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**Article:**
Hardy-Werbin, M., Rocha, P., Arpi, O. et al. (13 more authors) (2019) Serum cytokine levels as predictive biomarkers of benefit from ipilimumab in small cell lung cancer. OncoImmunology, 8 (6). e1593810. ISSN 2162-4011

https://doi.org/10.1080/2162402x.2019.1593810

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**TITLE**:

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

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**Disclosure statement:**

E.A. and C.O. have received honoraria for consultancy and lectures from Bristol-Myers Squibb outside of the current work. The remaining authors report no conflict of interest.

**Ethics approval and consent to participate**:

Sample collection and data analyses were approved by the local ethics committee of the participating institutions and informed consent of each study participant. The study was conducted in accordance with the European Good Clinical Practice requirements (Declaration of Helsinki).

**Funding:**

This work was supported in part by a grant from (1) Fundació La Marató de TV3 (666/C/2013); (2) ISCiii/FEDER (CIBERONC CB16/12/00241, RD12/0036/0051, PIE15/00008, PI15/00146, PI16/00591, PI13/00140); (3) Xarxa de Bancs de Tumors (XBTC); (4) Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR), Generalitat de Catalunya (2014SGR740), (5) Fundació Cellex and (6) Cancer Research UK grant: C491/A12135

## ABSTRACT

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

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

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

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

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

**Keywords**:

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

## INTRODUCTION

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

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

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

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

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

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

## RESULTS

### Patients' characteristics and outcomes

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

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

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

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

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

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

We next studied how cytokine levels changed once treatment had been started in each cohort. Patients treated with chemotherapy alone, showed a decrease of GM-CSF, IFNγ, Mip-1α, IL-1β, IL-2, IL-6 and IL-8 median concentration from baseline to tumor response; TNFα, IL-5 and IL-10 showed an increase from baseline to tumor response; and IL-4 levels showed no significant changes. Patients treated with immunochemotherapy showed a global increase of all cytokines assessed (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, TNFα, Mip-1α and GM-CSF) (Figure 2A and 2B) (Supplementary Table S3). These differences between cohorts were statistically significant for all cytokines except for GM-CSF and IL-8.

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

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

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

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

**Baseline IL-2 levels predict sensitivity to ipilimumab, while IL-6 and TNFα predict resistance**

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

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

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

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

## DISCUSSION

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

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

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

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

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

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

cohort and were different from those observed in the chemotherapy only arm, were considered predictive of ipilimumab-linked effects. For instance, serum IL-2 behaved as a predictor of benefit to ipilimumab, and elevated baseline levels identified patients with a significant longer OS. No such a difference was observed in patients treated with chemotherapy alone. IL-2 is a cytokine that promotes the proliferation of T cells, supporting the initiation and maintenance of immune response [31]. Moreover, it stimulates the proliferation of natural killer cells and enhances their activity [32]. As the regulation of T-cell activation through binding of CTLA4 to B7 may affect IL-2 secretion [33], the release of this blockade with ipilimumab would increase IL-2 concentration enhancing the immune response, and could explain the observed better outcome. In contrast, IL-6 and TNF$\alpha$ behaved as predictors of resistance to ipilimumab: patients with higher baseline concentrations treated with immunochemotherapy had a shorter OS. Our data are consistent with observations in other solid cancers: IL-6 has been associated with tumor progression in lung cancer [34] and to a lack of benefit from ipilimumab in melanoma [35,36]. Moreover, it has been tested as a target in cancer in vivo [37]. TNFα has pro-tumorigenic activity in cancer [38] and has been linked to MAPK inhibitor resistance in melanoma when secreted by macrophages. Increased serum TNF$\alpha$ might reflect an immunosuppressive tumor microenvironment explaining the observed associated resistance to ipilimumab. Although these mechanisms seem plausible, they require further validation.

As serial sampling was available in both cohorts, we evaluated if changes in the cytokine serum levels could predict for benefit from ipilimumab. Our results showed that IL-4 levels were not significantly modified in patients treated with chemotherapy alone. In patients treated with chemotherapy in whom IL-4

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

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

## PATIENTS AND METHODS

### Patients and study design

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

### Sample collection

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

## Cytokine assessment

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

## Cytokine cut-off calculation

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

## Statistical analyses

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

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**Figure legends:**

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

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

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

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

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

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

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

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