MOLECULAR PATHOLOGY OF SYSTEMIC LUPUS ERYTHEMATOSUS IN ASIANS

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SCHOOL OF MEDICINE AND HEALTH SCIENCES

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Abstract

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease affecting various parts of the body. Polymorphisms in genes involved in toll-like receptor (TLR)/interferon (IFN) signalling pathways have been reported previously to be associated with SLE in many populations. This study aimed to investigate the role of seven single nucleotide polymorphisms (SNPs) within *TNFAIP3* (rs2230936 and rs3757173), *STAT4* (rs7574865, rs10168266, and rs7601754), and *IRF5* (rs4728142 and rs729302), that are involved in upstream and downstream pathway of type I IFN production, in the Malaysian SLE. Genotyping of 360 Malaysian SLE patients and 430 normal healthy individuals revealed that *STAT4* rs7574865 and rs10168266 with their minor T alleles [p=0.001, odds ratio (OR)=1.40 and p=5.75x10^{-4}, OR=1.43, respectively] and *TNFAIP3* rs3757173 with its C allele (p=0.017, OR=1.66) were associated with elevated risk of SLE in the Malaysian patients, as well as in the Malays and Chinese. The minor G allele of *TNFAIP3* rs2230926 was found to reduce the SLE risk (p=0.021, OR=0.53) in the Malaysian patients, particularly in the Malays. No association was observed for *STAT4* rs7601754 and SNPs in *IRF5* gene. Besides having haplotype TT (p=1 x 10^{-4}, OR=1.53) and CG (p=0.02, OR=0.76) being significantly associated with SLE susceptibility, *STAT4* rs7574865 and rs10168266 also formed the best model for high-risk group in multifactor dimensionality reduction (MDR) test. In conclusion, polymorphisms in *STAT4* and *TNFAIP3* genes could be potential genetic risk factors for SLE development in the Malaysian individuals.

Genotyping analysis of two recently reported *HLA* variants demonstrated that the frequency for minor allele G and its homozygous GG of *HLA-DRB1/HLA-DQA1* rs9271366 significantly higher in the Malaysian SLE patients (p=5.78 x 10^{-5}, OR=1.63 and p=0.001, OR=3.30, respectively), as well as in the Malays and Chinese. Whereas, the minor allele T (p=1.93 x 10^{-5}, OR=0.58) and the heterozygous CT (p=3.65 x 10^{-4}, OR=0.54) of *HLA-DQB1/HLA-DQA2* rs9275328 conferred protection to SLE in the Malaysians, including the Malays and Chinese. Both SNPs did not show associations with SLE in the Indians. Haplotype GC and AT were significantly associated with SLE (p<5.0 x 10^{-4}) after 10000 permutations. The MDR test clustered the genotype combinations of GG and CC, and AG and CC of rs9271366 and rs9275328, accordingly, as high-risk group. These results consolidated the importance of *HLA* gene, which involved in the immune complex processing, in the Malaysian SLE.

The seeking of potential SLE/lupus nephritis (LN)-associated protein biomarkers was attempted using two-dimensional difference gel electrophoresis (2D-DIGE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) approaches. A total of 30 spots from 18 high-abundant (HAP)-depleted plasma proteins, 26 spots from 11 plasma HAPs, and 59 spots from 25 urinary proteins were identified to have differential expressions. Serum constitutional and transport proteins, including haptoglobin and histidine-rich glycoprotein, occupied the largest portion among both plasma and urinary proteins (~28%). Several proteins were newly discovered in the present study, such as afamin, hemopexin, retinol-binding protein 4, and vitamin D-binding protein. Group-specific proteins were also determined for each group of SLE/LN, for instance up-regulation of plasma alpha-1-antitrypsin and down-regulation of serum amyloid P-component were unique to SLE without kidney involvement, while decreased urinary protein AMBP was specific to LN class V. These proteins may have prospective diagnostic value to discriminate the types of SLE and classes of LN. Most differentially expressed plasma proteins returned to normal levels upon LN remission, while most urinary proteins remained at their abnormal levels.

Interactions between SLE/LN-associated proteins analysed using IPA software summarized the networks or pathways responsible for the pathogenesis of each patient group. Network functions in cellular movement, inflammatory response, and cancer was associated with SLE without kidney involvement, whereas the top scored network generated for LN class II involved in cellular movement, haematological system development and function, and immune cell trafficking. Abnormal lipid metabolism, small molecule biochemistry, molecular transport, and vitamin and mineral metabolism were responsible for the development of LN class III. LN class IV was related to network implicated in cell cycle, cell death and survival, and tumor morphology. Connective tissue disorders, inflammatory disease, and skeletal and muscular disorders might increase the susceptibility to LN class V. Interestingly, networks having functions in immune and inflammatory responses were not as important as expected.

Mycophenolate mofetil (MMF) is one of the promising immunosuppressants with less toxicity used in the treatment of LN. After 8 months of MMF treatment, LN class III patients showed level changes in 18 protein fragments derived from 10 plasma proteins, such as angiotensinogen, and 6 protein fragments from 4 urinary proteins, such as lysosomal alpha-glucosidase. The results might have uncovered the mechanism of action of MMF other than that has been described previously.

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General Declaration

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. No copyright legislation stated under the Copyright Act is violated during the course of this work and shall be infringed by any party. I also declare that this thesis is no more than 100,000 words in length including quotes and exclusive of the tables, figures, references, appendices, and footnotes.

This thesis includes one (1) review paper and one (1) original paper published in peer reviewed journals, and one (1) unpublished publication. The core theme of the thesis is molecular pathology of systemic lupus erythematosus in Asians. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Medicine and Health Sciences, Monash University (Sunway Campus) under the supervision of Professor Maude Elvira Phipps and Professor Iekhsan Othman.

In the case of Chapter 1, 2, 3, and 4, my contribution to the work involved the following:

[If this is a laboratory-based discipline, a paragraph outlining the assistance given during the experiments, the nature of the experiments and an attribution to the contributors could follow.]

Thesis chapter	**Publication title**	**Publication status***	**Nature and extent of candidate's contribution**
1 (**Section 1.3**)	Genetic risk factors of systemic lupus erythematosus in the Malaysian population: a minireview	Published *Clin Dev Immunol*, 2012, 963730. (doi: 10.1155/2012/963730)	Article review, data collection, manuscript preparation
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[* For example, 'published'/ 'in press'/ 'accepted'/ 'returned for revision']

I (have)/ have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

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Monash University (Sunway Campus)

Date: ……

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Abbreviations

°C	degree Celsius
µg	microgram
l	litre
ml	millilitre
µl	microlitre
mM	millimolar
µM	micromolar
cm	centimetre
µm	micrometre
nm	nanometre
mmol	millimole
nmol	nanomole
pmol	picomole
fmol	femtomole
mA	milliampere
2D-DIGE	two-dimensional difference gel electrophoresis
2-DE	two-dimensional electrophoresis
A2M	alpha-2-macroglobulin
AACT	alpha-1-antichymotrypsin
AAT/SERPINA	alpha-1-antitrypsin
ACE	angiotensin converting enzyme
ACR	American College of Rheumatology
AFM	afamin
AGP1	alpha-1-acid glycoprotein 1
ALB	serum albumin
AMBP	protein AMBP
ANA	antinuclear antibody
ANCA	cytoplasmic antigen autoantibodies
ANXA2	annexin A2
ApoA-I	apolipoprotein A-I
ApoE	apolipoprotein E
APS	antiphospholipid syndrome
ARMS-PCR	tetra-primer amplification refractory mutation system–polymerase chain reaction
ASHG	alpha-2-HS-glycoprotein
BPC	base peak chromatogram
BSA	bovine plasma albumin
BVA	biological variation analysis
C4A	complement C4-A
C4B	complement C4-B
C4BPA	C4-binding protein alpha chain
CBB	coomassie briliant blue
CCL2	chemokine 2
CFB	complement factor B
CFH	complement factor H
CFI	complement factor I
CI	confidence interval

CLU	clusterin
CNS	central nervous system
cont.	continue
CP	ceruplasmin
CRP	C reactive protein
CVC	cross-validation consistency
CVID	common variable immunodeficiency
Cys	cysteine
D’	D prime
ddH_2O	double distilled water
DEP	differentially expressed protein
DHEA	dehydroepiandrosterone
DIA	differential in-gel analysis
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dpi	dots per inch
DPLN	diffuse proliferative lupus nephritis
dsDNA	anti-double-stranded DNA
DTT	dithiothreitol
e.g.	exempli gratia (for example)
ECC	extracted compound chromatogram
EDTA	ethylenediaminetetraacetic acid
EIC	extracted ion chromatogram
ENA	extractable nuclear antigen
ERSD	end-stage renal disease
ESI	electrospray ionization
ESR	erythrocyte sedimentation rate
EZH2	histone-lysine N-methyltransferase
FCGR1A	FcgammaRIa
FCN3	ficolin-3
FcγR	FcgammaR
FDA	Food and Drug Administration
FDH	familial dyalbuminemic hyperthyroxinemia
FGB	fibrinogen beta chain
FGG	fibrinogen gamma chain
FT-ICR	fourier transform ion cyclotron resonance
FTL	ferritin light chain
GBM	glomerular basement membrane
GC	vitamin D-binding protein
GFR	glomerular filtration rate
Gln	glutamine
GSN	gelsolin
GWAS	genome-wide association studies
h	hour
HAP	high abundant protein
HDL	high density lipoprotein
His	histidine
HLA	human leucocyte antigen
HNF1A	hepatocyte nuclear factor 1-alpha
HP	haptoglobin
HPX	hemopexin

HRG	histidine-rich glycoprotein
hsp90	90 kDa heat shock protein
HSPG2	heparan sulfate proteoglycan core protein
HUPO	Human Proteome Organisation
HUS	hemolytic-uremic syndrome
HWE	Hardy-Weinberg equilibrium
ICs	immune complexes
IEF	isoelectric focusing
IFN	interferon
IgA	immunoglobulin A
IgG	immunoglobulin G
IGHA1	Ig alpha-1 chain C region
IGHA2	Ig alpha-2 chain C region
IGHG	Ig gamma chain C region
IGKC	Ig kappa chain C region
IgM	immunoglobulin M
IL-1	interleukin-1
IL-6	interleukin-6
IMPDH	inosine monophosphate dehydrogenase
iNOS	inducible nitric oxide synthase
IRF5	interferon regulatory factor 5
ISN/RPS	International Society of Nephrology/Renal Pathology Society
ITIH4	inter-alpha-trypsin inhibitor heavy chain H4
IVIG	intravenous immunoglobulins
KNG1	kininogen-1
LCAT	lecithin cholesterol acyltransferase
LC-MS	liquid chromatography–mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LD	linkage disequilibrium
LN	lupus nephritis
LPL	lipoprotein lipase
m/z	mass:charge ratio
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MASP1	mannan-binding lectin serine protease 1
MASP2	mannan-binding lectin serine protease 2
MCP-1	monocyte chemoattractant protein1
MCP-1	monocyte chemoattractant protein-1
MCTD	mixed connective tissue disease
MDR	multifactor dimensionality reduction
MGB	minor groove binder
MHC	major histocompatibility complex
min	minute
MMF	mycophenolate mofetil
MPA	mycophenolic acid
MRM	multiple reaction monitoring
MudPIT	multidimensional protein identification technology
MW	molecular weight
NFQ	non-fluorescent quencher
NHS	N-hydroxysuccinimide
NO	nitric oxide

NPV	negative predictive value
NSAIDs	non-steroidal anti-inflammatory drugs
OR	odds ratio
OSM	oncostatin-M
oxLDL	LDL oxidation
PBMCs	peripheral mononuclear cells
PCR	polymerase chain reaction
Phe	phenylalanine
p*I*	isoelectric point
PMN	polymorphonuclear leucocyte
PMT	photomultiplier tube
PPI	protein-protein interaction
PPP	Plasma Proteome Project
PPV	positive predictive value
PTECs	proximal tubular epithelial cells
PTMs	post-translational modifications
qPCR	quantitative real-time PCR
r^2	r square
RBP4	retinol-binding protein 4
RCLB	red cell lysis buffer
rpm	revolutions per minute
s	second
SAP	serum amyloid component P
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SELDI-TOF-MS	surface enhanced laser desorption/ionisation time-of-flight mass spectrometry
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SRM	single reaction monitoring
ssDNA	single-stranded DNA
STAT4	signal transducer and activator of transcription 4
TEMED	tetramethylethylenediamine
TF	serotransferrin
TG	triglycerides
Th1	T helper 1 lymphocyte
Th17	T helper 17 lymphocyte
Th2	T helper 2 lymphocyte
TIC	total ion chromatogram
TIFF	tagged image file format
TLR	toll-like receptor
T_m	melting temperature
TNF	tumour necrosis factor
TNFAIP3	tumour necrosis factor alpha-induced protein 3
UMMC	University of Malaya Medical Centre
V	volt
Vh	volt hour
VLDL	very low density lipoprotein
WHO	World Health Organisation
x g	times gravity
ZAG	zinc-alpha-2-glycoprotein

Chapter 1

Introduction

1.1 Systemic Lupus Erythematosus

1.1.1 Background

Systemic lupus erythematosus (SLE) is a complex and chronic autoimmune disease, characterised by autoantibody production, complement activation and immune complex deposition leading to diverse clinical manifestations and target tissue damage. The autoantibodies produced are predominantly non-organ specific directed against several self molecules present in the nucleus, cytoplasm and cell surface, and the deposition of antibody-containing immune complexes in blood vessels will cause inflammation in the heart, joints, skin, lungs, liver, kidneys and nervous system. Hence, SLE can affect virtually any organ system with diverse presentations ranging from rash and arthritis through anaemia and thrombocytopaenia to serositis, nephritis, seizures and psychosis (Hom *et al.*, 2008, Rahman and Isenberg, 2008). The course of the disease normally involves periods of relative quiescence and periods of exacerbations (Hochberg, 1997, Guidelines, 1999). The mortality rate of SLE patients is a least three times higher than the general population (Abu-Shakra *et al.*, 1995). Survival rates are ~80% at 10 years after diagnosis and ~65% at 20 years (Boumpas *et al.*, 1995, Gladman, 1996). Deaths in the early course of SLE are usually due to the active disease and infections, while deaths occur later are often caused by atherosclerotic vascular disease (Urowitz *et al.*, 1976).

1.1.2 Epidemiology

The prevalence of SLE is estimated to be between 40 and 400 cases per 100,000 individuals (Helmick *et al.*, 2008). Severity, acquisition risk and clinical manifestations of the disease can vary by ethnicity, geography and sex, with a prevalence that is higher in some non-European populations such as African Americans, Hispanics and Asians (Lau *et al.*, 2006). Hence it is not surprising to observe a huge difference in the prevalence of lupus between Northern Europeans and blacks, which ranges approximately 40 to more than 200 cases per 100,000 persons, respectively (Johnson *et al.*, 1995). In Asians, the prevalence of

SLE generally falls within 30 to 50 cases per 100,000 individuals, with Chinese communities being more susceptible than those in India and tropical Africa (Frank, 1980).

The disease happens nine times more frequently in women than men, especially in women in child-bearing ages of 15 to 35. Childhood SLE affects girls more often than boys, with girls outnumbering boys 4:1. In the United States, SLE can be reported in 5000 to 10,000 children (Lehman *et al.*, 1989). Although SLE can happen at any age, it becomes more frequent after five years of age and is increasingly prevalent after the first decade of life (Lehman *et al.*, 1989). Similar to the adult SLE cases, Asian, Black and Hispanic children are more frequently affected than their Caucasian peers.

1.1.3 Classification criteria

Since the aetiology of SLE is unclear and heterogeneity of clinical manifestations could be involved, it is imperative to have a standard set of classification criteria that enables the diagnosis of the disease to be made. A patient is diagnosed to have SLE when he/she meets at least four out of eleven SLE classification criteria derived by the American College of Rheumatology (ACR). The preliminary criteria were initially published in 1971 and they were subsequently revised in 1982, with a few updates added in 1997 (Cohen *et al.*, 1971, Tan *et al.*, 1982, Hochberg, 1997). The classification criteria cover various disorders, from organs such as skin, oral, joints, lungs, heart, kidneys and nervous system, to haematologic and immunologic systems, as well as an abnormal titre of antinuclear antibody (**Figure 1.1**).

However, the criteria may cause possibility of misclassification of the patients. The patients with SLE having less than four criteria could be classified non-SLE and vice-versa. The diagnosis becomes more complicated when a patient with possible SLE also has manifestations of, or even meets criteria for another rheumatic disease (Smith and Shmerling, 1999). Clinicians may have different thresholds for determining whether or not a patient has a particular criterion, as well as for serologic testing for antinuclear antibodies (ANAs) and antibodies directed against double-stranded DNA or Smith antigens which have variable thresholds as a result of evaluations that have not been methodologically standardized (Smith and Shmerling, 1999, Tan *et al.*, 1999). Several reports also demonstrated the discrepancy between outcomes of classifying patients as SLE clinically and according to criteria (Fries and Siegel, 1973, Michet *et al.*, 1985). For example, Calvo-

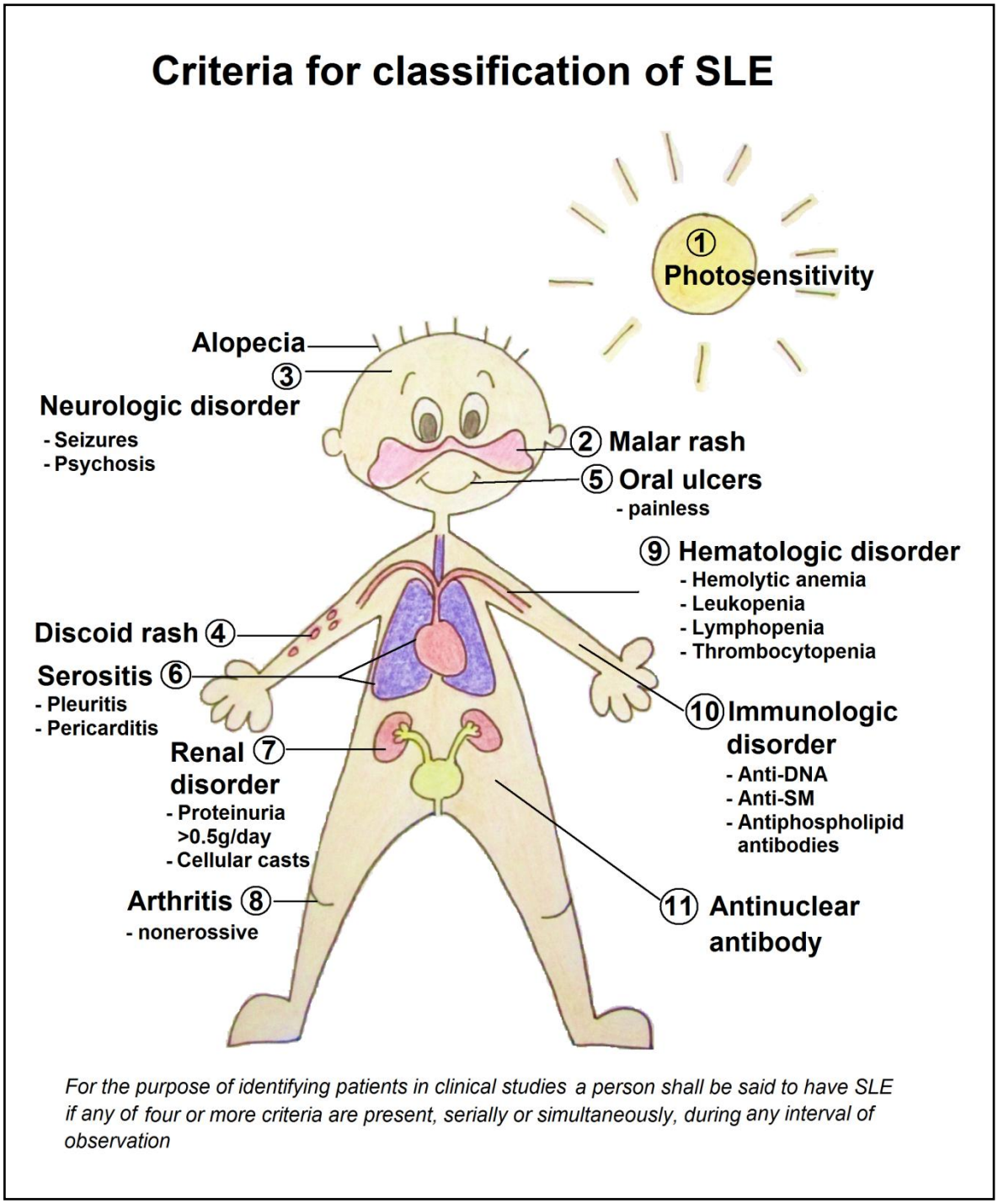


Figure 1.1. Criteria for classification of SLE.

(Drawn as adapted from Smith and Shmerling, 1999 and Tan *et al.*, 1982)

Alén and colleagues reported that 22 of 112 patients clinically diagnosed with SLE did not meet criteria (Calvo-Alen *et al.*, 1995). The prevalence of patients diagnosed with SLE may be much higher in the community than predicted by the criteria. Subsets of patients with SLE who do not meet criteria are described by some researchers as 'latent lupus', 'incomplete lupus', lupus-like disease, and 'probable lupus' (Ganczarczyk *et al.*, 1989, Greer and Panush, 1989, Asherson *et al.*, 1987). These group of patients generally have less severe disease that is less likely to involve organ systems, have more insidious onset, and are more like to be older at disease onset (Fries and Siegel, 1973, Smith and Shmerling, 1999). It is suggested that the classification criteria may bias towards more severe and longer duration disease (Lom-Orta *et al.*, 1980). The criteria also may not always distinguish SLE from other systemic rheumatic diseases correctly, especially patients with antiphospholipid syndrome (APS) (Smith and Shmerling, 1999). Hence, there is no single set of criteria that may ever fulfil the needs of different professions such as clinicians, educators and researchers.

Seeing that the ACR classification criteria have inherent limitations, in 1984, Clough and colleagues introduced weights for each criterion to improve the sensitivity and specificity of the classification criteria (Clough *et al.*, 1984). The probability of a patient having SLE increased with increasing score. However, these have not been widely used due to their complexity.

In conclusion, the ACR classification criteria for SLE should be revised periodically to improve their specificity and sensitivity in distinguishing SLE from other systemic rheumatic diseases, and so that they will better serve the requirements of clinicians, teachers, researchers, and patients.

1.1.4 Laboratory Tests

Autoantibodies involved in SLE are generally targeted against intracellular antigens of the cell nucleus (double- and single-stranded DNA), histones and extractable nuclear antigens (ENAs). Most of these autoantibodies are not exclusive for SLE and might be produced non-specifically as a result of polyclonal B cell activation (Egner, 2000). Thus an ideal test for SLE requires high degree of specificity (detects only those with disease), sensitivity (detects all those with disease), positive predictive value (PPV) (where most positives have disease), and negative predictive value (NPV) (where most negative

do not have disease) (Egner, 2000). No single test presently has all these features and therefore collective information obtained from several tests would be more useful to conclude if a person is having SLE.

Antinuclear antibody immunofluorescence test can be performed to screen patients with clinical features of SLE, although it will also detect most anti-single-stranded DNA (ssDNA), anti-double-stranded DNA (dsDNA), ENAs, and other autoantibodies. Seeing that false positives are common in ANA test, ANA positive samples should be subjected to more specific assays, such as a combination of ENA (Ro/La/Sm/RNP) and dsDNA assays that manage to detect most patients with SLE, to confirm the presence of the disease (Clough and Chang, 1990, Froelich *et al.*, 1990). The erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) measurements might be useful in providing additional information. Other autoimmune diseases such as Sjogren's syndrome and mixed connective tissue disease (MCTD) may produce overlapping serology with SLE, and anti-dsDNA titres are occasionally found in autoimmune hepatitis and rheumatoid arthritis. Therefore, it is essential that the results produced by all assays are interpreted parallel to the clinical details to determine the status of SLE. For monitoring the disease, a combination of quantitative assays, including anti-dsDNA, C3, C4, CRP, and ESR, would give the most comprehensive information.

1.1.5 Treatment

At present there is no cure for SLE. Several therapies are used to suppress symptoms and relieve discomfort. Treatment of SLE varies depending on the severity of the disease and also clinical manifestations involved. Several types of drugs that are being to treat SLE are summarised in **Table 1.1**.

In general, for cutaneous and mild SLE, steroidal and sunblock creams are important to relieve skin lesions and for sun protection. Non-steroidal anti-inflammatory drugs (NSAIDs) that can block prostaglandins are used for relief of joint pain and swelling and muscle pain. Antimalarial drugs are prescribed for discoid lupus or mild lupus where skin and join problems being the predominant symptoms. As for the treatment of severe SLE, corticosteroids are used to suppress the inflammatory process and relieve symptoms caused by anaemia and kidney involvement. Immunosuppressant drugs are prescribed when kidney or neurological involvement or acute blood vessel inflammation

Table 1.1. Drugs used for treatment of SLE.

Type of drug	Drug	Mechanism/Usage	Side effects
Creams	Steroid creams, vitamin A (Tegison), sunblock creams	For skin lesions and sun protection	
Non-steroidal anti-inflammatory drugs (NSAIDS)	Aspirin, ibuprofen, naproxen, ketoprofen, diclofenac, tolmetin, dexibuprofen	Block prostaglandin that dilates blood vessels and cause inflammation and pain. To relieve joint pain and swelling, muscle pain	Gastric erosions/ulcers/bleeding, decrease in renal function, increase in liver enzyme levels, aseptic meningitis, hypertension
Antimalarial drugs	Hydroxychloroquine, chloroquine, quinacrine	For discoid lupus or mild lupus when skin problems and joint pain are predominant	Skin rash, gastrointestinal problems, headache, hair loss, muscle aches, macular damage
Corticosteroids	Prednisone, methylprednisone, hydrocortisone, dexamethasone	Relieve many complications and symptoms, including anaemia and kidney involvement	Osteoporosis, cataracts, diabetes, susceptibility to infections, weight gain, hypertension, menstrual irregularities
Immunosuppressant drugs	Cyclophosphamide	For treatment of lupus nephritis	Nausea, vomiting, hair loss, myeloproliferative disorders, malignancy, hemorrhagic cystitis, secondary infertility, infections
	Mycophenolate mofetil		Diarrhoea, miscarriage and birth defects if used during pregnancy
	Azathioprine		Myelosuppression, hepatotoxicity, lymphoproliferative disorders
	Cyclosporin		Diarrhoea, peptic ulcers, pancreatitis, fever, vomiting

(Adapted and modified from Guidelines, 1999)

is present (Guidelines, 1999).

New approaches for SLE treatment have also been developed. B-cells are deemed to be a critical key component for the development of SLE. The new therapeutic agent have therefore focused on functions of B-cells in taking up autoantigens and presenting them via specific cell surface immunoglobulins to T-cells, subsequently affecting T-cell-dependent immune responses (Looney *et al.*, 2004a, Looney *et al.*, 2004b, Ginzler and Dvorkina, 2005). Monoclonal antibodies developed for this purpose include epratuzumab and belimumab. Intravenous immunoglobulins (IVIG) can be considered for patients who do not respond to other SLE treatments. Intravenous immunoglobulins (IVIG) are a blood product containing pooled, polyvalent, IgG extracted from plasma of blood donors. Intravenous immunoglobulins may form a type of immune complex that will interact with activating Fc receptors and subsequently mediate anti-inflammatory effects (Clynes, 2005, Siragam *et al.*, 2006). It may also react with membrane receptors on T-cells, B-cells, and monocytes (Bayry *et al.*, 2003). Another treatment using male sex hormones (dehydroepiandrosterone, DHEA) was developed based on rationale that SLE is more prevalent in female of child-bearing age (Chang *et al.*, 2002). Immunoablation with high-dose immunosuppressant followed by rescue with autologous hematopoietic stem-cell transplantation has allowed short-term remission in some patients. (Traynor *et al.*, 2002, Lisukov *et al.*, 2004). Finally, phototherapy and plasmapheresis are also developed for SLE treatments, whereby ultraviolet A-$_1$ is used to block inflammatory immune factors and plasma containing inflammatory antibodies is removed from blood, respectively.

1.1.6 Aetiology

The aetiology of SLE still remains elusive, although orchestra of various environmental and genetic factors has been considered to be responsible for the onset and progression of the disease.

A. Environmental Factors

Environmental exposes that may be potential factors in the pathogenesis of SLE include infectious agents, chemicals or other compounds that are able to modulate immune responses (Cooper *et al.*, 1999, Edwards, 2005) for instance occupational or environmental

pollutants or drugs, and behavioural factors such as smoking and diet. These environmental triggers could initiate the disease and bring about the production of autoreactive T-cells and autoantibodies, the stimulation of pro- and anti-inflammatory cytokines, and finally organ damage (Sarzi-Puttini *et al.*, 2005). Infectious agents may cause certain responses by molecular mimicry and interfere with immunoregulation; diet influences the production of inflammatory mediators; drugs/toxins alter cellular responsiveness and immunogenicity of self antigens; and physical/chemical agents cause inflammation, increased cellular apoptosis and tissue damage (Mok and Lau, 2003). Environmental/exogenous oestrogens may induce or unmask SLE and cause precipitate flares (Meier *et al.*, 1998), whereas long-term tobacco smoking potentially impair the secretion of pro-inflammatory cytokines and reduce the activity of natural killer cells (Sarzi-Puttini *et al.*, 2005).

B. Genetic factors

Pathogenesis of SLE is associated with functional deficiency of multiple immunological components, including innate immune system, altered immune tolerance mechanism, hyperactivation of T- and B-cells, reduced ability to clear immune complexes and apoptotic cells, and defect of multiple immune regulatory networks (Firestein, 2008). The failure of these mechanisms could be due to the variants of SLE susceptibility genes. To date, many different genes have been found to contribute to the disease susceptibility. In a small proportion of patients (<5%), a single gene could become the key player for this disease (Mok and Lau, 2003), however multiple genes are found to be implicated in most patients. It is estimated that at least four susceptibility genes or loci are needed for the development of the disease (Schur, 1995). The susceptibility genes that have been most extensively studied are within the major histocompatibility complex (MHC). It is believed that variants of human leucocyte antigen (HLA) class II gene are very important. Before 2007, the discovery of SLE susceptibility genes had been rather slow, with only nine genes identified via candidate gene studies or linkage approaches. The introduction of genome-wide association studies (GWAS) has not only helped us to support the findings from previous candidate gene studies, the number of candidate genes has exponentially swelled to more than 30, and many more novel genetic loci are yet to be unveiled (Moser *et al.*, 2009). The candidate genes recently discovered can be clustered into three main groups: i) *IRF5*, *STAT4*, *TNFAIP3* and *TREX1* that are involved in the innate immune response including the TLR/interferon signalling pathway; ii) *HLA-DR*, *PTPN22*, *PDCD1*, *LYN*,

BLK and *BANK1* that are involved in immune signal transduction of B, T and antigen-presenting cells; and iii) C2, C4, FCGRs, CRP and *ITGAM* that are involved in immune complex clearance mechanism (Harley *et al.*, 2009, Moser *et al.*, 2009, Lee and Bae, 2010).

The genetic information obtained from GWAS has allowed many researchers to investigate specific variants for particular genetic loci using a variety of other approaches such as RFLP-PCR, tetra-primer ARMS-PCR, and real-time genotyping PCR. This in turn has enabled the replication of these experiments and confirmed those associated polymorphisms with SLE in different populations. Genetic heterogeneity is common among populations in SLE, especially between Caucasians and Asians. For instance, *PTPN22* which demonstrated significant association with SLE in Caucasians, was not found to be associated with some ethnicities in Asia (Kochi, 2010). The identification of genetic heterogeneity may enhance our understanding of mechanisms that lead to SLE pathogenesis in certain populations and subsequently may permit more precise diagnosis, prognosis and treatment for the patients.

1.2 Lupus nephritis

1.2.1 Background

Systemic lupus erythematosus that involve renal manifestations is known as lupus nephritis (LN) and it is the major cause of death in SLE patients. The abnormalities of kidneys occur within the first few years of after the diagnosis of lupus is made and it is important that early renal biopsy is done in SLE patients with an abnormal urinalysis and/or reduced glomerular filtration rate, so that proper management and therapeutic decisions can be made. The biopsy serves as vital prognostic information according to histological categorisation of different types of lupus nephritis, the degree of activity, chronicity and the immunopathogenesis (Ortega *et al.*, 2010).

The epidemiology of LN varies depending on the studied population. The LN cumulative incidence is relatively higher in people of Asian (55%), African (51%), and Hispanic (43%) ancestry as compared with Caucasians (14%) (Ortega *et al.*, 2010). The 5- and 10-year LN survival rates in the 1990s ranged between 83-93% and 74-84%, respectively, and up to 25% of LN patients develop end-stage renal disease (ERSD) 10 years after onset of renal disorder (Mok, 2010).

1.2.2 Pathogenesis and Classification

The pathogenesis of LN is described as the autoantibody binding of intrinsic antigens in the kidneys, such as extracellular matrix components or cell surface glycoproteins (Weening *et al.*, 2004). The renal damage in LN could result from the binding of autoantibodies either to circulating antigens, forming circulating pre-formed immune complexes, or to antigens deposited from the circulation in glomerular and vessel walls, forming *in situ* immune complexes, as has been seen for nucleosomes and anti-dsDNA autoantibodies (Berden, 1997, Weening *et al.*, 2004). Subsequently, Fc receptor and complement binding take place, triggering inflammatory and cytotoxic reactions. In addition, anti-neutrophil cytoplasmic antigen autoantibodies (ANCA) have also been reported to cause vasculitis and glomerulonephritis in lupus nephritis patients via "pauci-immune" neutrophil-dependent mechanisms (Marshall *et al.*, 1997).

There are six classes of LN that are associated with three patterns of glomerular injury: (i) LN class I and II involve mesangial pattern of injury that leads to a syndrome of microscopic haematouria and sub-nephrotic proteinuria with well-preserved or minimally reduced glomerular filtration rate (GFR); (ii) LN class III and IV involve endothelial pattern of injury that characterised by an acute reduction in GFR, haematouria, and mild to moderate proteinuria; and (iii) LN class V involves epithelial pattern of injury that is associated with significant proteinuria with nephritic syndrome, and with preservation or gradual reduction in GFR. Lupus nephritis class VI that initially was not included in 1974 WHO classification system was introduced in 1982 to denote advanced sclerosing glomerulonephritis (Weening *et al.*, 2004). The classification systems for LN are summarised in **Table 1.2**, with guidelines on how classification can be made. The pathogenesis of mesangial proliferation and endothelial injury in LN class II-IV is due to the release of proteases as a result of chemotaxis of monocytes and polymorphonuclear cells (de Zubiria Salgado and Herrera-Diaz, 2012). Type I interferon produced by dendritic cells in adaptive immune response elicits maturation and activation of infiltrating T cells, as well as amplification of T-helper-2 (Th2), T-helper-1 (Th1), and T-helper-17 (Th17)

Table 1.2. Common classifications for lupus nephritis.

LN classes	1995 WHO classification	ISN/RPS 2004 classification
Class I	Normal, deposits on immunohistology and/or electron microscopy	Minimal mesangial LN, deposits on immunohistology and/or electron microscopy
Class II	Mesangial widening and cell proliferation	Mesangial proliferative LN
Class III	Focal segmental mesangiocapillary proliferative glomerulonephritis (<50% glomeruli)	Focal proliferative LN (<50% glomeruli)
Class IV	Diffuse proliferative severe mesangial/mesangiocapillary proliferation	Diffuse proliferative LN (>50% glomeruli)
Class V	Diffuse membranous glomerulonephritis	Membranous LN (with or without co-existing class III or class IV LN)
Class VI	Advanced sclerosing glomerulonephritis	Advanced sclerosing LN (>90% globally sclerotic glomeruli)

The 1995 WHO and 2004 ISN/RPS classifications.

(Adapted and modified from Ortega *et al.*, 2010)

lymphocytes. All these stimulate B-cells to amplify and activate macrophages to release more pro-inflammatory molecules, thus generating more effector cells that can no longer be modulated by regulatory T cells. In the end, epithelial proliferation and fibrosis occur in LN class V and VI (de Zubiria Salgado and Herrera-Diaz, 2012).

1.2.3 Diagnosis

For reliable classification, adequacy of the tissue specimen and histopathology techniques are compulsory (**Figure 1.2**). Tissues should be optimally preserved and processed by skilled technician. Histopathology techniques used are light microscopic examination, immunofluorescence and electron microscopy. For light microscopic analysis, the biopsy should contain a minimum of 10 glomeruli in order to exclude a focal lesion, and stained with special stains for assessment of glomerular number, cellularity and capillary wall alterations (Corwin *et al.*, 1988). Immunofluorescence analysis on renal biopsy include staining of IgG, IgA, and IgM isotypes, kappa and lambda light chains, and complement components C3 and C1q. Electron microscopy is essential in ultrastructural evaluation of renal cortical tissue (Weening *et al.*, 2004).

1.2.4 Treatment

Histological patterns of LN serve as the basis for therapeutic guidelines and decisions to prevent target organ damage. Patients with LN class I and II do not require directed immunosuppressive treatment; instead, they just need to maintain adequate blood pressure control and blockade of the rennin angiotensin aldosterone system (de Zubiria Salgado and Herrera-Diaz, 2012).

Patients with active proliferative LN are usually treated with induction therapy, whereby they are initially introduced to a pulse of intravenous steroid followed by a high-dose oral steroid, or by this method in conjunction with other immunosuppressive agents. These include cyclophosphamide, mycophenolate mofetil, and azathioprine. Pulsed intravenous cyclophosphamide for six consecutive months used to be the most common prescription for treating active proliferative LN, however mycophenolate mofetil has gained more popularity due to reduction of side effects. The active metabolite of mycophenolate mofetil suppresses B- and T-cell proliferation because the salvage pathway necessary for DNA synthesis is absent.

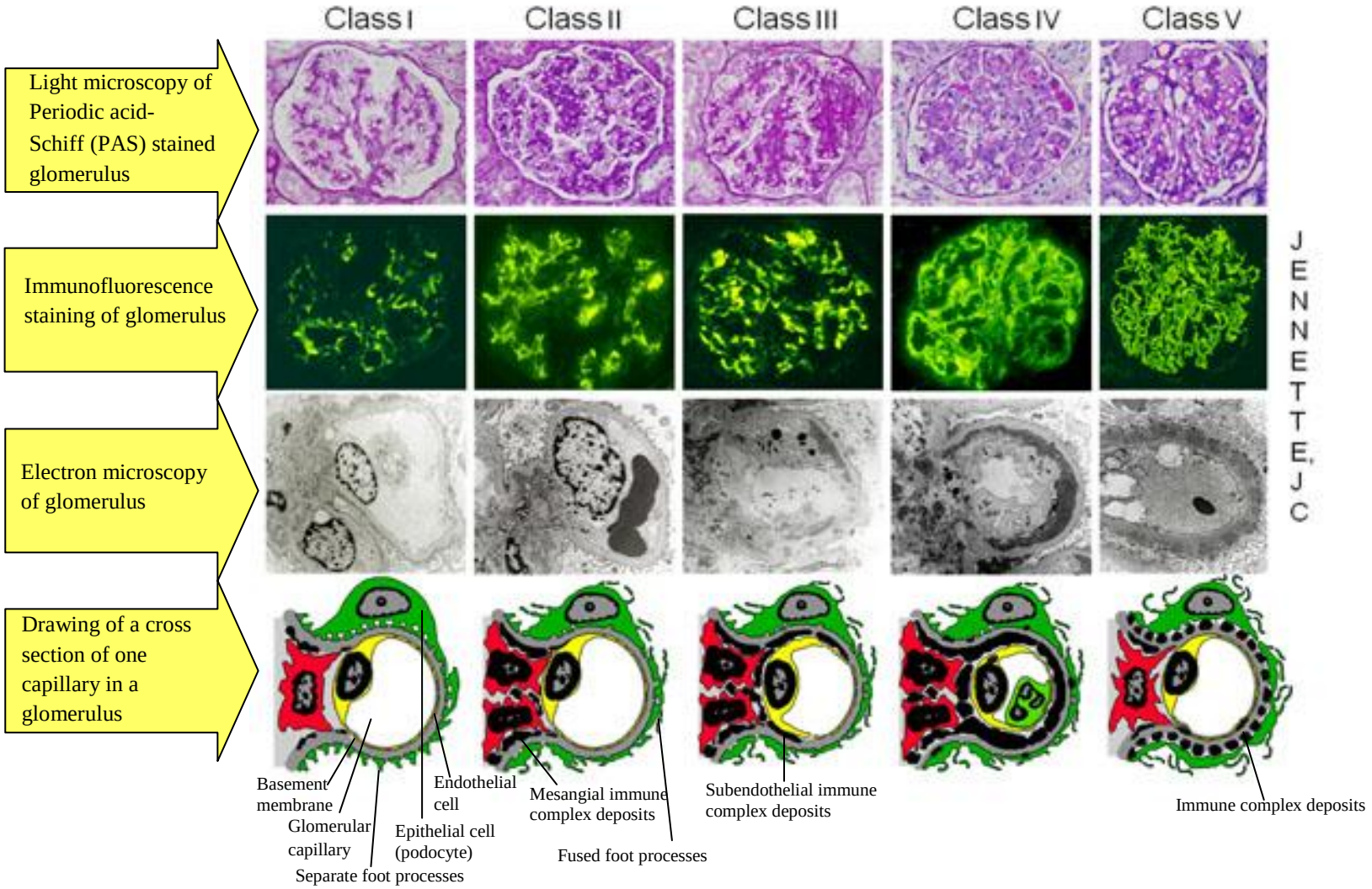


Figure 1.2. Histological features of glomerular pathology in lupus nephritis.

(Adapted from http://www.unckidneycenter.org/kidneyhealthlibrary/lupus.html with permission from the author, Professor J. Charles Jennette)

Another recent treatment for LN is tacrolimus, a macrolide calcineurin inhibitor that potently suppresses human T-cell proliferation. (Miyasaka *et al.*, 2009). For maintenance therapy for proliferative LN that aims to maintain renal remission previously achieved in the induction and to avoid flares or relapses, azathioprine or corticorsteroids may be prescribed (Houssiau *et al.*, 2010). In addition, a recent systematic review has shown an impressive response by LN patients to a relatively new therapeutic agent, rituximab, which is a chimeric half murine-half human monoclonal antibody directed against the B-cell marker CD20 (Ramos-Casals *et al.*, 2009).

1.3 SLE and LN in Malaysia

Malaysia is a multiracial country populated by groups or various ethnicities. However, the Malays (55.1%), Chinese (24.3%) and Indians (7.4%) form the majority. A prevalence of 43/100,000 individuals in Malaysia has been reported (Wang *et al.*, 1997, Osio-Salido and Manapat-Reyes, 2010). Chinese have the highest prevalence of SLE in Malaysia (57/100,000), followed by Malays (33/100,000) and Indians (14/100,000) (Yap et al., 1999, Chua et al., 2008a). The overall 5-year and 10-year survival rates were reported as 82% and 70%, respectively (Wang *et al.*, 1997), whereas the overall mortality rate was 20.2% (Yeap *et al.*, 2001). Renal involvement seems the highest incidence among the Malaysian patients compared to the other clinical manifestations (Wang *et al.*, 1997). However, the major cause of death in Malaysian SLE patients was reported to be from infections (Yeap *et al.*, 2001).

Several groups of researchers have been actively investigating genes that are associated with SLE susceptibility in the Malaysian population by screening possible reported candidate genes across the SLE patients and healthy controls. These candidate genes included MHC genes and genes encoding complement components, TNF, FcγR, T-cell receptors and interleukins. However, most of the polymorphisms investigated in these genes did not show significant associations with susceptibility to SLE in Malaysian scenario, except for those occurring in MHC genes, such as HLA A*11 (Mohd-Yusuf *et al.*, 2011), and genes coding for TNF-α (Azizah *et al.*, 2004, Chua *et al.*, 2008b), IL-1β (Chua *et al.*, 2009b), IL-1RN (Lau *et al.*, 2009) and IL-6 (Chua *et al.*, 2009a).

Lupus nephritis is one of the common renal diseases in Malaysia. Renal biopsies from patients of the University Hospital Kuala Lumpur between 1982 and 1991 uncovered

LN as the most prevalent and accounted for 24.9% of the renal diseases, followed minimal change nephritis (20.7%), IgA nephropathy and other renal disorders (Looi, 1994). In general, peak age of incidence is between 20-30 years old and female shows more preponderance to LN than male, with female:male ratio being 6-10.3:1 (Looi, 1994, Yahya, 2008). Although Looi (1994) reported that Chinese was the most affected ethnic in LN, Yahya (2008) revealed no racial predilection. World Health Organisation class IV is the predominant histological pattern of LN encountered, in both adults and children (Looi, 1994, Khoo *et al.*, 2005, Yahya, 2008). The 5-year patient and renal survival rates in children are 84% and 75%, respectively (Khoo *et al.*, 2005).

1.4 Biomarkers or proteins associated with SLE

The classical pathogeneses of SLE have been recognised as deficiency of complement system, deregulation of cytokine secretion and inefficiency of CRPs that subsequently bring about the abnormalities of apoptosis, clearance deficiency and finally intolerance to body's self antigens. Levels of IFN-α in the serum of SLE patients were first reported 32 years ago and it potentially induces normally quiescent monocytes to differentiate into autoantigens presenting dendritic cells (Hooks *et al.*, 1979). Anti-dsDNA and complement C1q, C3, and C4 are conventional biomarkers that are widely used as serological tests for the diagnosis of SLE. The circulating levels of B lymphocyte stimulator (BAFF) are elevated in SLE patients and the levels are also correlated with the changes of SLE disease activity and anti-dsDNA titers (Cheema *et al.*, 2001, Petri *et al.*, 2008). Belimumab was developed by Human Genome Sciences to against BAFF and it was the only drug approved by the US Food and Drug Administration (FDA) (Mitka, 2011). Other proteins involved in the pathogenesis of SLE include the over-expression of 90 kDa heat shock protein (hsp90) that subsequently induces the reactivity of antibodies (Latchman and Isenberg, 1994, Ripley *et al.*, 2001), as well as elevated level of autoantibodies against serum amyloid component P (SAP) (Zandman-Goddard *et al.*, 2005, Zhang *et al.*, 2011) and ribosomal P proteins in CNS lupus patients (Elkon *et al.*, 1985, Reichlin, 2003).

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or biological responses to a therapeutic intervention (Group, 2001, Herbst *et al.*, 2012). Biomarkers of SLE can be

characterised into several classes: i) diagnostic biomarkers; ii) prognostic biomarkers; iii) predictive biomarkers; iv) pharmacodynamic biomarkers; and v) surrogate biomarkers. Diagnostic biomarkers, such as serum anti-phospholipid and anti-ribosomal P protein, are used to identify the disease, whereas prognostic biomarkers are used to categorise patients based on degree of risk for disease occurrence or progression. In the case of SLE, serum anti-dsDNA and C3a are used to predict flares of SLE. The predictive biomarkers are important in grouping patients by their likelihood for response to particular therapeutic intervention, and the example for SLE is serum anti-dsDNA which is used predicate favourable response to belimumab in patients. Blood level of type I IFN gene transcript acts as pharmocodynamic biomarker that aid in dose selection or regimen for clinical trials of anti-IFN-α therapy in SLE. Lastly, surrogate biomarkers are used to substitute for a clinical efficacy endpoint and in the clinical trials of rituximab, serum anti-dsDNA which has negative correlation with clinical response was evaluated (Herbst *et al.*, 2012).

In the proteomic study of SLE, several differentially regulated proteins expressed by peripheral mononuclear cells (PBMCs), including immunoglobulin J chain, apolipoprotein A-IV precursor, glutathione S-transferase, calprotection L1H, and zinc finger protein subfamilyl A, has been reported in SLE patients (Dai *et al.*, 2008). Newly discovered serum autoantibodies against cystallin αB, esterase D, and APEX nuclease associated with SLE patients with CNS syndrome were also reported (Katsumata *et al.*, 2011). While surface enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS) approach enabled the identification of urinary proteins with m/z of 3340 and 3980 to be distinctive between active and inactive lupus nephritis (Mosley *et al.*, 2006), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) approach also detected novel SLE familial associated proteins as having m/z of 9342.23, 4094.03, 5905.35, and 7973.53 (Wu *et al.*, 2009).

1.5 Research approaches used in this study

1.5.1 Genetics

1.5.1.1 Tetra-primer amplification refractory mutation system–polymerase chain reaction (ARMS-PCR)

Tetra-primer ARMS-PCR is a simple and economical SNP genotyping method involving a PCR reaction followed by gel electrophoresis. The technique was developed based on certain principles of the tetra-primer PCR method and ARMS (Newton *et al.*, 1989, Ye *et al.*, 1992) (**Figure 1.3**). In the figure, the single nucleotide polymorphism having G→A substitution is used as an example to describe the principle of tetra-primer ARMS-PCR method. Two outer primers and two allele-specific inner primers are employed in the reaction. In the end, G allele-specific or/and A allele-specific amplicons will be generated, together with a definite presence of non-allele-specific amplicons produced by the two outer primers. The two outer primers should be placed at different distance from the polymorphic nucleotide so that the two allele-specific amplicons differ in length and can be discriminated by gel electrophoresis. Two bands will be seen on the agarose gel for homozygotes of G and A alleles while three bands for heterozygote. The primers should be around 30 or more bases long and both inner primers are introduced with a deliberate mismatch at position -2 from the 3'-terminus in order to increase the allelic specificity (Newton *et al.*, 1989, Ye *et al.*, 2001). Because it is simple, rapid, low-cost, and high-throughput, tetra-primer ARMS-PCR method has been applied particularly in studying SNPs or genetic determinants of complex diseases such as coronary artery disease, spinal muscular atrophy and breast cancer (Baris *et al.*, 2010, Masud and Qureshi, 2011, Gomes *et al.*, 2012).

1.5.1.2 Real-time PCR for Taqman SNP genotyping assay

Real-time PCR, which also known as quantitative real-time PCR (qPCR), is a PCR technique that amplifies and simultaneously quantifies a targeted DNA molecule. The amplified DNA is detected as the reaction progresses in real time. The two commonly used methods for detection of amplified products in real-time PCR are: i) non-specific fluorescent dyes that intercalate in between any double-stranded DNA, and ii) sequence-specific DNA probes that are labelled with a fluorescent reporter.

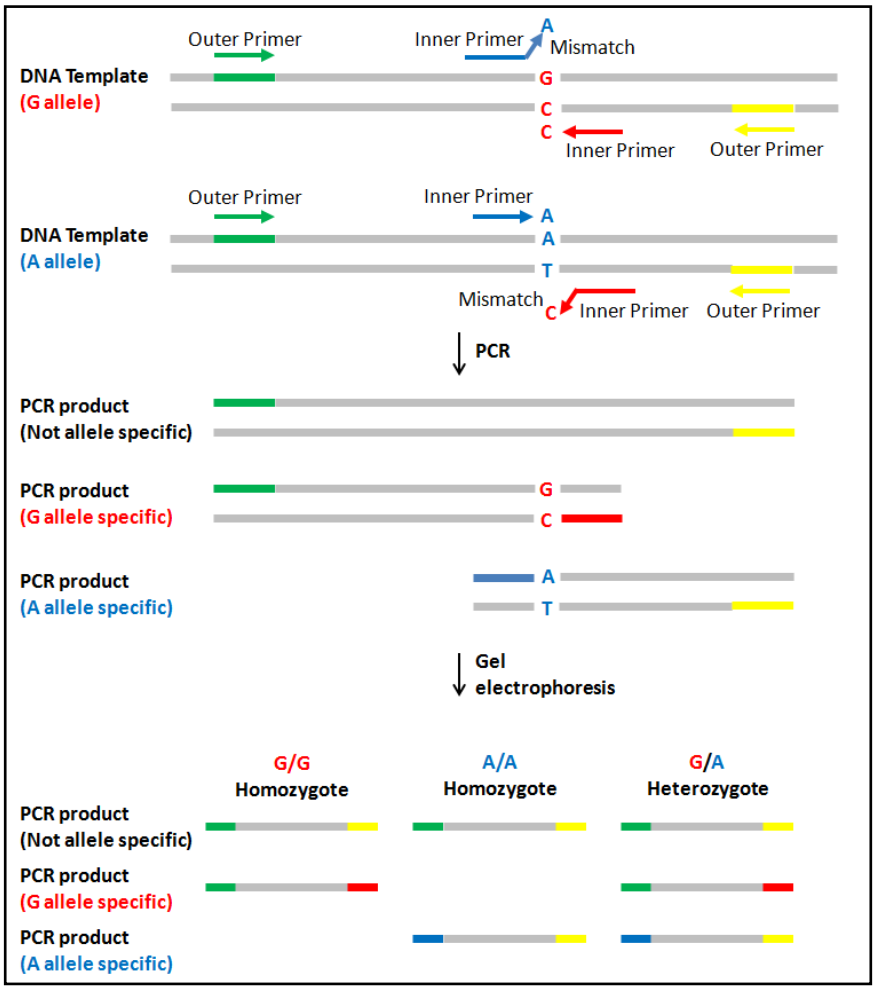


Figure 1.3. Schematic illustration of the tetra-primer ARMS-PCR method.

(Redrawn as adapted from Ye *et al.*, 2001)

Taqman SNP genotyping assay employ the second detection method and allows rapid, simple and specific way of allelic discrimination. It is ideal for genotyping applications including screening, association, candidate region, candidate gene, and fine-mapping studies. The assay consists of a pair of unlabeled PCR primers and at least one Taqman SNP genotyping assay employ the second detection method and allows rapid, simple and specific way of allelic discrimination. It is ideal for genotyping applications including screening, association, candidate region, candidate gene, and fine-mapping studies. The assay consists of a pair of unlabeled PCR primers and at least one allele-specific Taqman probe with FAM or VIC dye label on the 5' end, and minor groove binder (MGB) nonfluorescent quencher (NFQ) on the 3' end. The MGB molecule binds to the minor groove of the DNA helix and increases binding stability between probe and template. This permits the use of probes as short as 13 bases for improved mismatch discrimination and greater flexibility when designing assays for difficult or variable sequence. The principle of Taqman SNP genotyping assay is illustrated in **Figure 1.4**. Initially, the

fluorescence of the probe is quenched by NFQ. When the allele-specific probe hybridises with its complementary sequence on DNA template, 5' nuclease chemistry by means of exonuclease cleavage of the allele-specific 5' dye label takes place and generates the permanent assay signal. The coupling of real-time PCR and Taqman SNP genotyping assay enables rapid genotyping by amplification of the target DNA, hybridisation of probe, and detection of fluorescent signal.

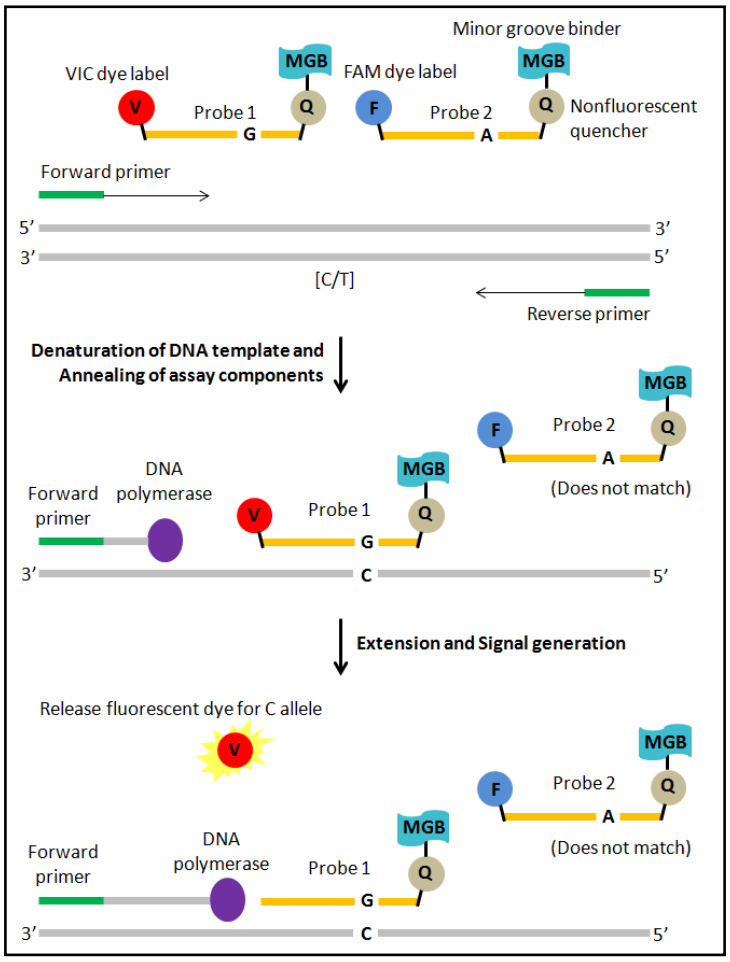


Figure 1.4. Schematic illustration of the principle of the Taqman SNP genotyping assay.

1.5.1.3 Linkage disequilibrium (LD)

Linkage disequilibrium is an important tool both at the end stages of positional cloning studies to map genes of particular interest and in reconstruction of population histories (Zhao *et al.*, 1999). With the rapid development of molecular biology, LD has been intensely used to facilitate the mapping of complex disease loci through GWAS (Ardlie *et al.*, 2002). Instead of having to examine each and every polymorphism, markers that are representative of the LD landscape of the region are selected and screened. The excluded markers are in high LD with one or a combination of selected markers. As such,

if the causal locus is not genotyped, the association with disease could still be assessed by correlating it with the genotyped marker loci (Wray, 2005). In short, LD measures the haplotype distribution at a pair of loci and subsequently describes an association between a pair of chromosomal loci in a population.

Linkage disequilibrium is the non-random association of alleles at adjacent loci (Ardlie *et al.*, 2002). When a particular allele at one locus is found closely located on the same chromosome with another allele at the second locus, they are most likely to be in disequilibrium. The commonly used measures in LD interpretation are D' and r^2. D is one of the earliest measures of disequilibrium that indicates the deviation of the observed haplotype frequencies from the expected. But due to its dependency on the frequencies of the alleles, Lewontin suggested normalising D by dividing it by the theoretical maximum for the observed allele frequencies, to obtain D' value (Lewontin, 1964). D' value ranges from 0 to 1, whereby D'=1 is called complete LD. This occurs if, only if, two SNPs have not been separated by recombination (or recurrent mutation or gene conversion) during the history of the sample (Ardlie *et al.*, 2002). In this case, there are at most 3 of the 4 possible haplotypes present in the population. On the other hand, when D'<1, it indicates that the complete ancestral LD has been disrupted. Since D' may be inflated in small sample size, hence r^2 is used as complementary to D'. R^2 is the correlation coefficient of the frequencies. It is more robust and is preferred over D' seeing that it is able to summarise both recombinational and mutational history compared with D' which measures only recombinational history. R^2 values fall between 0 and 1, and the case r^2=1 is known as perfect LD. Perfect LD happens if, and only if, the two SNPs have not been separated by recombination, but also have the same allele frequencies. In this case, observations at one marker provide complete information about the other marker, making the two redundant.

Linkage disequilibrium can be influenced by several factors: i) genetic drift; ii) population growth, iii) admixture or migration; iv) population structure; v) natural selection; vi) variable recombination rates across the genome; vii) variable mutation rates; and viii) gene conversion (Ardlie *et al.*, 2002).

1.5.1.4 Multifactor dimensionality reduction (MDR)

Multifactor dimensionality reduction method was developed by Ritchie, Moore, Hahn, and their team in the effort to solve the mathematical and computational problems

that hindered the detection, characterisation, and interpretation of gene-gene interactions or epistasis in the studies of human disease susceptibility (Ritchie *et al.*, 2001, Hahn *et al.*, 2003, Ritchie *et al.*, 2003, Hahn and Moore, 2004, Moore, 2004). It is inspired by the combinatorial-partitioning method, a data-reduction method for the exploratory analysis of quantitative traits (Nelson *et al.*, 2001). With MDR method, high-dimensional genetic data is collapsed into a single dimension and hence permitting the construction of gene-gene interactions in relatively small sample sizes. Briefly, multilocus genotypes are pooled into high-risk and low-risk groups, effectively reducing the genotype predictors from 'n' dimensions to one dimension. The new, one-dimensional multilocus-genotype variable is evaluated for its ability to classify and predict disease status through cross-validation and permutation testing (**Figure 1.5**). The MDR method is model free, whereby it does not assume any particular genetic model; and is nonparametric, in which it does not estimate any parameters (Ritchie *et al.*, 2001). Thus it is suitable to be used with genotyping data which is nonparametric.

In step 1 of **Figure 1.5**, a set of *n* genetic and/or discrete environmental factors is selected from the pool of all factors. In step 2, the *n* factors and their possible multifactor classes or cells are represented in *n*-dimensional space. The ratio of the number of cases to the number of controls is estimated within each multifactor class. In step 3, each multifactor cell in *n*-dimensional space is labelled either as "high-risk," if the cases:controls ratio meets or exceeds some threshold (e.g., $\geqslant$1.0), or as "low-risk," if that threshold is not exceeded. In this way, a model for both cases and controls is formed by pooling high-risk cells into one group and low-risk cells into another group. This reduces the *n*-dimensional model to a one-dimensional model (i.e., having one variable with two multifactor classes—high risk and low risk). In this initial implementation of MDR, balanced case-control studies are required. In step 4, the prediction error of each model is estimated by 10-fold cross-validation. Here, the data (i.e., subjects) are randomly divided into 10 equal parts. The MDR model is developed for each possible 9/10 of the subjects and then is used to make predictions about the disease status of each possible 1/10 of the subjects excluded. The proportion of subjects for which an incorrect prediction was made is an estimation of the prediction error. To reduce the possibility of poor estimates of the prediction error that are due to chance divisions of the data set, the 10-fold cross-validation is repeated 10 times, and the prediction errors are averaged.

Single best multifactor models are picked from among the models for each of the three- to *n*-factor combinations. Among this set of best multifactor models, the combination of loci and/or discrete environmental factors that minimises the prediction error is selected. Thus, the classification errors and the prediction errors estimated by 10-fold cross-validation are used to select the final multifactor model. Hypothesis testing for this final model can then be performed by evaluating the consistency of the model across cross-validation data sets—that is, how many times the same MDR model is identified in each possible 9/10 of the subjects. The reasoning is that a true signal (i.e., association) should be present in the data regardless of how they are divided.

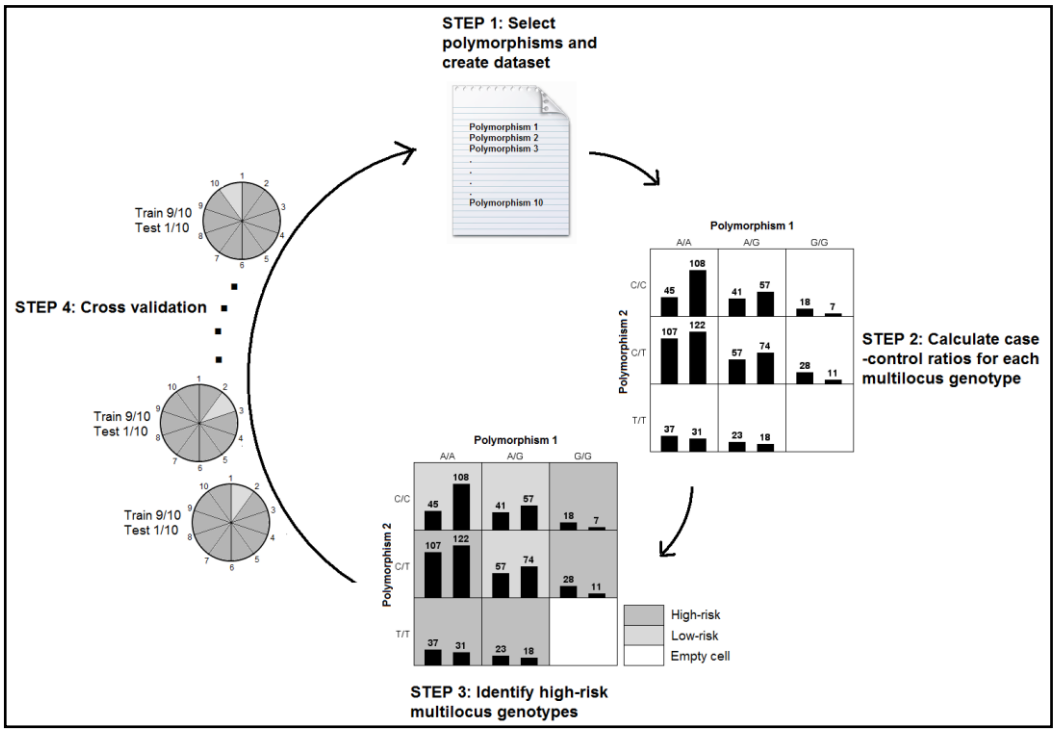


Figure 1.5. The principle and steps involved in the MDR method.

(Redrawn as adapted from Ritchie *et al.*, 2001)

1.5.2 Proteomics

The word "proteome" originated in 1994 when Marc Wilkins coined the term to describe the set of all PROTEins expressed by genOME (Wilkins *et al.*, 1996). While genomics and transcriptomics provide basic information on DNA sequence, regulatory elements, and gene expression, proteomics provides insight into quantitative information on the total protein profile of a cell, tissue or organism. Proteomics allows the level of protein expression to be evaluated and can be used to determine the presence of protein

isoforms and post-translational modifications (PTMs) or to examine protein-protein interaction (PPI) (Macarthur and Jacques, 2003). In brief, the aim of proteome analysis is to not only identify all proteins in a cell, but to also create a complete three-dimensional map of the cell showing the location proteins.

A proteome map is very complex and constituted by several thousands of proteins. The proteome is dynamic, not static as genome. The proteome of a cell will reflect the immediate environment in which it is studied (Graves and Haystead, 2002). Therefore, during an experiment, a series of samples is analysed, and the quantitative alterations of expression levels are monitored. This approach is now widely used in drug discovery, diagnostics, therapy, and agricultural research, where up- or down-regulation of proteins is investigated in cells that are stimulated by gene deletion or over-expression, drug treatment, nutrient withdrawal, or by physical or chemical stimulation (Westermeier and Naven, 2002).

1.5.2.1 Plasma and urinary proteomics

Plasma and urine are two commonly studied body fluids. Pathophysiological level of plasma and urine proteins may indicate certain diseased states and these proteins could be used as biomarkers for the diagnosis of the diseases or to develop more effective drugs against the target proteins with fewer side effects.

Human plasma is the primary clinical specimen that not only represents the largest and deepest version of the human proteome, but also contains all tissue proteins (as leakage markers) plus numerous immunoglobulin sequences. Plasma or serum protein profiles has been studied for the past 70 years, since before we knew the existence of genes (Anderson and Anderson, 2002), and plasma proteins related to various diseases such as multiple sclerosis (Rabiner *et al.*, 1954), haematological disorders (Mitbander *et al.*, 1958), and pulmonary tuberculosis (Radanov *et al.*, 1968) has been of interest since 1950's. From time to time, protein profile of plasma has been developed and improved in conjunction with the emergence of more advance technologies with higher resolution power. The nature of plasma having 90% of high abundant proteins (HAPs) and many proteins with multiple isoforms and post-translational modifications make the proteomic study of plasma proteins even more challenging (Lee *et al.*, 2006, Cho *et al.*, 2008). Special treatments for HAP removal or fractionation of plasma proteins are necessary to unshed lower abundant

proteins. No single method can be used to develop a complete protein profile of plasma. Two-dimensional electrophoresis (2-DE) is able to separate up to more than 1000 plasma proteins to date. MS approaches such as liquid chromatography–mass spectrometry (LC-MS), SELDI-TOF and multidimensional protein identification technology (MudPIT) permit resolution of even larger number of plasma proteins, up to a few thousands. However, different techniques may generate different sets of protein profile and it is required to combine a few techniques to produce a more complete plasma protein profile. Plasma Proteome Project (PPP) initiated by Human Proteome Organisation (HUPO) in 2002 was able to construct a publicly-available database with 3020 non-redundant proteins recruited from the pilot phase with 35 collaborating laboratories from 13 countries, using a wide variety of methods which included LC-MS/MS, MALDI-MS, fourier transform ion cyclotron resonance (FT-ICR)-MS, SELDI-MS, and 2-DE (Omenn *et al.*, 2005, States *et al.*, 2006).

Urine is composed of proteins filtered from plasma, as well those resulting from the physiological process of secretion form tubular epithelial cells of all segments along the nephron (Kerjaschki and Farquhar, 1982, Birn and Christensen, 2006). Given that urine is abundant and easily sampled without invasive procedures, it is considered the ideal specimen for biomarkers discovery in diseases. The studies on normal urine composition began in 1979, whereby the first 2-DE separation of urine proteins was reported by Anderson and co-workers (Anderson *et al.*, 1979a, Anderson *et al.*, 1979b). Starting from 1997, the existence of MS and evolution of methodological approaches to protein chemistry and detection has brought about extensive studies not only on the protein composition of normal, but also urinary biomarkers of a myriad of diseases, especially renal diseases such as diabetic glomerulopathy (Mischak *et al.*, 2004), acute renal injury (Nguyen *et al.*, 2005), and IgA nephropathy (Haubitz *et al.*, 2005), and other diseases including cancers (Ward *et al.*, 2008, Abdullah-Soheimi *et al.*, 2010, Liu *et al.*, 2010) and coronary artery disease (Zimmerli *et al.*, 2008, Delles *et al.*, 2010). Yet, the complex nature of human urine having low protein content and presence of salts tightly bound to proteins or other interferences molecules, such as glycosaminoglycan, may impede high-quality resolution and reproducibility of protein profiles in 2-DE analysis by creating problems such as smears. Hence, removal of these interference substances is essential. So far, Candiano (2010) reported a separation of 1118 spots in a normal urine samples, with 275 have been characterised as isoforms of 82 proteins while the others remain unknown.

1.5.2.2 Two-dimensional electrophoresis (2-DE)

Two-DE was first introduced by P.H. O' Farrell (1975) and J. Klose (1975). Two-DE is performed by coupling isoelectric focusing in the first dimension with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in the second dimension, which enables the separation of complex mixtures of proteins according to their isoelectric point (p*I*), molecular weight (MW), solubility, and relative abundance.

Before performing 2-DE, it is essential to make sure the sample preparation is appropriately done. Denatured, disaggregated, reduced, and solubilized proteins are very important to produce 2-D proteome maps with high resolution and also to ensure each spot representing an individual polypeptides (Gorg *et al.*, 2004). Thus, the lysis and rehydration solutions which are used to extract or dissolve proteins should contain certain components to assure complete solubilisation and denaturation of proteins prior to IEF: urea as denaturant; CHAPS for sample solubilisation and prevention of aggregation through hydrophobic interactions; dithiothreitol (DTT) as reducing agent; and carrier ampholytes (IPG buffer) for protein solubilisation by minimising aggregation due to charge-charge interaction. Protease inhibitors are required to inactive proteases in the sample that can result in degradation and subsequently cause artefact spots and loss of high MW proteins. Contaminants such as salt ions, polysaccharides, nucleic acids, lipids, phenolic compounds and some other insoluble materials must be removed to avoid inferences in isoelectric focusing (IEF).

Proteins are amphoteric molecules which carry positive, negative, or zero net charge depending on their surrounding pH. In IEF, the presence of pH gradient under the influence of an electric field causes a protein to move to the site in the gradient where its net charge is zero (p*I*). Thus IEF is able to separate proteins according to their p*I*. In the second dimension (SDS-PAGE), SDS, an anionic detergent, in the polyacrylamide gel masks the charge of the proteins to form anionic complexes, which have a roughly constant net negative charge per unit mass. This enables the proteins to migrate from cathode to anode under an electric field, and at the same time, separation of proteins according to their MWs across polyacrylamide gel without being affected by their own charges.

For visualisation of 2-DE gels, the most commonly used staining method is Coomassie Briliant Blue (CBB) which is low cost, easy to use, and highly compatible with most downstream protein analysis and characterisation methods such as mass spectrometry

(MS). The detection limit of classical CBB stain ranges from 200-500 ng protein per spot, which may not be sufficiently sensitive in the detection of low abundance proteins. However, recently, there have been many commercially available CBB stains on the market, with the detection limit being improved to below 20 ng and water is used to destain the gel instead. To detect proteins with much lower abundance, silver staining is normally employed. It is far more sensitive than CBB stain, with detection limit as low as 0.1 ng protein per spot. Nevertheless, silver stain is much less reproducible and may post some problems such as noise in MS. Some other staining methods which are also widely used include reverse staining, fluorescent staining (e.g. SYPRO and RuBPS dyes), and radiolabelling of protein.

1.5.2.2.1 Two-dimensional difference gel electrophoresis (2D-DIGE)

Two-D DIGE technique is more powerful than the classical 2-DE by combining novel propriety technologies in fluorescence, sample multiplexing, and image analysis. The types of CyDye Fluor Dyes are used in this technique: minimal dyes for normal applications, and saturation dyes for very small amounts of samples. CyDye Fluor minimal dyes have an NHS ester reactive group that will form a covalent bond with the epsilon amino group of lysine residues in proteins via an amide linkage. The dyes are made sure to label approximately 1-2% of lysine residues so that each labelled protein carries only one dye and is visualised as a single protein spot. The dyes afford sensitivity down to 25 pg protein per spot, and a linear response to protein concentration up to five orders of magnitude (10^5). The use of CyDye DIGE Fluor dyes, which are available in 3 dyes (Cy2, Cy3, and Cy5), enables multiplexing of up to three separate protein mixtures on the same. The incorporation of internal standard in every gel and randomization of samples eliminate gel-to-gel variation and thereby significantly increasing accuracy and reproducibility without needing to replicate gels for the same sample. The multiplexing ability of 2D-DIGE also allows accurate measure of very small protein differences with high confidence by using specially designed and fully automated image analysis softwares, i.e. DeCyder 2D software and ImageMaster 2D platinum software. In long run, this technique offers a time- and cost-effective, less laborious, and more accurate method in investigation of differentially expressed proteins.

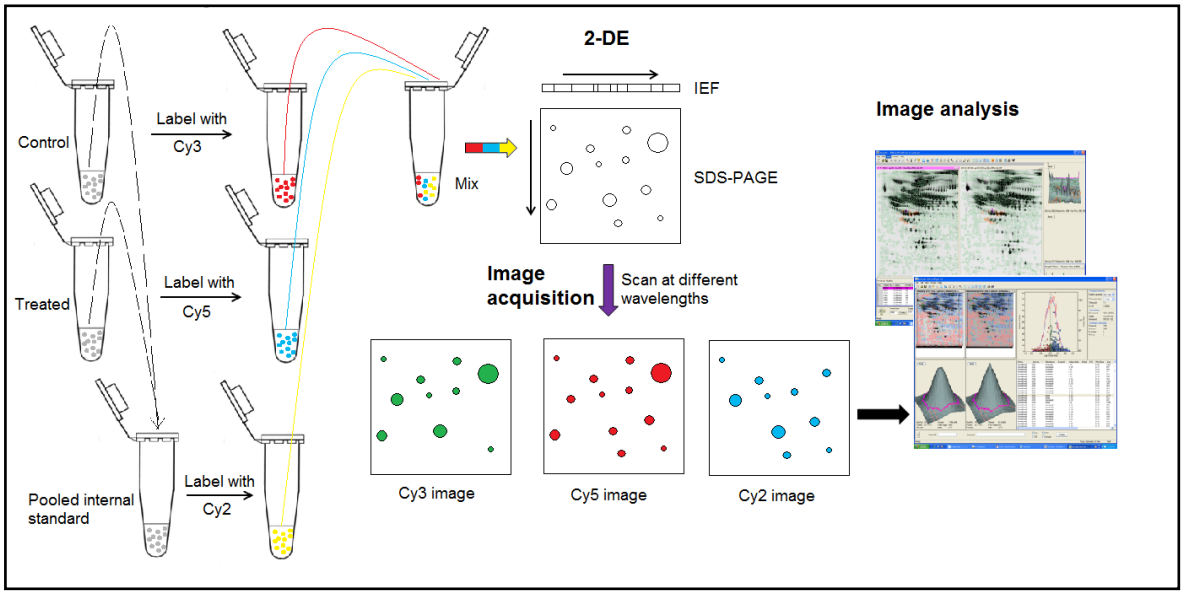


Figure 1.6. Brief workflow of 2D-DIGE technique. The samples and pooled internal standard labelled with Cydyes are mixed together and undergo 2-DE. The images obtained from samples labelled with Cy3, Cy5, and Cy2 show protein spots of green, red, and blue colours, respectively, when scan under their respective wavelengths. The overlay of the three images helps in comparison of protein profiles on the same gel. Spot detection and statistical analysis can be carried out using DeCyder 2D software.

(Redrawn as adapted from GE Healthcare)

1.5.2.3 Peptide and protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Mass spectrometry is by far the most common technique used in proteomic analysis for the identification of unknown proteins, although alternatives such as N-terminal amino acid analysis are also available. Mass spectrometry is an analytical technique that measures the MW of molecules based on the motion of a charged particle in an electric pr magnetic field. The sample molecules are ionised in the gas phase and separated according to their mass:charge ratio (m/z) (Westermeier and Naven, 2002). MS is gaining its popularity in the identification of protein excised from 2-DE gels given that it is very sensitive, requires very small amounts of samples (femtomole to attomole concentration), and allows high sample throughput.

