Aus dem Institut/der Klinik für Dermatologie, Venerologie und Allergologie
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DISSERTATION

Analysis of mast cells and mast cell-mediator-related histological features in cholinergic urticaria

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Abbreviations

Abbreviations and terms	Description
MCs	Mast cells
IgE	Immunoglobulin E
SC	Stratum Corneum
SG	Stratum Granulosum
SS	Stratum Spinosum
SB	Statum Basale
H1R/HR1R	Histamine 1 receptor
H2R/HR2R	Histamine 2 receptor
H3R/HR3R	Histamine 3 receptor
H4R/HR4R	Histamine 4 receptor
NK cell	Natural killer cell
DCs	Dendritic cells
CholU	Cholinergic urticaria
EAACI	European Academy of Allergology and Clinical Immunology Dermatology Section, the EU-funded network of excellence
GA(2)LEN	Global Allergy and Asthma European Network
EDF	European Dermatology Forum
UNEV	Urticaria network e.V
PCE	Pulse controlled ergometry
UAS	Urticaria activity score
CholUAS7	Cholinergic urticaria activity score 7
DLQI	Dermatology Life Quality Index
CholU-Qol	Cholinergic Urticaria Quality of Life Questionnaire
AGH	Acquired generalized hypohidrosis
CHRM3	Cholinergic receptor muscarinic 3
AchE	Acetylcholine Esterase
sgAHs	Second generation antihistamines
CSU	Chronic Spontaneous Urticaria

nsAH	Non-sedating antihistamine
EDTA	Ethylendiamantetraessigsaure
ICH	International Conference on Harminization
GCP	Good Clinical Practice
SOPs	Standard Operating Procedures
CholUSI	Cholinergic Urticaria Severity Index
UCT	Urticaria Control Test
HE	Hematoxylin Eosin
AS-D	Naphthol AS-D Cholroacetate for Specific Esterase Kit
VAS	Visual analoge scale
HR	Heart rate
M. globosum	Malassezia globosum
AD	Atopic dermatitis
ASwST	Autoperspiration sweat skin test
RT	room temperature
PBS	Phosphate Buffered Saline,
QT	4-acetyloxybutanoyl 4 acetyloxybutanoate
QF	methyl methacryla crosspolymer.
TBS	Phosphate Buffered Saline
TBST	TBS with 0.05% Tween
SD	Standard Definition
IHC	immunohistochemistry

1. Abstract

Mast cells (MCs) are thought to be key effector cells in chronic urticaria including cholinergic urticaria (CholU). Although the pathophysiology of CholU is not well understood, degranulation of mast cells upon exercise and sweating with subsequent release of histamine and other mediators are held to provoke the elicitation of small wheals, flare and often severe itch. However, as of now, only little data support this theory.

Accordingly, this study aimed to better characterize the role of MCs in CholU. To this end, we clinically characterized patients with CholU, collected histological samples of the skin before and after puls-controlled provocation testing (PCE), analyzed them for skin MC numbers, analyzed MC activating factors like total and specific IgE, analyzed MC products such as tryptase, investigated the expression of MC product-related receptors like histamine receptors, and looked for non-IgE mediated mast cell activation pathways and mechanisms including acethylcholine/achethylcholine esterase dysregulation.

We characterized 13 CholU patients and 13 matched healthy controls. Clinically, patients with CholU in our cohort had a long duration of disease and rated themselves as mostly moderately affected. PCE testing showed comparable onset of sweating in patients with CholU and healthy persons, but most of the patients with CholU had decreased sweating responses. Atopic predisposition was frequent in patients with CholU, and total IgE serum levels were elevated. Specific IgE against the skin resident fungi Malassezia was only seen in CholU patients and may be a marker for more prolonged courses of disease. Our results showed that MC numbers were increased in the skin of CholU patients compared to healthy controls, but we failed in detecting degranulation of MCs upon exercise challenge. The MC mediator tryptase did not increase in the serum upon provocation in CholU patients, despite often severe skin symptoms. Skin of CholU patients expressed low levels of H1 and H2 receptors, high levels of H4 receptors, but no H3 receptors.

In summary, the increase in skin MC numbers in CholU patients points towards a role of MC in the pathology of the disease, albeit the exact role remains unclear, as we failed to prove degranulation. It is worth nothing that differences in cutaneous histamine receptor expression were seen, but for meaningful conclusions, further analyses in a larger number of patients are needed.

2. Abstrakt

Mastzellen wird eine zentrale Rolle in der Pathophysiologie der chronischen Urtikaria einschließlich der cholinergischen Urtikaria (CholU) zugeschrieben. Obwohl die genaue Rolle von Mastzellen bei der CholU nicht gut verstanden ist, wird davon ausgegangen, dass Anstrengung und Schwitzen zu deren Degranulation führt. Mastzell-Mediatoren wie Histamin werden für die typischen Symptome der CholU, wie Quaddeln, Rötungen und oftmals starken Juckreiz verantwortlich gemacht. Obwohl die CholU eine schon lange bekannte Erkrankung ist, die klinisch gut charakterisiert ist, gibt es nur sehr wenige Daten die die Rolle von Mastzellen in der Pathogenese der CholU stützen.

In dieser Studie hatten wir daher zum Ziel, die Rolle von Mastzellen bei CholU besser zu chrakterisieren. Von klinisch sehr gut charakterisierten Patienten wurden daher Haut- und Blutproben vor und nach einer Puls-kontrollierten Ergometrie (PCE) gewonnen, die Anzahl der Mastzellen in der Haut bestimmt, Mastzell-aktivierende Faktoren wie gesamt-IgE und spezifisches IgE untersucht, Mastzell-Produkte wie Tryptase quantifiziert, die Expression von Rezeptoren für Mastzell-Produkte ermittelt und nicht-IgE vermittelte Mastzell-Aktivierungswege und -Mechanismen wie Acethylcholine/Achethylcholine-Esterase Dysregulierung untersucht.

In dieser Studie wurden insgesamt Proben von 13 CholU Patienten und 13 passenden gesunden Kontrollpersonen untersucht. Die CholU Patienten in unserer Kohorte wiesen einen langen Krankheitsverlauf auf und waren meist moderat von der Erkrankung betroffen. In der PCE wiesen Patienten mit CholU ein zeitlich ähnliches aber geringeres Schwitzverhalten im Vergleich zu den gesunden Kontrollen auf. CholU Patienten hatten mehr atopische Stigmata und erhöhte gesamt-IgE Spiegel auf. Einige Patienten wiesen spezifisches IgE gegen den Hautkeim Malassezia auf, das mit längerer Krankheitsdauer assoziiert war. Die Anzahl der Mastzellen in der Haut von CholU Patienten war signifikant erhöht im Vergleich zu gesunden Kontrollen, jedoch konnte wir keine Degranulation der Mastzellen detektieren. Im Serum der Patienten zeigte sich keine Erhöhung der Tryptase, trotz oft stark ausgeprägten Hautbeschwerden nach Provokationstestung. In der Haut von CholU Patienten zeigte sich eine niedrige Expression von Histamin H1 und H2 Rezeptoren und eine hohe Expression der H4 Rezeptoren. H3 Rezeptorexpression konnte nicht nachgewiesen werden.

Zusammengefasst ergab unsere Studie eine signifikante Erhöhung der Anzahl kutaner Mastzellen, was auf eine wichtige Rolle in der Pathophysiologie der Erkrankung hinweist, auch wenn die genaue Rolle weiterhin unklar bleibt. Außerdem sahen wir unterschiedliche Histaminrezeptorexpressionsmuster, jedoch müssen hier noch weitere Untersuchungen folgen, um konkrete Schlussfolgerungen ziehen zu können.

3. Introduction

3.1 Mast cells

Mast cells (MCs), first described by Paul Ehrlich in 1877 [1], are known to be primary effector cells in allergy, particularly, during the early and acute phases. Using aniline blue/methyl Blue staining, Ehrlich observed highly granulated cells present in the intestinal mucosa and named them MCs. He also found that these cells increase in numbers in pathological conditions involving chronic inflammation. However, the central role of MCs in allergy was revealed only when they were shown to release histamine upon immunoglobulin E (IgE)-induced cell activation, results which were published in 1953 and 1966 [2,3], nearly a century after Ehrlich's first discovery [4].

MCs are found in all tissues, primarily within tissues that connect to the outside environment such as the intestinal tract, lungs and skin where pathogens and allergens are frequently encountered. MCs, together with dendritic cells (DCs), are one of the first responders to harmful toxins, pathogens, parasites, allergens and environmental antigens. They play a key role in innate immunity and adaptive immunity, and their malfunction can result in chronic disease, mainly allergic disease and autoimmune disease [5]. MCs are multifunctional immune cells and the primary responders in allergic reactions, orchestrating strong immune responses within minutes of encountering allergens and other IgE-dependent or independent stimuli [5,6].

Within minutes of the activation of MCs, degranulation occurs whereby MCs release preformed mediators, including histamine, from cytoplasmic granules [7]. Histamine is the best known biogenic amine and was one of the first functional active MC mediators to be described [8,9].

In the second phase, newly synthesized lipid mediators, such as prostaglandins and leukotrienes are released by MCs followed by a late phase release of cytokines and chemokines, usually several hours after activation. Importantly, not all stimuli trigger a full-blown response. Thus, depending on the type of stimulus involved and the type of receptor encountered, MCs run through various response programs.

The skin is the largest organ in the human body, which functions primarily as a barrier to the environment, thereby protecting the body from pathogens, environmental agents, hot or cold, stress, and water loss. In the skin, MCs are located in all vascularized tissues, such as the dermis and the subcutaneous layers. Yet, the distribution of MCs is not homogeneous. In the skin, MCs are predominantly found close to sensory nerve fibers, hair follicles, blood and lymphatic vessels and subcutaneous nerve plexus (Fig.1) [10,11]. There is also a gradient of cutaneous MC numbers from the top to the bottom, with higher MC frequencies in the superficial layers of the skin and the lowest frequencies in the subcutaneous layers. Moreover, higher numbers of MCs are found at peripheral sites of the body like the hands, feet and face compared to the back skin or trunk. Collectively, these data show that the local microenvironment, i.e. tissue cells and signals, is of critical importance for shaping MC phenotypes. Since all tissue signals and cell populations, including MCs, exhibit non-homogenous distribution patterns, it suggests that MCs within a certain tissue are heterogeneous and show differences based on their localization within the tissue.

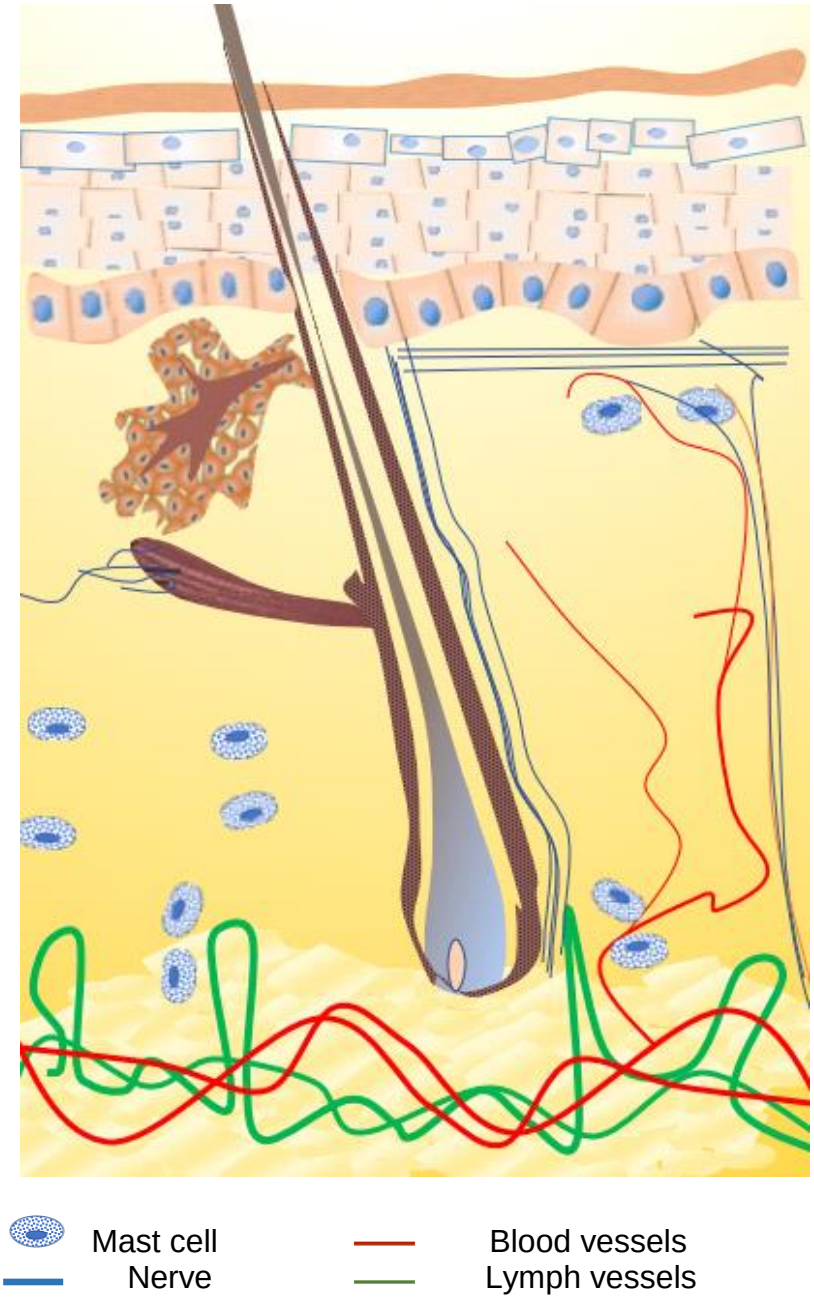


Fig. 1 Skin localization of mast cells.
Abbreviations: SC - Stratum Corneum, SG - Stratum Granulosum, SS - Stratum Spinosum, SB - Statum Basale. (Modified from Wong DJ, Chang HY., 2008-2009)

Histamine and Histamine receptors

MCs generate and release histamine during anaphylactic reactions, and pharmacological evidence exists to show that histamine regulates this process via specific receptors [12]. Histamine is a multifunctionally active mast cell mediator and it is ubiquitously released from activated tissue MCs and blood basophils. Histamine can produce powerful physiological effects and its actions are mediated through specific receptors located on

target cells. The four histamine receptors include H1R, H2R, H3R, and H4R [13–17]. Stimulation of H1R increases the heart rate, cardiac output, vascular permeability, production of nasal mucus, bronchial and intestinal smooth muscle contractions, and T cell, neutrophil and eosinophil chemotaxis [11,18]. Stimulation of H2R increases gastric acid secretion, airway mucus production, and inhibits neutrophil and eosinophil influx into tissues [18,19]. H3R have been found in the brain in a variety of neurologic disorders [20], expressed in the central nervous system and to a lesser extent the peripheral nervous system, where they act as autoreceptors in presynaptic histaminergic neurons, and also control histamine turnover by feedback inhibition of histamine synthesis and release [21]. H4R is preferentially expressed on immune cells, such as eosinophils [22,23], basophils [24], natural killer (NK) cells, DCs, monocytes [25], T cells [26] and MCs [27–29]. The H4R was highlighted as a promising therapeutic target in atopic dermatitis, chronic arthritis and asthma [27,30]. The H4R has also been implicated in the regulation of other non-hematopoietic systems [30]. The functional link from the activation of individual histamine receptors to the resulting cellular processes is yet not completely understood.

3.2 Urticaria

3.2.1 Epidemiology

Urticaria is a skin disease, also known as hives. The symptoms are sudden whealing and/or deep swellings, associated with local redness of the skin and itching. In severe cases, urticaria may also burn and sting. Urticarial patches often start in various body areas and show a high fluctuation around the body. In most cases, urticaria lasts for several days (acute urticaria), disappearing without irreversible damage. In less than 5% of cases, symptoms continue to develop for more than six weeks (chronic urticaria) [31].

It is estimated that around 20% - 25% of people are affected by urticaria in their lifetime [32]. Adults are held to be more vulnerable than children to chronic urticaria. In adults, females are more commonly affected than males. However, no distinct difference between males and females has been reported in the pediatric population. The duration of disease tends to be longer in females. Generally, middle-aged females tend to suffer most from the signs and symptoms of chronic urticaria [33].

Often, acute urticaria occurs after a viral infection or from an allergic reaction to drugs or food. Other factors like psychological stress or exercise can exacerbate urticaria. A history of hay fever or asthma is an important risk factor [34].

3.2.2 Categorization of urticaria

The group consensus meeting [include EAACI (European Academy of Allergology and Clinical Immunology) Dermatology Section, the EU-funded network of excellence, GA2LEN (Global Allergy and Asthma European Network), the EDF (European Dermatology Forum) and UNEV (urticaria network e.V.)] gave new advice on the definition and diagnosis of chronic urticaria in December 2008 [35]. In 2017, the EAACI/GA^{2}LEN/EDF/WAO guideline on urticaria was updated and revised and currently recommends that urticaria is classified based on its duration as acute ($\leqslant$ 6 weeks) or chronic (> 6 weeks) [36].

Chronic urticaria subtypes:

i) Chronic spontaneous urticaria:

Spontaneous appearance of wheals, angioedema or both > 6 weeks due to known (e.g. autoreactivity: the presence of MCs activating autoantibodies) or unknown causes.

ii) Chronic inducible urticaria:

Symptomatic dermographism (also called urticaria factitia or dermographic urticaria); cold urticaria (also called cold contact urticaria); delayed pressure urticaria (also called pressure urticaria); solar urticaria; heat urticaria (also called heat contact urticaria); vibratory angioedema; cholinergic urticaria; contact urticaria; aquagenic urticaria.

Chronic spontaneous urticaria

Recurrent urticaria, i.e. wheals, angioedema or both, for longer than 6 weeks is classified as chronic urticaria, either chronic spontaneous urticaria or chronic inducible urticaria. Inducible urticarias account for 25% of all chronic forms of urticaria and occur more frequently in young adults [37].

Inducible urticarias

Inducible urticaria is defined as a unique subpopulation of chronic urticaria in which urticaria can be induced reproducibly by different specific physical stimuli on the skin, such as cold contact, heat contact, electromagnetic radiation (solar radiation) and

mechanical triggers (friction, pressure, vibration. There are also forms of inducible urticaria, where symptoms are provoked by exercise (cholinergic urticaria) or water contact (aquagenic urticaria).

Cholinergic urticaria

Cholinergic urticaria (CholU) is a common form of urticaria, which is clinically characterized by pinpoint-sized wheals, large flares and severe itch. It is a subtype of chronic inducible urticaria, symptoms of which are evoked by sweating [38] [38,39]. Symptoms can often be induced by exercise, passive warming (environmental heat, warm baths), emotional stress, and in some cases, hot and spicy food [40]. In the past, there were reports that a rise in body temperature of more than 0.7°C or an increase in the core body temperature is needed to provoke CholU [41,42]. However, some reports have shown that there is no correlation between the time to wheal formation and the time to increase either core or mean body temperature (MBT) [38]. Wheals in cholinergic urticaria are usually smaller (1-2 mm diameter pruritic wheals surrounded by a red flare) than those in spontaneous urticaria and of shorter duration. The itching, erythema and wheals normally subside within 30 minutes [43,44]. Cholinergic urticaria induced by exercise usually occurs after 6 minutes of onset of exercise. Increased clinical symptoms and physical signs occur approximately 12 to 25 minutes later [41].

3.3 Cholinergic Urticaria

3.3.1 Epidemiology

As described before, CholU is a frequent form of chronic inducible urticaria, where symptoms of wheals, flares and itch are evoked by sweating, upon environmental heat, warm bath, emotional distress, exercise and, in some patients, by some hot and spicy food [40].

Since 1924, when Duke first described CholU [45], many studies [46–49] have characterized this disease, mainly based on clinical features. CholU can occur at any age, but young adults are most commonly affected. In this population, CholU affects up to 20%, but only 0.2% of all dermatological patients present with CholU [43], indicating that most CholU patients do not seek the help of specialized physicians. CholU is a chronic disease,

usually lasting for several years, with a serious impact on the quality of life. Severely affected patients are not able to study, work or do housework [40].

CholU generally has a favorable prognosis [50]: Hessman reported that only 31% of patients had symptoms lasting longer than 10 years. Sibbald reported that the average duration of symptoms was estimated to be 7.5 years and ranged from 3 to 16 years [51]. The peak incidence of young adults is between 10 to 30 years of age [44,52–54].

3.3.2 Pathomechanism

CholU forms a heterogeneous disease group, and the underlying pathomechanisms are not completely understood [55]. Recently, Japanese studies suggested four distinct CholU subclasses [55,56]:

i) CholU with poral occlusion: caused by poral occlusion, occlusion of superficial acrosyringium due to keratotic plugs and dilatation of sweat ducts are evident by histological examination. Most cases of this type have been reported in Asia; this type of CholU is very rarely reported in Europe. Thus, CholU due to poral occlusion may be related to hot and humid climates.

ii) CholU with hypohidrosis: CholU is sometimes accompanied by acquired generalized hypohidrosis (AGH), suggesting that the sweat itself is not essential for the initiation of CholU. In patients with AGH, the inhibited sweat production may cause the elevation of local acetycholine levels. Consequently, excess acetylcholine could stimulate sensory nerve endings to evoke pain and act on muscarinic receptors on MCs in the vicinity of sweat glands to cause wheals. In one case of CholU, a patient with AGH showed an absence of muscarinic receptor expression on MCs [57]. Additionally, a recent study found that the expression of acetylcholine esterase (AchE) was decreased in exocrine epithelial cells, and acetylcholine was not completely degraded due to the lack of AchE [58]. Consequently, acetylcholine is thought to spill over to neighboring MCs and stimulate them to degranulate.

iii) CholU with possible sweat allergy: passive transfer experiments suggest a pathophysiological role of IgE in physical urticaria, but clear evidence is still lacking [59]. In CholU, there is evidence that the symptoms are induced by an allergy to components of human sweat. Specific IgE to sweat is present in patients with CholU, but not in normal controls [53]. Basophils from these patients reportedly reacted to autologous sweat and

released high amounts of histamine *in vitro* [53]. In addition, recent reports suggest that antigens of *Malassezia globosa*, a skin resident fungus, may be contained in human sweat, which acts as an allergen to induce histamine release from mast cells and basophils in patients with CholU [60].

iv) Idiopathic CholU: Previous reports described the three major causes of CholU, described above however, there still remain patients with CholU who do not fit into these categories. CholU in these patients, CholU is categorized tentatively as idiopathic [56].

Table 1 CholU: pathogenesis-based categorization and its treatment options

Sub type	Season (severe)	Intradermal test for Cholinergic agent	Sweat allergy	Hypohidrosis	Treatment
i	Winter	Typically negative	Negative	Occasional	Bathing, keratolytic agents
ii	Perennial	Positive	Negative	Always	Systemic steriod therapy
iii	--	Positive	Positive	None	Anti-IgE therapy desensitization
iv	--	Negative	Negative	None	Antihistamine drugs

3.3.3 Signs and Symptoms

CholU is the only form of chronic urticaria, where wheals are very small and pin point sized, usually surrounded by large flare reactions and often accompanied by a severe itch. The wheals typically appear on the upper body and usually remain for a few minutes up to an hour before they spontaneously disappear [61–63].

