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Neurovascular and Neuroimmune Aspects in the Pathophysiology of Rosacea

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Abstract

Rosacea is a common skin disease with a high impact on quality of life. Characterized by erythema, edema, burning pain, immune infiltration, and facial skin fibrosis, rosacea has all the characteristics of neurogenic inflammation, a condition induced by sensory nerves via antidromically released neuromediators. To investigate the hypothesis of a central role of neural interactions in the pathophysiology, we analyzed molecular and morphological characteristics in the different subtypes of rosacea by immunohistochemistry, double immunofluorescence, morphometry, real-time PCR, and gene array analysis, and compared the findings with those for lupus erythematosus or healthy skin. Our results showed significantly dilated blood and lymphatic vessels. Signs of angiogenesis were only evident in phymatous rosacea. The number of mast cells and fibroblasts was increased in rosacea, already in subtypes in which fibrosis is not clinically apparent, indicating early activation. Sensory nerves were closely associated with blood vessels and mast cells, and were increased in erythematous rosacea. Gene array studies and qRT-PCR confirmed upregulation of genes involved in vasoregulation and neurogenic inflammation. Thus, dysregulation of mediators and receptors implicated in neurovascular and neuroimmune communication may be crucial at early stages of rosacea. Drugs that function on neurovascular and/or neuroimmune communication may be beneficial for the treatment of rosacea.

INTRODUCTION

Rosacea is a common chronic inflammatory skin disease primarily characterized by transient or persistent facial erythema, telangiectasia, papules, pustules and/or edema, and burning pain, possibly resulting in fibrotic, phymatous rosacea (Marks, 1989; Wilkin *et al.*, 2002;

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CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

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Powell, 2005). As the etiology and pathogenesis remains uncertain, treatment mainly targets the symptoms instead of modulating the pathophysiological process (Crawford *et al.*, 2004).

Four different subtypes have been defined based on typical clinical characteristics: erythematotelangiectatic rosacea (ETR), papulopustular rosacea (PPR), phymatous rosacea (PhR), and ocular rosacea (Wilkin *et al.*, 2002).

Endogenous key factors of this complex pathogenic interplay—blood vessels, lymphatic vessels, fibroblasts (FBs), and cells of the immune system—have been identified (Marks, 1969; Jansen and Plewig, 1997; Aroni *et al.*, 2004; Crawford *et al.*, 2004; Gomaa *et al.*, 2007). However, the underlying mechanisms of onset and maintenance of molecular and cellular alterations, and the connection between involved cells, are not yet understood (Crawford *et al.*, 2004).

Several triggers that exacerbate rosacea have been identified: UV radiation, temperature changes (heat and cold), chemical irritation, strong emotions, alcoholic beverages, and spicy food (Jansen and Plewig, 1997), and probably microbial agents (Lacey *et al.*, 2007; Whitfeld *et al.*, 2011). Rosacea patients appear susceptible to certain banal stimuli. This modification of cutaneous sensitivity indicates the relevance of the sensory and/or autonomic nervous system in the pathogenesis of the disease (Steinhoff *et al.*, 2011; Guzman-Sanchez *et al.*, 2007). Interestingly, neurogenic inflammation, a condition evoked by released neuropeptides after stimulation of sensory nerve endings (Cevikbas *et al.*, 2007), resembles clinical features of rosacea such as local erythema, edema, hyperemia, and recruitment of leukocytes to the site of inflammation. In addition, stimulation of mast cells (MCs) by neuropeptides and consecutive release of histamine, tryptase, and other mediators mediates inflammation, as well as itchy and/or burning sensations (Steinhoff *et al.*, 2000; Arck *et al.*, 2006; Ikoma *et al.*, 2006).

With respect to sensory and autonomic nerves in this disease, one problem is that the RNA is harbored in ganglia and is therefore not detectable by skin biopsies. Another limiting factor is that the local concentration of released neuromediators is often under the detection limit of protein assays or immunohistochemistry. Therefore, little investigation in this aspect of rosacea has been made until now. For a comprehensive approach, we analyzed the neurovascular and neuroimmune alterations at different clinical stages of rosacea, both on the RNA and protein level by quantitative real-time RT-PCR (qRT-PCR), and by immunohistochemistry using markers for nerves, blood vessel, endothelial cells, lymphatic vessels, FBs, and MCs.

RESULTS

Anatomical association of sensory nerves, MCs, and blood vessels in rosacea

To determine the anatomical association of facial unmyelinated sensory nerves with the vascular and immune system (MCs) in rosacea, we performed double immunofluorescence (IF, Figure 1). A close colocalization was observed between PGP9.5-positive sensory nerves and blood vessels, as well as MCs. Some myofibroblasts were colocalized with free nerve endings. Lymphatic vessels were rarely colocalized with unmyelinated nerves.

Marked vasodilatation, not angiogenesis, in rosacea

The aim of this study was to quantify vessel number and circumference in rosacea. Staining with CD31, a marker for blood vessel endothelium, showed grossly dilated vessels in all subtypes of rosacea (Figure 2a–h). Morphometrical analysis demonstrated statistically significant enhancement of CD31-positive tissue in ETR ($P<0.01$) and PhR ($P<0.05$), as well as perimeter enlargement ($P<0.05$). The number of vessels was not increased

significantly when compared with healthy skin (HS). Accordingly, gene array studies combined with qRT-PCR revealed no upregulation of angiogenic key genes in ETR and PPR and only slight changes in PhR (Figure 6a).

Dilatation of lymphatic vessels, not lymphangiogenesis, in rosacea

The task was to determine the number and circumference of lymphatic vessels in rosacea. Staining with podoplanin, a marker for lymphatic vessels, showed vasodilation in all subtypes (Figure 2i–p). Morphometric analysis showed a statistically significant augmentation in vessel surface ($P<0.01$) and perimeter ($P<0.05$) exclusively in ETR. The number of vessels was not altered significantly in any subtype of rosacea. Investigation of lymphangiogenic key genes by gene array analysis and qRT-PCR revealed upregulation of podoplanin in all subtypes, whereas *LYVE1* was down-regulated and *VEGFC* or *PROX1* were unchanged when compared with HS (Figure 6b).

Slightly enhanced number of myelinated nerve fibers in subtypes of rosacea

We next sought to address the question of whether myelinated (neurofilament-positive, NF-200) and/or unmyelinated (PGP9.5-positive) nerve fibers are anatomically closely associated with other skin structures in rosacea skin in comparison with lupus erythematosus (LE) and HS (Figure 3). Unfortunately, PGP staining is hard to interpret by light microscopy because of the thin, irregular positive staining under these conditions. Here, IF is the better technique. Therefore, we first determined whether myelinated nerves, which are ultimately involved in pain transmission, are found in increased numbers in rosacea patients. Double IF results for unmyelinated nerves (PGP9.5) were analyzed qualitatively and showed close anatomic association (Figure 1a–d, other data not shown). Staining with NF-200 showed a higher density of neural structures, especially in the upper dermis (Figure 3). Morphometrical analysis demonstrated an increase of nerves, especially in ETR, but not PPR and PhR. Although clear differences in nerve numbers were observed between rosacea subtypes and HS, the data did not reach statistical significance.

