



(51) International Patent Classification:

C12Q 1/6886 (2018.01) C12Q 1/68 (2018.01)
C07K 14/47 (2006.01) G01N 33/50 (2006.01)
C12N 15/11 (2006.01) G01N 33/574 (2006.01)
C12N 15/12 (2006.01) G01N 33/68 (2006.01)

SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(21) International Application Number:

PCT/US2023/035230

(22) International Filing Date:

16 October 2023 (16.10.2023)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/478,900 06 January 2023 (06.01.2023) US

(71) Applicants: UNIVERSITY OF MASSACHUSETTS

[US/US]; One Beacon Street, 31st Floor, Boston, Massachusetts 02108 (US). TRUSTEES OF TUFTS COLLEGE [US/US]; Ballou Hall, Medford, Massachusetts 02155 (US).

(72) Inventors: RICHMOND, Jillian M.; 131 Hartford Pike,

Dayville, Connecticut 06241 (US). OLAYINKA, Jadesola; 515 W 59th St., New York, New York 10019 (US). ALMELA, Ramon M.; 33 Latura Street, Shrewsbury, Mississippi 01545 (US).

(74) Agent: DEYOUNG, Janice Kulger; P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

(81) Designated States (unless otherwise indicated, for every

kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every

kind of regional protection available): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE,

(54) Title: DIAGNOSIS OF SKIN CONDITIONS IN VETERINARY AND HUMAN PATIENTS

(57) Abstract: Described herein are methods for diagnosing and treating mycosis fungoides (MF) and cutaneous T cell lymphoma (CTCL), e.g., for making a differential diagnosis of CTCL/MF versus other skin conditions.

WO 2024/147809 A1

DIAGNOSIS OF SKIN CONDITIONS IN VETERINARY AND HUMAN PATIENTS

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Patent Application Serial No. 63/478,900, filed on January 6, 2023. The entire contents of the foregoing are hereby incorporated by reference.

5

TECHNICAL FIELD

Described herein are methods for diagnosing and treating mycosis fungoides (MF) and cutaneous T cell lymphoma (CTCL), e.g., for making a differential diagnosis of CTCL/MF versus other skin conditions.¹

BACKGROUND

10

Cutaneous T cell lymphomas (CTCL) are a heterogenous group of non-Hodgkin's lymphomas characterized by the proliferation of neoplastic T-lymphocytes in the skin.

SUMMARY

15

Mycosis Fungoides (MF) is the most common subtype of Cutaneous T Cell Lymphoma (CTCL), a cancer of T cells that migrate to the skin. Clinically, the lesions caused by MF can look very different and mimic other skin conditions such as vitiligo, atopic dermatitis and others, particularly in skin of color patients. Early and accurate diagnosis is vital to improve prognosis. Described herein are methods for diagnosis of CTCL/MF, e.g., for making a differential diagnosis of CTCL/MF versus other skin conditions.

20

Thus, provided herein are methods comprising: obtaining a sample comprising skin from a subject; and determining a level of expression of one, two, three, or all four of cathepsin W (CTSW), T Cell Receptor Associated Transmembrane Adaptor 1 (TRAT1), killer cell lectin like receptor K1 (KLRK1; also known as NKG2D), and/or XCL1/XCL2 (lymphotactin) in the sample.

25

Also provided herein are methods of diagnosing a subject as having cutaneous T cell lymphoma. The methods include obtaining a sample comprising skin from a

subject; determining a level of expression of one, two, three, or all four of cathepsin W (CTSW), T Cell Receptor Associated Transmembrane Adaptor 1 (TRAT1), and killer cell lectin like receptor K1 (KLRK1), and/or XCL1/XCL2 (lymphotactin) in the sample; comparing the level of expression of CTSW, TRAT1, KLRK1 and/or
5 XCL1/XCL2 in the sample to a reference level, and identifying the subject as having CTCL wherein a level of expression of CTSW, TRAT1, KLRK1 and/or XCL1/XCL2 at or above the reference level indicates that the subject.

In some embodiments, the sample comprises skin from a lesion, e.g., a nodule, tumor, plaque, ulcerated, or rashy patch of skin that is suspected of being CTCL. In
10 some embodiments, the subject is a mammal. In some embodiments, the subject is a human, e.g., a human subject having skin of color. In some embodiments, the subject has Fitzpatrick phototype IV, V, or VI skin.

In some embodiments, the subject is a canine, feline, or equine.

In some embodiments, the methods further comprise selecting and optionally
15 administering a treatment for CTCL to a subject identified as having CTCL. In some embodiments, the treatment comprises a skin-directed therapy, optionally topical steroids, involved-site radiation therapy (RT), total skin electron-beam therapy (TSEBT), or phototherapy/photopheresis, or systemic therapies, optionally immunotherapies (retinoids, interferon, chimeric antigen receptor T cells, or
20 forodesine), small molecules (histone deacetylase inhibitors), antibodies (optionally anti-CCR4, anti-programmed cell death protein 1, anti-CD47, or anti-CD30), chemotherapy, or allogeneic haematopoietic stem cell transplantation.

Also provided herein are kits comprising reagents for detecting the biomarkers described herein, e.g., kits comprising probes or primers that bind to the biomarkers
25 described herein, and one or more control sequences, e.g., a housekeeping gene. In some embodiments the kits comprise microarrays. In some embodiments, the kits comprise fewer than 10 or 20 probes or primers.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to
30 which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their

entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

5

DESCRIPTION OF DRAWINGS

FIG. 1. Clinical presentations of epitheliotropic lymphoma in dogs. Top row left to right: widely disseminated lesions with hair loss associated with severe scale-crusting in an English bulldog and a retriever mix. Mild crusting with depigmentation, loss of nose cobblestone appearance and erythema on the muzzle of a beagle mix.

10 Middle row left to right: alopecia, erythema and crusting on the nasal planum and hind legs of a schnauzer mix; depigmentation, loss of nose cobblestone appearance and erythema on the muzzle of a beagle mix with footpad involvement with crusting and ulcerations. Note the depigmentation on the nasal planum and muzzle, which can be mistaken for other immune-mediated processes. Bottom row left to right:

15 multifocal lesions with erythema and crusting and without notable hair loss in a pitbull mix. Erythematous alopecia patch and hyperkeratotic plaque lesions on the dorsum of a shepherd mix. Please note that breed is not associated with a particular presentation of the disease.

FIGs. 2A-D. Gene expression in epitheliotropic lymphoma (EL) versus healthy margin controls reveals CD8+ cytotoxic T cell signatures. A. Sample H&E photomicrographs of healthy and EL tissue. B. Volcano plot of differentially expressed genes (DEGs) between healthy and EL. C. Heatmap of DEGs generated with Rosalind software. D. Principal component analysis (PCA) of cases versus healthy margins generated with ClusVis software. (n=6 EL and 5 healthy controls, 20 Padj <0.05 considered significant).

25

FIGs. 3A-D. Gene expression in EL versus other interface dermatitis conditions reveals CTSW and TRAT1 can distinguish between malignancy and other immune mediated processes. A. Volcano plot of EL versus other interface dermatitis conditions including cutaneous lupus erythematosus, pemphigus, and erythema multiforme spectrum disorders. B. Heatmap generated in Rosalind software. CTSW gene highlighted by a rectangle. C. Raw counts of the top DEGs between EL and ID (one-way ANOVA with Tukey's post tests significant as indicated). D. Receiver 30

operator characteristic (ROC) curves of CTSW, TRAT1 and KLRK1. (n=6 EL and 31 other interface dermatitis conditions; Padj <0.05 considered significant.)

FIGs. 4A-E. Validation cohort confirms CTSW and TRAT1 as potential biomarkers for distinguishing EL and ID. A. Volcano plot of EL versus other interface dermatitis conditions. B. Heatmap and Gene Set Analysis generated in Rosalind software. C. Raw counts of the top DEGs between EL and ID (one-way ANOVA with Tukey's post tests significant as indicated). D. Receiver operator characteristic (ROC) curves of CTSW, TRAT1, and XCL1/XCL2. E. Multi-ROC curve combining CTSW, TRAT1 and KLRK1 normalized counts for distinguishing EL from ID in the discovery and validation cohorts (discovery cohort: n=6 EL and 31 other interface dermatitis conditions; validation cohort: n=6 EL and 9 other interface dermatitis conditions; Padj <0.05 considered significant.)

FIGs. 5A-D. Comparative transcriptomics of human MF and canine EL versus ID DEGs. A. Geo2R reanalysis of GSE143382 human MF dataset. DEGs from dermatitis vs early MF were used as they represent the closest clinical match. B. DEG overlap between the canine validation cohort (canine IO panel) and human dataset (myeloid v2 panel) using the 327 common denominator genes from the respective NanoString probesets. C. Comparison of previously published up- and downregulated genes in canine and human datasets. D. Analysis of TOX vs TRAF1, which was used to distinguish human MF from ID, or CTSW vs TRAT1 in canine samples.

FIG. 6. Immunohistochemical analysis of CTSW using a human/murine reactive antibody.

FIGs. 7A-B. Immunohistochemical analysis of CD244 using a human/murine reactive antibody. A. EL. B. Discoid lupus erythematosus (DLE).

FIGs. 8A-B. qPCR of CTSW does not work reliably on FFPE RNA. qPCR was performed on RNA from FFPE skin (A) or frozen lymph node aspirates (B). CTSW set 2 may be used to detect transcript in frozen but not FFPE RNA samples.

FIG. 9. Truncated microarray diagnostic tool works reliably on FFPE RNA. A truncated microarray diagnostic tool was developed using four biomarkers of interest (*CTSW*, *TRAT1*, *KLRK1*, and *Loc490356/lymphotactin/XCL1/XCL2*) and five housekeeping gene controls (*B2M*, *RPL13A*, *CCZ1*, *HPRT1*, and *GAPDH*). This tool was able to sensitively and specifically detect EL in six dogs apart from six ID cases (RNA was pulled from both discovery and validation cohort samples).

DETAILED DESCRIPTION

The World Health Organization-European Organization for Research and Treatment of Cancer (WHO-EORTC) created a classification consensus recognizing 19 distinct subtypes of CTCL.¹ The most common subtype is mycosis fungoides (MF), which is known for its progression of three stages: patches, plaques, and tumors²—of which their mushroom-like appearance inspires the name. Patients can present with all three stages simultaneously, or may experience one stage for years. Other important subtypes of CTCL include Sézary syndrome, which is the leukemic variant of CTCL characterized by malignant Sézary cells in the plasma, and primary cutaneous CD30+ lymphoproliferative disorders, which include lymphomatoid papulosis and CD30+ primary cutaneous anaplastic large cell lymphoma.⁴⁰ Along with MF, these subtypes have indolent clinical behavior, while Sézary syndrome has aggressive clinical behavior.¹

In humans, CTCLs often present with lesions such as red patches, plaques, nodules, ulcerations, or other skin rashes, and can evolve into cutaneous tumors and/or progress to visceral involvement. In the United States, the overall annual age-adjusted incidence of CTCL was 6.4 per million persons over the time period of 1973 to 2002, with an annual incidence increase of 2.9×10^{-6} .³ Incidence is greater in males than in females, and in Black people than in White people.

MF has a chronic, relapsing course, and patients often try several therapies to control the course of their disease⁴³. The prognosis of MF depends highly upon the stage at which a patient is diagnosed: for stage IA in patients who respond well to therapy, their overall survival rate is similar to age matched controls. However, for stage IIB to stage III, patients have a median survival rate of 3.2-6 years⁴⁴. Stage IV, in which patients develop extracutaneous symptoms, patients have a survival rate of less than 1.5 years. Historically, CTCL has been considered an “old white man’s disease,” primarily affecting Caucasian men over age 60⁴⁵. However, CTCL affects African Americans (AA) and other skin of color (SOC) patients, with up to 70% female bias and a younger age at presentation for the MF subtype (mean age 44 years)⁴⁶⁻⁴⁹. MF disproportionately affects SOC patients: patients are often diagnosed at a later stage, making treatment more difficult and less effective at controlling disease⁵⁰⁻⁵³. Thus, SOC patients have worse prognoses than white patients. Further, SOC patients are less likely to be referred to extracorporeal photopheresis, a standard treatment for CTCL⁵⁴. A better understanding of MF pathogenesis in SOC patients is

needed both for early identification and effective treatment, as many treatments to date have been tested and developed in predominantly white patient populations⁵⁵.

In addition to humans, other mammals including dogs, cats, and horses can also develop cutaneous lymphomas, including T and B cells lymphomas.^{56,57,64-66} In canine cutaneous epitheliotropic lymphoma (EL), neoplastic lymphocytes also infiltrate the skin and mucosa.⁴ Canine epitheliotropic T cell lymphoma (T-EL) has different subtypes when described with the same standards as humans, including MF, Sézary syndrome, and pagetoid reticulosis⁵. While CD4+ helper T-cells predominantly drive disease in human CTCL, canine EL is predominantly a disease of CD8+ cytotoxic T-cells.⁵ The disease progression in both human CTCL and canine EL can be very similar. Human CTCL usually involves progression from patch stage to plaque stage to tumor stage, whereas in canine patients with MF, the variety of lesions can present at any time during disease development and each lesion type does not necessarily represent a progressive disease stage. Clinical presentations of both diseases involve lesions such as exfoliative erythroderma, ulceration, depigmentation, plaques, and nodules, and both diseases are difficult to diagnose in earlier stages due to similar clinical presentation to inflammatory or benign processes⁶⁶. While CD4+ helper T-cells predominantly drive disease in human CTCL, canine T-EL is predominantly a disease of CD8+ cytotoxic T-cells⁵.

MF in SOC can mimic other conditions, potentially leading to misdiagnosis of patients and hence delayed treatment start^{49,58}. MF presentation varies widely⁴⁹: it can present as flat or raised lesions, plaques with an annular morphology, scale, or violaceous plaques, psoriasiform lesions, hyper- and/or hypo-pigmented lesions, ulcerations, or nodules. In SOC patients, depigmented MF is one of the most common presentations, which may be mistaken for vitiligo, fungal infection, or other dermatitis conditions.

Forms of CTCL that express CD8+ phenotype in humans have been reported³¹: in some rare cases, well-defined types of CTCL (such as MF) express CD8+ and have similar clinical presentation and disease prognosis as the more common CD4+ cases. Outside of these, studies suggest separate groups of more aggressive CD8+ cytotoxic CTCLs, including an epidermotropic type and a panniculitis-like subcutaneous T cell lymphoma type.

One challenge in the field is distinguishing early-stage MF in humans, particularly human subjects with skin of color, and dogs from other immune-mediated

interface dermatitis conditions. The present results expand understanding of these disorders and provide biomarkers for diagnosis disease, improving prognosis and treatment response, and allows the development of targeted therapies, e.g., repurposing known drugs, for veterinary and human medicine. As shown herein, *CTSW*, *KLRK1* and *TRAT1* may be used to distinguish EL from other forms of interface dermatitis (ID) in humans and veterinary subjects in both a discovery and validation cohort. *XCL1/XCL2* was also highly significant at distinguishing EL from other forms of ID in the validation cohort. These genes may be particularly important to prevent misdiagnosis in both veterinary and human patients, in order to distinguish CTCL from clinical mimickers.

CTSW is a member of the cathepsin S family that is a papain-like protease. Cathepsins have been reported to regulate cancer progression and therapeutic responses⁸. Cathepsin S is upregulated in follicular lymphoma, and an activating mutation Y132D drives lymphomagenesis through alterations in antigen presentation⁹ and a pro-tumorigenic microenvironment¹⁰. Cathepsin W is known to be expressed in human CD8+ T cells¹¹ and Natural Killer (NK) cells.^{12,13} Similarly, family member Cathepsin G is 2-fold upregulated in early disease stages (IA/IB).¹⁴ In breast cancer, increased expression of Cathepsin B and Cathepsin L is associated with poorer prognosis, greater mortality, and greater disease metastases.⁸ In colorectal cancer, elevated Cathepsin B and Cathepsin L are associated with increased disease metastasis and poorer prognosis¹⁵, while elevated Cathepsin S predicts both decreased survival when treated with surgery alone, with potential benefit from adjuvant 5-fluorouracil and folinic acid treatment. High Cathepsin B expression in lung, ovarian, pancreatic neuroendocrine cancers and pancreatic adenocarcinomas, is negatively correlated with survival and positively associated with recurrence, invasion, and/or tumor grade. Upregulation of Cathepsin K in osteosarcomas predicts poor prognosis and disease metastasis. Generally, it seems that the tumor microenvironment helps activate cathepsins, which in turn activate oncogenesis: mutant HRAS in mammary epithelial cells upregulates *CTSB* and *CTSL*: the *HER2* oncogene drives expression of *CTSB* through the transcription factor myeloid zinc finger 1 (MZF1), and *CTSB* is a functional driver of the invasive phenotype.

TRAT1 is a 30 Kd type III transmembrane protein expressed by human T lymphocytes and natural killer (NK) cells. *TRAT1* regulates T-cell receptor expression.¹⁶ It consists of an extracellular domain, transmembrane region, and

cytosolic tail. TRAT1 facilitates CTLA-4 shuttling from the trans Golgi network to the T-cell surface where it stabilizes the T-cell antigen receptor and CD3 complex. TRAT1 knockdown experiments demonstrated reduced CTLA-4 mediated cytokine release as well as CTLA-4 cell surface expression and subcellular distribution. When
 5 TRAT1 is overexpressed in Jurkat T cells, there is an increase in T-cell receptor expression, however this is not the case in regular T cells.¹⁷ By contributing to the structural integrity of the TCR/CD3 complex, TRAT1 has a significant role in TCR functions, which include triggering antigen-specific T-cell responses.¹⁸ In non-blood cancers (solid tumors), *TRAT1* expression is important for a good prognosis. However
 10 in T cell lymphomas, *TRAT1* can be highly expressed in tumor cells. It remains unknown how this relates to prognosis.

