

*Helicobacter pylori* infection has a detrimental impact on the efficacy of cancer immunotherapies

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## 1 Abstract

**Objective** In this study, we determined whether *Helicobacter pylori* (*H. pylori*) infection dampens the efficacy of cancer immunotherapies.

**Design** Using mouse models, we evaluated whether immune checkpoint inhibitors or vaccine-based immunotherapies are effective in reducing tumour volumes of *H. pylori*-infected mice. In humans, we evaluated the correlation between *H. pylori* seropositivity and the efficacy of the programmed cell death protein 1 (PD-1) blockade therapy in patients with non-small-cell lung cancer (NSCLC).

**Results** In mice engrafted with MC38 colon adenocarcinoma or B16-OVA melanoma cells, the tumour volumes of non-infected mice undergoing

anticytotoxic T-lymphocyte-associated protein 4 and/or programmed death ligand 1 or anti-cancer vaccine treatments were significantly smaller than those of infected mice. We observed a decreased number and activation status of tumour-specific CD8<sup>+</sup> T cells in the tumours of infected mice treated with cancer immunotherapies independent of the gut microbiome composition. Additionally, by performing an in vitro co-culture assay, we observed that dendritic cells of infected mice promote lower tumour-specific CD8<sup>+</sup> T cell proliferation. We performed retrospective human clinical studies in two independent cohorts. In the Dijon cohort, *H. pylori* seropositivity was found to be associated with a decreased NSCLC patient survival on anti-PD-1 therapy. The survival median for *H. pylori* seropositive patients was 6.7 months compared with 15.4 months for seronegative patients (p=0.001). Additionally, in the Montreal cohort, *H. pylori* seropositivity was found to be associated with an apparent decrease of NSCLC patient progression-free survival on anti-PD-1 therapy.

**Conclusion** Our study unveils for the first time that the stomach microbiota affects the response to cancer immunotherapies and that *H. pylori* serology would be a powerful tool to personalize cancer immunotherapy treatment.

## 2 Data availability statement

Data are available upon reasonable request.

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4      Significance of this study

5      What is already known on this subject?

- Previous preclinical and clinical studies demonstrated that the large and small intestinal microbiota affect the efficacy of cancer immunotherapies.

6      What are the new findings?

- Our study unveils for the first time that the stomach microbiota of *Helicobacter pylori* infected hosts affects the response to cancer immunotherapies.

7      How might it impact on clinical practice in the foreseeable future?

- *H. pylori* serology would be a powerful tool to personalise cancer immunotherapy treatment.

## 8 Introduction

Immune checkpoint inhibitors (ICIs) aim to reinvigorate the immune system toward cancer cells. However, despite unprecedented results, the majority of patients do not respond to ICIs and several individualistic factors have been associated with resistance such as the composition of the gut microbiota and tumour immune contexture.<sup>1</sup>

Vétizou *et al*<sup>2</sup> demonstrated that the antitumour effects elicited by anticytotoxic T-lymphocyte-associated protein 4 (CTLA4) blockade in mice were dependent on the presence of *Bacteroides fragilis*, *Bacteroides thetaiotaomicron* and *Burkholderiales* in the gut microbiota. Other research groups have identified additional immune-potentiating bacteria in the gut such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii* and *Bifidobacterium* species present in those who respond to immunotherapy compared with those who do not.<sup>3,4</sup> More importantly, human data clearly demonstrated that the composition of the intestinal microbiome had a considerable influence on the effectiveness of anticancer immunotherapies<sup>3-5</sup> and that the use of antibiotics compromised the efficacy of programmed cell death protein 1 (PD-1) blockade therapy.<sup>6</sup> Paradoxically, no common bacterial taxa were clearly identified as key indicators and/or modulators of ICI responses across published studies.<sup>1</sup> This may be due to technical issues linked to microbiome analysis or to geographical factors such as diet and lifestyle. Another possibility is that the analysis of the patient's faecal samples solely may be overlooking key information. Indeed, the small intestine<sup>7</sup> and/or stomach microbiota may also profoundly affect responses to ICIs.

*Helicobacter pylori* (*H. pylori*), which colonises the stomach mucosa of 50% of the world population, actively manipulates host tissues to establish an immunosuppressive environment that maintains chronic infection.<sup>8</sup> *H. pylori* dampens the effector functions of CD4<sup>+</sup> T cells,<sup>8</sup> dendritic cells (DCs)<sup>9-12</sup> and macrophages<sup>8</sup> and promotes the generation of regulatory T cells (Tregs)<sup>10</sup> and myeloid-derived suppressor cells.<sup>13</sup> Previous observations demonstrated that systemic inflammatory disorders such as asthma, lupus, inflammatory bowel disease and

eosinophilic esophagitis are negatively associated with *H. pylori* infection in the human population<sup>14–17</sup> and in animal models.<sup>18–21</sup> This suggests that *H. pylori* may mitigate unbalanced systemic immune responses. The negative impact of *H. pylori*-mediated immunomodulation on a large number of immune cell types associated with antitumour immunity allows for the important consideration that *H. pylori* infection may decrease the response to cancer immunotherapies.

## 9 Results

### 10 *H. pylori* infection decreases the effectiveness of cancer immunotherapies in preclinical models

In order to test whether *H. pylori* infection diminishes the effectiveness of ICIs, we first evaluated whether *H. pylori* decreases the efficacy of anti-CTLA4 therapy using an MC38 colon adenocarcinoma model (see [online supplemental figures S1A](#)). Interestingly, the tumour volumes of non-infected mice undergoing anti-CTLA4 treatment were significantly smaller than those of *H. pylori*-infected mice ([figure 1A](#)). We next evaluated whether *H. pylori* decreases the efficacy of anti-CTLA4/programmed death ligand 1 (PD-L1) combination therapy<sup>22</sup> (see [online supplemental figures S1B](#)). As shown in [figure 1B](#), the tumour volumes of non-infected mice undergoing anti-CTLA4/PD-L1 treatment were significantly smaller than those of infected mice. These results demonstrate that *H. pylori*-infected mice are less responsive to CTLA4 blockade alone or in combination with anti-PD-L1. Furthermore, by using a B16-OVA melanoma model, we evaluated whether *H. pylori* decreases the efficacy of a vaccine-based cancer immunotherapy. Tumour-bearing mice were transferred with OVA-specific CD8<sup>+</sup> T cells (OT-1 cells) and were immunised with OVA peptide (SIINFEKL) emulsified in CpG<sup>23</sup> (see [online supplemental figures S1C](#)). Remarkably, the tumour volumes of vaccinated non-infected mice were significantly smaller than those of vaccinated infected mice ([figure 1C](#)), demonstrating that *H. pylori*-infected mice are less responsive to anti-cancer vaccination. In addition, we observed that the infection of the stomach mucosa with *Helicobacter felis* (*H. felis*), a *Helicobacter* species lacking a number of the virulence factors of *H. pylori* such as Cag pathogenicity island and vacuolating

