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Neutrophil Extracellular Traps (NETs) profiles in patients with incident SLE and lupus nephritis

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Author contribution

GMG was the PI of the study. He was involved in study design and coordination, patients' recruitment, data managing and supervision, manuscript writing and discussion. **MB** had a key role in lab analysis, proteomics, supervision, statistics and data managing; **AB** was involved the patient recruitment, data collection, manuscript discussion, coordination of the study; **RB, MG, GC** were involved in lab analysis; **PR** and **PB** performed STED analysis; **AP** performed the mass spectrometry analysis; **AV, PC, FP, PM, SV** were involved in some experimental work and discussed critically the manuscript. **LS, BS**, **LC, GM, FF, MF, GP, LA, RAS, GP, MB, AM, GAR, FP, FP, SN, FM, GE, GG, DS, FS, AR, AT** were involved in patient recruitment, data managing, manuscript discussion.

Clinical trial registration number: The Zeus study was registered at https://clinicaltrials.gov (study number: NCT02403115).

Competing interests. Authors declare no conflicts of interest. A patent on the use of anti-enolase antibodies in diagnosis of LN is pending.

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Abstract

Objective.—Neutrophil Extracellular Traps (NETs) expose modified antigens for auto-antibodies in vasculitis (SVV). Little is known on levels and removal pathways of NETs in Systemic Lupus Erythematosus (SLE), especially in lupus nephritis (LN). We determined circulating levels and defined kinetics of NETs removal in large subsets of incident SLE patients, a part with newly onset nephritis.

Methods.—Serum levels of NETs (ELISA), DNase1/DNase1L3 (ELISAs) and DNase activity (functional assay) were determined in 216 incident SLE patients, 103 had incident LN, in 25 with primary Membranous and IgA nephropathy respectively and in healthy controls. The presence of DNase inhibitors and DNases1L3 mutations were in parallel evaluated.

Results.—Serum NETs were very high in iSLE/LN compared to all groups of controls; incident LN that occurred after iSLE onset had the highest levels. DNase activity was decreased in iLN compared to SLE and controls despite similar serum levels of DNase1/DNase1L3; 20% of LN patients had one half of DNase activity. In these cases, pre-treatment of serum with Protein A restored DNase efficiency; one patient was homozygous for a c.289_290delAC variant in *DNase1L3.* The amount of *ex vivo* NETs production by neutrophils purified from LN, SLE and normal controls was similar in all cases.

Conclusions.—iLN patients have increased circulating NETs and reduced DNase activity. The presence of inhibitory substances and/or of rare DNase1L3 mutations explain the decreased DNase activity. Accumulation of NETs is associated and may contribute to disease severity in SLE, in particular to the development of renal lesions.

Keywords

Neutrophyl Extracellular Traps; DNase activity; DNase level; DNase mutations

INTRODUCTION

The release of Neutrophil Extracellular Traps, or NETosis, is one of the first defense line utilized by neutrophils against bacteria, virus, protozoa and other pathogens (1–3). It starts with the de-condensation and release of nuclear chromatin outside the cell and leads to the formation of a physical net where pathogens are entrapped and killed by elastase, defensin and reactive oxygen species (ROS) (1, 4, 5).

Despite their beneficial effects in host defense, NETs occur at the expense of potential injury to the host. The formation and removal of NETs should be timely regulated and failure to do so may lead to unfavorable consequences. NETosis may be, in particular, implicated in the pathogenesis of autoimmune conditions since DNA and post-translational modified proteins in the NETs may become antigenic (6–11). This view is also supported by the observation that pathogens, which activate NETosis, frequently function as trigger or enhancing factor for autoimmune diseases (12).

In early studies, patients with systemic lupus erythematosus (SLE) were shown to display higher NET levels than healthy controls, possibly due to a defective NET degradation (13). Within SLE patients, NETs levels and NET-degrading ability have been associated with disease severity (14) where, in small series of patients, higher NETs levels correlate with the development of lupus nephritis (LN). Nonetheless the importance of the topics, only few studies have focused on the kinetics of NETs in SLE and data on levels of circulating NETs in LN are still scanty. Our study fills up these gaps: taking advantage of the large cohort of patients with SLE and lupus nephritis (LN) recruited within the Zeus project(15), we measured circulating NETs remnants, kinetics of NETs formation and removal by DNase in large cohorts of SLE and LN patients. Potential factors modifying DNase activity (ie. circulating inhibitors, mutations of *DNASE*1) were, in parallel, analyzed.

PATIENTS AND METHODS

Study design.

Samples from SLE patients were obtained from the bio-bank of the Zeus study, a prospective, multicenter study collecting blood samples and clinical information from SLE patients (15) (NCT02403115). A written consent was obtained before sampling. The data base and samples collection is located at the Giannina Gaslini Institute of Genoa (I). Diagnosis of SLE was done according to the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus (SLICC) (16). Controls were

50 healthy donors of the hospital staff (19–50 yrs); 20 patients with IgA, Glomerulonephrites (GN) and 30 with Membranous nephropathy were also studied. All SLE were incident patients (i). Also LN patients were incident; they were recruited immediately at the onset of renal symptoms that coincided, in general, with the time of renal biopsy. Blood samples were obtained at this time. The iLN cohort included two populations: a first group presented the renal pathology as first symptom (iLN as onset), a second group presented the renal flare after 1 or more years from the SLE diagnosis (iLN in SLE) (see Table 2).

