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Auto-antigenic Properties of the Spliceosome as a Molecular Tool for Diagnosing Systemic Lupus Erythematosus and Mixed Connective Tissue Disease Patients

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

AUTO-ANTIGENIC PROPERTIES OF THE SPLICEOSOME AS A MOLECULAR
TOOL FOR DIAGNOSING SYSTEMIC LUPUS ERYTHEMATOSUS AND MIXED
CONNECTIVE TISSUE DISEASE PATIENTS

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Annia Mesa

2014

To: Dean Kenneth G. Furton
College of Arts and Sciences

This dissertation, written by Annia Mesa, and entitled Auto-antigenic Properties of the Spliceosome as a Molecular Tool for Diagnosing Systemic Lupus Erythematosus and Mixed Connective Tissue Disease Patients, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Annia Mesa is approved.

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Florida International University, 2014

DEDICATION

I dedicate this research work to my parents: Silvia Celika Avila León and Aldo Benito Mesa Dias. For as long as I can remember, they have been determined to provide me with opportunities and resources to improve my education and reach my goal to become a Ph.D. For that, I will be endless in debt.

Gracias Mami y Papi.

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ABSTRACT OF THE DISSERTATION

AUTO-ANTIGENIC PROPERTIES OF THE SPLICEOSOME AS A MOLECULAR
TOOL FOR DIAGNOSING SYSTEMIC LUPUS ERYTHEMATOSUS AND MIXED
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by

Annia Mesa

Florida International University, 2014

Miami, Florida

Professor DeEtta K. Mills, Major Professor

Systemic Lupus Erythematosus (SLE) and Mixed Connective Tissue Disease (MCTD) are chronic, autoimmune disorders that target overlapping autoantigens and exhibit similar clinical manifestations. Despite 40 years of research, a reliable biomarker capable of diagnosing these syndromes has yet to be identified. Previous studies have confirmed that components of the U1 small nuclear ribonucleoprotein complex (U1 snRNP) such as U1A are 1000 fold more autoantigenic than any other nuclear component in SLE patients. Based on these findings, I hypothesize that models derived from the U1 snRNP autoantigenic properties could distinguish SLE from MCTD patients. To test this hypothesis, 30 peptides corresponding to protein regions of the U1 snRNP were tested in triplicates by indirect ELISA in sera from SLE or MCTD subjects. In addition laboratory tests and clinical manifestations data from these patients were included and analyzed in this investigation. Statistical classification methods as well as bioinformatics pattern recognition strategy were employed to determine which combination, if any, of all the variables included in this study provide the best segregation power for SLE and MCTD.

The results confirmed that the IgM reactivity for U1 snRNP and U1A have the power to significantly distinguish SLE from MCTD patients as well as identify kidney and lung malfunctions for these subjects ($p \leq 0.05$). Furthermore, the data analysis revealed eight novel classification rules for the segregation of SLE and MCTD which are a better classification tool than any of the currently available methods ($p \leq 0.05$). Consequently, the results derived from this study support that SLE and MCTD are indeed separate disorders and pioneer the description of eight novel classification criteria capable of significantly discerning between SLE and MCTD patients ($p \leq 0.05$).

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ABBREVIATIONS AND ACRONYMS

+	Positive
Ø	No significant correlation
3D	Three-Dimensional
ANA	Activated Antinuclear Assay
ACR	American College of Rheumatology
BAFF	B cell Activated Factor
BLR	Binomial Logistic Regression
BSA	Bovine Serum Albumin
C-score	Confidence Scores
CPK	Creatine Phosphate Kinase
CNS	Central Nervous System
DNA	DeoxyriboNucleic Acid
dsDNA	Double Stranded DeoxyriboNucleic Acid
e.g.	<i>Exempli Gratia</i>
ELISA	Enzyme Linked Immunosorbent Assays
FANA	Fluorescent Activated Antinuclear Assay
FN	False Negative
FP	False Positive
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL	Interleukin
IRB	Institutional Review Board
MCTD	Mixed Connective Tissue Disease
n	sample number
NCBI	National Center for Biotechnology Information
NGAL	Neutrophil Gelatinase Associated Lipocalin
NMR	Nuclear Magnetic Resonance
OD	Optical Density
OD%	Percentage of Optical Density
P	Peptide
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with 0.05% Tween-20
PDB	Protein Data Bank
PMSF	Phenylmethanesulphonyl Fluoride
PM	Polymyositis
Pr (<i>SLE</i>)	Probability of Systemic Lupus Erythematosus
Pr (<i>MCTD</i>)	Probability of Mixed Connective Tissue Disease
rAlarcón-Segovia	Reduced Alarcón-Segovia model
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
rKahn	Reduced Kahn model
rKasukawa	Reduced Kasukawa model

RMSD	root mean square deviation
ROC	Receiving Operating Characteristics
RRM	RNA Recognition Motif
rSharp	Reduced Sharp model
Scl70	Topoisomerase 1
SLE	Systemic Lupus Erythematosus
SLICC	Systemic Lupus International Collaborating Clinics
Sm	Smith protein
snRNP	Small Nuclear Ribonucleoprotein Particle
SS	Systemic Sclerosis
SSA	anti-Ro antibodies
SSB	anti-La antibodies
SmD	Smith protein D
SPSS	Statistical Product and Service Solutions
TN	True Negative
TP	True Positive
U1-70K	U1-70K protein
U1Ap	U1-A protein
U1C	U1-C protein
U1 snRNP	U1 Small Nuclear Ribonucleoprotein Particle
U2 snRNP	U2 Small Nuclear Ribonucleoprotein Particle
U4 snRNP	U4 Small Nuclear Ribonucleoprotein Particle
U5 snRNP	U5 Small Nuclear Ribonucleoprotein Particle

U6 snRNP	U6 Small Nuclear Ribonucleoprotein Particle
VCAM	Vascular Cell Adhesion Molecule
χ	Chi square
$\bar{X}$ OD of sample	Optical Density Value of the Sample Group
$\bar{X}$ OD of control group	Optical Density Value of the Control Group

I. INTRODUCTION

Autoimmune diseases develop when the immune system stops recognizing self antigens and produces aberrant antibodies capable of developing equally over-reactive responses to self as well as non-self antigens (Carroll, 2004). In general, individuals diagnosed with these syndromes represent a heterogeneous population characterized by a broad number of clinical manifestations that have unpredictable courses and often cannot be associated with a unique autoimmune illness (Konforte et al., 2012). It is without doubt that early and accurate recognition of these diseases could dictate the patient's treatment which in turn has the potential to improve the prognosis as well as reduce the financial burden to the ill subject and family (Bodolay et al., 2007). Nevertheless, the lack of recognition and categorization methods for autoimmune syndromes exhibiting clinical and molecular features common in other well characterized autoimmune illnesses has hampered diagnosis. As result, patients exhibiting characteristic overlapping clinical manifestations observed in different autoimmune syndromes are often misdiagnosed and go through years of clinical investigations and laboratory tests before obtaining the accurate diagnosis (Adsay et al., 2005; Simao, 2010; Nițescu et al., 2011; Quinkler, 2012; Bertias et al., 2013; Pena et al., 2013).

Mixed Connective Tissue Disease (MCTD) is one of the most commonly known syndromes showing overlapping characteristics with other autoimmune disorders (Rebora and Parodi, 1990; Aringer et al., 2005; Swanton and Isenberg, 2005; Nowicka-Sauer et al., 2012). Sharp et al. (1972) initially described MCTD as a systemic autoimmune disease characterized by elevated antibodies directed to subunits of the U1 small nuclear ribonucleoprotein particle (snRNP). Since then, diverse research groups defined it as a

non-specific autoimmune syndrome with clinical and molecular features observed in others syndromes such as Systemic Lupus Erythematosus (SLE), Systemic Sclerosis (SS), Rheumatoid Arthritis (RA) and Polymyositis (PM) (Mukerji and Hardin, 1993; Egner, 2000; Haustein, 2005). The initial, but not always reliable, description of MCTD as a benign disease that transforms into other well known autoimmune disorders has limited its recognition during the study of SLE and other cohorts of autoimmune patients (Sharp et al., 1972; Tan et al., 1982; Hochberg et al., 1997; Lundberg, 2005; Petri et al., 2012; Shi et al., 2012). Recent studies have shown lung disease seems to be specifically associated with MCTD but not SLE patients (Shirai et al., 2012; Watanabe et al., 2012; Gunnarsson et al., 2013). By contrast, renal disorders have long been a characteristic prevalent in SLE but not the MCTD population (Everett and Harrell, 1956; Natali et al., 1972; Cavagna et al., 2013; Lin et al., 2013). The fact that independent studies have described that SLE and MCTD patients are associated with malfunction of different organs highlights the relevance of the recognition of MCTD concept as a clinical tool to identify patients that could develop lung disease as oppose to kidney malfunction, for example. Given that four different criteria sets currently coexist to identify MCTD patients illustrates the significant challenges in uncovering clinical symptoms and/or biomarkers that could be specifically associated with this autoimmune syndrome (Sharp, 1987; Kasukawa et al., 1988; Kahn et al., 1991; Amigues et al., 1996).

During the past six decades, extensive studies have provided evidence to support the following: first, MCTD exhibited a considerable number of overlapping features with SLE making virtually impossible the segregation of some patients using currently available classification criteria sets (Sharp et al., 1972; Tan et al., 1982; Amigues et al.,

1996; Hochberg et al., 1997; Lundberg, 2005; Petri et al., 2012; Shi et al., 2012). Second, the concept of MCTD has clinical relevance since malfunction of vital organs has been reported to be prevalent in this syndrome when compared to SLE, for example (Everett and Harrell, 1956; Natali et al., 1972; Shirai et al., 2012; Watanabe et al., 2012; Gunnarsson et al., 2013). Third, new classification rules with significant power to distinguish between SLE and MCTD patients need to be developed given that available classification criteria sets were designed to identify either SLE or MCTD subjects but not to segregate between these two autoimmune disorders (Sharp, 1987; Kasukawa et al., 1988; Kahn et al., 1991; Amigues et al., 1996; Hochberg et al., 1997; Petri et al., 2012). Prompted by the clinical need for developing accurate methods to segregate between SLE and MCTD patients, the present study explores auto-antigenic properties of the spliceosome; a potential novel molecular tool for the discrimination of these two autoimmune syndromes.

The spliceosome is a macromolecule composed of five distinguished RNA and protein complexes known as U1 small nuclear ribonucleoprotein (U1 snRNP), U2 snRNP, U4 snRNP, U5 snRNP and U6 snRNP (Moore et al., 1993). All these snRNP composites are arranged around the nascent pre-mRNA to remove introns and link exons through the splicing reaction producing a final mature mRNA (Staley and Guthrie, 1998). Earlier reports have confirmed the auto-antigenic properties of U1 snRNP in SLE and MCTD patients. The U1 snRNP is the first spliceosomal subunit binding the pre-mRNA and is composed of three specific U1 snRNP proteins (U1-70K, U1Ap and U1C) and seven Smith (Sm) proteins (Pomeranz Krummel et al., 2009). Specifically, MCTD subjects show prevalence of autoimmune response to U1 snRNP specific proteins

(snRNP) while SLE individuals have a predominance response to Sm proteins (Luyckx et al., 2005). Interestingly, the majority of previous analyses have focused on the auto-antigenic properties of U1-70K and Sm proteins but not on U1A and U1C (McClain et al., 2004; Luyckx et al., 2005). Likewise, the immunoglobulin G (IgG) immune response for these and other nuclear antigens have been extensively documented in SLE and MCTD patients while the potential role of other immunoglobulins (IgM, IgD, IgE and IgA) has been limited (Zhang et al., 1995; Vlachoyiannopoulos et al., 1996; Witte et al., 1998; Palafox Sanchez et al; 2009).

The present study will apply novel classification rules specifically designed for SLE and MCTD discrimination by exploring the IgM response for 15 U1A and 15 additional U1 snRNP peptides, as well as analyzing the frequency and correlations of 183 clinical symptoms and biomarkers recorded in patients with these autoimmune syndromes. The data analyses confirmed previous findings that the immune response for U1A and U1 snRNP peptides mature from IgM to IgG in MCTD patients while SLE subjects appear to retain IgM responses for these autoantigens. In addition, the results revealed that models derived from the IgM reactivity for U1 snRNP and IgM anti-U1A titers have the power to significantly segregate between SLE and MCTD patients as well as identify kidney and lung malfunctions in subjects diagnosed with any of these two illnesses ($p \leq 0.05$), respectively. Furthermore, eight novel classification rules for the segregation of SLE and MCTD were described and showed better classification power than any of the currently available methods ($p \leq 0.05$). Consequently, the results derived from this study provide evidence to support the notion that SLE and MCTD are indeed separate disorders while pioneer the description of eight novel classification criteria sets

capable of discerning between SLE and MCTD patients ($p \leq 0.05$). Recognizing that validation of these findings is required, the data described have the potential to improve the accurate clinical diagnosis of SLE and MCTD which in turn could increase the patient's prognosis and life quality.

II. CHAPTER 1

Uncovering the IgM autoimmune response for U1Ap
in patients diagnosed with SLE or MCTD

Introduction

The small nuclear ribonucleoprotein (snRNP) A (referred to here as “U1Ap”), in combination with U1-70K and U1C, is a specific U1 RNP polypeptide that, along with the U1-RNA and Sm proteins, forms an active U1 snRNP complex. The complex, in turn, plays an essential role in pre-mRNA processing as a functional unit of the spliceosome (Moore and Sharp, 1993). The spliceosomal composite is formed of two RNA recognition motifs (RRM) located at the N- and C-terminal of the U1Ap and has been reported to be conserved across the Eukaryote domain (Scherly et al., 1989; Chen et al., 2007; Somarelli et al., 2010). The N-terminal RRM domain (RRM1) of U1Ap has been extensively studied and has been shown to be necessary and sufficient to bind U1-RNA via its stem loop II and facilitate the splicing process (Scherly et al., 1989, Jessen et al., 1991, Somarelli et al., 2010). By contrast, little is known about the potential function of the U1Ap C-terminal RRM domain (RRM2) despite similar characteristics to other RNA binding domains including U1Ap RRM1 (Scherly et al., 1989, Tang and Rosbash, 1996). The RRM2 of U1Ap exhibits unusual RNA binding properties since it does not bind to U1, U2 or U5 stem loops or interact with random RNA sequences (Lu and Hall, 1995).

Autoimmune responses to U1 snRNP specific proteins, including U1Ap, have been described in patients diagnosed with Systemic Lupus Erythematosus (SLE) and Mixed Connective Tissue Disease (MCTD) (de Wildt et al., 1997; Faig and Lutz , 2003; McClain et al., 2004; Sato et al., 2010). In some patient cohorts, anti-U1Ap responses have been reported to be the first anti-U1-snRNP to develop (McClain et al., 2004). Major organ involvement, including lung and kidney, are common in these individuals (Gutsche et al., 2012; Braun-Moscovici et al., 2013). Though MCTD was described as a

separate disorder (Sharp et al., 1972), there is much controversy regarding its independent nature because of the great number of overlapping auto-antigens and clinical manifestations shared with SLE (Rebora and Parodi, 1990; Swanton and Isenberg, 2005; Nowicka-Sauer et al., 2012). Nevertheless, there are auto-antigens and clinical symptoms that are more frequently exhibited in one disease group than the other. For example, SLE patients show elevated immunoglobulin G (IgG) autoimmune responses to Sm proteins while those with the autoimmune response directed to snRNP subunits are frequently MCTD individuals (Luyckx et al., 2005). Clinically, kidney damage is more frequent in SLE while lung malfunction is often observed in MCTD patients (De Clerck et al., 1989; Sawai et al 1994; Yoshida et al., 1994; Watanabe et al., 2012). Previous studies have described elevated IgG autoimmune response to U1Ap fragments in both SLE and MCTD patients (Sato et al., 2010). In addition, Vlachoyiannopoulos et al. (1996) reported that SLE but not MCTD patients showed a predominant immunoglobulin M (IgM) response to snRNP subunits, including U1Ap. Furthermore, a recent study revealed that U1Ap antigenicity against IgM could aid in the differentiation between these two autoimmune disorders (Mesa et al., 2013).

The aim of the present study is to examine the IgM responses to U1Ap as a molecular indicator to assess differences between SLE and MCTD, and to uncover potential relevance of anti-U1Ap IgM reactivity in predicting organ targeting. To accomplish the goal, a total of 17 U1Ap peptides encompassing most of the protein's sequence were monitored for their IgM antigenicity in sera from SLE and MCTD patients with or without kidney and/or lung involvement. The results support previous findings that SLE but not MCTD patients have prevalent IgM responses to U1Ap peptides

(Vlachoyiannopoulos et al., 1996, Mesa et al., 2013). Furthermore, IgM reactivity to specific U1Ap fragments has potential to identify patients with kidney and lung involvement with an accuracy of 72% and 71%, respectively ($p \leq 0.005$). Consequently, this investigation provides evidence to support that the IgM response to U1Ap peptides could be employed as a predictive blood marker for kidney and lung damage in patients diagnosed with either SLE or MCTD while contributing additional molecular evidence to support the separate etiology of these autoimmune syndromes.

