Elucidating pathogenesis and improving clinical management of benign cutaneous inflammation in urticaria and malignant inflammation in Cutaneous T-Cell Lymphomas.

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ABSTRACT

English:

Introduction: Chronic urticaria (CU) is defined when hives occur for at least 6 weeks and affects 0.1–0.3% of children. It can be physical (PU), spontaneous (CSU), or of a different nature. When no identifiable cause can be found, this condition is classified as chronic spontaneous urticaria (CSU). Approximately 40-50% of CSU patients are now considered to have an autoimmune etiology. The presence of autoantibodies that are capable of inducing mast cell (and basophil) degranulation can be established *in vivo* with the use of the autologous serum skin test (ASST) or *in vitro* with the Basophil Activation Test (BAT) measuring CD63 expression. Up to date, only a few studies were conducted in children with CSU assessing for autoimmunity. Only limited data on the natural history and prognostic variables of chronic urticaria and its subtypes is available in pediatric population. Because of its chronicity, CU in children results in substantial impairment of health-related quality of life (QoL) and significantly lower school performance compared with other allergic diseases. With current treatment guidelines, the effect of CU on the QoL in children is not clear.

While urticaria represents benign inflammation, Cutaneous T-Cell Lymphomas (CTCL) represents malignant inflammation that is driven by neoplastic $CD4^+$ T cells that are localized to the skin. Although many patients with CTCL presenting with stage I disease enjoy an indolent course and normal life expectancy, about 15% to 20% of them progress to higher stages and ultimately succumb to their disease. Currently, it is not possible to predict which patients will progress and which patients will have a stable disease. It is also often challenging to diagnose this cancer since it often masquerades as chronic eczema, psoriasis or other benign inflammatory dermatoses. Based on our previous reports it may be possible to identify a set of molecular markers that may be used to help prognosticate this disease and distinguish it from benign mimickers.

Methods: During my thesis work on urticaria we have created a cohort of 139 children affected by this condition and entered their clinical and laboratory parameters as well as questionnaire findings into a database. All patients were followed for ~2 years. We have studied the utility of BAT to help diagnose and monitor response to treatment for autoimmune CSU in children. We also evaluated the natural history of the disease and assessed for predictors of disease resolution using established validated tools including the weekly urticaria activity score (UAS7) and the Chronic Urticaria quality of life questionnaire (CU-Q2oL)

For CTCL research we used the RT-PCR and tested gene expression in 60 CTCL skin biopsy samples and in 11 patient-derived cell lines to discover novel diagnostic and prognostic molecular markers and to gain additional insight in the molecular etiology of malignant inflammation and how it differs from benign inflammatory dermatoses that often mimic CTCL (e.g., eczema, psoriasis etc.). We also for the first time tested the expression of Embryonic Stem Cell (ESC) genes in this cancer.

Results: Based on the log-normal distribution of CD63 values in control subjects, the reference range and the cut-off for positive CD63 BAT values was established to be 1.2% to 1.8% (95%CI) and 1.8%, respectively. Children with CSU showed significantly elevated and significantly more increased BAT values as compared to healthy controls (Wilcoxon rank test p-value <0.001). In contrast, no difference was found between BAT results in controls and PU patients. In pediatric CSU patients, higher disease activity was associated with higher BAT values. Using the BAT we documented that 57% of children in our cohort had autoimmune CSU. CSU resolved in 43 patients, with the rate of resolution of 10.3% per 100 patient-years. Positive BAT and basopenia were statistically associated with earlier disease resolution. Furthermore, in our study of patients being treated with omalizumab, BAT has proven to be useful in predicting response to treatment. CU-Q2oL at time of recruitment suggested that itch, sleep impairment and physical appearance were the most important factors affecting QoL. The mean UAS7 at first follow up was 8.23 and reduced to 3.62 on next follow up suggesting an overall good control of symptoms with current management strategies.

Our research findings on CTCL demonstrated that 17 genes (CCL18, CCL26, FYB, T3JAM, MMP12, LEF1, LCK, ITK, GNLY, IL2RA, IL26, IL22, CCR4, GTSF1, SYCP1, STAT5A, and TOX) are able to both identify patients who are at risk of progression and also distinguish CTCL from benign mimickers (e.g., chronic eczema, psoriasis, etc.) Furthermore, our findings for the first time demonstrated that many critical ESC genes including NANOG, SOX2, OCT4 (POU5F1) and their upstream and downstream signaling members are expressed in CTCL. Furthermore, select ESC genes (OCT4, EED, TCF3, THAP11, CHD7, TIP60, TRIM28) were preferentially expressed in CTCL samples when compared to benign skin biopsies.

Conclusions: Chronic urticaria in children, similar to adult CU, is associated with increased prevalence of autoimmune comorbidities in patients and family members. Relative

frequency of autoimmune urticaria in children is similar to adults and if proven in the future, may be a good prognostic factor predicting earlier disease resolution. BAT may serve as an effective laboratory tool to help diagnose autoimmune CSU and monitor response to treatment in moderate/severe cases. Current treatment strategies are effective in improving disease symptoms and the quality of life in children.

Our gene expression findings in CTCL, combined with other gene expression analyses, prepare the foundation for the development of personalized molecular approach toward diagnosis and prognostication of this cancer. Furthermore, our findings suggests that ESC genes are ectopically expressed together with CT genes, thymocyte development genes and B cell-specific genes and may be working in concert to promote tumorigenesis in CTCL.

Résumé en français:

Introduction : l'urticaire chronique (CU) est définie lorsque l'urticaire récidive durant au moins 6 semaines et affecte 0,1 à 0,3 % des enfants. Elle peut être physique, spontanée (CSU), ou d'une autre nature. Lorsqu'aucune cause ne peut être identifiée, cette condition est désignée urticaire spontanée chronique (CSU). Environ 40 à 50 % des patients avec CSU sont maintenant considérés avoir une étiologie auto-immune. La présence d'autoanticorps capables d'induire la dégranulation des basophiles et des mastocytes peut être établie *in vivo* avec l'utilisation du test cutané avec le sérum autologue (ASST) ou *in vitro* avec plusieurs tests incluant le test d'activation des basophiles (BAT) mesurant l'expression du marqueur CD63. À ce jour, seules quelques études ont été menées chez les enfants évaluant l'implication de l'auto-immunité dans la CSU. Peu de données sur l'histoire naturelle et le pronostic de l'urticaire chronique et ses sous-types sont disponibles en pédiatrie. En raison de sa chronicité, l'urticaire chronique chez les enfants affecte considérablement la qualité de vie (QOL) et réduit de façon significative la performance scolaire, par rapport aux enfants avec d'autres maladies de nature allergiques. Avec les lignes directrices actuelles, l'effet de la maladie sur la qualité de vie des enfants n'est pas clair.

Alors que l'urticaire représente une inflammation bénigne de la peau, le lymphome cutané à cellules T (CTCL) représente une inflammation maligne qui est causée par les cellules T CD4 positives néoplasiques localisées à la peau. Bien que de nombreux patients atteints de CTCL se présentent avec une maladie au stade I et profitent d'une maladie indolente avec une espérance de vie normale, environ 15 % à 20 % d'entre eux vont progresser à un stage plus avancé et éventuellement succomber à la maladie. En ce moment, il n'est pas possible de prédire quels patients progresseront et ceux qui auront une maladie stable. Il est également souvent difficile de diagnostiquer ce cancer puisqu'il mime des conditions cutanées bénignes telles l'eczéma, le psoriasis ou d'autres dermatoses inflammatoires. Sur la base de nos travaux précédents, il peut être possible d'identifier un ensemble de marqueurs moléculaires qui peuvent être utilisés pour aider le clinicien à prédire le pronostic de cette maladie et la distinguer des imitateurs bénins.

Méthodes : au cours de mon travail de thèse sur l'urticaire nous avons créé une cohorte de 139 enfants touchés par CU et avons recueilli leurs paramètres cliniques, leurs bilans biologiques ainsi que les résultats des questionnaires dans une base de données. Tous les

patients ont été suivis pendant environ 2 ans. Nous avons étudié l'utilité de BAT pour aider à mieux diagnostiquer et surveiller la réponse au traitement de l'urticaire auto-immune chez les enfants. Nous avons également évalué l'histoire naturelle de la maladie et des facteurs prédictifs de la résolution de l'urticaire à l'aide des outils validés tels le score de l'activité hebdomadaire de l'urticaire (UAS7) et le questionnaire sur la qualité de vie (CU-Q2oL).

Dans le projet de recherche sur le lymphome cutané, nous avons utilisé le PCR en temps réel et l'expression des gènes sur les échantillons de la peau de 60 patients atteints de CTCL et sur 11 lignées de cellules dérivées de patients atteints de la maladie afin de découvrir de nouveaux marqueurs diagnostiques et pronostiques et à mieux comprendre l'étiologie moléculaire du CTCL. Nous avons également testé pour la première fois l'expression des gènes de cellules souches embryonnaires (ESC) dans ce cancer.

Résultats : en fonction de la distribution logarithmique normale des valeurs de CD63 chez les enfants témoins (sans CU), on a établi que l'intervalle de référence et le seuil positif pour le test BAT utilisant l'expression du marquer CD63 sont de 1,2 % à 1,8 % (intervalle de confiance à 95 %) et 1,8 %, respectivement. Les valeurs du BAT chez les enfants avec la CSU ont été significativement plus élevés que chez les témoins sains (test Wilcoxon rank, valeur prédictive $p<0,001$). En revanche, il n'y avait pas de différence entre les résultats du BAT chez les témoins et les patients avec la PU. Le BAT a été associé avec une maladie plus symptomatique. À l'aide du BAT, nous avons établi que 57 % des enfants dans notre cohorte avaient une origine auto-immune probable pour leur CSU. Après deux ans de suivi, la maladie a régressé dans 43 cas, avec un taux de résolution annuel de 10,3 %. Le résultat positif du BAT et la basopénie étaient statistiquement associés à une résolution plus rapide. En outre, dans le cadre de notre étude de patients traités avec l'omalizumab, le BAT s'est avérée utile pour prédire la réponse au traitement. Le CU-Q2oL au moment du recrutement a suggéré que la démangeaison, les troubles du sommeil et l'apparence physique sont les principaux facteurs affectant la qualité de vie chez les enfants avec la CU. Le score UAS7 a été réduit entre la première et la seconde visite de suivi (de 8.23 à 3,62) suggérant une bonne maîtrise des symptômes avec les recommandations de prise en charge actuelles.

Les conclusions de notre recherche sur le CTCL ont démontré que 17 gènes (CCL18, CCL26, FYB, T3JAM, MMP12, LEF1, LCK, ITK, GNLY, IL2RA, IL26, IL22, CCR4, GTSF1, SYCP1, STAT5A, et TOX) sont en mesure d'identifier les patients à risque de progression et

aussi distinguer le CTCL des imitateurs bénins. De plus, nos résultats ont démontré pour la première fois que des gènes de la famille des cellules souches embryonnaires (ESC) tels NANOG, SOX2, OCT4 (POU5F1) sont exprimés dans le CTCL. De plus, certains de ces gènes (OCT4, EED, TCF3, THAP11, CHD7, TIP60, TRIM28) ont été exprimés préférentiellement dans des échantillons de CTCL et étaient absents dans les maladies cutanées bénignes.

Conclusions : l'urticaire chronique chez les enfants, semblable à la CU chez les adultes, est associée à une augmentation de la prévalence des maladies auto-immunes comorbides chez les patients et lesmembres de leur famille. La fréquence relative de l'urticaire auto-immune chez les enfants est similaire à celle de l'adulte et, si confirmée par les études futures, la présence d'autoanticorps peut suggérer un meilleur prognostic avec une résolution plus rapide de la maladie. Le BAT peut servir comme outil de laboratoire pour diagnostiquer la CU auto-immune et surveiller la réponse au traitement dans les cas plus sévères. Les stratégies de traitement actuelles sont efficaces pour améliorer les symptômes de la maladie ainsi que la qualité de vie chez les enfants.

Nos résultats d'expression des gènes dans le CTCL préparent le terrain pour le développement de l'approche personnalisée pour le diagnostic et le pronostic de ce cancer. En outre, nos résultats suggèrent que les gènes ESC, cancer-testis ainsi que ceux impliqués dans le développement des thymocytes et des cellules B peuvent travailler de concert pour promouvoir dans la tumorigénèse dans le CTCL.

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PREFACE AND CONTRIBUTIONS OF AUTHORS:

As part of my graduate studies I have led the project on the study of chronic idiopathic urticaria under the supervision of Dr. Ben-Shoshan and Dr. Sasseville and I have contributed significantly through original research and data analysis to the research project on Cutaneous T-Cell Lymphomas that was led by Dr. Sasseville and his resident, Dr. Litvinov. All of the aforementioned scientists are co-authors on the attached publications.

Chapter 1. Introduction and Literature Review

Inflammation of the skin is a major source of morbidity and in some cases mortality in dermatology patients. Inflammatory dermatoses could be classified as being benign (e.g. urticaria, psoriasis, eczema, drug eruptions etc.) or malignant (e.g. Cutaneous T-Cell Lymphoma, Cutaneous B cell lymphomas, Leukemia/Lymphoma cutis, etc). The pathogenesis of these forms of inflammation differs greatly depending on the nature of the cell driving the process. To achieve control of inflammation and to improve care for our patients we must improve our understanding of the molecular pathogenesis of these conditions.

Urticaria

Chronic urticaria (CU) is defined as the occurrence of transient wheals lasting more than 6 weeks. Physical urticaria (PU) occurs when the CU is associated with a specific physical stimulus that will produce urticarial plaques (e.g., cold urticaria, solar urticaria, delayed pressure urticaria, localized heat urticaria, dermographic urticaria, or vibration urticaria)[1]. The point prevalence of CU is 0.5–1% of general population[2-4] and it affects 0.1–0.3% of children[5]. Although no studies assessing the prevalence of CU in Canadians have been published so far, assuming a similar 0.3% lifetime prevalence, it is likely that 101,620 Canadian children will be affected by CU this year[6].

In 80% of CU cases, hives occur spontaneously and are not attributable to a physical stimulus or an identifiable cause. This is classified as chronic spontaneous urticaria (CSU)[7]. While the exact pathogenesis of CSU remains to be elucidated, patients can be divided into at least two subgroups: those who are truly idiopathic, and those who were previously diagnosed with idiopathic disease, but are now reclassified as having autoimmune CSU (this occurs in approximately 40–50% of adults and children)[8-10].

It is increasingly acknowledged that all forms of urticaria and especially the more chronic forms have substantial societal impact. However, the current medical literature regarding its pathogenesis and prognosis is severely limited particularly in the pediatric population. Urticaria (acute and chronic) is the fourth most prevalent allergic condition after rhinitis, asthma and drug allergy, but the number of patients who visit emergency departments due to urticaria is higher (54.4%) compared to other allergic diseases (24.6%) ($p<0.001$)[11]. Recent studies suggest increased prevalence of all cases of urticaria in the UK and Australia[4,12,13]. A recent Australian study reported that hospital admissions for urticaria were approximately 3 times higher in children aged ≤4 years than for any other age group. This study revealed that between 1993-1994 and 2004-2005, there

were significant increases in the rate of hospital admissions for urticaria in all age groups and that the greatest increase was in those aged 15 to 34 years (7.8% per year)[13].

Adults and children with CU have a substantial impairment of health-related quality of life (QoL)[14] and significantly lower school performance compared with other allergic diseases (p =0.029)[11]. Patients suffering from CU scored in the lowest 25th percentile on physical impact questionnaire. CU is associated with absence from school in 7.4% of cases with an average of 7.5±18.5 missed days per year. Interestingly, 3.3% of children with CU also caused their parents to take days off work[11]. In a US study it was estimated that patients with CU consumed a mean of $2047±$1483 in health care costs annually. Because CU is primarily an outpatient disease, medication costs alone accounted for 62.5% ($1280) of the total annual cost, while indirect costs (e.g. earnings lost owing to travel to outpatient visits and absences from work) accounted for 15.7% ($322) of the total cost. Thus, it was concluded that high medication costs, followed by total indirect costs, result in a significant economic burden among patients with CU and that low-income patients are at risk for suboptimal treatment and increased burden due to poorly controlled disease[15]. Furthermore, it is unknown what is the effect of CU on the quality of life (QoL) in children and what are the factors associated with poor QoL.

Autoimmune factors have been recently identified as being pivotal to the pathogenesis of CSU in a substantial group of patients. However, most of these studies involved adults and no studies up to this point explored autoimmune factors in physical urticarias. In a recent study on 2,523 patients with non-autoimmune CU (defined through a negative autologous serum skin) it was found that physical urticarias accounted for 17.1% of cases, infection for 7.7% and autoimmune thyropathy for 7.3% of cases. Moreover, in more than 60% of cases no identifiable cause could be found[16]. This study included only adults and excluded those with autoimmune CU. Other studies have supported this conclusion and highlighted that although mast cells undoubtedly play a role in CSU, CSU is not considered an allergic disease given that an allergic cause for CSU is rarely identified.

Regardless of its cause, autoimmune CSU is characterized by the presence of IgG antibodies towards the alpha chain of the high-affinity IgE receptor and rarely (up to 10% of cases) towards IgE itself[17,18]. In addition, *Puccetti et al.*[19] have demonstrated that FcεRII/CD23 may be another relevant autoantigen in patients with CSU. CD23 is a low-affinity IgE receptor expressed on eosinophils and is able to induce histamine secretion (?) indirectly through the release of major

basic protein. Antibodies against CD23 were found in 65.4% of patients[19]. Furthermore, it was recently suggested that a sizeable subgroup of adult CSU patients expresses IgE antibodies against thyroid peroxidase (which correlates with the level of IgG against thyroid peroxidase in these patients) that may cause an "autoallergic" mast cell activation[20].

Up to this point, most studies on the autoantibodies to FcεRI, FcεRII, IgE and thyroid gland as etiology of CSU, were performed primarily in adults and suggest that these occur in at least 40-50% of patients with CU[19,21]. However, extensive epidemiological studies have not been conducted. Further, in children, only a few reports were done in prospective approaches and estimated that autoimmune CSU affects about half of pediatric CU cases in Europe[22] and almost 40% of children with CU in Thailand[23]. No similar studies were conducted in North America. Moreover, no studies to date had explored any association between autoimmune factors and physical urticaria.

Thyroid autoantibodies have a prevalence of 3–6% in the general population versus 15–30% in CSU patients and 30% of adults with CU have Hashimoto's thyroiditis[24]. It was also shown using an immortalized human mast cell line (LUVA) displaying high concentrations of FcεRI that epitopic cross-reactivity does not explain the increased prevalence of thyroid autoantibodies found in CU patients[24]. Given that in a recent study it was shown that 50% of CSU patients exhibited significantly (i.e. more than 4 fold) elevated levels of total serum IgE (>100 IU/ml) as compared to only 13% of healthy control subjects[25], it was suggested that in CSU mast cells may be activated by allergens that engage specific IgE antibodies bound to their high affinity IgE receptor, FcεRI. In line with this, it was shown that more than 50% of patients with CSU exhibit IgE antibodies against self, i.e. against thyroid peroxidase. These IgE-anti-TPO autoantibodies, when bound and activated on the surface of mast cells, could cause 'autoallergic' mast cell degranulation, a novel pathogenic pathway of urticaria induction. However, none of these studies had explored this association in the pediatric age group.

The presence of autoantibodies that are capable of inducing mast cell (and basophil) degranulation can be tested either *in vivo* with the use of the autologous serum skin test (ASST) or *in vitro* with various methods including the basophil activation test (BAT)[26].

Basophils represent <1 % of circulating leukocytes and are the substrate for the BAT[27]. Structurally and functionally, basophils are similar to tissue mast cells, which are central to the pathogenesis of CSU. Similarly to mast cells, basophils express the high-affinity IgE receptor

(FcεRI) on their surface. Cross-linking of the FcεRI by IgE and antigen or an antibody itself results in cell activation, the release of preformed granules and the secretion of cytokines, chemokines, and lipid mediators[28].

Upon FcεRI-mediated stimulation, basophils also upregulate the expression of distinct activation markers[29]. In 1991, *Knol et al.* demonstrated that tetraspanin (also known as CD63), a cell surface marker, is upregulated on degranulating basophils[30]. It was further shown that basophil activation via FcεRI leads to the fusion of CD63-positive secretory granules, which can be measured by flow cytometry and represents an indirect marker of histamine release[27,31,32]. BAT, which is based on CD63 cell surface expression is a sensitive and specific tool in the diagnostic work up for a wide range of IgE-mediated processes including food and drug allergies[29]. Recently, the BAT has also been shown to correlate with the ASST as well as the results of basophil histamine release assays and was proposed as a reliable functional assay in the diagnosis of autoimmune urticaria[33].

Data on the natural history and prognostic variables of chronic urticaria and its subtypes are scarce. Chronic urticaria is considered a self-limited disease, yet it resolves spontaneously in only 30-55% of patients within 5 years, and 20% continue having the disease for more than 10 years[34,35]. However, a more recent study suggests that some patients may have a highly refractory course of the disease. In a historical cohort study of 544 patients who visited a tertiary referral centre at a university hospital between 1968 and 1990 the proportion that cleared after 5 and 10 years was 29% and 44%, respectively. At the same time, only 11% of patients with cold urticaria (n=35) spontaneously cleared 10 years after onset of symptoms. In this study dermographism showed the best prognosis, and cold urticaria the worst. Only a few studies explored the natural history of physical urticaria and these were limited to only a few subsets. In children, a single retrospective study was done in the UK on 53 children over a 3-year period (1999-2002). In the Kaplan-Meier analysis, the number of children becoming urticaria-free was 11.6% (95% CI: 5.4%-23.9%) at 1 year post-onset and 38.4% (95% CI: 25.9%-54.3%) at 5 years post-onset. A history of allergic conditions and more frequent episodes of urticaria were associated with a worse prognosis[36]. These studies had a relatively small sample size. Furthermore, none of them were conducted in North America.

Cutaneous T-Cell Lymphoma (CTCL)

While urticaria is an example of benign often reactive in nature inflammatory reactions, Cutaneous T-Cell Lymphomas (CTCL) represent malignant inflammation of the skin[37]. CTCL is a rare, but potentially devastating malignancy. These cancers are characterized by localization of neoplastic T lymphocytes to the skin. Mycosis Fungoides (MF), its leukemic form, Sézary Syndrome (SS) and primary cutaneous anaplastic large cell lymphoma (cALCL) are the most common variants and account for ~80% of all cutaneous lymphomas[38].

Previous epidemiologic studies based on the Surveillance, Epidemiology and End Results (SEER) databases documented that until recently, CTCL was on the rise in the United States and around the world[39]. Multiple studies have shown a threefold increase in CTCL incidence during 1970-2000 but, interestingly, in the last decade the incidence of CTCL in the United States has stabilized and is estimated to affect ~10 individuals per million population per year[39,40]. Recent epidemiologic studies documented geographic clustering of cases of this rare cancer in Texas[41,42] and non-random distribution in Pittsburgh, Pennsylvania[43], therefore suggesting that there might be an important epidemiologic trigger for this malignancy. Recent work implicated immunosuppression, hydrochlorothiazide diuretics, low levels of Vitamin D and toxins secreted by *Staphylococcus aureus* as possible triggers/promoters of this this malignancy[44,45].

At early stages this cancer typically presents with erythematous patches and plaques on the trunk following a bathing suit distribution. Patch/plaque lesions are typically persistent and progressive[46]. Lesions can vary in shape, size, distribution and morphology. Typically lesions exhibit erythematous annular or serpiginous border and central atrophy (cigarette paper-like skin). Patients may have pruritus or burning sensation of the skin. Patients may have several non-diagnostic biopsies and it may take many years before a definitive diagnosis is established[46]. Classic histologic features that are typically seen in plaque MF disease include a band-like infiltrate of lymphocytes in the upper dermis, epidermotropism in the epidermis, where atypical T-cells with cerebriform nuclei form Pautrier microabscesses[46].

Advanced disease, SS was first described by Albert Sézary in 1938[47]. It is a distinct CTCL leukemic disorder characterized by erythroderma, debilitating pruritus, lymphadenopathy and detection of circulating atypical T cells in the blood[46]. Alopecia, palmoplantar keratoderma, onychodystrophy, and ectropion of the eyes are also commonly seen in SS patients[46]. The diagnosis is often based on one or more of the following criteria[46]: 1) expanded $CD4^+$ population resulting in a CD4/CD8 ratio >10 by flow cytometry; 2) absolute Sézary count $\geq 1000/mm^3$; 3)

aberrant expression of pan-T-cell antigens (such as loss of expression of any or all: CD2, CD3, CD4, CD5); (4) positive T-cell clonality detected by PCR or other molecular methods; (5) variable loss of expression of CD26 and CD7 markers on T cells.

The diagnosis of CTCL is often challenging since this cancer can masquerade clinically as other entities such as chronic eczematous dermatitis, psoriasis, pityriasis rubra pilaris, drug eruptions or fungal infections[46]. Even histopathology and PCR studies for T cell receptor clonality are sometimes insufficient for a definitive diagnosis, particularly at early and erythrodermic stages of the disease[46]. Based on available estimates in literature, it takes on average 6 years to diagnose this malignancy since its initial presentation[48]. Recent discoveries describing the use of high-throughput T cell receptor sequencing instead of traditional PCR methods to detect clonality promise to significantly shorten the delay in the diagnosis of CTCL [48,49]. This powerful method may also be suitable to help distinguish drug, viral or other reactive skin eruptions from Mycosis Fungoides in CTCL patients[48,49].

Furthermore, previous research from our laboratory demonstrated that it might be feasible to distinguish CTCL from its benign mimickers based on a set of gene expression markers (e.g., TOX, POU2AF1, GTSF1, ITK, FYB, LEF1, CCR4 etc.,) the elevated expression of which may additionally confer poor disease prognosis[50,51]. Similarly, expression profiling of microRNAs yielded potential valuable diagnostic/prognostic markers including miR-21, miR-155, miR-203, miR-205, miR-214, miR-488[52,53].

In recent years great strides were made to elucidate the cellular and molecular pathogenesis of this cancer. Specifically, the cellular origin of CTCL was demonstrated to be very important[54]. Mycosis Fungoides was proposed to arise from skin resident memory T (T_{RM}) cells, whose normal function is to defend the body from external pathogens. These cells express cutaneous lymphocyte-associated antigen (CLA) and CCR4, but often lack L-selectin and CCR7 expression, which would enable them to access lymph nodes and circulation[54]. Sézary Syndrome and leukemic-CTCL on the other hand are believed to arise from skin-tropic central memory T cells (T_{CM}) that express L-Selectin and CCR7 as well as CLA and CCR4, which enables them to affect, skin, lymph nodes and blood resulting in greater morbidity and mortality to the host[54]. Consequently, while skin-limited Mycosis Fungoides often exhibits indolent clinical behavior and does not impact survival, leukemic variants of CTCL and Sézary Syndrome often have a median disease survival of only 2-4 years[40,55-57]. Notably, it was found that Sézary cell count was a powerful predictor of disease-

related mortality where patients with <1000 Sézary cells/L had an overall survival of 7.6 years, while patients with >10,000 Sézary cells/L in blood survived only 2.1 years[57].

Recent research further aided our understanding of underlying molecular pathogenesis for CTCL. Specifically, large-scale mutational genome profiling analyses identified genomic alterations in a number of putative oncogenes and tumor suppressor genes including CARD11, CCR4, TP53, NF-κB and Janus Kinase signaling members[58-62]. Some of these results may have important clinical relevance, where for instance, Mogamulizumab (anti-CCR4 antibody) recently showed promising clinical response in advanced CTCL patients[63,64]. It was most striking, however, that there was very little overlap with respect to genomic alterations/mutations between these large-scale genome profiling studies, therefore highlighting this cancer genetic heterogeneity[58-62]. Similar molecular heterogeneity was also seen previously with respect to chromosomal translocations, where only few chromosomal alterations were seen across multiple studies (e.g., loss of 10q24, 10p12.1-726.3 9p21, 17p13.2-p11.2 or gains of 17p11.2-q25.3, and 8q24.1-q24.3 etc.), while most chromosomal alteration were only reported to occur sporadically [58,65-69].

In addition to the highlighted difficulties in diagnosing this cancer, it also remains very challenging to prognosticate this disease. Clinical disease stage at the time of diagnosis for early stage MF as well as clinical disease stage combined with age (≥60), presence of large cell transformation, elevated lactate dehydrogenase for advanced stages of CTCL were established as powerful prognostic markers and are very useful in a clinical setting[40,55-57,70]. In addition to the aforementioned criteria, the presence of folliculotropic/syringotropic disease on histology and the ability to detect the same T cell receptor clone longitudinally also confers a grave cancer prognosis[71,72]. Most patients (i.e., 70-80%) present with limited skin stage I patch/plaque disease and only ~15-20% of stage I patients progress to higher stages. There remains a critical unanswered challenge to accurately predict which patients will progress compared to those who will experience an indolent disease course. Multiple reports highlighted that high expression of *TOX* [73,74] as well as miR-155, miR-21 and let-7i microRNAs[53,60] may predict poor disease outcome.

Chapter 2. Studying the use of CD63 Basophil Activation Test (BAT) to define and monitor chronic idiopathic urticaria in children. (Full published manuscript is presented in Appendix 1.)

2A. INTRODUCTION

Chronic urticaria (CU) is a mast cell-driven disease and is defined as the recurrence of transient wheals and/or angioedema for at least 6 weeks. In most cases, hives and angioedema occur spontaneously and are not attributable to a specific trigger (chronic spontaneous urticaria; CSU)[75]. Up to 40% of adults and children with CSU are considered to have a type II autoimmune basis for their disease[8,10,76]. In these patients, autoantibodies are believed to induce mast cell (and basophil) degranulation. Patients can be screened for the presence of these autoantibodies either *in vivo* with the use of the autologous serum skin test (ASST) or *in vitro* via the basophil activation test (BAT)[77-81].

Basophils represent <1 % of circulating leukocytes and are the substrate for the BAT[27]. Structurally and functionally, basophils are similar to tissue mast cells, which are central to the pathogenesis of CSU. Similarly to mast cells, basophils express the high-affinity IgE receptor (FcεRI) on their surface. Cross-linking of FcεRI by IgE and antigen or an antibody results in cell activation, the release of preformed granules and the secretion of cytokines, chemokines, and lipid mediators[28].

Upon FcεRI-mediated stimulation, basophils also upregulate the expression of distinct activation markers[29]. In 1991, *Knol et al.* demonstrated that tetraspanin, CD63, a cell surface marker, is up-regulated on degranulating basophils[30]. It was further shown that basophil activation via FcεRI leads to the fusion of CD63-positive secretory granules, which can be measured by flow cytometry and represents an indirect marker of histamine release[27,31,32]. BAT using CD63 expression is a sensitive and specific tool for the diagnostic work up of a wide range of IgE-mediated processes including food and drug allergies[29]. Recently, the BAT has also been shown to correlate with the ASST as well as with the results of basophil histamine release assays and was proposed as a reliable functional assay in diagnosis of autoimmune urticaria[33]. In contrast to the ASST, the BAT 1) does not bear the risk of accidental infection by injections of heterologous serum, 2) is not influenced by the intake of antihistamines, 3) provides quantifiable results, 4) is suited for the monitoring of treatment, 5) can distinguish between patients who express anti-IgE

and anti-Fc FcεRI antibodies, and 6) can be performed in patients of any age including infants[82-84].

The definition of a positive BAT outcome and the interpretation of BAT results in CSU and other patient populations are directly dependent on the reference values established in control populations, e.g. healthy individuals. Ideally, such control populations should match the patient population in gender and age distribution as well as the rate of atopic individuals. At present, BAT reference values and cut-off criteria are derived from limited numbers of adults, but have not yet been determined in children. BAT results in children may be different from those in adults, and pediatric reference values and cut-off criteria are, therefore, needed. The aim of this experiment was to establish the reference range of CD63 expression-based BAT results in children without CSU, to compare their BAT results with those of pediatric CSU patients and physical urticaria (PU) patients and to assess the effects of age, gender, ethnicity, atopy, disease activity and IgE levels on BAT results.

2B. MATERIALS AND METHODS.

Control subjects and patients with CSU or PU

The study was conducted between January 2014 and December 2015 in Montreal, Canada. Subjects were recruited at the allergy and immunology clinic of the Montreal Children's Hospital. The study population included eighty non CSU control children recruited from established registries in our allergy clinic, 105 children with CSU and 23 with PU (Table 2.1). Data was collected on age, gender, ethnicity, IgE levels and atopy (asthma, atopic eczema, food allergy, and allergic rhinitis) as previously defined[85].

