Protocol title

An observational study of Cardiovascular complications of Carfilzomib treatment in clinical practice.

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Contents

Study Synopsis

Study Title:	An observational study of Cardiovascular complications of Carfilzomib treatment in clinical practice.
Methods:	This is an, observational, non interventional, study in patients with relapsed or refractory myeloma treated with carfilzomib (CFZ), according to the approved indications. Patients will be evaluated prospectively for different parameters of vascular function, blood pressure and cardiac function in conjunction with studies of proteasome inhibition and function. The aim of this study is to provide insights into the effects of carfilzomib on vascular function and the mechanisms of UPS inhibition on cardiovascular complications of proteasome inhibitors.
Objectives:	Primary • to describe cardiovascular complications associated with the use of carfilzomib and investigate the role of the UPS inhibition, in patients treated with carfilzomib and dexametahsone, on atheromatosis and vascular inflammation and function Secondary • to outline the clinical significance of carfilzomib toxicity in hemodynamic parameters and cardiovascular function and vascular structure
Endpoints	Primary • Changes in hemodynamic markers (peripheral and aortic office blood pressure and 24 hour ambulatory BP monitoring parameters) and in peripheral vascular function (endothelial function, arterial stiffness, arterial wave reflections) before, during and after study drug administration Secondary • Changes in subclinical atherosclerosis markers (carotid intima-media thickness and vascular wall and plaque echogenicity) • changes in markers of cardiac function (ejection fraction,

	systolic and diastolic strain and strain rate) • changes in circulating cardiac and vascular inflammatory biomarkers (NtproBNP, cTnT, C-reactive protein, interleukin 6, vascular cell and intercellular adhesion molecule-1, E-selectin, and monocyte chemoattractant protein-1) before and after study drug administration
Population:	Patients with relapsed or refractory myeloma treated with carfilzomib as per approved indications
Phase:	This is an observational, non interventional, prospective study
Number of Sites:	This is a single center study
Description of Study assessments:	Patients with an indication to receive carfilzomib & dexamethasone will be treated according to approved dose and schedule A. Vascular studies: Vascular measurements will be performed at baseline before and after dexamethasone, before and after CFZ administration, 2 hours and 24 hours after CFZ administration. These measurements will be repeated on days 8,9,15 and 16 of cycle 1, on days 1,2 of cycles 2 and 3 and days 1,2,15 and 16 of cycle 6 or earlier if the patient develops progression of disease. Vascular measurements include assessment of arterial stiffness, arterial wave reflections, subclinical carotid and femoral atherosclerosis and carotid plaque composition, using non-invasive well-validated techniques. Blood pressure will be evaluated using 24h hour ambulatory monitoring at baseline and on days 1 and 8 of cycle 1and at the beginning of cycles 2, 3 and 6. B. In vitro studies: In parallel and at each time point, the activity of UPS and intracellular levels of ubiquitin conjugates will be measured in peripheral blood mononuclear cells (PBMCs) and red blood cells (RBCs) using enzymatic proteasome activity assays and western blot techniques, respectively.
Study Duration:	2 years

1. Background

Proteasome inhibitors (PI) have emerged as highly effective drugs on the treatment of multiple myeloma (MM) and actually they are currently standard of care at all stages of the disease. After the initial approval of bortezomib, the first-in-class PI, by the U.S. Food and Drug Administration (FDA) on 2003 for the treatment of relapsed/refractory MM, the role of proteasome has been extensively explored and the ubiquitin-proteasome pathway has been clearly documented as a major target for myeloma therapy. “Second” generation PIs, such as carfilzomib (CFZ), were developed with improved efficacy and safety profiles, expanding the therapeutic spectrum for patients with progressive MM. Carfilzomib is an epoxyketone proteasome inhibitor that binds selectively and irreversibly to the β- subunit of the 20S proteasome and more specifically targets the chymotrypsin catalytic subunit. However in the clinical studies of carfilzomib (either single agent or in combination with other agents) a low but constant signal of potential cardiotoxicity or hypertension, have been observed, but the mechanisms have not been studied prospectively and in depth (1). In the ENDEAVOR study a subgroup of patients followed prospectively with cardiac echocardiography did not reveal either prognostic or predictive factors associated with the risk of cardiotoxicity or hypertension. Furthermore, other proteasome inhibitors also affect cardiac function (such as bortezomib or ixazomib) but this has not been studied and probably their effect has been underestimated by the fact that the duration of therapy with bortezomib is significantly shorter(2).

Homeostasis of the proteome (proteostasis) is critical for normal cellular function and thus for the overall health span of the organism. To ensure proteome stability cells have developed a complex proteome quality control, the so-called proteostasis network (PN) (3). PN is responsible for normal proteome synthesis and recycling, while in parallel it suppresses proteotoxic stress by identifying and either rescuing or degrading damaged polypeptides (4). Central role to the PN play two main proteolytic pathways, the autophagy- lysosome system (ALS) and the ubiquitin proteasome system (UPS). UPS is mainly responsible for the removal of both normal short-lived ubiquitinated proteins and of oxidized or abnormal proteins, whereas the ALS acts through degradation of long-lived proteins, aggregated ubiquitinated proteins and through recycling or damaged organelles.

