immuno

MDPI

Article

LDH Isotyping for Checkpoint Inhibitor Response Prediction in Patients with Metastatic Melanoma

Sandra van Wilpe [1,*], Sofie H. Tolmeijer [1], I. Jolanda M. de Vries [2], Rutger H. T. Koornstra [3] and Niven Mehra [1]

[1] Department of Medical Oncology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands; Sofie.Tolmeijer@radboudumc.nl (S.H.T.); Niven.Mehra@radboudumc.nl (N.M.)
[2] Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands; Jolanda.deVries@radboudumc.nl
[3] Department of Medical Oncology, Rijnstate Hospital, 6815 AD Arnhem, The Netherlands; RKoornstra@Rijnstate.nl
* Correspondence: Sandra.vanwilpe@radboudumc.nl

Abstract: Serum lactate dehydrogenase (LDH) levels are inversely related with response to immune checkpoint inhibitors (ICIs) in patients with metastatic melanoma. LDH is a key regulator of glycolysis, a pathway known to be upregulated in malignant tumors and to negatively affect antitumor immunity. We hypothesized that LDH isotype distribution in peripheral blood better reflects tumor glycolytic activity than total LDH levels and might therefore contribute to immunotherapy response prediction. LDH isotyping was performed in blood of 40 patients with metastatic melanoma and elevated LDH levels, of which 22 were treated with ipilimumab plus nivolumab. LDH-1 levels were decreased in 57.5% of patients. The percentage of LDH-2, -3 and -4, on the other hand, was elevated in 35%, 67.5% and 37.5% of patients, respectively. There was no difference in LDH isotype distribution between patients with versus patients without clinical benefit of ICIs, except for a numerically lower percentage of LDH-1 in patients without clinical benefit (median 13.3% vs. 17.6%, $p = 0.1295$). The percentage of LDH-1 correlated with total LDH levels and tumor burden and is therefore not likely to have strong, independent predictive value for response to ICIs. In conclusion, LDH isotyping does not contribute to ICI response prediction in melanoma patients with elevated LDH levels.

Keywords: lactate dehydrogenase; checkpoint inhibitors; melanoma; biomarkers

Citation: van Wilpe, S.; Tolmeijer, S.H.; de Vries, I.J.M.; Koornstra, R.H.T.; Mehra, N. LDH Isotyping for Checkpoint Inhibitor Response Prediction in Patients with Metastatic Melanoma. *Immuno* **2021**, *1*, 67–77. https://doi.org/10.3390/immuno1020005

Academic Editors: Toshihiko Torigoe and Stéphane Terry

Received: 11 March 2021
Accepted: 8 April 2021
Published: 12 April 2021

1. Introduction

Immune checkpoint inhibitors (ICIs) that antagonize the inhibitory receptors programmed cell death protein 1 (PD-1) or cytotoxic lymphocyte-associated antigen 4 (CTLA-4) have significantly improved the survival of patients with metastatic melanoma. The combination of PD-1 inhibitor nivolumab and CTLA-4 inhibitor ipilimumab induces objective responses in 58% of patients, with median overall survival (OS) not reached after 60 months [1]. Unfortunately, not all patients benefit from ICIs. Biomarkers that can predict response to ICIs are urgently needed. Although several biomarkers have been associated with response to ICIs in melanoma, such as tumor mutational burden [2], $CD8^+$ T cell infiltration [3] and lactate dehydrogenase (LDH) levels, robust biomarkers that can accurately distinguish responders from non-responders remain to be identified.

Elevated LDH levels occur in approximately 40% of patients with metastatic melanoma [4] and are associated with lower response rates to ICIs. Objective response rates for the combination of ipilimumab and nivolumab are respectively 65.3%, 44.7% and 37.8% in patients with LDH levels below, 1 to 2 times above and more than 2 times above the upper limit of normal (ULN) [1,5]. There are several biological mechanisms that may account for elevated serum LDH levels in melanoma patients. Elevated LDH levels may result

from tumor necrosis, which is usually more extensive in patients with high tumor burden. Additionally, altered tumor metabolism may contribute to elevated LDH levels [6]. LDH is a major player in glucose metabolism. It is a tetrameric enzyme composed of LDH-A and LDH-B subunits. Five isoforms exist: LDH-1 (4B), LDH-2 (3B1A), LDH-3 (2B2A), LDH-4 (1B3A) and LDH-5 (4A) (Figure 1). As the number of LDH-A subunits increases (LDH-4 and LDH-5), the LDH isoenzyme becomes more efficient in catalyzing the conversion of pyruvate to lactate, thereby facilitating glycolysis. In contrast, isoforms consisting predominantly of LDH-B (LDH-1 and LDH-2) preferentially catalyze the reverse reaction. Malignant tumors commonly exhibit an altered glucose metabolism, characterized by an increase in glucose uptake and glycolytic activity, regardless of oxygen availability. Interestingly, this altered glucose metabolism is thought to hamper antitumor immunity due to the immune suppressive effects of glucose deprivation [6–8] and acidity [9–11].