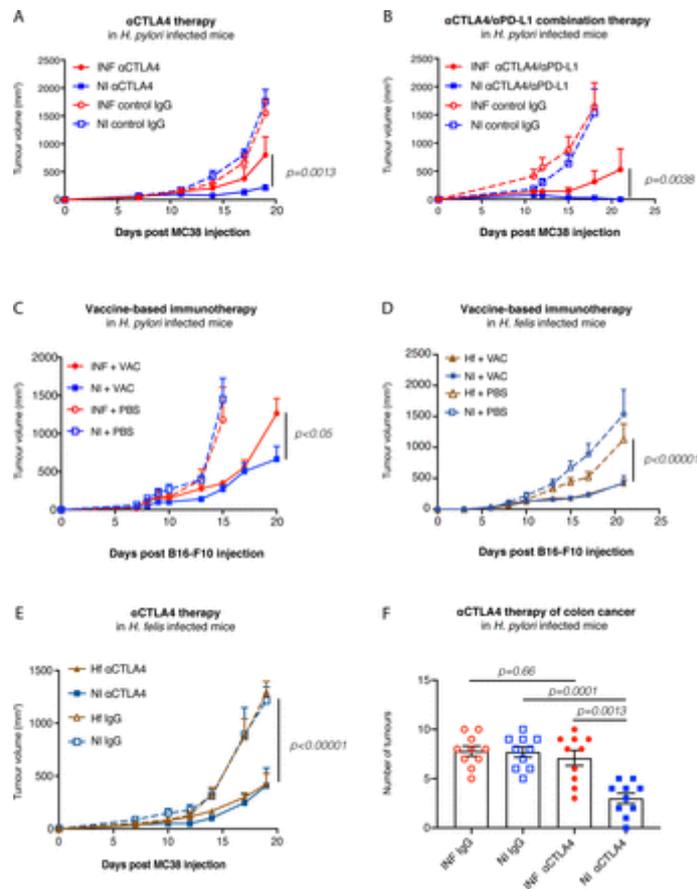
cytotoxin (VacA),<sup>24</sup> does not decrease the effectiveness of anti-cancer vaccination (figure 1D) or anti-CTLA4 therapy (figure 1E). Lastly, we evaluated the effect of *H. pylori* infection on the effectiveness of immunotherapy in tumours developing in situ. We selected the model of azoxymethane (AOM)/dextran sodium sulfate (DSS) colon cancer, as it has recently been shown that anti-CTLA4 therapy reduces the tumour burden in this model (see [online supplemental figures S1D](#)).<sup>25</sup> Remarkably, the number of colon tumours in non-infected mice undergoing anti-CTLA4 treatment was significantly lower than those of *H. pylori*-infected mice (figure 1F). Collectively, we provide evidence demonstrating that the presence of *H. pylori* in the gastric microbiota specifically jeopardises the efficacy of cancer immunotherapies.

## 11 Supplemental material

[\[gutjnl-2020-323392supp001.pdf\]](#)

## 12 Supplemental material

[\[gutjnl-2020-323392supp002.pdf\]](#)



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## Figure 1

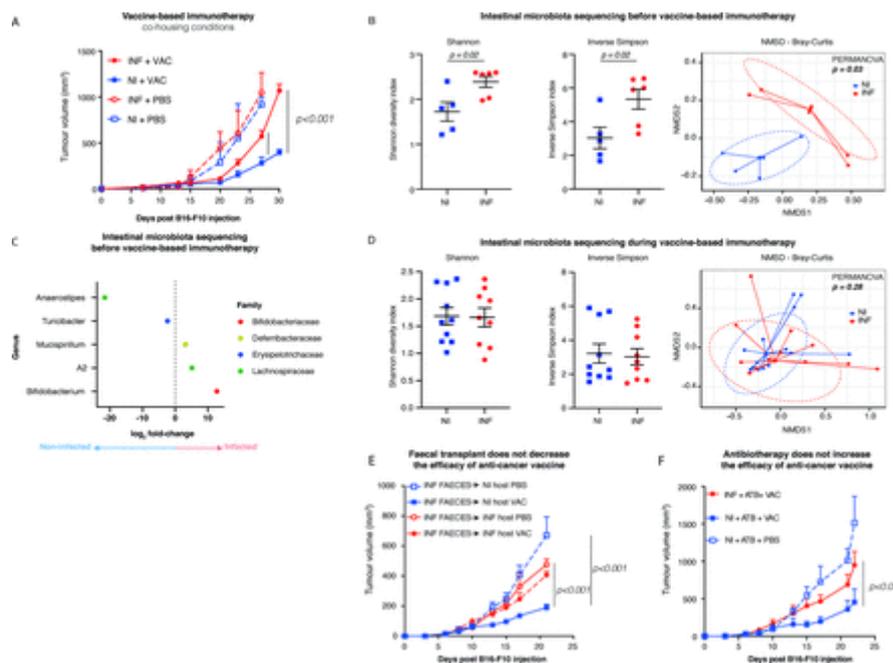
*Helicobacter pylori* infection decreases the effectiveness of cancer immunotherapies in preclinical models. (A) Mice were injected with MC38 colon adenocarcinoma cells and intraperitoneally injected with anti-CTLA4 ( $\alpha$ CTLA4) or IgG2b isotype as control. At day 19, tumour volumes of non-infected (NI) and infected (INF) mice treated with anti-CTLA4 therapy were significantly different ( $p < 0.01$ , two-way analysis of variance (ANOVA)). Experimental groups included seven mice. (B) MC38 tumour growth kinetics of NI and INF mice treated with anti-CTLA4/PD-L1 ( $\alpha$ CTLA4/ $\alpha$ PD-L1) combination therapy. Anti-CTLA4/PD-L1 treatment resulted in tumour rejection in seven of eight NI mice, whereas anti-CTLA4/PD-L1-treated INF mice showed tumour rejection in two of six mice. At day 21, the tumour volumes of NI and INF mice treated with anti-CTLA4/PD-L1 antibodies were statistically different ( $p = 0.009$ , two-way ANOVA). Experimental groups included five to eight mice. (C) B16-OVA tumour growth kinetics of NI and INF mice treated with an anti-cancer vaccine (VAC) or phosphate-buffered saline (PBS) as a control. Vaccination resulted in decreased tumour growth in NI mice, however, did not efficiently limit tumour growth in INF mice. At day 20, tumour volumes of vaccinated NI and INF mice were statistically different ( $p < 0.05$ , two-way ANOVA). Experimental groups included five to eight mice. (D) B16-OVA tumour growth kinetics of NI and *H. felis* (*H.f*)-infected mice treated with an anti-cancer vaccine or PBS as a control. Vaccination resulted in statistically significant decrease in tumour growth in NI and *H.f*-infected mice ( $p < 0.001$ , two-way ANOVA). Experimental groups included nine to ten mice. (E) MC38 tumour growth kinetics of NI and *H.f*-infected mice treated