Liquid chromatography-MS combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. It is highly sensitive and selective. It is usually applied in the detection and identification of

chemicals or proteins in a complex mixture. Electrospray ionisation (ESI) is the commonly used interface in LC-MS to produce ions from the liquid containing the analytes of interest, whereby the liquid is dispersed by electrospray into a fine aerosol of charged droplets which subsequently undergo evaporation and fission to release ions (Kebarle and Verkerk, 2009). The ability of tandem MS-MS to select certain ions for further fragmentation is very useful for proteomic analysis of complex samples where peptide masses may potentially overlap (Wysocki *et al.*, 2005).

Figure 1.7 illustrates the flow involved in peptide and protein identification using LC-MS/MS approach. The proteins from a sample are first proteolytically cleaved into small peptides. The digested sample is then subjected to LC-MS/MS, whereby peptides are ionized and selected ions are fragmented to produce MS-MS spectra. Computational tools such as database search are implemented to assign a peptide to each acquired MS-MS spectrum. Protein identification is achieved by compiling the results of all peptide assignments. The protein identification results need to be validated since the peptides may correspond to more than a single entry in the protein sequence database. Database search softwares nowadays normally come with autovalidation function, which greatly ease the process of protein identification.

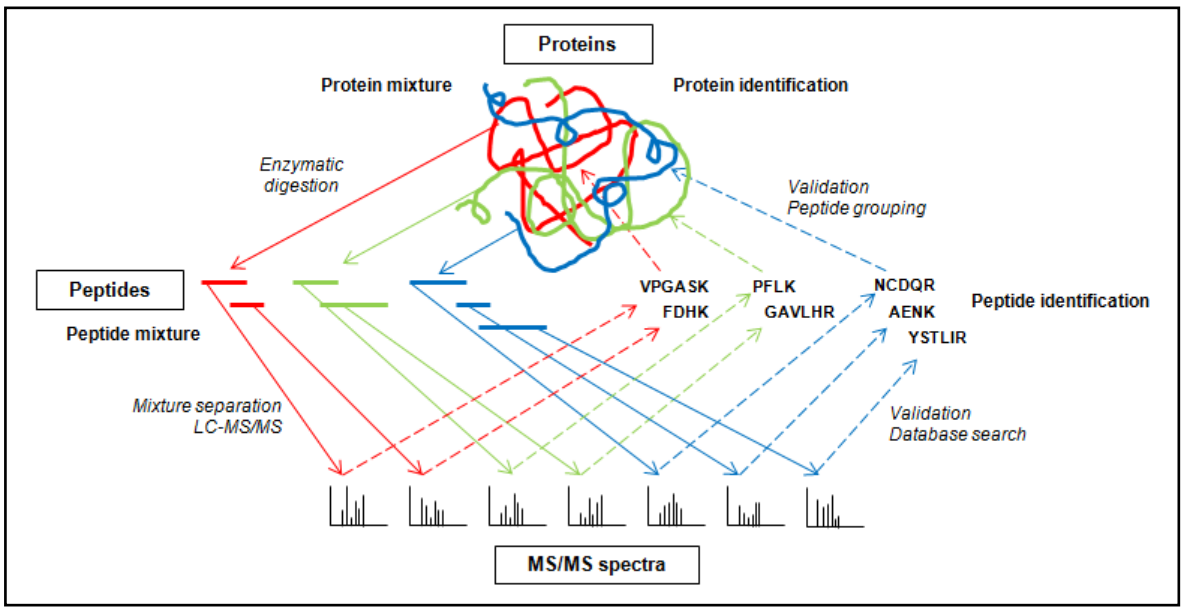


Figure 1.7. Experimental flow of protein identification from a complex mixture using LC-MS/MS approach.

(Redrawn as adapted from Nesvizhskii and Aebersold, 2004)

1.6 Significance and objectives of the study

Systemic lupus erythematosus is a heterogeneous, polygenic and severely debilitating disease. It is therefore important to investigate in a comprehensive way, the major genetic components, their expression in terms of proteins and biomarkers that underlie the molecular pathogenesis of this disease and its progression. While there may be several types of life threatening complications which affect the heart, lungs and central nervous system, a significant proportion of patients who present with SLE, eventually progress to LN and die of kidney failure. This is still a major cause of morbidity.

To date, many genetic loci have been reported for SLE. These loci include the MHC and genes that encode for cytokines, immune receptors and other major players. Of late, many new SNPs have emerged and been linked to SLE. These include polymorphisms occur in *TNFAIP3*, *STAT4*, and *IRF5*, which are genes involved in toll like receptor/interferon (TLR/IFN) signalling pathways. They participate in upstream and downstream pathways of type I IFN production. The *STAT4* gene consists of 24 exons that spread over a 120 kb region on chromosome 2q32.3. It encodes a transcription factor that mediates signals induced by IL-12, IL-23, and type I IFN and activates the production of IFN-γ and IL-17. It also directs the differentiation of helper T cells toward the proinflammatory T-helper type 1 and T-helper type 17 lineages that have been shown to play a critical role in the pathogenesis of SLE. The *STAT4* null allele in lupus-prone mouse model confers reduced autoantibody production and glomerulonephritis, indicating that *STAT4* may be involved in multiple SLE-associated phenotypes (Xu *et al.*, 2006). There are a few studies involving *STAT4*-deficient lupus-prone mice which demonstrate the role of *STAT4* in autoantibody production only (Jacob *et al.*, 2003, Singh *et al.*, 2003). Polymorphisms in the *STAT4* gene have been found to be strongly associated with SLE susceptibility, in particular rs7574865 (Kawasaki *et al.*, 2008, Yuan *et al.*, 2010).

TNFAIP3, or tumor necrosis factor alpha-induced protein 3 gene, encodes A20 protein which is a negative regulator of the NF-κB signalling pathway, an essential pathway in the pathogenesis of SLE. A20 is a ubiquitin-editing enzyme required for effective termination of NF-κB-mediated proinflammatory responses induced by TLRs, TNF receptor, IL-1 receptor and NOD2 (Lee and Bae, 2010). A meta-analysis and imputation study identified a 109 kb risk haplotype spanning *TNFAIP3* region with LN and hematologic manifestation (Bates *et al.*, 2009). A non-synonymous mutation (c.380T>G), rs223092, in *TNFAIP3* gene which causes phenylalanine-to-cysteine change at position

127 of A20 protein has been consistently linked with SLE various ethnic groups. Another candidate gene, *IRF5*, which is IFN regulatory factor 5, is a transcription factor that mediates inflammatory and immune responses (Honda *et al.*, 2005). This factor stimulates the production of the proinflammatory cytokines TNF-α, IL-12 and IL-6 following TLR signalling as well as transactivation of type I IFN and IFN-induced genes (Barnes *et al.*, 2001, Takaoka *et al.*, 2005). Polymorphisms in *IRF5* cause functional changes in messenger RNA, which in turn alter *IFR5*-mediated transcription resulting in elevated SLE risk (Graham *et al.*, 2006).

Human leucocyte antigen (HLA)-DRB1/HLA-DQA1 rs9271366 and *HLA-DQB1/HLA-DQA2* rs9275328 are two newly reported HLA variants and Yang and co-workers (2010) were the first to associate both SNPs with Asian SLE patients. Single nucleotide polymorphism (SNP) rs9271366 is located in the intergenic region between the *HLA-DRB1* and *HLA-DQA1* (Ruiz-Narvaez *et al.*, 2011). All these and possibly new players, which this study hopes to elucidate in the process of investigations, are believed to be central for proper function of a normal immune system. It is imperative that we understand the underlying biology of what distinguishes 'self' from 'non-self'. This knowledge will inform efforts directed at managing and finding more effective therapies for SLE.

Some of the above genetic polymorphisms seem to feature only in Caucasian patients, others in non-Caucasians such as Chinese, Japanese, Hispanics, and African Americans. So far, association between these SNPs and SLE has not been reported in Malaysians (i.e. Chinese, Malays, and Indians). Therefore, it is essential to look at whether or not these SNPs display any association with SLE in these ethnic groups so that we can gain new knowledge of the pathogenesis and progress. Samples recruited will encompass 50% Chinese, 40% Malays, and 10% Indians, and these resemble the SLE distribution in Malaysian population. It would be expected that if these SNPs were major players in the SLE scenario, they would feature across all ethnic groups. Minor SNPs having unique geographic and ethic distributions are envisaged to contribute more toward mitigating this disease than directly causing it.

Proteins play very important role in the immune response. Differential regulation and uncontrolled expression of proteins as a consequence of mutation or other aberrations result in disease. Again, many proteins have been associated with diagnosis and are hallmarks of SLE. Several studies using proteomic approach have been conducted by

Suzuki *et al.* (2007), Dai *et al.* (2008), Zhang *et al.*, (2011) and they reported several predictive biomarkers of SLE as well as LN. However, these studies mostly involve discovery of high-abundant proteins (HAP) such as albumin and ceruloplasmin, and these proteins have been long recognised to be SLE-associated. Furthermore, no protein has been found to be solely associated with either SLE or LN to date. All potential biomarkers reported are shared by other autoimmune diseases such as rheumatoid arthritis, Sjögren's syndrome, and diabetes mellitus type 1. Thus, in this study, the searching for HAP-depleted plasma proteins, as well as urine proteins, that are associated with SLE and LN, will be attempted using a more advance techniques coupling 2D-DIGE and LC-MS/MS. Same techniques will be applied in the longitudinal study of patients under mycophenolate mofetil (MMF) treatment in order to gain more understanding on the mechanism of actions of MMF in LN.

It is hypothesised that SNPs occurred in *STAT4*, *TNFAIP3*, and *IRF5*, as well as *HLA-DRB1/HLA-DQA1 rs9271366* and *HLA-DQB1/HLA-DQA2 rs9275328* are associated with susceptibility of Malaysian SLE. In addition, it is also hypothesised that SLE or LN patients differentially express proteins that are more specific to the diseases than the other autoimmune diseases. The significance of the study mentioned above calls for the following objectives:

Objectives of the project

1. To investigate the association of genetic polymorphisms in *HLA-DRB1/DQA1*, *HLA-DQB1/DQA2*, and genes involved in TLR/IFN signalling pathways (i.e. *TNFAIP3*, *STAT4*, and *IRF5*) with SLE and LN of the three major ethnic groups in Malaysia.

2. To identify plasma and urine proteins that are differentially expressed in SLE and LN patients.

3. To generate and study protein-protein interactions between proteins that are associated with each class of LN.

4. To study the effect of mycophenolate mofetil (MMF) on LN by carrying out a longitudinal study on LN patients under MMF treatment and identifying MMF-associated plasma and urine proteins.

Expected outcomes

1. Significant associations of SNPs in *HLA-DRB1/DQA1*, *HLA-DQB1/DQA2*, *TNFAIP3*, *STAT4*, and *IRF5* region with SLE of the three major ethnic groups in Malaysia.

2. New knowledge of SNPs associated with SLE in the Malaysians compared to in Hispanics, Caucasians, and African Americans.

3. Newly discovered proteins associated with each class of LN and SLE without kidney involvement, as well as MMF treatment.

4. Pathways or networks involved in the pathogenesis of each class of LN and SLE without kidney involvement.

Chapter 2

Materials and Methods

2.1 Genotyping Study

2.1.1 Subjects and sample collection

Blood samples were collected from patients diagnosed with SLE according to 4 out of the ACR criteria and healthy volunteers recruited at the University of Malaya Medical Centre (UMMC), Kuala Lumpur, in compliance with requirements as stipulated by the UMMC Medical Ethics Committee (UMMC Ethics Approval Code: 733.19) (**Appendix I**). A total of 910 Malaysians, which comprised of 380 SLE patients and 530 healthy controls were recruited. However, in order to fulfil Hardy-Weinberg equilibrium (HWE), as well as after excluding those that could not have their results reproduced in the genotyping study, a final total of 790 subjects (360 patients and 430 healthy controls) were included in this study. The sample size was determined based on the number of samples available from the UMMC, as well as calculation using parameters such as confidence interval of 5%, confidence level of 95%, population size of 30000000, and response distribution of 50% (http://www.raosoft.com/samplesize.html). The minimum recommended sample size was 385. Given that the clinics were small and the time limited for this study, it was not practical to select only patients and controls that were age- and sex-matched.

2.1.2 DNA extraction

Peripheral blood was collected from each patient or healthy control into a 3 ml EDTA tube (BD), as summarised in **Figure 2.1.1**. Conventional phenol-chloroform DNA extraction method was carried out to isolate DNA from peripheral blood samples. Three millilitres of each blood sample was added into 40 ml of cold 1X red cell lysis buffer (RCLB) in a 50 ml centrifuge tube. The solution was mixed gently by inversion for 1 min and then centrifuged at 3500 rpm for 10 min at 10 ºC. Supernatant was discarded and pellet was resuspended with 20 ml of cold 1X RCLB. The solution was mixed again by inversion

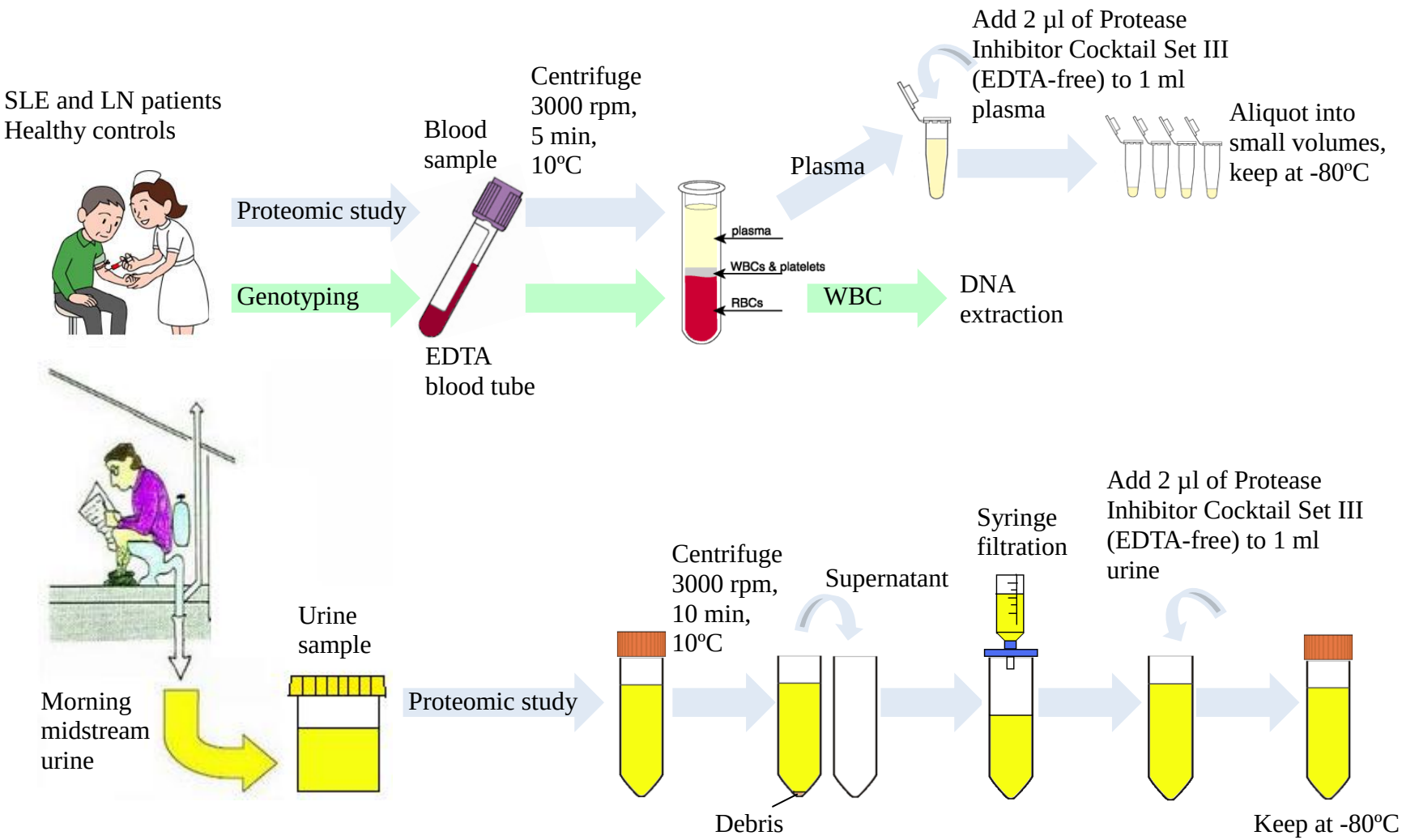


Figure 2.1.1. Flowchart summarizing the sample collection and processing for genotyping and proteomic study.

for 1 min and centrifuged at 3500 rpm for 10 min at 10 ºC. Supernatant was removed and pellet was dried by inverting the tube for 1 min. After that, the pellet was resuspended with 160 µl 5X proteinase K buffer, 40 µl 20% SDS (w/v), 40 µl 10 mg/ml proteinase K and 300 µl double distilled water (ddH_2O). The mixture was then incubated overnight at 37 ºC with gentle shaking at 60 rpm. On the next day, the mixture was cooled down to room temperature before addition of 200 µl of 6 M NaCl. The mixture was vortexed for 15-30 sec to mix and subsequently transferred into two 1.5 ml microcentrifuge tubes. Eight hundred microlitres of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (Amresco, Solon, OH, USA) were added into each tube and the mixture was vortexed until it became milky. The mixture was centrifuged at 13000 rpm for 20 min at 10 ºC, and three layers were formed after the centrifugation step. The top aqueous layer was transferred to a new 1.5 ml microcentrifuge tube and added with 900 µl chilled absolute ethanol. The mixture was inverted for 2 min and then centrifuged at 13000 rpm for 5 min at 10 ºC. After pouring off supernatant, the pellet was rinsed with 1 ml of 70% (v/v) ethanol and followed by centrifugation at 13000 rpm for 1 min at 10 ºC. Supernatant was removed and the remaining DNA pellet was air-dried or dried in a speed vac. Finally, the DNA pellet was dissolved in 100 µl of TE buffer added with 2 µl of RNase A (Fermentas, Ontario, Canada) and incubated for 30 min at 37 ºC before it was stored at -70 ºC. The concentration and purity of the extracted DNA samples were quantified by measuring their absorbance values at 260 nm and 280 nm via a NanoPhotometer (Implen, München, Germany).

2.1.3 SNPs for investigation

A total number of nine SNPs were selected to study their associations with SLE in Malaysian population. Among the SNPs, three of them were located in *STAT4* region, two in *TNFAIP3* region, another two in *IRF5* region, and the final two in *HLA* region. The information of the SNPs has been summarised in **Table 2.1.1**. The selection of SNPs was based on the findings reported by Han *et al.*, (2009), Cai *et al.*, (2010), and Yang *et al.*, (2010), whereby the selected SNPs were found to be significantly associated with SLE.

2.1.4 Primer design

Inner and outer primers for amplifying each SNPs were designed using an internet accessible program at http://cedar.genetics.soton.ac.uk/public_html/primer1.html designed by Ye *et al.* (2001). For each SNP, target DNA sequence was inputted, the polymorphic site was specified, and the criteria for the primers (*T*m, %GC, length, and complementarity) and product sizes. The primer sequences were listed in **Table 2.1.2**.

Table 2.1.1. SNPs that were investigated in this study on their associations with SLE.

Genes	SNP	Chromosome	Position	Alleles	References
STAT4	rs7574865	2	191672878	G/T	(Han *et al.*, 2009, Yang *et al.*, 2010)
	rs10168266	2	191644049	C/T	(Yang *et al.*, 2010)
	rs7601754	2	191648696	A/G	(Yang *et al.*, 2009)
TNFAIP3	rs2230936	6	138237759	T/G	(Han *et al.*, 2009,
	rs3757173	6	138231847	T/C	Cai *et al.*, 2010, Yang *et al.*, 2010)
HLA-DRB1/ HLA-DQA1	rs9271366	6	32694832	A/G	(Yang *et al.*, 2010)
HLA-DQB1/ HLA-DQA2	rs9275328	6	32774800	C/T	
IRF5	rs4728142	7	128361203	G/A	(Yang *et al.*, 2010)
	rs729302	7	128356196	A/C	

2.1.5 Tetra-primer amplification refractory mutation system–polymerase chain reaction (ARMS-PCR)

Tetra-primer ARMS-PCR was performed in the genotyping of rs10168266 and rs7601754 in *STAT4* region, rs2230926 and rs3757173 in *TNFAIP3* region, and rs4728142 in *IRF5* region. The procedure for performing tetra-primer ARMS-PCR was modified according to that described by Ye *et al.* (2001). For each PCR reaction, a total volume of 10 µl PCR mixture consisting of 50 ng of template DNA, 10mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08% (v/v) Nonidet P40, 0.2 mM of dNTPs (Fermentas), appropriate concentration of $MgCl_2$, appropriate concentration of inner and outer primers and 0.75 U of *Taq* DNA polymerase (Fermentas). The concentration of $MgCl_2$ and inner and outer primers used for amplification of each SNP were summarised in **Table 2.1.2**.

In order to increase the specificity of amplification, the mixture was then subjected to touchdown PCR. It was incubated for 5 min at 95ºC, followed by 10 cycles of

Table 2.1.2.PCR primers*.

SNP	primer sequence	*T*m	Final concentration	Inner/outer primers ratio	Mg^{2+}	Annealing temperature
STAT4						
rs10168266	Forward inner primer (T allele) (29 bp) 5’-CAAAGTAGTAGCTATTGACTACATGAGAT	57 ºC	1.0 µM	4	2.5 mM	55 ºC
	Reverse inner primer (C allele) (27 bp) 5’-GTTATTACTACGGGTGGGTAGACATTG	62 ºC	1.0 µM			
	Forward outer primer (28 bp) 5’-AAAAGTATAGAATTTGGAGGAAGAGAGT	59 ºC	0.25 µM			
	Reverse outer primer (28 bp) 5’-TATTGGGGTATACTGAAAAGAAAGAGTA	59 ºC	0.25 µM			
rs7601754	Forward inner primer (A allele) (21 bp) 5’-GGGTGAAGAAAAGGAACTCCA	60 ºC	1.0 µM	5	1.25 mM	55 ºC
	Reverse inner primer (G allele) (23 bp) 5’-CAAGGTCTTAGTATCATCTTGGC	57 ºC	1.0 µM			
	Forward outer primer (5' - 3') (28 bp) 5’-GGAGGTGATTACTATATTTCTAGGCTAA	58 ºC	0.2 µM			
	Reverse outer primer (5' - 3') (27 bp) 5’-AAAAATTAAAAATTAGTTGGCTATGGT	58 ºC	0.2 µM			
TNFAIP3						
rs2230936	Forward inner primer (G allele) (28 bp) 5’-CAGACTTGGTACTGAGGAAGGCGCTATG	69 ºC	1.0 µM	4	2.5 mM	62 ºC
	Reverse inner primer (T allele) (23 bp) 5’-GTCTGTTTCCTTGAGCGTGCCGA	69 ºC	1.0 µM			
	Forward outer primer (5' - 3') (28 bp) 5’-CTGAAAACCTTTGCTGGGTCTTACATGC	69 ºC	0.25 µM			
	Reverse outer primer (5' - 3') (29 bp) 5’-GACCTAGTCCATCAGATGCTACCAGAGGG	69 ºC	0.25 µM			

rs3757173	Forward inner primer (T allele) (26 bp) 5’-GACCTTATTCCCTTCCCTGAAATGAT	64 ºC	1.0 µM	4	2.5 mM	53 ºC
	Reverse inner primer (C allele) (27 bp) 5’-CCTTAGCTGCAGACTAAGGTGGTATTG	64 ºC	1.0 µM			
	Forward outer primer (5' - 3') (28 bp) 5’-TTAAACCATTCAGTCCCCTAGAATAGCA	64 ºC	0.25 µM			
	Reverse outer primer (5' - 3') (28 bp) 5’-TAAAATCTTCCTACTGCCCATCTCTTTC	64 ºC	0.25 µM			
IRF5						
rs4728142	Forward inner primer (A allele) (26 bp) 5’-GTCACACCCCAAAAAGCTCTGAGACA	68 ºC	2.0 µM	5	1.25 mM	55 ºC
	Reverse inner primer (G allele) (26 bp) 5’-CCTTCCTCCCCATTTCTTACTAACCCC	68 ºC	2.0 µM			
	Forward outer primer (5' - 3') (28 bp) 5’-GAAAGGTGGAGACTCCGAGTGTAGAGGT	68 ºC	0.2 µM			
	Reverse outer primer (5' - 3') (28 bp) 5’-GACAGAGCGATACTCCGTCTCAAAAGAA	68ºC	0.2 µM			

* developed independently during the course of this work, hitherto unpublished. © ChaiHwaChia

denaturation at 95ºC, annealing at temperature 10ºC higher than appropriate annealing temperature (**Table 2.1.3**) in the first cycle and decreased by 1ºC per cycle, and extension at 72ºC. After that, the reaction was continued by 20 cycles of denaturation at 95ºC, annealing at appropriate temperature (**Table 2.1.3**) and extension at 72ºC. The reaction was ended with final extension at 72ºC for 10 min. The duration for each step was also summarised in **Table 2.1.3**.

2.1.6 Agarose gel electrophoresis

After completing PCR, two microlitres of 6X loading dye (Fermentas) were added to 10 µl of each PCR product and loaded into one of the wells on 2% (w/v) agarose gel containing 0.5 µg/ml of ethidium bromide. PCR product migrated from cathodic to anodic end in the presence of 1X TBE buffer (1st BASE, Selangor, Malaysia), under voltage of 150 V for 20 min. The gel was transferred into a gel documentation unit (Major Science, Saratoga, CA, USA) for visualisation under UV and image acquisition.

2.1.7 Real-time PCR

Predesigned and custom designed TaqMan SNP genotyping assays were used to genotype SNPs where tetra primers could not be designed for ARMS-PCR (probe ID: *STAT4* rs7574865, C_29882391_10; *IRF5* rs729302, C_2691216_10; *HLA-DRB1/HLA-DQA1* rs9271366, C_33416976_20; *HLA-DQB1/HLA-DQA2* rs9275328, AX39XV7; Applied Biosystems, Foster City, CA, USA). Fifty nanograms of template DNA was mixed with 2X Taqman GTXpress master mix (Applied Biosystems) and 20X Taqman genotyping assay (Applied Biosystems) to make up to a total volume of 10 µl. Real-time PCR reaction was initiated with pre-PCR read step at 60 °C for 1 min, followed by DNA polymerase activation at 95 °C for 20 s, 40 cycles of denaturation (95 °C for 3 s) and annealing/extension (60 °C for 30 s), and ended with a final extension step at 60 °C for 1 min. Fluorescence was detected using an Applied Biosystems 7500 Fast Real-Time PCR System. The results were verified by sequencing.

Table 2.1.3. Touchdown PCR conditions*.

SNP	Initial denaturation	First 10 cycles	Remaining 20 cycles	Final extension	Amplicon size
STAT4					
rs10168266	95ºC, 5 min	Denaturation: 95ºC, 45 sec Annealing: 65-56ºC, 45 sec Extension: 72ºC, 45 sec	Denaturation: 95ºC, 45 sec Annealing: 55ºC, 45 sec Extension: 72ºC, 45 sec	72ºC, 10 min	257 bp (T allele) 369 bp (C allele) 571 bp (from two outer primers)
rs7601754	95ºC, 5 min	Denaturation: 95ºC, 30 sec Annealing: 65-56ºC, 30 sec Extension: 72ºC, 30 sec	Denaturation: 95ºC, 30 sec Annealing: 55ºC, 30 sec Extension: 72ºC, 30 sec	72ºC, 10 min	324 bp (A allele) 178 bp (G allele) 459 bp (from two outer primers)
TNFAIP3					
rs2230936	95ºC, 5 min	Denaturation: 95ºC, 45 sec Annealing: 72-63ºC, 45 sec Extension: 72ºC, 45 sec	Denaturation: 95ºC, 45 sec Annealing: 62ºC, 45 sec Extension: 72ºC, 45 sec	72ºC, 10 min	237 bp (G allele) 176 bp (T allele) 362 bp (from two outer primers)
rs3757173	95ºC, 5 min	Denaturation: 95ºC, 45 sec Annealing: 63-54ºC, 45 sec Extension: 72ºC, 45 sec	Denaturation: 95ºC, 45 sec Annealing: 53ºC, 45 sec Extension: 72ºC, 45 sec	72ºC, 10 min	150 bp (C allele) 270 bp (T allele) 371 bp (from two outer primers)
IRF5					
rs4728142	95ºC, 5 min	Denaturation: 95ºC, 30 sec Annealing: 65-56ºC, 30 sec Extension: 72ºC, 30 sec	Denaturation: 95ºC, 30 sec Annealing: 55ºC, 30 sec Extension: 72ºC, 30 sec	72ºC, 10 min	204 bp (A allele) 118 bp (G allele) 270 bp (from two outer primers)

* developed independently during the course of this work, hitherto unpublished. © ChaiHwaChia

2.1.8 Statistical analyses

A. Polymorphisms and SLE risk

Allele and genotype frequencies were calculated, followed by performance of the χ^2 goodness-of-fit test to evaluate whether or not the observed genotype frequencies of each polymorphisms were departures from (HWE) in SLE patients and control subjects separately (*p* values >0.05). An open access HWE calculator developed by Rodriguez *et al.*(2009) was used. As Malaysia is a multiracial country, Fisher's exact test was conducted on 2x2 contingency table using SPSS software to assess the association of each SNP with SLE susceptibility in the Malaysian population as a whole, as well as according to Malay, Chinese or Indian ethnicity. *P* values <0.05 were regarded as significant and odds ratios (OR) with 95% confidence intervals were calculated. Adjusted ORs were computed using logistic regression, whereby major allele and major homozygous genotype of each SNP was set as reference group and their ORs were adjusted to 1.

B. Linkage disequilibrium (LD)

Single nucleotide polymorphisms (SNPs) that were found to be significantly associated with SLE in the **Section A** was assessed for their LD by using Haploview 4.2, an open-source software downloaded from http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview (Barrett *et al.,* 2005). LD plot, D', and r^2 values between the SNPs, haplotype frequencies and odds ratios, and permutation *p* values (for 10000 replicates) were obtained from the analysis.

C. SNP-SNP interaction

To detect, characterise, and interpret interactions or epistasis between the SNPs significantly associated with SLE (**Section A**), a nonparametric and genetic model-free method termed multifactor dimensionality reduction (MDR) test was carried out using open-source MDR software 2.0 beta 8.4 developed by Moore *et al.*(2006). In MDR, data are randomly divided into a training set (9/10 of the data) and an independent testing set (1/10 of the data) for cross-validation consistency (CVC). Once the best combination of

factors is identified, the final step of MDR is to determine which genotypes are of high-risk or low-risk. The best combination of SNPs that concurrently hit the highest balanced accuracy and the highest average CVC was identified in this study. Interaction among SNPs was evaluated from the resulting entropy and combinations of genotypes that were predicted to bring about SLE were also identified.

The overall workflow designed for genotyping study was summarised in flowchart shown in **Figure 2.1.2**.

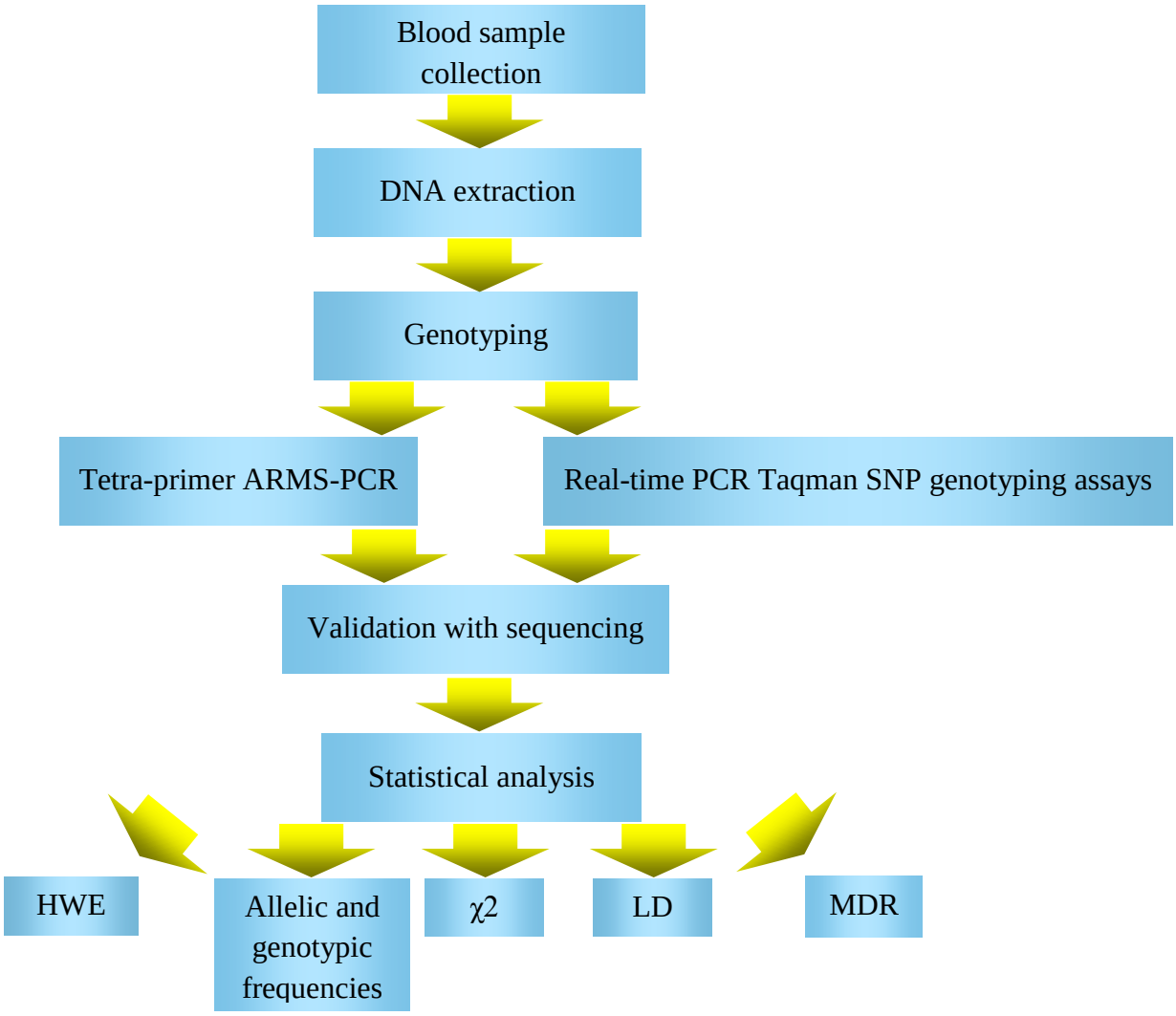


Figure 2.1.2. Flowchart summarising the workflow of genotyping study.

2.2 Proteomic Study

2.2.1 Subjects

A. Normal healthy individuals vs. SLE patients

Blood and urine samples were collected from patients diagnosed with SLE according to 4 out of ACR criteria and healthy volunteers recruited at the University of Malaya Medical Centre (UMMC), Kuala Lumpur, in compliance with requirements as stipulated by the UMMC Medical Ethics Committee (UMMC Ethics Approval Code: 733.19) (**Appendix I**). Patients being recruited included those with SLE but without kidney involvement, as well as those with lupus nephritis (LN) of different classes and in remission. Due to small sample size for the duration of the study, it was not practical to select only age or sex matched subjects.

B. Treatment follow-up

Two LN class III patients, a 55-year-old female and a 22-year-old male, who were undergoing mycophenolate mofetil (CellCept) treatment were followed up. Serial plasma and urine samples were collected from a total of five visits: first visit being diagnosed as LN and before treatment, subsequent four after-treatment visits with interval of around two months between two visits.

2.2.2 Sample collection

Peripheral blood was collected from each patient or healthy control in a 3 ml EDTA tube (BD), as shown previously in **Figure 2.1.1**. Blood sample was centrifuged immediately at 3000 rpm for 5 min at 10 ºC to separate plasma from blood cells. Plasma was transferred to new microcentrifuge tubes, where 2 µl of Protease Inhibitor Cocktail Set III (EDTA-free) (Calbiochem, La Jolla, CA, USA) was added to every 1 ml of plasma so that plasma would contain 0.5 µM AEBSF, 0.16 µM aprotinin, 10 µM bestatin, 3 µM E-64, 4 µM leupeptin, and 2 µM pepstatin A. Plasma was aliquoted into small volumes of 50 µl and kept at -80ºC until further use.

Morning midstream urine was collected from each subject in a urine container. The urine was then poured into a 50 ml centrifuge tube and spun down at 3000 rpm for 10

min at 10 ºC in order to bring down any possible debris. The cleared urine was then transferred to a new 50 ml centrifuge tube and filter-sterilized with 0.45 µm cellulose acetate membrane syringe filter (Sartorius Stedim Biotech, Aubagne Cedax, France). Every 1 ml of the filtered urine was added with 2 µl of Protease Inhibitor Cocktail Set III (EDTA-free) (Calbiochem) and stored at -80 ºC until further use.

2.2.3 Plasma high-abundant proteins (HAPs) removal

Since plasma contained at least 14HAPs that would potentially obscure the appearance of lower-abundant proteins on two-dimensional electrophoresis (2-DE) gels, depletion of HAPs was performed using Agilent Human 14 Multiple Affinity Removal System spin cartridge (Agilent Technologies, Santa Clara, CA, USA). Proteins that were removed included albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha-2-macroglobulin, alpha-1-acid glycoprotein, IgM, apolipoprotein A-I, apolipoprotein A-II, complement C3, and transthyretin. The procedure began with diluting 8-10 µl of each plasma sample with Buffer A to a final volume of 200 µl and filtering through a 0.22- µm spin filter (Agilent Technologies) by centrifugation at 10000 rpm for 5 min to prevent clogging of spin cartridge frits. Then filtered plasma sample was added to Agilent Human 14 Multiple Affinity Removal System spin cartridge (Agilent Technologies), which was already placed in a screw-top collection tube, and centrifuged for 1 min at 100 x g to collect the flow-through fraction (F1). The spin cartridge was removed from the centrifuge and allowed to sit for 5 min at room temperature. Four hundred microlitres of Buffer A was added to the top of the resin bed and centrifuged for 2.5 min at 100 x g for washing purpose. The flow-through fraction was collected into the same F1 collection tube. The spin cartridge was placed into a new collection tube for second washing step, whereby another 400 µl of Buffer A was added to the top of the resin bed followed by centrifugation for 2.5 min at 100 x g. The flow-through fraction was collected as F2. The spin cartridge was removed from the F2 collection tube and Luer-Lock adaptor was attached to the cartridge top. A 5-ml plastic Luer-Lock syringe (labelled “B”) filled with 2.5 ml of Buffer B was attached to the spin cartridge via Luer-Lock adapter and Buffer B was slowly pushed through the spin cartridge in order to elute bound HAPs into a new collection tube. Finally, the resin bed was re-equilibrated with 5 ml of Buffer A using a 5-ml syringe (labelled “A”). The entire process was repeated for four times for each plasma sample in order to obtain sufficient amount of HAP-depleted proteins for 2-DE. For storage, the resin

bed was left wet with Buffer A and a layer of Buffer A was left above the top frit. The spin cartridge was stored at 4ºC by having its both ends tightly recapped. Again, every 1 ml of flow-through fractions F1 and F2 that contained the HAP-depleted proteins was added with 2 µl of Protease Inhibitor Cocktail Set III (EDTA-free) (Calbiochem) and was stored at -80ºC until used. The same procedure was also applied to the eluent that contained HAPs.

2.2.4 Sample concentration

Flow-through fractions F1 and F2 for each sample were combined and concentrated using Vivaspin 2 (Sartorius Stedim Biotech, Goettingen, Germany) with 5000 molecular weight cutoffs. Before use, membranes were pre-rinsed in ddH_2O and fitted to concentrator. The concentrator was then filled up to 3 ml of solutions F1 and F2 and centrifuged at 4000 x g for 30 min at 10 ºC or until the sample volume became 200 µl. The concentrated sample was reversed spun into the concentrate recovery cap at 4000 x g and 10ºC for 5 min and transferred into a new microcentrifuge tube. The sample was kept at -80 ºC until further use.

As for urine, Vivaspin20 (Sartorius Stedim Biotech) with 5000 molecular weight cutoffs was used instead. The membranes fitted to concentrator was pre-rinsed with dH_2O and subsequently, the concentrator was filled up to 25 ml of urine followed by centrifugation at 4000 x g and 10 ºC until the sample volume became 200 µl. The concentrated urine sample was transferred into a new mirocentrifuge and kept at -80 ºC until further use.

2.2.5 Sample clean-up

The procedure was performed as recommended by manufacturer using 2-D Clean-Up Kit (GE Healthcare, Uppsala, Sweden). All steps were carried out in an ice bucket unless otherwise specified. Three hundred microlitres precipitant was added to 100 µg protein sample in a 1.5 ml microcentrifuge tube. The solution was mixed well by vortexing or inversion and left on ice for 15 min. A volume of 300 µl of co-precipitant was then added to the mixture and mixed briefly by vortexing. The tube was centrifuged at 13,000 rpm for 5 min at room temperature and removed from the centrifuge as soon as the centrifugation was complete. The supernatant was rapidly removed by decanting or careful

pipetting without disturbing the pellet. The remaining supernatant was removed by repeating centrifugation and pipetting. Without disturbing the pellet, 40 µl of co-precipitant was layered on top of the pellet and left on ice for 5 min. The tube was centrifuged again, with the cap-hinge and pellet facing outward, at maximum speed. The supernatant was removed with pipette tip and discarded. A volume of 25 µl of distilled water was then added to the top of the pellet and vortexed for 5-10 s. This was followed by 1 ml of wash buffer (pre-chilled at -20 ºC for at least 1 h) and 5 µl of wash additive. The mixture was vortexed in order to fully disperse the pellet. The tube was incubated at -20 ºC for at least 30 min and vortexed for 20-30 s once every 10 min.

After incubation, the tube was centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was air dried briefly for 5 min.

2.2.6 Determination of protein concentration

Protein quantitation was carried out using 2-D Quant Kit (GE Healthcare) by following the protocol recommended by manufacturer. The assay was performed at room temperature, including centrifugation. Prior to performing the assay, appropriate volume of working colour reagent was prepared by mixing 100 parts of colour reagent A with 1 part of colour reagent B. Each individual assay required 1 ml of working reagent.

To start the assay, tubes consisting of 0, 10, 20, 30, 40 and 50 µg of bovine plasma albumin (BSA) were prepared for the purpose of producing standard curve. Each sample with volume of 3 µl was prepared in duplicate. Five hundred microlitres of precipitant was added to each tube, including the standard curve tubes. The tubes were vortexed briefly and incubated for 2-3 min, followed by addition of 500 µl co-precipitant. The tubes were mixed briefly by vortexing or inversion and subsequently centrifuged at 13,000 rpm for 5 min. The tubes were removed from centrifuge immediately after centrifugation was complete and supernatants were decanted. Rapidly, the tubes were repositioned in the mircrocentrifuge with the cap-hinge and pellet facing outward and centrifuged briefly in order to bring down any remaining liquid to the bottom of the tubes. The remaining supernatant was removed using a pipettor, with no more visible liquid remained in the tubes. After that, 100 µl of copper solution and 400 µl of distilled water were added to each tube and the mixture was vortexed briefly. One millilitre of working colour reagent was added rapidly to each tube and mixed by inversion followed by

incubation at room temperature for 15-20 min. The absorbance of each sample and standard was then read at 480 nm using NanoPhotometer (Implen), with water as the reference. The absorbance was read within 40 min of the addition of working colour reagent. A standard curve was generated by plotting the absorbance of the standards against quantity of protein. The protein concentration of the samples was determined by referring their absorbance to the standard curve.

2.2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.7.1 Preparation of 10% polyacrylamide gel

Five millilitres of 10% polyacrylamide gel (resolving gel) solution (**Appendix II**) was poured and filled to 3 cm below the top of the rectangular glass plate [(inner) 7.3 am x 10.2 cm, (outer) 8.3 cm x 10.2 cm] (Bio-Rad, Hercules, CA, USA). Water was added to overlay the gel and to prevent its exposure to oxygen. The gel was allowed to polymerise for at least 1 h. After the gel was set, the overlay was rinsed off the gel sandwich with distilled water. Two millilitres of stacking gel solution (**Appendix II**) was then applied over the resolving gel. A comb was introduced onto the stacking gel solution. This was done carefully to avoid formation of air bubbles and the gel was left to polymerise for at least 1 h.

2.2.7.2 Preparation of protein samples

Five microlitres of each sample containing 10 µg protein (HAPs and HAP-depleted proteins) were mixed with 5 µl of 2X solubilising buffer (**Appendix II**), composing 0.125 M Tris-HCl (Amresco) (pH 6.8), 20% (v/v) glycerol, 0.2 M DTT (Amresco), 0.02% (v/v) bromophenol blue (GE Healthcare), in a tube and boiled for 3-5 min. After boiling, the protein sample was placed on ice until use.

2.2.7.3 Electrophoresis

Protein samples were separated with SDS-PAGE mini-gels utilising Bio-Rad Mini-Protean II System as described by Laemlli (1970). The comb was slowly removed from the gel and each well was rinsed with SDS cathode buffer (**Appendix II**) which

consisting of 0.025 M Tris-base (Amresco), 0.193 M glycine (Amresco) and 0.1% (w/v) SDS (Amresco). Sample was loaded gently by pipetting into each well that was initially filled with SDS cathode buffer. Broad range prestained protein marker, PageRuler (Fermentas), was included for electrophoresis in each gel. The proteins were electrophoresed at 100 V in SDS cathode buffer for 90 min or until the front dye reached the bottom of the gel. The gels were stained with Blue-BANDit (Amresco), a commercially available Coomassie blue comparable stain, for visualization.

2.2.7.4 Coomassie blue staining

Blue-BANDit (Amresco) staining procedure was performed as recommended by manufacturer. Each gel was pre-washed with deionized water for 15 min, followed by submerging completely in adequate volume of Blue-BANDit staining solution for 1 h. The gel was destained in double distilled water for at least 30 min until background became clear. The gel was stored in distilled water prior to gel image documentation.

2.2.7.5 Image acquisition and analysis

All stained gels were transferred to a densitometer, ImageScanner^{TM} III (GE Healthcare), and scanned at a 300 dpi resolution in a tagged image file format (TIFF). The images were acquired with Labscan^{TM} 6.01 (GE Healthcare) and retrieved for analysis using ImageQuant TL^{TM} 7.0 (GE Healthcare).

2.2.8 Two-dimensional electrophoresis (2-DE)

2-DE was performed as described by Görg *et al.* (2000).

2.2.8.1 First-dimensional isoelectric focusing (IEF)

Rehydration of IPG strip was performed prior to IEF. A volume of 250 µl of each sample containing 50 µg (for silver staining) or at least 1-2.5 mg (for Coomassie blue staining) of proteins was loaded to the slot of Immobiline DryStrip reswelling tray (GE Healthcare). A precast 13 cm IPG DryStrip pH 3-10 or pH 4-7 (GE Healthcare), with the

gel sided down, was then placed in the protein solution. Air bubbles were avoided form being trapped under the IPG strip. Each IPG strip was overlaid with 3 ml of DryStrip cover fluid (GE Healthcare) to minimise evaporation and urea crystallization. The IPG strip was allowed to rehydrate at room temperature for 10-15 h.

The rehydrated IPG strip was then transferred to Ettan IPGphor strip holder (GE Healthcare), with gel faced up. Two paper wicks, about 5 mm long each, were wetted with deionized water, blotted almost completely, and placed on both sides of the IPG strip. Electrode was slid down on top of each paper wick and the IPG strip was overlaid with DryStrip cover fluid to prevent evaporation and precipitation of urea.

Ettan IPGphor III (GE Healthcare) was connected to an external computer via the serial port to control and monitor the electrical conditions. The strip holder, together with the IPG strip, was placed onto the IPGphor to undergo IEF under the following parameters:

1. Step and hold: 100 V for 500 Vh
2. Step and hold: 500 V for 1000 Vh
3. Gradient : 4000V for 6750 Vh
4. Gradient : 8000 V for 12000 Vh
5. Step and hold: 8000 V for 32000 Vh

Total : 52250 Vh (overnight)

Temperature: 20ºC

Following IEF, second-dimensional electrophoresis was performed immediately, or the IPG strip was stored in screw-cap tubes at -80ºC.

2.2.8.2 Equilibration of IPG strip

After IEF, the IPG strip was subjected to equilibration step, where each strip was immersed in 10 ml of equilibration buffer with 1% (w/v) of dithiothreitol (DTT) (Amresco), and subsequently in another 10 ml of equilibration buffer with 2.5% (w/v) of iodoacetamide (Amresco) for 15 min each. Equilibrated IPG strip was then dipped and lubricated in the SDS cathode buffer.

2.2.8.3 Second-dimensional SDS-PAGE

Second-dimensional SDS-PAGE was performed in 12.5% homogenous polyacrylamide gel using the Hoefer SE600 (GE Healthcare) as described by the manufacturer.

To cast a gel, the gel cassette was assembled according to instructions given by the manufacturer (GE Healthcare). Gel solution was prepared based on recipe in **Appendix II**, without tetramethylethylenediamine (TEMED). The solution was stirred with magnetic bar. TEMED was then added and the solution was gently swirled. The 25 ml gel solution was immediately poured into the gel cassette (18 cm x 16 cm, 1.0 mm think glass plates) to 5 mm below the top. The gel was overlaid with water to minimise gel exposure to oxygen and to create flat gel surface. The gel was left overnight at room temperature to allow complete polymerization.

The polymerised gel was rinsed several times with SDS cathode buffer before IPG strip was positioned onto its surface with the plastic backing against one of the glass plates. Warm (60 ºC) agarose (GE Healthcare) sealing solution was slowly pipetted to the amount required to seal the IPG strip in place. Sample application piece (GE Healthcare) loaded with 10 µl of broad range prestained molecular weight marker, PageRuler (Fermentas) was promptly inserted beside the IPG strip. The agarose was left for a minimum of 1 min to cool and solidify.

The gels were electrophoresed in a Hoefer SE600 (GE Healthcare) tank filled with SDS cathode buffer at 10 mA per gel for 20 min, followed by 30 mA per gel for 3 h at room temperature.

2.2.8.4 Coomassie blue and silver staining

Coomassie blue staining was carried according to the method described in **Section 2.2.7.4**.

Silver staining procedure was initiated by soaking the electrophoresed gel in the adequate volume of fixing solution containing 40% (v/v) ethanol and 10% (v/v) acetic acid for 10 min. The gel was then rinsed with double distilled water for 10 min and subsequently submerged in fixing/sensitising solution, which consisted of 0.05% (v/v)

glutaraldehyde, 0.01% (v/v) formalin, and 40% (v/v) ethanol, for 5 min. The gel was rinsed with 40% (v/v) ethanol and followed by double distilled water, 20 min each. The gel was sensitised in adequate volume of 0.2 g/L sodium thiosulfate for 1 min and after that rinsed twice with double distilled water for 1 min each. Silver nitrate solution of 0.1% (w/v) was added to the gel and incubated for 20 min. The gel was rinsed again with double distilled water for 1 min before developed in developing solution consisting of 2.5% (w/v) sodium carbonate and 0.04% (v/v) formalin for 4-15 min. Development was terminated with stopping solution containing 0.04 M disodium EDTA.

2.2.8.5 Image acquisition and analysis

All stained gels were transferred to a densitometer, ImageScanner III™ (GE Healthcare), and scanned at a 300 dpi resolution in an ImageMaster™ format (mel). The images were acquired with Labscan™ 6.01 (GE Healthcare) and retrieved for analysis using ImageMaster™ 2D Platinum 7.02(GE Healthcare).

2.2.9 Two-dimensional difference gel electrophoresis (2D-DIGE)

For comparison of protein profiles between SLE patients and normal individuals, 2D-DIGE was used. This technique enables multiplexing of up to three separate protein mixtures on the same second dimension SDS PAGE gel, thus it was used in order to reduce time consumption and eliminate gel-to-gel variation. Three different CyDye DIGE Fluor dyes (GE Healthcare) Cy2, Cy3, and Cy5 were used to label protein samples prior to loading to each gel.

2.2.9.1 Experimental design

Randomisation of protein samples across gels is necessary to removes any bias from experiments such as experimental conditions, sample handling and labelling. It is important to distribute individual experimental samples evenly between different CyDye DIGE Fluor dyes and different gels to avoid for instance systematic errors.

Cy2 was used to label internal standard, while protein samples were labelled with either Cy3 or Cy5. Samples were randomised using an internet accessible randomiser at

http://www.randomizer.org/. The distribution of randomised samples (plasma HAPs and HAP-depleted proteins and urine proteins) across gels and CyDye DIGE Fluor dyes for comparison of protein profiles between normal healthy individuals and SLE patients, as well as treatment follow-up study, are listed in **Table 2.2.1** and **Table2.2.2**.

Volume needed for labelling of each sample with either Cy3 or Cy5 was 50 µg. Cy2 was used to label internal standard that was included in each gel. To create internal standard, an aliquot of 25 µg from each sample was mixed together in a vial and 50 µg was used for each gel.

A. Normal healthy individuals vs. SLE patients

Table 2.2.1. Distribution of samples across gels and CyDye DIGE Fluor dyes for comparing protein profiles between normal healthy individuals and SLE patients.

Gel	Cy2	Cy3	Cy5
1	Mixture of all samples	S4	LNR5
2		N4	LN(3)2
3		LN(5)2	LN(4)2
4		N2	S5
5		LN(4)5	LNR3
6		LN(4)1	LN(3)3
7		S2	LN(2)2
8		N1	S1
9		S3	N5
10		LNR2	LN(2)1
11		LNR4	LN(2)3
12		LNR1	LN(4)4
13		N3	LN(3)1
14		LN(5)3	LN(4)3
15		LN(5)1	

Note:
N - Normal individual (5 samples)
S – SLE patient (5 samples)
LNR – Lupus nephritis patient in remission (5 samples)
LN(2) – LN Class II patient (3 samples)
LN(3) – LN Class III patient (3 samples)
LN(4) – LN Class IV patient (5 samples)
LN(5) – LN Class V patient (3 samples)

B. Treatment follow-up

Table 2.2.2. Distribution of samples across gels and CyDye DIGE Fluor dyes for investigating protein profile changes along the course of mycophenolate mofetil (CellCept) treatment.

Gel	Cy2	Cy3	Cy5
1		A2 (Sample A, 2nd visit)	B3 (Sample B, 3rd visit)
2		A4	B1
3	Mixture of all samples	B4	A3
4		A1	B5
5		B2	A5

2.2.9.2 Reconstitution of CyDyes

To reconstitute CyDyes, 99.8% anhydrous dimethylformamide (DMF) (Sigma-Aldrich) less than three-months-old from day of opening was used. This is to ensure amine compounds that were produced as a result of DMF degradation reacted with the N-hydroxysuccinimide (NHS) ester CyDye, reducing the concentration of dye available for protein labelling.

The 5 nmol of each CyDye minimal dye solid compound were taken out of -20 ºC and left for 5 min at room temperature. It was then reconstituted with 5 µl DMF to a concentration of 1 nmol/µl. All stock solutions were vortexed vigorously for 30 s and centrifuged for 30 s at 12,000 x g. The stock solutions of Cy2, Cy3, and Cy5 presented colours of deep yellow, deep red, and deep blue, respectively. The stock solutions were stored at -20 ºC and stable for 3 months.

The stock solution was diluted into working solution by adding 1 volume of the stock solution with 1.5X volumes of DMF to create 400 pmol/µl. The working solutions were stored at -20 ºC and stable for 2 weeks.

2.2.9.3 CyDye labelling

Upon sample clean-up based on method mentioned in **Section 2.2.5**, protein samples were reconstituted with DIGE lysis buffer, composing of 7 M urea (Amresco), 2 M thiourea (GE Healthcare), 4% (w/v) CHAPS (GE Healthcare), and 30 mM Tris

(Amresco) and adjusted to pH 8.5, to a concentration of around 2.5 µg/µl. Samples were vortexed and spun briefly.

For efficient labelling, the pH of protein samples should be optimally 8.5, or must be above pH 8.0. Hence, pH of each sample was checked by pipetting 2 µl sample on a pH indicator paper (Merck) and reading out the pH value immediately. If necessary, the pH value was adjusted to pH 8.5 with 100 mM NaOH solution.

One microlitre of Cy3 or Cy5 working solution was added to 20 µl of each sample equivalent to 50 µg proteins. Likewise, one microlitre of Cy2 working solution was added 20 µl of internal standard consisting of 50 µg protein. The mixture was vortexed and centrifuged briefly. It was then left on ice for 30 min in the dark. The reaction was terminated with 1 µl of 10 mmol/l lysine. The solution was mixed, spun briefly and left for 10 min on ice in the dark.

2.2.9.4 Preparation for loading samples onto IPG Strips

The labelled samples and internal standard were combined according to the experimental designed. One gel included one Cy3-labelled sample, one Cy5-labelled sample and Cy2-labelled internal standard, 50 µg each. After combining, total volume of mixture was 60 µl. An equal volume, which was 60 µl, of freshly prepared DIGE 2X lysis buffer [DIGE lysis buffer with 2% DTT (Amresco) and 2% (v/v) pH4-7 IPG buffer (GE Healthcare)] was added to each combined mixture and left on ice for 10 min. The final volume of each combined mixture was 120 µl.

2.2.9.5 First-dimensional isoelectric focusing (IEF)

Anodic cup loading method was used for this part of study.

Immobiline pH gradient (IPG) DryStrips of 13 cm and pH 4-7 (GE Healthcare) was selected based on result obtained from optimization. Prior to performing IEF, rehydration of IPG strips was done as mentioned in **Section 2.2.8.1**, except that samples were not included in the rehydration buffer.

After rehydration for 10-15 h, each IPG strip was transferred from Immobiline DryStrip reswelling tray (GE Healthcare) to Ettan IPGphor strip holder (GE Healthcare)

with the gel side facing up. A loading cup (GE Healthcare) was positioned on anodic end the gel, in a distance approximately 1 cm from the end of the gel portion of the IPG strip. The loading cup was pushed down with finger to ensure that the feet of the cup were properly seated at the bottom of the channel of strip holder. Leakage was tested by adding some DryStrip cover fluid (GE Healthcare) into the cup.

Pre-wetted paper wicks (GE Healthcare) were placed on both ends of the IPG strip. Electrode was slid down on top of each paper wick and the IPG strip was overlaid with DryStrip cover fluid.

Finally, protein sample was loaded into the cup and overlaid with some DryStrip cover fluid. The entire set-up was placed on the Ettan IPGphor III (GE Healthcare) connected to an external computer and focused under the same parameters mentioned in **Section 2.2.8.1**. Following IEF, second-dimensional electrophoresis was performed immediately, or the IPG strip was stored in screw-cap tubes at -80ºC.

2.2.9.6 Equilibration of IPG strip

Equilibration of IPG strip was done as described in **Section 2.2.8.2**.

2.2.9.7 Second-dimensional SDS-PAGE

Second-dimensional SDS-PAGE was done as mentioned in **Section 2.2.8.3**, except that normal glass plates were replaced with low fluorescent glass plates. Ten microlitres of ECL Plex Fluorescent Rainbow Markers (GE Healthcare) was included in every gel.

2.2.9.8 Image acquisition

Typhoon^{TM} FLA 9000 biomolecular imager (GE Healthcare) was turned on and left to warm up for at least 30 min prior to scanning. The gel, together with glass plates, was taken out of Hoefer SE600 (GE Healthcare) when second-dimensional SDS-PAGE completed. Glass plates were rinsed with water and wiped with lint-free paper. After ensuring the glass plates were clean, dry and free from lint, the entire glass plate assembly

was positioned on the low fluorescent glass plate stage of the imager. The stage was slid into the imager, followed by closing of the imager lid.

Image scanning was operated using Typhoon Scanner Control software. To detect CyDyes, fluorescence mode was selected [**Figure 2.2.1(A)**]. Scan area was selected so that software was able to recognize where the gel was located. The gel was initially scanned with a fast 1000 µm prescan function and photomultiplier tube (PMT) voltage of 500 for all three emission filters to obtain a rapid overview of the gel image. This step assisted in adjustment of scanning settings, such as PMT voltages, for optimal detection of emission wavelength of each CyDye. When suitable PMT voltages were identified, the gel was scanned at a pixel size of 100 µm and scanned image was saved for further analysis.

2.2.9.9 Image and statistical analysis

2D-DIGE images were analysed using DeCyder 2D version 7.0 software (GE Healthcare), an automated image analysis software suite which enables detection, quantification, matching and analysis of 2D-DIGE gels.

Firstly, scanned gel images were imported into a project so that they were accessible for other modules. After that, differential in-gel analysis (DIA) was carried out, whereby protein spots were detected and quantitated on a set of images from the same gel following background subtraction, in-gel normalisation and gel artefact removal [**Figure 2.2.2(A)**]. Normalised volume indicates the volume normalised across the three dyes and across the gel. One of the outputs provided by DeCyder is the ratio of the normalised volumes, also known as the standardised abundances,

$$\begin{cases} R_{pg} = VolCy5_{pg}/VolCy2_{pg}, \\ G_{pg} = VolCy3_{pg}/VolCy2_{pg}, \end{cases}$$

(Fodor *et al.*, 2005)

for each spot p and gel g in the experiment. $VolCy5_{pg}$ refers to the normalised volume of spot p on gel g in the Cy5 sample and same for the other two dyes. Log standard abundance, defined as the log10 of the standardised abundance, for each spot was calculated.

To match and compare multiple images from different gels, workspaces were created and images were assigned into groups in biological variation analysis (BVA)

module [**Figure 2.2.2(B)**]. Statistical analysis was performed by combining two measures: i) average abundance ratio (fold change), and ii) *p* value from the Student's *t*-test. If $\bar{S}_{p1}$ and $\bar{S}_{p2}$ denote the average standardized abundance of protein *p* in groups*i* = 1 and 2, respectively,

$$\bar{S}_{pi} = \frac{\sum_{Rpg} \epsilon_{\text{Group}i} R_{pg} + \sum_{Gpg} \epsilon_{\text{Group}i} G_{pg}}{|R_{pg} \epsilon \text{Group}_i| + |G_{pg} \epsilon \text{Group}_i|} ,$$

Then the corresponding average abundance ratio (fold change) is

$$F_p = \begin{cases} +\bar{S}_{p1}/\bar{S}_{p2} \text{ for } \bar{S}_{p1} > \bar{S}_{p2}, \\ -\bar{S}_{p2}/\bar{S}_{p1} \text{ for } \bar{S}_{p1} < \bar{S}_{p2}. \end{cases}$$

(Fodor *et al.*, 2005)

In this study, protein spots having average abundance ratio of ≥ 1.5 or ≤ -1.5 in a group compared with another group, with *p* value ≤ 0.05, were identified. The position of the selected protein spots on the gels was also identified.

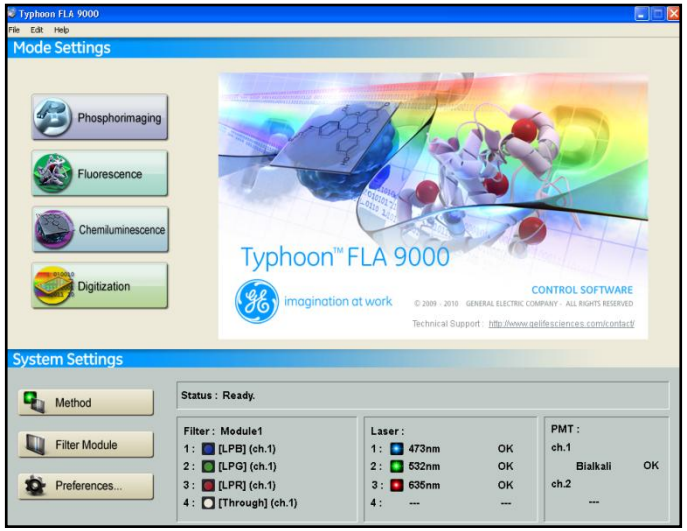
2.2.10 Protein identification

2.2.10.1 In-gel trypsin digestion

In-gel trypsin digestion procedures as described by Shevchenco *et al.*(1996) was used in this study, with some modifications. Protein spots were selected according to results obtained from image analysis.

Selected protein spots were picked from Coomassie blue-stained gels using pasteur pipette and placed into clean microcentrifuge tubes. A volume of 50 µl of acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) was added to each gel piece and left for 15 min. During this step, gel pieces were dehydrated, shrank and became opaque. The supernatant was removed and the spot was rehydrated for 10 min by adding 25 µl of 25 mM ammonium bicarbonate (Sigma-Aldrich). The supernatant was then removed and the spot was dehydrated again with 50 µl of acetonitrile for 15 min. After that, the rehydration and dehydration steps were repeated twice to give a total of three washes. All liquid was removed and spot was dried in a speed vac. Each dehydrated gel piece was digested with 5 µl of 10 ng/µl porcine trypsin solution (Sigma-Aldrich) and rehydrated on ice for 45 min.

(A)



(B)

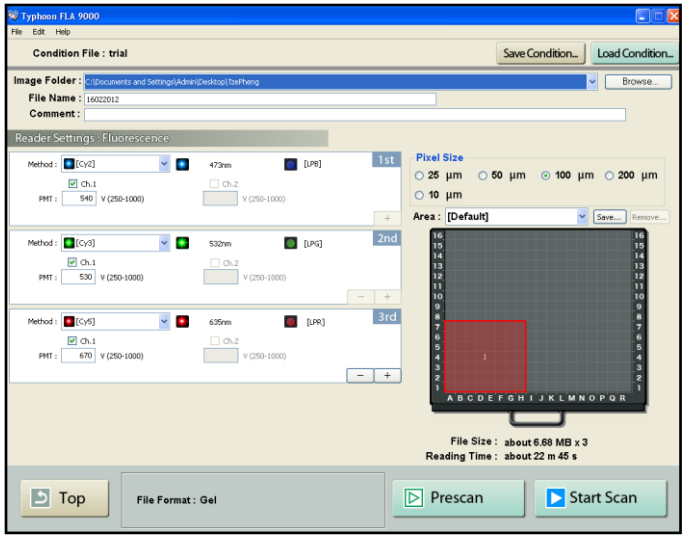
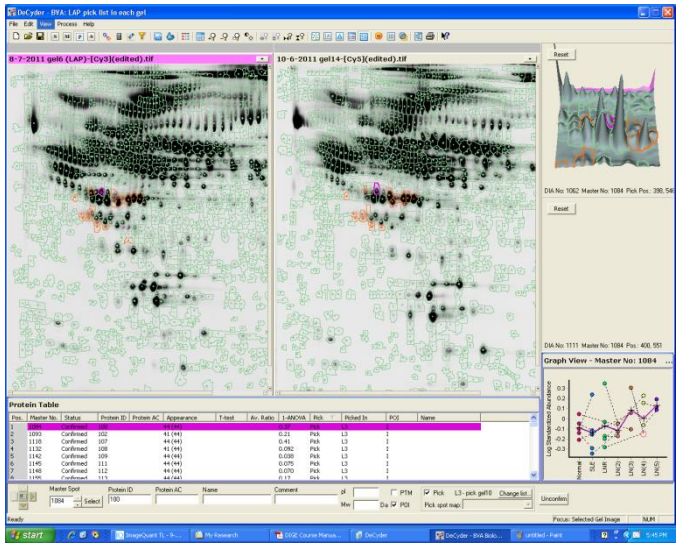


Figure 2.2.1. Typhoon Scanner Control windows. **(A)** allows mode and system settings, while **(B)** is for PMT voltage, pixel size, and area settings before gel scanning.

(A)



(B)

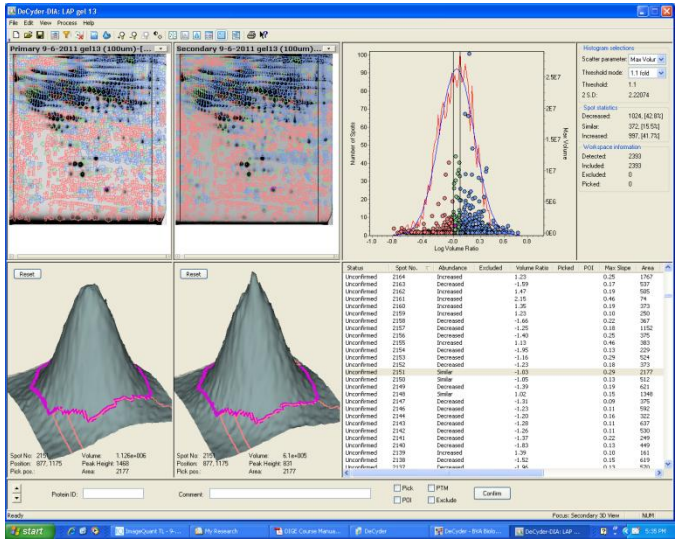


Figure 2.2.2. Image analysis of 2D-DIGE gels using DeCyder 2D version 7.0 software (GE Healthcare). **(A)** shows DIA window and **(B)** is BVA window.

2.2.10.2 Peptide extraction

Upon tryptic digestion, the solution was transferred to a clean tube two. Each gel piece was added with 10 µl of 5% (v/v) formic acid (Sigma-Aldrich) in 50% (v/v) acetonitrile so that it was just covered. The whole thing was vortexed for 20-30 min, spun and sonicated for 15 min. The liquid was removed and added to digest solution in tube two. The gel piece was added again with 10 µl of 5% formic acid in 50% acetonitrile. The whole thing was vortexed for 15 min, spun and sonicated for 15 min. The liquid was removed and added to digest solution in tube two. The solution was vortexed, spun and dried by using speed vac.

2.2.10.3 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Instrument used for LC-MS/MS analysis was the Agilent 1200 HPLC-Chip/MS interface coupled with Agilent 6520 Mass Q-TOF LC/MS (Agilent Technologies). Sample pellet was resuspended with 8 µl of 0.1% (v/v) formic acid in water and1 µl was injected into the instrument, having the large capacity chip packed with Zorbax 300SB-C18 (Agilent Technologies) as the column. Solvents used were 0.1% (v/v) formic acid in water and 90% (v/v) acetonitrile in water with 0.1% (v/v) formic acid. The other parameters for LC-MS/MS setting were documented in **Appendix III**.

2.2.10.4 Data analysis

Data obtained from LC-MS/MS was processed with Agilent Spectrum Mill for MassHunter Workstation (Agilent Technologies), whereby MassHunter Qualitative Analysis Software Version B.03.01 (**Figure 2.2.3**) was used for chromatogram and MS spectral data acquisition while Spectrum Mill Rev A.03.03.084 SR4 (**Figure 2.2.4**) was for protein identification via database search. MS/MS spectral of each sample were searched against *Homo sapiens* proteins in SwissProt database with some other settings listed in **Appendix III**. Carbamidomethylation (C) modification was included during the search.

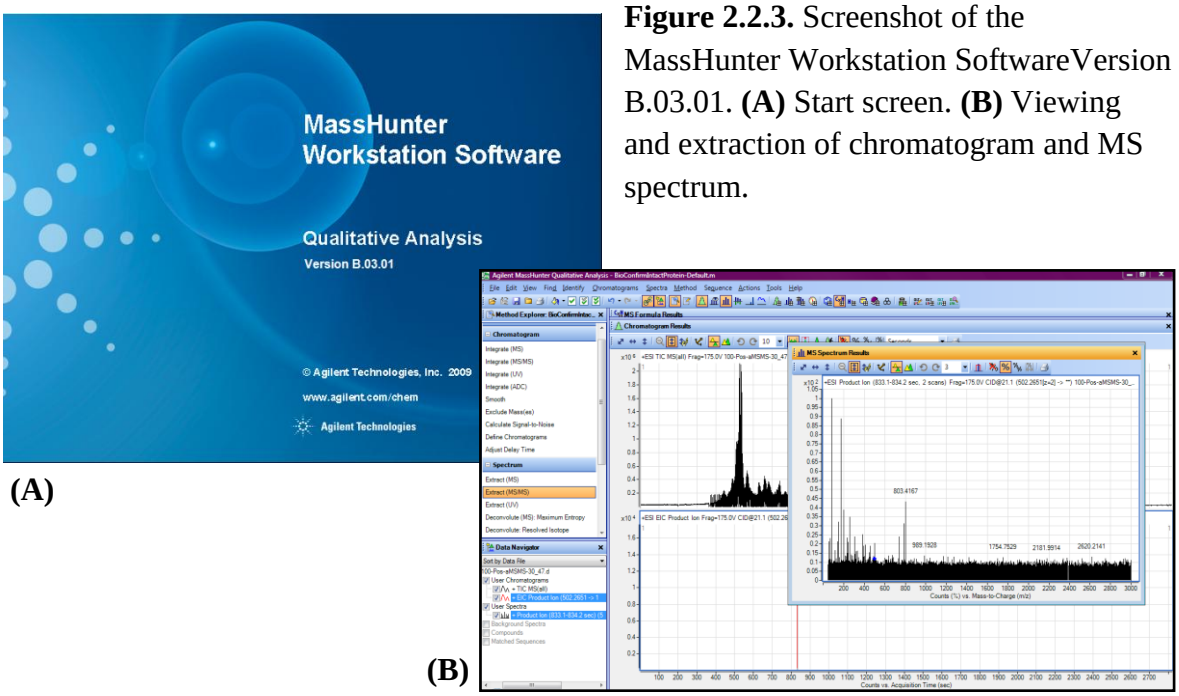


Figure 2.2.3. Screenshot of the MassHunter Workstation SoftwareVersion B.03.01. **(A)** Start screen. **(B)** Viewing and extraction of chromatogram and MS spectrum.

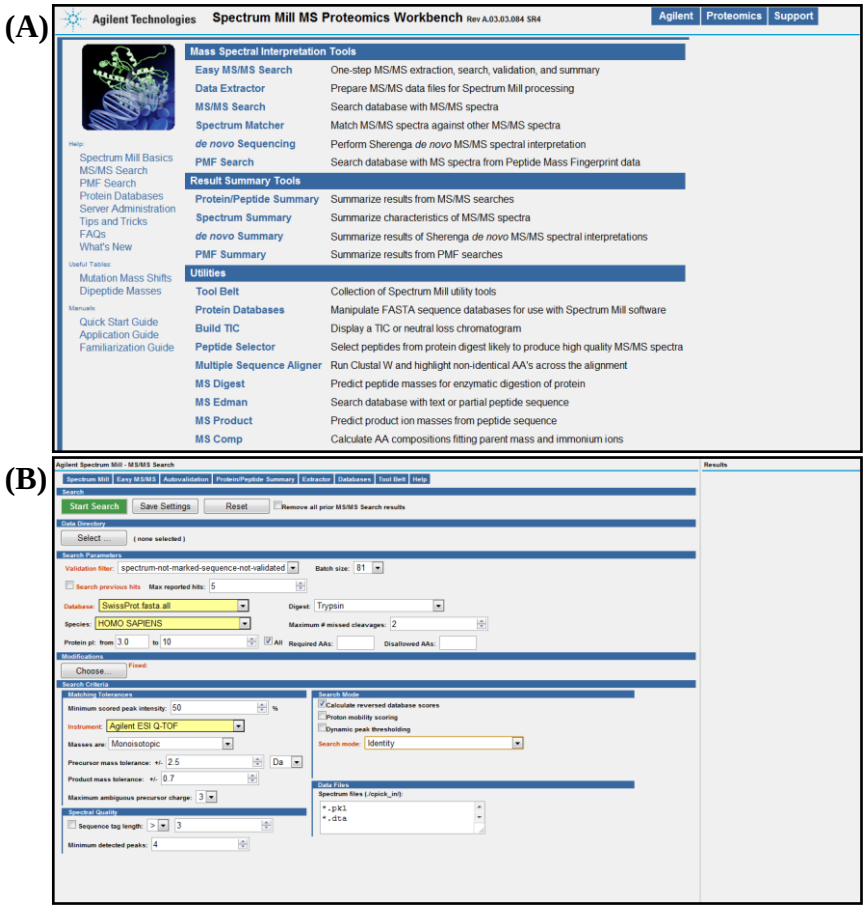


Figure 2.2.4. Screenshot of the Spectrum Mill MS Proteomics Workbench Rev A.03.03.084 SR4. **(A)** Start screen showing all the tools, utilities, and guidelines. **(B)** Page for database

2.2.10.5 Protein-protein interaction (PPI)

PPI network was constructed for each SLE group by connecting proteins associated to each group using IPA software (Ingenuity Systems, www.ingenuity.com) (**Figure 2.2.5**). A data set consisting of proteins with their gene identifiers and corresponding expression values was uploaded into application. Each identifier was mapped to its corresponding object in the Ingenuity Knowledge Base. Graphical representation of the molecular relationships between molecules in a network was generated, together with the functional analysis of the network. All relationships were supported by at least one reference from literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base. Different functional classes of proteins/gene products were represented by different shapes of nodes. The intensity of the node colour indicated the degree of up- (red) or down- (green) regulation.

The overall workflow designed for proteomic study was summarised in flowchart shown in **Figure 2.2.6**.

