



Soluble CTLA-4 as a favorable predictive biomarker in metastatic melanoma patients treated with ipilimumab: an Italian melanoma intergroup study

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Abstract

CTLA-4 blockade by means of ipilimumab (IPI) potentiates the immune response and improves overall survival (OS) in a minority of metastatic melanoma (MM) patients. We investigated the role of soluble CTLA-4 (sCTLA-4) as a possible biomarker for identifying this subset of patients. sCTLA-4 levels were analyzed at baseline in sera from 113 IPI-treated MM patients by ELISA, and the median value (200 pg/ml) was used to create two equally sized subgroups. Associations of sCTLA-4 with best overall response (BOR) to IPI and immune-related adverse events (irAEs) were evaluated through logistic regression. Kaplan–Meier and Cox regression methods were used to analyze OS. A remarkable association between sCTLA-4 levels and BOR was found. Specifically, the proportion of patients with sCTLA-4 > 200 pg/ml in irSD or irPD (immune-related stable or progressive disease) was, respectively, 80% (OR = 0.23; 95%CL = 0.03–1.88) and 89% (OR = 0.11; 95%CL = 0.02–0.71) and was lower than that observed among patients in irCR/irPR (immune-related complete/partial response). sCTLA-4 levels increased during IPI treatment, since the proportion of patients showing sCTLA > 200 pg/ml after 3 cycles was 4 times higher (OR = 4.41, 95%CL = 1.02–19.1) than that after 1 cycle. Moreover, a significantly lower death rate was estimated for patients with sCTLA-4 > 200 pg/ml (HR = 0.61, 95%CL = 0.39–0.98). Higher baseline sCTLA-4 levels were also associated with the onset of any irAE (*p* value = 0.029), in particular irAEs of the digestive tract (*p* value = 0.041). In conclusion, our results suggest that high sCTLA-4 serum levels might predict favorable clinical outcome and higher risk of irAEs in IPI-treated MM patients.

Keywords Soluble CTLA-4 · Ipilimumab · Best response · Adverse events · Overall survival

Abbreviations

ANC	Absolute neutrophil count
BOR	Best overall response
CTLA-4	Cytotoxic T lymphocyte antigen-4
dALC	Derived lymphocyte count
dNLR	Derived neutrophil–lymphocyte ratio

EAP	Expanded access program
flCTLA-4	Full-length CTLA-4
HR	Hazard ratio
IPI	Ipilimumab
irAE	Immune-related adverse events
irCR	Immune-related complete response
irPD	Immune-related progressive disease
irPR	Immune-related partial response
irSD	Immune-related stable disease
MLR	Multinomial logistic regression
MM	Metastatic melanoma
OR	Odds ratio
sCTLA-4	Soluble CTLA-4
SNV	Single nucleotide variants

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Tconv Conventional T cells
Treg Regulatory T cells

Introduction

Ipilimumab (IPI), the human monoclonal antibody targeting CTLA-4 immune checkpoint, was approved for the treatment of metastatic melanoma (MM) after the demonstration that it improves overall survival (OS) in both pre-treated and untreated MM patients [1, 2]. However, the benefit in terms of long-lasting disease control and long-term survival was limited to a relatively small fraction of patients (about 20%) [1, 3].

The antitumor mechanism of action proposed for IPI relies on the blockade of CTLA-4 binding to CD80 and CD86 ligands on the antigen presenting cells (APC), resulting in T-cell activation, intratumoral infiltration, and tumor-specific effector functions [4, 5]. Indeed, IPI may prevent the physiological inhibitory function exerted by CTLA-4, upon binding to ligands, on cell proliferation, interleukin-2 production and cell cycle progression of activated T cells [6, 7]. Several molecular mechanisms, either cell intrinsic or cell extrinsic, have been proposed for CTLA-4 inhibitory function operating in both conventional T (Tconv) and regulatory T (Treg) cells. Among those, competition with the costimulatory CD28 homologue for binding to the shared ligands CD80/CD86 on APC is likely to be the major mechanism along with downregulation of CD80 and CD86 on APC via trans-endocytosis [reviewed in 8].

Most functional studies on CTLA-4 have focused on the membrane-bound receptor isoform (full-length; flCTLA-4), but other CTLA-4 isoforms can also be expressed, including a soluble form of CTLA-4 (sCTLA-4) which originates from a spliced mRNA transcript that lacks the transmembrane sequence [9]. sCTLA-4 has been detected in normal human serum [10]; higher serum levels have been reported in autoimmune diseases [11] and in tumors such as pediatric B-acute lymphoblastic leukemia [12], breast cancer [13], lung, esophageal, and other cancers [14], thus supporting its important role in T-cell-mediated immune regulation.

Indeed, sCTLA-4, that can be secreted by both Tconv and Treg cells [15], is able to bind CD80/CD86 ligands on APC and might interfere with their interaction with flCTLA-4 or CD28 on T-cell surface [16] leading to upregulation or downregulation of the immune response [16, 17].