The central though protein degradation mechanism in eukaryotic cells is the Ubiquitin Proteasome System. It is responsible for the non-lysosomal degradation of the majority of the intracellular proteins playing a key role in cellular processes such as regulation of cell cycle

progression, division, development and differentiation, protein quality control, apoptosis, cell trafficking, and modulation of the immune and inflammatory responses (5-6).It involves the activation, transfer, and binding of multiple moieties of ubiquitin to protein substrates by action of E1, E2, and E3 enzymes, respectively (7). Labeled by a polyubiquitin chain, proteins are recognized and degraded by the 26S proteasome complex. This complex is composed of a 20S core particle that embodies the catalytic activity and 19S regulatory particles (8).

Besides protein degradation the UPS has been attributed a significant role in the activation of various transcription factors, such as nuclear factor Kb (NF-κB) a pivotal mediator in atherosclerosis; genes controlled by NFκB include (among others) those that encode for vascular cell adhesion molecule-1, E-selectin, and monocyte hemoattractant protein-1. Thus UPS may play a part in maintaining a fine balance in the atherosclerotic process as a direct regulator of pro-inflammatory and/or anti-inflammatory genes and as a regulator of cell survival and proliferation (9-10).

The role of the ubiquitin-proteasome system (UPS) in vascular biology and function is under investigation with controversial results. Over the past decade, accumulating evidence supports the hypothesis of a pathophysiological role of the UPS in the process of atherosclerosis and vascular function. However, the effect of UPS on this process is not well defined. Experimental, clinical and autopsy observational studies have demonstrated that low UPS activity and increased ubiquitin conjugates are associated with unstable coronary, aortic and carotid lesions and vascular aging (11-13). However, experimental studies have yielded contradicting results about the role of UPS inhibition and atherosclerosis. Specifically, UPS inhibition induced deterioration in endothelial function of coronary arteries of hypercholesterolemic pigs and promoted an unstable plaque phenotype in apolipoprotein (Apo)E deficient mice (14-15). On the other hand, UPS inhibition in human endothelial cells induced a protective stress response in endothelial cells through up-regulation of antioxidant enzyme expression and of eNOS, an enzyme with pivotal role in endothelial function, suggesting a potential anti-inflammatory and/or anti-oxidant effect (16-18). Similarly, UPS inhibition up-regulated eNOS expression and activity, which was associated with improved renal blood flow (19). Taken together, depending on the environment, dose and duration, cell type and organ studied, proteasome inhibitors can have diverse and broad effects and can act as ‘poisons or remedies’ in several pathophysiological processes including vascular function and atherosclerosis (20).

In order to clarify this controversial issue, the net effect of UPS inhibition on vascular function and atherosclerosis should be assessed prospectively. To the best of our knowledge, besides animal and autopsy based studies, no clinical human studies have been so far conducted in order to assess UPS inhibition on vascular function. This project will be the first to describe the acute and chronic effect of a proteasome inhibitor on vascular structure and function. Such data would *1*. improve our understanding on the pathophysiological pathways linking UPS function and atherosclerosis; *2*. provide therapeutic implications on the possible utility of UPS modulation in the prevention and/or treatment of atherosclerosis and *3*. improve

current knowledge on possible hemodynamic and vascular toxicity of carfilzomib in multiple myeloma.

2. **Potential risks and benefits**

This is an observational, non interventional, prospective study. Patients with relapsed or refractory multiple myeloma who have received at least one prior line of therapy and who receive carfilzomib with dexamethasone according to the approved dose and schedule (2) will be included in this observational study. No specific treatment intervention related to the study is going to be performed. No specific harms or benefits are expected due to study investigations.

3. Objectives and Purpose

The present study will assess patients with multiple myeloma receiving the proteasome inhibitor carfilzomib (CFZ), in order to investigate, *in vivo*, a wide spectrum of human atherosclerosis indices, from cardiovascular risk factors to subclinical atherosclerosis at multiple successive stages in a human model under conditions of global UPS inhibition, and correlate with cardiovascular complications associated with carfilzomib therapy, in patients receiving carfilzomib with dexamethasone.

3.1 Primary objective

- to investigate cardiovascular complications and the role of the UPS inhibition on atheromatosis and vascular function and inflammation, in patients with relapsed or refractory myeloma who are receiveing carfilzomib and dexametahsone.

3.2 Secondary objective(s)

- to outline the clinical significance of carfilzomib toxicity in hemodynamic parameters and vascular function and structure in humans

4. Study design and study end points

4.1 Description of the Study Design

The present study will assess patients with multiple myeloma receiving the proteasome inhibitor carfilzomib (CFZ), in order to investigate*, in vivo*, a wide spectrum of human atherosclerosis stages, cardiovascular risk factors and subclinical atherosclerosis at multiple successive stages in a human model under conditions of global UPS inhibition.

