Supplementary material

Material and methods

Skin collection. All facial skin material was obtained from previously taken diagnostic biopsies (Rosacea, Lupus) and plastic surgery (HS) at the Dept. of Dermatology, University Hospital Münster, Germany. The clinical diagnosis of rosacea subtypes was performed according to the classification system of the National Rosacea Society (Wilkin et al., 2002) Based on that we classified and investigated five different groups of patients: for morphometric stainings Erythemotelangiectic rosacea (ETR): 9 patients (3 male/ 6 female, mean age 56 years); Papulopustular rosacea (PPR), 9 patients (6 male/ 3 female, mean age 52 years), phymatous rosacea, 9 patients (7 male/ 2 female, mean age 67 years). The control groups consisted of 9 patients (1 male/ 8 female, mean age 44 years) suffering from lupus erythematodes (LU). 10 patients (6 male/ 4 female, mean age 56 years) whose facial skin was not affected were described as healthy skin (HS) by the dermatopathologist. For gene analytic studies four different groups were investigated: ETR (n= 11), PPR (n= 11) and PhR (n= 6) and HS (n=12, face). 4 mm punch biopsies were taken almost entirely from the paranasal area, controls form sun-unexposed facial skin (retroauricular), and embedded overnight within an automated closed system (Tissue-Teck® V.I.P., Vogel; Gießen, Germany). Permission for human studies was given by the Ethical Committee of the University of Muenster Germany, in accordance with the ethical standards of the 1964 Declaration of Helsinki.

Antibodies. The used antibodies for immunohistochemistry and double immuno-fluorescence are listed in S 1.

Double immuno-fluorescence. Histological staining with double immunofluorescence was completed according to standard protocol (Collins AB et al., 2002). Slides were pretreated with Target Retrieval Puffer pH 6,1 (S1699, DAKO, Germany) and incubated at 90° C for 40 Min. For double-immunoflu orescence, Anti-Protein Gene Product 9.5 antibody (Polyclonal-Rabbit Anti- PG 9.5, 1:100, Chemicon (Millipore) P09936) was incubated by 4℃ over night. Next day, slides were washed in PBS buffer and incubated with the secondary antibody Alexa Fluor 488 chicken anti-rabbit IgG (1:200, Molecular Probes, Inc) for 60 min by room temperature. For co-staining slides were performed together with additional antibodies (Tab.1) and were

incubated. Slides were washed with PBS buffer and again incubated with the secondary antibody Alexa Fluor 555 donkey anti-mouse IgG (1:200, Molecular Probes, Inc) for 60 min by room temperature. After washing in a dark chamber, slides were mounted in Vectashield (Vector, CA, USA). In controls, primary polyclonal antibodies were pre-incubated 24 - 48 h with corresponding Control Rabbit IgG (DAKO, Germany, X0936) to elucidate background staining.

Immunohistochemistry. 6-7 µm sections were cut from the paraffin blocs using a microtome (MicromInternational GmBH; Walldorf, Germany) and mounted on coated glass slides (SuperfrostPlus, Menzel; Braunschweig, Germany). The produced sections were dried in an oven at 60℃ for 30min. S hortly ahead of the immunostaining procedure the dried sections were deparaffinized in XEM200 2-times for 15 min each, then washed by graded alcohols (96%, 70%, 50%, 2-times, 10 min each) and finally rinsed in aqua bidest 2-times for 5 min. According to the characteristics of the different markers the necessity of section pretreatment is altered as is noted in Tab.1. Buffered Citraet pH 6: For heat-induced epitope retrieval, the tissues used for either CD31- or neurofilament-staining were immersed in a 1:10 dilution of concentrated target retrieval solution and heated in a water bath (95–99 ℃). Subsequently, the sections were cooled down to room-temperature and rinsed in PBS, pH 7.4.

Immunohistochemical stains were prepared using an automated system (Autstainer 480, LabVisionCorp., Fremont, USA) that employs the streptavidin-biotin method for labelling. After incubation with the respective primary antibody for 1 h (see Tab. 1), slides were washed with PBS containing 0.05% (v/v) Tween 20. Then, samples were incubated for 30 min with a biotinylated secondary antibody (PolyLink anti goat antibody). Labelling was performed applying a streptavidin-horseradish peroxidase conjugate (HRP-Label) for 30 min. Following thorough PBS rinsing (0.05% (v/v) Tween 20) for 3x15 min, the antibody localization was accomplished by incubation with 3-Amino-9-Ethylcarbazol (AEC-Substrat kit) for 15 min, optimized by prvious antibody dilution experiments.

Subsequently, sections were rinsed once with PBS (0.05% (v/v) Tween 20) and once with pure PBS, subsequently counterstained with hematoxylin (15sec., Mayers Hämalaun) and transferred to fresh tap water for 10 min to optimize cell nucleus staining for improved morphological distinction. Finally, specimen were

mounted with thin cover glass using either Kaiser's Glycerin gelantine (immunofluoreacence) or Vectashield embedding solution.

