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

Immunologische Effekte und potentielle Pathomechanismen der autologen Serumtherapie bei chronischer spontaner Urtikaria

Immunological effects and potential mechanisms of action of autologous serum therapy in chronic spontaneous urticaria

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Abbreviations and important definitions

Abbreviation	Description
AH	Antihistamine
ASST	Autologous serum skin test
AU	Acute urticaria
BHHCT	Fluorescent sensitizer for Eu3+- labeling
BHRA	Basophil histamine release assay
BW	Body weight
CD4	Cluster of differentiation 4
CholU	Cholinergic urticaria
CINDU	Chronic inducible urticaria
CR	Complete response
CST	Cold stimulation test
CSU	Chronic spontaneous urticaria
CU	Chronic urticaria
DLQI	Dermatology life quality index
DNA	Deoxyribonucleic acid
DPU	Delayed pressure urticaria
ELISA	Enzyme-linked immunosorbent assay
FcεRI	Fc epsilon receptor 1
GA2LEN	Global allergy and asthma European network
HRF	Histamine releasing factors
HSA	Human serum albumin
IFNγ	Interferon gamma
IgE	Immunoglobulin E
IgG	Immunoglobulin G

IL	Interleukin
IL20-R	Interleukin 20 receptor
MCID	Minimal clinically important difference
MDA	Melanoma differentiation-associated
NR	Non response
NSAIDs	Non-steroidal anti-inflammatory drugs
PGD2	Prostaglandin D2
PR	Partial response
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SEM	Standard error of the mean
SHIP	SH2 domain-containing inositol 5-phosphatase
STAT	Signal transducer and activator of transcription
TBS	Tris-buffer saline
TempTest	Determine the critical temperature thresholds
TGFβ	Tissue growth factor beta
Th	T helper cell types
TNF	Tumor necrosis factor
UAS	Urticaria activity score

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1 Abstract

Background Chronic spontaneous urticaria (CSU) is characterized by spontaneously occurring itchy wheals, angioedema, or both for a period of at least 6 weeks. Autoimmunity is thought to be one of the most frequent causes of CSU. Recent studies have shown that autohemotherapy can be effective in the treatment of CSU. It has been postulated that Autologous Serum Skin Test (ASST)-positive CSU patients may show better responses than ASST-negative patients. The mechanisms of action of autohemotherapy are unknown, but may include tolerizing or desensitizing effects. Very recently, interleukin 24 (IL-24) has been identified as a specific and functional IgE-autoantigen in CSU.

Objective To investigate the immunological effects and potential mechanisms of action of autohemotherapy, i.e. autologous serum injections, by analyses of the changes in serum autoreactivity, as assessed by ASST and basophil histamine release test, and in serum concentrations of IgE-anti-IL-24 and IgG-anti-IL-24 in ASST-positive CSU patients.

Material and methods In total, 66 ASST-positive CSU patients were treated with weekly intramuscular injections of autologous serum for 8 weeks and followed up for 12 additional weeks. Disease activity was assessed with the Urticaria Activity Score (UAS7), Dermatology Life Quality Index (DLQI) and the use of on demand antihistamines. The ASST was done at baseline, week 9 and week 21. Serum samples obtained at baseline and weeks 9, 13 and/or 21 were analysed for concentrations of IgE-anti-IL-24 and IgG-anti-IL-24 via ELISA and the ability to release histamine in basophils via basophil histamine release assay (BHRA).

Results Therapy with autologous serum markedly reduced CSU disease activity and quality of life impairment after 8 and 20 weeks. 28 % (14/50) and 34 % (17/50) of patients were turned to ASST-negative in week 9 and week 21, respectively. No significant changes in basophil histamine releasing activity were observed. Changes in the ASST and BHRA were not linked to the clinical response assessed by the UAS7. Autologous serum treatment resulted in a significant decrease of IgE-anti-IL-24 serum concentrations, but did not result in changes of IgG-anti-IL-24 serum concentrations. The mean concentrations of IgE-anti-IL-24 were correlated with both UAS7 and DLQI at week 21.

Conclusions Our findings suggest that the immunological effects of autohemotherapy include a reduction of skin reactivity to serum factors and concentrations of IgE autoantibodies against IL-24, both of which are held to contribute to the pathogenesis of

CSU. Further studies are needed to better characterize the relevance of these effects for the mechanism of action of autohemotherapy in CSU.

2 Zusammenfassung

Hintergrund: Die chronische spontane Urtikaria (csU) ist charakterisiert durch das spontane Auftreten von Quaddeln und/oder Angioödemen über einen Zeitraum von mindestens 6 Wochen. Es wird angenommen, dass Autoimmunität eine der häufigsten Ursachen für CSU ist. Aktuelle Studienergebnisse konnten aufzeigen, dass eine Eigenbluttherapie eine effektive Therapie der csU sein kann. So wurde postuliert, dass Autologe Serum Haut-Test- (ASST)-positive csU Patienten möglicherweise besser auf eine Eigenbluttherapie ansprechen als ASST-negative. Der Wirkmechanismus einer Eigenbluttherapie ist unklar, wobei vermutlich eine Toleranzentwicklung bzw. eine Desensitivierung eine Rolle spielen könnte. Erst kürzlich wurde mit Interleukin-24 (IL-24) ein häufiges, spezifisches und funktionales IgE-Autoantigen identifiziert.

Zielstellung: Die vorliegende Studie untersucht die immunologischen Effekte und potentiellen Wirkmechanismen einer Eigenbluttherapie mit autologem humanen Serum durch die Analyse von Veränderungen des autoreaktiven Profils anhand der Veränderungen des ASST und der Histamin-Ausschüttung von Basophilen des Serums der csU Patienten. Zusätzlich sollen Serum-Konzentrationen von IgE-anti-IL-24, IgE-anti-IL-24 und IgG-anti-IL-24 unter der Therapie analysiert werden.

Material und Methoden: 66 ASST-positive csU Patienten wurden über 8 Wochen mit wöchentlichen intramuskulären Injektionen mit autologem Serum behandelt und über weitere 12 Wochen verfolgt. Die Krankheitsaktivität mittels Urtikaria Aktivitäts-Score 7 (UAS7), der Dermatologische Lebensqualität-Index (DLQI) und die Einnahme von Anthistaminika wurde über diesen Studienzeitraum erfasst. Der ASST wurde zu Therapiebeginn, in Woche 9 und Woche 21 erhoben. Serumproben zu Therapiebeginn, in Woche 9, 13 und in Woche 21 wurden mittels ELISA bezüglich der Konzentrationen für IgE-anti-IL-24, IgE-anti-IL-24 and IgG-anti-IL-24 sowie der Basophilen Histamin-Ausschüttung (BHRA) analysiert.

Ergebnisse: Die autologe Serumtherapie führte zu einer deutlichen Reduktion der Krankheitsaktivität und Verbesserung der Lebensqualität nach 8 und 20 Wochen. 28 % (14/50) und 34 % (17/50) der Patienten wurden ASST-negativ in Woche 9 und 21. Es zeigten sich keine Veränderungen in der Histamin-Ausschüttung der Basophilen. Zudem führte die Autologe Serumtherapie zu einer signifikanten Reduktion der IgE-anti-IL-24 Serumkonzentrationen, wobei sich keine Veränderungen der IgG-anti-IL-24

Konzentrationen zeigten. Dabei korrelierten die IgE-anti-IL-24 Konzentrationen mit dem UAS7 und DLQI in Woche 21.

Schlussfolgerung: Die Ergebnisse der Studie zeigen auf, dass eine Eigenbluttherapie immunologische Effekte im Sinne einer Reduktion der Hautreaktivität auf Serumfaktoren und Abnahme von IgE-Autoantikörpern gegen IL-24 bewirkt, die möglicherweise zur Pathogenese der CSU beitragen. Weitere Studien werden benötigt, um die Relevanz dieser Effekte für den Wirkmechanismus der Eigenbluttherapie bei cSU besser zu charakterisieren.

3 Introduction

3.1 Urticaria

Urticaria is a common skin disease characterized by the sudden development of pruritic wheals, erythematous papules or plaques, with or without angioedema (**Figure 3.1**). It is the fourth most important disease seen by allergists and dermatologists after rhinitis, asthma and drug allergy[1]. Nearly 20 % of the population will suffer from urticaria at least once in their lifetime[2]. According to the frequency and duration of symptoms, urticaria can be classified into acute and chronic urticaria[3]. Chronic urticaria is defined as occurrence of the symptoms for more than 6 weeks and can be divided into the chronic spontaneous form (CSU) and the chronic inducible form (CINDU).

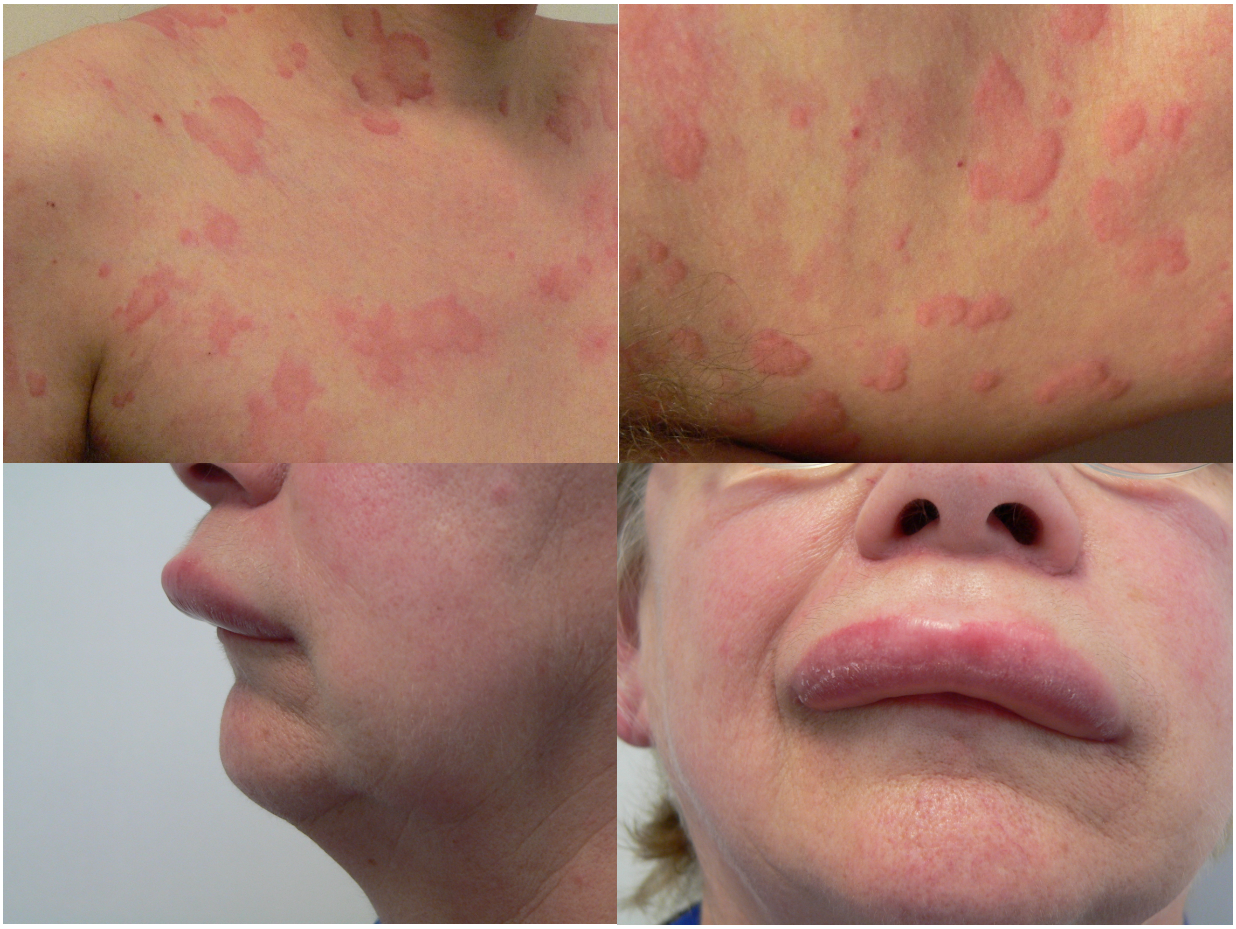


Figure 3.1 Urticaria wheals and angioedema

3.1.1 Epidemiology and prevalence

Urticaria occurs in both adults and children with a prevalence from 0.11 % to 0.6 %[4]. Acute urticaria accounts for up to 16-20 % of all urticaria patients and occurs more widely in infants and young children[5,6]. The incidence of CSU and other chronic forms of urticaria

is up to 1 % in most European countries[7,8] and it appears to be more common in adults, with females affected twice as often as males[1,6,7]. CSU is thought to affect at least 0.1 % of the population[9]. The chronic inducible forms including physical urticaria and other types of inducible urticaria are not that common. Physical urticaria comprises up to 25 % amongst cases of chronic urticaria[10].

3.1.2 Classifications

3.1.2.1 Acute urticaria

Acute urticaria (AU) is defined as the occurrence of wheals for less than 6 weeks. In up to 50 % of AU patients, no cause can be identified[11]. Other attacks may be associated with food, drugs, inhalants and infections. The attack of acute urticaria may co-occur with or without angioedema, and the wheals come in different sizes and shapes.

3.1.2.2 Chronic urticaria

Chronic urticaria can be classified into chronic spontaneous urticaria (no specific eliciting factor involved) and chronic inducible urticaria (specific eliciting factor involved)[6](**Table 3.1**).

Table 3.1 Classification of chronic urticaria

Chronic Urticaria Subtypes	
Chronic spontaneous urticaria (CSU)	**Chronic inducible urticaria (CINDU)**
	Symptomatic dermographism
	Cold urticaria
	Cholinergic urticaria
	Solar urticaria
	Heat urticaria
	Vibratory urticaria
	Delayed pressure urticaria
	Aquagenic urticaria

- **Chronic spontaneous urticaria (CSU)**

Chronic spontaneous urticaria (CSU) is characterized by the spontaneous occurrence of

itching and short-lived wheals with or without angioedema lasting for more than 6 weeks[6,12]. Although CSU is a non-life-threatening disease, patients often have a poor quality of life and the burden of disease impacts the patients at work or school[13]. In up to 90 % of the cases, the cause of the disease could not be figured out by routine clinical practice[6,14]. Nowadays, autoimmunity is discussed as playing a critical role in the pathogenesis of CSU (discussed in the next chapter).

- **Chronic inducible urticaria (CINDU)**

(i) Symptomatic dermographism

Symptomatic dermographism, also known as factitial urticaria, is defined as the appearance of a wheal and flare reaction shortly (within seconds to few minutes) after applying shearing force to the skin[15]. It can be confirmed by stroking the skin with a blunt-pointed object, which causes an immediate linear red itching wheal response[5].

(ii) Cold urticaria

Acquired cold urticaria is characterized by a localized or diffuse eruption of wheals after the exposure of the skin to cold objects, air or liquids[16]. The ice cube test is a simple test to diagnose cold urticaria by stimulating the skin on the volar surface of the forearm for 5 min with an ice cube[17]. If patients develop wheals after rewarming for 5-10 min, the test is considered as positive.

(iii) Cholinergic urticaria

Cholinergic urticaria (CholU), a subgroup of chronic inducible urticaria is characterized by pinpoint-sized (surrounded by macular erythema) and highly pruritic wheals that are provoked by elevation of the body's core temperature such as sweating in exercise, spicy foods, strongly emotional stress, and hot baths[18].