A**. Investigation of the role of the UPS inhibition on vascular inflammation and atheromatosis**

Circulating biomarkers of vascular inflammation (CRP, IL-6, ICAM-1) and vascular function (endothelial function, arterial wave reflections) and structure (intima media thickness, peripheral atheromatous plaques, plaque echogenicity and arterial stiffness) will be measured at baseline at pre-specified short and long-term time points after treatment. The acute response of vascular function and inflammatory markers to CFZ as well as long term changes in vascular structure after treatment will be assessed. These changes will be examined in comparison to the degree of change in UPS activity by validated techniques.

B. Outline of the clinical significance of carfilzomib therapy in hemodynamic parameters and vascular function and structure

In the same population and under the same design, changes in 24hour ambulatory blood pressure (BP) parameters as well as in vascular parameters as described above will be assessed focusing on time points with significant BP increments and vascular dysfunction. Subsequently, we will examine whether the magnitude of deterioration in these parameters of interest is associated with the degree of UPS activity.

5. Study enrollment and withdrawal

5.1 Participant Inclusion Criteria

1. Males and females at least 18 years of age.
2. Voluntary written informed consent before performance of any study-related procedure.
3. Subject must have documented relapsed or refractory multiple myeloma in need of therapy, after at least one previous line of therapy for myeloma.
4. Eastern Cooperative Oncology Group (ECOG) performance status score of ≤ 2.
5. Willingness and ability to participate in study procedures.

5.2 Participant Exclusion Criteria

1. Anti-myeloma treatment within 2 weeks prior to Cycle 1, Day 1.
2. Cumulative dose of corticosteroids greater than or equal to the equivalent of 140mg prednisone for ≥4 days or a dose of corticosteroids greater than or equal to the equivalent of 40 mg/day of dexamethasone for ≥4 days within the 2-week period prior to Cycle 1, Day 1.
3. Previous allogeneic stem cell transplant; or Autologous Stem Cell Transplantation (ASCT) within 12 weeks before Cycle 1, Day 1.
4. Clinical signs of meningeal involvement of multiple myeloma.
5. Clinically significant cardiac disease, including:
   a) Myocardial infarction within 6 months, or unstable or uncontrolled condition (e.g., unstable angina, congestive heart failure, New York Heart Association Class III-IV).
   b) Cardiac arrhythmia (CTCAE Grade 2 or higher) or clinically significant ECG abnormalities.

   c) ECG showing a baseline QT interval as corrected by Fridericia's formula (QTcF) >470 msec.
6. Known active hepatitis B, or C.
7. Known HIV infection.
8. Prior or concurrent malignancy, except for the following:
   a) Adequately treated basal cell or squamous cell skin cancer.
   b) Any cancer (other than in-situ) from which the subject has been disease-free for 3 years prior to study entry.
9. Any of the following laboratory test results during Screening:
   a) Absolute neutrophil count ≤1.0 × 10^9/L;
   b) Hemoglobin level ≤7.5 g/dL (≤5 mmol/L);
   c) Platelet count <75 × 10^9/L in patients in whom <50% of bone marrow nucleated cells are plasma cells and <50x10^9/L in patients in whom more than 50% of bone marrow nucleated cells are plasma cells;
   d) Alanine aminotransferase level ≥2.5 times the upper limit of normal (ULN);
10. Pregnant or nursing women.

5.3 Duration of the study

Patients will receive therapy until disease progression or as per physician's decision regarding the patient's best interest and according to the approved indications. Study accrual and collection of data will be completed in two years. The study can be terminated for any reason and at any time by the Sponsor.

5.4 Participant Withdrawal or termination

5.4.1 Reasons for Withdrawal or Termination

Every patient has the right to discontinue study participation at any time, for any reason, and every patient may be discontinued from the study for any reason beneficial to his/her well-being.

Subjects MUST discontinue for any of the following reasons:

- Withdrawal of informed consent.
- Any adverse event (AE), laboratory abnormality or intercurrent illness that, in the opinion of the investigator, indicates that continued participation in the study is not in the best interest of the subject.
- Pregnancy.
- Progressive Disease.

5.4.2 Handling of Participant Withdrawals or Termination

All patients, except those who withdraw consent, will be followed up according to the study procedures.

5.5 Premature Termination or Suspension of Study

The study can be terminated for any reason and at any time by the Sponsor.

6. Study research strategy - Technical description

6.1 Population and treatment

6.1.1 Population

The study will include patients with relapsed or refractory multiple myeloma who have received at least one prior line of therapy (2) and who according to physicians decision are treated with carfilzomib dexamethasone in the approved indication, doses and schedule. Briefly, patients aged 18 years or older with relapsed or refractory multiple myeloma, Eastern Cooperative Oncology Group performance status of 0 to 2, at last one prior treatments, will be included (see section 5.1 & 5.2).