KLRK1 is also known as *NKG2D*. It is a killer type lectin receptor expressed on NK cells and cytotoxic T cells.¹⁹ *KLRK1* is upregulated in human peripheral T cell lymphomas.²⁰ A recent study demonstrated that benign T cells drive inflammation in
 15 MF tumors in humans²¹: an influx of CD8+ T cells following immunotherapy in CD4-driven tumors was protective. Therefore, caution should be exercised in fully characterizing the tumor (e.g. CD4 or CD8; does the tumor itself bear *KLRK1*, or is it expressed by the infiltrating T cells²²) to ascertain whether *KLRK1* both as a biomarker and a potential treatment target is beneficial or detrimental.²¹

LOC490356 was a top DEG in our validation cohort. This locus encodes *lymphotactin*, also known as *XCL1/XCL2*. This chemokine is homeostatically expressed by NK cells and has antimicrobial activity.²³ *XCL1* is downregulated in human Sezary Syndrome,^{24,25} but was expressed by T-3B cells in a case report of a patient with concurrent T and B cell cutaneous lymphomas²⁶ and by T cells in a
 25 patient with lymphoproliferative disorder.²⁷ As with *KLRK1*, it is unclear whether *XCL1* could be protective or pathogenic in the EL setting. In murine tumor models, injection of XCL1-expressing myeloma cells in Balb/c and nude mice resulted in tumor regression.²⁸ An XCL1 fusion peptide improved tumor rejection in a mouse B16 model of melanoma via recruitment of XCR1+ dendritic cells to the tumor.²⁹
 30 This also raises the point that some of the associated biomarkers identified for EL and other forms of CTCL may be expressed by infiltrating immune cells and not the tumor itself. Nevertheless, these seem to serve as sensitive and specific biomarkers for identifying early stage EL apart from other forms of interface dermatitis.

Other biologically relevant genes identified in our datasets correspond to both tumor and immune cell function. *CD6*, *CD86*, and *CD8A* were all upregulated, and are implicated in T-cell activation and regulation. *MS4A1*, a gene that encodes the B-cell marker CD20, was upregulated in EL. *PTPRC* was also upregulated, and is essential for T- and B-cell antigen receptor signaling. Immunoregulatory cytokines and their receptors were upregulated including *IL6* and *IL21R*. *S100A12* regulates inflammatory and immune responses, and was upregulated in EL. Tumor necrosis factor alpha (TNF-alpha) has been implicated in the pathogenesis of CTCL.³⁰ *TNF*, *TNFRSF17*, *CD27*, and *FASLG* were all significantly upregulated in canines with EL. *CD27*, a TNF family member required for the maintenance of T-cell immunity, was significantly upregulated. *FoxP3* has been reported to be variably expressed depending on the stage of disease in CTCL.³¹ *FoxP3* was not significantly differentiated in the EL canines compared to the healthy controls; however two canines, Sample 3 and Sample 4, had relatively greater counts than all other cases. They represent cases with significant dermal neoplastic involvement and patch/plaque stage, respectively. Last, given that *CCL22* was upregulated, *CCR4* depleting antibodies like mogamulizumab may be helpful for treating canine EL.

We also observed downregulated genes that may be biologically significant in EL. *VGLL3* has been recognized as a tumor suppressor gene in serous ovarian carcinomas^{32,33} and stomach adenocarcinoma.³⁴ *VGLL3*, which regulates the Hippo pathway,³⁵ was downregulated in EL skin. *IL16* was also downregulated, reflecting the pattern observed in severe Sézary Syndrome.³⁶ Reintroduction of pro-IL16 in MOLT4 tumors can induce regression in nude mice.³⁷ Therefore, adding back tumor suppressor genes may serve as a therapeutic option for EL and should be considered in future veterinary clinical trials.

Forms of CTCL that express CD8+ phenotype in humans have been reported³¹; in some rare cases, well-defined types of CTCL (such as MF) express CD8+ and have similar clinical presentation and disease prognosis as the more common CD4+ cases.³⁸ Outside of these, studies suggest separate groups of more aggressive CD8+ cytotoxic CTCLs,³⁹ including an epidermotropic type and a panniculitis-like subcutaneous T cell lymphoma type. Therefore, it is possible that, genetically and/or transcriptionally, canine EL matches human CD8+ lymphomas more closely than MF as a general subtype. Future comparative oncology studies

should further characterize these rare CD8+ tumors to better understand which condition is most closely modeled in dogs.

Subjects

The present methods can be used to diagnose and treat CTCL (such as MF) in mammals, e.g., in veterinary subjects such as dogs, cats, or horses, or in humans, e.g., humans of color, e.g., humans who have skin of Fitzpatrick phototype IV, V, or VI. Human subjects will generally have a lesion, e.g., a skin rash that includes patches of exfoliative erythroderma, ulceration, plaques, and nodules; veterinary subjects can have depigmentation, crusting, erythema, ulceration and/or alopecia. Disease progression usually moves from patch stage to plaque stage to tumor stage.

Methods of Diagnosis or Monitoring

The present methods rely on detection of two, three, four, or more genes selected from cathepsin W (CTSW), KLRK1 (NKG2D), TRAT1, and XCL1/XCL2 as biological makers of CTCL. The methods can include obtaining a sample from a subject, and evaluating the presence and/or level of two, three, four, or more of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 in the sample, and comparing the presence and/or level with one or more references, e.g., a control reference that represents a normal level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2, e.g., a level in an unaffected subject, and/or a disease reference that represents a level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 associated with CTCL, e.g., a level in a subject having CTCL.

As used herein the term “sample”, when referring to the material to be tested for the presence of a biological marker using the method of the invention, includes inter alia tissue, e.g., a skin biopsy sample from a lesion suspected of being CTCL. Various methods are well known within the art for the identification and/or isolation and/or purification of a biological marker from a sample. An “isolated” or “purified” biological marker is substantially free of cellular material or other contaminants from the cell or tissue source from which the biological marker is derived i.e. partially or completely altered or removed from the natural state through human intervention. For example, nucleic acids contained in the sample can be isolated according to standard methods, for example using lytic enzymes, chemical solutions, or isolated by nucleic acid-binding resins following the manufacturer’s instructions.

The present methods can include determining levels of either protein or mRNA encoding one, two, three, four, or more CTSW, KLRK1, TRAT1 and/or XCL1/XCL2. In some embodiments, the methods can also include determining levels of protein or mRNA encoding the aforementioned genes. Exemplary sequences for human, canine, feline, and Equine CTSW and TRAT1 are available in NCBI GenBank, e.g., as shown in Table A.

Table A. Exemplary sequences for CTSW, TRAT1, and KLRK1 (NKG2D)

mRNA	protein	gene name	species
NM_001335.4	NP_001326.3	cathepsin W preproprotein	<i>Homo Sapiens</i>
NM_016388.4	NP_057472.2	T-cell receptor-associated transmembrane adapter 1 isoform 1	<i>Homo Sapiens</i>
NM_001317747.2	NP_001304676.1	T-cell receptor-associated transmembrane adapter 1 isoform 2	<i>Homo Sapiens</i>
NM_007360.4	NP_031386.2	NKG2-D type II integral membrane protein	<i>Homo Sapiens</i>
NM_002995.3	NP_002986.1	X-C motif chemokine ligand 1/lymphotactin precursor	<i>Homo Sapiens</i>
NM_003175.4	NP_003166.1	X-C motif chemokine ligand 2/cytokine SCM-1 beta precursor	<i>Homo Sapiens</i>
XM_038424941.1	XP_038280869.1	cathepsin W	<i>Canis lupus familiaris</i>
XM_038444882.1	XP_038300810.1	T-cell receptor-associated transmembrane adapter 1	<i>Canis lupus familiaris</i>
XM_038439018.1	XP_038294946.1	NKG2-D type II integral membrane protein isoform X1	<i>Canis lupus familiaris</i>
XM_038439017.1	XP_038294945.1	NKG2-D type II integral membrane protein isoform X1	<i>Canis lupus familiaris</i>
XM_038439019.1	XP_038294947.1	NKG2-D type II integral membrane protein isoform X2	<i>Canis lupus familiaris</i>

mRNA	protein	gene name	species
XM_038671439.1	XP_038527367.1	lymphotactin isoform X1	<i>Canis lupus familiaris</i>
XM_038671435.1	XP_038527363.1	lymphotactin isoform X2	<i>Canis lupus familiaris</i>
XM_038671436.1	XP_038527364.1	lymphotactin isoform X3	<i>Canis lupus familiaris</i>
XM_038671437.1	XP_038527365.1	lymphotactin isoform X4	<i>Canis lupus familiaris</i>
XM_038671438.1	XP_038527366.1	lymphotactin isoform X5	<i>Canis lupus familiaris</i>
NM_001122871.1	NP_001116343.1	cathepsin W precursor	<i>Felis catus</i>
XM_006936023.5	XP_006936085.1	T-cell receptor-associated transmembrane adapter 1 isoform X1	<i>Felis catus</i>
XM_045036901.1	XP_044892836.1	T-cell receptor-associated transmembrane adapter 1 isoform X2	<i>Felis catus</i>
XM_003988411.4	XP_003988460.3	NKG2-D type II integral membrane protein isoform X1	<i>Felis catus</i>
XM_045061359.1	XP_044917294.1	NKG2-D type II integral membrane protein isoform X2	<i>Felis catus</i>
XM_001494633.4	XP_001494683.1	cathepsin W	<i>Equus caballus</i>
XM_005602016.3	XP_005602073.1	T-cell receptor-associated transmembrane adapter 1	<i>Equus caballus</i>
XM_014740713.2	XP_014596199.2	NKG2-D type II integral membrane protein	<i>Equus caballus</i>
XM_005610937.3	XP_005610994.2	NKG2-D type II integral membrane protein	<i>Equus caballus</i>
XM_001490960.4	XP_001491010.1	lymphotactin	<i>Equus caballus</i>

Where the sequence given above is a precursor, proprotein, or preproprotein, the methods can include detecting the mature sequence (e.g., amino acids 128-376 of NP_001326.3, which represents the mature human CTSW protein).

Preferably the presence and/or level of CTSW, KLRK1, TRAT1 and/or
5 XCL1/XCL2 nucleic acids are evaluated, e.g., using methods known in the art, e.g.,

using polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), quantitative or semi-quantitative real-time RT-PCR, digital PCR i.e. BEAMing ((Beads, Emulsion, Amplification, Magnetics) Diehl (2006) Nat Methods 3:551-559); RNase protection assay; Northern blot; various types of nucleic acid sequencing (Sanger, pyrosequencing, NextGeneration Sequencing); fluorescent in-situ hybridization (FISH); or gene array/chips) (Lehninger Biochemistry (Worth Publishers, Inc., current addition; Sambrook et al., *Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001); Bernard (2002) Clin Chem 48(8): 1178-1185; Miranda (2010) Kidney International 78:191-199; Bianchi (2011) EMBO Mol Med 3:495-503; Taylor (2013) Front. Genet. 4:142; Yang (2014) PLOS One 9(11):e110641; Nordstrom (2000) Biotechnol. Appl. Biochem. 31(2):107-112; Ahmadian (2000) Anal Biochem 280:103-110. In some embodiments, high throughput methods, e.g., protein or gene chips as are known in the art (see, e.g., Ch. 12, Genomics, in Griffiths et al., Eds. Modern genetic Analysis, 1999, W. H. Freeman and Company; Ekins and Chu, Trends in Biotechnology, 1999, 17:217-218; MacBeath and Schreiber, Science 2000, 289(5485):1760-1763; Simpson, *Proteins and Proteomics: A Laboratory Manual*, Cold Spring Harbor Laboratory Press; 2002; Hardiman, *Microarrays Methods and Applications: Nuts & Bolts*, DNA Press, 2003), can be used to detect the presence and/or level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2. Measurement of the level of a biomarker can be direct or indirect. For example, the abundance levels of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 can be directly quantitated. Alternatively, the amount of a biomarker can be determined indirectly by measuring abundance levels of cDNA, amplified RNAs or DNAs, or by measuring quantities or activities of RNAs, or other molecules that are indicative of the expression level of the biomarker. In some embodiments a technique suitable for the detection of alterations in the structure or sequence of nucleic acids, such as the presence of deletions, amplifications, or substitutions, can be used for the detection of biomarkers of this invention.

RT-PCR can be used to determine the expression profiles of biomarkers (U.S. Patent No. 2005/0048542A1). The first step in expression profiling by RT-PCR is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction (Ausubel et al (1997) Current Protocols of Molecular Biology, John Wiley and Sons). To minimize errors and the effects of sample-to-sample variation, RT-PCR is usually performed using an internal standard, which is

expressed at constant level among tissues, and is unaffected by the experimental treatment. Housekeeping genes, such as GAPDH, B2M, cg14980/CCZ1, HPRT, and RPL13A, are commonly used.

Gene arrays can be prepared by selecting probes that comprise polynucleotide sequences that bind to CTSW, KLRK1, TRAT1, and/or XCL1/XCL2 and then immobilizing such probes to a solid support or surface. For example, the probes may comprise DNA sequences, RNA sequences, co-polymer sequences of DNA and RNA, DNA and/or RNA analogues, or combinations thereof. The probe sequences can be synthesized either enzymatically *in vivo*, enzymatically *in vitro* (e.g. by PCR), or non-enzymatically *in vitro*.

Alternatively, the presence and/or level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 proteins can be evaluated using methods known in the art, e.g., using standard electrophoretic and quantitative immunoassay methods for proteins, including but not limited to, Western blot; enzyme linked immunosorbent assay (ELISA); biotin/avidin type assays; protein array detection; radio-immunoassay; immunohistochemistry (IHC); immune-precipitation assay; FACS (fluorescent activated cell sorting); mass spectrometry (Kim (2010) Am J Clin Pathol 134:157-162; Yasun (2012) Anal Chem 84(14):6008-6015; Brody (2010) Expert Rev Mol Diagn 10(8):1013-1022; Philips (2014) PLOS One 9(3):e90226; Pfaffe (2011) Clin Chem 57(5): 675-687). The methods typically include revealing labels such as fluorescent, chemiluminescent, radioactive, and enzymatic or dye molecules that provide a signal either directly or indirectly. As used herein, the term "label" refers to the coupling (i.e. physical linkage) of a detectable substance, such as a radioactive agent or fluorophore (e.g. phycoerythrin (PE) or indocyanine (Cy5), to an antibody or probe, as well as indirect labeling of the probe or antibody (e.g. horseradish peroxidase, HRP) by reactivity with a detectable substance.

In some embodiments, an ELISA method may be used, wherein the wells of a microtiter plate are coated with an antibody against which the protein is to be tested. The sample containing or suspected of containing the biological marker is then applied to the wells. After a sufficient amount of time, during which antibody-antigen complexes would have formed, the plate is washed to remove any unbound moieties, and a detectably labelled molecule is added. Again, after a sufficient period of incubation, the plate is washed to remove any excess, unbound molecules, and the presence of the labeled molecule is determined using methods known in the art.

Variations of the ELISA method, such as the competitive ELISA or competition assay, and sandwich ELISA, may also be used, as these are well-known to those skilled in the art.

In some embodiments, an IHC method may be used. IHC provides a method
5 of detecting a biological marker *in situ*. The presence and exact cellular location of the biological marker can be detected. Typically a sample is fixed with formalin or paraformaldehyde, embedded in paraffin, and cut into sections for staining and subsequent inspection by confocal microscopy. Current methods of IHC use either direct or indirect labelling. The sample may also be inspected by fluorescent
10 microscopy when immunofluorescence (IF) is performed, as a variation to IHC.

Mass spectrometry, and particularly matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS), is useful for the detection of biomarkers of this invention (See U.S. Patent No. 5,118,937; 5,045,694; 5,719,060; 6,225,047).

15 In some embodiments, the level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 is comparable to or above the level of the nucleic acids or protein(s) in the disease reference, then the subject can be diagnosed with CTCL.

Suitable reference values can be determined using methods known in the art, e.g., using standard clinical trial methodology and statistical analysis. The reference
20 values can have any relevant form. In some cases, the reference comprises a predetermined value for a meaningful level of CTSW, KLRK1, TRAT1, and/or XCL1/XCL2 e.g., a control reference level that represents a normal level of CTSW, KLRK1, TRAT1, and/or XCL1/XCL2 e.g., a level in an unaffected subject or a subject who is not at risk of developing a disease described herein, or in a normal
25 tissue (skin biopsy sample) of from the same subject, and/or a disease reference that represents a level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 associated with CTCL.

The predetermined level can be a single cut-off (threshold) value, such as a median or mean, or a level that defines the boundaries of an upper or lower quartile,
30 tertile, or other segment of a clinical trial population that is determined to be statistically different from the other segments. It can be a range of cut-off (or threshold) values, such as a confidence interval. It can be established based upon comparative groups, such as where association with risk of developing disease or presence of disease in one defined group is a fold higher, or lower, (e.g.,

approximately 2-fold, 4-fold, 8-fold, 16-fold or more) than the risk or presence of disease in another defined group. It can be a range, for example, where a population of subjects (e.g., control subjects) is divided equally (or unequally) into groups, such as a low-risk group, a medium-risk group and a high-risk group, or into quartiles, the
5 lowest quartile being subjects with the lowest risk and the highest quartile being subjects with the highest risk, or into n-quartiles (i.e., n regularly spaced intervals) the lowest of the n-quartiles being subjects with the lowest risk and the highest of the n-quartiles being subjects with the highest risk.

In some embodiments, the predetermined level is a level or occurrence in the
10 same subject, e.g., at a different time point, e.g., an earlier time point.

Subjects associated with predetermined values are typically referred to as reference subjects. For example, in some embodiments, a healthy control reference subject does not have a disorder described herein (e.g. CTCL).

A disease reference subject is one who has (or has an increased risk of
15 developing) CTCL. An increased risk is defined as a risk above the risk of subjects in the general population.

Thus, in some cases the level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 in a subject being greater than a healthy (disease-free) reference level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 is indicative of a clinical status (e.g., indicative
20 of the presence of CTCL). In other cases the level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 in a subject being less than or equal to the healthy reference level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 is indicative of the absence of disease or normal risk of the disease. In some embodiments, the amount by which the level in the subject is the less than the reference level is sufficient to distinguish a subject
25 from a control subject, and optionally is a statistically significantly less than the level in a control subject. In cases where the level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 in a subject being equal to the reference level of CTSW, KLRK1, TRAT1, and/or XCL1/XCL2 the “being equal” refers to being approximately equal (e.g., not statistically different).

30 The predetermined value can depend upon the particular population of subjects (e.g., human subjects) selected. Accordingly, the predetermined values selected may take into account the category (e.g., sex, age, health, risk, presence of other diseases) in which a subject (e.g., human subject) falls. Appropriate ranges and

categories can be selected with no more than routine experimentation by those of ordinary skill in the art.

In characterizing likelihood, or risk, numerous predetermined values can be established.

5 In some embodiments, the methods include monitoring a treatment for efficacy, e.g., by determining a first level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2; administering a treatment for CTCL, e.g., as described herein, and then determining a second level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2. If the second level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 is below the first level,
10 the treatment has been successful. In some embodiments, if the second level of level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 is less than or equal to a healthy control level, the healthcare provide can consider discontinuing the treatment. The methods can also include identifying the subject as having CTCL based on the first level of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2 being above a healthy control
15 reference level.

