

The Impact of Vitamin D Supplementation as an Adjuvant Therapy on the Abundance of Treg Cells in Patients with Atopic Dermatitis

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Abstract

Background

Atopic dermatitis (AD) is a chronic, inflammatory, and severely Pruritus cutaneous disease that has an immunologic base. The most common treatments are topical steroids, which bring many adverse side effects. Vitamin D can adjust the immune system; therefore, in this study, we investigated the effect of vitamin D on the abundance of Treg cells in AD patients.

Methods

In this interventional study, 40 subjects who completed the intervention were enrolled, including 20 AD patients (as the experimental group) and 20 healthy subjects (as the control group). Accordingly, the subjects whose vitamin D levels was less than 30 ng/ml were assigned to receive vitamin D (1000 IU) daily for a 2-month duration. The severity of AD was evaluated based on SCORAD (Scoring Atopic Dermatitis) and the amount of CD4+CD25+Foxp3+Treg cells was also assessed using Flow cytometry.

Results

Average serum level of vitamin D before performing the treatment in the experimental ($14/90 \pm 4/5$) and control groups ($15/95 \pm 5/0$) was lower than that of after the treatment (experimental group $24/60 \pm 5/2$ and control group $23/60 \pm 7/3$) ($P < 0.001$). As well, there was a significant difference between the average scores of SCORAD after the vitamin D intervention ($P < 0.001$). The amount of Treg cells significantly increased in the experimental group ($P = 0.002$, Diff=0.35) after performing the intervention.

Conclusion

it was found that besides conventional medicines, Vitamin D can be adjusted as an effective complementary therapy by modulating the immune system.

Trial registration: IRCT20150716023235N13, Registered 11 Feb 2018, <https://www.irct.ir>

Introduction

Atopic dermatitis (AD) is a chronic, refractory, inflammatory, and severely pruritic cutaneous disease, which is common among children. However, it can also occur among adults at any time, and it is often accompanied with the elevated IgE levels and personal or family atopic history. (1, 2) Almost 5-20% percent of children suffer from AD (3, 4). Of whom about 40-60% become an adult form of AD (5). In most of these patients, the disease starts before the age of 5 years old and a scant precedence in females (6, 7). The disease can be seen in any race and geographical area, but its prevalence rate in urban parts and developed countries, especially in west, is higher (8, 9).

The pathophysiology of Atopic dermatitis is complex, which may be influenced by many factors such as skin barrier disorders, genetically immune deficiency, immune response aberration to Th2, and impaired cutaneous microbial flora (10, 11). Since AD is a multifactorial disease, so it is expected to be triggered by some environmental factors in genetically susceptible individuals. As well, many studies have previously pointed out several susceptibility loci to confirm the strong genetic predisposition of Atopic dermatitis like FLG (Filaggrin) gene, which can code an important epidermal structural protein. Additionally, the “loss of function” mutations in this gene indicate a poor prognosis. In this regard, the researchers believe that as one loci mutation could increase at least 3-fold risk of developing AD, so it is the strongest risk factor for AD. (12, 13) Up to now, no definitive treatment has been found for atopic dermatitis and AD cure is centred on a systematic multilateral approach such as moisturizing the skin, topical anti-inflammation medicines or restricting inflammatory factors. Moreover, a systematic cure includes antibiotics and medicines, which are effective on immune system activity. Many different factors, including the reduced use of restrictive diets and identification of the stimulus factors are necessary to prevent the disease as well as its relapse. Although these treatments are effective, they cannot be used for a long-term because of having some potential serious side effects and systemic adverse effects. Thus, mental issues follow the stability of the disease and it is necessary to support it using a more beneficial method with less adverse effects (14).

