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Identification of biomarkers in the treatment of chronic spontaneous urticaria

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Zusammenfassung

Die chronisch spontane Urticaria (CSU) ist eine entzündliche Hauterkrankung mit einer Prävalenz von ca. 1%, die durch das rezidivierende Auftreten von Quaddeln und/oder Angioödemen ohne eruierbaren Trigger über den Zeitraum von 6 Wochen gekennzeichnet ist. Das spontane Auftreten von Symptomen begleitend von einem quälenden Juckreiz führt zu einer hohen Einschränkung der Lebensqualität. Aktuell ist die Pathogenese der CSU nicht vollständig geklärt. Die Aktivierung von Mastzellen, die, z.B. über das IgE *Cross-linking*, Histamin freisetzt, führt zu einer Vasodilatation und in der Folge zur Bildung von Quaddeln und Angioödemen. Jedoch wurde über die letzten Jahre die Bedeutung anderer Formen der Mastzellaktivierung und die Beteiligung anderer Zellen (wie T-Zellen, basophile und eosinophile Granulozyten) sowie der Komplement- und Koagulationskaskaden deutlich. Heutzutage ist die *in-label* Therapie begrenzt auf Antihistaminika und den anti-IgE-Antikörper Omalizumab. Diese Studie zielt auf die Erforschung des Pathomechanismus der CSU über die Mastzell- und IgE-vermittelte Histaminausschüttung hinaus. Wir untersuchten eine Patientenkohorte im ersten Monat der Omalizumab-Behandlung und fokussierten uns zum einen auf die Änderung der IgE-Spiegel und zum anderen auf die Änderung von Gen- und miRNA-Expressionen im peripheren Blut. Die Kohorte wurde basierend auf dem Ergebnis des Urtikaria Kontrolltests in *Responder* und *Non-Responder* unterteilt und mit gesunden Probanden verglichen. Unsere Ergebnisse zeigten sich übereinstimmig zu bisherig publizierten Studien, die IgE-Spiegel als einen potentiellen Biomarker postulieren. Jedoch konnten wir keine spezifischen Änderungen bezogen auf das Therapieansprechen feststellen, was die Notwendigkeit weiterer Biomarker verdeutlicht. In unseren mRNA und miRNA Analysen konnten wir die größten Änderungen auf Expressionsebene an Tag 2 nach Omalizumab-Einleitung sehen. Dies legt eine Änderung auf Transkriptionsebene in den ersten Tagen nach Therapieinitiierung nahe und korreliert mit der Symptombesserung einiger Patienten bereits zu Beginn der Therapie. Wir selektierten einzelne Gene für unsere Validierungsstudie mittels qPCR-Analyse. Hierbei identifizierten wir Kandidatengene, von denen die meisten bisher nicht in Verbindung mit CSU gebracht wurden und die z.B. auf eine Beteiligung von T-Zellen oder auch Thrombozyten hinwiesen. Die größte Limitation der Studie ist die kleine Kohortengröße. Jedoch wurde das vorliegende *Setting*, unseres Wissens, bisher nicht zur Untersuchung von mRNA und miRNA in der CSU angewendet und könnte so nützliche Erkenntnisse zur CSU-Pathogenese beitragen. Weitere Studien in größeren Kohorten sind nötig, um unsere Daten zu validieren.

Summary

Chronic spontaneous urticaria (CSU) is a debilitating inflammatory skin disease with a prevalence of approximately 1% of the population characterised by recurrent itchy wheals and/or angioedema for more than 6 weeks with no known trigger. The spontaneous development of the symptoms and the agonising itch lead to a high quality of life impairment. So far, the pathogenesis of CSU remains not fully understood. The mast cell is the key effector cell which releases histamine upon activation, for example via IgE cross-linking, leading to vasodilation and subsequently to the development of wheals and angioedema. The mast cell is the key effector cell which releases histamine upon activation, for example via IgE cross-linking, leading to vasodilation and subsequently to the development of wheals and angioedema. However, over the past years, the importance of other forms of mast cell activation and the involvement of other cells (such as T cells, basophils and eosinophils) as well as of the complement and coagulation cascades has become clear. Today, in-label treatment options are limited to antihistamines and the anti-IgE-antibody omalizumab. This study aimed to explore the pathomechanism of CSU beyond mast cells and IgE-dependent histamine release and to identify possible biomarkers for the disease and its treatment. We investigated a patient cohort in its first month of omalizumab treatment by, on one hand, looking at the change of IgE levels and, on the other hand, exploring changes of gene and miRNA expression in peripheral blood. The cohort was divided into responders and non-responders (depending on the score of the Urticaria Control Test) and was compared to a group of healthy controls. Our results proved to be coherent with previously published studies showing that IgE is a potential biomarker in CSU treatment. However, we could not observe specific behaviour regarding responders and non-responders emphasising the fact that further biomarkers are needed. In our mRNA and miRNA microarray analysis we observed the greatest changes of expression levels at day 2 after the first omalizumab dose. This proposes changes on transcription levels leading to symptom amelioration as soon as the first days of treatment initiation in some patients. We chose several candidate genes and miRNAs that were analysed in our validation study via qPCR analysis. Thus, we identified several genes and miRNAs of interest, most of which have not been described to be linked to CSU so far, underlining, for example, T cell involvement or even suggesting platelet involvement. The biggest limitation of this study is the small sample size. However, to our knowledge, our study setting has not been used so far to investigate mRNA and miRNA in CSU and with such may provide new valuable input in CSU pathogenesis. Further research in larger cohorts is needed to further validate our results.

Abbreviations

AE	Angioedema
BTK	Bruton’s tyrosine kinase
cDNA	Complementary DNA
CIndU	Chronic inducible urticaria
CR	Complete Responder
CRH-R1	Corticotrophin-releasing hormone receptor 1
CS	Corticosteroid(s)
CSU	Chronic spontaneous urticaria
CU	Chronic urticaria
CU-Q2oL	Chronic Urticaria Quality of Life Questionnaire
C5a	Complement component 5a
DEG	Differentially expressed gene
DNase	Desoxyribonuclease
DLQI	Dermatology Quality of Life Index
EPO	Eosinophil peroxidase
g	Gravitational force
h	Hour(s)
H4R	Histamine 4 receptor
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL	Interleukin
JAK	Janus kinase(s)
KIT	KIT proto-oncogene receptor tyrosine kinase
lncRNA	Long non-coding RNA
LTC4	Leukotriene C4
MC	Mast cell
MBP	Major basic protein
MRGPRX2	Mas-related G protein-coupled receptor X2
min	Minute(s)
miRNA	Micro RNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NR	Non-Responder
NSAID	Non-steroidal anti-inflammatory drug
PAF	Platelet-activating factor
PAR2	Proteinase-activated receptor-2

PR	Partial responder
PROM	Patient-reported outcome measure
qPCR	Quantitative polymerase chain reaction
R	Receptor
RNA	Ribonucleic acid
SCF	Stem cell factor
Siglec 8	Sialic acid-binding immunoglobulin-like lectin 8
STAT	Signal transducer and activator of transcription
ST2	Serum stimulation-2
TGF	Transforming growth factor
T_H	T helper cell
TNF-α	Tumor necrosis factor - α
TPO	Thyroid peroxidase
T_{reg}	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
U	Unit(s)
UCT	Urticaria Control Test
VEGF	Vascular endothelial growth factor

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1 Introduction

Urticaria is a common inflammatory skin disease characterised by the appearance of wheals or angioedema or both (1). 20% of the population can develop symptoms of urticaria at some point during their life, however the symptoms rarely persist over the period of 6 weeks and thus become chronic. **Chronic spontaneous urticaria (CSU)** is a subtype of urticaria, which has a worldwide prevalence of approximately 1% (2). Even though people have been affected by this disease from ancient times, the aetiology still remains not fully understood (1). Patients with CSU suffer from severe quality of life impairment and the burden of disease is considerable not only for the patients but also for their families and for the respective economy (3-5). Thus, a treatment to achieve symptom control is crucial. In order to achieve this goal, a higher number of or more targeted therapeutical options are needed and therefore a better understanding of the pathomechanism of the disease and the identification of possible biomarkers.

1.1 Definition

CSU is characterised by recurrent itchy wheals or angioedema or both over the time period of 6 weeks with no specific triggers (Fig. 1) (1). Concomitant symptoms like nausea or emesis are less common (3).

A. **B.**

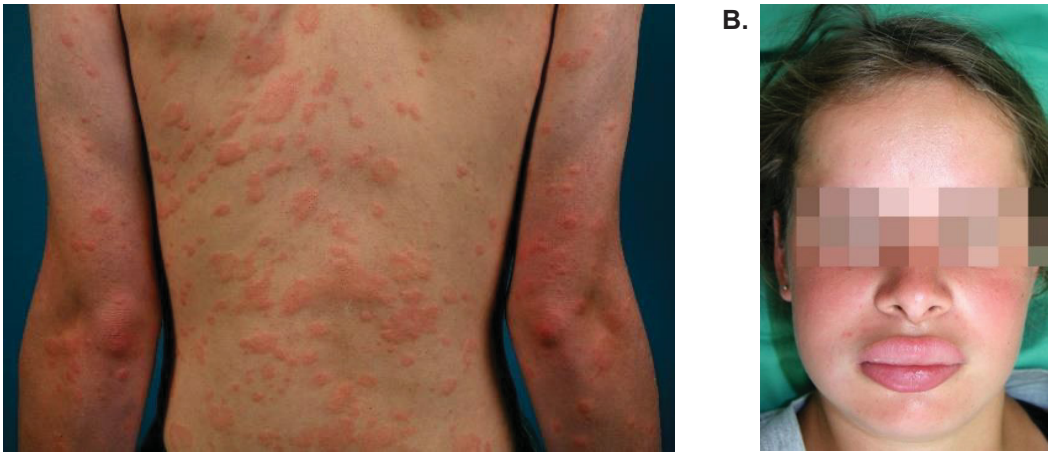

Fig. 1: **Clinical presentation of patients with urticaria.**

Representative images of typical urticaria symptoms: hives (A) and angioedema (B). Patients suffer from severe itching caused by these.

Wheals describe itchy swellings of the upper dermis with surrounding reflex erythema which can remain at one given location for < 24 h. Angioedema is a swelling of the deeper skin layers (i.e. lower dermis/ subcutis) or mucous membranes which causes an itching, burning or even painful sensation for the patients suffering from it. It usually takes under

72 h to resolve. Importantly, the skin (or mucous membrane respectively) returns to its normal appearance afterwards (no scarring) (1).

1.2 Differential diagnoses

CSU is a diagnosis of exclusion, which can be made by physical examination and taking a detailed medical history regarding the occurrence of the symptoms.

The duration of the recurrence of wheals and/or angioedema makes it possible to differentiate between an acute or chronic urticaria. Urticaria recurring during a time of up to 6 weeks is called acute urticaria, whereas the recurrence of the symptoms for longer than 6 weeks classifies the urticaria as chronic. Chronic urticaria (CU) consists of two subtypes (Table 1): chronic inducible (CIndU) and chronic spontaneous (CSU) (1).

Chronic inducibale urticaria (CIndU)	**Chronic spontaneous urticaria (CSU)**
Aquagenic urticaria	For > 6 weeks recurring wheals and/or angioedema with no attributable trigger due to known or unknown causes
Cholinergic urticaria	
Cold urticaria	
Contact urticaria	
Delayed pressure urticaria	
Heat urticaria	
Solar urticaria	
Symptomatic dermographism	
Vibratory angioedema	

Table 1: **Subtypes of chronic urticaria**

Chronic urticaria (CU) characterised by recurrent wheals and/or angioedema > 6 weeks can be further subdivided into chronic inducible (CIndU) and chronic spontaneous urticaria (CSU). CIndU can be provoked by a specific stimulus listed above. If there is no identifiable trigger, the CU is classified as CSU. It is postulated that CSU can have known and unknown causes referring to a possible autoimmune aetiology of CSU. Modified from: *EAACI/GA²LEN/EDF/WAO* urticaria guideline, Zuberbier et. al, Allergy 2022 (1)

In contrast to CSU, CIndU has an attributable trigger as, for example, heat, water, pressure, exercise or cold. If such a stimulus cannot be identified, i.e. one cannot provoke the symptoms and they appear “out of nowhere”, the diagnosis of CSU can be made. However, ever since the 2014 EAACI guidelines for urticaria it is said that CSU might have known and unknown causes meaning there could be autoimmune causes for

the development of CSU (please refer to 1.6 for a detailed explanation of the known pathomechanism of CSU) (1, 6, 7).

To rule out potential other diseases causing wheals or angioedema one should follow the diagnostic algorithm first published by Maurer *et al.* in 2013 and then modified for the 2014 guideline and still recommended in the latest 2022 guideline (Fig. 2): Hives with concomitant fever or malaise could be an indication for autoinflammatory diseases as Schnitzler's syndrome, whereas hives persisting for > 24 h at the same location lead towards the diagnosis of urticaria vasculitis. Angioedema only can also be bradykinin-mediated and thus a manifestation of e.g. acquired or hereditary angioedema (1, 6-8).

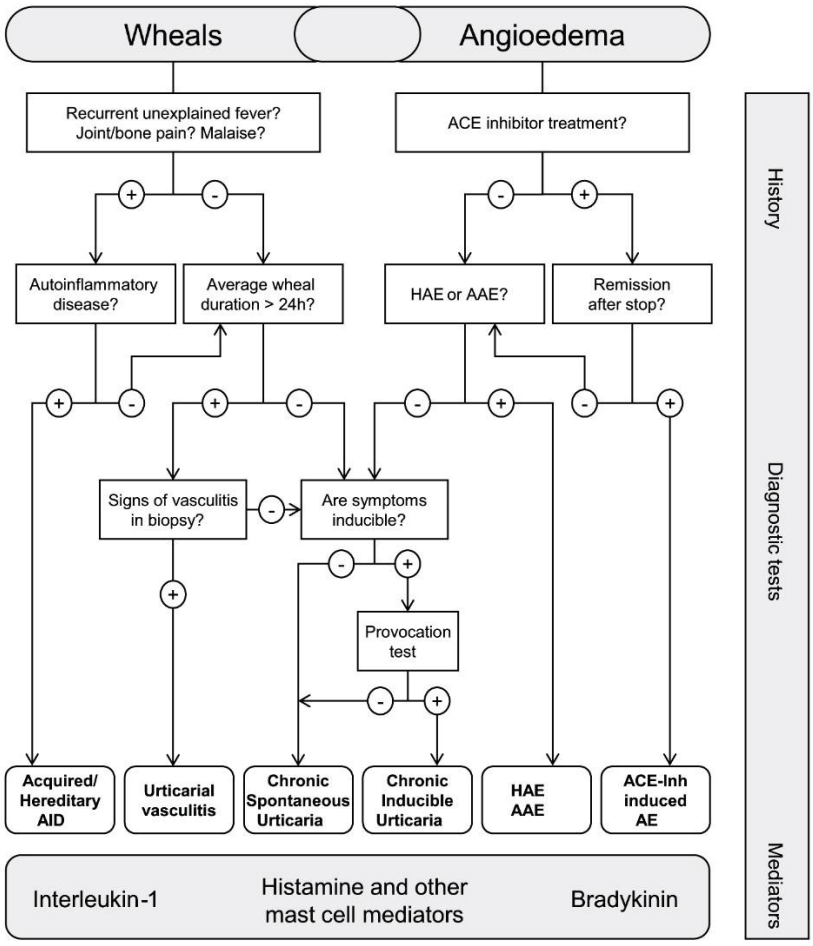

Fig. 2: **Diagnostic algorithm for patients with wheals and/or angioedema**

The algorithm first published by Maurer *et al.* in 2013 and later modified for all three subsequent international guidelines of 2014,2018 and 2022 shows a diagnostic path for patients with urticaria symptoms > 6 weeks. Both wheals and angioedema can occur in chronic spontaneous urticaria and chronic inducible urticaria. With hives alone one should ask for concomitant symptoms like fever or joint pain to rule out rare autoinflammatory diseases (AID). If the wheals remain localised at one site for > 24h one should consider taking a biopsy to rule out urticaria vasculitis. Angioedema alone can be a manifestation of bradykinin-mediated diseases such as hereditary angioedema (HAE) or acquired angioedema (AAE) or the far more common ACE-Inhibitor-induced angioedema. Abbreviations: ACE= angiotensin converting enzyme. Modified from: *EAACI/GA²LEN/EDF/WAO* urticaria guideline, Zuberbier et. al, Allergy 2022 (1)

As part of the diagnostic workup in CSU, a differential blood count and CRP (C-reactive protein) and/or ESR (erythrocyte sedimentation rate) could help to rule out possible underlying chronic inflammation. Apart from that, IgG anti-TPO (thyroid peroxidase) and total serum IgE should be measured. If there are any further suspected causes in the medial history/ blood test results, e.g. infectious diseases (for example *helicobacter pylori)*, mastocytosis, thyroid gland disorders, those should be further investigated (1, 9, 10). An extended diagnostic workup with no clear indication in the patient's medical history or laboratory results is not recommended (1).

1.3 Epidemiology

The prevalence of CSU is approximately 1 % of the population (11). Regarding the population in Germany, a recently published study observing a database of approximately 3,5 Mio patients showed a prevalence of 0,5 % for CU, of which 71,2% were diagnosed with CSU and 9,1 % with CSU and concomitant CIndU respectively (5). Approximately 37 % of CSU patients suffer from both angioedema and wheals, 57 % from hives only and 6 % from angioedema only (12). Mostly 20-40 y/o persons are affected, the number of affected females is twice as high as males (2, 13). CSU is associated with autoimmune diseases with studies showing that up to one third of CSU patients have an autoimmune comorbidity (1, 14-16). Most common are hypothyroidism (10%), especially in female patients, and also rheumatoid arthritis (1,9% of female CSU patients), others include diabetes mellitus type I, coeliac diseases, systemic lupus erythematosus or Sjögren syndrome (15, 17). An association with thyroid autoimmunity has been first described by Leznoff and Sussmann in 1989 and has been topic of multiple investigations ever since, with a most recent meta-analysis by Tienforti *et al.* showing a five-to-seven fold likelihood of TPO-antibodies in CU-patients (14-16, 18-23).

1.4 Burden of disease

The appearance of wheals and angioedema leads to self-consciousness in patients, but it is especially the intractable itch that makes the disease unbearable for the ones suffering from it. Due to the unforeseeable appearance of the symptoms in CSU the patients have an even higher quality of life impairment as they cannot e.g. avoid certain triggers as with CIndU. CSU may cause sleeplessness and affects the patients' job performance and social interactions thus leading to a burden of health care and society (4) (3, 24-26).

1.5 Patient-reported outcome measures

CSU is characterised by the fluctuating appearance of its core symptoms – wheals and/or angioedema. Often patients do not have any symptoms at their clinical presentation making it difficult to objectify their burden of disease. In recent years, patient-reported outcome measures (PROMs) in form of questionnaires, simple yes/no-questions or rating scales have not only become an essential tool in clinical trials but also in clinical care (17, 27, 28). Patients achieve certain scores by answering questions which can then help to objectify the patients' view on disease activity, disease control and quality of life. In dermatology the most used PROM instrument is the *Dermatology Life Quality Index* (DLQI). However, over the past years several other tools have been developed specifically for CU and CSU (17). The following will describe the questionnaires used in this study, which are also some of the most used instruments in CSU for patients presenting with wheals and angioedema.

1.5.1 Urticaria Control Test

The *Urticaria Control Test* (UCT) is a 4-question tool with a recall period of 28 days used to assess disease activity and control in CU patients. It was first published in 2014 by Weller *et al.* with a study proving the tool's validity and reliability and ever since became one of the guideline-recommended instruments to evaluate disease control (1, 6, 29). Especially in such a disease as CSU where symptoms appear spontaneously, it is crucial to assess disease activity retrospectively starting at first clinical presentation to determine treatment objectives. The test's 4 questions cover the symptoms of urticaria, quality of life, treatment and disease control (supplemental Fig. 1). Each question has 5 possible answers which result in 0-4 points each. The total score that can be achieved is 16 – meaning the disease is well controlled and the patient had no urticaria symptoms (itch, hives, swelling) over the past 28 days. The cut-off value is 12, patients with a score <12 are considered to have a poorly controlled disease and need change in treatment. Patients who have a recorded increase of minimum 3 points of the UCT value compared to the previously documented value are considered partial responders to the respected treatment (1, 29).

1.5.2 Chronic Urticaria Quality of Life Questionnaire

The *Chronic Urticaria Quality of Life Questionnaire* (CU-Q2oL) is a PROM tool with a recall period of 14 days. It was first published in 2005 by Baiardini *et al.* and has been recommended to assess quality of life impairment in CSU patients since the 2014 guideline (1, 6, 7, 30, 31). The CU-Q2oL has been specifically developed for CSU and

is thus more precise in assessing CSU patients' well-being than the later discussed *Dermatology Life Quality Index*. The questionnaire consists of 23 questions applying to six different domains: functioning, sleep, itching/ embarrassment, mental status, swelling/ eating and limited looks (supplemental Fig. 2). Each question offers an answer on a 4-point-scale (1= "not at all" to 4= "very much") with a maximum of 115 achievable points and minimum of 14 points. As different domains are supposed to have a different impact on patient's lives, the calculation should be performed through a specific conversion table. Of course, a limitation of this instrument is that in a clinical setting, a precise calculation would be overly time consuming. However, in general, a higher score corresponds with a decreased quality of life due to CSU and simply identifying a response-pattern also helps to navigate through the questionnaire. There is no cut-off value in contrast to the UCT or the DLQI. The CU-Q2oL was translated into German in 2009 and after that in many other languages including Polish, Turkish, Persian, Hebrew and recently also Chinese and Arabic – different languages may highlight each domain differently according to cultural differences (32-35).

1.5.3 Dermatology Life Quality Index

First published in 1994, the Dermatology Life Quality Index (DLQI) with a recall period of 7 days has been used to assess quality of life impairment in dermatological diseases for over 25 years now (36). This PROM toll has also been validated for the use in urticaria patients (37, 38). There are 10 questions covering six domains: symptoms, daily activities, leisure, work/school, personal relationships and treatment of disease (supplemental Fig. 3). Each question has 4 alternative answers rating from "not at all" (score: 1) to "very much" (score: 4) with a possible answer of "not relevant" (score: 0). The maximum score is 30 – a value higher than 10 corresponds to a moderate impairment due to the disease and should result in change of treatment. By using the tool in follow-up visits, a change of 4 points in the total score correlates with a clinically significant change in quality of life impairment. The DLQI is a standard instrument in dermatological practise and, in contrast to the CU-Q2oL described above, less time-consuming for the patient to fill in and for the clinician to evaluate.

1.6 Pathophysiology

CSU is considered to be a mast cell-driven disease (1, 12). In its core, mediators released from activated mast cells (MCs) lead to vasodilation and plasma extravasation which in turn lead to the development of typical urticaria lesions – hives and angioedema i.e. swelling of the upper and lower dermis/subcutis respectively. The mediators also act

as activators of sensory nerves resulting in the debilitating itch or burning sensation for the patient. Over the past years research has opened new insights on MC activation and other cells involved in the pathogenesis of the disease (Fig. 3). Still, the aetiology remains not fully understood and is lacking specific biomarkers to e.g., assess treatment possibilities (1). In the following, the focus will be on key effector cells and their proposed predominant functions in CSU.

1.6.1 Mast cells

Skin MCs play a key role in urticaria. MCs are part of the innate immunity and can be divided into 2 types - MC_T (tryptase-positive but chymase-negative) and MC_{Tc} (tryptase and chymase-positive) (39, 40). MC_{Tc} are predominantly located in the skin and are thus considered to be the primary effector cells in urticaria (41, 42). Up to date there are several known pathways of MC activation and degranulation.

1.6.1.1 IgE-dependent mast cell activation and autoimmune CSU theory

In allergic diseases, but also in CSU there is IgE-dependent MC activation through IgE cross-linking and binding to FcεRI, the high affinity IgE receptor found on the surface of MCs (43, 44). Studies have shown that many CSU patients have elevated serum levels of total and free IgE (45-47). The percentage of patients with high total IgE levels differs according to the respective study; in a recent review article Altrichter *et al.* showed a cohort with 50% of the patients having high total IgE levels (>100 IU/ml). However, there are also patients with low total IgE levels – with Altrichter *et al.* showing percentages of 25 % for total IgE levels < 30 IU/ml and 2% for < 2 IU/ml (47). Apart from the regular IgE-dependent MC activation with properly functioning IgE, the idea of an autoimmune component in the aetiology has been strengthened over many years (48-52). Today, also as part of the international guideline, there is a differentiation of two types of autoimmunity: Type I autoimmune CSU is linked to IgE autoantibodies and type IIb autoimmune CSU is associated with IgG or/and IgM autoantibodies against FcεRI and IgE itself (1, 53). Type I autoimmune CSU is also called autoallergic CSU due to IgE antibodies being directed to autoallergens – most common are IgE-autoantibodies against thyroid peroxidase (TPO) and interleukin 24 (IL-24) (53). Regarding type IIb autoimmunity over 200 IgE-type autoantigens have been described (54). The autoimmunity theory in CSU is strengthened by the high number of associated autoimmune diseases of CSU patients (14). Cases of overlapping type I and IIb autoimmunity in CSU patients have been described (55).