In addition, for the CSU cohort, we measured disease activity by using the weekly urticaria activity score (UAS7), the gold standard for assessing CSU activity (based on daily recordings of wheal number and itch intensity)[86].

We excluded patients with an active infection, autoimmune diseases (only for the control group), a recent allergic reaction and those who were taking drugs that could lead to direct mast cell/basophil degranulation (e.g. opiates). Patients receiving oral corticosteroids were also not included in the study. For CSU and PU patients, their treatment regimen was not interrupted prior to recruitment.

The study protocol and consent forms were approved by the institutional review board of the McGill University Health Centre and were in accordance with the guiding principles of the Declaration of Helsinki. Written informed consent was obtained from children's parents or legal guardians prior to conducting any study-related investigation. In addition, patients older than 7 years were requested to sign an assent form. All data used in this study remained anonymous.

Basophil Activation Test

Commercially available BAT kits were purchased from Bühlmann (Flow Cast ®, Bühlmann, Switzerland). The CD63 expression on the surface of donor basophils was determined by flow cytometry according to the protocol provided by the company and adapted for use in CSU patients as previously described.[82] Briefly, 50 μL of healthy donor blood was mixed with 50 μL of patients' serum, 100 μL of stimulation buffer (which contains IL-3) and 20 μL of antibody mix (contains fluorescein isothiocyanaten-labeled anti-CD63 antibody and phycoerythrin-labeled anti-CCR3 antibody) and incubated for 30 minutes at 37°C. Anti-FcεRI antibody (50 μL) and N-formyl-methionyl-leucyl-phenylalanine (fMLP, 50 μL) served as positive controls. As a negative control, patients' serum was replaced by 50 μL of stimulation buffer. Basophils were identified by their CCR3 expression on a FACScalibur. The percentage of activated basophils was determined based on the expression of CD63.

Statistical analyses

Statistical analyses were conducted using R version 2.12.0 of XLSTAT software. Given the relatively small sample size, the reproducibility of BAT results from different basophil donors was assessed based on the non-parametric Wilcoxon signed rank test with continuity correction. The normality of BAT results in the control population was assessed visually by a histogram as well as by the Kolmogorov–Smirnov test. Box plot was used to identify and exclude outliers. Values 1.5*IQR (interquartile range) above the third quartile or below the first quartile of control BAT values were considered outliers and were removed from the analysis for the calculation of the cut off value only. This procedure resulted in exclusion of one outlier of CD63 BAT (natural log of CD63 0.01% (-4.61); fourteen patients had this value out of eighty recruited. Reference values for CD63 in controls were defined by the central 95% confidence interval from the obtained log-normal distribution. The cut-off value for abnormal (high) CD63 was established as being

above the 95th percentile of the rate of CD63^{+} cells induced by the non-CSU control patients' sera, similar to published studies in adult CSU patients[33].

The Wilcoxon rank test was used to compare crude BAT means between the groups (controls, CSU and PU patients) as it does not require the data to follow a pre-specified parametric distribution (e.g. Normal). To test for possible variations related to age, gender, ethnicity (Caucasian, Non-Caucasian) and atopy (asthma, food allergy, atopic eczema or hay fever), univariate and multivariate linear regression analyses were performed using a logarithmic data transformation (log-normal distributions) to assess for potential confounders.

To determine the CD63 cut-off value for predicting disease activity we used the CUTOFF finder software (Charité University)[87]. UAS7 was used to separate CSU patients into those with mild (UAS7 < 15) *vs.* moderate to severe (UAS7 ≥15) disease according to the expert opinion[88].

2C. RESULTS

Demographic characteristics and reference range of BAT results of control children

The current study included 80 control subjects, 53.4% were male and the median age was 10 years (25% interquartile (IQ), 5; 75% IQ, 15). The majority were Caucasians and the most common atopic condition was food allergy (Table 2.1). Mean IgE levels were 1,562 μg/L (640 kU/L; standard deviation (SD) 889 kU/L). Patient demographic characteristics remained similar after the removal of the outliers for the CD63 cut off calculation (Table 2.1).

The median percentage of activated basophils as assessed by CD63 expression in the control population was 1.28% (25%IQ, 0.50; 75%IQ, 2.42). The Kolmogorov–Smirnov test of normality and the histogram (Figure 2.1a) indicated that the distribution of CD63 BAT was skewed. Hence, values were converted to the natural logarithmic scale and outliers were removed (Figure 2.1b). Log-normal distribution of CD63 was used to establish the reference range, and the cut-off for high CD63 was defined as 1.8% of CD63^{+} cells (1.46%; 95%CI: 1.20%, 1.77%) once converted to numerical scale. In the control population (80 patients), no association was found between CD63 levels and age, sex, ethnicity or presence of atopy using univariate and multivariate regression analyses (Table 2.2).

BAT results are elevated and more commonly increased in children with CSU

A total of 105 pediatric patients with CSU (48.6% male, median age 8 years, 25% IQ, 5; 75% IQ, 12.5) were included in the study (Table 2.1). The vast majority (96.2 %) was treated with an antihistamine, mostly on demand. The mean IgE value was 352.1 μg/L (144.3 kU/L; SD, 215.6k U/L)). The median CD63 expression was 2.1% (25%IQ, 1.16; 75%IQ, 5.34), significantly higher than in the control populations (Wilcoxon rank 2131.5, $p<0.001$, Figure 2.2).

High BAT results in children with CSU are associated with high disease activity and the absence of atopy

In multivariate analyses of log-normal distributed CSU patients, two factors were associated with CD63 levels: atopy and disease activity as assessed by the UAS7 score (Table 2.2). For every increase in UAS7 by one score point there was an increase in CD63 by 0.1%. To further assess the association between CD63 expression and the UAS7 score – CUTOFF finder software was used with CD63 as a marker and UAS7 as an outcome measure. Using this method, CD63 levels above 3.35% were statistically significantly associated with higher disease activity (i.e. UAS7 ≥15, sensitivity: 64.3%, specificity: 80.5%, $p < 0.01$; Figures 2.3). Atopy was found to be inversely associated with CD63, and the presence of atopy reduced CD63 levels by 0.75 %, on average.

BAT values in pediatric patients with physical urticaria are similar to those in healthy controls

A total of 23 pediatric patients with PU (47.8% male, median age 11 years, 25% IQ, 5; 75% IQ, 16) were included in the study (Table 2.1). Most took antihistamines on demand (91.3%). The mean IgE value was 947.4 μg/L (388.3 kU/L; SD, 713.6 kU/L)). The median CD63 expression was 1.88% (25%IQ, 0.89; 75%IQ, 3.34). The distribution of individual CD63 values for PU patients is illustrated in Figure 2.2c BAT values did not differ statistically between PU patients and controls (Wilcoxon rank sum difference of 840, $p = 0.23$).

BAT assay donor basophils

In this study, a healthy non-atopic volunteer, a 40 year-old male, served as the basophil donor for the entire study. The reactivity of the donor cells was tested by incubation with anti-FcεRImAb and N-formyl-methionyl-leucyl-phenylalanine (fMLP) (50 μL, Flow Cast ®,

Bühlmann, Switzerland). In order to test the reproducibility of the results, BAT analyses of 46 randomly chosen patients were repeated using basophils from a second healthy non-atopic volunteer donor matched for age and sex. There was no significant difference between BAT results obtained from basophil donor 1 and 2 (Wilcoxon rank test, p-value = 0.9) (Table 2.3).

2D. DISCUSSION AND CONCLUSIONS

We present here the first pediatric and the largest to date study assessing CD63 levels in non-CSU controls. Our study is the first to provide the reference range for the BAT, CD63 test, in non-CSU atopic and non-atopic pediatric patients and to determine factors associated with increased levels in CSU children.

The presence of pathogenic auto-antibodies in CSU was first demonstrated in 1988 with the discovery of IgG antibodies against IgE[17]. Additional IgG antibodies against the high-affinity IgE receptor (FcεRI) were identified in 1993 and their capability of inducing histamine release from mast cells and basophils was documented[10]. Current data indicate that up to 40 % of adult and pediatric CSU cases may be explained by the presence of these mast cell activating antibodies[8,10,76].

Currently, the autologous serum skin test (ASST) is used as a screening tool to detect the presence of pathogenic autoantibodies in CSU patients[89]. However, this method has several pitfalls and potential limitations especially in pediatric populations as discussed elsewhere[82]. Hence, attempts were made to develop *in vitro* diagnostic methods for the detection of pathogenic autoantibodies in CSU patients' sera[90]. Blood basophils and/or skin mast cells from healthy donors have been used *in vitro* to determine the presence and levels of functional anti-FcεRIα or anti-IgE autoantibodies using the basophil histamine release assay (BHRA) and basophil activation test (BAT)[26]. There is good concordance between assays using basophils and mast cells. Because basophils are much easier to obtain than tissue mast cells, BHRAs are currently the gold standard for the detection of functional autoantibodies in autoimmune CSU[26]. In 2006, *Szegedi et al.* demonstrated a strong correlation between the BHRA and BAT using CD63 molecule as the marker for activated basophils. The advantages of BAT include its low cost, rapid performance, and the availability of ready to purchase kits, which permit standardization and reproducibility of the results (Flow Cast ®, Bühlmann, Switzerland; CU Index test, IBT Laboratories, Lenexa, Kan;

Anti-IgE Receptor Ab, ADx Laboratories, Denver, Colo; HR-Urticaria Test, RefLab, Copenhagen, Denmark)[33,82].

Several BATs have been assessed in adult CSU patients, and CD63 and CD203c are believed to be the most reliable markers[26]. The advantages of CD63 include that it is the most studied marker in CSU and shows the best correlation to basophil degranulation[82]. In addition, several studies found a very high correlation (up to 95.5%) between the CD63 expression on basophils and the ASST in the diagnostic work up of autoimmune CSU[91,92]. Furthermore, CD63 expression has been suggested to be suitable for monitoring disease activity and response to therapy in patients with urticaria and in patients with atopic disease[83,91,93,94]. Hence, BAT using CD63 as the activation marker is a valuable tool in the diagnostic work up of the CSU patients.

As of now, no published results of BAT studies in pediatric CSU patients are available and the reference range for normal CD63 expression in children is unknown. Studies in adult CSU patients defined the BAT cut-off as the 95th percentile of CD63^{+} cells induced by the serum of healthy atopic or non-atopic volunteers[77,82,91,92]. However, most studies do not specify if the distribution of their control values were normally distributed.

Our results demonstrate that pediatric CSU patients have significantly higher levels of CD63 BAT than non-CSU controls. In contrast, there was no significant difference in CD63 expression between controls and PU patients, probably because the latter exemplify episodic urticaria only in response to a particular trigger. Age, gender, ethnicity or presence of atopy (asthma, eczema, hay fever or food allergies) did not influence CD63 levels in non-CSU patients. However, atopic CSU patients appeared to have lower CD63 scores than their non-atopic peers.

Using a cut-off of 1.8% of positive CD63 cells (>95% CI of logarithmic form), 58% of our CSU cohort showed a positive BAT, one of the positivity criteria for autoimmune CSU. This is in line with previous studies in adults that used the CD63 BAT and found a 40-50% prevalence of autoimmune CSU[33,77,91]. Previous adult studies assessed smaller cohorts, with often less than 10 non-CSU controls, to establish the cut-off for autoimmune CSU, and the type of value distribution was generally not defined[33,77,82,89,91,92].

In children, the prevalence of autoimmune CU was suggested to be similar to that in adults, based on BHRA and ASST[8,76,95]. To our knowledge, BAT was not yet validated as a diagnostic test for autoimmune CSU in children. Hence, the use of CD63 BAT as a diagnostic test for autoimmune CSU needs to be established by future studies conducted specifically in pediatric

patients. Nonetheless, we demonstrate that CD63 expression is significantly higher in pediatric CSU patients compared to non-CSU controls and PU patients and that its expression may be affected by the presence of atopy in CSU patients. Further, CD63 values above 3.4 % have a sensitivity of more than 60% and a specificity of more than 80% for predicting moderate to high disease activity. This may be clinically useful to monitor responses to therapy objectively, in addition to the use of the UAS7, which requires patient compliance. Taken together, we suggest that the CD63 BAT may be a useful test to diagnose and monitor autoimmune CSU in children.

One should keep in mind that different CD63 BAT methodologies exist and may influence the outcome. Triple labelled flow cytometric detection of $CD63^+$, $CCR3^+$ and HLA-DR^- cells was shown to be preferable, because it allows for a high basophil recovery[26]. To further reduce basophil degranulation variability a single basophil donor should be used if feasible, or basophil donors with similar characteristics. In our study, a single donor was used and results were reproduced using a second basophil donor. In contrast to atopic donor basophils, basophils from healthy donors need to be primed with IL-3, a transcription activator known to enhance specific basophil degranulation, to achieve comparable degranulation and histamine release[82,96]. Optimum IL-3 concentrations have been previously defined and are now integrated into available CD63 BAT kits[82].

Our study has some potential limitations. The available patient sera volumes were too low to perform the gold standard BHR assay to validate CD63 BAT as a diagnostic test for autoimmune CSU in children. Future studies need to address this. Duplicate samples were only tested on 46 patients in total using basophils from different donors and, while the finding of similar results is reassuring, the lack of duplicate testing on all patients constitutes a limitation in our study. It is impossible to predict, if duplicate or triplicate analysis would have prevented outliers' elimination from the study analysis. All eliminated outlier values from CD63 cut-off calculation had CD63 expression of 0%, hence potential inclusion of these values would have resulted in further decreased CD63 cut-off value. The rate of atopy, especially food allergy, was higher in our control group than in the CSU and PU groups. However, linear regression showed that atopy was not associated with CD63 expression in non-CSU subjects. Finally, given that different CD63 BAT kits may show different results, our results may only be applicable for the CD63 BAT kit we used in this study.

In conclusion, the CD63 BAT appears to be a feasible and useful tool for the diagnostic work up in CSU. Its specificity and sensitivity for diagnosing autoimmune CSU in children should be assessed by a future study that includes CD63 BAT and BHRA measurements, the ASST and quantification of anti-IgE and anti-FcεRI autoantibodies. The cut-off for positive CD63 likely differs in adult population and should be addressed by future studies. The established CD63 BAT cut-off of 1.8% in children, if confirmed by future studies, may be used in the future for research and clinical practice. CD63 BAT may also be used to assess disease activity in CSU. We are currently using the CD63 BAT to monitor disease activity and response to treatment in our center.

2E. FIGURES AND TABLES

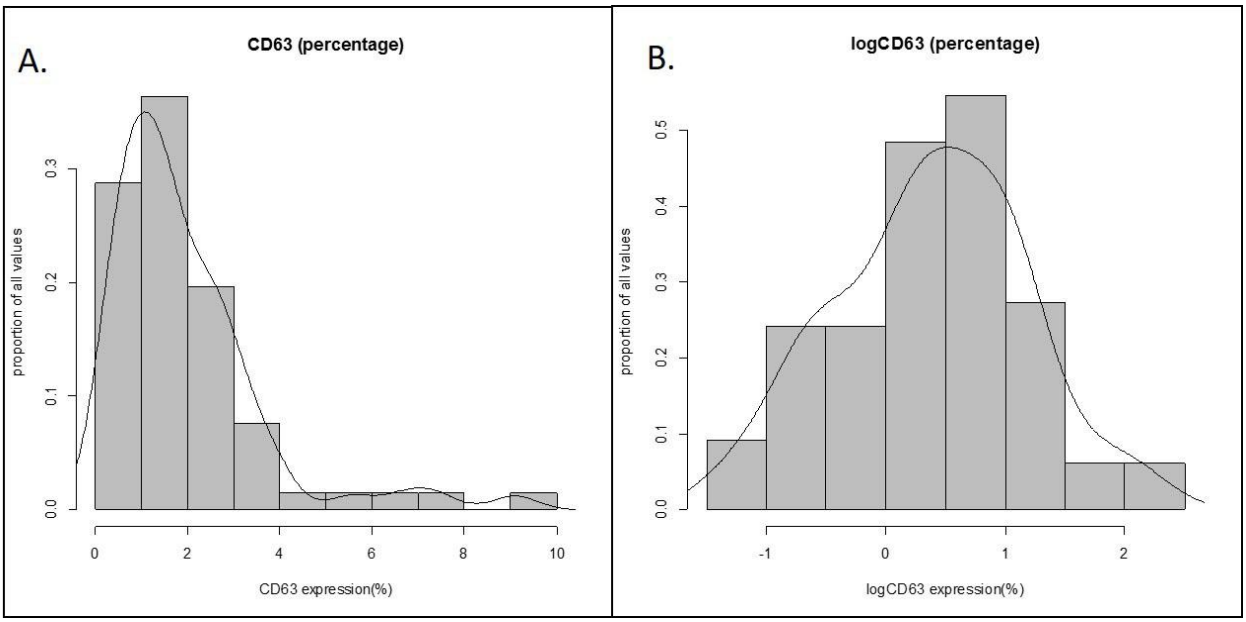


Figure 2.1A-B. Distribution of CD63 expression in non-CSU control subjects using numerical (A) and logarithmic (B) scales.

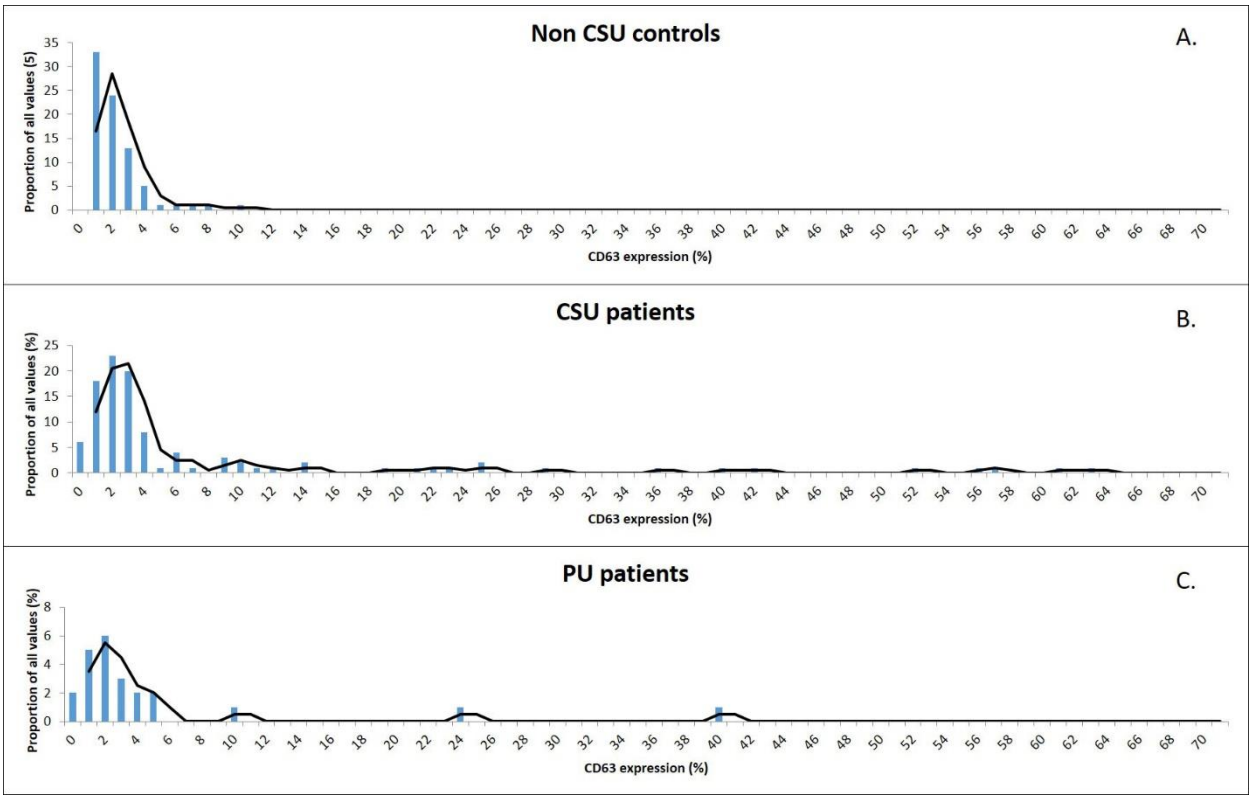


Figure 2.2 A-C. CD63 distribution for controls (A), CSU patients (B) and PU patients (C). More than 50% of CSU patients show BAT values above 1.8%. CSU, chronic spontaneous urticaria; PU, physical urticaria.

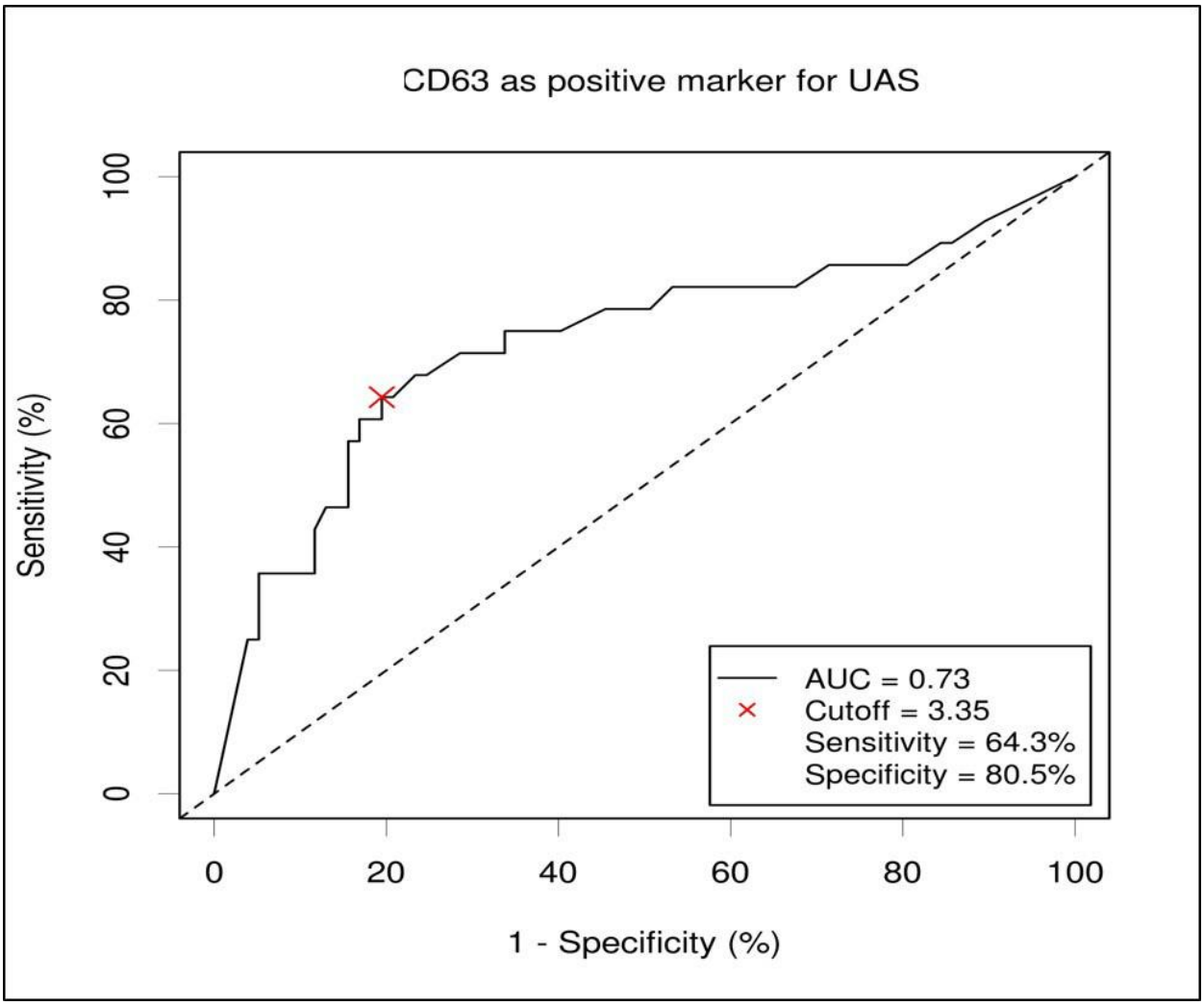


Figure 2.3. Area under the curve (AUC) with sensitivity and specificity for CD63 as outcome measure predicting worse disease severity. UAS, (weekly) urticaria activity score.

	Controls (n=80)	Controls with outliers removed (n=66)	CSU pts (n=105)	PU pts (n=23)
Median age in years (IQR)	10 (5,15.0)	9.5 (5,15.0)	8.0 (5.0,12.5)	11.0(5,16)
Male gender in % (95% CI)	53.4 (42.6,64.9.0)	54.5 (42.2,66.9)	48.6 (38.9,58.3)	47.8(25.7-69.9)
Ethnicity (% Caucasian)	86.3 (78.5,94.0)	86.4 (77.9,94.9)	65.7(56.5,74.9)	91.3(78.9-103.8)
Atopy % (95% CI)	78.8 (70,87.9)	80.3 (70.5,90.2)	21.9 (13.9,30.0)	26.1(6.7,45.5)
Food allergy % (95% CI)	68.8 (58.4,79.1)	69.7 (58.3,81.1)	3.8 (0.9,7.5)	8.7(-3.8,21.2)
Asthma % (95% CI)	13.8 (6.0,21.5)	15.2 (6.3,24.0)	10.5 (4.5,16.4)	13.0(-1.8,27.9)
Hay fever % (95% CI)	3.75 (-0.5,8.0)	4.5 (-0.6,9.7)	6.67 (1.8,11.5)	8.7(-3.8,21.2)
Eczema % (95% CI)	12.5 (5.1,19.9)	13.6 (5.1,22.1)	12.4 (6.0,18.8)	17.4(0.63,34.2)
Drug allergy% (95% CI)	15.0 (7.0,23.0)	16.7 (7.4,25.9)	3.8 0.9,7.5)	8.7(-3.8,21.2)
Recent positive SPT	33.8 (23.1,44.4)	36.4 (24.4,48.3)	0	0
Type of PU				
Cold				69.6(49.2,89.9)
Cholinergic	N/A	N/A	N/A	26.1(6.7,45.5)
Solar				13.0(-1.8,27.9)
Other				4.35(-4.7,13.4)
Subjects with antihistaminic mediation in % (95% CI)	0	0	96.2 (92.5,99.9)	91.3(78.8,103.8)
Median CD63 level in % (IQR)	1.3 (0.5, 2.4)	1.4 (0.9,2.6)	2.1 (1.2,5.3)	1.9(0.9,3.3)
Mean IgE level in µg/L (SD)	1561.5 (2169.9)	1690.4 (2308.5)	352.1 (526.1)	947.4(1741.1)

Table 2.1. Subjects' demographics and clinical characteristics.

IQR, interquartile range; CI, confidence interval; CSU, chronic spontaneous urticaria; PU, physical urticaria; N/A, not applicable; IgE, immunoglobulin E; SD, standard deviation; pts, patients; SPT, skin prick test.

	Control patients (80)				CSU patients (105)					
	Univariate (log)		Multivariate (log)		Univariate (log)		Multivariate (log)		Multivariate (exp)	
Variable	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Age	0.02	-0.02,0.06	0.06	-0.05,0.05	-0.05	-0.1,0.03				
Gender (male)	0.2	-0.2,0.6			-0.03	-0.8,0.8				
Ethnicity (Caucasian)	-0.2	-0.8,0.3	-0.7	-1.7,0.2	-0.1	-1.0,0.7				
Food allergy	0.1	-0.3,0.5								
Asthma	0.2	-0.3,0.8								
Eczema	-0.4	-0.9,0.2								
Hay fever	0.04	-0.9,1.0								
Atopy	0.1	-0.4, 0.6	0.1	-1.3,1.3	-1.3*	-2.2,-0.4	-1.4*	-2.3,-0.5	0.25*	0.1,0.61
SPT	-0.1	-0.5,0.3	0.6	-0.6,0.7						
IgE	-0.0002	-0.0001,0.001	-0.00006	-0.00001,0.00001	0.0004	-0.0004,0.001				
UAS7					0.06*	0.02,0.09	0.06*	0.02,0.09	1.06*	1.02,1.10
* p<0.05										

Table 2.2. In pediatric CSU, high UAS7 (≥ 15) is associated with higher levels of CD63, atopy is associated with lower CD63 levels. CD63 BAT values in control subjects are not associated with age, gender, ethnicity, IgE serum levels, or the presence of allergic diseases, positive skin prick test reactions or atopy. UAS7, weekly urticarial activity score; SPT, skin prick test; IgE, immunoglobulin E; CI, confidence interval; log, logarithmic expression; exp, exponential of log; β, beta coefficient of regression.

Sample	Basophil donor #1	Basophil donor #2	Mean ((%)	StdDev
1	3.24	1.87	2.56	0.685
2	10.00	10.20	10.10	0.1
3	1.50	2.15	1.83	0.325
4	1.75	0.50	1.13	0.625
5	1.39	3.66	2.53	1.135
6	2.10	2.82	2.46	0.36
7	8.96	7.35	8.16	0.805
8	13.70	11.40	12.55	1.15
9	1.61	2.42	2.02	0.405
10	2.16	5.22	3.69	1.53
11	45.80	37.90	41.85	3.95
12	2.70	4.65	3.68	0.975
13	13.10	10.11	11.61	1.495
14	1.75	1.37	1.56	0.19
15	2.97	3.75	3.36	0.39
16	1.65	3.16	2.41	0.755
17	35.60	41.22	38.41	2.81
18	2.06	2.94	2.50	0.44
19	2.44	3.85	3.15	0.705
20	0.00	2.25	1.13	1.125
21	2.82	0.00	1.41	1.41
22	94.40	90.90	92.65	1.75
23	37.30	42.00	39.65	2.35
24	1.37	3.85	2.61	1.24
25	1.22	0.00	0.61	0.61
26	0.00	4.85	2.43	2.425
27	5.21	5.00	5.11	0.105
28	1.30	4.80	3.05	1.75
29	2.74	4.10	3.42	0.68
30	3.70	4.01	3.86	0.155
31	2.70	0.00	1.35	1.35
32	2.99	0.00	1.50	1.495
33	2.70	5.56	4.13	1.43
34	0.00	0.00	0.00	0
35	3.03	0.00	1.52	1.515
36	5.56	4.00	4.78	0.78
37	2.33	0.00	1.17	1.165
38	1.27	3.67	2.47	1.2
39	1.23	3.85	2.54	1.31
40	2.86	5.50	4.18	1.32
41	1.37	4.33	2.85	1.48
42	3.70	6.25	4.98	1.275
43	0.00	0.00	0.00	0
44	0.00	1.25	0.63	0.625
45	1.35	2.33	1.84	0.49
46	0.00	0.00	0.00	0

Positive control (basophil donor 1)		0.24
Positive control (basophil donor 1)		1.27
Negative control		0.00

Table 2.3. Basophil activation test (CD63) results in 46 patients tested using basophils from 2 different basophil donors. StdDev, standard deviation.

Chapter 3. Study of clinical management of pediatric chronic spontaneous and physical urticaria patients using omalizumab and BAT CD63 assay. (Full published manuscript is presented in Appendix 2.)

3A. INTRODUCTION

It is believed that up to 50% of CSU cases are associated with pathogenic auto-antibodies, which can be detected *in vivo* by autologous serum skin test or *in vitro* by basophil activation test (BAT) reflected by increased CD63 expression or histamine release. The first line treatment for CSU is second-generation antihistamines and if not effective, the dosage can be increased up to four times in adults and children[75]. Unfortunately, 25-50% of patients do not respond to this treatment regimen and require other treatment modalities including anti-leukotrienes, cyclosporine and more recently omalizumab[97].

Omalizumab is a recombinant humanized monoclonal anti-IgE antibody that was recently approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for antihistamine refractory CSU patients who are at least 12 years-old[98,99]. It binds free serum IgE, prevents its attachment to the cognate high-affinity receptor on mast cells (FcεRI) and decreases receptor expression[99].

The Urticaria Activity Score (UAS) is a validated tool used in both clinical practice and trials for the assessment and monitoring of disease activity in CSU patients. It measures key urticarial symptoms (wheals and pruritus) on a daily basis. The UAS7 is the weekly sum score with a range between 0 (no wheals, no itch) and 42 (maximum disease activity) per week[75].

