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ORIGINAL ARTICLE

Impact of acetaminophen on the efficacy of immunotherapy in cancer patients

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Background: Acetaminophen (APAP) use has been associated with blunted vaccine immune responses. This study aimed to assess APAP impact on immunotherapy efficacy in patients with cancer.

Patients and methods: Exposure to APAP was assessed by plasma analysis and was correlated with clinical outcome in three independent cohorts of patients with advanced cancer who were treated with immune checkpoint blockers (ICBs). The immunomodulatory effects of APAP were evaluated on a preclinical tumor model and on human peripheral blood mononuclear cells (PBMCs) from healthy donors.

Results: Detectable plasma APAP levels at treatment onset were associated with a significantly worse clinical outcome in ICB-treated cancer patients, independently of other prognostic factors. APAP significantly reduced ICB efficacy in the preclinical MC38 model, as well as the production of PD-1 blockade-related interferon-γ secretion by human PBMCs. Moreover, reduction of ICB efficacy *in vivo* was associated with significantly increased tumor infiltration by regulatory T cells (Tregs). Administration of APAP over 24 h induced a significant expansion of peripheral Tregs in healthy individuals. In addition, interleukin-10, a crucial mediator of Treg-induced immune suppression, was significantly up-regulated upon treatment with ICB in cancer patients taking APAP.

Conclusions: This study provides strong preclinical and clinical evidence of the role of APAP as a potential suppressor of antitumor immunity. Hence, APAP should be used with caution in patients treated with ICB.

Key words: acetaminophen, immune checkpoint inhibitors, immunotherapy, cancer

INTRODUCTION

Pain is the most common symptom experienced by patients with advanced cancer. Acetaminophen (APAP, commonly known as paracetamol) alone or in combination with a weak opioid, such as codeine or tramadol, is usually considered as the first-line strategy to manage mild-to-moderate pain in this setting.[1] Although generally considered to be safe, evidence suggests that APAP may have negative immunomodulatory effects. Indeed, preclinical studies demonstrated that APAP can inhibit the proliferation of immune cells and the T-cell-dependent antibody response.[2,3] Moreover, pioneer clinical studies have suggested that APAP inhibits viral clearance and/or neutralizing antibody response in patients infected with chicken pox or rhinovirus.[4] More recently, randomized studies have shown that APAP has a negative impact on vaccination response with decreased antibody levels in subjects receiving APAP for fever prophylaxis.[5,6] Given its potential to impair vaccine effectiveness, the World Health Organization stated in 2015 that administration of APAP before or at the time of vaccination is not recommended.[7]

The development of immune checkpoint blockers (ICBs) was a revolutionary milestone in the field of immuno-oncology. Anti-Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and anti-programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) therapies, such as ipilimumab, nivolumab, or pembrolizumab, are now available for treating various malignancies, including non-small-cell lung cancer, melanoma, and bladder cancer.[8,9] The objective of our study was to investigate whether APAP may impair the efficacy of ICB in patients with advanced cancer.

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METHODS

Patients, treatments, and evaluation

Study design, eligibility criteria, and treatment were previously described for the CheckMate 025 trial[10] (NCT01668784, sponsor: Bristol-Myers Squibb). The main inclusion criteria for the BIP (NCT02534649, sponsor: Institut Bergonié) and PREMIS studies (NCT03984318, sponsor: Gustave Roussy) were age ≥18 years, histologically proven malignant tumor, unresectable and/or metastatic disease, and at least one tumor evaluation by imaging after immunotherapy onset. All patients included in the BIP and PREMIS studies were treated with anti-PD-L1 either in monotherapy or combined with anti-CTLA-4 antibodies, either within clinical trials, or in the context of approved indications of the European Medicine Agency, or within early access programs. The best response to treatment was evaluated according to the Response Evaluation Criteria In Solid Tumors (RECIST) guidelines.[11] Routine follow-up and treatment beyond progression therapeutic options were similar within the two studies. To investigate the pharmacodynamic impact of APAP on peripheral immune cells, four healthy volunteers received 1000 mg of APAP orally every 6 h, over a period of 24 h (total dose: 4 g). Peripheral blood mononuclear cells (PBMCs) were collected at baseline and 2 h after the last administration of APAP. This study was approved by the institutional ethics review board of the Institut Bergonié (Bordeaux, France). Written informed consent was obtained from all subjects involved in the study.