Materials and Methods

Patients recruitment

In this study, SLE (n = 56) and MCTD (n = 26) individuals presenting in either outpatient or inpatient settings to the Division of Rheumatology at the University of Miami Miller School of Medicine, consented to participate in research studies following IRB-approved protocols between 2005 and 2010. Healthy individuals (n = 10) also consenting to participate following IRB-approved guidelines were included as negative controls. The American College of Rheumatology (ACR) classification criteria (Hochberg et al., 1997) and the Alarcon Segovia criteria (Amigues et al., 1996) were used to classify patients as having SLE and MCTD, respectively. In cases where both criteria sets were satisfied, patient diagnoses were determined by the chart-documented diagnoses of their clinical rheumatologists. All SLE and MCTD individuals included in this investigation represent well characterized patients and have been subjects of previous studies (Maldonado et al., 2006; Perkins et al., 2008; Somarelli et al., 2011, Mesa et al., 2013).

Medical records selection

Kidney involvement was defined following the American College of Rheumatology SLE Classification Criteria's renal criterion (Hochberg et al., 1997) and/or renal biopsy. Sufficient information was available to make a determination in 70 of the 82 study patients. Kidney involvement was present in 22 patients (19 SLE and 3 MCTD), and was absent in 48 patients (26 SLE and 22 MCTD). Lung disease was designated to be present if pulmonary fibrosis was confirmed by chest X-ray or CT-scan, or if pulmonary artery pressure by right heart catheterization or right ventricular systolic pressure estimated by echocardiography meter exceeded 40 mmHg. Sufficient information existed to make a determination about lung involvement in 52 patients, of whom 26 patients had lung involvement (SLE = 18 and MCTD = 8) and 26 did not have lung involvement (SLE = 20 and MCTD = 6). All the medical records were obtained following the IRB protocols from University of Miami (IRB numbers: 20030724 and 20040286) and Florida International University (IRB number: 040308-00).

Selection and synthesis of U1Ap peptides

Auto-antigenic U1Ap peptides known to elicit an IgG autoimmune reaction in SLE patients were obtained from literature review to be tested for IgM antigenicity (Barakat et al., 1991; Arbuckle et al., 1998; Talken et al., 2001; Poole et al., 2009; Somarelli et al., 2011). In addition, amino acid sequences corresponding to each of the two RNA recognition motifs (RRMs) were included as peptides (P4 and P14, Table 1). All peptides were commercially synthesized by BioMatik Corporation (Wilmington, DE, USA) and purified by high performance liquid chromatography to $\geq 90\%$ purity.

Assessing IgM autoimmune response for U1A protein peptides

Whole blood from SLE (67 samples from 56 patients), MCTD (29 samples from 26 patients) and healthy (11 samples from 10 persons) individuals were obtained at the moment of the recruitment and subsequent follow up visits, when applicable. Supernatant serum from each patient was diluted 1:100 in phosphate buffered saline (1X PBS) containing 10 mg/ml bovine serum albumin (BSA) and 0.5 mM phenylmethanesulphonyl fluoride (PMSF) and subsequently stored at -80°C until tested. Diluted sample sera from SLE, MCTD and healthy individuals were used to determine IgM reactivity for U1Ap peptides via indirect-ELISA as previously described (Somarelli et al., 2011, Mesa et al., 2013). Goat anti-human IgM horseradish peroxidase conjugated second antibody (Southern Biotech, Birmingham, AL, USA) used at 1:2000 in BSA/PBS containing 0.05% Tween-20 (BSA/PBS-T). All ELISAs were performed in triplicate. Each ELISA plate contained no peptide, no serum, no conjugate and no substrate controls. The optical density (OD) value of the IgM anti-U1Ap titers in each patient was normalized by the average IgM anti-U1Ap reactivity in the healthy group per peptide examined and expressed as OD% following established methods (Muñoz-Paredes *et al.* 1999).

Generating and evaluating the U1Ap three-dimensional structure

A three-dimensional (3D) structural model of the full-length U1Ap (amino acids 1-282) was required to analyze the IgM anti-U1Ap titers given that currently the nuclear magnetic resonance (NMR) structure for human U1Ap only includes residues 2 to 102 (Protein Data Bank (PDB) ID number: 1DZ5). To generate a complete U1Ap 3D structure, the U1Ap linear sequence was retrieved from the National Center for

Biotechnology Information (NCBI) protein data bank (accession number: P09012) and submitted to the I-TASSER server for protein structure and function prediction (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) (Roy et al., 2010 and 2012). A total of five models were generated for U1Ap with confidence scores (C-score) ranging from -2.19 to -3.98. Each of the U1Ap 3D models was superimposed on the U1Ap NMR 3D structure (PDB ID: 1DZ5 containing 12 ensemble structures) using PyMOL (version 1.30) to obtain the most accurate structure possible. The best U1Ap model was selected by finding the highest ratio of overlaid atoms to root mean square deviation (RMSD) during super-positioning on each of the NMR structures in the ensemble. The I-TASSER model 2 paired with ensemble structure 12 given that the assemble yielded the highest ratio ($565:1.679=318.0$; detailed protocol for generating and evaluating U1Ap 3D models is included in Appendix 1).

Mapping IgM response onto U1A protein 3D structure in SLE and MCTD patients

The observed IgM responses for U1Ap peptides in SLE and MCTD patients were mapped independently onto the U1Ap 3D model. To construct the IgM reactivity gradient for each peptide, the average value of the healthy group was adjusted to a baseline of 0.5. Subsequently, the average IgM reactivity recorded in each of the SLE and MCTD groups was expressed as a proportion relative to that baseline. Reactivity lower or higher than the healthy patient averages per peptides resulted in proportional values below or above 0.5, respectively. On the basis of the proportional value, each peptide was mapped on a heat map with the scale ranging from blue for the lowest antigenicity, to green, yellow, and finally red for the highest antigenicity.

Statistical analysis

Significant differences in the average peptide antigenicity against IgM, in SLE and MCTD patients were assessed by independent sample *t*-test. Correlation analyses were performed to verify the IgM responses for U1Ap peptides were not correlated to peptide size ($p \leq 0.05$). A Chi-square test was employed to compare clinical and laboratory categorical variables between SLE and MCTD patients. Spearman's correlation was employed to assess the association between IgM anti-U1Ap titers and kidney or lung involvement. Binomial Logistic Regression (BLR) was used to assess which combination of IgM reactivity for U1Ap peptides enhanced the discrimination between patients presenting with kidney or lung involvement versus those lacking these manifestations. Peptides that were found to be not significant in BLR analysis were not included in subsequent tests. Receiving Operating Characteristics (ROC) curves were used to confirm results obtained by BLR and determine the cut off values to ensure the best sensitivity and specificity for kidney and lung models which were 0.5 and 0.6, respectively. All the statistical analyses were performed using PASW statistical Data Editor, version 18, with $p \leq 0.05$ set as the standard for statistical inference for all the tests executed.

Results

U1Ap peptides showed contrasting IgM antigenicity in SLE and MCTD patients

Indirect ELISAs were employed to assess IgM responses for U1Ap peptides in sera from SLE, MCTD and healthy individuals. In general, SLE patients exhibited higher IgM anti-U1Ap titers when compared to the healthy individuals (P1-P11 and P13, Figure

1). By contrast, except for P5, P8 and P9, MCTD patients had lower IgM responses to U1Ap peptides than healthy individuals (Figure 1). Interestingly, IgM anti-U1Ap-P16 IgM reactivity was significantly lower in both SLE and MCTD patients compared to healthy controls ($p \leq 0.04$) (Figure 1). SLE patients showed trends toward higher IgM reactivity for U1Ap subunits than MCTD patients (P4, P6, P7, P10, P11 and P13 were the most antigenic U1Ap fragments against IgM with 1.3 fold higher levels in SLE than MCTD patients), but the differences were not significant ($p > 0.05$, Figure 1). Specifically, P7 and P17 represented the biggest (137%) and smallest (96%) differences between the SLE and MCTD subsets.

Auto-antigenic IgM response is directed to non-RNA recognition domain regions of U1Ap

To compare the IgM reactivity in the RNA recognition domain versus non-RNA recognition domain fragments of U1Ap, the average peptide reactivity corresponding to regions identified as RNA recognition motif (RRM) 1 (P1-P4), RRM2 (P14-17) and the non-domain area (P4-P13) were calculated in SLE, MCTD and healthy populations. Patients showed elevated IgM anti-U1Ap responses to non-domain areas of the U1Ap (P5-P13) when compared to RRM1 (P1-P4) and RRM2 (P14-P17) (Figure 1). However, only the SLE and not the MCTD patient subgroup exhibited significantly elevated average IgM response to peptides in U1Ap non-domain regions when compared to the average IgM reactivity for peptides covering RRM1 or RRM2 ($p \leq 0.003$). The IgM response to the U1Ap C-terminal end, which encompasses RRM2, was noticeably lower in both SLE and MCTD patients than in control samples (Figure 1).

IgM derived U1Ap 3D epitope maps for SLE and MCTD patients

The IgM reactivity for each U1Ap peptide monitored in sera from SLE and MCTD patients was used to create two independent epitope maps of U1Ap (Figure 2 and detailed protocol in Appendix 1). In general, the IgM reactivity was not always directed to exposed fragments of the U1Ap, given that superficial peptides appeared to have an equal chance to be highly antigenic (P7 in SLE and P9 in MCTD) or not (P17 in SLE and P4 in MCTD) (Figure 2). Likewise, different molecular structures like α -helices (P4), β -sheets (P15) and loops (P8) seemed to elicit antigenic IgM responses to U1Ap. However, β -sheets were the least antigenic of all three forms (P15 and P16 in Figure 2). When comparing the SLE and MCTD subgroups, higher U1Ap IgM reactivity was directed to superficial peptides with α -helical structure in both SLE (P7) and MCTD (P4) (Figure 2). The lowest IgM responses were observed for peptides corresponding to exposed β -sheet fragments of the U1Ap in both SLE and MCTD (e.g., P16 in both SLE and MCTD patients in Figure 2).

IgM anti-U1Ap titers discriminate between patients with and without kidney involvement

The IgM responses to specific U1Ap peptides (P1, P2, P4, P5, P12, P15 and P16) across the entire patient group significantly correlated with kidney involvement ($p \leq 0.05$). BLR analyses using all 17 U1Ap peptides confirmed that the combined IgM reactivity for P2, P8, P11, P14, P15 and P17 (referred to as “U1Ap kidney model”) represented the best predictor to identify patients with kidney disease ($p \leq 0.0001$) (Figure 3A). Specifically, ROC analysis confirmed that the U1Ap kidney model had significant power (AUC = 0.828) to separate between patients with kidney and without

kidney disease with 85% specificity, 45% sensitivity and 73% overall accuracy to correctly categorize patients ($p \leq 0.005$) (Figure 3B). Furthermore, the analyses revealed that the capacity of the U1Ap kidney model to distinguish an SLE patient with or without kidney disease was not significantly different than that observed in patients with MCTD ($p > 0.05$).

IgM anti-U1Ap reactivity patterns and lung involvement

No anti-U1Ap peptide responses in univariate analyses were found to significantly correlate with lung damage. However, BLR analysis revealed that the combined IgM response to peptides P4, P9, P12 and P15 (the “U1Ap lung model”) distinguished between patients with or without lung involvement similarly to the performance of the kidney model above (AUC = 0.822) (Figure 4A). The overall accuracy of the U1Ap lung model to classify patients was 79% with 81% sensitivity 77% specificity (Figure 4B). As expected, the capacity of the U1Ap lung model to identify a patient with or without lung disease was not significantly different when SLE or MCTD subsets were contrasted.

Discussion

New markers that differ between SLE and MCTD patients are being described as the understanding of these autoimmune syndromes increases (Sasaki et al., 2011; Lage et al., 2012; Mesa et al., 2013; Jin et al., 2013). In the present study, the IgM reactivity for peptides covering the full length of the U1Ap was explored to find potential novel U1Ap antigens unique to either autoimmune disorder as well as to determine associations that

might exist between IgM responses to U1Ap and organ targeting. The present investigation reported differences between SLE and MCTD patients regarding their anti-U1Ap responses, provided the first IgM derived epitope mapping of U1Ap for these autoimmune disorders, and describe two patterns of IgM anti-U1Ap peptide reactivity that are associated with kidney or lung disease.

The mapping of the IgM response onto the predicted 3D structure of the full length U1Ap represented a unique approach to examine the behavior of the autoimmune reaction for this protein in SLE and MCTD patients (Figure 2), especially since only the U1Ap's RRM1 NMR has been described (Varani et al., 2000). In concordance with a previous report (McClain et al., 2004), U1Ap fragments that do not participate in protein or RNA binding show the highest IgM antigenicity in both SLE and MCTD (P5-P13 in Figures 1 and 2). Nevertheless, only SLE but not MCTD patients showed significant differences when domains RRM1 and RRM2 were compared to non-domain areas (U1Ap amino acids 87-206, $p \leq 0.05$). The present study revealed that bound and unbound region of U1Ap are capable of elicit an autoimmune response and therefore suggest that unbound regions of U1Ap are not sufficient to elicit IgM antigenicity. Consequently, other factor(s) like molecular mimicry from defective immune response to viral infections described by Migliorini et al. (2005) could be essential in promoting anti-U1Ap IgM responses in SLE but not MCTD patients. Contrary to the common believe that antigenic fragments are located on superficial protein regions (McClain et al., 2004), the data suggest that the IgM response to U1Ap does not appear to rely only on peptide accessibility in the intact form of the protein. Consequently, it is reasonable to hypothesize that the IgM response in SLE and MCTD patients could form prior to U1Ap

reaching its native state or after deficient clearance of U1Ap apoptotic material like that reported by the deleterious role of granzyme B in autoimmune diseases (Casciola-Rosen et al., 1999; Mevorach, 2003). The fact that SLE and MCTD patients' sera were unable to produce an autoimmune reaction for U1Ap RRM2 (P14-P17 in Figures 1 and 2) when compared to U1Ap RRM1 (P1-P4) was not surprising given that RRM2 sequence differs by 78% from that of RRM1 and its binding capacity has been shown to not resemble that of a typical RNA binding protein (Lu and Hall, 1995). Taken all together, the 3D epitope map not only allows for visualization of the IgM antigenicity of U1Ap fragments determined by their location and molecular structure but also provides a basis to develop a new hypothesis regarding the IgM response to U1Ap (Figure 2).

The binomial analysis revealed that the combined IgM reactivity of six U1Ap peptides (P2, P8, P12, P14, P15 and P17) allowed the detection of kidney damage in either SLE or MCTD patients with an overall accuracy of 72% ($p \leq 0.005$) (Figure 3). Interestingly, the power of the U1Ap kidney model did not significantly differ between SLE and MCTD with kidney malfunction ($p \leq 0.05$). The present study describes for the first time an U1Ap model as a predictive serological biomarker for kidney disease. However, levels of neutrophil gelatinase-associated lipocalin (NGAL), soluble vascular cell adhesion molecule 1 (VCAM-1), Interleukin 6 (IL-6) and nucleosome have been described as molecular determinants for kidney disease only for SLE but not reported for MCTD patients (Yang et al., 2012; Pizarro et al., 2007; Brugos et al., 2012; Simón et al., 2004). Currently, NGAL is considered the gold standard for the diagnosis and prognosis of kidney injury with sensitivity (70.8%) and specificity (87.5%) in SLE patients (Haase et al., 2009; Yang et al., 2012). Nevertheless, previous research derived from NGAL has

not recognized the inclusion of MCTD patients therefore its power to detect kidney disease in these unrecognized illness is unknown. Consequently, the proposed U1Ap kidney model represents the only available blood marker for kidney disease for patients diagnosed with either SLE or MCTD.

