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Microbiota and oxidative-antioxidant balance in systemic lupus erythematosus

Microbiota y balance oxidante-antioxidante en lupus eritematoso sistémico

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RESUMEN

Introducción: el lupus eritematoso sistémico as una enfermedad inflamatoria crónica en la que está implicado el estrés oxidativo.

Objetivo: evaluar la concentración de antioxidantes de la dieta y sanguíneos, así como de la microbiota sobre las concentraciones de malondialdehído y proteína C reactiva en 21 pacientes de lupus y 21 controles pareados por edad y sexo.

Métodos: los parámetros bioquímicos de rutina y proteína C reactiva se determinaron a través de métodos enzimáticos: cobre, zinc y selenio por espectrometría de masas, malondialdehído y capacidad antioxidante total por métodos espectrofotométricos, la microbiota fecal por técnicas metagenómicas y la dieta a través de cuestionarios de frecuencia de consumo.

Resultados: no se han observado diferencias en la dieta en los pacientes con lupus respecto al grupo control, excepto en la ingesta de ácidos grasos trans, siendo mayor en el grupo de lupus. En estas pacientes se observaron mayores niveles circulantes de cobre y menores de zinc. La concentración de cobre en suero se relacionó directamente con los niveles de proteína C reactiva y esta proteína, a su vez, con la proporción de *Lentisphaerae*, *Proteobacteria* y *Verrucomicrobia* en heces. Además, mientras que los niveles de malondialdehído se asociaban inversamente con la proporción de *Cyanobacteria* y *Firmicutes*, con *Actinobacteria* se encontró una correlación positiva. La presencia de anti-SSA/Ro en lúpicas se relaciona con mayores concentraciones de zinc.

Conclusión: estos resultados podrían ser útiles para profundizar en el futuro conocimiento de esta compleja enfermedad.

Palabras clave: Malondialdehído. Proteína C reactiva. Lupus. Antioxidantes. Microbiota.

ABSTRACT

Background: Systemic lupus erythematosus (SLE) is a chronic inflammatory disease of autoimmune nature, in which oxidative stress is implicated.

Aim: compare the concentrations of dietary and blood antioxidants, as well as gut microbiota, with serum malondialdehyde (MDA) and C reactive protein (CRP) in 21 subjects suffering from non-active systemic lupus erythematosus (SLE) and 21 age and gender-matched controls.

Methods: General biochemical parameters and CRP were determined by enzymatic methods: copper, zinc and selenium by inductively coupled plasma mass spectrometry (ICP-MS), MDA and total antioxidant capacity (TAC) by spectrophotometric methods, gut microbiota by metagenomic analyses and dietary intake by means of food frequency questionnaire.

Results: no significant differences were found in diet between lupus patients and the control group, with the exception of trans fatty acids intake, which was higher in patients. In addition, higher concentration of serum copper and lower of zinc in SLE were found. Serum copper was positively associated with CRP and also, this protein with the proportion of *Lentisphaerae*, *Proteobacteria* and *Verrucomicrobia* in feces. Moreover, MDA levels displayed inverse correlations with the *Cyanobacteria* and *Firmicutes* groups, while

Actinobacteria showed a positive association. The lupus subjects with presence of anti-SSA/Ro were related to higher levels of serum zinc.

Conclusion: These results could be useful in the future to go deeper into the understanding of this complex disease.

