Diagnostic and prognostic markers in tumor stage mycosis fungoides and Sézary syndrome

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Evaluation of immunophenotypic and molecular biomarkers for Sézary syndrome using standard operating procedures: a multicenter study of 59 patients

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Journal of Investigative Dermatology 2016;136:1364-72

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ABSTRACT

Differentiation between Sézary syndrome and erythrodermic inflammatory dermatoses can be challenging and a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells that could be useful as additional diagnostic criteria. In this European multicenter study, the sensitivity and specificity of these immunophenotypic and recently proposed but unconfirmed molecular biomarkers in Sézary syndrome were investigated. Peripheral blood CD4+ T cells from 59 patients with Sézary syndrome and 19 patients with erythrodermic inflammatory dermatoses were analyzed for cell surface proteins by flow cytometry and for copy number alterations and differential gene expression using custom-made quantitative PCR plates. Experiments were performed in duplicate in two independent centers using standard operating procedures with almost identical results. Sézary cells showed *MYC* gain (40%) and *MNT* loss (66%); up-regulation of *DNM3* (75%), *TWIST1* (69%), *EPHA4* (66%) and *PLS3* (66%); and down-regulation of *STAT4* (91%). Loss of CD26 (≥ 80% CD4+ T cells) and/ or CD7 (≥ 40% CD4+ T cells) and combination of altered expression of *STAT4*, *TWIST1*, and *DNM3* or *PLS3* could distinguish, respectively, 83% and 98% of patients with Sézary syndrome from patients with erythrodermic inflammatory dermatoses with 100% specificity. These additional diagnostic panels will be useful adjuncts in the differential diagnosis of Sézary syndrome versus erythrodermic inflammatory dermatoses.

INTRODUCTION

Sézary syndrome (SS) is a rare and aggressive type of cutaneous T-cell lymphoma that is derived from CD4+ skin-homing memory T cells and characterized by erythroderma, generalized lymphadenopathy and neoplastic T cells (Sézary cells) in the skin, lymph nodes, and peripheral blood.[1]

Differentiation between SS and erythrodermic inflammatory dermatoses (EID) may be extremely difficult, both clinically and histopathologically, but is very important from therapeutic and prognostic perspectives. For a long time the diagnosis was based on demonstration of atypical T cells, so-called Sézary cells, in blood smears.[2;3] However, it was demonstrated that Sézary cells can also be observed in the peripheral blood of patients with EID and even in healthy control subjects.[4;5] Demonstration of at least 1000 Sézary cells per mm^3 was often used as a decisive criterion, but this was not generally agreed on.[1] To prevent patients with EID being misclassified as having SS and being treated as such, in 1997 the European Organization for Research and Treatment of Cancer group proposed the demonstration of clonal T cells and the presence of an expanded CD4+ T-cell population resulting in a CD4/CD8 ratio above 10 as additional criteria for a diagnosis of SS.[6]

At present, the diagnosis of SS is based on clinical presentation (erythroderma and lymphadenopathy) and demonstration of a T-cell clone in the peripheral blood (preferably the same clone in the skin), in combination with one or more of the following criteria: an absolute Sézary cell count greater than 1000 cells per mm^3; loss of T-cell markers CD2, CD3, CD4, and /or CD5; and /or an expanding population of CD4+ T cells leading to a CD4/CD8 ratio of more than 10.[7;8] However, distinction between SS and EID can still be difficult, because T-cell clonality can be observed in a substantial proportion of patients with EID as well, and not all SS patients have a CD4/CD8 ratio of greater than 10 at first presentation.[9;10]

To solve this diagnostic problem, a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells. Flow cytometry studies reported loss of CD7 and CD26 expression by Sézary cells and suggested CD4+CD7− cells of at least 40% and CD4+CD26− cells of at least 30% as tentative diagnostic criteria in those difficult cases.[10-22]

In addition, recent studies described expression of killer cell immunoglobulin-like receptors CD158a, CD158b, and CD158k and the “central memory” T cell phenotype (CD27+, CD45RA−, CD45RO+) as characteristic features of Sézary cells.[14;16;17;23-30] Molecular investigations identified gain of *JUNB*, *MYC*, and loss of *MYC* antagonists *MNT* and *MXI1* as recurrent genetic lesions in the SS genome.[31-33] Gene expression studies showed increased expression of *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *JUNB*, *TWIST1*, *EPHA4*, and *MYC* and decreased expression of *STAT4* in Sézary cells.[32;34-39]

However, the diagnostic value of these biomarkers in diagnosing SS has not been investigated thoroughly. Moreover, most biomarkers were identified in small, single-center studies with a limited number of patients and controls and have not been confirmed in large independent studies. In addition, flow cytometry studies have used widely differing protocols, which impedes interpretation and comparison of results from

different studies.

The goal of this European Organization for Research and Treatment of Cancer multicenter study was to investigate the sensitivity and specificity of these biomarkers for SS in a large group of well-defined SS patients compared with EID patients using standard operating procedures (SOPs).

METHODS

DESIGN OF THE STUDY

To achieve sufficient power for the study, a consortium of six European Organization for Research and Treatment of Cancer centers with extensive experience with SS was formed including centers from Helsinki, Finland; London, United Kingdom; Leiden, The Netherlands; Mannheim, Germany; Turin, Italy; and Paris, France. At time of inclusion peripheral blood samples were collected for investigation of (i) expression of cell surface proteins by flow cytometry, (ii) copy number variation (CNV), and (iii) gene expression (GE) profiles. The markers were selected based on the literature and are presented in **Supplementary Table S1**.

To optimize standardization and to prevent interdepartmental differences, SOPs were produced for the workflow of blood sampling, isolation of peripheral blood mononuclear cells (PBMCs), and enrichment for CD4+ T cells (SOP 001), DNA isolation (SOP 002), RNA isolation (SOP 003), complementary DNA synthesis (SOP 004), CNV and GE assays (SOP 005), flow cytometry experiments (SOP 007), and the freezing and shipment of samples (SOP 008) (**Supplementary Figure S1** and **Supplementary Materials** online). Much effort was put into standardizing flow cytometry analysis, because this technique has been shown to have limited reproducibility in multicenter studies because of limited standardization of laboratory procedures, instrumental settings, and interpretation of results.[40;41]

To test if the use of SOPs leads to increased reproducibility, the flow cytometry experiments were performed in duplicate in Leiden and Paris on all samples, and assays for CNV and GE were performed in Leiden and repeated in London for a selected number of samples.

In all participating centers the study was approved by the local institutional ethical review boards, and written informed patient consent was obtained. Consensus meetings to compare experimental results were held on August 31, 2012, in Paris and October 31, 2013, in Leiden.

PATIENT SELECTION AND CLINICAL CHARACTERISTICS

Between September 2009 and October 2013 a total of 103 subjects were enrolled with the following diagnosis: SS (n = 72), EID (n = 27), and healthy controls (n = 4).

Inclusion criteria for the SS patients were diagnosis of SS based on the recent World Health Organization - European Organization for Research and Treatment of Cancer criteria and available complete clinical data. Inclusion criteria for patients with EID were

presentation with erythroderma and blood test results not meeting the SS blood criteria.