3B. CASE SERIES

In the current study we describe four pediatric cases presenting with severe CSU with or without delayed pressure urticaria with complete remission after treatment with subcutaneous omalizumab injections and propose the use of $CD63^+$ as a potential serum marker that could guide treatment (Table 3.1). All patients and/or their parents have signed an informed consent and the study was approved by the Ethic Review Committee of McGill University Health Centre.

Case 1: A 4 year-old girl presented with CSU (wheals and angioedema, UAS7 score of 21) for the last two years. She reported to our emergency department and allergy clinic every 2 weeks due to persistent hives and pruritus despite regular treatment with twice the daily recommended

dose of second generation anti-histamines. She was treated in the emergency department with systemic corticosteroids and epinephrine. Her investigations were within normal limits including a complete blood count, CRP (C reactive protein) and tryptase levels. In addition, due to an episode of syncope associated with her hives, she had a Computer Tomography scan of the head and an EEG that did not reveal any pathology. Bone marrow biopsy did not show any evidence of mastocytosis. She achieved complete remission within one week of the first dose of omalizumab (150 mg subcutaneous injection) and was able to discontinue all anti-histamines. Pretreatment and post treatment BAT results (based on CD63 expression) were comparable and within the previously reported normal range[33].

Case 2: A 16-year-old boy presented with CSU and delayed pressure urticaria resistant to 4 times the daily dose of antihistamines and antileukotrienes for 8 years (UAS7 of 28) (Figure 3.1). He frequently missed school and avoided all sporting activities because of his symptoms. BAT results were similar and within normal range pre and post treatment. He achieved complete remission and stopped all anti-histamines within one week of omalizumab (300 mg) treatment and resumed all physical activities.

Case 3: A 5-year-old girl with CSU (wheals and angioedema) was followed in our clinic for 3 years (UAS7 of 42), unresponsive to double dose of antihistamines and additional use of ketotifen and anti-leukotrienes. She was treated twice with oral corticosteroids leading to short lasting remissions. She achieved complete remission within 5 days following the first dose of omalizumab (150 mg) and anti-histamines were tapered. Pre and post treatment BAT was within previously described normal range[33].

Case 4: A 10-year-old girl was diagnosed with antihistamine-resistant CSU of three years duration (UAS7 of 21). She required short courses of systemic corticosteroids twice a year due to urticaria exacerbations. Pretreatment BAT revealed high percentage of $CD63^+$ basophils (62.16%, Figure 3.2) which decreased to 40.45% one month following omalizumab administration. She achieved only partial improvement (UAS7 of 10) after the second dose (300 mg). Furthermore, she experienced an exacerbation (UAS7 of 42) after her 4^{th} dose. At that time her CD63 positivity, as measured using BAT was 82.75%. Hence, after 4 months of therapy we elected to change her management and administer subcutaneously omalizumab 300mg every 2 weeks rather than monthly. Within a few days, her hives ceased to occur and $CD63^+$ levels went down to 69.86%

after 2 weeks of this new regimen. $CD63^+$ levels further decreased to 27.7% 1 month later and the patient successfully discontinued anti-histamines at that time.

None of these patients experienced any adverse effects and all of them remain currently on treatment (treatment duration 3-11 months). The cases described suggest an important role for omalizumab in the management of severe CSU/delayed pressure urticaria. Further, we report, for the first time, a potential role for the use of BAT in predicting the clinical efficacy of omalizumab management in CSU.

3C. DISCUSSION AND CONCLUSIONS

Crosslinking of the high-affinity IgE receptor (FcεRI) which is expressed on all basophils and mast cells leads to cellular activation and degranulation of preformed granules containing vasoactive mediators such as histamine[100]. Up to 50% of CSU patients are reported to have an autoimmune etiology due to the presence of mast cell activating antibodies (IgG type) against the IgE receptor or IgE itself[10]. IgE type autoantibodies against autoantigens such as thyroperoxidase or DNA have also been reported to be frequent in CSU patients[101]. CD63 molecule is expressed on activated basophils, correlates with cell degranulation, can be measured by flow cytometry and is suggested to correlate with the presence of auto-antibodies activating mast cells and basophils[100]. CD63 measurements were performed in all patients before initiating the treatment with omalizumab and within 2 weeks after administration of the SC injections and at the time of clinical improvement using a previously described protocol[82]. Cutoff for positive BAT was defined as 2.2%, representing the mean CD63 expression in healthy controls plus two standard deviations as previously published[33].

Although all four patients achieved complete remission, the patient with high percentage of activated basophils required change of protocol involving more frequent injections to achieve remission. Consistent with other reports in CSU and pollen allergy, decrease in the CD63 expression was noted after omalizumab[91,93,94], but we are the first to exemplify that cases that are more resistant to omalizumab treatment (Figure 3.1) are associated with increased CD63 levels and that a decrease in these levels might be associated with a delayed and less pronounced clinical response. Because omalizumab has been shown to reduce the level of free IgE by 96% within 3 days and decrease FcεRI expression on basophils by 73% after 7 days[93], we hypothesize that in CSU patients with very high load of circulating antibodies and/or FcεRI density reflected by high

% of CD63 expression, omalizumab treatment may be more challenging and require adjustment in the standard recommended protocol.

To our knowledge, this is the first report of successful and safe use of omalizumab in children <12 years of age for CSU and the first report of successful treatment of delayed pressure urticaria in children. Our small case series demonstrates that omalizumab is very effective in CSU patients with normal CD63 levels and that high CD63 expression level may indicate more persistent forms that may require more frequent injections or higher dosage. Larger studies are required to establish the role of CD63 expression as a potential marker for CSU management.

3D. FIGURES AND TABLES

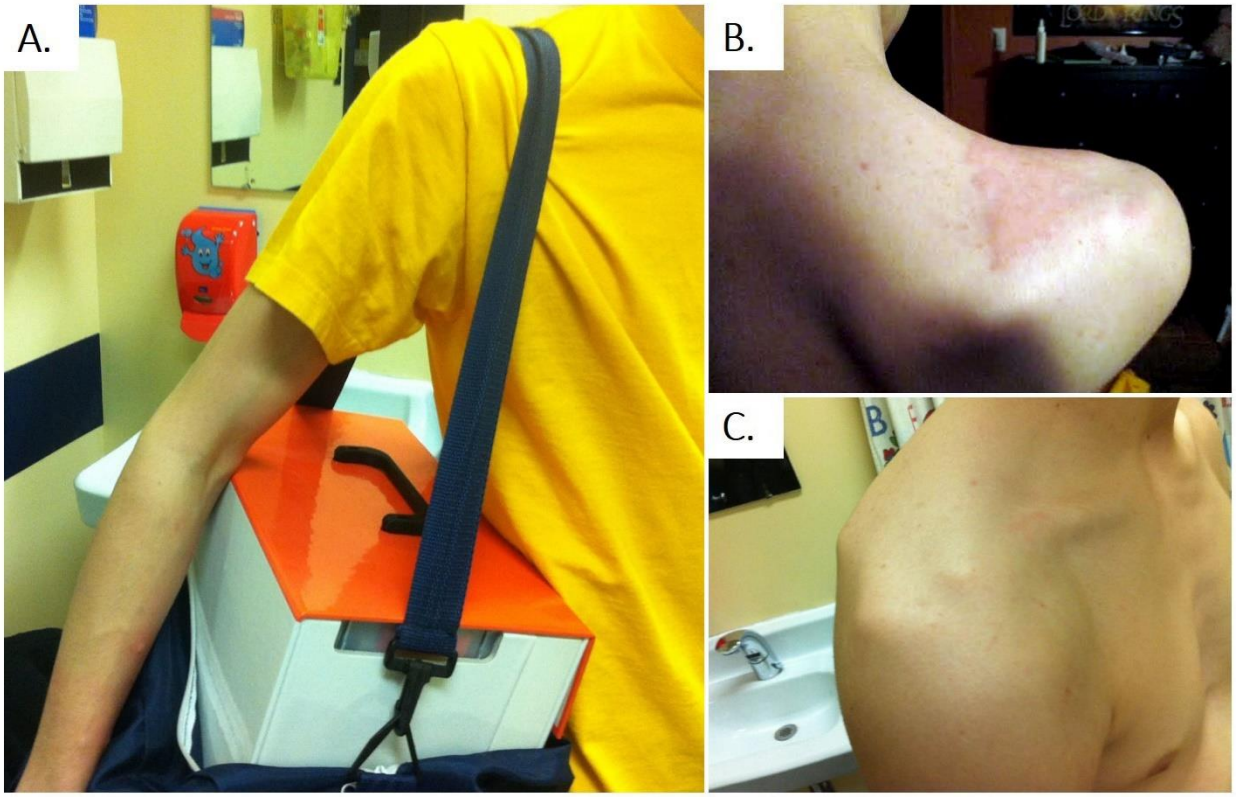


Figure 3.1. 16-year-old boy presented with CSU and delayed pressure urticaria resistant to 4 times the daily dose of antihistamines and antileukotrienes for 8 years.

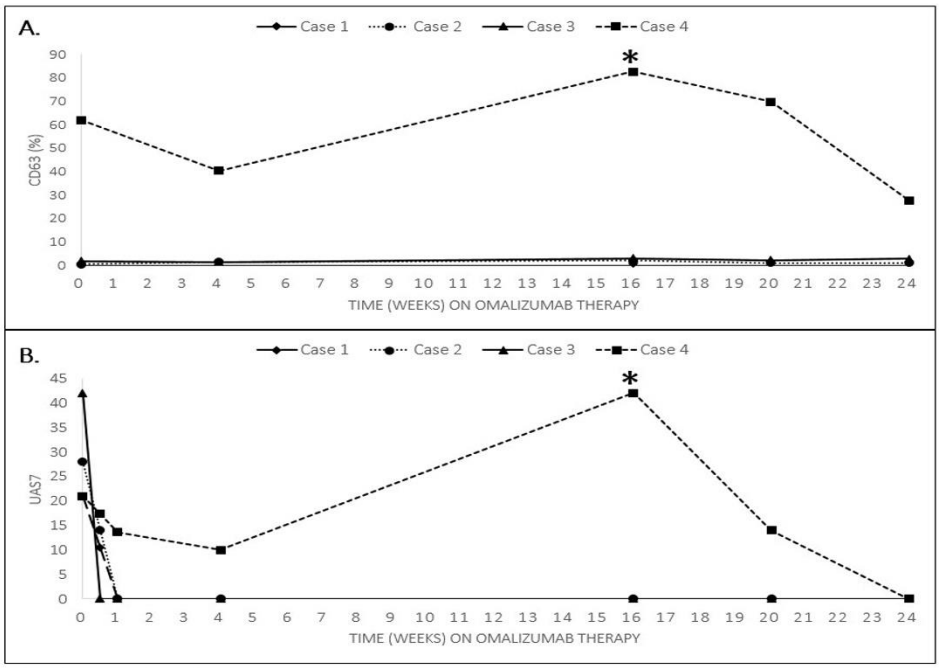


Figure 3.2. CD63 Basophil Activation assay and correlating clinical urticarial activity scores for the presented 4 cases in the study show strong correlation.

#	Age (years)	Sex	CSU duration (years)	Weekly UAS at baseline	Type of urticaria	Total IgE (U/ mL)	CD63 expression (%) at baseline	Dose and interval of omalizumab administration	Time to complete response (weeks)
1	4	F	2	21	CSU (wheals and angioedema)	919	0.86	150 mg monthly	1
2	16	M	8	28	CSU (wheals) and Delayed pressure urticaria	4080	0.48	300 mg monthly	1
3	5	F	3	42	CSU (wheals and angioedema)	65	1.90	150 mg monthly	< 1
4	10	F	1	21	CSU (wheals)	41	62.16	300 mg monthly for 4 months followed by 300 mg every second week	20

Table 3.1. Patients' demographics, pertinent laboratory finding and response to treatment.

Chapter 4. Investigating pathogenesis and risk factors of Chronic Urticaria (CU) in children, its societal impact and natural history.

4A. INTRODUCTION

Chronic urticaria (CU) is defined as the occurrence of transient wheals lasting more than 6 weeks. Physical urticaria (PU) occurs when the CU is associated with a specific physical stimulus that produce urticarial plaques (e.g. cold urticaria, solar urticaria, delayed pressure urticaria, localized heat urticaria, dermographic urticaria, or vibration urticaria[1]). The point prevalence of CU is 0.5–1% of general population[2-4] and it affects 0.1–0.3% of children[5]. Although no studies assessing the prevalence of CU in Canadians have been published so far, assuming a similar 0.3% lifetime prevalence, it is likely that ~101,000 Canadian children are be affected by CU each year[6].

In 80% of CU cases, hives occur spontaneously and are not attributable to a physical stimulus or an identifiable cause and this is classified as chronic spontaneous urticaria (CSU)[7]. While the exact pathogenesis of CSU remains to be elucidated, patients can be divided into at least two subgroups: those who are truly idiopathic, and those, who were previously diagnosed with idiopathic disease, but are now reclassified to have an autoimmune CSU (this affects ~ 40–50% of adults and children)[8-10]. The presence of autoantibodies that are capable of inducing mast cell (and basophil) degranulation can be established either *in vivo* with the use of the autologous serum skin test (ASST) or *in vitro* with various methods including the basophil activation test (BAT) using CD63 activation marker[26]. In children, only a few studies were done in a prospective manner that suggested that autoimmune CSU affects about half of pediatric CU cases in Europe[8,22] and Turkey[102], while in Thailand autoimmune CSU affects~40% of children[23]. We have recently addressed the use of BAT using CD63 expression for the diagnosis of autoimmune urticaria in Canadian children. As discussed in chapter 2, 58% of our pediatric CSU cohort had a positive BAT (defined based on 1.8% activation cutoff) therefore, suggesting autoimmune-driven urticaria[103]. Also, in that analysis, we found that BAT was significantly higher in patients with CSU compared to healthy volonteers. Notably, there was no statistical difference between BAT results in controls and patients with inducible forms of urticaria (e.g., physical urticaria and dermatographism).

Furthermore, it was recently suggested that a sizeable subgroup of adult CSU patients expresses IgE antibodies against thyroid peroxidase (correlating with the level of IgG against

thyroid peroxidase) that may cause "autoallergic" mast cell activation[20]. In 15-30% of adult CSU cases thyroid autoantibodies may be detected and up to 30% may develop thyroiditis (most commonly Hashimoto thyroiditis)[104-108]. Limited information is available with respect to autoimmune thyroiditis and thyroid autoantibodies in children. In addition, recent reports suggested a possible genetic predisposition to CSU and occasional studies suggested a possible association with various infectious triggers and comorbid autoimmune conditions[75,109].

Data on the natural history and prognostic variables of chronic urticaria and its subtypes are scarce. Chronic urticaria is considered a self-limited disease, yet it resolves spontaneously in only 30-55% of patients within 5 years, while 20% of patients suffer with this disease for more than 10 years[34,35,76].

Children with CU have a substantial impairment of quality of life (QoL)[14] and significantly lower school performance compared with individuals affected by other allergic diseases[11]. CU is associated with absence from school in 7.4% of cases with an average of 7.5± 18.5 missed days per year. However, it is not clear what is the effect of CU on the quality of life (QoL) in children and what are the factors associated with poor QoL. The European Academy of Allergy and Clinical Immunology (EAACI), the Global Allergy and Asthma European Network (GA(2)LEN), the European Dermatology Forum (EDF), and the World Allergy Organisation (WAO) recommendto use the disease specific QoL questionnaire (CU-Q2oL), and the weekly urticaria activity score (UAS7) as the standard measurements for disease-related QoL and disease severity, respectively[75].

The aim of this study was to assess the association of pediatric CU with autoimmunity, thyroiditis, and other comorbid conditions and to determine the QoL and natural history of CU in North American children.

4B. MATERIALS AND METHODS

Patients

The study protocol and consent forms were approved by the institutional review board of the McGill University Health Centre and were in accordance with the guiding principles of the Declaration of Helsinki. Written informed consent was obtained from children's parents or legal guardians prior to conducting any study-related investigations. In addition, patients older than 7 years were requested to sign an assent form. Participants aged from 0 to 17 years were recruited prospectively from the urticaria and dermatology clinics at the Montreal Children's Hospital from

December 2013 to December 2015. Complete history and physical examination were performed for each patient at the time of study entry and during each follow up visit. CU was defined as the recurrence of itchy wheals with or without angioedema for >6 weeks with hives present most days of the week. Physical urticarias were identified by proper investigations (i.e. suggestive history for physical triggers and appropriate confirmatory provocation testing, as recommended[110]). Other potential causes of urticaria (i.e. parasitoses) were identified by history and/or confirmatory stool testing for ova and parasites. As part of the standard of care, all patients had a complete blood count (CBC) (including absolute basophil count) and C Reactive Protein (CRP) levels at the of study ently[75]. In addition, serum total immunoglobulin E (IgE) and tryptase were obtained at baseline. Additional investigations (e.g. antinuclear antibody serology, skin biopsy, etc) were performed only if there were clinical features suggestive of a comorbid autoimmune disease.

Treatment regimen was based on the EAACI, GA(2)LEN, EDF and WAO recommendations[75]. All patients were prescribed initially a second generation non-sedating antihistamine such as desloratidine or cetirizine once daily according to age recommendation for approximately 2 weeks, and the dose was increased up to fourfold of the recommended initial dose after 2 weeks in presence of persistent symptoms. Those with persistent urticaria despite maximal dosage of antihistamines after 4 weeks were asked to contact the treating physician in order to be evaluated and potentially change/upgrade the treatment regimen. Third line treatment options included ketotifen[111], leukotriene receptor antagonist (montelukast) or omalizumab[83]. Anti-parasitic medications were given in case of positive stool culture only.

Assessment of autoimmunity and thyroiditis

In order to characterise patients with an auto-immune aetiology, basophil activation test using CD63 marker expression was performed. Values above 1.8% of basophil activation were considered significant as previously described[103]. In addition, in order to assess for the development of autoimmune thyroiditis levels of thyroid stimulating hormone (TSH), free thyroxine (FT4) and thyroid peroxidase antibodies (IgG anti-TPO) were verified[20].

Assessment of natural history of the disease

Disease resolution was defined when hives had not occurred for at least 1 year without treatment (the date of resolution being the last day of active hives) as described in previous

studies[23]. All patients were followed prospectively and assessed for disease resolution. In case of loss to follow up, patients were called on annual basis to verify if the disease was still persistent. Cox model was used to address the rate of resolution and sociodemographic, clinical and laboratory parameters predicting prognosis.

Assessment of disease severity and QoL

The CU-Q2oL is a 23-item questionnaire divided into six scales: pruritus (two items), impact on daily activities (six items), sleep problems (five items), limits (three items), appearance (five items) and swelling (two items). Patients indicate, on a Likert scale with multiple options (1: not at all; 5: very much), how much they have been troubled by each problem in the last 2 weeks. Scores for each scale and a total index derived by summing the scales are calculated and transformed to a 0–100 scale (indicating the percent of maximum possible), with 100 indicating the worst QoL. It has been validated in Canada. It is available in English and French languages and is considered to be the gold standard in clinical practice and research[35,86,112,113]. While not validated for the use in pediatric population, CU-Q2OL has been consistently used in individuals ≥12 years of age to assess QoL at baseline and after treatment.[114-117] In the current study, CU-Q2oL has been administered at the time of study entry for adolescents (≥12 years). Disease severity was assessed using weekly urticaria activity score (UAS7) (weekly average for the previous 12 weeks) at each clinical follow up visit as previously described[75,83,103]. Mild disease was defined as UAS7 <15 and moderate to severe disease was defined as UAS7 ≥15[103]. To improve compliance, in case patients and/or parents forgot to bring their UAS7 forms on follow up, they were given an opportunity to send it to the principal investigator by email or bring it to the next follow up visit. As a measure of last resort, patients were called by one of the investigators and asked to verbally provide the recorded daily numbers for the period of 12 weeks. If patient or parent did not record numbers and did not remember the scores, UAS7 was not calculated and was judged as a missing value.

Statistical Considerations

Cox model was used to calculate the rate of resolution per year and to determine factors associated with resolution (i.e. demographic data including age, gender, presence of co-morbid

auto-immune disorders and laboratory characteristics including CRP, absolute basophil count and BAT findings).

4C. RESULTS

Demographic data

One hundred thirty nine children with CU were recruited (Table 4.1). The gender distribution was equal. Almost 70% of children were of Caucasian race. The mean age of disease onset was 6.7±4.7 years of age (ranged from 0–17 years of age). The mean duration of CU was 2.0±1.8 years (range 0.3-8.7 years). Most common type of CU was CSU (78%). Twenty two percent of patients had PU, of those, approximately a quarter had CSU as well. The most common subtype of PU was cold-induced urticaria (15.8%) followed by cholinergic (6.5%), sun-induced (2.2%) and delayed pressure (0.7%) urticarias. A quarter of patients had concomitant angioedema symptoms (28% of CSU patients). Almost all patients (95%) required a treatment for their symptoms consisting of second generation antihistamines alone or, in resistant cases, in combination with ketotifen (13%) and/or antileukotriene (1.4%) and/or omalizumab (5%).

Regarding comorbidities, the most common was atopy (28%) (usually asthma or atopic dermatitis). The rate of atopy in family members was similar (21.6%). Six patients were diagnosed with a comorbid autoimmune disease: two with type 1 diabetes mellitus, three with autoimmune hypothyroidism and one child with systemic lupus erythematosus (SLE). Five children were known for other not related chronic health conditions (Table 4.1). Interestingly, in 17% of children with CU there was a familial history of autoimmune disease with autoimmune thyroiditis being the most common (10.1%). Other conditions included SLE, autoimmune arthritis, inflammatory bowel disease and multiple sclerosis. One patient was adopted and, hence, no family history was available. Furthermore, in 12% of children, one or more immediate family members had a history of CSU.

Laboratory investigations

Most patients had their blood drawn (n=126). In vast majority of patients, the results were completely normal (Table 2). Thyroid peroxidase antibodies were positive in four patients. Of those, 3 were diagnosed with Hashimoto thyroiditis and were being treated with thyroid replacement therapy. Thyroid function tests (TSH and FT_4) were normal in all patients. C reactive

protein (CRP) was found to be elevated in 10 patients (8%), while 8 patients (6%) had peripheral blood eosinophilia (none had met the criteria for hypereosinophilic syndrome). In one of those patients, stool parasites were found and urticaria resolved following antiparasitic treatment. Immunoglobulin E levels were high in 40% of patients with a mean of 552 μg/L±1522.3μg/L. Serum tryptase was normal in all patients. A BAT analysis on this cohort of patients was previously described and showed that 57% of them had positive findings suggesting auto-immune urticaria[103].

Natural history

In total, 43/135 CU cases have resolved during the course of the study giving a resolution rate of 10.3 % per year (Figure 4.1a). Four patients were lost to follow up and their disease resolution could not be assessed. Cox model was used to assess for association between likelihood of disease resolution and the age, gender, urticaria activity score, personal and family histories of autoimmune disease, high CRP, positive BAT and basophil counts. Two variables were found to have statistically significant associations. Higher BAT (Figure 4.1b) and lower basophil count (Figure 4.1c) were associated with earlier resolution of urticaria. Patients with positive BAT were twice more likely to resolve after one year (hazard ratio (HR) of 2.2, $p < 0.05$), while patients with increased basophils were 60% less likely to see their disease resolve (HR 0.43, $p < 0.05$).

Disease severity and quality of life

At the time of study entry, eligible patients (≥12 years old) were asked to complete a CU-Q2oL questionnaire and all patients were given the weekly urticaria activity score (UAS7) form to complete for the next follow up visit. At the initial visit, 77.4% of children who answered the QOL questionnaire were already taking antihistamines prescribed by their primary care physician. Based on their responses, their quality of life was found to be most affected by pruritus (42.5%) followed by the impact of hives on their appearance (24.2%) and sleep impairment (23.7%) (Table 4.2). Symptoms of angioedema manifested by lip and/or eye swelling only rarely caused significant morbidity (4.5%). A total of 8 UAS7 were administered to each patient over the 24-month follow up period (Table 4.3). Most patients (95%) were actively treated since the beginning of the study. The first average UAS7 score was of 8.4±9.7 (maximal score 42) indicating only a mild disease. While compliance was excellent for the first set of UAS7 (137 responders or 98.6%),

it rapidly and consistently decreased during subsequent follow up visits, where only 3 patients reported their UAS7 scores for during the 8th follow up.

4D. DISCUSSION AND CONCLUSIONS

In this study we for the first time describe a North American cohort of pediatric urticaria patients (n=139) from a single university center that was followed over a period of 2 years. Based on our analyses CU was as common in girls as in boys. Approximately 20% of our patients had a proven physical trigger (usually cold) for their hives, a rate similar to previously published in literature[1]. Among our patients, only 1 case was associated with infectious trigger/cause (stool parasite infection). Almost 30% of patients had atopic diathesis, a prevalence grossly similar to that in general population[118]. Importantly, in over half of our patients (57%), the BAT using CD63 expression was positive suggesting an autoimmune etiology. Thyroid peroxidase antibodies were positive in 4 patients (3.5%), of those 3 were already known and treated for Hashimoto hypothyroidism (2.1%). Three additional patients had a concomitant diagnosis of an autoimmune condition such as type 1 diabetes mellitus (1.4%) or SLE (0.7%). However, given the small sample size, it is difficult to draw definitive conclusions. Notably, we also observed a higher rate of autoimmune disease (17%) and especially autoimmune thyroiditis (10%) in family members of our patients. The prevalence of autoimmune diseases in the general population has been raising over the last few decades, and recent reviews estimate the cumulative prevalence of autoimmune diseases to be between 7.6 and 9.4%[119]. Furthermore, interestingly 10% of patients reported family medical history of chronic urticaria.

CSU resolution rate in our cohort was 10.3 patients-year. Similar rates were reported in adult and pediatric literature[14,36,76]. However, this rate reflects CSU cases seen in a pediatric tertiary care center and may not be applicable for CU cases seen in a primary care setting. Also, in Montreal, the waiting time to be evaluated by a specialist ranges form 2-6 months. Hence it is likely that a number of CU patients whose disease resolved prior to their appointment were missed.

We assessed for possible predictors of resolution including the age, gender, disease severity (UAS7), personal and family histories of autoimmune disease, high CRP, positive BAT and basophil count. Surprisingly, higher BAT and lower basophil count were associated with earlier spontaneous resolution of urticaria. We hypothesize that this finding could be due to transient, infection induced, auto-antibody production. Infections, especially viruses are common in children

and are well accepted pathogenic players in up to 80% of cases of acute urticaria[120-122]. Furthermore, acute viral infections in children and adults have been proposed to be able to induce transient auto-antibodies against self, due to 1) mimicry between the virus and self or 2) virus induced cell apoptosis revealing a self neo-antigen. Antibodies produced in such cases are usually low title and transient, but can be high title, disease specific and pathogenic[123]. Clinical disease can occur when auto-antibodies bind and alter the function on a self-antigen or generate immune complexes which lead to tissue damage. Clinical examples of such interactions include, but are not limited to ebstein barr virus (EBV) and SLE, hepatitis C virus and cryoglobulinemic vasculitis, mycoplasma and cold agglutinins, etc. This infection-induced autoimmunity will often resolve within months, but can also trigger a chronic disease[124].

In support of this notion *Kulthanan et al.* performed a retrospective chart review of 337 CSU adult patients and demonstrated that in adults CSU resolved faster in patients with autoimmune urticaria (56.5% in 1.2 years) compared to idiopathic forms (34.5 % in 1 year),

Given its chronic nature and associated pruritus, CU may have a profound impact of patients' QoL. Earlier studies have suggested that CU QoL impact is comparable to that of diabetes mellitus type 1 and epilepsy.[125] In this study, we wanted to assess the disease severity and QoL impairment in the setting of new treatment strategies and guidelines[75]. The average weekly UAS7 at the time of the first follow up visit (3 months after enrolling into a study for the majority of patients) was 8.4±9.7 indicating mild symptoms. However, the third interquartile UAS7 was 14, indicating that up to 25% of patients had at least moderate disease severity.

At that visit, treatment was adjusted. Fortunately, the values of third interquartile have significantly improved on subsequent follow up visits, but the response rate dropped significantly. While it is difficult to comment on disease severity in patients who did not fill the UAS7 score on follow up, in general these patients had well controlled disease symptoms and often did not come for their follow up.

In conclusion, CU in children, similar to adults, is associated with increased prevalence of autoimmune comorbidities in patients and family members. Relative frequency of autoimmune urticaria in children is similar to adults and if proven in the future, may be a good prognostic factor predicting earlier disease resolution. Quality of life and disease severity improve in children with current treatment strategies, which appear to be effective based on this study findings.

4E. FIGURE AND TABLES

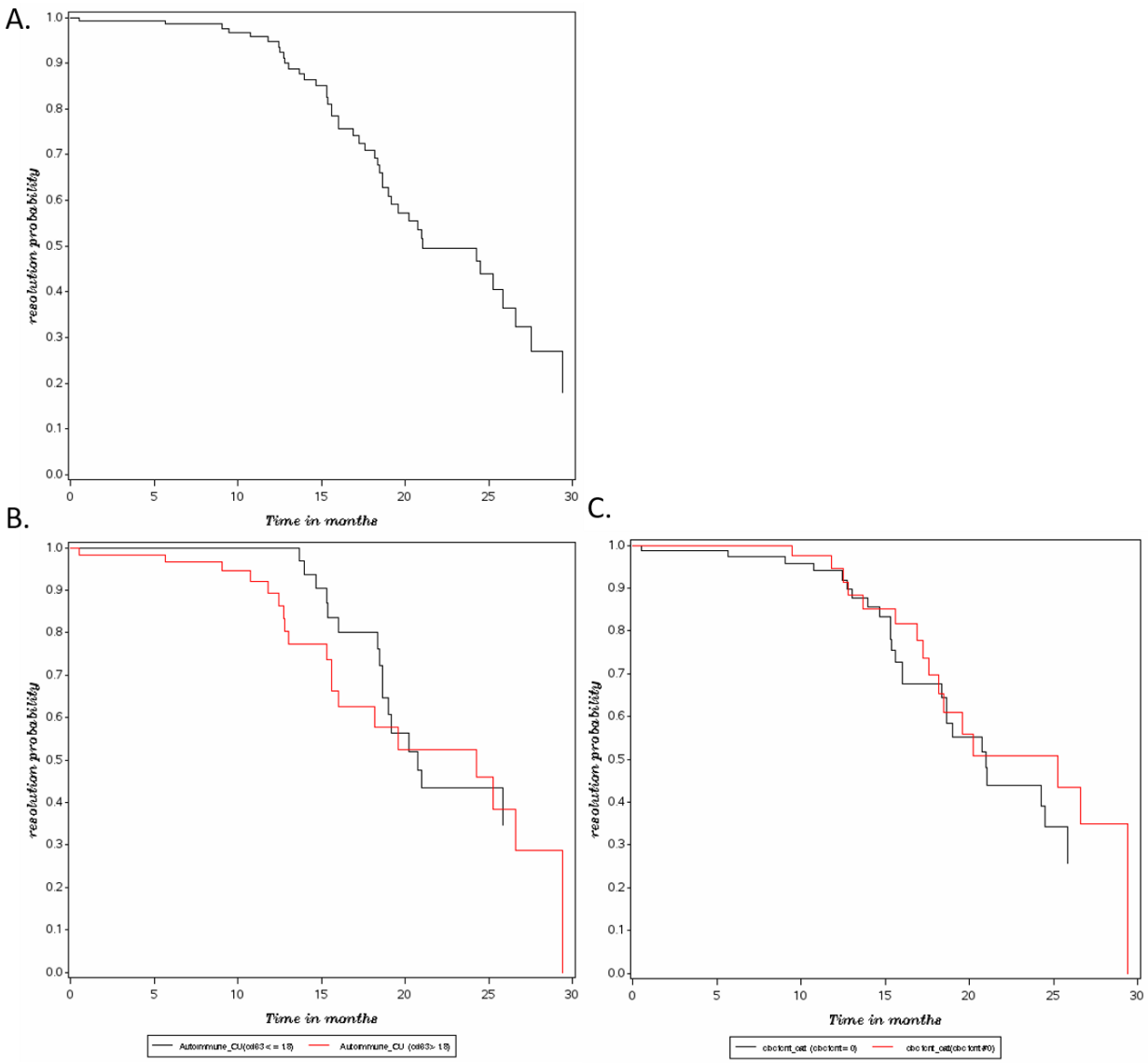


Figure 4.1. Survival analysis of chronic urticarial resolution rates. A. Overall disease resolution of 10 per 100 patients-year **B.** Faster resolution was observed in patients with autoimmune urticaria **C.** Basopenia had weak association with faster resolution of CSU.