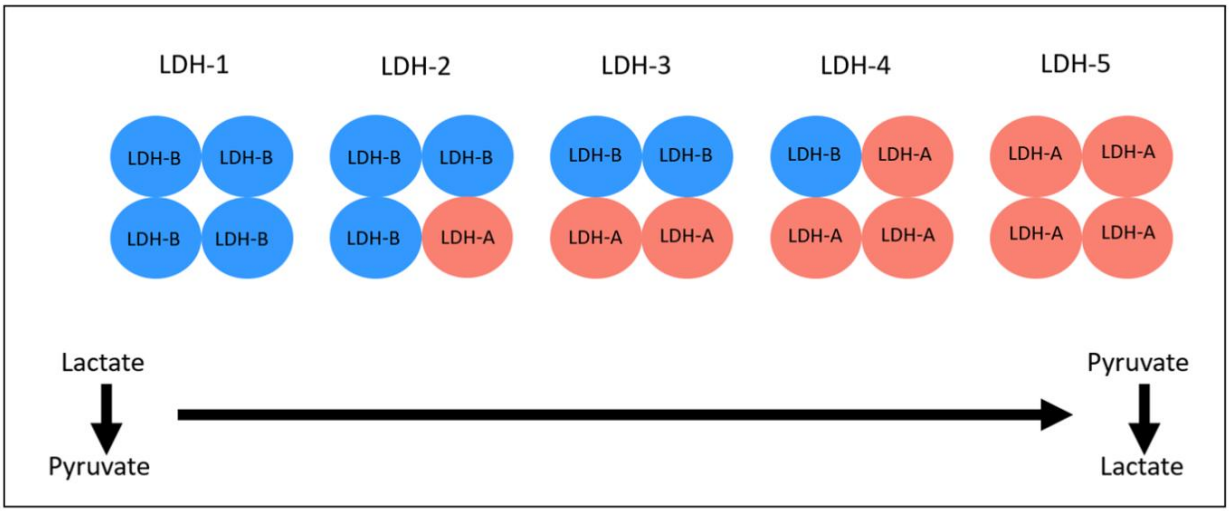

Figure 1. Lactate dehydrogenase (LDH) isotypes. LDH is a tetrameric enzyme composed of LDH-A and LDH-B subunits. Five isoforms exist: LDH-1 (4B), LDH-2 (3B1A), LDH-3 (2B2A), LDH-4 (1B3A) and LDH-5 (4A). As the number of LDH-A subunits increases (LDH-4 and LDH-5), the LDH isoenzyme becomes more efficient in catalyzing the conversion of pyruvate to lactate, thereby facilitating glycolysis. In contrast, isoforms predominantly consisting of LDH-B (LDH-1 and LDH-2) preferentially catalyze the reverse reaction.

Previous research has shown that the expression of LDH-5 and LDH-A are increased in melanoma cells compared to healthy tissue [12,13]. In primary melanoma, the expression of LDH-5 was associated with poor survival [12]. The predictive value of LDH isotype distribution in tumor tissue or peripheral blood for response to ICIs is unexplored. However, a recent study in primary non-small cell lung cancer described a significant inverse relationship between tumor LDH-5 expression and PD-1 expressing tumor-infiltrating lymphocytes [14], suggesting a relationship between LDH-5 expression and antitumor immunity.

We hypothesized that the LDH isotype distribution in peripheral blood reflects glycolytic activity of the tumor and postulated that patients with relatively high LDH-4 and LDH-5 levels compared to LDH-1 and LDH-2 levels would benefit less from ICIs. To study this, we performed LDH isotyping in blood samples of patients with metastatic melanoma and elevated LDH levels.

2. Materials and Methods

2.1. Patient Population

Blood samples of 42 melanoma patients were collected between March 2017 and January 2019. All patients had histologically or cytologically confirmed, BRAF V600E/K mutated, stage IV melanoma and were naïve for ICIs and BRAF/MEK inhibitors. Patients had elevated LDH levels at inclusion ($\geq$250 U/l). Twenty-four patients were treated with a combination of ipilimumab 3 mg/kg and nivolumab 1 mg/kg every 3 weeks (cohort 1).

After 4 cycles of therapy, patients with disease control (either stable disease, partial response or complete response) received nivolumab maintenance therapy (240 mg every 2 weeks) unless unacceptable toxicity occurred. Main aim of this cohort was to study the relation between LDH isotypes and response to ICIs. To be able to relate LDH isotype distribution to total LDH levels and tumor burden in a larger cohort, we also performed LDH isotyping in 18 patients that were treated with BRAF/MEK inhibitors (cohort 2). These patients received vemurafenib 960 mg twice daily and cobimetinib 60 mg once daily in a 21-day-on, 7-day-off schedule.

This study was reviewed and approved by the medical ethics committee region Arnhem-Nijmegen (dossier number 2016–2769). Written informed consent of all study participants was obtained prior to blood collection. The study was performed in accordance with the relevant guidelines and regulations.

2.2. Response Assessment

All patients had measurable disease at baseline. Response to treatment was evaluated according to RECIST 1.1 [15]. All patients underwent planned response evaluation by CT-scan after 6, 12 and 18 weeks of therapy and subsequently every 3 months. Clinical benefit from ICIs was defined as a complete response, partial response or stable disease for at least 6 months.

2.3. Blood Collection and Plasma Collection

Blood samples were collected at baseline and after 6 or 12 weeks of therapy. At each timepoint, 30 mL blood was collected in EDTA tubes. Plasma isolation was performed on the same day. Blood was centrifuged at room temperature in three steps. Whole blood was first centrifuged at 120× *g* for 20 min to separate plasma from blood cells. The plasma was then centrifuged at 360× *g* for 20 min to remove platelets. Subsequently, the platelet-poor plasma was centrifuged at 14,000× *g* for 10 min to remove remaining cellular debris. The plasma was stored as 1 mL aliquots at −80 °C.

2.4. Total LDH Levels and LDH Isotyping

Total LDH levels were measured both directly after blood withdrawal, as part of routine laboratory assessment, as well as in the cryopreserved plasma samples at the moment of LDH isotyping. LDH isotyping was performed in an ISO 15189 accredited clinical laboratory, using 1 mL of plasma. Samples were thawed at room temperature and visually inspected for hemolysis. Hemolytic samples were excluded, because hemolysis induces a relative increase in LDH-1 and LDH-2 levels [16,17]. LDH isoenzymes were identified and quantitated by agarose gel electrophoresis on the semi-automated HYDRASYS system using the Hydragel 7 ISO-LDH-kit (#4110, Sebia). The five LDH isoenzymes are presented as percentage activity to total LDH activity.

2.5. Circulating Tumor DNA

To investigate whether LDH isotype distribution might be an independent predictor for response to ICIs, we studied the relationship between LDH isotype distribution, total LDH levels and tumor burden. As most patients included in our study had extensive disease, with multiple metastases including non-measurable lesions, tumor burden assessment by CT scan was considered suboptimal. Previous studies have shown a strong correlation between ctDNA and tumor burden in melanoma patients [18]. In our cohort, ctDNA analyses had previously been performed in 38 of 40 with successful LDH isotyping. Therefore, ctDNA was used as surrogate marker for tumor burden.

