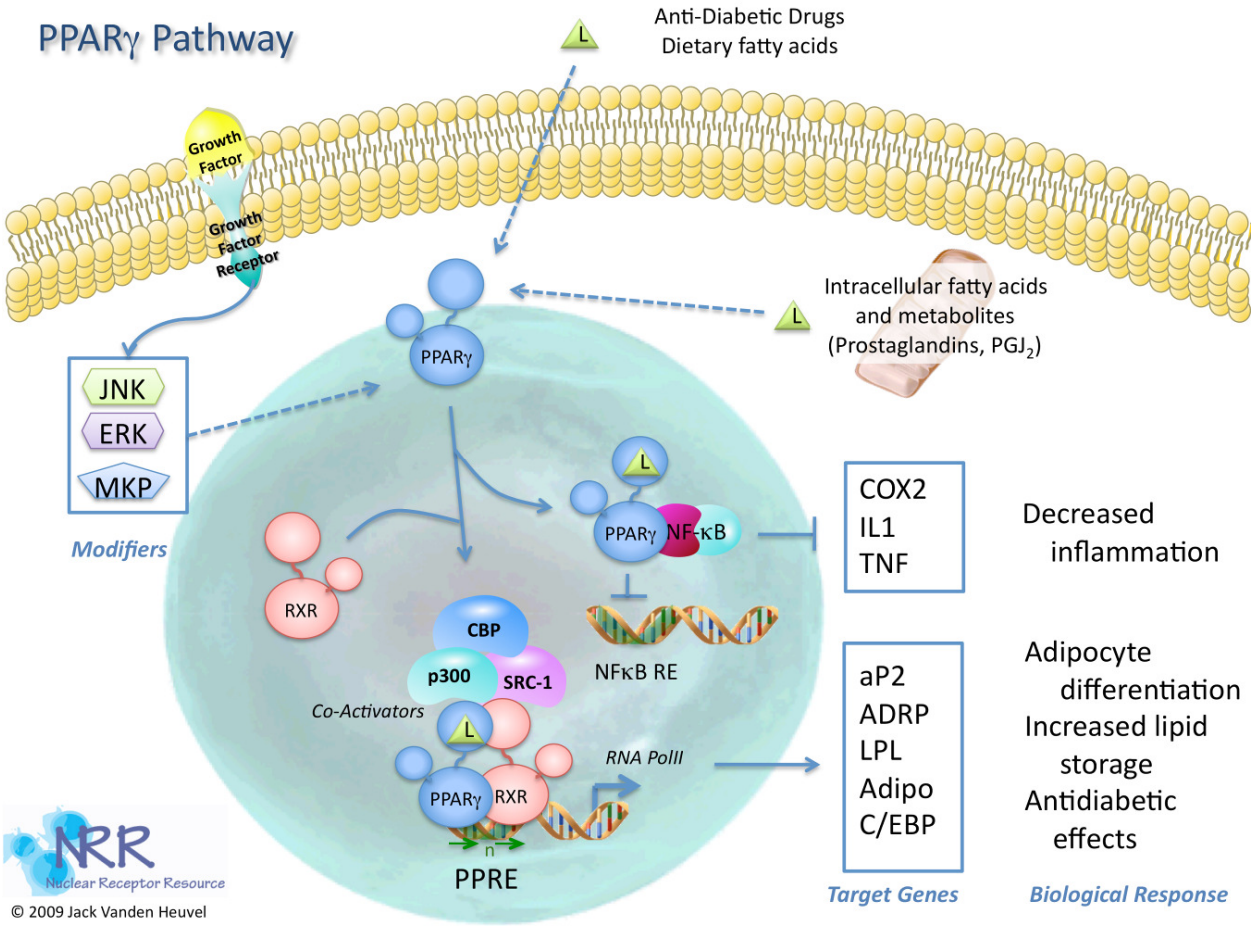


Figure 4. PPARγ regulated cellular pathways. Source: http://nrresource.org

Also, PPARγ was described as a regulator molecule apart from lipogenesis in number of cellular processes, like inflammation, atherosclerosis, bone morphogenesis and cancer. Also, ligands, which regulate the molecule were developed. Among them thiazolidione (TZD) class of drugs (trogitazone, rosiglitazone, etc.), which was applied for diabetic patients to induce insulin sensitivity on peripheral cells. However, modest ligand binding potency, and

severe side-effects (fluid retention, edema and congestive heart failure) were reported in type 2 diabetes, led to development of tyrosine based ligands.

Virtually all known activators and inhibitors of adipogenesis act at least in part by regulating the expression or activity of PPARγ, and cells that lack PPARγ cannot be converted into adipocytes, not even if other powerfully pro-adipogenic factors are ectopically expressed. PPARγ is the only transcription factor that is absolutely necessary and to be sufficient for adipogenesis (Barak et al., 1999; Cuaranta-Monroy & Nagy, 2015). In cultured cellular models of adipogenesis, PPARγ mRNA is directly induced by factors that act early in the cascade, such as C/EBPβ, C/EBPδ, EBF1, and KLF5. Conversely, early repressors of adipogenesis such as GATA2, KLF2 act in part by reducing PPARγ expression. Other factors, such as SREBP1c may act to increase PPARγ activity.

As functions of PPARγ is concerned, the receptor itself induces genes of terminal differentiation, including lipid handling enzymes and other mediators of adipocyte physiology. Interestingly, PPARγ protein is required for normal placentation; thus PPARγ -/- embryos die relatively early in embryogenesis.

As it is seen, lipid production of adipocytes are complex and regulated via multiplex molecular pathways. Next, we go back to our lipid producing cells in skin, the sebocytes, to study their structure and function, also molecular mechanisms which regulate these cells.

V. ANATOMY AND FUNCTIONS OF SEBACEOUS GLAND IN HUMANS

1. Structure of sebaceous glands and its position in human skin

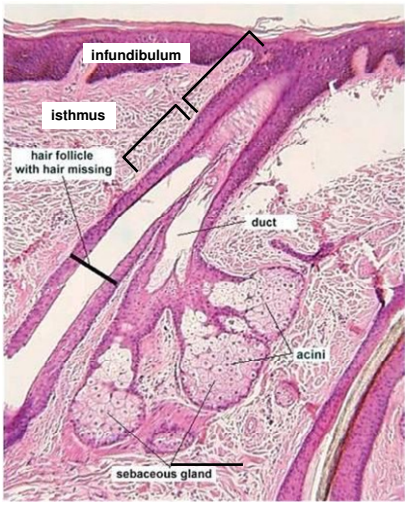


Figure 5. Human skin, sebaceous gland, histopathology.

Sebaceous glands (SG) are holocrine glands, built up of modified keratinocytes. SG has two parts, glandular part (lobule or acinus) and duct. The gland consists of basal, dividing cells and mature, suprabasal and central sebocytes. The mature cells produce sebum continuously, as they synthesize lipids, these cells die, and the whole cells shed to the lumen of duct. Sebocyte turnover time estimated to be 14 days. The sebaceous gland duct is connected to a hair follicle mostly, and form pilosebaceous unit, rarely connected directly to the skin surface. Isthmus is part of hair follicle from the junction of sebaceous gland to the junction of apocrine sweat gland. Hair follicle from junction of sweat gland to epidermis is called infundibulum (Figure 5). Cells of hairshaft, which are in infundibulum shed to epidermis. The ductal cells, located under infundibulum, are called infrainfundibular cells, and shed to sebum. There are special, SG rich

part of human skin: scalp, face, chest, axillas, genital area, upper outer extent of arms. None are found on the palms and soles.

Formerly, it was a concept in dermatology, that sebaceous glands have important function only in animals, but not in human skin, and it was thought that sebaceous glands were living fossils. However, the lack of a good model for the detection of sebaceous gland function detection was the reason for the lack of information about SG biology. In the last 20 years, new models of skin physiology, signaling mechanisms, molecules were developed. In addition, novel research methods, mostly from molecular biology, were applied in basic skin research.

2. Research models for investigating sebaceous glands

Models for sebaceous glands are 1. Human morbidities, questionnaires, non-invasively collected sebum samples and human blood / tissue samples, derived from healthy individuals or patients 2. animal models 3. in vitro cultured sebocytes.

Firstly, as most of the experiments start with pilot studies, or observations, it is worth mentioning, that certain human diseases are also serve as models for sebaceous gland function. For example in polycystic ovary syndrome (PCO) , a morbidity in women, with hirsutism-acne-multiplex cysts in ovaria- amenorrhea, elevated androgen level is measured, which lead to the concept of SG is regulated by androgens. Also in other syndromes, which are associated with acne, like

congenital adrenal hyperplasia (CAH), seborrhoea-acne-hirsutism-androgenetic alopecia syndrome (SAHA), synovitis-acne-pustulosis-hyperostosis-osteitis (SAPHO) and hyperandrogenism-insulin resistance acanthosis nigricans (HAIR-AN), focused studies to the effects of androgenes, and insulin on SG. In addition, several well-established questionnaires are useful to bring us closer to several external influencing factors of SG and its diseases. Human SG lipid production can be quantitatively measured in vivo on humans, by various instruments, types of so called 'Sebumeter'. This method is based on photometry, which measures the quantity of sebum, blocking the skin with a filter paper/plastic strip, and measuring the weigh of sebum on the skin surface. It is a fast, non-invasive, and relatively cheap method; vast number of patients can be involved in the measurements. However, the information through sebometry is limited; it provides data only about the whole surface lipid content and only from a small area of the skin. The quality of sebum is a more difficult to analyze. In a recent study, in acne patients profiles of neutral lipids were acquired with rapid-resolution reversed-phase/ high pressure liquid chromatography- time of flight – mass spectrometry (HPLC-TOF-MS) (Camera et al., 2016).

Human blood/ skin samples, derived from healthy individuals and patients, are also interesting, as sebaceous gland/ sebaceous gland mechanism models. Limitating factor is that, gathering a good number of samples is not easy. In one hand, if the disease is frequent, like acne vulgaris, for the clinical diagnosis no routine biopsy is needed. On the other hand, if it is a rare disease, like SG

carcinoma, the number of patients, therefore, number of samples is limited. Isolating SG from multiple healthy donors, and pooling them, as did group ofm Thiboutot (Thiboutot et al., 2003), gave valuable data, however it is a very difficult and time consuming procedure. Comparing samples from acne involved, non-involved SG and SG healthy individuals, microarray based studies and immunhistochemistry based studies are also available (Trivedi et al., 2006; Alestas et al., 2006). However, these samples are good for descriptive studies, and give information about only one time point of the lifetime of SG. The advantage of these studies is that samples are from humans, in situ, gathered from their natural environment, and work with whole sebaceous glands.

However, to get information about physiology of SG, for functional studies, in vivo experiments are also needed. For that murine models exist. Also, primary animal cells are used, for example hamster preputional sebaceous glands. However, the problem is that SGs are different in human SGs, and also, density of SG in human skin and lipid production is different from animal skin.

Several laboratories attempted to use primary human sebocytes by isolating sebaceous glands, and then using the proliferatory ability of the basal cells of SG (Zouboulis et al., 1991; Xia et al., 1989). However the work needed sophisticated and difficult treatment of cells, and sebocytes proliferated only for maximum 6 passages, thus only a few thousands of cells were growing from each SG. Thus, pooling these cells from multiple donors were the only way to get any statistically relevant experimental results.

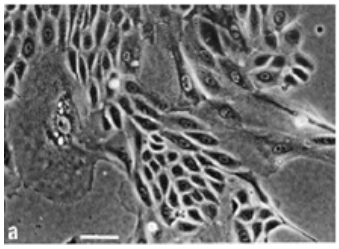


Figure 6. SZ95 immortalized human sebocytes in cell culture dish. Native, polarized microscopy. Scale bar:30um. Zouboulis and Seltmann, 1999

20 years ago, Zouboulis and Seltmann initiated the blooming of sebaceous gland research, when they established the first immortalized human sebocyte cell line in 1995 (Figure 6.). They cultured human facial sebocytes from a 87-year-old woman and transfected them with the simian virus-40 large T antigen, which resulted in the first human immortalized sebocyte cell line (Zouboulis et al., 1999). Since then, two other cell lines, SEB-1 by the group of Thiboutot in USA (Thiboutot et al., 2003) and SEB-E6_E7 by the group of Lo Celso in Japan established immortalized sebocyte cells (Lo Celso et al., 2008). The three cell lines were compared in 2009, (Xia et al., 2009) and summarized that all preserved the characteristics of sebocytes, namely lipid droplet production; however, in expression of proteins some differences were detected, such as the expression of surface proteins.

3. Functions of sebaceous glands

3.1 Lipid production

On SG rich skin areas lipids on the surface are supplied by sebum mainly, on areas that have less SG, epidermal keratinocytes produce the majority of lipids.

Sebum is a pale yellow viscous fluid composed of glycerides and free fatty acids (57%), squalenes (12%) wax and sterol esters (29%), and free sterols (2%) (Burgdorf et al, 2009). These lipids are called major lipids, as vast of sebum consist of these lipids. Depending on race, age of human, the quality and quantity of these lipids varies, but only in slight ranges. Sebum production at birth is relatively high, because of maternal hormones. In childhood it is low, and radically increase in puberty. Hormones, cytostatic drugs, anabolic steroids influence production of these lipids. The main controlling hormones seem to be androgens, as these hormones influence development and lipid production. By the end of puberty the level of androgens decrease, and in line with sebum production, remains low during adult life, and further decreases in old ages. There are also lipids, which are produced in small amounts; however, limited data are available about these ones.

3.2 Immune and communication function

In the past few years, newly detected molecules in sebaceous glands gave new aspects in skin research. Alestas et. al. described that sebocytes produce

cytokines such as IL-6,IL-8, IL-1β, TNFα. They investigated also the leukotriene pathway, which is involved in inflammatory reactions in acne, also prostaglandin (PG) pathway, PGE2, leukotriene B4 (LTB4) might have a role, and also COX enzymes (Alestas et al., 2006) in acne pathomechanism. Toll like receptor (TLR) genes TLR2 and TLR4 are upregulated in kertinocytes of acne lesions (Kim et al., 2002), also, TLR2 is expressed on surface of monocytes around acneic SG (Jugeau et al., 2005). In addition, distinct strains of Propionibacterium acnes (P. acnes) induced selective IL-8 and β-defensin-2 expressions in human keratinocytes through TLRs (Nagy et al., 2006).

Androgen receptor (AR) is also expressed on sebocytes (Zouboulis, 2003). In addition, 5-α-reductase enzyme was found in SG.

In sebocytes, insulin like growth factor receptor, IGFR-1 is expressed. IGF-1 activates IGFR-1, increases AR transcriptional activity, and it has a role in the enhancement of lipid production (Smith et al., 2006). Fibroblast growth factor (FGFR), belongs to androgen dependent tyrosine kinases, expressed in sebocytes as well (epidermal cells, too). Melanocortin 5 receptor (MC5R) was expressed on fully differentiated sebocytes (Zhang et al, 2003). Its ligand is α- MSH . MC1R is also expressed on human sebocytes (Bohm et al., 2006).

NF-κB, and activator protein-1, also matrix metalloproteinases (MMPs) are expressed in sebocytes. MMPs have been shown as molecular mediators, targets of fibroblast growth factor receptor 2 (FGFR2) signaling pathway (Steinberg et al., 2005), and present in acne involved SG. Forkhead Box Transcription Factor

Class O1A gene is nutrient- and growth factor sensing factor, also found on SG, and controls AR activity, and basic cell life processes (Zouboulis et al., 1998).

Sebocytes have several pathological conditions. Some of them are originated from SG; many of them are part of a syndrome, which involve the whole skin or the whole organ. Here I describe the ones, which we investigated in our present research.