Our analysis of expression levels of genes involved in neurovascular and neuroimmune interactions revealed upregulation in pituitary adenylate cyclase-activating polypeptide (PACAP), *vasoactive intestinal peptide (VIP)*, HTR3A (5-hydroxytryptamine (serotonin) receptor 3A), *nerve growth factor beta, adrenergic, alpha-1D-, receptor (ADRA1D)*, *adrenomedullin 2*, and *cathelicidin antimicrobial peptide* (Figure 6c). Downregulation was detected in *VIP receptor-1*, *PACAP receptor-1*, *nerve growth factor receptor (trkA)*, *kallikrein-related peptidase-5*, diverse adrenergic receptors *(ADRB2/3, ADRA2C/1B)*, *neuropeptide Y*, and *tachykinin*, *precursor 1*/*substance P*.

Increased MC numbers in all subtypes of rosacea

MCs are functionally closely associated with both blood vessels and nerves, and altogether form a so-called microvascular unit (Steinhoff *et al.*, 2003). We sought to determine the number of MCs in the various subtypes of rosacea, and to analyze the potential increase of MC associated with vascular structures, nerves, and FBs (Figure 4).

Quantitative analysis of tryptase staining revealed a statistically significant increased density of MC in all subtypes of rosacea, especially in PPR ($P<0.05$) followed by ETR ($P<0.01$) and PhR ($P<0.05$).

To analyze the genes associated with MC function, we investigated the mRNA expression levels of histamine receptors (HRH1–4; Figure 6d). Our gene array data showed upregulation of *HRH2* and *HRH3*. Gene expression of *HRH1* was not altered, and *HRH4* was downregulated.

Increased staining of Vimentin-positive cells in rosacea

Fibrocytes/blasts have an essential role in the induction of skin fibrosis. Quantitative analysis of vimentin staining, a marker for fibrocytes/blasts and mesenchymal structures of blood vessels, demonstrated a statistically significant higher density of FB in PPR ($P<0.01$), followed by ETR ($P<0.05$) as compared with controls (Figure 5). Accordingly, qRT-PCR showed strong upregulation of genes involved in matrix remodeling as well, especially in PPR and PhR. Upregulation of *matrix metalloproteinase (MMP)-1* and *-12* was strongest, followed by upregulation of *MMP-10*, *-3*, and *-9*. All MMP inhibitors were downregulated at the RNA level (Figure 6e).

DISCUSSION

The involvement of neuroimmune and neurovascular communication in the pathophysiology of rosacea is currently indicated only by clinical observation. Therefore, we examined the morphological and molecular correlation of various cells involved in the pathophysiology of rosacea and their specific pattern in the various subtypes of rosacea as compared with LE and HS. Our results show a close anatomic association of sensory nerves, blood vessels, and immune cells, as well as signs of neuroimmune and neurovascular communication such as vasodilation, rather than angiogenesis, dilated lymphatic vessels, and a strong increase in MC and FB numbers. In accordance with that finding, our qRT-PCR data demonstrate upregulation of receptors that are targets for mediators released by MC or sensory nerve endings. Taken together, our results strongly suggest substantial neurovascular and neuroimmune interaction in the pathophysiology of rosacea. In comparison with rosacea, LE also showed increased vasodilatation of blood vessels and lymphatic vessels, but no increase in nerves, MC, or FB numbers.

Our findings of significant vasodilatation in all subtypes of rosacea correlate well with symptoms such as flushing and erythema. Some authors proclaimed that angiogenesis has an important role in the pathogenesis, and suggested that the increase in vascular tissue, in particular, was due to this (Aroni *et al.*, 2008; Gomaa *et al.*, 2007). Morphologically, we could not find an increase in the vessel number in any subtype, whereas significant vasodilation was obvious. This finding correlates well with our gene array and qRT-PCR data, in which expression of angiogenic key genes was rarely modulated. PhR showed slight upregulation as well as downregulation of angiogenic genes as a sign of increased tissue remodeling.

The mechanism(s) that induce rapid flushing and erythema in rosacea are still unknown (Sobottka and Lehmann, 2009). Despite the prominent telangiectasia, blood vessels maintain their ability to respond to vasoactive stimuli (Guarrera *et al.*, 1982), suggesting that changes are not structural or due to irreversible damage. However, our molecular investigation indicates a marked upregulation of genes that are involved in vasodilatation. Thus, sensory nerves may induce vasodilatation by activating high-affinity receptors for vasoregulatory neuropeptides on endothelial cells and/or smooth muscle cells surrounding vessels. In LE, lymphatic vessels were extremely dilated, whereas rapid flushing such as in rosacea is rarely observed in LE. This may explain the differences with respect to the strong neurovascular association in early rosacea but not in later subtypes and LE.

Recently, the lymphatic system has attracted growing interest as an important contributor during chronic inflammation (Huggenberger *et al.*, 2010). Our results suggest that lymphatic vessels are already involved in the initiation process of rosacea but not in later subtypes, although clinically visible signs of edema are described at later stages (Crawford *et al.*, 2004). Although the early involvement of lymphatic tissue was suggested before, augmentation of lymphatic tissue was previously attributed mainly to lymphangiogenesis

(Gomaa *et al.*, 2007). Our morphometric results show no enhancement in vessel number when compared with HS. Furthermore, our RT-PCR results showed that most of the genes involved in growth and elongation of lymphatic capillaries were only slightly or not at all upregulated. *LYVE1*, a gene having a key role in metabolism, binding, and transport of hyaluronic acid from tissues to lymphatic vessels and in transplacement of leukocytes in lymphatic vessels and lymph nodes (Jackson, 2009), was even downregulated (Figure 6b).

Little data exist about a possible influence of the nervous system on lymph vascular tissue. Some studies showed that neuropeptides affect the function of lymphatic vessels. For example, substance P induces the reduction in diastolic and systolic vessel diameter, stroke volume, and increase in contraction frequency (Amerini *et al.*, 2004), and calcitonin gene-related peptide dose dependently inhibits the vasomotion of lymph vessels (Hosaka *et al.*, 2006). Which of the neuropeptides involved in rosacea pathophysiology affect lymphatic function has to be clarified in detailed studies.