DNA was extracted from 2 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and eluted in 30uL low-TE buffer. DNA quantity was determined using Qubit (Thermofisher). DNA Quality was checked on a fragment analyzer (Agilent high sensitivity genomic DNA kit, #DNF-488-0500) to rule out any contamination by genomic DNA. ctDNA copies were measured using the droplet digital PCR *BRAF* V600 screening

kit (#12001037, Bio-rad), which is able to detect *BRAF* p.V600E [c.1799T > A], p.V600R [c.1798_1799delinsAG] and p.V600K [c.1798_1799delinsAA] mutations.

2.6. Statistics

Differences in LDH isotype ratio and the percentage of individual isotypes between patients with and without clinical benefit were visualized in boxplots. A non-parametric Levene's test was used to verify the equality of variances between groups. Baseline differences between groups were assessed using a Wilcoxon Rank-Sum test.

Spearman's Rank-Order Correlation was used to analyze correlations between LDH levels, LDH isotypes and ctDNA (copies/mL) among all baseline samples (both cohorts). For visualization purposes, not normally distributed data were plotted on log-transformed (LDH-1), square root transformed (LDH-5) or (reversed) reciprocal axes (total LDH).

To assess how changes in total LDH levels and tumor burden affected LDH isotype distribution, patients were grouped based on their response at the time of blood collection and the change in total LDH levels (>25% versus <25% decline). Differences between paired samples obtained before and after 6 or 12 weeks of therapy were assessed using a Wilcoxon signed-rank test. In all tests, a p-value below 0.05 was considered statistically significant. Statistical analyses were performed in R version 3.6.3.

3. Results

3.1. Patient Characteristics and Samples

Blood samples of 42 patients with metastatic melanoma were collected. Of the 42 patients, 2 patients had hemolytic baseline samples. Baseline characteristics of the remaining 40 patients are shown in Table 1 and Supplementary Materials Table S1. Twenty-two patients were treated with ipilimumab and nivolumab (cohort 1). Main aim of this cohort was to study the relation between LDH isotype distribution and response to ICIs. In this study, clinical benefit was defined as progression-free survival for at least 6 months. Of the 22 patients in cohort 1, 15 patients experienced clinical benefit. In these patients, the best objective response at 18 weeks was a partial response (PR; n = 13) or stable disease (n = 2). Seven patients experienced progression within 6 months. Most of these patients did not have an initial response, except for 1 patient who initially had a PR, but developed new lesions after 4 months of treatment. Although all patients had elevated LDH levels at inclusion (>250 U/l), LDH levels had normalized at the time of baseline blood collection in 2 out of 22 patients (LDH levels of resp. 219 and 248). To be able to relate LDH isotype distribution to total LDH levels and tumor burden in a larger cohort, we also performed LDH isotyping in 18 patients that were treated with BRAF/MEK inhibitors (cohort 2).

Table 1. Patient characteristics.

	Cohort 1 (*n* = 22)	**Cohort 1 + 2 (*n* = 40)**
Age–median (range)	69 (28–80)	63 (28–80)
Sex–no. (%)		
Male	15 (68.2)	28 (70)
Female	7 (31.8)	12 (30)
ECOG–no. (%)		
0	13 (59.1)	24 (60)
1	6 (27.3)	13 (32.5)
2	3 (13.6)	3 (7.5)
M status–no (%)		
M1a	3 (13.6)	8 (20)
M1b	-	-
M1c	14 (63.6)	23 (57.5)
M1d	5 (22.7)	9 (22.5)
Lymph node or lung metastases–no (%)	21 (95.5)	39 (97.5)
Lymph node metastases–no. (%)	15 (68.2)	31 (77.5)
Lung metastases–no (%)	15 (68.2)	23 (57.5)
Liver metastases–no. (%)	8 (36.4)	14 (35)
LDH (U/l)–median (range) *	366 (219–1197)	358 (219–1400)
ALAT (U/l)–median (range)	22.5 (10–175)	22 (8–370)
ASAT (U/l)–median (range)	25.5 (14–86)	28 (13–314)
Bilirubin (µmol/L)–median (range)	7 (4–23)	7 (3–277)

* Total LDH levels at baseline according to routine laboratory assessment.

On-treatment LDH isotyping data were available for 33 of 40 patients. On-treatment samples were missing in 7 patients, because no blood had been collected (*n* = 3) or samples were hemolytic (*n* = 4). An overview of the available samples per cohort is shown in Figure 2.

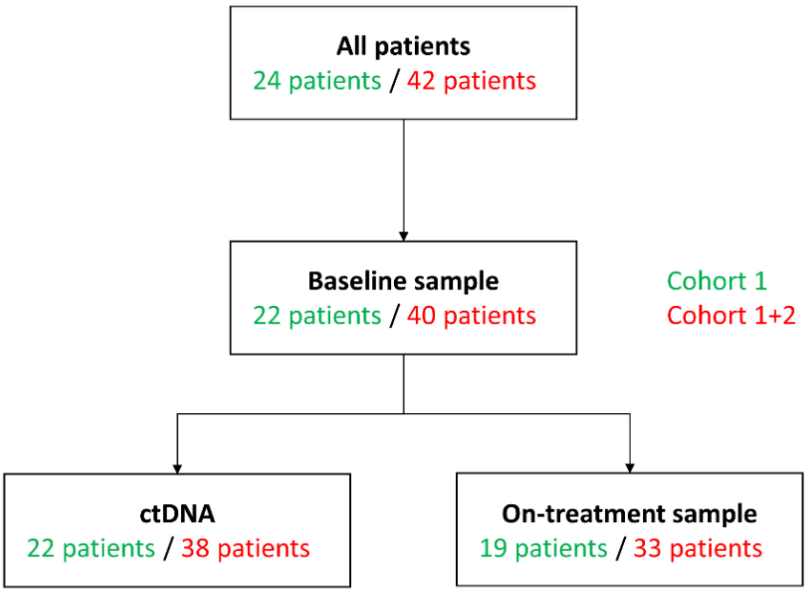

Figure 2. Overview of available samples.