Kits

Also provided herein are kits for use in a method described herein. Such kits can includes reagents for detecting the biomarkers described herein, e.g., probes or primers that bind to two, three, or all four of CTSW, KLRK1, TRAT1 and/or
20 XCL1/XCL2, and optionally one or more housekeeping genes for use as controls, e.g., B2M, RPL13A, CCZ1, HPRT1, and/or GAPDH. In some embodiments, the kits don't include probes or primers that bind to any other biomarkers. In some embodiments, the kits comprise a microarray. In some embodiments, the probes or primers bind to at least 20 consecutive nucleotides of a sequence shown in Table 4, or
25 a human homolog thereof. In some embodiments, the probes or primers are detectably labeled (e.g., with a fluorophore or other detectable moiety) or comprise a barcode or other unique molecular identifier that allows the presence of the target sequence to be identified.

Methods of Treatment

30 Once a subject has been identified as having CTCL using a method described herein, a treatment can be selected and/or the subject can be treated accordingly. Therapy options can comprise skin-directed therapies, such as topical steroids,

involved-site radiation therapy (RT), total skin electron-beam therapy (TSEBT), or phototherapy/photopheresis, and systemic therapies, such as immunotherapies (e.g., retinoids, interferon, chimeric antigen receptor T cells), small molecules (histone deacetylase inhibitors), antibodies (e.g., anti-CCR4 (e.g., mogamulizumab), anti-programmed cell death protein 1, anti-CD47, or anti-CD30 (e.g., brentuximab vedotin)), or chemotherapy. Allogeneic haematopoietic stem cell transplantation can also be used, see, e.g., Dummer et al., Nat Rev Dis Primers. 2021 Aug 26;7(1):61; Kempf and Mitteldorf, Hematol Oncol. 2021 Jun;39 Suppl 1:46-51; Weiner et al., J Am Acad Dermatol. 2021 Mar;84(3):597-604; and Mehta-Shah, J Natl Compr Canc Netw. 2020 May;18(5):522-536.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Materials & Methods:

The following materials and methods were used in the Examples herein.

Clinical samples: Skin biopsies from the biorepository at Tufts Cummings School were selected based on pathology reports. H&E sections were reexamined by a board-certified veterinary pathologist to confirm diagnoses and absence of obvious infectious disease, and clinical notes were reexamined by a board-certified veterinary dermatologist. Healthy control samples were obtained from leg margin biopsies from amputations. For the discovery cohort, six epitheliotropic lymphoma samples were obtained from shave and/or punch biopsies of dogs as noted in the case presentation section. Samples were deposited with written owner consent in the Tufts biobank at the time the veterinary patients were seen at the hospital, spanning the years 2011-2019. For the validation cohort, eight EL samples were obtained from the biobank at Colorado State University, seven of which yielded enough RNA for downstream analyses.

Isolation of RNA from FFPE blocks: 30µm curls were cut from the blocks and stored in Eppendorf tubes at ambient temperature. RNA was isolated using the Qiagen FFPE RNeasy kit per the manufacturer directions. Briefly, razor blades were treated with RNase, excess paraffin was removed, and tissues were sliced into thin strips (5µm) to create more surface area prior to incubation with deparaffinization

solution (Qiagen). The manufacturer protocol was followed and RNA was quantified using a nanodrop.

Nanostring cartridge and processing: A custom Nanostring canine gene panel of 160 genes including cytokine, chemokine, and immune genes, as well as skin and immune cell specific transcripts was created. We used *B2M*, *RPL13A*, *CCZ1* and *HPRT* as housekeeping genes for this study. For the validation cohort, the NanoString canine Immune-Oncology (IO) panel was used. RNA was hybridized using a BioRad C1000 touch machine, and samples were loaded into Nanostring cartridges and analyzed with a Sprint machine. Gene expression data are deposited on GEO under Accession # GSE213087.

nSolver analysis: NanoString's software, nSolver was used for all NanoString analysis. Raw counts were plotted with GraphPad Prism. Advanced analysis was used for the "cell Type Score", which is a summary statistic of the expression of the marker genes for each cell type. It is the geometric mean of the log2-transformed normalized counts for each set of marker genes. These scores were validated against FACS and IHC, and are a robust method of quantifying relative cell type abundance.⁶

Immunohistochemistry (IHC): IHC was performed on 5µm sections using rabbit-anti-canine *CD244*, *CTSW* (US Biological) or isotype control (Biolegend) at 1:100 dilution using a Dako automated slide staining machine. All sections were counterstained with hematoxylin. H&E images were taken using an Olympus BX51 microscope with Nikon NIS Elements software version 3.10, and IHC images were taken using an Olympus BX40 microscope with cellSens Entry software version 1.14.

qPCR: qPCR for *CTSW* was performed on remaining RNA samples or frozen lymphoma samples using BioRad iScript cDNA synthesis kit and iTaq universal SYBR green supermix per the manufacturer's protocols. A 60 degree qPCR program was run on QuantStudio (Applied Biosystems) or BioRad CFX96 machine.

Comparison to human CTCL: The human CTCL dataset from Nielsen et al.⁷ (GSE143382) was analyzed using Geo2R. Gene lists were truncated in Microsoft Excel using the formula "`=IF(ISERROR(VLOOKUP(cell,reference,1,FALSE)), FALSE,TRUE)`" and shared DEGs between human and canine were analyzed with BioVenn.

Statistics: To assess the statistical significance of our results, we performed DEG analysis using Rosalind software and/or nSolver software. We also analyzed raw and/or normalized counts between groups using nSolver and GraphPad Prism

software version 9 to examine potential differences in previously identified genes pertinent to EL and CTCL pathogenesis. Normality tests were performed in GraphPad Prism. Normally distributed data were analyzed using student's t test and non-normally distributed data were analyzed using Mann-Whitney U test. Receiver
 5 Operator Characteristic (ROC) curves were calculated in GraphPad Prism. Multi-ROC curves were calculated by a biostatistician in SPSS. A statistically significant difference was considered as $p < 0.05$.

**Example 1. Cathepsin W, T-cell receptor-associated transmembrane adapter 1 (TRAT1) and killer cell lectin like receptor K1 (KLRK1) are sensitive and
 10 specific RNA biomarkers of canine epitheliotropic lymphoma**

RNA from FFPE skin curls were isolated from six canines with EL, and five healthy canine patients. Using the NanoString nCounter software, we performed comparative transcriptomics studies on 160 genes from lesional skin biopsies from 6 canine EL cases and 5 healthy canine cases. Our NanoString probeset targets included
 15 cytokine, chemokine, and immune related genes, as well as skin associated genes and neuroendocrine genes. These 160 genes were examined based on their known cellular functions. We then compared our EL findings to a previously published CTCL dataset (Human Dataset GSE143382). Principal component analysis (PCA) revealed methods to predict disease status.

Example 1.1. Canine gene expression analysis reveals significant increases and decreases in key immune and skin genes in EL versus healthy controls

Canine EL can present with different features including depigmentation, crusting, erythema, ulceration and/or alopecia (**FIG. 1**). We analyzed tissue blocks leftover from diagnostic biopsies to assess gene expression (**FIG. 2A, Table 1**). Using
 25 a NanoString platform that was optimized for FFPE RNA analysis, we performed targeted transcriptomics studies on 160 custom curated genes from lesional skin biopsies from 6 canine EL cases and 5 healthy canine cases. Our NanoString probeset targets included cytokine, chemokine, and immune related genes, as well as skin associated genes and neuroendocrine genes. Advanced cell type analysis revealed
 30 cytotoxic T cells were significantly abundant ($p=0.0001$; not shown). Comparing the EL samples to healthy margins revealed 32 upregulated differentially expressed genes (DEGs) and 7 down regulated DEGs with $\text{Padj} < 0.01$ (**FIGs. 2B & C**). An array of

genes involved in cytotoxic processes were all upregulated, including *GZMA*, *KLRB1*, *KLRD1*, *KLRK1*, and *PRF1*. *CPA3*, which is involved in proteolysis and degradation of endogenous proteins, was upregulated in EL lesions. Principal component analysis revealed that EL cases could be readily distinguished from healthy controls in 95% confidence intervals, with the exception of one early relapse case which fell in between the EL and healthy skin gene signatures (**FIG. 2D**).

Table 1. Discovery cohort signalments.

Case & Diagnosis	Signalment*	Breed	Notes (relevant features in addition to neoplastic process)
EL Case 1	11 yo, MN	Dachshund	Patch/plaque stage
EL Case 2	13 yo, MN	Labrador cross	Multifocal ulceration with moderate neutrophilic inflammation
EL Case 3	11 yo, FS	Labrador Retriever	Significant eosinophilic infiltrate accompanying the neoplastic population; multifocal ulceration, serocellular crusts and intracorneal neutrophilic pustules
EL Case 4	9 yo, FS	Bloodhound	Significant dermal neoplastic involvement
EL Case 5	11 yo, FS	Olde English Bulldogge	Significant dermal neoplastic involvement with epitheliotropism restricted to the adnexa with relative sparing of the epidermis
EL Case 6	13 yo, MN	Golden Retriever	Early stage epitheliotropic lymphoma with minimal to absent dermal involvement. This is a recurrence from epitheliotropic lymphoma that had undergone remission
Healthy 1	8 yo, FS	Labrador Retriever	NA
Healthy 2	11 yo, FS	Siberian Husky cross	NA
Healthy 3	11 yo, MN	Golden Retriever	NA
Healthy 4	12 yo, MN	German Shepherd cross	NA
Healthy 5	6 yo, FS	Alaskan Malamute	NA

*age at time of biopsy; FS = female spayed, MN = male neutered

Example 1.2. CTSW can distinguish EL from other interface dermatitis conditions

Next, we compared EL gene expression to other forms of interface dermatitis (ID) including lupus erythematosus, pemphigus subtypes, and erythema multiforme spectrum conditions. Seventeen DEGs were significantly downregulated and 14 were significantly upregulated with $P_{adj} < 0.05$ (**FIGs. 3A & B**). To evaluate whether any of these DEGs could serve as potential diagnostic biomarkers for EL, we analyzed RNA

counts of the highest DEGs singly (**FIG. 3C**). Of these, *CTSW* exhibited no overlap between cases and cleanly distinguished EL from other potential clinical mimickers. Receiver operator characteristic (ROC) curves of *CTSW* was 100% sensitive and specific for counts >1092 (**FIG. 3D**). *TRAT1* and *KLRK1* were also highly significant (p<0.0001), and ROC analysis revealed 83.3% sensitivity and 96.77% specificity for *TRAT1* counts >213, and 100% sensitivity and 96.77% specificity for *KLRK1* counts >292.5.

To confirm this finding, we performed analysis on a validation cohort of 6 EL and 9 ID samples using the NanoString canine IO panel (**FIGs. 4A & B; Table 2**). *CTSW* exhibited 100% sensitivity and 88.9% specificity to distinguish the two conditions at a count >121.8 (**FIGs. 4C & D**). *TRAT1* exhibited 88.89% sensitivity and 83.33% specificity to distinguish EL from ID at a count >59.7. *KLRK1* was 100% sensitive and specific at counts >346.9. The difference in absolute counts between the discovery cohort and the validation cohort is likely due to the 2 different platforms used (160 custom gene codeset versus canine IO ~800 gene codeset). We noted that an additional biomarker was identified by the canine IO panel: lymphotactin also called *XCL1/XCL2*, though we were unable to verify this gene in our discovery cohort because it was not included in the 160 gene codeset. Last, we examined whether combining biomarkers could be more powerful than using them singly. Multi-biomarker ROC analysis revealed that combining *CTSW*, *TRAT1* and *KLRK1* is highly sensitive and specific for identifying EL as compared to other forms of ID (**FIG. 4E**).

Table 2. Validation cohort signalments.

Case & Diagnosis	Signalment*	Breed
EL Validation Case 1	13 yo spayed Female	Golden Retriever
EL Validation Case 2	17 yo spayed Female	Yorkshire Terrier
EL Validation Case 3	10 yo spayed Female	Shih Tzu
EL Validation Case 4	10 yo spayed Female	Labrador
		Australian Shepherd
EL Validation Case 5	8 yo spayed Female	Cross
EL Validation Case 6	13.6 yo neutered Male	Golden Retriever

*age at time of biopsy; yo, year old

Example 1.3. Comparative analysis of canine EL and human CTCL reveals shared inflammatory and immunoregulatory gene expression signatures

We also compared our EL findings to a previously published human CTCL dataset (Human Dataset GSE143382⁷). We focused on the DEGs between early MF

and ID (**FIG. 5A**). First, we truncated the datasets to a common denominator gene list of 327 based on the NanoString panels (canine IO and human Myeloid v2). Next, we compared which of these genes had a $P < 0.05$ and found 87 overlapping DEGs (**FIG. 5B**). We examined specific genes identified in our DEG overlap as well as other published genes of interest and found similar expression trends in human and canine datasets (**FIG. 5C**). We examined whether the two-gene classifier identified in human MF would also distinguish canine EL from ID, and found that while *TOX* and *TRAF1* could separate the cases (green) from controls (black), better separation is achieved with *CTSW* and *TRAF1* (**FIG. 5D**).

Example 1.4. *CTSW* and *CD244* are expressed at the protein level in canine EL and ID lesions

To confirm protein level expression of key genes, we performed IHC for *CTSW* and *CD244* using antibodies that react to human and murine proteins with predicted homology to canine amino acid sequences, as no canine specific antibodies are commercially available. IHC staining did not differentiate EL from ID lesions, indicating possible cross-reactivity of the antibodies to other epitopes and/or discordance between RNA transcripts and protein level expression in the different conditions (**FIGs. 6 & 7A-B**). Similarly, qPCR for *CTSW* was not effective at distinguishing EL from ID in FFPE samples, which may be due to the highly fragmented RNA, but was informative in frozen samples. (**FIGs. 8A-B**; primers **Table 3**).

Thus, we developed a truncated custom nanostring nCounter microarray diagnostic tool with our 4 biomarkers (canine *CTSW*, *TRAF1*, *KLRK1*, and *Loc490356/lymphotactin/XCL1/XCL2*) and five housekeeping gene controls (canine *B2M*, *RPL13A*, *CCZ1*, *HPRT1*, and *GAPDH*) (target sequences shown in **Table 4**). This tool was able to sensitively and specifically detect EL in six dogs apart from six ID cases in FFPE samples (RNA was pulled from both discovery and validation cohort samples) (**FIG. 9**).

Table 3. qPCR Primers used for these studies.

Name	Sequence	SEQ ID NO:
CTSW set 1 F	GCT TCC TGC TAC CCA CAC TT	1
CTSW set 1 R	AAG ATG TCC AGA CGA CGA GC	2
CTSW set 2 F	ACC CAT TCT TGG GGA ACA CC	3
CTSW set 2 R	AAC CCA CAA GCA GGA CAG AG	4
GAPDH F	GAT GGG CGT GAA CCA TGA G	5
GAPDH R	TCA TGA GGC CCT CCA CGA T	6

Gene	NCBI Accession No.	Target Sequence (<i>Canis familiaris</i>)	SEQ ID NO.
CTSW	XM_540846.5	CCAGCCACTGGAGCTGAAACAGGTCTTT GCGTTGTTCCAGATCCAGTACAACCGGA GTTACTCAAACCCAGAAGAATATGCTCG TCGTCTGGACATCTTT	7
TRAT1	XM_535733.5	GTACAGTTACTCTGAAGACTATATTCCCA GGGAGGAGGAGTATTATGTTGAAGACAC ACCAATTTATGGTAACTTAGATGACATGG TCCCAGAACCAGTG	8
KLRK1	XM_005637160.1	CCTTAATTATAACTCTGAACCTGTAACAC ATGGTACTTCTCCACGAAGGCCAAAAGAG AAAGCCTACATTAATCACCAGCAAATGTG GAGAAAACCTCATCT	9
Loc490356/ lymphotactin/XCL1/XCL2	XM_022421696.1	TTTCTCACTGAATTCATCGTGGAAGGTGT GGGGAGTGAAGTCCTAGAAAGGAGCAT CTGTATGAGTCTGACGACCAAGAGACTG CCAGTTAAAAGCATCA	10
B2M	NM_001284479.1	TCTTCTGGTCCACACTGAATTCACCTCCCA ATGAGCAGGATGAGTTTAGCTGCCGTGT AAAGCATGTTACTCTCAGTGAGCCCCAG ATCGTTAAGTGGGAC	11
RPL13A	NM_001313766.1	GGCCGGAAGGTTGTAGTCGTGCGCTGT GAAGGCATCAACATTTCTGGCAATTTCTA CAGAAACAAGTTGAAGTACCTGGCCTTC CTCCGCAAGCGGATGA	12
CCZ1	XM_536878.6	GCCGAGAAAAGTACAATTCACATGAGGA AAACACCTAGTGTGTCACTTACATCTGTG CATCCGGACTTAATGAAGATTCTGGGTG ACATCAACAGTGATT	13

HPRT1	NM_0010033 57.1	CCATCACATCGTAGCCCTCTGTGTGCTC AAGGGAGGCTATAAATTCTTTGCTGACC TGCTGGATTATATCAAAGCACTGAACAG AAATAGTGATGGATCC	14
GAPDH	NM_0010031 42.1	CAGTGACACCCACTCTTCCACCTTCGAC GCCGGGGCTGGCATTGCCCTCAATGAC CACTTTGTCAAGCTCATTTCTGGTATGA CAATGAATTTGGCTAC	15

References

1. Willemze R, Cerroni L, Kempf W, Berti E, Facchetti F, Swerdlow SH, et al.
5 The 2018 update of the WHO-EORTC classification for primary cutaneous
lymphomas. *Blood*. 2019;133:1703–14.
2. Muñoz-González H, Molina-Ruiz AM, Requena L. Clinicopathologic
Variants of Mycosis Fungoides. *Actas Dermosifiliogr*. 2017;108:192–208.
3. Criscione VD, Weinstock MA. Incidence of Cutaneous T-Cell Lymphoma
10 in the United States, 1973-2002. *Arch Dermatol*. 2007;143:854–9.
4. Fontaine J, Bovens C, Bettenay S, Mueller RS. Canine cutaneous
epitheliotropic T-cell lymphoma: a review. *Vet Comp Oncol*. 2009;7:1–14.
5. Moore PF, Olivry T, Naydan D. Canine cutaneous epitheliotropic
lymphoma (mycosis fungoides) is a proliferative disorder of CD8+ T cells. *Am J*
15 *Pathol*. 1994;144:421–9.
6. Danaher P, Warren S, Dennis L, D'Amico L, White A, Disis ML, et al.
Gene expression markers of Tumor Infiltrating Leukocytes. *J Immunother Cancer*.
2017;5:18.
7. Nielsen PR, Eriksen JO, Lindahl LM, Wehkamp U, Bzorek M, Andersen G,
20 et al. Diagnostic Two-Gene Classifier in Early-Stage Mycosis Fungoides: A
Retrospective Multicenter Study. *J Invest Dermatol*. 2021;141:213–7.e5.
8. Olson OC, Joyce JA. Cysteine cathepsin proteases: regulators of cancer
progression and therapeutic response. *Nat Rev Cancer*. 2015;15:712–29.
9. Dheilly E, Battistello E, Katanayeva N, Sungalee S, Michaux J, Duns G, et
25 al. Cathepsin S Regulates Antigen Processing and T Cell Activity in Non-Hodgkin
Lymphoma. *Cancer Cell*. 2020;37:674–89.e12.
10. Bararia D, Hildebrand JA, Stolz S, Haebe S, Alig S, Trevisani CP, et al.