In all patients, disease activity and type of organ involvement were scored according to the SLEDAI 2000–2K index (17). Lupus nephritis was defined according to WHO classification on the basis of immunofluorescence for IgG, IgA, IgM, C1q, C3 deposition and classical histology stainings (hematoxylin/eosin, Masson's trichrome, silver methenamine, and periodic–acid Schiff). Patients with severe infections, malignancies, positivity for chronic hepatitis B, HBV or Hepatitis C virus, breast-feeding or pregnant were excluded. Therapies manly consisted in steroids and hydroxylchloroquine in SLE and steroids plus cytotoxic drug in LN patients (Table 2).

Permission and Registration.

We obtained written approval of the protocol by the local Independent Ethics Committee (Comitato Etico Regione Liguria) on October 24th, 2014 (n 407REG2014). The study was approved by the Italian Drug Agency (AIFA) and was registered at https://clinicaltrials.gov (study number: NCT02403115).

Serum NET remnants quantification.

Levels of NET remnants were determined in serum and plasma utilizing an ELISA assay in accord to Hakkim et al. (13) with some modification (18). The assay determines the myeloperoxidase (MPO)-DNA complex and consists in blocking MPO with specific antibodies on solid phase and determining the free edge of the complex with unconjugated anti-dsDNA antibodies (3519 DNA, AbCam, Cambridge, UK). Anti-MPO monoclonal antibody (5 μg/ml) (Clone 2A11, Serotec, Bio-Rad, CA, USA) were coated overnight at 4°C in 96-well maxi-sorp-nunc-immuno plates (ThermoFisher Scientific, MA, USA) in 3% BSA in PBS. One-hundred μl of (1:50) diluted sera were added per well and incubated overnight at 4°C. After 3 washes with PBS and 0.05% v/v of tween-20 (PBS-T), samples were incubated 4 hours with anti-double-stranded DNA polyclonal antibody (Abcam, Cambridge, UK). After three washes with PBS-T, HRP anti-Human IgG were added and incubated 1 hour and washed again three times with PBS before adding the peroxidase substrate (TMB, Bio-Rad). Absorbance at 450 nm was measured using Mark microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, Ca).

DNase activity and Protein A for removing inhibitors.

DNase activity was determined with a one-step assay based on the decrease of fluorescence intensity of degrading Picogreen DNA dye/double-stranded DNA (dsDNA) complex in solution. Serum and plasma (100 μl) diluted 1:50 with 100 mM Tris-HCl, 20 mM $CaCl_2$ and 2 mM $MgCl_2$ (pH 6.8) were placed in ultraviolet light-transmissive high-quality 96-well

micro-plates (Brand, Wertheim, Germany). After adding high polymeric dsDNA (SIGMA-Aldrich, MI, USA) and Picogree DNA dye (ThermoFisher Scientific, MA, USA) pre-heated at 37°C, a first fluorescent measurement was performed after 5 minutes (T_0). Then, the plate was incubated overnight at 37°C, sealed from the top with adhesive sticker to inhibit evaporation during incubation step and protected from light until the final measurement after 16 hours (T_{16}). Measurements of Picogreen DNA dye fluorescence intensity were performed in a fluorescence reader at 520 nm (three reads with 20 μs of integration time). The percentage of decrease of fluorescence intensity was determined by subtracting the value obtained at T_0 with the value at T_{16}. Results are given as % of degraded DNA per well.

DNase activity in 'low activity samples' was also measured after Protein A (staphylococcus aureus) treatment to remove potential inhibitors of DNases. In this case, 100 μl of Protein A (Sigma Aldrich, St Louis, MO) in PBS were incubated with 100 μl of serum for 2 hours. Unbound material was recovered after centrifugation (800 g × 30 min) and re-tested for DNase activity.

DNASE1 and DNASE1L3 quantification.

A home-made ELISA assay was utilized to test DNAse1 serum levels. Anti-DNAse1 rabbit polyclonal antibodies (Abcam) were coated in 96-well maxi-sorp-nunc-immuno-plates (ThermoFisher Scientific)and maintained overnight at 4°C. After blocking in 3% BSA in PBS, 100 μl of standard or 1:50 diluted sera were added to each well and incubated overnight at 4°C. Wells were washed three times with PBS and 0.05% v/v of tween-20 (PBS-T). Then, plates were incubated 4 hours with anti-DNase1 mouse polyclonal antibody (Abnova, Taipei, Taiwan). Each well was, then, washed again, added with 100 μl of substrate solution and blocked with 50 μl of stop solution; reaction was read at 450 nm as above. Results are given as ng/ml. The assay was validated by comparison with a commercial kit for DNase1 (Catalog n LS-F27682 LSBio Inc., Seattle, USA).

For DNASE1L3 we utilized a specific for DNase 1L3 ELISA (kit purchased from LSBioInc., Seattle, USA) according to the manufactureŕs instructions; details are given in the Supplementary methods. Results are given as ng/ml.