Vitamin D is a member of a group of fat-soluble vitamins, which is largely synthesized in lower layers of skin epidermis. Moreover, it can be gathered from foods and nutritional supplements. The role of vitamin D is very complex, because it plays a vital role in normal skin physiology; immune response modulating; and regulation of keratinocytes proliferation, differentiation, and apoptosis (causes stratum corneum), and it also synthesizes a barrier that is penetrable by fat (15). In addition, vitamin D stimulates the production of cathelicidin antimicrobial peptide, which is reduced during the treatment of Atopic dermatitis. Due to the existence of nuclear receptor of vitamin D in all types of immune cells, it is clear that vitamin D is responsible for expressions of genes, which can reproduce dendritic cells, macrophages, and other antigen presenting cells. Vitamin D also inhibits the syntheses of both IL-12 and IL-23, which reduce the number of Th1 cells as a result. Furthermore, this vitamin could increase the expression of some cytokines like IL-10 or differentiate the Treg cells, which consequently lead to the decreased allergic reactions (16, 17). Considering that the pathogenesis of Atopic dermatitis includes a number of disorders in epidermal barrier dysfunction or the impaired immune responses, and given the fact that vitamin D is a regulatory factor for both of these processes, so there is a possibility that serum levels of vitamin D and the severity of Atopic dermatitis may be correlated (18, 19). Therefore, the present study aimed to investigate the effects of vitamin D on clinical symptoms and the abundance of Treg cells among patients suffering Atopic dermatitis Display quotations of over 40 words, or as needed.

Methods

Ethical approval and recruitment

This intervention study was approved by the Ethics Committee of Mashhad University of Medical Sciences and also registered in the International Clinical Trials Registry Platform with the reference code of IRCT20150716023235N13. This was performed at Qaem (Aj) Hospital of Mashhad University of medical sciences from November 2017 to May 2018. Written informed consent forms were obtained from the patients or their parents before the initiation of the study.

Patient population

Atopic dermatitis patients' ages were between 1 month and 50 years old. Two cc venous blood sample was taken from each subject to identify the serum levels of vitamin D, and then those with vitamin D deficiency were selected for the study (Vitamin D deficiency level was set at lower than 30 ng/ml).

Those that disused edible and topical medicines to control Atopic dermatitis were enrolled in the experimental group. They were then checked in terms of the inclusion criteria. As well, existence of either other allergic diseases or other cutaneous diseases except Atopic dermatitis, a history of inflammatory autoimmune diseases (specially asthma, noticeable cardiovascular disease, autoimmune disease, malignancy, mental disorders or other chronic disease), using any systemic or topical Corticosteroids or calcineurin inhibitor as well as vitamin D for at least 2 months, and existence of any infectious or other cutaneous diseases were considered as the exclusion criteria

The inclusion criteria for entering the control group were as follows: Patients without Atopic dermatitis aged between one 1 month and 50 years old, those with low serum levels of vitamin D (lower than 30 ng/ml), and disuse of edible medicine for a specific disease. Moreover, the exclusion criteria for the control group were the existence of an allergic disease; a family history of Atopic dermatitis; any noticeable cardiovascular disease, autoimmune disease, malignancy, mental disorders or other chronic disease); usage of any systemic or topical Corticosteroids or calcineurin inhibitor and vitamin D for at least 2 months; and existence of any infectious or other cutaneous diseases.

Intervention

Vitamin D (1000 IU) pill was prescribed from Vitabiotics Company (in order to homogenize the medicine's dosage and formulation's impacts on its effectiveness). In this regard, the patients (aged more than 2 years old) received vitamin D 3 1000 IU/day pill , and children aged under 2 years old received oral drop 1000 IU.

In this study, the severity of eczema was evaluated using the commonly used SCORAD classification for the patients in the experimental group. As well, the amount of the administered medicine was recorded and 2 cc of Heparin venues blood was then taken. Moreover, the cytokines' serum concentrations such as the percentage of CD4+CD25+Foxp3+ and Treg cells, were determined by the flow cytometric method. Later, all the included subjects aged more than 2 years old were given 60 pills of vitamin D 1000IU and a vitamin D 1000 IU oral drop was also given to the subjects aged under 2 years old. Notably, the subjects of both groups received 1 ml of the oral drop and a pill daily for a 2-month duration. After this period, it

was the time for the second visit, so 2 cc of heparin blood was taken to identify the amount of CD4+CD25+Foxp3+Treg along with 2 cc for the assessment of serum levels of vitamin D.

