CLINICAL RESEARCH PROTOCOL

Study Title: *A randomized phase II/III trial of doxycycline vs. standard supportive therapy in newly-diagnosed cardiac AL amyloidosis patients undergoing bortezomib-based therapy*

Protocol Number: AC-012-EU
Investigational Product: Doxycycline
EudraCT Number: 2017-002210-31

Indication: Light Chain (AL) Amyloidosis

Sponsor: Scientific Institute Policlinico San Matteo, University of Pavia, Italy
Institution: Biochemistry, Biotechnology and Advanced Diagnostics Laboratories, Amyloidosis Research and Treatment Center, Foundation Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, and Department of Molecular Medicine, University of Pavia, Pavia, Italy

Principal Investigator: Giovanni Palladini, MD, PhD

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Statistician: Catherine Klersy, MD

Development Phase: Phase 2/3

Date of Original Protocol: 30 May 2017, Version 1.0

SIGNATURE PAGE

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I have read the forgoing protocol and agree to conduct this study as in accordance with the current protocol.

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Investigator Signature Date

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Investigator Print Name

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1 SUMMARY

Systemic amyloidoses are rare diseases affecting approximately 1 in 100,000 persons each year. In systemic amyloidoses abnormal proteins deposit in bodily organs and severely impair their function, causing death if not treated effectively. Light chain (AL) amyloidosis is caused by a usually small population of plasma cells (the cells that produce antibodies). These cells produce part of antibodies, the light chains (LC) that form amyloid deposits. Almost every organ, with the exception of the brain, can be affected by AL amyloidosis. The heart is involved in three fourths of patients and is responsible for almost all the deaths occurring in the first 6 months after diagnosis.

Current therapy of AL amyloidosis is based on drugs targeting the plasma cells producing the amyloid-forming LC. At present, most patients receive a powerful anti-plasma cell drug, bortezomib, as part of their initial treatment. However, bortezomib-based therapy, can improve heart involvement only in less than one third of patients with AL amyloidosis, and many patients (approximately one third) still die within 12 months from diagnosis. Early cardiac deaths remain an acute unmet need and the major determinant of overall outcome in this disease. Thus, there is the need of alternative means to treat heart involvement in AL amyloidosis.

Doxycycline is a widely used, well tolerated, antibiotic that has been marketed for decades and used to treat a number of different infectious diseases caused by bacteria. This molecule has been extensively studied in the laboratory, in animal models and, more recently, in small studies involving patients, for its potential of improving cardiac damage in amyloidosis. These studies showed that doxycycline disrupts amyloid deposits, reduces the amyloid load in a mouse model, and counteracts the toxicity exerted by amyloid-forming LCs on C. elegans, a worm whose pharynx is used as a model resembling human heart. In a small clinical study, doxycycline was given to patients with cardiac AL amyloidosis during treatment for their underlying plasma cell disease. This resulted in a remarkable improvement of survival compared to “matched historical controls” (i.e. similar patients who had received only anti-plasma cell therapy without doxycycline in the past).

Based on these promising preliminary results, we designed the present clinical trial to assess whether the addition of doxycycline to anti-plasma cell therapy can improve survival in patients with cardiac AL amyloidosis who were not previously treated. The rate of survival at 12 months will be compared in patients receiving doxycycline and in controls receiving standard antibiotic therapy, together with anti-plasma cell therapy.

Patients will be assessed for parameters of plasma cell disease, heart involvement and possible involvement of other organs, as well as for quality of life. To make sure that patients who will receive doxycycline and those who will not have comparable severity of cardiac disease, patients will be stratified according to the stage of cardiac involvement. Patients with very advanced heart dysfunction will not be enrolled in the trial, because preliminary data indicate that doxycycline is of little or no benefit in these subjects.

Patients will be randomized to receive doxycycline or standard antibiotics in combination with anti-plasma cell therapy. Bortezomib-based treatment directed against plasma cells will be delivered according to each participating institutions’ guidelines. Doxycycline will be administered at a dosage of 100 mg two times a day, which is usual in the treatment of bacterial diseases. Standard antibiotics will be delivered according to each participating institutions’ guidelines (provided that drugs of the same class as doxycycline are not administered) in the control arm. Patients will be provided a diary to record possible adverse events and will be instructed accordingly. Patients will be evaluated at trial centers every 2 months for treatment efficacy and toxicity. In case of unsatisfactory response second-line therapy will be initiated. In the absence of unacceptable toxicity, doxycycline administration will be continued for the entire duration of follow-up (12 months).

2 PROTOCOL SYNOPSIS

Principal/Coordinating Investigator	Giovanni Palladini, MD, PhD Foundation "Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo", Amyloidosis Research and Treatment Center Viale Golgi, 19 – 27100 Pavia, Italy Telephone: +39-0382-502994 Fax: +39-0382-502990 E-mail address: giovanni.palladini@unipv.it
Title of clinical trial	*A randomized phase II/III trial of doxycycline vs. standard supportive therapy in newly-diagnosed cardiac AL amyloidosis patients undergoing bortezomib-based therapy*
Clinical trial type and phase	*This will be an open-label parallel-group randomized (1:1) trial. Control patients receive standard supportive therapy.*
Objective(s)	*The trial aims at establishing whether the addition of the antibiotic doxycycline to anti-plasma cell therapy can reduce early mortality in newly-diagnosed patients with cardiac AL amyloidosis.* *Cardiac involvement is responsible for almost all the early deaths in AL amyloidosis. Therapy solely aimed at the underlying disease can rescue only a minority of patients with cardiac AL amyloidosis, whose treatment remains a largely unmet need. The severity of cardiac involvement is accurately assessed by a staging system based on cardiac biomarkers. Patients with stage II/IIIa cardiac involvement will be enrolled in this study. The 12-month cumulative proportion survival of stage II/IIIa patients treated with bortezomib-based combination is approximately 65%, ranging from 60% to 70% in different published series including more than 1,000 patients (Palladini, et al. Blood 2015; Palladini, et al. ASH 2015).* *Pre-clinical studies indicate that doxycycline 1) disrupts amyloid fibrils in vitro, 2) reduces the amyloid load in a transgenic mouse model, and 3) is capable of counteracting the proteotoxicity of amyloidogenic light chains in a C. elegans model (Cardoso, et al. FASEB J 2006; Diomede, et al. Blood 2014). A recent retrospective matched case-control study showed that the addition of doxycycline to specific therapy improved survival of stage II/IIIa patients to 95% (Wechalekar, et al. ASH 2015).* The **primary objective** is the 1-year survival. **Secondary objectives are:** • Safety of doxycycline treatment • Rate of infections • Cardiac and renal response • Hematologic response
Intervention(s)	**Experimental intervention:** doxycycline (100 mg bid)

	Control intervention: antibiotic prophylaxis as per standard practice (tetracyclines prohibited) **Duration of intervention per patient:** 12 months **Duration of follow-up per patient:** 6 months
Key inclusion and exclusion criteria	**Key inclusion criteria:** • newly-diagnosed AL amyloidosis • stage II/IIIa heart involvement • age ≥18 years • planned bortezomib-based therapy • total bilirubin <1.5 × upper reference limit (url), patients with Gilbert disease who have a total bilirubin, predominantly unconjugated >1.5 × url without any other liver function test abnormalities are still eligible • alkaline phosphatase <5 × url • alanine aminotransferase <3 × url • not eligible for ASCT. Patients who are eligible for high-dose chemotherapy and ASCT but decline the procedure, can be enrolled in the study **Key exclusion criteria:** • non-AL amyloidosis • previous treatment for AL amyloidosis • pregnant or nursing women • uncontrolled infection • active malignancy • known hypersensitivity to doxycycline, bortezomib, boron, or mannitol • treatment with drugs potentially affecting doxycycline absorption • significant acute gastrointestinal symptoms • active peptic ulceration and/or esophageal reflux disease • contraindications to bortezomib based therapy
Endpoint(s)	**Primary endpoint:** • proportion surviving at 12 months **Secondary endpoints:** • safety, i.e. rate of severe (CTCAE v5.0) grade 3 or greater adverse events) • rate of infective adverse events of any grade • cardiac response (as per consensus criteria) at 2, 4, 6 and 12 months

	• hematologic response (as per consensus criteria) at 2, 4, 6 and 12 months • renal response (as per consensus criteria) at 2, 4, 6 and 12 months
Sample size	This is a multicenter trial. Number of patients to be assessed for eligibility: 180 Number of patients to be allocated to the trial: 120 Number of patients to be analysed: 120
Statistical analysis	**Calculation of sample size:** The sample size was computed based on the primary endpoint according to the following hypotheses: - a type I error of 5% (2-tailed) - a power of 80% - an attrition of 10% - a proportion of patients surviving at 12 months in the control arm 65% - an expected proportion surviving at 12 months in the active arm of 88% (HR 0.28; 26 events). Based on these hypotheses 120 patients (60 per arm) will need to be enrolled. **Randomization:** A randomization list with random block size will be generated with Stata (Stata Corp, College Station, TX, USA). Randomization will be stratified by stage (II and IIIa) and will be web-based. **Description of the primary efficacy analysis and population:** The ITT population includes all patients randomized and will be used for the analysis of secondary endpoints. The per protocol population includes patients randomized and without major deviations from the protocol and will be used for a sensitivity analysis for the primary endpoint. Cumulative survival at 12 months will be computed together with 95% confidence interval with the Kaplan Meier method. The stratified logrank test will be used to compare survival. The hazard ratio and 95% confidence interval (CI) will be computed from a Cox model to assess the primary endpoint. The stratification factor used at randomization will be accounted for. **Safety:** The rate of severe adverse events will be tabulated as count and percent, separately for each treatment arm. **Secondary endpoints:** The rate of infective events will be reported with Poisson 95%CI separately for each treatment arm and compared with a Poisson (or negative binomial regression in case of overdispersion); incidence

	rate ratios (IRR) and 95%CI will be reported. The stratification factor used at randomization will be accounted for. The proportion of patients with cardiac, hematologic and renal response at 3, 6 and 12 months will be summarized by treatment arm. The difference between proportions and 95%CI will be computed with a generalized binomial linear model. The stratification factor used at randomization will be accounted for. Patients dying will be considered as non-responders. Stata 14 (Stata Corp, College Station, TX, USA) will be used for computation.
Trial duration	First patient in to last patient out (months): 29 Recruitment period (months): 17 Duration of the entire trial (months): 30
Key words	*Amyloidosis, cardiomyopathy, heart failure, doxycycline, therapy, unmet needs*

3 INTRODUCTION

3.1 THE MEDICAL PROBLEM

Light chain (AL) amyloidosis is a protein conformational disease, caused by a small bone marrow plasma cell clone producing light chains (LCs) that undergo conformational changes, aggregate and deposit in tissues in the form of amyloid fibrils. This process causes dysfunction of the organs involved and leads to death if not effectively treated. The great majority of early deaths are due to heart involvement, and the prognosis of AL amyloidosis is determined by the presence and severity of heart dysfunction (Merlini, *et al* 2013). Accurate staging systems exist that are based on biomarkers of cardiac dysfunction and damage, N-terminal pro-natriuretic peptide type-B (NT-proBNP) and troponins and are used for patient stratification in clinical trials (Table 1) (Dispenzieri, *et al* 2004, Wechalekar, *et al* 2013).

Table 1. Prognostic stratification of patients with AL amyloidosis based on cardiac biomarkers

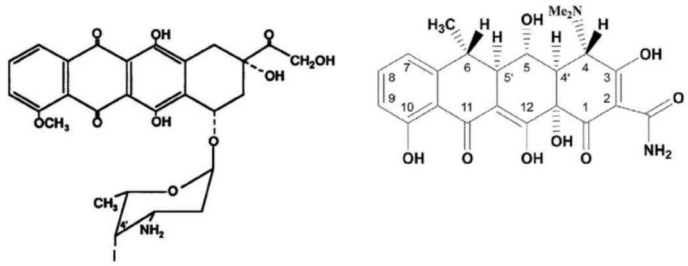
Markers and thresholds	Stages
• NT-proBNP >332 ng/L [>8500 ng/L to discriminate between stages IIIa and IIIb] • cTnT >0.035 ng/mL (or cTnI > 0.1 ng/mL or hs-cTnT >77 ng/L)	I. no markers above the cutoff II. one marker above the cutoff IIIa. both markers above the cutoff and NT-proBNP ≤8500 ng/L IIIb. both markers above the cutoff and NT-proBNP >8500 ng/L

Until now, the treatment of AL amyloidosis has been based on anti-plasma cell treatment. Despite a progressive improvement in long-term outcome due to the introduction of novel agents, particularly bortezomib combinations, the rate of early cardiac deaths has not improved, and the treatment of cardiac AL amyloidosis remains a largely unmet need (Merlini and Palladini 2013). Currently, the most commonly used therapeutic approaches for cardiac AL amyloidosis patients are combinations of alkylating agents, more frequently cyclophosphamide, dexamethasone and bortezomib (CyBorD). We have assessed the efficacy of this combination in a large study including 230 patients (Palladini, *et al* 2015). In this study the outcome was largely dependent on the severity of heart involvement, and a response of amyloid cardiac dysfunction was observed in less than one third of patients with this combination. Thus, there is the need of exploring and developing novel alternative treatment strategies for this condition.

3.2 BACKGROUND + ADDENDUM 1

In 1995 we serendipitously discovered the anthracycline 4'-iodo-4'-deoxydoxorubicin (IDOX) as the prototype of a class of compounds able to inhibit protein aggregation in vitro and in animal model of systemic amyloidosis (Merlini, *et al* 1995) and in patients (Gertz, *et al* 2002, Gianni, *et al* 1995). The first study showed that IDOX presented high affinity for all types of amyloid deposits, and would have been a candidate for the treatment of all types of amyloidoses (Merlini, *et al* 1995). Accordingly, an international patent on the use of IDOX in all types of amyloidosis was deposited (n. WO9504538). In consideration of the cytotoxicity of IDOX, in the following years, tetracycline antibiotics were investigated on the basis of structural homologies with the aglycone moiety of the anthracyclines, as shown in Figure 1.