(iv) Solar urticaria

Solar urticaria is a very rare photodermatosis triggered by light exposure within minutes to a few hours. The disease is defined as itching, wheals and erythema induced by ultraviolet, visible, or even infrared radiation[19,20]. Phototesting/photoprovocation is used to confirm the diagnosis.

(v) Heat urticaria

Heat urticaria is recognized by the formation of wheals and itchy erythema after direct

contact with heat (objects, liquids, air). A diagnosis is made by a skin provocation test such as a hand water bath of 40℃ water for 10 min until an urticarial response is observed.

(vi) Vibratory urticaria

Vibratory urticaria is a rare form of physical urticaria characterized by pruritus and swelling at the site of skin under exposure to vibration including motorcycling, playing the trumpet or using electric drills or lawnmowers[6,21].

(vii) Delayed pressure urticaria

Delayed pressure urticaria (DPU) is characterized by the development of erythematous wheals after sustained pressure is applied to the skin. Compared to other subtypes of urticaria, DPU patients usually suffer from rather burning and severe pain[20].

(viii) Aquagenic urticaria

Aquagenic urticaria was first described by Shelley *et al*. (1964)[22] and is defined as a distinctive folliculopapular urticaria induced by contact with water at any temperature[6].

The etiology of the different CINDUs is widely unknown, but autoimmune and/or autoallergic mechanisms were thought to play a role in the pathogenesis of CINDU.

3.1.3 Etiology, trigger factors and pathogenesis of CSU

3.1.3.1 Etiology

The etiology of urticaria is unknown, and most reviews failed to identify a cause in the majority of CSU patients. However, some hypotheses can be given, such as autoimmunity, thyroid disorder, malignancy and hereditary disorders[23,24]. Recent studies suggested that the incidence of CSU was higher among first-degree relatives[25] and the association with human leukocyte antigen class II proved a genetic background of this skin disease[26].

3.1.3.2 Trigger factors

- **Medications and food**

Pseudo-allergy (non-allergic hypersensitivity reactions) against foods, food additives or drugs (such as non-steroidal anti-inflammatory drugs (NSAIDs)) has been described to trigger or aggravate preexistent CSU[6]. Type-I reactions to food are a rare cause of CSU,

e.g. younger children showed symptoms after having cow's milk, eggs and seafood. For adults, the most common foods were seafood and nuts[27,28].

- **Infection**

The causal relationship between urticaria and infectious disease has been discussed for more than 100 years[29]. Several chronic persistent bacterial infections (helicobacter pylori, streptococcus, staphylococcus, mycoplasma pneumonia and brucella)[30], viral infections (cytomegalovirus, parvovirus B19, norovirus, hepatitis B virus)[31], parasitic infections (ascaris, filarial, schistosoma, toxocara)[32] or fungal infections (candida albicans)[33] were suspected to trigger urticarial symptoms in patients.

- **Psychological factors**

Psychological factors were studied as critical triggers in patients with urticaria. Most reports suggested that anxiety, emotional distress, severe depression and anger played a role in worsening CSU.[34,35]

3.1.3.3 Pathogenesis

The cellular effects, inflammatory mediators and autoimmunity are all crucial to the pathogenesis of CSU. But none of the theories have been conclusively established[26,36].

- **Skin histopathology**

Skin mast cells degranulate in the dermis and cellular perivascular infiltrates such as neutrophils, eosinophils and lymphocytes were found to be the major components of histopathology in urticaria[37,38]. The histology of CSU was characterized as a non-necrotizing perivascular infiltrate composed of monocytes and CD4+ T lymphocytes[39]. The clinical symptoms and the leukocyte infiltrate shown in chronic urticaria are similar to the late phase of allergen-induced reactions. However, compared to allergen late phase, chronic urticaria lesions have a Th0 rather than Th2 cytokine profile[38]. Hermes *et al*. (1999)[40] demonstrated that endothelial cells increased expression of tumor necrosis factor α and interleukin 3 in lesional and non-lesional skin of chronic urticaria patients.

- **Cellular effects**

Degranulation of mast cells and basophils is a main event in urticaria[38] and the levels of histamine in skin biopsied are elevated[41,42]. Some findings reported an increase in mast cell numbers, which underlined the complex nature of the pathogenesis of urticaria[6].

However, some studies reported that no difference of mast cell numbers was observed in lesional, non-lesional or healthy skin[43]. The elevation of histamine levels[42] might be associated with either increased quantitative release of histamine from skin mast cells or blood basophil infiltration of CSU skin tissues[44]. Blood basophils have also been investigated for decades in CSU, especially when Greaves *et al.* (1974)[45] and Kern *et.al.* (1976)[46] identified a reduction in total available histamine-release ability after IgE-receptor activation. Blood basophils IgE receptor responses of patients with CSU have been segregated into two basophil phenotypes: CSU responders and CSU non-responders. These two phenotypes are stable in active disease and have different expression in some features. In contrast to the responders, the non-responder basophils do not degranulate to *ex vivo* IgE receptor activation and possess elevated concentrations of the IgE receptor regulating inhibitory phosphatases, SH2 domain-containing inositol 5-phosphatase (SHIP)-1 and SHIP-2.

- **Autoimmune mechanisms**

Autoimmunity is thought to be one of the most frequent causes of CSU. The autoimmune theory was first originated from the high correlation between the presence of autoimmune serum factors and thyroid autoimmunity. The occurrence of thyroid autoantibodies and thyroid dysfunction in CSU patients is two times higher than that of the general population[47,48]. In the 1980s, this theory was developed based on an *in vivo* skin test (autologous serum skin test, ASST), in which 58 % (7/12) of CSU patients had wheal and flare reactions at the site of intradermal injections of their own serum within 30 min[49]. Also, around 30 - 40 % of (overlapping) CSU patients showed circulating IgG autoantibodies to either IgE or the α subunit of the high affinity IgE receptor that could induce mast cell and basophil degranulation[36].This autologous serum skin test (ASST) has been proven to be one of the most useful screening tests for autoimmune urticaria[6,50] and the circulating histamine-releasing factors are responsible for the wheal and flare symptoms in ASST-positive patients[51]. Interestingly, ASST-positive patients have more associated systemic symptoms and autoimmune conditions than ASST-negative patients[52]. In addition, a study demonstrated CSU serum could induce histamine release from basophils of healthy donors because of the IgG autoantibodies targeting the Fc part of IgE[53]. This activity was termed basophil histamine release assay (BHRA).

Two types of autoimmune reactions (IgE to autoallergens and IgG autoantibodies to IgE or its receptor, respectively) have been claimed to be relevant to autoimmune CSU

patients so far[54] (**Figure 3.2**). Rosman *et al*. (1962)[55] first described that CSU could be caused by a hypersensitivity type I reaction. In this reaction, mast cells and basophils can release histamine and other proinflammatory mediators after IgE molecules crosslink on their surfaces with (auto) antigens. There is evidence that autoreactive CSU patients have much higher concentrations of IgE autoantibodies against double-stranded DNA and thyroid antigens such as thyroid peroxidase / thyroglobulin than normal subjects[48,56,57]. In the type II reaction, 30 - 50 % of CSU patients express autoantibodies that can induce degranulation of mast cells and basophils[58] including circulating IgG autoantibodies directed against the high-affinity receptor for IgE (IgG-anti-FcεRI) or the IgE molecule (IgG-anti-IgE)[59-61].

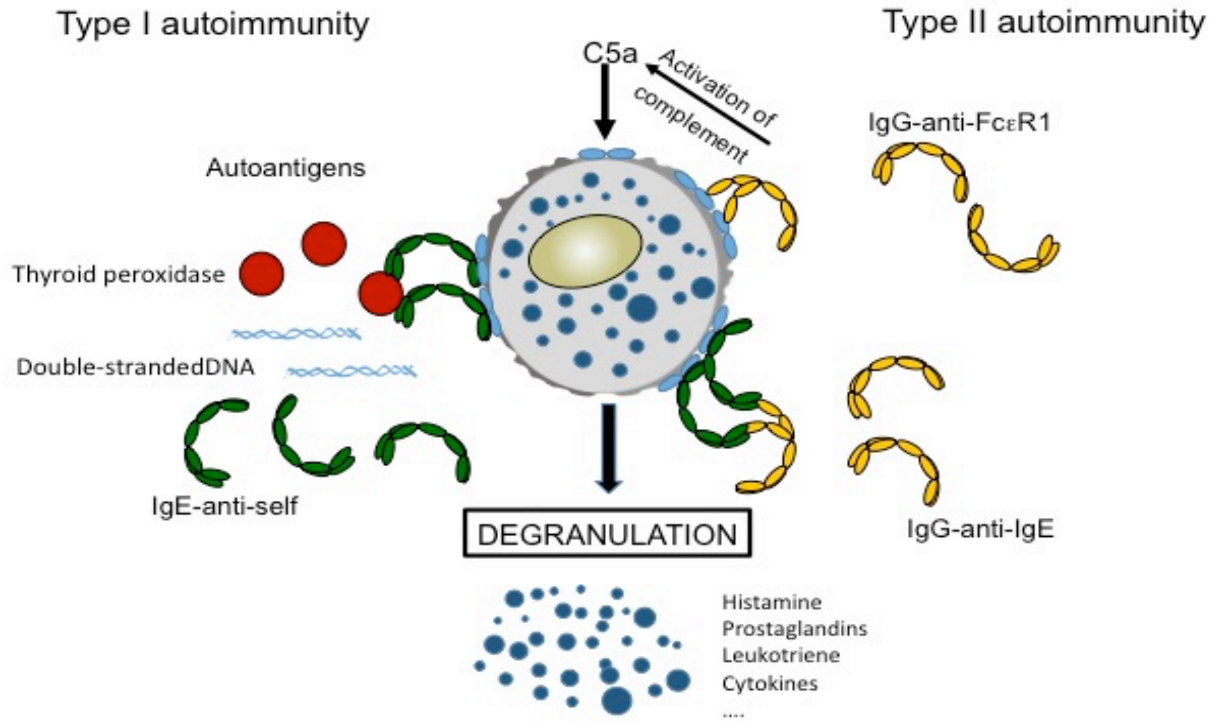


Figure 3.2 Proposed pathomechanisms of autoimmune CSU: Two types of proposed autoimmune reactions

3.1.3.4 Interleukin 24 (IL-24)

Interleukin 24 (IL-24) is a member of the interleukin 10 family of cytokines. It forms signals upon linking to one of two heterodimeric receptors: IL-20R1/IL-20R2 and IL-22R1/IL-20R2[62,63]. IL-24 was first known as melanoma differentiation-associated 7 (mda-7) because of its tumor-suppressing activity in healthy melanocytes by multiple pathways[64,65]. IL-24 can selectively control and inhibit the apoptosis, proliferation, invasion and metastasis of cancer cells via binding to its receptors, then rapidly activate

signal transducers and activators of transcription 1 (STAT-1) and STAT-3 transcription factors. IL-24 can be secreted by monocytes, macrophages, keratinocytes and activated T helper 2 cells. The major target tissues of IL-24 are non-haematopoietic tissues, e. g., skin, lung and reproductive tissues[66,67]. IL-24 is expressed in the human immune system, including the thymus, spleen and peripheral blood leukocytes. It plays important roles in wound healing, arthritis and cancer. Furthermore, IL-24 is also shown at increased levels in psoriasis[64,66]. Very recently, IL-24 was identified as a common, specific and functional autoantigen of IgE autoantibodies in CSU. Interestingly, IgE-anti-IL-24 was detected in all patients with CSU, but it was undetectable in idiopathic anaphylaxis patients or healthy subjects. Furthermore, the concentrations of IgE-anti-IL-24 were found to be associated with the urticaria disease activity[68].

3.1.4 Management of chronic spontaneous urticaria

3.1.4.1 Diagnostic approach

- **Confirm a diagnosis of CSU**

The lesions of CSU may vary in shape and size, present as itchy raised wheals surrounded by an erythematous base and central pallor (**Figure 3.1**). Diagnosis can be confirmed by a detailed history such as signs and symptoms, aggravating factors, and past medical history. Normally, laboratory investigations are not required for diagnosis of CSU. The EAACI/GA²LEN/EDF/WAO guideline recommend physical exams, full blood count (FBC), C-reactive protein (CRP) and further individual tests such as antinuclear antibodies (ANA) to identify underlying causes[3].

- **Assess the disease activity in CSU**

The guideline suggests using the urticaria activity score (UAS) to assess disease activity[6]. Daily urticaria activity scores (UAS, range 0-6) are calculated as the sum of wheal and itch scores as follows (**Table 3.2**). The weekly UAS (UAS7, range 0-42) is calculated by adding the daily scores over 7 days.

Table 3.2 The UAS7 for assessing disease activity in CSU

UAS7 score	Wheals	Pruritus
0	No wheals	None
1	< 20 wheals	Mild itch (Present but not annoying)
2	20-50 wheals	Moderate itch (Troublesome but does not interfere with normal daily activity or sleep)
3	>50 wheals	Severe itch (Sufficiently troublesome to interfere with normal daily activity or sleep)

3.1.4.2 Treatment

The first therapeutic option is to avoid eliciting factors such as food and NSAIDs. The main goal of therapy in CSU is to achieve complete symptom control. Evidence-based guidelines support that the most effective, first-line treatment for CSU are non-sedating second generation antihistamines, since the older first-generation sedating antihistamines have side effects on central nervous system[3]. A number of non-sedating antihistamines were shown to be effective in clinical studies, e. g., cetirizine, desloratadine, fexofenadine, levocetirizine, loratadine, ebastine, rupatadine and bilastine[6,69-71]. Considering that up to 50 % of patients[72] still suffer from symptoms with the use of standard-dosed non-sedating agents, an up-dosing up to the four-fold dose is recommended as second-line treatment[73]. Around two thirds of non-responders to standard doses were shown to benefit from antihistamine up-dosing[74]. Although this treatment is off-label, antihistamine updosing has not been associated with severe adverse events or safety concerns[75].

Omalizumab, a humanized anti-IgE antibody has recently been shown to be very effective and safe in the treatment of CSU[76,77]. It is approved for CSU, suitable for long-term treatment and can markedly improve the patients' quality of life. Maurer showed that omalizumab diminished clinical symptoms in CSU patients who had no response to

approved doses of H1-antihistamine[77]. The guidelines recommend adding omalizumab as the third-line treatment in CSU patients unresponsive to second-generation H1-antihistamines. Ciclosporin A has been used for many years in refractory CSU and is known to inhibit mast cell mediator release[78]. In combination with second-generation H1 antihistamines it has exhibited beneficial effects in patients with severe disease refractory to any dose of antihistamines[79]. However, this drug is off-label for urticaria and may be associated with a number of side effects such as hypertension, hypertrichosis and headache. In summary, the guidelines suggest that omalizumab should be tried before ciclosporin A[6].

3.2 Autohemotherapy for chronic spontaneous urticaria

Autohemotherapy, i.e., repeated intramuscular or intravenous injections of autologous serum or autologous whole blood, has been used for over 150 years. Autohemotherapy is not a standard treatment of CSU, but it has been reported to be effective in patients with chronic urticaria in clinical trials and real-life practice. There are different forms of autohemotherapy used in urticaria treatment, such as autologous whole blood vs. autologous serum injections and intramuscular vs. subcutaneous injections. Godse *et al.* [80] reported that subcutaneous autologous serum therapy was effective and better than intramuscular applications in the treatment of CSU. Panchami *et al.* (2014)[81] confirmed that autologous serum therapy was a useful adjunct in the treatment of CSU. They showed that autohemotherapy reduced the intake of antihistamines and improved the quality of life. More recently, Staubach *et al.* [82] and Elazab *et al.* [83] reported that autohemotherapy is very effective in autoimmune CSU patients, with ASST-positive patients showing a better clinical response than ASST-negative patients in both studies. In CSU treatment, most studies applied one injection per week for a period of 8-10 weeks. Although few studies reported an increase of infection risks e.g., hepatitis C[84,85], it appears to be safe to perform autohemotherapy using a direct withdrawal-and-injection approach without any procedure in between. Since the autohemotherapy procedure is safe and simple to perform and inexpensive, it is highly recommended for less developed countries.

