Detailed Description of Project Protocol

1. Title: Investigating interactions between the triad of (I) Myeloma disease activity, (II) Immune-competence (iii) Infections and Health Care Associated Infections to stratify patients for risk of infection, guide use of prophylactic antibiotics and improve response to anti-myeloma therapies

2. Background

2.1 Brief description of study protocol

The main study this application relates to is the NIHR-Health Technology Assessment Programme 08/116/69 - Tackling Early Morbidity and Mortality in Myeloma: Assessing the benefit of antibiotic prophylaxis and its effect on healthcare associated infections. The objective of this randomised, double-blind, placebo-controlled multi-centre phase 3 trial (TEAMM) is to assess the risks, benefits and cost effectiveness of a prophylactic antibiotic for 12 weeks in patients with newly diagnosed symptomatic multiple myeloma (MM). The trial hypotheses that Levofloxacin will reduce infection related morbidity and mortality, increase response to anti-myeloma therapy and improve patient survival, without significant increase in Health Care Associated infection (HCAI). The study population is newly diagnosed symptomatic MM patients, (median age 68yrs IQR 60 - 75), within 2 weeks of starting anti-myeloma therapy. Patients are allocated to receive either Levofloxacin 500 mg or placebo once daily orally for 12 weeks. At entry, 4, 8, 12 & 16 weeks samples are taken for central laboratory analysis of stools and nasal swabs for microbiology; blood and urine for myeloma response and immune function. The primary outcome is number of febrile episodes in the first 12 weeks from randomisation defined as a single oral temperature ≥38° C and that the patient is then given anti-infectives. The secondary outcome measures include: days on anti-infective therapy for treatment of infection; carriage and invasive infections with *S. aureus, C. difficile* and ESBL coliforms; number of days in hospital and deaths from baseline to 12 weeks; response to anti-MM therapy and its relationship to infection. The full TEAMM protocol (ISRCTN51731976) can be found here: http://www2.warwick.ac.uk/fac/med/research/ctu/trials/cancer/teamm/

TEAMM started in November 2011 and recruitment of 800 patients is set to end in November 2015, with all study samples being collected by February 2016. 686 patients had been randomised by 31st March 2015 out of a planned 566 with an average of 26 patients per month across last 3 months (121% of target). Therefore, we anticipate the target sample size of 800 patients being completed prior to the projected end date.

2.2 Existing research

MM is a cancer of bone marrow plasma cells that causes profound immunosuppression and recurrent infections. Infection is a major cause of morbidity and mortality in patients with active MM [1, 2], particularly in the first 12 weeks from diagnosis as anti-MM therapy gradually brings the disease under control. An analysis of all patients registered onto UK MRC trials from 1980 to 2002 showed that 10% of patients died within 60 days of trial entry, and almost half of these were due to bacterial infection [3]. Although survival has greatly improved with modern anti-MM therapies, this high early death rate remains unchanged. The objective of the TEAMM trial is to tackle the early mortality rate in MM through infection prevention via antibiotic prophylaxis.

Active MM results in wide-ranging immunodeficiency and immune dysregulation [1]. Interaction between MM tumor cells and the immune system via cytokines promotes tumor

cell survival and drug resistance [2, 4]. Evidence from in vitro studies suggests infection may amplify this damaging relationship, causing inflammation and the release of cytokines that further stimulates the growth and survival of MM cells and resistance to anti-MM therapy [5, 6]. Infections also delay and disrupt anti-MM therapy. Although this complex cycle between the immune system, infection, and MM activity has been outlined in several articles, the specific mechanism by which the risk of infection is increased in the presence of active MM disease is not well understood. A range of factors and cytokines are implicated in MM pathogenesis and infection. However, currently there is no one factor or criteria to identify those at greater risk of infection following diagnosis. Consequently, this project aims to use established biomarkers of immune-competence, infection and inflammation to stratify infection risk in newly diagnosed patients. Further, as TEAMM is the first large study of its kind in MM, the mechanisms connecting Levofloxacin to patient outcomes have yet to be investigated.

Polyclonal immunoglobulins (Igs, normal antibodies) are reduced in MM and this reduction, known as immunoparesis, is typically linked with bacterial respiratory tract infections especially streptococcus pneumoniae, one of the most frequent infections that occur in these patients [1]. Total levels of polyclonal Igs may provide an indication of susceptibility to infection. However levels of antibody against a range of pertinent bacterial antigenic targets will provide more specific information regarding protection against bacterial infections like Streptococcus pneumonia, but have yet to be measured in a RCT assessing infections. In addition, anti-viral Igs, against influenza and also latent viruses, represent an important marker of immune-competence, but their relationship with other aspects of immune function and patient outcomes in a RCT such as TEAMM have yet to be explored. Neutrophil function has been shown to be reduced with ageing [7, 8] and is implicated in the high incidence of bacterial infections in older adults [9]. Interleukin-10 (IL-10) is regarded as an anti-inflammatory cytokine that down regulates innate immune function, including those of neutrophils [10, 11]. Further, IL-10 has been shown to be elevated in MM patients, and correlates significantly with disease progression [12]. Therefore IL-10 may be implicated in both susceptibility to infection and active MM and be a therapeutic target. These markers of immune competence have not been studied in the context of antibiotic prophylaxis and infection in myeloma. We aim to use these markers to characterise infection risk and guide treatment stratification.

TEAMM already assesses serum C Reactive Protein (CRP) as a marker of inflammation. Procalcitonin (PCT) becomes elevated in response to pro-inflammatory stimuli of bacterial origin, which is of key interest in newly diagnosed MM patients and TEAMM. PCT has been shown to be more sensitive in identifying community-acquired pneumonia compared with CRP [13]. A recent systematic review of RCTs has shown that PCT to confirm bacterial infection is generally associated with reduced antibiotic use, without an increase in mortality or treatment failure [14]. The use of PCT in differential diagnosis of febrile states in MM has been documented in case studies [15], but the use of PCT to confirm bacterial infection in a study as large as TEAMM has not taken place. This project will utilise this measure as a sensitive marker of infection induced inflammation.

