Neutrophil Extracellular Traps (NETs) constitute a network of DNA and proteins released by neutrophils in response to infectious and immunologic triggers. NET proteins are recognized as autoantigens in ANCA vasculitis; limited knowledge is available in other autoimmune pathologies. The composition of NETs produced ex vivo by resting and Phorbol-myristate acetate (PMA) stimulated neutrophils was analyzed by high-throughput Fusion Orbitrap technology in 16 patients with Systemic Lupus Erythematosus/Lupus nephritis (9 SLE/7 LN) and in 11 controls. Seven-hundred proteins were characterized and specific fingerprints discriminated LN from SLE. We focused on methyl-oxidized α enolase (methionine sulfoxide 93) that was markedly increased in NETs from LN and was localized in NET filaments in tight connection and outlying DNA. The isotype of anti-α enolase antibodies was IgG2 in LN and IgG4 in other autoimmune glomerulonephritis (Membranous Nephropathy, MN); serum anti-α enolase IgG2 were higher in LN than in SLE and absent in MN. The same IgG2 antibodies recognized 5 epitopes of the protein one containing methionine sulphoxide 93. In conclusion, specific NET protein fingerprints characterize different subsets of SLE; methyl-oxidized α enolase is over-expressed in LN. Circulating anti-α enolase IgG2 recognize the oxidized epitope and are high in serum of LN patients. Post-translational modified NET proteins contribute to autoimmunity in patients with LN.
are main examples\textsuperscript{23,24}. In vasculitis, myeloperoxidase (MPO) and proteinase-3 (PR3), the two auto-antigens recognized by anti-neutrophil cytoplasm antibodies (ANCA), are, in fact, released from NETs\textsuperscript{21}. Systemic Lupus Erythematosus (SLE)\textsuperscript{25} is another condition in which NETs have been shown to be formed\textsuperscript{13,26,27} and NETs implication in SLE pathogenesis hypothesized.

Though NETosis is recognized as a key protective and pathophysiological mechanism, no studies have utilized high throughput proteomics to identify and characterize structural features of the soluble protein components of the NETs complex. The present study investigates protein composition of NETs and their post-translational modifications in patients affected by clinical relevant SLE. Patients were subdivided according to the presence of renal complications (Lupus nephritis, LN), the most severe long term evolution of SLE.

Results

NETs composition in different clinical settings. Controls, SLE and LN. A total of 697 proteins were identified in PMA stimulated neutrophils (Supplement Table S1). Among these, 404 (57.9\%) proteins overlapped among the 3 different groups of interest and only 53 (7.6\%), 18 (2.6\%) and 222 (31.9\%) were exclusive for LN, SLE and Control neutrophils respectively (Fig. 1a). The majority of the 697 NET components corresponded to proteins associated with autoimmune disease (477), some of them had been previously described in relation with SLE (169); 140 were specific of neutrophils (UniProt, Open Target and Atlas database). Sub-cellular localization was similar: 28–30\% of proteins were localized in membranes, 32–34\% in cytoplasm/cytoskeleton, 15\% in organelle and 23–24\% in nucleus (Fig. 1a).

MDS analysis allowed to distinguish 3 clusters relative to the 3 different clinical conditions with marginal overlap of areas at 95\% of confidence interval (CI) between SLE and LN (Fig. 1b). Volcano plot reports proteins with at least a two-fold increment and P-values $\leq$ 0.05 (after correction for multiple interactions) as limit for significance (Fig. 2a). The first Volcano considered SLE and LN together as compared with control neutrophils: a total of 137 proteins were differently expressed by the two cohorts (Supplement Table S2). Among these, 56 and 81 proteins were enriched respectively in Control and SLE/LN groups. The proteome profile that includes the differences above is highlighted by the heat map after Z-score shown in Supplement Fig. S1a. These 137 proteins were classified on the basis of their cellular components (CC) and molecular function (MF) according the available GO signatures: 35\% of proteins were annotated as cytosol/cytoskeleton, 31\% as nuclear, 28\% as organelle and 6\% as membrane proteins. Based on their molecular function, 40\% proteins were classified as binding proteins, 39\% as protein with catalytic activity, 12\% as structural molecules, 4\% as proteins with antioxidant activity, 1\% as signal transducers and 2\% as transporters and receptors (Supplement Fig. S1b). Signatures for Controls, SLE or LN derived from all identified proteins and their relative abundance are showed in Fig. 2b. In this diagram reports the significant GO signatures of Control (x-axis), LN (y-axis) and SLE (z-axis). Non-hierarchical cluster analysis with K-means of this plot shows two distinct clusters characterized by the GO enriched in Control (black circles) or in SLE and LN (red circles). The ellipse shows the cluster area at 95\% of confidence interval (CI).

SLE vs LN. The same statistical approach was adopted to characterise the differences of NETs composition in supernatants of SLE or LN patient-derived neutrophils (Supplement Table S2): 15 proteins were found to
discriminate between the two conditions and are reported in details in Table 1 and in Fig. 2a. Among these, 4 and 11 proteins were over-expressed in SLE and LN supernatants respectively. The differences above are shown by the heat map after Z-score in Fig. 2c. The 15 proteins above were classified on the basis of CC and MF according the available GO reference signatures: 36% of proteins were annotated as cytosol, 32% as nuclear, 27% as membrane and 5% as organelle components. Based on MF, 50% proteins were classified as binding proteins, 33% as catalytic activity, 7% as transcription factor and 2% as transporter (Fig. 2c).