DNASE1L3 sequencing.

The analysis was done with Next Generation Sequencing in the Laboratory of Neurogenetics and Neuroinflammation at the Image-Institut des maladies genetiques, Paris (Prof Y. Crow) (19).

Anti-DNA antibodies.

Anti-DNA antibodies determination was done with 2 methods, one was the commercial assay DNA-DIAMEDIX (Delta Biologicals, Rome, Italy) and the second was a home-made western-blot. Details are given in the Supplements Methods. The agreement between DIAMEDIX and the home-made assay was measured using the Cohen kappa (k=0.61; 0.50–0.72 CI at 95%) and the Sperman's correlation coefficients (0.67 with 0.59–0.75 CI at 95%). Sensitivity, and specificity were 90% and 70%, respectively.

Ex vivo NET production.

The study on *ex vivo* NET production was done in representative part of patients recruited for the main study groups above (ie. 18 with active iLN, 15 with iSLE and 27 controls). Neutrophils were isolated from heparinized peripheral blood under sterile conditions, using dextran sedimentation followed by Ficoll gradient centrifugation (20) (see supplement methods for more details). Neutrophil suspensions were allowed to adhere onto 24-well plastic dishes and were cultivated as described in supplementary methods. NET release was determined in resting and Phorbol Myristate Acetate (PMA) stimulated cells.

To quantify NETs production, it was used the Cayman's NETosis assay kit (cat. No 601010, Cayman Chamical, MI, USA) according to the manufactureŕs instructions. Briefly, 100 µl of standard or culture supernatants per well, pre-heated to 37°C, were incubated with 100 µl of the 1:30 diluted NET assay neutrophil elastase substrate for 2 hours at 37°C before reading at 405 nm.

Statistical analysis.

Comparison of data sets were done using Mann-Whitney or Kruskal–Wallis tests respectively for two or more of two unpaired samples. Spearman correlations were calculated for defining any relationship among biochemical parameters and biomarkers of lupus activity. Two-tailed P-values ≤ 0.05 were considered significant.

In Receiver operating characteristic (ROC) analysis, proteins with an area under the curve (AUC) ≤ 0.5 were excluded.

Normal Limits.

Normal limits for all the parameters above were calculated from ROC curves; the Cut Off represented the value that minimizes the geometric distance from 100% sensitivity and 100% specificity on the ROC curves (21, 22).

RESULTS

Clinical features of the different groups of patients.

The main clinical differences between the 3 sub-groups of patients (incident SLE, incident LN as onset, incident LN after SLE) are reported in Table 1 and 1B. Age was comparable excepting for 3 pts under 16 years in the group with iLN as onset. With the exception of the renal involvement that was uniquely present in all iLN and joints that were comparable in iSLE and iLN, there were a few minimal disparities in other organ pathologies (Table 1). With reference to renal parameters (histology, proteinuria, renal function etc.) no difference was observed in iLN with nephritis occurring as onset of the disease or after at least 1 year from the diagnosis of SLE.

Circulating NETs remnants are high in SLE and lupus nephritis.

Serum NETs remnant levels were significantly higher in patients with iLN and iSLE compared to healthy controls and to patients with other primary glomerulonephritis ($p<0.0001$) (Figure 1A, Supplement Figure 1). NETs levels were comparable in the two sub-

groups of iLN and, in both, were higher compared to SLE ($p<0.02$). Serum and plasma NETs were correlated and comparably modified in the cohorts of patients as above(not shown). ROC curves showed AUCs of 0.82 and 0.92 of the DNA-MPO assay (measuring NETs remnants) to diagnose SLE or LN, respectively (Figure 1B) (21, 22).

Serum NETs did not correlate with biomarkers of SLE activity including C3/C4 and SLEDAI (Supplement Table 1) nor with age and therapies and in particular with steroid doses. A

DNase activity is reduced in lupus nephritis.

DNase activity was reduced in iLN patients compared to SLE and controls (Figure 2A) reaching, in some cases, levels one half the normal activity. The two subgroups of iLN had comparable DNase activity. The lowest levels were found in patients with high circulating NETs; 20% of LN patients with serum NETs > 0.5 (RU/ml) had DNase under the limit of normality (Figure 2B).

Sera with DNase activity in the lower range (that are indicated in squares in Figure2b) were pre-treated with Protein A and G to remove potential inhibitors. This pre-treatment increased DNase activity up to normal levels in 5 samples, implying that the removal of serum elements with affinity for the dye had restored DNase activity in patients with very low functional levels (Figure 2C).

Mutations of *DNASE1L3.*

One patient (indicated with a triangle in Figure 2a) was found to carry the a c.289–290delAC homozygous variant of *DNASE1L3*. He was a boy of 10 yrs who presented a severe form of lupus nephritis that progressed very rapidly to end stage renal failure. After a few months of hemo-dialysis he received a renal transplant. Details on the clinical outcome and on therapies will be reported separately from here.

Circulating levels of DNASE1 and DNASE1L3.

The serum levels of these two enzymes that are deputed to NETs removal (23, 24) were similar in LN and SLE patients and in healthy controls (Figure 3A, 3B). DNASE1 and DNASE1L3 levels were not correlated with serum NETs nor with DNase activity (Supplement Figure 2A, 2B, 2C).