Metabolomic profiling of plasma samples from the BIP study

Detection of APAP and of its metabolite APAP glucuronide was carried out for the patients included in the institutional profiling program BIP (NCT02534649; sponsor: Institut Bergonié) via liquid chromatography-mass spectrometry. Details are provided in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2022.05.010.

Quantitative dosage of APAP

Quantitative dosage of APAP and of its metabolite APAP glucuronide was carried out for the patients included in the institutional profiling program PREMIS (NCT03984318; sponsor: Gustave Roussy) via liquid chromatography-mass spectrometry. Details are provided in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2022.05.010.

Human PBMC profiling

PBMCs were isolated from whole blood of healthy donors before and 24 h after APAP dosing using density gradient centrifugation with Lymphoprep (STEMCELL Technologies, Vancouver, BC, Canada) as per the manufacturer's instructions. PBMCs (5×10^5 cells) were first stained with Zombie NIR viability kit (Biolegend, San Diego, CA) for 10 min and then blocked for 5 min with Human TruStain FcX (Biolegend) and True-Stain Monocyte Blocker (Biolegend) solutions. Cells were incubated for 15 min at 4°C with cell surface marker antibodies, washed in magnetic-activated cell sorting buffer (2 mM EDTA, 0.5% bovine serum albumin, 1× phosphate-buffered saline), fixed for 30 min at 4°C with Foxp3 Fixation/Permeabilization solution (eBioscience, Waltham, MA), and permeabilized with Permeabilization buffer (eBioscience) for 5 min at room temperature. The cells were finally incubated with intracellular marker antibodies for 30 min at 4°C, washed twice, and resuspended in magnetic-activated cell sorting buffer for analysis on the Novocyte Quanteon flow cytometer (Agilent Technologies, Santa Clara, CA). Two different antibody panels were used for PBMCs immunophenotyping: (i) Zombie/CD45/CD3/CD4/CD8/CD19/CD25/CD56/PD1/CTLA4/TIGIT/TIM3/FoxP3/LAG3, and (ii) Zombie/CD45/CD3+CD19/CD11c/CD11b/CD14/CD56/HLA-R/CD123/PDL1/CD15/IDO1/Arginase1 (Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2022.05.010). To normalize the signals obtained before and after APAP dosing, PBMCs from a healthy donor were stained in parallel of the samples and served as shared control for CytoNorm normalization.[12] Normalization and cell phenotyping were both carried out on FlowJo v10.8 (BD Biosciences, Franklin Lakes, NJ). Differences in cell populations and marker expression between pre- and post-treatment samples were tested using paired Student's *t*-test. Only significant changes in marker expression were illustrated on the heatmaps (pheatmap R package v1.0.12).

Plasma proteomics

Proteomic profiling of plasma samples from cancer patients included in the institutional profiling program PREMIS (NCT03984318; sponsor: Gustave Roussy) was assessed using the Olink Target 96 Inflammation panel (Olink Proteomics AB, Uppsala, Sweden) according to the manufacturer's instructions. Details are provided in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2022.05.010. Differences in plasma collected at baseline and week 6 were assessed by paired Student's *t*-test using the limma R package (v3.48.3), and proteins with a fold change >1.25 and a Benjamini–Hochberg-adjusted *P* value <0.05 were extracted. Venn diagram was drawn using the ggvenn R package (v0.1.9).

In vitro *functional assays*

PBMCs from three different healthy donors were isolated by Lymphoprep density gradient centrifugation. Cells (1×10^5) were seeded in 96-well plates and treated with anti-CD3 (1 μg/ml, Biolegend) with or without nivolumab (1 μg/ml, Selleckchem) along with increasing doses of APAP (Sigma-Aldrich). After 72 h, supernatants were collected and interferon-γ release was assessed by homogeneous time-resolved fluorescence using the Human IFN gamma kit (Cisbio, Codolet, France).