The mechanism of action of autohemotherapy is still not well understood. Several lines of evidence point to the effects on immune mechanisms: Bocci *et al*. (1994)[86] claimed that “autohemotherapy may represent a physical stimulation for reprogramming the

immune system". They suggested the effects of autohemotherapy were linked to the apoptosis of autocytotoxic T lymphocytes caused by IFN*γ* or other immunosuppressive factors such as IL-10, TGF*β* antagonists and eicosanoids. In contrast, Sheikhi *et al*. (2014)[87] suggested a potential mechanism where the induced anti-idiotype antibodies could react against IgE autoantibodies and subsequently block IgE autoantibodies to bind with their high affinity receptors (FcεRI) of mast cells or basophils. Interestingly, some clinical studies, but not all, suggested that serum autoreactivity in CSU patients had a high concordance with the response to autohemotherapy. For example, Staubach *et al*. (2006)[82] proposed that ASST-positive CSU patients, but not ASST-negative CSU patients, benefited greatly from autologous whole blood therapies due to the induction of tolerance to circulating histamine-releasing factors. Zhang *et al*. (2014)[88] have suggested that the mechanism of action of autohemotherapy is possibly related to the down-regulation of IL-4 and IgE in patients. Based on these reports, we investigated the effects of autohemotherapy in ASST-positive patients with CSU on two distinct immunological features observed in many patients with CSU, serum autoreactivity and IgE autoantibodies.

4 Aim of the study

Recent small studies and case reports have shown that patients with CSU can benefit from autohemotherapy. However, the treatment regimes were not standardized and results from larger patient cohorts are lacking. Also, the mechanism of action of this therapeutic option remains elusive so far. We hypothesize that autohemotherapy is effective and safe in treating patients with autoimmune CSU. Thus, we aim to assess whether autologous serum injections decrease the disease activity in CSU and can even cure a proportion of patients of their condition. We postulate that the therapeutic effects of autohemotherapy in CSU are due to tolerization/desensitization processes induced by the injections of autologous serum. To investigate the effects of autohemotherapy in CSU patients, we will:

- Assess serum reactivity before, during and after eight weeks of autohemotherapy by autologous serum skin tests and basophil histamine release assay
- Monitor the concentrations of IgE (and IgG) against Interleukin 24
- Assess changes in serum autoreactivity and IgE-anti-IL-24 concentrations for their clinical relevance and correlate them with changes in clinical outcome parameters, i.e. the weekly urticaria activity score (UAS7), the on demand use of antihistamines, and quality of life (DLQI)
- Discuss our findings in the context of the known scientific literature

5 Methods

5.1 Trial design and selection of patients

This was an observational study during the period from September 2010 to December 2013, which intended to include all available prospective CSU patients with a known positive autologous serum skin test who had received autologous serum therapy. As a multicenter project it was attended by the GA^{2}LEN Urticaria Centers of Reference and Excellence in Berlin, Germany and Istanbul, Turkey as well as the Nashik Urticaria Clinic, India. Patients were recruited by all participating centers.

The study on autohemotherapy is a pilot study in 66 patients. The sample size is considered sufficient to adequately investigate the objectives of this exploratory study. This assumption is based on the investigator's experience with minor pilot studies about the treatment of urticaria[89–93]. With the unknown variability of the treatment effect in this population, statistical power considerations are unwarranted in principle. Formally, by assuming an effect size of 0.20 for changes in UAS7, 134 patients will be needed for the analysis to reach a power of 80% with a level of significance of 0.05 (one sample size t-test). With an effect size of 0.30, there will be 74 patients required. With an effect size of 0.50, the minimum patient number should be 28 for the analysis, with an effect size of 0.80, the number of patients needed will be only 12 (calculated by G*Power 3.1).

For selection of patients, the following criteria had to be fulfilled:

Inclusion criteria:

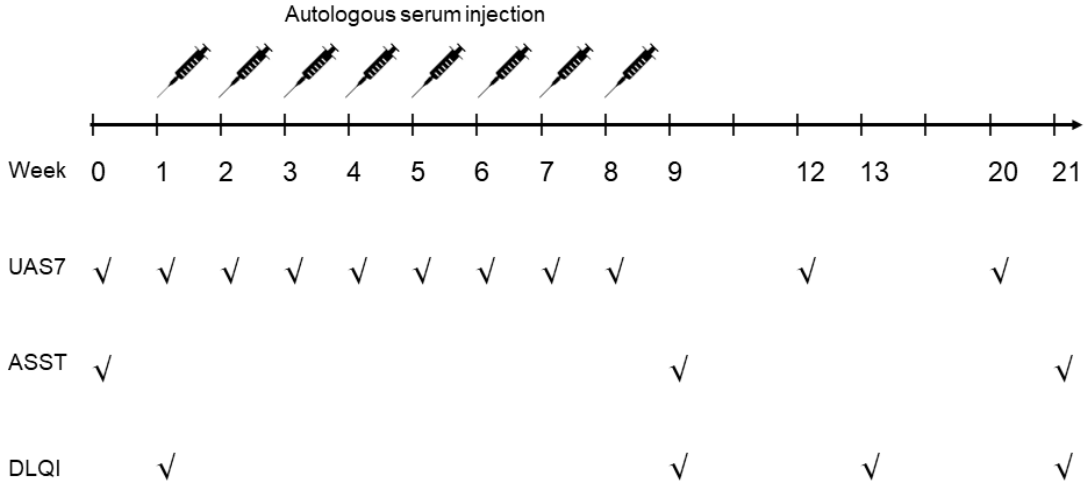
1 Male and female outpatients, aged between 18 and 75 years
2 Approved diagnosis of chronic spontaneous urticaria (duration > 6 weeks) with a positive autologous serum skin test (ASST-positive)
3 UAS 7> 6 in baseline week
4 History of beneficial effects of antihistaminic treatment
5 Signed and dated informed consent of the patient before the start of specific protocol procedures
6 Patients must be able and willing to record symptom scores and to document them in diaries.
7 Ability to understand the scope and nature of the trial and to understand and write and fill in the patient diary.

Exclusion criteria:

1. Chronic severe diseases, especially those affecting the immune system, except autoimmune chronic urticaria
2. Acute urticaria (history of urticaria less than 6 weeks) or mainly physical urticaria
3. Any pathologic alterations in the area of autologous serum application which may interfere with intramuscular injections
4. History or presence of epilepsy, significant neurological disorders, cerebrovascular attacks or ischemia.
5. Therapy with anticoagulants, e.g. warfarin or phenprocoumon
6. Constant therapy with acetylsalicylic acid
7. Presence of active cancer which requires chemotherapy or radiation therapy
8. Presence of alcohol abuse or drug addiction
9. Permanent use of oral corticosteroids or other immunosuppressants / immunomodulators within 3 weeks prior to start or at any time during the study
10. Emergency use of oral corticosteroids >12 times in the last year
11. Pregnancy or breast-feeding
12. Significant changes in naturopathic or complementary treatments that may affect chronic urticaria such as special diets, fasting, microbiological therapies or acupuncture within 6 weeks prior to start or at any time during the study.

Treatment and data collection schedule:

Table 5.1 Schedule of treatment



All patients provided written and oral consent and the study was approved by the ethics

committee of the Charité-Universitätsmedizin Berlin.

5.2 Assessment of disease activity and impact

Disease activity assessment was based on the weekly Urticaria Activity Score (UAS7) and the use of on-demand antihistamines (AH-demand). Disease impact on quality of life was assessed with the Dermatology Life Quality Index (DLQI). UAS7 scores, AH-demand data and DLQI values were obtained by use of patient diary documentation. The schedules of visit and treatment are shown in **Table 5.1**.

5.2.1 Calculation of UAS7

Patients recorded the number of wheals and the intensity of pruritus daily during weeks 0-8, 12, and 20. The weekly urticaria activity scores (UAS7) were calculated as the sum of wheal and itch scores over 7 days as shown in **Table 3.2**. If less than 7 days are available for rating at a visit, missing days will be replaced by the average of the daily values available from the diary (**Table A.1,** Appendix).
Response to the treatment was defined by a reduction of UAS7 compared to week 0. “Complete response” was defined as a reduction of UAS7 equal to or more than 90 %, “partial response” as a reduction of between 30 - 90% and “non response” as equal to or less than a 30 % reduction in UAS7.

5.2.2 Calculation of on-demand use of antihistamines

The antihistamine demand (AH-demand) was determined weekly. During weeks 0-8, 12, and 20, antihistamines should be administered only when needed, at all other time points patients were free to use antihistamines either on-demand or as prophylaxis. The weekly AH-demand score was calculated as the sum of all antihistamine tablets (one point per tablet) used per week. Patients were advised to use cetirizine first line. However, if cetirizine was not efficient or tolerated, other antihistamines were permitted. Antihistamines were not allowed within three days before the ASST (**Table 5.1** and **A.1,** Appendix).

5.2.3 Calculation of DLQI

The quality of life of patients was investigated by the Dermatology Life Quality index (DLQI). The DLQI was developed in 1994 by Finlay and Kahn as quality of life

questionnaire for patients with dermatological disease[94,95]. The DLQI consists of 10 questions across six domains: symptoms/feelings, daily activities, leisure, work/school, personal relationships and treatment. Each question is scored from 'very much' (score = 3) to 'not at all' (0), and an overall score (0-30) is calculated by totaling the individual domain scores. Higher scores indicate higher quality of life impairment.

The DLQI was applied in weeks 1, 9, 13 and 21. A minimal clinically important difference (MCID) of 3-4 points has been estimated to be meaningful for the DLQI in patients with CSU[96]. The MCID is the minimum change considered important by the patient and mandating a change in management. The proportion of patients whose change in the total DLQI score from baseline reached an MCID of ≥ 4 was also assessed at week 9, 13 and 21 **(Table 5.1** and **Table A.2,** Appendix**).**

5.3 Autologous serum skin test

This very simple and quick test is used as a screening instrument to identify the presence of circulating histamine-releasing factors in CSU patients. In each patient, 2ml of venous blood was drawn from the cubital vein and the serum was separated by centrifuge at 1300 rpm for 15 min at room temperature. 50 µl of normal saline (negative control), histamine (10 µg/mL) (positive control) and serum, serum diluted (saline) 1:1 and serum diluted 1:10 were injected intradermally 3 to 5 cm apart into the volar forearm of the same patient (**Figure 5.1**). The test area should be free of lesions. The maximum vertical (d1) and horizontal (d2) diameters of the wheals were measured with a transparent ruler after 10 min. The average diameter (D) was calculated [D = (d1 + d2) / 2] (**Figure 5.2**). A positive test was defined as a serum-induced wheal response at any concentration with an average diameter of more than 2 mm than the negative control. ASST was performed at week 0, 9 and 21, with no antihistamine intake three days prior to testing **(Table 5.1).**

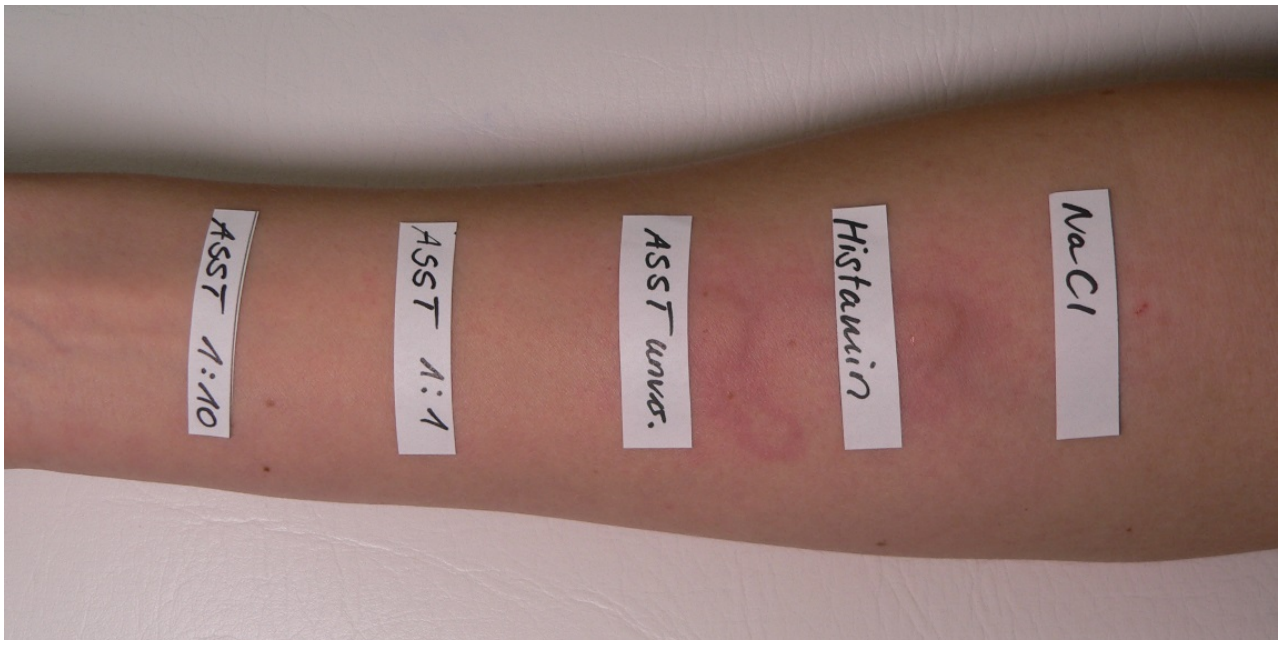


Figure 5.1 ASST-positive CSU patient: The ASST is positive with the injections of undiluted serum, but remained negative at serum diluted with 1:1 and 1:10 with saline.

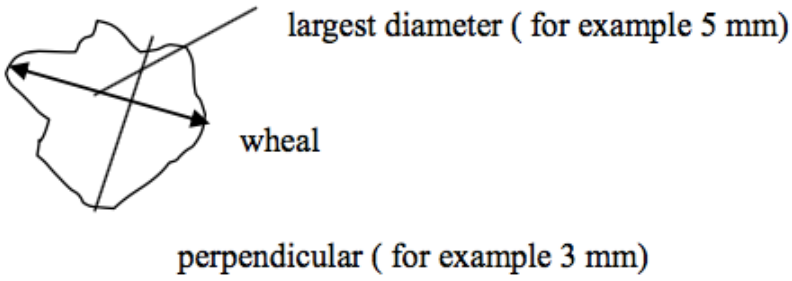


Figure 5.2 Measure and calculate the wheals

5.4 Autologous serum injections

Venous blood was drawn from the cubital vein and the serum was separated by centrifuge at 1300 rpm for 15 min at room temperature. The obtained autologous serum was reinjected into the gluteal muscle of the patient immediately without any further manipulation at week 1 to week 8. The volume of the first injection was 0.025 ml / kgBW (with a maximum of 2.5ml in total); in all further treatments 0.05 ml / kgBW (with a maximum of 5 ml in total) per injection was applied. Altogether, there were eight injections at alternating sides, once a week over eight weeks. **(Table 5.1** and **Figure 5.3).**

Figure 5.3 Timetable for taking blood

5.5 Blood samples

Blood samples for ELISA and BHRA were taken at time points 0, 9, 13 and 21 **(Table 5.1 and Figure 5.3).** Samples were centrifuged at 1300g for 15 minutes at room temperature to separate serum and plasma. Serum and plasma were portioned in 500 µl Eppendorf microtubes and stored at -80 °C.