3.2. LDH at Baseline

First, we evaluated total LDH levels and isotype distribution at baseline (cohort 1 and 2). Median total LDH level, as measured in cryopreserved plasma, was 308 U/l (range 206–1243 U/l). There was a strong correlation between routine laboratory LDH results and total LDH levels in cryopreserved plasma (hereinafter referred to as total LDH levels) (R = 0.94, $p < 2.2 \times 10^{-16}$, Figure 3a), arguing against LDH degradation in our cryopreserved plasma samples.

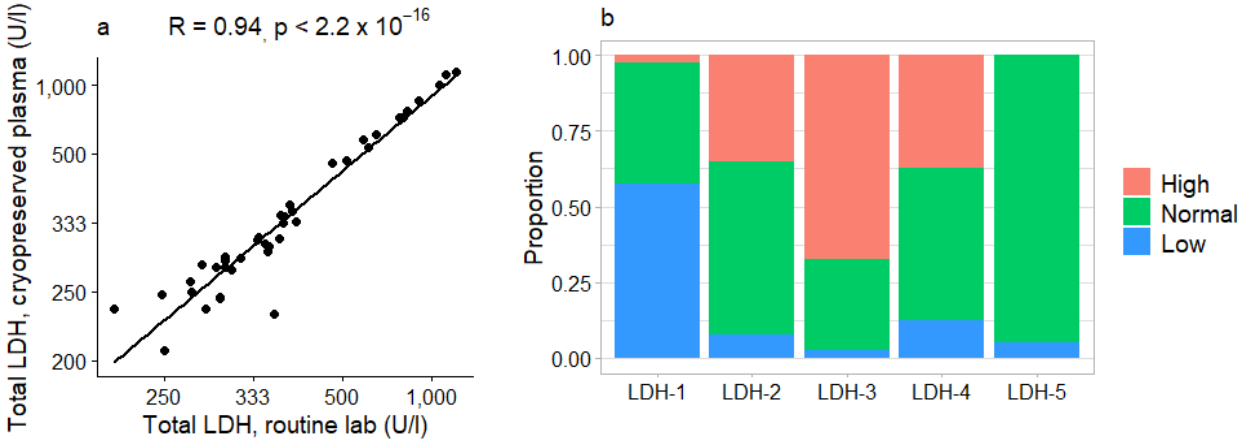

Figure 3. Total LDH levels and LDH isotypes at baseline. (**a**) Correlation between total LDH levels as assessed by routine laboratory assessment and total LDH levels in cryopreserved LDH levels. (**b**) Isotype distribution at baseline. Reference values are 16.1–31.5% for LDH-1, 29.2–41.6% for LDH-2, 17.0–26.2% for LDH-3, 5.9–12.3% for LDH-4 and 3.2–17.3% for LDH-5.

At baseline, the median percentages (+ range) of LDH-1 to LDH-5 were 15.0% (7.8–32), 38.6% (26.1–53), 27.2% (11.4–35.4), 10.8% (2.3–28) and 6.3% (1.3–17), respectively (see Figure 3b for reference values). Of all patients, 57.5% had abnormally low LDH-1 levels. The percentage of LDH-2, -3 and -4, on the other hand, was elevated in 35%, 67.5% and 37.5% of patients. Surprisingly, the percentage of LDH-5 was elevated in none of the patients (Figure 3b).

3.3. Relationship between LDH Isotypes and Response to ICIs

Next, we assessed the differences in LDH isotype distribution between patients with versus without clinical benefit from ICIs (cohort 1). We hypothesized that the ratio between LDH-4/5 and LDH-1/2 would be lower in patients who benefited from ICIs. Nevertheless, the ratio did not clearly differ (Figure 4). We did observe a non-significant difference in total LDH levels between patients with and without clinical benefit (median 285 vs. 527, $p = 0.056$). When looking at the individual isotypes, we observed that the percentage of LDH-1 was numerically lower in patients that did not benefit (median 13.3% vs. 17.6%, $p = 0.1295$), whereas the percentage of other isotypes did not differ.