The evidence that IPI may be clinically efficacious in only a fraction of MM patients highlights the need to identify baseline biomarkers allowing to select patients who may benefit most from IPI treatment.

Several potential biomarkers including those of the systemic inflammatory response, neutrophil–lymphocyte ratio, soluble factors, lactate dehydrogenase (LDH), absolute

lymphocyte count (ALC) [18], and epigenetic features of melanoma and tumor microenvironment [19] have been proposed.

We recently reported on CTLA-4 gene variants (SNVs) as possible biomarkers predictive of IPI efficacy and improved long-term survival [20] as well as of onset of endocrine adverse events [21] in a cohort of IPI-treated MM patients.

In the present study, we measured the serum levels of sCTLA-4 in a subgroup of this previous MM cohort and evaluated their correlation with response to IPI therapy, immune-related adverse events (irAEs) and OS. In some patients we also investigated serum sCTLA-4 levels during the course of IPI treatment to evaluate their possible variations.

Materials and methods

Patient characteristics and serum collection

This study included 113 patients, aged 24–85 years, presenting stage IV MM; these patients, whose pre-treatment sera were available for testing sCTLA-4, are part of the previous cohort of 173 patients we analyzed for CTLA-4 SNVs [20, 21]. Among the 113 patients, 89 were enrolled between July 2010 and January 2012 through the Italian Expanded Access Program (EAP) [22], whereas 24 patients were enrolled between February 2012 and September 2013 when IPI became available in Italy.

Patients were treated intravenously with IPI (3 mg/kg) every 3 weeks for a maximum of 4 doses as previously described [20]. Clinical response was assessed as the best overall response (BOR) using immune-related response criteria (irRC) [23] and classified as immune-related complete response (irCR), partial response (irPR), stable disease (irSD), or progressive disease (irPD).

All patients were monitored for onset of adverse events (AEs), including immune-related adverse events (irAEs), graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3, 2006 [24]. These included irAEs of skin (rash and pruritus), digestive tract (gastro-intestinal: diarrhea, nausea and vomiting; liver and pancreas disorders: hepatitis, transaminases or amylase and lipase increase) and endocrine system (hypophysitis, hypothyroidism and thyroiditis).

Serum samples were collected from 113 patients at baseline (before starting the first IPI cycle) and from 59 (52.2%) of them also at each IPI cycle before the infusion (patients before cycle 2: $N=47$; before cycle 3: $N=10$; before cycle 4: $N=2$).

We also collected serum samples from 108 healthy blood donors recruited at the participating centers as controls (HC, 56.5% male, median age: 48.3; range = 24.0–66.2).

Detection of sCTLA-4 by ELISA

Aliquots from MM patient and HC sera were centrifuged (1500g for 10 min at 4 °C), and supernatants were stored at –80 °C until the sCTLA-4 analysis was performed.

sCTLA-4 was measured using an sCTLA-4-specific enzyme-linked immunosorbent assay (ELISA) kit (Bender MedSystems, Milan, Italy) according to manufacturer's protocol. Each sample was tested undiluted (100 µl/well) in duplicate with deviation between duplicates lower than 10% for any value reported. The lowest sensitivity threshold was 100 pg/ml.

CTLA-4 genotyping

Genotyping of CTLA-4 SNVs – 1661A > G (rs4553808), – 1577G > A (rs11571316), – 658C > T (rs11571317), – 319C > T (rs5742909), + 49A > G (rs231775) and CT60G > A (rs3087243) was performed as previously described [20].

Statistical analysis

Patient and disease characteristics were explored using descriptive statistics. Discrete variables (e.g., gender, ECOG-PS) were expressed as relative frequencies (percentages), while continuous variables (e.g., age and time from diagnosis) were summarized using median and range values. Analysis of contingency tables and related Chi-square test was used to assess the associations between categorical variables, while the nonparametric median test was applied to compare distributions of continuous variables among subgroups of subjects.

In all analytical contexts, baseline sCTLA-4 levels were used as a dichotomous factor, a priori categorized according to 200 pg/ml, which was the median value of MM patients. This can be considered a reasonable threshold given the quite small sample size and the fairly high frequency of sCTLA-4 zero values ($N = 43$, 38.1%) among MM patients. In addition, the median guarantees two equally sized subgroups.

Multinomial logistic regression (MLR) modelling was used to analyze the association between sCTLA-4 levels and the best overall response (BOR: irCR + irPR, irSD and irPD) while adjusting for individual baseline characteristics: age at 1st IPI cycle, gender, time from diagnosis, LDH levels, brain, liver, and cutaneous metastases, participating center and the derived neutrophil-to-lymphocyte ratio (dNLR). The dNLR, calculated as $ANC/(WBC-ANC)$, was used in agreement with the previous studies [25, 26], where only the absolute neutrophil count (ANC) and white cell count (WBC) were recorded.