Cathepsin S Alterations Induce a Tumor-Promoting Immune Microenvironment in Follicular Lymphoma. *Cell Rep.* 2020;31:107522.

11. Linnevers C, Smeekens SP, Brömme D. Human cathepsin W, a putative cysteine protease predominantly expressed in CD8⁺ T-lymphocytes. *FEBS Lett.* 1997;405:253–9.

12. Wex T, Bühling F, Wex H, Günther D, Malfertheiner P, Weber E, et al. Human cathepsin W, a cysteine protease predominantly expressed in NK cells, is mainly localized in the endoplasmic reticulum. *J Immunol.* 2001;167:2172–8.

13. Stoeckle C, Gouttefangeas C, Hammer M, Weber E, Melms A, Tolosa E. Cathepsin W expressed exclusively in CD8⁺ T cells and NK cells, is secreted during target cell killing but is not essential for cytotoxicity in human CTLs. *Exp Hematol.* 2009;37:266–75.

14. Shin J, Monti S, Aires DJ, Duvic M, Golub T, Jones DA, et al. Lesional gene expression profiling in cutaneous T-cell lymphoma reveals natural clusters associated with disease outcome. *Blood.* 2007;110:3015–27.

15. Kuester D, Lippert H, Roessner A, Krueger S. The cathepsin family and their role in colorectal cancer. *Pathol Res Pract.* 2008;204:491–500.

16. Kirchgessner H, Dietrich J, Scherer J, Isomäki P, Korinek V, Hilgert I, et al. The transmembrane adaptor protein TRIM regulates T cell receptor (TCR) expression and TCR-mediated signaling via an association with the TCR zeta chain. *J Exp Med.* 2001;193:1269–84.

17. Valk E, Leung R, Kang H, Kaneko K, Rudd CE, Schneider H. T cell receptor-interacting molecule acts as a chaperone to modulate surface expression of the CTLA-4 coreceptor. *Immunity.* 2006;25:807–21.

18. Boćko D, Bilińska M, Dobosz T, Zoledziewska M, Suwalska K, Tutak A, et al. Lack of association between an exon 1 CTLA-4 gene polymorphism A(49)G and multiple sclerosis in a Polish population of the Lower Silesia region. *Arch Immunol Ther Exp.* 2003;51:201–5.

19. Lanier LL. NKG2D Receptor and Its Ligands in Host Defense. *Cancer Immunol Res.* 2015;3:575–82.

20. Iqbal J, Weisenburger DD, Greiner TC, Vose JM, McKeithan T, Kucuk C, et al. Molecular signatures to improve diagnosis in peripheral T-cell lymphoma and prognostication in angioimmunoblastic T-cell lymphoma. *Blood.* 2010;115:1026–36.

21. Vieyra-Garcia P, Crouch JD, O'Malley JT, Seger EW, Yang CH, Teague

JE, et al. Benign T cells drive clinical skin inflammation in cutaneous T cell lymphoma. *JCI Insight*. 2019;4.

22. Kamarashev J, Burg G, Mingari MC, Kempf W, Hofbauer G, Dummer R. Differential expression of cytotoxic molecules and killer cell inhibitory receptors in
5 CD8+ and CD56+ cutaneous lymphomas. *Am J Pathol*. 2001;158:1593–8.

23. Yang D, Chen Q, Hoover DM, Staley P, Tucker KD, Lubkowski J, et al. Many chemokines including CCL20/MIP-3 α display antimicrobial activity. *J Leukoc Biol*. 2003;74:448–55.

24. Hahtola S, Tuomela S, Elo L, Häkkinen T, Karenko L, Nedoszytko B, et
10 al. Th1 response and cytotoxicity genes are down-regulated in cutaneous T-cell lymphoma. *Clin Cancer Res*. 2006;12:4812–21.

25. Rindler K, Jonak C, Alkon N, Thaler FM, Kurz H, Shaw LE, et al. Single-cell RNA sequencing reveals markers of disease progression in primary cutaneous T-cell lymphoma. *Mol Cancer*. 2021;20:124.

26. Jonak C, Alkon N, Rindler K, Rojahn TB, Shaw LE, Porkert S, et al. Single-cell RNA sequencing profiling in a patient with discordant primary cutaneous B-cell and T-cell lymphoma reveals micromilieu-driven immune skewing. *Br J Dermatol*. 2021;185:1013–25.

27. Visentini M, Carbonari M, Ghia E, De Propriis S, Guarini A, Girmenia C,
20 et al. A lymphotactin-producing monoclonal T-cell lymphoproliferative disorder with extreme lymphocytopenia and progressive leukoencephalopathy. *Leuk Lymphoma*. 2006;47:1421–3.

28. Cairns CM, Gordon JR, Li F, Baca-Estrada ME, Moyana T, Xiang J. Lymphotactin expression by engineered myeloma cells drives tumor regression:
25 mediation by CD4+ and CD8+ T cells and neutrophils expressing XCR1 receptor. *The Journal of Immunology*. 2001;167:57–65.

29. Mizumoto Y, Hemmi H, Katsuda M, Miyazawa M, Kitahata Y, Miyamoto A, et al. Anticancer effects of chemokine-directed antigen delivery to a cross-presenting dendritic cell subset with immune checkpoint blockade. *Br J Cancer*.
30 2020;122:1185–93.

30. Alpdogan O, Kartan S, Johnson W, Sokol K, Porcu P. Systemic therapy of cutaneous T-cell lymphoma (CTCL). *Chin Clin Oncol*. 2019;8:10.

31. Berti E, Tomasini D, Vermeer MH, Meijer CJ, Alessi E, Willemze R. Primary cutaneous CD8-positive epidermotropic cytotoxic T cell lymphomas. A

distinct clinicopathological entity with an aggressive clinical behavior. *Am J Pathol.* 1999;155:483–92.

32. Gambaro K, Quinn MCJ, Wojnarowicz PM, Arcand SL, de Ladurantaye M, Barrès V, et al. VGLL3 expression is associated with a tumor suppressor phenotype in epithelial ovarian cancer. *Mol Oncol.* 2013;7:513–30.

33. Cody NAL, Shen Z, Ripeau J-S, Provencher DM, Mes-Masson A-M, Chevrette M, et al. Characterization of the 3p12.3-pcen region associated with tumor suppression in a novel ovarian cancer cell line model genetically modified by chromosome 3 fragment transfer. *Mol Carcinog.* 2009;48:1077–92.

34. Zhang L, Li L, Mao Y, Hua D. VGLL3 is a prognostic biomarker and correlated with clinical pathologic features and immune infiltrates in stomach adenocarcinoma. *Sci Rep.* 2020;10:1355.

35. Hori N, Okada K, Takakura Y, Takano H, Yamaguchi N, Yamaguchi N. Vestigial-like family member 3 (VGLL3), a cofactor for TEAD transcription factors, promotes cancer cell proliferation by activating the Hippo pathway. *J Biol Chem.* 2020;295:8798–807.

36. Richmond J, Tuzova M, Parks A, Adams N, Martin E, Tawa M, et al. Interleukin-16 as a marker of Sezary syndrome onset and stage. *J Clin Immunol.* 2011;31:39–50.

37. Richmond J, Finkel M, Studwell A, Little F, Cruikshank W. Introduction of pro-interleukin-16 inhibits T-lymphoblastic leukemia growth in mice. *J Cancer Res Clin Oncol.* 2011;137:1581–5.

38. Querfeld C, Guitart J, Kuzel TM, Rosen ST. Primary cutaneous lymphomas: a review with current treatment options. *Blood Rev.* 2003;17:131–42.

39. Gormley RH, Hess SD, Anand D, Junkins-Hopkins J, Rook AH, Kim EJ. Primary cutaneous aggressive epidermotropic CD8+ T-cell lymphoma. *J Am Acad Dermatol.* 2010;62:300–7.

40. Pulitzer M. Cutaneous T-cell Lymphoma. *Clin Lab Med.* 2017;37(3):527-546.

41. Miteva M, El Shabrawi-Caelen L, Fink-Puches R, et al. Alopecia universalis associated with cutaneous T cell lymphoma. *Dermatology.* 2014;229(2):65-69.

42. Willemze R, Jansen PM, Cerroni L, et al. Subcutaneous panniculitis-like T-cell lymphoma: definition, classification, and prognostic factors: an EORTC Cutaneous Lymphoma Group Study of 83 cases. *Blood*. 2008;111(2):838-845.
43. Jawed, S. I., Myskowski, P. L., Horwitz, S., Moskowitz, A. & Querfeld, C.
 5 Primary cutaneous T-cell lymphoma (mycosis fungoides and Sézary syndrome): part II. Prognosis, management, and future directions. *J. Am. Acad. Dermatol.* 70, 223.e1–17; quiz 240–2 (2014).
44. Smith, B. D. & Wilson, L. D. Management of mycosis fungoides. Part 1. Diagnosis, staging, and prognosis. *Oncology* 17, 1281–1288 (2003).
45. Redmond, W. J. & Rahbari, H. Mycosis fungoides: a retrospective study.
 10 *J. Am. Acad. Dermatol.* 1, 431–436 (1979).
46. Criscione, V. D. & Weinstock, M. A. Incidence of Cutaneous T-Cell Lymphoma in the United States, 1973-2002. *Arch. Dermatol.* 143, 854–859 (2007).
47. Wilson, L. D., Hinds, G. A. & Yu, J. B. Age, race, sex, stage, and
 15 incidence of cutaneous lymphoma. *Clin. Lymphoma Myeloma Leuk.* 12, 291–296 (2012).
48. Balagula, Y., Dusza, S. W., Zampella, J., Sweren, R. & Hinds, G. A. Early-onset mycosis fungoides among African American women: a single-institution study. *J. Am. Acad. Dermatol.* 71, 597–598 (2014).
49. Hinds, G. A. & Heald, P. Cutaneous T-cell lymphoma in skin of color. *J. Am. Acad. Dermatol.* 60, 359–75; quiz 376–8 (2009).
50. Imam, M. H., Shenoy, P. J., Flowers, C. R., Phillips, A. & Lechowicz, M. J. Incidence and survival patterns of cutaneous T-cell lymphomas in the United States. *Leuk. Lymphoma* 54, 752–759 (2013).
51. Balagula, Y., Dusza, S. W., Zampella, J., Sweren, R. & Hinds, G. A. Early-onset mycosis fungoides among young African American women: A single-institution study. in *JOURNAL OF INVESTIGATIVE DERMATOLOGY* vol. 134 2059–2059.
52. Zampella, J. G. & Hinds, G. A. Racial differences in mycosis fungoides: a
 30 retrospective study with a focus on eosinophilia. *J. Am. Acad. Dermatol.* 68, 967–971 (2013).
53. Sun, G. et al. Poor prognosis in non-Caucasian patients with early-onset mycosis fungoides. *J. Am. Acad. Dermatol.* 60, 231–235 (2009).

54. Agi, C., Kuhn, D., Chung, J., Zampella, J. & Hinds, G. Racial differences in the use of extracorporeal photopheresis for mycosis fungoides. *J. Dermatolog. Treat.* 26, 266–268 (2015).

55. Su, C. et al. Racial disparity in mycosis fungoides: An analysis of 4495 cases from the US National Cancer Database. *J. Am. Acad. Dermatol.* 77, 497–502.e2 (2017).

56. Pariser and Gram, “Feline cutaneous lymphocytosis: case report and summary of the literature,” *Journal of Feline Medicine and Surgery* (2014) 16, 758–763

57. Miller, “Classification and clinical features in 88 cases of equine cutaneous lymphoma,” *J Vet Diagn Invest.* 2015 Jan;27(1):86-91

58. Wheat, C. M. & Okoye, G. A. Cutaneous T-Cell Lymphoma. in *Clinical Cases in Skin of Color: Medical, Oncological and Hair Disorders, and Cosmetic Dermatology* (eds. Love, P. B. & Kundu, R. V.) 111–124 (Springer International Publishing, 2016). doi:10.1007/978-3-319-23615-5_10.

59. Egbeto IA, CJ Garelli, C Piedra-Mora, NB Wong, CN David, NA Robinson & JM Richmond. “Case Series: Gene Expression Analysis in Canine Vogt-Koyanagi-Harada/Uveodermatologic Syndrome and Vitiligo Reveals Conserved Immunopathogenesis Pathways Between Dog and Human Autoimmune Pigmentary Disorders.” *Front Immunol.* 2020; 11, doi: 10.3389/fimmu.2020.590558.

60. Garelli CJ, NB Wong, C Piedra-Mora, LM Wrijil, G Scarglia, CN David, RM Almela, NA Robinson & JM Richmond. “Shared inflammatory and skin-specific gene signatures reveal common drivers of discoid lupus erythematosus in canines, humans and mice.” *Curr Res Immunol.* 2021; (2) 41-51, doi.org/10.1016/j.crimmu.2021.03.003

61. Wex T, Bühling F, Wex H, Günther D, Malfertheiner P, Weber E, Brömme D. Human cathepsin W, a cysteine protease predominantly expressed in NK cells, is mainly localized in the endoplasmic reticulum. *J Immunol.* 2001;167(4):2172-8.

62. Stoeckle C, Gouttefangeas C, Hammer M, Weber E, Melms A, Tolosa E. Cathepsin W expressed exclusively in CD8+ T cells and NK cells, is secreted during target cell killing but is not essential for cytotoxicity in human CTLs. *Exp Hematol.* 2009;37(2):266-75.

63. Kuester D, Lippert H, Roessner A, Krueger S. The cathepsin family and their role in colorectal cancer. *Pathol Res Pract*. 2008;204(7):491-500.

64. Brachelente C, Affolter VK, Fondati A, Porcellato I, Sforna M, Lepri E, et al. CD3 and CD20 Coexpression in a Case of Canine Cutaneous Epitheliotropic T-
5 Cell Lymphoma (Mycosis Fungoides). *Vet Pathol*. 2016;53:563–6.

65. Meuten DJ. *Tumors in Domestic Animals*. Wiley & Sons, Limited, John; 2016.

66. Gross TL. *Skin diseases of the dog and cat : clinical and histopathologic diagnosis*. Ames, Iowa: Blackwell Science; 2005.

10

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the
15 scope of the following claims.

WHAT IS CLAIMED IS:

1. A method comprising:
obtaining a sample comprising skin from a subject, and
determining a level of expression of one, two, three, or all four of cathepsin W (CTSW), T Cell Receptor Associated Transmembrane Adaptor 1 (TRAT1), killer cell lectin like receptor K1 (KLRK1; also known as NKG2D), and/or XCL1/XCL2 (lymphotactin) in the sample.
2. A method of diagnosing a subject as having cutaneous T cell lymphoma, the method comprising:
obtaining a sample comprising skin from a subject;
determining a level of expression of one, two, three, or all four of cathepsin W (CTSW), T Cell Receptor Associated Transmembrane Adaptor 1 (TRAT1), and killer cell lectin like receptor K1 (KLRK1), and/or XCL1/XCL2 (lymphotactin) in the sample;
comparing the level of expression of CTSW, TRAT1, and/or KLRK1 in the sample to a reference level, and identifying the subject as having CTCL wherein a level of expression of CTSW, TRAT1, and/or KLRK1 above the reference level indicates that the subject.
3. The method of claims 1 or 2, wherein the sample comprises skin from a lesion, optionally a nodule, tumor, plaque, ulcerated, or rashy patch of skin that is suspected of being CTCL.
4. The method of claims 1 to 3, wherein the subject is a mammal.
5. The method of claim 4, wherein the mammal is a human, optionally a human subject having skin of color.
6. The method of claim 5, wherein the subject has Fitzpatrick phototype IV, V, or VI skin.
7. The method of claim 4, wherein the subject is a canine, feline, or equine.

8. The method of claim 2, further comprising selecting and optionally administering a treatment for CTCL to a subject identified as having CTCL.
9. The method of claim 8, wherein the treatment comprises a skin-directed therapy, optionally topical steroids, involved-site radiation therapy (RT), total skin electron-beam therapy (TSEBT), or phototherapy/photopheresis, or systemic therapies, optionally immunotherapies (retinoids, interferon, chimeric antigen receptor T cells, or forodesine), small molecules (histone deacetylase inhibitors), antibodies (optionally anti-CCR4, anti-programmed cell death protein 1, anti-CD47, or anti-CD30), chemotherapy, or allogeneic haematopoietic stem cell transplantation.
10. A kit comprising reagents for detecting the biomarkers comprising or consisting of two, three, or all four of CTSW, KLRK1, TRAT1 and/or XCL1/XCL2, and optionally one or more controls.
11. The kit of claim 10, wherein the reagents comprise probes or primers that bind to the two, three, or all four biomarkers and optionally one or more controls.
12. The kit of claims 10 or 11, wherein the controls are housekeeping genes, optionally one, two, three, four, or all five of B2M, RPL13A, CCZ1, HPRT1, and/or GAPDH.
13. The kit of claims 10 or 11, which comprises a microarray.
14. The kit of claims 10-13, wherein the probes or primers bind to at least 20 consecutive nucleotides of a biomarker sequence shown in SEQ ID NO:7-10, or a human homolog thereof.