VI. SELECTED PATHOLOGICAL CONDITIONS OF SEBACEOUS GLANDS

1. Tumors

Skin adnexal tumors with sebaceous differentiation are uncommon, difficult to classify, and may be controversial. The main controversy concerns the microscopic features, which vary from well to poorly differentiated and sometimes undifferentiated varieties.

1.1 Benign tumors

Benign tumors of the skin are frequent. Mostly clinical diagnosis is enough to identify the structure, biopsy/excision is only necessary when malignancy is suspected.

1.1.1 Sebaceous hyperplasia

Sebaceous hyperplasia is a common skin finding in aging adults, reported to occur in approximately 1% of the healthy US population, begin to appear in

middle age (fifth decade). However, the prevalence of sebaceous hyperplasia has been reported to be as high as 10-16% in patients receiving long-term immunosuppression with cyclosporin A (Boschnakow et al., 2003). The difference in prevalence between men and women is unknown. Sebaceous hyperplasia is very common in newborns. Of 1000 consecutive neonates examined in an Iranian prospective cohort study, 43.7% had sebaceous hyperplasia (Moosavi & Hosseini, 2006).

Etiology: As androgen levels decrease with advancing age, the sebocyte turnover begins to slow down. This decrease in cellular turnover results in crowding of primitive sebocytes within the sebaceous gland, causing a benign enlargement of the sebaceous gland, or sebaceous hyperplasia. This is particularly apparent in the areas where sebaceous glands are concentrated, such as the face. Although the hyperplastic glands are often inflated up to 10 times their normal size, they secrete very little sebum. Histology shows expansion of normal lobular sebaceous gland architecture without thickening of peripheral germinative layer of sebocytes.

Although more commonly found in the older population, premature or familial cases have been reported in which younger individuals are affected with multiple lesions, suggesting a genetic predisposition. In these cases of premature familial sebaceous hyperplasia, extensive sebaceous hyperplasia appears at puberty and tends to progress with age (Dupre et al., 1983; Grimalt et al., 1997; Wang et al., 2011).

1.1.2 Sebaceous adenoma

A rare type of adnexal tumors, presenting as a well circumscribed flesh-coloured or yellow papule or nodule, mainly located on the face, scalp or trunk. Histopathology shows nodular lobulated growth of cells in the deep dermis. The tumour consists of basaloid cells and sebaceous cells with cytoplasmic lipid vacuoles, but the lesion is not as organoid as sebaceous hyperplasia. Often cyst formation is observed. Proportion of sebocytes is variable, but it is minor compared to basaloid component.

1.2 Malignant tumor

Diagnosis of malignant tumor of sebaceous gland is not only clinically but histologically difficult. To detect proliferating cells, detection of excess of active cell cycle markers, like Ki-67, or p63 proteins are used. In addition, sebocyte origin antigens, like androgen receptors (AR) can be used.

1.2.1 Sebaceous carcinoma

This is a rare tumor, presenting most often on the eyelids. The tumor presents on middle aged or elder patients. Women are affected more than men, cc.55-77%. The tumors presenting on skin has two forms: ocular (80%) and extraocular (20%) (face, neck, genital area, trunk) localization. Seldom, it is localized extracutaneously, mainly in parotid gland. The etiology is not known, however a genetic syndrome Torre-Muir syndrome is described with the presence of multiple sebaceous carcinomas, adenomas, basal cell carcinoma with

sebaceous differentiation and malignant visceral tumors. Straightly, a genetic predisposition leads to the presence of the tumor. In this syndrome the mutation of DNA mismatch repair proteins, MLH1 and MSH2 was described (Schwartz & Torre, 1995). The tumor is soft, skin-colored, a few mm sized. Histologically it shows atypical epidermal cells with pagetoid invasion in normal epidermis. Carcinoma cells are polygonal, diverse in morphology and form lobules in the dermis. Central necrosis can be detected. Carcinoma cells express ARs. Ocular type of sebaceous carcinoma is the most aggressive type, with higher metastatic potential (Doxanas & Green, 1984; Pang & Rodriguez-Sains, 1985; Rao et al., 1982).

Every benign or malignant tumors originated from sebaceous glands might associate with other internal tumors in Torre Muir syndrome where intestinal carcinoma or prostate carcinoma or ovarian carcinoma may present with the skin tumors. Thus, when multiplex sebaceous tumors present, a search for internal tumor is important, and follow up of the patients for internal and skin tumors as well. In addition, sebaceous tumors might associate with Lynch syndrome (hereditary nonpolyposus colorectal cancer).

PPARγ has roles in various tumors: prostate, uroepithelial carcinoma, breast cancer, non-small cell lung cancer, and colon cancer (Segawa et al., 2002; Nakashiro et al., 2001; Avis et al., 2001; Lee et al., 2003). However the clear mechanism of PPARγ in tumor genesis is not yet defined (Varga & Nagy, 2008).

2. Inflammation of SG

2.1 Acne vulgaris

Several types of acne are known. Most of them are characterized with inflammation of the pilosebaceous unit. The most common is acne vulgaris, a multifactorial disease, starting in puberty. Acne vulgaris appears as seborrhea, comedones (open or closed), hyperemic papules, pustules or nodes, localized typically on surfaces which are rich in sebaceous glands. Acne vulgaris typically starts at puberty, as an androgen dependent process (Burgdorf et al, 2009).

Epidemiology

Almost all people are affected, early in puberty, most symptoms resolve by early adulthood. More severe forms present in men, but women tend to have acne vulgaris for decades in association with their period, which prove the involvement of hormones in progression of this dermatosis. In developed countries it is more common. Dietary factors, like high fat, high sugar or high milk and dairy product intake are predisposing factors to gain acne (Melnik 2011; Ghodsi et al., 2009). These also support the complexity of regulation of sebaceous glands.

Pathomechanism

Classical steps of acne formation consist of abnormal infundibular hyperkeratinization of sebaceous gland or follicular unit, elevated production of sebum, which stucks under the plug, and then a secondary inflammatory reaction

provoked, also P. acnes might have role in the process as well. In contrast, some authors describe acne formation as a primary inflammatory, autoinflammatory disease, which is triggered by proinflammatory sebum lipid fractions (Zouboulis et al., 2013). The extending knowledge about the molecules play role in the dermatopathy probably will help to understand better the pathomechanism.

Sebum in sebaceous glands consists of trigliceride, cholesterol esthers, diacylglicerides, cholesterol, wax esthers, squalaene (Zouboulis et al., 1999), but sterile sebum contains no free fatty acid (FFA). According to a South Korean study, sebum level change induces inflammatory acne formation on face (Choi et al., 2011). Agents, which inhibit sebum production, improve acne. Others, which promote sebum production, promote acne. Recent study from Italy showed that in sebum of acne patients, the majority of different lipid species were diacylglycerols (DGs), followed by fatty acyls, sterols, and prenols. These data indicated an association between the clinical grading of acne and sebaceous lipid fingerprints and highlighted DGs are more abundant in sebum from adolescents affected with acne (Camera et al., 2016). In addition, P. acnes and Staphylococcus epidermidis are bacteria of normal skin flora, however, if sebum is in excess, specific subtypes of these bacteria might spread and cause inflammation, and take part in acne formation. These bacteria have lipases which cut the sebum to FFA, and the bacteria also attract chemotactic proteins, and activate neutrophil cells.

Although in most cases acne vulgaris is a sole dermatosis, in some patients,

it is part of a complex syndrome. Investigating the hormonal background of these syndromes, like CAH, SAHA syndrome highlight the role of androgen steroids, while polycystic ovary (PCO) and hyperandrogenism-insulin resistance acanthosis nigricans (HAIR-AN) syndromes indicate insulin resistance in acne. Apert syndrome with increased FGFR2 signaling results in follicular hyperkeratinization and sebaceous gland hypertrophy in acne. SAPHO and pyogenic arthritis-pyoderma gangrenosum-acne (PAPA) syndromes raise role of immunological factors in acne.

Pathomechanism of dermatoses involving SG are complex. And to help our patients with these dermatoses is a complex deal, too. To develop better therapies, it is important to get more information about the regulation of sebocytes.

VII. REGULATORY MOLECULES OF SEBACEOUS GLANDS

1. Hormones

Puberty is the main age of acne, when androgens are produced in high level, as above mentioned. In addition, several hormonal regulation defects which result in elevated androgen level, are accompanied by acne vulgaris (Cinar et al., 2014). Also, elevated AR activity present in for example, CAH syndrome, due to shorter CAG or GGN repeats in AR coding gene; also in raised gonadal or adrenal androgen production, or modification of androgen co regulators (Heemers & Tindall, 2007). Interestingly, first months of life some infants have

acne, called ‘acne infantum’ or ‘newborn acne’, which is probably due to high androgen level of the mother. These hormones are primarily testosterones, ovarian and adrenal hormones. How do these act? In men testosterone is inactive, 5-α- reductase activates the hormone to 5-α-dihidrotestosterone (DHT) (Davison & Bell, 2006). Sebaceous glands express androgen receptors and also 5-α-reductase enzyme in the cytoplasm. Testosterone gets to SG cytoplasm, and converts to DHT, which activates the AR. Androgen receptor translocates to nucleus and regulate expression of certain genes. In women, DHT is produced from androstenedione. The level of DHT in both sexes are enough to activate SG for maximal sebum production. Interestingly, production of DHT in acneic skin is 30x higher than in normal skin.

Insulin-like hormones also have role in acne formation, as recent studies highlighted. Dietary factors with high glycaemic index like milk, and frequent sugar, cake and pastry intake were identified in mild and moderate acne compared to people with normal skin (Aksu et al., 2011; Grossi et al., 2014 ; Melnik 2013; Schaller et al., 2016). Presence of acne in endocrinological changes with high serum insulin/ IGF-1 level like PCO and HAIR-AN syndromes also indicate high insulin level in acne. Growth hormone in puberty stimulates the hepatic secretion of IGF-1 protein, which might be another regulatory description of higher acne prevalence in this age group. Group of Thiboutot (Cappel et al., 2005) showed positive correlation with level of insulin like growth factor-1 and number of acne lesions in women and they also showed the possible molecular

mechanism behind the phenomenon (Smith et al., 2006) on SEB-1 human immortalized sebocytes. In addition, studies from cell cultures proved that insulin-like growth factors induce lipid production (Smith et al., 2006). High dose insulin activated IGF-1, which attach and activates SREBP-1 pathway which evolves lipogenesis in sebocytes.

Another growth factor receptor, FGFR belongs to androgen dependent tyrosine kinases, that is expressed in sebocytes, too (epidermal cells, too). FGFR deletion leads to SG atrophy, marking its important role in SG genesis (Grose et al., 2007). Apert syndrome, an acrosyndactyly syndrome, results from missence mutation in FGFR2 coding gene (Ser252Trp or Pro 253Arg). Apert syndrome is often associated with severe acne (Anderson et al., 1998).

Steroids, when administered in systemic and chronic course, also induce acne formation, probably via the above-described SREBP-1 involving pathway. Corticotrophin releasing hormone (CRH) / CRH-receptors are widely distributed in skin, and expressed on sebaceous glands. Excess of CRH is detected in acne involved SGs (Slominski & Mihm, 1996), also shown, that it regulates lipid production by regulation of DHT to testosterone formation. CRH modulate inflammation as a proinflammatory molecule, in a direct paracrine effect on mast cells. In central nervous system, CRH is responsible for production of IL1, IL6, TNF-a. These cytokines also highly expressed in SG with acne vulgaris.

Together with high expression of CRH, CRH-receptor 1 it suggests the role of CRH in acne evolution as well, as a regulatory molecule. This hypothesis was suggested as „the brain of the skin: sebaceous gland" by Zouboulis C.C. (Pappas et al., 2015).

2. Cytokines

Alestas et. al. described that sebocytes produce cytokines: IL-6, IL-8, IL-1β, and TNFα. They also investigated PG pathway (COX enzymes) (Alestas et al., 2006), and PGE2, LTB4 molecules in acne pathomechanism, as in acne high enhancement of LT pathway, IL-6 and IL-8 cytokines was observed. Recently positive association was found between the minor T allele of the IL1A +4845(G>T) SNP and acne, and the severity of inflammatory acne symptoms correlated with the ratio of individuals carrying the homozygote T/T phenotype (Szabo et al., 2010). Proinflammatory cytokines also play important role in acne pathogenesis. Two SNPs of TNF regulatory region have been detected recently, TNFα-857 minor T-allele was found to act as a protective factor in Hungarian study population in acne, but higher occurrence of minor-308 A allele was found in female acne (Szabo et al., 2011). Toll like receptor (TLR) genes TLR2 and TLR4 are upregulated in KCs of acne lesions (Kim et al., 2002). TLR2 is also expressed on monocytes surface around acneic SG (Jugeau et al., 2005). In addition, distinct strains of P. acnes induced selective IL-8 and β-defensin-2 expressions in human keratinocytes through TLRs (Nagy et al., 2006). IL1R and

TLR has great structural homology, which might describe the IL-1α- induced upregulation of TLR – mediated immune responses in acne vulgaris.

3. Transcription factors

Investigations of SG includes research on the transcriptional mechanisms that regulate lipid metabolism and inflammatory responses of sebocytes (Toth*, et al.,* 2011). Identification of specific regulatory mechanisms and key actors in these processes is essential to fully understand the normal physiological function and to clarify the pathophysiological involvement of SGs in dermatoses. A particularly relevant area of research is the regulation of gene expression by transcription factors responding to changes in fatty molecules and in that way linking metabolic processes to gene regulation.

Many of the transcription factors which play role in adipocytes have role in SG cellular processes. Expression of C/EBPβ, and C/EBPα was described in sebocytes in situ and in cultured SZ95 sebocytes as well (Chen et al., 2003). KLF5 mRNA and protein is expressed in sebocytes (Sur et al., 2002). Early B cell factors (EBFs) were not described in sebocytes, however, expression of an indirect target gene of EBFs SREBP-1 and many of steroidogenic enzymes were detected in sebocytes (Thiboutot et al., 2003). A role of Melanocortin receptor 5 (MC5R) was demonstrated in lipid production in animal models (Bohm et al., 2006). Its ligand is α-MSH, which had a modulatory role in SG inflammation. PPARs are also important factors in sebaceous lipogenesis.

4. PPARs in skin and sebocyte biology

PPARs, as nuclear hormone receptors were investigated in SG, and in whole skin. All three subtypes are expressed in follicular units, in keratinocytes and sebocytes. In cultured sebocytes mRNA of PPARδ expressed the greatest amounts, as compared to PPARγ, and PPARα (Alestas et al., 2006). PPAR molecules are involved in the regulation of lipid synthesis in sebocytes. Agonists of PPARs stimulated lipid synthesis: PPAR agonists increased lipid production in SEB-1 sebocytes, also patients treated with fibrates or tiazolidinediones had significant increase in sebum production (Trivedi et al., 2006). Linoleic acid, a ligand of PPARδ, regulated synthesis of dihidrotestosterone (Makrantonaki & Zouboulis, 2007) and stimulated lipid formation in sebocytes and epidermal KC (Rosenfield et al., 1999; Chen et al., 2003).

PPARγ is also important for SG and hair follicle development. Murine model of PPARγ conditional knockout gives a direct evidence for the essential role of PPARγ in SG development. In a murine model of targeted hair follicle stem cell deletion of PPARγ, many of the histological findings including loss of normal SG and gene expression findings was observed (Zouboulis et al., 2014). In lichen planopilaris, a cicatrical alopecia, increase of inflammatory cells, loss of hair, also loss of normal SG presents with lower expression of PPARγ.

