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1 **TITLE:**

2 Serum cytokine levels as predictive biomarkers of benefit from ipilimumab in
3 small cell lung cancer

4

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4 no conflict of interest.

5

6 **Ethics approval and consent to participate:**

7 Sample collection and data analyses were approved by the local ethics
8 committee of the participating institutions and informed consent of each study
9 participant. The study was conducted in accordance with the European Good
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11

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1 **ABSTRACT**

2

3 **Background.** Immunotherapy has shown efficacy in small cell lung cancer
4 (SCLC), but only a subset of patients benefits. Surrogate biomarkers are urgently
5 needed. Our aim was to evaluate serum Th1, Th2 and proinflammatory cytokines
6 in two cohorts of SCLC patients before and during treatment with chemotherapy
7 with or without ipilimumab and to correlate them with survival.

8 **Patients and methods.** Two cohorts of SCLC patients were studied: patients
9 treated with chemotherapy (n=47), and patients treated with chemotherapy plus
10 ipilimumab (n=37). Baseline, on-treatment and after-treatment serum samples
11 were evaluated for the presence of IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-
12 gamma, TNFalpha, GM-CSF and Mip-1alpha using a Luminex assay. Differential
13 changes of cytokines between cohorts were analyzed. Associations between
14 cytokine levels and their changes with overall survival were evaluated.

15 **Results.** Patients treated with ipilimumab showed a global increase of all
16 cytokines after treatment initiation. A high level of IL-8 at baseline was associated
17 with worse prognosis regardless of treatment. Baseline increased IL-2 levels
18 predicted sensitivity to ipilimumab, while high IL-6 and TNFalpha predicted
19 resistance. An on-treatment increase in IL-4 levels in patients treated with
20 immune-chemotherapy was associated with a better overall survival.

21 **Conclusions.** The addition of ipilimumab to standard chemotherapy in SCLC
22 modulates the serum levels of cytokines. Baseline levels and their change over
23 time relate to overall survival. Blood based biomarkers are convenient for patients

1 and our results support prospective validation of cytokines as predictive
2 biomarkers for ipilimumab in SCLC.

3

4 **Keywords:**

5 Small cell lung cancer, ipilimumab, cytokines, immunotherapy, biomarkers

6

7

1 INTRODUCTION

2 Small cell lung cancer (SCLC) is the most aggressive type of lung cancer.
3 Platinum-based chemotherapy has been the standard of care for the last three
4 decades and unfortunately varying combinatorial systemic approaches have not
5 improved survival ^{1,2}. The substantial incidence of autoimmune paraneoplastic
6 immune events ³ and the high tumor mutational burden ⁴ suggest that immune
7 modulation is a promising strategy in SCLC ⁵.

8 Consistent with these concepts, immune checkpoint inhibitors have shown
9 some activity in SCLC ⁶⁻¹⁰. Ipilimumab, a fully human immunoglobulin G1
10 monoclonal that blocks CTLA-4 ¹¹, showed a trend to improved overall survival
11 (OS) when combined with standard chemotherapy in a phase II trial ⁶. Although
12 the confirmatory phase III failed to confirm an improvement in OS ²,
13 combination of anti-CTLA4 and anti-PD1 agents showed a significant antitumor
14 activity in SCLC patients in second line of treatment, particularly when
15 ipilimumab is included in the regime ⁷.

16 However, two more recent studies of the combination in the maintenance and
17 second line settings have failed to demonstrate benefit over standard
18 approaches ^{12,13}. These failed trials have not used any biomarkers for selection
19 of patients with higher likelihood of benefit and unfortunately this may preclude
20 these drugs to get to the clinic. Despite this, there is a subset of patients who
21 benefit from immunotherapy and have long term outcomes when this strategy is
22 used ^{2,9}. Predictive biomarkers to select patients who will benefit from
23 immunotherapy are therefore urgently needed. In SCLC additionally the limited

1 tissue available for biomarker studies ¹⁴ makes blood-based tests particularly
2 interesting and relevant.