Key words: Malondialdehyde. C-reactive protein. Lupus. Antioxidants. Microbiota.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by the presence of autoantibodies against self-antigens, especially those directed to double-stranded DNA and other nuclear components, resulting in tissue damage (1,2). As occurs with other autoimmune diseases, inflammation and oxidative stress are frequent in the course of SLE (3,4). According to this, some authors have reported higher levels of the biomarker of inflammation, C-reactive protein (CRP), in these chronic patients compared to controls (4-6). Although the cause of this pathology is unknown, accumulating evidence suggests that its development is conditioned by genetic, hormonal and environmental factors (7,8), including gut microbiota. Strong evidence in the last years suggests a connection between lupus and the composition of our gut commensals (9). Microbiota might have different mechanisms of action over the host balancing anti- and pro-inflammatory responses (10). In line with this, even though it is not clear if oxidative stress is a cause or a consequence of this pathology; recent studies have reported higher levels of the lipid peroxidation subproduct, malondialdehyde (MDA), in lupus patients (11-14). As both, oxidative stress and inflammation, may be implicated in SLE pathogenesis, they may be affected by the intake of oxidants and antioxidants and the antioxidant capacity from serum (15). In this regard, although it has been hypothesized that copper and selenium could be related with an adaptive response against oxidative stress and inflammation in rheumatoid arthritis, by means of an increase in ceruloplasmin and glutathione peroxidase respectively (15,16), data on the role of these trace elements in lupus is scarce. Recently, lower serum levels of zinc and selenium in SLE patients respect to healthy controls have been reported, being serum copper concentrations inversely associated with the disease activity (17,18). Thus, the present study was designed to compare the concentrations of

antioxidants, pro-oxidants, major microbial groups, MDA and CRP in SLE patients and healthy controls.

SUBJECTS AND METHODS

Volunteers

The study sample comprised 21 patients of SLE selected from the updated Asturian Register of Lupus (19). All of them fulfilled at least four of the American College of Rheumatology criteria for SLE (20), were women of Caucasian origin aged between 27 and 70 years, and had non-active disease at the time of sampling (Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score ≤ 8). This study is framed within a multidisciplinary project entitled "Towards a better understanding of gut microbiota functionality in some immune disorders", whose main aim was to characterize the intestinal microbiota composition in SLE patients. For this reason, only those subjects who had not used antibiotics, glucocorticoids, immunosuppressive drugs, monoclonal antibodies, or other immunotherapies during the last three months were recruited for the study. Information on cumulative clinical manifestations was obtained by reviewing clinical records, whereas specific antinuclear antibodies (ANA) were analyzed at the time of sampling (Table II). Twenty-one age-matched healthy women from the same population were recruited as controls.

Ethics approval for this study (reference code AGL2010-14952) was obtained from the Bioethics Committee of CSIC *(Consejo Superior de Investigaciones Científicas)* and from the Regional Ethics Committee for Clinical Research *(Servicio de Salud del Principado de Asturias)* in compliance with the Declaration of Helsinki. All determinations were performed with fully informed written consent from all participants involved in the study.

Nutritional assessment

Dietary intake of the previous year was registered with a food frequency questionnaire (FFQ) of 160 food items, designed *a priori* for this project and validated with a 24 h dietary for the intake of dietary biocompounds (21). Experts noted down detailed information about menu preparation and other information relevant to the study on fiber intake, for example, the consumption of fruit peeled or with skin. At the time of the interview, volunteers were asked about the frequency of consumption and amount they ate of each

food. They could choose from up to seven serving sizes. To record the consumption of alcoholic beverages, each volunteer was asked if he/she regularly consumed wine, beer, cider, and/or liquors, as well as the type and amount, using household measures (a glass, a bottle, etc.). Methodological issues concerning dietary assessment have been detailed elsewhere (21). Food intake was analyzed for energy, macronutrients, and total dietary fiber content by using the nutrient Food Composition Tables developed by the *Centro de Enseñanza Superior de Nutrición Humana y Dietética* (CESNID) (22). In addition, the following fiber components were ascertained using Marlett et al. food composition tables (23). The phenolic compound content in foods was completed using the Phenol Explorer database. It contains more than 35,000 content values for 500 different polyphenols in over 400 foods. For recipes, polyphenol content was calculated on the basis of the contents of the ingredient and its polyphenol composition. All of this information was mainly determined by high-performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE) and was obtained from more than 1,300 publications (24).

Anthropometric measures

Weight was measured in lightweight clothing and barefoot on a scale with an accuracy of ± 100 g (Seca, Hamburg, Germany). Height was registered using a stadiometer with an accuracy of ± 1 mm (Año-Sayol, Barcelona, Spain). Subjects stood barefoot, in an upright position and with the head positioned in the Frankfort horizontal plane. Body mass index (BMI) was calculated using the formula weight (kg)/height $(m)^2$. Waist circumference was measured using an inextensible and non-deformable tape. The measurement was taken between the lower costal margin and the top of the iliac crest after normal expiration with the subject standing and unforced position. Body fat percentage was estimated by electrical impedance (Tanita, Tokyo, Japan), with the subject barefoot and with the skin in contact with the electrodes.