PPARs are activated by specific molecules, some of them are known ligands, others are molecules, which induce these ligands. On sebocytes the effect of some of PPARγ ligands were observed. In SEB-1 sebocytes PPARγ ligand

rosiglitazone and PPAR panagonist GW4148 elevated lipid synthesis. In line with that, sebum production in thiazolidinedione treated type II diabetes patients was higher compared to control group (Trivedi et al., 2006). Substance-P upregulated PPARγ protein expression in cultured sebocytes (Lee et al., 2008). In addition, peroxidated squalene induced upregulation of PPARα (Ottaviani et al., 2006). Endocannabinoids via cannabinoid receptor 2 enhanced lipid synthesis and apoptosis of human sebocytes, which upregulated PPARs and PPAR mediated target genes (Dobrosi et al., 2008).

Certain molecules work together with PPARs in SG. PPARs cooperate with LXR (liver X receptor), which was reported to regulate lipid metabolism in SZ95 cells (Jiang et al., 2008). FoxO1 as a cosuppressor, antagonizes PPARγ activity, and inhibits PPARγ expression as well (Fan et al., 2009).

Arachidonic acid (AA) markedly enhances lipid synthesis in SZ95 sebocytes (Wrobel *et al.*, 2003; Alestas *et al.*, 2006; Tóth *et al.*, 2009; Géczy *et al.*, 2012) probably throught PPARγ (Alestas *et al.*, 2006). Furthermore, AA is metabolized via various pathways to a large variety of derivates (Harizi *et al.*, 2008), and selected metabolites act as potent endogenous ligands of PPARs, especially of PPARγ, in fibroblasts and adipocytes (Forman *et al.*, 1995; Kliewer *et al.*, 1997). However, the nature and identity of endogenous ligands that activate PPARγ in the skin are not known. Potential ligands of PPARs include fatty acids, various eicosanoids, and other lipids found in the skin and also in SGs (Trivedi*, et al.*, 2006). Previous studies have shown that AA can activate PPARγ; however, it is

highly likely that not AA, but some of its metabolites are the main bioactive mediators of PPARγ activation (Alestas et al., 2006; Trivedi et al., 2006). Endogenous ligands for the PPARs include free fatty acids and eicosanoids. PPARγ is activated by PGJ_2 (a prostaglandin) and certain members of the 5-HETE family of arachidonic acid metabolites including 5-oxo-15(S)-HETE and 5-oxo-ETE in non-sebocyte, MDA-MB-231, MCF7, and SKOV3 cancer cells lines (O'Flaherty et al., 2005).

Although, it was shown that PPARγ was expressed in sebocytes, little is known about its expression profile and activity during sebocyte differentiation, because not known the expression profile of target genes of PPARγ in sebocytes in vitro and in situ SG. In addition, there is lack of data of PPARγ expression pattern in normal SG and in various sebaceous gland pathologic conditions.

Little is known about the mechanism/molecules, which play role in the change of lipid production in SG - normal and pathologic ones. In addition, there is a lack of information about which lipid fractions are regulated by PPARγ in SG.

VIII. HYPOTHESIS AND RESEARCH QUESTIONS

1. Hypotheses

- PPARγ is expressed and active in sebocytes,

- PPARγ has endogenous activator ligands in sebocytes, and these are specific AA derivates,

- PPARγ has a role in sebocyte differentiation and lipid production, and has an effect on the regulation of various lipid fraction production,

- PPARγ is expressed in pathological sebaceous glands, but its expression pattern differs from healthy SG,

- PPARγ target genes are expressed in pathological SG, and their expression varies from normal SG expression pattern,

- In diseases of sebaceous glands, endogenous ligand activated PPARγ activation plays a role.

2. Research questions

- What is the expression pattern of PPARγ in normal and pathologic sebaceous glands?

- Which are the target genes of PPARγ in sebaceous glands?

- What is the nature and identity of endogenous ligands that activate PPARγ, are these among AA derivates?

- What can be the role of PPARγ in differentiation and lipid metabolism?

- Which particular lipids are affected by PPARγ activation in sebocytes?

- What is the expression pattern of PPARγ in SG of acne vulgaris patients and the pattern of potential endogenous PPARγ ligands?

IX. MATERIALS AND METHODS

1. Cell culturing

Human immortalized sebocytes SZ95 (Zouboulis et al., 1999) were cultured in Sebomed basal medium (Biochrom, Berlin, Germany) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 5 ng/ml epidermal growth factor (Sigma, St. Loius, MO, USA), 50 U/ml penicillin and 50 U/ml streptomycin (both from Biogal, Debrecen, Hungary) and 1 mM $CaCl_2$. The medium was changed every other day and cells were sub-cultured at 80-90% confluence.

2. Detection of viable cells

For quantitative measurement of viability, MTT (3- (4,5- dimethylthiazol) 2,5-diphenyl tetrazolium bromide) assay was carried out as described previously. Briefly, SZ95 sebocytes (10,000 cells/well) were cultured in 96-well plates in quadruplicates and were treated with compounds for 24 hrs. Subsequently, supernatants were discarded, cells were incubated with 0,5 mg/ml MTT, and the concentration of formazan crystals that is proportional with viable cell number, was measured colorimetrically (Dobrosi et al.2008, Bodó et al. 2005).

3. Determination of intracellular lipids

For the detection of sebaceous neutral lipids, SZ95 sebocytes were cultured on glass cover slips and treated with various compounds for 24 hrs. Cells were then washed in phosphate-buffered saline (PBS), and stained with 1 μg/ml Nile

red (Sigma-Aldrich) solution in PBS for 20 minutes at 37 ^{o}C and the fluorescence released was determined by fluorescence microscope (Leica microsystems). For quantitative measurement of lipid content, SZ95 sebocytes (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in quadruplicates and were treated with compounds for 24 hrs. Subsequently, supernatants were discarded, cells were washed twice with PBS, and 100 µl of a 1 µg/ml Nile red (Sigma-Aldrich) solution in PBS was added to each well. The plates were then incubated at 37°C for 20 min, and fluorescence was measured on a Molecular Devices FlexStation^{384} II Fluorescence Image Microplate Reader (FLIPR, Molecular Devices, San Francisco, CA USA). Results were expressed as percent of the relative fluorescence units (RFU) in comparison with the controls using 485 nm excitation and 565 nm emission wavelengths for neutral lipids, and 540 nm excitation and 620 nm emission wavelength for polar lipids (Alestas et al., 2006). Experiments were repeated 3 - 5 times.

4. Determination of lipid species by direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS)

For quantitative measurement of membrane lipid classes, SZ95 sebocytes (4 million/ flask) were cultured in T75 flasks in triplicates and were treated with compounds for 6 -24 hrs. Subsequently, supernatants were discarded, cells were washed twice with PBS, and collected by scraping in Eppendorf tubes. Protein

content was determined with BCA Protein assay (Thermo Scientific, USA) according to the manufacturer instructions, and the measurement was made on plate reader (562nm). Lipids were quantified by laboratory of Clinical Chemistry, at University of Regensburg, Germany, by direct flow injection electrospray ionization tandem mass spectrometry in positive ion mode using the analytical setup and strategy described previously (Liebisch et al., 2004; Liebisch et al., 2006). A precursor ion of *m/z* 184 was used for phosphatidylcholine (PC), sphingomyelin (SM) (Liebisch et al., 2004) and lysophosphatidylcholine (LPC) (Liebisch et al., 2002). Neutral loss fragments were used for the following lipid classes: Phosphatidylethanolamine (PE) 141, phosphatidylserine (PS) 185, phosphatidylglycerol (PG) 189 and phosphatidylinositol (PI) 277 (Brugger et al., 1997; Matyash et al., 2008). PE-based plasmalogens (PE-pl) were analyzed according to the principles described by Zemski-Berry et al. (Zemski-Berry et al., 2004). Sphingosine-based ceramides (Cer) and hexosylceramides (HexCer) were analyzed using a fragment ion of *m/z* 264 (Liebisch et al., 1999).

5. Detection of major neutral lipid classes

Composition of SZ95 sebocytes in major neutral lipid classes was investigated by separating crude lipid extracts with high performance thin layer chromatography (HPTLC). Measurements were made by Mauro Picardo and Emmanula Camera, (Istituto Dermatologico San Gallico, Rome Italy). Silica gel 60 plates 20x10 cm (Merck, Darmstadt, Germany) were used as the stationary

phase. 20 µL of the lipid extract from each sample were loaded onto the HPTLC plate. The separation was achieved by running the HPTLC with two consecutive eluent systems. The plate was first run in benzene/hexane 70/30 (v/v). After drying, the plates were run in hexane/diethylether/acetic acid 50/50/2 (v/v/v). Again the plates were dried and dyed with a 10% solution of copper sulfate in water/methanol 80/20 (v/v). The plates were heated at 120°C for 60 minutes to develop grey-to-black spots. Densitometric analyses were performed to assess spot intensities. Arbitrary units were normalized by the protein content of the cell extract. To confirm the HPTLC results, relative abundance of representative species was computed and compared between treatments. Relative abundance was normalized by protein content in the respective sample. Results were reported as average ± SD of four replicate experiments. Profiles of abundance of triglycerides (TG) and diglycerides (DG) and quantification of cholesterol (CH) and squalene (SQ) were performed with HPLC-MS with a time of flight MS detector (ToF) as previously reported (Camera et al., 2010). Quantitative determination of arachidonic acid (AA) was performed with a triple quadrupole mass detector (MSD, Agilent Technologies, CA, USA) following HPLC separation. Before the lipid analyses, cells were extracted as previously reported. Briefly, cells were cracked by thaw-freezing. Cells were pelleted after suspension in 50 µL MQ water. Protein content was determined in the supernatant with Bradford assay and cell lipids were extracted with ethyl acetate after suspension of cell debris in aqueous ethanol containing 200 ng of deuterated cholesterol as

the internal standard (ISTD, cholesterol-2,2,3,4,4,6-d_6, CDN isotopes INC., Pointe-Claire, Quebec, Canada). After extraction with ethyl acetate, the organic phase was evaporated to dryness under gaseous nitrogen and dissolved in 100 µL of acetone/methanol/isopropanol mixture. 20µL were loaded onto the HPTLC plate for the TLC separation, whereas 20 µL were diluted 1:5 in acetone/methanol/isopropanol in presence of 2 µM deuterated fatty acid as the ISTD and analyzed by HPLC-MS. For the quantitative determination of AA, CH, and SQ, calibration curves were run in presence of the respective ISTD. Quantitative results of AA, CH, and SQ were normalized by protein concentration and were expressed as pmole/mg protein, µg/mg protein, and ng/mg protein, respectively. Results were reported as average ± SD of four replicate experiments.

6. Detemination of eicosanods / docosanoids using HPLC-MS-MS

For quantitative determination of eicosanoids and docosanoids in treated SZ95 cells, the HPLC-MS-MS methodology was used; the analytical sample preparation procedure is based on an established method used for retinoid quantification (Rühl *et al.*, 2006) and was recently published for organs and serum analysis (Szklenar et al.,). Measurements were made by Ralph Rühl, University of Debrecen.

7. RNA isolation, Real Time –quantitative PCR (RT-qPCR)

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacture's protocol. Reverse transcription was performed at 42°C for 30 min from 100 ng of total RNA using Superscript II reverse transcriptase (Invitrogene, Carlsbad, CA, USA). Quantitative PCR was performed on ABI PRISM 7900 instrument (Applied Biosystems Foster City, CA, USA), 40 cycles of 95° C for 12s and 60°C for 1 min using TaqMan assays (Applied Biosystems Foster City, CA, USA). All PCR reactions were done in triplicate with one control containing no RT enzyme. The comparative Ct method was used to quantify transcripts and normalize for human cyclophilin A. Cyclophilin A levels did not vary between treatments. Primers and Fam/Tamra labelled probes were designed for measuring the following genes (table 3.)

Human Gene	Probe	Forward	Reverse
PPARγ	FAM-CAAACCTGGGCGGTCTCCACTGAG	GATGACAGCGACTTGGCAA	CTTCAATGGGCTTCACATTCA
PPARδ	FAM-CAGCCACACGGCGCCCTTTG	AGCATCCTCACCGGCAAAG	CCACAATGTCTCGATGTCGTG
PPARα	FAM-CAAACCTGGGCGGTCTCCACTGAG	CATTACGGAGTCCACGCGT	ACCAGCTTGAGTCGAATCGTT
RXRβ	TCTGTCAGCACCCGATCAAAGATGG	CCCATTCAGCAGGAGTAGG	CTCATGTCACGCATTTTGGA
ADRP	FAM-TCTGTGGTCAGTGGCAGCATTAACACA-TAMRA	TGACTGGCAGTGTGGAGAAGA	ATCATCCGACTCCCCAAGA
PGAR	FAM-TTGGAATGGCTGCAGGTGCCA-TAMRA	TCCGCAGGGACAAGAACTG	CGGAAGTACTGGCCGTTGA
cyclophilin A	FAM-CGCGTCTCCTTTGAGCTGTTTGCA-TAMRA	ACGGCGAGCCCTTGG	TTTCTGCTGTCTTTGGGACCT
RXRα	TaqMan gene expression assay for human : Hs00172565_m1 (Applied Biosystems)		

Table 3. Sequences of probes and primers used for RT-qPCR reactions.

The comparative Ct method was used to quantify transcripts and normalize for human cyclophilin A. Mean and SD were calculated for both the normalized and the normalizer values for all experiments. Data shown are mean±SD of three biological replicates measured in triplicates. *$p<0.05$ was considered as significant change compared to vehicle treated control, or ligand treated cells. Comparative q-RT-PCR analyses were carried out using dilutions of target amplicons for PPARα, γ and β/δ (data not shown).

For laser microdissection (LCM) derived samples the RT and qPCR reactions were made in one single step using the Cells Direct One Step RT-qPCR kit (Invitrogene, Cat. No: 11754-100, Carlsbad, CA, USA). Reverse Transcription reaction was for 10minutes 50°C, 2 minute 95°C for hot start of DNA polymerase, and qPCR is performed for 40cycles of 12s 95°C and 60°C for 1min.

8. Statistical analysis

All data are presented as mean ± SD. When applicable, data were analyzed using a two tailed un-paired t-test and $P<0.05$ values were regarded as significant differences. In addition, statistical differences were further verified using 1-way ANOVA with Bonferroni and Dunnett post hoc tests, resulting in similar results.

9. Ligands

Cells were treated with the following ligands: PPARγ selective agonist rosigliazone (RSG), antagonist GW9662 (Alexis Biochemicals), RXR

panagonist LG100268, a gift from R. Heyman (Ligand Pharmaceuticals), arachidonic acid (Sigma), 5-KETE, 12-KETE, 15-KETE (Cayman Biochemicals).

10. Patient samples

The study was approved by the Institutional Research Ethics Committee *(number: DE OEC RKEB/IKEB 2337-2005)* and adhered to Declaration of Helsinki Guidelines. Formalin-fixed and paraffin embedded tumor tissues from 9 sebaceous carcinoma and 10 sebaceous hyperplasia, 10 sebaceous adenoma cases and ten sections from normal human skin (of 10 donors) were used for immunohistochemistry (IHC) obtained from the archive blocks of Departments of Dermatology and Pathology, University of Debrecen. The human tissue samples were all handled as anonymous in respect of patients' dignity. One sebaceous adenoma and one sebaceous hyperplasia sample was cut, and half of the samples were stored at -80°C degrees and used for laser microdissection as described below.