From the initial 72 patients with SS 13 were excluded because of inferior sample quality (n = 10) or insufficient clinical data (n = 3). From the initial 27 EID patients eight were excluded because of inferior sample quality (n = 3) or insufficient clinical data (n = 5).

The final study group consisted of 59 patients with SS, 19 patients with EID, and 4 healthy controls. The SS group consisted of 32 patients with newly diagnosed SS and 27 patients with known SS. Thirty-six SS patients received treatment at the time of blood sampling (10 newly diagnosed with SS and 26 with known SS). The treatment consisted of psoralen plus UVA therapy (n = 2), extracorporeal photopheresis as monotherapy or combined with immunomodulatory agents (n = 12), prednisone alone or in combination with chlorambucil (n = 9), monotherapy with interferon alfa, bexarotene, methotrexate, or acitretin (n = 11), and polychemotherapy (n = 2).

The EID group included nine patients with atopic erythroderma, five patients with erythrodermic psoriasis, two patients with erythrodermic drug eruption, two patients with idiopathic erythroderma, and one patient with paraneoplastic erythroderma secondary to a cholangiocarcinoma. None of the EID patients developed a lymphoma during follow-up (median follow-up = 22 months, range = 8-38 months).

WORKUP BLOOD SAMPLES

PBMCs were isolated from peripheral blood and stored in liquid- or vapour-phase nitrogen. Part of the fresh PBMCs were enriched for CD4+ T helper cells by depletion of non-CD4+ T cells, resulting in greater than 95% purity for the CD4+ T-cell population, using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) for CNV and GE assays.

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored at -20 °C.

RNA was isolated using the RNeasy Mini Kit (Qiagen), which included on-column DNase digestion. Two µg of RNA was reverse-transcribed in triplicate with the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA), using random priming in a final volume of 20 µL. After synthesis, complementary DNA samples were stored at -20 °C. A detailed description of this workup of blood samples is found in the **Supplementary Materials** (SOPs 001-004 and 008).

FLOW CYTOMETRY

In Leiden and Paris flow cytometry was performed for the following antigens: CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD27, CD45RA, CD45RO, CD158a, CD158b, and CD158k, including isotype-specific controls (**Supplementary Table S2** and SOP 007 in the **Supplementary Materials**). Lymphocytes were gated from forward and sideward scatter patterns; next, antigen expression was assayed from CD4+ gated lymphocytes. Specific antigen expression was observed relative to autofluorescence and (non-)specific signals obtained from each individual patient and a PBMC control sample. This control sample was derived from two healthy donors and functioned as internal reference sample each

flow cytometry session. Samples were analyzed in a blinded setting.

Specific antigen expression in the population of gated cells (expression or loss) was displayed in percentages (**Figure 1a and b**). Antigen expression was considered dim if

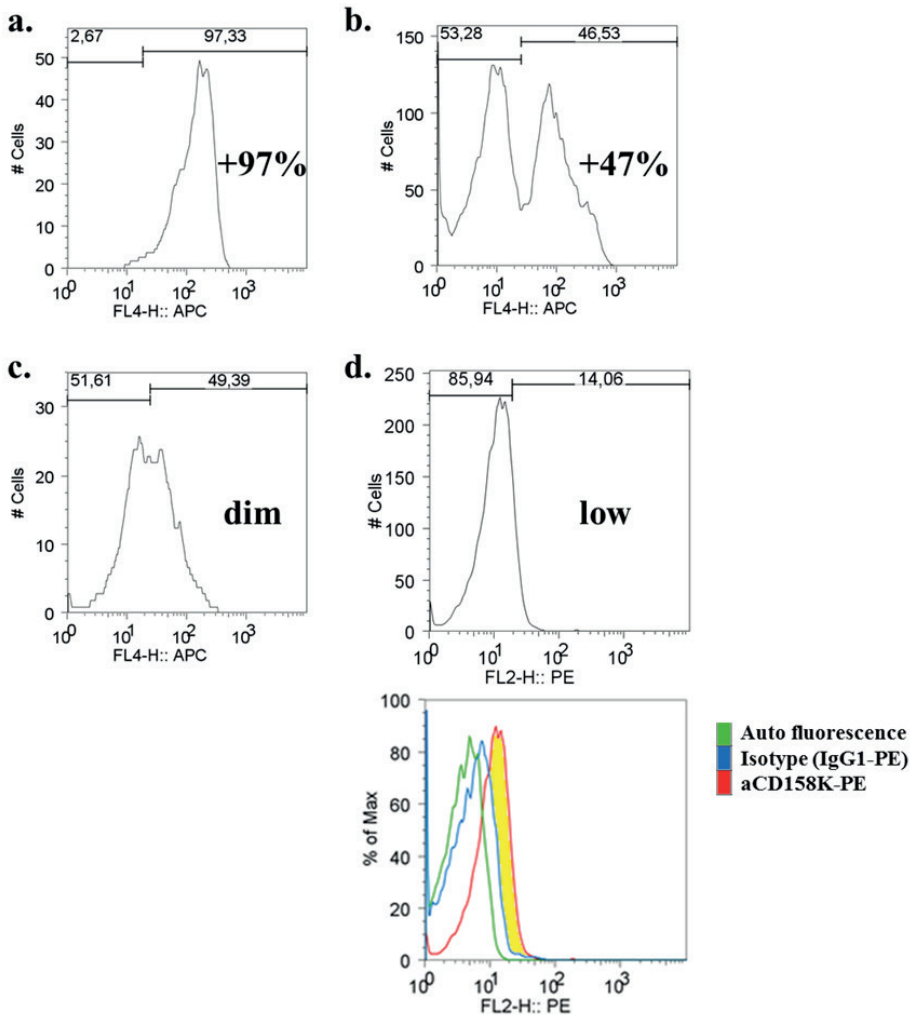

Figure 1. Interpretation of flow cytometry results.
a. A single peak located on the right side of the determined threshold represents a single population with positive staining for CD7 in 97% of the gated cells; b. Two distinct populations of the gated cells, in which 47% of the cells show positive expression for CD7 and 53% show negative staining; c. Diminished expression for CD7 of a single population of gated cells, surrounding a determined threshold, is indicated as "dim". d. The specific CD158k antigen expression signal (indicated in red) is slightly shifted to the right compared to its auto fluorescence and isotype control signals (green and blue lines), as is indicated in yellow. This implicates that the gated cells do express CD158k but at very low level, indicated as "low".

all gated cells showed diminished expression around the determined threshold (**Figure 1c**). For CD158a, CD158b, and CD158k, expression below 5% of the gated cells was considered as no expression, but when intermediate expression of a single population of gated cells, surrounding a determined threshold, was found, this was characterized as low-expressing antigen (for example, CD158k^{low}) (**Figure 1d**).

COPY NUMBER VARIATION ASSAY

Quantitative PCR assays with FAM labelled hydrolysis MGB probes (Life Technologies, Carlsbad, CA) were developed for target genes *JUNB*, *TWIST1*, *MYC*, and *MNT* and reference genes *ABT1*, *ARG2*, and *DNM3* (**Supplementary Table S3**). Reference genes were selected from different large copy number-stable chromosomal regions in SS, selected from array-based comparative genomic hybridization experiments on 20 SS samples.[33] Amplification efficiency was evaluated in triplicate, using eight 4-fold serial dilution points ranging from 3 ng/µL to 183 fg/µL DNA concentration, under optimised primer and hydrolysis probe concentrations. Assays with amplification efficiency value between 90% and 100% and a correlation coefficient above 0.98 were accepted for CNV analysis. Assays were performed on custom-made PCR plates (Life Technologies) following SOP 005 (**Supplementary Materials** online).