Animal studies

All animal studies were carried out under protocols approved by the Institutional Animal Care and Use Committee of the University of Bordeaux (Bordeaux, France).

Details are provided in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2022.05.010.

Statistical analysis

The cut-off date for statistical analysis of baseline demographic data and clinical outcome was by 30 June 2021. Descriptive statistics were used to describe the distribution of variables in the population. Progression-free survival (PFS) was defined as the time from the start of treatment until disease progression, death, or last patient contact. Overall survival (OS) was defined as the time from the start of treatment until death or last patient contact. Survival rates were estimated using the Kaplan−Meier method (survival R package, v3.2-13). Patients from the CheckMate 025 and BIP studies were classified either as 'high' or 'low' based on a threshold value (1×10^5 mass spectrometry intensity), while patients from the PREMIS study were classified as 'presence' or 'absence' according to the quantitation of APAP and APAP glucuronide. Differences between groups were evaluated by the chi-square test for categorical variables and analysis of variance with Tuckey tests or Wilcoxon tests for continuous variables. Prognostic factors were planned to be identified by univariate and multivariate analyses using a Cox regression model. Variables tested in univariate analysis included age, sex, tumor type, presence of liver metastasis, presence of bone metastasis, number of metastatic sites, antibiotic use, steroid use, performance status, number of previous lines of treatment, lactate dehydrogenase levels, and presence of detectable levels of APAP in the plasma. Variables associated with PFS and OS with a P value <0.05 in the univariate analysis were planned to be included in the multivariate analysis. Analyses were carried out in R using the survival analysis package (v0.2.0). All statistical tests were two-sided, and $P < 0.05$ indicated statistical significance.

RESULTS

Since self-medication with APAP is highly prevalent among the general population, analysis of medical records is not appropriate to accurately determine APAP exposure. We therefore analyzed the publicly available serum metabolomics data from 297 patients with advanced renal cell carcinoma and treated with nivolumab in the context of the randomized phase III trial CheckMate 025 (NCT01668784).[10] We found that patients with detectable levels of APAP or APAP glucuronide had significantly worse OS than patients without detectable APAP levels at treatment onset (Figure 1A and B, Supplementary Figure S1A, available at https://doi.org/10.1016/j.annonc.2022.05.010). To note, correlations with objective response rate and PFS were not analyzed as these data were not available. We then used the same untargeted mass spectrometry-based metabolomics approach to analyze plasma samples of 34 patients included in the institutional profiling program BIP (NCT02534649) and treated with ICB for advanced disease. Their characteristics are described in Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2022.05.010. Significant levels of APAP or APAP glucuronide were detected in 50% of these patients. As shown in Figure 1E, patients exposed to APAP had significantly lower objective response rate (0% versus 29.4%; $P = 0.015$), and tended to have worse PFS [median PFS, 1.87 versus 4.72 months; 95% confidence interval (CI) 0.30-1.32 months; $P = 0.219$; Figure 1C] and OS (median OS, 7.87 versus 16.56 months; 95% CI 0.3-1.63 months; $P = 0.412$; Figure 1D) than patients without detectable APAP levels at treatment onset.

To investigate the robustness of these results, we also evaluated the levels of APAP using a quantitative mass spectrometry approach in plasma samples collected from 297 patients enrolled in the PREMIS study (NCT03984318). Their characteristics are described in Table 1. We found that patients with detectable APAP levels had significantly worse PFS (median PFS, 2.63 versus 5.03 months; 95% CI 0.53-0.91 months; $P = 0.009$; Figure 1F) and OS (median OS, 8.43 versus 14.93 months; 95% CI 0.32-0.69 months; $P < 0.0001$; Figure 1G) compared with patients with APAP-free plasma. Objective response rate was also numerically higher in the APAP-negative group than in the APAP-positive group (28.9% versus 20.7%; $P = 0.106$; Figure 1H), although the difference did not reach statistical significance (except in patients with poor performance status, Supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2022.05.010). On multivariate analysis, APAP plasma levels remained independently associated with both PFS and OS (Table 2).