Analysis of fecal microbiota

Fresh fecal material (between ten and 50 grams per person) was collected in sterile containers and immediately manipulated and homogenized within a maximum of three hours from defecation. During the waiting period, from defecation to homogenization,

samples were kept at 4 °C. Thirty ml of RNA latter solution (Applied Biosystems, Foster City, CA) were added to ten grams of the sample and the mixture was homogenized in sterile bags, using a stomacher apparatus (IUL Instruments, Barcelona, Spain) (three cycles at high speed, one minute per cycle). Homogenized samples were then stored at -80 °C until use. For DNA extraction, samples were thawed and the QIAamp DNA Stool Mini kit was used (Qiagen Ltd, Strasse, Germany), as previously described (25). Fecal DNA extraction, 16S rRNA amplification, sequencing of 16S rRNA gene-based amplicons and sequence-based microbiota analysis were reported elsewhere (26). Sequences were deposited in the NCBI Short Read Archive (SRA) with the accession numbers SRP028162 and PRJNA276631.

Biochemical analyses

Each volunteer was asked to provide a blood sample, drawn after a 12-hour fast and subsequently centrifuged, divided in aliquots and immediately frozen and stored at -80 °C until further analyses.

Serum glucose, total cholesterol and high-density lipoproteins (HDL), triglycerides and CRP were determined by enzymatic methods. Low-density lipoprotein (LDL) was calculated from the Friedewald formula (27). All these determinations were carried out in an independent laboratory. Serum concentrations of copper, zinc and selenium were measured by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) in the Scientific and Technical Services of the University of Oviedo.

MDA concentrations in serum were determined with the spectrophotometric method of lipid peroxidation LPO-586 (Byoxytech, Oxis International, S.A., France). This kit uses the reaction of a chromogenic reagent with MDA, without interference from 4-hydroxyalkenals (hydrochloric acid solvent procedure), in aqueous samples at 45 °C. One molecule of MDA reacts with two molecules of reagent to yield a stable chromophore with maximal absorbance at 586 nm (28). The within-run coefficient of variation ranged from 1.2% to 3.4%, depending on the concentration of MDA.

Total antioxidant capacity (TAC) in serum was determined with the colorimetric assay P40117 (Innoprot, Innovative Technologies in Biological Systems, S.L., Spain). In this method, Cu^{2+} is converted to Cu^{+} by both small molecules and protein. The reduced ion is chelated with a colorimetric probe giving a broad absorbance peak around 450 nm, proportional to the TAC (29).

Statistical analysis

Statistical analysis was performed using IBM-SPSS version 22.0 (SPSS-Inc., Chicago, USA). Goodness of fit to normal distribution was investigated by Kolmogorov-Smirnov test. For descriptive purposes, continuous variables were presented on untransformed mean ± standard deviation (SD) and percentage for categorical ones. In order to elucidate the differences in dietary compounds intake and serum parameters between SLE and control subjects Student's t-tests were calculated. Pearson's bivariate correlation was used to investigate the association between the levels of serum trace elements and CRP, as well as serum C reactive protein and malondialdehyde levels with fecal microbiota between systemic lupus erythematosus patients and controls. Heatmap was generated under R version 3.3.3 package heatmap.2. The conventional probability value for significance (0.05) was used in the interpretation of results.

RESULTS

General characteristics of SLE patients and controls are listed in table I. SLE sample could be defined as a group of women with a mean age of 48.14 ± 11.53 years old, BMI indicative of moderate overweight and a body fat percentage over the recommendations (30). Only a small percentage of the sample was smoker and less than 50% was sedentary. There were no differences in any of the studied variables between SLE and controls (Table I). Clinical features of SLE patients are described in table II. The most frequent manifestations were: presence of ANA positivity, photosensitivity, malar rash and hematological disorders, whereas others, such as neurological disorders, were found in a small percentage of the sample. No significant differences were found regarding diet, with the exception of the intake of trans fatty acids, which was higher in SLE subjects (Table III). When comparing the serum levels of the biochemical parameters in SLE and controls, a higher concentration of copper and lower of zinc in the patients was found, while the concentration of glucose, total cholesterol, HDL, LDL, triglycerides, MDA, CRP, selenium and TAC were similar in these groups (Table IV). Pearson's bivariate correlations were performed in order to explore into the association between serum trace elements and CRP (Fig. 1). From the evaluated components, it was only found a positive correlation between serum copper and CRP in SLE patients ($r = 0.503$; $p = 0.024$). Furthermore, the serum levels of trace elements were

