7ο ΚΡΗΤΟ-ΚΥΠΡΙΑΚΟ ΣΥΜΠΟΣΙΟ ΡΕΥΜΑΤΟΛΟΓΙΑΣ
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Κύπρος 23 Οκτωβρίου-25 Οκτωβρίου 2015

i. Gene expression and regulation in SLE

ii. Autophagy and SLE

Ελένη Α. Φράγκου, Νεφρολόγος

25/10/2015

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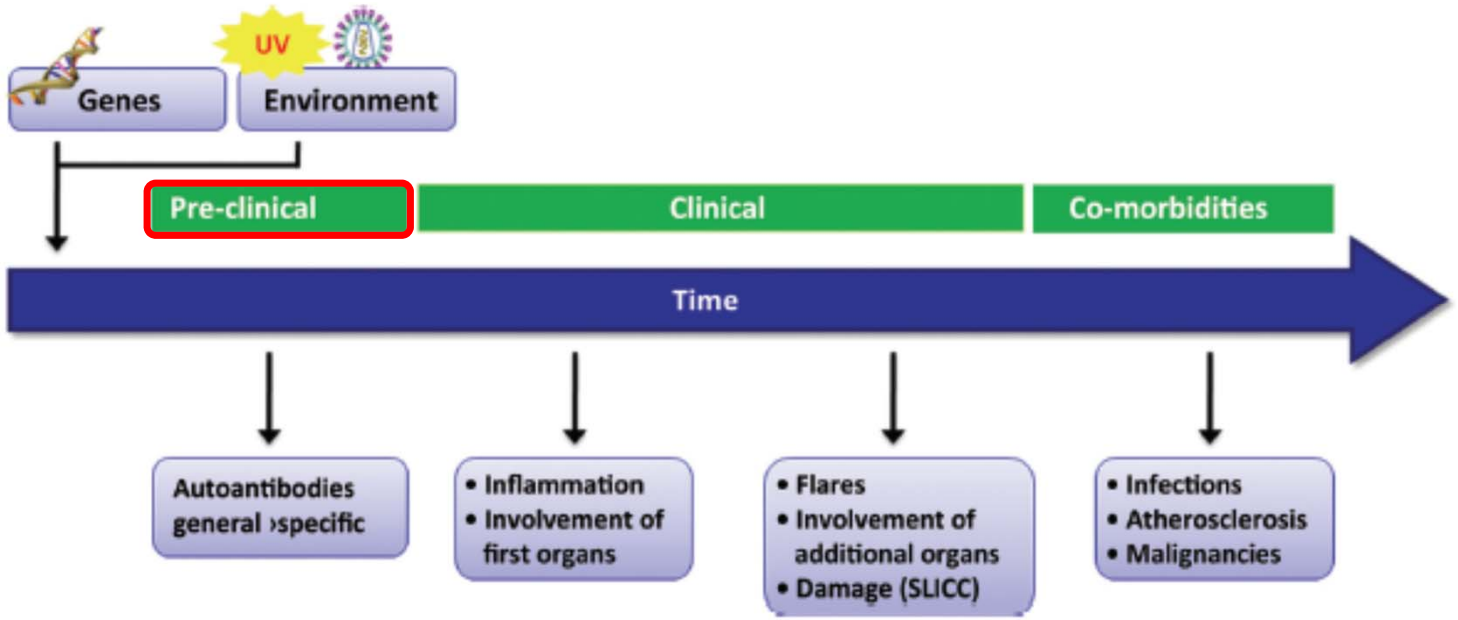
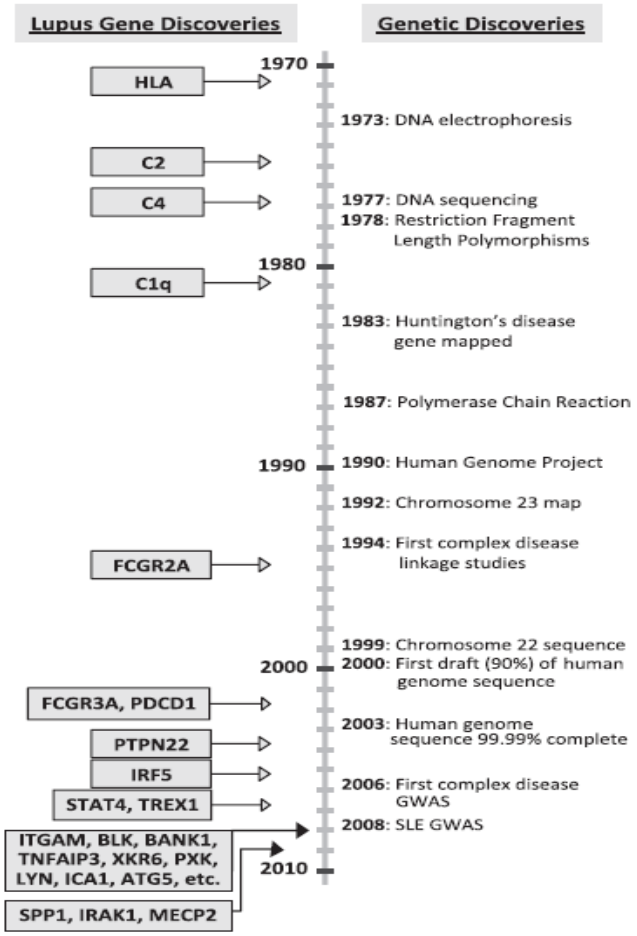
SLE PATHOGENESIS IS COMPLEX

Lupus often starts at a young age. Very long disease course...



Ann Rheum Dis 2010;69:1603–11.

GENETIC CONTRIBUTION TO SLE IS ~30%



GENOME-WIDE ASSOCIATION STUDIES (GWAS)

>50 SLE-associated genes/loci

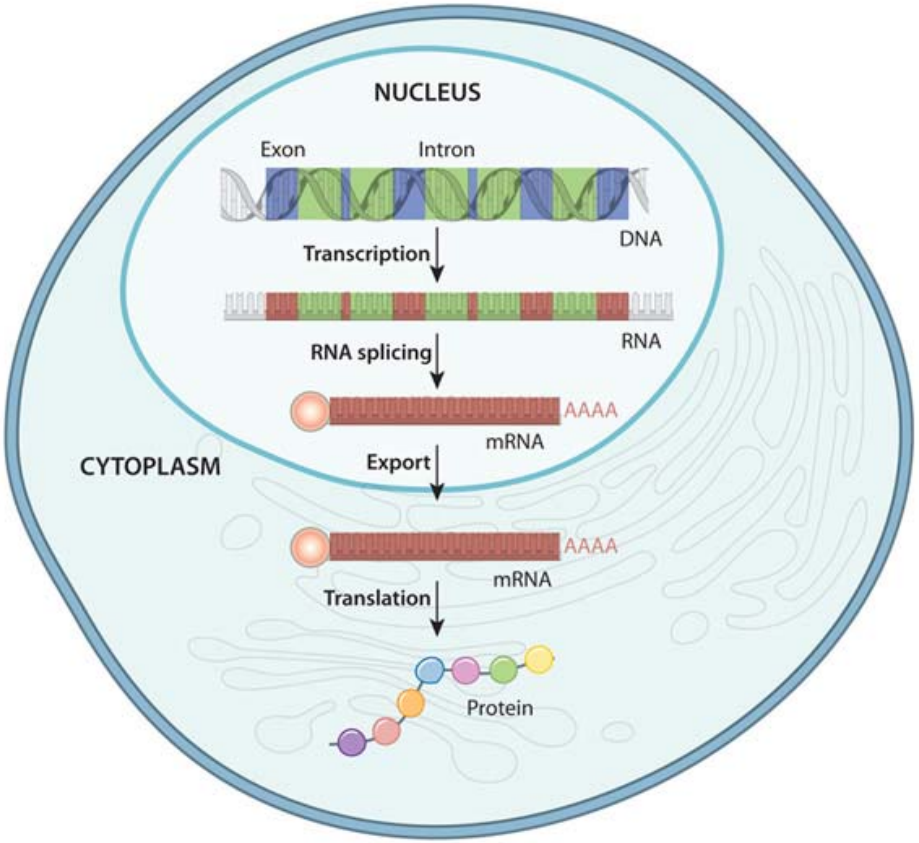
However,

- They explain only 10-20% of heritability
- Each variant has a small contribution to genetic risk
- Causal variants have not been identified

Genes Immun 2009, Arthritis Res Ther 2012

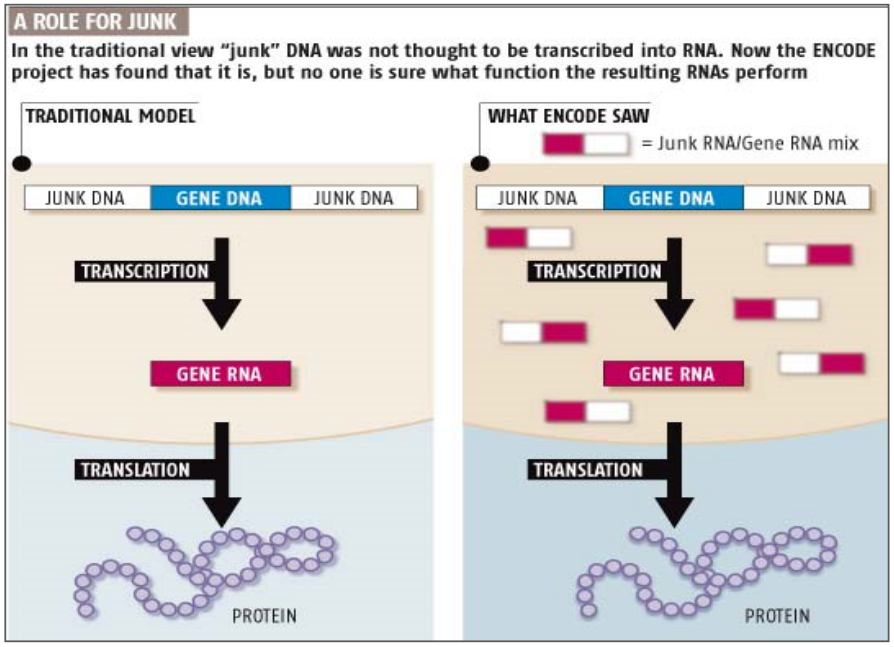
GENETIC CONTRIBUTION TO SLE IS ~30%

Genetic information is transferred from DNA to proteins through mRNA



GWAS – ENCODE Project

- 76% of human genome is transcribed
- **>90% of variants** associated with complex diseases **are located in non-coding regions**
- **SLE-associated variants:**
  strongly enriched in enhancers
  highly active in SLE-associated cell-types

