Running title: diabetes and immune checkpoint inhibitors

Type 2 diabetes mellitus and efficacy outcomes from immune checkpoint blockade in patients with cancer.

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Abstract.

Background: No evidence exists as to whether type 2 diabetes (T2DM) impairs clinical outcome from Immune Checkpoint Inhibitors (ICI) in patients with solid tumors.
Methods: In a large cohort of ICI recipients treated at 21 institutions from June 2014 to June 2020, we studied whether patients on glucose lowering medications (GLM) for T2DM had shorter OS and PFS. We used targeted transcriptomics in a subset of patients to explore differences in the tumor microenvironment of patients with/without diabetes.
Results: A total of 1395 patients were included. Primary tumors included NSCLC (54.7%), melanoma (24.7%), renal cell (15.0%) and other carcinomas (5.6%). Following multivariable analysis, patients on GLM (n=226, 16.2%) displayed an increased risk of death (HR 1.29, 95%CI:1.07-1.56) and disease progression/death (HR 1.21, 95%CI:1.03-1.43) independent of number of GLM received. We matched 92 metformin exposed with 363 controls and 78 patients on other oral GLM or insulin with 299 control patients. Exposure to metformin, but not other GLM was associated with an increased risk of death (HR 1.53, 95%CI:1.16-2.03) and disease progression/death (HR 1.34, 95%CI:1.04-1.72). T2DM patients with higher pre-treatment glycaemia had higher neutrophil-to-lymphocyte ratio (p=0.04), while exploratory tumoral transcriptomic profiling in a subset of patients (n=22) revealed differential regulation of innate and adaptive immune pathways in T2DM patients.
Conclusions: In this study patients on GLM experienced worse outcomes from immunotherapy, independent of baseline features. Prospective studies are warranted to clarify the relative impact of metformin over a pre-existing diagnosis of T2DM in influencing poorer outcomes in this population.

Keywords: diabetes, metabolic syndrome, immune checkpoint inhibitors, cancer, immunotherapy, immune suppression, inflammation, tumor micro-environment.

Statement of translational relevance:

In this study we highlight how patients with advanced solid tumors and concomitant type 2 diabetes (T2DM) experience worse outcome from Immune Checkpoint Inhibitors (ICI) independent of baseline clinicopathologic characteristics. In view of the increasing global burden of type 2 diabetes and the constantly expanding clinical indications of ICI-based therapies, the identification of metabolic host factors as determinants of immune response in patients with cancer has relevant implications for clinical practice. Prospective studies should investigate whether receipt of certain glucose-lowering medications such as metformin as opposed to quality of diabetes control might be modifiable factors to improve outcomes from immunotherapy.

Introduction.

Immune checkpoint inhibitors (ICI) have led to a significant increase in the survival of patients affected by a widening variety of malignancies[1]. Whilst re-invigoration of an immune-exhausted effector T-cell response is at the basis of the mechanism of action of ICI, several host characteristics have been increasingly recognised for their capacity to enhance or blunt ICI efficacy[2-4]. Concomitant medications, patients' body mass index and the presence of a sub-clinical pro-inflammatory response are amongst the accumulating traits to have emerged in the recent past as key modulators of immunotherapy efficacy[2,5].

The complex relationship existing between metabolic syndrome, type 2 diabetes mellitus (T2DM) and cancer has been known for a long time[6]. T2DM is a highly prevalent comorbidity affecting up to 15% of patients at the time of cancer diagnosis[7]. In an increasingly ageing and more co-morbid population, cancer and T2DM share common risk factors[8] and mechanistic evidence has highlighted an increased risk of cancer among patients with a pre-existing diagnosis of diabetes[9].

On the other hand, the complex metabolic changes that characterise the progression of diabetes may exert multiple immune-suppressive effects potentially impairing anti-cancer immunity[10]. Studies on peripheral blood mononuclear cells (PMBC) have shown how hyperglycaemia leads to loss of Interleukin-10 (IL-10) secretion by myeloid cells and to reduced production of Interferon gamma (IFN-γ) and Tumor Necrosis Factor alpha (TNF-α) by T-cells[11], along with lower production of IL-12 and IFN-γ in PBMC cultures after

exposure to pathogens[12]. Hyperglycaemia can also cause neutrophil dysfunction, including defects in reactive oxygen species (ROS) production, immunoglobulin-mediated opsonization and degranulation[13-15]. The role of diabetes in promoting immune dysfunction is further supported by the finding that hyperglycaemia can induce macrophage polarisation towards a pro-tumorigenic M2 phenotype[16,17] alongside functional defects in NK cells degranulation capacity[18].

In a therapeutic landscape characterised by a continuously expanding list of indications where ICI have been proven effective[19] it is of the utmost importance to establish whether a concomitant diagnosis of T2DM carries a negative impact on ICI efficacy, in order to identify patients at risk of worse outcome and inform clinical practice.