1 / 29

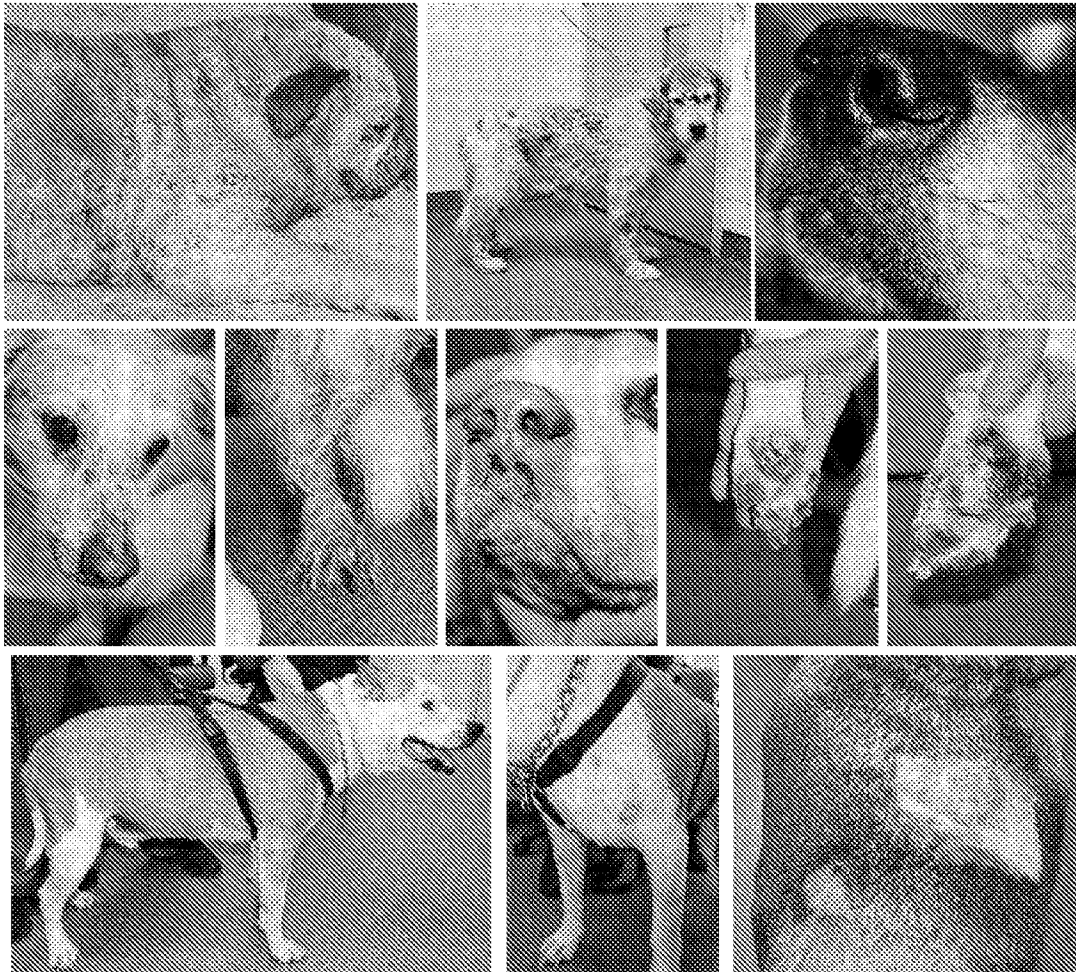


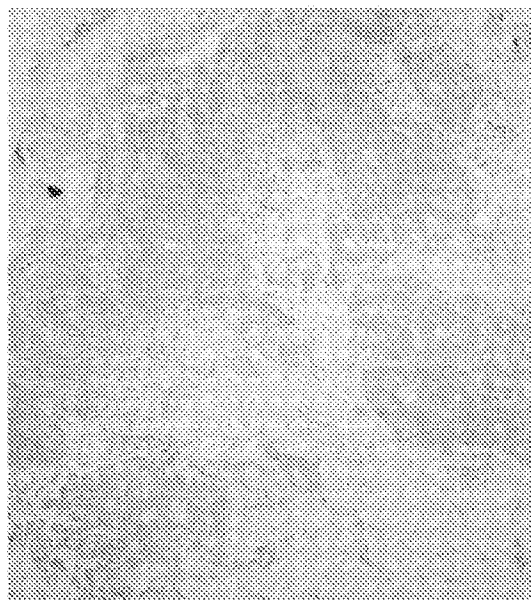
FIG. 1

2 / 29

EL Case 1



EL Case 2



EL Case 3



EL Case 4



FIG. 2A

3 / 29

EL Case 5



EL Case 6 – Early relapse



Healthy margin skin

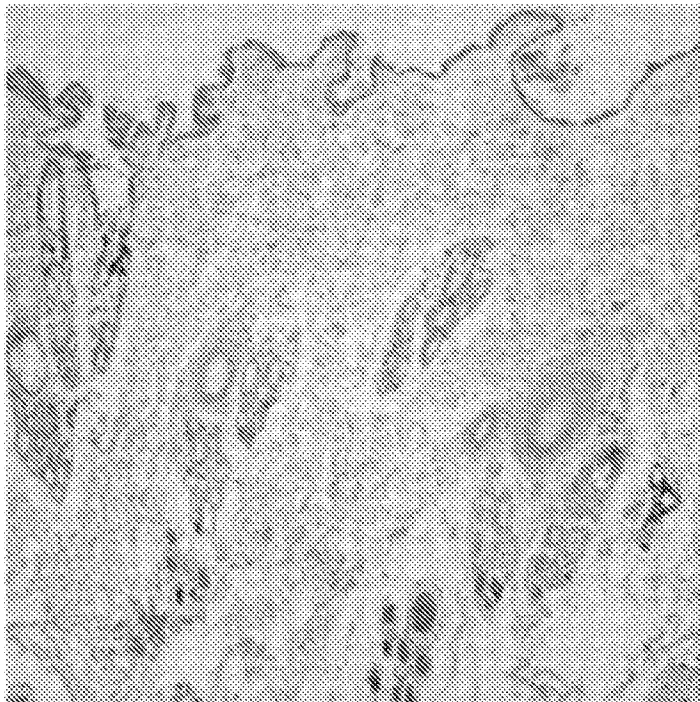


FIG. 2A, continued

EL vs Healthy Margin

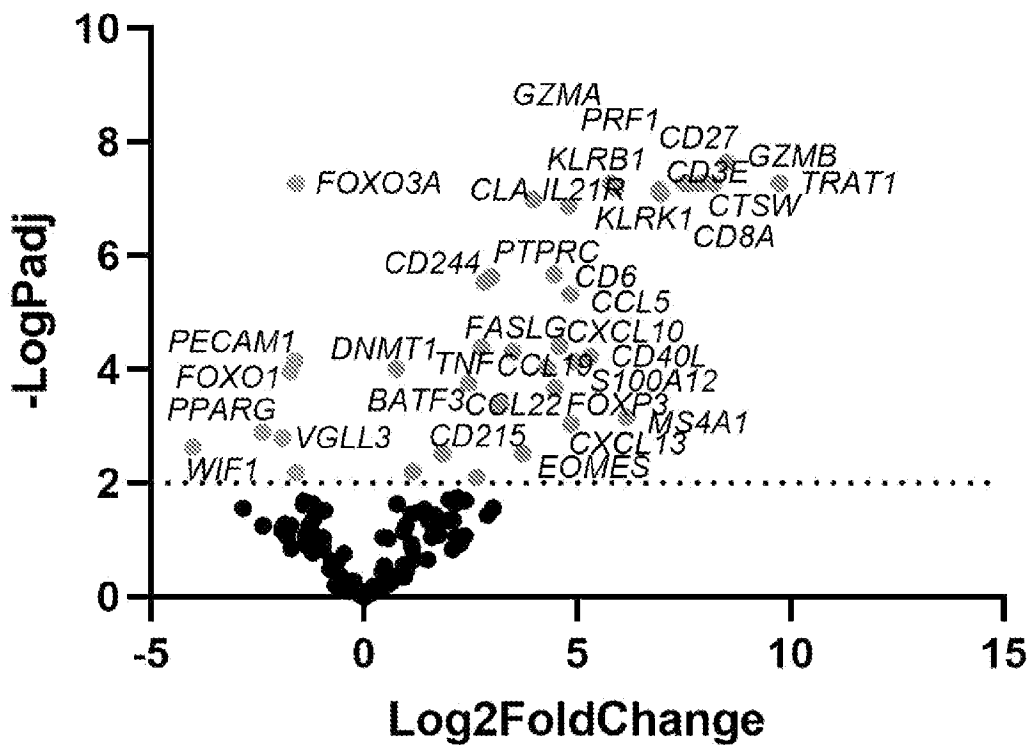


FIG. 2B

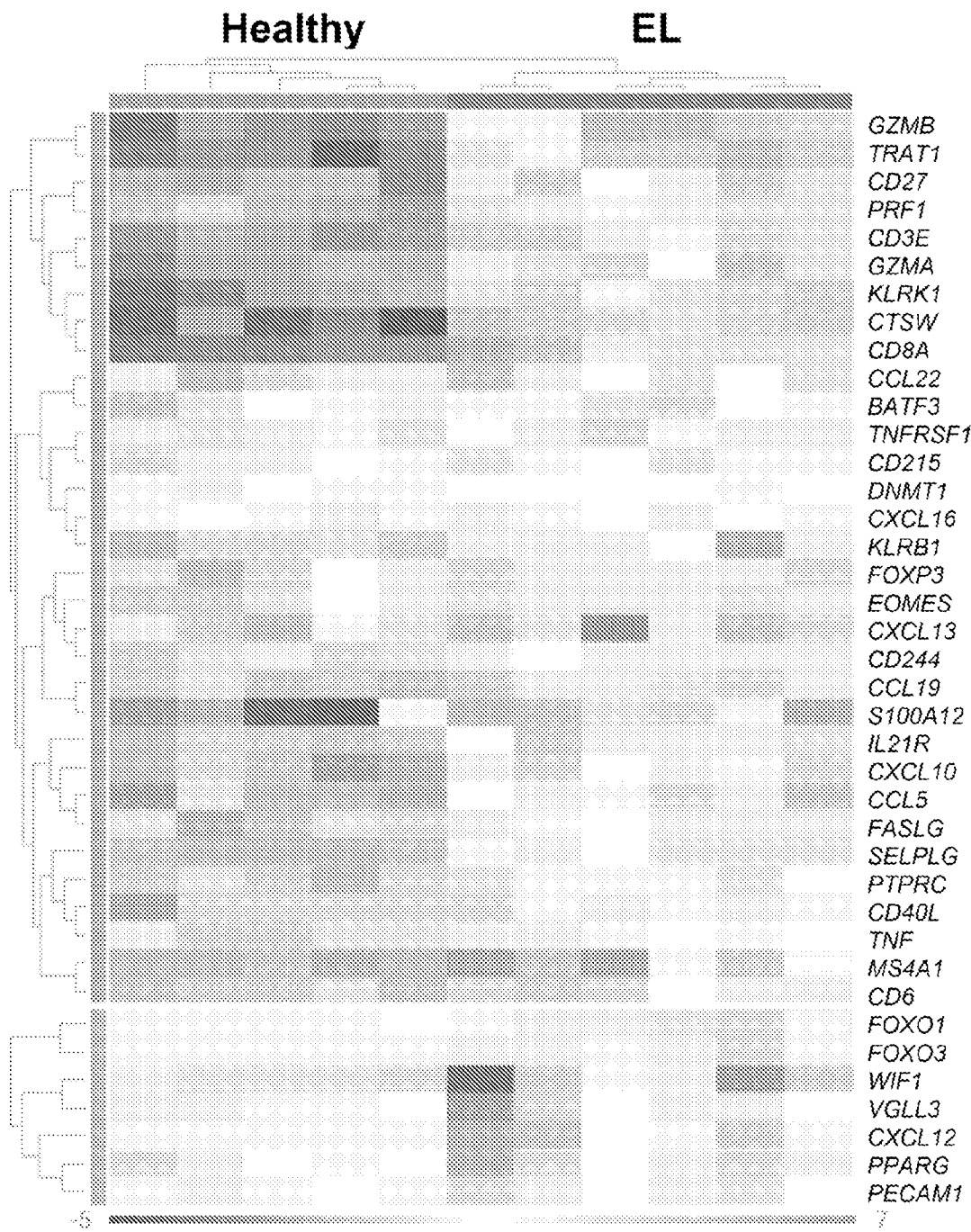


FIG. 2C

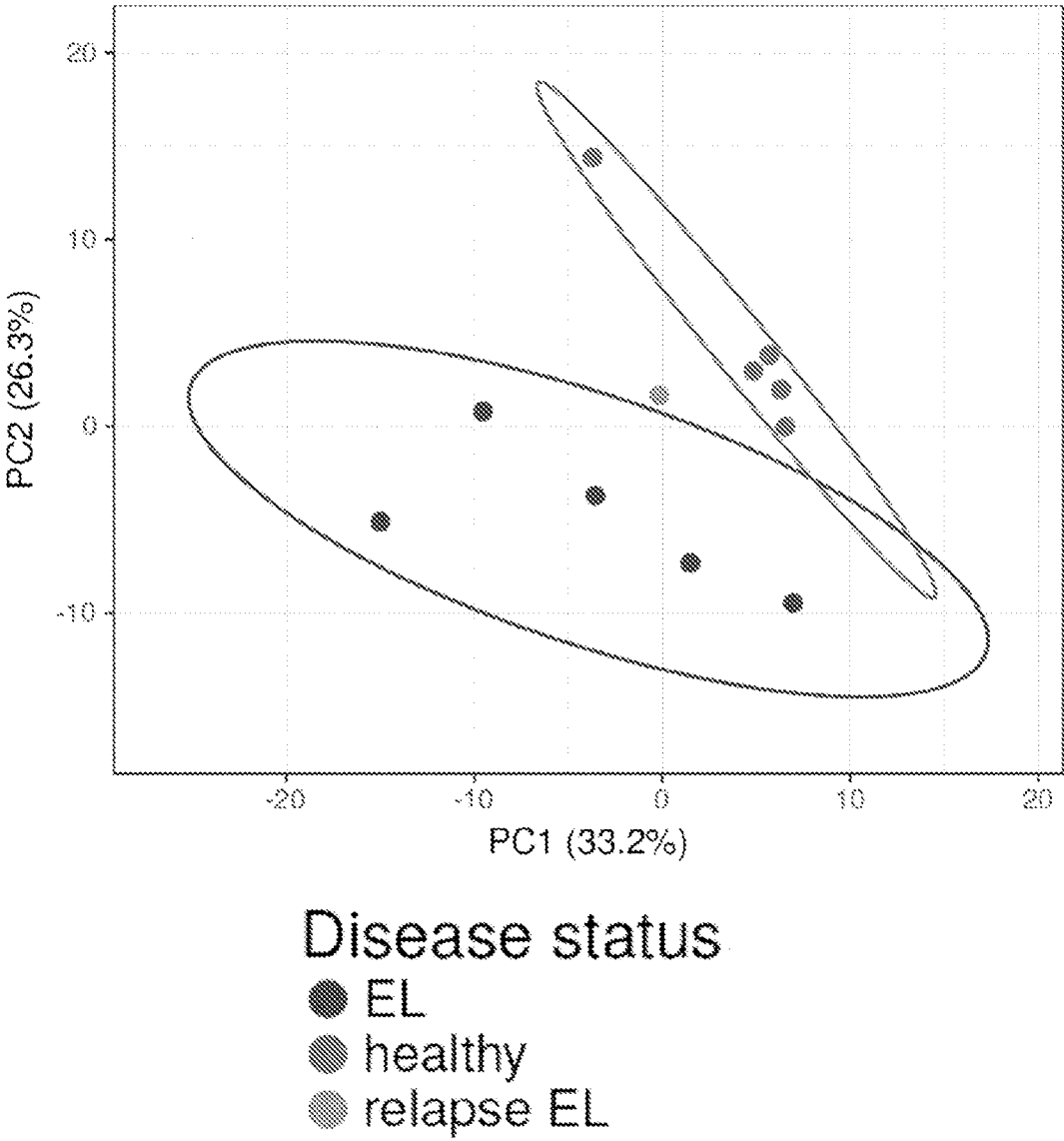


FIG. 2D

EL vs Other Interface Dermatitis

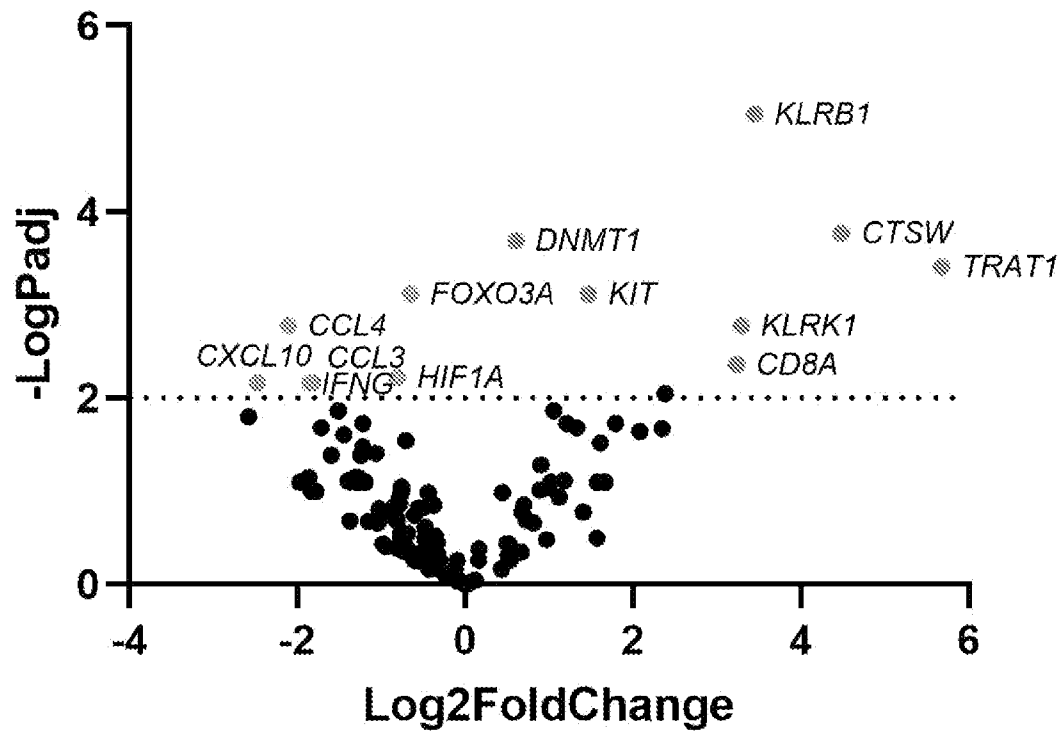


FIG. 3A