3.3.4 Diagnosis

The diagnosis of CholU is based on the patient history and provocation testing. Typical features of CholU, in contrast to other forms of urticaria, include small wheal size and short duration of the wheals. Usually patients do not display symptoms when they present to their physician. Accordingly, provocation tests are useful for diagnosis.

Provocation tests for CholU are based on increasing the body temperature either through exercise (e.g. on a treadmill or stationary bicycle) or by using a hot bath or sauna [35,64,65]. Illig reported that a medium rise in MBT of 0.7°C was necessary to cause whealing [64]. Furthermore, the EAACI/GA^{2}LEN/EDF/UNEV consensus panel recommendation for confirming the diagnosis of CholU is to achieve a rise of ⩾1.0°C in core body temperature by a passive warming test consisting of sitting for up to 15 minutes in a bath full of water at 42°C [35]. These tests allow the diagnosis of CholU, but not grading of disease severity.

More recently, our team has developed and published a specific pulse-controlled ergometry (PCE) protocol for standardizing the diagnosis of CholU and to objectively assess disease activity. This approach also includes an assessment of the provocation UAS (Urticaria Activity Score), which allows a grading of the resulting symptoms.

Patient-reported outcomes

In a recent publication, our working group has introduced several patient-reported outcome measurements, which allow for the characterization and quantification of CholU symptoms. For disease activity, the CholU Activity Score (CholUAS7), and for quality of life, the CholU Quality of Life (CholU-Qol) Questionnaire has been developed.

3.3.5 Mast cell-related physiopathology in CholU

Although the underlying causes of CholU are still not completely understood, it is believed that the activation of MCs in the skin and their release of histamine and other proinflammatory mediators are the most relevant factors. Histologically, wheals and edema occur on the skin, and molecules released by MCs cause blood vessels to dilate and fluids to penetrate the skin. MCs play an important role in most patterns of urticaria via the release of histamine and other inflammatory mediators such as cytokines, chemokines and leukotrienes (Figure 2). Histamine acts on cutaneous nerve endings to induce characteristic pruritus. In chronic urticaria, it has been demonstrated that MC activation can not occur only via specific IgE-allergen interactions, but also via autoreactive mechanisms, like functional autoantibodies against IgE and high-affinity IgE receptors on MCs and basophils, which lead to cross-linking of the high-affinity IgE receptors and subsequent mediator release.

Tryptase is a serine protease that is primarily produced and stored in MCs and less abundantly, in blood basophils. The basal serum tryptase level in healthy individuals is caused by the constant release of enzymes from mature tissue MCs [66]. The median serum tryptase level in healthy adults averages about 5 ng/ml and ranges from < 1 to 30 ng/ml. Serum tryptase levels are less than or equal to 15 ng/ml in more than 99% of healthy people [67–69]. Despite this, in commercially available tests, the normal range is usually set at < 11.4 ng/ml or even < 10 ng/ml [70]. Serum tryptase levels in healthy individuals increase with age [68,71], and many healthy individuals have serum tryptase levels between 10 and 15 ng/ml. Even healthy human serum tryptase levels can exceed 25 ng/ml.

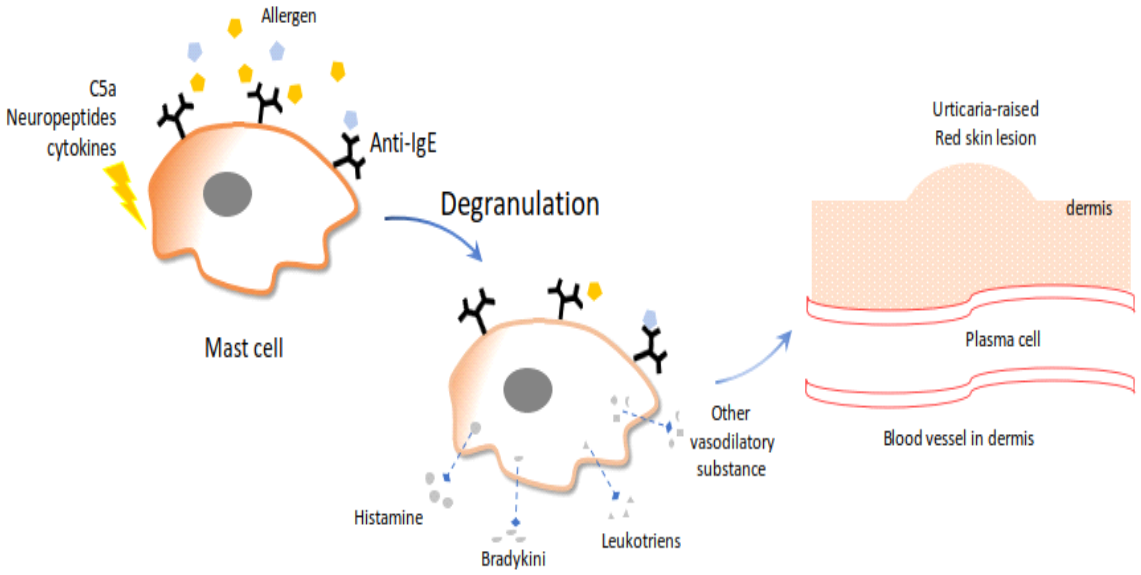


Fig. 2 Physiology of mast cell-mediated urticaria

3.3.6 Management

Urticaria guidelines

The current urticaria guidelines (shown in Table 2) give treatment recommendations for all forms of chronic urticaria including CholU.

Non-sedating H1 antihistamines, and within this group, specifically second generation antihistamines (sgAHs) are recommended as the first-line treatment of mild to moderate chronic urticaria [72], but many patients do not respond to standard sgAH doses [40]. In

severe cases of urticaria, if the standard dose for refractory urticaria is ineffective, there is evidence that increasing the dose up to four times may control the symptoms without compromising the patient's safety [6,73], and this is the recommended second line treatment option. But there is still a large patient cohort that remains symptomatic despite this updosing [74]. To date, there are no other licensed drugs available for the treatment of patients with CholU. Omalizumab, an anti-IgE monoclonal antibody, which is licensed for the treatment of chronic spontaneous urticaria (CSU), has also been shown to be effective in some case reports in CholU, but this treatment is currently not available for most patients.

Table 2 Recommended treatment algorithm for chronic urticaria including CholU

Second-generation H1 antihistamine (nsAH*)
In case of insufficient control after 2-4 weeks or sooner if symptoms are intolerable.
Updosing of nsAH (up to 4 times the standard dose)
In case of insufficient control after 2-4 weeks or sooner if symptoms are intolerable.
In addition to nsAH: omalizumab
In case of insufficient control after 6 months or sooner if symptoms are intolerable.
In addition to nsAH: cyclosporine

*nsAH: non-sedating antihistamine

Further treatment options:

In earlier case reports, anticholinergic substances such as clidinium bromide [75] or scopolamine butylbromide [76,77] were used to relieve symptoms. In recent years, there have been reports of successful treatments of severely affected patients who were refractory to antihistamines, using methylvaleronitrile, an anticholinergic drug [78]. Methatheliniumbromide is currently licensed in Germany for the treatment of hyperhidrosis and has been shown to be effective in CholU in a case report [78].

3.3.7 Current problems in the treatment of CholU

Antihistamines are the first-line treatment for CholU. These are the only licensed drugs for this disease, but they are often ineffective. The next treatment step (updosing) is off-label, and only very limited publications regarding this treatment algorithm are available for CholU. Some drugs like omalizumab and methantheliniumbromide have been shown to be effective, but these drugs are not licensed and are not available for most patients.

The limited available therapeutic options result from the limited understanding of the pathophysiology of the disease and the little known drivers of different clinical characteristics of patients with CholU. Currently, it is unclear whether patients show differences at various ages in the clinical features of CholU, duration of disease, comorbidities and response to treatment.

The role of MCs is also not completely understood and needs to be investigated in more detail, it could be beneficial for this patient cohort to understand if MC-directed therapies. Specifically, it is not clear, if MC activation can occur via specific IgE-mediated mechanisms or via acethylcholine-receptor aberrations, if MC numbers are altered in CholU, if MCs release histamine and tryptase upon whealing in CholU, and if patients with CholU have aberrations in the downstream histamine pathyway (e.g. histamine-receptor). In order to address some of these questions, the following study has been performed.

3.4 Aim of the study

The main goal of this project was to explore the role and relevance of MCs in the pathophysiology of patients with CholU compared to healthy controls.

To achieve this goal we aimed to:

1) clinically characterize patients with CholU and compare them to a matched, healthy control group.

2) assess MC activating factors in CholU by:

A) assessing the prevalence and relevance of atopy in the two groups.

B) investigating possible the MC triggers, such as total and specific IgE.

C) analyzing possible correlations between these MC triggers and clinical parameters.

3) analyze aberrations in the MC status of the skin in patients with CholU by:

A) studying the difference of MC number and degranulation status between patients with CholU and a healthy control group.

B) comparing the variations of MCs in amount and degranulation status after motion stimulation between the two groups.

4) look for MC products (tryptase levels before and after the provocation between the two groups) and their correlation with clinical markers.

5）analyze possible dysregulations of mediator-receptor interactions possibly involved in CholU:

A) histamine receptors

B) ACH (acetylcholine) esterase

4. Materials and Methods

The appliances, chemicals, antibodies, media, buffers, kits and disposables used in this study are listed below. The concentrations and specific applications of the listed materials are specified in the corresponding method section.

4.1 Appliances

All the appliances and instruments used for this doctoral thesis are listed in table 3.

Table 3 List of appliances employed for this study

Appliance	Model	Distributor
Caliper	Series No 10-4987015-BR	Bruune flexlineal, Germany
Centrifuge	Megafuge 1.0 STR	Heraeus, Hanau, Germany
Microscope	Zeiss Axioplan 2	Zeiss, Germany
Microscope	Confocal DMI 6000	Leica, Germany
Traying cabinet	Hera cell	Heraeus, Hanau, Germany
PH-Meter	pH-Fix 4.5-10.0	Laborbedarf & ROTH
Power Supply	Power Pac 300	Biobad, Munchen, Germany
Tissue embedding machine	Shandon CitadelTM 1000	Thermo Fisher Scientific, Walldorf, Germany
Walter bath	WBT 222	Medingen, Dresden, Germany
Suctions bulb	pipetus	Hirschmann Laborgerate, Eberstadt, Germany
Thermo Mixer	Thermomixer R	Eppendorf, Hamburg, Germany
Incubator	Wärmeschrank	Memmert, Germany

4.2 Chemicals

The chemical and reagents used for the development of this doctoral thesis are listed alphabetically in table 4.

Table 4 List of chemicals and reagents employed for this study

Chemicals/Reagents	Distributor
Ethylendiamintetraessigsaure (EDTA)	Merck, Darmstadt, Germany
Natriumhydroxid (NaOH)	Merck, Darmstadt, Germany
PBS w/o Ca2+ and Mg2+	PAA, Pasching, Osterreich
Tris-buffer saline(10x)	Sigma-Aldrich, Steinheim, Germany
Tween-20	Sigma-Aldrich, Steinheim, Germany
H_2O，steril	Ampuwa, Fresenius Kabi, Germany
Formaldehydlosung 37%	Merck, Darmstadt, Germany
0.01M sodium citrate buffer, PH6.0	Sigma-Aldrich, Steinheim, Germany

4.3 Antibodies and probes for histology

The antibodies and probes used for skin pathological staining and immunohistochemistry for the development of this doctoral thesis are listed below (Table 5).

Table 5 List of antibodies and probes employed for the study

Antigen	Isotype	Clone	Dilution	Distributor
H4R	Rabbit IgG	NLS3775	1:90	Novus Biologicals Germany
H1R	Rabbit IgG	LS-A1167	1:90	Lifespan Biosciences Germany
Anti-ACHE	Rabbit IgG	LS-B6676	1:90	Lifespan Biosciences Germany
H1R type 1	Probe	VA1-3000210-VT	1:40	Thermo Fisher Scientific Germany
H2R type 1	Probe	VA1-3000201-VT	1:40	Thermo Fisher Scientific Germany
H3R type 1	Probe	VA1-11070-VT	1:40	Thermo Fisher Scientific Germany
H4R type 1	Probe	VA1-11039-VT	1:40	Thermo Fisher Scientific Germany
Tryptase type 6	Probe	VA6-19985	1:40	Thermo Fisher Scientific Germany
Secondary antibody	Labelled polymer-HRP anti-rabbit	10068282	Ready to use	Dako, United States

4.4 Patient samples and data acquisition

Data were collected from March 2017 until April 2018 at the Department of Dermatology and Allergy, Charité – Universitätsmedizin , Berlin, Germany. In total, 13 healthy volunteers and 13 patients with a history of CholU were recruited. The presence of CholU was confirmed in the patients using PCE provocation testing [38]. The study was approved by the Ethics Committee of Charité-Universitatsmedizin, Berlin, Germany (approval #EA1/242/15) and was registered with the German Clinical Trials Register (DRKS-ID: DRKS00012755). It was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonization (ICH), Good Clinical Practice (GCP) Guidelines, national laws and regulations, and wih the Standard Operating Procedures (SOPs) of the Department of Dermatology and Allergy, Charite - Univer-sitätsmedizin, Berlin, Germany. All participants gave signed informed consent at the beginning of the study.

4.5 Clinical characterization of the patients

In total, 13 patients with CholU and 13 healthy controls participated in this study. One participant in the healthy control group did not complete the study for personal reasons. Overall, there were 12 women (4 women in the CholU group, 8 women in the healthy group), and 13 men (9 men in the CholU group, 4 men in the healthy group) participating in the study. None of the women were pregnant or lactating. None of the participants had taken corticosteroid at least 2 weeks before the provocation test. Patients were advised not to take antihistamines at least 3 days before provocation. Nevertheless, 4 patients had taken 1 antihistamine tablet within the last 3 days of provocation. No participants had taken corticosteroids or other MC stabilizers within the past two weeks. All participants, expect one healthy control, who terminated the study before the provocation, could be provoked. None of the participants were allergic to iodine.

As seen in the following table, the healthy control group and the CholU group were comparable regarding sex, age and body mass index. There were no significant differences between the two groups (Table 6).

Table 6 Participant demographics and results summary.

Parameter	Healthy controls	Patients with CholU
Number	12	13
Sex (m/f)	8/4	9/4 *
Age (years)	35.6 (22 - 59)	34(19 - 53) *
Body mass index (kg/m^2)	24.9 (21.1 - 34.7)	24.5 (18.5 - 31) *

Data are presented as median with range.

* P > 0.05 no difference between healthy control group and CholU group.

4.6 Measurements

Thirteen healthy controls and 13 patients with CholU were assessed for atopic skin diathesis (atopic predisposition) by using of the Erlangen Atopy Score [79]. Both groups were assessed for disease severity via the Visual Analogue Scale (VAS), Likert scale and Cholinergic Urticaria Severity Index (CholUSI) [38] (Table 7) and disease activity (CholUAS7) (Table 8), quality of life impairment (Dermatology Life Quality Index [DLQI]) (Table 9) [80], Cholinergic Urticaria Quality of Life Questionnaire (CholU-QoL)(Table 10) [81]], urticaria control test (Table12), seasonal exacerbation, global score 7, total and specific serum IgE, and comorbidities.

4.6.1 Assessment of disease severity

We validated the diagnosis of CholU and determined disease severity using a PCE challenge test. That is, all the patients performed a static bicycle ride for 30 minutes in a

pulse-controlled manner. Patients were instructed to speed up or slow down their pedaling so that the pulse rate eventually reached 160 beats per minute. The time of initiation of treatment was recorded as a sign of the severity of the disease. Observations continued during the 10 minutes recovery period after the 30 minutes of exercise.

The CholU Severity Index (CholUSI) was used to assess CholU severity [38]. The CholUSI is a sum score that takes into account the frequency of CholU symptoms (< once a month = 0 point; once a month = 1 point; > once a month = 2 points; once a week = 3 points; > once a week = 4 points; daily = 5 points; > daily = 6 points), eliciting factors (one point each for: physical exercise, hot bath, hot shower, emotional stress, hot food, sauna, other), duration of skin lesions (< 5 min = 0 point; 5 – 10 mins = 1 point; 10 ‑ 20 mins = 2 points; 20 ‑ 30 mins = 3 points; 30 ‑ 60 mins = 4 points; < 1 hour = 5 points) and itch (none = 0 point; mild = 1 point; moderate = 2 points; severe = 3 points). Accordingly, the CholUSI score ranges from 0 to 21 points: < 5 points: very mild CholU; 5 ‑ 9 points: mild CholU; 10 ‑ 15 points: moderate CholU; > 15 points: severe CholU.

Table 7 CholUSI (Cholinergic Urticaria Severity Index)

Frequency: Less than once a month = 0, Once a month = 1, More than once a month, less than once a week = 2, Once a week = 3, More than once a week = 4, Every day = 5, Several times a day = 6
Trigger: exercise / effort = 1, Hot baths = 1, Hot showers = 1, Emotional stress = 1, Spicy food = 1, Sauna = 1, Other = 1
Duration of skin symptoms: Less than 5 minutes = 0, 5-10 minutes = 1, 10-20 minutes = 2, 20-30 minutes = 3, 30-60 minutes = 4, More than an hour = 5

(Clinics for dermatology, venereology and allergy Consultation hours for urticaria, angioedema and mastocytosis by Prof. Dr. med. M. Maurer.)

VAS and Likert scale:

Evaluation of patients with urticaria of current illness situation in the past 2 weeks:

VAS: in the following, please assess how severe your urticaria symptoms (your disease activity) have been over the past 2 weeks. For this, we ask you to mark on the line recorded below in the most suitable place for you. The very left end of the line indicates that you did not have any urticaria symptoms. A cross on the right side of the line means that you had maximum conceivable urticaria symptoms. A cross in the middle would mean a moderate level of complaints.

No urticarial complaints Maximum imaginable urticaria complaints

Please rate below how much your overall quality of life has been affected by your urticaria over the past 2 weeks.

No restriction in your quality of life

maximum conceivable restriction in your quality of life

Likert scale: in addition, we request that patients make the same documentation for their urticaria complaints in the past 2 weeks with the following options:

No (complaints)	Mild	Moderate	Severe

In addition, we would ask patients to make the same assessment of their quality of life in the last 2 weeks with the following possible answers:

No (restriction)	Mild	Moderate	Severe

4.6.2 Assessment of disease activity

Urticaria is assessed with urticaria activity score (UAS). The UAS measures two symptoms – number of wheals and intensity of itching – each on a 0 - 3 scale each day. The UAS was recorded by each patient daily and was obtained from the patients weekly. For both the number of wheals and the severity of itch, on a given day for every of the day in of a given week to get the weekly UAS. The possible weekly aggregate UAS ranged from 0 to 42. Score-based health states were defined as follows: urticaria-free = 0; well controlled urticaria = 1 – 6; mild = 7 – 15; moderate = 16 – 27; and severe urticaria = 28 – 42.

Table 8 CholUAS: Urticaria Activity Score

Wheals	Pruritus	Score
None	None	0
Mild (< 20 wheals / 24hours)	Mild (present but not annoying or troublesome)	1
Moderate(20 - 50 wheals/ 24 hours)	Troublesome but does not interfere with sleep	2
Intense(> 50 wheals/ 24 hours or large confluent areas of wheals)	Severe pruritus, which is sufficiently troublesome to interfere with normal dairy activity or sleep	3

Cholinergic urticaria is assessed with CholUAS. In daily diaries, in order to better assess disease activity, we require patients to record their signs and symptoms of CholU and triggering factors on the last 7 days before provocation. Specifically, patients recorded a pruritus intensity (itch day:

no = 0, mild = 1, moderate = 2, severe = 3) and their treatment intensity (weal day: no = 0, mild = 1, moderate = 2, severe = 3) intensity of excitation (intensity of elicitor day: no exposure to inducer = 4, exposure to mild elicitor = 3, exposure to moderate elicitor = 2, exposure to strong elicitor = 1). CholUAS7 is calculated as the sum of 7 days of [(weal day + itch day) x elicitor day strength], reaching a maximum of 168 points. A comparison of different composite scores has identified this score as the best score for determining the activity of CholU disease.

4.6.3 Quality of life instruments

Quality of life was assessed using the DLQI questionnaire and the Chronic Urticaria Quality of life questionnaire, following recent recommendations [80]. The DLQI measures skin health-related quality of life, has a recall period of 7 days, consists of 10 questions and has a score that can range from 0 to 30, with 0 – 1 points: no impairment, 2 – 5 points: low impairment, 6 – 10 points: moderate impairment, 11 – 20 points: severe impairment and > 20 points: extreme impairment. The aim of this questionnaire is to measure how much your skin problem has affected your life over the past week.

Table 9 DLQI

Item No.	Over the last week
1	Over the past week, how itchy, sore, painful or stinging has your skin been?
2	Over the past week, how embarrassed or self-conscious have you been because of your skin?
3	Over the past week, how much has your skin interfered with you going shopping or looking after your home or garden?
4	Over the past week, how much has your skin influenced the clothes you wear?
5	Over the past week, how much has your skin affected any social or leisure activities?
6	Over the past week, how much has your skin made it difficult for you to do any sport?
7	Over the past week, has your skin prevented you from working or studying?
8	Over the past week, how much has your skin created problems with your partner or any of your close friends or relatives?
9	Over the past week, how much has your skin caused any sexual difficulties?
10	Over the past week, how much of a problem has the treatment for your skin been, for example by making your home messy, or by taking up time?

(very much = 3, a lot = 2, a little = 1, not at all = 0)

4.6.4 Assessment of disease-related quality of life

The German CholU-QoL assesses disease-related quality of life (symptoms, functional life, social interaction, therapy, emotions) from a 5 domain structure. It has 28 questions and each question is answered with a five-level Likert scale. The total CholU-QoL score has a range from 0 to 100, with higher scores indicating higher levels of impairment in quality of life.

It shows the items of the German translation, in the order in which they appear on the questionnaire, along with an English equivalent and an abbreviated name. Each statement or question is scored on a 5-point scale, as explained. Items are preceded by the question: the five choices presented are: gar nicht, wenig, mittel-mäßig, stark, sehr stark (not at all, little, medium, severe, very severe).