As acne vulgaris do not need histological examination in everyday practice, we collected samples from volunteers. Patients with acne vulgaris were examined according to the Global Acne Grading System Score (GAGS) to evaluate the severity of acne vulgaris (Doshi et al., 1997). Patients, selected for biopsy, were categorized in GAG 1 group (mild acne). The biopsies were obtained from the upper back sites, non-acne involved skin from 9 donors with acne vulgaris (NAS

– acne, n=9), and acne-involved skin samples on acneic papules from the same donors (AS – acne, n=9). Skin of 5 healthy donors (H, n=5) as reference (control) were used. The biopsied skin was frozen in liquid nitrogen and stored on –80 °C degrees until analysis.

11. Immunohistochemistry (IHC), immuncytochemistry and double immunofluorescence (IF)

IHC for PPARγ was carried out using the biotin-free and tyramid-based CSAII kit (Dako, Glostrup, Denmark) according to the manufacturer's instruction and as described previously (Szanto et al, 2004; Szatmari et al., 2006; , Gogolak et al, 2007). For immuncytochemistry sebocytes were cultured on coverslip slides, and fixed with 1% paraformaldehyde. Single IHC staining for androgen receptor (AR), and p63, which demonstrates the myoepithelial-basal layers of sebocytes (Tsujita-Kyutoku *et al,* 2003), and for the common proliferation marker Ki67, which is expressed in cells during active cell cycle –and predominantly in basal immature sebocytes of SG (Cottle et al., 2013*)* were all detected with standard immune-peroxidise method using an EnVision^{+}-HRP (horse-raddish-peroxidase) detection kit or an automated immune-stainer (Bond-Leica, UK) utilizing VIP (purple) or DAB (brown) chromogenic substrates for microscopic visualisation. Monoclonal antibody (mab) to PPARγ (Santa-Cruz, E8; 1:75), mab-AR (1:50), and mab-p63 (1:50) both from Novocastra (Newcastle, UK), and rabbit monoclonal antibody (r-mab) to ki67 (1:30; clone

EPR3611, BioGenex, San Ramon, CA, USA) were used. To check the specificities of antibodies positive controls were also included in each IHC run: for AR, p63 and ki67, normal prostate glands were used where the latter two markers show characteristic basal-myoepithelial nuclear positivity (not shown). For PPARγ white adipose tissue was used as positive control. For negative controls, isotype-matched control immunoglobulins (Dako) were used in place of the primary antibody resulting in no staining (not shown). The immunostained slides were then evaluated semi-quantitatively for sebaceous glands/SG tumors, reflecting the % of positive cells: -, negative; *1*+, 0-20%; *2*+, 20-50%; *3*+, 50-75%; *4*+, 75-100%. When double IHC immune-peroxidase staining for the simultaneous detection of PPARγ and Ki67 was made, a standard sequential labelling method was applied. Nuclear counterstaining was with methyl-green or Meyers hematoxylin. For double IF detection, tissues for PPARγ protein was visualized with mab to PPARγ and anti-mouse-HRP IgG followed by tyramid-TMR (tertamethyl-rhodamine; red fluorescence) treatment which was then stained further with r-mab Ki67 using biotinylated-anti-rabbit IgG and streptavidin-FITC (fluorescent iso-thiocyanat; green fluorescent) of the TSA-TMR/FITC system (Perkin Elemer, USA), essentially as described previously (Szanto et al., 2010) and the protocols provided by the vendor. For IF, the nuclear counterstaining was made with DAPI.

12. Laser Capture Microdissection (LCM) and RNA preparation

Normal tissues collected from surgical specimens for the microdissection study exhibited neither tumor nor inflammation around and within the sebaceous glands. Skin samples were frozen in liquid nitrogen, and stored at -80°C until use for the microdissection and morphology procedures, as previously described (Jenkins et al., 2005). For each specimen, serial sections were made in a cryo-microtome maschine (Leica) to collect 12 x 5μm thick identical sections mounted onto the polyethilene slide-membrane (Olympus Europe and Molecular Machines and Industries, Hamburg, Germany) for LCM followed by an immediate dehydration and fixation in alcohol at room temperature using nuclease free water for the diluent of 70% ethanol. The first and the last section of each sample were immunostained for PPARγ (Figure 8.) or stained with HE to make the identification easier of the structures that needed to be removed, albeit the use of stain was not always necessary since sebaceous gland and epidermis were clearly identifiable in most of the cases. The slides were observed with MMI-Olympus Cellcut Laser Capture Microdissection system (Olympus Europe and Molecular Machines and Industries, Hamburg, Germany). The region of interest was cut by the computer-guided laser beam attached to the microscope then transferred to the lifting cap of the sterile RNAse-free eppendorf tube to collect the samples for molecular biology analyses.

RNA was extracted from the laser-capture microdissected cells using the "Absolutely RNA Nanoprep kit" (cat. No: 400753, Stratagene, La Jolla, CA,

USA) according to the manufactures instructions. Briefly, cells on the cap of 0.5 ml tube were solubilized with 100µl of lysis buffer of the kit. As a final step, the microdissected cells were then eluted in 20ul of elution buffer, and diluted with nuclease free water to 20-25x, so that 8-10 genes could be measured from each sample. Samples were stored on -70 ºC for long-term usage.

X. RESULTS

1. PPARγ and its target genes are expressed in normal human SGs in situ

First, we determined the expression level of PPARγ and markers of its activity in human SGs in situ. Immuno-labeling identified PPARγ protein expression predominantly in the nuclei of normal SGs of skin biopsies obtained from healthy volunteers (n=10) (Figure 7.), similarly to previous reports (Zhang *et al.*, 2006; Trivedi *et al.*, 2006; Alestas *et al.*, 2006).

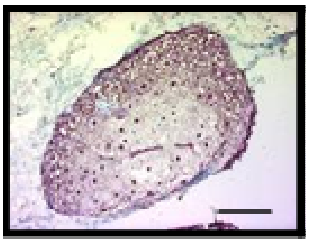


Figure 7. PPARγ expression in human sebaceous gland. Scale bar:100μm

In addition, SGs were isolated from donors of healthy (n=5) and pathologic (n=2) human skin tissues by laser-microdissection and mRNA levels of PPARγ were examined by quantitative real-time PCR (qPCR). The expression of PPARγ mRNA was higher in healthy SG than in pathologic samples, although the latter was available only in limited numbers (Figure 8.).

It is generally accepted that PPARγ activity is indicated by the mRNA expression of its target genes including the adipose differentiation related protein (ADRP) and PPARγ angioprotein related protein (PGAR), as established in adipocytes, macrophages and dendritic cells in previous studies (Forman *et al.*, 1995; Szanto *et al.*, 2004; Nagy and Szanto, 2005; Szatmari *et al.*, 2006; Varga and Nagy, 2008). We also found that laser-microdissected SG samples from

healthy individuals express ADRP and PGAR mRNA at higher levels than SGs from pathologies (Figure 8.). These data suggest that PPARγ and also its target genes are present in human SGs.

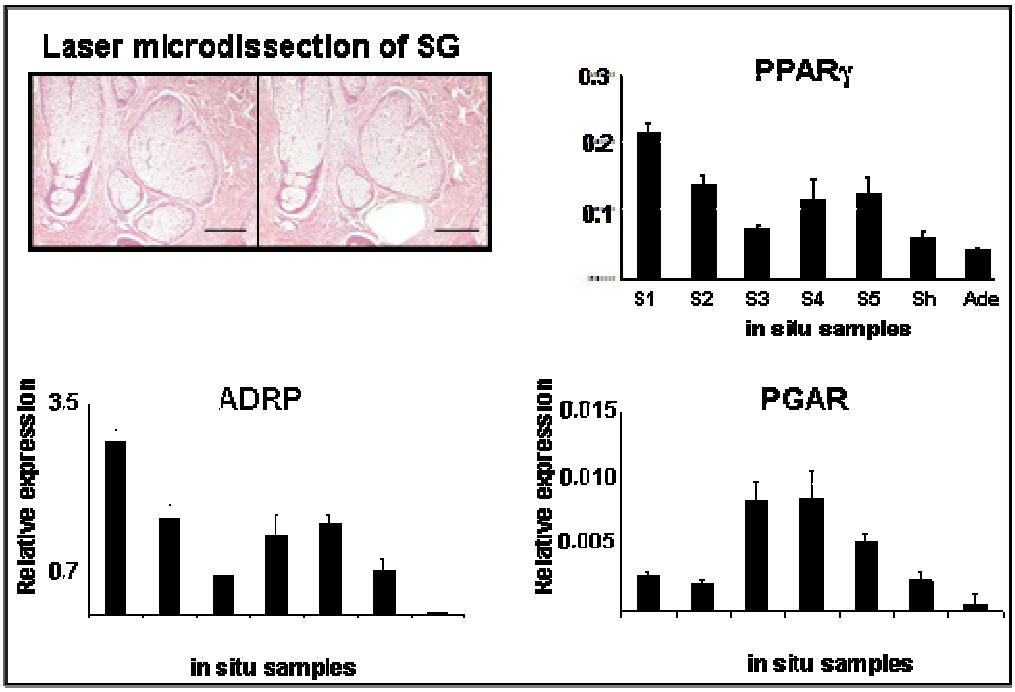


Figure 8. mRNA expression of PPARγ and its target genes in healthy sebaceous glands.In normal (n=5, S1-S5 from 5 different volunteers), and in two pathological samples, SG hyperplasia (Sh), SG adenoma(Ade) SG cells were laser microdissected and collected for mRNA analyses. RT-qPCR measurements showed that PPARγ, ADRP and PGAR were expressed. Values shown are means ±SD of three measurements of each sample.

2. Active PPARγ is present in human SZ95 sebocytes

To further explore how PPARγ functions in sebocytes we used a well-established human SG cell line, SZ95 sebocytes (Zouboulis *et al.*, 1999). Expressions of the three PPAR isoforms were detected at the mRNA level in

SZ95 sebocytes (Figure 9.). Remarkably, PPARγ mRNA expression was highest among the PPAR isoforms. We measured the mRNA levels of RXR isoforms, which are the heterodimerization partners of PPARγ. Confirming a previous report (Tsukada et al., 2000) RXRα appeared to be the dominant RXR subtype in SZ95 sebocytes (Figure 9.).

Next, we investigated PPARγ activity in SZ95 sebocytes. We treated SZ95 sebocytes with the synthetic PPARγ agonist rosiglitazone (RSG) and measured the mRNA levels of two target genes, PGAR and ADRP. RSG (1-10 μM) induced significant upregulation of PGAR (ca. 32-fold) and ADRP (ca. 3-fold). The RXR agonist LG100268 (20-200 nM) also increased PGAR (ca. 46-fold) and ADRP (ca. 2-fold) expressions, respectively (Figure 9.). Furthermore, co-administration of the two agonists resulted in even higher PGAR gene expression elevations. Administration of a PPARγ inhibitor GW9662 markedly abrogated the RSG-induced target gene induction in a dose-dependent manner (1-10 μM). These data collectively suggest that PPARγ is active in human SZ95 sebocytes. This confirms our previous observations (Dobrosi *et al.*, 2008). Our observations also suggest that PPARγ and RXRα are likely to act on the same molecular pathways although synergy could not be detected.

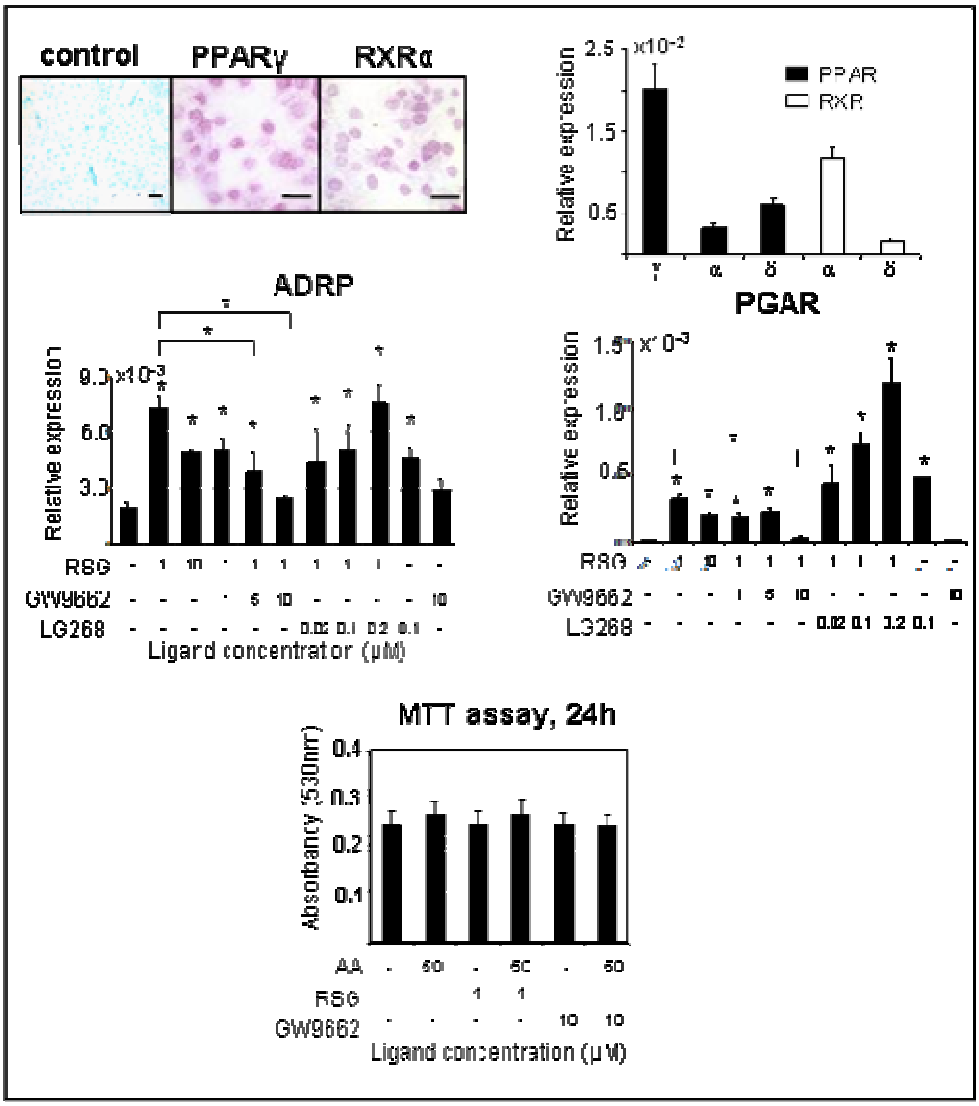
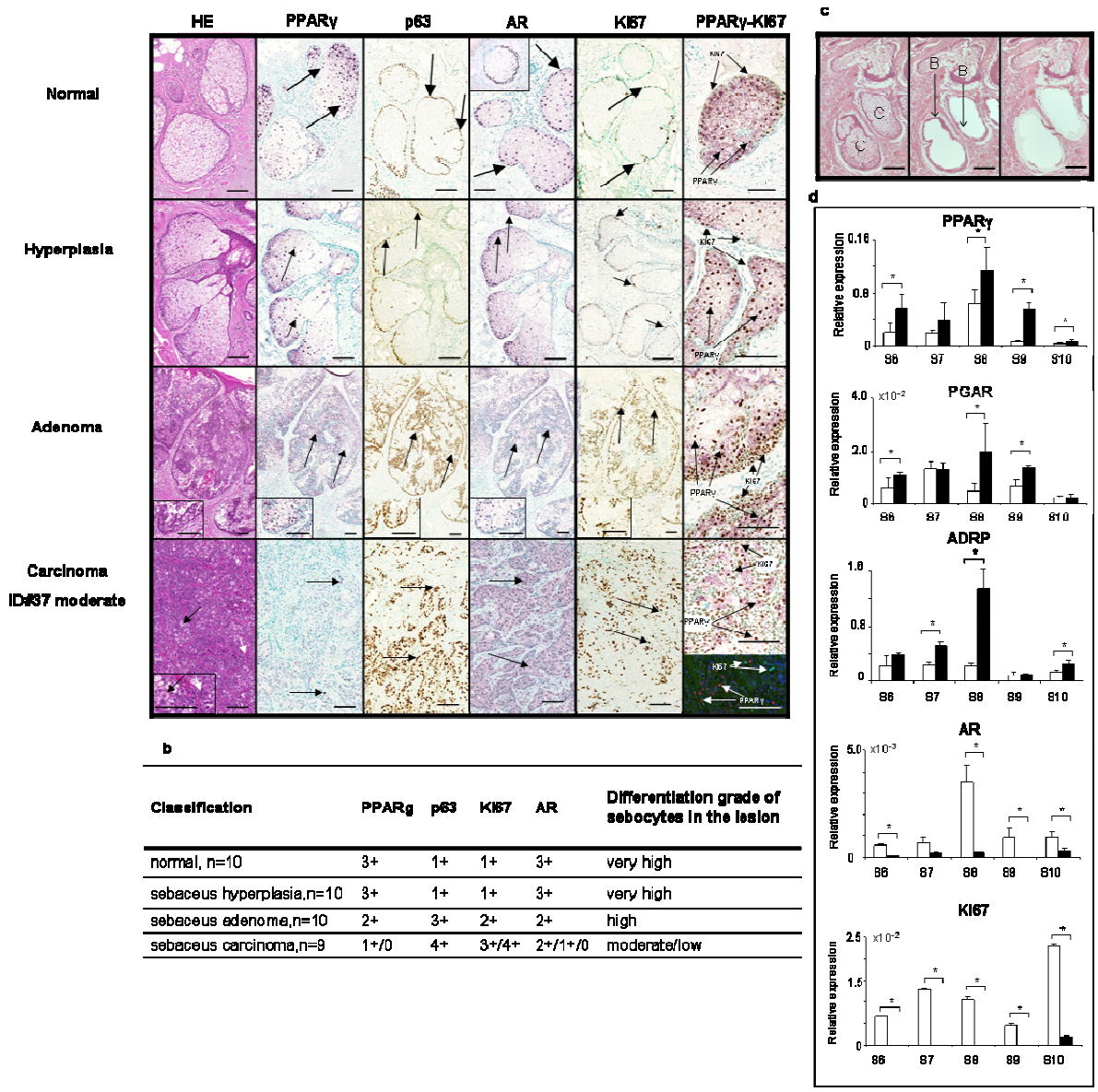