The combination of IgM reactivity for four U1Ap subunits (P4, P9, P12, and P15) had significant power to identify patients with lung disease diagnosed with either SLE or MCTD with an overall accuracy of 79% ($p \leq 0.002$) (Figure 4). The U1Ap lung model did not show significant difference in predicting lung damage when SLE and MCTD were compared. However, lung biomarker homogeneity like the one observed in the present study has been reported in patients suffering from autoimmune disorders (Nishimaki et al., 1999). The proposed lung model is congruent with previous studies given that auto-antigenicity against U1Ap has been associated with lung disease in SLE and MCTD patients (Nishimaki et al., 1999; Bertoli et al., 2007; Cojocaru et al., 2011; Lian et al., 2012) and has even been shown to be elevated in three different types of lung cancer (Zhang et al., 2005 and 2008). Aside from these associations, no study has recorded the predicted accuracy of these correlations and, as a result, chest radiography has been the suggested method to diagnose lung injury (Levitt et al., 2013). Since evidence of lung damage is reported in both of these autoimmune syndromes and indicated to be the leading cause of mortalities in MCTD patients (Prakash, 1992; Pehlivan and Inanc, 2010; Allen et al., 2012), the U1Ap lung model should be considered as a new serological predictive factor for lung disease with the sensitivity and specificity of 81% and 77%, respectively, in patients with either SLE or MCTD (Figure 5).

In summary, the results are in agreement with previous studies given that there is a prevalence of IgM anti-U1Ap reactivity in SLE but not MCTD patients (Figures 1). The IgM derived U1Ap epitope map showed that peptide accessibility is not sufficient to justify the contrasting autoimmune reactions observed in SLE and MCTD patients (Figure 2) and the data suggests that molecular mimicry from previous infection and/or deficient clearance of U1Ap apoptotic fragments are candidate factors for this difference. Consequently, these findings constitute additional molecular evidence to support the different etiology of these autoimmune disorders. Furthermore, for the first time, this work described two serological biomarkers with the predicted capacity of 72% of detecting kidney damage and 71% of identifying lung injury in patients diagnosed with either SLE or MCTD (Figures 4 and 5). With acknowledgement that additional validation of the U1Ap proposed models for identification of kidney and lung malfunction is required, future research on biomarkers associated with organ malfunction in SLE population should strongly consider the recognition of MCTD patients given the great number of overlapping characteristics shared by these two autoimmune syndromes.

Table 1: U1A protein peptides characterization

Peptide Number	Peptide Position	Peptide Sequence	Peptide length	Reference
P1	1–11	MAVPETRPNHT	11	Barakat et al. (1991)
P2	35–58	SQFGQILDILVSRSLKMRGQAFVI	24	Barakat et al. (1991)
P3	47–59	RSLKMRGQAFVIF	13	Arbuckle et al. (1998)
P4	60–95	KEVSSATNALRSMQGFPHYDKPMRIQYAKTDSIIA	36	No previously tested
P5	96–103	KMKGTFFE	8	Poole et al. (2009)
P6	112–119	KPKSQETP	8	Somarelli et al. (2011)
P7	118–127	TPATKKAVQG	10	Poole et al. (2009)
P8	143–154	GMPPMTQAPRIM	12	Poole et al. (2009)
P9	159–178	GQPPYMPPPGMIPPPGLAPG	20	Poole et al. (2009)
P10	165–172	PPPGMIPP	8	Talken et al. (2001)
P11	178–185	GQIPPGAM	8	Somarelli et al. (2011)
P12	180–193	IPPGAMPPQQLMPG	14	Poole et al. (2009)
P13	196–203	PPAQPLSE	8	Somarelli et al. (2011)
P14	204–235	NPPNHILFLTNLPEETNELMLSMLFNQFPGFK	32	No previously tested
P15	236–242	EVRLVPGR	8	Poole et al. (2009)
P16	239–251	LVPGRHDIAFVEF	13	Arbuckle et al. (1998)
P17	257–282	AGAARDALQGFKITQNNAMKISFAKK	26	Barakat et al. (1991)

Figures Legends

Figure 1: SLE but not MCTD patients show predominant IgM response to U1Ap subunits. The diagram under U1A protein is a graphical representation of the linear structure of this protein where RNA recognition motif 1 (RRM1) and 2 (RRM2) are labeled. The amino acids covered by each of the peptides (P1-P17) are indicated under peptide position. The red, blue and gray columns correspond to SLE (n = 56), MCTD (n = 26) and healthy (n = 10) individuals, respectively. The lines on top of each column represent the standard error.

Figure 2: U1A protein epitope map for IgM antigenicity in SLE and MCTD patients. The IgM optical density percentage (OD%) based on the healthy group IgM reactivity obtained from SLE (top) and MCTD (bottom) patients was mapped onto the 3D structure of U1A protein. The IgM antigenic scale is included at the bottom of the figure where blue and red represent the lowest and highest IgM reactivity for U1A protein, respectively. Gray areas on the SLE and MCTD epitope maps correspond to regions of unknown IgM antigenicity since no peptide covers these specific fragments. The rectangle embedded in the middle represents the U1A protein 3D structure where RNA recognition motif 1 (RRM1) is labeled in red, RRM2 is colored in blue while the gray fragments are regions free of RNA and protein interactions.

Figure 3: IgM reactivity for U1Ap is a candidate marker for kidney damage. The observed IgM response to U1A protein (U1Ap) peptides was analyzed by binomial logistic regression (BLR) to assess which peptide combination increase could

significantly identify patients with kidney damage in either SLE or MCTD populations ($p \leq 0.05$). The classification ability of each of these peptides and predicted models were corroborated by receiving operating characteristic (ROC) curves analysis ($p \leq 0.05$) **A. *ROC curves revealed the power of U1A peptides in classifying patients with kidney damage.*** In the graph, the peptides and predicted U1Ap kidney model are on the x axis while the area under the curve (AUC) resulting from ROC curves analysis is indicated on the y axis. The larger the number, the higher the probability of identifying an individual with kidney disease compared to a healthy individual. The lines on top of each column correspond to standard deviation. The p values ≤ 0.05 , ≤ 0.001 and 0.0001 are represented with one (*), two (**) or three (***) asterisks, respectively. **B. *Distribution of patients with kidney damage based on the U1Ap kidney model.*** Patients with evidence of kidney damage and healthy individuals are on the x axis while the predicted probabilities obtained from BLR analysis are on the y axis. The black and white dots indicate true positive (TP) and true negatives (TN) while the crosses represent either false positives (FP) or false negatives (FN). A cut off value of 0.5 (from a range of 0 to 1) was selected to allow equal chances to FP and FN ($p \leq 0.05$).

Figure 4: IgM anti-U1Ap titers are potential biomarkers for lung damage. The IgM reaction for U1A protein (U1Ap) peptides was analyzed by binomial logistic regression (BLR) to uncover peptide combinations with power to discern between patients with lung damage and healthy individuals in either SLE or MCTD populations ($p \leq 0.05$). The grouping capability of each of these peptides and predicted models were corroborated by receiving operating characteristic (ROC) curves analysis ($p \leq 0.05$) **A. *ROC curves***

showed the classification power of the U1Ap lung model. In the graph, the peptides and predicted U1Ap lung model and area under the curve (AUC) resulting from ROC curves analysis are on the x and y axes. The bigger the AUC, the higher the classification power for lung disease. The lines on top of each column correspond to standard deviation. The asterisks (***) represent $p \leq 0.0001$. **B. Segregation of patients with lung damage utilizing the U1Ap lung model as classifier.** In the plot, patients and predicted probabilities of kidney damage are on the x and y axes. The black and white dots indicate true positive (TP) and true negatives (TN), respectively, while the crosses represent either false positives (FP) or false negatives (FN). A cut off value of 0.5 (from a range of 0 to 1) was selected to allow equal chances to FP and FN ($p \leq 0.05$).

Figure 1: SLE but not MCTD patients show predominant IgM response to U1Ap subunits

Figure 2: U1A protein epitope map for IgM antigenicity in SLE and MCTD patients.

Figure 3: IgM reactivity for U1Ap is a candidate marker for kidney damage.

Figure 4: IgM anti-U1Ap titers are potential biomarkers for lung damage.

III. CHAPTER 2

Production of spliceosomal derived biomarkers for identification of SLE and MCTD

Introduction

Systemic Lupus Erythematosus (SLE) and Mixed Connective Tissue Disease (MCTD) are systemic autoimmune disorders with overlapping clinical manifestations that possess aberrant immune responses against common auto-antigens (Zdrojewicz et al., 1999; Egner 2000; Greidinger and Hoffman , 2001; Riemekasten and Hahn, 2005; Neogi et al., 2006; Liu and Ahearn, 2009). Despite its description as an independent autoimmune disease (Sharp et al., 1972), the classification of MCTD as distinct from SLE remains controversial because of the high number of common clinical features between SLE and MCTD patients (López-Longo et al., 1994; Aringer et al., 2005; Swanton et al., 2005; Venables, 2006; von Bierbrauer et al., 2008; Nowicka-Sauer et al., 2012). Nevertheless, the concept of MCTD has been reported as a useful definition in clinical practice (Zdrojewicz et al., 1999; Venables, 2006; von Bierbrauer et al., 2008; Nowicka-Sauer et al., 2012), and clinical and serological features segregate the two illnesses (Isenberg et al., 2007; Breda et al., 2010). The American College of Rheumatology (ACR) has created universal classification parameters for SLE (Hochberg, 1997); however, four different criteria sets exist for MCTD patients, with the Alarcón-Segovia criteria being the most widely accepted (Amigues et al., 1997).

Currently, there is no single test with sufficient specificity and sensitivity to discriminate between SLE and MCTD, which has hampered the identification of MCTD as a separate syndrome (Egner 2000; Reveille, 2004; Perkins et al., 2008; Liu and Ahearn, 2009; Hoffman et al., 2012). A positive diagnosis by any set of criteria requires a patient to exhibit at least four clinical symptoms and/or tests out of those included in each list, which can take years to develop (Liu and Ahearn, 2009; Hoffman et al., 2012).

Moreover, traditional laboratory tests are performed with numerous commercially available kits that can vary in principle and cut-off values, which may alter the final results and diagnoses (Egner 2000; Reveille, 2004; Mahler et al., 2005; Isenberg et al., 2007; Chiaro et al., 2011). These and other factors complicate proper diagnosis of these two closely related and overlapping illnesses.

Previous investigations have demonstrated that SLE and MCTD patients often exhibit 1000-fold greater auto-reactivity to subunits of the U1 small nuclear ribonucleoprotein particle (snRNP) than to any other cellular component (Hoet et al., 1993; Vlachoyiannopoulos et al., 1996). The U1 snRNP is an RNA-protein complex that is responsible for pre-mRNA processing and is composed of 10 proteins (U1-70K, U1A, U1C and seven Smith antigen (Sm) proteins) (Luyckx et al., 2005; Mesa et al., 2008; Buratti et al., 2010; Somarelli et al., 2010). In general, previous studies aimed at finding biomarkers for SLE and MCTD have focused on IgG-specific responses to nuclear components, including the U1 snRNP; however, some studies have revealed differential IgM reactivity for nuclear components in SLE and MCTD patients (Zhang et al., 1995; Vlachoyiannopoulos et al., 1996; Witte et al., 1998; Palafox Sánchez et al., 2009). Yet, the potential use of the IgM response as a molecular tool to classify SLE and MCTD patients has not been fully explored.

To determine whether SLE and MCTD represented distinct disorders and test whether the two patient groups can be segregated, the IgG- and IgM-specific responses of patients with SLE and MCTD and healthy individuals against 15 different U1 snRNP peptides (named P1-15) were evaluated by indirect enzyme-linked immunosorbent assays (ELISAs). Interestingly, higher IgG-specific reactivity for U1 snRNP peptides was

observed in individuals with SLE or MCTD compared to healthy individuals, but elevated IgM responses in SLE patients compared to those with MCTD and healthy adults. The IgM response to two peptides, P4 and P10 (P4/P10), exhibited 71.3% accuracy in segregating between these two autoimmune disorders ($p \leq 0.05$). In summary, these data support the notion that SLE and MCTD are, indeed, distinct disorders and highlight the potential clinical use of the IgM anti-U1 snRNP system as a molecular tool to assist in the classification of SLE and MCTD patients.

Materials and Methods

Collection and preparation of sample sera

Sera were obtained from whole blood of 122 patients previously diagnosed with SLE (n=81) or MCTD (n=41) and 31 healthy individuals. Samples were collected following the Institutional Review Board (IRB) accepted protocols of the University of Miami (IRB numbers: 200307-24 and 200402-86) and Florida International University (IRB number: 040308-00). The SLE and MCTD patients (collectively refer as ill or patient group) were clinically diagnosed according to the American College of Rheumatology (ACR) criteria and the Alarcón-Segovia criteria, respectively, along with clinician judgment (Amigues et al., 1996; Hochberg, 1997). The laboratory tests considered in the present study were commercially performed by Quest Diagnostic Incorporated and their positive values are included in Table 3. Details of the flare or remission period in these SLE and MCTD patients were not recorded at the moment of whole blood collection and therefore disease activity for these SLE and MCTD patients is not been considered in this study.

Selection of U1 snRNP peptides

The U1 snRNP peptides included were previously reported in Somarelli et al., (2011) and commercially synthesized by BioMatik Corporation (Wilmington, DE, USA). The observed IgM reactivity for each of the U1 snRNP peptides was ranked from most (1) to least (15) antigenic for each disease state (Table 3).

Monitoring IgM reactivity for U1 snRNP peptides by indirect ELISAs

The indirect ELISA protocol employed to assess IgM reactivity for each peptide and sample included was previously described (Somarelli et al., 2011). The average IgM derived OD value for each peptide was normalized using the average OD value of the healthy group per peptide examined and was expressed as OD% based on the following formula (Muñoz-Paredes et al., 1999):

$$OD\% = \left(\frac{\bar{X} \text{ OD of sample in } P_x}{\bar{X} \text{ OD of control group in } P_x} \right) \times 100$$

where “ $\bar{X}$ OD of sample in P_x ” is the average OD value of the sample group (SLE or MCTD) and “ $\bar{X}$ OD of control in P_x ” indicates the average OD of the control group (healthy group) from each of the peptides included in this study (P1-P15). To evaluate the relative reactivity contributed by IgM and IgG in SLE, MCTD and healthy populations, the average OD values from IgG-specific ELISAs previously reported by Somarelli *et al.*, (2011), which used the same samples and U1 snRNP peptides included in this study, were re-analyzed and converted to OD% using the equation described above (Muñoz-Paredes et al., 1999).

Statistical analyses

Significant differences in IgG and IgM reactivity between patients (SLE and MCTD) and healthy groups and between SLE and MCTD individuals for each of the peptides was assessed using independent sample *t*-tests. Clinical tests and symptoms were evaluated by independent sample *t*-tests (numerical data) or Chi (*X*) squared tests (nominal data). Receiver Operating Characteristic (ROC) curves were generated with the PASW software package (version 18). Forward binary logistic regression (BLR) analyses using the IgM and IgG anti-U1 snRNP titers in ill (SLE and MCTD) and healthy individuals as well as SLE and MCTD patients were performed with the PASW software package (version 18). P-values ≤ 0.05 were considered statistically significant for all tests. Correlations between each IgM anti-U1snRNP peptide titers and IgM anti-Rheumatoid Factor (RF) antibody titers were performed using PASW software package (version 18), however; since none of them were significantly correlated, they were not further considered in this study.

Results

IgM anti-U1 snRNP reactivity is elevated in SLE but not MCTD patients

The IgM response to U1 snRNP peptides was monitored via indirect ELISAs and reported as OD% (Figure 5A). IgM anti-U1 snRNP titers were significantly higher in the SLE group than either the MCTD population or healthy individuals ($p \leq 0.05$). In fact, in many instances, IgM responses to U1 snRNP peptides in MCTD patients were equal to or below those exhibited by healthy individuals (P3, P4 and P9-P15 in Figure 5A). The discrimination capacity of IgM-anti-U1 snRNP peptide ELISAs was assessed by ROC

curve analysis and indicates that IgM reactivity for P1 and P13 provides significant power to classify SLE and MCTD patients; however, none of the IgM responses were sufficient to discern SLE and MCTD from non-disease controls with statistical significance (Figures 1C-D and Appendix 2).

SLE and MCTD patients exhibit an elevated IgG response for U1 snRNP peptides

As previous studies have reported (Fries et al., 1984; Nishimaki et al., 1999; Lindorfer et al., 2001; Routsias et al., 2010; Ryu et al., 2011; Somarelli et al., 2011;), the IgG-mediated reactivity for each of the U1 snRNP peptides was significantly higher in both SLE and MCTD populations than in the healthy group; however, IgG reactivity does not differ between the two autoimmune disorders (Figure 5B). ROC curve analyses on IgG anti-U1 snRNP titers per peptide ascertain their individual ability to discern between patients (SLE and MCTD) and healthy individuals and between SLE and MCTD patients (Figure 5C-D, respectively; and Appendix 2). As previously reported (Somarelli et al., 2011), all but IgG anti-P2 responses were capable of significantly discriminating ill (SLE and MCTD) and healthy individuals with IgG anti-P4 being the best ($p \leq 0.05$); however, none of the IgG anti-U1 snRNP titers had a statistically significant ability to classify SLE and MCTD patients (Figure 5D).