3 Cytokines are soluble molecular messengers with a crucial role in immune
4 response signaling ¹⁵. While Th1 cytokines (IL-2, IFN γ and TNF α) elicit cell-
5 mediated responses, Th2 cytokines (IL-4, IL-5, and IL-10) direct the T-cell
6 response away from a protective Th1 phenotype ^{16,17}. The Th1/Th2 cytokine
7 balance is disrupted in malignant tumors ^{18–20} favoring an immunosuppressive
8 microenvironment. There are preliminary data supporting a prognostic role of
9 inflammatory cytokines such as IL-6 and IL-8 in NSCLC ^{21,22}. However, the
10 biological impact of cytokine levels has to date not been evaluated in SCLC.

11 We analyzed serum Th1, Th2 and inflammatory cytokines in two independent
12 cohorts of SCLC patients treated with standard chemotherapy with or without
13 the anti-cytotoxic T-lymphocyte antigen-4 (CTLA-4) monoclonal antibody
14 ipilimumab. Our goal was to evaluate if baseline levels of cytokines or changes
15 induced by therapy would predict benefit from the addition of ipilimumab in
16 SCLC and allow insights into the immunological consequence of the addition of
17 a checkpoint inhibitor.

18

1 RESULTS

2 Patients' characteristics and outcomes

3 We included 84 SCLC patients. Cohort 1 consisted of 47 patients treated with
4 platinum – etoposide; cohort 2 included 37 patients treated with platinum –
5 etoposide in combination with ipilimumab 10mg/kg. Patients' characteristics are
6 summarized in Supplementary table S1. Cohort 1 included more men (74.5% vs
7 64.9%), patients with performance status (PS)=2 (7% vs 0%) and patients with
8 limited disease (25% vs 0%). Median progression free survival (PFS) was 6.8
9 months (m) in cohort 1 and 6.9m in cohort 2; median overall survival (OS) was
10 13.3m in cohort 1 and 17m in cohort 2.

11

12 Serum Th1, Th2 and pro-inflammatory cytokines are lower in SCLC 13 patients than in a cohort of healthy individuals

14 We evaluated pre-treatment serum cytokine levels in SCLC patients (both
15 cohorts combined) in comparison to healthy volunteers. IL-1 β , IL-5, Mip-1 α and
16 TNF α were significantly lower in SCLC patients compared with healthy
17 volunteers (Figure 1; Supplementary Table S2). The remaining cytokines except
18 IL-6, were also numerically lower in SCLC but the difference was not statistically
19 significant. When we restricted the analyses to patients with extensive disease,
20 serum levels of IL-1 β , IL-4, IL-5 and Mip-1 α were significantly lower in SCLC
21 patients when compared with healthy volunteers.

22

1 **Baseline cytokine levels correlate with age, PS and stage**

2 We assessed the correlation between the level of cytokines (both cohorts) and
3 clinical features. Baseline levels of $TNF\alpha$ were significantly higher in patients
4 over 60 years old; IL-5 was significantly higher in patients with PS 0 vs PS 1/2
5 and IL-2 was significantly higher in female patients compared with male
6 (Supplementary Figure S1). IL-4 and Mip-1 α were significantly lower in patients
7 with extensive disease when compared to those with limited disease
8 (Supplementary Figure S2).

9

10 **Ipilimumab globally increases Th1, Th2 and inflammatory cytokines**

11 We next studied how cytokine levels changed once treatment had been started
12 in each cohort. Patients treated with chemotherapy alone, showed a decrease
13 of GM-CSF, $IFN\gamma$, Mip-1 α , IL-1 β , IL-2, IL-6 and IL-8 median concentration from
14 baseline to tumor response; $TNF\alpha$, IL-5 and IL-10 showed an increase from
15 baseline to tumor response; and IL-4 levels showed no significant changes.

16 Patients treated with immunochemotherapy showed a global increase of all
17 cytokines assessed (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, $IFN\gamma$, $TNF\alpha$, Mip-1 α
18 and GM-CSF) (Figure 2A and 2B) (Supplementary Table S3). These differences
19 between cohorts were statistically significant for all cytokines except for GM-
20 CSF and IL-8.

21 We calculated the percentages of cytokine variation from the first to the second
22 time-point using previously log₂ transformed data. A heatmap of the changes in
23 cytokine levels after treatment, compared to baseline is displayed in Figure 2C.