Figure 9. Expression of PPAR, RXR mRNA isoforms in SZ95 sebocytes, and potential PPARγ target genes. Immunohistochemistry demonstrates that PPARγ and RXRα are expressed at the protein level in SZ95 sebocytes. mRNA expression levels of PPARα, PPARδ, PPARγ, RXRα, and RXRβ in SZ95 sebocytes. Cells were treated with PPARγ agonist RSG (1-10 μM), PPARγ antagonist GW9662 (1-10 μM), RXRα, β, γ pan-agonist LG100268 (0.02-0.1 μM) for 6 hours. mRNA expressions of ADRP and PGAR were induced by RSG and LG100268, notably, GW9662 was able to inhibit the induction. None of the ligands used was cytotoxic (MTT assay, 24h). Values shown are means ±SD of three separate experiments. P< 0.05 was considered as significant change; *compared to vehicle treated control; # compared to 1 μM RSG treatment.

3. PPARγ expression correlates with the differentiation stage of sebocytes

Next we sought to define the role of PPARγ in the process of sebocyte differentiation. Therefore, we investigated SGs from human biopsies obtained from patients with benign or malignant SG lesions. Immuno-histochemical analyses revealed the nuclear expression of PPARγ in normal SGs, which is in line with previous reports (Alestas *et al.*, 2006; Trivedi *et al.*, 2006) (Figure 10.a). Remarkably, PPARγ protein was hardly detectable in basal cell layers of SG (n=10) in immature (non-differentiated) sebocytes. In order to document the differentiation stages of sebocytes within SG, we immunostained sections and measured mRNA for Ki67, a well known marker of active cell cycle, and for the androgen receptor (AR). Also, the grade of differentiation of sebocytes within the SG was classified. Immunostaining was scored semi-quantitatively according to previous reports (Cottle et al., 2013; Tsujita-Kyutoku et al., 2003) (Figure 10. a,b and Supplementum table 1.).



b

Classification	PPARg	p63	KI67	AR	Differentiation grade of sebocytes in the lesion
normal, n=10	3+	1+	1+	3+	very high
sebaceus hyperplasia,n=10	3+	1+	1+	3+	very high
sebaceus adenoma,n=10	2+	3+	2+	2+	high
sebaceus carcinoma,n=9	1+/0	4+	3+/4+	2+/1+/0	moderate/low

Figure 10. Comparative protein and mRNA expression patterns of PPARγ in conjunction with compartment-associated reference markers for human normal and pathological sebaceous glands (SG) and laser-microdissected healthy SG compartments. a, HE-stained histology and the corresponding immunoprofiles in human skin tissues for PPARγ, p63, AR and Ki67 nucleo-protein expressions of various sebaceous gland (SG) types, respectively. Black arrows indicate representative positive cells (brown or purple nuclei). Last coloumn: PPARγ-Ki67 double-labeled images by means of both immuno-peroxidase and immunofluorescence methods (lower right images). c, Basal and subrabasal

(white column) and central (black column) cell layers of normal SG samples were laser microdissected (n=5 samples from 5 donors, ie.S6:sample6), for mRNA analysis. Scale bar 100μm. d, PPARγ, ADRP, PGAR, AR and Ki 67 expression at the mRNA level. The values shown are mean ±SD of three separate measurements. * P< 0.05 was considered as significant change.

In order to extend these analyses using reference markers, we also included p63 protein, known to be expressed in the nuclei of basal immature sebocytes of SGs (Tsujita-Kyutoku et al, 2003). PPARγ was present at high levels in sebocytes of all samples (n=10) from patients with SG hyperplasia (Figure 10.a). Both the Ki67 and p63 markers stain nuclei of immature sebocytes located at the basal layers of normal SG, whereas AR was expressed in a bit wider range of normal sebocytes incorporating predominantly the basal and suprabasal cell layers, and to a lesser extent the central cells, as well. By contrast, PPARγ was detectable in terminally differentiated mature sebocytes located in the central regions of normal SGs, but this protein was hardly detectable in immature cells of the basal layer. Double immunolabeling for Ki67 along with PPARγ clearly demonstrated the predominantly different localizations for these two nuclear proteins discriminating the immature (Ki67 positive) cells from the mature (PPARγ positive) ones, which appeared to be the rule for these marker expressions in normal and hyperplastic SGs. We also analyzed samples of SG-tumors. When compared to normal and hyperplastic SGs, cells of sebaceous adenomas (n=10) generally expressed decreased amounts of PPARγ, which appeared to decrease further in malignant tissues and sebaceous carcinomas (n=9) and down to hardly

detectable levels in poorly differentiated neoplastic cells. In the same SG carcinoma samples, the un-affected normal tissue parts with intact sebaceous glands retained the significantly high PPARγ-specific nuclear protein expression levels (Figure 11.).

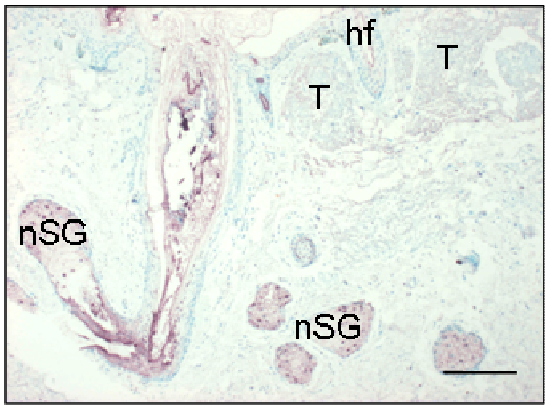


Figure 11. Expression of PPARγ in low differentiated SG carcinoma and in healthy sebaceous glands. scale bar:100μm PPARg is stained with deep red colour, counterstaining is with methilenegreen.

Decreased level or the absence of PPARγ protein expression levels correlated with an increasing number of Ki 67 and p63 positive neoplastic cells of sebaceous carcinoma cases, reflecting the ratio of more differentiated (i.e., PPARγ-positive cells with lipid droplets) and the less mature (poorly differentiated, basaloid) cellular components of the tumors in all the cases studied. This could be clearly demonstrated with double immunostaining with Ki67 and PPARγ, confirming that the more differentiated carcinoma cells expressed preferentially PPARγ without Ki67, and the Ki67 positive proliferating pleomorphic neoplastic cells did not exhibit PPARγ (Figure 10, last image at lower right). In contrast, the expression pattern of AR at protein levels generally did not show such an obvious and clear distinction in well differentiated and poorly differentiated cellular components of sebaceous carcinomas studied so far. However it was expressed in high proportion of adenoma cells in all cases (Figure 10.a, middle).

We then corroborated our results by investigating the PPARγ expression pattern and its signaling pathway during normal sebocyte differentiation. Therefore, terminally differentiated normal SG cells (i.e., from the central zone of the gland) and immature cells (from the basal-parabasal zones) were collected separately by in situ laser microdissection from fresh frozen human skin tissues; all samples were from the facial region of 5 different donors. Subsequently, mRNA expressions of PPARγ, ADRP and PGAR, Ki67, AR were measured (Figure 10.c). PPARγ, ADRP and PGAR were expressed at low levels in basal cells, whereas higher expression was detected in the well-differentiated sebocytes of the central zone 4/5 samples (PPARγ), 3/5 samples (ADRP, PGAR). In contrast Ki67 and AR mRNA was expressed high level in basal cell layer, and less or not detectable in central cells (AR 4/5 samples, Ki67, 5/5 samples). These data together with findings from the 10 samples analyzed with immunohistochemisty indicated that PPARγ may be employed as a potential marker of sebocyte differentiation.

4. AA regulates sebocyte differentiation, in part via PPARγ-coupled signaling pathways

To further explore the biological consequences of PPARγ stimulation on SG biology, we also investigated the functional role of PPARγ in SZ95 sebocytes. Sebocyte differentiation is characterized by the synthesis of various lipids, among which neutral lipids represent the major part (Zouboulis *et al.*, 1999; Rosenfield *et al.*, 1998).

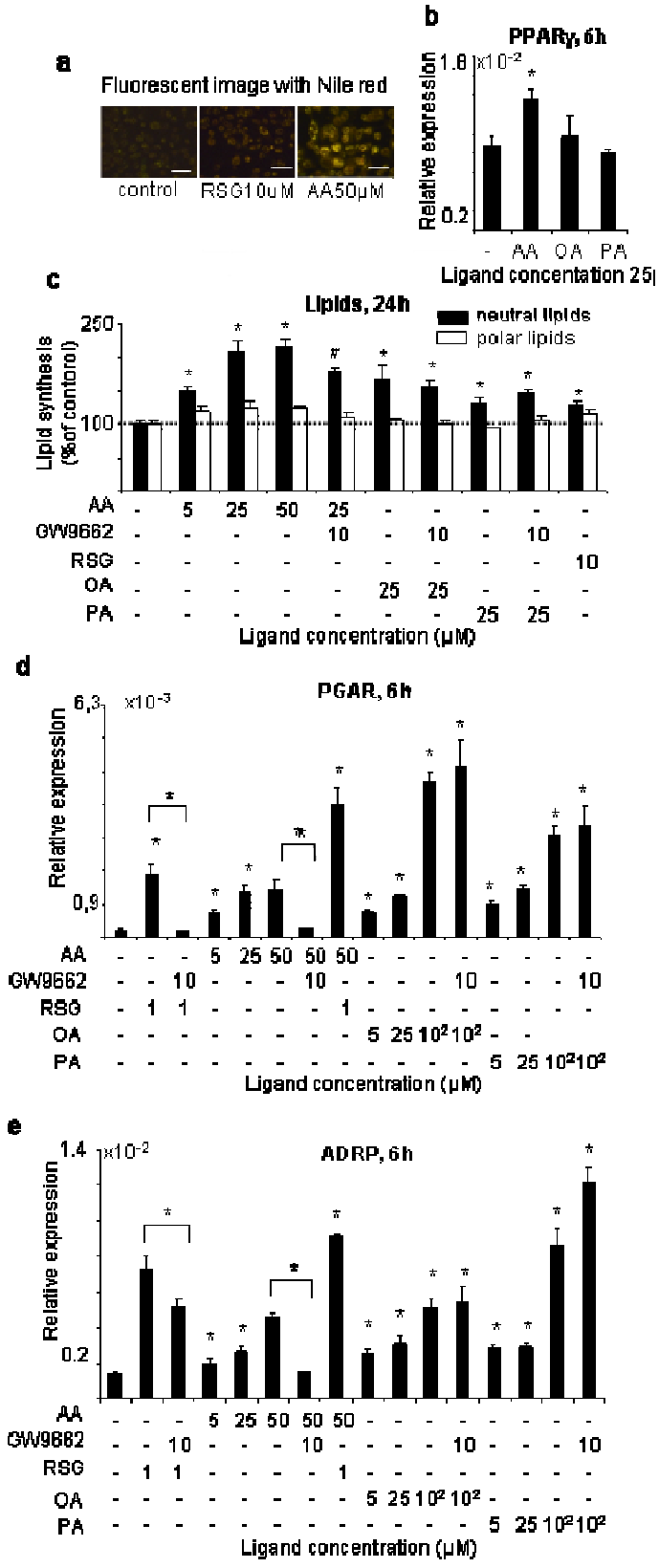


Figure 12. AA induces upregulation of PPARγ and PPARγ target genes resulting in differentiation of SZ95 sebocytes. (a-c) SZ95 sebocytes were treated for 24h or (d-e) 6h with 1-10 µM RSG, 1-10 µM GW9662 (PPARγ inhibitor), 5-50 µM AA, 5-100 µM OA, 5-100 µM PA and combinations of these ligands. a)Intracellular lipids were assessed by Nile red labeling following fluorescence microscopy. Scale bar: 50 µm b) Quantitative measurement of neutral and polar lipids by FLIPR fluorescent microplate reader. Data (means ± SD) are presented as percentage of mean values of quadruplicates; the value of the vehicle treated control group was defined as 100%. Two additional experiments yielded similar results. c-e) mRNA expression of PPARγ, PGAR, and ADRP. AA increased the mRNA expression of PGAR and ADRP in a dose-dependent manner which was inhibited by GW9662. Mean±SD of three biological replicates measured in triplicates. * P< 0.05 was considered as significant change compared to vehicle treated control, or ligand treated cells.

Confirming our previous results (Toth *et al*, 2009; Alestas *et al.*, 2006), first we showed that AA, one of the most effective differentiation inducer of SZ95 sebocytes, markedly enhanced lipid synthesis of the cells (Figure 12.a). Quantitative Nile red-based fluorescence assay was performed, which showed that AA induced neutral lipogenesis in SZ95 sebocytes in a dose-dependent manner (Figure 12.a and c.).

Intriguingly, the PPARγ agonist RSG also induced sebaceous neutral lipid synthesis in SZ95 sebocytes (albeit at a lower potency than AA) (Figure 12. a and c). We then assessed the involvement of PPARγ in AA induced biological changes. AA treatment of the cells resulted in a marked upregulation of the mRNA expression levels of the PPARγ target genes (PGAR and ADRP) which effect could be fully abrogated by a PPARγ specific antagonist GW9662 (Figure 12.d-e). Moreover, the lipid synthesis-promoting activity of AA was also inhibited by a PPARγ antagonist. However, it must be noted that GW9662 was able to only partially (albeit significantly) prevent the effect of AA. Finally, we found that AA treatment also upregulated the mRNA level of PPARγ (Figure 12.b).