Variable	Value (Std. Dev.)

Gender **(**Male, in %) (Female, in %)	 48.9 51.1
Ethnicity (Caucasian, in %)	**68.4**
Age of onset, (mean in years)	6.7 (4.7)
Disease duration, (mean in years)	2 (1.8)
Parental marital status (two parents, in %)	78.4
Parental education (college and above, in %)	79.1
Personal history of atopy (%) Asthma Eczema Food allergy Allergic rhinitis	28.1 16.6 14.4 6.5 6.5
Personal history of autoimmunity (%) Thyroiditis Systemic lupus erythematosus Insulin dependent diabetes mellitus	4.2 2.1 0.7 1.4
Other comorbidities Cystic fibrosis IgA nephropathy Autistic spectrum disorder Epilepsy	 0.7 0.7 0.7 1.4
Treatment (%) Antihistamines Ketotifen Montelukast Omalizumab	 95.0 12.9 1.4 5.0
Type of urticaria (%) **CSU** **CSU + PU** **PU** Cold Sun Cholinergic Delayed pressure	 84.9 7.2 22.3 15.8 2.2 6.5 0.7
Associated angioedema	23.7
Family history (%) Chronic urticaria Atopy Thyroid disease Lupus Autoimmunity other	 12.2 21.6 10.1 0.7 6.1
Abbreviation: CSU, chronic spontaneous urticaria; PU, physical urticaria; IgA, immunoglobulin A; Std. dev., standard deviation.	

Table 4.1. Patients' demographics, pertinent clinical findings, treatment and co-morbidities.

Investigations	**Number (%)**

Abnormal thyroid function tests	0 (0.0)
Positive thyroid antibodies (> 9 IU/mL)	4 (3.5)
BAT (CD63 expression > 1.8%)	59 (57)
Positive stool examination for parasites	1 (0.7)
CRP (> 5 mg/L)	10 (8.2)
IgE (> 240 μg/L)	49 (39.8)
Eosinophils (> 4%)	8 (6.1)
Tryptase (>13.5μg/L)	0 (0.0)
Upper limit for normal values is included in parenthesis.	

Table 4.2. Results of laboratory investigations.

Visit number	Number of responders	Mean	Standard deviation	Range	IQ1	IQ3
1	137	8.4	9.7	(0.0-35.0)	0.7	14
2	116	3.6	6.7	(0.0-35.0)	0	4.7
3	69	2.4	5.8	(0.0-42.0)	0	2.5
4	45	5.5	10.5	(0.0-42.0)	0	7
5	21	2.7	4.6	(0.0-16.0)	0	3.3
6	14	4.4	7.6	(0.0-24.0)	0	4.4
7	5	0.3	0.7	(0.0-1.5)	0	0
8	3	6.2	10.7	(0.0-18.6)	0	18.6
Abbreviation: IQ, interquartile.						

Table 4.3. Variation of weekly urticaria activity score (UAS7) over time

Chapter 5. Analysis of expression of Embryonic Stem Cell (ESC) genes in CTCL. (Full published manuscript is presented in Appendix 3.)

5A. INTRODUCTION

The molecular pathogenesis of CTCL remains only partially understood. While many studies focused on investigation of known oncogenes and tumor suppressor genes in CTCL[37,74,126,127], recent reports suggest that it is also important to look for genes that could be erroneously expressed in this cancer[128]. Ectopic expression of oncodevelopmental genes (e.g. α-Fetoprotein, H19 and Sonic Hedgehog signaling genes), embryonic stem cell (ESC) genes (e.g. OCT4, DOX2, NANOG, EED, SUZ12, etc.) and cancer testis (CT) genes have been reported in various solid and lymphoproliferative malignancies, where they are believed to play a central role in tumorigenesis and cancer progression. The expression status of these genes in CTCL remains unknown.

Recent reports demonstrated that CT genes are ectopically expressed in this malignancy. It was previously suggested that CT antigens during carcinogenesis may play an important role in maintaining cell survival (i.e., inhibition of apoptosis)[129-131], promoting resistance to various forms of chemo- and radio-therapy[132,133] and contributing to oncogenesis by targeting p53 and p21 tumor suppressor genes[134,135]. Also, since SYCP1, SYCP3, DMC1 and REC8 CT antigens under normal conditions regulate generation of double strand DNA breaks during crossing over in meiosis, it was suggested that these genes may promote aneuploidy and genomic instability in cancers by producing aberrant chromosomal recombinations[136].

Most importantly, in recent years a concept of Cancer Stem Cells (CSC) has emerged, where these cells share many characteristics with normal stem cells, specifically, the enhanced self-renewal capacity, resistance to apoptosis, ability to maintain undifferentiated state, overcome cellular senescence and give rise to all cell types found in a particular tumor. Like normal stem cells, CSCs divide infrequently and, therefore, are often spared by therapies that target rapidly dividing populations of cells. These CSCs are proposed to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. Hence, understanding the expression of these embryonic genes in CTCL may help us better understand the pathogenesis of this cancer and identify novel diagnostic markers and therapeutic targets.

During this project working with Dr. Litvinov and Dr. Sasseville I have investigated the expression for a panel of ESC genes in CTCL and summarized expression patterns for CT antigens, thymocyte development, B cell-specific and other ectopically expressed genes in the historic cohort of 60 CTCL patients that our laboratory described in other publications[51,137].

5B. MATERIALS AND METHODS

Patients and Samples

All patients were enrolled in the IRB-approved study protocol with informed consent in accordance with the Declaration of Helsinki[51,137]. CTCL patients were recruited from the Cutaneous Lymphoma Clinic at the Dana Farber Cancer Institute (DFCI)/Brigham and Women's Hospital (BWH). All tissue samples were obtained and processed as previously described[137].

Cell Culture

HH, H9, Hut78, MJ and Hut102 patient-derived CTCL cell lines were previously described[138,139] and were purchased from the American Tissue Culture Collection (ATCC). H9 is a clonal derivative of Hut78 cell line[140]. MyLa, PB2B, Mac2A, SZ4, SeAx, Sez4 were a generous gift from professors K. Kaltoft and N. Ødum (Copenhagen, Denmark) and were previously described elsewhere[141-145]. MJ, Hut78 cells were serially passaged in IMDM media (Invitrogen, Catalog # 12440-079) containing 10% fetal bovine serum (FBS) (Invitrogen, Catalog # 26140-079). HH, H9, Hut102, MyLa, Mac2A and SZ4 cells were grown in RPMI media containing 10% FBS. Finally, Sez4 and SeAx cells were grown in RPMI media (Invitrogen, Catalog # 11875-093) containing 10% FBS, 5 ng/mL of recombinant human IL-2 (Catalog # 202-IL-010 from R&D Systems) and IL-4 (Catalog # 204-IL-010 from R&D Systems). All cells were grown in 5% CO_2, 95% air humidified incubator at 37^{o}C.

Quantitative Real-Time Reverse Transcription-PCR Gene Expression Analysis.

Gene expression was tested via RT-PCR in CTCL patients' lesional skin, normal skin form healthy volunteers and lesional skin from patients with benign inflammatory dermatoses as previously described[51]. Primer pair sequences for tested genes and control housekeeping genes are listed in Table 5.1. RT-PCR was performed utilizing the obtained cDNA from patients and iScript RT-PCR mix (Bio-Rad, Catalog # 170-8893) on Bio-Rad iCycler as previously described[51,126,127].

The expression was standardized using genorm method[146] utilizing ACTB, B2M, SDHA, YWHAZ and HMBS housekeeping genes.

5C. RESULTS

Identification and function of CSCs have recently become the focus of cancer research. While the expression of many embryonic stem cell and putative CSC markers has been extensively studied in other cancers, very little experimental data is available for CTCL. To address this, we tested the expression of 26 ESC markers in CTCL lesional skin samples. These results demonstrate that, while few genes are not expressed or infrequently expressed in CTCL (e.g., SALL4, ESRRB and DAX1/ NR0B1), the majority of ESC markers are heterogeneously expressed in CTCL (Figure 5.1 and Table 5.2). Furthermore, a number of genes that are critical for maintenance of pluripotency in ESCs (e.g., EED, SUZ12, EZH2, MTF2, OCT4, CHD7 and TIP60) are almost universally expressed in CTCL samples. Normal function of these genes and their potential roles in carcinogenesis are summarized in Table 5.2.

Any given CTCL lesional skin biopsy contains numerous cell types including keratinocytes, fibroblasts, merkel cells, melanocytes as well as infiltrating malignant and reactive $CD4^+$ T cells. To confirm that the above-observed ectopic expression of embryonic stem cell genes takes place in malignant T cells we tested the expression of these 26 ESC genes in a panel of 11 patient-derived immortalized CTCL cell lines (Figure 5.1B). Our findings confirm that many of these genes are also ectopically expressed in patient-derived CTCL cell lines.

We further highlight that a number of CT antigens are ectopically expressed in CTCL lesional skin, where cTAGE1 was expressed in all samples, SYCP1 and GTSF1 were frequently expressed in CTCL, while SYCP3, REC8 and DMC1 were heterogeneously expressed in CTCL lesional skin (Figure 5.2A).

Finally, we analyzed the expression of genes that are usually not expressed in mature $CD4^+$ T cells. TOX and EVA1 are usually expressed in thymocyte development, but are subsequently downregulated in mature T cells[147,148], while BLK and POU2AF are usually specific to B cells and should not be expressed in T cells[149,150]. PLS3 (Plastin 3) is an actin binding protein, which is also normally not expressed in T cells[74,151]. Our analysis confirms that BLK, POU2AF, TOX, EVA1 and PLS3 are ectopically expressed in CTCL lesional skin biopsies (Figure 5.2B).

5D. DISCUSSION AND CONCLUSIONS:

Our results for the first time document the expression of embryonic stem cell genes in CTCL lesional skin biopsies and patient-derived cell lines. From this extensive list of genes (Table 5.2) few classes especially stand out. Polycomb Repressive Complex 2 (PRC2) genes (EZH2, EED and SUZ12) were previously shown to promote pluripotency in normal stem cells, enhance self-renewal capacity, maintain de-differentiation and resist apoptosis in normal ESCs[152-154]. A schematic diagram on the role of these genes in carcinogenesis is presented in Figure 5.3B. Polycomb group proteins are key regulators of chromatin structure, cell identity, and development[152]. Indeed, it was suggested that reprogramming of somatic cells toward pluripotency would involve extensive chromatin reorganization and changes in gene expression. These genes were documented to be ectopically expressed in multiple malignancies, where they establish/promote a cancer stem cell-like state[152-154]. Such ectopic expression of PRC2 components often correlates with disease severity[152,154]. As highlighted in Figure 5.3A, EZH2, EED and SUZ12 are strongly expressed in CTCL samples. Similarly, our study indicates that JARID2, a protein that often associates with the PRC2 complex[155], as well as PHC1 and RNF2 members of the PRC1 complex[156,157], are also ectopically expressed in CTCL lesional skin (Figure 5.3A). All three genes were shown to be important to maintain embryonic stem cell identity (Table 5.2).

NANOG, SOX2 and OCT4 are another group of key transcription factors involved in the maintenance of pluripotency of embryonic stem cells[158-160]. These genes are essential for maintaining the self-renewing undifferentiated state of the inner cell mass of the blastocyst[161]. OCT4, SOX2 and NANOG are ectopically expressed in many malignancies, where they are often associated with aggressive disease and poor cancer survival[162-168]. Similarly to EZH2, EED and SUZ12, these genes work in concert to induce pluripotent embryonic stem cell-like state and promote cancer stem cell phenotype[169,170]. Based on our expression results these genes are heterogeneously expressed in CTCL lesional skin. Furthermore, consistent with the above findings, upstream and downstream members of NANOG signaling are also expressed in CTCL. ZFX, a transcription factor, is known to transactivate the promoters of NANOG and SOX2[171,172], while DAX1 and MTF2 pluripotency markers are induced by NANOG[161]. All of the aforementioned genes are heterogeneously expressed in CTCL lesional skin. Overexpression of ZFX contributes to the "stemness" and pluripotent behavior of cancers[171,172]. Our study further demonstrates the expression of other pluripotency embryonic stem cell markers including CNOT3, KLF4, TBX3 and TRRAP. Detailed description of these genes and their biological activities is

described in Table 5.2. On the other hand, few ESC genes (e.g. SALL4 and ESRRB) were not expressed in CTCL lesional biopsies.

Looking at other classes of genes, we and others have previously documented that several CT genes were ectopically expressed in CTCL[128]. The normal function of these genes is to regulate meiosis, synaptonemal complex assembly and generation of double strand DNA breaks during crossing over[128]. Ectopic expression of these genes in cancer may promote genomic instability and be an important driving force behind aneuploidy and generation of balanced and unbalanced chromosomal translocations. Hence, while ESC genes may be reprogramming cancer stem cell-like phenotype, CT genes may be promoting genomic instability enabling these cells to express important oncogenes and disrupt critical tumor suppressor genes.

Finally, recent studies highlighted that malignant T cells in CTCL are able to express few B cell-specific genes one of which is the Src kinase BLK [149]. Importantly, BLK is constitutively active in malignant T cells and appears to be a *bona fide* oncogene that drives malignant T cell proliferation *in vitro* and tumor formation *in vivo*[149,173]. In addition, recent translational experimental work revealed that TOX expression, which is usually silenced in mature T cells, can be used as a robust prognostic and diagnostic marker for MF and SS[174,175], while PLS3 actin-binding protein, which is usually not expressed in T cells is consistently expressed in CTCL[74,151,176,177]. A growing body of literature documents that ectopic expression of these genes in CTCL is not a mere indication of deregulated cellular processes, but an important mechanism of tumorigenesis and cancer progression. Our study confirms that select thymocyte development genes (e.g., EVA1 and TOX), B cell–specific genes (POU2AF and BLK) and PLS3 are ectopically expressed in CTCL. A summary of different classes of ectopically expressed genes in this cancer is summarized in Figure 5.4.

In summary, our work highlights the importance of ectopic expression in CTCL of ESC genes, CT antigens, B cell-specific and thymocyte development genes. Further analysis of how ECS genes reprogram CTCL and promote cancer stem cell phenotype will greatly enhance our fundamental understanding of this cancer and will help us develop novel therapeutic targets.

5E. FIGURES AND TABLES.

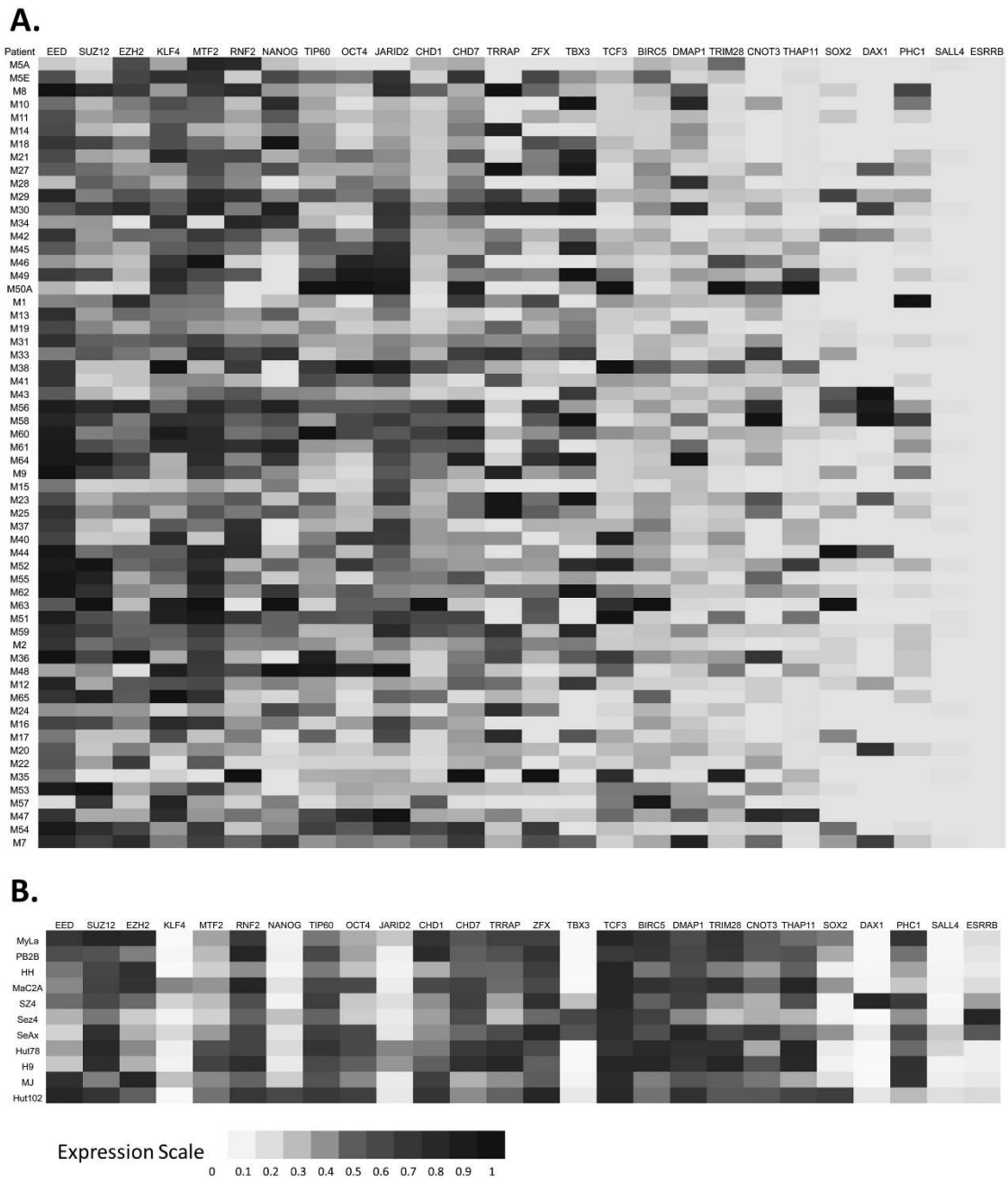


Figure 5.1. A. Expression of embryonic stem cell genes in CTCL lesional skin biopsies. **B.** Expression of embryonic stem cell genes in patient-derived CTCL cell lines.

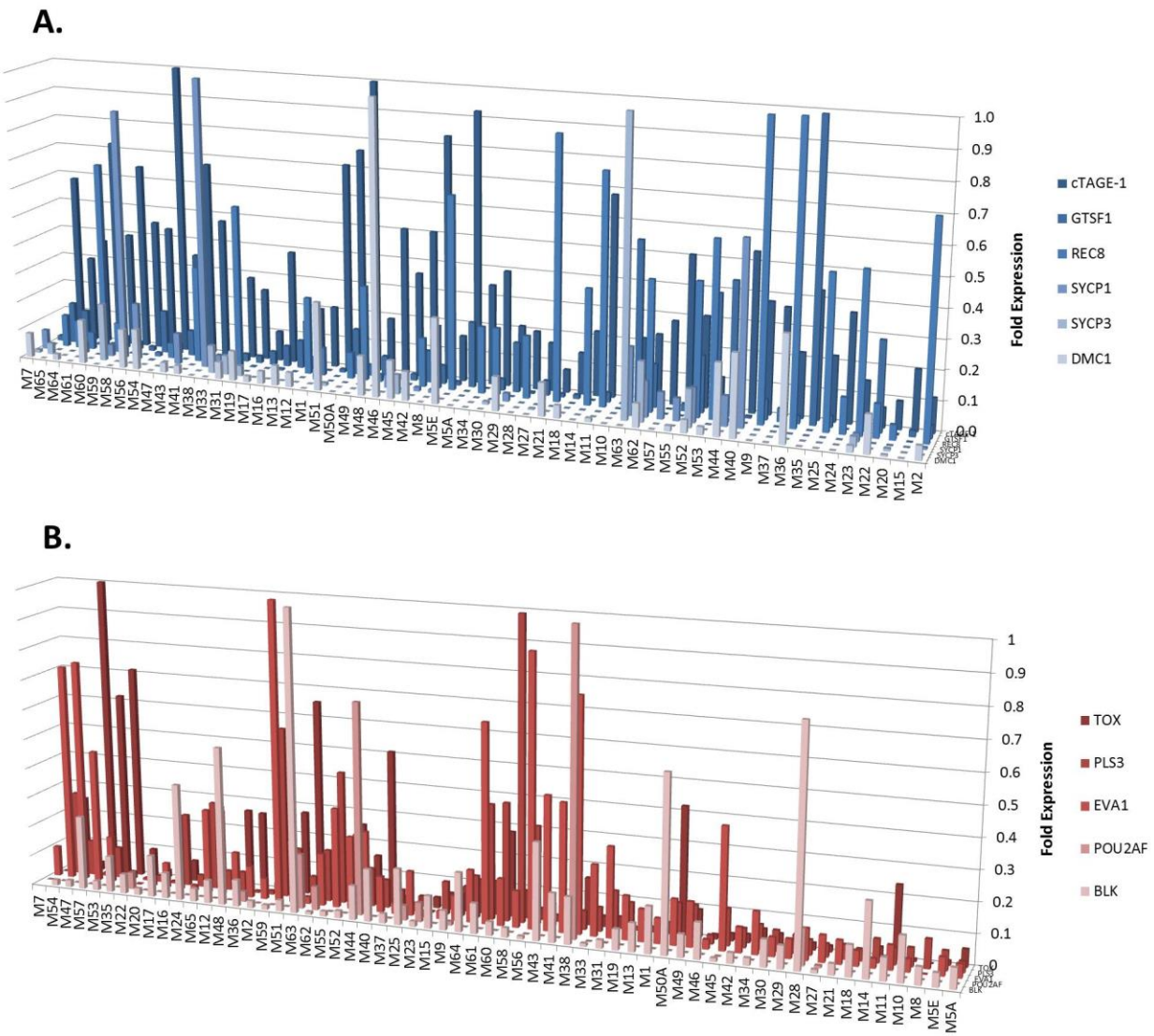


Figure 5.2. A. Expression of cancer testis antigens in CTCL lesional skin. **B.** Expression of thymocyte development genes (EVA1 and TOX), B cell-specific genes (BLK and POU2AF) and PLS3 in CTCL.

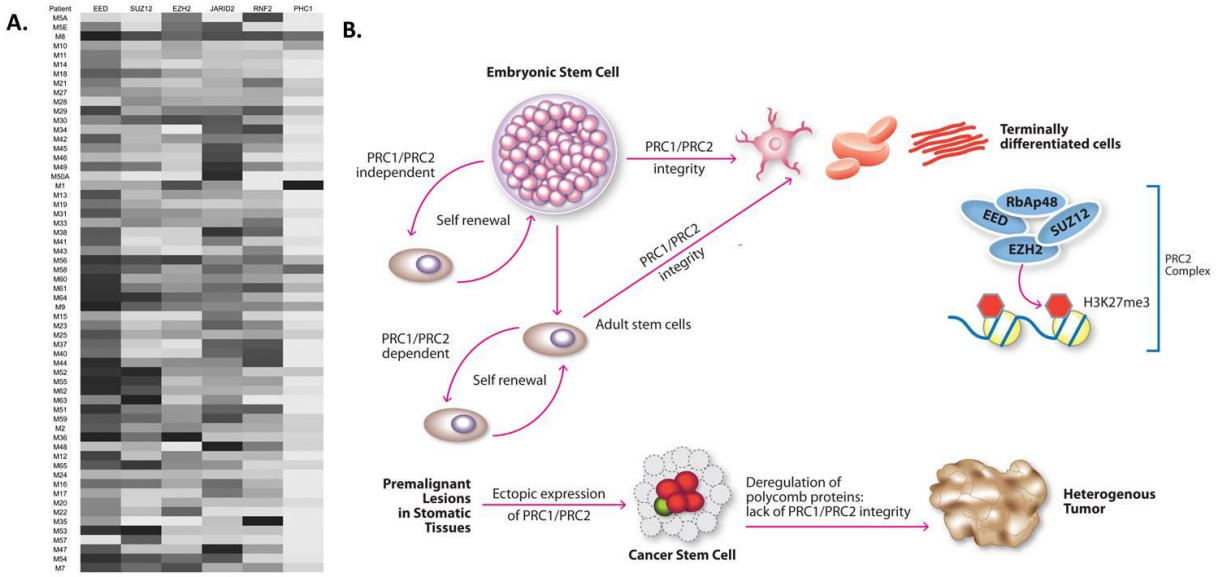


Figure 5.3. A. PRC2 complex genes (EED, SUZ12 and EZH2) are heterogeneously expressed in CTCL. JARID2 protein associates with PRC2 complex while PHC1 and RNF2 proteins are members of the PRC1 complex. JARID2, PHC1 and RNF2 are all expressed in CTCL lesional skin. **B.** Integrity of PRC1 and PRC2 signaling is critical for embryonic and adult stem cells. Ectopic expression of PRC1 and PRC2 in cancer may reprogram somatic cells towards cancer stem cell phenotype. Deregulation of these genes in somatic adult tissues may play a critical role in tumorigenesis.

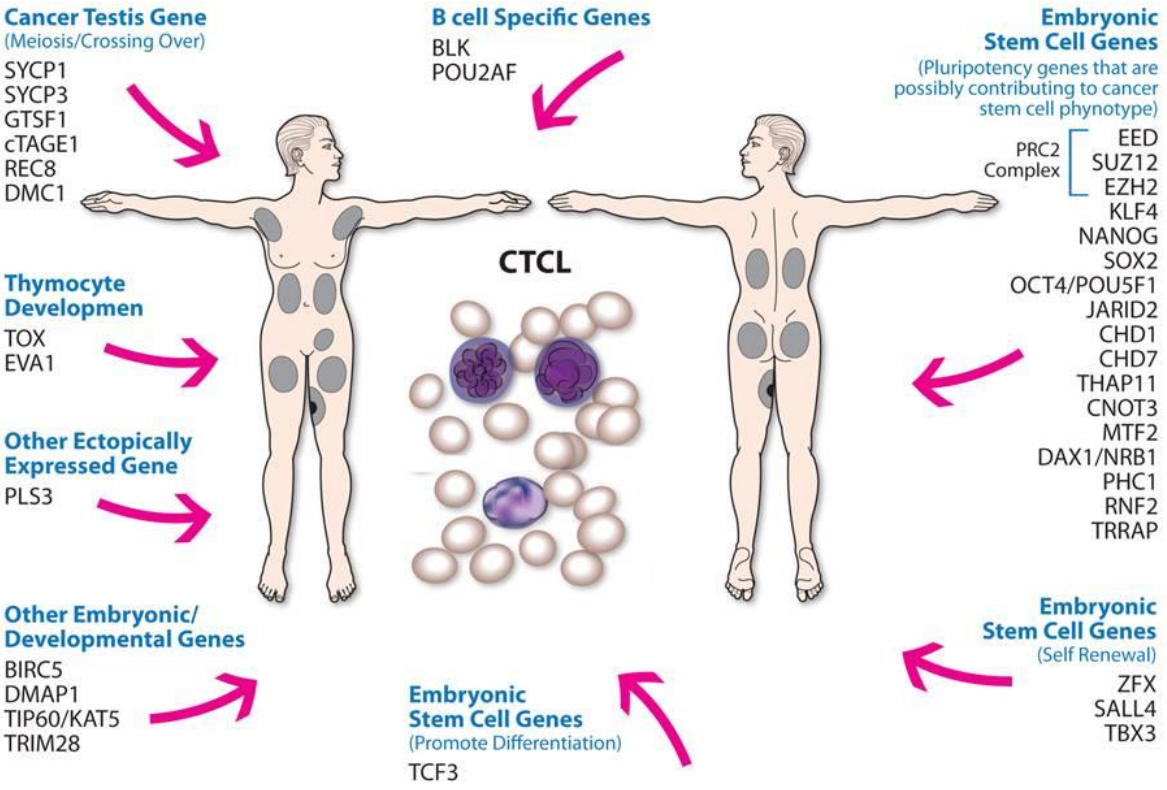


Figure 5.4. Ectopic expression of embryonic stem cells genes, cancer testis genes, B cell-specific genes and thymocyte development genes may work in concert to promote tumorigenesis and cancer progression in CTCL. Specifically, while embryonic stem cell genes may be reprogramming cancer stem cell phenotype, cancer testis genes may be contributing to aneuploidy and genomic instability by producing aberrant chromosomal recombinations.

Gene Name	Forward Primer	Reverse Primer
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
BIRC5	TTCTCAAGGACCACCGCATCT	AGTGGATGAAGCCAGCCTCG
BLK	TCGGGGTCTTCACCATCAAAGC	GCGCTCCAGGTTGCGGATGA
CHD1	AGACCGACATCAGGGAGATTCTTACA	CCTGTGATCATCCAGTTTTCTGTGTTTC
CHD7	GGCACAGCTCCACCCATCAC	CTGAGTCATATCCGGCACTGGTTT
CNOT3	CGTCCGTCTCCAAGAGAGTATGAAGA	CAAACTGCTCCACGCCCTCG
CTAGE1	TCCTTACCGTCCCCCAAGACCT	GCTGTCGTTCTGGATGTTCAGCA
DMAP1	GCGCGGATGTACGGGACATTC	CCTCGGGCCTCTTGAAAGTCAG
DMC1	TGCAATGTCAAAGGACTCTCAGAAGC	CCCGGTGGTGATATGGAAAACCA
EED	TGCGGCCAAGAAGCAGAAGC	TGCATTTGGCGTGTTTGTAGGTG
ESRRB	CGGGGACATTGCCTCTGGCTA	TGATCTCGCACTCGTTGGTGGC
EZH2	AAAATTATGATGGGAAAGTACACGGGGA	CTTCTCTTTCTTCAGGATCGTCTCCATC
GTSF1	GCAGACCAGCACCCCATTTGTC	GGCAGAGATTTGGGAACTCGCA
HMBS	GCTTCACCATCGGAGCCATCT	TGGCAGGGTTTCTAGGGTCTT
JARID2	AGGCTAGTGGAAGAGAAGGACTGC	CCTGTGTTATTGGGGAGGACGG
KAT5	TCCTGAGCGTGAAGGACATCAGT	GCCTCTTTCTTGGGGAACTGGATC
KLF4	ATCTCAAGGCACACCTGCGAA	ATCTGAGCGGGCGAATTTCCAT
MPZL2	TGGGTTTCCCTCATGTATGGCAAG	CATTAACAGCCTCCAGCACCCG
MTF2	ACTGAGGGAACTGCACATTCATCC	GGCCAAGATCTTCCTGTACGCG
NANOG	TGGATCCAGCTTGTCCCCAAA	AGGCCCACAAATCACAGGCATA
NR0B1	AGGGGACCGTGCTCTTTAACCC	AGTTCGATGAATCTGTCATGGGGC
PHC1	CCAAACACCAGCACTACACAGCA	GCACAGATTGGGTCAAGGTGGT
PLS3	ACTCTCTTGGTGTCAATCCTCACGT	TCCCAGTTTCGGGTATGGAGGT
POU2AF1	GGGGCTCAGATAAGTCCTCTCTGG	GTGGTTTGCCCACAGCTAATTTTCA
POU5F1	TGCAAAGCAGAAACCCTCGT	TCGGGCACTGCAGGAACAAAT
REC8	TGATGGAGACCCTAGAAGATGCTCC	ACTCTCTCTGGGATTGCAGCCT
RNF2	GCCTCATCCCACACTTATGGAAAAAGA	AGTTCTTCTAAAGCTAACCTCACAGCC
SALL4	ACCCCAGCATCTGGCTAAAACAC	GTGGCTTCATCCTCACTCGCCA
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
SOX2	TGAACCAGCGCATGGACAGTTA	CATCATGCTGTAGCTGCCGTT
SPO11	ACAGAGCAACACTTATGCAACCAAAAG	ACTCCTCCTTGACACTTTTAACATGCA
SUZ12	TCATCGCCAACCTGGATTTGCT	ATGTTCTTTGCTGTTCTACTTCCCCAT
SYCP1	CCACCAGCTTCTCATCTTTGTGTCA	AGCAATTACAGCCCAACGGTCC
SYCP3	ACCAAGGCTTCTCTCAAAACTAGTAACC	ATCCCACTGCTGAAACAAAGTCAGA
TBX3	CTGGAGGCTAAAGAACTTTGGGATCA	ATCCAGCCCAGAACATCTCACTTTAAAT
TCF3	CCTGTTTGAAACGGCGAGAAGA	TGGGGAGCTGAAAGCACCAT
THAP11	CCAAAGGACGCTGAGTTGCGG	CGTACCGTGTAGGTCTTGCGG
TOX	TGAGCATGACAGAGCCGAGCC	CAGCGAGTGGTCTGGGAGGG
TRIM28	CCCCACAGGAGTTTGCCCAG	GCACAGCAGAGAACTTGGTGTCA
TRRAP	GTCCACGCTGATGTTGGAGCA	AGGGAGTAAAGCTCCGCAAGGG
YWHAZ	TCCCCAATGCTTCACAAGCAGA	TCTTGTCATCACCAGCGGCAA
ZFX	GTTGAACTGCTTGATCAGAACAGCAG	TCGGCATGAAGGTTTTGATTTCATTGTC

Table 5.1. Gene primers used for RT-PCR experiments.