Table 10 CholU - QoL: Chronic Urticaria Quality of Life Questionnaire

Item No.	Statement	Translation
	Wie sehr haben Sie in den letzten 14 Tagen unter den folgenden Beschwerden Ihrer Nesselsucht (Urticaria) gelitten?	How much have you suffered from the following symptoms of hives (urticaria) in the past 14 days?
1	Quaddeln	Wheals
2	Hautrötungen	Erythema
3	Juckreiz	Itching
4	Hitzegefühl	Feeling hot
5	Müdigkeit	Fatigue
	Wie sehr waren Sie in den letzten 14 Tagen in den folgenden Bereichen des täglichen Lebens furch Ihre Nesselsucht (Urticaria) beeinträchtigt?	How were you been affected by urticaria in the following 14 days in the following areas of daily life?
6	Schule/Studium/Beruf	School / Education / Occupation
7	Freizeitgestaltung	Recreation
8	Tägliche Aktivitäten (z.B. Haushalt / Einkaufen / Gartenarbeit)	Daily activities (e.g., household / shopping / gardening)
9	Körperliche Aktivitäten / Sport	Physical activities / sports
10	Aktivitäten mit anderen Menschen	Activities with other people
11	Wahl der Kleidung	Choice of clothes
	Mit den folgenden Fragen möchten wir näher auf Schwierigkeiten und Probleme eingehen,die mit Ihrer Nesselsucht (Urticaria) in den letzten 14 Tagen verbunden waren:	With the following questions, we would like to elaborate on the difficulties and problems associated with your hives (urticaria) in the past 14 days:

12	Fühlten Sie sich wegen Ihrer Nesselsucht in Ihrer Leistungsfähigkeit eingeschränkt?	Did you feel limited in your performance because of your hives?
13	Waren sie wegen Ihrer Nesselsucht beeinträchtigt, anstrengende Tätigkeiten zu verrichten?	Was your ability to perform difficult work impaired?
14	Hat es Ihnen Ihre Nesselsucht erschwert aktiv zu sein?	Does your urticaria make it difficult for you to be active?
15	Haben Sie wegen Ihrer Nesselsucht Situationen oder Tätigkeiten gemieden, in denen Sie ins Schwitzen kommen?	Have you avoided situations or activities that make you sweat because of your hives?
16	Waren Sie wegen Ihrer Nesselsucht in Ihrer Mobilität eingeschränkt?	Were you restricted in your mobility because of your hives?
17	Haben Sie unter der Sichtbarkeit Ihrer Nesselsucht gelitten?	Have you suffered from the visibility of your hives?
18	Hat die Nesselsucht Ihnen Wunsch eingeschränkt, mit anderen Menschen zusammen zu sein?	Has hives limited your desire to be with other people?
19	Haben Sie wegen Ihrer Nesselsucht dazu geneigt häufiger zu Hause zu bleiben?	Do you tend to stay home more often because of your hives?
20	Haben Sie sich wegen Ihrer Nesselsucht in der Öffentlichkeit unwohl gefühlt?	Did you feel uncomfortable with your urticaria in public?
21	War Ihnen Ihre Nesselsucht peinlich?	Was your urticaria embarrassing?
22	War es Ihnen unangenehm, wenn andere Menschen Ihre Nesselsucht oder damit verbundenes Kratzen bemerkt haben?	Did you feel uncomfortable when other people noticed your hives or the associated scratching?
23	Haben Sie sich Sorgen gemacht, Ihre Nesselsucht könnte sich verschlimmern?	Have you worried your hives could get worse?
24	Haben Sie unter Ungewissheit gelitten, wann Ihre Nesselsuchtbeschwerden auftreten?	Did the uncertainty about when the hives complaints would occur worry you?
	Mit den folgenden Fragen möchten wir näher auf Schwierigkeiten und Probleme eingehen, die mit Ihrer Behandlung der Nesselsucht	With the following questions we would like to find out more about the difficulties and problems associated with your treatment of hives (urticaria) in the past 14 days:

	(Urticaria) in den letzten 14 Tagen verbunden waren:	
25	Stellte die Behandlung Ihrer Nesselsucht für Sie eine Belastung dar?	Did the treatment of your hives put a strain on you?
26	Hatten Sie Sorge vor möglichen Nebenwirkungen Ihrer Nesslsuchtbehandlung?	Did you worry about possible side effects of your hives treatment?
27	Haben Sie unter Nebenwirkungen Ihrer Nesselsuchtbehandlung gelitten?	Have you suffered from side effects of your hives treatment?
	Fühlten Sie sich in den letzten 14 Tagen aufgrund Ihrer Nesselsucht (Urticaria)...?	Did you feel in the past 14 days due to your hives (Urticaria):
28	Frustriert	Frustrated?
29	Deprimiert	Depressed?
30	Genervt	Annoyed?

4.6.5 Assessment of atopy

The Erlanger criteria for the assessment of an atopic skin diathesis were developed to analyze, independently of the current skin disease, the atopic diathesis with respect to the target organ skin. Despite subjective freedom from symptoms, several of the features contained in the score added to skin-damaging factors.

This score is broken down into the following 6 areas of atopy criteria: atopic family history, atopic self-history, atopic minimality, atopic stigmata, dermal neurovegetative, and laboratory values. These ranges include the features listed in Table 11. In the case of unclear or weak findings, only half the score can be awarded for the respective characteristic.

The scoring system for this questionnaire is Category 1, no atopy (score 0–3 points); Category 2, atopy unlikely but cannot be excluded (score 4–9 points); Category 3, atopic (score 10–14 points); Category 4, clear atopic skin diathesis (score 15-19 points)，Category 5, very strong atopic skin diathesis (score >20 points).

Table 11 Erlanger Atopic score

Category	Characteristic
Family medical history	Eczema
	Rhinitis / bronchial asthma
Own medical history	Rhinitis / conjunctivitis
	Asthma allergy
	Cradle cap

	Itching on uninfected skin
	Textile intolerance
	Metal intolerance
	Photophobia
Minimal forms	Xerosis
	fissures
	Dyshidrosis
	Pityriasis alba
	Pulpitis sicca
	Nipple eczema
	Perleche
Stigmata	Palmar hyperlinearity
	Sign of hertoghe
	Dirty neck
	Keratosis pilaris
Neurovegetative	White dermographism
	Acrocyanosis
Laboratory values	IgE > 100 U/ml
	IgE > 200 U/ml
	positive phadiatop(sIgE)

For a laboratory assessment of atopy (total IgE serum level), patient serum was collected by venous puncture and sent to the central laboratory of the clinic (Labor Berlin Inc, Berlin, Germany). Total IgE was determined using the Immuno CAP System Phadia Laboratory Systems, Thermo Fisher Scientific Inc, Uppsala, Sweden.
We also sent the serum to the same laboratory for assessing tryptase and *Malassezia globosum* serum level.

The quantification of the total tryptase concentration with ImmunoCAP Tryptase leads to a risk assessment for severe allergic reactions. ImmunoCAP Tryptase measures the concentration of tryptase released into the serum by the MCs. MCs play a key role in allergic reactions and increase in number under inflammatory conditions. When activated, they release a variety of mediators that cause signs and symptoms of allergic reactions, such as B. anaphylaxis. These mediators also include tryptase and histamine. A transient increase in blood tryptase concentration following anaphylactic shock in a patient helps to determine and assess the extent of the response. A persistent elevated basal concentration of tryptase is an indication of a possible mastocytosis. (Serum 1.0 ml, MethodeImmuno-CAP fluorescence assay, reference range < 11.4 µg/l). Serum from all patients was analyzed for IgE against Malassezia specific IgE by the same central laboratory of the clinic. They calculated the results as 7 levels shown in the table below.

CAP class 0	< 0.35 IU/ml
CAPclass 1	≥ 0.35 IU/ml and < 0.70 IU/ml
CAP class 2	≥ 0.70 IU/ml and < 3.5 IU/ml
CAP class 3	≥ 3.5 IU/ml and < 17.5 IU/ml
CAP class 4	≥ 17.5 IU/ml and 50 IU/ml
CAP class 5	≥ 50 IU/ml and 99.99 IU/ml
CAP class 6	≥ 100 IU/ml

4.6.6 Urticaria control test (UCT)

The following questions should help patients with CholU to understand their current illness. For each question, we gave a score. The total score (UCT) is 16, we separated the UCT to two levels (UCT < 12, not controlled, UCT ≥ 12 well controlled).

Table 12 Urticaria Control Test

To what extent have you suffered from the physical complaints of the urticaria (itching, wheals and / or swelling) over the past 4 weeks? 0=very severely 1= severely 2=moderately 3=mild 4=not at all
2. How was your quality of life affected by urticaria in the past 4 weeks? 0=very severely 1= severely 2=moderately 3=mild 4=not at all
How often has therapy for your urticaria in the past 4 weeks been insufficient to control your urticaria symptoms? 0=very severely 1= severely 2=moderately 3=mild 4=not at all
How well did you control your urticaria in the past 4 weeks? 0= Not at all 1= barely 2= moderately 3= well 4= very well

4.6.7 Seasonal exacerbation

Patient were assessed anamnestically to defermine, whether their symptoms increased in summer, winter, or unchanged over the year.

4.6.8 Global score 7

A weekly score (global symptom score 7) was calculated as a sum of the daily scores.

How would you rate your complaints today?

No = 0, Mild, tolerable moderate = 1, Some restriction = 2, Very severe, severely noticeable, significant restriction = 3

4.6.9 Pulse-controlled ergometry (PCE) test

The PCE test [38] involves a 30-min static bicycle ride at an ambient temperature of 20 – 22.8°C. Twenty minutes acclimatization time was allowed before tests, during which time participants were fitted with a double sensor device attached to the forehead (double sensor, TDS, Draegerwerk AG, Luebeck, Germany). Starch-iodine powder (according to Minor' sweat test) [82] was applied to the lower back to detect sweating. For the provocation test, the individual was seated on the bicycle and remained stationary until the sensors measured stable results for at least 1 minute. During exercise, the times of the start of the sweating, defined as the appearance of the first blue dots in the starch-iodine powder, and the start of reddening/ whealing were recorded. Physician rated the sweating grading by 5 levels at the end of PCE test.

Score	Sweating	Starch-iodine powder
0	Non (sweating)	No change
1	Very little	Sweating only in small points or small areas
2	Less than normal	Not all areas blue
3	Normal	All blue
4	More than normal	All blue, sweat running down

CholU patients were rated after the PCE test on their number of wheals and their itch severity, resulting in a UAS_{provo} ranging from 0 to 6 points.

a) Numbers of wheals (0 = 0; 1 to 20 wheal = 1; 20 to 50 wheal = 2; > 50 wheal = 3)

b) Itching (none = 0, mild = 1, moderate = 2, severe = 3)

4.6.10 Skin biopsy

The full skin (from epidermis to fat layer) was punched before and after the PCE test. After the PCE test, the wheal (lesional skin) was punched in the CholU patient group. Normal skin (non-lesional biopsy) was punched in both CholU group before the PCE test and in the healthy control group (before and after the PCE test). Skin specimens were fixed using 4% buffered formaldehyde (Herbeta Arzneimittel, Berlin, Germany).

4.6.11 Histology

The embedding of the section in paraffin was carried out using the Shandon Citadel 1000 (Waltham, USA) according to the following protocol (Table13). After embedding, 5 µm thick tissue sections were cut using a Microtome Finesse 325 from Shandon (Waltham, USA). These sections were first stained by hematoxylin-eosin staining (HE staining) (Table14) to visualize the nuclei and cytoplasm non-specifically.

Table 13 The embedding of the cuts in paraffin

Medium	Time of application (minutes)
Aqua distilled	20
70% ethanol	50
70% ethanol	60
96% ethanol	50
96% ethanol	60
Absolute ethanol	50
Absolute ethanol	60
Absolute ethanol	60
xylene	50
xylene	60
paraffin	120
Paraffin	Unlimited

Table 14 HE staining on paraffin sections

Coloring	Step time (minutes)
3 x xylene	10
2 x absolute ethanol	5
2 x 96% ethanol	5
2 x 70% ethanol	5
3 x distilled water	5
Hematoxylin (freshly filtered)	1
rinse with running water	10
Rinsing in aqua distill	1
Eosin	1
Rinse 4 x in distilled water	1
70% ethanol	3
96% ethanol	3
absolute alcohol	3
xylene	3
Cover with Clarion environmentally safe permanent mounting medium	

Number of MCs and in the skin-sections were assessed by the following MC specific stainings:

i) 2-Naphthalenecarboxamide, 3-(acetyloxy)-N-(2-methylphenyl)- (Naphthol AS-D) Chloroacetate for Specific Esterase Kit (for MC numbers)

ii) Toluidine blue (alternative staining for MC numbers)

iii) Giemsa (staining, which allows detection of MC granules).

Table 15 AS-D staining on paraffin sections

Prepare AS-D solution: 1ml sodium nitrite solition + 1ml fast red violet LB base + 5 ml Trizmal 6.3 buffer concentante + 1ml Naphtol AS-D chlorocicete	
Coloring	Step time (minutes)
2 x xylene	10
2 x absolute ethanol	2
1 x 96% ethanol	2
1 x 70% ethanol	2
2 x Aqua distilled (37°C)	2

Table 16 Toluidine blue

Coloring	Step time (minutes)
2 x xylene	5
2 x absolute ethanol	2
2 x 96% ethanol	2
2 x 70% ethanol	2
Aqua distilled	5
0.5% Toluidine blue	overnight
Rinsing in aqua distilled	1

AS-D solution avoid light (37°C)	15
1 x Aqua distilled (37°C)	2
Hematoxylin solution	2
Rinse in tap water and cover with slopping mounting medium	

2 x 95% alcohol	2
2 x absolute alcohol	
2 x xylene	
Cover with slopping mounting medium	

Table 17 Giemsa staining on paraffin sections

Coloring	Step time (minutes)
3 x xylene	10
2 x absolute ethanol	5
2 x 96% ethanol	5
2 x 70% ethanol	5
3 x Aqua distilled	5
Giemsa Azur Eosin ethylene blue undiluted and filtered	1
0.1% acetic acid	10 seconds
Rinsing in aqua distilled	10
3 x absolute alcohol	3
3 x xylene	5
Cover with Clarion environmentally safe permanent mounting medium	

Sections were analyzed using microscope of Zeiss Axioplan2, in average 5 high-power fields (HPF 40X) x 5 times and counting under microscope, the mean value was calculated.

In-situ-hybridization

Ribonucleic acid (RNA) histamine 1, 2, 3, 4 receptor expression staining and Tryptase-RNA expression staining was performed with a selected number of patients and healthy control samples (Table 18). Histamine 1 and 4 receptors were also stained via antibody-staining to analyze protein-expression level (Table 19).

Table 18 Histamine receptors and tryptase stainings on paraffin sections (view RNA)

Coloring	Step time (minutes)	Step temperature
3 x xylene	5	RT
2 x absolute ethanol	5	RT
Air dry	5	RT
PapPen-air dry	30	RT
Heat-Pretreatment solution 1:100 in Aqua distill	5	92°C
2 x Aqua distill,1 x PBS	1	RT
Protease QF 1:100 in 40°C PBS	20	40°C
2 x PBS	1	RT
Probe- 1:40 in prewarmed Probe-set-Diluent QT	40 hours	40°C
3 x wash buffer* with shaking	2	RT
Preample Mix QT	25	40°C

3 x wash buffer* with shaking	2	RT
Amplifier Mix QT	15	40°C
3 x wash buffer* with shaking	2	RT
Label-Probe 1/6-AP 1:1000 in Label-Probe-Diluent QF	15	40°C
3 x wash buffer* with shaking	3	RT
Fast Red substrate in the dark	30	40°C
PBS	5	RT
Cover with slopping mounting medium		

* wash buffer: wash comp1 + wash comp2 + Aqua distilled, RT: room temperature, PBS:Phosphate Buffered Saline, QT: 4-acetyloxybutanoyl 4 acetyloxybutanoate, QF: methyl methacryla crosspolymer.

Table 19 Histamine receptors staining on paraffin sections (antibody)

Coloring	Step time (minutes)	Step temperature
3 x xylene	5	RT
3 x absolute ethanol	3	RT
2 x 95% ethanol	3	RT
1 x 80% ethanol	3	RT
Rinse slides in gentle running distilled water	5	RT
Steam slides in 0.01 M sodium citrate buffer, pH6.0	20	99-100°C
Remove from heat and let stand in buffer	20	RT
Rinse in 1 x TBST*	1	RT
Apply a universal protein block	20	RT
Drain protein block from slides, apply diluted primary antibody	45	RT
Rinse slides in 1 x TBST	1	RT
Apply a biotinylated secondary antibody appropriate for the primary antibody	30	RT
Rinse slides in 1 x TBST	1	RT
Apply alkaline phosphatase streptavidin	30	RT
Rinse slides in 1 x TBST	1	RT
Apply alkaline phosphatase chromogen substrate	30	RT
Rinse slides in distilled water	1	RT

*TBS: Phosphate Buffered Saline TBST: TBS with 0.05% Tween

Photos were taken and sections were analyzed using a confocal microscope of Leica DMI 6000 (High Performance Fortran: HPF 20X) and counting was done using Image J program.

4.7 Statistical analyses

Statistical analysis was done using Microsoft Excel Version 2006 and Graphpad Prism 5. As not all the data were normally distributed, they are all presented as median (with range) and mean (with standard deviation [SD]) and the statistical significance of differences between patient groups was calculated using Student's t-test for independent means, as

this test is considered suitable for small sample sizes. Correlations were calculated using the Pearson correlation test. $P < 0.05$ was considered to indicate a significant difference.

5. Results

5.1 CholU patient characteristics

5.1.1 Patients with CholU had a long duration of disease.

Patients with CholU had a mean duration of their disease until presentation of 11.1 ± 10 years (median 7.5; 0.8 to 29 years) (Fig. 3).

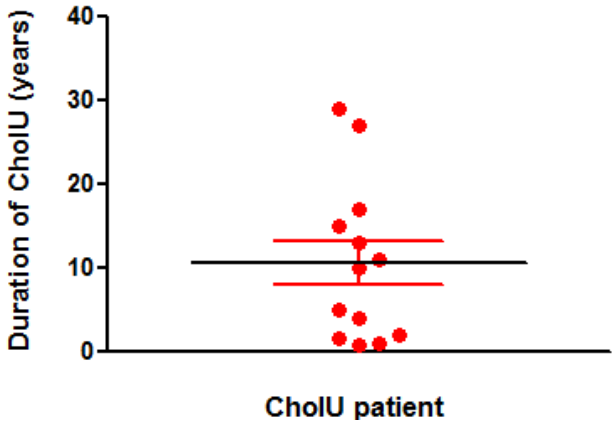


Fig. 3 Duration of disease in CholU patients group.
Individual patients are shown as dots. Blank line shows mean, red whiskers represent standard deviation.

5.1.2 Patients rated themselves as mostly moderately affected

The majority of patients rated their disease severity (using a 3 dimensional Likert scale) as moderately affected (Fig. 4). Using this score, patients had in a mean of 1.9 ± 0.8 points (range 1 to 3) in the last 2 weeks. All patients had at least one complaint.

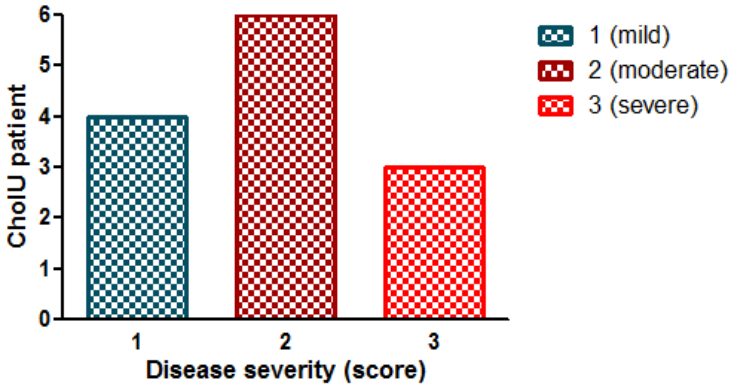


Fig. 4 Disease severity in CholU patients.
Bar graph shows patients' numbers in each category.

Comparable results were seen using the VAS assessment of the same questions. Patients with CholU had a median level of urticaria of 4.9 ± 2.2 (range 2.3 to 9.3) (Fig. 5A). The patient rating of VAS (disease severity) correlated with the results of the Likert scale. The VAS scores of the severe group were significantly higher compared to the mild disease group (P = 0.04) and showed a trend towards the moderate disease group (P = 0.08). There was no significant difference between the moderate and the mild disease groups (P > 0.05) (Fig. 5B).

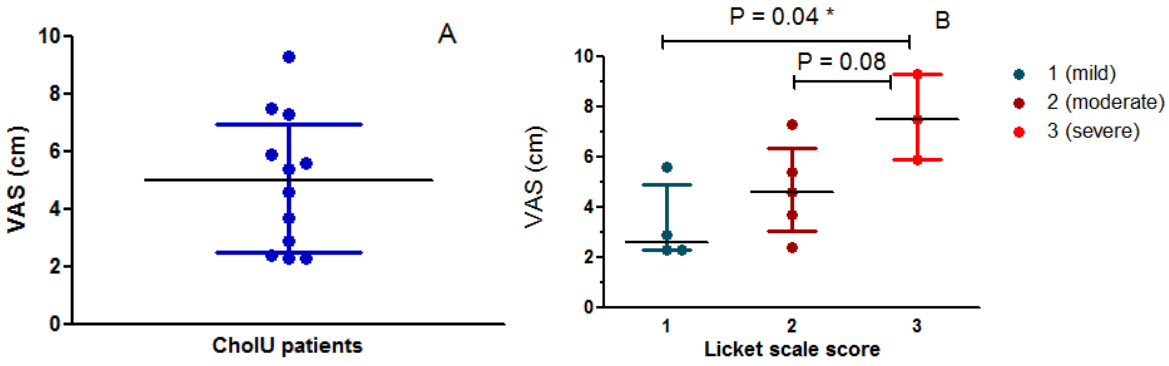


Fig. 5 VAS assessment in the CholU patient group (A) and Correlation of Likert and VAS scale assessment of disease severity (B).

Individual patients are shown as dots. Black line shows mean, colored whiskers represent standard deviation. Asterisk marks significant differences, as calculated by t-test.

5.1.3 Patients with CholU were in the mean moderately affected, using clinical scores

Using newly implemented disease-specific measures, the disease severity was assessed by using a daily diary, as described above, documenting wheal and itch severity and calculating the CholU activity score 7 (CholUAS7), by implicating the elicitor on the respective day. Patients also rated their daily symptoms using a 3-dimensional Likert scale, scoring their daily symptoms form none to severe (global symptom score). A weekly score (global symptom score 7) was calculated as a sum of the daily scores. Furthermore, the CholUSI as a marker for disease severity was assessed in these patients. Results are given in Table 20.

Table 20 CholUAS7 and CholUSI in CholU patients group

Patient characteristics	Mean±SD (median; [range])
Duration of disease	11.1±10 (7.5; 0.8 to 29)
Wheal 7	6.8±5.6 (5.5; 0 to 18)
Itch 7	7.8±5.9 (7.5; 0 to 19)
CholUAS 7*	38.2±31.7 (32； 0 to 98）
CholUSI	12.2±2.3 (11; 9 to 16)
Global symptom score 7	6.8±5.6 (5.5; 0 to 18)

* 7-d Sum of ([$wheal_{day}$ + $Itch_{day}$] x Intensity of $elicitor_{day}$) SD: Standard Deviation

CholUAS: Cholinergic Urticaria Activity CholUSI: Cholinergic Urticaria Severity Index

5.1.3.1 CholUAS7

The established CholUAS7 correlated very well with the patients' rating of their disease severity (r = 0.9, P< 0.001). The CholUAS7 was significantly higher in the severe, compared to the mild disease group (P = 0.01) and the moderate disease group (P = 0.03) (Fig. 6A).