6.1.2 Carfilzomib treatment

According to the approved indications patients will be given carfilzomib at a dose of 20 mg/m^2 on days 1 and 2 of cycle 1; and thereafter at a dose of 56 mg/m^2, in a 30 min intravenous infusion, on days 1,2,8,9,15 and 16. Dexamethasone (40mg IV) will be administered 24-48 hours before first CFZ infusion in order to assess the individual vascular effects of each drug separately; subsequently dexamethasone will be given IV at a dose of 20 mg on days 1,2,8,9,15 and 16, and orally on days 22 and 23 of a 28-day cycle. The rationale for using these doses is based on the results of the ENDEAVOR study and the approved indications and dosing of KYPROLIS. Intravenous hydration (250-500 mL before and after dose administration) is going to be given during cycle 1 and at the investigator's discretion thereafter.

6.1.3 Sample size

This is an observational study with non interventional design. Sample size calculation is made on the basis of previous data indicating that at least 2% absolute difference in Flow mediated dilatation (FMD) (corresponding to 20-30% relative difference) is considered significant and clinically relevant. With the null hypothesis being that CFZ/Dex therapy is associated with <10% relative difference in FMD and the alternative hypothesis being that a ≥30% difference is considered as significant, and with an a=0.05, a sample of 40 patients is required for 90% power of detecting these differences (all tests are 2-sided). A dropout rate of 15% is assumed. A total number of 46 patients is thus calculated to be enrolled.

6.1.4 History record

A detailed history will be obtained from every subject and all possible risk factors that could be associated with an increased risk of cardiac and vascular adverse events such as history of hypertension, coronary artery disease, arrhythmias, diabetes mellitus, dyslipidemia, smoking, renal disease. All medication that patients are receiving at the beginning of the study will also be recorded. Thorough clinical examination will also be performed along with

ECG and cardiac echocardiography to ensure that every patient has an ejection fraction ≥40% thus being eligible for enrollment. Data will be captured in a specifically designed CRF.

6.2 Functional and structural assessment of the vasculature

6.2.1 Assessment of arterial stiffness

Arterial stiffness in the aorta by measurement of pulse wave velocity (PWV)

Carotid-femoral pulse wave velocity (c-f PWV) is an established index of aortic stiffness and an independent predictor of worse cardiovascular prognosis(21, 22). It is considered as the gold standard for assessing aortic stiffness non-invasively and is calculated from measurements of pulse transit time and the distance travelled between 2 recording sites with a validated non-invasive device (Complior, Artech Medical, France). Two different pulse waves are obtained at the same time transcutaneously with the patient in a supine position at 2 sites, at the right common carotid artery and the right femoral artery (i.e. 'carotid-femoral' PWV) by using pressure-sensitive transducers. The distance traveled by the pulse wave is measured over the body surface and calculated by subtracting the carotid (sternal notch from the carotid)-femoral distance as distance/time (m/s)(23). Distance should be measured precisely because small inaccuracies may influence the absolute value of PWV (24). Moreover, the femoral pressure waveform may be difficult to record accurately in patients with metabolic syndrome, obesity, diabetes, and peripheral artery disease. The velocity of the pulse wave as it traverses the arterial tree is critically dependent on characteristics of the conduit artery wall, with pulse travelling at a higher velocity through stiffer arterial vessels (25). The principal determinants of PWV are described by the Moens–Korteweg equation that was derived in the 1920s, and relates the velocity of pulse wave travel in a vessel to the distensibility of that vessel where co is the wave speed, E is Young's modulus in the circumferential direction, h is the wall thickness, R is the vessel radius and ρ is the density of fluid. In a given blood vessel filled with blood of fixed density, PWV is proportional to the square root of the Young's modulus of elasticity of the vessel. In other words, the stiffer the vessel, the faster the PWV.

Central blood pressures and reflected waves in the aorta

Non-invasive estimation of aortic pressure waveforms and reflected waves by pulse wave analysis (PWA) will be performed in the Angiology laboratory by the SphygmoCor System (AtCor Medical Pty Ltd, Sydney, Australia). The radial artery is gently and steadily compressed against the underlying bone, thus flattening it and equalizing circumferential pressures, allowing radial pressure waves to be recorded by a high fidelity micromanometer placed on the tip of a hand – held tonometer the size of a pen (Millar). Optimal recording is obtained if the wrist is bent outward and supported by using a small cushion or the operator's hand. The following indices are measured: a. augmentation index (AI, percentage) normalized for the heart rate of 75 bpm, expressed as a percentage of the aortic pulse pressure, b. central systolic and diastolic pressures (cBP), c. time to the beginning of the reflected wave (in milliseconds) and d. blood pressure amplification calculated as the ratio of peripheral pulse pressure: central pulse pressure. Central pressure waves are

derived by use of transfer functions and augmentation index (AIx) is used as a measure of reflected waves. As described before in the case of stiff arteries, PWV rises and the reflected wave arrives back at the central arteries earlier, adding to the forward wave and augmenting the systolic pressure. This phenomenon is quantified through the augmentation index—defined as the difference between the first and second systolic peaks expressed as a percentage of the pulse pressure (difference between systolic and diastolic blood pressure)(26).