with anti-CTLA4 ( $\alpha$ CTLA4) therapy. Anti-CTLA4 treatment resulted in decreased tumour growth in both NI and *H.f* mice. At day 19, the tumour volumes of NI and *H.f* mice treated with anti-CTLA4 antibodies were not statistically different ( $p=0.65$ , two-way ANOVA). Experimental groups included six mice. (F) Number of colon tumours of NI and *H. pylori* INF-infected mice treated with anti-CTLA4 ( $\alpha$ CTLA4) or IgG2b isotype as control. Anti-CTLA4 injection resulted in decreased tumour number in NI mice but not in INF mice. At sacrifice, tumour numbers of anti-CTLA4-injected NI and INF mice were statistically different ( $p<0.0013$ , Mann-Whitney test). Experimental groups included ten mice. For the experiments described in [figure 1A–F](#), the infectious status of each individual mouse was confirmed at sacrifice by performing rapid urease tests and/or colony forming units on the stomach (see [online supplemental figures S2A–F](#)). CTLA4, cytotoxic T-lymphocyte-associated protein 4; PD-L1, programmed death ligand 1.

13 *H. pylori*-mediated immunosuppression of cancer immunotherapies is independent of the *H. pylori*-induced modification of the faecal microbiota

We investigated whether *H. pylori*-mediated immunosuppression relies on the modulation of the composition of the gut microbiota. To evaluate the role of the gut microbiota in *H. pylori*-induced immunosuppression, we performed three types of experiments. As a first approach, we performed co-housing experiments. Due to the coprophagia behaviour of mice, the composition of the faecal microbiota of co-housed mice is very similar. However, co-housing does not allow for *H. pylori* transmission from infected to non-infected neonate/adult mice (see [online supplemental figures S2A](#)). Using a B16-OVA melanoma model, we observed that the tumour volumes of vaccinated non-infected mice were significantly smaller than those of vaccinated infected mice ([figure 2A](#)). Similarly, infected mice were less responsive to anti-CTLA4 therapy in the MC38 model (data not shown). Next, we performed 16S rRNA gene sequencing of the intestinal microbiota both before and during vaccine-based immunotherapy administration (see [online supplemental figures S1C](#)). We observed that the intestinal microbiota of non-infected and infected mice differed at steady state ([figure 2B](#)), confirming the work of

Kienesberger *et al.*<sup>26</sup> Notably, we found that *H. pylori* infection leads to a decreased bacterial colonisation of *Lachnospiraceae* and *Erysipelotrichaceae* genera and to an increased bacterial colonisation of the *Bifidobacterium* genus (figure 2C). Infected mice displayed higher alpha diversity compared with non-infected mice. Very interestingly, similarly increased alpha diversity has been described in *H. pylori*-infected individuals compared with matched *H. pylori*-negative controls.<sup>27</sup> Paradoxically, high alpha diversity and high prevalence of *Bifidobacterium* are commonly observed in mice which develop a favourable anti-cancer immune response following immunotherapy administration.<sup>1,28</sup> Interestingly, the faecal microbiota of non-infected and infected mice were similar in the vaccine-based immunotherapy setting (figure 2D). Lastly, we transplanted faeces from *H. pylori*-infected mice to non-infected mice and performed cancer immunotherapy using the B16-OVA melanoma model (see online supplemental figures S1E). We observed that the vaccinated non-infected mice, transplanted with faeces of infected mice, maintained the ability to efficiently control tumour growth compared with non-vaccinated counterparts (figure 2E). Collectively, co-housing experiments, 16S rRNA gene sequencing and faecal transplantation demonstrate that *H. pylori*-mediated immunosuppression of cancer immunotherapies is independent of the *H. pylori*-induced modification of the faecal microbiota.



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## Figure 2

*Helicobacter pylori*-mediated immunosuppression of cancer immunotherapies is independent of the *H. pylori*-induced modification of the faecal microbiota. (A) Co-housing experiments, non-infected (NI) (n=9) and *H. pylori*-infected (INF) (n=10) mice were subcutaneously injected with B16-OVA tumour cells and vaccinated. Vaccination (VAC) showed very limited tumour growth inhibition in INF mice. At days 27 and 30, the tumour volumes of vaccinated NI and INF mice were statistically different ( $p < 0.001$ , two-way analysis of variance (ANOVA)). At sacrifice, we performed rapid urease tests on the stomach of NI and INF co-housed mice. We only detected *H. pylori* infection in INF and not in NI mice, confirming that co-housing does not allow for *H. pylori* transmission (see [online supplemental figures S2A](#)). (B) Shannon, Inverse Simpson and Bray-Curtis based Non-metric Multidimensional Scaling (NMDS) analysis of 16S rRNA gene sequencing of the intestinal microbiota before the initiation of the vaccine-based immunotherapy. 16S rRNA analyses were performed in non-co-housing conditions. At steady state, INF mice displayed higher alpha diversity compared with NI mice ( $p = 0.02$ , Mann-Whitney test). (C) 16S rRNA gene sequencing of the intestinal microbiota before the initiation of the vaccine-based immunotherapy. Differential abundance analysis performed at the genus taxonomic level comparing 16S rRNA sequencing of INF vs NI mice at steady state. Only bacteria with an adjusted p value  $< 0.05$  are displayed (Benjamini-Hochberg method). (D) Shannon, Inverse Simpson and Bray-Curtis based Non-metric Multidimensional Scaling (NMDS) analyses of 16S rRNA gene sequencing of the intestinal microbiota during vaccine-based immunotherapy. The