Data were normalized against reference genes and relative to the common reference using the ΔΔCq method and are presented as relative copy number, where 2 stands for diploid DNA.[42] The following thresholds were maintained for the CNV data: 1.5-2.5 was considered as diploid (normal) DNA, greater than 2.5 as gain in copy number, and less than 1.5 as loss in copy number.

GENE EXPRESSION ASSAY

GE quantitative PCR assays with FAM labelled hydrolysis MGB probes (Life Technologies) were developed and validated, as described for CNV quantitative PCR assays, for target genes *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC*, *JUNB*, *TWIST1*, *EPHA4*, and *STAT4* and reference genes *ARF5*, *ERCC3*, and *TMEM87A* (**Supplementary Table S3**). Stably expressed reference genes were selected from microarray experiments on SS samples and validated in SS and EID samples according to the GeNorm method.[37;38;43] Assays were performed on custom-made PCR plates (Life Technologies) following SOP 005. Receiver operating characteristic curve analysis was used to determine fixed cut-off thresholds for each individual gene expression quantitative PCR assay with a specificity of 100% and an accuracy above 0.80. An one-tailed Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. *P*-values below 0.05 were regarded as statistically significant.

RESULTS

PATIENT CHARACTERISTICS

Clinical characteristics at diagnosis of the 59 SS patients and 19 EID patients are summarized in **Table 1.**

The patients with SS had a T-cell clone in the peripheral blood (59 of 59), a CD4/CD8 ratio above 10 (53 of 57) and/or a Sézary cell count above 1000 per mm^3 (34 of 43, including all four patients with a CD4/CD8 ratio lower than 10).

One EID patient showed a T-cell clone in the peripheral blood, and another EID patient had a CD4/CD8 ratio above 10 because of very low numbers of CD8+ T cells, but none had a Sézary cell count above 1000 per mm^3 (**Table 1**).

FLOW CYTOMETRY

Flow cytometry experiments were performed both in Leiden and Paris for all 59 SS patients and 19 EID patients. Differences in flow cytometry results between Leiden and Paris were less than 20% in 99.8% of individual assays; and in these cases an average was used in further analysis. In only 2 of 1027 assays (0.2%) did the differences in results exceeded 20%, and these were therefore excluded from further analysis.

In this study, 87% of the SS patients (46 of 53) had a CD4/CD8 ratio above 10 at inclusion, compared with 8% of the EID patients (1 of 12) (sensitivity = 87%, specificity = 92%).

The CD4+ gated lymphocytes were CD3+ and CD8−. In the CD4+ T-cell population, 7 of 59 (12%) SS patients showed loss for CD2 (median = 45%, range = 32-100%) and 4 (7%) patients showed diminished expression for CD2 ($CD2^{dim}$), whereas this was never observed in the 19 EID cases. One SS patient showed 90% CD5 loss, compared with none of the EID patients.

In the CD4+ T-cell population, a percentage of CD4+CD7− cells above 40% was found in 32 of 59 (54%) SS patients but never in EID patients (sensitivity = 54%, specificity =

Table 1. Clinical characteristics at diagnosis of the 59 patients with Sézary syndrome and 19 patients with erythrodermic inflammatory dermatoses.

Values at diagnosis SS versus erythroderma EID	SS, n=59	EID, n=19
Male-female ratio	37:22	16:3
Age in years, median (range)	65 (32-89)	67 (29-86)
Erythroderma	46/52 (88%)	19/19 (100%)
Pruritus	45/52 (87%)	13/19 (68%)
Ectropion	6/52 (12%)	1/19 (5%)
Hyperkeratosis hand/feet	21/52 (40%)	3/19 (16%)
Palpable lymphadenopathy	21/52 (40%)	4/19 (21%)
Lymphadenopathy confirmed by CT scan	17/41 (41%)	1/7 (14%)
Leukocytes ≥10.0 $x10^9$/L	40/56 (71%)	5/14 (36%)
CD4/CD8 ratio ≥10.0	53/57 (93%)	1/12 (8%)
Absolute Sézary cell count ≥1000 per mm^3	34/43 (79%)	0/10 (0%)
T-cell clone in peripheral blood	59/59 (100%)	1/17 (6%)
Identical T-cell clone in blood and skin	32/38 (84%)	0/3 (0%)

EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome

100%). In addition, $CD7^{dim}$ was found in 2 of 59 (3%) SS patients, compared with none of the EID patients.

In the CD4+ T-cell population a percentage of 30% or more CD4+CD26− cells was found in 51 of 59 (86%) SS patients but also in 10 of 19 (53%) EID patients (sensitivity = 86%, specificity = 47%). When shifting the percentage to 80%, 39 of 59 (66%) SS patients but none of the EID patients had CD4+CD26− cells of 80% or more in the CD4+ T-cell population (sensitivity = 66%, specificity = 100%). In addition, $CD26^{dim}$ was found in 5 of 59 (8%) SS patients and in 1 of 19 (5%) EID patients.

Loss of CD26 by more than 80% and/ or loss of CD7 by more than 40% of CD4+ T cells was found in 49 of 59 (83%) SS patients but was never observed in the EID patients (sensitivity = 83%, specificity = 100%).

Investigations on CD158k expression showed that more than 5% of these CD4+ T cells expressed CD158k or $CD158k^{low}$ in 19 of 58 (33%) SS patients, compared with 1 of 19 (5%) EID patients (sensitivity = 33%, specificity = 95%). The results (including expression of CD158a and CD158b) are summarized in **Table 2**.

No major difference was observed in the expression of CD27, CD45RA and CD45RO, by CD4+ T cells between SS and EID patients (data not shown).

COPY NUMBER VARIATION

CNV experiments were performed in 58 SS patients and 17 EID patients in Leiden. Duplicate experiments for 14 samples were performed in London, which gave identical results (**Supplementary Figure S2**).

In 47 of 58 (81%) SS patients, alterations in copy number were found compared with none of the 17 EID patients. Gain of *MYC* was observed in 23 of 58 (40%) SS patients (sensitivity = 40%, specificity = 100%). *MNT* loss was found in 38 of 58 (66%) SS patients (sensitivity = 66%, specificity = 100%) and one (2%) patient showed gain of *MNT* (**Figure 2**). Gain of *MYC* and/ or loss of *MNT* was found in 76% (44 of 58) of SS patients (sensitivity = 76%, specificity = 100%). Copy number alterations of *JUNB* and *TWIST1* were found in only a minority of SS patients (**Figure 2**).

Table 2. Overview of the tested flow cytometry markers in 59 patients with Sézary syndrome and 19 patients with erythrodermic inflammatory dermatoses at inclusion in the study.