**Biostatistical analysis.** Interactions of proteins/peptides over expressed by LN and SLE NETs is presented in Fig. 3a–c. Four proteins over-expressed in NETs of LN (ie ENO1, ANXA1, DSG1 and ESD) are hubs for cellular and extra-cellular functions. In particular ENO1 is involved in the energetic metabolism (glycolysis and gluconeogenesis with GAPDH, PKD and PARP9), has a function in the transport of hexoses (traslocation of GLUT4...
in membrane CALM2 and ACTG1), in biosynthesis of nucleotides (MNE4, RNASE3, CLAM2 and NF1), in the response to infections (GSTO1, SH3BGR13, TXN, CAT, ESD, TRPM8, RNASE2, LCN2, GAPDH, CTSG, ELANE, Mnda, MPO, CAP1, SPRED1, ACTB, PFN1, MYH9, ROCK1, ACTG1, CALM2, HSPA8, ELANE, GAPDH) and, finally, it is involved in cytokinesis and cell motility (ACTG1, ACTB, VIM, MYH9, ACTN4, TLN1, TPM1, ANXA6, ANXA1, CALM2, DSG1, ROCK1, Cadsps, Gsn and MSN). ANXA1 is connected with regulation of cytokinesis, in regulation of apoptosis and in interleukin signaling. DSG1 is directly linked with the cleavage of apoptotic proteins and via ROCK1 with MAPK signaling and infectious diseases (WDR1). ESD is directly linked with detoxification processes, and indirectly (MPO,TRPM8) with defence against bacteria and fungi. To describe more in general the interconnections, we drew two ‘bubble diagrams’ of significant top GO signatures highlighted by previous analysis (Fig. 3b,c). Interestingly, many proteins were enriched in biochemical processes associated with inflammation, autoimmunity and lupus diseases and the major biochemical pathways were implicated in regulation of cellular process, interleukins and JAK-STAT signaling, vitamin and metabolic cofactors and oxidation.

**Post-translational modifications of NETs components.** Results of the characterization of post-translational modifications (PTM) of the NET peptides are presented in Fig. 4a. We evaluated all types of PTM, grouping them in: methionine (sulfoxide), thiol oxidation (sulfonic acid), deamination of N, Q and K aminoacids (citrullination) and all other types of PTM.

A total of 1931 peptides with at least one PTM were identified (Fig. 4a) corresponding to 440 proteins. The majority of PTM was found on Control peptides (1296) followed by LN (877) and SLE (662). Only a few of these PTM were exclusive for LN (16.1%) and SLE (8.5%) samples or (8.2%) were in common with the two. Despite some overlapping, SLE and LN showed a peculiarity of PTM peptide composition and profile intensity that allowed to clear distinguish these samples from control NETs (Supplement Fig. S2). To better describe these differences we utilized univariate statistical analysis, PLS-DA and SVM. A total of 123 peptides with at least one PTM were found when Control cells was compared to SLE and LN as considered together (Supplement Fig. S3a). The profile of these highlighted PTM peptides were showed by means of heat map after Z-score (Supplement Fig. S3b).

The same analysis was performed to describe the differences between SLE and LN. A total of 34 peptides were highlighted by volcano plot (Fig. 4b). The profile of these highlighted peptides were shown by means of heat map after Z-score (Fig. 4c left side); among this, 21 PTM peptides identify a core of shared peptides, when Control cells were compared to SLE and LN together and SLE was compared to LN (Fig. 4c right side). This core panel of peptides allows to distinguish simultaneously CTR, SLE and LN NETs samples (Supplement Table S3) and the corresponding proteins are in relationship with autoimmune and SLE disease (Uniprot, Disgenet, Open Target and Atlas database).

**Oxidised α-enolase in NETs discriminates LN vs SLE and controls.** LN patient-derived NETs were characterized by a high expression of α-enolase (ENO1, 2 isoforms: P06733 and P06733-2) (see Table 1). PTM relative to α-enolase and characterized by Fusion Orbitrap in supernatant of NETs are outlined in Fig. 5a. Major PTM that characterized LN NETs was methionine sulfoxide in place of methionine 93 that is included in the E85-K105 peptide. The expression of the modified E85-K105 containing methionine sulphoxide was significantly increased in LN patients vs SLE; all controls had normal methionine 93 with null expression of the modified peptide (Fig. 5b). The localization of α-enolase in the NET filaments was typically outlying DNA (Fig. 5c).

| Protein IDs | Protein name | Gene name | Fold Change SLE/LN vs CTR | Fold Change SLE/LN vs CTR | Fold Change LN vs SLE | Fold Change LN vs SLE |
|-------------|--------------|-----------|--------------------------|--------------------------|----------------------|----------------------|
| H7C571      | Transcription cofactor vestigial-like protein 3 | VGLL3 | 2.199 | −4.698 | 8.683 |
| F5H6P7      | Protein mago nashi homolog | MAGOH1B | −2.589 | 2.279 | −4.649 | 5.547 |
| F6TLX2      | Glyoxalase domain-containing protein 4 | GLOD4 | 1.386 | 1.496 | 3.599 |
| H7C3U4      | E3 ubiquitin-protein ligase MYCBP2 | MYCBP2 | 6.549 | 5.216 | 2.736 |
| O75083      | WD repeat-containing protein 1 | WDR1 | −1.464 | 3.179 | 2.897 |
| P04083      | Annexin A1 | ANXA1 | 1.402 | 2.829 | 3.289 |
| P06733      | Alpha-enolase | ENO1 | 7.085 | 6.042 | 6.906 |
| P06733-2    | Alpha-enolase; MBP-1 | ENO1 | 5.149 | 8.477 | 5.128 |
| P10768      | S-formylglutathione hydrolase | ESD | 2.824 | 2.307 | 2.775 |
| P61970      | Nuclear transport factor 2 | NUTF2 | 2.661 | 3.303 | 3.631 |
| P78417      | Glutathione S-transferase omega-1 | GSTO1 | 2.586 | −4.711 | 7.646 |
| Q02413      | Desmoglein-1 | DSG1 | 1.507 | 3.239 | 6.108 |
| Q4VX76-2    | Synaptotagmin-like protein 3 | SYTL3 | 2.797 | 4.011 | 2.901 |
| Q6WKZ4-3    | Rab11 family-interacting protein 1 | RAB11FIP1 | 3.928 | 5.995 | 8.906 |
| Q9ULU8      | Calcium-dependent secretion activator 1 | CADPS | 2.449 | −7.085 | 6.906 |