Interleukin-6 (IL-6) increases in the bloodstream during bacterial and viral infection. IL-6 is also a pro-inflammatory cytokine and a range of studies have confirmed this cytokine

promotes MM cell growth, survival, and drug resistance [4, 16]. Classic IL-6 signalling occurs when IL-6 binds to membrane-bound IL-6R + gp130, an alternative IL-6 signalling pathway termed IL-6 'trans-signalling' occurs. In this condition, IL-6R is shed from the surface of host cells during MM resulting in elevated soluble IL-6R, followed by the formation of soluble IL-6/IL-6R complexes that bind membrane-bound gp130 expressed on tumour cells, resulting in tumour growth [17, 18]; the increased soluble IL-6R has been associated with a poor response to chemotherapy [19]. Interleukin 8 (IL-8), a pro-inflammatory chemokine, is a known inducer of angiogenesis in myeloma and has been implicated in the pathophysiology of the disease [20, 21]. This chemokine correlated with MM disease activity [22] and has also been shown to contribute to bone marrow-induced NF-κB activity; this activity is associated with treatment resistance to bortezomib [23]. During inflammation and infection, IL-8 plays a role in the recruitment and activation of neutrophils [24]. These cytokines have been separately acknowledged in their role in infection and MM disease activity; however, their potential simultaneous roles in infection-induced inflammation, active MM and treatment resistance have yet to be investigated. Investigating these biomarkers in TEAMM would uncover their importance for these interactions, and thereby help identify patients who may be at risk of poor treatment response, who may be most susceptible to infection, and those who may most benefit from Levofloxacin and/or additional treatment pathways during induction therapy.

Measuring the ratio of malignant monoclonal Ig versus uninvolved polyclonal Ig can provide more accurate and sensitive information than just monoclonal component on its own [25]. More advanced assays are now available to identify changes in disease activity, and these can be utilised by this project to monitor myeloma response and its relationship with immune competence and infection. In addition to myeloma protein changes, we will measure CD138 (Syndecan-1) which is a proteoglycan that is shed from myeloma cells that has effects on tumor cell growth, survival, adhesion and invasion and on bone cell differentiation [26, 27]. Elevated levels of serum soluble syndecan-1 (sCD138) correlate with increased tumor mass and decreased patient survival [26, 28]. More recently, a large cohort study demonstrated that the level of sCD138 at presentation and its reduction with treatment were powerful prognostic indicators for overall survival in MM [29]. Measurement of CD138 in TEAMM at presentation and during therapy induction will enable sensitive measurement of speed and depth of response, and help establish how Levofloxacin may be associated with improved response and survival.

This project builds on this body of existing research by planning to investigate the links between the triad of MM disease activity, immune competence and infection, to better understand the relationship between the intervention and patient outcomes in TEAMM. Examining more sensitive markers of MM activity, response to therapy and bacterial infection will support this objective and provide additional benefits for patient monitoring and stratification.

2.3. Risks and benefits

There are no new potential risks posed by this study. This study is analysing stored serum samples that have been collected during TEAMM; no further patient contact is required. This project represents a unique opportunity to produce outcomes that will deliver benefits to MM patients, other cancer patients and the NHS. The project objectives are driven by impacting

on clinical practice and improving patient care in three main areas: informing patient management following diagnosis via risk stratification; improving infection identification; informing efficacy of therapy and decisions regarding switching treatment; identifying patients who may benefit from additional available targeted therapies.

Risk stratification for infection at diagnosis will enable targeted prescription of antibiotics and avoid blanket prescription in an environment of antibiotic resistance. This will divert patients who may not benefit from prophylactic antibiotics to just education and close monitoring and help manage the risk of health care associated infections. In high risk patients levofloxacin aims to reduce number of infections, and their consequent impact on disease activity and anti-MM therapy, and improve patient prognosis.

More sensitive measurements of speed and depth of MM response will enable rapid identification of poor responders to therapy and enable early therapy change. Gaining control over active disease via therapy switching will simultaneously reduce the risk of infection and its associated complications, including mortality risk. Response during induction therapy predicts final response and survival, therefore switching from sub-optimal therapy at an early stage offers clear long-term benefits for patients, reducing the chances of residual disease and relapse. This strategy can avoid adverse events and NHS expenditure related to ineffective therapies. In addition to identifying patients most at risk of infection and poor treatment response, understanding interactions between infection, inflammation and active MM could help stratify patients further towards other targeted therapies. This has the potential to maximize the effectiveness of anti-MM and improve patient outcomes following diagnosis.

Introducing risk stratification and more sensitive monitoring into MM management will enable more personalised, targeted and responsive care and clinical decisions. This in turn should reduce patient's risk of infection and improve their chance of successful therapy during induction, thus avoiding early mortality and improving long-term prognosis. In addition to these benefits for MM patients, this research is also relevant to other groups who are susceptible to infection, such as those undergoing chemotherapy for other cancers or the general elderly population. Therefore the outcomes of this project could potentially lead to benefits for a variety of patients.

2.4 Rationale for the proposed study

MM is one of the top 20 most common cancers in the UK, diagnosed in nearly 5,000 patients each year. MM is an incurable disease that requires long-term monitoring and predominately affects individuals aged 60 and over. The incidence of MM has increased by over 11% in the last decade, and is likely to further increase in the next decade due to an ageing population and improving long-term survival. Therefore, providing the most effective healthcare for MM patients is imperative and will be of sustained interest in the up-coming years. As such, this study seeks to generate knowledge and outputs to improve MM patient care and disease monitoring.

As shown in Figure 1 (Appendix), interactions between the triad of myeloma disease activity, immunity and infection are central to TEAMM. Active MM suppresses immunity, resulting in a high incidence of infections following diagnosis. These infections delay anti-MM therapy and infection-induced inflammation in turn nurtures active MM and promotes resistance to

anti-MM therapy. This cyclical relationship contributes to the early mortality observed in MM. However, the mechanisms driving these interactions have yet to be explored. Factors that suppress immunity, promote active MM and are implicated in infection-induced inflammation have been separately identified, although they have yet to be examined together to understand their role in the cycle of active disease and treatment response following diagnosis. Uncovering disease-related mechanisms following diagnosis and during induction therapy will facilitate stratification and intervention to improve responses early in the disease and long-term prognosis.