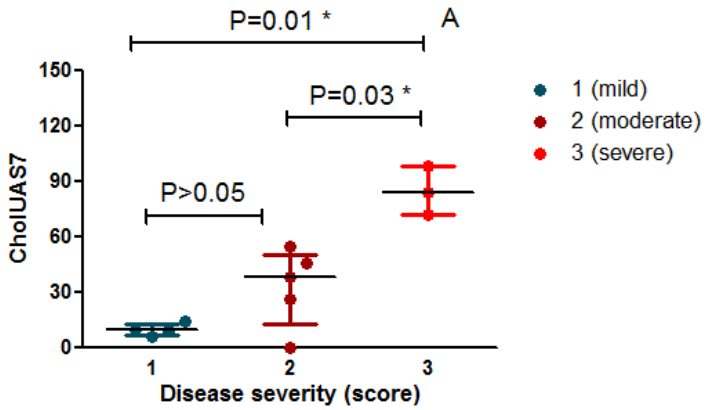


Fig. 6 CholUAS7 and correlation of CholUAS7 with VAS and global symptom score7.

Fig. 6A CholUAS7 was positively correlated with the patients' rating of disease severity. Individual patients are shown as dots. Black line shows median, colored whiskers show interquartile range. Asterisk marks significant differences, as calculated by t-test.

Furthermore, the CholUAS7 was positively correlated with the patients' VAS rating of their disease severity (Fig 6B), and with the patients' Likert scale of their disease severity (r = 0.9, P < 0.001, data not shown), and with global symptom score 7 (r = 1.0, P < 0.0001, Fig. 6C). However, there was no correlation with the duration of their disease (r = 0.4, P > 0.05).

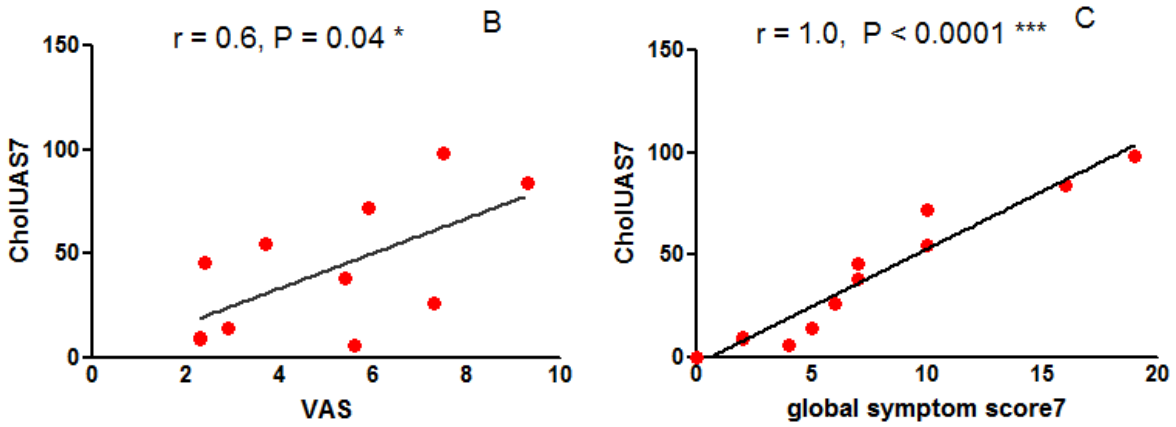


Fig. 6 B/C. Correlation of CholUAS7 with VAS (patient rating of disease severity) (B), and correlation of CholUAS7 with global symptom score7 (C). Individual patients are shown as dots. Black line shows the correlation. Asterisk marks significant differences as calculated by the Pearson test.

5.1.3.2 CholUSI: most CholU patients had moderate CholU.

In line with previous results, the majority of patients with CholU (n = 9, 69%) could be grouped into the moderate group using the CholUSI. Of the remaining four, two (15%) of them fell into the mild CholU group and the other two (15%) were in the severe CholU group regarding the CholUSI (Fig. 7A).

The CholUSI was not significantly correlated with diseases severity (VAS) (r = 0.6, P> 0.05) and the CholUAS7 (r = 0.4, P > 0.05), but there was a trend towards the patients' rating of disease severity (Likert scale) (r = 0.6, P = 0.05) and global symptom score7 (r = 0.5, P = 0.07). Interestingly, a longer duration of disease was significantly correlated with the CholUSI score (r = 0.8, P < 0.001) (Fig. 7B).

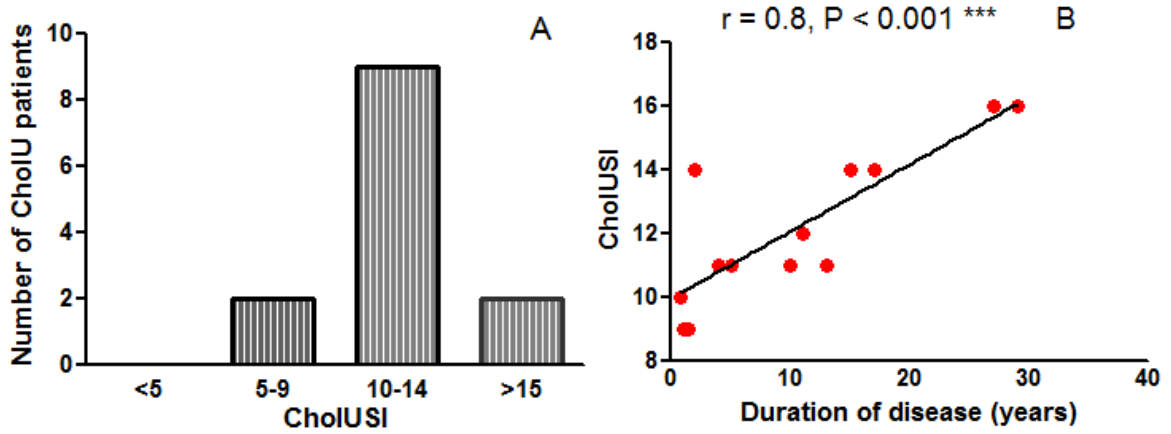


Fig. 7 CholUSI and correlation with duration of disease.

Distribution of patients with CholU regarding their CholUSI (A) Bar graph shows patient numbers in each category and correlation of CholUSI with duration of disease (B) Individual patients are shown as dots. Black line shows the correlation. Asterisk marks significant differences as calculated by the Person test.

5.1.4 Quality of life

5.1.4.1 Patients rated themselves as mostly moderately for current disease situation in the past 2 weeks

Patients rated their quality of life impairment using a 3-dimensional Likert scale in the mean as moderately affected (1.9 ± 0.8, range 1 to 3) within the last 2 weeks before presentation (Fig. 8B). Comparable results were seen in the VAS assessment of the same question. The CholU patient group had a median level of quality of life impairment of 5.0 ± 2.5 (range 1.3 to 8.4) (Fig.8A).

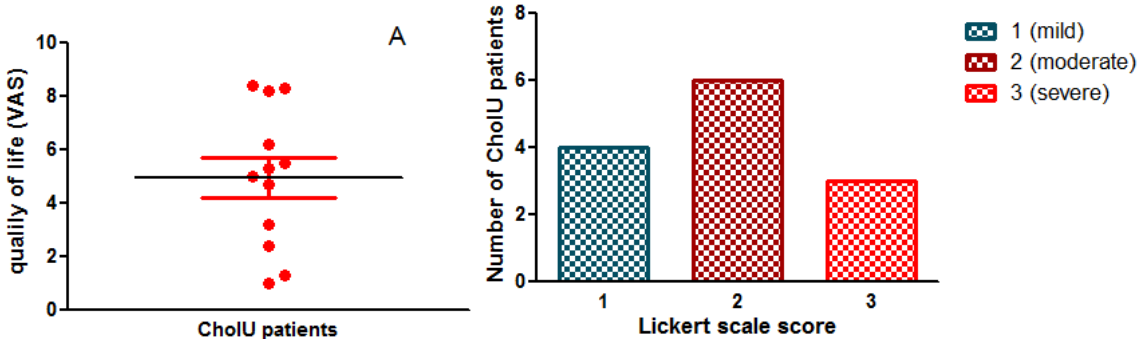


Fig. 8 Quality of life in the CholU patient group. (A) Individual patients are shown as dots. Black line shows mean, colored whiskers represent the standard deviation. (B) Bar graph shows patient numbers in each category.

Most patients had correlation scores between their disease severity rating and their quality of life impairment rating (Fig. 9). Only four patients scored in different categories in the 3-dimensional Likert scale of self-rating.

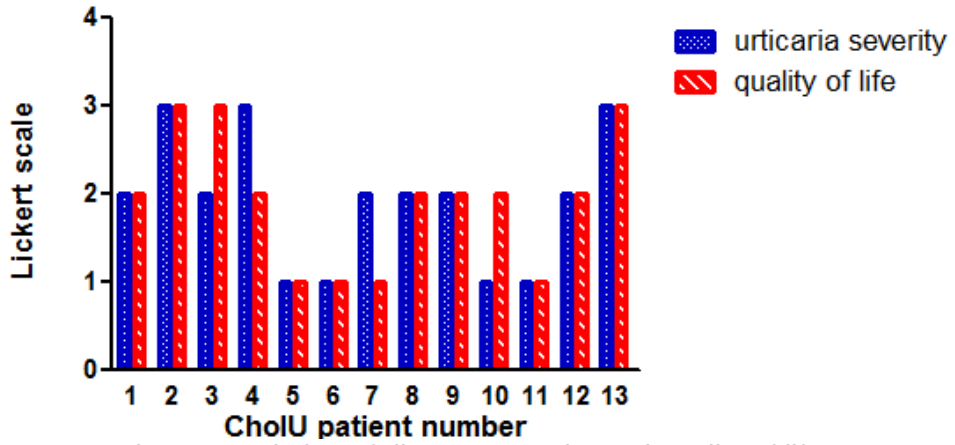


Fig. 9 Correlation of disease severity and quality of life.

Bar graph shows urticaria severity and quality of life of Likert scale score in each patient.

5.1.4.2 DLQI assessment showed moderate to strong impairment in most patients with CholU patients

Quality of life was assessed using the DLQI questionnaire and the CholU-Qol, following recent recommendations[80,81]. The mean DLQI of the CholU patient group was12.4 ± 5.1 (7 to 16 range). Most patients in our patient group had moderate or strong impairment. One patient scored extremely high, and none scored no or low impairment. (Fig. 10).

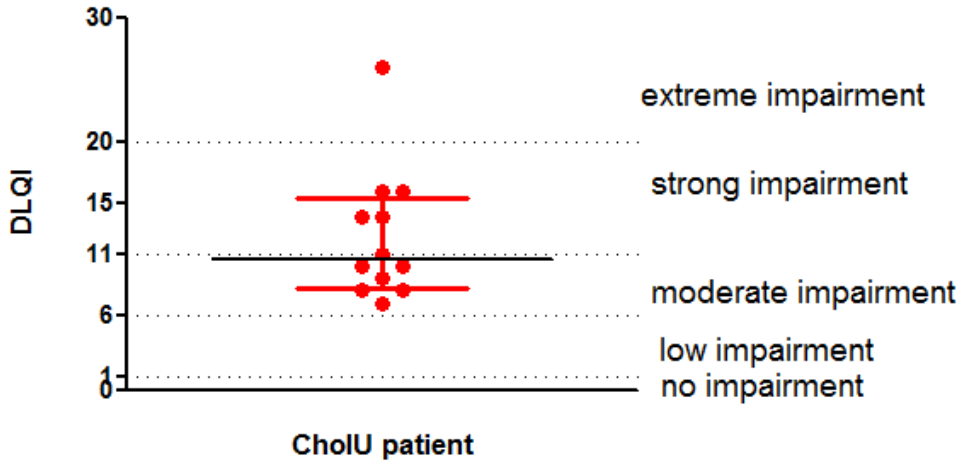


Fig. 10 DLQI assessed quality of life in patients with CholU.

Individual patients are shown as dots. Black line shows median, colored whiskers show interquartile range. Dotted line represents cut-offs between different categories (as described on the right side of the figure).

The DLQI was highly correlated with the patients' ratings of the quality of life (VAS and Likert scale) in the CholU patient group. DLQI was positively correlated with the VAS (r =

0.7, P = 0.02) (Fig. 11A) and also positively correlated with the Likert scale rating (r = 0.7, P = 0.004) (Fig. 11B).

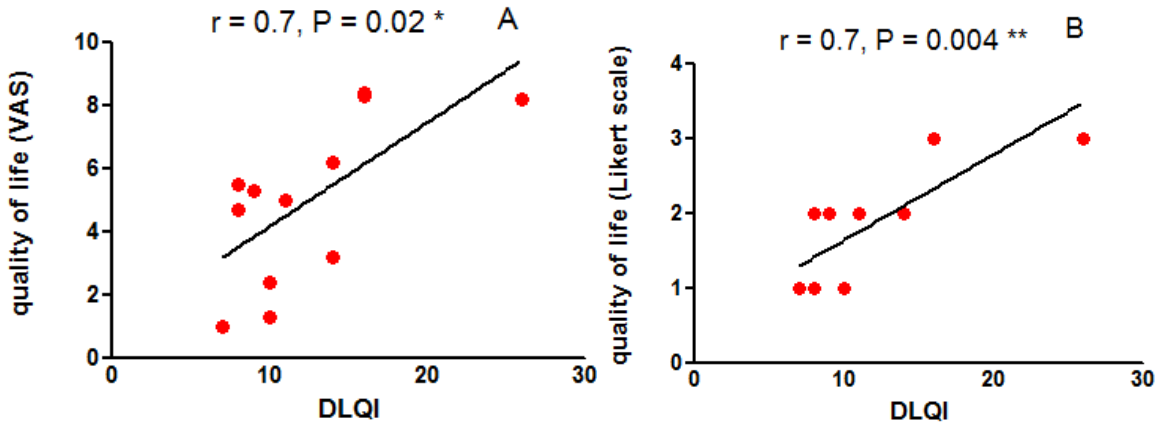


Fig. 11 Correlations: DLQI with VAS (A) and DLQI with the Likert scale (B).

Individual patients are shown as dots. Black line shows the correlation. Asterisks mark significant differences as calculated by the Person test.

5.1.4.3 CholU-Qol of patients with CholU showed that the quality of life impairment has a broad range

The mean CholU-QoL score was 42 ± 12.2 in the CholU patient group, with a broad range of 25 to 67.5 (Fig. 10A). This disease-specific score correlated overall very well with the DLQI (r = 0.7, P = 0.01) (Fig. 12B).

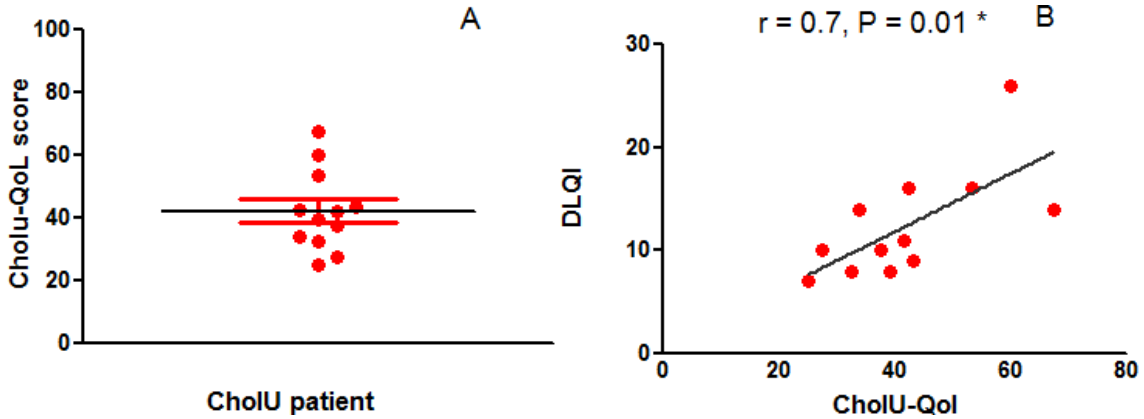


Fig. 12 CholU-Qol scores and correlation with DLQI.

CholU-Qol scores of patients with CholU (A) and the correlation with DLQI in the CholU patient group (B). Individual patients are shown as dots. Black line shows mean, colored whiskers represent standard deviation. Black line shows the correlation. Asterisk marks significant differences as calculated by the Person test.

The CholU-QoI also correlated significantly with the patients' ratings of quality of life impairment using VAS (r = 0.8, P = 0.004) and the Likert scale (r = 0.6, P = 0.03) in the CholU patient group (Fig. 13A and B).

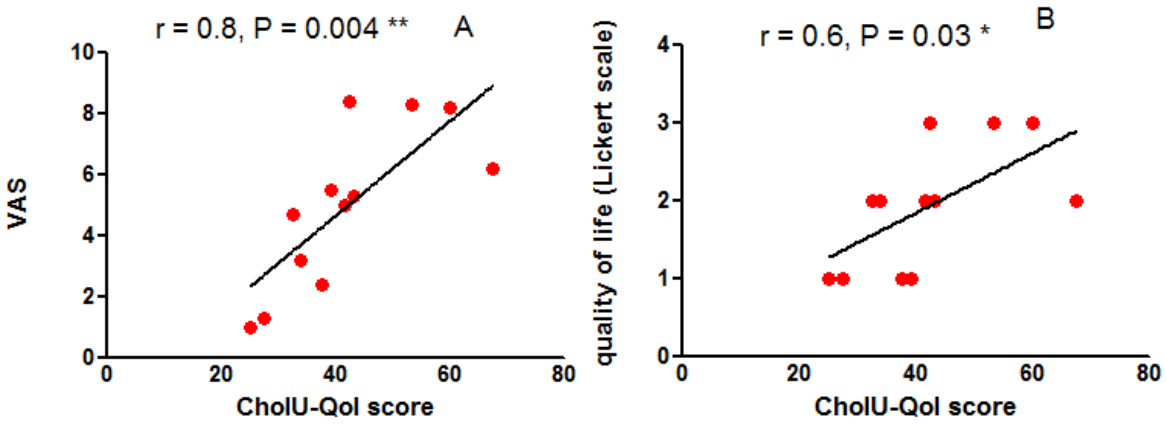


Fig. 13 Correlations: CholU-QoI with VAS (A) and Likert scale (B).

Individual patients are shown as dots. Black line shows the correlation. Asterisks mark significant differences as calculated by the Person test.

5.1.5 Treatment / Antihistamines

Patients documented their antihistamine intake in the last week before presentation. On average, patients took 3.9 ± 3.2 tablets per week (median 5.0; 0 to 9) (Fig. 14). Overall two groups of patients can be separated: patients that took antihistamines (n = 8) and patients who did not (n = 5).

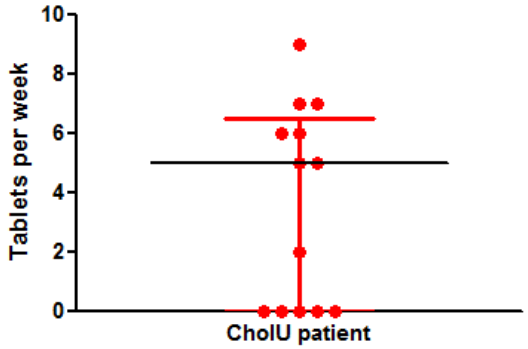


Fig. 14 Antihistamine intake per week of patients with CholU.

Individual patients are shown as dots. Black line shows median, colored whiskers show the interquartile range.

When analyzing the correlation between antihistamine intake and disease severity or quality of life impairment, we saw no correlation of antihistamine intake per week with

CholUAS7 (r = 0.008, P > 0.05), DLQI (r = 0.02, P > 0.05) or CholUSI (r = 0.001, P > 0.05) or CholU-Qol (r = -0.01, P > 0.05).

5.1.6 CholU, in almost all patients was not well controlled

The urticaria controlled test was designed to understand the effect of the current treatment of patients (UCT < 12 urticaria not controlled, UCT ⩾ 12 urticaria well controlled). Of the 13 patients in the CholU group, 12（92%）indicated that their disease was not well controlled (Fig. 15).

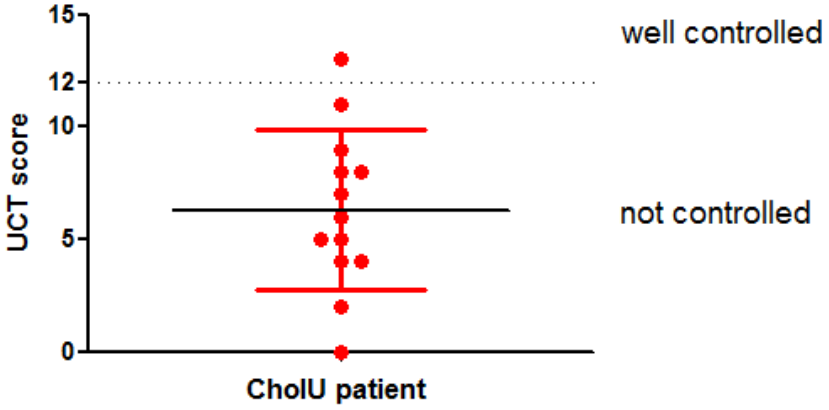


Fig. 15　UCT score of patients with CholU.

Individual patients are shown as dots. Black line shows mean, colored whiskers represent standard deviation. Dotted line represents cut-offs between different categories (as described on the right side of the figure).

Eight patients had taken antihistamines in the last week (1-9 tablets per week) before the assessment; the remaining patients had no such therapy. We divided them into an antihistamine-treated group and a group without antihistamine therapy. We found that the mean UCT of the antihistamine group was comparable to the non-antihistamine group (P > 0.05), indicating no significant influence of this therapy in controlling the disease (Fig. 16).

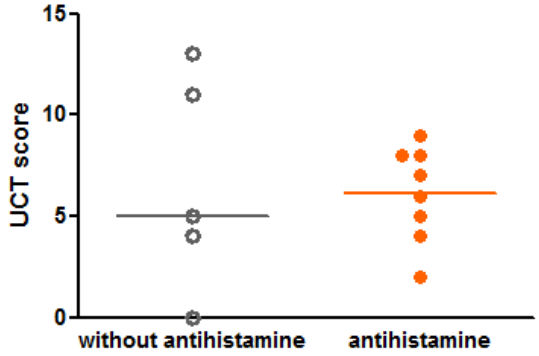


Fig. 16 The correlation between UCT and antihistamine treatment.

The lines show median.

5.1.7 Seasonal distribution of CholU aggravation showed winter was the worst season

One of the theories of the pathophysiology of CholU was the theory of poral occlusion, which is meant to be most severe in winter. Accordingly, we asked the patients about their symptom severity in respect to the seasonal changes. Almost half (46%) of patients with CholU reported that their CholU symptoms were unchanged over the whole year, 39% of them had more severe symptoms in winter, and only 15% had worse symptoms in summer. Interestingly, in this study all (4) of the female CholU patients reported no seasonal changes (Fig. 17).

