Brief Report

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Vitamin D Deficiency Is Associated With Endothelial Dysfunction and Increases Type-1 Interferon Gene Expression in a Murine Model of SLE

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Abstract

Objective

Patients with Systemic Lupus Erythematosus (SLE) have an increased risk of cardiovascular disease (CVD) and impaired endothelial repair. Although vitamin D deficiency is associated with increased CVD risk in the general population, a causal relationship has not been demonstrated. We aimed to determine whether vitamin D deficiency directly modulates endothelial dysfunction and immune responses in a murine model of SLE.

Methods

Vitamin D deficiency was induced in lupus-prone MRL/*lpr* mice by dietary restriction for 6 weeks. Endothelium-dependent vasorelaxation was quantified using aortic ring myography and endothelial repair mechanisms by quantifying the phenotype and function of bone marrow endothelial progenitor cells (EPCs) and an *in vivo* Matrigel plug model. Lupus disease activity was determined by expression of interferon-sensitive genes (ISGs) in splenic tissue, serum autoantibodies and renal histology. To validate the findings, expression of ISGs was also measured in whole blood from vitamin D deficient and replete SLE patients.

Results

Vitamin D deficiency resulted in impaired endothelium-dependent vasorelaxation and decreases in neoangiogenesis without a change in total number of EPCs. There were no differences in anti-dsDNA titers, proteinuria or glomerulonephritis (activity or chronicity) between deficient or replete mice. Vitamin D deficiency was associated with a trend towards increased ISG expression both in mice and in SLE patients.

Conclusion

Vitamin D deficiency is associated with hampered vascular repair and reduced endothelial function, and may modulate type I IFN responses.

Patients with systemic lupus erythematosus (SLE) have an increased risk of cardiovascular disease (CVD) which is not explained by traditional risk factors (1). As a surrogate of vascular risk, endothelium-dependent vasorelaxation is impaired in lupus patients compared to matched controls indicating endothelial dysfunction, a predictor of atherogenesis (2). As a mechanism underlying endothelial dysfunction in SLE, we have proposed that an imbalance develops in these patients, where accelerated endothelial cell apoptosis is coupled with decreased vascular repair (as manifest by fewer endothelial progenitor cells (EPCs) and a reduced ability to differentiate into mature EPCs). We have also proposed that type I interferons (IFNs), cytokines increased in SLE patients, are main drivers of the decreased vascular repair and atherosclerosis risk in this disease (3).

Vitamin D deficiency may promote premature CVD in this patient group. Lupus patients have lower serum 25-hydroxyvitamin D (25(OH)D) compared to healthy subjects, which may be due in part to sunlight avoidance (4). Low serum 25(OH)D is associated with endothelial dysfunction in the general population (5) and with increased aortic stiffness in SLE patients (6). However evidence for a causal relationship between vitamin D deficiency and premature CVD in SLE is currently lacking. Furthermore, while vitamin D may be immunomodulatory *in vitro*, the association between vitamin D deficiency and disease activity in SLE patients remains controversial.

We investigated whether induction of vitamin D deficiency modified endothelial function, neoangiogenesis and progression of disease in lupus-prone MRL/*lpr* mice and also analyzed the association between vitamin D levels and the type I IFN signature in SLE patients.

Methods

Animals

MRL/*lpr* mice (000485 MRL/MpJ-*Fas^{lpr}*/J) and control MRL/mpj (000486) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and fed standard chow until 8 weeks of age. Mice were then changed onto a vitamin D-deficient diet (TD.89123) or calcium- and phosphate-matched control diet (TD.89124) (both from Harlan Laboratories, WI, USA). Mice were euthanized after 6 weeks on the specialized diet and serum was obtained at this time. Plasma samples for parathyroid hormone (PTH) analysis were collected from the tail vein 1 week prior to euthanasia. Protocols were approved by the NIAMS Animal Care and Use Committee.