evaluated according to the presence or absence of clinical features, and it was found that patients suffering from renal disorders had lower levels of zinc (near to statistically significance p = 0.092), and, contrary, those with presence of anti SSA/Ro had higher levels of this trace element (Fig. 2). No associations were found for the rest of clinical features. In order to evaluate whether gut microbial composition may be related with serum levels of MDA and CRP, a Pearson's bivariate correlation analysis was assessed. Among the phyla analyzed, MDA levels displayed inverse correlations with *Cyanobacteria* and *Firmicutes* and positive with *Actinobacteria* only in SLE group (Fig. 3). A positive association between CRP and *Lentisphaerae, Proteobacteria* and *Verrucomicrobia* was also observed in SLE, but not in controls (Fig. 3).

DISCUSION

The results of this study do not support the existence of higher oxidative stress in non-active patients of systemic lupus erythematosus. The detection of a lower concentration of zinc and higher of copper in SLE patients compared to controls, as well as the association of these components with the concentration of C-reactive protein and some clinical features of this pathology, is the most important finding of this paper together with the detection of a direct association between the concentration of this inflammatory biomarker and the fecal proportions of *Lentisphaerae, Proteobacteria* and *Verrucomicrobia*.

Although the literature about this topic is scarce, lipid peroxidation has been reported in other studies with SLE patients (11,12,31,32). We are aware that a single marker is not sufficient to denote oxidative stress, but MDA is one of the most abundant products of lipid peroxidation and, probably, the most frequently used in humans (33). The similar levels of MDA found between our lupus patients and controls do not confirm previous evidence, being one of the possible reasons that patients were in a non-active phase of the disease at the time of sampling. In line with this, some authors have found higher levels of this lipoperoxidation marker (9.23 µM) in active SLE patients (SLEDAI score around 40) of similar age (12), so it seems feasible that the severity of the disease could determine the degree of oxidative damage in SLE. In this regard, to the best of our knowledge the negative correlation between the phylum *Firmicutes* and the MDA levels in lupus patients cannot be explained through a direct metabolic activity of the gut microbiota members on lipid peroxidation. Rather, it could indicate that the local intestinal environment in SLE

patients is responsible for the lower levels of these bacteria, which are extremely oxygen sensitive. In fact, these bacteria are more sensitive to oxidative stress processes than other members of the microbiota. In relation to this, it has been documented that the gut microbiota displays low levels of *Firmicutes* in some diseases involving intestinal or extra-intestinal inflammation and oxidative stress, such as Crohn's disease (34), ulcerative colitis (35) and other immune-mediated inflammatory diseases (36). Indeed, the microbiota of inflammatory bowel disease (IBD) patients has a low abundance of *Faecalibacterium prausnitzii*, a member of this phylum with a known and well characterized anti-inflammatory effect (34-37). In spite of the fact that the absence of differences in total antioxidant capacity between SLE and controls is in agreement with previous studies in SLE subjects (38,39), there is no consensus about it in the literature. While some authors have reported lower levels in autoimmune diseases (40,41), others have found higher ones (42). Since it has been reported an increase in the serum levels of total antioxidant capacity to act against oxidative stress (42), it is likely that this parameter is dependent on the oxidative stress status.

On the other hand, the lower levels of serum zinc found in SLE subjects compared to controls were similar to those reported in other studies carried out with SLE population (17,18). It has been hypothesized that this decrease could be a result of a defense mechanism of the body against oxidative stress, given that the use of zinc as a cofactor for the antioxidant enzyme superoxide dismutase might compromise the amount of this trace element available in blood (32,43-46). In addition, as it has been described, SLE subjects in our sample with specific disease features and/or anti SSA/Ro negative had lower levels of serum zinc in comparison with the rest of the patients, the latter being similar to those reported in the controls (47-49). Therefore, although the number of subjects and the nature of the study do not allow establishing causality or directionality, these results could be useful in future for other studies aimed at clarifying the role of zinc in the presence of these clinical features.