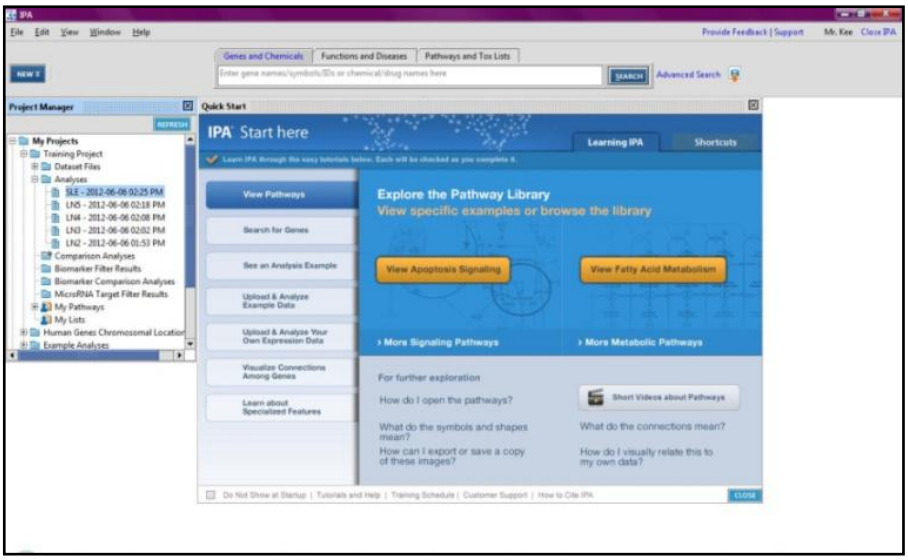


Figure 2.2.5. Start screen of IPA software used for protein-protein interaction analysis.

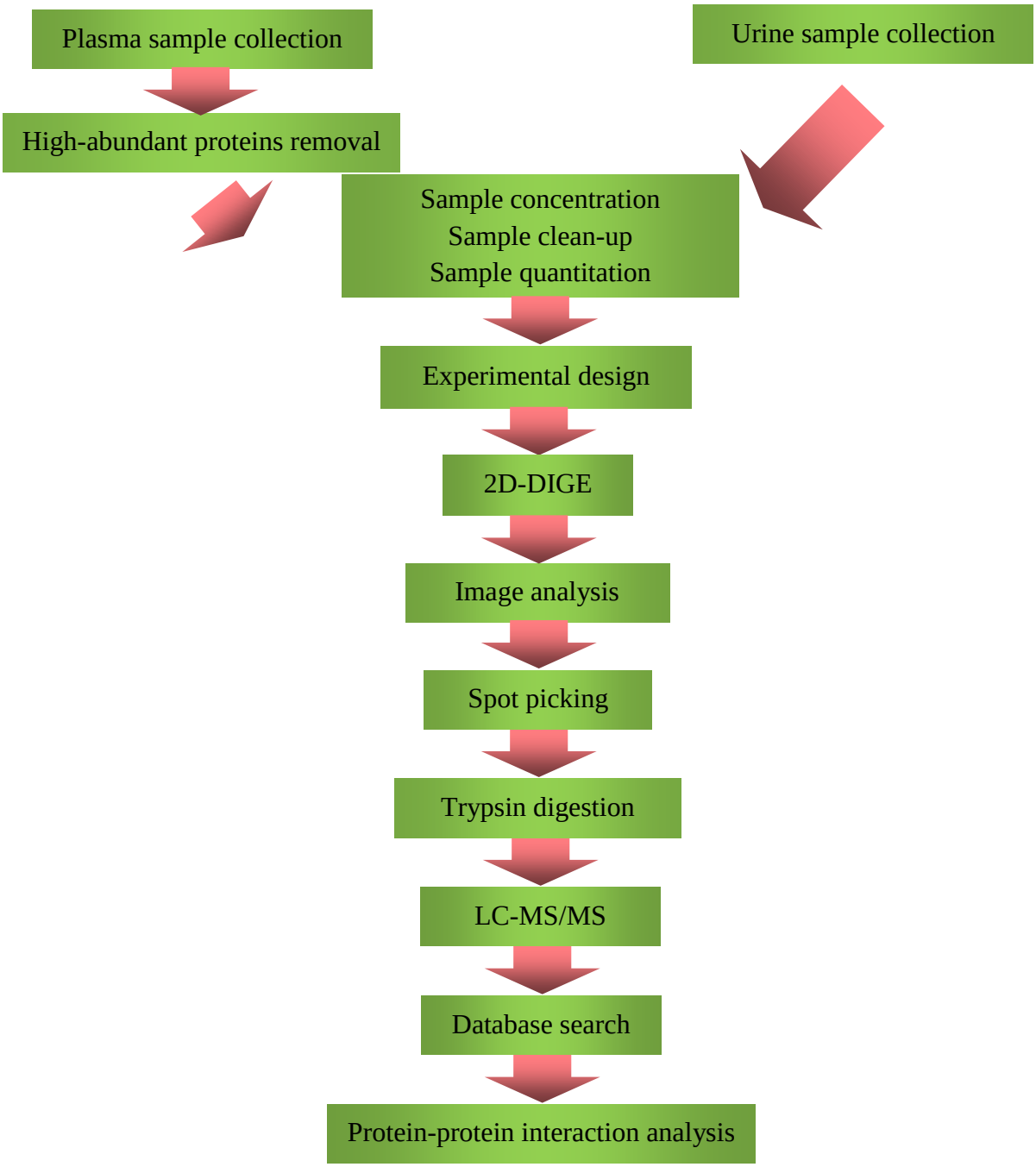


Figure 2.2.6. Flowchart summarising the workflow of proteomic study.

Chapter 3

Results

3.1 Genotyping Study

3.1.1 Subject recruitment

The demographic data of the individuals being recruited was summarised in **Table 3.1.1**. The ages of the subjects ranged from 15 to 75 years old. Patients and healthy controls recruited were ethnic and gender matched, as indicated by the *p* values >0.05.

Table 3.1.1. Demographic data of individuals recruited in this study.

	SLE patients	**Healthy controls**	***P* value**
Total	360	430	
Age range (years)	15-75	16-55	
Malay	93 (25.8%)	110 (25.6%)	>0.05
Chinese	245 (68.1%)	294 (68.4%)	>0.05
Indian	22 (6.1%)	26 (6.0%)	>0.05
Female	328 (91.1%)	391 (90.9%)	>0.05
Male	32 (8.9%)	39 (9.1%)	>0.05
Female: Male	10.25: 1	10.03: 1	

3.1.2 Method optimisation

3.1.2.1 Tetra-primer ARMS-PCR conditions

Single nucleotide polymorphisms (SNPs) that were genotyped using the tetra-primer ARMS-PCR approach were *STAT4* rs10168266 and rs7601754, *TNFAIP3* rs2230936 and rs3757173, and *IRF5* rs4728142. Touchdown PCR method was used and the PCR condition for genotyping of each SNP was optimised by adjusting: 1) annealing temperatures according to melting temperatures given during primer design; 2) concentration and ratio between outer and inner primers; 3) concentration of $MgCl_2$; and 4) duration of each PCR step. The final PCR recipe and touchdown PCR protocol for genotyping of each SNP is listed in **Table 2.1.2** and **2.1.3**. **Figure 3.1.1** to **3.1.5** shows the patterns of genotypes for each SNP that appeared on the 2% (w/v) agarose gels.

In total 910 subjects were genotyped. However, there were 120 subjects who did not genotype well or reproducibly. This was to be expected in SNP genotyping analysis. Therefore, these subjects were removed from the analysis and a total of 790 subjects were included.

3.1.2.2 Real-time PCR for Taqman SNP genotyping assays

Real-time PCR for predesigned and custom designed Taqman SNP genotyping assays were applied to *STAT4* rs7574865, *IRF5* rs729302, *HLA-DRB1/ HLA-DQA1* rs9271366, and *HLA-DRB1/ HLA-DQA1* rs9271366 because tetra-primers could not be appropriately designed to amplify and discriminate these two SNPs. Two unlabeled PCR primers (forward and reverse), one VIC dye-MGB labelled probe detecting the Allele 1 sequence, and one 6-FAM dye-MGB labelled probe detecting the Allele 2 sequence were included in each reaction. The primers were designed to be 20-24 bases in length with melting temperatures (T_m) of 55 to 60 ºC, producing amplicons with sizes of ~100 bp or not exceeding 300 bp. The probes were 20-30 bases in length, with their 5' ends must not be a guanosine (G) residue as it could quench the fluorescent signal even after hydrolysis. Their T_m's were between 65-67 ºC, which were approximately 10 ºC higher than the primer T_m.

Fluorescence is the property of some atoms and molecules to absorb light (be excited) at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, known as the fluorescence lifetime. VIC dye is a fluorescent dye which has an absorption (excitation) wavelength at 538 nm and emits green fluorescence at wavelength of 554 nm when Allele 1 is detected. The presence of homozygous Allele 1 gave red signal on the allelic discrimination graph (**Figure 3.1.6** to **3.1.9**). As for 6-FAM dye, its excitation occurs at 595 nm and emission at 521 nm when Allele 2 is detected. Homozygous Allele 2 was presented as blue signal on the allelic discrimination graph, while heterogenous showed blue signal.

The standard PCR reaction mix preparation and real-time PCR protocol recommended by manufacturer (**Section 2.1.7**) worked well in amplification and discrimination of all SNPs and therefore did not require any optimisation.

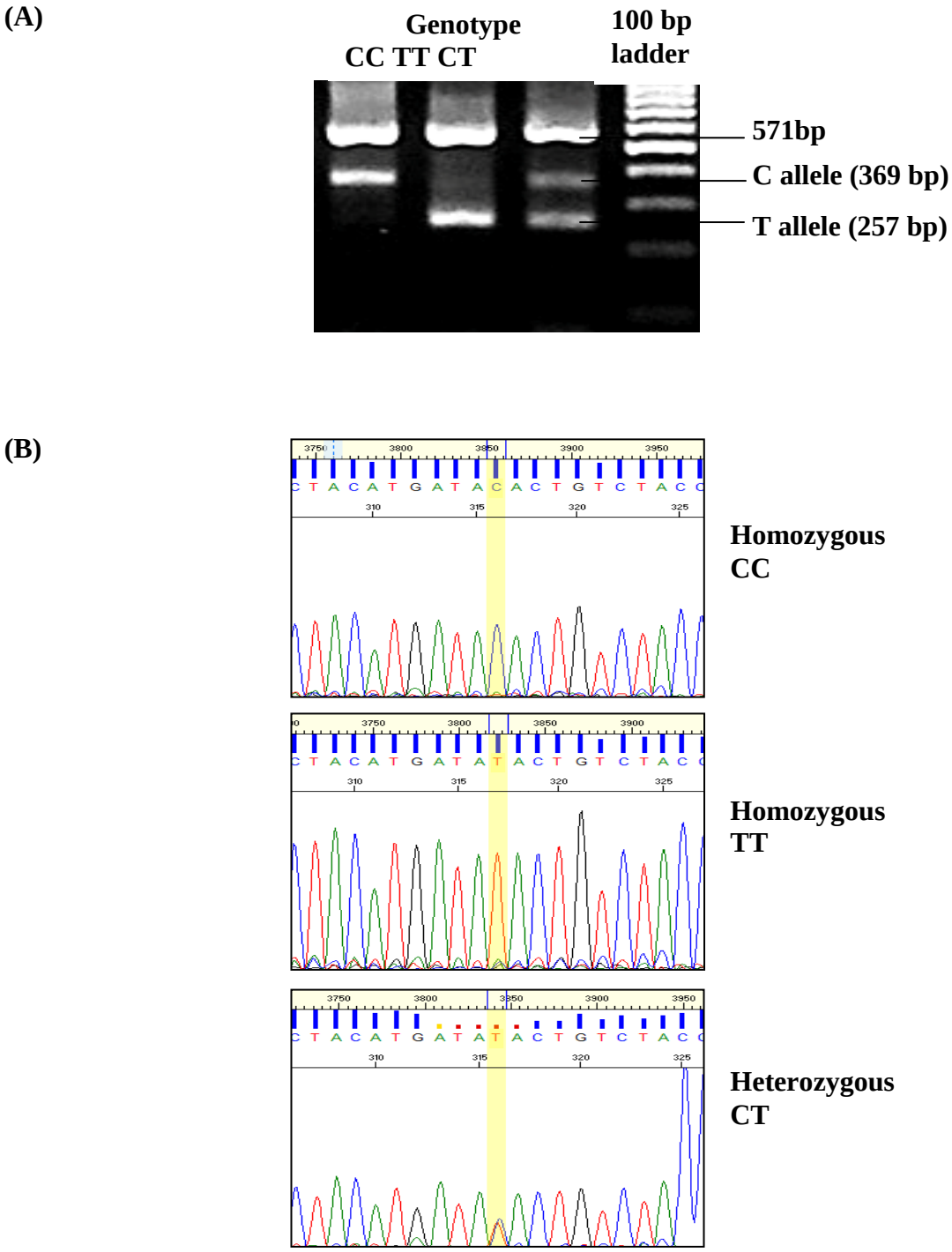


Figure 3.1.1. Genotyping of *STAT4* rs10168266 using the tetra-primer ARMS-PCR approach. (A) Gel profile for each genotype of *STAT4* rs10168266 on the 2% (w/v) agarose gels. Bands with 571 bp resulted from the outer primers and were seen on every lane. C and T alleles produced bands with sizes of 369 and 257 bp, respectively. Thus homozygous CC showed 2 bands with 571 and 369 bp; homozygous CC showed 2 bands with 571 and 257 bp; and heterozygous CT showed all the 3 bands. **(B)** Validation of genotyping results by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous CC and TT produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous CT emitted overlapped signals of both alleles.

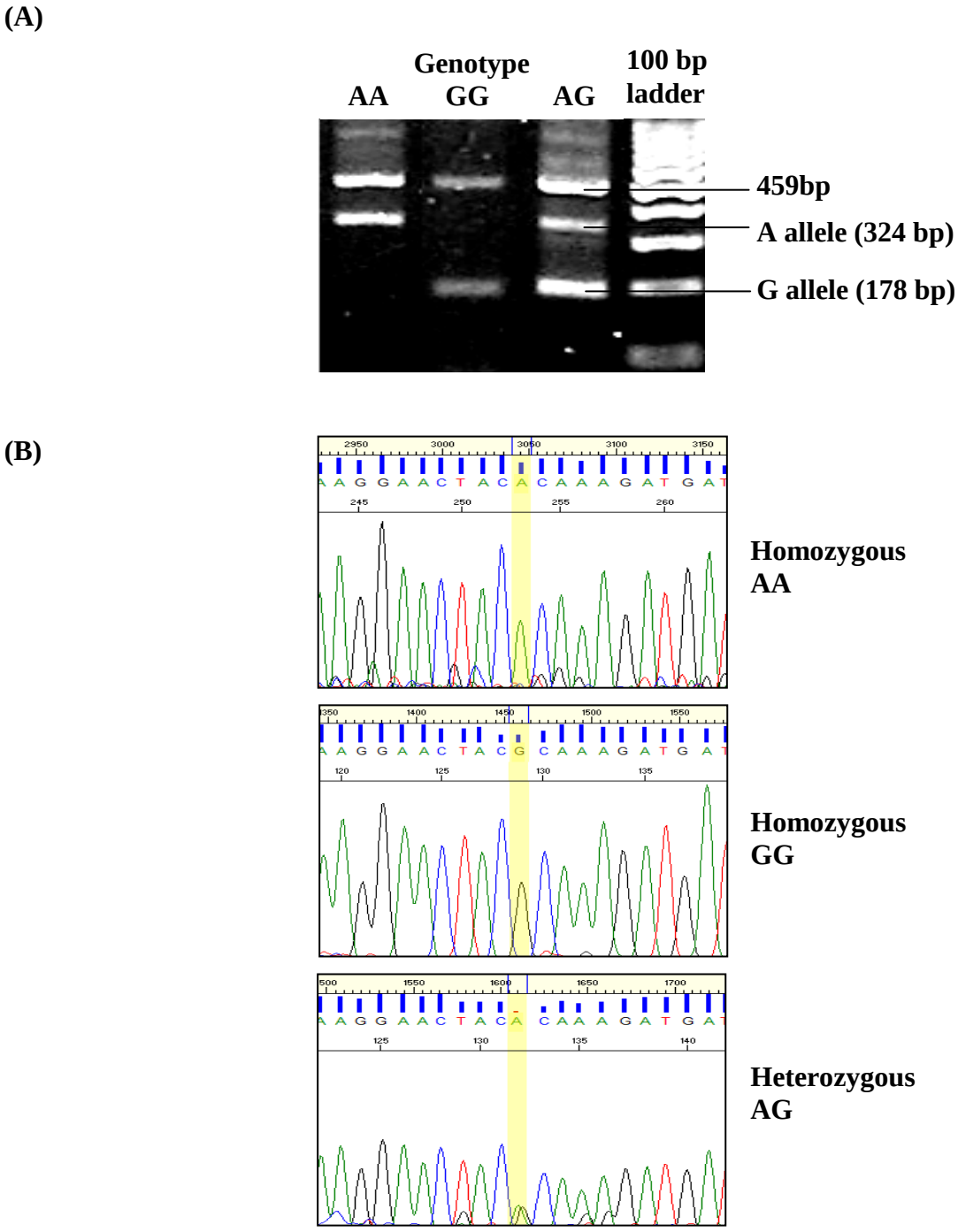
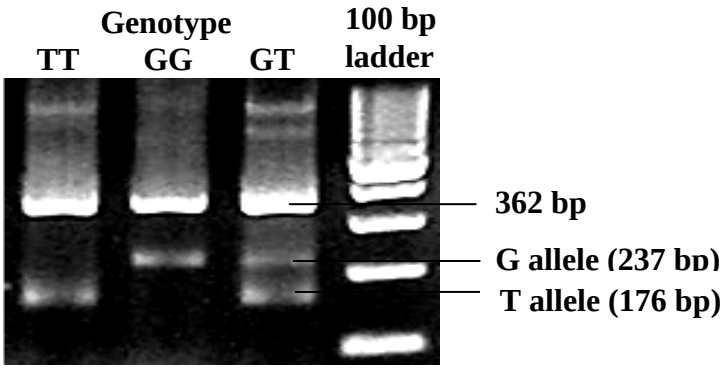


Figure 3.1.2. Genotyping of *STAT4* rs7601754 using the tetra-primer ARMS-PCR approach. (A) Gel profile for each genotype of *STAT4* rs7601754 on the 2% (w/v) agarose gels. Bands with 459bp resulted from the outer primers and were seen on every lane. A and G alleles produced bands with sizes of 324 and 178 bp, respectively. Thus homozygous AA showed 2 bands with 459 and 324 bp; homozygous GG showed 2 bands with 459 and 178 bp; and heterozygous AG showed all the 3 bands. **(B)** Validation of genotyping results by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous AA and GG produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous AG emitted overlapped signals of both alleles.

(A)



(B)

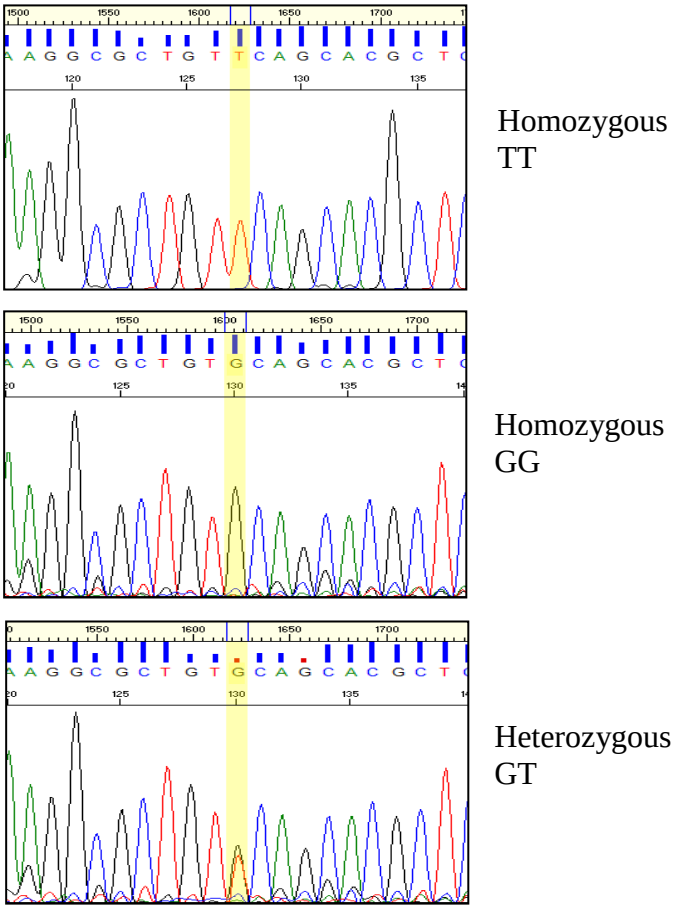


Figure 3.1.3. Genotyping of *TNFAIP3* rs2230936 using the tetra-primer ARMS-PCR approach. (A) Gel profile for each genotype of *TNFAIP3* rs2230936 on the 2% (w/v) agarose gels. Bands with 362 bp resulted from the outer primers and were seen on every lane. G and T alleles produced bands with sizes of 237 and 176 bp, respectively. Thus homozygous GG showed 2 bands with 362 and 237 bp; homozygous TT showed 2 bands with 362 and 176 bp; and heterozygous GT showed all the 3 bands. **(B)** Validation of genotyping results by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous GG and TT produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous GT emitted overlapped signals of both alleles.

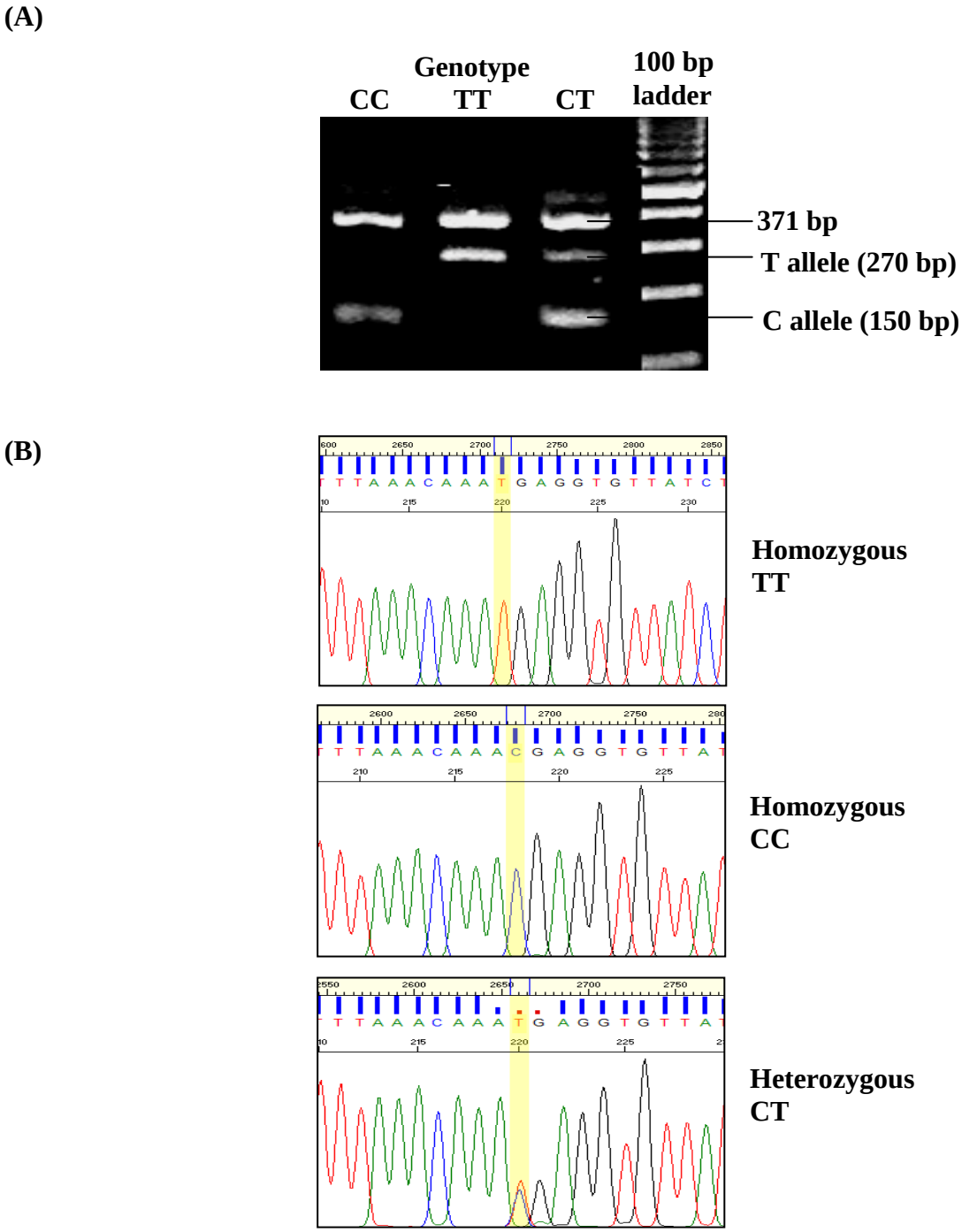
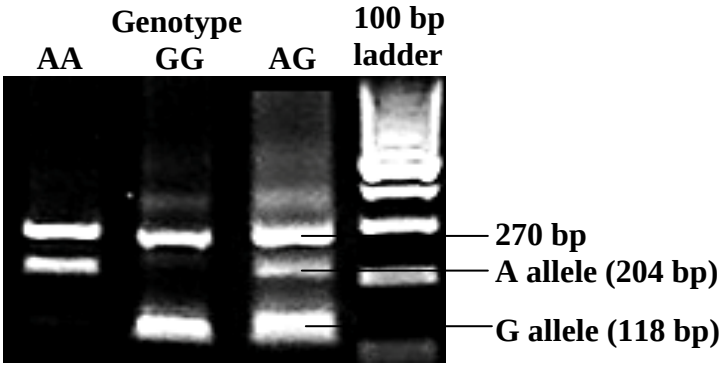


Figure 3.1.4. Genotyping of *TNFAIP3* rs3757173 using the tetra-primer ARMS-PCR approach. (A) Gel profile for each genotype of *TNFAIP3* rs3757173 on the 2% (w/v) agarose gels. Bands with 371bp resulted from the outer primers and were seen on every lane. C and T alleles produced bands with sizes of 150 and 270 bp, respectively. Thus homozygous CC showed 2 bands with 371 and 150 bp; homozygous TT showed 2 bands with 371 and 270 bp; and heterozygous CT showed all the 3 bands. **(B)** Validation of genotyping results by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous CC and TT produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous CT emitted overlapped signals of both alleles.

(A)



(B)

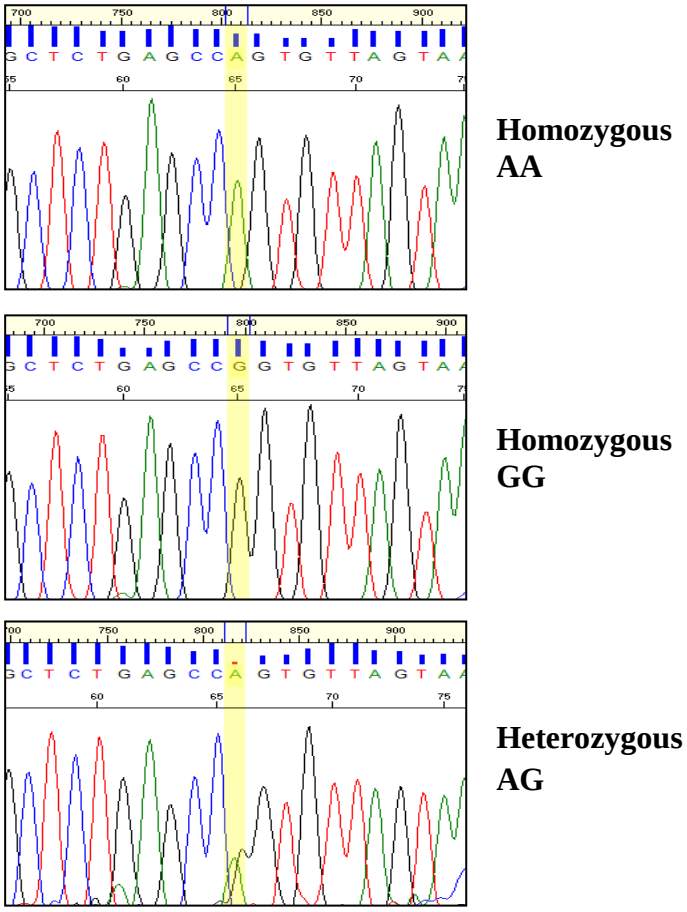


Figure 3.1.5. Genotyping of *IRF5* rs4728142 using the tetra-primer ARMS-PCR approach. (A) Gel profile for each genotype of *IRF5* rs4728142 on the 2% (w/v) agarose gels. Bands with 270 bp resulted from the outer primers and were seen on every lane. A and G alleles produced bands with sizes of 204 and 118bp, respectively. Thus homozygous AA showed 2 bands with 270 and 204 bp; homozygous GG showed 2 bands with 270 and 118 bp; and heterozygous AG showed all the 3 bands. **(B)** Validation of genotyping results by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous AA and GG produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous AG emitted overlapped signals of both alleles.

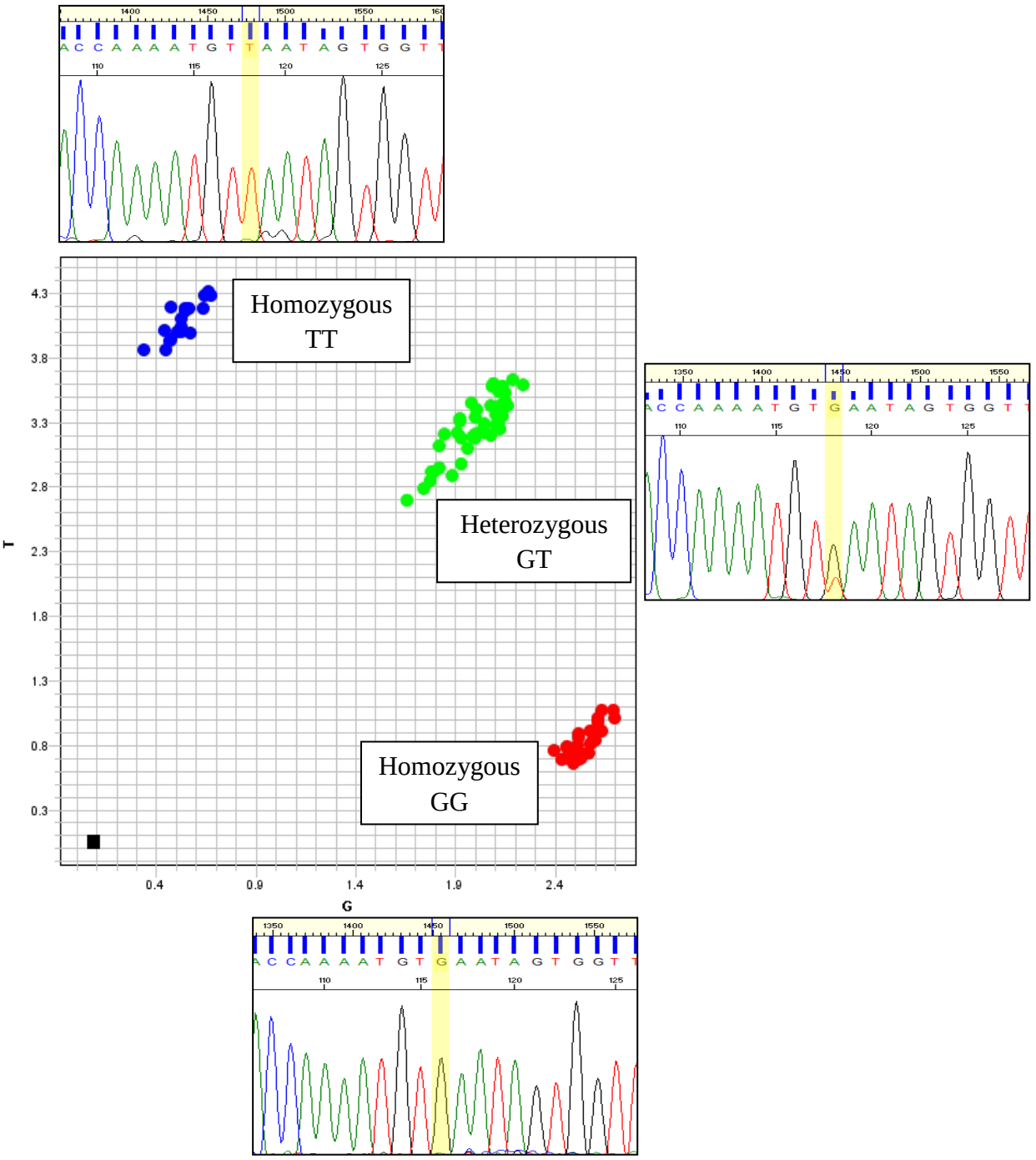


Figure 3.1.6. Genotyping of *STAT4* rs7574865 with Taqman SNP genotyping assay. Allelic discrimination plots show distribution of fluorescent signals according to genotypes of *STAT4* rs7574865. Validation of genotyping results was done by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous TT and GG produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous GT emitted overlapping signals of both alleles.

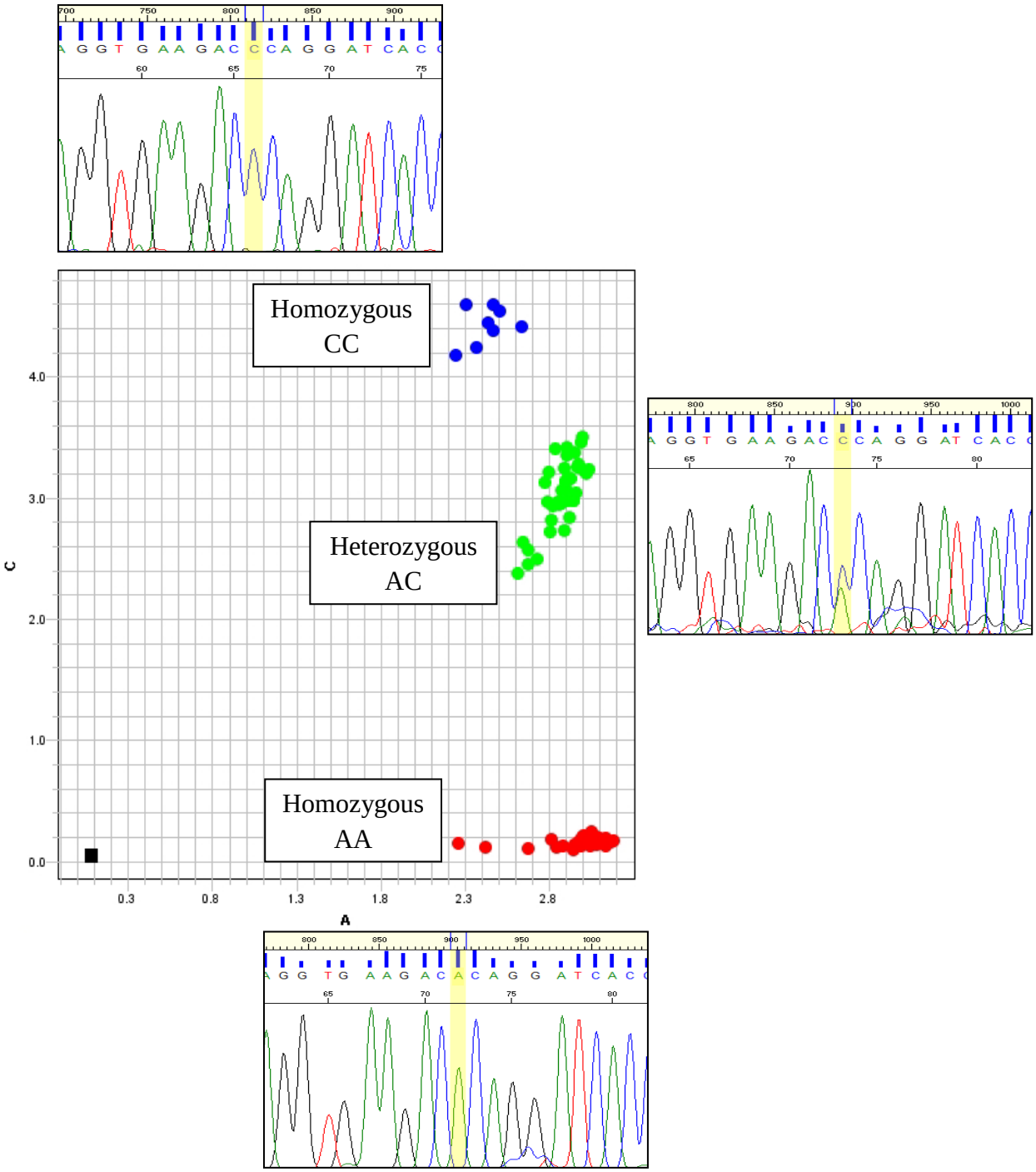


Figure 3.1.7. Genotyping of *IRF5* rs729302 with Taqman SNP genotyping assay. Allelic discrimination plots show distribution of fluorescent signals according to genotypes of *IRF5* rs729302. Validation of genotyping results was done by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous AA and CC produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous AC emitted overlapping signals of both alleles.

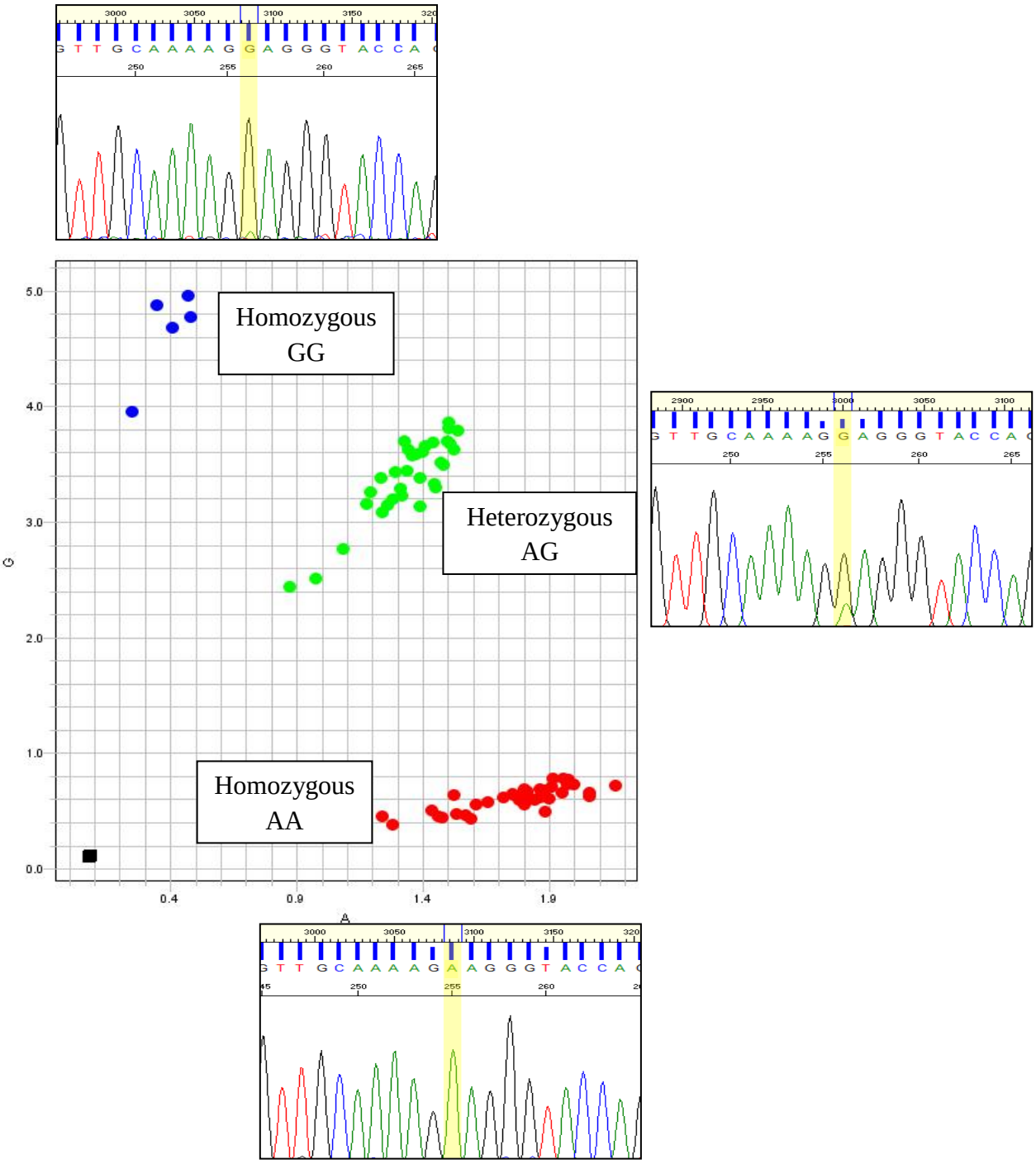


Figure 3.1.8. Genotyping of *HLA-DRB1/HLA-DQA1* rs9271366 with Taqman SNP genotyping assay. Allelic discrimination plots show distribution of fluorescent signals according to genotypes of *HLA-DRB1/HLA-DQA1* rs9271366. Validation of genotyping results was done by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous AA and GG produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous AG emitted overlapping signals of both alleles.

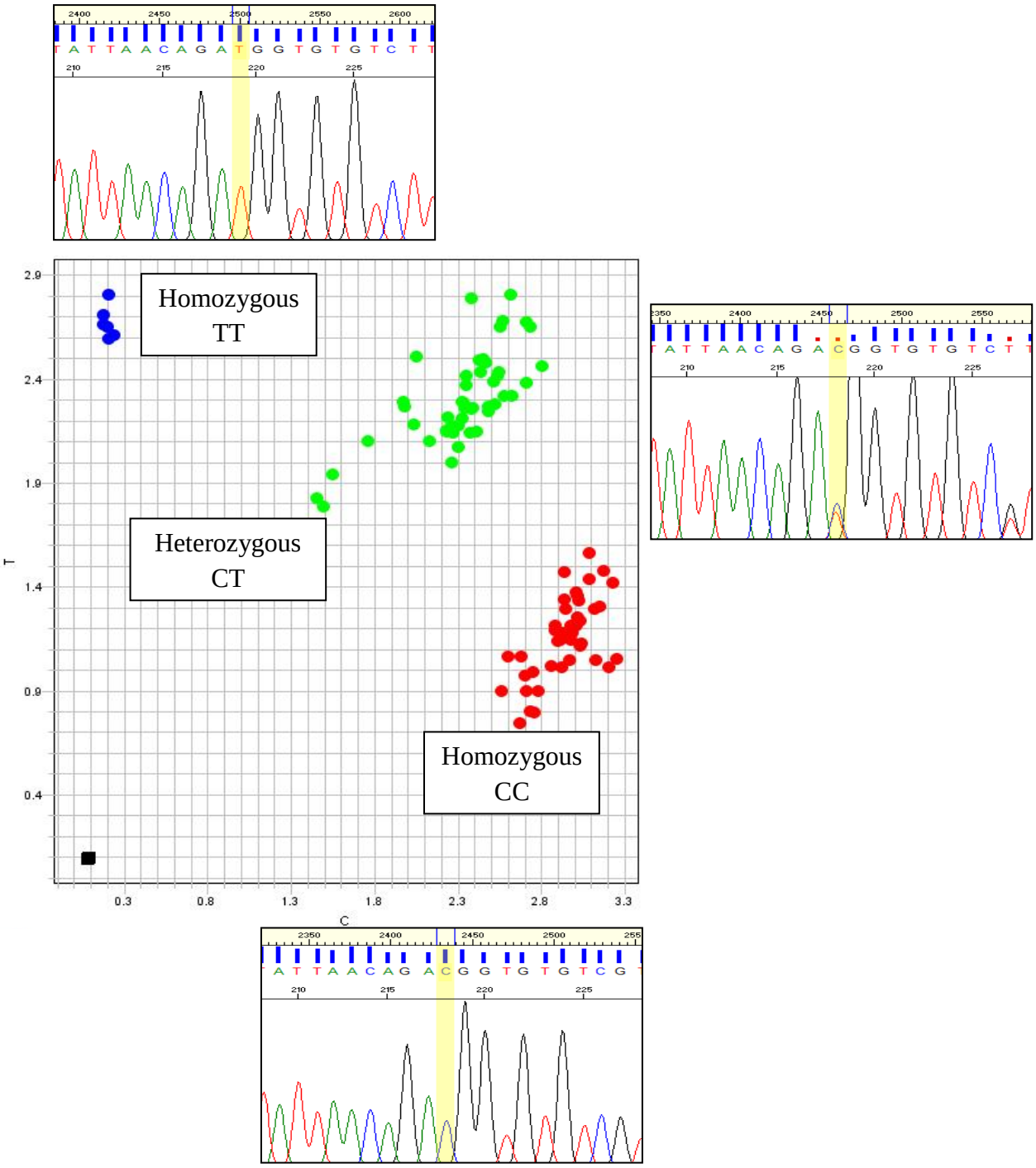


Figure 3.1.9. Genotyping of *HLA-DQB1/HLA-DQA2* rs9275328 with Taqman SNP genotyping assay. Allelic discrimination plots show distribution of fluorescent signals according to genotypes of *HLA-DQB1/HLA-DQA2* rs9275328. Validation of genotyping results was done by sequencing. Ten samples from each genotype were sent for sequencing. Homozygous CC and TT produced one fluorescent signal corresponding to their respective allele at the polymorphic site, while heterozygous CT emitted overlapping signals of both alleles.

3.1.3 Gene Polymorphisms involved in Toll-like Receptor/Interferon signalling pathways in SLE

3.1.3.1 Polymorphisms and SLE risk

The χ^2 goodness-of-fit test demonstrated that all polymorphisms investigated in this study fulfilled HWE in both SLE and control groups. Fisher's exact test revealed rs7574865 and rs10168266 of *STAT4* as well as rs2230936 and rs3757173 of *TNFAIP3* were significantly (p<0.05) associated with SLE susceptibility in Malaysians (**Table 3.1.2**). The minor alleles of *STAT4* rs7574865 and rs10168266 and *TNFAIP3* rs3757173 significantly increased the risk for SLE in Malaysians by having OR>1. However, for *TNFAIP3* rs2230936, the minor allele showed protective effect for SLE risk instead (OR=0.53, 95% CI: 0.30-0.92). The minor homozygous genotypes of *STAT4* rs7574865 and rs10168266 and heterozygous genotype of *TNFAIP3* rs3757173 elevated SLE risk in the Malaysians (p<0.05, ORs>1.5), while those bearing heterozygous genotype of *TNFAIP3* rs2230936 gained significant protection against SLE (p=0.032, OR=0.54, 95% CI: 0.31-0.96). The remaining SNPs did not show significant associations with SLE in our Malaysian cohort.

The frequency of minor allele T of *STAT4* rs7574865 was significantly increased in Malay and Chinese SLE patients (p=0.019, OR=1.62, 95% CI: 1.08-2.43; p=0.004; OR=1.42, 95% CI: 1.12-1.82, respectively). The minor homozygous TT was found to elevate risk of SLE in the Chinese only (p=0.046, OR=2.01, 95% CI: 1.21-3.33). As for the other SNP in *STAT4*, rs10168266, its minor allele T was observed to be significantly linked to SLE susceptibility in Malays (p=0.005, OR=1.80, 95% CI: 1.20-2.71) and Chinese (p=0.014,OR=1.36, 95% CI: 1.07-1.73). The minor homozygous TT was associated with risk of SLE in the Chinese only (p=0.047, OR=1.93, 95% CI: 1.15-3.24). In the case of *TNFAIP3* rs2230936, only the Malays having minor allele G (p=0.025, OR=0.33, 95% CI: 0.12-0.91) and heterozygous TG (p=0.021, OR=0.31, 95% CI: 0.11-0.88) were observed to reduce the risk of SLE. Indians were not affected by any of the SNPs studied.

Table 3.1.2. Frequencies of alleles and genotypes for SNPs of *STAT4*, *TNFAIP3*, and *IRF5* genes in patients with SLE and in healthy control subjects.

Ethnic	Locus	Frequency		χ^2 (*p* value)	OR (95% CI)
		SLE patients	Healthy controls		
	***STAT4* rs7574865**				
Malaysian		**n=360**	**n=430**		
	Allele				
	G†	397 (55.1%)	544 (63.3%)	-	1.00
	T	323 (44.9%)	316 (36.7%)	10.72 (0.001)**	1.40 (1.14-1.71)
	Genotype				
	GG†	107 (29.7%)	172 (40.0%)	-	1.00
	GT	183 (50.8%)	200 (46.5%)	1.47 (0.226)	1.47 (1.08-2.01)
	TT	70 (19.4%)	58 (13.5%)	5.12 (0.024)*	1.94 (1.27-2.96)
Malays		**n=93**	**n=110**		
	Allele				
	G†	104 (55.9%)	148 (67.3%)	-	1.00
	T	82 (44.1%)	72 (32.7%)	5.52 (0.019)*	1.62 (1.08-2.43)
	Genotype				
	GG†	29 (31.2%)	51 (46.4%)	-	1.00
	GT	46 (49.5%)	46 (41.8%)	1.19 (0.276)	1.76 (0.95-3.24)
	TT	18 (19.3%)	13 (11.8%)	2.21 (0.137)	2.44 (1.04-5.68)
Chinese		**n=245**	**n=294**		
	Allele				
	G†	263 (53.7%)	366 (62.2%)	-	1.00
	T	227 (46.3%)	222 (37.8%)	8.08 (0.004)*	1.42 (1.12-1.82)
	Genotype				
	GG†	69 (28.2%)	114 (38.8%)	-	1.00

	GT	125 (51.0%)	138 (46.9%)	0.89 (0.345)	1.50 (1.02-2.20)
	TT	51 (20.8%)	42 (14.3%)	4.00 (0.046)*	2.01 (1.21-3.33)
Indian		**n=22**	**n=26**		
	Allele				
	G†	30 (68.2%)	30 (57.7%)	-	1.00
	T	14 (31.8%)	22 (42.3%)	1.12 (0.290)	0.64 (0.27-1.47)
	Genotype				
	GG†	9 (40.9%)	7 (26.9%)	-	1.00
	GT	12 (54.5%)	16 (61.5%)	0.24 (0.624)	0.58 (0.17-2.01)
	TT	1 (4.6%)	3 (11.6%)	0.76 (0.382)	0.26 (0.02-3.06)
	***STAT4* rs10168266**				
Malaysian		**n=360**	**n=430**		
	Allele				
	C†	400 (55.6%)	551 (64.1%)	-	1.00
	T	320 (44.4%)	309 (35.9%)	11.86 (5.75 x 10^{-4})**	1.43 (1.17-1.75)
	Genotype				
	CC†	104 (28.9%)	172 (40.0%)	-	1.00
	CT	192 (53.3%)	207 (48.1%)	2.12 (0.146)	1.53 (1.12-2.10)
	TT	64 (17.8%)	51 (11.9%)	5.52 (0.019)*	2.08 (1.34-3.23)
Malays		**n=93**	**n=110**		
	Allele				
	C†	104 (55.9%)	153 (69.5%)	-	1.00
	T	82 (44.1%)	67 (30.5%)	8.06 (0.005)*	1.80 (1.20-2.71)
	Genotype				
	CC†	26 (28.0%)	53 (48.2%)	-	1.00
	CT	52 (55.9%)	47 (42.7%)	3.51 (0.061)	2.26 (1.22-4.16)
	TT	15 (16.1%)	10 (9.1%)	2.31 (0.128)	3.06 (1.21-7.73)

Chinese		**n=245**	**n=294**		
	Allele				
	C†	266 (54.3%)	363 (61.7%)	-	1.00
	T	224 (45.7%)	225 (38.3%)	6.10 (0.014)*	1.36 (1.07-1.73)
	Genotype				
	CC†	69 (28.2%)	108 (36.7%)	-	1.00
	CT	128 (52.2%)	147 (50.0%)	0.27 (0.604)	1.36 (0.93-2.00)
	TT	48 (19.6%)	39 (13.3%)	3.95 (0.047)*	1.93 (1.15-3.24)
Indian		**n=22**	**n=26**		
	Allele				
	C†	30 (68.2%)	35 (67.3%)	-	1.00
	T	14 (31.8%)	17 (32.7%)	0.01 (0.929)	0.96 (0.41-2.27)
	Genotype				
	CC†	9 (40.9%)	11 (42.3%)	-	1.00
	CT	12 (54.5%)	13 (50.0%)	0.10 (0.753)	1.13 (0.35-3.67)
	TT	1 (4.6%)	2 (7.7%)	0.20 (0.654)	0.61 (0.05-7.88)
	***STAT4* rs7601754**				
Malaysian		**n=360**	**n=430**		
	Allele				
	A†	641 (89.0%)	745 (86.7%)	-	-
	G	79 (11.0%)	115 (13.4%)	2.10 (0.148)	0.80 (0.59-1.08)
	Genotype				
	AA†	285 (79.2%)	322 (74.9%)	-	1.00
	AG	71 (19.7%)	101 (23.5%)	1.63 (0.201)	0.79 (0.56-1.12)
	GG	4 (1.1%)	7 (1.6%)	0.38 (0.537)	0.65 (0.19-2.23)

Malays		**n=93**	**n=110**		
	Allele				
	A†	168 (90.3%)	189 (85.9%)	-	1.00
	G	18 (9.7%)	31 (14.1%)	1.85 (0.174)	0.65 (0.35-1.21)
	Genotype				
	AA†	76 (81.7%)	80 (72.7%)	-	1.00
	AG	16 (17.2%)	29 (26.4%)	2.45 (0.118)	0.58 (0.29-1.15)
	GG	1 (1.1%)	1 (0.9%)	0.01 (0.906)	1.05 (0.07-17.13)
Chinese		**n=245**	**n=294**		
	Allele				
	A†	433 (88.4%)	509 (86.6%)	-	1.00
	G	57 (11.6%)	79 (13.4%)	0.79 (0.375)	0.85 (0.59-1.22)
	Genotype				
	AA†	191 (78.0%)	221 (75.2%)	-	1.00
	AG	51 (20.8%)	67 (22.8%)	0.30 (0.581)	0.88 (0.58-1.33)
	GG	3 (1.2%)	6 (2.0%)	0.54 (0.462)	0.58 (0.15-2.40)
Indian		**n=22**	**n=26**		
	Allele				
	A†	40 (90.9%)	47 (90.4%)	-	-
	G	4 (9.1%)	5 (9.6%)	0.01 (0.929)	0.93 (0.22-4.01)
	Genotype				
	AA†	18 (81.8%)	21 (80.8%)	-	1.00
	AG	4 (18.2%)	5 (19.2%)	0.01 (0.924)	0.93 (0.22-4.01)
	GG	0 (0%)	0 (0%)		0 (0-NaN)

	TNFAIP3 **rs2230926**			
Malaysian	**n=360**	**n=430**		
Allele				
T†	701 (97.4%)	818 (95.1%)	-	1.00
G	19 (2.6%)	42 (4.9%)	5.32 (0.021)*	0.53 (0.30-0.92)
Genotype				
TT†	341 (94.7%)	389 (90.5%)	-	1.00
TG	19 (5.3%)	40 (9.3%)	4.59 (0.032)*	0.54 (0.31-0.96)
GG	0 (0%)	1 (0.2%)	0.84 (0.360)	0 (0-NaN)
Malays	**n=93**	**n=110**		
Allele				
T†	181 (97.3%)	203 (92.3%)	-	1.00
G	5 (2.7%)	17 (7.7%)	5.00 (0.025)*	0.33 (0.12-0.91)
Genotype				
TT†	88 (94.6%)	93 (84.5%)	-	1.00
TG	5 (5.4%)	17 (15.5%)	5.30 (0.021)*	0.31 (0.11-0.88)
GG	0 (0%)	0 (0%)		0 (0-NaN)
Chinese	**n=245**	**n=294**		
Allele				
T†	476 (97.1%)	563 (95.7%)	-	1.00
G	14 (2.9%)	25 (4.3%)	1.49 (0.222)	0.66 (0.34-1.29)
Genotype				
TT†	231 (94.3%)	270 (91.8%)	-	1.00
TG	14 (5.7%)	23 (7.8%)	0.93 (0.335)	0.71 (0.36-1.42)
GG	0 (0%)	1 (0.4%)	0.84 (0.361)	0 (0-NaN)

Indian		**n=22**	**n=26**		
	Allele				
	T†	44 (100%)	52 (100%)	-	1.00
	G	0 (0%)	0 (0%)	49.33 (0)	0 (0-NaN)
	Genotype				
	TT†	22 (100%)	26 (100%)	-	1.00
	TG	0 (0%)	0 (0%)	24.67 ($1.81x10^{-5}$)	0 (0-NaN)
	GG	0 (0%)	0 (0%)		0 (0-NaN))
	***TNFAIP3* rs3757173**				
Malaysian		**n=360**	**n=430**		
	Allele				
	T†	666 (92.5%)	820 (95.3%)	-	1.00
	C	54 (7.5%)	40 (4.7%)	5.68 (0.017)*	1.66 (1.09-2.53)
	Genotype				
	TT†	308 (85.6%)	391 (90.9%)	-	1.00
	TC	50 (13.9%)	38 (8.9%)	5.05 (0.025)*	1.67 (1.07-2.61)
	CC	2 (0.5%)	1 (0.2%)	0.54 (0.462)	2.40 (0.23-28.13)
Malays		**n=93**	**n=110**		
	Allele				
	T†	171 (91.9%)	209 (95.0%)	-	1.00
	C	15 (8.1%)	11 (5.0%)	1.58 (0.209)	1.67 (0.75-3.72)
	Genotype				
	TT†	78 (83.9%)	99 (90.0%)	-	1.00
	TC	15 (16.1%)	11 (10.0%)	1.70 (0.193)	1.73 (0.75-3.98)
	CC	0 (0%)	0 (0%)		0 (0-NaN)

Chinese		**n=245**	**n=294**		
	Allele				
	T†	455 (92.9%)	561 (95.4%)	-	1.00
	C	35 (7.1%)	27 (4.6%)	3.21 (0.073)	1.60 (0.95-2.68)
	Genotype				
	TT†	212 (86.5%)	268 (91.2%)	-	1.00
	TC	31 (12.7%)	25 (8.5%)	2.47 (0.116)	1.57 (0.90-2.74)
	CC	2 (0.8%)	1 (0.3%)	0.55 (0.460)	2.53 (0.23-28.07)
Indian		**n=22**	**n=26**		
	Allele				
	T†	40 (90.9%)	50 (96.2%)	-	1.00
	C	4 (9.1%)	2 (3.8%)	1.12 (0.290)	2.50 (0.44-14.35)
	Genotype				
	TT†	18 (81.8%)	24 (92.3%)	-	1.00
	TC	4 (18.2%)	2 (7.7%)	1.20 (0.274)	2.67 (0.44-16.20)
	CC	0 (0%)	0 (0%)		0 (0-NaN)
	***IRF5* rs4728142**				
Malaysian		**n=360**	**n=430**		
	Allele				
	G†	625 (86.8%)	768 (89.3%)	-	1.00
	A	95 (13.2%)	92 (10.7%)	2.34 (0.126)	1.27 (0.93-1.72)
	Genotype				
	GG†	271 (75.3%)	343 (79.8%)	-	1.00
	GA	83 (23.1%)	82 (19.1%)	1.88 (0.170)	1.28 (0.91-1.81)
	AA	6 (1.6%)	5 (1.1%)	0.36 (0.547)	1.52 (0.46-5.03)

Malays		**n=93**	**n=110**		
	Allele				
	G†	158 (55.9%)	190 (86.4%)	-	1.00
	A	28 (44.1%)	30 (13.6%)	0.17 (0.685)	1.12 (0.64-1.96)
	Genotype				
	GG†	66 (71.0%)	81 (73.6%)	-	1.00
	GA	26 (28.0%)	28 (25.5%)	0.16 (0.687)	1.14 (0.61-2.13)
	AA	1 (1.0%)	1 (0.9%)	0.01 (0.906)	1.23 (0.08-20.00)
Chinese		**n=245**	**n=294**		
	Allele				
	G†	436 (89.0%)	540 (91.8%)	-	1.00
	A	54 (11.0%)	48 (8.2%)	2.55 (0.111)	1.40 (0.93-2.10)
	Genotype				
	GG†	195 (79.6%)	249 (84.7%)	-	1.00
	GA	46 (18.8%)	42 (14.3%)	1.97 (0.160)	1.40 (0.88-2.21)
	AA	4 (1.6%)	3 (1.0%)	0.39 (0.532)	1.70 (0.38-7.70)
Indian		**n=22**	**n=26**		
	Allele				
	G†	31 (70.5%)	38 (73.1%)	-	1.00
	A	13 (29.5%)	14 (26.9%)	0.08 (0.776)	1.14 (0.47-2.78)
	Genotype				
	GG†	10 (45.5%)	13 (50.0%)	-	1.00
	GA	11 (50.0%)	12 (46.2%)	0.07 (0.790)	1 .19 (0.37-3.81)
	AA	1 (4.5%)	1 (3.8%)	0.02 (0.903)	1.30 (0.07-23.43)

	***IRF5* rs729302**			
Malaysian		**n=360**	**n=430**	
	Allele			
	A†	481 (66.8%)	564 (65.6%)	-	1.00
	C	239 (33.2%)	296 (34.4%)	0.26 (0.609)	0.95 (0.77-1.17)
	Genotype			
	AA†	160 (44.4%)	184 (42.8%)	-	1.00
	AC	161 (44.7%)	196 (45.6%)	0.06 (0.810)	0.95 (0.70-1.27)
	CC	39 (10.9%)	50 (11.6%)	0.12 (0.725)	0.90 (0.56-1.43)
Malays		**n=93**	**n=110**		
	Allele				
	A†	128 (68.8%)	143 (65.0%)	-	1.00
	C	58 (31.2%)	77 (35.0%)	0.66 (0.416)	0.84 (0.56-1.28)
	Genotype				
	AA†	43 (46.2%)	49 (44.5%)	-	1.00
	AC	42 (45.2%)	45 (40.9%)	0.37 (0.542)	1.06 (0.59-1.91)
	CC	8 (8.6%)	16 (14.5%)	1.71 (0.191)	0.57 (0.22-1.46)
Chinese		**n=245**	**n=294**		
	Allele				
	A†	320 (65.3%)	384 (65.3%)	-	1.00
	C	170 (34.7%)	204 (34.7%)	0 (1.000)	1.00 (0.78-1.29)
	Genotype				
	AA†	106 (43.3%)	121 (41.2%)	-	1.00
	AC	108 (44.1%)	142 (48.3%)	0.96 (0.328)	0.87 (0.61-1.25)
	CC	31 (12.7%)	31 (10.5%)	0.58 (0.445)	1.14 (0.65-2.00)

Indian		**n=22**	**n=26**		
	Allele				
	A†	33 (75.0%)	37 (71.2%)	-	1.00
	C	11 (25.0%)	15 (28.8%)	0.18 (0.672)	0.73 (0.23-2.30)
	Genotype				
	AA†	11 (50.0%)	14 (53.8%)	-	1.00
	AC	11 (50.0%)	9 (34.6%)	1.16 (0.281)	1.56 (0.48-5.08)
	CC	0 (0%)	3 (11.6%)	2.71 (0.100)	0

†reference category,*p<0.05, **p<0.002

3.1.3.2 Linkage disequilibrium (LD)

From the genotyping analysis carried out in **Section 3.1.3.1**, four SNPs were found to be significantly associated with SLE and these SNPs were then subjected to LD analysis.*STAT4* rs7574865 and rs10168266 of chromosome 2 were grouped as an LD block by having D' value of 0.835 and r^2 value of 0.679 (**Figure 3.1.10**). Haplotype TT was significantly associated with SLE (p=5.94 x 10^{-5}, OR=1.53, 95% CI:1.24-1.88) and remained significant after 10000 permutations (p=1 x 10^{-4}) (**Table 3.1.3**). Haplotype CG, on the other hand, significantly conferred protection against SLE by having p value of 0.006 and OR value of 0.76 (CI:0.62-0.92), and remained significant after 10000 permutations (p=0.02). D prime (D') and r^2 values between *TNFAIP3* rs2230936 and rs3757173 were 0.383 and 0.093, respectively, and they did not form an LD block.

3.1.3.3 SNP-SNP interaction

The potential gene-gene interaction among SNPs that were significantly associated with SLE in **Section 3.1.3.1** was evaluated using the MDR method. It is essential to look into how the SNPs will interact with each other to cause the disease. The best models for main effects to SLE-associated SNP combinations are shown in **Table 3.1.4**. The final best model was selected for having the highest balanced accuracy and CVC and the two-SNP combinations comprising of rs7574865 and rs10168266 of *STAT4* gene fulfilled these criteria. Frequencies of cases and controls for the different multi-locus genotypes for this model are illustrated in **Figure 3.1.11**. Multifactor dimensionality reduction (MDR) test provided if-then rules to predict the occurrence of SLE by looking at the case:control (SLE/No) ratio for each genotype combination of these two SNPs. This meant if the genotype combinations had case:control ratio of >1, which were also the high-risk groups, then they could be used to predict the presence of SLE in an individual. In the present study, genotypes for *STAT4* rs7574865 and rs10168266 in combinations of GT and CT, and TT and TT, accordingly, were assigned to high-risk group by obtaining case:control ratio of >1 and could be used for SLE prediction (**Table 3.1.5)**. Although GT and CT combination had case:control ratio of 0.99 only, maybe because of the relatively higher number of controls compared with cases, it was still classified as high-risk group by the software. However, due to this reason, as well as the fact that these two genotypes were not significant for SLE susceptibility from the previous Fisher's exact test, the present

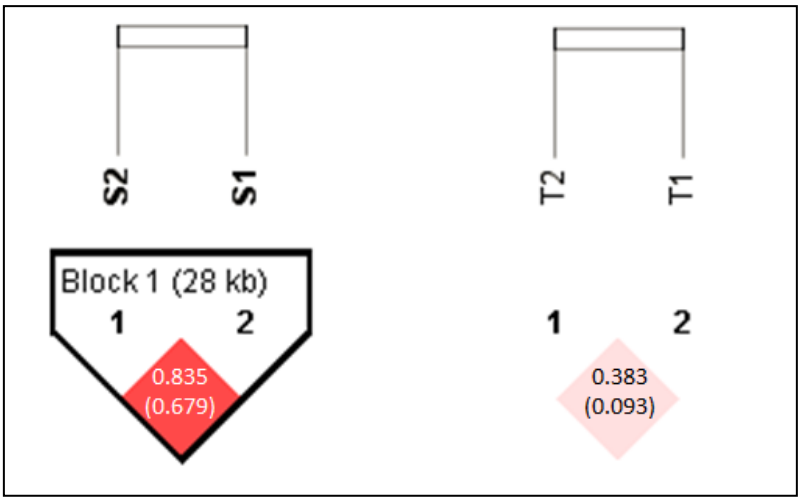


Figure 3.1.10. Linkage disequilibrium analysis of the SLE-associated SNPs of *STAT4* and *TNFAIP3*. The analysis clustered *STAT4* rs7574865 (S1) and rs10168266 (S2) as an LD block, having D' value of 0.835 and r^2 value of 0.675. *TNFAIP3* rs2230936 (T1) andrs3757173 (T2) which had D' value of 0.383 and r^2 value of 0.093 were not identified as an LD block. The top magnitude in each square indicates the D' value while the bottom one in bracket represents r^2 value.

Table 3.1.3. Haplotype analysis of *STAT4* rs7574865 and rs10168266. Frequency of each haplotype constituted by *STAT4* rs7574865 (S1) and rs10168266 (S2) is shown, as well as the permutation test results.

Haplotype (Block : S1, S2)	Frequency					
	Case (n=720)	Control (n=860)	χ^2	*p* value	*p* value after permutation	OR (95% CI)
TT	0.519	0.588	7.57	5.94×10^{-5}	$1 \times 10^{-4*}$	1.53 (1.24-1.88)
CG	0.412	0.315	16.12	0.006	0.02*	0.76 (0.62-0.92)
CT	0.037	0.053	2.35	0.126	-	-
TG	0.033	0.045	1.54	0.215	-	-

* Significantly associated with SLE after 10000 permutations.

Table 3.1.4. MDR analysis of SLE-associated SNPs of *STAT4* and *TNFAIP3*. The best combination of SNPs is identified as having the highest balanced accuracy, and the highest average cross validation consistency.

Model	Balanced accuracy[a]	Average CVC[b]
S1	0.5298	5/10
S1 S2*	0.5669	6/10
T1 T2 S1	0.5649	5/10
T1 T2 S1 S2	0.5615	10/10

S1-*STAT4* rs7574865; S2–*STAT4* rs10168266; T1-*TNFAIP3* rs2230936; T2-*TNFAIP3* rs3757173; CVC-cross-validation consistency

*Final best model, *p* value = 0.0002

[a]Balanced accuracy is the accuracy of classification of cases and controls in the whole dataset.

[b]Average CVC is the number of times the model was selected as the best model after 10-fold cross-validation runs.

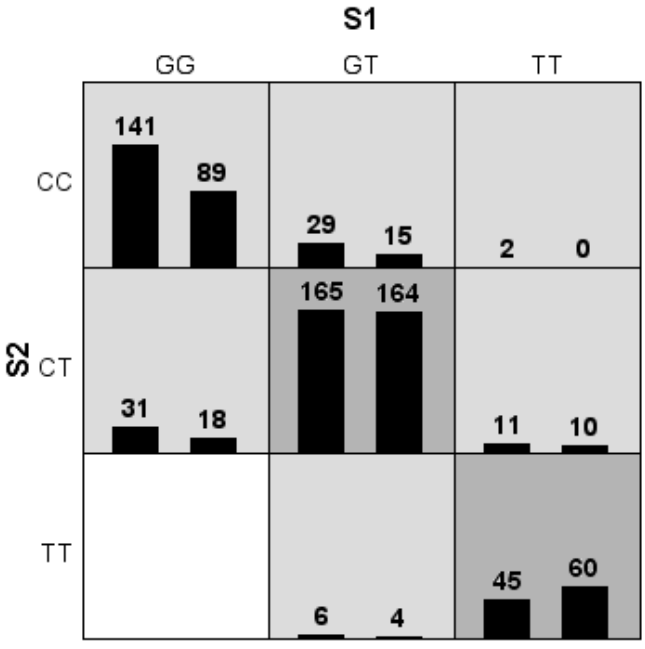


Figure 3.1.11. Frequencies of cases and controls for the different multi-locus genotypes of the best model obtained by MDR analysis (combination of rs7574865 and rs10168266 of *STAT4* gene). For each cell, the left bar represents the number of controls and the right bar the number of cases. Cells shaded in dark gray denote high-risk multi-locus genotypes, light gray low-risk multi-locus genotypes. S1-*STAT4* rs7574865, S2-*STAT4* rs10168266

Table 3.1.5. Prediction for occurrence of SLE under each genotype combination of rs7574865 and rs10168266 of *STAT4* gene. The prediction was determined by case/control (SLE/No) ratio. Genotype combination having SLE/No ratio of >1 was predicted to confer higher risk for SLE.

Combination (S1, S2)	Class 'SLE'	Class 'No'	SLE/No ratio	Predicted 'class'
GT, CT	164	165	0.99	SLE
GT, TT	4	6	0.67	No
GT, CC	15	29	0.52	No
TT, CT	10	11	0.91	No
TT, TT*	60	45	1.33	SLE
TT, CC	0	2	0	No
GG, CT	18	31	0.58	No
GG, CC	89	141	0.63	No

*most relevant genotype combination, S1- *STAT4* rs7574865, S2-*STAT4* rs10168266

study decided to exclude GT and CT combination from the high-risk group. Thus, it was concluded that a person who bears genotype combination of TT and TT for *STAT4* rs7574865 and rs10168266 may be predicated to have higher risk for SLE.

The interaction entropy graph for the four SLE-associated SNPs is illustrated in **Figure 3.1.12**. The entropy removed by each SNP and interactions between SNPs was small. *STAT4* rs7574865 and rs10168266 removed the most entropy, which were 1.00% and 1.16%, respectively. Only one synergistic interaction effects were observed, i.e. between *TNFAIP3* rs2230936and rs3757173 which explained 0.22% of the entropy. The remaining interactions showed redundancy by having negative entropy value, including interaction between *STAT4* rs7574865 and rs10168266 (-0.86%). Interaction between *TNFAIP3* rs2230936and *STAT4* rs10168266, however, removed the most entropy (-0.90%). The redundancy is an indication of the presence of linkage disequilibrium between these SNPs. The interaction dendogram obtained with the MDR software is shown in **Figure 3.1.13**. In accordance to the results obtained from the entropy graph, redundant interaction between *STAT4* rs7574865 and rs10168266 was also observed in the dendogram. *TNFAIP3* rs2230936and *STAT4* rs10168266 had the strongest interaction by being closely apart. *TNFAIP3* rs3757173 was on the separate and further branch from the other SNPs, indicating *TNFAIP3* rs3757173 had the least interaction with the other SNPs.

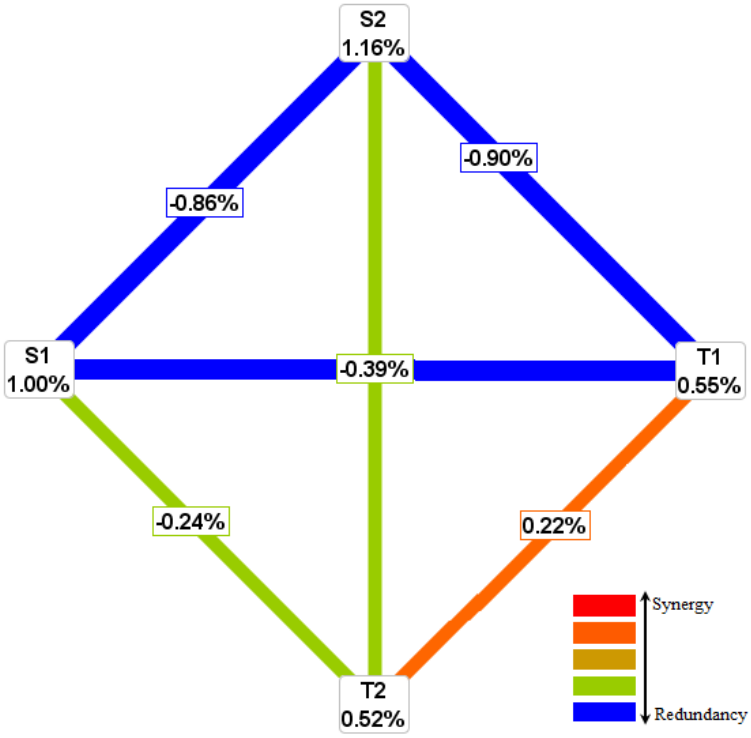


Figure 3.1.12. Entropy-based interaction graph of SLE-associated SNPs of *STAT4* and *TNFAIP3*. The percentage of entropy removed by each SNP is visualized in the boxes. The percentage of entropy removed by the two-way interactions between SNPs is shown by each connection. Positive entropy values indicate synergic interaction while negative entropy values indicate redundancy. S1-*STAT4* rs7574865; S2–*STAT4* rs10168266; T1-*TNFAIP3* rs2230936; T2-*TNFAIP3* rs3757173.

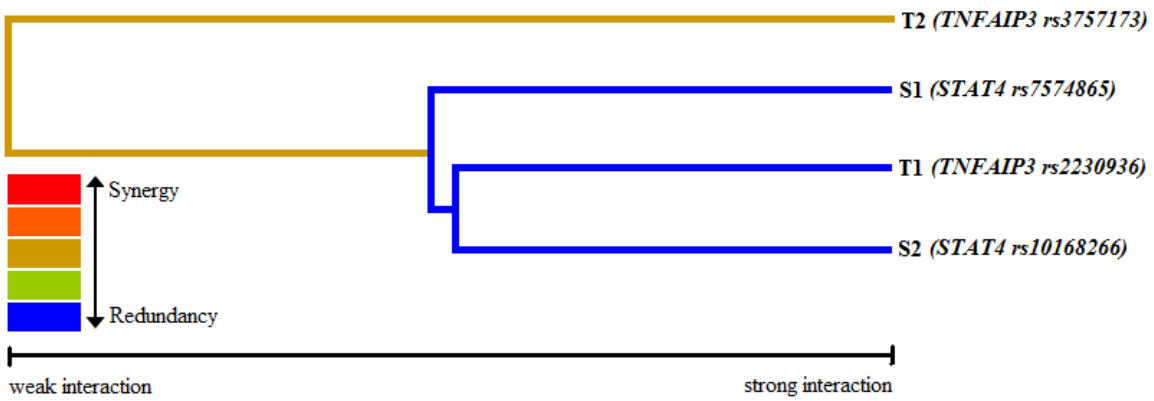


Figure 3.1.13. Interaction dendogram of SLE-associated SNPs of *STAT4* and *TNFAIP3*. Stronger interactions between SNPs are visualized by depicting SNPs more closely together at the leaves of the tree (right side of the graph).

