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# LOW SERUM LEVEL OF VITAMIN D IS ASSOCIATED WITH INCREASED NUMBER OF LOW-DENSITY GRANULOCYTES (LDGs) AND NEUTROPHIL EXTRACELLULAR TRAPS (NETs) FORMATION IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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## Abstract

The purpose of the study was to analyze the relationship of vitamin D level with the percentage of LDGs and NETs formation in SLE patients. We studied 28 female SLE patients and 15 matched healthy controls recruited from outpatient of Internal Medicine Department, Saiful Anwar Hospital, Malang. Vitamin D ($25OH_2)D_3$) serum level was assessed using ELISA, LDGs number (flowcytometri) and NETs formation (ELISA). Serum level of vitamin D significantly lower in SLE patients than healthy controls (23.17 ± 7.42 vs. 32.11 ± 14.44 ng/ml , p : 0.019 ). Number (%) of LDGs and NETs formation were significantly higher in SLE patients compared to healthy controls. SLE patients with vitamin D level < 20 ng/ml tended to have higher LDGs number and NETs formation than the SLE patients with vitamin D level > 20ng/ml and healthy controls. Low level of vitamin D is associated with increased LDGs number and NETs formation (r : -0.452 to -0.662) .

***Keywords***: SLE, LDGs, NETs, Vitamin D.

## 1. Introduction

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease characterized by multi organ inflammation. Although the 10 years life expectancy of patients with SLE increased to 90% in developing countries, our study showed that patients with SLE in Indonesia still have low life expectancy (Handono 2000). The pathogenesis of the disease is still unclear, however, there is evidence of deviation function of T cells, B cells, dendritic cells and regulatory T cells that ultimately lead to systemic inflammation and tissue damages (Jianxin et al., 2009 ). Neutrophils reported play a pivotal, but until recently undefined role in SLE (Brinkmann and Zychlinsky, 2012).

An abnormal neutrophil subset has been identified in PBMCs fractions from adult SLE patients. These low density granulocytes / LDGs play an important role in the pathogenesis of SLE by damaging endothelial cells and synthesizing more proinflammatory cytokines and type 1 IFNs (Villanueva et al., 2011). LDGs have enhanced capacity to form Neutrophil Extracelluler Traps (NETs) and upregulate expression of various neutrophil proteins and enzymes implicated in NET formation and autoimmunity induction. These NETs also expose dsDNA, an autoantigen considered key in lupus pathogenesis. Furthermore, lupus neutrophils and in particular LDGs elicit enhance endotelial cell cytotoxicity through NET formation. This phenomenon also appears to play role in the induction of IFN-α synthesis by pDCs. It has been reported that enhanced NETosis occur in vivo in SLE that related to skin and kidney pathology. Neutrophils from peripheral blood and skin of SLE patients frequently externalize IL-17 as part of the NETosis process, which contribute to tissue damage and immune dysregulation (Villaneuva et al., 2011) . Furthermore, the lupus patients with higher circulating LDGs numbers have increased skin involvement and or vasculitis. Skin and kidney tissue of patients with lupus infiltrated by neutrophils, which expose LL - 37 and ds-DNA. NETosis is associated with an increase level of anti - dsDNA antibodies in the serum of patients with SLE. These results clearly indicate the pathogenic role of aberrant neutrophil function and dysregulation of NET formation and the subsequent response in the pathogenesis of lupus (Lande et al., 2011).

It has been widely reported the relationship between increased the incidence of autoimmune disease with vitamin D deficiency (Adams and Hewison, 2008). Various studies have suggested the presence of a high percentage of SLE patients with vitamin D deficiency in a country with four seasons (Kuhn and Krammer, 2009). However, our previous study showed also a similar results, that 71% of Indonesian SLE patients have vitamin D defficiency. The low levels of vitamin D in these patients was associated with high disease activity score, decreased function of regulatory T cells, increased function of dendritic and B cells , with increased secretion of autoantibodies (Handono et al., 2012). Our study showed that vitamin D defficiency is related to increased number of LDGs and formation of the NETs in SLE patients.

## 2. Materials and Methods

### *2.1 Subjects*

The study conducted in 28 female SLE patients ( fullfil 2012 revised American College of Rheumatology criteria for SLE ) with active disease ( SLEDAI score ≥ 5 ) from outpatient of Internal Medicine, Dr. Saiful Anwar Hospital Malang, during July to October 2013. 15 female healthy controls were recruited by advertisement. The Faculty of

Medicine and Dr. Saiful Anwar Hospital review board aprroved this study and informed consent was obtained from all subyects.