Differential auto-immune responses and symptoms are observed in SLE and MCTD patients

The results showed that SLE and MCTD patients exhibit significantly different IgM anti-U1 snRNP reactivity ($p \leq 0.05$) despite similar IgG-mediated antigenicity for

the same peptides (Figures 1A-B). To further support the idea that SLE and MCTD represent distinct auto-immune illnesses, statistical analysis of 42 standard laboratory tests were performed with blood samples from the SLE and MCTD patient cohort. These analyses revealed that 11 out of the 42 clinical tests were significantly different in SLE and MCTD patients ($p \leq 0.05$) (Table 2). Specifically, differences were observed in tests designed to detect nuclear auto-antigens (RNP, Sm, Scl70, dsDNA, elevated DNA), renal function (creatinine phosphokinase levels, renal proteinuria, renal hematuria) and immune system components (C3 and C4 complement levels) ($p \leq 0.05$). These findings support the idea that SLE and MCTD represent distinct autoimmune manifestations, with specific antigenic targets and antibody class reactivities.

Similarly, statistical assessment of 40 clinical symptoms from patients in the SLE and MCTD cohort indicated that 16 out of the 40 clinical characteristics evaluated were significantly different between SLE and MCTD patients (Table 4). Most of the significantly different clinical manifestations involved the skin and joints of these patients; however, the data also confirmed that neuropsychiatric disorders and problems in the circulatory system were also significantly different between the two groups. Once again, the fact that clinical symptoms differ in SLE and MCTD populations supports the hypothesis that these maladies may be clinically distinct.

Antibody class reactivities for U1 snRNP peptides segregate among SLE, MCTD and healthy individuals

The IgM and IgG responses for all U1 snRNP peptides were combined in a BLR to determine which peptide and auto-antibody combinations might provide the highest

segregation between patient (SLE and MCTD) and healthy populations. These analyses revealed that the combined IgG-specific response for P2, P4, P5, P10 and P13 has the greatest capacity to discern between sick and healthy individuals with an overall accuracy of 94% ($p \leq 0.05$) (Figure 6A) where the probability of correctly predicting a patient with either SLE or MCTD is higher than that for correctly predicting a healthy individual (96.7% and 83.9%, respectively).

Additional BLRs were performed with the individual IgG and IgM reactivities for each U1 snRNP peptide to assess which peptide and Ig class combination significantly discriminates between SLE and MCTD patients. These analyses indicated that only the combined IgM response for P4 (U1C) and P10 (U1A) significantly discriminate between SLE and MCTD patients, with an overall accuracy of 71.3% ($p \leq 0.05$) (Figure 6B). Remarkably, most of the classification power derives from the proper classification of SLE patients (95.1%) rather than proper grouping of MCTD patients (24.4%) (Figure 6B). Consequently, the data demonstrate that by first combining the IgG reactivity for P2, P4, P5 and P10 and then the titers for IgM anti-P4/P10, an overall accuracy of 73.9% at discriminating among SLE, MCTD and healthy groups can be achieved.

Comparing the power of IgM anti-P4/P10 with conventional clinical tests

To determine the classification power of the proposed IgM-specific P4/P10 ELISA-based system, ROC curves were used to compare this system with eight conventional clinical tests. The individual IgM reactivities for P1 and P13 were also included in the ROC curves analyses because they discriminate between SLE and MCTD (Figure 5D). The 11 laboratory tests that significantly differ between SLE and MCTD

patients were performed only in a small portion of each sub-population (Table 2). Not all tests could be analyzed as result of the reduced sample size. Instead, eight of the most frequently-used laboratory tests that are part of the classification criteria to diagnose SLE or MCTD were included in the ROC curve analysis (FANA titers, dsDNA ELISA, elevated serum DNA titers and positive results for RNP, Sm, SSA, SSB and Scl-70) (Egner, 2000; Mahler et al., 2005; Isenberg et al., 2007; Breda et al., 2010). When using the subset of individuals for whom clinical test results were available (SLE = 59 and MCTD = 24), the IgM anti-P4/P10 titers and IgM anti-P1 reactivity displayed the greatest discrimination capacity to classify SLE and MCTD patients ($p \leq 0.05$) (Figure 7 and Appendix 2). The ROC curves confirmed that among the conventional tests evaluated, elevated DNA and positive results for Sm are the third and fourth best at significantly segregating SLE and MCTD ($p \leq 0.05$).

Improving the discriminatory capacity of IgM anti-P4/P10 titers

The BLR analyses were performed to assess whether the combination of the IgM anti-P4/P10 system and any of the eight laboratory tests employed to diagnose SLE or MCTD (FANA titers, dsDNA ELISA, elevated serum DNA titers and positive results for RNP, Sm, SSA, SSB and SCL-70)^{4; 15-16; 21-22} might provide greater capacity to distinguish between these syndromes. The individual IgM reactivities for P1 and P13 were considered in this BLR analysis because they showed a significant ability to classify SLE and MCTD patients ($p \leq 0.05$) (Figure 5D). The BLR analyses indicated that the combination of the IgM-based reactivity for P4/P10 and an elevated DNA assay represent the best combination of variables to segregate SLE from MCTD when compared with

IgM anti-P4/P10, -P1, or-P13 and any single laboratory test examined ($p \leq 0.0001$) (Figure 7 and Appendix 2). None of the other clinical test combinations improved the power of discrimination between SLE and MCTD patients over that exhibited by the individual tests alone ($p \leq 0.05$). The analyses also suggest that, when combined with the standard elevated DNA test, the IgM response against P4/P10 may be useful in enhancing the current segregation of SLE from MCTD.

Discussion

Despite the fact that MCTD was described as a distinct rheumatic syndrome in 1972 (Sharp et al., 1972), placement of this disorder as a separate auto-immune illness remains controversial. Opinions are divided regarding classification of MCTD as a separate malady due to the number of auto-antigens and clinical symptoms that show overlap with SLE (López-Longo et al., 1994; Aringer et al., 2005; Swanton et al., 2005; Venables, 2006; von Bierbrauer et al., 2008; Nowicka-Sauer et al., 2012). The immune responses of SLE and MCTD patients for overlapping ‘self’ antigens coupled with the diversity of commercially available clinical tests with differing protocols, reagents and cut-off values have impeded the development of standard and uniform assays to segregate these syndromes (Egner, 2000; Mahler et al., 2005; Neogi et al., 2006; Liu and Ahearn, 2009). With the exception of a few studies (Vlachoyiannopoulos et al., 1996; Zhang et al., 1995; Witte et al., 1998; Palafox Sánchez et al., 2009), most investigations have focused on IgG-mediated reactivity toward specific antigens as potential molecular tools to differentiate between SLE and MCTD patients (Fries et al., 1984; Lindorfer et al., 2001; Routsias et al., 2010; Ryu et al., 2011). Given that SLE and MCTD patients are

characterized by elevated blood titers of multiple Ig classes, including IgM (Pollar and Tan. 1985; Kingsmore et al., 1989; Vlachoyiannopoulos et al., 1996), it was hypothesized that IgM responses to a number of U1 snRNP peptides may allow us to increase the present discrimination between SLE and MCTD and provide additional molecular evidence to claim the independent nature of these two disorders.

The data indicate that the combined IgM reactivity for fragments of U1C (P4) and U1A (P10) is capable of classifying SLE and MCTD patients with an accuracy of 71.3% (Figure 6B), a value higher than previously reported peptide-based immunoassays that have been used to segregate these disorders (Mahler et al., 2005). These findings are in concordance with previous reports, which revealed a preponderance of IgM anti-U1 snRNP antibodies in SLE, but not MCTD patients (Zhang et al., 1995; Vlachoyiannopoulos et al., 1996). Therefore, the present work is congruent with prior investigations and demonstrates the potential utility of differential Ig class responses as a classification tool for SLE and MCTD. The current work also provides molecular evidence to support the distinct etiology of these syndromes.

The binomial analyses identified combinations of laboratory tests and/or peptide reactivities that significantly discern between these maladies. Interestingly, the IgM anti-P4/P10 ELISA-based system provided the greatest capacity to segregate between SLE and MCTD disorders and eight other conventional laboratory tests ($p \leq 0.0001$) (Figure 7). Additionally, we revealed that the combination of IgM anti-P4/P10 antigenicity with the elevated DNA test segregated 79.8% of SLE and MCTD patients, even in the smaller subset of patients for whom clinical test results were available ($n = 59$ for SLE and $n = 24$ for MCTD) (Figure 7). It is not surprising that the dsDNA test contributes to the

differentiation of these diseases given that antibodies against DNA have been detected in approximately 70% of SLE patients and shows 95% specificity for this disorder (Reveille, 2004; Breda et al., 2010). Yet, the fact that the dsDNA test alone exhibits a lower ability to segregate SLE and MCTD patients (66.4%) than the IgM anti-P4/P10 system (73.1%), indicates the significant contribution of this ELISA-based system in discerning between these two maladies (Figure 7).

A total of 16 out of 40 clinical manifestations studied significantly differ between SLE and MCTD patients (Table 4). On average, MCTD patients exhibited hand/joint swelling and muscle weakness with 25% higher frequency than SLE patients. Similarly, malar and discoid rashes were found to be more prevalent in the SLE than the MCTD group (46% and 10% versus 13% and 0%, respectively). These findings are in concordance with previous studies that reported these clinical manifestations as key features in SLE or MCTD patients (Perkins et al., 2008; Hoffman et al., 2010). Evidence of mental illness was also found to be 32% higher in MCTD than SLE patients. Although selection bias of the clinicians diagnosing these disorders cannot be ruled out, the results obtained from a subset of SLE and MCTD patients suggest that the immune response of SLE patients seems to be directed to skin areas on the face while those suffering from MCTD appear to develop a more systemic immune response that attacks the skin, joints and muscles throughout various parts of the body. Furthermore, these findings highlight specific clinical manifestations that appear to differ between SLE and MCTD patients and should be considered as clinical evidence that they may be distinct diseases.

Overall, this study further highlights the current challenges in developing quantitative tests for the classification of SLE and MCTD and therefore the recognition of MCTD as

a separate entity (Egner, 2000; Mahler et al., 2005; Neogi et al., 2006; Liu and Ahearn, 2009). Here, a novel approach based on differential antibody class (IgM and IgG) responses has been described as a mechanism to discriminate between SLE and MCTD patients with better accuracy than conventional laboratory tests currently employed as part of the classification criteria to diagnose these syndromes. In addition, the data revealed contrasting frequencies of clinical symptoms characterizing these auto-immune syndromes whereby SLE patients showed a concentrated auto-immune manifestation directed to skin areas on the face while those suffering from MCTD developed more systemic immune responses that attack the skin, joints and muscles throughout various parts of the body. Consequently, these results provide further evidence to support the fact that there are molecular and clinical aspects of SLE and MCTD to indicate that these diseases are, indeed, two distinct autoimmune syndromes.

Table 2: Clinical tests evaluated in SLE and MCTD patients

	Clinical test name	SLE		MCTD		P value
		Positive	Total	Positive	Total	
SLE / MCTD specific	Fluorescence antinuclear Abs titers	76.56%	64	90.63%	32	0.3860
	Fluorescence antinuclear Abs pattern	86.30%	73	85.71%	35	0.4430
	IgG anticardiolipin positive	23.53%	68	23.81	21	0.8480
	IgM anticardiolipin positive	6.06%	66	18.18%	22	0.0870
	Rheumatoid factor titer by latex	8.33%	12	20.00%	5	0.0870
	IgM anti-rheumatoid factor Abs by ELISA	41.86%	43	38.10%	21	0.8480
	IgM anti-rheumatoid factor Abs titer	20.93%	43	23.81%	21	0.9040
	RNP positive	84.00%	75	100%	40	0.0080
	Sm positive	60.27%	73	28.21%	39	0.0010
	SSA positive	58.11%	74	47.22%	36	0.2820
	SSB positive	21.62%	74	11.11%	36	0.1800
	SCL 70 positive	1.45%	69	16.13%	31	0.0040
	Elevated serum DNA titer	64.10%	78	29.73%	37	0.0010
	Anti-dsDNA positive	49.35%	77	27.03%	37	0.0010
	IgG anti U1-70K Abs*	37.84%	37	55.00%	20	0.2130
	IgG anti SmB/B' Abs*	44.12%	34	50.00%	20	0.7700
	IgG anti SmD Abs*	73.53%	34	63.16%	19	0.7260
	Anemia	25.00%	80	22.50%	40	0.7630
	Hemolytic anemia	3.23%	62	8.33%	12	0.4120
	White blood count	8.77%	57	6.06%	33	0.2780
	Leukopenia	11.54%	78	12.50%	40	0.8780
	Lymphopenia	36.71%	79	42.5%	40	0.5400
	Thrombocytopenia	10.13%	79	2.50%	40	0.1370
	Thrombocytosis	5.13%	78	2.56%	39	0.5180
	Creatine phosphokinase positive	91.07%	56	74.29%	35	0.4770
	Creatine phosphokinase elevated	7.02%	57	25.71%	35	0.0120
	Serum creatinine	10.53%	76	5.26%	38	0.4500
	Renal cellular cast	10.67%	75	2.56%	39	0.1280
	Renal proteinuria	40.85%	71	13.51%	37	0.0040
	Renal hematuria	18.92%	74	2.70%	37	0.0180
	C reactive protein	20.83%	72	18.18%	33	0.4850
	Elevated C reactive protein	23.61%	72	20.59%	34	0.7290
	Low C3 complement	41.77%	79	15.00%	40	0.0030
	C3 complement level	56.96%	79	82.50%	40	0.0001
	Low C4 complement	48.10%	79	30.77%	39	0.0730
	C4 complement level	44.30%	79	69.23%	39	0.0001
	Erythrocyte sedimentation rate	57.89%	79	52.27%	40	0.8530
	Elevated erythrocyte sedimentation rate	56.48%	76	55.00%	40	0.8710
General	IgG anti-rheumatoid factor Abs	41.86%	43	42.86%	21	0.9400
	IgG anti-rheumatoid factor Abs titer	41.86%	43	38.10%	21	0.8480
	Immunoglobulin isotypes for RF factor	79.07%	43	76.19%	21	0.5300
	Lymphocyte absolute value	74.14%	58	75.86%	29	0.3460

Table 3: Overview of U1 snRNP peptides used in the study

Peptide number	U1 snRNP protein	Peptideregion (amino acid range)	Peptide sequence	Observed IgM reactivity ranked	
				<i>SLE</i>	<i>MCTD</i>
1 ^{†*}	U1A	196-203	PPAQLSE	1	6
2 ^{†*}	Sm E	63-70	EIHSKTKS	3	2
3 ^{†*}	Sm F	46-53	NTEEYIDG	12	9
4 ^{†*}	U1C	90-97	GMMPAPHM	11	8
5 [*]	U1-70K	337-344	PDGPDGPE	6	4
6 ^{†*}	Sm B	83-90	EGPPPKDT	2	3
7 ^{†*}	Sm G	1-8	MSKAHPPE	5	5
8 ^{†*}	Sm D3	20-27	CETNTGEV	4	1
9 ^{†*}	Sm F	77-84	EEEEEDGEM	9	12
10 ^{†*}	U1A	112-119	KPKSQETP	13	13
11 ^{†*}	Sm D2	14-21	EELQKREE	10	11
12 ^{†*}	Sm D1	22-29	GTQVHGTI	8	10
13 ^{†*}	U1A	178-185	GQIPPGAM	14	14
14 ^{†*}	U1-70K	325-332	APPDDGPP	15	15
15 ^{†*}	U1C	66-73	PFSAPPPA	7	7

The peptide designation, region and sequences as well as the U1 snRNP protein column displayed in this table (first four columns) were previously published by Somarelli et al. (2011). The observed IgM antigenicity (columns 5 and 6, from left to right) was ranked from 1 to 15 where “1” represents the peptide with the highest IgM antigenicity and “15” indicates the peptide with the lowest IgM antigenicity. Daggers (†) indicate IgM peptide antigenicities that significantly differ between SLE and MCTD patients while the asterisks (*) represent IgG reactivities for U1 snRNP peptides that significantly differ between ill (SLE and MCTD) and healthy individuals ($p \leq 0.05$).