3.1.4 Polymorphisms in HLA region

3.1.4.1 Polymorphisms and SLE risk

The χ^2 goodness-of-fit test demonstrated that the two polymorphisms investigated in this study fulfilled HWE in both SLE and control groups. Fisher's exact test showed that the *HLA-DRB1/HLA-DQA1* rs9271366 was significantly associated with SLE risk in the Malaysian population (p=5.78 x 10^{-5}, OR=1.63, 95% CI: 1.28-2.06) in general. Specifically, it increased risk in the Malays (p=0.044, OR=1.54, 95% CI: 1.01-2.34)and Chinese (p=2.05 x 10^{-4}, OR=1.79, 95% CI: 1.31-2.43). SLE susceptibility was conferred by the minor allele G and its homozygous genotype GG (**Table 3.1.6**). Interestingly in the Chinese, the blending of minor risk allele G into the genotypes seemed to gradually increase the risk for SLE from homozygous AA, heterozygous AG (p=0.026, OR=1.65, 95% CI: 1.13-2.40), and homozygous GG (p=0.009, OR=3.92, 95% CI: 1.50-10.25).

In the case of *HLA-DQB1/HLA-DQA2* rs9275328, the minor allele T was associated with reduced SLE risk in the Malaysians (p=1.93 x 10^{-5}, OR=0.58, 95% CI: 0.45-0.74), Malays (p=0.004, OR=0.51, 95% CI: 0.32-0.82) and Chinese (p=2.71 x 10^{-4}, OR=0.56, 95% CI: 0.40-0.76) (Table 2). The Malaysians, including Malays and Chinese, bearing the heterozygous genotype CT presented significant protection against SLE by having ORs of around 0.50, with p values of <0.05. Generally, the Indians are routinely included in our studies but often their low numbers do not result in any statistical significance. Thus it was not surprising to find that both SNPs did not show association with SLE in the Indians. However, there is a departure from this trend whereby the homozygous GG of rs9271366 and heterozygous CT of rs9275328 were significantly associated with SLE risk and protection, respectively, in these three populations.

3.1.4.2 Linkage disequilibrium (LD)

Linkage disequilibrium (LD) analysis showed that rs9271366 and rs9275328 were in the same LD block, by having a very high D' value of 0.941 (**Figure 3.1.14**). However, the low r^2 values of 0.065 indicated that both SNPs hardly correlated with each other. Haplotypes in this LD block that were significantly associated with SLE even 10000 permutations were applied were GC and AT (p<5.0 x 10^{-4}), in which haplotype GC was a

Table 3.1.6. Frequencies of alleles and genotypes for rs9271366 and rs9275328 polymorphisms in patients with SLE and in healthy control subjects.

Ethnic	Locus	Frequency		χ^2 (*p* value)	OR (95% CI)
		SLE patients	Healthy controls		
	***HLA-DRB1/HLA-DQA1* (rs9271366)**				
Malaysian		**n=360**	**n=430**		
	Allele				
	A†	525 (72.9%)	700 (81.4%)	-	1.00
	G	195 (27.1%)	160 (18.6%)	16.17 (5.78 x 10^{-5})**	1.63 (1.28-2.06)
	Genotype				
	AA†	197 (54.7%)	284 (66.0%)	-	1.00
	AG	131 (36.4%)	132 (30.7%)	2.86 (0.091)	1.43 (1.06-1.94)
	GG	32 (8.9%)	14 (3.3%)	11.34 (0.001)**	3.30 (1.71-6.34)
Malays		**n=93**	**n=110**		
	Allele				
	A†	117 (62.9%)	159 (72.3%)	-	1.00
	G	69 (37.1%)	61 (27.7%)	4.07 (0.044)*	1.54 (1.01-2.34)
	Genotype				
	AA†	40 (43.0%)	55 (56.4%)	-	1.00
	AG	37 (39.8%)	49 (33.6%)	0.47 (0.494)	1.04 (0.58-1.87)
	GG	16 (17.2%)	6 (10.0%)	7.20 (0.007)*	3.67 (1.32-10.20)
Chinese		**n=245**	**n=294**		
	Allele				
	A†	374 (76.3%)	501 (85.2%)	-	1.00
	G	116 (23.7%)	87 (14.8%)	13.78 (2.05 x 10^{-4})**	1.79 (1.31-2.43)
	Genotype				
	AA†	145 (59.2%)	213 (72.4%)	-	1.00
	AG	84 (34.3%)	75 (25.5%)	4.95 (0.026)*	1.65 (1.13-2.40)

	GG	16 (6.5%)	6 (2.0%)	6.88 (0.009)*	3.92 (1.50-10.25)
Indian		**n=22**	**n=26**		
	Allele				
	A†	31 (70.5%)	40 (76.9%)	-	1.00
	G	13 (29.5%)	12 (23.1%)	0.52 (0.472)	1.40 (0.56- 3.49)
	Genotype				
	AA†	12 (54.5%)	16 (61.5%)	-	1.00
	AG	7 (31.8%)	8 (30.8%)	0.01 (0.938)	1.67 (0.51-5.50)
	GG	3 (13.6%)	2 (7.7%)	0.45 (0.502)	0
	***HLA-DQB1/ HLA-DQA2* (rs9275328)**				
Malaysian		**n=360**	**n=430**		
	Allele				
	C†	609 (84.6%)	653 (75.9%)	-	1.00
	T	111 (15.4%)	207 (24.1%)	18.25 (1.93 x 10^{-5})**	0.58 (0.45-0.74)
	Genotype				
	CC†	260 (72.2%)	248 (57.7%)	-	1.00
	CT	89 (24.7%)	157 (36.5%)	12.70 (3.65 x 10^{-4})**	0.54 (0.40-0.74)
	TT	11 (3.1%)	25 (5.8%)	3.43 (0.064)	0.42 (0.20-0.87)
Malays		**n=93**	**n=110**		
	Allele				
	C†	150 (80.6%)	150 (68.2%)	-	1.00
	T	36 (19.4%)	70 (31.8%)	8.12 (0.004)*	0.51 (0.32-0.82)
	Genotype				
	CC†	61 (65.6%)	50 (45.5%)	-	1.00
	CT	28 (30.1%)	50 (45.5%)	5.02 (0.025)*	0.46 (0.25-0.83)
	TT	4 (4.3%)	10 (9.0%)	1.80 (0.180)	0.33 (0.10-1.11)

Chinese		**n=245**	**n=294**		
	Allele				
	C†	421 (85.9%)	454 (77.2%)	-	1.00
	T	69 (14.1%)	134 (22.8%)	13.26 (2.71 x 10^{-4})**	0.56 (0.40-0.76)
	Genotype				
	CC†	182 (74.3%)	175 (59.5%)	-	1.00
	CT	57 (23.3%)	104 (35.4%)	9.35 (0.002)*	0.53 (0.36-0.77)
	TT	6 (2.4%)	15 (5.1%)	2.51 (0.113)	0.39 (0.15-1.01)
Indian		**n=22**	**n=26**		
	Allele				
	C†	38 (86.4%)	49 (94.2%)	-	1.00
	T	6 (13.6%)	3 (5.8%)	1.74 (0.188)	2.58 (0.61-10.99)
	Genotype				
	CC†	17 (77.3%)	23 (88.5%)	-	1.00
	CT	4 (18.2%)	3 (11.5%)	0.42 (0.516)	1.80 (0.36-9.14)
	TT	1 (4.5%)	0 (0%)	1.21 (0.272)	0 (0-NaN)

†reference category,*p<0.05, **p<0.002

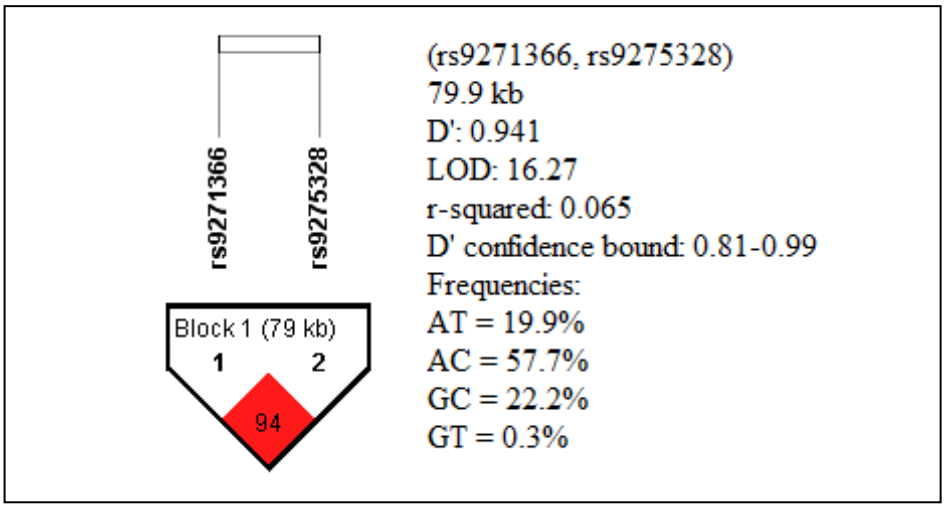


Figure 3.1.14. Linkage disequilibrium analysis of rs9271366 and rs9275328. LD plot of rs9271366 and rs9275328 shows that both SNPs are in the same LD block by having high D' value (0.941) but have low correlation to each other by having low r^2 value (0.065).

SLE predisposing factor (OR=1.63, 95% CI: 1.29-2.07) while haplotype AT, conversely, was found to provide protection against SLE (OR=0.57, 95% CI: 0.44-0.74) (**Table 3.1.7**).

3.1.4.3 SNP-SNP interaction

In MDR test, genotype combinations of AG and CC, as well as GG and CC of rs9271366 and rs9275328, respectively, were clustered as high-risk group by having case: control ratio of >1 [**Figure 3.1.15(A)**]. The OR of the genotype combinations was 1.79, with CI of 1.32 to 2.43 and *p* value of 0.0002. The interaction entropy graph illustrated that rs9271366 and rs9275328 removed 1.54% and 1.71% of the entropy, respectively, and their redundant interaction explained 1.96% of the entropy [**Figure 3.1.15(B)**].

Table 3.1.7. Haplotype analysis of rs9271366 and rs9275328. Frequency of each haplotype in Block 1 in SLE patients and normal individuals.

Haplotype (Block 1)	Frequency Case (n=720)	Frequency Control (n=860)	χ^2	*p* value	*p* value after permutation	OR (95% CI)
AC	0.578	0.576	0.005	0.943	1.00	1.01 (0.82-1.23)
GC	0.268	0.183	16.194	5.42×10^{-5}	$<5.00 \times 10^{-4*}$	1.63 (1.29-2.07)
AT	0.151	0.238	18.458	1.74×10^{-5}	$<5.00 \times 10^{-4*}$	0.57 (0.44-0.74)

* Significantly associated with SLE after 10000 permutations.

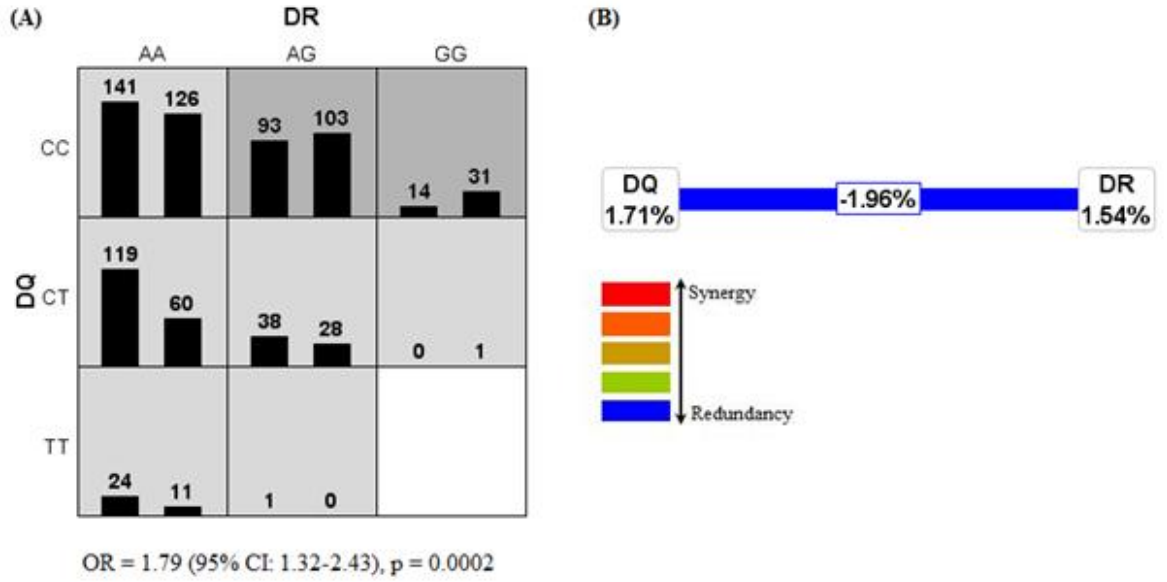


Figure 3.1.15. MDR analysis of rs9271366 and rs9275328. Frequencies of cases and controls for the different multi-locus genotypes of rs9271366 (DR) and rs9275328 (DQ) obtained by MDR is shown in (**A**). For each cell, the left bar represents the number of controls and the right bar the number of cases. Cells shaded in dark gray denote high-risk multi-locus genotypes, light gray low-risk multilocus genotypes. Entropy-based interaction graph between rs9271366 and rs9275328 was shown in **(B)**. The percentage of entropy removed by each SNP is visualized in the boxes. The percentage of entropy removed by the two-way interactions between SNPs is shown by each connection. Positive entropy values indicate synergic interaction while negative entropy values indicate redundancy. Here, rs9271366 and rs9275328 demonstrate redundant interaction by explaining 1.96% of the entropy.

3.2 Detection of SLE-associated proteins using proteomic approaches

3.2.1 Subject recruitment

A total of 29 normal healthy individuals and SLE/LN patients were selected for this study and the demographic data of these individuals is summarised in **Table 3.2.1**. The clinicopathological information of the patients is shown in **Table 3.2.2**.

Table 3.2.1. Individuals selected for this study.

Individual	n	Age range (years old)	Gender
Normal healthy individuals	5	23-30	
SLE without kidney involvement	5	42-50	
Lupus nephritis			
Class II	3	20-53	Female
Class III	3	22-55	
Class IV	5	19-35	
Class V	3	18-37	
Remission	5	28-53	
Total	29		

3.2.2 Optimisation of protein extraction and 2-DE

3.2.2.1 High-abundant plasma protein removal

After removal of the 14 high-abundant proteins (HAPs), which included albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha-2-macroglobulin, alpha-1-acid glycoprotein, IgM, apolipoprotein A-I, apolipoprotein A-II, complement C3, and transthyretin, the resulting HAPs and HAP-depleted proteins were analysed on 10% polyacrylamide gel (**Figure 3.2.1**). The molecular weights of the 14 HAPs are listed in **Table 3.2.3**. Each lane loaded with HAPs consisted of approximately 10 bands, where the thickest band was expected to be albumin. Some HAPs with close MWs might share the same protein bands and that could be the reason for less than 14 bands seen on the HAP lanes. Lanes loaded with HAP-depleted proteins demonstrated numerous fine bands. Proteins with very low molecular weights might not be resolved because of the separation limit of the 10% polyacrylamide gel.

Table 3.2.2. Clinicopathological information of patients in this study. The diagnoses were done at Clinical Diagnostic Lab, UMMC, and the results were obtained from Nephrology Unit, UMMC.

		Frequency					
Component	**Level**	**SLE (without kidney involvement) (n=5)**	**LN class II (n=3)**	**LN class III (n=3)**	**LN class IV (n=5)**	**LN class V (n=3)**	**LN in remission (n=5)**
Serum albumin (g/l)	<35	1	3	3	2	3	0
	35-60*	4	0	0	3	0	5
Serum creatinine (µmol/l)	<62	4	2	2	1	2	3
	62-115*	1	1	1	3	1	1
	>115	0	0	0	1	0	1
ESR (mm/hr)	2.0-10*	1	0	1	0	0	1
	>10	4	3	2	5	3	4
Total cholestrol (mmol/l)	<5.2*	2	0	1	1	1	3
	>5.2	3	3	2	4	2	2
LDL (mmol/l)	<2.59*	0	1	0	1	1	1
	>2.59	5	2	3	4	2	4
Urine erythrocyte (cell/µl)	0-1*	3	1	1	3	2	3
	>1	2	2	2	2	1	2
Microalbumin:creatinine (mg/mmol creatinine)	0-3.5*	4	0	0	0	0	1
	>3.5	1	3	3	5	3	4
C3 (mg/dl)	<86	1	1	3	0	3	3
	86-184*	4	2	0	5	0	2
C4 (mg/dl)	<20	3	1	3	1	2	3
	20-59*	2	2	0	4	1	2
Anti-dsDNA (iu/ml)	0-200*	0	1	1	2	1	1
	>200	5	2	2	3	2	4

*Normal level

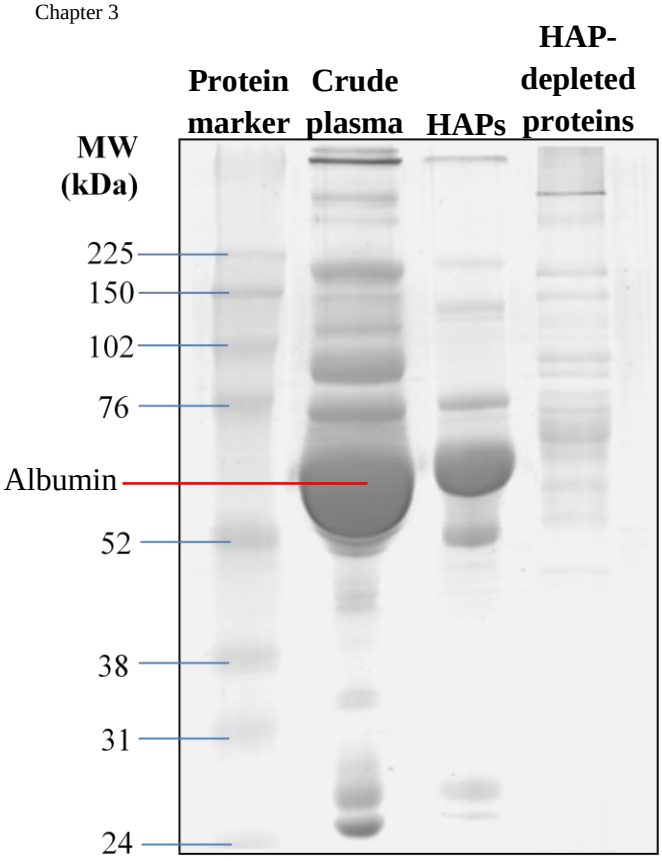


Figure 3.2.1. Separation of crude plasma proteins, high-abundant proteins (HAPs), and HAP-depleted plasma proteins on 10% polyacrylamide gel and stained with Coomassie Blue. Fourteen (14) HAPs were removed from crude plasma using Agilent Human 14 Multiple Affinity Removal System spin cartridge, resulting in HAP-depleted plasma proteins. The thick protein band pointed with red line is expected to be albumin. MW-molecular weight

Table 3.2.3. Molecular weights of the 14 high-abundant proteins that were removed from plasma.

High-abundant plasma protein	Molecular weight (kDa)
Complement C3	187
Alpha-2-macroglobulin	163
Transferrin	77
Albumin	69
Transthyretin	58
Fibrinogen	52-94
Antitrypsin	47
Haptoglobin	45
Apolipoprotein A-I	31
Alpha-1-acid glycoprotein	24
Apolipoprotein A-II	11
IgG	160 53 [heavy gamma (γ) chain] 25-30 [light kappa/lambda (κ/λ) chain]
IgM	900 76-92 [heavy mu (μ) chain] 25-30 [light kappa/lambda (κ/λ) chain]
IgA	150 50 [heavy alpha (α) chain] 25-30 [light kappa/lambda (κ/λ) chain]

3.2.2.2 Two-dimensional electrophoresis (2-DE)

First-dimensional isoelectric focusing (IEF) of plasma and urine samples was optimised according to several aspects: (i) sample preparation; (ii) pH range of IPG DryStrip; (iii) concentration of IPG buffer; (iv) position of loading cup; and (v) running protocol.

Running protocol for IEF was standardised mentioned in **Section 2.2.8.1** and almost all protein samples were able to achieve the volt hours set, as shown in **Figure 3.2.2**. Adjustment of the IEF running protocol did not make any changes or improvements to the unsatisfactory protein images.

Plasma proteins did not pose much problems during IEF. Immobiline pH gradient (IPG) DryStrip of pH4-7 was selected because most proteins were found to be concentrated within this pH range. The observation that 90% of plasma proteins were comprised of high-abundant protein (HAP) and these proteins might potentially interfere with identification of other lower-abundant proteins on the 2-DE gels, which can be seen in **Figure 3.2.3(A)**, removal of HAPs was done. Judging by the images obtained, most of the HAPs captured by and eluted from the spin cartridge [**Figure 3.2.3(C)**] had been removed from the plasma. This resulted better flow-through of increased number of other remaining protein spots [**Figure 3.2.3(B)**].

Urine proteins required more optimisation because streaking occurred on the 2-DE gels, especially on the anodic side, most likely due from the presence of salts tightly bound to proteins or other interfering molecules, such as glycosaminoglycan (Lafitte *et al.*, 2002, Oh *et al.*, 2004). Therefore, adjustments were made during sample preparation and obvious improvement was observed after urine proteins were cleaned up using method mentioned in **Section 2.2.5** compared to just loading crude protein sample, as seen in **Figure 3.2.4**. Position of the loading cup during IEF was fixed on the anodic side of the IPG DryStrip, given that basic cup loading would cause disappearance of the basic proteins [**Figure 3.2.4(D)**]. Concentration of IPG buffer at 0.5% in the rehydration buffer led to streaking on anodic half of the 2-DE gel, while 1.0% and 1.5% of IPG buffer were able to reduce the streaking [**Figure 3.2.4(B)**, **(C)** and **(E)**]. In conclusion, the best 2-DE image was achieved when urine sample was cleaned up and subsequently focused on pH4-7 IPG DryStrip using anodic cup loading method, with concentration of the IPG buffer in the

Figure 3.2.2. Graph showing the IEF running protocol on the Ettan IPGphor 3 apparatus. The blue line was the running protocol set on the instrument, whereas the red line recorded the real-time voltage and current during the exact run for monitoring purpose.

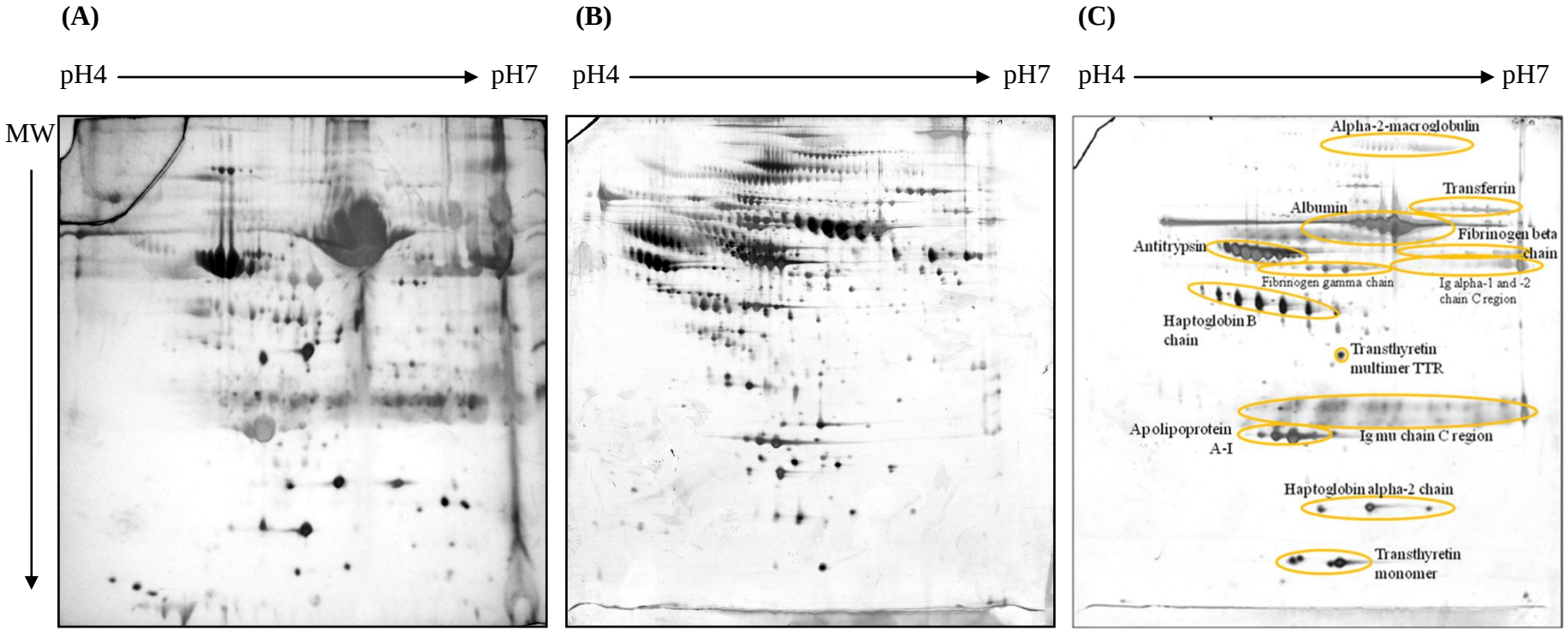


Figure 3.2.3. 2-DE of plasma proteins after removal of high-abundant proteins (HAPs). 2-DE was carried out on Immobiline DryStrip of pH 4-7 and 12.5% polyacrylamide gel, as described in **Section 2.2.8**. Silver-stained images show the separation of proteins from **(A)** crude plasma (1 mg), **(B)** the flow-through (HAP-depleted proteins) (100 µg), and **(C)** the elute (HAPs) (100 µg).

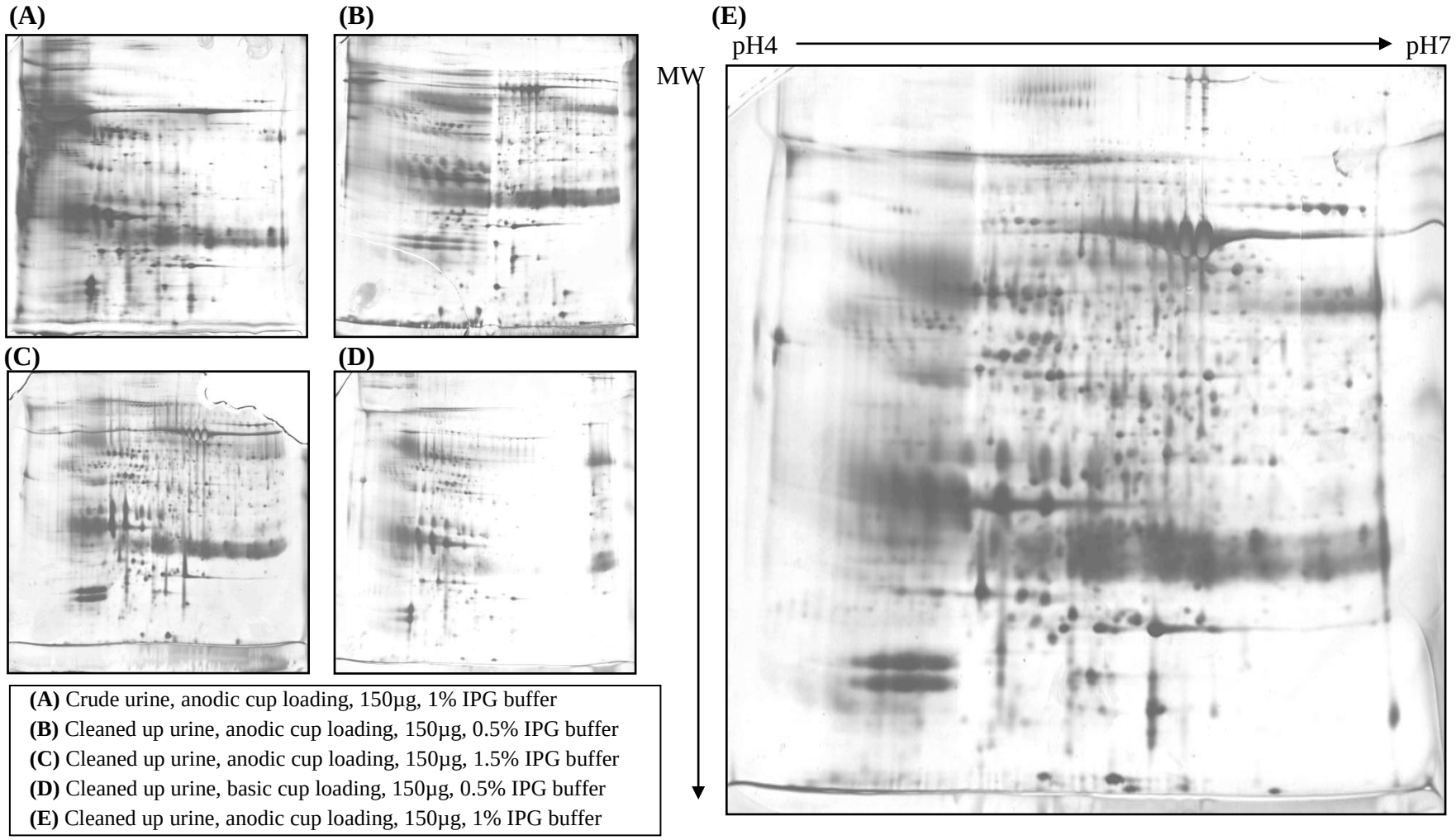


Figure 3.2.4. Optimisation of 2-DE for urine proteins. (A) to **(D)** are silver stained 2-DE images, pH 4-7, after different treatments and running parameters of urine proteins. The best separation was achieved in **(E)** and 2-DE of all urine samples were performed according to the corresponding parameters.

rehydration buffer being 1.0% [**Figure 3.2.4(E)]**. These parameters were used for all urine samples throughout the experiment.

3.2.2.3 Two-dimensional difference gel electrophoresis (2D-DIGE)

Labelling of protein samples with CyDyes was performed according to procedure mentioned in **Section 2.2.9.3**. Upon electrophoresis, the 2D-DIGE gels were scanned using the fluorescence mode of Typhoon FLA 9000 (GE Healthcare). Adjustment of photomultiplier tube (PMT) voltage for each CyDye was required in order to obtain similar intensity for the three protein images in the same gel. Initial PMT voltage was set as 500V for all three emission filters to obtain a rapid overview of the gel image and subsequent adjustment of PMT voltage was done accordingly. It was observed that Cy2-labelled image had higher intensity and lower PMT voltage ~450V was sufficient enough, while PMT voltage of Cy3-labelled image was optimised as ~550V. The Cy5-labelled had the lowest intensity and required higher PMT voltage at ~700V in order to achieve similar intensity with images labelled with the other two CyDyes. Slight adjustment of each PMT voltage was necessary in every experiment, by referring to the optimised set points. **Figure 3.2.5, Figure 3.2.6** and **Figure 3.2.7** show 2D-DIGE images of CyDye-labelled plasma and urine proteins, respectively. Proteins labelled with different CyDyes would emit fluorescence of different colours: Cy2 dye gave blue fluorescence, Cy3 dye gave green fluorescence, and Cy5 dye gave red fluorescence. Overlaying of images labelled with all three CyeDyes on a same gel is shown in **Figure 3.2.5(D)**, **Figure 3.2.6(D)** and **Figure 3.2.7(D)**. Cy2 dye was normally used for labelling of the internal standards. Overlaying of images labelled with Cy3 and Cy5 dyes allowed approximate comparison of two protein samples loaded on the same gel: green- or partial green-fluorescent spots indicated the proteins had relatively higher expression level in Cy3-labelled sample; red- or partial-red fluorescent spots showed higher expression levels of proteins in Cy5-labelled sample; and yellow-fluorescent spots indicated that the particular proteins had similar expression levels in both samples [**Figure 3.2.5(E)**, **Figure 3.2.6(E)** and **Figure 3.2.7(E)**].

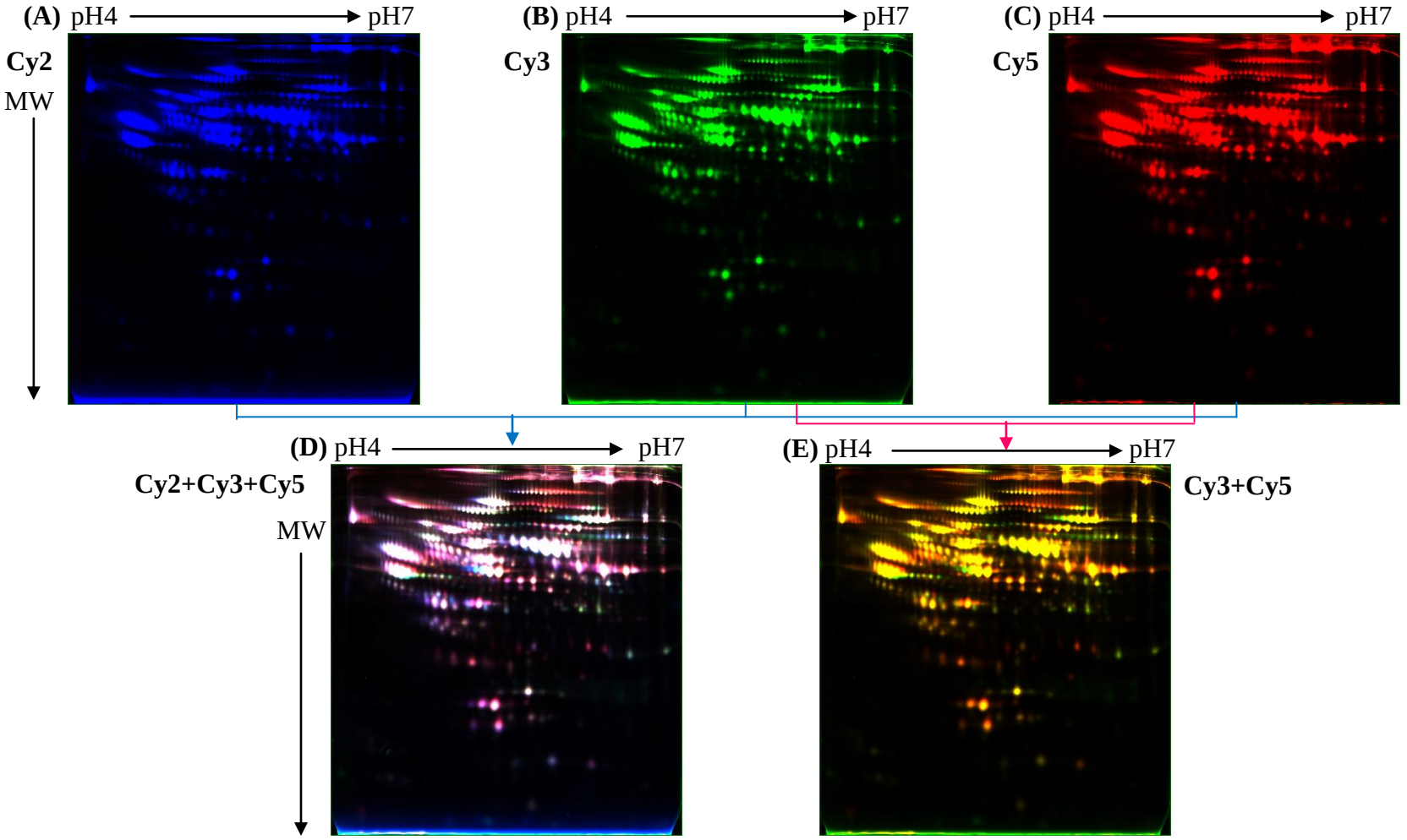


Figure 3.2.5. 2D-DIGE images of various samples after depletion of HAPs. (A) shows internal standard proteins labelled with Cy2 dye, **(B)** shows proteins of a patient/normal individual labelled with Cy3 dye, and **(C)** shows proteins of another patient/normal individual labelled with Cy5 dye. **(D)** and **(E)** show overlapping of images labelled with all three CyDyes and with Cy3 and Cy5 dyes, respectively.

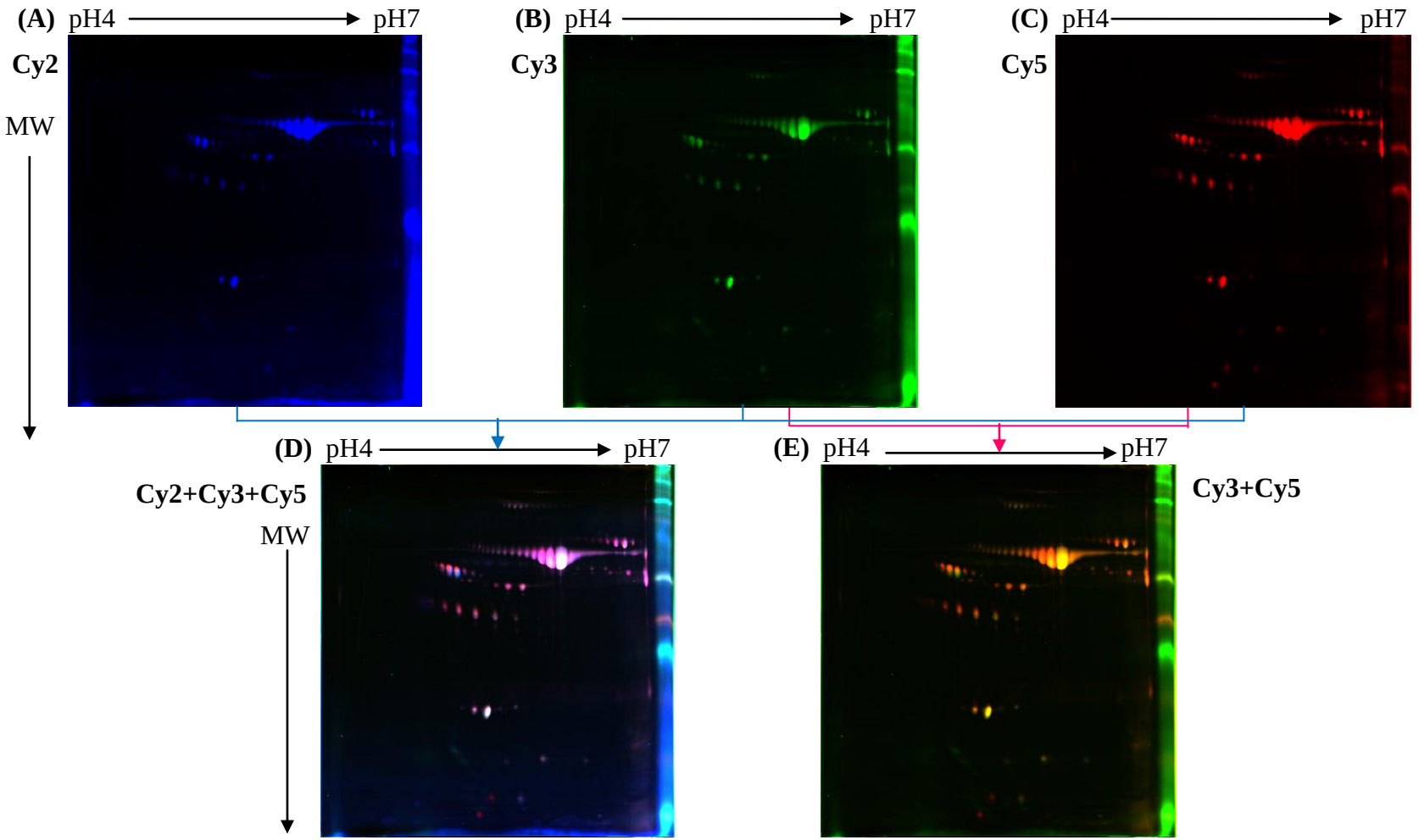


Figure 3.2.6. 2D-DIGE images of plasma HAPs from various samples. (A) shows internal standard labelled with Cy2 dye, **(B)** shows proteins of a patient/normal individual labelled with Cy3 dye, and **(C)** shows proteins of another patient/normal individual labelled with Cy5 dye. **(D)** and **(E)** show overlapping of images labelled with all three CyDyes and with Cy3 and Cy5 dyes, respectively.

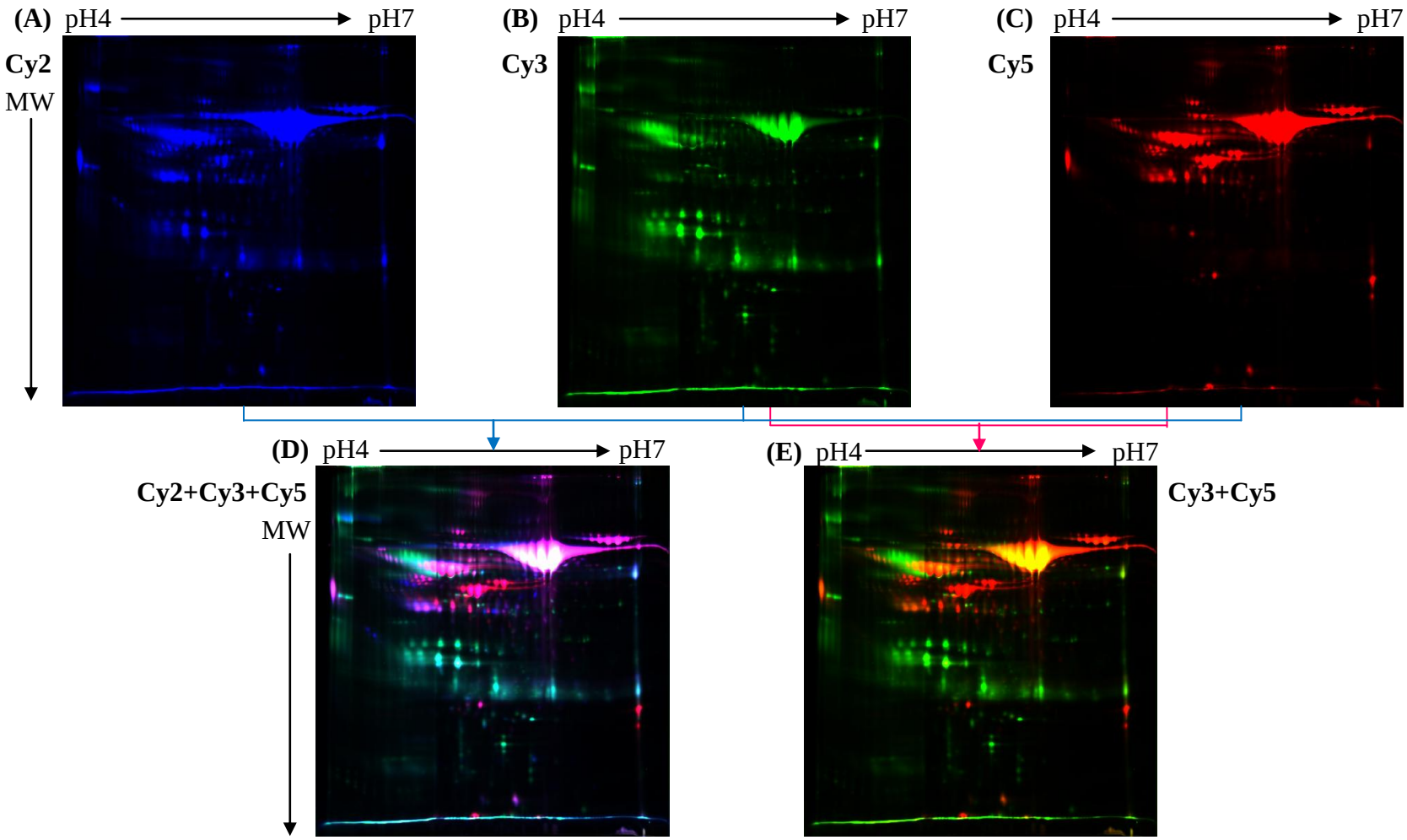


Figure 3.2.7. 2D-DIGE images of urine proteins from various samples. (A) shows internal standard labelled with Cy2 dye, **(B)** shows proteins of a patient/normal individual labelled with Cy3 dye, and **(C)** shows proteins of another patient/normal individual labelled with Cy5 dye. **(D)** and **(E)** show overlapping of images labelled with all three CyDyes and with Cy3 and Cy5 dyes, respectively.

3.2.3 Image analysis of protein profiles between normal individuals and SLE patients

Image analysis was done using DeCyder 2D version 7.0 software (GE Healthcare) according to procedure mention in **Section 2.2.9.9**. Spot detection was performed using Differential In-gel Analysis (DIA) module. For each gel, the spot intensity of the two gel images loaded with experimental specimen proteins was normalized against that of internal standard before spot detection was carried out. The representative gel image after spot detection is shown in **Figure 3.2.8**. At the end, 2D-DIGE of HAP-depleted plasma proteins resulted in an average of 1227 protein spots, plasma HAP 377 protein spots, and urine protein 1319 protein spots (**Table 3.2.4**).

Comparison of protein profiles between normal individuals and SLE patients was carried out using Biological Variation Analysis (BVA) module and protein spots having average abundance ratio of ≥ 1.5 or ≤ -1.5 in the patient groups compared with normal individual group, with p value ≤ 0.05, were identified as differentially expressed protein (DEP) spots. The average abundance ratio gives the standardised volume ratio between the patient and normal individual groups. **Figure 3.2.9** represents differential expression analysis graph for one of the protein spots. Standard abundance of the spot in each gel was shown in the graph, as well as the mean values for each experimental group. Standard abundance shows the DIGE ratio and how much the abundance of each spot varies from the standard.

3.2.3.1 Plasma proteins

i. HAP-depleted proteins

Figure 3.2.10 shows the image analysis result of HAP-depleted proteins. SLE patients without kidney manifestations were found to have 36 protein spots differentially expressed compared with normal individuals. Among the LN patient groups, LN patients in remission had the lowest number of DEP spots (18 spots), while LN class III patients had the highest (70 spots).

ii. High-abundant proteins

Image analysis result is shown in **Figure 3.2.11**. Comparison of protein profiles demonstrated that SLE patients without kidney manifestations had 6 protein spots which were expressed differentially compared with normal individuals. Among the LN groups, LN patients who were already in remission had lower number of DEP spots (5 spots) than patients with active LN, with LN class V patients having the most number of DEP spots (16 spots).

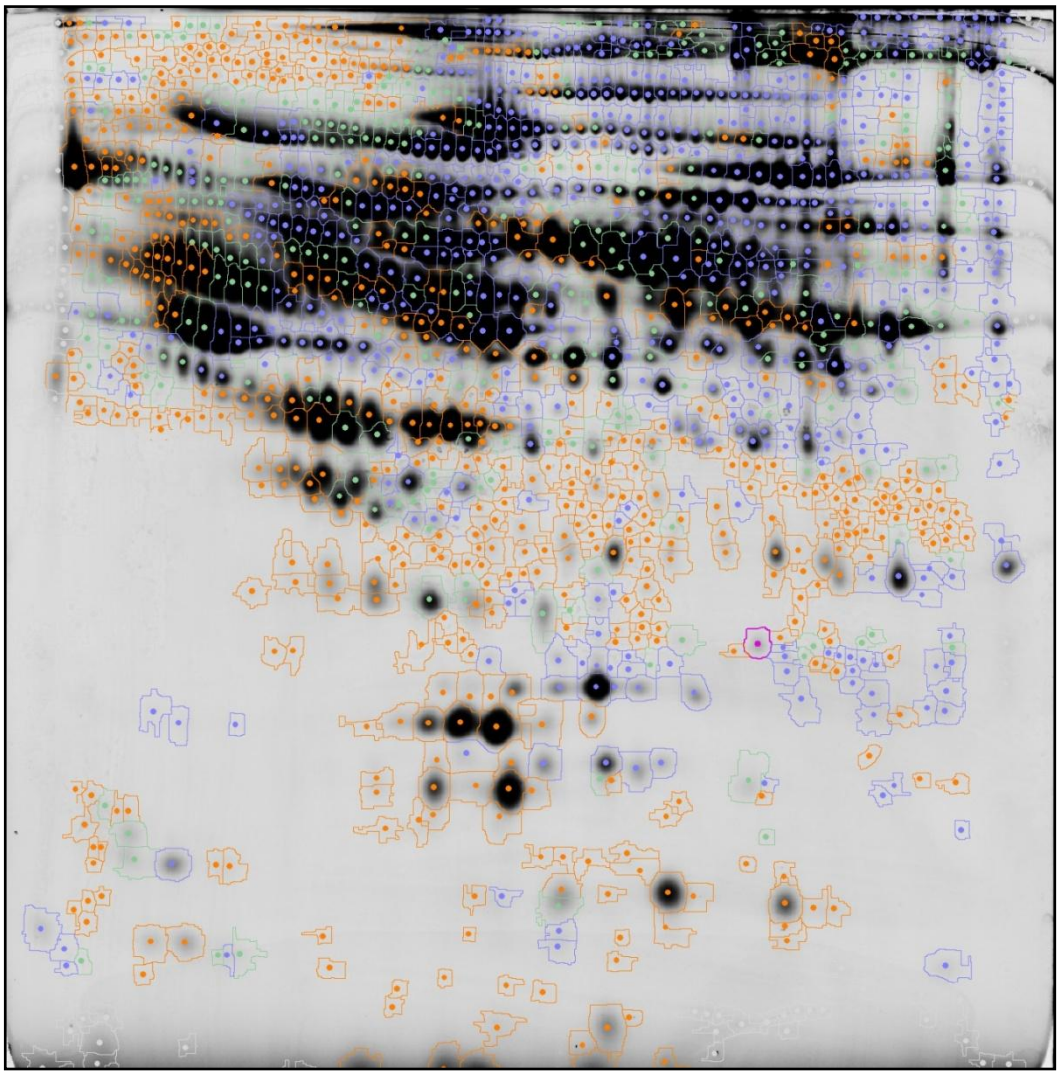


Figure 3.2.8. Representative gel image after spot detection. The 2D-DIGE image was obtained from separation of Cy3-labelled HAP-depleted proteins of a normal healthy control on Immobiline DryStrip pH4-7 and 12.5% polyacrylamide gel. Spot detection was performed using Differential In-gel Analysis (DIA) module of DeCyder 2D version 7.0 software. Detected spots were circled with dots in the middle which denoted the peaks of the protein spots. In this image, a total of 1443 protein spots were detected.

Table 3.2.4. Number of protein spots detected on each gel as a result of 2D-DIGE of HAP-depleted plasma protein, plasma HAP, and urine protein samples.

	No. of spots		
Gel No.	**HAP-depleted plasma proteins**	**Plasma HAPs**	**Urine proteins**
1	1183	500	1000
2	892	495	1370
3	1244	432	1275
4	1257	401	1211
5	1157	339	1337
6	1282	384	1416
7	1231	416	1278
8	1372	423	1375
9	1236	343	1290
10	1048	229	1398
11	1231	407	1384
12	1305	310	1488
13	1443	315	1392
14	1223	358	1378
15	1307	300	1186
Average	**1227**	**377**	**1319**

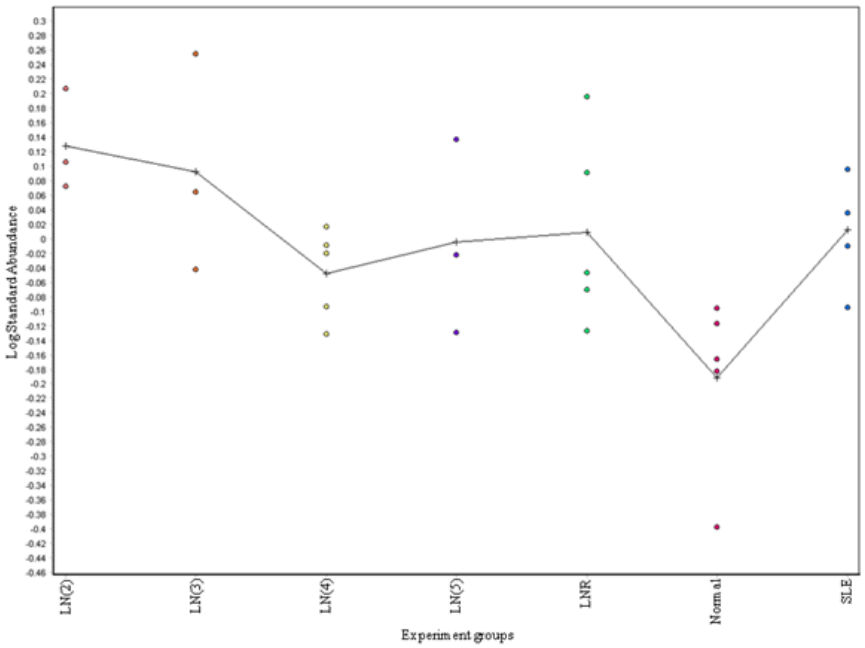


Figure 3.2.9. Representative differential expression analysis graph generated by BVA module for one of the protein spots. The log standard abundance is the log abundance of a spot after normalised with internal standards and it was denoted with spots of different colours based on its experiment group. The average of log standard abundance for each group was indicated with (+). LN(2) – LN class II patients; LN(3) – LN class III patients; LN(4) – LN class IV patients; LN(5) – LN class V patients; LNR - LN patients in remission; Normal – normal individuals; SLE – SLE patients without kidney involvement

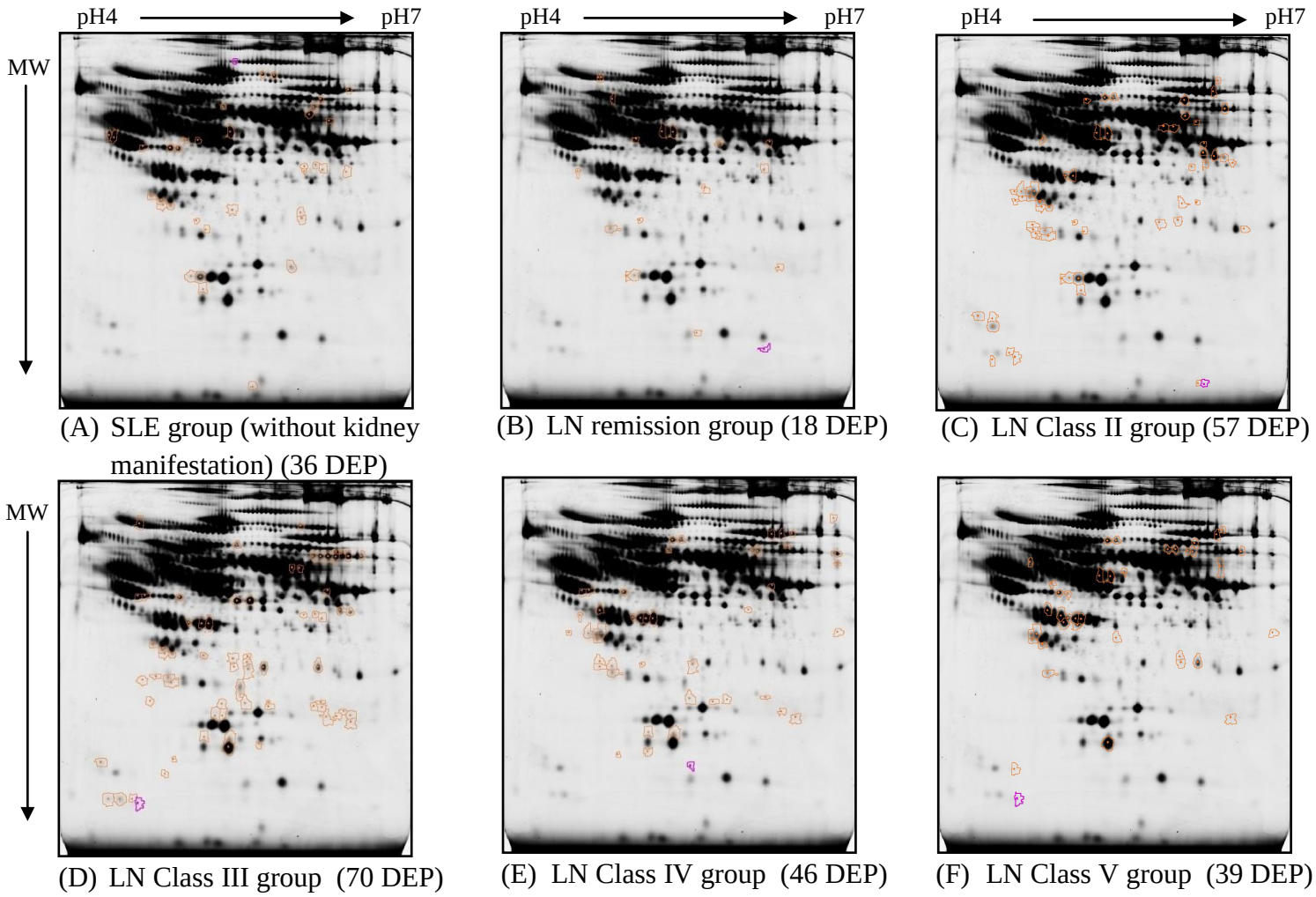


Figure 3.2.10. HAP-depleted plasma proteins differentially expressed in each SLE group (with or without kidney manifestation) compared with normal individual group. Differentially expressed proteins are circled in orange. DEP – differentially expressed protein spots compared with normal samples

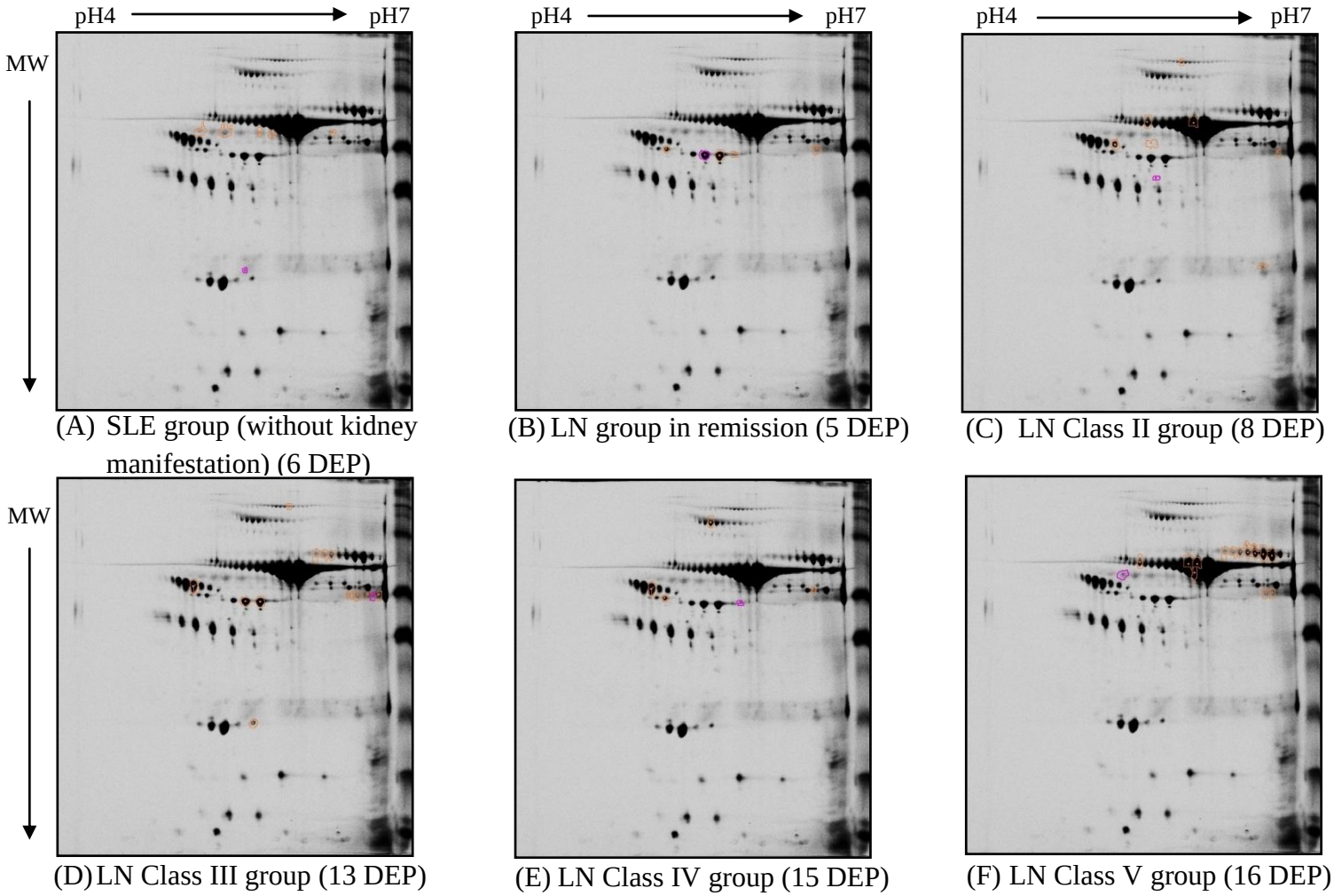


Figure 3.2.11. Plasma HAPs differentially expressed in each SLE group (with or without kidney manifestation) compared with normal individual group. Differentially expressed proteins are circled in orange. DEP – differentially expressed protein spots compared with normal samples

3.2.3.2 Urine proteins

It was observed that urine protein profiles of LN patients were distinctly different from those of normal individuals and SLE patients without kidney manifestations. Urine of LN patients had relatively higher abundance of albumin spots on 2-DE gel images as a result of kidney damage (**Figure 3.2.12**). This was also seen in urine protein separation on SDS-PAGE gel, where albumin band of LN patients was significantly much thicker than that of normal individuals and SLE patients without kidney involvement (**Figure 3.2.13**). This caused the urine protein profiles of LN patients contained fewer protein spots and looked similar to that of crude plasma because the huge albumin spots had suppressed the protein spots of lower abundance from appearing on the gel.

Biological variation analysis (BVA) results showed that urine protein profiles between SLE patients without kidney manifestations and normal individuals did not differ significantly, with only 35 DEP spots observed (**Figure 3.2.14**). However, the higher number of protein spots in LN patients showed significant variation in expression levels compared to normal individuals. LN class II and III patients displayed the most number of DEP with 245 and 246 spots, respectively.

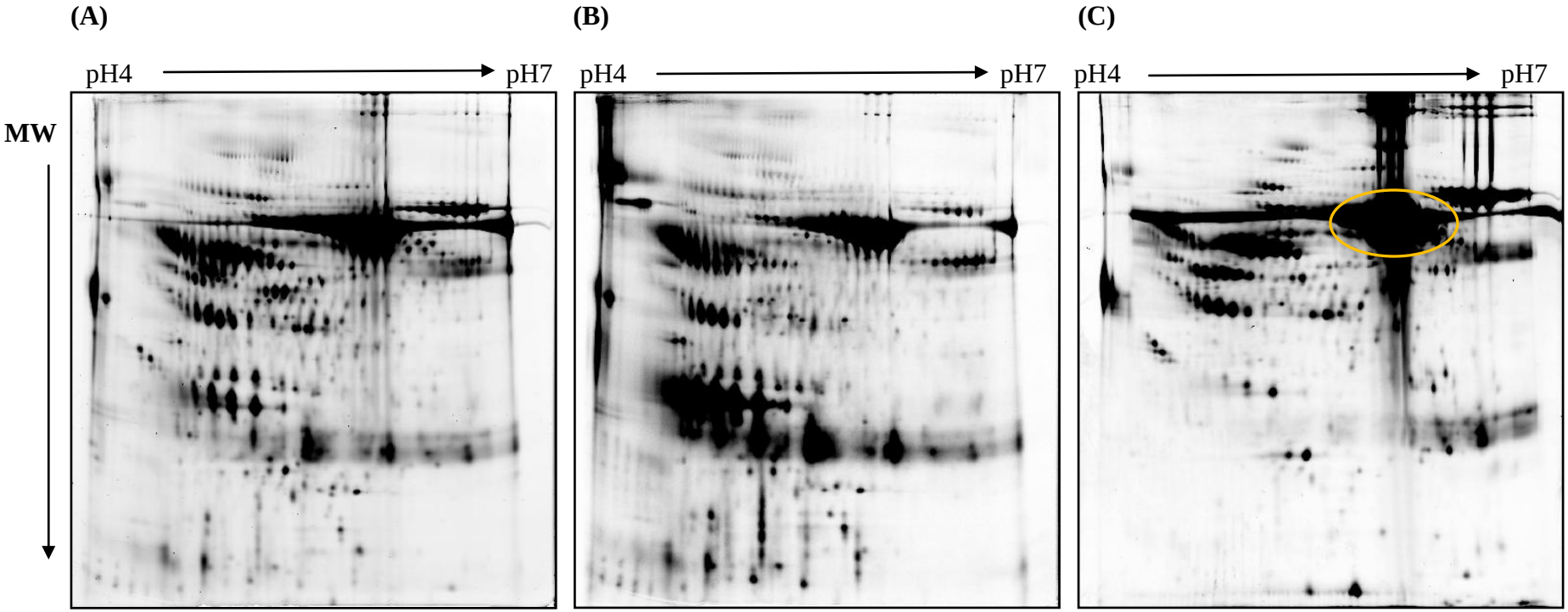


Figure 3.2.12. 2D-DIGE images of urine proteins from normal individual and patient groups. Images shown were produced by separation of 50 µg of urine proteins from **(A)** normal individuals, **(B)** SLE patients without kidney manifestations, and **(C)** lupus nephritis class IV patients on Immobiline DryStrip pH4-7 and 12.5% polyacrylamide gel. Normal individuals and SLE patients without kidney manifestations have similar protein profiles, whereas that of lupus nephritis patients shows relatively fewer protein spots with a very high abundance of albumin (circled in orange) due to kidney damage.

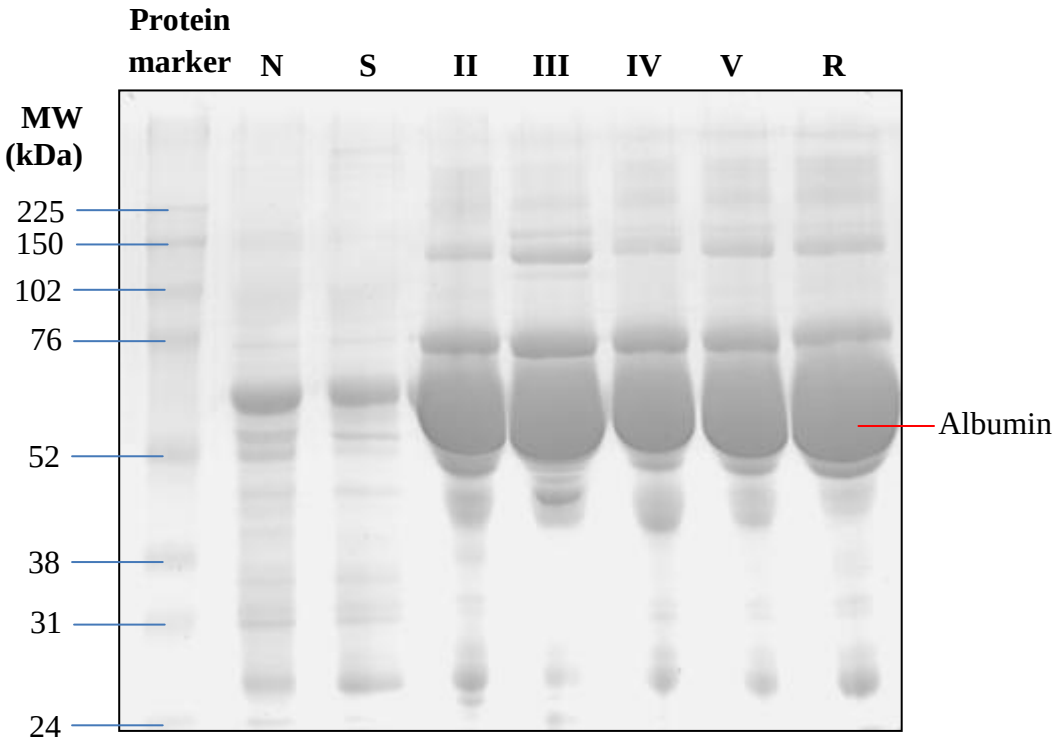


Figure 3.2.13. SDS-PAGE of urine proteins from normal individual and SLE patients. Ten microlitres of urine proteins from each normal individual and patient were separated on 10% polyacrylamide gel. The thick protein band pointed with red line is expected to be albumin. Apparently, LN patients had relatively higher level of albumin excreted in urine compared with that of normal individuals and SLE patients without kidney involvement. N-normal individuals; S-SLE patients without kidney involvement; II-LN class II patients; III-LN class III patients; IV-LN class IV patients; V-LN class V patients; R-LN patients in remission.

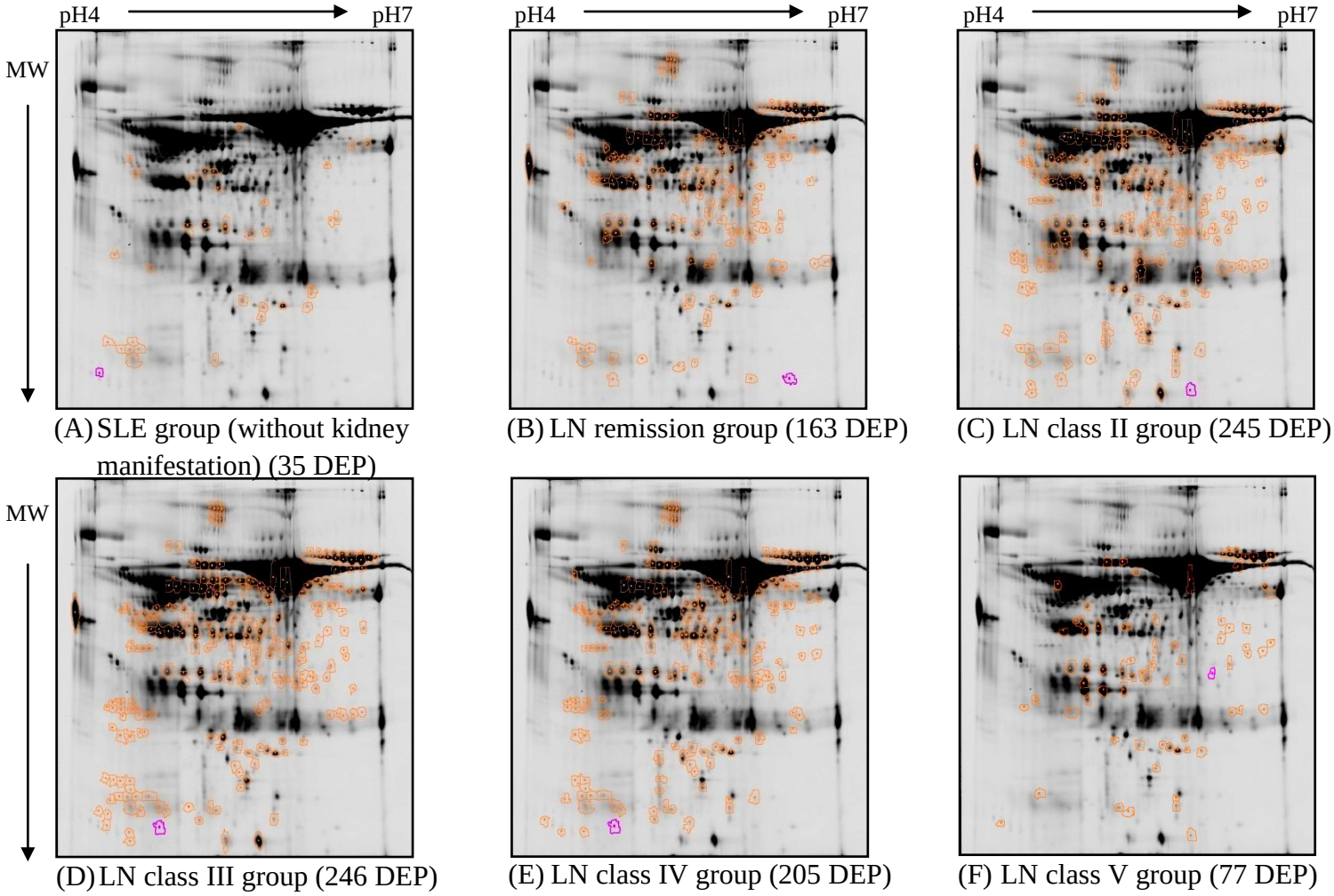


Figure 3.2.14. Urine proteins differentially expressed in each SLE group (with or without kidney manifestation) compared with normal individual group. Differentially expressed proteins are circled in orange. DEP – differentially expressed protein spots compared with normal samples

3.2.4 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Upon analysis of LC-MS/MS, the data files were transferred to the analysis software for extraction of chromatogram and MS spectrum results. In this study, Agilent MassHunter Qualitative Analysis Software Version B.03.01 (Agilent Technologies) described in **Section 2.2.10.4** was used. The various types of chromatograms that could be generated included total ion chromatogram (TIC), base peak chromatogram (BPC), extracted ion chromatogram (EIC) and extracted compound chromatogram (ECC) (**Figure 3.2.15**). These chromatograms could be applied on all MS scans (combination of MS and MS/MS scans), MS scans, and MS/MS product ion scans. Chromatograms for specific m/z value(s) or m/z range of product ion scans could be also obtained.

The MS spectrum was derived directly from the MS chromatogram and for more complete MS and MS/MS spectra, compound-specific mass spectra and chromatograms were extracted from data files that had been acquired using auto MS/MS. In Auto MS/MS operation, the instrument was set to first acquire a single-stage MS spectrum. The decision engine in the acquisition software examined this spectrum for the presence of "qualifying peaks" according to criteria set in the acquisition method. If one or more qualifying peaks were found, the instrument automatically switched to MS/MS and did one product ion scan for each qualifying peak, using an m/z derived from that peak as the precursor ion m/z value. Negative and positive controls were included in every run to check the performance of the equipment. The negative control was a blank sample and its MS/MS results were checked to ensure no hit to any protein during the database search. The positive control contained 100 fmol BSA and its MS/MS should confirm the correct protein identification in the database search (**Figure 3.2.16**).

Figure 3.2.17(A) shows the overlaying of MS/MS spectra of 300 largest compounds of a protein, while **Figure 3.2.17(B)** shows the individual MS and MS/MS spectra of one of the compounds. MS spectrum could be extracted at defined time points for retention from the chromatogram, or vice-versa, chromatogram could be generated for selected peak from MS spectrum.

