Title: Biomarkers of efficacy and safety of the academic BCMA-CART ARI0002h for the treatment of refractory multiple myeloma

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Conflict of Interest Disclosures

AOC declares support for attending meetings from Janssen. MER declares no conflict of interest. AZ declares no conflict of interest. VGC declares receiving honoraria from Janssen, Pfizer, Bristol Myers Squibb (BMS)/Celgene, and GSK; support for attending meetings or travel from Janssen and GSK; and participation on data safety monitoring or advisory board from Janssen. SN declares no conflict of interest. SI declares no conflict of interest. ALD declares no conflict of interest. VC declares receiving honoraria from Janssen, BMS, Sanofi, GSK, and Amgen; support for attending meetings or travel from Janssen; and participation on data safety monitoring or advisory board from Janssen, Sanofi, and Amgen. NLM declares no conflict of interest. PRO declares receiving honoraria from consulting activities from BMS, Janssen, Sanofi, Abbvie, Pfizer, Roche, and GSK; honoraria from lectures from BMS, Janssen, Sanofi and GSK; support for attending meetings or travel from Abbvie; and participation on data safety monitoring or advisory board from Janssen. JLR declares receiving consulting fees from Janssen; honoraria from Janssen, Amgen, Sanofi, Kite/Gilead, Novartis, and BMS; support for attending meetings or travel from Kite/Gilead; and participation on data safety monitoring or advisory board from Janssen, BMS, and Novartis. DFM declares receiving honoraria and travel grants from Janssen. NMC declares receiving

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Word statement of translational relevance

There is an increase in demand for CART therapy, which will presumably grow even more with CARTs moving to earlier lines of treatment. Demand overcomes availability, and the costs of CART therapy are unaffordable for some countries and public health systems. In this sense, academic CARTs could be a promising option to palliate these problems, allowing a strategy of point-of-care that could be implemented worldwide.

Despite impressive outcomes observed with BCMA-CART in patients with heavily pretreated multiple myeloma, this therapy is not curative and myeloma patients continue to relapse. Understanding the mechanisms of failure of CART therapy and finding biomarkers of response that could be addressed to improve efficacy is of main importance.

Abstract

Background

BCMA-CARTs improve results obtained with conventional therapy in the treatment of relapsed/refractory multiple myeloma. However, the high demand and expensive costs associated with CART therapy might prove unsustainable for health systems. Academic CARTs could potentially overcome these issues. Moreover, response biomarkers and resistance mechanisms need to be identified and addressed to improve efficacy and patient selection. Here, we present clinical and ancillary results of the 60 patients treated with the academic BCMA-CART, ARI0002h, in the CARTBCMA-HCB-01 trial.

Methods

We collected apheresis, final product, peripheral blood and bone marrow samples before and after infusion. We assessed BCMA, T-cell subsets, CART kinetics and antibodies, B-cell aplasia, cytokines, and measurable residual disease by next generation flow cytometry, and correlated these to clinical outcomes.

Results

At cutoff date March 17th 2023, with a median follow-up of 23.1 months (95%CI 9.2-37.1), overall response rate in the first 3 months was 95% (95%CI 89.5-100); cytokine release syndrome (CRS) was observed in 90% of patients (5% grades≥3) and grade 1 immune effector cell-associated neurotoxicity syndrome was reported in 2 patients (3%). Median progression-free survival was 15.8 months (95%CI 11.5-22.4). Surface BCMA was not predictive of response or survival, but soluble BCMA correlated with worse clinical outcomes and CRS severity. Activation marker HLA-DR in the apheresis was associated with longer progression-free survival and increased exhaustion markers correlated with poorer outcomes. ARI0002h kinetics and loss of B-cell aplasia were not predictive of relapse.

Conclusion

Despite deep and sustained responses achieved with ARI0002h, we identified several biomarkers that correlate with poor outcomes.

Introduction

The introduction of B-cell maturation antigen (BCMA) directed chimeric antigen receptor T-cell (CART) therapy to the treatment of relapsed/refractory (R/R) multiple myeloma (MM) is undoubtedly revolutionary, with the achievement of high response rates and median survivals that overcome those achieved with conventional therapy in this setting[1–3]. These outstanding results led to the approval of idecabtagene vicleucel (ide-cel)[4] and ciltacabtagene autoleucel (cilta-cel)[5], and results of several CARTs targeting BCMA for R/R MM have been reported so far, including different single-chain variable fragment (scFv) origins (mouse, llama, humanized and human) and T-cell origin (autologous vs. allogeneic)[6–8]. Also, GPRC5D has emerged as an active immunotherapeutic target with promising results[9]. Consequently, the demand for this therapy overcomes availability and, in some countries and health systems, the costs of this therapy are unaffordable. These unmet needs could be improved by academic CARTs.

Our institution developed ARI0002h, a second generation 4-1BB-based CAR, with a humanized scFv directed against BCMA[10]. An academic, pivotal, single-arm, open label clinical trial (CARTBCMA-HCB-01) for the treatment of R/R MM patients was started. Results of the first cohort of 30 patients showed an overall response rate (ORR) of 100%, with 67% achieving complete responses (CR). After a median follow-up of 18 months, progression-free survival (PFS) was 14.5 months and overall survival (OS) was not reached, with a PFS rate at 12 months of 70%. Cytopenias were the most common side effect and although 80% of patients developed cytokine release syndrome (CRS), all cases were grades 1-2. No cases of neurotoxicity or late neurologic events were observed[11].

Despite the clinical outcomes obtained with BCMA-CARTs, patients continue to relapse and the absence of a plateau in the survival curves reported by different constructs does not suggest a definitive cure. This, added to the cost that CART therapy signifies in the health care systems of many countries, emphasizes the importance of finding predictive factors and mechanisms of resistance in order to guide patient selection. Some of the proposed features that could impact CART function are BCMA antigen density, soluble BCMA levels (including the possibility of a BCMA antigen escape), T-cell subsets (stem cell memory vs. effector), T-cell exhaustion, and the effect of non-human scFv leading to anti-CAR antibodies[12].

Here, we report the clinical results from a planned interim analysis with cut-off date March 17th 2023 of 60 patients treated with ARI0002h, including longer follow-up of the initial cohort and 30 additional patients from cohort 2. We also describe ancillary studies performed on patients samples evaluating potential mechanisms of efficacy and resistance to ARI0002h.

Methods

Study design and subjects

CARTBCMA-HCB-01 is a pilot, single-arm, open-label study performed in 7 Spanish centers. Patients aged 18-75 years old with RRMM were eligible if they had measurable disease, received ≥2 prior regimens, including a proteasome inhibitor, an immunomodulatory drug and an anti-CD38 antibody, and were refractory to the last line of treatment. A booster dose of up to $3x10^6$ CAR+cells/kg was planned at least 3 months after the first dose in patients with any kind of response and no limiting side effects. Prior to the booster dose, LD was repeated following the same scheme only when CART cell persistence was ruled out (Suppl.Figure 1). The study was registered with EudraCT (2019-001472-11) and ClinicalTrials.gov (NCT04309981). The study was performed in accordance with the Declaration of Helsinki Ethical Principles for Medical Research involving Human Subjects and the protocol was approved by the Ethics Committee of Hospital Clinic de Barcelona. All subjects provided written informed consent.

Samples

Apheresis and final product (FP) samples were obtained and analyzed. Peripheral blood (PB) and bone marrow (BM) samples were obtained at the established timepoints; PB at inclusion, prior to LD, on days 0, 3, 7, 14, 28, 70, 100 and months 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24; BM at inclusion, days 28 and 100, and months 6, 12, 18, 24. When patients were withdrawn from the study, end-of-treatment (EOT) samples from the PB and BM, if possible, were collected. Potency was analyzed in the FP by flow cytometry, assessing killing capacity of CAR-T cells against the U266 tumor cell line. Measurable residual disease (MRD) was assessed by next generation flow. Kinetics of ARI0002h were measured by qPCR in the PB and by flow cytometry in the BM. B-cell kinetics were measured by flow cytometry. BCMA molecules/cell on plasma cells (PC) were quantified by flow cytometry. Soluble BCMA was quantified in serum by ELISA. T-cell subsets were analyzed by flow cytometry. Serum levels of cytokines were

measured by ELISA. Anti-CAR antibodies were determined by flow cytometry (Figure 1). Detailed information is described in the Supplementary Methods.

Clinical results

Primary endpoints were overall response rate (ORR) within the first 3 months and rate of cytokine release syndrome (CRS) and/or neurotoxicity in the first 30 days. Response was assessed as per International Myeloma Working Group (IMWG) criteria[13] and BM minimal residual disease (MRD)[14] was analyzed by next-generation flow. Adverse events (AEs) were graded using CTCAE v5.0. CRS and neurotoxicity were graded according to the American Society for Transplantation and Cellular Therapy (ASTCT) criteria[15].

Statistical analysis

Categorical variables were reported in number or proportions and continuous variables were reported as mean and standard deviation or median and interquartile range. Comparisons between categorical values were performed with χ^2 or Fisher's exact test. The Student's t-test or the Wilcoxon test (when appropriate) were used to analyze the continuous variables. The log-rank test was used to analyze the differences in survival curves, and the Kaplan-Meier method to graphically depict the PFS and OS. A Cox proportional hazards model was used to calculate the hazard ratio (HR). Pearson or Spearman correlation tests were used when appropriate.

Data availability

All materials, data, and protocols described in the manuscript will be made available upon request, if the request is made within 6 years of publication. A summary of the study protocol is included in the Supplementary data of this article.

Results

Clinical outcomes of ARI0002h

As of March 17th 2023, 72 patients with R/R MM were screened, of which 69 underwent apheresis and 61 received LD, with 60 patients finally receiving ARI0002h (Figure 2A). This is the population that was evaluated in the studies described herein. The main patient characteristics and response data are described in Table 1. Bridging therapy was administered to 48% of patients. The ORR in the first 3 months was 95% (≥ very good partial response (VGPR) in 77%). Median time to first response was 1 month. Responses deepened over time, with 58% achieving CR (55% stringent CR –sCR-), 30% VGPR, 7% partial response. Only 1 patient (2%) was refractory, and 2 patients (3%) died prior to first evaluation (septic shock and macrophage activation syndrome (MAS)).

With a median follow-up of 23.1 months (95%CI 9.2-37.1), estimated median PFS was 15.8 months (95%CI 11.5-22.4) (Figure 2B). A higher baseline level of serum M protein correlated with shorter PFS (HR 1.018; p=0.022). Median OS was not reached with OS rates at 12 and 18 months of 81% and 69%, respectively (Figure 2C). Eighteen of 60 (30%) patients died. Causes of death were disease progression (n=13), COVID19 (n=2), cranial trauma, septic shock and MAS (n=1 each).

CRS was observed in 90% with 5% grades ≥ 3. Median time to CRS was 7 days (range 1-14) with a median duration of 4.5 days. Eight (13%) patients did not receive the third fraction due to CRS. ICANS grade 1 was reported in only 2 patients (3%) with no late neurologic events. 6 patients (10%) developed a MAS (four grades 1-2, one grade 3, one death). Tocilizumab and steroids were administered in 68% (mainly for persistent grade 1 CRS) and 30% of patients, respectively.

The booster dose had been administered to 44 out of 55 eligible patients (80%), with no CRS, ICANS or MAS or other toxicities. Median time after first infusion was 4.4 months (interquartile range (IQR) 4.0-5.1); 34% received a second LD regimen. Response was evaluable in 42 patients; 45% (n=19) were already in sCR, 29% (n=12) maintained the previously achieved response and 26% (n=11) improved their response. Six patients (10%) developed neoplasias after ARI0002h infusion. Detailed information is provided in Suppl.Table 1.

B-cell maturation antigen role in efficacy

BCMA data were obtained from two sources: (1) quantification of BCMA molecules on the surface of myeloma vs. normal PC in the BM and (2) measurement of soluble BCMA in the serum.

Mean BCMA molecules on the surface of myeloma PC at inclusion was 1261 molecules/cell (SD 838, range 225-4040). A decrease in number was observed between inclusion and relapse in myeloma PC (17 samples available; p=0.019) (Figure 3A), although none of the patients became BCMA negative in the BM. Conversely, BCMA in normal PC decreased only in 1 of 4 patients with paired samples available. We found elevated sBCMA in all patients at both inclusion (252,661 pg/mL; SD 411,464) and relapse (142,887; SD 402,826), except for a patient. However, BCMA was detected in the bone marrow PC of this subject. The only patient who was primarily refractory to ARI0002h showed an increase in sBCMA between baseline and day 28 (Figure 3B).

The amount of BCMA molecules on the surface of myeloma PC was equivalent between patients who did or did not achieve a CR on days 28, 100 or as their best response (Figure 3C); survival (PFS and OS) was not correlated to the quantity of

BCMA either. In terms of sBCMA, baseline values were lower in patients who achieved CR (days 28, 100 or CR as best response), although differences were not statistically significant. Interestingly, sBCMA measurements on day 28 were predictive of CR achievement at all three timepoints (Figure 3D). Additionally, comparing baseline and day 28 sBCMA with the best response achieved, without segregating into CR/<CR, an association was observed (Figure 3D). Moreover, a higher sBCMA at baseline was correlated with a shorter time to event in DOR ($p<0.001$), PFS ($p=0.003$) and OS ($p=0.008$). Differences in baseline sBCMA were observed between patients who developed CRS grades 1-2 vs. higher grade CRS ($p=0.041$) (Figure 3E). sBCMA levels did not differ according to the presence of plasmacytomas. The number of BCMA molecules on myeloma PC or the quantity of sBCMA did not affect ARI0002h persistence or peak magnitude.

Manufacturing and potency

All products were obtained at first attempt but two. In one the apheresis was repeated and the other one was manufactured using cryopreserved T-cells that were available. Both products were successfully manufactured in the end. Mean T-cell transduction was 76%; dose was calculated based on CAR+ cells, therefore all patients received the same quantity of CARTs and all patients had $3x10^6$ CAR+ cells/kg available for the first infusion. In the case of the booster dose, 38/44 (86%) received $3x10^6$ CAR+ cells/kg in a unique infusion (n=2 received $1.2x10^6$ CAR+ cells/kg and n=4 received $1.8x10^6$ CAR+ cells/kg; which was the dose available). Median CART production and turnaround times were 9 days (IQR 8-10) and 32 (IQR 27-37), respectively. Median vein-to-vein time was 41 days (IQR 34-51), with differences between patients who did or did not receive bridging therapy (48 vs. 36 days;$p=0.004$). No impact on PFS and OS was observed according to manufacturing time. *In vitro* killing capacity of the CAR T-cells was tested in all FP, but no correlation was found between potency and clinical outcomes, in line with previously reported data[16–18],

T-cell subsets in the apheresis and final product

T-cell populations in the apheresis and the final product (FP) (in CAR+ and CAR- cells) are depicted in Figure 4A. We found an inverted CD4/CD8 ratio in the apheresis and FP, but greater expansion of CD4 cells during manufacturing was observed, with an increased CD4/CD8 ratio in the FP compared to after apheresis (0.83 vs 0.43;$p=0.0011$). The FP was enriched in central memory cells (15.5% vs 3.7%;$p<0.001$), whilst naïve plus stem cell memory T-cells were increased in CD8 (25.5% vs. 5.7%;$p<0.0001$) but decreased in CD4 (10.1% vs. 15.9%;$p<0.001$). After

cell expansion during manufacturing, the proportion of Th1 (20.9% vs. 11.8%;p<0.0001) and Treg (11.9% vs. 1.5%;p<0.0001) populations were also increased, accompanied by a large increase in cells expressing the activation marker HLA-DR (CD8: apheresis 15.2% vs FP 72%,p<0.0001; CD4: apheresis 9.3% vs FP 68.2%,p<0.0001).