### *2.2 Vitamin D [25 (OH)2D3] measurement*

Serum was obtained from 3 cc whole blood of the patients and serum level of vitamin D [$25(OH)_2D_3$] was measured by ELISA (Alegria ; Orgentec Diagnostica GmbH-Germany). Serum level of vitamin D > 30 ng/ml was considered normal and <30 ng / ml was considered hypovitamin D .

### *2.3 Isolation of PBMC and granulocytes*

10 cc of EDTA blood samples was added to 10 cc polymorphrep in a centrifuge tube. Centrifuged of 1400 rpm for 33 min at room temperature. PBMC (upper ring formed from the centrifugation) taken with caution, transferred into a new tube. Granulocytes (bottom ring) were taken and transferred to a new tube. Cells were washed with 10 cc sterile PBS and centrifuged at 1200 rpm for 10 min. Washing process was repeated twice. Supernatant was discarded and the pallet was taken as an isolated PBMCs and granulocytes.

### *2.4 Calculation of percentage LDGs*

Low Density Granulocyte (LDGs) are granulocytes cells that express low CD14+ and high CD10+ in PBMC fractions. $1x10^4$ PBMCs were incubated with 10 ul of PE/Cy5 labeled anti-CD10 and 2.5 ul of FITC-labeled anti-CD14 antibodies ( R&D Systems). The percentage of LDGs was calculated using flowcytometry based on cells expressing low CD14 + and CD 10 +.

### *2.5 Induction NETs Formation*

Formation of neutrophil extracellular traps (NETs) is a releasing cytoplasmic protein containing granular chromatin (DNA and histones) and protein elastase, IL-17, Ro / SSA, La / SSB. $5 \times 10^6$ cells / ml of isolated granulocytes were cultured in 500 µl complete RPMI medium (RPMI 1640 and fetal bovine serum) supplemented with 2% AS, incubated at $37\ ^0C$ for 18-24 hours. 20 nM PMA was added or left unstimulated, incubated for 2 hours at 37 °C. The supernatant was separated for quantification of NETs.

### *2.6 Detection of NETs activity*

Neutrophil ($1\text{-}2x10^5$cells/ml) were isolated as above were seeded in poly-L-lysine coverslips eight – well chamber slides, and allowed to settle and incubated at 37 ° C, 5% CO2 for 15 min. Cells were washed with ice-cold PBS and either fixed right away with 4% paraformaldehyde and than blocked overnight at 4 ° C with 10% FBS / 1% BSA/0.05% Tween 20 and 2 mM EDTA / PBS or incubated for 2 h in RPMI 1640 / glutamine / 2% BSA in the presence or absence of 20 nM PMA to induce NET formation, followed by fixation and overnight blocking at 4 °C. NETs were detected by washing and fixed cells wth ice cold 10% FBS/PBS and incubating with anti-human etalase (1 : 100) for 45 min at 4 C, followed by incubation with secondary fluorochrome-conjugated Abs for 45 min at 4 C. Coverslips were mounted in Prolong Gold Antifade reagent (Invitrogen) and analyzed using an Olympus, Bio Imaging Navigator, FSX 100.

### *2.7 Quantification of NETosis*

Neutrophil Extracellular Traps ( NETs ) was measured through the release of cytoplasmic proteins (MPO), which binds to the DNA. NETs formation was assayed by comparing the absorban of MPO - DNA released from stimulated and unstimulated neutrophils (controls) using ELISA method. Supernatant from cultured neutrophils were used as sample. 50 µl of anti - MPO mAb ( Upstate , catalog no. 07-496 ) coated in 96 - well plates (1:500 dilution) overnight at 4 °C. After washed, 20 µl sample was added to the wells with 80 µl incubation buffer containing peroxidase - labeled anti - DNA mAb (Cell Death ELISAPLUS , Roche ; dilution 1:25), incubated for 2 hours by shaking 300 rpm at room temperature. 100 µl peroxidase substrate ( ABTS ) was added and absorbance at 405 nm was measured after 20 min incubation at room temperature . NETs values was calculated as absorbance values compared to controls ( Caudrillier et al . , 2012).

### *2.8 Statistical analysis*

The collected data will be analyzed with SPSS 17 version. The data were expressed as means. Differences of the percentage of LDG and the NETs formation between SLE patients and healthy controls, between SLE patients with vitamin D levels <20 ng / ml and > 20ng/ml tested with independent t test and ANOVA. Correlation of vitamin D levels and percentage of LDG and NETs formation tested with Pearson correlations. $P < 0.05$ was considered statistically significant.