Ectopically Expressed Genes in CTCL	
Cancer Testis antigens	
cTAGE1	This cancer testis antigen was documented to be ectopically expressed in CTCL. cTAGE1 (Cutaneous T-Cell Lymphoma-Associated Antigen 1) is robustly expressed in a majority of CTCL patients and patient-derived cell lines[128,178].
DMC1	DMC1 (disrupted meiotic cDNA 1) meiotic recombination protein plays a critical role in executing meiotic recombination. The name was derived during gene discovery which involved knock-out mutation experiments of meiotic genes. DMC1 is upregulated in a number of lymphoma cell lines after induction of mitotic catastrophe after a genotoxic insult[136].
GTSF1	GTSF1 (Gametocyte Specific Factor 1) is a cancer testis antigen ectopically expressed in CTCL[128] and was reported to be a part of a molecular signature that is specific to this cancer[179]. This gene is highly conserved among 27 species, including humans[180].
REC8	REC8 is a cancer testis antigen that was previously shown to be ectopically expressed in CTCL[128]. This gene's normal function is required for synaptonemal complex formation, crossing over recombination, and sister chromatid cohesion in meiosis[181].
SYCP1	SYCP1 (Synaptonemal complex protein 1) is a cancer testis antigen ectopically expressed in CTCL[128]. The normal function of this gene is to regulate crossing over in meiosis[128].
SYCP3	SYCP3 (synaptonemal complex protein 3) is a cancer testis antigen that was shown to be ectopically expressed in a subset of CTCL[128]. Normal function of this gene is essential for the formation of synaptonemal complex and for recombination of homologous chromosomes. SYCP3 mutations were identified in females with recurrent pregnancy losses[182].
B cell specific genes	
BLK	BLK **(**B-lymphoid kinase) is exclusively expressed in B cells and thymocytes but not in mature T cells and was shown to be ectopically expressed in CTCL[149,173]. This gene is important for B-cell receptor signaling and B-cell development.
POU2AF1	POU2AF1 is a B cell-specific transcriptional factor. This gene is essential for B cell maturation and germinal center formation[150]. Previous research demonstrated ectopic expression of B cell-specific genes in this cancer, as in the case of BLK gene[149].
Thymocyte development genes	

EVA1	EVA1 (Epithelial V-like antigen 1), also known as MPZL2 is expressed in the thymus early in embryogenesis and subsequently is downregulated during thymocyte developmental progression. This gene is believed to contribute to the earliest phases of thymus organogenesis[183].
TOX	TOX is a transcription factor, which is essential for early development of $CD4^+$ T cells and is normally not expressed in mature $CD4^+$ T cells. Previous studies demonstrated that malignant infiltrating T cells in CTCL ectopically express TOX, which causes the proliferation/apoptosis balance to shift toward proliferation by suppressing the transcription of several tumor suppressors. It was previously proposed that targeting TOX activity may be a promising treatment strategy for CTCL. TOX expression was independently found by two separate laboratories to be a robust diagnostic and prognostic marker for this cancer[148,175,184].
Embryonic Stem Cell Genes	
BIRC5	BIRC5, also known as Survivin, is a member of the inhibitor of apoptosis (IAP) gene family. This gene is strongly expressed during fetal development and in variety of tumors, where it is associated with advanced and progressive disease. It is often not expressed or only weakly expressed in normal adult tissues[185].
CHD1	CHD1 (Chromodomain-helicase-DNA-binding protein 1) is an embryonic gene, whose activity is required to maintain open chromatin in pluripotent mouse embryonic stem cells[186].
CHD7	CHD7 (Chromodomain-helicase-DNA-binding protein 7) is one of nine members of the chromodomain helicase DNA-binding domain family of ATP-dependent chromatin remodeling enzymes. It is believed together with PBAF during embryogenesis to promote neural crest gene expression and cell migration. CHD7 function is important for chromatin remodeling, transcriptional regulation and expression of pluripotency genes. Consistent with this gene function, CHD7 is known to be mutated in CHARGE syndrome (Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, and Ear abnormalities and deafness)[187-189].
CNOT3	CNOT3 is known as CCR4-NOT transcription complex, subunit 3. CCR4-NOT complex is one of the major cellular mRNA deadenylases. CNOT3 together with CNOT1 and CNOT2 play a critical role to maintain embryonic stem cell identity and inhibit differentiation into organ-specific lineages. These genes are usually enriched in the inner cell mass of a blastocyst and are downregulated during differentiation [190].
DMAP1	DMAP1 participates in DNA repair by directly interacting with PCNA. DMAP1-depleted cells in p53-deficient background demonstrate chromosomal instability and tumor formation in mice. Recent reports indicate that DMAP1 acts as a tumor suppressor by maintaining chromosomal integrity[191].

EED, EZH and SUZ12	EED (embryonic ectoderm development) together with SUZ12 (the suppressor of zeste-12 protein) and EZH2 (the enhancer of zeste protein-2) proteins form the Polycomb Repressive Complex 2 (PRC2), whose role is to repress gene expression via di- and tri-methylation of lysine 27 on histone H3. Reprogramming of somatic cells toward pluripotency would involve extensive chromatin reorganization and changes in gene expression. Polycomb group proteins are key regulators of chromatin structure, cell identity, and development[152]. Activity of this complex promotes "cancer stem cell" state, enhances self-renewal capacity and lack of complete cellular differentiation. The contribution of aberrant EZH2, EED and SUZ12 expression to carcinogenesis by directing cells toward a cancer stem cell state is being increasingly recognized[153,154]. PRC2 was also shown to play a critical role in the establishment as well as maintenance of aberrant silencing of tumor suppressor genes during cellular transformation. PRC2 is overexpressed in numerous cancer types including breast, prostate, lung and hematologic malignancies. Such ectopic expression of PRC2 components often correlates with disease severity[152,154].
ESRRB	Estrogen-related receptors (ERRs) are orphan nuclear hormone receptors expressed in metabolically active tissues and modulate numerous homeostatic processes. ESRRB is believed to be an important target of the GSK3/TCF3 axis regulating embryonic stem cell self-renewal. ERRs do not bind the ligand estrogen, but they are able to bind the estrogen response element (ERE) embedded within the ERR response elements to regulate transcription of genes [192]. Based on our analysis this gene is not expressed in CTCL lesional.
JARID2	JARID2 is a member of a family of chromatin modifiers with histone demethylase activity. JARID2 activity is essential for the development of multiple organs. Recent studies have demonstrated that this gene is a component and/or associates with the polycomb repressive complex 2 (PRC2) and is required for embryonic stem cell differentiation[155]. JARID2 is a putative target of miR-155[193].
KAT5/TIP60	KAT5 (K(lysine) acetyltransferase 5) is also known as TIP60 is a member of MYST family of histone acetyl transferases. This gene was shown to be involved in DNA repair and apoptosis. TIP60 histone acetyltransferase activates genes required for proliferation and silences genes that promote differentiation. TIP60 is a critical subunit of a protein complex that responds to DNA damage by inducing apoptosis. It acetylates p53 and stimulates transcription of proapoptotic genes[194,195].
KLF4	KLF4 (Kruppel-Like Factor 4) is a transcription factor that can act as an activator and as a repressor. It transactivates key transcription factors during embryonic development and plays an important role in maintaining embryonic stem cell phenotype. KLF4 is highly expressed in epithelial tissues, where it regulates terminal epidermal differentiation. Depending on the p21Cip1 status of a malignant cell it can act as a tumor suppressor or an oncogene. KLF4 was shown to be a potent oncogene in breast cancer, where it promotes stem cell-like state as well as cell migration and invasion[196].

MTF2	MTF2 (Metal Response Element Binding Transcription Factor 2) is also known as PLC2 (Polycomb-like gene). MTF2 is a pluripotency marker induced by NANOG[161]. This gene forms a complex with PRC2 and in a PRC2-dependent manner represses the expression of developmental Hox genes. It was also shown to synergistically promote the activity of PRC1[197].
NR0B1/DAX1	NR0B1 (nuclear receptor subfamily 0, group B, member 1) is also known as DAX1 (dosage-sensitive sex reversal – congenital adrenal hypoplasia critical region on X chromosome), acts as a dominant-negative regulator of transcription which is mediated by the retinoic acid receptor. DAX1 lacks a classic DNA binding domain and instead acts as a coregulatory protein that inhibits the transcriptional activity of other nuclear receptors through heterodimeric interactions. This protein also functions as an anti-testis gene by acting antagonistically to Sry. Mutations in this gene result in both X-linked congenital adrenal hypoplasia and hypogonadotropic hypogonadism. It is also believed to be important for maintenance of embryonic stem cell pluripotency**.** DAX1 is highly expressed in mouse embryonic stem cells and this expression is controlled by STAT3, OCT3, OCT4 and NANOG transcription factors. In knockdown experiments loss of DAX1 induces upregulation of differentiation genes belonging to the three embryonic germ layers. Recently, DAX1 expression was observed in various tumors including ovarian, breast, prostate, lung carcinomas and Ewing sarcoma, where DAX-1 may was proposed to be involved in the maintenance of cancer stem cells phenotype[198,199].
PHC1	PHC1 (polyhomeotic homolog 1), also known as HPH1, is a component of a Polycomb group multiprotein PRC1-like complex. It is required to maintain the transcriptional repression several genes (including Hox genes), throughout development. This complex plays a crucial role in the regulation of embryonic development and regulation of the cell cycle and hematopoiesis. HPH1/PHC1 was found to be expressed in Hodgkin's disease and prostate cancer[156].
RNF2	RNF2 (ring finger protein 2) is a E3 ubiquitin-protein ligase that is a member of the Polycomb group of proteins. RNF2 is a central component of the PRC1 complex[157]. Polycomb group of proteins form complexes and repress the expression of various genes (including Hox genes) involved in development and cell proliferation. Studies in mice suggested that RNF2 gene is important for specification of anterior-posterior axis in early development[200].
SALL4	SALL4 (Sal-Like 4) is a human homologue of the Drosophila spalt homeotic gene. This C2H2 zinc-finger transcription factor plays a key role in the maintenance and self-renewal of embryonic and hematopoietic stem cells. SALL4 expression usually diminishes gradually during development and is silenced by adulthood. SALL4 acts as an oncogene in carcinogenesis and was shown to be ectopically expressed in lymphoma, plasma cell myeloma, acute lymphoblastic leukemia, ALK-positive anaplastic large cell lymphoma, hepatocellular carcinoma and other cancers. Furthermore,

	SALL4B-transgenic mice develop acute myeloid leukemia[201-203]. Based on our analysis this gene is not expressed in MF/SS lesional skin.
SOX2 POU5F1/OCT4 and NANOG	SOX2 [SRY(sex determining region Y)-box 2], is a transcription factor of the SRY-related high mobility group box (SOX) family. POU5F1 (POU domain, class 5, transcription factor 1) also known as OCT4 (Octamer-binding transcription factor 4) plays an important role in embryonic development, especially during early embryogenesis. NANOG, SOX2 and OCT4 are the key transcription factors involved in the maintenance of pluripotency of embryonic stem and embryonal carcinoma cells[158-160]. OCT4, SOX2 and NANOG transcription factor are capable of inducing the expression of each other, and are essential for maintaining the self-renewing undifferentiated state of the inner cell mass of the blastocyst and in embryonic stem cells[161]. Many malignancies were previously documented to express OCT4, SOX2 and NANOG and in many cases, their expression is associated with aggressive behavior and poor cancer survival[162-168]. In cancer these transcription factors working in concert can induce pluripotent embryonic stem cell-like state[169,170]. Ectopic expression of these genes helps to resist apoptosis and overcome cellular senescence[170]. It has also been documented that cancer cells under stressful and hypoxic conditions may be reprogrammed to re-express different pluripotency-associated stem cell-like markers including OCT3, OCT4, NANOG and SOX2[204]. The factors, similarly to PRC2 complex may be contributing towards cancer stem cell phenotype.
TBX3	TBX3 is a member of T-box gene family. This gene was reported to play critical roles in embryonic development, maintenance of pluripotency and cell fate determination. TBX3 is overexpressed in several cancer cells including breast cancer, bladder carcinoma and melanoma[205,206].
TCF3	TCF3 (Transcription factor 3) is a member Tcf/Lef transcription factor family. LEF1 and TCF1 are required for transactivation of Wnt signaling genes, while TCF3 functions predominantly as a transcriptional repressor. Notably, TCF3 is expressed in different types of stem cells including embryonic and hair follicle stem cells. TCF3 promotes differentiation of embryonic stem cells by counteracting Wnt-mediated maintenance signals[207]. TCF3 is believed to be an important negative regulator of embryonic stem cell self-renewal[208]. Chromosomal translocation t(1;19)(q23;p13.3) which leads to a production of the TCF3-PBX1 (E2A-PBX1) fusion protein was observed in **A**cute Lymphoblastic Leukemia**[209]**. Recurrent mutations in TCF3 that promote PI3-kinase signaling were documented in human Burkitt's lymphoma samples[210]. While this gene is upregulated in CTCL it is not known if it is also mutated.
THAP11	THAP11 (Thanatos-associated protein 11), also known as Ronin, is essential for the self-renewal of embryonic stem (ES) cells. This gene contributes to ES cell pluripotency by regulating the transcription of genes involved in the metabolic processes that sustain the growth of self-renewing ES cells[211].
TRIM28	TRIM28 (tripartite motif-containing protein 28), also known as KAP1, KRAB-associated protein 1 or TIF1β is highly expressed in embryonic stem

	cells, whose activity is critical for the silencing of endogenous retroviruses (ERVs) in embryonic stem cells[212]. This gene is crucial for early mouse development, since TRIM28 knock-out embryos arrest after implantation and do not gastrulate. This gene was also shown to be important in T cell development [213,214]. It is also required for maintenance of imprinting marks. Embryonic stem cells rapidly die or undergo differentiation if TRIM28 is downregulated. In colorectal cancer patients high epithelial to stromal TRIM28 expression ratio was associated with shorter overall survival[215]. In lung cancer patients, expression of this gene by circulating metastatic cells is associated with shorter tumor-specific survival[216].
TRRAP	TRRAP (Transactivation/Transformation Domain-Associated Protein) is a common component of several HAT (Histone Acetyl Transferase) complexes including TIP60/NUA4. TIP60-p400 HAT and TRRAP were suggested to be involved in the maintenance of stem cell identity. Also, like other embryonic genes, TRRAP was found to play an important role in maintaining a tumorigenic, stem cell-like state[217].
ZFX	ZFX (Zinc finger protein X-linked) is a member of Zfy family of zinc finger proteins. Its main function as a transcriptional regulator in embryonic and hematopoietic stem cells is to maintain self-renewal and inhibit differentiation[218]. This gene is also upregulated in several human cancers including hepatocellular carcinomas, glioblistomas and various leukemias, where its activity is to promote self-renewal and tumorigenic potential[171,172,218]. In cancers ZFX was shown bind and transactivate promoters of Nanog and SOX2 as well as lead to overexpression of c-Myc. Overexpression of ZFX contributes to the stemness and pluripotent behavior of cancers[171,172].
Other ectopically expressed genes	
PLS3	PLS3 (Plastin-3) is an actin-binding protein, which is not normally expressed in T cells. It was shown that the promoter for this gene is demethylated in cancer. The normal function is to regulate actin structure elongation. This gene was shown to be expressed in CTCL and is associated with SS cell survival and migration. Many studies documented that PBMCs and SS $CD4^+$ T cells overexpress PLS3[74,151,176,177].

Table 5.2. Detailed description of ectopically expressed genes in CTCL.

Chapter 6. Utilizing gene expression profiling to improve personalized diagnosis and management of Cutaneous T-Cell Lymphoma (CTCL). (Full published manuscript is presented in Appendix 4.)

6A. INTRODUCTION

As highlighted in the introduction section of this thesis, today clinical disease stage at the time of diagnosis remains the best predictor of survival and progression for CTCL. Early stages (i.e., stage IA and IB) often exhibit an indolent disease course, with normal or near normal life expectancy[219]. In contrast, advanced stages and/or SS are associated with recalcitrant disease and poor 5-year survival rate[220]. The majority of CTCL patients present with an early stage (i.e., IA or IB) disease[219]. However, ~10-20% of these patients will progress to higher stages and may ultimately succumb to their cancer[219]. At the same time, while most patients with advanced CTCL stages will experience an aggressive course of disease progression, a small minority of these patients survive for much longer than 5 years.

Improving our ability to effectively diagnose CTCL (i.e. being able to distinguish it molecularly from benign mimickers) and, most importantly, developing molecular tools to identify patients at risk of progression at early disease stages will enable us to personalize our management approach towards diagnosis and treatment of this cancer.

To discover novel prognostic molecular markers and to gain additional insight in disease etiology our laboratory previously performed a microarray and subsequent RT-PCR analyses of gene expression in biopsy specimens from 60 stage I-IV MF/SS patients[51,137]. These patients were initially followed for 6 years. The original gene expression analyses revealed three distinct transcription profile clusters (i.e. clusters 1, 2 and 3), where clusters 1 and 3 contained a mix of stage I–IV disease patients, while cluster 2 contained mostly stage I and only a few cases of advanced disease patients[51,137]. All stage IV MF/SS patients fell into clusters 1 and 3[51,137]. The described three distinct transcription profile clusters were associated with different clinical courses. Cluster 2 genes corresponded to the best clinical outcome and good response to therapy while cluster 1 and 3 molecular signature patterns were associated with the worst and intermediate clinical outcomes, respectively, and poor response to therapy[51,137]. Unfortunately, due to relatively short initial clinical follow up period (6 years) these trends at that time did not reach statistical significance[51,137].

In the current project working with Dr. Sasseville and Dr. Litvinov I have performed a literature review that highlighted ~240 genes whose expression and function is believed to be important in CTCL pathogenesis, diagnosis and/or treatment. We subsequently tested the expression of these genes by RT-PCR in our patient population to identify additional genes that fit

into the above described three cluster prediction model. A number of genes that fit this model were then selected for further testing and their expression patterns were compared between CTCL lesional skin *vs.* normal skin from healthy volunteers *vs.* lesional skin from patients affected by benign dermatoses that often masquerade as CTCL (e.g. chronic eczema, psoriasis and pityriasis rubra pilaris). This was done to identify which prognostic markers may also have a molecular diagnostic value in this cancer.

6B. MATERIALS AND METHODS:

Patients and Samples

All patients were enrolled in the IRB-approved study protocol with informed consent in accordance with the Declaration of Helsinki[51,137]. CTCL patients were recruited from the Cutaneous Lymphoma Clinic at the Dana Farber Cancer Institute/Brigham and Women's Hospital. All tissue samples were obtained and processed as previously described[137]. Briefly, six-mm punch biopsies from involved skin were collected from patients between January 26, 2003 and June 1, 2005. The obtained 6 mm biopsies were immediately snap-frozen in liquid nitrogen. Tissue was powdered in liquid nitrogen (Cryo-Press; Microtec Co, Chiba, Japan), and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and converted to cDNA using the iScript RT-PCR kit (Bio-Rad, Hercules CA) according to the manufacturer's instructions.

The historic cohort of patients from Boston (n=60), which was initially reported in 2007[137], was at the heart of extensive research that led to multiple publications in the field[51,73,126-128,137,221]. For these patients, 11 years of clinical follow-up data was analyzed in the same way as in previous reports[51,73,126-128,137,221]. The biopsy samples analyzed in this study are the same samples that were analyzed in our previous papers[51,73,126-128,137,221]. The diagnosis and clinical staging were established according to the diagnostic criteria of CTCL[38].

Similarly, volunteers with normal healthy skin (n=6) and benign inflammatory dermatoses (n=17) were recruited from the outpatient dermatology clinic of the University of British Columbia (Vancouver, Canada) with informed consent[73]. Full-thickness lesional skin punch biopsies were obtained under local anesthesia as previously described[51,73,137].

Quantitative Real-Time Reverse Transcription-PCR Gene Expression Analysis.

Before initiating this study, we searched PubMed, Medline and Web of Science databases using terms "genetic", "gene expression", "gene expression profiling" and "CTCL", "Mycosis

Fungoides" or "Sézary Syndrome" to identify studies published in English. Based on this search >400 studies were reviewed and 241 genes of interest were selected. Gene expression was tested via RT-PCR in CTCL patients' lesional skin, normal skin form healthy volunteers and lesional skin from patients with benign inflammatory dermatoses as previously described[51,73,128]. Primer pair sequences for tested genes and control housekeeping genes are listed in table 6.1. RT-PCR was performed utilizing the obtained cDNA from patients and iScript RT-PCR mix (Bio-Rad, Mississauga, Ontario) on Bio-Rad iCycler as previously described[51,126,127]. The expression was standardized using genorm method[146] utilizing ACTB, SDHA, YWHAZ and HMBS housekeeping genes. For every analyzed gene the highest expression value in our samples was set as 1 fold of expression similarly to the protocol in our previous studies[51,128].

Statistical analyses

Disease progression and disease-specific survival was analyzed using XLSTAT (Addinsoft, New York, NY) software to obtain Kaplan-Meier curves as previously described[51]. p values were calculated using the logrank test[222]. Patient multivariate analysis was performed using the Cox proportional hazards regression method taking into account multiple progression events for each patient.

6C. RESULTS

The three signature gene expression model identifies novel prognostic markers for CTCL.

One of the important criticisms of the previous microarray and RT-PCR gene expression studies that were performed in our laboratory[51,137] was that they did not include numerous genes that were reported in literature by other authors to be important in the pathogenesis, diagnosis and treatment of CTCL. To address this concern, in the current study we conducted a literature search to identify ~240 genes (Table 6.1) that were previously reported to play an important role in CTCL and tested their expression in our cohort of patients for which extensive clinical follow up is available. This analysis demonstrated that a number of previously reported genes can be classified into cluster 1, cluster 2 or cluster 3 expression patterns (as shown in Figures 6.1-3) and such expression is congruent with their suggested biological role in CTCL pathogenesis.

Proto-oncogenes, inflammatory cytokines, cell cycle, novel cancer testis genes were expressed in aggressive disease clusters 1 and 3 (Figure 6.1, Figure 6.3 and Table 6.2). In total, 33 out of ~240 genes tested fit into cluster 1 (poor prognosis cluster) expression pattern with partial

overlap with cluster 3 (intermediate prognosis cluster) patients (Figure 6.1), while 7 gene were preferentially expressed in cluster 3 patients (Figure 6.3). In these clusters we observed the expression of cell survival and cell cycle genes CCND2, NFKB1, PLK1, NAIP; putative oncogenes JUNB, TOX, AHI1; novel cancer testis genes GTSF1, SYCP1 as well as embryonic stem cell genes TCF3, EVA1, CHD1; genes promoting inflammatory T cell signaling ITK, LCK, FYB, GNLY, CCL18, CCL26, E-Selectin; skin homing chemokine receptor CCR4; cytokines (and their cognate receptors) that were reported to be secreted by the Th17 cells IL-26, IL-17A, IL-17F, IL-21 and IL-21R; the IL-22 cytokine; actin binding protein PLS3; matrix metalloproteinase MMP12; downstream positive regulator of WNT/β-catenin signaling LEF1; transcription factors STAT5A, MXI and POU2AF; markers of T cell activation TFRC, IRF4; and other signaling genes including T3JAM, FOSL1, SHD1A, SERPINB4 (Figure 6.1, Figure 6.3 and Table 6.2). As described in detail in table 6.2 many of these genes were reported to play cancer promoting roles in CTCL and other cancers. Moreover, several of them (e.g. CCR4, IL2RA and TOX) are recognized or proposed as therapeutic targets in CTCL.

On the other hand, putative tumor suppressor genes CDKN1C, BCL7A DLEU1, miR-205 and CST6; epidermal differentiation genes LCE2B, LOR; TGF-β signaling gene LTBP4; WNT/β-catenin pathway antagonist WIF1 (WNT Inhibitory Factor 1) and psoriasis susceptibility gene PSORS1C2 were expressed in a favorable prognosis cluster 2 (Figure 6.2). Also, IL-18 cytokine, which is known to induce interferon-γ response (possibly targeting malignant infiltrating T cells), and its downstream target IL1F7 (also known as IL-37) were also upregulated in this cluster of patients (Figure 6.2). As discussed in table 6.2 many of these genes were reported to act as tumor suppressors in CTCL and other cancers and were shown to be downregulated in neoplasia.

We previously demonstrated, based on 6 years of clinical follow up, that these three signature gene expression patterns were associated with different clinical outcomes in CTCL patients[51]. However, at that time these trends did not reach statistical significance[51]. In the current work we extended the clinical follow up of our patients until 2014 (11 years of clinical follow up).

The new extended 11-year clinical analysis of CTCL progression confirms our previous observations and documents that cluster 2 had much fewer number of progression events (i.e., advancement to a higher CTCL stage and/or death) than clusters 1 and 3 (Figure 6.4A). Logrank test of the presented Kaplan-Meier analysis documents that these three clusters are statistically different ($p=0.005$). Similarly, with respect to survival, cluster 2 patients enjoyed a favorable 11-

year survival, while clusters 1 and 3 patients experienced an overall poor survival (Figure 6.4B). Statistical significance was observed for survival differences between the three clusters (p=0.034). All 60 patients were analyzed in the above-described analyses.

Since each cluster had a large number of stage I disease patients (i.e., 11/19 stage I patients in cluster 1, 18/20 patients in cluster 2 and 14/21 patients in cluster 3), we specifically analyzed the progression of these patients towards more advanced disease (i.e., stage≥II) with respect to their genetic clusters. As presented in Figure 6.4C, Cluster 1 and 3 stage I patients had the highest 11-year progression rates. Strikingly, none of the cluster 2 stage I patients have progressed towards advanced disease (i.e., progression rate of 0%) in the period of 11 years. In addition, we conducted Kaplan-Meier comparisons for each individual cluster pair. p values for these analyses are presented in Figure 6.4D.

Finally, for this patient cohort we conducted a multivariate analysis of disease progression based on gender, age and clinical disease stage at the time of diagnosis. As we expected, the clinical stage at the time of diagnosis was a strong predictor of cancer progression in our patients (Table 6.3). Specifically, stage ≥III patients had a ~12 fold risk of progressing to higher stages and/or dying from their disease, when compared to stage I disease patients. Stage II disease patients had a 4.7 fold risk of progression and/or death. Also, consistent with trends reported in the literature, a weak association was documented between male sex and disease progression (Table 6.3). Based on our analysis, age alone was not an independent risk factor for disease progression (Table 6.3).

Comparison of gene expression between CTCL, normal skin and lesional skin from benign inflammatory dermatoses patients.

The early stages of MF are often difficult to distinguish clinically from other benign entities including chronic eczema, psoriasis and pityriasis rubra pilaris[46]. Furthermore, detection of T cell clonality in itself is also not diagnostic of CTCL since a number of benign dermatoses (e.g. lichen planus, pityriasis lichenoides, lichen sclerosus and pigmented purpura) too can have a dominant T cell clone[46]. Histological diagnosis is often difficult since in early patch disease stages malignant lymphocytes represent only 5-10% of the total inflammatory infiltrate. Even in advanced stages, using all available clinical and laboratory tools, it is often difficult to distinguish patients with advanced erythrodermic MF and SS disease from patients presenting with non-malignant erythrodermas secondary to psoriasis, pityriasis rubra pilaris and atopic dermatitis[46]. Hence, new

genetic markers are urgently needed to distinguish CTCL from various benign inflammatory dermatoses.

To address this we compared the expression of the above described candidate genes between CTCL lesional skin (n=60), skin biopsies from healthy volunteers and patients with chronic eczema, psoriasis, pityriasis rubra pilaris (PRP). Strikingly numerous genes that were upregulated in clusters 1 and 3 CTCL patients (Figures 6.1 and 6.3) were preferentially expressed in CTCL, but not in benign skin samples (Figure 6.5, Table 6.4). Specifically, this analysis demonstrated that CCL18, CCL26, FYB, T3JAM, MMP12, LEF1, LCK, ITK, GNLY, IL2RA, IL-26, IL-22, CCR4, GTSF1, SYCP1, STAT5A and TOX can jointly be used as diagnostic and poor prognostic markers in CTCL patients. This comparative expression analysis further revealed that select genes (SERPINB13 and BCL7A) were preferentially upregulated in benign skin conditions, but not in CTCL (Figure 6.6A). PSORS1C2 and WIF1 cluster 2 genes were expressed in normal skin and in indolent CTCL cases (Figure 6.6B and Figure 6.7).

6D. DISCUSSION AND CONCLUSIONS:

This study summarizes many years of research from our laboratory and in detail describes the findings for the Boston CTCL cohort of patients. In this work we have completed a comprehensive gene expression analysis for ~240 genes that were identified in our prior studies and/or were suggested by previous literature reports to play an important role in CTCL carcinogenesis. Expression of these genes was analyzed in the context of the three-signature pattern prediction model that we previously described[51,137]. Based on 11 years of clinical follow up, we document that 52 of these genes are preferentially expressed in various genetic clusters that correlate with different disease outcomes (i.e., overall progression, disease-specific survival and progression of stage I patients to more advanced stages). We further compare the expression of these genes between CTCL and benign inflammatory dermatoses that often mimic CTCL and identify 22 of these genes that are specific for CTCL and 5 genes that are preferentially expressed in benign dermatoses or in indolent CTCL and benign dermatoses.

These results, combined with other expression profiling and meta-analysis studies[72,174,179] lay the groundwork for the development of personalized molecular approach towards diagnosis and management of CTCL. As highlighted in our findings, a panel of 17 genes, CCL18, CCL26, FYB, T3JAM, MMP12, LEF1, LCK, ITK, GNLY, IL2RA, IL-26, IL-22, CCR4, SYCP1, GTSF1,

STAT5A and TOX, can serve a dual role to diagnose CTCL and instantaneously predict a poor clinical disease course, which can be a powerful tool for clinicians. While before clinicians often made their decisions by evaluating a single diagnostic/prognostic marker (e.g., PSA, CEA or HER2/neu), new technologies in medicine will soon enable us to follow a panel of multiple cancer genes in our patients[223].

From the above gene list it is notable that TOX expression was also independently found by two separate laboratories to be a robust diagnostic and prognostic marker for this cancer[73]. STAT5A was documented to promote carcinogenesis starting in the early stages of CTCL by activating the oncogenic microRNA miR-155[224]. IL-22, a Th22/Th17 cytokine, was proposed to be a dominant cytokine in CTCL tumor microenvironment[225]. Highly expressed in SS and MF the skin-homing chemokine receptor CCR4 is an investigational CTCL therapeutic target of Mogamulizumab, a humanized anti-CCR4 antibody[226]. IL2RA (IL-2Rα) is expressed in up to 50% of MF/SS cases. Interleukin-2 diphtheria toxin fusion protein (denileukin diftitox) was designed to target this receptor in CTCL[227]. CCL26 was shown to correlate with the clinical itch burden in CTLC patients[228], while another potent T cell chemoatractant, CCL18, has been consistently shown to be upregulated in MF and correlate with the types of skin lesions (i.e., patch *vs.* plaque *vs.* tumors)[229]. As evident from this brief overview, molecular markers identified in our study are not of mere academic interest, but have a direct clinical correlation to disease symptoms and treatment. By combining this knowledge with similar studies that identified critical molecular diagnostic and prognostic markers[72,174,179] we hope to improve our ability to effectively manage this cancer.