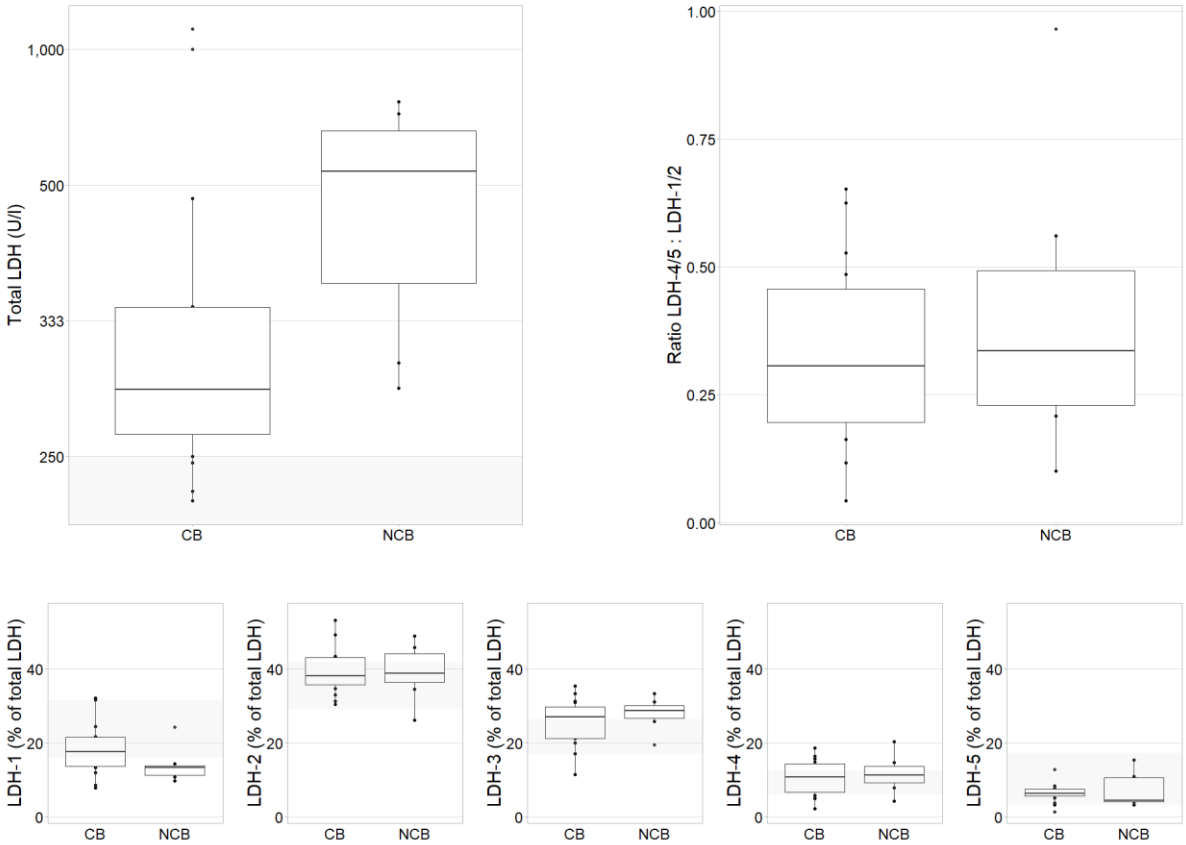

Figure 4. Differences between patients with versus without clinical benefit to immune checkpoint inhibitors. The large plots in the upper part of the figure depict the difference in total LDH levels and the ratio between LDH-4/5 and LDH-1/2. The differences in individual isotypes are shown below. Grey areas indicate the normal range. Abbreviations: CB = Clinical benefit; NCB = No clinical benefit.

3.4. LDH Isotypes in Relation to Tumor Burden and Total LDH Levels

As we observed a trend towards lower percentages of LDH-1 in patients without clinical benefit, we questioned whether this might be an independent predictor for response to ICIs, or merely reflected a difference in total LDH levels and/or tumor burden. We decided to use circulating tumor DNA (ctDNA) as a surrogate marker for tumor burden because radiological assessment of tumor burden was complicated by the presence of non-measurable lesions in some patients [18]. Moreover, we already had ctDNA data available for 38 of 40 patients. When we combined the two cohorts, we discovered that the percentage of LDH-1 was inversely correlated with total LDH levels ($R = -0.46$, $p = 0.0031$, Figure 5a) and ctDNA concentration ($R = -0.55$, $p = 0.00031$, Figure 5b). These results suggest that the percentage of LDH-1 is not an independent predictor for response to ICIs. While an inverse correlation was found for LDH-1, the percentage of LDH-3 was positively correlated with total LDH levels ($R = 0.35$, $p = 0.028$). There was also a weak association between LDH-3 and ctDNA concentration ($R = 0.3$, $p = 0.065$). Additionally, ctDNA concentration was positively correlated with the percentage of LDH-4 ($R = 0.35$, $p = 0.03$).

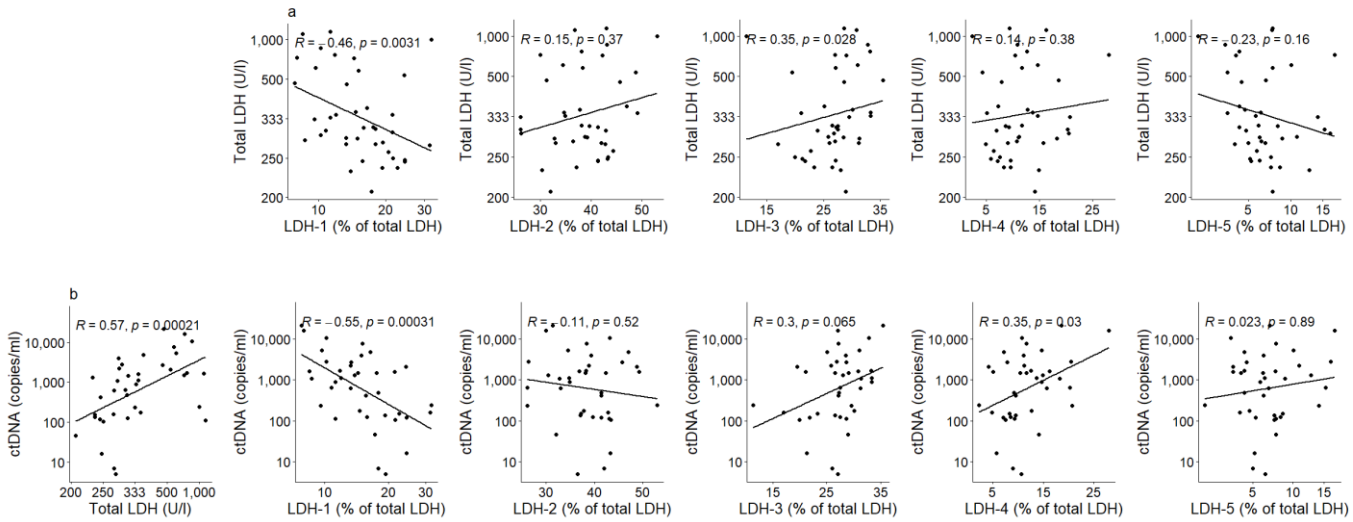

Figure 5. Correlation between LDH isotypes, total LDH levels and ctDNA levels. (**a**) Correlation between LDH isotypes and total LDH levels at baseline. (**b**) Correlation between LDH isotypes and ctDNA at baseline. ctDNA levels are shown on a log-transformed axis. One patient had undetectable ctDNA levels at baseline. For visualization purposes, the ctDNA copy number of this patients was replaced by 5 (~lowest value in this plot).