A recent theory that received greater emphasis in the pathophysiology of rosacea suggests the involvement of mechanisms of neurogenic inflammation, which may reflect the early and late clinical features of the disease including flushing, erythema, and induction of leukocyte infiltration, especially of MCs (Steinhoff *et al.*, 2003; Roosterman *et al.*, 2006; Reich *et al.*, 2010). Although statistically not significant, we found a clear tendency of increased nerve density, especially in ETR, followed by a decrease in PhR. Reflecting those morphometrical findings, sensations such as “pricking, burning, or pain” are predominantly known in ETR and PPR, followed by less sensation in PhR (Crawford *et al.*, 2004; Sobottka and Lehmann, 2009).

To further investigate the role of sensory nerves in the context of intercellular communication, we performed double IF staining, which showed a close anatomical association of sensory nerves, especially with blood vessels and MCs. Pathways of neurovascular and neuroimmune interactions in the pathophysiology of rosacea are still unknown; therefore, we analyzed gene expression levels of neuromediators and neuroreceptors at the RNA level. Note that these genes must be expressed by non-neuronal cells, because the neuronal genes are located in the dorsal root ganglia.

So far, suggested communicating neuropeptides involved in rosacea include *calcitonin gene-related peptide* and *substance P* (*tachykinin, precursor 1*; Powell *et al.*, 1993; Lonne-Rahm *et al.*, 2004). Unexpectedly, neither of these neuropeptides nor their receptors was upregulated at the RNA level, but were rather downregulated (Figure 6c). According to our results, their relevance in rosacea is rather marginal. In contrast, we recognized some new molecular pathways, which we do not believe to have been linked to the pathophysiology of rosacea until now.

Gene analysis showed upregulation of serotonin receptor *HTR3A*. Serotonin (5-hydroxytryptamine) is an important inflammatory and neurosensory mediator that is released from platelets and MCs, thereby contributing to nociception and vasoregulation (Oliveira *et al.*, 2007). Expressed by primary afferent nerve fibers, HTR3A conveys their excitation and sensitization. 5-Hydroxytryptamine and the responding receptors have already been investigated for other inflammatory skin diseases (Nordlind *et al.*, 2006), but, to our knowledge, contribution of 5-hydroxytryptamine in the pathophysiology of rosacea has not been studied. It has been reported that HTR3A antagonist Ondansetron led to good remissions of cutaneous symptoms in rosacea patients (Wollina, 1999). Thus, HTR3A antagonization may be a promising future therapeutic approach.

Cathelicidin antimicrobial peptide, a recently described antimicrobial peptide, was upregulated in all subtypes. Processed by kallikrein-5, cathelicidin antimicrobial peptide has

proinflammatory activity, and promotes angiogenesis and chemotaxis. Yamasaki et al, (2007, 2011) suggest cathelicidin antimicrobial peptide to be one factor eliciting an exacerbated response of the innate immune system (Morizane *et al.*, 2010).

Moreover, our molecular studies demonstrated that vasoactive neuropeptides such as *PACAP*, *VIP*, or *adrenomedullin (ADR2)* were significantly enhanced. PACAP has already been linked to the pathophysiology of psoriasis and atopic dermatitis, where it may mediate vasodilatation and plasma extravasation, and influence neurogenic inflammation via activation of VPAC receptors (Steinhoff *et al.*, 1999; Seeliger *et al.*, 2010). Both PACAP and VIP are capable of stimulating MC degranulation (Peters *et al.*, 2006; Lenti *et al.*, 2009). Upon stimulation, neuromediators such as PACAP can also be released by endothelial cells under inflammatory conditions, suggesting an important role of PACAP, VIP, or transient receptor potential channels, as well as adrenergic receptors, in rosacea pathophysiology.

MCs have long been known to be key effector cells in neurogenic inflammation, immune defense, and fibrosis (Metz and Maurer, 2007). A recent study discussed the important role of MCs in rosacea's evolution to a chronic stage (Aroni *et al.*, 2008).

Our results clearly indicate a marked upregulation of MC density in all subtypes of rosacea, with the greatest increase in PPR. In LE, MCs were not significantly increased compared with MCs in HS, which indicates that the high density of MCs in rosacea is more than a sign of skin inflammation in general.

Being activated by neuropeptides such as PACAP or VIP, MCs could be important interconnecters amplifying the neural impulses and conveying them via histamine or tryptase release to vasculature and immune cells (Steinhoff *et al.*, 2003). Our morphometric and gene analysis data, however, indicate a strong interaction between nerves and MCs. *HRH2*, a receptor that mediates vasodilatory effects of histamine, was upregulated in rosacea, especially in PhR, when, according to our immunohistochemical findings, vasodilatation is most impressive. In addition, we observed a positive correlation between the increase of MCs and FBs in dermal structures of rosacea tissues, suggesting a strong interaction, e.g., by MC tryptase, which is known to have a chemotactic and mitotic effect on FBs (Gruber, 2003). Recent studies show that MC/FB communication appears to be involved in skin fibrosis (Monument *et al.*, 2010).

Skin fibrosis and phymatous changes are characteristics of late-stage rosacea. In our study, we observed a significantly higher distribution of vimentin-positive cells in rosacea patients, with a maximum in PPR patients. This finding is consistent with clinical symptoms such as phymatic changes and previous histological findings (Jansen and Plewig, 1997). Activation of FB seems to be a specific feature in rosacea, as number of FBs is significantly different from that found in LE. Clinically, the skin in ETR is described to be fine in texture without obvious signs of fibrosis and fibrotic chances occur particularly in PhR (Crawford *et al.*, 2004). Unexpectedly, however, according to our data FB occurrence in skin is vice versa predominantly in ETR and PPR. This observation leads us to conclude that fibrotic processes in rosacea start much earlier than is expected on the basis of clinical observations.

Our qRT-PCR experiments showed massive upregulation of MMP, especially in PPR and PhR, indicating that a strong process of remodeling is taking place. MMP inhibitors were found rather downregulated (Figure 6e). In addition to their involvement in tissue destruction and fibrosis, MMPs have recently been found to be involved in angiogenesis and apoptosis (Amălinei *et al.*, 2010). In rosacea, MMP-9 and -2 are known to be involved in the development of neuropathic pain (Kawasaki *et al.*, 2008) and fibrotic processes. Taken together, morphometrical and molecular results, effective inhibition of MMP, e.g., by tetracyclines (Gu *et al.*, 2010; Lipowsky *et al.*, 2011), may provide a novel therapeutic

approach for the treatment of fibrosis and pain (stinging and burning) associated with rosacea. The unexpected early detection of FB activation is very important with respect to timing rosacea treatment. Here, detailed studies using various markers of the different FB differentiation stages are necessary to further dissect the role of FB activation.