Currently, other than the decision to assign patients to high/low-dose therapy, patient stratification is not evident in myeloma care provision. TEAMM will tell us about the benefits and risks of prophylactic antibiotics, but not about the mechanisms linking antibiotics and patient outcomes. Our mechanistic study will provide outputs and information that will take advantage of the knowledge surrounding benefits and risks that arise from TEAMM. Patients prescribed antibiotics may experience less infections and better patient outcomes. Given the current challenges surrounding antibiotic resistance and the possibility of healthcare associated infections, antibiotic prophylaxis may not be appropriate to give to all patients. As such, there is a need to stratify risk of infection at diagnosis. Presently we know that MM patients are at greater risk of infections, and we know that this negatively affects patient outcomes, but we do not have the tools to predict which patients are most susceptible to infection and thus most likely to benefit from levofloxacin; this project seeks to establish these tools to inform key decisions during induction therapy.

Myeloma patients secrete their own monoclonal immunoglobulin (MI) that provides a cancer biomarker unique to each patient. Currently, a range of techniques are required to screen, characterise and quantitate MI. Not only is this expensive and slow, but significant between method and between laboratory variability exists in the quantitation of MI as seen in external quality control schemes (NEQUAS). In addition, due to natural biological variation in MI between patients it is difficult to make comparisons across different types of myeloma. As such, using a disease marker that is present regardless of individual differences in MI, CD138, will enable more reliable inter-patient comparisons and more effective stratification of patients. We propose to employ more sensitive techniques and markers to assess active myeloma/response to therapy to identify non-/sub-optimal responders and inform early therapy change. This is important as response during induction therapy predicts final response. Sensitive measurements of the speed and depth of response are also important to support addressing questions surrounding active myeloma and immune-competence and infection outlined in this proposal, facilitating more accurate myeloma response comparisons between the treatment and control arms.

3. Research objectives

Objective 1: Measuring markers of immune competence to develop risk-stratification of patients for infection so that the decision to prescribe antibiotic prophylaxis can be personalised.

We will analyse measures of immune-competence and their relationship with infections during TEAMM to develop immune profile criteria to identify patients at disease presentation who are most at risk of infection. This objective represents phase one of the work scheme outlined in the gantt chart. A total of 14 months has been allocated to conducting the laboratory analyses of immune-competence markers followed by 2 months for statistical

analyses to establish infection risk criteria/threshold of immune-competence below which the likelihood of bacterial infection is significantly increased. The purpose of creating infection risk stratification criteria at disease presentation is to target use of prophylactic antibiotics. These criteria should ensure that patients at high risk of infection receive prophylactic antibiotics to prevent infection related morbidity and mortality and improve response to anti-myeloma therapy. By identifying patients who are unlikely to benefit from prophylactic antibiotics there will be reduced use of antibiotics and their associated risk of health care associated infections.

Objective 2: Measuring markers of myeloma activity to sensitively measure speed and depth of myeloma response.
Using new, clinically-validated analytical laboratory methods we will measure markers of active myeloma and response to therapy at each time point in TEAMM. This will form phase 2 of the project, and consist of 8 months of laboratory analyses and 2 months of statistical analyses assessing speed and depth of response in relation to levofloxacin and placebo patients, infection and mediators of myeloma tumour growth. The purpose of these myeloma response markers is to provide more accurate speed of response comparisons between the treatment and control arms to assess if levofloxacin does improve response to anti-myeloma therapy and across the trial to see if infection reduces response to anti-myeloma therapy. Secondly to provide more precise profiling of disease response to rapidly identify patients who do not respond adequately to therapies, and inform change to alternative therapies.

Objective 3: Measuring markers of inflammation to identify patients who may be at risk of poor treatment responses and patients who may benefit from additional targeted therapies.
Markers of inflammation associated with active myeloma and induced by infection will be measured at all TEAMM timepoints as part of the third phase of study data collection; this has been scheduled to take place over the course of 4 months, followed by 2 months of statistical analyses investigating their relationship with levofloxacin, infection and responses to anti-myeloma therapy. The purpose of assessing these markers is to confirm bacterial infection and to identify patients who may be at risk of poor treatment response, who may be most susceptible to infection, and those who may most benefit from Levofloxacin and/or additional treatment during induction therapy.

4. Research design

This project is a retrospective analysis of samples collected during TEAMM, a randomised, double-blind, placebo-controlled phase 3 trial in which placebo or Levofloxacin was allocated on a 1:1 basis. This study will assess a range of markers not measured in the original trial to assess the mechanism of action of Levofloxacin. As samples will have already been collected and stored, this study will feature laboratory and statistical analyses only.

5. Study population

The mechanistic study will include analyses of all participants who entered the TEAMM trial, providing they did not go on to withdraw from the study at a later date. All participants are required (placebo/treatment, those who did/did not develop infections, those who did/did not survive) in order to compare patients and fully investigate the outcomes of the proposed mechanistic study.

6. Proposed outcome measures

Three main blocks of outcome measures are proposed from study entry to 16 weeks

Markers of immune-competence

- Anti-bacterial and anti-viral antibodies
- Neutrophil function
- IL-10

Markers of active myeloma and response to therapy

- Class specific heavy/light chain ratios to provide measurement of malignant versus un-involved immunoglobulin
- Syndycan-1 (CD138)

Markers of infection-induced inflammation

- IL-6 family
- IL-8
- Procalcitonin

The timepoints to be measured for each of these variables, the number of samples to be analysed, the assay to be used, and supplier, and costs are fully broken down in Table 1 (appendix). The expected physiological ranges for these biomarkers are shown in Table 2 (appendix).

7. Proposed sample size

All possible samples collected at trial entry, 4, 8, 12 & 16 weeks for the 800 patients recruited into the TEAMM trial will be used to assess markers. The first 250 patients samples will be enough to produce statistical models for immune-competence, infection-induced inflammation and myeloma activity and response which can be validated by the remaining set. It is estimated that 98% of baseline samples will be available for the 800 TEAMM patients and not more that 25% attrition at 16 weeks.

Assuming 30% of patients will experience a febrile episode, a sample of 250 patients is sufficient, for example to detect odds ratios of at least 1.001 in continuous biomarkers with a 5% significance level and at least 95% power and an odds ratio of at least 1.11 for a binary variable with a 20:80 split and 80% power. Power calculations were performed using PROC power in SAS version 9.3 using the Shieh-O'Brien approximation.

Table 1 provides sample sizes and patients to be tested in relation to each of our research objectives. Initially, a minimum of 250 patients will be required to generate statistical models, however, the remaining patients will be required to test and validate these models. Therefore, the samples numbers stipulated in table 1 will have to be completed to validate the analyses. We already have data at all time points on polyclonal immunoglobulins, C-reactive protein and conventional measures of myeloma paraprotein. In addition to the analyses relating to study end points and biomarkers from our three research areas, associations between the biomarkers to be measured in this mechanistic study and the routine variables already collected will be explored. Due to the heterogeneity of MM, a minimum of 250 patients is required to ensure a high enough sample size in the groups of interest, e.g. infection groups, myeloma response groups.