5.6 Outcome measures

Reduction of disease activity is assessed by UAS7 including number of wheals and intensity of itch (week 0 vs. week 8). UAS7 was calculated using the data documented in the patient´s diary **(Table 5.1 and Table A.1,** Appendix**).**
Long-term (week 12 and 20, resp. vs. week 0) efficacy in reduction of urticaria symptoms (UAS7, wheal number and size, pruritus duration), quality of life, autologous serum skin test reaction (size and duration), and reduction of antihistamine demand were assessed by patient and investigator **(Table 5.1 and Table A.1,** Appendix**).**

5.7 Measurements of autoantibodies against IL-24

IgE-anti-IL-24 serum concentrations were assessed by a site-directed IgE capture ELISA as reported recently[68]. In brief, 16 µg of anti-IgE antibody (MHE18, Biolegend) was dispensed in 10 ml Coating Buffer (0.1 M carbonate at pH = 9, Sigma) to make a coating solution. Each well of a black Maxisorb plate (Thermofisher) was coated overnight at 4 °C with 25 µl of the coating solution. After blocking with 2 % HSA in TBS-Tween overnight at 4 °C, 25 µl of serum was added into each well and incubated at 4 °C overnight. After the removal of unbound protein from the plate by three washing steps with TBS-T and water, respectively, 25 µl of the antigen-solution (20 µl IL-24-BHHCT-Eu per 10 ml 50 mM Tris buffer pH = 8) per well was added and the plate was incubated at 4 °C for 2 h. After ten washing steps, 25 µl DELFIA enhancer solution (Perkin Elmer, Waltham, USA) was added

to each well and the plate was incubated for 5 min at room temperature. Finally, the plates were measured in a Victor V reader (Perkin Elmer) with standard Europium settings.
For the measurement of IgG-anti-IL-24, 16 µg of anti-IgG antibody (anti-human IgG Fc, Biolegend) were dispensed in 10 ml Coating Buffer to make a coating solution.

5.8 Basophil histamine release assay

The serum induced histamine release method aims at detecting autoantibodies in patient sera directed against both the free IgE receptors and the cell bound IgE by the use of blood bank buffy coat donor blood containing 1-2 % basophils. An optimal histamine release response is obtained by partial removal of basophil bound IgE by the stripping procedure described below. As the cell response varies between different donors, four blood bank buffy coats were collected from the blood bank the day before performing the test. They must origin from blood that has been drawn and fractionated within the same day. The buffy coats were mixed with equal volumes of RPMI. IL-3 in a final concentration of 1 ng / mL was added and the samples were stored overnight at 2-8 °C. The next day the buffy coats were centrifuged to remove plasma and RPMI. Thereafter the samples were washed with physiological saline and subsequently exposed to low pH using a stripping buffer, pH = 3.6 from RefLab, Denmark, Copenhagen to partially remove IgE from the basophils. Finally, the IgE-stripped samples were resuspended in Pipes buffer from RefLab before incubation with patient sera. Each serum was tested at a dilution of 20 % and 10 %. Total histamine content was determined after lysis of cells with 7 % HClO4.
The following standard controls (pools) were included in each testing and the histamine release criteria stated below were fulfilled to include the results:
Pool negative: sera collected from healthy volunteers. Histamine release < 5 %.
Pool I: sera from CSU patients. Histamine release between 60 % and 80 %.
Pool II: sera from CSU patients. Histamine release between 30 % and 50 %.
Pool III: sera from CSU patients. Histamine release between 20 % and 30 %.
Pool Positive: Anti-IgE from KPL, USA, 4 ng / mL. Histamine release > 30 %.
Next, patient sera and stripped buffy coat cells were incubated in a total volume of 100 µL for 60 minutes at 37 ˚C. After centrifugation, 25 µL supernatants were transferred to glass fiber coated microtiter plates RLA210 and histamine was measured according to RefLab instructions. A triplicate histamine determination of each sample was performed. Assay variation was < 7 %. Assay sensitivity was 5 ng histamine / mL. The release of

histamine in cell supernatants was expressed as the percentage of total histamine content from lysed donor cells. A positive BHRA is defined as 16.5 % above the spontaneous histamine release[97].

5.9 Assessment of the outcome parameters and schedule

In summary, for UAS7 and AH-demand, the results of measurements were determined at week 0 (at baseline), 8 (immediately after end of treatment), 12 (4 weeks after the end of treatment) and 20 (12 weeks after end of treatment). The measurements for DLQI were determined at week 1, 9, 13, and 21. ASST was assessed at baseline (week 0) and additionally in the weeks 9 and 21 **(Table 5.1).**

5.10 Statistical Analysis

Statistical analysis of the data was performed using GraphPad Prism 6.0 and IBM SPSS statistics 23 software. Sample size calculation was performed by G*Power 3.1. Data were analysed by calculating values for the mean and standard error of the mean (= SEM), and mean or standard deviation (= SD). Two related paired non-parametric data were compared using the Wilcoxon singed-rank test. Two independent data were compared using Mann Whitney U test. Paired and categorical data (e.g. data from the same patients obtained at different time points) were compared using the McNemar test. One or more categorical data were compared using the Chi-squared test. Correlations were determined by the Spearman test. In the statistical tests, a p-value ≤ 0.05 was considered as statistically significant.

6 Results

6.1 Study participants and availability of samples and data

We included 66 CSU patients in the study. Of the 66 patients, 21 were from Berlin, 33 from Istanbul, and 12 from Nashik. Based on the fact that this was an observational study, serum samples and clinical data were not available from all time points for all participants. Data shown are from all patients with available data at the specific timepoints. For example, for analyses of disease activity scores UAS7, there were only 46 complete data sets available for each timepoint to compare.

Since this was a multiple centers study, 10 patients' blood samples were damaged during transportation and 41 patients' sera were missing from certain study centers or certain timepoints. So there were only 15 patients' serum samples available for all timepoints for the analysis of IgE-anti-IL-24 and IgG-anti-IL-24 antibodies.

6.2 Effects of autohemotherapy in ASST-positive CSU patients

6.2.1 Changes in disease activity

Autologous serum therapy markedly reduced the mean ± SEM CSU disease activity as assessed by UAS7 from 23.3 ± 1.2 before treatment to 14.8 ± 1.7 at the end of the 8-week treatment period (~ 36 %, $p \leq 0.0001$, **Figure 6.1 a+b**). Disease activity was first significantly reduced, to 17.9 ± 1.4, after 2 weeks of treatment (~ 23 %, $p \leq 0.0001$). UAS7 values continued to decrease after the end of treatment, to 12.8 ± 1.6 (~ 45 %, $p \leq 0.0001$) and 12.6 ± 1.8 (~ 46 %, $p \leq 0.0001$) at week 12 and 20, respectively (**Figure 6.1 a+b**). Since the UAS7 values showed a skewed distribution, **Figure c** demonstrates the Mode value of different time points, which also means that most patients decrease the disease activity assessed by UAS7.

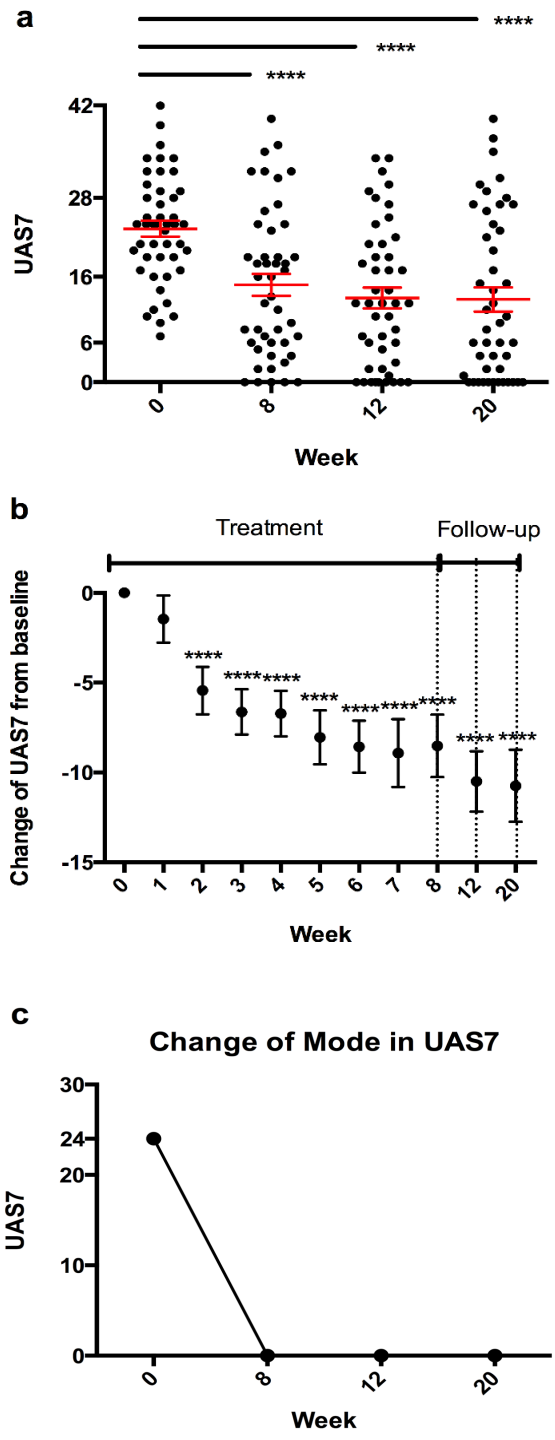


Figure 6.1 Effects of autologous serum therapy on disease activity in patients with CSU and a positive ASST. UAS7 scores are shown for each patient and displayed as mean and standard error of the mean (SEM) (**Figure a+b**) and mode value (**Figure c**). **** p ≤0.0001 (Wilcoxon signed-rank test) compared to week 0; UAS7 = Weekly Urticaria Activity Score. N = 46.

6.2.2 Changes in antihistamine demand

Autologous serum therapy significantly reduced the antihistamine demand (AH-demand) in autoimmune CSU patients. **Figure 6.2** indicates that the use of on-demand antihistamine treatment was significantly decreased in week 8 after the treatment (from 2.9 ± 0.6 to 1.5 ± 0.4 tablets per week, $p \leq 0.05$) and continued to decrease thereafter, to 1.4 ± 0.4 tablets per week ($p \leq 0.01$, in week 20).

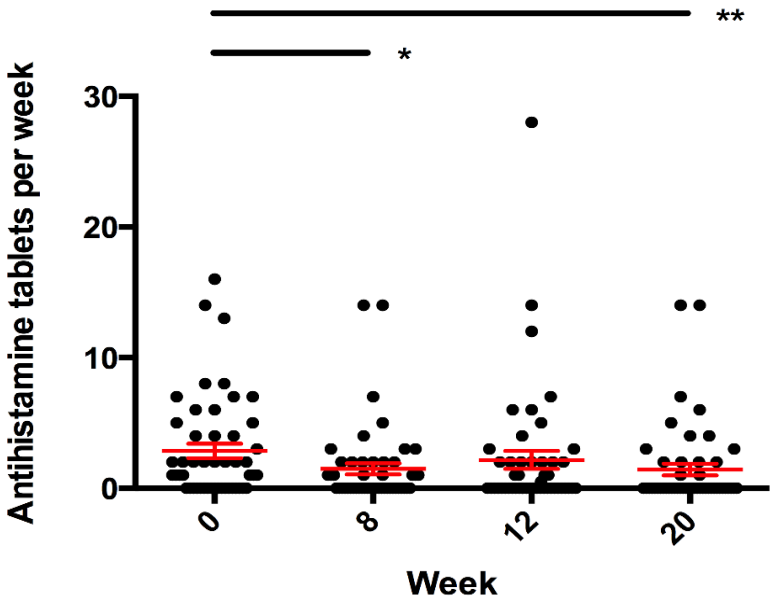


Figure 6.2 Effects of autologous serum therapy on the use of on-demand antihistamine medication are shown for each patient and displayed as mean and standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$(Wilcoxon signed-rank test) compared to week 0; N = 49.

6.2.3 Changes in quality of life

Treatment with autologous serum lead to a long-term increase in the quality of life assessed by the DLQI **(Figure 6.3a).** Quality of life was significantly improved from 8.8 ± 1.2 (at baseline, corresponding to moderate impairment) to 6.3 ± 1.1 (at week 9, corresponding to moderate impairment, $p \leq 0.05$), 6.0 ± 1.2 (at week 13, corresponding to moderate impairment, $p \leq 0.01$) and 5.1 ± 1.1 (at week 20, corresponding to mild impairment, $p \leq 0.001$) after the start of treatment. Furthermore, the results demonstrated that the quality of life even continued to improve after 12 weeks in the follow-up period.

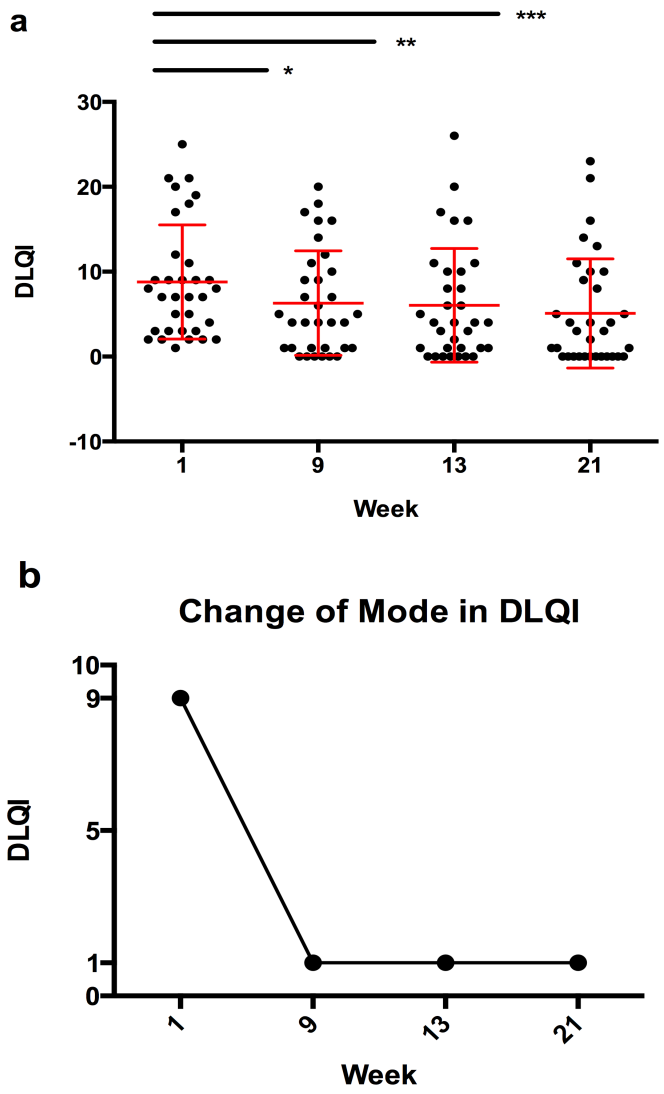


Figure 6.3 Effects of autologous serum therapy on the DLQI are shown for each patient and displayed as mean and standard error of the mean (SEM) (**Figure a**) and mode value (**Figure b**). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Wilcoxon signed-rank test) compared to week 0; DLQI = Dermatology Life Quality Index. N = 33.