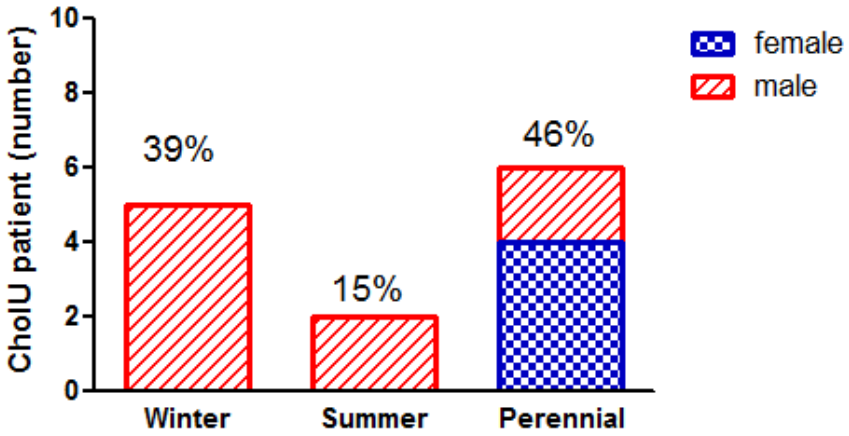


Fig. 17 Seasonal and sex distribution of the CholU group.
Bar graph shows patient numbers in each category.

5.1.8 Provocation test

5.1.8.1 CholU patients and healthy controls have comparable markers at the onset of sweating

All CholU patients and healthy controls took the PCE test. In the healthy control group, the median time to onset of sweating was 17.5 min (range 13 to 25), which was comparable to the CholU patient group (median 16.6 min, range 11 to 22, $P > 0.05$) (Fig. 18A). Also the heart rate (HR) at onset of sweating (healthy: median 122.4, range 98 to 149) was similar (CholU: median 120.4, range 102 to 138, $P > 0.05$) (Fig. 18B).These results indicate that patients with CholU do not have a problem with the onset of sweating.

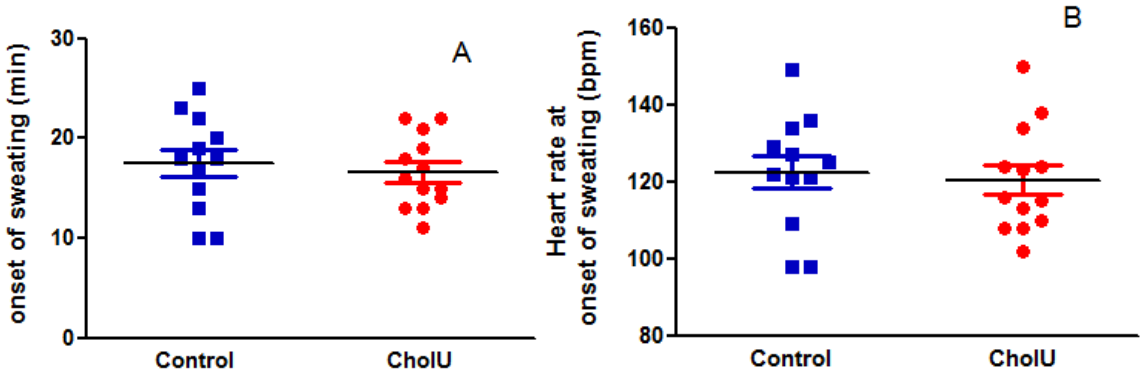


Fig. 18 PCE threshold levels of the healthy control group and the CholU patient group. Individual patients are shown as dots. Black lines show mean, colored whiskers represent standard deviation.

Using physician assessment of the sweat grading in the sweat test according to Minor, we saw that ten patients with CholU had very little or decreased sweating behavior and three patients had normal sweating. None showed above-average sweating behavior (Fig. 19).

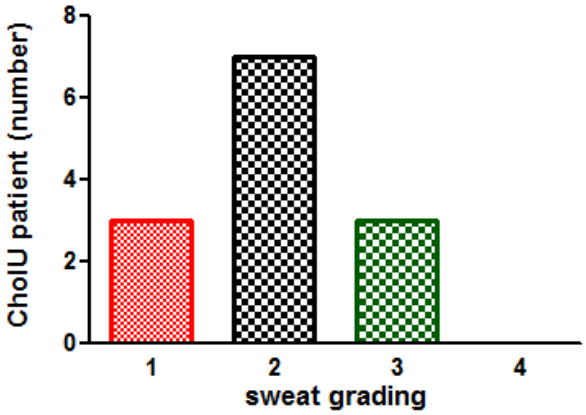


Fig. 19 Sweat grading in the CholU patient group. Bar graph shows patient numbers of each category. Patients who had very little sweating were represented in red, less than normal in black, and normal in green.

5.1.8.2 Patients with CholU develop wheals after the onset of sweating

In patients with CholU, the development of skin reddening (flare reaction) (median 20.7 minutes, range 14 to 27) and whealing (median 23.8 minutes, range 14 to 35 minutes) always occurred after the onset of sweating (median16.6 minutes, range 11 to 22) (Fig. 20). There was no such skin reaction in the healthy control group.

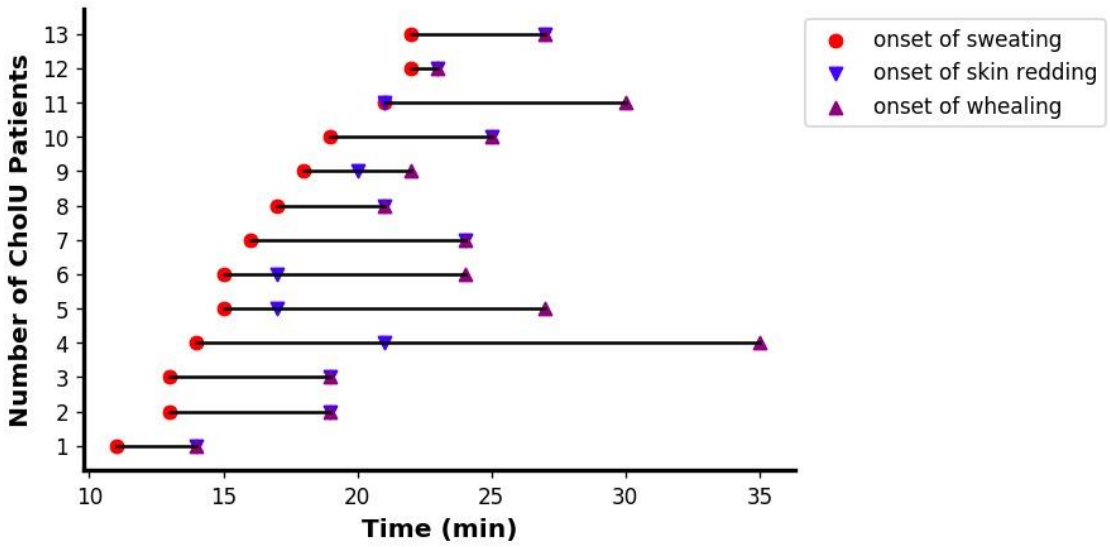


Fig. 20 Time between sweating and skin reddening/ whealing in the CholU patient group. Onset of sweating is represented by red dots, onset of skin reddening by blue triangle, and onset of whealing by purple triangles.

Patients started whealing after a mean time of 7.2 ± 4.9 minutes after the onset of sweating. Six (46%) patients started to wheal 4-7 minutes after onset of sweating and in four (31%) patients, sweat was more delayed after 8-12 minutes. Two (15%) patients had a very short time of two minutes between sweating and whealing and one patient had a very long time of 21 minutes.

5.1.8.3 Provocation UAS score

Patients with CholU had a mean UAS_{provo} score of 3.9 ± 0.4 (range 1 to 6) (Fig. 21A). The UAS_{provo} correlated very well with the patients' ratings of disease severity (Likert scale). The group with high disease severity had significantly higher UAS_{provo} compared with the mild group ($P = 0.04$). There was a trend towards higher UAS_{provo} in the moderate compared to the severe group, but this did not reach significance ($P = 0.08$) (Fig. 21A). There was also a strong correlation with the VAS patient ratings of their disease severity and the UAS_{provo} ($r = 0.8$, $P < 0.001$).

When looking at the correlation of the symptoms provoked by PCE (UAS_{provo}) and the daily dairy scores of the patients (CholUAS7), we saw a significant, but rather weak correlation ($r = 0.4$, $P = 0.03$) (Fig. 21B).

Interestingly, the UAS_{provo} of the PCE was highly correlated with the quality of life measures used in these patients. Patients had high correlations with their self-rated VAS and Likert scale of life quality impairment (Fig.21 C/D), DLQI (Fig. 21E) and CholU-Qol (Fig. 21F). There were no differences in the UAS_{provo} regarding seasonal exacerbation ($P > 0.05$) or the sex of patients ($P > 0.05$).

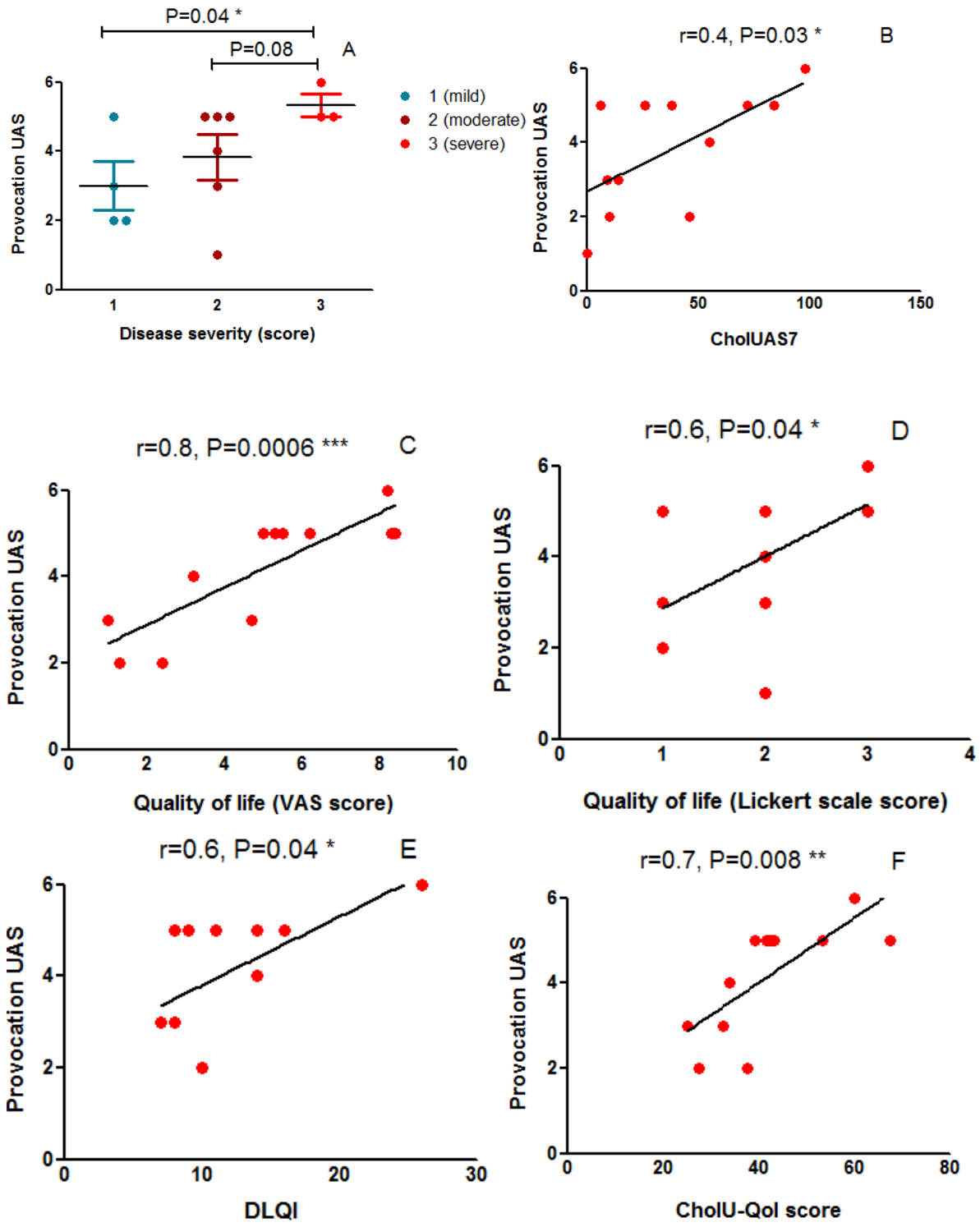


Fig. 21 Provocation UAS score correlations.

Individual patients are shown as dots. (A) Black line shows median, colored whiskers show the interquartile range. Asterisks mark significant differences as calculated by the t-test. (B, C, D, E, F) Black line shows the correlation. Asterisks mark significant differences as calculated by the Person test.

5.1.8.4. Influence of Antihistamines

Eight (62%) patients with CholU had taken antihistamines within one week, four (31%) patients from the CholU group had taken antihistamines within 24 hours before assessment. None of the patients had taken corticosteroids of other MC stabilizers for the past two weeks.

To analyze if the intake of antihistamines was influencing the disease severity, patients were divided into two groups: i) CholU patients with antihistamine intake (23%) and ii) CholU patients without antihistamine intake (77%) (Fig 22). Five (56%) of the patients who had not taken antihistamines had developed symptoms in the last 24 hours before provocation testing, whereas only one (25%) of patients who took antihistamines had symptoms, but this difference was not significant ($P > 0.05$) (Fig 22). There was no significant difference between the two patient groups regarding CholUAS7 ($P > 0.05$), CholU-Qol ($P > 0.05$), DLQI ($P > 0.05$), and CholUSI ($P > 0.05$).

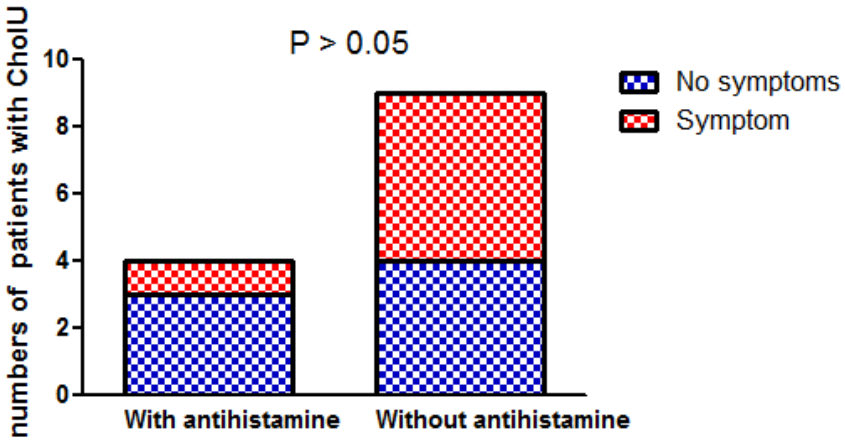


Fig. 22 Treatment with antihistamines could have an influence on disease symptoms .
Bar graph shows patient numbers in each category. Patients who had symptoms are represented in red and those who did not are in blue (unpaired t-test).

Interestingly, patients numbers 04, 06, 09 and 11 had taken antihistamines in the last 24 hours before the PCE test (Fig. 20). In these patients, the mean delay of the onset of whealing after the start of sweating was higher compared to those who did not take antihistamines in the last 24 hours (4.1 ± 3.0). This difference was not significant, the

patients who took antihistamines showed a trend towards longer delay of onset of symptoms compared to the patients without antihistamine (P = 0.09), possibly due to low patient numbers (Fig. 23).

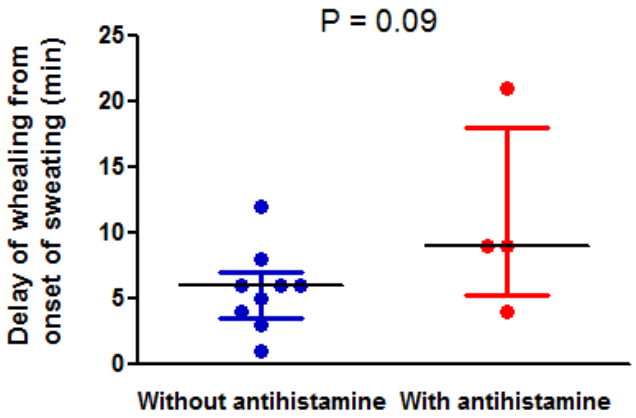


Fig. 23 Time from sweating to onset of whealing in patients who did / did not take antihistamines in the last 24 hours before PCE.
Individual patients are shown as dots. Black line shows median and colored whiskers show the interquartile range.

Patients with or without antihistamine intake were not different regarding their onset of sweating (P > 0.05) or onset of whealing (P > 0.05).

5.2 Investigation of possible MC triggers

As whealing is the key feature of CholU, MCs are thought to play a key role in the pathophysiology of the disease, we investigated possible clinical MC triggers in patients with CholU.

5.2.1 Atopic predisposition is frequent in patients with CholU

Using the Erlangen atopy score, the mean atopy score was significantly higher (P = 0.001) in the CholU group (7.9 ± 3.0) compared to the healthy control group (3.7 ± 2.3) (Fig. 24A). Separating the patients and healthy controls into the proposed five categories, seven of the 12 controls (58%) were found to have no atopy (category 1), the remaining five healthy controls (42%) fell into category 2 (atopy unlikely but cannot be excluded), and no one was found to be atopic or severely atopic. In the CholU group, one patient (8%) was found to have no atopy (category 1), eight (62%) fell into “atopy unlikely but cannot be excluded” (category 2) and the remaining (31%) were considered to be atopic (Fig. 24B).

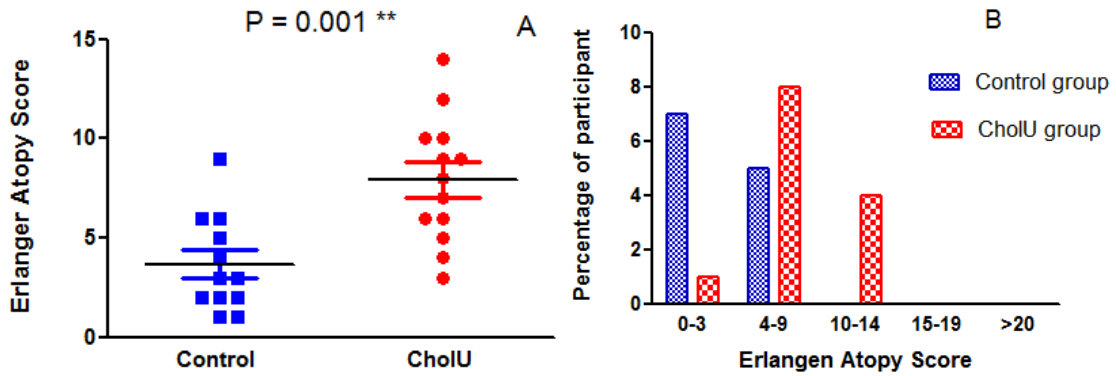


Fig. 24 Patients with CholU have a higher mean Erlangen atopy score (A) and atopy is more frequent in the CholU group than in the healthy control group (B).
(mean and standard deviation, t-test) (A) Individual patients are shown as dots. Blank line shows mean and colored whiskers represent standard deviation. (B) Bar graph shows patient numbers in each category. Erlangen atopy scores are divided into: Category 1, no atopy (Score 0 – 3 points); Category 2, atopy unlikely but cannot be excluded (score 4 – 9 points); Category 3, atopic (score 10 – 14 points); Category 4 clear atopic skin diathesis (score 15 – 19 points), Category 5 very strong atopic skin diathesis (score > 20 points).

5.2.2 Total IgE and specific IgE

The mean total IgE serum levels in the CholU patient group (190.2 ± 207.9) was higher than in the healthy control group (137.3 ± 237.5), but this difference was not significant (P > 0.05) (Fig. 25).

Seven (54%) patients with CholU had elevated total serum IgE levels (> 100 kU/mL) and in five (38%) of them, the total serum IgE was > 200 kU/mL. In comparison, in the healthy control group only two (17%) participants, displayed a total IgE of over 100 kU/mL. There was no significant difference (P > 0.05).

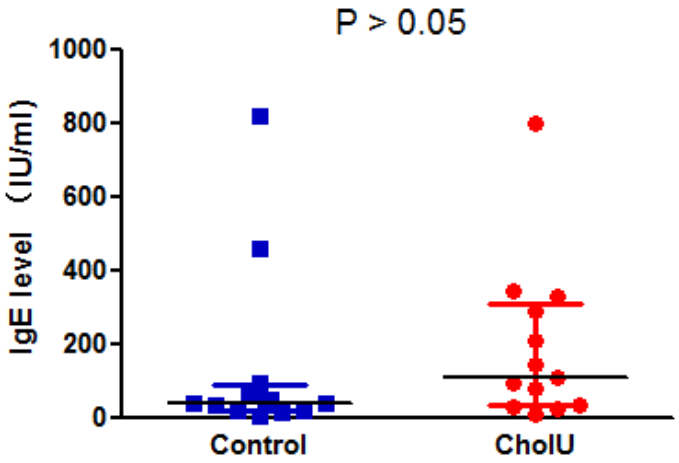


Fig. 25 The total IgE levels of the two groups.

Individual patients are shown as dots. Black line shows median and colored whiskers show the interquartile range.

5.2.3 Malassezia-specific IgE may be a marker for more severe and more prolonged disease

As IgE directed against a subform of Malassezia (Malassezia globosa) had been described as a relevant pathogenetic mechanism in CholU patients, we assessed Malassezia-specific IgE in both patients with CholU and healthy controls (Fig. 26). Three out of 13 patients had detectable levels of IgE anti Malassezia, but only one patient had values above the cut off of 0.35 IU/ml.

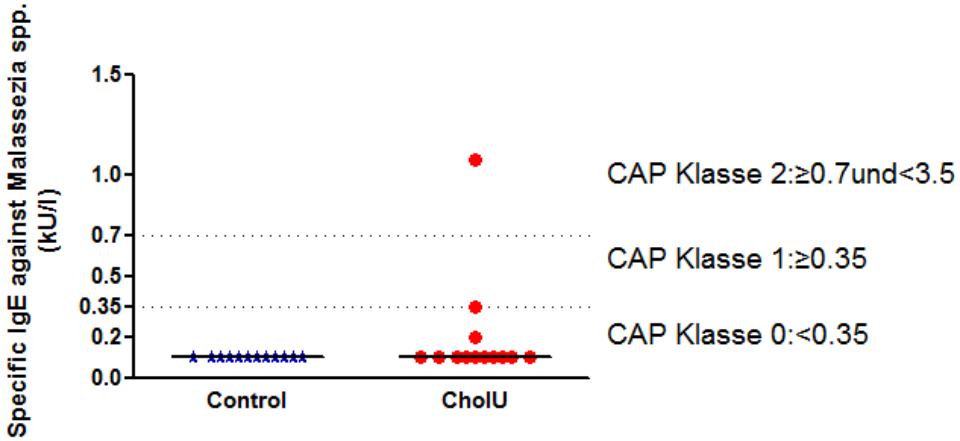


Fig. 26 Two groups of Malassezia spp-specific IgE distribution.