1.6.1.2 IgE-independent mast cell activation

MCs can be activated by various other, non-IgE-dependent mechanisms via several different receptors. Over the past years Mas-related G protein-coupled receptor X2 (MRGPRX2) has shown to be a key receptor for MC activation. The expression of MRGPRX2 is increased in CSU patients and the activation of MCs via this receptor is thought to be more rapid (56, 57). MRGPRX2 is a receptor for e.g. major basic protein (MBP) and eosinophil peroxidase (EPO), but also substance P, vasoactive intestinal peptide (VIP) and IL-31 (58). Other relevant receptors of IgE-independent MC activation or differentiation and proliferation respectively are complement component 5a (C5a) receptor (C5aR) via the complement cascade, neurokinin 1 and 2 receptors (NK1R and NK2R) via neuropeptides and KIT (a tyrosine kinase growth receptor) via stem cell factor (SCF) as well as IL-4 receptor α (IL-4Rα) via IL-13 but also histamine-4-receptor (H4R) via histamine (12, 59-62).

It is known that emotional stress leads to aggravation of chronic spontaneous urticaria (2, 63, 64). On a cellular level this aggravation could be explained by an increased neuropeptide-mediated MC activation but also via corticotrophin-releasing hormone (CRH) binding to CRH-R1, which was shown to be upregulated in CU-tissue (64).

An example of an inhibitory MC receptor is sialic acid-binding immunoglobulin-like lectin 8 (siglec 8), which leads to MC silencing (65).

1.6.1.3 Intracellular pathways and degranulation of mediators

There are several intracellular pathways of MC activation. The stimulation of FcεRI for example leads to the activation of e.g. the intracellular spleen tyrosine kinase (SYK) and Bruton's tyrosine kinase (BTK) phosphorylating downstream signaling molecules and subsequently leading to activation of transcription factors such as NF-κB (66, 67). BTK is thought to be the predominant kinase of FcεRI-dependent MC stimulation (66). Activation of H4R leads to MC chemotaxis, increased FcεRI expression and MC activation/degranulation via intracellular calcium mobilisation (62, 68, 69).

MC degranulation involves various mediators. Histamine is a key mediator released from MCs leading to the typical vasodilation along with e.g. leukotriene C4 (LTC4) and platelet-activating factor (PAF) as well as prostaglandin D2 (PGD2) (42, 44). Histamine stimulates type C unmyelinated neurons and thus leads to the typical itch/burning sensation CSU patients experience. These sensory nerves lead to the secretion of neuropeptides, e.g. substance P, which subsequently leads to a so called axon reflex – a reflex-like vasodilation of arterioles surrounding the oedema of the formed wheal. The

development of vasodilation in terms of a “red spot”, the previously described axon reflex – “flare” –, and oedema leading to the formation of a “wheal” are the three parts of the “triple response of Lewis”. The symptoms were named after T. Lewis who was the first to describe these observations after intracutaneous histamine injection in the 1920s (70). As mentioned above, histamine binds to H4R and thus leads to an autocrine loop, however it also binds to the H4R on basophils. Another autocrine loop works via released MC tryptase binding to the G protein–coupled receptor (GPCR) proteinase-activated receptor-2 (PAR2) . The release of nerve growth factor (NGF) not only promotes itch (and pain) sensations by enhancing the development of sensory nerves, but also leads to an enhanced immune response by T helper (T_H) 2 cells and MCs themselves (42, 71).

The number of released cytokines is vast and so are their effector cells. This underlines the fact that, even though MCs are thought to be the predominant key effector cells, various other cells are involved in urticaria as well (44). For example, the release of IL-33 and IL-31 not only leads to a direct activation of sensory neurons inducing itch, but it also leads to the induction of a T_H2-dependent immune response (in the case of IL-31 it also creates to an autocrine loop via promoting the release of IL-4 and IL-13) (72-75). Furthermore, IL-31 promotes IgE-production by B cells (54). Other promotor cytokines of T_H2 immune response released by MCs are IL-25 and TSLP (both members of the IL-17 cytokine family) (42, 76). MCs also release IL-5 which is a key cytokine for the activation of eosinophils (42). It is thought that MCs also release IL-3, which influences basophil growth (44).

1.6.2 Basophils and Eosinophils

Both basophils and eosinophils are key effector cells in the pathogenesis of CSU. There are several similar mechanisms to MC activation. Both cells have H4R and thus are activated by histamine released by MCs. Basophils also release histamine themselves (44). Both cells express a receptor of the IL-1-receptor superfamily called ST2 (serum stimulation-2) whose ligand is IL-33, which is released, for example, by MCs and macrophages (77). In basophils the activation of ST2 also leads to the recruitment of yet another cell type – neutrophils – via the chemokine CXCL1 (78). Both eosinophils and basophils express MRGPRX2 with similar activation mechanism as mentioned above. Interestingly IL-3 and IL-5, also released by MCs, lead to an increased expression of MRGPRX2 which subsequently would create an enhanced immune response as IL-3 is thought to be a key interleukin for basophil activation and IL-5 for eosinophil activation respectively (57, 79). Just as MCs, basophils also release IL-31, IL-4 and IL-13. As mentioned above, IL-4 and IL-13 release is, among others, induced by IL-31. IL-4 is

thought to influence MC growth and differentiation as well as enhance the expression of FcεRI on MCs (80, 81). FcεRI is also expressed on basophils and thus can be activated by IgE cross-linking or via possible autoimmune pathways described above (53). A major chemoattractant for eosinophils is eotaxin released by MCs (82). Upon activation, eosinophils release interleukins such as IL-6 and IL-8 (upon activation via tryptase released by MCs and via IL-31 regarding IL-6 release) but also MBP and EPO, which subsequently enhance histamine release via activation of MRGPRX2 (44, 83). Eosinophils further release SCF which binds to KIT on MCs leading to the enhancement of MC development (12). Eosinophils also release tissue factor that leads to an involvement of the coagulation and complement cascades via thrombin and C5a and their respective binding to MCs but also to eosinophils themselves. Furthermore, eosinophils release vascular endothelial growth factor (VEGF) leading to an increased vascular permeability and activation of the coagulation cascade (83). MCs and eosinophils also communicate with one another via physical contact and paracrine signalling with eosinophils leading to a lower threshold of MC activation via IgE (44).

1.6.3 T Cells

Even though MCs are considered key effector cells in CSU, the infiltrate of skin biopsies taken from CSU patients mostly shows T cells, hereby T helper (T_H) 2 cells being the most prominent ones with some T_H1 and T_H17 cells. Studies focusing on this group of cells are undergoing and much needed (83).

Of the released cytokines listed above a key interleukin for T_H2 cells is IL-33, an alarmin, which induces T_H2 immune response (84). Apart from the typical T_H2 cytokines secreted by MCs (IL-31, IL-4, IL-13, IL-25, IL-5 and TSLP), PDG2, leukotriene E4, NGF as well as histamine also lead to the proliferation, activation and survival of T_H2 cells. T_H2 cells are main contributors to mostly IL-4, IL-5 and IL-13 as well as to IL-33, IL-31, IL-24 and IL-9 (12, 44, 54, 83). As mentioned above IL-4 and IL-13 promote the production of IgE via class switch of B cells (85). Interestingly, IL-24 is a member of the IL-10 family, which is also involved in autoimmunity. To highlight the idea of CSU as an autoimmune disease, it can be noted that one of the IgE-autoantigens was found to be IL-24 (86).

T_H17 expression was shown to be increased in the skin of CSU patients (87). T_H17 is known to be part of chronic inflammatory diseases and, especially regarding CSU pathogenesis, leads to production of IgE in B cells (88). A key interleukin for T_H17 differentiation is IL-23 and upon activation this lymphocyte releases several cytokines such as IL-17, IL-6, IL-8, IL-22, IL-23 and tumor necrosis factor (TNF)-α (83). Of these, most data regarding CSU cytokines can be found on IL-6 at the moment. However, it

should be noted that only a small part of IL-6 is thought to be released from T_H17 cells, most of IL-6 is secreted by innate immune cells in CSU – predominately MCs and eosinophils (but also macrophages, dendritic cells) (89, 90). TNF-α is also released by MCs and macrophages and has a broad range of effector mechanisms. In CSU, TNF-α

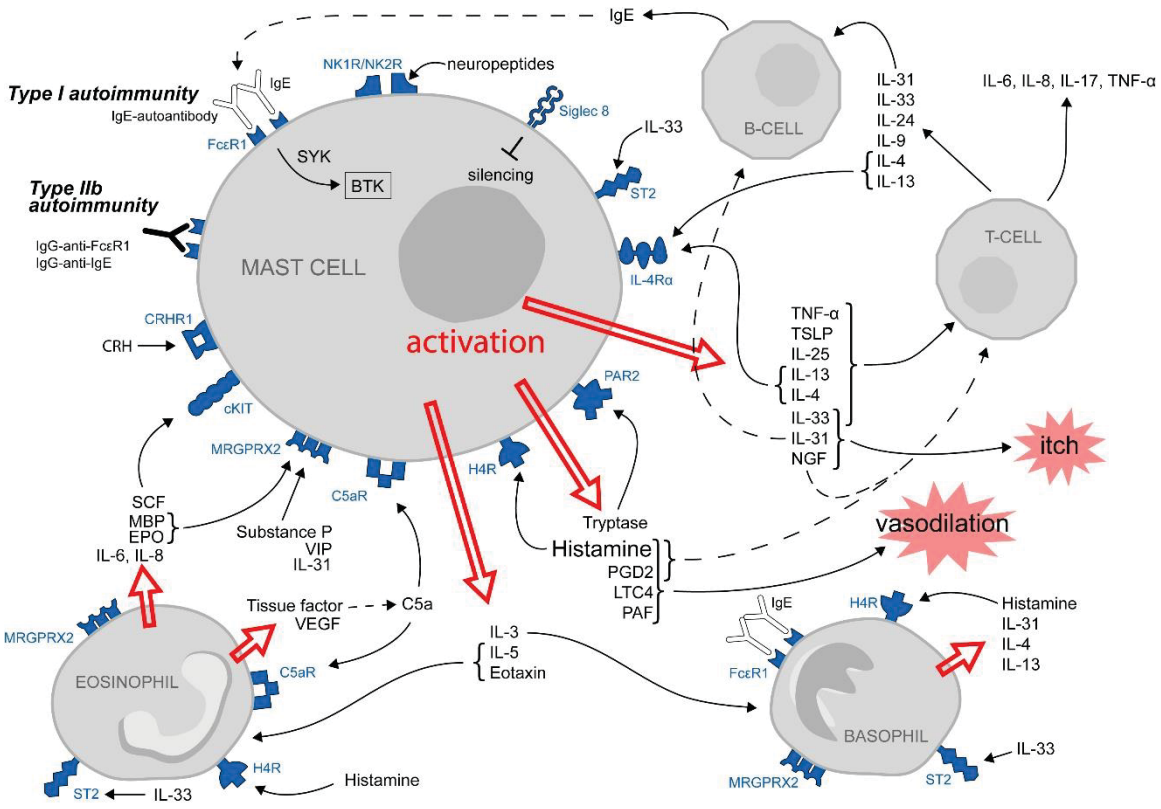

Fig. 3: **A schematic model of key factors in CSU pathogenesis based on current findings**

Depicted are several known pathways in CSU pathogenesis. The mast cell (MC) is considered to be the key effector cell in CSU. MCs release, among others, histamine upon their activation, which leads to vasodilatation and the development of wheals and angioedema. Depicted are further basophils, eosinophils as well as T cells and B cells with their respective mediators. Abbreviations: BTK = Bruton's tyrosine kinase, CRH = corticotropin-releasing hormone, EPO = eosinophil peroxidase, H4R = histamine 4 receptor, IL = interleukin, LTC4 = leukotriene C4, MBP = major basic protein, MC = mast cell, MRGPRX2 = mas-related G protein-coupled receptor-X2, NGF = nerve growth factor, NK1R/NK2R = neurokinin-1/2 receptor, PAF= platelet-activating factor, PAR2 = proteinase-activated receptor-2, PGD2 = prostaglandin D2, R =receptor, SCF = stem cell factor, Siglec 8 = sialic acid-binding immunoglobulin-like lectin 8, ST2 = serum stimulation-2, TNF-α = tumor necrosis factor α, VEGF = vascular endothelial growth factor

not only leads to MC proliferation and expression of a more reactive phenotype of MCs but is also thought to promote neutrophilic infiltrate in CSU patients' skin and to lead to lower T_{reg} cell induction (83). T_{regs} are thought to balance immune response inducing tolerance to self-antigens. Supporting the idea of an autoimmune aetiology in CSU, one study also described reduced circulating T_{regs} in CSU patients (91). However, so far data

on IL-10 (which is a key cytokine released from T_{regs} beside TGF-β) is inconsistent just as the limited data on T_H1 cells and its main released cytokine IFN-γ (83).

Apart from activation via cytokines, MCs and T cells have physical contact in form of heterotypic adhesion via CD80/CD86 and CD54 (ICAM-1) expressed on MCs and leukocyte function-related antigen-1 (LFA-1) expressed on respective T lymphocytes leading to a direct activation of these cells (92, 93). T cells also secrete microvesicles which promote MC activation and e.g. the production of IL-24 (94, 95).

1.6.4 mRNA and miRNA

Over the past years research in medicine has focused on finding biomarkers for different diseases in order to get an insight on prognostic factors and personalise therapeutic approach. Some of this research focuses on molecular interactions via creation of molecular networks which was enabled by omics technologies among others. Part of this networks consists of micro RNA (miRNA) and their interaction with messenger RNA (mRNA) (96-98). MiRNA are composed of 22-24 nucleotides and belong to the group of non-coding RNAs, i.e. they do not translate into polypeptides. MiRNAs form a complex with Argonaute proteins called miRNA-induced silencing complexes (miRISCs). Through the respective miRNA these complexes bind to their target mRNAs at the 3' untranslated region (3' UTR) (99). This leads to repression and breakdown of the respective RNA and by such to a negative post-transcriptional regulation of gene expression. One miRNA can target several mRNAs (100). MiRNAs are thought to have a regulatory effect on both the adaptive and the innate immune systems, in particular on macrophages and granulocytes (101, 102). Modified miRNA expressions were shown for different conditions, including skin and allergic diseases (96, 103). It was demonstrated that miRNAs lead to changes in inflammatory cytokines affecting their development and release by effector cells. Interestingly, a changed expression of mRNA was discovered in autoimmune diseases (97, 104). Regarding research on miRNAs, it is of note that they can be found in all body fluids and remain relatively stable for further assessments with obtaining the blood sample being less invasive than obtaining biopsies (105). With such, mRNAs and non-coding RNAs, especially miRNAs, have recently become a topic of interest in CSU as well.

First it was Lin *et al.* in 2017 who identified 16 differently expressed miRNAs in plasma of 12 patients with CSU. Possible target genes were linked to identified upregulated genes associated with CSU published by Patel *et al.* in 2015 (106). Thus 13 genes were identified with roles in, among others, the TGF-β - and glucocorticoid receptor signaling pathways (107). Zhang *et al.* demonstrated an upregulation of miRNA-125a-5p in serum

of 20 CSU patients compared to 20 healthy controls with validation of this results via qPCR in 59 patients and 58 HC. MiRNA-125a-5p is described to have an association with autoimmune disease and is thought to influence TGF-β signaling pathway and further T_{regs} via IL-6 and STAT3 showing that upregulated miRNA-125a-5p corresponds to downregulated T_{regs} (105). Via research on mRNA and long non-coding RNA (lncRNA, like miRNA part of the non-coding RNA family), Liang and collaborators focused on the IP3/DAG pathway known to be part of MC activation and found T264761 lncRNA to have an association with this disease pathway (108). Peng *et al.* explored a so far uncommon direction in CSU by investigating pyroptosis-related genes. Pyroptosis describes inflammasome-mediated cell death, which was shown to be linked to several inflammatory diseases. The study by Peng *et al.* identified several pyroptosis-related genes to be differently expressed in CSU - among these, *IL1B* was proposed to play a role in MC activation (109). In the most recent published study Prosty *et al.* applied RNA deconvolution to broadly investigate T cell pathways in CSU patients strengthening the above-mentioned idea of T_H2, T_H17 as well as T_{reg} contribution to CSU activity.

1.7 Systemic Treatments in CSU

The systemic treatment of CSU follows the suggested algorithm of the current international guideline published in 2022 and included antihistamines, omalizumab and ciclosporin (Fig. 4). The approval of omalizumab for the use in CSU in 2014 changed the recommended therapeutical options: Drugs like montelukast and dapsone fade into the background, whereas omalizumab is now a well-established and so far, only approved add-on therapy for patients not adequately controlled by antihistamines. Ciclosporin remains an off-label treatment option for omalizumab non-responders (1, 6, 7).

To note is that CSU can have spontaneous remissions and thus disease activity should be closely monitored and ongoing treatment should be re-evaluated every 3 to 6 months (1). All treatments are symptomatic, no curative treatment is available for CSU today.

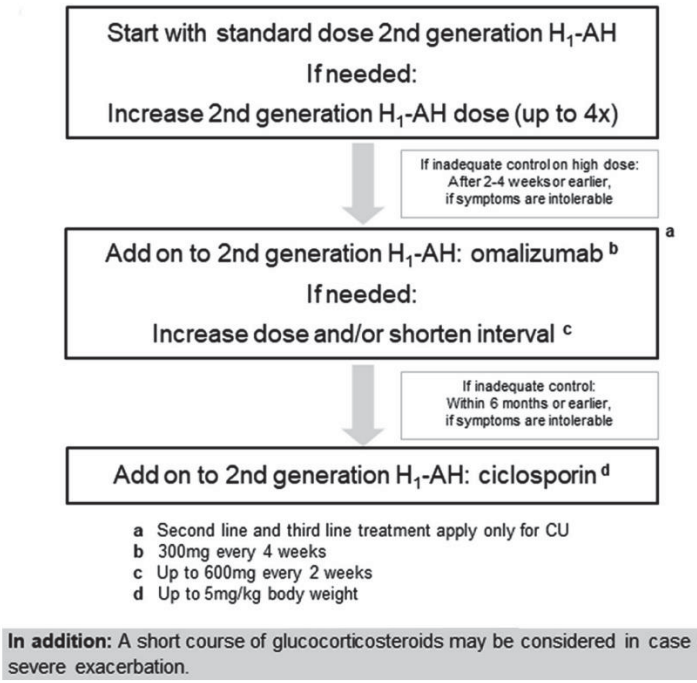

Fig. 4: **Treatment algorithm for CSU from the international 2022 guideline**

As a first step in CSU treatment, the urticaria guideline recommends a standard dose of a 2nd generation antihistamine, i.e., once daily in the recommended dose of the respective antihistamine. If there is no symptom control, it is recommended to up-dose the antihistamine – up to fourfold daily (off-label). If the patient does not show significant improvement and the disease is still poorly controlled after 2-4 weeks (or earlier if symptoms are unbearable) the guideline recommends the add-on treatment with the anti-IgE-antibody omalizumab (300mg every 4 weeks). If not sufficiently controlled, an off label up-dosing of omalizumab is recommended (up to 600mg every 2 weeks). Patients who do not respond adequately to omalizumab treatment are recommended to switch to off-label ciclosporin after 6 months' time or earlier if needed. Ciclosporin is known to achieve good disease control, however, may have severe side effects omalizumab does not show. Short term corticosteroid use remains a possibility for serious exacerbations, however, should not exceed 10 days of treatment. Modified from: *EAACI/GA²LEN/EDF/WAO* guideline 2022, Zuberbier *et al*. (1)

1.7.1 Antihistamines

Antihistamines have been a key basic urticaria treatment more many years now (110). They act as inverse agonists at the H1-receptor of the histamine and thus stabilise it in its inactive form. This leads to the reduction of the histamine effects leading to lessened wheal and oedema formation as well as to reduced pruritus. 1st generation antihistamines like dimenhydrinate are now strongly recommended against as they can cross the blood-brain-barrier and with such are more likely to cause adverse effects as drowsiness and nausea or even more severe adverse effects when overdosed (30). Nowadays, there are 2nd generation antihistamines that are minimally or non-sedating showing a good safety profile. Examples of 2nd generation antihistamines are (alphabetically): bilastine, cetirizine, desloratadine, ebastine, fexofenadine, levocetirizine, loratadine and rupatadine. It is not recommended to combine different

antihistamines. However, a switch to another antihistamine can be considered. The treatment algorithm recommends a standard dose of an antihistamine i.e. one dose daily with or without urticaria symptoms (111). If there is no symptom improvement (in up to 61%), it is recommended to increase the antihistamine dose off-label up to its fourfold daily intake (112-115). A large number of patients (up to 45-50% %), however, does not sufficiently respond even to the up-dosed antihistamine treatment (115). This corresponds with the notion that other factors but histamine play an important role in the pathogenesis of the disease.

1.7.2 Omalizumab

If there is no sufficient disease control under treatment with antihistamines, the guideline algorithm recommends the use of omalizumab, a monoclonal anti-IgE-antibody approved for CSU since 2014 (6, 116-119). Currently, it remains the sole licensed add-on therapy for CSU and can be used in patients 12 years of age and older (1).

In 2003, the agent received FDA (Food and Drug Administration) approval for its use in moderate to severe allergic asthma – the EMA (European Medicines Agency) approval followed in 2005 after publication of the INNOVATE trial results by Humbert *et al*. (120, 121). Since 2020 Omalizumab has also been approved for its third treatment indication – chronic rhinosinusitis with nasal polyps (122).

The effect on patients with urticaria was discovered by coincidence in 2006: A patient with allergic asthma showed improvement of her cold urticaria symptoms under treatment with omalizumab (123). Case reports for CSU followed shortly, for example by Sands *et al.* in 2007 and by Spector *et al.* in 2008 (124, 125).

Phase II trials and randomised controlled phase III (ASTERIA I und II, GLACIAL) trails demonstrated the efficacy and safety of omalizumab use in CSU patients (116, 117, 126, 127). Trial endpoints were, among others, the reduction of wheal and angioedema development and the reduction of pruritus. Quality of life was measured by the above mentioned DLQI and over time, after its introduction into clinical use, by the CU-Q2oL. Different trails demonstrated complete remission rates ranging from 70 to 83% (19, 128, 129). Most patients receiving omalizumab had already responded to treatment after the first application. In 2014, Metz et. al demonstrated a complete remission of symptoms after 1 week of treatment in 57% of evaluated patients in 2014 (128). Kaplan *et al.* focused on the response rate of the patients treated in the above-mentioned phase III trials: 15-24% of the evaluated patients showed complete response and 37-51% a well-controlled disease 4 weeks after treatment initiation. Furthermore, the study underlined the possibility of late-responders – showing improvement 24 weeks after the first

omalizumab dose. The average time to response was shown to be 12-13 weeks (130). The current guideline suggests that even though omalizumab leads to mostly fast improvement of urticaria symptoms or even to complete remission, the drug should be used for at least 6 months' time to cover the possibility of late treatment response. The recommended dose is 300 mg (2 150 mg syringes at a time) subcutaneously every 4 weeks. If the CSU is inadequately controlled, the guideline recommends increasing omalizumab dose up to 600 mg and/ or to shorten the interval to every 2 weeks off-label. Treatment should be re-evaluated regularly, e.g. every 6 months, and treatment discontinuation can be discussed if patient is symptom free (1). If a relapse occurs, omalizumab can be reintroduced and shows again good efficacy (131). Reported adverse effects were injection side reaction, infections of the upper respiratory tract (esp. nasopharyngeal) and headache – however, similar to those in the place groups of the above-mentioned phase III trials.

Omalizumab binds to free IgE via its Cε3 domain which is the domain through which IgE interacts with FcεRI leading to MC (and basophil) activation (132). As omalizumab forms complexes with free IgE, free IgE levels are reduced, there is less IgE cross-linking and interacting with FcεRI which over time is thought to lead to FcεRI downregulation and reduced MC releasability. Regarding the proposed autoimmunity in CSU patients, Omalizumab is thought to reduce activity of IgG-autoantibodies against IgE and FcεRI as well as IgE autoantibodies, but it is essential to underline that omalizumab is also effective in patients with no observed autoantibodies. Of note is that apart from autoimmunity, the classical IgE-binding mechanism does not explain the rapid onset of symptom relief in the patients responding to omalizumab after one week of initiation (133).