Image analysis. Using a 200x magnification five pictures were taken within each section from randomly chosen areas moving from epidermis to dermis. The positive stained area of the dermis was analyzed quantitatively by using specific image analysis software (Cell^D 2,6 (Build 1200), Olympus Soft Imaging Solutions GmbH; Münster, Germany). By previously defined color thresholds stained areas were detected and counted by particle analysis. The automated analysis was restricted to prior manually defined regions of interest (ROI) which contained all requested structures but excluded background irritations, defects of the tissue, epidermis, hair follicle and sebaceous glands.

For blood and lymph vessel investigation additional manual studies were performed. In order to avoid incorrect measurements through longitudinal sliced vessels only structures containing a lumen were chosen. Vessel circumference was evaluated by measurement of manually drawn outlines around the lumen of the vessels. Additionally, number of vessels per slide was counted.

Statistical analysis All statistical analysis was performed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, Illinois). Statistical significance was determined by using a Mann and Whitney U-Test. Differences were considered significant at a P-value below 0.05.

Quantitative rtPCR The samples have been placed in RNAlater (Qiagen Inc.) for preservation of the RNA. mRNA expression of the genes was evaluated using semi-quantitative PCR technology (qRT-PCR – Taqman Low Density Arrays). Total RNA was extracted using RNeasy extraction kits (Qiagen Inc.) according to manufacturer's protocol. RNA Quantity was measured using Quant-it RNA assay kit (Molecular Probes) and the quality was monitored by following the electrophoresis behaviour of RNA using a 2100 Bioanalyser (Agilent). 800 ng of extracted RNA of good quality [RNA indication number (RIN) ≥ 7] was then used for synthesizing cDNA using high capacity cDNA archive kits (Applied Biosystems).

TaqMan low density array (TLDA) analysis. Gene expression analysis was performed using TLDA arrays containing PCR primers for genes of interest and housekeeping genes. A triplicate determination was performed for each sample.
Used ready-to-use TaqMan® Gene Expression Assays are listed in S2.

Synthesized cDNA (50ng of cDNA per column) was added to the PCR master mix, and the mixture was loaded by centrifugation into the wells of the array containing the lyophilized primer sets (Applied Biosystems). The wells were sealed and the PCR reactions performed on ABI 7900HT (Applied Biosystems). PCR threshold cycle (Ct) numbers at which the fluorescent signal of the generated nascent DNA exceeds a threshold value were determined. The Ct number of a given gene in a given sample was normalized by first subtracting the average Ct of the housekeeping genes (GAPDH, ACTB, HPRT) in the same sample, and then adding back the average Ct of the housekeeping genes across all samples.

Statistical analysis. The fold modulation of gene expression of rosacea samples versus samples of healthy volunteers was defined as $2^{(\text{mean CtHV} - \text{mean CtRo})}$, with Ct_{HV} and Ct_{Ro} depicting the Ct values of healthy volunteer and rosacea samples, respectively.

To identify genes that were significantly modulated in the different Rosacea subtype samples, one-way ANOVA with Benjamini-Hochberg multiplicity correction was performed using JMP 7.0.1 (SAS Institute) and irMF 3.5 (National Institute of Statistical Sciences, NISS) software.

Antibody	*Species*	*Dilution*	*Pretreatment*	*Source*	*Localization*	*Remark*
IMMUNOHISTOCHEMISTRY						
CD 31 (PECAM-1)	Monoclonal Mouse Anti-Human	1:200	Citrate pH 6	DAKO, Denmark M0823	Endothelial cells	Weak reaction with monocytes, thrombocytes and lymphocytes
Neurofilament		1:50	Citrate pH 6	DAKO, Denmark M0762	Central and peripheral axons (neurons)	Recognizes myelinated nerves
Podoplanin (D2-40)		1:300	BSA	DAKO, Denmark M3619	Lymphatic endothelial tissue	Fetal germ cells
Tryptase		1:2000	BSA, Tween-20	Chemicon (Millip.),US MAB1222	Mast cell tryptase	Low reactivity to basophils
Vimentin		1:100	BSA	DAKO, Denmark M0725	Cells of mesenchymal origin (fibrocytes)	Reactivity to smooth muscle cells and lipocytes
DOUBLE IMMUNOFLUORESCENCE						
PGP9.5	Polyclonal Rabbit Anti-Human	1:100	Heat induced epitope retrieval, pH 6,1	Chemicon (Millip.),US P09936 .	Neurons and cells of neuroendocrine system	Recognizes unmyelinated nerves
CD31 (PECAM-1)	Monoclonal Mouse Anti-Human	1:50		DAKO, Germany M0823; Clone JC70A	Blood endothelial cells	Weak reaction with monocytes, thrombocytes and lymphocytes
Podoplanin (D2-40)		1:50		DAKO, Germany M3619	Lymphatic endothelial tissue	Fetal germ cells
Tryptase		1:300		DAKO, Germany M7052	Mast cell tryptase	Low reactivity to basophils
SMA		1:300		DAKO, Germany M0851; Clone 1A4	Myofibroblasts	Smooth muscle cells, myoepithelial cells

S 1. Antibodies used for Immunohistochemistry and double immunofluorescence