Figure 1. Structures of iododoxorubicin and doxycyclin showing the resemblance of the polycyclic conjugated structure.



4'-iodo-4'-deoxydoxorubicin Doxycycline

Figure 1 Structures of iododoxorubicin and doxycycline

A considerable and growing body of evidence exists supporting the role of the marketed antibody doxycycline as a possible non-chemotherapy drug targeting amyloid deposits and their precursors in several unrelated forms of amyloidosis including AL amyloidosis. The following anti-amyloid effects have been demonstrated for doxycycline in vitro and in animal models by us and other groups:

- disruption of transthyretin amyloid fibrils in vitro, resulting in complete disaggregation of the fibrils (Cardoso, *et al* 2003b);
- disruption of transthyretin amyloid deposits and reduction of the amyloid load in a transgenic mouse model (Cardoso, *et al* 2010, Cardoso and Saraiva 2006);
- prevention of amyloid β fibrillization and toxicity in a Drosophila melanogaster model of Alzheimer disease (Costa, *et al* 2011);
- capability of counteracting the proteotoxicity of amyloidogenic LCs a C. elegans model in which amyloidogenic LCs from patients with heart involvement induce a reduction of the pumping of the worm's pharynx, an orthologue of vertebrates' heart with autonomous contractile activity, reminiscent of cardiac myocytes (Diomede, *et al* 2014).

3.3 STUDY RATIONALE

Based on this pre-clinical data, small preliminary clinical studies have been performed to assess the role of doxycycline in AL amyloidosis.

- In a retrospective study involving 106 cases and 349 controls, Kumar et al. showed that doxycycline used as post stem cell transplant antibacterial prophylaxis improved survival in patients with AL amyloidosis, compared to standard antibiotic prophylaxis with penicillin (Kumar 2012). In this study, enrolling stem cell transplant-eligible patients at low-risk of early death, there was a late survival benefit in the doxycycline arm.
- Investigators from the UK reported that the addition of doxycycline to chemotherapy improved survival of patients with stage II/IIIa cardiac AL amyloidosis in a retrospective matched case-control study, involving 30 cases and 73 controls (, Wechalekar, *et al* 2017). In this report, early deaths were almost abolished in stage II/IIIa patients receiving doxycycline, with a 95% survival at 6 and 12 months, compared with only 60% in subjects who did not receive the drug. Unfortunately a similar improvement was not observed in patients with very advanced, probably irreversible heart involvement.

This independent, concordant pre-clinical and clinical evidence prompted us to design the present study to assess the impact of the addition of doxycycline to standard therapy for cardiac AL amyloidosis in a controlled, prospective clinical trial. Doxycycline is a marketed antibiotic that has been used to treat bacterial infections for decades. Its toxicity profile is very well established and favorable. In the clinical studies in AL amyloidosis, doxycycline was used at a dosage (100 mg bid) that is commonly used in clinical practice. The drug can be safely administered to patients with heart dysfunction. Antibiotic prophylaxis is commonly used during anti-plasma cell therapy in AL amyloidosis. The cost of doxycycline is low compared to that of current anti-amyloid therapy. Based on these premises, the introduction of doxycycline in the treatment of AL amyloidosis could be done with a minimal change in practice, with an expected low added toxicity, and at a very low cost. Yet, if the preliminary data will be confirmed by the present prospective study, repurposing doxycycline in the treatment of AL amyloidosis could result in a significant reduction of early mortality, eventually addressing one of the most important unmet needs in this disease.

3.3.1 Clinical Safety

Doxycycline is a tetracycline antibiotic agent approved for use in several common bacterial infections. Its use is well established and the drug is marketed and readily available throughout Europe. The dosage proposed in the present study (100 mg every 12 hours) is recommended for approved indications. Administration at this dosage for long period of times (up to 9 months) is indicated in several diseases (anthrax prophylaxis, bartonellosis, periodontitis).

Adverse effects of doxycycline are listed in Tables 1 and 2.

Table 2 Common adverse effects of doxycycline

Adverse event	Frequency
Nausea	8-13%
Vomiting	8%
Myalgia	6%
Bacterial vaginosis (chlamidia)	3%

Table 3 Severe adverse effects of doxycycline

Adverse event	Frequency	Notes
Drug hypersensitivity syndrome	rare	Drug rash with eosinophilia and systemic symptoms (DRESS) has occurred with tetracycline therapy
Erythema multiforme	rare	
Photosensitivity	rare	Doxycycline has the potential for causing phototoxic reactions, and photosensitivity reactions have been reported in patients taking tetracycline antibiotics
Stevens-Johnson syndrome	rare	
Toxic epidermal necrolysis	rare	
Clostridium difficile diarrhea	rare	Clostridium difficile-associated diarrhea (CDAD), ranging from mild diarrhea to fatal colitis, has been reported with use of almost every antibiotic, including doxycycline.
Hepatotoxicity	rare	
Anaphylaxis	rare	Hypersensitivity reactions such as urticaria, angioneurotic edema, anaphylaxis, anaphylactoid purpura, pericarditis, exacerbation of systemic lupus erythematosus, and serum

		sickness have been reported in patients taking tetracycline antibiotics
Superinfection	rare	Overgrowth of fungi and other non-susceptible organisms may occur with almost every antibiotic treatment
Arrest of bone development and/or growth	rare	A decrease in fibular growth rate was seen in premature infants treated with tetracycline 25 mg orally every 6 hours, which was reversible upon drug discontinuation
Pseudotumor cerebri	rare	Intracranial hypertension (pseudotumor cerebri), manifesting with symptoms of headache, papilledema, blurred vision, diplopia, and vision loss, has developed with the use of doxycycline and other tetracyclines. Women of childbearing age who are overweight or with a history of intracranial hypertension are at increased risk. Intracranial hypertension usually resolves after treatment discontinuation, but pressure may remain elevated for weeks.

Precautions for doxycycline photosensitivity

In the present study patients will be instructed to avoid excessive exposure to sunlight and artificial ultraviolet light, and to use sunscreens. Doxycycline should be discontinued if erythema greater than grade 2 occurs.

Precautions for Clostridium difficile diarrhea

Patients will be instructed to report the occurrence of diarrhea during treatment. Patients who experience diarrhea following doxycycline use, including occurrence more than 2 months after administration, will be evaluated for CDAD.

4 STUDY PLAN

4.1 DESCRIPTION OF INTERVENTION

In this study the experimental intervention will be the administration of doxycycline at the dosage of 100 mg bid. This dosage was used and proved effective in the preliminary clinical studies in cardiac AL amyloidosis (Wechalekar, *et al* 2017). This is also the standard dosage of this antibiotic that is safely administered in antibacterial therapy. Doxycycline will be administered concomitantly with anti-plasma cell therapy for AL amyloidosis. In the control arm, non-tetracycline standard antibiotic prophylaxis will be administered (Table 3).

Bortezomib-based combinations with risk-adapted patient-tailored schedules are the most common anti-plasma cell therapies currently used to treat AL amyloidosis with heart involvement (Merlini, *et al* Blood 2013; Palladini, et al. Blood 2015). The choice of the specific combination and dosage in each patient is done according to standard institutional practice.

The choice of the standard antibiotic prophylaxis in the control arm will be made according to institutional guidelines. Tetracyclines will not be allowed.

Table 4 Scheme of intervention

Treatment arm	Drug	Dose	Route of administration
Intervention	Doxycycline	100 mg bid	oral
Control	Standard antibiotic	per institutional protocol	oral

The trial primary endpoint is survival at 12 months. The duration of intervention will be 12 month. Cycles of bortezomib-based anti-plasma cell therapy will be continued based on response to be evaluated every 2 months according to current validated criteria (Palladini, *et al.* J Clin Oncol 2012). In case of unsatisfactory response (i.e. less than very good partial response or partial response plus organ response) a switch to a second line treatment regime will be allowed. In the absence of unacceptable toxicity, doxycycline administration will be continued for the entire duration of follow-up (12 months). Organ responses after frontline chemotherapy can occur as late as one year after treatment initiation. Since the activity of doxycycline therapy is expected to facilitate cardiac response and increase its rate, we elected to continue administration during the whole time period heart responses can occur (Muchtar et al., Leukemia 2018).

The study is based on the hypothesis that the addition of doxycycline to standard therapy directed against the amyloidogenic plasma cells can significantly improve survival in cardiac AL amyloidosis by reducing the proportion of patients who die in the first few months after diagnosis.

The primary endpoint of the trial will be survival at 12 months. Cumulative proportion survival at 12 months in the intervention and control groups will be compared as described in the statistical analysis section.

The secondary endpoints will be safety (with special attention to the rate of infective adverse events), and hematologic and organ response to treatment in the two arms.

For safety assessment, the rate of CTC grade 3 or greater adverse events will be compared in the two groups. To take into account the possible effect of different antibiotic prophylaxis, the rate of infective events of any grade will also be compared in the two groups.

Hematologic and organ response will be assessed at 2, 4, 6 and 12 months as per current, validated consensus criteria (Table 4).

Table 5 Validated criteria for early response assessment in AL amyloidosis (Palladini, et al. J Clin Oncol 2012; Palladini, et al. Blood 2014)

Response criteria	Definition
Hematologic response • Complete response • Very good partial response • Partial response • No response	 • Negative serum and urine immunofixation and normal FLC ratio • dFLC <40 mg/L • FLC decrease >50% compared to baseline • All other patients
Cardiac response	Decrease of NT-proBNP by >30% and 300 ng/L (if baseline NT-proBNP >650 ng/L), or at least 2 point decrease of NYHA class (if baseline NYHA class is III or IV)
Renal response	At least 30% decrease in proteinuria or drop below 0.5 g/24 hour, in the absence of renal progression defined as a >25% decrease in eGFR

• eGFR, estimated glomerular filtration rate; dFLC, difference between involved (amyloidogenic) and uninvolved circulating free light chain; FLC, circulating free light chain; NT-proBNP, N-terminal pro-natriuretic peptide type-B; NYHA, New York Heart Association.

4.2 STUDY DESIGN

Treatment-naïve patients with stage II/IIIa cardiac AL amyloidosis will be enrolled in this trial. After written informed consent is obtained, patients will be assessed for eligibility. Baseline evaluation will include assessment of the amyloidogenic plasma cell clone (serum

and urine immunofixation, measurement of circulating free light chains, bone marrow studies), amyloid related organ involvement (echocardiography, cardiac biomarkers, estimated glomerular filtration rate, proteinuria, liver function tests), and quality of life. Patients will be stratified according to cardiac stage (II or IIIa) and randomized to receive doxycycline or standard antibiotic prophylaxis during anti-plasma cell therapy. Staging is based on the cardiac biomarkers NT-proBNP (cutoff 332 ng/L) and troponin (cutoffs 0.1 ng/mL for cTnI, 0.035 ng/mL for cTnT, 77 ng/L for high-sensitivity cTnT), Stage II and III patients have one or both biomarkers above the cutoff, respectively. Stage III patients whose NT-proBNP is >8500 ng/L (stage IIIb) are not eligible. Treatment directed against the amyloidogenic plasma cell clone will be delivered according to each participating institutions' guidelines. Doxycycline will be administered at a dosage of 100 mg bid. Standard antibiotic prophylaxis will be delivered according to each participating institutions' guidelines (provided that tetracyclines are not administered) in the control arm. To minimize discomfort for patients, administration of therapy at local hospitals is allowed. Patients will be provided a diary to record possible adverse events and will be instructed accordingly. Patients will be evaluated at trial centers at least every 2 months for response and toxicity. Response will be assessed according to current consensus criteria (Palladini, et al J Clin Oncol 2012). In case of unsatisfactory response (i.e. <very good partial response or partial response plus organ response) a switch to a second line treatment regime will be allowed. In the absence of unacceptable toxicity, doxycycline administration will be continued for the entire duration of follow-up (12 months).

4.3 ENDPOINTS

4.3.1 Primary

- proportion surviving at 12 months

4.3.2 Secondary

- safety, i.e. rate of severe (CTCAE v4.0) grade 3 or greater adverse events
- rate of infective adverse events of any grade
- cardiac response (as per consensus criteria) at 2, 4, 6 and 12 months
- hematologic response (as per consensus criteria) at 2, 4, 6 and 12 months
- renal response (as per consensus criteria) at 2, 4, 6 and 12 months

4.4 NUMBER OF SITES AND SUBJECTS

The study is a multicenter study and will involve 120 subjects. Centers participating are listed below.

1. Amyloidosis Research and Treatment Center, Foundation "IRCCS Policlinico San Matteo", Pavia, Italy
2. Cross Cancer Institute, University of Alberta; Edmonton AL, Canada
3. Service d'Hématologie et de Thérapie Cellulaire, Centre de Référence des Amyloses Primitives et des Autres Maladies par Dépôts d'Immunoglobuline, Limoges, France
4. Amyloidosis Center, University Hospital, Heidelberg, Germany
5. Department of Hematology, Hospital Clínic, Barcelona, Spain
6. Istanbul University Cerrahpasa Faculty of Medicine, Department of Internal Medicine, Division of Hematology, Istanbul, Turkey
7. Alexandra Hospital, University of Athens School of Medicine, Department of Clinical Therapeutics, Athens, Greece
8. National Amyloidosis Centre, University College London (Royal Free Campus), London, UK

4.5 ESTIMATED STUDY DURATION

Each subject's study duration may be up to 19 months, consisting of a Screening/Baseline phase (1 month), a Treatment phase (12 months), and a Follow-up phase (6 months). A subject's participation in the study will begin with a 28-day Screening period; if assessed as eligible for enrollment, the subject will receive study drug. Subjects will remain on study until study completion, which will occur when the subject completes the 6-Month Follow-up Visit.

The duration of the entire trial will be 30 months.