This study further highlighted ectopic expression of novel cancer testis genes and embryonic genes GTSF1, SYCP1, TCF3 and CHD1 that were expressed in poor prognosis cluster 1 patients, while cTAGE1, GTSF1 and THAP11 were preferentially expressed in CTCL, but not in benign skin samples. EVA1 (also known as MPZL2), another poor prognosis marker, is expressed early on in the thymus, but then is strongly downregulated by thymocyte developmental progression[183]. Also, previous work suggested that a B cell specific gene, B-lymphoid kinase or BLK, is constitutively active in malignant T cells and appears to be a *bona fide* oncogene which drives malignant T cell proliferation *in vitro* and tumor formation *in vivo*[173]. In this study we confirm the expression of the aforementioned genes in CTCL and demonstrate that another B cell-specific transcriptional factor POU2AF1 is expressed in poor prognosis cluster 1 patients. Other

important putative CTCL oncogenes confirmed by this study include JUNB, PLS3, AHI and PLK1.

For favorable prognosis genes this study highlights putative tumor suppressor genes BCL7A, CKDN1C, miR-205, DLEU1, IL-18 and WIF1. BCL7A and CDKN1C were previously proposed by our laboratory and others to play important roles in CTCL pathogenesis[126,127,230,231]. miR-205 microRNA was documented to act as a tumor suppressor in melanoma and other cancers and has the ability to distinguish CTCL from other benign entities[232]. DLEU1 (Deleted in Lymphocytic Leukemia 1) long non-coding RNA putative tumor suppressor gene is frequently deleted in B-cell chronic lymphocytic leukemia (B-CLL)[233] and is for the first time reported in this study to play an important role in CTCL. IL-18 inflammatory cytokine is known to induce expression of interferon-γ and promote Th1 immune response, both of which are associated with disease clearance[234]. WNT/β-catenin signaling inhibitor, WIF1 (WNT Inhibitor Factor 1), was previously shown to be downregulated in salivary gland carcinomas, acute lymphoblastic leukemias (ALL)[235] and acute myeloid leukemias (AML)[236]. Our study also for the first time suggests that loss of this gene may also be important for CTCL carcinogenesis. Finally, STAT4 transcription factor appears to play a dual role in CTCL, where, when compared to benign dermatoses, this gene is upregulated in lesional skin[74,221], but then as disease progresses this gene is lost in aggressive/recalcitrant disease[74,177,221]. Previous reports documented that loss of STAT4 expression is associated with a switch from Th1 towards Th2 phenotype in CTCL[221]. Loss of STAT4 expression was shown to be a robust and reliable diagnostic marker for SS[177].

In summary, this study combined with other gene expression profiling analyses prepares the groundwork for the development of personalized molecular approach towards diagnosis and treatment of CTCL. Further analyses of gene expression and function for the above described genes may hold the promise of developing effective diagnostic and prognostic molecular tests for our patients.

6E. FIGURES AND LEGENDS

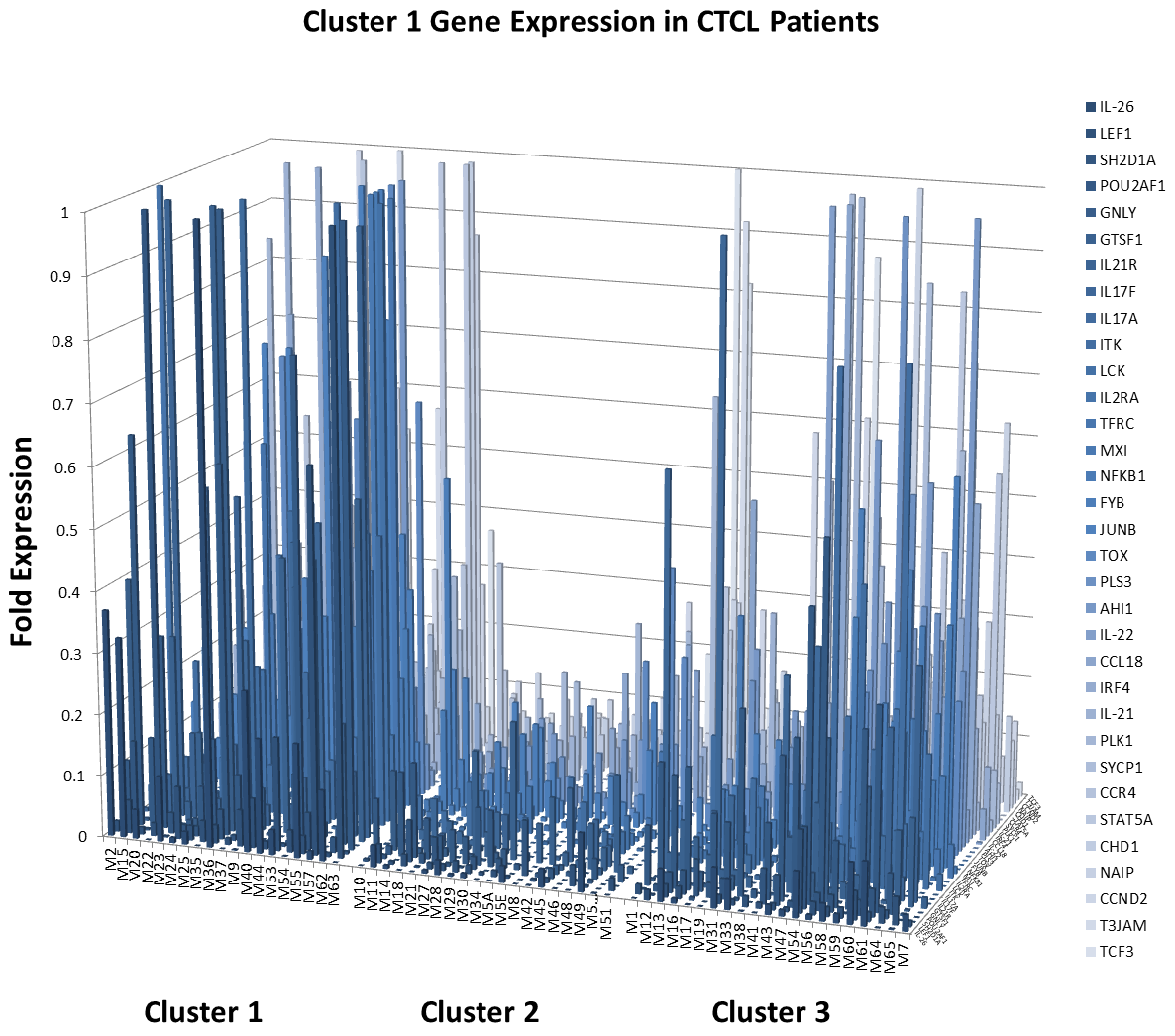


Figure 6.1. Expanded RT-PCR analysis of gene expression reveals 33 genes that are expressed in poor and intermediate prognosis cluster 1 and 3 patients, but not in favorable prognosis cluster 2 patients.

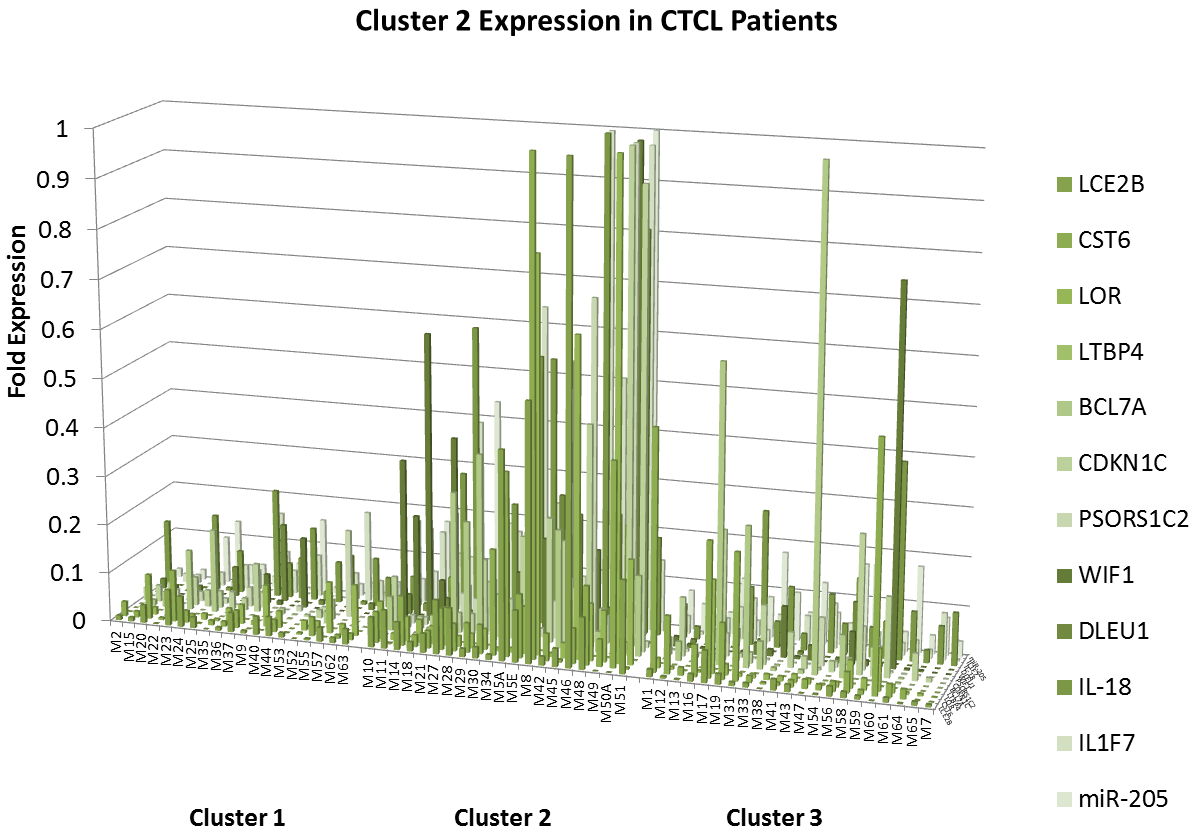


Figure 6.2. RT-PCR analysis of gene expression reveals 12 genes that are expressed in favorable prognosis cluster 2 patients and partially overlapping with patients in Cluster 3.

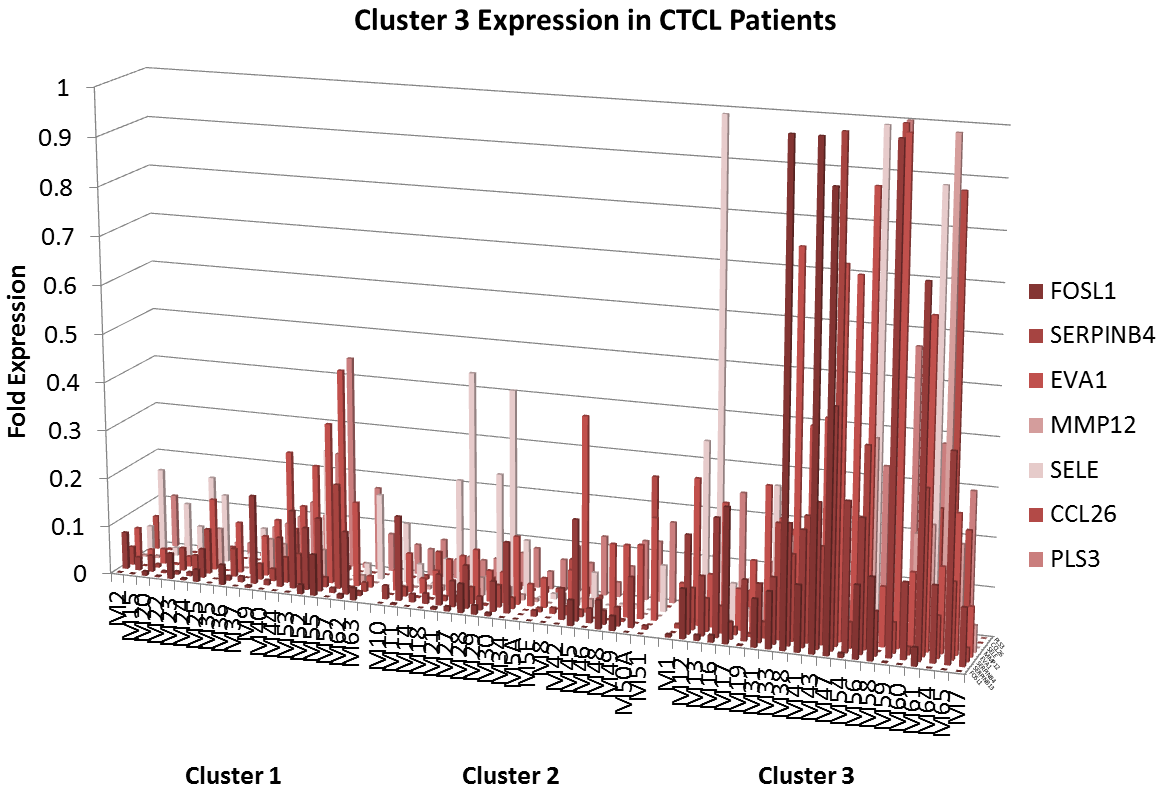
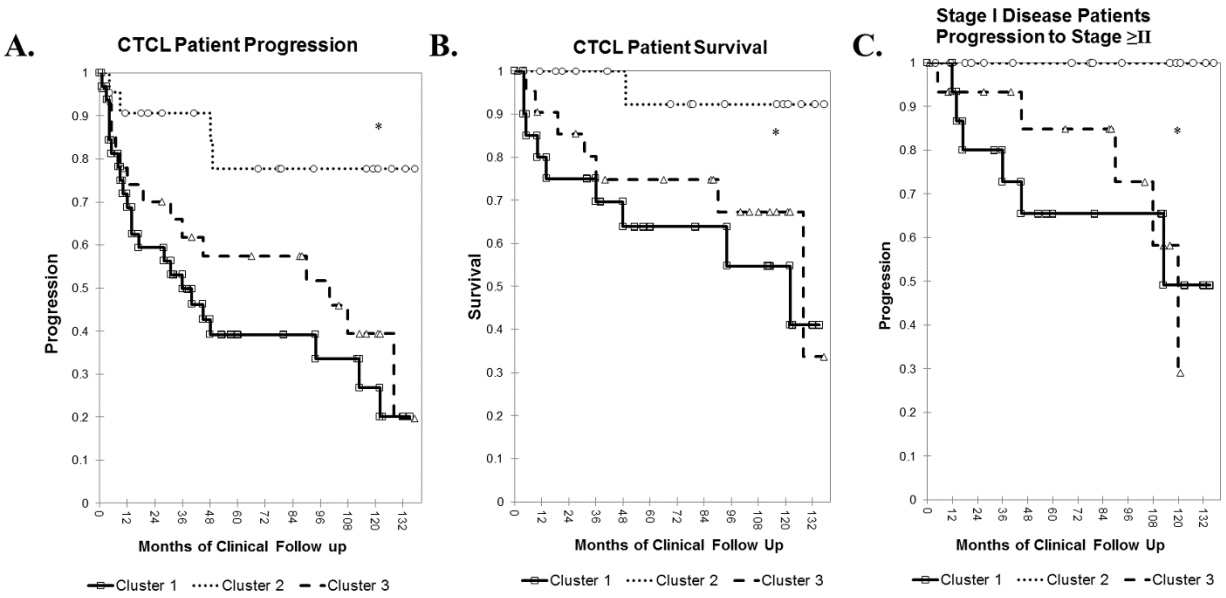


Figure 6.3. RT-PCR analysis of gene expression reveals 7 genes that are preferentially expressed in the intermediate prognosis cluster 3 patients.



D.

	All Clusters	Clusters 1 & 2	Clusters 1 & 3	Clusters 2 & 3
CTCL Patient Disease Progression	p=0.005	p=0.001	p=0.301	p=0.017
CTCL Patient Disease-Specific Survival	p=0.034	p=0.008	p=0.482	p=0.038
Stage I Patient Progression to Stage ≥II	p=0.028	p=0.008	p=0.762	p=0.009

Figure 6.4. Kaplan-Meier analysis of CTCL patient disease outcomes **A**. CTCL patient overall disease progression (defined as progression to a higher clinical stage and/or death, p=0.005) **B.** CTCL patient disease-specific survival, p=0.034 **C.** Progression of patients with stage I disease to more advance stages (i.e., stage ≥II). **D.** p-Values for Kaplan-Meier comparisons for each individual cluster pair.

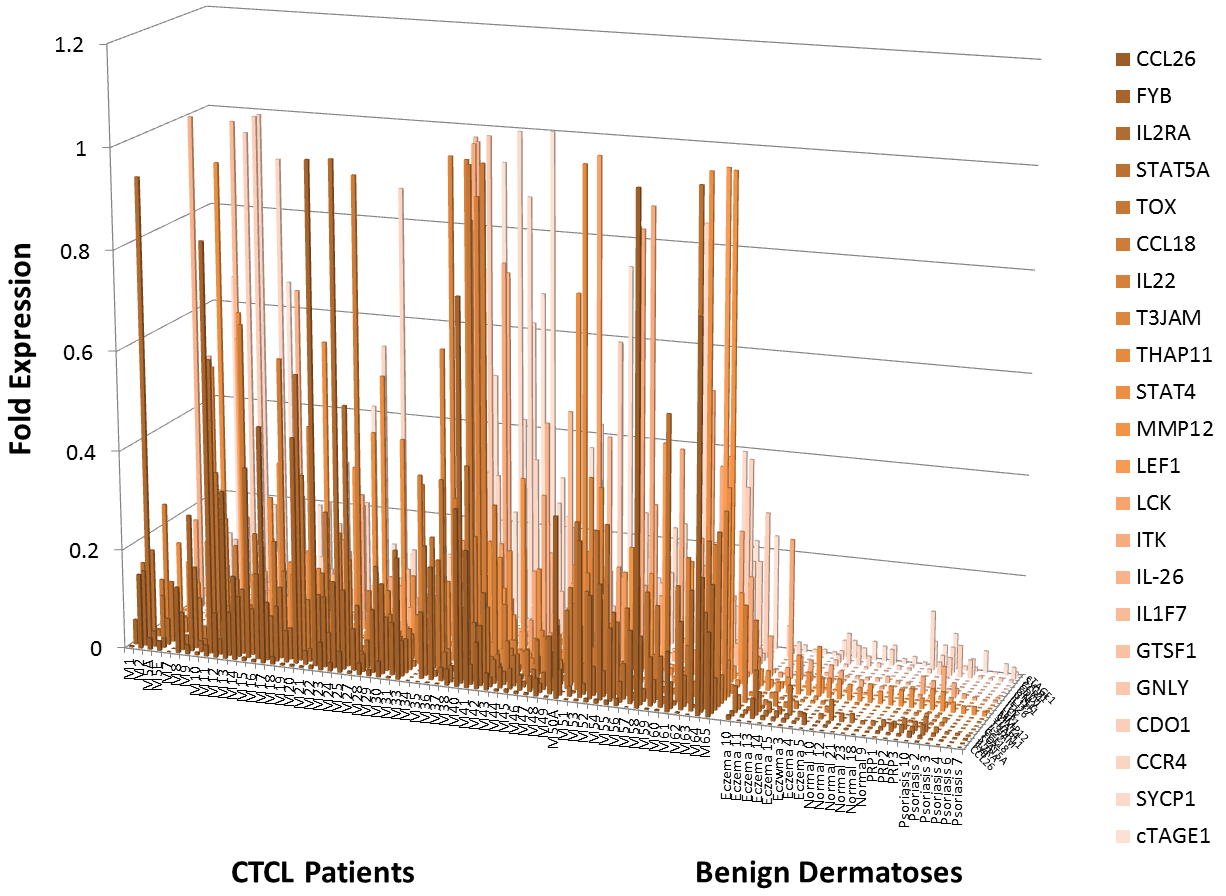


Figure 6.5. RT-PCR analysis of gene expression identified 22 genes that are preferentially expressed in CTCL, but not in normal skin from healthy volunteers or benign inflammatory dermatoses that often masquerade as CTCL (e.g., psoriasis, chronic eczema and pityriasis rubra pilaris or PRP). Notably, 17 of the above genes are upregulated in cluster 1 and 3 patients and potentially can serve as diagnostic and prognostic markers at the same time.

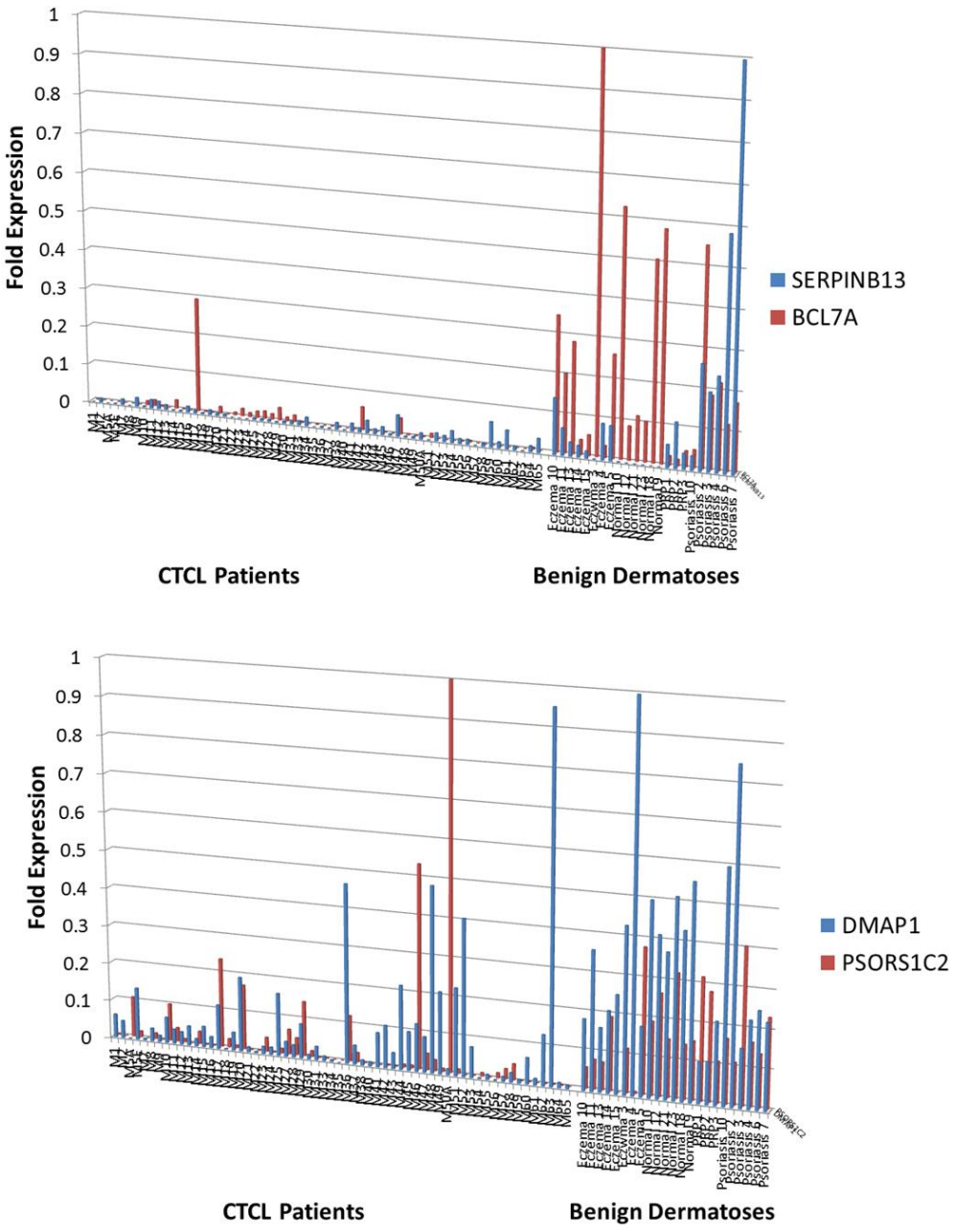


Figure 6.6. RT-PCR analysis of gene expression identified several genes that are (A) upregulated in normal skin and benign dermatoses, but not in CTCL or (B) upregulated in benign skin samples and in indolent/stable CTCL.

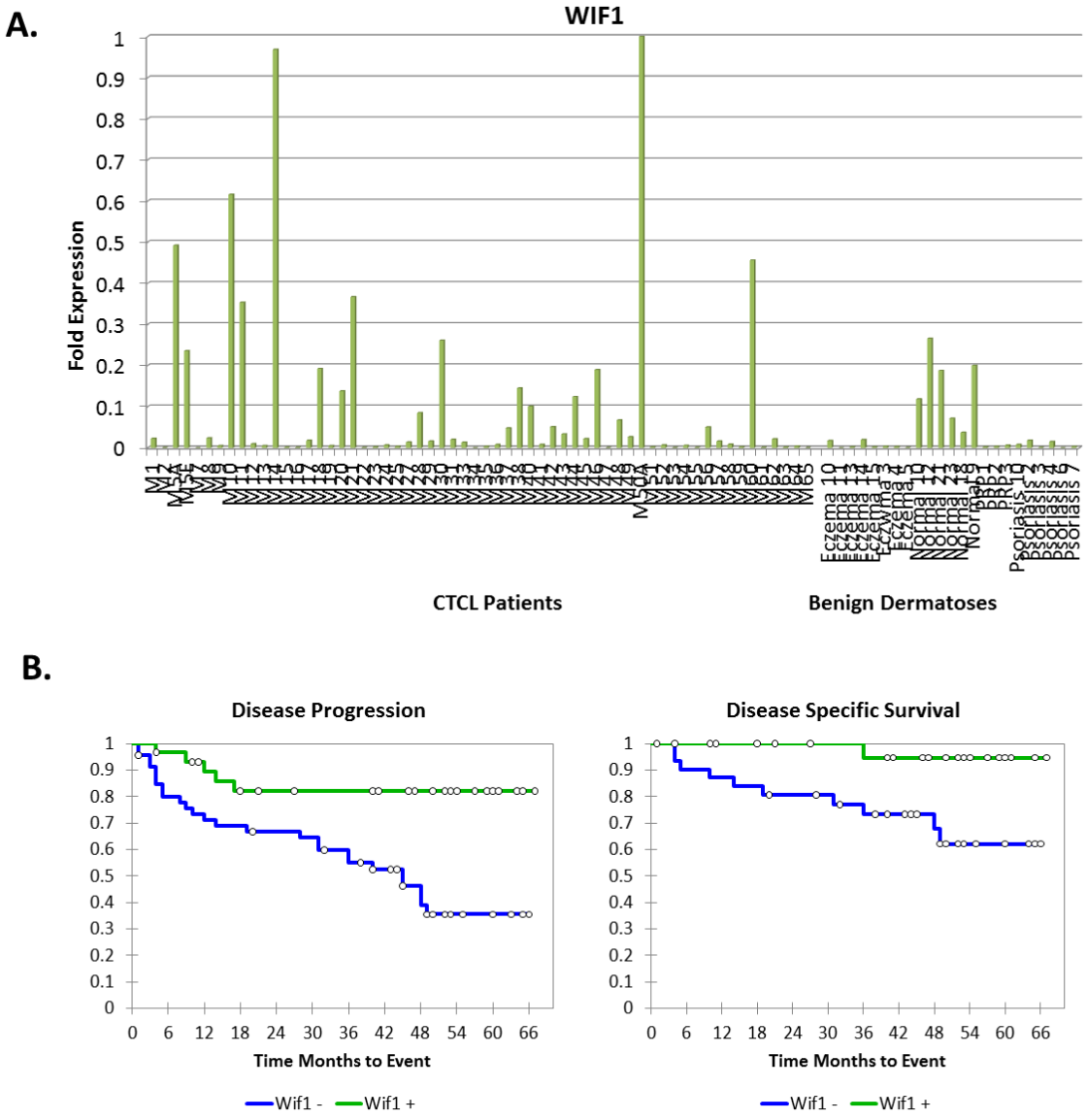


Figure 6.7. A. WIF1 (Wnt Inhibitory Factor 1) gene is expressed in normal skin and CTCL lesional skin in a subset of patients. **B.** Correlation of WIF1 expression in CTCL patients with disease progression. Kaplan-Meier analysis documents that loss of WIF1 expression is associated with poor CTCL disease progression (left panel, p=0.002). Disease progression is defined as a progression to a higher clinical stage and/or death. Loss of WIF1 is also associated with poor cancer-specific survival (right panel, p=0.012).