Measurement of vitamin D and PTH levels

Serum samples obtained at euthanasia were stored at -80°C until use. 25-hydroxyvitamin D (25(OH)D) levels were measured using by immunoassay following manufacturer's instructions (Enzo 25(OH) Vitamin D ELISA kit, Enzo Life Sciences (UK) LTD, UK). Plasma PTH was measured using a murine intact (1-84) PTH ELISA kit (ALPCO, New Hampshire, USA) following the manufacturer's instructions.

Aortic ring myography

Endothelium-dependent vasorelaxation was measured by aortic ring myography as previously described (7). Following euthanasia, 2mm rings were carefully cut from the thoracic aorta to ensure that the endothelium remained intact. The aortic rings were mounted onto a myograph (Danish Myo Technology, Aarhus, Denmark) and bathed in warmed, aerated physiological salt solution (PSS). The rings were equilibrated prior to

contraction with PSS containing 100mmol/l KCl (KPSS). Cumulative concentrations of phenylephrine (PE) (10^{-9}mol/l up to 10^{-5}mol/l) were added to produce a dose-response curve. The rings were washed and contracted again with PE (at the EC_{50}). Cumulative concentrations of acetylcholine (ACh) (10^{-9} mol/l to 10^{-5}mol/l) were added to produce a relation dose-response curve. The ACh-mediated relaxation was calculated as the % of PE contraction.

EPC quantification and differentiation into mature endothelial cells (ECs)

EPCs were obtained from bone marrow and their capacity to differentiate into ECs was quantified using fluorescent microscopy as described previously (3). Briefly, femurs and tibias were flushed with a buffer solution (15mM EDTA in Hanks Buffered Salt Solution) and bone marrow cells were separated on Histopaque 1083, and plated in triplicate onto fibronectin-coated plates (1.3×10^6/cm^2) in MCDM 131 media supplemented with EGM-MV2 bullet kit (Lonza, Switzerland) and 5% FBS. Cells were cultured for 1 week with a media change every 3 days. On day 7, cells were incubated with 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (diI)–acetylated LDL (dil-ac-LDL, Biomedical Technologies, USA) and FITC-conjugated *Bandeiraea (Griffonia) Simplicifolia* Lectin I (BS-1, Vector Laboratories, USA) for 4 hours. Images from 3 random fields per well were obtained using a Leica DMIRB fluorescent inverted microscope (Bannockburn, USA) with an objective magnification of ×10 and an Olympus DP30BW camera (Olympus Corporation, Tokyo, Japan). The number of dual positive cells was quantified using Cell C software (The Mathworks Inc, USA) and expressed as mean number per high powered field (HPF).

In vivo Matrigel plug angiogenesis assay

Matrigel plug assays were performed as described previously (3). Briefly, after 5 weeks on the diet, 2 plugs comprising 500μl growth factor-reduced Matrigel (Becton Dickinson, USA) containing 20nM basic fibroblast growth factor (R&D systems, USA) were injected subcutaneously into each animal. Plugs were removed 1 weeks later and the haemoglobin (Hb) concentration was quantified using the 3,3',5,5'-tetramethylbenzidine method and normalized to the weight of the plug.

Disease activity assessment

Serum concentration of anti-double-stranded (ds)-DNA and total IgG was measured using the Mouse Anti-dsDNA IgG ELISA kit and Mouse IgG ELISA kit, respectively (both from Alpha Diagnostic International, Texas, USA), following manufacturer's instructions.

The expression of interferon sensitive genes (ISGs) was determined in the spleen using real-time RT-qPCR. Splenic tissue was harvested at euthanasia and stored at -80°C in RNAlater (Qiagen, Germany). RNA was extracted from homogenized tissue using the mRNA Mini kit (Qiagen). RNA was reverse transcribed using iScript RT Supermix kit (Bio-Rad, California,USA). Real-time qPCR was carried out using an ABI 7500 machine and Bio-Rad SYBR green iQ™ kit (Bio-Rad). The following primers were used: *MCP1* (forward; AGGTCCCTGTCATGCTTCTG, reverse; GGATCATCTTGCTGGTGAAT), *IRF7* (forward; TGCTGTTTGGAGACTGGCTAT, reverse; TCCAAGCTCCCGGCTAAGT), *ISG15* (forward; CAGAAGCAGACTCCTTAATTC, reverse; AGACCTCATATATGTTGCTGTG), *IFNG* (forward; AGCGGCTGACTGAACTCAGATTGTA, reverse; GTCACAGTTTTCAGCTGTATAGGG), *ACTB* (forward; TGGAATCCTGTGGCATCCTGAAAC, reverse; TAAAACGCAGCTCAGTAACAGTCCG). Gene expression was normalized to the reference gene (*ACTB*) and then to samples obtained from the MRL/mpj control strain using the ΔΔCt method.