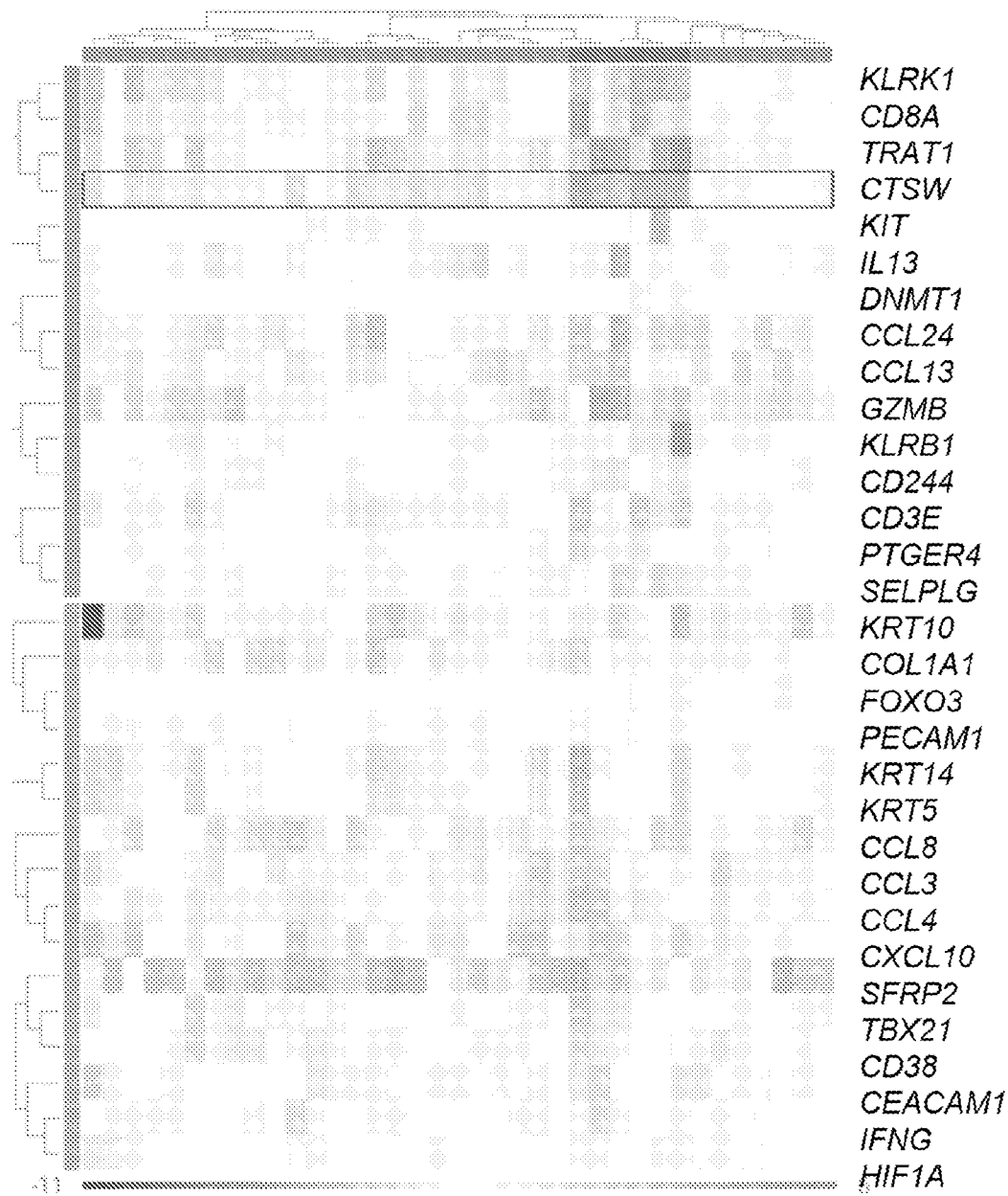


FIG. 3B

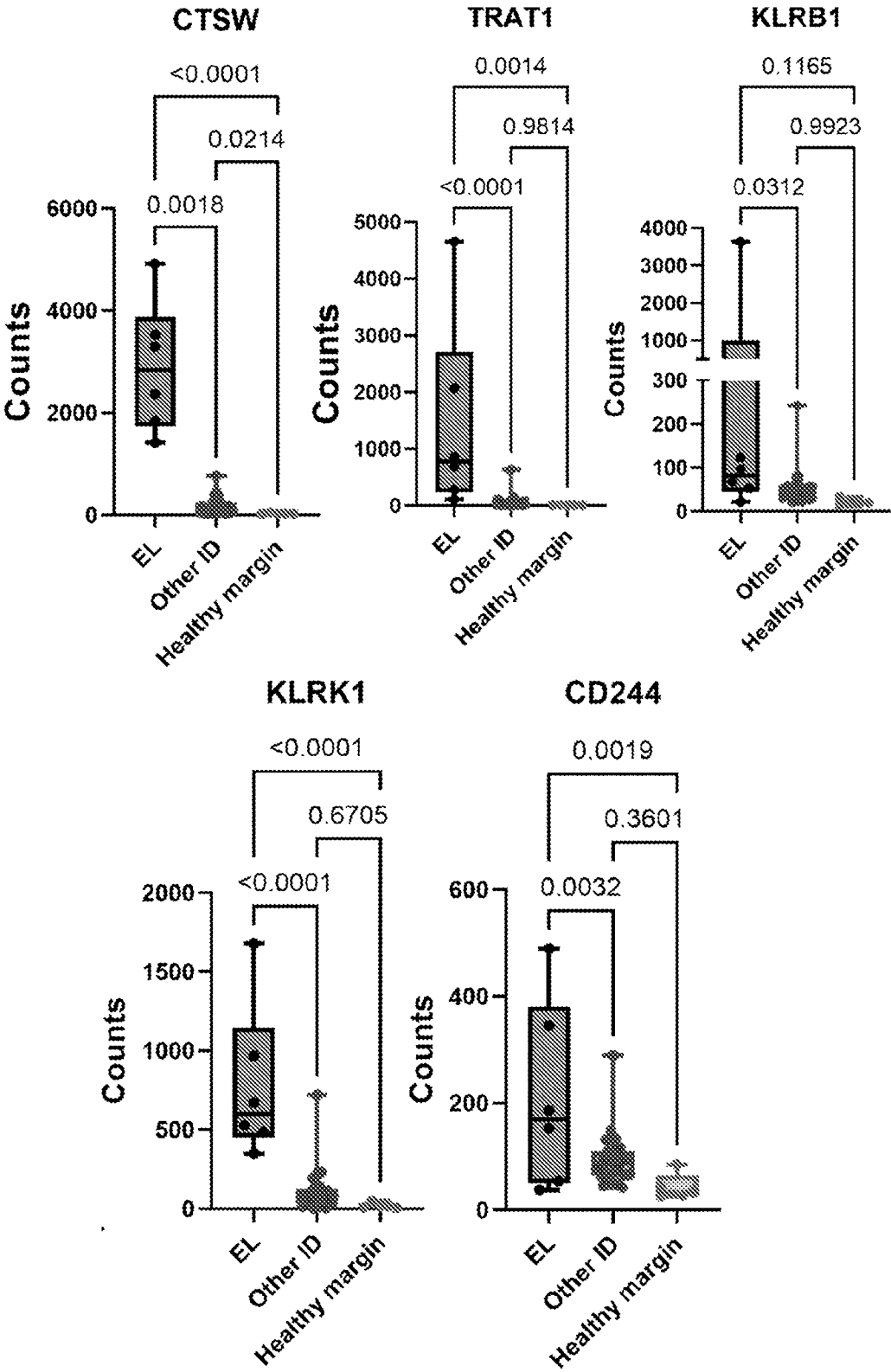
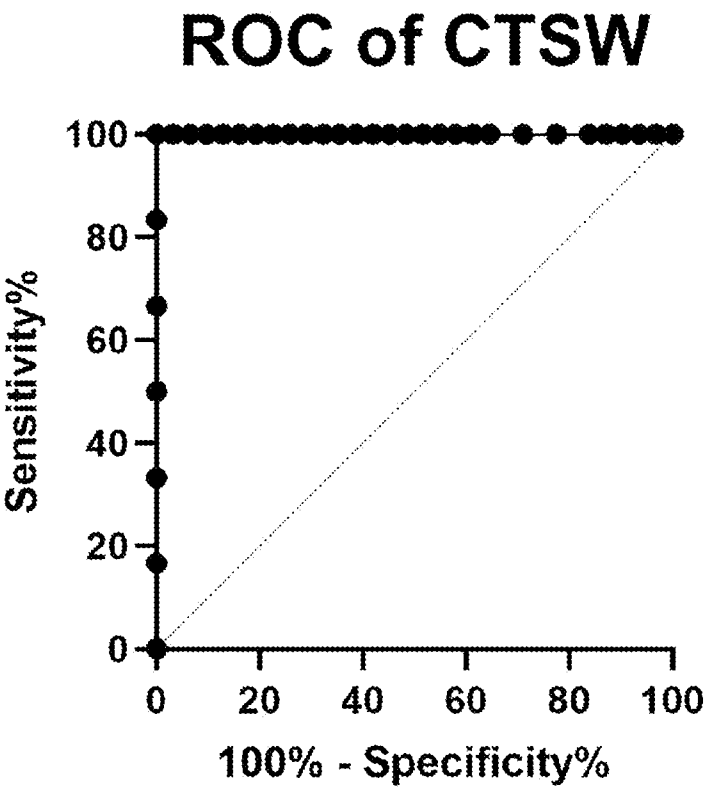
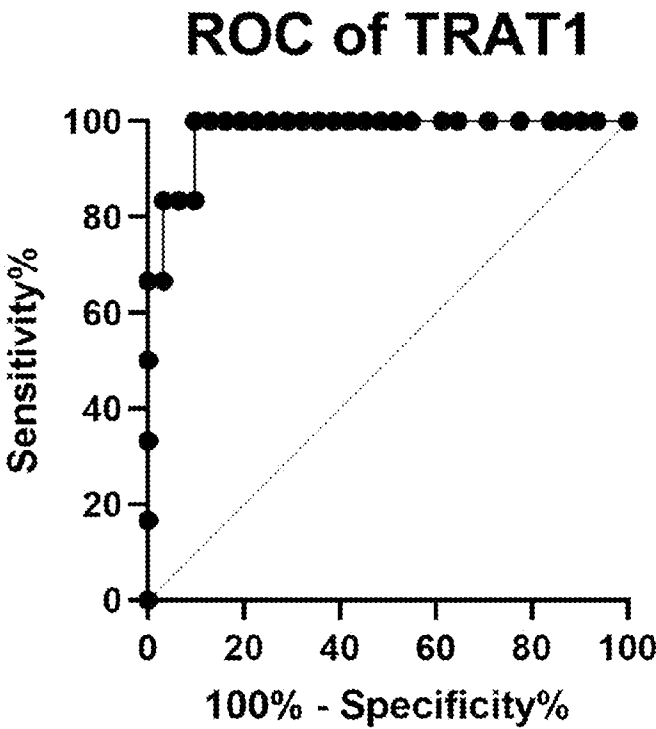


FIG. 3C



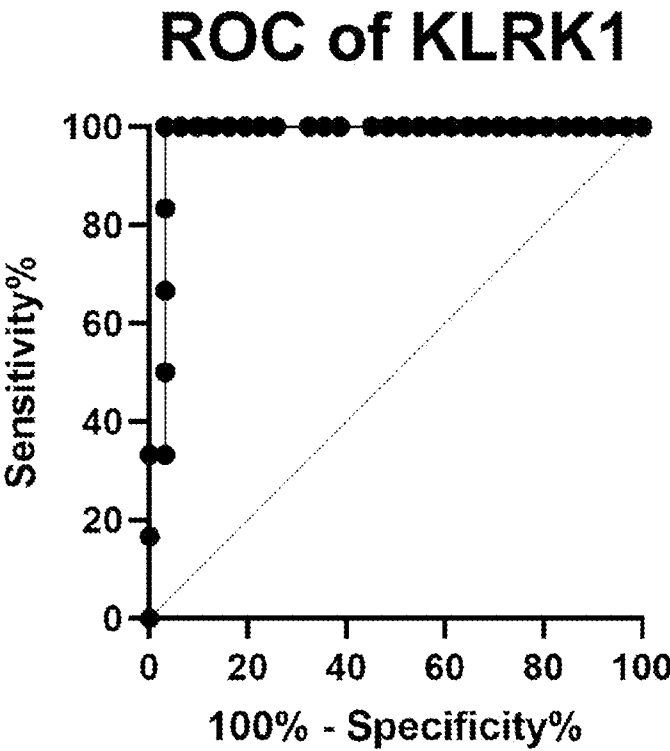
Count	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 1092	100	60.97% to 100.0%	100	88.97% to 100.0%	

FIG. 3D



Count	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 213.0	83.33	43.65% to 99.15%	96.77	83.81% to 99.83%	25.83

FIG. 3D, continued



Count	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 292.5	100	60.97% to 100.0%	96.77	83.81% to 99.83%	31