In this study, we analysed a large multicentre cohort of patients with advanced cancers treated with chemotherapy-free ICI-based regimens to evaluate whether use of glucose lowering medications as a surrogate for a prior history of T2DM might be associated with clinical outcome from ICIs in patients with solid tumors.

Materials and Methods

Study objectives and design.

The aim of this analysis was to describe the potential impact of pre-existing T2DM on clinical outcomes from ICI-based treatments in a large multicenter cohort of patients with advanced solid tumors treated outside clinical trials[20-27].

Overall, 21 Institutions from Italy and the United Kingdom participated to the data collection (**Supplementary Table 1**) and retrospectively included patients with stage IV malignancy treated with ICIs as 1st or subsequent line from June 2014 to June 2020, with a data cut-off period of 31st of December 2020. Patients on ICI-based combinations, such as chemo-immunotherapy and targeted-therapy-ICIs, were excluded.

Programmed Death-1/Programmed Death-Ligand 1 (PD-1/PD-L1) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) inhibitors were administered at doses and schedules indicated in the respective summary of product characteristics.

Clinical outcomes of interest included progression free survival (PFS), defined as the time from treatment initiation to disease progression or death (whichever occurred first) and overall survival (OS), defined as the time from treatment initiation to patients' death or loss to follow-up. Periodic tumor re-assessment was performed at the discretion of treating clinicians with frequency ranging from 12 to 16 weeks. Investigators were asked to provide

disease progression information according to RECIST (V. 1.1) criteria[28]. For PFS as well as for OS, patients without events were considered as censored at the time of the last follow-up.

To reproducibly assess the effect of T2DM on ICI outcomes, we used the receipt of any glucose lowering medications (GLM) at the moment of ICIs initiation as a surrogate of a diagnosis of T2DM and define the population of interest. GLMs started at any time prior to and taken until immunotherapy initiation were grouped in accordance to international guidelines and recommendations[29] as metformin, other oral diabetes medications (including sulfonylureas, meglitinides, thiazolidinediones, α-glucosidase inhibitors, DPP-4 inhibitors, SGLT2 inhibitors, and cycloset) and insulin therapy.

We first assessed the impact of diabetes on OS and PFS with univariable and multivariable analyses. In addition, considering the differential distribution of baseline patients' characteristics between patients with and without diabetes, we also performed a propensity score matching (PSM) between the two groups and explored OS and PFS across the matched populations.

Subsequently, we conducted two additional PSM sub-analyses among patients with NSCLC and melanoma, to explore the association between the receipt of baseline GLMs and OS/PFS in the two matched cohorts.

Baseline exposure to each class of antidiabetic medication was also verified for their association with OS and PFS following ICI therapy. We then stratified diabetic patients according to the receipt of one class versus multiple classes of GLM at the time of ICI commencement, a methodology that allowed us to infer potential association between oncological outcomes and surrogates of diabetes severity and duration.

In an attempt to verify the independence between the diagnosis of diabetes and type of anti-diabetic treatment received, we performed two separate PSM procedures between metformin-exposed patients (after the exclusion of patients on any non-metformin anti-diabetic drug), patients on other oral antidiabetic drugs/insulin therapy only (after the exclusion of patients on metformin) and those without diabetes.

To investigate whether chronic hyperglycemia is associated with systemic inflammation in patients with cancer we computed the median baseline glycaemia (MBG) from up to 3 random blood sugar test samples performed within 3 months prior to ICI initiation. We described the association between MBG and the pre-treatment neutrophil-to-lymphocyte ratio computed from routine full blood counts test taken within 30 days prior to ICI therapy initiation.

In an ancillary translational analysis and to complement our clinical findings, we intended to establish whether the tumor micro-environment (TME) of patients with pre-existing diabetes was associated with significantly different features in the intra-tumoral immune infiltrate. Following total RNA extraction of macrodissected unstained sections containing >20% of tumor tissue, targeted transcriptome profiling was performed on a subset of primary tumor samples of diabetic patients and non-diabetic controls extracted from the Imperial College London cohort, using the NanoString PanCancer Immune Profiling panel on an nCounter® Analysis System (NanoString Technologies, Seattle). Methodology of targeted transcriptomic analysis follow established protocols[30] with details reported as **Supplementary Methods**.

The procedures followed were in accordance with the precepts of Good Clinical Practice and the declaration of Helsinki. Written informed consent was obtained from alive patients at the moment of data collection, while it was waived by competent authorities due to anonymized nature of patient data and retrospective design of the study for deceased patients. The study was approved by the respective local ethical committees on human experimentation of each institution, after previous approval by the coordinating center (University of L'Aquila, Internal Review Board protocol number 32865, approved on July 24th, 2018).