After an 8-week therapy with autologous serum, the proportion of patients that reached an MCID of ≥ 4 in total DLQI scores was 35 % (15/43) compared to baseline. The percentage increased steadily to 41 % (19/46) in week 13 and 47 % (20/43) in week 21. However, there was no significant difference between week 9, week 13 and week 21. (McNemar test, $p > 0.05$) (**Figure 6.4**).

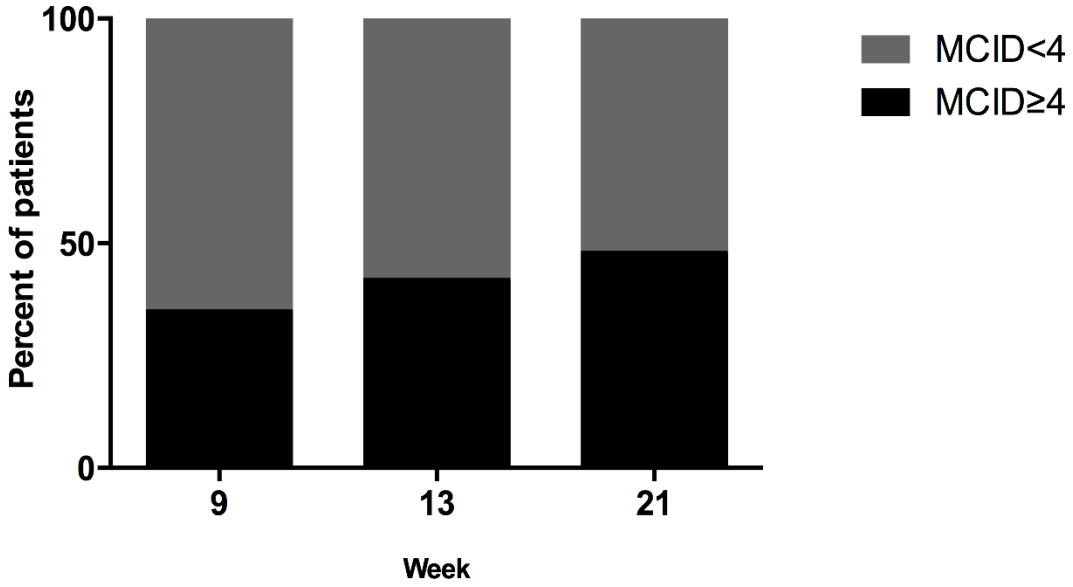


Figure 6.4 Distribution of patients who reached or did not reach MCID ≥ 4 on the DLQI after autohemotherapy (a). No significant difference was observed by McNemar test ($p>0.05$). MCID = Minimal clinically important differences. N = 43

6.2.4 Correlations between UAS7 and DLQI

Treatment with autologous serum for 8 weeks, UAS7 in week 0, 8, 12 and 20 correlated with DLQI values in week 1, 9, 13 and 21, respectively. (Spearman test, $p = 0.047$, 0.0071, 0.0006 and ≤ 0.0001) **(Figure 6.5 a, b, c, and d)**

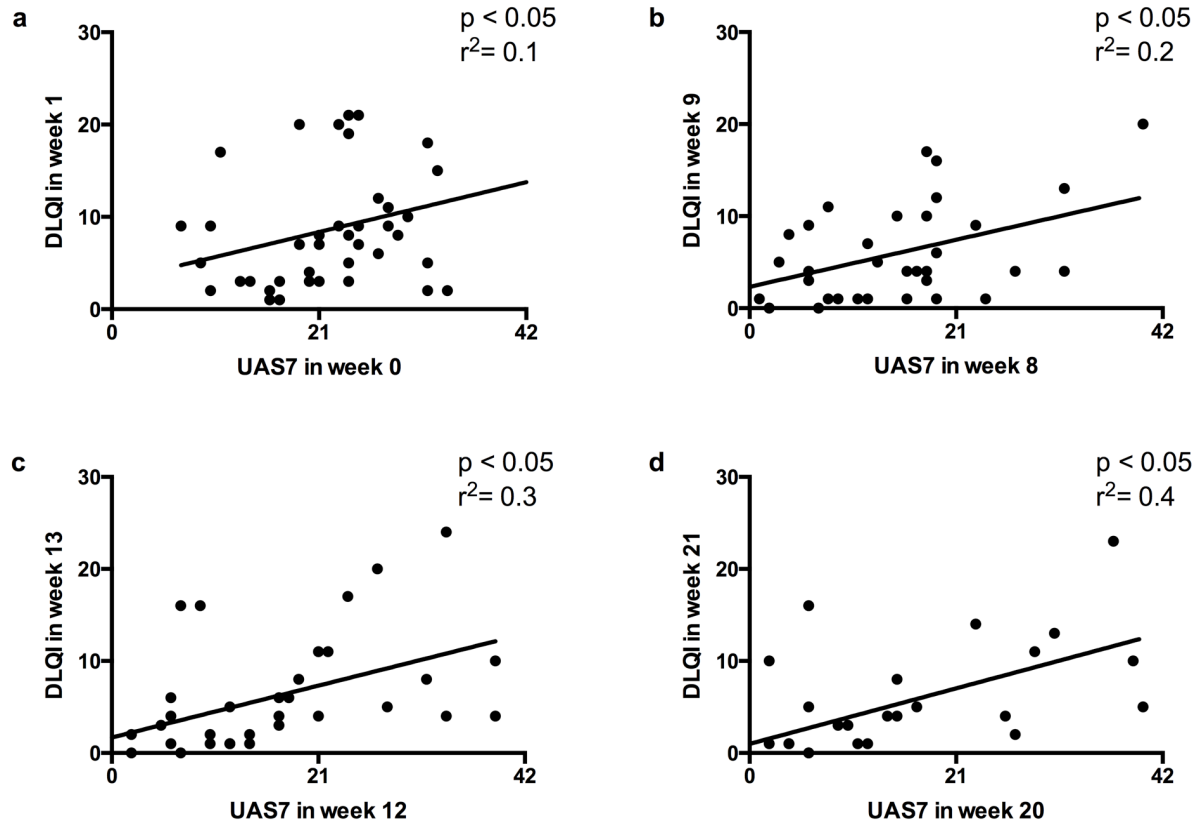


Figure 6.5 Correlations of UAS7 and DLQI at different time points were calculated by Spearman test (regression line calculated by Pearson test for better visual understanding). UAS7 = Weekly Urticaria Activity Score. DLQI = Dermatology Life Quality Index. N = 41, 39, 41 and 34 for a, b, c and d, respectively.

6.2.5 Distribution and kinetics of response

According to the reduction of UAS7, patients were grouped into complete response (CRs), partial response (PRs) and non-response (NRs). At the end of the treatment phase (in week 8), the rate of complete response and partial response was 13 % and 48 %, respectively. The response rate increases constantly within the 8 weeks of weekly treatment and continues to increase after treatment. At the end of the follow up, in week 20, nearly two-thirds of patients showed complete and partial response to autologous serum therapy (**Figure 6.6**).

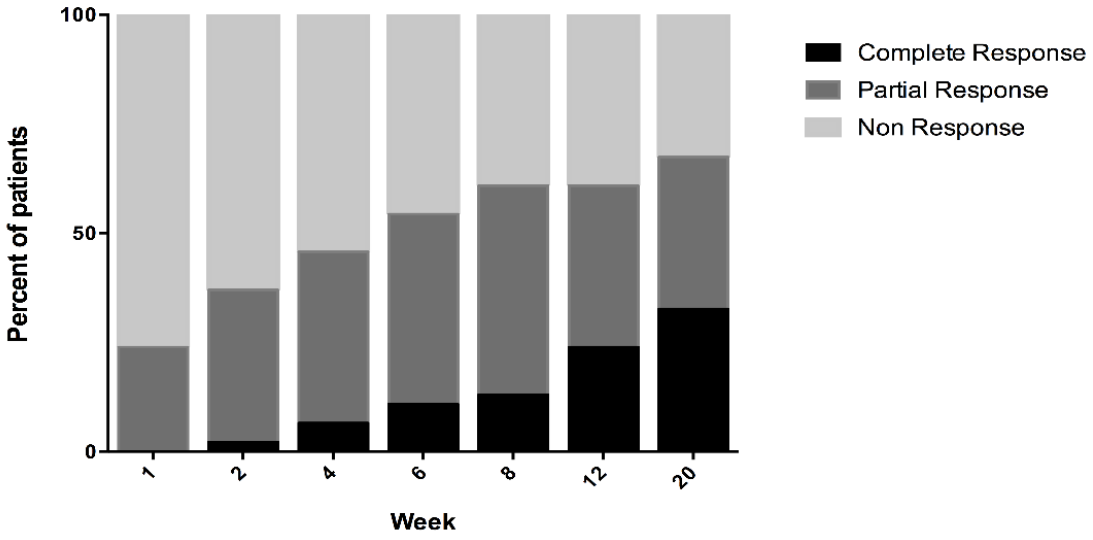


Figure 6.6 Distribution of patients with complete, partial and non-response to treatment with autologous serum during the treatment phase (week 1-8) and the follow-up period (week 12 and week 20). N = 46.

6.3 Changes in ASST status and clinical response after treatment with autohemotherapy

As part of the inclusion criteria, all patients were tested ASST-positive at baseline (week 0). After 8 weeks of treatment with autologous serum, 28 % (14/50, $p \leq 0.0001$ as compared to baseline) of patients were ASST-negative. In week 21, the last week of the follow-up period, the rate of ASST negative patients was 34 % (17/50, $p \leq 0.0001$ as compared to baseline) **(Figure 6.7a)**.

Each response group (CR, PR, NR) contained patients who turned ASST-negative. There was no significant difference comparing complete responders (CRs), partial responders (PRs) and non-responders (NRs) for their rates of patients who became ASST-negative (Chi-square test, $p > 0.05$). 40 % (2/5) and 54.5 % (6/11) of CRs, 22.2 % (4/18) and 30.8 % (4/13) of PRs, and 26.7 % (4/15) and 21.4 % (3/14) of NRs were ASST-negative at week 9 and week 21, respectively (**Figure 6.7b**). There was also no significant difference when comparing patients who became ASST-negative with patients who remained ASST-positive for their rates of CR, PR and NR (**Figure 6.7c**).

In addition, patients who experienced significant quality of life (QoL) improvement (MCID

≥ 4) did not show different rates of turning ASST-negative than those who did not improve in their QoL (MCID < 4). Also, patients who showed a change in their ASST status did not differ in their rates of showing marked QoL improvement as compared to patients who remained ASST-positive.

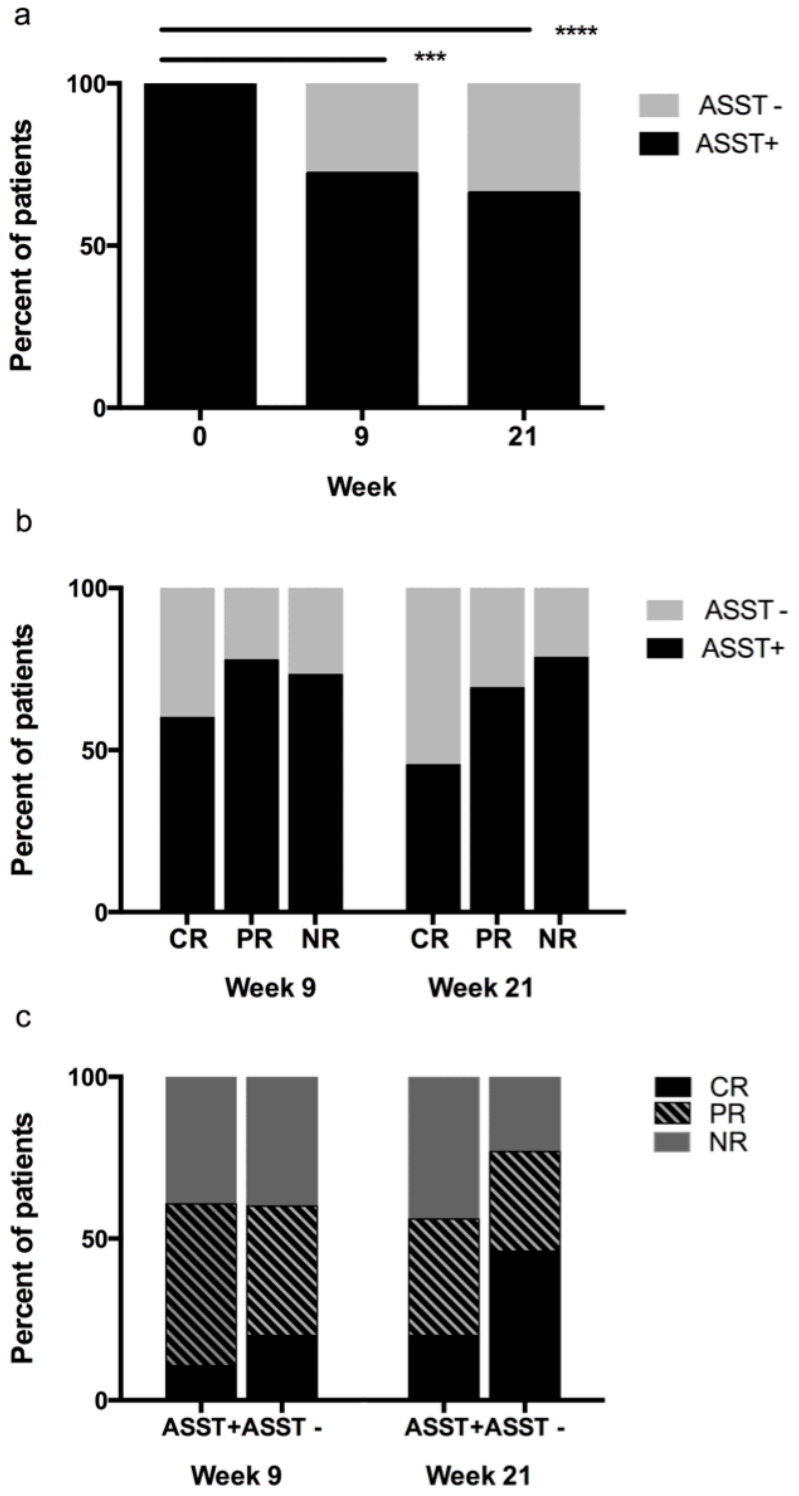


Figure 6.7 Comparison of changes in ASST (a) and proportions of ASST positive and negative patients in complete, partial and non-responders at week 9 and 21 (b and c). At baseline (week 0), all patients were ASST-positive. *** $p \leq 0.001$, **** $p \leq 0.0001$ (McNemar test). ASST +/- = Autologous Serum Test Positive/ Negative. CR = Complete response, PR = Partial response, NR = Non response. N = 50, 38 and 38 in Figure a, b and c respectively.

6.4 Response to treatment and changes in BHRA

Before the start of autohemotherapy, 5 of 41 patients were BHRA-positive, and these 5 patients were similar in their treatment responses as compared to BHRA-negative patients. After 8 weeks of weekly treatment with autologous serum injections, 4 of the 5 BHRA-positive remained BHRA-positive, and the one patient who became BHRA-negative was a non-responder and did not achieve a significant QoL improvement in week 9. There was no significant difference in the rate of patients who were BHRA-positive before and after the therapy (McNemar test, $p>0.05$) (**Figure 6.8**). In addition, no link was observed between the changes in ASST and BHRA (Chi-squared test). There was no difference found between the clinical responses with BHRA status (Mann Whitney U test, $p>0.05$).