Table 1. List of the fifteen proteins what maximize the discrimination between SLE and LN NETs samples.
Based on this finding we finally sought to characterize the specific epitopes on α-enolase recognized by antibodies and in particular by IgG2 that is the isotype of circulating anti-α-enolase autoantibodies (see below). Six peptides of decreasing MW from 14 to 0.5 KDa generated by CNBr cleavage of α-enolase (Fig. 5a) were recognized. Noteworthy, the peptide containing the methionine sulfoxide in place of methionines 93, corresponding to CNBr fragment G58-M93, was recognized by circulating autoantibodies (Fig. 5d). That findings support the recognition by immune system of α-enolase post-translational modified epitopes.

Circulating anti-α-enolase auto-antibodies in patients with LN and in Membranous nephropathy. To further strengthen the relevance of α-enolase antibodies in patients with LN, serum levels of autoantibodies against α-enolase were assessed in a large cohort of 103/116 patients having LN/SLE. In a preliminary approach, isotype specificity of anti-α-enolase antibodies was tested in 20 patients with LN and in 20 with Membranous Nephropathy (MN) a well characterized condition of idiopathic autoimmune glomerulonephritis: anti-α-enolase IgG2 were uniquely found in LN while IgG4 were specific for anti-α-enolase in MN patients (Fig. 6a) therefore clearly showing a high isotype specificity of these antibodies in different autoimmune
conditions. According to the above finding, anti-α-enolase IgG2 levels were increased in serum of LN patients (Fig. 6b) and to a minor extent in SLE patients compared to control sera. Sensitivity and specificity were very high in both cases (Fig. 6c).

Discussion
This study sought to characterize the protein composition and post-translational modifications of NETs produced ex vivo by resting and PMA-stimulated neutrophils isolated from blood of healthy donors, patients with SLE or with LN. Results reveal a complex composition of post-translational modified NET proteins and suggest their relevance in autoimmunity. PMA, a substance that stimulates NAPH-oxidase activity and increases oxygen...
radical production\textsuperscript{10–12}, is credited as the \textit{in vitro} model of NETs activation\textsuperscript{10}. We utilized PMA to maximize the production of NETs and the differences between the studied conditions. Our data cast a new light on NET composition in different clinical settings extend the knowledge beyond DNA and histones and potentially provide a springboard for further mechanistic studies in autoimmune conditions such as SLE and vasculitis.

NETs include overall almost 700 proteins, 50% of the total corresponding to proteins already described in association with autoimmunity, inflammation and SLE. Moreover, it is here shown that neutrophils respond to an oxidative stimulus (PMA) by producing components that could be immunogenic: the 90% are membrane, cytosol and cytoskeleton proteins and present phosphorylation, methyl/thiol oxidation and oxidative deamination as major post-translational modifications. Our study, therefore, vastly extends the number of proteins present in NETs from the original description of 25 reported by Urban \textit{et al.}\textsuperscript{16}; this evolution was allowed by the use of high-throughput Fusion Orbitrap that is a new technology of analysis with much enhanced sensitivity in respect to previous mass spectrometry approaches.

This new finding on NETs composition is of particular interest since recent observations indicate that circulating NETs are increased in patients with LN nephritis and that their levels correlate with complement consumption and with levels of other classic antibodies and biomarkers of SLE (\textit{i.e.}, anti-DNA, etc)\textsuperscript{26,28}. Increased NETs levels in LN, prompted us to investigate NET post-translational modified proteins generated by neutrophils in subsets of patients with SLE since it was hypothesized that they could represent potential auto-antigens recognized \textit{de novo} by the immune system. As the results demonstrate, there are, in fact, NET associated proteins that are highly specific for LN (\(n = 11\)) and for SLE (\(n = 4\)); in addition to specific NET-associated proteins, we also

---

**Figure 5.** Oxidised \(\alpha\)-enolase: characterization, expression and localization. (a) Post-translational modifications of \(\alpha\)-enolase in NETs were characterized by Fusion Orbitrap; the E85-K105 peptide containing methy-sulfoxide methionine 93 was found in NETs limited to LN patients; three-dimensional structure of \(\alpha\)-enolase where the epitopes for IgG2 interaction (see below) are reported in green and the merge between G58-M93 and E85-K105 is shown in yellow-red. (b) Intensity of the E85-K105 peptide containing methy-sulfoxide methionine 93 in NETs from LN and SLE. (c) Stimulated emission depletion microscopy (STED) analysis of filaments. (d) The epitopes of \(\alpha\)-enolase that are recognized by anti-\(\alpha\)-enolase IgG2 purified from serum of LN patients. The protein was digested by CNbr and peptides deriving from digestion were separated by western-blot.
observed post-translational modifications specific for SLE and LN including oxidative changes and new deamination residues.

Among the most expressed NET-proteins that were produced by neutrophils deriving from LN patients there were two, i.e. Annexin A1 and α-enolase, that have a recognized regulatory role within the immune system and were, for this reason, further characterized. Annexin A1 (AnxA1) is a 37 KDa protein with phospholipid–binding properties that is expressed in cytoplasm of nucleate cells of blood; it has multifunctional roles in innate and adaptive immunity mainly in the control and resolution of inflammation. Annexin A1 levels are regulated by glucocorticoid and play many of their anti-inflammatory effects; it also modulates neutrophil apoptosis and promotes their phagocitosis by macrophages. Increasing evidence indicates that Annexin A1 plays anti-inflammatory effects in Rheumatoid Arthritis and promotes breast cancer progression and metastasis. Anti-Annexin A1 antibodies have been detected in association with SLE and Rheumatoid Arthritis and have been proposed as diagnostic markers of discoid lupus.