Basophils are proposed to be a key target of omalizumab treatment in CSU patients also by the IgE-binding effect described above. Studies demonstrated FcεRI downregulation as early as at day 8 after treatment initiation which is thought to possibly be an explanation for rapid-responders. However, research on basophils in CSU is conflicting and its role in the disease is not yet fully understood (133, 134).

Omalizumab was also observed (by binding free IgE) to reduce IgE interaction with the low affinity IgE receptor, FcεRII commonly known as CD23, found on e.g. B cells or eosinophils (133, 135).

Interestingly, Omalizumab has also shown to influence T cell related cytokines. Altrichter *et al.* demonstrated an association of a good response rate to the reduction of serum IL-31 in 2016 (136). Rauber *et al.* showed in a study of 15 patients treated with omalizumab

an association of symptom relief to the reduction of IFNγ-, IL-10 and IL-31 secreting cells (137).

Still, the mechanisms of how omalizumab leads to amelioration of symptoms in CSU patients remain not completely understood, especially regarding the rapid responders and possible ways other than binding to IgE. And even though omalizumab has revolutionised CSU treatment, there are still patients who do not respond to this treatment or do not respond sufficiently (depending on the source, up to 40 % not sufficiently responding and 11,8% not responding) (138). These patients are in need of different treatment options.

1.7.3 Off-label add-on-treatment

Before Omalizumab was introduced to CSU patients, other therapies had already been in use. The calcineurin-inhibitor ciclosporin, an immunosuppressant with a proposed negative effect on T cells and its mediators, has presented a treatment option for CSU for many years now (139-141). However, due to a high possibility of adverse effects, ciclosporin is solely recommended for patients who have an insufficient response to omalizumab and is off-label (1). Other treatment options that are, however, not recommend, include leukotriene receptor antagonists (most data available for montelukast), sulphones like dapsone or immunosuppressants like methotrexate or mycophenolate motefil. These could be used in cases where all recommended options fail/ are not tolerated or if these are not available (1).

1.7.4 Corticosteroids

Systemic corticosteroids (CS) treatment is effective in CSU patients, however it should be used as a short term treatment only (142). CS have a broad mode of action through, among others, alternation of DNA replication as well as downregulation of NFκB and leukocyte migration. Thus, possible effects of CS in CSU include reduction of inflammation, vasoconstriction and suppression of the immune system. Due to a known vast number of possible adverse effects in long-term CS treatment, the medication should not be used for longer than 10 days in a row with a suggested dose of 20-50 mg of prednisone or equivalent in CSU patients (1).

1.7.5 New treatments for CSU

As there is unmet clinical need for patients not responding to recommended guideline treatments, research focusing on new agents for CSU has been abundant over the past years. Several agents are now in clinical trials or have shown to be effective through

case reports and open label studies. The different mechanisms of action of these drugs strengthen the idea of CSU having multiple key effector cells in its pathogenesis. **Dupilumab**, an anti-IL4Rα-antibody inhibiting the receptor's activation via IL-4 and IL-13, which has already been approved for atopic dermatitis, asthma, chronic rhinosinusitis with nasal polyps, eosinophilic oesophagitis and latest for prurigo nodularis, showed to be effective in CSU. However, the current phase III trial for this indication has been stopped due to interim analyses showing its futility to meet trial endpoints (115). The IL-5 signaling pathway presents as another target for possible CSU treatment options: **benralizumab**, an anti-IL-5Rα-antibody licensed for eosinophilic asthma, and **mepolizumab**, an anti-IL-5-antibody approved for asthma, chronic rhinosinusitis with nasal polyps as well as hypereosinophilic syndrome and granulomatosis with polyangiitis, are currently under investigation for CSU. Another agent currently in phase trial 2 is **tezepelumab,** an anti-TSLP-antibody licensed for asthma and eosinophilic oesophagitis (138). All the above-mentioned antibodies target pathways directly related to T_H2 signalling. Regarding T_H17 signalling, an anti-IL-17A-antibody, **secukinumab**, showed to be effective in CSU patients (143).

A different approach currently under development for CSU is directly targeting KIT and siglec 8 expressed on MCs via the monoclonal antibodies **barzolvolimab** and **lirentelimab** currently in phase 3 trials. Targeting these pathways lead to a stop in MC development or even MC depletion (12, 144, 145).

Finally, a novel target is located inside MCs – BTK – to reduce downstream signaling and thus also reduce MC degranulation. As BTK is also part of intercellular B-cell signalling it is thought to also have an effect of IgE production relevant for CSU patients. Thinking of the proposed autoimmune aspect of CSU, targeting B-cells is thought to reduce possible autoantibodies. The BTK-inhibitor **remibrutinib** is currently under investigation in a phase 3 trial (66, 115, 138).

The development and investigation of all the above-mentioned treatment options in CSU again underlines the multifaced pathogenesis of the disease, which remains not quite understood and thus it is currently not possible to determine patients' response to a specific treatment before well into its initiation.

1.8 Aims of study

This study aimed to explore CSU pathomechanism beyond MCs and IgE-dependent histamine release. We planned to investigate a patient cohort in its first month of omalizumab treatment by, on one hand, looking at the change of IgE levels and, on the

other hand, exploring changes of gene and miRNA expression in peripheral blood. We chose to look at patients receiving omalizumab because, even though, omalizumab is an anti-IgE-antibody, it is thought to have a broader mode of action in CSU than solely its effect on IgE. Our objective was to explore what kind of genes and miRNAs are differentially expressed in CSU patients and whether these could be linked to pathways beyond IgE-mediated histamine release. We aimed to investigate whether there are differences in gene and miRNA expression levels at baseline comparing responders, non-responders and healthy controls. We also planned to investigate, whether systemic treatment with omalizumab leads to changes in gene and miRNA expression levels and whether these changes correlate with the response to omalizumab. This would be achieved by first identifying differentially expressed genes and miRNAs via microarray analysis and then selecting candidate genes and miRNAs for a validation study via qPCR and comparing these findings to a cohort of healthy controls.

By this, we planned at achieving a better understanding of pathways involved in CSU. We aimed at identifying biomarkers which are much needed in CSU to eventually determine a tailored treatment in future or better understand how the disease will evolve with each patient.

2 Materials and Methods

2.1 Ethical clearance

The study was approved on July 22nd 2014 by the ethics committee of the medical faculty of the Heinrich-Heine-University (approval number: 4676, study title in German: “Identifikation von Biomarkern bei der Behandlung der chronischen spontanen Urtikaria”).

2.2 Study population

Patients were recruited from the dermatology department of the university hospital of Duesseldorf between 2014 and 2018. A total of 24 patients with antihistamine-resistant CSU before initiation of omalizumab treatment were recruited initially, of which 17 patients were included in the study (for detailed explanation see below; overview of patient characteristics in table 2). CSU diagnosis was made by taking a detailed medical history and physical examination based on the criteria of the latest EAACI/GA²LEN/EDF/WAO guideline at that time. Written informed consent was obtained prior to study participation.

Patients were treated with omalizumab and as it is an add-on therapy, patients could take 2nd generation antihistamines during the study period. Peripheral blood was obtained prior to the first omalizumab dose Day (D) 0, 2 days (D2) and 14 days (D14) after treatment initiation, and prior to the second omalizumab dose at day 28 (D28).

Patients	Sex	Age [years]	Symptoms	CSU Duration [months]
P1	F	35	W	5
P2	F	58	W +AE	108
P3	M	38	W +AE	48
P4	F	63	W +AE	12
P5	F	32	W	3
P6	F	19	W +AE	8
P7	F	46	W +AE	300
P8	F	32	W +AE	7
P9	M	46	W +AE	36
P10	F	69	W +AE	19
P11	M	58	W +AE	96
P12	F	32	W	3
P13	F	23	W	10
P14	M	51	W +AE	120
P15	M	43	W +AE	4
P16	M	57	W	9
P17	F	41	W	3

Table 2: **Patient characteristics**

17 patients diagnosed with CSU were included in the study: 11 female and 6 male patients with a mean age value of 44 years and an age range of 19-69 years. 11 patients suffered from the development of wheals and angioedema; 6 patients solely presented with wheals. The duration range was 3-300 months, the mean duration value was 47 months and the mean value 10 months. Abbreviations: CSU = chronic spontaneous urticaria, P = patients, F = female, M = male, W = wheals, AE = angioedema

Disease control and quality of life impairment were documented by patients filling in the German versions of the UCT, CU-Q2oL and DLQI at D0 and D28. Responders were

defined by the disease control test score of the UCT being ≥ 12. Patients who had a recorded increase of minimum 3 points of the UCT value at D28 were considered partial responders. Patients with no documented increase or even a documented decrease in the UCT score at D28 were considered non-responders (please refer to 1.5.1 for further details on the UCT). Of the 24 initially recruited patients, 7 patients were discontinued from the study as they missed out on the D2 sample collection time point or on 2 of 4 collection time points, or the collection time points differed too much from the appointed ones. Thus, the study population consisted of 17 patients in total: 11 female and 6 male patients; age range: 19-69 years, mean: 44 years, median: 43 years; 6 patients with wheals only, 11 patients with wheals and AE, no patients with solely AE. With these patients, still there was some deviation from the chosen collection time points in several cases: For D2, 2 patients had blood drawn on day 3 after starting omalizumab treatment and 5 patients on day 4. For D14 it was day 14 with a range of -7 to + 9 days (3 patients – day 7, 1 patient – day 13, 3 patients – day 15, 1 patient each on day 19 and day 25), whereas 2 patients missed out on the appointment. Regarding D28, the deviation was up to – 4 to + 21 days (1 patient each on day 24, 29, 30 and 49; 2 patients each on day 32 and 39). Furthermore, peripheral blood was drawn from 8 healthy controls – subjects with no known history of CSU or urticaria in general and no history of atopic diseases aged > 18 years.

2.3 Reagents and materials

Reagent	Content/Concentration	Company	Cat.Nr.
SuperScript II Reverse Transcriptase	SuperScript® II Reverse Transcriptase 200 U/µl (10000 U total)	Invitrogen; Carlsbad, USA	18064014
	First-strand buffer (5x)		
	Dithiothreitol (DTT) (100 mM)		
Rnasin® Plus Rnase Inhibitor	40 U/µl (10000 U total)	Promega; Madison, USA	N2615
Dnase I Recombinant	10 U/µl (10000 U total)	Roche; Basel, Switzerland	04716728001
Oligo deoxythymidine (dT) 12-18 Primer	0.5 µg/ml (25 µg total)	Invitrogen; Carlsbad, USA	18418012
Random Primer	500 µg/ml (20 µg total)	Promega;	C118A

		Madison, USA	
Desoxy nucleoside triphosphate (dNTP)	2.5 mM (1 ml total)	Bioline; London, UK	BIO-39053
Nuclease-free water	-	Roth; Karlsruhe, Germany	T143.3

Table 3: **List of reagents used for cDNA synthesis**

Reagents/materials	Content/Concentration	Company	Cat.Nr.
Ribosomal RNA Control Reagents (VIC ™ Probe)	Ribosomal Probe (VIC) 40 µM	Life technologies; Austin, USA	4308329
	Ribosomal Forward Primer 10 µM		
	Ribosomal Reverse Primer 10 µM		
Power SYBR® Green PCR Master Mix	5 ml	Applied Biosystems; Warrington, United Kingdom	4367659
TaqMan® 2X Universal PCR Master Mix	5 ml	Applied Biosystems; Warrington, United Kingdom	4318157
Nuclease-free water	-	Roth; Karlsruhe, Germany	T143.3
CELLSTAR® 96-well plate		Greiner Bio-One; Frickenhausen, Germany	650180
Plate seal for 96-well plate		NUNC™ by ThermoScientific; Roskilde, Denmark	236269
MicroAmp™ Optical 96-well reaction plate with Barcode		Applied Biosystems; Warrington, United Kingdom	4306737
StarSeal Advanced Polyolefin Film		STARLAB; Hamburg, Germany	E2796-9795

Table 4: **List of reagents used for qPCR**

Gene	Sequence forward	Sequence reverse	Note
CD28	gagaagagcaatggaaccattatc	tagcaagccaggactccaccaa	SYBR
CD52	cctcttcctcctactcaccatc	ctggtgtcgttttgtcctga	SYBR
CD69	caagttcctgtcctgtgtgc	gagaatgtgtattggcctgga	SYBR
CLEC2B	tggggctttaagagtgaagg	ttgggtaaagccagttagcaa	SYBR
COMMD6	ccaagtgctttgaaatgacg	agcagaaaggagactggaggt	SYBR
COX7B	agcgcactaaatcgtctcca	gaaaatcaggtgtacgtttctgg	SYBR
FcεR1A	acctgtctaccaccgagcat	tcatggactcctggtgctta	SYBR
GIMAP2	ggaccaaaatgaacacagtcac	cgattcaaatgcttgcttcc	SYBR
IL6R	gactgtgcacttgctggtggat	acttcctcaccaagagcacagc	SYBR
IntegrinB3	ccatgatcggaaggagtttgct	aaggtggatgtggcctctttatac	SYBR
LTF	gtgtccaggctgacagaagtt	cgcaccactgaacactcct	SYBR
COMMD8	Hs01060714_m1		Assay on demand
CXCR1	Hs00174146_m1		Assay on demand
CXCL5	Hs01099660_g1		Assay on demand

Table 5: **List of qPCR primers (genes)**

Gene	Catalogue number	Note
UniSp6 (Control)	YP00203954	SYBR
hsa-let-7e-5p	YP00205711	SYBR
hsa-miR-3609	YP02107756	SYBR
hsa-miR-486-3p	YP00204107	SYBR
hsa-miR-6716-5p	YP02115284	SYBR
hsa-miR-6808-3p	YP02118534	SYBR

Table 6: **List of qPCR primers (miRNAs)**

2.4 Measurement of total serum IgE

Blood sera taken at D0 and D28 were used for the measurement of total serum IgE levels by immunoassay (ImmunoCap®; Phadia/ Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. One sample was excluded due to a quality failure, thus, total IgE levels of 16 patients were investigated. For each measurement 100 µl of blood serum was needed drawn using a Vacutainer® blood collection tube (Becton, Dickinson and Company; Plymouth, United Kingdom).

2.5 RNA Isolation

Total RNA (including mRNA and miRNA) was isolated using the PAXgene® Blood miRNA Kit (PreAnalytiX; Hombrechtikon, Switzerland) according to the manufacturer's instructions. For each patient at each appointed time point as well as healthy controls one PAXgene® Blood RNA tube was used (blood draw volume equals 2.5 ml, additive volume – 6.9 ml allowing RNA stabilisation). Prior to any further procedures the tube was incubated for at least 2 h at room temperature (18-25°C) to allow the proprietary reagent of the tube to lyse blood cells. If the tube was stored at 2-8°C, -20°C or -70°C, it was again incubated at room temperature for 2 h before starting RNA isolation.

In the first step the PAXgene® Blood RNA tube was centrifuged for 10 min at 4000 x *g*. After removing the supernatant, the pallet was washed with 4 ml RNase-free water. This was followed by vortexing the pallet until its dissolution and again centrifuging the tube for 10 min at 4000 x *g*. Subsequently, 350 µl buffer BM1 were added before again vortexing until the pallet was visibly dissolved. The sample was then transferred into a 1,5 ml Eppendorf® tube (Eppendorf AG; Hamburg, Germany) before adding 300 µl Puffer BM2 and 40 µl proteinase K. After vortexing the sample, it was incubated for 10 min at 55°C in a shaker-incubator (Thermomixer comfort – Eppendorf AG; Hamburg, Germany) at 850 rpm. This mixture was transferred into a PAXgene® Shredder Spin column located in a 2 ml processing tube and centrifuged for 3 min at full speed (13200 × *g*). Thereafter, the supernatant of the Shredder spin column was transferred into a new 1.5 ml microcentrifuge without disturbing the pellet in the processing tube. In the next step, 700 µl isopropanol (100 %) were added and the sample vortexed. Subsequently, the sample was transferred into a PAXgene® RNA spin column binding RNA located in a 2 ml processing tube before being centrifuged for 1 min at 8000 × *g* twice with discarding flow-through and placing the spin column onto a new processing tube in-between. Thereafter, 350 µl buffer BM3 (with the respective amount of ethanol added beforehand as BM3 is presented as a concentrate) were added onto the RNA spin column and centrifuged for 15 s at 8000 × *g*. The flow-through was again discarded and the RNA spin column placed

in a new 2 ml processing tube. In a separate 1.5 ml Eppendorf® tube a DNAse I incubation mix was prepared by adding DNAse I stock solution (prepared in advance by dissolving 1500 Kunitz units of solid DNase I in 550 µl RNase-free water) to 70 µl Buffer RDD. 80 µl of this DNAse I incubation mix were then pipetted directly on the RNA spin column before incubation on the benchtop for 15 min. In the following steps buffer solutions (350 µl buffer BM3 in the 1st round and 500 µl buffer BM4 – with added respective amount of ethanol beforehand – in 2nd and 3rd rounds respectively) were added to the RNA spin column and then the mix was centrifuged at 8000 × *g* for 15 s in rounds 1 and 2 and for 2 min in round 3 respectively. Each time the respective processing tube with the flow-through was discarded and the RNA spin column placed in a new 2 ml tube before finally being centrifuged for 1 min at 8000 × *g* to remove residual liquid, especially ethanol. The processing tube with flow-through was discarded again and the RNA spin column placed in a new 1.5 ml microcentrifuge tube. The following 2 steps elute RNA by adding 50 µl buffer BR5 directly onto the RNA spin column membrane each time and centrifuging the sample for 1 min at 8000 × *g*. The eluate was then incubated in the shaker-incubator for 5 min at 65°C before being put on ice instantly. Finally, the RNA concentration at 280nm was determined with the use of NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific; Waltham, USA) while buffer BR5 was used to zero the spectrophotometer.

2.6 Microarray analysis

Microarray analysis to screen for differentially expressed genes in CSU patients was performed using a DNA MicroArray Chip (Type: Affymetrix human PrimeView 2.0, Thermo Fisher Scientific, Waltham, USA) at the “Biologisch-Medizinisches Forschungszentrum” (BMFZ) of the Heinrich Heine University. For the analysis, 6 patients with clinically significant symptom improvement under omalizumab treatment were initially chosen (i.e. UCT value increase > 3 points comparing D0 and D28); however, due to quality failures only 4 patients were included in the final analysis. Investigated time points were D0, D2 and D14. For each analysis the BMFZ needed an aliquot of the patients’ RNA samples with a concentration of 100 ng/µl and a total volume of 10 µl. In the second part of the study miRNA microarray analysis was performed using miRNA MicroArray Chip (Type: Affymetrix GeneChip miRNA 4.0 Array, Thermo Fisher Scientific, Waltham, USA). For this analysis, initially, 4 patients were chosen, however, 1 patient was withdrawn due to quality failure. Here D0, D2, D14 and D28 were compared.

2.7 cDNA synthesis

In order to validate microarray results via quantitative PCR (qPCR) during the course of the study the previously isolated RNA and miRNA initially needed to be transcribed into complementary DNA (cDNA).

The reagents used in the following steps of the cDNA synthesis for future gene expression analysis are listed in table 3 (paragraph 2.3). For some reagents defreezing was needed before further usage. Afterwards these and other reagents, including the previously isolated RNA were chilled on ice for all steps described in the following. Prior to starting the procedure DNase Mix I was prepared by adding 2.5 µl nuclease-free water, 1.5 µl 5 × first strand buffer (Invitrogen; Carlsbad, USA), 1 µl RNasin® Plus (Promega; Madison, USA) and 1 µl DNase I recombinant (Roche; Basel, Switzerland) in a 1.5 ml Eppendorf® tube per each RNA sample. As the first step, 4 µg RNA was diluted in nuclease-free water up to a total volume of 10 µl in a 0.5 ml thin-walled PCR tube. Thereafter, 6 µl DNase Mix I was added and the mixed sample then placed into a thermal cycler (TRIO Thermoblock with a heated lid, Biometra; Göttingen, Germany). The incubation lasted 30 min in total – the first part was 20 min at 37°C, the second part was 10 min at 70°C – before the samples were chilled down to 4°C. During the incubation period, 2 further solutions were prepared: Mix II consisting of 1 µl Oligo dT (Invitrogen), 0.4 µl Random Primer (Promega) as well as 2.6 µl nuclease-free water per each RNA sample; and Mix III consisting of 4.5 µl *5* × first strand buffer, 1.5 µl dNTP Mix (Bioline; London, United Kingdom), 1 µl DTT (Invitrogen), 0.5 µl RNasin® Plus, 1 µl SuperScript® II Reverse Transcriptase (Invitrogen) and 1.5 µl nuclease-free water per each RNA sample. After the incubation, 4 µl Mix II were added to each RNA sample. Next, the sample was again placed in the thermal cycler for an incubation period of 10 min at 70°C before cooling down to 4°C. Subsequently, 10 µl Mix III were transferred to each sample. Thereafter, the samples were placed in the cycler for the last incubation period: 50 min at 42°C, then 10 min at 70 °C before cooling down to 4°C. In the last step of cDNA synthesis, the now eluted cDNA was diluted to a concentration of 10 µg/ml adding 370 µl nuclease-free water to a final volume of 400 µl. As a preparation for the following qPCR, the cDNA was transferred into a round-bottom 96-well cell culture plate (Greiner Bio-One; Frickenhausen, Germany) with the dilution of 1:4 meaning 50 µl cDNA being added to 150 µl nuclease-free water. Thus, a concentration of 250 ng/ml cDNA was achieved.

For cDNA synthesis of miRNA the miRCURY® LNA® RT Kit (Qiagen; Venlo, Netherlands) was used. The following steps were performed as indicated in the kit handbook. First, each RNA sample was diluted to 5 ng/µl using nuclease-free water. The next steps were

performed on ice. 2 µl of each diluted RNA sample was transferred in a PCR-tube (Eppendorf) and added the each of following reagents provided in the kit: 2 µl 5x miRCURY® RT Probe Reaction buffer, 4.5 µl RNase-free water, 1 µl 10x miRCURY® RT Enzyme Mix and 0.5 µl UniSp6 RNA spike-in (synthetic RNA spike-in, which had prior been resuspended by adding 80 µl nuclease-free water). The samples were then placed into the above described thermal cycler (TRIO Thermoblock). The incubation lasted 65 min in total – the first part was 60 min at 40°C, the second part was 5 min at 95°C – before the samples were cooled down to 4°C. Thereafter, preparing for the following qPCR, the cDNA was transferred into a round-bottom 96-well cell culture plate (Greiner Bio-One) with the dilution of 1:80 meaning 1 µl cDNA was added 79 µl nuclease-free water.

For both cDNA synthesis processes (mRNA and miRNA), the prepared 96-well plates sealed with a NUNC™ plate seal (Thermo Fisher Scientific) as well as the residual cDNA in the Eppendorf® tubes were chilled down to -20°C or -80°C respectively for storage until any further procedures.

2.8 Quantitative PCR

Quantitative PCR (qPCR) was used to validate the microarray results by analysing gene and miRNA expression and its changes at the chosen time points of D0, D2, D14 and D28 as well as investigate gene and miRNA expression for the healthy controls.

The reagents used for the gene analyses are listed in table 4 in paragraph 2.2. Specific primers were designed using the online “Universal Probe Primer Design Service” from Roche, which was discontinued in December 2020 (Table 5).

For the analysis of some genes, TaqMan primers (Applied Biosystems; Warrington United Kingdom) were required. For the preparation of a primer mix 12.5 µl TaqMan Mastermix, 0.55 µl nuclease-free water, 0.75 µl specific target probe as well as 0.6 µl specific target forward and reverse primers respectively were added. If an assay on demand (AOD) primer was used, the primer mix consisted of 10 µl TaqMan Master Mix and 1 µl AOD primer. If preparing the primer mix for the ribosomal 18S control gene 12.5 µl TaqMan Mastermix, 2.05 µl nuclease-free water as well as 0.15 µl of Ribosomal Probe (Life technologies; Austin, USA), Ribosomal Forward Primer (Life technologies) and Ribosomal Reverse Primer (Life technologies) respectively were added. For each sample, 15 µl of the primer mix were transferred into a 96-well MicroAmp Optical qPCR plate (Applied Biosystems) before adding 10 µl of the previously diluted cDNA (25ng). One well was left as a negative control and was added 10 µl nuclease-free water. The

qPCR plate was sealed with a StarSeal Advanced Polyolefin Film (STARLAB; Hamburg, Germany before being centrifuged for 2 min at 1200 rpm (Laborfuge 400, Heraeus by Thermo Fisher Scientific). In a final step, the qPCR plate was put in a QuantStudio 6 Flex qPCR System (Applied Biosystems by Thermo Fisher Scientific) which was set to a TAMRA quencher and a VIC reporter beforehand. The system was adjusted to 40 cycles with an initial warming up of 2 min at 50°C and then 10 min at 95°C and subsequential 40 cycles consisting of 15 s at 95°C followed by 1 min at 60°C.