MLR can be considered as an extension of the more widely used binomial logistic regression modelling (BLR)

for dichotomous outcome (i.e., responders vs non-responders) in that it allows to perform simultaneously two binary comparisons: irSD vs irCR + irPR and irPD vs irCR + irPR. Within each comparison, odds ratio (OR) point estimate, along with 95% confidence limits (95%CL), was computed and considered as an index of association between sCTLA-4 levels and the clinical outcome.

BLR modelling with a random intercept was used to assess the relationship between the longitudinal trajectory of dichotomized sCTLA-4 repeated measurements and the number of IPI cycles administered [27]. This method is particularly appropriate for analyzing longitudinal data given its capability to attenuate some statistical drawbacks due to the correlation among sCTLA-4 measurements performed on the same subject.

To evaluate the correlation between sCTLA-4 levels and irAE development, a BLR analysis was performed using the presence/absence of specific side effects as a binary response. In addition, in this case, regression results were adjusted for baseline patient characteristics.

Analysis of correlation of sCTLA4 levels with OS, as estimated from the date of first IPI cycle to the date of last contact or death from any cause, was carried out using the Kaplan–Meier method, while the Cox regression modelling was applied to estimate the correlation between cumulative death rates and sCTLA4 levels controlling for imbalances in baseline patient characteristics. Death rate (hazard) ratio (HR) and corresponding 95%CL were used as an index of association [28].

Statistical inference on HR/OR was carried out using the likelihood ratio test, and a two-tailed p value < 0.050 was considered as statistically significant. All analyses were performed using Stata (StataCorp. Stata Statistical Software. Release 13.1. College Station, TX (USA), 2013).

Results

Patient features, disease characteristics, and treatment regimens

Overall, 113 MM patients were enrolled from July 2010 to September 2013. The observation period ended by June 30th, 2015. Based on tumor location, the primary melanoma subtype was cutaneous ($N = 90$, 79.7%) or mucosal ($N = 12$, 10.6%). For the remaining 11 (9.7%) patients, the primary tumor site was unknown.

Table 1 shows the main patient features, disease characteristics, and pre/post-IPI treatment regimens. Namely, at the time of the first IPI cycle, 23 (20.4%) patients had brain metastases and 38 (33.6%) had liver metastases. Among them, 7 patients (6.2%) presented metastases at both sites. The other 59 (52.2%) patients had metastases at sites

Table 1 Patient and disease characteristics, and treatment regimens

Characteristics	N (%)
Age, years, at 1st IPI [‡] cycle (median, range)	58.4 (28.4–85.0)
Gender	
Male	62 (54.9)
Female	51 (45.1)
Primary melanoma subtype	
Cutaneous	90 (79.8)
Mucosal	12 (10.6)
Unknown	11 (9.7)
ECOG [§] performance status	
0	73 (64.6)
1	31 (27.4)
2	8 (7.1)
Unknown	1 (0.9)
Serum LDH [¶]	
Normal level	44 (38.9)
≥ Upper limit of normal (ULN)	59 (52.2)
Unknown	10 (8.9)
Number of metastasis sites at 1st IPI cycle	
1	42 (37.2)
2	33 (29.2)
≥ 3	38 (33.6)
Brain metastases	
Present	23 (20.4)
Absent	90 (79.7)
Liver metastases	
Present	38 (33.6)
Absent	75 (66.4)
Metastases other than brain and/or liver ^δ	
Yes	59 (26.7)
No (only brain or/and liver)	54 (24.4)
Number of therapies prior to IPI	
1	66 (58.4)
2	33 (29.2)
≥ 3	14 (12.4)
Chemotherapy ^{&} prior to IPI	
Yes	99 (87.6)
No	14 (12.4)
Immunotherapy [#] prior to IPI	
Yes	25 (22.1)
No	88 (77.9)
Targeted therapy [†] prior to IPI	
Yes	14 (12.4)
No	99 (87.6)
Therapy post-IPI [*]	
Yes	42 (37.2)
No	71 (62.8)
Derived neutrophil-to-lymphocyte ratio	
≤ 2.2	52 (46.0)
> 2.2	53 (46.9)
Unknown	8 (7.1)

Table 1 (continued)

Characteristics	N (%)
Whole sample	113 (100.0)
[‡] IPI ipilimumab	
[§] ECOG-PS Eastern Cooperative Oncology Group-Performance Status	
[¶] LDH, lactate dehydrogenase, ^δ one or more sites of distant disease other than brain and/or liver (mostly lung and lymph node)	
^{&} Including mainly: dacarbazine, temozolomide and fotemustine	
[#] Including mainly interferon alpha and interleukin-2	
[†] BRAF/MEK inhibitors including vemurafenib and dabrafenib	
[*] Including IPI, chemotherapy, BRAF/MEK inhibitors and anti-PD-1	

different from brain and/or liver, mostly lung ($n = 57$, 50.4%) and lymph nodes ($n = 42$, 37.2%). Overall, about one-third of all patients ($n = 38$, 33.6%) presented distant disease at 3 or more sites.