faecal microbiota of INF and NI mice were similar on day 8 post CpG/OVA vaccination (no statistical difference, Mann-Whitney test). (E) Faecal transplantation of INF faeces into NI or INF recipient mice. Recipient mice were treated with antibiotics and orally administered with 100 mg of faeces from INF mice on a daily basis for 5 days. Two weeks after the last oral gavage, vaccine-based immunotherapy was initiated on B16-OVA tumour-bearing mice ([online supplemental figures S1E](#)). Remarkably, vaccinated NI mice transplanted with INF faeces (n=9) are still capable to very efficiently control tumour growth compared with non-vaccinated (PBS) counterparts (n=7) ( $p<0.001$ , two-way ANOVA). Additionally, vaccinated NI mice transplanted with INF faeces (n=9) show better tumour growth control compared with vaccinated INF mice transplanted with INF faeces (n=4) ( $p<0.001$ , two-way ANOVA). We observed that faecal transplantation of INF faeces to NI recipient mice do not allow for *H. pylori* infection ([online supplemental figures S2C](#)). (F) Antibiotherapy (ATB) does not increase the efficacy of anti-cancer vaccination. Mice were infected with *H. pylori* during the neonatal period and treated with ATB at 6 weeks of age. One-month post ATB, mice were engrafted with B16-OVA melanoma cells and vaccinated ([online supplemental figures S1F](#)). We observed that the eradication of *H. pylori* infection by ATB ([online supplemental figure S2B](#)) did not substantially increase the efficacy of cancer vaccination ( $p<0.05$ , two-way ANOVA). For panels A–D and F, data shown are representative of two independent experiments. For the experiments described in [figure 2A–D](#), the infectious status of each individual mouse was confirmed at sacrifice by performing rapid urease tests and/or colony forming unit on the stomach (see [online supplemental figures S2A–C](#)).

14 Eradication of *H. pylori* infection by antibiotherapy does not increase the efficacy of vaccine-based immunotherapy  
We evaluated whether the eradication of *H. pylori* infection by antibiotherapy reverts the *H. pylori*-induced decreased efficacy of cancer immunotherapies. Antibiotic administration does not substantially rescue the efficacy of vaccine-based cancer immunotherapy in infected mice ([figure 2F](#)). Together with the known detrimental effect of antibiotic

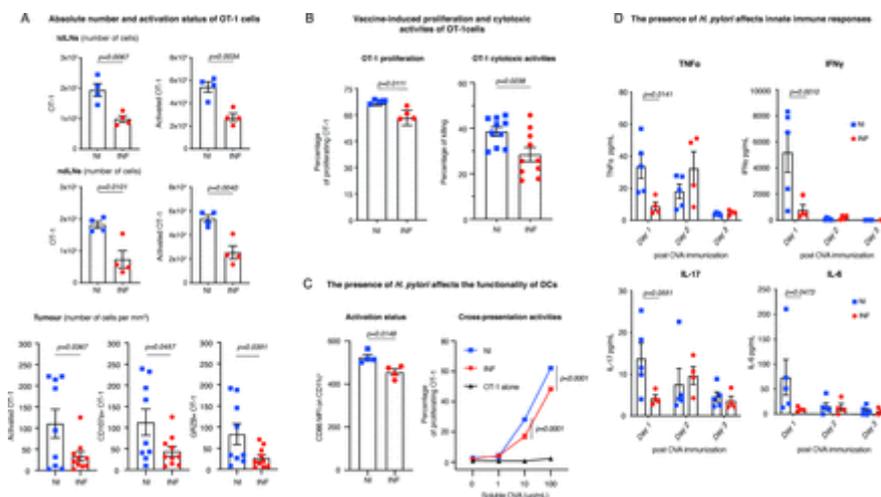
administration on the efficacy of immunotherapies in cancer patients,<sup>6</sup>  
<sup>29</sup> this result suggests that antibiotic administration to cure *H. pylori* infection is most likely a poor option to increase the efficacy of cancer immunotherapies in patients.

15 *H. pylori* jeopardises tumour specific immune responses

We next studied the impact of *H. pylori* on the functionality of the immune system. Using flow cytometric analysis, we analysed the absolute cell number and activation states of CD8<sup>+</sup> and CD4<sup>+</sup> T cells, Tregs, migratory and resident DC1 cells, DC2 cells, monocyte-derived DCs, macrophages and monocytes in the spleen and lymph nodes of non-infected and infected mice at steady state. We also analysed T cell subsets and monocytes in the blood. We identified that *H. pylori* infection does not impact the amount nor the activation states of the aforementioned cell types (see [online supplemental figures S3 and S4](#)). Identifying no impact of *H. pylori* on the immune cells at steady state, we next evaluated whether *H. pylori* affects the previously described immune cells in the context of cancer. We observed similar absolute numbers of immune cells in the blood, spleen, non-draining and tumour-draining lymph nodes (ndLNs and tdLNs, respectively) and tumour(s) of non-infected and infected mice (see [online supplemental figure S5](#)). Importantly, flow cytometric analysis of immune cells isolated from the aforementioned organs/tissue/tumour(s) of MC38 tumour-bearing mice showed that the presence of *H. pylori* alone is unable to substantially affect the immune cell populations associated with antitumour immunity (see [online supplemental figures S6 and S7](#)). Lastly, we analysed the immune cells in MC38 tumour-bearing mice undergoing anti-CTLA4 treatment. We observed similar numbers of tumour infiltrating immune cells between non-infected and infected mice (see [online supplemental figure S8](#)). In addition, absolute cell numbers and activation status of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the tumours, tdLNs, ndLNs and blood were similar in non-infected and infected mice (see [online supplemental figure S8](#) and data not shown). It is noteworthy to indicate that we observed a substantial reduction in the number of myeloid cells, notably, a tendency of reduction in the number of activated monocytes, in the tumours of infected mice compared with non-infected mice undergoing anti-CTLA4 treatment (see [online supplemental figure S8](#)). These results are reminiscent of the

work of Iida *et al.*,<sup>30</sup> showing that the gut microbiota can modulate the infiltration of monocyte-derived cells into the tumours, impacting the response to cancer therapy.

The efficacy of cancer immunotherapies relies on the generation of tumour-specific immune responses. We next evaluated whether *H. pylori* jeopardises tumour-specific immune responses, first by studying the absolute cell number and differentiation status alongside the effector functions of OT-1 cells extracted from the tumour(s), ndLNs and tdLNs of vaccinated B16-OVA tumour-bearing mice. We observed a decreased number and activation status of tumour-specific T cells in the ndLNs and tdLNs of infected mice ([figure 3A](#)). The decreased number of OT-1 cells in LNs is in part due to a lower vaccine-induced proliferation of OT-1 cells observed in the LN draining the vaccination site in infected mice compared with their non-infected counterparts ([figure 3B](#)). Moreover, we observed a decreased number of activated OT-1 cells in the tumours of infected mice ([figure 3A](#)). Remarkably, a similar number and activation status of OT-1 cells were found in tumour and tdLNs of *H. felis*-infected or non-infected mice (see [online supplemental figure S9A](#)). Lastly, by performing in vivo killing assay, we observed that the cytotoxic activities of OT-1 cells generated by vaccination in *H. pylori*-infected mice were lower than those generated in non-infected or *H. felis*-infected mice (see [figure 3B](#) and [online supplemental figure S9B](#)). Collectively, our data show that vaccine-primed tumour-specific immune responses are specifically reduced in *H. pylori*-infected mice.



