Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network

Trans-MAPP Study of Urologic Chronic Pelvic Pain: Symptom Patterns Study (SPS)

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Table of Contents

1. INTRODUCTION 5
1.1. Background 5
1.2. Mission and Structure of the MAPP Research Network 5
2. TRANS-MAPP SYMPTOM PATTERNS STUDY 8
2.1. Objectives 8
2.2. Aims and Hypotheses for Trans-MAPP Symptom Patterns Study 9
2.3. Bi-directional Link with MAPP Phase II Translational Animal Models Consortium (M2-TMC) 12
3. STUDY DESIGN 13
3.1. Overview of the Trans-MAPP Symptom Patterns Study 13
3.2. Screening Visit and Run-in Period 14
3.3. Baseline Deep-Phenotyping Visit 14
3.4. Biomarker and Microbiome Protocols 15
3.5. Neuroimaging 17
3.5.1. Identifying Brain Regions and Networks Related to UCPPS Symptom Change 18
3.5.2. Number of Scans Per Participant 18
3.5.3. Number of Participants 19
3.6. Quantitative Sensory Testing 19
3.6.1. QST Core Battery 20
3.7. ATLAS Module 22
3.7.1. Eligibility of Time Intervals for ATLAS Modules 23
3.7.2. Pre-specified ATLAS Therapies 23
3.7.3. Notification Procedures for ATLAS Modules 24
3.7.4. Sample Size Considerations for ATLAS Comparisons 25
3.8. Trans-MAPP SMARTPHONE APPLICATION To Assess Pelvic Pain: The (M)APP Study 26
4. STUDY POPULATION 27
4.1. Study Participants and Subgroup Targets 27
4.2. Recruitment Timetable and Targets by Discovery Sites 28
4.3. Eligibility Criteria 28
4.3.1. Inclusion Criteria 28
4.3.2. Exclusion Criteria 29
4.3.3. Deferral Criteria 30
5. MEASURES AND FOLLOW-UP 31
5.1. Risk Factors and Outcome Measures 31
5.1.1. General Measures of Sociodemographics, Health, and Quality of Life 31
5.1.2. UCPPS Symptoms Measures 32
5.1.3. Non-urological Symptom Measures 32
5.1.4. Trait-like Personal Factors 33
5.1.5. Assessment of UCPPS Symptom Flares 33
5.1.6. Biological Specimens 33
5.2. Contact Schedule and Participant Procedures 34
5.2.1. Screening/Eligibility Visit (Week 0) 34
5.2.2. Run In Visits (Week 1-3) 36
5.2.3. Baseline Visit (Week 4) 37
5.2.4. Monthly Internet Medication Questionnaires 39
5.2.5. Quarterly Assessments 39

5.2.6. Deep Phenotyping In-clinic Visits at 6, 18 and 36 Months 40
5.2.7. Optional Clinic Visits at 12, 24 and 30 Months....41
5.2.8. Internet Site Design and Operation....41
6. STATISTICAL CONSIDERATIONS AND ANALYTIC PLANS....42
6.1. Sample Size/Power Considerations for the Symptom Patterns Study....42
6.2. General Statistical Methods....43
6.2.1. Baseline Statistical Analyses:....43
6.3. Additional Analytical Considerations Specific to the Aims 43
6.3.1. Primary Outcomes for UCPPS Symptoms....43
6.3.2. Characterizing Longitudinal Variability:....45
6.3.3. Variability as a Predictor of Symptom Outcomes: 46
6.3.4. Longitudinal Data Analysis of UCPPS Symptom Patterns....46
7. HUMAN SUBJECT CONSIDERATIONS 51
7.1. Symptom Patterns Study Participant Considerations....51
7.1.1. Symptom Patterns Study Participant Recruitment 51
7.1.2. Screening and Enrollment 51
7.1.3. Participant Follow-up 51
7.1.4. Participant Retention 52
7.1.5. Participant Withdrawal 52
7.1.6. Participant Reimbursement 52
7.2. Ethical Issues....52
7.2.1. Potential Risks to Participants....52
7.2.2. Informed Consent....54
7.2.3. Consent for Genetic Testing and DNA Storage....55
7.2.4. Storage and Archival of Study Data....55
7.2.5. HIPAA Authorization....55
8. PARTICIPANT CONFIDENTIALITY....56
9. STUDY ORGANIZATION AND OVERSIGHT 57
9.1. Discovery Sites....57
9.2. Data Coordinating Core (DCC) 59
9.3. Tissue Analysis and Technology Core (TATC)....60
9.4. NIDDK Program Staff....61
9.5. MAPP Steering Committees and Subcommittees....61
9.6. External Experts Panel 61
10. STUDY MANAGEMENT 62
10.1. Discovery Site Responsibilities....62
10.1.1. Discovery Site Director and Investigators 62
10.1.2. Institutional Review Board 62
10.1.3. Record Retention....62
10.2. Data Coordinating Core responsibilities 62
10.2.1. Quality Assurance....62
10.2.2. Website Enhancements....63
10.2.3. Data Security 63
10.3. Tissue Analysis and Technology Core Responsibilities....64
10.3.1. Personnel Training....64
10.3.2. Specimen Kit Distribution, Banking, Annotation/Blinding....64
10.3.3. Biorepository Collection, Management and Distribution 64

10.3.4. Specialized Assay Platforms 65
11. References 66

Appendix 1: Biomarker Protocol ..... [A1] 1 – [A1] 50
Appendix 2: Microbiome Protocol ..... [A2] 1 – [A2] 27
Appendix 3: Neuroimaging Protocol ..... [A3] 1 – [A3] 22
Appendix 4: Quantitative Sensory Testing Protocol ..... [A4] 1 – [A4] 25
Appendix 5: ATLAS Module ..... [A5] 1 – [A5] 12
Appendix 6: MAPP Phase II SPS Visit Schedule ..... [A6] 1 – [A6] 2
Appendix 7: (M)APP Protocol ..... [A7] 1 – [A7] 30

Table 1: ATLAS Total Sample Size for Specified Effects (1-sided a=0.05, power=80%). 25
Table 2: Proposed Composition of MAPP Phase II Symptom Patterns Study Compared to MAPP Phase I UCPPS Participants by Target Recruitment Factors 27
Table 3: Schedule of Biologic Specimen Collection 33
Table 4: Sample Size Requirements for Detecting Specified Risk Ratios Under Selected Study Design Characteristics: Type I Error Rate of 5%, Power of 90% 42
Table 5: Variability Across Weeks 4-46 as Predictor of Symptom Severity at Week 48 46
Table 6: Concordance of Functional Cluster Membership with K=3 for Pain Severity and Urinary Severity 50

Figure 1. Timeline for MAPP Phase II Symptom Patterns Study 13
Figure 2. Timeline for MAPP Phase II Symptom Patterns Study: Screening and Run-in Period 14
Figure 3. Timeline for MAPP Phase II Symptom Patterns Study: Baseline and Follow-up Period 15
Figure 4. Convergence of MAPP-I neuroimaging results 17
Figure 5. Protocol Overview of Bladder Filling Paradigm Based on Resting State Changes 18
Figure 6. Number of scans per patient 18
Figure 7. Analysis of sample size. 19
Figure 8. Testing Sites 20
Figure 9. MAST Handset 21
Figure 10. Timeline for MAPP Phase II Symptom Patterns Study: ATLAS Module 22
Figure 11. Examples of Common Targeted Therapies for ATLAS Monitoring 24
Figure 12. CHOIR Body Map Modified for Regional Specificity of "Pelvic Pain Only" 27
Figure 13. Data Domains: Demographic, Urologic, Non-urologic and Biopsychosocial 31
Figure 14. Visit Schedule for Screening (Week 0), Run-in (Weeks 1–3), Baseline (Week 4) and Follow-up 34
Figure 15. Sequence of MAPP II SPS Screening Visit Data, Physical Exam and Biospecimen Collection 35
Figure 16. Sequence of MAPP Phase II Study Baseline Data and Biospecimen Collection and Procedures 37
Figure 17. Representative Participant PID #100677: Female, BPS:Yes, Baseline SYM-Q1:Mild (0-3) 43
Figure 18. Distribution of Longitudinal Pain Severity Score Sub-domains by Sex and BPS Subtypes 44
Figure 19. Distribution of Longitudinal Urinary Severity Score Sub-domains by Sex and BPS Subtypes 44
Figure 20. Longitudinal Distribution of Pain Severity and Urinary Severity Scores by Sex and BPS Subtypes 45
Figure 21. Longitudinal Six-week Pain Severity Standard Deviation by Sex in UCPPS Patients. 45
Figure 22 PID # 100690, Female, BPS:Yes, PID # 101221, Female, BPS:Yes, SYM-Q1: Moderate (4-6) – "Improving" SYM-Q1: Mild (0-3) – "Worsening" 48
Figure 23. Functional Clustering into K=3 Classes of Pain Severity Patterns (Left panel; Cluster 2=Improvers) and Functional Clustering into K=3 Classes of Urinary Severity Patterns (Right panel; Cluster 1=Improvers) 50

1. INTRODUCTION

1.1. Background

Urologic chronic pelvic pain syndrome (UCPPS) encompasses two highly prevalent non-malignant urologic disorders, interstitial cystitis/bladder pain syndrome (IC/BPS) and chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), primarily characterized by chronic and often debilitating pain in the pelvic region and/or genitalia, and typically a spectrum of defects in bladder and lower urinary tract function.[1, 2] As with many chronic pain disorders, UCPPS are poorly understood and characterized, and treatment is mostly empirical and unsatisfactory. Estimates of prevalence of the syndromes vary widely. In 1990, IC/BPS was thought to affect as many as 500,000 U.S. citizens, with 25% of the patients under age 25.[3] In the U.S., the prevalence of IC/BPS symptoms has been estimated to be 2.7% in women[4]; whereas the prevalence for analogous symptoms is estimated to be 1.3% in men.[5] Prevalence estimates for CP/CPPS in men vary between 1.8 – 6.4%, depending upon case definitions and screening methods.[6, 7]

The impact and burden of IC/BPS and CP/CPPS are substantial. Patients suffer considerable morbidity resulting in a significant decrease in quality of life for both the patient and his/her partner due to the physical and psychological impact of the condition. In fact, the quality of life of IC patients has been characterized as being worse than that of patients undergoing dialysis.[8]

1.2. Mission and Structure of the MAPP Research Network

To better understand the etiology and treated natural history of UCPPS, and to identify clinical factors and research measurements to define clinically relevant sub-groups of these patients for future clinical trials, and to inform symptom management, the NIDDK established the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network in 2008[9] (http://www.mappnetwork.org/). The primary clinical research effort carried out by this network in Phase I was a prospective cohort study, the Trans-MAPP Epidemiology/Phenotyping (EP) Study.[10] From 12/14/2009 through 12/14/2012 1,039 men and women were enrolled, including persons with UCPPS (n=424); persons with other co-morbid illnesses, including fibromyalgia, irritable bowel syndrome, and chronic fatigue syndrome (n=200 for all conditions); and healthy controls (n=415). Study participants were extensively characterized (i.e., phenotyped) at baseline, and UCPPS participants were followed for 12 months. During follow-up, using a web-based symptom data capture system, a small battery of self-report questionnaires were repeated on a bi-weekly schedule for 48 weeks. In addition, neuroimaging was performed in a subset of participants (n=279) at baseline, and biological samples were collected on all participants at baseline and follow-up for studies to identify plasma and urine biomarkers and potential infectious agents. This study was complemented by a number of Discovery Site-Specific clinical research studies (i.e., studies generally conducted at a single site) and investigations of animal models developed by multiple sites to mimic symptoms of human UCPPS.

Initial analyses of these data have identified a number of provocative findings. There are strong indications those certain subgroups of participants (albeit with small sample sizes) with urinary and non-urinary symptoms tend to improve over time; whereas other subgroups tend to worsen over time. These patterns of improving or worsening are differentially expressed according to sex, subtype of bladder pain syndrome (BPS), and pain location (localized to the pelvic region vs pain reported in the

pelvic region and beyond). Further details describing a "functional clustering" approach to the discovery and validation of these subgroups, characterized on the basis of longitudinal change into 1) improving, 2) stable and 3) worsening, are provided in Section 6.3.4.4 in Figure 23.

The second phase of the MAPP Network is designed to conduct a prospective, observational study of men and women with UCPPS, referred to as the **S**ymptom **P**atterns **S**tudy (SPS), enriched with pre-defined subgroups, with longer follow-up, in order to further investigate clinical and biologic factors associated with worsening and/or improvement of reported urinary and non-urinary symptoms.

The MAPP Phase II SPS also presents the opportunity to apply many of the most promising research methods in the pain field (e.g. functional, chemical and structural neuroimaging, quantitative sensory testing) during the course of the study (at baseline and then longitudinally) to better characterize men and women with UCPPS. Most of these measures were only collected at a single point in time in the Trans-MAPP EP Study of Phase I, and within a sample of UCPPS patients not enriched with predefined subgroups. Further phenotyping in the second phase study will allow us to better determine which of these measures, or any other measures identified during Phase I (e.g., urinary or serum biomarkers), might identify individuals most likely to have spontaneous improvement of their symptoms, versus transition to a more "peripheral" (pelvic pain only) or "centralized" form (pelvic pain and beyond) of urinary and non-urinary pain.

Phase I of the MAPP focused on frequent data collection over a 12-month time period in order to examine and categorize symptom patterns and their predictors. While information about UCPPS treatments was collected, those data were not specific enough to identify which treatments were effective in individual UCPPS participants. This information will be critical to MAPP efforts because one of the main goals of the MAPP is to identify subtypes of UCPPS patients that have differing underlying mechanisms for their disease, and thus would be expected to respond differently to treatments targeting those different mechanisms. To address this gap, in Phase II we will follow participants for a longer period of time (3 years), and will longitudinally track UCPPS therapies and non-UCPPS medications.

Within the SPS, MAPP network investigators propose to conduct an **A**nalysis of **T**herapies during the **L**ongitudinal **A**ssessment of **S**ymptoms (ATLAS) protocol. The rationale for the ATLAS protocol is that through exposure of UCPPS patients to pre-defined therapeutic interventions with accompanying phenotyping, a relationship between response (or lack of response) to a given therapy and specific phenotypic profiles may be established. If successful, this would identify sub-sets of UCPPS patients with characteristic phenotypes (i.e., disease marker profiles, degree and patterns of symptoms, influence of symptom flares, presence of co-morbid syndromes, neurological structure/function, etc.) that preferentially respond to specific treatments. This could eventually lead to "personalized analgesia", by allowing the design of future clinical trials that would specifically test UCPPS therapies restricted to patients with UCPPS "endophenotype" that are expected to respond to a treatment.

This concept of treating chronic pain patients based on the underlying mechanism(s) rather than disease has been suggested for some time[11], but is just now beginning to be realized in other patient populations. For example, studies suggest that several genetic polymorphisms (e.g. in COMT, opioid receptors) may predict subsequent responsiveness of beta-blockers in temporomandibular joint

disorder (TMJD), and opioid analgesics in cancer pain, respectively.[12-15] Quantitative sensory testing measures (e.g., reduced conditioned pain modulation or DNIC) have recently been shown to identify neuropathic pain patients more likely to respond to the drug duloxetine.[16] Functional and chemical neuroimaging techniques also show great promise in identifying endophenotypes of patients that are likely to respond to certain therapies, including surgical procedures aimed at eliminating pain.[17-19] Since all of these techniques are planned to be used in MAPP Phase II, a major goal of the Symptoms Pattern Study is to obtain the necessary information to enhance the ability of investigators conducting future clinical trials of UCPPS patients to tailor patient sub-groups (using clinical and research measures) to therapies that they will likely respond to with the hope of improving the efficiency and likelihood of showing a beneficial effect, with the ultimate goal of utilizing more effective therapies within the clinical setting.

The first step to identify endophenotypes that are associated with differential treatment outcomes is to test whether individuals with UCPPS with symptoms confined to the bladder (“peripheral” endophenotype) preferentially respond to therapies anticipated to work more peripherally, and conversely whether individuals with more systemic features of disease (i.e. “centralized” endophenotype) preferentially respond to those that work more centrally.

2. TRANS-MAPP SYMPTOM PATTERNS STUDY

2.1. Objectives

Building on the findings from the Trans-MAPP EP Study conducted during Phase I of the MAPP Research Network, a second larger and longer prospective observational study of UCPPS patients, the Trans-MAPP SPS was designed. The primary objectives of this study are to:

1. Identify clinical features, participant-reported characteristics, and biological measurements (e.g., biomarkers, CNS structure/function, microbial populations and patterns) assessed at baseline, and during follow-up, that predict, or are strongly associated with, UCPPS symptom patterns (either improvement or worsening) over time.
2. Characterize potential biological changes (e.g., regional versus systemic pain profiles, inflammatory state, function of the central nervous system) associated with changes in symptoms to reveal underlying physiological contributors to improvement or worsening.
3. Further characterize and validate definitions of UCPPS symptom patterns as a clinically significant and patient-valued measure.
4. Establish and validate definitions of phenotypic subgroups of UCPPS patients based on symptom patterns and biological (pathophysiological) features.
5. Develop statistical models for clinical use in predicting differential longitudinal patterns of UCPPS symptoms.

The Trans-MAPP SPS is designed to correlate clinically important longitudinal symptom patterns with phenotypic characteristics. This study expands previous research conducted by the MAPP Research Network's investigators during the first five years to address clinically important questions of etiology and natural history of UCPPS. Novel features of this study include:

1. A screening visit (week 0) to confirm eligibility, followed by a run-in period of three (3) weekly on-line symptom assessments, in advance of the deep-phenotyping baseline in-clinic visit at week 4, in order to adjust for regression-to-the-mean and confirm subtype classifications for enrichment sampling of target subgroups.
2. Study of the cohort enriched for individuals likely to develop distinct longitudinal symptom patterns (either improvement or worsening) over time.
3. Stratification of study participants at baseline based on candidate predictors (identified in Phase I) of symptom patterns (either improvement or worsening) of symptom progression.
4. Sufficient sample size, resulting from enriched recruitment of targeted subgroups, to provide adequate statistical power to detect baseline and longitudinal phenotypic factors with utility for discriminating patient subgroups with differing symptom patterns.
5. Development and evaluation of a multivariable statistical model based on phenotypic characteristics associated with symptom progression (either improvement or worsening) patterns. The goal is to develop a tool for clinical use that may predict disease course.
6. Application of additional, novel biological measurements (e.g., central nervous system structure/function at baseline and during the follow-up period) to correlate physiological findings with symptom progression profiles.
7. Use of additional novel measures of acute symptom change or flares (e.g., studies of induced flare, new questionnaires to better characterize flares) to complement above assessment (#5).

8. Extended duration of follow-up to permit better characterization of the long-term natural history of these syndromes, including durability of symptom patterns.
9. Expanded assessment of transition from regional pain (i.e., restricted to pelvis) to systemic pain phenotype in UCPPS and relevance to symptom change and presence of co-morbid syndromes.

2.2. Aims and Hypotheses for Trans-MAPP Symptom Patterns Study

AIM 1: To characterize distinguishable longitudinal urological and non-urological symptom patterns in a cohort of UCPPS patients enriched for pain restricted to the pelvis (i.e., regional pain) at baseline.

Hypothesis 1a: *For a cohort of UCPPS patients with pain restricted to the pelvis (i.e., regional pain) at baseline, 25% will progress to symptom improvement, 25% will progress to symptom worsening, and 50% will show minimal variability (stable) over time, as measured through assessments of pain (SYMQ-1, GUPI Pain Subscale), lower urinary tract function (GUPI Urinary Subscale, ICSI-Total) and non-urological pain (SYMQ-6).*

Hypothesis 1b: *Male and female UCPPS patients, when stratified by bladder pain symptoms, will show similar longitudinal symptom profiles of UCPPS symptom improvement and worsening.*

Hypothesis 1c: *Worsening or improvement of urinary and non-urinary symptoms will be associated with improvement or worsening of other clinically important outcome measures including quality of life, SF-12, pain intensity, and severity of sleep disturbance.*

AIM 2: To identify associations between baseline clinical factors and urinary and non-urinary symptom progression in a cohort of UCPPS patients enriched for pain restricted to the pelvis (i.e., regional pain) at study entry (baseline).

Hypothesis 2a: *Individuals with a past history of chronic pain in one or more non-pelvic body regions (but at present having pain only confined to the pelvis) will be more likely to have a chronic than self-limited course of UCPPS illness.*

Hypothesis 2b: *The past or present history of other somatic symptoms (e.g., fatigue, memory problems, sleep disturbances), other somatic syndromes (e.g., chronic fatigue syndrome, irritable bowel syndrome, fibromyalgia), and psychological comorbidities (e.g., mood and cognitions) at baseline will be associated with a higher likelihood of UCPPS symptom worsening over time.*

Hypothesis 2c: *Individuals with a history of greater numbers of flares reported at baseline will show an increased likelihood of UCPPS symptom worsening over time.*

Hypothesis 2d: *Factors associated with symptom progression will differ between patients with UCPPS alone and patients with UCPPS plus co-morbid syndromes (e.g., CFS, IBS, and FM).*

Hypothesis 2e: *UCPPS patients that improve or worsen will have differing physiological changes over time, which are preferentially associated with UCPPS symptom improvement, worsening, or other observed progression profiles.*

AIM 3: To characterize transitions between regional pain (pelvic pain only) and systemic pain (pelvic pain and beyond) for UCPPS over time, and the corresponding association with symptom improvement or worsening and selected diagnosed co-morbid Non-Urological

Associated Syndromes (NUAS) disorders (e.g., fibromyalgia, irritable bowel syndrome, chronic fatigue syndrome)

Hypothesis 3a: *The treated natural history of UCPPS involves a transition from pelvic pain (i.e., regional) to systemic pain over time and this corresponds with a transition from pathophysiology at peripheral locations to involvement of changes in the central nervous system.*

Hypothesis 3b: *Symptom worsening will be associated with progression from pelvic (i.e., regional) to more systemic pain over time.*

Hypothesis 3c: *Symptom worsening will be associated with manifestation of co-morbid syndromes over time.*

AIM 4: To identify associations between baseline objective quantitative pain sensitivity measures and symptom progression in a cohort of UCPPS patients enriched for pain restricted to the pelvis at baseline.

Hypothesis 4a: *Quantitative measures of generalized and segmental pain sensitivity, altered endogenous pain modulation, and/or global sensory sensitivity are associated with symptom variations within UCPPS patient subgroups and can be used to predict longitudinal outcomes and treatment response.*

AIM 5: To create accurate, non-invasive diagnostic and prognostic tests, based on specific, definable and validated levels of biomarker(s) that will objectively identify and stratify UCPPS patients and guide the diagnosis, treatment and long-term management of patients with UCPPS.

Hypothesis 5a: *Within this context, these validated biomarkers will, in an unbiased manner:*

- Have prognostic value for treatment response and onset of flares
- Aid in clinical management and identify new therapeutic avenues for patients with UCPPS

Hypothesis 5b: *Overarching Microbiome Project Hypothesis:*

- Microbiome characteristics of the lower urinary tract at baseline and dynamic longitudinal changes in the microbial profile will be associated with phenotype symptom patterns and phenotypic progression (worsening or improvement of symptoms, as well as changing symptom phenotype patterns).

AIM 6: To identify brain signatures that remain stable, while other brain signatures are correlated with symptom change over time.

Hypothesis 6a: *Brain functional differences (or, functional signatures) that distinguished UCPPS patients from healthy controls were identified in MAPP Phase I [20]. In Phase II, it is hypothesized that UCPPS symptom severity will correlate with patterns of resting state brain activity in a UCPPS subtype--specific manner.*

Hypothesis 6b: *Neocortical gray matter longitudinal changes are predicted to correlate with pain and urinary symptom changes over time, including the progressive worsening of UCPPS symptoms and the improvement of these symptoms following successful treatment. Similarly, UCPPS subtypes are expected to exhibit different configurations of regional gray matter change.*

Hypothesis 6c: *Distinct features of axonal (white matter) integrity will reflect neuroplasticity related to pain maintenance versus predisposition to develop UCPPS pain. White matter integrity has been shown to prospectively predict individuals who will go on to develop disease, suggesting that white matter properties may indicate predispositions to develop chronic pain. Alternately, in the*

MAPP I phase both increases and decreases in white matter correlated with UCPPS symptoms severity in women, and we hypothesized that white matter increases may play a direct role in UCPPS symptom maintenance.

Hypothesis 6d: *Central peripheral interactions mediating UCPPS symptoms will be revealed through within-participant correlation of functional and gray matter brain markers of UCPPS and peripheral blood and target urine biomarkers sampled over time, and these interactions will be differentially expressed across distinct UCPPS subtypes.*

AIM 7: To determine the relative effectiveness (in the peripheral vs. central phenotypes) of UCPPS therapies administered to MAPP participants

Hypothesis 7a: *Drugs with the classes of tricyclics antidepressants and opioids will be preferentially effective, as measured by a clinically meaningful improvement after 12 weeks of therapy, in patients with central compared to peripheral endophenotype of UCPPS.*

Hypothesis 7b: *Pelvic Floor Physical Therapy, pentosanpolysulfate, and alpha blockers will be preferentially effective, as measured by a clinically meaningful improvement after 12 weeks of therapy, in patients with a peripheral compared to central UCPPS endophenotype.*

Hypothesis 7c: *Exploratory analyses will identify other baseline clinical endophenotypes that preferentially respond (or do not respond) to the targeted therapies.*

AIM 8: To characterize and stratify UCPPS patients by their flare signature patterns.

Hypothesis 8a: *Flares will be more common, of longer duration, and of greater intensity for individuals characterized as pelvic pain and beyond than for individuals with pelvic pain only.*

Hypothesis 8b: *Flares of the pelvic pain only group will be correlated with changes in the microbiome characteristic of the lower urinary tract; whereas, the pelvic pain and beyond group will demonstrate less of a relationship.*

Hypothesis 8c: *For the pelvic pain and beyond group, flares will be associated with greater affective distress; whereas, the pelvic only group will not demonstrate this association.*

AIM 9: To develop and test instruments and statistical models that use selected measurements noted above to parsimoniously predict in clinical settings (i.e. rapid screening in routine clinical practice, more intensive screening in sub-specialty clinics) a likely future symptom pattern in individual patients.

Hypothesis 9: *Multivariable models can be developed that predict a reasonable degree of variance in whether individuals with similar clinical presentation of UCPPS symptoms will experience either worsening or improvement of their symptoms.*

2.3. Bi-directional Link with MAPP Phase II Translational Animal Models Consortium (M2-TMC)

The MAPP Research Network marshals impressive clinical and epidemiologic resources to increase our understanding of UCPPS patient phenotypes. Although this new understanding may contribute to improved patient care, novel therapies still require mechanistic insights that are difficult to glean from clinical studies alone. Indeed, the true power of MAPP derives from state-of-the-art clinical studies and mechanistic interrogation of clinically relevant UCPPS animal models within the network, to foster bi-directional collaboration between clinicians and basic scientists.

The MAPP Phase II Translational Animal Models Consortium (M2-TMC) is a multi-site, highly collaborative effort that builds on the clinical observations of the first phase of the MAPP Study, to examine relevant mechanistic pathways in animal models, and to provide insights as to whether putative biomarkers identified in the human studies are mechanistically involved in nociception and urinary dysfunction in animals. By adopting uniform preclinical methods to assess pelvic nociception and urinary dysfunction, use of a selected panel of animal models to study UCPPS, close integration with the MAPP Biomarker Working Group, the M2-TMC is poised to investigate clinically relevant mechanistic pathways that emerge from Phase I studies and the Symptom Patterns Study with the ultimate goal of identifying novel treatments for UCPPS.

For MAPP Phase II, the Animal Models consortium propose an unprecedented level of validation and translational alignment of the animal models with the emerging human data from the MAPP study. The objectives of the M2-TMC are to work as a team: (1) to advance the mechanistic understanding of UCPPS, and (2) to enhance bi-directional translation between animal studies and clinical findings to allow *in vivo* mechanistic studies of greater relevance to the observed clinical condition. This bi-directional interaction between the different MAPP Working Groups will add significant overall value to the cooperative network.

3. Study Design

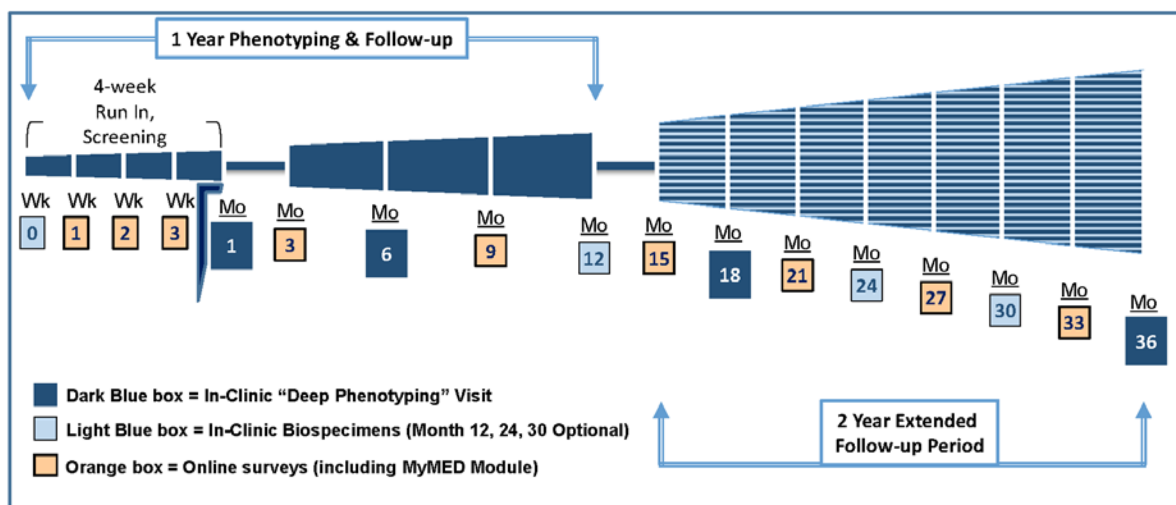
3.1. Overview of the Trans-MAPP Symptom Patterns Study

As summarized in Figure 1, the overall design of the Trans-MAPP SPS includes screening and enrollment of eligible UCPPS patients during an initial clinic visit (week 0), followed by a run-in period of 3-weekly internet-based sessions for capture of primary symptom data. These same data are then assessed at a "deep phenotyping" baseline visit at week 4, and then quarterly during an extended follow-up period, until a final in-clinic visit at 36 months. The run-in period is designed to account for regression-to-the mean effects, as well as assessment of variability for selected urologic symptoms and location of pain sites reported on the body map, in order to establish baseline subgroup classifications of each UCPPS participant at the baseline in-clinic visit (month 1), involving a "deep-phenotyping" battery of symptom measures, biospecimen collection, quantitative sensory testing (QST) procedures and neuroimaging scans (for those meeting neuroimaging eligibility criteria).

Following the "deep phenotyping" symptom assessment at week 4, each participant begins the longitudinal follow-up with internet-based quarterly symptom assessments for a total of 36 months. Each participant also returns for in-clinic reduced ("lite") phenotyping data and biospecimen collection every 6 months, as illustrated in Figure 1.

The target sample size for enrollment in the SPS is n=640* (320 males, 320 females), of which 50% of each sex will endorse pain in the Pelvic region only (PP Only Furthermore, n=240 (37.5%) of the target sample size (60 in each sex by PP Only subgroup) will endorse BPS: No, defined by the absence of pain that worsens with bladder filling (RICE Q4), and urinary urgency due to pain/pressure/discomfort (RICE Q2/Q3), as illustrated further in Section 4.1 (Table 2). The eligibility criteria for the SPS have been simplified slightly, relative to the enrollment criteria into the EP study in MAPP Phase I, requiring a "feeling of pain, pressure, or discomfort in the lower abdomen or pelvic area having been present for the majority of the time during the most recent 3 months" for all enrollees.   However, these target subgroup proportions of 50% (PP Only) and 37.5% (BPS: No) represent enrichment recruitment, relative to the realized proportions in MAPP Phase I, as illustrated further in Section 4.1 (Table 2).

Figure **1**. Timeline for MAPP Phase II Symptom Patterns Study



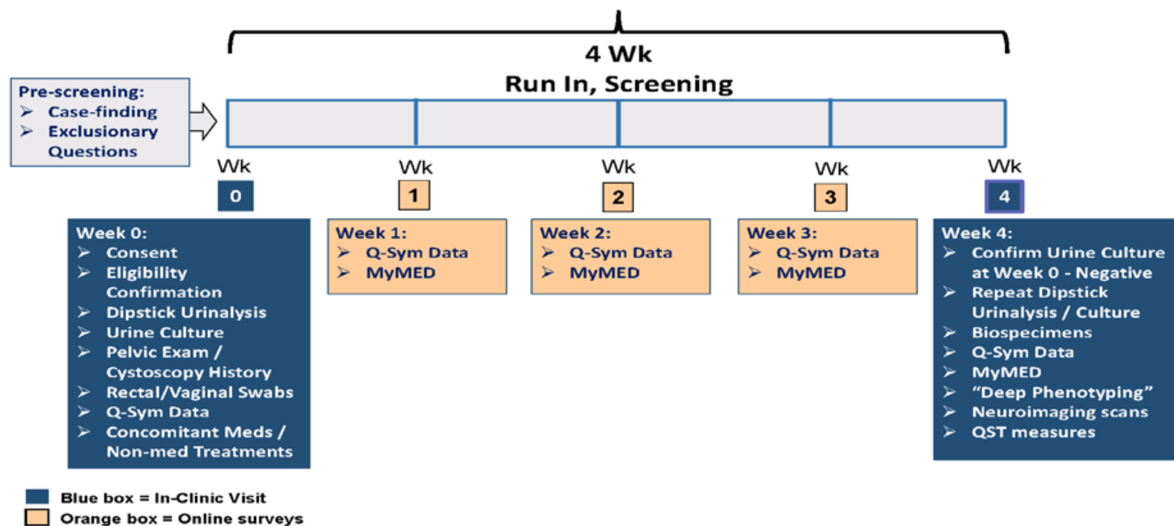
While the target recruitment remains 640 participants, the study group will cease to recruit if 600 participants are recruited by December 31, 2018.

As described in further detail in the following sections, this Symptom Patterns Study also includes an embedded module of “deep-phenotyping” aimed at “**A**nalysis of **T**herapies during the **L**ongitudinal **A**ssessment of **S**ymptoms (ATLAS)”. The rationale for this ATLAS module is that by conducting targeted “deep-phenotyping” at the beginning of exposure to therapeutic interventions, and repeated again at 12 weeks after initiating new therapies, a relationship between response (or lack of response) to a given therapy and specific phenotypic profiles may be established. This ATLAS module is aimed at subgroup identification of UCPPS patients with characteristic phenotypes, for example, disease marker profiles, degree and patterns of symptoms, influence of symptom flares, presence of co-morbid syndromes, and/or neurological structure/function, that preferentially respond to specific treatments.

3.2. Screening Visit and Run-in Period

At the initial screening visit, potential study participants will be consented, eligibility criteria will be assessed, a diagnostic urine specimen will be collected for dipstick analysis and culture assessment, a pelvic exam will be performed (including a rectal/vaginal swab), and data will be collected about prior cystoscopy/hydrodistention findings and concomitant medications. In addition, the internet-based quarterly symptom (Q-Sym) data will be administered. Participants meeting the eligibility criteria will be scheduled for their 4-week in-clinic “deep-phenotyping” visit, and will be instructed to begin their weekly internet-based symptom data capture to assess variability over the following 4 weeks before the “deep-phenotyping” session (Figure 2).

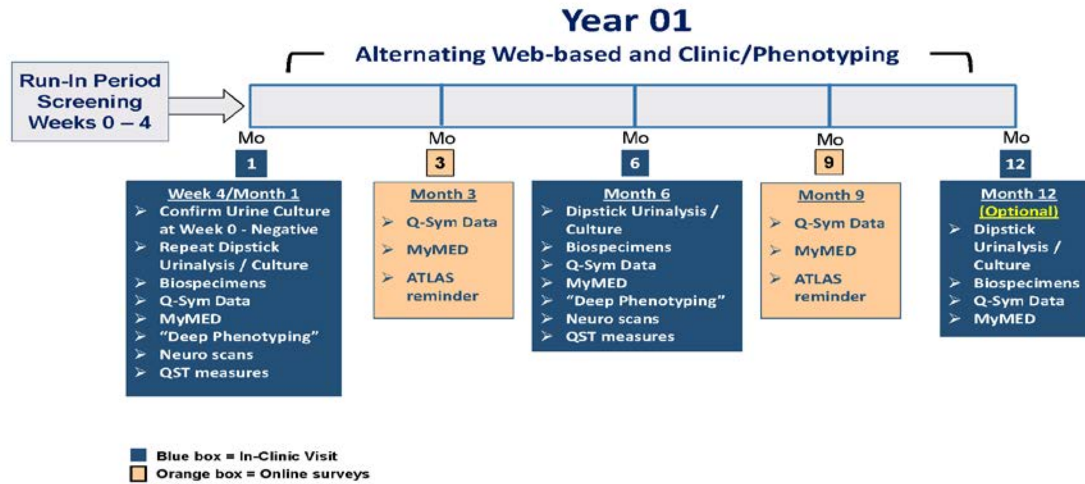
Figure **2**. Timeline for MAPP Phase II Symptom Patterns Study: Screening and Run-in Period



3.3. Baseline Deep-Phenotyping Visit

At week 4, after confirming a negative urine culture from the week 0 specimen, and another negative dipstick urinalysis, eligible UCPPS patients will undergo a “deep phenotyping” symptom assessment during an extensive in-clinic visit, including biospecimen collection, QST procedures and neuroimaging scans (Figure 2).

Figure **3**. Timeline for MAPP Phase II Symptom Patterns Study: Baseline and Follow-up Period



Following this baseline "deep-phenotyping" visit at week 4, enrolled participants will provide internet-based quarterly symptom (Q-Sym) data capture and medication updates, and semi-annual in-clinic visits for biomarker specimen collection (Figure 3).

3.4. Biomarker and Microbiome Protocols

Biomarker and microbiome studies proposed for Phase II were designed to validate and extend ongoing findings from MAPP Phase I. To achieve the goals outlined within the Biomarker and Microbiome Working Group protocols included as Appendices 1 and 2, a comprehensive set of biologic specimens will be collected using the clinic visit schedule set out in the SPS study. Longitudinal assessment of biomarker and microbiome composition will be used to analyze the correlation with specific phenotypes, change in symptom patterns, response to therapy, and neuroimaging and QST measures. In addition, the collection of different specimens (blood, urine, saliva, vaginal and rectal swabs) and its derivatives (Genomic, Bacterial, and fungal DNA) at the same time point will allow for comparison between the different measures and allow for both systemic and more organ specific measures.

In MAPP Phase I we identified a number of urinary biomarkers that correlated with UCPPS symptom severity. The most promising biomarkers, MMP-2, MMP-9, MMP-9/NGAL complex, NGAL, VEGF, and VEGF Receptor-1, will be analyzed using established ELISA assays on urine specimens collected from SPS participants. This panel will be extended based on the results of the directed mass-spectrometry (MS) approach to refine the set of 45 candidate urinary biomarkers identified by the global discovery analysis using MS in MAPP Phase I. Additional and complementary efforts to expand our discovery efforts of MAPP Phase I proteomic analysis of urine and plasma specimens will performed using Sequential Window Acquisition of Theoretical Mass Spectra (SWATH) applications. SWATH is a hybrid between *de novo* discovery and multiple reaction monitoring (MRM) assays, in which thousands of proteins are identified and quantified in a single run providing a time and cost-effective assessment of the broad proteome on large sample sets. The same technology will be used to analyze post-translational modifications (PTM).

Analyses of a site-specific study in MAPP Phase I identified differences between the IL-1β and IL-6 response of the stimulation of UCPPS patient's peripheral blood mononucleated cells (PBMCs) with either TLR-2 (PAM) or TLR-4 (LPS) agonists and the response seen in control participants.[21] In MAPP Phase II we will assess TLR-2/TLR-4 responsiveness using the TruCulture System from Myriad RBM. Three 1 mL samples of whole blood will be drawn into special TruCulture tubes containing (a) LPS (TLR-4 agonist), (b) FLS-1(TLR-2 agonist), and (c) Null, no treatment. Tubes will be incubated on site at 37°C for 24 hours. After 24 hours the supernatant will be separated from the cells by using the special valve separator included with the TruCulture system. The tubes will be capped and frozen at -80°C prior to shipment to TATC. An aliquot of the specimen will be used for analysis with two Human CustomMap panels containing 32 cytokine/chemokine markers including IL-6 and IL-1β. The same site-specific study also found that the hypothalamic-pituitary-adrenal axis was dysregulated in UCPPS patients compared to controls. Salivary cortisol will be measured twice daily (morning (4-9am) and bedtime (8pm-12am)). To establish a baseline measure, saliva specimens will be collected at home by the participant during the 3 day period following the week-4 Baseline Deep-Phenotyping visit as well at 7 day periods following the Months 6 and 18 visits. This will allow us to simultaneously consider the role of cellular inflammation (TLR-2/4 responsiveness) and endogenous control of inflammation (HPA axis). The TLR-2 and TLR-4 are bound by bacteria and mediate the host response to infection[22] which puts the response of the TLR stimulation in context with the participant's urinary microbiome.

In MAPP Phase II we plan to study the changes in overall complexity of commensals and opportunistic pathobionts using several complementary approaches. The T-5000 Universal Biosensor technology[23] which was used in MAPP Phase I was able to show a greater presence of fungi (e.g. Candida sp.) in female UCPPS participants who experience a flare compared to those who did not. We will expand on the findings in MAPP Phase I to investigate whether the microbiome is correlated with clinical phenotype and/or longitudinal change in symptoms. Since the panel used in MAPP Phase I had only limited coverage for fungi we will expand our analysis to include a more comprehensive fungi screen. To validate and confirm findings we will use Deep 16S rRNA sequencing. As a complementary approach to Ibis we will use next generation sequencing of species-specific ribosomal DNA sequences for both bacteria as well as fungi. Findings will be validated using a high throughput quantitative PCR platform. In addition, participants will have the option to provide vaginal (female) and rectal (female and male) swab specimens. DNA isolated from these specimens will be included in the analysis to determine their contribution to the urine microbial profile and association with participant phenotype and/or symptoms pattern.

Investigation of PTM, in particular S-acylation (palmitoylation) of urinary proteins involved in the host response and microbial interaction in combination with the microbial profile will be used to determine its association and correlation with participant's symptoms pattern and onset of flare. In MAPP Phase I we used a metabolomics approach using a liquid chromatography-mass spectrometry (LC-MS) platform to study the biochemical processes that contribute to urinary symptoms. We identified etiocholan-3a-ol-17-one sulfate (Etio-S), a 5-β reduced isomer of testosterone as a potential urine marker correlated with the UCPPS participant subgroup with high symptom score and multiple extra-urinary tract symptoms. In MAPP Phase II we will try to identify additional metabolites (or metabolite profiles) that are associated with specific UCPPS phenotypes and/or symptom patterns. For Etio-S analysis we have

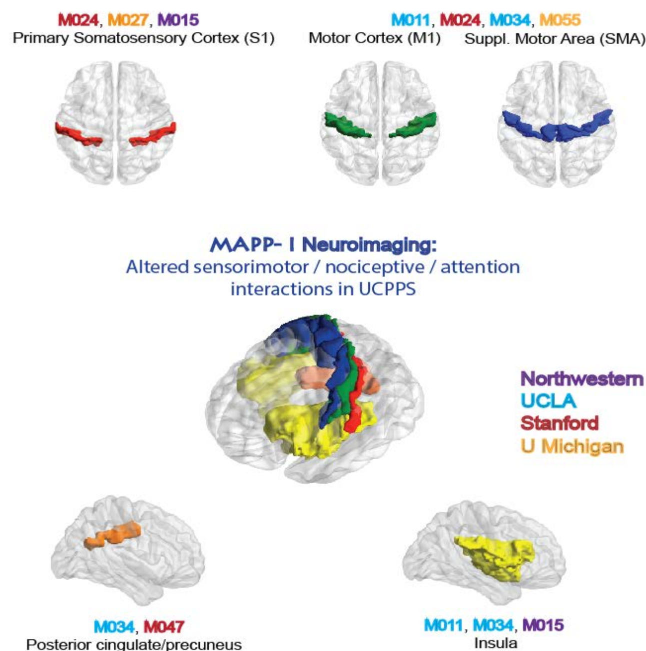
develop a more specific assay that allow us to examine larger sample sets. The same approach will be used for newly discovered metabolites.

A plasma vacutainer will be collected at all scheduled SPS clinic visits, and buffy coats from these collections will be used to isolate genomic DNA. These DNA specimens will be used to study systemic genetic and epigenetic alterations in UCPPS participants. DNA methylation has been shown to be dynamic and inducible by environmental factors and stress. Genome-wide profiling of CpG methylation in peripheral lymphocytes will be performed using the Illumina Humanmethylation450 Beadchip to investigate its potential role in UCPPS. Pro-inflammatory and pro-oxidant environmental factors can accelerate age-dependent LTR attrition. To determine whether this plays a role in UCPPS we will analyze Leucocyte Telomere Length by quantitative PCR using a single copy control gene as normalizer to produce a relative measurement (called the T/S ratio).

3.5. Neuroimaging

The neuroimaging findings obtained during the Trans-MAPP EP Study clearly demonstrated the feasibility of combining structural and resting state brain images obtained at different sites with different scanners. They also provided converging evidence from different types of analyses to support a unique pattern of brain abnormality which focus on the sensory and motor systems, which appears to differ from the neuroimaging literature on chronic somatic pain, and which may provide the basis for novel treatment strategies (Figure 4).

Figure **4**. Convergence of MAPP-I neuroimaging results

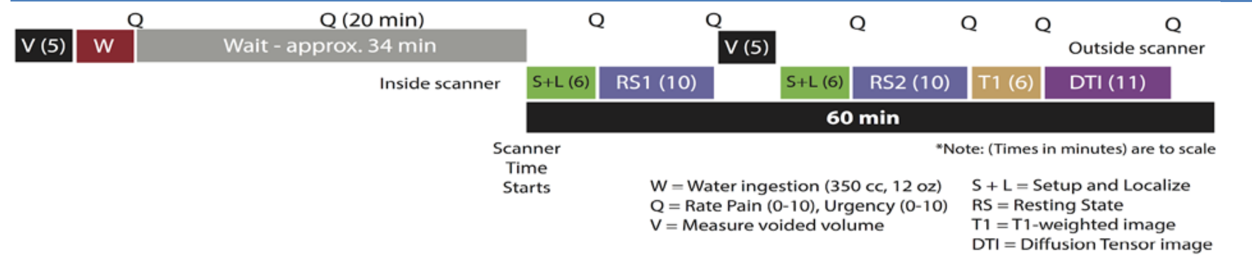


Given the prevalence of bladder-related pain in UCPPS conditions, as well as the importance of motor networks observed in MAPP Phase I neuroimaging data, we hypothesize that functional and structural alterations may be localized in brain networks related to urine storage. These networks have been extensively studied and summarized in Fowler, Griffiths, deGroat, 2008[24]

Based on the Neuroimaging discoveries of MAPP Phase I, The MAPP Phase II Neuroimaging protocol aims to develop a more comprehensive model of the neural representation of UCPPS symptoms and the brain-body interaction that mediates these symptoms in different UCPPS subgroups. To further define the findings of MAPP Phase I, the Phase II Neuroimaging protocol is designed to specifically look at the performance of the bladder filling network longitudinally in a well phenotyped sample of patients with UCPPS.

The Single Session Neuroimaging Protocol will be performed with all MAPP Phase II eligible participants at the 1, 6, 18, and 36 months visits as well as at any ATLAS Study visits. The Single Session Neuroimaging Protocol includes Structural, Resting State, and Bladder Evoked Response. The standard single session for the fixed volume, fixed time evoked procedure is shown in Figure 5 below. The participant voids before the start of the scanning session, and is then setup in the scanner. The participant is queried (Q) to rate pain (0 -10) and urgency (0 -7) at fixed time points.

Figure **5**. Protocol Overview of Bladder Filling Paradigm Based on Resting State Changes



The neuroimaging modalities acquired in the MAPP Phase I study, including structural images of gray matter (T1), white matter (DTI), and fMRI images of brain function in resting state (RS) will be acquired in the MAPP Phase II Single Session Neuroimaging Protocol. However, these data will be collected at multiple time-points in MAPP II participants. Another difference between the MAPP I and MAPP II neuroimaging protocols is the acquisition of an additional RS scan after water ingestion, which when compared to the first RS scan will facilitate the identification of brain networks mediating painful bladder filling in UCPPS participants.

3.5.1. Identifying Brain Regions and Networks Related to UCPPS Symptom Change

In the analysis of longitudinal neuroimaging data, single session clinical symptom data will be correlated with brain imaging metrics derived from the individual or combined modalities. If $X(t)$ represents a clinical parameter of interest collected close to the time of scan (t), and $Y_i(t)$ represents the brain imaging metric associated with voxel i, brain imaging analysis in standard software will seek to identify clusters of voxels for which the correlation between $X(t)$and $Y_i(t)$ is significant. In another layer of analysis, differences in the association between $Y_i(t)$and $X(t)$will be determined between the different participant subgroups.

3.5.2. Number of Scans Per Participant

Proposed MAPP Phase II sample sizes for the SPS are based on an analysis of symptom worsening and improving across the MAPP Phase I cohort, and predicts n=640 participants. This analysis did not directly address the probability that participants will be observed at different symptom states given a scanning schedule, or estimate the expected change in symptoms.

Figure **6**. Number of scans per patient

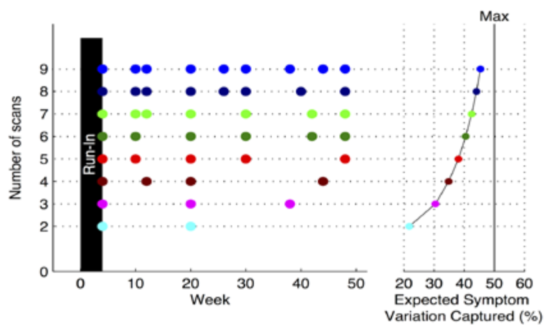
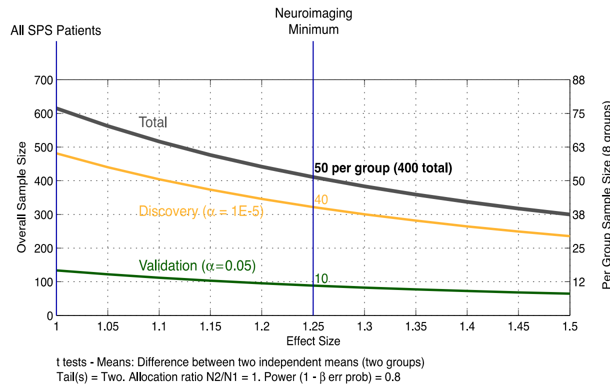


Figure 6 shows the results of analysis of variability in the ISCI symptom questionnaire (sampled every 2 weeks from MAPP Phase I participants). For a given number of scans, the optimal weeks to have these scans obtained (to maximize the difference in reported symptom across the set of scans) are shown. There is a large increase in the expected symptom change when the number of scans is increased from 2 to 3 (20% symptom variation is expected for 2 scans and 30% symptom variation is expected for 3 scans), with a diminishing increase in additional variation for additional scans. Scanning every 6 months (after 4 week run in, at week 24, and week 48) also produced 30% symptom variation, indicating that it was a nearly-optimal scan schedule for a total of 3 scans.

3.5.3. Number of Participants

All participants in the SPS, that meet the neuroimaging study criteria and are willing to participate, will be enrolled in longitudinal neuroimaging procedures, and thus we can estimate the minimum effect size that is needed to detect group differences for a given sample size, power, and significance level. Representative effect sizes observed in MAPP Phase I ranged from 0.75 (Kilpatrick et al., 2014 - frequency analysis[20]) to 1.25 (Kutch et al., M034 in preparation - functional connectivity analysis; structural analysis[25]). Of 640 SPS participants, the minimum size of a MAPP-II recruitment cell is 60, which allows for two-tailed unpaired t-test between two groups of 60. Assuming that an analysis is focused on the validation of a MAPP-I result, we calculate that for a power of 0.8 we would be able to validate effect sizes (alpha error probability = 0.05) of 0.51, clearly less than the expected effect sizes of MAPP-I. These parameters will allow us reach (alpha error probability 5e-05) effect sizes of 0.92, which are consistent with MAPP-I effect sizes. In summary, the sample size of the MAPP-II SPS appears to be adequate for both validation and discovery purposes.

Figure **7**. Analysis of sample size.



Analysis of sample size (without it being fixed to 640 *a priori* – Figure 7), indicates that the neuroimaging sample size should be larger than 400 to capture effect sizes that were observed in MAPP Phase I across 8 different recruitment groups (males/females, PP only/PP & beyond, BPS:Yes, BPS:No).

The neuroimaging protocol will be implemented (and associated neuroimaging CRF will be completed) on 10-12 healthy participants twice (6 months apart) at each site.

Further details are provided in the full protocol for Neuroimaging in Appendix 3.

3.6. Quantitative Sensory Testing

To better evaluate the role that the CNS is playing in pain and sensory sensitivity among individuals with UCPPS, a broader and more comprehensive set of QST methods will be implemented in MAPP Phase II. QST will be conducted at the week 4 (baseline) and 6, 18, and 36 month deep phenotyping in-clinic visits, as well as during all ATLAS modules (pre-therapy and post-therapy), on all SPS participants. The MAPP Phase II QST protocol consists of a "core" battery of four methods (see below) that assess generalized and segmental pain sensitivity and modulation. As in MAPP Phase I, participants will be assessed for generalized mechanical sensitivity using the University of Michigan (UM) MAST system. In addition, the QST core battery will include an evaluation of spinal segmental mechanical sensitivity at the most common referral site of pelvic pain (the suprapubic area), as well as tests of temporal summation and conditioned pain modulation (CPM).

Procedures for QST will adhere to standardized experimental protocols. Equipment will undergo regular calibration to maintain reliability and consistency across multiple testing sites. Instructions will be scripted and participants will undergo extensive training before testing. All procedures have been evaluated for reliability and safety, and are well tolerated by chronic pain patients, causing no more than temporary mild discomfort. However, participants can stop testing at any time if a procedure

becomes unbearable. It is anticipated that the entire QST core battery will require approximately 90 minutes to complete, including training and rest intervals.

The University of Michigan will provide all equipment required to perform the core battery to all discovery sites. Training for MAPP research personnel will be conducted at the face-to-face steering committee meetings and/or onsite by the QST Working Group. Ongoing feasibility testing of the core battery will be conducted prior to the Year 1 recruitment launch. Adjustments to the protocol will be made as necessary during feasibility testing to achieve optimal efficiency and an acceptable level of participant and staff burden. No changes will be made that increase the length of burden of the testing.

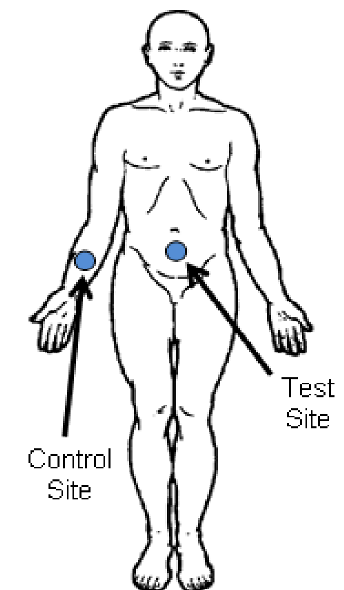
3.6.1. QST Core Battery

a. Generalized Mechanical Sensitivity.

Consistent to MAPP Phase I, generalized, or global, mechanical sensitivity will be evaluated using the computer-controlled MAST system.[26, 27] The MAST system will deliver an ascending series of discrete pressures (5-s duration; 4 kg/cm^2/s ramp rate) at 20-s intervals staring at 0.25-0.50 kg/cm^2 to the dominant thumbnail using a 1 cm^2 rubber probe. Pain intensity will be rated after each stimulus on a 0-100 NRS displayed on the participant interface (0 indicating “no pain” and “100” indicating “most intense pain imaginable”). The test will be terminated when participants reach their maximum tolerable pain level and request the test to stop (tolerance), a pain intensity rating of $\geq$ 80/100 is recorded, or the maximum allowable pressure of 10 kg/cm^2 is reached.

b. Segmental Mechanical Sensitivity.

Figure **8**. Testing Sites



Segmental mechanical sensitivity will be assessed following a method modified from Lai et al.[28] A handheld, analog algometer with a 1 cm^2 flat rubber probe (FPK Algometer, Wagner Instruments, Greenwich, CT) will be used to deliver quantifiable pressure stimuli to the suprapubic area (midway between the pubic symphysis and the umbilicus; Figure 8). Algometry will also be performed at a nonsymptomatic control site on the ulnar forearm midway between the wrist and elbow on the dominant body side (Figure 8).

Participants will first empty their bladder prior to testing to reduce the chance of algometer stimulation provoking unanticipated urine voiding. Next, a random sequence of fixed intensity pressures (2 or 4 kg/cm^2, 5-s duration, 20-s inter-stimulus interval) will be applied, 3 times each, first to the forearm control site and then to the suprapubic test site, with the participant in the supine position. Thus, a total of 12 pressure stimuli will be delivered (six at each site). Pressure will be increased at rate of approximately 0.5 kg/cm^2/s (equivalent to 50 kPa/s). A ticking 1Hz frequency metronome with earphones will be used to help control the rate of applied pressure to reduce examiner variability.[29] Participants will be asked to rate the pain intensity of each pressure using a 0-100 NRS. The mean of three NRS ratings for each pressure level at each testing site will be used for analysis.

Pressure pain threshold will also be assessed bilaterally at a second control site. Pressure will applied manually using the algometer at a steady rate of 0.3-0.5 kg/cm^2/s to the center of the trapezius muscle until the participant indicates an initial sensation of pain.[44] The pressure level at the time of this verbal report will be recorded as the PPT. Pressure will not exceed 8 kg/cm^2. This procedure will be repeated 2-5x and the mean threshold will be used for analysis.

c. Temporal Summation.

Temporal summation, also referred to as windup pain, will be evaluated using a method similar to that of the German Neuropathic Pain Network[29] and the OPPERA study.[30] In this test, a single pinprick stimulus at a fixed intensity of 256 mN (PinPrick Stimulator, MRC Systems GmbH) will be applied perpendicularly to the skin for approximately 0.5 s. Following a 5 s pause, a train of 10 identical pinprick stimuli (256 mN) will be applied with a frequency of 1Hz within an area of 1 cm^2. Immediately following the single stimulus and the train of 10 stimuli, participants will be asked to report the pain intensity of the pinprick sensation using a 0-100 NRS. This testing paradigm (a single stimulus followed by a train of 10 stimuli) will be conducted 3 times with the same 256 mN stimulator at the suprapubic region and at the forearm control site while the participant is in the supine position. For each testing site, the mean pain rating of the three stimulus trains will be divided by the mean pain rating of the single stimuli to calculate a wind-up ratio (WUR); a WUR of >1 indicates temporal summation.[31]

d. Conditioned pain modulation (CPM).

Participant responses to a painful test stimulus during painful conditioning vs. neutral (non-painful) conditioning stimulation will be assessed as a measure of endogenous pain inhibition.

Figure **9**. MAST Handset

Painful pressure delivered using the MAST system to the dominant thumbnail will serve as the test stimulus (Figure 9). Immersion of the contralateral foot into a circulating water bath with either body temperature water (32.0-33.0°C) or moderately painful hot water (44.0-46.5°C)[32] will serve as the neutral and painful conditioning stimuli, respectively. The water bath is a custom-made, 14 L polycarbonate tank (L x W x D, 18x8x8") with a perforated foot rest positioned 1 inch from the bottom surface of the tank to permit water flow around all surfaces of the foot. Water temperature and flow rate is maintained using a laboratory-grade thermal immersion circulator (LX Immersion Circulator, PolyScience, Niles, IL).

For the assessment of CPM, continuous or pulsed test pressure will be applied for 30-s to the dominant thumbnail at each participant's pre-determined Pain30 or Pain40 level (i.e., the estimated pressure intensity that evokes a pain intensity rating of 30/100 or 40/100, respectively). Participants will rate the pain intensity of the pressure 3X (10-, 20-, and 30-s) using a 0-100 NRS. Conditioning stimulation will begin 5-10 min after the test stimulus by immersing the contralateral foot up to malleolus area into the water bath. The foot will be immersed for approximately 60-s; perceived pain and heat intensity of the water will be rated 5-, 10- and 20-s after foot immersion and immediately following foot withdrawal. The neutral conditioning stimulus will be applied first, followed by the painful conditioning stimulus, with a 5-10 min rest between

each session. The foot will be removed from the water bath between sessions. Parallel to the last 30-s of conditioning, the same test stimulus will be reapplied to the dominant thumbnail for 30-s and the participants will be again asked to rate the intensity of the pressure at 10-, 20-, and 30-s. CPM magnitude will be calculated as the difference in the mean of the three pain ratings given to the test stimulus prior to the conditioning stimuli and the three pain ratings of the test stimulus given during the conditioning stimuli.

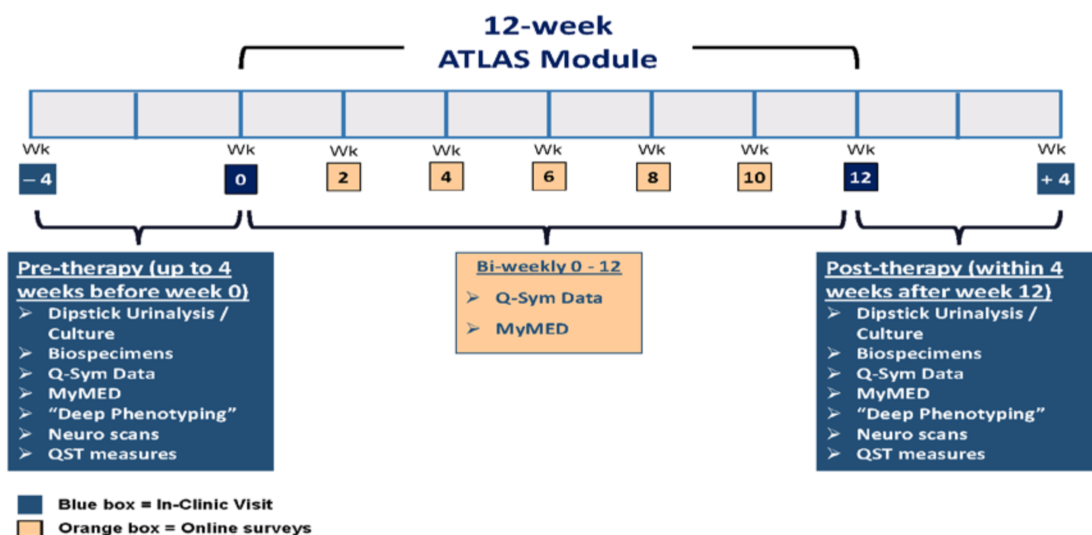
As an alternate CPM procedure, thumbnail pressure will be replaced with either the trapezius PPT or the forearm temporal summation procedures described above. The conditioning stimuli will not change and the test stimulus will be presented during the last 30 to 40-s of conditioning. This alternate procedure will be employed if the MAST system is unavailable or if participants are unable to tolerate sustained thumbnail pressure.

Further details for the QST Protocol are provided in Appendix 4.

3.7. ATLAS Module

"Deep-phenotyping", the "**A**nalysis of **T**herapies during the **L**ongitudinal **A**ssessment of **S**ymptoms ATLAS will be implemented in the SPS at the beginning of initiation of pre-specified new therapeutic interventions, so that a potential relationship between response (or lack of response) to a given therapy and specific phenotypic profiles of subgroups of UCPPS patients may be established. Upon completion of the 6-month in-clinic visit, each MAPP SPS participant will be asked to notify the study coordinators of any planned treatment change for their UCPPS symptoms. This notification of intent to seek treatment change to add a pre-specified ATLAS treatment of interest will trigger a phenotyping battery of tests, as summarized in Figure 10, to be conducted within 8 weeks before and up to 8 weeks after a 12-week more intensive bi-weekly internet-based symptom data capture. Taken together, the 'pre-treatment' ATLAS phenotyping, biweekly ATLAS symptom assessments, and 'post-treatment' ATLAS phenotyping are referred to as an 'ATLAS module'.

Figure **10**. Timeline for MAPP Phase II Symptom Patterns Study: ATLAS Module



The **ATLAS** module will be initiated, upon notification from the participant of prescribed treatment change, to add a pre-specified ATLAS treatment, within the longitudinal follow-up period of the

Symptom Patterns Study. This longitudinal follow-up period will begin at any time after the participant completes the in-clinic visit at 6 months, and prior to 30 months of follow-up (with sufficient time remaining in follow-up to carry out the data collection noted in above figure). All medications and all UCPPS therapies (medications, procedures, and nontraditional therapies) will be tracked prospectively on a monthly basis throughout the 36-month SPS. As summarized in Figure 10, initiation of specific treatments (ATLAS treatments) will be preceded by an in-clinic assessment of functional/structural neuroanatomy, collection of blood and urine for biomarker and microbiome assessment, trait and state measures, and urologic symptom profiles using the standard Q-Sym data module. In an effort to reduce participant burden, if ATLAS module occurs within 8 weeks before or after a core MAPP in-clinic visit (for Month 6, only 8 weeks after), the MAPP core visit will count as the ATLAS pre-treatment in-clinic visit.