Renal histology and urinalysis

Immune complex deposition was assessed by immunofluorescence staining of IgG and C3 on frozen kidney sections as described previously (7) and were semi-quantitatively scored by a renal pathologist (AZR). Renal activity and chronicity indexes were scored blindly by a renal pathologist (AZR) on formalin-fixed sections stained with Trichrome and PAS as previously reported. Urine albumin and creatinine were quantified using the mouse Albuwell ELISA and Creatinine Companion Kit (Exocell, Pennsylvania, USA) and albumin:creatinine ratios were calculated.

Patient recruitment

Patients with SLE were recruited from a single UK center (Central Manchester University Hospitals NHS Foundation Trust). Serum vitamin D was measured using liquid chromatography-mass spectrometry (LC-MS). Vitamin D deficiency was defined as 25(OH)D<20ng/ml (50nmol/l) and replete levels were defined as 25(OH)D>30ng/ml (75nmol/l). Disease activity was measured using the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) score (8). Informed consent was obtained from all study participants, in compliance with the Declaration of Helsinki and the study was approved by the North West 1 Research Ethics Committee (11/NW/0008).

Gene expression analysis

Peripheral blood from 10 vitamin D-deficient and 10 vitamin D-replete SLE patients was collected into PAXgene tubes which were kept at room temperature for 24-48 hours, then stored at -80°C until analyzed. RNA was extracted and quantitative RT-PCR was performed as described previously (9). Total RNA was extracted from whole blood using the PAXgene

RNA Isolation Kit (PreAnalytix, Switzerland). Quantitative RT-PCR was carried out using TaqMan Universal PCR Mastermix (Applied Biosystems, UK). TaqMan probes were used for the ISGs; *IFI27* (Hs01086370_m1), *IFI44L* (Hs00199115_m1), *IFIT1* (Hs00356631_g1), *ISG15* (Hs00192713_m1), *RSAD2* (Hs01057264_m1) and *SIGLEC1* (Hs00988063_m1), and for the reference genes; *HPRT1* (Hs03929096_g1) and *18s* (Hs999999001_s1). The relative abundance of each gene was normalized to the expression of *HPRT1* and *18s* using the Applied Biosystems StepOne software (version 2.1). The values were also expressed as a total ISG score as described previously (9).

Statistical analysis

Statistical analyses (Student's t-test) were carried out using Prism v6.04 (GraphPad, USA).

Results

Dietary absence of cholecalciferol rapidly depletes serum vitamin D in mice

The growth rate for the mice exposed to vitamin-D deficient diet was comparable to mice on the control diet over the course of the study and there were no phenotypic differences in the mice in terms of body weight, cardiac weight or alopecia (n=10 in each group, data not shown).

Compared to the control diet, mice on the vitamin D-deficient diet had significantly lower serum 25(OH)D after 6 weeks (mean [sd] 26.1 [1.6] vs. 65.0 [2.5] nmol/l, p<0.0001). Plasma PTH levels after 5 weeks were also higher in mice receiving the vitamin D deficient diet (179.5 [91.8] vs. 76.8 [11.3] pg/ml, p=0.006). Serum calcium levels after 6 weeks did not differ between the groups (0.33 [0.05] vs. 0.35 [0.04] mg/ml, p=0.376).