Table 4: List of clinical symptoms observed in SLE and MCTD

Clinical symptom name	SLE		MCTD		<i>p</i> -value
	<i>Positive</i>	<i>Total</i>	<i>Positive</i>	<i>Total</i>	
Skin telangiectasia	5%	79	15%	40	0.0650
Skin nasal/oral ulcers	29%	79	23%	39	0.4880
Raynaud's syndrome	53%	80	85%	40	0.0001
History of hand swelling	41%	81	61%	41	0.0120
Observed hand swelling	19%	81	39%	41	0.0140
Acrosclerosis	4%	80	26%	38	0.0001
Skin digital pitting	8%	80	8%	39	0.9700
Proximal scleroderma	3%	79	0%	39	0.3160
Skin alopecia	58%	80	72%	39	0.1310
Malar rash	46%	78	13%	39	0.0001
Discoid rash	10%	78	0%	39	0.0380
Skin rash	33%	78	38%	39	0.5840
Skin photosensitivity	57%	76	58%	38	0.8940
Skin calcinosis	1%	79	5%	38	0.2000
Sicca, xerophthalmia and xerostomia	49%	81	66%	41	0.1330
Erosive inflammatory arthritis	43%	23	45%	20	0.9200
Lymphadenopathy	24%	79	20%	40	0.6180
Fever	22%	78	15%	40	0.7780
Proximal muscle weakness	29%	76	49%	39	0.0360
Myositis	6%	79	27%	33	0.0020
Myalgia	54%	80	48%	40	0.8970
Morning stiffness	53%	73	64%	36	0.3000
Swelling of three or more joints	42%	78	63%	40	0.0380
Joint tenderness	35%	79	69%	39	0.0010
Joint swelling	29%	78	63%	40	0.0010
Symmetric swelling	27%	78	64%	39	0.0001
Rheumatoid nodule	6%	77	10%	40	0.5000
Arthralgia	82%	79	84%	38	0.7950
Neuropathy	31%	77	28%	40	0.6810
Seizure	4%	80	0%	40	0.2150

Table 4, continuation

Clinical symptom name	SLE		MCTD		<i>p</i>-value
	<i>Positive</i>	<i>Total</i>	<i>Positive</i>	<i>Total</i>	
Psychosis	3%	79	0%	39	0.3160
Neuropsychiatric disorder	19%	78	51%	39	0.0001
Hypomotility in cine deglutition esophageal	41%	78	58%	40	0.0890
Pulmonary fibrosis	22%	45	23%	26	0.6330
Pleuritic pain or rubbing heard	37%	78	23%	39	0.1250
Pericarditis	30%	73	18%	39	0.1610
Avascular necrosis	3%	74	0%	33	0.3040
Clotting	1%	75	12%	34	0.0160
Myocardial infarction	0%	73	11%	35	0.0030
Stroke	6%	81	2%	41	0.3570

Figure legends

Figure 5: Contrasting IgM-specific anti-U1 snRNP peptide responses observed in SLE and MCTD patients. (A) and (B) represent the average percent optical density (OD%) values for the IgM class and IgG classes, respectively. Peptide number and OD% are on the x and y axes, respectively. The black, gray and white bars symbolize the average OD% of SLE, MCTD and healthy groups, respectively. (†) and (*) indicate significantly different OD% between SLE and MCTD as well as patients and healthy populations, respectively ($p \leq 0.05$). (C) and (D) correspond to the area under the curve (AUC), derived from ROC curves, for ill (SLE and MCTD) vs. healthy individuals as well as SLE vs. MCTD patients, respectively. Peptide number per Ig class and their AUC values are indicated on the x and y axes, respectively. (●) and (◆) symbolize significantly different AUC between patients and healthy individuals as well as SLE and MCTD patients, respectively ($p \leq 0.05$). The dotted lines in C and D indicate the cut-off value (0.5). Black bars in all graphs represent standard error of the mean.

Figure 6: Identification of a two-step ELISA system for classification of SLE, MCTD and healthy individuals. (A) The combination of IgG-mediated anti-P2/P4/P5/P10/P13 provides the best segregation between SLE and MCTD vs non-disease controls. The distribution of ill (SLE and MCTD) and healthy individuals and the predicted combined IgG-mediated reactivity are represented on the x and y axes, respectively. Gray and white circles indicate true positives (TP). (B) Combined IgM-anti-P4/P10 can classify SLE and MCTD patients. The distribution of SLE and MCTD patients' combined IgM anti-P4/P10 predicted values are on the x and y axes,

respectively. Black and gray diamonds indicate true positive (TP) samples for SLE and MCTD patients, respectively. The crosses represent false negatives (FN) or false positives (FP). Predicted values were obtained using binomial logistic regression (BLR) with a cut-off of 0.5 ($p \leq 0.05$).

Figure 7: Area under the curve analysis reveals the classification power of IgM anti-P4/P10. Receiver operating characteristic (ROC) curves were generated using peptide antigenicities or laboratory tests. The columns in the graph represent the area under the curve (AUC) in the y axis for each variable tested. The bars on top of each column indicate standard error of the mean. FANA titers, dsDNA, ↑DNA (elevated serum DNA) and positive results for RNP, Sm, SSA, SSB and SCL are clinical tests used during SLE and MCTD diagnosis. The “IgM anti-P4/P10” indicates the combined IgM anti-P4/P10 titer while “IgM anti-P4/P10 + ↑DNA” represents the combination of the IgM anti-P4/P10 ELISA and the elevated DNA assay. The “*” and “**” indicate significant differences in classifying SLE and MCTD with p values of ≤ 0.05 and ≤ 0.0001 , respectively.

Figure 5: Contrasting IgM-specific anti-U1 snRNP peptide responses observed in SLE and MCTD patients

Figure 6: Identification of a two-step ELISA system for classification of SLE, MCTD and healthy individuals.

Figure 7: Area under the curve analysis reveals the classification power of IgM anti-P4/P10

IV. CHAPTER 3

Developing novel classification rules customized to distinguishing
between SLE and MCTD patients

Introduction

Mixed Connective Tissue Disease (MCTD), also known as Sharp's syndrome, was first described in 1972 as an autoimmune disease characterized by high titers of antibodies to U1 small nuclear ribonucleoprotein particle (snRNP) (Sharp et al., 1972). Since then, the concept of MCTD as a distinct clinical entity has been challenged due to the number of common characteristics share mainly with Systemic Lupus Erythematosus (SLE) (Aringer et al., 2005; Nowicka-Sauer et al., 2012) and less often with other autoimmune syndromes such as Polymyositis (PM), Systemic Sclerosis (SS) and Rheumatoid Arthritis (RA) (Mukerji and Hardin, 1993; Egner, 2000; Haustein, 2005). Even though the Alarcón-Segovia criteria is currently the most accepted criteria for MCTD, the fact that four different MCTD classification criteria sets currently coexist further add to the complexity observed in the diagnosis of these patients (Sharp, 1987; Kasukawa et al, 1988; Kahn et al., 1991; Amigues et al., 1996). Furthermore, the initial description of MCTD as a benign autoimmune disease (Sharp et al., 1972) most likely has jeopardized its relevance as independent identity. Nevertheless, involvement of vital organs such as lung and heart in MCTD patients has long been reported by several independent investigations (Badui et al., 1984; Smolen and Steiner, 1998; Venables, 2006; Watanabe et al., 2012; Gunnarsson et al., 2013), however; these findings have not caught sufficient attention from the research community. Consequently, new efforts to improve current classification methods for MCTD that could facilitate its early diagnosis and potential treatment have been limited.

Despite the lack of support MCTD has had to be recognized as an independent syndrome rather than an SLE *like* disease, there are a number of serological and clinical

characteristics that support the independent nature of these autoimmune disorders. For example, typically MCTD patients express elevated autoantibodies targeting U1 small nuclear ribonucleoprotein (snRNP) specific proteins known as U1-70K, U1A and U1C while those with SLE show anti-Smith (Sm) and anti-dsDNA antibodies (Luyckx et al., 2005). Moreover, even though some SLE patients develop anti-U1 snRNP response; they are able to retain IgM reactivity against these antigens while those with MCTD class switch to IgG response instead (Vlachoyiannopoulos et al., 1996; Somarelli et al., 2011; Mesa et al., 2013). Likewise, severe renal and central nervous system (CNS) manifestations are observed in SLE patients (Zidan et al., 2013) while lung and heart malfunctions are frequent in MCTD subjects (Watanabe et al., 2012; Gunnarsson et al., 2013).

As a result of contrasting clinical characteristics and specific organ involvement reported in SLE and MCTD patients, the recognition of MCTD remains useful in clinical practices (Madisson, 2000; Venable 2006; Ortega-Hernandez et al., 2012). Currently there is no available method capable of distinguish between these systemic autoimmune syndromes given that the two classification criteria sets for SLE and four classification rules for MCTD were designed to recognize either of these diseases but not to segregate between them (Sharp, 1987; Kasukawa et al, 1988; Kahn et al., 1991; Amigues et al., 1996; Hochberg et al., 1997; Petri et al., 2012). The aim of this study is to develop novel classification criteria specifically designed to segregate between SLE and MCTD patients. To do this, 183 clinical and traditional laboratory test variables, 33 experimental factors from cytokine and IgM response to antigens as well as six currently used classification criteria sets were evaluated in the patient cohort (121 SLE and 41 MCTD).

Analysis of the results revealed eight new classification rules for SLE and MCTD distinction which are better classifiers than currently existent methods ($p \leq 0.0001$). Furthermore, these analyses corroborated that the combination of Malar rash and positive results for double stranded DNA (dsDNA), FANA titers, elevated creatine proteinase kinase (CPK) and IgM reactivity for Smd2_{amino-acids 14-21} (novel model 1) represents the best classification criteria for SLE and MCTD patients with an overall accuracy of 80% ($p \leq 0.0001$). Additionally, we provide two panels of blood biomarkers that correlate with specific organ involvement in either SLE or MCTD patients. Likewise, we highlight a number of laboratory tests currently used to assist in the diagnosing of SLE or MCTD but lacked classification power for any of these autoimmune syndromes. In summary, this research described for the first time classification rules customized for the distinction of SLE and MCTD. Despite required validation, these findings could assist in the early diagnosis of patients with either SLE or MCTD which in turn may potentially allow the development of a specific medical treatment to prevent damage of vital organs frequently affected in these patient populations.

Patients and Methods

Selection of SLE and MCTD patients

In this study, a total of 165 patients previously diagnosed with either SLE (121 samples from 98 individuals) or MCTD (41 samples from 27 individuals) were obtained following the Florida International University and University of Miami Institutional Reviewed Board (IRB) accepted protocols (IRB numbers: 040308-00 as well as 20030724 and 20040286, respectively). Individuals were diagnosed with SLE by

employing the American College of Rheumatology (ACR) criteria for lupus while Alarcón-Segovia criteria were utilized to diagnose those with MCTD (Amigues et al., 1996; Hochberg et al., 1997). All the individuals included represent well characterized patients that have been the subject of previous publications (Maldonado et al., 2006; Perkins et al., 2008; Somarelli et al., 2011; Mesa et al., 2013).

Collection of clinical data

A total of 183 clinical variables were obtained from the 165 patients, when available (Appendix 3). These variables include 74 clinical symptoms, 76 traditional laboratory tests and 33 experimental blood markers. All the clinical variables were recorded on the same date blood and/or urine samples were collected from the patient. The traditional laboratory tests refer to standardized commercial laboratory assays performed during the diagnosis of patients with SLE or MCTD. Experimental blood markers variables include 18 cytokines and IgM reactivity for 15 different peptides derived from U1 small nuclear ribonucleoprotein particle (snRNP). Detailed description of each of the clinical manifestations as well as normal range and cut off values for traditional and experimental laboratory tests are listed in Appendix 3.

Evaluation of classification criteria sets for SLE and MCTD patients

Two classification criteria sets available for diagnosing of SLE and four class criteria set for MCTD identification were compared regarding their capability to segregate patients with either autoimmune syndrome. The classification criteria sets for SLE are known as ACR and the recently described Systemic Lupus International

Collaborative Clinics (SLICC) (Hochberg et al., 1997; Petri et al., 2012) while those for MCTD are named Alarcon-Segovia, Sharp, Kasukawa and Kahn (Sharp, 1987; Kasukawa et al., 1988; Kahn et al., 1991; Amigues et al., 1996). All clinical and laboratory tests available from the 183 computerized medical records were employed to assess the number of SLE and MCTD subjects from the patient cohorts that could fulfill each of the six classification criteria sets. The accuracy, sensitivity and specificity of each of the classification criteria sets were estimated using the standard clinical methods.

Construction of reduced classification criteria models for SLE and MCTD segregation derived from established class criteria sets

The variables composing each of the two classification criteria sets for SLE and four class criteria sets for MCTD diagnosis were employed in six independent forward Binomial Logistic Regression (BLR) analyses using SPSS (version 18). These analyses reveal which combinations of variables improve the segregation of SLE and MCTD when compared to each of the individual variables included per classification criteria evaluated ($p \leq 0.05$). As result, six reduced models were obtained in which each of them correspond to a smaller version of each of the six classification criteria considered in the analysis. The accuracy, sensitivity and specificity for each of the reduced methods to classify SLE and MCTD patients were calculated. Receiving Operating Characteristics (ROC) curves were employed to rank the reduced models based in their power to segregate between SLE and MCTD patients.

Identifying reduced number of variables to develop new classification rule for SLE and MCTD discrimination

Since this study includes 183 clinical variables but only 165 patients, an initial selection of variables was required to maintain stability and robustness of any subsequent statistical analysis performed. Knowing that the objective was to develop a new classification rule customized for the discrimination of SLE and MCTD patients, all the variables showing significant difference between patients with these autoimmune disorders were selected ($p \leq 0.05$). Likewise, all clinical and laboratory features included in each of the reduced classification criteria models were also chosen given that forward BLR demonstrate their improved power to discriminate between SLE and MCTD patients ($p \leq 0.05$). The variables selected to build a new classification rule include 21 clinical manifestations, 12 traditional laboratory tests and four experimental blood markers (Appendix 4). Valvular heart disease, laboratory blood tests for calcium, albumin/globulin and creatine kinase as well as the interleukin 17A (IL-17A) experimental assay were initially selected but could not be included in the subsequent statistical analysis given the reduced number of patients with available values for these variables.

Developing novel classification criteria models for SLE and MCTD segregation

A total of 37 pre-selected variables which include clinical symptoms as well as traditional and experimental laboratory tests (Appendix 4) were considered to develop new classification models tailored to segregate between SLE and MCTD patients using forward BLR analysis in SPSS (version 18). All 37 variables were considered to

construct classification Model 1 while only clinical manifestations and standardized laboratory tests (33 variables) were evaluated to build classification Model 2. The probability of a patient to be classified as SLE or MCTD based on Model 1 or 2 are defined by the following equations, respectively:

$$\text{Model 1} = \log\left(\frac{\text{Pr}(SLE)}{\text{Pr}(MCTD)}\right) = -35 + 54(\text{Malar rash}) + 53(\text{dsDNA}) - 35(\text{FANA titer}) \\ - 55(\text{CPK elevated}) + 36(\text{IgM anti-SmD2 reactivity})$$

$$\text{Model 2} = \log\left(\frac{\text{Pr}(SLE)}{\text{Pr}(MCTD)}\right) = 19 + 4(\text{Malar rash}) - 22(\text{Synovitis}) + 3(\text{dsDNA}) - \\ 24(\text{Scl70}) + 44(\text{ANA})$$

where each of the models symbolize the log ratio of the combined variables in the SLE group divided by that of the MCTD cluster ($p \leq 0.05$). The probability of the sample to be SLE or MCTD in each model is calculated by plotting the values of each of the characteristics listed within parenthesis in each the independent equations. For each of the models, a probability >0.5 indicates the patients belong to the SLE group while a probability <0.5 classify the patient as MCTD.

Statistical analysis

Significant differences between SLE and MCTD patients for each of the 187 clinical variables included in this study were determined by Chi (X) square or independent sample T test in SPSS (version 18) when the value was nominal or numerical, respectively ($p \leq 0.05$). Correlations between laboratory tests and clinical

manifestations observed in either SLE or MCTD patients were determined by T-test/ANOVA with repeated measurements within patients for test with numerical values while a generalized linear mixed model was applied to assays with binary values ($p \leq 0.05$). The resulting p-values for each of the correlations were used to construct a heatmap for each autoimmune syndrome in R where significant correlations before and after Bonferroni correction as well as variables with no correlation are color coded in white, blue and red, respectively. Alopecia, swelling of neck lymph nodes, rheumatoid nodule, hemolytic anemia, avascular necrosis, pulmonary vascular lesions, renal clot, renal arterial stenosis, chorea and psychosis were not included during the correlation analysis because insufficient sample size and/or lack of sufficient variability in SLE and/or MCTD cohorts. Receiving Operating Characteristic (ROC) curves were performed in SPSS (version 18) to corroborate and compare classification power of each of the models resulting from forward BLR ($p \leq 0.05$).