In many cases, a larger number of patients are required, particularly at baseline, and there are a range of specific reasons justifying this decision for each of the research questions.

For example, for markers of immune-competence all patients at baseline would need to be analysed. Immuno-competence markers are being used to predict a range of patient outcomes: infection, admissions to hospital due to infection, deaths from infection, total hospital admissions and total deaths. We have previously examined immunosuppression of polyclonal immunoglobulins in relation to survival outcomes in other MM trials and it is evident that large sample numbers are required to power these analyses. We will be analysing immune-competence biomarkers in relation to TEAMM outcomes initially for those who did/did not have an infection, followed by the different treatment arms of the trial. From the first 400 patients in TEAMM, there is roughly a 30% rate of infection and 10% death rate, with a third of these being due to infection. Therefore, all 800 patients at baseline will ensure sufficient patient numbers across these groups and statistical power for our prognostic analyses across the range of outcome variable of interest.

For markers of active MM and response to therapy, all patients will be measured at all time points where samples area available. This is because we expect to see differences in responses between each of these 4-week intervals as cycles of treatment are typically 3-4 weeks long. Due to clonal heterogeneity in MM, not all patients have a good response during induction therapy, linked to worse patient outcomes. Based on other trials using the same modern biological therapies as TEAMM, at 16-weeks we anticipate 30-40% of patients will have a complete or very good response, 20-30% will be poor responders, and the remaining patients will have a partial response. These groups, based on International Response Criteria, will be analysed in relation to infection and inflammation. Baseline and 16 weeks will enable assessment of overall response, following 4-5 cycles of induction therapy. Disease activity and response at 4-weeks will be used to predict overall response and assess speed of response. Samples at 8- and 12- weeks will be used to assess the rate of tumor kill response over the course of the cycles of treatment.

Markers of infection-induced inflammation will be assessed at various time points. Procalcitonin will be measured at baseline, 4-, 8- and 12-weeks in order to support the identification of bacterial infections versus viral infections throughout the trial. Inflammatory cytokines will be measured in all patients at baseline to identify and predict those who may be at risk of poor treatment response, who may be most susceptible to infection, and those who may most benefit from Levofloxacin and/or additional treatment during induction therapy. In a smaller cohort of 250 patients (selected to include range of response criteria) will be analysed in order to investigate interactions between inflammation, response to therapy and potential treatment resistance

8. Statistical analysis

Exploratory data analyses will be carried out to determine associations between markers and outcome measures. Pre-specified cut-points for each marker will be used to stop data dependent inflation of estimates (table 1). Associations and correlations of markers will also be explored against standard variables collected routinely within the trial. In addition analysis on serial samples will be performed to assess differences across time using random effects models. Analysis will be carried out to assess markers for immune-competence, infection-induced inflammation and myeloma activity and response.

Analyses will be carried out using validated statistical procedures in SAS version 9.3, such as logistic regression assessing effects of biomarkers on binary outcomes and linear models for continuous outcomes. Cox-regression will be used to assess the effects biomarkers for survival.

These data will be examined by the Data and Safety Monitoring Committee appointed for the main TEAMM phase III trial. In this way data from both the main trial and the translational

study can be evaluated simultaneously. A detailed SAP will be developed and agreed with the Data and Safety Monitoring Committee appointed for the main TEAMM phase III trial and will be informed by the

9. Ethical arrangements

The study is using stored serum samples from TEAMM. No new or additional patient contact is required for this study; all patients have provided informed consent for the use of their samples collected within the TEAMM trial and as such current ethical approval covers work that will be done in this project.

10. Research Governance

The original TEAMM trial is sponsored by the University of Birmingham and co-sponsored by University of Warwick. The lead applicant, University of Birmingham, will be the sponsor for this mechanistic application. The TSC chair for TEAMM supports this study and has provided written approval. This study will not infringe on or require any changes to the governance arrangements for the main clinical evaluation.

11. Project timetable and milestones (updated on July 2017)

Milestones	Start – End dates	Activities	Objectives
Grant Start Feb 2017	02/17 – 06/17	Preparation of patient samples and recruitment and training of research staff (1 research Fellow and 2 MLAs)	- Bank of 977 patient serum samples x 5 time-points - Training for study-specific tests and assays
Milestone 1	04/17 – 12/17	Laboratory analyses of: - Phase 1 - Anti-bacterial antibodies - Phase 1 – IL-10 - Phase 3 – IL-6 and IL-8 - Phase 3 – PCT	- Sample analysis complete - Results entered into databased - Statistical analysis of research questions relevant to Phase 3
Milestone 2	11/17 – 07/18	Laboratory analyses of: - Phase 1 – Neutrophil function - Phase 1 – Anti-viral antibodies - Phase 2 – CD 138	- Sample analysis complete - Results entered into databased - Statistical analysis of research questions relevant to Phase 1
Milestone 3	06/18 – 11/18	Laboratory analyses of: - Phase 2 – Amount of malignant and uninvolved immunoglobulin	- Sample analysis complete - Results entered into databased - Statistical analysis of research questions relevant to Phase 2
Grant completion Feb 2019	11/18 – 02/19	Final statistical analyses Preparation of final report to EME Draft of first manuscript to be	- Report submitted to EME - Draft of first

		submitted	manuscript written and ready to be submitted to an open-access peer-reviewed journal
Post-grant		Continued study dissemination - Preparation of manuscripts with data produced from the study - Submission of abstracts to relevant conferences and committee meetings - Web page summarising the main findings of the study on the TEAMM website - Communication of results via University of Birmingham outreach pathways - Promotion and discussion of study findings to key decision makers in myeloma care via relevant national and international channels involving clinical experts	- Successful manuscript publication - Modification to stratification, monitoring and clinical management of patients - Input to national guidelines