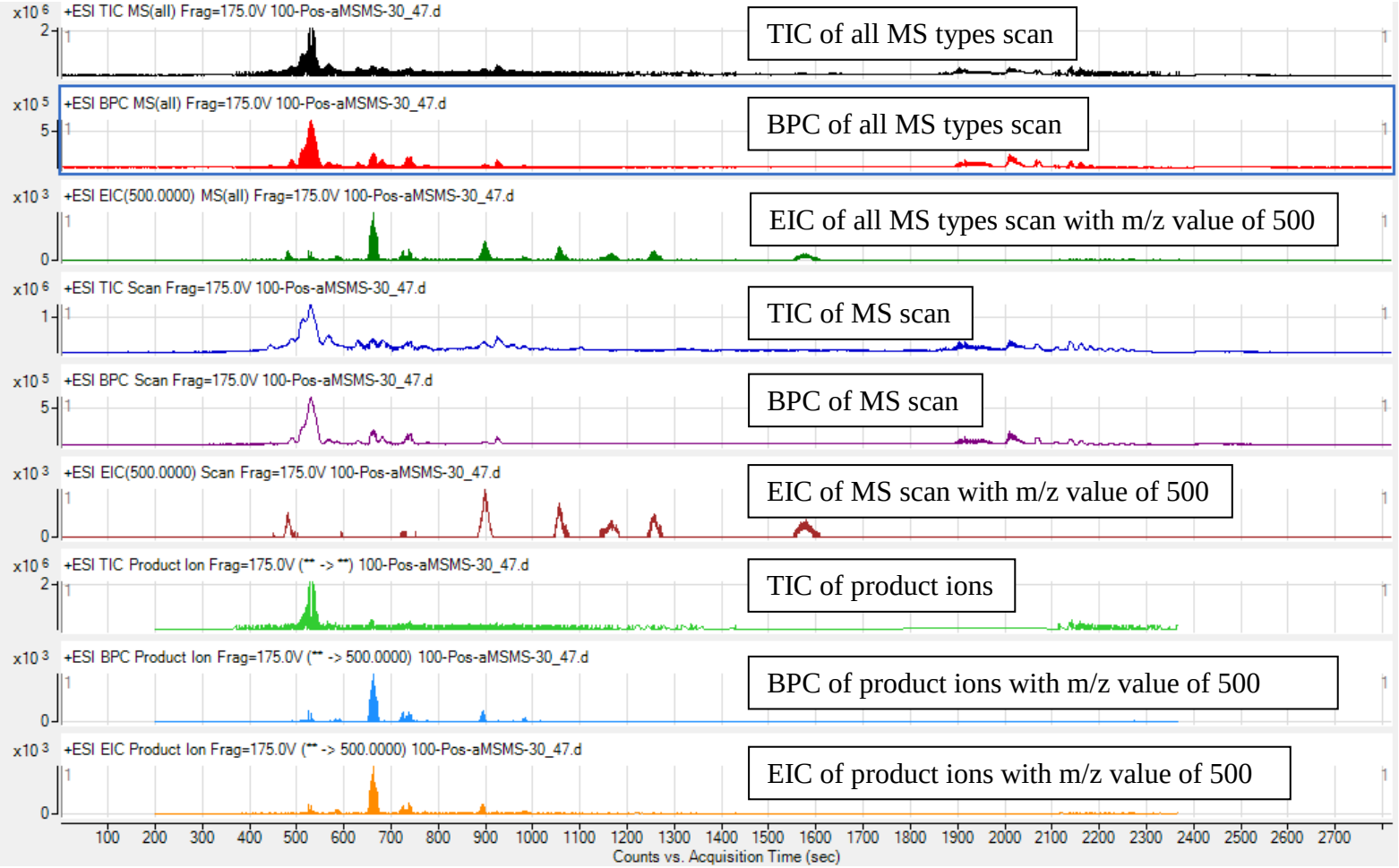
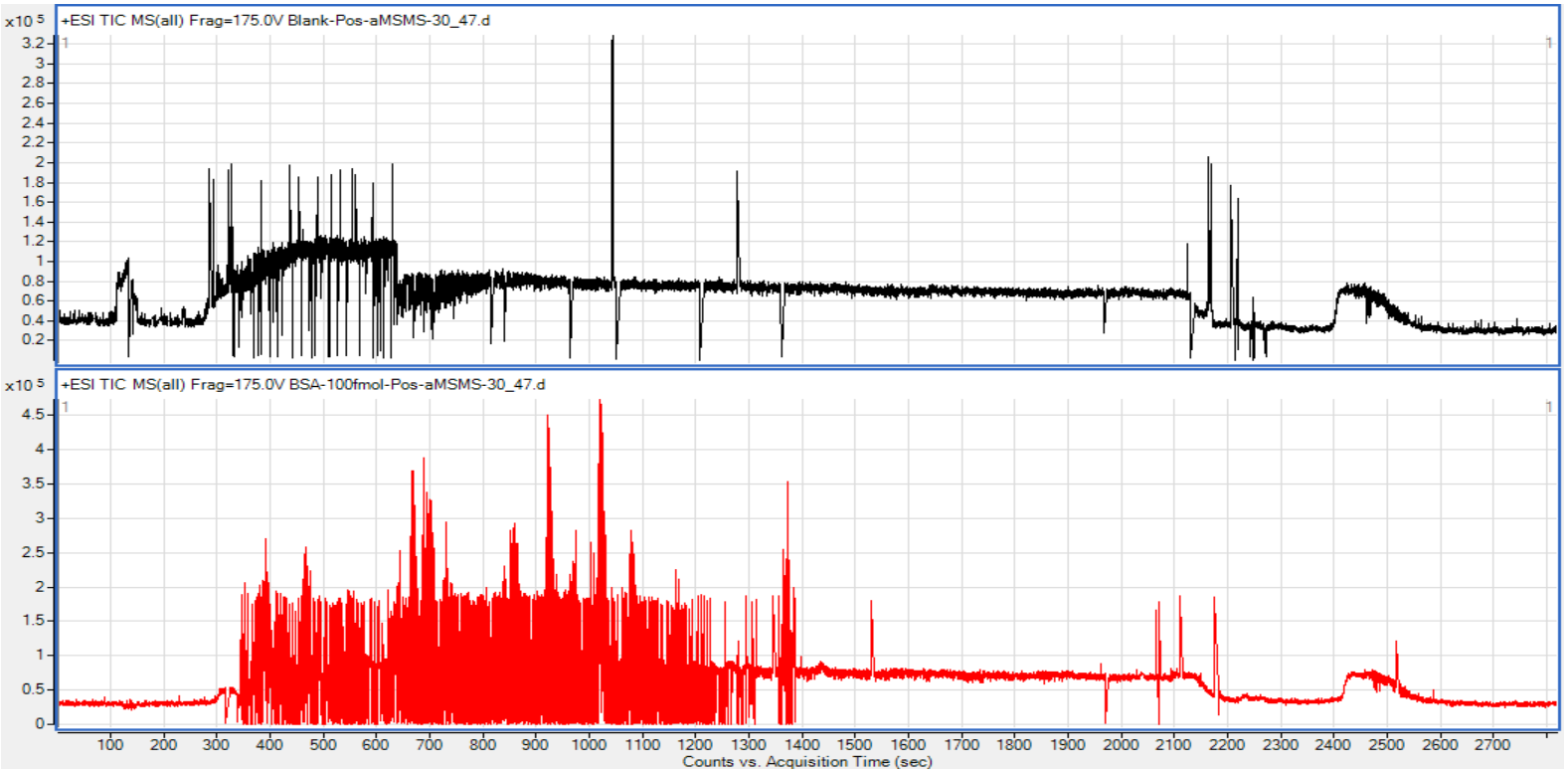


Figure 3.2.15. Types of chromatograms generated with LC-MS/MS result. TIC-total ion chromatogram; BPC-base peak chromatogram; EIC-extracted ion chromatogram; ECC-extracted compound chromatogram

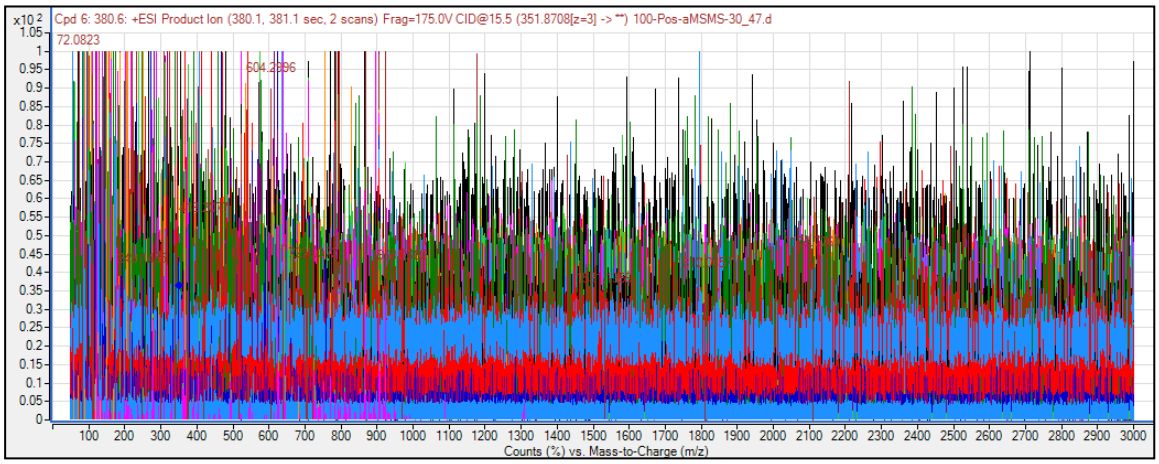


Negative control: Blank

Positive control: 100 fmol BSA

Figure 3.2.16. Chromatograms of negative (blank) and positive (100 fmol BSA) controls. One microlitre of trypsin digested protein sample was injected into Agilent 6520 Accurate-Mass Q-TOF LC/MS and passed through large capacity chip packed with Zorbax 300SB-C18 (Agilent Technologies). Solvents used were 0.1% (v/v) formic acid in water and 90% (v/v) acetonitrile in water with 0.1% (v/v) formic acid. Flow rates used were 4uL/min from Agilent 1200 Series capillary pump and 0.3uL/min from Agilent 1200 Series nano pump. Fragmentor voltage was set at 175V.

(A)



(B)

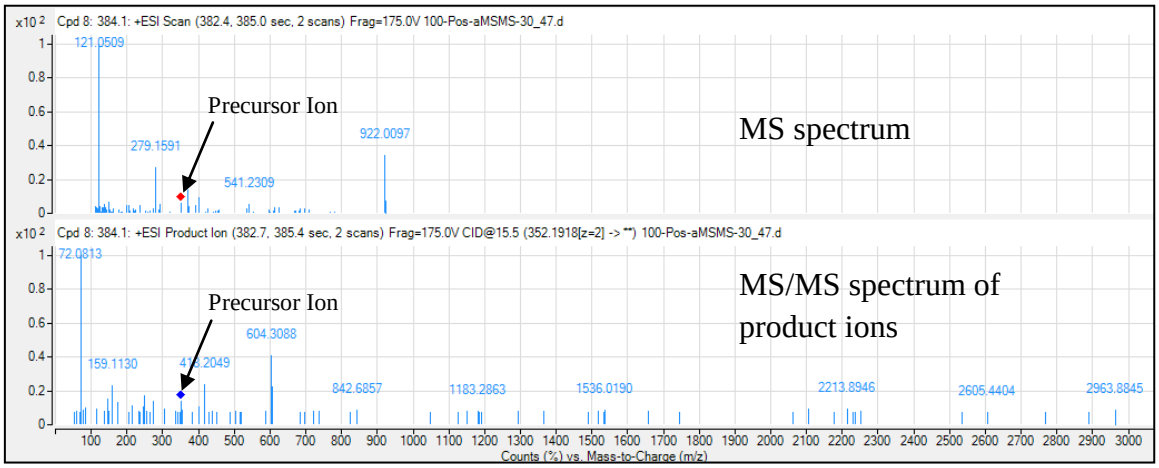


Figure 3.2.17. MS and MS/MS spectrum of trypsin in-gel digested haptoglobin. (A) Overlaid mode of all MS/MS spectra of the 300 compounds, **(B)** List mode shows MS and MS/MS spectra of one of the compounds. MS scan range was set between 110-3000 m/z, while MS/MS scan range was 50-3000 m/z.

3.2.5 Peptide/Protein identification

Spectrum Mill for MassHunter Workstation Version B.03.01 (Agilent Technologies) is the peptide search engine that enables quick and large scale identification of proteins and peptides via fast database searches. It does an assessment of the quality of the spectra and only searches the appropriate spectra. It also validates the spectra both automatically and visually.

In this study, the MS/MS spectral were extracted by Spectrum Mill from the data files prior to conducting the search. Database used was SwissProt and MS/MS spectral were searched against *Homo sapiens* proteins. **Figure 3.2.18** shows an example of protein/peptide summary generated after the search. In addition to displaying protein names for top database hits, the summary also contains other details such as peptide validation, z, score, forward-reverse score, spectrum intensity, sequence, MH^+ matched, number of spectra and distinct peptides, MS/MS search score, percentage of amino acid coverage, mean peptide spectral intensity etc., depending of the user's preference. In order to confirm the identity of a particular protein, the number of distinct peptides and MS/MS search score of the hit should be as high as possible. The colour code was applied to distinguish the number of distinct peptides detected for each protein, where darker colours (i.e. red) indicated higher number while lighter colours (i.e. yellow) indicated lower number. Colours in between (i.e. orange) represented moderate number of distinct peptides detected. The protein name that was listed first in the protein summary was usually considered as the identity of the sample because it had the highest score and the most number of distinct peptides among the proteins, but did not necessarily possess the highest percentage of amino acid coverage and mean peptide spectral intensity.

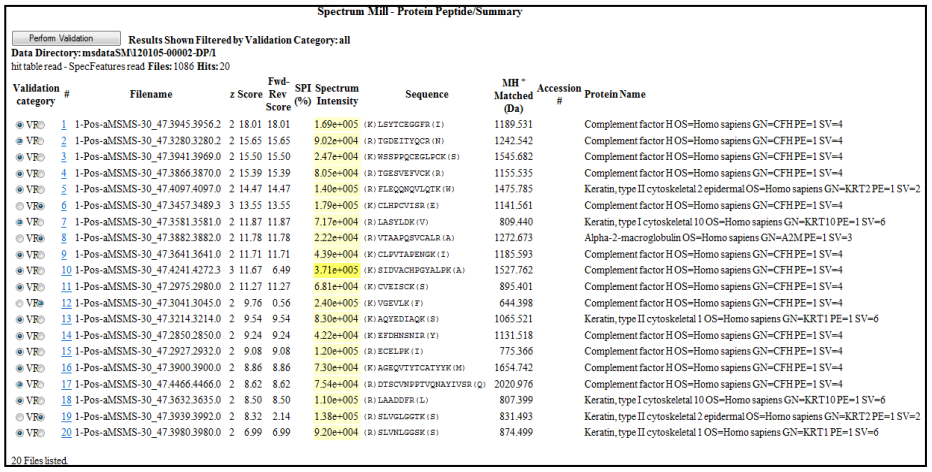
3.2.5.1 Plasma proteins identification

i. HAP-depleted plasma proteins

A total of 215 protein spots were identified from 2D-DIGE images to be differentially expressed in SLE without kidney involvement and LN patients compared to normal individuals (**Figure 3.2.19**). However, only 124 spots were able to be picked from the matched Coomassie blue gels for trypsin in-gel digestion and thus for LC-MS/MS analysis (**Figure 3.2.20)**. Protein spots that could not be picked from the first Coomassie blue-stained gel [**Figure 3.2.20(A)**] were extracted from the second gel [**Figure 3.2.20(B)**]. Further reduction to 102 protein spots could proceed for identification when 22 protein spots failed to produce spectra during LC-MS/MS. The protein identification results for these protein spots are listed in **Table 3.2.5**, whereby they were found to belong to 33 different proteins. The proteins that were identified included adiponectin, afamin, alpha-1-antichymotrypsin, alpha-2-HS-glycoprotein, apolipoproteins, clusterin, haemopexin, and vitamin D-binding protein.

The 22 protein spots failed to produce MS/MS spectral could be due to the very low abundance in the sample and they appeared as very faint spots on the 2-DE gels. Likewise, protein spots gained hits with very low scores (e.g. <50) as well as low number of spectra (e.g. 1) and distinct peptides (e.g. 1) in the protein identification could be also because of the same reason. The identities of the protein spots were verified by matching their locations on the 2-DE gel with some established protein profiles (Fountoulakis *et al.*, 2004, Herosimczyk *et al.*, 2006, Desrosiers *et al.*, 2007, Candiano *et al.*, 2010).

(A)



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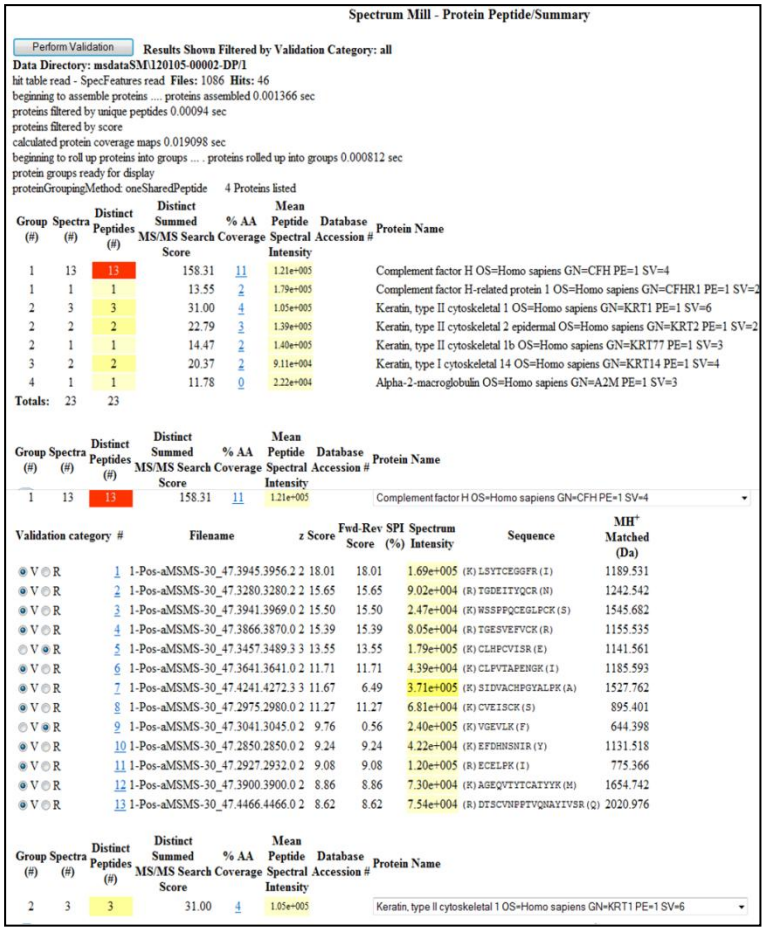
VR	2	1-Pos-aMSMS-30_47.3280.3280.2	2	15.65	15.65		9.02e+004	(R)TGDEITYQCR(N)	1242.542		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	3	1-Pos-aMSMS-30_47.3941.3969.0	2	15.50	15.50		2.47e+004	(K)WSSPPQCEGLPCK(S)	1545.682		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	4	1-Pos-aMSMS-30_47.3866.3870.0	2	15.39	15.39		8.05e+004	(R)TGESVEFVCK(R)	1155.535		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	5	1-Pos-aMSMS-30_47.4097.4097.0	2	14.47	14.47		1.40e+005	(R)FLEQQNQVLQTK(W)	1475.785		Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2
VR	6	1-Pos-aMSMS-30_47.3457.3489.3	3	13.55	13.55		1.79e+005	(K)CLHPCVISR(E)	1141.561		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	7	1-Pos-aMSMS-30_47.3581.3581.0	2	11.87	11.87		7.17e+004	(R)LASYLDK(V)	809.440		Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
VR	8	1-Pos-aMSMS-30_47.3882.3882.0	2	11.78	11.78		2.22e+004	(R)VTAAPQSVCALR(A)	1272.673		Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3
VR	9	1-Pos-aMSMS-30_47.3641.3641.0	2	11.71	11.71		4.39e+004	(K)CLPVTAPENGK(I)	1185.593		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	10	1-Pos-aMSMS-30_47.4241.4272.3	3	11.67	6.49		3.71e+005	(K)SIDVACHPGYALPK(A)	1527.762		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	11	1-Pos-aMSMS-30_47.2975.2980.0	2	11.27	11.27		6.81e+004	(K)CVEISCK(S)	895.401		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	12	1-Pos-aMSMS-30_47.3041.3045.0	2	9.76	0.56		2.40e+005	(K)VGEVLK(F)	644.398		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	13	1-Pos-aMSMS-30_47.3214.3214.0	2	9.54	9.54		8.30e+004	(K)AQYSDIAQK(S)	1065.521		Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
VR	14	1-Pos-aMSMS-30_47.2850.2850.0	2	9.24	9.24		4.22e+004	(K)EFDHNSNIR(Y)	1131.518		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	15	1-Pos-aMSMS-30_47.2927.2932.0	2	9.08	9.08		1.20e+005	(R)ECELPK(I)	775.366		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	16	1-Pos-aMSMS-30_47.3900.3900.0	2	8.86	8.86		7.30e+004	(K)AGEQVTYTCATYYK(M)	1654.742		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	17	1-Pos-aMSMS-30_47.4466.4466.0	2	8.62	8.62		7.54e+004	(R)DTSCVNPPTVQNAYIVSR(Q)	2020.976		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
VR	18	1-Pos-aMSMS-30_47.3632.3635.0	2	8.50	8.50		1.10e+005	(R)LAADDFR(L)	807.399		Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
VR	19	1-Pos-aMSMS-30_47.3939.3992.0	2	8.32	2.14		1.38e+005	(R)SLVGLGGTK(S)	831.493		Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2
VR	20	1-Pos-aMSMS-30_47.3980.3980.0	2	6.99	6.99		9.20e+004	(R)SLVNLGGSK(S)	874.499		Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6

20 Files listed.

(B)



Spectrum Mill - Protein Peptide/Summary

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calculated protein coverage maps 0.019098 sec

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protein groups ready for display

proteinGroupingMethod: oneSharedPeptide 4 Proteins listed

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	13	13	158.31	11	1.21e+005		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4
1	1	1	13.55	2	1.79e+005		Complement factor H-related protein 1 OS=Homo sapiens GN=CFHR1 PE=1 SV=2
2	3	3	31.00	4	1.05e+005		Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
2	2	2	22.79	3	1.39e+005		Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2
2	1	1	14.47	2	1.40e+005		Keratin, type II cytoskeletal 1b OS=Homo sapiens GN=KRT77 PE=1 SV=3
3	2	2	20.37	2	9.11e+004		Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4
4	1	1	11.78	0	2.22e+004		Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3
Totals:	23	23					

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	13	13	158.31	11	1.21e+005		Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4

Validation category	#	Filename	z	Score	Fwd-Rev Score	SPI (%)	Spectrum Intensity	Sequence	MH+ Matched (Da)
V R	1	1-Pos-aMSMS-30_47.3945.3956.2	2	18.01	18.01		1.69e+005	(K)LSYTCEGGFR(I)	1189.531
V R	2	1-Pos-aMSMS-30_47.3280.3280.2	2	15.65	15.65		9.02e+004	(R)TGDEITYQCR(N)	1242.542
V R	3	1-Pos-aMSMS-30_47.3941.3969.0	2	15.50	15.50		2.47e+004	(K)WSSPPQCEGLPCK(S)	1545.682
V R	4	1-Pos-aMSMS-30_47.3866.3870.0	2	15.39	15.39		8.05e+004	(R)TGESVEFVCK(R)	1155.535
V R	5	1-Pos-aMSMS-30_47.3457.3489.3	3	13.55	13.55		1.79e+005	(K)CLHPCVISR(E)	1141.561
V R	6	1-Pos-aMSMS-30_47.3641.3641.0	2	11.71	11.71		4.39e+004	(K)CLPVTAPENGK(I)	1185.593
V R	7	1-Pos-aMSMS-30_47.4241.4272.3	3	11.67	6.49		3.71e+005	(K)SIDVACHPGYALPK(A)	1527.762
V R	8	1-Pos-aMSMS-30_47.2975.2980.0	2	11.27	11.27		6.81e+004	(K)CVEISCK(S)	895.401
V R	9	1-Pos-aMSMS-30_47.3041.3045.0	2	9.76	0.56		2.40e+005	(K)VGEVLK(F)	644.398
V R	10	1-Pos-aMSMS-30_47.2850.2850.0	2	9.24	9.24		4.22e+004	(R)EFDHNSNIR(Y)	1131.518
V R	11	1-Pos-aMSMS-30_47.2927.2932.0	2	9.08	9.08		1.20e+005	(R)ECELPK(I)	775.366
V R	12	1-Pos-aMSMS-30_47.3900.3900.0	2	8.86	8.86		7.30e+004	(K)AGEQVTYTCATYYK(M)	1654.742
V R	13	1-Pos-aMSMS-30_47.4466.4466.0	2	8.62	8.62		7.54e+004	(R)DTSCVNPPTVQNAYIVSR(Q)	2020.976

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
2	3	3	31.00	4	1.05e+005		Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6

Figure 3.2.18. Example of protein identification result on the Spectrum Mill for MassHunter Workstation Version B.03.01. Data obtained from LC-MS/MS analysis were search against *Homo sapiens* database in SwissProt. Carbamidomethylation modification was included during the search. **(A)** Peptide summary and **(B)** protein summary were generated by the software. In this example, the protein was identified as complement factor H because it had the highest score, distinct peptides, and amino acid coverage percentage.

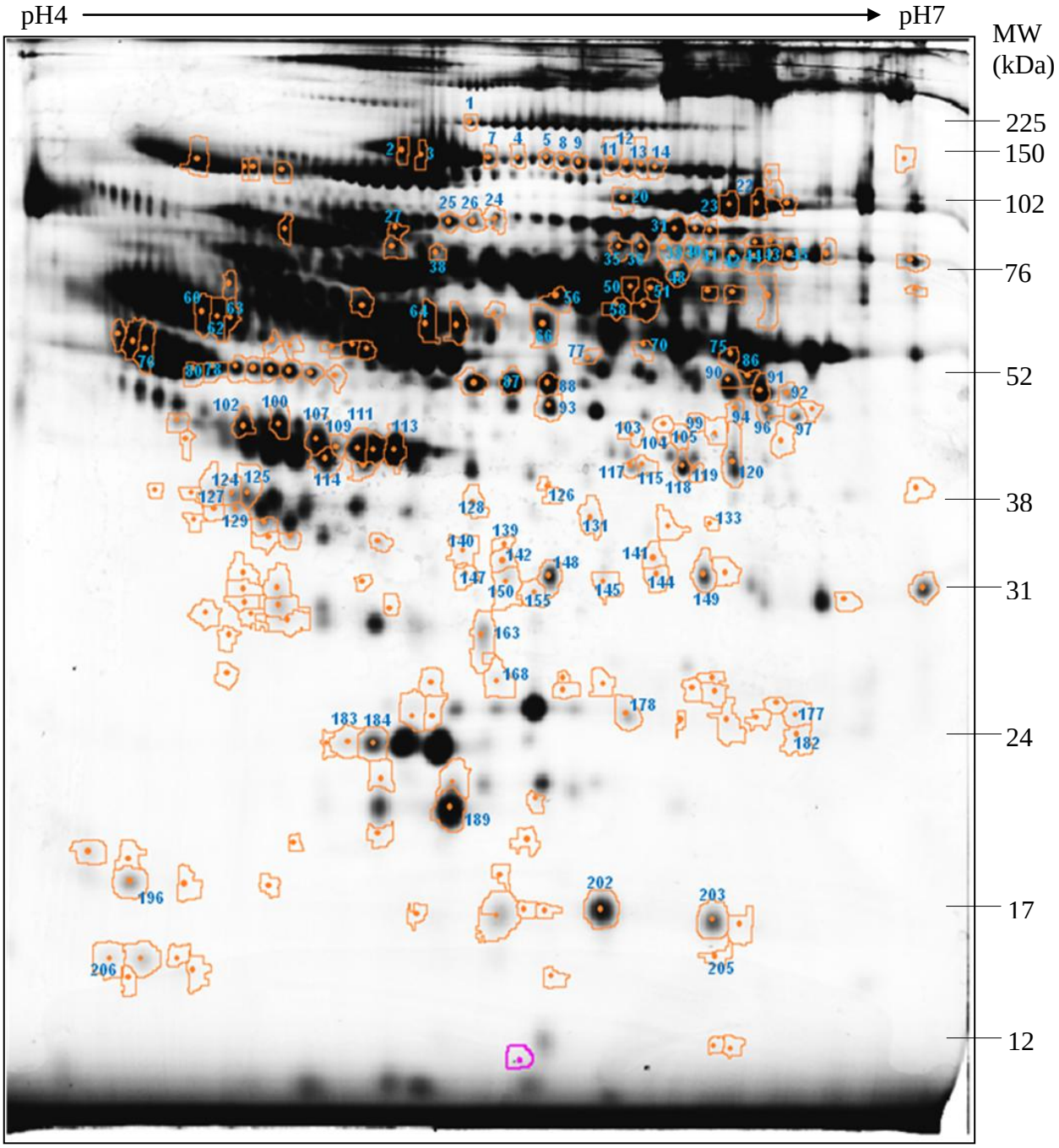


Figure 3.2.19. HAP-depleted protein spots being selected on 2-D DIGE gel after image comparison. These included all protein spots that were differentially expressed in patients with SLE without kidney involvement and with all LN classes compared with normal healthy individuals. Spots circled in orange are the proteins of interest while those labelled with blue numbers are the ones managed to be picked from the Coomassie blue-stained 2-DE gel for trypsin in-gel digestion and successfully identified with LC-MS/MS (**Figure 3.2.15**).

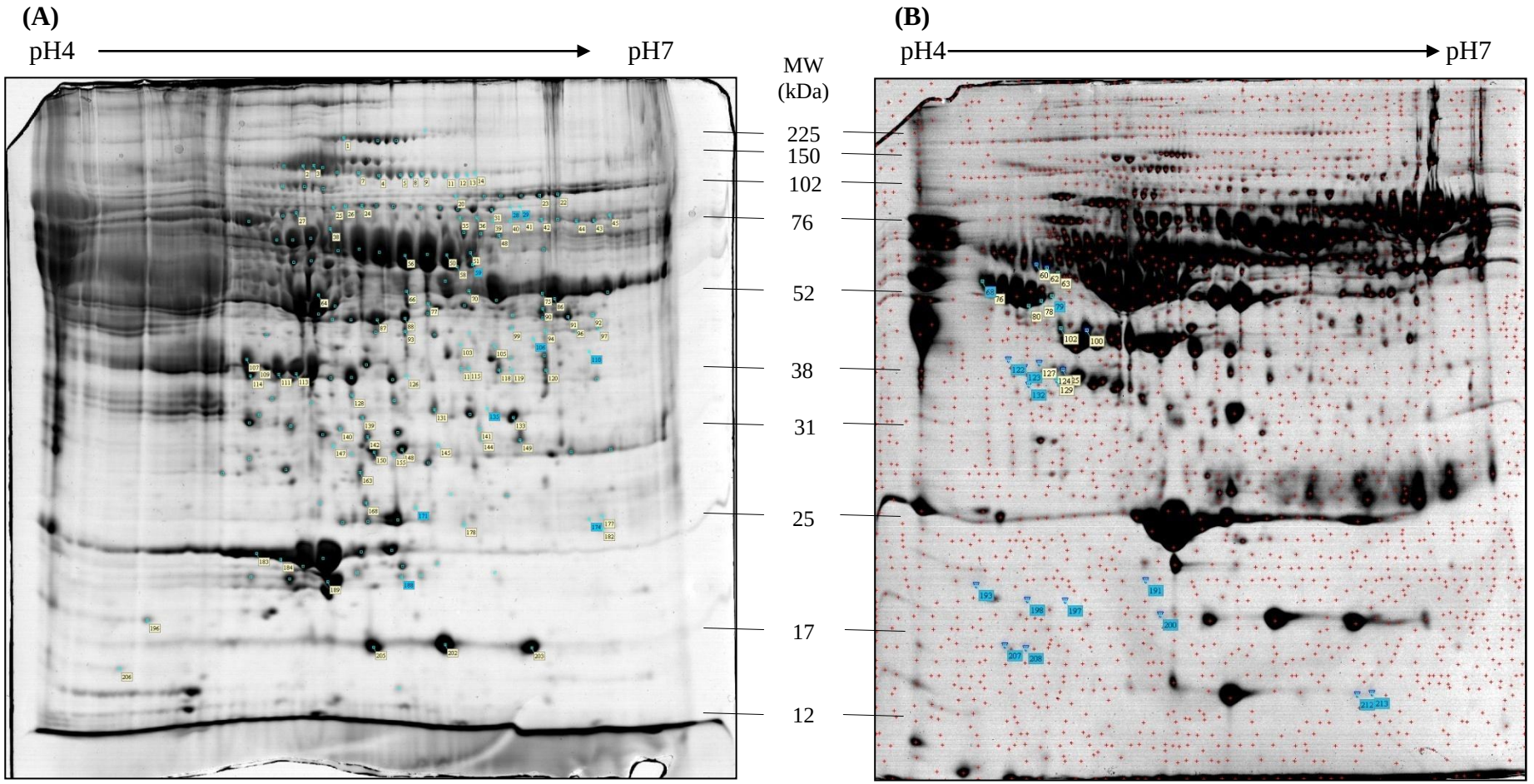


Figure 3.2.20. HAP-depleted plasma proteins of interest on Coomassie blue-stained 2-DE gels. The gels were matched with 2-D DIGE gel in **Figure 3.2.19** using ImageMaster™ 2D Platinum 7.02 (GE Healthcare). Protein spots that could not be picked from gel **(A)** were extracted from gel **(B)**. Two milligrams of pooled plasma protein samples from all normal healthy individuals and patients in this study were separated on each gel and proteins of interest that were picked for trypsin in-gel digestion are labelled in boxes. Protein spots that were able to be identified with LC-MS/MS are labelled in yellow boxes.

Table 3.2.5. Protein identification results obtained from Spectrum Mill analysis for SLE-associated HAP-depleted plasma proteins of all patient groups.

No.	Protein	Map	Protein ID	Spectra	Distinct Peptides	MS/MS search	% AA Coverage
1	**163**	1590	Adiponectin	2	1	20.98	6
2	**27**	591	Afamin	39	30	447.74	44
3	**60**	835	Alpha-1-antichymotrypsin	6	6	74.87	11
4	**76**	930	Alpha-2-HS-glycoprotein	3	3	43.48	7
5	**78**	954		5	5	79.66	10
6	**80**	956		7	6	98.12	13
7	**4**	390	Alpha-2-macroglobulin	24	19	297.66	15
8	**5**	392		15	13	175.69	9
9	**7**	397		13	12	143.46	8
10	**8**	407		15	14	167.36	10
11	**9**	413		16	13	165.05	10
12	**11**	416		7	7	82.94	5
13	**12**	419		8	8	98.64	6
14	**13**	420		2	2	22.04	1
15	**14**	421		1	1	12.2	1
16	**183**	1810	Apolipoprotein A-I	36	24	424.27	69
17	**184**	1811		39	24	461.01	67
18	**109**	1142	Apolipoprotein A-IV	26	19	302.57	39
19	**111**	1145		36	27	436.1	64
20	**113**	1155		60	37	649.6	77
21	**140**	1420	Apolipoprotein E	20	15	218.35	50
22	**141**	1441		5	4	41.71	17
23	**142**	1446		37	26	358.36	64
24	**147**	1480		5	4	37.72	13
25	**150**	1486		20	14	183.42	47
26	**148**	1482		55	35	566.65	80
27	**66**	869	Apolipoprotein H	12	9	157	35
28	**70**	902		16	11	194.12	36
29	**86**	973		38	15	286.78	50
30	**94**	1041		4	4	43.62	14
31	**48**	731	C4b-binding protein alpha chain	11	10	148.91	20
32	**2**	375	Ceruloplasmin	22	20	302.97	20
33	**3**	386		26	21	301.78	22
34	**38**	668		14	12	181.47	14
35	**124**	1264	Clusterin	3	3	32.84	9
36	**125**	1265		7	6	76.32	11
37	**127**	1269		1	1	12.64	2
38	**129**	1303		2	1	18.18	2
39	**24**	567	Complement C1r subcomponent	11	10	121.96	14
40	**26**	580	Complement C4-A & B	3	3	23.71	1
41	**25**	577		20	14	195.38	21
42	**115**	1186		12	10	131.19	7
43	**117**	1193		13	8	134.8	7
44	**118**	1197		24	12	206.1	9
45	**119**	1200		6	4	66.05	3
46	**120**	1202		18	11	183.21	9
47	**168**	1700		9	4	75.02	2
48	**20**	20	Complement factor B	2	2	15.84	3
49	**22**	44		28	21	332.32	29
50	**23**	47		37	29	411.13	35

51	**189**	915	Complement factor B	1	1	21.26	3
52	**1**	76		13	13	158.31	11
53	**133**	340		29	18	309.36	17
54	**103**	103	Complement factor H-related	4	4	58.73	14
55	**105**	108	protein 1	3	3	41.4	14
56	**77**	42	Complement factor I	14	9	148.27	17
57	**177**	767	Dermcidin	4	3	35.6	18
58	**182**	791		2	2	31.9	12
59	**75**	25	Fibrinogen beta chain	24	20	288	45
60	**87**	85	Fibrinogen gamma chain	33	19	330.08	45
61	**88**	87		37	21	349.54	53
62	**144**	475	Ficolin-3	2	1	19.95	4
63	**145**	478		2	1	19.43	4
64	**149**	483		7	6	88.42	20
65	**31**	13	Gelsolin	29	20	288.3	22
66	**100**	84	Haptoglobin	13	11	158.96	18
67	**107**	118		26	16	285.16	33
68	**126**	266		4	3	40.63	8
69	**128**	288		21	13	201.37	35
70	**202**	92		32	11	207.71	21
71	**203**	114		23	12	212.64	23
72	**205**	185		20	12	177.95	21
73	**50**	77	Haemopexin	49	29	428.25	51
74	**51**	79		24	19	303.1	40
75	**56**	99		60	26	402.95	48
76	**58**	25		28	19	277.43	46
77	**90**	91		11	8	133.69	21
78	**91**	14		19	12	201.93	27
79	**92**	16		8	7	89.03	17
80	**96**	50		5	5	60.56	12
81	**97**	66		7	5	76.12	16
82	**99**	81		6	4	66.47	14
83	**196**	31		1	1	19.35	2
84	**35**	54	Histidine-rich glycoprotein	6	5	70.54	9
85	**36**	59		4	4	55.56	10
86	**62**	39	Kininogen-1	6	6	91.72	7
87	**63**	46		5	5	72.98	7
88	**93**	40	Pigment epithelium-derived	20	15	221.13	31
89	**131**	328	Plasma kallikrein	5	4	68.39	6
90	**139**	402	Retinol-binding protein 4	11	7	103.88	35
91	**39**	72	Serotransferrin	3	3	32.66	5
92	**40**	74		2	2	19.95	2
93	**41**	75		6	6	60.94	11
94	**42**	77		3	3	40.13	4
95	**43**	78		19	18	250.46	24
96	**44**	81		22	18	258.64	25
97	**45**	82		12	10	133.66	17
98	**155**	516	Serum amyloid P-component	5	3	48.76	14
99	**178**	772		5	4	60.13	17
100	**64**	63	Vitamin D-binding protein	47	31	549.35	65
101	**102**	93	Zinc-alpha-2-glycoprotein	4	3	45.35	8
102	**114**	173		12	10	148.22	34

ii. High-abundant plasma proteins (HAPs)

A total of 48 HAP protein spots were identified from 2D-DIGE images to be differentially expressed in SLE and LN patients compared to normal individuals [**Figure 3.2.21(A)**]. Forty-four (44) spots were able to be picked from the matched coomassie blue gels for trypsin in-gel digestion and LC-MS/MS analysis [**Figure 3.2.21(B)]** but MS spectrum could not be obtained for one of the spots. The protein identification results for the remaining 43 protein spots are listed in **Table 3.2.6**, whereby they were found to belong to 12 proteins encompassing alpha-1-antitrypsin, alpha-2-macroglobulin, apolipoprotein A-I, haptoglobin, fibrinogen, immunoglobulins, serotransferrin and serum albumin, in line with the affinity column used to separate these proteins from plasma. The identities of the protein spots were once again verified by matching their locations on the 2-DE gel with some established protein profiles (Fountoulakis *et al.*, 2004, Herosimczyk *et al.*, 2006, Desrosiers *et al.*, 2007, Candiano *et al.*, 2010).

3.2.5.2 Urine proteins identification

A total of 399 protein spots were identified from 2D-DIGE images to be differentially expressed in SLE and LN patients compared to normal individuals [**Figure 3.2.22(A)**]. However, only 222 spots were able to be picked from the matched Coomassie Blue gels for trypsin in-gel digestion and for LC-MS/MS analysis [**Figure 3.2.22(B)**]. Finally, 195 protein spots managed to generate MS spectra which belonged to 41 proteins, as listed in **Table 3.2.7**. Various proteins were identified. Some were also detected in plasma, such as serotransferrin, haemopexin, serum albumin, fibrinogens, and alpha-1-antitrypsin, while some of them were the kidney secretory and structural proteins, for instance polymeric immunoglobulin receptor, vesicular integral-membrane protein VIP, and gelsolin. Likewise, protein spots gaining hits with very low scores as well as low number of spectra and distinct peptides were not omitted from the results and their identities were verified by matching their spot locations on the 2-DE gel with some established protein profiles (Fountoulakis *et al.*, 2004, Herosimczyk *et al.*, 2006, Desrosiers *et al.*, 2007, Candiano *et al.*, 2010).

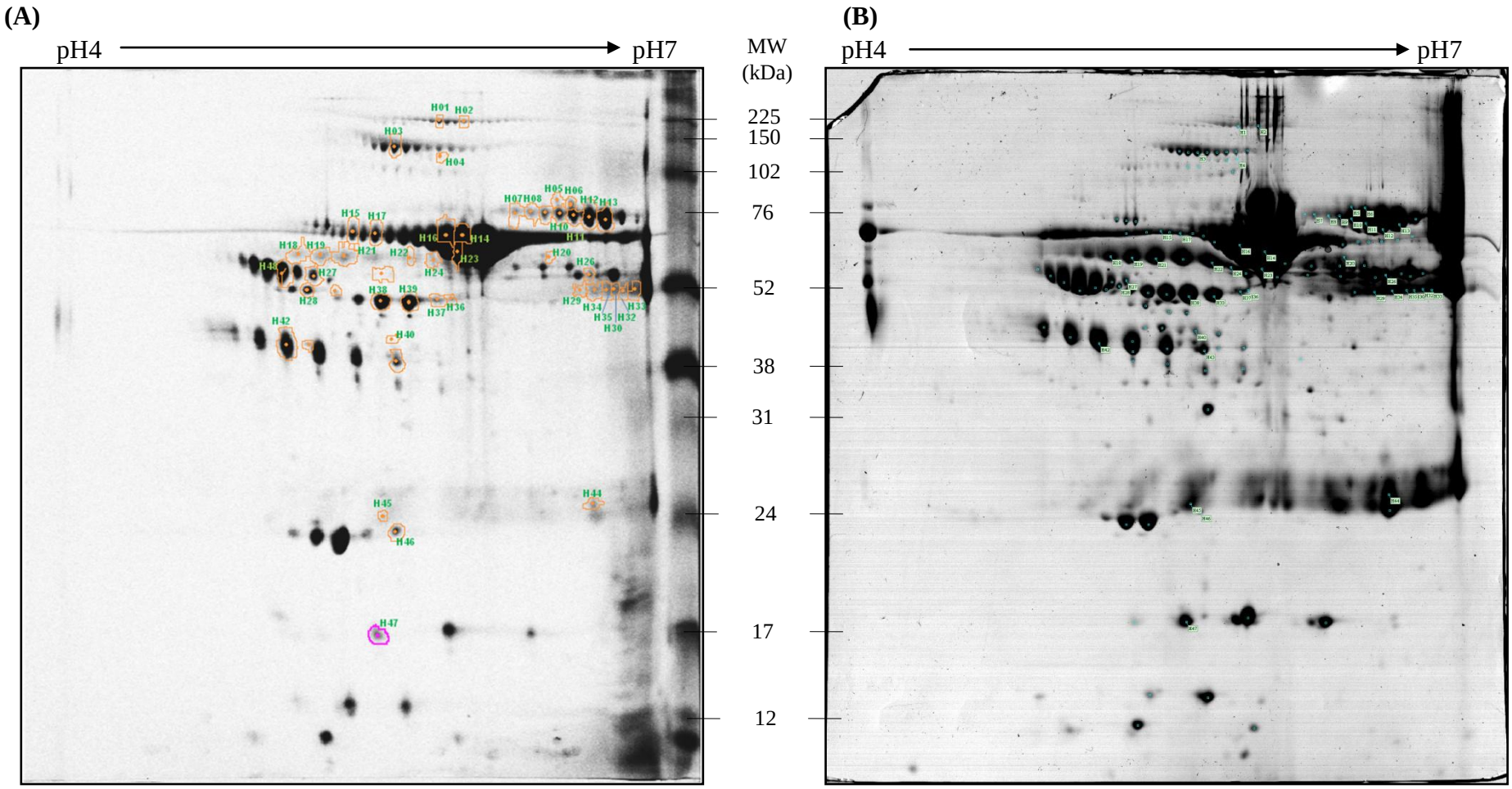


Figure 3.2.21. Selection and picking of HAPs of interest from 2-D DIGE and Coomassie blue-stained gels. SLE-associated HAP spots of all patient groups were compiled and labelled on a 2-D DIGE gel **(A)** and picked from a matched Coomassie blue-stained 2-DE gel **(B)** for in-gel digestion. Both gels had been matched using ImageMaster^{TM} 2D Platinum 7.02 (GE Healthcare). The Coomassie blue-stained gel was produced by 2-DE of 1 mg pooled HAP samples from all normal individuals and patients in this study.

Table 3.2.6. Protein identification results obtained from Spectrum Mill analysis for SLE-associated HAPs of all patient groups.

No.	Protein no.	Map no.	Protein ID	Spectra	Distinct Peptides	MS/MS search score	% AA Coverage
1	H27	722	Alpha-1-antitrypsin	7	7	107.01	16
2	H01	2547	Alpha-2-macroglobulin	3	3	29.11	2
3	H02	1936		13	11	160.23	15
4	H03	1582		14	10	139.67	8
5	H04	1543		1	1	11.46	1
6	H20	819		2	2	27.75	1
7	H47	212	Apolipoprotein A-I	5	5	53.49	23
8	H26	724	Fibrinogen beta chain	6	5	69.53	13
9	H28	716	Fibrinogen gamma chain	7	5	72.84	11
10	H36	610		2	2	16.38	3
11	H37	601		1	1	12.46	2
12	H38	590		21	12	221.84	25
13	H39	583		19	13	206.94	26
14	H42	572	Haptoglobin	14	11	176.5	22
15	H44	539		8	6	97.35	13
16	H48	210		7	6	72.87	21
17	H18	821	Ig alpha-1 and -2 chain C region	6	6	89.5	14
18	H19	820		11	8	121.06	23
19	H21	816		17	10	156.8	30
20	H22	791		13	10	148.87	32
21	H24	773		8	7	103.32	16
22	H29	714	Ig gamma chain C region	4	3	48.39	10
23	H30	706		3	3	48.43	9
24	H33	653		7	6	91.01	20
25	H34	651		4	4	65.8	13
26	H35	650		4	4	69.09	13
27	H45	346	Ig kappa chain C region	10	5	92.18	79
28	H46	318		9	5	70.42	79
29	H07	1024	Ig mu chain C region	5	4	48.65	7
30	H08	1007		3	3	33.15	6
31	H05	1497	Serotransferrin	2	2	35.14	3
32	H06	1062		7	7	105.52	9
33	H10	860		20	17	254.66	25
34	H11	856		39	30	482.18	40
35	H12	854		34	29	460.97	38
36	H13	851		34	29	443.33	35
37	H32	654		4	4	41.27	6
38	H14	832	Serum albumin	91	54	938.04	68
39	H15	829		18	14	228.47	24
40	H16	828		57	40	653.54	57
41	H17	825		14	12	189.97	19
42	H23	775		103	55	985.88	72
43	H40	578		11	8	105.03	12

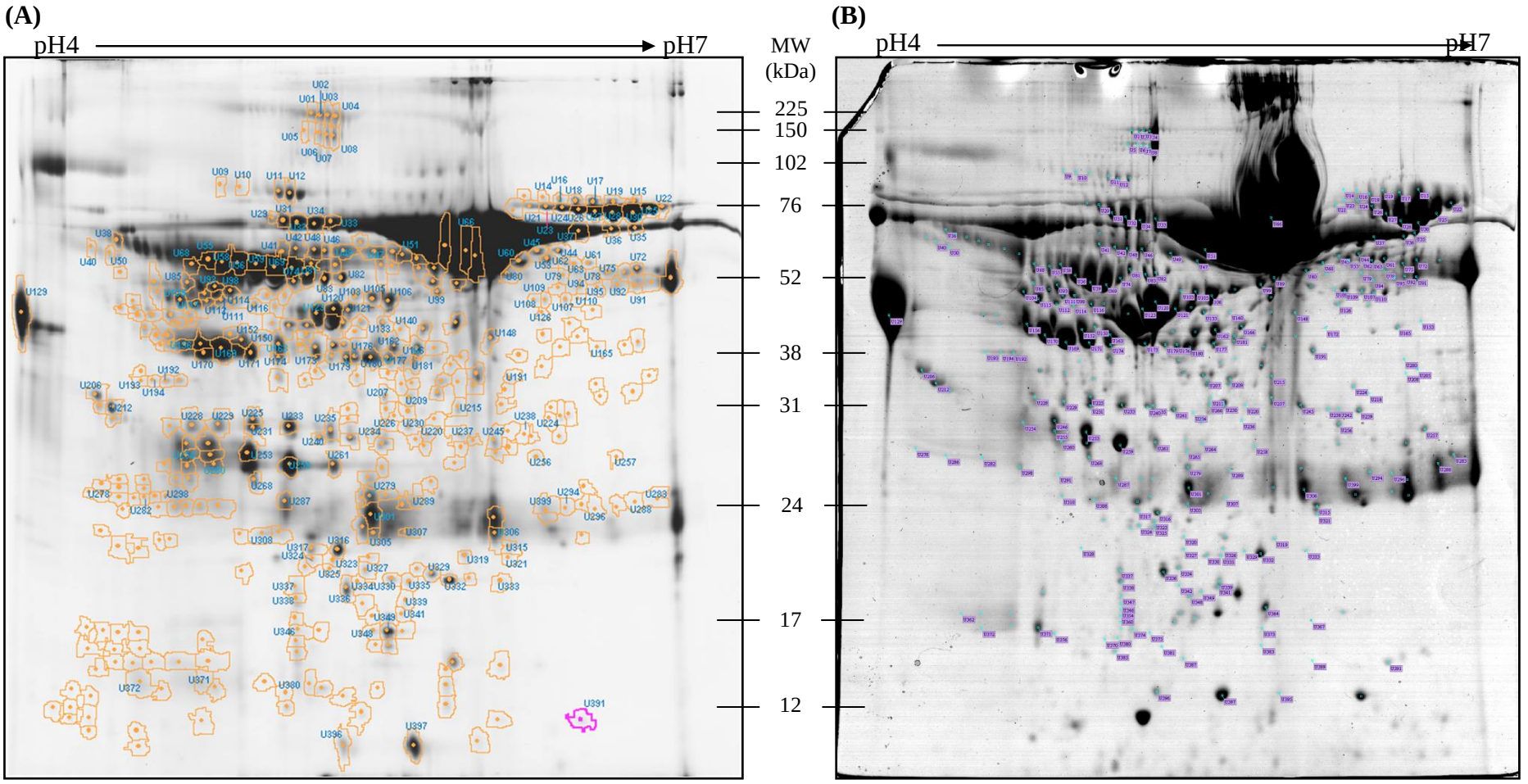


Figure 3.2.22. Selection and picking of urine proteins of interest from 2D-DIGE and Coomassie Blue-stained gels. SLE-associated urine spots of all patient groups were compiled and labelled on a 2-D DIGE gel **(A)** and picked from a matched Coomassie blue-stained 2-DE gel **(B)** for in-gel digestion. Both gels had been matched using ImageMaster™ 2D Platinum 7.02 (GE Healthcare). The Coomassie blue-stained gel was produced by 2-DE of 1 mg pooled HAP samples from all normal individuals and patients in this study. Proteins of interest are circled in orange while those labelled with blue numbers on **(A)** and purple boxes on **(B)** are protein spots that were able to be picked and identified with LC-MS/MS.

Table 3.2.7. Protein identification results obtained from Spectrum Mill analysis for SLE-associated urine proteins of all patient groups.

No.	Protein no.	Map no.	Protein ID	Spectra	Distinct Peptides	MS/MS search score	% AA Coverage
1	U11	508	Afamin	11	8	114.59	15
2	U12	514		4	3	27.41	5
3	U129	955	Alpha-1-acid glycoprotein 1	13	6	118.69	30
4	U38	659	Alpha-1-antichymotrypsin	43	18	302.38	30
5	U40	685		35	14	213.34	27
6	U50	711		13	12	204.74	26
7	U56	728	Alpha-1-antitrypsin	49	25	468.87	51
8	U58	732		34	24	386.18	51
9	U59	733		52	25	471.98	51
10	U69	757		49	30	528.86	60
11	U74	770		34	22	371.46	47
12	U85	810		20	15	254.75	35
13	U93	831		30	20	355.32	44
14	U98	853		31	21	349.05	48
15	U104	870		30	21	336.61	45
16	U111	892		20	14	238.28	31
17	U112	897		21	15	250.09	36
18	U114	899		20	13	196.5	29
19	U115	902		33	21	373.99	44
20	U116	908		30	21	354.42	49
21	U206	1198		12	9	130.98	19
22	U212	1236		14	8	127.55	21
23	U319	1707		5	5	54.15	10
24	U391	2087		5	4	70.46	8
25	U29	594	Alpha-1B-glycoprotein	21	16	235.17	32
26	U31	600		21	17	221.74	29
27	U32	603		39	18	304.87	33
28	U33	604		23	15	235.67	28
29	U34	606		35	19	337.06	41
30	U316	1695	Apolipoprotein A-I	22	17	227.32	48
31	U317	1699		9	9	110.36	31
32	U325	1747		2	2	25.5	8
33	U278	1511	Apolipoprotein D	2	2	16.51	9
34	U282	1521		2	2	25.42	9
35	U330	1779	Basement membrane-specific	14	11	179.5	2
36	U332	1782	heparan sulfate proteoglycan core	17	11	203.64	2
37	U333	1785	protein	9	7	96.87	1
38	U334	1787		1	1	14.34	0
39	U335	1798		3	3	31.39	1
40	U371	2019	CD59 glycoprotein	2	2	31.71	15
41	U01	218	Ceruloplasmin	5	4	43	4
42	U02	227		4	3	33.19	4
43	U03	228		9	8	127.94	10
44	U04	237		5	4	57.79	4
45	U341	1869	Ferritin light chain	3	3	43.78	21
46	U150	1015	Gelsolin	8	7	95.14	8
47	U152	1019	Haptoglobin	16	13	202.99	24
48	U163	1046		17	12	189.3	22
49	U173	1072		16	14	205.61	24

50	U176	1077	Haptoglobin	10	9	114.55	18
51	U177	1078		1	1	12.08	2
52	U41	691	Ig alpha-1 chain C region	12	10	160.57	32
53	U42	693		11	7	107.97	24
54	U46	699		16	11	178.95	38
55	U47	700		21	14	213.67	42
56	U48	702		19	9	133.5	32
57	U49	703		19	11	179.35	33
58	U47(2)	700	Ig alpha-2 chain C region	14	10	147.19	31
59	U48(2)	702		10	8	112.95	34
60	U49(2)	703		11	8	124.46	20
61	U91	826	Ig gamma-2 chain C region	5	5	83.47	14
62	U92	830		6	5	83.62	14
63	U94	837		3	3	54.96	7
64	U305	1641	Ig kappa chain C region	7	4	63.57	64
65	U306	1644		8	4	68.11	63
66	U308	1651	Ig kappa chain V-I region	1	1	24.13	14
67	U289	1540	Ig kappa chain V-III region	2	2	29.8	23
68	U315	1689		2	2	18.23	23
69	U399	2213	Ig lambda chain C regions	6	3	43.66	41
70	U288	1536		11	4	69.28	48
71	U294	1554		6	5	63.5	55
72	U296	1556		4	3	40.66	41
73	U307	1646	Ig lambda chain V-IV regions	1	1	16.84	16
74	U225	1287	Inter-alpha-trypsin inhibitor	8	4	51.96	4
75	U229	1293	heavy chain H4	3	2	20.9	3
76	U231	1295		7	4	60.48	4
77	U233	1300		7	4	52.88	5
78	U235	1313		5	4	39.88	4
79	U193	1147	Kallikrein-1	2	1	11.92	4
80	U194	1148		1	1	16.42	4
81	U55	724	Kininogen-1	17	15	208.6	25
82	U68	756		14	12	177.08	19
83	U337	1810	Lithostathine-1-alpha and beta	2	1	19.16	6
84	U338	1839		2	2	21.86	14
85	U346	1908		1	1	15.97	6
86	U372	2021		2	1	12.6	6
87	U380	2037		1	1	15.43	6
88	U05	283	Lysosomal alpha-glucosidase	1	1	11.97	1
89	U06	298		1	1	13.05	1
90	U07	304		2	1	14.13	1
91	U08	308		3	2	26.63	2
92	U298	1558	Lysosomal protective protein	2	2	24.1	3
93	U348	1913	Mannan-binding lectin serine	4	3	46.77	4
94	U349	1914	protease 2	3	2	28.8	4
95	U234	1312	Napsin-A	2	1	15.84	5
96	U72	763	Pancreatic alpha-amylase	12	10	150.91	21
97	U09	475	Polymeric immunoglobulin	5	4	45.59	6
98	U10	479	receptor	9	7	86.85	11
99	U279	1512	Prostaglandin-H2 D-isomerase	2	1	12.53	8
100	U287	1532		2	1	15.3	8
101	U301	1576		2	2	32.96	12
102	U192	1141	Protein AMBP	2	2	29.01	7
103	U228	1292		2	2	36.21	7
104	U253	1376		12	9	159.33	16

105	U254	1382	Protein AMBP	3	3	42.66	7
106	U259	1409		16	10	167.69	16
107	U260	1413		2	2	28.93	5
108	U261	1420		1	1	14.87	2
109	U268	1463		1	1	13.84	2
110	U336	1807	Retinol-binding protein 4	6	4	66.42	20
111	U14	523	Serotransferrin	5	3	32.85	3
112	U15	525		38	31	504.81	42
113	U16	527		6	6	77.56	9
114	U17	529		24	21	332.91	24
115	U18	533		20	16	235.72	20
116	U19	536		20	16	228.57	20
117	U22	560		50	40	624.78	45
118	U23	564		6	6	80.09	10
119	U24	570		13	11	151.92	15
120	U25	573		83	53	950.95	50
121	U26	575		30	24	362.78	35
122	U27	580		63	44	726.56	46
123	U28	593		85	54	968.85	51
124	U30	595		72	51	863.12	56
125	U165	1050		6	5	59.93	5
126	U257	1389		6	6	79.49	6
127	U21	555	Serum albumin	20	16	228.81	21
128	U35	627		42	30	490.67	45
129	U36	636		61	45	751.32	59
130	U37	644		65	45	758.73	57
131	U44	695		31	22	374.19	31
132	U45	698		26	18	278.17	24
133	U51	712		92	55	978.45	72
134	U53	721		30	24	370.23	34
135	U60	736		27	19	301.28	26
136	U61	737		50	33	542.85	49
137	U62	742		36	27	426.82	40
138	U63	743		46	33	509.09	45
139	U66	751		118	63	1162.51	81
140	U75	771		26	20	311.49	26
141	U78	778		8	8	100.28	12
142	U79	784		5	5	64.37	8
143	U80	786		39	29	474.45	45
144	U82	794		27	21	355.31	28
145	U99	855		38	32	501.86	50
146	U103	868		18	15	273.31	24
147	U105	874		35	25	402.56	35
148	U106	880		57	40	679.34	46
149	U107	884		20	17	224.2	24
150	U108	886		6	6	69.44	10
151	U109	887		36	23	376.78	38
152	U110	890		21	15	246.66	23

153	U120	929	Serum albumin	61	38	679.77	45
154	U121	932		60	37	629.72	45
155	U123	940		57	37	634.73	43
156	U126	946		7	7	94.34	12
157	U133	966		23	18	320.45	25
158	U140	983		13	12	179	16
159	U148	1013		18	15	246.37	20
160	U162	1044		33	31	493.58	41
161	U166	1052		24	20	278.53	23
162	U179	1081		20	17	248.87	23
163	U180	1082		32	23	380.63	34
164	U181	1096		10	9	100.72	15
165	U191	1138		13	11	148.77	19
166	U207	1204		14	12	198.96	18
167	U209	1212		11	11	133.67	17
168	U215	1244		9	9	126.65	14
169	U220	1272		5	5	69.51	6
170	U224	1283		6	5	63.45	9
171	U226	1290		7	7	105.84	11
172	U237	1322		13	12	137.76	18
173	U238	1324		4	4	59.94	5
174	U239	1327		9	8	132.09	10
175	U245	1340		20	18	254.28	24
176	U256	1383		10	10	139.69	12
177	U283	1523		12	10	136.65	16
178	U321	1715		4	4	55.35	5
179	U323	1729		1	1	19.12	1
180	U324	1746		1	1	16.94	1
181	U339	1841		2	2	29.7	2
182	U327	1754	Tetranectin	2	1	11.18	5
183	U329	1771		2	1	15.46	5
184	U230	1294	Transthyretin	6	4	57.97	17
185	U396	2132		3	2	33.91	12
186	U397	2142		11	6	94.62	37
187	U240	1328	Vesicular integral-membrane protein VIP36	3	3	40.92	8
188	U241	1329		2	2	25.88	3
189	U81	787	Vitamin D-binding protein	18	15	267	37
190	U83	798		25	16	284.79	40
191	U156	1030	Zinc-alpha-2-glycoprotein	16	13	219.39	38
192	U169	1062		27	18	320.56	48
193	U170	1064		25	16	297.99	48
194	U171	1066		21	15	266.59	51
195	U174	1074		9	7	100.43	18

3.2.6 SLE-associated proteins

SLE-associated proteins were categorized according to clinical manifestations, i.e. SLE without kidney manifestation and lupus nephritis (LN), as well as classes of LN. Protein spots identified with scores less than 45 in the previous section were omitted. All resulting SLE-associated protein spots had average abundance ratios of ≥ 1.5 or ≤ -1.5 relative to normal individuals, with *p* value of ≤ 0.05 in T-test analysis. Some proteins had numerous isoforms involved so higher cut-off abundance ratios were applied to these proteins in order to reduce the number of isoforms as well as to make the results more significant.

3.2.6.1 Plasma proteins

Protein identification results of HAPs and HAP-depleted proteins were combined to give an overview picture of plasma proteins that were associated with SLE. In general, 56 isoforms from 29 plasma proteins that were differentially regulated were found to be correlated with SLE regardless of the clinical manifestations (**Table 3.2.8 and Table 3.2.9**). Besides afamin, retinol-binding protein 4, serum amyloid P-component, vitamin D-binding protein, apolipoprotein A-I, clusterin, C4b-binding protein alpha chain, ficolin-3, Ig kappa chain C region, ceruloplasmin, fibrinogen beta chain, alpha-1-antitrypsin, alpha-2-macroglobulin, and gelsolin, the remaining proteins had more than one isoforms involved. Haemopexin isoforms were filtered at higher average abundance ratio (≤ -2 or ≥ 2) because considerably large number of isoforms was noticed. **Figure 3.2.23** illustrates the relative expression levels of these protein isoforms in each SLE groups compared to normal individuals. The SLE-associated plasma proteins could be clustered according to their functional criteria: 27.6% of them were serum constitutional and transport proteins, 13.8% were lipoprotein metabolism, another 17.2% were involved in complement factors, 13.8% were metal binding proteins, another 10.3% were immunoglobulins and other immune proteins, 6.9% were coagulation factors, another 6.9% were proteases and inhibitors, and 3.4% were kidney secretory and structural proteins (**Figure 3.2.24**).

When zooming in to plasma proteins associated to SLE patients without kidney manifestation, eight isoforms from eight proteins were observed, with up-regulation in four isoforms/proteins and down-regulation in the other four isoforms/proteins (**Table 3.2.8, Table 3.2.9, and Figure 3.2.25**). Alpha-1-antitrypsin and serum amyloid P-component

were distinctively up- and down-regulated, respectively, in SLE patients without kidney manifestation, not in other groups.

As for LN patients, a total 54 isoforms from 27 proteins were observed to be abnormally regulated compared with normal individuals (**Table 3.2.8 and Table 3.2.9**):

- Down-regulation of serum constitution and transportation proteins (except for retinol-binding protein 4), complement fractions (except for ficolin-3), and immunoglobulins and other immune proteins (except for Ig kappa chain C region) were consistently seen in all LN groups, whilst up-regulation was observed for proteins involved in lipoprotein metabolism.
- Most proteins associated with LN class II were responsible in serum constitution and transportation and complement pathways, with numerous haemopexin isoforms were found. Down-regulation of afamin and gelsolin was specific to LN class II only.
- Up-regulation of proteins responsible in lipoprotein metabolisms seemed to be important in LN class III and up-regulation of retinol-binding protein 4 and adiponectin was distinctive to this class only.
- LN class IV, which was a more severe form of kidney damage similar to LN class III, on the other hand had fewer associated isoforms/proteins whilst metal-binding proteins were found to be more prominent. Up-regulation of Ig kappa chain C region was specific to LN class IV only.
- Serum constitutional and transport proteins were shown to have more important role in LN class V, with down-regulation of histidine-rich glycoprotein being specific to this class. Down-regulation of fibrinogen beta-chain was also found to be unique to LN class V.
- LN patients who were already in remission while this study was conducted were formerly LN class IV patients. When comparing relative protein abundance between these two groups of patients, it was found that many isoforms/proteins that were aberrantly expressed in active LN class IV group had returned to normal levels after remission (**Table 3.2.10, Figure 3.2.26(A), and Figure 3.2.27**). These proteins included apolipoprotein A-IV, clusterin, complement C4-A & B, complement factor B, Ig kappa chain C region, alpha-2-HS-glycoprotein, and ceruloplasmin. Serotransferrin and zinc-alpha-2-glycoprotein remained up-regulated and down-regulated, respectively, as in active LN class IV group. In

addition, down-regulation of isoforms from haptoglobin and haemopexin, as well as up-regulation of isoforms from apolipoprotein A-I and Ig alpha-1 and -2 chain C region, which were not observed in active LN class IV occurred in patients in remission (**Figure 3.2.26(B) and Figure 3.2.27**).

Table 3.2.8. Plasma protein isoforms associated with non-renal SLE and each class of lupus nephritis (LN), identified from LC-MS/MS analysis.

No.	Protein No.	Protein name	Gene name	Accession (Swiss-Prot)	Average Abundance Ratio* SLE (Non-LN) (n=5)	LN Class II (n=3)	LN Class III (n=3)	LN Class IV (n=5)	LN Class V (n=3)
		Serum constitutional and transport proteins							
1	27	Afamin	AFM	P43652		↓-1.56§			
2	H42	Haptoglobin (1)	HP	P00738			↓-1.88		↓-1.92
3	H44	Haptoglobin (2)							↓-1.89
4	51	Haemopexin (1)	HPX	P02790			↓-2.08		
5	58	Haemopexin (2)				↓-2.58			
6	91	Haemopexin (3)				↓-2.03			
7	92	Haemopexin (4)				↓-2.32			
8	35	Histidine-rich glycoprotein (1)	HRG	P04196					↓-2.12§
9	36	Histidine-rich glycoprotein (2)							↓-2.4§
10	139	Retinol-binding protein 4	RBP4	P02753			↑1.74§		
11	H14	Serum albumin (1)	ALB	P02768					↓-2.35
12	H15	Serum albumin (2)					↓-2.3		↓-2.37
13	H16	Serum albumin (3)					↓-1.8		
14	H17	Serum albumin (4)				↓-1.89	↓-2.21		
15	H40	Serum albumin (5)					↓-2.07		↓-2.46
16	178	Serum amyloid P-component	SAP	P02743	↓-1.64§				
17	64	Vitamin D-binding protein	VDB	P02774		↓-1.56			↓-1.66
		Lipoprotein metabolism							
18	H47	Apolipoprotein A-I	ApoA-I	P02647			↑1.87		
19	109	Apolipoprotein A-IV (1)	APOA4	P06727		↑2.16		↑2.07	↑2.23
20	111	Apolipoprotein A-IV (2)						↑2.13	↑2.42
21	113	Apolipoprotein A-IV (3)					↑1.83	↑1.72	
22	140	Apolipoprotein E (1)	ApoE	P02649			↑2.01		
23	150	Apolipoprotein E (2)					↑3.27		
24	125	Clusterin	CLU	P10909		↑1.94		↑1.68	↑1.57
		Complement fractions							
25	48	C4b-binding protein alpha chain	C4BPA	F2Z2V7	↓-1.79	↓-1.57			
26	25	Complement C4-B & A (1)	C4A &	P0C0L4 &		↓-2.72		↓-2.59	↓-2.5
27	168	Complement C4-B & A (2)	C4B	P0C0L5			↓-4.22	↓-2.02	
28	22	Complement factor B (1)	CFB	P00751		↓-2.61		↓-1.56	↓-1.78

29	23	Complement factor B (2)						↓-1.79	
30	1	Complement factor H (1)	CFH	P08603	↑1.75				
31	133	Complement factor H (2)				↓-1.95			
32	149	Ficolin-3	FCN	O75636			↑1.97		↑1.79
		Immunoglobulins and other immune proteins							
33	H19	Ig alpha-1 and -2 chain C region (1)	IGHA1 &	P01876 &			↓-2.16		
34	H22	Ig alpha-1 and -2 chain C region (2)	IGHA2	P01877		↓-1.92			
35	H24	Ig alpha-1 and -2 chain C region (3)				↓-1.89			
36	H29	Ig gamma chain C region (1)	IGHG1	P01857	↑2.32				
37	H30	Ig gamma chain C region (2)							↓-1.64
38	H33	Ig gamma chain C region (3)							↓-2.2
39	H34	Ig gamma chain C region (4)							↓-1.66
40	H35	Ig gamma chain C region (5)				↓-1.94			↓-2.27
41	H46	Ig kappa chain C region	IGKC	P01834				↑1.93§	
		Metal binding proteins							
42	78	Alpha-2-HS-glycoprotein (1)	AHSG	P02765	↓-2.64		↓-2.32	↓-2.34	
43	80	Alpha-2-HS-glycoprotein (2)					↓-2.6	↓-2.42	
44	38	Ceruloplasmin	CP	P00450	↓-1.91			↑1.64	
45	H10	Serotransferrin (1)	TF	P02787			↑1.57		
46	H11	Serotransferrin (2)					↑1.53		
47	H13	Serotransferrin (3)						↑1.92	
48	102	Zinc-alpha-2-glycoprotein (1)	AZGP1	P25311				↑1.79	
49	114	Zinc-alpha-2-glycoprotein (2)							↑1.63
		Coagulation factors							
50	H26	Fibrinogen beta chain	FGB	P02675					↓-3.72§
51	H28	Fibrinogen gamma chain (1)	FGG	P02679	↑2.68				
52	H38	Fibrinogen gamma chain (2)							↓-2.91
53	H39	Fibrinogen gamma chain (3)							↓-2.53
		Proteases and inhibitors							
54	H27	Alpha-1-antitrypsin	AAT	P01009	↑3.52§				
55	H03	Alpha-2-macroglobulin	A2M	P01023			↑1.88§		
		Kidney secretory and structural proteins							
56	31	Gelsolin	GSN	P06396		↓-1.81§			

Note:

* p value ≤ 0.05, average abundance ratio ≤ -1.5 or ≥ 1.5 relative to normal individuals, except for haemopexin which was cut off at average abundance ratio of ≤ -2 or ≥ 2

↑ - up-regulation, ↓ - down-regulation,

§ proteins specific to the corresponding class of SLE/LN only

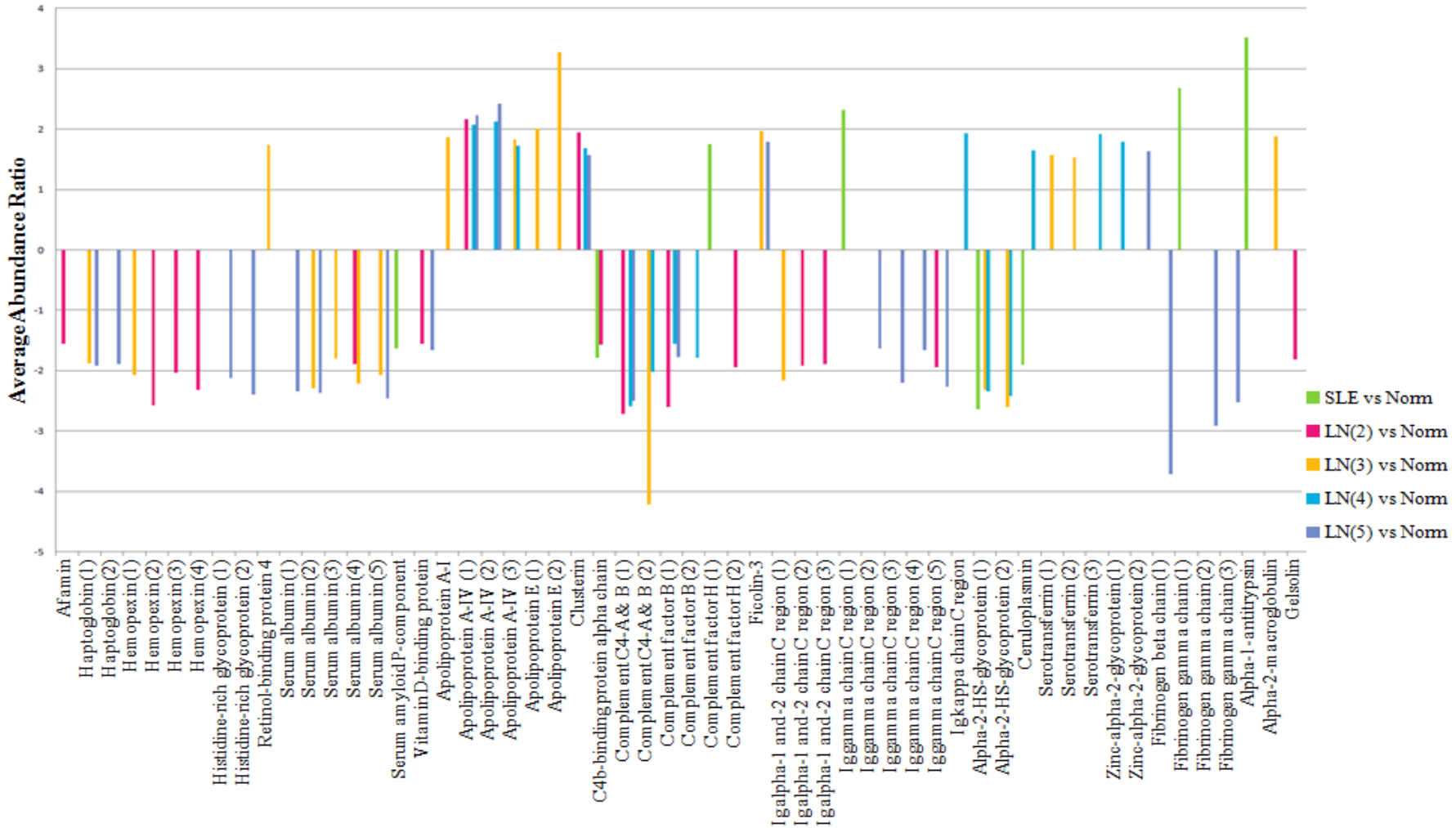


Figure 3.2.23. Column chart showing average abundance ratios of plasma proteins/isoforms associated with SLE without kidney involvement and LN class II to class V. Norm – Normal individuals; vs - versus; LN(2) - LN class II; LN(3) - LN class III; LN(4) - LN class IV; LN(5) - LN class V

Table 3.2.9. Summary of plasma proteins associated with non-renal SLE and each class of lupus nephritis (LN).

No.	Protein name	Gene name	Accession (Swiss-Prot)	Molecular weight (Da)	Average Abundance Ratio* SLE (Non-LN) (n=5)	LN Class II (n=3)	LN Class III (n=3)	LN Class IV (n=5)	LN Class V (n=3)
	Serum constitutional and transport proteins								
1	Afamin	*AFM*	P43652	69,069		↓§			
2	Haptoglobin	*HP*	P00738	45,205			↓		↓
3	Haemopexin	*HPX*	P02790	51,676		↓	↓		
4	Histidine-rich glycoprotein	*HRG*	P04196	59,578					↓§
5	Retinol-binding protein 4	*RBP4*	P02753	23,010			↑§		
6	Serum albumin	*ALB*	P02768	69,367 47,360		↓	↓		↓
7	Serum amyloid P-component	*SAP*	P02743	25,387	↓§				
8	Vitamin D-binding protein	*VDB*	P02774	52,964 39,542		↓			↓
	Lipoprotein metabolism								
9	Apolipoprotein A-I	*ApoA-I*	P02647	30,778			↑§		
10	Apolipoprotein A-IV	*APOA4*	P06727	45,399		↑		↑	↑
11	Apolipoprotein E	*ApoE*	P02649	36,154			↑§		
12	Clusterin	*CLU*	P10909	57,833 53,643 52,495 48,803 32,364		↑		↑	↑
	Complement fractions								
13	C4b-binding protein alpha chain	*C4BPA*	F2Z2V7	13,092	↓	↓			
14	Complement C4-A & B	*C4A & C4B*	P0C0L4 & P0C0L5	192,771		↓	↓	↓	↓
15	Complement factor B	*CFB*	P00751	85,533 68,872		↓		↓	↓
16	Complement factor H	*CFH*	P08603	139,096 51,034	↑	↓			
17	Ficolin-3	*FCN*	O75636	32,903 31,678			↑		↑
	Metal binding proteins								
18	Alpha-2-HS-glycoprotein	*AHSG*	P02765	39,325	↓		↓	↓	
19	Ceruloplasmin	*CP*	P00450	122,205	↓			↑	
20	Serotransferrin	*TF*	P02787	77,064			↑	↑	
21	Zinc-alpha-2-glycoprotein	*AZGP1*	P25311	34,259				↑	↑
	Immunoglobulins and other immune proteins								
22	Ig alpha-1 and -2 chain C region	*IGHA1 & IGHA2*	P01876 & P01877	37,655		↓	↓		
23	Ig gamma chain C region	*IGHG1*	P01857	36,106	↑	↓			↓
24	Ig kappa chain C region	*IGKC*	P01834	11,609				↑§	
	Coagulation factors								
25	Fibrinogen beta chain	*FGB*	P02675	55,928					↓§
26	Fibrinogen gamma chain	*FGG*	P02679	51,512 49,497	↑				↓

	Proteases and inhibitors						
27	Alpha-1-antitrypsin	*AAT*	P01009	46,737 40,263 34,755	↑§		
28	Alpha-2-macroglobulin	*A2M*	P01023	163,291			↑§
	Kidney secretory and structural proteins						
29	Gelsolin	*GSN*	P06396	85,698 80,641 81,941		↓§	

Note:

* *p* value ≤ 0.05, average abundance ratio ≤ -1.5 or ≥ 1.5 relative to normal individuals, except for haemopexin which was cut off at average abundance ratio of ≤ -2 or ≥ 2

↑ up-regulation, ↓ down-regulation

§ proteins specific to the corresponding class of SLE/LN only

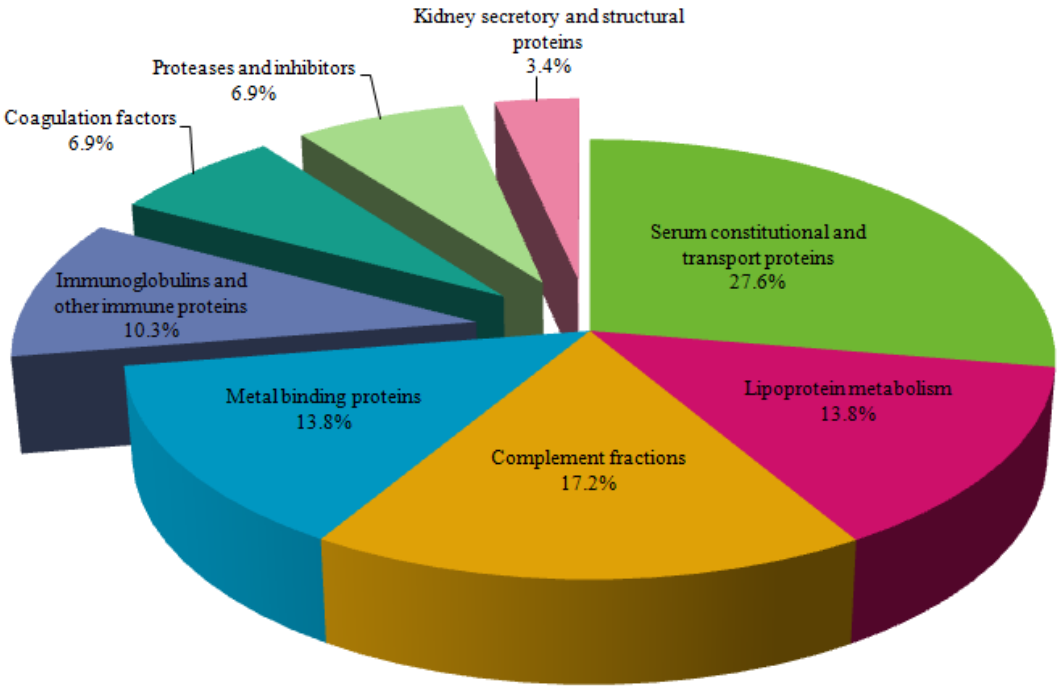


Figure 3.2.24. Functionalities of SLE-associated plasma proteins of all patient groups.