FIG. 3D, continued

EL validation cohort

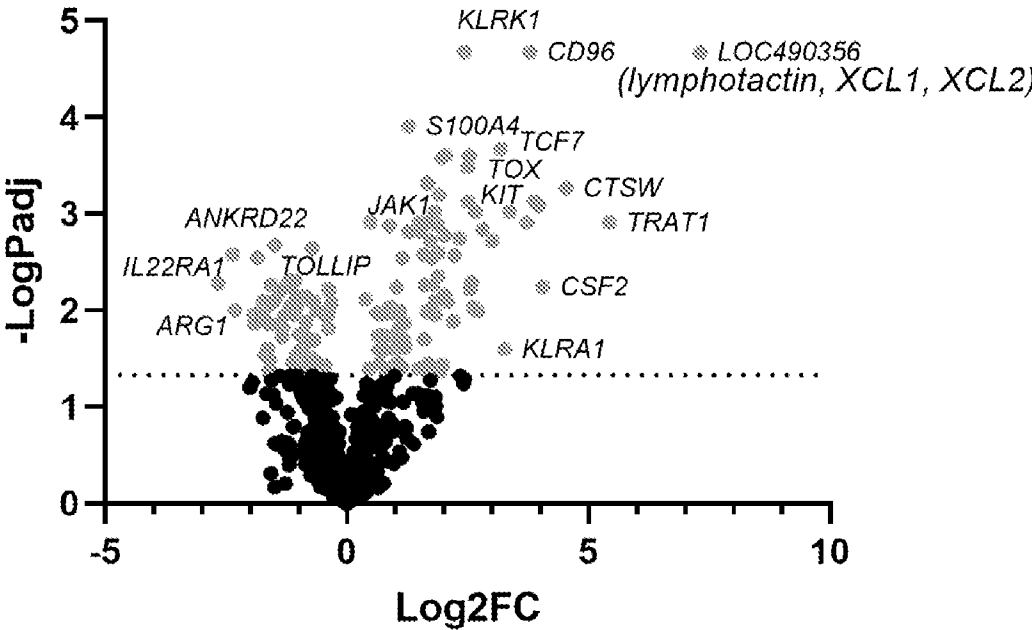


FIG. 4A



FIG. 4B

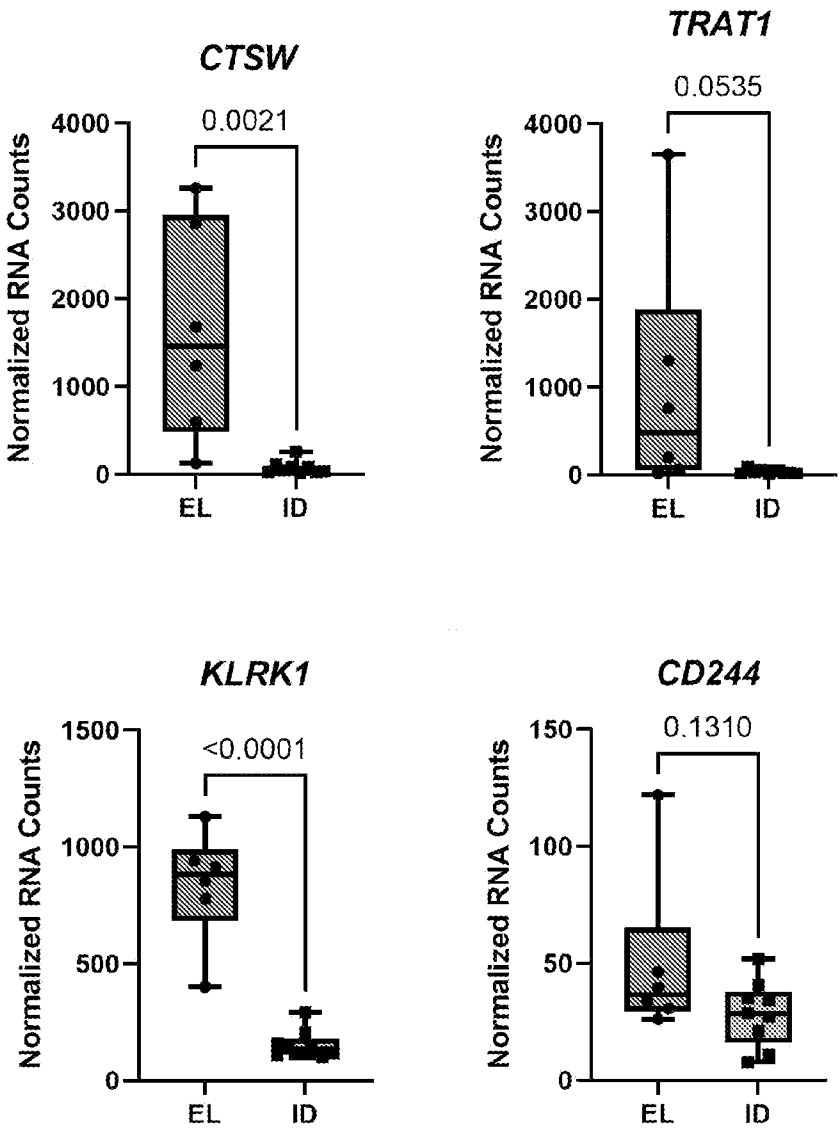


FIG. 4C

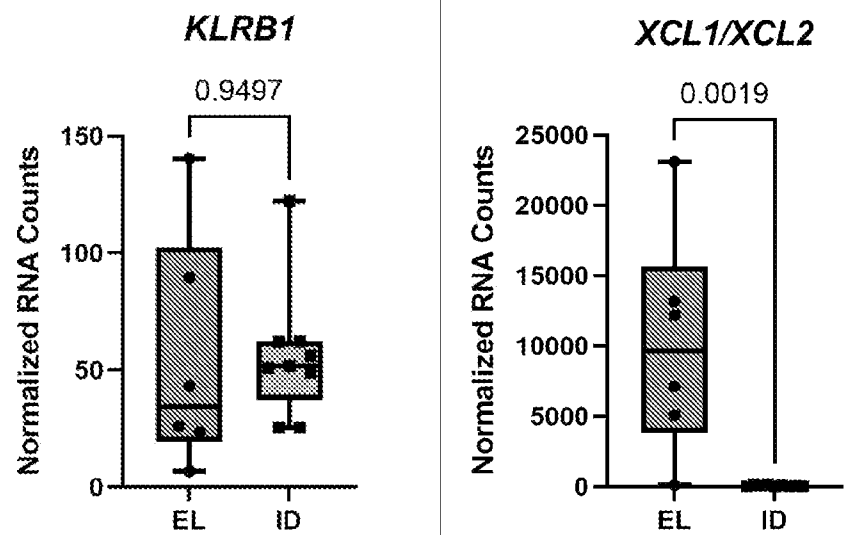
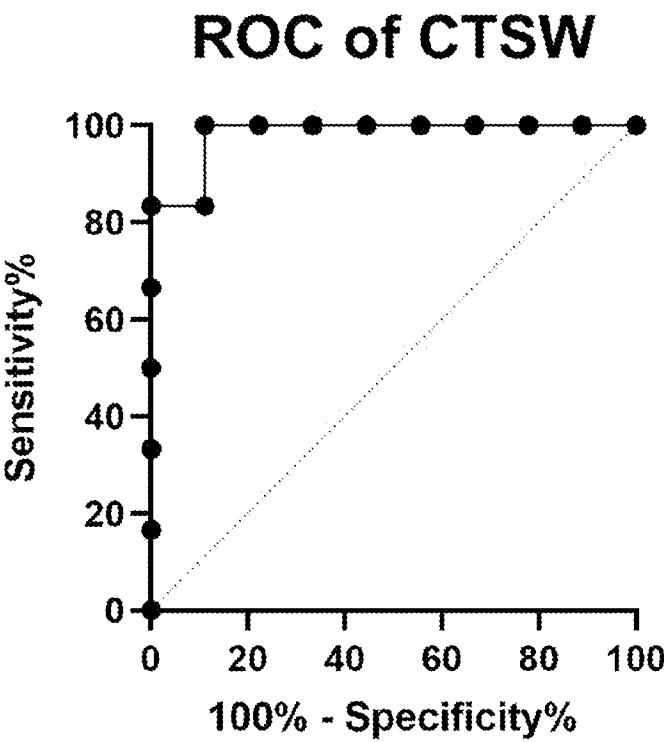
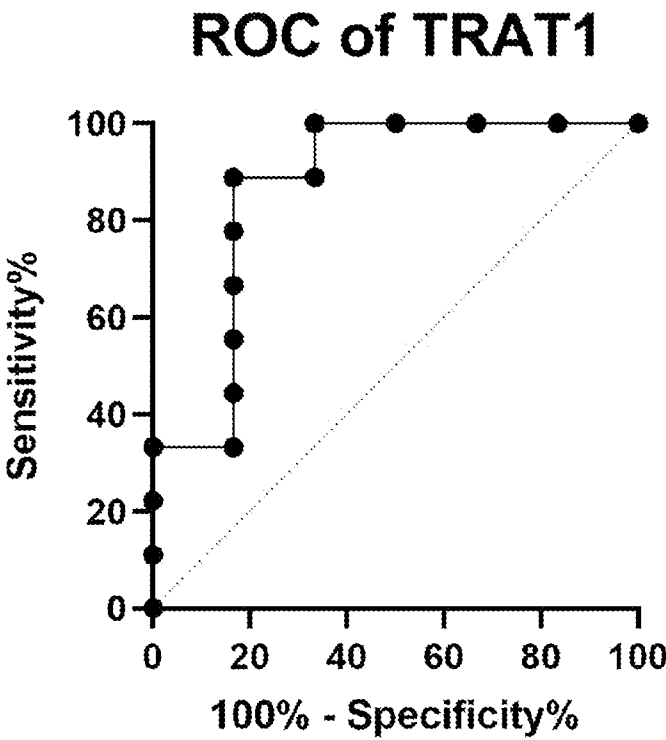


FIG. 4C, continued



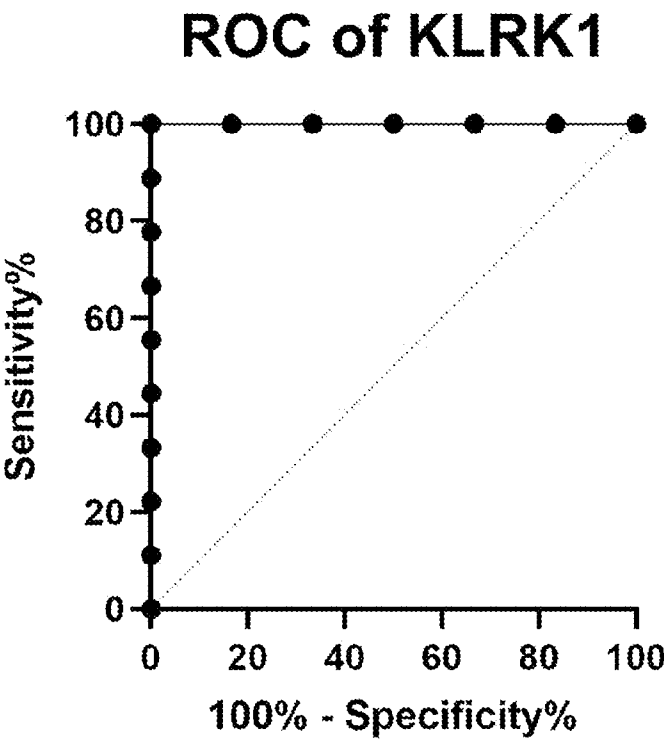
Count	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 121.8	100	64.57% to 100.0%	88.89	56.50% to 99.43%	9

FIG. 4D



Count	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 59.70	88.89	56.50% to 99.43%	85.71	48.69% to 99.27%	6.222

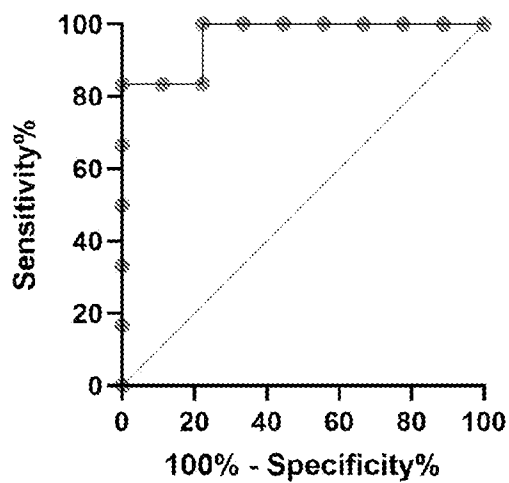
FIG. 4D, continued



Count	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 346.9	100	70.09% to 100.0%	100	64.57% to 100.0%	

FIG. 4D, continued

ROC of XCL1/XCL2



	Sensitivity%	95% CI	Specificity%	95% CI
> 2619	83.33	43.65% to 99.15%	100.0	70.09% to 100.0%

FIG. 4D, continued

ROC plot for epitheliotropic lymphoma - Normalized

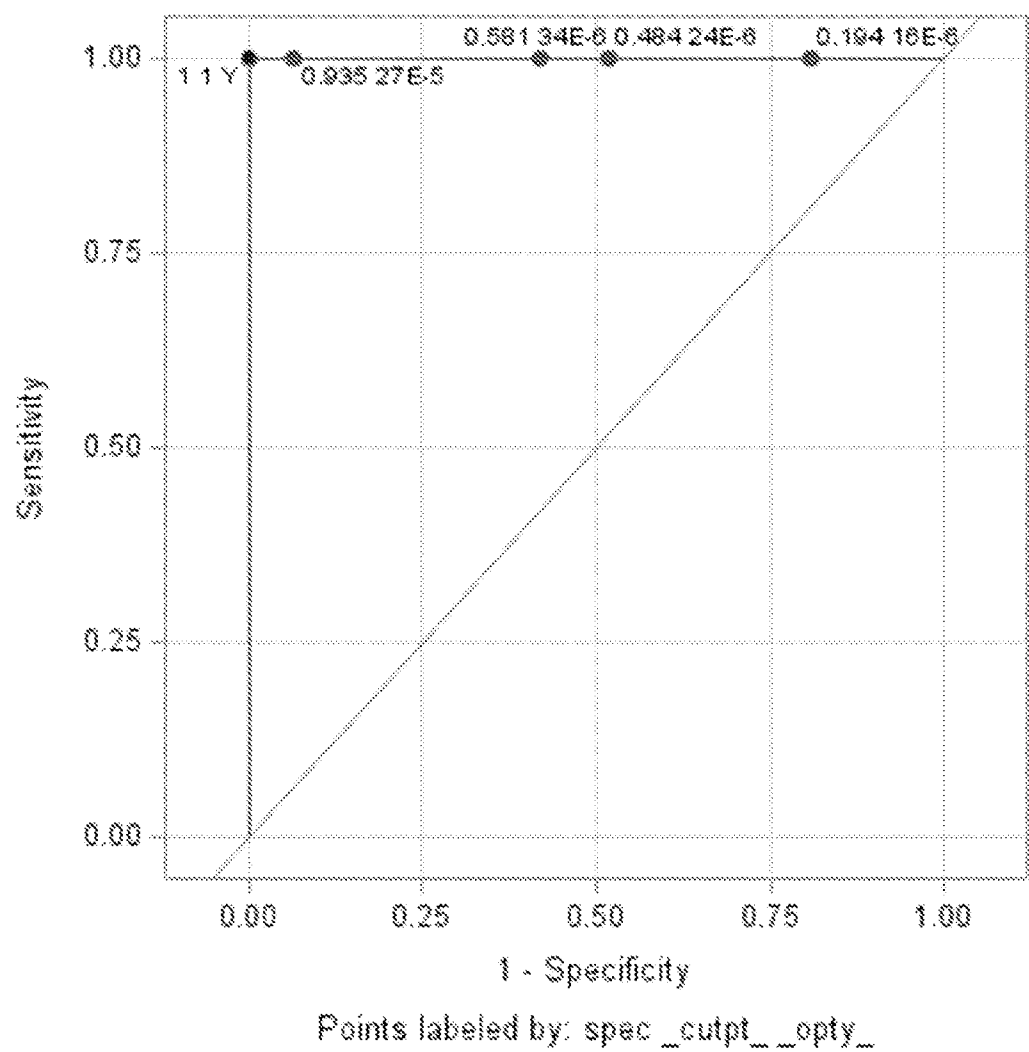


FIG. 4E

GSE143382: limma, Padj<0.05

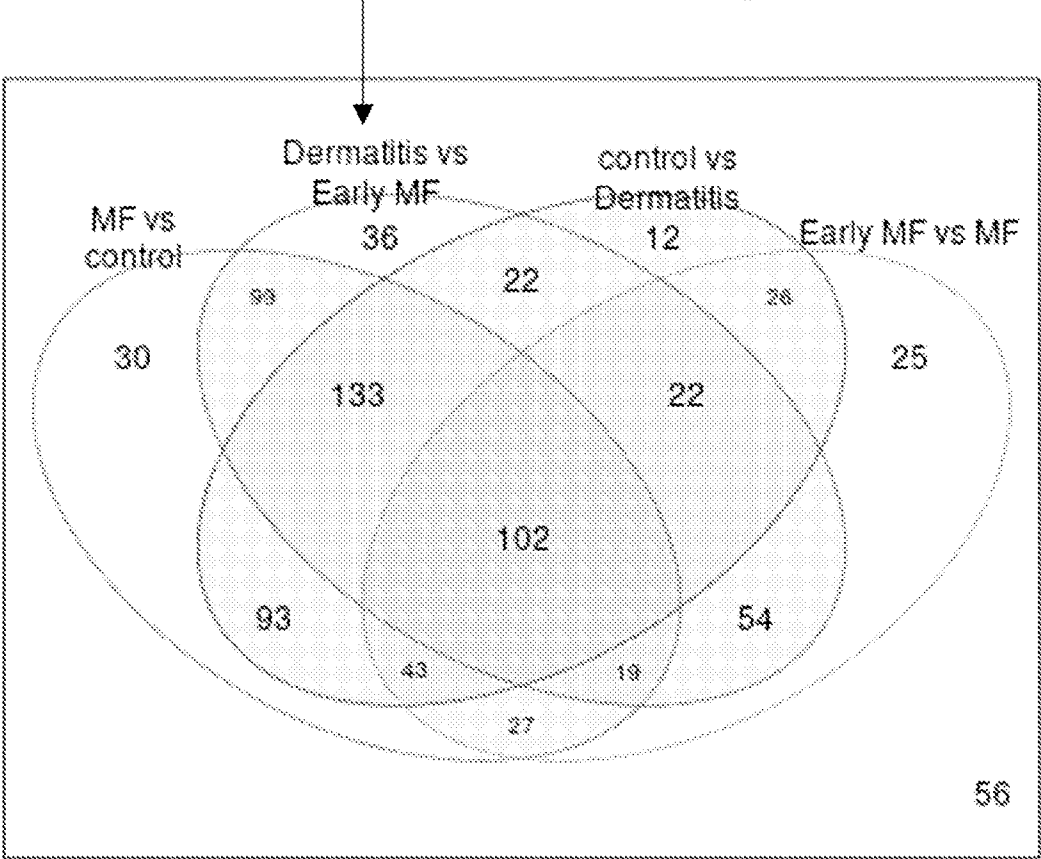


FIG. 5A

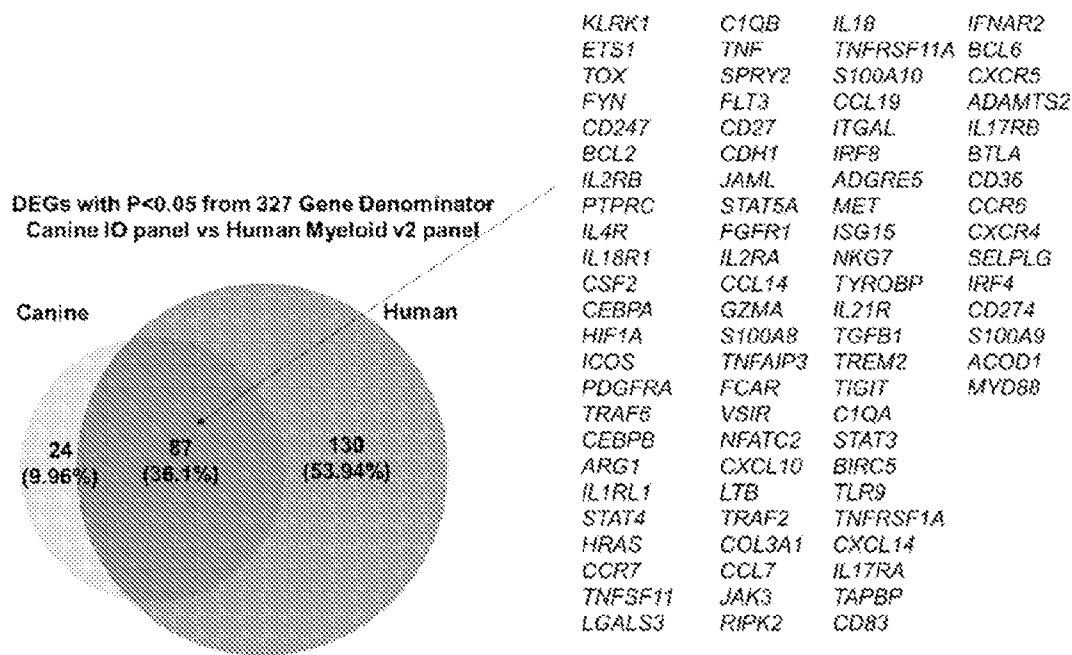
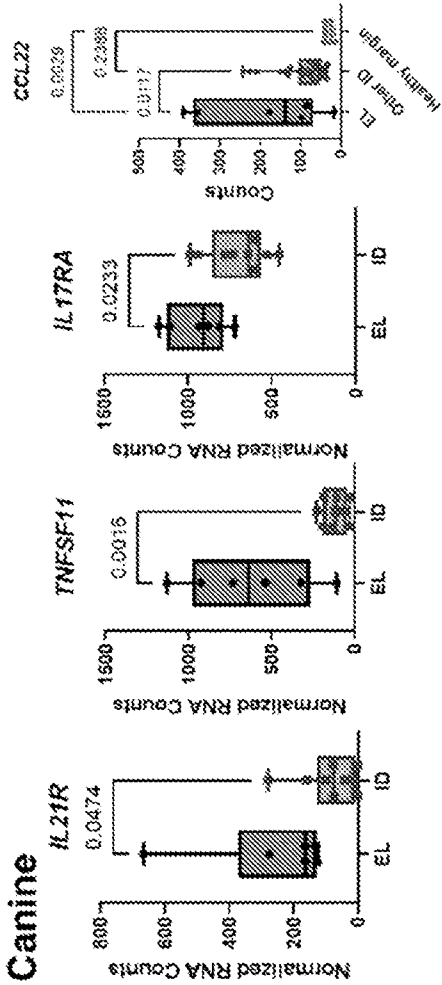
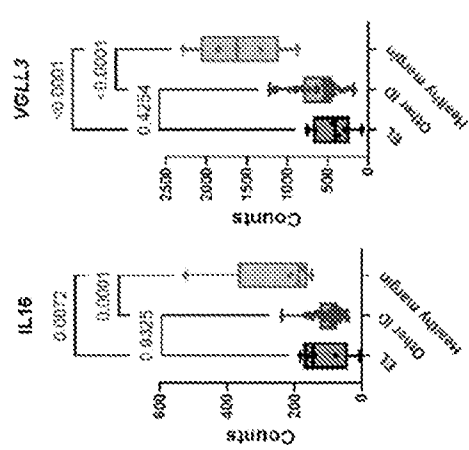