3.7.1. Eligibility of Time Intervals for ATLAS Modules

The eligible time interval to initiate an ATLAS module during longitudinal follow-up within the SPS was designed to not impact the ongoing usual clinical care for UCPPS symptom management, but to maximize the potentially relevant phenotypic measurements, prior to initiating a treatment change, during the new therapy period, and again at 12 weeks post-therapy change. A maximum of two ATLAS modules may be implemented for each SPS participant, subject to the following criteria:

- 1st ATLAS module may begin only after the 6 month in-clinic visit is completed. However, if participants begin an ATLAS treatment within 7 days prior to the Month 6 visit, the Month 6 SPS visit may serve as the ATLAS pre-treatment phenotyping visit.
- Any ATLAS module may not begin later than month 30, and must be completed by month 35
- No minimum time period between 1st and 2nd ATLAS module, but if the start of the 2nd module is within 2 months of the end of the 1st module, then the post-therapy measurements from the 1st module are permitted to serve as pre-therapy measurements for the 2nd module
- 36-month final in-clinic visit must occur regardless of timing of last ATLAS module
- If an ATLAS module overlaps with scheduled SPS data collection (e.g., on-line quarterly symptom assessments, scheduled deep phenotyping visits), ATLAS data collection will supersede SPS data collection.
- On-line quarterly data collection increases to bi-weekly frequency during the 12-week ATLAS therapy period
- Ideally the ATLAS therapy module will include a 4-week window pre-therapy change and 4-week post-therapy for data collection, procedures and testing from participants; however, this may vary slightly based on the timing of the participant's pre-ATLAS therapy initiation visit.

3.7.2. Pre-specified ATLAS Therapies

Network investigators identified six treatments in men and five treatments in women that will be the focus of ATLAS study investigations. These choices were based on the treatment patterns observed in MAPP Phase I as well as the desire to examine treatments which may improve symptoms through 'centrally-acting' and 'peripherally-acting' mechanisms, recognizing that this categorization scheme dichotomizes the presumed mechanisms of these interventions based on our interpretation of available information. Feasibility concerns were also taken into account. For instance, NSAIDs were commonly taken by MAPP Phase I study participants; however, investigators felt that this likely represented patient self-medication for a wide range of medical conditions rather than for UCPPS. Similarly, bladder instillations were a commonly reported therapy, but these are frequently used as 'rescue' therapy for flares at the time of clinic visits, which would not permit sufficient time to conduct pre-therapy ATLAS assessments. The treatments that will initiate more in-depth assessment (Figure 11) are:

1) ***Oral opioids*.** This will be categorized as a centrally-acting therapy, and will be examined in both men and women. While these agents may be utilized intermittently by patients, MAPP network investigators feel that the more common practice pattern at MAPP sites is to provide prescriptions to be used on a regular (e.g. daily) basis as opposed to very intermittent use.
2) ***Tricyclic antidepressants*.** This will be categorized as a centrally-acting therapy, and will be examined in both men and women.
3) ***Pelvic floor physical therapy*.** This will be categorized as a peripherally-acting therapy, and will be examined in both men and women.
4) ***Alpha-adrenergic blockers*.** This will be categorized as a peripherally-acting therapy, and will be examined in men only.
5) ***Oral pentosan polysufate (PPS)*.** This will be categorized as a peripherally-acting therapy, and will be examined in both men and women.
6) ***Neuropathic pain medications.*** This will be categorized as a centrally-acting therapy, and will be examined in both men and women.
7) ***Cystoscopy with Hydrodistention****.* This will be categorized as a peripherally-acting therapy, and will be examined in ATLAS studies in both men and women.

Figure **11**. Examples of Common Targeted Therapies for ATLAS Monitoring

Example Targeted ATLAS Therapies by Treatment Type Updated

*<u>*PLEASE NOTE*</u>: For these examples each number matches the corresponding brand name and generic name for medications. An *extended* list of Brand & Generic Names will be provided to Participants for reference in case of ATLAS med. additions.

Targeted ATLAS Therapies

All Participants	All Participants	All Participants	All Participants	All Participants	All Participants	Males only
Oral opioids (Medication)	**Tricyclic antidepressants (Medication)**	**Pelvic floor physical therapy (Non-Medication)**	**Elmiron / Oral pentosan polysulfate (Medication)**	**Neuropathic pain Treatments (Medication)**	**Cystoscopy with Hydrodistention**	**Alpha-adrenergic blockers (Medication)**
Common Brand Names	Common Brand Names	Common Names	Brand Name	Common Brand Names	Common Names	Common Brand Names
1.Endocet 2.Lortab 3.Oxycontin 4.Percocet 5.Percodan 6.Vicodin 7.Tylenol w/ Codeine	1.Elavil 2.Tofranil 3.Treyzafagit 4.Sensoval	1.Pelvic Physical Therapy 2.Pelvic Floor Physical Therapy 3.Pelvic Floor Dysfunction Therapy 4.Pelvic Floor Myofascial Release 5.Pelvic Floor Rehabilitation	1.Elmiron	1.Lyrica 2.Neurontin	1. Cystoscopy with Hydrodistention	1.Flomax 2.Hytrin 3.Cardura 4.Rapaflo
Common Generic Names	Common Generic Names		Generic Names	Common Generic Names		Common Generic Names
1.Acetaminophen& Oxycodone 2.Acetaminophen& Hydrocodone 3.Oxycodone 4.Acetaminophen& Oxycodone 5.Aspirin& Oxycodone 6.Acetaminophen& Hydrocodone 7.Acetaminophen& Codeine	1.Amitriptyline 2.Imipramine 3.Desipramine 4.Nortriptyline		1.Pentosan Pentosan Polysulfate Pentosan Polysulfate Sodium	1.Pregabalin 2.Gabapentin		1.Tamsulosin hydrochloride 2.Terazosin 3.Doxazosin 4.Silodosin

3.7.3. Notification Procedures for ATLAS Modules

Upon completion of the 6-month in-clinic visit, each MAPP participant will be requested to notify the MAPP research coordinators immediately when they intend to change their UCPPS treatment regimen, including prescription medications, nonprescription agents, procedures, therapies, and 'nontraditional' treatments. This notification will be done either via telephone to a dedicated MAPP telephone number, or via an on-line notification form. Any change, including starting a new UCPPS therapy or stopping an

existing UCPPS therapy will be recorded. Participants will be educated and reminded about the therapies that are of specific interest to MAPP for ATLAS modules.

When a new ATLAS therapy is identified, participants will have the option to participate by completing the bi-weekly online ATLAS questionnaires only or in addition to the online questionnaires, undergo a battery of 'pre-treatment' phenotyping tests, conducted in person at the clinic. It is ideal that this testing be conducted before the new ATLAS treatment is initiated. A 8-week window will be allowed to complete the 'pre-treatment' ATLAS studies prior to starting the ATLAS therapy. During the ATLAS therapy, biweekly on-line symptom assessments will be completed by participants for 12 weeks. After 12 weeks of ATLAS therapy, participants will have the option, in addition to completing the online questionnaires, to return to the clinic foran additional set of 'post-treatment' phenotyping tests including a neuroimaging scan, QST assessments, and biospecimen collection. A 4-week window will be allowed to complete the 'post-treatment' phenotyping after the 12 weeks of ATLAS therapy. The 'pre-treatment' ATLAS phenotyping, biweekly ATLAS symptom assessments, and 'post-treatment' ATLAS phenotyping are referred to as an 'ATLAS module'.

3.7.4. Sample Size Considerations for ATLAS Comparisons

Approximately n=256 study participants (40% of SPS target enrollment of 640) are expected to experience an ATLAS event, based on MAPP Phase I treatment and medication reporting. To illustrate the sample size/power considerations further, consider that comparisons of the response rate in the "preferential effect" subgroup will be compared with the "proposed non-responder" subgroup, for which we are enriching the "preferential effect" group for responders and enriching the "proposed non-responder group" for non-responders.

Accordingly, as summarized in the following sample size table (Table 1), a subgroup of n=34 study participants who initiated a selected class of ATLAS treatments, such as "Tricyclic antidepressants", will provide 80% power to detect a responder rate of 40% among those who are enriched for the "preferential effect" subgroup, compared to a responder rate of 5% among those enriched for the "non-responder" subgroup. Similarly, a subgroup of n=32 study participants who initiated a selected class of ATLAS treatments, such as "Tricyclic antidepressants", will provide 80% power to detect a responder rate of 50% among those who are enriched for the "preferential effect" subgroup, compared to a responder rate of 10% among those enriched for the "non-responder" subgroup.

Table **1**: ATLAS Total Sample Size for Specified Effects (1-sided α=0.05, power=80%).

Responder Rate for Subgroup Enriched for Non-response	**Responder Rate for Subgroup Enriched for Response**					
	0.25	0.30	0.35	0.40	0.45	0.50
0.05	78	56	42	34	28	24
0.10		98	68	50	40	32
0.15			114	78	56	44

Further details for the ATLAS module of the SPS protocol are provided in Appendix 5.

3.8. Trans-MAPP SMARTPHONE APPLICATION To Assess Pelvic Pain: The (M)APP Study

Participants enrolled in the MAPP SPS study will have the option to participate in the (M)APP study following completion of the Months 6, 18 and 36 SPS clinic visits. Participants will be prompted up to four times a day for 14 days to answer a brief set of questionnaires on their smart phone. Participation in (M)APP assessments is optional.

The purpose of this study is to use a mobile smartphone application for assessing pelvic pain variation throughout the day in patients with chronic pelvic pain. The MAPP Discovery Site teams from the University of Iowa and Northwestern University have expertise in the technical intricacies of computerized assessment and will help to create a system in which assessment data can be rapidly and easily collected and subsequently transferred to clinicians and researchers for rapid analysis.

The data obtained from the MAPP mobile phone application, (M)APP, will complement, but not replace, the participant-reported questionnaire data obtained from the SPS. Data on pain severity, pain location, anxiety, and depression that are collected in the SPS will be examined both cross-sectionally and longitudinally with respect to the (M)APP dataset to ensure that the newly-develop app is a valid assessment. These comparisons will help to inform the hypothesis as outlined below. In addition, there are novel questions in the app that are relevant to chronic pain, but not included in the SPS (e.g., day-to-day stress, alcohol use).

Please see Appendix 7, (M)APP Protocol for additional information regarding the MAPP smartphone application protocol.

4. Study Population

4.1. Study Participants and Subgroup Targets

Appealing to the sample size/power considerations, summarized subsequently in Table 4 (Section 6.1), the Trans-MAPP Symptom Patterns Study (SPS) will recruit 640 UCPPS patients meeting the eligibility criteria, recruited from 6 clinical sites. Approximately one-half of the participants will be male, and one-half will be enriched to meet the body map pain location criteria of PP Only, as illustrated in Figure 12.

Table **2**: Proposed Composition of MAPP Phase II Symptom Patterns Study Compared to MAPP Phase I UCPPS Participants by Target Recruitment Factors

Actual Dist'n: MAPP Phase I

BPI Body Map Location	Sex	UCPPS Subtype				Total
		BPS: No	BPS:Yes			
		Neither	Painful Filling	Painful Urgency	Both	
PP Only	Males	14 (24.56)	2 (3.51)	17 (29.82)	24 (42.11)	57
	Females	7 (13.73)	4 (7,84)	14 (27.45)	26 (50.98)	51
PP and Beyond	Males	33 (24.63)	14 (10.45)	33 (24.63)	54 (40.30)	134
	Females	20 (10.99)	16 (8.79)	34 (18.68)	112 (61.54)	182
Total		74	36	98	216	424

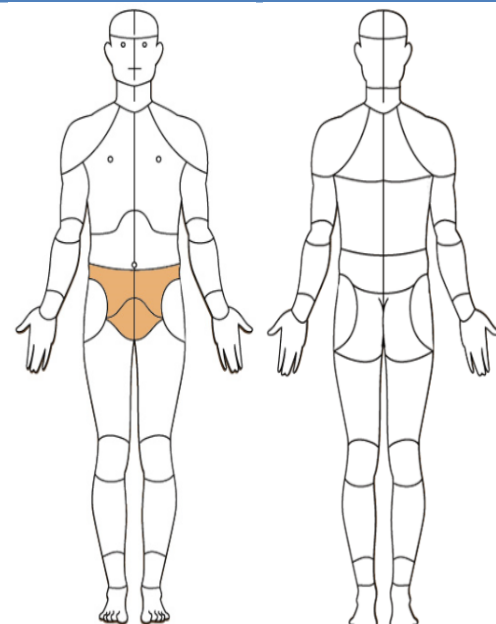
Proposed Dist'n: MAPP Phase II

BPI Body Map Location	Sex	UCPPS Subtype				Total
		BPS: No	BPS:Yes			
		Neither	Painful Filling	Painful Urgency	Both	
PP Only	Males	60	40		60	160
	Females	60	40		60	160
PP and Beyond	Males	60	40		60	160
	Females	60	40		60	160
Total		240	160		240	640

Furthermore, as illustrated in Table 2 (right-hand panel), these subgroup proportions of 25% (n=160) in each of the four row totals represent enrichment sample size targets, relative to the subgroup composition recruited within the MAPP Phase I EP Study (left-hand panel). In particular, the MAPP Phase II recruitment strategy will target 50% of males and females within the "PP Only" subgroup.

This "PP Only" localization region will be determined from a body map, in which the participant endorses pain only in one or more of the four highlighted regions on the front view of the Collaborative Health Outcomes Information Registry (CHOIR) (http://snapl.stanford.edu/choir/) body map, modified with the abdominal midline to increase the specificity of the PP Only region, as shown in Figure 12. To meet these subgroup criteria, participants will need to leave all other body regions blank on the front view, as well as all body regions blank on the back view.

Figure **12**. CHOIR Body Map Modified for Regional Specificity of "Pelvic Pain Only"



In addition, enrichment recruitment of 240 UCPPS patients (120 males; 120 females) will also be targeted for those who endorse BPS:No. Specifically, these items inquire about the presence of pain that worsens with bladder filling (RICE Q4), and urinary urgency due to pain/pressure/ discomfort (RICE Q2/Q3), as shown in Table 2. Participants in MAPP Phase I who did not endorse the presence of pain that worsens with bladder filling (RICE Q4), nor urinary urgency due to pain/pressure/ discomfort (RICE Q2/Q3), were classified as BPS:No. These enrichment subgroup criteria are based on data-driven results arising from extensive longitudinal symptom patterns analyses of MAPP Phase I data currently underway. These baseline factors appear to be associated with differential likelihoods of being an "Improver", although the sample sizes are quite limited,

as shown in Table 2 (left-hand panel). However, as shown subsequently in the sample size/power results in Table 4 (Section 6.1), the sample size requirements are minimized when the key risk factor distributions are 50:50 for absent:present. With sample sizes of approximately 320 males and 320 females, this Symptom Patterns Study will have 90% power to detect RR=2.0 for these promising risk factors, separately within males and females.

In addition, all UCPPS participants from MAPP Phase I will be contacted, and invited to participate in the Symptom Patterns Study, and will be assessed in the identical manner as new participants, as outlined in the eligibility criteria in Section 4.4. These MAPP Phase I re-enrollees into MAPP Phase II will provide even longer-term data regarding symptom progression, although there will be variable length gaps between their 48-week symptom assessment visit and their re-entry into the long-term follow-up study currently underway.

4.2. Recruitment Timetable and Targets by Discovery Sites

Under the following assumptions:

- the subgroups illustrated in Table 2 will be recruited;
- recruitment will commence within the first months of 2015;
- all participants will be recruited over a42 month period;
- recruitment targets (n=107) will be identical at each of the 6 Discovery Sites.

Depending on when participants are recruited into the study, some will be followed for up to 3 years, at which time their data will be censored for primary analyses. Participants enrolled in the study later in the recruitment phase will have less follow up time. However, participants who reach the 3-year endpoint will continue to be followed at 3-month intervals for secondary analyses.

4.3. Eligibility Criteria

4.3.1. Inclusion Criteria

Patients are eligible for the SPS if they meet the following criteria:

1. Participant has signed and dated the appropriate Informed Consent document.
    a. Agreed to participate in ALL required Symptoms Patterns Study procedures (including Biospecimen collections, and Quantitative Sensory Testing).
    b. Gave permission for use of DNA for genetics studies.
2. Gender recorded in Participant Registration module.
3. Participant is at least 18 years of age.
4. Participant is able to speak, read, and understand English.
5. In the past 3 months participant has had a feeling of pain, pressure, or discomfort in the lower abdomen or pelvic area -- that is, the part of the body that is above the participant's legs and below the belly button. (This criteria does not apply to returning MAPP I participants)
6. These symptoms have been present for the majority of the time during the most recent 3 months.
7. Participant reports a response of at least 1 on the pain, pressure or discomfort scale (on the SYM-Q-Screening Q1) for UCPPS symptoms during the past 2 weeks. (This criteria does not apply to returning MAPP I participants)

8. Participant has received a ***clinical diagnosis*** of either or both IC/BPS or CP/CPPS (per AUA guidelines) or a clinician familiar with UCPPS criteria confirms participant meets UCPPS evaluation criteria per-protocol. (This criteria does not apply to returning MAPP I participants)

Inclusion criteria exceptions for MAPP I participants:

MAPP I participants may be enrolled even if they do not have symptoms in the past 3 or most recent 3 months and/or score 0 on SYM-Q-Screening Q1.

Please note: For **reference**, *NOT Inclusion Criteria*, document if the participant is under the ongoing care of a MAPP Clinical Investigator.

Inclusion criteria exceptions for Neuroimaging criteria:

Potential participants who are ***ineligible*** for the MRI criteria but meet all other study criteria are eligible to participate in the study.

4.3.2. Exclusion Criteria

Any patient meeting any one of the following criteria will not be eligible for enrollment in the Symptom Patterns Study. However, participants who develop any of these exclusion criteria during the follow-up phase of the study will continue to be followed. It will be recorded in the follow-up data if a patient has developed any of the exclusion criteria.

1. Participant has an on-going symptomatic urethral stricture.
2. Participant has an on-going neurological disease or disorder affecting the bladder or bowel fistula.
3. Participant has a history of cystitis caused by tuberculosis, radiation therapy or Cytoxan/cyclophosphamide therapy.
4. Participant has augmentation cystoplasty or cystectomy.
5. Participant is currently undergoing dose titration or medication adjustments for a poorly controlled autoimmune or infectious disorder (such as Crohn’s Disease, Ulcerative Colitis, Lupus, Rheumatoid Arthritis, Multiple Sclerosis, or HIV) which in the opinion of the Investigator could impact bladder symptoms.
6. Participant has a history of any pelvic malignancy (e.g. GI, GU, Gyn).
7. Participant is having ongoing systemic treatment/therapy for any type of cancer.
8. Participant has current major psychiatric disorder or other psychiatric or medical issues that would interfere with study participation (e.g. dementia, psychosis, upcoming major surgery, etc.).
9. Participant has severe cardiac, pulmonary, renal, or hepatic disease that in the judgment of the study physician would preclude participation in this study.

Exclusion Criteria for Males Only

1. Diagnosis of unilateral orchalgia, without pelvic symptoms.
2. History of transurethral microwave thermotherapy (TUMT), transurethral needle ablation (TUNA), balloon dilation, prostate cryo-surgery, or laser procedure.

4.3.3. Deferral Criteria

<u>Deferral Criterion – Treatment and History</u>

- If participant has had definitive treatment for acute epididymitis, urethritis, vaginitis, the participant will be deferred for at least 6 weeks from resolution of symptoms.
- If participant has history of unevaluated hematuria, this will require the evaluation of a study physician to determine if this has been appropriately evaluated.
- If participant has had cystoscopy with hydrodistention or kenalog injection, the participant will be deferred for at least 3 months from the date of the procedure.

<u>Deferral Criterion – Prostate related (Males *ONLY)*</u>

- If male participant has had a prostate biopsy or transurethral resection of the prostate (TURP) within the last three months, he will be deferred for 3 months following prostate biopsy or TURP.

<u>Deferral Criteria - Urine test results*</u>

A clean-catch midstream urine specimen (VB2) will be obtained from all male and female participants during the initial phase of eligibility confirmation, so that a urine dipstick analysis and urine culture (minimum of 24 hours incubation) can be done for all participants, and a urine pregnancy test can be conducted for females of child bearing age (excluding post-menopausal and those with a history of hysterectomy).

- If participant has an abnormal dipstick urinalysis indicating abnormal levels of nitrites and/or occult blood that in the opinion of the Principal Investigator, warrants a deferral, participant will be deferred until normal level of nitrites from dipstick urinalysis is confirmed.
- If participant has had a positive urine culture in the past 6 weeks, or currently has a midstream urine culture (VB2) ($\geq$100,000 CFU/ml), with a single uropathogen, the participant will be treated and deferred for at least 6 weeks from the date of positive urine culture result. (Must be documented on Urine Culture Result – UCR form).

* Repeat urine dipstick analysis and minimum 24-hour urine culture will be performed at all subsequent clinic visits for purposes of data collection and not deferral.

<u>Deferral Criterion – Pregnancy Test (Females of childbearing potential *ONLY)*</u>

- If a female participant has a positive urine pregnancy test she will be deferred until after delivery.

  (If a female participant becomes pregnant during the study, she will be withdrawn from the study at the time the pregnancy is identified; data from prior to the pregnancy will be included in the analyses).

5. MEASURES AND FOLLOW-UP

5.1. Risk Factors and Outcome Measures

Extensive data on risk factors and outcomes measures will be collected. These measures can be classified into a number of primary domains as described below (Figure 13). Measures will be collected on one of four schedules; the contact schedule being described in more detail subsequently in Section 5.2.

5.1.1. General Measures of Sociodemographics, Health, and Quality of Life

Data on age, gender, race/ethnicity, education and income will be collected at the Screening and Eligibility Confirmation visit, Week 0. A physical exam will include weight, height, and a brief pelvic evaluation. A more extensive standardized pelvic exam will be performed to assess pelvic musculature tenderness. At each in-clinic phenotyping visit (screening, baseline, 6 months, 18 months, and 36-months) participants will be asked to list all prescription and over-the-counter drugs they are taking as well as all non-medication therapies they are receiving.

A Quality of Life (QOL) assessment will be performed at each in-clinic visit and semi-annual and annual online contacts using the revised Medical Outcomes Health Survey Short Form-12 (SF-12).[33]

Figure **13**. Data Domains: Demographic, Urologic, Non-urologic and Biopsychosocial

Data Domains: Urological

Screening/ Run-In Weeks 0-3	Deep Phenotyping / ATLAS Visits	Quarterly	Annually / Semi-annually Optional In-clinic visit@12,24,30 months
Consent			
Eligibility			
Urine Dip/Cult	Urine Dip/Cult		Urine Dip/Cult
SYM-Qs/ Flare Status	SYM-Qs Flare Status	SYM-Qs Flare Status	SYM-Qs Flare Status
GRA (Run-In only)	GRA	GRA	GRA
RICE	RICE	RICE	RICE
GUPIs	GUPIs	GUPIs	GUPIs
IC-SI/PI	IC-SI/PI	IC-SI/PI	IC-SI/PI
Phys.Exam/ Pelvic Exam/ CystoHx (wk.0)			**Pelvic Exam/ CystoHx (18mo.)**
Med. History/ Inf.Hx. /Fam.Hx/ Hx Antibiotics (wk.0 only)	Hx Antibiotics		Hx Antibiotics
MyMeds/PTHX (wk. 0 only)	MyMEDS/ PTHX	MyMEDS *(monthly)*	MyMEDS/ PTHX
	M/F SEAR		
	FSFI		
	IIEF, UWMSFS		
	Bladder Impact		
BioSpecs	BioSpecs		BioSpecs
	NeuroScans		
	QST Measures		

Data Domains: Non-Urological

Screening/ Run-in Weeks 0-3	Deep Phenotyping / ATLAS Visits	Quarterly	Annually / Semi-annually
BPI/CHOIR (Body Map)	BPI/CHOIR (Body Map)	BPI/CHOIR (Body Map)	BPI/CHOIR (Body Map)
Pain: PainDetect	PainDetect	PainDetect	PainDetect
	McGill Pain		
	Gracely Box Scales		
CMSI checklist & Fibromyalgia module	***NUAS***: CMSI checklist (Incl. CMSI modules)	CMSI checklist & Fibromyalgia module	CMSI checklist & Fibromyalgia module
QOL: WHO-DAS	WHO-DAS, SF12	WHO-DAS	WHO-DAS, SF12
	IPAQ		IPAQ
	WPAI		WPAI
	PANAS		PANAS
Mood: HADS	HADS	HADS	HADS
PROMIS:Fatigue	PROMIS:Fatigue	PROMIS:Fatigue	PROMIS:Fatigue
PROMIS:Sleep	PROMIS:Sleep	PROMIS:Sleep	PROMIS:Sleep
	MASQ		MASQ
Perceived Stress	Perceived Stress	Perceived Stress	Perceived Stress
	Personality: TIPI (Wk. 4 only)		
	Catastrophizing		Catastrophizing
	Trauma: CTES (Wk.0 only) RTES (Wk.0 & Foll.up)		

Health care resource utilization data will be collected at Screening Week 0, Online Run-In contacts, Baseline Week 4, and all clinic and online follow-up contacts, using brief questions related to seeking medical care due to urologic or pelvic pain symptoms in the past 2 weeks.

At the Baseline Week 4 visit a directed medical history will be obtained. Participants will also be asked about family members' medical history. Family members will include first-degree blood relatives only, these include: parents, grandparents, aunts, uncles, siblings, and children. Data will be collected for family members' history of chronic pain disorders and psychiatric disorders on the Family Medical History Questionnaire (FAMHX).

The comprehensive approach to phenotyping implemented in in the Phase I Trans-MAPP EP Study will be continued in the SPS. The data domains of interest and the actual assessment tools that will be used in the SPS are displayed in Figure 13.

Urologic-specific assessments will remain essentially unchanged from the Phase I Trans-MAPP EP Study capturing concepts such as urinary frequency, urgency, pain, bother, aspects of sexual functioning and

quality of life, also adding the 6-item RAND Bladder Symptom Impact scale (RICE BSI-6).[34] The non-urological domains, (i.e., type of pain, co-morbid symptom clusters, QOL, mood, personality and trauma history) have undergone some revision based upon MAPP Phase I data analyses that expand particularly informative domains, and eliminate or streamline other areas found to be less informative to our understanding of UCPPS. Specifically, the qualitative assessment of pain has been expanded to include measures such as the PainDetect[35] and 2011 Fibromyalgia Survey Criteria[36] which have been validated to help differentiate pain from peripheral nociceptive input from neuropathic or centralized pain. The areas of QOL and functional assessment were also expanded to include WPAI[37] and the International Physical Activity Questionnaire-Short Form (IPAQ-SF),[38] so that activities of daily living and work attendance/productivity can be assessed.

5.1.2. UCPPS Symptoms Measures

Pain, urgency, and frequency symptom severity measures will be collected at Screening Week 0, Run-In online contacts, Baseline Week 4, and at all in-clinic and online contacts. Participants will also be queried about flares of their urologic or pelvic pain symptoms.

Standardized urologic measures using the case definition questionnaire from the Rand Interstitial Cystitis Epidemiology (RICE) Study[39], Interstitial Cystitis Symptom Index (ICSI)[40], Interstitial Cystitis Problem Index (ICPI)[40], and the Female Genitourinary Pain Index (FGUPI)[41]/Male Genitourinary Pain Index (MGUPI)[41] will be collected at all in-clinic and online contacts.

Additional urologic measures including the AUA Symptom Index (AUASI)[42], Female Self-Esteem and Relationship (FSEAR) Questionnaire[43], Male Self-Esteem and Relationship (MSEAR) Questionnaire[43], Female Sexual Function Index (FSFI)[44], International Index of Erectile Function (IIEF)[45] and the University of Washington Ejaculatory Function Scale (EFS)[46, 47] will be obtained at the Baseline Week 4 contact and all bi-annual (in-clinic and online) phenotyping contacts.

5.1.3. Non-urological Symptom Measures

Data on non-urological pain, utilizing a body map with numbered regions, will be obtained at each in-clinic visit and online contact, using the Body Pain Index (BPI)[48] tools. Additional pain domain data will be collected using the PAIN *Detect* questionnaire. Mental and physical health data will be collected on the same schedule using the Medical Outcomes Health Survey (SF-12)[33]. Data on sleep and fatigue using the PROMIS scales[1], and perceived stress using the Perceived Stress Scale (PSS)[49] will also be collected at each in-clinic and online contact. A self-reported Complex Medical Symptom Inventory (CMSI) [50] checklist that assesses a broad spectrum of symptoms often found in UCPPS patients will also be administered at all in-clinic and online assessments. At the Baseline Week 4 assessment visit, the CMSI Co-morbid Diagnostic Syndrome Modules will also be administered by the evaluating clinician. These modules utilize syndrome-specific diagnostic symptoms to assess for the probable presence of several syndromes commonly found in UCPPS patients, including FM[51], CFS[52], IBS[53], vulvodynia[54], migraine[55], and temporomandibular joint disorder (TMJD)[56].

Data on anxiety and depression symptoms, using the Hospital Anxiety and Depression Scale (HADS)[57], will be collected. Moreover, at each of the bi-annual phenotyping visits (Baseline Week 4, 6, 12, 18, 24, 30 and 36 months), mood data using the Feelings and Emotions Questionnaire within the PANAS

[1] PROMIS version 1.0 item banks were used. For further information, please refer to www.nihpromis.org

instrument[58], coping data on catastrophizing, using the Catastrophizing scale (CSQ:CAT)[59], and cognition using the Multiple Ability Self-Report Questionnaire (MASQ)[60], will be obtained.

The Fibromyalgia scale (CMSI2-FM2) will be repeated at each in clinic visit at 6, 18, and 36 months.

5.1.4. Trait-like Personal Factors

Data on trait-like personality and trauma history factors will be collected once at the in-clinic Baseline Week 4 phenotyping visit using the Childhood Traumatic Events Scale (CTES).[61]

5.1.5. Assessment of UCPPS Symptom Flares

Characteristics of flares of urologic or pelvic pain symptoms, defined as “symptoms much worse than usual”, will be assessed at each in-clinic visit, as well as at each online quarterly symptom assessment. In addition to reporting whether the participant is currently experiencing a flare, participants will be asked about the frequency and duration of flares in the past reporting period. At the screening visit, each participant will also be asked to compare the intensity of pain and urinary frequency during their waking hours during a typical flare, compared to their usual UCPPS symptoms, as well as the extent to which their typical flare interferes with routine daily responsibilities, pleasurable activities and sleep. Furthermore, at the Screening/Week 0 clinic visit, each participant will also be asked to characterize the details of a flare management plan, if they have one. If a participant reports a current flare at any of the in-clinic visits he/she will be asked to provide an additional “flare” urine samples.

5.1.6. Biological Specimens

Biologic specimens collected during the course of the study include urine, blood, saliva, and rectal and vaginal swabs. The schedule of these collections is summarized in Table 3.

Table **3**: Schedule of Biologic Specimen Collection

<table>
<tr><td rowspan="2">Blood Collection</td><td colspan="2">Blood specimen 10mL ACD plasma vacutainers
(Plasma and Buffy Coat DNA)</td><td colspan="2">4 weeks, 6, 12, 18, 24, 30, 36 months and ATLAS visits</td></tr>
<tr><td colspan="2">STIM TUBES
Blood for stimulated cytokines
3 x 1 mL whole blood in TruCulture tubes pre-loaded with LPS, FSL-1 or Null (no treatment)</td><td colspan="2">Monday, Tuesday, Wednesday, and Thursday clinic visits only
4 weeks, 6, 18, 36 months and ATLAS visits</td></tr>
<tr><td>Biomarker Urine</td><td colspan="2">Spot urine specimen Biomarker/VB2 (up to 90mL)</td><td colspan="2">4 weeks, 6, 12, 18, 24, 30, 36 months and ATLAS Visits</td></tr>
<tr><td>Saliva</td><td colspan="2">Salivary cortisol samples</td><td colspan="2">2 times a day, waking and bedtime
• 3 day period after week 4 visit
• 7 day period after 6 and 18 Month visits
• 7 day period after 36 Month visit IF one or more scheduled collections were missed</td></tr>
<tr><td rowspan="3">Microbiome</td><td>Urine (VB1 ~20 mL, VB2 and VB3[1] ~30-40mL each)</td><td colspan="2">Female:
0 weeks and 18 months: VB1 and VB2
6, 12, 24, 30, 36 months, and ATLAS visits: VB2
At flare during any clinic visit: VB1 and VB2</td><td>Male[1]:
0 weeks and 18 months: VB1, VB2, and VB3
6, 12, 24, 30, 36 months, ATLAS: VB2
At flare during any clinic visit: VB1, VB2, and VB3 (if clinician available to obtain sample)</td></tr>
<tr><td>Rectal Swabs[2]</td><td>Female/Male:</td><td colspan="2">0 weeks, 18 months,</td></tr>
<tr><td>Vaginal Swabs[3]</td><td>Female:</td><td colspan="2">0 weeks, 18 months,</td></tr>
</table>

[1]VB3 Urine collection in males following prostate massage is optional
[2]Collection of rectal swabs is optional at visits that include physical exam
[3]Collection of vaginal swabs is optional at visits that include physical exam
12, 24, 30 months visits are optional.

5.2. Contact Schedule and Participant Procedures

Participants who meet eligibility criteria, and enroll in the study at the Week 0 Screening/Eligibility visit, will be followed up quarterly with internet-based questionnaires, as well as in-clinic visits at 6, 18, and 36 months. At months 12, 24 and 30 participants will have the option of having a clinic visit that includes the completion of online questionnaires, as well as the collection of biospecimens. A complete listing of data elements to be collected at the follow up visits is provided in Appendix 6: MAPP Phase II SPS Visit Schedule.

As illustrated in Figure 14, discrete modules of measures and procedures are scheduled for each in-clinic study visit, as well as quarterly on-line modules of data capture. These include measures that do not change over time, such as demographic information, "trait measures" (e.g., personality), and early life history measures. In general, these measures are collected at the initial in-person Week 0 Screening and Eligibility Confirmation clinic visit, although some are collected in the second in-person Week 4 Baseline clinic visit to reduce participant burden at Screening Week 0.

Figure **14**. Visit Schedule for Screening (Week 0), Run-in (Weeks 1–3), Baseline (Week 4) and Follow-up

	Run-In	Baseline and Follow-up Month												
PROCEDURES	0	1	3	6	9	12	15	18	21	24	27	30	33	36
Eligibility Confirmation Sequence of Procedures	X													
Microbiome/Biomarker Specimen Collection Microbiome ____ M Biomarker ____ B Swabs§ ____ S Cortisol ____ C	M S	B C		M B C		M B		M B S C		M B		M B		M B C^Δ
Clinical Procedures & Data Collection Meds & Tx. Collection ____ M Pelvic/Phys. Exam ____ P CystoHx ____ C	M P C	M		M		M		M P C		M		M		M C
MyMED On-line Module		X	XX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX	XXX
Online Data Collection Screening Visit Surveys ____ S Q-Sym On-line Survey/Flare Status ____ Q Semi/Annual Phenotyping Surveys ____ P Deep Phenotyping Clinic Contacts ____ D	S	Q P D	Q	Q P D	Q ATLAS module →	Q P	Q	Q P D	Q ATLAS module →	Q P	Q	Q P	Q	Q P D
Deep Phenotyping Clinic Procs*		X		X				X						X

§optional rectal/vaginal swabs; specific details in protocol

*Deep Phenotyping Visit: Biospecimen collection, Neuroimaging scans; Expanded questionnaire battery; QST Procs

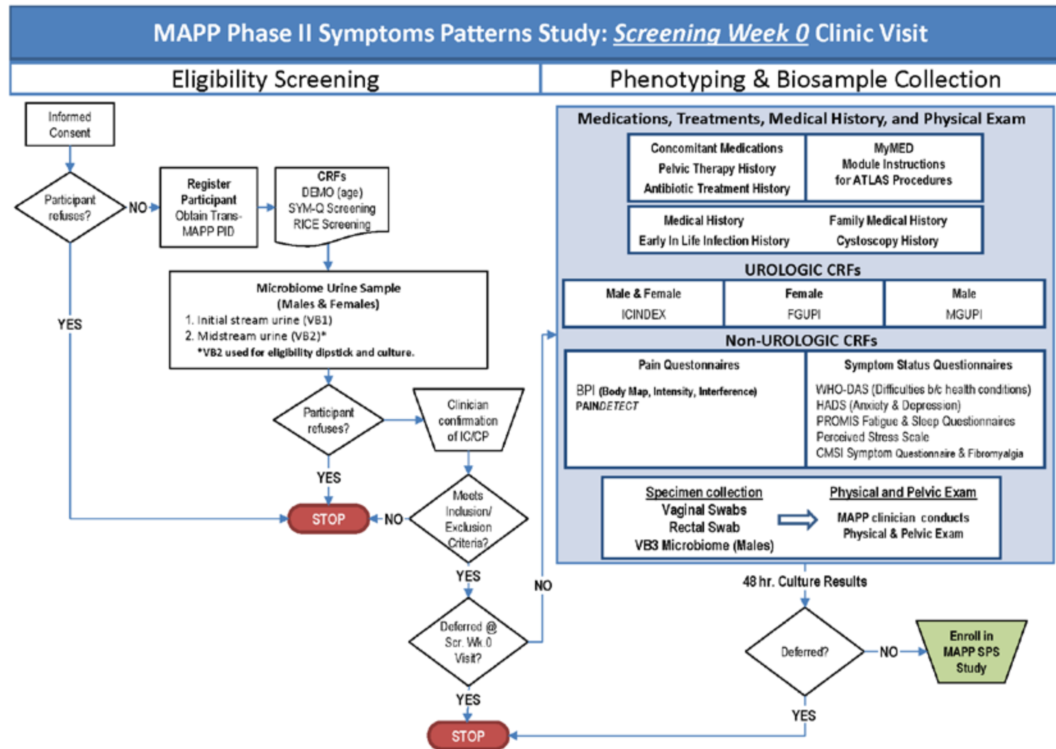
Δ Cortisol collection at Month 36 PRN. Following Month 1 collection cortisol is collected 2 more times as needed.

5.2.1. Screening/Eligibility Visit (Week 0)

Potentially eligible participants will be scheduled for an eligibility screening visit that will include completion of informed consent, eligibility Case Report Forms (CRFs), physical exam, a pelvic exam, collection of a urine sample, microbiome specimen collection, the completion of a minimal subset of CRFs related to the participant's urological symptoms and current medications. The entire screening visit is expected to take approximately 90 minutes to complete. As illustrated in Figure 15, the eligibility screening session (left-hand panel) is intended to collect the minimally sufficient data to confirm

eligibility, so that the extensive baseline phenotyping visit (right-hand panel, Figure 15) and corresponding assessments (biospecimen specimen collection, neuroimaging, and QST) is initiated only for participants highly likely to be confirmed eligible after the minimum 24 hour urine culture results are known.

Figure **15**. Sequence of MAPP II SPS Screening Visit Data, Physical Exam and Biospecimen Collection



At this screening/eligibility visit (week 0), the following sequence of steps will occur:

- Layered informed consent process; consent obtained for various levels of specimen collection (ICF)
- Contact information provided (Site documentation)
- Demographic information recorded (DEMO)
- Symptom assessment via Symptom and Healthcare Utilization Questionnaire (SYM-Q-Screening) – including questions regarding Flares.
- RICE Case Definition Questionnaire for Screening & Eligibility Confirmation (RICE_Screening)
- Eligibility Confirmation form (ELIG2) – to be completed (Inclusion, Exclusion, Deferral Criteria) prior to participant-entered questionnaire battery.
- Neuroimaging Eligibility Assessment (ELIG_SCAN2)
- Urine Culture Result (UCR) – confirm negative urine culture from the microbiome VB2 specimen within 48 hours of the Week 0 screening visit
    - a clean catch midstream urine specimen will be obtained for the urine dipstick test and minimum 24 hour urine culture (see further details in Appendix 2);
  - a urine dipstick test will be performed for the presence of leukocyte esterase, nitrites, and hematuria;

  - If the dipstick is positive for nitrites, the participant will be treated and deferred until urine culture results are available, and deferred for three months from the date of positive urine culture test result.
  - For females of childbearing potential, a pregnancy test is performed.

Assuming that the participant meets all eligibility criteria that can be known prior to the results of the minimum 24 hour urine culture (confirmed on ELIG2 form), the participant will begin the Phenotyping, Physical Exam, and additional microbiome specimen collection (VB3).

- Physical and Pelvic Exam (EXAM2 and PEX): A physical exam and a pelvic exam will be performed by a MAPP physician (or designated trained clinician) to assess pelvic musculature tenderness. While the pelvic exam conducted during the Screening/Eligibility physical exam is required, both the prostate –massage and the VB3 urine sample acquired immediately following the prostate massage (males only), as well as the rectal and vaginal swabs are optional and not required to participate in the study.

- Medical History (MEDHX2)
- Family Medical History (FAMHX)
- Pelvic Therapy History (PTHX)
- Cystoscopy History (CYSTO-2)
- Antibiotic Treatment History (ABHX)
- Concomitant Medications (CMED2) – Documentation of current medications

During the Screening/Eligibility Visit, participants will be asked to complete information regarding their current medications which will be utilized during the course of the study to track medication changes via the online “My Med” module as well as a trigger for initiation of an ATLAS Study visit (see Section 3.7.3).

After a minimum 24 hour negative urine culture result is confirmed, the Enrollment Confirmation (ENROLL) form will be completed, confirming that all Eligibility Criteria have been met.

The participant will complete the following online symptom questionnaires at the screening (week 0) visit:

- MyMED (Ongoing and newly received medications and non-medication treatments)
- Early In Life Infection History (EIL-INF)Interstitial Cystitis Symptom Index (ICSI) and Problem Index (ICPI) form – (ICINDEX)
- Male Genitourinary Pain Index (MGUPI)/ Female Genitourinary Pain Index (FGUPI) utilized in determining eligibility for females.
- Complex Medical Symptoms Inventory (CMSI2_Screening)
- Fibromyalgia symptoms module (CMSI2_FM2)
- Brief Pain Inventory: Body Map, Intensity, Interference (BPI2 Male/Female)
- PAIN Detect (PAIN)
- WHO Disability Assessment Schedule (WHO-DAS)
- Hospital Anxiety and Depression Scale (HADS)
- PROMIS Fatigue
- PROMIS Sleep
- Perceived Stress Scale (PSS)

5.2.2. Run In Visits (Week 1-3)

During the run-in period, study participants will complete a brief online module each week for weeks 1, 2 and 3, assessing symptoms, health care utilization, flare status, and quality of life in a weekly time frame via the following questionnaires:

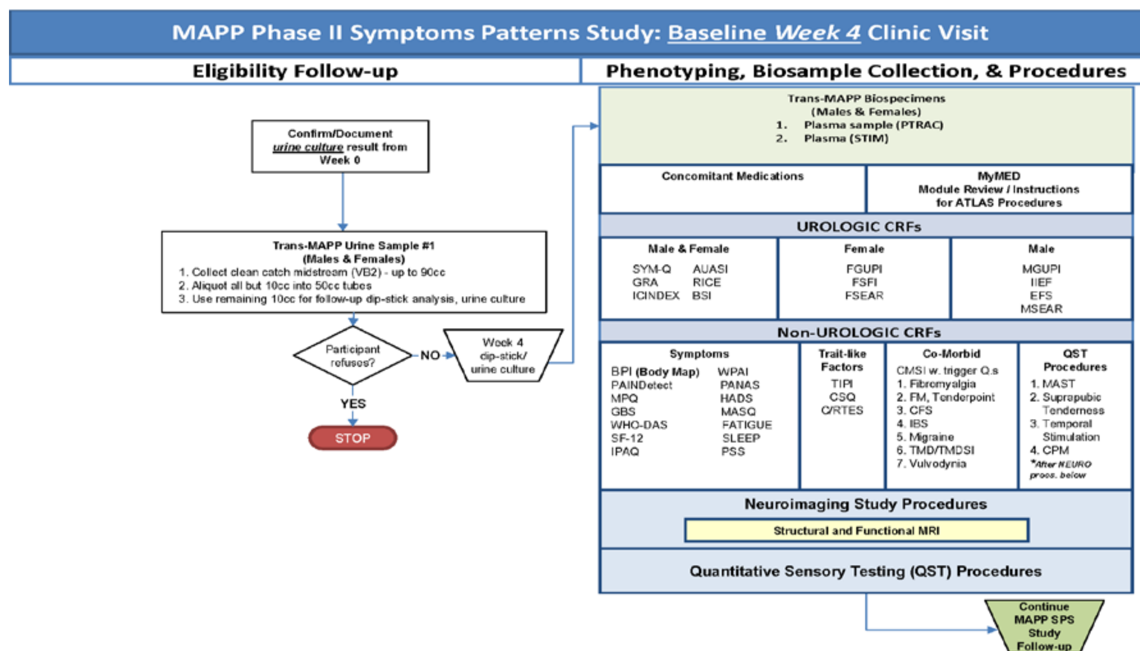
- MyMED (Ongoing and newly received medications and non-medication treatments)
- Symptom, Healthcare Utilization, and Flare Status Questionnaire: (SYM-Q_Run-In)
- Global Response Assessment (GRA)
- Interstitial Cystitis Symptom and Problem Index (ICINDEX Run-In)
- RICE Case Definition Questionnaire (RICE Run-In)
- Female/Male Genitourinary Pain Index (FGUPI/MGUPI)
- Complex Medical Symptoms Inventory (CMSI2_Run-In)
- Fibromyalgia symptoms module (CMSI2_FM2)
- Brief Pain Inventory: Body Map, Intensity, Interference (BPI2 Male/Female)
- PAIN Detect (PAIN)
- WHO Disability Assessment Schedule (WHO-DAS_R.A.)
- Hospital Anxiety and Depression Scale (HADS)
- PROMIS Fatigue
- PROMIS Sleep
- Perceived Stress Scale (PSS_Run-In)

5.2.3. Baseline Visit (Week 4)

At the Baseline (Week 4) visit, the following sequence of steps will occur (Figure 16):

- Eligibility Follow up / Dipstick Urinalysis and Urine Culture Result
  - a clean catch midstream urine (VB2) specimen will be obtained for the urine dipstick test and minimum 24 hour urine culture (see further details in Appendix 2);
  - a urine dipstick test will be performed for the presence of leukocyte esterase, nitrites, and hematuria;

Figure **16**. Sequence of MAPP Phase II Study Baseline Data and Biospecimen Collection and Procedures



- Reconfirm Neuroimaging Eligibility
- Research Coordinator will review with the participant his/her medical history and history of family member diagnosis of IC/BPS and/or CP/CPPS.

- The participant will begin the Deep Phenotyping on-line survey and Biospecimen Sample Collection phase (Figure 16) of the Symptoms Patterns Study.
- Brief clinical diagnostics will also be collected via the **CDX** form including: Height, weight, waist circumference, blood pressure, urine culture results, current flare status/specimen collection, and negative pregnancy test confirmation.

The Deep Phenotyping On-line Survey at the Baseline (Week 4) visit will include the following questionnaires and is expected to take approximately one hour to complete:

- MyMED (Ongoing and newly received medications and non-medication treatments)

Urological Phenotyping Case Report Forms:

- Symptom, Healthcare Utilization, and Flare Status Questionnaire: (SYM-Q_Baseline)
- Interstitial Cystitis Symptom Index (ICSI) and Problem Index (ICPI) form) – (ICINDEX)
- Global Response Assessment (GRA)
- AUA Symptom Index (AUASI)
- Rand Interstitial Cystitis Epidemiology (RICE_Follow-up) Study – IC Case Definition Questionnaire from the RICE study
- RICE Bladder Symptom Impact (BSI)
- Concomitant Medications (CMED2) – Documentation of current medications
- Pelvic Therapy History (PTHX)

Female Participants only:

- Female Genitourinary Pain Index (FGUPI)
- Female Sexual Function Index (FSFI-2)
- Female Self-Esteem and Relationship (FSEAR) Questionnaire

Male Participants only:

- Male Genitourinary Pain Index (MGUPI)
- International Index of Erectile Function (IIEF)
- University of Washington Ejaculatory Function Scale (EFS)
- Male Self-Esteem and Relationship (MSEAR) Questionnaire

As shown in Figure 16 (right-hand panel), all SPS participants will be providing biospecimens, including urine and blood (plasma)

Participants will also be presented with an online log (My-Med) of the medications that they reported taking at the previous visit and will complete online assessments to inform the study if they've stopped taking any of these initial medications or if they've started taking a new medication and if so, the name of the new medication.

Biospecimen Exam CRFs:

- Blood specimen 10mL ACD plasma vacutainers (Plasma and Buffy Coat DNA)
- STIM TUBES – Three 1 mL blood samples for stimulated cytokines
- Spot urine specimen Biomarker/VB2 (up to 90ml)

Non-Urological Phenotyping CRFs:

Symptoms and Illness Impact	• PAIN *Detect* (PAIN) • McGill Pain Questionnaire (MPQ) • Gracely Box Scales (GBS) • Brief Pain Inventory (BPI2) + revised body map • WHO Disability Assessment Schedule (WHO-DAS) • Medical Outcomes Health Survey – (SF-12) • International Physical Activity Questionnaire (IPAQ) • Work Productivity & Activity Impairment Questionnaire (WPAI) • Feelings and Emotions Questionnaire (PANAS) • Hospital Anxiety and Depression Scale (HADS) • Fatigue, and Sleep Questionnaires (PROMIS) • Multiple Ability Self-Report Questionnaire (MASQ) • Perceived Stress Scale (PSS)
Traits and Early Life Experience	• Ten Item Personality Inventory (TIPI) • Catastrophizing Sub-scale (CSQ:CAT) • Childhood Traumatic Events Scale; Recent Traumatic Events Scale (C/RTES)
Co-morbid Symptoms	• Complex Medical Symptoms Inventory Questionnaire (CMSI2_Baseline) • CMSI, Fibromyalgia (CMSI2_FM2) • CMSI, Chronic Fatigue Syndrome (CMSI2_CFS2) • CMSI, Irritable Bowel Syndrome (CMSI2_IBS2) • CMSI, Migraine (CMSI2_MI2) • CMSI, Vulvodynia (CMSI2_VDYN) • CMSI, Current TMD Symptoms (CMSI2_TMD2) • Gonzalez TMJD Questionnaire (TMDSI)

During the baseline visit participants will undergo an initial series of Quantitative Sensory Tests (QST) as well as neuroimaging scans(participants that are eligible for a neuroimaging scan).

At the conclusion of the visit participants will be provided with collection and shipping materials for the 3 day post baseline visit Cortisol collection.

5.2.4. Monthly Internet Medication Questionnaires

Throughout the course of the 36-month study, starting at the Week 0 (Screening/Eligibility) visit, participants will provide information regarding current medications and targeted non-medication treatments. This information will be utilized to track medication and non-medication treatment changes. After the Screening/Eligibility visit, participants will provide information about medication and non-medication treatment changes via internet questionnaires, each month. Participants will be presented with an online log of the medications that they reported taking at the previous clinic visit and asked to complete online questionnaires to inform the study if they have stopped taking any of the initial medications or started taking a new medication and if so, the system will prompt the participant to enter the name of the new medication.

5.2.5. Quarterly Assessments

Participants will provide self-reported longitudinal symptom data via web-based internet tools. The following questionnaires will be completed at each quarterly assessment (CRF names provided in parentheses):

- (Ongoing and newly received medications and non-medication treatments)

Urologic Questionnaires
- Symptom and Health Care Utilization Questionnaire (SYM-Q_Follow-up)
- Global Response Assessment (GRA)
- Interstitial Cystitis Symptom Index and Problem Index (ICINDEX)
- RICE Case Definition Questionnaire (RICE_Follow-up)

Female Participants only:
- Female Genitourinary Pain Index (FGUPI)

Male Participants only:
- Male Genitourinary Pain Index (MGUPI)

Non-Urologic Questionnaires:
- Complex Medical Symptoms Inventory (CMSI2_Follow-up)
- Fibromyalgia symptoms module (CMSI2_FM2)
- Brief Pain Inventory: Body Map, Intensity, Interference (BPI)
- Pain Detect (PAIN)
- WHO Disability Assessment Schedule (WHO-DAS)
- Hospital Anxiety and Depression Scale (HADS)
- PROMIS Fatigue (FATIGUE)
- PROMIS Sleep (SLEEP)
- Perceived Stress Scale (PSS)

5.2.6. Deep Phenotyping In-clinic Visits at 6, 18 and 36 Months

Participants will come into the clinic at months 6, 18, and 36. During these visits biospecimens will be collected and the participant will have QST and Neuroimaging assessments (if eligible for the neuroscan). Participants will also be asked to take part in the (M)APP protocol at each visit.

A brief set of clinical and diagnostic procedures and forms will also be administered at months 6, 18, and 36 including:

- **CDX** form procedures: Height, weight, waist circumference, blood pressure, urine culture results, current flare status/specimen collection, and negative pregnancy test confirmation.
- Pelvic Therapy History (PTHX)
- Antibiotic Treatment History (ABHX)
- Concomitant Medications (CMED2) – Documentation of current medications

In addition, a more extensive set of questionnaires is administered at these visits, as compared to the limited subset administered for the quarterly non-clinic visits. This assessment will also be administered via the internet.

This set of questionnaires includes all of the CRFs from the quarterly assessments, plus the additional CRFs listed subsequently:

Urological Phenotyping CRFs:
- AUA Symptom Index (AUASI)
- RICE Bladder Symptom Impact (BSI)

Female Participants only:
- Female Sexual Function Index (FSFI-2)
- Female Self-Esteem and Relationship Questionnaire (FSEAR)

Male Participants only:
- International Index of Erectile Function (IIEF)
- University of Washington Ejaculatory Function Scale (EFS)
- Male Self-Esteem and Relationship Questionnaire (MSEAR)

Non-Urological Phenotyping CRFs:

- Complex Medical Symptoms Inventory (CMSI2_Follow-up)
- Fibromyalgia symptoms module (CMSI2_FM2)
- Brief Pain Inventory (BPI2) plus Revised Body Map
- PAIN Detect (PAIN)
- McGill Pain Questionnaire (MPQ)
- Gracely Box Scales (GBS)
- WHO Disability Assessment Schedule (WHO-DAS)
- Medical Outcomes Health Survey – (SF-12)
- International Physical Activity Questionnaire (IPAQ)
- Work Productivity & Activity Impairment Questionnaire (WPAI)
- Feelings and Emotions Questionnaire (PANAS)
- Hospital Anxiety and Depression Scale (HADS)
- Anger, Fatigue, and Sleep Questionnaires (PROMIS)
- Multiple Ability Self-Report Questionnaire (MASQ)
- Perceived Stress Scale (PSS)
- Catastrophizing Sub-scale (CSQ)
- Recent Traumatic Events (RTES)

At the conclusion of the 6 and 18 Month visits, participants will be provided with collection and shipping materials for the 7 day Cortisol collection. If, at the 36 Month visit, one or both of the previous scheduled collections were missed, provide participant with the collection and shipping materials for the 7 day cortisol collection.

If participant agreed to take part in the (M)APP protocol, provide the participant with information regarding initiation of the MAPP phone application.

5.2.7. Optional Clinic Visits at 12, 24 and 30 Months

Participants will complete a series of online questionnaires, as well as have the option of including an in-clinic visit at months 12, 24, and 30 months, primarily for biospecimen collection. This set of questionnaires includes all of the CRFs from the quarterly assessments and also the PTHX, plus the additional CRFs listed subsequently:

Non-Urological Phenotyping CRFs:

- Medical Outcomes Health Survey – (SF-12)
- International Physical Activity Questionnaire (IPAQ)
- Work Productivity & Activity Impairment Questionnaire (WPAI)
- Feelings and Emotions Questionnaire (PANAS)
- Multiple Ability Self-Report Questionnaire (MASQ)
- Catastrophizing Sub-scale (CSQ)

5.2.8. Internet Site Design and Operation

The Trans-MAPP internet site will be the primary data collection tool for longitudinal assessment for the online surveys. A number of steps will be taken to assist in participant tracking and retention:

- The site will be designed to be attractive, engaging, and as user-friendly as possible
- Places to contact, via phone or email, for help for site operation or anything else regarding the study will be clear on every page

- If a participant misses a data collection time-point, the primary enrollment site will be notified by the DCC to follow-up with the participant via email, phone, or mail
- Reminders for upcoming assessments will be emailed from the Discovery Sites to each participant, with easy link to participant's own data collection page. Two weekly follow-up emails will be sent automatically after a missed contact
- Repeated attempts will be made by the Discovery Sites to contact difficult-to-reach participants, and at each time-point, participants will be asked for their most recent phone number, email and physical address

6. STATISTICAL CONSIDERATIONS AND ANALYTIC PLANS

6.1. Sample Size/Power Considerations for the Symptom Patterns Study

Although a broad set of symptoms, including urological, non-urological and biopsychosocial measures will be evaluated in the SPS, a set of simplifying assumptions are necessary in order to provide sample size estimates. Without loss of generality, suppose that each UCPPS participant in the SPS is classified as either an "Improver" or a "Not Improver", for a primary outcome measure, such as pain severity or urinary severity (or a composite of UCPPS symptoms), using a within-person slope criteria from a mixed effects model described subsequently. Now suppose that Pr{Improver|Factor=Absent} is the underlying probability of being classified as an "Improver" among the subgroup for which a selected risk factor is absent. For example, UCPPS participants in MAPP Phase I follow-up, whether male or female, who were classified as BPS=No for urologic subtype, were less likely to be classified as an "Improver" than those classified as BPS=Yes. Under the assumption that the likelihood of being classified as an "Improver" is higher for the subgroup for which the risk factor is present, the required sample sizes displayed in Table 4 were derived under a hypothesized risk ratio = 2.00 (column 1) and risk factor prevalence projections (column 2), assuming 90% power to detect the specified differences with a 5% type I error rate.

Table **4**: Sample Size Requirements for Detecting Specified Risk Ratios Under Selected Study Design Characteristics: Type I Error Rate of 5%, Power of 90%

		Sample Sizes for Risk Factor Absent (n_1) : Present (n_2) = Total		
Underlying Risk Ratio (RR)	Percent of Sample (n_1:n_2)	Pr{Improver|Factor} (0.10 vs. 0.20)	Pr{Improver|Factor} (0.15 vs. 0.30)	Pr{Improver|Factor} (0.20 vs. 0.40)
RR = 2.00	10:90	152 : 1,368 = **1,520**	91 : 819 = **910**	61 : 549 = **610**
	20:80	171 : 684 = **855**	103 : 412 = **515**	69 : 276 = **345**
	30:70	194 : 453 = **647**	117 : 273 = **390**	79 : 185 = **264**
	40:60	224 : 336 = **560**	136 : 204 = **340**	91 : 137 = **228**
	50:50	266 : 266 = **532**	161 : 161 = **322**	109 : 109 = **218**

In particular, suppose that the underlying probability of being classified as an "Improver" is 15% among the subgroup for which a selected risk factor is absent, as indicated in Table 4 (column 4), and the probability of being classified as an "Improver" is 30% among the subgroup for which the selected risk factor is present, corresponding to a risk ratio of 2.0 (0.30/0.15). This configuration of 15% vs 30% is directly analogous to the differential in proportion of "Improvers" for the males with BPS=No vs BPS=Yes Then it can be noted in Table 4 that a total sample size of 322 would provide 90% power to detect this 2-fold increase, provided that the prevalence of the risk factor is 50%. If on the other hand, the prevalence of the risk factor present is 80% (20:80 in row 2, column 2), the required sample size to detect this same effect size (RR=2.00) is increased to 515.

6.2. General Statistical Methods

The DCC will continue to provide biostatistical design expertise and will lead targeted data analyses in support of the MAPP Phase II SPS, as well as multiple related studies.

6.2.1. Baseline Statistical Analyses:

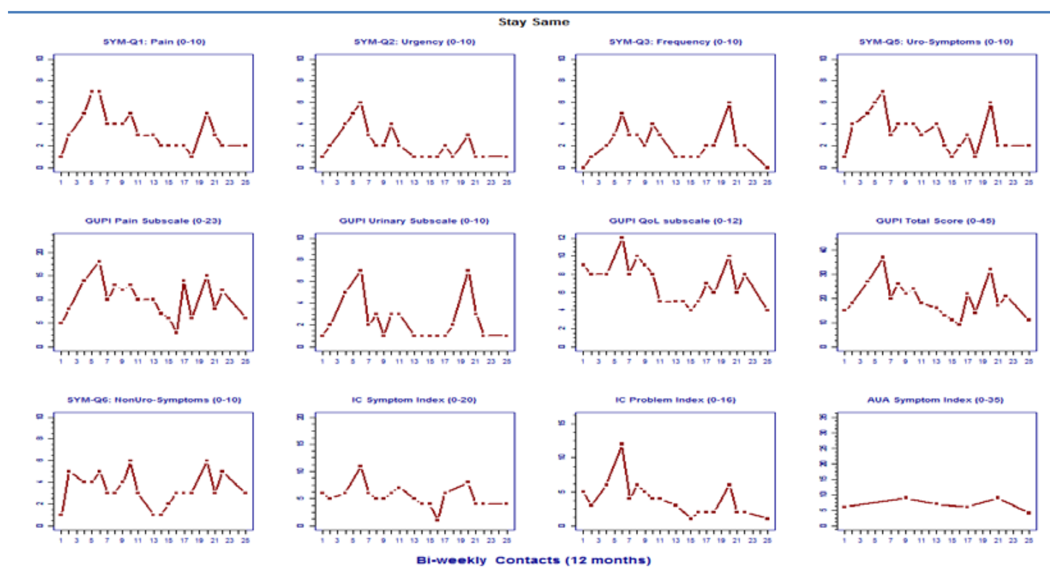
Before proceeding with analyses, all relevant measures are fully described, including aspects of data quality. For both predictor variables and outcomes, a summary of each variable, or group of variables, is produced. Sample means and standard deviations are reported for continuous variables; relative frequencies are calculated for categorical variables. Graphical methods are used extensively to identify potential outliers and to examine assumptions (such as normality) underlying statistical models. Hypothesis testing is conducted using a two-sided significance level (type I error) of $\alpha=0.05$, with actual p-values reported whenever possible. Continuous variables are compared using the two-sample t-test; whereas, Fisher's exact test is used to compare dichotomous variables. Ordinal variables are assessed using the Wilcoxon rank-sum test. Multivariable linear and logistic regression models are used to adjust for potential confounding variables in comparisons of continuous and binary measures, respectively. Analyses are performed using SAS 9.4.

6.3. Additional Analytical Considerations Specific to the Aims

6.3.1. Primary Outcomes for UCPPS Symptoms

Multiple self-reported symptoms from urological, non-urological and psychosocial domains, measured either bi-weekly or bi-monthly during MAPP Phase I, were available to characterize and validate longitudinal change, as illustrated in Figure 17 for a representative participant. This series of 12 symptoms, arrayed in a panel of 3 x 4 individual graphs, are organized according to 4 of the SYM-Q measures (pain, urgency, frequency, overall pelvic symptoms) in the top row, the 4 GUPI scales (pain, urinary, QoL, total) in the middle row, and the SYM-Q6 (non-urological pain), IC-SI, IC-PI and AUA scores in the bottom row. Early analytical investigations focused on the potential to create composite outcomes that would characterize an "improver" as patients meeting selected "change criteria" on the urological symptoms, while not worsening on the non-urological symptoms (SYM-Q6).

Figure **17**. Representative Participant PID #100677: Female, BPS:Yes, Baseline SYM-Q1:Mild (0-3)

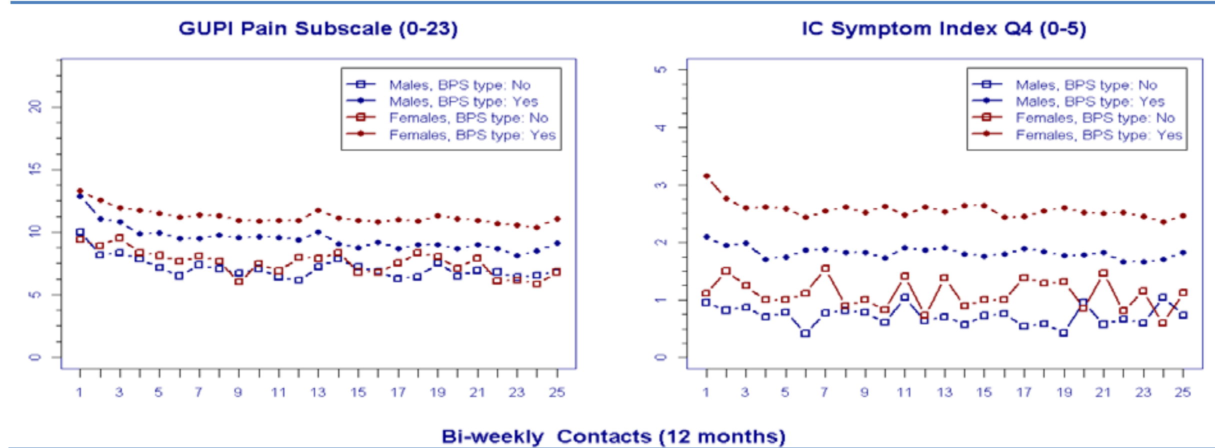


However, selection of primary outcome(s) among these multidimensional UCPPS conditions poses many challenges, especially in the search for relevant subgroups with prognostically different longitudinal symptom patterns, which undoubtedly differ for various symptom domains. As a result, dimension reduction methods of Principal Components Analysis (PCA) and Factor Analytic methods were applied to a large item bank of urological pain, frequency and urgency measures from MAPP Phase I participants at baseline, to investigate the minimal number of factors, and specific item composition, that would capture a large portion of the total variability among these multiple measures. In particular, two primary UCPPS outcome variables were constructed, as follows:

1) **Pain Severity Score**, constructed from the GUPI pain subscale score (0-23) and ICSI Q4: Bladder pain score (0-5), summed to create a primary outcome for UCPPS pain, ranging from 0 to 28.

As illustrated in Figure 18, the longitudinal profile for each of these pain subscales is more severe for females than males, within each BPS subtype (No, Yes).