4.6 DEFINITION OF END OF STUDY

The study will end when the last subject discontinues study treatment and completes the 6-Month Follow-up Visit.

4.7 TERMINATION OF THE CLINICAL STUDY

If the Investigator becomes aware of conditions or events that suggest a possible hazard to subjects if the clinical study continues, then the clinical study may be terminated.

Conditions that may warrant termination of the clinical study include, but are not limited to:

- The discovery of an unexpected, relevant, or unacceptable risk to the subjects enrolled in the clinical study
- A decision by the Sponsor to suspend the study, or to suspend or discontinue development of the study drug, for any reason.

4.8 BIAS PROTECTION

This is a controlled randomized clinical trial with parallel groups (interventional and control). To assure ethical conduct of the study, the control group will receive state-of-the-art treatment plus standard antibiotic prophylaxis, while the interventional group will receive the proposed treatment (doxycycline) on top of the state-of-the-art-treatment.

A randomization list with random size blocks will be prepared by the Scientific Department of the San Matteo Hospital, Service of Biometry and Statistics. The randomization list will be implemented in Stata (StataCorp, College Station, TX, USA) and the corresponding code will be saved in a "do-file" stored in a password-protected computer. A predefined seed will be used to allow repeatability of the random list generation.

The randomization will be stratified by cardiac stage (stages II and IIIa allowed), which is by far the most relevant prognostic factor in AL amyloidosis.

Randomization will be web-based. The investigator will need to confirm that all eligibility criteria are satisfied and that the informed consent has been signed before being able to access to the randomization phase. A document with the group assignment will be generated for each patient and will need to be printed and stored by each investigator.

Blinding was not deemed possible in the present study, because patients in the control arm will receive standard antibiotic prophylaxis which will vary according to institutional guidelines. The type and dosages of antibacterial treatment in the control arm will be recorded. To account for possible interference, the rate of infective adverse events of any grade will be attentively recorded and analyzed.

The primary endpoint being all-cause death, biased assessment of the results is not possible. As for secondary endpoints, the statistician performing the analysis will be blinded.

5 SELECTION, DISCONTINUATION AND WITHDRAWAL OF SUBJECTS

5.1 INCLUSION CRITERIA

Subjects must meet ***all*** of the following criteria:

1. Age ≥ 18.
2. newly-diagnosed AL amyloidosis.
3. Confirmed diagnoses of AL amyloidosis by the following:
    a) histochemical diagnoses of AL amyloidosis determined by polarizing light microscopy of green birefringent material in Congo red stained issue specimens OR characteristic electron microscopy appearance AND
    b) Confirmatory electron microscopy immunohistochemistry OR mass spectroscopy of AL amyloidosis. Confirmation of amyloid type can be omitted in patients with a clear-cut clinical evidence of AL amyloidosis (e.g. cardiac and renal involvement, soft tissue involvement) in the presence of a monoclonal component.
4. Cardiac involvement as defined by ALL of the following:

    a) Either an endomyocardial biopsy consistent with AL amyloidosis OR an echocardiogram demonstrating a mean left ventricular wall thickness in diastole >12 mm in the absence of other causes (e.g., severe hypertension, aortic stenosis) which would adequately explain the degree of wall thickening .

    b) Cardiac stage II disease: either cTnT > 0.035 ng/mL (or in place of cTnT the cTnI > 0.10 ng/mL or hs-cTnT >77 ng/L) or simultaneous NT-proBNP >332 ng/L OR patients with cardiac stage IIIa: both cTnT > 0.035 ng/mL (or in place of cTnT the cTnI > 0.10 ng/mL or hs-cTnT >77 ng/L) and simultaneous NT-proBNP >332 ng/L and NT-proBNP ≤8500 ng/L.
5. Planned bortezomib-based therapy.
6. Total bilirubin <1.5 × upper reference limit (url), patients with Gilbert disease who have a total bilirubin, predominantly unconjugated >1.5 × url without any other liver function test abnormalities are still eligible.
7. Alkaline phosphatase <5 × url.
8. Alanine aminotransferase <3 × url.
9. Systolic blood pressure 90-180 mmHg.
10. Women of childbearing potential (WOCBP) must have a negative serum pregnancy test within 14 days prior to the first administration of study drug and perform a pregnancy test every 4 weeks to rule out pregnancy, they must agree to use highly effective physician-approved contraception 30 days prior to the first study drug administration.

    Highly-effective contraceptive methods with a Pearl Index lower than 1 are: Oral hormonal contraception (‘pill’) (as far as its efficacy is not expected to be impaired during the trial, e.g. with IMPs that cause vomiting and diarrhoea or interfere with hormone metabolism, adequate safety cannot be assumed), Dermal hormonal contraception (e.g. contraceptive patch), Vaginal hormonal contraception (NuvaRing®), Long-acting injectable contraceptives, Tubal ligation (female sterilisation), Double barrier methods.

    This means that the following are not regarded as safe: condom plus spermicide, simple barrier methods (vaginal pessaries, condom, female condoms), copper spirals, the rhythm method, basal temperature method, and the withdrawal method (coitus interruptus).

    The following duration of highly effective contraception is necessary: Bortezomib: during and until 3 months after the end of therapy, Melphalan: during and 6 months after the end of therapy, Cyclophosphamide: during and 12 months after the end of therapy

11. Males must be surgically sterile or must agree to use highly effective physician-approved contraception from 30 days prior to the first study drug administration to 90 days following the last study drug administration.
12. Ability to understand and willingness to sign an informed consent form prior to initiation of any study procedures.
13. Patient was assessed to determine ineligibility for ASCT. Patients who are eligible for high-dose chemotherapy and ASCT but decline the procedure, can be enrolled in the study.

5.2 EXCLUSION CRITERIA

Subjects must meet ***none*** of the following criteria:

1. Non-AL amyloidosis.
2. Stage IIIb (NT-proBNP >8500 ng/L and cTnI >0.1 ng/mL, or cTnT >0.035 ng/mL, or hs-cTnT >77 ng/L.
3. Previous treatment for AL amyloidosis.
4. Clinically overt multiple myeloma with lytic bone lesions.
5. Symptomatic orthostatic hypotension that in the medical judgment of the Investigator would interfere with subject's ability to safely receive treatment or complete study assessments.
6. Patients with uncontrolled infection or active malignancy with the exception of adequately treated basal cell or squamous cell skin cancer, in situ cervical cancer, adequately treated Stage I cancer from which the patient is currently in complete remission, or any other cancer from which the patient has been disease-free for 5 years.
7. Known HIV positive.
8. Pregnant or nursing women.
9. Known hypersensitivity to doxycycline, bortezomib, boron, or mannitol.
10. Treatment with drugs potentially affecting doxycycline absorption.
11. Significant acute gastrointestinal symptoms.
12. Active peptic ulceration and/or esophageal reflux disease.
13. Patients with serious medical or psychiatric illness likely to interfere with participation in this clinical study.
14. Contraindication to bortezomib based therapy

5.3 EARLY TREATMENT DISCONTINUATION

If the subject discontinues study drug prior to the EOT Visit, they should return for an ETD Visit 28-35 days after their final administration of study drug.

Early treatment discontinuation will occur if:

- Subject experiences unacceptable toxicity
- Subject experiences immunologic reaction
- Subject becomes pregnant

5.4 EARLY TERMINATION FROM THE STUDY

Subject participation in this study will continue until the end of the study (i.e., 12 months of treatment and 6 months of follow-up). Early termination occurs if the subject fails to complete the entire study, through the 6-Month Follow-up Visit. Subjects may withdraw their consent to participate in this study at any time without prejudice. The Investigator must

withdraw from the study any subject who requests to be withdrawn. A subject's participation in the study may be discontinued at any time at the discretion of the Investigator in accordance with his/her clinical judgment.

Early termination from the study may occur if:

- In the opinion of the Investigator, the subject cannot safely participate in the procedures required by the protocol
- Subject withdraws consent
- Subject is unwilling or unable to comply with the study requirements
- Unacceptable toxicity
- Subject is lost to follow-up

6 INVESTIGATIONAL MEDICINAL PRODUCT AND TREATMENT

6.1 DOSAGE, ADMINISTRATION AND SCHEDULE

Doxycycline administration will be scheduled 100 mg everyday twice daily.

Sites will be responsible of buying their own investigational product supplies.

6.2 CONCOMITANT THERAPY

Allowed

- Radiation therapy for the removal of local amyloid deposits is allowed.

Prohibited

- ASCT
- Other investigational agents

7 STUDY PROCEDURES

All assessments will be conducted in-person at the clinic.

7.1 STUDY EVALUATIONS

Patients will be evaluated with complete history and physical examination within 4 weeks of registration and with tests and studies at each visit as delineated below. Baseline history and physical examination will be performed by the patient's physician during the each visit and will include orthostatic vital signs, height and weight, and performance status assessment.

Evaluations include assessment of organ system involvement with amyloid and performance status assessment. Tests and studies will also be obtained to assess the clonal plasma cell disease. Genetic tests for hereditary amyloidosis as indicated will be performed at national coordinating centers as screening procedures.

Baseline pathology tests will include:

- abdominal fat pad aspirate or
- minor salivary gland biopsy or
- rectal biopsy or
- involved organ biopsy.

Amyloid tissue typing will be obtained by mass-spectrometry or by immunohistochemistry.

Blood tests will include:

- freelite test;
- complete blood count with differential;
- PT/PTT;
- comprehensive panel plasma glucose, serum creatinine, urea, ALT, AST, alkaline phosphatase, bilirubin, albumin and γGT;
- cardiac troponin T (cTnT) or cardiac Troponin I (cTnI) or high-sensitivity Troponin T (hs-cTnT), N-terminal pro-natriuretic peptide type B (NT-proBNP);
- serum pregnancy test;
- blood test for HIV.

Cardiac and pulmonary tests will include:

- echocardiogram to estimate mean left ventricular wall thickness and assess systolic and diastolic function and evidence of restrictive physiologic changes;
- electrocardiogram;

Renal tests will include:

- creatinine;
- 24 hour urine collection for total volume, total protein, urinary albumin to creatinine ratio, protein electrophoresis, and immunofixation.

Neurologic evaluation will include:

- Orthostatic vital signs;
- Neurologic exam;

Other tests, such as formal autonomic or endocrine testing, cardiac stress test or electrophysiologic studies, or ultrasound or CT or MRI for liver size in patients with hepatomegaly, 24-hour Holter monitor and chest x-ray may be obtained as clinically indicated.

Cardiac stage II disease: either cTnT > 0.035 ng/mL (or in place of cTnT the cTnI > 0.10 ng/mL or hs-cTnT >77 ng/L) or simultaneous NT-proBNP >332 ng/L and patients with cardiac stage IIIa: both cTnT > 0.035 ng/mL (or in place of cTnT the cTnI > 0.10 ng/mL or or hs-cTnT >77 ng/L) and simultaneous NT-proBNP >332 ng/L and NT-proBNP ≤8500 ng/L

The Investigators may elect to repeat seemingly spurious results.

7.1.1 Amyloid-related organ involvement

At study entry, the extent of amyloid-related organ involvement is evaluated in each patient according to standard criteria. On follow-up evaluations after treatment, amyloid-related organ involvement is assessed as improved, stable or worsened.

- Renal involvement is defined as non-Bence Jones proteinuria >0.5 g/day (predominantly albumin) in a 24-hour urine collection.
- Cardiac involvement is defined as

    a) Either an endomyocardial biopsy consistent with AL amyloidosis OR an echocardiogram demonstrating a mean left ventricular wall thickness in diastole >12 mm in the absence of other causes (e.g., severe hypertension, aortic stenosis) which would adequately explain the degree of wall thickening

b) Cardiac stage II disease: either cTnT > 0.035 ng/mL (or in place of cTnT the cTnI > 0.10 ng/mL) or simultaneous NT-proBNP >332 ng/L OR patients with cardiac stage IIIa: both cTnT > 0.035 ng/mL (or in place of cTnT the cTnI > 0.10 ng/mL) and simultaneous NT-proBNP >332 ng/L and NT-proBNP ≤8500 ng/L.

- Hepatic involvement is defined as hepatomegaly on physical exam or alkaline phosphatase > 1.5 × u.l.n..
- GI involvement is defined with confirmation by tissue biopsy.
- Peripheral neuropathic involvement is defined based on clinical history in the presence of symmetric sensory-motor peripheral neuropathy.
- Autonomic nervous system involvement is defined by gastric emptying disorder, pseudo obstruction and/or voiding disorders not related to direct organ infiltration.
- Soft tissue and lymphatic involvement may be ascertained based on classic physical exam findings (macroglossia, shoulder pad sign, raccoon eyes, carpal tunnel syndrome, synovial enlargement, firm enlarged lymph nodes, etc.) or biopsy.

The Investigators may elect to repeat seemingly spurious results.

7.1.2 Evaluation of the clonal plasma cell disease

Evaluation of the clonal plasma cell disease involves the following studies:

- serum immunofixation, quantitative immunoglobulins, and serum free light chains;
- 24 hour urine for total volume and total protein, immunofixation;
- a bone marrow examination with a core marrow biopsy or aspirate;
- a skeletal survey for lytic lesions.

7.1.3 Quality of life assessment

Quality of Life QoL questionnaires will be administered to patients at each visit. The SF-36 and QLQ-C30 surveys will be used to register patient-reported outcomes.

7.2 SUBJECT REGISTRATION AND RANDOMIZATION PROCEDURE

7.2.1 Stratification

To make sure that patients who will receive doxycycline and those who will not have comparable severity of cardiac disease, patients will be stratified according to the stage of cardiac involvement.

- Cardiac stage II disease: either cTnT > 0.035 ng/mL (or in place of cTnT the cTnI > 0.10 ng/mL or hs-cTnT >77 ng/L) or simultaneous NT-roBNP >332 ng/L.
- Cardiac stage IIIa: both cTnT > 0.035 ng/mL (or in place of cTnT the cTnI > 0.10 ng/mL or hs-cTnT >77 ng/L) and simultaneous NT-proBNP >332 ng/L and NT-proBNP ≤8500 ng/L.