Statistical analysis

Baseline patients' characteristics were reported with descriptive statistics as appropriate. The $\chi 2$ and test was used to compare categorical variables. PFS/OS were evaluated and compared using the Kaplan-Meier method and the log-rank test. Duration of follow-up was calculated according to the reverse Kaplan-Meier method. Cox proportional hazards regression was used for the univariable and multivariable analysis of the risk of disease progression/death and death, and to compute the hazard ratios (HR) with 95% confidence intervals (CIs).

Fixed multivariable models were used including all the variables already known to significantly impact clinical outcomes in the cohort including primary tumor types (non-small cell lung cancer - NSCLC, melanoma, renal cell carcinoma and others), age (continuous), biological sex (male *vs* female), Eastern Cooperative Oncology Group-Performance Status (ECOG-PS) (0-1 *vs* ≥ 2), burden of disease (number of metastatic sites ≤ 2 *vs* > 2), treatment line (first *vs* second *vs* further lines), body mass index (BMI – continuous), corticosteroids at immunotherapy initiation (dose ≥10 mg prednisone daily or

equivalent - yes vs no), and systemic antibiotics at immunotherapy initiation (yes vs no) (both taken within 30 days prior to ICIs initiation)[20-26,31].

Acknowledging that data-source consisted of 21 different institutions, which could represent a source of bias, a centre-specific conditional interpretation by using frailty models was applied to correct all the 95%CIs from multivariable Cox regressions.

To respectively compare the outcome of patients on metformin only and those on other oral antidiabetic drugs/insulin therapy only with those without diabetes, separated propensity score matching (PSM) procedures with nearest method, 1:4 ratio and a caliper of 0.2 were performed, including all the above mentioned clinical characteristics[32]. The balancing ability of the PSM were estimated through the standardized mean differences (SMD) of the matched characteristics. Considering differences in sample size and prevalence of patients with diabetes between different primary tumor groups, a 1:1 ratio, 0.1 caliper and 1:3 ratio, 0.1 caliper were used for the PSM in the NSCLC and melanoma cohorts, respectively[33].

The Kruskal-Wallis test was used to compare MBG between diabetic and non-diabetic patients. Linear regression and logistic regression with odds ratio (OR) and 95%CIs were used to the associations between the MBG and the NLR.

All P-values were 2-sided and confidence intervals set at the 95% level, with significance pre-defined to be at <0.05. Analyses were performed using the R-studio software, R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, and the MedCalc® Statistical Software version 20 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021).

Data availability statements

The datasets used during the present study are available from the corresponding author upon formal reasonable request and after approval of the study steering committee.

Results.

Patients' characteristics.

Overall, 1395 consecutive patients with advanced solid tumors treated with nivolumab (766, 54.9%), pembrolizumab (499, 35.8%), atezolizumab (71, 5.1%), ipilimumab (35, 2.5%) and other ICIs (24, 1.7%) were included in the analysis. As reported in **Table 1**, median age was 68 years (range: 21–91), male/female ratio was 888/507 and primary tumors were: NSCLC (54.7%), melanoma (24.7%), renal cell carcinoma (15.0%) and others (5.6%). In total, 226

patients (16.2%) were on GLM, of which 147 (65.0%) on metformin, 125 (55.3%) on other oral diabetes medication and 76 (33.6%) patients on insulin therapy. Details of diabetes medications are summarized in **Supplementary Table 2**. Of note, 41 patients had pre-existing autoimmune disorders (8 cases of thyroid dysfunction, 10 skin disorders, 4 inflammatory bowel disease, 2 vasculitis, 2 neurological disorders, 15 others). There were no cases of pre-existing type 1 diabetes.

Patients with diabetes were older (median age 71 vs 68 years, p <0.0001), more likely males (73.9% vs 61.7%, p=0.0005), with higher BMI (median 25.6 vs 24.9, p=0.0075). Patients with diabetes more frequently presented with a low-burden disease (≤2 metastatic sites 41.2% vs 49.3%, p=0.0253).

At the median follow-up of 32.5 months (95%CI: 31.1-34.0) the median OS and PFS for the overall population were 17.7 months (95%CI: 15.5-19.5; 832 events) and 8.2 months (95%CI: 7.3-9.2; 1057 events).

Pre-existing type 2 diabetes is associated with worse outcome from ICIs.

In the overall population, patients receiving GLM displayed an increased risk of death (HR 1.23, 95%CI: 1.03-1.47 – **Figure 1A**) but not of disease progression/death (HR 1.14, 95%CI: 0.97-1.33 – **Figure 1B**) in comparison to the control group. Considering the differential distribution of baseline features between the two groups, multivariable analyses were performed for both the clinical endpoints. After adjustment for all the available confounders (**Table 2**), receipt of GLM resulted to be independently associated with an increased risk of death (HR 1.29, 95%CI: 1.07-1.56) and disease progression/death (HR 1.21, 95%CI: 1.03-1.43).