Regarding the association of the analyzed T-cell subsets and clinical outcomes, a higher proportion of CD8 central memory cells in the FP (but not in the apheresis) was correlated with the achievement of CR on day 100 (p=0.045). Several populations in the apheresis showed a mild-intermediate correlation with the proportion of CD8 central memory cells in the FP (Suppl.Table 2), suggesting that a starting material enriched in CD4 naive and CD4 CM cells, could have a roll helping CAR- T central memory differentiation during cell culture. We could not find a correlation with Tregs or other T-cell subsets, although the CAR- population of the FP was enriched in Treg (30%), compared to the CAR+ fraction (11.9%). In contrast, a higher percentage of the activation marker HLA-DR in the apheresis (but not in the FP) was associated with an increased PFS (CD8: HR 1.050,p=0.046; CD4: HR 1.063,p=0.041); for OS only a trend towards significance was found (CD8: HR 1.056,p=0.084; CD4: HR 1.055,p=0.050).

Exhaustion markers

We analyzed exhaustion markers (PD-1, TIGIT, TIM-3, LAG-3) in the apheresis, FP, in the PB on day 28, and in the BM on days 28, 100 and EOT. We observed a marked increase in PD-1 and TIGIT from the FP to days 28 and 100 in the BM (p<0.001), and in PD-1 from the FP to day 28 in the PB (p<0.001) (Figure 4B). TIM-3 and LAG-3 expression increased from apheresis to the FP (p<0.001), but a decrease was observed in the PB and BM on day 28 (p<0.001). From day 28, TIM-3 started to increase in CAR+ cells of the BM, but LAG-3 expression in the BM remained low.

In terms of impact on efficacy, patients who achieved CR on day 28, 100 and as best response had a lower expression of TIGIT in the BM on day 28 (p=0.011, p=0.013, p=0.011, respectively; Figure 4C). We did not find a correlation between PD-1 expression and response. Higher levels of PD-1 and TIGIT in the apheresis, TIM-3 and TIGIT in CAR+ CD8 T cells in the PB on day 28 and TIM-3 in CAR+ CD4 T cells in the PB on day 28 correlated with shorter PFS and OS (Suppl.Table 3;Suppl.Figure 2).

Kinetics of ARI0002h

Persistence of ARI0002h was measured in both PB and BM. Of note, BM samples were obtained less frequently, potentially introducing a sampling bias. Median

persistence of ARI0002h in the PB and BM was 4.9 (95%CI 3.8-5.9) and 5.6 months (95%CI 3.6-7.7), respectively. The mean peak was 10.4 copies/genome (SD 11.4; range 0.68-68.8) (Figure 5A) and occurred on day 14 and 28 in 70% and in 18% of patients, respectively. Persistence in the PB at each timepoint is depicted in Suppl.Figure 3. We analyzed persistence and the magnitude of the peak according to baseline tumor burden, measured as proportion of BM PC and baseline sBCMA, with no significant differences (Suppl.Figure 4&5).

Of the 44 patients who received the booster dose, expansion data were evaluable from 39. Expansion in the PB was observed in 61.5% of patients (n=24), not observed in 35.9% and the result was uncertain in one patient. Median persistence after booster dose in patients who expanded was 3.7 months (95%CI 1.4-6.0). Half of the expansions (12/24) occurred within the first seven days after the booster dose. The peak observed after the booster dose was lower (mean 2.74 copies/genome; SD 6.6) than after the initial expansion (Figure 5B), although samples after 14 days (median time to peak observed after the first infusion) were not available. Second LD was administered to 6 of 14 patients in the non-expansion group and to 5 of 24 of the expansion group (p=0.266). In 6 patients, a CART expansion in the PB was observed prior to MM relapse, including 2 patients who simultaneously became MRD positive. ARI0002h was detectable in 32.1% (9/28) of the patients with samples at relapse (Figure 5C).

To establish the potential impact of PB CART persistence in DOR, PFS and OS, we performed a survival analysis setting a landmark time at 3 and 4.9 months (the median persistence of this cohort) from infusion. A log-rank comparison categorizing persistence at both timepoints showed no differences (Suppl.Figure 6). Triple- or penta-refractory patients had a similar persistence of ARI0002h. For patients with prior allogeneic stem cell transplantation (alloHSCT) (n=5), ARI0002h persistence was high, but was not statistically longer compared to those who had not undergone alloHSCT in either the PB (11.1 vs 4.9 months;p=0.417) or BM (11.1 vs 5.4 months;p=0.315) (Suppl.Figure 7).

The magnitude of the peak of expansion had no impact on PFS or OS, but did correlate with the probability of CRS occurring (CRS vs. no CRS: 11.1 vs 3.7 copies/genome;p=0.030). A trend to higher proportion of CD4 T-cells on peak day was observed in patients who were in CR on day 100 (CR 19.0 vs <CR 12.5;p=0.046) and who achieved a CR as best response (CR 25.7 vs <CR 18.8;p=0.056).

B-cell aplasia

The disappearance of normal B cells is a well-known on-target/off-tumor effect of BCMA targeted CARTs. All 60 patients treated with ARI0002h had B cells in the PB at inclusion (mean 8.1%, SD 12.2%) and all of them eventually developed B-cell aplasia. On the day of infusion, 31.6% (n=12/38) of patients lacked B cells, probably due to the lymphodepletion regimen, although in 4 of these cases we observed a brief recovery. Median time from infusion to the start of B-cell aplasia was 3 days (IQR 12, range 0-29) and the median duration was 3.3 months (95%CI 2.6-3.9). Amongst the 28 patients who developed disease progression, 5 (17.9%) maintained a continuous B-cell aplasia in the EOT samples; 1 patient was not evaluable and in the remaining 22 patients median time from B-cell recovery to relapse was 10.3 months (IQR 12.6, range 0.5-24.3) (Suppl.Figure 8). Of the 9 patients who relapsed with CART in PB, B-cells were undetectable in only 4.

Cytokines

Cytokine results are only available for the first cohort of 30 patients. Serum quantities of different cytokines are depicted in Supple.Figure 9A. See also Suppl.Table 4 and Suppl.Figure 10. Patients who experienced CRS of any grade, compared to those who did not, showed significant differences in baseline IFNγ (CRS 4.1 vs no CRS 8.4 pg/ml;p=0.029), granzyme B on day 14 (CRS 365.9 vs no CRS 39.6 pg/ml;p=0.040), IL-6 on day 14 (CRS 370.8 vs no CRS 8.1 pg/ml;p=0.014) and IL-6 on day 28 (CRS 310.2 vs no CRS 5.5 pg/ml;p=0.020) (Suppl.Figure 9B).

CART antibodies

We detected antibodies against the CART in 60% (n=36/60) of patients. Although we detected antibodies in consecutive samples in few patients, in most of them, anti-CAR antibodies were not sustained over time. The detection of anti-CAR antibodies at each timepoint is summarized in the Suppl.Table 5. Ten patients had a positive antibody measurement prior to the booster dose, and of those, 5 (50%) expanded, 3 did not expand (30%), and in 2 expansion was not evaluable. We analyzed survival setting a landmark time at 3 months, comparing patients with or without antibodies at that timepoint, with no differences in PFS (p=0.435) or OS (p=0.824). Anti-CAR antibodies were detected in the EOT sample of 25% (7 of 28) of patients (Suppl.Table 6), but they were also detected in the 5 patients who have not relapsed after more than 20 months (Suppl.Table 7).

Measurable residual disease by next generation flow cytometry

MRD analysis results obtained for the 60 patients at different timepoints are shown in Suppl.Table 8. MDR-negative rates in evaluable samples on days 28 and 100 were 98% and 96%, respectively. Of note, 24% (n=13/54) of the samples were not evaluable on day 28 due to hemodilution. MRD was evaluable at the expected sensitivity of 10^{-6} in approximately half of the samples in the initial timepoints (days 28 and 100) and in 60-75% of samples at later timepoints (months 6 to 24). A sensitivity of at least 10^{-5} was achieved in 87.5-100% of all samples at all timepoints. No differences were observed in PFS according to MRD-negative sensitivity (Suppl.Figure 11).

In 12 of 28 patients (43%) who developed disease progression, MRD positivity was observed early, prior to overt relapse (Figure 5C). This premature detection is probably underestimated due to the frequency of BM sampling. In 2 patients the positivity was evident at a sensitivity of 10^{-4}, in 8 patients at 10^{-5} and in 2 at 10^{-6}. Median time from MRD positivity to relapse was 3.1 months (IQR 3.4) (Suppl.Figure 12). When analyzing MRD in 19 available end-of-treatment (EOT) samples, 3 were MRD negative: 2 had extramedullary disease progressions and 1 patient withdrew for alloHSCT.

Other clinical correlations

When analyzing tumor burden markers other than sBCMA, we found that M-protein correlated with shorter PFS (p=0.013), while the proportion of BM PC did not. No differences in terms of PFS were observed according to the presence of triple-refractoriness, high-risk cytogenetics or extramedullary disease, when accounting for all plasmacytomas. There was a trend to shorter PFS when plasmacytomas were extramedullary (Suppl.Figure 13A-D). Interestingly, International Staging System (ISS) was a better predictor calculated at baseline rather than at diagnosis of MM (Suppl.Figure 13E-F). In a multivariate analysis, all three variables, sBCMA, M-protein and ISS at baseline retained statistical significance (Suppl.Table 9).

The impact of MRD in PFS could not be analyzed in this study since almost all patients were MRD negative on days 28 and 100. An involved serum free-light chain below the lower limit of normality was found in 80% and 84.3% of patients on days 28 and 100 and a correlation with PFS was observed (p=0.004) with day 100 measurements (Suppl.Figure 14).

Discussion

In this study, previously reported ARI0002h preliminary outcomes[11] were confirmed, with 30 additional patients and longer follow-up. One distinctive feature of this study was the booster dose. Both expansion and an improvement of responses were

observed after booster dose administration. It is unknown whether patients who received the booster dose in sCR (45%) had a longer DOR. Expansion results were similar between patients receiving or not a second LD, which suggests that it may not be mandatory. Still, a bias is present, since LD was administered according to CART persistence prior to booster dose, and therefore groups were not balanced. Since the study lacks a randomized comparison group, it is uncertain whether the booster dose is fully responsible for the benefits in terms of efficacy. Despite that, we believe that the feasibility of the booster dose manufacturing, administration and the lack of side effects warrant further investigation of this strategy.

The fractionated administration of ARI0002h may have contributed to a slightly delayed profile of CART kinetics and cytokine expression, compared to other reported BCMA-CART that are administered in a single infusion[4,8,19]. In ARI0002h, the peak of expansion was mainly observed in days 14 to 28. The highest levels of IL-6 and granzyme B were observed also from day 7, onwards. This behavior might have a different effect on immunological parameters and could explain the lower neurologic events observed in ARI0002h, although these observations can only be confirmed with a randomized study.

MAS/hemophagocytic lymphohistiocytosis (HLH) related deaths have been reported after cilta-cel and ide-cel[5,20]. In another study[21], 21.8% of patients treated with BCMA-CART met criteria for MAS/HLH. In our study, MAS/HLH was screened along follow-up, especially since one patient died due to MAS. All cases who met any of the available criteria, even if only laboratory criteria were found, were reported as MAS. All cases presented a previous CRS (4 grades 1 and 2 grades 2).

Factors associated with the duration of response in patients with MM treated with CARTs against BCMA can be related to the host (age, preforming status), baseline disease (tumor burden, cytogenetics, extramedullary disease) and with the CART itself. In our study, the ISS calculated at baseline rather than at diagnosis was a better predictor of outcomes. The finding that an involved serum FLC below the limit of normality in day 100 was predictive of longer PFS could be useful in patients who achieve a negative MRD or present hemodiluted BM samples.

The quantity of BCMA molecules on the surface of plasma cells did not impact response or survival in this cohort. However, sBCMA levels at baseline were lower in complete responders and seemed to have a response prediction capacity when measured on day 28. Of note, higher levels were also associated with CRS grade of severity, which would be inconsistent considering that no effector activity is performed against sBCMA. For this reason, we believe that the worse outcomes of high sBCMA levels found in this study in terms of both efficacy and toxicity have to do with sBCMA

as a surrogate marker of tumor burden, rather than a sink for ARI0002h, although clinical data have reported a benefit in blocking cleavage of BCMA[22,23]. Also, our data suggest that sBCMA levels could be used as a tumor marker in BCMA positive patients, as suggested by several other studies[24–29]. We did not find any evidence of BCMA negative relapse in this series. Since the expression was significantly lower at relapse, a mechanism associated with this low BCMA reservoir population cannot be discarded.

Reported data on T-cell subpopulations in [30–34] treated with CART suggest that long-lived self-renewing early memory phenotypes are of vital importance for CART expansion and promote profound and sustained tumor responses. Most studies are focused on the apheresis product, but a few studies that analyzed the FP report similar findings[19,35,36]. ARI0002h T-cell subpopulation distribution showed that CART cells in the final product were not terminally differentiated, retaining effector functions while maintaining memory phenotypes with proliferation ability. In our cohort, only CD8 central memory cells in the FP correlated with response on day 100. We did not find clinical correlations regarding regulatory T cells, apheresis subpopulations, or populations other than CD8 central memory cells in the FP. In one study, a lower expression of HLA-DR was observed in non-responders to CART for acute lymphoblastic leukemia[32]. In our data, the activation marker HLA-DR in both CD8 and CD4 correlated with longer PFS.

As previously observed for cilta-cel and ide-cel[37,38], the persistence of ARI0002h cells seems to be unrelated to the probability of progression. In this study, median persistence of the CART was 4.9 months, while median PFS was 15.8 months. Similarly, the loss of B-cell aplasia occurred soon before relapse in the majority of patients. In contrast, more than 30% of patients had detectable levels of ARI0002h at relapse. We observed a clear increase in the expression of exhaustion markers, particularly PD-1 and TIGIT in the BM, but also TIM-3 from day 28, and markers remained high in the two BM samples at relapse with detectable CART. Moreover, in some patients, the detection of CARTs at relapse did not coexist with a B-cell aplasia. All of this highlights the role that exhaustion may play in ARI0002h cells. Mechanisms to overcome exhaustion could include obtaining a higher percentage of long-lasting cells in the FP, such as central memory T-cells[39], and perpetuating T-cell activity with immune checkpoint blockade. A booster dose was administered with the aim of improving efficacy, with CARTs not exposed to MM, and therefore not exhausted, but based on exhaustion markers kinetics observed, we could consider an earlier administration.

Despite the humanized nature of the scFv, the development of anti-CAR antibodies was observed. Yet, their neutralizing ability and the potential deleterious impact on ARI0002h efficacy remains unclear and further studies are ongoing. The antibody detection at several timepoints in 5 patients who remain in response after more than 20 months of follow-up, the CART expansion observed after the booster dose in patients with anti-CAR antibodies, and the lack of impact in terms of PFS and OS in patients with a positive detection at 3 months, supports the idea that these antibodies may not have a clinical impact.

MRD negativity was achieved in the first 100 days by almost all patients treated with ARI0002h, which makes comparisons between MRD status and PFS or OS unfeasible. Despite that, the MRD negativity rates in the BM were higher compared to the CR rates determined by the classic IMWG criteria[14] that include serum M protein and free light chain evaluation. Therefore, the use of NGF or other high sensitivity techniques to analyze MRD could detect responders faster as reported elsewhere[40,41]. In addition, MRD positivity was observed in some patients prior to relapse.

One limitation of the study is that the follow-up of cohort 2 is relatively short. Furthermore, we were not able to perform genomic studies on the samples, although mechanisms of CART resistance mediated by genomic alterations are infrequent, unlike for T-cell enhancers[42].

In summary, ARI0002h preliminary outcomes were confirmed. An increase in exhaustion markers in the BM was observed and correlated with poor outcomes, together with low HLA-DR marker, high tumor burden and sBCMA.

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Authorship Contributions

AOC, MER, AZ, SN, LR, JD, MJ, AUI, BP, MP and CFdL were involved in the conception and design of this study. MER, AZ, SI, ALD, MJ, BP and MP provided laboratory data. AOC, VGC, VC, NLM, PRO, JLR, JSP, EO, EG, SV and MSG provided clinical data. AOC, MER, AZ, SI, ALD, DFM, NMC, LGRL, LLC, LP, BMA, LR, JC, NT, MLP and ASS contributed to data collection. AOC, MER, AZ, SI, ALD, SV, VOM, FP, JML, JMM, MVM, AUI, BP, MP and CFdL collaborated in data analysis and interpretation. AOC, MER, AMB and CFdL were involved in the writing of the manuscript. All authors reviewed the final manuscript and approved its last version to be published.