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## Figure 3

*Helicobacter pylori* jeopardises tumour-specific immune responses. (A) Absolute cell number and activation status of OT-1 CD8<sup>+</sup> T cells isolated from the tdLN, ndLN and tumour(s) of non-infected (NI) and infected (INF) vaccinated B16-OVA tumour-bearing mice (days 10 and 15 post B16 inoculation, respectively, for LNs and tumours). Activated OT-1 cells that were defined as CD44<sup>+</sup>CD62L<sup>-</sup>. (B) Left panel: percentage of proliferating OT-1 cells in the tdLN of NI and INF mice evaluated by carboxyfluorescein succinimidyl ester staining. Right panel: in vivo cytotoxic activities of OT-1 cells in vaccinated NI and INF mice. (C) The presence of *H. pylori* affects the functionality of DCs. Left panel: expression of the activation marker CD86 on CD11c<sup>+</sup>CD11b<sup>-</sup>CD8a<sup>+</sup> DCs in the tdLN of NI and INF mice. Right panel: frequency of proliferating OT-1 cells on ex vivo stimulation with DCs isolated from the spleen of NI or INF mice. Data are representative of three independent experiments (NI, n=9; INF, n=10) (p<0.05, unpaired t-test). (D) The presence of *H. pylori* affects innate immune responses, as determined by inflammatory cytokine (TNF $\alpha$ , IFN $\gamma$ , IL-6 and IL-17) levels in the serum of NI and INF mice. NI and INF B16-OVA tumour-bearing mice were injected with OT-1 cells and vaccinated with OVA in CpG. The serums were recovered on days 1, 2 and 3 postvaccination. For panels A–C, data shown are representative of three independent experiments. For panel D, data shown are representative of two independent experiments. For the experiments described in [figure 3A–D](#), the infectious status of each individual mouse was confirmed at sacrifice by performing rapid urease tests and/or colony forming unit on the stomach (see [online supplemental figures S2E](#)). DCs, dendritic cells; MFI, mean

**fluorescence intensity; ndLN, non-draining lymph nodes; tdLN, tumour-draining lymph node.**

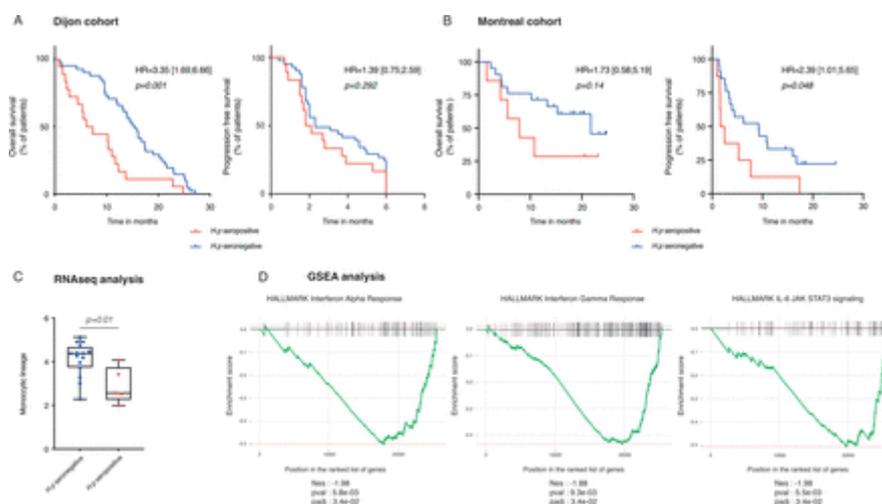
It has previously been described that the gut microbiota can modulate DC function, which impacts CD8<sup>+</sup> T cell priming.<sup>28 31</sup> With this, we reasoned that the reduction of tumour immune responses may be associated with *H. pylori*-induced DC defects.<sup>9–12</sup> We indeed detected defects in activation of DC1 and DC2 cells in the tdLNs of infected mice in as little as 24 hours post B16-OVA engraftment ([figure 3C](#)). Next, by performing an in vitro co-culture assay, we observed that splenic DCs of infected mice promote lower antigen-specific proliferation of OT-1 cells ([figure 3C](#)). Taken together, these results demonstrate that *H. pylori* decreases DC activation processes,<sup>12 32</sup> their cross-presentation activities and jeopardises the tumour-specific immune responses initiated by vaccination.

Lastly, we observed a significant decrease of inflammatory cytokine production in the serum of infected mice on day 1 post OVA/CpG vaccination ([figure 3D](#)). Notably, IFN $\gamma$ , a cytokine playing a pivotal role in the efficacy of tumour vaccination (see [online supplemental figure S10](#)), was drastically reduced in the serum of infected mice ([figure 3D](#)). Altogether, it can be concluded that by dampening innate immune responses (DCs cross-presentation activities and vaccine-induced inflammatory cytokine production), *H. pylori* decreases the tumour-specific immune responses initiated by vaccine-based immunotherapy, leading to a decrease in its efficacy.

16 *H. pylori* seropositivity is associated with reduced effectiveness of anti-PD1 immunotherapy in patients with NSCLC

We performed a retrospective study in two independent cohorts of patients with non-small cell lung cancer (NSCLC) to evaluate the correlation between *H. pylori* seropositivity and the effectiveness of cancer immunotherapies. *H. pylori* seropositivity was determined using validated commercial ELISA tests. In the first cohort of 60 patients with NSCLC from Dijon (France), we detected *H. pylori* antigen-directed IgG antibodies in the serum of 18 patients. In the second cohort of 29 patients with NSCLC from Montreal (Canada), we detected *H. pylori* antigen-directed IgG antibodies in the serum of 8 patients. Remarkably, in the