Markers for SS described in literature	SS, n=59	EID, n=19	Sensitivity (%)	Specificity (%)
CD4/CD8 ratio ≥ 10	46/53	1/12	87	92
CD4+CD7− ≥ 40%	32/59[1]	0/19	54	100
CD4+CD26− ≥ 30%	51/59[2]	10/19	86	47
CD158a*	2/58	0/19	3	100
CD158b*	13/59	1/19	22	95
CD158k*	19/58	1/19	33	95

EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome
*Low expression and expression of 5% or more of the CD4+ lymphocytes.
[1]Including 4/7 SS patients with a CD4/CD8 ratio below 10.
[2]Including 5/7 SS patients with a CD4/CD8 ratio below 10.

GENE EXPRESSION

GE analyses were performed on 55 SS, 19 EID, and 4 healthy control cases in Leiden. Thirty samples were also analyzed in London, with identical results in 28 samples (**Supplementary Figure S3**); two samples could not be analyzed because of a technical error.

DNM3, *TWIST1, EPHA4*, *PLS3*, and *STAT4* were the most differentially expressed genes in SS patients compared with EID patients and healthy controls with 100% specificity ($P < 0.001$) (**Table 3, Supplementary Figure S4**). Up-regulation of *DNM3*, *TWIST1, EPHA4*, and *PLS3* was found in 66−75% of SS patients, and *STAT4* was down-regulated in 91% of SS patients (**Figure 3**). Up-regulation of *DNM3*, *TWIST1, EPHA4*, and *PLS3* was found in 41 of 55 (75%), 38 of 55 (69%), 36 of 55 (66%), 36 of 55 (66%) of SS patients, respectively, and *STAT4* was down-regulated in 50 of 55 (91%) of SS patients. Combining alterations in gene expression (*STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3*), we could

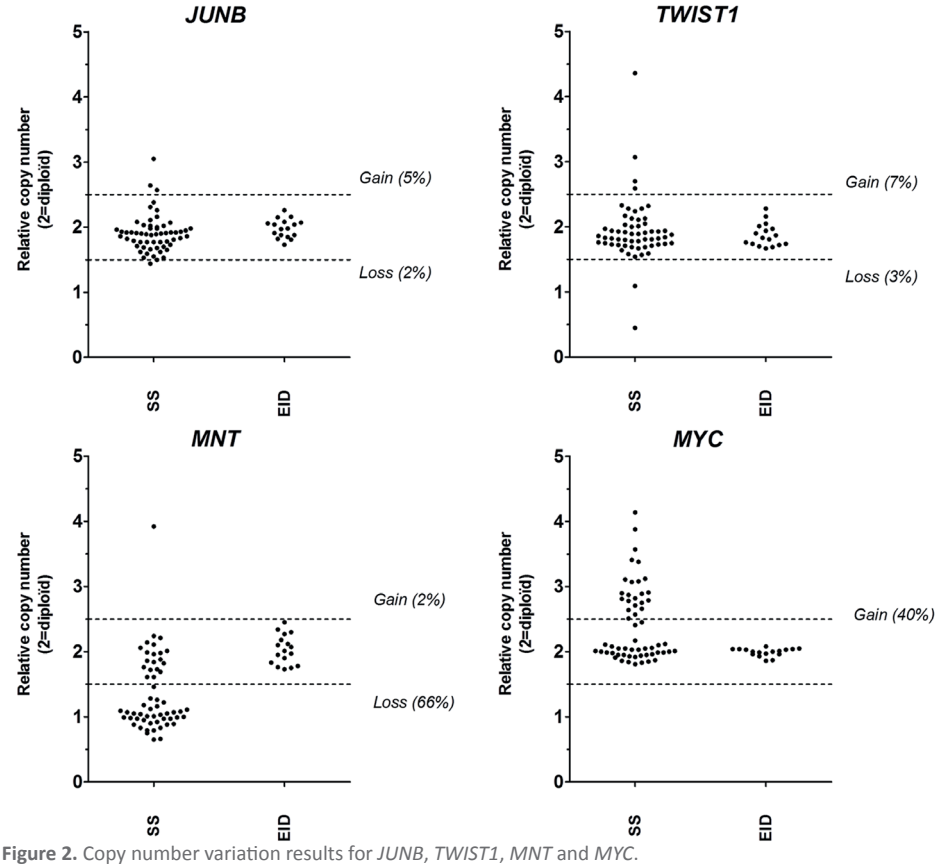

Figure 2. Copy number variation results for *JUNB*, *TWIST1*, *MNT* and *MYC*.
The gains and losses in copy number in 58 SS compared to 17 EID cases are shown as normalized relative copy number, where 2 represents diploid DNA. The dotted lines signifies the chosen thresholds for gain and loss, 2.5 and 1.5, respectively. EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome

distinguish 54 of 55 (98%) SS patients from all EID patients (sensitivity = 98%, specificity = 100%).

Aberrant gene expression of *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC* and *JUNB* was found in only a minority of SS patients (**Table 3**).

DISCUSSION

In the present multicenter study, we investigated the diagnostic sensitivity and specificity of immunophenotypic and molecular biomarkers in SS using SOPs. We show that by using SOPs, it is possible to obtain highly reproducible results for flow cytometry, CNV, and GE analysis and show that loss of CD7 and CD26 by CD4+ T cells; gain in copy number of *MYC* and loss of *MNT*; increased expression of *DNM3, TWIST1, EPHA4*, and *PLS3*; and decreased expression of *STAT4* are highly characteristic for Sézary cells.

In the current study the most SS patients have a CD4/CD8 ratio above 10, but we also show that a significant minority of patients (13%) does not fulfil this diagnostic criterion. For these patients additional immunophenotypic markers are clearly needed.

Comparison of results from previous flow cytometry studies is hindered by the use of different protocols and cell populations. In the present study we focused on CD4+ gated T cells because CD4 is rarely lost by Sézary cells, facilitating the comparison of expression levels of different immunophenotypic markers.

Previous studies reported that a CD4+CD26− cell population greater than 30% had a sensitivity of 97% and specificity of 100% in diagnosing peripheral blood involvement

Table 3. Results of aberrant gene expression in all tested genes in 55 patients with Sézary syndrome relative to 19 patients with erythrodermic inflammatory dermatoses and 4 healthy control subjects.
With the receiver operating characteristic curve analysis a threshold was established at a specificity of 100% and an accuracy above 0.80. *PLS3*, *DNM3*, *TWIST1*, *EPHA4* and *STAT4* and were found to be useful diagnostic markers in Sézary syndrome.

