

SUPPLEMENTAL MATERIALS

TITLE: Blood monitoring of circulating tumor plasma cells by next generation flow in multiple myeloma after therapy

AUTHORS: Luzalba Sanoja-Flores¹, Juan Flores-Montero¹, Noemi Puig², Teresa Contreras-Sanfeliciano³, Roberia Pontes⁴, Alba Corral-Mateos¹, Omar García-Sánchez², María Díez-Campelo², Roberto José Pessoa de Magalhães Filho⁵, Luis García-Martín⁶, José María Alonso-Alonso⁷, Aranzazú García-Mateo⁸, Carlos Aguilar-Franco⁹, Jorge Labrador¹⁰, Abelardo Barez-García¹¹, Angelo Maiolino⁵, Bruno Paiva¹², Jesús San Miguel¹², Elaine Sobral da Costa⁴, Marcos González², María Victoria Mateos², Brian Durie¹³, Jacques van Dongen¹⁴ and Alberto Orfao on behalf of the EuroFlow consortium.

AFFILIATIONS:

1. Cancer Research Center (IBMCC-CSIC/USAL-IBSAL); Cytometry Service (NUCLEUS) and Department of Medicine, University of Salamanca, Salamanca, Spain (USAL). Centro de Investigación Biomédica en Red de Cáncer, Instituto Carlos III, Madrid, Spain. CIBER-ONC number CB16/12/00400.
2. Department of Hematology, University Hospital of Salamanca, IBSAL; IBMCC (USAL-CSIC), Salamanca, Spain (HUSA). CIBER-ONC number CB16/12/00233.
3. Department of Biochemistry, University Hospital of Salamanca, Salamanca, Spain.
4. Department of Pediatrics, Institute of Pediatrics and Puericulture Martagão Gesteira (IPPMG), Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

5. University Hospital Clementino Fraga Filho, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil.
6. Department of Hematology, Hospital of Zamora, Zamora, Spain.
7. Department of Hematology, Hospital of Palencia, Palencia, Spain.
8. Department of Hematology, Hospital of Segovia, Segovia, Spain.
9. Department of Hematology, Hospital of Soria, Soria, Spain.
10. Department of Hematology, Hospital of Burgos, Burgos, Spain.
11. Department of Hematology, Hospital of Ávila, Ávila, Spain.
12. Clinica Universidad de Navarra; Applied Medical Research Center (CIMA), IDISNA, Pamplona, Spain (UNAV). CIBER-ONC number CB16/12/00369 and CB16/12/00489
13. Cedars-Sinai Samuel Oschin Cancer Center, Los Angeles, United States.
14. Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, Netherlands.

MATERIALS AND METHODS

Patients and samples. Multiple myeloma (MM) patients were treated out of clinical trials and grouped according to time of minimal residual disease (MRD) assessment¹ into: (1) cases evaluated during (active) treatment (n=30) -immediately prior to autologous stem cell transplantation (ASCT) or after induction (high-dose therapy based on: bortezomib+ thalidomide+ dexamethasone (VTD), bortezomib+ lenalidomide+ dexamethasone (VRD), carfilzomib+ lenalidomide+ dexamethasone (KRD), bortezomib+ cyclophosphamide+ dexamethasone (VCD) or bortezomib+ melphalan+ prednisone (VMP) regimens)- and, (2) after the end of (active) ASCT therapy (n=107). Overall, 118/137 patients received or were candidates to undergo an ASCT. At the time of bone marrow (BM) MRD plus blood circulating tumor plasma cells (CTPC) analyses, patients were categorized by the 2016 International Myeloma Working Group (IMWG) response criteria² into: cases in stringent CR (sCR)/CR (n=71), very good partial response (VGPR; n=33), partial response (PR; n=10), stable disease (SD; n=2), and progressive disease (PD; n=21). Those 54 patients in whom sequential follow-up blood samples were evaluated for both serum immunofixation (sIF) and CTPC (median interval between sequential analyses of 10 months) included: (1) MM cases in which either blood CTPC or sIF were persistently negative (negative/negative; 36 and 29 cases, respectively) or they turned negative after a first positive evaluation (positive/negative; 6 and 11 cases, respectively); and (2) MM patients who were either CTPC or sIF positive in the last evaluation, regardless of their previous status (negative/positive, 6 and 5 cases, respectively; and, positive/positive, 6 and 9 cases, respectively).

Prior to entering the study, written informed consent was given by each patient according to the Declaration of Helsinki. All samples were received and processed at the different participating centers (USAL-HUSA, UFRJ-IPPMG).