Alpha-enolase is a glycolytic enzyme with multiple localizations and functional implications that go behind the metabolic role. In eukaryotes, α-enolase is expressed mainly in cytoplasm of cells but it is also present in the outer membrane of several epithelial, endothelial and hematopoietic cells where it acts as plasminogen receptor and activator. Circulating anti-α-enolase antibodies have been described in association with LN and NETs, α-enolase became a main focus of our study. A first finding was that in NETs produced by LN cells, α-enolase is modified for the presence of sulphoxide methionine that is constant in all LN patients compared to few SLE (with a highly statistical difference). We hypothesized that oxidation of α-enolase in NETs contributes to break tolerance and leads to the formation of anti-α-enolase antibodies. It is, in fact, currently accepted that NET formation, beside determining externalization of nucleosome and DNA, produces post-translational modifications in other nucleosome components inducing the de novo formation of potential auto-antigens. Evidence of NETs as a source of auto-antigens has been documented in Small Vessels Vasculitis where concomitant increased of NET production is associated with the presence of ANCA-associated autoantibodies against MPO and proteinase 3 that are two components of NETs. Therefore, modified proteins in NETs should be considered as trigger of autoimmunity in terms of increased production of autoantigens.

Our main focus was LN, a severe complication of SLE developing in almost 50% of patients and leading to renal failure (ESRD). In fact, though anti-DNA antibodies are considered the main culprit for disease onset in SLE patients, other autoantibodies have also been detected in the kidney of those patients who develop LN and anti-α-enolase represent a major nephritogenic auto-antibodies in this condition.

In the validation study we focused some aspects related to the specificity of anti-α-enolase antibodies in LN in comparison with other autoimmune conditions targeting the kidney and results add something to elucidate the mechanisms leading to their formation. In fact, circulating anti-α-enolase antibodies have been also described in MN, an idiopathic autoimmune conditions linked with the presence in serum of circulating auto-antibodies versus several glomerular basement membrane proteins here including anti-α-enolase. We observed a rigid isotype specificity of anti-α-enolase antibodies in LN versus MN, since IgG2 were the unique antibodies in LN and IgG4 in MN. High serum levels of anti-α-enolase IgG2 in patients with LN suggest that the adaptive immune response leading to their formation involves TLR8 and TLR9 that are the TLRs driving an IgG2 isotype switching.
Experiments are now in course to demonstrate a direct effect of oxidised α-enolase deriving from NETs in stimulation of TLRs 8/9 and in anti-α-enolase IgG2 production. Two ancillary observations presented here indirectly support this possibility: one is the localization of α-enolase contiguous and external to DNA in NET filaments suggesting it is of easy accessibility to TLRs and B cells; the second is that IgG2 purified from sera of LN patients interact with the epitope G58-M93 that overlaps with the site of α-enolase oxidation (i.e. sulphoxide methionine 93). It must be stressed here that, in spite generation of anti-α-enolase IgG2 should be linked to oxidized α-enolase and TLR8/9 stimulation, anti-α-enolase antibodies react also with non oxidized α-enolase as in our ELISA assay, that utilizes the unmodified protein.

Conclusions
This study reveals, for the first time, the vast complexity of the protein composition of Neutrophil Extracellular traps produced ‘ex vivo’ by cells purified from patients with SLE and LN compared to cells extracted from healthy donors. NETs protein composition is highly specific for the different clinical settings and methyl-oxidized α-enolase (with sulphoxide methionine 93) is highly characteristic for NET of LN vs SLE patients and absent in controls. We also showed that sulphoxide methionine 93 is included in the peptide recognized by specific IgG2 that are increased in sera of LN patients and represent autoantibodies highly characteristic of this pathology. Overall, data here presented support the idea that modification of NET α-enolase is the starting mechanism leading to the formation of circulating auto-antibodies specific for LN that deposit within glomeruli and determine lupus nephritis, the most serious complication of lupus erythematosus.

Materials and Methods

Study design. ‘Ex vivo’ cell NETs production studies were performed in 9 LN, 7 SLE and 11 normal subjects. All the patients of the present study were out of therapy at the time of blood collection.

Diagnosis of SLE was done according to the American College Rheumatology criteria (SLICC) as outlined with details in the Validation study (see below). Lupus nephritis was defined according to WHO classification (see below). We obtained written approval of the protocol by the local Independent Ethics Committee (Comitato Etico Regione Liguria) on October 24, 2014. All methods were performed in accordance with the relevant guidelines and regulations.

Methods. Isolation of neutrophils. Polymorphonuclear cells (PMN) were isolated from -EDTA peripheral blood under sterile conditions using a dextran sedimentation technique followed by Ficoll gradient centrifugation as previously described33. Briefly, 1 volume EDTA blood was mixed with 0.8 volume of Dextran Plander 70000 solution (Fresenius Kabi Italia s.r.l, IsoladellaScala, VR, Italy) and RBC were allowed to sediment for 45 minutes at room temperature. The granulocyte-rich supernatant was then collected, layered onto Ficoll-Histopaque 1077 and centrifuged at 800 g for 30 minutes. Residual RBC were removed from granulocyte-containing pellet by hypotonic lysis. PMNs were finally re-suspended in RPMI medium supplemented with 1 mM Calcium Chloride and 1% Human Serum Albumin solution (Albital 200 g/l, Kedron, CastelvecchioPascoli, Borgo, LU, Italy) for further investigations.