Results

Contrasting clinical and serological features are exhibited by SLE and MCTD patients

Each of the 183 clinical variables included in this research (Appendix 3) were evaluated to assess significant differences between SLE and MCTD populations. We identified 35 variables that significantly differ between these autoimmune diseases including clinical symptoms related to skin, muscle, kidney and heart tissues as well as a 18 serological assays ($p \leq 0.05$) (Figure 8). Interestingly, the skin derived variables (Raynaud's Malar and Discoid rashes) tend to be higher in SLE than MCTD patients ($p \leq 0.05$). Likewise, the study of muscle features showed that SLE patients appear to have

greater inflammation of the joints and extremities while MCTD individuals exhibit prevalence of myositis and muscle weakness ($p \leq 0.05$). As expected, renal disease and renal hematuria are elevated in SLE but not MCTD patients ($p \leq 0.05$). Additionally, myocardial infarction is elevated in MCTD patients while valvular heart disease seems to be more frequent in SLE individuals ($p \leq 0.05$). Evaluation of the serological assays revealed that with the exception of creatine phosphate kinase and positive antibodies for topoisomerase (Scl-70 +), all the traditional laboratory tests were higher in the SLE population when compared to the MCTD group ($p \leq 0.05$). Similarly, all the experimental tests which include identification of interleukin 17A and IgM reactivity for U1A, SmD1 and SmD2 are elevated only in SLE but not MCTD individuals ($p \leq 0.05$). Consequently, this analysis showed that SLE and MCTD patients exhibit unique clinical manifestations and molecular markers ($p \leq 0.05$).

Molecular markers associations with unique tissue damage are specific to either SLE or MCTD patients

Each of the laboratory tests analyzed in this study was individually correlated with clinical symptoms presented in patients diagnosed with SLE or MCTD (Figures 2 and 3). The resulting p -value heatmap uncovered the SLE cohort seven clinical manifestations significantly associated with IL33 (oral ulcers, leukopenia and lymphopenia), IL12p40 (telangiectasia and joints deformity), BAFF (Thrombosis) and positive protein in urine (renal disease) ($p \leq 0.05$) (Table 5 and Figure 9). Different significant correlations were detected in the MCTD population where seven molecular markers were significantly associated with nasal ulcers (BAFF), oral ulcers (IL33), anemia (C3 and C4 level as well

as IgM and IgG rheumatoid factor ELISA titer) and pleuritis (IL17A) ($p \leq 0.05$) (Table 5 and Figure 10). It is noteworthy that IL33 correlates with oral ulcers equally in SLE and MCTD ($p \leq 0.05$). Interestingly, 44% (4/9) of the biomarkers that show significant correlations with specific clinical symptoms correspond to new experimental blood antigens that showed reactivity in SLE and/or MCTD patients. In this way, the p -value heatmaps resulting from correlation analysis suggest that correlations of molecular markers and clinical manifestations are unique to either SLE or MCTD patients ($p \leq 0.05$).

Classification criteria sets are not designed for segregating SLE and MCTD patients

Each of the six criteria sets for the classification of either SLE or MCTD was applied to the patient cohorts to evaluate their capacity to segregate between these autoimmune syndromes (Table 6). These analyses revealed that there is virtually no difference in the accuracy of the new (Systemic Lupus International Collaborating Clinics (SLICC)) and old (ACR) SLE classification criteria sets ($p \leq 0.05$) (Table 6). This is an important observation considering SLICC is composed by 25 additional variables that are not listed in the ACR class criteria (Hochberg et al., 1997; Petri et al., 2012). Specifically, SLICC and ACR showed elevated sensitivity (93.26% and 85.39%, respectively) but both have deficient specificity (13.33, and 24.44%, respectively) (Table 6). When evaluating all the MCTD class criteria sets, the Alarcón-Segovia showed the best accuracy (72.39%) at discriminating between SLE and MCTD even though Sharp, Kasukawa and Kahn class criteria sets also exhibited significant segregation between the two autoimmune disorders ($p \leq 0.05$) (Table 6). In contrast to SLICC and ACR

classification criteria, the Alarcón-Segovia exhibits moderate sensitivity (64.44%) and specificity (76.40%) in distinguishing between MCTD and SLE patients ($p \leq 0.05$) (Table 6). Taken all these together, the data showed that the six established methods for the classification of SLE (ACR and SLICC) and MCTD (Alarcón-Segovia, Sharp, Kasukawa and Kahn) lack power to segregate patients diagnosed with either these syndromes.

Regression analysis of existent classification criteria sets uncover essential factors required for SLE and MCTD segregation

Six independent forward BLR analyses with variables corresponding to those listed in each of the six classification criteria were performed to construct six reduced models. Evaluation of the reduced models exposed 6 combinations from 18 essential features with significant power to differentiate between SLE and MCTD patients (Table 7). Comparison of all six reduced models reveals that the reduced Kasukawa (rKasukawa) shows the highest accuracy (85.5%) at segregating between these autoimmune syndromes which power is derived by the combination of Raynaud's phenomenon, Malar rash, adenopathies, sclerodactyly and muscle weakness ($p \leq 0.05$) (Table 7). Specifically, the rKasukawa not only showed superior sensitivity (75%) and specificity (90.5%) when compared to the other five reduced models but seems a better classifier than the Alarcón-Segovia classification criteria to discriminate between SLE and MCTD patients (Table 7). Indeed, all the reduced models are better classifiers for SLE and MCTD patients when compared to their corresponding *complete* class criteria (Tables 2 and 3). Consequently, the forward BLR revealed four groups composed by combination of 18 core variables with the capacity to segregate between SLE and MCTD ($p \leq 0.05$).

ROC curves confirmed the classification power of reduced class criteria models

A total of six individual ROC curves analyses were performed to confirm the segregation power of the variable per criteria set when compared to the corresponding *complete* classification criteria (Table 6) as well as proposed reduced models (Table 7). As expected, the ROC analysis confirmed that all reduced models are a better classifier for these autoimmune syndromes than any of the *complete* class criteria or any variable included in them ($p \leq 0.0001$) (Figure 11). Likewise, the analysis revealed that two laboratory tests previously identified by BLR (positive dsDNA and RNP in Table 7) have individual power to distinguish between SLE and MCTD patients ($p \leq 0.05$) (Figure 11). Also, eight of the clinical symptoms predicted by BLR (synovitis, Malar rash, non-erosive arthritis, acrosclerosis, Raynaud's, esophageal hypomotility, sclerodactility and muscle weakness in Table 7) showed to have individual power to segregate between SLE and MCTD ($p \leq 0.05$) (Figure 11). In addition, the ROC analysis uncovered that swollen hands and negative laboratory results for Sm (Sm-) each have independent capability to separate SLE from MCTD patients ($p \leq 0.05$) (Figure 11). Therefore, the ROC analysis exposed ability of swollen hands and Sm- results in segregating between these autoimmune disorders as well as confirmed the power of 10 out of the 18 variables identified by BLR in differentiating between SLE and MCTD patients ($p \leq 0.05$).

Construction of novel class criteria sets to assist diagnosing of SLE and MCTD

A total of 37 pre-selected variables (Appendix 4) were employed in forward BLR analysis to develop new predicted models tailored specifically for SLE and MCTD classification. When considering experimental and traditional laboratory assays as well as

clinical symptoms, the analysis revealed that the combination of Malar rash, positive results for dsDNA and FANA titers, elevated creatine phosphate kinase (CPK) and IgM anti-SmD2_{amino-acids 14-21} reactivity (referred to as Model 1) exhibits an overall accuracy of 80% in discriminating between SLE and MCTD patients with sensitivity and specificity of 92% and 74%, respectively ($p \leq 0.0001$) (Table 8). A separate forward BLR analysis was performed utilizing only traditional laboratory tests and clinical manifestations from patients with either SLE or MCTD. The data confirmed that the combination of Malar rash, synovitis as positive results for dsDNA, topoisomerase (Scl70) and anti-nuclear assay (ANA) (named Model 2) showed a 74% accuracy at discerning between these two autoimmune syndromes with 83% sensitivity and 69% specificity ($p \leq 0.0001$) (Table 8). In summary, the BLR analysis uncovered two novel class criteria sets (Models 1 and 2) which were custom built for discriminating between SLE and MCTD patients ($p \leq 0.0001$).

Model 1 represents the best classification rule to discern between SLE and MCTD patients

Novel proposed classification Models 1 and 2 (Table 8) were included in ROC curves analysis along with each of the classification criteria for SLE and MCTD (Table 6) and their corresponding reduced models (Table 7) to compare their classification power for SLE and MCTD patients. The analysis confirmed that Model 1 exhibits the highest capacity to discern between SLE and MCTD with AUC = 0.930 ($p \leq 0.0001$) (Figure 12). Likewise, rKasukawa model, rACR model, rSharp model, Model 2, rSLICC model, rAldarcon-Segovia model and Sharp classification criteria occupy the second to

eight place in having power to classify SLE and MCTD patients ($p \leq 0.05$) (Figure 12). The rKahn model and the rest of the classification criteria sets (Kasukawa, Alarcon-Segovia, Kahn, ACR and SLICC) were unable to segregate patients diagnosed with any of these autoimmune syndromes (Figure 12). Therefore, ROC curves analysis confirmed that the combination of variables included in Model 1 are the best classification rule for SLE and MCTD patients when compared to Model 2, all six classification criteria sets available for these autoimmune disorders as well as their corresponding reduced models ($p \leq 0.0001$).

Discussion

Since its initial description by Sharp et al. (1972), the recognition of MCTD as an unique disease has been constantly challenged, mainly due to the number of overlapping characteristics shared by patients diagnosed with SLE (Aringer et al., 2005; Nowicka-Sauer et al., 2012). Currently, in cases where the concept of MCTD is acknowledged, it is often portrayed as a subset of SLE patients with good prognosis despite exhibiting features listed in SLE and MCTD classification criteria sets and prevalence of lung and heart problems (Badui et al., 1984; Smolen and Steiner, 1998; Venables, 2006; Watanabe et al., 2012; Gunnarsson et al., 2013; Bertsias et al., 2013). The reported contrasting organs involvement in patients with diagnosis of MCTD (lung and heart) when compared to those with SLE (kidney and CNS) provides evidence of the clinical relevance of the MCTD concept to prevent and/or treat organ malfunction in these autoimmune syndromes, regardless if MCTD is recognized as a separate illness or a SLE subtype. Given that available classification criteria sets were developed to identify either SLE or

MCTD subjects but not to segregate between them, it seems imperative to design classification rules specifically tailored to discriminate SLE and MCTD patients since early class group identification could influence the type of medical plan developed to impede or reduce organ damage, thus potentially improving the patient's prognosis. This investigation provides, for the first time, eight different classification rules strategically derived for the segregation of SLE and MCTD patients. Analysis of the resulting data confirmed that all eight proposed models exhibit a higher power to correctly identify SLE or MCTD patients than any other available class criteria set ($p \leq 0.0001$). Despite a required validation step, the proposed models highlight clinical and molecular features with power to segregate SLE and MCTD subjects while providing additional evidence to support the independent etiology of these autoimmune syndromes.

In congruency with previous reports (Vlachoyiannopoulos et al., 1996; Luyckx et al., 2005; Somarelli et al., 2011; Watanabe et al., 2012; Gunnarsson et al., 2013; Mesa et al., 2013; Zidan et al., 2013), the analysis of 187 medical records documented for SLE and MCTD patients revealed that 35 clinical manifestations and molecular features are significantly different in the two disease states (Figure 8). The prevalence of skin rashes in SLE while myositis and muscle weakness was presented in MCTD was expected since these are typical clinical manifestation associated with each of these autoimmune illnesses (Uthman et al., 1996; Belibou et al., 2012; Szodoray et al., 2012). Like others (Watanabe et al., 2012; Gunnarsson et al., 2013; Zidan et al., 2013), the data showed that specific organ malfunction was involved with each of these syndromes where renal disease was elevated in SLE cohort while myocardial infarction was more frequently in the MCTD population ($p \leq 0.002$). Additionally, the correlation analyses support the idea

that SLE and MCTD are indeed separate disorders given that unique correlations of molecular markers and clinical symptoms are observed in either SLE or MCTD cohorts (Figures 2 and 3). An interesting association revealed was between IL17A and pleuritis observed only in MCTD but not SLE subjects (Table 5). In addition to representing a novel potential blood biomarker, the association of IL17A and pleuritis might indicate pleural inflammation related to bacterial infections as has been recently reported (Monguillott Dalmarco et al., 2011; Kollintza et al., 2013). It is noteworthy to mention that the frequency of lung disease in the SLE and MCTD cohorts does not differ (44% and 48% respectively). Given that some studies have reported the predominance of lung disease in MCTD patients (Sasaki et al., 2011; Gunnarsson et al., 2012) while others have uncovered that lung malfunction in these patients is lower than expected (Gunnarsson et al., 2013), these results suggest that additional investigations encompassing bigger MCTD samples and inclusion of other autoimmune syndromes are required to assess if lung disease should be considered as a target organ during the diagnosis and treatment of MCTD. Consequently, the data provide grounds to suggest that new classification rules customized for SLE and MCTD segregation should be developed to prevent and/or reduce organ involvements in these patients.

The evaluation of ACR, SLICC, Alarcón-Segovia, Sharp, Kasukawa, and Kahn criteria sets revealed the average capability of segregating SLE and MCTD patients (Table 6). Specifically, the misclassification rate of patients using any of these methods ranges from 36% to 87% for MCTD subjects (16/45 and 39/45 MCTD incorrectly grouped by Alarcón-Segovia and SLICC, respectively) while varies from 7% to 56% for SLE (6/89 and 48/85 SLE mistakenly clustered by SLICC and Kasukawa, respectively).

This deficiency of classification power was expected given that the inclusion of MCTD patients was not recognized during the development of SLE classification criteria sets (Tan et al., 1982; Hochberg et al., 1997; Petri et al., 2012). By contrast, patients diagnosed with SLE and other autoimmune syndromes were included during the production of each of the four classification criteria sets for MCTD (Amigues et al., 1996). Nevertheless, these studies did not include well characterized MCTD cases and rather choose the presence of positive anti-U1 snRNP antibodies as patient inclusion criteria despite knowing these antigens elicit a response in SLE and MCTD subjects (Amigues et al., 1996; Sato et al., 2010; Nagai et al., 2012; Mesa et al., 2013). Consequently, these findings provide evidence to suggest that currently available SLE and MCTD classification criteria sets do not exhibit sufficient power to correctly identify SLE or MCTD patients (Table 6) (Figure 11).

The regression analysis identified eight different novel classification rules (Tables 3 and 4) which exhibit significantly higher capability to segregate SLE and MCTD patients than any other classification criteria sets available ($p \leq 0.05$) (Figure 12). Particularly, Model 1 composed by the presence of Malar rash, elevated CPK and positive results for dsDNA, FANA and IgM anti-SmD (Table 8), represents the best classifier to identify SLE and MCTD patients with a significant AUC of 0.930 ($p \leq 0.0001$) (Figure 12). The segregation power of the combination of variables in Model 1 was predicted given that, with the exception of elevated CPK; all the other clinical characteristics and lab tests have been repetitively reported elevated in SLE patients but reduced in MCTD subjects (Vlachoyiannopoulos et al., 1996; Luyckx et al., 2005; Somarelli et al., 2011; Mesa et al., 2013). The misclassification rate of proposed Model 1

attains 8% for MCTD patients (2/24 incorrectly clustered) and 26% for SLE subjects (11/42 falsely grouped). In this way, the overall classification power for SLE and MCTD shown by Model 1 is significantly improved when contrasted with existent validated classification criteria sets ($p \leq 0.0001$) (Figure 12). Additionally, the results revealed that rKasukawa, rACR, rSharp, Model 2, rSLICC and rAlarcón-Segovia follow Model 1 in their power to segregate between SLE and MCTD patients ($p \leq 0.05$) (Figure 12). The ROC curves confirmed that Sharp classification criteria exhibit significant ability to discern between these autoimmune syndromes however the average AUC (0.684) highlights the average power of this criteria set ($p \leq 0.05$) (Figure 12).

Taking all of this together, this study shows evidence to support the relevance of recognizing the MCTD concept while providing, for the first time, eight different classification rules tailored for the specific classification of SLE and MCTD patients. Given that these results and others have identified specific organ involvement in either SLE or MCTD patients (Figure 8) (Vlachoyiannopoulos et al., 1996; Luyckx et al., 2005; Watanabe et al., 2012; Gunnarsson et al., 2013; Mesa et al., 2013; Zidan et al., 2013), it highlights the clinical value of MCTD diagnosis given that the type of medical regime delivered to a patient will rely on the potential development of specific organ(s) malfunction. Aware of the lack of methods designed to segregate between these autoimmune disorders (Table 6 and Figure 11), we described eight different novel classification rules (Tables 3 and 4) from which Model 1 (derived by the combination of Malar rash, elevated CPK and positive results for dsDNA, FANA and IgM anti-SmD) represents the best classifier from those evaluated with a power of 93% to distinguish between SLE and MCTD patients (Figure 12). Noteworthy the limitations and the need

for a validation step of the current findings, we believe that the time has come to recognize the clinical relevance of the MCTD concept to prevent and/or treat organ malfunction. Consequently, the proposed classification rules presented in this research pioneers the efforts to attain the development of new consensus criteria custom made for segregating between SLE and MCTD patients.