	SLE (non-LN)	LN class II	LN class III	LN class IV	LN class V
Up-regulated	Complement factor H Ig gamma chain C region Fibrinogen gamma chain Alpha-1-antitrypsin*	Apolipoprotein A-IV Clusterin	Retinol-binding protein 4* Apolipoprotein A-I Apolipoprotein E Ficolin-3 Serotransferrin Alpha-2-macroglobulin*	Apolipoprotein A-IV Clusterin Ceruloplasmin Serotransferrin Zinc-alpha-2-glycoprotein Ig kappa chain C region*	Apolipoprotein A-IV Clusterin Ficolin-3 Zinc-alpha-2-glycoprotein
Down-regulated	Serum amyloid P-component* C4b-binding protein alpha chain Alpha-2-HS-glycoprotein Ceruloplasmin	Afamin* Hemopexin Serum albumin Vitamin D-binding protein C4b-binding protein alpha chain Complement C4-A & B Complement factor B Complement factor H Ig alpha-1 and -2 chain C region Ig gamma chain C region Gelsolin*	Haptoglobin Hemopexin Serum albumin Complement C4-A & B Alpha-2-HS-glycoprotein Ig alpha-1 and -2 chain C region	Complement C4-A & B Complement factor B Alpha-2-HS-glycoprotein	Haptoglobin Histidine-rich glycoprotein* Serum albumin Vitamin D-binding protein Complement C4-A & B Complement factor B Ig gamma chain C region Fibrinogen beta chain* Fibrinogen gamma chain

Figure 3.2.25. Summary of abnormally regulated plasma proteins involved in each group of SLE patients (lupus nephritis and without kidney involvement). Proteins labelled with (*) are group-specific proteins.

Table 3.2.10. Comparison of plasma protein isoform levels between patients with active LN class IV and those in remission (formerly LN class IV patients).

No.	Protein No.	Protein name	Gene name	Accession (Swiss-Prot)	Average Abundance Ratio*	
					LN Class IV (n=5)	LN Remission (n=5)
		Serum constitutional and transport proteins				
1	H44	Haptoglobin (2)	HP	P00738		↓-1.97#
2	51	Haemopexin (2)	HPX	P02790		↓-2.27#
		Lipoprotein metabolism				
3	183	Apolipoprotein A-I (1)	ApoA-I	P02647		↑2.35#
4	109	Apolipoprotein A-IV (1)	APOA4	P06727	↑2.07†	
5	111	Apolipoprotein A-IV (2)			↑2.13†	
6	115	Apolipoprotein A-IV (3)			↑1.72†	
7	125	Clusterin	CLU	P10909	↑1.68†	
		Complement fractions				
8	25	Complement C4-A & B (1)	C4A & C4B	P0C0L4 & P0C0L5	↓-2.59†	
9	168	Complement C4-A & B (2)			↓-2.02†	
10	22	Complement factor B (1)	CFB	P00751	↓-1.56†	
11	23	Complement factor B (2)			↓-1.79†	
		Immunoglobulins and other immune proteins				
12	H21	Ig alpha-1 and -2 chain C region (4)	IGHA1 & IGHA2	P01876 & P01877		↑1.53#
13	H46	Ig kappa chain C region	IGKC	P01834	↑1.93†	
		Metal binding proteins				
14	78	Alpha-2-HS-glycoprotein (1)	AHSG	P02765	↓-2.34†	
15	80	Alpha-2-HS-glycoprotein (2)			↓-2.42†	
16	38	Ceruloplasmin	CP	P00450	↑1.64†	
17	H11	Serotransferrin (2)	TF	P02787		↑1.51
18	H13	Serotransferrin (3)			↑1.92	
19	H12	Serotransferrin (4)				↑1.62
20	102	Zinc-alpha-2-glycoprotein (1)	AZGP1	P25311	↑1.79	
21	114	Zinc-alpha-2-glycoprotein (2)				↓-1.51

Note:

* *p* value ≤ 0.05, average abundance ratio ≤ -1.5 or ≥ 1.5, except for haemopexin which was cut off at average abundance ratio of ≤ -2 or ≥ 2

↑ up-regulation, ↓ down-regulation

† abnormally regulated in patients with LN class IV only

abnormally regulated in LN patients in remission only

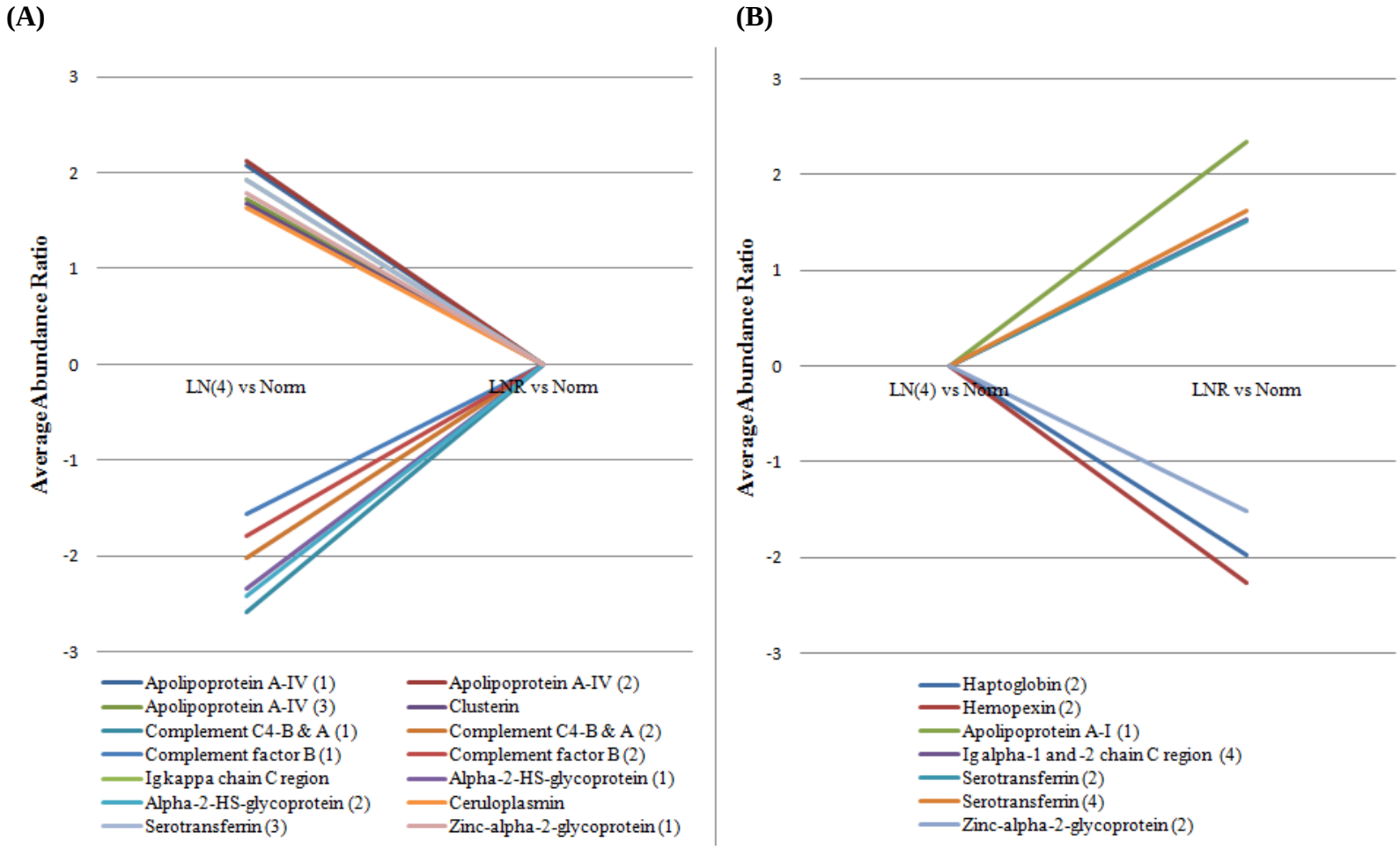


Figure 3.2.26. Changes of plasma protein isoform levels between active LN class IV and LN in remission. The protein isoform levels are the average abundance ratios, whereby the protein isoform levels for both disease states were measured relative to those of normal individuals. 0 denotes normal level X-axis denotes disease states. (**A)** shows the presence of abnormally regulated protein isoforms in patients with active LN class IV but became normal in patients in remission whilst **(B)** shows the otherwise. LN(4) – LN class IV; LNR – LN in remission; vs – versus; Norm – Normal individuals

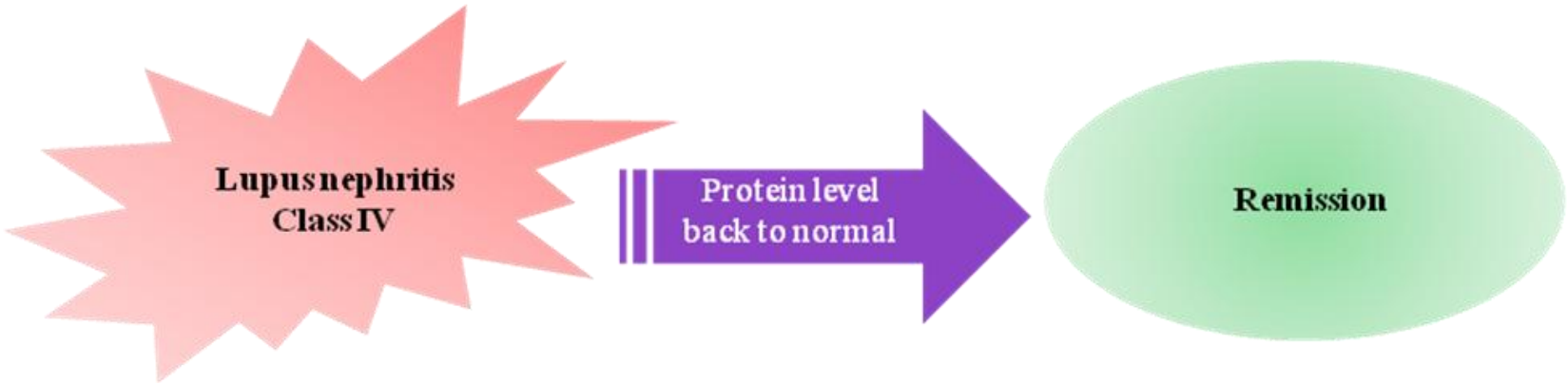


Figure 3.2.27. Summary of changes in plasma protein levels along transition from lupus nephritis (LN) class IV to remission. Most proteins with aberrant expressions in LN class IV had become normal upon achieving remission. There were still a few proteins that were up- (↑) or down- (↓) regulated in LN remission patients (average abundance ratio of ≤ -1.5 or ≥ 1.5, or ≤ -2 or ≥ 2 for haemopexin**)** compared with normal individuals, with *p* value ≤ 0.05. Serotransferrin (highlighted in yellow) that was up-regulated in LN class IV remained highly expressed during remission, whereas zinc-alpha-2-glycoprotein that was initially up-regulated became down-regulated during remission.

3.2.6.2 Urine proteins

Overall, 59 isoforms from 25 urine proteins were found to be associated with SLE regardless of the clinical manifestations (**Table 3.2.11 and Table 3.2.12**). Besides afamin, alpha-1-acid glycoprotein 1, retinol-binding protein 4, Ig kappa chain C region, alpha-1-antichymotrypsin, mannan-binding lectin serine protease 2, kininogen-1, pancreatic alpha-amylase, and gelsolin, the remaining proteins had more than one isoforms involved. For those proteins which had too many isoforms involved, such as alpha-1B-glycoprotein, haptoglobin, and albumin, higher cut-off points for average abundance ratio were applied in order to reduce the number of isoforms as well as to make the results more significant. **Figure 3.2.28** illustrates the relative excretion levels of these protein isoforms in each SLE groups compared to normal individuals. When clustering according to functions, 28% of the SLE-associated urine proteins were serum constitutional and transport proteins, 4% were responsible in lipoprotein metabolism, another 4% were complement fractions, 12% were metal binding proteins, 16% were immunoglobulins and other immune proteins, 24% were proteases and inhibitors, and 12% were kidney secretory and structural proteins (**Figure 3.2.29**).

Systemic lupus erythematosus (SLE) patients without kidney manifestation had only three protein isoforms (2 isoforms from inter-alpha-trypsin inhibitor heavy chain H4 and one from gelsolin) observed to be at lower urine levels than normal (**Table 3.2.10, Table 3.2.12, and Figure 3.2.30**). As for the case of LN patients, still a total of 59 isoforms from 25 urine proteins associated proteins were observed. In spite of reduced urinary excretion of a few proteins/isoforms such as retinol-binding protein 4, mannan-binding lectin serine protease 2, inter-alpha-trypsin inhibitor heavy chain H4, and basement membrane-specific heparan sulfate proteoglycan core protein, almost all proteins/isoforms were increasingly excreted in urine of LN patients. Most proteins associated with LN class II were serum constitutional and transport proteins, proteases and inhibitors, and immune proteins, with reduced excretion of Ig kappa chain C region and increased excretion of kininogen-1 being specific to LN class II only. Besides that, decreased in urine mannan-binding lectin serine protease 2 was also found to be distinctive to LN class II. Urinary excretion of serum constitutional and transport proteins, metal binding proteins, and kidney secretory and structural proteins seem to be more affected in LN class III patients. However, no specific protein was discovered for LN class III. LN class IV had fewer proteins affected, with elevated level of urine retinol-binding protein 4

and reduced urine level of alpha-1-antichymotrypsin being distinctive to LN class IV only. Only 7 isoforms from 5 proteins, mostly of proteases and inhibitors, were associated with LN class V and protein AMBP appeared to be specific to LN class V.

LN patients who were already in remission in this study were previously LN class IV patients. When comparing relative urinary abundance of isoforms between these two groups, 7 isoforms that were aberrantly excreted in active LN class IV group had returned to normal level upon remission [**Figure 3.2.31(A)**]. But at the end, only 2 proteins (i.e. afamin and retinol-binding protein 4) had actually recovered to normal urinary level after remission (**Table 3.2.13 and Figure 3.2.32**). Most LN class IV-associated proteins remained abnormally excreted after remission [**Table 3.2.13, Figure 3.2.31(B) and Figure 3.2.32**]. An additional of 6 affected isoforms, which at the end refined as 2 proteins, were initially absent in active LN class IV but observed in patients in remission [**Figure 3.2.31(C) and Figure 3.2.32**]. The proteins were alpha-1-acid glycoprotein 1, ferritin light chain and Ig lambda chain C regions.

Table 3.2.11. Urine protein isoforms associated with non-renal SLE and each class of lupus nephritis (LN), identified from LC-MS/MS analysis.

No.	Protein No.	Protein name	Gene name	Accession (Swiss-Prot)	Average Abundance Ratio* SLE (Non-LN) (n=5)	LN Class II (n=3)	LN Class III (n=3)	LN Class IV (n=5)	LN Class V (n=3)
		Serum constitutional and transport proteins							
1	U11	Afamin	AFM	P43652		↑3.41		↑2.4	
2	U37	Albumin (1)	ALB	P02768			↑20.54		
3	U45	Albumin (2)				↑34.89			
4	U66	Albumin (3)				↑25.39			
5	U103	Albumin (4)					↑20.64		
6	U129	Alpha-1-acid glycoprotein 1	AGP1	P02763		↑10.15	↑9.96		
7	U29	Alpha-1B-glycoprotein (1)	A1BG	P04217		↑28.69			
8	U31	Alpha-1B-glycoprotein (2)					↑10.2		
9	U32	Alpha-1B-glycoprotein (3)				↑11.83			
10	U34	Alpha-1B-glycoprotein (4)				↑10.09			
11	U152	Haptoglobin (1)	HP	P00738			↑22.53		
12	U163	Haptoglobin (2)					↑25.44		
13	U173	Haptoglobin (3)					↑19.2		
14	U336	Retinol-binding protein 4	RBP4	P02753				↓-3.08[§]	
15	U81	Vitamin D-binding protein (1)	VDB	P25311		↑10.25	↑6.51	↑4.49	
16	U83	Vitamin D-binding protein (2)				↑7.31	↑3.65		
		Lipoprotein metabolism							
17	U316	Apolipoprotein A-I (1)	ApoA-I	P02647		↑7.83	↑5.33		
18	U317	Apolipoprotein A-I (2)				↑3.52	↑2.34		
		Complement fractions							

19	U348	Mannan-binding lectin serine protease 2	MASP2	O00187		↓-5.02§			
		Metal binding proteins							
20	U03	Ceruloplasmin (1)	CP	P00450			↑16.66	↑4.81	
21	U04	Ceruloplasmin (2)					↑11.5	↑3.06	
22	U25	Transferrin (1)	TF	P02787			↑33.23		
23	U28	Transferrin (2)					↑33.44		
24	U30	Transferrin (3)					↑34.42		
25	U156	Zinc-alpha-2-glycoprotein (1)	AZGP1	P25311			↑13.58	↑12.28	
26	U169	Zinc-alpha-2-glycoprotein (2)				↑11.43	↑19.85	↑13.32	
27	U170	Zinc-alpha-2-glycoprotein (3)					↑17.24	↑14.23	
28	U171	Zinc-alpha-2-glycoprotein (4)					↑16.35	↑10.22	
29	U174	Zinc-alpha-2-glycoprotein (5)					↑12.72		
		Immunoglobulins and other immune proteins							
30	U41	Ig alpha-1 and -2 chain C region (1)	IGHA1	P01876		↑6.2			
31	U42	Ig alpha-1 and -2 chain C region (2)	&	&		↑3.54	↑3.67		
32	U46	Ig alpha-1 and -2 chain C region (3)	IGHA2	P01877		↑4.03	↑4.5		
33	U47	Ig alpha-1 and -2 chain C region (4)				↑2.33	↑4.39		
34	U48	Ig alpha-1 and -2 chain C region (5)				↑3.48	↑4.25		
35	U49	Ig alpha-1 and -2 chain C region (6)				↑2.41	↑3.41		
36	U91	Ig gamma-2 chain C region (1)	IGHG2	P01859		↑2.45		↑2.1	
37	U92	Ig gamma-2 chain C region (2)				↑2.66		↑2.01	
38	U94	Ig gamma-2 chain C region (3)				↑2.43			
39	U306	Ig kappa chain C region	IGKC	P01834		↓-1.78§			
40	U288	Ig lambda chain C regions (1)	IGLC1	P0CG04		↑2.51	↑2.78		↑2.03
41	U294	Ig lambda chain C regions (2)				↑1.87	↑2.23		
		Proteases and inhibitors							
42	U38	Alpha-1-antichymotrypsin	AACT	P01011				↑2.89§	
43	U56	Alpha-1-antitrypsin (1)	AAT	P01009		↑21.45			
44	U59	Alpha-1-antitrypsin (2)				↑27.98	↑28.34		
45	U69	Alpha-1-antitrypsin (3)				↑21.75	↑22.87		
46	U115	Alpha-1-antitrypsin (4)				↑26.4			
47	U319	Alpha-1-antitrypsin (5)				↑24.24			
48	U225	Inter-alpha-trypsin inhibitor heavy chain H4 (1)	ITIH4	Q14624					↓-7.53
49	U231	Inter-alpha-trypsin inhibitor heavy chain H4 (2)			↓-1.79	↓-8.89	↓-4.5	↓-4.06	
50	U233	Inter-alpha-trypsin inhibitor heavy chain H4 (3)			↓-2.47	↓-11.54	↓-4.97	↓-3.71	↓-10.28
51	U55	Kininogen-1	KNG1	P01042		↑2.87§			
52	U72	Pancreatic alpha-amylase	AMY2A	P04746		↓-1.51	↓-2.69		↓-2.05
53	U253	Protein AMBP (1)	AMBP	P02760					↓-3.04§
54	U259	Protein AMBP (2)							↓-2.79§
		Kidney secretory and structural proteins							
55	U330	Basement membrane-specific heparan sulfate proteoglycan core protein (1)	HSPG2	P98160			↓-6.01	↓-4.3	
56	U332	Basement membrane-specific heparan sulfate proteoglycan core protein (2)				↓-10.11	↓-3.05	↓-3.16	↓-16.35
57	U150	Gelsolin	GSN	P06396	↓-1.91		↑2.23		
58	U09	Polymeric immunoglobulin receptor (1)	PIGR	P01833			↓-4.19	↓-2.38	
59	U10	Polymeric immunoglobulin receptor (2)				↓-2.26	↓-3		

Note:

↑ increased excretion, ↓ decreased excretion

§ proteins specific to the corresponding class of SLE/LN only

* *p* value ≤ 0.05, average abundance ratio ≤ -1.5 or ≥ 1.5, except for the following proteins/isoforms which were cut off at higher average abundance ratios:

Proteins/isoforms	**Average abundance ratio**	
	≥	≤
Alpha-1B-glycoprotein	10	-10
Haptoglobin	10	-10
Albumin	20	-20
Transferrin	30	-30
Zinc-alpha-2-glycoprotein	10	-10
Alpha-1-antitrypsin	20	-20

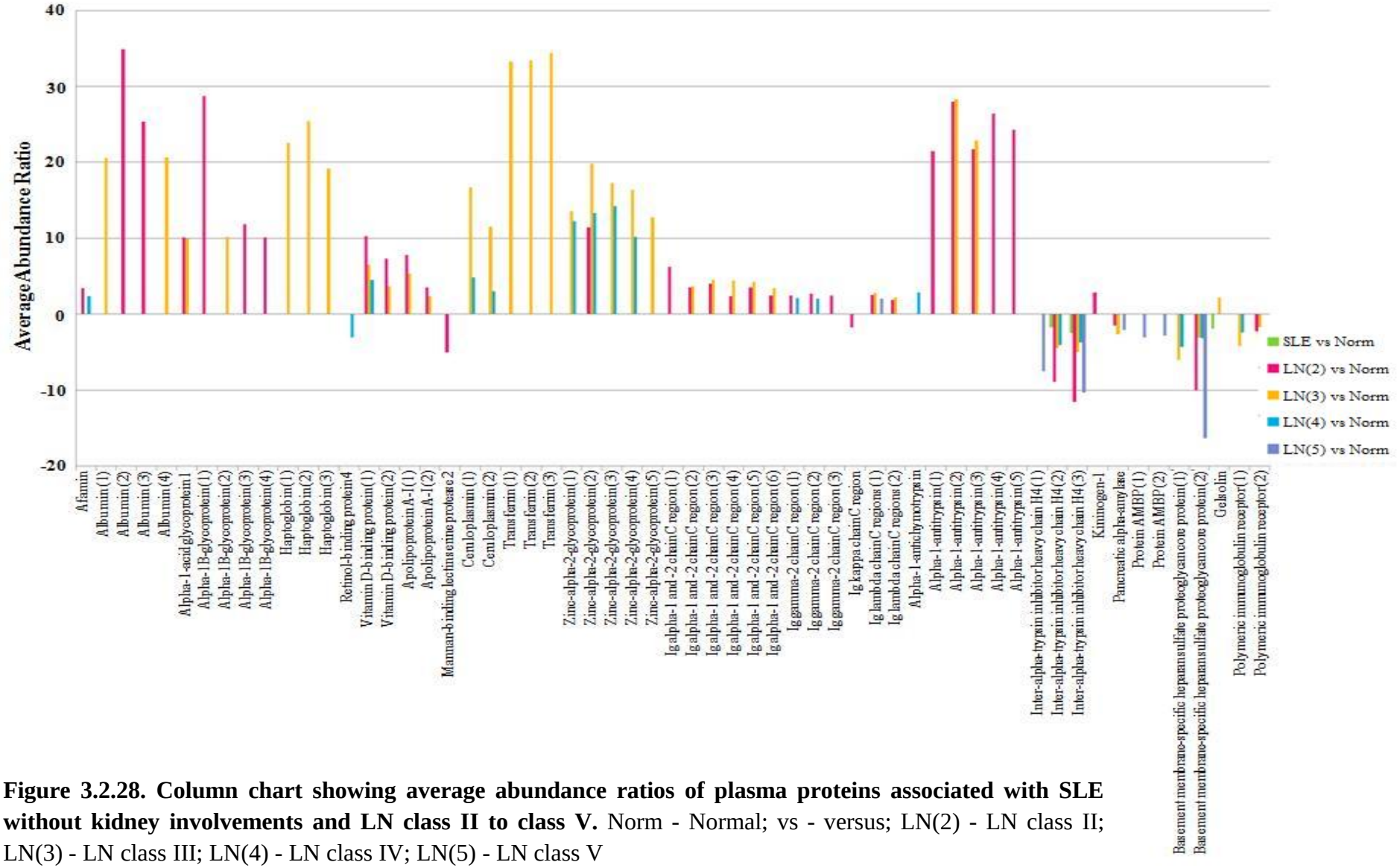


Figure 3.2.28. Column chart showing average abundance ratios of plasma proteins associated with SLE without kidney involvements and LN class II to class V. Norm - Normal; vs - versus; LN(2) - LN class II; LN(3) - LN class III; LN(4) - LN class IV; LN(5) - LN class V

Table 3.2.12. Summary of urine proteins associated with non-renal SLE and each class of lupus nephritis (LN).

No.	Protein name	Gene name	Accession (Swiss-Prot)	Molecular weight (Da)	Average Abundance Ratio* SLE (Non-LN) (n=5)	LN Class II (n=3)	LN Class III (n=3)	LN Class IV (n=5)	LN Class V (n=3)
	Serum constitutional and transport proteins								
1	Afamin	*AFM*	P43652	69,069		↑		↑	
2	Albumin	*ALB*	P02768	69,367 47,360		↑	↑		
3	Alpha-1-acid glycoprotein 1	*AGP1*	P02763	23,512		↑	↑		
4	Alpha-1B-glycoprotein	*A1BG*	P04217	54,254 40,718		↑	↑		
5	Haptoglobin	*HP*	P00738	45,205		↑	↑		
6	Retinol-binding protein 4	*RBP4*	P02753	23,010				↓§	
7	Vitamin D-binding protein	*VDB*	P25311	52,964 39,542		↑	↑	↑	
	Lipoprotein metabolism								
8	Apolipoprotein A-I	*ApoA-I*	P02647	30,778		↑	↑		
	Complement fractions								
9	Mannan-binding lectin serine protease 2	*MASP2*	O00187	75,702 20,629		↓§			
	Metal binding proteins								
10	Ceruloplasmin	*CP*	P00450	122,205			↑	↑	
11	Transferrin	*TF*	P02787	77,064		↑	↑		
12	Zinc-alpha-2-glycoprotein	*AZGP1*	P25311	34,259		↑	↑	↑	
	Immunoglobulins and other immune proteins								
13	Ig alpha-1 and -2 chain C region	*IGHA1* & *IGHA2*	P01876 & P01877	37,655		↑	↑		
14	Ig gamma-2 chain C region	*IGHG2*	P01859	35,901		↑		↑	
15	Ig kappa chain C region	*IGKC*	P01834	11,609		↓§			
16	Ig lambda chain C regions	*IGLC1*	P0CG04	11,348		↑	↑		↑
	Proteases and inhibitors								
17	Alpha-1-antichymotrypsin	*AACT*	P01011	47,651 24,034 10,717				↑§	
18	Alpha-1-antitrypsin	*AAT*	P01009	46,737 40,263 34,755		↑	↑		
19	Inter-alpha-trypsin inhibitor heavy chain H4	*ITIH4*	Q14624	103,357 101,241	↓	↓	↓	↓	↓
20	Kininogen-1	*KNG1*	P01042	71,957 47,883		↑§			
21	Pancreatic alpha-amylase	*AMY2A*	P04746	57,707		↓	↓		↓
22	Protein AMBP	*AMBP*	P02760	38,999					↓§
	Kidney secretory and structural proteins								
23	Basement membrane-specific heparan sulfate proteoglycan core protein	*HSPG2*	P98160	468,830		↓	↓	↓	↓
24	Gelsolin	*GSN*	P06396	85,698 80,641 81,941	↓		↑		
25	Polymeric immunoglobulin receptor	*PIGR*	P01833	83,284		↓	↓	↓	

Note:

↑ increased excretion, ↓ decreased excretion

§ proteins specific to the corresponding class of SLE/LN only

* p value ≤ 0.05, average abundance ratio ≤ -1.5 or ≥ 1.5, except for the following proteins/isoforms which were cut off at higher average abundance ratios:

Proteins/isoforms	**Average abundance ratio**	
	≥	≤
Alpha-1B-glycoprotein	10	-10
Haptoglobin	10	-10
Albumin	20	-20
Transferrin	30	-30
Zinc-alpha-2-glycoprotein	10	-10
Alpha-1-antitrypsin	20	-20

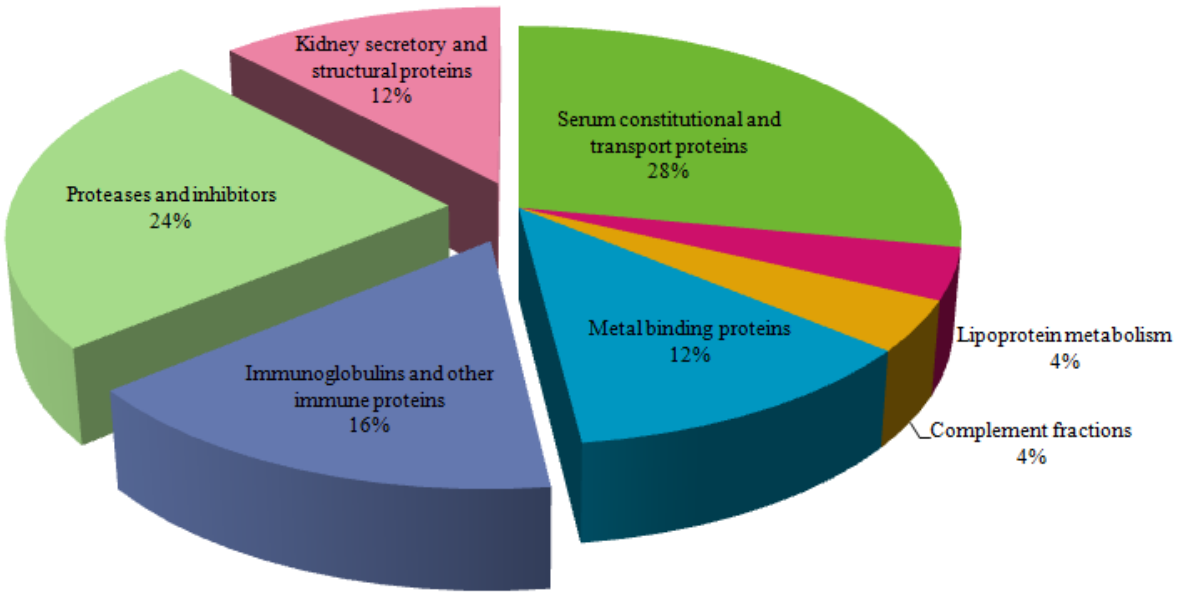


Figure 3.2.29. Functionalities of SLE-associated urine proteins of all patient groups.

	SLE (non-LN)	LN class II	LN class III	LN class IV	LN class V
Increased excretion		Afamin Albumin Alpha-1-acid glycoprotein 1 Alpha-1B-glycoprotein Haptoglobin Vitamin D-binding protein Apolipoprotein A-I Transferrin Zinc-alpha-2-glycoprotein Ig alpha-1 and -2 chain C region Ig gamma-2 chain C region Ig lambda chain C regions Alpha-1-antitrypsin Kininogen-1*	Albumin Alpha-1-acid glycoprotein 1 Alpha-1B-glycoprotein Haptoglobin Vitamin D-binding protein Apolipoprotein A-I Ceruloplasmin Transferrin Zinc-alpha-2-glycoprotein Ig alpha-1 and -2 chain C region Ig lambda chain C regions Alpha-1-antitrypsin Gelsolin	Afamin Vitamin D-binding protein Ceruloplasmin Zinc-alpha-2-glycoprotein Ig gamma-2 chain C region Alpha-1-antichymotrypsin*	Ig lambda chain C regions
Decreased excretion	Inter-alpha-trypsin inhibitor heavy chain H4 Gelsolin	Mannan-binding lectin serine protease 2* Ig kappa chain C region* Inter-alpha-trypsin inhibitor heavy chain H4 Pancreatic alpha-amylase Basement membrane-specific heparan sulfate proteoglycan core protein Polymeric immunoglobulin receptor	Inter-alpha-trypsin inhibitor heavy chain H4 Pancreatic alpha-amylase Basement membrane-specific heparan sulfate proteoglycan core protein Polymeric immunoglobulin receptor	Retinol-binding protein 4* Inter-alpha-trypsin inhibitor heavy chain H4 Basement membrane-specific heparan sulfate proteoglycan core protein Polymeric immunoglobulin receptor	Inter-alpha-trypsin inhibitor heavy chain H4 Pancreatic alpha-amylase Protein AMBP* Basement membrane-specific heparan sulfate proteoglycan core protein

Figure 3.2.30. Summary of abnormally regulated urine proteins involved in each group of SLE patients (lupus nephritis and without kidney involvement). Proteins labelled with (*) are group-specific proteins.

Table 3.2.13. Comparison of urine protein isoform levels between patients with active LN class IV and those in remission (formerly LN class IV patients).

No.	Protein No.	Protein name	Gene name	Accession (Swiss-Prot)	Average Abundance Ratio*	
					LN Class IV	LN in Remission
		Serum constitutional and transport proteins				
1	U11	Afamin	AFM	P43652	↑2.4†	
2		Albumin isoforms	ALB	P02768	↑<20	↑<20
3	U129	Alpha-1-acid glycoprotein 1	AGP1	P02763		↑5.21#
4	U29	Alpha-1B-glycoprotein (1)	A1BG	P04217	↑<10	↑<10
5	U341	Ferritin light chain	FTL	P02792		↑1.94#
6		Haptoglobin isoforms	HP	P00738	↑<10	↑<10
7	U336	Retinol-binding protein 4	RBP4	P02753	↑3.08†	
8	U81	Vitamin D-binding protein (1)	VDB	P25311	↑4.49	↑2.82
		Metal binding proteins				
9	U03	Ceruloplasmin (1)	CP	P00450	↑4.81	↑3
10	U04	Ceruloplasmin (2)			↑3.06	
11		Transferrin isoforms	TF	P02787	↑<30	↑<30
12	U156	Zinc-alpha-2-glycoprotein (1)	AZGP1	P25311	↑12.28	↑5.28
13	U169	Zinc-alpha-2-glycoprotein (2)			↑13.32	↑6.24
14	U170	Zinc-alpha-2-glycoprotein (3)			↑14.23	↑6.81
15	U171	Zinc-alpha-2-glycoprotein (4)			↑10.22	↑4.45
		Immunoglobulins and other immune proteins				
16	U91	Ig gamma-2 chain C region (1)	IGHG2	P01859	↑2.1	↑2.4
17	U92	Ig gamma-2 chain C region (2)			↑2.01	
18	U288	Ig lambda chain C regions (1)	IGLC1	P0CG04		↑2.44#
19	U294	Ig lambda chain C regions (2)				↑1.6#
		Proteases and inhibitors				
20	U38	Alpha-1-antichymotrypsin (1)	AACT	P01011	↑2.89	
21	U50	Alpha-1-antichymotrypsin (2)				↑2.24
22		Alpha-1-antitrypsin isoforms	AAT	P01009	↑<30	↑<30
23	U231	Inter-alpha-trypsin inhibitor heavy chain H4 (2)	ITIH4	Q14624	↓-4.06	
24	U233	Inter-alpha-trypsin inhibitor heavy chain H4 (3)			↓-3.71	↓-2.2
		Kidney secretory and structural proteins				
25	U330	Basement membrane-specific heparan sulfate proteoglycan core protein (1)	HSPG2	P98160	↓-4.3	
26	U332	Basement membrane-specific heparan sulfate proteoglycan core protein (2)			↓-3.16	↓-2.48
27	U09	Polymeric immunoglobulin receptor (1)	PIGR	P01833	↓-2.38	↓-2.49
28	U10	Polymeric immunoglobulin receptor (2)				↓-2.19

Note:

↑ increased excretion, ↓ decreased excretion

† abnormally excreted by patients with LN class IV only

abnormally excreted by LN patients in remission only

* p value ≤ 0.05, average abundance ratio ≤ -1.5 or ≥ 1.5, except for the following proteins/isoforms which were cut off at higher average abundance ratios:

Proteins/isoforms	Average abundance ratio	
	≥	≤
Alpha-1B-glycoprotein	10	-10
Haptoglobin	10	-10
Albumin	20	-20
Transferrin	30	-30
Zinc-alpha-2-glycoprotein	10	-10
Alpha-1-antitrypsin	20	-20

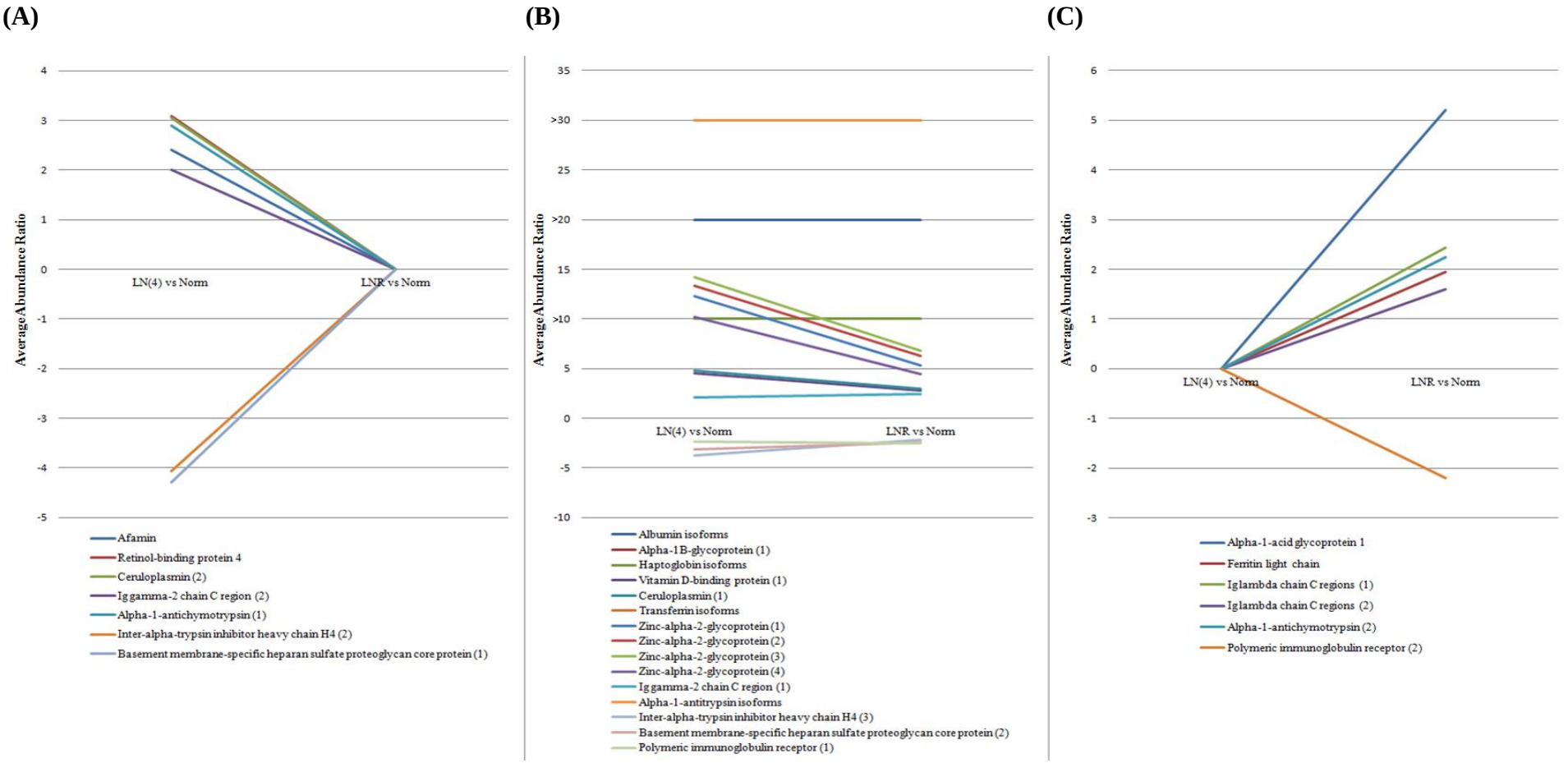


Figure 3.2.31. Changes of urine protein isoform levels between active LN class IV and LN in remission. The protein isoform levels are the average abundance ratios, whereby the protein isoform levels for both disease states were measured relative to those of normal individuals. 0 indicates normal level and X-axis denotes disease states. (**A)** shows the presence of abnormally regulated protein isoforms in patients with active LN class IV but became normal in patients in remission whilst **(C)** shows the otherwise. **(B)** shows the persistence of abnormal levels of protein isoforms in both disease states. LN(4) – LN class IV; LNR – LN in remission; vs – versus; Norm – Normal individuals

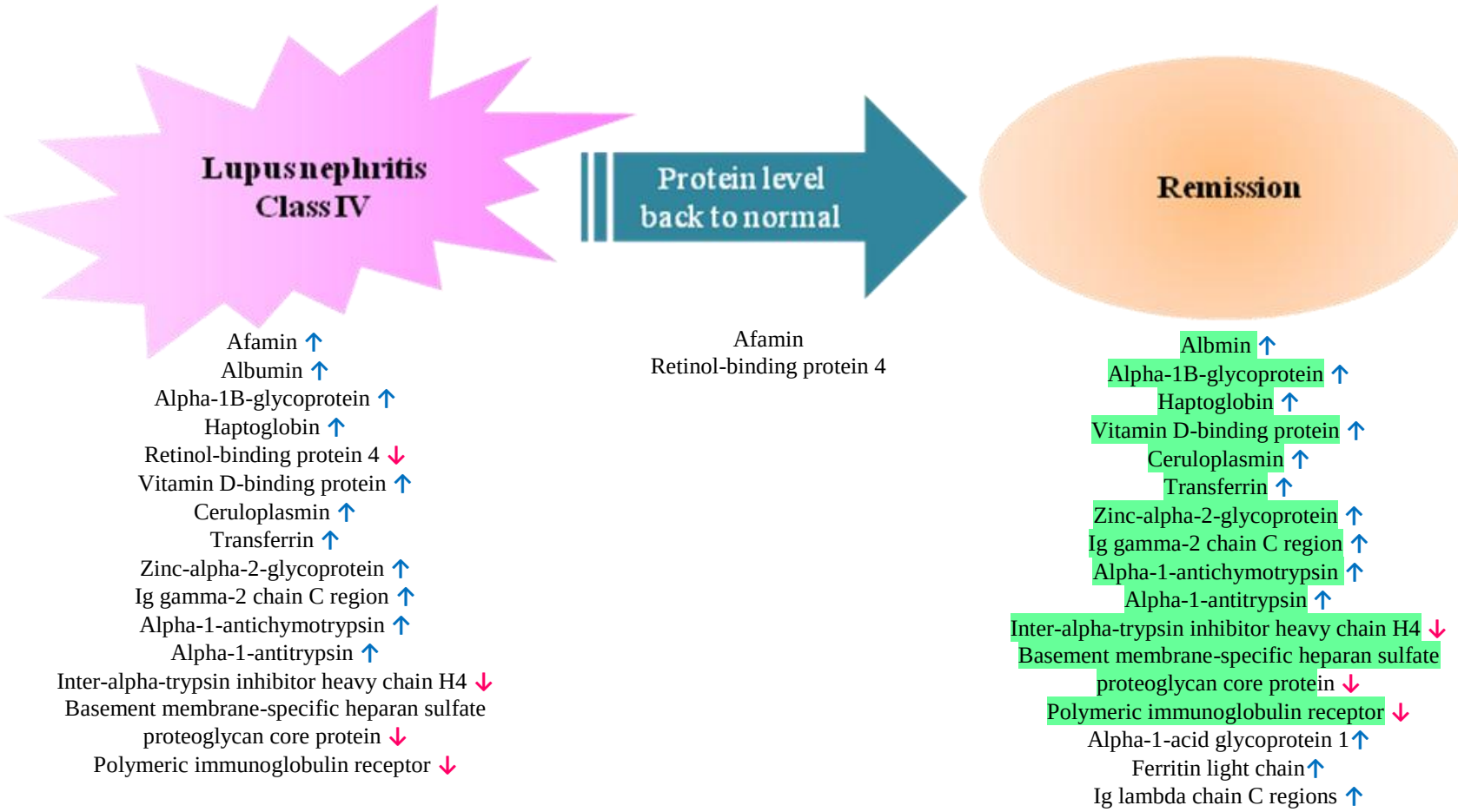


Figure 3.2.32. Changes of urine protein levels along transition from lupus nephritis (LN) class IV to remission. Most proteins abnormally excreted in LN class IV still remained at their abnormal levels in urine upon achieving remission (highlighted in green), except for afamin and retinol-binding protein 4. Additional proteins, i.e. alpha-1-acid glycoprotein 1, ferritin light chain and Ig lambda chain C regions, which were at normal urine level in active disease state appeared to be abnormally excreted in urine during remission.

3.2.7 Protein-protein interaction of SLE-associated proteins

Protein-protein functional linkages and interactions were predicted and constructed using IPA software (Ingenuity Systems). Protein-protein interaction (PPI) networks were constructed for each SLE group based on the group-associated proteins (**Table 3.2.9**). Only plasma proteins were used for this study purpose as some of the abnormally excreted proteins in patients' urine might be merely due to impaired filtration of the glomerulus and did not represent the pathophysiological role of the proteins in the disease. The interactions between SLE-associated plasma proteins and other related proteins included expression regulation, inhibition, activation, and protein-DNA interaction.

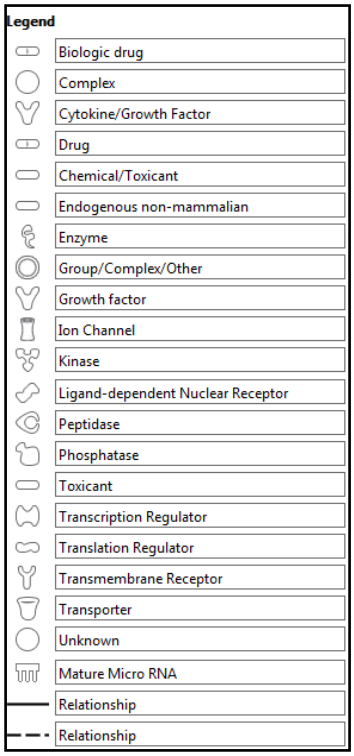
Protein-protein interaction (PPI) analysis results of SLE patients without kidney manifestations gave only one network with the score of 20 and this pathway involved all group-associated proteins. The network mainly functions in cellular movement, inflammatory response, and cancer (**Figure 3.2.34**).

As for LN class II, four networks were generated and the top scored network involved group-associated proteins such as clusterin (CLU), haemopexin (HPX), and complement factor B (CFB), which interacted with other related proteins in cellular movement, haematological system development and function, and immune cell trafficking (**Figure 3.2.35**). Network 2 illustrates how C4-binding protein alpha chain (C4BPA) was connected with histone-lysine N-methyltransferase (EZH2) to function in developmental disorder, hereditary disorder, cellular movement. Vitamin D-binding protein (GC) interacted with annexin A2 (ANXA2) and CD44 in Network 3, which functions in cell-to-cell signaling and interaction, connective tissue development and function, and tissue development. Network 4, which was responsible in cell morphology, humoral immune response, and inflammatory response was constituted by interaction between Ig gamma chain C region (IGHG1) and three other related proteins.

Protein-protein interaction (PPI) networks of LN class III mainly involved lipid metabolism, small molecule biochemistry, molecular transport, and vitamin and mineral metabolism (score 32) (**Figure 3.2.36**). Interaction between ficolin-3 (FCN3) and mannan-binding lectin serine protease 1 and 2 (MASP1 and MASP2) was also seen in Network 2, which functions in humoral immune response, inflammatory response, and cardiovascular disease.

Four PPI networks were constructed for LN class IV, with network implicated in cell cycle, cell death and survival, and tumour morphology (**Figure 3.2.37**) being the top ranked network (score 11). Network 2, which involved interaction of alpha-2-HS-glycoprotein (ASHG) with hepatocyte nuclear factor 1-alpha (HNF1A) functions in connective tissue development and function, organ development, and skeletal and muscular system development and function. Interaction between ceruplasmin (CP) and other related proteins forms Network 3, while Network 4 resulted from interaction of C4-binding protein (C4B) with related proteins.

The top scored (score 17) PPI network of LN class V was constituted by an orchestra of LN class V-associated proteins, including haptoglobin (HP), fibrinogen chains (FGB and FGG), and CFB, with other related proteins such as tumour necrosis factor (TNF) and interleukin-6 (IL-6) to function in connective tissue disorders, inflammatory disease, and skeletal and muscular disorders (**Figure 3.2.38**). Histidine-rich glycoprotein (HRG) interacted with FcgammaRIa (FCGR1A) and performed in cancer, haematological disease, and respiratory disease in Network 2. Interaction between FCN3 and MASP, which was seen for LN class III also occurs in LN class V. Likewise, networks involving GC and IGHG1 which appeared in LN class II are also found in LN class V.



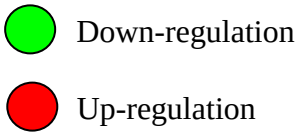
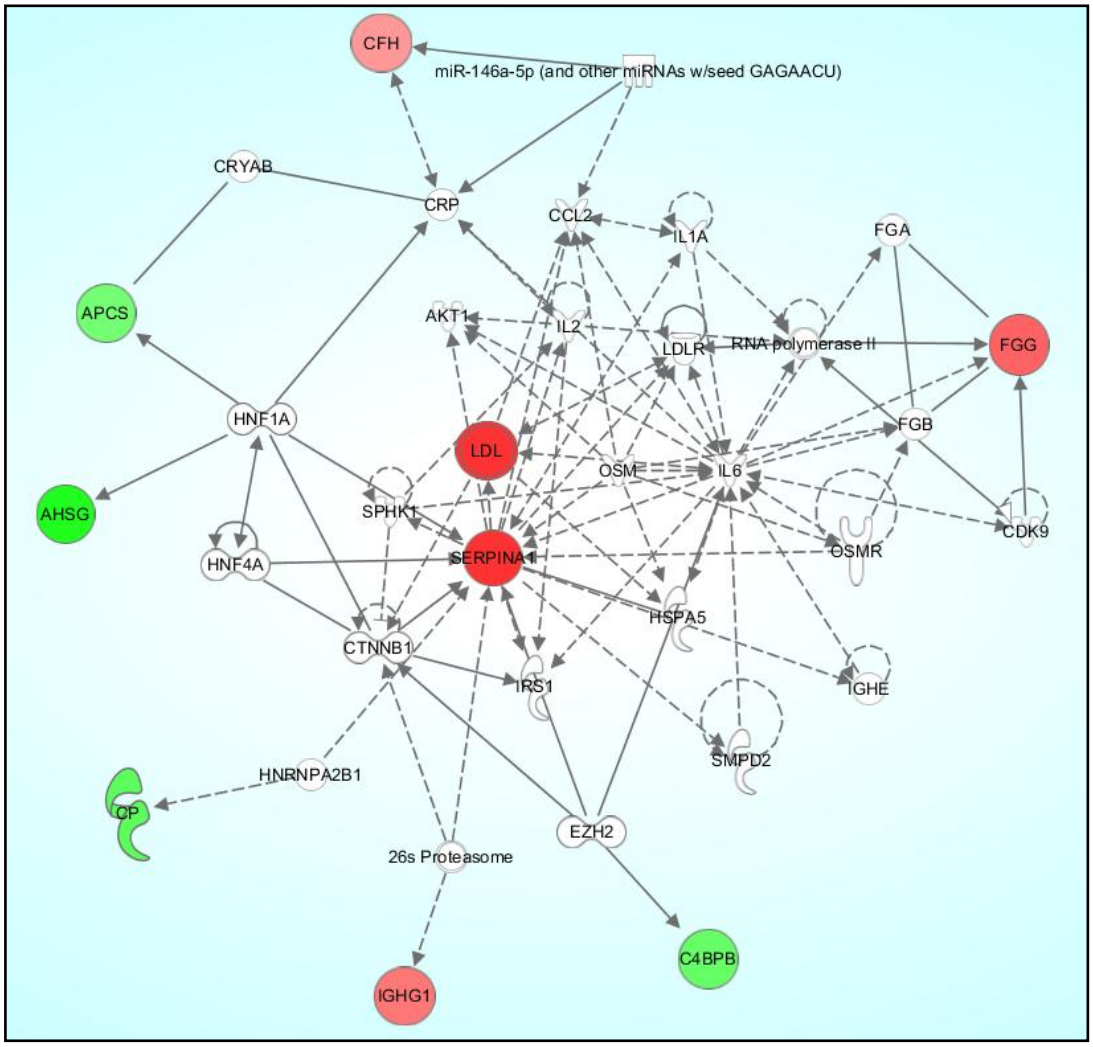


Figure 3.2.33. Symbols and labels that would be applied on the following figures (**Figure 3.2.34** to **Figure 3.2.38**).

(i) SLE without kidney manifestation



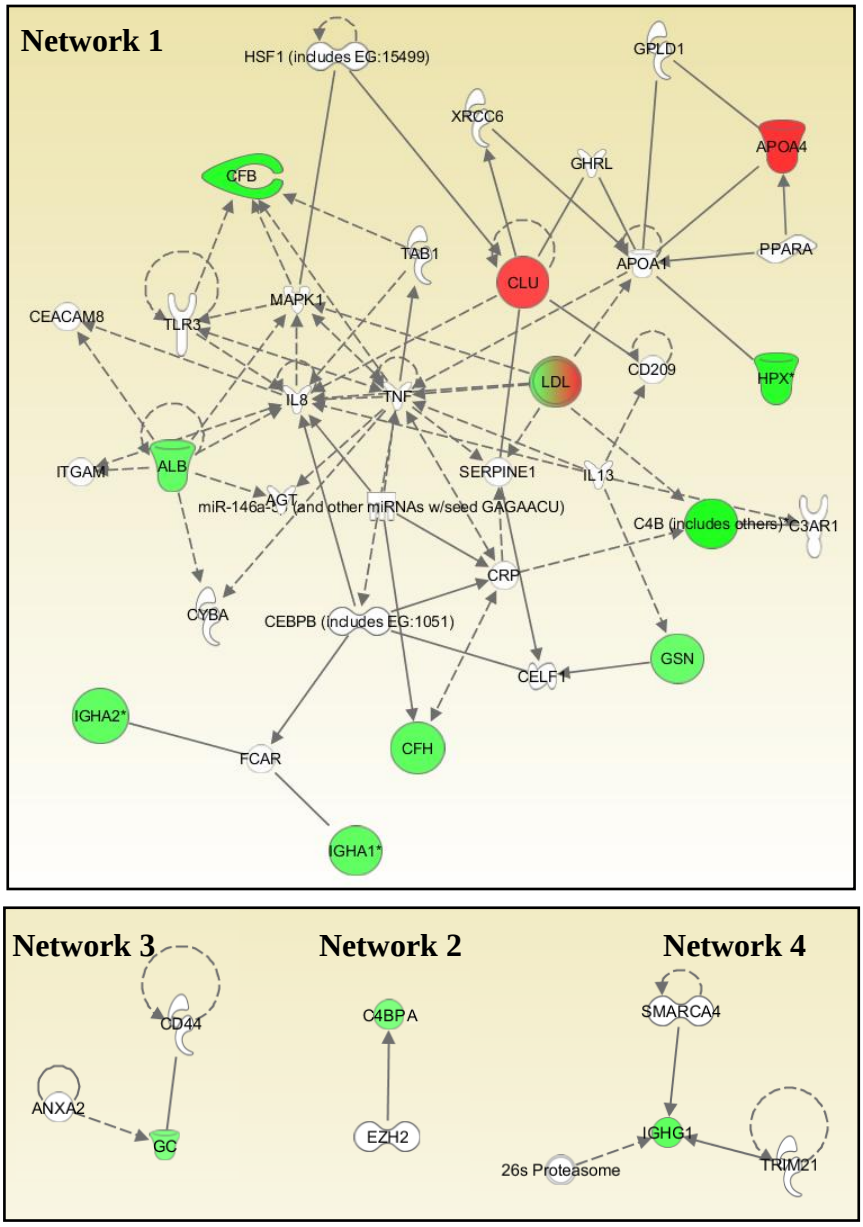
Score: 20

Top Functions:

Cellular movement, Inflammatory response, Cancer

Figure 3.2.34. Protein-protein interaction networks of SLE group without kidney manifestation.

(ii) LN class II



Score: 22

Top Functions:

Cellular movement, Hematological system development and function, Immune cell trafficking

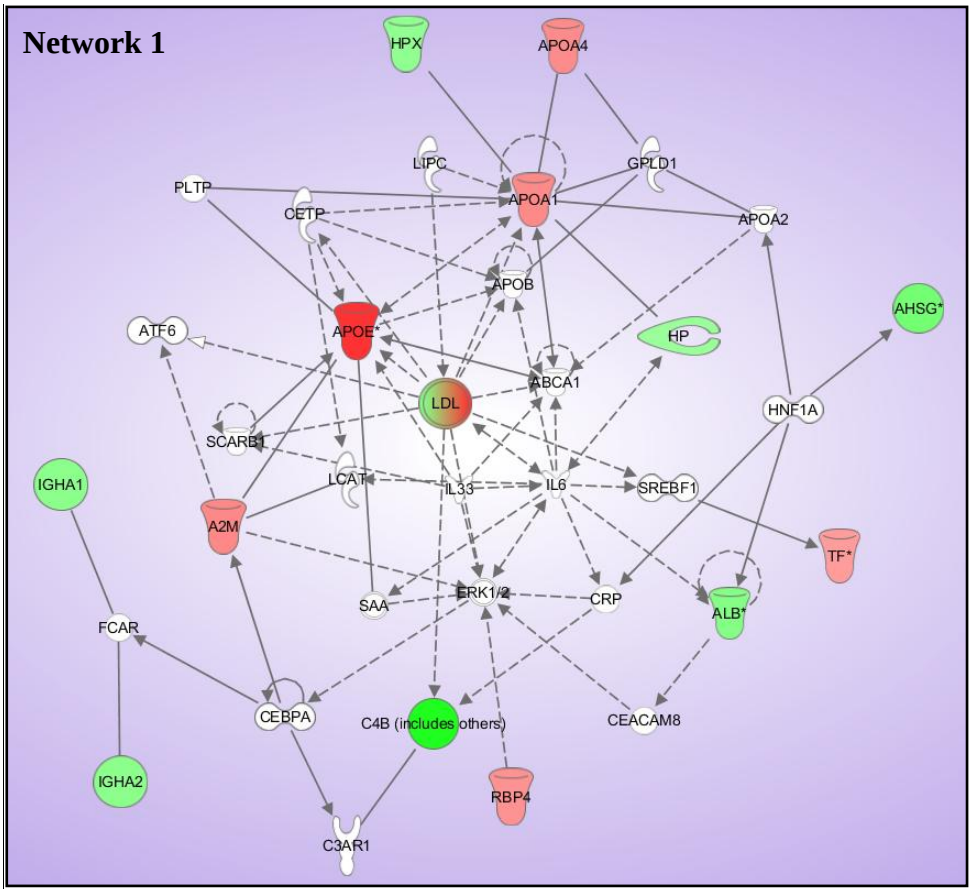
Network 2
Score: 3
Top functions: Developmental Disorder, Hereditary Disorder, Cellular Movement

Network 3
Score: 2
Top functions: Cell-To-Cell Signaling and Interaction, Connective Tissue Development and Function, Tissue Development

Network 4
Score: 2
Top functions: Cell Morphology, Humoral Immune Response, Inflammatory Response

Figure 3.2.35. Protein-protein interaction networks of LN class II.

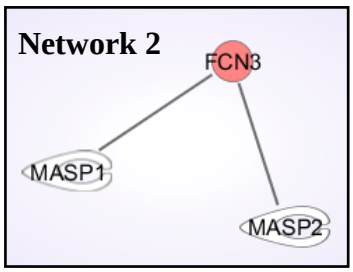
(iii) LN class III



Score: 32

Top Functions:

Lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism



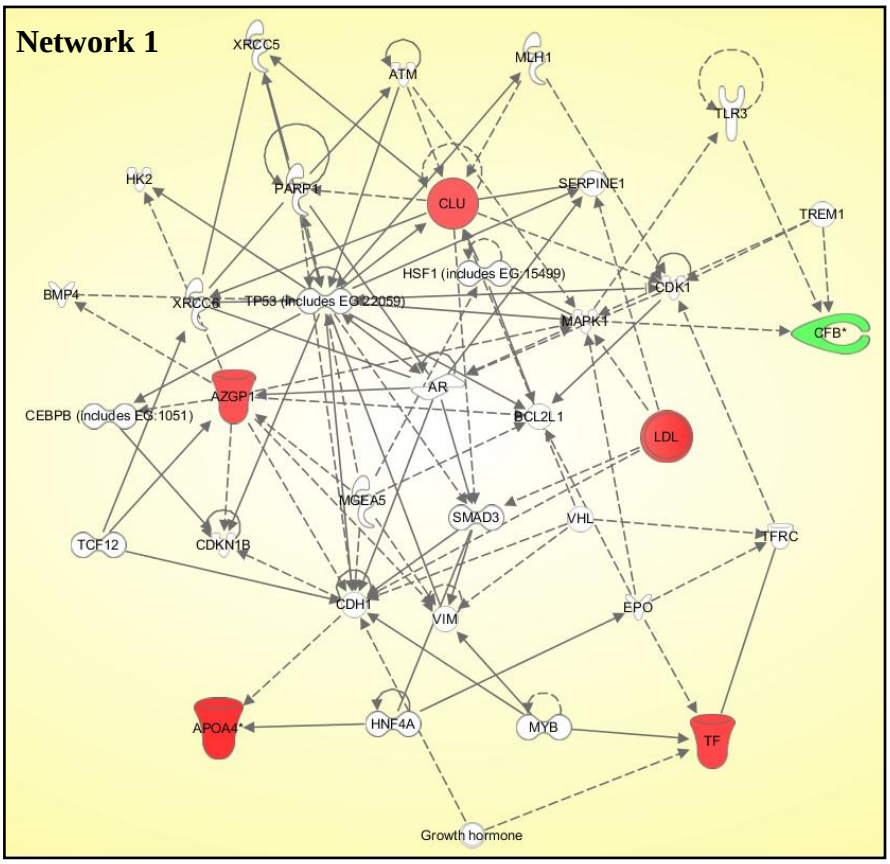
Score: 2

Top Functions:

Humoral immune response, inflammatory response, cardiovascular disease

Figure 3.2.36. Protein-protein interaction networks of LN class III.

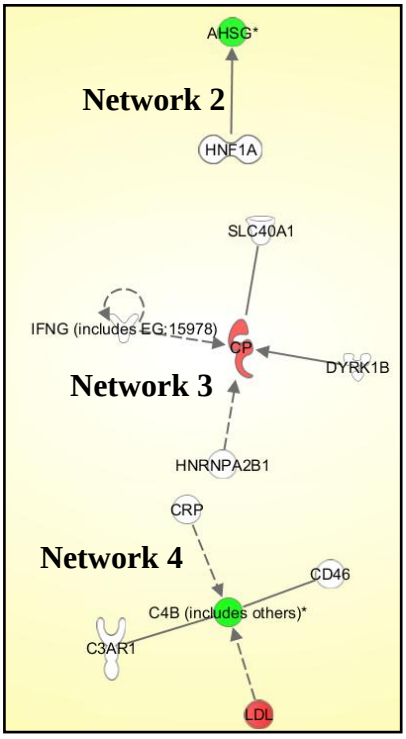
(iv) LN class IV



Score: 11

Top Functions:

Cell cycle,
Cell death and survival,
Tumor morphology



Network 2

Score: 3

Top Functions: Connective tissue development and function, organ development, skeletal and muscular system development and function

Network 3

Score: 2

Top Functions: Molecular transport, cellular function and maintenance, small molecular biochemistry

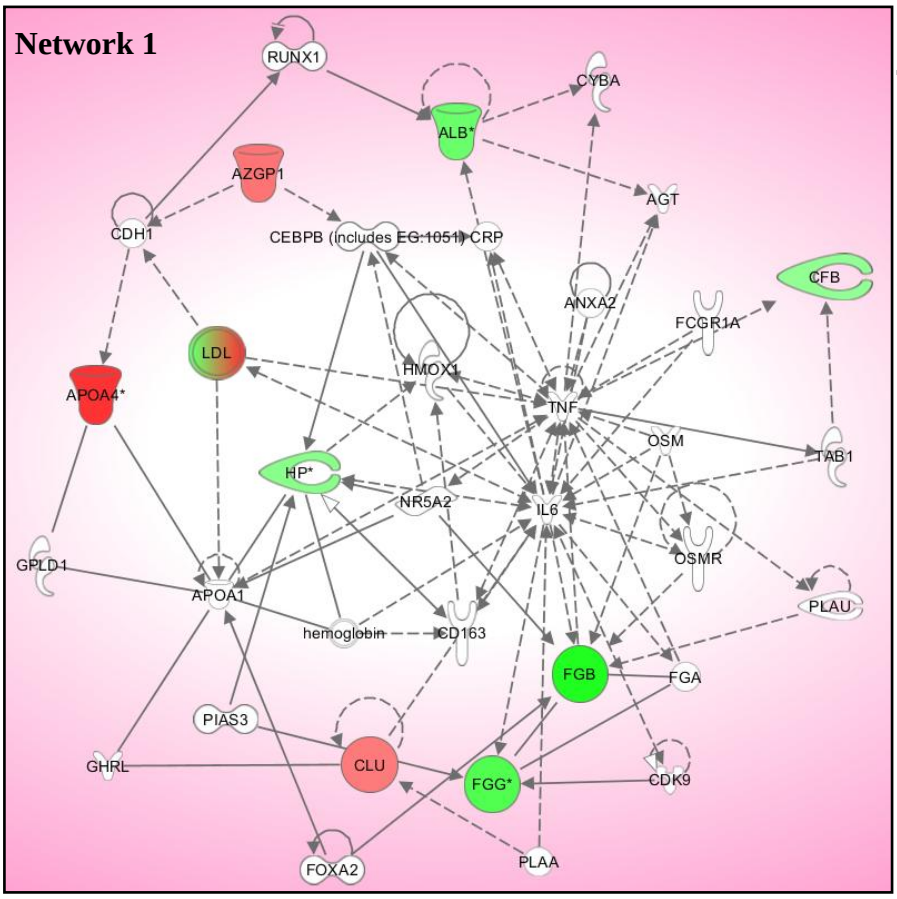
Network 4

Score: 2

Top Functions: Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking

Figure 3.2.37. Protein-protein interaction networks of LN class IV.

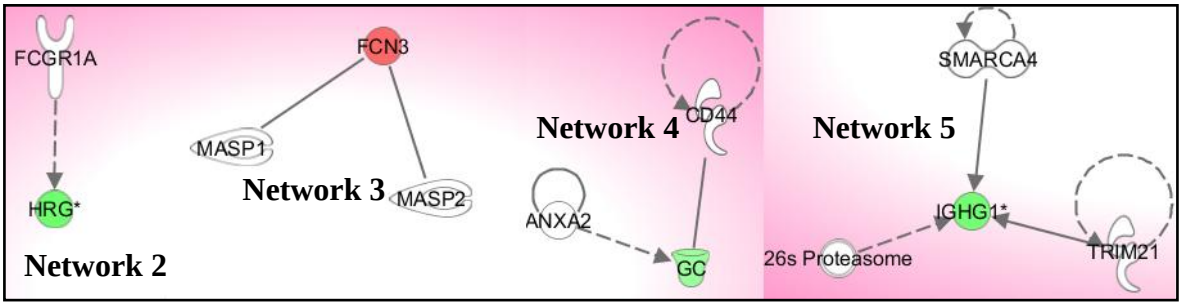
(v) LN class V



Score: 17

Top Functions:

Connective tissue disorders, Inflammatory disease, Skeletal and muscular disorders



Network 2
Score: 3
Top Functions:
Cancer, Hematological disease, respiratory disease

Network 3
Score: 2
Top Functions:
Humoral immune response, inflammatory response, cardiovascular disease

Network 4
Score: 2
Top Functions:
Cell-to-cell signaling and interaction, connective tissue development and function, tissue development

Network 5
Score: 2
Top Functions:
Cell morphology, humoral immune response, inflammatory response

Figure 3.2.38. Protein-protein interaction networks of LN class V.

3.3 Protein level changes during mycophenolate mofetil (Cellcept) treatment

Mycophenolate mofetil (MMF) (Cellcept) is one of the treatments prescribed to LN patients in the University Malaya Medical Centre (UMMC). In this study, two LN class III patients, one male and one female, were followed up along their course of mycophenolate mofetil treatment to study protein level changes in their plasma and urine. Plasma and urine samples collection was started on the day they were diagnosed having LN but before taking any treatment. Samples were then collected every time they came for doctor's visit, which was about every two months, and samples were collected for four visits. The patients were in remission when final sample collection was done. Samples were processed according to the same procedures mentioned in previous sections and 2D-DIGE was carried out based on standard protocol. Image analysis was performed to identify protein spots which had abundance level changes along the treatment. Protein spots having average abundance ratios of < or > 1.0 on 5th visit compared to 1st visit (before treatment), with p value of ≤ 0.05, and showing increasing or decreasing trend in protein levels were selected for trypsin in-gel digestion and LC-MS/MS analysis. These protein spots might not show smooth increasing or decreasing trends, rather, fluctuation of protein levels was observed along the treatment (**Figure 3.3.1)**. Finally, protein identification was done.

3.3.1 Plasma proteins

Figure 3.3.2 shows plasma protein spots, including 27 HAP-depleted protein spots and 12 HAP spots, which fulfilled the criteria mentioned above. However only 11 HAP-depleted protein spots and 7 HAP spots were able to be identified and the results are listed in **Table 3.3.1**. Protein spots possessing hits with very low scores as well as low number of spectra and distinct peptides were not omitted from the results and their identities were verified by matching their spot locations on the 2-DE gel with some established protein profiles (Fountoulakis *et al.*, 2004, Herosimczyk *et al.*, 2006, Desrosiers *et al.*, 2007, Candiano *et al.*, 2010). Generally, 10 plasma proteins were involved in level changes during MMF treatment. The graphs showing abundance level trend of all MMF treatment-associated plasma protein spots were illustrated in **Figure 3.3.3**. Three isoforms of angiotensinogen were observed, where two of these isoforms demonstrated decreasing trends while one showing increased trend, albeit with some

fluctuations. Apolipoproteins, which were apolipoprotein A-IV and apolipoprotein E, decreased in levels along the treatment. Contrariwise, MMF consumption caused both ceruloplasmin and complement C4-A & B to increase upon the 5th hospital visit. Fibrinogen gamma chain involved two isoforms with increasing trends. For gelsolin, a general decreasing trend was noticed, although the level fluctuated up at the 4th visit and went down again at the 5th. A total of six isoforms were identified for haptoglobin, whereby all of these demonstrated the similar decreasing patterns. Similar decreasing trend was also applied to serum albumin. Tetranectin level, on the other hand, drop drastically in the first 2 months (before 1st visit) after MMF consumption but increased gradually after that, with its level being 1.29 fold higher than the initial level at the 5th visit.

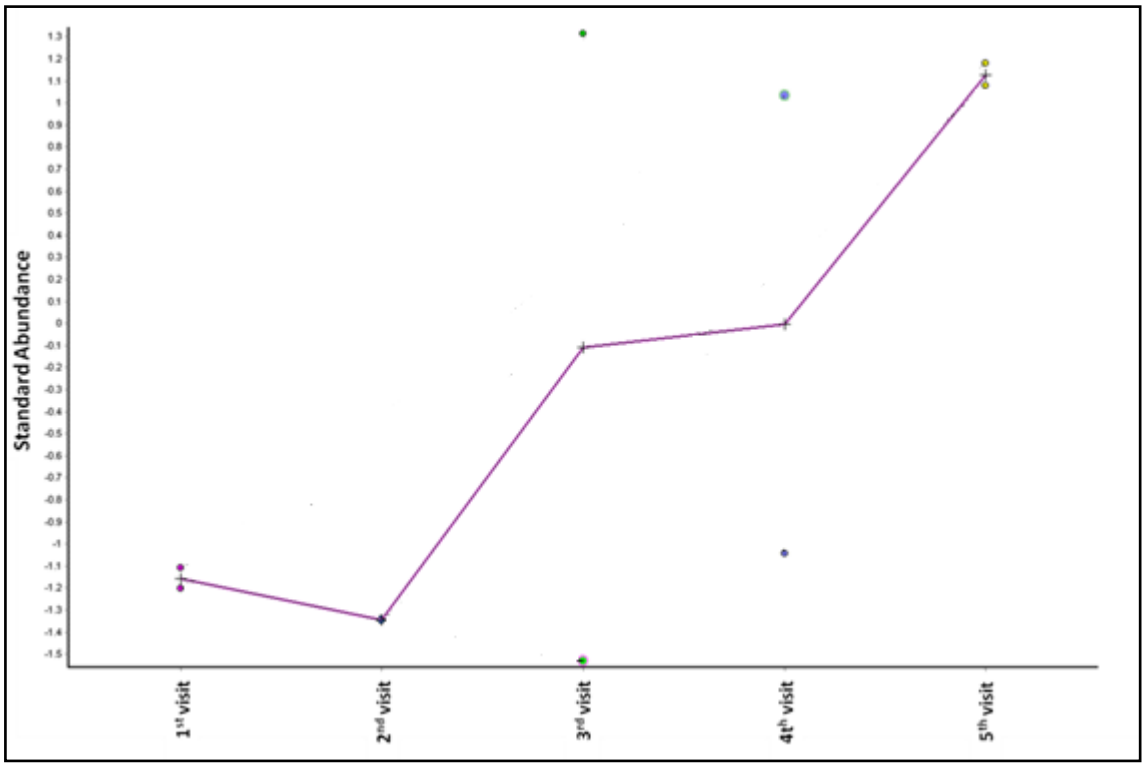


Figure 3.3.1. Example showing trend of protein abundance level from 1st to 5th visits. The coloured spots indicate the standard abundance of a particular protein spot of each patient. The average of protein standard abundance at each doctor visit was indicated with (+) and linked with a purple line. Protein spots exhibiting such trend, i.e. having ≤ -1.0 or ≥ 1.0 of average abundance ratio on 5th visit compared with 1st visit, were selected for identification with LC-MS/MS.