Ex vivo NETs formation.—We studied *ex vivo* NETs production by neutrophils (release of elastase and DNA) obtained from subsets of patients recruited in the main study (neutrophils from 15 iSLE, 18 iLN and 27 normals). Resting neutrophils from patients with SLE and LN released lower levels of elastase than control cells; after stimulation with phorbol-12-myristate-13-acetate (PMA) stimulation, the elastase release increased significantly in the three groups, control neutrophils still presenting the major increment (Figure 4A). DNA and elastase released from stimulated neutrophils were highly correlated (Figure 4B) implying that elastase reflects the DNA-NET complex. Altogether, these data suggest that increased levels of NETs in SLE patients are not due to increased production by neutrophils and indirectly support the concept about the impairment of removal.

DISCUSSION

Modifications of the mechanisms regulating NETosis have been associated with autoimmunity based on the concept that DNA and post-translational modified proteins in NETs may become antigenic(6–8). Failure of NETs removal has been, in particular, considered a trigger for developing renal lesions (LN) in patients with SLE(13). Lupus nephritis is an autoimmune condition that develops in about 50% of all SLE patients(25, 26) and causes end stage renal failure in a significant portion of this cohort. In this study, we investigated NETs formation/removal in SLE patients seeking to define whether kinetics of NETs is different in those patients who develop LN. For this study it was utilized a large population of patients with incident SLE/LN that included patients recruited at the time of the onset of symptoms that for incident LN corresponded to the time of renal biopsy. For the large number of patients, incident LN was also subdivided in cases with LN as first symptoms or incident LN occurring in patients with an already known diagnosis of SLE.

We report here new findings that provide elements of interest on NETosis and its correlation with autoimmunity and with renal manifestations of SLE: ***1***-circulating serum NET remnants were high in lupus nephritis and SLE compared to other control groups; the increment was more evident in incident LN and, in particular, in those patients who presented iLN after having already developed other SLE localization; ***2***-serum NETs did not correlate with parameters of lupus activity not they were correlated with SLEDAI-2k; ***3***-DNase activity was reduced in LN patients for the presence of inhibitory substances in a few sera whereas serum DNASE1/DNASE1L3 levels were normal; ***4***- one boy with the lowest levels of DNase activity had a c.289_290delAC homozygous variant in *DNASE1L3.*

To the best of our knowledge, this is the first study showing a marked increase of circulating NETs in a large cohort of patients with incipient SLE and in particular in patients with incipient lupus nephritis. The later finding is of particular interest suggesting that serum NETs are in some way involved or participate in the pathogenesis of renal lesions. On the contrary, NETs levels did not correlated with signs of lupus acute activity such as the SLEDAI-2k index or with C3/C4 thus representing a parameter indicating activity in the long term. On a more clinical vein, serum NET levels could be utilized as a potential new biomarker of SLE evolving towards nephritis.

A second main finding is that DNase activity is defective in patients with LN. Digestion of circulating DNA and of DNA in NETs is mediated by DNASE1 and DNASE1L3 (27). In our patients, serum DNASE1 and DNASE1L3 levels were normal (albeit within a broad range in different cohorts) and no correlation was found between serum DNASE1 and/or DNASE 1L13 levels and DNase activity. The only study available in the literature reported low DNASE1 activity serum in 23 patients with SLE who were studied in combination with patients with microscopic polyangiitis (28). No data are available relative to serum levels of DNASE1IL3. Other Authors (13, 29) have, instead, shown the existence of circulating anti-NET antibodies that inhibit DNASEs in a subset of patients with SLE, defined as non-degraders, who were prone to develop LN. Genetic data in humans carrying mutations of *DNASEs* (19, 30–32) and results deriving from molecular manipulation of *DNASEs* in mice

(27, 33–35) indicate an association between mutations of *DNASEs*, reduced DNase activity and autoimmune activity, in particular, with the development of renal lesions.

We could confirm here, in a large cohort of patients with Lupus nephritis, that both mechanisms (ie. presence of inhibitors and genetic defects) modify DNase activity *in vivo* and that they are associated with a specific phenotype of SLE with renal lesions. Therefore, mechanisms for DNase functional failure are multifactorial and can be observed in a significant part of patients with LN having low enzyme activity. Actually, DNase activity was restored by ProteinA suggesting that circulating inhibitors of DNASEs are probably IgG that have been removed by this protein; this finding confirms and strengthens the original hypothesis made by Hakkim (13) about the existence of circulating antibodies that reduce NETs removal in SLE patients prone to develop nephritis. A c.289_290delAC homozygous variant in *DNASE1L3* has been detected in one young boy (the youngest of the entire cohort) who presented a very early development of LN and rapid evolution to end stage renal failure (clinical details and therapy will be presented and discussed elsewhere). The same mutation of DNASE1L3 has been already described in few other children with early-onset hypocomplementaemic urticarial vasculitis with glomerulonephritis (30). Overall, it seems reasonable that molecular defects should be detected in young people, whereas circulating inhibitors could occur at an older age. Considering the different clinical approaches that should be utilized in the two different conditions (ie. in presence of inhibitory substances vs. molecular molecular defects) we propose here to introduce both the determination of serum NET remnants and the functional analysis of DNase as screening tests in clinical settings; in those cases who present low DNase activity (and we suggest only in this subset of patients) it would be useful to proceed with the characterization of circulating inhibitors and with molecular sequencing of *DNASE1* and *DNASE1L3.*