Up-/down-regulation	SS, n=55	Sensitivity (%)
PLS3	36/55	66
DNM3	41/55	75
CDO1	20/55	36
TRAIL	4/55	7
CD1D	6/55	11
GATA3	2/55	4
MYC	0/55	0
JUNB	9/55	16
TWIST1	38/55	69
EPHA4	36/55	66
STAT4	50/55	91

SS, Sézary syndrome

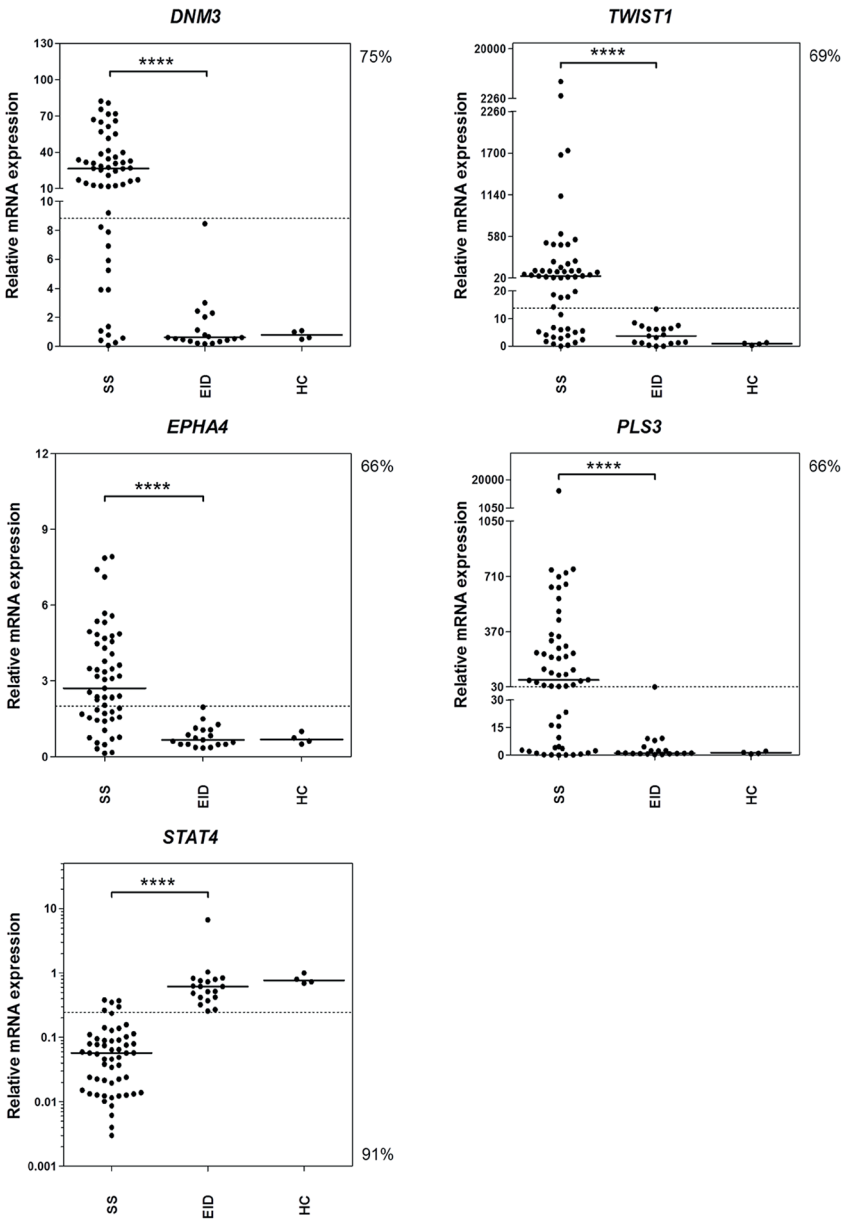

Figure 3. Gene expression results for *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and *STAT4*.
The differential gene expression is shown as relative normalized mRNA levels in 55 SS compared to 19 EID and 4 HC cases. **** represents the statistical significant difference in gene expression in SS compared to EID and HC ($P < 0.001$). The dotted lines represent the thresholds for differential expression, determined with receiver operating characteristic curves with a specificity of 100%. The Y-axis represents the relative mRNA expression with varying scale in all figures. EID, erythrodermic inflammatory dermatoses; HC, healthy control; mRNA, messenger RNA; SS, Sézary syndrome

and suggested this cut-off point as tentative diagnostic criterion for SS.[10;18] Indeed, loss of CD26 in more than 30% of CD4+ T cells was found in 86% of SS patients but also in 53% of EID patients, resulting in a specificity of 47%. However, when using a percentage of 80% as the cut-off point, we found that 39 of 59 (66%) SS patients but none of the EID patients met this criterion.

These discrepant results can be explained by different flow cytometry protocols. We evaluated CD26 expression on CD4+ gated T cells, whereas Bernengo et al looked at CD4+CD26− cells on gated total lymphocytes. Indeed, when looking at the CD4+CD26− cells of 30% or more on total lymphocytes, similar results were found (sensitivity = 80%, specificity = 95%; data not shown).

A level of CD4+CD7− cells of more than 40% has also been suggested as tentative criterion in the diagnosis of SS.[21] Consistent with literature, we found that loss of CD7 above 40% of the CD4+ T cells is highly specific (100% specificity) but not a sensitive marker (sensitivity = 54%).[10;11;44] Similar results were found for 40% or more CD4+CD7− cells on total lymphocytes (sensitivity = 42%, specificity = 100%; data not shown).

Flow cytometry results show that in 83% of SS patients, CD4+ T cells display loss of CD26 by more than 80% and/or loss of CD7 by more than 40%, whereas this was never observed in EID patients. These observations are relevant because they can readily be included in immunophenotypic testing of erythrodermic patients.

Previous studies reported expression of killer cell immunoglobulin-like receptor CD158k in 65–97% of SS patients.[16;25;26] Flow cytometry analysis, performed in both Leiden and Paris, showed expression of CD158k in only 33% of SS patients (19 of 58 cases), and in most of these SS patients (18 of 19) the CD158k antigen was expressed at low levels. This discrepancy can be explained by the fact that the present study was performed on frozen PBMCs instead of freshly isolated PBMCs. Indeed, a recent study on freshly isolated PBMCs from SS patients showed high CD158k expression in Sézary cells.[45]

For CNV and GE analysis the use of SOPs and custom made PCR platforms led to highly reproducible results as well. Gain of *MYC* and/ or loss of its antagonist *MNT* was found in 76% of SS patients but never in EID. Gain of *TWIST1* and *JUNB* was detected in only a small minority of SS patients.

In line with the literature, we found up-regulation of *DNM3*, *TWIST1, EPHA4*, and *PLS3* and down-regulation of *STAT4* in most of SS patients.[34-39] In contrast, only a minority of SS patients showed up-regulation of *GATA3*, *CD1D*, *TRAIL*, *CDO1*, *JUNB*, and *MYC*, implying that these genes are not useful diagnostic markers. Why gain of *MYC* and loss of *MNT* which is observed in most of patients, does not lead to up-regulation of *MYC* expression is as yet unexplained.

Combined alterations in gene expression of *STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3* could distinguish 98% of SS patients from EID patients, suggesting that this diagnostic panel will be useful as additional molecular criterion in the diagnostic differentiation between SS and EID.

In the present study 27 patients were diagnosed with SS before inclusion in the study. However, no significant differences were found in the prevalence of the previously

described markers between 27 patients already diagnosed with SS before inclusion in the study and the 32 SS patients newly diagnosed at time of inclusion (**Supplementary Table S4**). Similarly, the prevalence of investigated markers was similar in 36 SS patients who received treatment during sample collection at inclusion and the 23 SS patients who did not receive any form of treatment (**Supplementary Table S5**). These observations argue that the observed immunophenotypic and molecular changes are stably expressed in Sézary cells.