‘In vitro’ NETs induction. PMN suspensions were allowed to adhere for 1 hour at 37 °C onto 24-well plastic dishes at the density of 10 × 10⁶ cells/ml in RPMI medium, supplemented with Calcium Chloride and Human Serum Albumin as above described. NET formation was thereafter induced by treating PMNs for 3 hours with 20 nM Phorbol myristate acetate (PMA). Cells were then washed with PBS and incubated with 15U/ml S7 Nuclease for 15 minutes at 37 °C. Reaction was stopped with 2 mM EDTA. Cellular debris were then pelleted by centrifugation at 300 g and supernatants saved for Elastase assay (see below).

Determination of NETs production. To quantify NETs production, it was used the Cayman’s NETosis assay kit (cat. No 601010, Cayman Chemical, MI, USA) according to the manufacturer’s instructions. Briefly, 100 ul of standard or culture supernatants per well, pre-heated to 37 °C, were incubated with 100 ul per well of the 1:30 diluted NET assay neutrophil elastase substrate in PBS. The 96-well plate was covered and incubated 2 hours at 37°C. Finally, the cover was removed and the plate was read at 405 nm.

Sample preparation for Mass Spectrometry, NanoLC and Mass Spectrometer setup. Pellets obtained by acetone precipitation were re-suspended in 25 μl of lysis buffer (6 M GdmCl, 10 mM TCEP, 40 mM CAA, 100 mM Tris pH 8.5). The samples were reduced and alkylated and lastly digested in a single step and then loaded into StageTip. Peptides were analyzed by nano-UHPLC-MS/MS using an Ultimate 3000 RSLC with EASY spray column (75 μm × 500 mm, 2 μm particle size, Thermo Scientific) and with a 180 minute non-linear gradient of 5–45% solution B (80% CAN and 20% H₂O, 5% DMSO, 0.1% FA) at a flow rate of 250 nl/min. Eluting peptides were analyzed using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific Instruments, Bremen, Germany). Orbitrap detection was used for both MS1 and MS2 measurements at resolving powers of 120 K and 30 K (at m/z 200), respectively. Data dependent MS/MS analysis was performed in top speed mode with a 2 sec. cycle-time, during which precursors detected within the range of m/z 375–1500 were selected for activation in order of abundance. Quadrupole isolation with a 1.4 m/z isolation window was used, and dynamic exclusion was enabled for 45 s. Automatic gain control targets were 2.5 × 10⁵ for MS1 and 5 × 10⁴ for MS2, with 50 and 60 ms maximum injection times, respectively. The signal intensity threshold for MS2 was 1 × 10⁴. HCD was performed using 30% normalized collision energy. One microscan was used for both MS1 and MS2 events. For all the MS1 scans the option ETD internal Calibration was selected.

MaxQuant software, version 1.5.5.30, was used to process the raw data, setting a false discovery rate (FDR) of 0.01 for the identification of proteins, peptides and PSM (peptide-spectrum match), moreover a minimum length
of 6 amino acids for peptide identification was required. Andromeda engine, incorporated into MaxQuant software, was used to search MS/MS spectra against Uniprot human database (release UP00005640_9606 February 2016). Two different elaborations were made to identify the PTMs in order to limit the false positives. In the first processing, variable modifications are Acetyl (Protein N-Term) and Phospho (STY). The second processing step includes Oxidation (M), Deamidation (NQ), Trioxidation (C) and Carbamido-methyl (C). Finally, in order to overcome the common limitations of search engine based PTM analysis, we used the unbiased PTM Dependent Peptide search option, taking advantage of high mass accuracy data collected in high resolution mode with an internal calibration (MS1 error < 1 ppm). The intensity values were extracted and statistically evaluated using the different Site Table, DP table or Protein Groups table. Algorithm MaxLFQ was chosen for the protein quantification with the activated option ‘match between runs’ to reduce the number of the missing proteins.

MS proteomics RAW data, Peptides and Proteins Table are available at the ProteomeXchange Consortium database via the Proteomics Identifications (PRIDE) partner repository, under data set IDs PXD007754 Reviewer account details:

Username: reviewer49884@ebi.ac.uk
Password: RmJZrfxD

**Purification of serum enolase and CNBr fragmentation.** The experiments for defining the epitopes of α-enolase recognized by anti-α-enolase IgG2 were done with the protein purified from sera of 5 LN patients. Fifty ml of pooled serum were overall processed. Anti-α-enolase monoclonal antibody (Abcam) were immobilized on 50 ml ProteinA Sepharose (Bio-Rad) and washed with PBS. Sera were loaded and re-circulated overnight at 4°C and after several washes with the PBS buffer, bound substances were eluted in Glycine–HCL buffer pH2.5. The elute was utilized for CNBr digestion according to the conditions below: 1-purified enolase in 0.4 M ammonium bicarbonate was incubated with 1% v/v 2-mercaptoethanol at room temperature for 1 h in a dark box; 2–the sample was dried in speed vacuum and re-suspended in 5 µL of deionized water, 15 µL of trifluoroacetic acid (TFA) and 5 µL of 5 M CNBr in acetonitrile (ACN). The tube was wrapped in aluminum foil and left overnight at 4°C; 3–the reaction was stopped by drying down under vacuum; 4–finally, the sample was re-suspended in Tris-HCl 62.5 mM pH 6.8, 2% w/v SDS and 10% glycerol, loaded in polyacrylamide gel and transferred on PVDF membrane. After saturation the membrane was incubated with a pooled sera of LN patients, diluted 1:50 in PBS-T and 1%w/v BSA, rinsed with PBS-T and then incubated with anti-human IgG HRP-conjugated diluted 1:3000 in PBS-T and 1%w/v BSA. Chemiluminescence was use for detection.