Vitamin D deficient lupus-prone MLR/lpr mice have impaired endothelium-dependent vasorelaxation

Impaired endothelium-dependent vasorelaxation develops rapidly in MRL/*lpr* mice compared to the background strain (7). Following 6 weeks of vitamin D deficient diet, MRL/*lpr* mice had significantly impaired endothelium-dependent vasorelaxation compared to the mice on the control diet (figure 1).

Given that reduced endothelium-mediated vasorelaxation is associated with a reduction in the capacity of EPCs to differentiate into mature ECs, and with impaired neoangiogenesis, we investigated whether either of these parameters was impaired in vitamin D-deficient

mice. The mean number of mature ECs per HPF, following proangiogenic stimulation of EPCs, was similar in the vitamin D-deficient mice compared to the replete mice (105 [37] vs. 123 [35], p=0.309) (figure 1). EC differentiation was enhanced when EPCs were exposed *in vitro* to the active form of vitamin D (10nM 1,25(OH)$_2$D$_3$) in the deficient, but not the replete mice (mean change of 14.1 [7.4] vs. -5.0 [9.8], p=0.067).

Neoangiogenesis was measured using an *in vivo* Matrigel plug assay. These experiments were carried out in a separate group of mice to ensure that components of the Matrigel did not affect the myography experiments. The mice on the vitamin D-deficient diet had significantly impaired angiogenesis compared to those on the control diet (mean concentration of Hb/gram plug 226 [93] vs. 583 [96] μg/ml per g plug, p=0.0228).

Overall, these results indicate that vitamin D deficiency impairs endothelium-dependent vasorelaxation and neoangiogenic capacity in lupus-prone mice.

Effects of vitamin-D deficiency on renal disease and autoantibodies

At euthanasia, there were no differences in splenic weight or lymphadenopathy between experimental groups. There was no difference between vitamin D deficient and replete mice in the levels of anti-dsDNA (3.63[0.75] vs. 2.99[0.42]10^6 U/ml, p=0.471) or total IgG (98.3[10.6] vs. 98.9[5.0]mg/ml, p=0.955). Similarly, there was no difference in the anti-dsDNA:IgG ratio (figure 2).

No differences were observed between deficient and replete mice in the urinary albumin concentration (2.58[2.3] vs. 5.47[5.9]μg/ml, p=0.194) or albumin:creatinine ratio (1.80[2.9] vs. 1.36[1.2], p=0.699). Mice in both groups developed a similar degree of histological

glomerulonephritis activity and chronicity (using semi-quantitative lupus nephritis activity and chronicity score) and similar degrees of immune complex (IgG/C3) deposition (figure 2).

Vitamin D-deficient lupus-prone mice and SLE patients have upregulation of ISGs

We have previously shown that type I IFNs drive endothelial dysfunction and atherosclerotic plaque burden in murine models (3). We therefore investigated whether vitamin D deficiency modified ISG expression in MRL/*lpr* mice as a putative mechanism to explain how vitamin D modulates angiogenesis and vasomotor function. Splenic expression of the ISGs *MCP1*, *IRF7*, *ISG15* and *IFNG* was increased in the vitamin D deficient group compared to the control mice, although only the expression of *MCP1* reached statistical significance (p=0.036 for *MCP1*) (figure 3).

We then examined the association between vitamin D levels and ISG expression in a small number of patients with clinically stable SLE (defined as not requiring any change in therapy). Whole blood expression of ISGs was measured in 10 deficient and 10 replete patients who had a mean (sd) serum 25(OH)D of 30.3 (10.8) and 101.4 (23.8) nmol/l respectively. The mean (sd) SLEDAI-2K score was 1.3 (1.9) in deficient patients and 1.4 (1.7) in replete patients (p=0.863). An ISG score was calculated as the median fold change of the 6 ISGs compared to the median of 20 healthy controls. The mean ISG score showed a trend toward being higher in the deficient patients compared to the vitamin D-replete patients (mean [sd] 9.20 [7.5] vs. 4.54 [4.7], p=0.115) (figure 3). Similarly to the observation in the mice, the expression of all of the ISGs (*IFI27, IFI44L, IFIT1, ISG15, RSAD2, SIGLEC1*) tended to be higher in the vitamin D deficient patients.