For the analysis of some genes, SYBR Green primers (Applied Biosystems) were used. As a first step a primer mix for the specific target gene was prepared by adding 10 µl of forward and reverse primer each to 480 µl nuclease-free water achieving a concentration of 2 µM. 2,5 µl of this primer mix was added to 12.5 µl SYBR Green Master Mix resulting in a total volume of 15 µl per sample. The n–xt step - i.e. transferring the primer mix and the cDNA into the qPCR plate - was identical to the TaqMan-based preparations described above. Regarding the setting for the qPCR system there was, however, a difference as it was adjusted to a SYBR reporter with no quencher. Of note is, that qPCR cycles of SYBR Green primers have a melt curve stage and thus the 40 consecutive cycles were set to an additional temperature increase at the end of each cycle meaning 15 s at 95°C followed by 1 min at 60°C and then 15 s at 95°C.

For the miRNA qPCR analysis, the previously described miRCURY® LNA® RT Kit (Qiagen) was used. The catalogue numbers for the respective primers are listed in table 6; all primers were SYBR® Green primers. First, a PCR master mix for each specific target miRNA was prepared by adding 5 µl 2x miRCURY® SYBR® Green Master Mix to 1 µl primer mix resulting in a total volume of 6 µl per sample. Thereafter, the PCR master mix and 4 µl of respective cDNA were transferred into the qPCR plate. 4 µl nuclease-free water was added into a separate well as a negative control. The following steps of the qPCR analysis and prior preparations were identical to the SYBR®-based qPCR described above.

2.9 Statistical analysis

2.9.1 Analysis of microarray data

All arrays were submitted to RNA-normalisation and underwent ArrayQualityMetrics quality-control. The analysis for differentially expressed genes (DEG) was conducted using Limma (Linear models of MicroArray data) for linear modelling of the DEGs. The graphic presentation of the data was done using heatmaps showing gene expression at

each analysed time point for each patient and venn diagrams showing DEGs that were overlappingly differentially expressed at the analysed time points.

2.9.2 Analysis of PROMs, total IgE levels and qPCR data

The questionnaires filled in by the patients as well as total serum IgE levels and qPCR results were analysed and figures drawn using GraphPad Prism Version 5.03 (GraphPad Software; San Diego, USA). Due to quality failures or missed time points by the patients, there were missing data for some items. For the present cohort a normal distribution could not be assumed and thus non-parametric tests were used. For PROM results and total serum IgE levels, the Wilcoxon test was used. Comparing responders to non-responders at D0, the Mann Whitney U test was applied, whereas comparing responders, non-responders and healthy controls at D0 the Kruskal-Wallis test with Dunn's corrections were used. P values ≤ 0.05 was considered significant. Comparing the changes in gene expression at D0 to D2, D14 and D28 respectively, the Wilcoxon-Test was performed for each comparison (with the application of the Bonferroni correction the P value was adjusted for these tests and set to ≤ 0.0167).

3 Results

3.1 Patient characteristics

17 patients were included in the study with a male to female ration of 6 to 11 (overview presented in table 7). The age range was 19-69 years with a mean of 44 years. The duration of ongoing CSU disease varied significantly with a range of 3 to 300 months, a median of 10 months and a mean of 46.53 months. During the course of the disease, all patients had been put on a standard dose of antihistamines initially, which had then been up-dosed up to 4-fold of the standard dose. Antihistamines used (alphabetically) were cetirizine, desloratadine, ebastine, fexofenadine, loratadine and rupatadine. All patients were inadequately controlled under antihistamines at study enrolment and start of Omalizumab treatment. All patients had received short term CS treatment for acute exacerbations of CSU at some point during the course of the disease, some patients even received long term CS treatment. 4 patients had additional montelukast (10mg daily) treatment and were still insufficiently controlled upon commencing omalizumab therapy. In terms of a search for an underlying cause for the disease, 3 patients had been diagnosed with a *helicobacter pylori* infection, treated for it and still suffered from CSU. 3 patients had concomitant thyroid disease; 1 patient suffered from rheumatoid

arthritis as another concomitant autoimmune disease. 2 patients had reported non-steroidal anti-inflammatory drug (NSAID) intolerance. 4 patients had an allergy or

Patient characteristics	
Sex ratio [male to female]	6:11
Age [range in years]	19-69
Duration of CSU [range in months]	3-300
Wheals and AE [# of patients]	11
Wheals only [# of patients]	6
On treatment with 4-fold standard dose of AH [# of patients]	17
Previous treatment with systemic CS [# of patients]	17
On treatment with montelukast	4
Thyroid disease	3
NSAID intolerance [# of patients]	2
Type I hypersensitivities/intolerances [# of patients]	4
Treatment for *h. pylori* infection [# of patients]	3
No known concomitant diseases/medications/allergies [# of patients]	6

Table 7: **List of anamnestic characteristics of the patient cohort**

The patient cohort for the study consisted of 17 patients in total – 11 female patients and 6 male patients – with a range of 19-69 years and a disease duration range of 3-300 months. 11 patients had wheals and angioedema, 6 patients – wheals only. All 17 patients received a 4-fold standard dose treatment with antihistamines and systemic corticosteroid treatment. 4 patients also had montelukast treatment. 3 patients suffered from thyroid disease, 2 – from NSAID intolerance. 4 patients had different type I hypersensitivities. 3 patients received treatment for *h. pylori* as a diagnostic work up in CSU, however with no benefit for the CSU itself. 6 patients had no reported concomitant diseases/medications/allergies. Abb.: AE = angioedema, # = number, AH = antihistamines, CS = corticosteroids, NSAID = non-steroidal anti-inflammatory drug, *h. pylori* = *helicobacter pylori*

intolerance history: 3 patients described pollen allergy (type I hypersensitivities) leading to allergic rhinitis, with one patient also suffering from related oral allergy syndrome. One of these patients was also allergic to cats (type I hypersensitivity) and another patient of this group also suffered from asthma. A dif^{fe}rent, 4th patient, was anamnestically lactose intolerant. 6 patients had no know allergies or intolerances and no concomitant diseases or medications and were anamnestically healthy apart from their CSU diagnosis.

3.2 PROM scores underline effectiveness of omalizumab in cohort and determine non-responders

To validate the chosen patient group as well as to determine patients who improved under omalizumab therapy for further analysis, PROM tools were used at D0 and D28. Patients answered the following standardized questionnaires at both visits: UCT, CU-Q2oL and DLQI (for details on these PROM tools, please refer to 1.5).

The UCT was used to define responders and non-responders for this study group (Fig. 5). At baseline no patient had a UCT score of 12 underlining the need for further therapy with the mean score being 3.6 [range: 0-8, median: 4]. The results of the UCT at D28 showed a significant improvement (p=0.0004) in respect to disease control: the mean value was 9.6 [range: 0-16] with a mean change of 6.1 points and a change range of up to 14 points to a decrease in 5 points (Fig. 4). 6 patients (35.29%) achieved a score of ≥12 meaning complete response, 2 patients even had a top score of 16 corresponding to a complete disease control. 8 patients (47.05 %) showed an increased UCT score by ≥ 3 making them partial responders. However, 3 patients (17.63 %) had either the same score as at D0 or even a decrease in UCT value. Thus, these 3 patients were determined as non-responders (NR) for all further analyses; the complete and partial responders (CR/PR) were all determined as responders for further investigations.

To further validate the patient cohort, quality of life measurement tools – the CU-Q2oL and DLQI – were used (Fig. 5). Quality of life significantly improved with respect to both PROMs with the change in DLQI score being slightly more significant than the change of the CU-Q2oL score ($p = 0.0029$ vs. $p = 0.0157$). The mean DLQI value at D0 was 14.7 [range: 1-22] compared to 8.5 [range: 0-22] at D28 with 3 patients having a DLQI score of 0 (17.63 %) at D28. The mean change value was - 5.79 with a range –f up to - 26 points to solely -1 point to no change. The NRs showed either no change or a change by 1 or 3 points respectively corresponding to the UCT score. The results of the CU-Q2oL corresponded to the DLQI and UCT results, however, were less pronounced. The mean baseline value was 71.4 [range: 34-100] decreasing to a mean of 55.9 [range 23-100] at D28. The mean change value was - 15.47, however, the NRs again showed either no change or even an increase in the total CU-Q2oL score.

Additionally, overall, the number of patients taking AH also changed significantly: at D0 all 17 patients were on (4-fold standard dose) AH compared to 10 (58.82%) patients at D28.

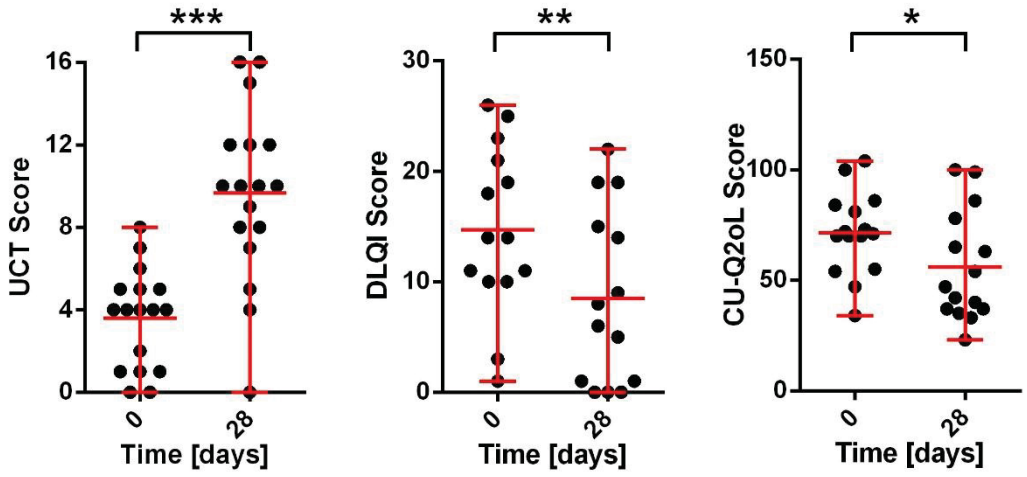

Fig. 5**: Increase of total UCT score and reduction of DLQI and CU-Q2oL scores at D28 compared to D0**

Disease control and quality of life impairment in CSU cohort at D0 and D28 for UCT (n=17), DLQI (n=14) and CU-Q2oL (n=15). UCT score range: 0-16, 16 indicates complete disease control, > 12 corresponds to a well-controlled disease defining complete treatment response and an increase by ≥ 3 defines partial response. Patients with no change or decrease in UCT value at D28 are considered non-responders. For DLQI and the more urticaria specific CU-Q2oL, 0 indicates no quality-of-life impairment, 30 or 100 respectively – maximum impairment. The dots with error bars represent means with range. Statistical analysis was performed using Wilcoxon matched pairs signed-rank test (*** equals P ≤ 0,001, ** equals P ≤ 0.01, * equals P ≤ 0.05). Abbreviations: UCT= Urticaria Control Test, DLQI = Dermatology Life Quality Index, CU-Q2oL = Chronic Urticaria Quality of Life Questionnaire, CSU = chronic spontaneous urticaria

3.3 Total serum IgE levels increase at D28 under omalizumab treatment further validating cohort

Total serum IgE levels were measured for 16 patients at D0 and D28 showing a significant increase after 4 weeks of omalizumab treatment ($p< 0.0001$, Fig. 6). At baseline the range of total serum IgE levels was 3.97 to 1867 and the distribution was as follows: 1 patient < 10 kU/l, 5 patients 10 < x < 40 kU/l, 1 patient 40 < x < 100 kU/l, and 9 patients > 100 kU/l with 6 patients 100 < x < 200 kU/l, and 2 patients > 1500 kU/l. Comparing this distribution to the clinical response it should be pointed out that is was indeed 1 NR who had a D0 total serum IgE level < 10 kU/l., however both other NR were in the group between 100 and 200 kU/l. Notably, 3 of the CR were in the group of total serum IgE levels between 10 and 40 kU/l and the other 3 in the group between 100 and 200 kU/l.

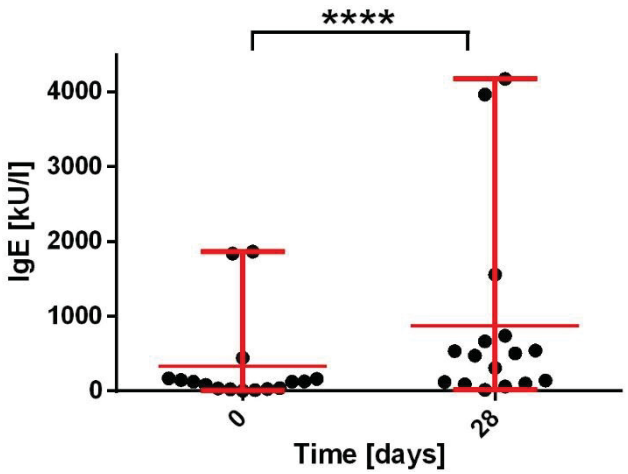

Fig. 6: **Increase in total serum IgE levels at D28 and D28**

Depicted are total serum IgE levels in kU/l at baseline compared to D28 (n=16). The dots with error bars represent means with range. Statistical analysis was performed using Wilcoxon matched pairs signed-rank test (**** equals P ≤ 0.0001). Abbreviation: IgE = immunoglobulin E

Both patients with IgE levels > 1500 kU/l were PR. All but 1 patient (a PR) showed an increase in total serum IgE levels at D28 [range: 16.5-4177, mean: 875.15 and median: 490 kU/l]. The range of increase varied between 12.53 and 2337 kU/l, the median increase was by 3.7-fold [range: 2.12. - 42.35]. For the 3 NR no tendency could be shown: they all showed an increase in total IgE levels by either 2.43, 3.9 or 4.16-fold – so it cannot be said that they showed a lesser increase than PR or CR. The biggest change of a 42.35-fold increase was indeed shown by a CR, however other CR showed an increase by as small as 3.29-fold.

In the described cohort, no correlation between the total IgE levels and age or disease severity (comparing to UCT, CU-Q2oL and DLQI scores at baseline) was observed.

3.4 DNA microarray depicts a complex regulation of gene expression

Four responders were included in the DNA microarray analysis, where changes between D0 to D2 and D0 to D14 under omalizumab treatment were compared (Fig. 7). Both analyses showed mostly upregulation in DEGs, especially for the comparison of D0 to D2. For visualisation of the top 150 regulated genes heat maps were used. The top regulated genes could be divided into clusters with similar gene expression patterns. One cluster with a smaller amount of DEGs showed mostly downregulation, the other 2 clusters showed mostly upregulation of DEGs, e.g. CD52, COX7B, CLEC2B, COMMD8, GIMAP2 or FcεR1A. The validated patients P1, P6, P12, P15 were responders based on

their UCT at D28. At D2 the biggest changes in DEGs were shown for P6 followed by P15, P1 and P12 (Fig. 7A). Overall, the change of gene regulation at D14 was less prominent than at D2. Here the biggest change in DEGs is shown for P1 followed by P6 and P12. DEGs for P15 at D14 appear to be mostly unchanged in their expression with some up- and downregulation (Fig. 7B). Clinically, P1 was a CR with a UCT score of 12 at D28 and showed the biggest improvement under omalizumab compared to P6, P12, P15 who were PR with an increase of the UCT score ≥3 up to 10 at D28. None of the patients needed to take any antihistamines under omalizumab treatment underlining their response to therapy.

In order to narrow down the number of possible candidate genes for further validation via qPCR analysis, the regulated genes with a cut-off log fold change of 0,5 at D2 and D14 compared to D0 were analysed and thus 160 overlapping genes were found to be regulated at both time intervals. The analysis is illustrated in a venn diagram (Fig. 8), the full list of the overlapping DEGs and corresponding Gene Ontology Terms (GO-Terms) can be found in the supplemental tables 1 and 2.

All the genes were looked up in pubmed database with special interest regarding involvement in T cell pathways and any known involvement in urticaria. Thus, the following genes of special interest were identified fur further assessment: *CD52, COX7B, COMMD6, FcεR1A, CLEC2B, GIMAP2, LTF* and *COMMD8. CD69, CD28, IntegrinB3, IL6R,* as well as *CXCR1* and *CXCL5* were chosen as additional candidate genes due to their possible involvement in T cell pathways or to have a MC association.

Fig. 7A

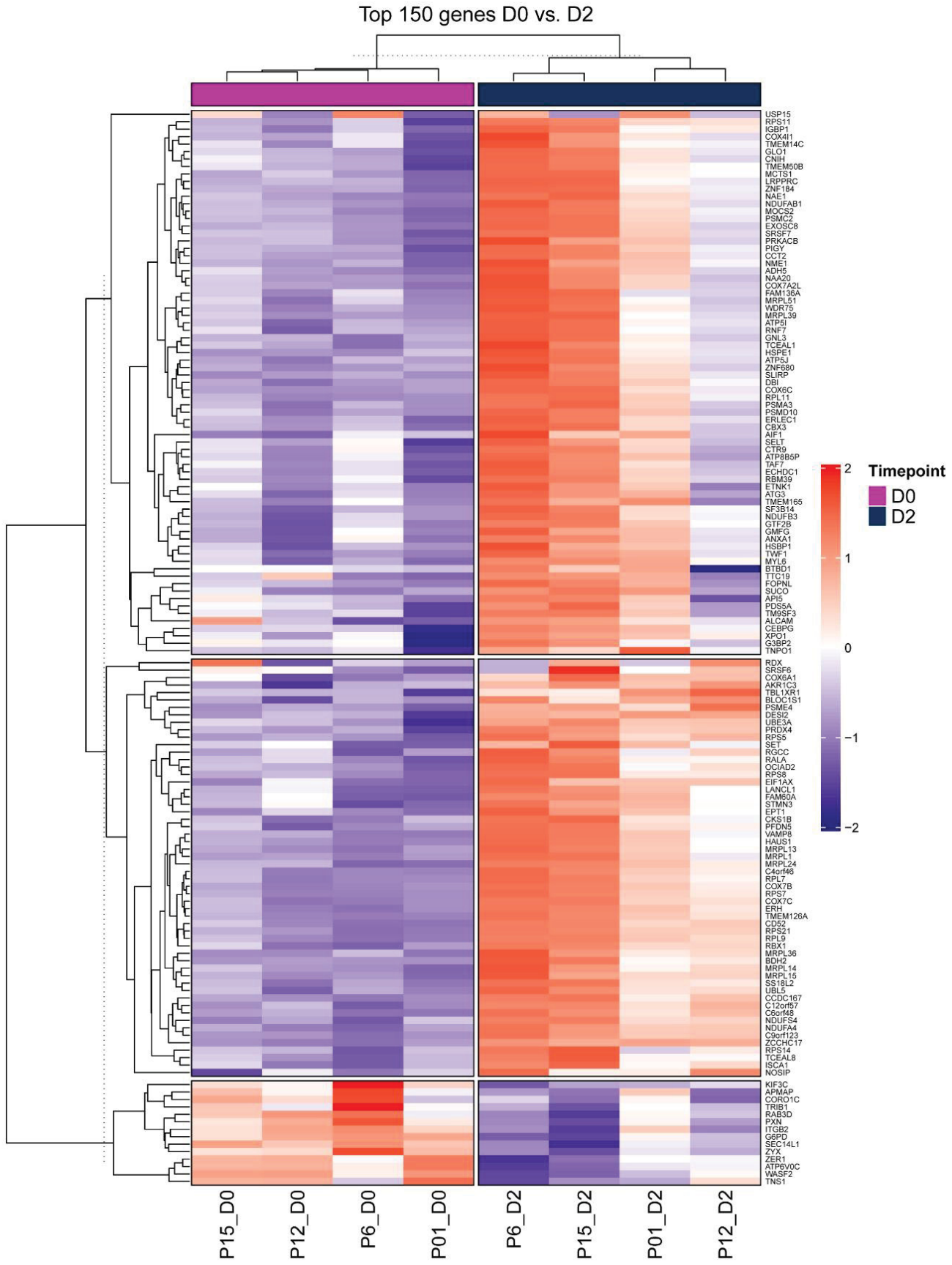
Top 150 genes D0 vs. D2
Timepoint
D0
D2
2
1
0
−1
−2
P15_D0
P12_D0
P6_D0
P01_D0
P6_D2
P15_D2
P01_D2
P12_D2

Fig. 7B

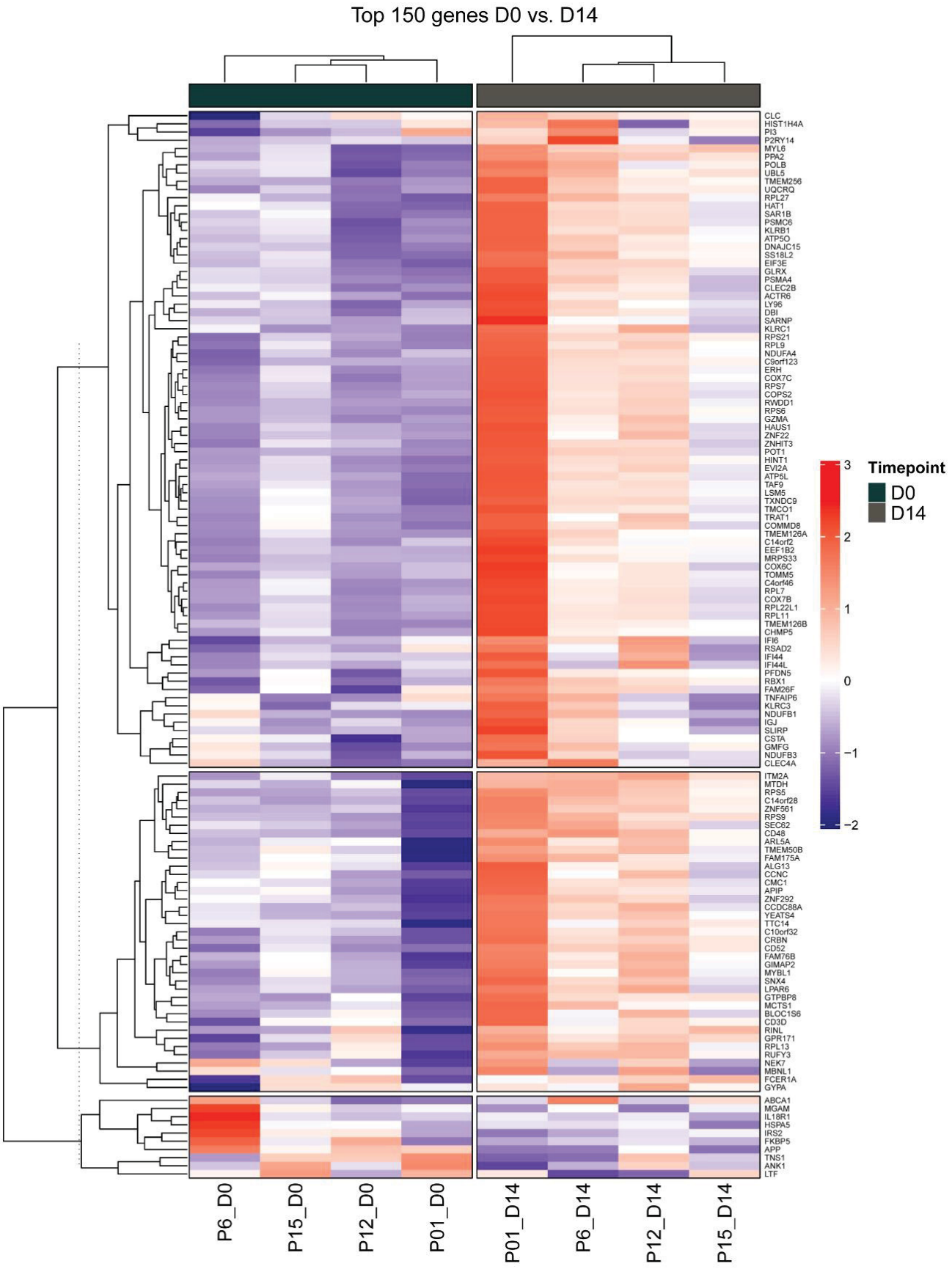
Top 150 genes D0 vs. D14
Timepoint
D0
D14
3
2
1
0
−1
−2
CLC
HIST1H4A
PI3
P2RY14
MYL6
PPA2
POLB
UBL5
TMEM256
UQCRQ
RPL27
HAT1
SAR1B
PSMC6
KLRB1
ATP5O
DNAJC15
SS18L2
EIF3E
GLRX
PSMA4
CLEC2B
ACTR6
LY96
DBI
SARNP
KLRC1
RPS21
RPL9
NDUFA4
C9orf123
ERH
COX7C
RPS7
COPS2
RWDD1
RPS6
GZMA
HAUS1
ZNF22
ZNHIT3
POT1
HINT1
EVI2A
ATP5L
TAF9
LSM5
TXNDC9
TMCO1
TRAT1
COMMD8
TMEM126A
C14orf2
EEF1B2
MRPS33
COX6C
TOMM5
C4orf46
RPL7
COX7B
RPL22L1
RPL11
TMEM126B
CHMP5
IFI6
RSAD2
IFI44
IFI44L
PFDN5
RBX1
FAM26F
TNFAIP6
KLRC3
NDUFB1
IGJ
SLIRP
CSTA
GMFG
NDUFB3
CLEC4A
ITM2A
MTDH
RPS5
C14orf28
ZNF561
RPS8
SEC62
CD48
ARL5A
TMEM50B
FAM175A
ALG13
CCNC
CMC1
APIP
ZNF292
CCDC88A
YEATS4
TTC14
C10orf32
CRBN
CD52
FAM76B
GIMAP2
MYBL1
SNX4
LPAR6
GTPBP8
MCTS1
BLOC1S6
CD3D
RNL
GPR171
RPL13
RUFY3
NEK7
MBNL1
FCER1A
GYPA
ABCA1
MGAM
IL18R1
HSPA5
IRS2
FKBP5
APP
TNS1
ANK1
LTF
P6_D0
P15_D0
P12_D0
P01_D0
P01_D14
P6_D14
P12_D14
P15_D14