Table 5: List of molecular markers associated with clinical manifestations in SLE and MCTD patients

Tissue affected	Molecular markers	SLE	MCTD
Skin	<i>BAFF</i>	∅	Nasal ulcers
	<i>IL33</i>	Oral ulcers	Oral ulcers
Joints	<i>IL12p40</i>	Telangiectasia	∅
		Deformity	∅
Blood	C3 level	∅	Anemia
	C4 level	∅	
	IgM RF ELISA titer	∅	
	IgG RF ELISA titer	∅	
	<i>IL33</i>	Leukopenia	∅
		Lymphonia	∅
	<i>BAFF</i>	Thrombosis	∅
Heart	<i>IL17A</i>	-	Pleuritis
Kidney	Urine protein +	Renal disease	∅

Experimental molecular markers are in *italics* while traditional lab tests are not. All the markers listed are associated with each of the clinical symptoms with a p -value ≤ 0.05 and Bonferroni correction. The empty symbol ($\emptyset$) represents no significant correlation determined with $p \leq 0.05$ after Bonferroni correction. Interlukins are denoted as follow: IL33, IL12p40, BAFF and IL17A). “RF” stands for rheumatoid factor while urine protein + corresponds to protein detection in urine samples.

Table 6: Evaluating SLE and MCTD classification criteria sets in patient cohort included in the present study

	Classification criteria sets	SLE		MCTD		<i>p</i> -value
		Correctly classified	Incorrectly classified	Correctly classified	Incorrectly classified	
S L E	<i>SLICC</i>	83	6	6	39	0.207
	<i>ACR</i>	76	13	11	34	0.161
M C T D	<i>Alarcón-Segovia</i>	68	21	29	16	<0.0001
	<i>Sharp</i>	48	41	37	8	<0.0001
	<i>Kasukawa</i>	37	48	42	3	<0.0001
	<i>Kahn</i>	82	7	11	34	0.008

SLICC and ACR stands for Systemic Lupus International Collaborating Clinics and American College of Rheumatology, respectively. The analysis were performed in SPSS (version 18) and included 89 SLE patients and 45 MCTD individuals.

Table 7: Description of reduced models for each SLE and MCTD classification criteria

	Reduced models for each classification criteria	Accuracy	p-value	Features included in the reduced models
SLE	<i>rSLICC</i>	79.6%	<0.0001	Discoid rash, joint synovitis, leukopenia, ANA+, dsDNA+, Sm+ and phospholipid +
	<i>rACR</i>	74.4%	<0.0001	dsDNA+, hemolytic anemia, Malar rash and Non-erosive arthritis
	<i>rAlarcón-Segovia</i>	74.2%	<0.0001	Synovitis and acrosclerosis
MCTD	<i>rSharp</i>	78.4%	<0.0001	Severe myositis, Raynaud's or esophageal hypomotility, RNP+, Sm+
	<i>rKasukawa</i>	85.5%	<0.0001	Raynaud's, adenopathies, Malar rash, sclerodactyly and muscle weakness
	<i>rKahn</i>	69.7%	<0.0001	Raynaud's and synovitis

SLICC and ACR stands for Systemic Lupus International Collaborating Clinics and American College of Rheumatology, respectively. Anti Nuclear Antibody positive (ANA+) as well as positive antibody detection of double stranded DNA (dsDNA+), Smith protein (Sm+) phospholipid (phospholipid +) and Ribo-Nucleo-protein (RNP+) are indicated. The analysis were performed using the binomial logistic regression (BLR) function in SPSS (version 18) and included 89 SLE patients and 45 MCTD individuals.

Table 8: Description of novel models for segregation of SLE and MCTD patients

Proposed models	Features included in each model	Cut-off	Accuracy	<i>p</i>-value
Model 1	Malar rash, dsDNA+, FANA titers, elevated CPK and <i>IgM reactivity for SmD2_{amino-acids 14-21}</i> .	0.42	80.30%	<0.0001
Model 2	Malar rash, synovitis, dsDNA+, Scl70+, ANA+	0.28	73.64%	<0.0001

The models were obtained using binomial logistic regression (BLR) function in SPSS (version 18) when clinical variables as well as traditional and experimental laboratory tests from SLE and MCTD patients were considered (**Appendix 4**). Model 1 was built using experimental and traditional variables from 42 SLE and 24 MCTD patients while only traditional variables derived from 75 SLE and 35 MCTD patients were considered for Model 2. Values under the cut off points will be classified as SLE while those above it will be identified as MCTD. Positive antibody detection of double stranded DNA (dsDNA+), topoisomerase (Scl70+) and Anti Nuclear Antibody positive (ANA+) are indicated. The experimental variable is denoted in italics.

Figure legends

Figure 8: SLE and MCTD differ in the prevalence of clinical characteristics and molecular factors. In each graph, the inner circle represents the MCTD cohort while the outside circle consists of the SLE population. Positive and negative values for each of the variables are represented by green and red, respectively. History of proximal muscle weakness, observed proximal muscle weakness, observed joints swelling, symmetric joints swelling, low extremity swelling are denoted by “H prox muscle weakness”, “O prox muscle weakness”, “O joints swelling”, “S joints swelling” and “L extremity swelling”, respectively. Positive laboratory tests for ribonucleoprotein (RNP), Smith proteins (Sm), double stranded DNA (dsDNA), Fluorescent Antinuclear Antibodies (FANA), anti-La antibodies (SSP) and topoisomerase (Scl) are indicated with “+”. Experimental antigens as opposed to traditional laboratory tests are in italics.

Figure 9: Proposed biomarker panel for clinical manifestations observed in SLE patients. In the plot, the clinical symptoms and laboratory tests are on the “x” and “y” axis, respectively. The white, blue and red boxes indicate significant correlations, significant correlations after Bonferroni corrections and no correlations, respectively ($p \leq 0.05$). Hand swelling, proximal scleroderma, any heart clot, valvular heart diseases, pulmonary hypertension, pulmonary fibrosis, gastric reflux, lymph nodes swelling, morning stiffness, myocard infarction and interleukin receptor BAFFR were initially considered in the analysis but not included due to the reduced sample size for each of these variables.

Figure 10: Specific blood markers correlate with clinical symptoms in MCTD patients. Laboratory tests performed in MCTD subjects are displayed in the “y” axis while clinical manifestations exhibit in this patient population are on the “x” axis. The white, blue and red boxes indicate significant correlations, significant correlations after Bonferroni corrections and no correlations, respectively ($p \leq 0.05$). Renal proteinuria, hematuria and cell cast, stroke, venous heart clot, lung disease, synovitis, symmetric swelling of the joints, observed proximal muscle weakness and calcinosis were initially considered for the correlations but not included in the final analyses given the reduced sample size available in the MCTD cohort for these variables.

Figure 11: Reduced models for each SLE and MCTD classification criteria exhibit better power in discriminating between SLE and MCTD patients. Each of the reduced models were obtained by applying binomial logistic regression (BLR) in SPSS (version 18) when all the variables per classification criteria were considered in SLE ($n = 89$) and MCTD ($n = 45$) patients. The Systemic Lupus International Collaborating Clinics (SLICC) and American College of Rheumatology (ACR) classification criteria sets for SLE diagnosis are denoted with **A** and **B**, respectively. The Alarcón-Segovia (**C**), Sharp (**D**), Kasukawa (**E**) and Kahn (**F**) represent the classification criteria sets for diagnosing MCTD patients. In each plot, the area under the curve (AUC) from Receiving Operating Characteristics (ROC) curves are on the y axis. Each of the columns represent characteristics and the reduced model included per classification criteria. The lines on top of each column are standard error. Significant difference between SLE and MCTD

patients with p -values ≤ 0.05 , ≤ 0.005 and ≤ 0.0001 are denoted with “*”, “**” and “***”, respectively.

Figure 12: Proposed Model 1 exhibits the highest discrimination power in identifying SLE and MCTD patients. The x axis includes the classification criteria for SLE and MCTD, their correspondent reduced models as well as the new proposed models (Model 1 and Model 2) for segregation of patients with these autoimmune syndromes. The y axis represents the area under the curve (AUC) resulting from the Receiving Operating Characteristics (ROC) curves analysis. Variables with significant power to differentiate between SLE and MCTD with p -values of 0.05, 0.005 and ≤ 0.0001 are indicated with “*”, “**” and “***”, respectively.

Figure 8: SLE and MCTD differ in the prevalence of clinical characteristics and molecular factors

Figure 9: Proposed biomarker panel for clinical manifestations observed in SLE patients

Figure 10: Specific blood markers correlate with clinical symptoms in MCTD patients

Figure 11: Reduced models for each SLE and MCTD classification criteria exhibit better power in discriminating between SLE and MCTD patients

Figure 12: Proposed Model 1 exhibits the highest discrimination power in identifying SLE and MCTD patients

V. CONCLUSIONS

The recognition of MCTD as a separate disorder from SLE has remained debatable since its initial description in 1972 (Sharp et al., 1972; Aringer et al., 2005; Nowicka-Sauer et al., 2012). Nevertheless, the clinical relevance of the MCTD concept to identify individuals with different organ malfunction from patients that fulfill the SLE classification criteria has been documented (Badui et al., 1984; Smolen and Steiner, 1998; Venables, 2006; Watanabe et al., 2012; Gunnarsson et al., 2013; Bertias et al., 2013). This investigation provides clinical and molecular evidence to support the independent etiology of SLE and MCTD. The experimental approach suggests that models resulting from the IgM anti-U1 snRNP titers could be considered as a potential blood biomarkers capable of differentiating SLE from MCTD patients as well as molecular indicators for lung and kidney disease in patients suffering from any of these disorders ($p \leq 0.005$). Likewise, derivation of the data analysis resulted in the description of novel classification rules for SLE and MCTD with significant improved classification power when compared to available methods ($p \leq 0.005$). Even knowing that these findings must be validated and recognizing the limitations of the approached utilized in this research, the results strongly suggest that the described biomarkers and classification criteria sets might have clinical relevance during the accurate identification of MCTD therefore potentially improving patients' prognosis.

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APPENDICES

APPENDIX 1

Protocol for generating a 3D protein structure and applying a heat scale to residues of interest

This protocol assumes that the amino acid sequence of a protein of interest is available.

Generating a heat map scale

The goal for generating a heat map scale is to pick one group as a baseline and then compare how far above or below that baseline some other group performs. The resulting deviation should range from 0 to 1. In general, one picks the control group (i.e. healthy subjects) as the baseline. For a four color heat scale of blue to green to yellow to red, with blue being least intense and red being most intense, the following procedure can be used.

1. For each value measured, calculate deviation by the test group from the control group using the formula:

$$\text{Deviation} = 0.5 + \text{Test} - \text{Control}$$

2. Calculate the appropriate RGB values for the deviation.
 - a. In Excel, place the value for Deviation in cell B2.
 - b. To calculate the Red RGB value, place the following code in cell C2:
 - `=IF(B2>0.67,255,IF(B2>0.33,255*(3*B2-FLOOR(3*B2,1)),0))`
 - c. To calculate the Green RGB value, place the following code in cell D2:
 - `=IF(B2>0.67,-255*(3*B2-FLOOR(3*B2,1))+255,IF(B2>0.33,255*(3*B2-FLOOR(3*B2,1))))`

- d. To calculate the Blue RGB value, place the following code in cell E2:
- =IF(B2>0.33,0,-255*(B2*3-FLOOR(B2*3,1))+255)
3. Use the resulting RGB values as custom colors in some other application. An example is Case 1 step 5 below.

Generating 3D structures

In terms of an experimentally determined 3D structure of the protein, there are three cases that are addressed: (1) no such 3D structure is available; (2) a partial structure is available; or (3) a complete structure is available.

Case 1

1. Submit the amino acid sequence to the I-TASSER protein structure prediction server to generate 3D structures of your protein. The server is located at:
<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>.
2. Download and install PyMOL to visualize the predicted structures. It can be obtained at: <http://www.pymol.org/>.
3. Open the I-TASSER model of interest (usually the one with the highest C-score) in PyMOL.
4. Define your residues of interest using the select command.
 - Example, to define residues 10 through 20 by the name p1, at the PyMOL> prompt type:
 - select p1, resi 10-20

Define your custom colors from the heat map scale using the set_color command.

- Example, to define a custom color with RGB values of R=127, G=255, B=212, type:
- `set_color p1_color = [127, 255, 212]`

Assign your custom colors to your residues of interest.

- For the example residues and color above, type:
- `color p1_color, p1`

Repeat steps 4 through 6 for each set of residues of interest.

Choose how you would like your structure represented. From the PyMOL viewer next to “all”, click S for Show, then “as” and choose either lines, sticks, ribbon, or cartoon.

To change the background color to white, type:

- `bg_color white`

Orient the structure as desired, or click the Orient button in the PyMOL console.

If planning to create an animation, be sure to leave sufficient white space around the structure.

To save a high quality image of the structure, type:

- `ray`
- Click on File->Save Image As->PNG... and select your filename

For a rotated image, type:

- `rotate y, 180`
- Repeat step 11

Open your image in a graphics package (for example, Photoshop) to add labels.

To create an animated GIF of your structure, first install ImageMagick. It can be obtained at: <http://www.imagemagick.org/script/index.php>.

Create a series of still images to serve as frames for your animation. From the PyMOL prompt, type:

- `mset 1 x60`
- `util.mrock 1,60,180`
- `set ray_trace_frames=1`
- `mpng frame`

Copy your frames to a temporary folder and open a command prompt. Type:

- `convert -delay 1 -loop 0 frame*.png animated.gif`

Case 2

1. Follow steps 1 and 2 as in Case 1.
2. Compare your predicted structures to your experimentally determined structure in PyMOL by superposition.
 - a. Open your experimentally determined structure and your I-TASSER models in PyMOL.
 - b. For each comparison, where model is the name of your predicted structure and experimental is the name of your experimentally determined structure, type:
 - c. `super model, experimental`
 - d. Find the best ratio of retained atoms to RMS value from the final line of output.

Using only the best superposition, follow steps 4 through 15 as in Case 1.

Case 3

1. Follow steps 2 and steps 4 through 15 as in Case 1.

APPENDIX 2

**ROC curves for ill (SLE/MCTD) vs healthy
using IgM and IgG reactivity**

Variables	IgM reactivity			IgG reactivity		
	AUC	SE	<i>p</i> value	AUC	SE	<i>p</i> value
P1	.562	.055	.286	.863	.036	<.0001
P2	.570	.057	.227	.613	.050	.053
P3	.567	.061	.251	.796	.043	<.0001
P4	.522	.059	.710	.925	.024	<.0001
P5	.575	.058	.197	.933	.029	<.0001
P6	.579	.058	.173	.842	.037	<.0001
P7	.558	.059	.321	.787	.044	<.0001
P8	.598	.060	.091	.857	.039	<.0001
P9	.508	.060	.890	.653	.047	.008
P10	.547	.059	.424	.867	.035	<.0001
P11	.509	.058	.874	.817	.041	<.0001
P12	.517	.056	.773	.850	.040	<.0001
P13	.560	.056	.305	.804	.046	<.0001
P14	.613	.053	.052	.895	.033	<.0001
P15	.532	.060	.583	.849	.043	<.0001

APPENDIX 2, continuation

ROC curves for SLE vs MCTD using IgM and IgG

Variables	IgM reactivity			IgG reactivity		
	AUC	SE	<i>p</i> value	AUC	SE	<i>p</i> value
P1	.631	.053	.018	.587	.054	.119
P2	.547	.053	.402	.534	.056	.540
P3	.551	.054	.358	.536	.054	.517
P4	.537	.054	.500	.505	.056	.922
P5	.570	.053	.210	.549	.054	.374
P6	.581	.053	.147	.510	.055	.852
P7	.561	.054	.269	.516	.054	.780
P8	.548	.054	.387	.527	.057	.624
P9	.577	.053	.164	.538	.053	.498
P10	.585	.052	.124	.528	.056	.745
P11	.574	.052	.185	.502	.056	.978
P12	.584	.052	.128	.536	.056	.512
P13	.610	.051	.048	.569	.055	.214
P14	.600	.052	.072	.556	.055	.316
P15	.605	.053	.060	.545	.056	.422

APPENDIX 2, continuation

ROC curves for SLE vs MCTD using IgM reactivity
and clinical tests

Variables	AUC	SE	<i>p</i> value
IgM P1 OD	.664	.061	.016
IgM P13 OD	.618	.063	.084
IgM P4 OD + IgM P10 OD	.731	.057	.001
FANA titers	.553	.066	.437
RNP positive	.589	.063	.189
Sm positive	.638	.065	.043
SSA positive	.554	.068	.428
SSB positive	.561	.066	.374
SCL positive	.565	.070	.338
DNA elevated	.665	.065	.023
ds DNA titers	.605	.062	.123
IgM P4/P10 OD + DNA elevated	.798	.052	<.0001