We show that standardization of flow cytometry, CNV, and GE procedures leads to strong reproducibility of results. We argue that to facilitate comparison of results from different centers, it will be important to closely define the subset of cells that was investigated, and based on the present study we suggest gating on CD4+ T cells in future studies.

For patients in whom the distinction between SS and EID still cannot be made using the current diagnostic criteria, we propose that these two additional diagnostic panels can be used: (i) loss of CD26 (≥ 80% CD4+ T cells) and/ or loss of CD7 (≥ 40% CD4+ T cells) for immunophenotypic analysis and (ii) combination of altered gene expression of *STAT4*, *TWIST1*, and *DNM3* or *STAT4*, *TWIST1*, and *PLS3* for molecular analysis.

ACKNOWLEDGEMENTS

This study was supported by research funding from the Dutch Cancer Society to M.H. Vermeer, the Helsinki University Central Hospital Research Funds grant TYH2012232 to A. Ranki and the INCa-DGOS-Inserm grant to M. Bagot. In addition, this research was supported by grants from the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health. We would like to thank all participating patients, Kaija Järvinen, technician at the Department of Dermatology and Allergology, University of Helsinki and Skin and Allergy Hospital, Helsinki University Central Hospital, Finland and Anneliese Pfisterer, technician at the Department of Dermatology, Venereology and Allergy, University Medical Center Mannheim, Ruprecht-Karls-University of Heidelberg, Germany, for their technical help, Francette Jean-Louis, technician at the Skin Research Institute, Hospital Saint-Louis, France, for analyzing data and Emma Kent from St. John's Institute of Dermatology, Division of Genetics and Molecular Medicine, Faculty of Life Sciences & Medicine, King's College London, United Kingdom, Dr. H.B. Thio of the department of Dermatology, Erasmus Medical Center, Netherlands and P.R. Nijboer, dermatologist in the Gemini Hospital, Netherlands, for providing clinical data.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Biomarkers for Sézary syndrome selected from literature that are investigated in the present study. The arrows indicate the reported expression in Sézary syndrome.

Protein expression	Copy number variation	Gene expression
CD7↓	*JUNB*↑	*PLS3*↑
CD26↓	*TWIST1*↑	*DNM3*↑
CD27+	*MYC*↑	*CDO1*↑
CD45RA−	*MNT*↓	*TRAIL*↑
CD45RO+		*CD1D*↑
CD158a↑		*GATA3*↑
CD158b↑		*MYC*↑
CD158k↑		*JUNB*↑
		TWIST1↑
		EPHA4↑
		STAT4↓

Supplementary Table S2. List of monoclonal antibodies.

CD marker	Clone	Isotype	Fluorochrome	Source
CD2	S5.2*/ RPA-2.10#	mouse IgG2a	APC*/ PE-Cy5#	BD Pharmingen
CD3	SK7*/ UCHT1#	mouse IgG1	APC*/ FITC#	BD Pharmingen
CD4	SK3*/ SFCI12T4D11#	mouse IgG1	PerCP-Cy5.5*/ PC7#	BD Pharmingen/Beckman Coulter
CD5	UCHT2	mouse IgG1	PE*/ PE-Cy5#	BD Pharmingen
CD7	eBio124-1D1*/ M-T701#	mouse IgG1	APC*/ PE-Cy5#	eBioScience/ BD Pharmingen
CD8	SK1	mouse IgG1	FITC	BD Pharmingen
CD26	M-A261	mouse IgG1	FITC	BD Pharmingen
CD27	M-T271	mouse IgG1	APC*/ PE#	BD Pharmingen
CD45RA	L48	mouse IgG1	FITC	BD Pharmingen
CD45RO	G155-178*/ UCHL1#	mouse IgG2a	PE*/ PE-Cy5#	BD Pharmingen
CD158a	HP-3E4	mouse IgM	FITC*/ PE#	BD Pharmingen
CD158b	CH-L	mouse IgG2b	PE	BD Pharmingen
CD158k	AZ158	mouse IgG1	PE	Innate Pharma

*Antibody used in Leiden

#Antibody used in Paris

Supplementary Table S3. List of optimised copy number variation and gene expression primers and hydrolysis probes used in quantitative PCR assays.

Assay	Gene	Reference	Forward primer (5'-3')	(nM)
CNV	*TWIST1*		CCGCTAGGGAGAGCAGTCTC	900
	MNT		CACGCCTGTCCTGACCATAG	300
	MYC		GCCGCATCCACGAAACTTTG	900
	ABT1	YES	TGCATGTCCTGTTGCTTCGC	900
	ARG2	YES	AAGAGAAGCAAAGTGGGGAGTAG	900
	DNM3	YES	CTAAACACCTCTGCTGATTTCTGC	300
	JUNB			
GE	*PLS3*		CGGGCTGGCAAAAAATTAAC	900
	DNM3		GGATGACTCCTGGATACAGCACT	300
	CDO1		GCAATGTACGCCAAGTTCGA	300
	TRAIL		GCTCACATAACTGGGACCAGAGGAAG	900
	CD1D		ACCAGGGACAAGCAGGTAAGAG	300
	GATA3		ACAACCTGGTCCCGTTTTATTCTGC	300
	STAT4		CGTGTCAACCAACGATTCCCAGAA	900
	MYC		GGTGCTCCATGAGGAGACAC	900
	ERCC3	YES	ATATCCAAGGTAGGTGACACTTCG	900
	TMEM87A	YES	CATCTGGACAACCATGAAGTTCAG	300
	ARF5	YES	TGCTGATGAACTCCAGAAGATGC	900
	JUNB			
	TWIST1			
	EPHA4			

CNV, copy number variation; GE, gene expression

Reverse primer (5'-3')	(nM)	Probe (5'-3')	(nM)
TGACCCTGGGTGTCTCTGTC	900	CCCTCCTGTCACGCACACTCACGC	200
TTCACTGGATTTGACTTCTTCAGC	900	TGCCAACTTCAGGGTCCCCAGCGT	150
GTCCTTGCTCGGGTGTTGTAAG	900	CAAAGTGCCCGCCCGCTGCTATGG	250
CCAGCATCCTCACAGACTGATTC	900	CCAGCCAGGAGCCAAGCACCGC	250
GTGTGATCAAACATACAGCCTCAG	900	AGCCGTGGTCCCAGGTCTAACCCC	250
CCGCCTTTCATGATGCCAATG	900	TGAGCCACCCCTTGCGAATCACCT	250
Assay: Hs00357891_s1 (Life Technologies)			
GCGATTTGATTGAGAAGATGGAA	900	TTTAGTGCTGACATCAAGGATTCCAAAGCCTA	250
GCCGGATGTGGGCCTT	900	AGGTCACCTCCTCCAAGCCCCAC	200
TGTCCTTCACCCCAACAGAGA	900	CGAAATCTTGTGGATCAAGGAAATGGAAA	200
AGTTGCTCAGGAATGAATGCCCAC	900	AAGGCTCTGGGCCGCAAAATAAACTCCTG	250
ACGGTTAAAGCCAAGCCAGG	900	CTGGAACACACATGTCCTATCCAAAGGAATCAGC	250
GCTGGGAAGCAAAGGTGAGCAAAG	900	CCCTTCTTCTCTTTGCTAAACGACCCCTCC	250
AAACTGCCAGCTCATCACCTCCAG	900	TTCTTTAATAATCCTCCACCTGCCACATTGAGTCA	250
CAGCAGAAGGTGATCCAGACTC	900	CCACCACCAGCAGCGACTCTGAGG	250
TTGTACTCTTCTGCAACCATCCC	900	CGAAGCACCCGCCCTAGCCTTTGG	200
AGGATCATGGAGAACAGCAAGC	900	ATCGTCTACCCACAGCTCCCGCCA	200
CGGCTGCGTAAGTGCTGTAG	900	TGTCAGTCAGCTCGCTCACGGGCA	200
Assay: Hs00357891_s1 (Life Technologies)			
Assay: Hs00361186_m1 (Life Technologies)			
Assay: Hs00953175_m1 (Life Technologies)			

Supplementary Table S4. Overview of the biomarkers in patients who were diagnosed with Sézary syndrome before inclusion in the study compared with patients who were newly diagnosed at time of inclusion of the study. The Chi-squared test was performed to test for statistical significant differences in marker distribution between these two groups.