In summary, our combined morphological and molecular study indicates a meaningful role of neurovascular and neuroimmune networks in the development of rosacea. We demonstrated, both at the histological and molecular levels, that neurogenic inflammation is an important part of the pathophysiology, resulting in vasodilation, but not in angiogenesis, and contributes to the fibrotic processes observed in this chronic inflammatory disease. Furthermore, our study detected some promising pathways of conduction, which remain to be clarified and validated in detailed studies. To gradually get to know the different involved parameters and interaction pathways opens a wide range of new and finally cause-related pharmacological targets for therapeutic intervention.

MATERIALS AND METHODS

For detailed descriptions see Supplementary Material online. The antibodies used are listed in Supplementary Material S1 online. The ready-to-use TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) are listed in Figure 6.

Skin collection

All facial skin material was obtained from previously taken diagnostic biopsies (Rosacea, Lupus) and plastic surgery (HS) in the Department 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). On the basis of that, we investigated five different groups of patients: for morphometric stainings ETR (n = 9), PPR (n = 9), PhR (n = 9), HS (n = 10), LE (n = 9); and for gene analytic studies we investigated the following groups: ETR (n = 11), PPR (n = 11), PhR (n = 6), HS (n = *12*, face). Patients were informed about the possible use of tissue leftover from surgery for investigation, and gave their written consent. Permission for human studies was given by the Ethical Committee of the University of Münster Germany, in accordance with the ethical standards of the 1964 Declaration of Helsinki Principles.

Double immunofluorescence

Histological staining with double IF was completed according to the standard protocol (Collins *et al.*, 2002). Frozen sections of skin samples were processed for double IF staining. Blocking was performed with Target Retrieval Buffer, pH 6.1 (S1699, DAKO, Hamburg, Germany) at 90 °C for 40 minutes. Antibody pools were incubated overnight at 4 °C. After secondary antibodies (1:400) were washed, they were applied for 60 minutes at room temperature.

Immunohistochemical analysis

Immunohistochemistry was performed as described (Rattenholl *et al.*, 2007). Paraffin sections of skin samples were deparaffinized and processed for immunohistochemical staining. Blocking was performed according to the specific characteristics of the different markers. Antibody pools were incubated for 1 hour. After secondary antibodies were washed, they were applied for 30 minutes at room temperature.

Image analysis

Using × 200 magnification, we took five pictures within each section, 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).

Quantitative RT-PCR

qRT-PCR was performed as previously described (Ständer *et al.*, 2004). In short, mRNA expression was evaluated using semiquantitative PCR technology (qRT-PCR–Taqman Low Density Arrays). After extraction of total RNA, cDNA was synthesized using high-capacity cDNA archive kits (Applied Biosystems). Gene expression analysis was performed using TLDA arrays containing PCR primers for genes of interest and housekeeping genes. Synthesized cDNA (50 ng of cDNA per column) was added to the PCR master mix, and PCR reactions were performed on ABI 7900HT (Applied Biosystems).

Statistical analysis

Statistical analyses for morphometry were performed using SPSS for Windows, version 17.0 (SPSS, Chicago, IL). Statistical significance was determined by using *t*-tests. Differences were considered significant at a *P*-value ≤ 0.05.

The fold modulation of gene expression of rosacea samples versus samples of healthy volunteers was defined as $2^{(\text{mean } C_{\text{tHV}} - \text{mean } C_{\text{tRo}})}$, with C_{tHV} and C_{tRo} depicting the C_t values of healthy volunteer and rosacea samples, respectively.

To identify gene modulation between rosacea subtypes, one-way analysis of variance with Benjamini–Hochberg multiplicity correction was performed using JMP 7.0.1 (SAS Institute, Cary, NC) and irMF 3.5 (National Institute of Statistical Sciences, NISS, Triangle Park, NC) software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ETR	erythematous rosacea
FB	fibroblast
HS	healthy skin
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A
LE	lupus erythematosus
MC	mast cell
MMP	matrix metalloproteinase
PACAP	pituitary adenylate cyclase-activating polypeptide
PhR	phymatous rosacea
PPR	papulopustular rosacea

qRT-PCR	quantitative real-time RT-PCR
SP	substance P
VIP	vasoactive intestinal peptide

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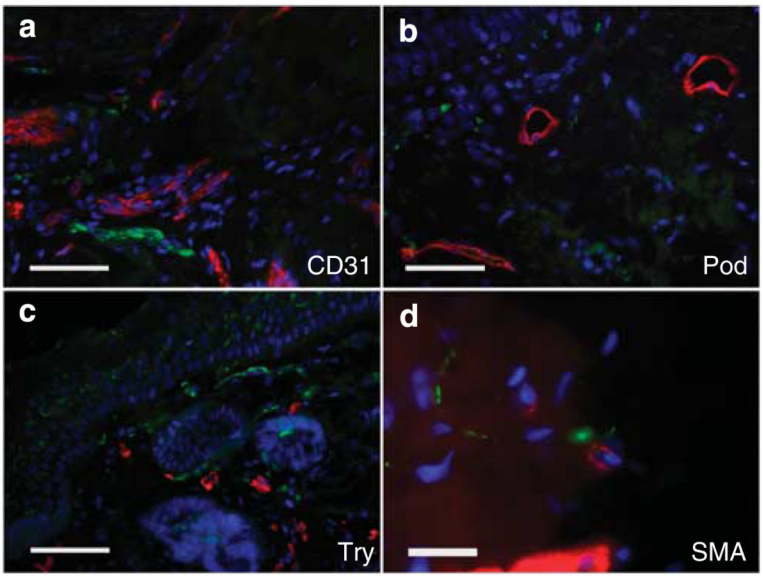


Figure 1. Association of different vascular and immune structures with sensory nerves in human facial skin of rosacea patients, as shown by double immunofluorescence staining

Colocalization of sensory nerves (PGP9.5) was determined in combination with CD31 for blood vessels (**a**), podoplanin (Pod) for lymphatic vessels (**b**), tryptase (Try) for mast cells (**c**), and smooth muscle actin (SMA) for myofibroblasts or blood vessels (**d**). Our data show a close anatomical association of unmyelinated nerves, especially with blood vessels and mast cells, and less with lymphatic vessels or myofibroblasts (bar = 300 μm; **a**–**c** and bar = 100 μm; **d**).

