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Review Article

Evidence of Immunological Abnormalities in Sitosterolemia

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Received: September 07, 2021; **Accepted:** October 07, 2021; **Published:** October 14, 2021

Abstract

Objectives: Mutations involving the ABCG8 gene cause sitosterolemia (STSL), a rare metabolic disease due to an abnormal plasmatic plant sterols level. STSL hematological findings include stomatocytic hemolysis and macrothrombocytopenia. So far, few cases of leukopenia have been reported and there are no studies that consider inflammatory activity. The purpose of our study is to evaluate immunological abnormalities in STSL and the effect of dietary and pharmacological treatment.

Study Design: In a sitosterolemic patient presenting leukopenia and increased inflammatory indexes were evaluated *in vitro* interleukin 1B secretion from monocytes and type 1 interferon (IFN) signature. Besides, Stimulation Indexes (SI) of patient's and two Healthy Controls' (HCs) T-helper lymphocytes in response to proliferative stimuli were assessed, when incubated in enriched medium alternately with patient plasma or with AB Rh^+ group control plasma. SI (compared to the same HCs) and IFN signature have been evaluated again three (T3) and six (T6) months apart, after patient received therapy.

Results: Following treatment with Ezetimibe (EZE) and adequate diet, lower plasmatic plant sterols levels and progressive improvement of hematological and immunological abnormalities were observed. These data were confirmed by the negativization of IFN signature at T3 and by the progressive normalization at T3 and T6 of SI in patient's and HCs' T-helper lymphocytes incubated in patient's plasma-enriched medium.

Conclusion: High plasmatic phytosterols levels due to STSL seems to affect white blood cells proliferation and trigger pro-inflammatory process, justifying leukopenia and increased phlogosis indexes observed in our patient. These hypotheses are supported by normalization of clinical and laboratory data during EZE therapy.

Take-home Message: Immunological abnormalities should be considered among the hematological manifestations of sitosterolemia, moreover high levels of plasma phytosterols could be a pro-inflammatory trigger. Treatment with ezetimibe seems to reverse these manifestations.

Keywords: Sitosterolemia; Inflammation; Plant sterols; Immunological abnormalities; Type I interferon signature; ABCG8

Abbreviations

Hb: Hemoglobin; PLT: Platelet Count; MPV: Mean Platelet Volume; WBC: White Blood Cells; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; CRP: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate

Background

The mechanism of intestinal mucosa transporters and regulating systems of plant sterols' absorption have been recently described. Physiologically, plasma levels of phytosterols are controlled by a balance between the absorption and efflux systems represented by the transporters sterolin-1 and -2 [1]. These efflux pumps, expressed on enterocytes and biliary canalicular membranes, contain ATP Binding Casette (ABC) proteins encoded by ABCG5 and ABCG8 [2]. Homozygous or compound heterozygous mutations of these genes lead to a rare disease named Sitosterolemia (STSL), characterized by an excessive accumulation of phytosterols, especially β-sitosterol. Clinically it presents huge phenotypic heterogeneity from completely asymptomatic forms to advanced atherosclerosis and premature cardiac death [3,4], Patients are also known to present immunological abnormalities and signs of systemic inflammation [5]. Alteration of lipid metabolism might influence inflammatory pathways, the most relevant example being deficit of sterol synthesis pathway due to mevalonate kinase deficiency, an autosomal recessive inherited metabolic disease clinically characterized by recurrent fever and elevation of inflammatory markers due to dysregulation of the inflammatory cytokine secretion interleukin 1 beta (IL-1 β) in monocytes [6]. Of note, macrophage activation via toll-like receptors (TLR) 4 agonist is modulated by β –sitosterol [7].

Characteristic hematologic manifestations of STSL include stomatocytic anemia, macrothrombocytopenia, splenomegaly and abnormal bleeding [8]. So far, only anecdotal cases of leukopenia have been reported [9] and there are no studies that consider inflammatory activity, which is often described in patients with STSL. Treatment with Ezetimibe (EZE) allows reduction of plasma levels of phytosterols and so the normalization of platelets count, Mean

Citation: La Rosa A, Ivaldi F, Penco F, Bocca P, Volpi S and Di Rocco M. Evidence of Immunological Abnormalities in Sitosterolemia. Austin Med Sci. 2021; 6(2): 1053.

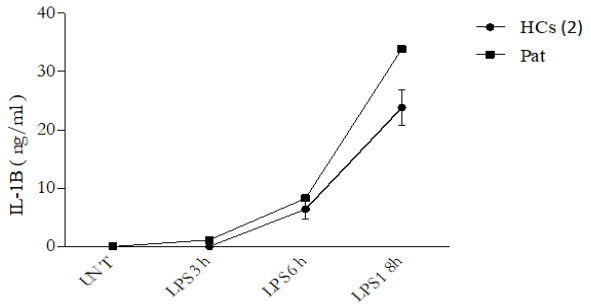

Figure 1: Dosage of IL-1 β (ng/ml) secretion by macrophage after *in vitro* stimulation of TLR4 using LPS at three, six and eighteen hours respectively in Pat, before therapy, and in two HCs. Data shows a moderately increased IL-1 β production by the patient's macrophages, suggestive of higher inflammatory activity. UNT: Untreated; IL-1 β: Interleukin 1 beta; TLR4: Toll-Like Receptor 4; LPS: Lipopolysaccharide; Pat: Patient; HCs: Healthy Controls.