After the PSM procedure, 225 patients on GLM were matched with 808 patients from the control group, with an optimal balancing ability (**Supplementary Table 3**). Within the matched cohorts, the receipt of GLM was associated with an increased risk of death (HR 1.25, 95%CI: 1.04-1.50 – **Figure 1C**) and a tendence towards and increased risk of disease progression/death (HR 1.17, 95%CI: 0.99-1.38 – **Figure 1D**).

Among 763 patients with NSCLC, 138 (18.1%) were on baseline GLM. After PSM, 135 of them were matched with 135 patients from the control group with a good balancing ability (**Supplementary Table 4**). Within the matched NSCLC cohorts, the receipt of baseline GLM was associated with an increased risk of death (HR 1.49, 95%CI: 1.11-2.01 – **Supplementary**

Figure 1A), alongside a non-significant effect on the risk of disease progression/death (HR 1.17, 95%CI: 0.89-1.33 - **Supplementary Figure 1B**).

Among 345 patients with melanoma, 49 (16.5%) were on baseline GLM. These were propensity score matched with 128 patients from the control group with a good balancing ability (**Supplementary Table 5**).

The median OS of patients receiving GLM was 22.9 months (95%CI:12.0-NR, 25 events) while the OS of the control group was not reached (52 events) with a tendence towards an increased risk of death (HR 1.39, 95%CI: 0.86-2.23 – **Supplementary Figure 1C**). Similarly, the median PFS of patients exposed to GLM was 11.4 months (95%CI: 4.9-23.4, 37 events) while that of the control group was 13.8 months (95%CI: 8.7-26.0, 77 events; HR 1.35, 95%CI: 0.91-2.01 – **Supplementary Figure 1D**).

Increasing GLM burden does not impact clinical outcome from immunotherapy.

Among 226 patients on treatment for diabetes, 102 (45.1%) were receiving GLM monotherapy, whilst 124 (54.9%) were receiving a combination treatment. We sought to determine whether diabetes medication burden was associated with a progressive detrimental impact on clinical outcomes. However, we found that only patients on monotherapy experienced an increased risk of death in comparison to the control group (HR 1.29, 95%CI: 1.01-1.65), while no significant effect was associated with being on multiple diabetes medications (**Supplementary Figure 2A**). Similarly, neither monotherapy, nor combination therapy were associated with worse PFS (**Supplementary Figure 2B**).

Differential effect of metformin and other anti-diabetes medications on clinical outcomes.

Overall, 147 patients were on metformin and 134 were on other oral antidiabetic drugs/insulin therapy. On univariable analysis, receipt of metformin therapy was associated with an increased risk of death (HR 1.35, 95%CI: 1.09-1.66, **Supplementary Figure 3A**) and disease progression/death (HR 1.23, 95%CI: 1.02-1.49, **Supplementary Figure 3B**). On the contrary, being on other oral antidiabetic drugs/insulin therapy was not associated with both the OS and PFS (**Supplementary Figure 4**).

Stratifying patients into those who were on baseline metformin either alone or in combination and those who were on other than metformin diabetes medications only, we reported similar trends for OS (log-rank p-value = 0.018) and PFS (log-rank p-value = 0.086) but without

significant differences between exposure to metformin and other diabetes medications only (**Supplementary Figure 5**).

After the exclusion of 54 patients (23.9%) on metformin, other oral hypoglycemic and insulin therapy combinations, and 1 patient (0.4%) on metformin and insulin therapy combination, 92 patients (40.7%) on metformin monotherapy and 79 (34.9%) on other antidiabetic medications (of which 21 -26.6%- on other oral hypoglycemic medications, 11 -13.9%- on insulin monotherapy, and 47 -59.5%- on combinations of both) were included in the respective PSM analysis.

Compared to patients who were not taking diabetes medications, those on metformin only were older (median age 71 vs 68 years, p=0.0035) and more frequently males (66.3% vs 61.7%, p=0.0384, **Supplementary Table 6**). After the PSM procedure, 92 patients on metformin only were matched with 363 patients from the control group, with an optimal balancing ability (**Supplementary Table 7**). Within the matched cohorts, being on metformin only was associated with an increased risk of death (HR 1.53, 95%CI: 1.16-2.03 – **Figure 2A**) and disease progression/death (HR 1.34, 95%CI: 1.04-1.72 - **Figure 2B**).

Compared to the control group, patients on other oral antidiabetic drugs/insulin therapy only were older (median age 72 vs 68 years, p<0.0001), with a higher BMI (median 25.9 vs 24.9, p=0.0108) and a higher burden of metastatic sites (63.3% vs 50.7%, p=0.0306); they also were more likely males (78.5% vs 61.7%, p=0.0028) and with a higher proportion of NSCLC (72.2% vs 53.5%, p=0.0143) (**Supplementary Table 8**).