Dijon cohort, *H. pylori* seropositivity was found to be associated with a clearly defined decrease of NSCLC patient survival on anti-PD-1 therapy ([figure 4A](#),  $p=0.001$ ). Survival medians were 6.7 months for *H. pylori* seropositive patients vs 15.4 months for seronegative patients. Additionally, in the Montreal cohort, *H. pylori* seropositivity was found to be associated with an apparent decrease of NSCLC patient progression-free survival on anti-PD-1 therapy ([figure 4B](#),  $p<0.05$ ) and a numerical difference for overall survival (9.3 months for *H. pylori* seropositive patients compared with 21.7 months for seronegative patients). These data obtained from two independent cohorts of patients with NSCLC on anti-PD-1 therapy clearly demonstrate that *H. pylori* seropositivity is associated with lower effectiveness of anti-PD-1 immunotherapy. Additional prospective studies will further validate our data. Lastly, we performed RNAseq analysis of RNA extracted from formalin-fixed paraffin-embedded tumours from patients with NSCLC from the Dijon cohort.<sup>33</sup> Although we analysed a limited number of patients, we observed a significantly decreased number of cells from the monocyte lineage ([figure 4C](#)) and a substantially decreased expression of genes induced by type I interferon, IFN $\gamma$  and IL-6 in the tumours of infected patients compared with non-infected patients ([figure 4D](#)). These results are reminiscent of the preclinical data ([figure 3D](#) and see [online supplemental figure S8C1](#)), suggesting that the dampening of the innate immune responses by *H. pylori* in infected hosts may decrease the efficacy of cancer immunotherapies.<sup>30</sup>



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## Figure 4

***Helicobacter pylori* seropositivity is associated with reduced effectiveness of anti-PD-1 immunotherapy in patients with non-small cell lung cancer (NSCLC). (A) Dijon cohort: overall survival and overall progression-free survival of patients with NSCLC from the Dijon cohort (60 patients). Kaplan-Meier curves depicting the overall survival of patients with NSCLC treated with nivolumab or pembrolizumab (anti-PD-1 monoclonal antibodies). *H. pylori* seropositivity was found to be associated with a clearly defined decrease of NSCLC patient survival on anti-PD-1 therapy ( $p=0.001$ , Wald test as part of a univariate of Cox model). (B) Montreal cohort: overall survival and overall progression-free survival of patients with NSCLC from the Montreal cohort (29 patients). Kaplan-Meier curves depicting the overall survival of patients with NSCLC treated with nivolumab or pembrolizumab. Log-rank (Mantel-Cox) test  $*p<0.05$ . (C) *H. pylori* infection substantially affects myeloid cells in the tumour(s) of patients with NSCLC. RNAseq analysis was performed on the tumours of patients from the discovery cohort. MCP-counter software was used to determine the tumour infiltration of CD3<sup>+</sup> T cells, CD8<sup>+</sup> T cells, cytotoxic lymphocytes, NK cells, B lymphocytes, cells originating from monocytes (monocytic lineage), myeloid-derived dendritic cells, neutrophils, endothelial cells and fibroblasts. We observed that in the tumours of *H. pylori* seronegative patients, there was a substantially higher expression of genes expressed by cells originating from monocytes compared with *H. pylori* seropositive patients ( $p=0.01$ , Wilcoxon test). (D) Gene-set enrichment analyses (GSEA) were performed on the RNAseq data and revealed that *H.***

***pylori* infection substantially decreases the expression of genes that are controlled by type I IFN, IFN $\gamma$  and IL-6 in the tumours of patients with NSCLC undergoing immunotherapy. PD-1, programmed cell death protein 1.**

## 17 Discussion

In this study, we show that *H. pylori* infection partially blocks the activity of ICIs and vaccine-based cancer immunotherapy in murine models.

Mechanistically, we observed that *H. pylori* dampens the innate and adaptive immune responses of infected hosts and, more specifically, inhibits the antitumoural CD8<sup>+</sup> T cell responses by altering the cross-presentation activities of DCs. In addition, we report that *H. pylori* seropositivity is associated with a reduced effectiveness of anti-PD1 immunotherapy in patients with NSCLC.

It has already been reported that *H. pylori*-infected mice have defective DC activation processes.<sup>9–12</sup> These DC defects were observed in vivo within the gastric mucosa, mesenteric lymph nodes as well as in the lungs of asthmatic mice.<sup>12</sup> Remarkably, this *H. pylori*-induced DC hypoactivation leads to decreased activation of allergen-specific Th2 cells in asthmatic mice.<sup>19 34 35</sup> In this study, we provide evidence that the *H. pylori*-induced DC defects also lead to decreased activation of tumour-specific CD8<sup>+</sup> T cells. *H. pylori* infection not only affects DC function but also that of monocytes and/or macrophages. Indeed, in humans, we observed a decreased number of cells from the monocyte lineage and a substantially decreased expression of genes induced by type I interferon, IFN $\gamma$  and IL-6 in the tumours of infected patients with NSCLC undergoing anti-PD1 treatment ([figure 4C](#)). In addition, we observed a tendency of reduction in the number of activated monocytes in the tumours of infected mice undergoing anti-CTLA4 treatment ([online supplemental figure S8](#)) and a blunted production of inflammatory cytokines in the serum of vaccinated infected mice ([figure 3D](#)). Different virulence factors produced by *H. pylori* such as VacA,  $\gamma$ -glutamyl transpeptidase, neutrophil-activating protein of *H. pylori* and urease have been described to impair myeloid cell activities.<sup>36</sup> Additional experiments will determine whether those *H. pylori*-

derived factors are key in decreasing the effectiveness of cancer immunotherapies.

An informative and puzzling finding of our study is that the eradication of *H. pylori* infection by antibiotherapy does not revert the *H. pylori*-induced hyporesponsiveness to cancer immunotherapy. This could be a consequence of the non-specific antibiotherapy-mediated reduction of immune-potentiating bacteria and/or *H. pylori*-induced neonatal imprinting<sup>37</sup> of the immune system that persists even after its eradication. Such neonatal imprinting that persistently modulates the functioning of the immune system has already been reported in mice.<sup>38 39</sup> Thus, the eradication of *H. pylori* using a strategy that spares the microbiota, such as vaccination, would likely prove to be unsuccessful, as the immune system would have been previously imprinted.