Gene Name	Forward Primer	Reverse Primer
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
ACVRL1	ACATGAAGAAGGTGGTGTGTGTGG	CGGGCAGAGGGGTTTGGGTA
ADAMDEC1	GGGGCCAGACTACACTGAAACATT	ACCCGTCACAAGTACTGATGCTG
AHI1	GTCCAAAACTACCCCATCAAGGCT	GCAGCACAGGAACGTATCACCT
ANGPT2	TGGCAGCGTTGATTTTCAGAGG	GCGAAACAAACTCATTTCCCAGCC
ANPEP	TGAAGAAGCAGGTCACACCCCT	AACTCCGTTGGAGCAGGCGG
APOA1	GCCGTGCTCTTCCTGACGG	TGGGACACATAGTCTCTGCCGC
ATXN7	CACCGCCCACTCTGGAAAAGAA	GGGTGCAGGGCTTCTTGGTG
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
BAG4	AGGTTCCAGGATATCCGCCTT	TCGGTCCTGATTGTGGAACACT
BCL2	ACAACATCGCCCTGTGGATGA	CCGTACAGTTCCACAAAGGCAT
BCL2L14	GCTCAGGGTCAAAGGACGTTGG	TCAGCTACTCGGTTGGCAATGG
BCL7A	GAACCATGTCGGGCAGGTCG	CCCATTTGTAGATTCGTAGGGATGTGT
BIN1	TGCTGTCGTGGTGGAGACCTTC	GCCGTGTAGTCGTGCTGGG
BIRC3	TGCTATCCACATCAGACAGCCC	TCTGAATGGTCTTCTCCAGGTTCA
BIRC5	TTCTCAAGGACCACCGCATCT	AGTGGATGAAGCCAGCCTCG
BLK	TCGGGGTCTTCACCATCAAAGC	GCGCTCCAGGTTGCGGATGA
BTRC	CCAAATGTGTCATTACCAACATGGGC	GCAGCACATAGTGATTTGGCATCC
BUB3	CGGAACATGGGTTACGTGCAGC	CCAAATACTCAACTGCCACTCGGC
CAGE1	TCCAAAATGCACAGTCTTCTGGCT	GGAGGCTCTTCAGTTTTTGCAGC
CASP1	CCTGTTCCTGTGATGTGGAGGAAA	GCTCTACCATCTGGCTGCTCAA
CASP3	AGCGAATCAATGGACTCTGGAATATCC	GTTTGCTGCATCGACATCTGTACCA
CCL5	TCATTGCTACTGCCCTCTGCG	ACTGCTGGGTTGGAGCACTTG
CCL18	CCCTCCTTGTCCTCGTCTGCA	GCACTGGGGGCTGGTTTCAG
CCL26	TTCCAATACAGCCACAAGCCCC	GGATGGGTACAGACTTTCTTGCCTC
CCND2	TCAAGTGCGTGCAGAAGGACAT	CTTCGCACTTCTGTTCCTCACA
CCND3	TGGCTGCTGTGATTGCACATGA	GATGGCGGGTACATGGCAAAGG
CCR3	ACGCTGCTCTGCTTCCTGG	TCCTCAGTTCCCCACCATCGC
CCR4	AGCATCGTGCTTCCTGAGCAA	GGTGTCTGCTATATCCGTGGGGT
CCR7	AGACAGGGGTAGTGCGAGGC	CTGGAAAATGACAAGGAGAGCCACC
CD164	AAACCTGTGAAGGTCGAAACAGCT	AGTCTGTCGTGTTCCCCACTTGA
CD1D	CACGTTCAGCGACCAGCAGTG	CTCACAGCCAGCGGACACCT
CD22_A	CATCCTCATCCTGGCAATCT	CTCTGCATCTCCAGTTCGTG
CD22_B	TTTTTGAGCACCCTGAAACC	CGGATACCCATAGCAGGAGA
CD274	ACCAGCACACTGAGAATCAACACA	GTCCTTTCATTTGGAGGATGTGCCA
CD52	CCTCCTACTCACCATCAGCCTCC	TGCTACCAAAGCTGCCTCCTGG
CD7	GGACAACCTGACTATCACCATGCA	TCCGAGCATCTGTGCCATCCTT
CD70	TGGGACGTAGCTGAGCTGCAG	GTCGTGGAGGAGCAGATGGC
CDKN1C	AGATCAGCGCCTGAGAAGTCGT	CTCGGGGCTCTTTGGGCTCT
CDKN2A	GGCACCAGAGGCAGTAACCAT	AGCCTCTCTGGTTCTTTCAATCGG
CDKN2B	GGGGCTGGAACCTAGATCGCC	CGGTCGGGTGAGAGTGGCA
CDO1	ACAGTCCACCTTTTGATACATGCCA	GCCCCTTAGTTGTTCTCCAGCG
CHD1	AGACCGACATCAGGGAGATTCTTACA	CCTGTGATCATCCAGTTTTCTGTGTTTC
CHD7	GGCACAGCTCCACCCATCAC	CTGAGTCATATCCGGCACTGGTTT
CLU	CGCCACAACTCCACGGGCTG	GTCAACCTCTCAGCGACCTGGA
CNOT3	CGTCCGTCTCCAAGAGAGTATGAAGA	CAAACTGCTCCACGCCCTCG
CR592140	ACCAGTGAAATTGACCTGCCCG	GCCCCAACCGAAATGTTTAACGC

CST6	CTGACGATGGAGATGGGGAGCA	GCCAGGGAACCACAAGGACC
CTAG1A	CGTGCCAGGGGTGCTTCTGA	TGCGTGATCCACATCAACAGGG
CTAG2	CAGGGGCAGCAAGGGCC	GCGACGAGAAAGGCATCGTGAT
CTAGE1	TCCTTACCGTCCCCCAAGACCT	GCTGTCGTTCTGGATGTTCAGCA
CTLA4	AGCTGAACCTGGCTACCAGGAC	ACACAAAGCTGGCGATGCCT
CTNNB1	TGGATACCTCCCAAGTCCTGTATGAG	TGCCCTCATCTAATGTCTCAGGGA
CXCL9	TGCTGGTTCTGATTGGAGTGCAA	AGGAAGGGCTTGGGGCAAATTG
CXCL11	CAGCAGCACCAGCAGCAACA	TGCAAAGACAGCGTCCTCTTTTGA
DDX53	TGGAAGAGGGCGGAGGCTAATC	AAAGCAGAGTGGTGGTTCACGG
DMAP1	GCGCGGATGTACGGGACATTC	CCTCGGGCCTCTTGAAAGTCAG
DMC1	TGCAATGTCAAAGGACTCTCAGAAGC	CCCGGTGGTGATATGGAAAACCA
DNM3	CTCCAGCCAACACTGATCTTGCA	CTGGCATCCGTTCCTTCATCCA
DPP4	TGGTATCAGATGATCTTGCCTCCTCA	CCACTTCCTCTGCCATCAAAGCT
E2F1	ATGGTGATCAAAGCCCCTCCT	TCGATCGGGCCTTGTTTGCT
E2F4	AGATACCCTCTTGGCCATCCG	GTGAATCTGGTACTTCTTCTGCCC
EED	TGCGGCCAAGAAGCAGAAGC	TGCATTTGGCGTGTTTGTAGGTG
EP400	TGCCCCCACCAAACCACAGA	TGCTTTCCTCAGCTCCGCAATG
EPHA4	GGCAGATGGTGAATGGCTGGT	GAGTAGCTGTGGGGTGGGCA
ESRRB	CGGGGACATTGCCTCTGGCTA	TGATCTCGCACTCGTTGGTGGC
EZH2	AAAATTATGATGGGAAAGTACACGGGGA	CTTCTCTTTCTTCAGGATCGTCTCCATC
FAS	AACCATGCTGGGCATCTGGA	GTTGATGTCAGTCACTTGGGCA
FASTK	AGGAAACGCAACTCAGCAGCAA	TGCCACCCCTGCTTCCCGA
FCRL3	CCCAGCACAGTCATGGAGTGAG	GTGTCATCCTCGTGATAAAACCAGTACA
FLT4	CCTGACACGCTCTTGGTCAACA	CCGGTCATCCCACACCACCT
FOSL1	CGGAGGAAGGAACTGACCGACT	TTCCAGCACCAGCTCTAGGCG
FOXP3	AGCTCCTACCCACTGCTGGC	TGCCCTGCCCTTCTCATCCAG
FYB	CCTCCCTTGTTTACCTTGGGTCC	GTGGAGGTGGTGGCAGGGAA
GAGE6	ACAGCCTCCTGAAATGATTGGGC	TGCTCCCTCATCCTCTCCCTCC
GATA3	GGCAACCTCGACCCCACTGT	CGTCCCTGCTCTCCTGGCTG
GATA6	TGAACCCGTGTGCAATGCTT	TTTCATAGCAAGTGGTCTGGGC
GNLY	TGGTCTTCTCTCGTCTGAGCCC	CCCAGCTCCTGTGTTTTGGTCA
GTSF1	GCAGACCAGCACCCCATTTGTC	GGCAGAGATTTGGGAACTCGCA
HCK	GGGGTGCATGAAGTCCAAGTTCC	CTGGTGTGTTGCTGTTGTGGCT
HDAC1	CGATGGCCTGTTTGAGTTCTGTCA	TCGGACTTCTTTGCATGGTGCA
HDAC2	TGGCGTACAGTCAAGGAGGCG	AGCAAGTTATGGGTCATGCGGATT
HIF1A	ACCCTAACTAGCCGAGGAAGAACTATGA	AGGTGGTTTCTTATACCCACACTGAGG
HMBS	GCTTCACCATCGGAGCCATCT	TGGCAGGGTTTCTAGGGTCTT
IFI35	TGGGGCTGAGAGAGACCACAG	CTGAAGGGCGTGGAGGGC
IFNG	GCATCGTTTTGGGTTCTCTTGGC	CCGCTACATCTGAATGACCTGCA
IGFL2	TCTGTCTCCTCCTCTTGTGTCCAA	CGGGTCTCGCTCAGGGACA
IK	CCCATGGACGTTGACAAAGGA	CAGCAGACCCAGCAAACTTTTCA
IL1F7	GGACAAAGTCATCCATCCCTTCAGC	CCGACTCCAGCATGTTCCAGG
IL1RN	GCCGACCCTCTGGGAGAAAAT	TGGTTGTTCCTCAGATAGAAGGTCT
IL2	CCAAACTCACCAGGATGCTCACA	ACGTTGATATTGCTGATTAAGTCCCTGG
IL2RA	GAAAGACCTCCGCTTCACTGCC	GGATCTCTGGCGGGTCATCGT
IL4	GCAGTTCCACAGGCACAAGCA	GGTTGGCTTCCTTCACAGGACA
IL5	GCTGATAGCCAATGAGACTCTGAGG	TCCACAGTACCCCCTTGCACA
IL7R	AGTGAATGGATCGCAGCACTCAC	AAATTCAGGCACTTTACCTCCACGA

IL9	TGACCAGTTGTCTCTGTTTGGGC	TGGGTATCTTGTTTGCATGGTGGT
IL10	AGGAGGTGATGCCCCAAGCTG	GCCTTGCTCTTGTTTTCACAGGG
IL12A	TGGCAGTTATTGATGAGCTGATGCA	AGCATGAAGAAGTATGCAGAGCTTGA
IL13	GCATGGTATGGAGCATCAACCTGA	CCTCTGGGTCTTCTCGATGGCA
IL15	AGGCATTGTGGATGGATGGCTG	AACACAAGTAGCACTGGATGGAAATACT
IL17A	GTCAACCTGAACATCCATAACCGGA	GCACTTTGCCTCCCAGATCACA
IL17F	TCACGTAACATCGAGAGCCGC	TGGAGATGTCTTCCTTTCCTTGAGCA
IL17RA	TGCCCACACCCAACAAGGAGA	ACTCAAACCTGACGCACAAACGT
IL17RB	CGAGCTTCAGTGGTGATTCCAGT	GCCTGTTTGTGGGCAGAGCA
IL17RC	ACTGGACCGCAGATCATTACCTTG	CTGAAGGGGCAGATGTTCGTCC
IL18	TCATTGACCAAGGAAATCGGCCTC	TCACACTTCACAGAGATAGTTACAGCCA
IL21	TCTGCCAGCTCCAGAAGATGTAGA	TCTCCCTGCATTTGTGGAAGGTG
IL21R	AAGGAAGGCTGGAACCCTCACC	GGGGCATGAAGAACCGCTCAG
IL22	CCCTTGAAGAAGTGCTGTTCCCT	TCAGCTTTTGCACATTCCTCTGGA
IL23A	TGCTCCCTGATAGCCCTGTGG	TTTGAAGCGGAGAAGGAGACGC
IL23R	AGTGCCCAAGACCATAATTTATTGGGAT	TCCAAGTAGAATTCTGACTGTTGCACA
IL26	TCCTGTGCTTCATCAGCTAGAGAGA	GGCTTTGGTTTACTGACTGCTTTCC
IL32	CCTTGGCTCCTTGAACTTTTGGC	CATTCGGGCCTTCAGCTTCTTCA
IRF1	CGCTGTGCCATGAACTCCCTG	AGCATCTCGGCTGGACTTCGAC
IRF3	CCAGCCAGACACCTCTCCGG	GCAGGGCTCAGGGGCTACAG
IRF4	TATGCTTGTGCCCCACCTGAGT	ACGTGGTCAGCTCCTTCACGA
IRF7	CTGGGCTTCGGGCAGGAC	AGGGAAGACACACCCTCACGC
ITK	GGCTCAACAAGGACAAGGTGGC	TCCAGGCACACCCCATACAGC
JARID2	AGGCTAGTGGAAGAGAAGGACTGC	CCTGTGTTATTGGGGAGGACGG
JUNB	GAACGCCTGATTGTCCCCAACA	CGAAGCCCTCCTGCTCCTCG
KAT5	TCCTGAGCGTGAAGGACATCAGT	GCCTCTTTCTTGGGGAACTGGATC
KIR3DL2	TGCATGTTCTGATTGGGACCTCAG	TTCACTGTTCTGTCCCCCGCA
KIT	GCACCGAAGGAGGCACTTACAC	GCTGCCACACATTGGAGCATG
KLF4_	ATCTCAAGGCACACCTGCGAA	ATCTGAGCGGGCGAATTTCCAT
KLHDC5	GGATGCGTGGAATTTTGTGGCG	CATGTCGGAAGAGGGGCAGTAC
LCE2B	TGCTCCTGCGTGTGACCAGG	GGGGCAGGCATTTAGGGGGAC
LCK	GGAGATCTGGGCTTTGAGAAGGGG	GCCACAAAATTGAAGGGGATGAAGC
LEF1	AGCGAATGTCGTTGCTGAGTGT	AGCTGTCTTTCTTTCCGTGCTAATTCA
LIF	TGCCATACGCCACCCATGTCA	CAGGTTGTTGGGGAACGGCT
LOR	CTCTCCTCACTCACCCTTCCTGG	CCACCGCCGCCAGAGGTCTT
LTA	ACAGCACCCTCAAACCTGCT	CGGTCCGTGTTTGCTCTCCA
LTBP4	CGGCATCTGTACCAACACCGAC	CTGCGACCCGCACAGGG
MAGEA9B	AACCAGGAGGACAGGAGCCC	CATCAGGCCCAAGTCCTCTCCT
MAL	CTTGCCCGACTTGCTCTTCATCT	CCACGAAGCAGAACACAGACACG
MAX	ACGGGCTCATCATAATGCACTGG	TGTGGCTTTGTCTAGGATTTGGGC
MCL1	AAGGACAAAACGGGACTGGCT	CACATTCCTGATGCCACCTTCT
MDM2	CCGTGAAGGAAACTGGGGAGTC	CGAAGCTGGAATCTGTGAGGTGG
MIR155	CTGTTAATGCTAATCGTGATAGGGGT	AATGCTAATATGTAGGAGTCAGTTGGAG
MIR203	TGGGTCCAGTGGTTCTTAACAGTTC	TCGCTGTCGCCGCGC
MIR205	AAAGATCCTCAGACAATCCATGTGCTT	TGTCAGCTCCATGCCTCCTGAA
MMP2	CACTGCGGTTTTCTCGAATCCA	TTACCGTCAAAGGGGTATCCATCG
MMP9	TTCGACGATGACGAGTTGTGGT	CGAAGATGAAGGGGAAGTGGCA
MMP12	GCCGTAATGTTCCCCACCTACAA	TCAGGATTTGGCAAGCGTTGGT

MOS	TGCTGTGCTTCCAGACACCCT	CCTGCTTGGTAGTCATTTGCCAGA
MPZL2	TGGGTTTCCCTCATGTATGGCAAG	CATTAACAGCCTCCAGCACCCG
MRC2	CAGGACTACGGCAAAGACGAGC	GCCTCCCTCCACGACAGCGT
MTF2	ACTGAGGGAACTGCACATTCATCC	GGCCAAGATCTTCCTGTACGCG
MXI1	CCCGGCACACAACACTTGGTTT	CGCCACTTTAAAAATCTCTGTTCTCGTT
MYB	GTCATGTTCCATACCCTGTAGCGTT	CTGCTATCCCCTCATTCAAGCACA
MYC	GAGACACCGCCCACCACCAG	TCCAGCAGAAGGTGATCCAGACT
NAIP	TTCCTGGGTCCAGAGAGAATTACC	TCCAGCCGTAGTTCTTCGTAAGC
NANOG	TGGATCCAGCTTGTCCCCAAA	AGGCCCACAAATCACAGGCATA
NAV3	ACCTCTGGTTTCCCCTTCTGCC	AGTCTTGGGCTGGGATGCTGTT
NEDD4L	AGGATCTCGGACCAGCCCTCA	TGACACTGCATGACCTCAACCTTG
NFKB1	TGCAACTATGTGGGACCAGCAA	AGTGTTTTCCCACCAGGCTGT
NFKB2	GAGATGGAGGAGCTGGGGTTGG	CAGAAGGAGGCGGGTGAGGG
NKG7	GCCTCCACACCCCCAGATCC	TCTGCTCACAAGGTTTCATAGCCAG
NKIRAS2	TGCTCAAGAAGGAGATTGACAAATCCA	ACACCTCCCACAGCTTCACCTT
NOTCH1	AGCTGGACCCCATGGACG	GGTGGCACTCTGGAAGCACT
NR0B1	AGGGGACCGTGCTCTTTAACCC	AGTTCGATGAATCTGTCATGGGGC
NUB1	GTTGCAAGGCAATTGAGCGTGG	GCCAGTGATGTGCAATCGGGTC
PALM2-AKAP2	TGCCGAAGAGGAGGAAGCCAG	TCTGGGACTCTTCACTTTCTAGCGT
PDCD1	TCGTCTGGGCGGTGCTACAA	GGTGAAGGTGGCGTTGTCCC
PHC1	CCAAACACCAGCACTACACAGCA	GCACAGATTGGGTCAAGGTGGT
PIP5K1B	ACACTGTTTCTGTTCATAGACCAAGCT	AGGGCGGCGATTGAATTGCAC
PLK1	AGTACCTGCACCGAAACCGAGT	GGGTCTTCTTCCTCTCCCCGTC
PLS3	ACTCTCTTGGTGTCAATCCTCACGT	TCCCAGTTTCGGGTATGGAGGT
POU2AF1	GGGGCTCAGATAAGTCCTCTCTGG	GTGGTTTGCCCACAGCTAATTTTCA
POU5F1	TGCAAAGCAGAAACCCTCGT	TCGGGCACTGCAGGAACAAAT
PRDM2	ACACTACTGAGCCTGTGGCGG	TGCCTTTTAAAATTGGTTTAGTGGCCC
PSMD3	GGCCCTAGACCTTGTAGCCGC	CATGCCGAAGCGTAGCTGTCC
PSORS1C2	CAGCTTTGGGGGCCAGTACAT	CCTCTGCGGGTGGGTGAGAG
PTEN	ATTCCCAGTCAGAGGCGCTAT	TCATCTTGTGAAACAACAGTGCCA
PTGS2	ACAGGCTTCCATTGACCAGAGC	ACCATAGAGTGCTTCCAACTCTGC
PTPN6	GGGCATGGTGCAGACGGAGG	TGGCTGGGGGATAGGTGATGTT
PTPN7	CCAGGACATGAAAGAGTGCCCAG	AGCTGATTCTGGTGTCTGATGGTCT
PTPRG	TGGAAGCCATTCCTGTCAAACAGT	TGCAGTGATGTTCATATCAGCAGTACA
RAC2	GCCAATGTGATGGTGGACAGCA	TGACGAGGGAGAAGCAGATGAGG
RASA1	ACGATAGCAGAAGAACGCCTCA	AAAGTACAAAGGACCCTGGCCTC
RB1	CAGATGGTATGTAACAGCGACCGT	TCAGTGGTTTAGGAGGGTTGCTTC
REC8	TGATGGAGACCCTAGAAGATGCTCC	ACTCTCTCTGGGATTGCAGCCT
RHOF	GCGTGACCGTTGGCAGCA	CGTTGTCGTAGCTGGTGGGATT
RNF2	GCCTCATCCCACACTTATGGAAAAAGA	AGTTCTTCTAAAGCTAACCTCACAGCC
SALL4	ACCCCAGCATCTGGCTAAAACAC	GTGGCTTCATCCTCACTCGCCA
SCPEP1	TGCAGAGCAAGTACTGAATGCCG	TCCATTGTAGACGTGGGAGTGCT
SDC4	GGCCCTGAAGTTGTCCATCCCT	CATCCTCACTCTCTTCAACGGGTG
SDHA_1	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
SDHA _2	TGTTGATGGGAACAAGAGGGCA	GCCTACCACCACTGCATCAAAT
SELL	CAGGCAAATGGAACGATGACGC	ACCCCACATCACAGTTGCAGGT
SERPINB4	ACCAGTGTGGAATCTACTGATTTTGCA	TCGTATCATTGCCAATAGTCCCATCAG
SERPINB5	CTCACTGAAACTAATCAAGCGGCTC	CCTTTGCATACGGTCTCTTCGT

SERPINB13	TGTGCTTCTGCCCAACGACATC	ACCGTCCTCCACCTCAAACCG
SH2D1A	AGTCCTCAGCTAGAAGTACACAAGGT	TGCATTTGTAGCTCACCGAACTGT
SKAP1	TTTGGATCGGAGTGGCAGAAGC	GCCATCCGTACACCGTAGCCC
SMAD1	CAAGAATTTGCTCAGTTATTGGCACAGT	TGGCGGTGGTATTCTGCTCCC
SMAD2	GCTTGAGAAAGCCATCACCACTC	CTCAGTCCCCAAATTTCAGAGCA
SOCS3	AGCCCCAAGGACGGAGACTTC	CGGGAAACTTGCTGTGGGTGAC
SOX2	TGAACCAGCGCATGGACAGTTA	CATCATGCTGTAGCTGCCGTT
SPO11	ACAGAGCAACACTTATGCAACCAAAAG	ACTCCTCCTTGACACTTTTAACATGCA
ST8SIA1	TGTTGGCTCTACATCTTCCCCGT	GGTCGCAGCAGTCTTCCATTTGT
STAG3	TGACAGGGACTCAAACCATACCTCA	TGTTTTCGGTGGTCGTTTTGCTG
STAT1	TGATCTCCAACGTCAGCCAGC	GCCAACTCAGCACTTCTGAAAGC
STAT2	CATTGGAGGGCGCGGGGACT	TCGAATGTCCACAGGCAGGAGG
STAT3	ATGCGGCCAGCAAAGAATCA	AGCGGCTATACTGCTGGTCAAT
STAT4	GGAAATTCGGCATCTGTTGGCC	TTCTCTTTGGAAACACGACCTAACTGT
STAT5A	TGGCAGTGGTTTGACGGGGT	GTCGGGCTTGTTGATGAGCAGG
STAT5B	ACTGAAGATCAAGCTGGGGCA	ACAATATATGGCGGATGCAGCG
STAT6	GGCCACTTTCAGACAAATACTTCAAGGA	TGCAGCCTCCGCAAGCCT
SUZ12	TCATCGCCAACCTGGATTTGCT	ATGTTCTTTGCTGTTCTACTTCCCCAT
SYCP1	CCACCAGCTTCTCATCTTTGTGTCA	AGCAATTACAGCCCAACGGTCC
SYCP3	ACCAAGGCTTCTCTCAAAACTAGTAACC	ATCCCACTGCTGAAACAAAGTCAGA
SYK	TCTTTTTCGGCAACATCACCCG	GCGCAGCAAATAAAGCCCATCA
TBX3	CTGGAGGCTAAAGAACTTTGGGATCA	ATCCAGCCCAGAACATCTCACTTTAAAT
TBX21	ACGCTTCCAACACGCATATCTTTACT	GTTCTCCCGGAATCCTTTGGCA
TCF3	CCTGTTTGAAACGGCGAGAAGA	TGGGGAGCTGAAAGCACCAT
TCF7L1	ACCGTATTACCCACTCTCTCCCG	ATCGAGGCGTTCATGGCGAG
TGFB1	AGTTGTGCGGCAGTGGTTGA	CTTGCAGTGTGTTATCCCTGCT
THAP11	CCAAAGGACGCTGAGTTGCGG	CGTACCGTGTAGGTCTTGCGG
THBS4	AGTTCAGCCACCATCTTCGGTCT	AACCACCAAATGCACCTTCCCA
TIMP1	AGATCCAGCGCCCAGAGAGA	AGCAACAACAGGATGCCAGAAG
TLR7	TGCTCTCTTCAACCAGACCTCTACA	AGTTTTAGGAAACCATCTAGCCCCAAG
TNFRSF4	ACACCTACCCCAGCAACGACC	CACGGCTTGGAGCTGACCAC
TNFRSF8	AGCTCCACCTGTGCTACCCG	CGTTGAGCTCCTCCTGGGTCTG
TNFSF10	TGCAGTCTCTCTGTGTGGCT	GCCACTTTTGGAGTACTTGTCCTG
TNFSF11	TGGTGGATGGCTCATGGTTAGATCTG	CAAGAGGACAGACTCACTTTATGGGAAC
TNFSF13B	TGCAGGGCCACCACGCG	TGCTGTTCTGACTGGAGTTGCC
Tox	TGAGCATGACAGAGCCGAGCC	CAGCGAGTGGTCTGGGAGGG
TP53	ACCATGAGCGCTGCTCAGATA	CCACACGCAAATTTCCTTCCAC
TP63	CGGAGGTGGTGAAGCGGTGC	GCACACTCTGTCTTCCTGTGATGG
TP73	AGCTCGGGAGGGACTTCAAC	AGGGTCATCCACATACTGCGA
TRAF2	ACCGGTACTGCTCCTTCTGC	TCGTGAACACAGGCAGCACA
TRAF3IP3	TGACCACCTCTCCTCACAGGCT	TTGGTTTGCTGACTGGCATCGT
TRIM28	CCCCACAGGAGTTTGCCCAG	GCACAGCAGAGAACTTGGTGTCA
TRIP13	GAGTCGCCAACGGTCCACGT	AAGGTTCATCAAACTCAGTCCATGTGT
TRRAP	GTCCACGCTGATGTTGGAGCA	AGGGAGTAAAGCTCCGCAAGGG
TTR	AGTCTGGAGAGCTGCATGGGC	CGGAGTCGTTGGCTGTGAATACC
TWIST1	TCCATGTCCGCGTCCCACTA	AGCTCCATCCTGGTGTACCTT
VEGFC	GCCAACCTCAACTCAAGGACAGA	CCCCACATCTATACACACCTCCCG
WIF1	GAATTCCTGTCCTTGCGCTCCC	CTGCCACCCCATCCTGTTTTCC

DIABLO	TGCAGTTGGTCTTTCAGAGATGGC	AGCTTGGTTTCTGCTTTCCGGG
XTP6	AGAGTGGAGGCTGGAAGGATGG	TCAGCACAAGGCAAGGATGCTC
YWHAZ	TCCCCAATGCTTCACAAGCAGA	TCTTGTCATCACCAGCGGCAA
ZAP70	ACCCGAATGCATCAACTTCCGC	CTTGCCCTGCTCGATGAAGGC
ZBTB16	CCTGGATAGTTTGCGGCTGAGAA	ATGGGTCTGCCTGTGTGTCTCC
ZFX	GTTGAACTGCTTGATCAGAACAGCAG	TCGGCATGAAGGTTTTGATTTCATTGTC

Table 6.1. Primers used for RT-PCR experiments.

Gene Name	Putative biological role and importance in cancer/CTCL.
Part A. Genes upregulated in Cluster 1 poor prognosis patients.	
IL-26	IL-26 is one of the cytokines produced by the Th17 cells[237].
IL-21 and IL21R	IL-21 is one of the cytokines produced by the Th17 cells[237]. Autocrine IL-21 stimulation was shown to be involved in the maintenance of constitutive STAT3 activation in SS[238].
IL-17A and IL-17F	Both cytokines are typically produced by the Th17 cells[237]. As addressed in the discussion, there remains an uncertainty whether IL-17 signaling plays an important role in CTCL or whether it is seen as a secondary epiphenomenon. Aberrant constitutive activation of JAK/STAT signaling has been documented in CTCL[239]. This constitutive activation of signaling was shown to result in IL-17 production[239].
IL-22	IL-22 was initially thought to be produced by Th17 cells. Later it was shown that IL-22 production can also occur in an unique Th22 subset of cells that lack the ability to produce IL-17 and interferon-γ[240]. IL-22 was proposed to be a dominant cytokine in the tumor microenvironment of CTCL lesional skin[225].
IL2RA	IL-2Rα chain constitutes a part of the high affinity three chain IL-2 receptor. IL-2Rα expression is restricted to T cells that recently encountered an antigen, while in healthy individuals the majority (i.e. >95%) of peripheral T cells are IL-2Rα negative. IL-2Rα was shown to be expressed in patients rejecting allografts, in autoreactive T cells from patients with autoimmune conditions[227]. CTCL malignant cells were shown to constitutively express this protein, which is driven by STAT3 activation[241]. In MF skin and SS circulating T cells IL-2Rα is upregulated in up to 50% of cases. Interleukin-2 diphtheria toxin fusion protein (denileukin diftitox) is designed to target IL-2 receptor in CTCL[227].
ITK	ITK (Interleukin-2 inducible T-cell kinase) is a member of the Tec kinase family of non-receptor tyrosine kinases. It plays an important role in T-cell signaling. Together with other T-cell specific tyrosine kinases (e.g. LCK and ZAP-70) ITK amplifies signals transmitted via a T-cell receptor cascade. Abnormal activity of this cascade was shown to lead to autoimmune disorders and inflammation. ITK-SYK translocations were observed in Peripheral T-cell lymphomas (PTCL)[242].
LCK	LCK **(**lymphocyte-specific protein tyrosine kinase) is a member of the Src family of protein tyrosine kinases and plays an important role to amplify signals transmitted via a T-cell receptor cascade. Previous cytogenetic studies demonstrated that LCK gene can fuse together with the TCRβ subunit to produce a fusion protein in T-cell acute lymphoblastic leukemia via t(1;7)(p34;q34) translocation. This gene is overexpressed in PTCL. LCK was shown to be able to identify SS from normal control samples[243].
FYB	FYB is an adaptor protein involved in T cell signal transduction pathways. This gene is expressed in T cells, myeloid cells and is believed to promote positive regulation of T cell activation, integrin-mediated adhesion and IL-2 production[243].
GNLY	Granulysin is a cytolytic granule protein, which is expressed in cytotoxic T cells and NK cells. Granulysin is implicated in a variety of diseases including infection, cancer, transplantation, autoimmunity and drug eruptions. Cytolytic proteins including granzyme B and perforin were previously documented to play critical pathophysiological roles in NK/T cell lymphomas. Increased levels of this gene were detected in NK/T cell lymphomas and anaplastic large cell lymphomas (ALCL) cases[244].
GTSF1	GTSF1 (Gametocyte Specific Factor 1) is a cancer testis antigen ectopically expressed in CTCL[128] and was reported to be a part of a molecular signature that is specific to this cancer[179].
SYCP1	SYCP1 (Synaptonemal complex protein 1) is a cancer testis antigen ectopically expressed in CTCL[128]. The normal function of this gene is to regulate crossing over in meiosis[128].

POU2AF1	POU2AF1 is a B cell-specific transcriptional factor. This gene is essential for B cell maturation and germinal center formation[150]. Previous research demonstrated ectopic expression of B cell specific genes in CTCL, as in the case of BLK gene[149].
CHD1	CHD1 (Chromodomain-helicase-DNA-binding protein 1) is an embryonic gene, whose activity is required to maintain open chromatin of pluripotent mouse embryonic stem cells[186].
TOX	TOX is a transcription factor, which is essential for early development of $CD4^+$ T cells and is normally not expressed in mature $CD4^+$ T cells. Previous studies demonstrated that the $CD4^+$ T cells in CTCL ectopically express TOX, which causes the proliferation/apoptosis balance to shift toward proliferation by suppressing the transcription of several tumor suppressors. It was previously proposed that targeting TOX activity may be a promising treatment strategy for CTCL. TOX expression was independently found by two separate laboratories to be a robust diagnostic and prognostic marker for this cancer[73,184].
TCF3	TCF3 (Transcription factor 3) is a member of Tcf/Lef transcription factor family. LEF1 and TCF1 are required for transactivation of Wnt signaling genes, while TCF3 functions predominantly as a transcriptional repressor. Notably, TCF3 is expressed in different types of stem cells including embryonic and hair follicle stem cells. TCF3 promotes differentiation of embryonic stem cells by counteracting Wnt-mediated maintenance signals[207]. TCF3 is believed to be an important negative regulator of embryonic stem cell self-renewal. Chromosomal translocation t(1;19)(q23;p13.3) which leads to a production of the TCF3-PBX1 (E2A-PBX1) fusion protein was observed in Acute Lymphoblastic Leukemia. Recurrent mutations in TCF3 that promote PI3-kinase signaling were documented in human Burkitt's lymphoma samples[210]. While this gene is upregulated in CTCL it is not known if it is also mutated.
LEF1	LEF1 (Lymphoid enhancer-binding factor 1) is a downstream member of the Wnt/β-catenin signaling pathway. Increased LEF1 expression has been reported as a poor prognostic marker in various hematologic malignancies[245].
NFKB1	NFKB1 is a master transcription factor that plays a major role in inflammatory and immune responses. Activation of the NFκB signaling pathway plays a critical role in the development and progression of many types of cancer. NFKB1 was shown to be overexpressed in MF patients and may correlate with poor disease outcome[246].
JUNB	c-JUN and c-FOS were initially identified as viral oncoproteins and their roles in tumorigenesis are well established. Previous studies documented gain in copy numbers of JUNB in 54% of c-ALCL and 26% of SS/MF patients with strong nuclear expression of JUNB protein in these cancers. Dysregulation of AP-1 expression in CTCL was also shown to be associated with genomic amplification of JUNB in this cancer. JUNB is an important transcriptional regulator of IL-4 expression and is associated with the Th2 phenotype in the advanced CTCL[247].
CCL18	CC chemokine ligand (CCL) 18 is produced by monocytes and dendritic cells and was shown to act as a potent chemoattractant for T and B cells. CCL18 expression is upregulated in atopic dermatitis and bullous pemphigoid. The CTCL lesional skin was shown to express elevated levels of CCL18 mRNA in comparison to normal skin. Further studies showed that dermal macrophages and dendritic cells in CTCL skin were responsible for CCL18 production. Serum levels of this protein were elevated in CTCL and correlate with the types of skin lesions. Patients with high serum levels of CCL18 had more aggressive disease course than patients with low CCL18 levels[229].
CCR4	CC chemokine receptor 4 (CCR4) is highly expressed in SS and MF skin in all CTCL subtypes. Expression of CCR4 is limited in non-malignant cells as it is absent in neutrophils, monocytes, B cells and naïve T cells. It is also expressed in fewer than half of all memory T cells. Based on these

	findings Mogamulizumab, humanized anti-CCR4 antibody, was proposed as an attractive therapeutic target for CTCL and is currently being tested in patients[226].
STAT5A	Upregulation in STAT5 signaling occurs in early CTCL stages. A growing body of experimental evidence suggests that this gene is important for expression of anti-apoptotic proteins (bcl-2 and bcl-x), cell cycle genes (Cyclin D and c-myc) and oncogenic miR-155 microRNA, all working in concert to promote cancerogenesis[37,248].
SH2D1A	SH2D1A (SH2 domain-containing protein 1A) was shown to be associated with X-linked lymphoproliferative disease[249].
TFRC (CD71)	Iron is transported in serum by transferrin and enters the cell via the transferrin receptor (TFRC). In replicating cells iron is required for DNA synthesis and cytochrome function. To meet this need, as proliferating cells exhaust available intracellular iron, they increase their surface expression of TFRC. Activated T cells express surface receptors including CD25 (the IL-2 receptor) and TFRC/CD71. Previous reports document upregulation of T cell activation markers including TFRC/CD71 in CTCL[250].
MXI1	MXI1 protein is a basic helix-loop-helix, leucine zipper transcriptional factor that can dimerize with Max protein and bind to specific DNA sequences and suppresses the transcription of genes that are typically transactivated by a c-Myc/Max dimer[251].
AHI1	Abelson Helper Integration site 1 (AHI1) and downstream signaling members, (e.g. CDKN1C), were suggested to play an important role in CTCL carcinogenesis[19,91]. AHI1 is typically activated by provirus insertional mutagenesis in various murine leukemias and lymphomas. Overexpression of this gene was demonstrated in Hut102 and Hut78 CTCL cell lines. One of the putative functions of AHI1 is to suppress CDKN1C[126,252].
IRF4	The IFN regulatory factor 4 gene, also known as multiple myeloma antigen 1 (MUM1), is normally expressed in plasma cells, melanocytes, subset of B cells, and in activated T cells. It is required for B-cell development, plays an important role in Th2, Th17 T-cell differentiation and T-cell cytotoxic function. In some multiple myeloma, IRF4 is involved in t(6;14)(p25;q32) reciprocal translocation, which leads to the juxtaposition of this gene next to the immunoglobulin heavy chain locus. IRF4 rearrangements were previously documented is a subset of diffuse large cell B-cell lymphoma, splenic marginal zone lymphoma, chronic B-cell lymphoid leukemia, transformed MF and ALCL cases. IRF4 locus amplification was observed in a subset of c-ALCL and transformed MF/ SS cases[253].
PLK1	Polo-like kinases belong to the serine/threonine kinase family and is critical for mitosis and DNA integrity. PLK1 is one of the most studied members of this family and was found to be upregulated in a variety of cancers. It is also upregulated in the G2/M phase of mitosis. PLK1 was found to be overexpressed in advanced lesions of CTCL and in several CTCL cell lines including HH, Hut78, MyLa, SeAx and SZ4. Downregulation of this gene results in decreased malignant cell proliferation and viability[254].
NAIP	NLR apoptosis inhibitory protein (NAIP) is homologous to two baculovirus inhibitor of apoptosis proteins (IAP) and is able to suppress apoptosis induced by various signals[255]. Resistance to apoptosis is believed to be one of the cardinal features of CTCL[255].
CCND2	CCND2 (G1/S-specific cyclin-D2) upregulation was documented in a subset of CTCL cases and in a variety of leukemias and lymphomas[256].
T3JAM	T3JAM (TRAF3-interacting JNK-activating modulator) is expressed in bone marrow, spleen and thymus and was shown to promote specific activation of JNK signaling[257].
Part B. Genes upregulated in Cluster 2 favorable prognosis patients.	