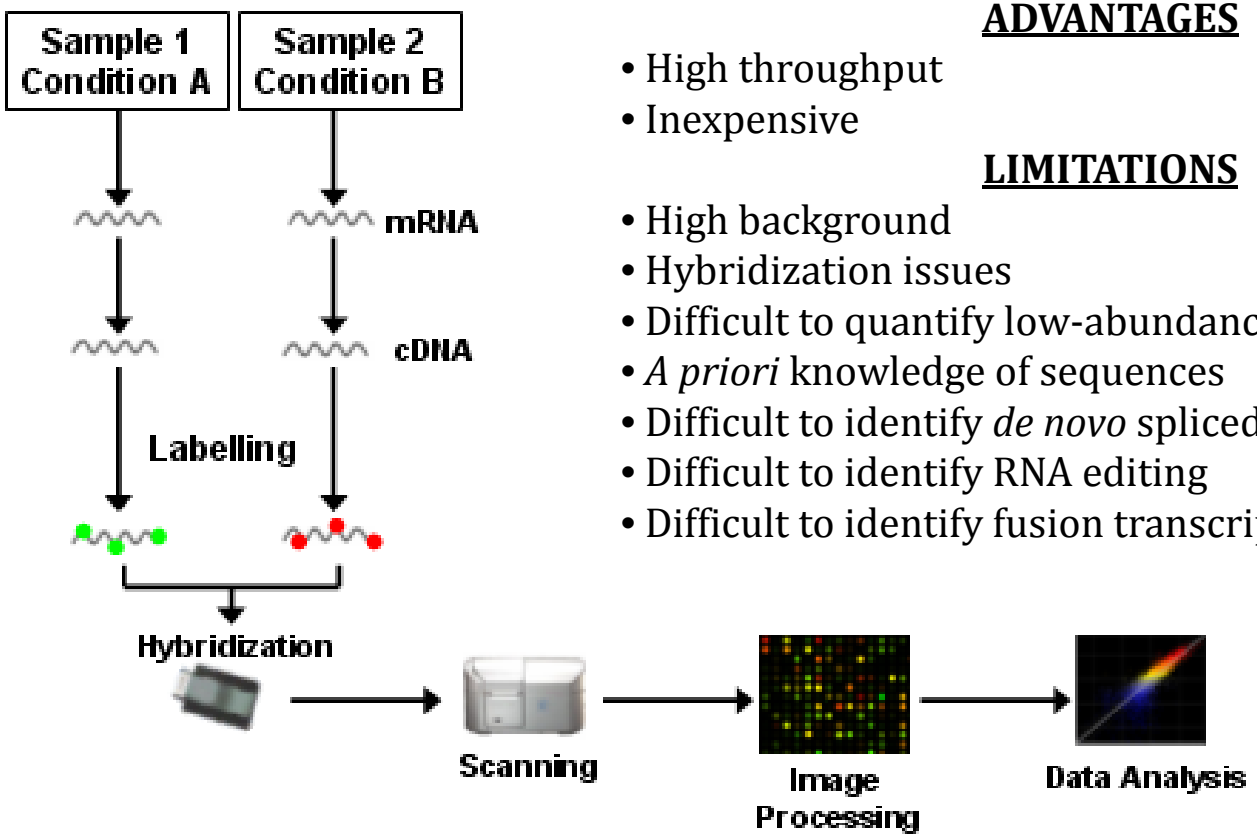


✓ **Non-coding and regulatory genomes are important in human disease**

NOVEL APPROACHES TO CHARACTERIZE NON-CODING AND REGULATORY GENOMES IN HUMAN DISEASE

NOVEL APPROACHES TO CHARACTERIZE NON-CODING AND REGULATORY GENOMES IN HUMAN DISEASE

A. HYBRIDIZATION-BASED TECHNOLOGIES (MICROARRAYS)



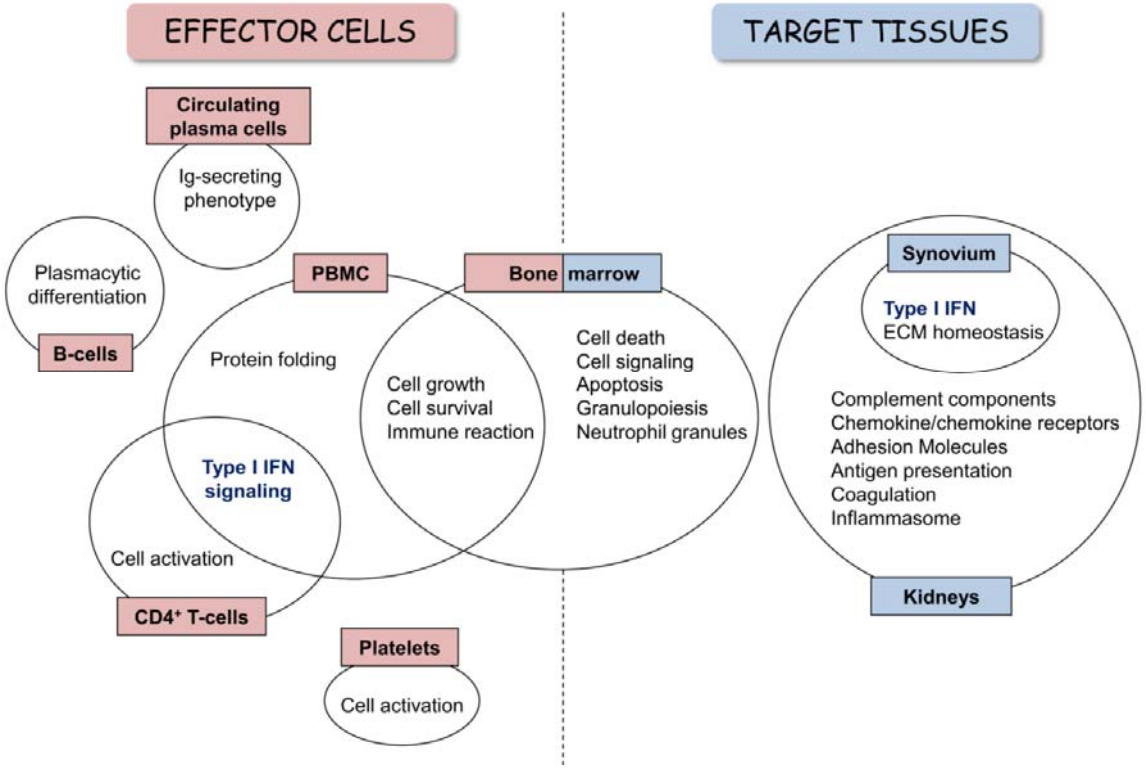
ADVANTAGES

- High throughput
- Inexpensive

LIMITATIONS

- High background
- Hybridization issues
- Difficult to quantify low-abundance transcripts
- *A priori* knowledge of sequences
- Difficult to identify *de novo* spliced isoforms
- Difficult to identify RNA editing
- Difficult to identify fusion transcripts

MOLECULAR SIGNATURE OF SLE

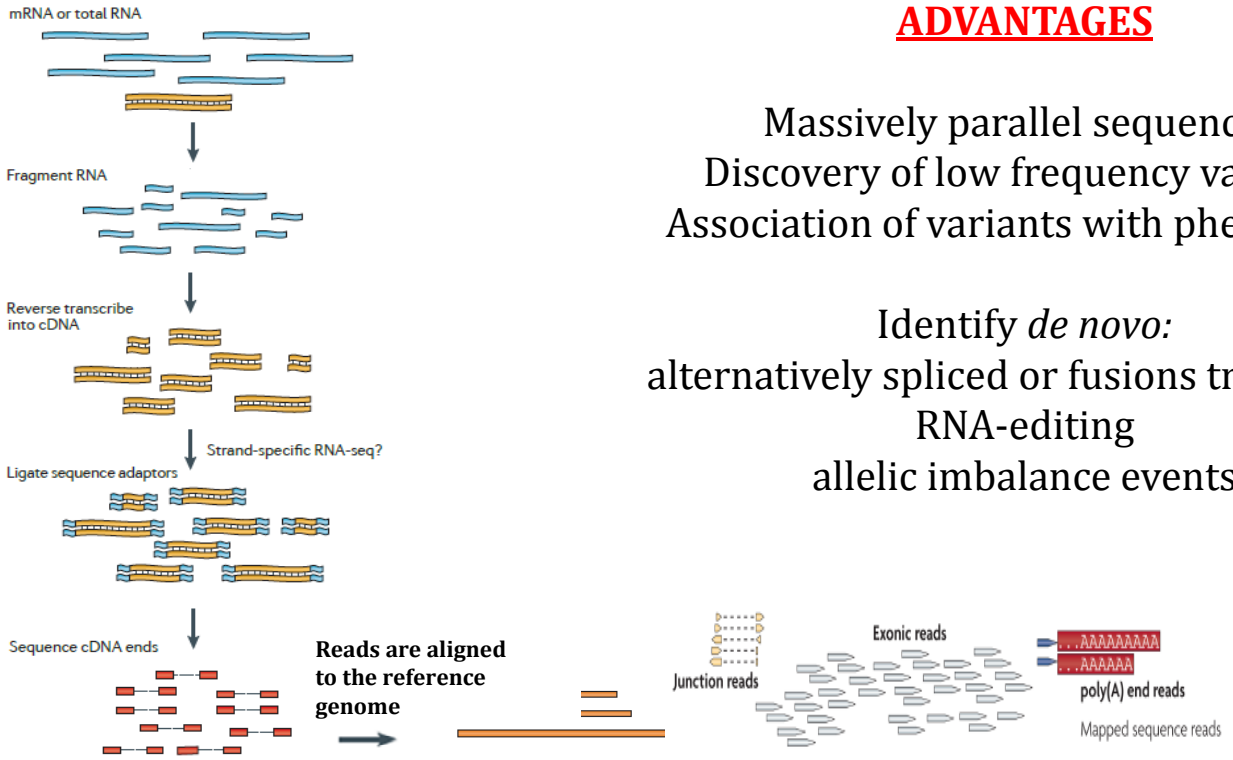


✓ **Unanswered questions and unmet needs still exist**

Frangou E et al. Eur J Clin Invest 2013

NOVEL APPROACHES TO CHARACTERIZE NON-CODING AND REGULATORY GENOMES IN HUMAN DISEASE

B. NEXT-GENERATION SEQUENCING TECHNOLOGIES: RNA-Seq



ADVANTAGES

Massively parallel sequencing
Discovery of low frequency variants
Association of variants with phenotypes