	Diagnosis SS before inclusion, n=27		**New diagnosis SS at inclusion, n=32**		***P*-value**
Flow cytometry markers					
CD4/CD8 ratio ≥ 10	16/21	(76%)	30/32	(94%)	0.065
CD4+CD7− ≥ 40%	14/27	(52%)	18/32	(56%)	0.735
CD4+CD26− ≥ 30%	21/27	(78%)	30/32	(94%)	0.074
CD158a*	1/26	(4%)	1/32	(3%)	0.881
CD158b*	4/27	(15%)	9/32	(28%)	0.219
CD158k*	10/26	(38%)	9/32	(28%)	0.404
Copy number variation markers					
JUNB gain	2/26	(8%)	1/32	(3%)	0.383
TWIST gain	3/26	(12%)	1/32	(3%)	0.212
MYC gain	9/26	(35%)	14/32	(44%)	0.479
MNT loss	17/26	(65%)	21/32	(66%)	0.650
Gene expression markers					
DNM3 up-regulation	17/23	(74%)	24/32	(75%)	0.927
EPHA4 up-regulation	15/23	(65%)	21/32	(66%)	0.975
PLS3 up-regulation	14/23	(61%)	22/32	(69%)	0.544
TWIST1 up-regulation	16/23	(70%)	22/32	(69%)	0.949
STAT4 down-regulation	21/23	(91%)	29/32	(91%)	0.931

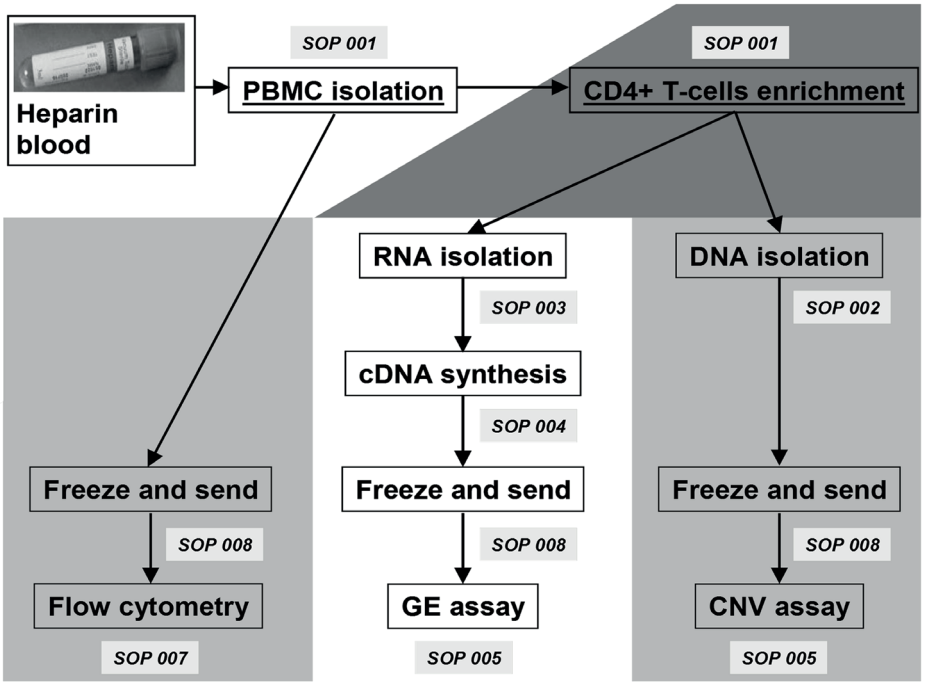
SS, Sézary syndrome

*Low expression and expression of 5% or more of the CD4+ lymphocytes.

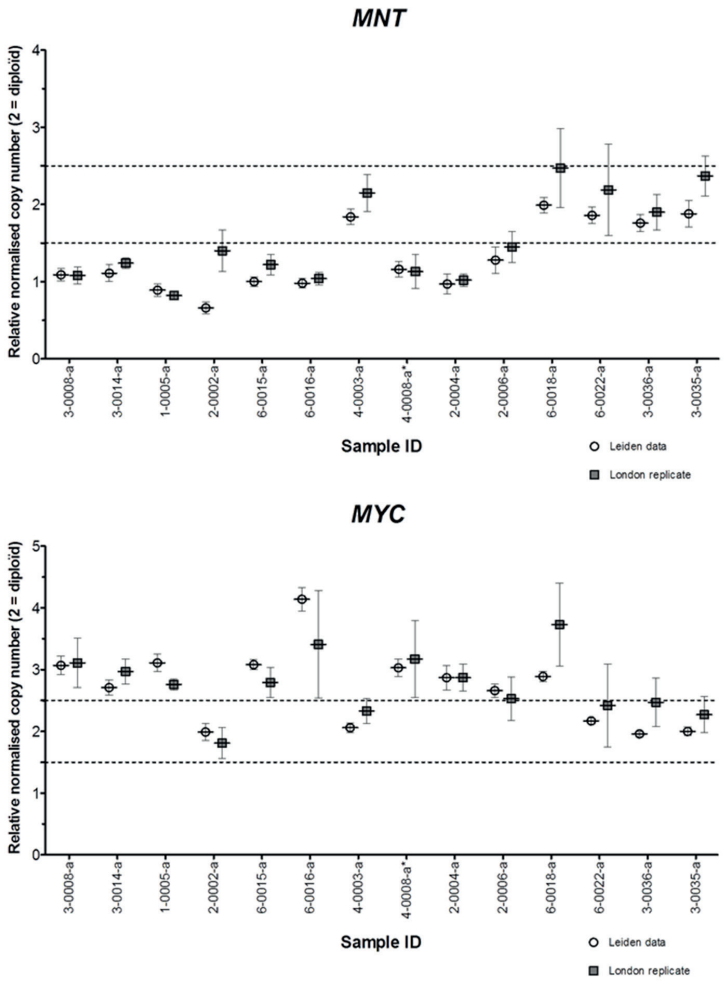
Supplementary Table S5. Overview of the biomarkers in patients with Sézary syndrome without any treatment at time of the inclusion in the study compared with those patients who had treatment at time of inclusion. The Chi-squared test was performed to test for statistical significant differences in marker distribution between these two groups.