FIG. 5B

Downregulated Genes



Human

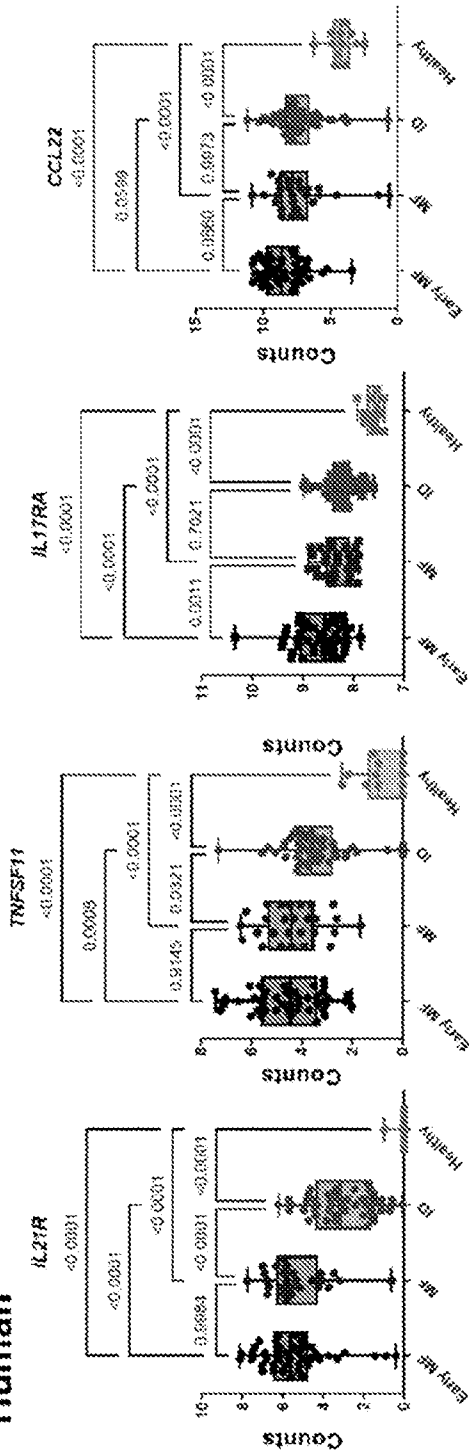


FIG. 5C

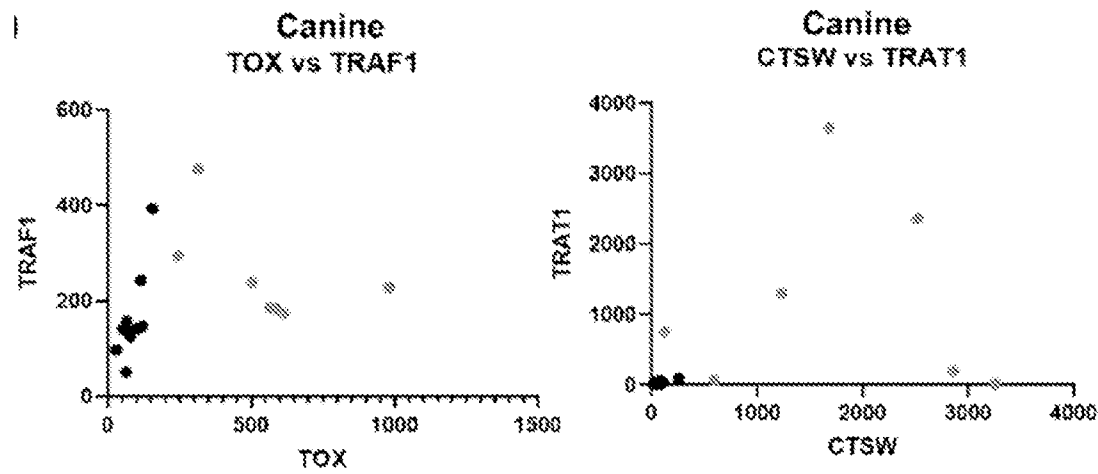


FIG. 5D

26 / 29

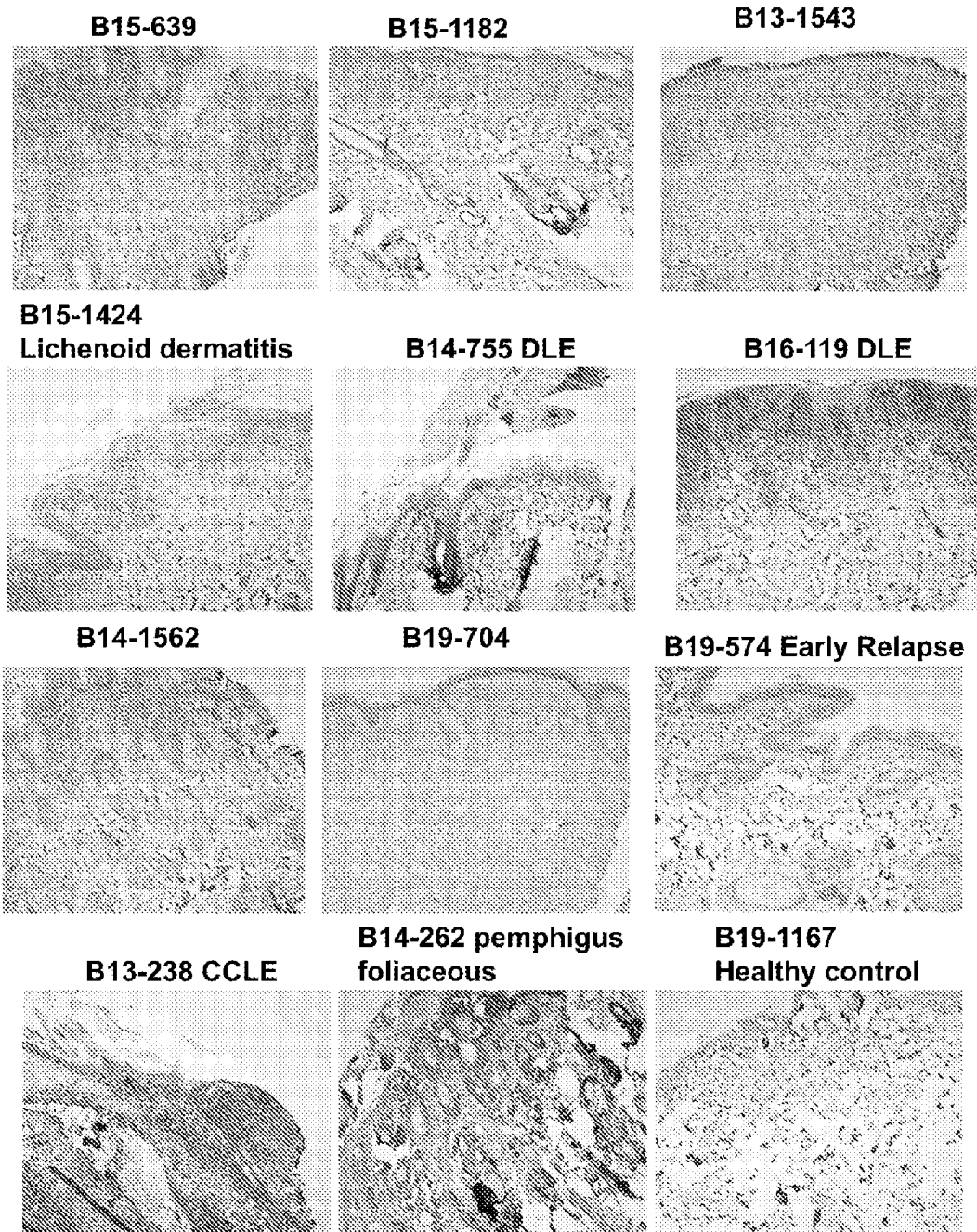


FIG. 6

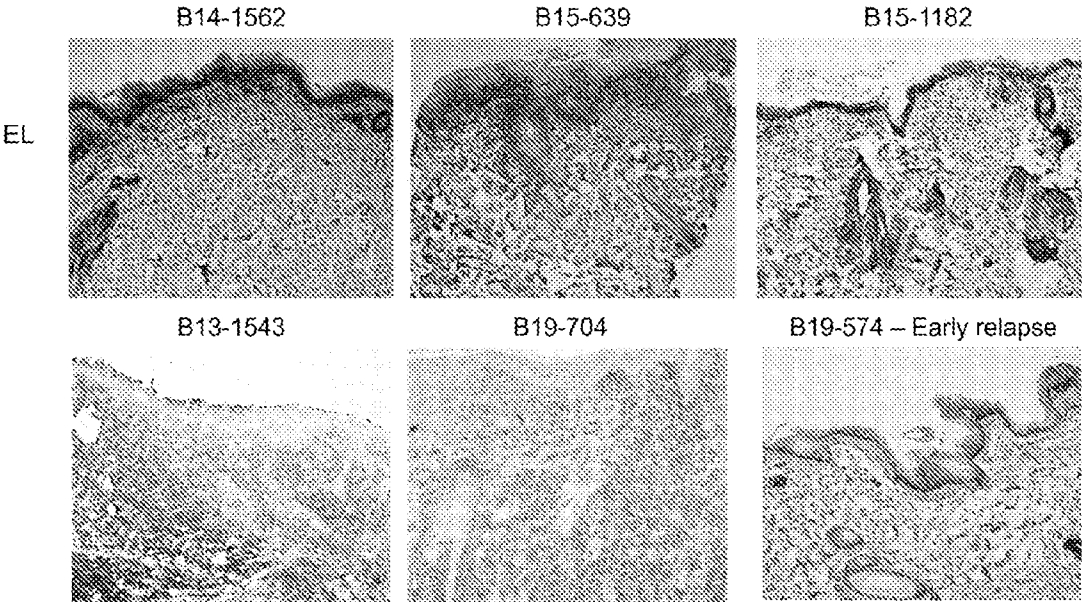


FIG. 7A

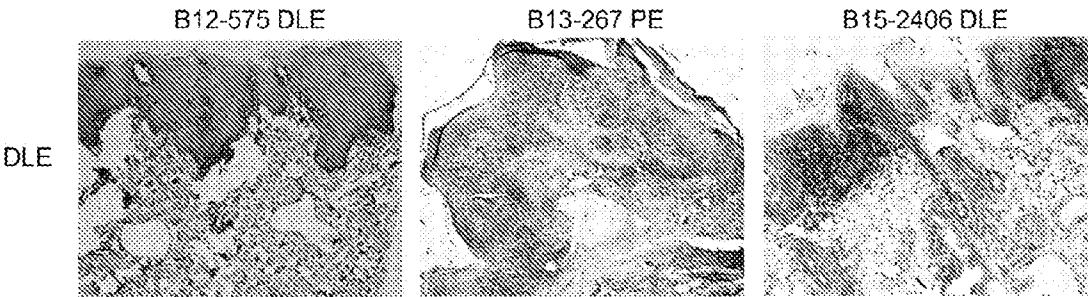


FIG. 7B

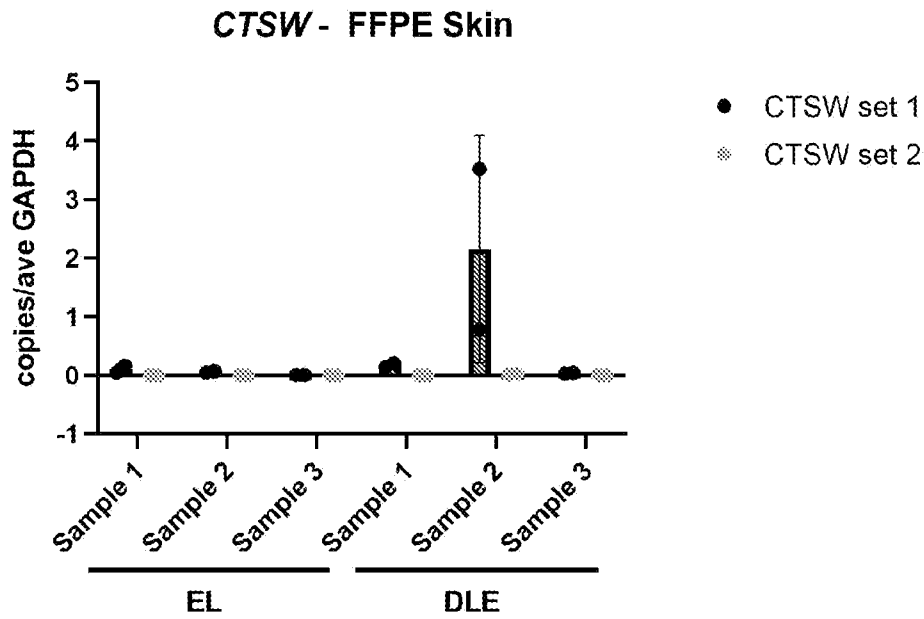


FIG. 8A

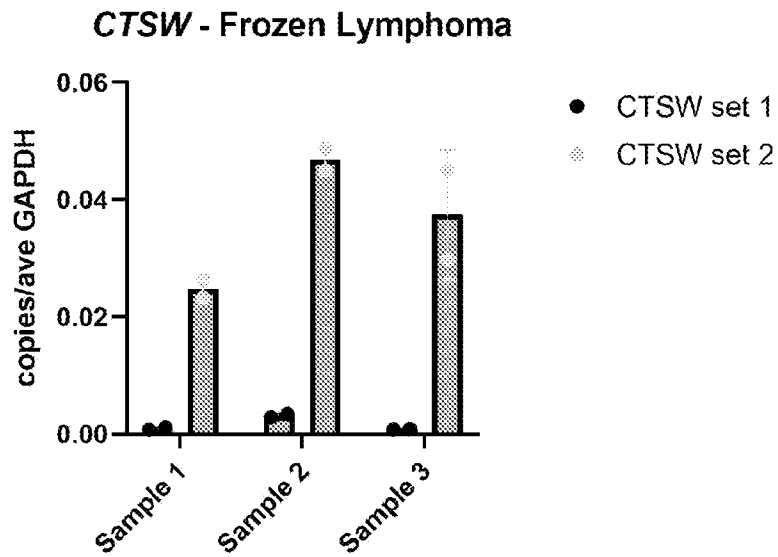


FIG. 8B

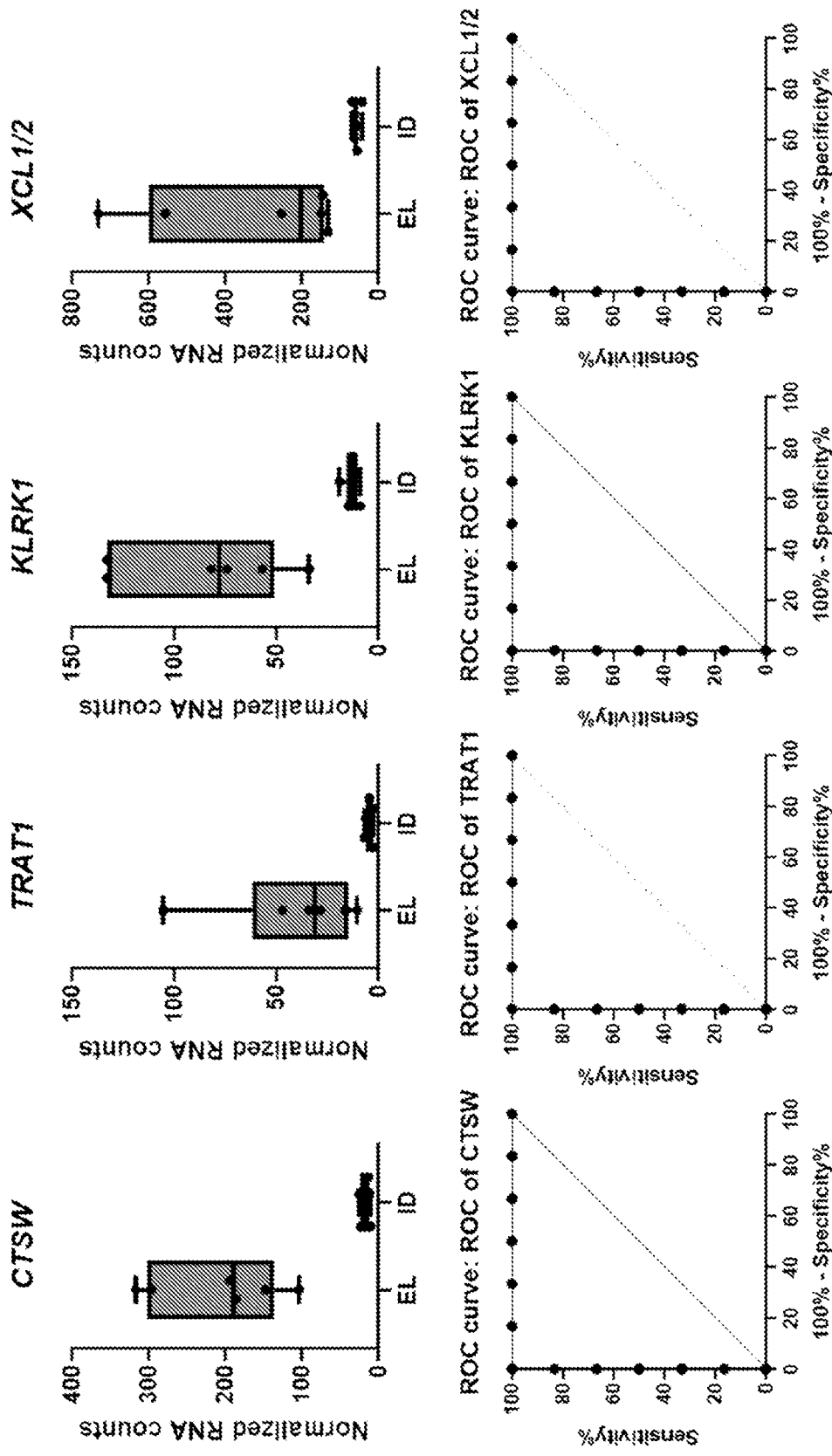


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2023/035230

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - INV. - C12Q 1/6886 (2023.01)

ADD. - C07K 14/47; C12N 15/11, 15/12; C12Q 1/68; G01N 33/50, 33/574, 33/68 (2023.01)

CPC - INV. - C12Q 1/6886; C07K 14/4748; G01N 33/57484 (2023.08)

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2022/256549 A2 (H. LEE MOFFITT CANCER CENTER AND RESEARCH INSTITUTE INC. et al.) 08 December 2022 (08.12.2022) entire document	1-3, 8, 9
X	US 2021/0301023 A1 (GENENTECH INC.) 30 September 2021 (30.09.2021) entire document	10-13
A	EGBETO et al. "Case Series: Gene Expression Analysis in Canine Vogt-Koyanagi-Harada/Uveodermatologic Syndrome and Vitiligo Reveals Conserved Immunopathogenesis Pathways Between Dog and Human Autoimmune Pigmentary Disorders," Front Immunol, 15 December 2020 (15.12.2020), Vol. 11, 590558, Pgs. 1-10. entire document	1-3, 8-13
E, X	OLAYINKA et al. "Cathepsin W, T-cell receptor-associated transmembrane adapter 1, lymphotactin and killer cell lectin like receptor K1 are sensitive and specific RNA biomarkers of canine epitheliotropic lymphoma," Front Vet Sci, 03 November 2023 (03.11.2023), Vol. 10, 1225764, Pgs. 1-11. entire document	1-3, 8-13

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 December 2023

Date of mailing of the international search report

FEB 13 2024

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Taina Matos

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/035230

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed.
 - b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/035230

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-7, 14
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.