Individual patients are shown as stars in the healthy control group and dots in the CholU patient group. Black line shows the median. Dotted line represent cut-offs between different categories (as described on the right side of the figure).

5.2.4. Correlation with clinical markers

Combined with other clinical data, we found that the increase in Malassezia-specific IgE was associated with a longer duration of illness (Fig. 27), but not with the severity of the disease (CholUAS7, P = 0.3; CholUSI, P = 0.4). And there was no correlation between Malassezia-specific (Malassezia spp.) IgE and CholU-Qol (r = 0.06, P = 0.5)/ DLQI (r = 0.2, P = 0.2)/ Provocation UAS (r = 0.04, P = 0.5)/ VAS (r = 0.1, P = 0.3)/ CholUAS7 (r = 0.3, P = 0.1)/ CholUSI (r = 0.2, P = 0.2).

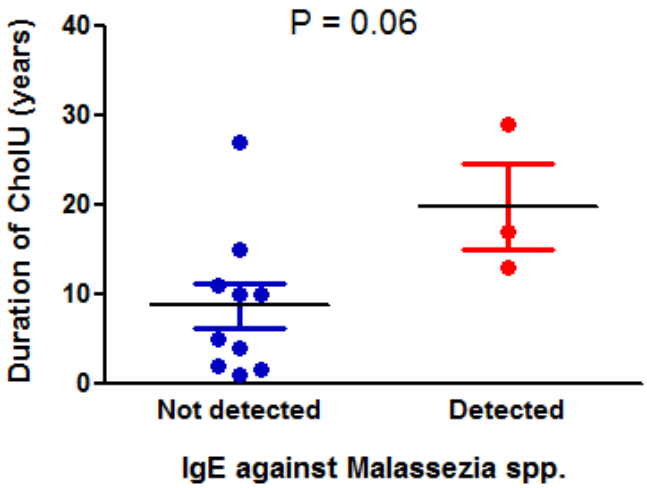


Fig. 27 Malassezia with duration and severity of CholU.

Individual patients are shown as dots. Black line shows the median and, colored whiskers show the interquartile range.

5.3 MC numbers and features

Here, we investigated if MCs in the skin have different features in patients with CholU compared to healthy controls.

5.3.1 MC numbers: Histological findings in patients with CholU compared to healthy controls

5.3.1.1 Absolute number of MCs

To further elucidate the role of MCs in the pathophysiology of CholU, we analyzed the number of MCs in the skin of both healthy controls and patients with CholU. To assess MC numbers, we compared naphthol AS-D chloroacetate for Specific Esterase Kit (AS-D staining) and toluidine blue staining in skin biopsies of unaffected skin before provocation (Fig. 29). There were significantly higher absolute numbers of MCs in the skin of patients with CholU compared to healthy controls (P = 0.03) (Fig. 28).

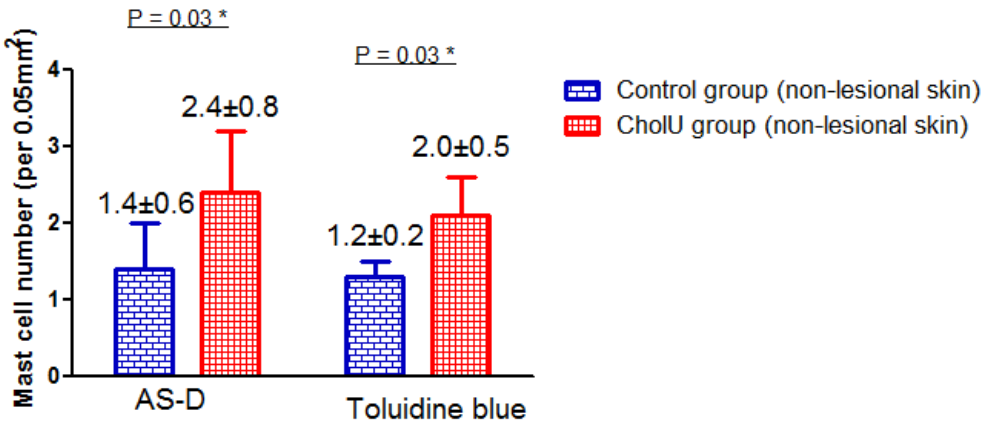


Fig. 28 Different staining of MCs between two groups before provocation.

Bar graph shows mean numbers in each group and whiskers show the standard deviation. Control group is represented in blue and the CholU group is in red (Control group n = 12, CholU group n = 13).

When analyzing the MC distribution in the dermal layers of the skin sections, we saw that MC numbers were significantly increased throughout all dermal layers, with the biggest and most significant differences in the uppermost layer and in the deeper layers (Table 21).

Table 21 MCs in the layers before provocation

Cells / 400 x HPF	Control	CholU	P value
Layer 1	4.4±2.2	6.1±2.3	0.03 *
Layer 2	3.2±1.8	3.8±2.9	0.08
Layer 3	1.6±1.1	2.6±2.5	0.003 **
Layer 4	1.2±1.0	1.8±1.1	0.1
Layer 5	0.7±0.9	1.6±1.8	0.03 *

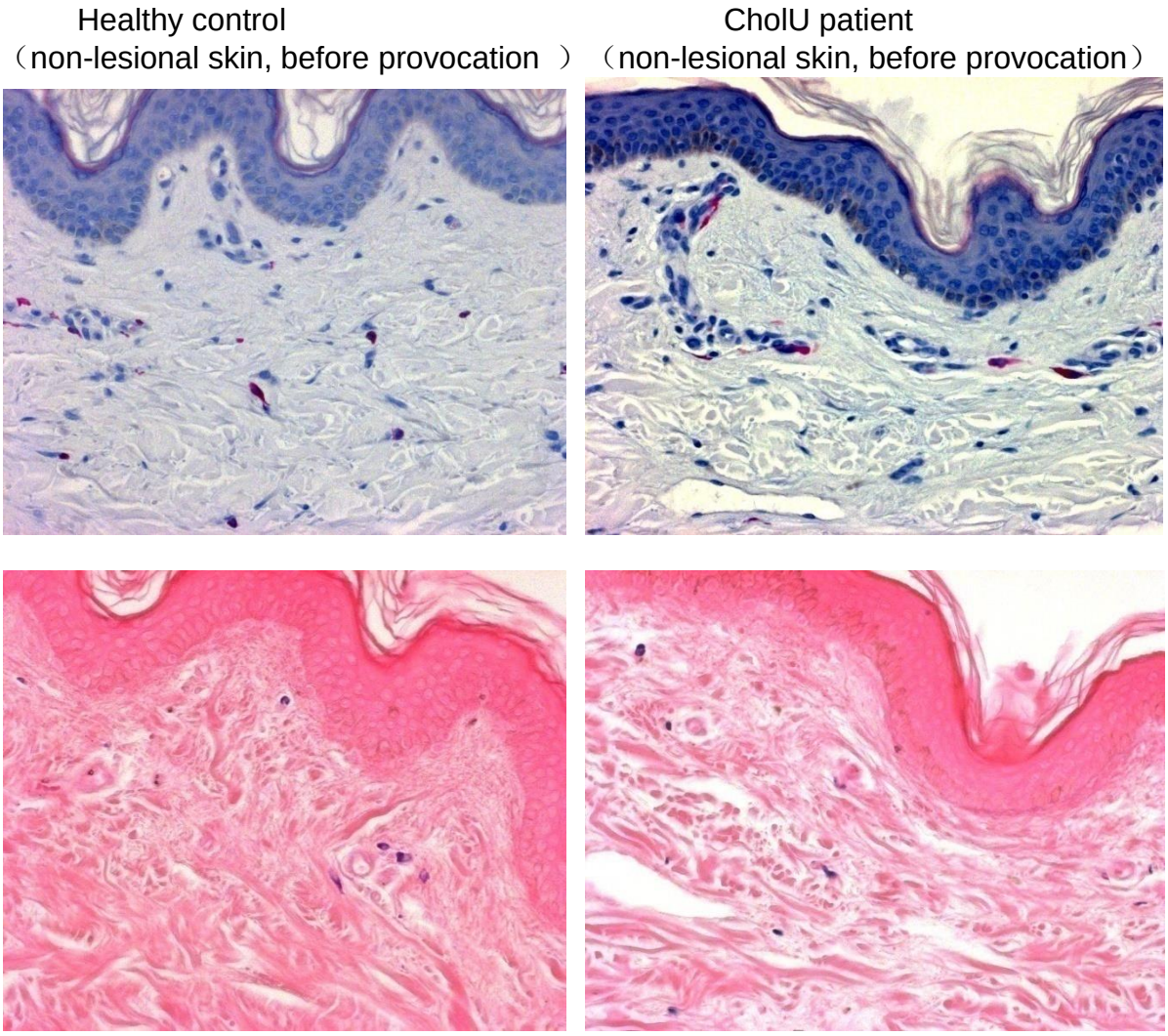


Fig. 29 Examples of AS-D / toluidine blue stainings in skin biopsies before provocation.
Examples of AS-D / toluidine blue stainings in skin biopsies of unaffected skin before provocation in a healthy control and in a CholU patient (from a single patients in each group), the biopsies in both of these two patients were non-lesional skin (pictures shown at 200X magnification).

Regarding MC distribution, we did not see differences between patients with CholU and healthy controls. MCs were often localized near vessels, subcutaneous glands and nerves, but also dispersed in the tissue.

MC number in lesional skin of CholU patients are slightly elevated compared to non-lesional skin

When we performed the provocation test, we also took biopsies from whealing skin in patients with CholU and from comparable skin regions of healthy controls (no skin reaction) (Fig. 31). When we analyzed the MC number using the same techniques in the

same skin samples of wheals in patients with CholU (lesional) and skin samples of healthy controls after provocation (non-lesion), we saw comparable results as seen in the skin before provocation. There were significantly higher numbers of MCs in patients with CholU compared to healthy controls (Fig. 30, P = 0.02). As before, we examined the MC distribution in the dermal layers of two groups (Table 22). The lesional skin after provocation showed higher numbers of MCs in all dermal layers compared to non-lesional skin in healthy controls after provocation with highly significant.

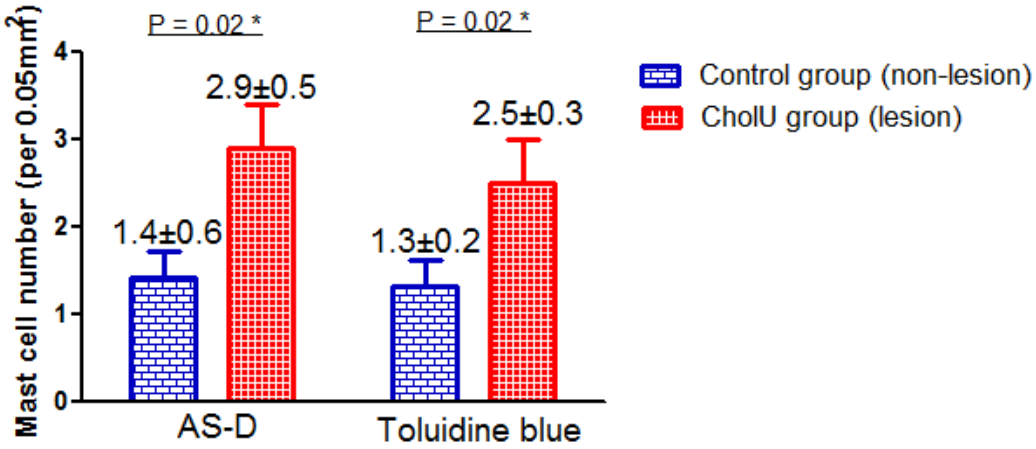


Fig. 30 Different staining of MCs between the two groups after provocation.

Bar graph shows mean numbers in each group and whiskers show the standard deviation. Control group is represented in blue and the CholU group is in red (Control group n = 12, CholU group n = 13).

Table 22 Number of MCs in the skin layers after provocation (mean, SD)

cells/ 400x HPF	Control	CholU	P value
Layer 1	4.4±1.7	7.3±3.3	0.001 ***
Layer 2	3.1±2.0	5.5±2.5	0.007 **
Layer 3	1.7±0.9	2.9±1.4	0.007 **
Layer 4	1.2±1.0	2±1.0	0.007 **
Layer 5	0.6±1.0	1±0.8	0.01 *

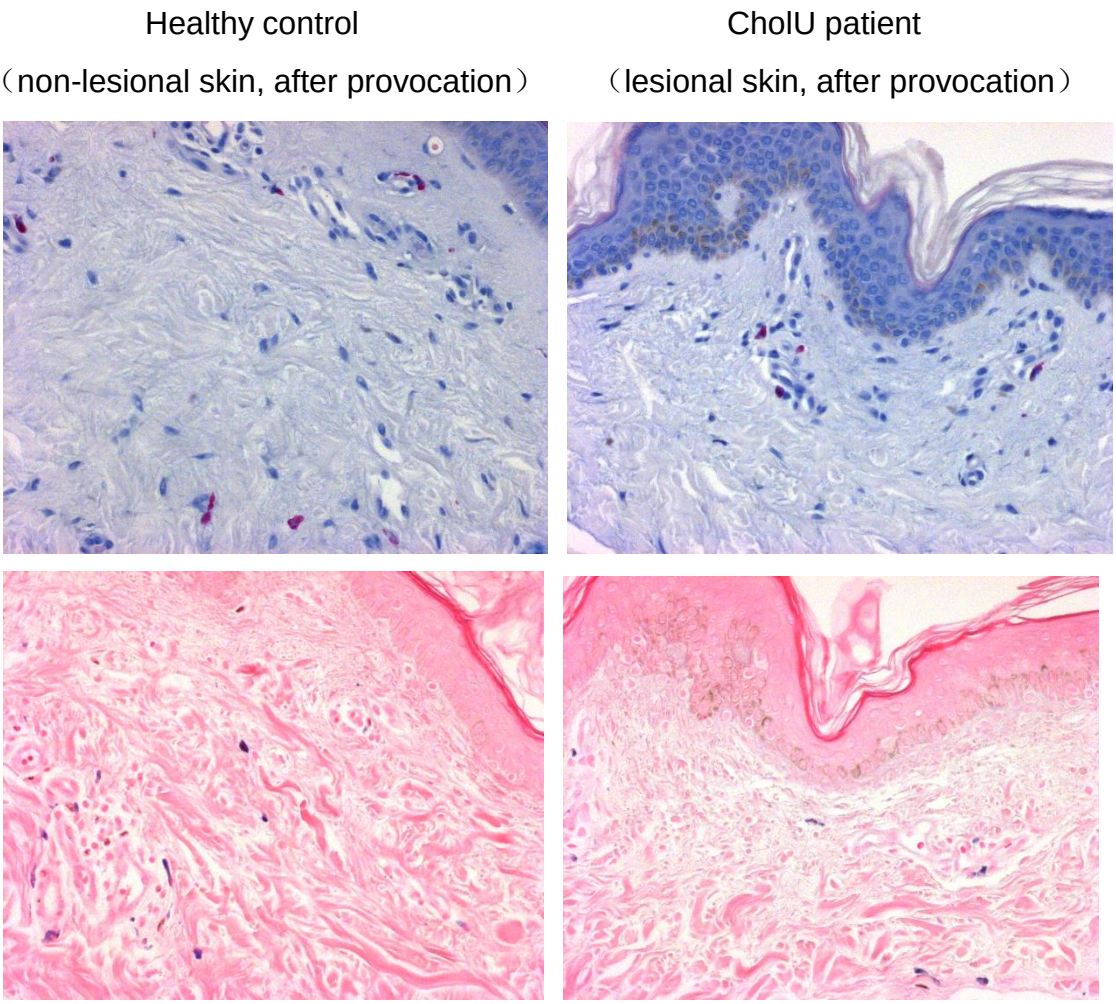


Fig. 31 Examples of AS-D / toluidine blue stainings in skin biopsies after provocation

Examples of AS-D / toluidine blue stainings in skin biopsies of unaffected and affected skin after provocation in a healthy control and in a patient with CholU. The biopsies in the healthy control group were non-lesional and in the CholU group they were lesional (pictures shown at 200X magnification).

Differences in MC numbers in the skin before and after provocation

We compared the MC number between the control group and CholU group before and after provocation. We found that the number of MCs in the healthy control group was comparable before and after provocation, whereas in the CholU group, there was a tendency towards even higher MC numbers in lesional (whealing) skin after provocation of patients (Fig. 32, P = 0.1).

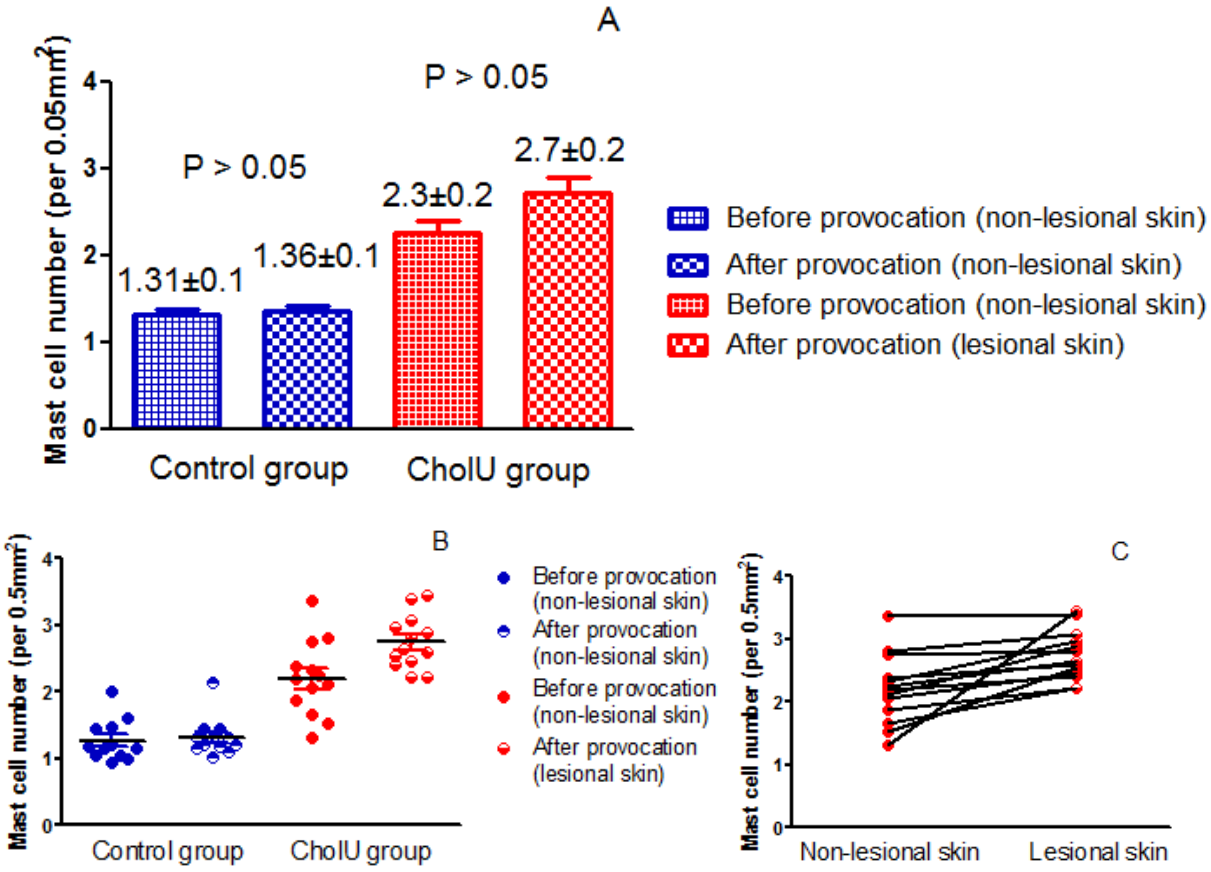


Fig. 32 MC numbers in the skin before and after provocation

The average of absolute MC numbers of two stainings +/- SD (A) and The number of MCs between the two groups before and after provocation (B) and the MC numbers of the CholU patient group before and after group (C).

5.3.1.2 Correlation with clinical data

MC numbers in the skin (in the affected skin after provocation) did not correlate with clinical features, such as CholUAS7 (r = -0.2, P = 0.5), onset of sweating (r = 0.03, P = 0.9), provocation UAS (r = 0.3, P = 0.4), DLQI (r = -0.2, P = 0.4) and CholU-Qol (r = 0.02, P = 0.3).

Interestingly, MC numbers were also not correlated with total serum IgE (r = 0.06, P = 0.8) or IgE against Malassezia spp. (r = -0.02, P = 0.9) or Erlangen Atopy score r = -0.02, P = 0.9).

5.3.2 MC degranulation upon provocation

We aimed to assess the degranulation status of MCs before and after provocation. We found that there was MC degranulation in both the control group and in the CholU patient group before and after provocation (Fig. 33). No clearly visible differences in the degranulation status were visible.

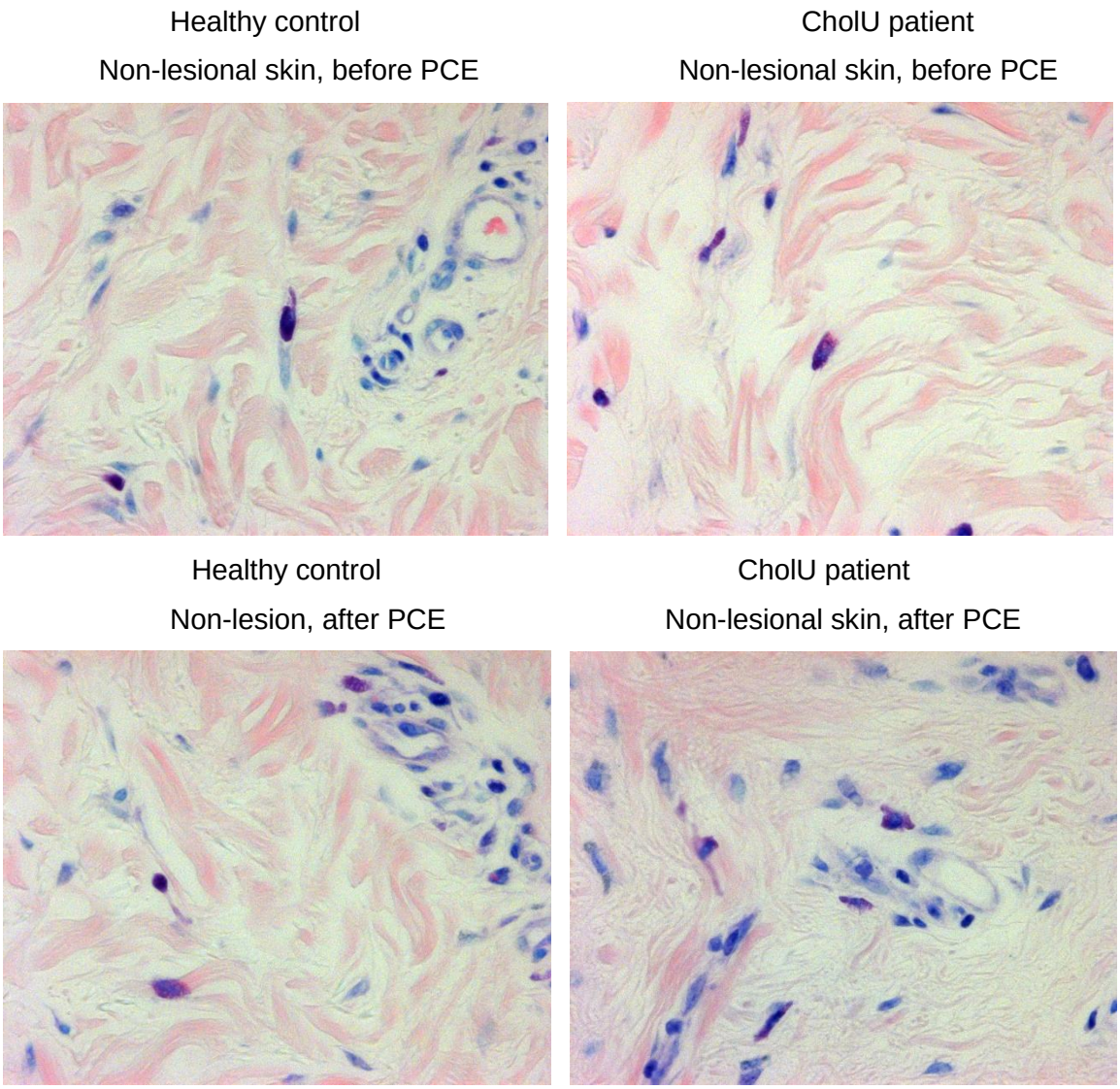


Fig. 33 Examples of Giemsa staining in skin biopsies

Examples of Giemsa staining in skin biopsies of unaffected and affected skin before and after provocation in a healthy control and in a patient with CholU. The biopsies in the healthy control group were non-lesional, in the CholU group before provocation, they were non-lesional and in the CholU group after provocation they were lesional (pictures shown at 400X magnification).