## 3. Result

The clinical data and demographic characteristic of these subyects are sumarized in table 1. The mean age of SLE patients was 31.39 ± 11.44 years with duration of illness was 24.4 ± 8.6 months and SLEDAI score was 12.4 ± 4.4. The mean age healthy controls was 33.07 ± 11.71 years. There were no differences in age, sex between SLE patients and healthy controls. Clinical manifestations that most SLE patients respectively were arthritis (79%), nephritis (58%) and malar rash (54%). The mean levels of vitamin D in SLE patients significantly lower than healthy controls (23.17 ± 7.42 vs. 32.11 ± 14.44 ng / ml, $p < 0.05$). The mean number (%) of LDGs and NETs formation in the SLE patients was significantly higher than in healthy controls (Table 1).

Table 1: Characteristics of the sample.

| Characteristics | SLE Patients, N = 28 | Healthy controls, N = 15 | P |
|---|---|---|---|
| Age (mean ± SD, years) | 31.39 ± 11.44 | 33.07±11.71 | 0.62 |
| Duration of illness ( mean ± SD, months) | 24.40 ± 8.60 | | |
| Clinical manifestations | | | |
| Arthritis (%) | 79% | | |
| Nephritis (%) | 58% | | |
| Malar Rash (%) | 54% | | |
| Discoid Rash (%) | 43% | | |
| SLEDAI score (mean± SD, %) | 12.4 ± 4.4 | | |
| Vitamin D level (mean± SD, ng / ml) | 23.17 ± 7.42 | 32.11 ±14.44 | 0.019* |
| Number of LDGs ( mean± SD, %) | 44.15±11.23 | 26.22±14.16 | 0.043* |
| Absorbens of NETs (mean±SD) | 0.69 ± 0.16 | 0.51±0.12 | 0.037* |

* p <0.05: significantly

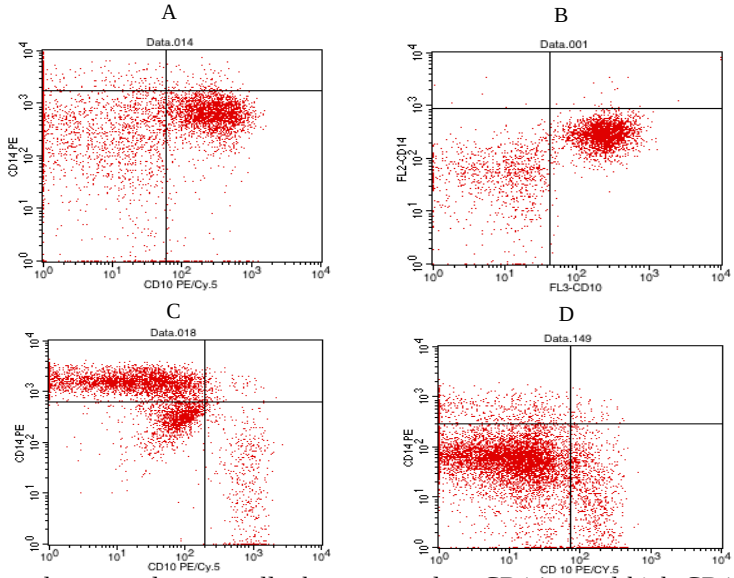


Figure 1: LDGs are showed as granulocytes cells that express low CD14 + and high CD10 + in PBMC fractions. The percentage of LDGs in SLE patients (A and B) compare in healthy controls (C and D)

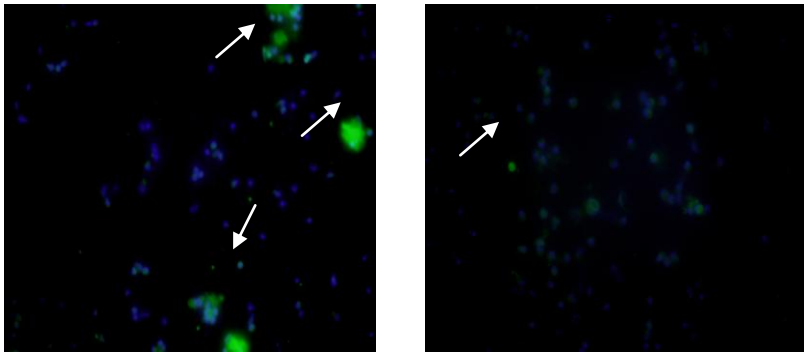

Figure 2 : NETs was detected by anti-human etalase - fluorochrome-conjugated Abs, analyzed using an Olympus, Bio Imaging Navigator, FSX 100. A). NETs formation in SLE patient. B) NETs formation in healthy control

Our result showed that in 28 SLE patients studied, 9 (32.14%) patients had vitamin D levels <20 ng / ml and 19 (67.86%) patients had vitamin D levels > 20 ng / ml. While mean levels of vitamin D in healthy controls was was 32.11 ± 14.44 ng/ml (table2).