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Tables

Table 1. Main features of patients treated with ARI0002h.

Features	ARI0002 treated patients (n=60)
Age (years); median (range)	58 (36-74)
Sex (F/M)	26/34
Heavy chain isotype IgG/IgA/IgD/Only light chain (%)	52/27/3/18
Light chain isotype kappa/lambda (%)	55/45
ISS stage at baseline I/II/III (%)	49/23/28
Plasmacytomas (%)	50
Extramedullary location	18
Serum M protein (g/L); mean (range)	10.5 (0-90)
Bone marrow plasma cells (%); median (IQR)	13 (2-31)
High-risk cytogenetics (%)*	28
Number of previous lines; median (range)	3 (2-10)
Prior **autologous** stem cell transplantation (%)	90
Prior **allogeneic** stem cell transplantation (%)	8
Prior drug exposure (%) / **refractoriness** (%)	
Bortezomib	100/52
Lenalidomide	100/78
Anti-CD38 monoclonal antibody	100/93
Carfilzomib	57/45
Pomalidomide	52/48
Bridging therapy (%)	48
Overall response in the first 100 days (%)	95
≥ Complete response (stringent CR)	44(40)
Very good partial response	33
Partial response	18
Refractory	2
Death prior to evaluation	3
Measurable residual disease negative; n negative/n evaluable (%) in total; % of 10^{-6}, 10^{-5}, 10^{-4}	
Day 28	40/41 (98%)^; 47.5, 42.5, 10
Day 100	49/51 (96%)^; 55.1, 34.7, 10.2
Cytokine release syndrome; n, (%)	54 (90)
Grade 1-2	51 (85)
Grade ≥ 3	3 (5)
Neurotoxicity; n, (%)	
Immune-effector cell associated neurotoxicity syndrome	2 (3)
Late neurologic events	0

* del(17p), t(4;14), t(14;16); ^Three of the unavailable samples were due to: one patient was primary refractory and 2 patients died before first evaluation.

Figures and figure Legends

Figure 1. Sample assessment workflow. Summary of biomarker analysis performed on samples according to its order of appearance: (1) features at inclusion, (2) apheresis, manufacturing and final product analysis, (3) follow-up findings and (4) evaluation of response.

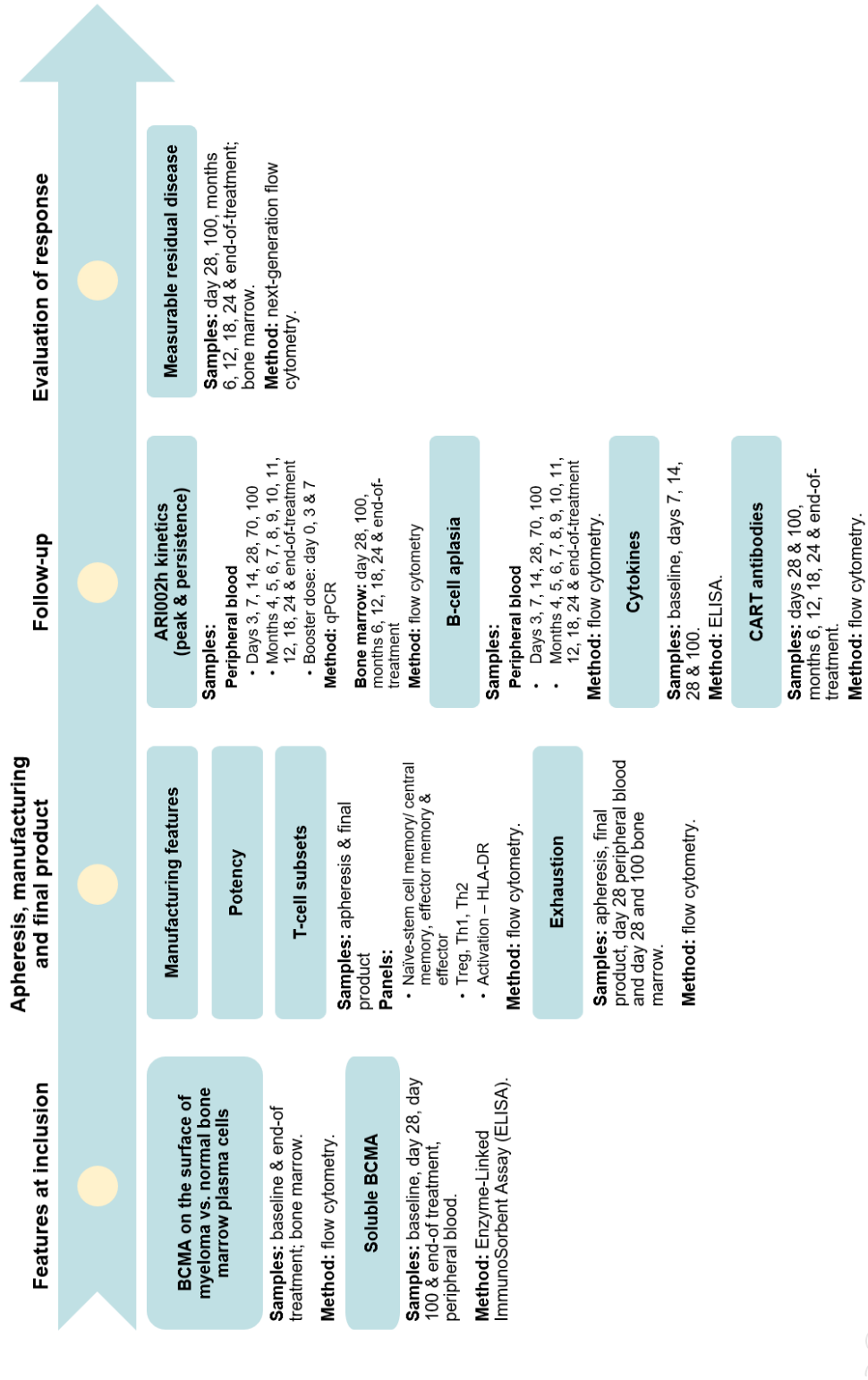


Figure 2. ARI0002h clinical outcomes (n=60). (A) Consort diagram of CARTBCMA-HCB-01 at cutoff date 17th March 2023. **(B)** Progression-free and **(C)** overall survival of the 60 patients treated with ARI0002h with a median follow-up of 23.1 months (95%CI 9.2-37.1).

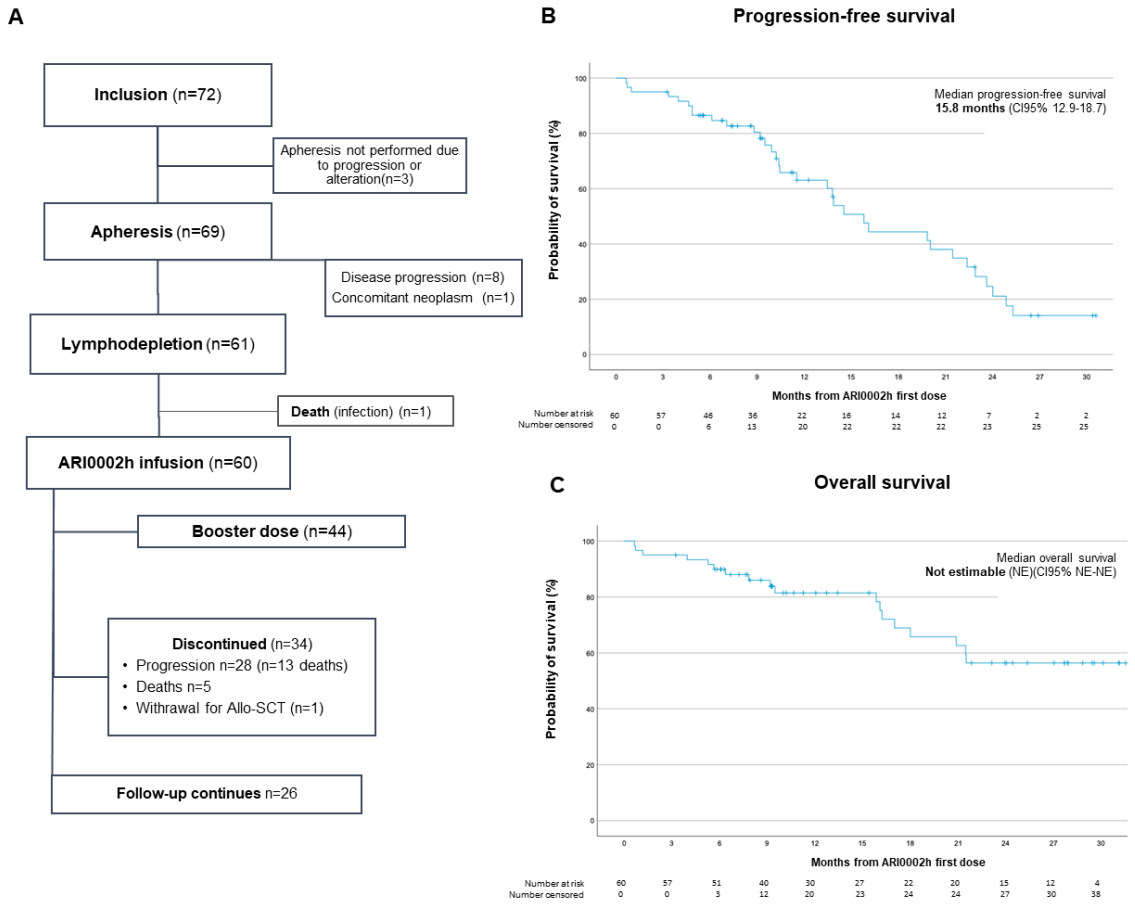


Figure 3. BCMA behavior in ARI0002h treated patients. (A) BCMA molecules/cell on myeloma and normal plasma cells at baseline and relapse in all patients (left) and only in paired samples (right). **(B)** Soluble BCMA (sBCMA) levels at different timepoints. A decrease is observed in all responding patients, with an increase at relapse. The only patient who was refractory developedan increase of sBCMA levels from baseline to day 28. **(C)** The density of BCMA molecules on myeloma plasma cells was not different in complete responders vs. patients who achieved less than a complete response. **(D)** Baseline sBCMA was lower in complete responders vs. less than a complete response on days 28 and 100, and in best response achieved, although differences were not statistically significant. sBCMA levels on day 28 were lower in complete responders at all three timepoints. When analyzing sBCMA at baseline and day 28, we found a correlation with the response achieved (CR: complete response; VGPR: very good partial response; PR: partial response; PD: progressive disease). **(E)** sBCMA levels in patients according to severity of cytokine release syndrome.

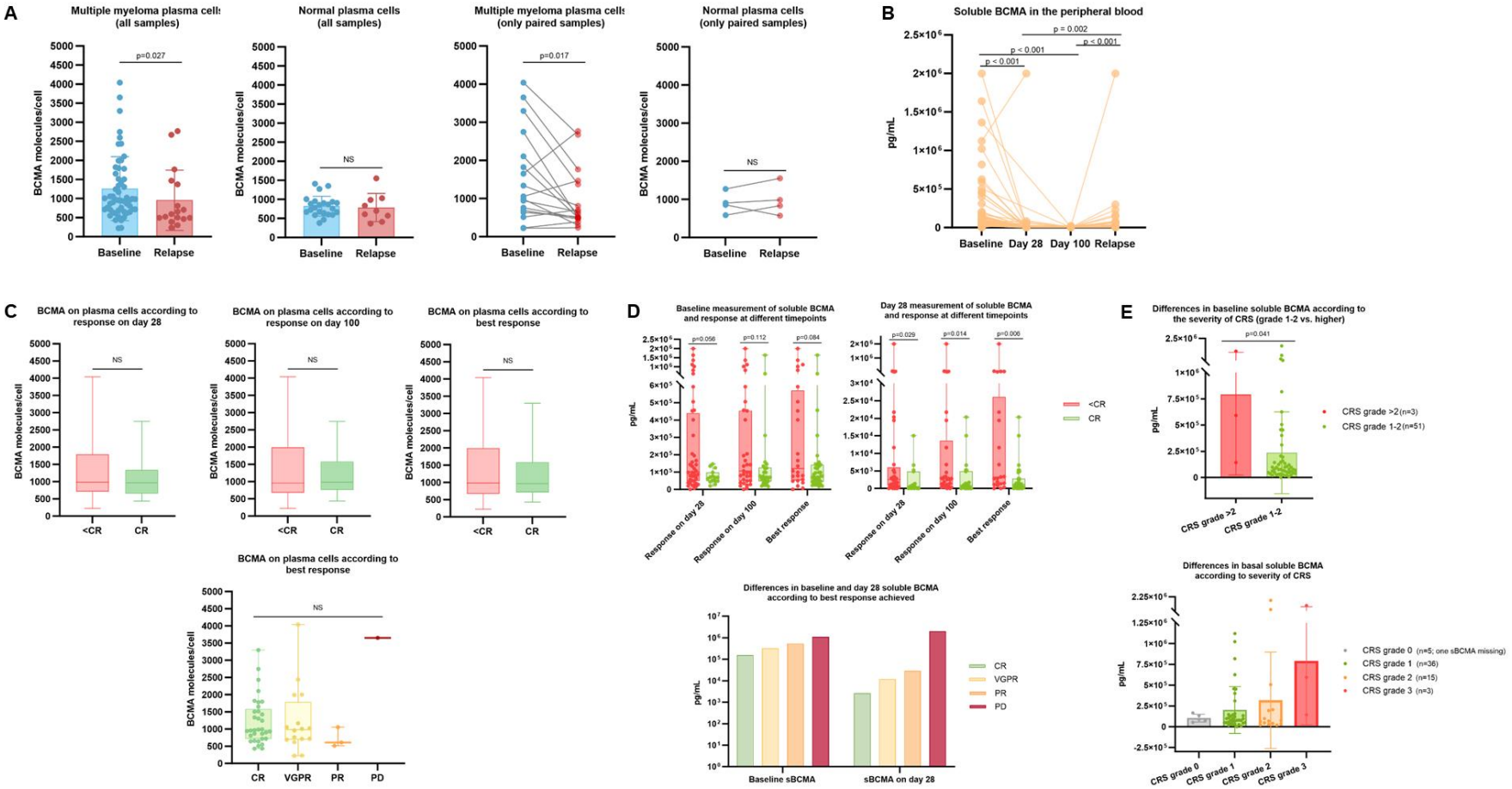
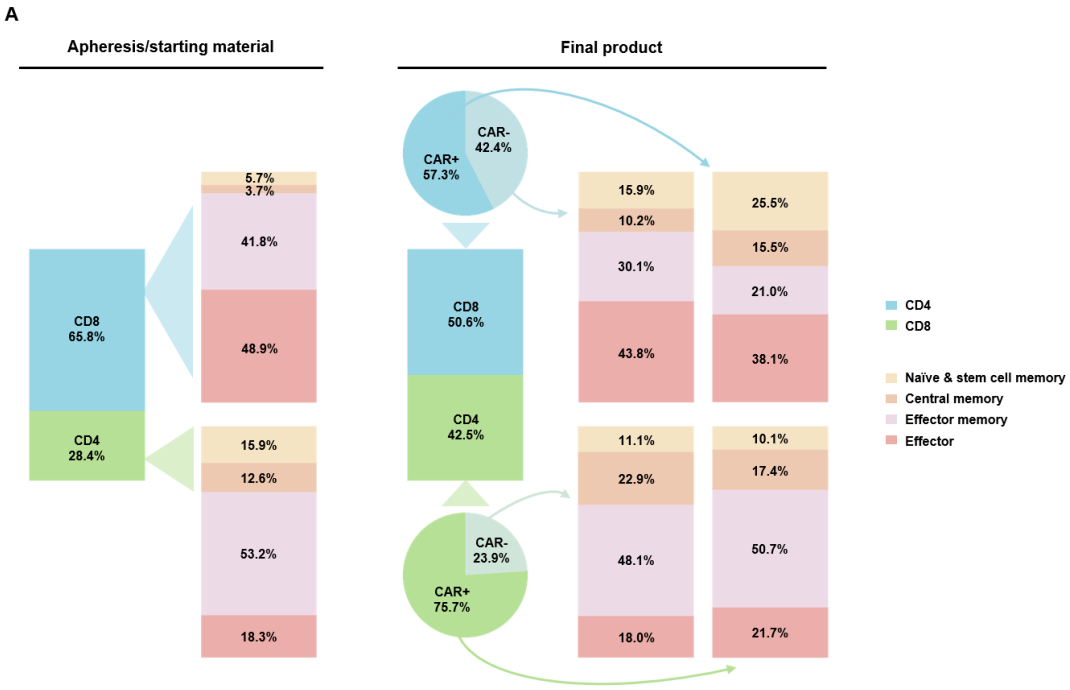
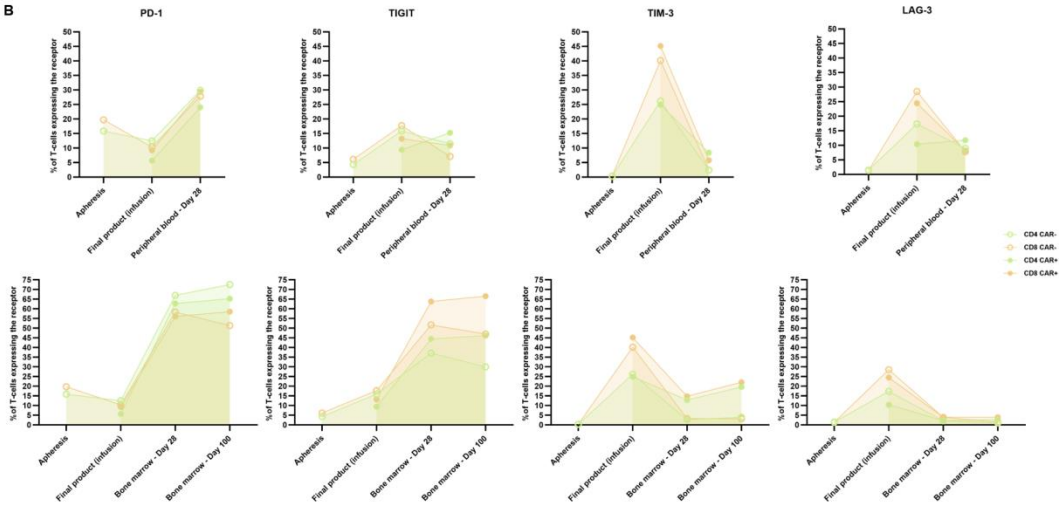