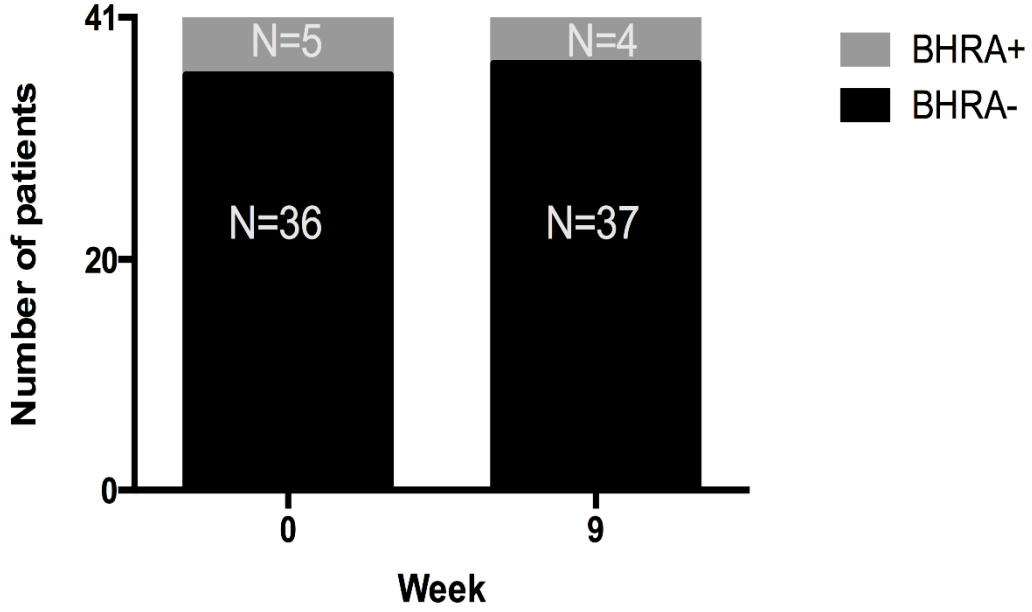


Figure 6.8 The histamine release of basophils by the serum of the patients as assessed at week 0 and week 9. Positive or negative BHRA was defined by a cut-off value of 16.5 %. No significant difference was observed by McNemar test ($p>0.05$). BHRA +/- = BHRA

positive/negative. BHRA = Basophil histamine release assay. N = 41;

6.5 Changes in the concentrations of IgE-anti-IL-24 and IgG-anti-IL-24

6.5.1 Serum concentrations of IgE-anti-IL-24 were reduced by treatment, but IgG-anti-IL-24 were not

Therapy with autologous serum reduced the serum concentrations of IgE-anti-IL-24 by 18% and 22%, from 161,457 ± 14,294 counts (before treatment) to 133,024 ± 17,455 counts (at week 9, $p \leq 0.001$) and 126,355 ± 13,431 counts (at week 21, $p \leq 0.001$), respectively (**Figure 6.9a**). In contrast, serum concentrations of IgG-anti-IL-24 did not change significantly ($p > 0.05$, **Figure 6.9b**).

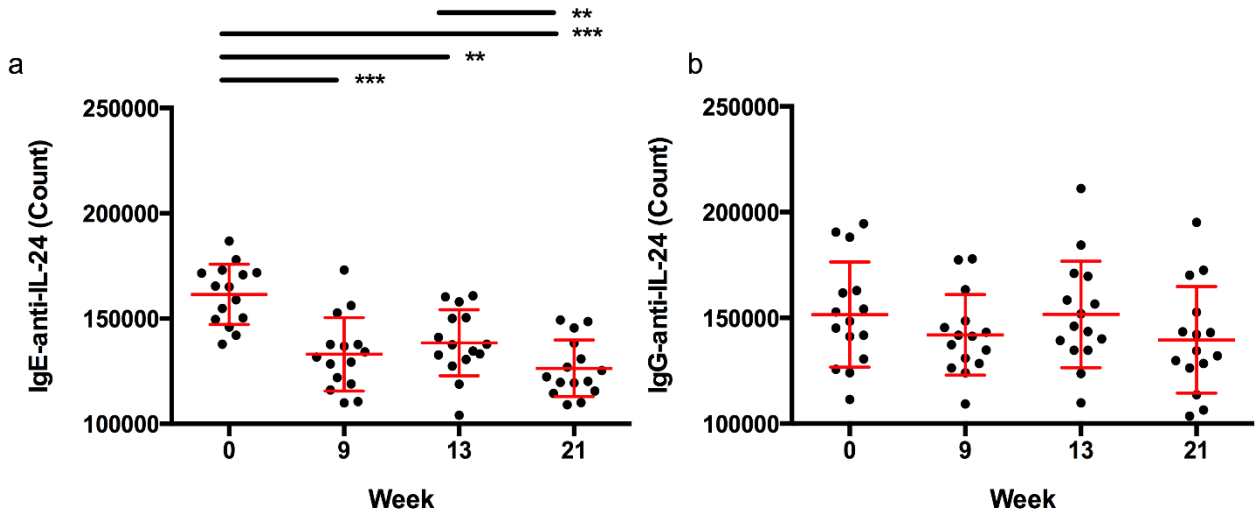


Figure 6.9 Comparison of IgE-anti-IL-24 and IgG-anti-IL-24 concentrations at different time points during treatment with autologous serum. The serum concentrations of IgE-anti-IL-24 and IgG-anti-IL-24 were measured via ELISA. Data is displayed as mean and standard deviation (SD). IL-24 = Interleukin 24. ** P ≤ 0.01, ***P ≤ 0.001(Wilcoxon signed-rank test); N = 15.

6.5.2 Correlations between the concentrations of IgE-anti-IL-24 and IgG-anti-IL-24

As shown above, autohemotherapy reduced the serum concentrations of IgE-anti-IL-24 and IgG-anti-IL-24. **Figure 6.10** indicated the correlation between these two parameters

at each time point (Spearman test, regression line calculated by Pearson test for better visual understanding). The link between the concentrations of IgE and IgG antibody against IL-24 was observed in week 0 (**Figure 6.10 a,** p=0.02). However, there were no correlations found at other time points.

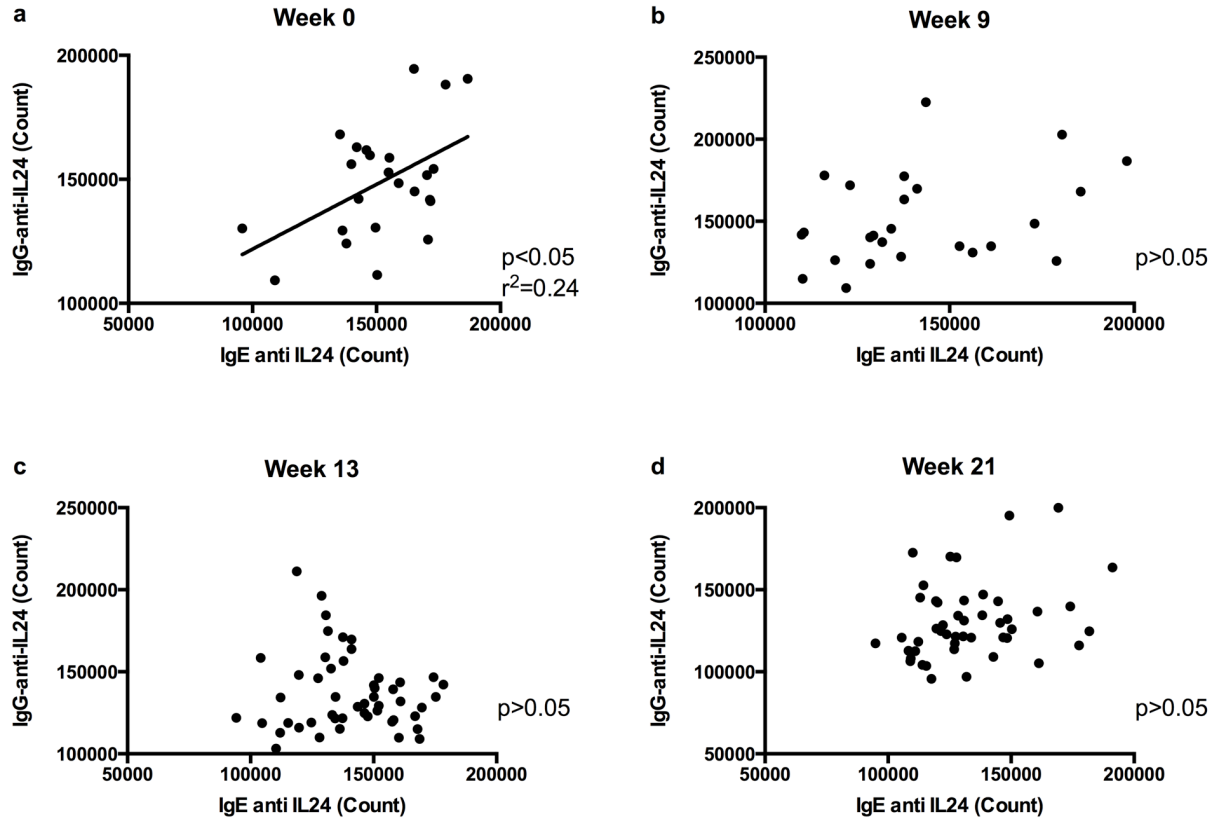


Figure 6.10 Correlations of IgE-anti-IL-24 and IgG-anti-IL-24 concentrations at different time points in treatment with autologous serum. The serum concentrations of IgE-anti-IL-24 and IgG-anti-IL-24 are measured via ELISA. Data are displayed as mean and standard deviation (SD). N = 24, 25, 50 and 47 for a, b, c and d, respectively.

6.5.3 Correlations between UAS7, DLQI and IgE-anti-IL-24 and IgG-anti-IL-24 concentrations

After therapy with autohemotherapy for 8 weeks, the concentrations of IgE-anti-IL-24 in week 21 strongly correlated, with both UAS7 and DLQI nicely reflecting clinical responses. Inverse results were observed in IgG-anti-IL-24, the concentrations of IgG-anti-IL-24 in week 9 showed links to UAS7 and DLQI at the same time point (Spearman test, $p < 0.05$). These results may indicate that the concentrations of IgE-anti-IL-24 can reflect the clinical response assessed by UAS7 and DLQI at the end of the follow-up period, and

concentrations of IgG-anti-IL-24 can indicate the treatment effect after 8-week injections with the autologous serum. **(Figure 6.11 a, b, c and d)**.

After 8 weeks of treatment, there were high initial IgE-anti-IL-24 mean concentrations, positively correlated with the clinical response to autohemotherapy, suggesting that high concentrations of IgE- anti-IL-24 may predict beneficial response to autohemotherapy (Mann Whitney U test, $p < 0.05$)(**Figure 6.11 e**).

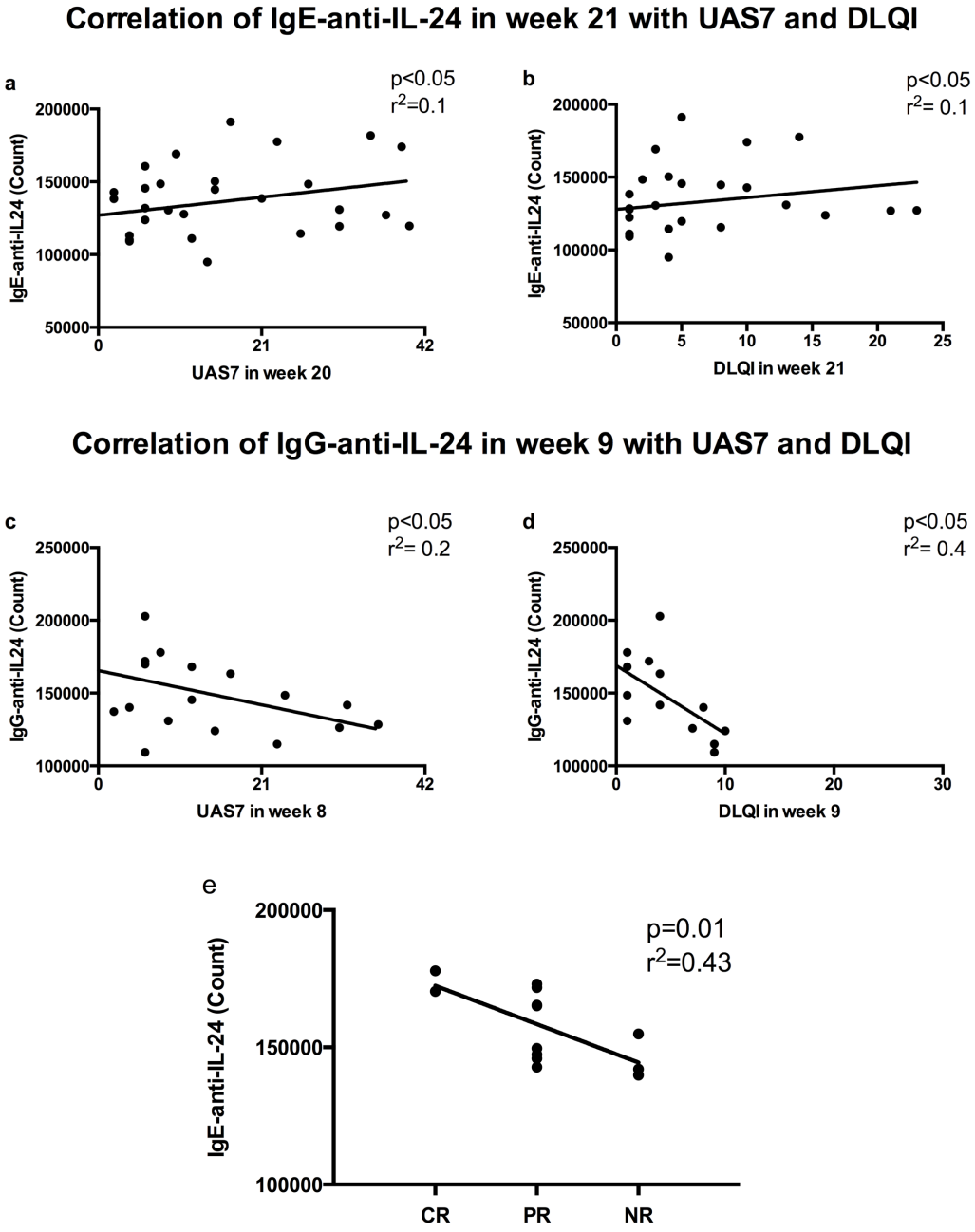


Figure 6.11 Correlation between the mean concentrations of IgE-anti-IL-24/IgG-anti-IL-

24 with UAS7 and DLQI (a, b, c and d). Correlation is determined by Spearman test ($p < 0.05$, regression line calculated by Pearson test for better visual understanding). The correlation of initial mean concentrations of IgE-anti-IL-24 and the response category (e). The initial mean concentrations of IgE-anti-IL-24 are the concentrations before treatment with autologous serum. The serum concentrations of IgE-anti-IL-24 (a, b, e) and IgG-anti-IL-24 (c, d) were measured via ELISA. Data is displayed as mean and standard deviation (SD). N = 39,37,19,15 and 12 in a, b, c, d and e, respectively.

6.5.4 The reduction of IgE-anti-IL-24 linked to the response to treatment

In different response groups, the mean concentrations of IgE-anti-IL-24 and IgG-anti-IL-24 all declined after treatment at week 9 (IgE-anti-IL-24: 158,821 ± 14,840 counts to 133,916 ± 11,586 counts in complete response and partial response; 158,884 ± 12,398 counts to 132,348 ± 24,717 counts in non response. IgG-anti-IL-24: 153,550 ± 24,230 counts to 148,372 ± 24,876 counts in complete response and partial response; 148,404 ± 14,979 counts to 143,418 ± 18,453 counts in non response) **(Figure 6.12**). In complete and partial responders to autohemotherapy, but not in non-responders, the mean concentrations of IgE-anti-IL-24 declined significantly from week 0 to week 9 ($p \leq 0.05$) **(Figure 6.12 a)**.