Identify *de novo:*
alternatively spliced or fusions transcripts
RNA-editing
allelic imbalance events

AIM OF THE STUDY

is to further investigate SLE etiopathogenesis

through a **comparative transcriptomic and genomic analysis** in

A. murine lupus model

B. in human LN

using RNA-Seq

(unbiased and not-requiring-*a priori* hypothesis NGS approach)

MATERIALS AND METHODS

1. **MURINE STUDIES**

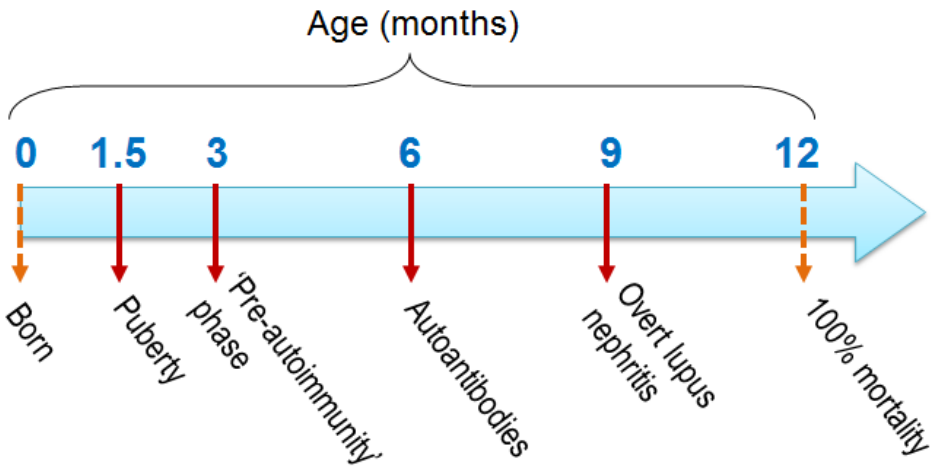
Female NZB, NZW, NZB/W F1, B6

B6: not autoimmune

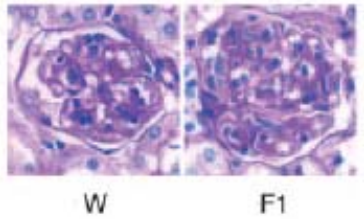
NZB, NZW: limited autoimmunity

NZB/W F1: lupus-like phenotype

classical model of human lupus

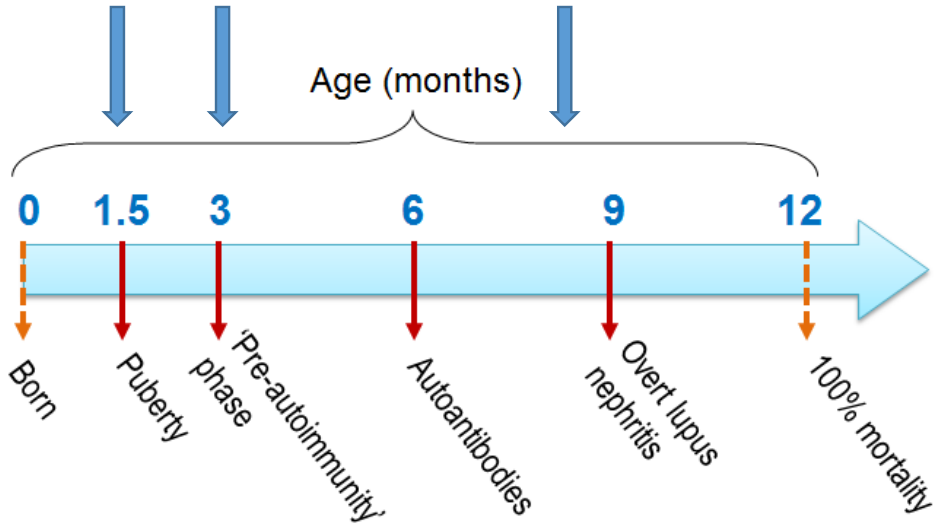


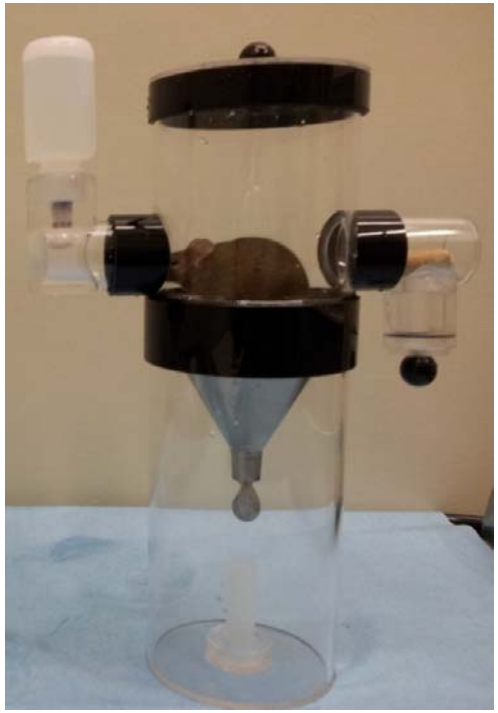
ANA
anti-dsDNA
Lymphadenopathy
Splenomegaly
Immune-complex GN



MATERIALS AND METHODS

1. MURINE STUDIES





NEPHRITIC STAGE

Proteinuria >300 mg/d for 3 consecutive days

MATERIALS AND METHODS

- **PERIPHERAL BLOOD COLLECTION** (serum autoantibody detection)
- **ANIMAL PERFUSION WITH PBS**
- **KIDNEYS, SPLEEN, BRAIN EXTRACTION, BM** (total RNA, protein, DNA)
- **KIDNEYS STORED** in paraffin blocks (RT), OCT (-80^{o}C)
- **TOTAL RNA EXTRACTION** (Trizol-based method for RNA-seq)
- **DNA EXTRACTION** (GWAS to generate SNP datasets)

MATERIALS AND METHODS

2. HUMAN STUDIES (MALES AND FEMALES)

Frozen kidneys from proliferative LN (n=30)

Frozen normal kidneys adjacent to cancer (n=30)

(Nephrology Department and Renal Histology Department
«Laiko» Hospital of Athens, University of Athens)

- **TOTAL RNA EXTRACTION** (Trizol-based method for RNA-seq)
- **DNA EXTRACTION** (GWAS to generate SNP datasets)

MATERIALS AND METHODS

3. RNA-SEQUENCING

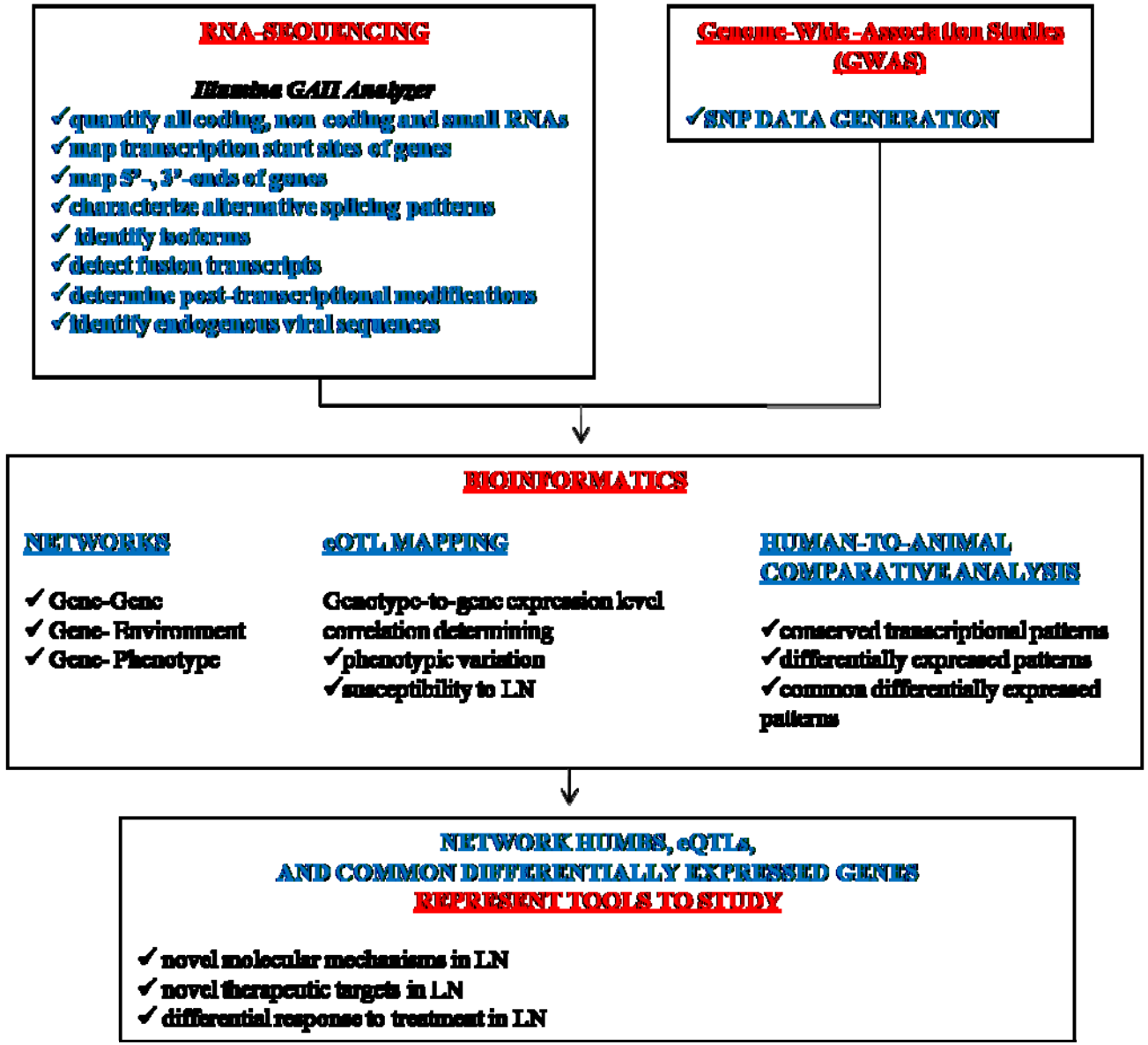
Illumina HiSeq Analyzer *(University of Geneva, Prof. E. Dermitzakis)*