A
Multiple myeloma plasma cells (all samples)
p=0.027
BCMA molecules/cell
Baseline
Relapse
Normal plasma cells (all samples)
NS
Multiple myeloma plasma cells (only paired samples)
p=0.017
Normal plasma cells (only paired samples)
NS
B
Soluble BCMA in the peripheral blood
p = 0.002
p < 0.001
p < 0.001
p < 0.001
pg/mL
Baseline
Day 28
Day 100
Relapse
C
BCMA on plasma cells according to response on day 28
NS
<CR
CR
BCMA on plasma cells according to response on day 100
BCMA on plasma cells according to best response
BCMA on plasma cells according to best response
CR
VGPR
PR
PD
D
Baseline measurement of soluble BCMA and response at different timepoints
p=0.056
p=0.112
p=0.084
Response on day 28
Response on day 100
Best response
Day 28 measurement of soluble BCMA and response at different timepoints
p=0.029
p=0.014
p=0.006
<CR
CR
Differences in baseline and day 28 soluble BCMA according to best response achieved
Baseline sBCMA
sBCMA on day 28
CR
VGPR
PR
PD
E
Differences in baseline soluble BCMA according to the severity of CRS (grade 1-2 vs. higher)
p=0.041
CRS grade >2 (n=3)
CRS grade 1-2 (n=51)
Differences in basal soluble BCMA according to severity of CRS
CRS grade 0 (n=5; one sBCMA missing)
CRS grade 1 (n=36)
CRS grade 2 (n=15)
CRS grade 3 (n=3)

Figure 4. T-cell subpopulations in the apheresis, final product and in the peripheral blood and bone marrow at different timepoints after infusion of ARI0002h. (A) Changes in T-cell subsets (naïve, central memory, effector memory and effector cells in CD4, CD8, CAR+ and CAR-) between the apheresis and final product. Gating was performed on CD3+ cells, CD8-CD4- cells and CD8+CD4+ cells were not included in the graph. **(B)** Changes in exhaustion markers (PD-1, TIGIT, TIM-3, LAG-3) on CD8 and CD4 CAR+ and CAR- cells between the apheresis, final product, and peripheral blood on day 28 (upper graphs), and bone marrow on days 28 and 100 (lower graphs). **(C)** Differences in TIGIT expression between patients who did or did not achieve a complete response on days 28, 100 and as best response.


A
Apheresis/starting material
Final product
CD8
65.8%
CD4
28.4%
5.7%
3.7%
41.8%
48.9%
15.9%
12.6%
53.2%
18.3%
CAR+
57.3%
CAR-
42.4%
CD8
50.6%
CD4
42.5%
CAR-
23.9%
CAR+
75.7%
15.9%
10.2%
30.1%
43.8%
25.5%
15.5%
21.0%
38.1%
11.1%
22.9%
48.1%
18.0%
10.1%
17.4%
50.7%
21.7%
CD4
CD8
Naïve & stem cell memory
Central memory
Effector memory
Effector


B
PD-1
TIGIT
TIM-3
LAG-3
% of T-cells expressing the receptor
Apheresis
Final product (infusion)
Peripheral blood - Day 28
Bone marrow - Day 28
Bone marrow - Day 100
CD4 CAR-
CD8 CAR-
CD4 CAR+
CD8 CAR+

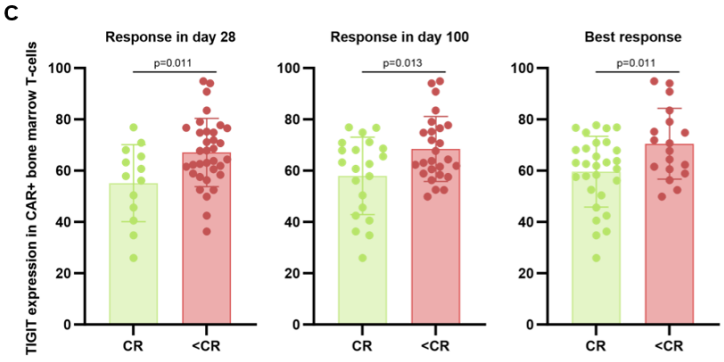
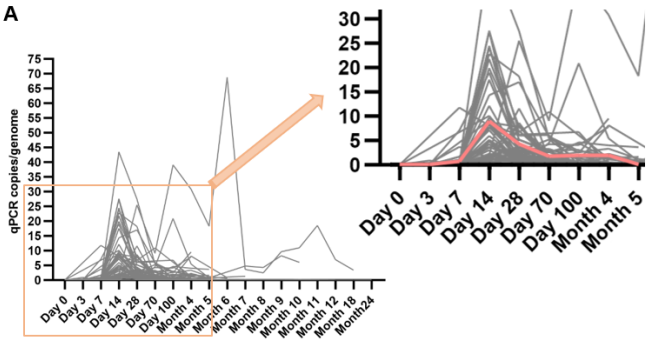
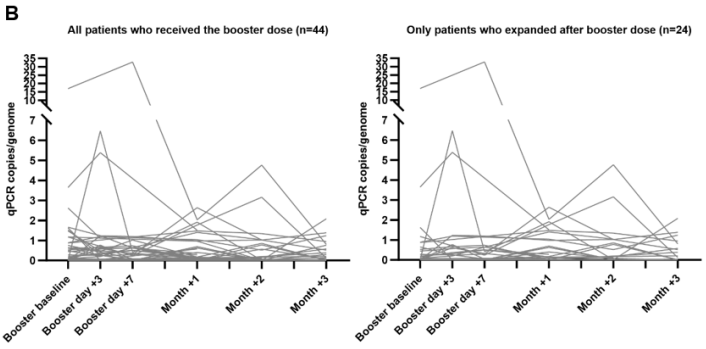
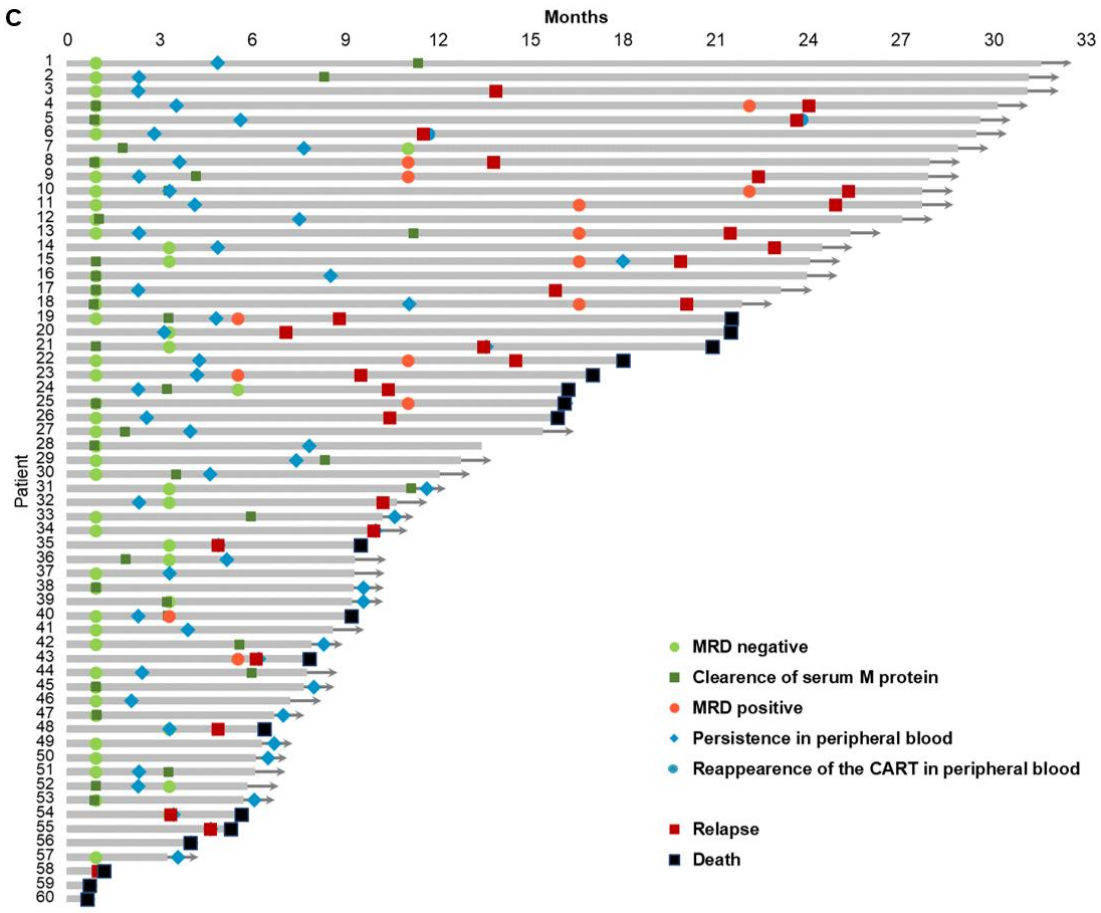

C
Response in day 28
Response in day 100
Best response
p=0.011
p=0.013
p=0.011
TIGIT expression in CAR+ bone marrow T-cells
CR
<CR

Figure 5. Kinetics of ARI0002h and B cells. (A) ARI0002h kinetics of individual subjects, from infusion to booster dose administration (measurements after booster dose of each individual patient are not depicted in this graph). Most patients present the peak of expansion on day 14. The red line represents the mean levels of ARI0002h. **(B)** ARI0002h kinetics after booster dose in all reinfused patients (n=44) and only in those in which ARI0002h expanded (n=24). **(C)** Swimmer plot showing the timing of MRD negativity, M protein clearence, MRD positivity, relapse and death for each patient. Persistence of the CART and reappearance (if occurred) are also depicted. Progression-free survival in patients with detectable CART in the end-of-treatment sample was (in months): 1 (refractory patient), 3.4, 4.6, 4.9, 6.1, 9.9, 11.5, 13.5, 23.6.






C
Months
0
3
6
9
12
15
18
21
24
27
30
33
Patient
MRD negative
Clearence of serum M protein
MRD positive
Persistence in peripheral blood
Reappearence of the CART in peripheral blood
Relapse
Death

832

Supplemental methods

Study design

CARTBCMA-HCB-01 is a pilot, single-arm, open-label study held in 7 Spanish centers. Patients aged 18-75 years old with RRMM were eligible if they had measurable disease, as assessed by M-protein (serum>10g/L or urine>200mg/24h) or serum free light chain levels (>100mg/L), received ≥2 prior regimens, including a proteasome inhibitor, an immunomodulatory drug and an anti-CD38 antibody, and were refractory to the last line of treatment. Prior BCMA-directed treatment was an exclusion criterion. ARI0002h was lentivirally transduced on autologous T-cells. Bridging therapy was allowed after apheresis according to investigator discretion. Lymphodepletion (LD) included cyclophosphamide (900mg/m^2 total dose) and fludarabine (90mg/m^2 total dose). The target dose (3x10^6/kg CAR+cells) was administered in a fractionated manner (10%/30%/60%), with at least 24h between infusions.

Samples

Patients' samples were obtained and processed at the following timepoints:

- **Apheresis**
- **Final product (FP)**
- **Peripheral blood:** baseline, prior lo LD, in days 0, 3, 7, 14, 28, 70, 100 and months 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, and 24.
- **Bone marrow:** baseline, days 28 and 100, and months 6, 12, 18, and 24.

The following table shows an overview of the analysis performed in each sample according to the compartment:

Apheresis		**Final product**	
Analysis	**Technique**	**Analysis**	**Technique**
T-cell subsets	Flow cytometry	T-cell subsets	Flow cytometry
-	-	Potency	Flow cytometry

Peripheral blood		**Bone marrow**	
Analysis	**Technique**	**Analysis**	**Technique**
Kinetics of ARI0002h	Quantitative polymerase chain reaction (qPCR)	Kinetics ARI0002h	Flow cytometry
-	-	Measurable residual disease detection	Next generation flow cytometry
Soluble BCMA	Enzyme-linked immunosorbent assay (ELISA)	BCMA quantification on the surface of plasma cells	Flow cytometry
T-cell subsets	Flow cytometry	T-cell subsets	Flow cytometry

B-cell kinetics	Flow cytometry	-	-
Anti-CAR antibodies	Flow cytometry	-	-
Cytokine measurement	Enzyme-linked immunosorbent assay (ELISA)	-	-

A FACSCanto (BD Biosciences, New Jersey, USA) flow cytometer and Infinicyt 2.0 software (Cytognos S.L., Salamanca, Spain) were used to perform flow cytometry analysis in BM samples. An Attune Next Flow flow cytometer (Thermofisher Scientific, Massachusetts, USA), the Attune Cytometric Software and FlowJo (V10.7.1) were used to analyze PB samples.

Specific methods used for each analysis are specified in the following paragraphs:

Potency

ARI0002h transduced T cells obtained after the manufacturing process (final product, FP) are challenged with the u266 multiple myeloma cell line (cells previously transduced with GFP) at a target/effector (tumor cell/T-cell) ratio of 2.5:1 in RPMI medium with 10% FBS, 1% glutamine and 1% penicillin-streptomycin. After 24 hours of co-culture, the quantity of live tumor cells was determined by flow cytometry and the percentage of T-cell mediated killing is calculated with respect to tumor cells alone, setup in the same assay, at the same quantity but without T-cell co-culture.

BCMA evaluation: soluble BCMA and plasma cell surface BCMA

After serum sample thawing and dilution (1:200), an enzyme-linked immunosorbent assay (ELISA) from (R&D Systems, Minneapolis, USA) was conducted according to the manufacturer's protocol. ELISA plates were analyzed using an EPOCH microplate spectrophotometer plate reader (BioTek Industries, Winooski, Vermont, USA) set to 450 nm. Data were processed with the BioTek Gen5 Data Analysis Software. The detection range was 2.62–20.000 pg/mL.