3.5. Changes during Therapy

To further dissect the relation between LDH isotypes, total LDH levels and tumor burden, we studied the changes in LDH isotype distribution during therapy with ICIs and BRAF/MEK inhibitors. On-treatment samples were available for 33 patients (cohort 1 and 2). Nineteen patients had an objective response according to RECIST 1.1 at the time of on-treatment blood sample collection. The percentage of LDH-1 significantly increased in patients with an objective response (median change: 4.3%; $p = 0.011$). Sixteen patients had a substantial decrease in total LDH levels during the first weeks of treatment, defined as a decrease more than 25% (LDH_{25}). Analogous to the patients with an objective response, patients with a LDH_{25} decline had a relative increase in LDH-1 levels (median change: 5.4%, $p = 0.0092$). The increase in the percentage of LDH-1 was highest in patients with both an objective response and a LDH_{25} decline (Figure 6). When considering the absolute changes in LDH isotypes (percentage × total LDH levels), we observed that absolute LDH-1 levels remained stable during therapy, whereas the absolute levels of other isotypes decreased (data not shown). No significant changes were seen in the percentages of other isotypes during therapy.

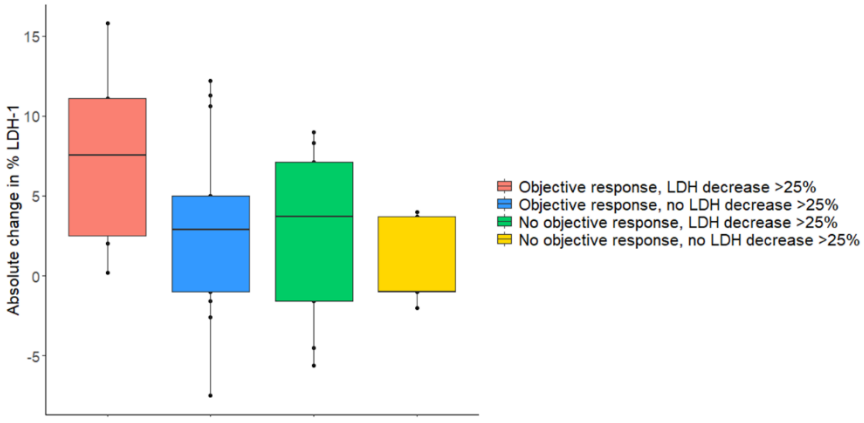

Figure 6. Changes in LDH-1 during therapy. The absolute changes in the percentage of LDH-1 are shown in the figure. Patients are grouped based on whether or not they had an objective response and/or a 25% decrease in total LDH levels at the time of on-treatment blood collection.

4. Discussion

This is the first study investigating the relationship between LDH isotype distribution in peripheral blood and response to ICIs. LDH is an important regulator of glycolysis, a process known to negatively affect antitumor immunity. We hypothesized that the ratio of LDH-4/5 over LDH-1/2 in peripheral blood reflects glycolytic activity in the tumor and might therefore be lower in patients who respond to ICIs. We measured LDH isotype distribution in patients with metastatic melanoma and elevated LDH levels and related it to response to ICIs. There was no significant difference between patients with versus patients without clinical benefit, neither in isotype ratio nor in the percentage of individual isotypes. There was a trend towards lower percentages of LDH-1 in patients that did not benefit. Further analysis, however, revealed that the percentage of LDH-1 correlated with total LDH levels and tumor volume. Therefore, it is unlikely that LDH-1 has utility as predictive biomarker for response to ICIs.

There is one other study available that performed LDH isotyping in patients with metastatic melanoma [19]. This study, including 49 patients, reported that patients with metastatic melanoma and high LDH levels have relatively low levels of LDH-1 and LDH-2 and abnormally high levels of LDH-3 and LDH-4. The percentage of LDH-5 did not correlate with serum LDH levels. According to the authors, the relative increase in LDH-3 and LDH-4 levels suggests that glycolysis is the primary metabolic pathway in melanomas of patients with elevated LDH levels. Although we also observed relatively high LDH-3 and LDH-4 levels and low LDH-1 levels here, the percentage of LDH-2 was elevated in many patients in our cohort. Because of the elevated LDH-2 levels and normal LDH-5 levels, we are not convinced that the overall LDH isotype distribution in our cohort is indicative of increased glycolytic activity in the tumor tissue.

There are several possible explanations for our finding that no clear difference in isotype distribution was apparent between patients who did versus patients who did not benefit from ICIs. First of all, LDH isotype distribution in peripheral blood may not be the optimal marker for intratumoral glycolytic activity. Several studies have shown that global glycolytic activity is increased in melanoma tissue [19–21]. However, it has been suggested that within tumors a metabolic symbiosis exists, with some cancer cells exhibiting high glycolytic activity and producing large amounts of lactate, while cells in other areas absorb this lactate, convert it to pyruvate and use it as an energy source. This concept is known as the 'lactate shuttle hypothesis' [19,22]. If most of the generated lactate is indeed reused by other cancer cells, not only the expression of glycolytic LDH isoenzymes (LDH-4 and LDH-5), but also the expression of non-glycolytic LDH isoenzymes (LDH-1 and LDH-2), would increase in glycolytic tumors. Hence, there might not be a shift in overall LDH isotype distribution in tumors with increased glycolytic activity.

Another reason why the data did not support our hypothesis could be that the isotype distribution of circulating LDH does not accurately reflect LDH isotype expression in the tumor. Circulating LDH does not necessarily originate from the tumor tissue itself. It might also derive from surrounding benign tissue that is destructed by tumor invasion. This might explain why the percentage of LDH-3, which is highly expressed in lymph nodes and lungs [17], was elevated in so many of our patients, as in our cohort all but one patient had lymph node or lung metastases. LDH-1 is primarily expressed in cardiac tissue and erythrocytes and its contribution to total LDH levels is not expected to increase either by tumor necrosis or destruction of surrounding benign tissue [17]. LDH-2 and -4 are expressed in various tissues, including lung tissue [17]. LDH-5 is highly expressed in liver and skeletal muscle [17]. If circulating LDH indeed originates for benign tissue near the tumor, you might expect LDH-5 to be elevated in some of our patients as 35% of patients in our cohort had liver metastases. However, LDH-5 has the shortest half-life of all isoenzymes (7 h versus 48, 31, 20 and 13 h for LDH-1, -2, -3 and -4, respectively) and that might (partly) explain why the percentage of LDH-5 remained normal [23].