After the PSM procedure, 78 patients on other oral antidiabetic drugs/insulin therapy only were matched with 299 patients from the control group, with an optimal balancing ability (**Supplementary Table 9**). Within the matched cohorts, being on other oral antidiabetic drugs/insulin therapy only was not associated with either the risk of death (HR 1.03, 95%CI: 0.75-1.41 – **Figure 2C**), nor that of disease progression/death (HR 0.99, 95%CI: 0.75-1.31 - **Figure 2D**).

Diabetes and poor glycemic control are associated with unopposed systemic inflammation and distinctive immune-suppressive features within the TME.

Overall, MBG data were available for 133 patients (**Supplementary Table 10**).

The median MBG value for the overall cohort was 5.7 mmol/L (range 4.1-19.9) and significantly different among diabetic (n=19, median 8.0 mmol/L, range: 5.6-19.9) and non-diabetic patients (n=114, median 5.6 mmol/L, range: 5.6: 4.1-8.7, p<0.0001). Median NLR

for the 133 patients evaluable for MBG was 3.8 (range 0.1-36.5). Increasing levels of MBG were significantly associated with increasing NLR values [$F(1,131)= 4.09$, $p = 0.04$] with an R^2 of 0.030 (**Supplementary Figure 6**). To discriminate the effect of concomitant corticosteroid therapy in influencing the relationship between MBG and NLR, we performed a multivariable logistic regression using the median NLR value as cut off. This model confirmed that baseline corticosteroid therapy was not associated with pre-treatment NLR (OR 1.87, 95%CI: 0.51-6.87), whereas increasing MBG was confirmed to be significantly associated with a high NLR (OR 1.58, 1.17-2.14).

In view of the negative association between T2DM and outcome from immunotherapy we performed an exploratory targeted transcriptomic profiling experiment in a small subset of 22 primary tumor samples selected from the Imperial College London cohort, including 11 controls and 11 diabetic patients. Clinical features of included patients are summarized in **Supplementary Table 11**. Using a bulk transcriptomic approach of macro-dissected tumor tissue we found that samples from patients with diabetes were characterized by distinctive characteristics suggestive of more profound immune suppression compared to non-diabetic controls (**Supplementary Figure 7**). In particular, directed gene set enrichment analysis suggested significant downregulation of a number of gene signatures involved in adaptive and innate immune responses in diabetic samples (**Figure 3**). Analysis of candidate genes highlighted the decreased expression of single transcripts belonging to the inflammatory response (CXCL9, CXCL11, BIRC5) and to the modulation of T-cell function (LAG3). (**Supplementary Figure 8A** and **8B**) in diabetic samples[34,35].

Discussion.

The wide therapeutic index of ICI has broadened the reach of systemic therapy in solid tumors, making it possible to safely treat elderly and multiply comorbid patients who may not qualify for cytotoxic or targeted therapies[36,37]. Polypharmacy and co-morbidities can however affect efficacy of ICI[25]. Despite being a highly prevalent co-morbidity in patients with cancer[38-40], and some preliminary descriptive findings in patients with lung cancer[41], there is no convincing evidence to suggest whether a coexisting diagnosis of diabetes leads to worse outcomes from immunotherapy.

In our large observational study of ~1400 ICI recipients, we were able to demonstrate that a concomitant diagnosis of T2DM at ICIs initiation was independently associated with inferior

outcomes from immunotherapy, a finding that relies on the use of multivariable models and PSM analyses.

Whilst hyperglycemia and T2DM are hallmarks of the metabolic syndrome, together with dyslipidemia, increased waist circumference and arterial hypertension[42], our study is the first to ~~clearly~~ suggest an opposite effect of T2DM compared to obesity in shaping ICI-mediated immune reconstitution. Obesity has been paradoxically associated with improved outcomes from ICIs[43], with pre-clinical and clinical evidence suggesting the presence of an obesity-related T-cell dysfunction that can be rapidly reversed upon checkpoint blockade[20,44].

Although we reported an association between GLM exposure and increasing BMI, our understanding of the relationship between obesity and response to ICIs has significantly evolved, calling into question a number of concurrent host factors[20]. Distribution of adiposity and body composition are more complex factors in dictating outcomes form immunotherapy, all imperfectly recapitulated by simple BMI computation. Obesity, dyslipidemia[43,45], chronic hyperglycemia and the development of peripheral insulin resistance could be interpreted as a progressive, time-dependent derangement of the host metabolic response, where high body weight and accumulation of subcutaneous fat precedes an increase in visceral adiposity, accumulation of intramuscular adipose tissue and secretion of adipocytokines, ultimately leading to progressive weight loss[46] in the context of active malignancy. Higher subcutaneous fat distribution is in fact associated with better outcomes from immunotherapy, whereas the opposite is true for inter-muscular fat and sarcopenic-obesity, traits that are increasingly associated with unopposed systemic inflammation and worse outcomes from ICIs[47-51].