Plasma cell surface BCMA was measured by flow cytometry at baseline and in MRD positive disease. A PE anti-human CD269 (BCMA) antibody (Cat. number: 357504; Biolegend, San Diego, USA) was used for staining and the BD QuantiBRITE™ Beads kit (BD Biosciences, New Jersey, USA) enabled molecule quantification (molecules/cell). In samples with positive measurable residual disease, BCMA molecules were stained by replacing CD56-PE with BCMA-PE in the EuroFlow protocol tube 1 (see above). For baseline measurements, in patients receiving bridging therapy, a second baseline sample was obtained after treatment to do the measurements.

T-cell subsets

Antibody panels applied to analyze T-cell subpopulations are shown in the following table:

Panel	Antigen	Label	Clone	Catalog number	Company
Quantification	CAR-T	BV421			
	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD25	FITC	2A3	345796	BD
	CD127	PE	A019D5	351304	Biolegend
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	TCRgd	PECy7	11F2	655410	BD
	CD19	PECy7	J3-119	IM3628	BC
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Subsets	CAR-T	BV421	M1310G	410704	Biolegend
	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD62L	FITC	SK11	347443	BD
	CD27	PE	L128	340425	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD45RA	PECy7	HI100	304126	Biolegend
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Exhaustion	CAR-T	BV421	M1310G	410704	Biolegend
	CD45	OC515	GA90	CYT-45OC	Cytognos
	TIM3	FITC	F38-2E2	345022	Biolegend
	TIGIT	PE	741182	FAB7898P	R&D
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	PD-1	PECy7	PD1.3	A78885	BC
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Cytotoxicity	CAR-T	BV421	M1310G	410704	Biolegend
	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD57	FITC	HNK-1	333169	BD
	cyGrzmB	PE	GB11	561142	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD56	PECy7	NCAM16.2	335826	BD
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Activation/ Inhibition	CAR-T	BV421	M1310G	410704	BD
	CD45	OC515	GA90	CYT-45OC	Cytognos
	HLADR	FITC	L243	347400	BD
	LAG3	PE	T47-530	565616	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD39	PECy7	A1	328212	Biolegend
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD

Kinetics of ARI0002h

In peripheral blood samples, genomic DNA was acquired with the MagNA Pure Compact Nucleic Acid Isolation Kit I on the MagNA Pure Compact System. Primers were designed against the vector transgene WPRE sequence (WPRE_F: 5'gtcctttccatggctgctc 3'; WPRE_R: 5'ccgaagggacgtagcaga 3') and the GATA2 gene – control (GATA2_F: 5'tggcgcacaactacatggaa 3'; GATA2_R: 5'cgagtcgaggtgattgaagaaga 3'). The number of transgene copies/cell was determined by quantitative real-time PCR using Light Cycler® 480 SYBRGreen® I Master (Roche, Basel, Switzerland).

In bone marrow samples, five 8-color combinations of monoclonal antibodies allowed T-cell subset determination (see T-cell subset section), including a panel with a Biotin-conjugated goat anti-mouse IgG F(ab')2 primary antibody (Jackson ImmunoResearch, Cambridge, UK) plus a BV421-Streptavidin secondary antibody, both used for CAR staining prior to flow cytometry analysis using a FACSCanto cytometer and the Infinicyt 2.0 software.

ARI0002h persistence was defined as the time between infusion and the first sample in which ARI0002h was no longer detected (qPCR result below 0.1 copies/genome in PB samples, and a negative result by flow cytometry in BM samples). For PB samples, at least two consecutive samples had to be reported as negative to confirm loss of persistence.

B-cell kinetics

The antibody panels applied to determine T-cell subsets include a *Quantification* panel, which contains a PE-Cy7 anti-CD19 antibody (Beckman Coulter, California, USA) that allows B-cell detection in the peripheral blood (see above).

Cytokine measurement

For cytokine analysis, serum levels of 14 cytokines were measured at 5 different timepoints (screening, day 7, 14, 28 and 100 after CART infusion). An enzyme-linked immunosorbent assay (ELISA) was performed using the commercially available Simple Plex assay from ProteinSimple (Biotechne, R&D Systems, Minneapolis, USA) according to manufacturer's protocol. The detection range for used immunoassays was as follows: IFN-g 0.17 – 4000 pg/mL, IL-1b 0.4 - 1,530 pg/mL, IL-2 0.54 - 2,050 pg/mL, IL-15 0.51 - 1,950 pg/mL, CCL3 1.05 - 4,000 pg/mL, granzyme B 1.31 - 5,000 pg/mL, IL-4 0.52 - 2,020 pg/mL, IL-6 0.28 - 2,652 pg/mL, IL-12 0.62 - 5,890 pg/mL, IL-10 0.58 - 2,212 pg/mL, IL-17a 1.05 - 10,000 pg/mL, CXCL10 0.6 - 920 pg/mL, IL-8 0.19 - 1,804 pg/mL and TNFa 0.3 - 1,160 pg/mL. The results were analyzed using the automated benchtop ELISA platform ELLA™ (Biotechne, R&D Systems, Minneapolis, USA). No levels of IL-4 were detected, therefore no information is reported regarding this cytokine.

Anti-CAR antibodies

To detect the presence of anti-CAR antibodies, a HEK-293T cell line was transduced with the ARI0002h lentivirus using a multiplicity of infection of 2 for 48 hours at 37ºC in DMEM-10% FBS supplemented with polybrene (Cat. number: TR-1003-G; Merck Millipore, Massachusetts, USA). ARI0002h expression was confirmed using a recombinant BCMA protein fused with a human Fc (Cat. number: ALX-522-026-C050; Enzo Life Sciences, New York, USA) and a BV421-anti-human IgG antibody (Cat. number: 409318; Biolegend, San Diego, USA). ARI0002h expressing HEK-293T cells were incubated with patient's serum and a FITC-anti-human IgG antibody (Cat. number: H10101C; Biolegend, San Diego, USA) was used to determine the presence of anti-CAR antibodies. A threshold of 20% was considered positive.

Measurable residual disease (MRD) by next generation flow (NGF) flow cytometry

Measurable residual disease (MRD) was assessed by 2-tube 8-colour next generation flow cytometry in accordance with the EuroFlow platform (see full protocol on the EuroFlow website: app.euroflow.org/downloads/public - protocol 1·3):

- **Tube 1:** CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, CD117-APC, and CD81-APCH7.
- **Tube 2:** as tube 1, but CyIgKappa-APC and CyIgLambda-APCH7 were included instead of CD117-APC and CD81-APCH7, respectively.

Limit of detection: 10^{-6}. Limit of quantification: $<5 \times 10^{-6}$.

To reach a sensitivity of 10^{-5}, 2 million cells were required. To reach a sensitivity of 10^{-6}, 10 million cells were required. Besides that, to consider a sample as evaluable, the presence of erythroblasts, B-precursor cells and mastocytes was also a requirement, since these populations are only found in the bone marrow. An absence of these populations suggests that the sample is hemodiluted. Anyway, the presence of clonal plasma cells, even in a hemodiluted sample, was given as positive MRD, since the expected number of clonal plasma cells would be higher if the sample was correct.

Clinical results

Duration of response (DOR) was defined as time between first response and progression (patients who were evaluable for response but died without progression were censored at the last follow-up). Progression-free survival (PFS) was defined as time between infusion and progression or death and overall survival (OS) was defined as time between infusion and death.

Statistical analysis

A two-sided p-value of <0.05 was considered statistically significant. Statistical analysis was performed with SAS System (v9.4), GraphPad Prism (v10.0.2) and IBM SPSS Statistics (v29.0).

Summary of the study protocol

Sponsor's Name:

Institut D'Investigacions Biomèdiques Agustí Pi i Sunyer (IDIBAPS).
C/Rosselló 149-153 08036 Barcelona, Spain.
Telephone number: 93 2275400.

Final product name:

ARI0002h cells. CARTBCMA_J22.9-h:CD8TM:4-1BB:CD3.

Investigational Medicinal Product (IMP)

ARI0002h cells. CARTBCMA_J22.9-h:CD8TM:4-1BB:CD3.
Adult differentiated autologous T-cells from peripheral blood, expanded and transduced with a lentivirus to express a chimeric antigen receptor with anti-BCMA (TNFRSF17) specificity conjugated to the 4-1BB co-stimulatory region and signal-transduction CD3z that has been humanized.

Study title:

Pilot study of the infusion of differentiates autologous T-cells from peripheral blood, expanded and transduced with a lentivirus to express a chimeric antigen receptor with anti-BCMA (TNFRSF17) specificity humanized conjugated with the co-stimulatory region 4-1BB and signal-transduction CD3z (ARI0002h) in patients with relapsed/refractory multiple myeloma with at least two prior line including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.

Investigator's team:

1. **Chief investigator (coordinator):**
   Carlos Fernández de Larrea
2. **Principal investigators at participating sites (Cohort 1):**
   - Carlos Fernández de Larrea - Hospital Clínic de Barcelona
   - Paula Rodríguez Otero - Clínica Universitaria de Navarra
   - Juan Luis Reguera Ortega - Hospital Virgen de Rocío
   - Mª Victoria Mateos - Instituto de investigación Biomédica de Salamanca
   - José Mª Moraleda- Hospital U. Virgen de la Arrixaca
3. **Principal investigators at participating sites (added to Cohort 2):**
   - Joaquín Martínez López - Hospital Universitario 12 de Octubre
   - Marta Sonia González Pérez – Complejo Hospitalario Universitario de Santiago

Study population:
Patients with relapsed/refractory multiple myeloma who have received treatment with at least 2 prior lines including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.

Phase and clinical trial design:
First-in-human, pilot, open, non-randomised, single-arm, prospective, national, multicentre, clinical trial.

Primary Objective:
To assess the safety and efficacy of CARTBCMA ARI0002h in patients with relapsed/refractory multiple myeloma who have received treatment with at least two prior lines including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.

Secondary objectives:
- To evaluate the effectiveness of ARI0002h
- To assess the duration of the response after the administration of ARI0002h
- To evaluate the overall survival after the administration of ARI0002h
- To evaluate the persistence of ARI0002h cells in peripheral blood after administration
- To assess the effect of ARI0002h treatment on the quality of life of patients
- To evaluate the adverse events occurring at 3 months and at one year

Primary Endpoints:
- **Efficacy primary endpoint:**

Overall response rate (ORR) in the first 3 months of the first infusion (defined as at least achieve partial response according to the criteria of the International Myeloma Working Group).
- **Safety primary endpoint:**

Rate of patients who develop a cytokine release syndrome and/or neurological toxicity in the first 30 days after the administration of ARI0002h, according to the criteria and gradation defined in the international consensus (Lee, Santomasso et al., 2019).*

Secondary Endpoints:
- Duration of the response (see criteria for second dose of ARI0002h)

- Response rate over the first year
- Complete response rate (CR) at 3 and 6 months after the first infusion.
- Overall response rate (ORR) at 6 months after the first infusion.
- Time to complete response.
- Time to better response.
- Negative measurable residual disease (MRD) rate in bone marrow at 3 and 6 months.
- Response rate of extramedullary disease by PET-TC at 3 months.
- Progression free survival (PFS), defined as the time elapsed between the administration of ARI0002h and the progression of the disease or death. Patients who are alive and in complete remission will be censored at the time of the last follow-up.
- Progression free survival at 12 months after the first infusion, defined as the time elapsed between the administration of ARI0002h and the progression of the disease or death. Patients who are alive and in complete remission at 12 months will be censored at the time of the last follow-up.
- Overall survival (OS) defined as the time elapsed between the infusion of ARI0002h and the death of the patient from any cause. Live patients will be censored at the time of the last follow-up.
- Presence of infusion reactions.
- Tumour lysis syndrome at any time after the administration of the treatment.
- Cytokine release syndrome, according to the criteria and grades defined in the international consensus (Lee, Santomasso et al., 2019).
- Neurological toxicity.
- Presence of prolonged cytopenia, defined as the reduction of neutrophil or platelet peripheral blood counts, grade 3 or 4 for more than 4 weeks after infusion.
- Persistence of CART BCMA ARI0002 in peripheral blood, which will be determined by flow cytometry and quantitative PCR of the transgene with monthly periodicity in the first 6 months and subsequently quarterly until 2 years after infusion.
- Expression of BCMA at diagnosis and at relapse, evaluated by multiparameter flow cytometry in bone marrow aspirate samples.
- Levels of soluble BCMA in serum pre-treatment and during treatment and its correlation with the degree of response of multiple myeloma.
- Quality of life at baseline, monthly basis the first 6 months and quarterly until 2 years from infusion.

Inclusion Criteria (main):

1. Patients between the age of 18 and 75 years with diagnosis of multiple myeloma.
2. Disease measurable** by monoclonal component in serum and/or urine or by free light chains in serum according to the eligibility criteria for clinical trials of the

International Myeloma Working Group.

3. Previous two or more lines of treatment. Patients must have received at least a proteasome inhibitor (such as bortezomib or carfilzomib), an immunomodulatory drug (lenalidomide or pomalidomide) and an anti-CD38 monoclonal antibody (such as daratumumab).
4. Refractory to the last line of treatment.
5. ECOG functional status ranging from 0 to 2.
6. Life expectancy over 3 months.
7. Patients who, after being informed, give their consent by signing the Informed Consent document.

Exclusion Criteria (main):

1. Previous allogeneic transplant in the prior 6 months to inclusion or GVHD that requires active systemic immunosuppressive treatment.
2. Previous treatment with CAR T-cell therapy or BCMA directed therapy.
3. Absolute lymphocyte count $<0.1x10^9$/L.
4. Previous neoplasia, except if patients have been in complete remission >3 years, except for cutaneous carcinoma (non-melanoma).
5. Active infection that requires treatment.
6. Active infection by HIV, HBV or HCV.
7. Uncontrolled medical disease.
8. Severe organic condition that meets any of the following criteria: EF 3 times normal value (except Gilbert syndrome).
9. Previous diagnosis of symptomatic AL amyloidosis.
10. Pregnant or lactating women. Women of childbearing age should have a negative pregnancy test in the screening phase.
11. Women of childbearing age, including those whose last menstrual cycle was in the year prior to screening, who cannot or do not wish to use highly effective contraceptive methods from the beginning until the end of the study.
12. Men who cannot or do not wish to use highly effective contraceptive methods from the beginning to the end of the study.
13. Contraindication to receive conditioning chemotherapy.

Representativeness of Study Participants

Cancer type(s)/subtype(s)/stage(s)/ condition	Relapse/refractory multiple myeloma (RRMM)
Considerations related to:	
Sex	In the United States (US) throughout 2017, 17,490 men and 12,790 women were diagnosed of MM, with a male/female ratio of 1.5. From patients treated with CAR-T cells (ide-cel) in real life world data in the US reported in 2023, 57% were male.
Age	In a recent survey in two regions of Spain (1994-2016), patients with MM were diagnosed at a median age of 72 years. However, the median age of the patients receiving ide-cel in the US was 64 years.
Race/ethnicity	There is a marked racial disparity in the incidence of MM and asymptomatic monoclonal gammopathies, with a two to threefold increased risk in blacks compared with whites, after adjusting for socioeconomic and other risk factors, suggesting a genetic predisposition.
Geography	The estimated newly diagnosed MM cases in the US throughout 2017 is 30,280, representing 1.8% of all new cancer, with an incidence rate (per 100,000 inhabitants and age-standardized to the 2000 United States standard population) of 8 and 5.2 respectively. Similar or slightly lower age-standardized incidence rates can be found in European countries. Conversely, in Asian countries, the incidence is particularly low. The incidence rates of MM in Spain, adjusted to the European population were 3.54 cases by 100.000/year for men and 2.54 cases by 100.000/year for women in 2005.
Overall representativeness of this study	The age and sex distribution of our study is similar to the average distribution of MMRR treated with CAR-T cells in real life. We do not have information about race/ethnicity in our trial in Spain.

Table 1. Patients diagnosed with a neoplasia.