Table 2: Serum vitamin D level, LDG number and formation of NET in SLE patients compared with healthy controls

| | SLE patients | | Healthy controls |
|---|---|---|---|
| | Vit D <20ng/ml | Vit D > 20ng/ml | |
| Age (mean ± SD, years) | 33.11±10.71 | 30.58±11.96 | 33.07±11.71 |
| Vitamin D level (mean± SD, ng/ml) | 16.92 ± 2.07 | 26.13±7.20 | 32.11 ±14.44 |
| LDG Number (mean± SD, %) | 48,24±14,10 | 39,24±22,12 | 26,22±14,16 |
| Formation of NETs (abs), (mean±SD) | 0,77±0,09 | 0,62±0,17 | 0,51±0,12 |

The differences of LDG number and NETs formation between the three groups above suggested that the percentage of LDG and NETs tended be higher in SLE patients with vitamin D levels <20ng/ml compared to the other two groups (Table 3).

Table 3: Analysis of variable differences between groups.

| | Comparison between groups | P |
|---|---|---|
| **LDGs Number (%)** | | |
| Group 1 | Group 2 | 0.806 |
| | Group 3 | 0.045* |
| Group 2 | Group 3 | 0.631 |
| **NETs Formation (abs)** | | |
| Group 1 | Group 2 | 0.09 |
| | Group 3 | 0.056* |
| Group 2 | Group 3 | 0.034* |

Group 1: SLE patients with vitamin D <20ng/ml; Group 2: SLE patients with vitamin D> 20ng/ml; Group 3: healthy control

The result showed that there was no difference in age between the SLE patients with vitamin D levels <20ng/ml and SLE patients with vitamin D levels> 20ng/ml. The number (%) of LDGs and formation of NETs tended to be higher in the group of SLE patients with vitamin D levels <20ng/ml compared to the other two groups, but SLE patients with vitamin D levels <20ng/ml had significantly higher number of LDG and NETs formation compared with healthy controls (table 4). Furthermore, it was found that there were negative correlation of vitamin D level with the number of LDG and formation of NETs (r: -0.452 and -0.662), (table 4).

Table 4: Correlation between vitamin D levels with LDG number and NETs formation

| | Variable | R | P |
|---|---|---|---|
| Levels of vitamin D | LDG Number | -0.452 | 0.051 |
| Levels of vitamin D | NETs Formation | -0.662 | 0.044 |

## 4. Discussion

Neutrophils are very important in shaping both innate and adaptive immune responses and have long been suspect to play role in SLE (Garca-Romo et al., 2011). In PBMCs of SLE patients, found that abnormal neutrophil subset called low-density granulocytes (LDGs). Neutrophils isolated from SLE patients have a greater ability to produce NETs particullary in response to antibody complexes. SLE patients, often produce antibodies against DNA, histones, and neutrophil proteins that are components of the NETs (Craft, 2011; Garcia Romo et al., 2011). It has been reported that vitamin D contribute to the immune respons regulation. Deficient of vitamin D has been associated with increased autoimmune disease such as SLE (Kamen et al., 2006). The relationship of neutrophils and vitamin D in the pathogenesis SLE is still needed to further clarify.

This study tried to analyzed the role of vitamin D in neutrophils regulation exspecially in the formation of NETs. Our study showed vitamin D levels in SLE patients significantly lower than healthy controls. This is consistent with our previous study (Handono et al., 2012). Low levels of vitamin D was associated with a failure of the immune regulation, including regulation of neutrophil activity. SLE patients studied showed higher number of LDG and NETs formation. This was thought to lead increase endothelial cell damage dan disease activity in SLE.

Recent evidence indicated that neutrophils may play important role in the induction of autoimmune responses and organ damage in SLE. Furthermore, microarray data indicate that abnormal neutrophils identified in PBMCs from lupus patients because of the cosegregation of low density granulocytes (LDGs) in mononuclear cell fractions. These LDGs reprsent a distinct neutrophil subset that is present in the peripheral blood of all adult SLE patients analyzed. Lupus LDGs are likely to be pathogenic, given their heightened capacity to induce vascular damage and synthesize type I IFNs upon exposure to specific stimulant, such as G-CSF and polyinosinic – polycytidylic acid, when compare with autologus lupus normal-density neutrophils anf healthy control neutrophils (Denny et al., 2010). Furthermore, lupus patients with higher circulating LDGs number have increase prevalence of skin involvement and/or vasculitis. Although currently no specific LDG surface markers have been identified that would allow them to be distinguished from normal density granulocytes, their nuclear morphology suggest that these cells present a more immature phenotype ( Bennet et al., 2003; Denny et al., 2010).