**Stimulated emission depletion microscopy (STED).** Other images (1024 × 1024 pixels, 8 bit) were acquired with a super-resolution laser scanning microscope based on stimulated emission depletion method STED microscope Leica SP5 TCS STED-CW gated (Leica-microsystems, Mannheim, Germany) equipped with an oil immersion HCX PL APO 100 × 1.4 NA objective set the pinhole to 1 Airy unit; time gating for the red channel was starting at 2 ns and ending at 10 ns. A series of confocal optical sections were taken at a z-step of 25 nm. The image acquisition through red (spectral window 514–553 nm) and green (spectral window 467–495 nm) channels were performed according to a time-sequential protocol to reject possible cross-talk artifacts. In particular, the excitation beams were at 514 nm (a pulsed, 80 MHz, super-continuum laser) and 458 nm (a continuous wave, CW laser) respectively. While the depletion beam was at 592 nm (CW laser) for both channels, the power was set at 120 mW and 350 mW respectively. The Leica Confocal Software program was used for image acquisition, storage and analysis. Illustrations were prepared using the freely available software ImageJ (rsb.info.nih.gov/jj), originally developed by NIH.

**Validation study. Sample size SLE/LN.** Overall, 216 incident SLE patients were included in the study; 103 patients of the SLE patients presented a glomerulonephritis at the time of recruitment. All the patients above were recruited in the frame of the Zeus study. The data base and samples collection is located at the Giannina Gaslini Institute of Genoa (I). None of the participants had a diagnosis before. Twenty-five healthy donors were recruited from the hospital staff (age 23–56 yrs). Patients were recruited prospectively and were out of therapy at the time of blood collection.

All gave their informed consent to the study protocol.

Inclusion Criteria were age between 4 and 65 years, any sex, the availability of informed consent. Diagnosis of systemic lupus erythematosus was done according to the American College Rheumatology systemic lupus classification criteria as revised by the Systemic Lupus International Collaborating Clinics (SLICC). Newly diagnosed lupus nephritis (stage I–VI according to WHO classification) were recruited among the large cohort of patients with lupus showing positivity of urinary elements such as hematuria, proteinuria and/or worsening of renal function in some cases as evaluated by the CPK-EPI formula. The diagnosis of LN was based on typical renal lesions with lupus showing positivity of urinary elements such as hematuria, proteinuria and/or worsening of renal function in some cases as evaluated by the CPK-EPI formula. The diagnosis of LN was based on typical renal lesions with lupus showing positivity of urinary elements such as hematuria, proteinuria and/or worsening of renal function in some cases as evaluated by the CPK-EPI formula. The diagnosis of LN was based on typical renal lesions with lupus showing positivity of urinary elements such as hematuria, proteinuria and/or worsening of renal function in some cases as evaluated by the CPK-EPI formula.

Membranous nephropathy. Twenty patients with Membranous Nephropathy were recruited within the Italian Consortium on Membranous Nephropathy that is chaired by Dr Ghiggeri at G. Gaslini Institute of Genoa. Criteria for enrollment were (1) a biopsy-based diagnosis of iMN, (2) a normal complement profile, (3) negative...
tests for ANA, nDNA, and ANCA and cryoglobulins and the absence of viral markers (hepatitis B surface antigen and HIV); (4) the absence, at the time of inclusion, of clinical and biochemical signs of cancer.

**Ethical committee.** Before initiation of the study, we obtained written approval of the protocol, Informed Consent Form and any information presented to potential subjects from the local Independent Ethics Committee (Comitato Etico Regione Liguria) on October 24, 2014. We also obtained approval from the Italian Drug Agency (Agenzia Italiana del Farmaco, AIFA). The study was registered at [https://clinicaltrials.gov](https://clinicaltrials.gov) (study number: NCT02403115).

**Isotype specificity.** Isotype specificity of anti-α-enolase antibodies in 20 sera of patients with LN and in 20 with MN was determined with Western blot as already described 46.

**Determination of anti-α-enolase IgG2.** A home made ELISA was utilized for determining serum levels of anti-α-enolase IgG2 as already described 42. Briefly, 100 ng of recombinant enolase (Abnova Corporation, Taipei, Taiwan) were put in MaxiPrep plate 96 wells, in PBS buffer and incubate at room temperature for 5 hours and then at 4°C overnight. Aliquots (200 μl) of blocking solution (PBS, 5% w/v BSA and 0.05% v/v Tween20) were put in each well. Serum samples (100 μl) diluted 1:50 in PBST (PBS – Tween20 0.05% v/v – BSA 1% w/v) was added and incubated for 4 hours at room temperature and then at 4°C overnight. After three washes in PBST, HRP-conjugated rabbit anti human IgG2 (Clone: HP6014- InVitrogen Corporation, Camarillo, CA) diluted 1:3000 in PBST and 1% w/v BSA were incubated at room temperature for 4 hours and after 3 washes in PBST, 100 μl of substrate TMB/H2O2 (10:1) was added and incubated up to 30 minutes. The reaction was stopped by adding 100 μl of 0.45 M of H2SO4 at any wells before reading absorbance at 450 nm. A standard curve was prepared utilizing HRP-IgG2 at different dilutions.

**Normal limits.** Normal limits for all the tests 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 53,54.

**Standard confocal and Stimulated Emission Depletion Microscopy (STED).** Imaging techniques based on standard microscopy and STED are described in Supplementary Information.

**Statistics and bioinformatic analysis.** After normalization, data obtained from mass spectrometry, were analyzed using unsupervised hierarchical clustering analyses, i.e. Multidimensional Scaling (MDS) to identify outlier and sample dissimilarity. Differences in protein and peptide expression between Control, SLE and LN NET cell supernatants were analysed using a non-parametric U-Mann Whitney test. P-values were adjusted using the Benjamini-Hochberg method. Results were considered significant with two fold change and adjusted for P-value ≤ 0.05 (to identify the significant fold change a power analysis was performed considering the number of all biological replicates and their variability). Volcano plot was used to visualize the statistical differences, in which case the cutoff lines were established using the function y = c/(x – x0). Non-linear support vector machine (SVM) and partial least squares discriminant analysis (PLS-DA) were utilized to identify maximal discrimination among groups. In SVM a Cross-validation approach a 4-fold increment limit was applied to estimate the accuracy of classification. The results of these analysis were summarized by mean of heat map graph.