Our clinical study was performed in two independent centres, one located in Europe and the other in North America. Results from both cohorts clearly show that the efficacy of PD-1 immunotherapies is lower in *H. pylori* seropositive patients with NSCLC. In the perspective that this result is confirmed in prospective studies, it has two important clinical implications. First, serological tests detecting *H. pylori* seropositivity may be a powerful tool in predicting the efficacy of ICIs for patients with NSCLC cancer. Future clinical studies will establish whether the same correlation between *H. pylori* seropositivity and the reduced effectiveness of cancer immunotherapies can be extended to patients suffering from other forms of cancer. Second, it may be very useful to consider *H. pylori* seropositivity as a confounding factor that may prevent the identification of bacterial taxa, in patient's faecal samples, affecting positively or negatively, the response to ICIs. Similarly, *H. pylori* seropositivity might also preclude any clinical benefits brought forth by faecal microbiota transplantation in ICI-treated patients.<sup>40-42</sup>

In summary, our study is in alignment with the recognised major role played by the microbiome on the efficacy of cancer immunotherapies.<sup>1</sup> However, our study unveils for the first time that the stomach microbiota affects the response to ICIs and that *H. pylori* serology would be a powerful tool to personalise the treatment of patients in the context of cancer immunotherapies.

## 18 Materials and methods

### 19 Mice

CD45.2<sup>+</sup> C57BL/6 mice were obtained from Charles River (Ecully, France), while CD45.1<sup>+</sup> OT-1 C57BL/6 mice were kindly provided by Dr Gregory Verdeil (University of Lausanne). Mice were bred under specific pathogen-free conditions. All animal experiments were performed in accordance with cantonal laws of animal protection.

### 20 *H. pylori* infection

*H. pylori* P49, a human clinical isolate adapted to mice and *H. felis* strain ATCC 49179<sup>43</sup> were grown in brain heart infusion (BHI) supplemented with 10% fetal bovine serum (FBS) in microaerophilic conditions. Mice were infected twice during the neonatal stage (4 and 5 days of age) with  $2.5 \times 10^8$  *H. pylori* P49 bacteria or once with  $5 \times 10^7$  *H. felis* (5 days of age). Bacteria were administered orally in 20  $\mu$ L of BHI. The control group received 20  $\mu$ L of BHI. For co-housing experiments, neonates belonging to the same litter were infected with *H. pylori* or treated with BHI as control and then weaned in the same cage. Infected and non-infected littermates received the same therapy.

### 21 Assessment of *H. pylori* colonisation

Rapid urease test (Cleartest Servoprax, Germany) and quantification of *H. pylori* colony forming units were used to assess infection status as previously described.<sup>44</sup>

### 22 MC38 tumour cells

Prof Greta Guarda (Biochemistry Institute, Lausanne, Switzerland) kindly provided MC38 colon adenocarcinoma cells. The cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO<sub>2</sub>.

### 23 B16-OVA tumour cells

B16-F10 mouse melanoma cells expressing OVA protein (B16-OVA) were kindly provided by Dr Gregory Verdeil.<sup>23</sup> The cells were cultured in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 10  $\mu$ g/mL G418 at 37°C, 5% CO<sub>2</sub>.

### 24 MC38 tumour model and ICI treatments

MC38 tumour cells ( $5 \times 10^5$ ) were injected subcutaneously into the shaved flank skin of adult male CD45.2<sup>+</sup> mice neonatally infected with *H. pylori* or treated with BHI as control. Mice received in 100  $\mu$ L PBS, either IgG2b isotype (clone MPC-11, BioXCell) or anti-mouse anti-CTLA4 (clone 9D9, BioXCell) alone or in combination with anti-mouse anti-PD-L1 (clone 10F.9G2, BioXCell) on days 7, 10, 13 and 16 post tumour engraftment. We injected intraperitoneally 200  $\mu$ g of antibody for the first injection and 100  $\mu$ g for the following three injections. Tumour volumes were measured according to length and width using Vernier callipers from day 7 following tumour engraftment and every 2–3 days thereafter (see [online supplemental figures S1A, B](#)).

## 25 AOM/DSS colon cancer model and ICI treatments

Infected and non-infected C57BL/6 mice were intraperitoneally injected with AOM at 8 weeks of age.<sup>45</sup> Mice were orally administered with three rounds of DSS. Ten days after the last administration of DSS, mice were injected with 100  $\mu$ g anti-CTLA4 antibodies (five injections, 3 days apart) and sacrificed 7 days after the last anti-CTLA4 injection (see [online supplemental figures S1D](#)). At sacrifice, we numerated the number of tumours within the colon.<sup>25</sup>

## 26 Cancer vaccination model

B16-OVA melanoma cells ( $2 \times 10^5$  cells) were injected subcutaneously into the shaved flank skin of adult female CD45.2<sup>+</sup> mice neonatally infected with *H. pylori* or *H. felis* or treated with BHI. Six days later, CD45.1<sup>+</sup> OT-1 CD8<sup>+</sup> T lymphocytes were isolated from the lymph nodes and spleen using CD8a<sup>+</sup> T Cell Isolation kit (MACS, Miltenyi Biotec GMBH, Solothurn, Switzerland). OT-1 CD8<sup>+</sup> T cells ( $1 \times 10^6$ ) were then adoptively transferred into recipient mice by intravenous injection. The following day, 10  $\mu$ g Ova peptide (SIINFEKL) and 50  $\mu$ g CpG provided by Dr Gregory Verdeil (University of Lausanne) in 100  $\mu$ L PBS were injected subcutaneously on the opposite flank to the tumour site in order to immunise the mice. Anti-IFN $\gamma$  neutralising antibodies (250  $\mu$ g/dose, XMG1.2 clone, BioXcell) were intraperitoneally administered the day of OVA+CpG vaccination and 24 hours later to evaluate the biological role of IFN $\gamma$  in the vaccine-induced tumour control (see [online supplemental figures S1G](#)). Tumour volumes were measured according to length and width using Vernier callipers from

day 7 following tumour engraftment and every 2–3 days thereafter (see [online supplemental figure S1C](#)). For flow cytometry analysis, mice were sacrificed 15 days post tumour engraftment.

## 27 Antibiotic treatment

*H. pylori* infection was eradicated by administering by oral gavage a combination of two antibiotics (amoxicillin (28.6 mg/kg/day) and clarithromycin (14.3 mg/kg/day)) and a proton pump inhibitor (omeprazole (400 µmol/kg/day)) (see [online supplemental figures S1F](#)).<sup>46</sup> The treatment lasted 7 days. The efficacy of eradication was controlled at sacrifice (see [online supplemental figure S2B](#)).

## 28 Faecal transplantation

Adult infected and non-infected mice were treated with enrofloxacin, amoxicillin and clavulanic acid for 3 weeks<sup>47</sup> and orally administered with 100 mg of faeces from infected mice on a daily basis for 5 days.<sup>48</sup> Two weeks after the last faeces administration, we started the B16-OVA melanoma model (see [online supplemental figure S1E](#)).