1 In patients treated with immunochemotherapy, the dominant effect is an
2 increase on cytokine levels in contrast to a reduction after chemotherapy alone.
3 Consistent with this, a principal component analysis (PCA) of fold change of
4 cytokine levels after treatment reveals that patients cluster according to the
5 treatment received (chemotherapy alone vs immunotherapy) (Figure 2D).

6

7 **Baseline IL-8 levels are an unfavorable prognostic marker in SCLC**
8 **regardless of treatment**

9 We inquired whether basal levels of cytokines correlated with survival in SCLC.
10 In both cohorts, patients with serum IL-8 concentration above cut-off, had a
11 worse OS. In cohort 1, patients with baseline IL-8 above cut-off had a median
12 OS of 9.2m vs 16.8m of those with lower levels ($p=0.028$); in cohort 2, patients
13 with baseline IL-8 above cut-off had a median OS of 5.3m vs 17m of those with
14 lower levels ($p=0.031$) (Figure 3). When restricting the analyses to the
15 advanced disease population we obtained the same outcome.

16

17 **Baseline IL-2 levels predict sensitivity to ipilimumab, while IL-6 and TNF α**
18 **predict resistance**

19 We evaluated the potential predictive role of cytokines in patients treated with
20 ipilimumab. Patients treated with immunochemotherapy with a serum IL-2
21 concentration above cut-off at baseline, had a median OS of 30.5m while those
22 with lower levels had a median OS of 8m ($p=0.015$) (Figure 4A). In contrast,
23 patients with a serum IL-6 above cut-off had a median OS of 9.5m while those

1 with lower levels was 18.5m (p=0.026) (Figure 4B). Patients with a serum
2 TNF α concentration above cut-off had a median OS of 7.8m while those with
3 lower levels was 18.5m (p=0.004) (Figure 4C). These associations were not
4 observed when patients were treated with chemotherapy alone. When we
5 restricted the analyses to the advanced disease population, all these results
6 were sustained. Similar results were found when the median cytokine serum level
7 was used as a cut-off (Supplementary Table S4). The multivariate analyses
8 showed that high levels of IL-2 were independently associated with sensitivity to
9 ipilimumab and high levels of IL-6 and TNF α were independently associated
10 with resistance to ipilimumab (Supplementary Table S5).

11

12 **Changes in IL-4 levels during treatment link to outcome in SCLC**

13 We hypothesized that quantitative changes in cytokine levels during treatment
14 could be associated with survival. Patients treated with chemotherapy alone
15 whose IL-4 increased more than 23% from baseline to response, had a
16 significantly worse OS (9.5m vs 16.3m; p=0.001). This finding was maintained
17 when analyzing only the advanced patients, although it lost statistical
18 significance (p=0.063). However, those treated with immunochemotherapy
19 whose IL-4 increased more than 32% had a significant better OS (18.5m vs
20 8.8m; p=0.042) (Figure 5).

21

22 **DISCUSSION**

1 To the best of our knowledge, this is the first study to assess how ipilimumab
2 affects serum levels of immunomodulatory cytokines in SCLC. Our access to
3 two cohorts of patients who were treated with either immunochemotherapy or
4 chemotherapy alone allowed us to assess the biological effect of the addition of
5 ipilimumab and to interrogate these data in the light of clinical outcomes.

6 An intriguing result is the observation of concordantly lower serum levels of
7 multiple cytokines in SCLC patients compared to healthy controls. In the
8 literature we only found 3 patients that had been evaluated in this way ²³; a
9 functional study on whole blood stimulated in vitro revealed a lower cytokine
10 release in cells from SCLC patients, perhaps offering an insight into the
11 underpinning biology of our observation ²⁴. More data are available on
12 circulating IL-6 levels: a study of 72 patients with SCLC identified that in both
13 limited and extensive disease, elevated IL6 levels could be detected, consistent
14 with our observation ²⁵.