Differentially expressed proteins in NETs samples highlighted by the combine use of univariate/multivariate statistical analysis and machine learning were analyzed according to GO terms for biological process, cellular component and molecular function in the database (http://www.geneontology.org/). To assess functional associations between proteins, differentially expressed between CTR/SLE/LN, R software was applied and visualized with Cytoscape. Pathways enrichment of proteins clusters were performed according to UniProt, Reactome, KEGG and ClueGO database. The results of GO analysis were shown three and bi-dimensional scatter plots.

Test performance in terms of sensitivity (ability of the test to identify true positive subjects) and specificity (ability of the test to identify true negative subjects) was evaluated for each parameter by Receiving Operating Characteristic (ROC). The proportion of patients correctly diagnosed is proportional to the area under the curve (AUC) where accuracy is absent for AUC ≤ 0.5, poor for 0.5 < AUC ≤ 0.7, moderate for 0.7 < AUC ≤ 0.9 and high for 0.9 < AUC < 1. A test is perfect for AUC = 1. The ROC curve also allows to identify the best cut off value that maximizes the difference between true positive subjects and false positives ones. To maximize sensitivity and specificity, the Youden’s index (Sensitivity + (1 − Specificity)) was applied. All statistical analysis were performed using software package R last version available at the time of experiments.

**References**

1. Brinkmann, V. et al. Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532–1535, [https://doi.org/10.1126/science.1092385](https://doi.org/10.1126/science.1092385) (2004).
2. Nathan, C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**, 173–182, [https://doi.org/10.1038/nri1785](https://doi.org/10.1038/nri1785) (2006).
3. Papayannopoulos, V. & Zychlinsky, A. NETs: a new strategy for using old weapons. *Trends Immunol* **30**, 513–521, [https://doi.org/10.1016/j.it.2009.07.011](https://doi.org/10.1016/j.it.2009.07.011) (2009).
4. Fuchs, T. A. et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**, 231–241, [https://doi.org/10.1083/jcb.200606027](https://doi.org/10.1083/jcb.200606027) (2007).
5. Urban, C. & Zychlinsky, A. Netting bacteria in sepsis. *Nat Med* **13**, 403–404, [https://doi.org/10.1038/nm0407-403](https://doi.org/10.1038/nm0407-403) (2007).
6. Steinberg, B. E. & Grinstein, S. Unconventional roles of the NADPH oxidase: signaling, ion homeostasis, and cell death. *Sci STKE* **2007**, pe11, [https://doi.org/10.1126/stke.3792007pe11](https://doi.org/10.1126/stke.3792007pe11) (2007).
41. Redlitz, A., Fowler, B. J., Plow, E. F. & Miles, L. A. The role of an enolase-related molecule in plasminogen binding to cells.

40. Pancholi, V. Multifunctional alpha-enolase: its role in diseases.

39. Meng, Z. et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog 5, e1000639, https://doi.org/10.1371/journal.ppat.1000639 (2009).

38. Bruschi, M. et al. Neutrophil Extracellular Traps (NETs) profiles in patients with incident SLE and lupus nephritis. J Rheumatol, in press (2018).

37. Iaccarino, L. The role of neutrophils and NETosis in autoimmune and renal diseases. Nature 497, 407–415 (2013).

36. de Graauw, M. et al. Glomerular autoimmune multicomponents of human lupus nephritis in vivo: alpha-enolase and annexin A1. J Am Soc Nephrol 25, 2483–2498, https://doi.org/10.1681/ASN.20130900987 (2014).

35. Yang, Y. et al. Human PAD4 regulates histone arginine methylation levels via demethylamination. Science 320, 279–283, https://doi.org/10.1126/science.1101400 (2004).

34. Yang, Y. & Hickey, M. J. Annexin A1 and the regulation of innate and adaptive immunity. J Exp Med 199, 353–358, https://doi.org/10.1084/jem.2001099 (2000).

33. Perretti, M. & Gavins, F. N. Annexin 1: an endogenous anti-inflammatory protein. J Immunol Res 2012, 902–920 (2012).

32. Sugimoto, M. A., Vago, J. P., Teixeira, M. M. & Sousa, L. P. Annexin A1 and the Resolution of Inflammation: Modulation of Neutrophil Recruitment, Apoptosis, and Clearance. J Immunol Res 2016, 6392358, https://doi.org/10.1155/2016/6392358 (2016).

31. Gavins, F. N. & Hickey, M. J. Annexin A1 regulates intestinal mucosal injury, inflammation, and repair. J Immunol 181, 5035–5044 (2008).

30. Gavins, F. N. & Hickey, M. J. Annexin A1 and the regulation of innate and adaptative immunity. Front Immunol 3, 354, https://doi.org/10.3389/fimmu.2012.00354 (2012).

29. Perretti, M. & Gavins, F. N. Annexin 1: an endogenous anti-inflammatory protein. News Physiol Sci 18, 60–64 (2003).

28. Bruschi, M. et al. Neutrophil Extracellular Traps (NETs) profiles in patients with incident SLE and lupus nephritis. J Rheumatol, in press (2018).

27. Bruschi, M. et al. Toll-like receptor 7 and TLR9 dictate autobody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunology 25, 417–428, https://doi.org/10.1016/j.imunis.2006.07.013 (2006).

26. Gavins, F. N. & Hickey, M. J. Annexin A1 and the regulation of innate and adaptive immunity. J Exp Med 199, 353–358, https://doi.org/10.1084/jem.2001099 (2000).