- Each RNA sample (**1-2 µg of total RNA, A260/A280 > 1.8**) will be sequenced with **37-bp paired end in one sequencing lane**
- Each paired-end read will be **quantified for individual exons and genes** given known transcripts (mouse and human alignment)
- Each paired-end read will be **normalized for insert size variability** using regression
- Transcript abundance will also be **quantified using the FluxCapacitor** method

4. GWAS (Whole genome)

High-density exome core chips

SNP dataset generation for NZB/W-F1 and humans


RNA-SEQUENCING
Illumina GAII Analyzer
✓quantify all coding, non coding and small RNAs
✓map transcription start sites of genes
✓map 5'-, 3'-ends of genes
✓characterize alternative splicing patterns
✓identify isoforms
✓detect fusion transcripts
✓determine post-transcriptional modifications
✓identify endogenous viral sequences
Genome-Wide -Association Studies (GWAS)
✓SNP DATA GENERATION
BIOINFORMATICS
NETWORKS
✓ Gene-Gene
✓ Gene- Environment
✓ Gene- Phenotype
eQTL MAPPING
Genotype-to-gene expression level correlation determining
✓phenotypic variation
✓susceptibility to LN
HUMAN-TO-ANIMAL COMPARATIVE ANALYSIS
✓conserved transcriptional patterns
✓differentially expressed patterns
✓common differentially expressed patterns
NETWORK HUMBS, eQTLs, AND COMMON DIFFERENTIALLY EXPRESSED GENES REPRESENT TOOLS TO STUDY
✓ novel molecular mechanisms in LN
✓ novel therapeutic targets in LN
✓ differential response to treatment in LN

DIFFERENTIALLY EXPRESSED GENE TRANSCRIPTS

1. represent tools to study **novel molecular mechanisms** and **novel therapeutic targets**

2. could be used as **biomarkers** of disease activity, severity, morbidity and response to therapy

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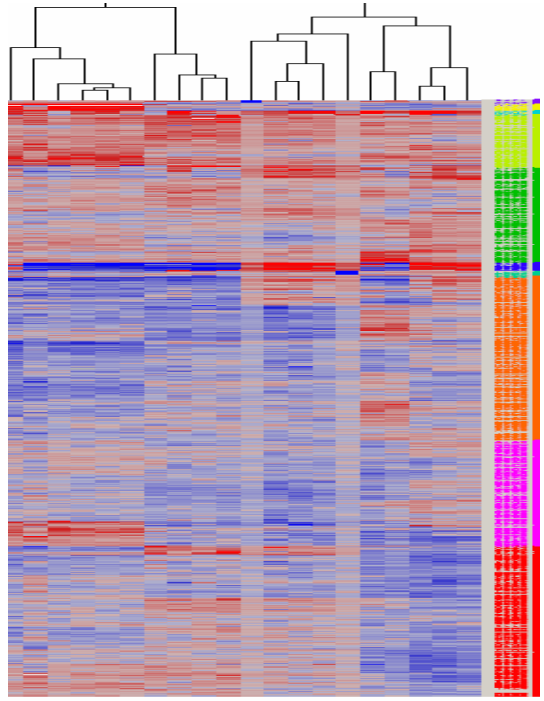
INTRODUCTION

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Active SLE patients express a strong neutrophil signature



cDNA microarray analysis (BM)

Granulopoiesis & products of activated PMNs

ELA2
LYZ
CD24
DEFA
CTG

Adhesion genes (Integrin family)

ITGβ2 (CD18)
MAP3K11
ACTB, ACTG1
ARPC3

Nakou et al. Arthr Rheum 2008

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Active SLE patients are characterized by

upregulation of **autophagy genes**

and by differential expression of **miRs targeting autophagy genes**

miR microarray analysis (PBMC)

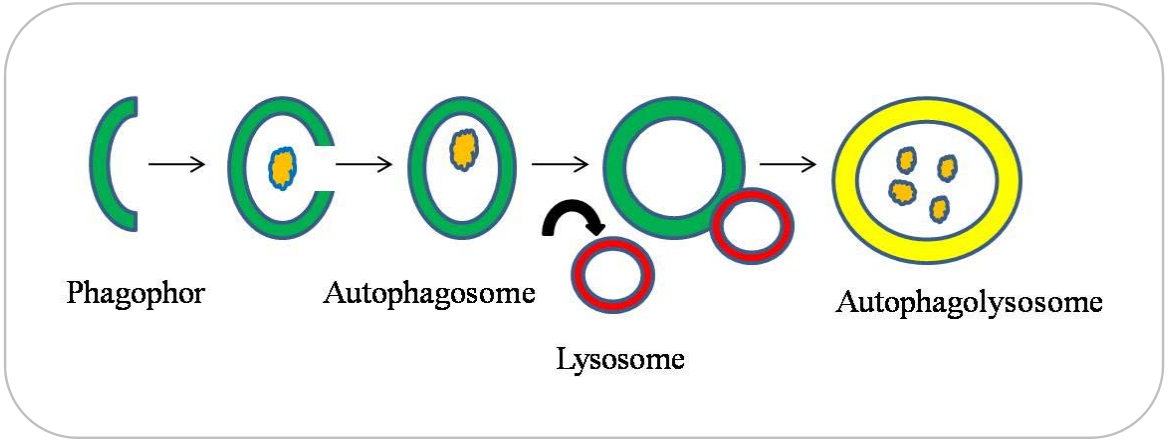
microRNA	Predicted gene targets
hsa-miR-296	HIPK1, EPN1, SRF
hsa-miR-196a	HOXC8, HOXA7, SOCS4
hsa-miR-17-5p	PPARA, E2F1, PKD2
hsa-miR-383	MAL2, MGA, IRF1
hsa-miR-184	SF1, PPP1CC, ELN
hsa-miR-379	GDF6, INSR, SEMA3A
hsa-miR-15a	BCL2, CDK6, FGF2
hsa-miR-16	BCL2, FGF2, CCND2
hsa-miR-150	ELK1, C-MYB, IRAK2
hsa-let-7a	STAT3, C-MYC, HMGA2
hsa-let-7d	HMGA2, IGF2BP1, TRIM7
hsa-let-7g	HMGA2, YOD1, TBFBR1
hsa-miR-98	EPHA4, FIGN, IGF1R
hsa-miR-532	CPEB3, PAK4, IRS2

microRNA	Predicted gene targets
hsa-miR-21	PDCD4, TPM1, PLAG1
hsa-miR-342	RASSF1, JMJD3, SOX6
hsa-miR-214	TRAF7, MNT, NARG1
hsa-miR-494	SOCS6, NOVA1, PTEN
hsa-miR-198	BMF, PBX1, BAX
hsa-miR-155	PU,1, FADD, C-MAF
hsa-miR-25	BIM, NFAT5, CD69
hsa-miR-106b	CDKN1A, BTG1, NFAT5
hsa-miR-373	MTF1, PARP8, PCAF
hsa-miR-324-3p	DAG1, TRAF7, CHES1
hsa-miR-544	FOXO1A, ABI2, TOX
hsa-miR-148a	KIS, DNMT3B, MEOX2
hsa-miR-148b	MITF, DNMT3B, MEOX2

Stagakis et al. Ann Rheum Dis. 2011; 70

AUTOPHAGY

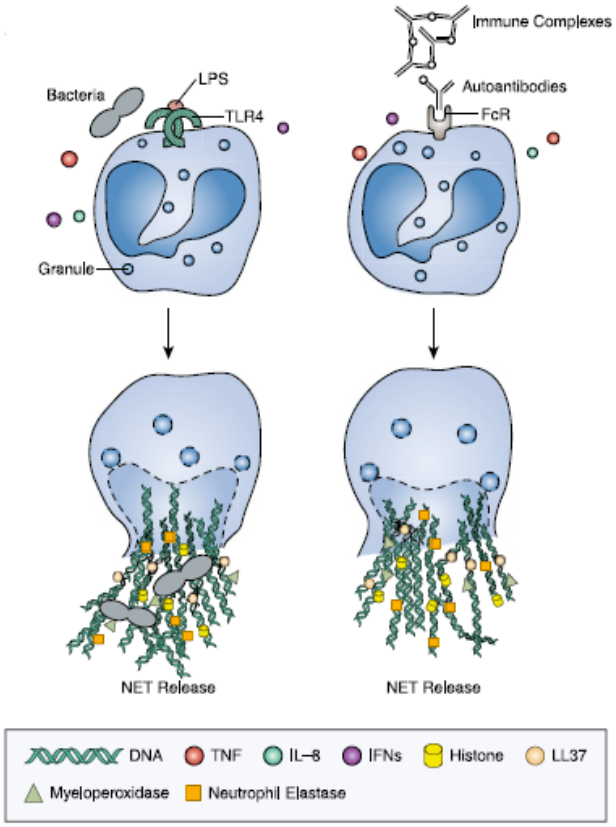
- **Homeostatic catabolic mechanism**
- Cells break their own components
- Is involved in cellular processes:
  cell cycle, energy regulation, **immune response**



WHAT IS THE MECHANISM OF TISSUE INJURY IN SLE?