6.2.2 Endothelial function by ultrasound measurement of endothelium-dependent flow-mediated dilatation (FMD)

Endothelial function will be assessed by Flow Mediated Dilatation (FMD) using a 7-14 MHz probe (Vivid 7 Pro, GE, USA) as previously described (27). After a 15-min rest in supine position in a quiet, climate-controlled room (22 ±1°C), the internal diameter of the brachial artery of the subject is assessed. Upper arm occlusion by supra-systolic level (50 mmHg above systolic blood pressure) is applied via a cuff fitted 8-cm distal to the brachial artery (in the antecubital fossa) and near the wrist for 5 min. The cuff is released following the 5-min inclusion to induce reactive hyperemia. This increase in shear stress results in endothelium-dependent flow-mediated dilation (FMD). Data are recorded 2 minutes before and immediately after cuff release to measure peak blood flow and brachial artery diameter for 2 min after cuff deflation. ECG-gated frames of brachial artery diameter are continuously recorded and brachial artery diameter is measured offline using dedicated border detection software (Brachial Analyzer for Research, MIA llc, USA). Flow-mediated dilatation is induced in response to reactive hyperemia and is expressed as the percentage change of internal diameter of the brachial artery from baseline. Flow-mediated dilatation has been previously shown to be nitric oxide dependent in humans and it is considered as a marker of endothelial function (28-29). Ultrasound analysis will be performed in each case manually by a qualified independent observer blinded to the clinical history of the subjects.

6.2.3 Carotid and Femoral Intima-Media Thickness (IMT)

B-mode ultrasound examination will also be performed, using a 14.0 MHz multi-frequency linear array probe attached to a high resolution ultrasound machine (Vivid 7 Pro, GE Healthcare, USA). All scans are going to be performed by the same operator as previously described(30). Carotid intima-media thickness (ccIMT) will be measured at the distal 1.0 cm of the common carotid proximal to the bifurcation. In each segment 3 measurements of the maximal IMT in the far wall will be averaged, after excluding plaque thickness. The average of the maximal IMT will be used in the analyses. Femoral IMT (fIMT) will be measured on each side, scanning a 1cm-long arterial segment proximal to the femoral bifurcation, defined as the common femoral artery segment and the average value of IMT of the far wall will be estimated. A cutoff value of >0.9 mm for mean ccIMT or fIMT will be considered increased. Plaques to carotid and femoral arteries are defined as a focal structure that protrudes into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value or demonstrates a thickness of >1.5 mm. The relative echogenicity of the atheromatous plaques and the IMT will be assessed by Artery Measurement Software (AMS), an automated piece of software for

dedicated analysis of gray scale median (GSM) (31) and carotid artery remodeling of the common carotid artery by calculating the arterial relative wall thickness (RWT) and the arterial cross-sectional are (CSA) (32).

6.2.4 Ankle Brachial Index (ABI)

The ABI is a simple, non-invasive diagnostic test for lower-extremity peripheral arterial disease (PAD) with high validity of the test for stenosis ≥50% in leg arteries (sensitivity ≈95% and specificity ≈100%) (33). The ABI can be easily measured by the means of ordinary blood pressure cuff and a Doppler ultrasonic sensor. The blood pressure cuff is used to measure systolic blood pressure in the brachial artery of both arms by use of the Doppler detector in the antecubital fossa. The blood pressure cuff is then applied to the ankle, and the Doppler probe is used to determine systolic blood pressure at the left and right posterior tibial arteries and dorsalis pedis arteries. The ABI for each leg equals the ratio of the higher of the 2 systolic pressures (posterior tibial or dorsalis pedis) in the leg and the average of the right and left brachial artery pressures, unless there is a discrepancy ≥10 mm Hg in blood pressure values between the 2 arms. An ABI <0.90 in either leg is considered evidence of PAD, and progressively lower ABI values indicate more severe obstruction. Besides an indicative marker for leg artery disease ABI has been shown to serve as a surrogate marker for atherosclerosis having at the same time predictive value future cardiovascular events, independent of established risk factors(34).

All the above mentioned vascular measurements will be performed at each time point, that is on baseline and days 1,2,8,9,15 and 16 of cycle 1 and on days 1,2 of cycle 2 and 3 before and 2 hours after drug administration. The B-Mode ultrasound of the carotid and femoral arteries will only be performed at baseline. On cycle 6 all vascular measurements will be repeated to assess the chronic effect of proteasome inhibition on vascular function and atherosclerosis. Subjects will be instructed to fast overnight, to refrain from smoking, ingesting alcohol or caffeine on the day of testing and to hold any vasoactive medications for 12 h before the imaging studies.

6.2.5 24-hour Ambulatory Blood Pressure Monitoring (ABPM)

Patients will have their blood pressure monitored for 24 hours during the 1st day of cycles 1, 2, 3 and 6.