5.4 MC products

Degranulation of MCs lead to release of histamine, leukotrienes, cytokines, chemokines and neutral proteases (chymase and tryptase). Histamine, prostaglandins and leukotrienes are not stable in serum and therefore difficult to measure. In contrast, tryptase is relatively stable in serum after centrifugation and was chosen as a MC degranulation marker in this study.

5.4.1 There was no significant difference in tryptase between CholU patients and healthy control subjects.

To analyze MC activation, we compared the tryptase between the healthy control group and the CholU patient group before and after provocation. The mean values in the CholU patient group before provocation (4.9 ± 2.3 kU/l) were slightly higher compared with the healthy group (4.5 ± 1.8 kU/l), but all of them were within the normal range. There was no significant difference between the two groups in the mean value of tryptase ($P > 0.05$). in the healthy group, there was no change in the average value before (4.5 ± 1.8 kU/;) and after (4.5 ± 1.8 kU/l) provocation (Fig. 34A) or in the CholU patient group (Fig. 27B), before (4.9 ± 2.3 kU/l) and after (4.8 ± 2.0 kU/l, $P > 0.05$) provocation. Among them, eight patients had slightly lower trypatse values after provocation, one patient had missing information of the pre-provocation value, and the values of the remaining patients were minimally higher than before provocation (Fig. 34B).

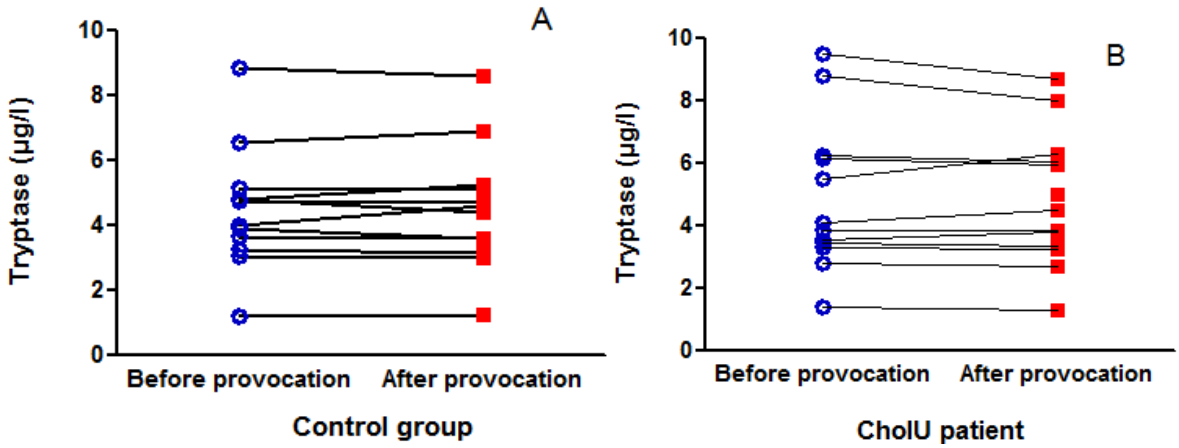


Fig. 34 The distribution of tryptase before and after provocation

The tryptase of before provocation is presented in blue circles and after provocation in red squares. Black lines connect the tryptase before and after provocation.

5.4.2 Changes in Mast cell mediator-related receptor expression

As histamine is the major MC mediator resulting in skin reddening, whealing and itching, we looked for changes in histamine receptor expression, which could explain stronger

symptoms in the patients with CholU. MCs generate and release histamine upon activation. Histamine exerts its action via specific receptors (H1-H4 receptors) on various cells, inducing vessel dilation, itch promotion and regulatory mechanisms on MCs. We wished to see if the expression of the histamine receptors is differently (regulated) in patients with CholU compared to healthy controls.

5.4.2.1 Histamine receptor expression in skin: higher expression and upregulation of H4 receptors in patients with CholU

Using RNA-hybridization we stained for H1 to H4 receptor expression in skin samples (Fig. 38). Unaffected skin of both healthy controls and patients with CholU expresses low levels of H1 and H2 receptors. H3 receptor was not detectable in any skin sample. In almost all the participants, H4 receptor RNA expression was detectable (see Fig. 35). The H4R expression was significantly higher in patients with CholU compared to healthy controls (P = 0.02) (n = 7).

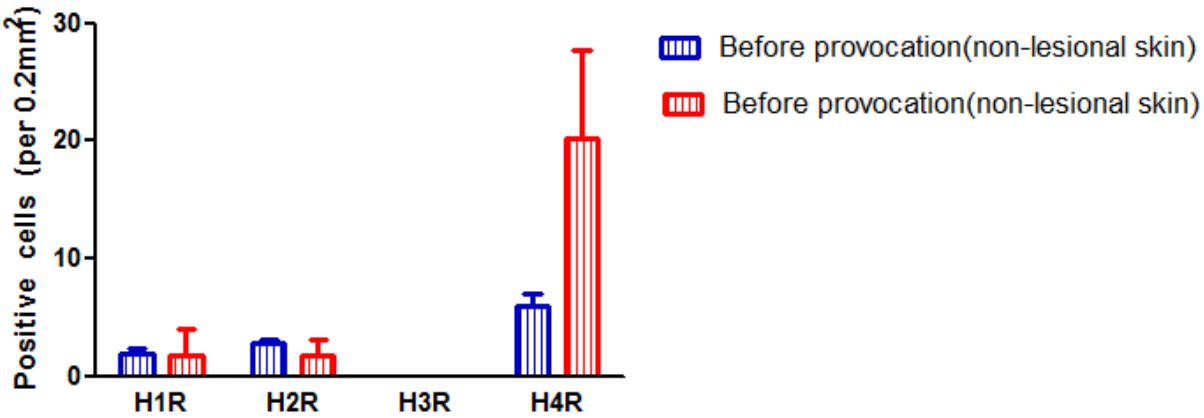


Fig. 35 Histamine receptors before provocation of the healthy control group and the CholU patient group.

In lesional skin of patients with CholU, after provocation, H4R expression was even higher, whereas exercise provocation alone in healthy controls did not upregulate H4R expression. In contrast, there was a higher number of H4R positive cells in lesional skin after provocation than before provocation in the CholU patient group (P = 0.047) (n = 7) (Fig. 36).

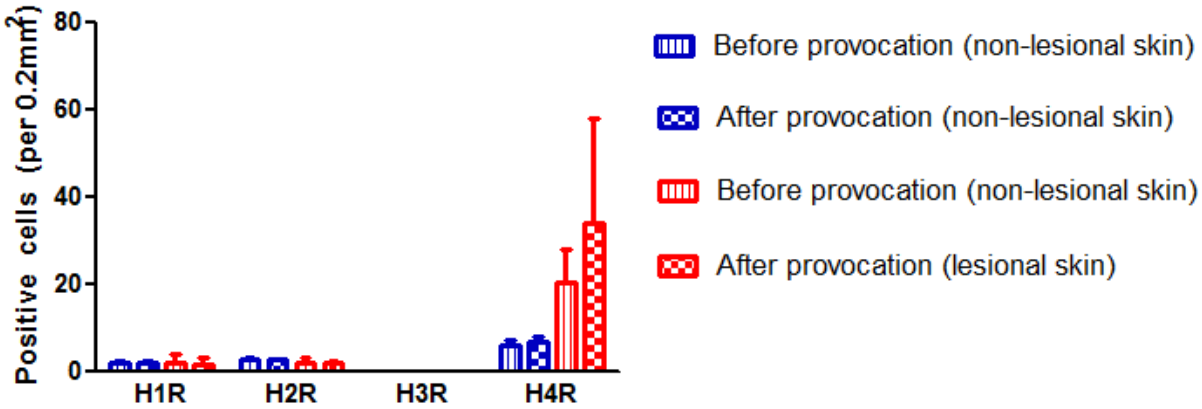


Fig. 36 Histamine receptors before provocation of the healthy control group and the CholU patient group

To compare histamine receptors expression with MC numbers, we stained for tryptase RNA in these skin samples and saw, as described above, higher expression in CholU patient skin and even higher expression in lesional skin after provocation (Fig. 37). We compared the H4 receptor and tryptase between the healthy control group and the CholU patient group before and after provocation. There was no distinct difference before and after provocation in healthy control group ($P > 0.05$) ($n = 5$).

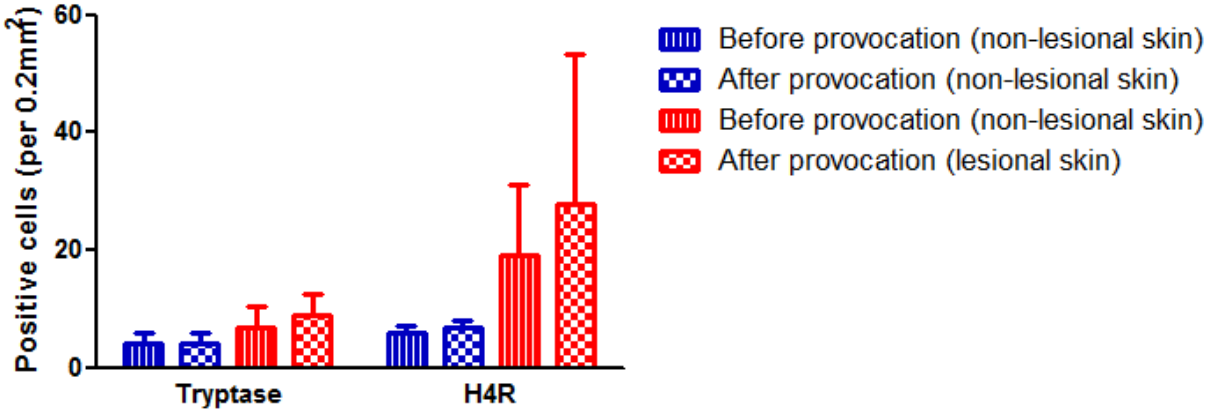
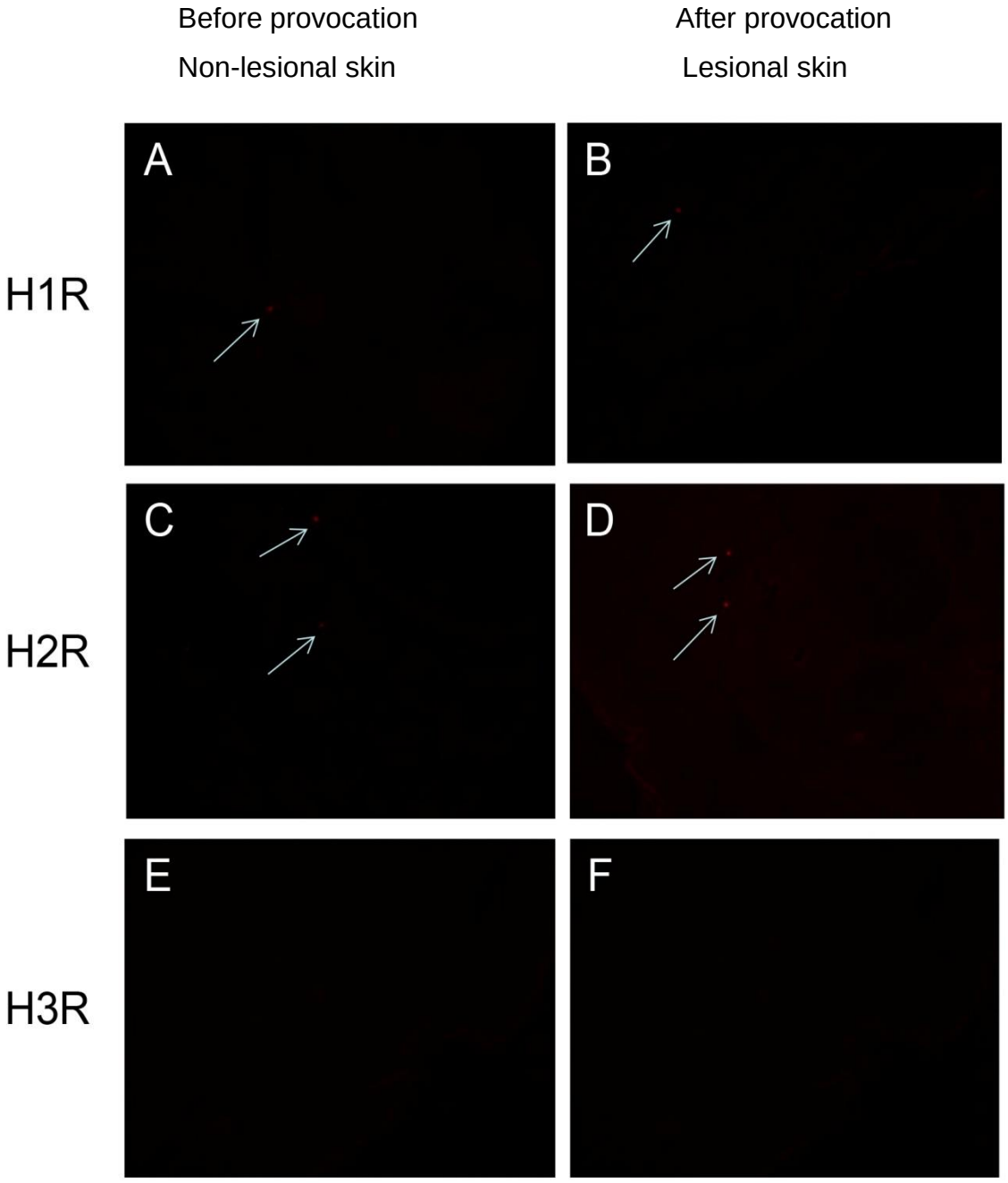


Fig. 37 The positive cells of H4R and tryptase between two groups before and after provocation

Overall, there were more H4R-positive cells than trpytase-positive cells. This difference was even more pronounced in patients with CholU compared to healthy controls. There was no significant difference between the number of tryptase-positive and H4R-positive cells ($r = -0.2$, $P > 0.05$.) ($n = 5$).


Before provocation
Non-lesional skin
After provocation
Lesional skin
H1R
H2R
H3R
A
B
C
D
E
F

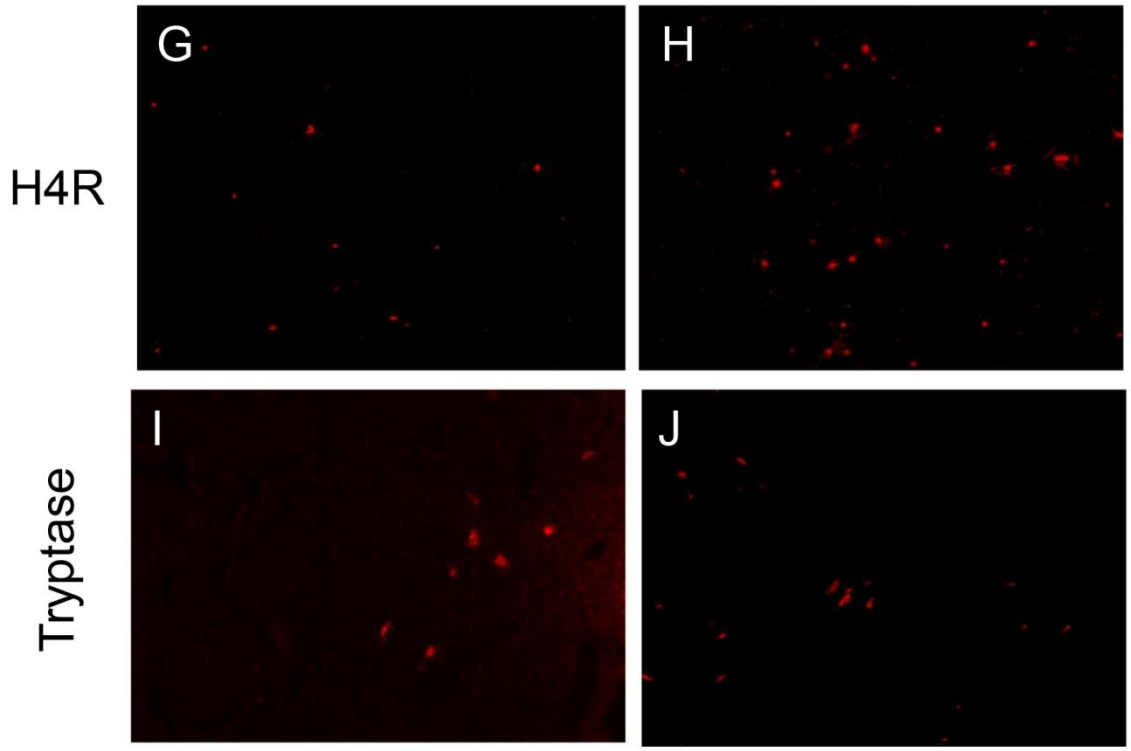


Fig. 38 Examples of histamine and tryptase stainings in skin biopsies

Examples of histamine and tryptase stainings in skin biopsies of unaffected and affected skin before and after provocation in the healthy control and the CholU patient group. (pictures shown at 400X magnification). CholU skin sections were stained for the different histamine receptor subtypes (red) (shown in A, B, E, F, G, H) and for the mast cell marker tryptase (red) (shown in I, J). Note that only few cells stained positive for H1R and H2R, the H3R staining was absent in cutaneous tissue. Due to the limited number of stainings, no statistical analysis had been performed.

5.4.2.2 Acetylcholine esterase expression

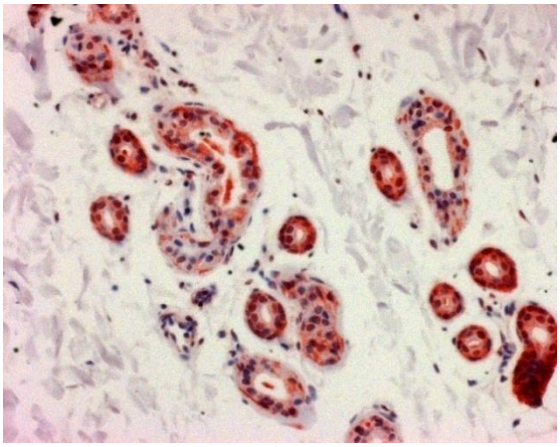
Aberrations in the AchE expression has been proposed as an underlying pathomechanism, therefore we were interested in the different expressions of this in healthy contols and patients with CholU. We specifically looked for AchE in the eccrine gland epithelial cells to see if there were differences in the non-lesional skin of patients with CholU before provocation and in the lesional skin after provocation.

In first staining of our patients and three healthy controls, we saw a decreased expression of AchE in the CholU patient group compared to the healthy control group, especially for

one patient who also had less sweating behavior upon PCE. Due to the limited number of staining, no statistical analysis had been performed.

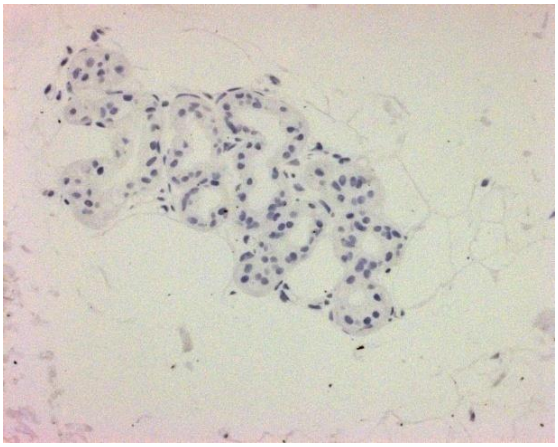
Healthy control

Non-lesional skin, before provocation



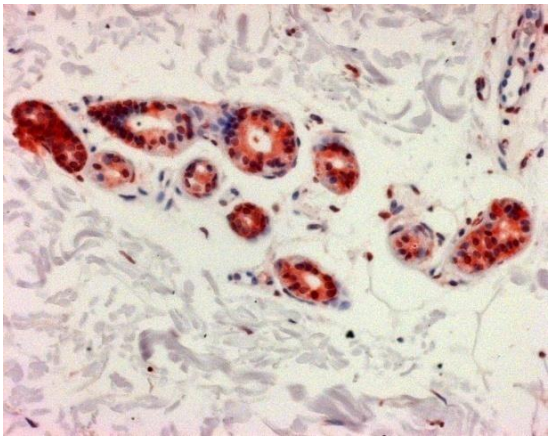
CholU patient

Non-lesional skin, before provocation



Healthy control

Non-lesional skin, after provocation



CholU patient

Lesional skin, after provocation

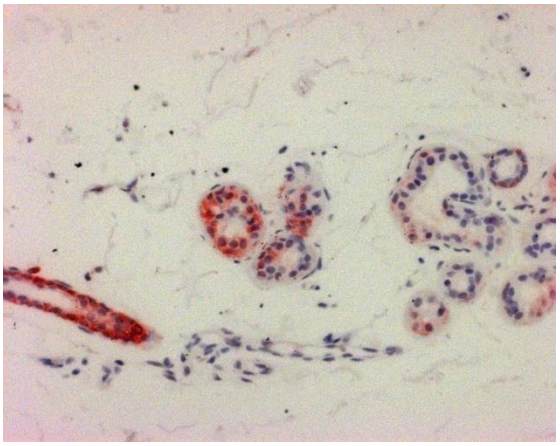


Fig. 39 Examples of AchE staining of sweat glands in skin biopsies

Examples of AchE staining of sweat glands in skin biopsies of unaffected and affected skin before and after provocation in healthy controls and in patients with CholU. (pictures shown at 200X magnification). The positive cells are shown in red color.