Laboratory Tests

The vitamin D status was determined using the ELISA (eBioscience) Kit and the abundance of regulatory T cells was also determined by the flow cytometric method. Firstly, in order to isolate T lymphocytes, total blood sample was poured into a test tube with a ratio of one volume of ficoll, which was then centrifuged for 30 minutes at 2500 RPM. Thereafter, cellular cloud was carefully separated. One million counted cells in the volume of 100 microliter are needed for each test tube. Two tubes are usually needed to identify regulatory T cells. Accordingly, one of the tubes are needed to identify regulatory T cells and another is a control sample or IgG2a K isotype control. Afterward, the following steps are performed:

1. 100 microliters of cells were added to each Flow cytometry tube. Later, 10 microliters of each surface antibody CD4 FITC and CD25 PE were added, and the tube was incubated for 30 minutes at 4 ° C in darkness. (this step was only applied on the test tube)
2. One cc of cold staining buffer was then added to the cells and after centrifuging for 5 minutes at 1200 RPM, the upper liquid was disposed.
3. One ml of a fresh diluted Fixation/Permeabilization buffer was added on sediment of the last step with a ratio between 1 and 9. Afterward, the tubes were incubated for 30 minutes at 4 ° C in darkness. (this step was applied on both the test and control tubes)
4. Two ml of fresh Permeabilization buffer was added to the tube samples from the last step, which were prepared with a ratio between 1 and 3 and it was centrifuged for 5 minutes at 1200 RPM. This step was repeated twice.
5. Ten microliter of Foxp3 PE CY5 antibody was added to the test tube after making the cells penetrable, and then 10 microliter of the control isotype IgG2a K was added to the control tubes. The samples were incubated for 30 minutes at 4 ° C in darkness.
6. Two ml of Permeabilization buffer was added to all the tubes, which were centrifuged for 5 minutes at 1500 RPM. This step was repeated twice.
7. The upper liquid was disposed. The cells were formed into the suspension state and then read by Flow cytometry device. As well, positive CD4 cells were chosen and then the percentages of positive CD25 and positive Foxp3 were calculated in the gate of positive CD4.

In the picture below (Fig1), Foxp3 is shown with the PE color(20):

Assessment of AD Severity

SCORAD is a system consisted of the following 3 parts: involvement range, severity, and subjective symptoms, which are known as diagnostic factors for the quality of life. Range of involvement is based on the rule 9, which is done in terms of the burn measurement (Berkow 1924). Disease's severity items include 6 criteria as follows: erythema, edema/papulation, oozing/crust, excoriation, lichenification, and skin dryness. In order to measure the severity, a part of the body, as a demonstrative of all the patient's lesions, is chosen for each item. Therefore, parts of the body with the highest involvement rates are not measured (European Task Force on Atopic 1993). In this regard, skin dryness must also be measured and then scored under normal conditions, not when acute lesion or lichenification happens. Correspondingly, each item is given a score of 0 to 3. (Absence of lesion = 0, mild = 1, moderate = 2, and severe = 3). Subjective symptoms, and itching and sleeping disorders were determined based on the previous 3 days and nights by the patient or the patient's parents. Accordingly, these two are scored in a scale ranged from 0 to 10. Ten is for the most severe state experienced by the patient. Later, these scores were evaluated online or by SCORAD (21).

Statistical Analysis

The present feasibility work is related to a randomized pilot study; therefore, no formal sample size calculation was undertaken. However, a proposed sample size as 40 (20 subjects per each group) was considered as an adequate size to meet the objectives of the study. All the statistical analyses were done using SPSS Statistics version 16. The mean and standard deviation were also used to describe quantitative data and tables or graphs were used for qualitative data. In addition, Chi squared test was used to compare qualitative data, and T test Mann-Whitney was applied to compare independent quantitative data considering the variable distribution. Additionally, Paired T test and Wilcoxon test were used to compare quantitative data once before and once after the test. P-value as 0.05 was considered statistically significant in all tests.