6.3 Cardiac Ultrasonography

Study participants will undergo a baseline echocardiographic examination before the initiation of the treatment. Standard protocol will be used and standard measurements from 2-D and Doppler echocardiography will be made. LV end-diastolic and end-systolic volumes, as well as ejection fraction will be derived from the apical 4- and 2-chamber views using the biplane Simpson’s rule. Left ventricular mass will be calculated according to Devereux’s formula.

Doppler examination will include interrogation of mitral inflow, and early (E) and late (A) peak diastolic velocities and deceleration time will be measured. Tissue Doppler analysis will include pulse wave interrogation of the medial and lateral mitral annulus, peak diastolic early E´ annular velocities will be obtained and the mean value and E/E' will be calculated. In addition, Speckle-tracking analysis will be applied to estimate LV rotational mechanics, and longitudinal strain parameters. Parasternal short-axis views at the level of the mitral valve and apex, and standard apical views (4-, 2-, 3-chamber) will be recorded for each study participant, according to the recommendations of the European and American Societies of Echocardiography. Five consecutive beats in each view will be stored digitally for off-line analysis. The frame rate will be set at between 50-100 frames/s, the sector width will be set as narrow as possible, and gain settings optimized. Global longitudinal strain and left ventricular twist will be assessed. Evaluation of cardiac function will be performed at baseline and thereafter at 6 months or earlier if a suspicious event occurs necessitating evaluation of cardiac function.

6.4 Study of Proteasome activity

In order to examine the molecular and cellular effects of therapeutic inhibitors in blood cells of MM patients, we will proceed to isolation of Red Blood Cells (RBCs) and Peripheral Blood Mononuclear Cells (PBMCs); these two cell types represent either a non-nucleate relatively "long-lived" proteome (RBCs) or cell lineages with the capacity to mobilize genome responses after proteasome inhibition (PBMCs). The blood samples which will be used in this research will be collected between specific time points of therapeutic proteasomal inhibitors administration. The first day of the treatment (no drug administration) will be used as a control time point.

Blood collection – isolation of cells

PBMCs and RBCs will be collected from whole blood of MM patients by using Ficoll-Paque (density 1.077 ± 0.001 g/mL at 20°C). Specifically, freshly collected heparinized blood will be transferred from each blood collection tube into a 15 mL tube. Blood will be diluted with phosphate-buffered saline [(PBS) 1:1 dilution]. The diluted blood will be carefully layered over the separation medium (2:1) and the two phases will be kept separated before the centrifugation. Subsequently, the tubes will be centrifuged at 400 xg for 30 min, at 20°C, acceleration 9, no brake (braking rate 0). Both cell types will be collected and washed with PBS.

RNA extraction and Quantitative Real-Time PCR (Q-RT-PCR) analyses

Total RNA will be extracted from selected patients PBMCs and will be quantified with BioSpec-nano spectrophotometer (Shimadzu Inc.). RNA (100 ng) will be then converted to cDNA, and Real-time PCR expression analyses of proteasomal genes, as well as of genes involved in cell proteostatic (e.g. autophagy or chaperones) or antioxidant modules, will be

performed using appropriate master mix and the PikoReal 96 Real-Time PCR System. Specific primers will be designed using the primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (35).

Measurement of proteasome peptidase activities in PBMCs and RBCs extracts

For measuring proteasome peptidase activities, blood cells will be lysed on ice by using a buffer suitable for the isolation of 26S proteasome (35-36) and lysates will be centrifuged at 19.000 xg (4 °C). Cleared lysates will be then adjusted with the Bradford method and the proteasome CT-L (LLVY) or C-L (LLE) peptidase activities will be measured by recording (excitation, 350 nm; emission, 440 nm) the hydrolysis of the fluorogenic peptides Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Leu-Glu-AMC (35).

Preparation of cell protein extracts, immunoblotting analyses and detection of protein carbonyl groups

Total proteins (from RBCs or PBMCs) will be extracted using a buffer containing 150 mMNaCl, 1% Nonidet P-40, 0.1 % SDS, 50 mMTris–HCl, pH 8.0. Cell lysate of each sample will be centrifuged for 10 min at 19,000 xg (4 °C). The protein content will be adjusted with the Bradford method and will be then analyzed by SDS-PAGE and immunoblotting [e.g. of total proteome ubiquitination (ubiquitin conjugates) or proteasomal subunits) will be performed as previously described (37). Primary and horseradish peroxidase-conjugated secondary antibodies will be incubated for 1 h at RT. Immunoblots will be developed by an enhanced chemiluminescence reagent kit. Analysis of blots quantification will be performed by scanning densitometry(38).

For the detection of proteome carbonylation (or detection of oxidized proteins) in cell extracts the OxyBlot protein oxidation detection kit (Millipore, Billerica, MA; #s7150) will be used, as per manufacturer's instruction (37-38).