## 29 CFSE labeling

OT-1 CD8<sup>+</sup> T cells pooled from the lymph nodes and spleen of OT-1 mice were washed twice in warm PBS and were then incubated with carboxyfluorescein succinimidyl ester (CFSE, Enzo Life Sciences, Lausen, Switzerland) diluted in warm PBS (2 µM) for 10 min at 37°C. Cells were then washed once in cold PBS and twice in RPMI supplemented with 10% FBS.<sup>49</sup> CFSE-labelled cells were used for in vivo OT-1 CD8<sup>+</sup> T cell proliferation experiments and for in vitro cross-presentation assays.

## 30 In vivo OT-1 CD8<sup>+</sup> T cell proliferation

Adult CD45.2<sup>+</sup> C57BL/6 mice received  $2 \times 10^5$  B16-OVA cells by subcutaneous injection. Six days later,  $1 \times 10^6$  CFSE-labelled CD45.1<sup>+</sup> OT-1 CD8<sup>+</sup> T cells were adoptively transferred by intravenous injection. The following day, mice were immunised with 10 µg OVA peptide (SIINFEKL) and 50 µg CpG in 100 µL PBS by subcutaneous injection on the opposite flank to the tumour. Three days postimmunisation, mice were sacrificed, and the tdLNs were collected and processed for flow cytometry analysis. For the analysis of CFSE dilution, cells were stained with antibodies

directed towards: CD45.2; CD45.1; CD3 and CD8, see Flow cytometry section in the [online supplemental methods](#) for details.

### 31 In vivo killing assay

The in vivo killing assay was performed by the intravenous injection of CFSE-labelled target cells as described by Trompette *et al.*<sup>50</sup> Briefly, splenocytes were loaded with OVA peptide (200  $\mu$ M) or left unloaded and labelled with 0.5  $\mu$ M or 5  $\mu$ M CFSE, respectively. A mixed 1:1 ratio between the two-labelled cell populations was intravenously injected into recipient mice (see [online supplemental figure S1H](#)). Fourteen hours postinjection, the spleens of recipient mice were recovered and the ratio between the CFSE<sup>low</sup> and CFSE<sup>high</sup> fraction was determined by flow cytometry to assess killing activity of OT-1 cells.

### 32 CD11c<sup>+</sup> DC isolation

The spleens of *H. pylori*-infected and non-infected CD45.2<sup>+</sup> C57BL/6 adult mice were collected and incubated with Collagenase IV (Sigma) and DNase (Sigma) for 20 min at 37°C. The spleens were then filtered through a 40  $\mu$ m cell strainer and washed in RPMI supplemented with 10% fetal calf serum (FCS). Cells were washed in MACS buffer and CD11c<sup>+</sup> DCs were isolated using a Pan dendritic cell isolation kit (MACS, Miltenyi Biotec GMBH).

### 33 Ex vivo cross-presentation assay

CFSE-labelled OT-1 CD8<sup>+</sup> T cells ( $2 \times 10^5$ ) were cultured in vitro with  $2 \times 10^5$  CD11c<sup>+</sup> DCs in RPMI supplemented with 10% FCS, 1% P/S; 1 mM sodium pyruvate, 0.05 mM  $\beta$ -mercaptoethanol and 10 mM HEPES in the presence of different concentrations of OVA protein (Ovalbumin, EndoFit, InvivoGen). After 72 hours of incubation at 37°C, cells were recovered and analysed by flow cytometry analysis.

### 34 Quantification of inflammatory cytokines in the serum

Using the kit instructions, a broad spectrum of cytokines were measured in the serum of non-infected and infected mice at different time points post OVA/CpG immunisation using the LEGENDPlex mouse inflammation panel kit (Biolegend, 740446). Data were acquired using LSRFortessa cell analyzer (BD, San Jose, California, USA) and analysed using Biolegend's LEGEND's plex data analysis software.

### 35 *H. pylori* IgG ELISA

*H. pylori* IgG antibodies were quantified in the plasma of patients, following the protocol for ELISA tests (*H. pylori* IgG ELISA Kit, Genesis Diagnostics). The optical density (OD) at 450 nm was read in a microplate reader (Tecan, Infinite M200 Pro).

### 36 Statistical analysis

Student's t-tests (unpaired, two tailed), Mann-Whitney or two-way analysis of variance tests were used to calculate the levels of significance between groups using GraphPad Prism V.8g. Graphs and figure legends are annotated with the level of significance between the tested groups.

### 37 Dijon patient cohort

This cohort contained 60 patients from three French university hospitals with stage IIIB or IV NSCLC who had previously received one or two lines of chemotherapy. All patients were treated with first-line platinum-based chemotherapy. None of the patients had epidermal growth factor receptor, anaplastic lymphoma kinase, B-Raf proto-oncogene serine/threonine kinase or ROS1 oncogenic driven tumours. On progression, they received 3 mg/kg nivolumab administered intravenously as a single agent every 2 weeks. Tumour response was evaluated by CT scan every four cycles. Tumours were collected, stored and used with informed and written consent from the patients. Clinical characteristics are shown in [online supplemental table 1](#).

### 38 **Survival curves**

Univariate and multivariate Cox proportional-hazards models were built to estimate HRs with a 95% CI. Among clinical variables, age, sex, WHO performance status and histology were evaluated and considered not significant (p value>0.05).

Survival probabilities were estimated using the Kaplan-Meier method and survival curves were compared using the log-rank test. Statistical analyses were performed using the R software (<http://www.R-project.org/>) and graphs were created using GraphPad Prism V.7.03.

### 39 Montreal patient cohort

Patients with metastatic NSCLC (n=29) undergoing treatment with ICIs were prospectively included ([online supplemental table 2](#)). Blood and

stool samples from these patients were collected through the CRCHUM lung cancer biobank (Ethics protocol 18.085–17.035, Montreal, Canada). All patients provided informed consent prior to study enrollment. Clinical characteristics are shown in [online supplemental table 2](#).

## 40 Data availability statement

Data are available upon reasonable request.

## 41 Ethics statements

### 42 Patient consent for publication

Not required.

### 43 Ethics approval

The study protocol was approved by the hospital's ethics committee. The study was performed in accordance with the guidelines of the Declaration of Helsinki.

## 44 Acknowledgments

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## 46 Supplementary materials

### • Supplementary Data

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## 47 Footnotes

- LV and ER contributed equally.
  - **Contributors** DV conceived the project and designed the study. PO, LV, ER, FG, BR, GV and DV analysed the data and prepared the manuscript. ER, PO, LV, BM, CB, CT, CR, MML, MM, EM and EL performed experiments. All authors interpreted the data, discussed the results and revised the manuscript.
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