Stratification will assure that stage II and IIIa patients are evenly distributed in the intervention and in the control arm, but no subgroup analysis is planned.

7.2.2 Randomization

Patients will be randomized (1:1) to receive doxycycline or standard antibiotics in combination with anti-plasma cell therapy.

7.2.3 Blinding

This is an open label study.

8 TREATMENT AND EVALUATION PLAN

8.1 TREATMENT

Patients will be screened and have baseline studies performed as in Section 7.1 above. After stratification, patients will then be randomized to receive either

ARM A

Doxycycline 100 mg/twice a day

OR **ARM B**

Standard antibiotic

Treatment should start within 3 weeks after randomization.

All subjects must receive concomitant standard of care chemotherapy, which must include bortezomib administered subcutaneously on a weekly basis for the initial, first-line chemotherapy regimen. The backbone is the treatment of bortezomib s.c. weekly. Cyclophosphamide or melphalan can be added at physician's decision.

Anti-plasma cell therapy treatment is continued until

- 2 cycles after achievement of a complete hematologic response or
- achievement of a very good hematologic response or partial hematologic response, without improvement after 2 additional cycles or
- less than a partial hematologic response after cycle 2 or
- completion of cycle 8
- progression of clonal plasma cell disease.

At the Investigator's discretion the subject may be administered chemotherapy at local hospital , rather than at Investigator's facility.
See Appendix 5 for standard antibiotic treatment schedule schemes (each center have to provide its own institutional standard antibiotic scheme).

8.1.1 Prophylactic and supportive treatment

During treatment prophylactic medications will include acyclovir 400 mg twice daily (or as per local standard of care) with dose adjusted for renal function, and a proton-pump inhibitor daily.

Patients with an HBV infection will receive prophylaxis with lamivudine 100 mg daily with dose adjusted for renal failure.

Supportive measures will include specific interventions and the use of standard medications. Screening for eligibility is permitted after supportive measures have been instituted.

Patients with congestive heart failure at diagnosis will be placed on diuretic therapy.

Patients with symptomatic orthostasis will be treated as per local protocols.

Amiodarone for arrhythmias.

Colony stimulating factors, erythropoietin, and transfusion of platelets and red cells are permitted. The use of G-CSF must be cautious due to possible fluid retention.

Loperamide is recommended for treatment of diarrhea, starting at the time of the first watery stool. The loperamide dose and regimen will be according to institutional guidelines. Prophylactic loperamide is not recommended. Therapies to prevent constipation (eg, adequate hydration, high-fiber diet, and stool softeners and Senna if needed) should be administered per institutional guidelines.

Prophylactic antiemetics are not routinely recommended but are allowed at the investigator's discretion.

Table 6 Test schedule

Test or procedure	**Prior to registration (≤28 days)**	**Month 1 (±15 days)**	**End of months 2, 4, 6, 8,10, 12 (± 15 days) End of treatment visit**	**Follow up**
Genetic studies to exclude hereditary amyloidosis[1]	x			
Medical history	x			x
Disease associated symptoms and signs	x		x	x
Quality of life assessment	x		x	x
Physical examination[2]	x		x	x
Performance status (ECOG scale)	x		x	x
NYHA class	x		x	x
Toxicity monitoring			x	x
Skeletal survey	x			
HIV test	x			
Complete blood count	x		x	x
PT and aPTT	x		x	x
Blood chemistry[3]	x		x	x
Bone marrow aspirate or biopsy[4]	x		(x)[5]	
Serum free light chains	x		x	x
Quantitative immunoglobulins	x			
Serum and urine immunofixation electrophoresis	x		x	x
Urine total protein, creatinine clearance and urinary albumin to creatinine ratio	x		x	x
NT-proBNP	x		x	x
BNP	x		x	x

[1] If indicated.
[2] Including weight and orthostatic vital signs and neurologic exam.
[3] Glucose, creatinine, urea, ALT, AST, alkaline phosphatase, bilirubin, albumin, γGT.
[4] Within 100 days prior to first day of therapy
[5] At the end of therapy in patients who meet immunofixation and FLC criteria of complete response.
[6] At the end of cycle 2 and at the end of treatment visit.
[7] To be repeated every 4 weeks.

cTnI or cTnT or hs-cTnT	x		x	x
12-lead ECG	x		x[6]	x
Echocardiogram	x		X[6]	x
Abdominal US, MRI or CT for liver size[1]	x		(x)[5]	x
Serum pregnancy test [7]	x	x	x	x
IMP dispensation		x		

8.1.2 Toxicity and side effects

Toxicities are to be assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE), version 4.0.

Treatment-related mortality may occur among patients on either arm provided that the etiology of death has been linked in common practice to the drugs the patient is receiving.

Potential side effects of doxycycline include epigastric burning and distress, abdominal discomfort, nausea, and vomiting, photosensitivity, hepatic or renal toxicity, pseudomembranous colitis, hypersensitivity or allergic reactions and cytopenia. Blood work for potential drug-related adverse events will be drawn monthly.

8.2 ASSESSMENT OF RESPONSE AND PROGRESSION

Amyloid-related organ response will be evaluated on the basis of the accepted criteria described below. The tests and studies listed in Section 7.0 will be repeated as appropriate to assess patients for both organ response and progression of disease.

The data demonstrating response to treatment in European patients should be validated by a referral center within each European country. Response will be assessed according to current consensus criteria (Palladini, et al J Clin Oncol 2012). In case of unsatisfactory response (i.e. <very good partial response or partial response plus organ response) a switch to a second line treatment regime will be allowed. In the absence of unacceptable toxicity, doxycycline administration will be continued for the entire duration of follow-up (12 months).

8.2.1 Organ response

An improvement of one or more affected organ(s) is defined by:

Kidneys

- A decrease in proteinuria by ≥30% or below 0.5 g/24 h without creatinine clearance decreased ≥25% over baseline.

Heart (echocardiograms must be performed at the same institution)

- reduction of NT-proBNP of 30% and >300 ng/L over the starting value.

Liver

- a ≥50% decrease of an initially elevated alkaline phosphatase level,
- or reduction in the size of the liver by at least 2 cm (determined by physical exam, US, CT or MRI).
- The Investigators may elect to repeat seemingly spurious results.

8.2.2 Organ progression

Worsening of one or more affected organ(s) is defined by:

Kidneys

- ≥25% worsening of creatinine or creatinine clearance.

Heart (echocardiograms must be performed at the same institution)

increase of NT-proBNP of 30% and >300 ng/L over the starting value.

Liver

- ≥50% increase in the alkaline phosphatase level above the lowest value.
- Worsening in organ function must be confirmed at the next visit.

The Investigators may elect to repeat seemingly spurious results.

8.2.3 Stable disease

Stable disease is defined when none of the criteria for improvement or for worsening disease are met.

8.2.4 Response and progression of the clonal plasma cell disease

Hematologic response will be categorized by consensus criteria described in Appendix 2 (Palladini 2012) and the frequency will be determined according to standard of care. For a specific organ (liver, kidney, heart), the corresponding response rate will be assessed only on subjects with involvement in that particular organ at Screening. Hematologic and organ response rates will be calculated, along with their associated 95% confidence intervals, by assigned treatment.

8.3 CRITERIA FOR REMOVAL FROM STUDY

Patients will be removed from study in case of:

- progression of clonal plasma cell disease and/or organ involvement as defined above or
- because of requested withdrawal or
- noncompliance or
- intercurrent illness that makes treatment assessment not possible or
- at the discretion of the treating physician and when it is deemed to be in the patient's best interest; in particular, patients can be removed from the study if they do not meet study criteria of a progression but they did not reach CR, VGPR or organ response and the local clinicians wish to give further treatment (these patients will be considered censored for the purpose of the survival analysis).

9 ADVERSE EVENTS/SERIOUS ADVERSE EVENTS AND REPORTING

Adverse events, both reported and observed, will be recorded in the source documents and on the appropriate section of the electronic case report form (eCRF) from the time initial eligibility is established during Screening/Baseline (upon signature of the informed consent form) through the 6-month Follow-up Visit.

9.1 DEFINITIONS

In accordance with Art.2 of the Directive 2001/20/EC, in this trial protocol the definitions reported in the table below apply.

TERM	DEFINITION
Adverse Event (AE)	Any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment[A]
Adverse Reaction (AR)	All untoward and unintended responses to an investigational medicinal product related to any dose administered
Medical Event	Any untoward medical occurrence in a patient or clinical trial subject after study enrolment and before any IMP administration
Serious Adverse Event (SAE) or Serious Adverse Reaction (SAR)	Any untoward medical occurrence or effect that at any dose: ▪ results in death ▪ is life-threatening ▪ requires hospitalisation or prolongation of existing hospitalisation ▪ results in persistent or significant disability or incapacity ▪ is a congenital anomaly or birth defect ▪ other medically important/clinically significant events[B]
Unexpected Adverse Reaction (UAR)	An adverse reaction, the nature or severity of which is not consistent with the applicable product information (e.g., investigator's brochure for an unauthorised investigational product or summary of product characteristics for an authorised product)

[A] An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, syndrome, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product. The AE may involve any organ or system and can be represented by the new onset, or the deterioration, of a disease, a syndrome, a symptom, or a physical sign, as well as by findings and results of instrumental examinations and laboratory tests. Any medically relevant and untoward change, including frequency or pattern changes for a fluctuating condition (e.g., migraine), occurring during the reporting period is considered an AE.

Examples include:

- The emergence of any signs and symptoms that were not present at baseline (an event present at baseline that has not changed is not considered an AE)
- Pre-existing conditions that are marked by a worsening from the subject's baseline/entry status (i.e., an increase in severity or frequency of the pre-existing abnormality or disorder)
- Reactions to study drug, abuse of drug, withdrawal phenomena, sensitivity, or toxicity to study drug
- Apparently unrelated illnesses
- Injuries or accidents

- Extensions or exacerbations or symptomatology, subjective events reported by the subject, or new clinically significant abnormalities in clinical laboratory tests, physiological tests or PE

[B] Important medical events that may not result in death, be life threatening, or require hospitalization may be considered an SAE when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in the definition of SAEs. Examples of such events are:

- Intensive treatment in an emergency room or at home for allergic bronchospasm
- Convulsions that do not result in hospitalization
- Development of drug dependency or drug abuse

An SAE may also include any other event that the Sponsor judges to be serious, or that suggests a significant hazard, contraindication, side effect, or precaution.

Important Note: The concepts of AEs and SAEs represent regulatory instruments used to evaluate and monitor the safety of clinical trial subjects. Therefore, these terms only apply in light of their regulatory definition. The term "serious," in a regulatory sense, does not necessarily mean "severe." All AEs (serious and non-serious) reported during a study will be taken into account when analyzing the study data and establishing the safety profile of the investigational drug.

Death: Death, in and of itself, is not an AE; it is only an outcome. The cause of death is the AE. Therefore, the Investigator should make every effort to obtain and document the cause of death for all subjects who die during the study. If, despite all efforts, the cause of death remains unknown, the SAE should be documented as an "unspecified fatal event."

Life-threatening AE: Any AE that places the subject, in the view of the Investigator, at immediate risk of death from the event as it occurred. This does not include an event that, had it occurred in a more severe form, might have caused death.

Hospitalisation: Hospitalisation is defined by the Sponsor as a full admission to the hospital for diagnosis and treatment for 24 hours or longer. This includes prolongation of an existing inpatient hospitalisation. Hospitalisation may include social hospitalization, defined as inadequate family support/care at subject's primary residence that results in subject being admitted to the hospital. Examples of visits to a hospital facility that do not meet the serious criteria for hospitalisation include:

- Emergency room visits (that do not result in a full hospital admission) for a period of less than 24 hours
- Outpatient surgery
- Preplanned or elective procedures
- Protocol procedures

A prescheduled elective procedure or a routinely scheduled treatment is not to be considered an SAE, even if the subject is hospitalized, provided the site stipulates that:

- The condition requiring the prescheduled elective procedure or routinely scheduled treatment was present before and did not worsen or progress between the subject's consent to participate in the clinical trial and the time of the procedure or treatment
- The prescheduled elective procedure or routinely scheduled treatment is the sole reason for admission and intervention

An untoward medical event occurring during the prescheduled elective procedure or routinely scheduled treatment should be recorded as an AE or an SAE, as appropriate.

Disability: A substantial disruption to a person's ability to conduct normal life functions.

Action taken with study drug: The action taken referred to the study drug must be described by choosing among:

- Drug withdrawn
- Dose reduced
- Dose increased
- Dose not changed
- Unknown
- Not applicable

Outcome: Each adverse event outcome must be described by choosing among:

- Recovered/Resolved
- Recovered/Resolved with sequelae
- Recovering/Resolving
- Not recovered/Not resolved/Ongoing
- Fatal
- Unknown

9.2 ASSESSMENT OF ADVERSE EVENTS

9.2.1 Severity

AEs will be assessed by the Investigator according to the NCI-CTCAE version 4.0. AEs that do not have a corresponding NCI-CTCAE term will be assessed according to their impact on the participant's ability to perform daily activities as listed below.

The severity of each AE should be characterized and then classified into one of five clearly defined categories as follows:

- Grade 1 (Mild): the AE does not interfere in a significant manner with the subject's normal functioning level; it may be an annoyance
- Grade 2 (Moderate): the AE produces some impairment of functioning, but is not hazardous to health; it is uncomfortable or an embarrassment
- Grade 3 (Severe): the AE produces significant impairment of functioning or incapacitation and is a definite hazard to the subject's health
- Grade 4 (Life threatening): Life threatening or disabling
- Grade 5 (Fatal): Causes death of the participant

These five categories are based on the Investigator's clinical judgment, which in turn depends on consideration of various factors such as the subject's reports, the Investigator's observations, and the Investigator's prior experience.