PMNs and NETosis

- **Novel form of cell death**
- PMNs release chromatin filaments decorated with proteins and enzymes released from granules (MPO, elastase, cathepsins etc)
- **N**eutrophil **E**xtracellular **T**raps **(NETs)**
- Implicated in several diseases: **SLE**

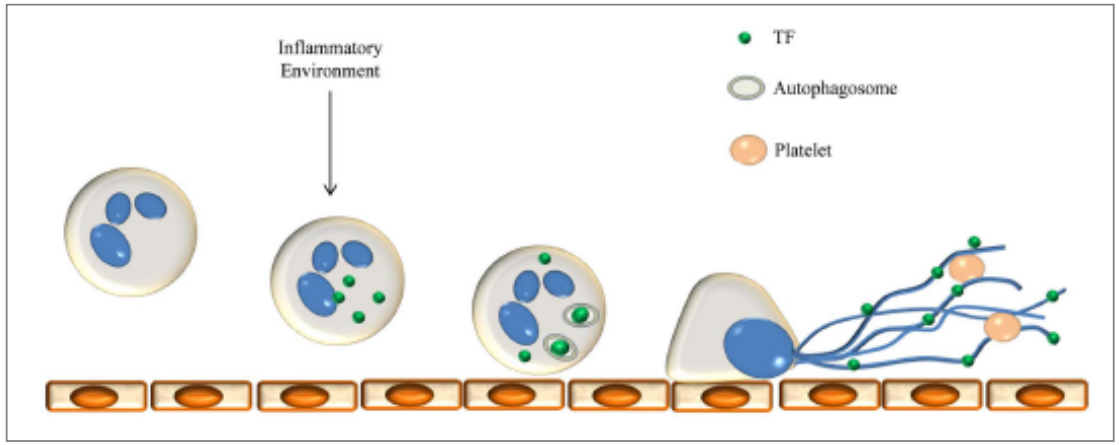


TISSUE FACTOR (TF)

is the main *in vivo* coagulation initiator

Through TF-thrombin axis: **coagulation cascade**

Through PAR activation: **inflammation**



In PMNs:

Intracellular localization of TF

Extracellular delivery of TF through NETs

Kambas K et al. Front Immunol 2012; 3: 385

HYPOTHESIS

In SLE patients, autophagy mediates the delivery of TF to NETs

AIM OF THE STUDY

is to investigate the role of TF-decorated NETs in SLE

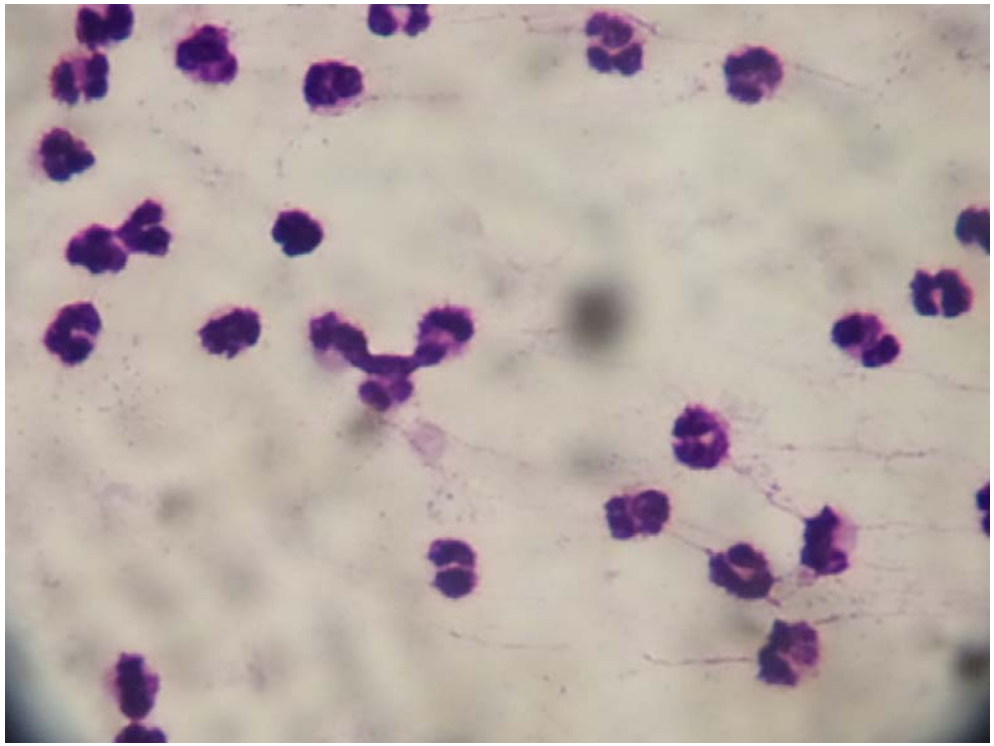
MATERIALS AND METHODS

Subjects enrolled *	Healthy Individuals	Active SLE patients	SLE patients on hydroxychloroquine
NUMBER	16	14	4
SEX			
Males	10 (63%)	1 (7%)	0 (0%)
Females	6 (37%)	13 (93%)	4 (100%)
MEAN AGE (years)	30.4	28.35	?
SELENA SLEDAI SCORE	-	>6	
Anti-PL abs	-	3	0

* Subjects receiving anti-PLTs or anticoagulants were excluded

MATERIALS AND METHODS

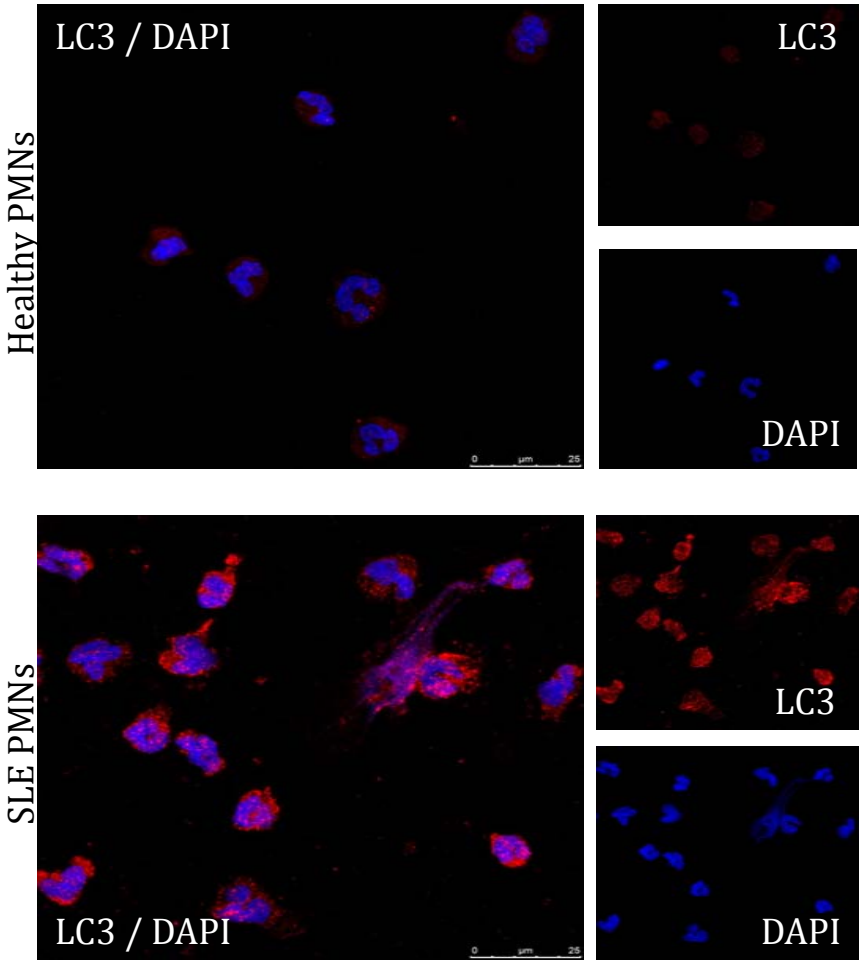
Serum and PMNs isolation (double gradient centrifugation - Ficolls)
PMNs viability >95% (Trypan blue staining)
PMNs purity >95% (**Giemsa staining**)



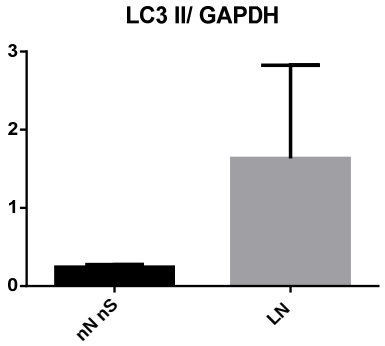
RESULTS

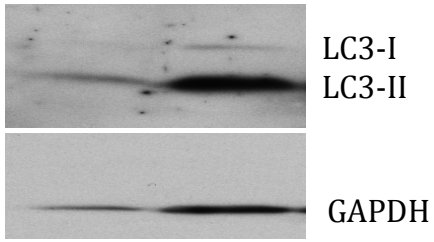
1. ACTIVE LUPUS PMNs EXPRESS INCREASED AUTOPHAGY LEVELS