15 The modulation of cytokine levels after treatment with immunochemotherapy
16 has not been previously reported in SCLC. We found that addition of ipilimumab
17 increased concentrations of the evaluated cytokines globally and appeared to
18 counteract the effect of chemotherapy that typically decreased cytokines. It is
19 recognized that SCLC cells may be the source of cytokines and therefore
20 successful treatment could reduce levels as observed for the majority of
21 cytokines. This might not be the main source, except for IL-6, as the
22 pretreatment levels in patients are already lower than in healthy controls.
23 Immune cells are also sources of cytokines and can be affected by
24 chemotherapy-induced apoptosis, likely contributing to the observed decrease.
25 Notably, however, patients treated with chemotherapy alone showed

1 stabilization or increase in levels of TNF α and Th2 cytokines. These data
2 suggest that the important compartment of cells contributing to the presence of
3 these cytokines might not be affected by chemotherapy, for example M2
4 macrophages in the tumor microenvironment ^{26,27}. In the absence of paired
5 samples of tumor tissue, we were unable to evaluate this directly. Ipilimumab
6 has previously been reported to increase secretion of IFN- γ , IL-2R, IL-12, and
7 IL-13 from PBMC in vitro exposure ²⁸, consistent with its proposed release of
8 activated T-cells from inhibition.

9 Next, we explored the prognostic and predictive role of cytokine levels at
10 baseline and during treatment. IL-8 is secreted by malignant cells and tumor
11 stroma cells; anti-IL-8 antibodies have shown activity in vitro and in vivo ²⁹ and it
12 is being currently tested in clinical trials (NCT02536469). We found that high
13 baseline levels of IL-8 were associated with worse OS regardless of treatment
14 type. This is consistent with previous literature and is probably a surrogate of
15 tumor burden as it showed a profound decrease with chemotherapy ³⁰.
16 Interestingly, IL-8 was minimally affected by the addition of ipilimumab. IL-8 has
17 been

18 To evaluate the predictive value of cytokine levels after immunochemotherapy,
19 we analyzed the changes in serum concentrations of each cytokine and
20 compared the effect on outcome in both cohorts. Although the cohorts have
21 differences in baseline clinical characteristics, the possibility of comparing the
22 effects of the combination to chemotherapy alone (standard treatment in SCLC
23 up to date) provided the opportunity to individualize the effects related to
24 ipilimumab. Only associations that were significant for the ipilimumab treated

1 cohort and were different from those observed in the chemotherapy only arm,
2 were considered predictive of ipilimumab-linked effects. For instance, serum IL-
3 2 behaved as a predictor of benefit to ipilimumab, and elevated baseline levels
4 identified patients with a significant longer OS. No such a difference was
5 observed in patients treated with chemotherapy alone. IL-2 is a cytokine that
6 promotes the proliferation of T cells, supporting the initiation and maintenance
7 of immune response ³¹. Moreover, it stimulates the proliferation of natural killer
8 cells and enhances their activity ³². As the regulation of T-cell activation through
9 binding of CTLA4 to B7 may affect IL-2 secretion ³³, the release of this blockade
10 with ipilimumab would increase IL-2 concentration enhancing the immune
11 response, and could explain the observed better outcome. In contrast, IL-6 and
12 TNF α behaved as predictors of resistance to ipilimumab: patients with higher
13 baseline concentrations treated with immunochemotherapy had a shorter OS.
14 Our data are consistent with observations in other solid cancers: IL-6 has been
15 associated with tumor progression in lung cancer ³⁴ and to a lack of benefit from
16 ipilimumab in melanoma ^{35,36}. Moreover, it has been tested as a target in cancer
17 in vivo ³⁷. TNF α has pro-tumorigenic activity in cancer ³⁸ and has been linked to
18 MAPK inhibitor resistance in melanoma when secreted by macrophages.
19 Increased serum TNF α might reflect an immunosuppressive tumor
20 microenvironment explaining the observed associated resistance to ipilimumab.
21 Although these mechanisms seem plausible, they require further validation.
22 As serial sampling was available in both cohorts, we evaluated if changes in the
23 cytokine serum levels could predict for benefit from ipilimumab. Our results
24 showed that IL-4 levels were not significantly modified in patients treated with
25 chemotherapy alone. In patients treated with chemotherapy in whom IL-4

1 increased we observed a worse overall survival. It is possible that this may be
2 reflecting an effect on macrophage M2 polarization ³⁹. Interestingly, IL-4
3 increased in the ipilimumab treated cohort and patients experiencing this
4 increase had a better outcome. This increase has been observed in mice
5 treated with ipilimumab but an association with outcome is not observed after
6 ipilimumab monotherapy ^{40,41}. However, the evidence of the pro or antitumoral
7 role of IL-4 in the literature is contradictory and its function seems to depend on
8 IL-4 levels and its association with other immunological modulators ⁴². Globally,
9 our results are novel and hypothesis generating, but warrant prospective
10 validation.