	No treatment at inclusion, n=23		**Treatment at inclusion, n=36**		***P*-value**
Flow cytometry markers					
CD4/CD8 ratio ≥ 10	22/23	(96%)	24/30	(80%)	0.095
CD4+CD7− ≥ 40%	12/23	(52%)	20/36	(56%)	0.799
CD4+CD26− ≥ 30%	21/23	(91%)	30/36	(83%)	0.383
CD158a*	0/23	(0%)	2/35	(6%)	0.243
CD158b*	6/23	(26%)	7/36	(19%)	0.548
CD158k*	6/23	(26%)	13/35	(37%)	0.380
Copy number variation markers					
JUNB gain	1/22	(5%)	2/36	(6%)	0.719
TWIST gain	1/22	(5%)	3/36	(8%)	0.165
MYC gain	8/22	(36%)	15/36	(42%)	0.689
MNT loss	16/22	(73%)	22/36	(61%)	0.222
Gene expression markers					
DNM3 up-regulation	17/22	(77%)	24/33	(73%)	0.705
EPHA4 up-regulation	13/22	(59%)	23/33	(70%)	0.418
PLS3 up-regulation	17/22	(77%)	19/33	(58%)	0.132
TWIST1 up-regulation	18/22	(82%)	20/33	(61%)	0.095
STAT4 up-regulation	20/22	(91%)	30/33	(91%)	1

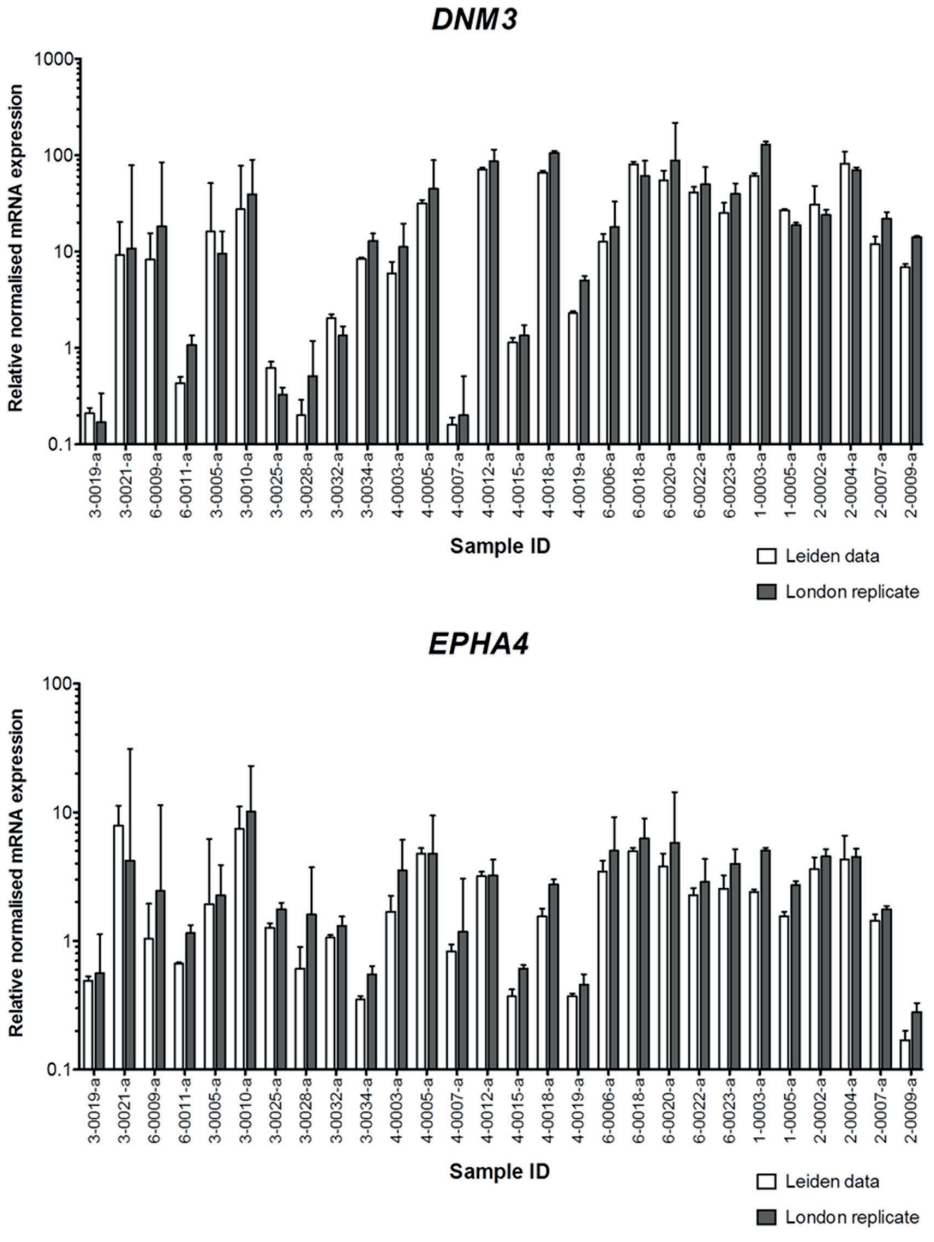
*Low expression and expression of 5% or more of the CD4+ lymphocytes.



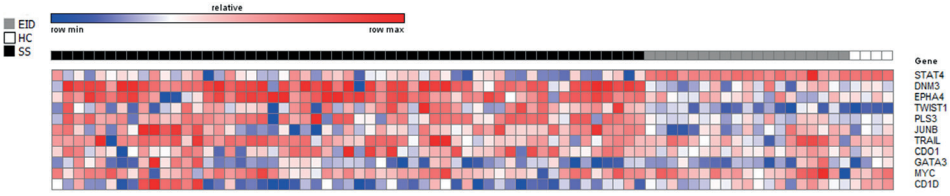
Supplementary Figure S1. Workflow of sample preparation.
This figure gives an overview of the workflow from blood sample collection to the eventual experiments using standard operating procedures (SOPs). CNV, copy number variation; GE, gene expression; PBMC, peripheral blood mononuclear cell



Supplementary Figure S2. Results for copy number analysis performed in Leiden and London. Examples are given of *MNT* and *MYC* for 14 samples.



Supplementary Figure S3. Results for gene expression analysis performed in Leiden and London. Examples are given for *DNM3* and *EPHA4* for 28 samples.



Supplementary Figure S4. Heatmap depicting relative normalized gene expression of evaluated biomarkers in 55 patients with Sézary syndrome, 19 patients with erythrodermic inflammatory dermatoses and 4 healthy control subjects.
Values are visualized as relative gene expression for each gene, normalized against the stably expressed reference genes *ARF5*, *ERCC3* and *TMEM87A* and log2 transformed. Red represents high expression and blue represents low expression. Gene ranking has been performed based on receiver operating characteristic curve analysis accuracy values. Varying from the highest accuracy ranked at top to the lowest placed at the bottom of the heatmap. EID, erythrodermic inflammatory dermatoses; HC, healthy control; SS, Sézary syndrome

Supplementary materials containing all the SOPs are available online at www.jidonline.org.