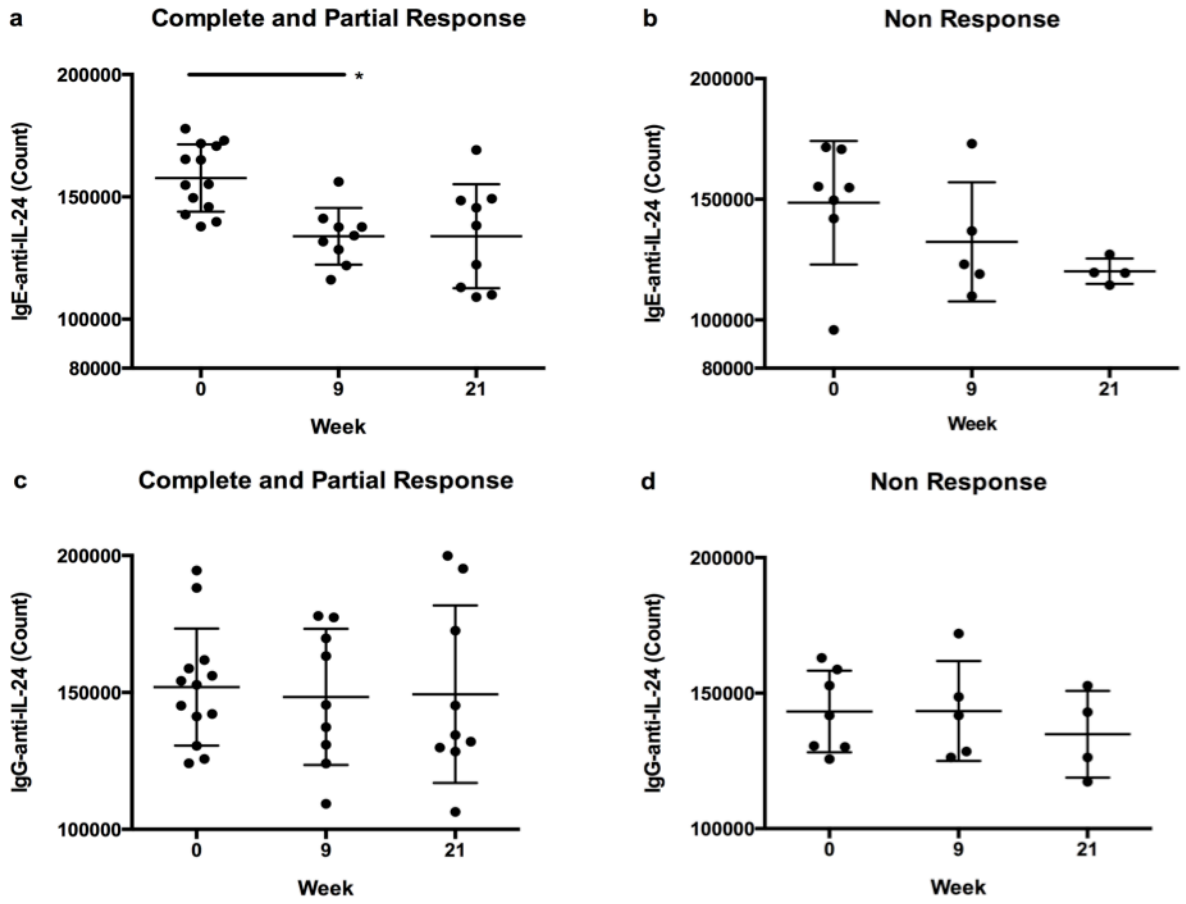


Figure 6.12 Comparison of IgE-anti-IL-24 and IgG-anti-IL-24 serum concentrations in different response groups before and after treatment. Data is displayed as mean and standard deviation (SD). Data shown are from all patients with available data at the specific time points. Statistical comparisons were done only for paired data available at baseline (week 0) and post treatment (week 9 or week 21). * P ≤ 0.05(Wilcoxon signed-rank test)**.** N = 9, 5, 9 and 5 for a, b, c, and d, respectively;

6.5.5 No link between the reduction of IgE-anti-IL-24 and ASST changes

In week 9 and week 21, some patients turned ASST-negative after the treatment and at the end of the follow-up period. There were no differences in serum concentrations of IgE-anti-IL-24 or IgG-anti-IL-24 in patients who became ASST-negative or not at any time point after autohemotherapy (**Figure 6.13**).

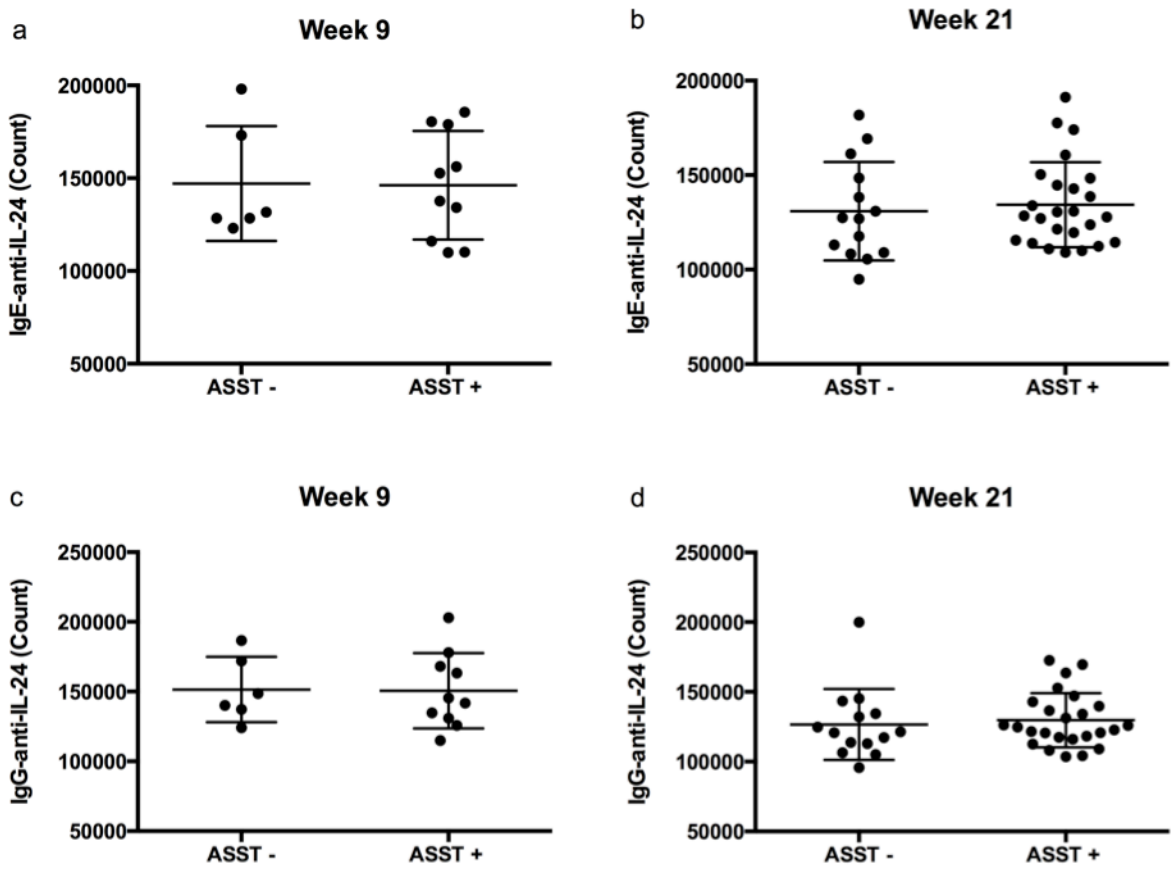


Figure 6.13 Comparison of IgE-anti-IL-24 and IgG-anti-IL-24 serum concentrations patients who become ASST negative with patients who remain ASST positive after treatment. Data are tested by Mann-Whitney U test and displayed as mean and standard deviation (SD). N = 6, 10 for a, c and 14, 25 for b, d, respectively.

7 Discussion

The main aim of this thesis was to explore the immunological effects and the potential mechanisms of action of autohemotherapy in CSU. Our findings confirm that autologous serum therapy has sustained clinical effects on ASST-positive CSU patients and highly suggests that autohemotherapy can induce changes in immunological mechanisms including a reduction of skin reactivity to serum factors and concentrations of autoantibodies. The mean concentrations of IgE-anti-IL-24 show a correlation with both UAS7 and the DLQI at week 21 Furthermore, the initial high concentrations of IgE anti-IL-24 of CSU patients could be possibly utilized to predict a positive treatment response to autohemotherapy.

7.1 Effects of autohemotherapy in ASST- positive CSU patients

CSU is frustrating for both physicians and patients. It not only reduces the patients' quality of life, but also affects their performance at work and school. The quality of life impairment of a CSU patient is comparable to that of a patient suffering from coronary artery disease reported by O' Donnell *et al*. (1997)[98]. CSU is a mast cell-mediated disease, in which mast cells can release histamine and other mediators, such as prostaglandin D2 (PGD2), TNF and cytokines. To date, many efforts have been made to identify the causes of CSU, but the pathomechanisms of CSU are still not fully understood. Two types of autoimmune mechanisms are held to be relevant in the pathogenesis of CSU patients: Type I autoimmunity (also called autoallergy) with IgE autoantibodies to autoantigens and Type II autoimmunity with IgG autoantibodies to IgE or its high affinity receptor, FcεRI[54]. As first described by Rosman *et al*. in 1962[55], CSU could be caused by a hypersensitivity type I reaction in which antigens crosslink IgE molecules on mast cells and basophils resulting in the release of histamine and other pro-inflammatory mediators. There is evidence that autoimmune CSU patients have significantly higher concentrations of IgE autoantibodies against double-stranded DNA and thyroid antigens such as thyroid peroxidase / thyroglobulin than normal subjects[48,56,57].

Autohemotherapy has been reported for over 100 years to treat various diseases including allergic rhinitis, atopic dermatitis, asthma, viral diseases and chronic urticaria[82,94-97]. It was firstly used for several dermatologic conditions by Ravaut (1913)[103] and Spiethoff (1913)[104]. However, after several decades, autohemotherapy was largely abandoned due to a lack of evidence and fear of side effects such as transmitting blood-

borne pathogens. Furthermore, some studies found soreness and/or bruising at the injection site, especially with autologous whole blood[105]. In the past few years, several researchers have started to rebuild the knowledge of autohemotherapy in the treatment of dermatologic diseases including urticaria with systematic and rigorous studies. Moreover, in a recent review by Brewer[90], autohemotherapy was not shown to cause major side effects.

In our study, 66 ASST-positive CSU patients were recruited to receive autohemotherapy. The first UAS7 score was measured before the treatment. In a recent review by Brewer (2014)[90] no association between the baseline symptom severity and the response to autohemotherapy was shown in the comparison of different studies. In our study, autohemotherapy reduced disease activity by 36 % after 8 weekly injections, led to sustained benefit at week 20 (46% reduction), and improved disease activity as early as after the second injection of autologous serum (**see Figure 6.1**). Also, the on-demand antihistamine consumption was significantly decreased by more than 50% as compared to baseline (**see Figure 6.2**). The results are in line with studies of Majid *et al*. (2015)[106] that showed a dramatic decline during the first few injections. Moreover, the scores were constantly decreased at week 20 ($p \leq 0.0001$), which indicates that autologous serum therapy can induce rapid and stable effects in CSU patients. Nearly two-thirds of all patients showed complete and partial response at the end of the follow-up period (see **Figure 6.6**). In this regard, our findings are completely in agreement with previous studies by Chen *et al*. (2012)[107], Godse *et al*. (2017)[80] and Abdallah *et al*. (2012)[105]. Abdallah *et al*. (2012)[105] performed a study to compare the efficacy of autologous whole blood injections and autologous serum injections among ASST-positive patients and found both modalities were equally effective. They observed that autohemotherapy reduced the pill burden and improved the quality of life. Chen *et al*. (2012)[107] found a 50 % reduction of UAS7 from baseline to the end of follow-up after autohemotherapy. Godse *et al*. (2017)[80] compared the effects of subcutaneous injections of autologous serum and saline control in treatment of CSU and suggested that subcutaneous autologous serum therapy significantly reduced the disease activity assessed by UAS7 and the requirement of antihistamines. More recently, Staubach *et al*. (2006)[82] and Elazab *et al*. (2017)[83] revealed that autohemotherapy could be very effective in autoimmune CSU patients, because ASST-positive showed a better clinical response than the ASST-negative control group in both studies.

CSU is known to have a substantial impact on patients' quality of life. In our study, the

quality of life of our patients was improved after the last autologous serum injection and further improved thereafter (see **Figure 6.3**). One in three patients and almost 50% of patients experienced significant quality of life improvement, i.e. a reduction of the DLQI by the minimal clinically important difference of 4 points, by the end of the treatment and follow up phase, respectively (see **Figure 6.4**). These results confirm the findings of an earlier study by Panchami *et al*. (2014)[108].

Comparing the duration of the treatment and follow-up period with the previous 7 studies[51,81,82,99,105,107,109], we chose the treatment length of 8 weeks (average treatment length was 8.7 weeks (from 5 weeks to 12 weeks)) and follow-up for 12 weeks (average follow-up period was 7.4 weeks (from 4 weeks to 16 weeks)). The frequencies of injections were all reported to be once per week.

In CSU, both UAS7 and DLQI are different measurements and critical factors to analyse the effects and impairment of the disease for patients. The improvement of disease activity and DLQI strongly correlated at each corresponding time point in our study. The good correlation between UAS7 and DLQI observed suggested robustness in our data. The combination of these two parameters enables better insight into the impact of the disease on social and everyday activities. Both of them are valuable instruments in measuring response to treatment (see **Figure 6.5**).

A minimal clinically important difference (MCID) of 4 points has been estimated for DLQI in CSU patients to be important for treatment decisions. The changes in MCID ≥ 4 mean that the patient experiences a meaningful change in QoL. We found that the proportion of patients that reached a MCID of ≥ 4 at week 9 and week 21 was 35 % and 47 %, respectively (see **Figure 6.4**). These results are also in line with the studies of Panchami *et al*. (2014)[108], who reported that the autologous serum therapy significantly improved UAS7 and DLQI from baseline. In contrast, a placebo controlled, randomized, single-blind study with CSU patients carried out by Kocatürk *et al*. (2012)[51] underlined that autologous whole blood injections and autologous serum injections are equally effective as placebo injections in reducing disease activity. To analyse the reason for symptom improvements in the placebo patients, they may be due to physiological effects caused by antihistamine on demand and psychological impacts[90]. For example, saline injections may also cause an immune response. This hypothesis is consistent with the observation from clinical trials with omalizumab that showed placebo injections to induce higher improvement than oral placebos in other trials[77,106-108]. Another reason for the improvement with placebo according to Brewer might also be associated with the inclusion of patients who were

presumably experiencing peak symptom severity that spontaneously declined during the study[90].

Taken together, our results and those of previous studies underline that autohemotherapy can have rapid, positive and persistent effects on disease activity and impact in patients with CSU, but they do not provide an explanation for why this happens.