Eighty-two (72.6%) patients received the 4 doses of IPI as scheduled; the remaining 31 (28.4%) patients received 3 or less doses.

Most patients ($n = 66$, 58.4%) received 1 line of systemic therapy prior to treatment with IPI, while 33 (29.2%) and 14 (12.4%) received 2 or ≥ 3 lines, respectively. Chemotherapy was the most frequent regimen used ($n = 99$, 87.5%) followed by immunotherapy ($n = 25$, 22.1%) and targeted therapy ($n = 14$, 12.4%). Among the patients receiving immunotherapy, 14 received interferon alone, 10 received chemotherapy associated with interferon and/or interleukin-2 and 1 received Mage-3 vaccine. Ipilimumab was never administered.

Following IPI therapy, 41 (39.4%) patients among the 104 who experienced disease progression received further systemic treatment including chemotherapy, BRAF/MEK inhibitors, anti-PD-1, or IPI re-treatment.

Detection of sCTLA-4 in MM serum samples at baseline and level correlation with prognostic factors

We investigated whether sCTLA-4 could be detected through ELISA assay in sera from the MM patients at baseline and in sera from HC subjects matched to MM patients by participating centers to increase the comparability between the two groups.

The contribution of participating centers in terms of number of recruited patients did not vary significantly (range = 11.5%–22.1%, Chi-square test p value = 0.168).

Overall, individual levels of sCTLA-4 ranged widely from 0 to 10660.0 pg/ml (mean ± SD = 876.9 pg/ml ± 1738.3 pg/ml) in the MM patients and from 0 to 5736.1 pg/ml (mean ± SD = 641.2 pg/ml ± 1055.5 pg/ml) in the HC subjects. sCTLA-4 median levels in MM patients were slightly

lower than those in the HC group (200.0 pg/ml vs 263.5 pg/ml), but this difference was not statistically significant.

Baseline serum sCTLA-4 levels were correlated with MM prognostic factors including LDH levels, ECOG, dNLR, and other patient characteristics (age at 1st IPI cycle, melanoma subtype, number of chemo- and immune therapies prior to IPI and number of metastatic locations) among which the dALC, a derived lymphocyte count (= WBC-ANC) which can be regarded as an estimate of the actual ALC [25, 26]. Spearman correlation analysis between sCTLA-4 levels and the aforementioned factors showed very low and statistically non-significant associations ranging from -0.19 (sCTLA-4 vs dNLR) to +0.17 (sCTLA-4 vs gender).

Correlation of baseline sCTLA-4 levels with BOR

Table 2 reports the joint distribution of BOR and sCTLA-4 levels, and the results of the MLR modelling. Of the 113 patients, 19 (16.8%) showed irPR + irCR, 16 (14.2%) irSD, and 78 (69.0%) irPD. Using irCR + irPR category as a base outcome, the frequency of irSD responses resulted to be about 80% lower in MM patients with sCTLA-4 > 200 pg/ml compared to those with sCTLA-4 ≤ 200 pg/ml (OR = 0.23; 95%CL = 0.03–1.88; p value = 0.169). In addition, a statistically significant reduction of about 90% was observed in patients with higher sCTLA-4 levels when irPD category was compared to irCR + irPR category (OR = 0.11; 95%CL = 0.02–0.71; p value = 0.020). We also performed the MLR analysis on the patient population deprived of the mucosal melanoma subgroup and we obtained completely overlapping results (Supplementary Table 1).

Moreover, higher values of sCTLA-4 were associated with a better disease responsiveness in the study population. In particular, sCTLA-4 median levels decreased from responders (irCR + irPR, 440.4 pg/ml) to patients with stable disease (irSD, 268.5 pg/ml) and then to progressors (irPD, 108.4 pg/ml; Fig. 1).