Immunophenotypic and immunofixation studies. Blood and BM aspirated samples were collected in tubes containing EDTA as anticoagulant and prepared following the EuroFlow bulk-lysis, surface membrane (sm)-only and sm-plus-cytoplasmic (cy) standard EuroFlow staining procedures, as described elsewhere³. The 2-tube/8-color EuroFlow MM MRD antibody panel “allows confirmation in a second independent measurement, of the clonal nature of suspicious (low numbers of) tumor plasma cells (TPC), through evaluation of the cytoplasmic kappa/lambda restriction of phenotypically aberrant PC which has previously proved to be required in a significant number of cases”³. As previously described in detail, in tube 1, cells were stained for: CD138-BV421, CD27-BV510, CD38ME-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, CD117-APC and CD81-APCC750; while in tube 2, the CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, Cy-immunoglobulin (Ig) Kappa-APC and CylgLambda-APCC750 staining were used³. Stained cells were measured in FACSCanto II flow cytometers -Becton/Dickinson Biosciences (BD), San Jose, CA- using the FASCDiVa software (BD). The percentage of tumor plasma cells (TPC) defined on immunophenotypic grounds was determined from all BM and blood nucleated cells, respectively, while absolute blood CTPC counts were calculated using a dual-platform approach, as previously described⁴. For flow cytometry data analysis, the *Infinicyt* software (version 2.0; Cytognos SL, Salamanca, Spain), was used. CTPC-negativity and MRD-negativity were defined as absence of TPC in blood or BM by NGF with a limit of detection of $<2 \times 10^{-6}$, respectively; while CTPC-positivity and MRD-positivity indicated presence of TPC in blood or BM by NGF above this cutoff level.

In parallel to CTPC NGF analyses, sIF using HYDRAGEL kits (HYDRASYS system, Sebia, Barcelona, Spain)⁵ was performed to detect the tumor M-component in fresh serum samples collected in tubes without anticoagulant, following the recommendations of the manufacturer.

Statistical methods. Crosstab tables were used to compare the distribution of cases presenting TPC by next generation flow (NGF) in paired blood vs. BM samples, and to assess the relationship between the blood CTPC status by NGF and sIF status. The Fisher's Exact test and the Wilcoxon test were applied to calculate the statistical significance of differences observed between groups for (paired) categorical and continuous variables, respectively. The Kaplan–Meier method and either the (two-sided) log-rank or the post-hoc tests were used to plot and compare progression-free survival (PFS) curves between two or more than two patient groups, respectively. PFS was calculated as the time lapse from assessment of response (BM MRD plus first blood CTPC analysis), to either disease progression or death for any reason. PFS hazard ratio (HR) values were estimated using Cox regression modelling by the forward Wald-stepwise method, after the proportional hazard assumption was checked for each covariable. Those covariables that showed (statistically) significant impact on PFS in multivariate analysis, were used to build a prognostic score. Statistical significance was set at p -values <0.05 . For all statistical analyses, the Statistical Package for Social Sciences (SPSS version 23; IBM, Armonk, NY), was used.

RESULTS

Blood CTPC vs BM MRD status. At time of analysis of blood CTPC plus BM MRD and sIF, 52% of all MM patients were in CR or sCR (71/137), while the other cases (66/137; 48%) reached lower quality of response to therapy (e.g. VGPR, PR, SD, PD) independently of their sIF status (4 were sIF⁻). Of note, those sCR/CR cases who had CTPC in blood at time of analysis, showed median percentage and absolute CTPC counts significantly lower than those observed within non-CR CTPC⁺ cases ($p=0.001$): 0.0002% (range: $<0.0001\%$ - 0.007%) and 17 CTPC/mL of blood (range: <5 -457

CTPC/mL) vs 0.005% (range: <0.0001%-0.6%) and 241 CTPC/mL of blood (range: <5-18,352 CTPC/mL), respectively (Supplemental Table 3).

Prognostic impact of PB CTPC vs. BM MRD. Overall, the blood CTPC status emerged as a prognostic factor independent of the phase of treatment at which it was assessed with median PFS of 17 vs 50 months for CTPC⁺ vs CTPC⁻ cases analyzed during therapy ($p=0.001$; Supplemental Figure 1A) and of 6 vs 32 months for those evaluated after discontinuation of active treatment ($p<0.0001$; Supplemental Figure 1D). Similarly, positivity for BM MRD showed a significant impact on PFS (vs. BM MRD⁻ cases) once assessed during or at the end of active treatment with median PFS of 19 vs 46 months ($p=0.02$; Supplemental Figure 1B) and of 18 vs 46 months ($p<0.0001$; Supplemental Figure 1E), respectively. Of note, the distribution of cases presenting blood CTPC according to their BM MRD and sIF status, was similar in MM patients investigated during and after active treatment vs. the whole series (Supplemental Table 4).