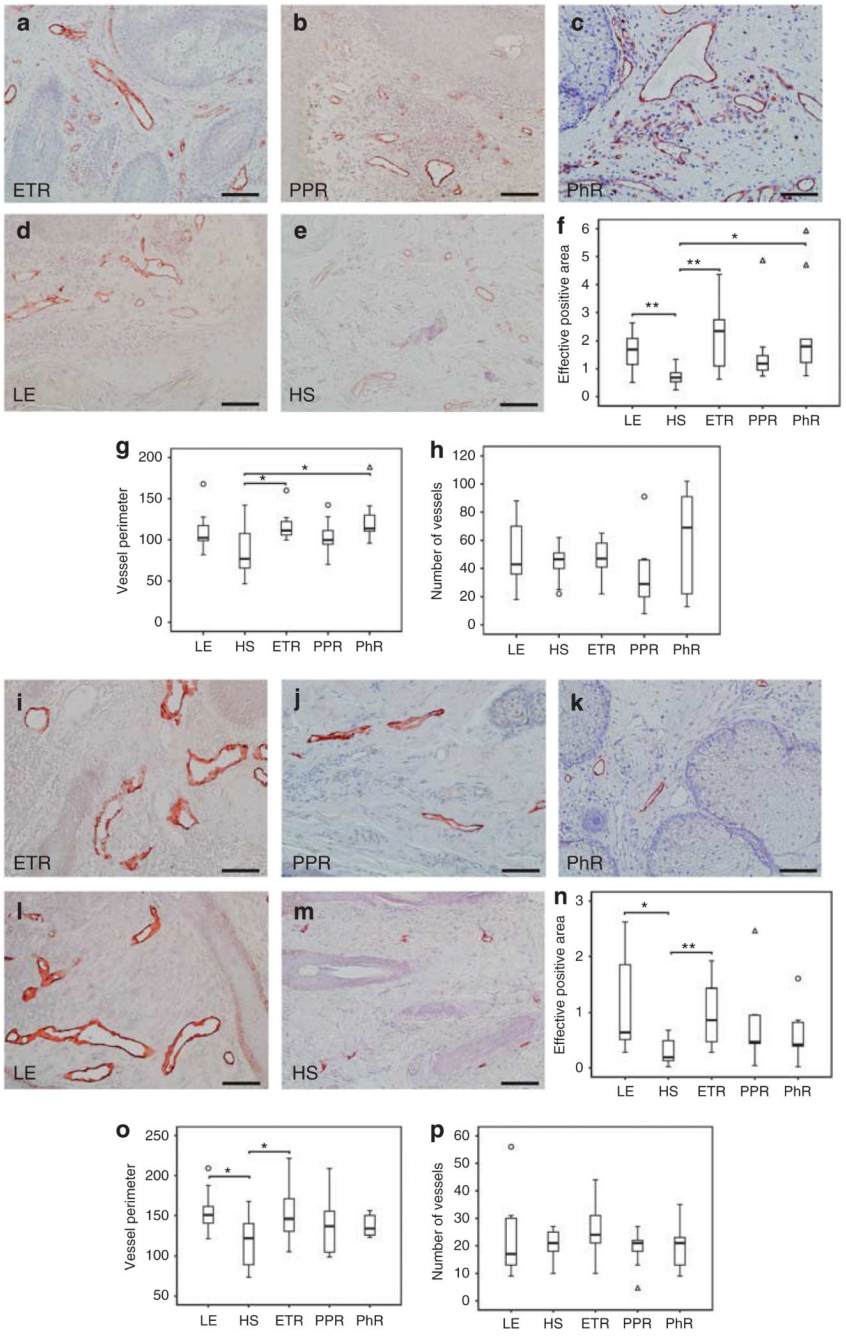


Figure 2. Density of blood and lymphatic vessels in human skin, as shown by immunohistochemistry and quantitative analysis of stained dermis

Immunoreactivity for CD31 and podoplanin was observed in erythematous rosacea (ETR, n = 9), papulopustular rosacea (PPR, n = 9), phymatous rosacea (PhR, n = 9), lupus erythematosus (LE, n = 9), and healthy skin staining (HS, n = 10; bar = 100 μm; **a**–**e** and **i**–**m**). (**f**) PhR showed strongest statistically significant augmentation of CD31-positive tissue, followed by ETR (**f**–**h**; **n**–**p**; *P<0.05; **P<0.01). (**g**) Vessel perimeter measurement showed significant vasodilation in ETR and PhR, whereas the number of vessels (**h**) was not increased. A tendency toward angiogenesis was only observed in PhR. Augmentation in lymph vessel surface was statistically significant exclusively in ETR (**n**). Lymph vessel

circumference measurements showed significant vasodilation in ETR (**o**). Number of lymphatic vessels was not increased in any subtype (**p**), unfilled triangles and circles represent outliers (**f**–**h**; **n**–**p**).

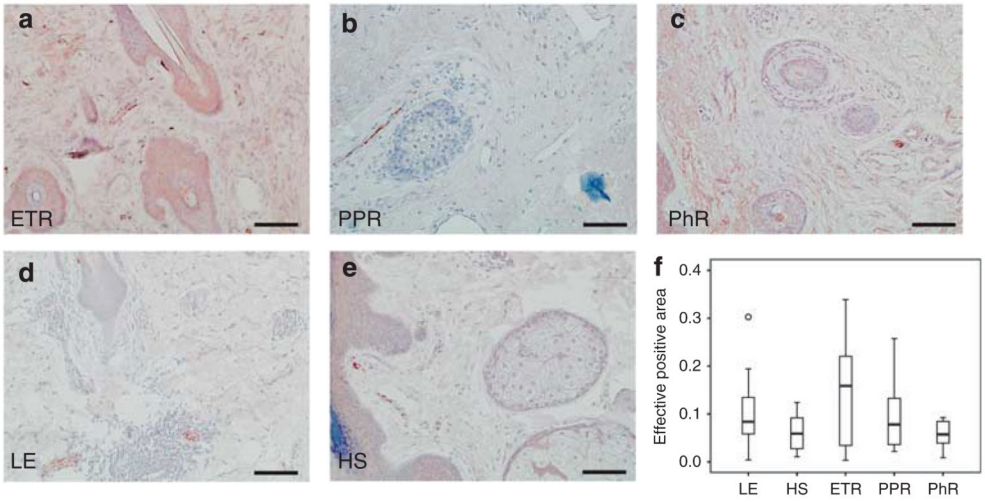


Figure 3. Localization and density of myelinated sensory nerves (NF200) in human skin, as shown by immunohistochemistry and quantitative analysis of stained dermis

Immunoreactivity for neurofilament was observed in erythematous rosacea (ETR, $n = 9$), papulopustular rosacea (PPR; $n = 9$), phymatous rosacea (PhR; $n = 9$), lupus erythematosus (LE; $n = 9$), and healthy skin (HS; $n = 10$; bar = 100 μm; **a**–**e**). There was a marked but not statistically significant increase of nerves in ETR (× 2.34) followed by a gradual decrease. Increase of neurofilament-positive nerves was comparable in PPR and LE (unfilled circle represents outlier) (**f**).