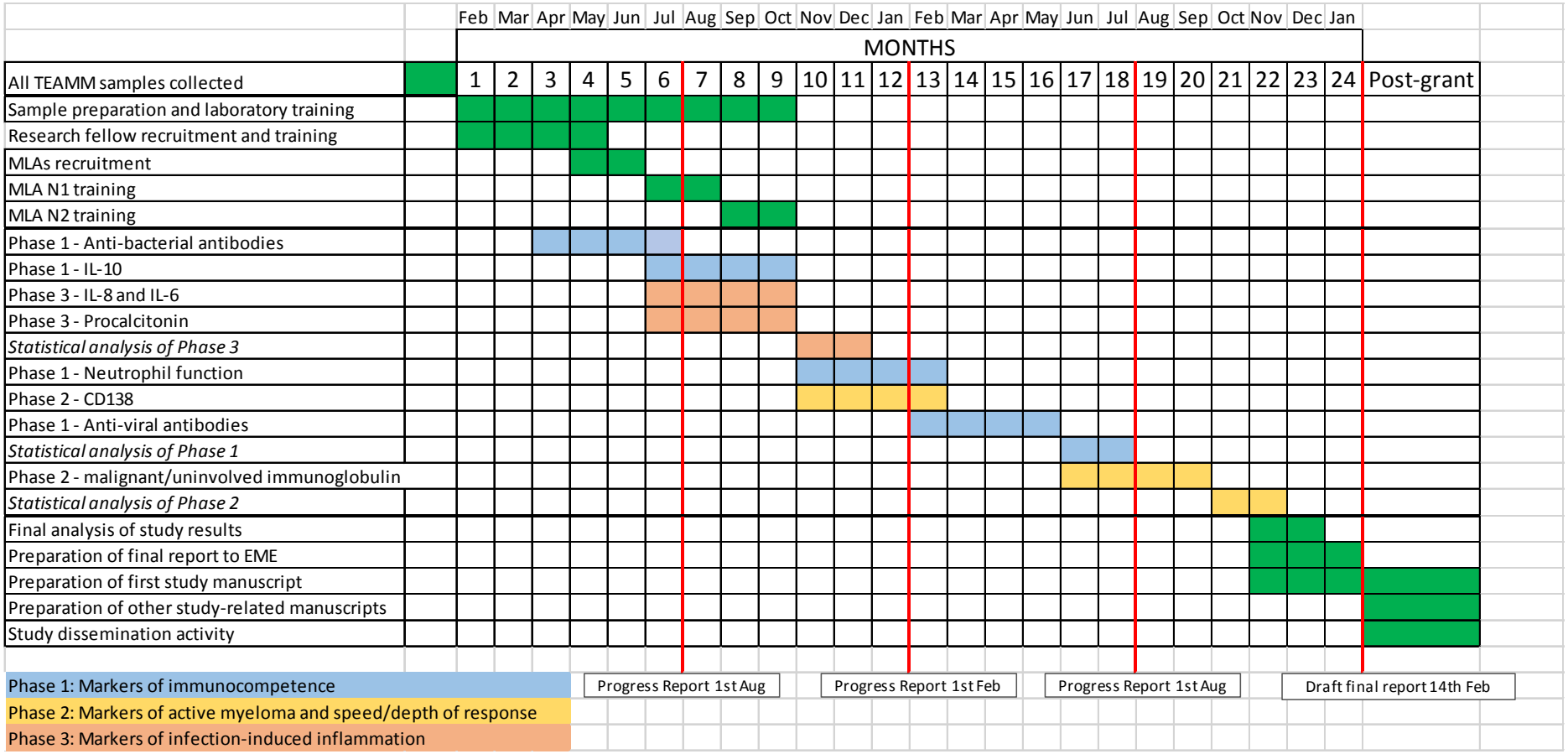
12. Deliverability

This study is dependent on the successful completion of the on-going TEAMM trial. The TEAMM trial is due to finish recruiting in November 2015. The recruitment target of 800 patients is being progressed ahead of schedule and the trial reached its half way mark 5 months ahead of schedule. Samples have been sent to the Clinical Immunology Service (CIS) throughout the trial and we already have nearly 2000 stored ready for analysis. The techniques we will use for sample analyses we already have set up in the CIS and we have experience of carrying out multiple tests and assays on large numbers of samples stored from clinical trials. Therefore, we do not anticipate any deliverability issues with this project.

13. Service Users

This study is mechanistic in nature and laboratory-based; it is utilising samples that have already been collected and stored and does not involve any further patient involvement. Eric Lowe from Myeloma UK is a co-applicant on the TEAMM trial and is a member of the TMG and will have updates on this EME translational grant. As such, we do not feel additional public involvement is applicable for this research application until results have been established. The implementation of these will require detailed discussions with patients and health care workers.

Gantt chart (version 2.0, updated on July 2017)