Confocal microscopy

Isolated PBMCs from selected patients will be fixed in 4% formaldehyde in PBS. They will then be transferred into poly-L-lysine coated slides and be permeabilized with 0.1% TritonX-100 as previously described with minor modifications (39). Blocked cells (1% BSA in PBS) will be incubated with primary anti-Ub antibodies (or antibodies against other cellular targets) and appropriate secondary antibody for 1 h at RT. For visualizing nuclei cells will be counterstained with DAPI. Samples will be imaged using a Nikon EZ-C1 confocal microscope equipped with a Nikon Plan APO 60.0×/1.40 oil immersion objective(40).

Measurement of Reactive Oxygen Species (ROS)

For the assessment of ROS production, cells will be incubated with 10 μM CM-H_2DCFDA dye (ThermoFisher Scientific) in PBS for 30 min at 37°C in the dark (35). Following dye removal, cells will be incubated for 10 min with PBS and then, will either be stained with DAPI and viewed at the NIKON C1 CLSM or the produced fluorescence will be measured using the Infinite 200 Tecan microtiter-plate photometer (Tecan Trading AG, Switzerland) at excitation and emission wavelengths of 490 and 520 nm, respectively.

6.5 Measurement of circulating cardiac and vascular inflammatory biomarkers

Plasma and serum samples will be obtained by venipuncture on admission and stored at -70^{o}C. Plasma probes will be centrifuged for 15 minutes at 10.000 g within 30 minutes of

collection. Probes will be aliquoted and stored at -70°C till analysis. The concentrations of C-reactive protein, interleukin 6, vascular cell and intercellular adhesion molecule-1, E-selectin, and monocyte chemoattractant protein-1 will be measured in ethylenediaminetetraacetic acid (EDTA)-plasma at the Oncology Laboratory of the Department of Clinical Therapeutics well-characterized enzyme immunoassays. N-terminal pro-B-type natriuretic peptide will be measured with a sandwich immunoassay on an Elecsys 2010 instrument (Roche Diagnostics). Serum cardiac troponin T concentrations will be measured using statndard procedures.

7. Timelines of the study

Vascular measurements and blood collection will be performed at baseline before and 2 hours after dexamethasone and carfilzomib administration. These measurements will be repeated as shown below:

Schedule of assessments

	Before Dexa	After Dexa	Before & after CarD	After CarD	Before & after CarD	After CarD	Before & after CarD	After CarD	Before & after CarD	After CarD	Before & after CarD	After CarD	Before & after CarD
Time points	d –2		d0	d2	d8	d9	d15	d16	C2d1	d2	C3d1	d2	C6d1or 6 months after baseline
Vascular studies	X	X	X†	X	X†	X	X†	X	X†	X	X†	X	X
24h ambuatory BP monitoring	X		X*		X*				X*		X*		X*
Cardiac US	X												X**
Blood for Proteasome activity	X		X	X	X	X	X?	X	X	X			X†
Serum collection for cardiac biomarkers	X		X†		X†		X†		X†		X		X

* During 24h of CarD

** Only before C6d1

† 2 measurements/ samples at this day

The subjects will be evaluated for AE at all visits.

8. Data presentation and statistical analysis

Data will be presented as mean ± standard deviation (SD). Continuous variables will be tested for normal distribution with the Kolmogorov-Smirnov test. Repeated measures ANOVA will be performed in order to assess significant variations of parameters of interest over time. Linear mixed models analysis will be performed in order to adjust for possible confounders over time. All tests will be two-tailed and statistical significance will be considered for P

values less than 0.05. All statistical analyses will be performed using SPSS version 21 for windows (Chicago, ILL, USA).

9. Safety reporting

9.1 Adverse Events Definitions

An adverse event (AE) is any untoward medical occurrence in a subject administered a medicinal product and that does not necessarily have a causal relationship with this treatment. An AE therefore can be any unfavorable and unintended sign (including laboratory finding), symptom or disease temporally associated with participation in a study, whether or not considered drug-related. In addition to new events, any increase in the severity or frequency of a pre-existing condition that occurs after the subject signs a consent form for participation is considered an AE. This includes any side effect, injury, toxicity, or sensitivity reaction.

The definition of an AE includes:

- Worsening of a pre-existing condition
- Events occurring from a medication error or overdose of a product(s), whether accidental or intentional
- Events occurring from abuse of a product(s)
- Events associated with the discontinuation of the use of a product(s), (eg, appearance of new symptoms)
- Any lack or loss of intended effect of the product(s)

Any condition, laboratory abnormality, or physical finding with an onset date prior to the subject signing consent for study participation is considered to be pre-existing in nature and part of the subject's medical history.