To confirm the impact of APAP on ICB efficacy and provide mechanistic insights, we conducted preclinical investigations using the MC38 colon tumor model, which has been shown to be responsive to PD-1/PD-L1-blocking antibodies.[13] Tumor rejection rates were significantly lower in mice treated with anti-PD-1/PD-L1 antibodies concomitantly with non-toxic doses of APAP than in mice treated with anti-PD-1/PD-L1 antibodies alone ($P = 0.045$) with a trend for OS benefit ($P = 0.17$) (Supplementary Figure S3A and B, available at https://doi.org/10.1016/j.annonc.2022.05.010). To investigate the mechanisms underlying APAP activity *in vivo*, we carried out a flow cytometry-based analysis of tumor-infiltrating leukocytes 13 days after tumor inoculation (8 days after treatment initiation). An increase in tumor infiltration by regulatory T cells (Tregs) was observed in mice treated with APAP and, to a higher and significant extent, in mice treated with both APAP and ICB (Supplementary Figure S3D, available at https://doi.org/10.1016/j.annonc.2022.05.010).

To evaluate the direct impact of APAP on immune cells, we exposed human PBMCs from healthy donors to anti-CD3 antibodies in the presence or absence of nivolumab (1 μg/ml) with increasing concentrations of APAP (0, 100, and 300 μM) for 72 h. As expected,[14,15] nivolumab increased the anti-CD3-induced interferon-γ secretion, an event that was drastically limited in the presence of APAP (Supplementary Figure S4, available at https://doi.org/10.1016/j.annonc.2022.05.010). Using flow cytometry and a gating strategy as illustrated in Supplementary Figure S5, available at https://doi.org/10.1016/j.annonc.2022.05.010, we then