	Neoplasia	Time from ARI002h infusion to diagnosis (months)
1	Skin – Basal cell carcinoma	4.2
2	Skin – Squamous cell carcinoma	4.9
3	Skin – In situ melanoma	9.2
4	Breast carcinoma	11.3
5	Papillary thyroid microcarcinoma	20.5
6	Colon adenocarcinoma	3.9

One additional patient was diagnosed with lung adenocarcinoma after inclusion and apheresis, but prior to lymphodepletion, and was withdrawn of the study.

Table 2. Correlation between T-cell subsets in the apheresis with the proportion of CD8 central memory cells in the final product (FP).

T-cell subset in the apheresis	P value	Spearman ρ (ro)
CD8	0.154	-
CD4	0.121	-
Naïve CD8	0.943	-
Central memory CD8	0.098	-0.26
Effector memory CD8	0.053	-0.3
Effector CD8	**0.029**	**0.34**
Naïve CD4	**0.007**	**0.41**
Central memory CD4	**0.045**	**0.31**
Effector memory CD4	**0.023**	**-0.35**
Effector CD4	**0.082**	**-0.27**

Table 3. Cox regression analysis of the impact of the expression of exhaustion markers in progression-free (PFS) and overall survival (OS).

Exhaustion marker	Sample, day & cell	Survival	B	Exp(B) (95%CI)	p value
PD-1	Apheresis	PFS	0.060	1.061 (1.024-1.100)	0.0010
		OS	0.052	1.053 (1.005-1.103)	0.030
TIGIT	Apheresis	PFS	0.223	1.25 (1.087-1.437)	0.0018
		OS	0.200	1.221 (1.009-1.478)	0.040
TIGIT	Day 28 PB CD8 CAR+	PFS	0.092	1.097 (1.038-1.159)	0.00099
		OS	0.089	1.094 (1.016-1.177)	0.017
TIM-3	Day 28 PB CD8 CAR+	PFS	0.137	1.147 (1.047-1.257)	0.0033
		OS	0.169	1.184 (1.057-1.326)	0.0036
TIM-3	Day 28 PB CD4 CAR+	PFS	0.064	1.066 (1.022-1.112)	0.0028
		OS	0.075	1.078 (1.020-1.138)	0.0077

Figure 1. Design of the study: timeline of lymphodepletion, ARI0002h fractionated infusion and booster dose.

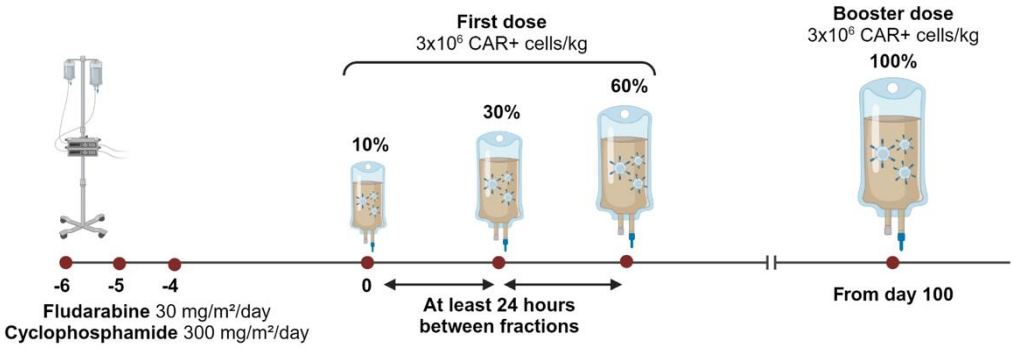


Figure 2. PD-1 and TIGIT expression in two patients ("A" and "B"), with available bone marrow samples across follow-up, including the end-of-treatment (EOT) sample. Of the 9 patients who relapsed with detectable CARTs in the peripheral blood, bone marrow EOT samples were available in these 2. Both patients had a high expression of both exhaustion markers, especially in CAR+ T-cells at all timepoints, including at relapse, which highlights the negative impact that exhaustion receptors may have in the efficacy of ARI0002h.

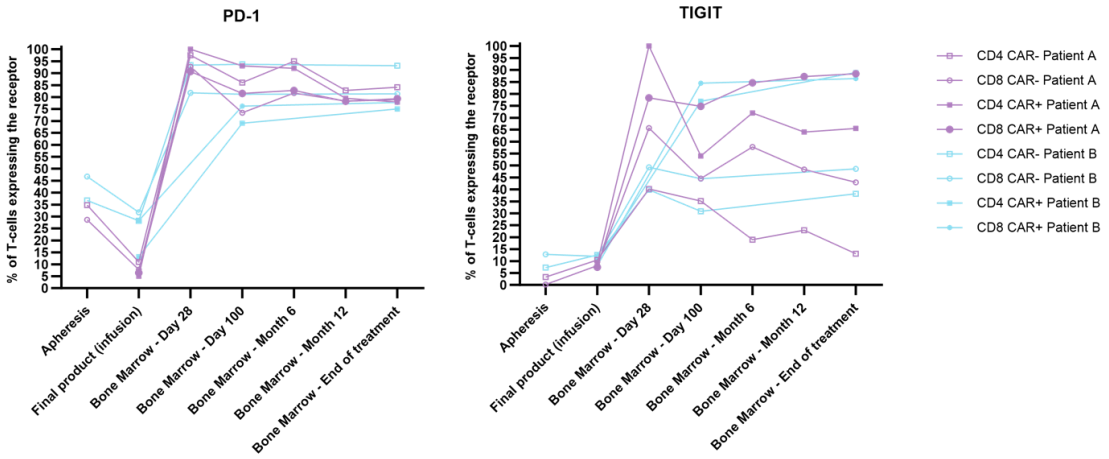
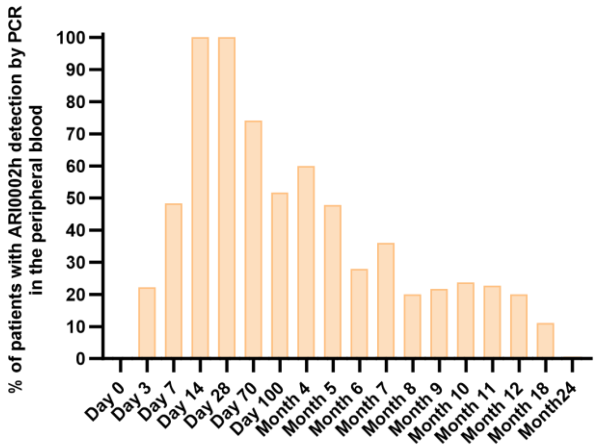


Figure 3. Persistence of ARI0002h in the peripheral blood at each timepoint. The table shows the number of positive / available samples at each timepoint.



	D3	D7	D14	D28	D70	D100	M4	M5	M6	M7	M8	M9	M10	M11	M12	M18	M24
Positive	6	14	27	29	20	15	15	11	7	9	5	5	5	5	4	1	0
Available samples	27	29	27	29	27	29	25	23	25	25	25	23	21	22	20	9	8
%	22,2	48,3	100,0	100,0	74,1	51,7	60,0	47,8	28,0	36,0	20,0	21,7	23,8	22,7	20,0	11,1	0,0

Figure 4. ARI0002h peak magnitude according to baseline tumor burden measured as proportion of bone marrow plasma cells and soluble BCMA. Baseline tumor burden was classified into low, medium, high and very high in the following manner: proportion of bone marrow plasma cells (low <10%, medium 11-20%, high 21-50%, very high > 50%) and levels of soluble BCMA (low <50.000pg/ml, medium 50.001-100.000pg/ml, high 100.001-500.000pg/ml, very high > 500.000pg/ml). No differences were observed either in terms of serum M protein (data not shown); 29 of 60 patients had less than 10 g/L and were included based on serum free-light chain. NS = non-significant.

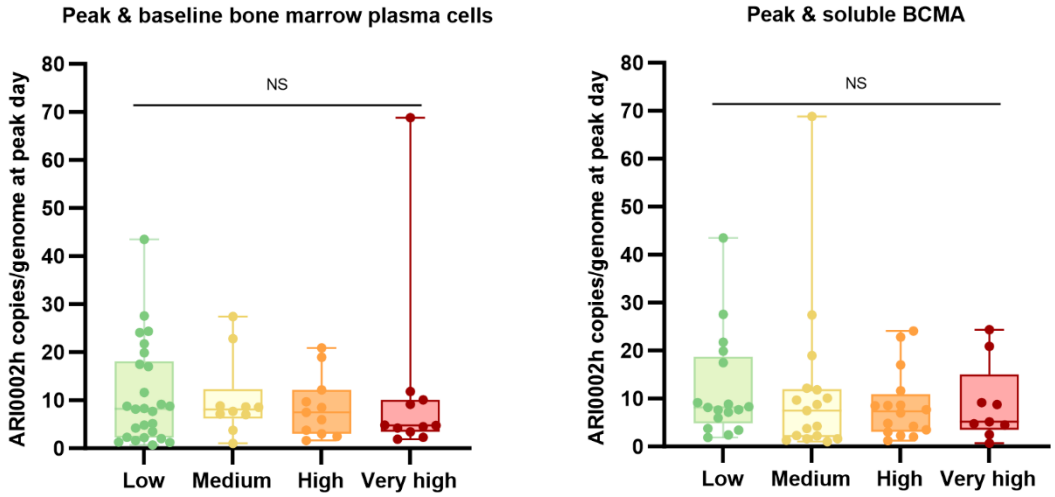


Figure 5. ARI0002h persistence according to baseline tumor burden measured as proportion of bone marrow plasma cells and soluble BCMA. Baseline tumor burden was classified into low, medium, high and very high in the following manner: proportion of bone marrow plasma cells (low <10%, medium 11-20%, high 21-50%, very high > 50%) and levels of soluble BCMA (low <50.000pg/ml, medium 50.001-100.000pg/ml, high 100.001-500.000pg/ml, very high > 500.000pg/ml). No differences were observed either in terms of serum M protein (data not shown). Low = green; medium = yellow; high = orange; very high = red. NE = not estimable.

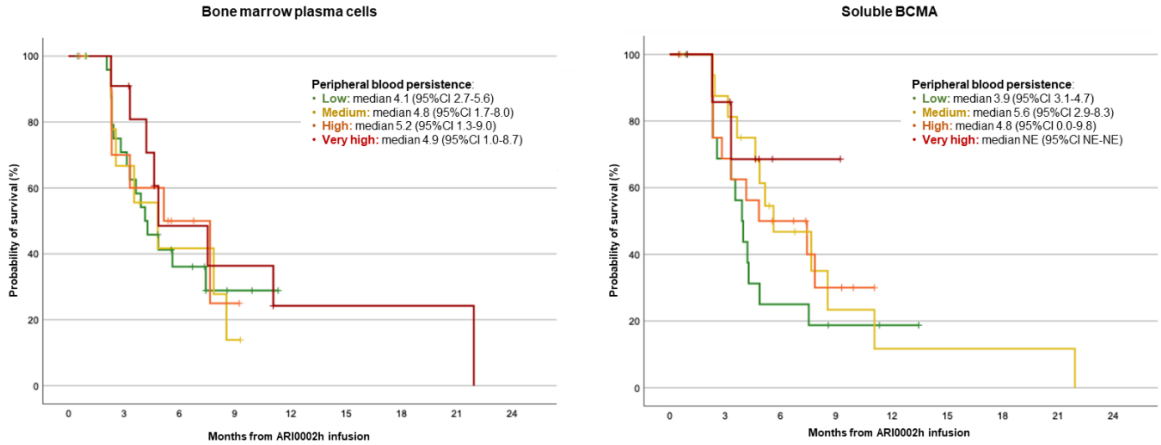
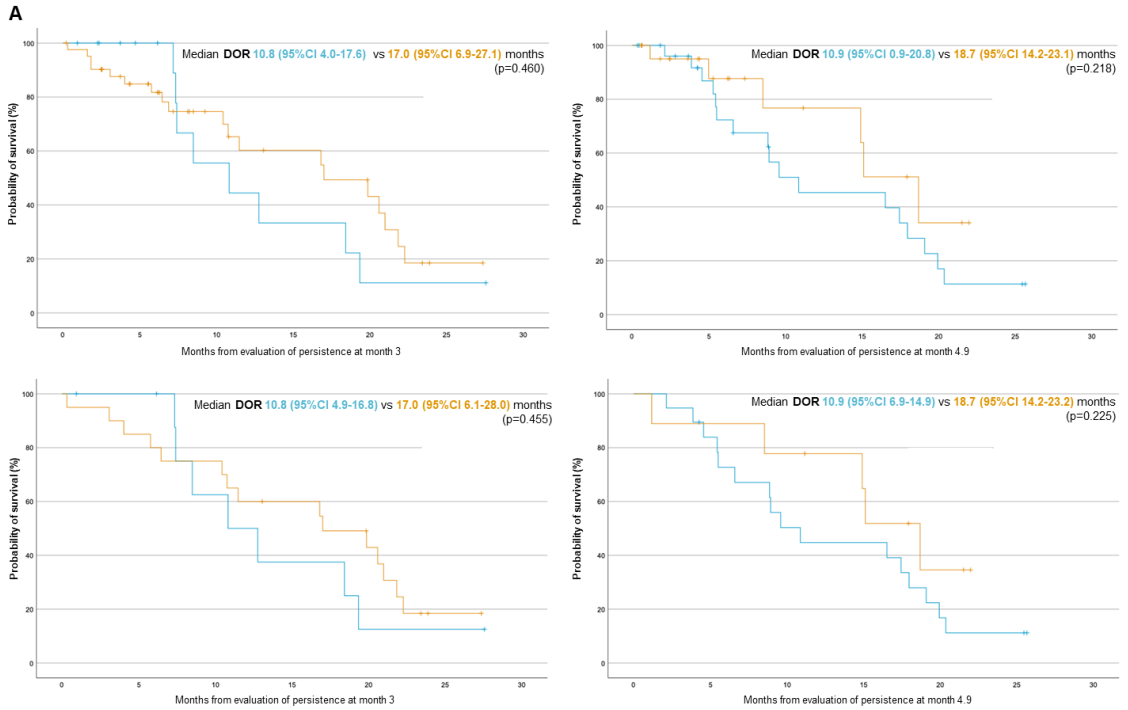
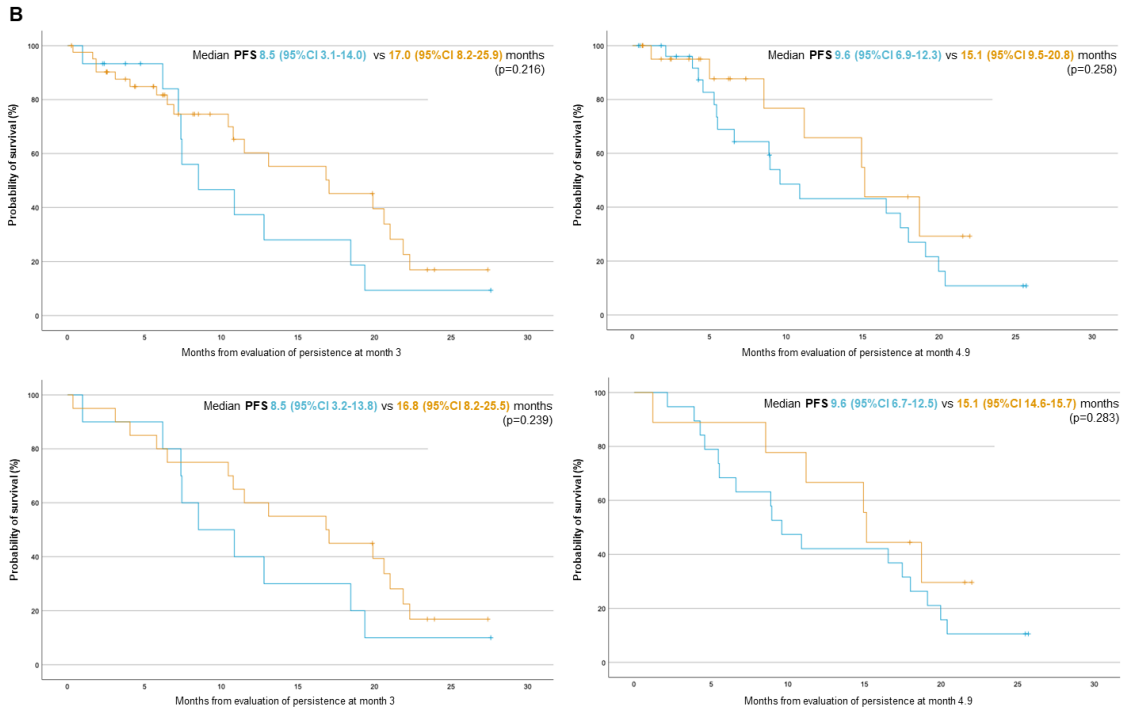


Figure 6. Impact of ARI0002h persistence in months 3 and 4.9 on **(A)** duration of response (DOR), **(B)** progression-free survival (PFS) and **(C)** overall survival (OS) in all patients (upper graphs) and in only cohort 1 (lower graphs). Patients with ARI0002h persistence (orange) vs. no persistence (blue).