We have not found statistically significant differences in the levels of CRP in SLE patients in respect to controls, probably because of their non-active disease at sampling. Moreover, although serum levels of CRP usually go in parallel with the disease activity in inflammatory states, the results from this point in SLE are inconclusive, being suggested that this autoimmune disease could be an exception (50). In this regard, while some authors have

reported moderate levels of CRP (2.1 mg/l) in lupus subjects (51), others have associated this pathology with a high increase (15-16 mg/l) in this acute-phase protein (12). The reasons for this disparity are not entirely clear, however, disease exacerbation could be a determinant factor in this situation. In regard to the positive association between *Proteobacteria* and *Verrucomicrobia* with CRP, it has been reported a higher abundance of some representative of these phyla, such as members of the family *Enterobacteriaceae* and *Akkermansia muciniphila*, in different inflammatory processes, including those associated with inflammatory bowel disease (34,52). Indeed, *Proteobacteria* and *Verrucomicrobia* members are Gram-negative microorganisms which contain lipopolysaccharide (LPS), a highly pro-inflammatory molecule located on the bacterial surface. LPS has been involved in a variety of inflammatory processes and could partially explain the association between the levels of CRP with a higher abundance of these bacteria (53).

Finally, as in other autoimmune diseases, our results revealed increased levels of serum copper in SLE subjects (16,17). This result could be explained by the increase in the synthesis of hepatic ceruloplasmin, and the subsequent release into the blood, in response to a higher production of some inflammation markers increased in this pathology, such as IL-6 and IL-1 (16). Our finding of a positive association between this element and CRP is in accordance with this hypothesis.

Despite the relatively limited statistical power, our analyses revealed the absence of increased levels of lipid peroxidation and CRP in SLE patients in a non-active phase of the disease. The identification of different concentrations of zinc and copper in serum in lupus, together with the association of these trace elements with some blood markers associated with this pathology could be useful in the future to go deeper into the understanding of this complex disease. Novelty results connecting microbiota with inflammation will be useful to generate new hypotheses to test dietary strategies to treat autoimmunity diseases.

CONFLICT OF INTEREST

All authors have declared no conflict of interest and read and approved the final submitted manuscript. No portion of the manuscript has been previously published.

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Table I. General characteristics in systemic lupus erythematosus (SLE) patients and controls

	SLE (n = 21)	*Controls (n = 21)*	*p*
Age (y)	48.14 ± 11.53	46.24 ± 9.45	0.561
BMI (kg/m^2)	25.23 ± 4.87	25.72 ± 4.67	0.738
Body fat (%)	33.62 ± 9.81	37.55 ± 11.50	0.245
Waist circumference (cm)	77.98 ± 10.14	81.77 ± 10.74	0.252
Smoking habit (%)	28.60	23.8	0.726
Alcohol consumption (%)	42.90	66.70	0.226
Sedentariness (%)[a]	38.90	47.60	0.584

BMI: Body mass index. [a]Declared as not making physical activity. Derived from Student´s t-test. Results presented as mean ± SD and percentage.

Table II. Clinical features of systemic lupus erythematosus (SLE) patients

	SLE (n = 21)
Disease duration (y)	9.81 ± 7.08
Clinical manifestations (%)	
Malar rash	10 (47.60)
Discoid lesions	7 (33.30)
Photosensitivity	15 (71.40)
Oral ulcers	9 (42.90)
Arthritis	11 (52.40)
Serositis	4 (19.00)
Renal disorder	4 (19.00)
Neurological disorder	1 (4.80)
Hematological disorder	10 (47.60)
ANA (%)	16 (76.20)
Anti-SSA/Ro (%)	10 (47.60)
Anti-dsDNA (%)	6 (28.60)
Anti-dsDNA titer (U/ml)	23.19 ± 34.22

ANA: Antinuclear antibodies; dsDNA: Double stranded DNA. Results presented as mean ± SD and number (percentage).