11 In conclusion, we have observed differential impact of ipilimumab in serum
12 cytokines in patients with SCLC. Baseline levels and changes on treatment
13 might serve as convenient predictive biomarkers of benefit from adding
14 ipilimumab to chemotherapy in a disease where tumor biomarkers studies are
15 challenging.

16

1 PATIENTS AND METHODS

2 Patients and study design

3 We retrospectively evaluated two independent cohorts of SCLC patients whose
4 outcomes we have previously reported ⁴³. Patients from cohort 1 were recruited
5 between November of 2009 and January of 2014 at the Hospital del Mar,
6 Barcelona and treated with platinum plus etoposide ^{44,45}. Cohort 2 included
7 patients recruited to a phase II trial of ipilimumab at 10mg/kg, platinum and
8 etoposide (ICE-trial) ⁹. We included a control donor population of healthy, age-
9 and sex-matched individuals (n=30). Sample collection and data analyses were
10 approved by the local ethics committee of the participating institutions and
11 informed consent of each study participant was obtained.

12

13 Sample collection

14 Serum samples were sequentially collected in each cohort: for cohort 1 at
15 baseline (before starting treatment), at first response evaluation (at 3 months
16 approximately) and at progression; for cohort 2 at baseline, at 3 and 6 months.
17 Whole blood samples were collected by standard venipuncture techniques
18 using serum separator tubes. Samples were allowed to clot for 30 minutes at
19 room temperature before centrifugation for 10 minutes at 1000 g at 4 C.
20 Following centrifugation, the supernatant (serum) was immediately removed
21 and assayed immediately or aliquoted and stored frozen at -80 C until further
22 use.

23

1 **Cytokine assessment**

2 Serum samples of all patients and healthy donors were evaluated using a
3 commercially Milliplex map Human High Sensitivity T Cell magnetic bead panel
4 (Millipore, Billerica, MA, USA) coupled with the Luminex xMAP platform. We
5 measured a panel of Th1 (IFN γ , IL-2, TNF α), Th2 (IL-4, IL-5, IL-10), and
6 inflammatory cytokines (GM-CSF, IL-1 β , IL-6, IL-8) plus MIP-1 α in accordance
7 with the manufacturer's instructions. Data was analyzed using five-parametric
8 curve fitting and assay controls included kit standards and Multiplex controls.
9 Intra-assay variabilities were less than 12%. Duplicate measurements with a
10 variability higher than 35% were excluded. These experiments were supervised
11 by technical personal of the Luminex Core Facility at IMIM.

12

13 **Cytokine cut-off calculation**

14 To evaluate the association of cytokines levels with survival, we evaluated the
15 impact of different cut-off methods, including medians (Supplementary Table
16 S4) and ROC curves (Supplementary Table S6 and Supplementary Figure S3).
17 Finally, as the endpoint for comparison was overall survival, we used the web-
18 based software Cut-off Finder ⁴⁶, previously used in the literature ^{47,48}. This
19 method takes into account this endpoint outcome: for each cytokine we
20 identified the threshold level at which a log-rank test allowed segregation of
21 patients into groups with good and poor outcomes (Supplementary Table S7).
22 Then we calculated the percentage of cytokine median concentration variation
23 from first to second time-point and considered >5% positive or negative
24 variations as significant changes (Supplementary Table S8).

1

2 **Statistical analyses**

3 Statistical analysis was carried out using Stata/MP 14 (StataCorp LLC, Texas,
4 USA) and Prism 7.0c (GraphPad Software, Inc.). Baseline values of cytokines
5 were compared among cohorts and healthy volunteers using the non-
6 parametric Mann-Whitney U-test. Overall survival, measured from date of start
7 of treatment until date of death or last visit, was plotted by the Kaplan-Meier
8 method and curves were compared with the log-rank test. All tests were
9 conducted at the two-sided test with 0.05 level of significance. R (v 3.4.3) was
10 used to log₂ transform data, compute cytokine variation and to generate heat
11 maps (using package gplots) and principal component analysis (PCA).