In conclusion, our results show a relationship between NET levels and evolution towards lupus nephritis in patients with SLE. Determining serum NET levels could represent an informative way to herald the development of renal lesions and have clinical implications. The present data on reduced DNase activity in patients with LN also support the idea that NETs accumulate in serum for a defective removal and circulating inhibitors of DNase activity are potentially responsible for this phenomenon. More rare mutations in *DNASE1L3* produce similar modifications. Altogether, these findings on NET remnants levels and their kinetics of production and removal represent a further advancement with new diagnostic and therapeutic potential implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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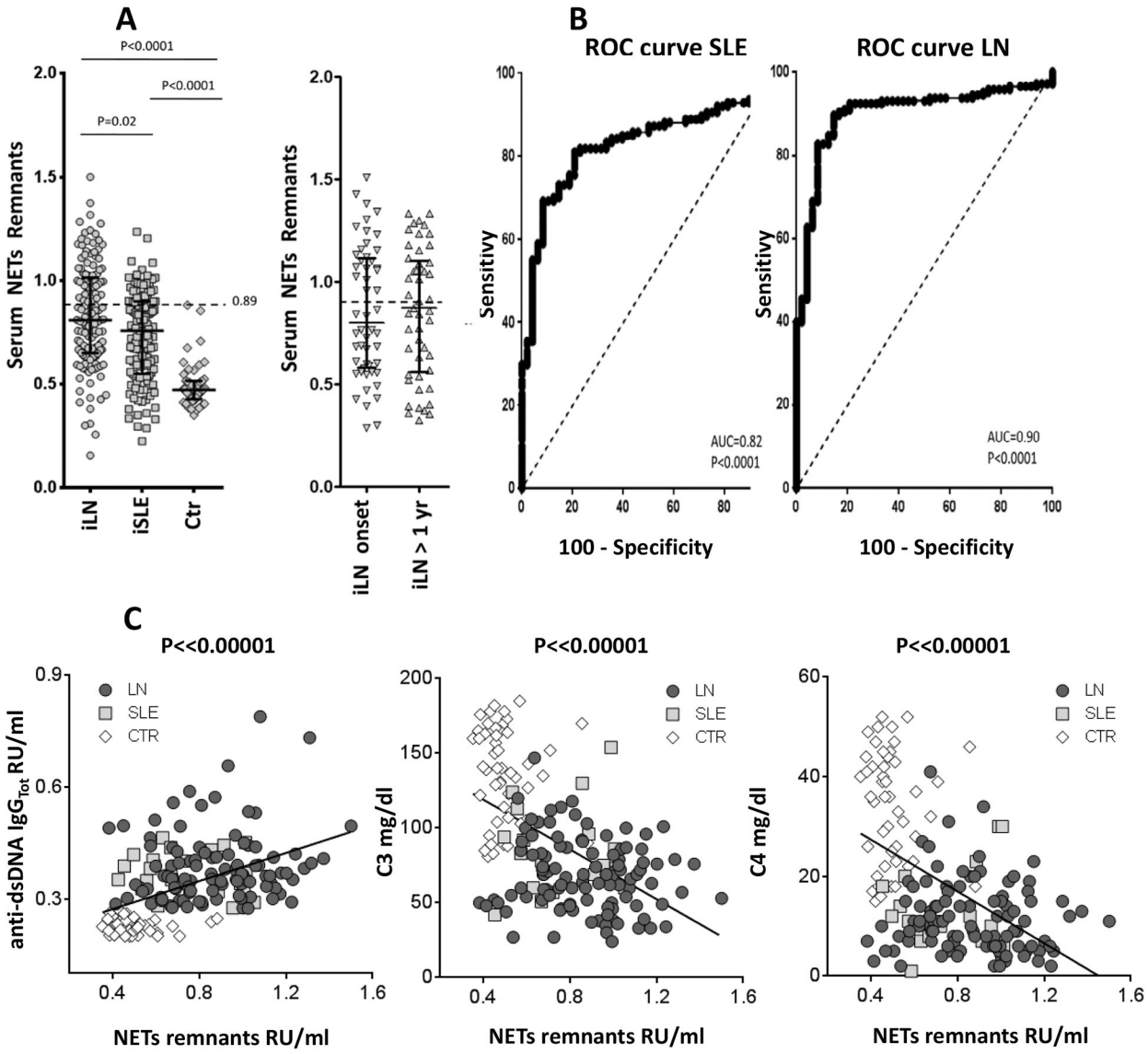

Figure 1. Circulating Neutrophil Extracellular Traps (NETs) Remnants.

(**A**) Serum NETs were determined using an ELISA measuring the DNA-MPO complex. Results are Relative Unit/ml given as median and interquartile range. The dotted line indicates the upper limit of normality (0.89). Here, it is reported serum NETs in all SLE, in all LN and in normal people. It is also, in parallel, shown NETs levels in LN patients split in the two subgroups divided according to the indications given in Materials and Methods (i.e. LN as onset, LN after 1year from the SLE onset).