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SUPPLEMENTAL FIGURES:

Supplemental Figure 1. PFS curves of MM patients grouped according to their PB CTPC, BM MRD status and both PB CTPC and BM MRD status during active treatment (A to C, respectively) and at the end of active therapy (D to F, respectively). (C and F) Cluster A (grey line) represents cases that were simultaneous negative for paired BM MRD and PB CTPC; Cluster B (black line) represents BM MRD positive and PB CTPC negative cases; and, Cluster C (red line) represents cases that were simultaneous positive for BM MRD and PB CTPC. PB, peripheral blood; BM, bone marrow; MRD; minimal residual disease; NGF, next generation flow; PFS, progression-free survival; CTPC, circulating tumor plasma cell; NR, not reached; MM, multiple myeloma.

Supplemental Table 1. Baseline MM Patient Characteristics (n=137).

Patients Characteristics	Distribution
Age, years, median (range)	63 (40-85)
Sex, males/females, (%)	58% / 42%
Hemoglobin, g/dL, median (range)	11.6 (4-16.3)
Serum calcium, mg/dL, median (range)	9.6 (7.1-14)
Serum creatinine, mg/dL, median (range)	0.9 (0.4-13.9)
Serum albumin, g/dL, median (range)	3.9 (2.6-5.1)
Serum β_2 -microglobulin, mg/L, median (range)	3.3 (1.1-33.3)
Bone marrow-plasma cell FISH, n (%)	96 (70%)
Abnormal cytogenetic profile*	45 (47%)
• t(4;14)	13 (21.3%)
• t(11;14)	7 (11.4%)
• t(14;16)	4 (6.5%)
• Del(17p)	13 (21.3%)
• Del(13q)	5 (8.2%)
• 1q gains	8 (13%)
• IgH translocation with unknow partner/deletion	9 (15%)
• Monosomy 13	2 (3.3%)
Normal FISH	51 (53%)

* ≥ 2 abnormal cytogenetic mutations could be present at the same patient.
 MM, multiple myeloma; y, years old; FISH, fluorescent in situ hybridization;
 t, translocation; Del, deletion; IgH, immunoglobulin heavy chain.

Supplemental Table 2. Blood CTPC Status Vs BM MRD And Serum IF Status Of MM Patients (n=137) Classified According To Response To Therapy.

	Blood CTPC Status			P
	Negative	Positive	Total	
BM MRD Status				
Negative	46/137 (34%)	0/137 (0%)	46/137 (34%)	
Positive	55/137 (40%)	36/137 (26%)	91/137 (66%)	<0.0001
Total	101/137 (74%)	36/137 (26%)	137/137 (100%)	
Serum IF Status				
Negative	60/137 (44%)	15/137 (11%)	75/137 (55%)	
Positive	41/137 (30%)	21/137 (15%)	62/137 (45%)	0.08
Total	101/137 (74%)	36/137 (26%)	137/137 (100%)	
BM MRD Negative				
sIF Negative	36/46 (78%)	0/46 (0%)	36/46 (78%)	
sIF Positive	10/46 (22%)	0/46 (0%)	10/46 (22%)	-
Total	46/46 (100%)	0/46 (0%)	46/46 (100%)	
BM MRD Positive				
sIF Negative	24/91 (26%)	15/91 (17%)	39/91 (43%)	
sIF Positive	31/91 (34%)	21/91 (23%)	52/91 (57%)	1.0
Total	55/91 (60%)	36/91 (40%)	91/91 (100%)	
From sCR/CR MM Cases				
BM MRD Negative	36/71 (51%)	0/71 (0%)	36/71 (51%)	
BM MRD Positive	23/71 (32%)	12/71 (17%)	34/71 (49%)	<0.0001
Total	59/71 (83%)	12/71 (17%)	71/71 (100%)	

CTPC, circulating tumor plasma cells; BM, bone marrow; MRD, minimal residual disease; sIF, serum immunofixation; MM, multiple myeloma; CR, complete response; sCR, stringent complete response; sCR/CR MM cases includes sCR and CR cases.

Supplemental Table 3. Relative And Absolute CTPC Counts In Blood Vs BM MRD Levels For The Whole MM Patient Cohort (n=137) And For MM Cases Classified According To Response To Therapy (sCR/CR Vs Non-CR Cases).