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4

1 **Figure legends:**

2 **Figure 1. Baseline Th1, Th2 and pro-inflammatory cytokines are lower in**
3 **SCLC patients than in healthy individuals.** Dot plots showing the difference
4 on cytokine titters between healthy volunteers (n = 30) and patients with small
5 cell lung cancer (n= 84). Top of grey box shows the median value. All cytokines
6 but IL-6 were decreased in SCLC patients when compared to a healthy
7 population, although only in the case of IL-1 β (p=0.014), IL-5 (p=0.0013), Mip-
8 1 α (p=0.0001) and TNF α (p=0.042) these differences were statistically
9 significant. Error bars show the interquartile range. GM-CSF, Granulocyte-
10 macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MIP,
11 Macrophage Inflammatory Protein; TNF, Tumor necrosis factor. *P<0.05,
12 ****P<0.0001

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1 **Figure 2. Evolution of cytokines levels in patients treated with**
2 **chemotherapy in combination with ipilimumab.** (A) Values correspond to
3 median cytokine concentration, and p values were obtained taking into account
4 the difference on the number of patients showing an increase vs decrease of
5 cytokines levels. B, baseline; R, response; P, progression (B) Bottom of green
6 bars reflects the variation of change of cytokine concentration from baseline to
7 second time-point of cohort 1. Top of orange bars reflect the variation of change
8 of cytokine concentration from baseline to second time-point of cohort 2. (C)
9 Hierarchical clustering of changes in cytokine levels in patients treated with
10 chemotherapy alone (top) and immunochemotherapy (bottom). The heatmap
11 depicts the fold-change of cytokines from first to second time-point, where blue
12 represents a decrease, and yellow and increase compared to baseline. The
13 dominant effect is of reduction of cytokines in patients exposed to
14 chemotherapy alone, compared to an increase in patients after
15 immunochemotherapy. (D) Principal component analysis (PCA) showing the
16 distribution of patients in three-dimensional space, according to changes in
17 cytokine levels after treatment. The PCA plot shows the clustering of patient
18 according to treatment type (chemotherapy alone, green;
19 immunochemotherapy, orange), visualizing similarities in patterns in changes in
20 cytokine levels. GM-CSF, Granulocyte-macrophage colony-stimulating factor;
21 IFN, interferon; IL, interleukin; MIP, Macrophage Inflammatory Protein; TNF,
22 Tumor necrosis factor.

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1 **Figure 3. Baseline IL-8 may be an unfavourable prognostic marker of**
2 **response to ipilimumab in SCLC.** Patients treated either with chemotherapy
3 alone or with ipilimumab harbouring a high baseline IL-8 had a worse OS than
4 those with a low baseline IL-8. mOS: median overall survival.

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1 **Figure 4. Baseline concentrations of cytokines may predict benefit from**
2 **ipilimumab in SCLC patients.** (A) IL-2 appears to predict specific benefit from
3 ipilimumab. (B) Patients with higher levels of IL-6 present worse OS when
4 treated with chemoimmunotherapy but not with chemotherapy alone,
5 suggesting a lack of benefit from ipilimumab in this subgroup. (C) Patients with
6 higher levels of TNF α had a worse OS. This difference in survival was not
7 replicated in patients treated with chemotherapy alone.
8

1 **Figure 5. Modulation of IL-4 during treatment in SCLC could predict**
2 **outcome.** Patients treated with chemotherapy alone whose IL-4 increased
3 more than 23% from first to second time-point had a shorter OS, while those
4 treated with immunochemotherapy whose IL-4 increased more than 32% had a
5 longer OS.

6

7

1 **Supplementary figure S1.** Baseline levels of TNF α were significantly
2 increased in patients over 60 years old [9.15 pg/mL vs 6.84 pg/mL (p=0.0067)];
3 IL-5 was significantly increased in patients with PS 1 or 2 vs PS 0 [2.83 pg/mL
4 vs 1.18 pg/mL (p=0.047)]; and IL-2 was significantly increased in female
5 patients compared with male [2.76 pg/mL vs 1.78 pg/mL (p=0.037)].

6

7 **Supplementary figure S2.** IL-4 and Mip-1 α were significantly decreased in
8 patients with extensive disease when compared to those with limited disease.

9

10 **Supplementary figure S3.** Among other methods, ROC curves were calculated
11 to evaluate its impact as a cut off parameter (see Supplementary Table S6).