(**B**) ROC curves showing specificity and sensitivity of the DNA-MPO assay for LN and SLE patients.

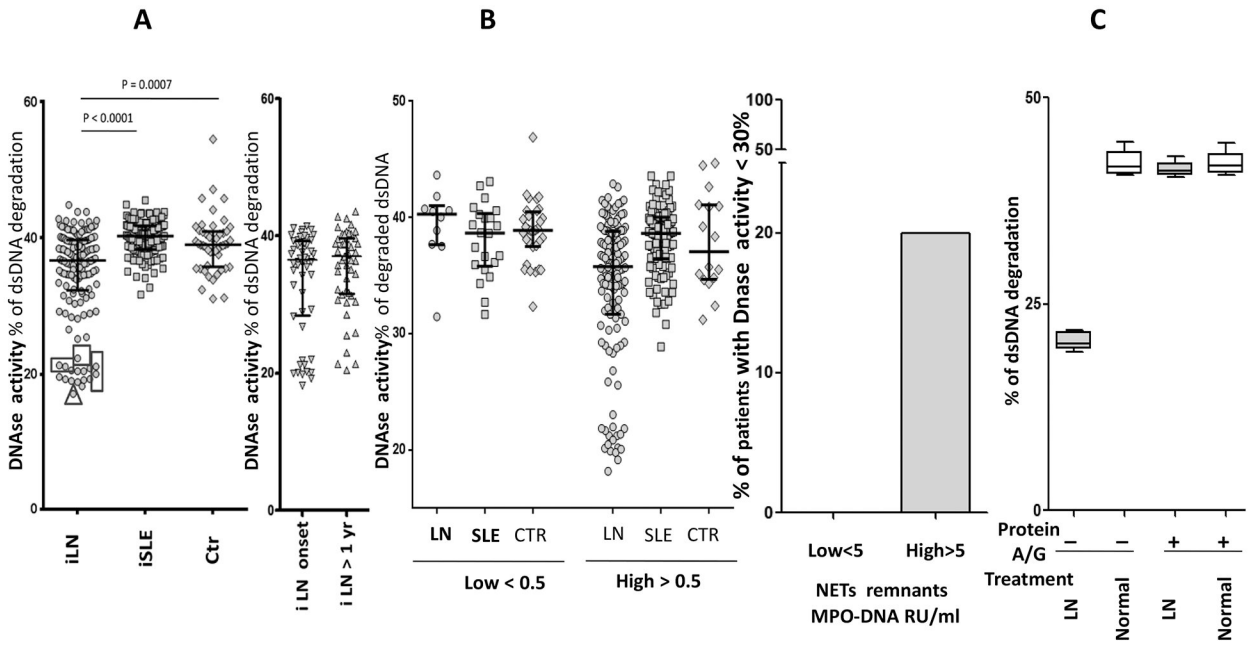

Figure 2. Serum DNase activity.

(**A**) DNase activity was determined with a one-step assay based on fluorescence decrease of degrading Picogreen DNA dye/double-stranded DNA (dsDNA). In patients indicated with a square serum DNase activity was re-tested after treatment with Protein A/G. The triangle indicates a boy who presented a c.289_290delAC homozygous variant in *DNASE1L3.* Here, it is also in parallel reported DNase activity in LN patients split in the two subgroups divided according to the indications given in Materials and Methods (i.e. LN as onset, LN after 1year from the SLE onset).

(**B**) patients with LN were subdivided according to their serum levels of NETs remnants (higher and lower than the normal level of 0.5 RU/ml).

(**C**) DNase activity in patients with high and low NETs remnants. All patients with DNase activity < 30% were patients in the high NETs group.