Table III. Differences in daily intake of the major dietary compounds in systemic lupus erythematosus (SLE) patients and controls

	SLE (n = 21)	*Controls (n = 21)*	*p*
Carbohydrates (g/day)	221.60 ± 64.37	195.14 ± 46.35	0.134
Dietary fiber (g/day)	25.56 ± 7.60	24.26 ± 8.95	0.617
Protein (g/day)	107.08 ± 25.49	97.82 ± 18.97	0.189
Vegetal protein (g/day)	29.11 ± 9.76	29.71 ± 15.01	0.879
Animal protein (g/day)	72.51 ± 22.30	65.40 ± 16.76	0.250
Lipids (g/day)	89.68 ± 37.63	79.11 ± 19.26	0.261
MUFA (g/day)	38.88 ± 19.62	33.17 ± 7.90	0.227
PUFA (g/day)	17.41 ± 8.06	15.74 ± 8.96	0.528
SFA (g/day)	25.99 ± 12.23	23.31 ± 5.71	0.367
Trans-fatty acids (g/day)	0.06 ± 0.05	0.02 ± 0.03	0.006
Polyphenols (mg/day)	1,811.99 ± 669.01	1,713.69 ± 625.31	0.625
Vitamin C (mg/day)	196.92 ± 97.23	192.44 ± 75.52	0.868
Vitamin E (mg/day)	14.15 ± 6.84	11.67 ± 3.90	0.157
Zinc (mg/day)	10.59 ± 2.56	9.26 ± 2.36	0.088
Copper (mg/day)	1.60 ± 0.49	1.45 ± 0.46	0.322
Selenium (µg/day)	127.95 ± 35.73	113.86 ± 28.33	0.165

Derived from Student's t-test. Results presented as mean ± SD. MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; SFA: Saturated fatty acids.

Table IV. Serum levels of biochemical parameters in systemic lupus erythematosus (SLE) patients and controls

	SLE (n = 21)	*Controls (n = 21)*	*p*
Glucose (mg/dl)	87.76 ± 8.89	93.38 ± 12.67	0.104
Total cholesterol (mg/dl)	194.29 ± 36.79	203.00 ± 37.22	0.450
HDL (mg/dl)	63.95 ± 16.54	65.86 ± 12.19	0.673
LDL (mg/dl)	115.00 ± 37.78	123.19 ± 32.23	0.454
Triglycerides (mg/dl)	75.14 ± 34.73	68.86 ± 25.47	0.507
Malondialdehyde (µM)	2.87 ± 0.42	2.77 ± 0.41	0.442
C- reactive protein (mg/l)	3.82 ± 4.17	2.53 ± 1.92	0.206
Selenium (µg/l)	140.20 ± 22.89	142.33 ± 29.23	0.797
Copper (µg/l)	1,287.40 ± 264.91	1,094.57 ± 246.26	0.021
Zinc (µg/l)	776.20 ± 146.11	922.90 ± 206.04	0.013
Total antioxidant capacity (mM)	0.40 ± 0.09	0.43 ± 0.10	0.318

HDL: High-density lipoproteins; LDL: Low-density lipoproteins. Derived from Student's t-test. Results presented as mean ± SD.