7.2 Changes in ASST status and clinical response after treatment with autohemotherapy

30-50 % of CSU patients express autoantibodies that can induce degranulation of mast cells and basophils including circulating autoantibodies directed against the high-affinity receptor for IgE (IgG-anti-FcεRI) or the IgE molecule itself (anti-IgE)[113,114]. The presence of these histamine releasing factors (HRFs) can be confirmed by both *in vivo* autologous serum skin test (ASST) and *in vitro* basophil histamine release assay (BHRA) [51]. After comparing the results of the ASST and BHRA, Sabroe *et al.* (2006) found that 60 % of the subjects showed ASST-positive, however, only half of ASST-positive serum activated basophils in BHRA. This finding demonstrates that the serum factors in ASST (mast cell specific) are different from those in BHRA (anti-IgE or anti-FcεRI)[115]. Meanwhile, the ASST has been proven to be one of the most useful, rapid and reliable clinical tests for differentiating between patients with and without circulating functional autoantibodies[116,117]. However, the methodology and the definition of a positive response are under debate and it is still unknown whether the injections of autologous serum can change the immunologic profiles of CSU patients.

Some studies showed the skin testing with autologous serum could elicit a wheal and flare response in most CSU patients during disease activity but not in the clinical remission period[49] and suggested the test should be regarded as a test for autoreactivity rather than specific test for autoimmune urticaria[118]. Magen *et al*. (2012)[119] found that the standard dose of antihistamines could not inhibit the wheal size induced by the intradermal injection of autologous serum in resistant CSU patients. Histology of ASST biopsies from CSU patients showed mixed infiltrates of neutrophils, mononuclear cells and eosinophils and the electron microscopy showed mast cell degranulation[120]. However, it is still unknown whether the injections of autologous serum can change the autoreactive profile of CSU patients.

Recently, the impact of positive or negative states of ASST on disease activity or

treatment decisions in CSU patients was discussed as being a controversial issue. Both Staubach *et al*. (2006)[82] and Elazab *et al*. (2017)[83] revealed that the ASST-positive patients show better effects to autohemotherapy than the ASST-negative autoimmune CSU patients. They proposed a mechanism where circulating histamine-releasing factors might induce tolerance. Since we primarily aimed at the mechanisms of action of autohemotherapy in CSU, we did not implement a placebo control and concentrated on the ASST status and the analysis of parameters in the serum of the patients within the treatment period. In Brewer's systematic review of autohemotherapy, he summarized that ASST-positive patients had 19 % less severe urticaria symptoms at the end of follow-up than ASST-negative patients in relevant studies[90].

In our study, the ASST became negative in only one third of the treated patients at the end of the follow-up phase, and this was not linked to the clinical response of patients. In other words, some patients (4/10 at week 9, 3/13 at week 21) without clinical benefit became ASST-negative, and some patients (17/28 at week 9, 14/25 at week 21) who had clinical benefit remained ASST-positive (see **Figure 6.7**). The latter has been described previously by Mori and Hashimoto[121], who reported CSU remission in a ASST-positive patient treated with autologous whole blood injections, without changes in the patient's ASST status. Panchami *et al*. (2014)[81] evaluated and compared the effectiveness of autologous serum therapy in autoreactive urticaria and non-autoreactive urticaria patients. He concluded that autologous serum therapy showed promising effects in both autoreactive and non-autoreactive CSU patients, and the effects are shown regardless of the presence of autoreactivity. You *et al*. (2015)[122] also demonstrated that antihistamine-resistant CSU patients did not respond significantly to autohemotherapy in spite of the skin test results to autologous serum. The above findings suggest that the status of ASST may not reflect the disease activity and the response to autologous hemotherapy in CSU patients.

7.3 Response to treatment and changes in BHRA

Basophil histamine release assay is a measurement of histamine release from healthy donors' basophils co-incubated with patients' serum[123]. It is used to detect functional autoantibodies, which have been shown to have good sensitivity and specificity for the presence of endogenous histamine-releasing factors[124]. Only about 50 % of ASST-positive CSU patients seem to have functional autoantibodies in the basophil assay. Bindslev-Jensen *et al*. (2000)[123] recommended that if the examined patients have positive

ASST results, the presence of HRFs should be confirmed by basophil histamine release. In accordance with those studies, we tested the serum autoreactivity by BHRA. In our study, we did not observe significant changes in BHRA response before and after autohemotherapy (see **Figure 6.8**). Moreover, changes in BHRA and ASST reactivity of the patients were not linked to the clinical response assessed by UAS7. Although the presence of HRFs was suggested to be confirmed by both ASST and BHRA, there was no correlation between these two methods in our study. In the study of Platzer *et al*. (2005), they explained the missing correlation between ASST and BHRA by the assumption that a low titre of HRFs or 'mast cell-specific' HRFs might be present in the serum of CSU patients who have a positive ASST but negative BHRA results. On the contrary, the results shown with negative ASST and a positive BHRA might be explained by some inherent variability of the ASST[124]. In our study, only one patient became BHRA negative and this patient was a non-responder. We did not have access to direct assays for measuring the concentrations of IgG autoantibodies and their changes in our patients. But even if we had measured antibody concentrations and had found a reduction in response to autohemotherapy, this would hardly be relevant pathomechanistically, as functional tests such as the ASST and the BHRA remained positive in the majority of patients and changes in the results of these functional tests were not linked to treatment responses.

7.4 Changes in the concentrations of IgE autoantibodies against IL-24 after autohemotherapy

Bajaj *et al*. (2008) and Schmetzer *et al*. (2018) suggested that CSU is an autoimmune disease in which IgE recognizes several autoantigens. IgE against many autoantigens was frequently found to be exhibited in CSU patients, while only eight of the autoantigens against IgE expressed symptoms in the skin, and only IgE autoantibodies against interleukin 24 (IL-24) were detectable in all CSU patients. IL-24 has been identified as a common, specific and functional autoantigen of IgE autoantibodies in CSU. Also, IgE-anti-IL-24 could not be detected in patients with idiopathic anaphylaxis or healthy subjects, rather only in patients with CSU. Here, the concentrations of IgE-anti-IL-24 were associated with the disease activity[68]. Based on this data, we especially analysed changes of IgE-anti-IL-24, as it seems to be a good candidate to observe concentrations and changes of IgE autoantibodies over time in CSU patients.

In this work, we measured the serum concentrations of IgE-anti-IL-24 by ELISA. Our results showed a significant reduction in the mean concentrations of IgE-anti-IL-24 after 8-week injections with autologous serum and the concentrations continually decreased till the end of follow-up period (see **Figure 6.9a**). This indicates that autohemotherapy has a significant effect on changing the concentrations of these autoantibodies. Based on these results, we further used the same ELISA protocol to measure the IgG antibody against IL-24 (see **Figure 6.9b**). Unlike the decrease in IgE-anti-IL-24 serum concentrations, the IgG-anti-IL-24 only displayed fluctuant changes and there were no remarkable changes observed at different time points.

In the results of IgE-anti-IL-24 serum concentrations, we also detected a correlation between concentrations of IgE-anti-IL-24 and clinical response as assessed by UAS7 and DLQI at the end of the follow-up period (see **Figure 6.11**). This is in agreement with results from the study of Schmetzer *et al*. (2018)[68]. The correlation between the clinical response at week 8 and the initial concentrations of IgE-anti-IL-24 in the patient group of Berlin (see **Figure 6.11e**) indicates that high initial concentrations of IgE-anti-IL-24 before treatment might predict a treatment response to autohemotherapy in CSU, which emphasizes the initial concentrations of IgE-anti-IL-24 as a potential prognostic marker. From the above findings, we considered that these different parameters may be used not only to predict the clinical response to autohemotherapy, but also to evaluate the therapeutic effects.

7.5 Proposed mechanisms of autohemotherapy

How does autohemotherapy work in CSU? Our hypothesis was that autohemotherapy, in patients who benefit from this treatment, reduces the concentrations and/or effects of these mast cell-degranulating IgE and IgG autoantibodies. Our results strongly suggest that autohemotherapy reduces the concentrations of IgE autoantibodies, but not the concentrations or effects of IgG autoantibodies.

The mechanism of action of autohemotherapy has not yet been well understood and only a few studies and hypotheses about potential mechanisms of autohemotherapy have been published so far. Some authors suggested that autohemotherapy could prompt the tolerization of patients to autoantigens in the blood circulation. For example, Alvarado-Flores *et al*. (2001)[125] indicated that there were tolerance-generating anti-idiotype antibodies to mast cell degranulation presented in autologous serum as assessed in

various autoimmune diseases.

Other researchers measured the changes in cytokines after injections of autologous serum, e.g., Liang *et al*. (2012)[126]. They treated 24 allergic rhinitis patients with acupuncture autohemotherapy while 21 patients were treated with western medications for 3 months for comparison. They measured their serum levels of interferon-gamma (IFN-gamma) and interleukin 12 (IL-12). The results showed that the therapy significantly relieved the clinical symptoms and the symptom relief correlated with the increase of IL-12 and IFN-gamma. Zhang *et al*. (2014)[88] demonstrated that the mechanism of action of autohemotherapy is related to the downregulation of IL-4 and total IgE in CSU patients.

Our results do suggest that autohemotherapy works in CSU, at least in part, because of its effects on IgE autoantibodies. First, we found that our patients treated with autohemotherapy showed a significant reduction of their IgE-anti-IL-24 concentrations after 8 weekly injections with autologous serum, and their concentrations continued to drop thereafter. Second, the drop in IgE-anti-IL-24 was only significant in responders, but not in non-responders to autohemotherapy although statistical comparisons in the analysis are based on small patient numbers in each response group. Taken together, these findings support the idea that IgE-autoantibodies such as IgE-anti-IL-24 are one of the targets of the mechanisms of action of autohemotherapy in CSU.

7.6 Limitations

Our study has several important strengths and limitations, and it points to interesting questions that need to be addressed by further studies. The limitations of our study include its uncontrolled design and relatively small sample size. Besides, the incomplete data sets with many missing values for certain time points and analyses may reduce the study quality and reproducibility, and may cause a risk of bias. In retrospect, we should not have limited inclusion into the study to ASST-positive patients. ASST positivity of our patient population, has undoubtedly biased our study group towards type IIb autoimmune CSU and against type I autoimmune CSU, in which autohemotherapy now has to be expected to be more effective than in type IIb autoimmune CSU. On the plus side, our patient population was fairly homogeneous, it was a multicenter study, and our key outcomes included major pathogenic drivers of type I and type IIb autoimmune CSU. Further studies are needed to better characterize the relevance and mechanisms of these effects of autohemotherapy. How is IgE-anti-IL-24 reduced by autohemotherapy: by

effects on its production, its clearance, or both? Are other IgE autoantibodies and concentrations of total IgE also reduced by autohemotherapy in CSU patients, ASST-positive and ASST-negative patients? Are total IgE concentrations affected by autohemotherapy?

7.7 Conclusion and outlook

In summary, our study demonstrates that autohemotherapy can lead to clinical benefit in part by its effects on concentrations of IgE autoantibodies against IL-24 in ASST-positive CSU patients. This supports the notion that specific and functional IgE autoantibodies contribute to the pathogenesis of CSU and suggests that these IgE autoantibodies represent a potential therapeutic target of autohemotherapy.

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Appendix

Table A.1 Patient diary

Week	Day	Number of Wheals				Intensity of Itch				Overall rating				Cetirizine				Triggers
		None	<20	20–50	>50	None	Mild	Moderate	Severe	No complaints	Mild complaints	Moderate complaints	Severe complaints	Morning	Noon	Afternoon	At night	e.g. Stress-Exercise -Food
1	1																	
	2																	
	3																	
	4																	
	5																	
	6																	
	7																	

Table A.2 Dermatology life quality index

DLQI

Centre Nr.: |__|__|__| Date: |__|__|__|

Score ☐

Patient Nr.: |__|__|__| T / M / J

Patients Initials |__|__|__| Diagnose : _____

The aim of this questionnaire is to measure how much your skin problem has affected your life OVER THE LAST WEEK. Please tick one box for each question.

1.	Over the last week, how **itchy**, **sore**, **painful** or **stinging** has your skin been?	Very much A lot A little Not at all	☐ ☐ ☐ ☐	
2.	Over the last week, how **embarrassed** or **self-conscious** have you been because of your skin?	Very much A lot A little Not at all	☐ ☐ ☐ ☐	
3.	Over the last week, how much has your skin interfered with you going **shopping** or looking after your **home** or **garden**?	Very much A lot A little Not at all	☐ ☐ ☐ ☐	 Not relevent ☐
4.	Over the last week, how much has your skin influenced the **clothes** you wear?	Very much A lot A little Not at all	☐ ☐ ☐ ☐	 Not relevent ☐
5.	Over the last week, how much has your skin affected any **social** or **leisure** activities?	Very much A lot A little Not at all	☐ ☐ ☐ ☐	 Not relevent ☐
6.	Over the last week, how much has your skin made it difficult for you to do any **sport**?	Very much A lot A little Not at all	☐ ☐ ☐ ☐	 Not relevent ☐

7.	Over the last week, has your skin prevented you from **working** or **studying**?	Yes No	□ □	 Not relevent □
	If "No", over the last week how much has your skin been a problem at **work** or **studying**?	A lot A little Not at all	□ □ □	
8.	Over the last week, how much has your skin created problems with your **partner** or any of your **close friends** or **relatives**?	Very much A lot A little Not at all	□ □ □ □	 Not relevent □
9.	Over the last week, how much has your skin caused any **sexual difficulties**?	Very much A lot A little Not at all	□ □ □ □	 Not relevent □
10.	Over the last 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 A lot A little Not at all	□ □ □ □	 Not relevent □

Please check you have answered EVERY question. Thank you.

Statutory Declaration

“I, [Linan Yu], by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [Immunologische Effekte und potentielle Pathomechanismen der autologen Serumtherapie bei chronischer spontaner Urtikaria / Immunological effects and potential mechanisms of action of autologous serum therapy in chronic spontaneous urticaria], independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the first supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

The data from this monograph were published in a peer-reviewed journal:

Linan Yu contributed the following to the below listed publications:
Publication 1: [Yu, L., Buttgereit, T., Stahl Skov, P., Schmetzer, O., Scheffel, J., Kocatürk, E., Zawar, V., Magerl, M. and Maurer, M.], [Immunological effects and potential mechanisms of action of autologous serum therapy in chronic spontaneous urticaria.], [J Eur Acad Dermatol Venereol], [2019].

Contributions:
Yu L conceived the presented study together with Buttgereit T, Magerl M, and Maurer M. Also, Yu L collected the clinical data, designed and carried out the measurements of IgE-anti-IL-24 and IgG-anti-IL-24. Yu L performed the statistical analysis and the analysis of the results, wrote the manuscript with support from Buttgereit T and Maurer M.

_____

Signature, date and stamp of first supervising university professor / lecturer

_____

Signature of doctoral candidate

Curriculum vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Certificate from an accredited statistician

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Bescheinigung

Hiermit bescheinige ich, dass Frau Linan Yu innerhalb der Service Unit Biometrie des Instituts für Biometrie und Klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- 12.10.2018: Schriftliche Beratung auf Basis des zugesandten Manuskripts mit Methoden und Ergebnissen/Grafiken

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Grafische Darstellung von Follow-up-Zeitpunkten für direkten Vergleich
- Korrektur der Auswertungsstrategie zu einigen Fragestellungen, da Schlussfolgerungen aus inadäquaten Vergleichen gezogen wurden
- Nennung der Korrelationskoeffizienten anstelle der p-Werte bei Regressionsanalysen
- Überprüfung der Grafiken auf inadäquate Darstellung von Mittelwert und SEM/SD

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und Klinische Epidemiologie übernimmt hierfür keine Haftung.

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