Fig. 7: **Heat map analysis of top regulated genes of 4 CSU patients at D2 and D14 compared to D0**

Heat map depicting DEGs at D2 and D14 compared to D0 based on DNA microarray analysis (A and B respectively). X-axis showing the 4 evaluated patients P1, P6, P12 and P15 at the time points of either D0 or D2 and D14 respectively (each patient corresponds to 1 column at either D0, D2 or D14). The y-axis depicts the top regulated genes arranged in dendrograms with similar gene expression patterns by hierarchical clustering. Red colour stands for gene upregulation, purple – for gene downregulation and white – for no regulation in gene expression. Abbreviations: CSU = chronic spontaneous urticaria, D (0,2,14) = day (0,2,14), DEG = differentially expressed genes, P (1,6,12,15) = patient (1,6,12,15)

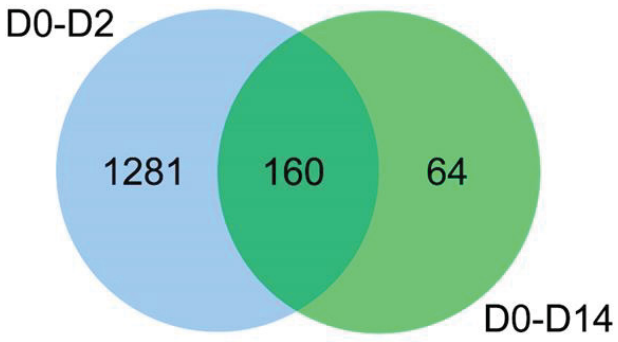

Fig. 8: **Venn diagram of overlapping DEGs in microarray analysis of gene regulation at D0 compared to D2 and D14**

Venn diagram illustrating on one side 1281 DEGs and on the other side 64 DEGs comparing gene regulation changes at D0 to D2 and D14 respectively (cut-off: 0.5 log fold change). The overlap of both sides shows that 160 DEGs are regulated in both analysed data sets (D0 to D2 and D0 to D14). For detailed list of the 160 overlapping DEGs please refer to the supplemental table 1. Abbreviations: D (0,2,14) = day (0,2,14), DEG = differentially expressed genes

3.5 miRNA microarray shows modest regulation changes

In the second part of the study, the goal was to identify candidate miRNAs for further assessments. In total 3 responders – P6, P9 and P15 – were investigated at D0 to D2, D14 and D28 respectively. In comparison to the DNA microarray results, the miRNA results were less prominent, but similarly showed mostly upregulation of miRNA expression. It should be noted, that, of course, the pool of possible miRNAs is smaller than of possible genes in DNA microarray analysis (approximately 6000 human miRNAs vs 20000 human genes). However, there was still a smaller number of noticeably regulated miRNAs through all investigated time points. The results were visualised in heatmaps (Fig. 9). For the comparison of D0 to D2, D14 and D28 respectively, the top expressed miRNAs could be divided into 2 clusters with similar expression patterns. For the comparison of D0 to D2, one cluster with a higher number of miRNAs showed mainly

upregulation, whereas the other cluster with a lesser amount of top expressed miRNAs showed mostly downregulation (Fig. 9A). The biggest changes in differentially expressed miRNAs were observed for P6, followed by P15 and then by P9. Interestingly, the most prominent relative changes of miRNA expression levels in P6 and P15 were upregulation, whereas in P9 a slight downregulation could be observed for most of the top expressed miRNAs. Comparing D0 to D14 and D28 respectively, the cluster with the bigger amount of the top differentially expressed miRNAs showed mainly downregulation (Fig. 9B and 9C). However, it should be noted that here, the changes in regulation overall are less prominent than in the first comparison of D0 and D2, with the least prominent regulation and even no regulation at D28. Clinically, P9 was a CR with a UCT value of 12 at D28 whereas P6 and P15 were PR with an increase of the UCT score ≥3 up to 10 at D28. None of the chosen patients needed to take additional antihistamines.

Fig. 9A

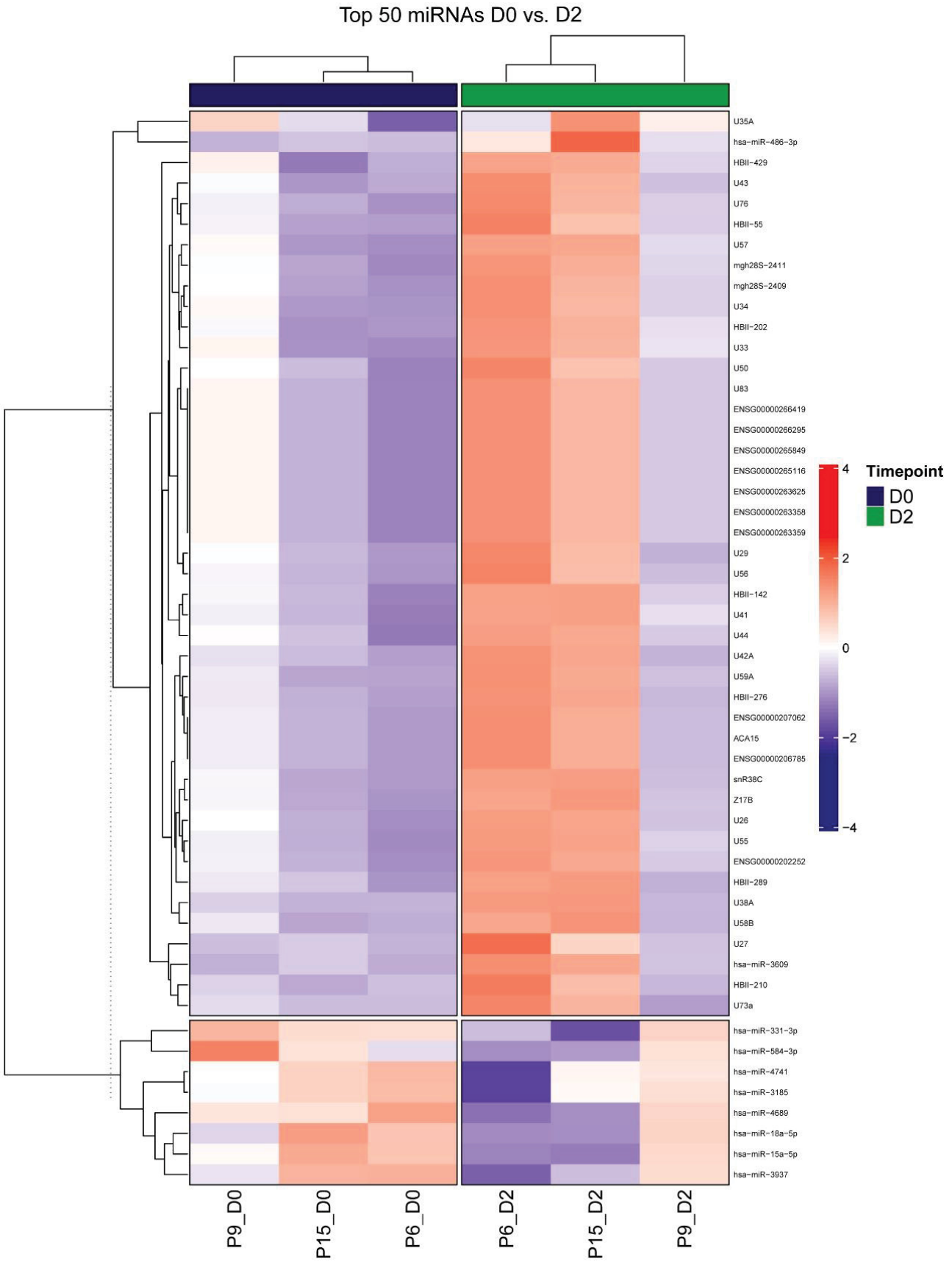
Top 50 miRNAs D0 vs. D2
U35A
hsa-miR-486-3p
HBII-429
U43
U76
HBII-55
U57
mgh28S-2411
mgh28S-2409
U34
HBII-202
U33
U50
U83
ENSG00000266419
ENSG00000266295
ENSG00000265849
ENSG00000265116
ENSG00000263625
ENSG00000263358
ENSG00000263359
U29
U56
HBII-142
U41
U44
U42A
U59A
HBII-276
ENSG00000207062
ACA15
ENSG00000206785
snR38C
Z17B
U26
U55
ENSG00000202252
HBII-289
U38A
U58B
U27
hsa-miR-3609
HBII-210
U73a
hsa-miR-331-3p
hsa-miR-584-3p
hsa-miR-4741
hsa-miR-3185
hsa-miR-4689
hsa-miR-18a-5p
hsa-miR-15a-5p
hsa-miR-3937
P9_D0
P15_D0
P6_D0
P6_D2
P15_D2
P9_D2
Timepoint
D0
D2
4
2
0
−2
−4

Fig. 9B

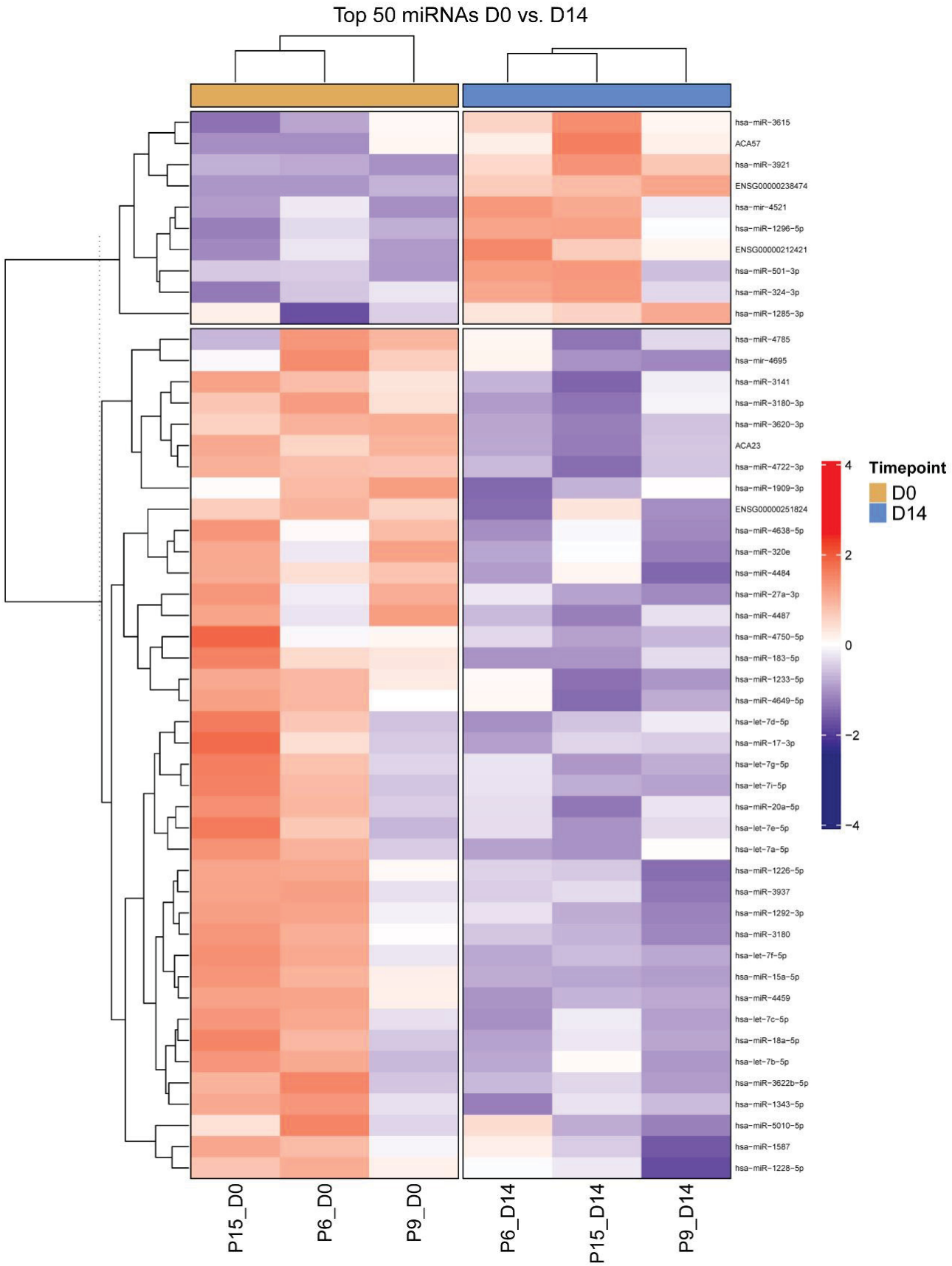
Top 50 miRNAs D0 vs. D14
hsa-miR-3615
ACA57
hsa-miR-3921
ENSG00000238474
hsa-mir-4521
hsa-miR-1296-5p
ENSG00000212421
hsa-miR-501-3p
hsa-miR-324-3p
hsa-miR-1285-3p
hsa-miR-4785
hsa-mir-4695
hsa-miR-3141
hsa-miR-3180-3p
hsa-miR-3620-3p
ACA23
hsa-miR-4722-3p
hsa-miR-1909-3p
ENSG00000251824
hsa-miR-4638-5p
hsa-miR-320e
hsa-miR-4484
hsa-miR-27a-3p
hsa-miR-4487
hsa-miR-4750-5p
hsa-miR-183-5p
hsa-miR-1233-5p
hsa-miR-4649-5p
hsa-let-7d-5p
hsa-miR-17-3p
hsa-let-7g-5p
hsa-let-7i-5p
hsa-miR-20a-5p
hsa-let-7e-5p
hsa-let-7a-5p
hsa-miR-1226-5p
hsa-miR-3937
hsa-miR-1292-3p
hsa-miR-3180
hsa-let-7f-5p
hsa-miR-15a-5p
hsa-miR-4459
hsa-let-7c-5p
hsa-miR-18a-5p
hsa-let-7b-5p
hsa-miR-3622b-5p
hsa-miR-1343-5p
hsa-miR-5010-5p
hsa-miR-1587
hsa-miR-1228-5p
P15_D0
P6_D0
P9_D0
P6_D14
P15_D14
P9_D14
Timepoint
D0
D14
4
2
0
−2
−4

Fig. 9C

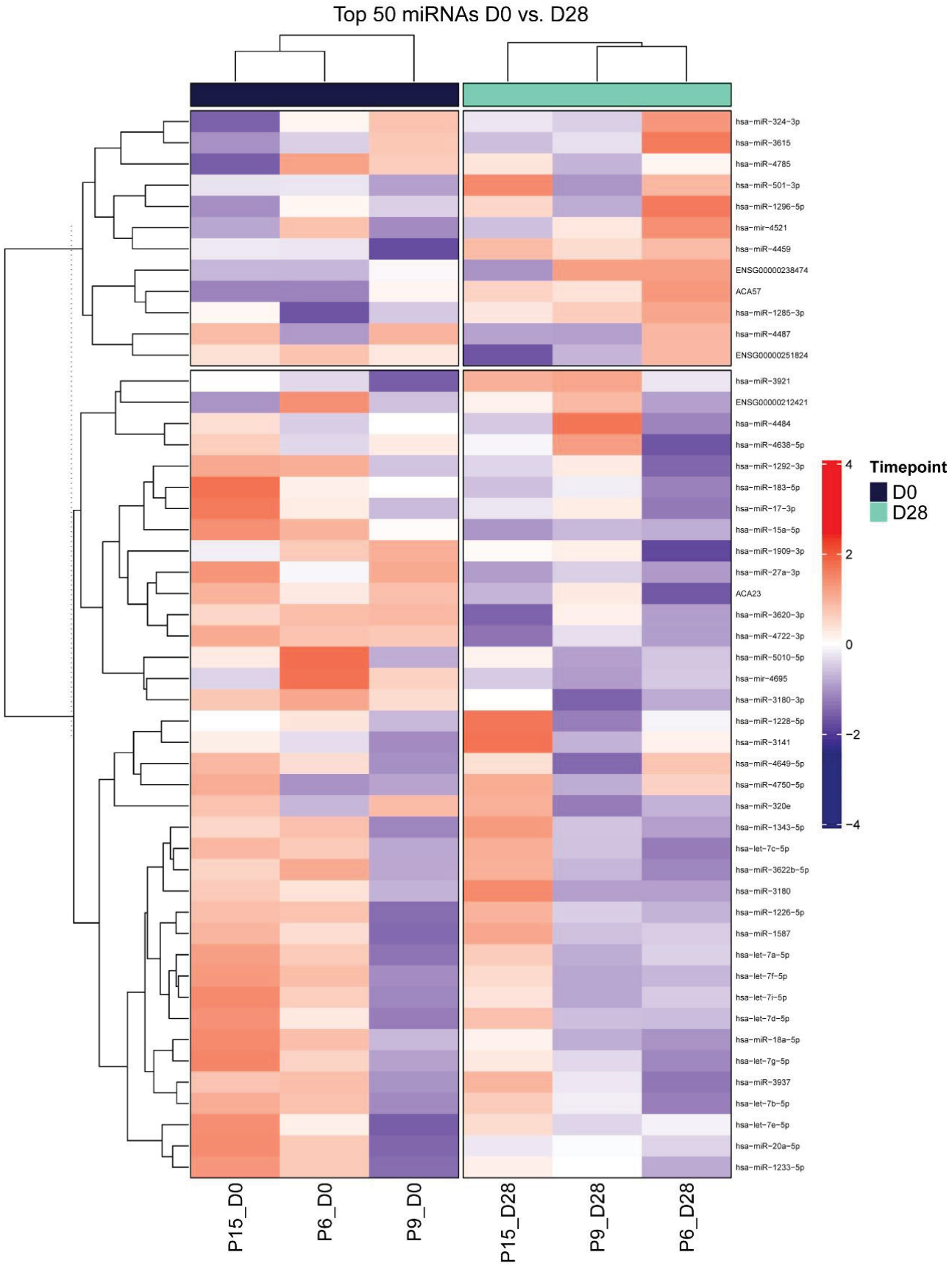
Top 50 miRNAs D0 vs. D28
hsa-miR-324-3p
hsa-miR-3615
hsa-miR-4785
hsa-miR-501-3p
hsa-miR-1296-5p
hsa-mir-4521
hsa-miR-4459
ENSG00000238474
ACA57
hsa-miR-1285-3p
hsa-miR-4487
ENSG00000251824
hsa-miR-3921
ENSG00000212421
hsa-miR-4484
hsa-miR-4638-5p
hsa-miR-1292-3p
hsa-miR-183-5p
hsa-miR-17-3p
hsa-miR-15a-5p
hsa-miR-1909-3p
hsa-miR-27a-3p
ACA23
hsa-miR-3620-3p
hsa-miR-4722-3p
hsa-miR-5010-5p
hsa-mir-4695
hsa-miR-3180-3p
hsa-miR-1228-5p
hsa-miR-3141
hsa-miR-4649-5p
hsa-miR-4750-5p
hsa-miR-320e
hsa-miR-1343-5p
hsa-let-7c-5p
hsa-miR-3622b-5p
hsa-miR-3180
hsa-miR-1226-5p
hsa-miR-1587
hsa-let-7a-5p
hsa-let-7f-5p
hsa-let-7i-5p
hsa-let-7d-5p
hsa-miR-18a-5p
hsa-let-7g-5p
hsa-miR-3937
hsa-let-7b-5p
hsa-let-7e-5p
hsa-miR-20a-5p
hsa-miR-1233-5p
P15_D0
P6_D0
P9_D0
P15_D28
P9_D28
P6_D28
4
2
0
−2
−4
Timepoint
D0
D28

Fig. 9: **Heat map analysis of top regulated miRNAs of 3 CSU patients at D2, D14 and D28 compared to D0**

Heat map depicting regulated miRNAs at D2, D14, D28 compared to D0 based on miRNA microarray analysis (A, B and C respectively). X-axis showing the 3 evaluated patients P6, P9 and P15 at the time points of either D0 or D2, D14 and D28 respectively (each patient corresponds to 1 column at either D0, D2, D14 or D28). The y-axis depicts the top regulated miRNAs arranged in dendrograms with similar expression patterns. Red colour stands for upregulation in miRNA expression, purple – for downregulation and white – for no regulation. Abbreviations: CSU = chronic spontaneous urticaria, D (0,2,14,28) = day (0,2,14,28), P (6,9,15) = patient (6,9,15)

To assess the modest differences and to identify possible candidate miRNAs, the most regulated miRNAs were arranged into 12 clusters of similar miRNA expression patterns (Fig. 10). This exploratory analysis was consistent with the DNA microarray analysis showing that most changes in regulation happen at D2. Out of the 12 clusters, cluster

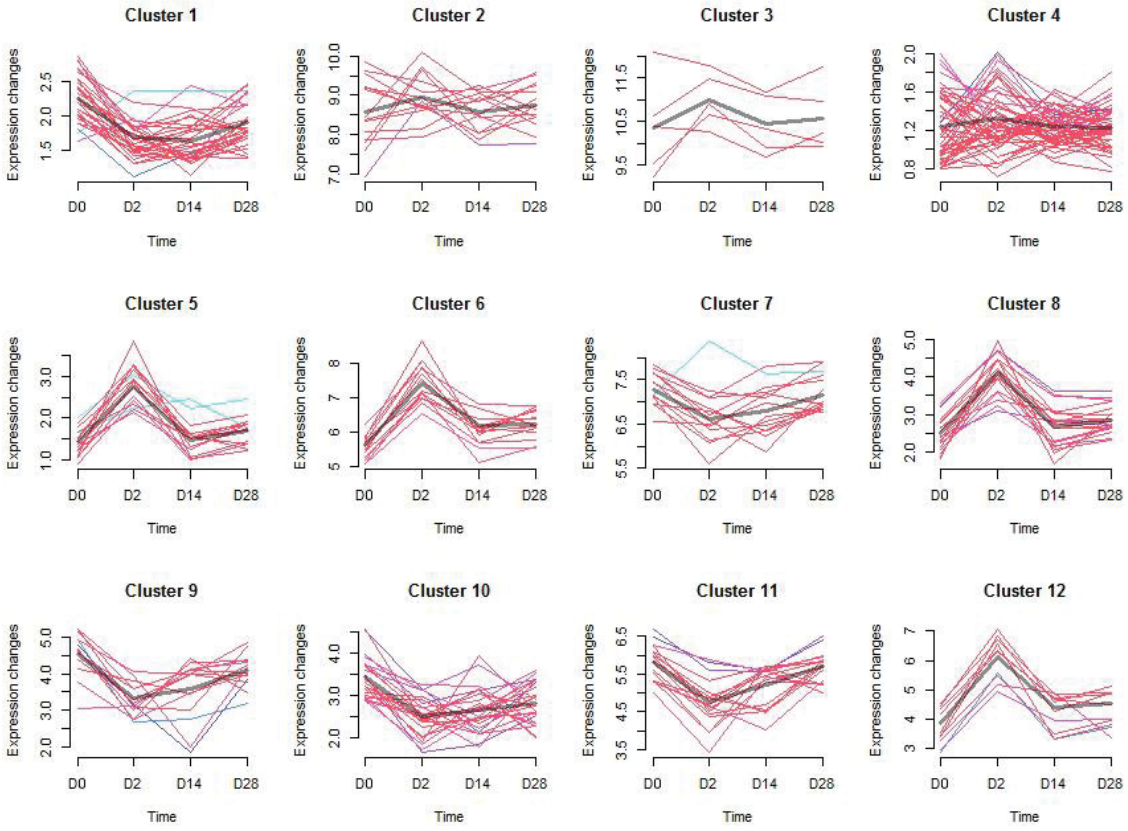

Fig. 10: **Expression changes of top regulated miRNAs at D0, D2, D14 and D28 arranged into clusters**

The figure illustrates expression changes of top regulated miRNAs of 3 CSU patients under treatment with omalizumab. The miRNAs are arranged into 12 clusters of similar miRNA expression patterns. The y-axis shows the expression change at the different time points depicted at the x-axis (D0, D2,D14, D28). Most prominent changes are seen at D2, most noticeable in the miRNA behaviour of cluster 5,6,8,10 and 12. In cluster 5,6,8 and 12 miRNA expression appears to be upregulated at D2, whereas in cluster 1o it appears to be downregulated. Abbreviations: D (0,2,14,28) = day (0,2,14,28), CSU = chronic spontaneous urticaria

5,6,8, 10 and 12 showed the most prominent behaviour with the most noticeable peak at D2 and lesser regulation changes at D14 and discreet changes at D28. Cluster 5,6,8, and 12 showed upregulation whereas cluster 10 showed downregulation of the respective miRNAs at D2. The miRNAs belonging to these clusters are listed in table 3 of the appendix. Furthermore, data from the DNA microarray analysis (with related mRNA) were correlated with the data from this miRNA microarray analysis. This analysis was illustrated by a co-expression network (Fig.11), which led to the identification of 5 miRNAs from the 5 prominent clusters chosen above: hsa-let-7e-5p, hsa-miR-3609, hsa-miR-6808-3p, hsa-miR-6716-5p and hsa-miR-486-3p.