Appendices

15. References

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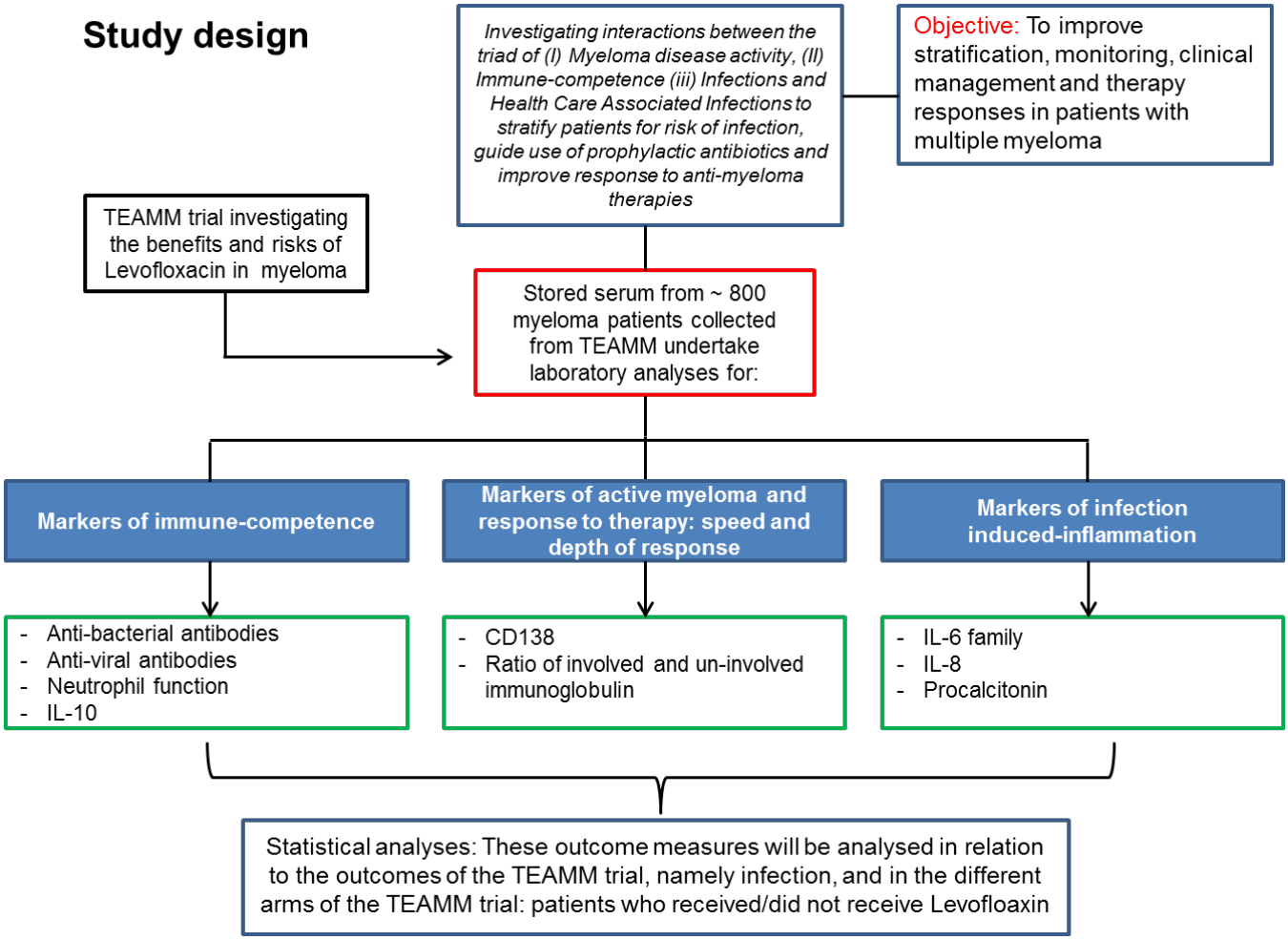
16. Flow diagram



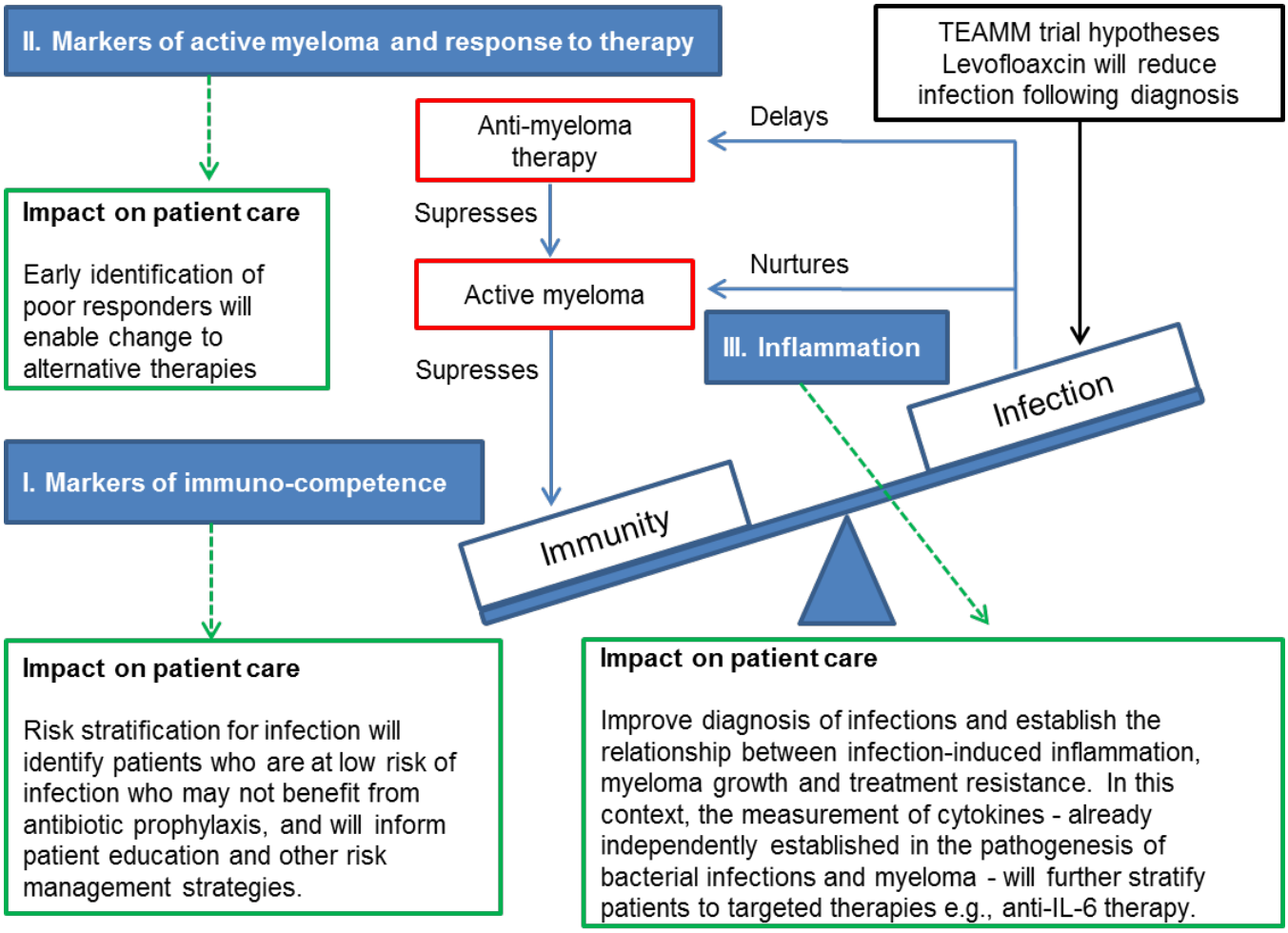


Figure 1. The cycle of immunity-infection-active disease in multiple myeloma. Active myeloma and anti-myeloma therapy supresses immunity. Infections delay administration of anti-myeloma therapy, and inflammation associated with infection is thought to nurture myeloma activity and resistance to anti-myeloma therapy. These interactions are central to TEAMM, which aims to prevent infections during anti-myeloma therapy and help break this cycle that fosters the maintenance of active myeloma. This project will measure 3 core aspects relating to this cycle (shown in the blue boxes) with identifiable routes to patient impact. The objective is to understand the mechanisms underlying these interactions in TEAMM to provide further routes and refinement of interventions through stratification and identification strategies.