9.2.2 Seriousness

When an AE occurs, the Investigator must first assess whether the event is serious using the definition provided.

If the event is serious, then an SAE Report Notification Form must be completed and the Safety Contact (SC) notified according to the procedure described in section 9.3.2.

9.2.3 Causality

The Investigator must assess the relationship of the AE to the study drug by using the following general guidelines:

- The temporal relationship of the AE to study drug administration
- The likelihood the AE can be attributed to concurrent disease
- The likelihood the AE can be attributed to the use of concomitant drugs
- Whether the event responds following withdrawal of study drug (de-challenge)

Each event will be assessed for its relationship to study drug as follows:

Definitely: event or laboratory test abnormality, with plausible time relationship to drug intake that cannot be explained by disease or other drugs, response to withdrawal plausible (pharmacologically, pathologically), event definitive pharmacologically or phenomenologically (i.e. an objective and specific medical disorder or a recognized pharmacological phenomenon).

Probably: event or laboratory test abnormality, with reasonable time relationship to drug intake, unlikely to be attributed to disease or other drugs, response to withdrawal clinically reasonable.

Possibly: event or laboratory test abnormality, with reasonable time relationship to drug intake, could also be explained by disease or other drugs, information on drug withdrawal may be lacking or unclear

Unlikely: event or laboratory test abnormality, with a time to drug intake that makes a relationship improbable (but not impossible), disease or other drugs provide plausible explanations

Not related: event or laboratory test abnormality, with a time to drug intake that makes a relationship improbable, disease or other drugs provide plausible explanations. There is no evidence of any causal relationship.

Not assessable: report suggesting an adverse reaction, cannot be judged because information is insufficient or contradictory, data cannot be supplemented or verified. **9.2.4 Expectedness**

If the event is a SAR, the SC will assess the expectedness of the event.

If a SAR is assessed as being unexpected, it becomes a suspected unexpected serious adverse reaction (SUSAR).

The definition of an unexpected adverse reaction (UAR) is a reaction not reported in the reference safety information (RSI) which in case of the AC-012-EU study will be the SmPC for *Miraclin 100 mg compresse*, LABORATORIO FARMACOLOGICO MILANESE S.R.L.

9.3 INVESTIGATOR'S RESPONSIBILITIES

9.3.1 Recording of adverse events

Subjects should be instructed to report to the study staff any AE that they experience, even those that may not be related to the study drug, and to discuss potential treatment. Subjects should be instructed to call the appropriate emergency response telephone number if they experience an AE that requires immediate attention or could, if untreated, have potentially serious consequences.

At each visit, the Investigator will prompt the subject with non-leading questions to determine if any AEs were experienced since the last visit. The Investigator must follow all AEs until the events have resolved or until the condition has stabilized and no further medical follow-up is warranted.

It is the responsibility of the Investigator to document all AEs that occur during the reporting period. Pre-existing conditions (noted before Screening/Baseline) should not be reported as AEs unless they worsen (i.e., become more severe or more frequent) after beginning participation in the study (upon signing the informed consent form).

Whenever feasible, AEs should be documented as medical diagnoses and a unifying diagnosis should be provided. For example, symptoms including fever, productive cough, opacity in the left lower lobe of the lung on x-ray would be reported as a single AE of pneumonia. Otherwise, if reported AEs do not appear clearly inter-related, individual signs or symptoms may be reported as separate AEs. Information recorded on the appropriate CRF will include the description of the AE, the date and time of onset and resolution (if applicable), severity, seriousness, relationship to the study drug, action taken, and the outcome.

When an AE resulting from disease progression meets the requirements to be considered serious the SAE verbatim term should be reported as the sign/symptom that best describes the event rather than as disease progression. For example, a subject presents with worsening shortness of breath due to a pleural effusion resulting from disease progression. The event term should be reported as "pleural effusion" instead of as disease progression.

9.3.2 Reporting of SAEs

All the AEs must be recorded in the eCRF reporting the diagnosis, a description of the event, whether it is considered serious (and if so the criterion satisfied), its duration (onset and resolution dates), its severity, its relationship to the IMP, any other potential causality factors, any treatment given or other action taken (including dose modification or discontinuation of the IMP) and its outcome.

The Investigators must notify **within 24 hours** the Safety Contact of any new AE with the features of SAE/SAR or of any new information on a previously reported SAE/SAR.

To this end, the Investigator should fill in the SAE Report Notification Form and send it by fax or email to the SC, using the following contacts.

Name	Telephone no.	Fax no.	E-mail
Safety Contact	+39 373 8530614	+39 080 9909321	pharmacovigilance@cvbf.net

The SAE Notification Form should be filled in as accurately as possible in all of their parts. For any new SAE, the following minimum information is required as initial notification:

- Clear identification of the Investigator with full contact information;
- Subject identification details (study number, site number, patient identification code with month/year of birth);
- IMP administration details (dose and dates);
- Diagnosis of the event with the description (or a brief description of signs/symptoms/clinical course if the diagnosis is not available) and the date of onset;
- Reason(s) for considering the event serious;
- Relationship of the event with the IMP or with the trial procedure (e.g., the causality according to the Investigator).

In addition, the Investigator must respond to any request for follow-up information or questions regarding the SAE within the same timelines as for initial reports.

The PI identifies a researcher in charge for the identification of possible AEs/SAEs and their management, and the preparation of reports, .

The Investigator must comply with any applicable requirements related to the reporting of SAEs involving his/her subjects to the Institutional Review Board/Independent Ethics Committee (IRB/IEC) that approved the study. In particular, all deaths must be immediately reported to the IRB/IEC that approved the study.

9.3.3 Reporting period

AEs are collected on an ongoing basis from the day of written informed consent/assent through the last study visit.

Expedited reporting (i.e. the notification of SAEs within 24 hours of awareness of the event) must be carried out

- from signature of informed consent up to first administration of first dose of the study drug if related to study participation (serious medical event)
- from administration of the first dose of the study drug up to the follow up visit (serious adverse event)
- with no time limitation if the event is likely to be related to study drug (serious adverse reaction)

9.4 SPONSOR'S RESPONSIBILITIES

The PharmacoVigilance Team (PVT) at CVBF, delegated by the Sponsor, will review all SAE reports received.

The causality assessment given by the Investigator cannot be overruled and in case of disagreement, both opinions will be provided in any subsequent reports.

The PVT will comply with country specific regulatory requirements related to safety reporting to regulatory authorities, IRBs/IEC/REB and Investigators and will also assure all Investigators are informed of any safety issues that arise during the course of the trial.

All SUSARs, which occur with the investigational medicinal products within this clinical trial will be submitted by CVBF in compliance with the timelines and standards for reporting SUSARs set out in the EU Directive 2001/20/EC [Directive 2001/20/EC of the European parliament and of the council of 4/April/2001] and linked guidance [European Commission, Enterprise and Industry Directorate General: Detailed guidance on the collection, verification and presentation of adverse reaction reports arising from clinical trials on medicinal products for human use, latest version]. The European Medicines Agency (EMA) and the concerned national health authorities (if applicable) will be informed through EudraVigilance, while the Ethics Committees and the investigators by CIOMS I form.

9.5 FOLLOW-UP OF ADVERSE EVENTS

All AEs experienced by a subject, regardless of the suspected causality, will be followed up until the event has resolved, until the Sponsor deems the event to be chronic or stable, until there is a satisfactory explanation for the changes observed, or until the subject is lost to follow-up.

9.6 PREGNANCY

The Sponsor has a responsibility to monitor the outcome of all pregnancies reported during the clinical study. Although pregnancy is not considered an SAE, an abnormal outcome of pregnancy is an SAE.

Each pregnancy must be reported by the Investigator to the PVT within 24 hours of becoming aware of the pregnancy through the SAE Notification Form.

The Investigator must follow-up and document the course and the outcome of all pregnancies even if the subject was withdrawn from the clinical study or if the clinical study has ended. Pregnancies must be followed until birth, termination of the pregnancy, or loss of the subject to follow-up.

9.7 QUALITY ASSURANCE AND SAFETY

The Sponsor will coordinate monitoring activity at sites. Peripheral centers must guarantee an adequate monitoring activity at their site and identify a monitor, whose name must be communicated to the Sponsor. The Sponsor will send documentation in order to make uniform the monitoring activities. A monitoring plan will be implemented before the start of the trial. Four visits are planned for each Center: the site initiation visit, a visit after 30% of the planned patients have been enrolled, a visit after 60% of the planned patients have been enrolled, and the closure visit. One hundred percent source data verification will be performed. Distance monitoring on the eCRF will also be planned to assess completeness, consistency and compliance with the study protocol with personalized e-queries sent to the Investigators. Rates of enrolment will be monitored and encouragement messages will be sent to the Investigators as needed. Centralized training of the study monitors will be performed at kick-off meeting and centralized coordination will follow with conference calls on a regular basis.

The sites will be requested to submit screening logs for the trial to the Sponsor Clinical Trial Center (CTC) on request. Sites will be requested to submit staff delegation logs for the trial to CTC at the frequency detailed in the trial monitoring plan or on request and these logs will be checked for consistency and completeness. Ensuring patient eligibility is the responsibility of the coordinator and Site PIs or other delegated Investigator(s). Checks of the criteria listed on the registration form will be undertaken by an appropriately trained staff member prior to registration.

Sites will be required to maintain a log of all patient informed consent forms that have been completed (regardless of whether the patient is subsequently registered to the trial). This log will include details of the versions of informed consent form/patient information sheet used, patient completion of the consent form, the name of the person taking consent, etc.

Copies of completed drug accountability logs will be collected at CTC for all trial patients. Sites will be required to submit logs on request. These will be monitored, as per the monitoring plan, to ensure completeness and correlation with data captured in the CRF.

Participating sites will be requested to conduct quality control checks of documentation held within the Investigator Site File and Pharmacy Site File at the frequency detailed in the trial monitoring plan. Checklists detailing the current version/date of version controlled documents will be provided for this purpose.

Data received at CTC will be subject to review.

Where central monitoring of data and/or documentation submitted by sites indicates that a patient may have been placed at risk (e.g. evidence of an overdose having been administered, indication that stopping rules were not observed following an adverse reaction, etc.), the matter will be raised urgently with site staff and escalated as appropriate.

Study safety will be monitored by the DMSB which will meet at regular, predetermined intervals to monitor the safety parameters and consider the adverse events, if any.

The trial will be conducted in compliance with pharmacovigilance regulations. The PharmacoVigilance Team at CVBF, delegated by the Sponsor, will ensure pharmacovigilance.

Data will be monitored for quality and completeness by the CTC. Missing data will be chased until it is received, confirmed as not available or the trial is at analysis.

Doxycycline, the study medication, is marketed in all the participating countries. The study medication will be purchased on the market with dedicated trial funding by each site. The investigational product will be labeled on site, according to the internal procedure. The sites will be responsible for drug accountability.

Every site involved in the Protocol will be responsible for an adequate insurance.

10 STATISTICAL CONSIDERATIONS

10.1 RECRUITMENT PLAN

This is a multi-center trial. The partners participated in all the large recent multicenter trials in AL amyloidosis

10.2 CALCULATION OF SAMPLE SIZE

The sample size was computed based on the primary endpoint (survival at 12 months). The 12-month cumulative proportion survival of stage II/IIIa patients treated with bortezomib-based combination is approximately 65%, ranging from 60% to 70% in different published series including more than 1,000 patients (Palladini, et al. Blood 2015; Palladini, et al. ASH 2015). The preliminary experience of the London and Pavia groups with the addition of doxycycline to bortezomib-based chemotherapy in patients with AL amyloidosis with moderate to severe heart involvement indicate that early (6-12 months) mortality can be reduced to 0-5%.

According to the following hypotheses:

- a type I error of 5% (2-sided)
- a power of 80%
- an attrition of 10%
- a proportion of patients surviving at 12 months in the control arm 65%
- an expected proportion surviving at 12 months in the active arm of 88% (HR 0.28; 26 events).

Based on these hypotheses 120 patients (60 per arm) will need to be enrolled. To reach this sample size, we expect that about 180 subjects will be assessed for eligibility.

10.3 ANALYSIS POPULATION

All randomized patients will be analyzed to the treatment arm originally assigned. This will represent the intention to treat population (ITT). All descriptive statistics and the primary analysis of the primary and secondary endpoints will be based on the ITT population. Patients without major protocol deviations will represent the per-protocol population (PP) and will be used for a sensitivity analysis of the primary endpoint. All patients receiving at least one dose of the prescribed treatment will represent the safety population. These will be used for the description of safety parameters.

10.4 PATIENTS DISPOSITION AND DESCRIPTION OF THE POPULATION

Patient disposition (from screening to analysis) will be described by means of a flow chart according to CONSORT guidelines. Descriptive statistics will be computed separately by treatment arm (mean and standard deviation, median and 25th-75th percentiles, minimum and maximum for continuous variables, counts and percent for categorical variables)

10.5 DESCRIPTION OF THE PRIMARY EFFICACY ANALYSIS

Cumulative survival at 12 months will be computed together with 95% confidence interval with the Kaplan Meier method separately for each treatment arm. The stratified log rank test will be used to compare survival. The hazard ratio and 95% confidence interval (CI) will be computed from a Cox model to assess the primary endpoint. The stratification factor used at randomization as well as the Center effect will be accounted for in the model.

The primary analysis will be based on the ITT population. To assess robustness of the results, the analysis will be repeated in the PP population. Follow-up will start at the date of randomization and end at the date of death or at the last date the patient was seen alive. Death from any cause will be considered.

10.6 SAFETY

The rate of severe adverse events (as described above) will be tabulated as count and percent, separately for each treatment arm.

10.7 SECONDARY ENDPOINTS

The rate of infective events will be reported with Poisson 95%CI separately for each treatment arm and compared with a Poisson (or negative binomial regression in case of over-dispersion); incidence rate ratios (IRR) and 95%CI will be reported. The stratification factor used at randomization as well as the center effect will be accounted for.