In our study, patients with diabetes experienced worse outcome independent of common clinicopathologic features of their oncological disease, including tumor site of origin and disease burden, giving credence to the hypothesis that diabetes may exert a pre-conditioning effect against ICI efficacy[10]. Despite the limited sample size and different prevalence of diabetes across different primary tumors, results of the survival analysis performed among the NSCLC and melanoma matched cohorts seem to support this, confirming a detrimental effect of pre-existing T2DM on OS for patients with NSCLC and a similar trend for patients with melanoma.

T2DM leads to an exquisitely immune-suppressive state. Patients with diabetes are less reactive to pathogens[12], with chronic hyperglycaemia leading to dysfunctional innate immune responses[13-15] and functional repercussions on all major immune cell subsets, including macrophages, dendritic cells, T-cells and NK cells[52]. Hyperglycaemia has also been

associated with the increase of circulating CD8+ PD-1+ T-cells in patients with T2DM, which show reduced glycolysis and impaired cytokine secretion[53].

Lack of detailed peripheral immune cell characterisation limits our ability to establish mechanistic links between T2DM and outcome. However, our study highlights a linear relationship between MBG and the patients' NLR, a solid and reproducible measure of systemic inflammation[54], postulating a link between T2DM and impaired ICI efficacy through defective modulation of innate immune pathways[55,56].

To provide further insight as to the mechanisms linked to inferior outcome from immunotherapy in ICI recipients, we performed an exploratory analysis of a small cohort of patients with and without diabetes with available pre-treatment archival tissue. Whilst limited by small sample size and exploratory intent, targeted transcriptomic analyses highlight downregulation of gene expression programmes involved in the innate and adaptive immune response in the TME of diabetic patients[57], in line with previous evidence showing worse T-cell exhaustion in diabetic patients with melanoma treated with ipilimumab[58].

The transcriptomic data presented in this study are hypothesis generating and cannot be viewed as exhaustive of all plausible explanations justifying inferior survival of patients with T2DM. Compositional changes in the gut microbiota can additionally be mentioned among potential underlying mechanisms to our findings, given that complex interplay existing between T2DM, metabolic dysfunction and perturbation of gut homeostasis[59]. A significant increase in the Bacteroidetes/Firmicutes ratio[60] and reduction in the presence of commensal bacterial species specifically associated with improved ICI efficacy, such as Akkermansia muciniphila[61-63], have been reported among patients with diabetes.

The increasingly appreciated role of concomitant medications as an alternative or perhaps complimentary cause of altered responsiveness to ICI raises the question of whether individual GLM classes may be important in influencing prognosis.

Whilst number of GLM was not associated with prognosis, stratification of outcome by GLM class suggested that the detrimental effect on clinical outcomes we observed was restricted to metformin recipients.

Whilst we cannot conclude whether the negative prognostic effect for metformin exposure is causative rather than associative, it is important to highlight that a consistent body of evidence supports metformin as preferred initial therapy for T2DM, along with a substantial patient-provider resistance to start diabetes combination treatments at metformin failure and poor adherence to insulin in western countries[64-68]. When these considerations are taken into

account it might be assumed that metformin exposure may capture patients with long-standing and potentially sub-optimally controlled diabetes. In fact, metformin was mainly given as monotherapy in our cohort, whereas other GLMs were mostly co-administered with insulin: a finding that makes it impossible to fully disentangle the effect of improved T2DM control associated with insulin therapy as opposed to a true mechanistic detrimental effect from metformin alone.

On the other hand tumour modulating role of metformin have been described for a long time in patients with cancer [69] [70], although evidence in support of an immune-modulating effect of metformin in the context of immunotherapy of cancer is scantly and mostly limited to the preclinical setting[71] [72,73].

Metformin may have immune-suppressive properties, through targeted inhibitory effect on leukocyte function including AMPK-induced mTORC1 inhibition and the reduction of mitochondrial ROS production[74,75]. In addition, multiple studies confirmed that metformin can lead to gut dysbiosis and gut microbial perturbation in healthy volunteers[76], which in turn are associated with gastrointestinal adverse effects following metformin intake[77]. A recent deep-learning multi-omics phenotyping study of 789 patients with newly diagnosed T2DM[78], reported an association between metformin and dysregulation of CXCL8 and CD177, which are involved in both the innate and adaptive anti-cancer immune response[79,80], alongside with a distinctive shift in gut metagenomics data.

Taken together, our data suggest a statistically significant and clinically meaningful difference in survival for patients receiving GLM for diabetes prior to ICI, with a greater effect observed for those exposed to metformin. Whilst hypothesis generating, these data require validation in prospective clinical studies before solid clinical recommendations are made, so that the relative contribution of metformin over adequacy and quality of T2DM control can be evaluated for their putative mechanistic linkage with outcome from immunotherapy.