Table 1. Breakdown of timepoints to be analysed, sample numbers, avilable suppliers and slected suppliers, and total cost for each of the biomarkers proposed in the study

Research Q area	Biomarkers	Number of patients to be tested	Time points to be tested	Number of samples to be tested	Kit/assay requirements	Potential suppliers	Selected supplier & justification	Total Cost for number of samples specified to be tested
Markers of immuno-competence	Anti-bacterial immunoglobulins: Pneumococcus, meningoccocus, haemophilus influenza B plus tetanus and diphtheria toxoids	800	Baseline	800	In house multi-plex Luminex assay	It is not currently possible to purchase a commercial assay that measures antibodies against 19 different bacterial antigens. To measure 19-different antibodies by ELISA would be 10-fold more expensive than Luminex multi-plex technology.	The Clinical Immunology Department, University of Birmingham, will test antibodies using an in-house Luminex method which has been validated and published (Whitelegg, A. et al., Journal of Immunological Methods, 2012) which enables simultaneous measurement of antibodies against 19 different antigens.	8,500
	Anti-viral immunoglobulins: Flu, CMV, EBV, varicella	800	Baseline	800	In-house heamaglutination assay and commercial ELISAs	**Influenza:** testing is restricted to a few specialist laboratories, but these are prohibitely expensive. Labs include major pharmaceutical companies including GlaxoSmithKline in Germany. **CMV:** established ELISAs are available from Genesis Diagnostics Biotech, Ebioscience, Abcam. **EBV:** ELISAs are available from Genway Biotech, Abcam, DiaSorin Corporation. **Varicella Zoster:** ELISAs are available from IBL International and Abcam	**Influenza**: The Clinical Immunology Department, University of Birmingham has developed a test in conjunction with the UK National Institute of Biological Standards and Controls, and has been validated and published (Long, J. et al., Brain Behavior and Immunity, 2012). **CMV**: Genesis Diagnostics Ltd provide a very sensitive assay that our group are accustomed to using (e.g., Turner, J. et al., 2010, Brain Behavior and Immunity) and this is the most widely used CMV ELISA on the market. **EBV**: Genway Biotech makes a very sensitive assay that we have used previously for other projects (e.g., Turner, J. et al., 2010, Brain Behavior and Immunity) and again, this is one of the most commonly used EBV ELISA on the market. **Varicella Zoster**: IBL International sell the most widely used and sensitive assay on the market. Again, we are accustomed to using this assay (e.g., Rector, J. et al., 2014, Brain Behavior and Immunity).	7,000
	Neutrophil function: - serum myeloperoxidase (degranulation) - cell free DNA (extracellular trap formation)	800	Baseline	800	In house assays	Assays to measure neutrophil function are, to our knowledge, not commercially or clinically available.	The Clinical Immunology Department, University of Birmingham, will utilise a well-established test developed in the University of Birmingham Medical School which measures neutrophil functions (Hazeldine, J. et al., 2014, Aging Cell).	2,000

	Interluekin-10	800	Baseline	800	Commercial multi-plex Luminex assay	Luminex multi-plex assay that includes IL-10 is available from: Bio-Rad, Millipore and RnD Systems	Bio-rad Corporation will be the provider for the luminex multi-plex assay used in this study. BioRad are the original inventors of this technology, and have the most reliable and widely used assays. Normal ranges for cytokines, including IL-10, measured by Luminex were established using Bio-Rad assays. The alternative to this approach is expensive. for example, ELISAs are available from numerous suppliers, but we have chosen to measure IL-10 by Luminex which enables us to measure a range of cytokines (Il-8, Il-6r, Il-6) at the same time, thus providing a more economical means of data collection.	3,000
Markers of active MM and response to therapy	Syndycan-1 (CD138)	800	x 5: baseline, 4-, 8-, 12- and 16-weeks	Up to 4,000	Commercial ELISA	ELISAs for the measurement of Syndecan-1 (CD138) are available from a number of commercial providers. These include: Diaclone, Abcam and RnD Systems.	We have identified Diaclone Ltd as the assay is sensitive, economical, and has been used in prior studies in myeloma, including a prior study from our research group (Lovell, R. et al., 2005; British Journal Haematology). Thus, in order to enable comparison to previous results in the field, it would be appropriate to use the same method of CD138 measurement from Diaclone.	7,000
	Class specific heavy/light chain ratios to provide measurement of malignant versus uninvolved immunoglobulin	800	x 5: baseline, 4-, 8-, 12- and 16-weeks	Up to 4,000	ELISA	Serascience Ltd	Seracsience have developed ELISA kits enabling quantitation of involved immunoglobulin alongside uninvolved immunoglobulin. Serascience will provide consumables and also laboratory support to conduct these analyses. An MTA is being developed ad will be in place prior to the start of the project.	0
Markers of infection-induced inflammation	Interluekin-8	800	Baseline: all patients 4-, 8-, 12-weeks: cohort of 250 patients selected based on range of myeloma responses	1,550	Commercial multi-plex Luminex assay	Luminex multi-plex assay that includes IL-8 is available from: Bio-Rad, Millipore and RnD Systems	As described above, Bio-rad Corporation will be the provider for the Luminex multi-plex assay used in this study. BioRad are the original inventors of this technology, and have the most reliable and widely used assays. Normal ranges for cytokines, including IL-8, measured by Luminex were established using Bio-Rad assays. The alternative to this approach is expensive. for example, ELISAs are available from numerous suppliers, but we have chosen to measure IL-8 by Luminex which enables us to measure a range of cytokines (Il-10, Il-6r, Il-6) at the same time, thus providing a more economical means of data collection.	7,520