The proportion of patients with cardiac, hematologic and renal response at 2, 4, and 6 months will be summarized by treatment arm. The difference between proportions and 95%CI will be computed with a generalized binomial linear model for repeated measures. The stratification factor used at randomization as well as the center effect will be accounted for. Patients dying will be considered as non-responders.

Stata 14 (Stata Corp, College Station, TX, USA) will be used for computation.

10.8 COVARIATES AND SUBGROUPS.

Cardiac stage (the stratification variable for randomization) will be included as covariate in all regression models, while lack of independence within Center in the calulation of standard errors**.**

No subgroup analyses are planned.

10.9 MISSING DATA

Description of baseline characteristics will be further summarized by treatment arm for patients completing the study and patients dropping out from the study. A multiple imputation of missing outcomes may be performed and a sensitivity analysis of the primary endpoint accounting for multiple imputation will be performed. Every effort will be made in the conduct of the study to avoid losses to follow-up.

10.10 INTERIM ANALYSES

No formal interim analysis for efficacy or futility are planned. However safety will be continuously monitored by the DMSB.

10.11 MULTIPLE TESTING

The trial is sized on the primary endpoint overall mortality. Statistical significance is set at a 2-side p-value<0.05. P-values deriving from the sensitivity analyses of the primary endpoint (per-protocol and after multiple imputation) and of the secondary endpoints of safety and response are to be considered exploratory.

10.12 PROTOCOL DEVIATIONS AND TREATMENT COMPLIANCE

The most relevant protocol deviation that is expected is inadequate treatment compliance, with patients not taking the study drug according to the prescribed schedule and for all the duration of the study. The patients will be thoroughly instructed at each visit on the treatment schedule, they will be given a diary to record any possible occasion they omitted to take the study drug, and they will be required to bring the unused study drug at each follow-up visit. Complience will be assessed by counting and recording the number of the returned drug capsules.

Major protocol deviations and compliance will be summarized separately per study arm.

11 STUDY AND DATA MANAGEMENT

11.1 SOURCE DOCUMENTS

Study data will be collected on source documents. The Principal Investigator (PI) is responsible for assuring that collected data are complete and accurate. Source documentation (the point of initial recording of a piece of data) should support data collected on the eCRF. Source documents include all recordings of observations or notations of clinical activities and all reports and records necessary for the evaluation and reconstruction of the clinical study.

11.2 DATA COLLECTION AND STUDY REPORT FORM MONITORING

All data obtained for this study will be entered into a local regulation (for reference: 21 CFR Part 11, USA) compliant Data Management System provided by the Sponsor. These data will be recorded with an Electronic Data Capture (EDC) system using eCRFs. The RedCap platform will be used for that purpose.

The Investigators will ensure the accuracy and completeness of the data reported to the Coordinating center. All data entry, modification or deletion will be recorded automatically in an electronic audit trail.

The Investigators will provide access to their original records to permit a representative from the Sponsor to verify the proper transcription of data. Data reported in the eCRFs should be consistent with and substantiated by the subject's medical record and original source documents. The eCRF data will be monitored by the Sponsor or designee. The final, completed eCRF Casebook for each subject must be electronically signed and dated by the Principal Investigator (PI) within the EDC system to signify that the Investigator has reviewed the eCRF and certifies it to be complete and accurate. The Sponsor will retain the final eCRF data and audit trail. A copy of all completed eCRFs will be provided to the investigator.

11.3 AVAILABILITY AND RETENTION OF RECORDS

The Sponsor must make study data accessible to the authorized representatives of IRB/IEC, and Regulatory Agency inspectors upon request. A file for each subject must be maintained that includes the signed Informed Consent Form (ICF) and the Investigator's copies of all source documentation related to that subject. The Investigator must ensure the reliability and availability of source documents from which the information on the CRF was derived.

Study documents should be retained for a minimum of 15 years after the end of the study. These documents should be retained for a longer period, however, if required by local regulations. There is no centralized laboratory in the present study. Thus, patients' samples will be handled as per institutional guidelines in compliance with local regulations.

11.4 SUBJECT CONFIDENTIALITY

The participants of the study will not be identified by name on any study documents to be collected by the Sponsor. In order to maintain subject confidentiality, only a site number and subject number will identify all study subjects on CRFs and other documentation.

12 ADMINISTRATIVE, ETHICAL, REGULATORY CONSIDERATIONS

12.1 SPONSOR, COORDINATING CENTRE(S) AND COMMITTEES

This is an Investigator Initiated Trial (IIT). The sponsor will be the Foundation "Scientific Institute Policlinico San Matteo, University of Pavia, Italy"

The Coordinating Center will

- represent the Consortium with Regulatory Authorities, funding institutions, and CRO;
- obtain first approval of the protocol and related documents from local Ethics Committee;
- include the trial in the Italian National Monitoring Centre of Clinical Trials, according to Italian Medicines Agency (AIFA) regulations, and in the EudraCT database;
- provide eCRFs;
- design and maintain the database;
- train monitors;
- coordinate monitoring visits;
- enroll and treat patients in Italy;
- perform statistical analysis;
- draft the manuscript(s) for publication.

A Steering Committee composed of the PIs of each partner will

- finalize the protocol;
- monitor compliance with milestones and with enrollment plan and predispose the necessary measures in case there is the risk of not meeting the milestones or in case of delays in enrollment.

An independent Data Safety Monitoring Board (DMSB) composed of three experts will be appointed by the Sponsor. This committee will receive and evaluate safety reports after one third and two thirds of patients will be enrolled. The members of the DSMB will serve in an individual capacity and provide their expertise and recommendations. The primary responsibilities of the DSMB are to 1) periodically review and evaluate the accumulated study data for participant safety, study conduct and progress and 2) make recommendations to the Steering Committee and Sponsor concerning the continuation, modification, or termination of the trial. The DSMB considers study-specific data as well as relevant background knowledge about the disease, test agent, or patient population under study. The DSMB is responsible for defining its deliberative processes, including event triggers that would call for an unscheduled review, stopping guidelines, and voting procedures prior to initiating any data review. The DSMB is also responsible for maintaining the confidentiality of its internal discussions and activities as well as the contents of reports provided. No member of the DSMB should have direct involvement in the conduct of the study. Furthermore, no member should have financial, proprietary, professional, or other interests that may affect impartial, independent decision-making by the DSMB.

12.2 ETHICAL CONDUCT OF THE STUDY

Patients will receive state-of-the-art therapy for AL amyloidosis in both the intervention and control arm. The investigational agent (doxycycline) will be administered on the top of standard treatment in the intervention arm, while standard antibiotic prophylaxis will be given in the control arm. Safety data will be constantly collected and periodically monitored by DSMB.

The trial will be conducted in accordance with the World Medical Association Declaration of Helsinki entitled 'Ethical Principles for Medical Research Involving Human Subjects' (2013 version) and in accordance with the terms and conditions of the ethical approval given to the trial.

There will be no compensation for patients enrolled in the trial.

The Sponsor and Investigators will preserve patient confidentiality and will not disclose or reproduce any information by which patients could be identified. Data will be stored in a secure manner.

Informed consent will be obtained from each patient before any study procedures is performed. This will include:

- consent from participants for access to their medical records by responsible individuals from the research staff or from regulatory authorities, where it is relevant to trial participation;
- consent from participants for the data collected for the trial to be used to evaluate safety and develop new research.

Participant name will be collected on the consent form when a participant is registered into the trial, but all other data collection forms will be coded with a trial number and will include two participant identifiers. If a participant withdraws consent from further trial treatment and / or further collection of data, their data and samples will remain on file and will be included in the final trial analysis. The data will then be further used according to the current laws.

Electronic data capture (EDC) will be based on the REDCap platform. REDCap is a novel workflow methodology and software tool that expedites the electronic collection of research data from a single site or multi-site clinical l research study. The software supports a secure web-based application for developing fully functional case report forms (CRFs) and surveys. In particular, through REDCap we will implement:

- full user authentication (log-on/password) to restrict users to study functions;
- real-time data validation, integrity checks for insuring data quality;
- de-identification options to be applied to data exports to remove fields that contain notes and other information that could identify patient;
- centralized, secure storage of research data with back-ups.

The study database will be resident on a server in a secure location within the Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.

All publications and presentations relating to the trial will be authorized by the Steering Committee (SC). The SC will form the basis of the writing committee and advice on the nature of publications. Contributing site investigators in this trial will also be acknowledged. Data from all sites will be analyzed together and published as soon as possible. Participating sites may not publish trial results prior to the first publication by the SC or without prior written consent from the SC.

The trial will be registered with an authorized registry, according to ICMJE Guidelines, prior to the start of recruitment.

The success of the trial depends upon the collaboration of all patients. For this reason, credit for the main results will be given to all those who have collaborated in the trial, through authorship and contributorship. Uniform requirements for authorship for manuscripts submitted to medical journals will guide authorship decisions. These state that authorship credit should be based only on substantial contribution to:

- conception and design, or acquisition of data, or analysis and interpretation of data
- drafting the article or revising it critically for important intellectual content
- and final approval of the version to be published
- and that all these conditions must be met (www.icmje.org).

In light of this the trial PI and members of the SC will be named as authors in any publication.

Publication of emerging safety data in the form of abstracts for oral or poster presentation, or journal letters is permitted. However no formal manuscript publication is permitted until the final analysis of the trial data has been completed. All abstracts and letters must be reviewed by at least the trial PI and trial statistician. The release of any data prior to the end of the trial must be approved by the Steering Committee.

12.3 REGULATORY APPROVAL

The Sponsor will make the appropriate applications to the Regulatory Authority and, if necessary, approval to import Investigational Product. The study will not start until the required regulatory approvals have been obtained in the appropriate jurisdiction.

12.4 INDEPENDENT REVIEW BOARD/ETHICS COMMITTEE APPROVAL

The Sponsor is responsible for obtaining Institutional Review Board/Ethic Committee (IRB/EC) approval for the final protocol, the ICF, and any materials provided to or used to recruit subjects. Written approval of these documents must be obtained from the IRB/EC before any subject may be enrolled at the site.

The Sponsor is also responsible for the following:

- Obtaining IRB/EC approval for any protocol amendments and ICF revisions before implementing the changes
- Providing the IRB/EC with any required information before or during the study
- Submitting progress reports to the IRB/EC, as required during the conduct of the study, requesting re-review and approval of the study, as needed.
- Notifying the IRB/EC of all serious and/or unexpected AEs related to the study medication

12.5 SUBJECT INFORMED CONSENT

The ICF documents the study-specific information the Investigator provides to the subject and the subject's agreement to participate. Among other things, the Investigator will fully explain in layman's terms the nature of the study, along with the aims, methods, potential risks, and any discomfort participation in the study may entail. The subject must voluntarily, personally sign and date the ICF before any study-related procedures are performed. The original and any amended, signed and dated ICF(s) must be retained in the subject's file at the study site and a copy of the signed ICF must be provided to the subject.

12.6 PROTOCOL AMENDMENTS AND STUDY TERMINATION

The Sponsor or designee may amend the protocol as needed to ensure that the clinical investigation is being conducted as intended. The Sponsor will initiate protocol amendments in writing if any change significantly affects the safety of subjects, the scope of the investigation, or the scientific quality of the study. Protocol changes must be submitted to the IRB/EC as a protocol amendment. If necessary, the ICF will be revised to reflect the changes in the amendment and will be submitted to the IRB/EC for review and approval. A copy of the amendment must be signed by the Investigator and returned to the Sponsor. Written documentation of IRB/EC approval is required before the amendment is implemented.

The Sponsor reserve the right to terminate the study, according to the study contract. The Sponsor should notify the IRB/EC in writing of the trial's completion or early termination.

13 REFERENCES

Cardoso, I., Martins, D., Ribeiro, T., Merlini, G. & Saraiva, M.J. (2010) Synergy of combined doxycycline/TUDCA treatment in lowering Transthyretin deposition and associated biomarkers: studies in FAP mouse models. *J Transl Med,* 8, 74.

Cardoso, I., Merlini, G. & Saraiva, M.J. (2003a) 4 '-iodo-4 '-Deoxydoxorubicin and tetracyclines disrupt transthyretin amyloid fibrils in vitro producing noncytotoxic species: screening for TTR fibril disrupters. *FASEB Journal,* 17, 803-809.

Cardoso, I., Merlini, G. & Saraiva, M.J. (2003b) 4'-iodo-4'-deoxydoxorubicin and tetracyclines disrupt transthyretin amyloid fibrils in vitro producing noncytotoxic species: screening for TTR fibril disrupters. *FASEB J,* 17, 803-809.

Cardoso, I. & Saraiva, M.J. (2006) Doxycycline disrupts transthyretin amyloid: evidence from studies in a FAP transgenic mice model. *FASEB J,* 20, 234-239.

Costa, R., Speretta, E., Crowther, D.C. & Cardoso, I. (2011) Testing the therapeutic potential of doxycycline in a Drosophila melanogaster model of Alzheimer disease. *J Biol Chem,* 286, 41647-41655.

Diomede, L., Rognoni, P., Lavatelli, F., Romeo, M., del Favero, E., Cantu, L., Ghibaudi, E., di Fonzo, A., Corbelli, A., Fiordaliso, F., Palladini, G., Valentini, V., Perfetti, V., Salmona, M. & Merlini, G. (2014) A Caenorhabditis elegans-based assay recognizes immunoglobulin light chains causing heart amyloidosis. *Blood,* 123, 3543-3552.

Dispenzieri, A., Gertz, M., Kyle, R., Lacy, M., Burritt, M., Therneau, T., Greipp, P., Witzig, T., Lust, J., Rajkumar, S., Fonseca, R., Zeldenrust, S., McGregor, C. & Jaffe, A. (2004) Serum cardiac troponins and N-terminal pro-brain natriuretic peptide: a staging system for primary systemic amyloidosis. *J Clin Oncol,* 22, 3751-3757.