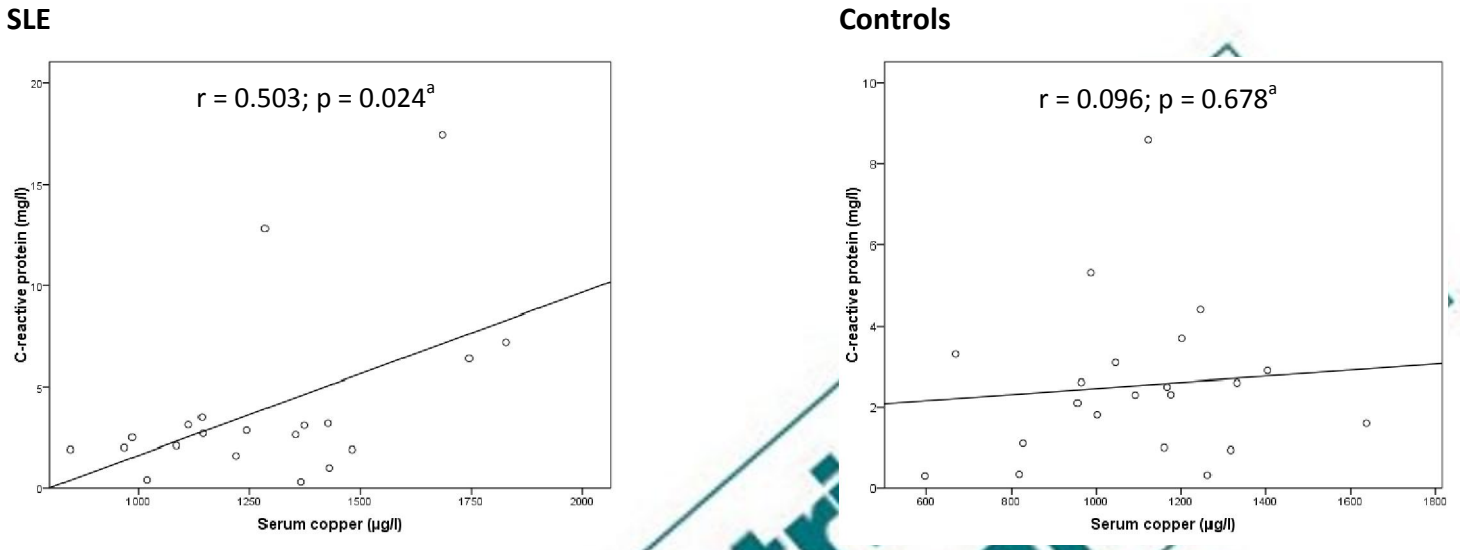

Figure 1. Dispersion graphic between serum levels of copper and C reactive protein in systemic lupus erythematosus (SLE) patients and controls. [a]Derived from Pearson's bivariate correlation analyses.