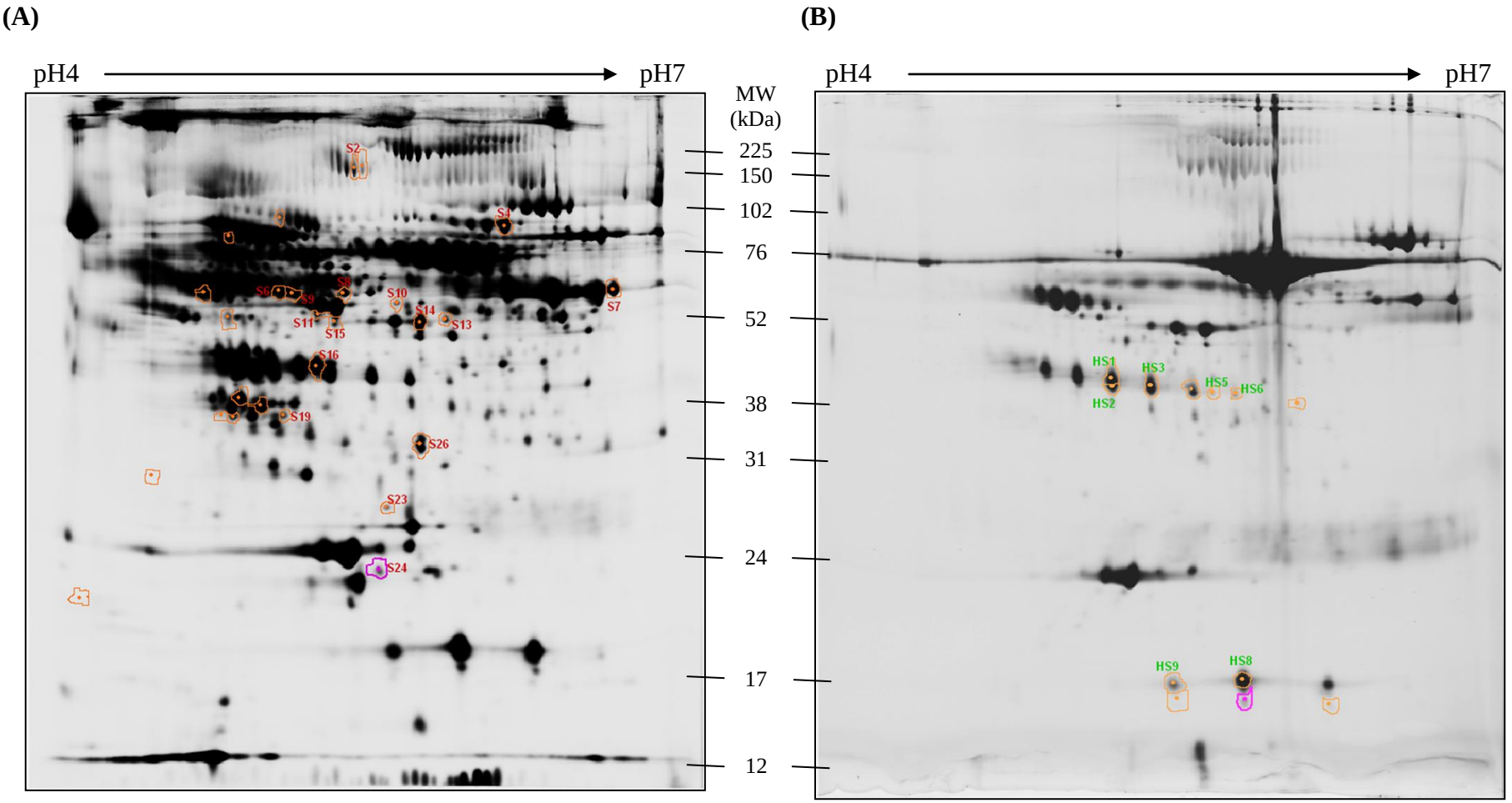


Figure 3.3.2. Plasma protein spots that are found to have abundance level changes in LN class III patients along receiving mycophenolate mofetil treatment. Protein of interest are circled in orange, while those labelled with red numbers on 2D-DIGE gel of low-abundant proteins **(A)**, and with green numbers on high-abundant proteins gel **(B)** are protein spots that were able to be picked for trypsin in-gel digestion and identification with LC-MS/MS.

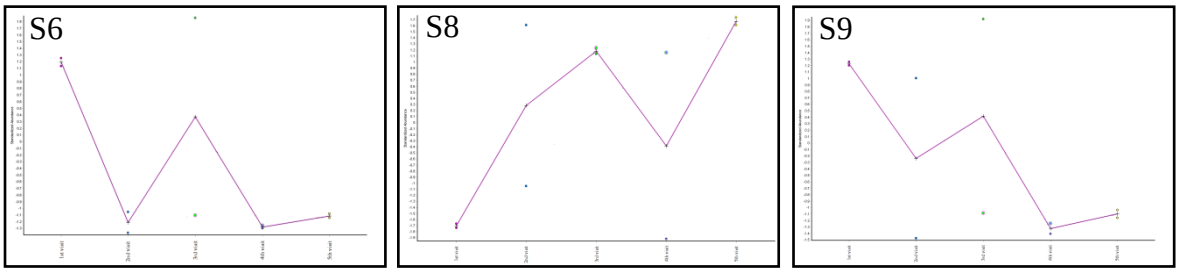
Table 3.3.1. Identification results of plasma protein spots associated with mycophenolate mofetil treatment.

Protein no.	Map no.	Protein ID	Spectra	Distinct Peptides	MS/MS search score	%AA coverage	Average Ratio[§]	T-Test *P* value*
S6	866	Angiotensinogen (1)	8	6	95.76	10	-1.32	0.039
S8	880	Angiotensinogen (2)	10	6	96.73	13	2.84	0.0017
S9	884	Angiotensinogen (3)	17	10	161.19	21	-1.34	0.037
S16	1182	Apolipoprotein A-IV	60	37	649.6	77	-2.27	0.023
S26	2421	Apolipoprotein E	55	35	566.65	80	-1.23	0.0068
S2	360	Ceruloplasmin	22	20	298.88	24	1.14	0.013
S23	1699	Complement C4-A & B	9	4	75.02	2	1.83	0.022
S11	975	Fibrinogen gamma chain (1)	6	6	63.62	20	1.32	0.027
S15	1018	Fibrinogen gamma chain (2)	14	12	177.69	32	1.18	0.022
S4	591	Gelsolin	29	20	288.3	22	-1.12	0.05
S24	1898	Tetranectin	8	7	101.94	38	1.29	0.025
HS1	1211	Haptoglobin (1)	12	11	163.28	20	-1.78	0.05
HS2	1234	Haptoglobin (2)	15	11	159.63	22	-1.8	0.043
HS3	1246	Haptoglobin (3)	16	12	189.91	23	-1.87	0.03
HS6	1267	Haptoglobin (4)	3	3	31.74	6	-1.97	0.0059
HS8	1960	Haptoglobin (5)	32	11	207.71	21	-2.04	0.014
HS9	1975	Haptoglobin (6)	20	12	177.95	21	-2.33	0.014
HS5	1256	Serum albumin	2	2	26.52	4	-1.31	0.0099

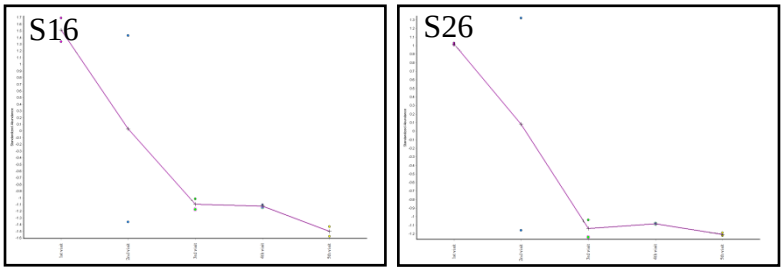
***P* value ≤ 0.05

[§]Average ratio of plasma protein level between samples collected at 5th and 1st visits.

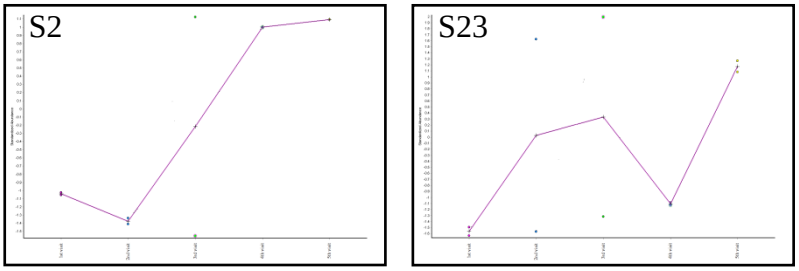
Angiotensinogen



Apolipoprotein A-IV **Apolipoprotein E**



Ceruloplasmin **Complement C4-A & B**



Fibrinogen gamma chain

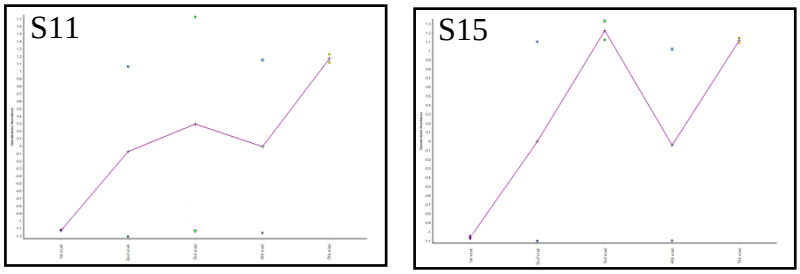
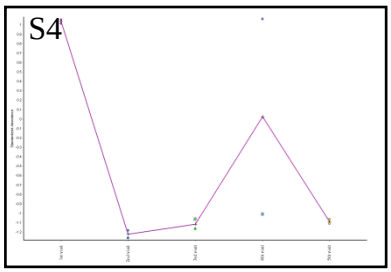
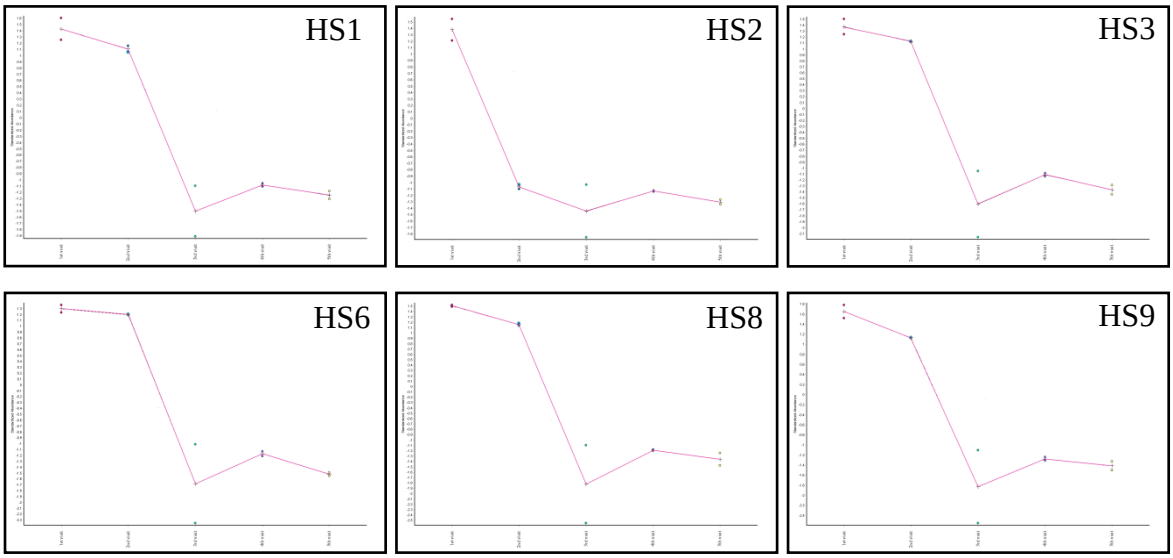


Figure 3.3.3. Changes in plasma protein levels during mycophenolate mofetil treatment. Plasma proteins which demonstrated increasing or decreasing trends of expression levels from 1st to 5th doctor visits are shown in the graphs.

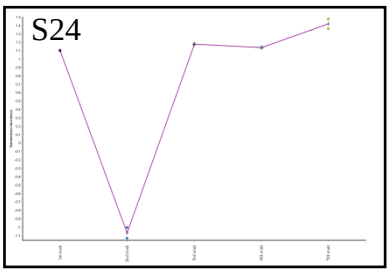
Gelsolin



Haptoglobin



Tetranectin



Serum albumin

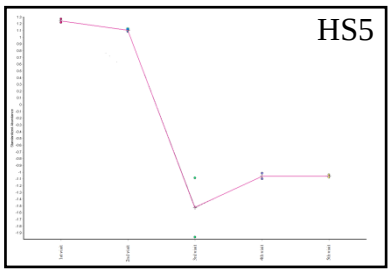


Figure 3.3.3 (Cont.). Changes in plasma protein levels during mycophenolate mofetil treatment. Plasma proteins which demonstrated increasing or decreasing trends of expression levels from 1st to 5th doctor visits are shown in the graphs.

3.3.2 Urine proteins

A total of nine urine protein spots were found to fulfil the criteria mentioned in **Section 3.3** and were shown in **Figure 3.3.4**. However only six urine protein spots were managed to be identified and the results are listed in **Table 3.3.2**. The other 3 protein spots could not produce spectrum during LC-MS/MS analysis and hence could not be identified. Protein spots possessing hits with very low scores as well as low number of spectra and distinct peptides were not omitted from the results and their identities were verified by matching their spot locations on the 2-DE gel with some established protein profiles (Fountoulakis *et al.*, 2004, Herosimczyk *et al.*, 2006, Desrosiers *et al.*, 2007, Candiano *et al.*, 2010). The six protein spots were belonged to four proteins, which were alpha-1-antichymotrypsin**,** Ig alpha-1 chain C region, lysosomal alpha-glucosidase, and serum albumin. Graphs showing abundance level trend of all MMF treatment-associated plasma protein spots were illustrated in **Figure 3.3.5**. All proteins/isoforms involved showed decreasing patterns, except for serum albumin which excretion level in the urine increased gradually and was 2.3-fold higher than the initial urinary level upon the 5th hospital visit. Lysosomal alpha-glucosidase demonstrated a great decrease along the MMF treatment, which ended up 4-fold lower than the initial urinary level at the 5th visit. Urine level of alpha-1-antichymotrypsin and Ig alpha-1 chain C region was affected by having 2 isoforms each displaying increasing trend within 8 month-course of MMF treatment.

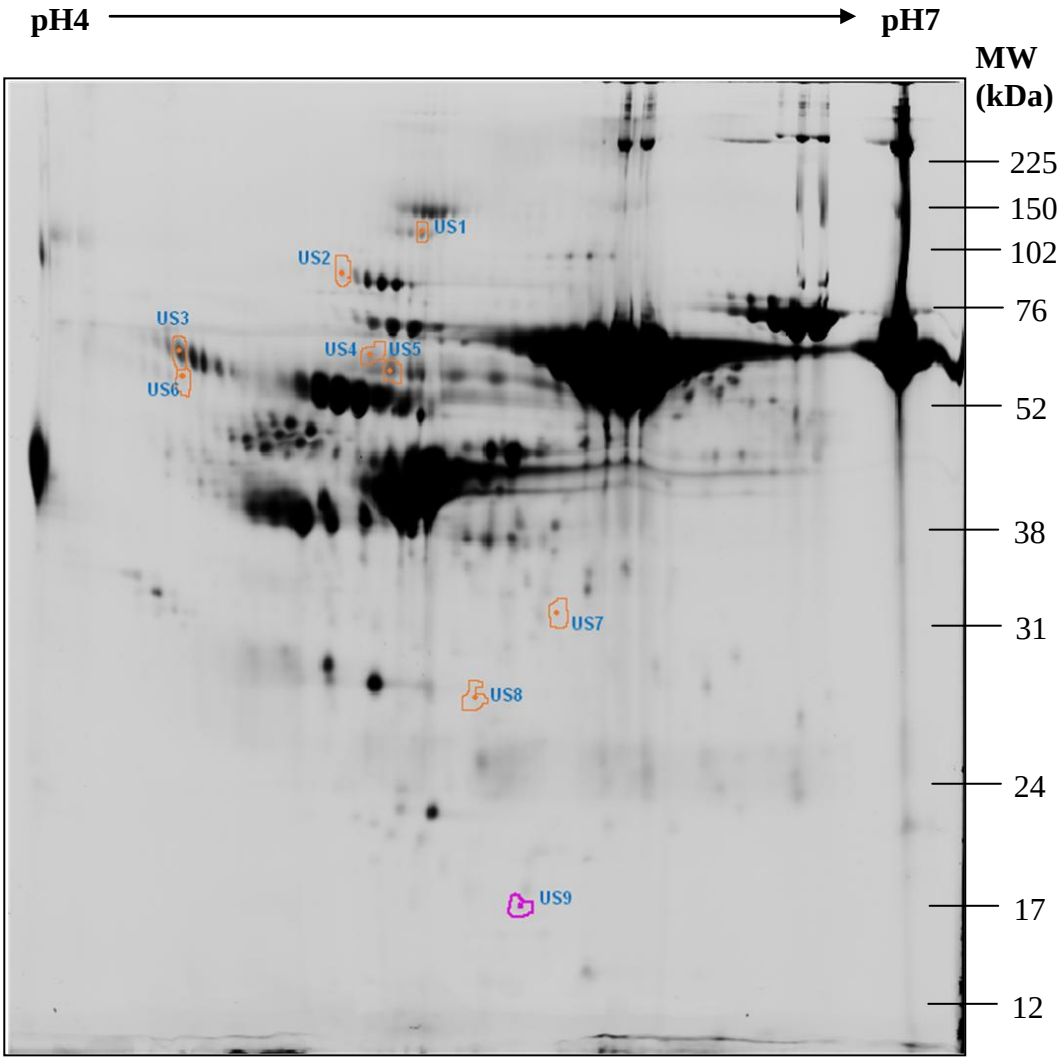


Figure 3.3.4. Urine protein spots that are found to have expression level changes in LN class III patients along receiving mycophenolate mofetil treatment. Proteins of interest are circled in orange, while those labelled with blue numbers are protein spots that were able to be picked for trypsin in-gel digestion and identification with LC-MS/MS.

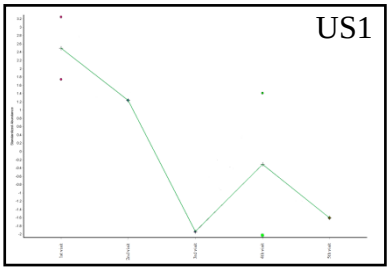
Table 3.3.2. Identification of urine protein spots associated with mycophenolate mofetil treatment by LC-MS/MS.

Protein no.	Map no.	Protein ID	Spectra	Distinct Peptides	MS/MS search score	%AA coverage	T-Test *P* value*	Average Ratio§
US1	336	Lysosomal alpha-glucosidase	1	1	13.05	1	0.05	-4
US3	631	Alpha-1-antichymotrypsin (1)	43	18	302.38	30	0.041	-2.31
US6	705	Alpha-1-antichymotrypsin (2)	13	12	204.74	26	0.033	-1.51
US4	639	Ig alpha-1 chain C region (1)	3	3	41.35	8	0.0078	-1.32
US5	673	Ig alpha-1 chain C region (2)	11	7	107.97	24	0.049	-1.99
US9	1767	Albumin	2	2	32.66	2	0.047	2.3

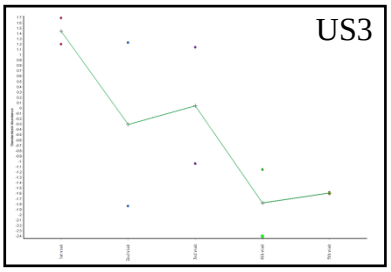
***P* value ≤ 0.05

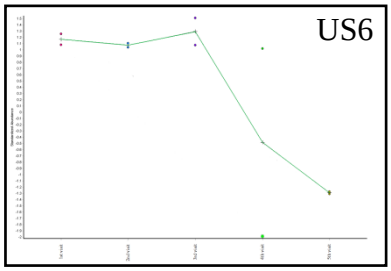
§Average ratio of urine protein level between samples collected at 5th and 1st visits.

Lysosomal alpha-glucosidase

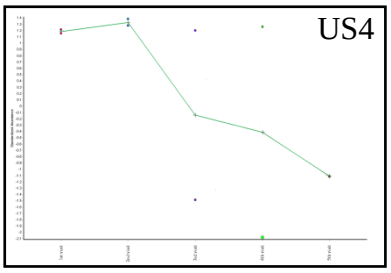


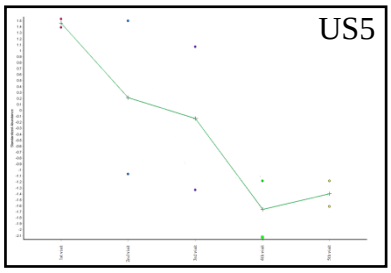
Alpha-1-antichymotrypsin





Ig alpha-1 chain C region





Serum albumin

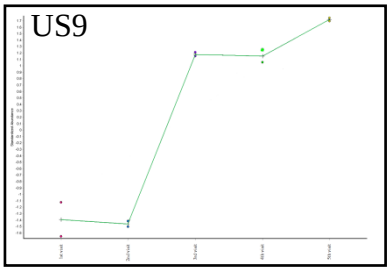


Figure 3.3.5. Changes in urine protein levels during mycophenolate mofetil treatment. Urine proteins which demonstrated increasing or decreasing trends of expression levels from 1st to 5th doctor visits are shown in the graphs.

Chapter 4

Discussion

4.1 Genomic investigations of SLE

4.1.1 Gene Polymorphisms involved in toll-like receptor/interferon signalling pathways in SLE

Systemic lupus erythematosus (SLE) is very a difficult disease to dissect pathologically. Recognition of self-nucleic acids by toll-like receptors (TLRs) TLR7 and TLR9 on plasmacytoid dendritic cells and B cells is believed to be an important step in its' pathogenesis (Barrat and Coffman, 2008). Increased anti-nuclear antibodies and production of type I IFN are both correlated with the severity of disease. *STAT4*, *TNFAIP3*, and *IRF5* are genes involved in regulating TLR/IFN signalling pathways. Single nucleotide polymorphisms (SNPs) investigated in this study, have consistently shown associations with SLE susceptibility in many populations, especially in Asians (Cai *et al.*, 2010, Ji *et al.*, 2010, Yang *et al.*, 2010, Yuan *et al.*, 2010, Hu and Ren, 2011). Single nucleotide polymorphisms (SNPs) rs7574865 and rs10168266 of *STAT4* and rs2230936 and rs3757173 of *TNFAIP3* were observed to have correlations with SLE in Malaysians generally. However, when the various ethnic groups were considered, only three SNPs (rs7574865, rs10168266 and rs2230936) of *STAT4* and *TNFAIP3* genes showed significance. Rs7574865 and rs10168266 of *STAT4* gene were significant in both Malays and Chinese. None of the SNPs seemed to influence SLE in Indians. Due to population demographics, the fewer Indians recruited in this study may have impacted the results. The SNPs of *IRF5* were not significant. This suggests that the *IRF5* genetic variants tested for in this study are not linked to SLE in our cohort and that there may be other variants that are more important.

TNFAIP3 rs2230936 and rs3757173 of chromosome 6 were not assigned to any LD block as their D' value was low. Thus their haplotype frequencies and association to SLE could not be analysed in spite the fact that they were individually associated with SLE in Malaysians in the previous section. The low D' and r^2 values indicates that the two SNPs had undergone recombination and random coupling of alleles occurred (Wray and Visscher, 2007).

Linkage disequilibrium (LD) analysis shows the importance of the two SNPs of *STAT4* gene with SLE susceptibility. The high D' value and moderate r^2 value demonstrated by *STAT4* rs7574865 and rs10168266 imply that they are inherited in the same ancestral LD block with minimal recombination. One of the SNPs was originally a monomorphic site with one allele and a new allele appeared on the background of the common allele at the other older SNP (Wray and Visscher, 2007, Ardlie *et al.*, 2002). This new polymorphism may be essential for the increased risk of SLE. Given that haplotype TT was consistently significant even after 10000 permutations were done its OR value was 1.53, this relationship may be potentially useful to predict the risk of SLE.

The SNP-SNP interaction results obtained from MDR test demonstrated that the genotypes TT for both rs7574865 and rs10168266, in the *STAT4* gene formed the best model and would increase the likelihood of developing SLE. This is in agreement with the outcome acquired from the LD analysis. The redundant information described by SNPs rs7574865 and rs10168266 indicates the presence of LD between them, as shown by the high D' value in the LD analysis. Interaction of several other polymorphic sites in the same region may be required in order to pose a larger or more explicit impact on the disease.

Therefore, it may be concluded from the present study that*STAT4* gene polymorphisms feature more prominently as the genetic risk factors in the Malaysian SLE rather than those polymorphisms in *TNFAIP3* and *IRF5*. Rs7574865 is located in the third intron of the *STAT4* gene. The minor/risk allele T has reported associations with other immune-mediated diseases such as rheumatoid arthritis, primary Sjögren's syndrome, type-1 diabetes, Crohn's disease and ulcerative colitis (Remmers *et al.*, 2007, Korman *et al.*, 2008, Martinez *et al.*, 2008, Zervou *et al.*, 2009). The association of this particular SNP with SLE susceptibility was observed in many populations, including both European and Asian populations (Yuan *et al.*, 2010). SNP haplotype in the third intron of *STAT4* marked by rs7574865 was found to be associated with SLE susceptibility and it could be responsible for splice variation or regulatory effects of *STAT4* (Remmers *et al.*, 2007, Kobayashi *et al.*, 2008).

Rs10168266, located in intron 5 of *STAT4* gene, is another variant that has been frequently related to SLE susceptibility (Kawasaki *et al.*, 2008, Hellquist *et al.*, 2010, Yang *et al.*, 2010). This was also reflected in findings of this study.

The next SNP that showed association with SLE in this study was *TNFAIP3* rs2230936. This coding SNP is a non-synonymous variant causing a phenylalanine-to-cysteine change at residue 127 of the A20 protein. It has been already proven that minor Cys127 is relatively stable compared to the Phe127 protein, causing it to be less effective at inhibiting TNF-induced NF-kB activity (Musone *et al.,* 2008). This reduced auto-inflammatory activity of A20 could result in excessive cellular response to TNF. Interestingly, as opposed to other findings suggesting that minor allele G was the risk factor of SLE, the results of this study demonstrated that it conferred protection against SLE in our cohort (Musone *et al.*, 2008, Bates *et al.*, 2009, Cai *et al.*, 2010, Kawasaki *et al.*, 2010, Lodolce *et al.*, 2010, Shimane *et al.*, 2010, Yang *et al.*, 2010, Fan *et al.*, 2011). This study speculates that, rather than rs2230936 itself, there might be other factors, for instance other SNPs adjacent to rs2230936 which are able to further alter the structure of A20 protein, which maybe actually responsible for SLE susceptibility in Malays. This speculation has yet to be validated. This study found *TNFAIP3* rs3757173 to be significantly associated with SLE in Malaysians as a whole but not specifically to any one ethnic group.

Finally, both SNPs in *IRF5* gene investigated in our study were not significant in the Malaysian patients although both are fairly established SLE risk factors for Europeans and some Asians (Vuong *et al.*, 2010, Kelly *et al.*, 2008, Harley *et al.*, 2008, Shin *et al.*, 2007). Presumably, other SNPs of this gene would have to be considered.

The present study was relatively small in contrast to larger studies of SLE by other researchers. Nevertheless, this study has found evidence to indicate that the genes involved in TLR/IFN signalling pathways especially *STAT4* polymorphisms are important in Asian SLE patients.

4.1.2 Genetic associations of HLA variants rs9271366 and rs9275328 with SLE

Yang and co-workers (2010) were the first to report the association of *HLA-DRB1/HLA-DQA1* rs9271366 with SLE susceptibility in Asians including populations from Hong Kong, China, and Thailand. This polymorphism in Hong Kong Chinese SLE patients and African American SLE women was also reported recently by Zhang *et al.* (2011) and Ruiz-Narvaez *et al.* (2011). These previous studies and the current one demonstrated the minor allele G as SLE risk factor. In Malaysia, in addition to the Chinese

and Malays, rs9271366 was also associated with SLE in the Indians. Interestingly, in the Chinese, the risk for SLE was increased according to the number of minor allele G present in the genotypes, which was from major homozygous genotype AA, heterozygous genotype AG to minor homozygous GG suggesting that minor allele G is a strong risk factor for SLE. This SNP has been associated not only with SLE, but frequently with other diseases such as multiple sclerosis (ANZgene, 2009).

HLA-DQB1/HLA-DQA2 rs9275328, which was initially reported as an SLE risk-associated SNP by Yang *et al.* (2010), has received less prominence than rs9271366. Its importance in SLE susceptibility has been documented in Hong Kong (Zhang *et al.*, 2011). Our findings are in agreement with the two studies, by showing the major allele C as the risk allele for SLE in the Malay and Chinese patients in Malaysia. The coupling of one minor allele T in the heterozygous genotype CT appears to reduce the susceptibility to SLE in these three ethnic groups. However, the homozygous TT genotype did appear to have a protective effect. This study did not find any significant association of these SNPs in the Indians, presumably due to the low numbers of participants in this study.

Linkage disequilibrium (LD) analysis is a test to evaluate the non-random association of alleles in adjacent loci. The D' value almost equalled to 1 indicated that the ancestral LD block comprising of rs9271366 and rs9275328 has been conserved with minimal historical recombination. Nevertheless, although they were inherited without disruption, they did not correlate with each other since the r^2 value was very low. The evolutionary explanation for this situation could be: one of the SNPs is a young occurred SNP that originally existed as a monomorphic site with one allele and the new polymorphic allele first turned up later on the background of the common allele at the other older SNP (Wray and Visscher, 2007). This may explain the tendency towards the presence of only three haplotypes and different allele frequencies of the coupled alleles. This is in accordance with our results, in which the frequency of haplotype GT was almost 0 and could be ignored. Haplotypes GC and AT were consistently significant after permutations was done. However, haplotype GC potentially predisposes a person to SLE risk, whereas haplotype AT may confer protection against SLE instead.

The MDR test also supported the LD result by showing the genotype combinations of GG and CC, and AG and CC of rs9271366 and rs9275328, respectively, being in the high-risk group. This means that these genotype combinations may increase the susceptibility to SLE and can be used to predict the occurrence of SLE. In spite of this,

both SNPs interactions may account for 1.96% of the entropy. This leads to speculation that several more polymorphic sites in the same region may be required to interact together to produce greater association with and impact on the susceptibility of SLE.

Although the correlation and interaction between the SNPs were low, this study would like to conclude that both *HLA-DRB1/HLA-DQA1* rs9271366 and *HLA-DQB1/HLA-DQA2* rs9275328 may play a part in influencing the susceptibility to SLE in the Malays and Chinese in Malaysia. However, the interplay of a larger number of SNPs or variants in HLA region may be necessary to look into in order to draw a better conclusion on how SLE can be predicted in an individual, as well as to refine pathological consequences that could lead to SLE.

4.1.3 Elucidating the molecular pathological and biological processes in SLE

Harley and co-workers (2009) proposed that most of the candidate SLE susceptibility genes are involved in three types of biological processes: 1) immune complex processing, 2) TLR function and type I IFN production, and 3) immune signal transduction in lymphocytes. The importance of candidate genes involved in immune complex processing, such as *HLA-DR,C1q*, *C4*, *FcγRIIA*, and *FcγRIIIA*, in the Malaysian SLE susceptibility is indubitable as they have been long and widely reported (Yap *et al.*, 1999a, Yap *et al.*, 1999b, Puah *et al.*, 2007, Chew *et al.*, 2008, Mohd-Yusuf *et al.*, 2011). The role of genes involved in TLR function and type I IFN production in the Malaysian SLE was recognised when the association between *HLA-DR* and the disease was observed (Mohd-Yusuf *et al.*, 2011).

The present study is the first to highlight the importance of genes involved in TLR/type I IFN pathway with SLE risk in the Malaysian patients by showing the associations of *STAT4* and *TNFAIP3* with the disease (**Section 3.1.3 and Section 4.1.1**). The associations of the new SNPs in *HLA-DRB1/HLA-DQA1*and *HLA-DQB1/HLA-DQA2* with SLE (**Section 3.1.4 and Section 4.1.2**) provide further evidence that immune-complex processing and immune signal transduction are highly important components in the development of SLE in the Malaysian.

The present genomic study has provided an insight that all the three biological processes are linked to SLE. More genes are yet to be studied in order to confirm the

involvement of these biological pathways in Malaysian SLE. This can help in the development of more effective treatment tailored specifically for Malaysian SLE patients, targeting on the significantly associated pathway. Importantly, the genotypes linked to SLE susceptibility can be investigated in patients so that better management can be carried out.

4.2 Proteomic analysis of SLE

4.2.1 Plasma proteins associated with SLE/lupus nephritis

The plasma proteins found to be associated with SLE or LN are tabulated in **Table 4.1**, together with their theoretical MWs, functions and references reporting their association with SLE and LN. In addition to some that had been previously reported, we report some new discoveries, such as afamin (AFM), haemopexin (HPX), retinol-binding protein 4 (RBP4), vitamin D-binding protein (GC), apolipoprotein A-IV (ApoA-IV), zinc-alpha-2-glycoprotein (ZAG), and gelsolin (GSN).

From the results in **Section 3.2.6.1** and **Figure 3.2.24**, the SLE-associated plasma proteins reported in this study basically could be clustered into a few groups according to their functions, i.e. serum constitutional and transport proteins which occupied the biggest portion (27.6%), complement fractions (17.2%), lipoprotein metabolism (13.8%), metal binding proteins (13.8%), immunoglobulins and other immune proteins (10.3%), coagulation factors (6.9%), proteases and inhibitors(6.9%), and kidney secretory and structural proteins (3.4%). How each of these proteins could be related to the development of SLE/LN will be discussed below. The hypoproteinaemia in LN results from massive and prolonged losses of major proteins in urine that are not counterbalanced by resynthesis. Amazingly, besides complement C4 and its related proteins, the classical or typical SLE-associated proteins such as C-reactive protein (CRP), complement C1q, and complement C3 that were usually tested and monitored in SLE diagnosis were found to be not significant in this study. This could be due to the statistical parameters set in this study. The importance of autoantibodies such as anti-dsDNA, anti-nucleosome, and anti-histone antibodies may be reflected by the significant presence of immunoglobulin chains observed in this study, albeit their functions could not be identified.

Several plasma proteins were found to be specific to certain SLE/LN group only and they are listed in **Table 4.2**. These proteins could be potentially used to diagnose and

Table 4.1. Previously reported and newly discovered SLE-associated plasma proteins.

Protein	Gene name	Reference	Function
Serum constitutional and transport proteins			
Haptoglobin	*HP*	(Rantapaa Dahlqvist *et al.*, 1988, Pavon *et al.*, 2006)	Haemglobin binding, cellular iron ion homeostasis
Histidine-rich glycoprotein	*HRG*	(Gorgani and Theofilopoulos, 2007, Blank and Shoenfeld, 2008)	Blood coagulation, fibrinolysis, haemstasis
Serum albumin	*ALB*	(Woch, 1977, Sheikh *et al.*, 2007, Sule *et al.*, 2007, Yip *et al.*, 2010)	Blood protein
Serum amyloid P-component	*SAP*	(Breathnach *et al.*, 1989, Sorensen *et al.*, 2000, Bijl *et al.*, 2004, Shoenfeld *et al.*, 2007, Voss *et al.*, 2008)	Acute-phase response, transport
Afamin*	*AFM*	-	Vitamin E binding protein, transport
Haemopexin*	*HPX*	-	Heme transport, host-virus interaction
Retinol-binding protein 4*	*RBP4*	-	Sensory transduction, transport, vision
Vitamin D-binding protein*	*GC*	-	Transport
Lipoprotein metabolism			
Apolipoprotein A-I	*APOA1*	(Lahita *et al.*, 1993, Delgado Alves *et al.*, 2003, Shoenfeld *et al.*, 2007, O'Neill *et al.*, 2010)	Lipid metabolism and transport
Apolipoprotein E	*ApoE*	(Feng *et al.*, 2007, Ma *et al.*, 2008)	Lipid metabolism and transport
Clusterin	*CLU*	(Moll *et al.*, 1998, Newkirk *et al.*, 1999, Wang *et al.*, 2004, Chauhan and Moore, 2006)	Complement pathway, innate immunity, apoptosis
Apolipoprotein A-IV*	*APOA4*	-	Lipid transport

Complement fractions			
C4b-binding protein alpha chain	*C4BPA*	(Daha *et al.*, 1983, Schifferli *et al.*, 1984, Barnum and Dahlback, 1990)	Complement pathway, innate immunity
Complement C4-A & B	*C4A, C4B*	(Dunckley *et al.*, 1987, Wilson *et al.*, 1988, Moulds *et al.*, 1993, Petri *et al.*, 1993, Yang *et al.*, 2004, Ittiprasert *et al.*, 2005)	Complement pathway, innate immunity, inflammatory response
Complement factor B	*CFB*	(Passwell *et al.*, 1988, Garlepp *et al.*, 1989)	Complement alternative pathway, innate immunity
Complement factor H	*CFHR1*	(Bao *et al.*, 2011, Zhao *et al.*, 2011)	Complement regulation, lipid metabolism
Ficolin-3	*FCN3*	(Andersen *et al.*, 2009)	Lectin complement pathway, innate immunity
Complement factor I*	*CFI*	-	Complement pathway, innate immunity, proteolysis
Metal binding proteins			
Alpha-2-HS-glycoprotein	*AHSG*	(Kalabay *et al.*, 1990)	Mineral balance
Ceruloplasmin	*CP*	(Denko and Gabriel, 1979, Jakab *et al.*, 1976)	Copper transport, ion transport
Serotransferrin	*TF*	(Denko and Gabriel, 1979)	Ion transport, iron transport
Zinc-alpha-2-glycoprotein*	*AZGP1*	-	Lipid catabolism
Immunoglobulins and other immune proteins			
Ig alpha-1 and -2 chain C region	*IGHA1,2*	(Cronin *et al.*, 1989, Jasin and Ziff, 1975, Cass *et al.*, 1968, Rankin and Isenberg, 1997, Cassidy *et al.*, 2007, Cunningham-Rundles and Bodian, 1999, Fernandez-Castro *et al.*, 2007, Song *et al.*, 2003,	Immune response
Ig gamma chain C region	*IGHG*		
Ig kappa chain C region	*IGKC*		

		Yong *et al.*, 2008)	
Coagulation factors			
Fibrinogen beta chain,	*FGB*,	(Nagayama *et al.*, 1992, Ames *et al.*, 2000)	Blood coagulation, haemstasis
Fibrinogen gamma chain	*FGG*		
Proteases and inhibitors			
Alpha-1-antitrypsin	*AAT*	(Karsh *et al.*, 1979, Zhang *et al.*, 1989, Lacki *et al.*, 1995, Perez Suarez *et al.*, 2002)	Acute-phase response, blood coagulation, haemstasis
Alpha-2-macroglobulin	*A2M*	(Panzironi *et al.*, 1997)	Protease inhibitor
Kidney secretory and structural proteins			
Gelsolin*	*GSN*	-	Actin polymerization, cilium biogenesis

* Newly discovered in this study

Table 4.2. Plasma proteins specific to SLE without kidney manifestation and each LN group.

Group	Specific protein(s)
SLE (non-renal)	Alpha-1-antitrypsin (AAT) ↑ Serum amyloid P-component (SAP) ↓
LN class II	Afamin (AFM)* ↓ Gelsolin (GSN)* ↓
LN class III	Apolipoprotein A-I (ApoA-I) ↑ Apolipoprotein E (ApoE) ↑ Retinol-binding protein 4 (RBP4)* ↑ Alpha-2-macroglobulin (A2M) ↑
LN class IV	Ig kappa chain C region (IGKC) ↑
LN class V	Histidine-rich glycoprotein (HRG) ↓
LN in remission	Complement factor I (CFI)*↓

*Newly reported

discern patients between SLE and LN, as well as among different groups of LN. Most of the proteins have been already documented by other studies. Complement factor I (CFI) which was observed to be specific to LN remission patients, AFM in LN class II patients, and RBP4 in LN class III patients, were newly identified in this study. Their interactions with other proteins will be also discussed.

4.2.1.1 Serum constitutional and transport proteins

Eight proteins involved in serum constitutional and transport were found to be associated with LN and SLE without kidney involvement in our cohort. The functions of these proteins and how their differential expressions may bring about SLE and LN are discussed in the following sections.

4.2.1.1.1 Haptoglobin (HP)

Haptoglobin (HP) is synthesized mostly by hepatocytes. Its function is to prevent loss of iron via kidneys and protect kidneys from damage by haemoglobin by combining with free plasma haemglobin, and at the same time, allowing degradation of haemglobin by enzymes (Langlois and Delanghe, 1996). It is also an acute-phase protein which can be induced as a result of infection, tissue injury, and malignancy, such as ulcerative colitis, acute rheumatic disease, and heart attack. Increased level of plasma HP in SLE patients has been reported by Rantapaa Dahlqvist *et al.* (1988) and Pavon *et al.* (2006). Decreased plasma HP level is otherwise associated with haemolytic anemia (Pintera, 1968), kidney dysfunction, as well as liver disease.

Several isoforms of HP were found in this study. These isoforms could result from post-translational modifications (PTM), which are glycosylation and disulfide bonds, at different positions of HP. The isoforms may also produced by genetic variants of HP, such as N129D in allele HP*1F (van der Straten *et al.*, 1984), that cause altered physic-chemical property of the protein. In this study, down regulation was found in cohorts with LN class III (isoform 1) and class V (isoform 1 and 2) and those in remission (isoform 2) (**Table 3.2.8 and Table 3.2.10**). Unfortunately, there is no information about the functional role of each HP isoform. However, since different proportion of HP isoforms may reflect a different HP phenotype, it could be concluded that LN class III is associated with HP phenotype with lower level of isoform 1, class V with lower level of isoform 1 and 2, and those in LN remission with lower level of isoform 2.

4.2.1.1.2 Histidine-rich glycoprotein (HRG)

HRG is a plasma glycoprotein that binds to ligands such as heme, heparin, heparin sulfate, thrombospondin, plasminogen, and divalent metal ions (Morgan, 1978, Jones *et al.*, 2004a, Jones *et al.*, 2004b, Simantov *et al.*, 2001, MacQuarrie *et al.*, 2011). It is involved in regulation of many processes including immune complex and pathogen clearance, cell adhesion, angiogenesis, coagulation and fibrinolysis (Gorgani *et al.*, 1999, Ohta *et al.*, 2009, Thulin *et al.*, 2009, Poon *et al.*, 2010). Association of HRG with SLE has been reported earlier, whereby significantly lower HRG level was observed (Gorgani and Theofilopoulos, 2007, Blank and Shoenfeld, 2008). This finding was also noticed in this study, and in addition, it only occurred in LN class IV (**Table 3.2.8**). This may be one of

the reason LN class V patients are predisposed to thrombotic complications, such as renal vein thrombosis and pulmonary embolus. On the other hand, elevation of plasma HRG level can lead to thrombophilia (Engesser *et al.*, 1987, Hoffmann *et al.*, 1993).

4.2.1.1.3 Serum albumin (ALB)

Serum ALB is the most abundant protein in plasma, accounting for approximately 80% of the plasma proteins. Its major function is to maintain the colloidal osmotic pressure of blood, and also as transporter of zinc, other metal ions, fatty acids, hormones, bilirubin and drugs in plasma (Lu *et al.*, 2008). Its association with SLE has long been discussed since 1977, whereby SLE patients were found to excrete altered form of albumin (Woch, 1977). Sheikh *et al.* (2007) suggested that reactive oxygen species damaged ALB could initiate autoimmunity in SLE. Reduced serum ALB level in both SLE and LN patients was reported, which was in accordance with the finding of this study (Sule *et al.*, 2007, Yip *et al.*, 2010). Serum ALB has also been observed to be a potential marker for assessment of disease activity on SLE (Yip *et al.*, 2010). The other disease that is caused by serum ALB deficiency is familial dyalbuminemic hyperthyroxinemia (FDH) (Ruiz *et al.*, 1982).

Several plasma ALB fragments were identified in this study, some with low MW. Donadio and colleagues (2012) hypothesized that proteolytic fragmentation of ALB is due to a higher susceptibility to proteases induced by oxidative stress, which can induce other structural modifications of ALB molecule and in turn affect some physiological functions of ALB and introduce pathophysiological roles in renal diseases. Alterations in binding and transport of compounds such as NO, hormones, xenobiotics, drugs, fatty acids, and amino acids, as well as impairment of antioxidant properties may be relevant to cause the renal diseases. Very low MW ALB fragments could be composed of two peptide sequences originating far apart in the ALB sequence, with a complete lack of intermediate amino acids (Candiano *et al.*, 2006, Hellin *et al.*, 2009).

4.2.1.1.4 Serum amyloid P-component (SAP)

Serum amyloid P-component (SAP) interacts with DNA and histones and is responsible in scavenging nuclear material released from damaged circulating cells. It is also involved in formation of all types of amyloid (Pepys *et al.*, 1982). Correlation between

SAP and SLE has been reported in many studies, including the study by Breathnach *et al.* (1989) that detected association of SAP with unusual globular dermal deposits of nuclear material in skin biopsies from SLE patients. The concentration of SAP-DNA complexes in SLE sera was also found significantly lower than in normal sera and particularly low in sera from patients with anti-DNA titres exceeding 50 (Sorensen *et al.*, 2000, Voss *et al.*, 2008). Although Bijl and colleagues (Bijl *et al.*, 2004) reported no alteration in SAP level in SLE patients, the current study did notice a significant reduction in SAP level in SLE groups, and it was specific for SLE patients without kidney involvement only.

4.2.1.1.5 Afamin (AFM)*

This current study is the first to report the association between decreased plasma AFM levels and LN class II patients.

Afamin (AFM) is one of the members of ALB gene family, together with ALB, alpha-fetoprotein (AFP), and vitamin D-binding protein (GC) (Lichenstein *et al.*, 1994). It is synthesized in liver and secreted in plasma and extravascular fluids such as follicular and cerebrospinal fluids (Jerkovic *et al.*, 2005). AFM is a vitamin E (alpha-tocopherol) binding glycoprotein and may take over in vitamin E transport in body fluids when lipoprotein system is not sufficient (Voegele *et al.*, 2002). It may be also involved in the homeostasis of vitamin E at blood-brain barrier (Kratzer *et al.*, 2009) and protect cortical neurons under apoptotic conditions (Heiser *et al.*, 2002). Decreased level of plasma AFM has been associated with ovarian cancers (Jackson *et al.*, 2007, Dieplinger *et al.*, 2009). Further study is required to delineate the mechanism of AFM in causing LN class II.

In the rat model (**Figure 4.1)**, AFM is targeted by various miRNA functional clusters from cytoplasm and regulated by IgG and transforming growth factor beta 1 (TGFB1), as well as drugs such as dextran, zomepirac and dexamethasone, to modulate the gene transcription of apolipoprotein E (*APOE*), apolipoprotein A-I (*APOA1*), apolipoprotein B (*APOB*), alpha-1-antitrypsin (*SERPINA1*), transferrin (*TF*), actin beta (*ACTB*), and tyrosine aminotransferase (*TAT*) (Pietrangelo *et al.*, 1992), where most of them are associated with SLE and LN.

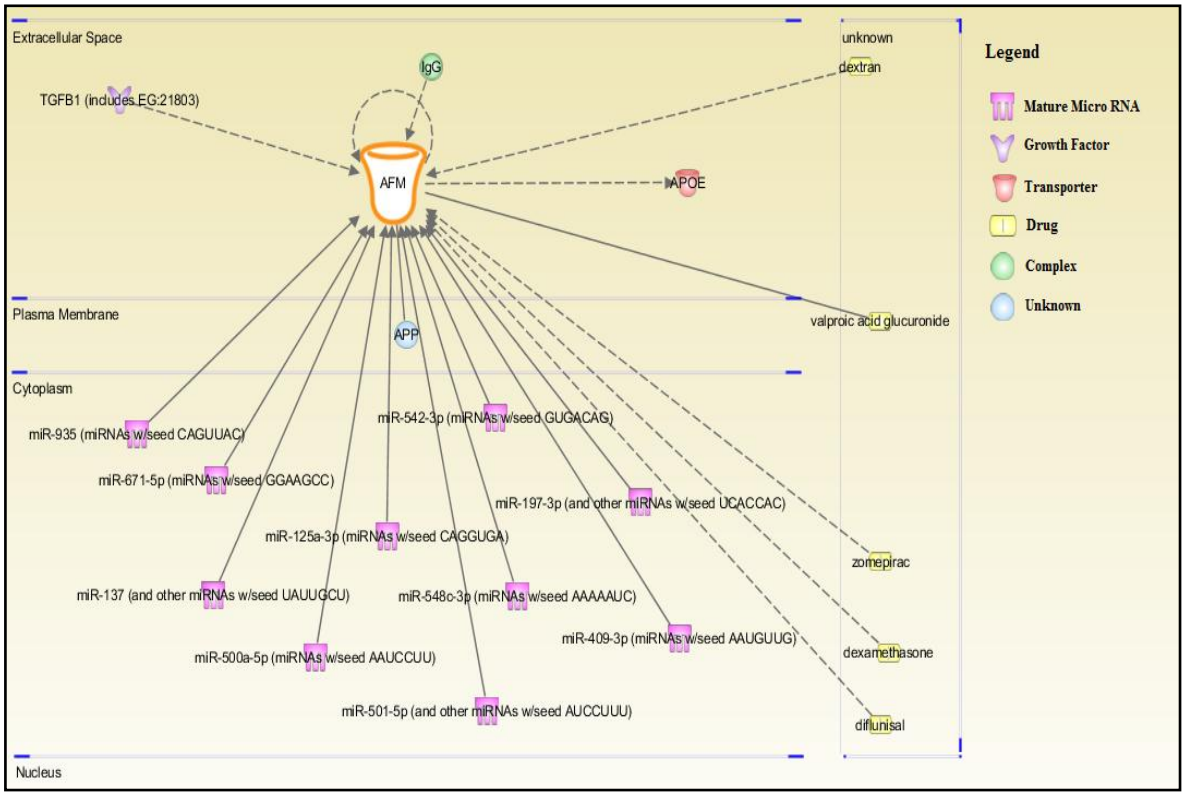


Figure 4.1. Example of functionality of AFM interaction pathway.

4.2.1.1.6 Haemopexin (HPX)*

Haemopexin (HPX) was another newly reported SLE/LN-associated protein in this study. HPX binds heme and transport it to liver for breakdown for the sake of protecting the body from the oxidative damage that can be caused by free heme (Tolosano and Altruda, 2002). Low HPX level in plasma usually indicates haemolytic anaemias, but it also occurs in renal disease where HPX concentrations reduce together with those of albumin and transferrin, presumably because they have similarly low molecular weights and are therefore excreted into the urine (Muller-Eberhard, 1970). Progressive liver disease can also lead to reduced HPX level as a result of synthesis disability by the failing liver. On the other hand, increased HPX level can be found in patients with diabetes mellitus, infections in severe rheumatoid arthritis and cancers (Muller-Eberhard, 1970).

In this present study, decrease in plasma HPX was seen in SLE patients without kidney involvements and this could be due to haemolytic anemia. However decreased levels observed in LN class II and class III patients could be due to small of protein that it had been excreted into the urine. Different HPX fragments involved in LN class II and

class III may indicate different alterations of HPX that cause different protease activity in these LN classes, as reported in the case of minimal change disease (Bakker *et al.*, 2005).

4.2.1.1.7 Retinol-binding protein 4 (RBP4)*

Retinol-binding protein 4 (RBP4) was one of the SLE/LN associated proteins reported first time in this study. The increased plasma RBP4 was also observed to be specific to LN class III patients.

The RBP binds to vitamin A and mobilizes it from liver stores to plasma in the form of the lipid alcohol retinol (Goodman, 1980). Elevation in plasma RBP4 level has been correlated with renal dysfunction in type II diabetes (Cabre *et al.*, 2007, Murata *et al.*, 2009), and this may be caused by exceeded tubular capacity and tubular dysfunction that could alter RBP4 homeostasis (Bangstad *et al.*, 1995, Salem *et al.*, 2002). This could be used to explain the presence of increased RBP4 level in the plasma of LN class III patients. Increased plasma RBP4 has also been reported in women with cervical dysplasia (Palan *et al.*, 1988) and cardiovascular disease in type II diabetes (Cabre *et al.*, 2007).

The interaction of RBP4 with other proteins or compounds is illustrated in **Figure 4.2.** In plasma, the RBP-retinol complex interacts with transthyretin (TTR) which prevents its loss by filtration through the kidney glomeruli (van Bennekum *et al.*, 2001). It is targeted by a wide variety of miRNA functional clusters and regulated by many endogenous chemicals (e.g. norepinephrine, nitric oxide), drugs (e.g. cisplatin, dexamethasone), transcription regulators [e.g. catenin (CTNNB1)], ligand-dependent nuclear receptor [e.g. [peroxisome proliferator-activated receptor alpha and gamma (PPARA and PPARG)] (Rosell *et al.*, 2012), and other proteins such as growth factor receptor-bound protein 2 (GRB2) and insulin. RBP4 modulate expression and activity of RBP1, insulin (Yang *et al.*, 2005), TGFB1, sonic hedgehog (SHH), enzymes [e.g. adenylatecyclase 6 (ADCY6)], kinase [e.g. mitogen-activated protein kinase 8 (MAPK80)], and other protein such as STAT5, ERK, and immunoglobulin (Berry *et al.*, 2011) (**Figure 4.2**).

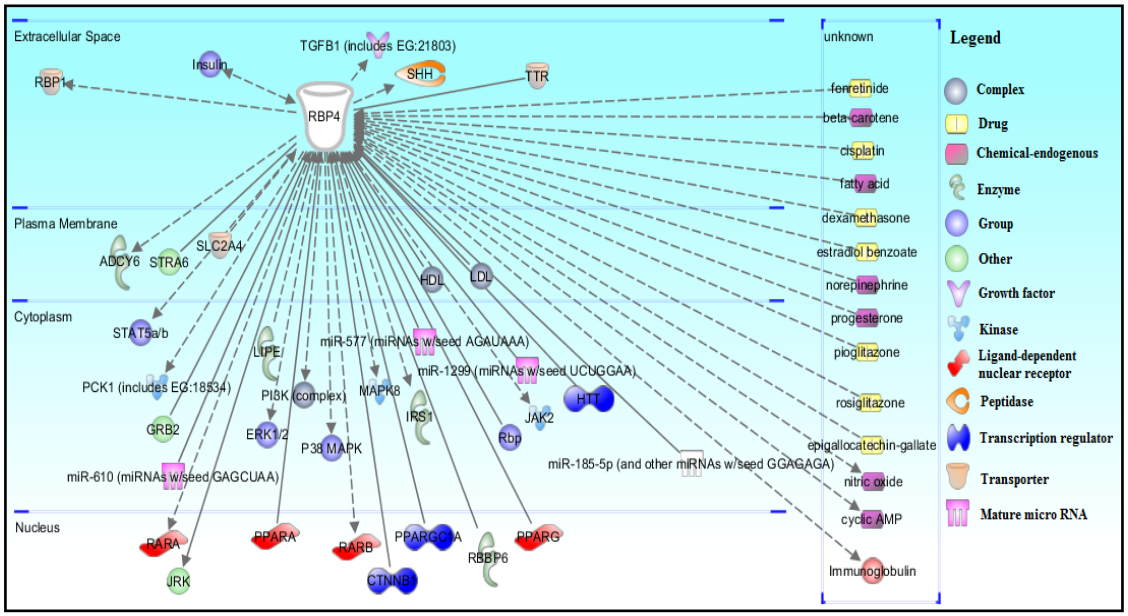


Figure 4.2. Example of functionality of RBP4 interaction pathway.

4.2.1.1.8 Vitamin D-binding protein (GC)*

Vitamin D-binding protein (GC) was another newly reported protein that was associated with SLE/LN in the present study.

Vitamin D-binding protein (GC) is another member of ALB gene family (Lichenstein *et al.*, 1994). It is the major serum transport protein for the vitamin D sterols and binds to actin monomers to prevent polymerization (Daiger *et al.*, 1978, Cooke and David, 1985, Van Baelen *et al.*, 1980). It can also be found on the surface of B-lymphocytes, subpopulations of T-lymphocytes, and the cytotrophobalsts of the placenta (Petrini *et al.*, 1984, Petrini *et al.*, 1985, Nestler *et al.*, 1987). Vitamin D deficiency has been frequently associated with SLE (Kamen *et al.*, 2006, Borba *et al.*, 2009), yet none has discussed about the relationship between GC and SLE. Low level of GC in plasma has been associated with type I diabetes and liver cirrhosis (Masuda *et al.*, 1989, Blanton *et al.*, 2011). In the case of acute tissue damage, GC act in concert with gelsolin (GSN) to neutralize the potential damaging effects of cellular actin released in the blood. GC sequesters the GSN-bound monomeric-G actin molecules that dissociate from the pointed filament ends of the actin polymer (Vasconcellos and Lind, 1993). This may induce an increased uptake and degradation of GC when bound to actin, and thus lead to decreased blood levels of GC upon acute tissue damage (Dueland *et al.*, 1990, Schiodt *et al.*, 1997). Therefore, in the present study, reduced levels of plasma GC in patients with LN class II and V, as well as in LN patients in remission, is understandable. Herein, it could also be

deduced from this study that increased levels of plasma GC is associated with milder forms of LN.

4.2.1.2 Lipoprotein metabolism and SLE

A total of four proteins involved in lipid metabolism were found to be associated with SLE/LN and their roles in the pathogenesis of diseases are described below.

4.2.1.2.1 Apolipoprotein A-I (ApoA-I)

Apolipoprotein A-I (ApoA-I) is the major component of plasma high density lipoprotein (HDL) and essential in lipid metabolism, whereby it reversely transports cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase (LCAT) (Minnich *et al.*, 1992). Significant elevated titre of anti-ApoA-I in SLE patients and its association with disease activity has been consistently reported in several studies (Delgado Alves *et al.*, 2003, Shoenfeld *et al.*, 2007, O'Neill *et al.*, 2010), which brings about the assumption that the resulting low ApoA-I level is in turn correlated with SLE (Lahita *et al.*, 1993). Nevertheless, the LN class III in the present study possessed increased level of plasma ApoA-I. Hence, it can be inferred from this study that the elevation of ApoA-I level serves as a counter effect against increased level of its autoantibody to prevent further damage caused by the autoantibody.

4.2.1.2.2 Apolipoprotein E (ApoE)

Apolipoprotein E (ApoE) was first identified in 1973 in human very low density lipoprotein (VLDL) (Shore and Shore, 1973). It also constitutes 1-2% of HDL. Besides transporting cholesterol and other lipids among various cells of the body, Apo-E also appears to have implication in the repair response to tissue injury, immunoregulation and modulation of cell growth and differentiation (Mahley, 1988). ApoE-deficient lupus murine models exhibited the association of ApoE defect with atherosclerosis in SLE (Feng *et al.*, 2007, Ma *et al.*, 2008). Pullmann *et al.* (2004) suggested an association between ApoE polymorphism and neuropsychiatric SLE in their study. In current study, significant

increase in Apo-E level was seen LN class III only. Again, this elevation may be explained as a counter effect against the induction of the autoantibody.

4.2.1.2.3 Clusterin (CLU)

Clusterin (CLU) (apolipoprotein J) functions in a variety of biological processes including protein aggregation inhibition, complement attack prevention, phagocyte recruitment, apoptosis induction and lipid metabolism (James *et al.*, 1991, Calero *et al.*, 1999, Poon *et al.*, 2000, Leskov *et al.*, 2003). Increase in CLU level has been reported to be associated mainly with renal SLE (Moll *et al.*, 1998, Chauhan and Moore, 2006), while otherwise has been observed in SLE with other complications such as skin ulcers, hair loss and thrombosis (Newkirk *et al.*, 1999, Wang *et al.*, 2004). Even so, Newkirk *et al.* (1999) also correlated low CLU level with proteinuria. In present study, significant increased level of CLU was shown in SLE patients with renal complications, except in LN class III which needs further explanation. Besides LN, CLU is also induced in various other renal diseases and has been suggested as a marker for renal injury. A study that demonstrated the ability of CLU to attenuate renal fibrosis deduced that up-regulation of CLU actually contributes a protective response against further renal obstruction (Jung *et al.*, 2012).

4.2.1.2.4 Apolipoprotein A-IV (ApoA-IV)*

The relationship between increased plasma ApoA-IV levels and LN was firstly revealed by this study. It was observed in all classes of LN patients, starting from the relatively milder LN class II.

Apolipoprotein A-IV (ApoA-IV) is synthesised mainly in intestine and released into plasma on the surface of chylomicrons (Tso and Liu, 2004). Fat absorption in intestine increases the synthesis and secretion of ApoA-IV. Besides that, ApoA-IV has been also linked with other functions, including as an antioxidant (Qin *et al.*, 1998), anti-inflammatory factor (Vowinkel *et al.*, 2004), anti-atherosclerotic factor (Cohen *et al.*, 1997), mediator of reverse-cholesterol transport (Dvorin *et al.*, 1986) and acute satiety factor (Fujimoto *et al.*, 1992). The increased level of plasma ApoA-IV in renal failure individuals has been associated with the anti-atherogenic properties of ApoA-IV and it started to elevate during the earliest phases of renal insufficiency (Dieplinger *et al.*, 1992,

Kronenberg *et al.*, 2002). This could be the reason for the presence of increased plasma ApoA-IV level in all classes of LN patients in the present study. The increased plasma ApoA-IV level could also be explained by the impaired metabolism of ApoA-IV by kidney (Haiman *et al.*, 2005).

A total of four isoforms of ApoA-IV were found, with different isoforms being associated with different LN classes. These isoforms could be derived from genetic variations, in which the two major isoforms are A-IV-2 and A-IV-2 with a G to T nucleotide change that substitutes His for Gln at position 360 (Menzel *et al.*, 1982, Utermann *et al.*, 1982, Lohse *et al.*, 1990a). Other isoforms could be due to insertions, other amino acid substitutions at other positions, and deletions (Lohse *et al.*, 1990b, Boerwinkle *et al.*, 1990, Hixson and Powers, 1991). Thus, it is possible that different proportions of these isoforms are responsible for the occurrence of different classes of LN.

4.2.1.3 Complement fractions

Six complement fractions were associated with SLE/LN in this investigation and their roles in the pathogenesis of the diseases are discussed below.

4.2.1.3.1 C4b-binding protein alpha chain (C4BPA)

C4b-binding protein alpha chain (C4BPA) is involved in classical and lection complement pathways by inhibiting the action of C4 (Hessing *et al.*, 1990). Reduced level of C4BP has been reported (Daha *et al.*, 1983, Schifferli *et al.*, 1984), as in agreement with the finding in this study for SLE without kidney involvement and LN class II, although elevated level has also been observed by other studies (Barnum and Dahlback, 1990).

4.2.1.3.2 Complement C4-A&-B (C4A & C4B)

Complement C4-A and –B is important in the activation of classical complement pathway. C4A and C4B are the acidic and basic form of complement factor 4, respectively. The proteolytic cleavage of complement C4, C4a anaphylatoxin, is a mediator of local inflammatory process (Moon *et al.*, 1981). Gene deficiency of these proteins has been reported to increase susceptibility to SLE in many populations, including Caucasoid,

Chinese, Japanese, black Americans, and Thai (Dunckley *et al.*, 1987, Wilson *et al.*, 1988, Moulds *et al.*, 1993, Petri *et al.*, 1993, Yang *et al.*, 2004, Ittiprasert *et al.*, 2005). In present study, decreased C4A and C4B levels were significant in all LN classes, with isoform 1 being associated with milder form of LN (class II and V), isoform 2 associated with LN class III, and both isoform 1 and 2 associated with LN class IV. This may imply that the involvement of different isoforms could reflect the classes of LN.

4.2.1.3.3 Complement factor B (CFB)

Complement factor B (CFB) is a one of the components involved in the activation of alternative complement pathway. Some studies using murine model of SLE showed association of decreased long CFB mRNA transcription and increased transcription of the short form with LN (Passwell *et al.*, 1988), whereas some reported no significant changes in CFB levels in SLE patients (Garlepp *et al.*, 1989). Correlation of declined plasma CFB level with LN, except for LN class III, was discovered in this study. It could be due to loss in the urine, which is also associated with decreased serum opsonic activity (McLean *et al.*, 1977, Reddingius *et al.*, 1993).

4.2.1.3.4 Complement Factor H (CFH)

Complement Factor H (CFH) acts as a cofactor in the factor I-medicated C3b cleavage and also accelerates the decay of C3bBb complex (C3 convertase) and (C3b)NBB complex (C5 convertase) in the alternative complement pathway. The significance of CFH in the pathogenesis of SLE has been discussed by Zhao *et al.* (2011) in genetic basis, and Bao *et al.* (2011) who suggested that CFH loss could enhance the development of LN. The current study found an increased level of a CFH isoform in SLE patients without kidney involvements, but decreased level of another CFH isoform in LN class II patients. Other diseases which are also associated with decreased CFB level are membranoproliferative glomerulonephritis type II (MPGN II) and haemlytic uremic syndrome atypical type 1, with both involving renal failure (Wyatt *et al.*, 1982, Hahn *et al.*, 2006). This may explain the reason for decreased CFH level in LN but increased in SLE without kidney involvements and it could be potentially used to distinguish between the two SLE conditions after they are further characterised.

4.2.1.3.5 Ficolin-3 (FCN3)

Ficolin-3 (FCN3) has implication in innate immunity via activation of the lectin complement pathway (Matsushita *et al.*, 2002). It is also known as thermolabile beta-2 macroglycoprotein or Hakata antigen, and was first reported to react with autoantibody from SLE patients in 1978 (Inaba and Okochi, 1978, Yae *et al.*, 1991, Akaiwa *et al.*, 1999). It mediates the clearance of late apoptotic cells, and thus is claimed to be involved in the maintenance of tissue homeostasis and subsequently exert a protective effect against the development of autoimmunity (Honore *et al.*, 2007). Elevation of FCN3 was discovered by Andersen and colleagues in SLE patients (Andersen *et al.*, 2009), which was also found significant in this study for LN class III and class V. This elevation may be owing to the effort of FCN3 to protect the kidneys against damage by autoimmunity.

4.2.1.3.5 Complement factor I (CFI)*

Complement factor I (CFI) was newly discovered in this study to be specifically associated with LN in remission.

Complement factor I (CFI) is a serine proteinase that is essential for regulating the complement cascade. It functions in the cleavage of the C3b and C4b in the presence of several specific cofactors and to modulates the activity of C3 convertase (Goldberger *et al.*, 1987). Mutations in the genes encoding CFI has been associated with atypical haemlytic-uremic syndrome (HUS), with an increase in autoantibodies against CFI present in the patients (Kavanagh *et al.*, 2012). Some cases of HUS have been reported to be accompanied by SLE and LN (Ogawa *et al.*, 2000, Al Shohaib *et al.*, 2000, Azharuddin and Bhatti, 2005). These could be the possible reasons for the LN patients in remission in the present study to have decreased level of plasma CFI.

In the interaction with other compounds, CFI is targeted by a wide variety of miRNA functional clusters and its expression and activation are regulated by Fe^{3+}4-(2-aminoethyl)benzenesulfonylfluoride, IL13, and drugs such as nitrofurantoin, methotrexate, and pirinixic acid (Tsiftsoglou and Sim, 2004) (**Figure 4.3)** (Blom *et al.*, 1999, Jarva *et al.*, 1999). It also inhibits the formation of immune complexes such as C2-C4b and C3-Cfb complexes.

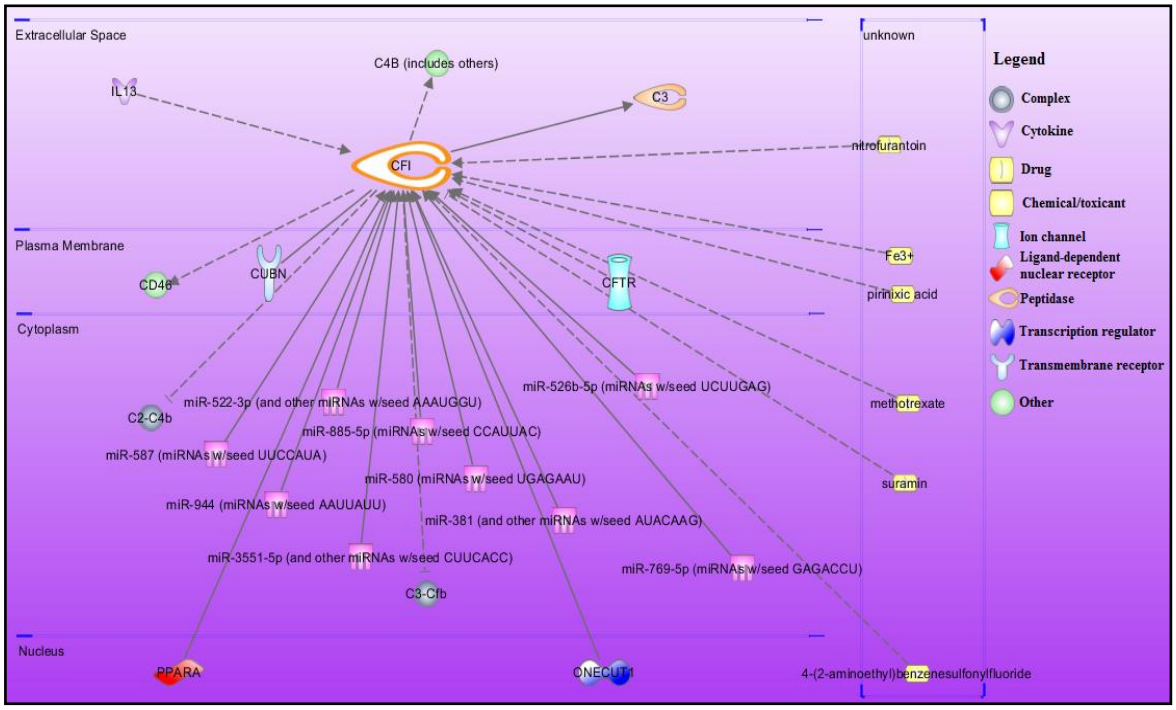


Figure 4.3. Example of functionality of CFI interaction pathway.

4.2.1.4 Immunoglobulins and other immune proteins

A total of three immunoglobulins and immune proteins were found to be associated with SLE/LN in this study. How the dysregulations of these proteins can lead to the diseases are discussed as follows.

4.2.1.4.1 Immunoglobulins

Since SLE is an autoimmune disease, an elevated level of all types of immunoglobulin in patients' plasma were expected, in conjunction with the increased of highly differentiated B lymphocytes and autoantibodies such as antinuclear and anti-cardiolipin antibodies (Jasin and Ziff, 1975). Hypergammaglobulinemia is a common laboratory finding in patients with active SLE and many studies have supported these findings, as well as the results from this study which showed an increase in Ig gamma chain C region in non-renal SLE. However, decreased level of Ig gamma chain C region was significant in LN class II and class V instead, involving different isoforms. These patients might be having hypogammaglobulinemia as have been previously reported to occur in SLE patients, as well as in selective IgA deficiency (Rankin and Isenberg, 1997, Cassidy *et al.*, 2007), common variable immunodeficiency (CVID) (Cunningham-Rundles

and Bodian, 1999, Fernandez-Castro *et al.*, 2007), drug-induced hypogammaglobulinemia (Song *et al.*, 2003), and hypogammaglobulinemia secondary to nephritic syndrome (Yong *et al.*, 2008). Significant decrease in Ig alpha-1 and -2 chain C regionin LN class II (isoform 2 and 3) and class III (isoform 1) in this study requires further investigation, whereas Ig kappa chain C region which is significantly higher in plasma of patients with LN class IV only can be of potential value as biomarker for diagnosis of this LN class.

4.2.1.5 Metal binding proteins

Four metal binding proteins were found to be associated with SLE/LN in our patients and their roles in the pathogenesis of the diseases are described below.

4.2.1.5.1 Serotransferrin (TF)

Serotransferrins (TFs) are plasma glycoproteins that regulate the level of free iron by binding iron very tightly, but reversibly (Crichton and Charloteaux-Wauters, 1987). Serotransferrin (TF) also has role in supporting cell proliferation by supplying cells with iron (Laskey *et al.*, 1988). Association between decreased level of TF and SLE was illustrated by Denko and Gabriel (Denko and Gabriel, 1979), which was in contrast with the results of current study, showing the association of elevated TF levels with LN class III (isoform 1 and 2) and class IV (isoform 3). Increased TF level occurs in patients suffer from iron deficiency anemia, and this study speculated that this clinical condition may come in parallel with LN class III and class IV. Further investigation is necessary to prove this speculation.

4.2.1.5.2 Ceruloplasmin (CP)

Ceruloplasmin (CP) is a copper-binding glycoprotein as well as a ferroxidase enzyme that oxidises of Fe^{2+} (ferrous iron) into Fe^{3+} (ferric iron) (Mzhel'skaya, 2000). It therefore assists TF which can only carry iron in ferric state in iron transport across the cell membrane. Correlation of increased CP level with SLE has been described (Denko and Gabriel, 1979, Jakab *et al.*, 1976), which supported the CP level elevation in LN class IV

patients in this study. However, reduced level of CP was observed in non-renal SLE patients in this study and the reason remains unclear.

4.2.1.5.3 Alpha-2-HS-glycoprotein (AHSG)

Alpha-2-HS-glycoprotein (AHSG) has role in biological processes such as endocytosis, brain development, and bone tissue formation (Arnaud *et al.*, 1988). Association of significantly low level of AHSG with SLE was observed by Kalabay *et al.* (1990), which was also seen in non-renal SLE and LN class III and IV patients in this study. This findings were somewhat predicted because AHSG is a negative acute-phase reactant (Lebreton *et al.*, 1979). Non-renal SLE had one AHSG isoform involved, while LN class III and IV had one additional isoform affected and this additional isoform may indicate the presence of more severe forms of LN.

4.2.1.5.4 Zinc-alpha-2-glycoprotein (ZAG)*

The associations of increased plasma ZAG levels with LN class IV and V were newly identified in this study.

Zinc-alpha-2-glycoprotein (ZAG) is involved in lipid mobilization by stimulating the depletion of fatty acids from adipose tissues (Bao *et al.*, 2005). It may participate in immune response seeing that it has similar structure with MHC class I antigen-presenting molecule (Sanchez *et al.*, 1999). Its role as tumuor marker (Freije *et al.*, 1991) and functions in RNase activity (Lei *et al.*, 1998), regulation of melanin production (Hale, 2002) and hindering tumour proliferation (He *et al.*, 2001) have been recognized. ZAG has been also suggested as a carrier protein of the nephritogenic renal glycoprotein in plasma (Shibata and Miura, 1982, Hassan *et al.*, 2008) and this could be the reason that increase in plasma ZAG was noticed in LN class IV and V patients in this study, with different isoforms being affected in both classes.

4.2.1.6 Coagulation factors

Two fibrinogen chains were found to be associated with SLE/LN in our patients and their roles in the pathogenesis of the diseases are described below.

4.2.1.6.1 Fibrinogen beta chain (FGB), Fibrinogen gamma chain (FGG)

Fibrinogen is a heterohexamer that consist of two sets of three non-identical chains (alpha, beta, gamma) (Doolittle, 1984). Fibrinogen is cleaved by thrombin to form fibrin and acts as a cofactor in platelet aggregation. Increased level of fibrinogen was noticed in SLE cohorts in study by Ames and *et al.* (2000), as also shown in current study for non-renal SLE. Decrease in fibrinogen levels was observed in LN class V patients in this study and it has been documented that abnormal blood coagulation occurs in more deteriorated kidney conditions such as LN class V (Nagayama *et al.*, 1992). The decrease may also result from increased predisposition of LN class V patients to thrombotic complications, such as renal vein thrombosis, by which increased cleavage of fibrinogen takes place.

4.2.1.7 Kidney secretory and structural proteins

Only one protein involved in kidney secretion and structure was found to be associated with SLE/LN in this study and how its differential expression can affect the diseases are discussed as follows.

4.2.1.7.1 Gelsolin (GSN)*

The current study is the first to report the connection of plasma GSN levels with LN. Its low plasma levels were also noticed to be unique to LN class II.

Gelsolin (GSN) serves as a calcium-regulated and actin-modulating protein that binds to the barbed ends of actin filaments to prevent monomer exchange (end-blocking or capping) (Harris and Weeds, 1984, Kilhoffer *et al.*, 1985). Decreased plasma GSN level has been reported in acute liver failure, myocardial infarction, septic shock, and myonecrosis patients (Suhler *et al.*, 1997), as well as in trauma and rheumatoid arthritis patients (Dahl *et al.*, 1999, Osborn *et al.*, 2008). It has been also recommended as a biomarker for distant organ metastasis of colorectal cancer, with its high level in plasma of the patients (Tsai *et al.*, 2012). Less evidence has shown the correlation between plasma GSN and LN or other renal diseases. Decline levels of plasma GSN in patients with chronic kidney disease (CKD) has been documented, with the presence of detectable

circulating actin in the blood (Lee *et al.*, 2009). Local depletion of plasma GSN resulted from tissue damage and exposure of cytoplasmic actin allows inflammatory mediators to locally execute adaptive defence and repair functions, while depletion of circulating plasma GSN could be due to the protective action of this protein to prevent the inflammatory mediators from injuring organs away from the primary site of damage (DiNubile, 2008, Lee *et al.*, 2009). But the presence of low GSN level in plasma of LN class II patients (non-inflammatory) only in this study remains intriguing. It could be due to other underlying clinical manifestations such as pulmonary inflammation and sepsis (Lee *et al.*, 2008, Oikonomou *et al.*, 2009).