Results

Quantitative data were checked in both groups (the experimental and control) using One-Sample KS test, and variables had normal distribution ($P > 0.05$). Of 20 cases in the patients' group, 9 cases were men (%45) and 11 cases were women (%55) with average age of 14.85 ± 14.8 years old). As well, of 20 cases of the control group, 9 were men (%45) and 11 were women (%55) with average age of 15.47 ± 14.5 years old). No significant difference was found in terms of age distribution between the two groups ($P > 0.99$). During the study, with proper justification and follow-ups, fortunately we had no drop in any of the study groups. Age and sex distributions in both groups are shown in table 1.

Table 1: Age distribution

	Cases (n=20)	Controls (n=20)	PV
Age, mean±SD	14.85±14.8	15.47±14.5	0.89
Sex, n (%)			
Male	9 (45%)	9 (45%)	> 0.99
Female	11 (55%)	11 (55%)	

The mean and standard deviation of the measured serum levels of vitamin D was 14.90±4.5 before the intervention in the experimental group and 15.95±5.0 in the control group. Additionally, no significant difference was observed between the two groups. As well, the mean and standard deviation of serum levels of vitamin D was 24.60±5.2 by passing two months from the intervention in the experimental group and 23.60±7.3 in the control group. As well, no significant difference was found between the two groups (P= 0.62). The measured serum levels of vitamin D before and after the intervention were examined in both groups by Paired-T test. The results showed a significant difference in serum levels of vitamin D before and after the intervention in both groups (P <0.001) (Table 2).

Table 2: Average level of serum levels of vitamin D in control and experimental groups before and after intervention

	Cases (n=20)	Controls (n=20)	PV
Vit D. (Before intervention)	14.90±4.5	15.95±5.0	0.49
Vit D. (After intervention)	24.60±5.2	23.60±7.3	0.62

Severity of the disease before and after the intervention was evaluated using SCORAD scoring system (Scoring Atopic Dermatitis). The maximum score before the treatment was set at 65.7 and the minimum one was 16.1. Moreover, 36.90±16.4 was estimated as the mean and standard deviation. These scores after performing the intervention were obtained as 56.7, 6, and 24.70±14.9, respectively. As shown in table 3, the results of Paired T-test reported that there was a significant difference in SCORAD before and after the intervention (P<0.001).

Table 3: Average SCORAD in the experimental group before and after the intervention

	Mean	Std. Deviation	PV
SCORAD			
Before intervention	36.90	16.4	<0.001
After intervention	24.70	14.9	

Severity of Atopic dermatitis in the patients was categorized into the following 3 groups: Mild (Less than 25), Moderate (25 to 50), and Severe (Higher than 50) (46). Correspondingly, this severity distribution in the experimental group is shown in table 4.

Table 4: Severity distribution in experimental group before and after the intervention

		Mild	Moderate	Severe
SCORAD	After intervention	7 (35%)	7 (35%)	6 (30%)
	Before intervention	12 (60%)	7 (35%)	1 (5%)

Severity alternations of the disease based on SCORAD scoring system before and after performing intervention for each individual patient are shown in Fig.2.

The mean and standard deviation of serum levels of vitamin D in the experimental group among the patients with low, average, and severe levels of disease before and after the intervention measured by ANOVA test, was significant. (P= <0.001) (Table 5).

Table 5: Average and standard deviation in serum levels of vitamin D based on severity of the disease in experimental group before and after intervention

experimental group	Mild	Moderate	Severe	pv
After intervention	26.00±2.6	24.00±6.5	12	0.02
SCORAD Before intervention	18.00±3.0	16.57±2.9	9.33±1.9	<0.001

The cytokines serum (CD4+CD25+FoxP3+ (Treg) cells) concentration in both groups before and after the intervention was determined using the flow cytometric method. Average percentages of Treg cells in the experimental group before and after the intervention were obtained as 0.356±0.23 and 0.705±0.41, respectively. Accordingly, in the control group, these were obtained as 0.516±0.29 and 0.663±0.30 after the intervention, respectively. Due to these results, Treg cell's percentage raised in both groups. These changes were significant over time in the experimental group, which was done by the Paired T-test. (P= 0.002). However, there was no significant difference in the control group (P= 0.164). The amounts of Treg cells before and after the intervention were compared in both group, and no significant difference was found in this regard. (P= 0.067, P= 0.716) (Table 6).