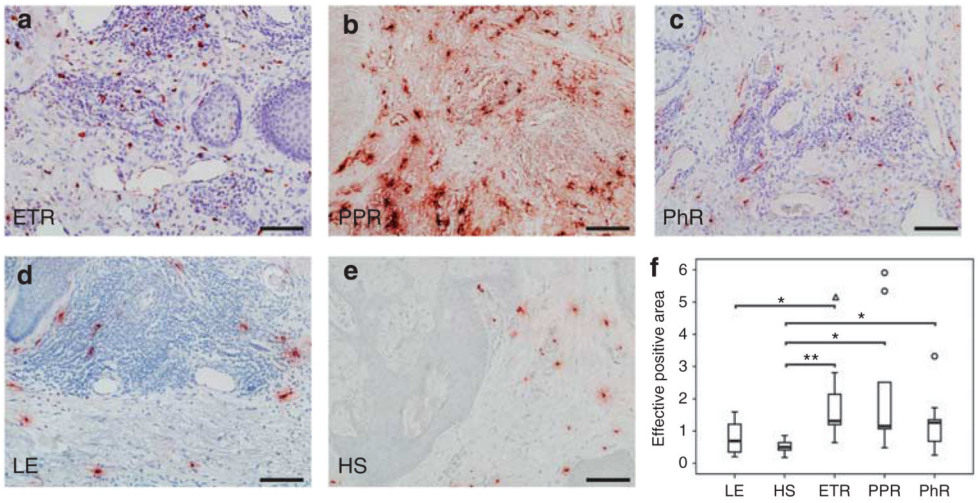


Figure 4. Localization and density of mast cells in human facial skin as shown by immunohistochemistry and quantitative analysis of stained dermis

Immunoreactivity for tryptase was observed in erythematous rosacea (ETR, $n = 9$), papulopustular rosacea (PPR, $n = 9$), phymatous rosacea (PhR, $n = 9$),lupus erythematosus (LE; $n = 9$), and healthy skin (HS, $n = 10$; bar = 100 μm; **a**–**e**). The increase in mast cell density was statistically significant for all subtypes (ETR × 3.98; PPR × 4.41; PhR × 2.54), whereas mast cell density did not increase in LE (*$P<0.05$, **$P<0.01$; unfilled circles and triangle represent outliers) (**f**).

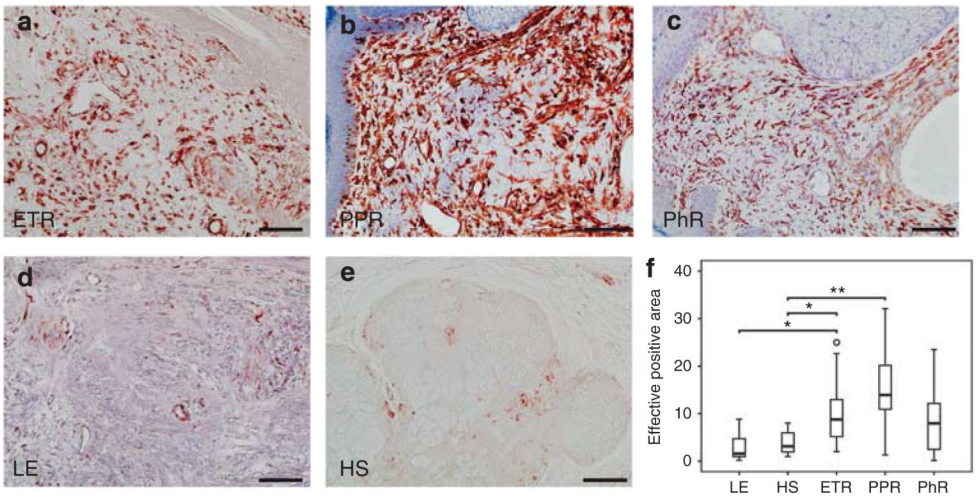
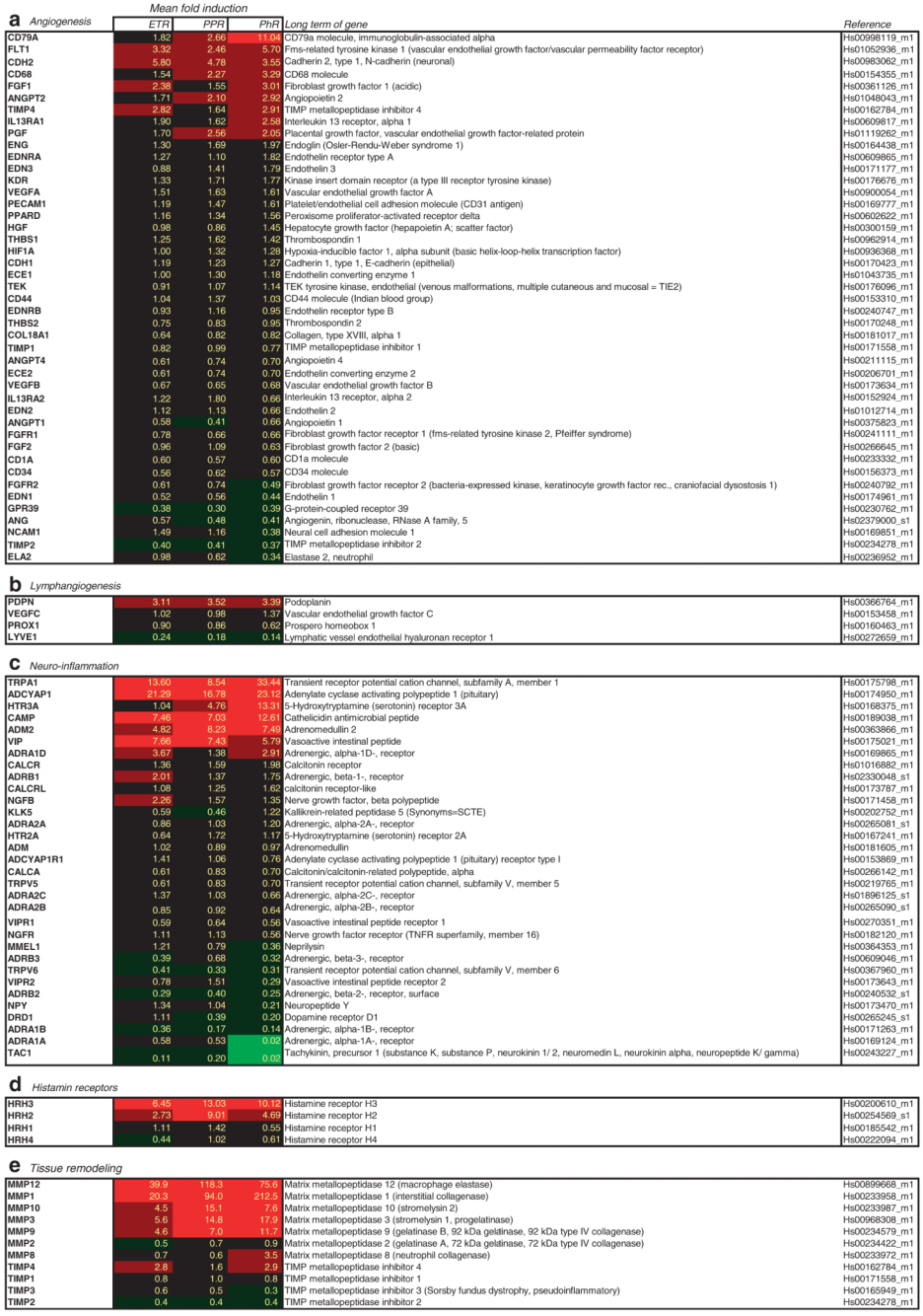