Gertz, M.A., Lacy, M.Q., Dispenzieri, A., Cheson, B.D., Barlogie, B., Kyle, R.A., Palladini, G., Geyer, S.M. & Merlini, G. (2002) A multicenter phase II trial of 4 '-iodo-4 ' deoxydoxorubicin (IDOX) in primary amyloidosis (AL). 9, 24-30.

Gianni, L., Bellotti, V., Gianni, A.M. & Merlini, G. (1995) New drug therapy of amyloidoses: resorption of AL-type deposits with 4'-iodo-4'-deoxydoxorubicin. *Blood,* 86, 855-861.

Kumar, S.D., A; Lacy, M; Haymann, S; Buadi, F; et al. (2012) Doxycicline used as post transplant antibacterica prophylaxisi improves survival in patients with light chain amyloidosis undergoing autologous stem cell transplant. *Blood,* ASH meeting 2012 poster 3138.

Merlini, G., Ascari, E., Amboldi, N., Bellotti, V., Arbustini, E., Perfetti, V., Ferrari, M., Zorzoli, I., Marinone, M.G., Garini, P. & . (1995) Interaction of the anthracycline 4'-iodo-4'-deoxydoxorubicin with amyloid fibrils: inhibition of amyloidogenesis. *Proc Natl Acad Sci U S A,* 92, 2959-2963.

Merlini, G. & Palladini, G. (2013) Light chain amyloidosis: the heart of the problem. *Haematologica,* 98, 1492-1495.

Merlini, G., Wechalekar, A.D. & Palladini, G. (2013) Systemic light chain amyloidosis: an update for treating physicians. *Blood,* 121, 5124-5130.

Muchtar E, Dispenzieri A, Leung N, Lacy MQ, Buadi FK, Dingli D, Grogan M, Hayman SR, Kapoor P, Hwa YL, Fonder A, Hobbs M, Chakraborty R, Gonsalves W, Kourelis TV, Warsame R, Russell S, Lust JA, Lin Y, Go RS, Zeldenrust S, Kyle RA, Rajkumar SV, Kumar SK, Gertz MA. Leukemia. 2018 Oct;32(10):2240-2249.

Palladini, G., Sachchithanantham, S., Milani, P., Gillmore, J., Foli, A., Lachmann, H., Basset, M., Hawkins, P., Merlini, G. & Wechalekar, A.D. (2015) A European collaborative study of cyclophosphamide, bortezomib, and dexamethasone in upfront treatment of systemic AL amyloidosis. *Blood,* 126, 612-615.

Palladini G, et al. New criteria for response to treatment in AL amyloidosis based on free light chain measurement and cardiac biomarkers: impact on survival outcomes. J Clin Oncol 2012; 30: 4541-4549.

Palladini G, et al. A staging system for renal outcome and early markers of renal response to chemotherapy in AL amyloidosis. Blood 2014; 124: 2325-2332.

Wechalekar, A., Whelan, C., Lachmann, H., Fontana, M., Mahmood, S., Gillmore, J. & Hawkins, P. (2015) Oral Doxycycline Improves Outcomes of Stage III AL Amyloidosis - a Matched Case Control Study. *Blood,* 126, 732.

Wechalekar, A., Whelan, C. Encouraging impact of doxycycline on early mortality in cardiac light chain (AL) amyloidosis . (2017) *Blood Cancer J,* 24, 546.

Wechalekar, A.D., Schonland, S.O., Kastritis, E., Gillmore, J.D., Dimopoulos, M.A., Lane, T., Foli, A., Foard, D., Milani, P., Rannigan, L., Hegenbart, U., Hawkins, P.N., Merlini, G. & Palladini, G. (2013) A European collaborative study of treatment outcomes in 346 patients with cardiac stage III AL amyloidosis. *Blood,* 121, 3420-3427.

14 APPENDICES

Appendix 1: Hematologic Response Criteria

Response Subcategory	Response Criteria
Complete Response (CR)	Normalization of free light chain ratio, negative serum and urine immunofixation
Very Good Partial Response (VGPR)*	Reduction in the dFLC to <40 mg/L
Partial Response (PR)*	A greater than 50% reduction in the dFLC
No Response (NR)	Less than a PR

Abbreviations: dFLC = difference between involved and uninvolved free light chains

*Only applicable for subjects with dFLC > 50 mg/L (5 mg/dL) at study entry.

Source: Palladini 2012

Appendix 2: Eastern Cooperative Oncology Group (ECOG) Performance Status

Grade	Eastern Cooperative Oncology Group
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead

Source: Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol 1982;5(6):649-55.

Appendix 3: New York Heart Association Functional Classification

NYHA Class	Symptoms
I	Cardiac disease, but no symptoms and no limitation in ordinary physical activity, e.g. shortness of breath when walking, climbing stairs etc.
II	Mild symptoms (mild shortness of breath and/or angina) and slight limitation during ordinary activity.
III	Marked limitation in activity due to symptoms, even during less-than-ordinary activity, e.g. walking short distances (20–100 m). Comfortable only at rest.
IV	Severe limitations. Experiences symptoms even while at rest. Mostly bedbound patients.

Abbreviations: NYHA = New York Heart Association.
Source: http://www.heart.org/HEARTORG/Conditions/HeartFailure/AboutHeartFailure/Classes-of-Heart-Failure_UCM_306328_Article.jsp

Appendix 4: Questionnaires

European Organization for Research and Treatment of Cancer **(EORTC QLQ-C30)** Survey

ENGLISH

EORTC QLQ-C30 (version 3)

We are interested in some things about you and your health. Please answer all of the questions yourself by circling the number that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

Please fill in your initials: ⌊ | | | ⌋
Your birthdate (Day, Month, Year): ⌊ | | | | | | | | ⌋
Today's date (Day, Month, Year): 31 ⌊ | | | | | | | | ⌋

	Not at All	A Little	Quite a Bit	Very Much
1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2. Do you have any trouble taking a long walk?	1	2	3	4
3. Do you have any trouble taking a short walk outside of the house?	1	2	3	4
4. Do you need to stay in bed or a chair during the day?	1	2	3	4
5. Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4

During the past week:	Not at All	A Little	Quite a Bit	Very Much
6. Were you limited in doing either your work or other daily activities?	1	2	3	4
7. Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8. Were you short of breath?	1	2	3	4
9. Have you had pain?	1	2	3	4
10. Did you need to rest?	1	2	3	4
11. Have you had trouble sleeping?	1	2	3	4
12. Have you felt weak?	1	2	3	4
13. Have you lacked appetite?	1	2	3	4
14. Have you felt nauseated?	1	2	3	4
15. Have you vomited?	1	2	3	4
16. Have you been constipated?	1	2	3	4

Please go on to the next page

ENGLISH

During the past week:	**Not at All**	**A Little**	**Quite a Bit**	**Very Much**
17. Have you had diarrhea?	1	2	3	4
18. Were you tired?	1	2	3	4
19. Did pain interfere with your daily activities?	1	2	3	4
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?	1	2	3	4
21. Did you feel tense?	1	2	3	4
22. Did you worry?	1	2	3	4
23. Did you feel irritable?	1	2	3	4
24. Did you feel depressed?	1	2	3	4
25. Have you had difficulty remembering things?	1	2	3	4
26. Has your physical condition or medical treatment interfered with your family life?	1	2	3	4
27. Has your physical condition or medical treatment interfered with your social activities?	1	2	3	4
28. Has your physical condition or medical treatment caused you financial difficulties?	1	2	3	4

For the following questions please circle the number between 1 and 7 that best applies to you

29. How would you rate your overall health during the past week?

1	2	3	4	5	6	7
Very poor						Excellent

30. How would you rate your overall quality of life during the past week?

1	2	3	4	5	6	7
Very poor						Excellent

SF-36 Questionnaire

Your Health and Well-Being

This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities. Thank you for completing this survey!

For each of the following questions, please select the one box that best describes your answer.

In general, would you say your health is:

Excellent
Very good
Good
Fair
Poor

Compared to one year ago, how would you rate your health in general now?

Much better now than one year ago
Somewhat better now than one year ago
About the same as one year ago
Somewhat worse now than one year ago
Much worse now than one year ago

The following question is about activities you might do during a typical day.

Does your health now limit you in vigorous activities, such as running, lifting heavy objects, participating in strenuous sports? If so, how much?

Yes, limited a lot
Yes, limited a little
No, not limited at all

The following question is about activities you might do during a typical day.

Does your health now limit you in moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf? If so, how much?

Yes, limited a lot
Yes, limited a little
No, not limited at all

The following question is about activities you might do during a typical day.

Does your health now limit you in lifting or carrying groceries? If so, how much?

Yes, limited a lot
Yes, limited a little
No, not limited at all

The following question is about activities you might do during a typical day.

Does your health now limit you in climbing several flights of stairs? If so, how much?

Yes, limited a lot
Yes, limited a little
No, not limited at all

The following question is about activities you might do during a typical day. Does your health now limit you in climbing one flight of stairs? If so, how much? Yes, limited a lot Yes, limited a little No, not limited at all
The following question is about activities you might do during a typical day. Does your health now limit you in bending, kneeling, or stooping? If so, how much? Yes, limited a lot Yes, limited a little No, not limited at all
The following question is about activities you might do during a typical day. Does your health now limit you in walking more than a mile? If so, how much? Yes, limited a lot Yes, limited a little No, not limited at all
The following question is about activities you might do during a typical day. Does your health now limit you in walking several hundred yards? If so, how much? Yes, limited a lot Yes, limited a little No, not limited at all

The following question is about activities you might do during a typical day. Does your health now limit you in walking one hundred yards? If so, how much? Yes, limited a lot Yes, limited a little No, not limited at all
The following question is about activities you might do during a typical day. Does your health now limit you in bathing or dressing yourself? If so, how much? Yes, limited a lot Yes, limited a little No, not limited at all
During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities? Cut down on the amount of time you spent on work or other activities as a result of your physical health All of the time Most of the time Some of the time A little of the time None of the time
During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities? Accomplished less than you would like as a result of your physical health All of the time Most of the time Some of the time A little of the time None of the time

During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities?

Were limited in the kind of work or other activities as a result of your physical health

All of the time
Most of the time
Some of the time
A little of the time
None of the time

During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities?

Had difficulty performing the work or other activities as a result of your physical health (for example, it took extra effort)

All of the time
Most of the time
Some of the time
A little of the time
None of the time

During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities?

Cut down on the amount of time you spent on work or other activities as a result of any emotional problems (such as feeling depressed or anxious)

All of the time
Most of the time
Some of the time
A little of the time
None of the time

During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities?

Accomplished less than you would like as a result of any emotional problems (such as feeling depressed or anxious)

All of the time
Most of the time
Some of the time
A little of the time
None of the time

During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities?

Did work or other activities less carefully than usual as a result of any emotional problems (such as feeling depressed or anxious)

All of the time
Most of the time
Some of the time
A little of the time
None of the time

During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

Not at all
Slightly
Moderately
Quite a bit
Extremely

How much bodily pain have you had during the past 4 weeks?

None
Very mild
Mild
Moderate
Severe
Very Severe

During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

Not at all
A little bit
Moderately
Quite a bit
Extremely

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks did you feel full of life?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks have you been very nervous?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks have you felt so down in the dumps that nothing could cheer you up?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks have you felt calm and peaceful?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks did you have a lot of energy?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks have you felt downhearted and depressed?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks did you feel worn out?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks have you been happy?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

This question is about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks did you feel tired?

All of the time
Most of the time
Some of the time
A little of the time
None of the time

During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)? All of the time Most of the time Some of the time A little of the time None of the time
How TRUE or FALSE is the following statement for you? I seem to get sick a little easier than other people. Definitely true Mostly true Don't know Mostly false Definitely false
How TRUE or FALSE is the following statement for you? I am as healthy as anybody I know. Definitely true Mostly true Don't know Mostly false Definitely false
How TRUE or FALSE is the following statement for you? I expect my health to get worse. Definitely true Mostly true Don't know Mostly false Definitely false

How TRUE or FALSE is the following statement for you?

My health is excellent.

Definitely true
Mostly true
Don't know
Mostly false
Definitely false

Appendix 5 Antibiotic Prophylaxis in Arm B

Patients who will be included in the Amyloidosis Center Heidelberg will receive antibiotic prophylaxis in arm B **during the first chemotherapy cycle** according to the SOP of our Center.

- Cotrimoxazole 960 mg BID or
- Ciprofloxacine 500 mg bid (if the patient is allergic to cotrimoxazole).

Addendum A Preclinical data

A1. Proposed indication

Use of doxycycline hyclate in the treatment of systemic amyloidosis caused by monoclonal immunoglobulin light chains (AL amyloidosis).

A2. Medical plausibility

A.2.1. Active substance: description of the medicinal product, pharmacological class and mode of action

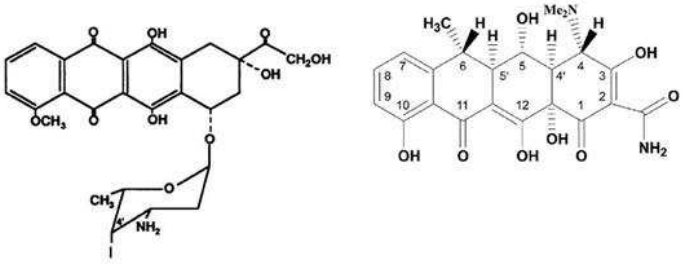
Doxycycline hyclate is a tetracycline class antibiotic. Doxycycline interferes with the third stage of bacterial protein synthesis. After amino acids are activated and attached to t-RNA (transfer RNA), the resulting amino acyl-t-RNA migrates to the bacterial ribosome for synthesis of proteins. Doxycycline binds to the 30S subunit on the ribosome and inhibits binding of the aminoacyl-t-RNA molecules. Please see section A.2.2. for mode of action in amyloidosis.