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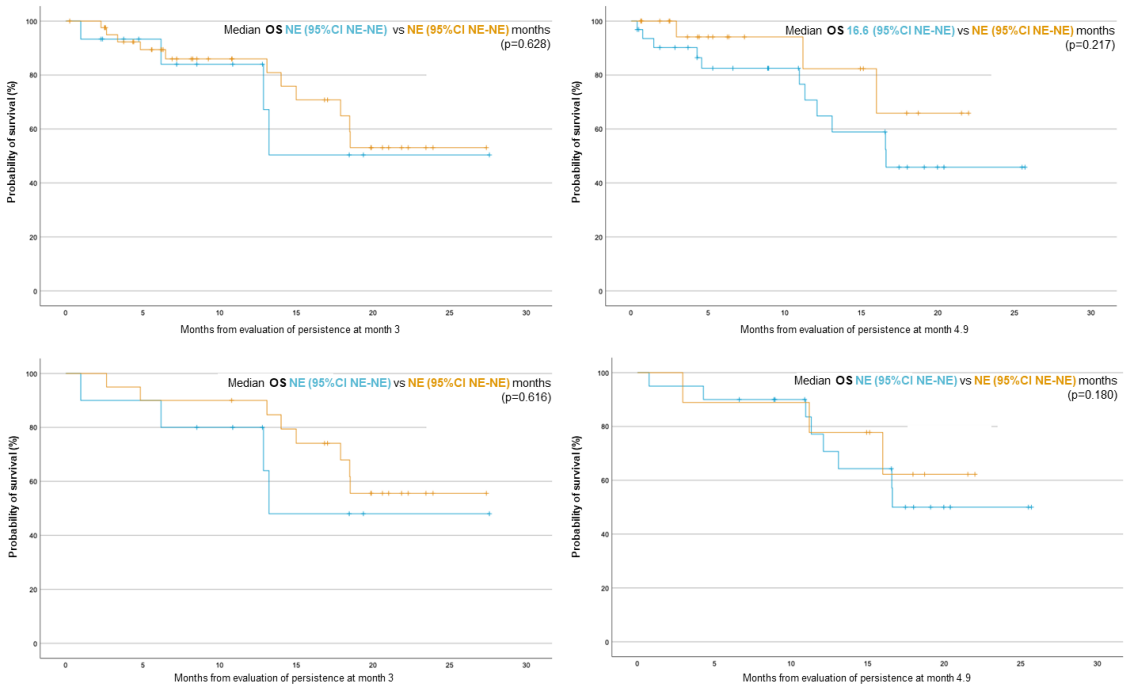


Events occurred before 3 month landmark			
	DOR	**PFS**	**OS**
Refractory	1	1	1
Progression	-	-	-
Death without progression	2	2	2
Not reached	-	-	-
Total missed events	3	3	3
Total included events	57	57	57
Events occurred before 4.9 month landmark			
	DOR	**PFS**	**OS**
Refractory	1	1	1
Progression	4	4	-
Death without progression	3	3	3
Not reached	1	1	1
Total missed events	9	9	5
Total included events	51	51	55

Figure 7. Persistence of ARI0002h in the peripheral blood according to **(A)** triple- and **(B)** penta-refractoriness (yes = orange, no = blue), and **(C)** prior allogeneic stem cell transplantation (yes = orange, no = blue).

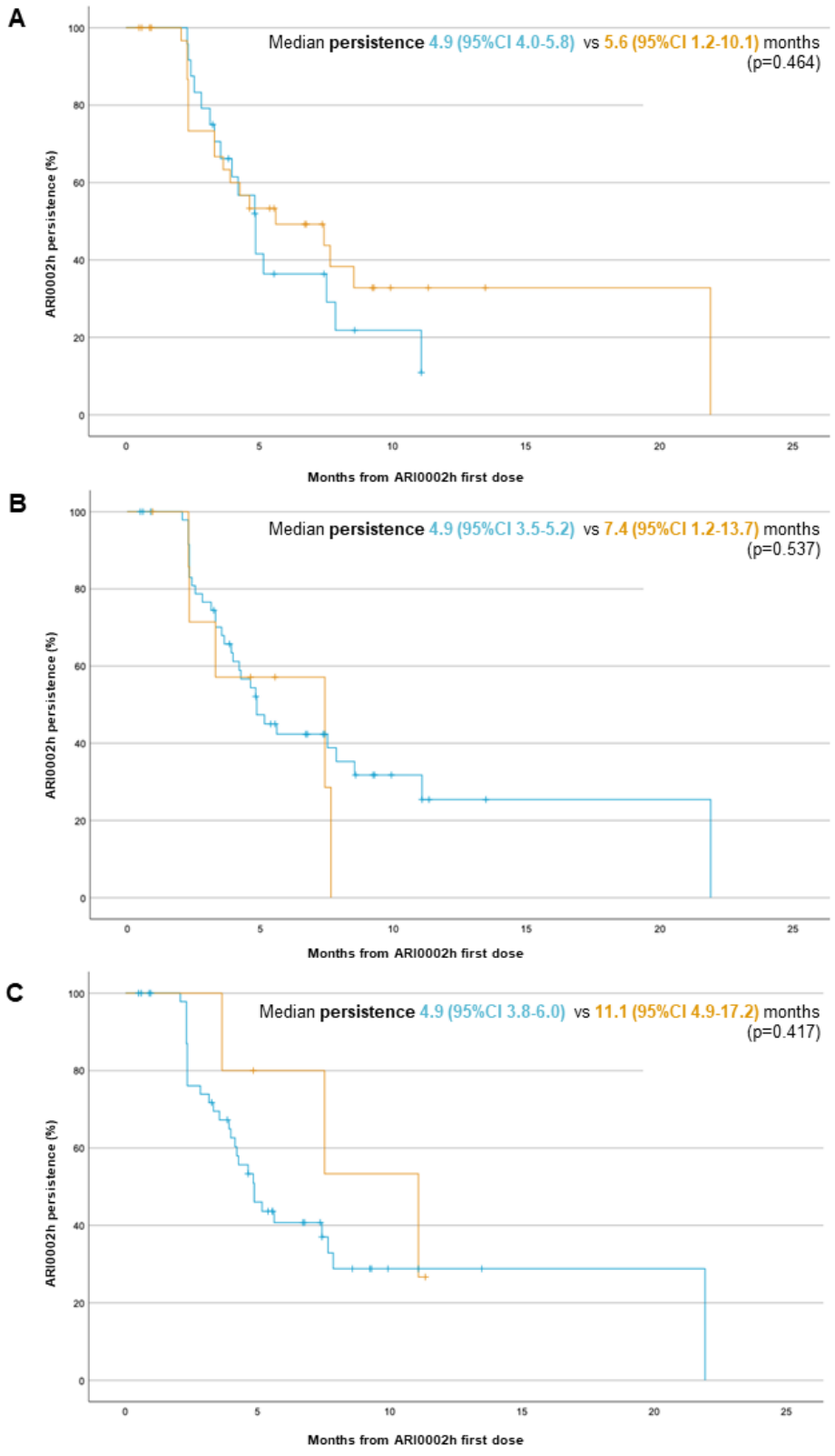


Figure 8. B-cell kinetics after ARI0002h infusion until study withdrawal. In the right-hand graph, B-cell levels are depicted only until booster dose, together with ARI0002h levels.

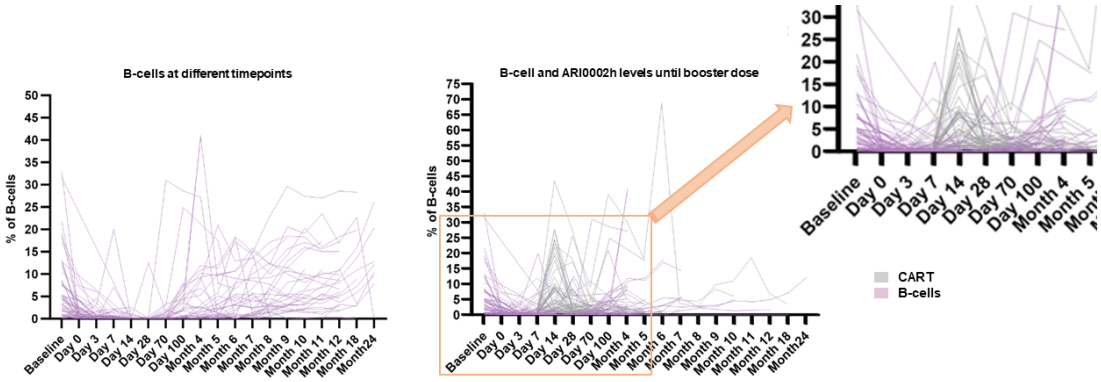


Figure 9. Cytokine levels in the peripheral blood of patients treated with ARI0002h. (A) Levels of cytokines and granzyme B at different timepoints, from baseline to day 100. (B) Differences in IFN-γ, granzyme B and IL-6 levels according to the development of CRS and CRS intensity (absence, grade 1 and grade 2).

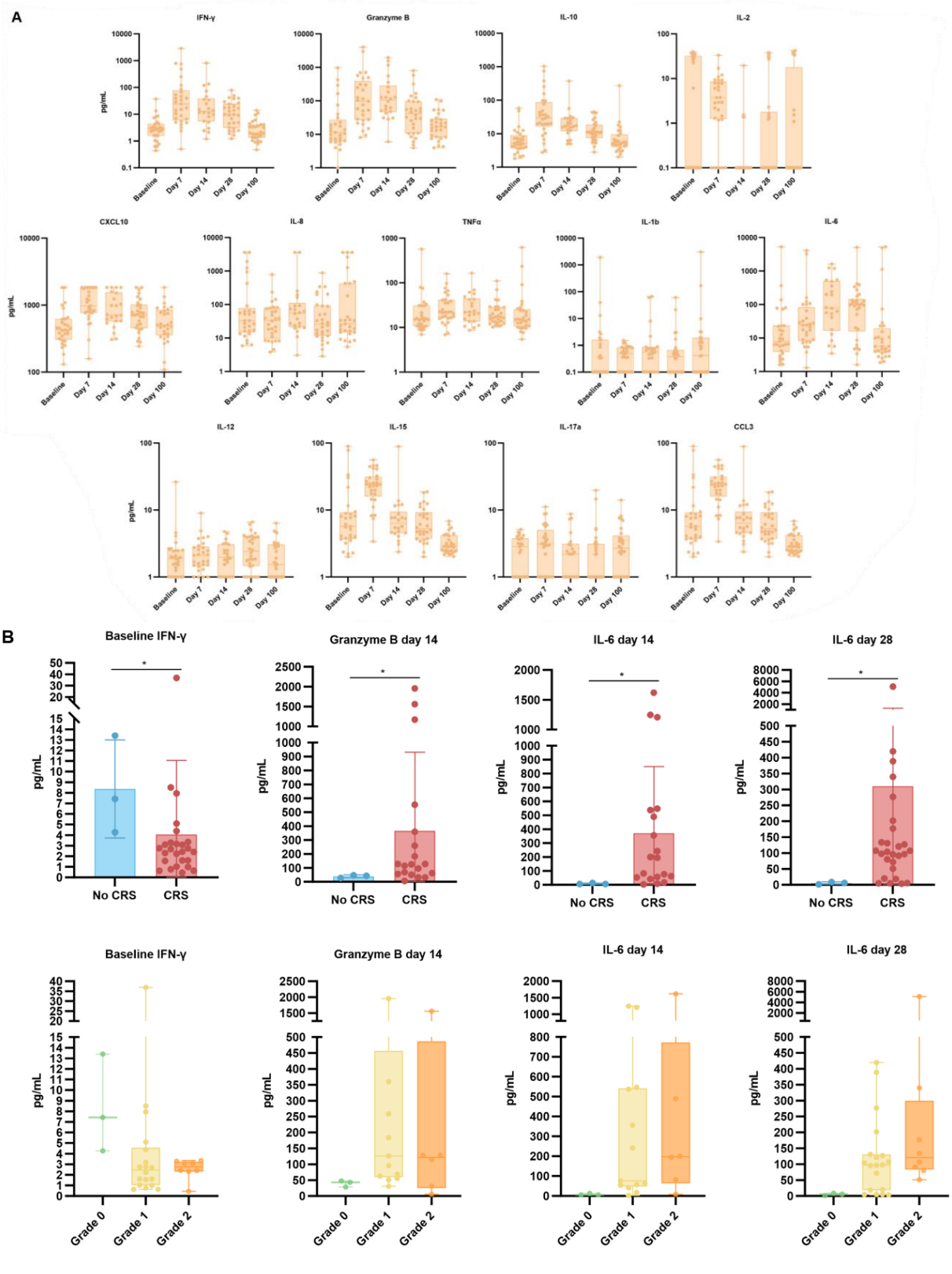


Table 4. Cox regression analysis of the impact of the levels of cytokines in progression-free (PFS) and overall survival (OS). Higher IFNγ, granzyme B and IL-10 on day 7, and IL-2 levels on day 100, were correlated with worse progression-free and overall survival. CXCL10 and IL-8 on day 7 were positively correlated with worse overall survival.

Cytokine	Survival	B	Exp(B) (95%CI)	p value
IFN-γ day 7	PFS	0.001	1.001 (1.00026-1.0018)	0.009
	OS	0.001	1.001 (1.00025-1.00183)	0.010
Granzyme day 7	PFS	0.00050	1.0005 (1.000072-1.00092)	0.022
	OS	0.00053	1.00053 (1.000092-1.000097)	0.018
IL-10 day 7	PFS	0.003	1.003 (1.001-1.005)	0.002
	OS	0.005	1.005 (1.002-1.008)	0.00057
IL-2 day 100	PFS	0.035	1.035 (1.009-1.063)	0.009
	OS	0.033	1.034 (1.004-1.065)	0.029
CXCL10 day 7	PFS	0.00073	1.00073 (0.999-1.0015)	NS 0.072
	OS	0.00124	1.00124 (1.000064-1.00241)	0.039
IL-8 day 7	PFS	0.0030	1.0030 (0.999-1.006)	NS 0.074
	OS	0.004	1.004 (1.001-1.007)	0.025

Figure 10. (A) Progression-free survival (PFS) and **(B)** overall survival (OS) curves obtained after dichotomizing the values of each cytokine (IL-10 on day 7, Granzyme on day 7, IFN-γ on day 7, IL-2 on day 100) according to the mean (lower=blue vs. higher=orange) in the study population at the selected time after infusion. To elucidate whether the elevation of these cytokines was caused by a higher tumor burden, we performed a correlation analysis with serum M protein or the percentage of BM PC that was not significant; sBCMA showed a positive correlation only with IL-2 levels on day 100 (r_s=0.418; p=0.024).