25. Kessenbrock, K. Neutrophil Recruitment, Apoptosis, and Clearance. J Immunol Res 2012, 902–920 (2012).

24. Gupta, S. & Kaplan, M. J. The role of neutrophils and NETosis in autoimmune and renal diseases. Nature Rev Nephrol 12, 402–413, https://doi.org/10.1038/nrneph.2016.71 (2016).

23. Giannakopoulos, B. & Krilis, S. A. The pathogenesis of the antiphospholipid syndrome. Arthritis Rheum 50, 883–896, https://doi.org/10.1002/art.20201 (2004).

22. Jennette, J. C. et al. Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. Arthritis Rheum 50, 976–984, https://doi.org/10.1002/art.20201 (2004).

21. Kessenbrock, K. et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med 3, 73ra20, https://doi.org/10.1126/scitranslmed.3001297 (2011).

20. Christensen, S. R. & Kaplan, M. J. The role of neutrophils and NETosis in autoimmune and renal diseases. Nature Rev Nephrol 12, 402–413, https://doi.org/10.1038/nrneph.2016.71 (2016).

19. Darrah, E. & Andrade, F. NETs: the missing link between cell death and systemic autoimmune diseases? Front Immunol 3, 428, https://doi.org/10.3389/fimmu.2012.00428 (2012).

18. Rohrbach, A. S., Slade, D. J., Thompson, P. R. & Mowen, K. A. Activation of PAD4 in NET formation. Front Immunol 3, 360, https://doi.org/10.3389/fimmu.2012.00360 (2012).

17. Metzler, K. D., Goosmann, C., Lubojemska, A., Zychlinsky, A. & Papayannopoulos, V. A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. Cell Rep 8, 883–896, https://doi.org/10.1016/j.celrep.2014.06.044 (2014).

16. Urban, C. F. et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood 117, 953–959, https://doi.org/10.1182/blood.2010-09-290171 (2011).

15. Li, P. et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog 5, e1000639, https://doi.org/10.1371/journal.ppat.1000639 (2009).

14. Garcia-Romo, G. S. et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med 3, 73ra20, https://doi.org/10.1126/scitranslmed.3001297 (2011).

13. Wang, Y. et al. Human PAD4 regulates histone arginine methylation levels via demethylamination. Science 306, 279–283, https://doi.org/10.1126/science.1101400 (2004).

12. Metzler, K. D., Goosmann, C., Lubojemska, A., Zychlinsky, A. & Papayannopoulos, V. A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. Cell Rep 8, 883–896, https://doi.org/10.1016/j.celrep.2014.06.044 (2014).

11. Metzler, K. D., Goosmann, C., Lubojemska, A., Zychlinsky, A. & Papayannopoulos, V. A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. Cell Rep 8, 883–896, https://doi.org/10.1016/j.celrep.2014.06.044 (2014).

10. Bouin, A. P., Grandvaux, N., Vignais, P. V. & Fuchs, A. P40(pbox) is phosphorylated on threonine 134 and serine 315 during activation of the phagocyte NADPH oxidase. Implication of a protein kinase c-type kinase in the phosphorylation process. J Biol Chem 273, 30097–30103 (1998).

9. Keshari, R. S., Verma, A., Barthwal, M. K. & Dikshit, M. Reactive oxygen species-induced activation of ERK and p38 MAPK mediates pMAF-induced NETs release from human neutrophils. J Cell Biochem 114, 532–540, https://doi.org/10.1002/jcb.24391 (2013).

8. Douda, D. N., Khan, M. A., Grasemann, H. & Palaniyar, N. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. Proc Natl Acad Sci USA 112, 2817–2822, https://doi.org/10.1073/pnas.1414055112 (2015).

7. Hakkim, A. et al. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat Chem Biol 7, 75–77, https://doi.org/10.1038/nchembio.496 (2011).
46. Denny, M. F. et al. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *J Immunol* **184**, 3284–3297, https://doi.org/10.4049/jimmunol.0902199 (2010).

47. Hakkim, A. et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci USA* **107**, 9813–9818, https://doi.org/10.1073/pnas.0909927107 (2010).

48. Tsokos, G. C. Systemic lupus erythematosus. *N Engl J Med* **365**, 2110–2121, https://doi.org/10.1056/NEJMra1100359 (2011).

49. Beck, L. H. Jr. et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. *N Engl J Med* **361**, 11–21, https://doi.org/10.1056/NEJMoa0810457 (2009).

50. Murtas, C. et al. Coexistence of different circulating anti-podocyte antibodies in membranous nephropathy. *Clin J Am Soc Nephrol* **7**, 1394–1400, https://doi.org/10.2215/CJN.02170312 (2012).

51. Weiss, J., Kao, L., Victor, M. & Elsbach, P. Oxygen-independent intracellular and oxygen-dependent extracellular killing of *Escherichia coli* S15 by human polymorphonuclear leukocytes. *J Clin Invest* **76**, 206–212, https://doi.org/10.1172/JCI111947 (1985).

52. Petri, M. et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* **64**, 2677–2686, https://doi.org/10.1002/art.34473 (2012).

53. Zweig, M. H. ROC plots display test accuracy, but are still limited by the study design. *Clin Chem* **39**, 561–577 (1993).

54. Zweig, M. H. & Campbell, G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* **39**, 561–577 (1993).

Acknowledgements
The study was supported by a grant from Fondazione Lupus Italia 2014. We acknowledge the support by Fondazione Malattie Renali nel Bambino (FMRB) and grant n ROL 9849 from Compagnia di San Paolo. Thank to all the Zeus study participants (doctors, nurses, laboratory personnel) and to all patients who accepted to be enrolled.

Author Contributions
G.M.G., G.C., M.P. and A.V. designed the experiments; M.B. and A.P. made the proteomics approach; M.P., L.S., F.P., P.M., R.B., C.L. and M.B. made the experiments; M.B. made statistics; G.M.G., M.P. and A.V. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-44379-w.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.