In addition, further research efforts should provide a more comprehensive evaluation of diabetes severity, including prevalence of micro and macrovascular complications, dietary habits, treatment adherence and baseline hemoglobin A1c levels[81,82] factors that cannot be reconstructed from our data due to the retrospective nature of our study.

Primary analyses in the whole study population were adjusted for primary tumor type, resulting in an optimal balancing ability. However, we acknowledge that the inclusion of different tumors is a significant source of heterogeneity. The separate PSM performed among

the NSCLC and melanoma cohorts suggest similar detrimental effects for pre-existing T2DM across different malignancies, even though the reduced sample size and a lower proportion of patients with diabetes within the melanoma group limited the analysis, which did not reach the statistical significance threshold.

In addition, despite the concordant trend of a reduced PFS for diabetic patients at the matched analysis, the lack of a statistically significant increase in the risk of disease progression/death (HR 1.17, 95%CI: 0.99-1.38, p=0.056) needs to be mentioned and might be related to the relatively small number of events across groups. Small sample size of the cohort included in the MBG and targeted transcriptomic analyses should also be considered in interpreting the results, which – although provocative – do not allow us to infer conclusive considerations about differential role of systemic inflammation and expression of immune-related genes in the TME of patients with diabetes.

Despite these limitations and the preliminary nature of our findings, our study is the first to our knowledge to report a clear detrimental effect of diabetes on clinical outcomes from ICIs in patients with solid tumors. In view of the constantly expanding clinical indications of ICI-based therapies across different cancer types[19] and the increasing global burden of metabolic syndrome, obesity and type 2 diabetes[83,84], our findings are of clinical importance and need to be carefully considered in the provision of cancer immunotherapy.

Further prospective research efforts are needed to fully elucidate the underlying mechanisms in support of our findings, to assess the putative detrimental role of metformin therapy and other GLM, and to investigate whether patients with cancer requiring an ICI-based treatment should be prioritized for optimization of T2DM therapy.

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Table 1: Baseline patients' characteristics for the overall population and according to the receipt of diabetes medications. ECOG-PS: eastern cooperative oncology group-performance status; NSCLC: non-small cell lung cancer; BMI: body mass index; PD-1/PD-L1: programmed death-1/programmed death-ligand 1; GLM: glucose lowering medications.

	Total	**No GLM**	**GLM**	
	N° (%) - 1395	**1169 - N° (%)**	**226 - N° (%)**	**p-value**
AGE, (years)				
Median	68	68	71	P < 0.0001
Range	21 – 91	21 – 91	22 – 88	
SEX				
Male	888 (63.7)	721 (61.7)	167 (73.9)	P = 0.0005
Female	507 (36.3)	448 (38.3)	59 (26.1)	
ECOG-PS				
0 – 1	1205 (86.4)	1011 (86.5)	194 (85.8)	P = 0.7963
≥ 2	190 (13.6)	158 (13.5)	32 (14.2)	
Primary Tumor				
NSCLC	763 (54.7)	625 (53.5)	138 (61.1)	
Melanoma	345 (24.7)	296 (25.3)	49 (21.7)	P = 0.0730
Renal cell carcinoma	209 (15.0)	185 (15.8)	24 (10.6)	
Others	78 (5.6)	63 (5.4)	15 (6.6)	
No. of metastatic sites				
≤ 2	726 (52.0)	593 (50.7)	133 (58.8)	P = 0.0253
> 2	669 (48.0)	576 (49.3)	93 (41.2)	
Treatment line of Immunotherapy				
First	519 (37.2)	422 (36.1)	97 (42.9)	P = 0.0522
Non-First	876 (62.8)	747 (63.9)	129 (57.1)	
BMI (kg/m^2)				
Median (range)	25.1 (13.6 – 50.8)	24.9 (13.6 – 50.8)	25.6 (16.4 – 43.2)	P = 0.0075
Underweight (≤ 18.5)	59 (4.2)	54 (4.6)	5 (2.2)	
Normal weight (18.5 - 25)	628 (45.0)	538 (46.0)	90 (39.8)	P = 0.0711
Overweight (25 -30)	508 (36.4)	415 (35.5)	93 (41.2)	
Obese (≥ 30)	200 (14.3)	162 (13.9)	38 (16.8)	
Baseline steroids				
No	1043 (74.8)	868 (74.3)	175 (77.4)	P = 0.3135

Yes	352 (25.1)	301 (25.7)	51 (22.6)	
Baseline systemic antibiotics No Yes	 1043 (74.8) 352 (25.1)	 1076 (92.0) 93 (8.0)	 199 (88.1) 27 (11.9)	 P = 0.0502
Metformin No Yes	 1248 (89.5) 147 (10.5)	-	 147 (65.0)	-
Other oral diabetes medications No Yes	 1270 (91.0) 125 (9.0)	-	 125 (55.3)	-
Insulin therapy No Yes	 1319 (94.6) 76 (5.4)	-	 76 (33.6)	-

Table 2: Fixed multivariable analyses for the risk of death and disease progression/death within the whole cohort. A centre-specific conditional interpretation by using frailty models was applied to correct all the 95%CIs. GLM: glucose lowering medications; BMI: body mass index; NSCLC: non-small cell lung cancer; ECOG-PS: eastern cooperative oncology group performance status.