LCE2B	LCE2B (Late Cornified Envelope Protein 2B) is one of at least 20 genes that are expressed during epidermal differentiation. This gene was found to be expressed in normal and psoriatic skin, but not in cultured keratinocytes or in other tested cell types or tissues[258].
CST6	CTS6 (Cystatin 6), also known as cystatin E/M, is a cysteine protease inhibitor that is downregulated in breast, cervical, glioma, prostate and gastric cancers. Loss of CST6 expression is attributed to promoter hypermethylation. Also, CST6 has been shown to be important for skin differentiation. This gene is proposed to act as a tumor suppressor gene by controlling the activity of a known oncogene, Legumain (LGMN)[259].
LOR	Loricrin is a major protein component of the cornified cell envelope that is responsible for protective barrier function of the stratum corneum. This gene is expressed in terminally differentiated keratinocytes. Mutations in this gene have been reported in Vohwinkel's keratoderma and progressive symmetric erythrokeratoderma[260].
LTBP4	The extracellular matrix protein LTBP4 (latent transforming growth factor β-binding protein 4) belongs to the fibrillin/LTBP family of glycoproteins. These proteins can covalently bind to TGFβ and play an important role in promoting the folding and secretion of this protein. Dysregulated expression of LTBP isoforms was shown to be associated with epithelial neoplasms. Specifically, LTBP1 is downregulated in neoplasms of the liver, ovaries and neuroendocrine tumors of the digestive system. LTBP2 is downregulated in esophageal squamous cell carcinoma and nasopharyngeal carcinomas. LTBP4 is downregulated in breast adenocarcinomas. Promoter hypermethylation was documented to be the mechanism of downregulation for this gene[261]
BCL7A	BCL7A (B-cell CLL/lymphoma 7A) putative tumor suppressor gene that was previously suggested by us and others to play an important role in CTCL carcinogenesis and progression[20,36,37]. It is often down-regulated in CTCL patients when compared to benign skin conditions. Several mechanisms for this loss of expression have been demonstrated, where BCL7A and the corresponding 12q24.31 region of the chromosome were lost in 56% of patients with CTCL. In another study, 48% of patients exhibited BCL7A promoter hypermethylation. Such promoter hypermethylation was preferentially observed in patients with aggressive CTCL. This gene may be an important prognostic marker in patients with early-stage disease[127,230,231].
CDKN1C	CDKN1C (Cyclin-Dependent Kinase Inhibitor 1C) belongs to the Cip/Kip family of cyclin-dependent kinase inhibitors, which negatively regulate cell cycle progression by inhibiting G1 cyclin-dependent kinases. Mutations in this gene were identified in patients with Beckwith-Wiedemann syndrome, which is characterized by an over-growth phenotype and an association with several cancers. Hence, loss-of-function of CDKN1C promotes cell proliferation giving rise to an over-growth phenotype[262]. Our previous work suggests that this gene is a downstream target of AHI1 oncogene and its loss may play an important role in CTCL carcinogenesis[126].
PSORS1C2	PSORS1C2 is a poorly characterized psoriasis susceptibility gene[263].
WIF1	WIF1 (WNT inhibitory factor 1) is a WNT/β-catenin signaling inhibitor and was previously shown to be downregulated in salivary gland carcinomas[264], acute lymphoblastic leukemias[235] and acute myeloid leukemias[236]. Our studies[51,137] for the first time suggest that loss of this gene may also be important for CTCL carcinogenesis.
DLEU1	DLEU1 (Deleted in Lymphocytic Leukemia 1) is a long non-coding RNA putative tumor suppressor gene and is frequently deleted in B-cell chronic lymphocytic leukemia[233] and may have tumor suppressing properties in CTCL[265].
IL-18	IL-18 proinflammatory cytokine is known to induce expression of interferon-γ and promote Th1 immune responses, both of which are associated with disease clearance[234].
IL1F7 (IL-37)	IL1F7 is a target gene upregulated by IL-18 signaling and acts as an inhibitor of innate immunity[266].

miR-205	miR-205 microRNA was documented to act as putative tumor suppressor gene that targets E2F1 in melanoma and other cancers and has the ability to distinguish CTCL from other benign entities[232].
Part C. Genes upregulated in Cluster 3 intermediate prognosis patients.	
FOSL1	FOSL1 (Fos-Like Antigen 1), also known as FRA1, forms a part of the AP-1 complex. Gain of function of this oncogene has been linked to the enhanced migration and invasion of colorectal breast, lung, bladder, head and neck, thyroid and brain carcinomas. FOSL1 expression is induced by RAS-ERK and Wnt/β-catenin pathways[267].
SERPINB4	SERPINB4, also known as squamous cell carcinoma antigen 2 (SCCA2), is a member of the ovalbumin family of serine proteinase inhibitors. It was originally isolated from metastatic cervical squamous cell carcinoma. SERPINB4 is expressed primarily in malignant cells and correlates with more aggressive tumors[268].
EVA1	EVA1 (Epithelial V-like antigen 1), also known as MPZL2 is expressed in the thymus early in embryogenesis and subsequently is downregulated during thymocyte developmental progression. It is believed to contribute to the earliest phases of thymus organogenesis[183].
MMP12	MMP12 (matrix metalloproteinase-12) also known as a macrophage metalloelastase or macrophage elastase is an enzyme involved in the breakdown of extracellular matrix in normal physiological and pathological processes. MMP12 may play a role in aneurysm formation, in the development of emphysema and was recently shown to promote migration and invasion in nasopharyngeal carcinoma[269].
SELE	SELE (E-selectin), also known as CD62 antigen-like family member E (CD62E), endothelial-leukocyte adhesion molecule 1 (ELAM-1), or leukocyte-endothelial cell adhesion molecule 2 (LECAM2), is a cell adhesion molecule expressed by activated endothelial cells. It plays an important role in inflammation. Previous studies that analyzed E-selectin staining in blood vessels showed differences between various disease groups and healthy controls, with the highest percentages being observed in CTCL patients[270].
CCL26	CC chemokine CCL26, also known as eotaxin-3, is a potent chemoattractant and was shown to correlate with the clinical itch burden in CTLC patients[228].
PLS3	PLS3 (Plastin-3) is an actin-binding protein, which is not normally expressed in T cells. It was shown that the promoter for this gene is demethylated in cancer. The normal function is to regulate actin structure elongation. This gene is associated with SS cell survival and migration and was shown to be overexpressed in SS cells by many studies[74].

Table 6.2. Detailed description of the 52 genes that fit into the three signature pattern expression model and their putative functions in CTCL and other malignancies.

Analysis of Maximum Likelihood Estimates (Cox Model)	
Patient Characteristics	Odds Ratio for Progression (p value)
Age	
< 40	1.77 (p=0.51)
40-59	1.48 (p=0.50)
≥ 60	1 (reference)
Sex	
Male	1.65 (p=0.25)
Female	1 (reference)
Clinical Stage at the Time of Diagnosis	
Stage I	1 (reference)
Stage II	4.7 (p=0.005)
Stage ≥ III	12.0 (p<0.0001)

Table 6.3. Multivariate analysis of patient characteristics that are associated with clinical disease progression.

Gene Name	Putative biological role and importance in cancer/CTCL.
Part A. Genes preferentially expressed in CTCL patients.	
CCL26	Please see Table 6.2C for more details.
CCL18	Please see Table 6.2A for more details.
FYB	Please see Table 6.2A for more details.
IL2RA	Please see Table 6.2A for more details.
LEF1	Please see Table 6.2A for more details.
LCK	Please see Table 6.2A for more details.
ITK	Please see Table 6.2A for more details.
TOX	Please see Table 6.2A for more details.
CCR4	Please see Table6. 2A for more details.
GNLY	Please see Table 6.2A for more details.
MMP12	Please see Table 6.2C for more details.
T3JAM	Please see Table 6.2A for more details.
IL1F7	Please see Table 6.2B for more details.
IL-22	Please see Table 6.2A for more details.
IL-26	Please see Table 6.2A for more details.
STAT5A	Please see Table 6.2A for more details.
SYCP1	Please see Table 6.2A for more details.
GTSF1	Please see Table 6.2A for more details.
cTAGE1	This cancer testis antigen was documented to be ectopically expressed in CTCL. cTAGE1 (Cutaneous T-Cell Lymphoma-Associated Antigen 1) is robustly expressed in the majority of CTCL patients and patient-derived cell lines[128].
CDO1	CDO1 (cysteine dioxygenase) was shown to be consistently overexpressed in SS patients and is the rate-limiting enzyme in the synthesis of taurine, an important semi-essential amino acid. CDO1 is not usually expressed in peripheral blood. The expression of this gene was documented in liver and brain. The CDO1 promoter, is believed to be under the regulation of c-myb, which is consistently overexpressed in PBMCs from SS, but not MF patients[151].
THAP11	THAP11 (Thanatos-associated protein 11), also known as Ronin, is essential for the self-renewal of embryonic stem (ES) cells. This gene contributes to ES cell pluripotency by regulating the transcription of genes involved in the metabolic processes that sustain the growth of self-renewing ES cells[211].
STAT4	STAT4 appears to be overexpressed in the early stages of CTCL when compared to benign skin diseases[265]. The expression of STAT4 is required for Th1 differentiation. The expression of this gene is lost in advanced CTCL with concomitant shift towards the Th2 phenotype[177,248,271]. Loss of STAT4 expression appears to be a robust and reliable diagnostic marker for SS[177].
Part B. Genes preferentially expressed in normal skin and in patients with benign inflammatory dermatoses.	
WIF1	Please see Table 6.2B for more details.
BCL7A	Please see Table 6.2B for more details.
PSORS1C2	Please see Table 6.2B for more details.
DMAP1	DMAP1 participates in DNA repair by directly interacting with PCNA. DMAP1-depleted cells in p53-deficient background demonstrate chromosomal instability and tumor formation in mice. Recent reports indicate that DMAP1 acts as a tumor suppressor by maintaining chromosomal integrity[191].
SERPINB13	SERPINB13 is an inhibitor of lysosomal cathepsin enzymes K and L and was shown to be downregulated in the head and neck cancers. Cathepsin K promotes cancer cell invasion via degradation of the extracellular matrix[272].

Table 6.4. Description of genes that are able to distinguish CTCL from benign dermatoses and their putative roles in CTCL and other malignancies.

Chapter 7. Literature review on the role of STAT signaling in Cutaneous T-Cell Lymphoma. (Full published manuscript is presented in Appendix 5.)

7A. LITERATURE REVIEW

We were invited to submit a review article to the *Cell Cycle* journal on the role of STAT signaling in CTCL. As part of this work I have performed a literature review on the role of STAT expression and function in CTCL[273]. Below is the text summarizing my literature review.

Signal transducers and activators of transcription (STAT) proteins have a dual role as cytoplasmic signaling proteins and nuclear transcription factors. Following activation by receptors with tyrosine kinase activity, phosphorylated STATs dimerize and translocate to the nucleus to induce expression of important genes essential for normal physiological functioning of the cell. Furthermore, STAT signaling plays a central role in mediating inflammation. Chronic inflammation is a critical hallmark of Cutaneous T-Cell Lymphoma (CTCL), a clinically heterogeneous group of lymphoproliferative malignancies that are characterized by localization of neoplastic T lymphocytes to the skin. Mycosis Fungoides (MF) and its leukemic variant Sézary Syndrome (SS) are some of the most common variants of CTCL[220]. Persistent activation of transcription factors of STAT protein family has been implicated in the pathogenesis of a wide variety of human cancers, including CTCL[274].

In early CTCL stages (Figure 7.1A), where disease presents with erythematous scaly patches and plaques, STAT4 appears to be overexpressed, when compared to non-malignant skin samples[265,274]. STAT4 activity, which is required for T helper (Th) 1 differentiation, is induced by IL-12 cytokine, which signals through JAK2 and TYK2 receptor tyrosine kinases[271]. STAT4 expression is subsequently lost at later clinical stages, where the disease acquires predominantly Th2 phenotype[176,177,271,275,276]. In fact, in leukemic CTCL/SS loss of STAT4 expression appears to be a robust and reliable diagnostic marker for this cancer[177]. Several molecular mechanisms were suggested to explain the observed STAT4 downregulation. Previous studies demonstrated that CTCL patients are profoundly deficient in IL-12 production[271,277,278]. Furthermore, while normal T cells express β1 and β2 chains of the IL-12 receptor, CTCL malignant Th2 cells only express IL-12R β1 chain and thus are not sensitive even to exogenous IL-12[271]. Moreover recent studies documented that downregulation of STAT4 could be indirectly driven by a constitutive activation of STAT5[224,274].

Upregulation of STAT5 signaling was shown to also occur in the early stages of CTCL[279,280]. STAT5 is known to be involved in the transcription of anti-apoptotic proteins (bcl-2 and bcl-x), cell cycle genes (Cyclin D and c-myc) and IL-4 cytokine[37,224,281], which work together to promote cancerogenesis. Inhibition of this protein signaling in CTCL results in inhibition of proliferation[224]. STAT5 can be activated via IL-2, IL-7 and IL-15 signaling (in the early disease stages) or via constitutively active JAK1 and JAK3 signaling in malignant cells [224]. A recent study demonstrated that one of the critical functions of STAT5 in CTCL is to upregulate oncogenic miR-155 microRNA[224]. miR-155 was dubbed as the "bridge between inflammation and cancer"[282] because transient elevation of this miRNA leads to activation of several types of immune cells, while constitutive upregulation of this gene results in the development of malignancies, by increasing genomic instability[224,282]. miR-155 was shown to be upregulated in a variety of hematological malignancies[53,283]. In their study Litvinov et al. demonstrate that STAT4 is one of the targets of miR-155[274]. Hence, STAT5-driven upregulation of miR-155 results in a subsequent loss of STAT4 expression and concomitant switch from Th1 to Th2 malignant phenotype. Furthermore this study indicates that STAT4 and STAT6 are inversely regulated and loss of STAT4 leads to upregulation of STAT6 expression and signaling, which further promotes Th2 phenotype in CTCL[274].

Recently, epigenetic changes became a significant focus of basic and clinical research in CTCL. A number of previous studies documented methylation/histone acetylation abnormalities in malignant CTCL cells[284,285]. In fact, two of the commonly used medications for advanced stages of this cancer are histone deacetylase (HDAC) inhibitors (Romidepsin and Vorinostat)[286]. By blocking the deacetylation of histones these agents promote an open (i.e., acetylated) chromatin structure and thereby upregulate the expression of a number of genes. The study by Litvinov et al. demonstrates that treatment of patient-derived CTCL cells with these inhibitors results in restoration of STAT4 and downregulation of STAT6 expression[274]. Downregulation of STAT6 by SAHA/Vorinostat was also previously documented by Zhang et al[285]. STAT6 is primarily activated by IL-4 and IL-13 for the development of the Th2 phenotype[287].

Finally, in advanced stages of this cancer (i.e., tumor and erythrodermic stages) constitutive expression of STAT3 has been consistently observed (Figure 7.1B). STAT3 can be activated by a wide variety of extracellular and intracellular stimuli including IL-2, IL-6, IL-7, IL-

9, IL-10, IL-15 and IL-21, and by intracellular tyrosine kinases such as *src* and *abl*[280,285,287,288]. Upregulation of STAT3 heralds progression from indolent to more aggressive disease course.

The question remains what is the predominant mechanism behind constitutive STAT3 activation in CTCL? Several papers suggested that constitutive STAT3 activation in advanced stages could be dependent on the presence of IL-7 and IL-15 cytokines in the microenvironment[280], development of pathogenic autocrine IL-21 signaling[288] and/or constitutive cytokine-independent activation of JAK1 and JAK3 signaling[241,289,290]. Recent evidence documents that, while IL-2, IL-7 and IL-15 may have a dual role to promote STAT3 and STAT5 signaling, IL-21 leads to a more specific activation of STAT3, which in turn directly upregulates IL-21 expression leading to potentiation of IL-21 autocrine signaling loop[288]. Hence, it is possible that initially STAT3 overexpression hinges on IL-2, IL-7 and IL-15 signaling, while later on autocrine IL-21 signaling and/or cytokine-independent JAK1 and JAK3 activity is sufficient to achieve constitutive STAT3 activation.

Constitutively active STAT3 can increase survival and resistance to apoptosis in malignant T cells (by promoting bcl-2 expression)[289], promote Th2 and Th17 phenotypes in advanced disease and induce expression of miR-21 oncogenic microRNA[291]. STAT3 induces cytokines involved in eosinophilia and erythroderma (e.g., IL-5). Constitutively activated STAT3 induces uncontrolled cellular growth *in vitro* and functions as an oncogene *in vivo*. STAT3 signaling in CTCL was also shown to upregulate VEGF[292], the anti-inflammatory cytokine IL-10[293] and a suppressor of cytokine signaling-3 (SOCS3)[294]. The latter was shown to confer resistance to IFN-α in malignant T cells[293,294].

Also, STAT3 upregulates IL-2RA (IL-2 receptor α), which results in increased sensitivity to IL-2 signaling[288]. In healthy individuals IL-2 receptor α expression is restricted to T cells that recently encountered an antigen and the majority (>95%) of peripheral T cells are IL-2RA-negative. Constitutive STAT3-mediated upregulation of IL-2RA in malignant T cells[241,288] is already being exploited for therapeutic benefit, where Interleukin-2 diphtheria toxin fusion protein (denileukin diftitox) can target and deliver this toxin to IL-2 receptor expressing CTCL cells[227].

While in recent years our knowledge and understanding of STAT signaling in CTCL has dramatically increased, several questions remain to be answered. Specifically, with respect to STAT5, there are two separate STAT5 proteins, STAT5A and STAT5B that are encoded by two closely related genes. While these proteins have ~94% sequence identity, gene knockout mouse

models showed that STAT5A and STAT5B have certain distinct, non-overlapping and sometimes even opposing functions. As recently documented in immortalized ALK[295] T cell lymphoma cell lines, STAT5A can act as a tumor suppressor gene, while STAT5B acts as an oncogene[295]. The exact roles of STAT5A and STAT5B signaling in MF and SS remain to be elucidated.

Also, spatial localization of STAT proteins in the cell may prove to be important. It was documented that ~10% of STAT3, STAT1 and STAT5 proteins were found in lipid membrane rafts[296,297]. Hence, genes that regulate STAT protein localization to these rafts may play a critical role in regulating STAT signaling in the cell[298].

Finally, as discussed above, in early disease autocrine and/or paracrine signaling via IL-2, IL-7 and IL-15 cytokines can lead to phosphorylation of STAT3 and STAT5[290], while in advanced disease cytokine-independent JAK1 and JAK3 constitutive signaling was documented to drive carcinogenesis[241,289,290]. Potent JAK/STAT3 inhibitor, Cucurbitacin I decreases STAT3 phosphorylation, results in apoptosis of Sézary cells and inhibits tumor growth in mice[290]. Several JAK/STAT3 signaling inhibitors were recently developed (e.g. AG490, CP690550 and AZD9150). Some of these inhibitors, CP690550/Tofacitinib and AZD9150, are in clinical trials for rheumatoid arthritis and non-Hodgkin lymphoma, respectively[37,299,300]. Based on the importance of STAT3 and STAT5 signaling in this cancer it would be of interest to test their clinical efficacy in CTCL.

In conclusion, STAT signaling appears to play a central role in the pathogenesis of this cancer, where early in the disease STAT5 upregulation drives the expression of miR-155 oncogene, which targets STAT4 and contributes to a switch from the Th1 to Th2 phenotype, while at later stages activation of STAT3 leads to increased survival and resistance to apoptosis, expression of miR-21 oncogenic microRNA and upregulation of IL-5, IL-10, IL-17 and autocrine IL-21 signaling, all working in concert to promote carcinogenesis. STAT4 and STAT6 are inversely regulated in CTCL and loss of STAT4 is a poor prognostic marker for early MF and can be a powerful diagnostic marker for leukemic CTCL/SS. Treatment with HDAC inhibitors is able to restore the balance between STAT4 and STAT6 expression. Further research into the molecular pathogenesis of JAK/STAT signaling in this cancer may enable us to develop effective therapies for our patients.

7B. FIGURE

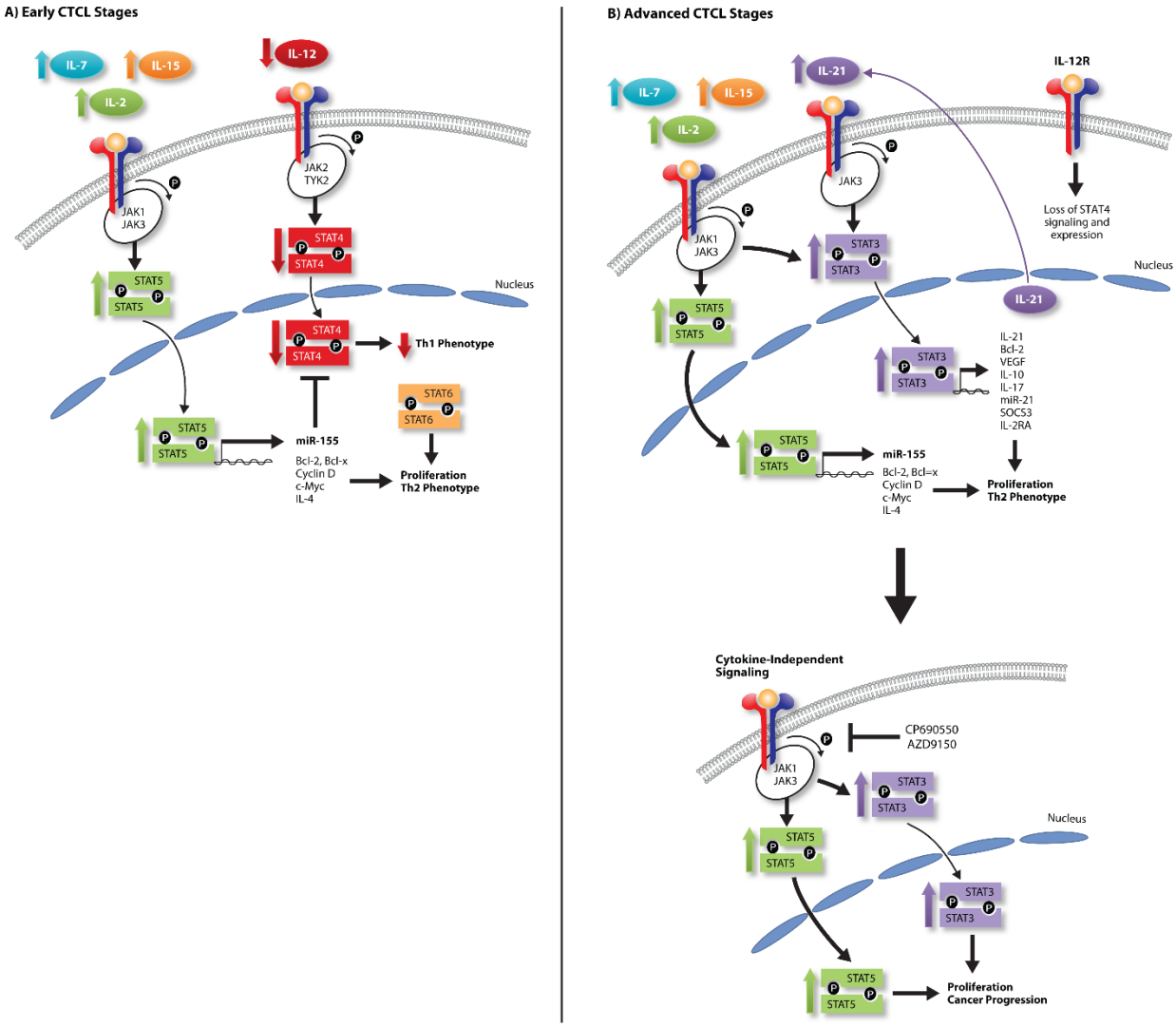


Figure 7.1. STAT signaling changes in CTCL. A. In early disease stages increased IL2RA expression together with augmented IL-2, IL-7 and IL-15 cytokine signaling leads to activation of STAT5, which upon phosphorylation, translocates into the nucleus and upregulates the expression of cell survival genes (Bcl-2 and BCL-x), cell cycle genes (Cyclin D and c-Myc) , Th2 cytokines (IL-4) and miR-155. One of the important functions of miR-155 is to downregulate STAT4 expression. This occurs concomitantly with decreased IL-12 cytokine levels in lesional skin and loss of IL-12 receptor β2 chain expression in malignant T cells all leading to the suppression of Th1 phenotype and switch towards the Th2 phenotype. **B.** In advanced disease, in addition to the upregulation in STAT5 signaling, establishment of IL-21 autocrine loop promotes STAT3

activation. Furthermore, in late disease stages STAT5 and STAT3 activation can become completely cytokine-independent being driven by constitutively active JAK1 and JAK3 kinases. CP690550 and AZD9150 inhibitors may be able to block such pathogenic JAK activation in CTCL.

Chapter 8. Conclusions

Throughout my graduate training I was able to study and publish original research on urticaria, a benign skin inflammatory condition, and CTCL, which exemplifies malignant inflammation of the skin. My research spanned the spectrum from molecular studies on gene expression profiling of ESC genes in CTCL to translational work of identifying potential diagnostic and prognostic markers for CTCL patients to clinical research where we established a cut-off for autoimmune CSU using the basophil activation test (BAT) in pediatric population and identified factors that are associated with faster remission rates of chronic urticaria.

Specifically, in my work I was able to test the utility of the BAT to diagnose children with autoimmune CSU based on the newly established 1.8% cut-off. We further documented in our omalizumab case series report that high BAT values indicate that a given patient may require higher doses and more frequent treatments to control his or her symptoms. We also for the first time established and followed a North American cohort of 139 children affected by CSU for 2 years. Our findings in this study demonstrated that this cohort of patients had a higher rate of associated autoimmune conditions including Hashimoto thyroiditis on personal and/or family history. This cohort also had a significantly higher family history of CSU in the parents of the affected children. Interestingly, while high BAT may predict greater intensity of symptoms based on our prior studies, the analysis of data from this cohort suggests that overall autoimmune CSU may resolve faster than other chronic urticaria. It was hypothesized that this could be due to pathogenic antibodies that are possibly triggered by a virus, the most common cause of urticaria in children, are transient in nature. As antibody levels fall the disease remits and symptoms subside. This hypothesis will need to be validated by future molecular and clinical studies.

Notably, the study of our cohort further indicates that overall the urticaria cases seen in our center are generally mild with the average UAS7 score of only 8.4 and most patients respond well to current treatments and are able to control their symptoms with first, second or, in rare cases, third line therapies.

While this is only the first presented analysis of this cohort of urticaria patients, we will continue to follow these patients for the next ~20 years to clarify whether they develop additional autoimmune diseases and whether their CSU symptoms recur.

In our research on CTCL we investigated the expression of Embryonic Stem Cell (ESC) genes in CTCL. Many of these genes are involved in the maintenance of pluripotency in embryonic stem cells. Their aberrant re-expression in this cancer may be promoting a cancer stem cell-like phenotype in this malignancy. In this analysis we for the first time tested the expression by RT-PCR for a panel of ESC genes in CTCL lesional skin biopsies and in immortalized cell lines. Our results highlight that select ESC genes are preferentially expressed in CTCL, but not in benign inflammatory dermatoses such as psoriasis, chronic eczema or pityriasis rubra pilaris that often mimic this malignancy. Finally, based on a number of recent reports, malignant T cells in CTCL are able to express few B cell-specific genes, cancer testis genes and thymocyte development genes (e.g., TOX). A growing body of literature documents that ectopic expression of these genes is not a mere indication of deregulated cellular processes, but an important mechanism of lymphomagenesis and cancer progression.

Importantly, as discussed above, Mycosis Fungoides (MF) and Sézary Syndrome (SS) often can masquerade as benign inflammatory dermatoses and can be difficult to diagnose. On average it takes 6 years to establish a diagnosis of CTCL. Histopathology and PCR studies for T

cell receptor clonality are sometimes not diagnostic. Most patients (~70%) present with stage I disease and about 15-20% of them progress to higher stages and succumb to their disease. Currently, it is not possible to predict which individuals are likely to progress. Our work based on 11 years of clinical follow up and large-scale RT-PCR gene expression analysis in MF/SS patient samples describes a 17-gene signature expression pattern that may help distinguish this cancer from its benign mimickers and identify patients at risk of disease progression. Such stratification may enable clinicians to detect and treat high-risk CTCL at an early stage, and spare the patients with low risk/indolent disease from exposure to toxic and expensive medications.

Also, from this analysis an important theme emerged showing that CTCL expresses higher levels of various inflammatory markers when compared to benign dermatoses such as eczema, psoriasis and pityriasis rubra pilaris. This correlates clinically with the observation that while it is often relatively easy to achieve control of benign inflammation with topical steroids, phototherapy and/or other treatment modalities, the same treatments often fail in controlling malignant inflammation in CTCL.

In addition, throughout my graduate studies I was able to publish 7 additional peer-reviewed publications that could not be discussed in the thesis due to space limitations. Copies of published articles are presented in appendix 6.

In conclusion, continued research on basic molecular, translational and patient-centered clinical platforms is likely to impact how we manage complex diseases such as urticaria or CTCL in dermatology in the near future.

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