A. Immunofluorescence for LC3B



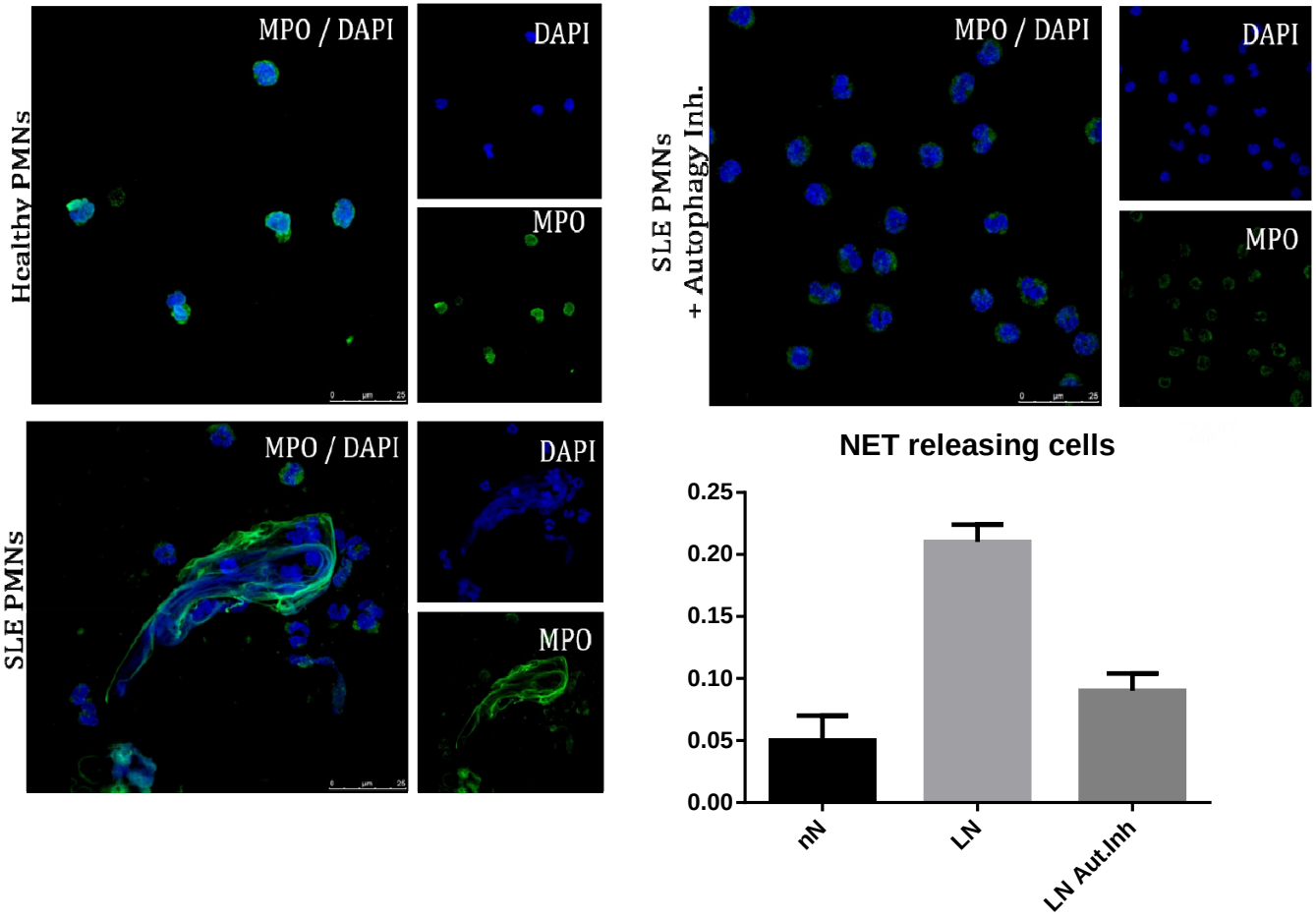
B. Immunoblotting for LC3



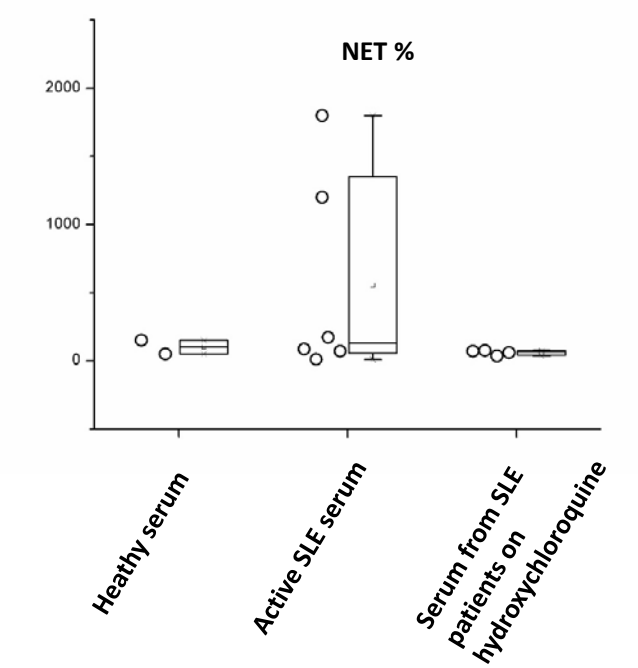


2. ACTIVE LUPUS PMNs UNDERGO INCREASED NETOSIS IN AN AUTOPHAGY-DEPENDENT MANNER

A. Immunofluorescence for MPO/DAPI

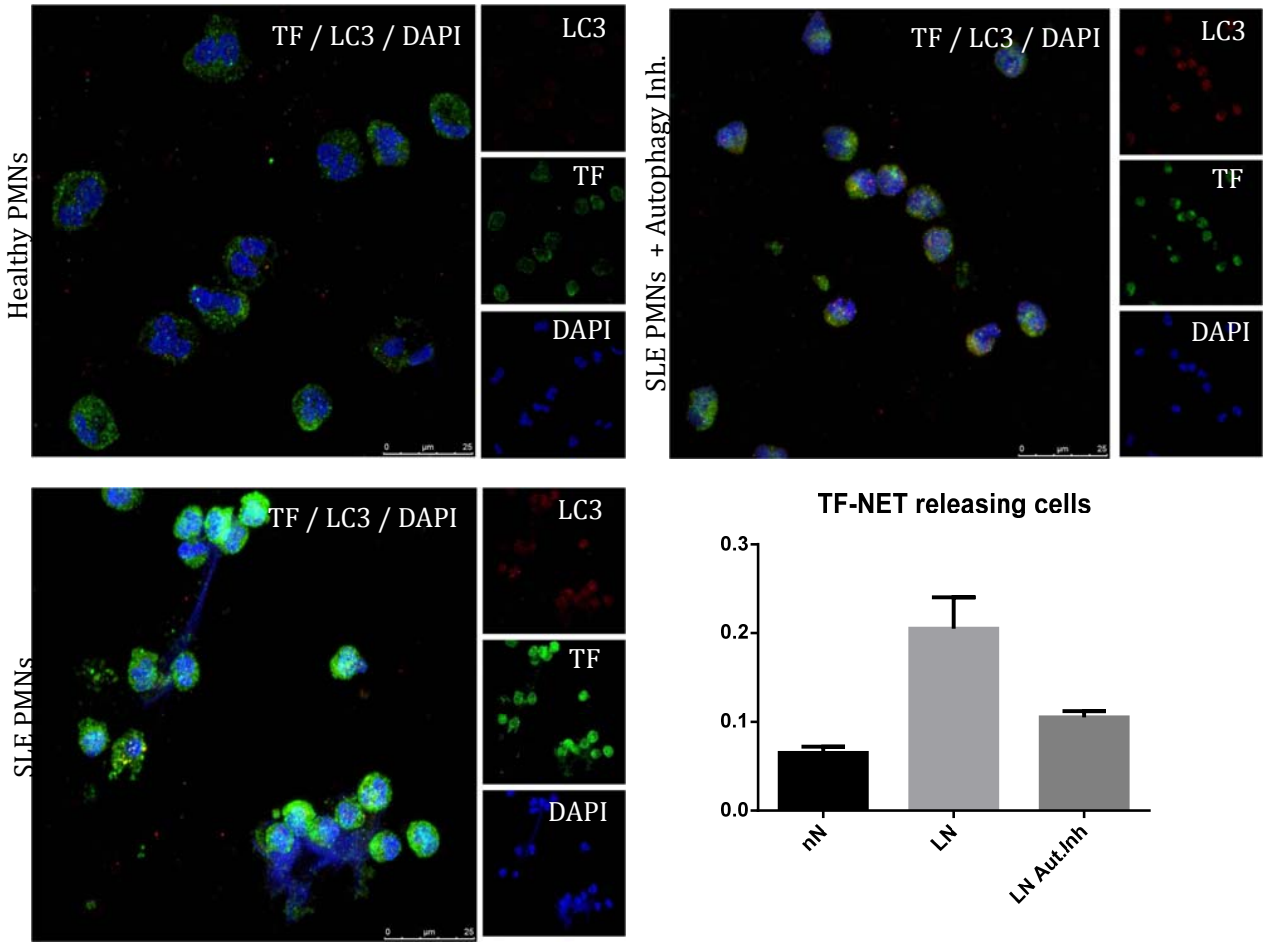


B. MPO/DNA complex ELISA in serum

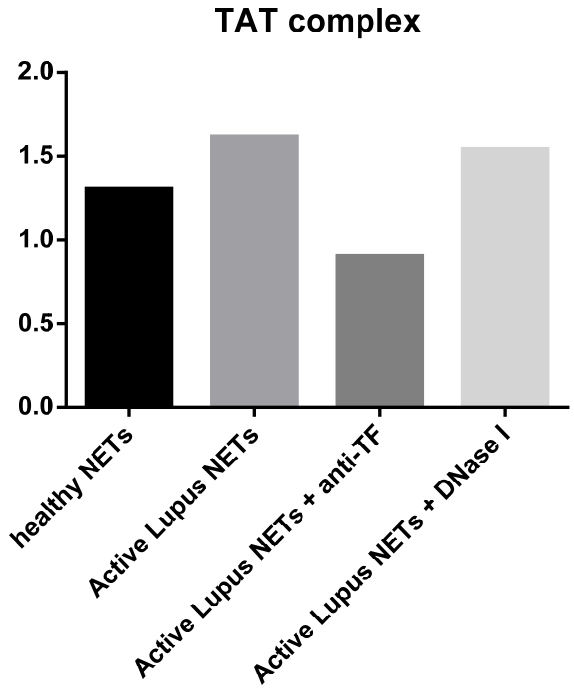


3. AUTOPHAGY MEDIATES THE RELEASE OF ACTIVE TF ON SLE NETs, LEADING TO TROMBIN GENERATION

A. Immunofluorescence for TF/LC3/DAPI



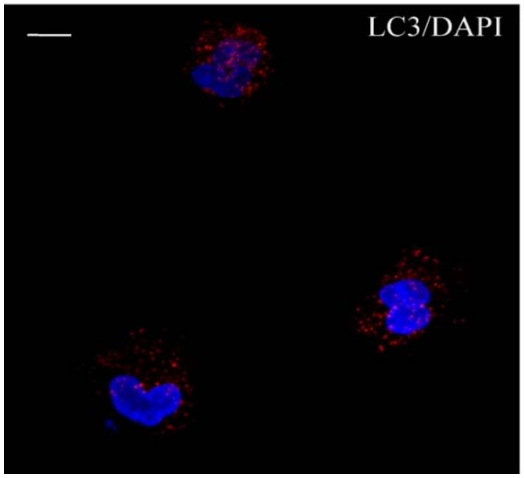
B. Thrombin-antithrombin complex assay



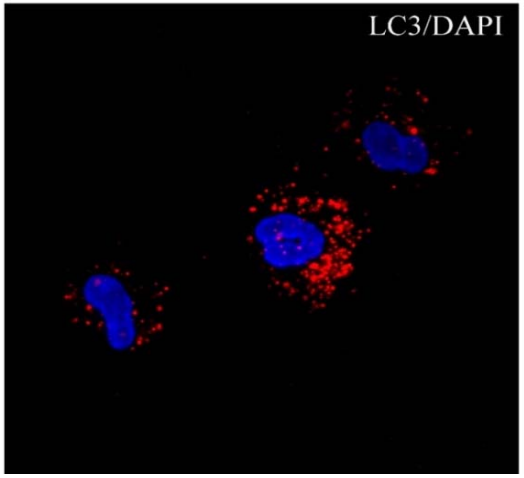
4. SLE SERUM INCREASES AUTOPHAGY IN HEALTHY PMNs