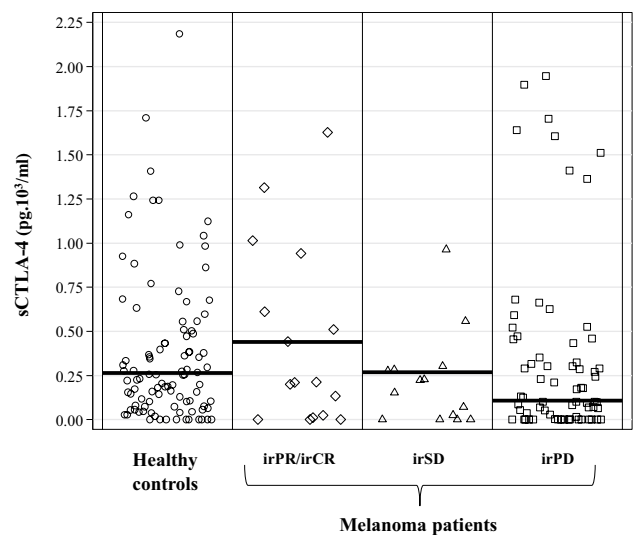


Fig. 1 Analysis of sCTLA-4 serum levels in healthy controls and IPI-treated MM patients classified according to the best overall response: irPR/irCR, irSD, irPD. sCTLA-4 levels were evaluated by ELISA testing in duplicate wells. Results were expressed as sCTLA-4 concentrations (pg × 10³/ml); the horizontal lines in each column represent median values of each subgroup. To point out the difference between median sCTLA-4 levels, only data in the range 0.00–2.25 pg × 10³/ml were plotted. *irPR/irCR* immune-related partial/complete response, *irSD* immune-related stable disease, *irPD* immune-related progressive disease

As we previously reported that BOR to IPI may be influenced by CTLA-4 SNVs which are known to affect the levels of sCTLA-4 mRNA expression [29], we also investigated the possible correlation between SNVs and sCTLA-4 levels in the MM patients, but we did not find any remarkable association.

Table 2 Association of sCTLA-4 serum levels with best overall response (BOR) in IPI-treated MM patients

sCTLA-4 levels	Total	irCR + irPR		irSD		irPD		irSD vs irCR + irPR			irPD vs irCR + irPR		
		N	%	N	%	N	%	OR	95%CL	p value	OR	95%CL	p value
≤ 200 pg/ml	57	7	36.8	7	43.8	43	55.1	1.00	(Ref.)	0.169	1.00	(Ref.)	0.020
> 200 pg/ml	56	12	63.2	9	56.3	35	44.9	0.23	0.03–1.88		0.11	0.02–0.71	
Whole sample	113	19	100.0	16	100.0	78	100.0	–	–	–	–	–	–

Association of sCTLA-4 serum levels with BOR was estimated through a multinomial logistic regression modelling using the value 200 pg/ml as cutoff point for serum sCTLA-4 levels. Detection of sCTLA-4 levels was performed by ELISA. All samples were tested in duplicate with deviation between duplicates lower than 10% for any reported value

IPI ipilimumab, *irCR* immune-related complete response, *irPR* immune-related partial response, *irSD* immune-related stable disease, *irPD* immune-related progressive disease, *OR* odds ratio adjusted for age at 1st IPI cycle, gender, time from diagnosis, LDH levels, dNLR pre-IPI immunotherapy, brain, liver and cutaneous metastases and participating center, *95%CL* 95% confidence, *p value* significance level of the likelihood ratio test, *Ref.* reference category

Longitudinal detection of sCTLA-4 in MM serum samples

Whenever possible, we assessed changes in serum sCTLA-4 levels during the course of IPI treatment. Thus, besides the 113 baseline serum samples, 59 additional sera were withdrawn prior to the sequential IPI cycles; out of these samples, 47 were collected before the 2nd cycle and the other 12 before the 3rd or the 4th cycle.

Logistic regression with a random intercept applied to the dichotomized sCTLA-4 (sCTLA-4 > 200 pg/ml vs sCTLA-4 ≤ 200 pg/ml) showed an increasing dose–response relationship between the number of IPI cycles and the proportion of patients with sCTLA-4 > 200 pg/ml. In particular, after three or more IPI cycles this proportion was more than four times higher than that observed after a single cycle (OR = 4.41, 95%CL = 1.02–19.1, *p* value for linear trend = 0.016) (Table 3).

Moreover, a statistically significant worsening in clinical conditions was found to be inversely correlated with higher levels of sCTLA-4. Specifically, compared to responsive disease (irCR + irPR), stable (irSD) and progressive (irPD) disease gave rise, respectively, to a proportion of patients with sCTLA-4 > 200 pg/ml close to 70%

Table 3 Joint effect of number of IPI cycles and best overall response on the proportion of MM patients with elevated (>200 pg/ml) sCTLA-4 serum levels

Factors and levels	sCTLA-4 levels > 200 pg/ml vs ≤ 200 pg/ml		<i>p</i> value
	OR	95%CL	
Number of IPI cycles			
1	1.00	(Ref.)	0.016
2	1.57	0.95–2.62	
≥ 3	4.41	1.02–19.1	
Best overall response			
irCR + irPR	1.00	(Ref.)	0.034
irSD	0.32	0.06–1.73	
irPD	0.17	0.04–0.68	

The association between the number of IPI cycles and BOR with sCTLA-4 serum levels (sCTLA-4 > 200 pg/ml vs sCTLA-4 ≤ 200 pg/ml) was estimated through a random-intercept binomial logistic regression modelling. Each OR represents the ratio between the proportion of patients with sCTLA-4 > 200 in a specific category and the same proportion in the reference category

IPI ipilimumab, irCR immune-related complete response, irPR immune-related partial response, irSD immune-related stable disease, irPD immune-related progressive disease, OR odds ratio adjusted for age, gender, time from diagnosis, LDH levels, dNLR, pre-IPI immunotherapy, brain, liver and cutaneous metastases, and participating center, 95%CL 95% confidence limit for OR, *p* value significance level of the likelihood ratio test for linear trend, Ref reference category

(OR = 0.32, 95%CL = 0.06–1.73) and 80% (OR = 0.17, 95%CL = 0.04–0.68) lower (Table 3).