In order to determine the specificity of AA treatment, we also treated the sebocytes with oleic acid (OA) and palmitoleic acid (PA). Interestingly both induced lipid accumulation and PGAR and ADRP expression, but neither effect was inhibited by PPARγ antagonist treatment (Figure 12. d-e) suggesting that PPARγ is not involved in these responses.

5. AA regulates the production of selected lipid molecules via PPARγ

To further explore how PPARγ activation affects lipid production in sebocytes, we treated the cells with AA (25 μM) and with a PPARγ antagonist GW9662 (10 μM) simultaneously for 24h and then analyzed the major neutral lipids and specific membrane lipids. We determined the relative abundance of major neutral lipid classes, such as TG, FFA, CE, CH, WE, and SQ using high performance thin layer chromatography (HPTLC) followed by densitometric analyses. Quantitative determinations of AA were performed by high pressure liquid chromatography coupled with tandem mass spectrometry (HPLC-QqQ/MS), whereas concentrations of SQ and CH and the relative abundance of AA-induced DG and TG were determined by HPLC-ToF/MS. HPTLC of lipid extracts of cell pellets resulted in the detection of CH, FFA, TG, WE and CE (Figure 13.a). SQ and DG were undetectable under these analytical conditions. Densitometric analyses of HPTLC spots showed that TG levels were significantly elevated by AA and this induction was inhibited by a PPARγ inhibitor (Figure 13. a). Substrate concentration of AA was determined quantitatively and exposing sebocytes to AA resulted in a significant elevation of AA concentration in the cells, while co-administration of GW9662 appeared not to influence these cellular levels of free AA (Figure 13.b).

Biosynthesis of TG is a multistep process that involves different lipid intermediates. To gain insight into the mechanisms involving PPARγ activation, we determined the relative abundance of AA-induced DG and TG by HPLC-MS analyses.

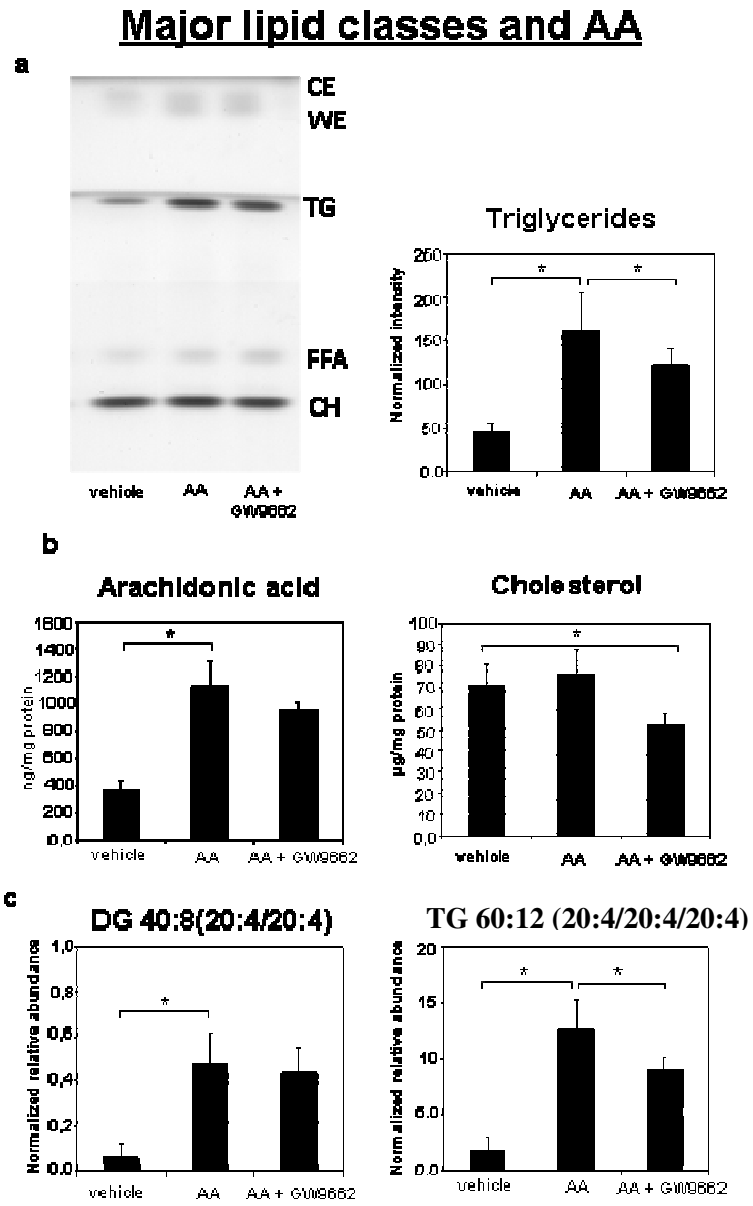


Figure 13. a-c: Arachidonic acid induced PPARγ activation changed selected major lipid classes in SZ95 sebocytes. Cells were treated for 24 h with 25 µM AA or AA 25 µM plus 10 µM GW9662. a, HPTLC and densitometric analysis of TG bands (average ± SD of normalized intensity); b, Quantitative determinations of AA and CH by HPLC-MS with triple quadrupole (QqQ) analyzer (average ± SD of normalized concentrations); c, HPLC-MS analyses with a time of flight MS detector (ToF) of AA containing DG 40:8 (20:4/20:4) and TG 60:12 (20:4/20:4/20:4). AA elevated the levels of DG and TG, but only TG 60:12 induction was significantly inhibited by the PPARγ antagonist GW9662. Measurements were made by Mauro Picardo and Emmanuela Camera (Istituto Dermatologico San Gallico, Rome Italy).

The AA containing DG 40:8 was significantly induced by AA, while GW9662 did not influence DG 40:8 levels. The levels of the TG 60:12, representative of the AA-containing TG were induced significantly (Figure 13.c).

When AA was co-administered with a PPARγ antagonist this induction of TG 60:12 was inhibited, which is consistently with the HPTLC results concerning the total TG (Figure 13. a-c).

HPTLC demonstrated that AA induced WE levels, however no effect of the PPARγ antagonist on this was observed. Additionally, AA had no significant effect on the total CE levels regardless of the presence of the PPARγ antagonist (densitometric results not reported). Interestingly, basal CH level was significantly decreased by the PPARγ antagonist GW9662 as demonstrated by the quantitative analysis of CH by HPLC-MS (Figure 13.b). In contrast, concentration of SQ determined by HPLC-MS was not affected by AA alone or in combination with the PPARγ antagonist GW9662 (data not shown).

Next, using direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS), nine main specific membrane lipid groups were assessed: phosphatidylinositol, lysophosphatidylcholine, sphingomyelin, dihydrosphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, ceramide and glucosylceramide. AA had no significant effect on ceramides and glucosylceramides. Among the other 7 measured membrane lipid groups, AA induced significant alterations in the levels of 136 of the 198 measured lipid species (Figure 14.).

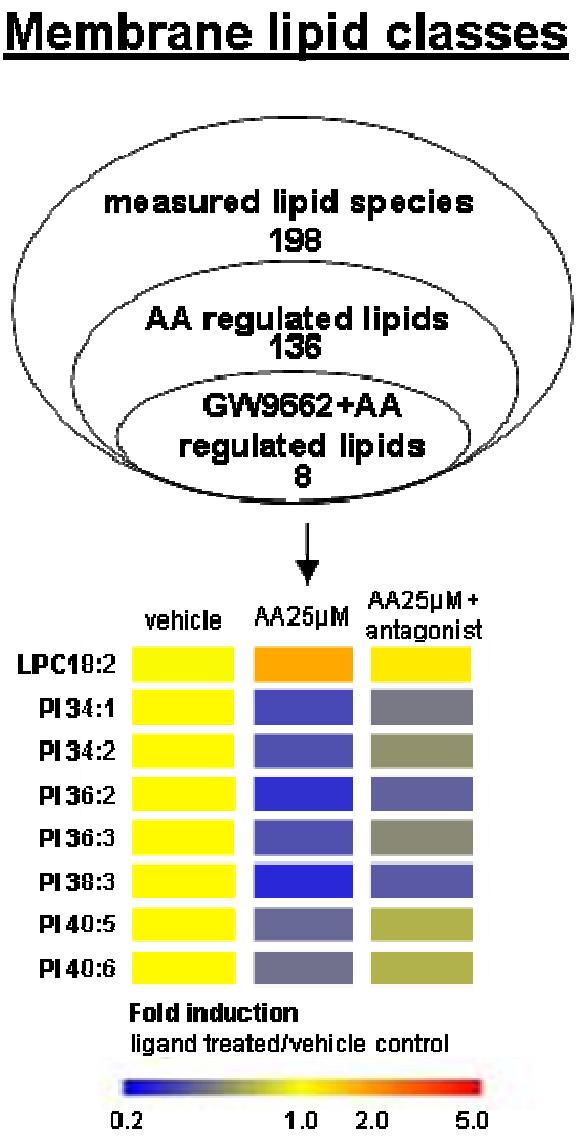


Figure 14. Arachidonic acid induced PPARγ activation changed selected membrane lipid classes in SZ95 sebocytes. ESI-MS/MS analysis of 9 membrane lipid classes: sphingomyelin, phosphatidylglycerol, phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine. AA changed the level of 136 lipid types significantly. Heat map of ESI-MS/MS of PI (phosphatidyl inositol) and LPC (lysophosphatidylcholine) 8 lipid species, where AA effect was inhibited by PPARγ antagonist GW9662. Color bar defines concentration fold change. Different fatty acid chain length and saturation of lipids are shown in numbers. Data are expressed as mean of three independent determinations of three independent biological replicates ($P<0.05$).Lipids were quantified by Gerhardt Liebisch, Laboratory of Clinical Chemistry, at University of Regensburg, Germany.

Importantly, the presence of the PPARγ inhibitor GW9662 significantly prevented the action of AA on 8 lipid species (Figure 14.). Among these molecules, AA decreased the level of seven types of phosphatidylinositol (PI) whereas it upregulated the expression of 18:2 lysophosphatidylcholine. These findings suggest that AA regulates the metabolism of these molecules in a PPARγ dependent fashion.

Therefore, the AA-coupled PPARγ pathway may contribute to the generation of major neutral lipids and membrane phospholipid production. With this, we comprehensively analyzed lipid changes upon AA treatment, and sorted out the ones requiring PPARγ activation.

The above data collectively suggest that i) PPARγ is indeed active in cultured SZ95 sebocytes where its activation resulted in a lipogenic action; ii) AA acts as a PPARγ agonist in SZ95 sebocytes; and iii) the effect of AA to enhance lipogenesis and hence sebaceous differentiation was only partially mediated by the activation of PPARγ and the related signaling (and hence other, PPARγ-independent signal transduction mechanisms are most likely involved) iiii) PGAR and ADRP can be regulated by other lipid mediators, and AA effect is in part PPARγ dependent. As an additional control we measured cell line viability with MTT, and none of the applied ligands induced cell death (Figure 10.).

6. AA metabolites activate PPARγ and induce lipogenesis in sebocytes

AA can be metabolized to a plethora of signal lipids (Harizi *et al.*, 2008). Therefore, we assessed whether certain AA metabolites can also induce similar cellular responses on human sebocytes. SZ95 sebocytes were treated with AA for 6 hrs and an HPLC-MS approach was then employed to quantitatively determine the levels of AA-derived signalling lipids.

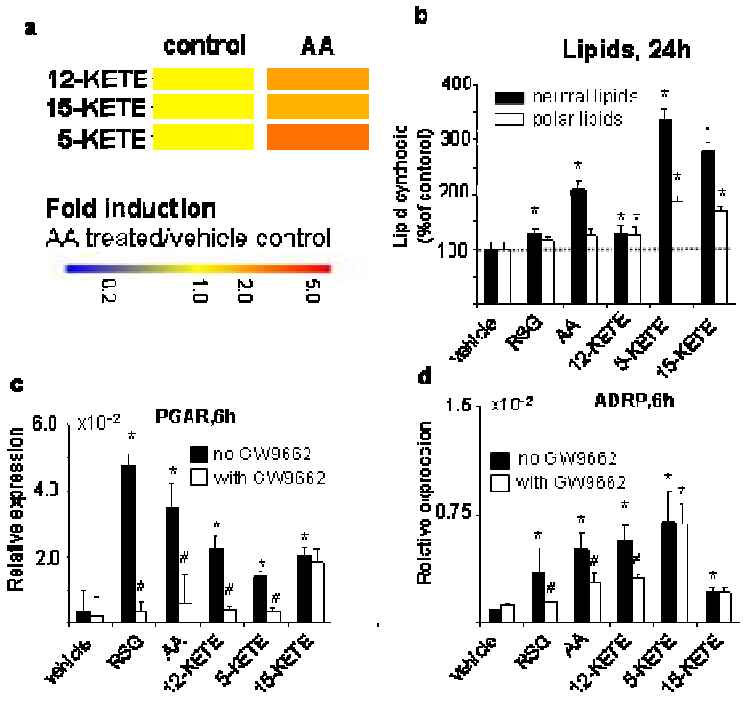


Figure 15. Arachidonic acid-derived metabolites 5-KETE and 12-KETE are potential endogenous lipogenic activators of PPARγ in SZ95 sebocytes. a) SZ95 sebocytes treated for 6 hours with 25 µM AA and then intracellular concentrations of its keto-metabolites (5, KETE, 12-KETE, 15-KETE) were measured with HPLC-MS technology. Heat map of concentrations; color bar defines concentration fold change. Measurements were made by Ralph Rühl, University of Debrecen. b-d) SZ95 sebocytes were treated with 1 µM RSG, 25 µM AA, 10 µM GW9662, 5 µM 5-KETE, 5 µM 12-KETE, 5µM 15-KETE and combination of these ligands with a PPARγ-antagonist. (b) Quantitative measurement of neutral and polar lipids by FLIPR fluorescent microplate reader after 24 h treatment. Data (means ± SD) are presented as percentage values; the value of the vehicle treated control group was defined as 100%. Two additional experiments yielded similar results. mRNA expression of PPARγ target genes (c) PGAR and (d) ADRP. Data are expressed as mean± SD of triplicate determinations of three independent biological replicates ($P<0.05$). $P< 0.05$ was considered as significant change; * compared to vehicle treated control; # compared to the treated group without the presence of GW9662.

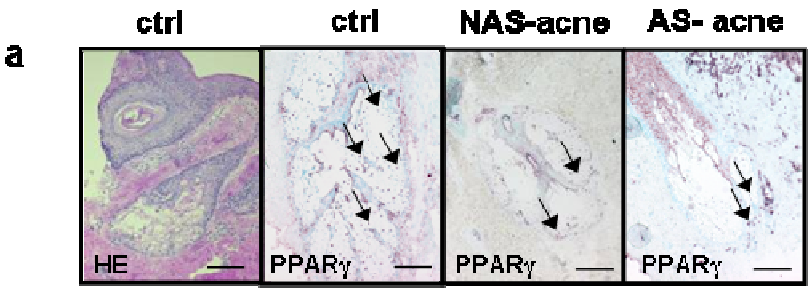
Among the several candidates, we primarily focused on eicosanoids which have a proven ability to activate PPARγ like 5-KETE, 12-KETE and 15-KETE. Upon AA

treatment, we detected increased concentrations of 5-KETE, 12-KETE, and 15-KETE (Figure 15. a).