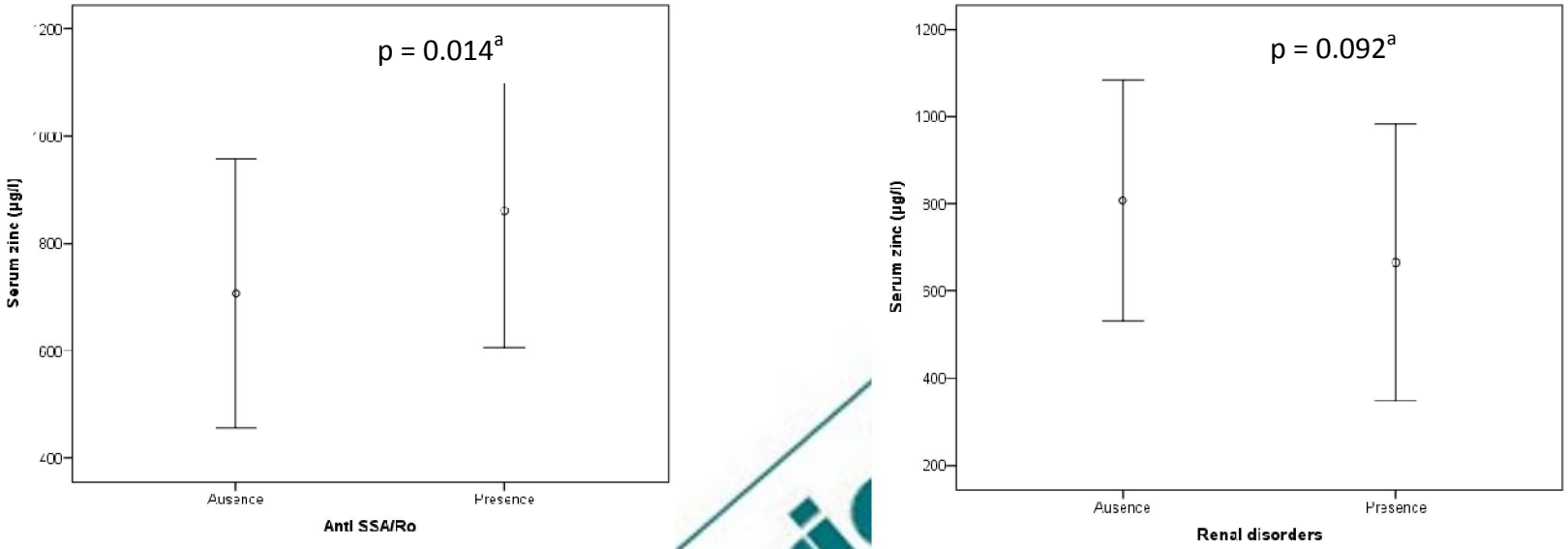

Figure 2. Serum concentrations of zinc according to the presence or absence of anti-SSA/Ro in systemic lupus erythematosus (SLE).

[a]Derived from Student's t-test. Results presented as mean ± 2 SD.

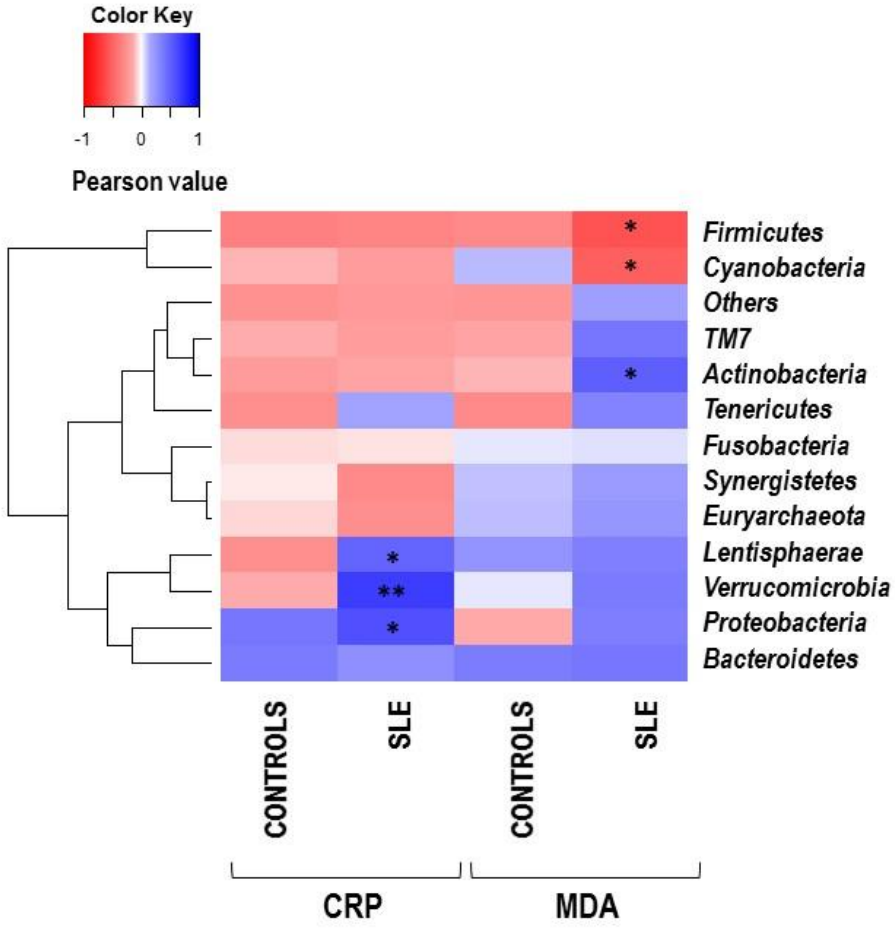

Figure 3. Correlation between serum C reactive protein (mg/l) and malondialdehyde levels (µM) and relative abundance of fecal microbiota (%) in systemic lupus erythematosus (SLE) patients and controls. Columns correspond to serum C reactive protein and malondialdehyde levels; rows correspond to fecal microbial phyla. Blue and red colors denote positive and negative association, respectively. The intensity of the colors

represents the degree of association between fecal microbiota and serum parameters by Pearson's bivariate correlations, and dots indicate significant associations. *$p < 0.05$; **$p \leq 0.01$.