Correlation of baseline sCTLA-4 levels with occurrence of irAEs

We evaluated potential correlations between sCTLA-4 levels at baseline and the development of irAEs. Twenty-eight out of the 113 patients (24.8%) developed at least one irAE of any grade. These included cutaneous (*n* = 13; 11.5%), endocrine (*n* = 5; 4.4%) and digestive tract (*n* = 18; 15.9%) irAEs.

Increased levels of baseline sCTLA-4 were significantly associated with the development of any irAE. Indeed, the percentage of patients who developed irAEs among patients with sCTLA-4 > 200 pg/ml was about twice that observed among patients with sCTLA-4 ≤ 200 pg/ml (30.5% vs 16.4%; Table 4).

BLR analysis confirmed the results obtained in descriptive analysis. After adjusting for baseline characteristics, patients with sCTLA-4 > 200 pg/ml showed a more than threefold increased risk of developing any irAE compared to patients with sCTLA-4 ≤ 200 pg/ml (OR = 3.63; 95%CL = 1.14–11.5, *p* value = 0.029). Similar results were also found when site-specific irAEs were considered: cutaneous irAEs (OR = 3.26; 95%CL = 0.68–15.6, *p* value = 0.140), endocrine irAEs (OR = 2.49; 95%CL = 0.25–25.1, *p* value = 0.439) and digestive tract irAEs (OR = 5.61; 95%CL = 1.07–29.4, *p* value = 0.041) (Table 4).

Correlation of baseline sCTLA-4 levels with overall survival

We estimated overall survival probabilities through the Kaplan–Meier method. During the follow-up period (median = 7.3, range = 0.1–52.9 months) 99 (87.6%) out of the 113 patients deceased. Although patients with higher sCTLA-4 levels showed a better life expectancy above all during the first 2 years of observation, differences in survival were not statistically significant. A similar trend was observed also in the patient population deprived of the mucosal melanoma subgroup (Supplementary Table 2).

To take into account potential imbalances in baseline patient characteristics, a multivariate Cox regression analysis was applied to compare cumulative death rates in the two sCTLA-4 subgroups (> 200 pg/ml vs ≤ 200 pg/ml). MM patients with sCTLA-4 > 200 pg/ml experienced a statistically significant better prognosis showing a 40% lower risk of death (HR = 0.61, 95%CL = 0.39–0.98, *p* value = 0.039) as compared to patients with sCTLA-4 ≤ 200 pg/ml (Fig. 2).

Table 4 Association between sCTLA-4 serum levels and immune-related adverse events in IPI-treated MM patients

Immune-related adverse events	sCTLA-4 levels				Total	OR	95%CL	p value
	≤ 200 pg/ml		> 200 pg/ml					
	N	%	N	%				
Any								
No	47	77.0	38	64.4	85	1.00	(Ref.)	0.029
Yes	10	16.4	18	30.5	28	3.63	1.14–11.5	
Cutaneous								
No	52	85.2	48	81.4	100	1.00	(Ref.)	0.140
Yes	5	8.2	8	13.6	13	3.26	0.68–15.6	
Endocrine								
No	55	90.2	53	89.8	108	1.00	(Ref.)	0.439
Yes	2	3.3	3	5.1	5	2.49	0.25–25.1	
Digestive tract								
No	51	83.6	44	74.6	95	1.00	(Ref.)	0.041
Yes	6	9.8	12	20.3	18	5.61	1.07–29.4	
Whole sample	61	100.0	59	100.0	113	–s	–	–

Association between sCTLA-4 serum levels (sCTLA-4 > 200 pg/ml vs sCTLA-4 ≤ 200 pg/ml) and immune-related adverse events was estimated through the binomial logistic regression modelling

IPI ipilimumab, OR odds ratio adjusted for age at 1st IPI cycle, gender, time from diagnosis, LDH levels, dNLR, pre-IPI immunotherapy, brain, liver and cutaneous metastases, and participating center; 95%CL 95% confidence limit, p value significance level of the likelihood ratio test Ref.: reference category