Discussion

Vitamin D deficiency is proposed to be an important risk factor for CVD development in the general population (10). In SLE, lower levels are associated with vascular stiffness (6) and increased traditional CVD risk factors (11). Epidemiological studies are not, however, able to demonstrate a causal relationship between low vitamin D and CVD. Our results provide the first evidence that vitamin D deficiency can accelerate the development of endothelial dysfunction and impair angiogenesis in murine lupus. These differences occurred at clinically relevant 25(OH)D concentrations and in a controlled environment, excluding common confounding factors such as concomitant medication, ethnic differences, UV exposure and disease duration Given the pleotropic effects of vitamin D, the mechanisms underlying our observations may be complex, and require further study.

The vitamin D deficient diet induced a state of hyperparathyroidism in the mice. Although the observed changes in vascular function could be due increased PTH, an interventional study by Sugden *et. al.* (2007) identified that vitamin D improved endothelial function in patients with type 2 diabetes without any change in PTH (12).

Importantly, two small clinical studies suggest that vitamin D therapy can modify endothelial function, and thus potentially reduce CVD risk, in SLE patients. A pilot study of 9 SLE patients demonstrated that treatment with cholecalciferol can improve endothelial function (13). In this study, patients with an increase in flow-mediated dilatation (FMD) had significantly higher post-treatment 25(OH)D levels, although there was no clear correlation between 25(OH)D and change in FMD. In a larger observational study of 40 patients, a significant association between changes in serum 25(OH)D and FMD improvement was

observed over 12 weeks, in association with increased numbers of pro-angiogenic myeloid cells, implicating that changes in endothelial repair were involved (14).

We have proposed that a failure of endothelial repair is an important contributor to premature CVD in SLE patients. While studies of circulating EPC numbers in SLE have shown conflicting results (reviewed in (15)), it is clear that EPCs are dysfunctional in SLE in terms of migratory and angiogenic capacity, and pro-angiogenic factor production). In the present study, while the number of EPCs and their capacity to differentiate into mature ECs was not influenced by vitamin D status, differentiation improved with *in vitro* vitamin D supplementation in depleted-mice. In addition, vitamin D deficient mice demonstrated reduced *in vivo* neoangiogenesis suggesting relevant functional impairments.

In both the murine model and the SLE patients, vitamin D deficiency was associated with increased expression of ISGs, although this was not statistically significant in the patients. The number of SLE patients included in this pilot study was small, and a larger cohort is needed to further investigate this finding. However, our findings support those of Mandal *et. al* (2014) who demonstrated an inverse association between serum 25(OH)D and both IFNα and SLEDAI score (16). Our results suggest that low vitamin D may drive type I IFN pathways resulting in increased ISG expression in the absence of other markers of active disease (anti-dsDNA levels, renal disease). Supporting the murine findings, the relationship between low 25(OH)D and increased ISG expression was observed in patients in clinical remission in our study, although this requires further validation. This suggests that vitamin D deficiency may contribute to "prime" the immune system in SLE even when there is no evidence of overt disease flare and that IFN may contribute to vascular damage perhaps independently of clinical disease activity.

Whilst we have demonstrated that vitamin D deficiency promotes endothelial dysfunction and IFN activation it cannot be assumed that vitamin D treatment reverses these processes. In a small study by Aranow *et. al.* (2105) (17), vitamin D therapy had no effect on the expression of ISGs in SLE patients. However, the effect of cholecalciferol may have been diluted in this study since only 16/33 (48%) patients were vitamin D-replete at the end of the study and the assessments were done only after 12 weeks of therapy. Further work is therefore needed to determine the reversibility of changes in endothelial function and disease activity due to vitamin D deficiency in SLE.

In summary we have shown that vitamin D deficiency can promote endothelial dysfunction, disruptions in neoangiogenesis, and may activate the type I IFN pathway in SLE. Vitamin D replacement may therefore offer a novel and safe strategy to reduce CVD risk in lupus. A well-conducted randomized trial is required to identify the optimal serum 25(OH)D level in this patient group and to determine the effects of vitamin D therapy on future clinical CVD events.