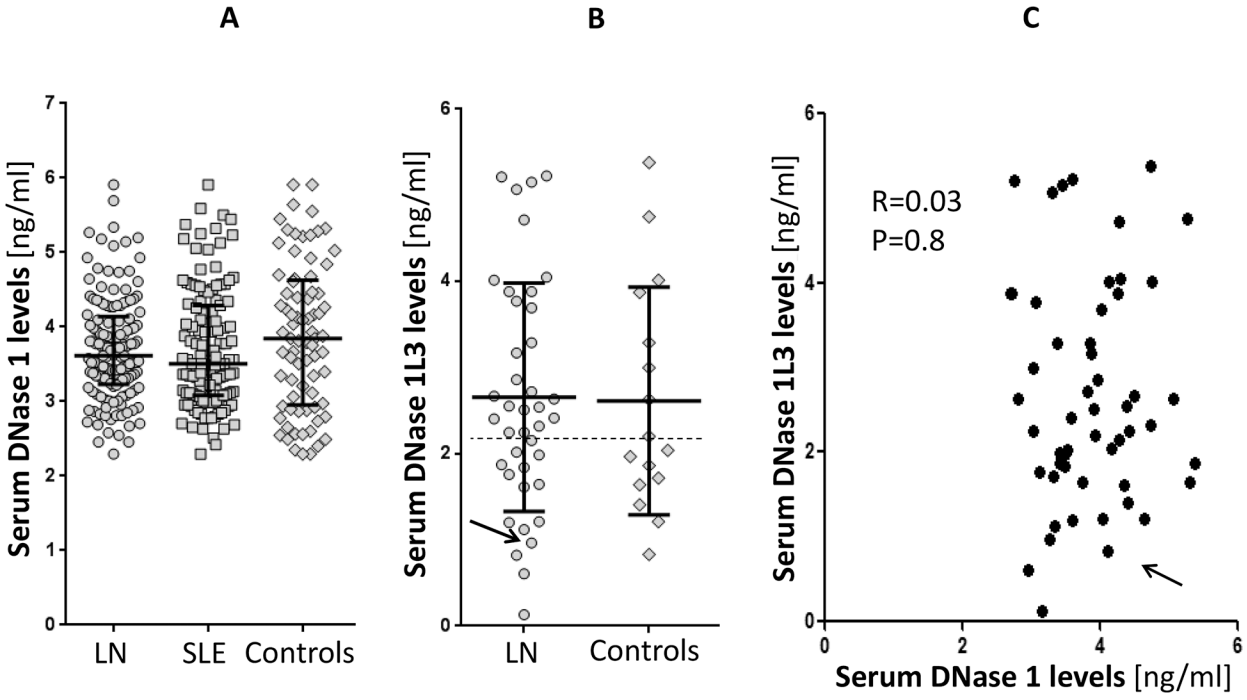

Figure 3. DNASE1 and DNASE1L3 levels.

(**A**) DNASE1 serum levels. A homemade ELISA assay has been utilized to test DNASE1. Results are expressed as ng/ml and represented as median and interquartile range.

(**B**) DNASE1L3 serum levels. For DNASE1L3 we utilized a commercial ELISA (LSBio kit, Seattle, USA). Results expressed as ng/ml are given as median and interquartile range. In this case, those LN patients who presented maximal variability in DNase activity were chosen for testing DNASE1L3 levels here including patients with low and patients with high DNase activity. For the broad distribution of values, ROC and normal limits were not calculated.