Figure 5. Localization and density of fibroblasts (FBs)/cytes and mesenchymal structures of blood vessels in human skin, as shown by immunohistochemistry and quantitative analysis of stained dermis

Immunoreactivity for vimentin was observed in erythematous rosacea (ETR, $n = 9$), papulopustular rosacea(PPR, $n = 9$), phymatous rosacea (PhR, $n = 9$), lupus erythematosus (LE; $n = 9$), and healthy skin (HS; $n = 10$; bar = 100 μm; **a**–**e**). Density of FBs was increased in all subtypes, but significantly so in ETR (× 2.9) and PPR (× 3.94). Skin of lupus patients showed decreased density of FBs/cytes as compared with healthy human skin (*$P<0.05$; **$P<0.01$; unfilled circle represents outlier) (**f**).



a *Angiogenesis*

	Mean fold induction ETR	PPR	PhR	Long term of gene	Reference
CD79A	1.82	2.66	11.04	CD79a molecule, immunoglobulin-associated alpha	Hs00998119_m1
FLT1	3.32	2.46	5.70	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	Hs01052936_m1
CDH2	5.80	4.78	3.55	Cadherin 2, type 1, N-cadherin (neuronal)	Hs00983062_m1
CD68	1.54	2.27	3.29	CD68 molecule	Hs00154355_m1
FGF1	2.38	1.55	3.01	Fibroblast growth factor 1 (acidic)	Hs00361126_m1
ANGPT2	1.71	2.10	2.92	Angiopoietin 2	Hs01048043_m1
TIMP4	2.82	1.64	2.91	TIMP metallopeptidase inhibitor 4	Hs00162784_m1
IL13RA1	1.90	1.62	2.58	Interleukin 13 receptor, alpha 1	Hs00609817_m1
PGF	1.70	2.56	2.05	Placental growth factor, vascular endothelial growth factor-related protein	Hs01119262_m1
ENG	1.30	1.69	1.97	Endoglin (Osler-Rendu-Weber syndrome 1)	Hs00164438_m1
EDNRA	1.27	1.10	1.82	Endothelin receptor type A	Hs00609865_m1
EDN3	0.88	1.41	1.79	Endothelin 3	Hs00171177_m1
KDR	1.33	1.71	1.77	Kinase insert domain receptor (a type III receptor tyrosine kinase)	Hs00176676_m1
VEGFA	1.51	1.63	1.61	Vascular endothelial growth factor A	Hs00900054_m1
PECAM1	1.19	1.47	1.61	Platelet/endothelial cell adhesion molecule (CD31 antigen)	Hs00169777_m1
PPARD	1.16	1.34	1.56	Peroxisome proliferator-activated receptor delta	Hs00602622_m1
HGF	0.98	0.86	1.45	Hepatocyte growth factor (hepapoietin A; scatter factor)	Hs00300159_m1
THBS1	1.25	1.62	1.42	Thrombospondin 1	Hs00962914_m1
HIF1A	1.00	1.32	1.28	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	Hs00936368_m1
CDH1	1.19	1.23	1.27	Cadherin 1, type 1, E-cadherin (epithelial)	Hs00170423_m1
ECE1	1.00	1.30	1.18	Endothelin converting enzyme 1	Hs01043735_m1
TEK	0.91	1.07	1.14	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal = TIE2)	Hs00176096_m1
CD44	1.04	1.37	1.03	CD44 molecule (Indian blood group)	Hs00153310_m1
EDNRB	0.93	1.16	0.95	Endothelin receptor type B	Hs00240747_m1
THBS2	0.75	0.83	0.95	Thrombospondin 2	Hs00170248_m1
COL18A1	0.64	0.82	0.82	Collagen, type XVIII, alpha 1	Hs00181017_m1
TIMP1	0.82	0.99	0.77	TIMP metallopeptidase inhibitor 1	Hs00171558_m1
ANGPT4	0.61	0.74	0.70	Angiopoietin 4	Hs00211115_m1
ECE2	0.61	0.74	0.70	Endothelin converting enzyme 2	Hs00206701_m1
VEGFB	0.67	0.65	0.68	Vascular endothelial growth factor B	Hs00173634_m1
IL13RA2	1.22	1.80	0.66	Interleukin 13 receptor, alpha 2	Hs00152924_m1
EDN2	1.12	1.13	0.66	Endothelin 2	Hs01012714_m1
ANGPT1	0.58	0.41	0.66	Angiopoietin 1	Hs00375823_m1
FGFR1	0.78	0.66	0.66	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	Hs00241111_m1
FGF2	0.96	1.09	0.63	Fibroblast growth factor 2 (basic)	Hs00266645_m1
CD1A	0.60	0.57	0.60	CD1a molecule	Hs00233332_m1
CD34	0.56	0.62	0.57	CD34 molecule	Hs00156373_m1
FGFR2	0.61	0.74	0.49	Fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor rec., craniofacial dysostosis 1)	Hs00240792_m1
EDN1	0.52	0.56	0.44	Endothelin 1	Hs00174961_m1
GPR39	0.38	0.30	0.39	G-protein-coupled receptor 39	Hs00230762_m1
ANG	0.57	0.48	0.41	Angiogenin, ribonuclease, RNase A family, 5	Hs02379000_s1
NCAM1	1.49	1.16	0.38	Neural cell adhesion molecule 1	Hs00169851_m1
TIMP2	0.40	0.41	0.37	TIMP metallopeptidase inhibitor 2	Hs00234278_m1
ELA2	0.98	0.62	0.34	Elastase 2, neutrophil	Hs00236952_m1