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Acknowledgements

Figure Legends

Figure 1: Reduced endothelial-dependent vasorelaxation and neoangiogenesis in vitamin D-deficient MRL/lpr mice

A, Reduced acetylcholine (ACh)-mediated vasorelaxation in vitamin D deficient MRL/lpr mice compared to those fed a vitamin D-replete diet. Values are the mean ± SEM for n=8 deficient and n=9 control mice, *p<0.05. **B,** Endothelial cell differentiation *in vitro* is no different in vitamin D deficient compared to replete mice (n=10 in each group). **C,** Reduced *in vivo* neoangiogenesis in vitamin D deficient MRL/lpr mice. Neoangiogenesis was quantified as the concentration of murine haemoglobin (Hb) in the subcutaneous Matrigel plug, adjusted for the plug weight (bar shows the mean of n=6 plugs from 3 deficient mice and n=7 plugs from 4 control diet mice), *p<0.05. EC_{50}=50% response contraction.

Figure 2: Autoantibody production and renal disease in MRL/lpr mice is not associated to vitamin D status in lupus-prone mice

A, Autoantibody production is not modulated by vitamin D status in MRL/*lpr* mice. The graph shows the means of the ratio of anti-dsDNA antibodies to total serum IgG for n=10 mice in each group. **B,** Urinary albumin:creatinine ratio was similar in both groups, horizontal bar shows the mean value of n=9 deficient n=8 control diet mice. **C,** Renal activity and chronicity scores were comparable in both groups, the horizontal bar shows the mean score of n=10 mice in each group. **D,** IgG and C3 complement deposition scores were similar in vitamin D deficient and replete mice. The bar shows the mean ± SEM deposition score (n=9 deficient and n=10 replete mice).

Figure 3: Vitamin D deficiency is associated with increased type-1 interferon signalling in MRL/lpr mice and SLE patients