Neutrophil immobilize and kill invading micobes extracellulary trough the formation of NETs. As a unique type of neutrophil cell death, recently described NETosis, this response is distinct from apoptosis and necrosis and characterized by the active release of nuclear chromatin fibers (Fuchs et al., 2007). NETosis is triggered by a variety of stimuli including microorganism, proinflammatory cytokines, activated platelets and endothelial cells. A variety of putative autoantigens are present within and attached to NETs chromatin fibers, including citrunillinated histones and various bactericidal protein and/or enzymes such as the cathalicidin LL-37, neutrophil elastase and myeloperoxidase (MPO).

Due to the potential role of netting neutrophils in externalizing autoantigens and DNA-modifying factors, thereby making these molecules more exposed to the adptive and innate immune systems, a putative link between NETosis and autoimmunity has been recently propose. It has been shown that neutrophils from patients with anti-neutrophilic cytoplasmic Ab (ANCA)-positive vasculitis release NETs enriched in MPO and LL-37. NETa are also present in the kidney from patients with ths disease, where they may provide a source of antigenic nucleosomes and promote immune complex formation. Further, impaired NET degradation has been identified in subset of SLE patients, secondary to Dnase

1 inhibitor and anti-NETAbs that prevent Dnase 1 access to NETs (Al-Mayouf et al., 2011). LDGs may represent an additional source of NETs, leading to heightened autoantigen exposure and modification of tissue damage. Recent evidence indicates that NETosis may be enhance in IFN-α-primed lupus neutrophils upon exposure to anti-ribonucleoprotein (RNP) Abs. This is accompanied by the release of LL-37 and high mobility group protein B1, which facilitate uptake and recognation of mammalian DNA by plasmacytoid dendritic cells (pDC). In addition, recent evidence indicates that NETs may be harmful to the endothelium and promote thrombosis

LDGs has an important role in lupus pathogenesis by damaging endothelial cells and increases the synthesis of pro-inflammatory cytokines and interferon (IFN) type I. Increased apoptosis of endothelial cells has a strong correlation with the formation of vascular malfunction and can predispose the formation of atherosclerosis . The most instrumental process is through NETosis that can improve the ability to damage the endothelium and stimulates the synthesis of IFN - a by plasmacytoid dendritic cells (PDCs) (Villanueva et al., 2011). PDCs is mediated through the activation of TLR - 9 is activated by the NETs and mediates innate immune responses to the adaptive immune system. It makes NETs able to initiate the autoimmune response (Garcia Romo et al., 2011; Lande et al., 2011).

Another report that the NETs play a role in the pathogenesis of SLE, there is a strong relationship between mutation DNase1 (Yasutomo et al., 2001) or DNase1 like 3 (Al Mayouf et al., 2011 ) which is a degrading enzyme of NETs. A cohort studies in European showed that lack of NETs degradation in sera of subpopulation of SLE patients was due either to the presence of DNase1 inhibitors or high titers of anti-NET antibodies (Hakkim et al., 2010) . The inability to degrade of NETs could be caused by complement activation and increased deposition of complement protein C1q , which inhibits DNase1 (Leffler et al., 2012).

In addition to the effects already described, neutrophils can secrete IL - 17 through NETosis in the skin and peripheral blood of patients with psoriasis (Lande et al., 2011). IL - 17 may play a role in the pathogenesis of SLE through its capacity to amplify local inflammation by increasing the recruitment of innate and adaptive immune cells. Neutrophils expressing IL – $17^{+}$ are sseen at significantly enhance levels in blood and affected skin from lupus patients. Hence, NETs formation by LDG has a strong relationship with the pathogenesis of SLE through various effects (Villanueva et al., 2011).

Most of our patients with SLE had lower vitamin D serum levels than healthy controls. SLE patients with vitamin D levels < 20ng/ml showed higher number of LDG and the NETs formation than patients with vitamin D levels > 20 ng / ml and healthy controls. Furthermore, there were a negative relationships between levels of vitamin D with number of LDGs NETs formation. It can be concluded that vitamin D plays a role in the pathogenesis of Indonesian SLE. Low levels of vitamin D will lead to increasing the number of LDGs and formation of NETs, which ultimately will be involved in endothelial cell damages.

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