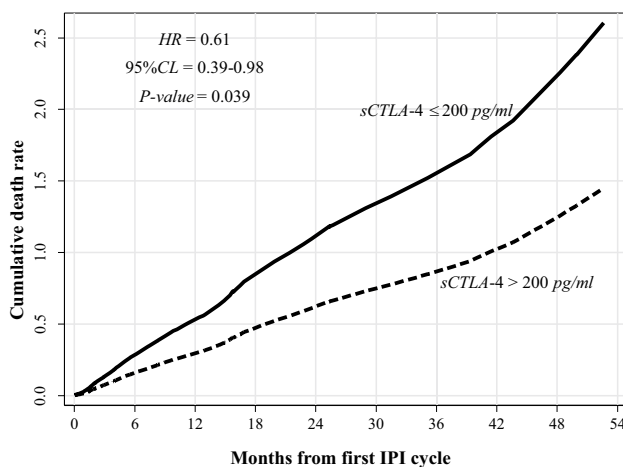


Fig. 2 Time distribution of cumulative death rates. Time distribution has been evaluated according to sCTLA-4 levels (> 200 pg/ml vs ≤ 200 pg/ml) by Cox regression modelling adjusted for age, gender, ECOG, LDH levels, dNLR, brain and liver metastases, and participating centers. HR hazard ratio

Discussion

Although CTLA-4 blockade has significantly improved the survival of MM patients only a minority of them achieves long-term disease control. Therefore, there is an urgent need of biomarkers to identify patients who potentially could benefit from CTLA-4 blockade treatment.

In the present study, we investigated the possible role of serum sCTLA-4 levels as predictive biomarker of response, survival and irAE occurrence in MM patients treated with IPI therapy.

The potential of sCTLA-4 as a predictive biomarker in the context of MM has not been investigated so far except for one preliminary study in a small cohort of MM patients who received IPI therapy [30] suggesting that higher pre-treatment serum CTLA-4 levels can be linked to clinical benefit.

The present study, based on a larger cohort of MM patients, further demonstrated that patients with sCTLA-4 serum levels higher than the median value at baseline (200 pg/ml) had better outcomes in terms of both response to IPI and survival as compared to patients with lower sCTLA-4 serum levels (≤ 200 pg/ml). However, they were at higher risk of developing irAEs. The observation of this favorable effect on response and survival was maintained if mucosal patients were excluded from the study population (see Supplementary Tables 1 and 2).

In particular, MLR analysis pointed out a remarkably greater share of responsive (irCR + irPR) patients with sCTLA-4 > 200 pg/ml with respect to stable (irSD) and progressive (irPD) patients.

Cox analysis showed a significant death risk reduction of about 40% in patients with sCTLA-4 levels > 200 pg/ml as compared to patients with levels ≤ 200 pg/ml.

Interestingly, sCTLA-4 median value in MM patients was slightly lower than that found in the healthy controls,

although not significantly. A reduced production of sCTLA-4 due to dysregulated T-cell activation in MM patients would probably lead to a poor competition with the membrane form flCTLA-4 on cytotoxic T cells, thus increasing T-cell downregulation. sCTLA-4 levels lower than that of HC have been found also in other tumors such as clear cell renal cell carcinoma [31] and colorectal cancer [32] which suggests a possible role of sCTLA-4 in promoting tumor immune tolerance.

The increased levels of sCTLA-4 expression that we here found in the IPI-responding MM patients might exhibit its effect in blocking the inhibitory function of flCTLA-4 and allow host cytotoxic T cells to attack the tumor cells, thereby enhancing the antitumor effect in synergy with IPI.

On the other hand, since sCTLA-4 could also inhibit T-cell costimulation by interfering with CD28/B7 ligand interaction on APC [11, 12], its role in preventing an adaptive anti-tumor immune response cannot be ruled out. However, we do not favor this hypothesis because of the association we observed between high sCTLA-4 levels and better BOR in our study population.

The analysis of the sera collected from the same patient at different time points during IPI therapy showed that the ability to produce circulating sCTLA4 increased with the number of IPI administrations. Indeed, as the number of IPI cycles increased, also the proportion of patients showing higher sCTLA-4 levels increased, whereas it decreased with the progression of disease.

The increase in sCTLA-4 serum levels along IPI therapy might reflect the activation status of T cells and thus a systemic response to the tumor antigens due to the blockade of CTLA-4/B7 interaction by means of IPI which potentiates T-cell costimulation.

sCTLA-4 found in patient serum may derive primarily from both effector Tconv and Treg circulating cells, although Tregs appear to be the predominant producers of sCTLA-4 [15]. However, sCTLA-4 may also be locally produced by melanoma cells and released directly into the blood. Indeed, we previously showed that melanoma cells are capable of expressing sCTLA-4 transcript and release sCTLA-4 in the culture supernatant [33].

Treatment with IPI antibody is typically associated with the onset of irAEs which are strongly linked to its immune-based mechanism of action. Previous studies have suggested that the presence of irAEs during IPI therapy may be associated with greater antitumor efficacy and clinical benefit [34]. However, conflicting results on this issue have also been reported [22].