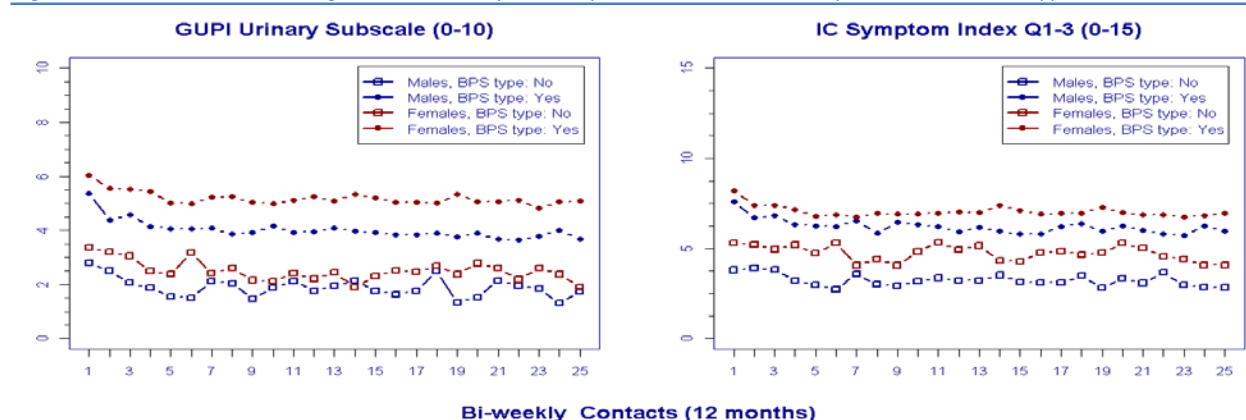
Figure **18**. Distribution of Longitudinal Pain Severity Score Sub-domains by Sex and BPS Subtypes



2) **Urinary Severity Score**, constructed from the GUPI urinary subscale score (0-10) and sum of ICSI Q1: Strong need to urinate (0-5), ICSI Q2: Frequency (0-5), and ICSI Q3: Frequency at night (0-5), summed to create a primary outcome for urinary symptoms, ranging from 0 to 25.

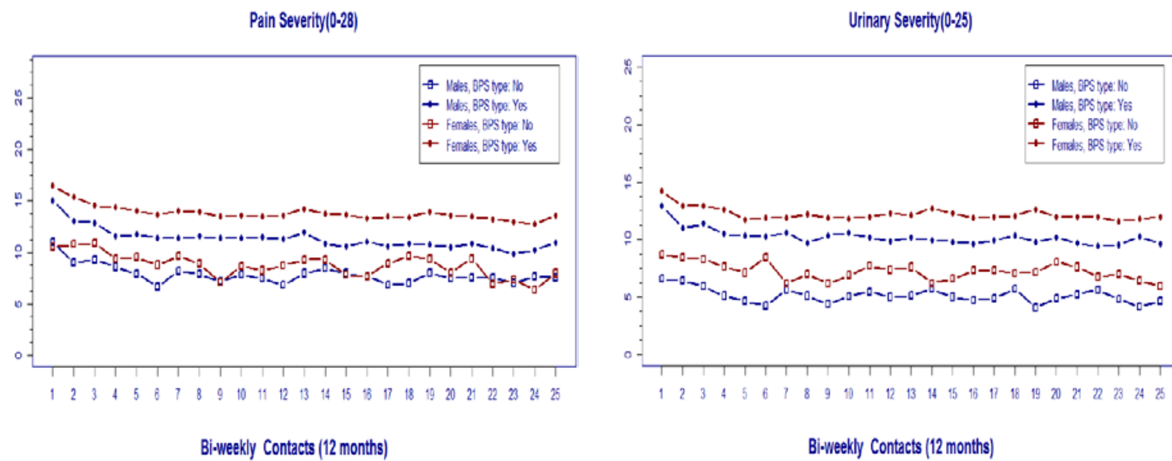
Similarly, as illustrated in Figure 19, the longitudinal profile for each of these urinary severity subscales is more severe for females than males, within each BPS subtype (No, Yes).

Figure **19**. Distribution of Longitudinal Urinary Severity Score Sub-domains by Sex and BPS Subtypes



Extensive analyses of these UCPPS symptom data, summarized in terms of the two primary outcomes: 1) Pain Severity Score and 2) Urinary Severity Score, using the longitudinal data from MAPP Phase I, as displayed in Figure 20, are well underway, focusing primarily on adjustments for "regression to the mean", examining the role of variability in predicting symptom levels at 48 weeks, and investigating the predictive role of UCPPS subtypes, body map pain region location and selected baseline risk factors in predicting longitudinal change in individual symptoms.

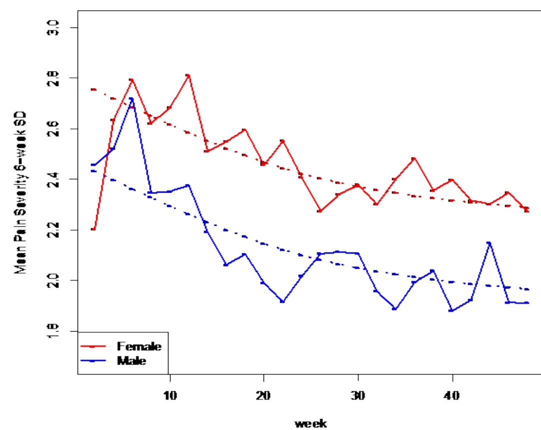
Figure **20**. Longitudinal Distribution of Pain Severity and Urinary Severity Scores by Sex and BPS Subtypes



6.3.2. Characterizing Longitudinal Variability:

Three measures of within-patient variability were derived, and evaluated, for correlation with demographic and clinical characteristics for each symptom: 1) variability over a hypothetical run-in period (weeks 0-4); 2) total standard deviation over 1 year (about the within-patient mean over time); and 3) time-varying 6-week standard deviation. The "run-in period" variability was measured by the within-patient standard deviation among the 3 measures at baseline, weeks 2 and 4. Secondly, the total longitudinal variability (excluding baseline and week 2) was estimated by the within-patient standard deviation (about the within-patient mean) across all of the patients' outcomes from weeks 4 to 48.

Figure **21**. Longitudinal Six-week Pain Severity Standard Deviation by Sex in UCPPS Patients.



The third type of variability was time-varying such that each participant had a unique value at each longitudinal visit. Six-week symptom standard deviation was defined within a moving window of 6 weeks. At week two, this was the standard deviation of symptoms at weeks 0 and 2, at week 4 symptoms in weeks 0, 2, and 4 contributed, and for weeks 6 and beyond the standard deviation was calculated using symptoms at that visit and the 3 prior visits. Time-varying pain severity variability is shown above by sex in UCPPS patients in Figure 21. Women reported more variable pain symptoms in men. In both sexes, pain variability decreased over the first six months of the study before stabilizing.

6.3.3. Variability as a Predictor of Symptom Outcomes:

Within-patient variability was also investigated as a predictor of subsequent health care utilization. In longitudinal analysis, 6-week pain and urinary severity standard deviation were used to predict whether participants engaged in several health care utilization behaviors, where health care utilization was assessed according to the same biweekly schedule as symptoms. Variability in both pain and urinary symptoms was evaluated as a potential predictor of utilization. Differences in the standard deviation of urinary symptoms were predictive of several health care utilization behaviors, including visiting a provider and having a medication changed (Table 5).

A second analysis considered variability over the “run-in period” (weeks 0-4) as a predictor of “Improving” or “Worsening”. For males, increased run-in variability was associated with a greater likelihood of improving on the Quality of Life GUPI subscore (OR 1.88, p=0.002). Increased run-in variability on SYMQ-1 was associated with significantly higher likelihood of worsening (OR=2.64, p=0.004); whereas larger run-in variability on GUPI Urinary was associated with a lower probability of worsening (OR=0.29, p=0.027). For females, no association was detected between run-in variability and longitudinal change for any symptom.

Table **5**: Variability Across Weeks 4-46 as Predictor of Symptom Severity at Week 48

	Overall (males and females combined)						
Symptom	Adjusted Beta	CI	p	Symptom	Adjusted Beta	CI	p
SYMQ-1	-0.25	(-0.63, 0.12)	0.185	GUPI Pain	-0.63	(-1.02, -0.23)	**0.002**
SYMQ-2	0.26	(-0.12, 0.63)	0.178	GUPI Urinary	-0.6	(-1.00, -0.21)	**0.003**
SYMQ-3	0.22	(-0.17, 0.6)	0.272	GUPI QoL	-0.76	(-1.09, -0.43)	**<0.001**
SYMQ-5	-0.25	(-0.62, 0.11)	0.177	GUPI Total	-0.69	(-1.08, -0.29)	**0.001**
SYMQ-6	0.26	(-0.07, 0.59)	0.118				
ICSI Total	-0.39	(-0.81, 0.03)	0.072				
ICPI Total	-0.64	(-1.07, -0.21)	**0.004**				

6.3.4. Longitudinal Data Analysis of UCPPS Symptom Patterns

In order to fully capitalize on the rich data structure underlying these multiple UCPPS symptom outcomes, such as the pain severity and urinary severity scores illustrated in Figure 20, we outline here a comprehensive analytical strategy for investigating longitudinal symptom patterns.

6.3.4.1 Univariate Longitudinal Data Analysis

We first model each UCPPS symptom trajectory over time separately by linear mixed effects models[62] and functional mixed effects models[63] depending on the complexity of the trajectories. Through this approach, we can also test whether the baseline covariates predict the subsequent symptom trajectories, such as sex, pelvic pain localization status, BPS subtype, and baseline severity.

Suppose that y_{ijl} $(i = 1 \cdots n,\ j = 1 \cdots m_i, l = 1 \cdots L)$ is the lth symptom of the ith subject measured at time t_{ijl} , and X_{il} denote the baseline covariates that are related to the lth outcome. The longitudinal mixed effects model takes the following form:

$$y_{ijl} = X_{il}\,\boldsymbol{\beta}_l(t_{ijl}) + Z_{il}\boldsymbol{\alpha}_{il}(t_{ijl}) + e_{ijl} \qquad (1)$$

where $\boldsymbol{\beta}_l\,(t) = \{\beta_{l1}\,(t), \cdots, \beta_{lp}\,(t)\}^T$ is a $p \times 1$ vector of fixed functions; $\boldsymbol{\alpha}_{il}\,(t) = \{\alpha_{1il}\,(t), \cdots, \alpha_{qil}\,(t)\}^T$ is a $q \times 1$ vector of random functions; $X_{il} = \{X_{il}[1], \cdots, X_{il}[p]\}(1 \times p)$ and

$Z_{il} = \{Z_{il}[1], \cdots, Z_{il}[q]\}(1 \times q)$ are design matrices that can include dummy variables as well as covariates; and e_{ijl} are measurement errors that can be correlated. We will first use polynomial functions (such as straight lines or quadratic curves) to model both the fixed functions $\boldsymbol{\beta}_l\ (t)$ and random functions $\boldsymbol{\alpha}_{il}(t)$. For example, when assuming the trajectories are linear and we only include one covariate x_{il}, such as the baseline severity score, the model is simplified to:

$$y_{ijl} = a_{0l} + \alpha_{1l}\, t_{ijl} + b_{0l}\, x_{il} + b_{1l}\, x_{il} * t_{ijl} + \alpha_{0il} + \alpha_{1il} t_{ijl} + e_{ijl} \qquad (2)$$

where $a_{0l} + \alpha_{1l}\, t_{ijl}$ is the overall mean trajectory over time, $b_{0l}\, x_{il} + b_{1l}\, x_{il} * t_{ijl}$ is the deviation from the mean trajectory due to x_{il}, and the random effect $\alpha_{0il} + \alpha_{1il} t_{ijl}$ accounts for the subject-specific deviations from the mean profile. The test of the covariate effect of x_{il} is the same as testing both $b_{0l} = 0$ and $b_{1l} = 0$, that can be tested by likelihood ratio test or approximate Wald test. The extension to higher order polynomials and multiple covariates are straightforward. If we discover that the trajectories are complex and difficult to be modeled as polynomials, we will use the nonparametric approach of Guo (2002)[63] in which both the fixed effects and random effects are modeled by smoothing splines.

6.3.4.2 Multivariate Mixed Effects Models

Despite the extensive array of longitudinal symptoms, this univariate approach models only one outcome at a time. However, as illustrated previously for a representative participant in Figure 17, these symptom patterns are strikingly similar across different symptoms within participant, giving rise to logical questions, such as: 1) whether a covariate has the same effect on different symptoms; and 2) whether all the symptoms of a specific participant tend to track within the same pattern over time.

Our second analytic approach is to jointly model the multiple longitudinal symptoms through multivariate mixed effects models.[64] The basic idea is to assume that the different longitudinal outcomes, such as 1) pain severity score and 2) urinary severity score, are independent, conditional on the joint random effects. The model takes a similar form as model (1), except that the random effects are assumed to have a joint distribution such as $\{\boldsymbol{\alpha}_{i1}(t)^T, \cdots, \boldsymbol{\alpha}_{iL}\ (t)^T\}^T \sim N(0,\ \sum(t))$. This multivariate model can be turned into a univariate linear mixed effects model by stacking the L symptom measures within each subject as L observations, and fitted using SAS Proc Mixed.[65] We can test whether different symptoms have similar overall mean trajectories by constraining the fixed effects in the multivariate model, such as $\boldsymbol{\beta}_l\ (t) = \boldsymbol{\beta}_{l'}\ (t)$, which means that the covariates have the same effect on the lth and l' th outcomes. We will use likelihood ratio test for these hypotheses. We can also compute the subject level correlation over time between the lth outcome and l'th outcome as

$$\rho_{i,l,l'}(t) = \frac{Z_{il} \sum_{l,l'}(t) Z_{il'}^T}{\sqrt{Z_{il} \sum_{l,l}(t) Z_{il}^T Z_{il'} \sum_{l',l'}(t) Z_{il'}^T}}$$

where $\sum_{l,l'}(t),\ \sum_{l,l}(t),\ and\ \sum_{l',l'}(t)$ are the corresponding blocks of the covariance functions. This can tell us the time-varying relationships among different symptoms within a participant.

6.3.4.3 A Longitudinal Latent Variable Model

As we can also observe in the plots of the multiple symptoms for a representative participant in Figure 17, a specific patient tends to display a very similar pattern over time. It is therefore natural to assume

that different symptoms may well be expressions of the same underlying disease progression. Our goal is to define the latent progression trajectory, and to explore whether its pattern can be predicted by baseline covariates. We will adapt the latent variable approach of Roy and Lin (2000)[66] into the following functional setting:

$$y_{ijl} = \mu_l + \gamma_l \, \eta(t_{ij}) + b_{il} + e_{ijl}$$

$$\eta(t_{ij}) = X_i \boldsymbol{\beta}(t_{ij}) + Z_i \boldsymbol{\alpha}_i(t_{ij}) + \epsilon_{ij} , \qquad (3)$$

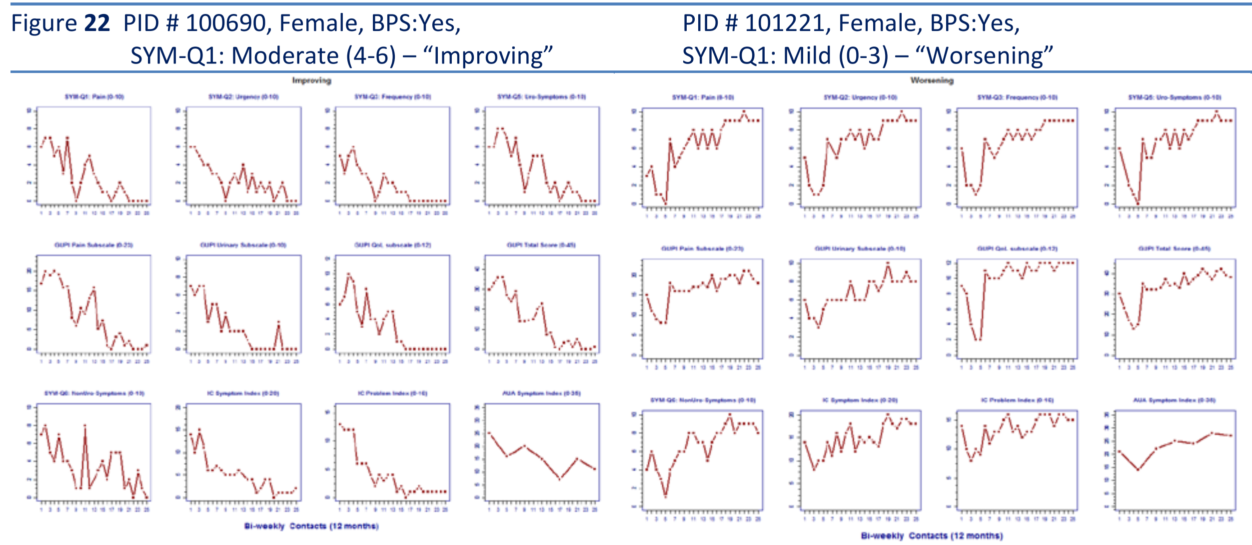
where $\eta(t)$ is the common latent trajectory over time measuring the symptom progression, γ_l is the corresponding loading factor for the lth outcome, μ_l is the mean of the lth outcome and b_{il} are the random intercepts, and we assume b_{il} are mutually independent. In the second model, $\boldsymbol{\beta}(t_{ij})$ are the functional fixed effects and $\boldsymbol{\alpha}_i(t_{ij})$ are the functional random effects. The second model takes the same form of the functional mixed effects model as in (1) except $\eta(t_{ij})$ is an unobserved latent variable. Similar to Roy and Lin (2000),[66] we will use an EM algorithm for estimation. We can then test the covariate effects through the functional fixed effects $\boldsymbol{\beta}(t)$.

The inference procedure and interpretations are essentially the same as those in the univariate longitudinal model, except that the covariates impact all the outcomes simultaneously through the common latent variable. Similar to the aforementioned univariate longitudinal approach, we will first use polynomial functions to model the trajectories, and then explore nonparametric approaches using smoothing splines.

6.3.4.4 A Functional Clustering Algorithm

More than 50% of the UCPPS participants in MAPP Phase I exhibit longitudinal symptom patterns that vary considerably, but relative to either “Improving” or “Worsening”, remain essentially stable or “No Change”, as illustrated in Figure 17. However, as shown in Figure 22, there are UCPPS participants who exhibit symptom patterns that are either “Improving” (left panel) or “Worsening” (right panel), for whom we would like to characterize their symptom patterns, and importantly, determine baseline risk factors are predictive of these differential symptoms over time.

Figure **22** PID # 100690, Female, BPS:Yes, SYM-Q1: Moderate (4-6) – “Improving” PID # 101221, Female, BPS:Yes, SYM-Q1: Mild (0-3) – “Worsening”



However, depending on which primary outcome is considered, nearly 25% of participants demonstrate patterns of "Improving", but there are many more varied symptom patterns, as a participant's symptoms can fluctuate differentially over time. The latent variable approach allows us to test whether a baseline covariate has a predictive effect on a pre-specified pattern in terms of the symptom progression.

Accordingly, we propose a functional clustering approach, based on the previously defined latent class trajectory, to detect uniquely different symptom progression patterns, and allowing the data to suggest the different patterns. We will extend the K-centers functional clustering approach of Chiou and Li (2007)[67] to include multiple longitudinal outcomes. This is very similar to the K-mean clustering algorithm. The outline of the algorithm follows.

Given an initial choice of K, and initial classification where we assume that $y_{ijl}^{(k)}$ belongs to the kth cluster, we can define the following multiple latent variables model;

$$y_{ijl}^{(k)} = \mu_l + \gamma_l \, \eta^{(k)}(t_{ij}) + b_{il} + e_{ijl}, \qquad (4)$$

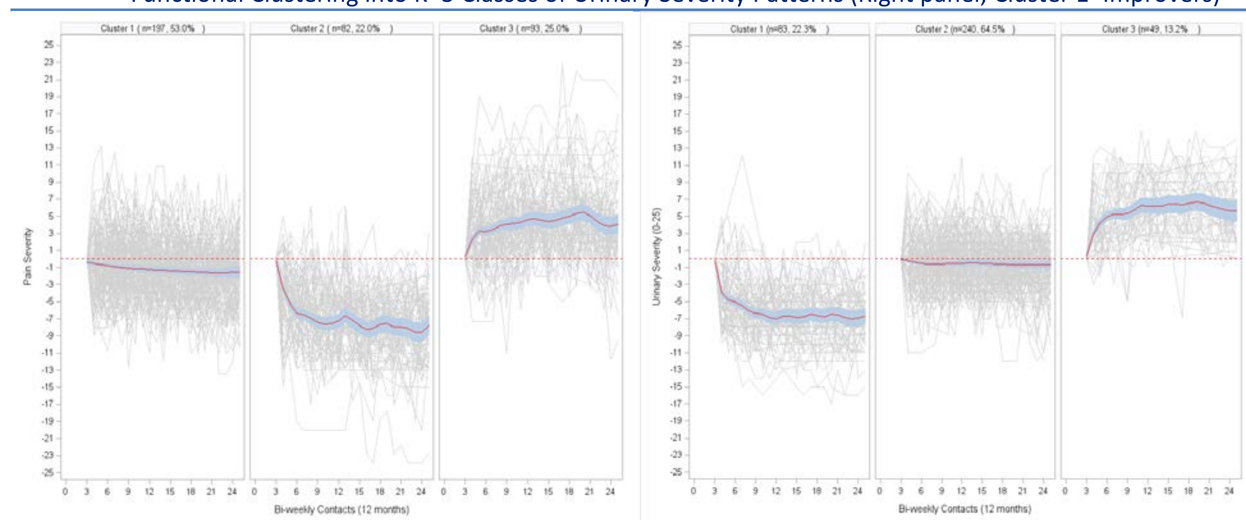
$$\eta^{(k)}(t_{ij}) = \beta^{(k)}(t_{ij}) + \varepsilon_{ij}^{*} \qquad (5)$$

where $\eta^{(k)}(t)$ is the latent trajectory for the kth cluster whose mean is $\beta^{(k)}(t)$ and the potentially correlated measure errors are ε_{ij}^{*} . We then model $\beta^{(k)}(t)$ through polynomials or smoothing splines. The model can be fitted using the similar approach as the previously proposed latent variable model.

We then leave out the ith subject, refit the K-cluster latent variable model. We calculate the l_2 distance of the ith subject to the estimated mean of kth cluster $\hat{\beta}^{(k)}(t)$. We then assign the ith subject to the nearest cluster. We repeat leaving out the ith subject, in sequence for each subject, and continue until till the assignments do not change. Finally, we vary K, and repeat whole process using a cross-validation criterion to select the optimal K.

This functional clustering algorithm was applied to each of the two primary outcomes for the subset of MAPP Phase I UCPPS participants with longitudinal symptom data measured at least through the 6-month visit.

Figure **23**. Functional Clustering into K=3 Classes of Pain Severity Patterns (Left panel; Cluster 2=Improvers) and Functional Clustering into K=3 Classes of Urinary Severity Patterns (Right panel; Cluster 1=Improvers)



This functional clustering approach allows us to objectively define the underlying symptom patterns separately for each primary outcome, and to select the optimal K. Once these patterns are defined, nominal logistic regression can be used to predict membership in these symptom pattern clusters using the baseline covariates. The initial values can be obtained by running a multivariate cluster algorithm based on the estimated subject-specific coefficients in the latent variable model.

Table **6**: Concordance of Functional Cluster Membership with K=3 for Pain Severity and Urinary Severity

Pain Severity Functional Cluster	Urinary Severity Functional Cluster			
	Worsening	**Stable**	**Improving**	**Total**
Worsening	26 6.99	59 15.86	8 2.15	93 25.00
Stable	19 5.11	147 39.52	31 8.33	197 52.96
Improving	4 1.08	34 9.14	44 11.83	82 22.04
Total	49 13.17	240 64.52	83 22.31	372 100.00

Upon further investigation of the K=3 cluster membership for each participant, separately on each of the Pain Severity symptom profile and the Urinary Severity symptom profile, we can note in Table 6 that 22% (82/372) of these participants were classified in the “Improver” cluster (Figure 23, Left-panel, Cluster 2) for the Pain Severity symptom pattern, and 22% (83/372) of these participants were classified in the “Improver” cluster (Figure 23, Right-panel, Cluster 1). However, only 12% (44/372) were classified as “Improver”, when considering both (pain symptom pattern, urinary symptom pattern) of these primary endpoints.

These preliminary results from the functional clustering analyses, applied separately to construct symptom patterns for each of 1) pain severity score and 2) urinary severity score, illustrates the rich potential to develop risk factor models for differential longitudinal symptom patterns for each of the primary outcome domains of symptoms.

7. HUMAN SUBJECT CONSIDERATIONS

7.1. Symptom Patterns Study Participant Considerations

7.1.1. Symptom Patterns Study Participant Recruitment

Participant recruitment will be conducted through the urology/urogynecology clinics at each of the designated clinical sites. In some cases, local newspapers and other media will be used to promote these MAPP research study opportunities. Participants may be self-referred or referred through their primary physician (either solicited or unsolicited by the urology/urogynecology clinics). Possible participants will be introduced to the protocol by study investigators and/or the research coordinator, and asked whether they are interested in participating in the study.

The process of securing local physician approval and contacting the screening candidate will depend on prevailing guidelines of local IRBs, the requirements of each medical facility and the governmental HIPAA Guidelines which became effective in April 2003. Typically, candidates will first learn of the study from an invitational letter signed by the local principal investigator and/or personal physician. Occasionally, some individuals may learn of the study during a routine encounter with a healthcare provider who has agreed to assist in recruitment. Those individuals who express preliminary interest in the study will have a screening telephone/clinic visit to confirm eligibility. Those who remain interested will be scheduled for the screening/baseline visit, at which point a written informed consent is obtained.

Once IRB approved the study will be registered on clinicaltrials.gov.

7.1.2. Screening and Enrollment

The screening and enrollment process will require one in-clinic visit. The visit is structured such that essential information required to assess eligibility is acquired prior to the conduct of more intensive and time-consuming procedures required of the visit [See Appendix 6]. Key eligibility criteria will be reviewed and confirmed, screening, demo, and key eligibility measures will be collected, including medication data and a urine sample will be obtained for assessing eligibility.

The screening/eligibility visit will be conducted as an in-person clinic visit to obtain informed consent for the entire protocol, confirm study eligibility, and provide participants with additional information about the study. Contact information and a questionnaire assessing eligibility will be completed and a urine dipstick and urine culture will be carried out. Those persons who are eligible after the initial screening process will be invited to complete the SPS “deep” phenotyping assessments. The completion of the screening/eligibility visit, followed by a negative minimum 24 hour urine culture result, defines enrollment in the Symptom Patterns Study.

7.1.3. Participant Follow-up

Participants who enroll in the study and complete a screening/baseline visit will be followed up through quarterly internet questionnaires, as well as in-person clinic visits at 6, 18, and 36 months following their initial baseline visit. A complete listing of protocol elements to be collected at the screening/eligibility and follow-up visits is provided in Appendix 6: MAPP Phase II SPS Visit Schedule.

During the course of the study, participants will be contacted by the Discovery Site Research Coordinators with email reminders to log on for their quarterly assessments, as well as to facilitate scheduling of the 6-, 18-, and 36 - month follow-up visits. Participants who do not complete their

scheduled quarterly contact assessments will be contacted by phone for further prompting or discussion of any issues with study participation.

7.1.4. Participant Retention

Retention of participants is central to internal validity of the study and will be an extraordinarily high priority of the investigators and staff. A key element is a pleasant, attentive and responsive staff that provides a reasonably flexible schedule. Other clinical center features that promote high retention rates include local tracking systems and frequent staff meetings.

7.1.5. Participant Withdrawal

It is anticipated that over the course of the 36-month follow-up period, participants may withdraw from the study. This may occur officially by formal notification from the participants to the investigator, or unofficially when a participant cannot be reached via the usual methods of contact. Every effort will be made to acquire complete data on all participants. However, a participant may withdraw consent for use of his or her data at any time.

Participants who relocate to an area from which it is no longer feasible to travel to the center for the in-clinic visits will be asked to continue participation in the study by completing the quarterly online assessments.

7.1.6. Participant Reimbursement

As compensation for their time and effort, participant reimbursement (provided by each site) should be provided. Appropriate amounts and actual schedule of reimbursements should be determined by each site.

7.2. Ethical Issues

7.2.1. Potential Risks to Participants

The protocol includes in addition to questionnaires, a physical examination, pelvic exams, urine specimen collection, blood draws, Neuroimaging studies, and Quantitative Sensory Testing (QST).

Minimal physical risk to discomfort to participants arises from the physical and pelvic exam, however, the Pelvic Muscle examination could increase pain during exam and transiently after the exam.

Any risks or discomfort from either procedure is temporary. The risks associated with other study procedures are as outlined below.

7.2.1.1 Risk Associated with Collection of the Biomarker Urine and Blood Specimens

Urine specimens are collected at all in clinic and ATLAS study visits. Urine specimen collection is routine patient care and should not present a burden to participants. Blood collection is also routine patient care but several more tubes than usually obtained in the evaluation of UCPPS will be requested. However, the number of actual needle sticks will not be significantly increased over routine care, and the volume of blood obtained poses no physiological risk. There is minimal risk of temporary mild pain, discoloration, bruising or infection at the place where the needle is inserted. The risks also include possible fainting. These risks are minimized by the use of trained personnel to draw your blood.

7.2.1.2 Risk Associated with Collection of the Optional Microbiome VB3 Urine Sample, Rectal, and Vaginal Swabs

Optional VB3 urine samples (male only), rectal, and vaginal swabs will be collected at week 0, 18 months and ATLAS study visits. While it may pose temporary discomfort, there are no known risks associated with the collection of the rectal and vaginal swabs. Male participants will be asked to collect VB3 specimens that require a prostate massage. Risk associated with a prostate massage includes temporary discomfort.

7.2.1.3 Risk Associated with Neuro Imaging Assessments

Neuroimaging studies are conducted at week 4 (baseline), 6, 18, and 36-month clinic visits as well as any ATLAS Study visits. The specific risks of the Neuroimaging procedures relate to the site-specific protocols, but in general, the physical risks associated with this study are those associated with MRI scanners. Functional Magnetic Resonance Imaging Scan does not involve injections or any radioactive tracers. Some people experience dizziness or a metallic taste in their mouth if they move their head rapidly in the magnet. However, this is only a temporary effect and is not experienced if the head is kept still. The scanner produces loud sounds at times and insulated earphones will be provided to reduce the noise you hear. Although the long-term risk of exposure to magnetic fields and radiofrequencies associated with MRI is not known. The possibility of any long-term risk is extremely low in view of the information accumulated over the past twenty years. If unrestrained iron or steel objects are accidentally brought near the MRI magnet, they can be pulled very quickly toward the magnet and can strike people in or near the magnet. Such an event is very unlikely because precautions are taken to prevent such objects from being brought near the magnet. Participants are screened for iron or steel implants or clips from surgery, or metallic objects such as shrapnel or metal slivers in their bodies and are excluded from the neuroimaging study if present. Dental fillings do not present a hazard.

There is a risk that the water ingestion protocol will generate pain, including a possible pain flare-up. The neuroimaging session contingency plan shows how this risk will be minimized so that the acquiring this data will not cause undue urgency in the participant.

7.2.1.4 Risk Associated with Quantitative Sensory Testing (QST)

Quantitative Sensory Tests (QST) are conducted at week 4 (baseline), 6, 18, and 36-month clinic visits as well as any ATLAS Study visits. The QST procedures may cause minor but temporary physical discomfort. Study personnel will be trained by the investigators to be sensitive to participant discomfort and concerns. Participants may inform the Research Coordinator to stop the QST anytime that the pain or unpleasantness of the task becomes unbearable.

Specifically, MAST testing may cause some temporary physical discomfort on the thumbnail. The MAST device incorporates a number of mechanical, electrical, and software safety features to prevent injury in the event of user error or device failure, including a safety pin that the participant can turn to immediately remove the pressure from the thumb.

The pressure algometer and pointed skin probe are commonly used in QST studies and will not cause tissue injury at the maximum forces applied in this study (8 kg/cm^2 and 256 mN, respectively). However, both instruments may cause minor physical discomfort in the areas of testing (forearm, shoulder, and pelvic region) that is expected to resolve within minutes of test completion. These instruments may also

cause small skin indentations and/or skin reddening that is expected to resolve within a couple hours. These tests will be halted automatically if the participant reports a pain rating of 100.

The heated water used in the CPM test will also cause some temporary discomfort of the foot and lower leg. The maximum temperature of the water (46.5°C) and the duration of immersion (1 minute) are below the recommended standards of the U.S. Consumer Product Safety Commission (Publication 5098) to prevent scalding injury in adults. The researcher who is conducting the test will stop any procedure immediately if the participant finds it too discomforting. Towels will be provided for drying the participant's feet after testing.

Risk/Benefit Assessment

This is a study to investigate the relationship between UCPPS and other chronic pain conditions to better understand the pathophysiology of these often disabling syndromes. Although there will not be any direct benefits to the participants, the information obtained from this study has considerable potential benefit to future patients and to society as a whole by providing new information about the pathophysiology of these conditions. This study may well lead to the discovery of common risk factors, symptoms, or potential biomarkers related to these complex disorders, and may, therefore, lead to improved management and treatment.

Gender and Minority Inclusion

This is a multi-center study recruiting a clinical population from numerous institutions across the United States. We estimate the racial/ethnic composition of participants to be approximately 85% White/Caucasian, 10% African American, and 5% Latino/Hispanic, Asian/Pacific Islander, and Other. We plan to enroll equal numbers of men and women.

7.2.2. Informed Consent

Interested participants will be asked to sign the informed consent form approved by the local Institutional Review Board (IRB). This form will provide consent for the screening and the follow-up procedures as well as permission to contact them in the future. Potential participants must sign written consent to participate prior to initiating screening/baseline visit data and/or specimen collection.

Each Discovery Site will prepare an informed consent form following the guidelines of their local IRB, and applicable regulations for Informed Consent. The form will, at a minimum, contain a description of the potential risks, benefits, expense to the participant, and alternative treatment. Prior to signing the informed consent, the Research Coordinator will review the details of the consent form orally with the participant, and answer any questions that the participant has concerning participation in the study. The original signed consent form will be kept in the participant study file at the clinical center, while a copy of the signed consent form will be given to the participant. Specifically, the following must be accomplished during the informed consent process:

- The participant must be informed that participation in the study is **voluntary** and that refusal to participate will involve no penalty or loss of benefits or negative impact on their medical care
- The participant must be informed of the **purpose** of the study and that it involves **research**
- The participant must be informed of any **alternative procedures**, if applicable
- The participant must be informed of any reasonably foreseeable **risks**

- The participant must be informed of any **benefits** from the research
- An outline of safeguards to protect participant **confidentiality** must be included as well as an indication of the participant's right to withdraw without penalty. This should be balanced with a discussion of the effect withdrawals have on the study, and the responsibility a participant has, within limits, to continue in the study if he or she decides to enroll
- The participant must be informed **whom to contact** for information about research participants' rights, information about the research study, and in the event of research-related injury
- The participant must be informed as to whether or not **compensation** is offered for participation in the study and/or in the event of a medical injury
- The participant must be informed that he/she will be notified of any significant **changes** in the protocol that might affect their willingness to continue in the study

The consent process may differ somewhat by clinical center according to local IRB guidelines. The informed consent document will be structured such that it enables potential participants to indicate which aspects of study they may not be willing to engage in. This form will cover all aspects of screening, baseline testing and subsequent follow-up visits.

7.2.3. Consent for Genetic Testing and DNA Storage

A separate signature will be required for consent to a blood specimen for genetic testing and storage of DNA. In order to proceed with eligibility confirmation, participants must sign consent for genetic studies. However, they may refuse long-term storage at the NIDDK Central Repository without consequence to study eligibility. Specimens will be stored at TATC, and eventually shipped for permanent storage at the NIDDK repository.

Participants will be informed that DNA and other biological samples may be used for many types of genetic and biomarker analyses, but that the confidentiality of this information will be ensured by (1) data security measures, both at the participating sites and DCC, and (2) at the point that their clinical information is combined with biological data (e.g., genetic studies) where these datasets will be de-identified.

7.2.4. Storage and Archival of Study Data

The University of Pennsylvania serves as the Data Coordinating Center for the study, which means that the study information from all research centers, after being stripped of identifying information, will be stored in secure electronic files at the University of Pennsylvania. All study data will be sent to the Data Coordinating Center by secured Internet connection. Only authorized members of the research study will have permission to see these data.

At the end of the study all data will be archived at the NIDDK Data Repository. Researchers who plan to use the data for future scientific study will be required to request and receive all of the necessary approvals or waivers from the NIDDK prior to gaining access to the data. Data will only be released to scientists who are qualified and prepared to conduct a research study.

7.2.5. HIPAA Authorization

In accordance with the mandated Federal HIPPA regulations, authorizations will be provided to all research participants at the time of presentation of consent that detail all potential risks of disclosure and individuals and organizations who may have access to participant research data.

8. PARTICIPANT CONFIDENTIALITY

Procedures to assure confidentiality will be strictly observed. All identifiable personal health information data will be (1) kept in confidential locked files; (2) identified by participant number only; and (3) kept separately from identifying information used for participant tracking and follow-up contacts. Identifying information will be kept in separate locked files. No identifying information will be disclosed in reports, publications or presentations.

Protection of participants depends on the joint activities of all Clinical Centers as well as the DCC. Extensive efforts will be made to ensure that participants' confidentiality is maintained. Each participant is assigned a unique study identification number and is never tracked through the study by name, social security number, medical record number, or other personal identifier. A log of the participant names, participant ID numbers, and pertinent registration information (e.g., home address, telephone number, and emergency contact information) is maintained in a locked area at each clinical site. The staff at the DCC does not have access to this log. Only the participant ID number and initials are given to the DCC staff and entered into the study database. Any communication between DCC and clinical sites regarding participant data occurs via the participant ID number. Any forms or documents sent to DCC, IRB or other regulatory authorities will have all personal information removed.

Authorized representatives of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), participating clinical institutions as well as the IRB have access to and may copy both medical records and records from participation in this study. Such access is necessary to ensure the accuracy of the findings, the safety and welfare of participants. If any publication or presentation results from this research, participants will not be identified by name or other personal identifier. All research reports, articles, and presentations will report only aggregate findings.

9. STUDY ORGANIZATION AND OVERSIGHT

9.1. Discovery Sites

Six (6) Discovery Sites participating in the Symptom Patterns Study will have primary responsibility for developing the study protocol, recruiting a sufficient number of study participants, maintaining high rates of follow-up and data collection, obtaining data of high quality, and interpreting, presenting, and publishing findings from the study. The 6 Discovery Sites, with Principal Investigators, are as follows:

1. University of California, Los Angeles (UCLA)/University of Southern California, Los Angeles, CA

Principal Investigators:

Emeran A. Mayer, M.D.
Professor and Executive Director
Center for Neurobiology of Stress at UCLA

Larissa V. Rodriguez, M.D.
Professor, Institute of Urology at USC

2. Northwestern University, Chicago, IL

Principal Investigators:

David J. Klumpp, Ph.D.
Associate Professor
Department of Urology

Anthony J. Schaeffer, M.D.
Herman L. Kretschmer Professor & Chairman
Department of Urology

3. Washington University, St. Louis, St. Louis, MO

Principal Investigators:

Gerald L. Andriole, M.D.
Professor of Surgery
Chair, Division of Urologic Surgery
Director, Prostate Study Center, Barnes-Jewish Hospital

H. Henry Lai, M.D.
Associate Professor of Surgery
Division of Urologic Surgery

4. University of Iowa, Iowa City, IA

Principal Investigator:

Karl J. Kreder, M.D., M.B.A.
Professor and Chair, Rubin H. Flocks Chair, Urology
Director, Urodynamics and Reconstructive Urology

5. University of Washington, Seattle, WA

Principal Investigator:	**Dedra Buchwald, M.D.** **Professor, General Internal Medicine**

6. University of Michigan, Ann Arbor, MI

Principal Investigators:	**Daniel J. Clauw, M.D.** **Professor of Anesthesiology & Medicine – Rheumatology, Director, Chronic Pain & Fatigue Research Center.** **J. Quentin Clemens, M.D., M.S.C.I.,** **Professor of Urology, Director, Division of Neurourology & Pelvic Reconstructive Surgery**

In addition to the 6 recruiting Discovery Sites listed above, the following Discovery Sites were funded to provide expertise in particular scientific and translational areas related to MAPP goals:

Harvard Medical School, Boston, MA

Marsha A. Moses, PhD
Julia Dyckman Andrus Professor, Harvard Medical School
Director, Vascular Biology Program Boston Children's Hospital
Department of Surgery Harvard Medical School and Boston Children's Hospital

Richard S. Lee, MD
Associate Professor of Surgery (Urology), Harvard Medical School
Director, Urologic Oncology, Co-Director, Urologic Trauma Boston Children's Hospital

Queen's University, Kingston Ontario

J. Curtis Nickel, MD, FRCSC
CIHR Tier 1 Canada Research Chair in Urologic Pain and Inflammation
Professor of Urology

Dean A. Tripp, PhD
Associate Professor

Garth D. Ehrlich, PhD
Professor of Microbiology and Immunology
Professor of Otolaryngology-Head and Neck Surgery,
Drexel College of Medicine, Philadelphia, PA

Cedars-Sinai Medical Center, Los Angeles, CA

Jennifer Anger, MD, MPH
Associate Professor of Urology
Associate Director of Urological Research

Michael Freeman, PhD
Professor in the CSMC Departments of Surgery, Medicine and Biomedical Sciences

Jayoung Kim, PhD
Associate Professor of Surgery and Biomedical Sciences

9.2. Data Coordinating Core (DCC)

The Data Coordinating Core (DCC) for the MAPP Research Network is located at the Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

Principal Investigator:	**J. Richard Landis, Ph.D.,** **Professor of Biostatistics, University of Pennsylvania**
Co- Investigator:	**Alisa Stephens-Shields, Ph.D.,** **Assistant Professor of Biostatistics, University of Pennsylvania**
Co-Investigator (IC/BPS):	**Robert M. Moldwin, MD,** **Professor, Department of Urology** **Hofstra North Shore-Long Island Jewish School of Medicine**
Co-Investigator (CP/CPPS):	**Michel Pontari, MD,** **Vice-Chairperson and Professor, Department of Urology,** **Temple University**

The DCC is responsible for the following:

- Set-up and maintenance of the MAPP longitudinal data collection website for patient-reported outcomes
- Providing biostatistical expertise in research design, outcome measures and analytic strategies for translational and clinical investigations of UCPPS
- Guiding and implementing statistical analyses, interpretation of findings, and supporting presentations and publication of results
- Facilitating the conduct of multi-disciplinary basic and translational research, by providing scientific leadership in the design and implementation of research projects across the MAPP Research Network
- Promoting network-wide quality assurance standards, practices and tools, including a comprehensive, secure www-based data management system (DMS) for collection and centralized storage of all multi-site study data
- Collaborating with the TATC Laboratory on best practices for data collection, specimen tracking and storage, as well as support technical processes between the DCC and TATC
- Providing comprehensive Data Coordinating Core administrative support for the MAPP Research Network, promoting effective communications, coordinating meetings, working groups, document development and management, and distribution of study proceedings
- Supporting the MAPP Research Network Ancillary Projects, assisting in their design, as well as implementing a process for the submission, review, and development of ancillary studies

9.3. Tissue Analysis and Technology Core (TATC)

The Tissue Analysis and Technology Core (TATC) is located at the University of Colorado, Denver School of Medicine, Department of Pathology, Aurora, CO

Principal Investigator: **M. Scott Lucia, M.D.,**
Professor of Pathology, University of Colorado

Co-Investigator: **Adrie van Bokhoven, Ph.D.,**
Assistant Professor of Pathology, University of Colorado

The TATC will be responsible for the following:

- Providing specimen collection, banking, annotation/blinding, distribution services across the MAPP Research Network
- Providing genomic and proteomic analyses and generate assay platforms for multi-site efforts and individual site efforts as needed
- Coordinating procedures for coding, shipping, processing, receipt and storage of biosamples at the TATC site and future transfer of the biorepository to the NIDDK Biorepository

9.4. NIDDK Program Staff

The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) will be responsible for oversight and administration of the scientific conduct of this research. Representatives from the NIDDK will work with the DCC and TATC to develop and implement the study.

NIDDK Program Staff:	**Christopher Mullins, Ph.D., Director,** **Basic Cell Biology Programs in Urologic & Kidney Disease** **John W. Kusek, Ph.D., Director, Kidney & Urological Clinical Trials** **Ziya Kirkali, MD, Senior Scientific Advisor** **Tamara Bavendam, MD, Program Director**

9.5. MAPP Steering Committees and Subcommittees

The primary governing body of the study is the Steering Committee, which is comprised of each of the Directors/Co-Directors at the Discovery Sites, DCC, TATC, and the NIDDK Project Scientists. Dr. J. Quentin Clemens from the University of Michigan is the Chair of the Steering Committee. The Steering Committee develops policies for the study pertaining to access to participant data and specimens, ancillary studies, performance standards, publications and presentations. They develop the study protocols and meet to discuss the progress of the study and resolve problems that arise.

A subset of the Steering Committee membership makes up the Executive Committee. This includes NIDDK MAPP Program staff, together with the Chair (Clemens) and Co-Chairs (Dr. R Moldwin and Dr. M. Pontari) of the Steering Committee, the DCC PI and co-investigators, and the TATC PI. The Executive Committee has frequent (typically weekly) teleconferences and makes the day-to-day decisions of the MAPP, consulting the larger Steering Committee or specific members where necessary.

In addition to the Steering and Executive Committees, subcommittees may be established on such areas as recruitment and quality control, publications, and ancillary studies. Small working groups may be established to prepare manuscripts and presentations. The following subcommittees have been established to address specific study issues:

- Biomarkers Working Group
- Microbiome Working Group
- Epidemiological Study Working Group
- Neuro-Imaging Working Group
- Organ Crosstalk Working Group
- Quantitative Sensory Testing Working Group
- Phenotyping Working Groups: Urological and Non-urological
- Study Design/Forms Review Subcommittee
- Quality Control Committee
- Publication Committee
- Patient Report Outcomes

9.6. External Experts Panel

An External Experts Panel (EEP) for the MAPP Research Network was appointed to review protocols and advises the NIDDK Program staff in the overall conduct of the MAPP Research Network. An independent group of experts in areas such as Urology, Rheumatology, Epidemiology, Ethics, Health Economics, and Biostatistics who are not otherwise involved in the study have been recruited by the NIDDK to evaluate the proposed protocol and periodically review the progress of the study.

10. Study Management

10.1. Discovery Site Responsibilities

10.1.1. Discovery Site Director and Investigators

Conduct of particular aspects of the study may be delegated to qualified personnel; however, it is the responsibility of each Discovery Site Director to oversee the overall study management. The Discovery Site staff must be trained in all study procedures.

Each Discovery Site is responsible to screen, recruit, enroll and retain a designated number of study participants. It is the responsibility of the Discovery Site study staff to assess their accrual, ensure participant confidentiality, maintain appropriate study documentation, enter and transfer data in a timely manner, and participate in the MAPP study meetings and conference calls.

10.1.2. Institutional Review Board

It is the responsibility of each Discovery Site to conduct the study according to the protocol, and to adhere to all applicable regulatory guidelines, and to provide the appropriate IRB with all pertinent material including a copy of the informed consent. Approval of the protocol and the informed consent form must be obtained, and forwarded to the DCC and TATC, prior to screening or enrolling participants. The Investigator also maintains the responsibility of initiating protocol re-approval, notification of protocol and/or consent form changes, notification of unanticipated events, and termination of the study according to the appropriate IRB requirements.

10.1.3. Record Retention

Investigators maintain study documents on-site and in an orderly fashion for a minimum of 6 years, and make available to the sponsor or the sponsor's representative: the signed study protocol, amendments, informed consent documents, and approval letters from the IRB, CRFs, all primary source documentation, and all letters of correspondence. The DCC maintains all study records for a period in accordance with their internal SOPs and applicable regulations.

10.2. Data Coordinating Core responsibilities

10.2.1. Quality Assurance

The DCC has developed written standard operating procedures (SOPs) to ensure that all aspects of the study are conducted in a standard and uniform manner. These procedures are organized into a Manual of Procedures (MOP), which complies with the protocol, Good Clinical Practice (GCP), and applicable regulatory requirements. The DCC will include a comprehensive Quality Assurance (QA) Plan in the MOP that will consist of the following activities:

Personnel Training and Certification: Prior to the Trans-MAPP SPS initiating participant enrollment, a comprehensive training session will be conducted with all study personnel that will encompass all aspects of the study, including communication, principles of GCP, study implementation and procedures, data entry and verification, test and specimen collection and transfer.

Clinical Protocol, MOP Adherence and Auditing Activities: The DCC will request and verify specific information from clinical centers, to ensure the application of study procedures as they apply to participant safety, required intervals for timely conduct of procedures, appropriate documentation of

data and specimens, and compliance with SOPs. This information will take the form of a written report, and may be acquired during clinical site monitoring visits.

Database Auditing: A comparison of a certain percentage of data written on CRFs to that entered into the electronic database provides information that describes and quantifies the accuracy of the data entry process and use of the data management system by personnel at each Discovery Site. This information will take the form of a written report.

Database Administration and Network Security: The DCC has SOPs established for authorizing and documenting secure access to the study website, study documents and the electronic Data Management System (DMS). These procedures ensure that only authorized personnel are able to view, access and modify study data.

Data Reporting: A set of standard reports will be developed to describe study activities that include accrual, study progress, and data quality. These reports will be developed using Oracle Reports and provided to investigators, NIDDK and designated committees as appropriate.

Preparation and Integrity of Analysis Datasets: The DCC Database Administrator will create a set of standard data access descriptor/view files, which will be used in the generation of SAS analysis datasets. As datasets are extracted from the main study database, they can be utilized separately from direct database processing, thereby, safeguarding the integrity of the data.

Data Management: The DCC provides overall coordination, logistical support, and implementation for all aspects of the study protocol including data collection, data processing, tracking of participant recruitment, tracking of specimens, training, quality assurance, and statistical analysis. The Clinical Research Computing Unit (CRCU), through its clinical data management, project management, and software systems developments, places into the field and maintains a state-of-the-art www-based data system that accommodates all scientific study data, and permits tracking and coordination of all Trans-MAPP Research Network activities within the framework of multidisciplinary project teams.

10.2.2. Website Enhancements

The DCC has developed a MAPP Network website (http://www.mappnetwork.org/) for study-wide communication management, data and document management, and activity management and coordination. The website provides general information to the public, single-point restricted access to tools and information for investigators and clinical center study personnel including study resources, communication tools as well as data entry and management tools. It also provides an additional level of restricted access for DCC study personnel.

10.2.3. Data Security

The research computing environment for the MAPP DCC is supported by a Biomedical Research Computing (BRC) group within the Clinical Research Computing Unit (CRCU) of the Center for Clinical Epidemiology and Biostatistics (CCEB) at the Perelman School of Medicine at the University of Pennsylvania. The BRC group is responsible to provide an integrated research computing and storage environment in a manner that supports the required confidentiality, integrity, and access of a common set of research data through all stages of its use, operated in a FISMA-compliant/FDA sensitive manner. The MAPP project is maintained within this compliant environment.

The CCEB General System Security Plan (CCEB-GSSP) is available on request to provide a quick read into the security within the CRCU, listing several of the security attributes most requested. Also available on request is a memo from Penn's Chief Scientific Officer affirming Penn's continuing commitment to meeting and maintaining its FISMA compliance. The CCEB/CRCU has performed a security and risk assessment using outside auditors to perform a gap analysis on its security measures against the FISMA recommended NIST SP800-18 and SP800-53 controls documents. The results of this assessment provided Penn Medicine's Chief Scientific Officer with the confidence to support and write a memo that is the AMC's equivalence of a federal "Authority to Operate (ATO) certification, as required for Federal agencies."

The CRCU database environment for MAPP utilizes Oracle's Advanced Security Option (ASO) with two primary foci: 1.) Strong encryption of the database transmissions to protect data traversing the data networks to and from the CRCU databases; and 2.) Internal database encryption of individual sensitive data elements, thus protecting ePHI data within the database. Both of these features are in use with the MAPP protocols and databases. The CRCU further utilizes a database monitoring tool that maintains an audit of all user session activities that occur against the protected MAPP databases. This tool is able to then recreate requested past user sessions to track all changes that occurred to data in the databases.

10.3. Tissue Analysis and Technology Core Responsibilities

10.3.1. Personnel Training

TATC along with the DCC will conduct a personnel training session and a certification session for staff who will perform clinical procedures before initiation of the protocol. This comprehensive training session includes all aspects of the protocol and MOP implementation such as specimen collection, handling, processing, and shipping. Periodic conference calls and training sessions will be conducted to uphold standard application of procedures.

10.3.2. Specimen Kit Distribution, Banking, Annotation/Blinding

TATC will generate and provide MAPP-specific collection kits for use by Discovery Sites as needed. Requests for kits will be done through an online ordering mechanism located on the MAPP Portal direct from TATC. The collection kits and components are bar-coded and will be linked with the participant at the time of registration of the participant with the DCC. Collected specimens will then be shipped to TATC for inventory into the biorepository.

The collection and handling procedures will follow the guidelines established by the NIH Best Practices Policies for biorepositories (www.biospecimens.cancer.gov). No participant identifiers will be used on the collection tubes and tracking forms. As specimens are received from sites, they will be scanned into the biorepository database, and archived in the appropriate freezer/storage unit until needed. Specimen tracking information will be entered into the database by TATC personnel.

10.3.3. Biorepository Collection, Management and Distribution

The TATC will act as a central repository for all body fluid and tissue specimens generated by the MAPP, its member Discovery Sites and other research entities as approved by the Network. To provide the highest quality non-biased patient samples, uniformly prepared and analyzed and to meet the needs of individual research teams, TATC will provide guidance and personnel training to collection sites on

protocol development and specimen collection and handling. The TATC will develop and distribute specialized specimen collection kits, and coordinate specimen collection, processing, annotation, bar-coding, shipping, banking, and distribution. The TATC will identify and implement best information technology architecture for the MAPP research network and provided access to its services through the MAPP Research Network portal hosted by the DCC. The biorepository will meet all NIH standards, and will provide specimens to researchers according to IRB, HIPAA and NIH procedures that protect the confidentiality of all consented participants whose tissue and blood are archived. The TATC will also work with the NIDDK Biorepository to coordinate procedures for collection, coding, storage and eventual transfer as directed by the NIDDK.

10.3.4. Specialized Assay Platforms

The TATC will generate and provide specialized assay platforms for specimen analysis such as protein and/or tissue arrays, DNA extractions and purifications as needed for individual Discovery Site efforts or ancillary/pilot projects.

Proteomics, Metabolomics, Transcriptomics: The TATC will provide centralized mass spectrometry services to assist the MAPP Research Network with proper collection and handling of specimens, consultative assistance for proteomics, metabolomics, and transcriptomics studies, and performance of a wide variety of assays, including chromatography-based proteome profiling, protein arrays, cytokine arrays, multiplexed ELISA, mass spectrometry and NMR-based targeted and mass spectrometry analyses (nanoLC ion trap and nanoLC hybrid quadrupole-linear ion trap).

Genomics, Genotyping: The TATC will provide consultative assistance and genomics services to assist the MAPP Research Network with advanced genotyping techniques. Methods for analysis of single nucleotide polymorphisms (SNPs) include single base extension assays with detection of incorporated base by fluorescence polarization, Taqman single SNP assays on 96- or 384-well real-time instruments or Taqman analysis of 32 or 64 SNPs on a nanoscale. Access to Sequenom and Illumina platforms are also available for larger scale studies.

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MULTIDISCIPLINARY APPROACH TO THE STUDY OF CHRONIC PELVIC PAIN (MAPP) RESEARCH NETWORK

APPENDIX 1: BIOMARKER PROTOCOL

Sponsored by: The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Department of Health and Human Services (DHHS)

PROTOCOL VERSION 1.1

Dated: October 10, 2016

This version replaces V1.0 dated November 10, 2014.

Collaborating Site: Harvard Medical School/Boston Children's Hospital, University of Iowa, University of Washington, Cedars-Sinai Medical Center, University of Colorado/TATC, University of Pennsylvania/DCC

Prepared by: Biomarker Working Group

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Table of Contents

1. Identifying Information ... 4
2. Brief Overview / Introduction ... 4
3. Rationale for Phase II Trans-MAPP Study ... 5
4. Describe Link to Trans-MAPP Symptoms Pattern Study (SPS) ... 9
5. Study Hypotheses ... 12
6. Specific Aims ... 13
6.1. Aims Related to Hypothesis 1 ... 13
6.2. Aims Related to Hypothesis 2 ... 14
6.3. Aims Related to Hypothesis 3 ... 14
6.4. Aims Related to Hypothesis 4 ... 14
7. Brief Description of Supporting Preliminary Data ... 14
7.1. Specific Aim 1: ... 14
7.2. Specific Aim 2 ... 20
7.3. Specific Aim 3 ... 20
7.3.a. UCPPS patients have an exaggerated inflammatory response compared to healthy controls. ... 20
7.3.b. Cortisol is dysregulated in UCPPS patients compared to healthy controls. ... 20
7.3.c. TLR-4 responsiveness is associated with pain in UCPPS. ... 21
7.3.d. Algorithm to predict Pain Symptoms in UCPPS patients. ... 21
7.4. Specific Aim 4 ... 21
7.4.a. DNA Methylation in Peripheral Blood Cells. ... 21
7.4.b. LTL in Peripheral Blood Cells. ... 22
8. Study Design and Methods ... 23
8.1. Specific Aim 1 ... 23
8.2. Specific Aim 2 ... 27
8.3. Specific Aim 3 ... 30
8.4. Specific Aim 4 ... 31
9. Description and Source of Patient Cohorts Beyond those from the SPS, including controls ... 32
10. Contact Schedule and Participant Procedures ... 33
11. Risk Factor and Outcome Measures ... 33
12. Biological Specimens ... 33
13. Other Measures ... 35
14. Analytic Plan ... 35
14.1. Specific Aim 1a ... 35
14.2. Specific Aim 1b ... 36
14.3. Specific Aim 1c ... 36
14.4. Specific Aim 2 ... 37
14.4.a. Specific Aim 2a and 2b ... 37
14.4.b. Specific Aim 2c ... 37
14.5. Specific Aim 3 ... 37
14.6. Specific Aim 4 ... 38
14.6.a. Analysis of Specific Aims 4a, 4b, and 4d ... 38
14.6.b. Analysis of Specific Aims 4c and 4d ... 38
15. Statistical Analysis and Sample Size Estimates ... 38
15.1. Specific Aim 1 ... 38
15.2. Specific Aim 2 ... 38
15.3. Specific Aim 3 ... 39

15.4. Specific Aim 4 40
16. Proposed involvement of MAPP Network Cores........ 40
17. Timeline for Completion of Data/Specimen Collection (see attached Appendix 1: SPS Protocol Timeline) 40
18. Anticipated Burden to Sites........ 40
19. Anticipated Risks to Participants 40
20. References........ 41
Appendix 1:SPS Protocol Timeline 50

1. Identifying Information

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2. Brief Overview / Introduction

The idiopathic nature of Urologic Chronic Pelvic Pain Syndrome (UCPPS) has prompted an intense search for diagnostic and prognostic clinical biomarkers of this syndrome. The development of clinically relevant biomarkers of UCPPS is critical to effective clinical management and to the understanding of UCPPS pathology, and to the goal of advancing current treatment and developing novel therapies. During MAPP I, we have taken advantage of the power of our interactive Trans-MAPP Network to successfully identify several sets of promising biologically-relevant urinary and blood biomarkers of UCPPS. Urinary biomarkers were identified using two distinct approaches: (1) a biologically-driven biomarker discovery strategy grounded in the basic biology and physiology of this disease and (2) a global proteomics biomarker discovery strategy. Six markers were identified using the first method and indicate that UCPPS significantly changes the urinary proteome as compared to that of healthy and positive controls and (2) UCPPS may alter the regulation of extracellular matrix proteins in a definable, biologically-relevant pattern. Validation work on the global proteomics project is ongoing and will complement the first set of markers once established. We are now in the process of completing the validation studies of those urinary proteins identified in our global proteomics work and intend to multiplex them with those discovered through our biologically-driven

discovery studies. Additional proteomics discovery will be conducted using Sequential Window Acquisition of all THeoretical Mass Spectra (SWATH) applications. SWATH is a hybrid between de novo discovery and MRM (Multiple Reaction Monitoring) assays, in which 1000s of proteins are identified and quantified in every sample with every single run without extensive fractionation. This time- and cost-effective technology allows reproducible assessment of the broad proteome of human body fluids on extremely large numbers of samples.

Other biomarker studies have found differences between a stimulation of a patient's peripheral blood mononucleated cells (PBMC) for either TLR-2 or TLR-4 and the response seen in controls. Importantly, TLR-4 stimulation also correlated with pain. Very few biomarkers have been found to correlate with pain in patients with UCPPS, and none are a stimulated biological response. Also, the hypothalamic-pituitary-adrenal axis was found to be abnormal in patients with UCPPS. This is similar to other systemic chronic pain conditions and leads support to the idea of a systemic disease process and not just an isolated bladder problem. Genetic changes both inherited and also as a response to environment, or epigenetic changes are postulated to play a role in development of UCPPS. Evidence for this has been provided in pilot studies showing changes in DNA methylation of peripheral blood cells that correlated to case status as a patient with UCPPS, and also with depression, one of the significant non-urologic comorbid conditions associated with pelvic pain. Also, alterations in telomere length were found among twins based on pain ratings with a full bladder induced by a water consumption task (shorter telomeres were associated with higher pain ratings).

Overall these findings represent novel discoveries in a field that has seen few such findings over the past several decades. These studies address the most contemporary theories in the etiology of not just UCPPS but chronic pain in general. They are covering the areas of both local bladder changes as investigated by the urinary markers, systemic markers of immune and endocrine dysfunction, and finally the importance of genetic alterations as a contributing factor. The idea of infection is also important given the TLR-4 response, and will complement the urinary microbiome project. Both the TLR receptors and several of the urinary biomarkers are also implicated in development of neuropathic pain, complementing neuroimaging studies underway and other studies of neural function such as quantitative sensory testing. The study of both local and systemic pathology complements the symptom pattern study as it looks at both bladder symptoms, the localization of symptoms to the pelvis or beyond, and what factors contribute to symptom progression.

3. Rationale for Phase II Trans-MAPP Study

The idiopathic nature of UCPPS has prompted an intense search for diagnostic and prognostic clinical biomarkers of this syndrome. The development of clinically relevant biologic markers of UCPPS is critical to implementing the most effective clinical management and to improve the understanding of UCPPS pathology with the goal of advancing current treatment and developing novel therapies. Current diagnostics are severely limited. Various biomarkers have been proposed in the past, however, validation of these markers has been disappointing. To date, there are (1) No definitive clinical tests for UCPPS that distinguish distinct groups of patients with different etiology and (2) No ability to determine who is at risk of progression or who will respond to specific therapy. Accurate noninvasive test(s) that are based on specific, definable levels of markers or response to provocative tests are essential to standardize clinical guidelines, diagnosis and management.

The formal, stated focus of MAPP II is to clarify, verify and investigate the distinct phenotypic groups established in MAPP I utilizing a multi-modality investigative effort that combines objective biological

biomarkers, neuroimaging, studies of the microbiome and advanced intensive phenotyping as well as patient reported outcomes. Through the Biomarker Working Group (BWG), we have developed the basis for continued interaction of the BWG members and the MAPP Research Network as a whole. The findings from MAPP I biomarkers coupled with the overall findings of MAPP I provide a clear basis for continuing these lines of investigation and expanding them as well.

Urinary biomarkers: The defining symptom in UCPPS is pelvic pain. Specific to Interstitial Cystitis /Bladder Pain Syndrome (IC/BPS) is the symptom of pain thought to be related to the bladder. Our entrance criteria reflect this as pelvic pain, thought to be related to the bladder. A subset of patients in MAPP I have been found to have either pain on bladder filling, relieved by bladder emptying, or urgency based on the desire to avoid pain by emptying the bladder. Although, as seen from our studies of pain beyond the pelvis, and many other non-urologic symptoms in these patients, bladder pain is what is driving the morbidity from this condition. Therefore, the focus on bladder/urinary biomarkers is an important component of the overall research effort.

As seen from MAPP I, patients with bladder pain can also have other systemic symptoms. Urinary biomarkers may be a reflection of these as well. MAPP II proposes that quantitative alterations in circulating urine protein concentrations will reflect the systemic and local changes in individuals with UCPPS. Due to the breadth of potential causes and states (*e.g.* flare vs. remission vs. treatment response), it is important that large and diverse population be analyzed during discovery in order to capture the continuum of symptoms. This will be addressed by the use of the SWATH technology.

Note: the SWATH technology will also be used to investigate serum proteins given the systemic nature of symptoms in many patients with UCPPS.

Post translational modifications (PTMs) will also be investigated. The specific PTMs that will be investigated are based on existing knowledge of chronic pain in different biological systems and include i) overall total redox modification of cysteine residues, the most susceptible amino acid residue to both ROS and RNS injury [1], and ii) phosphorylation, which reflects cellular signaling events via the activating phosphorylation of kinases and their modification of their downstream functional targets. Importantly, recent data from our and other laboratories that oxidation of cysteine residues can influence phosphorylation either directly modulating the kinase or altering the downstream targets, thus hindering the ability of a kinase to phosphorylate the protein [2-6]. We hypothesize that the disease-induced modifications that drive cellular response will be observed in the proteins present in urine and reflect, to some degree, the underlying molecular mechanisms of the source of a subject's pain.