Table 6: Average and standard deviation of Treg cells in control and experimental group before and after intervention

	Treg %(before intervention)	Treg. %(after intervention)	P
experimental group	0.356±0.23	0.705±0.41	0.002
Control group	0.516±0.29	0.663±0.30	0.164
P	0.067	0.716	

Discussion

Although many studies were done to examine the effects of vitamin D on AD so far, to the best of our knowledge, no study examined the effects of vitamin D on Treg cells in AD patients up to now. In our study, average serum levels of vitamin D before the intervention was found to be lower in the experimental group compared to the control group (14.90±4.5 vs. 15.95±5.0), but it had no significant difference (P= 0.49). In Sharma et al.'s study in 2017, average serum levels of vitamin D in AD group (30.38) was lower than that of the control group (53.46) (22). Additionally, in the Wang, El Taieb, and Han's studies, the average serum levels of vitamin D in the experimental group was lower than that of the control (23-25). However, in the Samochocki and Su's study, no significant difference was noticed, which was in line with our study (26, 27).

In our study, serum levels of vitamin D had no significant difference before the intervention in both groups (P= 0.49); however, there was a significant relationship between the statue of serum levels of vitamin D and SCORAD before and after the intervention (P<0.001 and P= 0.03). In this regard, the patients with lower vitamin levels had higher disease's severity. In 2013, El Taeib et al. showed that lower vitamin D level could lead to higher severity of disease in patients (23). These observations were also proven in the Udompataikul, Han T. Y, Ronceray, and Shin's studies (24, 28-30). But in the studies by Robl in 2016, Chiu in 2013, and Lee S. A in 2013, with the greater size of samples (105, 94, 157), no significant difference was reported between serum levels of vitamin D and SCORAD (19, 31, 32). These differences may probably be caused by some factors such as race, diet, sex, obesity, weather, and geographical area. In the Tsotra, Norizoe, and Sidbury's studies, after performing the intervention with vitamin D, they observed no significant difference in the severity of AD as determined by the SCORAD index. (P= 0.47, 0.38, >0.05) (33-35) However, in most of the studies similar to ours, the severity of AD was reported to have a reduction after the intervention. In another study done in the USA in 2015, Di Filippo et al. examined 39 Atopic dermatitis patients and 20 healthy subjects after 3 months of daily treatment with 1000 universal units of vitamin D. As a result, serum levels of this vitamin increased after the intervention. (29.41±10.73 vs 22.97±8.03 P=0.01) (36). In line with the above-mentioned studies, in our study, after 2 months of treatment with daily vitamin D IU1000 pills, an increase was observed in serum levels of vitamin D in both the control and experimental groups (P<0.001). The only study that had examined the percentage of Treg cells and serum levels of vitamin D among AD patients and controls, was the Lipinska's study in 2017 in which no intervention was applied. Accordingly, in this study, 19 Atopic dermatitis patients and 17 healthy

subjects were selected and the amount of Treg cells in AD group was calculated as 0.41 ± 0.2 , and in healthy group, it was 2.81 ± 2.1 .

The researches demonstrated that the amount of Treg cells in AD patients was lower than that of the healthy subjects ($P < 0.00001$) (37). In this study, even though the percentage of Treg cells in the experimental group was lower, it was not significant ($P = 0.067$). However, the abundance of these cells before and after the intervention in the AD group ($P = 0.002$) significantly altered, which is justifiable by considering AD background of the patients as well as variations in specific genes.

Conclusion

Besides conventional medicines and the routine treatments, vitamin D can be adjusted as a helpful and effective complementary therapy. As well, it is a cheap and accessible way used in order to control the symptoms of atopic dermatitis and to lower the symptoms by modulating the immune system. Additionally, due to its effect alongside topical Corticosteroids, low price, and few side-effects, it can be known as a proper option.

Declarations

Competing interests

The authors report no conflicts of interest for this work.