A, Expression of all 4 ISGs was higher in the vitamin D-deficient mice. Expression of *MCP1* (relative to the MRL/MpJ control strain) was significantly increased in the vitamin D deficient mice (bar show mean ± SEM expression of n=10 deficient mice and n=10 control diet mice, *p<0.05). **B,** Expression of each ISG was higher in vitamin D deficient patients. The bars show the mean ± SEM expression (normalised to 2 reference genes). **C,** The ISG score tended increased in vitamin D deficient SLE patients compared to vitamin D replete patients. Data points show the calculated ISG score for n=10 patients in each group.
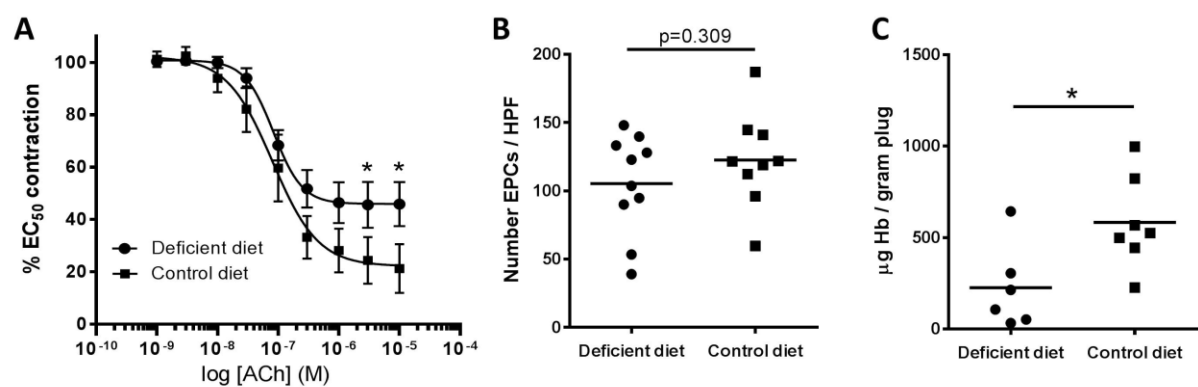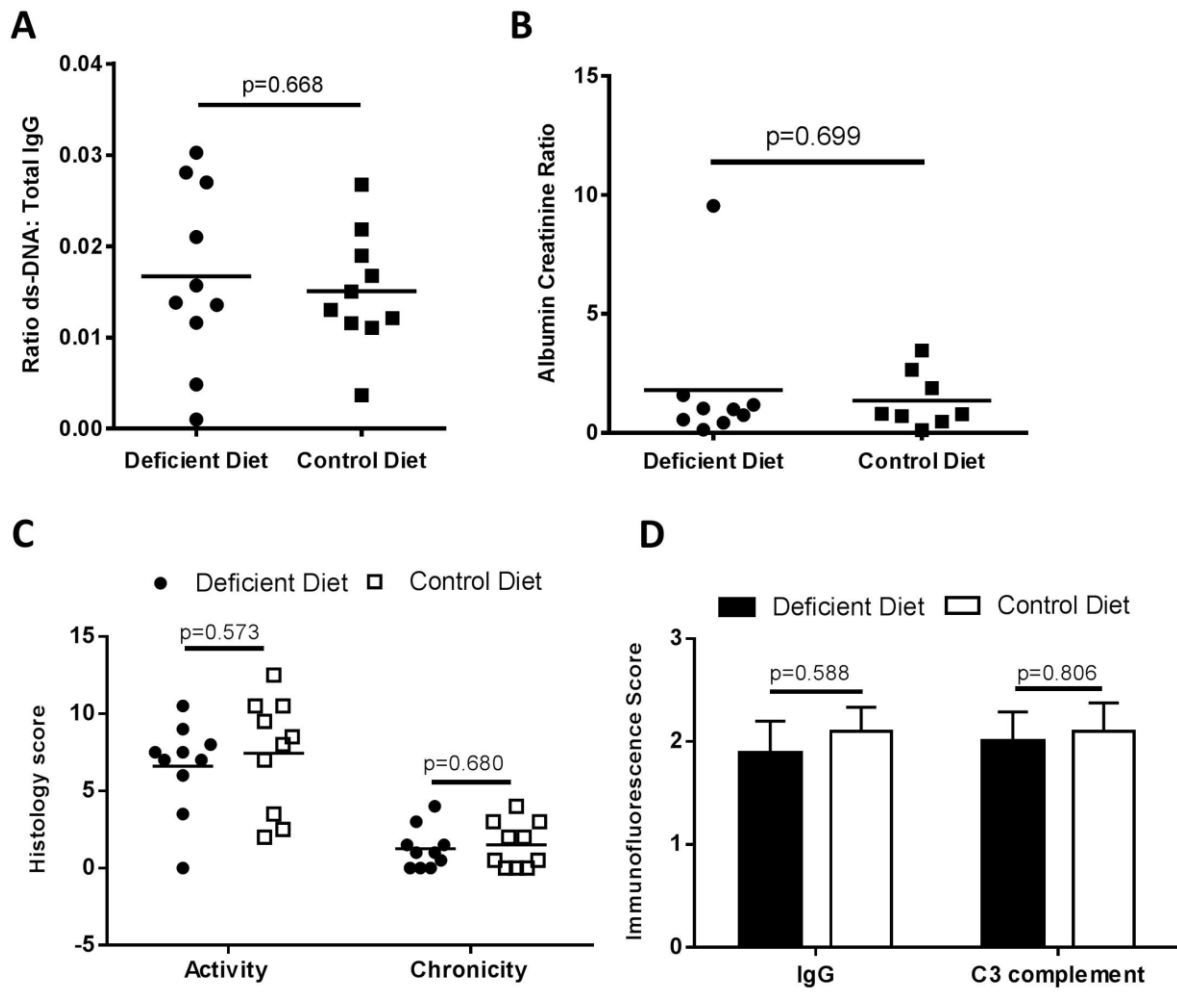
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Figure 2:

Figure 3: