Phenotypic Composition of Commercial Anti-CD19 CAR T Cells Affects *In Vivo* Expansion and Disease Response in Patients with Large B-cell Lymphoma

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ABSTRACT

Purpose: In clinical trials, the expansion and persistence of chimeric antigen receptor (CAR) T cells correlate with therapeutic efficacy. However, properties of CAR T cells that enable their *in vivo* proliferation have still to be consistently defined and the role of CAR T bag content has never been investigated in a real-life setting.

Experimental Design: Residual cells obtained after washing 61 anti-CD19 CAR T product bags were analyzed to identify tisagenlecleucel/Tisa-cel and axicabtagene ciloleucel/Axi-cel phenotypic features associated with postinfusion CAR T-cell *in vivo* expansion and with response and survival.

Results: While Tisa-cel was characterized by a significant enrichment in CAR$^+$CD4$^+$ T cells with central memory ($P < 0.005$) and

effector ($P < 0.005$) phenotypes and lower rates of CAR$^+$CD8$^+$ with effector memory ($P < 0.005$) and naïve-like ($P < 0.05$) phenotypes as compared with Axi-cel, the two products displayed similar expansion kinetics. *In vivo* CAR T-cell expansion was influenced by the presence of CAR T with a CD8$^+$ T central memory signature ($P < 0.005$) in both Tisa-cel and Axi-cel infusion products and was positively associated with response and progression-free survival ($P < 0.05$).

Conclusions: Our data indicate that despite the great heterogeneity of Tisa-cel and Axi-cel products, the differentiation status of the infused cells mediates CAR T-cell *in vivo* proliferation that is necessary for antitumor response.

Introduction

Following promising results in clinical trials, the CD19-targeted chimeric antigen receptor (CAR) T-cell products tisagenlecleucel (Tisa-cel) and axicabtagene ciloleucel (Axi-cel) have been approved for third-line treatment of relapsed/refractory (R/R) large B-cell lymphoma (LBCL; refs. 1–10).

A number of pretreatment patient clinical features including mainly parameters of lymphoma burden and levels of inflammatory cytokines were used to predict clinical response to CAR T-cell therapy (5, 11–13) but were not able to discriminate the outcome at the single patient level. On the other hand, *in vivo* CAR T-cell proliferation after infusion was found of central importance to clinical response and toxicities in acute lymphoblastic leukemia (ALL) and within clinical trials (4, 14) but has been less investigated in patients with lymphoma receiving Tisa-cel and Axi-cel as standard-of-care therapy. In addition, there are

no data correlating properties of Tisa-cel and Axi-cel infusion bag content and CAR T-cell expansion.

These infusion products differ in several aspects including the type of CAR construct used, the manufacturing process, the dose, the primary container and the fill volume. These characteristics, together with the quality of the T cells harvested from patients for genetic manipulation, the tumor burden and tumor microenvironment might all contribute to CAR T cells' fate *in vivo*. Recent evidence suggest that cellular and molecular features of infused CAR T-cell products are major factors accounting for the variability in efficacy among Axi-cel–treated lymphoma patients of the ZUMA-1 trial (15) and among patients with chronic lymphocytic leukemia (CLL) receiving CTL019 therapy (16). Of note, these data have been generated mainly as part of exploratory analysis of clinical trials using comprehensive and challenging methodologic approaches to identify predictive biomarkers in patients treated with specific products. Real-life evaluations assessing the phenotypic composition of the CAR T-cell infusion products and analyzing how these features affect CAR T-cell fate *in vivo* and outcome are scanty. Besides, no studies directly comparing Tisa-cel and Axi-cel characteristics and their impact on expansion kinetics have been reported yet.

In this single-center prospective study, we aimed at identifying key phenotypic Tisa-cel and Axi-cel CAR T-cell product characteristics associated with improved *in vivo* expansion and therefore tumor responses.

Materials and Methods

Patients

This single-center, prospective observational study conducted at the Hematology Division of the Fondazione IRCCS Istituto Nazionale dei Tumori (Milano, Italy) included 61 patients with R/R LBCL who received standard-of-care Tisa-cel or Axi-cel between December 2019

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Translational Relevance

Tisa-cel and Axi-cel showed exciting efficacy in relapsed/refractory B-cell lymphoma, but relapses are frequent and predictors of outcome are still inconsistently defined. Although CAR T-cell proliferation after infusion predicted response in clinical trials, Tisa-cel and Axi-cel kinetics have been poorly investigated in real-life settings and properties of CAR T cells that enable their expansion are unknown. Results of this single-center prospective study suggest that despite Tisa-cel and Axi-cel infusion products being different, their *in vivo* expansion kinetics are similar and are mediated by $CD8^+CAR^+$ T cells with central memory phenotypes within infused cells. In addition, for both products, numbers of circulating CAR T cells can be used as biomarkers of response. These observations indicate that Tisa-cel and Axi-cel product characteristics can be interrogated early to gain insight into the *in vivo* expansion capability of CAR T cells that is necessary for ultimate CAR T-cell efficacy and could prompt early intervention strategies.

and October 2021. All patients received lymphodepletion according to manufacturers (3, 17). The study (INT180/19) was approved by the local ethics committee and was carried out in accordance with the Helsinki Declaration and patients provided written informed consent. Patients were assigned to either Tisa-cel or Axi-cel based on slot production availability and histology, because patients with primary mediastinal B-cell lymphoma (PMBCL) can receive Axi-cel only. Disease status was assessed as per Lugano 2014 classification (18) by CT scan and PET/CT scan at day 30 (PET1) and at day 90 (PET3), or when clinically indicated. Total metabolic tumor volume (TMTV) was computed both by the 41% maximum standardized uptake value threshold and by an automatic delineation with the estimated threshold algorithm supplied with PET-Volume Computer Assisted Reading (VCAR) software (GE Healthcare). Immune effector cell associated neurotoxicity syndrome (ICANS) and cytokine release syndrome (CRS) related to CAR T cells were graded according to the American Society for Transplantation and Cellular Therapy Consensus Grading (19).

Identification of CAR T cells by flow cytometry

For CAR T-cell identification, cells were stained with the CD19 CAR detection reagent (Miltenyi Biotec) following the manufacturer's instructions. For infusion products, 50 µL from bag leftovers were used. For *in vivo* longitudinal CAR T-cell monitoring, 100 µL of peripheral blood samples (PB; refs. 20–22) collected at day 5, 7, 10, 14, 21 and monthly after infusion were used. After staining with the CAR detection reagent, for PB only, red blood cells (RBC) were lysed using the Ammonium Chloride Lysing solution (BD Biosciences). Then, for both PB and infusion products, cells were labeled with the following set of antibodies: biotin-PE (clone REA746, catalog no. 130–110–951), CD45-VioBlue (clone REA747, catalog no. 130–110–637, RRID: AB_2658243), CD3-FITC (clone REA613, catalog no. 130–113–138), CD4-VioGreen (clone REA623, catalog no. 0130–113–230), CD8-APC-Vio770 (clone REA734, catalog no. 130–110–681), CD14-APC (clone REA599, catalog no. 130–110–520), and 7-AAD (catalog no. 130–111–568) staining solution (all from Miltenyi Biotec). Additional information on CAR T-cell identification strategy are reported in Supplementary Fig. S1. For PB CAR T-cell detection, the protocol was optimized to eliminate the background signals generated by the biotin–PE antibody used to label the CD19 CAR Detection Reagent, which affected absolute CAR^+ cell enumeration. To assess the level of nonspecific background, 100 µL of PB were processed with RBC lysis, washed, and stained with: biotin-PE, CD45-VioBlue, CD3-FITC and 7-AAD staining solution. The amount of nonspecific signal detected was substracted to avoid the possible overestimation of circulating CAR^+ cells. Control samples were obtained from healthy volunteers who provided written informed consent.(Supplementary Figs. S2–S4). Data acquisition was performed with a MACSQuant Analyzer or a MACSQuant Analyzer MQ10 (Miltenyi Biotec); data were analyzed with the MACSQuantify software for absolute quantification of cells with custom analysis protocols (Express Mode Master Package, Miltenyi Biotec) or with predefined optimized settings for CAR T-cell analysis.

Differentiation of CAR T cells by flow cytometry

For CAR T-cell differentiation, at least 10^6 cells from infusion product leftovers were used. Cells were incubated with the CD19 CAR detection reagent (Miltenyi Biotec). After wash, the following antibodies were added: biotin-PE, CD3-FITC, CD4-VioGreen, CD8-APC-Vio770, CD45RO-APC (clone REA611, catalog no. 130–115–556), CD62L-Pe-Vio700 (clone 145/15, catalog no. 130–113–621) and CD197-VioBlue (clone REA546, catalog no. 130–117–353; all from Miltenyi Biotec). Data were acquired on BD FACSCanto II (BD Biosciences) or MACSQuant Analyzer MQ10 (Miltenyi Biotec) and analyzed using FlowJo software (RRID:SCR_008520), version 10. Additional information on differentiation status of CAR T cells within infusion products are reported in Supplementary Fig. S5.

Statistical analyses

Time of CAR T-cell administration was used as the origin in all time-to-event analyses. For progression-free survival, overall survival, and duration of response (PFS, OS, and DoR), Kaplan–Meier curves and the log-rank test were used. For group comparison of categorical data Fisher exact test or χ^2 test were used. Mann–Whitney test was performed for comparison of continuous variables. Multivariable logistic regression was used to study the association between different covariates to disease response. All the reported P values (P) are two-sided and were considered statistically significant when $P < 0.05$. Plots and statistical analysis were performed with the use of GraphPad Prism v.9.00 (RRID:SCR_002798), RAW Graph website and Adobe Illustrator (RRID:SCR_010279).

Data availability statement

The data generated in this study are available upon request from the corresponding author.

Results

Patient characteristics and clinical course

To rule out the possibility that the two groups of patients receiving either Tisa-cel or Axi-cel were significantly different, patient characteristics have been analyzed and are listed in **Table 1**. Axi-cel and Tisa-cel patients are comparable with the only difference being the presence of PMBCLs among Axi-cel recipients that explains a higher proportion of patients treated with check-point inhibitors (CPI) and a lower International Prognostic Index (IPI). As a whole, the population studied is similar to those described in other published real-life CAR T cells experiences except for a better performance status as a consequence of the fact that in Italy, Axi-cel and Tisa-cel can only be prescribed in performance status-Eastern Cooperative Oncology Group (PS-ECOG) 0 and 1 patients (5, 11, 23, 24).

Table 1. Patient characteristics.

No. of patients	Total = 61 pts	Axi-cel = 32 pts	Tisa-cel = 29 pts	P
Age (median)	56	55	56	
Males	36 (59%)	20 (62%)	16 (55%)	0.61
Histotypes				
DLBCL	31 (51%)	12 (37%)	19 (65%)	0.47
tFL	8 (13%)	3 (9%)	5 (17%)	0.46
HGBL	9 (15%)	4 (12%)	5 (17%)	0.72
PMBCL	13 (21%)	13 (41%)	0 (0%)	<0.001 *
Prior lines				
Prior lines ≥3	24 (39%)	11 (34%)	13 (45%)	0.44
Prior ASCT	18 (29%)	8 (25%)	10 (34%)	0.57
Prior CPI	9 (15%)	8 (25%)	1 (3%)	0.03 *
Primary refractory	46 (75%)	24 (75%)	22 (76%)	0.13
ECOG				
0	46 (75%)	27 (84%)	19 (65%)	0.13
1	13 (21%)	4 (12%)	9 (31%)	0.11
Stage				
≤II	14 (23%)	10 (31%)	4 (14%)	0.13
>II	45 (74%)	22 (69%)	23 (79%)	0.39
Extra-nodal sites ≥2	16 (26%)	7 (22%)	9 (31%)	0.06
IPI				
0–2	42 (69%)	26 (81%)	16 (55%)	0.05
3–5	13 (21%)	3 (9%)	10 (34%)	0.03[a]
TMTV[b] (median)	27.6 (0.7–389)	29.4 (1.35–256)	21.1 (0.7–389)	0.6
Bulky disease (>5 cm)	16 (26%)	10 (31%)	6 (21%)	0.39
ALC apheresis (median)	800 (230–3900)	810 (310–3900)	800 (230–2200)	
CRP at day 0 >ULN	30 (49%)	12 (37%)	18 (62%)	0.07
LDH at day 0 >ULN	18 (29%)	6 (19%)	12 (41%)	0.09
Ferritin at day 0 >ULN	37 (60%)	19 (59%)	18 (62%)	>0.99
Bridging therapy	48 (79%)	24 (75%)	25 (86%)	0.34
Status at infusion				
CR	7 (11%)	5 (16%)	2 (7%)	0.42
PR	11 (18%)	8 (25%)	3 (10%)	0.19
SD	4 (7%)	3 (9%)	1 (3%)	0.61
PD	36 (59%)	15 (47%)	21 (72%)	0.07

Abbreviations: ALC, all lymphocyte count; ASCT, autologous stem cell transplant; CPI, check-point inhibitors; CRP, C-reactive protein; DLBCL, diffuse large B-cell lymphoma; ECOG, Eastern Cooperative Oncology Group; HGBL, high grade B-cell lymphoma; IPI, International Prognostic Index; LDH, lactate dehydrogenase; PMBCL, primary mediastinal B-cell lymphoma; tFL, transformed follicular lymphoma; TMTV, total metabolic tumor volume; ULN, upper level normality; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
[a]Indicates that a value reaches statistical significance.
[b]Pre-lymphodepletion TMTV (41% threshold) data were available for 38 patients (22 Axi-cel and 16 Tisa-cel).

Safety

Any grade and grade ≥3 CRS occurred, respectively, in 74% (95% CI, 62%–83%) and 2% (95% CI, 0.1%–9%) of patients. Any grade and grade ≥3 neurotoxicity occurred in 13% (95% CI, 7%–24%) and 2% (95% CI, 0.1%–9%) of patients, respectively. Tocilizumab and steroid were used in 39% and 28% patients, respectively. No difference in terms of CRS and ICANS between Axi-cel and Tisa-cel reached statistical significance although a trend to higher neurotoxicity can be highlighted in Axi-cel recipients who in fact were characterized by higher use of steroids (Supplementary Table S1).

Response to therapy

All 61 patients were valuable for response at day 30 after infusion and 58 were valuable at day 90. Median follow-up from infusion was 9 months. The best overall response rate (ORR) and CR rate were 69% (95% CI, 56%–79%) and 52% (95% CI, 40%–64%) respectively. Median time to response was 30 days, and no patients achieved a response after day 90. Although there was a trend in response rates in favor of Axi-cel, we did not observed statistically significant differences in terms of best ORR and CR rates between Axi-cel and Tisa-cel (Supplementary Table S1).

Outcomes

Of the 61 recipients, the median PFS was 10.1 months (not reached for Axi-cel and 6.4 months for Tisa-cel). PFS at 1 year was 49% (56% for Axi-cel and 41% for Tisa-cel). Relapses after 6 months were rare. The median OS was not reached, OS at 1 year was 80%. According to the log-rank (Mantel–Cox) test, PFS and OS curves are not significantly different between Axi-cel and Tisa-cel (**Fig. 1**).

Collectively, there were no significant differences in terms of safety, response to therapy and outcomes between patients treated with Axi-cel and Tisa-cel (Supplementary Table S1).

Phenotypic composition of Tisa-cel and Axi-cel infusion products

Left-over cells collected after washing 61 infusion product bags [29 Tisa-cel (47.5%) and 32 Axi-cel (52.5%)] were analyzed by flow cytometry (FCM). In the infusion products analyzed, CD3+ cells were
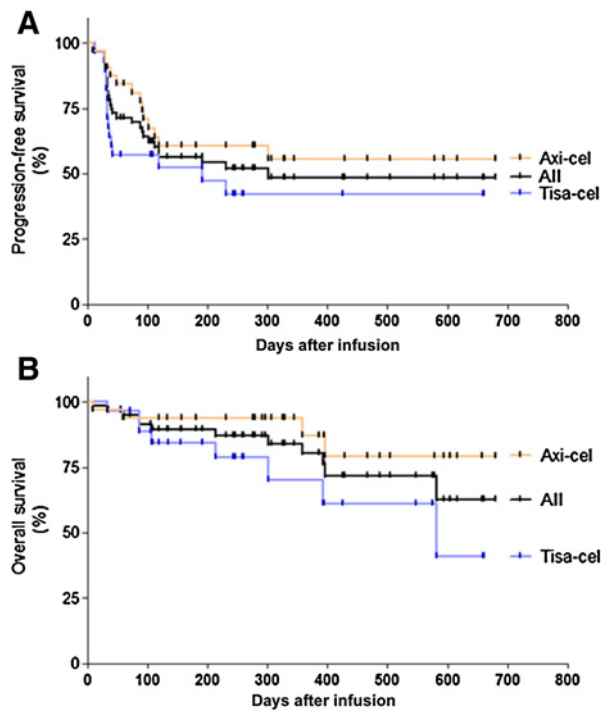
Figure 1.
Patients' survival. Kaplan–Meier curve showing PFS (**A**) and OS (**B**) according to the product infused. Overall, median PFS was 10.1 months, median PFS for Tisa-cel was 6.4 and not reached for Axi-cel. Overall, median OS was not reached, median OS for Tisa-cel was 19.4 and not reached for Axi-cel. According to the Log-rank test data were not statistically different.

73.2% of $CD45^+$ cells (median; range 25.2%–99.7%), CAR^+ cells were 55.2% of $CD3^+$ cells (median; range 5.8%–92.7%) and the $CD4^+$/$CD8^+$ ratio among CAR^+ cells was 1.94 (median; range 0.38–15.8).

When comparing Tisa-cel and Axi-cel infusion products, Tisa-cel was characterized by a lower fraction of $CD3^+$ among $CD45^+$ cells (median $CD45^+$/$CD3^+$: 63.8% vs. 78.5% for Tisa-cel and Axi-cel, respectively; $P < 0.005$) and of CAR^+ among $CD3^+$ cells as compared with Axi-cel (median $CD3^+$/$CD45^+$: 27.3% vs. 72.9% for Tisa-cel and Axi-cel, respectively; $P < 0.001$). On the contrary, Tisa-cel displayed a higher $CD4^+$/$CD8^+$ ratio among CAR^+ cells as compared with Axi-cel (median ratio $CD4^+$/$CD8^+$: 2.86 vs. 1.37 for Tisa-cel and Axi-cel, respectively; $P < 0.005$; **Fig. 2A–C**). The presence of different CAR^+ cell differentiation subsets in infusion products was then assessed (25–27). We analyzed the frequency of T central memory [T_{CM} ($CD45RO^+$/$CD197^+$/$CD62L^+$)], T naïve-like [T_{N-like} ($CD45RO^-$/$CD197^+$/$CD62L^+$)], T effector [T_E ($CD45RO^-$/$CD197^-$/$CD62L^-$)] and T effector memory [T_{EM} ($CD45RO^+$/$CD197^-$/$CD62L^-$)] subsets among $CD4^+$ and $CD8^+$ CAR^+ cells within Tisa-cel and Axi-cel infusion products ($n = 46$).

Collectively CAR T-cell infusion products were largely composed of differentiated T-cell subsets such as $CD4^+$ T_{EM} (21.9%), $CD8^+$ T_{EM} (12.4%) and of $CD4^+$ T_{CM} (18.4%), $CD8^+$ T_{CM} (11.4%), whereas T_{N-like} and T_E cells were less represented ($CD4^+$ T_E 0.01%, $CD8^+$ T_E 0.03%, $CD4^+$ T_{N-like} 0.04%, $CD8^+$ T_{N-like} 0.09%).

In Tisa-cel ($n = 19$), CAR^+ cells were enriched in $CD4^+$ cells with central memory and effector phenotypes (median CAR^+/$CD4^+$ T_{CM}: 37.7% in Tisa-cel vs. 14.1% in Axi-cel; $P < 0.005$ and median CAR^+/$CD4^+$ T_E: 0.056% in Tisa-cel vs. 0.005% in Axi-cel; $P < 0.005$) while

Axi-cel ($n = 27$) displayed an enrichment in $CD8^+$ cells with effector memory and naïve-like phenotypes (median CAR^+/$CD8^+$ T_{EM}: 6.07% in Tisa-cel vs. 23% in Axi-cel; $P < 0.005$ and median CAR^+/$CD8^+$ T_{N-like}: 0.04% vs. 0.15%; $P < 0.05$). Of note, no significant differences were found when comparing the frequencies of the other T-cell populations (CAR^+/$CD4^+$ T_{EM}, CAR^+/$CD4^+$ T_{N-like}, CAR^+/$CD8^+$ T_{CM} and CAR^+/$CD8^+$ T_E; **Fig. 2D–K**). All data are reported in Supplementary Table S2.

Infusion products phenotypic signatures associated with CAR T-cell expansion

To evaluate whether quantitative or qualitative features of CAR T-cell infusion products could affect *in vivo* CAR T-cell fate, expansion kinetics data of 53 patients with available longitudinal *in vivo* CAR T-cell records were analyzed. Cellular parameters, including concentration of CAR^+ cells at day 7 (C_7) and at day 10 after infusion (C_{10}), time to maximal expansion (T_{max}), concentration of CAR+ cells at T_{max} (C_{max}) and the magnitude of expansion up to 30 days (calculated as the area under the curve, extended, AUC_{0-30}) were evaluated. Overall, the kinetics curves displayed an initial increase in CAR T-cell numbers within the first 2 weeks of infusion, followed by a progressive decrease in all treated patients (**Fig. 3A**). Median T_{max} was 10 days for both products. Of note, both C_{max} and C_{10} were found to be strongly correlated with the AUC_{0-30} (Pearson correlation 95% CI; $r = 0.9588$; $P < 0.0001$ and $r = 0.8774$; $P < 0.0001$, respectively).

On the basis of this observation, the C_{10} was selected as the surrogate measure to quantify CAR T-cell expansion and the median C_{10} (24.5 CAR T cells/μL) was used as a cutoff to dichotomize the cohort of patients into "expanders" (patients with a $C_{10} \geq 24.5$ CAR T cells/μL) and "poor-expanders" (patients with a $C_{10} < 24.5$ CAR T cells/μL). When looking for possible differences in the infusion products administered to these two groups, we established that expanders had received infusion products significantly enriched in CAR^+CD8^+ cells with a central memory phenotype as compared with poor-expanders (median CAR^+/$CD8^+$ T_{CM}: 13.8% in expanders vs. 4.5% in poor-expanders; $P < 0.005$) thus suggesting that CAR^+/$CD8^+$ T_{CM} have the highest ability to expand (**Fig. 3B–I**; Supplementary Table S3). Of note, in our cohort total metabolic tumor volume (TMTV) values did not differ between expanders and poor-expanders (Supplementary Fig. S6). Similarly, the use of tocilizumab and steroids did not affect CAR T-cell expansion (OR, 2.301; χ^2 test 95% CI, 0.804–6.561; $P =$ ns; OR, 2.929; χ^2 test 95% CI, 0.670–7.400; $P =$ ns, respectively).

The fact that the CAR^+/$CD8^+$ T_{CM} population was the only population among all the tested ones positively associated with CAR T-cell expansion, but not differentially present in Tisa-cel and Axi-cel infusion products, is consistent with Tisa-cel– and Axi-cel–treated patients displaying similar expansion kinetics (**Fig. 3J** and **K**). In fact, no discrepancy in the number of CAR T cells/μL either at day 10 or at the T_{max} was observed (median C_{10}: 34 CAR T cells/μL for Tisa-cel vs. 22.9 CAR T cells/μL for Axi-cel; $P =$ ns and median C_{max}: 59.6 CAR T cells/μL for Tisa-cel vs. 54 CAR T cells/μL for Axi-cel; $P =$ ns); the magnitude of expansion was also similar in patients receiving the two products (median AUC_{0-30}: 128.8 for Tisa-cel and 113.3 for Axi-cel; $P =$ ns; **Fig. 3L**). Notably, CAR T-cell persistence was similar in Tisa-cel- and Axi-cel–treated patients (Supplementary Fig. S7A) and was not associated with a prolonged DoR (Supplementary Fig. S7B).

Association of CAR T cell *in vivo* expansion with response and survival

As CAR T-cell expansion after infusion has been correlated with clinical response in other studies (4, 14), we set to analyze whether this
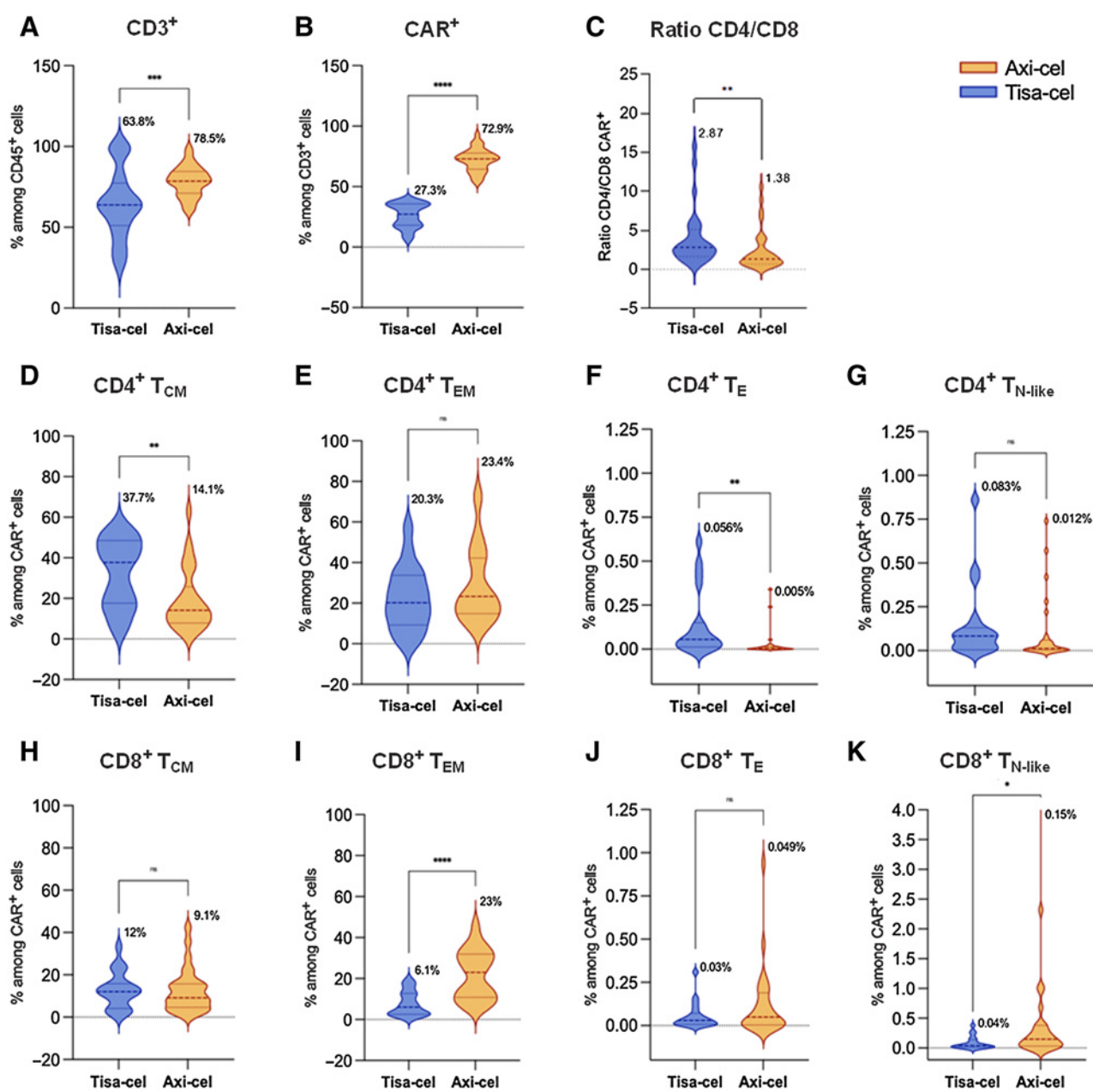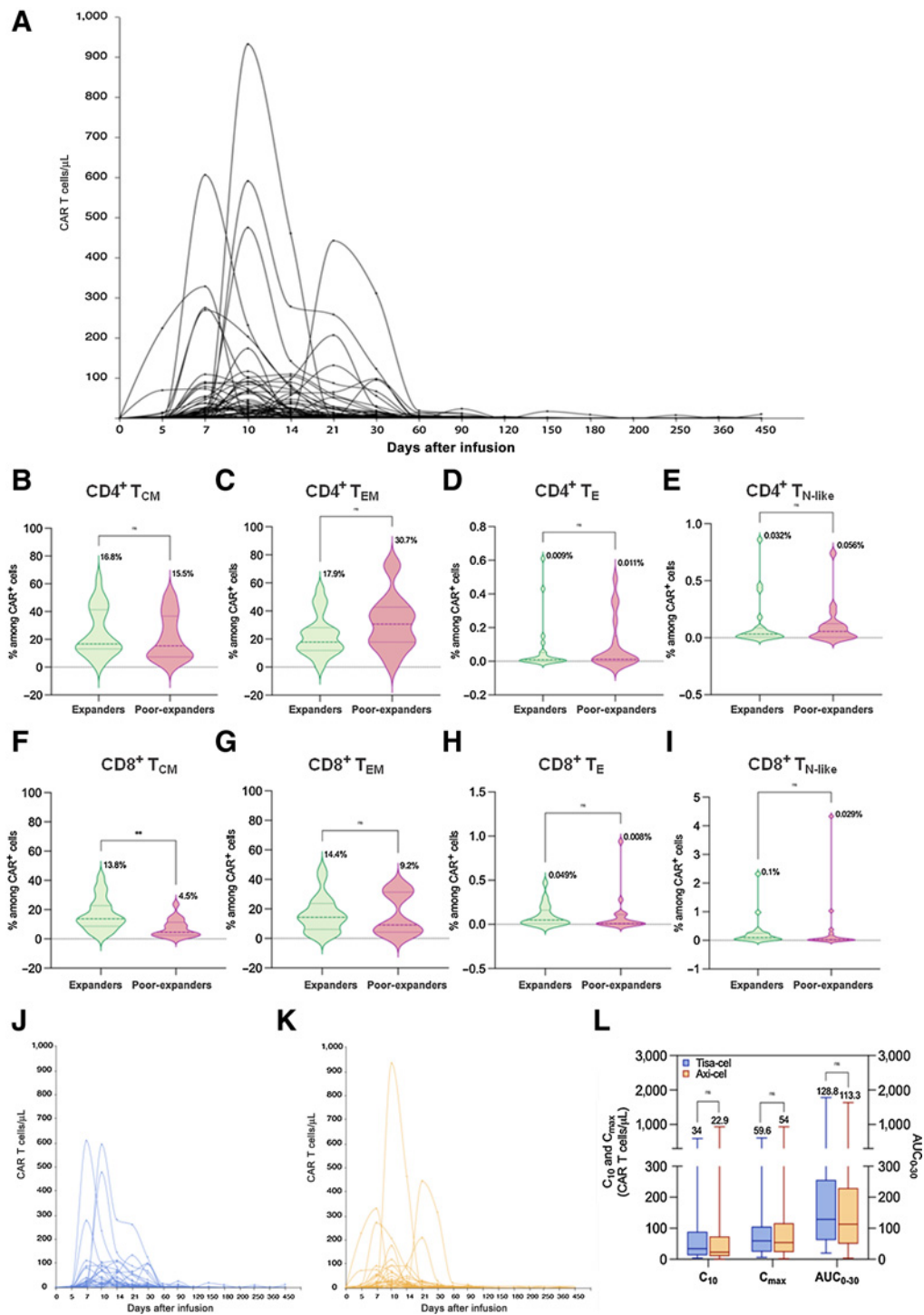
Figure 2.
Comparison of Tisa-cel and Axi-cel infusion products phenotypes by flow cytometry. Violin plots showing the comparison of CD3+ rates among CD45+ cells (**A**), CD19+CAR+ rates among CD3+ cells (**B**), and CD4+/CD8+ ratios (**C**) between Tisa-cel ($n = 29$) and Axi-cel ($n = 32$) infusion products. Violin plots showing the comparison of frequency distribution of T central memory (T_{CM}), T effector memory (T_{EM}), T effector (T_E), and T naïve-like (T_{N-like}) cells gated on CD4+ CAR+ cells (**D–G**) and on CD8+ CAR+ cells (**H–K**) between Tisa-cel ($n = 19$) and Axi-cel ($n = 27$) infusion products. Exact median values are reported. P values were calculated applying the Mann–Whitney test; *, $P < 0.05$; **, $P < 0.01$ or 0.005; ***, $P < 0.001$; ****, $P < 0.0001$.

was true also in our real-life setting. Patients who achieved complete response (CR) or partial response (PR) by day 90 after CAR T-cell infusion were defined as responders (RE); patients with stable, progressive disease (SD, PD) and patients who died for progression before day 90 were defined as nonresponders (NR). Accordingly, among the 53 patients with available longitudinal expansion assessment and clinical evaluation, 31 (59.1%) were RE (28 CR, 15 Tisa-cel and 13

Axi-cel; 3 PR, all Axi-cel) and 22 (40.9%) were NR (all PD, 11 Tisa-cel and 11 Axi-cel).

Consistent with previous reports, responders showed a superior expansion of CAR T cells as compared with NR, who exhibited reduced peak levels and a more rapid contraction (**Fig. 4A** and **B**). In detail, C_7, C_{10}, C_{max}, and AUC_{0-30} were detected in RE when compared with NR. In particular median C_7 was 22.8 CAR T cells/μL in RE versus 6.6 CAR

Figure 3.

In vivo CAR T-cell expansion by flow cytometry and association with infusion products phenotypes. **A,** Line chart of overall CAR T-cell expansion kinetics ($n = 53$). Violin plots showing the comparison of frequency distribution of T central memory (T_{CM}), T effector memory (T_{EM}), T effector (T_E), and T naïve-like (T_{N-like}) cells gated on $CD4^+$ CAR^+ cells (**B–E**) and on $CD8^+$ CAR^+ cells (**F–I**) between infusion products from expanders ($n = 21$) or poor-expanders ($n = 18$). Exact median values are reported. *P* values were calculated applying the Mann–Whitney test; **, $P < 0.01$. **J** and **K,** CAR T-cell expansion kinetics in patients receiving Tisa-cel (blue curves, $N = 26$) and Axi-cel (orange curves, $N = 27$). **L,** Box-and-whiskers plots showing the comparison of expansion parameters, namely concentration of CAR T cells at day 10 after infusion (C_{10}), peak concentration of CAR T cells (C_{max}) and magnitude of expansion by day 30 after infusion expressed as area under the curve (AUC_{0-30}) between patients receiving Tisa-cel or Axi-cel. Exact median values are reported. Comparison was made by the Mann–Whitney test; ns, $P > 0.05$, not significantly different.
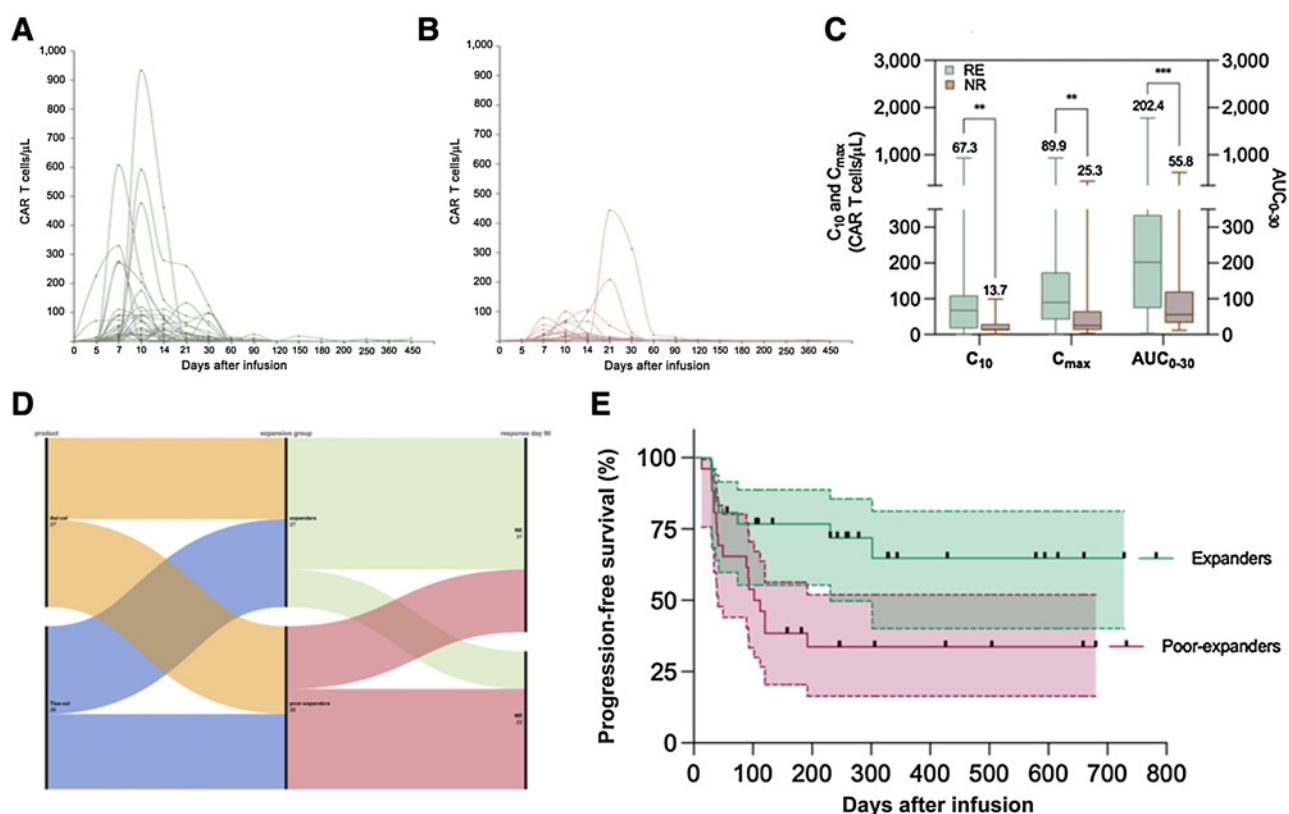
Figure 4.
In vivo CAR T-cell expansion by flow cytometry and association with response and survival. **A** and **B,** CAR T-cell expansion kinetics in responders to therapy by day 90 after infusion (RE, dark green curves, $n = 31$) and nonresponders (NR, dark red curves, $n = 22$). **C,** Box-and-whiskers plots showing the comparison of expansion parameters, namely concentration of CAR T cells at day 10 after infusion (C_{10}), peak concentration of CAR T cells (C_{max}), and magnitude of expansion by day 30 after infusion expressed as area under the curve (AUC_{0-30}) between RE and NR. Exact median values are reported. Comparison is made applying the Mann–Whitney test; **, $P < 0.01$ or 0.005; ***, $P < 0.001$. **D,** Alluvial plot representing how patients are distributed into the different groups analyzed. Tisa-cel ($n = 26$) and Axi-cel ($n = 27$) patients were equally divided into expanders [14 out of 26 Tisa-cel (53.8%) and 13 out of 27 Axi-cel (48.1%)] or poor-expanders [12 out of 26 Tisa-cel (46.2%) and 14 out of 27 Axi-cel (51.9%)], whereas the majority of expanders were RE by day 90 after therapy (21 RE out of 27 expanders, 77.8%) compared with poor-expanders (10 RE of 26 poor-expanders, 38.5%). **E,** Kaplan–Meier curve showing PFS according to CAR T-cell expansion. Median PFS for expanders was not reached, compared with 3.7 months for poor-expanders. Comparison is made applying the log-rank test; $P < 0.05$.

T cells/μL in NR (P ns), C_{10} was 67.3 CAR T cells/μL in RE versus 13.7 CAR T cells/μL in NR ($P < 0.01$), median C_{max} was 89.9 CAR T cells/μL in RE versus 25.3 CAR T cells/μL in NR ($P < 0.005$) and median AUC_{0-30} was 202.4 in RE versus 61.1 in NR ($P < 0.001$; **Fig. 4C**). Of note, we observed that among expanders, 21 (77.8%, 95% CI, 59.2%–89.4%) patients were RE, in contrast to 10 (38.5%; 95% CI, 22.4%–57.5%) in the poor-expander group. On the basis of these data, we found that expansion was significantly positively associated with response (OR, 5.600; χ^2 test 95% CI, 1.681–18.65; $P < 0.005$; **Fig. 4D**). This association remained significant even after correcting for other parameters linked with outcome in a multiple logistic regression analysis (C_{10} OR, 1.268; 95% CI, 1,062–1,676; $P < 0.05$; Supplementary Table S4; Supplementary Fig. S8). Consistently, expanders had significantly longer survival rates when compared with poor-expanders (PFS median, not reached vs. 3.7 months, respectively; $P < 0.05$; **Fig. 4E**).

Discussion

To the best of our knowledge, this is the first study performing correlative analysis of the T-cell immunophenotypic features of

Tisa-cel and Axi-cel infusion products and *in vivo* CAR T-cell expansion and efficacy. CAR T-cell expansion dynamics have been indicated as surrogate markers for tumor response and disease control in exploratory analysis of clinical trials involving patients with R/R lymphoma but properties of CAR T cells that enable their proliferation *in vivo* have still to be consistently defined in real-life settings. In the ZUMA-1 trial, peak CAR T-cell levels correlated with outcome but expansion of CAR+ cells was not relevant (4). These findings were further confirmed after normalizing peak CAR+ cells to tumor burden (28). In this study, poorly differentiated CAR T cells with a T naïve-like phenotype were enriched in expanders and early CAR T-cell expansion was associated with efficacy. In another retrospective analysis of biomarker data from Axi-cel–treated patients, cells from the infusion products of patients with ongoing CR at 3 months had an enrichment of CD8 central memory T-cell phenotypes compared with patients with PR/PD (15). This is in line with a previous study that evaluated CAR T cells with CD3ζ and 4–1BB signaling domains in the setting of CLL, in which a memory CD8 phenotype was associated with superior responses (16). In the Juliet trial, Tisa-cel expansion was positively correlated with the duration of response but no

correlative analysis on infusion product CAR T-cell characteristics and proliferation was reported (3).

In our study, we highlighted that an enrichment of $CD8^+$ T_{CM} phenotypes in CAR T-cell products is associated with improved in vivo CAR T-cell expansion. This is true for both Tisa-cel and Axi-cel and is consistent with what observed by Deng and Fraietta, but in contrast to Locke and colleagues who defined the CAR^+ T stem cell memory (T_{SCM}) population in infusion products as the one with the highest ability to expand (15, 16, 28). Of note, even in our cohort expanders have higher frequencies of $CD8^+CAR^+$ naïve-like memory cells expressing CD45RA, CD197, and CD62L when compared with poor-expanders although the difference did not reach statistical significance. Interestingly, transcriptomic analyses revealed that T_{SCM} cells are similar to T_{CM} and $CD45RO^+$ $CCR7^+$ T_{CM} cells are known to play a key role in antitumor immunity. The frequency of T_{CM} cells in blood was in fact a positive predictor for both response to anti–PD-1 immunotherapy and survival.

Collectively previous data and our current analyses point to the essential role of CAR^+ cells with a central memory phenotype in mediating in vivo CAR T-cell expansion which is required for their clinical efficacy. In fact, in our study cohort, the improved proliferation of circulating CAR^+ cells in vivo correlated with clinical outcome and progression free survival. Similarly Ayuk and colleagues have used the median CAR T-cell peak concentration to define "weak expanders" and "strong expanders" and showed a significantly increased survival of "strong expanders" among 21 patients treated with Axi-cel (10).

While estimating the AUC could be considered a valid tool to describe and quantify aspects of the concentration–time profile of CAR T cells in PB, for AUC_{0-30} to be calculated but also for the peak concentration to be defined, a number of longitudinal assessments within the first month postinfusion are needed. On the other hand, the C_{10} can be more conveniently used as a surrogate measure of CAR T-cell expansion. Given the high correlation we established among all the estimates of expansion analyzed (namely the AUC_{0-30}, C_{max} and C_{10}), in our study expanders and poor-expanders have been dichotomized based on the CAR T-cell concentration at day 10 post infusion which represents an early parameter of expansion that can be easily monitored in routine clinical practice.

Although target cell manipulation could provide better quality CAR T cells, to date, autologous cells from leukapheresis remain the most common starting material for CAR T-cell production as manufacturing defined composition of CAR T cells is difficult, costly and challenging (29). This is probably the reason why very limited information is provided when commercial products are released. These include the safety requirements and cell viability and cell dose for Tisa-cel but only the range of CAR^+ viable cells for Axi-cel ($0.4–2 \times 10^8$ cells). Nevertheless the use of out-of-specification products, designated as such because of viability <80%, were reported to be similarly effective and safe when compared with products with viabilities ≥80% (30). The dose range of Tisa-cel and Axi-cel was approved based on the pivotal trials, in the absence of formal dose finding first-in-human studies and there is no clear understanding either on the minimum effective CAR T-cell dose required or on the subtypes of infused CAR T cells mediating effective in vivo proliferation and efficacy.

The significantly different composition of Tisa-cel and Axi-cel infusion products we describe, with Tisa-cel being characterized by fewer CAR^+ cells among $CD3^+$ lymphocytes and less $CD8^+CAR^+$ cells, is surprising given their similar expansion kinetics in our cohort of patients. Notably, $CD8^+CAR^+$ central memory cells are among the few T-cell populations that were equally represented in Tisa-cel and Axi-cel infusion products, consistent with their role in mediating CAR T-cell expansion. In fact, another interesting finding of our study is that CAR^+ cells dynamics were similar in patients treated with Tisa-cel and Axi-cel, in line with their similar efficacy, despite the different manufacturing processes and costimulatory domains that are thought to induce CAR T-cell proliferation with distinctive kinetics (31, 32).

In conclusion, our study describes for the first time the clinical utility of characterizing the T-cell composition of commercial infusion products and of real-time enumeration of expanding CAR T cells in patients with lymphoma. Our results suggest that despite the fact that Tisa-cel and Axi-cel infusion products are significantly different, Tisa-cel and Axi-cel expansion kinetics are similar and are mediated by the presence of CAR T cells with central memory phenotypes in infusion products. Consistently, for both Tisa-cel and Axi-cel, the number of circulating CAR T cells, a surrogate of expansion, can be used as predicting biomarker of response. Despite requiring validation in a larger series of patients, these observations indicate that Tisa-cel and Axi-cel infusion product characteristics can be interrogated early to gain insight into the expansion capabilities of CAR T cells in vivo that are necessary for ultimate CAR T-cell efficacy.

Authors' Disclosures

Authors' Contributions

C. Monfrini: Data curation, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. F. Stella: Data curation, formal analysis, validation, investigation, writing–original draft, writing–review and editing. V. Aragona: Formal analysis, writing–original draft. M. Magni: Data curation, validation, investigation, visualization, writing–original draft, writing–review and editing. S. Ljevar: Data curation, formal analysis. C. Vella: Methodology. E. Fardella: Writing–review and editing. A. Chiappella: Clinical data collection. F. Nanetti: Writing–review and editing. M. Pennisi: Writing–review and editing. A. Dodero: Clinical data collection and analysis. A. Guidetti: Clinical data collection and analysis. P. Corradini: Conceptualization, resources, supervision, funding acquisition, writing–review and editing. C. Carniti: Conceptualization, supervision, validation, writing–original draft, project administration, writing–review and editing.

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Note

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