From **Figure 4.4**, it is noticed that GSN interacts with countless compounds. These include Ca^{2+} and polyphosphoinositide 4,5-bisphosphate (PIP_2), given that GSN is a Ca^{2+}- and PIP_2-regulated protein. PIP_2 is involved in phosphoinositide cycle that mediates signalling, cytoskeletal organization, and membrane trafficking. It regulates GSN by inactivating it. Gelsolin (GSN) is the only known Ca^{2+}-dependent severing protein identified by far, and the importance of Ca^{2+}-mediated actin severing has been reported during platelet activation (Hartwig, 1992). Besides that, interaction between GSN and caspase-3 (CASP3) is also found in **Figure 4.4**, as GSN is a substrate for CASP3 in apoptosis. Caspase-3 (CASP3) is the effector caspase in both the death receptor and mitochondrial apoptotic pathways (Kothakota *et al.*, 1997, Kamada *et al.*, 1998). Gelsolin (GSN) is cleaved by CASP3 and subsequently dismantles the membrane cytoskeleton to form blebs, which is a hallmark of apoptosis. Interactions of various actin-, PIP_2-, and caspase-related compounds with GSN can also be seen in the figure.

4.2.2 Protein-protein interactions and pathways involved in SLE/lupus nephritis

Protein-protein interaction (PPI) analysis of all key proteins in the SLE/LN groups revealed the existence of networks linked to various functions (**Figure 3.2.34 to 3.2.38**). For SLE patients without kidney involvement, only one network was generated and it is involved in cellular movement, inflammatory response, and cancer (**Figure 3.2.34**). Cellular movement may include the movement of cells as well as transport or homeostasis of ions ormolecules. Under inflammatory response, cellular movement could mean the migration of leukocytes, mainly neutrophils, along a chaemtactic gradient created by local cells to reach the site of injury and this is regulated by the interaction between IL-6 and

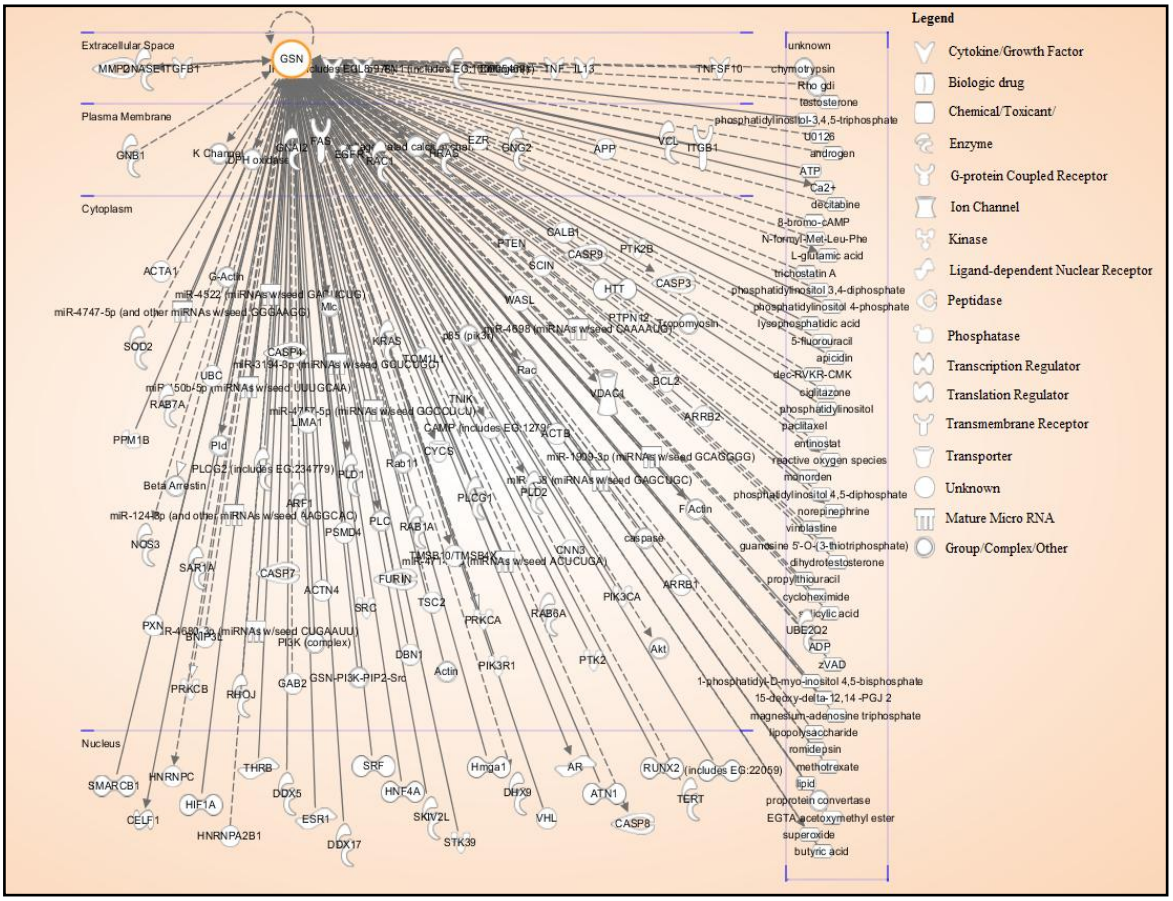


Figure 4.4. Example of functionality of GSN interaction pathway.

other proteins such as IL-1A and C-C motif chaemkine 2 (CCL2). The movement of coagulation factors such as fibrinogen (e.g. FGG) to the injury site during inflammation is also triggered by a series of interaction between the inflammatory mediators. Besides that, interactions involving CFH, C-reactive proteins (CRP), and C4BPB are also important in the activation of inflammatory response by acting as cofactors in the complement pathways. AAT (SERPINA) is an inhibitor of neutrophil elastase by which it protects the lower respiratory tract from destructive effects if neutrophil elastase during an inflammatory response. The cytokines oncostatin-M (OSM) and IL-6 have been shown to be responsible in the elevation of circulating levels of AAT during inflammation via the interaction of cytokine-inducible transcription factors with regulatory elements within the gene (Morgan *et al.*, 2002). Interaction of OSM, which is an IL-6 family cytokine, with other proteins may also promote breast cancer tumour invasion and metastasis (Douglas *et al.*, 1998).

Lupus nephritis (LN) class II pathologies are mainly affected by cellular movement, hematological system and function, and immune cell trafficking (**Figure 3.2.35**). The cellular movement and immune cell trafficking functions are similar and as

mentioned above, responsible for the migration of immune cells to the site of insult as a result of interactions and signalling of a series of inflammatory mediators. ALB and GSN have role in the hematological system and function by affecting the aggregation of blood platelet (Crane *et al.*, 2002), while HPX is essential for heme transport.

Interactions of proteins associated with LN class III mainly function in lipid metabolism, small molecule biochemistry, and vitamin and mineral metabolism (**Figure 3.2.36**). Abnormal lipid metabolism and dyslipidemia are often associated with cardiovascular risk in SLE (Nuttall *et al.*, 2003). In SLE, the activity of the key enzyme in lipid metabolism, i.e. lipoprotein lipase (LPL), might have been altered by autoantibodies such as anti-LPL and results in dyslipoproteinemia characterised by elevated levels of very low-density lipoprotein cholesterol (VLDL), and triglycerides (TG), and lower levels of high-density lipoprotein cholesterol (HDL) (Reichlin *et al.*, 2002, de Carvalho *et al.*, 2004, Borba *et al.*, 2006). In addition, enhanced production of cytokines involved in inflammation such as tumour necrosis factor (TNF), interleukin-1 (IL-1), and interferon (IFN)-gamma has been described to significantly down-regulate the activity of LPL (Ehnholm *et al.*, 1982, Beutler and Cerami, 1985, Semb *et al.*, 1987). Furthermore, monocyte chaemattractant protein-1 (MCP-1) and IL-6, the two other proatherogenic cytokines, were also found to be related with increased TG levels and decreased HDL levels, respectively (Asanuma *et al.*, 2006). Hypertriglyceridemia and low HDL in SLE may also contribute to an enhanced LDL oxidation (oxLDL) (i.e. Apo-B containing oxPL). Antibodies to oxLDL epitopes could possibly increase the uptake and accumulation of oxLDL to the endothelial wall (Vaarala *et al.*, 1993, Frostegard, 2005). The important role of high anti-apoA-1 IgG antibodies level and low HDL levels in SLE has also been reported (Lahita *et al.*, 1993). Besides that, long term use of corticosteroid in SLE has also been related to the elevated total plasma cholesterol and its fractions (LDL and HDL) levels, as well as TG, as a result of increased plasma insulin levels and lipid synthesis by the liver, and impaired lipid catabolism (Ettinger *et al.*, 1987, Bruce *et al.*, 1999, Sholter and Armstrong, 2000). The correlation between dyslipidemia and LN is not new (Appel *et al.*, 1985, Attman and Alaupovic, 1991, Frostegard *et al.*, 2005), and renal disease and renal failure has been well recognized as prominent risk factors for cardiovascular disease. Hence the involvement of lipid metabolism in LN, as observed in this study, is reasonably possible. Nevertheless, whether the aberrant lipid metabolism lead to the development of LN or it occurred secondary to LN could not be determined from this study. In addition,

defects in small molecule biochemistry and vitamin and mineral metabolism, such as retinol metabolism by RBP, are also associated with LN class III.

Lupus nephritis (LN) class IV is the relatively severe form of LN and it is related mainly to cell death and survival. In lupus, either a disturbed apoptosis or a reduced clearance of apoptotic cells may lead to the disruption of the balance between tolerance and autoimmunity resulted from the formation of autoreactive B and T cells and subsequently anti-nucleosome, anti-histone, and anti-DNA antibodies (Dieker *et al.*, 2008). In addition, dysregulation of autophagy in T cells may promote autoreactive T cell survival, or on the other hand, leads to an immunogenic cell death different from typical caspase-dependent apoptosis (Page *et al.*, 2011). In the current study, the dysregulation of CLU brings about the disruption of normal apoptosis, together with TF and IGKC. Secreted isoform of CLU protects cells from apoptosis and against cytolysis by complement, whereas nuclear isoforms enhance apoptosis. The abnormal apoptosis may potentially affect the cell cycle and tumour morphology.

Lupus nephritis (LN) class V is a milder form of LN compared to LN class II. The association of proteins involved in inflammatory disease with this disease this not surprising. However, the abnormal plasma level of ALB, C4BPA, CFB, CLU, FGB, HP, and IGHG1 that may lead to rheumatic disease have linked LN class V to connective tissue disorders. Arthritis is one of the manifestations of SLE. Whether or not this connection is caused by the underlying joint conditions of LN class V patients, or LN class V patients has a higher chance of having joint problem, further investigation is required.

4.2.3 Urine proteins associated with SLE/lupus nephritis

Some of the urine proteins have also been reported by previous studies to be associated with SLE or LN. These proteins are listed in **Table 4.3**, together with their theoretical MWs, functions and references reporting their association with SLE and LN. From the results in **Section 3.2.6.2** and **Figure 3.2.29**, the SLE-associated urine proteins reported in this study basically could be categorized into a few groups according to their functions, i.e. serum constitutional and transport proteins which accounted for the biggest portion (28%), proteases and inhibitors (24%), immunoglobulins and other immune proteins (16%), kidney secretory and structural proteins (12%), metal binding proteins (12%), lipoprotein metabolism (4%), and complement fractions (4%).

Table 4.3. Previously reported and newly discovered SLE-associated urine proteins.

Protein	Gene name	Reference	Function
Serum constitutional and transport proteins			
Alpha-1-acid glycoprotein 1	*ORM1*	(Watson *et al.*, 2012)	Acute-phase response, transport
Albumin	*ALB*	(Parving *et al.*, 1980, Yamada *et al.*, 1980, Terai *et al.*, 1987, Cottiero *et al.*, 1995, Batlle-Gualda *et al.*, 1997)	Blood protein
Ferritin light chain	*FTL*	(Nishiya *et al.*, 1989)	Iron storage, iron homeostasis, iron transport
Retinol-binding protein 4	*RBP4*	(Sesso *et al.*, 1994, Guy *et al.*, 1997)	Sensory transduction, transport, vision
Afamin*	*AFM*	-	Vitamin E binding protein, transport
Alpha-1B-glycoprotein*	*A1BG*	-	Unknown
Haptoglobin*	*HP*	-	Haemglobin binding, cellular iron ion homeostasis
Tetranectin*	*CLEC3B*	-	Bone mineralization, calcium ion binding
Vitamin D-binding protein*	*GC*	-	Transport
Lipoprotein metabolism			
Apolipoprotein A-I*	*APOA1*	-	Lipid metabolism and transport
Complement fractions			
Mannan-binding lectin serine protease 2*	*MASP2*	-	Complement pathway, immune response

Metal binding proteins			
Ceruloplasmin	*CP*	(Rovin and Zhang, 2009, Suzuki *et al.*, 2009)	Copper transport, ion transport
Transferrin	*TF*	(Suzuki *et al.*, 2009)	Ion transport, iron transport
Zinc-alpha-2-glycoprotein*	*AZGP1*	-	Lipid catabolism
Immunoglobulins and other immune proteins			
Ig alpha-1 and -2 chain C region	*IGHA1,2*	(Epstein, 1973, Hopper and Papagiannes, 1986, Hopper *et al.*, 1989, Tsai *et al.*, 1992)	Immune response
Ig gamma-2 chain C region	*IGHG2*		
Ig kappa chain C region	*IGKC*		
Ig lambda chain C regions	*IGLC1*		
Proteases and inhibitors			
Alpha-1-antichymotrypsin*	*AACT*	-	Acute-phase response, DNA binding, peptidase inhibitor activity, protein binding
Alpha-1-antitrypsin*	*AAT*	-	Acute-phase response, blood coagulation, haemstasis
Inter-alpha-trypsin inhibitor heavy chain H4*	*ITIH4*	-	Acute-phase response, calcium ion binding, endopeptidase inhibitor activity, hyaluronic acid binding
Kininogen-1*	*KNG1*	-	Blood coagulation, inflammatory response
Pancreatic alpha-amylase*	*AMY2A*	-	Carbohydrate metabolism
Protein AMBP*	*AMBP*	-	Cell adhesion, binding, transport

Kidney secretory and structural proteins			
Basement membrane-specific heparansulfate proteoglycan core protein*	*HSPG2*	-	Responsible for the fixed negative electrostatic membrane charge, cell adhesion; vascularization
Gelsolin*	*GSN*	-	Actin polymerization, cilium biogenesis
Polymeric immunoglobulin receptor*	*PIGR*	-	Epidermal growth factor receptor binding

* Newly discovered in this study

From **Table 3.2.12**, it was noticed that SLE patients had only 2 proteins abnormally excreted in the urine, which were inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and GSN. This is considered mild as compared to LN patients whose urine contained more than 10 abnormally excreted proteins. Such situation could be of similarity with microalbuminuria in subclinical nephropathy which occurs in patients without clinical renal disease (Parving *et al.*, 1980, Cottiero *et al.*, 1995, Batlle-Gualda *et al.*, 1997). Mesangial nephropathy has been considered responsible for this (Yamada *et al.*, 1980), but whether this can be used to predict the development of renal diseases in SLE patients is unclear (Batlle-Gualda *et al.*, 1997). Although different patient groups shared a number of abnormally excreted proteins, different fragments of these proteins were involved. For example, alpha-1B-glycoprotein were noticed in LN class II and class III but 3 of the protein fragments were affected in LN class II while 1 other fragment was affected in LN class III instead (**Table 3.2.11**). Considerable profound difference from normal level was observed for many urinary proteins/isoforms as compared to those of plasma, which was often more than 10-fold change, and involved numerous isoforms from the same proteins. Thus cut-off points for these proteins were set higher in order to make the results more significant. The more profound level of these urinary proteins/isoforms is most likely due to the deficiency in glomerular filtration and tubular reabsorption. Increased number of protein fragments or isoforms may arise from the tubular degradation of filtered protein and exocytosis of protein fragments towards the urinary side, in addition to genetic variants or alternative splicing of the gene. However, reports show that patients with renal disease and proteinuria may have progressive decrease in peptide excretion as a result of increasingly impairment of these kidney processes (Singh *et al.*, 2004, Prakash *et al.*, 2008). Unfortunately, the sequence or structure of each isoform in this study was not known and the information about their functions has not been documented. The excretory profile of the isoforms may reflect the patterns of kidney injury in each LN group. Lupus nephritis (LN) class V is a relatively milder form of LN that may present with few or no clinical or serologic manifestations of SLE (Donadio *et al.*, 1977). This form of LN is suggested to have renal pathogenesis different from that of proliferative LN (class III and class IV). Hence it is not surprising to find that the urinary protein levels were less affected for LN class V in this study.

Group-specific proteins shown in **Table 4.4** are proteins that were only found in the particular group of SLE/LN patients. Some of them have been linked to SLE/LN in previous investigations while some were newly discovered in the current study. These

Table 4.4. Urine proteins specific to SLE without kidney manifestations and each group of LN.

Group	Specific protein(s)
LN remission	Ferritin light chain (FTL)↑
LN class II	Mannan-binding lectin serine protease 2 (MASP2)↓
	Ig kappa chain C region (IGKC) ↓
	Kininogen-1 (KNG1)*↑
LN class IV	Retinol-binding protein 4 (RBP4)↓
	Alpha-1-antichymotrypsin (AACT)*↑
LN class V	Protein AMBP* (AMBP)↓

*Newly reported

proteins could be of potential use in the diagnosis as well as distinguishing the types of SLE in the future.

4.2.3.1 Serum constitutional and transport proteins

Serum constitutional and transport proteins generally were highly excreted in the urine of LN patients. This may be due to the reduced glomerular filtration rate (GFR) as a result of kidney damage and the serum proteins are insufficiently reabsorbed from the urine. Alpha-1-acid glycoprotein 1 (AGP1), albumin (ALB), retinol-binding protein 4 (RBP4), ferritin light chain (FTL) were among the proteins that have been documented to have association with SLE/LN, while the others were newly discovered in this present study (**Table 4.3**).

4.2.3.1.1 Alpha-1-acid glycoprotein 1 (AGP1)

Alpha-1-acid glycoprotein 1 (AGP1) is a transport protein in the blood which binds various ligands and synthetic drugs (Haughey *et al.*, 1985, Belpaire and Bogaert, 1990). It is also an acute phase reactant within increased in plasma level during acute inflammation (Schultz and Arnold, 1990). It plays a part in the coagulation of blood by having an antithromboplastic property (Nilsson and Yamashina, 1958). Increase in either

plasma or urinary AGP1 has been frequently linked with the occurrence of renal failure (Docci *et al.*, 1985, Rolan *et al.*, 1986, Vasson *et al.*, 1993, Vasson *et al.*, 1991) and the association between increased urinary AGP1 and juvenile-onset LN disease activity has been reported by Watson and colleagues (Watson *et al.*, 2012). Consistent with their findings, this present study also demonstrated an increased level of AGP1 excretion in the urine of LN class II and III as well as LN remission patients. The increase may be due to the response of AGP in protecting the glomerulus which is activated by a network of cytokines, including interleukin-1 beta, TNF-alpha, and interleukin 6 during the inflammation (Fournier *et al.*, 2000, Watson *et al.*, 2012).

4.2.3.1.2 Albumin (ALB)

Normal filtration fraction of ALB is reported in a range between 0.05 and 0.07% due to its size, shape, and negative electric charge which result in a very low glomerular sieving coefficient through normal glomeruli (Donadio *et al.*, 2012). Upon glomerular filtration, albumin will be almost completely reabsorbed by proximal tubular cells through the brush border via endocytosis. Hence, only very small amount is excreted unmodified into final urine (Donadio *et al.*, 2012).

A significantly high level of ALB in LN patients observed in this study was expected, seeing that albuminuria is one of the clinical presentations as a result of inflammation in the kidneys and damage to the glomerular filtration barrier with increased permeability to ALB. A less marked increase can also be attributed to a tubular dysfunction with reduced reabsorption of ALB (Donadio *et al.*, 2012). Most of the filtered ALB is returned to the peritubular blood supply by a retrieval pathway and albuminuria arises from dysfunction of this retrieval pathway (Comper *et al.*, 2008). Lupus nephritis (LN) patients who were already in remission also had high urinary ALB level and this may be due to persistent abnormalities in glomerular function, as reported by Cottiero and co-workers (1995).

Numerous ALB fragments were found excessively excreted in urine of LN patients. Most of the ALB isoforms derived from plasma, but a few were produced *in situ* by specific proteolysis (Candiano *et al.*, 2006). Candiano and co-workers (2006) suggested that the high concentration of ALB fragments in nephrotic urine indicates a preferential urinary excretion and reflects the partial maintenance of size selectivity properties of the

kidney. This can be used to explain the increased urinary level of different ALB fragments in different patient groups in present study, by having different preferential urinary excretion. Other studies reported that the kidney degrades large amounts of albumin and that the degraded fragments appear in the urine.

4.2.3.1.3 Retinol-binding protein 4 (RBP4)

Plasma RBP levels are low in patient with liver disease while are high in those with chronic renal disease (Goodman, 1980). These suggest that RBP is produced in liver and catabolized in kidneys. Urinary excretion of RBP4 in SLE patients was examined in a few studies (Sesso *et al.*, 1994, Guy *et al.*, 1997), however no clear conclusion on the association between RBP4 level and SLE was drawn, an increased level in LN patients was observed though. Urinary RBP has also been identified as a marker of renal disease activity, which progressively greater from without renal disease, stable renal disease, to active nephritis (Sesso *et al.*, 1994). Amazingly, the present study found a decreased RBP4 level in urine of LN class IV patients, with reason yet to be defined.

4.2.3.1.4 Ferritin light chain (FTL)

Ferritin is involved in iron storage and homeostasis. It is an acute-phase reactant that is increased in infection, inflammation, malignancy, and chronic disease (Ford *et al.*, 2009). Variation in composition of ferritin subunit potentially affects the rates of iron uptake and secretion in different tissues. FTL is the light subunit of the ferritin protein and the genetic defects has been associated with several neurodegenerative diseases (Luscieti *et al.*, 2010). Significantly elevated urinary ferritin in LN was reported by Nishiya and co-workers (Nishiya *et al.*, 1989), which was in accordance with findings in this study in the case of urinary FTL level. But this only occurred in LN patients who are already in remission. Other factors such as treatment and other SLE criteria may need to be taken into consideration to explain this situation and further study is required.

4.2.3.2 Lipoprotein metabolism

Significant increased urinary excretion of ApoA-I was observed from LN class II and class III patients in this study, involving 2 fragments. Although no literature has yet related the urinary level of ApoA-I with LN, its increased urinary level in patients with other nephrotic syndromes has been well recognised, which is as a result of inability of the impaired proximal tubules to reabsorb the large amounts of glomerularily filtered ApoA-I (Graversen *et al.*, 2008).

4.2.3.3 Complement fractions and Coagulation factors

Mannan-binding lectin serine protease 2 (MASP2) is a serine protease that activates the complement cascade via mannose-binding. Increase in urinary MASP2was reported in a study regarding contrast-induced acute kidney injury (Wang *et al.*, 2010). In contrast, decreased urinary MASP2 level was illustrated in microalbuminuric type 2 diabetic patients (Jin *et al.*, 2012), which may be used to support the findings of the current study. MASP2 regulates the maintenance of glomerular permeability and the pathogenesis of focal segmental glomerulosclerosis (Musante *et al.*, 2002). Low serum levels of MASP2 has been associated with several genetic polymorphisms, including four amino-acid tandem duplication (p.156_159dupCHNH), as well as SNPs p.R99C, p.R118C, p.D120G, p.P126L, and p.V377A (Thiel *et al.*, 2007). Seeing that the urinary reduction of MASP2 was only significant in LN class II in this study, the mentioned genetic polymorphisms could be also associated with patients with LN class II and the low serum levels may subsequently bring about the decreased excretion of MASP2 in urine. Anyhow, this is just a postulate and needs further investigations. Another possible reason for low levels of MASP2 is the abnormal complement system, one of the characteristics of SLE, which involve low C3 and C4 levels. Since MASP2 has a role in C4 cleavage and formation of C3 convertase, its levels could be also affected by these abnormalities.

4.2.3.4 Metal binding proteins

A general elevation in urinary levels of metal-binding proteins was seen in the present study. Association of transferrin (TF) and ceruloplasmin (CP) with SLE have been reported earlier while the zinc-alpha-2-glycoprotein (ZAG) has no record of association.

Urinary TF has been shown to increase in LN patients and have potential as biomarker of LN activity to help predicting the future course of LN (Suzuki *et al.*, 2009). The present study also found significantly and extremely high levels of urinary TF (>30-fold) in LN class III patients, involving 3 isoforms. This could be due to proteinuria as a result of kidney damage in LN.

Increased levels of urinary CP were noticed in LN patients compared to non-renal SLE patients and normal individuals in a study by Suzuki and co-workers (Suzuki *et al.*, 2009). The present study showed significant association between elevated urinary CP levels and LN class III and class IV, both are mesangiocapillary proliferative glomerulonephritis. The elevation could be because of proteinuria, or due to inflammation in the kidneys since CP is an acute-phase reactant. However, it is unclear why the excretion of both CP isoforms was higher in LN class III (focal and milder) compared with LN class IV (diffuse and more severe).

Urinary ZAG, although have not been associated with SLE or LN, was reported increased in patients with other renal diseases, including chronic glomerulonephritis and proximal tubular dysfunction (Ekman *et al.*, 1976). The increase may be due to increased glomerular permeability and defective proximal tubular reabsorption. In this study, a total of 5 fragments were found significant, with only 1 fragment being associated with LN class II, all 5 fragments with LN class III, and 4 fragments with LN class IV.

4.2.3.5 Proteases and inhibitors

Urinary excretion of proteases and inhibitors was another huge component which was found to LN patients. Studies suggested that proteases may result in glomerular injury by mechanisms dependent as well as independent of glomerular basement membrane (GBM) damage. Glomerular injury by proteolysis of the GBM are caused by polymorphonuclear leukocyte (PMN)-derived proteases (Cochrane *et al.*, 1965), whereas mechanisms that do not involve GBM damage include proteolysis of non-GBM proteins, proteinase-mediated alterations in the production of key glomerular metabolites, and proteinase-induced effects on cellular proliferation (Baricos and Shah, 1991). Increased urinary excretion of proteases has been reported for patients with various types of glomerulonephritis, such as diffuse proliferative glomerulonephritis (Sanders *et al.*, 1978) and membranous glomerulonephritis (Shibata and Nagasawa, 1977). However, besides

alpha-1-antichymotrypsin (AACT), alpha-1-antitrypsin (AAT), and kininogen-1 (KNG1), the urinary proteases found to be associated with SLE/LN in the present study had lower level instead. The reason for the decrease is yet to be investigated in the future. Significant increased of AACT and KNG1 excretion were exclusive in LN class IV and class II, respectively. The levels of the urinary protease inhibitors, such as inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and protein AMBP (AMBP) in this study, are expected to be decreased given that they are susceptible to degradation of proteolytic enzymes and thus potentially could result in glomerular injury via excess proteolytic enzymatic activity (Baricos and Shah, 1991). Declined urinary AMBP levels were unique to LN class V. None of the urinary proteins from the findings of the present study has been documented earlier to be correlated to LN.

4.2.3.6 Immunoglobulins(Ig) and other immune proteins

Normal human urine contains physiological amount of both kappa and lambda types of free light chains, as well as intact IgG and IgA (Rowe and Fahey, 1965). The presence of Fc fragment of IgG in normal human urine has also been reported after that (Berggard and Bennich, 1967). Due to their low molecular weights, these proteins which circulate in plasma are readily excreted into the urine, in contrast to the larger molecules such as IgM. A general significant increase of urinary excreted Ig chains were observed in all LN groups in the present study, mainly in LN class II and III cohorts, which included free light chains, heavy (alpha) chain of IgA and heavy (gamma) chain of IgG. These findings were consistent with those reported by previous investigations (Hopper and Papagiannes, 1986, Hopper *et al.*, 1989, Tsai *et al.*, 1992). Waldmann *et al.* (1972) showed in their turnover studies that only 60% of the newly synthesized light chains were eventually incorporated into complete antibody molecules; the remaining 40% were released into plasma and subsequently catabolized in the renal proximal tubules. In LN, the existence of urinary free light chains was attributed to both polyclonal B cell activation and renal tubule impairment, rather than just reflecting B cell Ig production in the normal state (Epstein, 1973). On the other hand, the presence of heavy chain in the urine of SLE patients could be associated with heavy chain diseases, which is characterized by the uncontrolled production of structurally incomplete Ig heavy chains (Husby *et al.*, 1998, Husby, 2000). Kappa and lambda light fragments may cause acute or chronic kidney damage by various mechanisms, including direct tubular toxicity, intratubular precipitation,

and interstitial deposition (Donadio *et al.*, 2012). As for why an elevation of urinary Ig kappa chain C regionwas seen only in LN class II patients in this study was not understandable and needs further investigation. And seeing that it is unique to LN class II, urinary Ig kappa chain C region could be a potential biomarker to discern this LN group from other LN classes.

4.2.3.7 Kidney secretory and structural proteins

Increased level of urinary kidney secretory and structural proteins are expected as autoantibodies and proteolytic enzymes may degrade GBM components and other structurally and/or functionally important intra- and extra- cellular proteins, such as receptors, cytoskeletal protein enzymes and so on, and are released in the urine (Gang *et al.*, 1970, Lubec *et al.*, 1980). Surprisingly, in contrary, the present study observed decreases in levels of almost all kidney secretory and structural proteins in urine from patients with SLE/LN, except for gelsolin. Gelsolinis involved in actin polymerization and maybe this was the reason for its increase in urine of LN class III patients who have sclerosis in their kidneys, despite a decrease was observed in SLE patients without kidney involvements. Significant and consistent decrease in urinary basement membrane-specific heparin sulfate proteoglycan core protein (HSPG2) was found in all LN groups, with 2 isoforms being associated with LN class III and class IV (more severe forms of LN) rather than 1 isoforms in LN class II and class V (milder forms of LN). Further investigation is required to determine the reason for this situation. Polymeric immunoglobulin receptor (PIGR) is an Fc receptor that facilitates the secretion and transport of IgA and IgM through renal epithelial cells in the humoral mucosa immune response of the kidneys. Decrease in urinary PIGR may indicate the increased incidence of urinary tract infection (Rice *et al.*, 1999). Two isoforms were found in this study: isoform 2 significantly decreased in LN class II, both isoforms 1 and 2 in LN class III, and isoform 1 in LN class IV. None of the urinary proteins found in this study has been reported by previous studies.

4.2.4 Comparative plasma and urine protein associated with lupus nephritis

Several proteins were found to have abnormal abundance levels in both plasma and urine of LN patients. These proteins included AFM, HP, ALB, RBP4, VDB, ApoA-I, CP, TF, ZAG, IGHA1 and 2, IGKC, and AAT, as shown in **Figure 4.5**.

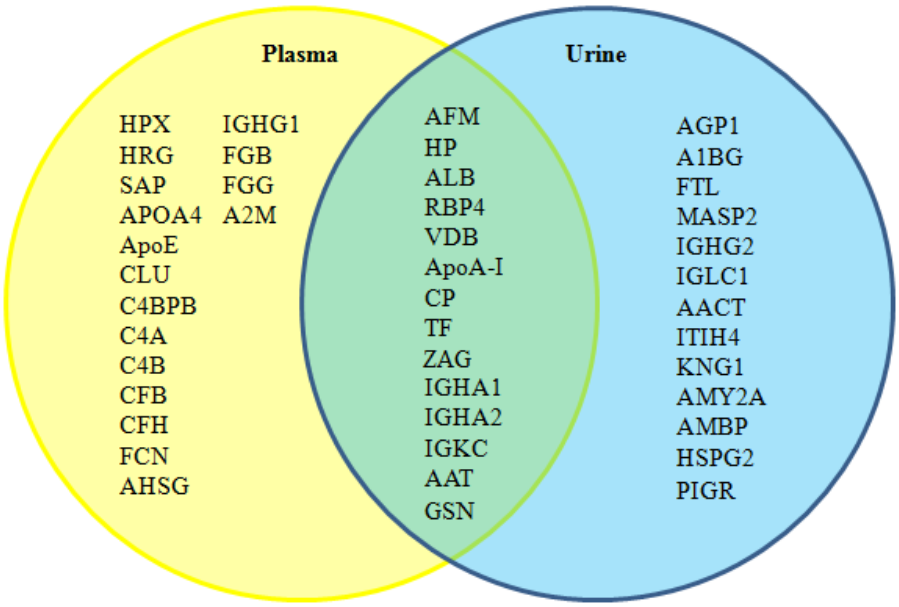


Figure 4.5. Comparative LN-associated plasma and urine proteins. The proteins listed in the overlapped area of the two circles were found to be at abnormal levels in plasma and urine of LN patients and SLE patients without kidney involvement.

Proteins that demonstrated higher plasma levels but lower urinary levels were RBP4 and IGKC. The increase in plasma levels may be due to the homeostasis of liver and immune response triggered by autoimmunity while the decrease in urinary levels needs further explanations. The proteins which levels were reduced in plasma but increased in urine of LN patients AFM, HP, ALB, VDB, IGHA1 and 2, and GSN. Various reasons may lead to the reduced plasma levels of these proteins, as mention in **Section 4.2.1**, such as acute-phase response. Impaired glomerular filtration and tubular reabsorption may increase their losses in the urine and this could be also one of the reasons for their low plasma levels. Proteins that showed elevated levels in both plasma and urine of LN patients were APOA-I, CP, TF, and ZAG. The elevated urinary excretion of these proteins may have triggered the homeostastic mechanisms of the liver in order to increase levels of these proteins in plasma.

Inflammation of kidneys and other organs could also concurrently raise the plasma and urinary levels of these proteins.

4.2.5 Changes of protein levels from active lupus nephritis to remission

Protein profiles of patients with LN class IV (active) was compared with those of patients who had already attained remission from previous LN class IV. The plasma levels of almost all abnormally regulated proteins in LN class IV have been successfully rectified upon progression to remission, except for TF which remained up-regulated as well as ZAG which was down-regulated instead. This could be a consequence of improved and controlled inflammatory and autoimmune responses in the patients that eventually brought the related plasma proteins back to their normal levels. However, there were several extra proteins found to be at their abnormal plasma levels in remission achieved patients, which were HP, HPX, ApoA-I, IGHA1 & 2, and CFI. The patients might have developed other types of SLE or the response to the proteins could be drug-induced. The low level of CFI is distinctive to LN in remission and therefore can be used to monitor the disease activity of the patients.

As for the urine proteins, only 2 out of 15 abnormally excreted urine proteins gained their normal levels after remission. The rest remained abnormally excreted, together with three additional proteins: AGP1, FTL, and IGLC1which were excessively excreted in patients in remission. No evidence has been found that could explain this situation, and this study postulated it as the result of sustained kidney damage and reduced GFR. Anyhow, further investigation is required to determine the correct explanation for these findings. On the other hand, increased urinary levels of AGP1 and FTL were specific for LN remission and it may be used as an indicator for monitoring the progression of LN to remission.

4.3 Longitudinal study of mycophenolate mofetil treatment

4.3.1 Mycophenolate mofetil and its mechanisms of action

Mycophenolate mofetil (MMF) is the morpholinoethyl ester of mycophenolic acid (MPA), which is its active metabolite. Mycophenolate mofetil (MMF) is initially recognised to be effective in prolonging survival of allografts and xenografts. Its

usefulness in autoimmune disease and SLE had been actively tested in late 1990s using murine models and it was proven to be able to suppress the development of lupus glomerulonephritis and albuminuria by reducing binding of immune complexes in the glomerular capillary wall in lupus nephritis (Corna *et al.*, 1997, Glicklich and Acharya, 1998). An open pilot trial study of MMF in SLE patients also showed that MMF is a promising alternative of immunosuppressive treatment for patients with moderate and severe SLE who were refractory to other immunosuppressants (Gaubitz *et al.*, 1999). Although the classical immunosuppressant, cyclophosphamide, gives excellent improvements in human LN, but seeing that they may increase the risk of malignancies and viral infections, it is slowly taken over by MMF which has less ovarian toxicity and studies has shown a more impressive efficacy of MMF compared with pulse intravenous cyclophosphamide therapy in treatment of diffuse proliferative lupus nephritis (DPLN) (Hu *et al.*, 2002, Mok and Lai, 2002). Besides LN, the possible role of MMF in the treatment of other SLE manifestations such as discoid lupus erythematosus (Goyal and Nousari, 2001), subacute cutaneous lupus erythematosus (Schanz *et al.*, 2002), haemolytic anaemia (Alba *et al.*, 2003), and immune thrombocytopenia (Vasoo *et al.*, 2003), as well as in other autoimmune diseases including vasculitis, myasthenia gravis, pemphigus vulgaris and inflammatory myopathy (Moder, 2003) have also been described.

Mycophenolate mofetil (MMF) inhibits proliferation of both B- and T-lymphocytes. Mycophenolic acid (MPA), the active agent of MMF, reversibly and non-competitively inhibits inosine monophosphate dehydrogenase (IMPDH) in lymphocytes. The depleting guanosine nucleotides thus inhibit purine synthesis and the depleting deoxyguanosine nucleotides subsequently inhibit the production of dATP (stimulated by dGTP) and dGTP, which are required for DNA synthesis and cell division (Allison, 2005). The cytotoxic effect is relatively more potent on lymphocyte compared with on fibroblast and other cell types (Allison and Eugui, 2000). MMF decreases antibody production and interferes with glycosylation of adhesion molecules and their *in vitro* expression (Adu *et al.*, 2001). Mycophenolate mofetil (MMF) also has non-immune effects on the kidneys and these include decreasing mesangial cell proliferation in experimental systems and inhibiting glomerular, tubular and interstitial cellular proliferation in animal model (Ardiles *et al.*, 2002, Pisoni *et al.*, 2005). Mycophenolate mofetil (MMF) is also important in inhibiting inducible nitric oxide synthase (iNOS) which produces high concentration of NO over a longer period and can damage tissues when NO forms procynitrite with

superoxide produced by cytokine activated monocyte/macrophage lineage cells and endothelial cells (Sugimoto *et al.*, 1999).

4.3.2 Mycophenolate mofetil-associated proteins

Most MMF-associated proteins did not show clear trends of level change during the treatment. Proteins which showed significant differential increase (≥1 fold) or decrease (≤1 fold) in plasma and urinary levels between the time before treatment and 5th doctor visit (8 month of MMF administration) were selected in this study. However slight fluctuations of protein levels were seen during the treatment. This could be due to the number of patient samples included in this study. More patient samples may provide more accurate average protein levels and reflect more proper trend of protein changes. Although the sample collection was done in the morning, no particular time was fixed and it was completely dependent on the time the patients arrived in the hospital. Since the protein expressions are dynamic, the levels of proteins could be affected by the time of sample collection, as well as the diet and activity of the patients before sample collection was carried out. The most appropriate time for sample collection (may not be in the morning) that can best reflect the protein levels influenced by MMF treatment is also yet to be identified.

4.3.2.1 Plasma proteins

Ten (10) plasma proteins were found to be associated with MMF treatment in this study and mechanisms of MMF that may cause these protein level changes were discussed below.

4.3.2.1.1 Apolipoproteins

A decreasing trend was observed for several apolipoproteins, i.e. apolipoprotein A-IV, E and H. Apolipoprotein A-IV and E were found elevated in plasma of LN class III patients in earlier section (**Table 3.2.5**). This indicated that MMF is able to bring the level of these two apolipoprotein back to normal by gradually reducing it. However, MMF caused down-regulation of apolipoprotein H which was initially normally expressed in LN

class III patients. The mechanisms of action of MMF in these conditions are yet to be elucidated.

4.3.2.1.2 Angiotensinogen

Angiotensinogen is a component of the renin-angiotensin system (RAS) which is involved the regulation of blood pressure, body fluid and electrolyte homeostasis (Tomita *et al.*, 1995). In response to reduced blood pressure, the enzyme renin cleaves angiotensinogen to yield angiotensin-1, which is a substrate of angiotensin converting enzyme (ACE) to produce angiotensin-2. The level of angiotensinogen, which did not show abnormal regulation in LN class III patients in the earlier experiment, was generally decreasing along the treatment in the present study. MMF has been demonstrated to have ACE inhibition activity (Moscoso-Solorzano *et al.*, 2009) and this present study speculated that this inhibition may cause accumulation of angiotensin-1 which signals the down-regulation of angiotensinogen. Further study is warranted to prove this speculation.

4.3.2.1.3 Ceruloplasmin (CP)

CP is a multicopper oxidase in the plasma. Increase in plasma CP levels potentially indicates an early progression of atherosclerosis in the condition through the mechanisms of increasing oxidized LDL and thus inhibiting nitric oxide (NO) production that can raise blood pressure (Daimon *et al.*, 2000). In this study, it was noticed that MMF increased the CP level and this brings up the question whether or not MMF will increase the risk of atherosclerosis in the patients. MMF has been said to inhibit NO production also, but these NO are the ones that cause tissue damage such as nephropathy rather than those acting on blood vessels (Allison, 2005).

4.3.2.1.4 Complement C4-A & B (C4A & C4B)

As mentioned earlier in **Section 4.2.1** that C4A and C4B deficiency with low level of C4A and C4B increases the susceptibility to SLE. In this study, MMF was found to improve the disease by elevating the C4A and C4B level in plasma of the patients.

4.3.2.1.5 Fibrinogen gamma chain (FGG)

Both up- and down-regulation of FGG were observed in this study upon MMF administration. Increased level of FGG has been associated with SLE/LN, or LN class III for this study, and down-regulation of FGG by MMF could be due to the MCA inhibition of mesangial cell activation and excess production of collagen and other matrix proteins to prevent progression of glomerulonephritis (Dubus *et al.*, 2002). Up-regulation of the other isoforms of FGG remains to be explained in further study.

4.3.2.1.6 Gelsolin (GSN)

Plasma GSN binds actin and bioactive mediators to localize inflammation. Low plasma GSN correlates with adverse outcomes in acute injury as well as with deteriorating renal function (Lee *et al.*, 2009). The effect of MMF in this study was ambiguous as it was supposed to suppress inflammation and bring up the level of plasma GSN.

4.3.2.1.7 Haptoglobin (HP)

As HP is an acute-phase reactant which is elevated during inflammation, the present study proved the action of MMF as an immunosuppressant in decreasing the plasma HP level in LN class III patients after the drug was taken.

4.3.2.1.8 Albumin (ALB)

Declined plasma ALB level is associated with SLE/LN and this was also shown in LN class III patients in the earlier experiment in **Section 4.2.1**. Nevertheless, the action of MMF in further decreasing the ALB level in LN class III patients in this study was obscure and requires further investigation. Moreover, only one spot among so many ALB spots was found to decrease and this may not represent the overall trend of this protein in response to MMF treatment.

4.3.2.1.9 Tetranectin (TN)

The pathophysiological role of TN in SLE/LN is unclear, yet decreased level of TN has been reported in rheumatoid arthritis patients, and its role in fibrinolysis was emphasized (Kamper *et al.*, 1997). LN class III patients in this study did not have TN abnormally expressed in their plasma. Thus, assuming that decreased level of plasma TN also occurs in SLE/LN patients, MMF may be able to recover its normal level by increasing it during the treatment, as seen in the present study. The interaction between TN and MMF requires further delineation.

4.3.2.2 Urine proteins

Alpha-1-antichymotrypsin, Ig alpha-1 chain C region, and lysosomal alpha-glucosidase were found to be generally elevated in urine of LN patients in the current study (**Section 3.2.6.2**). MMF showed its ability to recover their levels by increasing them in the urine of LN patients along the treatment. Alpha-1-antichymotrypsin is an acute-phase reactant and its decline in level was anticipated as MMF is an immunosuppressive agent. MMF inhibits the proliferation of B lymphocytes and hence a decrease in antibody production such as Ig alpha-1 chain C region was also expected. Lysosomal alpha-glucosidase is involved in the degradation of glycogen to glucose in lysosome. Delivery of this protein is enhanced by ICAM-1, an adhesion molecule that is overexpressed in tissues, including glomerular and tubular epithelium, of patients with SLE and other diseases during inflammation. Hence, the decrease in urinary lysosomal alpha-glucosidase in this study could be due to the reduced ICAM-1 as a result of the inflammation suppression by MMF (Lewis and D'Cruz, 2005, Hsu *et al.*, 2011). Further elevation of urinary albumin by MMF continues to be an issue to be investigated in the future study. However, since the result was produced by only one albumin spots, it may not be able to represent the whole picture of albumin level change along the MMF treatment.

In conclusion, other than mechanism of actions that had been described previously, the present study may give ways to exploration of novel mechanisms and effects of MMF on the SLE/LN patients. However, since this was the preliminary study for this objective, further study is required to better delineate its additional effects on the pathogenesis of SLE/LNthat can bring about the recovery of the diseases.

4.4 Limitations and future study

Several limitations were encountered in this study and they could be improved in the future study.

4.4.1 Sample size

In the genomic analysis, the sample size in this study might not be large enough to produce significant results that may arise from a much larger investigation. This was because the patients were only recruited from one hospital, the number of patients, nursing staff, rheumatologists and nephrologists interested enough to participate, were rather limited. Larger samples can be included in the future longitudinal studies by recruiting patients including more Indians, from other hospitals in Malaysia. This may increase the power for genetic association analysis and produce more convincing results.

In the longitudinal proteomic analysis of MMF treatment, at least 3 patients should be included. However, for the duration of this study, we were only able to recruit 2 patients due to incompliance of patients with their hospital appointments and earlier remission of these patients than expected. Hence, for further work, very close communication between researchers and patients should be established from the onset. The recruitment of a dedicated hospital staff would be recommended to ensure that patients comply with their treatments, meet appointment times, as well as to discuss how patients respond to the drug during the treatment.

4.4.2 Sample collection

Protein expression is dynamic and the protein levels can be affected by various factors, including diet, environment, daily activity, and emotion. Protein levels may vary if samples are collected at different points of time. Although patient samples in this study were collected in the morning, the collection time was not specified and was dependent solely on the arrival of patients in the hospital. Thus, in the current proteomic analysis, especially the longitudinal proteomic study of MMF treatment, fluctuation of protein levels was seen. To improve sample collection in the future study, patients may be requested to arrive at a certain time frame and the samples are processed in the lab as soon as possible

to avoid protein degradation. In addition, the sample collection time that can best reflect the protein levels affected by certain treatment should be also determined to avoid false results.

4.4.3 Clinical records

Since UMMC has only minimal information on the clinical manifestations of each SLE patient, association study between clinical manifestations of SLE and SNPs could not be carried out in any depth during this study. A more systematic record of patients' clinical manifestations should be implemented in the future study under closer collaboration with the clinicians so that the clinical records can be easily traced back for investigation.

4.4.4 Time constraints

Although this study managed to achieve all the objectives in the given time frame, it is conceivable that better results could have been obtained if more time was allowed for patient recruitment, MMF treatment follow-up, and validation. This represents a challenge in a developing country where brief clinical times for each patient in public hospitals, are dedicated to consultations and treatments rather than research which is viewed as a luxury. Also, time spent on chemical purchasing and long awaited delivery times had also slowed down the progress of the present study. More speedy arrangements with less preparatory paperwork would be preferable in future to maximise the time allocated for the study.

4.4.5 Application experts

The lack of application specialists and technical experts, especially in 2-DE and statistical analyses, had challenged the progress of the present study. As such, more time was required for troubleshooting and to explore the usage of various software options. Nevertheless, it was a learning exercise which taught me a great deal.

4.4.6 Validations and further approaches

Very limited validations could be performed on the results of the proteomic analyses due to time constraints. As such, these results presented here may be viewed as preliminary until further work. Validation of biomarker candidates can be performed using single or multiple reaction monitoring (SRM or MRM) analysis. It is a highly sensitive and selective method for the targeted quantitation of protein/peptide abundances in complex biological samples using mass spectrometry. Another approach that can be used for this purpose is Taqman Protein Assay (Applied Biosystems). It enables relative quantitation of protein using specific protein-binding antibodies.

For genomic analyses, with availability of more funds, the performance of genome-wide association study (GWAS) can be considered in future to examine larger number of SNPs as well as to discover novel SNPs that are associated SLE in these patients. Inclusion of more SNPs will also make the SNP-SNP interaction analysis more conclusive and meaningful, and allow more thorough understandings of molecular pathology of SLE in the Malaysian scenario. Current pricing for DNA microarray chips is still high.

Transcriptomic approaches can be carried out to complement the genomic and proteomic analyses of this study. These can be done by techniques such as RNA microarray and quantitative real-time PCR. It is interesting to investigate the whole process of protein expression, starting from discovery of SNPs associated with SLE susceptibility, transcription of genes bearing the SNPs, translation of mRNAs to proteins, and post-translational modifications of the proteins. Development of drugs that obstruct any step in the protein expression may be useful for the treatment of SLE.

Chapter 5

Conclusion

This investigation has provided insight into the molecular pathology of SLE in Asians. This was achieved by both genomic and proteomic approaches. Based on the significances and objectives proposed at the beginning of study, I would like to conclude this study with the following findings:

1. The association of genetic polymorphisms in *STAT4*, *TNFAIP3*, and *IRF5*, which are genes involved in TLR/IFN signaling pathways, with SLE in Malaysian patients of Chinese and Malay ethnicity has been determined. The presence of associations of polymorphisms in *STAT4* (rs7574865 and rs10168266) and *TNFAIP3* (rs3757173 and rs2230926) with SLE susceptibility in the Malays and Chinese, corroborated the role of TLR/IFN signaling pathways in this disease. The evidence of interaction between *STAT4* rs7574865 and rs10168266 in the LD and MDR tests further highlighted the importance of these polymorphisms as SLE genetic risk factors in both these ethnic groups in Asia. This study was also the first to investigate and report the connection between TLR/IFN signalling pathways and the Malaysian SLE scenario.

2. The association of new HLA variants, *HLA-DRB1/HLA-DQA1* rs9271366 and *HLA-DQB1/HLA-DQA2* rs9275328, with SLE has been determined. Both SNPs were found to be significant in the Malays and Chinese. There results further confirmed the role of HLA gene in SLE susceptibility in the Malaysian population. This study also provides new knowledge of SNPs associated with SLE in the Asians compared to that in Hispanics, Caucasians, and African Americans.

3. Plasma and urine proteins that were differentially expressed in patients with LN and SLE without kidney involvement have been identified using 2D-DIGE and LC-MS/MS approaches. These plasma proteins included some newly reported proteins such as retinol-binding protein 4, zinc-alpha-2-glycoprotein, and gelsolin. Most urine proteins were newly reported in this study. This explained the sensitivity of the approaches used in this study that managed to uncover relatively more and lower abundant proteins. This also brought about the identification of unique proteins that were differentially expressed or

excreted in each class of LN patients. These proteins can be of potential diagnostic values, supplementary to biopsy, in the discrimination of LN classes. The identification of urinary proteins can be of greater value since they can be used for the development of non-invasive diagnostic methods.

4. Protein-protein interactions between key proteins associated with each class of LN has been established. This study was able to recognise the networks or pathways responsible for the pathogenesis of each class of LN. For instance, pathway or network involved in cell cycle, cell death and survival, and tumor morphology might influence the development of LN class IV. The understanding of the pathogenesis of SLE is imperative for improvement of clinical management of this complex disease. This study was an important starting point towards the development of therapy solely targeting on the particular LN class by further studying its respective pathway, with less side effects.

5. Proteins that regained their normal levels upon LN remission have been identified. Most plasma proteins that were differentially expressed during active LN class IV regained their normal levels upon remission. However, most urinary proteins remained at their abnormal levels even LN remission was achieved. This suggests that permanent kidney damage occurs in LN class IV patients.

6. The effect of MMF treatment on plasma and urinary protein levels has been recognised. This may provide an initial picture on how the drug affects the protein levels that can bring active LN into remission. With further study, these findings may lead to the delineation of the existing effects of MMF, or to pave the way for exploration of new mechanism of actions of MMF and treatment options.

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Appendices

Appendix I

Ethics Approval Letter and Consent Form

UNIVERSITI MALAYA
KUALA LUMPUR
PUSAT PERUBATAN UM

JAWATANKUASA ETIKA PERUBATAN
PUSAT PERUBATAN UNIVERSITI MALAYA
ALAMAT: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEFON: 03-79494422 samb. 3209 FAKSIMILI: 03-79494638

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, University Malaya Medical Centre **ADDRESS:** LEMBAH PANTAI 59100 KUALA LUMPUR	**ETHICS COMMITTEE/IRB REFERENCE NUMBER:** 733.19
PROTOCOL NO: **TITLE:** Molecular Pathology Of Systemic Lupus Erythematosus In Asians	
PRINCIPAL INVESTIGATOR: Prof. Madya. Chua Kek Heng **TELEPHONE:** **KOMTEL:**	**SPONSOR:**

The following item [✓] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[✓] Borang Permohonan Penyelidikan	Ver date: 13 Jun 09
[✓] Study Protocol	Ver date:
[ ] Investigator Brochure	Ver date:
[✓] Patient Information Sheet	Ver date:
[✓] Consent Form	Ver date:
[ ] Questionnaire	
[✓] Investigator(s) CV's (Prof. Madya. Chua Kek Heng)	

and have been [✓]

[✓] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Comments:

i. *Investigator is required to follow instructions, guidelines and requirements of the Medical Ethics Committee.*
ii. *Investigator is required to report any protocol deviations/violations through the Clinical Investigation Centre and provide annual/closure reports to the Medical Ethics Committee.*

Date of approval: 22nd JULY 2009

s.k Ketua
Jabatan Perubatan Molekul

Timbalan Dekan (Penyelidikan)
Fakulti Perubatan, Universiti Malaya

Setiausaha
Jawatankuasa Penyelidikan Pusat Perubatan
Fakulti Perubatan, Universiti Malaya

[redacted]

.....
PROF. KULENTHRAN ARUMUGAM
Deputy Chairman
Medical Ethics Committee

CONSENT BY PATIENT FOR SAMPLE COLLECTION

I, …….……, Identity Card No..…….

(Name of Patient)

of ……..

(Current address)

hereby agree to take part in the study (research study/questionnaire study) specified below:

Molecular Pathology of Systemic Lupus Erythematosus in Asians

The nature and purpose of which has been explained to me by

Dr./ Mr./ Ms. .. ..…….……and interpreted by …….

(Name of Doctor/ Researcher) *(Name of Interpreter)*

to the best of his/her ability in …… language/dialect.

I have been told about the nature of the research study in terms of methodology, possible drawbacks and expected outcomes (as per patient information sheet). After knowing and understanding all the possible advantages and disadvantages of this research study, I voluntarily consent of my own free will to participate in the clinical research specified above.

I understand that I can withdraw from this research study at any time without assigning any reason whatsoever and in such a situation shall not be denied the benefits of usual treatment by the attending doctors.

Date:……...… Signature:…….…….……

(Patient)

IN THE PRESENCE OF

Name: ……

I/C No.: ……. Signature: ……..

Designation: …….. (*Witness for Signature of Patient)*

I confirm that I have explained to the patient the nature and purpose of the above-mentioned clinical research.

Date …… Signature: ……...

(Attending Doctor/ Researcher)

R.N.:_____ Name:_____ Sex: M/F Age: _____ Unit: _____

Appendix II

Preparation of Chemical Solutions

A. Two-dimensional electrophoresis and SDS-PAGE

1. Rehydration Buffer (to prepare 25 ml)

Chemicals	Amount	Final concentration
Urea	6.3 g	7 M
Thiourea	2.28 g	2 M
CHAPS	0.5 g	2% (w/v)
IPG buffer (pH 3-10/4-7)	125 µl	0.5% (v/v)
DTT	Add 2.8 mg per ml aliquot of rehydration stock solution prior to use	
Orange G	A few milligrams, for coloring	
Double distilled H_2O	To 25 ml	

Divide into 1 ml aliquots and store in freezer

2. DIGE Lysis Buffer (Labeling Buffer) (to prepare 25 ml)

Chemicals	Amount	Final concentration
Urea	6.3 g	7 M
Thiourea	2.28 g	2 M
CHAPS	1.0 g	4% (w/v)
Tris	0.09 g	30 mM
Double distilled H_2O	To 25 ml	

Divide into 1 ml aliquots and store in freezer

3. DIGE Stop Solution (to prepare 10 ml)

Chemicals	Amount	Final concentration
Lysine	18 mg	10 mM
Double distilled H_2O	To 10 ml	

Divide into 1 ml aliquots and store in freezer

4. DIGE 2X Lysis Buffer (to prepare 25 ml)

Chemicals	Amount	Final concentration
Urea	6.3 g	7 M
Thiourea	2.28 g	2 M
CHAPS	1.0 g	4% (w/v)
Orange G	A few milligrams, for coloring	
Double distilled H_2O	To 25 ml	

Divide into 1 ml aliquots and store in freezer

Before use add to 1 ml stock

IPG buffer (pH 3-10/4-7)	20 µl	2% (v/v)
DTT	20 mg	2% (w/v)

Prepare fresh

5. 4X Resolving Gel Buffer (Tris-HCl, pH8.8) (to prepare 1 L)

Chemicals	**Amount**	**Final concentration**
Tris-base	181.7 g	1.5 M
Double distilled H_2O	To 750 ml	
HCl	Adjust to pH 8.8	
Double distilled H_2O	To 1 L	

Store up to 3 months at 4 ºC in the dark

6. SDS equilibration buffer (to prepare 500 ml)

Chemicals	**Amount**	**Final concentration**
Tris-HCl, pH8.8	25 ml	50 mM
Urea	180.2 g	6 M
Glycerol (87% v/v)	172.5 ml	30% (v/v)
SDS	10.0 g	2% (v/v)
Double distilled H_2O	To 500 ml	

7. 10% SDS (to prepare 50 ml)

Chemicals	**Amount**	**Final concentration**
SDS	5.0 g	10% (w/v)
Double distilled H_2O	To 50 ml	

8. 10% Ammonium Persulfate (prepare just prior to use)

Chemicals	**Amount**	**Final concentration**
Ammonium persulfate	0.10 g	10% (w/v)
Double distilled H_2O	To 1 ml	

9. SDS Cathode Buffer (10X) (to prepare 1 L)

Chemicals	**Amount**	**Final concentration**
Tris-base	30.4 g	0.25 M
Glycine	144.0 g	1.92 M
SDS	10.0 g	1% (w/v)
Double distilled H_2O	To 1 L	

10. Agarose Sealing Solution (to prepare 100 ml)

Chemicals	Amount	Final concentration
Agarose (M or NA)	0.5 g	0.5% (w/v)
SDS cathode buffer (1X)	To 100 ml	

11. Casting of 12.5% Homogeneous Gel (to prepare 100 ml, 4 gels)

Chemicals	Amount
Monomer solution	41.7 ml
4X resolving gel buffer	25.0 ml
10% SDS	1.0 ml
Double distilled H_2O	31.8 ml
10% ammonium persulfate	500 µl
TEMED	33 µl

12. Casting of 10% Homogeneous Mini Gel (to prepare 20 ml, 2 gels)

Chemicals	Amount
Monomer solution	6.7 ml
4X resolving gel buffer	5.0 ml
10% SDS	0.2 ml
Double distilled H_2O	8.0 ml
10% ammonium persulfate	100 µl
TEMED	6.7 µl

13. 4X Stacking Gel Buffer (to prepare 50 ml)

Chemicals	Amount	Final concentration
Tris-base	3.0 g	0.5 M
Double distilled H_2O	To 40 ml	
HCl	Adjust to pH 6.8	
Double distilled H_2O	To 50 ml	

Store up to 3 months at 4 ºC in the dark

14. Stacking Gel Solution (to prepare 2 gels)

Chemicals	Amount
Monomer solution	0.88 ml
4X stacking gel buffer	1.66 ml
10% SDS	66 µl
Double distilled H_2O	4.06 ml
10% ammonium persulfate	33.4 µl
TEMED	3.3 µl

15. 2X Solubilizing Buffer (to prepare 10 ml)

Chemicals	Amount	Final concentration
4X stacking gel buffer	2.5 ml	0.125 M
10% SDS	4.0 ml	4% (w/v)
Glycerol	2.0 ml	20% (v/v)
Bromophenol blue	0.002 g	0.02% (w/v)
DTT	0.31 g	0.2 M
Double distilled H_2O	To 10 ml	

Store 0.5 ml aliquots at -20 ºC for up to 6 months

B. Silver Staining

16. Fixing Solution (to prepare 1 L)

Chemicals	Amount	Final concentration
Ethanol	400 ml	40% (v/v)
Acetic acid	100 ml	10% (v/v)
Double distilled H_2O	To 1 L	

17. Fixing/Sensitizing Solution (to prepare 1 L)

Chemicals	Amount	Final concentration
Glutaraldehyde	500 µl	0.05% (v/v)
Formalin	100 µl	0.01% (v/v)
Ethanol	400 ml	40% (v/v)
Double distilled water	To 1 L	

18. Rinse Solution (to prepare 1 L)

Chemicals	Amount	Final concentration
Ethanol	400 ml	40% (v/v)
Double distilled H_2O	To 1 L	

19. Sensitizing Solution (to prepare 1 L)

Chemicals	Amount	Final concentration
Sodium thiosulfate	0.2 g	0.02% (w/v)
Double distilled H_2O	To 1 L	

20. Silver Nitrate Solution (to prepare 1 L)

Chemicals	Amount	Final concentration
Silver nitrate	1 g	0.1% (w/v)
Double distilled H_2O	To 1 L	

21. Developing Solution (to prepare 1 L)

Chemicals	Amount	Final concentration
Sodium carbonate	25 g	2.5% (w/v)
Formalin	400 µl	0.04% (v/v)
Double distilled H_2O	To 100 ml	

22. Stop Solution (to prepare 1 L)

Chemicals	Amount	Final concentration
Disodium EDTA, dihydrate	14.6 g	0.04 M
Double distilled H_2O	To 100 ml	

C. In-gel trypsin digestion and peptide extraction

23. 25 mM Ammonium Bicarbonate (to prepare 100 ml)

Chemicals	Amount	Final concentration
Ammonium bicarbonate	0.2 g	25 mM
Double distilled H_2O	To 100 ml	

24. Trypsin Solution (to prepare 10 ml)

Chemicals	Amount	Final concentration
Porcine trypsin	0.1 g	10 ng/µl
Double distilled H_2O	To 10 ml	

25. 5% Formic Acid in 50% Acetonitrile (to prepare 100 ml)

Chemicals	Amount	Final concentration
Formic acid	5 ml	5% (v/v)
Acetonitrile	50 ml	50% (v/v)
Double distilled H_2O	To 100 ml	

Appendix III

LC-MS/MS Parameters

Instrument: Agilent 1200 HPLC-Chip/MS Interface, coupled with Agilent 6520 Accurate-Mass Q-TOF LC/MS

LC Parameters:

Column Used: Large Capacity Chip, 300 Å, C18, 160nL enrichment column & 75umx150mm analytical column
Agilent Part no: G4240-62010

Flow Rate: 4uL/min from Agilent 1200 Series Capillary pump and 0.3uL/min from Agilent 1200 Series Nano Pump

Solvents: 0.1% formic acid in water (A); 90% Acetonitrile in water with 0.1% formic acid (B)

Injection Volume: 1uL

Sample Analysis: Gradient with Agilent 1200 Series nanoflow LC pump as shown below.
method saved as: peptide-aMSMS-8-4-4-30(47)-HCchip.m

Time (min)	B(%)	
Initial	3	
30	50	
32	95	
37	95	
38	3	Stop time: 47 min

MS Parameters:

Ion Polarity: Positive

Vcap: 1980V

Fragmentor Voltage: 175V

Gas Temperature: 300ºC

Drying Gas Flow: 5 L/min

Data Acquisition: Spectra acquired in aMSMS mode: MS scan range 110-3000 m/z; MS/MS scan range 50-3000 m/z

Remarks:

Precursor Selection I: Precursor Abs. Threshold: 200

Precursor Selection II: 2, 3, >3 are included in the Precursor Charge State Selection and Preference

Preferred/Exclude: Exclude Precursor m/z of 922.009798 (Z=1) and 121.050873 (Z=1) (reference ions)

Data Analysis: Data was processed with
1) Agilent Spectrum Mill MS Proteomics Workbench software packages.

Spectrum Mill Settings:

MH+ Scan Range: 600 to 4000 Da

Scan Time Range for MS/MS Spectral: All

Database Search: SwissProt.MAR.2011.fasta

Species: Homo Sapiens

Modification: Carbamidomethylation (c)

Instrument: Agilent ESI Q-TOF

Search Mode: Identity

Mode to Summarize Result: Protein Summary Details (All, with validation status); Peptide (All, with validation status)

Filter by protein score: >11

Filter peptides by: Score: >6 ; **%SPI:** >60

Additional Procedure: Perform autovalidation with protein rules as below,

Rule	Precursor Charge	Score Threshold	% SPI Threshold	Fw-Rev Score Threshold	Rank 1-2 Score Threshold
1	2	6	60	2	2
2	1	6	70	2	2
3	3	8	70	2	2
4	4	8	70	2	2
5	5	12	70	2	2
6	2	6	90	1	1

Note: Default Values

Appendix IV

Publications and Presentation

Publications

1. CHAI, H. C., PHIPPS, M. E. & CHUA, K. H. 2012. Genetic risk factors of systemic lupus erythematosus in the Malaysian population: a minireview. *Clin Dev Immunol*, 2012, 963730. (doi:10.1155/2012/963730)

2. CHAI, H. C., PHIPPS, M. E., OTHMAN, I., TAN, L. P. & CHUA, K. H. HLA variants rs9271366 and rs9275328 are associated with systemic lupus erythematosus susceptibility in Malays and Chinese. *Lupus*, 2013, 2198-204. (doi: 10.1177/0961203312470183)

Presentation

1. Polymorphisms of genes involved in toll-like receptor/interferon signaling pathways in systemic lupus erythematosus. (Poster presentation) [In Autoimmunity Congress Asia (ACA), 17 to 19 November 2011]

Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 963730, 9 pages
doi:10.1155/2012/963730

Review Article

Genetic Risk Factors of Systemic Lupus Erythematosus in the Malaysian Population: A Minireview

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SLE is an autoimmune disease that is not uncommon in Malaysia. In contrast to Malays and Indians, the Chinese seem to be most affected. SLE is characterized by deficiency of body's immune response that leads to production of autoantibodies and failure of immune complex clearance. This minireview attempts to summarize the association of several candidate genes with risk for SLE in the Malaysian population and discuss the genetic heterogeneity that exists locally in Asians and in comparison with SLE in Caucasians. Several groups of researchers have been actively investigating genes that are associated with SLE susceptibility in the Malaysian population by screening possible reported candidate genes across the SLE patients and healthy controls. These candidate genes include *MHC* genes and genes encoding complement components, TNF, FcγR, T-cell receptors, and interleukins. However, most of the polymorphisms investigated in these genes did not show significant associations with susceptibility to SLE in the Malaysian scenario, except for those occurring in *MHC* genes and genes coding for TNF-α, IL-1β, IL-1RN, and IL-6.

1. Introduction

Systemic lupus erythematosus (SLE) is the prototypical autoimmune disease that is characterized by autoantibody production, complement activation, and immune complex deposition leading to diverse clinical manifestations and target tissue damage. The prevalence of SLE is estimated to be between 40 and 400 cases per 100,000 individuals [1]. While the precise etiology of SLE still remains vague, genetic predisposition and environmental and hormonal factors are deemed to play important roles in its pathogenesis. Severity, acquisition risk, and clinical manifestations of this disease can vary by ethnicity, geography, and sex, with a prevalence that is higher in women during their childbearing ages and some non-European populations such as African Americans, Hispanics, and Asians [2, 3].

In Asians, the prevalence of SLE generally falls within 30–50/100,000 individuals. SLE is more frequent among Chinese communities in Asia than it is in India and tropical Africa [4]. Malaysia is a multiracial country. In the peninsular, the Malays (55.1%), Chinese (24.3%), and Indians (7.4%) represent the largest ethnic groups. A prevalence of 43/100,000 individuals in Malaysia has been reported [5, 6]. Likewise, Chinese have the highest prevalence of SLE in Malaysia (57/100,000), followed by Malays (33/100,000) and Indians (14/100,000) [7, 8]. The overall 5-year and 10-year survival rates were reported as 82% and 70%, respectively [5], whereas the overall mortality rate was 20.2% [9]. Renal involvement is highest among the Malaysian patients [5]. However, the major cause of death in Malaysian SLE patients was reported to be from infection [9].

Pathogenesis of SLE is associated with functional deficiency of multiple immunologic components, including the innate immune system, altered immune tolerance mechanisms, hyperactivation of T and B cells, reduced ability of immune complexes and apoptotic cell clearance, and defects in multiple immune regulatory networks [10]. The failure of these mechanisms could be due to the influence of variants within SLE susceptibility genes. To date, many different genes have been found to contribute to disease susceptibility. In a small proportion of patients (<5%), a single gene could become the key player for this disease [11]; however, multiple genes have been implicated in most patients. It is estimated that at least four susceptibility

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CONCISE REPORT

HLA variants rs9271366 and rs9275328 are associated with systemic lupus erythematosus susceptibility in Malays and Chinese

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Background: Human leukocyte antigen (HLA) antigens and genes have long been reported associated with systemic lupus erythematosus (SLE) susceptibility in many populations. With the advance in technologies such as genome-wide association studies, many newly discovered SLE-associated single-nucleotide polymorphisms (SNPs) have been reported in recent years. These include *HLA-DRB1*/*HLA-DQA1* rs9271366 and *HLA-DQB1*/*HLA-DQA2* rs9275328. Our aim was to investigate these SNPs in a Malaysian SLE cohort. **Materials and methods:** SNPs rs9271366 and rs9275328 were screened across 790 Malaysian citizens from three ethnic groups (360 patients and 430 healthy volunteers) by Taqman SNP genotyping assays. Allele and genotyping frequencies, Hardy–Weinberg equilibrium, Fisher's exact test and odds ratio were calculated for each SNP and ethnic group. Linkage disequilibrium and interaction between the two SNPs were also evaluated. **Results:** The minor allele G and its homozygous genotype GG of *HLA-DRB1*/*HLA-DQA1* rs9271366 significantly increased the SLE susceptibility in Malaysian patients, including those of Malay and Chinese ethnicity (odds ratio (OR) > 1, $p < 0.05$). As for *HLA-DQB1*/*HLA-DQA2* rs9275328, the minor allele T and the heterozygous genotype CT conferred protective effect to SLE in Malaysians, as well as in Malays and Chinese, by having OR < 1 and p value <0.05. Both SNPs did not show associations to SLE in Indians. D′ and r^2 values for the two SNPs in LD analysis were 0.941 and 0.065, respectively, with haplotype GC and AT being significantly associated with SLE ($p < 5.0 \times 10^{-4}$) after 10,000 permutations were performed. The MDR test clustered the genotype combinations of GG and CC, and AG and CC of rs9271366 and rs9275328, accordingly, as high-risk group, and the two SNPs interacted redundantly by removing 1.96% of the entropy. **Conclusions:** Our findings suggest that in addition to some classical *HLA* variants, rs9271366 and rs9275328 are additional polymorphisms worth considering in the Malaysian and possibly in a larger Asian SLE scenario. *Lupus* (2012) **0**, 1–7.

Key words: Human leukocyte antigen; systemic lupus erythematosus; single nucleotide polymorphism; Malaysian

Introduction

Human leukocyte antigens (HLA) play major roles in the pathophysiology of systemic lupus erythematosus (SLE), an enigmatic autoimmune disorder. As such, HLA genes, especially class II and class III located on the short arm of the chromosome, are of paramount importance. The association of HLA antigens and genes with SLE has been reported since 1971, when Grumet et al. reported a possible relationship.[1] HLA class II genes (DP, DM, DOA, DOB, DQ and DR) encode cell-surface antigens that are presented from outside of the cells to T-cells and subsequently trigger the multiplication of T-helper cells and production of antibodies by B-cells. HLA class II genes have been correlated with the existence of certain autoantibodies such as anti-Sm, anti-Ro, anti-nRPN and anti-DNA antibodies, which are useful biomarkers in the diagnosis of SLE. HLA-DR2 has been consistently associated with SLE in Caucasian and Asian populations, and combinations of class II genes variants

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Polymorphisms of genes involved in toll-like receptor/interferon signaling pathways in systemic lupus erythematosus

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Introduction

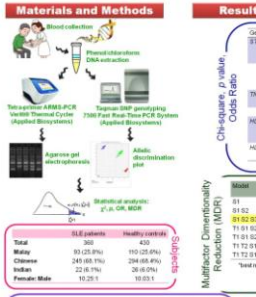
Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease affecting various parts of the body as a result of autoantibody production, complement activation, immune complex deposition and inflammation. Although the exact aetiology of this disease still remains unclear, a combination of genetic risk factors and environmental events are believed to the cause of the irreversible break in immunological self tolerance.

In general, one of the biological pathways that have been suggested to be involved in SLE is toll-like receptor (TLR)/interferon (IFN) signaling pathways (Harley *et al.*, 2009). Defect in TLR/IFN signaling pathways causes immune complexes containing self nucleic acids to interact with TLR7 and TLR9 inside plasmacytoid dendritic cells and B cells endosomes, resulting in the secretion of type I IFN and interleukin (IL)-6. The combined triggering of both B cell receptors and TLR leads to autoreactive B cell proliferation, whereby their differentiation into plasmablasts and autoantibody-secreting plasma cells are induced by type I IFN and IL-6, respectively (Banchereaau and Pascual, 2006).

Objectives

To investigate the association of several SNPs (Table 1) located in genes involved in TLR/IFN signaling pathways, i.e. *TNFAIP3*, *STAT4* and *IRF5*, with SLE in the Malaysian population. These SNPs were reported to be significantly associated with SLE in Asians. Two newly reported SNPs in *HLA* region were also studied.

Materials and Methods



Results

Discussion and Conclusion

SNPs of *STAT4* were found to have more important role in conferring risk to SLE in the Malaysian population as compared to those of *TNFAIP3* and *IRF5*.

STAT4 rs7601754, *IRF5* rs4728142 and rs729302 did not show significant associations with SLE in the Malaysian population, as opposed to the findings reported in Hong Kong, Mainland China, Thailand and Japanese populations (Yang *et al.*, 2010; Kawasaki *et al.*, 2008).

In conclusion, more SNPs of genes involved in TLR/IFN signaling pathways are yet to be investigated in order to determine their importance in SLE in the Malaysian population.

References:
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Yang W, Shen N, Ye DQ, Liu Q, Zhang Y, *et al.* (2010) Genome-wide association study in Asian populations identifies variants in ETS1 and IRF5 associated with systemic lupus erythematosus. *PLoS Genet* e1000841.
Kawasaki A, Kyogoku C, Ohashi J, Miyashita R, Hikami K, *et al.* (2008) Association of IRF5 polymorphisms with systemic lupus erythematosus in a Japanese population: support for a crucial role of intron 1 polymorphisms. *Arthritis Rheum* 58: 826-834.

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Appendix V

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1/19/13 Monash University (Student) Mail - Permission

MONASH University

Hwa Chai <hcchai18@student.monash.edu>

Permission

2 messages

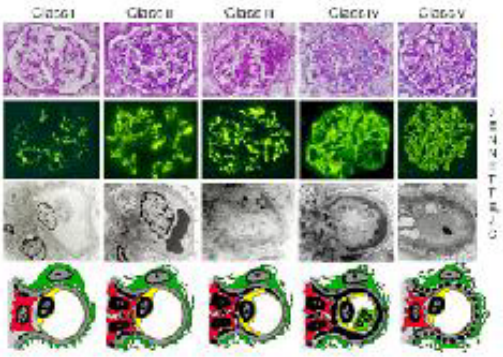
Hwa Chai [redacted] 15 January 2013 15:11
To: [redacted]

Dear Madam,

I am a PhD student from Monash University and working on research regarding lupus nephritis. I came across the following figure at http://unckidneycenter.org/kidneyhealthlibrary/lupus.html and found that it illustrates very well each class of lupus nephritis. Hence, I would like to seek permission from you to put the figure in my thesis in order to give better understandings to the readers on each class of LN as well as to add credits to the introduction of my thesis. I would be happy to share my thesis with you after it has been published later.

Thank you very much for your attention and looking forward to hearing from you soon.

Regards,
Chai Hwa Chia
School of Medicine and Health Sciences
Monash University (Sunway Campus)
Malaysia



Harward, Donna H [redacted] 15 January 2013 23:03
To: Hwa Chai [redacted]

Hello Hwa,

You may use the image, but you must cite Professor J. Charles Jennette, MD as its author, the date of use and credit the UNC Kidney Center web site as the resident source for the image. Good luck with your research.

Donna

Donna H. Harward
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F [redacted]
www.unckidneycenter.org
Remember to ask...HEY DOC, HOW ARE MY KIDNEYS?

From: Hwa Chai [mailto:[redacted]]
Sent: Tuesday, January 15, 2013 2:11 AM
To: Harward, Donna H
Subject: Permission

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