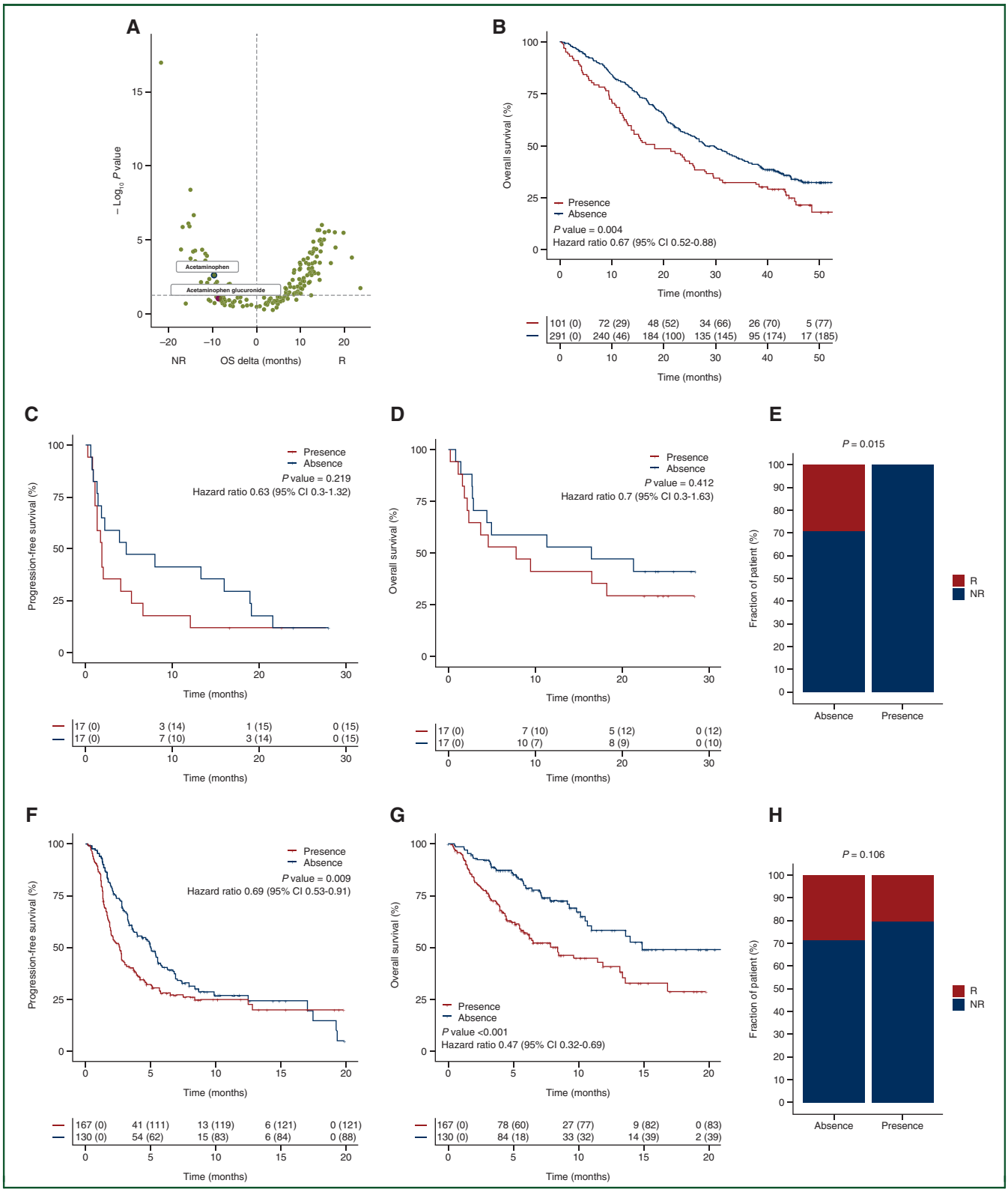

Figure 1. Acetaminophen (APAP) exposure impairs the efficacy of immune checkpoint blockers in patients with cancer.
(A) Volcano plot representation of the log-rank *P* values of OS (y-axis) and delta median OS (x-axis) associated with each plasmatic metabolite in the CheckMate 025 cohort. Optimal cut-off value for each metabolite marker was used to categorize the patients as 'high' or 'low' status. (B) Kaplan—Meier curve of OS according to baseline plasmatic APAP/APAP glucuronide levels in the CheckMate 025 cohort. (C,D,F,G) Kaplan—Meier curves of the progression-free survival (C,F) and OS (D,G) according to APAP/APAP glucuronide levels in the BIP (C,D) and PREMIS (F,G) cohorts. (E,H) Proportion of R and NR patients in the BIP (E) and PREMIS (H) cohorts according to their baseline plasmatic levels of APAP/APAP glucuronide classified as 'absence' and 'presence'. *P* value was calculated by the chi-square test.
CI, confidence interval; NR, non-responder; OS, overall survival; R, responder.

Table 1. Baseline clinical characteristics of patients from the PREMIS Cohort

Characteristics	Patients (N = 297)
Age, years	
Median (range)	63 (26-101)
Sex, *n* (%)	
Female	121 (40.7)
Male	176 (59.3)
ECOG performance status score[a] (%)	
0	106 (35.7)
1	142 (47.8)
≥2	48 (16.2)
NA	1 (0.3)
Antibiotic use, *n* (%)	
Yes	6 (2)
No	291 (98)
Steroids use, *n* (%)	
Yes	24 (8.1)
No	273 (91.9)
Number of previous treatment regimens, *n* (%)	
0-1	94 (31.6)
≥2	176 (59.3)
NA	27 (9.1)
Number of metastatic sites, *n* (%)	
0-1	69 (23.2)
≥2	228 (76.8)
Liver metastases, *n* (%)	
Yes	75 (25.2)
No	222 (74.8)
Bone metastases, *n* (%)	
Yes	91 (30.6)
No	206 (69.4)
Lactate dehydrogenase levels, *n* (%)	
≤ULN	241 (81.1)
>ULN	56 (18.9)
Type of immunotherapy, *n* (%)	
Anti-PD-1	164 (55.2)
Anti-PD-L1	102 (34.3)
Combination of immunotherapies	31 (10.4)
Type of cancer	
Non-small-cell lung cancer	111 (37.4)
Melanoma	24 (8.1)
Soft-tissue sarcoma	22 (7.4)
Renal cell carcinoma	18 (6.1)
Urothelial carcinoma	15 (5)
Others[b]	107 (36)
RECIST	
PD	186 (62.6)
SD	34 (11.4)
PR	63 (21.2)
CR	12 (4)
NA	2 (0.7)