TLR/endocrine studies: This work will inform clinical practice by simultaneously considering the roles of cellular inflammation (*i.e.* TLR-2/4 responsiveness) and endogenous control of inflammation (*i.e.* HPA axis), two highly interactive factors which are too often considered in isolation. Most importantly, identification and validation of neuroinflammatory pathways related to symptom patterns over time will have specific implications for novel targeted treatment approaches for subgroups, based on mechanistic considerations such as blocking TLR activation.

Central TLR Chronic Pain Hypothesis: Patients with UCPPS may well represent a heterogeneous group with respect to etiology but they nonetheless converge in the uniform expression of chronic pelvic pain, some associated with bladder filling and/or voiding. This fact suggests there is a final common pathway shared

among these patients. Recent developments in the field of chronic pain highlight the emerging role of non-neuronal glial and microglial cells in the facilitation of nerve impulses.[7-11] These immunoresponsive cells of the central nervous system (CNS) retain many of the same well-understood receptors and signaling pathways as their circulating monocytic counterparts.[12] The pathological expression of pro-inflammatory cytokines induced by the Toll-Like Receptors TLR-2 and TLR-4 (such as interleukin-1 beta [IL-1β], interleukin-6 [IL-6] and chemokines can actually enhance propagation and/or even generation of nociceptive signals.[13] These signals can be further influenced by the hypothalamic pituitary axis (HPA).[14] Cortisol, its major product, normally has a distinct diurnal pattern with high levels of secretion in the early morning, and a nadir at night [15]; however, in our pilot work described in section 7, UCPPS patients displayed flattened diurnal rhythms with nocturnal elevations, patterns similar to those observed in various inflammatory and functional disorders.[16-19] It has been shown that feedback loops between glucocorticoids and inflammation can be disrupted by chronic inflammation and even set the stage for chronic inflammation.[20,21] Although cortisol is generally considered to be anti-inflammatory, consistently high levels, such as occur with chronic inflammation and pain, can cause dysregulation of feedback systems and insensitivity of glucocorticoid receptors which normally should be inhibiting inflammatory signaling.[22-24] Moreover, glucocorticoids induced by both acute and chronic stress have been shown to prime central neuroinflammatory responses, resulting in persisting sensitization of microglia and sustained inflammation and pain.[25,26] Stress has also been associated with potentiation of pain [27] and the transition from acute to chronic pain.[28] Thus, the stress response and HPA both directly contribute to sustained potentiation of the central inflammatory response and to central sensitization in chronic pain. The data generated by investigation of baseline peripheral mononuclear cell (PBMC) TLR-2/4 responses in MAPP I patients (see preliminary data section 7) strongly points to a major role for TLRs and HPA dysregulation and indicates multiple forms of neuroinflammatory dysregulation in UCPPS.

Based on this literature, our central hypothesis is that TLR-4 activation is an underlying feature of UCPPS. It may begin peripherally from the bladder but then move progressively into the CNS where it is aberrantly perpetuated by microglial cells, evolving into a state of "central sensitization."[29] In murine models of systemic inflammation induced by lipopolysaccharide (LPS: a strong TLR-4 agonist), TLR-4 is differentially over-expressed in specific cortical regions of the brain.[30] In addition, nodes of the pain and fear networks have been shown to express TLR-4 receptors that may provide a basis for anatomical and functional changes seen in the MAPP neuroimaging studies [31], including increased presence of TLR-4 on the surface of microglia and macrophages in the amygdala, prefrontal cortex, and hippocampus resulting from psychosocial stress.[32] We further hypothesize that peripheral manifestations will be evident in circulating monocytes and macrophages which share strong homology with microglia.[33] Furthermore, since both acute and chronic stress can enhance secretion of cortisol[9], and potentiate inflammatory signaling peripherally and in central microglia [25,26], the extent of peripheral TLR-4 and HPA dysregulation *as they change over time* in response to acute or chronic stress (psychological or physical) may predict severity of pain, extent (propagation) of pain, frequency of flares, type of flare pattern and even the chronic worsening (or improvement) of pain. Thus this approach is directly relevant to the SPS protocol.

TLR Polymorphisms (PMs): Downstream Pro-Inflammatory Activity, and Symptoms in UCPPS Patients: As the quintessential pattern recognition proteins for initiating an innate immune response to a wide range of pathogen motifs, TLRs have been driven by selective environmental pressures to genetically favor certain structural alterations that promote species survival in areas of endemic infectious disease. Transition from

acute to chronic pain and disability: a model including cognitive, affective, and trauma factors. Pain [34] often in the form of a single nucleotide PM or SNP, a change in one nucleoside may transmute a key amino acid in the TLR protein that increases or decreases its ability to robustly transmit a signal to downstream pro-inflammatory pathways. As a general consequence and perhaps as a result of further endogenous TLR agonists released by tissue damage, classic autoimmune diseases such as rheumatoid arthritis, systemic lupus, and colitis have also been linked to TLR PMs.[35,36] As the emerging role of TLRs, especially TLR-4 and TLR-2, becomes clarified in mediating neuroinflammation and nociceptive pathways, it can be surmised that functional TLR PMs will similarly influence the course of chronic pain. The TLR-4 PM D299G, for instance, has been identified as a genetic risk factor for migraine.[37] Likewise, regulation of the immune-endocrine stress response (as measured by diminished ACTH release) during systemic inflammation was found to be influenced by the presence of TLR-4 SNP carriers heterologous for D299G and T399I resulting in reduced release of cytokines IL-8, IL-10 and GM-CSF.[38] While no studies to date have examined the role of TLR PMs in UCPPS, there have been multiple reports of the association of TLR-4 PMs with adult and pediatric urinary tract infections.[39-41] Thus, an analysis of TLR-2/4 pathway PMs should identify patients more susceptible to worsened UCPPS symptoms and long-term disease outcomes.

Genetic alterations in UCPPS: epigenetic modifications and telomere shortening: This project focuses on the possible role of central changes to inflammatory and other systems in UCPPS through novel epigenetic mechanisms. It examines UCPPS as systemic disorders by looking at epigenetic changes in circulating lymphocytes. This project can examine systemic changes in our fully phenotyped UCPPS patients that may help explain UCCPS co-morbidities as well.

Epigenetic Modifications: Epigenetics refers to processes that affect gene expression and function in the absence of any modifications in the actual DNA sequence. Such processes include histone modification, chromatin remodeling, and DNA methylation. The latter involves adding a methyl group to the fifth carbon of a cytosine base. It occurs most frequently within CpG islands (*i.e.*, genomic regions that contain a high frequency of CpG sites) in the promoter regions of many genes, and is typically associated with down-regulation of gene expression.[42] Unlike the DNA sequence itself, which is stable during development, DNA methylation is highly dynamic and can be induced by nutritional or behavior elements, chemical agents, and low-dose radiation.[43,44] These factors can directly affect methylation, chromatin remodeling, and other epigenetic pathways, altering the epigenome and subsequent gene expression and contributing to disease etiology.[43-46]

Although this research is in its infancy, DNA methylation may be related to key aspects of chronic pain, including regulation of peripheral inflammation and expression of genes related to pain processing.[47,48] The transition from acute to chronic pain may also be subject to epigenetic control. For example, one study noted that gene expression patterns change rapidly in response to injury, reporting > 1,000 genes activated in the dorsal root ganglion after nerve damage. Although gene expression patterns are not entirely determined by epigenetic processes, epigenetic control of inflammation and pain sensitivity (*i.e.*, glucocorticoid receptor function) is a critical part of the changes in gene activation.[47]

The role played by inflammation suggests that epigenetic alterations that affect systemic inflammation may also be important. In fact, epigenetic modification of inflammatory mediators could be a predisposing factor for UCPPS. Thus, we plan to investigate DNA methylation in peripheral lymphocytes to describe potential effects of systemic methylation patterns.

Telomeres: Human telomeres consist of TTAGGG tandem repeats and their binding proteins. Each time a somatic cell divides, its telomeres shorten because of the "end-replication problem."[49,50] After a finite number of cell divisions, telomeres reach a critical length that signals the replicative machinery to cease cell replication, a phenomenon known as "replicative senescence." In humans, embryonic stem cells and male germ line cells express telomerase, a reverse transcriptase that elongates telomeres,[51] but this enzyme is repressed during extra-uterine life in somatic cells, including hematopoietic stem cells.[52-54] As a result, the attrition of hematopoietic stem cell telomeres is expressed as age-dependent shortening of Leukocyte Telomere Length (LTL) [55,56], which is associated with aging-related diseases. Environmental circumstances can also shorten LTL.[57,58]

Specifically, pro-inflammatory and pro-oxidant environmental elements accelerate age-dependent LTL attrition. In vivo, inflammation accelerates LTL shortening by mediating increased replication of hematopoietic stem cells.[57,59] Oxidative stress, which represents an imbalance between the level of reactive oxygen species and their rate of detoxification, might augment the number of telomere repeats that are clipped off when each hematopoietic stem cell replicates. Accordingly, shorter LTL indicates a greater cumulative burden of inflammatory or oxidative stress, which could link epigenetic modification with LTL attrition in UCPPS.

Chronic pain can affect LTL by pro-inflammatory stress processes.[60-62] In a small study of chronic knee pain and perceived stress, the combination of high stress and pain was associated with shorter telomeres.[61] Similarly, among 66 women with fibromyalgia, worse pain was linked with shorter telomeres and with less gray matter in brain regions associated with pain processing.[60] One model proposes that shorter telomeres, as a function of genetics and environmental exposures such as stress, denote a vulnerability to chronic pain, which in turn contributes to physical and psychological processes relevant to telomere shortening.[60] These intriguing studies may link epigenetic modification, systemic inflammation, LTL and pain severity in our participants.

4. Describe Link to Trans-MAPP Symptoms Pattern Study (SPS)

The Trans-MAPP biomarkers studies will be the biological link to the symptoms and symptom patterns seen in the MAPP Network participants. The etiology and pathogenesis of UCPPS are largely unknown. The biomarkers will provide insight into the both possible causes of symptoms on which to base further treatments, as well as prognostic markers to aid in counseling our patients, and also guiding therapy. There are 9 aims of the SPS. Aim 5 lays out the intent to use the biomarkers. Aim 9 involves statistical modeling that can be applied to both symptoms and biomarkers. The first 5 aims include the intent to characterize bladder specific phenotypes (Aim 1). Other aims plan to characterize a cohort of patients with pain restricted to the pelvis including urologic and non-urologic symptoms (Aim 2), which of these symptoms predict progression (Aim 3), the biological markers associated with progression (Aim 5). Finally, Aim 4 proposes to characterize the transition between pelvic pain only and systemic or pelvic pain and beyond with respect to symptoms and non-urologic comorbid conditions. Another studies of symptom progression or improvement will also be carried out, the ATLAS (Analysis of Symptoms during Longitudinal Assessment of Symptoms) module of the central clinical protocol in which symptoms will be assessed during changes in therapy to look for endophenotypes of patients who respond to specific types of treatments and provide insights into concomitant biological changes.

The biomarker study findings are potentially applicable to all of these efforts. To date, as outlined in section 7, the major markers identified by the Boston group looking at predetermined biologic targets that will be studied in MAPP II are the matrix metalloproteinases (MMP) and their inhibitors, vascular endothelial growth factor (VEGF) and Lipocalin-2(LCN2;NGAL [neutrophil gelatinase-associated lipocalin]). Other markers are in study from the global discovery approach and will be included as they are validated. The Iowa group has focused on the role of the toll like receptors TLR-2 and TLR-4, and their interaction with the hypothalamic-pituitary-axis (HPA) and cortisol to modulate neuropathic pain. Other biomarker studies include epigenetic modulation of peripheral lymphocytes. Telomere length, which is affected by oxidative stress, will also be examined. Overall, these avenues of investigation well represent the scope of the MAPP, looking at the role of local pathology and symptoms in the bladder/pelvic region, and also the role of systemic factors beyond the pelvis including the role of neuropathic, psychological, immune and endocrine factors.

Biomarkers in characterization of bladder specific phenotypes (SPS Aim 1)

One of the clinical questions in UCPPS is whether there are patients with a specific bladder phenotype, in which there is pain with bladder filling and/or relieved by bladder emptying, or painful urgency, characterized by the need to urinate to avoid the feeling of pain as opposed to fear of wetting. The findings of MAPP I show that there are groups of these patients who have these symptoms, and make up about 2/3 of the MAPP cohort. The other third are patients with pelvic pain, and do not have changes in symptoms with bladder filling, or painful urgency. These patients are thought to have possibly pelvic floor dysfunction as a cause of their pain. Several of the biomarkers being studied have relevance to studying this distinction. MMPs have been implicated in a variety of biologic processes including cytokine and growth factor release, tumor growth, and the inflammatory responses.[63] Inflammation is proposed to play a role in some patients with UCPPS. Not all patients however have evidence of inflammation on markers or biopsy.[64] One question is whether the patients without bladder pain are those without or less bladder inflammation. The marker VEGF is involved in the vascular system and has been reported to be associated with immature vessel formation.[65] Alterations in bladder blood flow in patients with interstitial cystitis have been reported, pointing to a relative ischemia compared to asymptomatic controls.[66] The bladder ischemia phenotype could correlate to the bladder pain /painful urgency phenotype. TLR-2 and TLR-4 could also contribute to the bladder response to ischemia. These receptors are important in the cerebral response to ischemia and reperfusion injury, and are located on neural cells in the brain.[67] Bladder epithelial cells have been found to have many properties similar to neurons [68], and therefore the question becomes whether these receptors could play a similar role in the bladder. LCN2/NGAL is triggered by other inflammatory molecules including TNF-α [69,70], which may play a role in the pathogenesis of bladder changes seen in IC/PBS [71-73]. This also would suggest a link with NGAL and the bladder phenotype.

The patients without the bladder pain/painful urgency may have pelvic floor dysfunction as a cause of their pain [74]. One contributing factor to pelvic floor dysfunction is psychological stress. Patients with the altered cortisol response reflecting stress may be more likely to have this phenotype. This phenotype and biomarker findings will also have to correlate with the findings of the neuroimaging working group indicating altered connectivity between the cerebellum and areas of motor control which can result in dysfunction of the pelvic floor [75]. Epigenetic changes in the bladder epithelial cells could certainly produce local pathology resulting in pain with bladder filling. The biomarker working group will also have to collaborate with the urinary microbiome working group as infection may have several influences on the biomarkers under study. The TLR-2 and TLR-4 receptors are important as they are bound by bacteria and help mediate the host response to

infection [76]. The response of the TLR receptors must be put in to the context of the patient's urinary microbiome. Both commensal and pathogenic bacteria can also lead to epigenetic modification of cells [77], thus again the need for the results of the epigenetic studies on bladder epithelial cells to be put in the context of the urinary microbiome findings.

The investigation of post translational modifications (PTM) in proteins may indicate a link between the bladder and microbiome. PTM's are biologically important in the cellular function including cell-cell interactions; including those involved in host –pathogen interactions. This is important in light of the emerging hypothesis in UCPPS of the role of the microbiome influencing UCPPS – a concept now being investigated by this consortium. The hypothesis is that unidentified pathogenic bacteria and/or fungi are present within the urinary tract and are involved in the host cell-bacteria interactions that cause UCPPS and associated symptom flares. A corollary to this hypothesis is that these features, suggesting disease, will be converted to "normal" upon treatment. Thus, detection of host-bacterial or host-fungi protein interactions would be extremely specific and reflect severity and the underlying pathophysiology underlying each patient. MAPP II will be targeting cysteine residues, which also importantly include S-acylation. S-acylation is a PTM that occurs to proteins involved in **cell-to-cell adhesion,** including those involved in host (human) and microbial interactions. Thus, by targeting this specific subproteome *in the urine* we will be able to specifically identify those key proteins involved.

Characterization of a cohort of patients with pain restricted to the pelvis including urologic and non-urologic symptoms (SPS Aim 2), determine which of these symptoms predict progression (SPS Aim 3), the biological markers associated with progression (SPS Aim 5). Characterize the transition between pelvic pain only and systemic or pelvic pain and beyond with respect to symptoms and non-urologic comorbid conditions (SPS Aim 4).

As outlined above, all of the biomarkers under study may have a role in bladder pain, or pelvic floor dysfunction. They also may play a role in the distinction found between whether a patient has pain localized to the pelvis, thought to be a local process, or pelvic pain and beyond, thought to reflect a more wide spread alteration of nerve dysfunction and possible central sensitization [78-81]. MMPs have been shown to play a role in the early phase (MMP-9) and late phase (MMP-2) of development of neuropathic pain in an animal model of spinal nerve injury [82]. Studying these molecules in UCPPS may be important because therapy based on blocking the MMPs to treat neuropathic pain has been proposed [83]. NGAL has been shown to be important for the development of thermal hyperalgesia and mechanical allodynia in a model of inflammatory pain [84]. These changes are characteristic of central sensitization and have been reported in patients with UCPPS [81,85-87]. NGAL has been may also play a role in some of the psychological symptoms that will be measured including depression [88]. NGAL plays a role in CNS neuroplasticity and low levels of plasma NGAL are reported in depressed persons. Peripheral inflammation and psychological stressors also raise cerebral NGAL expression [89].

TLR-4 has been implicated in multiple CNS pathologic states [90] and therefore may be relevant to looking at CNS changes that produce extra pelvic symptoms in patients with UCPPS. The preliminary findings from Iowa show a correlation between TLR-4 response and pain. Certainly, alterations in the HPA pathway may be important as a feature of a systemic alteration in physiology that is not limited to one organ or part of the body. The current studies add to prior studies indicating alterations in the HPA axis in pelvic pain conditions [16]. As outlined in section 3, the TLR-4 pathway, and cortisol release are also under the influence of psychological stress, and therefore correlation with the battery of psychological tests administered to

patients is important. The data on TLR polymorphisms playing a role in other inflammatory conditions, including bowel inflammation [35,36] is important given the possible role in bladder inflammation, or any contribution of bowel inflammation to bladder symptoms given overlapping innervation of these two organs [91]. Another asset will be linking the biomarker studies to the use of smart phones. Assessment of a non-invasive stress- and inflammation-related biomarker in conjunction with symptom data by combining the smart phone application with cortisol assessments will enable us to combine methods across working groups to provide a multidisciplinary understanding of longitudinal changes in symptom patterns and biological precursors of changes in symptoms.

5. Study Hypotheses

The study will address the following hypotheses:

Hypothesis 1.	a) Patients with increased MMP levels will be those with bladder pain phenotype b) Elevation of urinary markers related to TNF-alpha signaling including NGAL will correlate with bladder symptoms c) Alterations in VEGF levels will be associated with the bladder symptom phenotype d) We hypothesize that the activity status, and changes in that status, of the urinary MMPs identified as biomarkers of UCPPS in our Phase I studies may be reflective of these clinical conditions. We hypothesize that by identifying the activity state of the urinary MMPs, we may identify yet a second, more specific group of related biomarkers, *i.e.* the latent and/or active form of these MMPs that can provide even more accurate assessment of disease status and stage.
Hypothesis 2	a) Specific proteins in the urine and/or plasma obtained from UCPPS patients reflect and capture the individual mechanistic diversity of UCPPS and that these can shift at the onset during flares and will revert to their individual baseline with effective treatment b) Post translational modifications in urinary proteins will correlate with changes in microbiome and predict symptom severity
Hypothesis 3.	a) Neuroinflammatory profiles observed at baseline derived from a) HPA diurnal profile and b) pro-inflammatory cytokine response to TLR-2 and TLR-4 stimulation (TLR-2/4 responsiveness) in peripheral blood will predict progression from pelvic (local) to systemic pain, flare frequency and severity, and likelihood of UCPPS patients improving or worsening during longitudinal follow-up. b) Patterns of change over time in these neuroinflammatory biomarkers will parallel symptom improvement and worsening and flare changes over time. Specifically, sustained elevations or increases in TLR-2/4 responsiveness and sustained or increased flatness of the HPA diurnal profile over time will be associated with symptom worsening and increased frequency, severity, and onset of flares. c) In UCPPS patients with pelvic pain only, neuroinflammatory biomarker profiles at baseline and over time will predict likelihood of progressing from local

	("centralized") to systemic pain (pelvic pain and beyond). In UCPPS patients with neither urinary urgency nor pain on bladder filling, neuroinflammatory biomarker profiles at baseline and over time will predict likelihood of progression of bladder pain syndrome (BPS) symptoms (painful filling, painful urgency). d) UCPPS patients with comorbid non-urological associated syndromes (NUAS: *e.g*, FM, IBS, migraines, vulvodynia) will have greater evidence of HPA and inflammatory dysregulation both at baseline and over time than UCPPS patients without comorbid syndromes. e) Flare onset/duration will associate with a flatter cortisol slope and increased TLR-2/4 responsiveness. Patterns of acute and chronic ongoing life stress will be associated over time with flatter cortisol slopes, and greater TLR-2/4 responsiveness. f) Specific polymorphisms in the TLR pathways will be associated with an increased or decreased likelihood of particular symptom complexes; the onset and severity of BPS symptoms, and the likelihood of flares and progression of symptoms over time g) Patients with altered cortisol or stress phenotype will have the non-bladder phenotype indicative of pelvic floor dysfunction, and also will correlate to neuroimaging findings of altered cerebellar connectivity h) TLR2 and 4 receptor genetic polymorphisms coupled with findings from the urinary microbiome will predict the bladder phenotype
Hypothesis 4.	a) We expect that DMRs of peripheral lymphocyte DNA will distinguish UCPPS patients with only pelvic pain (PP only) from those with pelvic pain and pain in other body regions (PP and beyond). b) We expect that LTL will discriminate among patients based on pain severity ratings. c) We expect that these biomarkers will be associated with pain variables, suggesting a mechanism that operates across related chronic pain disorders.

6. Specific Aims

Each of the above hypotheses motivates a series of specific aims that will be the focus of the Biomarker Trans-MAPP II study and are outlined below:

6.1. Aims Related to Hypothesis 1

Specific Aim 1a	**Patient with increased MMP levels will be those with bladder pain phenotype**
Specific Aim 1b	**To assess the clinical utility of multiplexing all biomarkers validated in our studies with biologic and clinical data identified and validated by other Trans-MAPP working groups**
Specific Aim 1c	**To elucidate the mechanism(s) underlying the presence of disease, disease flare, response to intervention and resolution**

6.2. Aims Related to Hypothesis 2

Specific Aim 2a **To identify clinically relevant non-invasive protein biomarkers of UCPPS through deep rolling proteomics analysis of urine and plasma to identify flare and the individual's response to clinical treatment**

Specific Aim 2b **To identify aberrant signaling pathways based on alterations to protein post-translational modifications that have been implicated in chronic pain.**

Specific Aim 2c **Determination of S-acylation (palmitoylation) of proteins involved in the host response and microbial interactions using Palmitoyl Protein Identification and Site Characterization (PalmPISC) in urine as an indicator of flare onset.**

6.3. Aims Related to Hypothesis 3

Specific Aim 2a **To prospectively characterize the role of neuroinflammation and stress pathways with respect to urological and non-urological longitudinal symptom patterns and flares in a Trans-MAPP cohort of UCPPS patients. This aim will identify biomarkers indicative of TLR activation and HPA dysregulation that predict UCPPS symptom patterns and their changes over time.**

Specific Aim 2b **To characterize TLR -2 and 4 genetic polymorphisms in patients and controls**

6.4. Aims Related to Hypothesis 4

Specific Aim 3a **Compare methylation patterns in peripheral lymphocytes obtained from two groups of UCPPS patients - PP only and PP and beyond - to determine the role of DMRs.**

Specific Aim 3b **Assess LTL among UCPPS patients based on pain severity ratings.**

Specific Aim 3c **Examine methylation patterns in peripheral lymphocytes as well as LTL in relation to pain intensity, pain duration, and psychological functioning among all participants.**

7. Brief Description of Supporting Preliminary Data

7.1. Specific Aim 1:

During the last funding period of this MAPP initiative (MAPP I), we have leveraged the power of our interactive MAPP-Research Network and our own extensive experience in the discovery and validation of noninvasive biomarkers of human diseases, to successfully identify a panel of urinary biomarkers for UCPPS using two distinct approaches.

We first focused on the urinary matrix metalloproteinase profile for the following reasons. Inflammation has been suggested to be an underlying pathophysiologic mechanism in UCPPS [92] and increased concentrations of pro-inflammatory cytokines (IL-1β, TNF-α, IL-8) have been reported in patients with UCPPS as compared with controls [93,94] In this respect, MMPs, distinguished by their zinc-and calcium-dependent proteolytic properties, are of particular interest[95,96]. Originally thought to be associated only with tissue remodeling and destruction, different MMPs expressed in humans have now been implicated in a variety of biologic processes including cytokine and growth factor release, tumor growth, and inflammatory responses [63]. MMP levels in urine have been shown by us, and now by others, to be elevated in patients with a variety of human diseases, including, but not limited to, cancer and other non-neoplastic diseases, and recent evidence suggests that MMPs may play an active role in the pathophysiology of intestinal inflammatory diseases as well [63]. During the inflammatory response, MMPs are released from connective tissue cells in response to the proinflammatory cytokines TNF-α and IL-1β [97]. Upregulation of MMP activity results in the recruitment

of proinflammatory cells to the site of tissue injury [98,99]. MMPs have also been implicated to be important players in the regulation of the immune response because of their ability to cleave inflammatory mediators and stimulate the clearance of inflammatory cells [100-103].

Given that MMPs may play an important role in UCPPS as a function of the inflammatory phenotype and the dysregulation of extracellular matrix (ECM) turnover present in this and other related diseases, we analyzed urine samples collected and banked by the MAPP consortium, a total of 502 samples, for the presence of a panel of MMPs that have been shown to play a role in inflammation and dysregulated ECM regulation as described above [104]. All samples provided by MAPP TATC were de-identified and were analyzed in our laboratory in a double-blinded manner using mono-specific ELISAs for these proteins [105,106]. These data and their analyses are discussed below [107].

In the course of our studies of UCPPS literature, we also learned that there is an interesting and potentially important link between the vascular endothelial growth factor (VEGF) and interstitial cystitis [108]. It has been reported that VEGF levels are increased in bladder biopsy samples from patients with interstitial cystitis (IC) compared to control patients, with levels correlated with pain severity [65,109]. Interestingly, there also appears to be a provocative and novel connection between neovascularization and IC, in that the new blood vessels in biopsy samples of IC patients show significantly lower levels of pericyte coverage of the nascent endothelium, evidence consistent with the previously known association between high levels of VEGF and immature vessel formation [65]. We have significant experience in the study of this and other angiogenic mitogens both *in vitro* and *in vivo* [110-112] and chose to add this protein as well as its receptor, VEGFR-1, to our list of candidate urinary proteins to be analyzed.

Finally, review of the literature as well as our laboratory's own experience, revealed that Lipocalin-2 (Lcn2; NGAL (neutrophil gelatinase-associated lipocalin)) levels are upregulated in numerous chronic and acute inflammatory conditions, including chronic kidney disease, ulcerative colitis and myocardial infarction [69,70]. There exists an intricate interplay between Lcn2 and other inflammatory cytokines as well, such that Lcn2 upregulation can be triggered by multiple inflammatory cytokines, including such as TNF-α, IL-1 β and IL-17 [69,70]. Moreover, Lcn2 itself can be a modulator of the levels of other inflammatory cytokines as well as of the behavior of inflammatory leukocytes [113-115]. In our own studies, we have demonstrated that Lcn2 is a significant positive stimulator of VEGF levels in breast tumor cells and can also independently induce the epithelial to mesenchymal transition in human breast cancer, as well as being a noninvasive biomarker of this disease [112,116]. For these reasons, we chose to ask whether Lcn2 might be present in the urine of individuals with UCPPS and whether it might provide useful clinical information. Using monospecific ELISAs that we had previously validated for use with human urines [105,106], we analyzed all 502 urine samples provided by the MAPP consortium as indicated above.

The analysis of our 6 biomarkers identified many highly specific biomarker patterns that appear to be sensitive to disease severity and gender. During the initial development of the cohorts to be analyzed, the MAPP-EP identified what appeared to be a phenotypic difference amongst participants, leading to the development of two specific cohorts. The first set of 251 participants included the samples from what were identified as the *"most" severe* of the UCPPS cohort in the MAPP-EP study and the second set of 251 participants were considered *"less" severe* as determined by specific phenotyping metrics. We performed separate biomarker analyses of the two different cohorts and identified considerable differences (Table 1 A-D, Table 2 E-G). In females with *more severe* UCPPS, urinary NGAL ($p = 0.014$) and VEGF ($p = 0.018$) are

significantly elevated in UCPPS as compared to normal controls (Table 1A). However, the females with less severe UCPPS had lower levels of urinary MMP-2 as compared to normal controls (Table 2E). When comparing female UCPPS to positive controls, both *more* and *less severe* female UCPPS demonstrated lower levels of urinary MMP-2 (Table 1C, 2G). This difference was much more significant in the *more severe* patients (p=0.002) (Table 1C). As for male patients with UCPPS, when compared to normal controls, urinary MMP-2 was lower (Table 2F), and VEGF-R1 was elevated only in male patients with *less severe* UCPPS (Table 2H). ROC curves for all of these data are provided in Figures 1 and 2. It is essential to note that the ROC curves presented are only univariate at this point in time. Overall, we have identified biomarkers that are specific to gender and the severity of UCPPS. Consistent with the identification of distinct clinical phenotypes during the MAPP-EP study, our findings further support distinct pathologic phenotypes and, in addition, we identify noninvasive urinary markers that may be used to monitor and predict disease severity and disease response to therapy in future intervention trials.

A. Urinary Biomarkers for Differentiating UCPPS and Healthy Controls (NC) – Females				
Biomarker	**UCPPS (N = 59)**	**NC (N = 27)**	**AUC**	**p-value**
MMP-2, ng/mL	0.28 (0.10-0.44)	0.20 (0.06-0.38)	0.554	0.42
MMP-9, ng/mL	0.22 (0.11-0.62)	0.31 (0.18-1.53)	0.549	0.47
NGAL, ng/mL	**7.9 (3.6-18.0)**	**3.39 (1.35-9.43)**	**0.665**	**0.014***
MMP-9/NGAL, ng/mL	0.07 (0.01-0.16)	0.09 (0.00-0.33)	0.529	0.67
VEGF, pg/mL	**85.3 (39.5-128.0)**	**40.1 (14.2-98.8)**	**0.660**	**0.018***
VEGF-R1, pg/mL	13.7 (8.0-32.2)	18.7 (11.0-50.1)	0.599	0.14
B. Urinary Biomarkers for Differentiating UCPPS and Healthy Controls (NC) – Males				
Biomarker	**UCPPS (N = 90)**	**NC (N = 24)**	**AUC**	**p-value**
MMP-2, ng/mL	0.20 (0.09-0.32)	0.31 (0.08-0.41)	0.579	0.23
MMP-9, ng/mL	0.08 (0.03-0.35)	0.13 (0.05-0.43)	0.524	0.72
NGAL, ng/mL	1.54 (0.26-4.82)	2.58 (1.06-6.59)	0.520	0.76
MMP-9/NGAL, ng/mL	0.06 (0.01-0.17)	0.11 (0.03-0.20)	0.550	0.45
VEGF, pg/mL	87.8 (42.5-158-2)	105.1 (72.6-159.0)	0.548	0.47
VEGF-R1, pg/mL	13.2 (6.2-28.7)	14.6 (4.9-33.7)	0.525	0.71

C. Urinary Biomarkers for Differentiating UCPPS and Positive Controls (PC) – Females				
Biomarker	**UCPPS (N = 59)**	**PC (N = 26)**	**AUC**	**p-value**
MMP-2, ng/mL	**0.28 (0.10-0.44)**	**0.51 (0.33-0.85)**	**0.711**	**0.002***
MMP-9, ng/mL	0.22 (0.11-0.62)	0.50 (0.11-1.52)	0.584	0.22
NGAL, ng/mL	7.9 (3.6-18.0)	6.35 (1.85-17.17)	0.564	0.35
MMP-9/NGAL, ng/mL	0.07 (0.01-0.16)	0.11 (0.03-0.35)	0.578	0.26
VEGF, pg/mL	85.3 (39.5-128.0)	77.8 (33.9-114.3)	0.560	0.38
VEGF-R1, pg/mL	13.7 (8.0-32.2)	25.7 (8.9-34.8)	0.573	0.29
D. Urinary Biomarkers for Differentiating UCPPS and Positive Controls (PC) – Males				
Biomarker	**UCPPS (N = 90)**	**PC (N = 25)**	**AUC**	**p-value**
MMP-2, ng/mL	0.20 (0.09-0.32)	0.26 (0.13-0.32)	0.532	0.62
MMP-9, ng/mL	0.08 (0.03-0.35)	0.25 (0.07-0.63)	0.571	0.28
NGAL, ng/mL	1.54 (0.26-4.82)	3.35 (1.22-8.77)	0.564	0.33
MMP-9/NGAL, ng/mL	0.06 (0.01-0.17)	0.06 (0.04-0.19)	0.526	0.69
VEGF, pg/mL	87.8 (42.5-158-2)	102.8 (54.5-174.8)	0.535	0.60
VEGF-R1, pg/mL	13.2 (6.2-28.7)	16.0 (6.6-38.5)	0.500	1.00

Table 1 A-D. Statistical analyses of the 6 biologically-driven urinary biomarkers on the first set of 251 patients from the MAPP-EP study. Tables are separated by healthy control (NC), positive control (PC=other non-urological-associated syndromes, including fibromyalgia, chronic fatigue syndrome and irritable bowel syndrome) and gender.

* Represent statistically significant p values, as further demonstrated in the ROC curves in Figure 1.

E. Urinary Biomarkers for Differentiating UCPPS and Healthy Controls (NC) – Females				
Biomarker	**UCPPS (N = 90)**	**NC (N = 49)**	**AUC**	**p-value**
MMP-2, ng/mL	**0.10 (0.00-0.39)**	**0.20 (0.07-0.57)**	**0.602**	**0.048***
MMP-9, ng/mL	0.07 (0.02-0.37)	0.08 (0.01-0.55)	0.502	0.97
NGAL, ng/mL	0.37 (0.23-1.08)	0.52 (0.21-2.14)	0.526	0.61
MMP-9/NGAL, ng/mL	0.00 (0.00-0.14)	0.05 (0.00-0.14)	0.572	0.16
VEGF, pg/mL	29.4 (18.9-44.4)	29.5 (16.9-43.7)	0.517	0.73
VEGF-R1, pg/mL	7.9 (4.4-12.9)	6.7 (3.7-13.1)	0.538	0.46
F. Urinary Biomarkers for Differentiating UCPPS and Healthy Controls (NC) – Males				
Biomarker	**UCPPS (N = 31)**	**NC (N = 25)**	**AUC**	**p-value**
MMP-2, ng/mL	**0.18 (0.08-0.30)**	**0.38 (0.02-0.84)**	**0.640**	**0.07†**
MMP-9, ng/mL	0.01 (0.00-0.06)	0.05 (0.01-0.14)	0.619	0.13
NGAL, ng/mL	0.29 (0.14-0.82)	0.40 (0.19-0.77)	0.563	0.58
MMP-9/NGAL, ng/mL	0.04 (0.00-0.11)	0.05 (0.00-0.13)	0.545	0.57
VEGF, pg/mL	59.0 (27.0-105.4)	57.3 (28.1-76.7)	0.539	0.62
VEGF-R1, pg/mL	8.8 (2.3-12.0)	9.7 (4.3-14.2)	0.521	0.79

G. Urinary Biomarkers for Differentiating UCPPS and Positive Controls (PC) – Females				
Biomarker	**UCPPS (N = 90)**	**PC (N = 49)**	**AUC**	**p-value**
MMP-2, ng/mL	**0.10 (0.00-0.39)**	**0.23 (0.03-0.52)**	**0.590**	**0.08†**
MMP-9, ng/mL	0.07 (0.02-0.37)	0.13 (0.00-0.71)	0.526	0.62
NGAL, ng/mL	0.37 (0.23-1.08)	0.48 (0.32-1.37)	0.576	0.14
MMP-9/NGAL, ng/mL	0.00 (0.00-0.14)	0.04 (0.00-0.15)	0.570	0.17
VEGF, pg/mL	29.4 (18.9-44.4)	26.8 (17.7-51.6)	0.513	0.80
VEGF-R1, pg/mL	7.9 (4.4-12.9)	7.0 (3.6-13.8)	0.532	0.54
H. Urinary Biomarkers for Differentiating UCPPS and Positive Controls (PC) – Males				
Biomarker	**UCPPS (N = 31)**	**PC (N = 7)**	**AUC**	**p-value**
MMP-2, ng/mL	0.18 (0.08-0.30)	0.09 (0.00-0.25)	0.618	0.34
MMP-9, ng/mL	0.01 (0.00-0.06)	0.02 (0.00-0.08)	0.528	0.82
NGAL, ng/mL	0.29 (0.14-0.82)	0.63 (0.28-1.51)	0.613	0.36
MMP-9/NGAL, ng/mL	0.04 (0.00-0.11)	0.00 (0.00-0.13)	0.558	0.64
VEGF, pg/mL	59.0 (27.0-105.4)	29.5 (30.6-108.8)	0.571	0.56
VEGF-R1, pg/mL	**8.8 (2.3-12.0)**	**0.0 (0.0-4.1)**	**0.869**	**0.003***

Table 2 E-G. Statistical analyses of the 6 biologically-driven urinary biomarkers on the **SECOND** set of 251 patients from the MAPP-EP study. Tables are separated by healthy control (NC), positive control (PC) and gender.

* Represent statistically significant p values, as further demonstrated on the ROC curves in Figure 2.

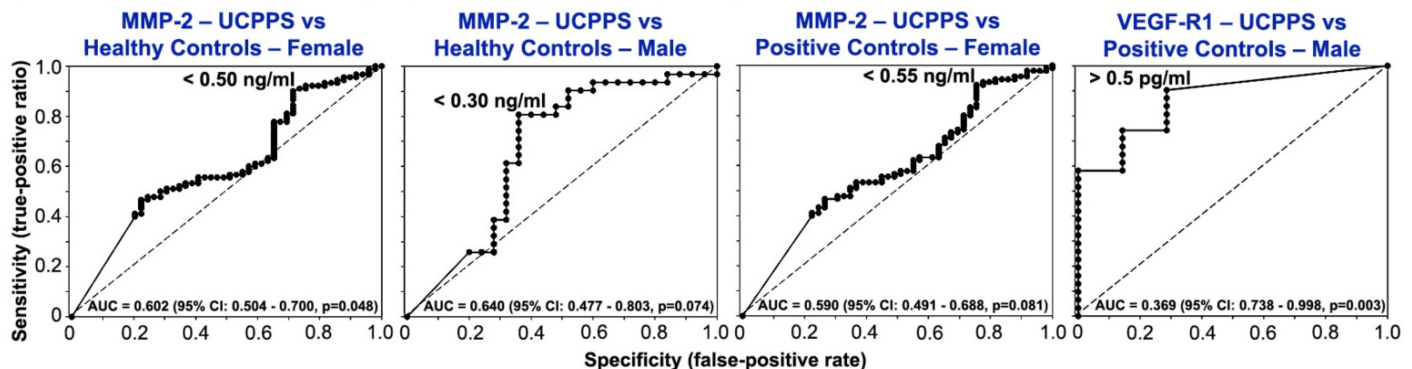

Figure 1. The initial analysis of the 251 "most" severe UCPPS patients identified in the Trans-MAPP EP demonstrated that in females, urinary MMP-2 distinguished between UCPPS patients and positive controls as well as between positive controls and healthy controls. Additionally, urinary NGAL and VEGF were elevated in female UCPPS vs. healthy controls.

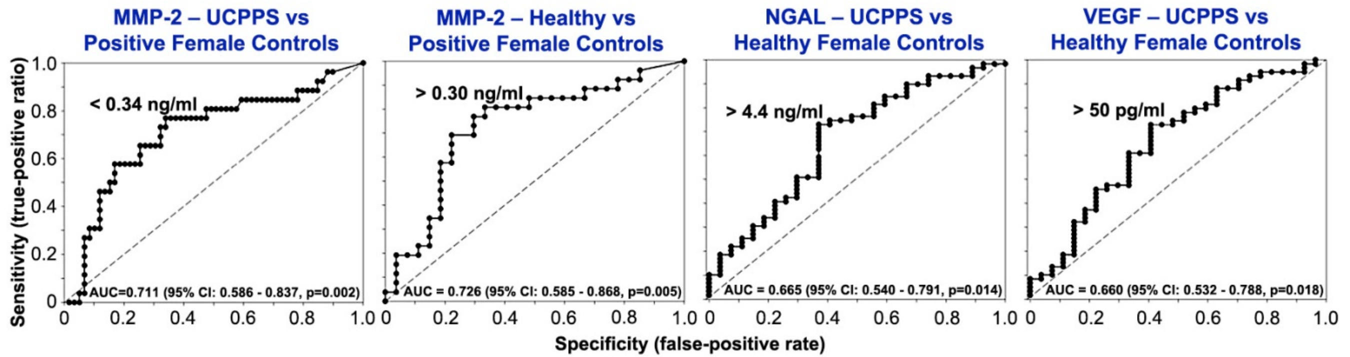

Figure 2. Analysis of the second set of patients (251) with "less" severe symptoms of UCPPS demonstrated that MMP-2 and VEGF-R1 are markers of UCPPS in this cohort of patients. In females, levels of urinary MMP-2 distinguished UCPPS from both normal and positive controls. In males of this cohort with less severe UCPPS, urinary MMP-2 was lower as compared to controls, and VEGF-R1 was higher as compared to positive controls. This significant distinction as compared to the "more" severe UCPPS patients is potentially highly relevant and may delineate a significant subtype of UCPPS.

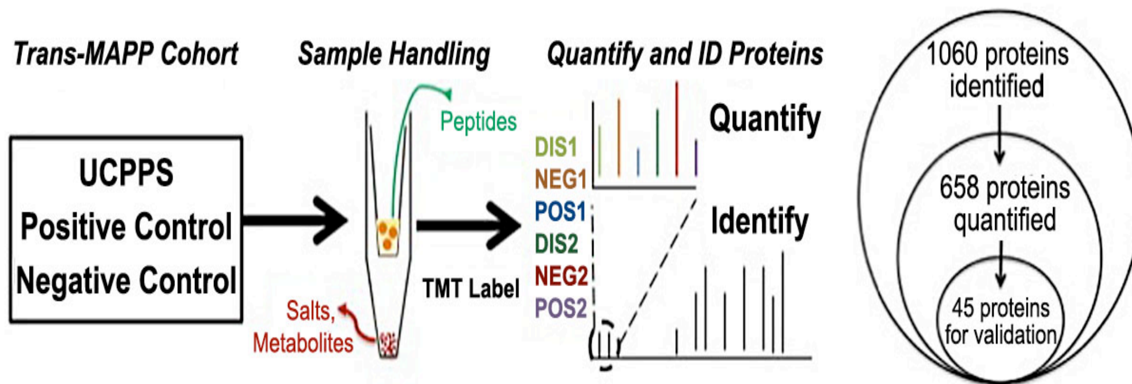

Figure 3. An unbiased global discovery quantitative proteomics analysis of 36 pairings of UCPPS and controls (N=108 samples) from the MAPP-EP study were used to identify new candidate markers of UCPPS. 1060 proteins were identified from which we identified 45 candidate biomarkers for further validation

Our second discovery approach was an unbiased global discovery effort of the urinary proteome of UCPPS patients and controls. A critical challenge of proteomic discovery studies is that protein quantities are often underestimated and their fold-change can be depressed [117,118]. Interrogating fluids that are in direct contact with the primary affected organ can enhance the discovery effort by identifying relevant proteins that would otherwise not be well characterized in secondary fluids (*e.g* serum). To identify the most promising UCPPS markers, we performed a three-way discovery study, in which we compared the urine from UCPPS vs. positive and negative controls (Figure 3). Using our previously described One-Step technology [119] we determined the relative protein quantitative differences to identify the most promising markers.

GeneID	UC:PC	UC:NC
K1C9	1.09	2.63
K2C1	1.01	2.59
K22E	1.23	2.50
UTER	2.48	2.47
CASPE	-1.24	1.75
TFF2	-1.74	-1.44
TETN	-1.10	-1.72
LAMP2	-1.05	-1.68
PEBP4	-1.25	-1.67
ZA2G	1.64	1.29
A2MG	1.63	1.12
GALNS	-1.18	-1.63
SEMG2	1.58	-1.13
LAMA4	-1.58	-1.20
K1C10	1.18	1.57

Table 3. Top 15 of the 45 candidate markers identified in the global discovery analysis and their respective quantitative fold change to the control groups. In addition to the fold change, markers demonstrated quantitative consistency across the entire cohort and were statistically significant in ≥60% or more case pairings. Directed MS analysis (Phase II) will significantly improve the quantification of all 45 markers to identify the best additional markers.

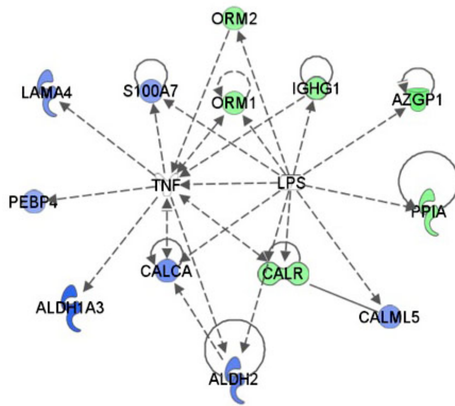

Figure 4. Most proteins that interact with LPS are increased (green) in UCPPS relative to controls, whereas the majority of proteins that interact with TNF-α are decreased (blue). In concert, these patterns may suggest increased LPS signaling and decreased TNF-α signaling in UCPPS.

Applying highly stringent MS criteria, we quantified 1060 proteins and found 288 proteins with significant quantitative differences (Figure 3). We identified 45 promising markers using multiple protein inclusion criteria such that these urinary markers are: 1) cohort-wide statistically significant; 2) identified in 4 or more case control pairings; and 3) demonstrated a quantitatively consistent trend in ≥60% of the pairings (Table 3).

To perform effective directed MS quantitation (Phase II), a rigorous and focused peptide target list representing the 45 potential markers is needed. To improve the selection of the unique peptide targets, we have performed a novel comparative analysis of the UCPPS data to a urine biomarker discovery study of kidney obstruction (R01 DK096238). Peptides that were observed in both urine studies were given priority for targeted assays, as the presence in these very different patient cohorts increases a peptide's robustness for MS detection. We removed those peptides with problematic modifications, limited abundant candidates (to a maximum of 5 peptide targets) and limited lower abundant candidates (to 7 peptide targets). For the potentially challenging targets, a small number of proteotypic peptides from Peptide Atlas (www.peptideatlas.org) were also selected based on empirically determined frequency of observation in other laboratories. Subsequently, we developed a set of 254 high fidelity peptide targets that represent the 45 potential biomarkers that will permit simultaneous unbiased directed quantitation in the directed MS assays.

To assess the biologic relevance of the identified markers, we performed an analysis of biological significance that revealed numerous interrelated pathways and common protein groups. This is an approach that is uniquely suited to discovery proteomics, as our in-depth analysis identified many of the proteins involved in highly interesting biological pathways and demonstrated significant quantitative differences in key proteins. For instance, a large subset of the candidate markers relate to each other in that they all are induced or inhibited by lipopolysaccharide or TNF leading to changes in leukocyte activation (7 proteins, p=0.0054) (Figure 4) and calcium trafficking (5 proteins, p=0.017).

These pathways and proteins are of particular relevance to multiple research groups within the MAPP Research Network (*e.g* Translational Animal Models Consortium and University of Iowa – LPS-stimulation studies). Particular examples include Orosomucoid-1 (ORM1/A1AG1), which interacts with both LPS and TNF. ORM1 is both a pro-inflammatory acute-phase response protein, in that it induces additional release of IL-1, TNF-α, IL-6 and IL-12 by leukocytes [120] and an anti-inflammatory in that it can prevent excessive inflammatory responses by inhibiting both neutrophil chemotaxis [121] and the generation of superoxide [122] and by inducing the secretion of soluble TNF- α receptor to inhibit further downstream signaling of TNF- α [123]. Considering the excessive inflammatory response in UCPPS urines, and the increased quantitative levels of ORM1 in UCPPS, it is possible that the anti-inflammatory efficacy of ORM1 is compromised in UCPPS. Another compelling example is uteroglobin. Uteroglobin is also an extremely interesting candidate as a potential selective urinary biomarker of UCPPS. Although the exact biological role of uteroglobin is unclear, uteroglobin is known to inhibit phospholipase A2 (PLA2) [124,125], which is critical

to the proinflammatory arachidonic acid pathway. Interestingly, PLA2 is induced by increased calcium, which is one of the molecules that appears to play a key role in our analysis. Inhibition of PLA2 by uteroglobin may indirectly decrease the production of arachidonic acid and disrupt the inflammatory pathway. Annexin A1 (ANXA1) is one of only 3 known genomic inhibitors of PLA2 and it was also identified as a significant marker in our studies. ANXA1 is known to have a substantial anti-inflammatory role relating to IL-10, nitric oxide synthesis, and inflammatory pain [126-131]. Overall, the biological and pathway analyses of our preliminary results have identified interesting targets of a highly integrated and dynamic protein network that underlies the disease pathology of UCPPS. These proteins may be relevant as diagnostics and/or therapeutic targets for the treatment of UCPPS.

7.2. Specific Aim 2

None

7.3. Specific Aim 3

Preliminary Work and Rationale. All data unless otherwise indicated are from Iowa site-specific research in MAPP I**.** For homogeneity of presentation, female data is presented below unless otherwise indicated; however, patterns for male patients paralleled those for females.

7.3.a. UCPPS patients have an exaggerated inflammatory response compared to healthy controls.

We screened 17 cytokines and chemokines following 72-hour PBMC stimulation with 7 different antigens and found the IL-1β and IL-6 response to TLR-2 (SAC-1) and TLR-4 (LPS) agonists differentiated most clearly between UCPPS and healthy controls (HC). LPS is from Gram-negative bacteria (endotoxin), and SAC-1 is from Gram-positive bacteria (staphlococci). TLR-2 stimulation in UCPPS patients (n=58) induced significantly greater IL-1 β (*p*=.04), and a trend to higher IL-6 (*p*=.09) compared to HC (n=28). (Figure 5). This suggests that with the greater power of the proposed MAPP II research we will observe differences in both TLR-2 and TLR-4 responsiveness between groups. UCPPS participants also had significantly elevated plasma IL-6 compared to HC's (*p*=0.04) suggesting an elevated level of basal inflammation. All analyses adjusted for BMI.

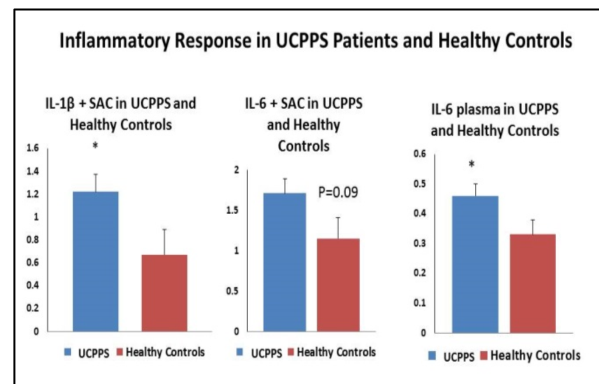

Figure 5.

7.3.b. Cortisol is dysregulated in UCPPS patients compared to healthy controls.

Salivary cortisol, a reliable assessment of the unbound, biologically active fraction of cortisol in general circulation [132], was collected at home in salivettes for 3 days on awakening, 4-6 pm, and bedtime. We had a high compliance rate (87.7%) with salivette completion and return. UCPPS patients had a significantly flatter cortisol slope (*p*=.01) and significantly diminished cortisol variability ([morning cortisol- nocturnal cortisol]/morning cortisol) compared to HCs (UCPPS: 62.5% decrease from morning to night vs. HC: 86.3%; *p*=.005), indicating a blunting of the diurnal cortisol rhythm in participants, with elevated nocturnal levels.

7.3.c. TLR-4 responsiveness is associated with pain in UCPPS.

Composite scores for stimulated cytokine responses were calculated by summing the z-scores for the IL-6 and IL-1β response in PBMCs following either TLR-4 or TLR-2 stimulation. (A z-score is a standardized score represented in units of the standard deviation; it is often used when making a composite score from two different variables). **The pro-inflammatory cytokine response to TLR-4 stimulation was a robust predictor of UCPPS symptoms, particularly pain severity and pain frequency.** The TLR-4 z-score was associated with significantly higher GUPI total scores (p=.005), GUPI pain scores (p=0.006), GUPI urinary symptom scores (p=.034), poorer FSFI sexual functioning (p=.002), and higher IC symptom index scores (p=0.037). Patients with longer symptom duration, and those with UCPPS plus NUAS, had greater TLR-4 responsiveness (p=.037, p=0.01, respectively). Notably, among patients reporting pelvic pain only significantly less TLR-4 responsiveness was noted as compared to patients with pelvic plus systemic pain (p<0.005). **These findings suggest that TLR-4 inflammatory responsiveness is associated with more broadly based and more long-lasting pain, consistent with the concept of central glial sensitization.** Higher TLR-4 z-scores were associated with significantly flatter cortisol slopes (p=.043), indicating greater inflammation is associated with more dysregulated diurnal cortisol rhythms, and suggestive of disrupted feedback. **The consistency of the association of TLR-4 responsiveness with pain suggests that this will be a useful model for examination of symptom and flare patterns over time.**

7.3.d. Algorithm to predict Pain Symptoms in UCPPS patients.

A combination of flatter HPA slope and TLR-4 responsiveness was predictive of pain symptoms in UCPPS patients. Specifically, those patients with the flattest cortisol slopes and the greatest TLR-4 responsiveness had the highest level of pain symptoms on a number of indicators of pelvic pain including GUPI pain frequency (p < .01), BPI pain intensity (p=0.02), and pain with sexual activity (p=0.001). As seen in Figure 6, the Body Map graphically illustrates that those patients with low nocturnal cortisol and low TLR-4 inflammation tend to have localized pain, whereas those with high nocturnal cortisol and high TLR-4 inflammation tend to have reports of moderate to severe systemic pain. These findings demonstrate that these biomarkers are clearly mapped onto UCPPS symptoms and indicate that a biomarker cluster will be useful in longitudinal predictions in MAPP II.

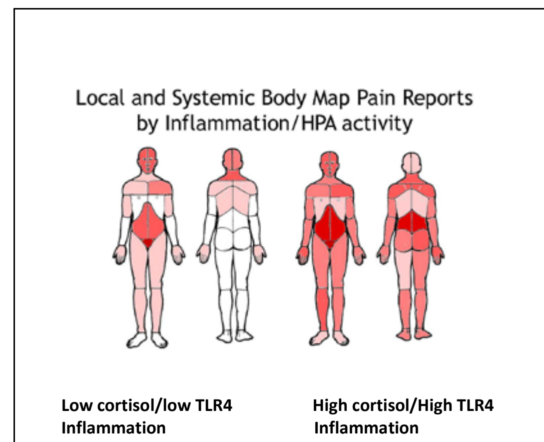

Figure 6

7.4. Specific Aim 4

7.4.a. DNA Methylation in Peripheral Blood Cells.

We conducted a pilot study in 8 pairs of MZ female twins discordant for UCPPS, as defined by MAPP criteria, and 4 pairs of healthy MZ controls. The index twin in each case pair reported an unpleasant sensation of pain, pressure, or discomfort in the bladder or pelvic region, which was associated with LUT symptoms such as painful filling or urgency. Symptoms were present on the day of the study visit and for most of the 3 months before enrollment. The co-twins in discordant pairs, and both twins in concordant control pairs, were healthy and pain-free. MZ twins are ideal for this type of study, because any differences within twin pairs are

by definition related to environmental exposures and not genetics, including the heritability of methylation itself.

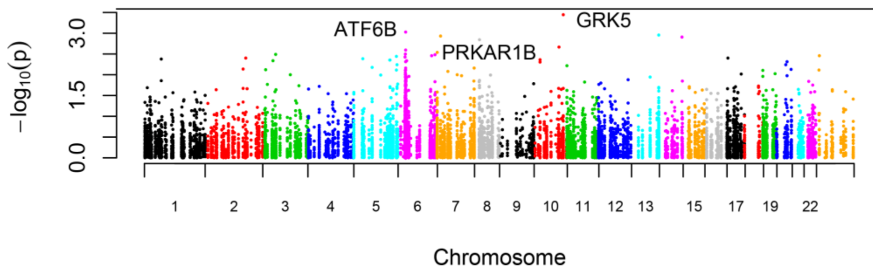

Figure 7. Methylation and case status

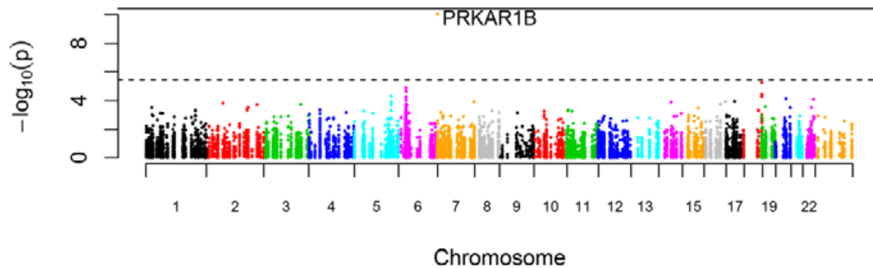

Figure 8. Methylation and depression

We assessed DNA methylation levels of peripheral blood cells by using the Illumina HumanMethylation450 Bead Chip. Samples from each of the 24 participants were randomly assigned to one of 2 chips for analysis, which was completed by using the MethLAB program. Given our small sample size, analysis of methylation sites was restricted to genomic locations identified as relevant to pain in the Algynomics Pain Research Panel v 2.0. Figure 7 shows the Manhattan Plot for methylation as a function of case status. Evaluation of the plot reveals a stacked peak of increasingly significant DMRs in the correlated CpG regions in Chromosome 6. No individual loci were significant after correcting for multiple comparisons, likely because of low power for dichotomous comparisons. Gene labels for the 2 most significant points ($p < 0.001$) are included. The association with PRKAR1B is included based on the next results.

After evaluating DMRs as a function of case status, we investigated whether methylation is related to depression, which is comorbid with UCPPS and other chronic pain conditions, as measured by the Hospital Anxiety and Depression Scale (HADS). Figure 8 shows the Manhattan Plot for methylation as a function of depression scores on HADS. Evaluation of the plot reveals a similar stacked peak in Chromosome 6 and a statistically significant relationship with PRKAR1B. These results suggest a set of DMRs that distinguish UCPPS cases from controls in peripheral blood cells and a similar set of DMRs that are associated with levels of depression. We did not have bladder epithelial cells available, but we believe that MAPP II will provide the ideal context to evaluate methylation hypotheses between cases and controls.

7.4.b. LTL in Peripheral Blood Cells.

As our next step, we conducted a pilot study with the same 8 pairs of female MZ twins discordant for UCPPS. During the study visit twins underwent an experimental bladder pain protocol to induce IC-like systems while undergoing brain MRI. Using a 10-point scale, they rated their pain after the scan but before voiding. LTL was measured by quantitative PCR. The amount of telomeric DNA was divided by the amount of control-gene DNA, producing the T/S ratio, a relative measurement of the sample’s LTL. Two control samples were run in each experiment to allow for normalization between experiments.

In 5 of 8 pairs, LTL was shorter in the UCPPS twins than in healthy co-twins (mean T/S ratio = 1.12, SD = 0.07 vs. 1.13, SD = 0.06). Mean differences were also in the hypothesized direction. Shorter telomeres were associated with pain ratings during the bladder instillation procedure (B = -0.029, p = .010) and with levels of HADS depression (b = -11.052, p < .0005). Figure 9 shows the association between LTL and bladder pain severity. As with the methylation data, the lack of statistical significance on the dichotomous case/control variable was not surprising, given our small sample of MZ twin pairs. Variability in LTL will always be greater between people with different genotypes, as was demonstrated in the statistically significant analysis of pain severity independent of case status.

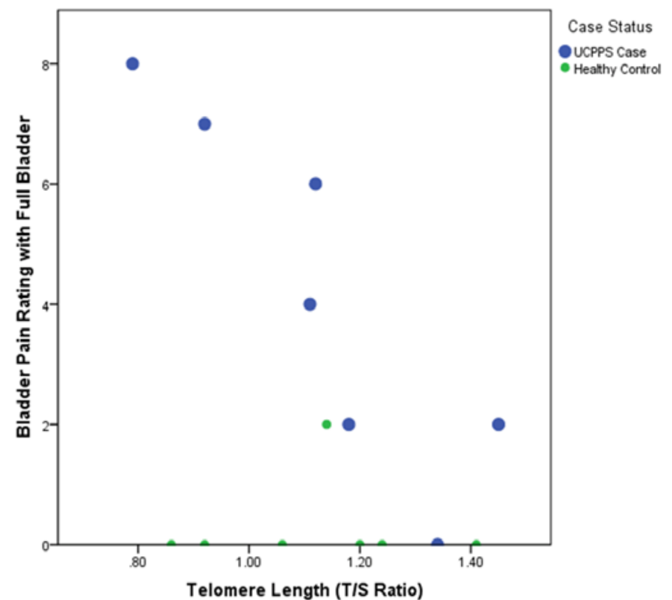

Figure 9. LTL and bladder pain ratings

These results are both promising and consistent with other LTL research on chronic pain and chronic stress. LTL has been associated with severity and duration of symptoms in affected individuals, even in studies that show no statistically significant differences in LTL associated with chronic pain or stress. [60-62] Studies that detect no differences between dichotomous groups are often underpowered for that purpose, but still have adequate power to detect differences in continuous variables such as severity or duration. MAPP Network resources will enable us to estimate the absolute difference in LTL associated with UCPPS, compared to healthy controls, and the association between LTL and symptom severity and duration in UCPPS patients.

8. STUDY DESIGN AND METHODS

8.1. Specific Aim 1

Specific Aim 1a To assess the utility of the identified and validated urinary biomarkers from Phase I in the clinical management of UCPPS

We hypothesize that directed quantitation of validated urinary biomarkers of UCPPS will identify a select panel of biomarkers that have clinical potential to diagnosis UCPPS, to predict disease status longitudinally, to guide therapy, and to detect disease improvement and resolution. Our preliminary data demonstrate our ability to identify quantitative differences in defined UCPPS cohorts using both biologically-driven directed analyses and global discovery quantitative proteomics. The next step in our discovery work is to refine the candidate list of 45 markers identified in our global discovery initiative by corroborating their quantitative presence in a larger *longitudinal* cohort. This “refined” list will be multiplexed with the existing, verified biologically-driven markers, in a longitudinal study of patients with UCPPS at 6, 12 months and during symptomatic flares. *As demonstrated in the Trans-MAPP EP and in our preliminary data, the highly variable disease presentation and inhomogeneity of UCPPS only further emphasize the importance of identifying objective markers of UCPPS that stratify and guide the management of patients*. Analysis of the highly unique flare and longitudinal biosamples will determine whether objective biological measurements can identify or

predict flares, whether they can be used as an independent marker of disease severity and/or as a measure of therapeutic success in prospective intervention trials. Additionally, mechanistic analyses of these samples will potentially identify new therapeutic targets (Aim 1c).

Objective 1 *To complete MAPP Phase 1 global discovery study*

The initial analysis will interrogate a cohort of 502 participants identified in collaboration with the Data Coordinating Center of the MAPP network. This exact same cohort was originally tested in our biologically-driven analysis during MAPP I (Preliminary Studies section 7), specifically, 270 UCPPS (121 males, 149 females), 107 (32 males, 75 females) positive controls (PC), and 125 (50 males, 75 females) negative controls (NC). In-depth phenotyping data and other biologically relevant data are readily available from the MAPP-EP.

Objective 2 *To determine whether the levels of our urinary biomarkers change during disease flares with subsequent recovery post-flare and whether they can predict these clinical stages*

Objective 3 *To determine whether these biomarkers can predict disease status at 6 month, 12 month and longer intervals of monitoring*

Objective 4 *To determine whether these markers can predict disease resolution*

Symptomatic flare and longitudinal (6, 12 months) urine samples from all UCPPS patients obtained from the MAPP-EP (~420) will be assessed by enzymatic (Aim 1b) and monospecific ELISAs for MMP-2, MMP-9, the MMP-9/NGAL complex, NGAL, VEGF, and VEGF-R1 as extensively studied by our group in MAPP I. For the refined global markers, if an established ELISA assay is not available, we will measure the ***absolute quantity*** of each new marker using high-throughput MS-based absolute quantification with Multiple Reaction Monitoring (AQUA-MRM) [133,134].

Specific Aim 1b To assess the clinical utility of multiplexing all biomarkers validated in our studies with biologic and clinical data identified and validated by other Trans-MAPP groups

Objective 1 *To determine whether multiplexing the verified panel of urinary biomarkers from Aim 1a with other biologic markers from the entire Trans-MAPP-RN increases the predictive power of the markers in distinguishing between disease, positive control and healthy control groups both at baseline and in a longitudinal and flare analysis*

In the studies proposed in Objective 1 of this Aim, we will multiplex the urinary biomarkers discovered in our own program (including those in Aim 1a) [107] with biologic data verified by other Trans-MAPP teams including those from the Iowa and TATC teams as well as the Microbiome and Neuroimaging groups. The compilation of these markers will be initially assessed on the well-established MAPP I EP study cohort (~ 1039 patients). For instance, the Iowa group has identified and will be validating plasma IL6, IL1 and MCP1 as potential biomarkers and Tissue Analysis and Technology Core has identified and will be validating urinary CRP and CD276 as potential biomarkers of UCPPS. These data and other objective data that will be forthcoming in the upcoming MAPP II will be multiplexed with the phenotyping data of all patients enrolled in the MAPP I EP study at baseline, longitudinally and at flare, to identify the best markers. The longitudinal and episodic flare testing are essential in order to determine which candidate markers are effective at stratifying patients with UCPPS and predicting outcome and response to intervention, since baseline entry samples may not fully reflect disease state. We hypothesize that this strategy will result in highly accurate predictors of UCPPS disease status and progression, therapeutic efficacy and response as measured by future Patient Reported Outcome (PRO) metrics.