6. Discussion

In this study, which included 13 patients with CholU from a specialized tertiary clinical urticaria center and matching healthy controls, we aimed to explore the role and relevance of MCs in the pathophysiology of patients with CholU c. To address this aim, patients were clinically characterized and histological samples of the skin before and after provocation testing were analyzed for MC numbers, MC activating factors, MC products and MC product related dysregulations.

6.1 Clinical data of patients with CholU

In our study, the CholU patient group comprised of four females and nine men, who had a mean age of 34 years. In 1994, Zuberbier *et al*. reported that the prevalence of CholU in women (12%) was slightly higher than that of men (10%) [52]. In our case, we recruited more male patients in our study. Whether men are more affected or are more likely to seek medical help and participate in clinical research remains unclear.

CholU is often regarded among physicians as "a young man's disease" [52], but recent publications also show patients with late onset of disease [63]. The average age in our patient group was 34 years, with a broad range from 19 to 53 years. However, most of the older patients had a long duration of CholU. On average, the duration of CholU was 11.1 years (SD = 10 years), with a range from 0.8 to 29 years, meaning most patients had an onset of disease at a young age.

Patients with CholU were mostly moderately affected by their disease

In our study, we analyzed patients who were mostly moderately affected, as rated by the patients and by using clinical scores. In this project, patients with CholU rated their disease severity globally with VAS, the Likert scale and global symptom score 7, and documented their symptoms in disease specific scores (CholUSI, CholUAS7). These disease-specific scores showed good correlation with the global severity measures, which corroborates the severity of disease. Only one of the 13 patients with CholU included in this study, rated herself as well-controlled, maybe because she had a long duration of disease (27 years) and she had adapted to the symptoms and learned how to control her condition in daily life.

Patients with CholU had a long duration of disease

Patients with CholU had a long duration of disease of up to 29 years (mean 11.1 years), which was much longer than in another report, where it was 48 months [61]. In the latter report, the authors stated that this short duration could be due to loss of follow up of the patients.

In our study, patients with long disease duration scored higher in CholUSI, which implicates the amount of trigger factors, frequency of symptoms and symptom duration, but was not connected with other clinical scores. CholUSI was introduced in a recent paper [74], but it has not been validated so far. The relevance of this score is not yet clear, but this correlation indicates that long-lasting disease is also associated with a higher disease burden.

Quality of life impairment parallels disease severity in CholU

For the quality of life impairment, the mean DLQI in our study among all patients was 12.4, which was consistent with previous reports of mean DLQI scores in CholU (mean DLQI 11.8 in 30 patients with CholU [74].The mean DLQI scores for psoriasis was reported as 9.7 [83], and for CSU it was around 10.9 [84], indicating that in our study, patients with CholU experienced an even higher level of impairment of their quality of life, then the average patient with psoriasis or CSU.

Furthermore, in this study, a newly introduced disease-specific quality of life instrument, CholU-Qol, was used for the quality of life assessment [81]. This score ranges from 0 to 100 and patients in this study showed a mean value of 42. This score was well correlated with the DLQI and patients' global ratings (VAS and Likert scale). The CholU-Qol has not yet been validated in lager studies, but it may serve as a valuable tool for clinical trials and improve routine patient management.

Antihistamine intake had little influence on disease severity and disease control

In this study, almost all the patients with CholU took one antihistamine tablet per day on a regular basis, but were advised to stop treatment for one week before the PCE test, and especially in the 24 hours before the PCE test. In order to avoid drug interference with clinical outcomes, we advised patients to try not to take antihistamines, MC stabilizes or glucocorticoids before assessment, if possible. Nevertheless, eight of the patients with

CholU took antihistamines in the last week before assessment and four of them took antihistamines in the last 24 hours before the PCE test.

Most of the patients with CholU who had taken one antihistamine tablet per day rated themselves as not having well-controlled symptoms. Currently, effective therapeutic approaches for the treatment of CholU are not well established. In a previous publication, there was only one clinical trial published that evaluated cetirizine as a treatment for CholU in 24 patients [85], it reported that taking cetirizine (10 mg or 20 mg) for three weeks was successful for the treatment of patients with CholU. Koch *et al.* reported that antihistamine updosing reduces disease activity in patients with difficult to treat CholU [74]. Antihistamines are the first line therapy in CholU [40]. Antihistamines have been shown to be effective in CholU treatment [85,86]. However, Koch reported that even higher antihistamine doses did not completely inhibit CholU symptom development. In the study, we saw that antihistamine intake did not correlate/influence clinical scores like CholUAS7, CholU-Qol, DLQI or CholUSI and that antihistamines did not improve the UCT score, indicating no major influence on the disease control. In out study with limited patient numbers, we only saw an influence on the delay of onset of symptoms upon sweating. CholU patients that had taken AH had a more delayed onset of the symptoms, but this difference just missed significance (P = 0.09). It was consistent with previous reports that antihistamine updosing delayed onset of the symtoms, but it is not significance [74].

Males, but not females, reported seasonal changes in CholU

Half of the patients with CholU rated their symptoms as unchanged over the whole year. Differences in seasonal deterioration have been reported in Japanese patients with more severe disease activity during winter season. CholU was in winter [56,87]. In our study, 46% of patients with CholU complained about CholU throughout the year, with the rest being more severe in winter than in summer. This is consistent with previous reports [56,78,87]. However, an Indian study of patients with CholU showed a moderate prevalence in October and November [54]. This may be because in India, October and November are hot and humid. In Germany this could be explained by the fact that, Europe does not have hot and humid weather throughout the year. The types of stimuli that elicit for CholU are climbing steps, sweating, walking, sports, mental stress, hot and spicy food, and alcohol. For some patients with CholU, symptoms were more severe in winter than in summer,

potently due to sweat gland blockage or acetylcholine/acetylcholine receptor abnormalities [55,88].

Upon provocation, patients with CholU had comparable onset of sweating, but probably a reduced amount of sweating

To objectively analyze the occurrence and severity of symptoms, a provocation test has been used for decades [35,64]. In our case, all the participants performed a static bicycle ride for 30 minutes using PCE [38]. Patients were instructed to speed up or slow down their pedaling so that the pulse rate increased three times per minute and to eventually reach 160 beats per minute. We compared the PCE threshold levels (onset of sweating time, increase in HR at sweating onset, increase in bpm at onset of sweating, and the time to the first discoloration of the iodine starch test according to Minor's sweat test from these two groups). There was no significant difference, indicating that patients with CholU do not have a dysfunction in the initiation of sweating.

Less sweating has been reported in previous publications [89]. We suspected that patients with CholU could have a sweat allergy. Thus, patients with CholU with acquired generalized hypohidrosis and with a sweat allergy may be classified as two distinct disease entities based on different pathogeneses.

Patients with CholU develop wheals after onset of sweating. However, there were no skin changes or wheals in the healthy control group. Interestingly, in the CholU patient group, the onset of wheals appeared after the onset of sweating and marked the beginning of skin changes. Thus, we hypothesized that patients with CholU may have a hypersensitivity to their own sweat. Previous reports have indicated that sweat is an exacerbating factor in atopic dermatitis (AD) [90–92] and that a sweat allergy exists in patients with CholU. This result was consistent with the results of sweat hypersensitivity, proven by positive ASwST (autoperspiration sweat skin test) and basophil histamine release test published by Jung in 2015 [93].

Pulse-controlled exercise provocation testing is both highly sensitive and specific for diagnosing CholU [38]. Wheals were produced in all patients with CholU but not in healthy controls. Additionally, it is the first protocol that allows assessment of disease severity, which is significantly correlated with the threshold time to whealing. In our case, the

correlation between the symptoms provoked by PCE and the daily diary scores of the patients (CholUAS7), was a significant, but rather weak connection with disease severity.

With the provocation UAS, we detected a positive correlation with disease-specific quality of life impairment (CholU-Qol) and a weaker correlation with DLQI. This confirms that PCE is a valid and valuable instrument for the diagnostic workup of patients with CholU, for confirming the diagnosis and assessing trigger thresholds. PCE also does both.

In our study, there was no significant difference in the onset of sweating upon PCE provocation, indicating that patients with CholU did not have problems with the onset of sweating, but most patients showed reduced rate of sweating in the test. Most the patients showed a decreased sweating behavior in the sweat test. This feature of CholU patients had been described by Sawada [89,94].

Symptoms upon provocation occur after sweating and are correlated with disease severity, quality of life impairment, CholU7, DLQI and CholU-Qol scores

Antihistamines could have an influence on the delay of whealing. Four patients with CholU had taken antihistamines in the last 24 hours before provocation. The mean delay of onset of whealing after the onset of sweating was higher in patients who had taken antihistamines compared to those who had not taken antihistamines in the last 24 hours. The difference is not significant, but trended towards this result, possibly due to the low patient numbers.

6.2 MC triggers

Atopic predisposition is frequent in CholU

Our study showed that patients with moderate to severe CholU showed high rates of atopy. In 1987, Hischmann *et al*. reported a high prevalence of atopy (34%) in patients with CholU [44]. Using the Erlangen Atopy score, 31% of our patient population was atopic and none of healthy control group were atopic. Using the Erlangen Atopy score, the previously reported higher scale value, perhaps due to an overall increase in atopic response due to a different assessment of specificity [95,96], or because we chose patients who were not particularly severe or who were being treated. A previous study has

reported an atopic patients population of 57% in a selected cohort, possibly due to the selected patient population of rather severely affected patients [97]. Atopy is associated with female sex, which is consistent with the recent German cohort study [96].

Patients with CholU exhibit elevated total IgE serum levels

Previous reports have shown that the total IgE levels are elevated in patients with chronic spontaneous urticaria [98] and in patients with CholU [97]. Atopic predisposition is thought to be associated with elevated total serum IgE levels, but there are few reports to prove this. Furthermore, no correlation of seasonal exacerbation and total or specific IgE levels have been seen in previous studies [56,87].

Thirteen patients with CholU (54%) had elevated total serum IgE levels, that is, > 100 kU/ml and in 38% of them the total IgE was > 200 kU/ml. However, in the healthy control group, 17% had total IgE levels > 100 kU/ml. Surprisingly, it was not only the CholU patient group but also the healthy control group that had high and comparable total serum IgE levels. There are several theories to explain this. Firstly, the increased total IgE levels may indicate that participants with or without CholU are in general 'allergy prone'. The test for this was originally designed for atopic skin diseases, it may not be detected by the Erlangen Atopic score. Secondly, elevated total IgE may be a sign of extrinsic reactivity to environmental allergies (classical atopy). However, it is also possible that autoantigens are the hallmark of the intrinsic reactivity of 'self-allergies'. This concept has also been proposed for CSU in which IgE has been detected [99–101].

According to previous reports, the higher total IgE was a marker for more severe allergic disease (e.g. acute AD and eczema) in any age [102,103].

Malassezia-specific IgE can be detected in selected patients with CholU

Malassezia-specific IgE is the a specific IgE that positively correlates to moderate to severe AD but this is controversial, because the clinical significance of this finding is unclear [91, 104]. It has been reported that Malasszia acts as a major histamine-releasing antigen in human sweat allergy. Moreover, serum levels of Malasszia-specific IgE in patients with AD and CholU were significantly higher than those in normal controls [90]. Currently, almost all published papers came from Japan. To our knowledge, no report in caucasion patients regarding this specific IgE had been published so far. To address the question, if our patient cohort also exhibit IgE against Malassezia, we measured the levels of Malasseria specific IgE in the serum from the CholU patient group and the control

group to compare the levels. As a result, in the CholU patient group, there was one participant who had a significant increase of specific IgE and two participants who had detectable levels of specific IgE. None from the healthy control group had detectable specific IgE levels to Malassezia. Combined with other clinical data, we found that the increase in Malassezia specific IgE in CholU patients was associated with a longer duration of disease, but most likely, this is due to the limited number of patients. This association did not reach significance (P = 0.06). No correlation with disease severity or quality of life markers could be seen, but again, the numbers of patients are very low and no final meaningful conclusions can be made here.

Despite the detection of specific IgE to Malassezia, this does not necessarily indicate the necessity of an antimycotic therapy [105]. Nevertheless, desenitisation therapy using autologous sweat or Malassezia globosa peptides has been shown to be beneficial for patients with intractable CholU due to sweat allergy [90].

6.3 MC numbers

The main goal of this study was to explore the role and relevance of MCs in patients with CholU. CholU is an edema of the skin, and molecules released by MCs cause blood vessels to dilate and fluids to penetrate the skin. MCs play an important role in most patterns of CholU, and release histamine and other inflammatory mediators. We analyzed the difference in MC numbers and degranulation status between the CholU patient group and the healthy control group. We then compared the variations in MC numbers and in degranulation status after motion stimulation between the two groups.

MC numbers are elevated in the skin of patients with CholU

We found that there were significantly higher absolute numbers of MCs in the skin of patients with CholU compared to healthy controls. MCs were found at all levels of the dermis, where they are grouped and often localized near vessels, subcutaneous glands and nerves, or dispersed in the tissue. There was no significant difference regarding this aspect between patients with CholU and healthy controls.

In 1950, the measurement of MC numbers in the skin at different sites of the body was reported for the first time [106]. In those early studies, the numbers of MCs in healthy

individuals were found to be between 44 and 50 MCs/mm^2 [107–109]. The normal range for the forearm was between 31.5 and 62.4 MCs/mm^2 [108,110].

In our study, there was no significant difference before and after provocation in healthy persons, and the MC numbers of healthy controls were between 20 and 60 MCs/mm^2. In the healthy controls MC numbers had a greater variation, maybe because the biopsies were taken from different parts of body (our skin biopsies were from the upper arm, upper leg or abdomen). We found that the MC numbers in the upper leg were higher than those of the upper arm (mean 45 MCs/mm^2) and the MC numbers of the upper arm (mean 38 MCs/mm^2) were higher than those of the abdomen (20 MCs/mm^2). These results were consistent with previous reports that human cutaneous MC populations are different depending on the region of the body as shown by MC mapping studies of the whole body [107,111].

In the CholU patients group, MC numbers in non-lesional skin before provocation showed significantly higher numbers of MC, compared to HC. Numbers of MC were almost doubled, compared to HC, but the population density of the MCs before provocation (non-lesional skin) were still within the normal range [107,111].

In CholU patients, there was a tendency towards even higher MC numbers in the site of lesional skin (after provocation) of the patients, but this difference was not significant. Also, most of the lesional skin biopsies were within the normal range, but all of them were in the high range of over 50 MCs/mm^2. Eleven (85%) were even close to the upper limit of 66.4 [112]. Two of the CholU patient samples were a little higher than the normal range.

In the literature, very little data about MC in the skin of CholU patients are available. In one case report of a patient with combined CholU and symptomatic dermographism, population density of MCs in pre-challenged skin and after provocation wheals were within the normal range, but also showed increased numbers of MCs post-challenge [112]. In the past, there was no systematic statistical analysis of the difference numbers of MCs in CholU between before and after provocation.

MCs were counted independently in several separate skin layers and we discovered that MC numbers were highest in the most superficial skin layers compared with the layers of

the subcutis, which consistently contained the lowest numbers of MCs. This was consistent with previous reports [111], which analyzed different layers in healthy human skin. In our study we saw no significant differences of the MC distribution from the superficial skin layers to the deeper skin layers in the skin from patients with CholU before and after provocation. Of special notice, there was a significant difference in each skin layer between healthy individuals and patients with CholU, always with higher numbers in ChoU patient skin. In further studies, we need to clarify if skin MC levels of CholU patients are linked to disease activity and if skin MC numbers decrease in response to effective or spontaneous remission of CholU.

6.4 MC products

Degranulation detection of mast cells upon exercise challenge failed

We aimed to compare the degranulation status of MCs before and after provocation. However, we could not find any visible differences in both the control group and the CholU group before and after provocation. This is different from previous reports [113] which they show that MC degranulation in the area of wheals has been demonstrated repeatedly by light and electron microscopy. This failure of detection of MC degranulation could be due to technical limitations of the Giemsa staining, the previous reports used molecular immunopathology methods.

In the serum of allergic patients MC degranulation e.g. in anaphylactic situations can be monitored by measurement of tryptase [114–116]. Tryptase is a serine protease that is primarily produced and stored in MCs. Changes of less than 1ng/ml are considered normal, increases of 1-15 ng/ml as non-specific or anaphylactoid, and changes of more than 15ng/ml as true anaphylactic reactions [117,118].

In our study, we compared serum tryptase levels between the healthy control group and the CholU patient group before and after provocation. There was no significant difference in tryptase levels between these two groups. Only two of our participants had small changes of more than 1ng/ml. None of them had changes of more than 2ng/ml. However, in other forms of urticaria, despite severe skin involvement significant raise in tryptase levels had been detected [119], probably because of the patients with CholU were in the mean moderately affected and some of them even also took antihistamines. .

Histamine receptor and Acethylcholine Esterase expression

H4R is upregulated in the skin of patients with CholU compared to healthy control skin

MCs generate and release histamine during anaphylactic reactions, and there is pharmacological evidence that histamine regulates this process via specific receptors. Histamine is multifunctional and ubiquitously released from tissues of MCs and blood basophils [12] and exerts its effect through activation of four histamine receptors (H1-H4). In human skin H1 and H2 receptors have been convincingly demonstrated to be expressed [120–122]. There is little or no evidence of expression of H3 receptors in human skin and was only shown to be present in human brain tissue[12]. The histamine H4 receptor is the newest member of this histamine receptor family, and is expressed throughout the gastrointestinal tract as well as in the liver, pancreas and bile ducts [123]. Recently, evidence of H4 receptors in human skin has also been reported [12].

Non-sedating H1 antihistamines and sgAHs are recommended as the first line treatment of mild to moderate chronic urticaria [72], but in the clinic, many patients do not respond to standard non-sedating H1 antihistamine doses [40,56]. Accordingly, the question is, if other histamine receptors are involved in the pathophysiology of the disease to provide new insights into the mechanisms controlling self- and paracrine-induced histamine-induced MC function.

As for the other histamine receptors, H1 and H2 receptors only exist in few cells in our biopsies, while H3 receptors could not be detected in our slides. This is consistent with a previously reported paper on human skin MC, where expression of H2 and H4, but not of H3 receptors had been shown [12]. However, in our study there was only a very weak expression of H1/H2 receptor. It is unclearly if this is similar in other forms of urticaria, as no reports in the literature found to confirm this.

According to our fluorescence imaging results, the cells stained with the H4 RNA probe are consistently high in the human skin tissues of both the CholU patient group and the healthy control group.

In our RNA and immunohistochemistry (IHC) results, cells expressing H4 were more frequently detected than cells stained with tryptase, indicating that H4 receptor is expressed in cells other than MCs. In a report of Lippert et al., double stainings, co-localizating tryptase and H4 receptors only accounted for a few cells of all stained cells, also indicating that the majority of H4 expressing cells in allergic patients are not mast cells [12].

We found that there were positive H4R cells in vascular structures of the skin, but in mice, it has been reported that H4 receptors are not involved in vascular responses [27]. Obviously, many of these conclusions were made before the discovery of H4R, and therefore, should be reconsidered.

In summary, the high number of H4 receptor expressing cells is consistent with previous reports in other allergic diseases, but their functional significance, especially in CholU, is still unclear.

Acethycholine esterase (AchE) expression is downregulated in the skin of patients with CholU compared to healthy control skin

Both direct and indirect theories in the interaction of Ach with MCs have been proposed in the sweating-associated histamine release from MCs [124]. The mechanisms underlying CholU remain unclear, as several controversial findings have been reported [124]. Ach had been shown to induce degranulation in rat MCs [125,126]. Acethylcholine is a very small molecule with short half life in the tissue. Acordingly Acethylcholine cannot be consistenly stained in histology [127].

Since acetylcholine is known to induce both sweating and wheals when injected intradermally, it has been considered that the cholinergic stimulus can elicit wheals as seen in CholU [128–130]. AchE is a degrading enzyme of acetylcholine, and changes in the AChE expression might contribute to the pathogenesis of CholU[131]. It was demonstrated by a binding assay that muscarinic cholinergic receptors and AchE are reduced in the skin of patients with CholU as compared with the healthy controls [132].

In our study we also found a decreased expression of AchE in some CholU skin samples compared to the healthy control group before provocation. Interrestingly, one CholU

patient who had almost no AchE expression also had less sweating in the PCE test, which is in line with Japanese publications about CholU with hypohidrosis or anhidrosis were decreased expression of AchE. Here, especially for the CholU patients with anhidrosis, almost no AchE expression has been shown [131].

Upon provocation, the AchE expression increased significantly in some CholU patients, but the significance of this finding is unclear. Also, AchE stainings had not been performed in all samples, and the limited number of patients and controls does not allow for meaningful conclusions.

6.5 Conclusions

1. Patients with CholU in our cohort had a long duration of disease and rated themselves as mostly moderately affected. Most patients with CholU had an onset of disease at a young age. Most of our patients with CholU reported moderate to strong impairment and almost all patients with CholU did not have a well-controlled disease status. Antihistamine intake had little influence on disease severity and symptoms. Interestingly, most patients reported seasonal aggravation of the symptoms in winter.

2. PCE testing is highly sensitive and specific for diagnosing CholU. Upon PCE, onset of sweating was comparable in patients with CholU and healthy persons. The PCE test showed that patients with CholU did not have a problem with the onset of sweating, but most of the patients with CholU had very little or decreased sweating behavior. Patients with CholU always developed wheals after the onset of sweating. The symptoms upon provocation (UAS_{provo}) were correlated with disease severity and quality of life impairment.

3. Atopic predisposition is frequent in patients with CholU. Patients with CholU showed higher rates of atopic stigmata compared to healthy persons, and patients with CholU exhibited an elevated total IgE serum level. Specific IgE against the skin resident fungi Malassezia may be a marker for a more prolonged courses of disease.

4. Mast cell numbers are elevated in the skin of patients with CholU. Our results showed that MC numbers are increased in the skin of CholU patients compared to healthy

controls, but we failed to detect degranulation of MCs upon exercise challenge. Further studies are needed to clarify if skin MC levels of CholU patients are linked to disease activity and if skin MC numbers decrease in response to effective or spontaneous remission of CholU.

5. The MC mediator tryptase did not increase in the serum upon provocation in CholU patients. There was no significant difference between patients with CholU and healthy persons in the serum before and after provocation. Tryptase expressing mRNA, resembling MCs, could be detected in both CholU patients and healthy controls. .

6. Human skin expresses H1, H2, H4 receptors, but not H3 receptors. In the analyzed skin samples, we detected few H1R and H2R at mRNA expression levels, H3R-specific mRNA was undetectable. Surprisingly, highest expression was seen for H4R mRNA levels. In the future, we would like to investigate whether these histamine receptors contribute to the pathophysiology of the disease.

7. Decreased expression of Acetylcholine esterase in CholU. Sweat glands of CholU patients showed decreased expression compared to sweat glands of healthy persons, and was associated in one case with reduced sweating behaviour. For meaningful conclusions, stainings in a larger number of patients and correlation with clinical features are needed.

7. Reference

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Datum Unterschrift

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