CR, complete response; ECOG, Eastern Cooperative Oncology Group; NA, not available; PD, progressive disease; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PR, partial response; RECIST, Response Evaluation Criteria In Solid Tumors; SD, stable disease; ULN, upper limit of normal.
[a]Performance status scores on the ECOG scale range from 0 (no disability) to 5 (death).
[b]Cervix carcinoma, colorectal cancer, gastric cancer, head and neck cancer, renal cancer, triple-negative breast carcinoma.

Table 2. Multivariate analysis of progression-free survival and overall survival in the PREMIS cohort

Independent variables	Progression-free survival		
	Hazard ratio	95% confidence interval	*P* value
Age (≥63 years, median value)	0.71	0.54-0.94	0.018
APAP exposure (yes)	1.43	1.07-1.91	0.015
LDH levels (>ULN)	1.55	1.17-2.06	0.002
ECOG status (≥2)	2.05	1.41-2.96	<0.001
Liver metastasis (yes)	2.10	1.54-2.87	<0.001
Independent variables	**Overall survival**		
	Hazard ratio	95% confidence interval	*P* value
Sex (male)	1.45	0.99-2.11	0.056
APAP exposure (yes)	1.78	1.18-2.68	0.006
LDH levels (>ULN)	1.91	1.30-2.81	0.001
Liver metastasis (yes)	2.60	1.76-3.85	<0.001
ECOG status (≥2)	3.57	2.27-5.60	<0.001

APAP, acetaminophen; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; ULN, upper limit of normal.

immunophenotyped PBMCs from four healthy donors who received 1000 mg of APAP every 6 h for a period of 24 h. As depicted in Supplementary Figure S6A and B, available at https://doi.org/10.1016/j.annonc.2022.05.010, APAP induced the expansion of Tregs in all donors, as well as the expression of the coinhibitory receptors LAG3 and TIM3, which are associated with a strong immunosuppressive phenotype.[16] Interestingly, APAP induced a concomitant expansion of myeloid and plasmacytoid dendritic cells (mDCs and pDCs, respectively), the latter being characterized by an overexpression of both indoleamine 2,3-dioxygenase 1 (IDO1) and arginase immunosuppressive enzymes (Supplementary Figure S6C and D, available at https://doi.org/10.1016/j.annonc.2022.05.010). Finally, to further assess the immunomodulation effect of APAP in cancer patients treated with ICB, the major surrogate cytokines were profiled in the plasma of patients included in the PREMIS study. In total, 92 cytokines were measured using the Olink Target 96 Inflammation panel. We found that interleukin (IL)-10, a crucial mediator of immune suppression induced by Tregs,[17] and Flt3-ligand, an essential growth factor for dendritic cells,[18-20] were significantly up-regulated upon treatment with ICB, exclusively in patients taking APAP (Supplementary Figure S7, available at https://doi.org/10.1016/j.annonc.2022.05.010).

DISCUSSION

In this study, we found that patients with advanced cancer taking APAP during immunotherapy experience worse clinical outcomes, which suggests that APAP decreases T-cell-mediated antitumor immunity. It is unlikely that our data are the result of bias or unmeasured confounding. Firstly, exposure to APAP was not inferred from medical records which could have introduced the possibility of recall bias. Secondly, association with adverse outcome was observed in three independent cohorts including two academic studies and one industry-sponsored clinical trial which enrolled patients who were hyperselected (45 inclusion/exclusion criteria). Thirdly, the multivariate analysis considered all the features that may be correlated with both prognosis and the need for taking acetaminophen including antibiotic or steroid use and presence of bone metastases. Fourthly, in agreement with our clinical findings, we showed that APAP reduces the efficacy of ICB in a well-characterized preclinical model of colorectal cancer.[13] Finally, we were able to demonstrate *in vitro*, using human PBMC-based

functional assays, that APAP significantly limits the anti-PD-1 therapy-associated effect on interferon-γ production. This observation is consistent with previous data showing that APAP decreases the interferon-induced antiviral responses of cultured mammalian cells to influenza virus.[21]