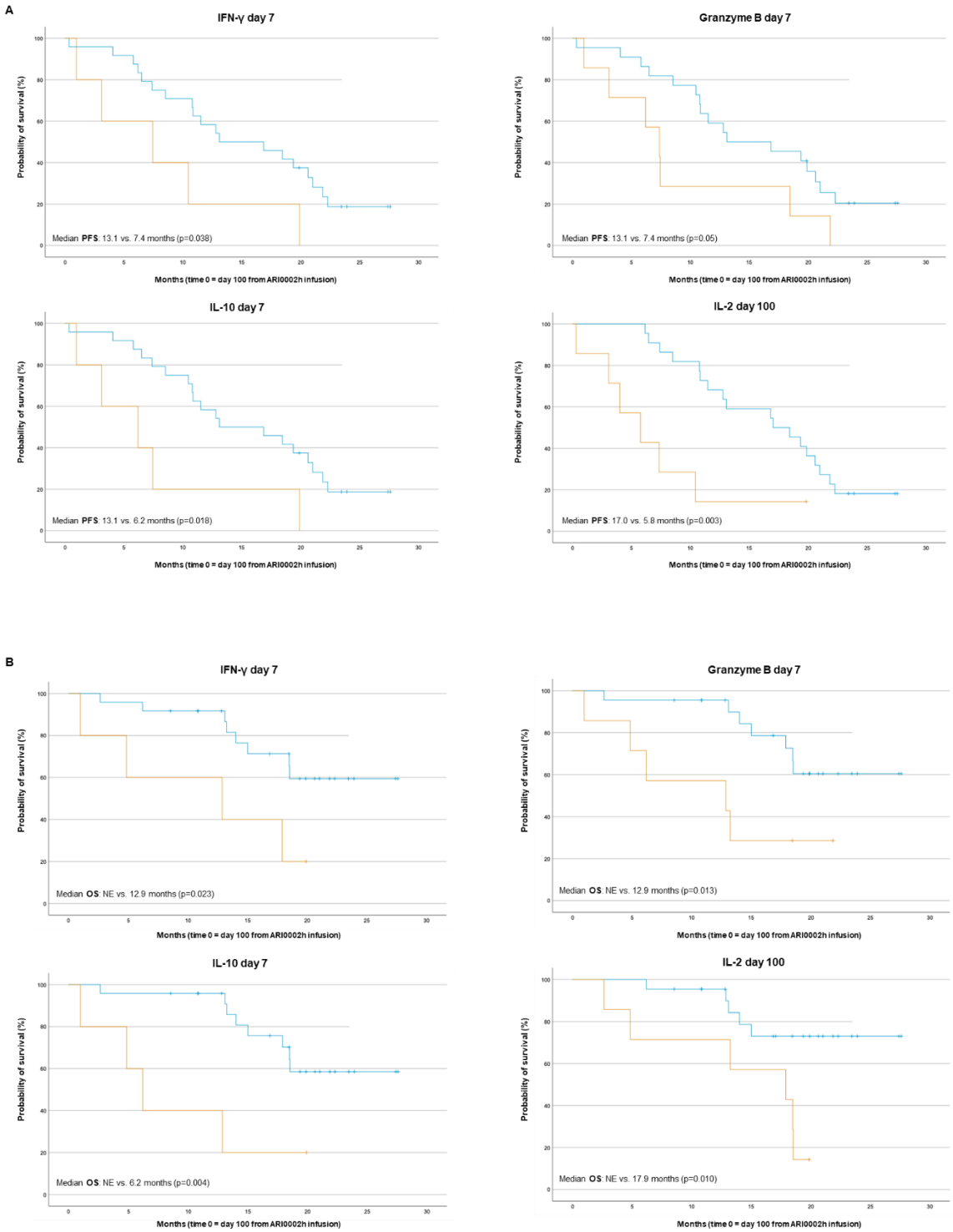


Table 5. Detection of CART antibodies at different timepoints.

	Day 28	Day 100	Month 6	Month 12	End of treatment
Patients with CART antibodies (n)	5	4	13	13	7
Samples available (n)	57	55	48	26	28
Patients with CART antibodies (%)	8.8	7.3	27.1	50	25

Table 6. Patients with positive antibodies against CART in the end-of-treatment (EOT) sample. In patient 1, several samples were positive before relapse; in patients 2 to 7 the positivity was in the EOT sample or very close to it. Note that one withdrawal was not due to relapse.

	Samples with positive CART antibodies	Time to withdrawal (months)	Reason for withdrawal
Patient 1	Months 12, 18, 24 and EOT.	24.0	Relapse
Patient 2	Month 12 and EOT.	12.3	AlloSCT
Patient 3	Only EOT.	13.8	Relapse
Patient 4	Only EOT.	11.5	Relapse
Patient 5	Only EOT.	10.2	Relapse
Patient 6	Only EOT.	8.8	Relapse
Patient 7	Only EOT.	4.6	Relapse

Table 7. Patients with positive antibodies against CART at some point that remain in response after a long follow-up (cohort 1).

	Samples with positive CART antibodies	Follow-up (months)
Patient 8	Day 28.	30.6
Patient 9	Months 6, 12, 18 and 24.	30.4
Patient 10	Month 18.	26.9
Patient 11	Month 18 and 24.	26.5
Patient 12	Month 12.	22.9

Table 8. Measurable residual disease results at different timepoints.

		Day 28	Day 100	Month 6	Month 12	Month 18	Month 24	End of treatment
Measurable residual disease general results								
Negative		40	49	41	21	8	6	3
Positive		1	2	4	4	5	3	16
Not evaluable		13	5	3	0	0	0	0
No sample		3	1	2	1	1	0	15
Out of study		3	3	8	17	25	30	-
Not reached		0	0	2	17	21	21	26
Total		60	60	60	60	60	60	60
Measurable residual disease negative results								
Negative (total n)		40	49	41	21	8	6	3
Sensitivity	**10^{-4}**	4	5	2	2	1	0	1
	10^{-5}	17	17	15	3	2	1	2
	10^{-6}	19	27	24	16	5	5	0
% negative at 10^{-6}		47.5	55.1	58.5	76.2	62.5	83.3	0
% negative at 10^{-5} and 10^{-6}		90	89.8	95.1	90.5	87.5	100	66.7

Figure 11. Progression-free survival according to MRD-negative sensitivity on day 28. Sensitivity of 10^{-6} = green, 10^{-5} = yellow, 10^{-4} = orange, hemodiluted = gray.

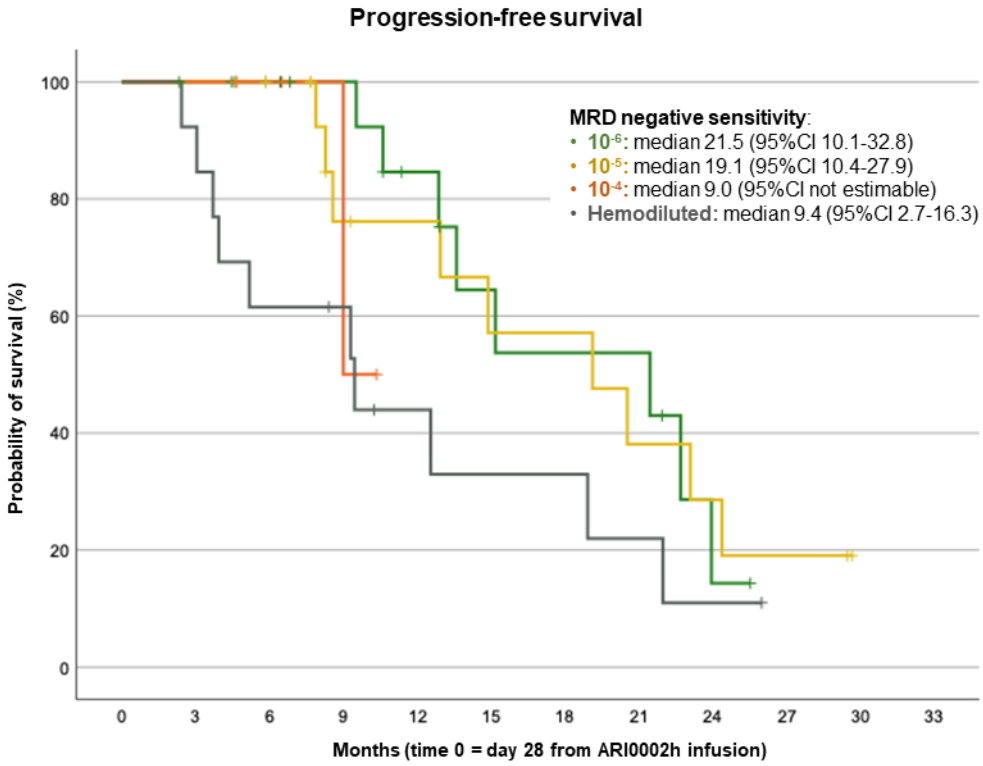


Figure 12. Time (months) from measurable residual disease positivity to overt relapse according to sensitivity of detection.

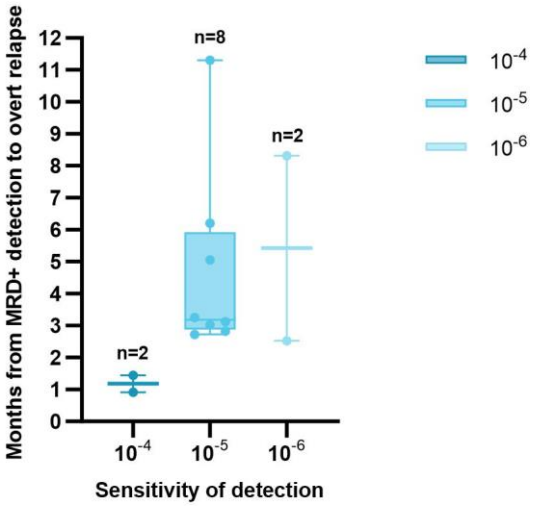
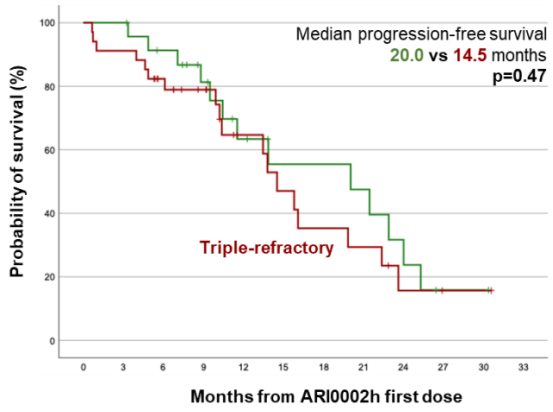


Figure 13. Progression-free survival according to **(A)** triple-refractoriness, **(B)** high-risk cytogenetics and the presence of extramedullary disease (**(C)** all plasmacytomas and **(D)** only those of extramedullary location). ISS was a better predictor when calculated at baseline **(E)**, rather than **(F)** at MM diagnosis.

A



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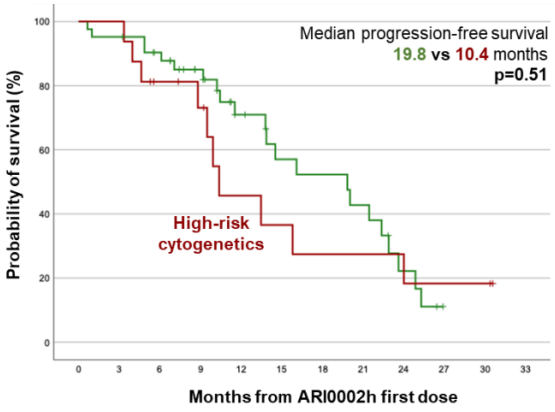

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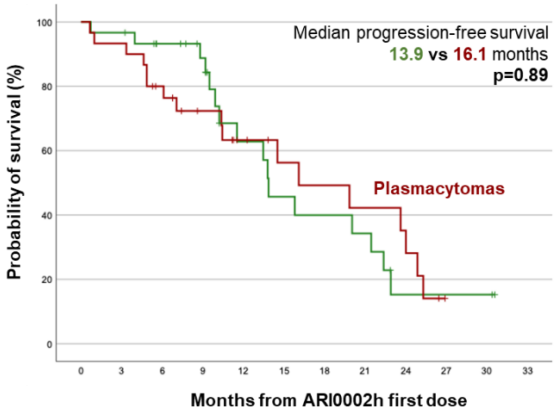

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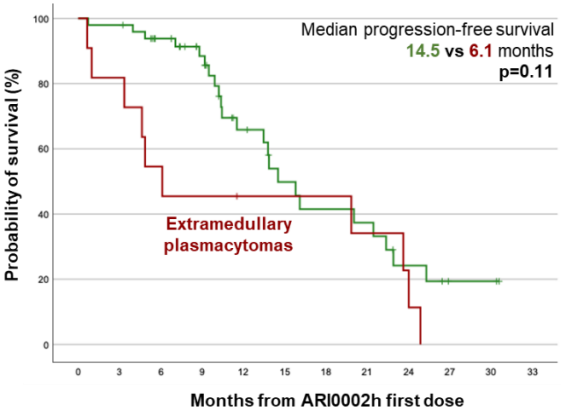

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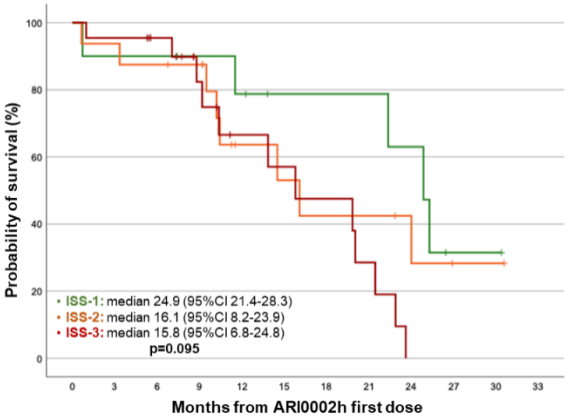

F

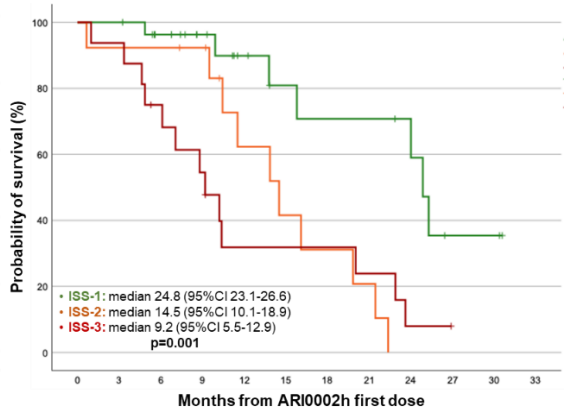

Table 9. Multivariate analysis of the impact in progression-free survival of clinical variables at baseline.

Variables at baseline	B	Exp(B) (95%CI)	p value
M-protein (g/L)	0.018	1.018 (1.001- 1.035)	0.039
Soluble BCMA (pg/mL)	0.0000012	1.0000012 (1.00000034-1.0000021)	0.006
ISS 2	1.427	4.165 (1.414-12.266)	0.010
ISS 3	1.276	3.582 (1.345-9.539)	0.011

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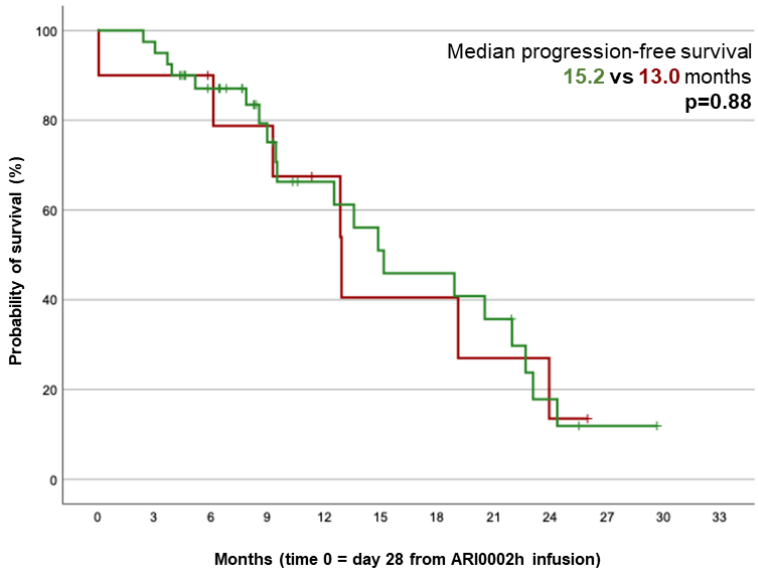
Figure 14. Progression-free survival according to involved serum free-light chain levels below the lower limit of normality (green) on **(A)** days 28 and **(B)** 100.

A



B