Furthermore, these endogenous AA metabolites administered at a concentration of 5 μM (Shiraki *et al.*, 2005; O'Flaherty *et al.*, 2005) significantly elevated sebaceous lipid synthesis (Figure 15.b). To explore whether these molecules also activate PPARγ in another set of experiments, SZ95 sebocytes were treated similarly and expressions of PPARγ target genes were then assessed. We found that 5-KETE, 12-KETE and 15-KETE significantly increased the gene expression levels of PGAR and ADRP. However, the PPARγ antagonist GW9662 was able to inhibit the induction of PGAR only in the case of 12-KETE and 5-KETE (Figure 15.c), and the induction of ADRP in the case of 12-KETE (Figure 15.d). Taken together, these findings indicate that AA-metabolites play a role in sebaceous lipogenesis and that 12-KETE and 5-KETE are likely to signal via PPARγ.

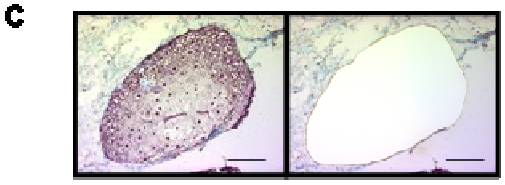
7. PPARγ and its target genes are expressed in situ both in normal SG, and in the non-acne-involved and acne-involved SGs of acne patients

Immunolabeling identified PPARγ protein expression predominantly in the nuclei of normal SGs obtained from healthy volunteers (3+), but low expression was detected in SG cells of acne patients, both in non-acne-involved (NAS) and in acne involved skin (AS) samples as well (Figure 16. a-b).



b

	ctrl n=5	NAS - acne n=9	AS - acne n=9
PPARγ positive cells	4 or 3+	2+	1+



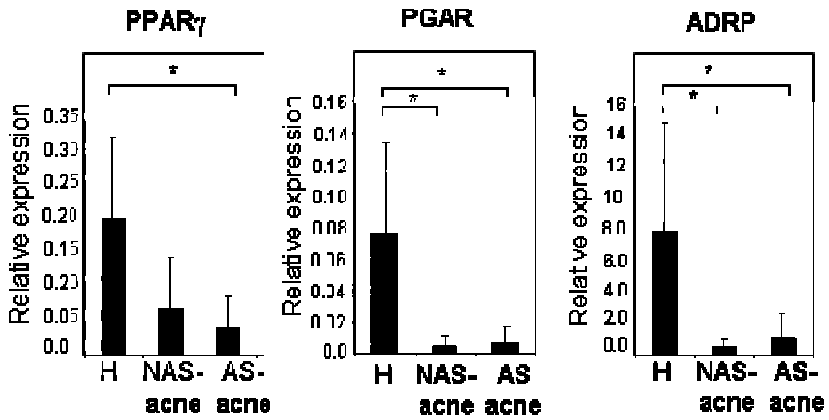


Figure 16. a-c Comparative protein and mRNA expression patterns of PPARγ in human normal sebaceous glands (SG) from healthy individuals (ctrl) n=5, non-acne involved (NAS-acne) n=9 and acne involved (AS-acne) SG n=9 from acne patients. a,b IHC: Tyramin amplification based immunostaining. The immunostained slides were counterstained with methyl-green or Meyers hematoxyllin, then evaluated semi-quantitatively for % PPARγ positive (black arrow) sebocytes of sebaceous glands, as: -, negative; *1*+, 0-20%; *2*+, 20-50%; *3*+, 50-75%; *4*+, 75-100%. c, Laser-capture microdissection of SG cells. mRNA expression determinated by RT-qPCR for PPARγ,

PGAR and ADRP. Both protein and mRNA expression of these molecules were decreased in acneic and non acneic samples of acne patients, compared to healthy controls. Data were analyzed using a two tailed un-paired t-test and *P<0.05 values were regarded as significant differences. In addition, statistical differences were further verified using 1-way ANOVA with Bonferroni and Dunnett post hoc probes, resulting in similar results. In real-time quantitative PCR experiments, the mean and SD were calculated for both the normalized and the normalizer values for all experiments. Normalizing gene is human cyclophyllin gene. Data shown are mean+-SD of 5 (n=5) or 9 (n=9) independent determinations performed in triplicate. P<0.05 vs control.

Then, SGs were in situ isolated from the cryosection samples by laser-microdissection technique (Figure 16. c) and mRNA level of PPARγ was examined from the collected cells using quantitative real-time PCR (RT- qPCR). The expression of mRNA – PPARγ, PGAR and ADRP was higher in SGs from healthy individuals, than in NAS and AS samples, respectively (Figure 16.c). These data suggest that PPARγ and its target genes are present in healthy SGs of acne patients and in SGs of age matched healthy donors.

8. Various eicosanoids are present in skin of acne patients

We determined the level of various PPARγ-activating eicosanoids in skin biopsies from patients. Due to limited amount of tissue available, we pooled 3 skin samples of 3 healthy donors, 3 NAS - acne and 3 AS-acne samples from 3 patients. 2 sets of data were analyzed. The level of PPARγ-activating eicosanoids was lowest in healthy skin samples, while NAS-acne skin samples had higher and AS-acne samples had the highest level of eicosanoids (Figure 17.). This might suggest, that the level of

PPARγ-activating eicosanoids are sufficient or even present in access in SG of acne patients, maybe due to the low level of PPARγ and feedback regulation in SG samples from acne patients.

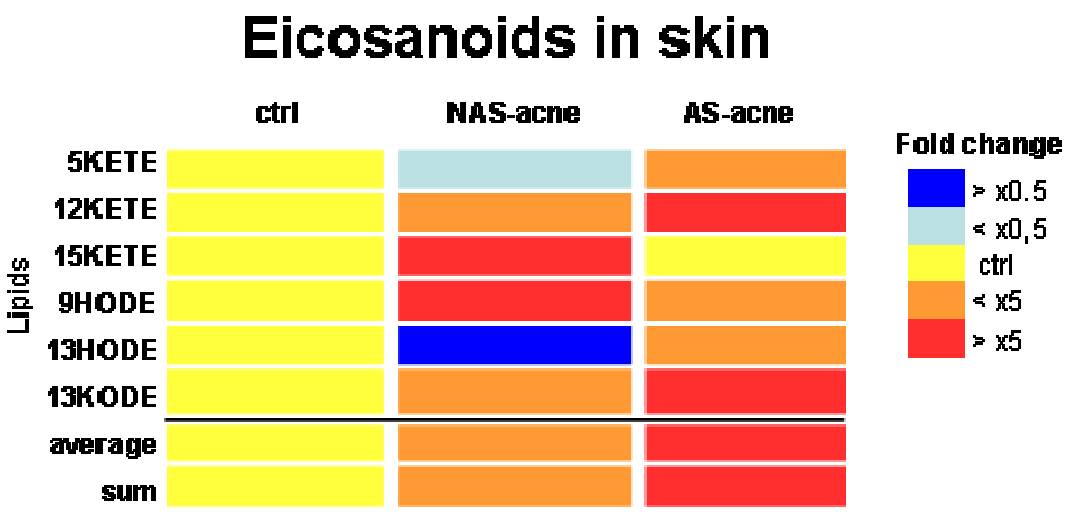


Figure 17. HPLC MS-MS analysis of eicosanoids in whole skin samples: healthy individuals (ctrl), non-acne involved (NAS-acne) and acne involved (AS-acne) skin from acne patients. Eicosanoids were elevated in NAS-acne, and even higher level in AS- skin of acne patients, compared to controls.3 samples were pooled and measured. Heat map reflecting of concentrations, color bar defines concentration fold change. Two sets of samples were measured in triplicates; data shown are average result of one set of samples. Measurements were made by Johanna Mihály and Ralph Rühl, University of Debrecen.

XI. DISCUSSION

Previous studies have shown that PPARγ is expressed in normal SG, in primary sebocytes and in SZ95 sebocytes (Chen *et al.*, 2003; Downie *et al.*, 2004; Kim *et al.*, 2001; Kliewer *et al.*, 1997; Michalik and Wahli, 2007; Rosenfield *et al.*, 1999; Thuillier *et al.*, 2002; Trivedi *et al.*, 2006; Zouboulis, 2003). However, very little is known on the expression level of this important transcription factor in situ. We intended to extend these studies by using laser microdissection. We found that less differentiated sebocytes express lower levels of PPARγ and its target genes as compared to more differentiated sebocytes (Figure 10.a). This was confirmed by using established markers of cell cycle activity and differentiation such as Ki67, p63 and AR molecules. Co-staining of Ki67 and PPARγ molecules showed that most of the cells which express Ki67 lack PPARγ expression and vice versa. AR was expressed mostly in basal and parabasal cell layers, and showed an overlap with PPARγ expression in the parabasal layer. We also compared the expression of these molecules in sebaceous hyperplasia and found that the expression of PPARγ and the other molecules detected were essentially the same as detected in normal SG. In contrast, the proportion of cells expressing PPARγ in sebaceous adenoma and sebaceous carcinoma is lower, but the proportion of Ki67, and p63 positive cells is higher (Figure 10.a). Recently, Cottle at al. described the expression of AR (basal, parabasal layer), Ki67 (some is basal layer) and PPARγ (intermediate, i.e.

outer central layer) in healthy SG of murine origin and gathered similar findings with AR in SG tumors. Additionally, the differentiation stage of carcinomas appeared to correlate with the presence of PPARγ in tumor cells (Figure 10.). In other words more differentiated tumors had higher PPARγ levels if compared to the ones with less differentiated cells. These findings are consistent with the results obtained using uroepithelial carcinoma cells, and also estrogen/progesterone receptor expression in breast cancers, in which the presence of PPARγ changed in line with differentiation state of carcinoma cells (Nakashiro et al., 2001; Payne et al., 2008). This suggests that PPARγ might be a reliable diagnostic marker of sebocyte differentiation state. In addition, we detected that PPARγ and its target genes ADRP and PGAR were expressed lower in sebocytes of AS from acne patients compared to sebocytes from healthy individuals. Interestingly, the receptor and its targets were detected at low level in non-acne involved SG from acne patients. These results appear to be consistent with the established anti-inflammatory role of PPARγ observed on inflammatory bowel disease (Mastrofrancesco *et al*., 2013) and also observed in lichen planopilaris (Price, 2011) and indicate that the inflammatory milieu is likely to contribute to reduced receptor levels and signaling.

In SZ95 sebocytes, one of the best available model systems for assessing human sebocyte function (Zouboulis *et al.*, 1999), we confirmed our previous results based on the observation that PPARγ mediated the expression of ADRP and PGAR target genes (Figure 11.) (Dobrosi *et al*, 2008). Nevertheless, this finding

only confirms the transcriptional regulatory event carried out by PPARγ and does not provide evidence regarding protein levels and/or functional roles for these genes in sebocytes. Using a model of AA-treated differentiated sebocytes (Alestas *et al.*, 2006; Wrobel *et al.*, 2003), we observed that the lipid rich SZ95 sebocytes expressed high levels of PPARγ and also its target genes PGAR and ADRP (Figure 10.), consistent with our previous finding, where the AEA (endocannabinoid) induced differentiation/lipid production was shown to be induced via PPARγ (Dobrosi *et al*, 2008). Also, the RXR agonist LG100268 induced ADRP and PGAR mRNA elevation. These data collectively suggest that both PPARγ and RXRα are active in human SZ95 sebocytes, and possibly are heterodimer partners, or act on the same molecular pathways synergistically (Figure 11.).

Prior art has shown that RSG-activated PPARγ has a role in sebaceous lipid production, a marker of sebocyte differentiation (Trivedi *et al.*, 2006). In our study both the natural ligand precursor, AA, and the synthetic ligand, RSG, induced sebaceous lipogenesis (Figure 12.b) similar to previous results (Trivedi *et al.*, 2006). In addition, we offer pharmacological evidence indicating that the specific PPARγ antagonist GW9662 dose - dependently inhibited this AA mediated target gene induction (Figure 13.c). This suggests that AA/ or its derivates activated PPARγ-induced gene activation coincides with neutral lipid production of sebocytes. Therefore, we analyzed the composition of neutral lipids, which are the major components of the sebaceous lipid mixture, along with membrane lipid species (Figure 13. and 14.). We observed that TG levels were significantly

elevated by AA, and this effect was inhibited by PPARγ antagonist by ca. 25%. This finding indicated that the elevation of TG by AA depended partly upon PPARγ activation. AA takes part in the TG formation as demonstrated by the elevation of AA-containing species among the total TG. Such experimental design allowed for the simultaneous investigation of the PPARγ activating and neutral lipid inducing properties of AA. The PPARγ inhibitor GW9662 also lowered the basal level of CH; however AA did not have an effect on cholesterol level. It is likely that, under our experimental conditions, AA caused remodelling of lipid species bearing fatty acid side chains, such as GLP and TG, and affected minimally the non-esterified lipid components.

It was described that the PPARγ ligand RSG induces TG production in sebocytes, however RSG also induced cholesterol accumulation, which was not prevented by an inhibitor GW9662 (Trivedi *et al*, 2006). Taken together, these data suggest that TG and cholesterol production is likely to be regulated by PPARγ in sebocytes, however AA in the dose we used had no effect on cholesterol levels. It is also likely that pathways leading to neutral lipid formation were regulated by AA in a selective manner.

In addition, we found that AA treatment also causes PPARγ dependent elevation of some minor lipids, like LPC and PIs (Figure 14.). In sebocytes, LPC is a minor phospholipid in membranes of lipid droplets consist of monolayer phospholipids (Corsini et al., 2003). Lipid droplets in a wide range of cell lines (i.e. adipocytes, keratinocytes) were shown to be involved both in deposition and

storage of neutral lipids (Brasaemle et al., 1997; Heid et al., 1998), and were shown to have ADRP protein on their surface. These data might indicate that PPARγ is involved in the formation of these lipid droplets, i.e. neutral lipid storage or production.

AA also significantly decreased the level of PIs in a PPARγ-dependent manner suggesting that enzymes, which are involved in the synthesis/degradation of PIs are also influenced by the nuclear receptor activation. The altered level of PIs, based on their versatile roles in various biological processes, might influence several functions of sebocytes, i.e. PIs are essential phospholipids in the formation of cellular membranes. Moreover, one group of their metabolic products, phosphoinositides, are key signal transduction molecules. The IP3 kinase pathway has a common part with the MAPK induced pathway, which was shown to be involved in AEA induced sebaceous lipid synthesis, in which PPARγ also played a role (Dobrosi *et al*., 2008).

Phospholipase A_2 (PLA2) is the enzyme type responsible for hydrolyzing fatty acids like AA from glycerophospholipids (Scott et al., 2007). Therefore, they represent the source for the AA production. PLA2 was detected in various cells of skin and in SZ95 sebocytes (Schagen *et al.* 2008). In addition it was shown, that treatment with active PLA2 increased neutral and polar lipid production of sebocytes (Schagen *et al.* 2008). Therefore, it seems possible, that PLA2 is one regulator of endogenous AA production in sebocytes, and it also determines the level of PPARγ activation in these cells at least in part. The identification of

endogenous AA metabolites (such as 5-KETE, 12-KETE) achieved in this study supports this scenario. In addition, the reduction of the PIs level also suggests that there is a feedback mechanism aimed at the regulation of AA production if it exogenously added.

Although PPARγ probably controls both the levels of major neutral lipids and level of LPC and PIs, the exact mechanism of action in sebaceous differentiation remains to be discovered.