A.2.2. Data with the specific product as applied for designation in specific models or in patients affected the condition

In 1995 we serendipitously discovered the anthracycline 4'-iodo-4'-deoxydoxorubicin (IDOX) as the prototype of a class of compounds able to inhibit protein aggregation in vitro and in animal models of systemic amyloidosis (Merlini, *et al* 1995) and in patients (Gertz, *et al* 2002, Gianni, *et al* 1995). The first study showed that IDOX presented high affinity for all types of amyloid deposits, and would have been a candidate for the treatment of all types of amyloidoses (Merlini, *et al* 1995). Accordingly, an international patent on the use of IDOX in all types of amyloidosis was deposited (international patent n. WO9504538).

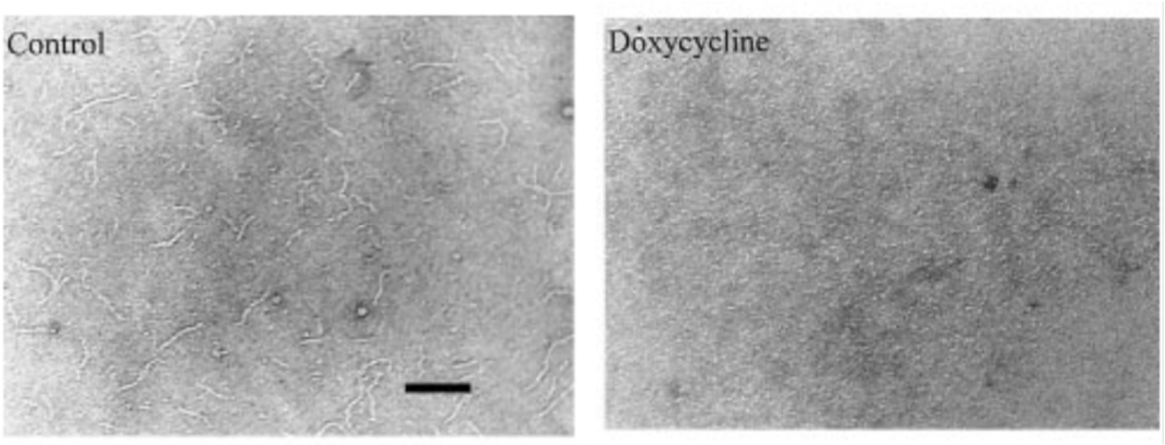
In consideration of the cytotoxicity of IDOX, in the following years, the tetracycline antibiotics were investigated on the basis of structural homologies with the aglycone moiety of the anthracyclines, as shown in Figure 1.

Structures of iododoxorubicin and doxycycline showing the resemblance of the polycyclic conjugated structure



We tested doxycycline for its ability to interfere with transthyretin (ATTR) amyloid formation and assessed its capacity to inhibit fibril formation and/or disrupt TTR fibrils (Cardoso, *et al* 2003). We found that doxycycline acted primarily as fibril disrupter since fibrils were observed in intermediate times (data not shown) before 17 days. After 17 days of incubation at 37°C with doxycycline (360 μM/100 μg fibrils), the sample clearly showed small round particles abundantly, no fibrils were visible after incubation (Figure 2). We concluded that doxycycline is therefore a good candidate in therapeutic approaches in amyloidosis.

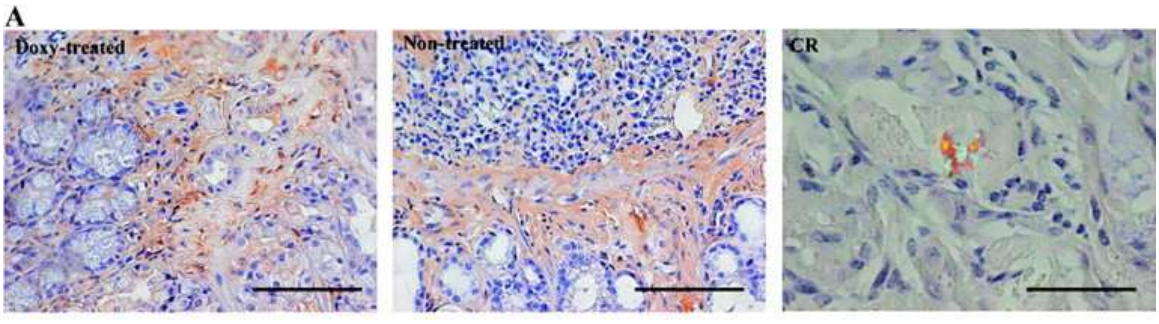
Doxycycline disrupts transthyretin amyloid fibrils in vitro



Effect of doxycycline on TTR Leu55Pro fibril assembly in vitro. After 17 days at 37°C, fibrils formed in the absence of drug were visualized by TEM (Control) whereas incubation with doxycycline (drugs 360 μM/100 μg fibrils) resulted in complete fibril disruption. Scale bar (control doxycycline) __ 100 nm. Adapted from Cardoso et al (Cardoso, *et al* 2003).

The Saraiva's group from Porto, Portugal, assessed the activity of doxycycline in vivo in the transgenic mouse model of TTR amyloidosis (transgenic mice for human TTR Val30Met in a TTR null background: TTR-Met30Val X TTR-KO mice). In this model, younger animals present only non-fibrillar TTR deposits, especially in the gastrointestinal tract and skin; with age, short fibrils are easily observed by electron microscopy, which are Congo red positive. Furthermore, TTR initial aggregates are cytotoxic, both in vivo and in vitro, as evidenced by the presence of increased amounts of proinflammatory cytokines and oxidative stress markers, such as nitrotyrosine, in tissues (Cardoso and Saraiva 2006). Doxycycline was administrated in the drinking water to 23- to 28-month-old mice over a period of 3 months. Immunohistochemistry analyses revealed no differences in nonfibrillar TTR deposition between treated (n=11) and untreated mice (n=11). However, Congo red positive material was observed only in the control group (untreated), whereas none of the animals treated with doxycycline was Congo red positive (Figure 3).

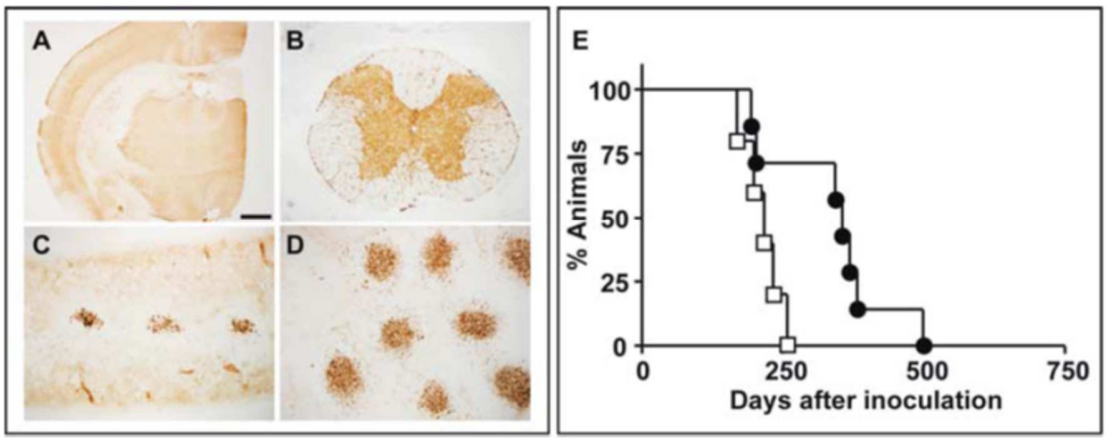
Doxycycline disrupts amyloid deposits and reduces the amyloid load in a transgenic mouse model



Immunohistochemistry for human TTR and Congo red binding in treated and non-treated transgenic mice. Left panel: Stomach slide representative of mice treated with doxycycline 40 mg/Kg/day for 3 months: TTR deposition presents similar load to the non-treated animals

(middle panel). Congo red positive TTR immunoreactive deposits were only observed in the control non-treated group (right panel). Scale bar: 100 μm (left and middle panels); 50 μm (right panel). Adapted from (Cardoso and Saraiva 2006).
Immunohistochemistry for several markers associated with amyloid, such as matrix metalloproteinase-9 (MMP-9) and serum amyloid P component (SAP), was performed. MMP-9 was altered with significantly lower levels in treated animals compared with the control group. Mouse SAP was absent in treated animals, being observed only in untreated animals presenting TTR congophilic deposits (Cardoso and Saraiva 2006). These results indicate that doxycycline is capable of disrupting Congo red positive amyloid deposits and decreases standard markers associated with fibrillar deposition, being a potential drug in the treatment of amyloidosis.
Dr. Saraiva's group in collaboration with our group reported that tauroursodeoxycholic acid (TUDCA), a biliary acid, administrated to the same transgenic mouse model was effective at lowering deposited non-fibrillar TTR, as well as the levels of markers associated with pre-fibrillar TTR, but only at young ages.
Different doxycycline administration schemes, including different periods of treatment, different dosages and different familial amyloid polyneuropathies (FAP) TTR V30M animal models were evaluated (Cardoso, *et al* 2010). It was observed that a minimum period of 15 days of treatment with a 8 mg/Kg/day doxycycline dosage resulted in fibril removal. This dosage corresponds to a human equivalent daily dose of 24 mg/m^2 or 0.65 mg/Kg according to (Nair and Jacob 2016). The possibility of intermittent treatments was also assessed and a maximum period of 15 days of suspension was determined to maintain tissues amyloid-free.
The efficacy of the treatment with tetracyclines has been evaluated also in other animal models of amyloidosis, such as peripheral and intracerebral prion infection in Syrian hamsters (Figure 4) (De Luigi, *et al* 2008).

Effects of doxycycline on prion infection in Syrian hamsters



PrP^{Sc} accumulated in all brain areas examined, except for the hippocampus (panel A), in spinal cord (panel B), in the white pulp of spleen (panel C) and in the Peyer's patches (panel D). Figure 4E shows that a single dose of doxycycline significantly ($p = 0.031$) increased median survival by 64%, from 217 days for controls to 355 days for the treated group. This protective effect was paralleled by the delayed onset of clinical signs of disease in all treated animals (data not shown).
Furthermore, it has been reported that tetracycline treatment retards the onset and slows the progression of diabetes in human amylin/islet amyloid polypeptide transgenic mice (Aitken, *et al* 2010).
Finally, it has been shown that doxycycline prevents amyloid β fibrillization and toxicity in a Drosophila melanogaster model of Alzheimer disease (Costa, *et al* 2011).

More recently, Saraiva and coworkers exposed to doxycycline-TUDCA acid treatment hTTR V30M transgenic mice (Teixeira, *et al* 2016). The animals were treated with both doxycycline (intra-peritoneal injection) and TUDCA (in the drinking water) for four weeks while an age-matched control group was treated with vehicles. More than 50% of the untreated animals presented TTR amyloid deposits, whereas only 9% of the treatment group exhibited amyloid deposition.

References:

Aitken, J.F., Loomes, K.M., Scott, D.W., Reddy, S., Phillips, A.R., Prijic, G., Fernando, C., Zhang, S., Broadhurst, R., L'Huillier, P. & Cooper, G.J. (2010) Tetracycline treatment retards the onset and slows the progression of diabetes in human amylin/islet amyloid polypeptide transgenic mice. *Diabetes,* **59,** 161-171.

Cardoso, I., Martins, D., Ribeiro, T., Merlini, G. & Saraiva, M.J. (2010) Synergy of combined doxycycline/TUDCA treatment in lowering Transthyretin deposition and associated biomarkers: studies in FAP mouse models. *J Transl Med,* **8,** 74.

Cardoso, I., Merlini, G. & Saraiva, M.J. (2003) 4 '-iodo-4 '-Deoxydoxorubicin and tetracyclines disrupt transthyretin amyloid fibrils in vitro producing noncytotoxic species: screening for TTR fibril disrupters. *FASEB Journal,* **17,** 803-809.

Cardoso, I. & Saraiva, M.J. (2006) Doxycycline disrupts transthyretin amyloid: evidence from studies in a FAP transgenic mice model. *FASEB J,* **20,** 234-239.

Costa, R., Speretta, E., Crowther, D.C. & Cardoso, I. (2011) Testing the therapeutic potential of doxycycline in a Drosophila melanogaster model of Alzheimer disease. *J Biol Chem,* **286,** 41647-41655.

De Luigi, A., Colombo, L., Diomede, L., Capobianco, R., Mangieri, M., Miccolo, C., Limido, L., Forloni, G., Tagliavini, F. & Salmona, M. (2008) The efficacy of tetracyclines in peripheral and intracerebral prion infection. *PLoS One,* **3,** e1888.

Gertz, M.A., Lacy, M.Q., Dispenzieri, A., Cheson, B.D., Barlogie, B., Kyle, R.A., Palladini, G., Geyer, S.M. & Merlini, G. (2002) A multicenter phase II trial of 4 '-iodo-4 ' deoxydoxorubicin (IDOX) in primary amyloidosis (AL). **9,** 24-30.

Gianni, L., Bellotti, V., Gianni, A.M. & Merlini, G. (1995) New drug therapy of amyloidoses: resorption of AL-type deposits with 4'-iodo-4'-deoxydoxorubicin. *Blood,* **86,** 855-861.

Merlini, G., Ascari, E., Amboldi, N., Bellotti, V., Arbustini, E., Perfetti, V., Ferrari, M., Zorzoli, I., Marinone, M.G., Garini, P. & . (1995) Interaction of the anthracycline 4'-iodo-4'-deoxydoxorubicin with amyloid fibrils: inhibition of amyloidogenesis. *Proc Natl Acad Sci U S A,* **92,** 2959-2963.

Nair, A.B. & Jacob, S. (2016) A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm,* **7,** 27-31.

Teixeira, C., Costelha, S. & Saraiva, M. (2016) *Doxycycline-Tauroursodeoxycholic acid treatment: effects in the heart of a Transthyretin V30M transgenic mouse model* Uppsala.