	Interleukin-6 and Interlukin-6 receptor	800	Baseline: all patients 4-, 8-, 12-weeks: cohort of 250 patients selected based on range of myeloma responses	1,550	In house ELISAs	Luminex multi-plex assay that includes IL-6 and IL6-R is available from: Bio-Rad, Millipore and RnD Systems.	As described previously, Bio-rad Corporation will be the provider for the luminex multi-plex assay used in this study. BioRad are the original inventors of this technology, and have the most reliable and widely used assays. Normal ranges for cytokines, including IL-6 and IL-6R, measured by Luminex were established using Bio-Rad assays. The alternative to this approach is expensive. For example, ELISAs are available from numerous suppliers, but we have chosen to measure IL-6 and IL-6R by Luminex which enables us to measure a range of cytokines (i.e., Il-10, Il-8) at the same time, thus providing a more economical means of data collection.	7,900
	Procalcitonin	800	x4: baseline, 4-, 8- and 12-weeks	3,200	Commercial ELISA	Commercial ELISAs are available from Abcam, Sigma and RayBiotech.	An ELISA from Abcam will be used for this study, as it is the most widely used ELISA for measuring procalcitonin due to its high sensitivity and reproducibility. Sensitivity is important for procalcitonin assays, as even small levels of detectable procalcitonin may indicate bacterial infection.	7,000
							Total Cost	**49,920**

Table 2. Physiological ranges of the biomarkers proposed within study

Markers of Immune-competence		
Anti-bacterial antibodies (against 19 antigens)	Total polyclonal antibody levels are routinely measured in MM; however antibody levels against specific bacteria are not, therefore ranges in patients are not currently available. Along with the aim of providing specific information against bacterial infections, the proposed study will also enable ranges to be established in these patients, which we expect to be significantly reduced compared to healthy controls, described below for IgG Healthy donors (n = 193), median (5–95th): x 12 pneumococcal serotypes: 1124 ng/mL (50–11100 ng/mL) X 4 meningococcal serotypes: 785 ng/mL (1–22920 ng/mL) Haemophilus B: 730 ng/mL (80–4900 ng/mL) Tetanus: 1.77 IU/mL (0.18–7.24 IU/mL) Diphtheria: 0.66 IU/mL (0.02–0.54 IU/mL)	J Immunol Methods. 2012. 30;377(1-2):37-46.
Anti-viral antibodies Flu CMV, EBV, Varicella	Due to the nature of anti-viral assays, they do not typically provide fully quantitative results, rather indicate positive/negatives, or antibody titres In this test, antibody titre represents the highest serum dilution to inhibit hemagglutination. An antibody titre of 40 represents at a 50% reduction in risk for influenza infection, compared to titres < 40 These tests are semi-quantitative, with a cut-off being applied (which depends on the assay used and varies between manufacturers of the same assay) to indicate a negative result, or positive result (current or previous infection)	Hannoun et al. Virus Res. 2004;103(1-2):133-8.
Neutrophil function - serum myeloperoxidase - NETs production	No data available for MM patients. For elderly individuals, of a similar age to MM patients, serum myeloperoxidase levels as follows have been reported: N = 3733; Median 258 pmol/L (interquartile range 168–424) Mean ± SEM: 327 ± 30 (n = 24) and 465 ± 52 (n = 24), pmol/L No normal ranges are currently available for serum-based NET formation and are in the process of being established	Walker et al. Hypertension. 2010;55(2):363-9 Tang et al. Am J Cardiol. 2009; 103(9): 1269–1274.
IL-10	MM patients (n= 17) Median 125 ng/mL (range) 26–434 MM patients (n =54) Mean (SD) 185.5±79.0 pg/mL MM patients (n =44) 185.1 ± 85.9 MM patients (n = 82)	Ameglio et al. Int J Oncol. 1995 Jun;6(6):1189-92. Alexandrakis et al. Pathol

	Median (range): 26.3 pg/mL (5.8–1579.9 pg/mL) Mean, SD: 48.3 ± 172.3 pg/mL	Oncol Res. 2015 Pappa et al. Cytokine. 2007;37(2):171-5 CIS laboratory data
Markers of active MM and response to therapy		
CD138	Multiple myeloma patients at disease presentation: 336 ng/ml (interquartile range: 143–1635 ng/ml)	Lovell et al. Br J Haematol. 2005;130(4):542–8.
Heavy/light chain ratios	IgG patients (typically 60 % of patients) *IgG kappa patients* Median (range): 93.52 (3.94–1334) 95% range: 5.13–864 *IgG lambda patients* Median (range): 0.018 (0.001–1.05) 95% range: 0.001-0.878 IgA patients (typically 20–25% of patients) *IgA kappa* Median (range): 462 (8.8–7352) 95% range: 11.2–6020 *IgA lambda* Median (range): 0.01 (0.001–0.32) 95% range: 0.018–0.255 Other ranges will be available specific to Serascience Ltd ELISA kits.	Wikilite. Binding Site http://www.wikilite.com/wiki/index.php/Clinical_utility_of_Hevylite_assays
Markers of infection induced inflammation		
IL-8	MM patients (n = 82) IL-8 median (range): 102.1 pg/mL (8.2–52365 pg/mL) Mean, SD: 2616.5 ± 7742.9 pg/mL	CIS laboratory data
IL-6 and IL-6R	MM patients (n = 121) *IL-6*, median (range): 14.1 (0–306) pg/mL *IL-6R*, median (range): 37.24 (16.95–129) pg/mL MM patients (n = 80) IL-6 median (range): 20 ng/mL (0–800 ng/mL) IL-6R, median (range): 36 ng/mL (10–120 ng/mL) MM patients (n = 82) IL-6 median (range): 13.1 pg/mL (4.1–5409 pg/mL) Mean, SD: 141.8 ± 685.6 pg/mL	Urbańska-Ryś et al. Eur Cytokine Netw. 2000 Sep;11(3):443-51. Kyrtsonis et al. Br J Haematol. 1996;93(2):398-400. CIS laboratory data
Procalcitonin	Procalcitonin levels are generally very low or undetectable in healthy individuals. Levels only increase in response to a bacterial stimulus	Schuetz et al. Expert Rev

	Levels of ≥ 0.25–< 0.5 ng/mL indicate a possible bacterial infection; levels of ≥ 0.5 ng/mL suggest the presence of a bacterial infection; levels of ≥ 2 ng/mL indicate a systemic infection is likely; levels of ≥ 10 ng/mL indicate severe sepsis or sepsis shock A small sample of MM patients has been previously tested in the CIS to assess feasibility of these assays. The majority of patients had minimal levels of procalcitonin, with some showing signs of infection and two patients presenting with levels indicative of a systemic infection Median (range): 0 ng/mL (0–8.6 ng/mL) Mean 0.039 ng/mL ± 0.28 ng/mL	Anti Infect Ther. 2010;8(5):575-87 CIS laboratory data