We found a significant association between higher sCTLA-4 levels at baseline and the onset of any irAE, in particular irAEs of the digestive tract (mainly gastro-intestinal and hepatic events). Indeed, patients with sCTLA-4 > 200 pg/ml showed a more than fivefold increased

risk of digestive tract irAEs as compared to patients with sCTLA-4 \leq 200 pg/ml. This finding is not surprising given that increased levels of serum sCTLA-4 have been found in patients with inflammatory bowel disease (e.g., Chron's disease) [35], ulcerative colitis [36], liver disease (e.g., chronic HBV infection) [37] and autoimmune pancreatitis [38], i.e., diseases with a pathogenesis related to the dysregulation of autoreactive T cells. In IPI-treated patients, high levels of sCTLA-4 which reflect an increased T-cell activation upon therapy might block interactions between flCTLA-4 expressed by autoreactive T cells, as well as Tregs, and B7 ligands and thereby enhance T-cell cytotoxicity and reduce Treg immunosuppressive function.

Moreover, in our cohort, stable and responsive patients showed a relative frequency of irAEs which was more than 5-times higher than that in progressive patients (adjusted OR = 5.42, 95%CL = 1.15–19.4, *p* value < 0.001). It is worth noting that this finding is consistent with the association of higher sCTLA-4 levels with both better BOR and higher irAE frequency and strengthens the predictive role of sCTLA-4. Unfortunately, the sample size of our study did not allow to assess the association between sCTLA-4 levels and irAE grades given the very low frequency of patients presenting low (1/2) or high (3/4) grade. In addition, grade 1 and 2 adverse events are noticeably under-reported in Expanded Access Programs.

All these findings support a potentially important role of high sCTLA-4 levels in improving the outcome of IPI-treated patients as compared to other biomarkers such as the low LDH levels [39], or those factors that show evidence of a pre-treatment activated T-cell infiltrate such as the IFN- γ signature [40] and the high density of CD8, PD-1 and PD-L1 expressing cells at the tumor invasive margin [41] or peripheral blood factors including the high relative eosinophil count (REC) and relative lymphocyte count (RLC), low LDH and limited visceral metastases [42] in melanoma patients treated with PD-1 blockade.

Our data are in line with previous reports by our group [43, 44] and by others [45] showing a favorable prognostic role of high CTLA-4 expression levels in different tumor types. Indeed, we previously demonstrated association of a better OS with high CTLA-4 levels in pleural effusions as well as in tumor tissues from malignant mesothelioma [43] and NSCLC patients [44]. Similar results for sCTLA-4 were found also in patients with different types of cancers treated with radiotherapy, chemotherapy, and/or chemoradiotherapy [14].

In conclusion, the present study evidenced a positive correlation between serum sCTLA-4 overexpression at baseline and clinical outcome of MM patients undergoing immunotherapy with ipilimumab, in terms of better response to IPI, higher frequency of irAEs and improved OS. Thus, CTLA-4 in its soluble isoform, might be used as a biomarker for

predicting the efficacy of IPI in patients with MM although further investigations are needed to ascertain this hypothesis and to investigate the underlying molecular mechanisms.

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Authors' contributions Study concept and design, and study supervision: MPP and Paola Queirolo. Recruitment and management of metastatic melanoma patients: Paola Queirolo, FF, LS, ET, PFF, EC, MG, FDG, GCAC, PM, Pietro Quaglino, PAA, ES, and FS. ELISA assay for detection of sCTLA-4: AM, SL, RC, and BB. SNV genotyping: AM, RC, BB, and MR. Acquisition, analysis, or interpretation of data: MPP, VF, AM, BD, MR, FS, and Paola Queirolo. Drafting of the manuscript: MPP, VF, BD, FS, and Paola Queirolo. Statistical analyses: VF and BD. Administrative, technical, or material support: AM, BB, PFF, CM, ST, EP, PF, SO-A, and MC. Critical revision of the manuscript for important intellectual content: MPP, VF, BD, PFF, MR, FS, and Paola Queirolo. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest Queirolo is member of the advisory board and consultant of Roche, Novartis, Bristol-Myers Squibb, Merck Sharp & Dohme, Amgen. Ferrucci participated to Bristol-Myers Squibb, Novartis and Roche advisory boards, has served as consultant and received travel support from Bristol-Myers Squibb, Roche, Novartis, Merck Sharp & Dohme. Ascierio has/had a consultant/advisory role for Bristol-Myers Squibb, Roche-Genentech, Merck Sharp & Dohme, Novartis, Ventana, Amgen, Array. He received also research grants from Bristol-Myers Squibb, Roche-Genentech, Ventana, Array. All other authors have declared no conflict of interest. The Expanded Access Program (EAP) was sponsored by Bristol-Myers Squibb.

Ethical approval Patients were recruited in six Italian centers. The study was approved by the local Ethics Committee (EC) of the Liguria Region (CE-IST OMA07.024 emended on 2 January 2011) and by the EC of each participating center. All procedures performed in the study were in accordance with the Helsinki declaration.


Informed consent All patients provided signed informed consent before enrolment. Additional informed consent was obtained from healthy blood donors in compliance with the Institutional regulations.

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