Utilizing the panel of markers identified in Aim 1a, we will perform a Trans-MAPP collaborative analysis of our urinary biomarkers with all available and verified biologic and clinical data, on the entire MAPP I cohort at baseline and at 6, 12 month and at flare (~1039 participants). The MAPP I cohort has undergone extensive state-of-the-art phenotyping analysis.

Objective 2 *To perform a prospective longitudinal and flare analysis of patients enrolled in the Trans-MAPP Symptoms Pattern Study (SPS)*

In Objective 2 of this Aim, the markers will then be further validated in the newly launched Trans-MAPP Symptom Patterns Study (SPS), which is a longer duration study that is specifically designed to enrich for targeted phenotypes. SPS is a three-year, prospective observational study of men and women with UCPPS to identify important factors associated with worsening and/or improvement of reported urinary and non-urinary symptoms. SPS is designed to specifically test the most promising research methods in the pain field at baseline, longitudinally, and flare to better characterize study participants. During MAPP I, most measures were collected only at a single point in time and were obtained from a cohort of UCPPS patients that were not enriched for either worsening or improvement of symptoms. Conversely, a study of the SPS longitudinal cohort will determine which of these markers, for example, particular level of a urinary biomarker combined with a specific neuroimaging marker, might serve as a biomarker panel for an individual most likely to have spontaneous improvement of their symptoms, versus transition to a more “centralized” or “chronified” form (pelvic pain and beyond) of urinary and non-urinary pain. This novel *multi-modal analysis* will lead to the development of various clinically useful biomarker panels that will identify specific subsets of patients, measure disease burden, predict prognosis and flare, and perhaps even lead to the development of novel therapeutics for UCPPS.

Specific Aim 1c To elucidate the mechanism(s) underlying the presence of disease, disease flare, response to intervention and resolution

Within the context of this Aim, we will work to understand the mechanisms underlying the presence of UCPPS, disease flare, response to intervention and disease resolution. We *hypothesize* that the *activity status*, and changes in that status, of the urinary MMPs identified as biomarkers of UCPPS in our Phase I studies may be reflective of these clinical conditions. We hypothesize that by identifying the activity state of the urinary MMPs, we may identify yet a second, more specific group of related biomarkers, *i.e.* the latent and/or active form of these MMPs that can provide even more accurate assessment of disease status and stage. It may also be that we have underestimated the power of our urinary MMP analysis in our samples since the ELISA data, by giving us *total* latent and active enzyme quantitation, could be masking the prominence of *either* the latent or the active form of MMP-2, MMP-9 or MMP-9/NGAL complex. In order to acquire this data, we will analyze all samples obtained from the network by substrate gel electrophoresis (gelatin zymography) to determine the activity states of these urinary enzymes.

In this Aim, we will also assess the presence of TIMPs as well as the MMP/TIMP ratios to determine whether this information increases the power and sensitivity of our biomarkers validated from Aim 1a and whether it unveils new biomarkers of UCPPS. These include MMP activity status *i.e.*, active or latent, as well as the presence and levels of the *endogenous inhibitors* of these enzymes, the tissue inhibitors of metalloproteinases (TIMPs). This will provide useful information because if the increase in the amount of active MMPs exceeds the capacity of the endogenous TIMPs to regulate MMP levels, then uncontrolled inflammation and tissue damage can ensue [135]. These data will then be analyzed to determine whether

they provide mechanistic insights into disease development and progression above. In addition, to having the potential to be additional patient-specific markers, the enzyme activity status may reflect the status of these enzymes *in vivo* which can be confirmed in the future *in vivo* animal studies. These results have the potential to ultimately inform the development of novel therapeutics for this disease by serving as therapeutic targets themselves.

We note here that we will be collaborating with MAPP's Translational Model Consortium to assess the utility of these and our other biomarkers in predicting disease status and stage in their collection of validated animal models of UCPPS. They will be providing us with urine samples from the different stages of disease progression and resolution in their models and we will analyze those samples for the presence of our validated biomarkers.

Objective 1 *To determine the activity status of urinary MMPs in the MAPP I samples*

All urine samples will be analyzed by gelatin zymography as previously reported by us [105,136-140]. The activity status of these proteases will be determined in a number of ways. *In vivo*, these MMPs are activated by a proteolytic cleavage of their pro-enzymes resulting in a molecular weight of the active form that is ~5-10 kDa smaller than the latent form. Since both forms appear on a zymogram as a function of SDS treatment of the sample before analysis, we can distinguish between the presence of these two forms for each MMP species [138]. Urine samples will be analyzed by gelatin zymography both before and after treatment with 1mM APMA (aminophenylmercuric acetate which exogenously cleaves the pro-enzyme portion of latent MMPs into their active form with a concomitant decrease in their molecular weight (MW). This decrease in size is clearly visible on a zymogram, as previously reported [138,141,142]. These are standard biochemical MMP assays with which we have extensive experience [138]. Taken together, the active and latent forms of these MMPs will be determined as a function of MW after activation, or lack thereof, by APMA. Quantitation of the levels of each of the MMP species will be determined via quantitative scanning densitometry as previously reported by us [143-145].

Objective 2 *To determine whether TIMPs, alone or in combination with MMPs, are noninvasive biomarkers of UCPPS*

Urine samples will be assessed for the presence of TIMPs using two distinct biochemical methods. Samples will be analyzed using mono-specific ELISAs for the TIMPs that have been shown to regulate the MMPs, *i.e.* TIMPs 1 and 2 [146]. We will confirm the presence and activity of these TIMPs by reverse zymography, a modification of the zymography previously described which confirms the presence of these TIMPs and their presence on the gel according to their distinctive MW. We have significant experience in assessing these TIMPs by these methods [138,146].

Objective 3 *To analyze and determine the statistical performance characteristics of the active and latent species of MMPs and urinary TIMPs, alone and in combination with each other and with the other biomarkers being multiplexed in Aim 1b, for their ability to provide useful clinical information regarding UCPPS*

Analyses will be conducted in collaboration with the MAPP DCC. These analyses are as described above in Aim 1a. Should specific active and latent forms of the urinary MMPs and/or TIMPs prove to be additional validated urinary biomarkers, we will multiplex them with the validated markers from the studies in Aim 2, Objective 1.

8.2. Specific Aim 2

Specific Aim 2a To identify clinically relevant non-invasive protein biomarkers of UCPPS through deep rolling proteomics analysis of urine and plasma to identify flare and the individual's response to clinical treatment.

We will carry out rolling SWATH to define and quantify 1000s of proteins simultaneously in samples from individuals from MAPP I who have flares and then to follow up as they undergo changes in therapy over time (MAPP II) and correlate there changes in the disease phenotype

Discovery and validation in urine (MAPP I):

Urine and plasma specimens collected from the same individuals during a clinic visit at baseline and during flares and when available those with 1+ flare and an ATLAS event (MAPP I and MAPP II, respectively). Furthermore, baseline samples will be compared to those of matched control urine from healthy individual (from MAPP I) and plasma and positive controls composed of individuals with non-UCPPS pain (from MAPP I).

Method for SWATH-peptide library construction:

The goal for the library is to represent the broadest set of proteins that are quantifiable across this cohort. Thus, the urine and also the plasma peptide library will be built using a two pooled samples: one from disease samples (individuals UCPPS at baseline and during flare) and a second control pool composed of samples from matched age and gender control and positive control individuals from MAPP I (50 ul each from individual samples to create the two pools).

For plasma each pool will undergo depletion of the top twelve highest abundant plasma proteins (using affinity chromatography. The protein concentration of the depleted samples (flow through) will be determined and equal amounts (~100 ug) will be desalted and separated into 20 fractions using reversed phase chromatography. The 20 fractions will be neutralized and undergo tryptic digestion using a sample preparation robotic system (Beckman Coulter) [147]. Quality control includes tracking efficiency of depletion using MRM assays for each of the high abundant plasma samples [147](Fu *et al*.) and 1DE to ensure reproducible depletion and complete digestion. For urine the same protocol employed by the previous biomarker team will be used. However, the digested samples will undergo basic reversed phase fractionation into 20 fractions. Each fraction originating from the pooled urine and plasma samples will be run on the 6600 Triple TOF MS instrument (AB Sciex), which was released in June 2014 and has been in the Van Eyk lab at CSMC since March. The peptide library is computationally created based on these data. Peptides representing human proteins and those fungal and bacterial proteins that will be identified based on the reduced field identified by the microbiome team at CSMC, using the Custom Knowledge-based Fungal Database (FunGu).

Rolling SWATH:

For the subsequent discovery portion and investigation of individual disease process and pathological variability, individual samples from MAPP I and those selected from MAPP II will undergo high abundant protein depletion for plasma and a single-step desalting with the same sample processing and QC measures as outlined above. Importantly, each individual's depleted plasma sample will not be further fractionated but will be directly analyzed on the 6600 Triple TOF using internal and endogenous retention and concentration standards. For urine, samples will be neutralized and desalted with no further fractionation prior to MS analysis. Samples will be blocked and the blocks blinded and randomized. Each block will consist of the

sample collected at baseline and during flares with their age- and gender-matched control and positive control samples. To track sample processing, C13 labeled beta-galactosidase (β-gal^{C13}) is added. C13 labeling ensures that the β-gal^{C13} added to the sample as a processing control can be distinguished from the endogenous protein. In order to quantify the amount of β-gal^{C13} we will use N15 labeled synthetic peptides used in the β-gal MRM assay. MRM and multiple reaction monitoring are MS-based ELISA-like assays that can be used to quantify precisely representative peptides. Based on the MRM data, the CV% and % recovery can be determined and any sample with CV% or % recovery greater than 15% will be reprocessed and rerun.

Potential Challenges for Aim 2a:

Technical issues around individual urine variability in pH, ionic strength and protein concentration will be handled by neutralization and dilution of urine prior to affinity chromatography depletion. Since the equivalent protein is loaded onto the reversed phase column, downstream processing will not be affected. We will determine protein differences based on total protein concentration (wt/wt) and also by amount of starting volume (vol/vol). Plasma does not have the same analytes chemistry differences as observed in urine. Rather we have to deal with the large dynamic range issue due to the fact that the top twelve abundant proteins comprise over 80$% of the plasma proteome. To achieve appropriate proteome coverage it is necessary to deplete these high abundant proteins using affinity chromatography. This step can introduce technical variability which due to tight SOP and QC monitoring assures we can reduce this challenge. We do not expect any issues to arise from the rolling swath pipeline especially as there are extensive QC steps in place. Yet, we recognize that the extent of samples to be analyzed is large and that there will be repeat analyses required. Based on our experience, we expect about 10% of samples will need to be repeated based on greater than 15 %CV or 15% recovery. We will ensure that these are carried out with the appropriate analytical controls.

Specific Aim 2b: To identify aberrant signaling pathways based on alterations to protein post-translational modifications in the urine proteome that have been implicated in chronic pain.

A subset of individuals in MAPP I who experience 1+ flare and their matched controls (and when possible, MAPP II individuals who have a flare and an associated Atlas event) and specifically, those subjects being analyzed in the Microbiome identification proposal by CSMC (See Microbiome Protocol Aim 2c).

Method for SWATH-peptide library construction:

The goal for construction of the peptide library is to obtain the broadest set of proteins that are quantifiable across this cohort. Thus, the peptide library will be built using a two pooled samples: one from disease samples (individuals during flare) and a second control pool composed of samples from age- and gender-matched control and positive control individuals from MAPP I (50 ul each from individual urine sample to create the two pools) but ultimately enriched for Ser/Thr phosphorylation or Cys oxidation. For the phosphorylation-library, the same method as outlined in Aim 1 will used except after digestion, the each fraction will undergo phospho-enrichment using TiOx affinity chromatography. For the Cys-OxPTM-library, urine will undergo a modified version of the classical biotin switch based on initial protein concentration: acetylated to block all free Cys residues and preserve the oxidative modification proteome prior to depletion. After fractionation and digestion with trypsin, each fraction will be reduced with DTT and the previously modified Cys residues will be labeled with biotin HDPD and subsequently enriched using streptavidin. Each

PTM-enriched fraction will be run on the 6600 Triple TOF MS and data analysis carried out as described in Aim 2a.

Rolling SWATH:

Digested samples from each individual will be enriched for TiOx or following biotin switch (no other fractionation will be required) and then analyzed on the 6600 Triple TOF as outline above. The same QC will be carried out.

Specific Aim 2c: Determination of S-acylation (palmitoylation) of proteins involved in the host response and microbial interactions using Palmitoyl Protein Identification and Site Characterization (PalmPISC) in urine as an indicator of flare onset.

A subset of individuals in MAPP I who experience 1+ flare and their matched controls (and when possible, MAPP II individuals who have a flare and an associated ATLAS event) and specifically, those subjects analyzed in Aim 2 and which are enriched in the Microbiome identification proposal by CSMC will be analyzed using Palmitoyl Protein Identification and Site Characterization-Tandom Mass Tag (PalmPISC-TMT) method. This will identify human and bacterial/fungi S-acylation proteins which have differences in the concentrations.

Methods:

PalmPISC, developed by the Freeman-Yang group, is an elegant alteration of the classical biotin switch method (which is used to identify different cysteine-based PTMs) for the selective enrichment of S-acylation proteins in samples. The Van Eyk group, in collaboration with Thermo Scientific, has developed a new MS reagent, Cys-TMT for the biotin switch assay (please refer our editorial on quantification of oxPTMs) for the unambiguous site-specific quantification of cysteine-modified PTMs, allowing site occupancy to be determined and up to 6 samples run simultaneously. We will combine these methods to allow for the quantification at the protein site of modification of S-acylation in urine using the Orbitrap ELITE MS instrument in duplicate. The dual identification of the protein and the exact modified cysteine residues is key to allow us to identify both host (human) and microbiome proteins which may be modified and altered in urine.

Potential problems, alternative strategies and benchmarks for success for Aims 2b and 2c.

We do not expect any insurmountable technical problems, as all proteomic methods described here are currently in use. However, we do recognize and have to deal with issues arising from differences in the proteome/subproteome in diverse biospecimens with distinct chemistry (e.g., urine). Our proteomics team (Van Eyk-Freeman-Yang) is very experienced and should be able to overcome these issues. We predict that UCPPS-associated biomarker candidates will emerge from these studies and that they will reflect multiple mechanisms underlying patient variability in symptoms and treatment response. Furthermore, we expect to identify the host proteins that may be responsible for the initiating events around the microbiome changes in UCPPS. *At a minimum*, we will be able to quantify the disease induced phosphorylation and redox PTMs and host-S-acylation proteins in able to determine the usefulness to increase specificity of the biomarkers. Depending on the concentration and timelines, we may also be able to quantify S-acylation-microbial entities that are involved at the protein level. We predicate that increases in either the host or specific bacteria or fungi will occur during symptom flares and may be detectable and that the underlying disease molecular mechanism will be reflected in the modification of specific cellular proteins. Potentially, blocking these

interactions could lead to effective therapy. Finally, Aim 2b and 2c are being carried out in a small but well-defined cohort and hence will have a longer validation process than Aim 2a.

8.3. Specific Aim 3

Specific Aim 3a **To prospectively characterize the role of neuroinflammation and stress pathways with respect to urological and non-urological longitudinal symptom patterns and flares in a Trans-MAPP cohort of UCPPS patients. This aim will identify biomarkers indicative of TLR activation and HPA dysregulation that predict UCPPS symptom patterns and their changes over time.**

Specific Aim 3b **To characterize TLR -2 and 4 genetic polymorphisms in patients and controls**

Building on our findings from MAPP I, Trans-MAPP studies will be implemented to validate and further characterize a fundamental role for TLR activation and central HPA dysregulation among patients with UCPPS. This will be fully integrated into the biospecimen collection scheme of the proposed prospective MAPP SPS. This approach will have a primary focus on identifying biomarkers individually or in combination that will characterize symptom progression over time in UCPPS and comorbid syndromes (NUAS). Our preliminary data suggest that the combination of HPA slope and TLR-4 responsiveness can be used to predict pain profiles. Although TLR-2 responsiveness differentiated UCPPS from controls, and activation of TLR-2 and TLR-4 is closely interrelated. Therefore, IL-1β and IL-6 cytokine responses to both TLR-2 and TLR-4 will be monitored in all phases of this Aim. **In MAPP I, inflammatory biomarkers were only assessed at the Iowa site and at baseline, such that subgroup analysis was not possible- thus the importance of bringing this work to the Trans-MAPP II setting to validate and extend our findings longitudinally.** Blood for stimulated TLR-2/4 assessments, unstimulated cytokines (*e.g.* IL-6) will be obtained at the week 4 Trans-MAPP 2 Baseline Phenotyping and Biospecimen Collection Clinic Visit along with all assessments outlined in Aim 1 for symptoms and covariates. Thereafter, blood for TLR-2/4 will be obtained at 6, 18, and 36 months and ATLAS visits at Trans-MAPP II clinic visits and salivary cortisol will be obtained for three consecutive days after the week 4 visit [148]. It will be important for cortisol and TLR-2/4 blood assessments to be obtained in conjunction with neuroimaging.

Buffy coats will be collected for genomic DNA extraction, thereby permitting assessment of TLR pathway polymorphisms (PMs). While there are over 100 different PMs in the TLR family, there are 24 that appear most relevant for this research because they specifically affect TLR-2 and TLR-4 signal transduction either directly at the ligand:receptor level (*e.g* TLR-2/4, MD-2, CD14) or indirectly via adapter proteins (*e.g* MyD88, TIRAP/Mal) or further downstream signal propagation proteins (*e.g* IRAK-1) or functional pro-inflammatory effector molecules like IL-6 and TNF-α. We will undertake a 2-pronged approach to assess the utility of TLR PMs in identifying patients at high risk for UCPPS development and progression. In the discovery phase we will use the MAPP I Iowa subset of patients for which we already have TLR-4 and TLR-2 activation data (n = 72) to identify (1) candidate TLR PMs which correlate with functional TLR-2/4 responsiveness and (2) those that distinguish UCPPS patients from healthy controls (HCs). These sets may or may not overlap. Based on prior clinical studies in infectious disease susceptibility [149] plus our data on TLR-4 activation in UCPPS, we expect the strongest associations to be with TLR-4 PMs. However, since TLR-2 activity in our research best distinguished UCPPS patients from HCs, it is likely that TLR-2 PMs will best identify individuals at greatest risk for acquiring UCPPS. Second, in the validation phase, we will assess candidate TLR PMs using the larger MAPP I and II cohorts. The MAPP I cohort contains distinct sets of HCs, positive controls, and UCPPS patients to investigate discriminatory TLR PMs. The MAPP II population, in contrast, will be prospectively assayed for

TLR-2/4 responsiveness along with baseline and longitudinal clinical parameters. In this manner we expect to be able to identify patients particularly at risk for worsening UCPPS due to TLR-4 aberrations.

Biomarker Analytic Methods.

Salivary cortisol will be collected in salivettes (Starsdedt) by participants at two time points (upon waking: 4-9am and bedtime: 8pm-12am) for three consecutive days (post baseline) and seven consecutive days (post two other follow up visits at 6, 18, and/or 36 Month). In our work with MAPP I participants, these two time points showed the greatest abnormalities in cortisol levels. Samples collected outside this time frame will be excluded to maintain homogeneity. Participants will be instructed not to eat, exercise or consume caffeine for thirty minutes prior to collecting a sample. Self-report of collection time has been demonstrated to be reliable and salivary cortisol is stable at room temperature [148]. Salivettes will be analyzed by chemiluminescence immunoassay (IBL, Hamburg, Germany) at the Technical University of Dresden. The lower detection limit is 0.41 nmol/L and inter-assay and intra-assay coefficients of variance are less than 10% [16].

To assess TLR-2/TLR-4 responsiveness we will use the TruCulture System from Myriad RBM. Three 1 mL of whole blood will be drawn into special TruCulture tubes containing (a) LPS (TLR-4 agonist), (b) FLS-1(TLR-2 agonist), and (c) Null, no treatment. Trans-MAPP tubes will be incubated on site at 37°C for 24 hours in a heating block. After 24 hours the specimen will be separated from the cells by using the special valve separator included with the TruCulture system. The tubes will be capped and frozen at -80°C prior to shipment to TATC. An aliquot of the specimen will be used for analysis with two Human CustomMap panels containing 32 cytokine/chemokine markers including IL-6 and IL-1β. While our earlier studies used processed PBMCs, we have since determined experimentally that non-processed whole blood using the TruCulture system will give nearly identical results, allowing us to expand this assay across the entire Trans-MAPP cohort.

Genomic DNA for TLR polymorphism analysis will be isolated from buffy coat collected at week-4 visit from a 10ml ACD plasma vacutainer. Polymorphism determination will be made using a high-throughput automated Fluidigm Dynamic array that exists in the University of Iowa DNA Sequencing Facility that permits simultaneous analysis of 24 different PMs in batches of 192 genomic DNA samples per plate at a fraction of the cost of conventional PCR methods [150].

8.4. Specific Aim 4

Specific Aim 4a	**Compare methylation patterns in peripheral lymphocytes obtained from two groups of UCPPS patients—PP only and PP and beyond--to determine the role of DMRs.**
Specific Aim 4b	**Assess LTL among UCPPS patients based on pain severity ratings.**
Specific Aim 4c	**Examine methylation patterns in peripheral lymphocytes as well as LTL in relation to pain intensity, pain duration, and psychological functioning among all participants.**

DNA Methylation:

The laboratory work will be conducted by Dr. Szyf's group at McGill University. Significant DMRs by tissue type will be identified by comparing the methylation patterns in our cases and controls, adjusting for age and sex. Genome-wide profiling of CpG methylation will be performed by the Illumina HumanMethylation450 BeadChip, which provides a quantitative determination of DNA methylation at a single CpG site resolution.

This platform offers comprehensive coverage, low sample input requirement (500ng genomic DNA), and high sample throughput capacity (~485,000 methylation sites per sample). Samples will be randomly assigned to 96-well plates. Bisulfite conversion of monocyte genomic DNA will be performed by using QIAGEN 96-well bisulfite conversion kits, which converts unmethylated cytosines to uracils, while methylated cytosines remain unchanged. Samples will then be desulphonated and eluted by using column preparation. The bisulfite-treated DNA samples will be hybridized to arrays, then methylated or unmethylated nucleotides will be detected by a single-base primer extension that includes different labels for methylated and unmethylated alleles. The fluorescently stained chip will be imaged by the Illumina BeadArray Reader, and fluorescence data will be analyzed by using BeadStudio software to assign site-specific DNA methylation β-values to each CpG site. The DNA methylation level at each CpG site will be computed by subtracting the background signal intensity of negative controls from each signal, which will then be calculated as a ratio (β-value) of the signal intensity from the methylated probes to the total intensity of both methylated and unmethylated probes. The β-values range continuously from 0 (unmethylated) to 1 (fully methylated). We will remove data points if their detection *P*-value is > 0.05, and probes if the proportion of missing data exceeds 5% across the study sample.

Leucocyte Telomere Length:

LTL will be measured by quantitative PCR (Q-PCR) in the laboratory of Dr. Rosana Risques at the University of Washington. Measuring LTL by Q-PCR is based on the principle that more telomeric DNA will produce more PCR amplification. For every sample, 2 PCRs are performed: the first to amplify telomeric DNA and the second to amplify a single-copy control gene (36B4, acidic ribosomal phosphoprotein PO). This approach provides an internal control to normalize the starting amount of DNA. A 4-point standard curve (2-fold serial dilutions from 10 to 1.25ng of DNA) is included in all PCRs to allow the transformation of cycle threshold into nanograms of DNA. All samples are run in triplicate and the median is used for calculations. The amount of telomeric DNA is divided by the amount of control-gene DNA, producing a relative measurement (called the T/S ratio) of LTL of the sample. Two control samples are run in each experiment to allow for normalization between experiments. Potential outlier samples with extremely long or short LTLs are repeated to confirm measurements.

9. Description and Source of Patient Cohorts Beyond those from the SPS, including controls

Patients will be participants in the MAPP SPS study. No patient cohorts beyond those from the SPS study are needed.

10. Contact Schedule and Participant Procedures

Collections will follow the Trans-MAPP SPS visit schedule. The schedule of specimen collection is listed in Table 4

Table 4. Schedule of Biologic Specimen Collection for Biomarker protocol (Appendix 1: SPS Protocol timeline), (see Microbiome Working Group Protocol for schedule of microbiome specimen collection)

Blood Collection	Blood specimen 10mL ACD plasma vacutainers (Plasma and Buffy Coat DNA)	4 weeks, 6, 12, 18, 24, 30, 36 months and ATLAS visits
	STIM TUBES Blood for stimulated cytokines3 x 1 mL whole blood in TruCulture tubes pre-loaded with LPS, FSL-1 or Null (control)	**Monday, Tuesday, Wednesday, and Thursday clinic visits only** 4 weeks, 6, 18, 36 months and ATLAS visits
Biomarker Urine	Spot urine specimen Biomarker/VB2 (up to 90mL)	4 weeks, 6, 12, 18, 24, 30, 36 months and ATLAS Visits
Saliva	Salivary cortisol samples	2 times a day, waking and bedtime • 3 day period after week 4 visit • 7 day period after 6 and 18 Month visits • 7 day period after 36 Month visit IF one or more scheduled collections were missed

- 12, 24, 30 months visits are optional.

11. Risk Factor and Outcome Measures

Common Data Elements (CDEs) from Trans-MAPP Epidemiology Study

We will use all the same questionnaires and data points from the Trans MAPP SPS study

Specialized Data Measures

No specialized data measures are needed. Biological findings from the BWG will be analyzed in the context of the questionnaires from the Trans MAPP SPS, neuroimaging and microbiome studies.

12. Biological Specimens

Biologic specimens collected during the course of the Trans-MAPP Study include urine (biomarker/microbiome, supernatant and DNA), blood (plasma and DNA), blood for stimulated cytokines, and salivary cortisol samples. The schedule of these collections is summarized in Table 4. Biospecimen collection kits will be used to simplify and standardize collection of body fluids for the expressed purpose of optimizing their collection for research. The TATC will provide MAPP Network-specific specimen collection

kits for use by Discovery Sites as needed. Requests for kits will be done through an online ordering system located on the DCC MAPP Network Portal and request will be direct the TATC. The collection kits and components are barcoded, and will be linked with the participant at the time of registration of the participant through the DCC MAPP Network Portal. Collected specimens will then be shipped to the TATC for processing, aliquoting, and inventory. The collection and handling procedures will follow the guidelines established by the NIH Best Practices Policies for biorepositories (www.biospecimens.cancer.gov). No patient identifiers will be used on the collection tubes and tracking forms. As specimens are received from sites, they will be scanned into the biorepository database, and archived in the appropriate freezer/storage unit until needed. Specimen tracking information will be entered into the database by TATC personnel.

Blood, ACD Plasma: All blood samples will be drawn into barcoded vacutainers supplied with the plasma collection kit. Blood collected in 10 ml ACD plasma vacutainers will be shipped overnight to TATC. Tubes received in the lab are spun at 1,000x *g* for 10 min resulting in separation of plasma, buffy coat, and red blood cells. The plasma will be separated into 0.5 mL aliquots and frozen. Buffy coat will be transferred to a cryotube and frozen until DNA is isolated using standardized kits and methods, quantity and quality will be assessed using Nanodrop ND-1000 and Qubit® 2.0 Fluorometer instruments. All samples are barcoded appropriately, catalogued in the database, and stored in a cryogenic freezing unit. Documentation of sample origin, acquisition, transportation, processing, and storage will be provided for each sample. Date/time tracking will be done to ensure proper processing times are followed and potential problem samples identified.

Blood, STIM Tubes: To assess TLR-2/TLR-4 responsiveness we will use the TruCulture System from Myriad RBM. Three 1 mL of whole blood will be drawn into special TruCulture tubes containing (a) LPS (TLR-4 agonist), (b) FLS-1(TLR-2 agonist), and (c) Null, no treatment. Trans-MAPP tubes will be incubated on site at 37°C for 24 hours in a heating block. After 24 hours the specimen will be separated from the cells by using the special valve separator included with the TruCulture system. The tubes will be capped and frozen at -80°C prior to shipment to TATC. Up on receipt at TATC the tubes are thawed and aliquoted into 0.25 mL aliquots and frozen. The cell fraction from under the separator will be transfer to a tube and frozen for possible future RNA isolation and analysis. All samples are barcoded appropriately, catalogued in the database, and stored in a cryogenic freezing unit. Documentation of sample origin, acquisition, transportation, processing, and storage will be provided for each sample. Date/time tracking will be done to ensure proper processing times are followed and potential problem samples identified.

Biomarker urine: For urine collection during clinic visits a 90 mL barcoded urine collection cup will be supplied with the kits. After clean-catch first-void urine collection, using an alcohol-free BZK wipe, a dip-stick analysis will be performed to include glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrite, and leukocytes. Two barcoded 50-mL conical tubes will then be filled, immediately frozen and stored at -80°C, and shipped to TATC in batches. Upon receipt by the TATC, the urine is thawed and spun at 2,500x *g* for 10 minutes, and the sediment is collected and discarded. After separation, the supernatant is frozen in separate 3 mL aliquots and stored at -80°C. Documentation of sample origin, acquisition, transportation, processing, and storage will be provided for each sample. Date/time tracking will be done to ensure proper processing times are followed and potentially problematic samples identified.

Cortisol: Salivary cortisol will be collected in salivettes (Starsdedt) at home by participants at two time points (upon waking: 4-9am and bedtime: 8pm-12am). Samples are stable at room temperature and will be returned to TATC.

13. Other Measures

No other new measures will be required

14. Analytic Plan

14.1. Specific Aim 1a

Objective 1: The median normalized levels of the proteins tested from directed MS will be compared between UCPPS, PC and NC using nonparametric Mann-Whitney *U*-tests with the appropriate FDR used to account for multiple comparisons and control the false positive rate [151]. Fold-change data will be analyzed to identify potentially important proteins that differentiate UCPPS, PC and NC from each other using a 2-fold difference as the effect size for each protein to determine sensitivity and specificity, with 95% confidence intervals constructed using the normal approximation to the binomial distribution [152]. Univariate comparisons will be used to identify candidates to be included in the multivariate logistic regression to determine the optimal set of independent predictors of UCPPS [153]. In addition, receiver-operating characteristic (ROC) curve analysis will be applied to determine area under the curve (AUC) for each significant multivariable protein biomarker in distinguishing cohorts and the composite AUC for combined predictive proteins [154]. Power analysis indicated that the sample size of 502 UCPPS, PC and NC subjects would provide 80% power to detect the clinically relevant 2-fold differences for the proteins being studied. Two-tailed values $p < 0.05$ with Bonferroni adjustment will be considered statistically significant. We will also apply classification and regression trees (CART) analysis to develop rule-based prediction of UCPPS based on the multivariate prediction biomarkers.

Objective 2: The biomarkers tested will be assessed for group differences using Mann-Whitney *U*-tests and logistic regression analysis to examine the combination of markers multiplexed together. ROC curves will be generated to identify optimal cut-off values for predicting clinically significant UCPPS from other pain states using the nonparametric min-max approach and then smoothed and diagnostic accuracy, sensitivity, specificity, and area under the curve determined with 95% confidence intervals [155,156]. Optimal cut-off values for significant biomarkers will be identified using the Youden index. Based on the final set of predictive biomarkers, an algorithm will be developed and the *c*-statistic used to assess the quality of prediction of UCPPS, with a model having c greater than roughly 0.70 indicating good clinical utility (a value of *c* of 0.5 indicates random predictions and a value of 1 indicates perfect prediction (*i.e.*, perfect separation of UCPPS and controls). Patients will be followed longitudinally with serial measurements and during episodic flares and changes in the biomarker profile will be compared over time to determine changes in patients relative to baseline levels using repeated-measures mixed-model analysis of variance (ANOVA) with a generalized estimating equations (GEE) approach [157] (73). The likelihood ratio test and Wald tests will be used to assess significance. These studies will be conducted by our longtime collaborator, Dr. David Zurakowski, in collaboration with the MAPP DCC.

14.2. Specific Aim 1b

Objective 1: Because of the inherent variability of the UCPPS cohort, a collaborative multivariate analysis will likely be needed to identify subset panels of novel biomarkers that can identify specific phenotypes of UCPPS. This will allow for stratification of patients and provide new objective measurements of disease response to intervention. Our longstanding colleague, Dr. Zurakowski will work with the DCC, as they have during MAPP I, to conduct these analyses. Once the validated panel is established, we will conduct K-clustering analyses of all objective biomarker data to identify patient clusters in a unbiased fashion.

The statistical strategy will involve several multivariate approaches including logistic regression, segmentation modeling, k-clustering and classification and regression trees (CART) to identify optimal combinations of urinary biomarkers for differentiating UCPP patients and controls with area under the ROC curve used as a measure of predictive accuracy and the Youden index applied to identify optimal cut-off values in order to maximize sensitivity and specificity (74). Odds ratios and 95% confidence intervals will be calculated for significant predictors to provide clinical assurance of the derived splitting and clustering rules from CART and logistic models. PROC LOGISTIC and GENMOD in SAS v.9.2 (SAS Institute, Cary, NC) will be used for logistic modeling and SPM v.7.0 Salford Predictive Modeler (Salford Systems, San Diego, CA).

Objective 2: The newly launched Trans-MAPP SPS is specifically designed to enrich for targeted phenotypes, as well as to specifically test the most promising research methods in the pain field at baseline, longitudinally, and flare to better characterize study participants. Utilizing the “best” panel of biomarkers identified in Objective 2 (above), all baseline, longitudinal and flare samples will be analyzed using techniques established during MAPP I. Subsequently, in combination with the DCC, regression-based strategies including multivariate logistic regression and classification and regression trees (CART) analysis as well as longitudinal data analysis using generalized estimating equations (GEE) with a binomial distribution and logit link function will be employed to develop a combined algorithm for predicting UCPPS by considering urinary biomarkers as well as changes in other defined clinical markers over time. These will include combining the significant independent predictive urinary biomarkers, neuroimaging, infectious etiology and other objective data with patient specific phenotype variables using multivariate logistic regression modeling to determine probability of UCPPS versus positive control and healthy control and applying maximum likelihood estimation to develop a multivariable predictive algorithm which will incorporate other biological and clinical data, such age, gender and neuroimaging data as covariates, with subgroup analysis to identify how biomarkers and patient-related sensory and pain characteristics can provide accurate prediction of UCPPS [158]. Based on the combined algorithmic statistical approach we will provide diagnostic characteristics including sensitivity, specificity, positive and negative predictive values (PPV and NPV) with 95% confidence intervals for all developed models. SAS and SPM software packages will be utilized for statistical analysis and regression modeling to help develop the predictive multivariate models of UCPPS.

14.3. Specific Aim 1c

Please see Section 6.

14.4. Specific Aim 2

14.4.a. Specific Aim 2a and 2b

For SWATH, data dependent acquisition files are imported into Peakview at a 5% protein FDR to generate the peptide spectral library. SWATH acquisition files will be searched against the peptide spectral library using the SWATH processing software within Peakview. Non-redundant peptides representative of specific proteins or their isoforms are extracted at 1% FDR in the SWATH processing software using a minimum of 5 transitions per peptide. Quantification and statistical analysis are performed in Markerview.

14.4.b. Specific Aim 2c

MS data obtained from the Orbitrap Elite MS instrument will be searched against the NCBI and/or Uniprot protein databases for human and subsets of bacteria and fungi, based on the results from the microbiome data obtained on these individuals We will utilize the PASS platform, which combines data obtained from 3 different search engines, X!Tandem, OSMA and Mascott, maximizing MS spectra assignment for the discovery based analysis. Data are filtered for high confidence identifications (<1% FDR).

14.5. Specific Aim 3

Statistical Analyses and Power. Cortisol values at each collection point will be regressed on the time of collection over the sampling period to calculate slope. Composite scores for stimulated cytokines will be calculated by summing the z-scores for the IL-6 and IL-1β response in whole blood following either LPS or FSL-1 stimulation as done previously. Symptoms will be assessed as outlined in the SPS; those of most interest include: pain (SYMQ-1, GUPI Pain Subscale), lower urinary tract function (ICSI-Total) and non-urological pain (SYMQ-6).

Hierarchical linear modeling, allowing for calculation of within-subject change and adjusting for covariates such as age, BMI, and sex will be used to assess relationships between the following variables: 1) HPA slope and TLR-2/4 z score at baseline with UCPPS symptom progression, flare frequency, severity, and improvement or worsening over time (**H_o:1a**); 2) Changes in HPA slope and TLR-2/4 z score over time and changes over time in UCPPS symptoms (improvement or worsening) and frequency, severity, and onset of flares over time; (**H_o:1b**) 3) Changes in HPA slope and TLR-2/4 z score over time and progression from regional to systemic pain (pelvic pain and beyond) over time (**H_o:1c**); 4) Changes in HPA slope and TLR-2/4 z-score over time in UCPPS patients with and without comorbid non-urological syndromes (**H_o:1d**) 5) Patterns of acute stress with initiation, severity, frequency, and duration of flares and chronic ongoing life stress (PSS over time) and HPA diurnal profile and TLR-2/4 responsiveness (**H_o:1e**). Best practices for disaggregation of within-subject and between-subject change will be employed [159]. For the TLR PMs, genotype frequency will be calculated and tested for Hardy-Weinberg equilibrium between UCPPS patients vs. controls in the entire MAPP 1 cohort. Allele and genotype frequency between UCPPS patients vs. controls will be investigated using a Chi-squared homogeneity test. The Kruskal-Wallis rank sum test will be used for analysis of the association between TLR PMs and TLR-2/4 responsiveness (TLR-2/4 Z score), cortisol slope, and graded measures of UCPPS intensity, worsening, and flares (**H_o:1f**). Odds ratios (ORs) and corresponding 95% confidence intervals will be calculated to measure the association between TLR PMs and the risk of UCPPS severity and evolution. Data analysis with be facilitated using the "Power for Genetic Association analyses" (PGA) package which comprises algorithms and graphical user interfaces for sample size and minimum

detectable risk calculations using SNP or haplotype effects under different genetic models and study constraints. The software accounts for linkage disequilibrium and multiple comparisons [160].

14.6. Specific Aim 4

14.6.a. Analysis of Specific Aims 4a, 4b, and 4d

These Aims propose to identify UCPPS and comorbid symptom-related DMRs. Regression models will be used to examine the association between DNA methylation and UCPPS status (Aims 3a and 3b), and between methylation and continuous ratings of pain intensity, pain duration, and psychological functioning (Aim 3d). We will evaluate dichotomous and continuous variables with by using logistic and linear regression, respectively. Models will adjust for potential confounders such as sociodemographic and behavioral variables, especially those associated with longitudinal symptom change in the Trans-MAPP SPS. Separate analyses will be conducted for each CpG probe or genomic region of interest by using MethLAB or similar software and adjusting for multiple comparisons by using standard methods in methylation.

14.6.b. Analysis of Specific Aims 4c and 4d

Aim 3c proposes to assess whether LTL is associated with UCPPS and with continuous ratings of pain intensity, pain duration, and psychological functioning (Aim 3d). We will use linear regression to examine the association between LTL and the independent variables of interest. We will evaluate dichotomous and continuous variables by using logistic and linear regression, respectively. Models will adjust for potential confounders.

15. STATISTICAL ANALYSIS AND SAMPLE SIZE ESTIMATES

15.1. Specific Aim 1

Power analysis indicated that the sample size of 502 UCPPS, PC and NC subjects would provide 80% power to detect the clinically relevant 2-fold differences for the proteins being studied. Two-tailed values $p < 0.05$ with Bonferroni adjustment will be considered statistically significant. We will also apply classification and regression trees (CART) analysis to develop rule-based prediction of UCPPS based on the multivariate prediction biomarkers.

15.2. Specific Aim 2

We will address what proteins are differentially expressed in UCPPS patients' urine and serum specimens compared to control and positive controls in MAPP I. We will use a two-sided two-sample T-Test with a false discovery rate (FDR) of 0.05. For each test the individual test alpha is 0.00096 (Power analysis performed with PASS 11 Software ENREF33). Furthermore, the association between clinical severity and phenotypes (such as central vs. pelvic vs. bladder) and subsets of protein changes in urine will be determined based on quantitative differences. In addition, protein changes will be ranked based on biological importance or potential interplay between groups of proteins and biological implications. This will be carried out i) every 50 samples and ii) as a continuous variable.

Validation of potential markers will be carried out during MAPP II for those individuals who have at least one flare event that is associated with a change in medication (a flare/ATLAS event). However, replication and the use of other appropriate independent cohorts is outside the scope of the current proposal. Our

recommendation for validation is that candidate proteins (up to 50) have their absolute quantity determined by MRM assays. As implied above, with MRM assays peptides that are unique to the target proteins are quantified based on the addition of a known amount of N^{15} stable isotopic labeled synthetic peptide (IS) that is composed of the same amino acid sequence as the targeted peptides. The advantage of MRM is that small volumes are required (10-25 ul per multiplex), does not require antibodies (unless sensitivity issue) and it is easy to multiple without any matrix effects. The MS data analysis will be handled using the same peptide library comprised in Aim 1.i. and combined with the phospho- and OxPTM SWATH library described.

15.3. Specific Aim 3

Most of the effect sizes noted in our pilot data on neuroinflammatory markers were at least medium to large. For example, the cross-sectional association between TLR-4 mediated inflammation and GUPI total scores and GUPI pain scores are R^2=.12 (GUPI total) and R^2= .08 (GUPI pain). Assuming that cross-sectional variability correlates at least moderately with longitudinal variability, with the accrual of the proposed 640 patients we anticipate that we should have more than adequate power (at least 80%) to conduct all proposed analyses examining symptom trajectories. If we cut the proposed sample size to 320 participants, this would translate to power of greater than .99 to detect a significant association between TLR-4 mediated inflammation and both GUPI total and pain subscale at any given time-point. If participants are further divided by gender or pain status (pelvic pain only vs. pelvic plus) resulting in groups of 160 participants, power would be .99 (GUPI total) and .96 (GUPI pain). If participants are divided on both gender and pain status, resulting in groups of 80 participants, power would still be .91 (GUPI total) and .75 (GUPI pain). Even if effect sizes were only half that anticipated, power would still be .99 (GUPI total) and .97 (GUPI pain) in the full group, and .89 (GUPI total) and .73 (GUPI pain) if groups were divided on either gender of pain status.

In analyzing risk factors for longitudinal symptom trajectories, it may be useful to conceptualize TLR-4 mediated inflammation as a categorical risk factor. Using simple median splits on TLR-4 mediated inflammatory score, total GUPI score, and GUPI pain score reveals that high TLR-4 mediated inflammation yields a risk ratio of 2.08 for being in the high total GUPI score category, and a risk ratio of 1.89 for being in the high GUPI pain score category. This means that a simple dichotomization of the patient sample on TLR-4 mediated inflammation, resulting in a sample with 50% present and 50% absent for the risk factor (TLR-4 mediated inflammation), yields substantial differences in the likelihood of a patient having high total GUPI symptoms or GUPI pain symptoms. In accordance with SPS Aim 1, it may be desirable to separate trajectories into categories, *i.e.*, “improvers” and “not-improvers”. If a risk ratio of 2.00 holds for TLR-4 mediated inflammation as a predictor of improvers vs. not-improvers, then following Table 3 in the longitudinal symptoms study document, a sample size of 320 will result in nearly 90% power at a=.05 if the prevalence of improvers vs not-improvers is 15% vs. 30%, and greater than 90% power if the prevalence is 20% vs. 40%.

Regarding TLR PM analysis, while no studies examining TLR PMs in UCPPS have ever been reported, in over 30 studies of similar or smaller magnitude populations involving various infectious diseases using just TLR-2/4 PMs, significant differences between diseased vs. control patients have been found in about half of the studies.[148] Typically these studies tested between 2-6 PMs, so by increasing our PM number to 24, we should be much more likely to find differences.

15.4. Specific Aim 4

Power. We present power estimates for a range of potential sample sizes that reflect the number of participating MAPP sites. For Aims 3a and 3b, **Table 5** shows the mean difference in DNA methylation between UCPPS patients and controls for which we will have at least 90% power to detect, assuming a range of standard deviation estimates. At the University of Washington (UW) site alone, we will have power to detect differences in methylation between 8% and 22%. If all MAPP sites participate, we will have 90% power to detect differences as small as 3%. Power was calculated at the genome-wide significance level ($\alpha = 1 \times 10^{-7}$, 0.05/485,000). For Aim 3c, we used data from the pilot study described in Section 7.3b and assumed mean (SD) LTL in the control subjects to be 1.13 (0.06); an alpha error rate of 0.05 was used. The UW site alone will have 90% power to detect a 4% difference in LTL between UCPPS patients and controls, as shown in **Table 6**. If all MAPP sites participate, we will have 90% power to detect as low as a 1% difference in LTL between groups. All power calculations were performed with G*Power 3.1.3 and based on 2-tailed independent samples t-tests

Table 5. Detectable mean difference between UCPPS patients and controls with 90% power

MAPP sites	Sample size	SD of methylation 0.05	0.10	0.15
1	96	0.08	0.15	0.22
3	288	0.04	0.08	0.12
6	576	0.03	0.06	0.09

Table 6. Detectable effect size with 90% power

MAPP sites	Sample size	Effect size	Detectable % difference
1	96	0.67	4%
3	288	0.39	2%
6	576	0.28	1%

16. Proposed involvement of MAPP Network Cores

a) Tissue Analysis and Technology Core (TATC)

The TATC will be responsible for providing standardized specimen collection kits, specimen processing, banking, annotation/blinding, distribution services across the MAPP Research Network. The TATC will also be involved in manuscript preparation.

b) Data Coordinating Core (DCC)

The Data Coordinating center will be responsible for data management and for providing data from the Trans MAPP study for the BWG studies. The DCC will also be involved in manuscript preparation.

17. Timeline for Completion of Data/Specimen Collection (see attached Appendix 1: SPS Protocol Timeline)

18. Anticipated Burden to Sites

Urine specimen collection is routine patient care and should not present a burden to sites. For the stimulated cytokine study sites are asked to collect three 1 mL tubes of blood per patient at the designated timepoints and incubate and process these specimens at the site.

19. Anticipated Risks to Participants

Urine specimen collection is routine patient care and should not present a burden to participants. Blood collection is also routine patient care but several more tubes than usually obtained in the evaluation of

UCPPS will be requested. However, the number of actual needle sticks will not be significantly increased over routine care, and the volume of blood obtained poses no physiological risk.

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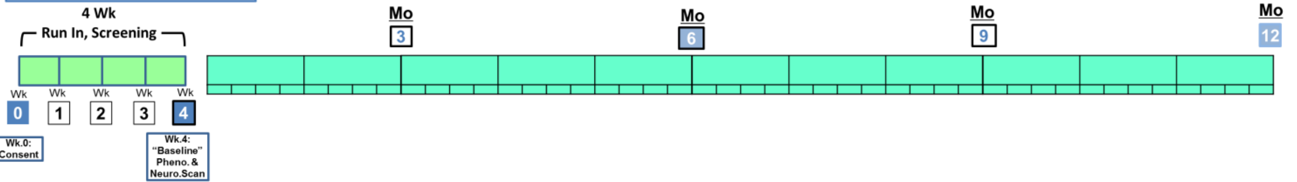
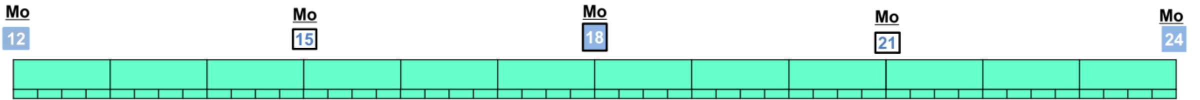
APPENDIX 1:SPS PROTOCOL TIMELINE

Trans-MAPP Symptoms Patterns Study
3-Year Timeline

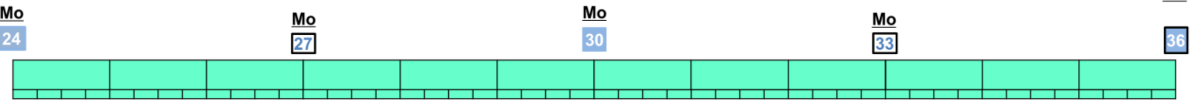
- Blue box = Semi-annual optional Clinic Visit (may be replaced by ATLAS clinic visit)
- Blue box w/ black border = Required Clinic Visit (can't be replaced)
- White box = Online surveys



Year 2 of SPS Follow-up



Year 3 of SPS Follow-up



MULTIDISCIPLINARY APPROACH TO THE STUDY OF CHRONIC PELVIC PAIN (MAPP) RESEARCH NETWORK

Appendix 2: Microbiome Protocol

Sponsored by: The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Department of Health and Human Services (DHHS)

PROTOCOL—VERSION 1.1

Dated: 06/05/2015

Collaborating Site: Queen's University, Washington University, Cedars-Sinai Medical Center, University of Colorado/TATC, University of Pennsylvania/DCC

Prepared by: Microbiome Working Group

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Table of Contents

1. Identifying Information ... 4
2. Brief Overview / Introduction ... 4
3. Rationale for Phase II Trans-MAPP Study ... 5
4. Describe Link to Trans-MAPP Symptoms Pattern Study (SPS) ... 8
5. Study Hypotheses ... 9
6. Specific Aims ... 9
6.1. Aims for Hypothesis 1 ... 9
6.2. Aims for Hypothesis 2 ... 9
6.3. Aims Related to Hypothesis 3 ... 10
7. Brief Description of Supporting Preliminary Data ... 10
7.1. Specific Aim 1 and 2 (interrelated aims): ... 10
7.1.a. Preliminary results from Male UCPPS trans-MAPP EP-Infectious Etiology (IE) study: ... 10
7.1.b. Preliminary results from Female UCPPS trans-MAPP EP-IE flare study: ... 10
7.1.c. Preliminary results from Female UCPPS trans-MAPP EP-IE study: ... 11
7.1.d. Additional and complementary studies by the Cedar-Sinai group. ... 11
7.2. Specific Aim 3 ... 12
7.2.a. Development of a new urinary metabolomic profiling approach. ... 12
7.2.b. Urinary Metabolomics Identifies a Molecular Correlate of Interstitial Cystitis/Painful Bladder Syndrome in Women (unpublished, manuscript in preparation). ... 12
8. Study Design and Methods ... 13
8.1. Queens/Drexel Methodology ... 13
8.1.a. Molecular Methodology ... 13
8.1.b. DNA extraction and Ibis eubacterial and fungal domain assays: ... 13
8.1.c. Deep 16 S rDNA sequencing: ... 14
8.2. Cedar-Sinai Methodology ... 14
8.2.a. To determine changes in bacterial and fungal profiles associated with flares in urine from UCPPS patients in MAPP I and II studies. ... 14
8.2.b. To identify the bacterial and fungal species in the vagina, prostate, and rectum of UCPPS patients to measure the contribution of the surrounding flora to bladder and urogenital pain symptoms. ... 15
8.2.c. To optimize and validate overall qualitative and quantitative bacterial and fungal load determination and profiling in UCPPS patients (section8.2b) using a high-throughput low-input quantitative PCR-based platform. ... 16
8.3. Specific Aim 3 ... 16
8.3.a. Specific Aim 3a - Identify the molecular pathway(s) responsible for the distinctive biochemical signature in the subgroup of female UCPPS patients with high symptom and extraurinary symptom scores. ... 16
8.3.b. Specific Aim 3b - Seek urinary biochemical correlates of UCPPS in male subjects. We will apply the approach developed in female patients to identify biochemical subgroups in a male patient cohort. ... 16
8.3.c. Specific Aim 3c - Follow urinary biochemical correlates of symptomatic flares through longitudinal follow-up. ... 17
9. Description and Source of Patient Cohorts Beyond those from the SPS, including controls ... 17
10. Contact Schedule and Participant Procedures ... 17
11. Risk Factor and Outcome Measures ... 18
12. Biological Specimens ... 18
13. Other Measures ... 19

14. Analytic Plan ... 19
14.1. Specific Aims 1 and 2 ... 19
14.2. Specific Aim 3 ... 20
15. Statistical Analysis and Sample Size Estimates ... 21
15.1. Specific Aims 1 and 2 ... 21
15.2. Specific Aim 3 ... 21
16. Proposed involvement of MAPP Network Cores ... 22
16.1. Tissue Analysis and Technology Core (TATC) ... 22
16.2. Data Coordinating Core (DCC) ... 22
17. Timeline for Completion of Data/Specimen Collection (see attached Appendix 1: SPS Protocol Timeline) ... 22
18. Anticipated Burden to Sites ... 22
19. Anticipated Risks to Participants ... 22
20. References ... 23
Appendix 1:SPS Protocol Timeline ... 27

1. Identifying Information

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J. Richard Landis, PhD., Alisa J. Stephens, PhD.	Data Coordinating Core

2. Brief Overview / Introduction

We plan to expand our initial and ongoing trans-MAPP Infectious Etiology Project, now as part of the newly named Trans-MAPP Microbiome Working Group, by determining the clinical implications and impact of the microbiota of the lower urinary tract on symptoms (symptom phenotype) and changing symptom patterns (flares) in patients with specified urologic chronic pelvic pain syndrome (UCPPS) phenotypes during its variable disease course. These aims will be realized by using novel, state-of-the-art culture-independent molecular methods (Ibis technology with planned confirmation of novel or important findings by 16S next generation DNA sequencing) to identify, characterize and correlate association of the microbiome (bacteria, fungi) with clinical symptom patterns as well as biomarker phenotyping in the trans-MAPP Symptom Pattern Study (SPS). This will be done by the Queen's/Drexel group. As a complementary supplemental approach we plan to identify and validate disease-specific longitudinal changes in bacterial and fungal communities in the urine in UCPPS patients using Next Generation Sequencing (NGS) and nano-fluidic qPCR.

Initially, we will characterize the hypothesized dysbiosis (changes in overall complexity or load of commensals and opportunistic pathobionts) between non-UCPPS (Control) and UCPPS patients from MAPP I (and its varying phenotypes) We will interrogate deeply the flare events already hypothesized with microbial changes in MAPP I, investigating significant changes in the microbial community between baseline, non-flare, and flare events captured for each UCPPS patient.

We expect to further characterize community differences between UCPPS phenotypes in this same population in MAPP II (*e.g.*, pelvic- vs. bladder-centric pain) at baseline, 6, 18 and 36 months, and at the time of Analysis of Therapies During the Longitudinal Assessment of Symptoms (ATLAS) visits (which, in many cases will also be flare events) in MAPP II. ATLAS modules, described in detail in the Trans-MAPP Symptom Patterns Study (SPS) protocol, are incorporated into the main SPS study to investigate the potential relationship between response (or lack of response) to a given therapy and specific phenotypic profiles of subgroups of UCPPS patients. Also, in MAPP II we will identify disease-specific fungal and bacterial

communities in the rectum, vagina, and prostate (VB3) of UCPPS patients to determine the potential contribution of these species to UCPPS symptoms. We will also correlate the presence of specific species in the vagina, rectum, and prostate (VB3) with the species identified in the bladder.

3. RATIONALE FOR PHASE II TRANS-MAPP STUDY

For decades, CP/CPPS and IC/PBS, here collectively referred to as UCPPS, were considered routine infectious diseases of bacterial origin and treated primarily with antibiotics [2,3]. Some argue that the biggest contribution of recent research to the clinical care of UCPPS has been to point out that routine indiscriminate treatment with antibiotics is not a correct uniform therapeutic approach. Although two RCTs of antibiotics in CP/CPPS failed to show an improvement over placebo [4,5], we have shown that some men with recent onset of prostatitis symptoms do have a significant and durable response to antibiotics [6,7]. Similarly the only controlled trial in IC/BPS showed minor but statistically significant improvement with antibiotics [8]. We have previously reviewed the evidence for and against the premise that traditional uropathogenic bacteria and organisms not considered uropathogens are implicated in the pathogenesis, maintenance and/or progression of UCPPS, particularly CP/CPPS [7,9-12]. We were the first to identify bacterial biofilms in culture negative patients with a past history of chronic bacterial prostatitis who had become refractory to antibiotics [13,14]. We believe that infection may be an etiological mechanism in a small but significant number of UCCPS patients.

We have come to understand that the tools we have used to study bacteria in UCPPS have been inadequate. The microbiologic diagnosis of infection in the bladder and prostate has been based upon the use of cultivation techniques in which bacteria are grown from voided urine or prostatic fluid based upon the provision of the nutritive and environmental conditions required to support growth. There are two major limitations of cultivation-based techniques. First, approximately 99% of bacteria in all natural ecosystems examined resist cultivation, and second, that bacteria preferentially grow in self-organized communities called “biofilms” which typically adhere to surfaces [15], but can also exist as self-assembled unattached rafts. Sessile bacteria in biofilms often fail to grow and produce colonies when placed on the surfaces of agar plates and so are underrepresented relative to planktonic microbes in traditional culture-dependent assays [16]. We have identified such biofilms in chronic prostatitis [13,14] while others have noted them in experimental cystitis models [17,18] (and have hypothesized that biofilms play a role in recurrent or chronic cystitis in humans [19]).

In recent years, however, molecular techniques have been developed to replace traditional culture techniques for the detection, identification and quantification of microorganisms [20]. One of these techniques, developed initially for the study of unculturable environmental microorganisms, is based on a molecular-phylogenetic approach using ribosomal (r) RNA gene sequences. This molecular approach to the study of microbial communities also has many potential applications in medicine [21], possibly including UCPPS. However, the inadequacy and conflicting results of standard culture based and earlier generation molecular diagnostic technologies to address the complexities associated with microbiomes of UCPPS are due to the fact that they are composed of vast numbers of species and strains – many of which may not be pathogenic. Although relatively few molecular studies have been performed and the results are somewhat conflicting, PCR-based approaches do detect bacteria in most prostatic samples. Shoskes *et al* [22,23] identified bacteria and bacterial signals in the EPS of a significant percentage of culture-negative prostatitis patients. Similarly, Krieger and colleagues [24,25] and Keay and coworkers [26] used PCR to detect bacteria in

transperineal biopsy tissues. Furthermore, Hochreiter *et al* [27] found no bacterial signal using PCR in prostate tissue from control organ donors but did find bacterial DNA in all radical prostatectomy specimens with concurrent histologic inflammation. In contrast, Leskinen *et al* [28] did not find evidence of bacteria in radical prostatectomy specimens done for prostate cancer, whether or not the patients had symptoms of prostatitis. By contrast, Xie et al [29] did find bacterial signals in organ donors who had concurrent prostatitis. There have been some attempts to employ similar techniques to search for the presence of causative organisms in patients with negative urine cultures and a diagnosis of IC/BPS [30-36], however the findings have also been inclusive. Furthermore, recent findings have indicated that bacterial presence even in the absence of inflammation can directly cause pain in both humans [37,38] and laboratory animals [34]. Chronic lower back pain in humans [37,38] has been linked to the presence of unculturable anaerobic microorganisms, specifically *Propionibacterium acnes;* and even more recently it has demonstrated through paw pad injection of bacteria into mice that pain is caused directly by the bacteria prior to any host inflammatory process occurring [39]. We describe our attempt to implicate microbial infection in the etiology of UCPPS in the Aim 2 Preliminary Work.

The NIH funded Human Microbiome Project (HMP) recognizes the need to characterize microbial communities found at multiple human body sites and to look for correlations between changes in the microbiome in human health and disease [40]. Unfortunately, there is a paucity of data for the urinary tract microbiome in health and disease [http://www.hmpdacc.org]. Based on conflicting data from previous research employing traditional plate-culture and standard 16S RNA PCR techniques, we propose in this application to use a suite of next-generation multi-component molecular diagnostic approaches to provide comprehensive microbial detection, identification, and enumeration over time in UCPPS patients and correlate identified or changing microbial patterns to symptom patterns and changes over time.

Impact of Lower Urinary Tract Microbiota on UCPPS Symptom Patterns. We were the first to identify bacterial biofilms in culture negative patients with a past history of chronic bacterial prostatitis who had become refractory to antibiotics [13,14]. We have discovered that some patients with symptoms may actually benefit with antibiotic therapy [6,7]. We have evaluated symptom associations with bacteriuria in patients with CP/CPPS [11,41] and IC/BPS [42]. This previous work has led to our hypothesis that the microbial ecology of the lower urinary tract may be an etiological mechanism and/or influence symptoms or symptom patterns in a small but significant number of UCCPS patients.

The application of older culture plate and molecular diagnostics to analyze specimens in the trans-MAPP SPS are unlikely give us definitive answers to our questions regarding pathogenesis, progression and symptom patterns. We have developed of an entire suite of new molecular diagnostic technologies that we believe will help provide the answers needed to meet our stated objectives. We have previously employed these technologies to characterize what were similarly confusing chronic infectious disease conditions of the human respiratory mucosa including: 1) otitis media with effusion, adenoiditis, cholesteatoma, chronic rhinosinusitus etc [1,43,44]; 2) orthopaedic conditions including atypical femoral fracture, bony nonunion, failure of arthroplastic implants, both those clearly associated with periprosthetic joint infections, but also including those clinically ascribed to “sterile loosening” and osteoarthritis of the knee [45-49] and more recently; 3) in UCPPS patients using baseline specimens from the first MAPP Network clinical study, the Trans-MAPP Epidemiology/Phenotyping (EP) Study (preliminary work section 7).

The next generation technology that we have used to analyze UCPPS specimens in the Trans-MAPP EP study was the Ibis T-5000 Universal Biosensor technology [50]. This technology uses a polymerase chain reaction – electron spray ionization–time-of-flight –mass spectroscopic-based technology (PCR-ESI-TOF MS) approach, which gives the exact base composition for each amplimer generated. Since multiple (8-16) amplimers are generated for each assay it is possible using a comprehensive database to provide a list of possible species matches for each amplimer. Then a triangulation approach in used in which all of the amplimers are considered together to arrive at a definitive species-level diagnostic for all known bacteria. In addition, the system will provide a most-closely-related match for unknown organisms. This technology does not require choosing what organism to test for as there is no need for an *a priori* hypothesis as to what species are present. The problem with all first generation DNA diagnostics were that the positive predictive values were high, but the low negative predicative values have made clinical implementation problematic. We believe that if we do identify novel findings in the microbiome, we must confirm it with an independent method. Thus, initially we used 454 LifeSciences-based massively parallel DNA sequencing, we plan to convert over to Pacific Biosciences based analyses as amplimer library methodologies are developed for this platform with enormous readlengths (> 10 Kb) that will provide for full 16S gene sequencing, thus permitting for the first time species level microbiome analyses, as contrasted with current genera and family-based taxonomies. With these technologies we are able to: 1) sequence millions of reads at a time - permitting both the routine sequencing and assembly to single contigs of multiple entire bacterial genomes on a daily basis [50,51] and 2) investigate simultaneously the qualitative and quantitative microbial makeup of up to 100 clinical specimens at the species level down to the 0.005% level.

Our strategy is to use the Ibis approach for screening and the deep 16S sequencing approaches as independent verification systems since the Ibis tests for many more genes than just the 16S (when we use the 454 system we are looking only at the 16S genes which give us species ID, but not strain ID – thus the Ibis can be used to identify particular strains – such as those with a particular antibiotic resistance (*e.g.* MRSA or MRSE, or vancomycin resistant enterococcus; or a particular virulence gene, *e.g. nuc, lukD,* or *PVL*). This apriori planned research strategy should and will include more than one method of assessment with each one supporting the other as and if necessary.

Rationale for Ibis approach (initial screening of specimens): The Ibis technology is designed to be used as a routine clinical diagnostic having a turn-around time of less than 24 hours at reasonable cost. Moreover, the Ibis technology has been demonstrated in numerous small scale clinical studies involving complex infectious processes including the first Trans-MAPP EP study to provide extremely accurate and comprehensive data. It is also important to have a research-based technology (deep 16S next generation DNA sequencing) against which the Ibis assays can be compared to determine if modifications to the current format would be needed to provide the broadest possible pathogen coverage for urological microbiome analysis. There is currently no expectation that next-generation DNA sequencing technologies will be used clinically as they are far too labor intensive, technically complex, time consuming, and would enormous require batching to make it economically feasible (including budget considerations for our planned Trans-MAPP study). However, the sequencing based technologies do provide enormous breadth and depth of coverage – attributes that are important when performing any confirmation studies such as described in our application. Thus, the two technologies serve different purposes, yet both can be used to support the other. As with any research program, it is advisable to have multiple independent methods of analysis when trying to convincingly demonstrate potentially novel observations. Project funding as outlined in the RFA will allow Ibis analysis on

all relevant urine specimens while confirmation of novel findings with be performed by next generation sequencing approach (see below).

Rationale for the Next Generation Sequencing Approach: UCPPS often has an acute onset. This sudden presentation, combined with the recent detection of resident, nonpathogenic bacteria and fungi in the bladder (unpublished data), raises the possibility that perturbations in the microbial flora of the bladder contribute to the symptomology of UCPPS.

Recent research from us and other groups indicates that complex bacterial commensal and pathobiont communities on human body surfaces and in body cavities, including the bladder (manuscript in preparation), co-exist with poorly characterized or uncharacterized fungal communities [52,53]. We and others have shown that changes in absolute quantity (load) or changes the relative abundance of the community (*e.g.* pathobionts outcompeting commensals for resources), which is also known dysbiosis, have been associated with lower and upper gut disease as well as other diseases [54].