The first reports on APAP as an immune response modulator were published in the early 1990s.[4] Nonetheless, Prymula et al. were the first to investigate this issue in detail in a randomized study that comprised 9- to 16-week-old healthy infants.[5] They reported reduced immunogenicity of common pediatric vaccines upon APAP use. The impact of APAP on immunogenicity was further supported by *post hoc* analyses from 10 previous trials with a reduction in antibody concentrations that was consistent across almost all the antigens studied.[5] Similarly, a recent systematic review of clinical studies comprising 2775 patients reported that prophylactic APAP administration negatively affected the immune response to pneumococcal conjugate vaccines in children.[22]

Data related to the potential impact of APAP on antitumor immunity remain almost non-existent. In a retrospective study, Kostner et al. analyzed the impact of body temperature achieved during IL-2 infusion in patients with advanced melanoma.[23] Interestingly, they found that IL-2-induced fever (peak temperature $\geq$39.5°C) was associated with an improved survival. However, when stratifying according to APAP use, the association between fever and improved survival was not present among patients routinely receiving APAP, thereby suggesting that APAP intake impairs antitumor immunity.

To date, the mechanisms involved in the immunomodulatory effect of APAP were unknown. As suggested by Kostner et al.,[23] data from vaccination studies indicated that prevention of inflammation and fever is unlikely. Indeed, these studies showed that immune responses (and effect of APAP) in children with and without fever were similar.[5,22] Our preclinical *in vivo* experiments indicated that the lower anticancer effects observed when APAP was combined with ICB were associated with a significant increase in tumor-infiltrating Tregs. These data are in line with a recent study in mice reporting that APAP has an impact on the maternal immune adaptation to pregnancy, by strongly increasing the proportion $FoxP3^+$ Tregs in uterus-draining lymph nodes.[24] $FoxP3^+$ Tregs, which are essential for promoting maternal immune tolerance toward the fetus, are also involved in antitumor immune response suppression. Similarly, in a mouse model of drug-induced liver injury, administration of APAP was shown to promote Treg induction and IL-10 production via a protective feedback loop that physiologically aims to alleviate APAP-induced liver injury, thus contributing to an immunosuppressive milieu.[25] Strikingly, high-throughput proteomic analysis of the plasma allowed us to identify IL-10, a crucial mediator of immune suppression induced by Tregs,[17] as being significantly up-regulated upon treatment with ICB exclusively in patients taking APAP. Moreover, immunophenotyping of PBMCs from healthy donors before and after APAP consumption showed a significant up-regulation of Tregs, particularly of the subset expressing LAG3 and TIM3 immune checkpoints, which are critical for Treg-mediated suppression.[26] In addition, APAP was also found to promote the expression of Flt3-ligand, an essential growth factor for DCs,[18-20] and a significant expansion of mDCs and pDCs expressing IDO1 and arginase, two subsets of immune cell populations known to be associated with induction of immunosuppressive Tregs.[27] Altogether, our data suggest that Tregs are key players that may underlie the immunomodulatory effect of APAP, thus compromising ICB efficacy.

While some controversies still exist concerning the potential predisposing effect of acetaminophen on cancer development, it is commonly considered as an innocuous drug.[28] By combining preclinical experiments and high-throughput profiling of human clinical samples, our study reports the most comprehensive picture of the immunomodulatory effects of APAP. A recent study raised concerns about the potential deleterious impact of APAP in patients with coronavirus disease 2019.[29] Our results confirm that more research should be carried out to understand the impact of APAP on immunity and present a compelling case for caution in using this drug in cancer patients treated with ICB. Whether this rule applies at immunotherapy onset or all over the treatment duration, to all antipyretics, all regimens (ICB combined with cytotoxic chemotherapy or with tyrosine kinase inhibitors), and to other immuno-oncology agents (antitumor vaccines, chimeric antigen receptor T cells), requires further investigations.

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DISCLOSURE

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