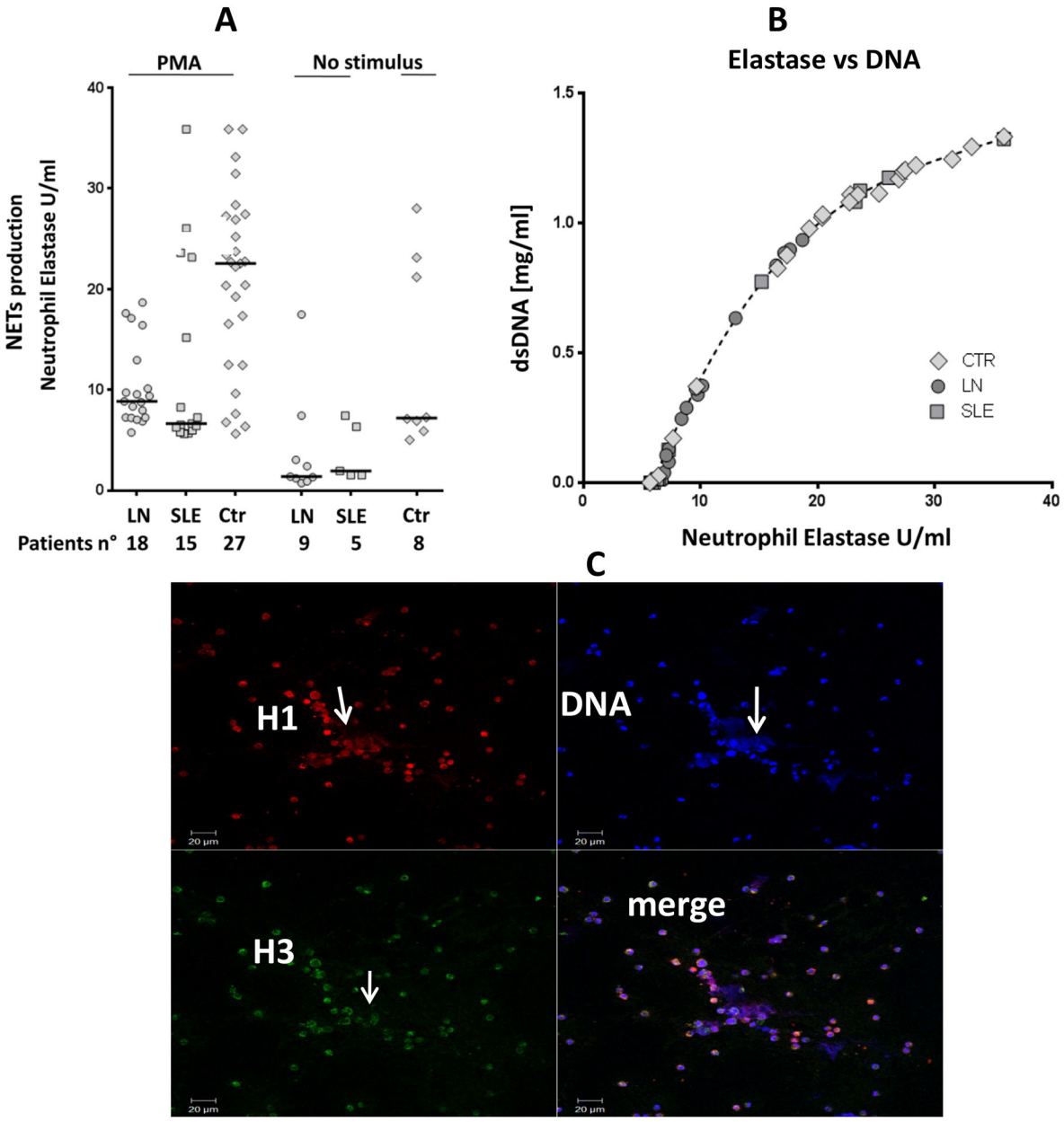

Figure 4. NETs production and protein composition.

(**A**) *Ex vivo* NETs formation was evaluated as elastase and DNA release by resting neutrophils purified from patients with SLE (n=5), lupus nephritis (n=9) and from healthy controls (n=8). More cells (15 SLE, 18 LN and 27 controls) were utilized for stimulation with phorbol-12-myristate-13-acetate (PMA). Kinetics of NETs formation was analyzed in all supernatants utilizing the elastase method(13).

(**B**) In all experiments the release of elastase from stimulated neutrophils was highly correlated with the release of DNA.

Table 1.

Characteristics of iSLE, iLN, IgA, MN and controls.

Patients	iSLE	iLN	CTR	IgA	MN
Number	113	103	50	20	30
Female/Male (n)	100/13	90/13	43/7	16/4	6/24
Age (years)	35 * (22–51)	25 * (8–43)	38 * (19–50)	28 * (15–30)	42 * (35–48)
yrs from diagnosis of SLE	<0,3	-	-	-	-
yrs from diagnosis of LN	<0,3	<0,3			
Organ involvement: n(%)					
Kidney	0	103(110)			
Joint	49 (43)	42(41)			
Hematologic **	65(58)	83(80)			
Cutaneous rush (no)	13(12)	0			
Neurologic	3(3)	0			
SLEDAI	8+/−4	6+/−6			
C3 mg%	81+/− 42	91+/−30			
C4 mg%	13+/−12	14+/−12			
Proteinuria (g/24 hours)					
at diagnosis	0.01(0.01–0.1)	2.41 (1.2–4.5)	NA	0.5	6.8
after 12 months	0.07 (0.02–0.1)	0.49 (0.2–1.1)	NA	0.4	2.4
Serum proteins (g/dl)					
at diagnosis	7.3 (6.9–7.7)	6.1 (5.3–6.8)	NA		
after 12 months	7.1 (6.6–7.7)	6.6 (6.3–7.08)	NA		
Therapy at diagnosis/after 12 months n(%)					
Steroids	82 (65)/26 (21)	78 (76)/62 (60)	NA/NA	12	0
Cytotoxic	0 (0)/0 (0)	25 (24)/0 (0)	NA/NA	-	20
Cyclosporine A	1 (1)/3 (2)	1 (1)/4 (4)	NA/NA	-	8
Azathioprine	5 (4)/0 (0)	6 (6)/21 (20)	NA/NA	-	
Mofetil Mycophenolate	3 (2)/0 (0)	7 (7)/14 (14)	NA/NA	-	
None/Hydroxychloroquine only	34 (31)/5 (4)	20 (19)/1 (1)	NA/NA	-	

Legend: NA= not available.

* data are given as median and interquartile ranges.

** For the presence of Hematologic changes, the SLEDAI 2K indication of WBC<3000 was considered

Table 2:

Renal and urinary characteristics in incident LN patients. Incident LN (iLN) was defined as the development of urinary symptoms of nephritis in patients with serum positivity for SLE markers and renal pathology typical of lupus nephritis. A sub-group of iLN presented the renal pathology as first symptom; a second sub-group presented the renal flare after 1 or more years from the SLE diagnosis.

Patients	**i LN as onset**	**i LN after SLE**
Number	53	50
Female/Male (n)	48/5	45/5
Age (years)	22 (8–40)	27 (18–43)
Pts <16yrs	3	0
yrs from LN	<0,3	<0,3
yrs from SLE	5	>1
Urinary abnormalities		
Casts	++	++
Proteinuria	2,5 (1,6–4,5)	2,1(1,3–4)
Red blood cells	+++	+++
Serum creatinine	0,7(0,5–1)	0,6(0,4–0,9)
Histological stage (%)		
I	0	0
II	7	10
III	25	28
IV	35	32
V	33	30
Therapy at diagnosis/after 12 months n(%)		
Steroids	40(76)/33 (60)	40(80)/10(20)
Cytotoxic	13(24)/0 (0)	15(30)/0(0)
Cyclosporine A	1 (1)/2 (4)	0(0)/0(0)
Azathioprine	3(6)/11(20)	0(0)/15(30)
Mofetil Mycophenolate	4 (7)/7 (14)	2(0,5)/10(20)
None/Hydroxychloroquine only	10 (19)/1 (1)	20(40)/5(10)