It is expected that urine, which traditionally was thought to be sterile, will contain many microorganisms not well characterized. In fact, we recently discovered some commensal fungal species in urine not previously identified in mouse or human gut using our unbiased NGS discovery-based approach. We seek to specifically better characterize the dysbiosis hypothesized in flare events in MAPP I and longitudinal changes in the Trans-MAPP SPS study. In addition, in the SPS study we will identify 1) rectal, vaginal, and prostate microorganisms that may be associated with UCPPS, 2) investigate the relationship between the non-bladder and bladder microbial communities, and 3) investigate the non-bladder microorganism contribution to the bladder community in UCPPS. Finally, the identification of specific microorganisms will help in the study of host-microbial interactions as described in second aim of the Biomarker Working Group protocol.

Metabolomics: UCPPS diagnosis and treatment are greatly limited by poor understanding of the biochemical processes that contribute to urinary symptoms. Without this level of understanding, efforts to better treat UCPPS cannot access the many of the molecular approaches that have improved treatment of other, better understood, diseases. As a syndromic illness, efforts to identify biochemical abnormalities must consider the possibility that UCPPS patients may represent multiple distinct pathophysiologic processes. We propose to identify these biochemical subgroups using mass spectrometry-based metabolic profiling approaches (metabolomics) [55]. The metabolomic approaches proposed here are intended to identify disease-associated molecules in an unbiased manner, making possible the discovery of biochemical processes that have not previously been associated with UCPPS. We propose to build upon findings from in MAPP I described in section 7 (preliminary data) while expanding this successful approach to the patients in the SPS study

4. Describe Link to Trans-MAPP Symptoms Pattern Study (SPS)

The Microbiome Study is integrated within the central Trans-MAPP SPS protocol. In this context we plan to correlate longitudinal microbiome composition and changes with specific phenotypes (*e.g.* Pelvic Only and Pelvic Beyond, pain descriptors), changing symptom patterns (*e.g.* flares and treatment initiation), treatment effects, biomarker profiles, psychosocial parameters and neuroimaging.

5. Study Hypotheses

The study will address the following hypotheses:

Hypothesis 1. *Noncultureable, unidentified, novel bacterial or non-bacterial microorganisms (fungal) or an imbalance or change of the microbial ecology or microbiome of the lower urinary tract is implicated in maintenance, symptom patterns, symptom progression and/or symptom changes over time ("symptom flares") in some patients (those with bladder centric or pelvic pain only phenotypes) diagnosed with UCPPS.*

Hypothesis 2. *The microbiome of the lower gut, vagina and prostate is associated with microbiome composition of the lower urinary tract and is associated with disease characteristics and longitudinal changes.*

Hypothesis 3. *Metabolic correlates of UCPPS disease onset and development can be identified by metabolomics.*

a) There exist sex-based biochemical differences among UCPPS patients.

b) Small molecule profiles of UCPPS patients can be used to identify periods of severe and less-severe symptoms.

6. Specific Aims

Each of the above hypotheses is described in greater detail in the following specific aims:

6.1. Aims for Hypothesis 1

Specific Aim 1a **Determine longitudinal changes of the microbiome in UCPPS patients enrolled in the trans-MAPP Symptom Pattern Study**

Specific Aim 1b **Assess microbiome differences between "bladder centric" phenotype and "non-bladder centric" phenotype as outlined in the trans-MAPP SPS protocol.**

Specific Aim 1c **Assess microbiome differences between "pelvic pain only" phenotype and "systemic or pelvic pain and beyond" phenotype as outlined in the trans-MAPP SPS protocol.**

Specific Aim 1d **Determine qualitative and quantitative changes in bacterial and fungal profiles associated with flares in urine from UCPPS patients**

Specific Aim 1e **Assess the pathogenicity of specific microbial patterns by their association with inflammatory biomarker patterns expressed in specific and predefined phenotypes in UCPPS patients enrolled in the trans-MAPP SPS study.**

Specific Aim 1f **Optimize and validate overall qualitative and quantitative bacterial and fungal load determinations and profiling in UCPPS patients identified by Ibis and NGS approaches using a high-throughput low-input quantitative PCR-based platform.**

6.2. Aims for Hypothesis 2

Aims of Hypothesis 2 are interrelated with those of Hypothesis 1

Specific Aim 2a **To identify the bacterial and fungal species in the vagina, prostate, and rectum in order to measure the association with and contribution to the surrounding flora to bladder and urogenital pain symptoms.**

6.3. Aims Related to Hypothesis 3

Specific Aim 3a **Identify the molecular pathway(s) responsible for the distinctive biochemical signature in the subgroup of female UCPPS patients with high symptom and extra-urinary symptom scores.**

Specific Aim 3b **Seek urinary biochemical correlates of UCPPS in male subjects.**

Specific Aim 3c **Identify urinary biochemical correlates of symptomatic flares through longitudinal follow-up.**

7. Brief Description of Supporting Preliminary Data

7.1. Specific Aim 1 and 2 (interrelated aims):

7.1.a. Preliminary results from Male UCPPS trans-MAPP EP-Infectious Etiology (IE) study:

Baseline VB1 and VB2 urine specimens were obtained from 110 CPPS cases and 115 matched controls (subjects with UCPPS symptoms). VB3 samples were provided by 67 CP/CPPS and 62 controls. A total of 78, 73 and 54 species (42, 39 and 27 genera) were detected in VB1, VB2 and VB3 respectively. Mean (SD) VB1, VB2 and VB3 species count per person was 1.62 (1.28), 1.38 (1.36) and 1.33(1.24) for cases and 1.75(1.32), 1.23(1.15) and 1.56 (0.97) for controls respectively. Overall species and genus composition significantly differed between CPPS patients and controls in VB1 only (p=0.008 species level, p=0.011 genus level, p=0.079 Gram-stain level) with overall differences driven by B. cenocepacia (RR=3.34, p=0.009), P. acnes (RR 0.38, p=0.01), and S. capitis/capare (RR=0.17, p=0.008). These differences (and similar trends at genus level) remained significant after adjustment for multiple comparisons. No significant differences were observed at the Gram-stain level in VB1 or at any level in VB2 or VB3. Based on our initial analyses described here, we were not able to attribute a bacterial etiology for male UCPPS, but we have not yet assessed the impact of the microbiome on symptom patterns. We are presently analyzing the data base with respect to microbial localization to the prostate (VB3 – VB2 + VB1 spectrum) to determine if microbiota localized to prostate plays a larger role in differentiation from controls and prediction of symptom patterns (*e.g.* pelvic only pain phenotype)

7.1.b. Preliminary results from Female UCPPS trans-MAPP EP-IE flare study:

Baseline urine specimens were obtained from 83 female UCPPS patients with reported flare and 127 UCPPS patients with no reported flare. Overall, 81 species (35 genera) were detected in VB1 and 73 in VB2 (33 genera). Mean (SD) VB1 and VB2 species count per person was 2.6 (1.5) and 2.4 (1.5) for flare patients and 2.8 (1.3) and 2.5 (1.5) for non-flare patients respectively. Overall species composition did not significantly differ between flare and non-flare patients at any level (p=0.14, 0.69 species level, p=0.94, 0.83 genus level, p=0.29, 0.50 Gram-stain level in VB1 and VB2, respectively) in multivariate analysis for richness. Multivariate analysis for the presence of gram-stain groups in VB2 specimens showed a marginally significant difference between flare and non-flare patients (p=0.049). Univariate analysis confirmed a significantly greater prevalence of fungi (Candida and Saccharomyces) in the flare group (15.7%) compared to the non-flare group in VB2 (3.9%) (p=0.006). When adjusted for antibiotic use and menstrual phase, women who reported a flare at baseline remained more likely to have fungi present in VB2 specimens than women who did not report a flare [OR= 8.3, CI=(1.7-39.4)]. This analysis suggests the microbiome may impact flare symptoms in a significant number of female patients with UCPPS. We plan to evaluate 6 and 12 month trans-MAPP-EP specimens to corroborate these findings.

7.1.c. Preliminary results from Female UCPPS trans-MAPP EP-IE study:

Initial pooled study results (male and female) were presented at the AUA annual meeting in San Diego May 2013 [56]. The results showed minor differences at some species composition levels between cases and controls. The data set has been enriched with the last years MAPP enrollment and new analyses are presently being undertaken.

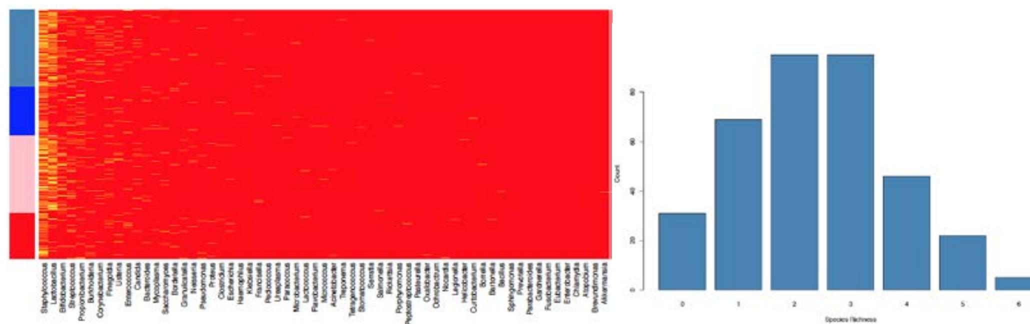

Figure 1: Representative heat map (genus distribution) and species richness graph[1]

The complete baseline data set is being prepared for 3 separate manuscripts that are in preparation. In summary, our preliminary findings showed some differences in microbiome (individual or patterns of microorganisms) between patients and controls and intriguing subpopulations were identified. Our work would suggest that UCPPS is not a microbial infection. However, we are intrigued by our initial observations that microbial composition or changes observed in the lower urinary tract could be implicated in symptom patterns or change of symptoms over time. For example, greater presence of fungi (e.g. Candida sp.) were detected in “flare” vs non-flare patients, even after we adjusted the analyses for antibiotic use. We further plan to explore our baseline data base of individual patient microbiota and the various clinical phenotypes (*e.g.* pelvis only pain vs systemic; bladder centric) being identified in the trans-MAPP Epidemiology/Phenotyping study to determine if any identifiable variations in the lower urinary tract microbiome is correlated with specific patient phenotypes.

7.1.d. Additional and complementary studies by the Cedar-Sinai group.

We will first focus on longitudinal studies of patients who participate in both MAPP I and MAPP II and experienced at least one flare event during MAPP I. Specifically, we will identify the bacterial and fungal dysbiosis in flare and non-flare events in UCPPS. We will use state-of-the-art resources in microbiome characterization through next generation sequencing of species-specific ribosomal DNA sequences. We will focus on characterizing the origin of the pathobionts in UCPPS urine samples in flare events as well as identifying the different microbial profiles of both “pelvic-” and “bladder-centric” UCPPS that are collected at baseline, 6, 18 and 36 months, and during ATLAS events in MAPP II. We will extrapolate and validate our findings using a high-throughput nano-fluidic qPCR system to characterize bacterial/fungal load and extract accurate quantitation for validation studies and translation into the clinic. The nanofluidic and NGS systems are currently unique to our team. These techniques will complement and validate findings noted in the MAPP I Microbial Etiology Study as well as complement and validate the Ibis T-5000 microbiome studies in MAPP II.

7.2. Specific Aim 3

7.2.a. Development of a new urinary metabolomic profiling approach.

Metabolomics offers direct insights into the chemical environment and metabolic pathways active at sites of human disease [57,58]. We have developed an untargeted metabolomics approach to identify disease-associated compounds in patient urine. We first applied this approach to differentiate urine from healthy volunteers from those with *E. coli* urinary tract infection. Metabolite profiles from minimally processed samples were obtained using an optimized liquid chromatography-mass spectrometry (LC-MS) platform capable of resolving ~2300 molecular features [55]. Principal components analysis (PCA) and discriminate analysis (PCA-DA) [59,60] readily distinguished patient groups and multiple supervised chemometric analyses resolved robust metabolic shifts between groups. This analysis revealed nine endocrine, catabolic, and lipid pathway products never previously associated with infection. Several of these signature metabolites may indicate microbial processing of host molecules. In another study, we detected a novel microbial molecule in the urine of patients infected with uropathogenic *E.coli* [61]. Overall, these studies highlight the ability of metabolomic approaches to discover new disease-associated urinary compounds.

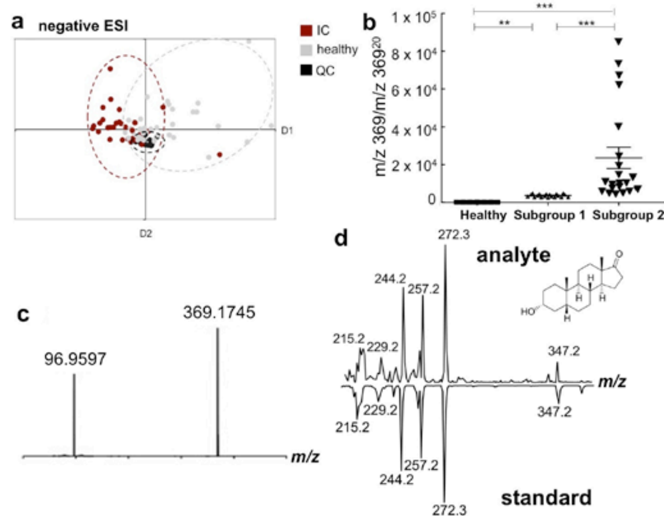

Figure 2 Metabolomic discovery of etiocholan-3a-ol-17-one sulfate as a high symptom UCPPS subgroup biomarker. (a) PCA-DA score plot of urines from a female UCPPS patient cohort. Patients with high symptoms (red) clustered separately from healthy controls (grey) and quality control samples (black). (b) Targeted analysis of a candidate biomarker identified from (a) expressed as *m/z* 369 to *m/z* 369^{20} differentiates high symptom UCPPS subgroup 2 from lower symptom subgroup 1 and healthy controls (p= 0.0003 and 0.0022, respectively, *t*-test). (c) High-resolution negative ion tandem ESI spectrum of the candidate biomarker is consistent with the empiric formula $C_{19}H_{30}O_5S$ for a (M-H at *m/z* 369.1745) that fragments to give a peak at *m/z* 96.9597, consistent with the presence of a sulfate group with the empiric formula SO_4H. (d) Matching GC-EI-MS spectra of this sulfatase-treated, TMS-derivatized analyte compared to a commercially available etiocholanolone reference standard. Retention time for both the analvte and standard was 21.8 minutes.

7.2.b. Urinary Metabolomics Identifies a Molecular Correlate of Interstitial Cystitis/Painful Bladder Syndrome in Women (unpublished, manuscript in preparation).

We selected female patients with high symptom scores and age-matched controls from the MAPP network for metabolomic discovery. The median age of the 80 study patients was 37±18 years. In IC/PBS patients, the mean symptom score was 26±5 versus 0.8±1 in controls. Patient urinary specimens were profiled using LC-MS. PCA and PCA-DA were used to overview the data and to identify molecular correlates of IC/PBS (Figure 2a). PCA-DA of urinary metabolite features revealed a principal component axis that defined two discrete subgroups (subgroups 1 and 2) within the IC/PBS cohort. Subgroup 2 exhibited distinctive clinical characteristics, including significantly higher localized pain and a higher number of painful body sites. Six (6) metabolites were identified as primary contributors to subgroup 2. Candidate biomarkers emerging from the discovery set were confirmed in subsequent targeted LC-MS/MS analyses. Of these, one was reliably quantified and was verified in confirmation studies as distinguishing the two subgroups (Figure 2b). Purification, accurate mass analysis, GC-MS, and comparison to commercial standard identified this biomarker as etiocholan-3a-ol-17-one sulfate, a 5-β reduced isomer of testosterone [62] (Figure 2c and 2d).

Etiocholanolone-sulfate (Etio-S) levels distinguish a UCPPS patient subgroup characterized by high symptom scores and multiple extraurinary tract symptoms. This marker was identified through unbiased biochemical profiling and suggests the existence of a biochemically distinct IC/PBS subgroup. Etio-S exerts immunostimulatory, pyrogenic and leukocystic effects in addition to exhibiting GABAA receptor positive neuromodulatory activity [63-67]. This marker suggests a new research direction and patient classification based upon differences in metabolism. This molecule's ability to stimulate local inflammation both suggest possible mechanistic connections with UCPPS symptoms.

8. Study Design and Methods

8.1 and 8.2: Specific Aims 1 and 2

8.1. Queens/Drexel Methodology

8.1.a. Molecular Methodology

We will use the Ibis T-5000 Universal Biosensor, a broad-spectrum, automated, high-throughput, molecular diagnostic technology (polymerase chain reaction –electron spray ionization–time-of-flight –mass spectoscopic-based technology) [51,68-73] that we have worked with for the past several years (described in preliminary work section) [45-51]. The Ibis technology will identify not only the predominant bacteria, but also fungi (we did not comprehensively assess all fungi in the first MAPP study) of the microbiome which may perturb the bacterial balance. A future phase 2 protocol will be developed to confirm any initial important or novel findings employing 454/Pacbio-based deep 16S bacterial sequencing. We have described the rationale in the preliminary work section for Aim 1. We now have extensive experience using these technologies to sequence culturable and unculturable human organisms from urine collected in UCPPS and control subjects in the first trans-MAPP EP infection etiology study. We will be using similar, but upgraded technology and approach, except that we will expand our studies to full fungal screening in addition to bacterial micro-organisms.

8.1.b. DNA extraction and Ibis eubacterial and fungal domain assays:

In brief, total DNA will be extracted from all samples, and the bacterial and fungal DNAs will be amplified by polymerase chain reaction (PCR) using the 16 primer pair BAC/Fungal detection systems developed by Ibis [70], and the individual amplicons will be "weighed" using the Ibis instrumentation for electrospray ionization (ESI) time-of-flight (TOF) mass spectrometer (MS). The species identities of the amplicons will then be revealed using a database containing base composition data on virtually all bacterial/fungal species sequenced to date. For all samples 3 ml of urine will be centrifuged at 10,000 rpm x 3 min, then 900 µl of supernatant will be removed and added to ATL lysis buffer and proteinase K. Nucleic acid from the lysed sample will be extracted using the Qiagen DNeasy Tissue kit (Qiagen cat# 69506). 10 µl of each sample will be loaded per well onto the BAC/Fungal detection PCR plates (Abbott Molecular). Each of these detection plates is a 96 well plate which contains 16 primer pairs per assay that survey all bacterial (or fungal) organisms by using: omnipresent loci (*e.g.* 16S/18S rDNA sequences); phylum/class/order specific loci; and some are targeted to specific pathogens of interest (*e.g.* the Staphylococcus-specific *tuf*B gene). The bacterial system also detects the presence of several key antibiotic resistance markers: van A and van B (vancomycin resistance) in Enterococcus species, KPC (carbapenem resistance) in Gram-negative bacteria, and mec A (methicillin resistance) in *Staphylococcus* species. An internal calibrant of synthetic nucleic acid template is also included for each primer pair in each assay, controlling for false negatives (*e.g.* from PCR inhibitors) and

enabling a semi-quantitative analysis of the amount of template DNA present. PCR amplification will be carried out as recommended by the manufacturer. The PCR products will then be desalted and sequentially electrosprayed into a TOF mass spectrometer. The spectral signals will be processed to determine the masses of each of the PCR products present with sufficient accuracy that the base composition of each amplicon could be unambiguously deduced. Using combined base compositions from multiple PCRs, the identities of the pathogens and a semi-quantitative determination of their relative concentrations in the starting samples will be established by using a proprietary algorithm to interface with the Ibis database of known organisms.

8.1.c. Deep 16 S rDNA sequencing:

Samples from some patients in these series (if clinically relevant findings require confirmation) will also be analyzed by the creation of 16S/18S amplimer clone libraries for deep sequencing. These clone libraries will then be sequenced in a massively parallel manner using molecularly bar-coded primers on either the GS titanium system developed by 454 Life Sciences where the bacteria will be identified to the genus level, or the Pacbio RS system where they will be identified to the subspecies/clade level. We have designed 114 different Multiplex Identifier (MID) tagged 16S PCR primers. The primers were generated by combining a sequencing primer key, an exclusive 10 base MID adaptor (454, 2009.), and a 16S rRNA targeted sequence. PCR will be performed in 50 µl reactions containing 1× High Fidelity PCR Buffer, 2.0 mM $MgCl_2$ solution, 0.8 mM dNTP Mix, 1 U Platinum Taq High-Fidelity (Invitrogen), 0.4 µM each primer (IDT) and 5 µl of DNA extract. Cycling will be performed with an initial denaturation step at 94 °C for 2 minutes followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 60 seconds, followed by a final extension of 7 minutes at 72 °C. Five µl of the PCR products will be visualized on 1% agarose gels. Positive PCR products will be purified using the Agencourt AMPure XP system (Beckman Coulter) and quantified using Quanti-iT PicoGreen dsDNA Assay Kit (Invitrogen). All primers that produce 16S PCR fragments will be diluted to equimolar concentrations (48 pg/ µl). PCR products will then be pooled into groups of samples to provide for massively multiplex sequencing of the libraries from individual specimens/patients. GS Titanium SV emPCR (LibA) and GS Titanium sequencing (454 Life Sciences), or Pacbio SMRT sequencing will then be performed on the amplicon libraries according to manufacturer's guidelines. Following conclusion of the sequencing runs, the reads from the multiple specimen libraries will be deconvoluted based on the molecular barcodes. These barcodes sequences will then be stripped from the reads and each sample's reads will then be analyzed using the RDP classifier online tool (http://rdp.cme.msu.edu/classifier/classifier.jsp). Following receipt of the data from RDP it will be parsed into phylum, class, family, genus and species views using the CGS-developed 16S phylogenetic parsing tool.

8.2. Cedar-Sinai Methodology

8.2.a. To determine changes in bacterial and fungal profiles associated with flares in urine from UCPPS patients in MAPP I and II studies.

Goal: Our initial goal is to characterize bacterial and fungal taxonomic differences longitudinally in MAPP I (i.e., using archived data/samples collected as part of the first phase Trans-MAPP Epidemiology/Phenotyping [EP] Study) between UCPPS patients and controls at baseline and during flare events. This will complement the work done by the Microbiome Working Group from MAPP I. We will focus our investigation on significant changes in baseline and UCPPS with multiple flare events (using data/samples from MAPP Phase I and II (i.e. the Trans-MAPP SPS) and compare these differences to healthy controls (MAPP I phenotypic data/samples). We will also assess differences in the microbial community in the patient at 6, 18 and 36 months, and ATLAS

events compared to baseline using data/samples collected as part of the MAPP II SPS. To determine bacterial and fungal species in flare events in UCPPS patients' urine, we will begin to use urine specimens collected in MAPP I. Where possible we will focus on longitudinal samples from patients with flare events that were al collected during clinic visits. Although the robust stability of microbial DNA means these techniques can also be readily applied to the flare and non-flare control specimens collected by UCPPS patients at home. Finally, the identification of specific microorganisms during the exact same flare events will increase significance of the host-microbial interactions that will be dissected in CSMC Biomarker working group protocol.

Experimental procedures:

10 mL of urine will be analyzed from healthy and positive control participants, and UCPPS patients to establish a baseline signature. 10 mL of VB2 specimens from each subsequent flare event will be assayed from each patient to capture patient-to-patient species changes. Those changes will be compared to each patient's baseline, and MAPP I patients with similar flare events will be compared to find overlapping changes in microbial signatures.

DNA preparation

After centrifugation of urine specimens, the bacterial and fungal amplicons will be generated from DNA extracted with a modified protocol that efficiently lyses the fungal cell wall and can be used for both bacterial and fungal DNA isolation. Sequencing libraries will be constructed from 10 ng DNA (10 ng before amplification). Phusion (NEB, MA) polymerase, which is optimized for high fidelity and robust amplification, will be used to amplify variable regions 1-3 (V1->V3) in the bacterial 16S gene according to a protocol that is based on the method of Ley et al. (48). The fungal library will be constructed by amplifying ITS1 sequences with universal ITS primers (ITS1F-ITS2). Insufficient amplification will be repeated until each patient sample contains 100 ng of amplified material. We recently introduced the TecanEvo 75 liquid handling robot for library construction, which will minimize cross-contamination and pipetting error and maximize throughput in amplification, purification, and library generation.

100 ng of template from each specimen will be used to construct a library using the TruSeq DNA prep as previously described. Each patient sample will be indexed with a unique nucleic acid bar code to allow for multiplexing during sequencing and separation of sequences in the data analysis. All libraries will be verified for enrichment and fragment length using an Agilent DNA bioanalyzer 1000. Sequencing will be performed on a 2x300 bp Illumina MiSeqPE flow cell per manufacturer's recommendations. Our data show that the median size of the bacterial libraries is 440 bp while the fungal library has more diversity and ranges from 350-700 bp.

8.2.b. To identify the bacterial and fungal species in the vagina, prostate, and rectum of UCPPS patients to measure the contribution of the surrounding flora to bladder and urogenital pain symptoms.

We will characterize the origin and/or association of pathogenic species with urine samples by also interrogating the microbial communities in vaginal swabs, rectal swabs, and VB3 specimens in the same patient. This will allow us to determine which organisms are the source of bladder-centric and pelvic-centric pain, and/or non-pelvic/non-bladder centric pain in UCPPS, and which reservoirs are most likely to be a possible source of bacterial and fungal communities that inhabit the bladder and may contribute to bladder pain. We will also more generally correlate the proportions of different bladder and fungal species in the

bladder, rectum, prostate, and vagina in individual patients and compare the distribution over time using the longitudinal assessments at baseline, 6, 18 and 36 months, and at ATLAS events.

Goal: We will assess the contribution of pathogenic contamination in microbial profiles from vaginal, rectal, and VB3 samples. We will identify shared pathobionts in vaginal, rectal and mid-stream voided (VB2) urine samples in female patients at baseline, while in male patients we will compare VB3, rectal, and VB2 to identify the potential origin of many of the pathobionts.

Experimental procedures: To determine bacterial and fungal species we will use urine, rectal and vaginal specimens collected through the SPS study as described in Table 2. Bacterial and fungal amplicons will be generated from DNA extracted with a modified protocol as described above from urine and vaginal and rectal swabs.

8.2.c. To optimize and validate overall qualitative and quantitative bacterial and fungal load determination and profiling in UCPPS patients (section8.2b) using a high-throughput low-input quantitative PCR-based platform.

Goal: We will assess the total microbial load associated with flare and baseline events and validate the changes in commensals/pathobionts by developing genus- and/or species-specific qPCR assays for the bacteria or fungal species that differ significantly in UCPPS patients identified in section n2a and 2b. This will be based on differences identified in section2a and 2b and so will only include a limited number of specific assays but may be used to extrapolate findings to include many more urine samples and test alternative hypothesis.

Experimental procedures: Up to thirty-three of the top differentially expressed bacterial and fungal species will be measured in separate samples not used for sequencing. Genus- or species-specific primers will be designed based on OTU's using a custom bioinformatics approach to ensure reduced overlap in other species in the Genus or Family. Primers are validated on our traditional VII7 before high throughput validation using the nanofluidic Fluidigm BioMark platform, which can assay the relative presence of 33 different species in triplicate for a fraction of the cost of traditional qPCR.

8.3. Specific Aim 3

8.3.a. Specific Aim 3a - Identify the molecular pathway(s) responsible for the distinctive biochemical signature in the subgroup of female UCPPS patients with high symptom and extraurinary symptom scores.

The MAPP I patient cohort will be used to identify additional metabolites associated with elevated Etio-S levels. To validate Etio-S and related biomarkers we will quantify these biomarker candidates in the MAPP II patient cohort. Based upon experience with MAPP I patients, this cohort is expected to contain at least 20 patients with similar clinical characteristics (GUPI score = 13±3, # non-urinary sites = 11±5) as the initial biomarker positive metabolomic discovery cohort.

8.3.b. Specific Aim 3b - Seek urinary biochemical correlates of UCPPS in male subjects. We will apply the approach developed in female patients to identify biochemical subgroups in a male patient cohort.

We will define a male patient cohort among MAPP I specimens similar to the approach used in the previous, female cohort study. Biomarker candidates identified in this cohort will be validated in male MAPP II

patients. Quantified biomarkers in male and female patients will be examined as correlates of gender-based metabolic or infection differences.

8.3.c. Specific Aim 3c - Follow urinary biochemical correlates of symptomatic flares through longitudinal follow-up.

We will identify longitudinally-collected MAPP urine specimens in which one of the specimens is categorized as a flare. We anticipate forty male and forty female patients will be sufficient to resolve flare-associated differences in the urinary metabolome.

All samples will be analyzed on an LC-MS platform optimized for maximal metabolite feature yield.

9. Description and Source of Patient Cohorts Beyond those from the SPS, including controls

Patients will be participants in the MAPP SPS study. No patient cohorts beyond those from the SPS study are needed.

10. Contact Schedule and Participant Procedures

Collections will follow the Trans-MAPP SPS visit schedule. The schedule of specimen collection is listed in Table 2

Table 2. Schedule of Biologic Specimen Collection for Microbiome protocol (See Trans-MAPP SPS timeline), (see Biomarker Working Group Protocol for schedule of biomarker specimen collection)

	Specimen	Schedule
Microbiome	Urine (VB1 ~20 mL, VB2 and VB3[1] ~30-40mL each)	**Female**: 0 weeks and 18 months: VB1 and VB2 6, 12, 24, 30, 36 months, and ATLAS visits: VB2 At flare during any clinic visit: VB1 and VB2 **Male[1]:** 0 weeks and 18 months: VB1, VB2, and VB3 6, 12, 24, 30, 36 months, ATLAS: VB2 At flare during any clinic visit: VB1, VB2, and VB3 (if clinician available to obtain sample)
	Rectal Swabs[2]	**Male/Female:** 0 weeks, 18 months
	Vaginal Swabs[3]	**Female**: 0 weeks, 18 months

[1]VB3 Urine collection in males following prostate massage is optional for patient
[2]Collection of rectal swabs is optional for patient at visits that include physical exam
[3]Collection of vaginal swabs is optional for patient at visits that include physical exam

- 12, 24, 30 months visits are optional.
- Collection/procedure order week 0, month 18, and ATLAS when swab collection takes place:
  - Males: VB1/VB2, physical exam/rectal swab, VB3 (saline wipes for all urine collections)
  - Females: VB1/VB2, Physical exam/rectal and vaginal swabs. (saline wipes for urine collections)

11. Risk Factor and Outcome Measures

a) Common Data Elements (CDEs) from Trans-MAPP Epidemiology Study

We will use all the same questionnaires and data points from the Trans MAPP SPS study

b) Specialized Data Measures

No specialized data measures are needed. Findings from planned studies will be analyzed in the context of the questionnaires from the Trans MAPP SPS, neuroimaging, and biomarker studies.

12. Biological Specimens

Biospecimen collection kits will be used to simplify and standardize collection of body fluids for the expressed purpose of optimizing their collection for research. The TATC will provide MAPP Network-specific specimen collection kits for use by Discovery Sites as needed. Requests for kits will be done through an online ordering system located on the DCC MAPP Network Portal and request will be direct the TATC. The collection kits and components are barcoded, and will be linked with the participant at the time of registration of the participant through the DCC MAPP Network Portal. Collected specimens will then be shipped to the TATC for processing, aliquoting, and inventory. The collection and handling procedures will follow the guidelines established by the NIH Best Practices Policies for biorepositories (www.biospecimens.cancer.gov). No patient identifiers will be used on the collection tubes and tracking forms. As specimens are received from sites, they will be scanned into the biorepository database, and archived in the appropriate freezer/storage unit until needed. Specimen tracking information will be entered into the database by TATC personnel.

Biologic specimens collected during the course of the Trans-MAPP Study for the microbiome protocol include biomarker and microbiome urine and vaginal and rectal swabs. The schedule of these collections is summarized in Table 1. Biomarker specimens are described in detail in the Biomarker Working Group protocol.

Microbiome urine: During the clinic visit participants will be asked to provide two clean–catch urine samples (first void ~20 mL(VB1) and mid-stream 30-40 mL (VB2)) using a saline wipe. In addition, males will be asked for an additional clean–catch first-void 30-40 mL urine sample (VB3) using a saline wipe immediately following a prostate massage. VB3 specimens will be optional for the patient. Urine samples will be immediately frozen and stored at -80ºC in 50 ml conical tubes, and shipped to TATC in batches. Upon receipt by TATC, all VB urine samples will thawed and aliquoted. Specimens will be stored at -80ºC. Documentation of sample origin, acquisition, transportation, processing, and storage will be provided for each sample. Date/time tracking will be done to ensure proper processing times are followed and potentially problematic samples identified.

Vaginal swabs: Up to 3 vaginal swabs will be collected from females. Vaginal swab collection will be optional for the patient. After collection the swabs will be stored at -80ºC and shipped to TATC in batches. Specimens will be stored at -80ºC. Documentation of sample origin, acquisition, transportation, processing, and storage will be provided for each sample. Date/time tracking will be done to ensure proper processing times are followed and potentially problematic samples identified.

Rectal swabs: Up to 3 rectal swabs will be collected from males and females. Rectal swab collection will be optional for the patient. After collection the swabs will be stored at -80ºC and shipped to TATC in batches.

Specimens will be stored at -80ºC. Documentation of sample origin, acquisition, transportation, processing, and storage will be provided for each sample. Date/time tracking will be done to ensure proper processing times are followed and potentially problematic samples identified.

13. Other Measures

No other new measures will be required

14. Analytic Plan

14.1. Specific Aims 1 and 2

Analysis Plan

Differences in species composition for various symptom patterns addressed in the Trans-MAPP SPS (bladder centric, pelvic pain only vs systemic pain, flare vs non-flare) will be assessed using "distance measure" methods from microbial ecology. PERMANOVA and Cochran-Mantel-Haenszel methods will be used to test differences by cohort type in the presence and/or richness of species. Tests of individual taxa within each level (species, genus, gram-stain) will adjust for multiple comparisons by controlling the False Discovery Rate [74]. Tests of overall composition at different levels (simultaneously considering all species/genera) will not be adjusted for multiple comparisons as they are likely correlated. Adjusted and unadjusted analyses will be undertaken, with adjusted analyses reported in final results. Analyses will adjust for demographic variables whose distributions differ between symptom pattern phenotypes. Tests for differences in overall microbial composition between pre-determined phenotype groups of UCPPS patients at the species, genus, and gram-stain level will be adjusted for antibiotic use and menstrual state (female only). We have worked closely with the DCC biostatisticians, and are part of the Microbiome Working Group, to develop these statistical methods in order to analyze trans-MAPP EP baseline data. The DCC statisticians have teamed up with other University of Pennsylvania statisticians with experience in microbial ecology studies and well as the Human Microbiome Project to develop specific statistical analyses plans in order to correlate identifiable microbiota patterns with the clinical phenotypes identified in the first trans-MAPP EP study. We will adapt this methodology to not only correlate microbiota variations with clinical phenotype but to also examine the impact of changes in the microbiome with fluctuating symptom patterns in the proposed study. See Innovations in Analyses of Human Microbiome (Section 3) for development of analysis algorithms.

Correlation with symptoms patterns and inflammatory biomarkers: We will correlate microbial pattern and symptom patterns with inflammatory biomarker identification (for example IL-6). These analyses will be carried out under the guidance of the TATC and DCC as the microbial and biomarker data becomes available and the symptom patterns are elucidated.

Data analysis specific to Cedar-Sinai methodology. Data analysis will be performed using general bioinformatics workflows including the QIIME pipeline v1.5.0. A number of filters will be used to generate high quality reads, which will be aligned to the Greengenes reference database (October 2012 release) using BLAST v2.2.22 with an identity percentage ≥97% to select the operational taxonomic unit (OTU). Taxonomy for each sequence is assigned using the Ribosomal Database Project (RDP) classifier v2.2. For fungal taxonomic assignment, filtered high-quality reads are aligned to our custom fungal reference database (v2.4)

of 1100 peer reviewed curated fungal sequences from human and mouse digestive tract, as well as to the UNITE database. We will require an identity percentage ≥97% for taxonomic assignment (Table 1).

Table 1. Performance of Custom Knowledge-based Fungal Database (FunGut) compared to reference databases

	Findley	Fungut	RTL	UNITE
Reads	8,214,115	8,214,115	8,214,115	8,214,115
Running time [(minutes)	731	214	248	1095

To determine the composition of bacterial and fungal species in the sample, OTUs will be compiled into genera and families. At this point, we will also exclude the possibility that results are affected by the sequencing depth through a rarefaction analysis. After the multiple sequence alignments with PyNAST v1.2, phylogenetic trees will be constructed using FastTree v2.1.3(49). Based on the calculated UniFrac distance metrics, a principal components analysis will be performed to explore the similarity in the bacterial profile among samples. To identify statistically significant differences between the control and disease groups, we will use the pvclust package in R (v2.15). Assuming there are significant differences, the most over-/under-representative genera and families will be shown in a two-way correlation clustering heatmap by using gplots package in R. We will also test the differences between microbial communities using either parametric Welch t test or non-parametric Mann-Whitney test. The false discovery rate (FDR) will be controlled by the Benjamini–Hochberg procedure. Finally, we will compare the bacterial profiling with previous studies to characterize the correlations/interactions among bacterial communities in the bladder and other body sites.

14.2. Specific Aim 3

Urinary metabolomic profiling. We will assay the global urinary metabolic profiles for each MAPP urine specimen using the LC-MS based metabolomics platform consisting of a fast flow HPLC (Shimadzu) equipped with a 2.1x100mm fused core phenylhexyl column (Supelco) capable of 6 second wide peaks interfaced with an industry-standard hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex QTrap 4000). This platform has been optimized to yield comprehensive, global metabolic profiles of >2,300 distinct molecular features from human urine specimens [55]. Ionization, chromatographic, and quality control conditions have been optimized, along with a sample processing protocol that requires minimal user handling. Triplicate determinations will be obtained for each specimen in both negative and positive ESI modes.

Urinary sulfated steroid profiling. To evaluate the hypothesis that changes in other sulfated steroid levels accompany the elevated Etio-S levels observed in high symptom female UCPPS patients, we will configure the mass spectrometer to detect other urinary sulfated steroids. This will be accomplished by a precursor scan in which the first quadrupole scans a *m/z* range of 100 and 500, the ions are dissociated in the second quadrupole, and the third quadrupole monitors for a sulfate ion signal at *m/z* 97. A comparable approach identified a new molecule in the urine of patients infected with uropathogenic *E.coli* [61]. This approach substantially increases analytic sensitivity for sulfated molecules and so has the potential to identify new biomarkers.

Targeted urinary biomarker analyses. When specific molecules are identified through metabolomic profiling or sulfated steroid profiling, the mass spectrometer will be configured to specifically detect these molecules at their corresponding chromatographic retention times. This multiple reaction monitoring (MRM) method will select and fragment only the biomarker ions of interest (MS/MS) and monitor their specific breakdown products. We routinely obtain analytic sensitivity down to the femtomole (10^{-15} moles/mL urine = 0.000001 μM) range for current urine biomarkers. Analyte levels will be quantified by peak integration and normalization to invariant urinary metabolites analogous to creatinine.

Biomarker identification. We will use multiple analyses to determine the chemical identity of the biomarkers identified through metabolomic analysis. We will first chromatographically fractionate urine to obtain a purified preparation of the molecule of interest. For this we will use a fraction collector-equipped fast flow HPLC (Shimadzu) equipped with a 2.1x100mm fused core phenylhexyl column (Supelco). After confirming the presence of the biomarker with LC-MS, we will determine the biomarker's chemical formula by analysis with a high resolution ("accurate mass") ESI-equipped mass spectrometer. This information will be used to generate a list of candidate biomarkers from a chemical database (Metlin, Scripps). We will further distinguish from among these candidates by fragmenting the ion using MS/MS, which may generate diagnostic molecular fragments. Further structural information will be generated using GC-EI-MS, which can extensively fragment molecules in a reproducible manner that has permitted the creation of highly reliable databases able to distinguish chemical isomers. Where sufficient biomarker material can be purified, we will also consider chemical structural analysis using proton (^{1}H) NMR.

15. Statistical Analysis and Sample Size Estimates

15.1. Specific Aims 1 and 2

Number of Patients (and Power). The sample size/power considerations determined by the Steering Committee for the Trans MAPP SPS is for recruitment of 640 UCPPS patients (320 male; 320 female) meeting the eligibility criteria, recruited from at 6 existing Discovery sites. Microbial data from our previously analyzed MAPP I subjects (see preliminary work Section 7.1) shows that we will have adequate sample size for evaluation with this number of subjects in each study group. This number may require "practical" readjustment based on objectives and aims of the trans-MAPP SPS protocol, budget constraints and our previous experience answering similar questions with fewer samples. Every subject enrolled in the trans-MAPP SPS will be a potential candidate for urinary microbiota assessment.

See section 14 for specific analytical plans for Aims 1 and 2.

15.2. Specific Aim 3

Data analysis. To identify patient-associated molecular features, full scan or sulfate precursor scan profiles from selected urinary specimens will be used for multivariate analysis by XCMS (Scripps). Differential biomarker expression among clinical subgroups will be identified by statistical analyses by both unsupervised (PCA) and supervised (OPLS-DA) methods. These analyses will be used to identify specific molecules associated with the condition of interest (symptoms, flares, high Etio-S). Biomarker candidates will be validated through traditional descriptive statistics (mean, standard deviation, median, range) between pre-defined clinical groups. For between-group comparisons, we will determine whether the responses are normally distributed using the Shapiro-Wilk test. If normally distributed, we will use independent samples t-

tests to determine whether mean siderophore production levels differ between groups. If these measures are not normally distributed, we will use the Mann-Whitney U test. Univariate analyses will be performed by χ^2 or Fisher's exact test where appropriate for categorical variables. For evaluation of biomarkers as diagnostics, we will construct Receiver-Operator Characteristic (ROC) curves with the assistance of the MAPP DCC.

Number of subjects. 40 marker discovery samples, their 6- and 12- month longitudinal samples (120 total), 40-60 validation samples, 40 flares, 40 healthy controls. An additional 120 samples have already been analyzed

16. Proposed involvement of MAPP Network Cores

16.1. Tissue Analysis and Technology Core (TATC)

The TATC will be responsible for providing standardized specimen collection kits, specimen processing, banking, annotation/blinding, and distribution services across the MAPP Research Network. The TATC will also be involved in manuscript preparation.

16.2. Data Coordinating Core (DCC)

The Data Coordinating center will be responsible for data management and for providing data from the Trans MAPP study for the MWG studies. The DCC will also be involved in manuscript preparation.

17. Timeline for Completion of Data/Specimen Collection (see attached Appendix 1: SPS Protocol Timeline)

18. Anticipated Burden to Sites

Urine specimen collection is routine patient care, VB1, VB2 and VB3 (males only) will be collected for this protocol which is an additional burden on the visit schedule. To minimize burden the whole set (VB1, VB2, and VB3) will only be collected at the 4 weeks visit and at the time a participant experiences a flare during the clinic visit. At the other visits only VB2 specimens will be collected.

19. Anticipated Risks to Participants

Male participants will be asked to collect VB3 specimens that require a prostate massage, these collections will be optional for the participant. In addition male and female patients will be asked to provide a rectal swab while female patients are asked to provide a vaginal swab. Vaginal and rectal swabs are optional for the patient.

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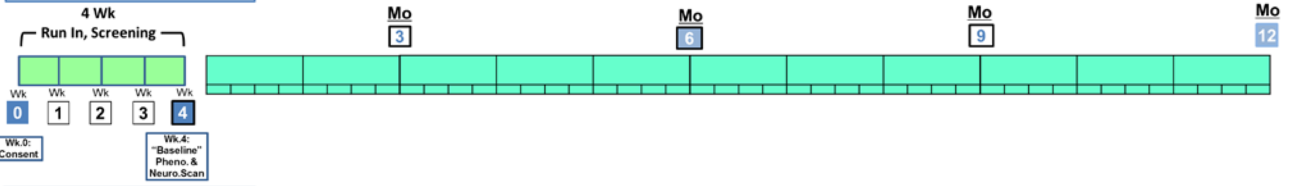
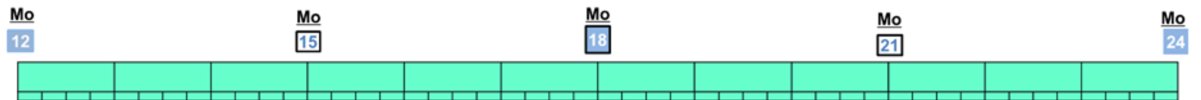
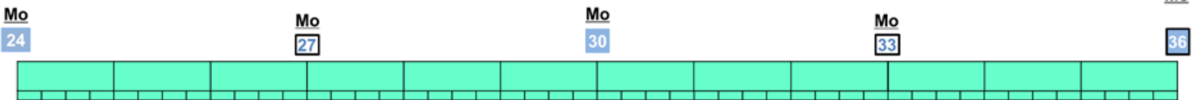
Appendix 1:SPS Protocol Timeline

Trans-MAPP Symptoms Patterns Study
3-Year Timeline







MULTIDISCIPLINARY APPROACH TO THE STUDY OF CHRONIC PELVIC PAIN (MAPP)

Appendix 3: Neuroimaging Protocol

Sponsored by:	The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Department of Health and Human Services (DHHS)
	PROTOCOL—VERSION 1.2
Dated:	06/16/2016
Collaborating Project Leaders:	
Prepared by:	The MAPP Neuroimaging Working Group
Confidentiality Notice:	This document is the confidential property of the MAPP Research Network Investigators. This document, either in part or in whole, may not be transmitted, reproduced, published, or used by persons outside the MAPP Network without prior written authorization from the MAPP Research Network Data Coordinating Center Principal Investigator.

Table of Contents

1. Identifying Information ... 3
2. Brief Overview / Introduction ... 3
3. Rationale for Phase II Trans-MAPP Study ... 3
4. Describe Link to Trans-MAPP Symptoms Pattern Study (SPS) ... 3
5. Study Hypotheses ... 4
5.1. Overarching Study Hypothesis ... 4
5.2. Specific Subhypotheses and Predictions ... 4
6. Specific Aims ... 6
7. Brief Description of Supporting Preliminary Data ... 6
8. Study Design and Methods ... 7
8.1. Single-Session Protocol ... 7
8.2. Image Acquisition Protocol ... 12
9. Description and Source of Patient Cohorts Beyond SPS, including Controls ... 14
10. Contact Schedule and Participant Procedures ... 15
11. Risk Factor and Outcome Measures ... 15
12. Biological Specimens ... 15
13. Neuroimaging Analytic Plan ... 15
13.1. Structural Analysis: Morphometry ... 15
13.2. Structural Analysis: Diffusion tensor imaging ... 15
13.3. Functional Analysis: Resting State ... 15
13.4. Functional Analysis: Evoked ... 16
13.5. Longitudinal Analysis of Brain Imaging Metrics ... 16
14. Statistical Analysis and Sample Size Estimates ... 16
15. Proposed Involvement of MAPP Network Cores ... 18
16. Timeline for Completion of Data/Specimen Collection (see attached Appendix 1: SPS Protocol Timeline) ... 19
17. Anticipated Burden to Sites ... 19
18. Anticipated Risks to Participants ... 20
19. References ... 20
Appendix 1: SPS Protocol timeline ... 22

1. IDENTIFYING INFORMATION

Working Group Chair:
Emeran Mayer, MD

Collaborating MAPP Network Discovery Sites:
Northwestern University; University of California, Los Angeles; University of Iowa; University of Michigan; University of Washington; Washington University, St. Louis

2. BRIEF OVERVIEW / INTRODUCTION

This document defines the hypotheses, aims, and protocols of the MAPP Phase-II neuroimaging study. This neuroimaging study aims to build on the neuroimaging discoveries of MAPP Phase-I to build a more comprehensive model of the neural representation of UCPPS symptoms, and the brain-body interaction that mediates these symptoms in different UCPPS subgroups.

3. RATIONALE FOR PHASE II TRANS-MAPP STUDY

Several brain differences between UCPPS patients and controls (brain signatures) were identified in the MAPP Phase-I study [3; 11; 13] (also MAPP manuscripts M027, M034, M037). These differences emerged in all three brain imaging modalities tested: T1-weighted imaging for gray matter, DTI imaging for white matter, and fMRI for resting state functional connectivity. While baseline brain imaging is available for many MAPP Phase-I patients for whom longitudinal symptom pattern data is available and is being analyzed to assess the predictive power of neuroimaging for UCPPS, an extremely limited number of patients had longitudinal brain imaging in MAPP Phase-I (1 year between scans). Longitudinal imaging data directly build upon objectives of the MAPP Phase-II Symptom Variability study in two ways: a) brain function and structure that co-vary with symptom severity and duration provide key indications of neuropathological mechanisms that maintain UCPPS, and b) invariant brain function and morphology that distinguish UCPPS from pain-free controls are potential biomarkers of predisposition to develop pelvic pain. Therefore, to more completely understand the UCPPS condition, and to delineate the central-peripheral interactions that may mediate UCPPS symptoms differently in distinct UCPPS subgroups, the optimal strategy is to execute a longitudinal study in which neuroimaging, inflammatory biomarkers, and symptom patterns are concurrently studied in a large cohort of patients across several years.

4. DESCRIBE LINK TO TRANS-MAPP SYMPTOMS PATTERN STUDY (SPS)

The neuroimaging protocol described below will be an integral part of the MAPP-II SPS, in that the standard set of phenotyping visits at 1, 6, 18, 36 months will included neuroimaging procedures.

5. Study Hypotheses

5.1. Overarching Study Hypothesis

In different UCPPS subtypes, certain brain signatures will be stable, and other brain signatures will be correlated with symptom change over time.

5.2. Specific Subhypotheses and Predictions

1. **Brain functional** differences (or*, functional signatures*) that distinguished UCPPS patients from healthy controls were identified in MAPP Phase I [11]. In Phase II, it is hypothesized that UCPPS symptom severity will correlate with patterns of resting state brain activity in a UCPPS subtype-specific manner.

    1.1 Longitudinal resting state data will reveal within-subject correlations between symptom severity and brain activity in regions implicated in nociceptive and emotional processing. Then, between-subject comparisons of these correlations will be used to articulate subtypes of UCPPS.

    1.2 Within-subject contrasts between baseline (post-voiding) resting state activity and post-water ingestion (high pain and/or urinary urgency) resting state activity will reveal enhanced functional connectivity in brain networks implicated in pain with bladder filling. Specifically, it is hypothesized that activity in the anterior paracentral lobule, and medial and ventral supplementary motor areas will exhibit enhanced functional connectivity to the midbrain and cerebellum, as we learned in MAPP I [11].

    1.3 The functional networks identified in (1.2) will be assessed across putative UCPPS subtypes with and without pain with bladder filling. Network functional connectivities will be compared between the pain with bladder filling subtypes, and a positive control group (no pain with bladder filling AND presence of pain and/or urinary dysfunction).

2. **Neocortical gray matter** changes are predicted to correlate with pain and urinary symptom changes over time, including the progressive worsening of UCPPS symptoms (i.e., [1]) and the improvement of these symptoms following successful treatment (i.e., [15]). Similarly, UCPPS subtypes are expected to exhibit different configurations of regional gray matter change [6; 14] (also MAPP manuscripts M024, M027, M055).

    2.1 Gray matter volume of distinct neocortical regions (excluding the subcortex and cerebellum) will correlate longitudinally with UCPPS symptom severity, indicating a role in pain maintenance. Given that gray matter changes may take weeks to develop after an inciting event, symptom profiles collected 3 months prior would best reflect measurable and statistically significant gray matter properties.

    2.2 Proposed UCPPS subtypes, including pelvic pain with and without bladder filling and pelvic pain only versus pelvic pain plus non-pelvic pain, will each show distinct configurations of gray matter changes that may serve as diagnostic biomarkers of pain

maintenance for each subtype. Additional subtypes suggested by the data will be explored in this manner, as well.

3. Distinct features of **axonal (white matter) integrity** will reflect neuroplasticity related to pain maintenance versus predisposition to develop UCPPS pain. White matter integrity has been shown to prospectively predict individuals who will go on to develop disease (Mansour, Alzheimer's study), suggesting that white matter properties may indicate predispositions to develop chronic pain. Alternately, in the MAPP I phase (Farmer et al, Submitted) both increases and decreases in white matter correlated with UCPPS symptoms severity in women, and we hypothesized that white matter increases may play a direct role in UCPPS symptom maintenance.

    3.1 Baseline white matter properties will be able to predict which individuals will continue to develop chronic pain, versus which individuals will improve over the longitudinal study.

    3.2 Longitudinal within-subject contrasts of fractional anisotropy will identify white matter regions that change across time, and these regions will be correlated to the core UCPPS symptoms at each time point to determine whether they robustly reflect changes in UCPPS symptoms over time.

    3.3 White matter regions that predispose individuals to further develop chronic pain will not overlap with regions that correlate longitudinally with symptoms severity.

4. **Central-peripheral interactions** mediating UCPPS symptoms will be revealed through within-participant correlation of functional and gray matter brain markers of UCPPS and peripheral blood and urine biomarkers sampled over time, and these interactions will be differentially expressed across distinct UCPPS subtypes [2; 5; 7; 11; 12]. Attention must be paid to time courses of these respective events, as functional imaging can reflect the present (or future predicted) pain state, gray matter can reflect the pain state weeks to months prior to measurement, and inflammation-related symptoms often develop days after the peak of colonization.

    4.1 At any single scan, functional resting state properties will be most closely correlated with enhanced peripheral blood and urine biomarkers of inflammation.

    4.2 Longitudinal analyses of within-subject scans will reveal individuals whose functional resting state data closely correlates with inflammatory markers, suggesting that these individuals are top candidates for inflammatory-based UCPPS subtypes.

6. SPECIFIC AIMS

Neuroimaging Overall Specific Aim **To determine if brain signatures, in conjunction with biomarkers and clinical exam data, moderate and/or mediate symptom variations within UCPPS patient subgroups, thereby identifying the brain-body networks generating the experience of UCPPS symptoms.**

Subaims:

1. To determine if structural (T1 and DTI) signatures of UCPPS are stable over a 3 year period.
2. To determine if functional (resting state fMRI) signatures of UCPPS, in conjunction with UCPPS biomarkers, mediate and/or moderate UCPPS symptom fluctuations over a 3 year period.
3. To extract bladder filling brain networks in UCPPS patients, and to determine if changes in this network mediate and/or moderate symptom fluctuations differentially in UCPPS subtypes over a 3 year period.

7. BRIEF DESCRIPTION OF SUPPORTING PRELIMINARY DATA

MAPP Phase-I data have, as of June 2014, yielded 12 TransMAPP manuscript proposals. The first of these, M014, was published in 2014 in the *Journal of Urology* [11]. Other manuscripts have since been submitted and are in various stages of revision. The figure below shows a summary of convergent evidence from these manuscripts that support alterations in how sensorimotor networks interact with attention and pain-generating mechanisms in the brain of patients with UCPPS.

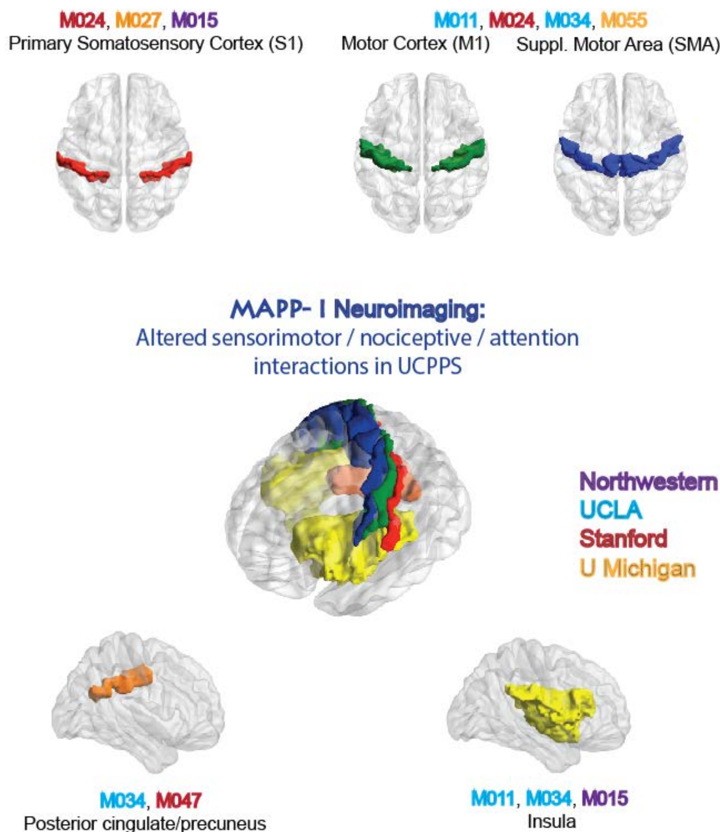


Figure 1. Convergence of MAPP-I neuroimaging results

Given the prevalence of bladder-related pain in UCPPS conditions, as well as the importance of motor networks observed in MAPP-I neuroimaging data, we hypothesize that functional and structural alterations may be localized in brain networks related to urine storage. These networks have been extensively studied and summarized in [8] (from which the figure below has been lifted):

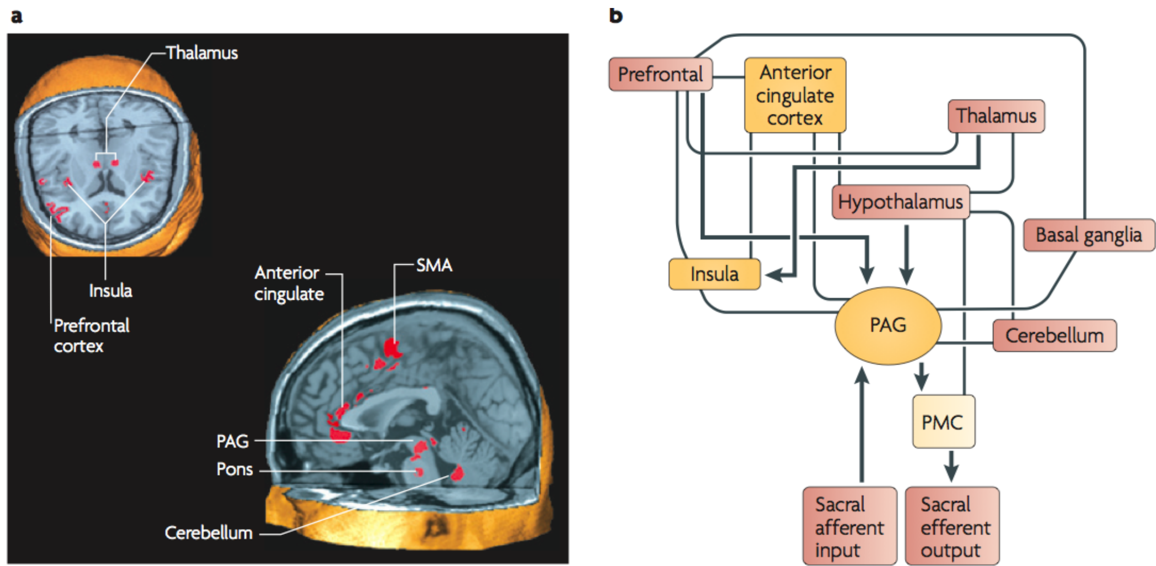


Figure 6 | **Brain areas involved in the regulation of urine storage. a** | A meta-analysis of positron-emission tomography and functional MRI studies that investigated which brain areas are involved in the regulation of micturition reveals that the thalamus, the insula, the prefrontal cortex, the anterior cingulate, the periaqueductal grey (PAG), the pons, the medulla and the supplementary motor area (SMA) are activated during the urinary storage. **b** | A preliminary conceptual framework, based on functional brain-imaging studies, suggesting a scheme for the connections between various forebrain and brainstem structures that are involved in the control of the bladder and the sphincter in humans. Arrows show probable directions of connectivity but do not preclude connections in the opposite direction. Part **a** reproduced, with permission, from REF. 64 © (2007) Backwell Science. Part **b** modified, with permission, from REF. 63 © (2005) Wiley-Liss.

Figure: The brain network regulating the storage of urine [8].

Neuroimaging data from MAPP Phase-I supports altered activity and connectivity of the SMA region associated with pelvic floor control in both men and women with UCPPS compared to sex-matched healthy controls. Other analyses of MAPP-I neuroimaging data have found structural and functional changes in the cerebellum, basal ganglia, precuneus, insula, and prefrontal cortex.

The MAPP Phase-II neuroimaging protocol is specifically designed to extend MAPP Phase-I data to specifically look at the performance of the bladder filling network longitudinally in a well-phenotyped sample of patients with UCPPS.

8. Study Design and Methods

8.1. Single-Session Protocol

The primary goal of the single session neuroimaging protocol, is to capture data relating to brain structure and function in resting state and subsequent to water consumption, and relate these measures to longitudinal measures of symptom changes within participant. The primary goal of the study is to acquire resting state fMRI (RS), T1-weighted structural images (T1), and diffusion tensor images (DTI) with the bladder as empty as possible, as in MAPP-I. A secondary goal is to capture a second RS scan with the participant reporting maximum urgency. A tertiary goal is to relate urgency at the second RS scan to the volume of urine voided after the second RS scan.

The specific procedure of the single session neuroimaging protocol is described below. This procedure is summarized in Figure 3.

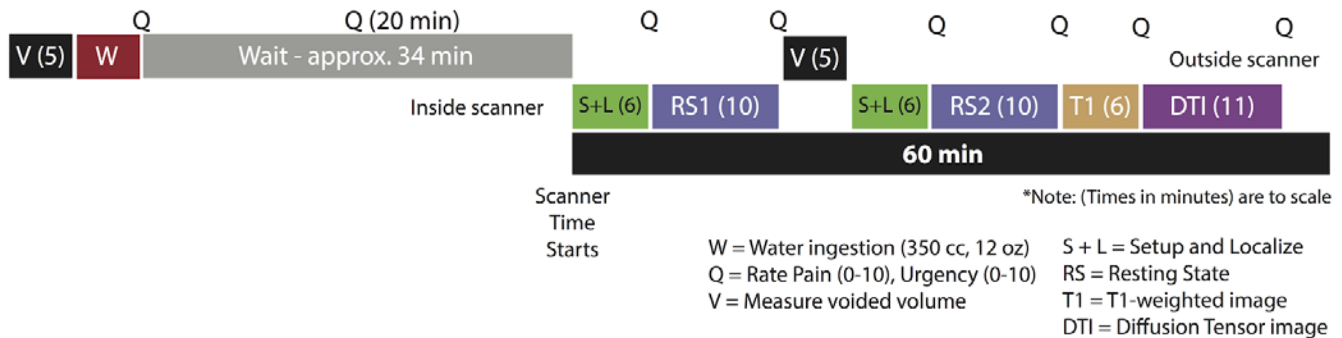


Figure 3. Protocol overview of bladder filling paradigm based on resting state changes

The standard operating procedure for the neuroimaging session is as follows. Items for the research coordinator to have:

- Copy of fMRI screening sheet
- Toilet hat (women) or urinal (men) specimen collector
- Gown or scrubs for participant (optional)
- Printed Neuroimaging CRF to record data
- Bottle of water (12 oz)
- Timer and watch
- Gloves

Plan to begin the participant void portion of the MRI task at approximately 44 minutes prior to when the imaging session is scheduled to begin. If you plan to ask the participant to change into a patient gown, plan to start earlier. Set your timer for 50 minutes. Watching the clock, start the time at 50 minutes prior to when your imaging session is scheduled to begin. Use this timer to coordinate the following steps.

1. Read to participant:: "*Now we'll start the MRI scan task. Your participation will help us understand how the brain responds to bladder discomfort and fullness. We will ask you to empty your bladder and drink 12 ounces of water. Then we will have you relax and answer a few questionnaires before the scans. You will undergo 4 scans, each about 10-15 minutes long. For the scans you will be asked to lie on your back in the scanner. Your head will be secured with a stabilizer that looks similar to an open football helmet. During the scans just relax and let your mind wander. Be sure to keep your head still during the scans, because moving in the scanner causes blurry, unusable images. You'll know when the scan is happening because the MRI will be making a series of loud banging noises. We will fit you with earplugs to block out the sound. We would like to image your brain when your bladder is as full as possible. You may experience increased pain and urgency, but we hope that this will help us to better understand pelvic pain. In between scans there is an intercom, where we can speak with you and you can let us know how you are doing. During the scans there is an emergency button you can press that tells us to stop the scan.* ***Do you have any questions?***"

2. (Optional) Approximately **49 minutes** before the start of the imaging session: Instruct participant to change into patient gown, including directions for the participant void below.

3. Approximately **44 minutes** before the start of the imaging session: Read to participant: *"We want to make sure you have an empty bladder before we start the task, so please try to urinate. If nothing empties from your bladder that is okay. If you are able to urinate, we want to measure the volume you void. To do this we will use a specimen collection (for females) hat/ (for males) urinal."* ***(For females, if participant is placing the hat)*** *"Here is the specimen collection hat. Lift the top toilet cover (if there is one) and the toilet ring, where you sit, and place the hat on the toilet, towards the rear of the toilet bowl. Lower the toilet ring to hold the hat in place and empty your bladder into the container. Leave the hat in the toilet when you're done."* (***For females, if coordinator is placing the hat***) *"Here is the specimen collection hat (show in the toilet). Empty your bladder into the container and leave the hat the in toilet when you're done."*(***For males***) *"Here is the specimen collection urinal. Empty your bladder completely into the cup and leave it here (show where you want it left) when you're done."* ***1. Void Time (H:M): ____________________ 2. Void Volume (cc): ___________________***

4. Approximately **39 minutes** before the start of the imaging session: Provide the water bottle to the participant and instruct them to try to finish the water in about 5 minutes. **3. Water drink start time (H:M): ____________________ 4. Water drink end time (H:M): ____________________ 5. Volume of ingested water (cc): ___________________**

5. Explain the rating of pain and urgency (show page demonstrating scales): "*Several times during the session we will ask you to rate pain and urgency to urinate. Please rate pain from 0 (no pain) to 10 (worst pain imaginable) and urgency from 0 (no urge to urinate) to 10 (worst urge imaginable)"*

6. Immediately **after water ingestion** ask the participant to rate their pain and urgency: **6. Time (H:M): _____________________ 7. Pain (0-10): _____________________ 8. Urgency (0-10): ____________________**

7. Approximately **20 minutes before the start of the imaging session:** Ask participant to rate their pain and urgency and record time.: **9. Time (H:M): _____________________ 10. Pain (0-10): _____________________ 11. Urgency (0-10): ____________________**

8. Perform all necessary pre-scanner checks (no metal, etc.).

9. Reassure the participant that they won't be in the scanner for very long with a full bladder: "*You will only be in the scanner for about 15 minutes at most with a full bladder. After that, you will be taken out of the scanner to urinate, and then return to the scanner for some additional scans with your bladder empty. Although it's important to stay in the scanner as long as possible, you are in control of these scans, and if you are* ***certain*** *that you can't hold your urine any longer, squeeze the emergency button and we will immediately remove you from the scanner."*

10. Approximately **5 minutes before the start of the imaging session:** Direct the participant towards the scanner room and have the MRI tech begin positioning the participant in the scanner.