Fig. 11A

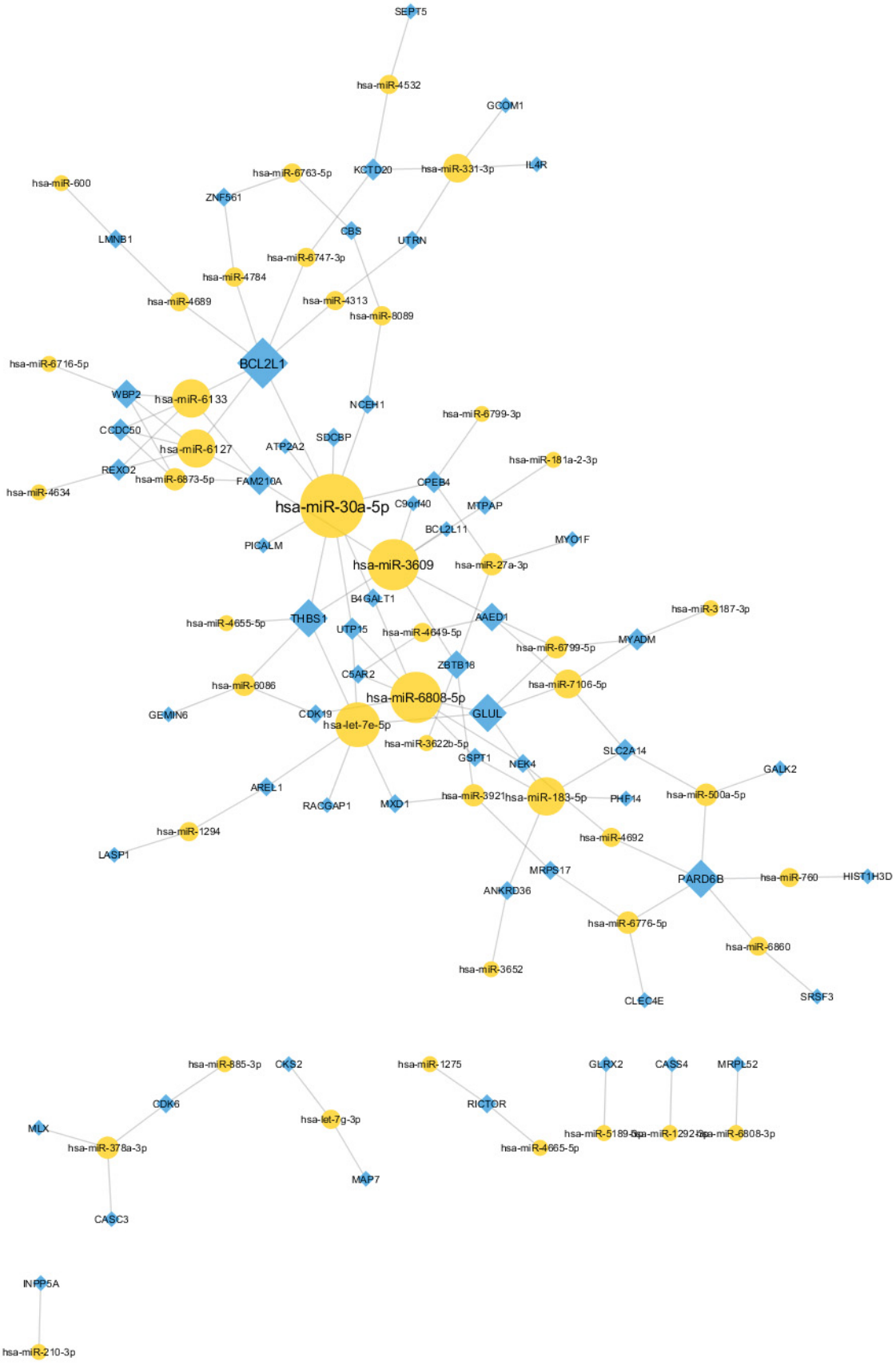
SEPT5
hsa-miR-4532
GCOM1
hsa-miR-6763-5p
KCTD20
hsa-miR-331-3p
IL4R
hsa-miR-600
ZNF561
LMNB1
CBS
UTRN
hsa-miR-6747-3p
hsa-miR-4784
hsa-miR-4689
hsa-miR-4313
hsa-miR-8089
hsa-miR-6716-5p
BCL2L1
WBP2
hsa-miR-6133
NCEH1
hsa-miR-6799-3p
CCDC50
hsa-miR-6127
ATP2A2
SDCBP
hsa-miR-181a-2-3p
REXO2
hsa-miR-6873-5p
FAM210A
CPEB4
hsa-miR-4634
hsa-miR-30a-5p
C9orf40
MTPAP
BCL2L11
MYO1F
PICALM
hsa-miR-3609
hsa-miR-27a-3p
B4GALT1
hsa-miR-3187-3p
hsa-miR-4655-5p
THBS1
UTP15
AAED1
hsa-miR-4649-5p
MYADM
hsa-miR-6799-5p
ZBTB18
C5AR2
hsa-miR-6086
hsa-miR-6808-5p
hsa-miR-7106-5p
GEMIN6
CDK19
GLUL
hsa-let-7e-5p
hsa-miR-3622b-5p
SLC2A14
GSPT1
NEK4
GALK2
AREL1
hsa-miR-3921
hsa-miR-183-5p
PHF14
hsa-miR-500a-5p
RACGAP1
MXD1
hsa-miR-1294
hsa-miR-4692
LASP1
MRPS17
PARD6B
hsa-miR-760
HIST1H3D
ANKRD36
hsa-miR-6776-5p
hsa-miR-6860
hsa-miR-3652
CLEC4E
SRSF3
hsa-miR-885-3p
CKS2
hsa-miR-1275
GLRX2
CASS4
MRPL52
CDK6
RICTOR
MLX
hsa-let-7g-3p
hsa-miR-4665-5p
hsa-miR-6808-3p
hsa-miR-378a-3p
MAP7
CASC3
INPP5A
hsa-miR-210-3p

Fig. 11B

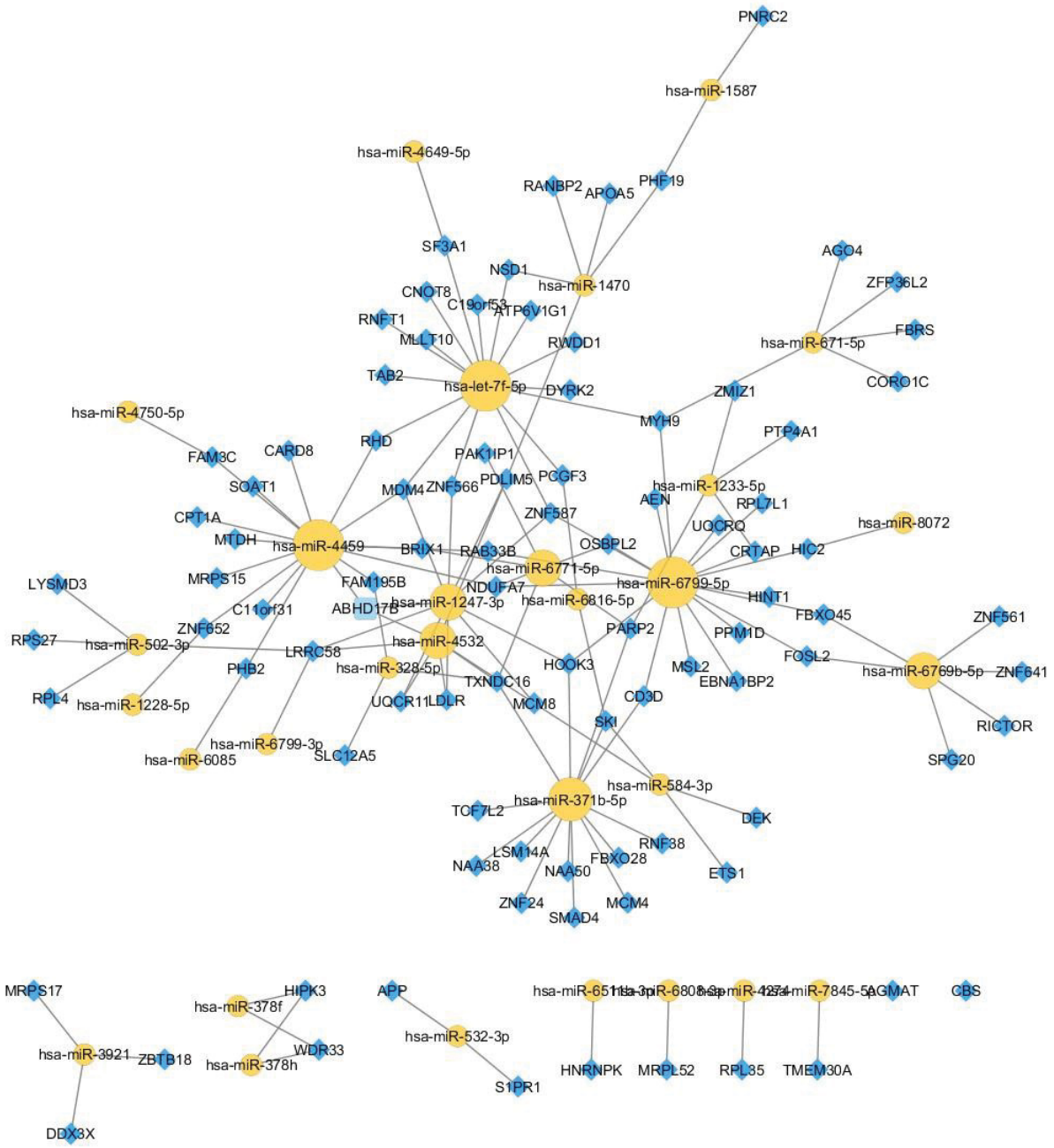

Fig. 11: **Co-expression network of mRNA and top regulated miRNA from DNA and miRNA microarray analyses at D0 to D2 and D14**

Analysis of both DNA and miRNA microarray results is illustrated by a co-expression network showing possible interactions of mRNAs and miRNAs at D0 to D2 (A) and D0 to D14 (B). Circles represent miRNAs, whereas rhomboids represent mRNAs. Lines indicate possible interactions. Abbreviation: D (0,2,14,28) = day (0,2,14,28)

3.6 Validation of chosen genes shows tendency to upregulation at D2 and significant upregulation of CD28

For the first gene validation study sera of all above described 17 patients and of 8 healthy controls were used. For each gene, gene expression levels of healthy controls as well as responders (n=14) and non-responders (n=3) at baseline (D0) were compared (Fig. 12). Furthermore, the change in gene expression levels at D2, D14 and D28 compared to D0 for the responder cohort was looked at (here the Bonferroni correction was applied, corrected p value was set to 0.0167). A general observation is the tendency to gene upregulation at D2 thus proving the microarray analysis results suggesting most regulation taking place at D2 comparing the proposed time points. Another general observation is that most gene expression levels stay upregulated at D28 if looking at the mean expression levels at D0 compared to D28. However, of the chosen genes, only the gene expression of *CD28* was significantly altered in respect to the expression levels at D2 showing a significant upregulation compared to D0 (p=0.0161). The qPCR results showed a significant downregulation at D14 compared to D0 for *IntegrinB3* (p=0.0093), which is different from the overall tendency to greatest visible regulation at D2. A relevant upregulation tendency at D2 compared to D0 (p=0.0266) could be observed for *CD52*. For expression levels of *COMMD6, CLEC2B*, *CD69* and *COX7B* no relevant changes can be observed, however looking at the behaviour of expression changes, an upregulation tendency at D2 can be noted followed by a visible downregulation at D14 and D28 compared to the mean expression value at D2. No relevant changes can be observed for the regulation of *FCεR1A* and *IL6R*.

Significant regulation can also be observed in the comparison of healthy controls, responders and non-responders with some of the chosen genes: For *CLEC2B*, gene expression levels of healthy controls could be seen to be significantly downregulated compared to the responders (p=0.0008), whereas for *FCεR1A* and *CD28*, the levels of healthy controls showed to be upregulated compared to the responders (p=0.0089 and p=0.0074 respectively). Overall, no clear behaviour could be detected regarding gene regulation of healthy controls and responders and non-responders at baseline apart from the above mentioned.

A smaller cohort was chosen for the validation of *CXCL5*, *COMMD8*, *LTF*, *GIMAP2* and *CXCR1* (Fig. 13). Here 10 (*COMMD8*, *LTF*, *GIMAP2*) or 7 (*CXCL5*, *CXCR1*) were compared to the 3 non-responders at D0 as well as the changes for the respective gene expression levels at D2, D14 and D28 compared to D0 for the responder cohort. Among these 5 chosen genes none proved to be significantly altered in their expressions at D2,

D14 and D28 compared to D0. Again, a tendency for the most upregulation can be observed at D2. No significant differences could be demonstrated between responders and non-responders at D0.

Fig. 12A

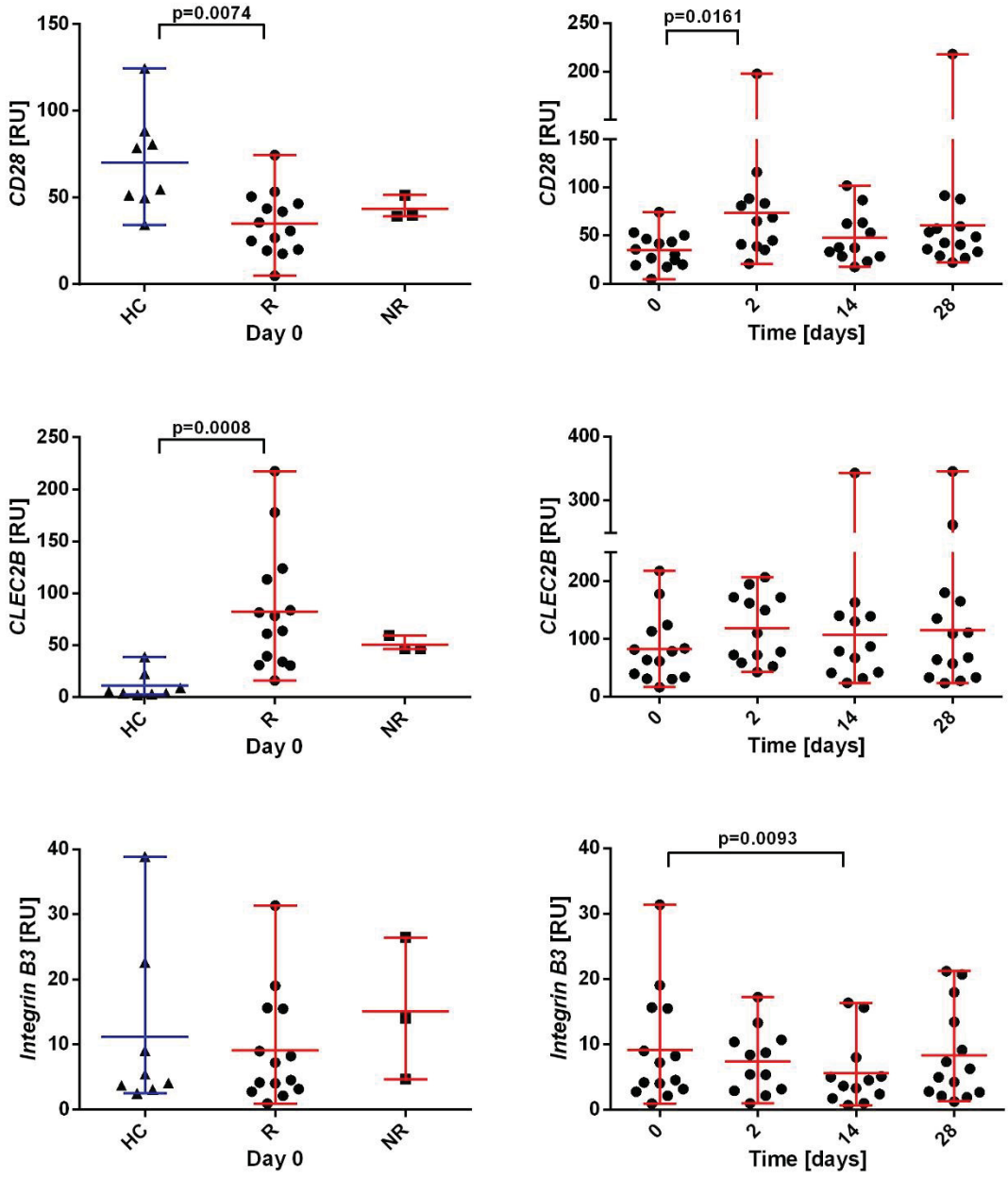

Fig. 12B

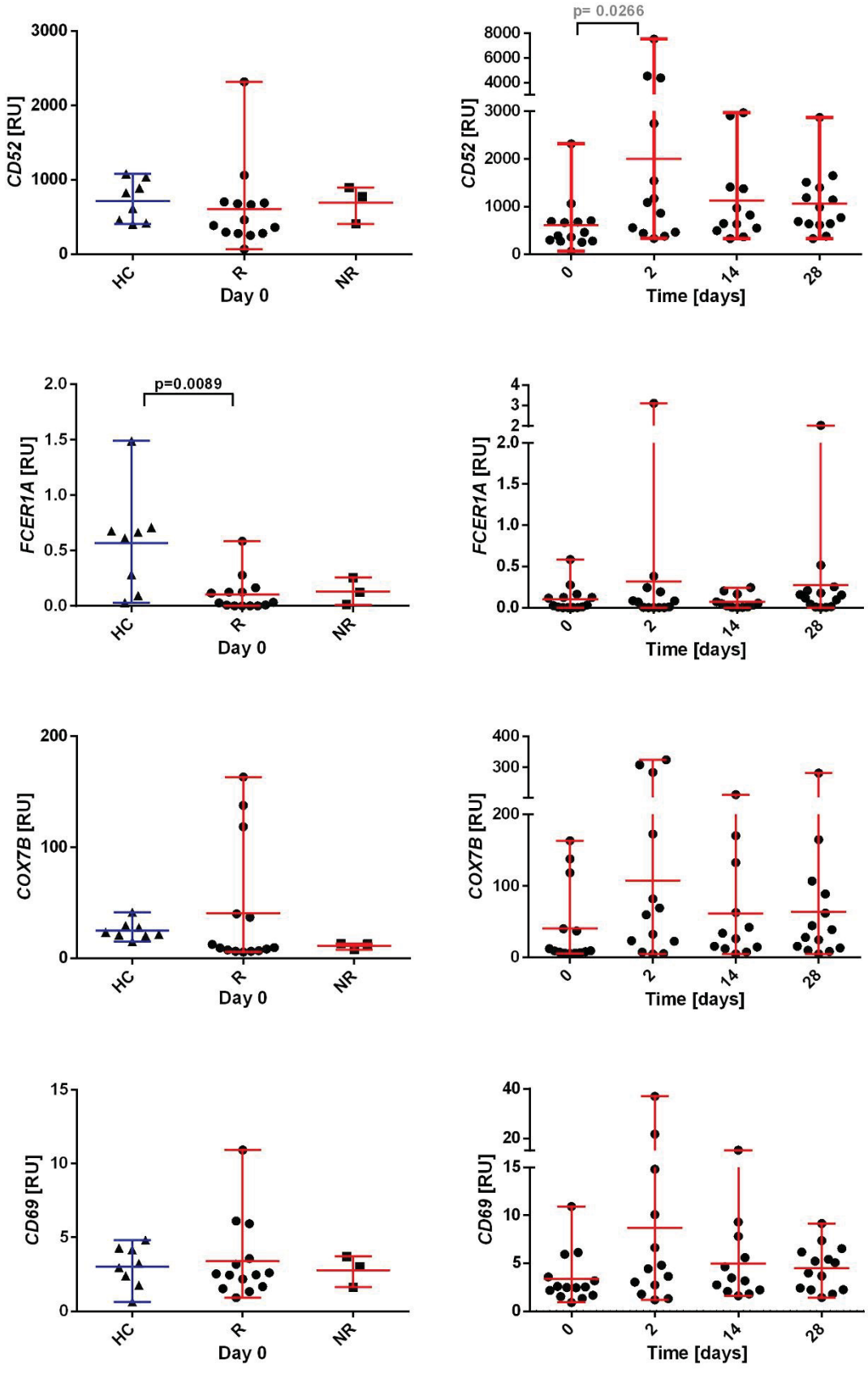
CD52 [RU]
3000
2000
1000
0
HC
R
NR
Day 0
p= 0.0266
8000
6000
4000
3000
2000
1000
0
0
2
14
28
Time [days]
FCER1A [RU]
p=0.0089
2.0
1.5
1.0
0.5
0.0
4
3
2
COX7B [RU]
200
100
400
300
CD69 [RU]
15
10
5
40
20

Fig. 12C

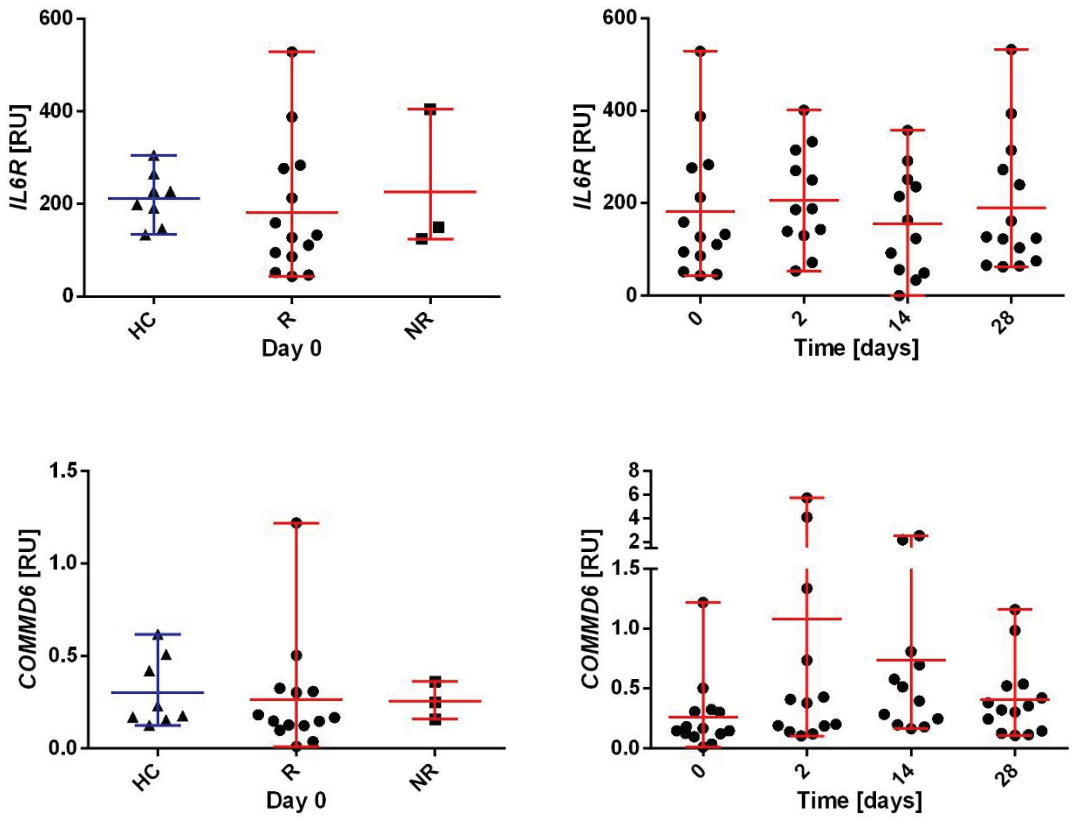

Fig. 12: **qPCR analysis of *CD28, CLEC2B, IntegrinB3, CD52, FcεR1A, COX7B, CD69 IL6R* and *COMMD6***