Ethics approval and consent to participate

The present study was approved by Research Council of Mashhad University of Medical Sciences (Grant Number: 951754). The ethics committee approval code number is (IR.MUMS.fm.REC.1396.607). This study has been registered in Clinical Trial Registry with the code of IRCT20150716023235N13. Informed written consent was obtained from the parents and all the procedures performed in the study involving human participants, were in accordance with the ethical standards.

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Consent for publication

All the authors gave consent to publication of the manuscript, in case of its acceptance of Clinical and Molecular Allergy journal.

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Availability of data and materials

All materials and data are available to publication

Authors' contributions

PP, FJA, MK, MR, and PL contributed to the conception and design of the study. PP, FJA, MR participated in data collection from the participants and laboratory analysis. PP, FJA, FI, MS supported the analysis and interpretation. FJA, MK, MR supervised the study. PP, FJA, FI participated in drafting and editing the manuscript. All authors read and approved the final manuscript.

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Figures

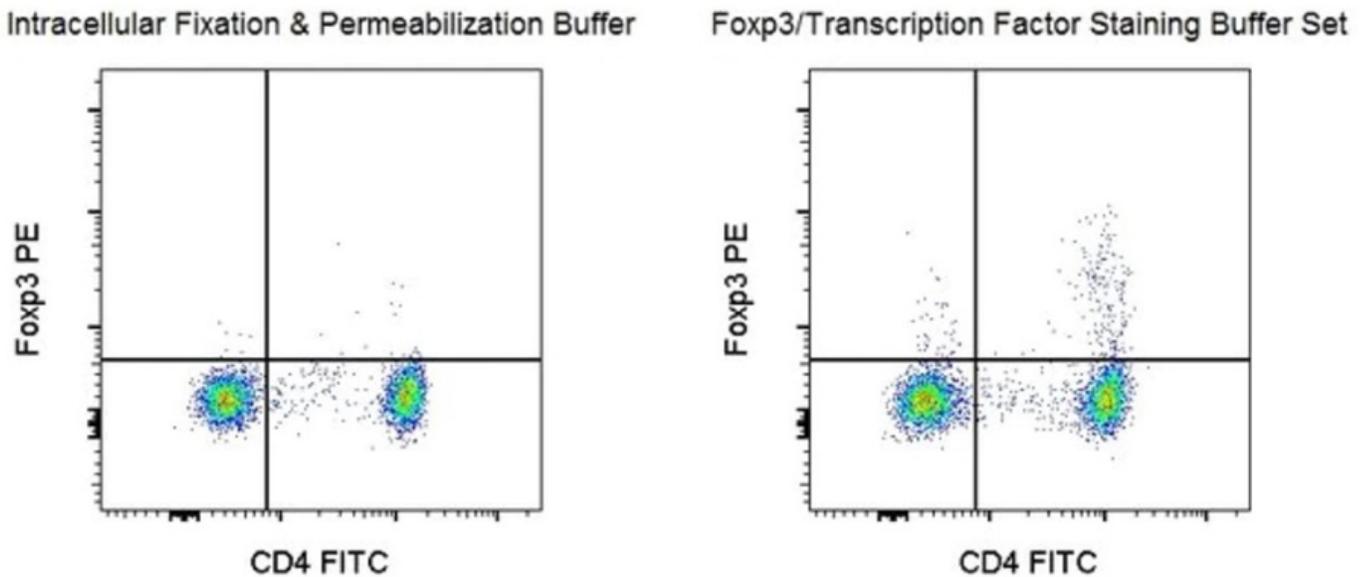


Figure 1

Human Foxp3 detected using Foxp3/Transcription Factor Staining Buffer Set Normal human peripheral blood cells were intracellularly stained with Anti-Human CD4 FITC and Anti-Human Foxp3 PE (clone 236A/E7) using the eBioscience Intracellular Fixation & Permeabilization Buffer Set and protocol (left) or the eBioscience Foxp3/Transcription Factor Staining Buffer Set and protocol (right). Staining for this

transcription factor is observed only when using eBioscience Foxp3/Transcription Factor Staining Buffer Set and protocol (20).