11. Have MRI technician perform **Localizer Scan**

12. Ask participant to rate pain and urgency and record time (prior to resting state 1): **12. Time (H:M): _____________________ 13. Pain (0-10): _____________________14. Urgency (0-10): ____________________**

13. Instruct participant: "*This first scan is 10 minutes. Rest with your eyes closed but do NOT go to sleep. Please keep your head as still as possible.*"

14. Have MRI technician perform **Resting State 1 Scan.**

15. Ask participant to rate pain and urgency and record time: **15. Time (H:M): _____________________ 16. Pain (0-10): _____________________ 17. Urgency (0-10): ____________________ 18. Did the participant go to sleep (Y/N): __________________**

16. Have MRI technician remove participant from scanner and instruct the participant to try to void if possible, using the same instruction as above for the urine collection hat/urinal. **19. Time (H:M): _____________________ 20. Void Volume (cc): ___________________**

17. Have MRI technician position the participant in the scanner

18. Have MRI technician perform **Localizer Scan.**

19. Ask participant to rate pain and urgency and record time: **21. Time (H:M): _____________________ 22. Pain (0-10): _____________________ 23. Urgency (0-10): ____________________**

20. Instruct participant: "*This next scan is 10 minutes. Rest with your eyes closed but do NOT go to sleep. Please keep your head as still as possible.*"

21. Have MRI technician perform **Resting State 2 Scan**

22. Ask participant to rate pain and urgency and record time:**24. Time (H:M): _____________________ 25. Pain (0-10): _____________________ 26. Urgency (0-10): ____________________ 27. Did the participant go to sleep (Y/N): __________________**

23. Instruct participant: *"The next scan will be about 6 minutes. Please keep your head as still as possible."*

24. Have MRI technician perform **T1 Scan**

25. Ask participant to rate pain and urgency and record time: **28. Time (H:M): _____________________ 29. Pain (0-10): _____________________ 30. Urgency (0-10): ____________________**

26. Instruct the participant: *"During the next scan, the table will shake like a massage table, and this is normal. This scan will be about 11 minutes. Please keep your head as still as possible."*

27. Have MRI technician perform **DTI Scan**

28. Ask participant to rate pain and urgency and record time: **31. Time (H:M): _____________________ 32. Pain (0-10): _____________________ 33. Urgency (0-10): ____________________**

29. **Were there any deviations from the protocol? No Yes → *If yes...***

30. **Participant needed to void after water ingestion prior to RS1 → RS1 not completed**

31. **Participant terminated scan to void mid-RS2, T1, or DT and returned to MRI:**

32. **Participant terminated scan and did not return to MRI.**

33. **Scan in progress during termination: ______________**

34. **Termination reason:**

    a. **Anxiety/Claustrophobia**

    b. **Too high of void frequency**

    c. **Refused to continue**

    d. **Other: ____**

35. **Other (*ex. problem with scanner; timeline delayed)***

    ____

36. Be sure that the scan data is named using this naming convention. Refer to pain.med.ucla.edu/mapp for the naming convention.

37. Inform appropriate person at your site to upload neuroimaging data to pain.med.ucla.edu/mapp

38. **How to Handle Deviations**

39. If the participant is certain they have to void prior to entering the MRI for the first localizer scan then allow the participant to void and begin with the second localizer scan and Resting State 2. Do not repeat the water ingestion.

40. If a participant terminates (asks for the MRI to stop) due to the need to void: During the first Localizer scan - allow the participant to exit the scanner, void, and return for the second Localizer, Resting State 2, T1, and DTI scans. Do not repeat the water ingestion. Mid-Resting State 1 scan - allow the participant to exit the scanner, void, and return for the second Localizer, Resting State 2, T1, and DTI scans. Do not repeat the water ingestion. During the second Localizer scan, Resting State 2, T1, and DTI scans - allow the participant to exit the scanner, void, and return to redo the scan from the beginning and complete any additional scans.

The above protocol (or similar variants) have been piloted at the University of Washington and Northwestern University as of June 2014. UCPPS patients have been found to tolerate the protocol.

8.2. Image Acquisition Protocol

The most up-to-date acquisition protocols should be downloaded according to scanner:

1. Siemens Trio
   a. PDF
   b. EDX

Structural Acquisition: Morphometry

The MP-RAGE pulse sequence should be used for T1-weighted 3D volume imaging. The equivalent pulse sequence on a GE scanner is 3D FSPGR IR. Basic parameters are:

TR = 2300 ms
TE = 2.98 ms
TI = 900 ms
Flip angle = GE: 11º, Siemens: 9º, Philips: 9º
FoV = 256 mm x 256 mm
Resolution = 256 x 256
Slices per volume = 240 (or maximum available while maintaining all other parameters)
Slice thickness = 1 mm
Inversion = Slice Selective
Phased array acceleration factor = 2
Phase encode direction = left-right and superior-inferior
Orientation = axial-oblique parallel to the line between the anterior and posterior commissures

Structural Acquisition: Diffusion tensor imaging

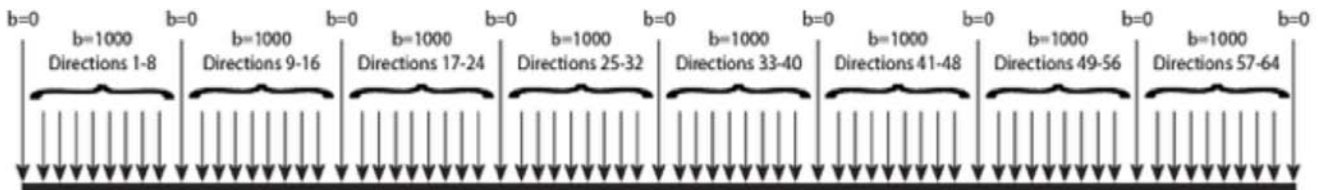
The diffusion tensor imaging (DTI) sequence chosen for MAPP-II is derived from a common q-ball imaging protocol involving 64 directions at equidistant angles on a sphere in q-space. The total number of directions (64) was chosen based on a balance between total acquisition time, the need for high angular resolution data for use in probabilistic tractography techniques, and commonly used gradient schemes from the MR system manufacturers. The 2mm isotropic resolution and 64 directional encoding scheme is also commonly used in neurosurgical studies and studies involving structural connectivity analysis.

The DTI protocol will use a diffusion-weighted single shot spin-echo EPI pulse sequence. The standard 64 directional diffusion encoding directions common to Siemens and Philips will be used on all scanners with research capabilities. For scanners with expanded research modes, a custom gradient table with 9 b=0 s/mm^2 images interspersed throughout the scan will be included for increased SNR and motion correction (see diagram below).

The following pulse sequence parameters should be used:

TR = > 8000ms (minimum to obtain 75 slices, or whole brain coverage)
TE = min (or between 85-100ms)
Flip angle = 90º
FoV = 256 mm x 256 mm
Matrix Size = 128 x 128

Slice thickness = 2 mm
Slice gap = 0
Slices per volume = >75
Phased array acceleration factor = 2
Bandwidth = maximum to accommodate resolution specifications
Orientation = axial-oblique parallel to the line between the anterior and posterior commissures
64 directions of diffusion sensitization b = 1000 s/mm^2
9 non-diffusion weighted images (b = 0 s/mm^2)



Functional Acquisition: Resting State

Resting state fMRI will a single shot echo planar imaging (EPI) pulse sequences with conventional rectangular Cartesian sampling. Basic pulse sequence parameters are as follows

TR = 2000 ms
TE = 30 ms
Flip angle = 77º
FoV = 220 mm x 220 mm
Resolution = 64 x 64
Phase encode direction = anterior -> posterior
Slice thickness = 4.0 mm
Slice gap = 0.5 mm
Slice acquisition = ascending (not interleaved)
Slices per volume = 34 – 40 to cover entire brain
Phased array acceleration factor = 2
Bandwidth = maximum to accommodate resolution specifications
Orientation = axial-oblique parallel to the line between the anterior and posterior commissures
Number of volumes = 300 (10 min acquisition)

During the resting state fMRI acquisition the subject should have his/her eyes covered. No audio or video presentation should be made during the scan. The subject should be instructed to rest quietly during this scan.

Image Acquisition Deployment Plan

Protocols for each scanner brand, model, and version will be distributed and made available for download. All scans will undergo review to ensure compliance and quality.

Protocol Testing Plan

We suggest implementing a traveling subject protocol to facilitate calibration of cross-site imaging: firstly, to instruct the scanner technicians and ensure proper adherence to the prescribed protocol (including scanner operation and the administration, timing, and management of fluid intake for the patient); secondly, to obtain scans that can be used for quality assurance and potentially contribute to assessing the inter-scanner variability inherent in multi-site studies. The traveling subject would be knowledgeable of the scan protocols and the bladder filling procedure, and he/she would be available to answer investigator and technician questions at each site.

Traveling subjects have been previously implemented in clinical studies using both fMRI [9] and DTI [16]. Acquisition of the full bladder-filling protocol and a second set of fMRI, DTI and structural scans would allow for test-retest analysis. Acquired data would be immediately uploaded to the central repository at UCLA to confirm functionality of the transfer mechanism. Once scans are received at UCLA, a quality control check would be performed that includes the following: inspection of scan parameters, check for values and proper reconstruction of additional images (such as ADC and FA images for DTI), check for dropped slices and motion, check of DTI tractography reconstruction, and check for artifacts and drastic distortion. The scan data will also be used to evaluate inter-scanner variability, whereas the test-retest scans will provide information on inter-session variability. In order to obtain an optimal estimate for inter-session variability, additional repeat scans of the same travelling subject would be performed at UCLA.

9. Description and Source of Patient Cohorts Beyond SPS, including Controls

The neuroimaging protocol will be run (and associated neuroimaging CRF will be completed) on 10-12 healthy participants twice (approximately 6 months apart).

Each site is requested to recruit controls that have an age and race/ethnicity distribution similar to that of the UCPPS subjects recruited in the MAPP SPS. The sites will also aim to recruit equal number of male/female healthy controls. All of the recruitment Discovery Sites for MAPP will participate in the Trans-MAPP Neuroimaging Protocol and therefore will all recruit 10-12 Control participants. These are:

- University of California, Los Angeles (UCLA)/University of Southern California (USC)
- Northwestern University, Chicago, Illinois
- Washington University, St. Louis, Missouri
- University of Iowa, Iowa City
- University of Washington, Seattle
- University of Michigan, Ann Arbor

For additional details regarding Control participants for the study, please see the MAPP Control Study protocol which includes details on the inclusion/exclusion criteria for the Control Participants as well as a detailed Control study visit schedule and other details.

10. CONTACT SCHEDULE AND PARTICIPANT PROCEDURES

(see attached list of current proposed Phase II/SPS Protocol CRFs)

11. RISK FACTOR AND OUTCOME MEASURES

12. BIOLOGICAL SPECIMENS

13. NEUROIMAGING ANALYTIC PLAN

The following sections define analysis methods appropriate to the various neuroimaging modalities being used in the MAPP-II study. Each of these analysis techniques define metrics of interest, which will be associated with symptom progression. The simultaneous analysis of longitudinal symptom progression and neuroimaging data is detailed in the last section.

13.1. Structural Analysis: Morphometry

Structural images will be processed according to methods used in MAPP-I (see [10] for details). Metrics of gray matter thickness, density, and similar will be carried forward into longitudinal analysis.

13.2. Structural Analysis: Diffusion tensor imaging

Image Preprocessing

Analyses are based on tools from the FMRIB Software Library (FSL) and in-house software. DTI images are first visually inspected volume by volume for obvious artefacts. Data are eddy current- and motion-corrected using affine registration to the first no-diffusion weighted volume of each subject using FMRIB's Diffusion Toolbox (FDT). Following skull extraction, a diffusion tensor model is fit to each voxel to determine voxel-wise fractional anisotropy (FA).

Analysis

Voxel-wise FA data is analysed for group differences using FSL's tract-based spatial statistics (TBSS). For each site, pooled FA data (patients and controls) is non-linearly aligned to standard space to generate a mean FA skeleton that represents a voxel-wise FA template of tracts that are common across all subjects. Aligned individual subject FA data is projected onto the common FA skeleton. Group contrasts are assessed using permutation methods (n=5000 permutations), with corrections for gender and age where appropriate. For regions exhibiting group FA differences, individual average FA values are extracted and correlated with clinical pain and urgency parameters to determine clinical significance. Component diffusivities, including mean, radial, and axial diffusivity, are examined for these FA regions to investigate potential mechanisms underlying altered white matter integrity.

13.3. Functional Analysis: Resting State

Frequency-based analyses

fMRI images will be processed to extract frequency-based measures of activity using methods defined in MAPP-I (see [11] for details). Metrics of activity in different frequencies bands will be carried forward into longitudinal analysis.

Functional connectivity analyses

Functional connectivity maps will be generated based on particular ROI using methods defined in MAPP-I (and [11] and M034 for details). Metrics of functional connectivity will be carried forward into longitudinal analysis.

13.4. Functional Analysis: Evoked

Functional connectivity and activity metrics derived from RS1 (low urgency) and RS2 (high urgency) will be contrasted from a single session, possibly normalized to the actual change in urgency reported by the participant. Contrast maps will be carried forward into longitudinal analysis.

13.5. Longitudinal Analysis of Brain Imaging Metrics

Identifying brain regions and networks related to UCPPS symptom change

In the most basic analysis of longitudinal neuroimaging data, single session clinical symptom data will be correlated with brain imaging metrics derived from the individual or combined modalities. If $X(t)$ represents a clinical parameter of interest collected close to the time of scan (t), and $Y_i(t)$ represents the brain imaging metric associated with voxel i, brain imaging analysis in standard software such as FSL will seek to identify clusters of voxels for which the correlation between $X(t)$and $Y_i(t)$ is significant. In another layer of analysis, differences in the association between $Y_i(t)$and $X(t)$will be determined between the different patient subgroups.

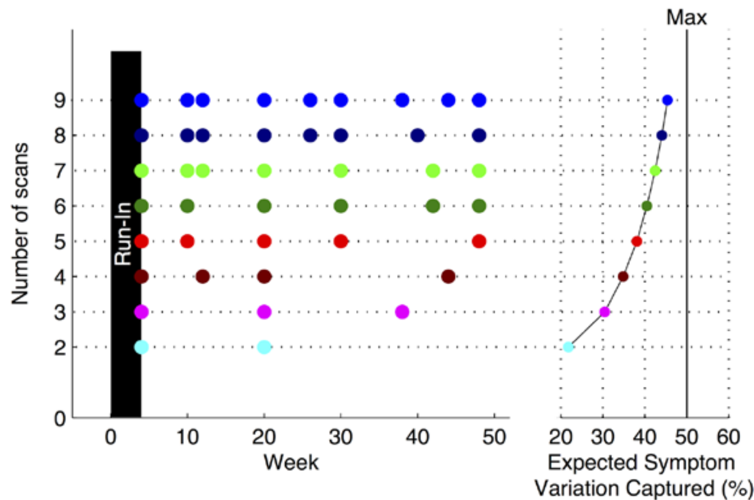
14. STATISTICAL ANALYSIS AND SAMPLE SIZE ESTIMATES

A statistical analysis to estimate necessary sample sizes for the proposed longitudinal neuroimaging study must take into account both the number of patients and the number of scans per patient.

Number of scans per patient

Proposed MAPP Phase-II sample sizes for the SPS are based on an analysis of symptom worsening and improving across the MAPP Phase-I cohort, and predicts n=640 patients. This analysis did not directly address the probability that patients will be observed at different symptom states given a scanning schedule, or estimate the expected change in symptoms.

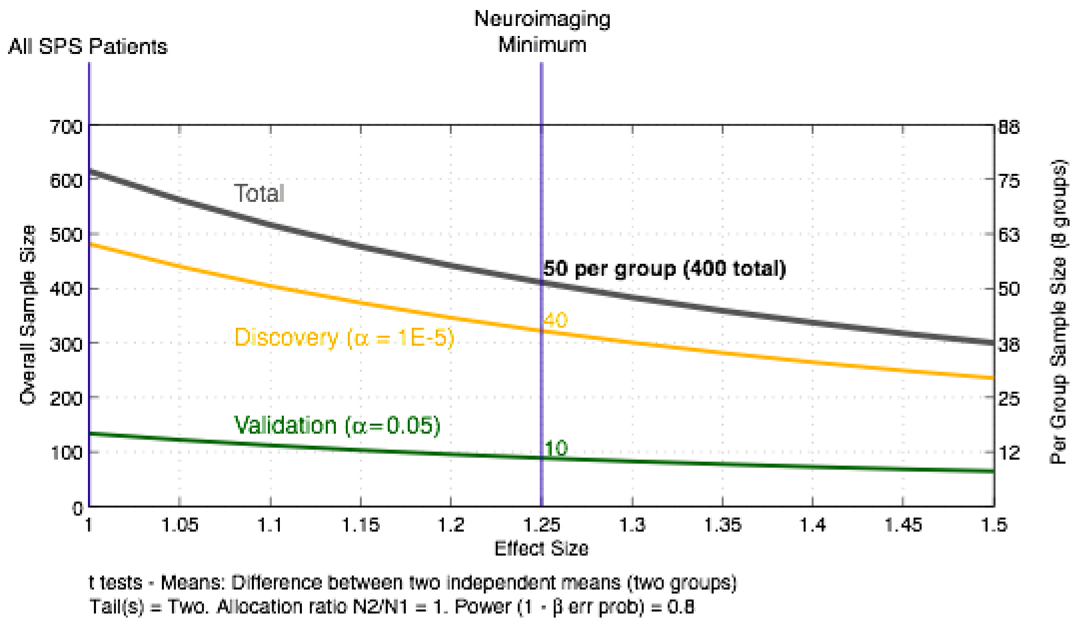
The figure below (Fig 4) shows the results of analysis of variability in the ISCI symptom questionnaire (sampled every 2 weeks from MAPP Phase-I participants). For a given number of scans, the optimal weeks to place these scans (to maximize the difference in reported symptom across the set of scans) is shown. There is a large increase in the expected symptom change when the number of scans is increased from 2 to 3 (20% symptom variation is expected for 2 scans and 30% symptom variation is expected for 3 scans), with a diminishing increase in additional variation for additional scans. Scanning every 6 months (after 4 week run in, at week 24, and week 48) also produced 30% symptom variation, indicating that it was a nearly-optimal scan schedule for 3 scans.



Number of Patients

All patients in the SPS will be enrolled in longitudinal neuroimaging procedures, and thus we can estimate the minimum effect size that is needed to detect group differences for a given sample size, power, and significance level. Representative effect sizes observed in MAPP-I ranged from 0.75 (Kilpatrick et al., 2014 - frequency analysis [11]) to 1.25 (Kutch et al., M034 in preparation - functional connectivity analysis; [10] - structural analysis). Of 640 UCPPS patients, the minimum size of a MAPP-II recruitment cell is 60, which allows for two-tailed unpaired t-test between two groups of 60. Assuming that an analysis is focused on the validation of a MAPP-I result, we calculate that for a power of 0.8 we would be able to validate effect sizes (alpha error probability = 0.05) of 0.51, clearly less than the expected effect sizes of MAPP-I. These parameters will allow us reach (alpha error probability 5e-05) effect sizes of 0.92, which are consistent with MAPP-I effect sizes. In summary, the sample size of the MAPP-II SPS appears to be adequate for both validation and discovery purposes.

Analysis of sample size (without it being fixed to 640 apriori), indicates that the neuroimaging sample size should be larger than 400 to capture effect sizes that were observed in MAPP-I across 8 different recruitment groups (males/females, PP only/PP & beyond, BPS:Yes, BPS:No).



15. Proposed Involvement of MAPP Network Cores

Data Coordinating Center (DCC)

In addition to storing all of the patient metadata associated with neuroimaging scans, we propose that in MAPP-II the DCC interacts with the individual scanning sites and the UCLA-PAIN neuroimaging center to ensure both 1) timely upload of all scans into the UCLA-PAIN repository (pain.med.ucla.edu/mapp) and 2) that quality assurance data generated in UCLA-PAIN reports is integrated into the DCC master MAPP-II database. We propose the following scheme for this interaction:

Data Coordinating Center (DCC)
U. Penn

1. Scanning site informs DCC of a new scan
2. DCC informs UCLA-PAIN to expect upload of new scan data
3. Scanning site uploads imaging data to UCLA-PAIN (UCLA-PAIN follows up with scanning site if upload does not occur within 1 week of scan date)
4. UCLA-PAIN perform quality assurance (QA) and generates report for each scan

5. UCLA-PAIN sends QA report to DCC for integration into database
6. DCC reports on-going QA results to scanning sites on neuroimaging teleconferences

16. Timeline for Completion of Data/Specimen Collection (see attached Appendix 1: SPS Protocol Timeline)

17. Anticipated Burden to Sites

Below is a diagram of the neuroimaging leads at each site. It has been divided into the overall neuroimaging lead, the protocol lead (concerned with the implementation and maintenance of the experimental protocol) and the technical lead (concerned with the implementation and maintenance of the image acquisition protocol). The 1.25 hour neuroimaging protocol has been extensively discussed within the MAPP Neuroimaging Working Group, and does not pose undue burden on the sites for completion.

18. ANTICIPATED RISKS TO PARTICIPANTS

Potential Risk to Participants

The specific risks of these procedures relate to the site specific protocols, but in general, the physical risks associated with this study are those associated with MRI scanners [4]. Functional Magnetic Resonance Imaging Scan does not involve injections or any radioactive tracers. Some people experience dizziness or a metallic taste in their mouth if they move their head rapidly in the magnet. However, this is only a temporary effect and is not experienced if the head is kept still. The scanner produces loud sounds at times and insulated earphones will be provided to reduce the noise you hear. Although the long-term risk of exposure to magnetic fields and radiofrequencies associated with MRI is not known. The possibility of any long-term risk is extremely low in view of the information accumulated over the past twenty years. If unrestrained iron or steel objects are accidentally brought near the MRI magnet, they can be pulled very quickly toward the magnet and can strike people in or near the magnet. Such an event is very unlikely because precautions are taken to prevent such objects from being brought near the magnet. Subjects are screened for iron or steel implants or clips from surgery, or metallic objects such as shrapnel or metal slivers in their bodies and are excluded from study if present. Dental fillings do not present a hazard.

There is a risk that the water ingestion protocol will generate pain, including a possible pain flare-up. The neuroimaging session contingency plan shows how this risk will be minimized so that the acquiring this data will not cause undue urgency in the participant.

Risk / Benefit Assessment

There are no direct benefits to subjects for participation in these studies except for monetary reimbursement. The risks as stated are minimal. There is a benefit to society in the expansion of scientific knowledge regarding disorders associated with pelvic pain.

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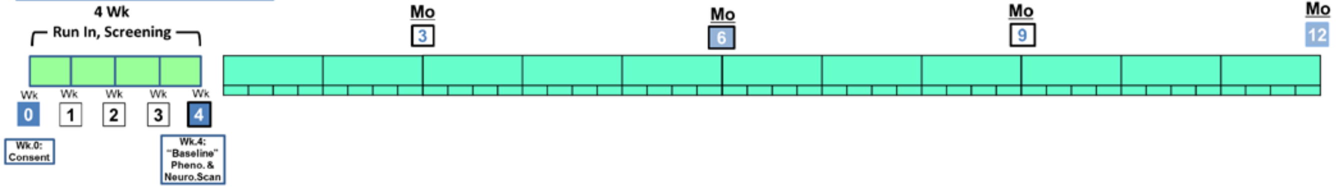
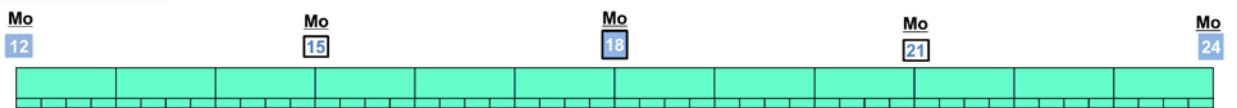
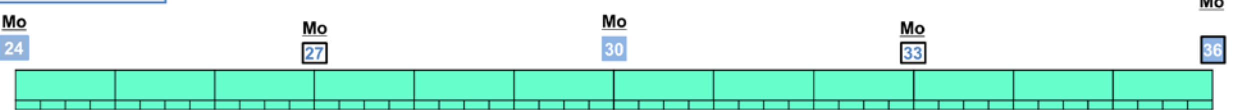
APPENDIX 1: SPS PROTOCOL TIMELINE

Trans-MAPP Symptoms Patterns Study
3-Year Timeline

Blue box = Semi-annual optional Clinic Visit (may be replaced by ATLAS clinic visit)
Blue box w/ black border = Required Clinic Visit (can't be replaced)
White box = Online surveys







MULTIDISCIPLINARY APPROACH TO THE STUDY OF CHRONIC PELVIC PAIN (MAPP)

Appendix 4: Trans-MAPP Quantitative Sensory Testing (QST) Study Protocol

Sponsored by: The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Department of Health and Human Services (DHHS)

PROTOCOL—VERSION 1.2

Dated: 06/16/2016

Collaborating Sites: University of Michigan, University of California Los Angeles, Washington University at St. Louis, University of Washington

Prepared by: Steven Harte, Daniel Clauw, Bruce Naliboff, Henry Lai, Simon Haroutounian, Timothy Ness

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Contents

CONTENTS 2

1. IDENTIFYING INFORMATION 3
2. BRIEF OVERVIEW / INTRODUCTION 3
3. RATIONALE FOR PHASE II TRANS-MAPP STUDY 4
4. DESCRIBE LINK TO TRANS-MAPP SYMPTOMS PATTERN STUDY (SPS) 6
5. STUDY HYPOTHESES 6
6. SPECIFIC AIMS 7
7. BRIEF DESCRIPTION OF SUPPORTING PRELIMINARY DATA 8
8. STUDY DESIGN AND METHODS 10
9. DESCRIPTION AND SOURCE OF PATIENT COHORTS BEYOND THOSE FROM THE SPS, INCLUDING CONTROLS 13
10. CONTACT SCHEDULE AND PARTICIPANT PROCEDURES 13
11. RISK FACTOR AND OUTCOME MEASURES 13
    a. Common Data Elements (CDEs) from Trans-MAPP Epidemiology Study 13
    b. Specialized Data Measures 14
12. BIOLOGICAL SPECIMENS 14
13. OTHER MEASURES 14
14. ANALYTIC PLAN 15
15. STATISTICAL ANALYSIS AND SAMPLE SIZE ESTIMATES 15
16. PROPOSED INVOLVEMENT OF MAPP NETWORK CORES 16
    a. TATC: none 16
    b. DCC: The DCC will be responsible for the following: 16
17. TIMELINE FOR COMPLETION OF DATA/SPECIMEN COLLECTION 16
18. ANTICIPATED BURDEN TO SITES 17
19. ANTICIPATED RISKS TO PARTICIPANTS 17
20. REFERENCES 17

APPENDIX 1: TRANS-MAPP SYMPTOMS PATTERNS STUDY 3 –YEAR TIMELINE 21

APPENDIX 2: CURRENT CRFS/SCHEDULE FOR TRANS-MAPP SYMPTOMS PATTERNS STUDY (SPS) 22

APPENDIX 3: QST CORE BATTERY VARIABLES V0.2 23

1. Identifying Information

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Collaborating MAPP Network Discovery Sites:

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University of California, Los Angeles, CA
Washington University, St. Louis, MO
University of Washington, Seattle, WA

2. Brief Overview / Introduction

The overall objective of this study is to provide the Phase II MAPP studies a broader and more comprehensive set of quantitative sensory testing (QST) methods. To this end, the QST Working Group proposes a "core" battery of four QST methods to assess pain sensitivity and modulation in UCPPS patients. The selection of these methods was based on MAPP-I multisite and site-specific data, literature review, and the objectives of the MAPP-II Symptom Pattern Study (SPS). It represents a consensus among the members of QST Working Group and offers the MAPP Network a comprehensive yet achievable approach to QST phenotyping. Data derived from the QST core battery will be used for development of patient subgroups, comparison with symptom, neuroimaging and biological data, and prediction of longitudinal symptom outcomes.

Building on the success of the pressure pain testing protocol in MAPP-I to differentiate UCPPS patients from control groups and predict symptom progression, we will continue to assess generalized mechanical sensitivity using the University of Michigan (UM) MAST system. In addition, the QST core battery will include an evaluation of spinal segmental mechanical sensitivity at the most common referral site of pelvic pain (the suprapubic area), as well as tests of temporal summation and conditioned pain modulation (CPM).

The core battery will be conducted at all discovery sites at week 4 (baseline) and 6, 18, and 36 month "deep phenotyping" in-clinic visits on all SPS participants starting in Year 1 of the MAPP-II life cycle. The core QST battery will also be executed during ATLAS modules at the pre-therapy and post-therapy in-clinic visits. A trans-MAPP "extended" QST battery, which tentatively includes tests of auditory and visual sensitivity, will be proposed during Year 2 as an amendment to the core battery protocol. The extended battery is being deferred because it 1) requires additional time for

methodological development, and 2) may only be feasible at a subset of sites due to availability and cost of required equipment.

As in MAPP-I, the UM will provide MAST systems to all discovery sites. In addition, UM will provide all other necessary equipment required to perform the core battery. The core battery requires approximately 90 minutes to complete. Participants will undergo a comprehensive familiarization and training procedure prior to data collection. Training for MAPP study personnel will be conducted at the face-to-face steering committee meetings and/or onsite by the QST Working Group. Ongoing feasibility testing of the core battery will be conducted prior to the Year 1 recruitment launch. Adjustments to the protocol will be made as necessary during feasibility testing to achieve optimal efficiency and an acceptable level of patient and staff burden. No changes will be made that increase the length of burden of the testing.

3. Rationale for Phase II Trans-MAPP Study

Many chronic pain patients report increased sensitivity to both somatic (e.g., mechanical, thermal) as well as non-somatic (e.g., auditory, visual, olfactory) sensory stimuli.[1-6] But relatively few studies have utilized QST to evaluate pain or sensory sensitivity in UCPPS and these have had primarily small and only female samples.[7-10] In one of the earliest such studies, Clauw et al., [7] demonstrated that female interstitial cystitis (IC) patients have significantly decreased pressure pain thresholds throughout the body at traditional fibromyalgia tender points compared to healthy controls.[7] Results from the remaining QST studies conducted on this patient population seem to depend largely upon the pain modality assessed. One group found hyperalgesia to bladder filling but no difference in cutaneous electrical thresholds between subjects with painful bladder syndrome and controls.[8] Ness et al. [9] showed that pressure pain and ischemic pain thresholds were significantly decreased in IC patients when measured at the forearm. More recently, Lai et al.[10] demonstrated increased pressure sensitivity in the suprapubic region of IC patients compared to controls. Thermal pain sensitivity has also been assessed; one group identified a significant *decrease* in sensitivity in 1 of 4 tested dermatomes among patients with painful bladder syndrome compared to controls,[11] while other studies failed to find any significant abnormalities in thermal pain sensitivity in IC patients.[9,10]

In addition to evaluating sensory sensitivity, QST can also be used to evaluate excitatory and inhibitory endogenous pain modulation. Endogenous pain inhibitory networks are activated in healthy humans and animals during application of a noxious stimulus, such that a noxious stimulus applied to one body area inhibits pain in other areas, resulting in a phenomenon termed conditioned pain modulation (CPM). Deficient CPM is associated with a variety of poor clinical outcomes, including increased postsurgical pain and analgesia requirements, and is believed to be a potential indicator of centralized pain.[12,13] A 2012 meta-analysis of studies that investigated CPM in multiple chronic pain conditions confirmed that CPM is attenuated in the majority of chronic pain

conditions.[14] Although UCPPS and related conditions were not included in the meta-analysis, a 2014 report identified deficient CPM in 14 females with IC/bladder pain syndrome using a thermal paradigm.[15]

Whereas QST to detect CPM is probing for the absence of inhibitory pain mechanisms in chronic pain states, temporal summation measures increases in excitatory pathways that lead to altered pain perception. Temporal summation refers to an increased perception of pain in response to sequential stimuli of equal physical strength. It is a QST model of neural plasticity and central hyper-excitability that is thought to reflect the progressive increase in neuronal firing of dorsal horn neurons in response to repetitive nociceptive C-fiber stimulation (i.e., windup).[16-20] Although temporal summation has not been assessed in UCPPS, it is increased in a number of other chronic pain conditions and it has been shown to be predictive of the development of pain.[20-24]

Finally, QST can also be used to show that some chronic pain patients, including those with UCPPS, exhibit increased sensitivity to non-somatic stimulation, including auditory and visual stimuli.[1,3,25-27] There is also evidence that somatic pain and auditory sensitivities are often interrelated in chronic pain reinforcing the overarching hypothesis that a global state of central nervous system (CNS) sensory amplification might play a role in the pathogenesis of many chronic pain disorders and these measures may mark an important individual patient phenotype.[1,2,25] The biological plausibility of this is further supported by the fact that functional imaging studies find the insula, a brain region that plays a polysensory integration function, is hyperactive in most individuals with chronic pain states.[28,29]

In Phase I of the MAPP, the University of Michigan-designed MAST system[30] was used to perform pressure-based QST as part of the trans-MAPP epidemiology study. Other sites had several site specific studies examining auditory and CPM measures in men and women with UCPPS. Initial analysis of the trans-MAPP data has shown increased pressure pain sensitivity in UCPPS patients at a non-symptomatic neutral site (the thumbnail) compared to healthy controls, and importantly, UCPPS somatic sensitivity *at baseline was predictive of longitudinal changes in symptom severity, a major endpoint being explored in MAPP II.* Smaller studies at UCLA have also shown increased auditory sensitivity in UCPPS compared to health controls and an association of CPM in UCPPS with self-reported non-urological pain. We propose to continue this work and to extend the use of QST in MAPP Phase II to better evaluate the role that the CNS is playing in pain and sensory sensitivity among individuals with UCPPS.

The overall objective of the MAPP Phase II QST protocol is to provide a broader and more comprehensive set of QST measures into the Phase II MAPP studies. The following paradigms will be used to evaluate pain processing at different levels of the neuraxis:

(1) *Generalized hypersensitivity* will be assessed using the MAST device on the thumb,

(2) *Spinal segmental hypersensitivity* will be assessed using a pressure algometer and pinprick stimulator at the suprapubic area, and

(3) *Conditioned pain modulation* will be used to determine the efficacy of supraspinal pain inhibitory mechanisms.

This structured testing strategy permits better characterization ("phenotyping") of UCPPS subjects into subgroups based on their somatosensory response to these different test procedures than thumb pressure testing alone. Moreover, different QST measures and modalities engage different aspects of pain perception and potentially different CNS mechanisms, thus permitting a more comprehensive investigation into the neurobiological mechanisms that underlie UCPPS sensory symptoms and the prediction of longitudinal symptom patterns.

4. Describe Link to Trans-MAPP Symptoms Pattern Study (SPS)

All SPS participants will complete the QST core battery. QST data will be compared to and correlated with symptom and biological data collected in the SPS.

5. Study Hypotheses

Overarching Study Hypothesis

Quantitative measures of generalized and segmental pain sensitivity, altered endogenous pain modulation, and/or global sensory sensitivity are associated with symptom variations within UCPPS patient subgroups and can be used to predict longitudinal outcomes and treatment response.

Specific Sub-hypotheses:

1. Individuals with UCPPS restricted to the pelvis (peripheral phenotype) at baseline will be more likely to progress to the centralized phenotype if they exhibit generalized hypersensitivity.
2. Individuals with UCPPS restricted to the pelvis (peripheral phenotype) at baseline that exhibit diminished CPM and/or enhanced temporal summation will show a greater likelihood of progression to the centralized phenotype.
3. Some QST measures will co-vary with symptoms and treatments and thus reveal underlying pathophysiological mechanisms (e.g., deficient endogenous pain control) in subsets of UCPPS patients. Other QST measures will not have these characteristics and will instead represent stable traits that may confer an increased risk of UCPPS but do not change over time.
4. UCPPS sensory profiles will correlate to brain functional, structural, and connectivity signatures.

5. **5.** The QST battery will identify sensory-based mechanistic patient subgroups (e.g., generalized vs. regional hypersensitivity).
6. **6.** QST measures will predict additional variance above and beyond other measures in the SPS, where multivariate models can be created that predict the largest variance in predicting symptom progression.
7. **7.** Subjects identified by QST as having greater generalized sensitivity (i.e., increased mechanical sensitivity at the thumb and/or forearm, and the pelvic region), and/or deficient CPM, and/or increased temporal summation will show a preferential response to central-acting treatments in the ATLAS study.
8. **8.** Subjects identified by QST with increased mechanical sensitivity only at the pelvic (suprapubic) region will show a preferential response to peripheral-acting treatments in the ATLAS study.

6. Specific Aims

The above hypotheses motivate a series of specific aims that will be the focus of the QST Trans-MAPP study and are outlined below:

QST Overall Specific Aim

To more fully characterize the sensory phenotype of UCPPS patients using a battery of quantitative measures to assess generalized and segmental sensory sensitivity and endogenous pain modulation.

Sub-aims:

1. To identify QST characteristics at baseline and follow-up that predict or are strongly associated with UCPPS symptom progression or regression.
2. To characterize potential changes in pain sensitivity and modulation associated with changes in symptoms to reveal underlying pathophysiological mechanisms of pain in UCPPS.
3. To determine the reliability of the QST measures and the stability of UCPPS sensory profile over a 3 year period.
4. To assess interactions between QST data and neuroimaging, biological, and ATLAS study outcomes.
5. To establish QST-based patient subgroups based on a multi-level sensory testing strategy.
6. To use QST data in conjunction with other SPS data in multivariate statistical models for predicting longitudinal symptom patterns.

7. Brief Description of Supporting Preliminary Data

The QST core battery is based on findings from MAPP-I and work conducted by investigators in the QST Working Group. These data are described below.

Pressure Pain Sensitivity (MAPP I). Evaluation of pressure pain sensitivity was conducted on a subset of MAPP Phase I participants (n = 346) at six discovery sites. The MAST QST system (described below) delivered an ascending series of discrete pressures to the dominant thumbnail. The thumbnail was chosen as a stimulation site because of its dense innervation of mechanical receptors and large representation in the primary somatosensory cortex.[31-33] In addition, the thumb is a "neutral site" that is not associated with pain symptoms in UCPPS or in other chronic conditions (e.g., it is not a fibromyalgia tender point or myofascial trigger point), and it is easily accessible for experimental testing. It was also previously demonstrated that experimental pain evoked by thumbnail pressure is associated with overall body tenderness [34], measures of clinical pain[35], functional neuroimaging[36], and brain levels of glutamate[37], and is lowered following analgesic

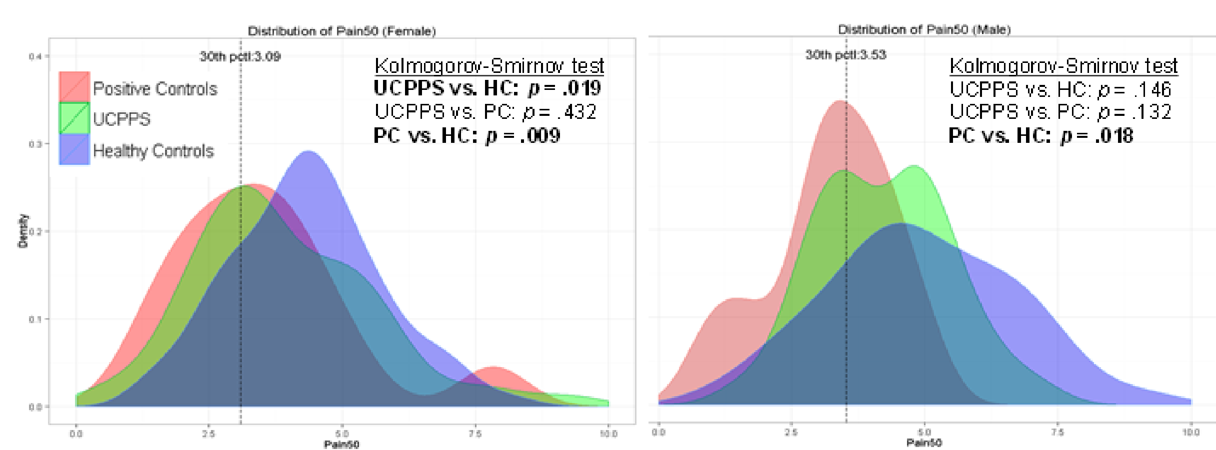

Fig. 1. Distribution of Pain50 values in MAPP Phase I.

treatment.[38]

Pain intensity was rated after each stimulus on a 0-100 numerical rating scale (NRS) displayed on the patient interface, and these ratings were used to compute a psychophysical function of each subject's pressure pain sensitivity. The midpoint between the minimum (pain threshold) and maximum (pain tolerance) stimulus intensity was estimated within-person using the SAS NLIN procedure to derive a measure of suprathreshold pressure pain sensitivity, referred to as Pain50. As shown on in **Figure 1**, female and male UCPPS and positive control patients (primarily fibromyalgia) exhibited significantly decreased Pain50 thresholds compared to healthy controls (*p*s < .02). Importantly, the Pain50 variable was not associated to potentially confounding psychological factors, including anxiety, depression, affect and coping. In addition to Pain50 findings, UCPPS females showed significantly decreased pressure pain detection thresholds (PPT) compared to healthy controls (p < .001), whereas UCPPS males showed decreased pain tolerance compared to

healthy controls (p = .01). These findings suggest possible sex differences in generalized pain sensitivity in UCPPS. Pain50 at baseline was also tested as a predictor of improvement or worsening of UCPPS over 6 months. Logistic regression indicated that patients who were less sensitive to pressure pain were more likely to improve on GUPI symptoms [(GUPI overall OR (95% CI) = 1.79 (1.02-3.13), p = .04; GUPI urinary = 2.05 (1.06-3.99), p = .03; GUPI QoL = 2.03 (1.09-3.77), p = .03)], whereas the ICINSEX symptom was less likely to worsen [(OR = .25 (.06-.99), p = .05]. These findings highlight the value of QST phenotyping in UCPPS patients and we will continue this pressure pain sensitivity assessment in MAPP Phase II to obtain larger sample sizes for additional subgroup analyses.

Conditioned Pain Modulation. Almost all paradigms for evaluating CPM incorporate both a conditioning stimulus (a noxious stimulus that evokes CPM) and a test stimulus (a noxious stimulus used to evaluate the analgesic effect of the conditioning stimulus). The magnitude of CPM is typically calculated as the difference in pain ratings of the test stimulus at baseline and during or immediately following application of the conditioning stimulus. A reduction in test stimulus rating by the conditioning stimulus implies functional or intact (inhibitory) CPM, and the degree of reduction expresses the efficiency of CPM mechanisms.

During Phase I of the MAPP, the UCLA site used a thermal CPM protocol based on previously validated procedures by Yarnitsky.[13] Data from 31 UCPPS subjects (14 F, 17 M) showed a significant relationship between CPM magnitude and SIMQ ratings of non-urological pain severity (r = -.33) and SIMQ ratings of negative mood (r = -.42). In both cases greater pain and negative mood were associated with decreased pain inhibition. Interestingly none of the urological symptom measures were associated with CPM supporting CPM as a potential useful measure of “centralization” in UCPPS. Group differences in CPM were not found between UCPPS and healthy controls, perhaps due to limited power in this small pilot sample. However, Ness et al.,[15] recently demonstrated diminished CPM in a sample of fourteen female IC/painful bladder patients compared to healthy controls.

Segmental Mechanical Hypersensitivity. Although not conducted as part of MAPP Phase I study, Lai et al.[10] recently investigated segmental mechanical hypersensitivity in ten female subjects with IC without comorbid fibromyalgia and ten matched healthy controls using a pressure algometer. Compared to controls, IC patients reported increased pain to fixed pressure stimuli (2 or 4 kg/cm^2) applied in a random sequence to the suprapubic (T11) area (**Figure 2**). This segmental hyperalgesia to mechanical pressure stimulation in the suprapubic area may be explained in part by peripheral sensitization or spinal central sensitization

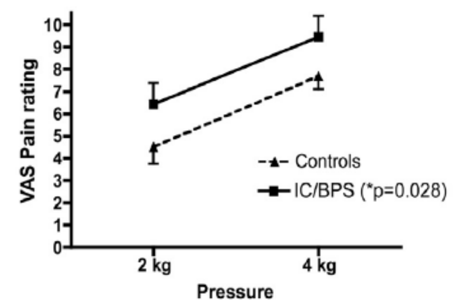

Fig. 2. Suprapubic mechanical hypersensitivity in interstitial cystitis. Adapted from Lai et al.[10]

in this patient population. The lack of global mechanical hypersensitivity in this study is in contrast to the MAPP I findings using the MAST system at the thumb and suggests that segmental pain testing offers a useful measure to further phenotype UCPPS patients into mechanistic subgroups.

8. Study Design and Methods

Procedures for QST will adhere to standardized experimental protocols. Equipment will undergo regular calibration to maintain reliability and consistency across multiple testing sites. Instructions will be scripted and participants will undergo extensive familiarization and training before testing, including practice sessions of each QST method (details included in QST MOP). All procedures have been evaluated for reliability and safety, and are well tolerated by chronic pain patients, causing no more than temporary mild discomfort. However, subjects can stop testing at any time if a procedure becomes unbearable. The order of tests will be as follows: Generalized Mechanical Sensitivity, Segmental Mechanical Sensitivity, Temporal Summation, Conditioned Pain Modulation. Pilot testing indicates that the entire QST core battery will require approximately 90 minutes to complete, including training and rest intervals between tests.

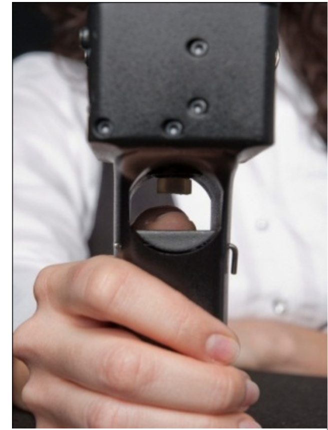

Fig 3. MAST handset.

QST Core Battery

a. *Generalized Mechanical Sensitivity.* Generalized, or global, mechanical sensitivity will be evaluated using the UM-designed MAST system.[30,39] The MAST system is a non-significant-risk investigational device that applies a computer-controlled pressure stimulus to the thumbnail at a precisely controlled intensity for a specified duration. The MAST system consists of two tablet computers, one of which is an experimenter-controlled server that manages the test procedure, and the other a touch-screen patient interface that can display instructions and that the participant uses to enter responses. The system also includes a hand-held force actuator, or handset, that applies pressure stimuli to the thumbnail bed (**Figure 3**). The MAST handset is a pistol-grip-style unit manufactured in cast urethane and ergonomically designed to be held comfortably in either hand by 95% of all U.S. adults with a slot into which the participant inserts his or her thumb. Pressure is applied to the participant's thumbnail by a conformal rubber probe with an area of 1 cm^2. The probe is attached to a cylindrical transducer driven by a miniature servo-motor. A dynamic, closed-looped control system uses digital load-cells to measure the exact pressures applied to the thumb, and self-adjusts motor output to the resistance of the thumb and any movement to ensure accurate and repeatable force delivery.

During testing, the MAST System will deliver an ascending series of discrete pressures (5-s duration; 4 $kg/cm^2/s$ ramp rate) to the dominant thumbnail at 16-24-s intervals, beginning at

0.25-0.50 kg/cm^2 and increasing in 0.25 or 0.50 kg/cm^2 steps. Pain intensity will be rated after each stimulus on a 0-100 NRS displayed on the patient interface (0 indicating "no pain" and "100" indicating "most intense pain imaginable"). The test will be terminated when subjects reach their maximum tolerable pain level and request the test to stop (tolerance), a pain intensity rating of $\geq$ 80/100 is recorded, or the maximum allowable pressure of 10 kg/cm^2 is reached.

Pain ratings from the MAST system will be used to compute a psychophysical function of each subject's pressure pain sensitivity, with pressure intensity and response magnitude represented on the x- and y-axes, respectively. These curves and their underlying data points will be used to compare single subject and group changes in pain sensitivity longitudinally throughout the SPS. As in MAPP I, the Pain50 variable will be also be calculated for each participant and used as the primary measure of generalized hypersensitivity. Pressure pain threshold (PPT) and pressure pain tolerance will also be determined for each subject. PPT is defined as the first pressure in a string of at least two consecutive pressures that elicited a NRS pain rating > 0. Tolerance is defined as the last pressure recorded in the stimulus response profile.

b. *Segmental Mechanical Sensitivity.* Segmental mechanical sensitivity will be assessed following a method modified from Lai et al.[10] A handheld, analog algometer with a 1 cm^2 flat rubber probe (FPK Algometer, Wagner Instruments, Greenwich, CT) will be used to deliver quantifiable pressure stimuli to the suprapubic area (midway between the pubic symphysis and the umbilicus; see **Figure 4)** – the most common referral site of pelvic pain. This type of algometer provides robust measurement and does not require extensive training.[40-42] Algometry will also be performed at a non-symptomatic control site on the ulnar forearm midway between the wrist and elbow on the dominant body side **(Figure 4**).

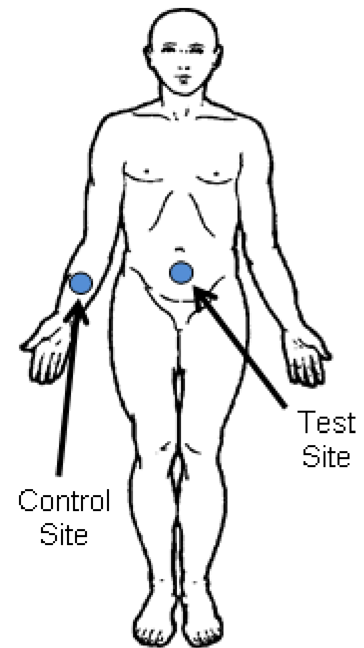

Fig. 4**.** Testing sites.

Patients will first empty their bladder prior to testing to reduce the chance of algometer stimulation provoking unanticipated urine voiding. Next, a random sequence of fixed intensity pressures (2 or 4 kg/cm^2, 5-s duration, 20-s inter-stimulus interval) will be applied, 3 times each, first to the forearm control site and then to the suprapubic test site, with the participant in the supine position. Thus, a total of 12 pressure stimuli will be delivered (six at each site). Pressure will be increased at rate of approximately 0.5 kg/cm^2/s (equivalent to 50 kPa/s). A ticking 1 Hz frequency metronome (Korg MA-1) with earphones will be used to help control the rate of applied pressure to reduce examiner variability.[43] Subjects will be asked to verbally rate the pain

intensity of each pressure using a 0-100 NRS. The mean of three NRS ratings for each pressure level at each testing site will be used for analysis.

Pressure pain threshold will also be assessed bilaterally at a second control site. Pressure will applied manually using the algometer at a steady rate of 0.3-0.5 kg/cm^2/s to the center of the trapezius muscle until the patient indicates an initial sensation of pain.[44] The pressure level at the time of this verbal report will be recorded as the PPT. Pressure will not exceed 8 kg/cm^2. This procedure will be repeated 2-5x and the mean threshold will be used for analysis.

c. *Temporal Summation*. Temporal summation, also referred to as windup pain, will be evaluated using a method similar to that of the German Neuropathic Pain Network[43] and the OPPERA study.[44] In this test, a single pinprick stimulus at a fixed intensity of 256 mN (PinPrick Stimulator, MRC Systems GmbH) will be applied perpendicularly to the skin for approximately 0.5-s. Following a 5-s pause, a train of 10 identical pinprick stimuli (256 mN) will be applied with a frequency of 1 Hz within an area of 1 cm^2. Immediately following the single stimulus and the train of 10 stimuli, participants will be asked to verbally report the pain intensity of the pinprick sensation using a 0-100 NRS. This testing paradigm (a single stimulus followed by a train of 10 stimuli) will be conducted 3 times with the same 256 mN stimulator at the suprapubic region and at the forearm control site (**Figure 4**) while the participant is in the supine position. At 15- and 30-s following the last train of 10 stimuli, participants will also be asked to rate any residual pain sensation (i.e., pain "after-sensations") in the testing area using the 0-100 NRS. For each testing site, the mean pain rating of the three stimulus trains will be divided by the mean pain rating of the single stimuli to calculate a wind-up ratio (WUR); a WUR of >1 indicates temporal summation.[45]

d. *Conditioned pain modulation.* Participant responses to a painful test stimulus during painful conditioning vs. neutral (non-painful) conditioning stimulation will be assessed as a measure of endogenous pain inhibition. The CPM procedure used here will follow the method of Yarnitsky[13,46,47] except that we will use pressure instead of thermal stimulation as a test stimulus.

Painful pressure delivered using the MAST system to the dominant thumbnail will serve as the test stimulus. Immersion of the contralateral foot into a circulating water bath with body temperature water (31.0-33.0°C) or moderately painful hot water (42.0-46.5°C)[46] will serve as the neutral and painful conditioning stimulus, respectively. The water bath is a custom-made, 14 L polycarbonate tank (L x W x D, 18x8x8") with a perforated foot rest positioned 1 inch from the bottom surface of the tank to permit water flow around all surfaces of the foot. Water temperature and flow rate is maintained using a laboratory-grade thermal immersion circulator (LX Immersion Circulator, PolyScience, Niles, IL).

For the assessment of CPM, continuous or pulsed test pressure will be applied for 30-s to the dominant thumbnail at each patient's pre-determined Pain30 or Pain40 level (i.e., the estimated pressure intensity that evokes a pain intensity rating of 30/100 or 40/100, respectively). Patients will rate the pain intensity of the pressure 3X (10-, 20-, and 30-s) using a 0-100 NRS. Conditioning stimulation will begin 5-10 min after the test stimulus by immersing the contralateral foot up to malleolus area into the water bath. The foot will be immersed for approximately 60-s; perceived pain and heat intensity of the water will be rated 5-, 10- and 20-s after foot immersion and immediately following foot withdrawal. The neutral conditioning stimulus will be applied first, followed by the painful conditioning stimulus, with a 5-10 min rest between each session. The foot will be removed from the water bath between sessions. Parallel to the last 30-s of conditioning, the same test stimulus will be reapplied to the dominant thumbnail for 30-s and the participants will be again asked to rate the intensity of the pressure 3X (10-, 20-, and 30-s). CPM magnitude will be calculated as the difference in the mean of the three pain ratings given to the test stimulus prior to the conditioning stimuli and the three pain ratings of the test stimulus given during the conditioning stimuli.

As an alternate CPM procedure, thumbnail pressure will be replaced with either the trapezius PPT or the forearm temporal summation procedures described above. The conditioning stimuli will not change and the test stimulus will be presented during the last 30 to 40-s of conditioning. This alternate procedure will be employed if the MAST system is unavailable or if patients are unable to tolerate sustained thumbnail pressure.

9. Description and Source of Patient Cohorts Beyond those from the SPS, including controls

None

10. Contact Schedule and Participant Procedures

The core battery will be conducted at all discovery sites at week 4 (baseline) and 6, 18, and 36 month "deep phenotyping" in-clinic visits on all SPS participants starting in Year 1 of the MAPP-II life cycle. The core QST battery will also be executed during ATLAS modules at the pre-therapy and post-therapy in-clinic visits. See the Trans-MAPP SPS Protocol Timeline for the complete test schedule (**Appendix 1**).

11. Risk Factor and Outcome Measures

a. Common Data Elements (CDEs) from Trans-MAPP Epidemiology Study

(see Appendix 2 for current proposed Phase II/SPS Protocol CRFs)

b. Specialized Data Measures

Stimulus-response data obtained during MAST testing will be transmitted directly to the DCC via secure file transfer protocol (SFTP) from MAST system computers immediately following testing. All other QST data will be recorded by study personnel on paper or electronic CRFs and entered into the MAPP electronic data capture system.

The primary QST measures of interest are listed below. (See **Appendix 3** for a complete listing of all collected data elements)

- Generalized Mechanical Sensitivity (MAST Test): Pain50; pressure pain threshold; pressure pain tolerance
- Segmental Mechanical Sensitivity (Algometer Test): suprapubic 2 kg and 4 kg mean pain ratings; forearm 2 kg and 4 kg mean pain ratings, trapezius PPT
- Temporal Summation (Pinprick Test): suprapubic WUR; forearm WUR
- Conditioned Pain Modulation: Relative CPM magnitude (painful conditioning - neutral conditioning)

12. Biological Specimens

Biologic specimens collected during the course of the Trans-MAPP Study include urine, blood (serum) and cheek swab for DNA. The schedule of these collections is summarized in Table 1. Please include any "new" specimen collection or specimen type needed not collected in Phase I

Table 1. Schedule of Biologic Specimen Collection in MAPP (See attached SPS timeline)

Measure	Implementation Schedule
Blood specimen	
Spot urine specimen	
Cheek Swab For DNA	

13. Other Measures

None

14. Analytic Plan

Analyses will be conducted by the DCC in collaboration with the QST Working Group. QST measures will be treated to the identical statistical methods applied to the SPS dataset. Primary analysis will consist of the within-subject prediction of longitudinal symptoms. Broadly speaking, we will test at least two hypotheses drawn directly from the Symptom Pattern Study:

Hypothesis 4a: *Quantitative measures of generalized and segmental pain sensitivity, altered endogenous pain modulation, and/or global sensory sensitivity are associated with symptom variations within UCPPS patient subgroups and can be used to predict longitudinal outcomes and treatment response.*

Hypothesis 8: *Multivariate models [that include QST data] can be developed that predict a reasonable degree of variance in whether individuals with similar clinical presentation of UCPPS symptoms have progression or regression of their symptoms.*

Prior to analysis, all relevant measures will be fully described, including aspects of data quality. For both predictor variables and outcomes, a summary of each variable, or group of variables, will be produced. Sample means and standard deviations will be reported for continuous variables; and relative frequencies will be calculated for categorical variables. Graphical methods will be used extensively to identify potential outliers and to examine assumptions (such as normality) underlying statistical models. Hypothesis testing will be conducted using a two-tailed significance level (type I error) of $\alpha = 0.05$, with actual p-values reported whenever possible. Continuous variables will be compared using the two-sample t-test; whereas, Fisher's exact test will be used to compare dichotomous variables. Ordinal variables will be assessed using the Wilcoxon rank-sum test. Multivariable linear and logistic regression models will be used to adjust for potential confounding variables in comparisons of continuous and binary measures, respectively.

15. Statistical Analysis and Sample Size Estimates

All subjects participating in the SPS will undergo QST. The Trans-MAPP SPS will recruit 640 UCPPS patients meeting the eligibility criteria, recruited from six clinical sites. Approximately half of the participants will be male, and half will be enriched to meet the body map pain location criteria of Pelvic Pain Only. Table 1 provides estimates of sample size estimates to achieve 90% power.

Table 1. Estimated sample size requirements to obtain 90% power.

	Correlation Between Pre- and Post-Assessments			
d	**0.1**	**0.3**	**0.5**	**0.7**
0.3	**213**	**166**	**119**	**72**
0.4	**121**	**94**	**68**	**42**
0.5	**78**	**61**	**44**	**28**
0.75	**36**	**29**	**21**	**14**
1	**21**	**17**	**13**	**9**

	Percent of Subjects in Group 1		
d	**25%**	**40%**	**50%**
0.3	**156/470**	**196/294**	**235/235**
0.4	**89/267**	**112/168**	**133/133**
0.5	**57/171**	**72/108**	**86/86**
0.75	**26/78**	**32/48**	**39/39**
1	**15/45**	**20/30**	**22/22**

16. Proposed involvement of MAPP Network Cores

a. **TATC:** none

b. **DCC:** The DCC will be responsible for the following:

- Providing statistical expertise in research design, outcome measures and analytic strategies;
- QST data management, including quality control, pre-processing, cleaning, and data storage;
- Providing mechanisms for data-sharing among sites and combining QST data with that collected in the SPS and neuroimaging protocols; and
- Interim and final data analysis and publication support in collaboration with the QST Working Group and other MAPP Investigators.

17. Timeline for Completion of Data/Specimen Collection

See Appendix 1: SPS Protocol Timeline

18. Anticipated Burden to Sites

Sites will be required to administer the QST battery. This will require training of study personnel, storage of testing equipment, as well as the space and time to perform testing. Under the direction and guidance of the QST Working Group, study personnel will also ensure that QST equipment is working properly and undergoes all scheduled and unscheduled calibration procedures and repairs, and that QST data are secured and uploaded to the DCC in a timely manner.

19. Anticipated Risks to Participants

QST may cause minor but temporary physical discomfort. Study personnel will be trained by the investigators to be sensitive to participant discomfort and concerns. Participants will be instructed that they can stop QST anytime that the pain or unpleasantness of the task becomes unbearable. Specifically, MAST testing may cause some temporary physical discomfort on the thumbnail. The MAST System incorporates a series of redundant mechanical, electrical, and software safety features to prevent patient injury in the event of user error or device failure, including a safety pin that the subject can turn to immediately remove the pressure actuator from his or her thumb. The pressure algometer and pin prick stimulus are commonly used in QST studies and will not cause tissue injury at the maximum forces applied in this study (8 kg/cm^2 and 256 mN, respectively). However, both instruments may cause minor physical discomfort in the areas of testing (suprapubic region, forearm, and trapezius) that is expected to resolve within minutes of test completion. These tests will also be halted if a participant reports a pain rating of 100. The heated water used in the CPM test will also cause some temporary discomfort of the foot and lower leg. The maximum temperature of the water (46.5°C) and the duration of immersion (1 minute) are below the recommended standards of the U.S. Consumer Product Safety Commission (Publication 5098) to prevent scalding injury in adults.

20. References

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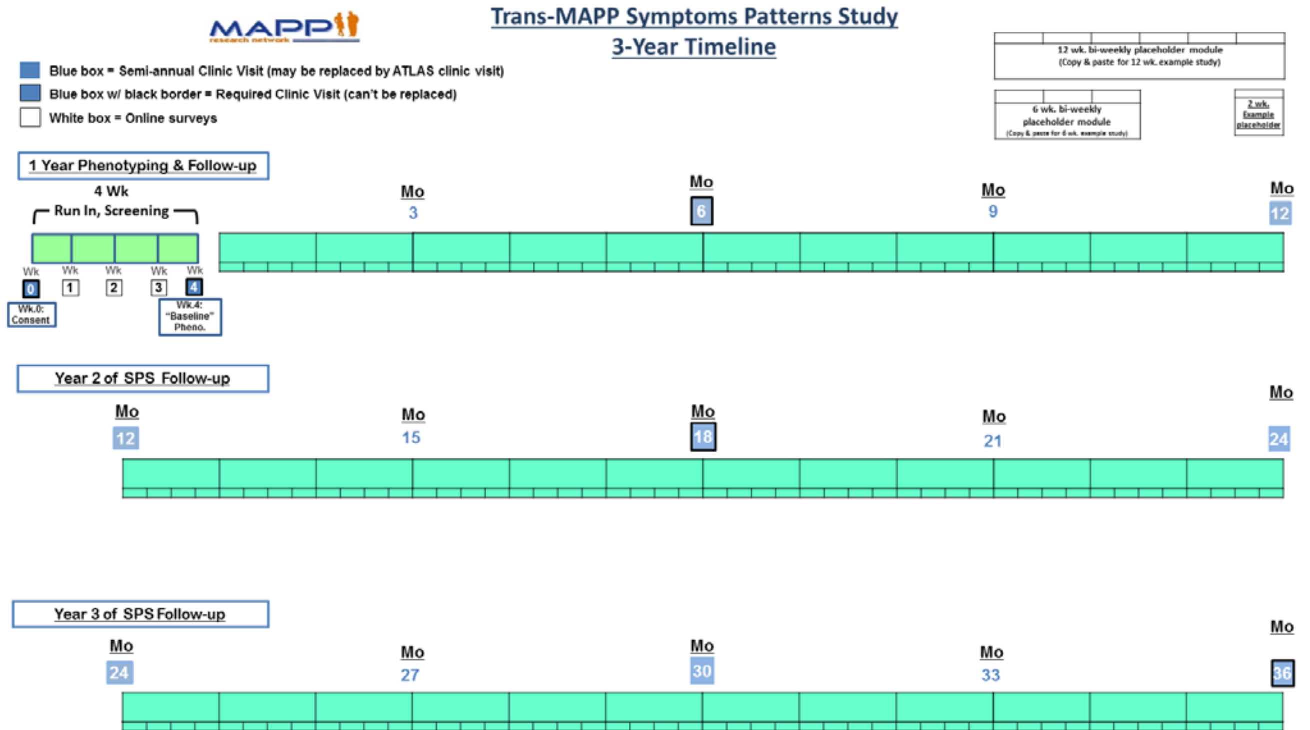
Appendix 1: Trans-MAPP Symptoms Patterns Study 3 –Year Timeline



Appendix 2: Current CRFs/Schedule for Trans-MAPP Symptoms Patterns Study (SPS)

(Please note: The current CRF list and schedule for administration is still under development and pending additional feedback from the various workgroups)

Appendix 3: QST Core Battery Variables v0.2

The Core Battery consists of four separate QST measures (in bold). Non-italicized variables are recorded on a CRF. Italicized variables need to be calculated. All pain intensity ratings will use a 0-100 numerical rating scale.