Patient Group	CTPC Cases Detected	N. Of CTPC/ mL Of Blood	% Of CTPC In Blood	% Of TPC In BM	P (% Blood CTPC Vs % BM MRD)
Whole MM Series (n=137)	CTPC - (n=101)	NA	NA	0.0001 (<0.0001-1.8)	-
	CTPC + (n=36)	86 (<5-18,352)	0.0014 (<0.0001-0.6)	0.14 (0.0005-14.3)	<0.0001
	Total	<5 (<5-18,352)	<0.0001 (<0.0001-0.6)	0.002 (<0.0001-14.3)	<0.0001
MM sCR/CR Cases (n=71)	CTPC - (n=59)	NA	NA	<0.0001 (<0.0001-1.8)	-
	CTPC + (n=12)	17* (<5-457)	0.0002* (<0.0001-0.007)	0.07 (0.0008-1.6)	0.002
	Subtotal	<5* (<5-457)	<0.0001* (<0.0001-0.007)	<0.0001* (<0.0001-1.8)	<0.0001
MM Non-sCR/CR Cases (n=66)	CTPC - (n=42)	NA	NA	0.004 (<0.0001-0.2)	-
	CTPC + (n=24)	241 (<5-18,352)	0.005 (<0.0001-0.6)	0.2 (0.0005-14.3)	<0.0001
	Subtotal	<5 (<5-18,352)	<0.0001 (<0.0001-0.6)	0.02 (<0.0001-14.3)	<0.0001

*P <0.01 vs MM cases who did not reach sCR/CR.

Results expressed as median (range) values. NA, not applicable; N; number; CTPC, circulating tumor plasma cell; TPC, tumor plasma cell; BM, bone marrow; MRD, minimal residual disease; MM, multiple myeloma; Non-sCR/CR MM patients includes VGPR, PR, SD, PD cases; sCR/CR MM cases includes sCR and CR cases; CR, complete response; sCR, stringent complete response; PR, partial response; VGPR, very good partial response; SD, stable disease; PD, progressive disease.

Supplemental Table 4. Blood CTPC status vs BM MRD and serum IF status of MM patients during (n=30) and after active (n=107) treatment.

From during active treatment patients				
	Blood CTPC status			
BM MRD status	Negative	Positive	Total	P
Negative	11/30 (37%)	0/30 (0%)	11/30 (37%)	
Positive	12/30 (40%)	7/30 (23%)	19/30 (63%)	0.03
Total	23/30 (77%)	7/30 (23%)	30/30 (100%)	
Serum IF status				
Negative	17/30 (57%)	3/30 (10%)	20/30 (67%)	
Positive	6/30 (20%)	4/30 (13%)	10/30 (33%)	0.2
Total	23/30 (77%)	7/30 (23%)	30/30 (100%)	
BM MRD negative				
sIF negative	10/11 (91%)	0/11 (0%)	9/11 (91%)	
sIF positive	1/11 (9%)	0/11 (0%)	1/11 (9%)	-
Total	11/11 (100%)	0/11 (0%)	11/11 (100%)	
BM MRD positive				
sIF negative	7/19 (37%)	3/19 (16%)	10/19 (53%)	
sIF positive	5/19 (26%)	4/19 (21%)	9/19 (47%)	0.7
Total	12/19 (63%)	7/19 (37%)	19/19 (100%)	
From after active treatment patients				
BM MRD status				
Negative	36/107 (34%)	0/107 (0%)	36/107 (34%)	
Positive	42/107 (39%)	29/107 (27%)	71/107 (66%)	<0.0001
Total	78/107 (73%)	29/107 (27%)	107/107 (100%)	
Serum IF status				
Negative	42/107 (39%)	12/107 (11%)	54/107 (50%)	
Positive	36/107 (34%)	17/107 (16%)	53/107 (50%)	0.2
Total	78/107 (73%)	29/107 (27%)	107/107 (100%)	
BM MRD negative				
sIF negative	26/36 (72%)	0/36 (0%)	26/36 (72%)	
sIF positive	10/36 (28%)	0/36 (0%)	10/36 (28%)	-
Total	36/36 (100%)	0/36 (0%)	36/36 (100%)	
BM MRD positive				
sIF negative	16/71 (22%)	12/71 (17%)	28/71 (39%)	
sIF positive	26/71 (37%)	17/71 (24%)	43/71 (61%)	0.8
Total	42/71 (59%)	29/71 (41%)	71/71 (100%)	

CTPC, circulating tumor plasma cells; BM, bone marrow; MRD, minimal residual disease; sIF, serum immunofixation; MM, multiple myeloma.

Supplemental Figure 1