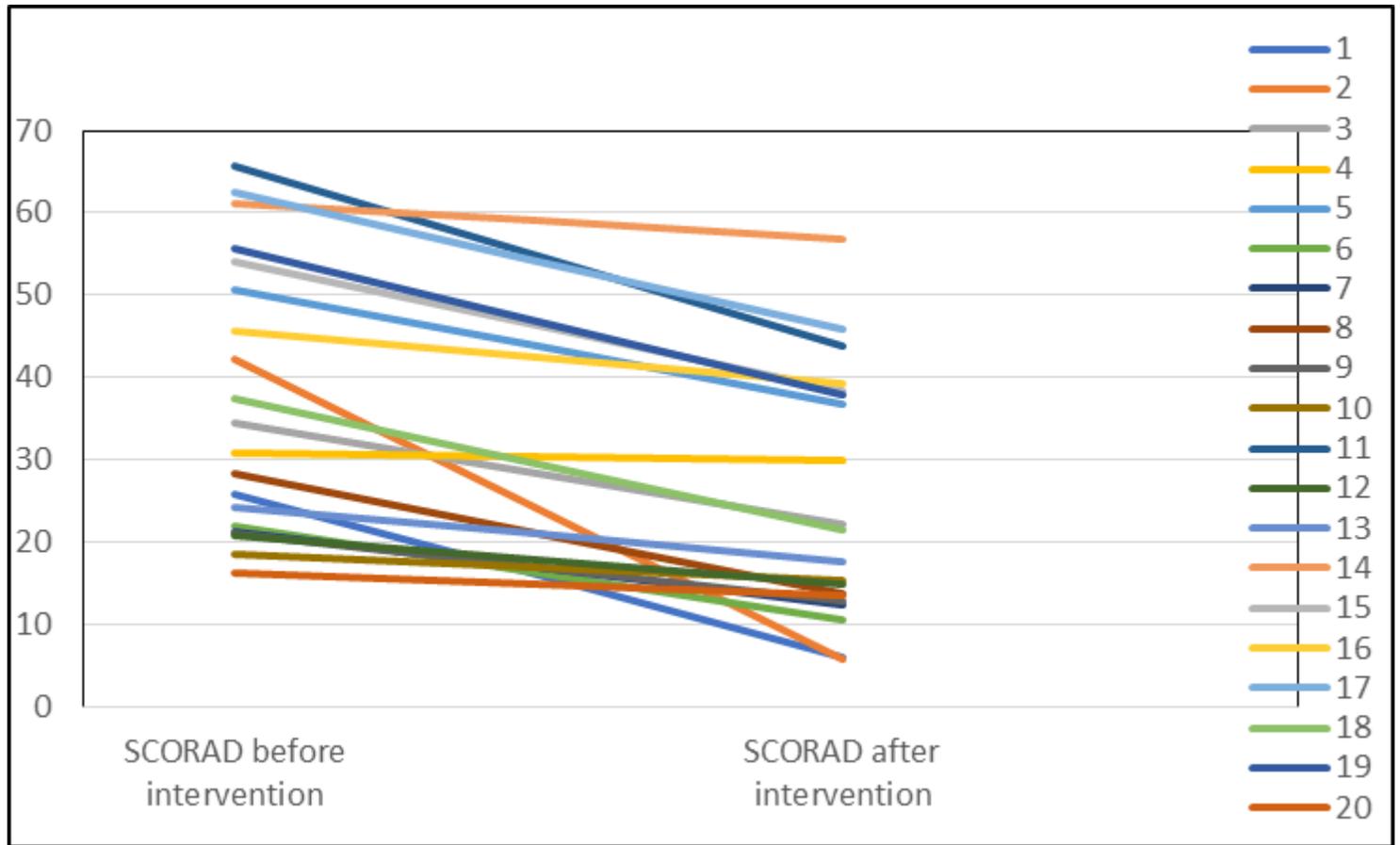


Figure 2

Severity alternations of the disease for each patient before and after the intervention based on SCORAD

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