1. **Generalized Mechanical Sensitivity (MAST Test)**
    a. Ratings of individual pressures (i.e., 0 .25 kg, 0.5 kg, 1 kg, … 10 kg)
    b. Calculated variables
        i. *Pain50*
        ii. *Pressure Pain Threshold*
        iii. *Pressure Pain Tolerance*
        iv. *% of Subjects Reporting Pain (at each pressure intensity)*
        v. *AUC*
        vi. *Slope*

2. **Segmental/Regional Mechanical Sensitivity (Algometer Test)**
    a. 2 kg fixed pressure – forearm (control)
        i. Rating 1
        ii. Rating 2
        iii. Rating 3
        iv. *Calculated mean of 3 ratings*
    b. 4 kg fixed pressure – forearm (control)
        i. Rating 1
        ii. Rating 2
        iii. Rating 3
        iv. *Calculated mean of 3 ratings*
    c. 2 kg fixed pressure – suprapubic
        i. Rating 1
        ii. Rating 2
        iii. Rating 3
        iv. *Calculated mean of 3 ratings*
    d. 4 kg fixed pressure – suprapubic
        i. Rating 1
        ii. Rating 2
        iii. Rating 3
        iv. *Calculated mean of 3 ratings*
    e. Pressure Pain Threshold – trapezius (control)
        i. Rating 1
        ii. Rating 2
        iii. Rating 3
        iv. Calculated mean of 3 ratings

3. **Temporal Summation (PinPrick Test)**

    a. Forearm (256 mN stimulator)
        i. Rating 1a – Single Stimulus
        ii. Rating 1b – 10 Stimuli
        iii. Rating 2a – Single Stimulus
        iv. Rating 2b – 10 Stimuli
        v. Rating 3a – Single Stimulus
        vi. Rating 3b – 10 Stimuli
        vii. *Calculated WUR (mean of a.ii, a.iv.,a.vi. / mean of a.i., a.iii., a.v.)*
        viii. After-sensation rating 15 s
        ix. After-sensation 30 s

    b. Suprapubic (256 mN stimulator)
        i. Rating 1a – Single Stimulus
        ii. Rating 1b – 10 Stimuli
        iii. Rating 2a – Single Stimulus
        iv. Rating 2b – 10 Stimuli
        v. Rating 3a – Single Stimulus
        vi. Rating 3b – 10 Stimuli
        vii. *Calculated WUR (mean of b.ii, b.iv.,b.vi. / mean of b.i., b.iii., b.v.)*
        viii. After-sensation rating - 15 s
        ix. After-sensation rating - 30 s

4. **Conditioned Pain Modulation**

    a. Test Stimulus Alone (30-s, Pain40 thumb pressure)
        i. Rating 1 - 10 s
        ii. Rating 2 - 20 s
        iii. Rating 3 - 30 s
        iv. *Calculated mean of 3 test stimulus ratings*
        v. *Calculated Pressure Summation (a.iii. - a.i.)*

    b. Test Stimulus + Neutral Conditioning Stimulus (60-s, neutral foot bath)

        i. Ratings of Neutral Conditioning Stimulus
            1. Rating 1 - 5 s
            2. Rating 2 - 10 s
            3. Rating 3 - 20 s
            4. Rating 4 - 60 s
            5. *Calculated mean of 4 ratings*

        ii. Ratings of Test Stimulus
            1. Rating 1 – 40 s
            2. Rating 2 – 50 s
            3. Rating 3 – 60 s
            4. *Calculated mean of 3 test stimulus ratings*

c. Test Stimulus + Painful Conditioning Stimulus (60-s, hot foot bath)

   i. Ratings of Painful Conditioning Stimulus
      1. Rating 1 - 5 s
      2. Rating 2 - 10 s
      3. Rating 3 - 20 s
      4. Rating 4 - 60 s
      5. *Calculated mean of 4 ratings*

   ii. Ratings of Test Stimulus
      1. Rating 1 – 40 s
      2. Rating 2 – 50 s
      3. Rating 3 – 60 s
      4. *Calculated mean of 3 test stimulus ratings*

d. CPM Magnitude (calculated variables)
   i. *Neutral Conditioning (b.ii.4. – a.iv.)*
   ii. *Painful Conditioning (c.ii.4. - a.iv.)*
   iii. *CPM relative effect (c.ii.4. – b.ii.4)*

MULTIDISCIPLINARY APPROACH TO THE STUDY OF CHRONIC PELVIC PAIN (MAPP)

APPENDIX 5: ATLAS MODULE

Sponsored by: The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Department of Health and Human Services (DHHS)

Dated: July 9, 2018
Incorporating:
Protocol Amendment 1, dated June 5, 2015
Protocol Amendment 3, dated October 10, 2016
Protocol Amendment 6, dated July 9, 2018

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Table of Contents

1. Background **3**
2. Objective **4**
3. Study Design **4**
3.1. Preliminary Data: Initiation of New UCPPS Treatments 4
4. Specific Aims **6**
5. Methods **7**
5.1. ATLAS Treatments 7
5.2. ATLAS Sequence of Measures 8
5.3. ATLAS Data Collection 9
5.4. ATLAS Tracking of Concomitant Medications and Treatments 9
5.5. Coordination of ATLAS Activities with Scheduled SPS Data Collection 10
5.6. Definition of ATLAS Treatment Response 10
5.7. Sample Size Analysis 10
6. References **12**

1. Background

Treatment and overall management of patients with interstitial cystitis/ bladder pain syndrome and chronic prostatitis/chronic pelvic pain syndrome, here collectively termed urologic chronic pelvic pain syndromes (UCPPS), is challenging despite the conduct of a large number of randomized, controlled clinical trials.

A major goal of the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network is to provide findings to develop future prevention or treatment strategies and thereby improve patient care. Despite successfully completing extensive phenotyping of a large number of UCPPS patients through the network's Trans-MAPP Epidemiology/Phenotyping (EP) Study, the MAPP Research Network recognizes there remains a significant gap in our understanding of how to apply this information to improve patient care. Specifically, we have very limited information about the impact of specific patient phenotypes on treatment response. To address this need, network investigators propose to implement an **A**nalysis of **T**herapies During the **L**ongitudinal **A**ssessment of **S**ymptoms (ATLAS) module within the MAPP II Symptoms Pattern Study (SPS). The rationale for this study is that through exposure of UCPPS patients to therapeutic interventions with accompanying phenotyping, a relationship between response (or lack of response) to a given therapy and specific phenotypic profiles may be established. If successful, this would identify subgroups of UCPPS patients with characteristic phenotypes (i.e., disease marker profiles, degree and patterns of symptoms, influence of symptom flares, presence of co-morbid syndromes, neurological structure/function, etc.) that preferentially respond to specific treatments. This could eventually lead to "personalized analgesia", by allowing us to subsequently design future clinical trials that would specifically test UCPPS therapies in just the UCPPS "endophenotype" that we expect to respond to a treatment with a certain mechanism.

This notion of treating chronic pain patients based on the underlying mechanism, rather than disease, has been suggested for some time[1], but is just now beginning to be realized. Studies suggest that several genetic polymorphisms (e.g. in COMT, opioid receptors) may predict subsequent responsiveness of beta-blockers in TMJD, and opioid analgesics in cancer pain, respectively.[2-5] Quantitative sensory testing measures (e.g., reduced conditioned pain modulation or DNIC) have recently been shown to identify neuropathic pain patients more likely to respond to the drug duloxetine.[6] Functional and chemical neuroimaging techniques also show tremendous promise in identifying endophenotypes of patients that are likely to respond to certain therapies, including surgical procedures aimed at eliminating pain.[7-9] Since we use all of these techniques in the MAPP Network, a major goal of this study is to obtain the necessary information to enhance the ability of investigators conducting future clinical trials of UCPPS patients to tailor patient sub-groups (using clinical and research measures) to therapies that they will likely respond to with the hope of improving the efficiency and likelihood of showing a beneficial effect of treatment.

The first step in validating that we can identify endophenotypes associated with differential treatment outcomes is to test whether individuals with UCPPS with symptoms confined to the bladder ("peripheral" endophenotype) preferentially respond to therapies felt to work more peripherally, and conversely whether individuals with more systemic features of disease (i.e. centralized endophenotype) preferentially respond to those that work more centrally.

2. Objective

The primary objective of the **ATLAS Study** is to identify potential biological profiles (endophenotypes) which predict clinical responses to common UCPPS therapies.

3. Study Design

The **ATLAS module** will employ a non-experimental (i.e. observational) longitudinal study design, incorporated within MAPP II Symptom Patterns Study (SPS). All medications and all UCPPS therapies (medications, procedures, nontraditional therapies) will be tracked prospectively on a monthly basis. In addition, MAPP II participants will be asked to notify the study coordinators at the time of any planned treatment change for their UCPPS symptoms. This process is analogous to the symptom flare study, which was successfully conducted within the central Trans-MAPP EP Study during MAPP I. Initiation of specific treatments (ATLAS treatments) will trigger a phenotyping battery of tests (ATLAS module), to be conducted before and during the initial three months of the ATLAS treatment. Concurrent with the administration of ATLAS treatments, UCPPS patients will be extensively phenotyped at initiation of treatment change, and will provide bi-weekly symptom data during the therapy, followed by extensive phenotyping again at 12 weeks post-ATLAS therapy initiation.

This design will permit extensive investigation of clinical and biological characteristics associated with patients who respond and do not respond to each targeted ATLAS class of therapies. Patients will be assessed for functional/structural neuroanatomy, biomarker profiles; trait and state measures; and urologic symptom profiles using a spectrum of outcome measures. This will provide sufficient information to correlate specific biologic profiles (phenotypes) with treatment response, while also accounting for concomitant therapies. The advantage of this design is that it will not impact ongoing clinical care for UCPPS, and does not introduce an experimental intervention. In other words, this is not a ‘treatment study’. However, since multiple ongoing therapies are allowed, it will be impossible to control for all potential confounding variables that would affect a clinical treatment response. Therefore, this study should be viewed as hypothesis generating with respect to effectiveness of treatments. However, we will be able to make more definitive statements regarding the biological subsets of individuals that preferentially respond (or not respond) to certain treatments, as well as make inferences regarding the mechanisms of action of treatments. Such insights may also inform on underlying etiology for UCPPS. We anticipate that information generated from this ATLAS module will be used to design future clinical trials by identifying biologic factors which potentially predict treatment response.

3.1. Preliminary Data: Initiation of New UCPPS Treatments

During MAPP I, all UCPPS participants were asked to list their current UCPPS treatments as part of the bi-monthly on-line data collection. These data were analyzed to identify the initiation of new therapies (therapies which were not mentioned by the participants at the previous data collection). These ‘new’ therapies would be potential candidates for ATLAS therapies. Results are listed below. Therapies which have been chosen as ATLAS module treatments are bolded (see Table 1 and Methods section 5.1 for more details). Note that these results reflect 12 months of follow-up; whereas the MAPP II SPS will have 36 months of follow-up, resulting in identification of significantly more ‘new’ therapies.

Table 1. MAPP Phase I UCPPS Treatments

Rank	Males	Treatment		Females	Treatment
1	42 (23%)	NSAIDS		69 (30%)	NSAIDS
2	**22 (12%)**	**Opioids**		**44 (20%)**	**Opioids**
3	**20 (11%)**	**Alpha Blockers**		30 (13%)	Bladder Instillations
4	15 (8%)	Pentosan polysulfate		**30 (13%)**	**Pelvic Floor PT**
5	**14 (8%)**	**Tricyclics**		**29 (13%)**	**Tricyclics**
6	**12 (7%)**	**Pelvic Floor PT**		**26 (11%)**	**Pentosan polysulfate**
7	11 (6%)	Benzodiazepines		26 (11%)	Antihistamines
8	8 (4%)	Antibiotics		25 (11%)	Benzodiazepines
9	7 (4%)	Gabapentin Agents		19 (8%)	Antibiotics
10	6 (3%)	Bladder instillations		25 (11%)	Gabapentin Agents

For pragmatic reasons, the bolded therapies above will be considered ATLAS therapies, that if spontaneously initiated during a participant's involvement in MAPP II, will trigger the ATLAS module. Most of these decisions were based on predicted sample size using data from MAPP I, i.e. a high enough proportion of individuals in MAPP I started these new treatments to suggest adequate power would be achieved from the MAPP II cohort. NSAIDs were not chosen for inclusion because, after extensive discussion, we determined that in many instances these are used very intermittently as OTC meds, and/or to treat non-pelvic symptoms, and thus would be very difficult to assess.

Categorizing each treatment as peripheral or centrally acting is imperfect, and any choices could be criticized since there are probably no pure peripheral or centrally acting treatments. For example, even though pelvic floor PT is clearly peripherally directed, the individuals receiving this treatment may feel that they are going to benefit, and thus experience a superimposed centrally mediated placebo response. Conversely, almost all drugs have known preclinical peripheral and central mechanisms of action. Thus we emphasize several points regarding classification:

- These categories represent the subset of UCPPS patients that, based on current evidence and clinical experience, we expect to preferentially respond to each treatment. These are not in any way the only potential mechanism of action (MOA) of each treatment.
- If we miscategorize a treatment, that will matter little regarding the ultimate benefit of ATLAS. For example, although we categorize opioids as being preferentially beneficial in individuals with the peripheral phenotype, our data will tell us whether this is true or not, and even allow exploratory hypotheses regarding different endophenotypes (i.e. that we did not *a priori* identify) that might be preferentially responsive, that could be further explored in future studies.
- In fact, one of the aims of ATLAS (Aim 3) will be to determine (in most cases for the first time) what the primary mechanism(s) of action is for of each of these commonly used treatments. This information is not only critical to better designing future trials in UCPPS, but can also allow us to perform exploratory analyses wherein a novel or unsuspected MOA is identified in Aim 2 or 3 that allows us to re-classify a treatment and/or identify a different biologically derived subset of individuals who differentially respond.
- Thus we are *a priori* characterizing one of the ATLAS treatments (tricyclic drugs) as being centrally acting, and the other four ATLAS therapies (PT, pentosan, alpha blockers, and opioids) preferentially working in individuals with symptoms confined to the pelvis.

4. SPECIFIC AIMS

Aim 1: To determine the relative effectiveness (in the peripheral vs. central phenotypes) of UCPPS therapies administered to MAPP study participants

Hypothesis 1: Tricyclics and opioids will be preferentially effective (increased odds ratio of a clinically meaningful improvement at 12 weeks) in the central vs. peripheral endophenotype of UCPPS.

Hypothesis 2: Pelvic floor PT, pentosanpolysulfate, and alpha blockers will be preferentially effective in the peripheral vs. central UCPPS endophenotype.

Hypothesis 3: Exploratory analyses will identify other baseline clinical endophenotypes that preferentially respond (or do not respond) to the above therapies.

Aim 2: To determine *baseline* biological profiles of study participants demonstrating differential targeted UCPPS treatment responses.

Hypothesis 1: UCPPS participants with baseline biomarker profiles suggesting a more peripheral/inflammatory disease process (e.g., TLR2/4 response, HPA-axis) will preferentially respond to peripheral therapies; whereas those with a biomarker profile suggesting a more central MOA will preferentially respond to those therapies.

Hypothesis 2: UCPPS participants with baseline QST profiles suggestive of centralized pain (lowered overall pain threshold, decreased CPM, globally increased sensory sensitivity) will preferentially respond to TCAs, and be less responsive to the peripherally directed therapies.

Hypothesis 3: UCPPS participants with baseline neuroimaging profiles suggesting centralized pain (increased connectivity between pro-nociceptive regions and the default mode network [DMN], decreased connectivity between anti-nociceptive regions and the DMN, changes on voxel based morphometry and DTI) will be most responsive to TCAs and relatively less responsive to the peripherally directed therapies.

Aim 3: To determine the mechanism of action of targeted UCPPS therapies, by comparing biological profiles at baseline to those following successful treatment.

Hypothesis 1: Biomarkers associated with the peripheral endophenotype will display the most change from baseline to post-treatment in those successfully treated with a peripherally directed therapy; whereas those associated with a more central MOA will exhibit the greatest change from baseline when successfully treated with TCAs.

Hypothesis 2: QST profiles most suggestive of a centralized pain state (above) will display the greatest change with successful TCA treatment and will not be expected to change with peripherally directed therapies.

Hypothesis 3: Neuroimaging profiles most associated with centralized pain (above) will display the greatest improvement with successful TCA (i.e. central) treatment and will not be affected by peripherally directed treatments.

5. METHODS

Prior to initiating an ATLAS module, eligible participants will have been enrolled into the MAPP II SPS, and will have already completed their 6-month follow-up clinic visit, including deep phenotyping, biospecimen collection, neuroimaging and QST assessments. The additional data collection required for the ATLAS module will be incorporated within the SPS framework as summarized below.

5.1. ATLAS Treatments

Network investigators identified seven treatments in men and six treatments in women that will be the focus of ATLAS study investigations. These choices were based on the treatment patterns observed in MAPP I (see Preliminary Data above) as well as the desire to examine agents which represent both 'centrally-acting' and 'peripherally-acting' mechanisms. Feasibility concerns were also taken into account. For instance, NSAIDs were commonly taken by MAPP participants, but the MAPP investigators felt that this likely represents participant self-medication rather than discrete prescription events. Similarly, bladder instillations were a commonly reported therapy, but these are frequently used as 'rescue' therapy for flares at the time of clinic visits, which would not permit sufficient time to conduct pre-therapy ATLAS assessments. The selected ATLAS treatments are:

- *Oral opioids*. This will be categorized as a centrally-acting therapy, and will be examined in ATLAS studies in both men and women. While these agents may be utilized intermittently by participants, MAPP network investigators feel that the more common practice pattern at MAPP sites is to provide prescriptions to be used on a regular (e.g. daily) basis as opposed to very intermittent use.
- *Tricyclic antidepressants*. This will be categorized as a centrally-acting therapy, and will be examined in ATLAS studies in both men and women.
- *Pelvic floor physical therapy*. This will be categorized as a peripherally-acting therapy, and will be examined in ATLAS studies in both men and women.
- *Alpha-adrenergic blockers*. This will be categorized as a peripherally-acting therapy, and will be examined in ATLAS studies in men only.
- *Oral pentosan polysufate (PPS)*. This will be categorized as a peripherally-acting therapy, and will be examined in both men and women.
- *Neuropathic pain medications*. This will be categorized as a centrally-acting therapy, and will be examined in both men and women.
- *Cystoscopy with Hydrodistention.* This will be categorized as a peripherally-acting therapy, and will be examined in ATLAS studies in both men and women.

Example Targeted ATLAS Therapies by Treatment Type Updated

******PLEASE NOTE***: For these examples each number matches the corresponding brand name and generic name for medications.
An *extended* list of Brand & Generic Names will be provided to Participants for reference in case of ATLAS med. additions.

Targeted ATLAS Therapies

All Participants	All Participants	All Participants	All Participants	All Participants	All Participants	Males only
Oral opioids (Medication)	Tricyclic antidepressants (Medication)	Pelvic floor physical therapy (Non-Medication)	Elmiron / Oral pentosan polysulfate (Medication)	Neuropathic pain Treatments (Medication)	Cystoscopy with Hydrodistention	Alpha-adrenergic blockers (Medication)
Common Brand Names	Common Brand Names	Common Names	Brand Name	Common Brand Names	Common Names	Common Brand Names
1.Endocet 2.Lortab 3.Oxycontin 4.Percocet 5.Percodan 6.Vicodin 7.Tylenol w/ Codeine	1.Elavil 2.Tofranil 3.Treyzafagit 4.Sensoval	1.Pelvic Physical Therapy 2.Pelvic Floor Physical Therapy 3.Pelvic Floor Dysfunction Therapy 4.Pelvic Floor Myofascial Release 5.Pelvic Floor Rehabilitation	1.Elmiron	1.Lyrica 2.Neurontin	1. Cystoscopy with Hydrodistention	1.Flomax 2.Hytrin 3.Cardura 4.Rapaflo
Common Generic Names	Common Generic Names		Generic Names	Common Generic Names		Common Generic Names
1.Acetaminophen& Oxycodone 2.Acetaminophen& Hydrocodone 3.Oxycodone 4.Acetaminophen& Oxycodone 5.Aspirin& Oxycodone 6.Acetaminophen& Hydrocodone 7.Acetaminophen& Codeine	1.Amitriptyline 2.Imipramine 3.Desipramine 4.Nortriptyline		1.Pentosan Pentosan Polysulfate Pentosan Polysulfate Sodium	1.Pregabalin 2.Gabapentin		1.Tamsulosin hydrochloride 2.Terazosin 3.Doxazosin 4.Silodosin

5.2. ATLAS Sequence of Measures

Throughout the 36-month SPS, all MAPP participants will be requested to notify the MAPP research coordinators immediately when there is any change in their UCPPS treatment regimen, including prescription medications, nonprescription agents, procedures, therapies, and 'nontraditional' treatments. This notification will be done either via telephone to a dedicated MAPP telephone number, or via an on-line notification form. Any change, including starting a new UCPPS therapy, stopping an existing UCPPS therapy, or changes in dosing will be recorded. Participants will be educated about the therapies that are of specific interest to MAPP for ATLAS studies.

When a new ATLAS therapy is identified, participants will have the option to participate by completing the bi-weekly online ATLAS questionnaires only or in addition to the online questionnaires, undergo a battery of 'pre-treatment' phenotyping tests, conducted in person at the clinic. Ideally, this testing should be conducted before the new ATLAS treatment is initiated. During the ATLAS therapy, biweekly on-line symptom assessments will be completed by participants for 12 weeks. After 12 weeks of ATLAS therapy, participants will have the option, in addition to completing the online questionnaires, to return to the clinic for an additional set of 'post-treatment' phenotyping tests including a neuroimaging scan, QST assessments, and biospecimen collection. Taken together, the 'pre-treatment' ATLAS phenotyping, biweekly ATLAS symptom assessments, and 'post-treatment' ATLAS phenotyping referred to as an 'ATLAS module'. Study participants are permitted to complete up to two ATLAS modules during the 36-month SPS study.

The pre and post ATLAS visits are identical and include the following:

- All deep phenotyping CRFs and participant questionnaires
- Complete QST battery
- Neuroimaging
- Biomarker specimen collection

During the pre and post ATLAS clinic visits, the Research Coordinator will enter the participant's ID and PIN to initiate the participant online survey. If the participant does not finish the survey during the study visit, the participant will have until the end of that calendar day (11:59pm) to complete the online questionnaires from home/other location of their choice. If the participant declines the Pre or Post-ATLAS module clinic visit, the RC will provide the participant with the necessary code to initiate the online bi-weekly and Post -ATLAS assessments from home.

Biweekly on-line assessments during ATLAS therapy will include MyMed and the quarterly symptoms questionnaires:

- SYM-Q_Follow-up
- GRA
- RICE_Follow-up
- CMSI2_Follow-up
- CMSI2_FM2
- BPI2
- PAIN
- GUPI
- ICSI and ICPI
- WHO_DAS
- HADS
- FATIGUE
- SLEEP
- PSS

5.3. ATLAS Data Collection

The intent is that all MAPP participants who begin an ATLAS therapy will complete the entire ATLAS module. However, the MAPP investigators acknowledge that this is probably not realistic. For instance, some MAPP participants may be unable to complete the entire 12 weeks of therapy due to side effects, lack of efficacy or other reasons.

5.4. ATLAS Tracking of Concomitant Medications and Treatments

At all in-person SPS protocol visits (baseline, 6 months, 18 months, 36 months, pre-treatment and post-treatment ATLAS visits) a comprehensive assessment of current UCPPS therapies (medication and non-medication, prescription and nonprescription) will be performed using standardized case report forms. Other medications (for non-UCPPS indications) will also be tabulated at these times. Throughout the 36 months of the SPS, participants will be requested to submit an update to these medications and therapies on a monthly basis via MyMed. Research Coordinators will contact participants on a monthly basis to remind them to complete the MyMed module. This will serve as a reminder for participants to report new UCPPS therapies (especially ATLAS therapies) and will also collect ongoing data about concomitant non-UCPPS medication use which could potentially confound data interpretation for ATLAS studies. This monthly data collection for medications and treatments will occur throughout the study, irrespective of ATLAS data collection efforts.

5.5. Coordination of ATLAS Activities with Scheduled SPS Data Collection

From baseline to 6 months, no ATLAS events will be conducted in order to provide a period of uninterrupted data collection for the SPS and to allow researchers and participants to become acclimated to the study procedures (including the deep phenotyping visits at baseline and month 6). The maximum number of deep phenotyping assessments that any MAPP participant would experience is 8. Each participant may complete no more than two ATLAS modules during the 36-month SPS study period. This limit was put in place to reduce complexity and individual study participant burden.

The first ATLAS module may begin only after the 6 month in-clinic visit is completed. However, if a participant begins an ATLAS treatment within 7 days prior to the Month 6 visit, the Month 6 SPS visit may serve as the ATLAS pre-treatment phenotyping visit.

Following completion of the Month 6 visit, to avoid participant burden, if a participant reports an ATLAS treatment change up to 8 weeks post completion of their 6 month visit, the 6 month visit may serve as the ATLAS pre-treatment phenotyping visit (prior to participant actually starting the new med).

In addition, after the Month 6 Clinic Visit there will be an 8 week ‘window’ before and after each scheduled SPS deep phenotyping visit that may serve as the ATLAS “pre-treatment” visit. This ‘window’ means that for any ATLAS therapy that occurs within 8 weeks of a regularly scheduled SPS deep phenotyping visit, prior to participant starting the new ATLAS med, the data from the deep phenotyping visit will be used as the ‘pre-treatment’ ATLAS phenotyping battery.

If a participant begins a new ATLAS treatment, the site will have up to 7 days after initiation or start of that new treatment to initiate an ATLAS module.

5.6. Definition of ATLAS Treatment Response

For each ATLAS module, response to therapy will be defined using the GRA instrument. Therapeutic success will be defined as a response of ‘Moderately improved’ or ‘Markedly improved’ at 12 weeks. Those who do not complete the 12 weeks of therapy will be defined as nonresponders.

5.7. Sample Size Analysis

Based on inferences from MAPP Phase I medication and treatment data logs, the total sample size of n=640 study participants selected for the MAPP Phase II SPS is estimated to result in approximately n=256 study participants (40% of n=640) with an ATLAS event. With a target of 50% males, this will provide n=128 ATLAS events among males and n=128 ATLAS events among females, that will be more than adequate for definitive studies addressing Aims 2 and 3, and will provide strong signals for testing the hypotheses within Aim 1. In particular, existing data suggests that there is not a prominent effect of sex on clinical treatment responses, so both sexes can be analyzed together in Aim 1; whereas they need to be analyzed separately in Aims 2 and 3 because of known sex effects on QST and neuroimaging. Aims 2 and 3 are based on previous studies that show that QST and imaging generally changes with n’s of 15-20 for imaging and 20- 30 for QST.

To illustrate the sample size/power considerations further, consider that comparisons of the response rate in the “preferential effect” subgroup will be compared with the “proposed non-responder”

subgroup. Most trials of successful analgesics have a 30% responder rate of 35- 50% in the active treatment group and a placebo response rate of 15- 30% less. What is most consistent across trials is the difference between the active and placebo response rate – this almost always ranges from 10 – 15% for effective therapies. If our hypotheses hold true, we predict we should be able to increase separation, because simultaneously

- we are enriching the “preferential effect” group for responders.
- we are enriching the “proposed non-responder group” for non-responders.

We can also assume that both groups should have somewhat lower than typical response rates because the regression to the mean that is a sizable component of treatment response in classic RCTs will be minimized using this design. Thus, projected power of the proposed studies should be constructed using the following estimates:

- Response rates in the preferential response group ranging from 30 – 50% (i.e. about what we typically see – this is higher than the typical RCT because of enriching for responders but lower than the typical RCT because of removing regression to the mean.)
- Response rates in the proposed non-responder group should range from 10 – 30%.
- The difference between these groups should not be less than 20% (otherwise enrichment did not work and our hypotheses are not being supported) and a more reasonable lower limit would be a 25% difference between groups – compared to the typical 10 – 15%.

Accordingly, as summarized in the following sample size table (Table 2), a subgroup of n=34 study participants who initiated a selected class of ATLAS treatments, such as “Tricyclic antidepressants”, will provide 80% power to detect a responder rate of 40% among those who are enriched for the “preferential effect” subgroup, compared to a responder rate of 5% among those enriched for the “non-responder” subgroup. Similarly, a subgroup of n=32 study participants who initiated a selected class of ATLAS treatments, such as “Tricyclic antidepressants”, will provide 80% power to detect a responder rate of 50% among those who are enriched for the “preferential effect” subgroup, compared to a responder rate of 10% among those enriched for the “non-responder” subgroup.

Table 2. ATLAS Total Sample Size for Specified Effects (1-sided α=0.05, power=80%)

Responder Rate for Subgroup Enriched for Non-response	Responder Rate for Subgroup Enriched for Response					
	0.25	0.30	0.35	0.40	0.45	0.50
0.05	78	56	42	34	28	24
0.10		98	68	50	40	32
0.15			114	78	56	44

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MAPP II Symptom Patterns Study Visit Schedule

Domain	INSTRUMENT	FORM CODE	Total Items	Week 0/ Screening & Elig. Conf.	Post-Screening Online Run-in (Wk.1,2,&3)	Week 4/ Post-Run-in Clinic Visit, Deep Pheno. Neuro. Scans & QST Procs.	Month 3 (Online data)	Month 6 (Clinic) Deep Pheno. Neuro. Scans & QST Procs.	Month 9 (Online data)	Month 12 (Opt.Clinic for Bio-spec.)	Month 15 (Online data)	Month 18 (Clinic) Deep Pheno. Neuro. Scans & QST Procs.	Month 21 (Online data)	Months 24 & 30 (Opt.Clinic for Bio-spec.)	Months 27 & 33 (Online data)	Month 36 (Clinic) Deep Pheno. Neuro. Scans & QST Procs.
				Visit #1, Screening/ Study Entry/ Phenotyping Visit	Weekly Follow-up (for the 3 wk.s post Screening)	Post-Run-in "Baseline" Clinic/ Deep Phenotyping/ Neuroimaging/ QST Clinic Visit	Quarterly Follow-up (Every 3 mon.)	6-month interval Phenotyping Clinic Visit	Quarterly Follow-up	Annual interval Phenotyping (Opt.Clinic) Visit	Quarterly Follow-up	6-month interval Clinic/ Deep Phenotyping/ Neuroimaging/ QST Clinic Visit	Quarterly Follow-up	Annual/ Semi-Annual interval Phenotyping (Opt.Clinic) Visits	Quarterly Follow-up	6-month interval Clinic/ Deep Phenotyping/ Neuroimaging/ QST Clinic Visit
Pre-screening	Pre-screening	PRESCR2	PRN													
Screening Procedures																
Consent	Informed Consent Form	ICF	PRN	X												
Demographics	Demographics	DEMO	12	X												
Symptom Assessment	Symptom, Health Care Utilization & Flare Status Questionnaire for: Screening, Baseline, Run-In, & Follow-up	SYM-Q Screening SYM-Q Run-In SYM-Q Baseline SYM-Q Follow-Up	12	X	X	X	X	X	X	X	X	X	X	X	X	X
	Global Response Assessment	GRA	2		X	X	X	X	X	X	X	X	X	X	X	X
Eligibility*	Eligibility	ELIG2	27	X												
	Neuroimaging Study Eligibility	ELIG_SCAN2	8	X												
	Urine Culture Result	UCR	3	X												
	Enrollment	ENROLL	3	X												
		Grand Total	67	65	14	14	14	14	14	14	14	14	14	14	14	14
Urologic CRFs (Females and Males):																
Symptoms	Interstitial Cystitis Symptom Index	ICINDEX ICINDEX Run-In	4	X	X	X	X	X	X	X	X	X	X	X	X	X
	Interstitial Cystitis Problem Index		4	X	X	X	X	X	X	X	X	X	X	X	X	X
	AUA Symptom Index	AUASI	7			X		X				X				X
	RICE Case Definition Questionnaire	RICE_Screening RICE_Run-In RICE_Follow-up	5	X	X	X	X	X	X	X	X	X	X	X	X	X
	RICE Bladder Symptom Impact	BSI	5			X		X				X				X
Medical History	Medical History	MEDHX2	21	X												
	Early In Life Infection History	EIL-INF	10	X												
	Family Medical History	FAMHX	1	X												
Treatment	Concomitant Medications	CMED2	PRN	X		X		X		X		X		X		X
	Pelvic Therapy History	PTHX	20	X		X		X		X		X		X		X
	My Medications (**Monthly Tx. Tracking**)	MyMED	PRN	X (Intro.)	X	X	X	X	X	X	X	X	X	X	X	X
	ATLAS Module Initiation	ATLAS_INIT	11					PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	
	ATLAS Module Stop	ATLAS_STOP	7					PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	
	Cystoscopy History	CYSTO	6	X								X				X
	Antibiotic Treatment History	ABHX	2	X				X		X		X		X		X
Physical Exam	Physical Exam	EXAM2	15	X				PRN		PRN		PRN		PRN		PRN
	Pelvic Exam, Female & Male	PEX_Female PEX_Male	9	X								X				
	Brief Clinical Diagnostics for Baseline & Follow-up	CDX	8			X		X		X		X		X		X
Study Stop/Withdrawal	Study Stop	SSTOP	3	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	X
	Consent Withdrawal	CONWITHDR2	6	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN
	Reinstatement of Consent	RECON2	2	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN
		Grand Total:	146	104	13	53	13	55	13	43	13	68	13	43	13	64
Urologic CRFs - Females only																
Symptoms	Female Genitourinary Pain Index	FGUPI	9	X	X	X	X	X	X	X	X	X	X	X	X	X
Sexual Function	Female Sexual Function Index	FSFI	19			X		X				X				X
	Female Self-Esteem & Relationship Questionnaire	FSEAR	12			X		X				X				X
		Grand Total:	40	9	9	40	9	40	9	9	9	40	9	9	9	40
Urologic CRFs - Males only																
Symptoms	Male Genitourinary Pain Index	MGUPI	9	X	X	X	X	X	X	X	X	X	X	X	X	X
Sexual Function	International Index of Erectile Function	IIEF	6			X		X				X				X
	U. Washington Ejaculatory Function Scale	EFS	3			X		X				X				X
	Male Self-Esteem & Relationship Questionnaire	MSEAR	14			X		X				X				X
		Grand Total:	32	9	9	32	9	32	9	9	9	32	9	9	9	32

MAPP II Symptom Patterns Study Visit Schedule

Domain	INSTRUMENT	FORM CODE	Total Items	Week 0/ Screening & Elig. Conf.	Post-Screening Online Run-in (Wk.1,2,&3)	Week 4/ Post-Run-in Clinic Visit, Deep Pheno. Neuro. Scans & QST Procs.	Month 3 (Online data)	Month 6 (Clinic) Deep Pheno. Neuro. Scans & QST Procs.	Month 9 (Online data)	Month 12 (Opt.Clinic for Bio-spec.)	Month 15 (Online data)	Month 18 (Clinic) Deep Pheno. Neuro. Scans & QST Procs.	Month 21 (Online data)	Months 24 & 30 (Opt.Clinic for Bio-spec.)	Months 27 & 33 (Online data)	Month 36 (Clinic) Deep Pheno. Neuro. Scans & QST Procs.
				Visit #1, Screening/ Study Entry/ Phenotyping Visit	Weekly Follow-up (for the 3 wk.s post Screening)	Post-Run-in "Baseline" Clinic/ Deep Phenotyping/ Neuroimaging/ QST Clinic Visit	Quarterly Follow-up (Every 3 mon.)	6-month interval Phenotyping Clinic Visit	Quarterly Follow-up	Annual interval Phenotyping (Opt.Clinic) Visit	Quarterly Follow-up	6-month interval Clinic/ Deep Phenotyping/ Neuroimaging/ QST Clinic Visit	Quarterly Follow-up	Annual/ Semi-Annual interval Phenotyping (Opt.Clinic) Visits	Quarterly Follow-up	6-month interval Clinic/ Deep Phenotyping/ Neuroimaging/ QST Clinic Visit
Non-Urologic CRFs																
Symptoms																
Symptom Test	Complex Medical Symptoms Inventory	CMSI2_Screening CMSI2_Run-In CMSI2_Baseline CMSI2_Follow-Up	41	X (3 Mon.last yr.)	X (Weekly)	X (1 Month)	X (3mon.)	X (3 mon.)	X (3mon.)	X (3 mon.)	X (3mon.)	X (3 mon.)	X (3mon.)	X (3 mon.)	X (3mon.)	X (3 mon.)
Syndrome Module	Fibromyalgia	CMSI2-FM2	4	X	X	X	X	X	X	X	X	X	X	X	X	X
Pain & Physical Function	BPI: Body map, Intensity, Interference	BPI2_Female BPI2_Male	10	X	X	X	X	X	X	X	X	X	X	X	X	X
	PAIN Detect	PAIN PAIN Run-In	16	X	X	X	X	X	X	X	X	X	X	X	X	X
	McGill Pain Questionnaire	MPQ	15			X		X				X				X
	Gracely Box Scales	GBS	2			X		X				X				X
Physical Function	WHO Disability Assessment Schedule	WHO-DAS WHO-DAS_R.A.	15	X	X	X	X	X	X	X	X	X	X	X	X	X
	SF-12 Health Status Questionnaire	SF-12	12			X		X		X		X		X		X
	International Physical Activity Questionnaire	IPAQ	7			X		X		X		X		X		X
	Work Productivity & Activity Impairment Questionnaire	WPAI	6			X		X		X		X		X		X
Mood	PANAS	PANAS	20			X		X		X		X		X		X
	Hospital Anxiety and Depression Scale	HADS	14	X	X	X	X	X	X	X	X	X	X	X	X	X
Cognition	Multiple Ability Self-Report Questionnaire	MASQ	38			X		X		X		X		X		X
Fatigue	PROMIS - Fatigue - Short Form	FATIGUE	7	X	X	X	X	X	X	X	X	X	X	X	X	X
Sleep	PROMIS - Sleep - Short Form	SLEEP	8	X	X	X	X	X	X	X	X	X	X	X	X	X
Stress	Perceived Stress Scale	PSS PSS_Run-In	10	X	X	X	X	X	X	X	X	X	X	X	X	X
		Grand Total:	225	125	125	225	125	225	125	208	125	225	125	208	125	225
Trait-like Personal Factors																
	Ten-Item Personality Inventory	TIPI	10			X										
Cat	Thoughts About Symptoms	CSQ	6			X		X		X		X		X		X
Trauma History	Childhood/Recent Traumatic Events Scale	CTES RTES	13			X		X (RTES)				X (RTES)				X (RTES)
		Grand Total:	29			29		19		6		19		6		19
Co-morbid Diagnostics																
Syndrome Modules	Chronic Fatigue	CMSI2-CFS2	19			PRN		PRN				PRN				PRN
	Irritable Bowel Syndrome	CMSI2-IBS2	10			PRN		PRN				PRN				PRN
	Vulvodynia	CMSI2-VDYN2	8			PRN		PRN				PRN				PRN
	Migraine	CMSI2-MI2	19			PRN		PRN				PRN				PRN
	Temporomandibular Joint Disorder	CMSI2-TMD2	8			PRN		PRN				PRN				PRN
	Gonzalez TMJD Questionnaire	TMDSI	3			PRN		PRN				PRN				PRN
		Grand Total:	67			PRN		PRN				PRN				PRN
Specimens and Procedures																
Plasma and DNA	Plasma Specimen Tracking	PTRAC2	PRN			X		X		X		X		X		X
	STIM TUBES	STIMTR2	PRN			X		X				X				X
	Home Saliva Collection	S3TRAC2	PRN			X										
		S7TRAC2	PRN					X				X				X (PRN)
Urine	Urine Specimen Tracking	UTRAC2	PRN			X		X		X		X		X		X
	Microbiome Spec. (Male/Female)	UMMBTR2 UFMBTR2 UUMBTR2	PRN	X				X		X		X		X		X
Rec./Vag. Swabs	Rectal & Vaginal Swabs	RSTRAC2/VSTRAC2	PRN	X								X				
Neuroimaging Procedures Confirmation		NEURO_SCAN2	8			X		X				X				X
Neuroimaging Data Collection		NEURO_CRF	47			X		X				X				X
Quantitative Sensory Testing Screening		QST_Screen	7	X												
Quantitative Sensory Testing Procedures		QST	12			X		X				X				X
Procedural or Unanticipated Problems		PUP	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN	PRN
		Grand Total:	74	7		67		67				67				67

Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network

APPENDIX 7: TRANS-MAPP SMARTPHONE APPLICATION TO ASSESS PELVIC PAIN: THE (M)APP STUDY PROTOCOL

Sponsored by: The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Department of Health and Human Services (DHHS)

PROTOCOL—VERSION 1.1

Dated: October 10, 2016

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Table of Contents

1 IDENTIFYING INFORMATION 1

2 BRIEF OVERVIEW / INTRODUCTION 2

2.1 Scientific rationale 2

3 DESCRIBE LINK TO TRANS-MAPP SYMPTOMS PATTERN STUDY (SPS) 3

4 STUDY HYPOTHESES 4

5 SPECIFIC AIMS 5

6 DESCRIPTION OF SUPPORTING PRELIMINARY DATA 6

7 STUDY DESIGN AND METHODS 7

7.1 Overview of (M)APP Study 7
7.2 Study-Wide Number of Participants s: Sample Size 7
7.3 Study-Wide Recruitment Methods 7
7.3.1 Recruitment 7
7.3.2 Identifying potential participants 7
7.3.3 Recruitment materials 7
7.4 Multi-Site Research 7
7.4.1 Communication across sites 8
7.5 Recruitment methods 8
7.5.1 Recruitment 8
7.5.2 Source of participants 8
7.5.3 Reimbursement 8

8 STUDY POPULATION 9

8.1 Eligibility Criteria 9
8.2 Inclusion Criteria (in addition to the MAPP II Symptom Pattern Study criteria) 9
8.3 Exclusion Criteria 9

9 STUDY PROCEDURES 10

9.1 Research procedures being performed to monitor participants for safety or minimize risks. 10
9.2 Procedures performed to lessen the probability or magnitude of risks. 10
9.3 Data and Specimen Banking 11
9.3.1 Data storage. 11
9.3.2 Specimen data. 11
9.4 Data Management 11
9.4.1 Power analyses 11
9.4.2 Data security 11
9.4.3 Quality control. 12
9.5 Research Setting 12
9.6 Consent Process 12
9.6.1 Process to Document Consent in Writing 12

10 RISK FACTOR AND OUTCOME MEASURES 13

10.1 Common Data Elements (CDEs) from Trans-MAPP Epidemiology Study 13
10.2 Specialized Data Measures 13

11 ANALYTIC PLAN 14

11.1 Aim 1 – 14
11.2 Aim 2 - 14
11.3 Aim 3 – 14

12 STATISTICAL ANALYSIS AND SAMPLE SIZE ESTIMATES 15

12.1 Power Analyses 15
12.2 Total number of Participants 15

13 PROPOSED INVOLVEMENT OF MAPP NETWORK CORES 16

13.1 Tissue Analysis and Technology Core (TATC)- 16
13.2 Data Coordinating Core (DCC) 16

14 TIMELINE FOR COMPLETION OF DATA COLLECTION 18

14.1 Timeline 18

15 ANTICIPATED BURDEN TO SITES 19

16 RISKS TO PARTICIPANTS 20

16.1 Anticipated Risks and Discomforts 20
16.2 Unforeseeable risks 20
16.3 Pregnancy 20
16.4 Risks to others 20
16.5 Provisions to Monitor the Data to Ensure the Safety of Participants 20
16.6 Provisions to Protect the Privacy Interests of Participants 20
16.6.1 Protection of privacy interests 20
16.6.2 Making Participants feel at ease 21
16.6.3 Research team permissions 21
16.7 Compensation for Research-Related Injury 21
16.8 Economic Burden to Participants 21

17 PARTICIPANT WITHDRAWAL 22

17.1 Stopping participation 22
17.2 Orderly termination 22

18 POTENTIAL BENEFITS TO PARTICIPANTS 23

19 SHARING OF RESULTS WITH PARTICIPANTS 23

20 REFERENCES 24

APPENDIX 1: VISIT SCHEDULE FOR TRANS-MAPP SPS SMARTPHONE APPLICATION 26

APPENDIX 2: CRFS FOR TRANS-MAPP (SPS) SMARTPHONE APPLICATION 27

1 Identifying Information

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University of Washington
Washington University

2 Brief Overview / Introduction

The purpose of this study is to use a mobile smartphone application for assessing pelvic pain variation throughout the day in patients with chronic pelvic pain. The MAPP Discovery Site teams from the University of Iowa and Northwestern University have expertise in the technical intricacies of computerized assessment and will help to create a system in which assessment data can be rapidly and easily collected and subsequently transferred to clinicians and researchers for rapid analysis.

2.1 Scientific rationale

Diary-based methods are commonly used in the urology clinic, but current tools do not capitalize on existing, widely-available technology.[7-10] Specifically, there is no mobile smartphone application (app) to measure symptoms in patients with chronic pelvic pain. With a high rate of mobile phone usage, there is the potential to make self-report assessment more convenient and accessible to patients, as well as to reach patients who do not have easy access to computers or clinics. The objective of this study is to develop an app for the assessment of symptoms in people with chronic pelvic pain. The newly-developed app will be rigorously tested for reliability, validity, and usability. **As a result of this study, the MAPP network will produce mobile technology that will facilitate clinical assessment.**

3 Describe Link to Trans-MAPP Symptoms Pattern Study (SPS)

The data obtained from the MAPP mobile phone application ((M)APP) will complement, but not replace, the patient-reported questionnaire data obtained from the Symptom Pattern Study (SPS). Data on pain severity, pain location, anxiety, and depression that are collected in the SPS will be examined both cross-sectionally and longitudinally with respect to the (M)APP dataset to ensure that the newly-develop app is a valid assessment. These comparisons will help to inform the hypothesis as outlined below. In addition, there are novel questions in the app that are relevant to chronic pain, but not included in the SPS (e.g., day-to-day stress, alcohol use).

4 Study Hypotheses

The study will address the following hypotheses:

	(M)APP Hypotheses	Method for testing
Hypothesis 1.	The (M)APP will show convergent validity with validated measurement tools (e.g., GUPI Pain) in the Symptom Pattern Study.	Levels and trajectories of pain measured by the (M)APP will be associated with levels and trajectories of the Pain Index in the symptom pattern study.
Hypothesis 2.	Intraday measurement of symptoms will predict the presence of flares.	Level of stress, mood, and alcohol consumption will be associated with the presence of flares.
Hypothesis 3.	Anxiety and depression assessed through validated PROMIS questionnaires will be associated with level and variability of pain and urinary symptoms.	PROMIS anxiety and depression will be regressed on pain and urinary symptom patterns as measured each day by the app.

5 Specific Aims

Each of the above hypotheses motivates a series of specific aims that will be the focus of the Smartphone Application Trans-MAPP study and are outlined below:

Aims Related to Hypothesis 1: To demonstrate that pain levels and trajectories can be efficiently and validly measured with a mobile app, using the SPS measures to show convergent validity with the app.

Aims Related to Hypothesis 2: To demonstrate the unique patterns of pain associated with "pain flares", as well as to identify predictors of pain flares (e.g., stress, negative mood).

Aims Related to Hypothesis 3: To use a smartphone assessment to show relationships between pain variability and level of depression, anxiety, mood, and alcohol use. Mental health concepts will be measured using PROMIS questionnaires, which allow for comparisons of the patient to the general U.S. population.

6 Description of Supporting Preliminary Data

Both Northwestern University and the University of Iowa have experience in software development including apps. The Department of Medical Social Sciences at Northwestern University is the software development team for the NIH PROMIS.[11-15] Our preliminary data using a tablet-based system already evinces that we can collect computer-based-data that shows relationships between pelvic pain symptoms and comorbid mental health symptoms (see Figure 1). These data also support strong relationships with comorbid symptoms, which will be investigated further in the smartphone study. Also, Griffith et al., among others, have shown the relationship between depression and pain in patients with UCPPS,[16] but this study will expand upon these findings by also looking at alcohol use, anxiety, and stress.

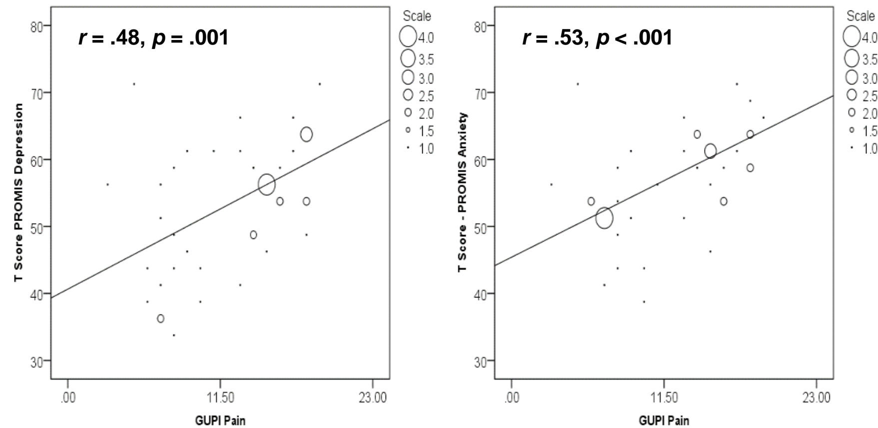

Figure 1: $N = 42$ pelvic pain patients; PROMIS depression (left panel) and anxiety scores (right panel) are significantly associated with Genitourinary Pain Index (GUPI); Linear regression line shown; Marker size indicates number of individuals at x-y.

7 Study Design and Methods

7.1 Overview of (M)APP Study

Participants enrolled in the MAPP SPS study will have the option to participate in the (M)APP study at three time points, immediately following the 6, 18, and 36 months SPS clinic visits. The app will be administered for two weeks, starting on the day after the 6-, 18-, and 36-month visits. Participation in the (M)APP assessments at any of these three time points is optional.

7.2 Study-Wide Number of Participants s: Sample Size

The app will be incorporated with the overall SPS and will represent use of the (M)APP study-wide (i.e. at all participating institutions). Thus, the sample size will be a subset of the SPS, depending on voluntary enrollment and possession of an appropriate smartphone.

7.3 Study-Wide Recruitment Methods

7.3.1 Recruitment

For the (M)APP study, the participants will be the same as for the MAPP SPS. Thus, this will be an added element to the SPS so all recruitment procedures will be the same. If a participant does not have an appropriate smartphone, or does not wish to participate, then they will not be asked to complete this part of the study.

7.3.2 Identifying potential participants

Participants will be identified by clinicians treating participants for pelvic pain and put in touch with the research team as in the SPS.

7.3.3 Recruitment materials

Standard MAPP recruitment material, including IRB approved flyers, will be used. No additional recruitment materials are suggested for this protocol.

7.4 Multi-Site Research

All discovery sites will ensure that:

- The most current version of the protocol, consent document, and HIPAA authorization will be used
- Procedures at all sites will be carried out as approved by the local IRBs
- All modifications will be communicated to sites, and approved (including approval by the site's IRB of record) before the modification is implemented

- All engaged participating sites will safeguard data as required by local information security policies
- All local site investigators conduct the study according to the protocol
- All non-compliance with the study protocol or applicable requirements will be reported in accordance with local policy

7.4.1 Communication across sites

Discovery sites will have at least monthly teleconferences about this study, which will be used to discuss problems and interim results.

7.5 Recruitment methods

7.5.1 Recruitment

Eligible patients will be referred to the study by collaborating MAPP Network physicians. All MAPP II SPS participants with a smart phone are eligible to participate.

7.5.2 Source of participants

Participants will be recruited through urology clinics and other recruitment venues affiliated with the MAPP Network discovery sites.

7.5.3 Reimbursement

As compensation for their time and effort, participant reimbursement (provided by each site) should be provided. Appropriate amounts and actual schedule of reimbursements should be determined by each site.

8 Study Population

8.1 Eligibility Criteria

To participate, individuals will need to meet all study criteria to participate in the MAPP II SPS study. All Participants enrolled in the MAPP II SPS study will be offered the opportunity to participate. Participants will be free to opt out if they do not wish to participate.

8.2 Inclusion Criteria (in addition to the MAPP II Symptom Pattern Study criteria)

Participant has smartphone that he or she is willing to use for the study.

8.3 Exclusion Criteria

Same as the MAPP II SPS Study. See main SPS protocol.

9 Study Procedures

If the participant agrees to participate in the (M)APP protocol, at the in-clinic visit the phone application will be loaded on their phone. This process will be conducted by a study coordinator:

- Android phones – (M)APP will be loaded on the phone using the Google Play store.
- Apple (iOS) phones – (M)APP will be loaded on the phone using the App Store.

The study coordinator will obtain the following information from the participant at the time of enrollment.

Note: These data will only be used to schedule phone prompts (aka “pings”).

- Gender (some questions will be sex specific)
- Date of enrollment (phones will be prompted for only the two weeks following study enrollment)
- Time Zone (phones will be prompted at standard times across all time zones)
- Preferred wake/bedtimes (phone prompts will be adjusted based on participant specific wake/sleep times to ensure they do not disrupt sleep)

Participants will be asked to use the app each day for two weeks. It is estimated that no more than 20 minutes (four times per day, 5 minutes per time) per day will be needed to complete the questions presented via the app.

9.1 Research procedures being performed to monitor participants for safety or minimize risks.

Participants will be instructed to call the research coordinator with any acute problems with the (M)APP. In addition, participants will be instructed on how to remove the (M)APP from their phone if necessary.

At the end of the study, participants will be told that they can remove and uninstall the app from their phone using normal procedures. They will be invited to contact the research team if they have difficulties doing so.

9.2 Procedures performed to lessen the probability or magnitude of risks.

All information transmitted by the phone ((M)APP) to the server at the University of Iowa is de-identified (i.e. no participant information is included). Were the information to inadvertently be obtained by a third party, it would not identify the participant. Site staff will encourage all users of the (M)APP to enable the password and/or fingerprint capabilities of the phone to ensure non-participants do not inadvertently use the (M)APP.

In addition, to ensure the application is being conducted appropriately, information on gender, time zone and wakeup/sleep times will be obtained.

9.3 Data and Specimen Banking

9.3.1 Data storage.

All data retained from this study will be de-identified and stored in secure databases at the University of Iowa. Each mobile device will have a unique identifier not attached to a participant's name or identify. The key to link the unique MAPP study ID with the phone ID will be stored separately in a secure file within the Department of Urology, University of Iowa. Eventually all de-identified data from this study will be transferred to the MAPP Data Coordinating Core at the University of Pennsylvania Perelman School of Medicine and eventually deposited into the NIDDK Data Repository.

9.3.2 Specimen data.

No biological specimens will be collected as part of this study.

9.4 Data Management

9.4.1 Power analyses

For within-time associations, statistical power should be ample. Figure 2 shows necessary sample size to detect significant correlations as a function of effect size, assuming desired power is .80, Type I error rate α = .05. As shown in the figure, even samples sizes as small as *N* = 200 have sufficient power to detect small effect size. Given that these studies are Trans-MAPP (i.e., planned for all sites), statistical power will be strong. The exact statistical power for latent growth curve models depends on what form the symptom trajectories take, but power calculations for the SPS suggested that for risk ratios of 2.0 or greater was adequate for many scenarios, and growth curve modelling will treat covariates as dependent measures as continuous, which should yield greater power as well as modelling flexibility. Thus, statistical power for the SPA should be adequate assuming that it is integrated into the Trans-MAPP efforts.

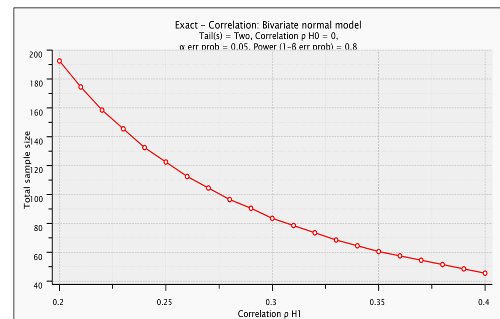

Figure 2 – Power to detect within time-point associations.

9.4.2 Data security.

All data security procedures for the MAPP II SPS will also be followed for this protocol. All research data will be stored on secure Iowa computers with no identifying information. Data transmitted from the app will also be encrypted, and our app will not collect identifying information. For research purposes, the only information that is required is the unique identifier of the person's mobile device. Even with these measures, there would be some risk of others looking at a participant's phone (e.g., if a participant completes a question about depression in a public place and someone looks over their shoulder). To reduce these risks, participants will be encouraged to use the app for this protocol in private, being mindful that people could attempt to look at their screen. This risk is similar to using

an ATM in public, but we will remind participants of this. Moreover, this is fully described in the consent form.

9.4.3 Quality control.

The system allows data to be examined at any time. At regular team meetings, we will look at the data to ensure that the number of subjects and assessments are correct, that data are being populated into our database, and that all values are "in range."

9.5 Research Setting

Participants will be recruited from MAPP Discovery Site clinicians. When participants are enrolled into this study, a research coordinator will install the app on the participant's phone. Data will be entered into the app by the participant at their convenience.

9.6 Consent Process

- Consent will take place in MAPP Discovery Site clinics at a time convenient for the participant
- Participants are free to revoke their consent at any time
- Only participants able to speak, read, and understand English will be included in this study
- Any participants who are cognitively impaired or unable to consent will be deemed ineligible for this study

9.6.1 Process to Document Consent in Writing

Although procedures involve no more than minimal risk, all Discovery Sites will collect written documentation of consent.

10 Risk Factor and Outcome Measures

10.1 Common Data Elements (CDEs) from Trans-MAPP Epidemiology Study

(see Appendix 2 for current proposed Phase II/SPS Protocol CRFs)

10.2 Specialized Data Measures

No new measures, other than the questions contained in the app, are required for this protocol.

11 Analytic Plan

11.1 Aim 1 –

Levels, standard deviations, and trajectories of pain measured by the (M)APP will be associated with levels and trajectories of the Pain Index in the SPS.

- Pain variation will be assessed by average score (of day/week), slope (daily, weekly) and standard deviation (daily, weekly).
- We will compare these pain data to end of week pain assessments as determined by the SPS. Level, variability, and trajectory of pain will be assessed using latent growth curve modelling.[17-19] We are aware, however, that alternative approaches are available (e.g., mixed models a.k.a. hierarchical linear models), and these will be explored as appropriate.

11.2 Aim 2 -

Level of stress, mood, and alcohol consumption will be associated with the presence of flares, as well as higher levels of pain.

- We predict that flare presence and intensity will be predicted by intraday mood, stress, and alcohol consumption ratings. Using mixed-model regression approaches, flare ratings on a particular day will be nested within persons. Within-day ratings of mood, alcohol consumption, and stress will be used as predictors.

11.3 Aim 3 –

PROMIS anxiety and depression will be regressed on pain and urinary symptom patterns as measured each day by the app.

- Participants will complete the app for two weeks at a time. Pain and Urinary mean and variability scores will be calculated for each individual week, nested within a person. End-of-week PROMIS depression and anxiety scores will serve as dependent variables in mixed-model regressions. We predict that higher levels of pain and more variability in pain will be significantly related to depression and anxiety.

12 Statistical Analysis and Sample Size Estimates

12.1 Power Analyses

As described above, statistical power should be ample even if we were to rely on a small subset of SPS participants (fewer than 200; see Figure 2, above). The exact statistical power for latent growth curve models depends on what form the symptom trajectories take, but power calculations for the SPS suggested that for risk ratios of 2.0 or greater was adequate for many scenarios, and growth curve modelling will treat covariates as dependent measures as continuous, which should yield greater power as well as modelling flexibility. Thus, statistical power should be adequate assuming that it is integrated into the Trans-MAPP efforts.

12.2 Total number of Participants

All participants enrolled in the MAPP SPS with a smartphone are eligible to participate in this study, therefore there could potentially be up to 640 participants enrolled. Although statistical power would be adequate with a smaller sample, latent growth curve models for longitudinal analyses work best with large samples, so we aim to enroll at least 300 participants throughout the MAPP.

13 Proposed involvement of MAPP Network Cores

13.1 Tissue Analysis and Technology Core (TATC)-

There will be no additional involvement with TATC because of this protocol.

13.2 Data Coordinating Core (DCC)

The Data Coordinating Core (DCC) for the MAPP Research Network is located at the Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

Principal Investigator:	J. Richard Landis, Ph.D., Professor of Biostatistics, University of Pennsylvania
Co- Investigator:	Alisa Stephens-Shields, Ph.D., Assistant Professor of Biostatistics, University of Pennsylvania
Co-Investigator (IC/BPS):	Robert M. Moldwin, MD, Associate Professor, Department of Urology Hofstra North Shore-Long Island Jewish School of Medicine
Co-Investigator (CP/CPPS):	Michel Pontari, MD, Vice-Chairperson and Professor, Department of Urology, Temple University

The DCC is responsible for the following:

- Providing biostatistical expertise in research design, outcome measures and analytic strategies for translational and clinical investigations of UCPPS
- Guiding and implementing statistical analyses, interpretation of findings, and supporting presentations and publication of results
- Facilitating the conduct of multi-disciplinary basic and translational research, by providing scientific leadership in the design and implementation of research projects across the MAPP Research Network
- Promoting network-wide quality assurance standards, practices and tools, including a comprehensive, secure www-based data management system (DMS) for collection and centralized storage of all multi-site study data
- Providing comprehensive Data Coordinating Core administrative support for the MAPP Research Network, promoting effective communications, coordinating meetings, working groups, document development and management, and distribution of study proceedings

- Supporting the MAPP Research Network Ancillary Projects, assisting in their design, as well as implementing a process for the submission, review, and development of ancillary studies

14 Timeline for Completion of Data Collection

14.1 Timeline

All data analyses should be complete within three months after the last participant has completed the study.

15 Anticipated Burden to Sites

We anticipate the burden to the participants to be minimal. The phone application can be easily downloaded from the App Store (for iOS users) or the Google Play store (for Android users).

The survey will be self-administered using the data provided to the programmers by the study coordinators (date of enrollment, sex, time zone, preferred wake/sleep time) and thus, will minimally burden coordinators.

Technical support will be provided by the programmers at the University of Iowa.

16 Risks to Participants

16.1 Anticipated Risks and Discomforts

This study will require participants to answer questions about pain and mental health symptoms on their mobile device. This could result in some anxiety, distress, embarrassment, and inconvenience from increased alerts from their phone. In addition, some participants might become upset answering questions about mental health. It will be made unmistakable to participants that they can discontinue the study if they feel uncomfortable without loss of benefit.

16.2 Unforeseeable risks

The use of mobile apps is commonplace, so we do not foresee significant risks with this study. If any risks are identified, however, local IRBs will be alerted immediately.

16.3 Pregnancy

Currently, there is no reason to believe that the use of a mobile app on a smartphone bears any risk to women if they become pregnant, or to an embryo or fetus, however pregnancy is an exclusion criteria for the MAPP SPS study and if a female becomes pregnant during the study she will be withdrawn from study participation.

16.4 Risks to others

The use of a mobile app to measure symptoms of pelvic pain bears no risks to others.

16.5 Provisions to Monitor the Data to Ensure the Safety of Participants

This study involves minimal risk insofar as we are asking standard questions about pain and other symptoms that have been asked in many other studies without harm to participants. The participants will have the opportunity to communicate with the research team if any problems arise. If any unexpected risks to the participant’s safety are observed, sites will immediately contact their local IRB.

16.6 Provisions to Protect the Privacy Interests of Participants

16.6.1 Protection of privacy interests

Participants will only interact with the MAPP Discovery Site research team members, and they will be seen in a private location within the Discovery Site clinics. The data that participants provide on the app will be transmitted to University of Iowa without any identifying information. All data will be kept on secure computers in de-identified format.

16.6.2 Making Participants feel at ease

MAPP Discovery site research team members have much experience discussing sensitive topics with research participants. Techniques that will be used include listening to participant's concerns, allowing participants to take their time, taking breaks as needed, and skipping any part of the research that is undesirable. Participants will have easy access to the research team throughout the study, which will facilitate their ability to express any concerns.

16.6.3 Research team permissions

The research team will need appropriate permissions and passwords to access study files, which will be overseen by designated MAPP Network personnel at each site. These procedures will help maximize the security of the data.

16.7 Compensation for Research-Related Injury

This study involves minimal risk and therefore there is no plan for compensation for injuries.

16.8 Economic Burden to Participants

Participants will need their own personal smartphone or mobile device and will be responsible for all charges for their own device, including use of data

17 Participant Withdrawal

17.1 Stopping participation

Participants will be terminated from study participation if they inform the study team that they no longer wish to participate. No other reasons are anticipated as to why a participant would be removed from the study. A protocol has been established to assist participants with removing the (M)APP from their phone during the study if desired, and for all participants at the end of the study.

17.2 Orderly termination

If a participant is removed from study, site staff will make a note in their contact database to not contact them again in the future. Discovery Site staff will also walk them through the process of removing the app from their mobile device.

18 Potential Benefits to Participants

There would be no direct benefits to participants as part of this study.

19 Sharing of Results with Participants

No results will be shared with participants.

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Appendix 1: Visit Schedule for Trans-MAPP SPS Smartphone Application

The app will be administered for two weeks, starting on the day after the 6-, 18-, and 36-month visits (i.e., the required visits).

Appendix 2: CRFs for Trans-MAPP (SPS) Smartphone Application