This study has some limitations. First of all, we used cryopreserved plasma samples for LDH isotyping. Usually, serum is used, because there are some concerns regarding

the stability of LDH in cryopreserved samples [24]. Nevertheless, the strong concordance between total LDH levels in cryopreserved plasma and LDH levels as assessed by regular laboratory assessment argue against loss of LDH activity in our cryopreserved plasma samples. Another limitation might be that we used ctDNA as surrogate marker for tumor burden. Although previous studies have shown a good correlation between ctDNA and tumor burden, the amount of ctDNA might also be influenced by other factors, including tumor metabolism [18]. A third limitation is the small sample size. It is possible that a small but significant difference in LDH isotype distribution would have been identified between patients with versus without clinical benefit had the study been much larger. Nevertheless, based on the results of our study, it is unlikely that a clinically meaningful difference exists. The differences between patients with and without clinical benefit observed in this study are too small to warrant further research.

5. Conclusions

LDH isotyping in peripheral blood of patients with elevated LDH does not contribute to response prediction in melanoma patients eligible for treatment with ICIs. Although we did not find LDH isotyping in peripheral blood to be a good predictor for response to ICIs in melanoma, more robust blood-, tissue- or imaging-based biomarkers associated with glycolysis might still have potential to serve as predictors for response to ICIs. Several studies have demonstrated the negative effects of glycolysis on antitumor immunity [6–11]. A recent study demonstrated that high expression of glycolysis-related genes was associated with low T cell infiltration and poor response to adoptive T cell transfer in melanoma [21]. Further research is warranted to elucidate the potential role of glycolytic activity in resistance to ICIs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/immuno1020005/s1, Table S1: Differences in baseline characteristics between patients with and without clinical benefit to checkpoint inhibitors.

Author Contributions: Conceptualization: S.v.W., R.H.T.K. and N.M.; formal analysis: S.v.W.; investigation: S.v.W. and S.H.T.; resources: N.M. and R.H.T.K.; writing—original draft preparation: S.v.W.; writing—review and editing: S.v.W., R.H.T.K., N.M., S.H.T., N.M., I.J.M.d.V.; visualization: S.v.W.; supervision: N.M. and R.H.T.K.; funding acquisition: S.v.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the C.J. Vaillantfonds (Landelijke Vereniging van Crematoria, the Netherlands). The funding organization had no role in the collection, analysis or interpretation of the data or in writing the manuscript.

Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the Radboudumc (protocol code 2016–2769, 15 December 2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and analyzed in this study are not publicly available because the current biomarker study was performed in participants of an ongoing (unpublished) clinical trial (NCT02968303). Datasets will be available from the principal investigator (RK) on reasonable request after the clinical trial is finished.

Acknowledgments: We thank all study participants for their participation in this study. We thank the department of clinical chemistry and hematology of the University Medical Center Utrecht for performing the LDH isotyping.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Larkin, J.; Chiarion-Sileni, V.; Gonzalez, R.; Grob, J.-J.; Rutkowski, P.; Lao, C.D.; Cowey, C.L.; Schadendorf, D.; Wagstaff, J.; Dummer, R.; et al. Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N. Engl. J. Med.* **2019**, *381*, 1535–1546. [CrossRef] [PubMed]