A. Immunofluorescence for LC3

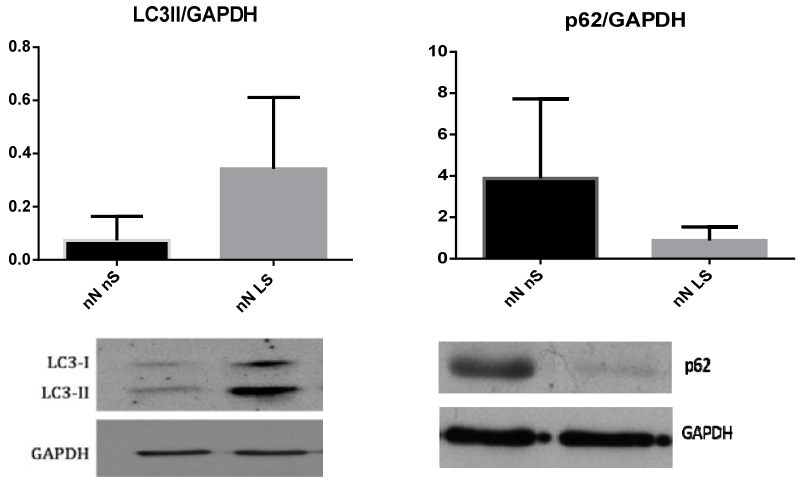
control



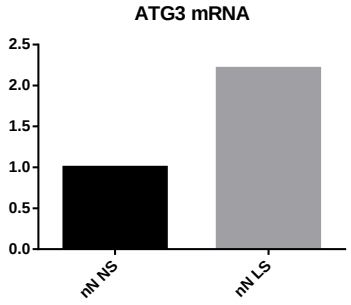
SLE serum



B. Immunoblotting for LC3 and p62

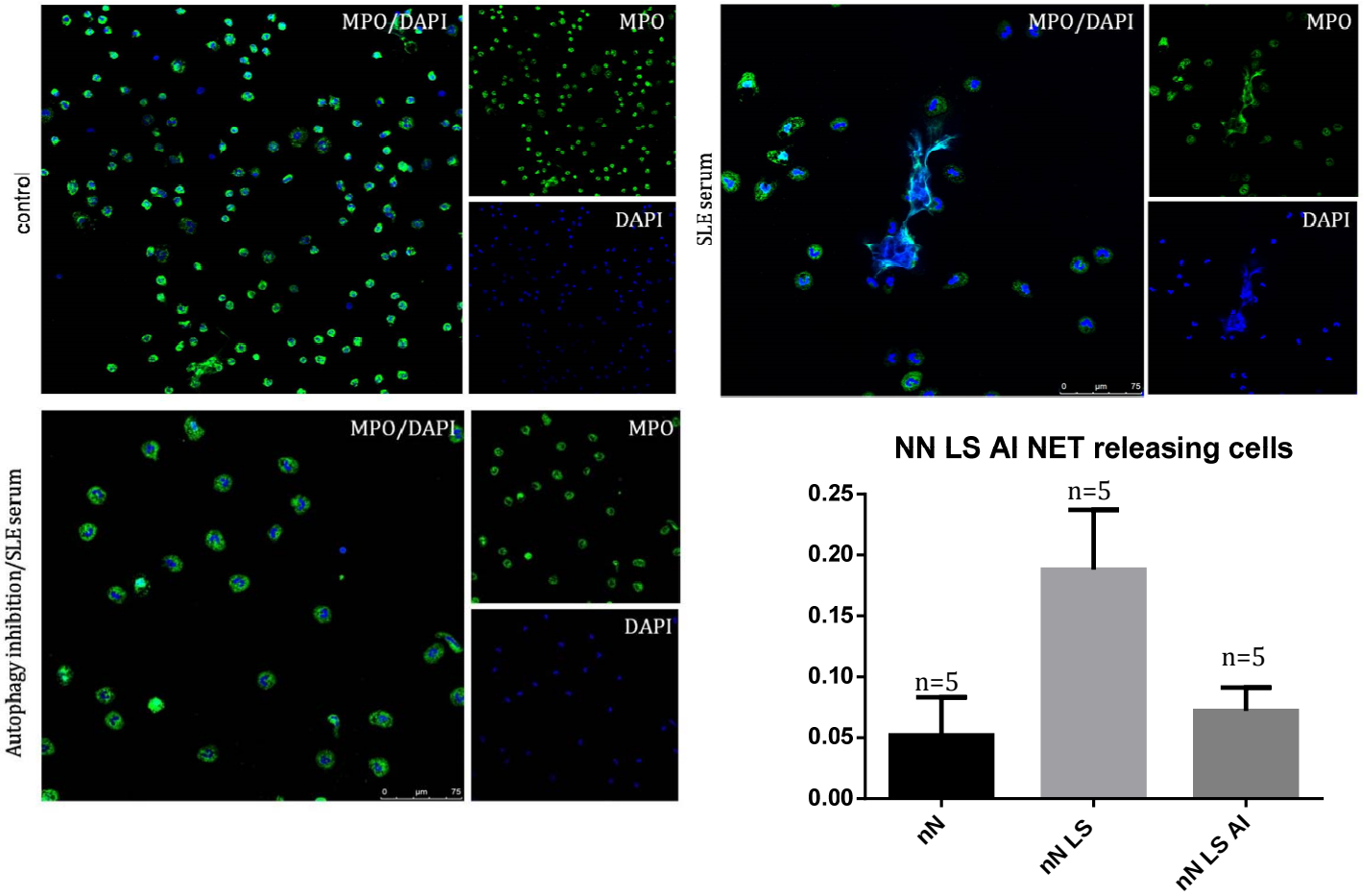


C. RT-PCR for ATG3 expression

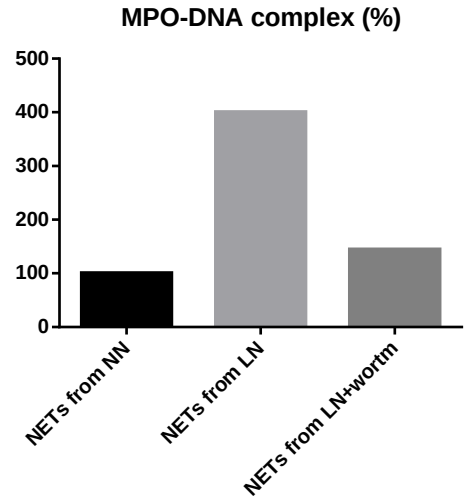


5. SLE SERUM INDUCES AUTOPHAGY-DEPENDENT NET RELEASE

A. Immunofluorescence for MPO/DAPI

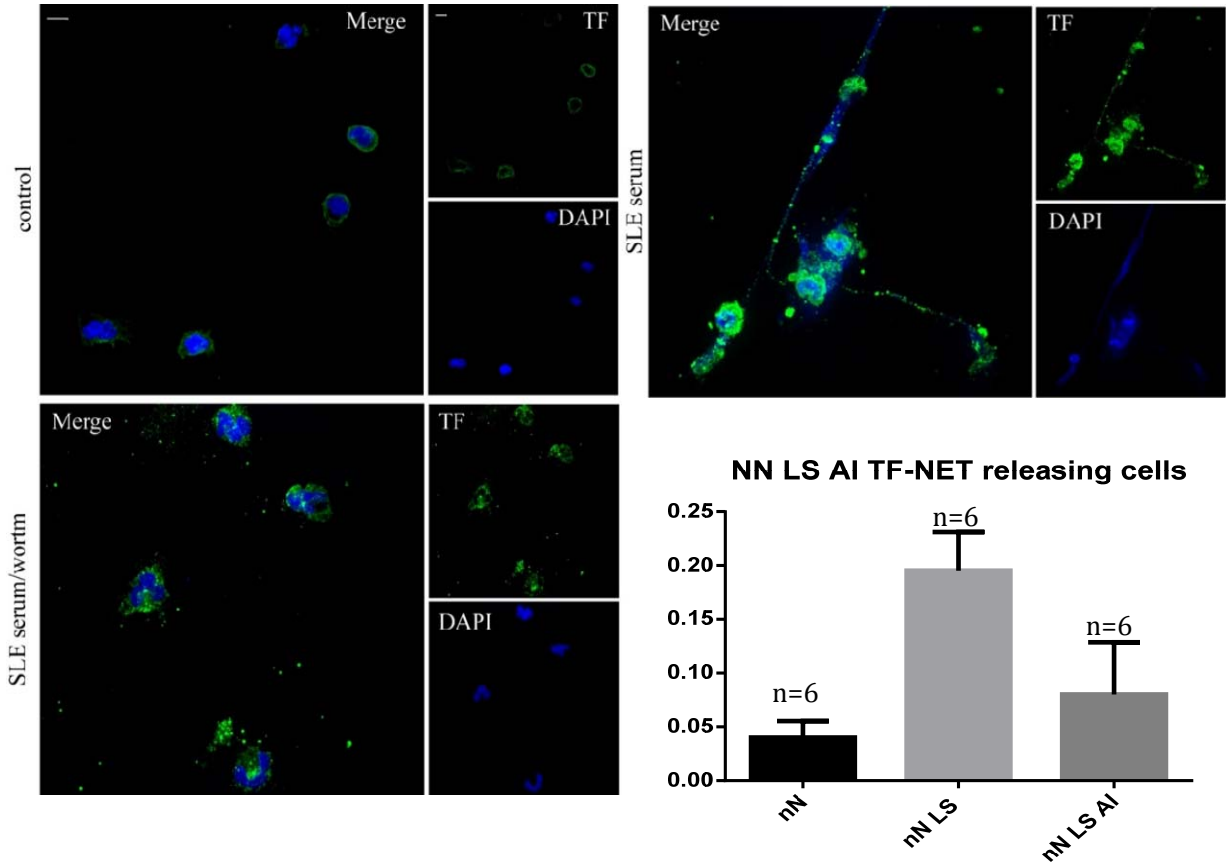


B. MPO/DNA complex ELISA in NET structures

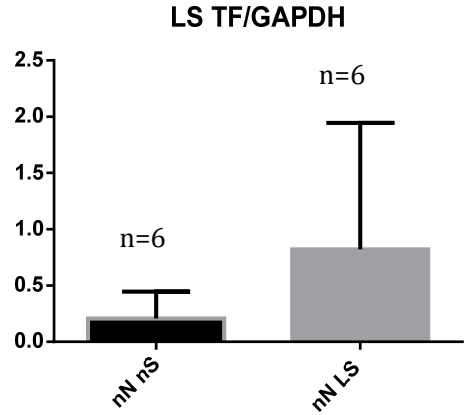


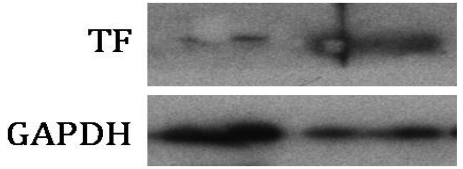
6. SLE SERUM UPREGULATES TF LEVELS IN HEALTHY PMNS AND INDUCES TF-DECORATED NETOSIS IN AN AUTOPHAGY-DEPENDENT MANNER

A. Immunofluorescence for TF/DAPI

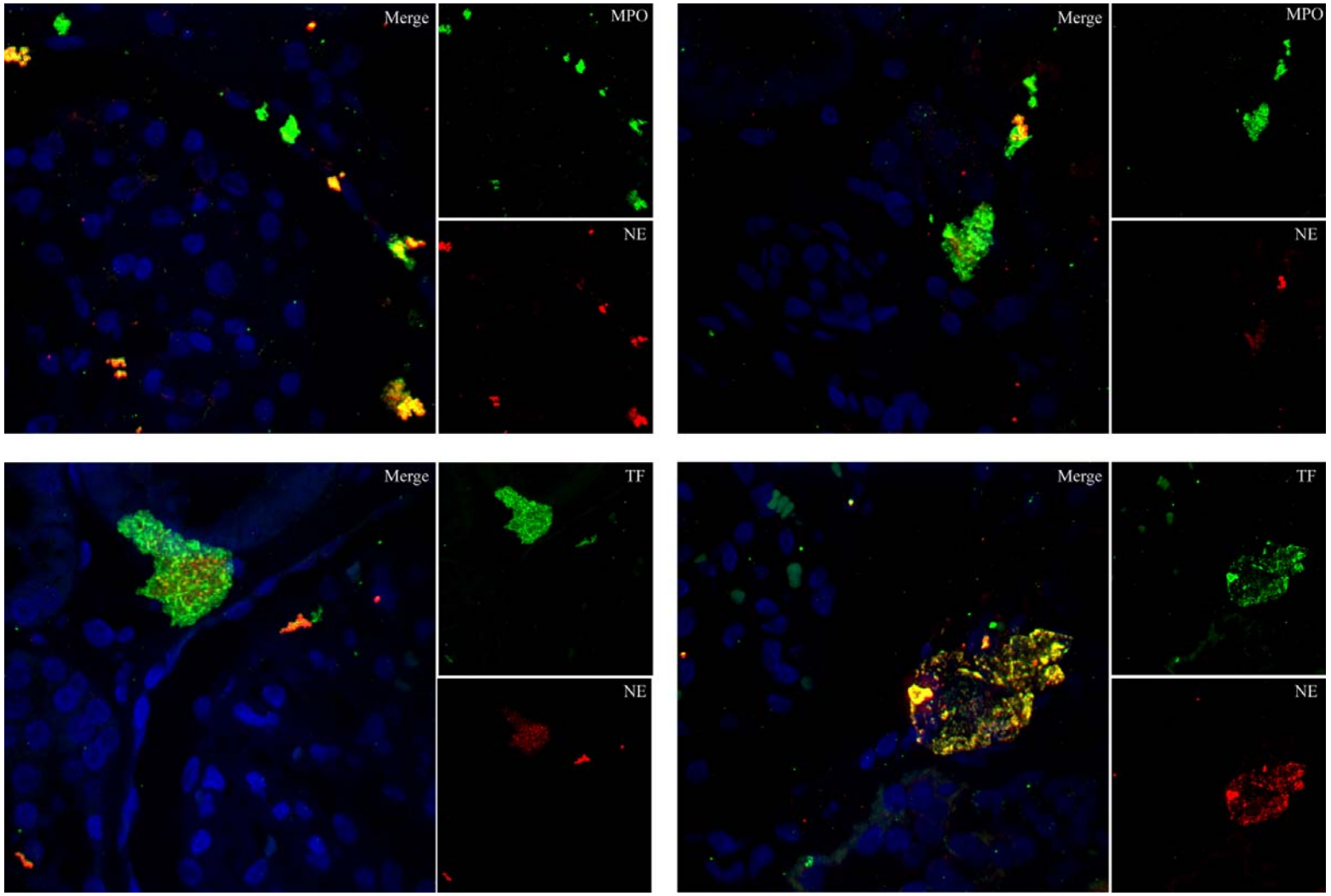


B. Immunoblotting for TF





7. TF-DECORATED NETs INFILTRATE THE KIDNEYS OF PATIENTS WITH LN