Another key goal of our study was to identify novel endogenous ligands, which might activate PPARγ in sebocytes. Various AA metabolites have been described as natural PPARγ ligands in the skin (Weindl at al., 2005), adipocytes (Oster et al., 2010), cardiomyocytes (Hovsepian et al., 2009) and in several cancers, including hepatocellular carcinoma cells (Maggiora, Oraldi, Muzio, & Canuto), non-small cell lung cancer (Li et al., 2012) and colon carcinoma cells (Dionne et al., 2010). Here, we show that the AA keto-metabolites (5-KETE and 12-KETE) act as endogenous PPARγ ligands in SZ95 sebocytes as they elevated sebaceous lipid synthesis in a PPARγ-dependent manner (Figure 16.b-d). Our data demonstrated that the same compounds are present in acne affected skin and their levels are increased. Therefore, it is tempting to speculate that this might be the part of a feed back regulation. However, other lipids or cytokines might contribute to regulation as well (Alestas et al., 2006). Nevertheless, our findings suggest that PPARγ might play a protective role in normal human sebocytes against excessive lipid accumulation and induction of inflammatory responses. In the skin of acne

patients, PPARγ is lower or even absent in inflammed sebaceous glands, which appear to be a major feature and maybe the reason of disregulated lipid homeostasis and inflammation. Confirming this theory, it is known that topical application of the PPARγ agonist azelaic acid transmits anti-inflammatory effects in acne vulgaris (Sieber & Hegel, 2014), which also supports the possible anti-inflammatory role of PPARγ in sebocytes.

XII. SUMMARY

SG produces 80% of lipids on the surface of the skin. These lipids play major role in epidermal barrier. Therefore it is a key question in skin research, how SG is regulated. In my work presented here, it is clearly shown, that PPARγ is differentially expressed in normal and pathological human sebocytes, and appears to have roles in their differentiation and lipid production.

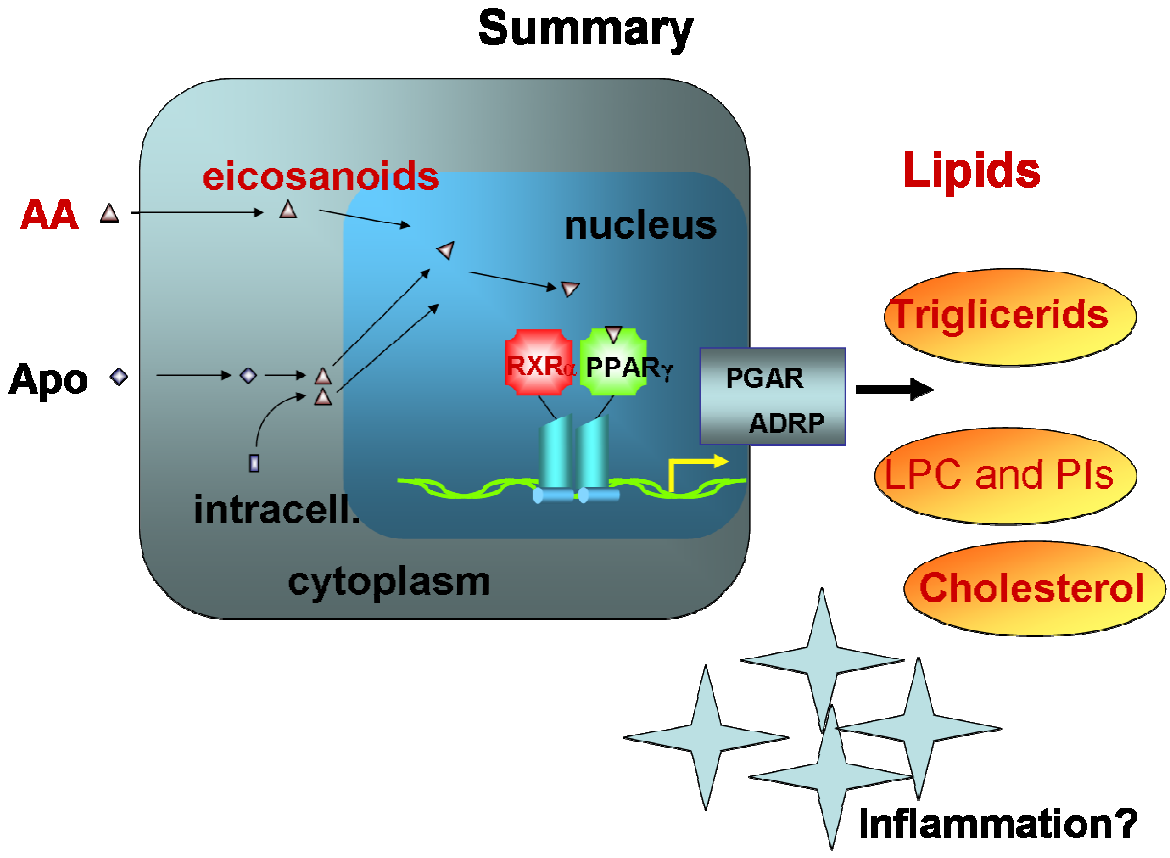


Figure 18. Summary. Role of PPARγ in sebocytes. Our findings are marked with red letters.

PPARγ and its target genes, ADRP and PGAR, expressed in sebocytes and shown association with SC differentiation. PPARγ is also present in normal and

hyperplastic SG, whereas its expression levels were decreased in cases obtained from SG adenoma and SG carcinoma cells, reflecting a maturation-linked expression pattern. Our data also clearly show that PPARγ levels and signaling activity in sebocytes from acne patients is decreased. Furthermore, in SZ95 sebocytes, naturally occurring lipids including arachidonic acid and arachidonic acid keto-metabolites (e.g. 5-KETE, 12-KETE) appear to regulate PPARγ signaling pathways, which in turn modulate phospholipid biosynthesis and induce neutral lipid synthesis (Figure 18.). Collectively, our findings highlight the importance of endogenous ligand-activated PPARγ signaling in human sebocyte biology and suggest that PPARγ might be a promising candidate for the clinical management of SG disorders.

XIII. NEW FINDINGS

1. PPARγ protein and mRNA is present at high level in sebaceous gland of skin from healthy individuals, but its expression is low in SG of sebaceous carcinoma and acneic SG. PPARγ protein expression is changed in line with sebocyte differentiation state in normal SG and SG with proliferative diseases.

2. PPARγ activity is indicated by the mRNA expression of its target genes like the adipose differentiation related protein (ADRP) and PPARγ angioprotein related protein (PGAR) in sebocytes.

3. PPARγ is activated by various eicosanoids in SG, namely 5-KETE, 12-KETE and 15-KETE.

4. PPARγ activity regulates production of TG, a major lipid in SG. Elevation of TG by AA depended partly upon PPARγ activation. Among polar lipids, LPC and PIs were regulated by PPARγ.

5. ADRP and PGAR levels,- referring to PPARγ activity - are low in sebocytes of acne patients, compared to normal SG.

6. PPARγ activator ligand eicosanoids are presented at high level in skin of acne patients.

7. Our data suggest that PPARγ might play a protective role in normal human sebocytes of healthy individuals against excessive lipid accumulation and inflammatory responses, which makes this molecule a possible therapeutic target in acne vulgaris.

XIV. ÖSSZEFOGLALÁS

A faggyúmirigyek termelik a bőrfelszíni lipidek 80%-át. Ezek a lipidek az epidermális barrierben fontos szerepet játszanak. Ezért a dermatológiai kutatások egyik kulcskérdése a faggyúmirigyek szabályozásának megismerése. Munkánkban a PPARγ molekula szerepét vizsgáltuk faggyúmirigysejtekben. A PPARγ molekula expressziója eltér az ép és a kóros sebocytákban, a molekulnak szerepe van a sebocyták differenciációjában és lipid termelésében. A PPARγ molekula és célgénjei, az ADRP és a PGAR gének expresszálódnak a sebocytákban, expressziós szintjük a differenciált sejtekben magasabb, mint az alacsonyan differenciált sebocytákban. A PPARγ molekulát kimutattuk ép és hyperplasiás faggyúmirigyekben, itt azonos szinten expresszálódnak, míg a faggyúmirigy adenoma és carcinoma sejtekben expressziójuk csökken, ez szintén azt támasztja alá, hogy a PPARγ molekula expressziója a sejt érettségétől függ. Emellett, eredményeink szerint a PPARγ molekula és célgénjei az acne vulgarisban szenvedő betegek faggyúmirigysejtjeiben alacsonyabb szinten expresszálódnak, mint az ép sebocytákban.

SZ95 immortalizált sebocytákban kimutattuk, hogy az endogén jelenlévő arachidonsav és arachidonsav keto-metabolitok (5-KETE, 12-KETE) a PPARγ molekula jelátviteli útvonalait szabályozzák, melyek befolyásolják a sejtek foszfolipid bioszintézisét és indukálják a neutrális lipid szintézisét. Eredményeink bemutatják, hogy az endogén-ligandok által szabályozott PPARγ molekulnak

kulcsszerepe van a sebocyták működésében, és így ígéretes gyógyászati célpont a faggyúmirigy betegségeinek kezelésében.

XV. LIST OF KEYWORDS

PPARγ, laser microdissection, sebaceous gland, eicosanoid, arachidonic acid, sebaceous tumor, acne vulgaris

XVI. KULCSSZAVAK

PPARγ, lézermikrodisszekció, faggyúmirigy, eikozanoid, arachidonsav, faggyúmirigy eredetű tumor, acne vulgaris

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XIX. LIST OF PUBLICATIONS RELATED TO DISSERTATION AND OTHER PUBLICATIONS

UNIVERSITY OF DEBRECEN
UNIVERSITY AND NATIONAL LIBRARY

Registry number: DEENK/218/2016.PL
Subject: PhD Publikációs Lista

Candidate: Anikó Dózsa
Neptun ID: AXTEDX
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Dózsa, A.**, Mihály, J., Dezső, B., Csizmadia, É., Keresztessy, T., Markó, L., Rühl, R., Remenyik, É., Nagy, L.: Decreased peroxisome proliferator-activated receptor [gamma] level and signalling in sebaceous glands of patients with acne vulgaris.
   Clin. Exp. Dermatol. 41 (5), 547-551, 2016.
   DOI: http://dx.doi.org/10.1111/ced.12794
   IF: 1.315 (2015)
2. **Dózsa, A.**, Dezső, B., Tóth, I. B., Bácsi, A., Póliska, S., Camera, E., Picardo, M., Zouboulis, C. C., Bíró, T., Schmitz, G., Liebisch, G., Rühl, R., Remenyik, É., Nagy, L.: PPARγ-Mediated and Arachidonic Acid-Dependent Signaling is Involved in Differentiation and Lipid Production of Human Sebocytes.
   J. Invest. Dermatol. 134 (4), 910-920, 2014.
   DOI: http://dx.doi.org/10.1038/jid.2013.413
   IF: 7.216

List of other publications

3. Paulenka, K., **Dózsa, A.**, Mórocz, I., Barna, T., Károlyi, Z.: Sister Mary Joseph csomó: colon adenocarcinoma metastasis a köldökben.
   Bőrgyógyász. Venerol. Szle. 92 (2), 82-84, 2016.
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4. **Dózsa, A.**, Károlyi, Z., Mórocz, I.: Sutton naevust utánzó melanoma.
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5. Tsakiris, I., Törőcsik, D., Gyöngyösi, A., **Dózsa, A.**, Szatmári, I., Szántó, A., Soós, G., Nemes, Z., Igali, L., Márton, I., Takáts, Z., Nagy, L., Dezső, B.: Carboxypeptidase-M is regulated by lipids and CSFs in macrophages and dendritic cells and expressed selectively in tissue granulomas and foam cells.
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12. Kemény-Beke, Á., Facskó, A., **Dózsa, A.**, Szatmári, I., Aradi, J., Berta, A.: Szemészeti tumorok telomerázaktivitása I.
Szemészet. 139 (1), 55-59, 2002.

Total IF of journals (all publications): 27,328
Total IF of journals (publications related to the dissertation): 8,531

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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XX. SUPPLEMENTUM

1. Table

Summary of immunostaining and differetiation grade of sebocytes in the lesion. Samples were evaluated semi-quantitatively for distinct sebaceous glands /SG tumors, reflecting the % of positive cells: -, negative; *1*+, 0-20%; *2*+, 20-50%; *3*+, 50-75%; *4*+, 75-100%.

Sample ID	Classification	PPARγ	P63	AR	Ki67	Differentiation grade of sebocytes in the lesion	Age, Gender (m:male, f:female)	localization
#1	normal	3+	1+	3+	1+	very high	64m	Head
#2	normal	3+	1+	3+	1+	very high	75m	Head
#3	normal	3+	1+	3+	1+	very high	68m	Head
#4	normal	3+	1+	3+	1+	very high	67m	Head
#5	normal	3+	1+	3+	1+	very high	72m	Head
#6	normal	3+	1+	3+	1+	very high	70m	Head
#7	normal	3+	1+	3+	1+	very high	67m	Head
#8	normal	3+	1+	3+	1+	very high	74m	Head
#9	normal	3+	1+	3+	1+	very high	56f	Head
#10	normal	3+	1+	3+	1+	very high	58m	Head
#15	sebaceus hyperplasia	3+	1+	3+	1+	very high	45m	Head
#16	sebaceus hyperplasia	3+	1+	3+	1+	very high	52m	Head
#17	sebaceus hyperplasia	3+	1+	3+	1+	very high	64m	Head
#18	sebaceus hyperplasia	3+	1+	3+	1+	very high	60f	Head
#19	sebaceus hyperplasia	3+	1+	3+	1+	very high	87m	Head
#20	sebaceus hyperplasia	3+	1+	3+	1+	very high	66f	Head
#21	sebaceus hyperplasia	3+	1+	3+	1+	very high	74m	Head
#22	sebaceus hyperplasia	3+	1+	3+	1+	very high	73m	Head
#23	sebaceus hyperplasia	3+	1+	3+	1+	very high	65f	Head

Sample ID	Classification	PPARγ	P63	AR	Ki67	Differentiation grade of sebocytes in the lesion	Age, Gender (m:male, f:female)	localization
#25	sebaceus adenoma	2+	3+	2+	2+	high	52m	Head
#26	sebaceus adenoma	2+	3+	2+	2+	high	52m	Back
#28	sebaceus adenoma	2+	3+	2+	2+	high	46m	Head
#29	sebaceus adenoma	2+	3+	2+	2+	high	61m	Head
#30	sebaceus adenoma	2+	3+	2+	2+	high	55m	Head
#31	sebaceus adenoma	2+	3+	2+	2+	high	55m	Back
#32	sebaceus adenoma	2+	3+	2+	2+	high	81m	Head
#33	sebaceus adenoma	2+	3+	2+	2+	high	63m	Head
#34	sebaceus adenoma	2+	3+	2+	2+	high	58m	Head
#36	sebaceus adenoma	2+	3+	2+	2+	high	46f	Head
#37	sebaceus carcinoma	1+	4+	1+	3+	moderate	46m	Head
#38	sebaceus carcinoma	0	4+	1+	4+	low	55m	Back
#39	sebaceus carcinoma	0	4+	2+	4+	low	86m	Head
#40	sebaceus carcinoma	0	4+	1+	4+	low	89f	Head
#41	sebaceus carcinoma	1+	4+	2+	3+	moderate	58m	Head
#44	sebaceus carcinoma	0	4+	0	4+	low	76m	Head
#45	sebaceus carcinoma	0	4+	1+	4+	low	56m	Head
#46	sebaceus carcinoma	0	4+	1+	4+	low	48m	Head
#47	sebaceus carcinoma	0	4+	1+	4+	low	57f	Head

2. Publications related to dissertation