b *Lymphangiogenesis*

PDPN	3.11	3.52	3.39	Podoplanin	Hs00366764_m1
VEGFC	1.02	0.98	1.37	Vascular endothelial growth factor C	Hs00153458_m1
PROX1	0.90	0.86	0.82	Prospero homeobox 1	Hs00160463_m1
LYVE1	0.24	0.18	0.14	Lymphatic vessel endothelial hyaluronan receptor 1	Hs00272659_m1

c *Neuro-inflammation*

TRPA1	13.60	8.54	33.44	Transient receptor potential cation channel, subfamily A, member 1	Hs00175798_m1
ADCYAP1	21.29	16.78	23.12	Adenylate cyclase activating polypeptide 1 (pituitary)	Hs00174950_m1
HTR3A	1.04	4.76	13.31	5-Hydroxytryptamine (serotonin) receptor 3A	Hs00168375_m1
CAMP	7.46	7.03	12.61	Cathelicidin antimicrobial peptide	Hs00189038_m1
ADM2	4.82	8.23	7.49	Adrenomedullin 2	Hs00363866_m1
VIP	7.66	7.43	5.79	Vasoactive intestinal peptide	Hs00175021_m1
ADRA1D	3.67	1.38	2.91	Adrenergic, alpha-1D-, receptor	Hs00169865_m1
CALCR	1.36	1.59	1.98	Calcitonin receptor	Hs01016882_m1
ADRB1	2.01	1.37	1.75	Adrenergic, beta-1-, receptor	Hs02330048_s1
CALCRL	1.08	1.25	1.62	calcitonin receptor-like	Hs00173787_m1
NGFB	2.26	1.57	1.35	Nerve growth factor, beta polypeptide	Hs00171458_m1
KLK5	0.59	0.46	1.22	Kallikrein-related peptidase 5 (Synonyms=SCTE)	Hs00202752_m1
ADRA2A	0.86	1.03	1.20	Adrenergic, alpha-2A-, receptor	Hs00265081_s1
HTR2A	0.64	1.72	1.17	5-Hydroxytryptamine (serotonin) receptor 2A	Hs00167241_m1
ADM	1.02	0.89	0.97	Adrenomedullin	Hs00181605_m1
ADCYAP1R1	1.41	1.06	0.76	Adenylate cyclase activating polypeptide 1 (pituitary) receptor type I	Hs00153869_m1
CALCA	0.61	0.83	0.70	Calcitonin/calcitonin-related polypeptide, alpha	Hs00266142_m1
TRPV5	0.61	0.83	0.70	Transient receptor potential cation channel, subfamily V, member 5	Hs00219765_m1
ADRA2C	1.37	1.03	0.66	Adrenergic, alpha-2C-, receptor	Hs01896125_s1
ADRA2B	0.85	0.92	0.64	Adrenergic, alpha-2B-, receptor	Hs00265090_s1
VIPR1	0.59	0.64	0.56	Vasoactive intestinal peptide receptor 1	Hs00270351_m1
NGFR	1.11	1.13	0.56	Nerve growth factor receptor (TNFR superfamily, member 16)	Hs00182120_m1
MMEL1	1.21	0.79	0.36	Neprilysin	Hs00364353_m1
ADRB3	0.39	0.68	0.32	Adrenergic, beta-3-, receptor	Hs00609046_m1
TRPV6	0.41	0.33	0.31	Transient receptor potential cation channel, subfamily V, member 6	Hs00367960_m1
VIPR2	0.78	1.51	0.29	Vasoactive intestinal peptide receptor 2	Hs00173643_m1
ADRB2	0.29	0.40	0.25	Adrenergic, beta-2-, receptor, surface	Hs00240532_s1
NPY	1.34	1.04	0.21	Neuropeptide Y	Hs00173470_m1
DRD1	1.11	0.39	0.20	Dopamine receptor D1	Hs00265245_s1
ADRA1B	0.36	0.17	0.14	Adrenergic, alpha-1B-, receptor	Hs00171263_m1
ADRA1A	0.58	0.53	0.02	Adrenergic, alpha-1A-, receptor	Hs00169124_m1
TAC1	0.11	0.20	0.02	Tachykinin, precursor 1 (substance K, substance P, neurokinin 1/ 2, neuromedin L, neurokinin alpha, neuropeptide K/ gamma)	Hs00243227_m1

d *Histamin receptors*

HRH3	6.45	13.03	10.12	Histamine receptor H3	Hs00200610_m1
HRH2	2.73	9.01	4.69	Histamine receptor H2	Hs00254569_s1
HRH1	1.11	1.42	0.55	Histamine receptor H1	Hs00185542_m1
HRH4	0.44	1.02	0.61	Histamine receptor H4	Hs00222094_m1

e *Tissue remodeling*

MMP12	39.9	118.3	75.6	Matrix metallopeptidase 12 (macrophage elastase)	Hs00899668_m1
MMP1	20.3	94.0	212.5	Matrix metallopeptidase 1 (interstitial collagenase)	Hs00233958_m1
MMP10	4.5	15.1	7.6	Matrix metallopeptidase 10 (stromelysin 2)	Hs00233987_m1
MMP3	5.6	14.8	17.9	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	Hs00968308_m1
MMP9	4.6	7.0	11.7	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	Hs00234579_m1
MMP2	0.5	0.7	0.9	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	Hs00234422_m1
MMP8	0.7	0.6	3.5	Matrix metallopeptidase 8 (neutrophil collagenase)	Hs00233972_m1
TIMP4	2.8	1.6	2.9	TIMP metallopeptidase inhibitor 4	Hs00162784_m1
TIMP1	0.8	1.0	0.8	TIMP metallopeptidase inhibitor 1	Hs00171558_m1
TIMP3	0.6	0.5	0.3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	Hs00165949_m1
TIMP2	0.4	0.4	0.4	TIMP metallopeptidase inhibitor 2	Hs00234278_m1

Figure 6. Detection of mRNA levels of genes relevant for neuroimmune and neurovascular interaction as determined by RT-PCR

(**a**) Fold induction of genes involved in angiogenesis. Modulation of expression was detectable in phymatous rosacea (PhR, $n = 6$), but not in erythematous rosacea (ETR, $n = 11$) and papulopustular rosacea (PPR, $n = 11$). (**b**) Fold induction of genes involved in lymphangiogenesis. RT-PCR showed upregulation of *podoplanin*, but downregulation of *LYVE1*. (**c**) Fold induction of genes involved in neurovascular interaction. RT-PCR showed marked modulation of gene expression of different neuropeptides and their corresponding receptors in all subtypes. (**d**) Fold induction of histamine receptors. *HRH3* was upregulated. (**e**) Fold induction of genes involved in tissue remodeling. RT-PCR shows evidence of a

strong enhancement of matrix metalloproteinases (MMPs), whereas their inhibitors are downregulated.