Depicted are gene expression levels of *CD28, CLEC2B, InegrinB3* (A), *CD52, FcεR1A, COX7B, CD69* (B) and *IL6R, COMMD6* (C) comparing either R, NR (n=3) and HC (n=8) or the changes of gene expression levels for R at D0, D2, D14 and D28. Number of responders at given time points varies due to missing time points or quality failures as follows: D0 – n=17, D2/D14 – n=15-16, D28 – n=16-17. Comparing R, NR and HC the Kruskal-Wallis test with Dunn's post-test were used. The changes of gene expression at D2, D14 and D28 each compared to D0 were analysed using the Wilcoxon test - significant P values in black (significant P value set to 0.0167 using Bonferroni correction), other relevant P values as depicted in grey. The dots with error bars represent means with range. Data shown in RU compared to 18S. Abbreviations: D (0,2,14,28) = day (0,2,14,28), RU = relative expression units, R = responder, NR = non-responder, HC = healthy control

Fig. 13A

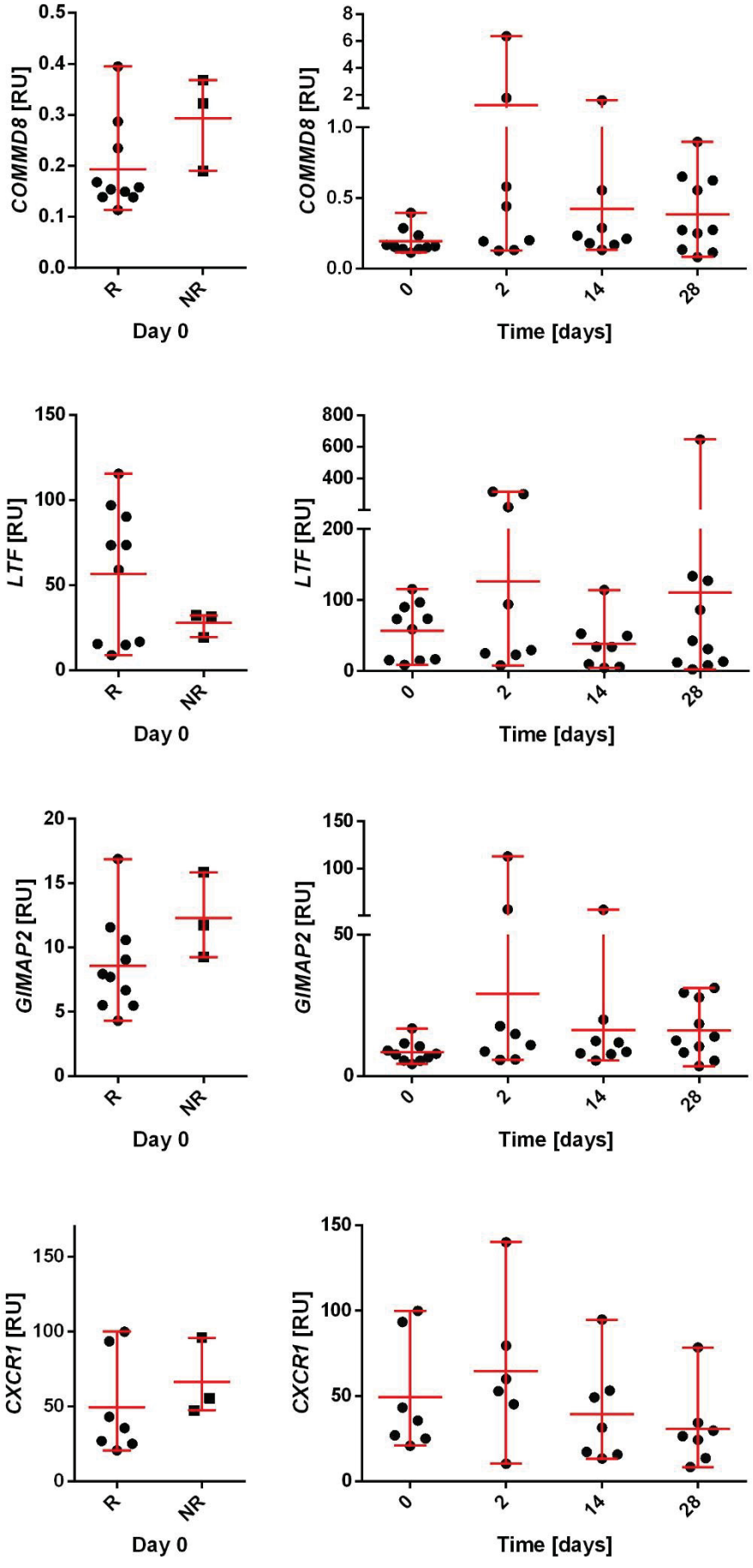
COMMD8 [RU]
0.5
0.4
0.3
0.2
0.1
0.0
R
NR
Day 0
COMMD8 [RU]
8
6
4
2
1.0
0.5
0.0
0
2
14
28
Time [days]
LTF [RU]
150
100
50
0
R
NR
Day 0
LTF [RU]
800
600
400
200
100
0
0
2
14
28
Time [days]
GIMAP2 [RU]
20
15
10
5
0
R
NR
Day 0
GIMAP2 [RU]
150
100
50
0
0
2
14
28
Time [days]
CXCR1 [RU]
150
100
50
0
R
NR
Day 0
CXCR1 [RU]
150
100
50
0
0
2
14
28
Time [days]

Fig. 13B

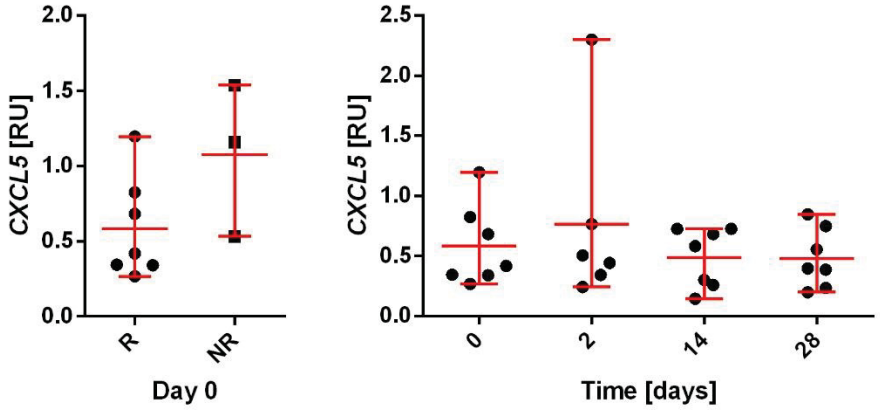

Fig. 13: **qPCR analysis of CXCL5, COMMD8, LTF, GIMAP2 and CXCR1**

Depicted are gene expression levels of CXCL5, COMMD8, LTF, GIMAP2 (A) and CXCR1 (B) comparing either R and NR (n=3) or the changes of gene expression levels for R at D0, D2, D14 and D28. Number of responders varies at given time points due to missing time points or quality failures as follows: D0/28 n=13 and D2/14 n=11-13 for COMMD8, LTF, GIMAP2; D0/14 n=10 and D2/28 n=9-10 for CXCL5, CXCR1. Comparing R and NR, Mann Whitney U test was used. The changes of gene expression at D2, D14 and D28 each compared to D0 were analysed using the Wilcoxon test with significant P value set to 0.0167 using Bonferroni correction. The dots with error bars represent means with range. Data shown in RU compared to 18S. Abbreviations: D (0,2,14,28) = day (0,2,14,28), RU = relative expression units, R = responder, NR = non-responder, HC = healthy control

3.7 Validation of chosen miRNA shows no significant changes

For the miRNA validation study the sera of the whole cohort (17 patients and 8 healthy controls) were used. Compared were either healthy controls, responders and non-responders or the changes in miRNA regulation at D0, D2, D14 and D28 for the responders (Fig. 14). Out of the 5 chosen, significantly regulated miRNAs from the microarray cluster and network analyses – hsa-let-7e-5p, hsa-miR-486-3p, hsa-miR-3609, hsa-miR-6808-3p and hsa-miR-6716-5p – none has shown significantly altered expressions over the course of the appointed time points. For hsa-let-7e-5p, a tendency of downregulation at D0 could be observed as proposed by the miRNA cluster it was taken from (i.e. cluster 10, please refer to 3.5, Fig. 9, and supplemental table 3), whereas for hsa-miR-486-3p, hsa-miR-3609 and hsa-miR-6808-3p – a tendency of upregulation could be seen at D2 as proposed by their respective clusters (i.e. clusters 5, 8 and 12). It should be noted that hsa-miR-6716-5p was barely expressed for most of the cohort, thus even though there was a significant change for 3 patients comparing D0 and D2 expression for responders, it was not noted down as the general expression level was almost non-existent. However, the comparison between responders, non-responders

and healthy controls showed significant differences for hsa-miR-3609 and hsa-miR-486-3p: For hsa-miR-3609 a significant difference in expression levels between healthy controls and responders was observed (p=0.0341), whereas for hsa-miR-486-3p – between healthy controls and non-responders (p=0.0368). However, it should be noted that the group of non-responders only consists of 3 patients.

Fig. 14A

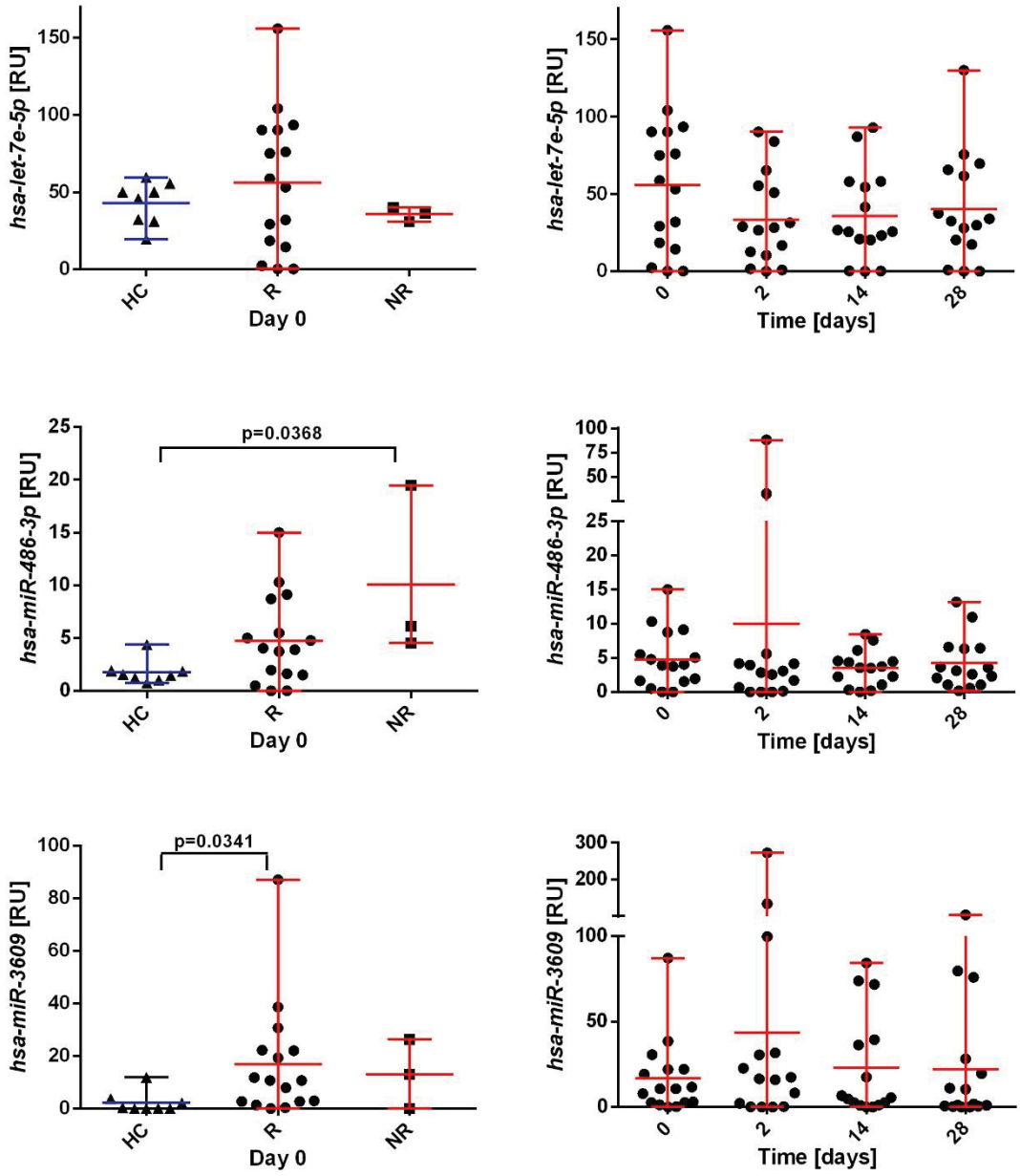

Fig. 14B

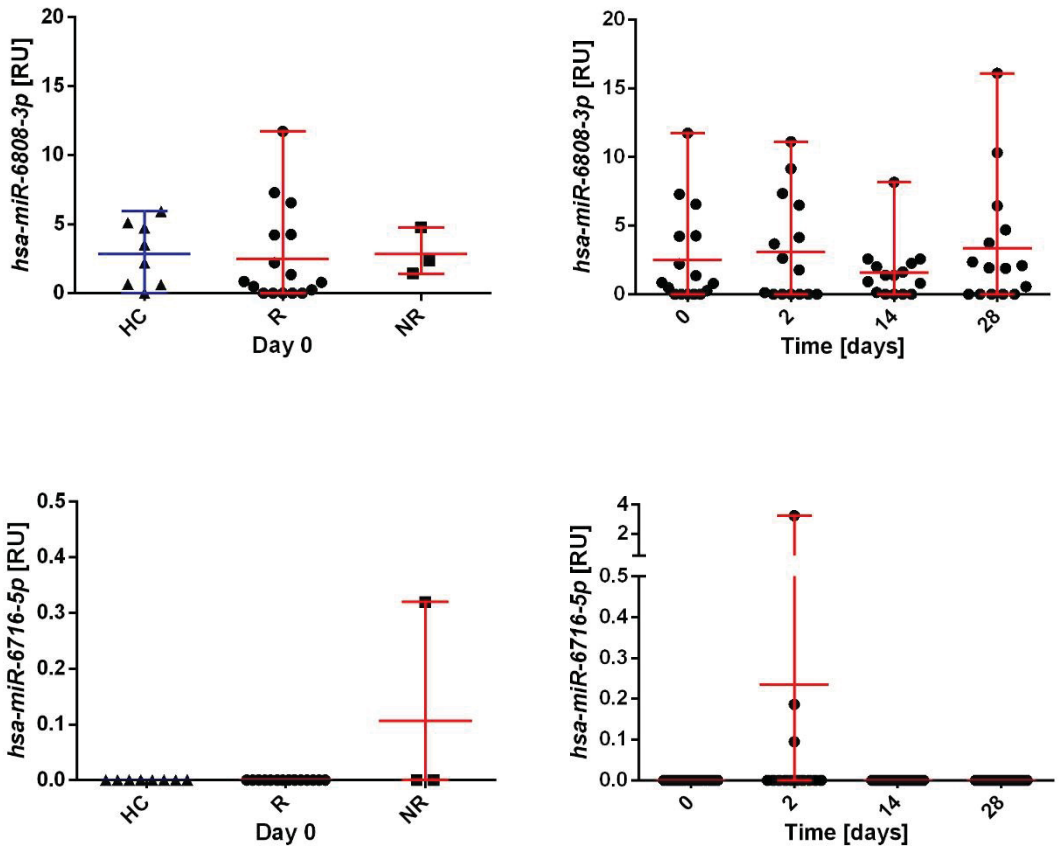

Fig. 14: **qPCR analysis of hsa-let-7e-5p, hsa-miR-486-3p, hsa-miR-3609, hsa-miR-6808-3p and hsa-miR-6716-5p**

Depicted are miRNA expression levels of hsa-let-7e-5p, hsa-miR-486-3p, hsa-miR-3609 (A) and hsa-miR-6808-3p, hsa-miR-6716-5p (B) in RU comparing either R, NR (n=3) and HC (n=8) or the changes of miRNA expression levels for R at D0, D2, D14 and D28. Number of responders at given time points varies due to missing time points or quality failures as follows: D0 – n= 17, D2/14/28 – n=16. Comparing R, NR and HC the Kruskal-Wallis test with Dunn's post-test were used. The changes of miRNA expression at D2, D14 and D28 each compared to D0 were analysed using the Wilcoxon test with significant P value set to 0.0167 using Bonferroni correction. The dots with error bars represent means with range. Data shown in RU compared to UniSp6 (control). Abbreviations: D (0,2,14,28) = day (0,2,14,28), RU = relative expression units, R = responder, NR = non-responder, HC = healthy control

4 Discussion

CSU is a common and debilitating disease with a pathogenesis not yet fully understood. Biomarkers are much needed to potentially identify sub-cohorts of patients, eventually identifying the most suitable current or future treatment option for each patient.

Currently, the only approved long-term treatment for CSU is the monoclonal anti-IgE-antibody omalizumab (1). In this study we investigated the changes in total serum IgE levels as well as mRNA and miRNA expressions during the first month of omalizumab treatment exploring the effects of the antibody and by this further deepening the understanding of CSU.

A much-discussed possible biomarker for CSU is the total serum IgE. In regard to omalizumab treatment it is thought that by binding to the antibody, the half-life of IgE is increased and thus an increase in total serum IgE can be observed especially in the beginning of the treatment with omalizumab (146). This overall, prominent increase in total serum IgE levels was observed in our cohort as well. However, what could not be observed in our cohort are relevant differences in baseline IgE levels between responders and non-responders meaning high total IgE at baseline significantly corresponding to a better omalizumab response, which was described by several studies (146-148). Back in 2010, Kessel *et al.* already reported that high total IgE levels in CSU patients correlate with a long disease duration and a higher disease severity compared to patients with a low total IgE (45). Again, this could not be shown in our patient group: For example, the two patients with the highest IgE levels had a disease duration under 12 months and the disease severity – if measured by baseline UCT, CU-Q2oL and DLQI – was not linked to baseline total IgE levels. In 2018, Straesser *et al.* described that low total IgE corresponds to younger CSU patients and that a total IgE <15 IU/ml correlates with omalizumab failure. We could not observe any age correlation in our cohort. However, we could observe that one of the non-responders had indeed a total IgE level of < 15 IU/ml at baseline, but then again, a complete responder also had a total IgE level of < 15 IU/ml. In a review article of 2021, Altrichter *et al.* looked into studies regarding total IgE and CSU and postulated that even though not all facets of total serum IgE in CSU are yet clear, high IgE levels correspond to a long disease duration, high disease activity and a better response to omalizumab (144). However, as our group, several studies did not share these findings and thus could not support these observations (149-152). To sum up, we could observe an overall increase in IgE coherent with previously published data. However, we could not observe a correlation with either disease duration, activity or response to omalizumab. As IgE does not prove be clear marker for

CSU and its response to omalizumab, it becomes apparent that, at least, IgE is not the only key element in CSU treatment with omalizumab. This further strengthens the idea that CSU pathogenesis is not only based on MC (and basophil) activation by IgE cross-linking via FcεR1. This is underlined by the fact that in CSU, the omalizumab dosage is not based on patients' total IgE levels (and weight) compared to the dosages in allergic asthma or chronic rhinosinusitis with nasal polyps (121, 122).

In this study, we investigated several differentially expressed mRNAs and miRNAs in omalizumab responders, first using microarray and then qPCR analyses. To the best of our knowledge, this study is the first one to look at the changes not only in mRNA but also in miRNA expression at different time points in the first month of treatment with omalizumab additionally comparing responders and non-responders to healthy controls at baseline.

Comparing expression levels at D0, D2, D14 and D28 by using microarray analysis, it was observed that upregulation outnumbered downregulation and that most upregulation took place at D2 under omalizumab treatment triggering a multitude of biological processes. This would correspond to clinically visible amelioration of symptoms as soon as a few days after treatment initiation in some of the patients.

In the first part of our validation study, we investigated changes in gene expression levels based on mRNA microarray results. *CD52* showed to be upregulated at D2. CD52 it is expressed on T and B cells as well as, to a lesser extent, on eosinophils and MCs and is known to be responsible for T cell co-stimulation and migration (153). A T cell involvement in CSU has been described by several groups. Recently Prosty *et al.* explored T_H2 and T_H17 pathways in CSU *in silicio* via RNA deconvolution proposing a dysbalance in these pathways as one of the reasons for CSU (154). A T_H2 involvement via elevated T_H2 related cytokines in CSU was described by several groups: Kay *et al.* observed elevated IL-33, IL-25 and TSLP levels and Ying *et al.* – increased mRNA expression of IL-4, IL-5 and IFN-γ in lesional skin of CSU patients, whereas Raap *et al.* described elevated IL-31 levels in patient sera and Lin *et al.* – elevated IL-17, IL-31 and IL-33 levels in patient plasma (75, 76, 155, 156). The importance of these pathways is underlined by the fact that treatment options targeting T cells or its cytokines showed to be effective in CSU: ciclosporin (a calcineurin-inhibitor which is thought to have a negative effect on T cells and its cytokine production), dupilumab (anti-IL4Rα-antibody), tezepelumab (anti-TSLP-antibody) and secukinumab (anti-IL-17A-antibody) (115, 143, 157). Omalizumab was reported to influence T cell cytokines: Altrichter *et al.* described a reduction of IL-31 serum levels in omalizumab responders after 6 months of treatment, whereas Rauber *et al.* observed a reduction of IL-10, IL-31 and IFN-γ secreting cells in

omalizumab responders looking at monthly changes during the course of a 5-months-treatment. Thus, for our group it was rather interesting whether our data indicates transcriptomic changes on T cell level during the first month of treatment and here especially during the first week. This is why, we chose several other genes associated with T cell pathways in addition to *CD52* to validate for our cohort: *IntegrinB3*, *CD28*, *IL-6R* and *CXCL5*. *CD28* showed to be significantly upregulated at D2 in our responders. *CD28* has an important role in signal amplification of T cell stimulation, affects T cell proliferation and differentiation and was already described in association to CSU by Brzoza *et al.* exploring CSU on a genetic level (158, 159). Integrin β3, a member of the integrin transmembrane receptor family, was described to play a role skin inflammation. It is partnered with Integrin αV, which is highly expressed on T cells. As Integrin $\alpha_v\beta_3$ it was shown to promote T cell migration, particularly T_H2, but also T_H1 and – in a more recent study – T_{reg} (160, 161). In our cohort, we observed a significant downregulation of *IntegrinB3* expression levels at D14, interestingly, at a later point than the overall tendency of highest regulation changes at D2. This could correlate with reduced inflammation seen under omalizumab therapy. IL-6, a known pro-inflammatory cytokine further promoting TNF-α and IL-1β secretion, was also shown to increase FcεR1-induced PDG2- production and VEGF production by McHale *et al* (162). It was described to be elevated in CSU patient sera and to be reduced as soon as during the first week of omalizumab treatment in a cohort of 8 patients by Grieco *et al.* (89). In our cohort, however, we observed neither significant changes in *IL-6R* expression in 14 omalizumab responders during the first month of treatment nor significant expression differences comparing responders, non-responders and healthy controls at baseline. Our findings also showed no significant changes in *CXCL5* expression – CXCL5 was previously reported to be linked to non-IgE-mediated MC activation (163).