APPENDIX 3

	Variable	Definition
Skin	Telangiectasia	Skin telangiectasia observed
	Nasal ulcers	Skin nasal ulcers observed
	Oral ulcers	Skin oral ulcers observed
	Raynaud	Raynaud: a pattern of color changes in the fingers as follows: pale/white followed by blue then red when the hands are warmed; color changes are usually preceded by exposure to cold or emotional upset
	Skin hand swelling history	Hand swelling by history
	Skin hand swelling observed	Hand swelling observed
	Sclerodactyly	Skin sclerodactyl / acrosclerosis
	Pitting	Skin digital pitting
	Alopecia	Skin alopecia-patchy or generalized
	Malar rash	Skin malar rash- fixed erythema, flat or raised, over the malar eminences, tending to spare the labial folds
	Discoid rash	Skin discoid rash- erythematous raised patches with adherent keratotic scaling and follicular plugging. Atrophic scarring seen in older lesions
	Skin rash	Skin rash other by history or exam
	Photosensitivity	Skin photosensitivity- skin rash as a result of unusual reaction to sunlight by history or exam
	Calcinosis	Skin calcinosis observed
	Proximal scleroderma	Skin proximal scleroderma / extend at or above wrist
	Sicca	Skin sicca- xerophthalmia and xerostomia
Muscle	Myositis	Myositis
	Myalgia	Myalgia-subjective muscle aching involving proximal muscles
	Arthralgia	Arthralgia
	Observed proximal weakness	Any objective weakness in the proximal muscles
	History proximal weakness	A subjective history of any proximal muscle weakness
	Muscular HAND SWELLING SPECIFIC	At least one area swollen in a wrist, mcp, or pip joint
	Muscular HAND SWELLING GENERAL	Muscular hand swelling
	Muscular HAND SWELLING HISTORY	Swelling of the hand by history
	Muscular HAND SWELLING OBSERVED	Swelling of the hands observed

Joints	Morning stiff	Morning stiffness lasting at least 1 hour before maximal improvement
	Join tender	Joint tenderness observed
	Synovitys	Synovitys
	Join swelling history	History of joint swelling
	Join swelling obs	Joint swelling observed
	Swelling joints > 3	Swelling of 3 or more joints (pip, mcp, wrist, elbow, knee, ankle and mtp)
	Symmetric swelling	Symetric swelling observed
	Joints pain	Joints pain
	Joints deformity	Joints deformity
Lymph nodes	Any lymphadenopathy	Any lymphadenopathy
	Lymfnodes swelling	Lymfnodes swelling
	Neck lymphnode Swelling	Neck lymphnode swellin
	Axila lymphnode Swelling	Axila lymphnode swellin
	Groin lymphnode Swelling	Groin lymphnode swellin
	Rheum nodule	Observed rheumatoid nodule- subcutaneous nodules over bony prominences or extensor surfaces, or in juxtaarticular regions
Gastro	Gastric reflux	Gastric reflux as diagnosed by patient symptoms of heartburn, acid reflux or chest pain and radiographic studies and/or endoscopy
Blood	Anemia	Anemia
	Anemia hemolyti	Hemolytic anemia
	Leukopenia	Leukopenia
	Lymphopenia	Lymphopenia, total lymphocyte count < 1500/mm
	Thrombopenia	Thrombocytopenia
	Thrombosis	Thrombosis
Heart	Pleuritis	Convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion
	Pericarditis	Pericarditis: documented by ecg or rub or evidence of pericardial effusion
	PLEURITIS and/or PERICARDITIS	Pleuritis and/or pericarditis: either or both of two above described
	Venous clot	Venous clot
	Arterial clot	Arterial clot
	Any clot	Any clot
	Myocard infarction	Myocardio infarction
	Valvular disease	Valvular heart disease
	Valvular disease site	Aortic, mitral, tricuspid, pulmonic
	Numb valvuar	Based on the valvular disease type

	Stroke	Stroke
	Avascular necrosis	Avascular necrosis
Lung	Pulmonary hyperten	Pulmonary hypertension ≥ 40 mmhg rvsp
	Pulmonaru vasc lesion	Pulmonary vascular disease by biopsy or autopsy
	Pulmonary fibrosis	Pulmonary fibrosis based on chest xray or ct scan
	Evidence of lung disease	Lung disease define by phtn (clin-p1) + and/or pulmonary fibrosis (clin-p3) +
Kidney	Evidence of renal disease	Renal disease define by acrenal + and/or renal biopsy +
	Renal cell cast	Renal cellular cast- red, hemoglobin, granular, tubular, or mixed
	Renal proteinuria	Renal proteinuria- persistent > 0.5 grams/24hrs or $> 3+$
	Renal hematuria	Renal hematuria- > 5 rbc per high powered field
	Renal clot	Reported renal clot
	Renal artery stenosis	Reported renal arthery stenosis
Neuro	Seizure	Seizure in the absence of offending drugs or known metabolic derangements
	Psychosis	Psychosis in the absence of offending drugs or known metabolic derangements
	Neuropathy	Any neuropathy
	Neuropsychiatric	Neuropsychiatric
	Chorea	Chorea
Lab tests - Metabolic Panel	Glucose	Glucose normal range: 65-99 mg/dl
	Ureanitrogen (BUN)	Urea nitrogen (bun) normal range: 7-25 mg/ml
	Creatine	Creatine normal range: 0.5-1.4 mg/dl
	Serum creatine	Serum creatine
	BUN/Creatine ratio	Bun/creatinine ratio normal range: 6-25 calc
	Sodium	Sodium normal range: 135-146 mmol/l
	Potasium	Potasium normal range: 3.5-5.3 mmol/l
	Chloride	Chloride normal range: 98-110 mmol/l
	Carbon dioxide	Carbon dioxide normal range: 21-33 mmol/l
	Calcium	Calcium normal range: 8.5-10.4 mg/dl
	Total protein	Total protein normal range: 6-8.3 g/dl
	Albumin	Albumin normal range: 3.7-5.1 g/dl
	Globulin	Globulin normal range: 2.2-4.2 g/dl
	Albumun/globulin	Albumun/globulin normal range: 0.8-2 calc
	Bilirubin	Bilirubin normal range: 0.2-1.5 mg/dl
	Alkaline phosphatase	Alkaline phosphatase normal range: 20-125 mg/dl
	Ast	Ast normal range: 3-35 mg/dl
	Alt	Alt normal range: 3-40 u/l

	Creatine kinase	Creatine kinase normal range: 0-165 u/l
Urinalysis	Urine glucose	Urine glucose normal range: 0
	Urine protein	Urineprotein normal range: 0
	Urine WBC	Urine wbc normal range: ≤ 5
	Urine RBC	Urine rbc normal range: ≤ 5
	Urine squamos eplithelial cells	Urine squamos eplithelial cells normal range: ≤ 5
	Urine bacteria	Urine bacteria normal range: 0
	Hyaline cast	Hyaline cast normal range: 0
Lab tests - Hematology	WBC count	Wbc count normal range: 3.8-10.8 thous/mcl
	RBC count	Rbc count normal range: 4.20-5.80 mill/mcl
	Hemoglobin	Hemoglobin normal range: 13.2-17.1 g/dl
	Hematocrit	Hematocrit normal range: 38.5-50%
	Mcv	Mcv normal range: 80-100 fl
	Mch	Mch normal range: 27-33 pg
	Mchc	Mchc normal range: 32-36 g/dl
	Rdw	Rdw normal range: 11-15%
	Platelet count	Platelet count normal range: 140-400 thous/mcl
	Absolute neutrophils	Absolute neutrophils normal range: 1500-7800 cells/mcl
	Absolute lymphocytes	Absolute lymphocytes normal range: 850-3900 cells/mcl
	Absolute monocytes	Absolute monocytes normal range: 200-950 cells/mcl
	Absolute eosinophils	Absolute eosinophils normal range: 15-500 cells/mcl
	Absolute basophils	Absolute basophils normal range: 0-200 cells/mhc
	Neutrophil %	Neutrophil % normal range: 35-80%
	Lymphocyte %	Lymphocyte % normal range: 18-44%
	Monocyte %	Monocyte % normal range: 0-10%
	Eosinophyl %	Eosinophyl % normal range: 0-3%
	Basophils %	Basophils % normal range: 0-1%
Lupus Specific lab tests	Ana +	Ana
	Anti-RNP +	Anti-rnp at hemagglutination titer $> 1:1600$
	Anti-Sm Abs +	Sm normal range: 0-20
	Anti-DNA Abs +	Dsdna titer normal range: 5-9 iu/ml
	Dna elevated	Elevated dna if ≥ 10 iu/ml
	FANA titer	Fana titer normal range: < 320
	Fana_pattr	Fana pattern. Homo , speckled nucleolar, homo/speckled, mixed , speckled/mixed, no-specific
	Ssa_result	Ssa normal range: < 20 eu/ml
	Ssb_result	Ssb normal range: < 20 eu/ml
	Scl_result	Scl normal range: < 20 eu/ml
	Anti-phospho-lipid Abs +	Anti-phospholipid abs + if > 10 gpl u/ml

	Anti cardiolipin antibody	Anti cardiolipin antibody if > 10 gpl u/ml
	Anticardiolipin igg LEVEL	Anticardiolipin igg normal ranger: < 10 gpl u/ml
	Anticardiolipin igg +	Anticardiolipin igg + if > 10 gpl u/ml
	Anticardiolipin igm Level	Anticardiolipin igm normal ranger: < 10 gpl u/ml
	Igm anticrd +	Igm anticrd + if > 10 gpl u/ml
	Anticardiolipin iga LEVEL	Anticardiolipin iga normal ranger: < 10 gpl u/ml
	Iga anticrd+	Iga anticrd+ if > 10 gpl u/ml
	C3 complement level	C3 complement normal range: 90 – 180 mg/dl
	C3_low	C3_low if < 90 mg/dl
	C4 complement level	C4 complement normal range: 16 – 47 mg/dl
	C4_low	C4_low if < 16
	Esr	Esr nomal ramnge: < 10 mg/dl
	Esr_elevated	Esr_elevated if >10 mg/dl
	C reactive protein	C reactive protein nomal range: 1.0 mg/dl
	Crp_elevated	Crp_elevated fi . 1.0 mg/dl
	RF latex Titer	Rf latex titer normal range: 14 iu/ml
	RF ELISA igm Titer	Rf elisa igm titer normal range: < 20 iu/ml
	RF ELISA igg Titer	Rf elisa igg titer normal range: < 20 iu/ml
	Cpk level	Cpk normal range: < 165 u/l
	CPK elevated	Cpkelevated: > 165 u/l
Experimental Lab test	Baff	Baff normal cytokine level < 1.19 ng/ml (based on healthy group average + sd of healthy group)
	Baffr	Baffr normal cytokine level < 0.09 ng/ml (based on healthy group average + sd of healthy group)
	Taci	Taci normal cytokine level < 0.02 ng/ml (based on healthy group average + sd of healthy group)
	Bcma	Bcma normal cytokine level < 1.64 ng/ml (based on healthy group average + sd of healthy group)
	Ifng	Ifng normal cytokine level < 13.13 pg/ml (based on healthy group average + sd of healthy group)
	Tnfa	Tnfa normal cytokine level < 7.58 pg/ml (based on healthy group average + sd of healthy group)
	Scd40l	Scd40l normal cytokine level < 1959.30 pg/ml (based on healthy group average + sd of healthy group)
	Il12p40	Il12p40 normal cytokine level < 61.68 pg/ml (based on healthy group average + sd of healthy group)
	Il12p70	Il12p70 normal cytokine level < 18.77 pg/ml (based on healthy group average + sd of healthy group)
	Il23	Il23 normal cytokine level < 348.34 pg/ml (based on healthy group average + sd of healthy group)
	Il17a	Il17a normal cytokine level < 3.88 pg/ml (based on healthy group average + sd of healthy group)
	Il17af	Il17af normal cytokine level < 19.40 pg/ml (based on healthy group average + sd of healthy group)
	Il17f	Il17f normal cytokine level < 38.00 pg/ml (based on healthy group average + sd of healthy group)

Il21	Il21 normal cytokine level < 13.5 pg/ml (based on healthy group average + sd of healthy group)
Il22	Il22 normal cytokine level < 31 pg/ml (based on healthy group average + sd of healthy group)
Il33	Il33 normal cytokine level < 12.3 pg/ml (based on healthy group average + sd of healthy group)
E-selectin	Eselectin normal cytokine level < 23.63 ng/ml (based on healthy group average + sd of healthy group)
Ngal	Ngal normal cytokine level < 79.10 ng/ml (based on healthy group average + sd of healthy group)
Igm anti-U1A (196-203) titer	P1-igm average normal range: 0-100
Igm anti-sme (63-70) titer	P2-igm average normal range: 0-100
Igm anti-smf (46-53) titer	P3-igm average normal range: 0-100
Igm anti-U1C (90-97) titer	P4-igm average normal range: 0-100
Igm anti-U1-70K (337-344) titer	P5-igm average normal range: 0-100
Igm anti-smb (83-90) titer	P6-igm average normal range: 0-100
Igm anti-smg (1-8) titer	P7-igm average normal range: 0-100
Igm anti-smd3 (20-27) titer	P8-igm average normal range: 0-100
Igm anti-smf (77-84) titer	P9-igm average normal range: 0-100
Igm anti-U1A (112-119) titer	P10-igm average normal range: 0-100
Igm anti-smd2 (14-21) titer	P11-igm average normal range: 0-100
Igm anti-smd1 (22-29) titer	P12-igm average normal range: 0-100
Igm anti-U1A (178-185) titer	P13-igm average normal range: 0-100
Igm anti-U1-70K (325-332) titer	P14-igm average normal range: 0-100
Igm anti-U1C (66-73) titer	P15-igm average normal range: 0-100
ELISA anti-snrbp	Based on healthy group average + sd of healthy group
Immunoblot anti-70KD	Based on healthy group average + sd of healthy group
Immunoblot anti-Sm	Based on healthy group average + sd of healthy group
Immunoblot anti-U1-A	Based on healthy group average + sd of healthy group

APPENDIX 4

Biological system		Variable
CLINICAL MANIFESTATIONS	SKIN	Telangiectasia
		Nasal ulcers
		Oral ulcers
		Hist hand swelling
		Obs hand swelling
		Sclerodactyly
		Pitting
		Alopecia
		Rash
		Photosensitivity
		Discoid rash
		Raynaud's
		Calcinosis
		Prox sclerodema
		Sicca
	MUSCLE	Myositis
		Myalgia
		Arthralgia
		Hist prox muscle weakness
		Obs prox muscle weakness
		Hist hand swelling
		Obs hand swelling
		Low extremety swelling
		Facial swelling
	JOINTS	Morning stiff
		Tender
		Synovitis
		Hist swelling
		Obs swelling
		Swelling (>3)
		Symmetric swelling
		Pain

	Deformity
LYMPH NODES	Any lymphadenopathy
	Swelling
	Neck swelling
	Axila swelling
	Groin swelling
	Rheumatoid nodule
GASTRO	Gastric reflux
BLOOD	Anemia
	Hemolytic anemia
	Leukopenia
	Lymphopenia
	Thrombopenia
	Thrombosis
HEART	Pericarditis
	Pleuritis
	Pericarditis/pleuritis
	Any clot
	Venous clot
	Valvular disease
	Avascular necrosis
	Myocardial infarction
	Stroke
LUNG	Hypertension
	Vascular lesions
	Fibrosis
	Lung disease
KIDNEY	Proteinuria
	Hematuria
	Clot
	Arterial stenosis
	Cell cast
	Renal disease
NERVOUS	Neuropathy
	Psychosis
	Chorea

APPENDIX 4, continuation

Lab tests		Variable
LABORATORY TESTS	URINALYSIS	Glucose +
		Protein +
		Wbc +
		Rbc +
		Squamos eplithelial cells +
		Hyaline cast +
	TRADITIONAL FOR LUPUS	Sm level
		Sm +
		Dsdna titer
		Dna +
		DNA elevated
		FANA titer
		Fana +
		Ssa +
		C3 level
		C3 low
		C4 level
		C4 low
		Esr
		ESR elevated
		Crp
		CRP elevated
		Igm-RF ELISA titer
		Igm-RF +
		Igg-RF ELISA titer
		Igg-RF +
		CPK level
		CPK elevated
	EXPERIMENTAL	II17a
		Igm-U1A ELISA (112-119)
		Igm-U1A ELISA (196-203)
		Igm-smd2 ELISA (14-21)
		Igm-smd2 ELISA (14-21)

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