	Multivariate Analysis	
	Risk of death	**Risk of disease progression/death**
VARIABLE	**HR (95% CI)**	**HR (95%CI)**
GLM No Yes	 1 **1.29 (1.07-1.56)**	 1 **1.21 (1.03-1.43)**
BMI Continous	 0.97 (0.96-0.99)	 0.98 (0.97-0.99)
Age Continuous	 0.99 (0.99-1.00)	 0.99 (0.99-1.00)
Primary Tumour NSCLC Melanoma Kidney Others	 1 0.72 (0.56-0.93) 0.55 (0.43-0.71) 0.89 (0.64-1.23)	 1 0.87 (0.68-1.10) 0.74 (0.59-0.92) 1.09 (0.82-1.44)
Sex Female Male	 1 1.14 (0.98-1.32)	 1 1.13 (0.99-1.29)
Treatment line First Non-first	 1 1.24 (1.05-1.46)	 1 1.24 (1.07-1.44)
N° of metastatic sites ≤ 2 >2	 1 1.57 (1.36-1.83)	 1 1.41 (1.24-1.62)
ECOG PS 0-1 ≥2	 1 2.32 (1.91-2.80)	 1 1.92 (1.61-2.30)
Baseline corticosteroids No	 1	 1

Yes	1.64 (1.39-1.93)	1.51 (1.30-1.75)
Baseline antibiotics		
No	1	1
Yes	1.44 (1.15-1.81)	1.35 (1.09-1.68)

Figures' legend:

Figure 1: Kaplan-Meier survival estimates according to the receipt of any diabetes medication. **A)** Overall Survival whole cohort; patients on any diabetes medication: 14.5 months (95%CI: 11.1 – 18.3; 148 events), patients not receiving diabetes medications: 18.9 months (95%CI: 15.9 – 21.5; 684 events). **B)** Progression Free Survival whole cohort; patients on any diabetes medication: 8.0 months (95%CI: 6.2 – 10.4; 185 events), patients not receiving diabetes medications: 8.2 months (95%CI: 7.1 – 9.4; 872 events). **C)** Overall Survival PSM cohort; patients on any diabetes medication: 14.4 months (95%CI: 11.2 – 18.7; 148 events), patients not receiving diabetes medications: 18.7 months (95%CI: 16.1 – 22.1; 466 events). **D)** Progression Free Survival PSM cohort; patients on any diabetes medication: 8.0 months (95%CI: 6.2 – 10.6; 185 events), patients not receiving diabetes medications: 8.4 months (95%CI: 7.5 – 10.1; 593 events). PSM: propensity score matching.

Figure 2: Kaplan-Meier survival estimates according to the receipt of metformin only after the exclusion of patients on other DM and insulin therapy. **A)** Overall Survival PSM cohort; patients on metformin only: 11.4 months (95%CI: 9.3 – 15.9; 66 events), patients not receiving metformin: 20.4 months (95%CI: 17.5 – 26.3; 363 events). **B)** Progression Free Survival PSM cohort; patients on metformin only: 7.9 months (95%CI: 4.3 – 11.4; 79 events), patients not receiving metformin: 8.9 months (95%CI: 7.3 – 10.9; 260 events). Kaplan-Meier survival estimates according to the receipt of other DM/insulin therapy only after the exclusion of patients on metformin. **C)** Overall Survival PSM cohort; patients on other DM/insulin therapy only: 19.3 months (95%CI: 14.7 – 24.8; 48 events), patients not receiving DM/insulin therapy: 18.1 months (95%CI: 14.8 – 21.9; 174 events). **D)** Progression Free Survival PSM cohort; patients on other DM/insulin therapy only: 10.1 months (95%CI: 6.9 – 16.5; 61 events), patients not receiving DM/insulin therapy: 8.2 months (95%CI: 6.6 – 11.6; 222 events). PSM: propensity score matching; DM: diabetes medications.

Figure 3: Gene set analysis showing the differential regulation of 22 gene expression signatures on the basis of diabetic status. Targeted transcriptomic analysis using NanoString PanCancer immune profiling was perform to compare patients with diabetes (n=11) with

non-diabetic controls (n=11). Methodological information for the gene set enrichment analysis and its interpretation is provided as **supplementary methods**.