COMMD6 was one of the DEGs proposed by our microarray analysis and showed an upregulation tendency at D2 in the validation study; the same tendency could be observed with *COMMD8*. These genes have not yet been described in association with CSU and could thus be considered a new finding. Both genes code for proteins of the copper metabolism gene MURR1 domain - family and act as NF-κB-inhibitors (164). COMMD6 has been described to be linked to the activation of NF-κB signalling pathway in tumors (165). COMMD8 has been investigated in venom immunotherapy and grass pollen allergen immunotherapy as possible inductor of NF-κB tolerance: Kempinski *et al.* proposed that COMMD8 could be linked to venom immunotherapy success leading to the stimulation of dendritic cells and further to the switch of naive T cells to T_{regs} and T_H2 cells to T_H1 cells (166). Romantowski *et al.* proposed a similar mode of action in grass pollen allergen immunotherapy with COMMD8 leading to IFN-γ mediated JAK/STAT

activation via NF-κB and by this to the switch of T_H2 cells to T_H1 cells and reduction of IgE production by B cells (167). Even though CSU is not considered an allergic disease, still, as described above, IgE is proposed to play an important role in this disease and there are several findings showing a T_H2 dysregulation. The activation of the JAK/STAT signaling pathway in CSU was thoroughly investigated by Feng *et al*. (168).

In our cohort *GIMAP2* showed an upregulation tendency at day 2, which could theoretically again lead to T cell involvement in CSU as GIMAP2 was described to influence lymphocyte maturation and lymphocyte apoptosis and was put into discussion as a marker in breast cancer (169, 170).

A very different direction in omalizumab treatment and thus CSU pathogenesis could be explored by looking at *CLEC2B*: It was one of the proposed DEGs in our microarray analysis, in the validation study an upregulation tendency at D2 of omalizumab treatment could be observed in our cohort. CLEC2B is found on platelets and is thought to be responsible for the preservation of vascular integrity, especially during inflammation by allowing vascular permeability. This is relevant for our cohort as, of course, wheal development is a central symptom of CSU. Interestingly, healthy controls showed significantly lower expression of *CLEC2B* in our study. In a recent study, Hide *et al.* also put emphasis on not neglecting the importance of the epithelial barrier, the coagulation and the complement cascade in the pathogenesis of CSU (59). Interestingly, the previously described Integrin β3 is expressed on platelets, as well. It can be activated by e.g. PAF in case of vascular damage and then acts in its activated form as $\alpha_{II}b\beta_3$ promoting haemostasis (171, 172). PAF is released by MCs, among others, and was recently proposed as a potential marker in CSU (173). A significant downregulation of *IntegrinB3* at D14 would correspond to reduced inflammation under omalizumab treatment.

COX7B was a candidate gene in our microarray analysis, and we could observe an upregulation tendency at D2 in our omalizumab responder cohort. Its possible meaning for CSU remains unclear as it is now mostly known to be a relevant player in mitochondrial disorders as cytochrome C oxidases are crucial for oxidative metabolism (174). However, it was described as a possible marker in psoriasis arthritis and ankylosing spondylitis by Zhang *et al.* and depicted to be linked to the adaptive immune response (175).

Interestingly, we could not prove *CD69* to have a relevant change in expression – only an upregulation tendency at D2 – in our cohort, which we would have assumed to find after our microarray analysis and as it was mentioned as one of the top regulated CSU genes involved in various immunological pathways by Patel *et al.* in 2015.

As a final finding of our gene validation study, we could, surprisingly, not identify any change in gene expression of *FcεR1* in our responders during the first month of treatment with omalizumab and even observed a significantly higher expression of *FcεR1* in healthy controls. The downregulation of *FcεR1* under omalizumab treatment was described as early as the beginning of its use in asthma treatment and then further established in CSU treatment (176, 177). However, it could be possible that in our cohort these changes would occur after a longer period of treatment, which we cannot prove in our study design.

The second part of our study explored candidate miRNAs from our microarray analysis. miRNAs are thought to be promising biomarkers due to their function as regulators of immunity and inflammation; they have been investigated especially as biomarkers in tumor genesis. Only a few studies so far have focused on possible relevant miRNAs for CSU. *Lin et al.* was the first one to look for promising miRNAs using plasma of 6 patients with active CSU for microarray analysis (107). Interestingly one of the described miRNAs – miR-6799-3p – was also found in our cohort. Lin *et al.* reported on a decreased expression of miR-6799-3p and in this study this miRNA appears to be downregulated at day 2 after initiation of omalizumab treatment in the cluster and network analysis of the microarray results.

Interestingly, the other top differentially expressed miRNAs identified in our microarray and network analyses have not been linked to CSU so far. Hsa-let-7e-5p was reported to be differentially regulated in oral lichen planus – a disease with a pathogenesis not yet fully understood, however were T cells play a crucial role (178). Furthermore, de la Rica *et al.* reported on the cluster of hsa-let-7e-5p and miR-125a-5p in the involvement of osteoclast differentiation by regulating by NF-κB (179). miR-125a-5p was observed to be significantly upregulated in CSU by Zhang *et. al* comparing miRNA microarray data of 20 CSU patients and 20 healthy controls (105). MiR-125a-5p was shown to be involved IL-6R and STAT3 expression and thus promoting T_{reg} cell differentiation (180, 181). It was also reported to be linked to autoimmune disease, such as rheumatoid arthritis (182). Of course, if thinking of CSU and its autoimmune component, this would be a further lead for miR-125a-5p association in autoimmune disease and possibly also for the investigated hsa-let-7e-5p assuming they form the above-described cluster. However, it should be noted that miR-125a-5p did not appear to be significantly regulated in our study.

Of the other differently expressed miRNAs in our cohort, little is known so far. Bianchi *et al.* investigated miR-486-3p and found it to promote granulocyte differentiation (183). This could be interesting in a CSU cohort as eosinophils and basophils also play an

important role in disease activity (44). Hsa-miR-3609, hsa-miR-6808-3p and hsa-miR-6716-5p were investigated in different tumor entities (pancreatic and breast cancers; lung cancer; prostate and colorectal cancers, respectively). So far there is, to this group's knowledge, no published research on their possible roles in inflammatory or autoimmune diseases (184-186). It should be noted that in our validation study via qPCR, we could not observe significant changes in the expression in the proposed miRNAs. This could be due to the small size of cohort or, of course, this could mean that these miRNAs are not as relevant as the microarray results suggested.

The differences in the proposed miRNAs in this miRNA microarray analysis compared to other miRNA candidates are possibly the ethnicity of the cohort (most of the currently published CSU miRNA studies investigated a Chinese population cohort) and the differences in the overall pool of miRNAs provided by the respective chip for the microarray analysis. Another crucial difference, of course, is the study layout: We investigated patients under omalizumab treatment, which the other studies did not. Furthermore, we specifically looked into miRNAs, but not specifically into lncRNAs as Liang *et al.* who reported on lncRNA T264761 to be differentially expressed in CSU and associated with MC activation (108).

This study has some limitations. The primary limitation is, of course, the small sample size, particularly regarding the number of non-responders. It was challenging to recruit patients for this study as it included additional visits at the clinic at D2 and D14 (especially, considering the fact that ours is a tertiary referral hospital, and we see many patients who travel from afar and, additionally, would have difficulties to take off from work). Even with the recruited patients it proved to be difficult to match the time points agreed on as some appointments were still missed or differed from the specified ones. However, thus our study presents data from real-life conditions with, at times, poor patient compliance. Skin biopsies at D0 and D28 might have strengthen our results, however, we had chosen against skin biopsies in this study as less patients might have agreed to participate to avoid possible complications and lasting scars. Another limitation is the relatively short time frame looked at in this study knowing that there are patients who are late responders to omalizumab. A bigger time frame would certainly provide more information on possible mRNA and miRNA regulations probably leading to a clearer determination of biomarkers in the treatment of CSU. It would also be interesting to further subdivide the cohort into partial and complete responders, disease duration and the appearance of wheals and angioedema vs. wheals or angioedema only, which would, of course, require a bigger cohort for validated results.

Nevertheless, to the knowledge of our study group, a study setting containing microarray analysis and validation at different time points of the first month of treatment with omalizumab has not yet been published and may thus provide new input for further research.

In conclusion, in our study we looked beyond the IgE-influence of omalizumab on CSU and further established its proposed effect on T cells by identifying and validating several possible mRNA and miRNA candidates. Our results also pointed to its possible effect on the vascular integrity, which has not been thoroughly investigated yet. Thus, our research shows new insights on omalizumab treatment and might provide potential perspectives in the search of biomarkers for CSU. Further studies investigating larger patient cohorts are needed to further deepen our knowledge of the disease.

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6 Appendix

Urticaria Control Test
Instructions: You have urticaria. The following questions should help us understand your current health situation. Please read through each question carefully and choose an answer from the five options that *best fits* your situation. Please limit yourself to *the last four weeks*. *Please don't think about the questions for a long time* and do remember to answer *all questions* and to provide *only one answer to each question*.
How much have you suffered from the **physical symptoms of the urticaria (itch, hives (welts) and/or swelling)** in the last four weeks? □ very much □ much □ somewhat □ a little □ not at all
How much was your **quality of life** affected by the urticaria in the last 4 weeks? □ very much □ much □ somewhat □ a little □ not at all
How often was the **treatment** for your urticaria in the last 4 weeks **not enough** to control your urticaria symptoms? □ very often □ often □ sometimes □ seldom □ not at all
Overall, how well have you had your urticaria **under control** in the last 4 weeks? □ not at all □ a little □ somewhat □ well □ very well

Supplemental Fig. 1: **Urticaria Control Test consisting of 4 questions**

Depicted is the Urticaria Control Test (UCT): Min. score – 0, max. score – 16. A higher value corresponds to better disease control. Cut-off value is 12 meaning the disease is well controlled. A value <12 complies to poor disease control. Modified from: Weller *et al*., J Allergy Clin Immunol, 2014 (29)

Dermatology Life Quality Index
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.
Over the last week, how **itchy**, **sore**, **painful** or **stinging** has your skin been? □ very much □ a lot □ a little □ not at all

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
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 relevant
Over the last week, how much has your skin influenced the clothes you wear? □ very much □ a lot □ a little □ not at all □ not relevant
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 relevant
Over the last week, how much has your skin made it difficult four you to do any **sport**? □ very much □ a lot □ a little □ not at all □ not relevant
Over the last week, has your skin prevented you from **working** or **studying**? □ a lot □ no □ not relevant 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
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 relevant
Over the last week, how much has your skin caused any **sexual difficulties**? □ very much □ a lot □ a little □ not at all □ not relevant
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 relevant

Supplemental Fig. 2: **Dermatology Life Quality Index consisting of 10 questions**

Depicted is the Dermatology Life Quality Index (DLQI): Min. score – 0, max. score – 30. A smaller value corresponds to better disease control. A value ≥ 10 complies to moderate to severe quality of life impairment and suggests treatment adjustment. Modified from: Finlay et Khan GK, Clin Exp Dermatol, 1994 (36)

Chronic Urticaria Quality of Life Questionnaire				
In the past 14 days, how much were you troubled by the following symptoms?				
Itch				
□ not at all	□ a little	□ rather	□ a lot	□ very much
Wheals				
□ not at all	□ a little	□ rather	□ a lot	□ very much
Eye swell				
□ not at all	□ a little	□ rather	□ a lot	□ very much
Lip swell				
□ not at all	□ a little	□ rather	□ a lot	□ very much
Indicate how often you were limited by your hives (urticaria) in the past 14 days in the following areas of daily life.				
Work				
□ never	□ rarely	□ sometimes	□ often	□ very often
Physical activities				
□ never	□ rarely	□ sometimes	□ often	□ very often
Sleep				
□ never	□ rarely	□ sometimes	□ often	□ very often
Free time				
□ never	□ rarely	□ sometimes	□ often	□ very often
Social relationships				
□ never	□ rarely	□ sometimes	□ often	□ very often
Eating				
□ never	□ rarely	□ sometimes	□ often	□ very often
In the following questions, we would like to know more about the difficulties and problems that could be related to your hives (urticaria) (regarding the past 14 days).				
Do you have difficulties falling asleep?				
□ never	□ rarely	□ sometimes	□ often	□ very often
Do you wake up at night?				
□ never	□ rarely	□ sometimes	□ often	□ very often
Are you tired during the day because you didn’t sleep well at night?				
□ never	□ rarely	□ sometimes	□ often	□ very often
Do you have difficulties concentrating?				
□ never	□ rarely	□ sometimes	□ often	□ very often

Do you feel nervous? □ never □ rarely □ sometimes □ often □ very often
Do you feel miserable? □ never □ rarely □ sometimes □ often □ very often
Do you have to limit your food choices? □ never □ rarely □ sometimes □ often □ very often
Are you bothered by the symptoms of hives (urticaria) that appear on your body? □ never □ rarely □ sometimes □ often □ very often
Are you embarrassed to go to public places? □ never □ rarely □ sometimes □ often □ very often
Is it a problem for you to use cosmetics (e.g. perfumes, creams, lotions, bubblebath, make up)? □ never □ rarely □ sometimes □ often □ very often
Do you have to limit your clothing choices? □ never □ rarely □ sometimes □ often □ very often
Are your sports activities limited because of your hives(urticaria)? □ never □ rarely □ sometimes □ often □ very often
Do you suffer side-effects from the medications you take for hives (urticaria)? □ never □ rarely □ sometimes □ often □ very often

Supplemental Fig. 3: **Chronic Urticaria Quality of Life Questionnaire consisting of 23 questions.**

Chronic Urticaria Quality of Life Questionnaire (CU-Q2oL) Min. score – 0, max. score – 115. A smaller value corresponds to better disease control. Modified from: Mlynek *et al.*, Allergy, 2009 (35)

Genes			
GIMAP2	SARNP	UQCRQ	RPS9
C17orf76-AS1/// SNORD49A/// SNORD49B/// SNORD65	COPS2	CHMP5	RPS29
GPR171	C11orf31	ZNF561	PSMC6
COMMD8	LPAR6	CCNC	ZNHIT3
RPS5	TMEM256	TOMM5	TMEM50B

RPL41	DBI	RPL13 /// SNORD68	TMEM167A
SLIRP	RSL24D1	RPS6	EIF3E
ANK1	RPS15A	ITM2A	ATP5L
PHF5A	CEP85L	RPL9 /// TIPIN	NDUFA4
CCDC88A	CD3D	RPL22L1	MRPL22
RPL35	NDUFB3	KLRC1 /// KLRC2	TXNDC17
TMA7	RPL21	RPL34	EEF1B2 /// SNORA41
PSMA2	LSM5	C4orf46 /// TOMM7	ALG13
CD52	NDUFB2	RPS27L	HAT1
COX7B	RPL7	C10orf32	FAM26F
LTF	CSTA	C14orf2	GLRX
PSMA4	COX6C	RPS24	SNX4
RPS10	KIAA0391 /// PSMA6	ERH	GYPA
ATP6V1G1	RPS21	RPS18	GTPBP8
KLRC1	C8orf59	GMFG	HAUS1
C14orf28	TMCO1	SNRPD2	FCER1A
RBM47	KIAA1704/// LOC100996395	YEATS4	FAU
TNS1	RPL35A /// ZNF391	POLB	COX7C
TRAT1	KLRB1	RPS3A /// SNORD73A	RPAIN
S100A8	SNHG8 /// SNORA24	POT1	DNAJC15
RPS10/// RPS10-NUDT3	ATP5J2 /// ATP5J2-PTCD1 /// PTCD1	RPL30	SAR1B
LSM3	COX7A2	ATP5C1	PPP2R5E
COQ5	ACTR6	SNRPG	RBX1
UHMK1	APIP	CLEC2B	POLR2K
SEC24A	GZMA	KLRC3	RUFY3
TMEM54 /// UQCR11	MGAM	TMEM126A	SPARC

KRBA2 /// RPL26	MRPL3	FAM175A	ATP5O
COMMD6	TMEM126B	CMC1	MYBL1
RWDD1	MRPS33	MIR1304 /// SNORA1 /// SNORA18 /// SNORA25 /// SNORA32 /// SNORA40 /// SNORA8 /// SNORD5 /// TAF1D	ZNF292
DPM1	LY96	RPS7	RPL39
RPL36A /// RPL36A-HNRNPH2	USMG5	MRPL32	SS18L2
RPL23 /// SNORA21	MRPL47	PDCD10	RPLP0
NDUFB1	C14orf142	MCTS1 /// PSIMCT-1	TAF9
CKS2	CD48	RPS27	FAM76B
TPT1	SUB1	EVI2A	TXNDC9

Supplemental table 1: **160 overlapping top regulated genes at D2 and D14 of the DNA microarray analysis.**

List of the 160 differentially expressed genes that were overlappingly top regulated in the DNA microarray analysis results of both D0/D2 and D0/D14. Abbreviation: D (0,2,14) = day (0,2,14)

GO Term	Description	Number of genes
GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	22
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	20
GO:0045047	protein targeting to ER	20
GO:0006613	cotranslational protein targeting to membrane	20
GO:0006412	translation	27
GO:0072599	establishment of protein localization to endoplasmic reticulum	20
GO:0070972	protein localization to endoplasmic reticulum	20
GO:0000956	nuclear-transcribed mRNA catabolic process	24
GO:0043043	peptide biosynthetic process	27
GO:0019083	viral transcription	20
GO:0006402	mRNA catabolic process	24
GO:0006413	translational initiation	21
GO:0006401	RNA catabolic process	24
GO:0006612	protein targeting to membrane	21
GO:0043604	amide biosynthetic process	27
GO:0006518	peptide metabolic process	27

GO:0034655	nucleobase-containing compound catabolic process	24
GO:0006605	protein targeting	22
GO:0006364	rRNA processing	21
GO:0016071	mRNA metabolic process	29
GO:0072594	establishment of protein localization to organelle	23
GO:0016072	rRNA metabolic process	21
GO:0090150	establishment of protein localization to membrane	21
GO:0046700	heterocycle catabolic process	24
GO:0044270	cellular nitrogen compound catabolic process	24
GO:0019439	aromatic compound catabolic process	24
GO:1901566	organonitrogen compound biosynthetic process	33
GO:0043603	cellular amide metabolic process	27
GO:1901361	organic cyclic compound catabolic process	24
GO:0072657	protein localization to membrane	21
GO:0044265	cellular macromolecule catabolic process	28
GO:0034470	ncRNA processing	21
GO:0009057	macromolecule catabolic process	29
GO:0033365	protein localization to organelle	22
GO:0006396	RNA processing	28
GO:0034660	ncRNA metabolic process	23
GO:0006886	intracellular protein transport	25
GO:1902580	single-organism cellular localization	24
GO:1902582	single-organism intracellular transport	30
GO:0046907	intracellular transport	33
GO:0034613	cellular protein localization	23
GO:0051649	establishment of localization in cell	34
GO:0070727	cellular macromolecule localization	23
GO:1902600	hydrogen ion transmembrane transport	10
GO:0061024	membrane organization	23
GO:1901564	organonitrogen compound metabolic process	34
GO:0044248	cellular catabolic process	29
GO:0015031	protein transport	29
GO:0045184	establishment of protein localization	30
GO:0044802	single-organism membrane organization	21
GO:0015992	proton transport	10
GO:0044085	cellular component biogenesis	10
GO:0006818	hydrogen transport	10
GO:0051641	cellular localization	25
GO:0044271	cellular nitrogen compound biosynthetic process	50
GO:0034641	cellular nitrogen compound metabolic process	64
GO:0022904	respiratory electron transport chain	8
GO:1901576	organic substance biosynthetic process	58
GO:0044249	cellular biosynthetic process	57
GO:0022900	electron transport chain	8
GO:1901575	organic substance catabolic process	29
GO:0034645	cellular macromolecule biosynthetic process	45
GO:0009059	macromolecule biosynthetic process	48
GO:0009058	biosynthetic process	58
GO:0022618	ribonucleoprotein complex assembly	10

GO:0006139	nucleobase-containing compound metabolic process	57
GO:0000028	ribosomal small subunit assembly	4
GO:0071826	ribonucleoprotein complex subunit organization	10
GO:0009056	catabolic process	30
GO:0008104	protein localization	29
GO:0022613	ribonucleoprotein complex biogenesis	6
GO:0033036	macromolecule localization	29
GO:0006807	nitrogen compound metabolic process	64
GO:0006123	mitochondrial electron transport, cytochrome c to oxygen	4
GO:0046483	heterocycle metabolic process	57
GO:0034654	nucleobase-containing compound biosynthetic process	43
GO:0006725	cellular aromatic compound metabolic process	57
GO:0016043	cellular component organization	60
GO:0071840	cellular component organization or biogenesis	60
GO:0044765	single-organism transport	42
GO:0018130	heterocycle biosynthetic process	43
GO:0044267	cellular protein metabolic process	44
GO:0019438	aromatic compound biosynthetic process	43
GO:0051234	establishment of localization	49
GO:0044237	cellular metabolic process	86
GO:0071702	organic substance transport	31
GO:1901362	organic cyclic compound biosynthetic process	43
GO:1901360	organic cyclic compound metabolic process	57
GO:0006810	transport	47
GO:0034622	cellular macromolecular complex assembly	16
GO:0090304	nucleic acid metabolic process	49
GO:1902578	single-organism localization	42
GO:0042274	ribosomal small subunit biogenesis	3
GO:0051179	localization	50
GO:0016070	RNA metabolic process	43
GO:0032774	RNA biosynthetic process	36
GO:0008152	metabolic process	90
GO:0002227	innate immune response in mucosa	3
GO:0006091	generation of precursor metabolites and energy	9
GO:0006120	mitochondrial electron transport, NADH to ubiquinone	4
GO:0042776	mitochondrial ATP synthesis coupled proton transport	3
GO:0002385	mucosal immune response	3
GO:0070125	mitochondrial translational elongation	5
GO:0019538	protein metabolic process	46
GO:0015672	monovalent inorganic cation transport	11
GO:0002181	cytoplasmic translation	3
GO:0070126	mitochondrial translational termination	5
GO:0002251	organ or tissue specific immune response	3
GO:0044260	cellular macromolecule metabolic process	67
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	5
GO:0006415	translational termination	5
GO:0015985	energy coupled proton transport, down electrochemical gradient	3
GO:0015986	ATP synthesis coupled proton transport	3
GO:0010257	NADH dehydrogenase complex assembly	4

GO:0097031	mitochondrial respiratory chain complex I biogenesis	4
GO:0032981	mitochondrial respiratory chain complex I assembly	4

Supplemental table 2: **Corresponding GO-Terms to the 160 overlapping top regulated genes at D2 and D14 of the DNA microarray analysis**

List of the GO-Terms that could be related to the 160 differentially expressed genes that were overlappingly top regulated in the DNA microarray analysis results of both D0/D2 and D0/D14. Abbreviation: D (0,2,14) = day (0,2,14)

Cluster 5	Cluster 6	Cluster 8	Cluster 10	Cluster 12
miRNA – term				
ENSG00000199411	ENSG00000263864	ACA44	hsa-let-7e-5p	ACA15
ENSG00000268237	ENSG00000264086	ENSG00000238936	hsa-let-7g-5p	ENSG00000202252
HBII-210	ENSG00000264202	ENSG00000252840	hsa-miR-15a-5p	ENSG00000206785
HBII-239	ENSG00000265732	ENSG00000265651	hsa-miR-183-5p	ENSG00000207062
hsa-miR-3921	HBII-142	HBII-135	hsa-miR-324-5p	ENSG00000263358
hsa-miR-4635	HBII-429	HBII-276	hsa-miR-3648	ENSG00000263359
hsa-miR-486-3p	hsa-miR-1275	hsa-miR-3609	hsa-miR-425-3p	ENSG00000263625
U105	hsa-miR-2115-5p	snR38C	hsa-miR-4484	ENSG00000265116
U26	mgh28S-2409	U105B	hsa-miR-4486	ENSG00000265849
U27	mgh28S-2411	U30	hsa-miR-4665-5p	ENSG00000266295
U28	U104	U35A	hsa-miR-4750-5p	ENSG00000266419
U54	U32A	U36C	hsa-miR-501-5p	HBII-289
U64	U44	U38A	hsa-miR-584-3p	HBII-55
U70	U48	U58A	hsa-miR-6127	hsa-miR-6808-3p
U78	U50	U58B	hsa-miR-6511b-3p	U29
U79	U55	U59A	hsa-miR-6716-5p	U41
U81	U57	U73a	hsa-miR-6778-5p	U42A
	U76	U75	hsa-miR-6799-3p	U56
		U83B	hsa-miR-6799-5p	U83
		Z17B	hsa-miR-6819-5p	
			hsa-miR-6820-5p	
			hsa-miR-6879-5p	
			hsa-miR-7106-5p	
			hsa-miR-877-5p	
			hsa-miR-885-3p	

Supplemental table 3: **Regulated miRNAs of the clusters with the most prominent expression changes at D2**

In the cluster analysis of the top regulated miRNAs at D0 to D2, D14 and D28 under omalizumab treatment, 5 clusters appeared to have the most prominent expression change, especially at D2. These 5 clusters – cluster 5,6,8,10 and 12 – are listed in this table with their respective miRNAs. Abbreviations: D (0,2,14,28) = day (0,2,14,28), CSU = chronic spontaneous urticaria

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