9.2 Causality

Using the following criteria, the relationship of the AE to a drug should be assessed as follows:

- Yes: The event is suspected to be related if:
  - there is a clinically plausible time sequence between onset of the AE and administration of treatment; and/or
  - there is a biologically plausible mechanism for the treatment to cause or contribute to the AE; and/or
  - the event responds to withdrawal of the medication (dechallenge) and/or recurs with rechallenge (when clinically feasible); and/or
  - the AE cannot be reasonably attributed to concurrent/underlying illness, other drugs, or procedures

- No:
  - the AE is more likely to be explained by the subject's clinical state, underlying disease, concomitant medication, study or non-study procedure; and/or
  - the time of occurrence of the AE is not reasonably related to administration of treatment; and/or
  - the event is unlikely to be related to the treatment(s)

9.3 Definition of Adverse Drug Reactions (ADRs)

AEs that are considered related to a product are classified as adverse drug reactions (ADRs).

9.3.1 Definition of Serious Adverse Events

A serious adverse event (SAE) is any AE as defined above that also:

- is fatal
- is life threatening (places the patient at immediate risk of death)
- requires in-patient hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability/incapacity
- is a congenital anomaly/birth defect
- is an "other significant medical hazard" that does not meet any of the above criteria

A hospitalization meeting the regulatory definition for "serious" is any in-patient hospital admission that includes a minimum of an overnight stay in a healthcare facility. "Other significant medical hazards" refer to important medical events that may not be immediately life-threatening or result in death or hospitalization, but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed in the definition above.

Any death occurring within 30 days of the subject receiving study drug, regardless of the subject having discontinued from the study must be reported to the Sponsor as an SAE.

9.3.2 Definition of Serious Adverse Drug Reactions (SADRs)

SAEs that are considered related to any product are classified as serious adverse drug reactions (SADRs).

9.3.3 Definition of Special Situations

Special Situations include:

Medication errors, overdose, misuse, or abuse, whether accidental or intentional, involving any product, regardless of whether associated with an AE, SAE, ADR and/or SADR

Pregnancy and lactation exposure regardless of whether associated with an AE, SAE, ADR and/or SADR

Transmission of infectious agents regardless of whether associated with an AE, SAE,ADR and/or SADR

Reports of uses outside the terms for authorized use of the product including off label use when associated with an AE, SAE, ADR and/or SADR

Occupational exposure

Product complaints

9.4 Adverse Events Reporting Procedures

All AEs (e.g., any new event or worsening in severity or frequency of a pre-existing condition or laboratory finding) with an onset date after the subject signs consent for study participation must be documented on the appropriate summary. Details of the event must include severity, relationship to study drug, duration, action taken, and outcomeAdverse drug reactions (ADRs) will be recorded on the appropriate form.

All AEs that are considered related to study drug must be followed to resolution or stabilization if improvement is not expected.

AEs should be reported from the time the subject signs consent through 30 days post-last dose of study drug or initiation of a new anti-cancer therapy, whichever occurs first. In addition, the Investigators should report any AE that may occur after this time period that is believed to have a reasonable possibility of being associated with study drug. AEs which completely resolve and then recur should be recorded as a new AE. For subjects who complete the end of study visit less than 30 days following their last dose of study drug, a follow up of ongoing AEs should be attempted by telephone, and documented in the subject's source. AEs continuing at 30 days post-last dose should have a comment in the source by the Investigators that the event has stabilized or is not expected to improve.

The Sponsor is responsible for evaluating all AEs, obtaining supporting documents, and determining that documentation of the event is adequate.

All Grade 3 and 4 laboratory abnormalities must be recorded as AEs on the CRF. Grade 1 and 2 abnormalities should only be recorded if they require treatment or are otherwise considered clinically significant by the Investigator.

9.4 Serious Adverse Event Reporting and Documentation Requirements

All SAEs occurring from the time that the subject signs consent for study participation through 30 days after the last administered dose of treatment will be reported. SAEs must be reported by Investigators to the Sponsor's designated point of contact by fax within 24 hours after the event was known to the investigator, using the SAE report form provided. This initial report should contain a minimum amount of information regarding the event, associated

treatment and patient identification, as described in the detail in the instructions. All SAEs regardless of relationship to study drug must be followed to resolution or to stabilization if improvement or resolution is not expected.

If a subject is permanently withdrawn from the study because of a SAE, this information must be included in the initial or follow-up SAE report as well as the appropriate form for Study Discontinuation.

The Sponsor and/or the Investigators are responsible for notifying the appropriate Regulatory Agencies, when required, and in accordance with applicable laws and regulations of any Expedited Safety Reports. Generally, these are all SAEs that are judged to be unexpected and related to study drug(s), as specified in ICH E2B guidelines. The Sponsor and/or Investigators are also responsible for notifying the Institutional Review Board (IRB) or Independent Ethics Committee (IEC) as applicable and in accordance with local regulations, of all SAEs.

The Sponsor is responsible to create a Final study Report (FSR) of the overall conduct of the specific study for distribution to the relevant EC(s) and Regulatory Agencies. The FSR will also be provided to Amgen at the end of the study.

9.6 Special Situations Reporting Requirements

All events of special situations should be recorded on the appropriate form and send to the Sponsor's designated point of contact by fax by the investigator within 24h. The sponsor will report all special situation event identified to the regulatory authorities as per local regulation

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