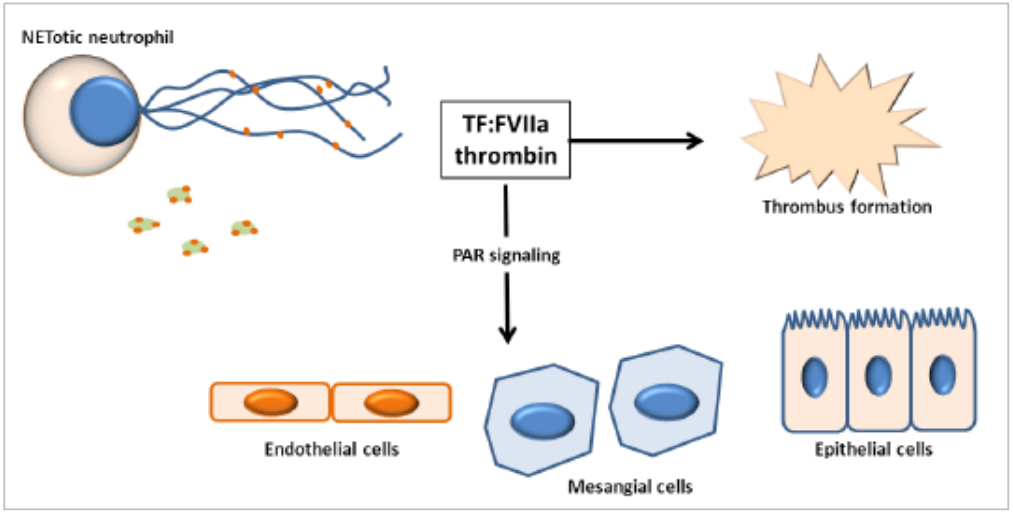


CONCLUSIONS

- ✓ PMNs from active SLE patients express **increased autophagy levels**
- ✓ PMNs from active SLE patients release **TF-decorated NETs in an autophagy-dependent** manner, leading to **thrombin generation**
- ✓ TF-decorated NETs **infiltrate the kidneys of LN patients**
- ✓ Ongoing experiments investigate the role of TF-decorated NETs on renal injury

DISCUSSION

- NETs are scaffolds with accumulated bioactive molecules
- NETs remain in target tissues even when PMNs are not present



- ✓ Inflammation and hypercoagulation in SLE
- ✓ Glomerular microthrombi deposition and glomerular endothelial injury

Kambas K Ann Rheum Dis. 2013

EULAR/ERA-EDTA recommendations for the management of LN. Bertsias GK. Ann Rheum Dis. 2012

ΕΥΧΑΡΙΣΤΩ

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