2. Wu, Y.; Xu, J.; Du, C.; Wu, Y.; Xia, D.; Lv, W.; Hu, J. The Predictive Value of Tumor Mutation Burden on Efficacy of Immune Checkpoint Inhibitors in Cancers: A Systematic Review and Meta-Analysis. *Front. Oncol.* **2019**, *9*, 1161. [CrossRef] [PubMed]
3. Tumeh, P.C.; Harview, C.L.; Yearley, J.H.; Shintaku, I.P.; Taylor, E.J.M.; Robert, L.; Chmielowski, B.; Spasic, M.; Henry, G.; Ciobanu, V.; et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* **2014**, *515*, 568–571. [CrossRef] [PubMed]
4. Petrelli, F.; Ardito, R.; Merelli, B.; Lonati, V.; Cabiddu, M.; Seghezzi, S.; Barni, S.; Ghidini, A. Prognostic and predictive role of elevated lactate dehydrogenase in patients with melanoma treated with immunotherapy and BRAF inhibitors: A systematic review and meta-analysis. *Melanoma Res.* **2019**, *29*, 1–12. [CrossRef]
5. Larkin, J.; Chiarion-Sileni, V.; Gonzalez, R.; Grob, J.; Cowey, C.; Lao, C.; Wagstaff, J.; Hogg, D.; Hill, A.; Carlino, M.; et al. 3303 Efficacy and safety in key patient subgroups of nivolumab (NIVO) alone or combined with ipilimumab (IPI) versus IPI alone in treatment-naïve patients with advanced melanoma (MEL) (CheckMate 067). *Eur. J. Cancer* **2015**, *51*, S664–S665. [CrossRef]
6. Van Wilpe, S.; Koornstra, R.; Den Brok, M.; De Groot, J.W.; Blank, C.; De Vries, J.; Gerritsen, W.; Mehra, N. Lactate dehydrogenase: A marker of diminished antitumor immunity. *Oncoimmunology* **2020**, *9*, 1731942. [CrossRef]
7. Chang, C.-H.; Curtis, J.D.; Maggi, L.B.; Faubert, B.; Villarino, A.V.; O'Sullivan, D.; Huang, S.C.-C.; Van Der Windt, G.J.; Blagih, J.; Qiu, J.; et al. Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis. *Cell* **2013**, *153*, 1239–1251. [CrossRef]
8. Chang, C.-H.; Qiu, J.; O'Sullivan, D.; Buck, M.D.; Noguchi, T.; Curtis, J.D.; Chen, Q.; Gindin, M.; Gubin, M.M.; Van Der Windt, G.J.; et al. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell* **2015**, *162*, 1229–1241. [CrossRef]
9. Brand, A.; Singer, K.; Koehl, G.E.; Kolitzus, M.; Schoenhammer, G.; Thiel, A.; Matos, C.; Bruss, C.; Klobuch, S.; Peter, K.; et al. LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells. *Cell Metab.* **2016**, *24*, 657–671. [CrossRef]
10. Husain, Z.; Huang, Y.; Seth, P.; Sukhatme, V.P. Tumor-Derived Lactate Modifies Antitumor Immune Response: Effect on Myeloid-Derived Suppressor Cells and NK Cells. *J. Immunol.* **2013**, *191*, 1486–1495. [CrossRef]
11. Colegio, O.R.; Chu, N.-Q.; Szabo, A.L.; Chu, T.; Rhebergen, A.M.; Jairam, V.; Cyrus, N.; Brokowski, C.E.; Eisenbarth, S.C.; Phillips, G.M.; et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* **2014**, *513*, 559–563. [CrossRef] [PubMed]
12. Zhuang, L.; Scolyer, R.A.; Murali, R.; McCarthy, S.W.; Zhang, X.D.; Thompson, J.F.; Hersey, P. Lactate dehydrogenase 5 expression in melanoma increases with disease progression and is associated with expression of Bcl-XL and Mcl-1, but not Bcl-2 proteins. *Mod. Pathol.* **2009**, *23*, 45–53. [CrossRef]
13. Feichtinger, R.G.; Lang, R.; Geilberger, R.; Rathje, F.; Mayr, J.A.; Sperl, W.; Bauer, J.W.; Hauser-Kronberger, C.; Kofler, B.; Emberger, M. Melanoma tumors exhibit a variable but distinct metabolic signature. *Exp. Dermatol.* **2018**, *27*, 204–207. [CrossRef]
14. Giatromanolaki, A.; Koukourakis, I.M.; Balaska, K.; Mitrakas, A.G.; Harris, A.L.; Koukourakis, M.I. Programmed death-1 receptor (PD-1) and PD-ligand-1 (PD-L1) expression in non-small cell lung cancer and the immune-suppressive effect of anaerobic glycolysis. *Med Oncol.* **2019**, *36*, 76. [CrossRef] [PubMed]
15. Schwartz, L.H.; Litière, S.; De Vries, E.; Ford, R.; Gwyther, S.; Mandrekar, S.; Shankar, L.; Bogaerts, J.; Chen, A.; Dancey, J.; et al. RECIST 1.1—Update and clarification: From the RECIST committee. *Eur. J. Cancer* **2016**, *62*, 132–137. [CrossRef] [PubMed]
16. Leung, F.Y.; Henderson, A.R. Influence of hemolysis on the serum lactate dehydrogenase-1/lactate dehydrogenase-2 ratio as determined by an accurate thin-layer agarose electrophoresis procedure. *Clin. Chem.* **1981**, *27*, 1708–1711. [CrossRef] [PubMed]
17. Wróblewski, F.; Gregory, K.F. Lactic dehydrogenase isozymes and their distribution in normal tissues and plasma and in disease states. *Ann. N. Y. Acad. Sci.* **1961**, *94*, 912–932. [CrossRef]
18. McEvoy, A.C.; Warburton, L.; Al-Ogaili, Z.; Celliers, L.; Calapre, L.; Pereira, M.R.; Khattak, M.A.; Meniawy, T.M.; Millward, M.; Ziman, M.; et al. Correlation between circulating tumour DNA and metabolic tumour burden in metastatic melanoma patients. *BMC Cancer* **2018**, *18*, 726. [CrossRef]
19. Ho, J.; De Moura, M.B.; Lin, Y.; Vincent, G.; Thorne, S.; Duncan, L.M.; Hui-Min, L.; Kirkwood, J.M.; Becker, D.; Van Houten, B.; et al. Importance of glycolysis and oxidative phosphorylation in advanced melanoma. *Mol. Cancer* **2012**, *11*, 76. [CrossRef] [PubMed]
20. Kluza, J.; Corazao-Rozas, P.; Touil, Y.; Jendoubi, M.; Maire, C.; Guerreschi, P.; Jonneaux, A.; Ballot, C.; Balayssac, S.; Valable, S.; et al. Inactivation of the HIF-1α/PDK3 Signaling Axis Drives Melanoma toward Mitochondrial Oxidative Metabolism and Potentiates the Therapeutic Activity of Pro-Oxidants. *Cancer Res.* **2012**, *72*, 5035–5047. [CrossRef]
21. Cascone, T.; McKenzie, J.A.; Mbofung, R.M.; Punt, S.; Wang, Z.; Xu, C.; Williams, L.J.; Wang, Z.; Bristow, C.A.; Carugo, A.; et al. Increased Tumor Glycolysis Characterizes Immune Resistance to Adoptive T Cell Therapy. *Cell Metab.* **2018**, *27*, 977–987.e4. [CrossRef] [PubMed]
22. Brooks, G.A. The Science and Translation of Lactate Shuttle Theory. *Cell Metab.* **2018**, *27*, 757–785. [CrossRef] [PubMed]
23. Boyd, J.W. The rates of disappearance of l-lactate dehydrogenase isoenzymes from plasma. *BBA Enzymol.* **1967**, *132*, 221–231. [CrossRef]
24. Zander, J.; Bruegel, M.; Kleinhempel, A.; Becker, S.; Petros, S.; Kortz, L.; Dorow, J.; Kratzsch, J.; Baber, R.; Ceglarek, U.; et al. Effect of biobanking conditions on short-term stability of biomarkers in human serum and plasma. *Clin. Chem. Lab. Med.* **2014**, *52*, 629–639. [CrossRef]