Platelet Volume (MPV) and Hemoglobin (Hb) levels [10], improving prognosis and long-term outcome [11].

The aim of our study is to focus on immunological abnormalities in sitosterolemia and the reverse after dietary and pharmacological treatment.

Study Design

A 17-year-old patient presenting a history of mild normocytic anemia and thrombocytopenia, hypergammaglobulinemia, hyperferritinemia, increased inflammatory indexs, arthralgias, partially responsive to analgesic therapy, and xanthomas on the extensor surfaces of joints was evaluated for STSL. Diagnosis was genetically confirmed by the presence of homozygous variant c.490 C> T p. (Arg164) of ABCG8 gene causative for STSL [12]. After obtaining informed consent, the patient was enrolled in the study. At diagnosis (T0) Hb, Platelet count (PLT), MPV, White Blood Cells (WBC), lipid profile and analysis of plasmatic phytosterols were evaluated.

To investigate the inflammatory state, C Reactive Protein (CRP), Erythrocyte Sedimentation Rate (ESR), peripheral blood type I interferon (IFN) signature and *in vitro* IL-1 β secretion from monocytes were evaluated. IFN signature [13] and *in vitro* IL-1 β secretion [14] have been performed as described. Briefly, for IFN signature, RNA was extracted from peripheral blood collected in PAXgene tubes using the PAXgene Blood RNA kit (Qiagen, Hilden, Germany). cDNA was retrotranscribed using SuperScript® VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, California, USA). Selected IFN-stimulated gene (*IFI27, IFI44L, IFIT1, ISG15, RSAD2, SIGLEC1*) expression was quantified by real-time PCR using gene-specific primers and probes (Roche) with the ddCt method relatively to a healthy donor calibrator using *HPRT* and *G6PD* as reference genes.

Peripheral Blood Mononuclear Cells (PBMCs) were harvested by density gradient from blood collected in heparin tubes. To assess IL-1 secretion, monocytes were isolated by cell adherence after one-hour incubation in 24 well plates and stimulated with Lipopolysaccharide (LPS) 100ng/ml; supernatants were collected before and after three, six and eighteen hours of stimulation; IL-1 β was measured by ELISA (R&D). To study lymphocyte proliferation, PBMCs were stained with Carboxyfluorescein Succinimidyl Ester (CFSE) [15]. 400.000 PBMCs were dispensed on a 96 well plates. Patient's PBMCs were incubated in complete RPMI 1640 medium enriched alternately with either 10% of the patient's plasma or 10% of group AB Rh^+ control plasma and stimulated with phytohaemagglutinin (PHA 1µg/mL, Sigma, St. Louis, MO) or anti-human CD3 and anti-human CD28 soluble (5µg/mL each, BD, USA). This procedure was performed simultaneously on two Healthy Controls' (HCs) PBMCs, selected by age and blood group homologous to the patient.

After four days of incubation, flow cytometry analysis was used to assess patient and HCs T-helper lymphocyte proliferation in response the different plasmas, using surface markers αCD3 (BV 420, BD, USA), αCD4 (APC, BD, USA) αCD8 (PE-Cy7, BD, USA) [16] The Stimulation Index (SI) was estimated as ratio of proliferated cells to unproliferated as a measure of CFSE response [15] This procedure was performed to assess the interference of high levels of circulating phytosterols with cell proliferation.

Blood tests, plasma plant sterols levels, SI (compared to the same HCs) and IFN signature have been valuated again three months (T3) apart, after patient received dietary and EZE therapy. SI and IFN signature were repeated one more at six months (T6) of treatment.

Results

We have analyzed hematological response to the dietetic and pharmacological treatment in a patient with a new diagnosis of STSL. At baseline, *in vitro* secretion of IL-1 β by monocytes following TLR4 stimulation was moderately increased compared to HCs (Figure 1). Table 1 reports the hematological findings at baseline and their variations at T3 and T6 after starting therapy with EZE. As expected, we assessed normalization of Hb and LDL, with progressive increase in WBCs count and reduction in plasmatic phytosterols about of 26% and inflammatory indexes (CRP and ESR).

Activation of type I IFN pathway was high in patient's peripheral blood as assessed at baseline and underwent complete normalization

Table 1: Patient hematological findings at baseline (T0) and after three (T3) and six (T6) months on Ezetimibe therapy.

	T0	T3	T6
Hb (g/dL)	11.9	14	14
PLT (10^3/mL)	107	84	107
MPV (fL)	15.3	16	15
WBC (10^3/mL)	3.04	3.1	4.2
Lymphocyte(10^3/mL)	0.59	0.9	0.9
HDL (mg/dL)	46	43	54
LDL (mg/dL)	308	96	69
Campesterol (µmol/L)*	176	127	/
Stigmasterol (µmol/L)**	19	14	/
Sitosterol (µmol/L)***	426	312	/
CRP (mg/dL)	11.4	1.6	1
ESR (mm/h)	47	6	9

*Reference Range 0 - 13 µmol/L; **Reference Range 0 - 4 µmol/L; ***Reference Range 0 - 29 µmol/L.

Abbreviations: Hb: Hemoglobin; PLT: Platelet Count; MPV: Mean Platelet Volume; WBC: White Blood Cells; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; CRP: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate.

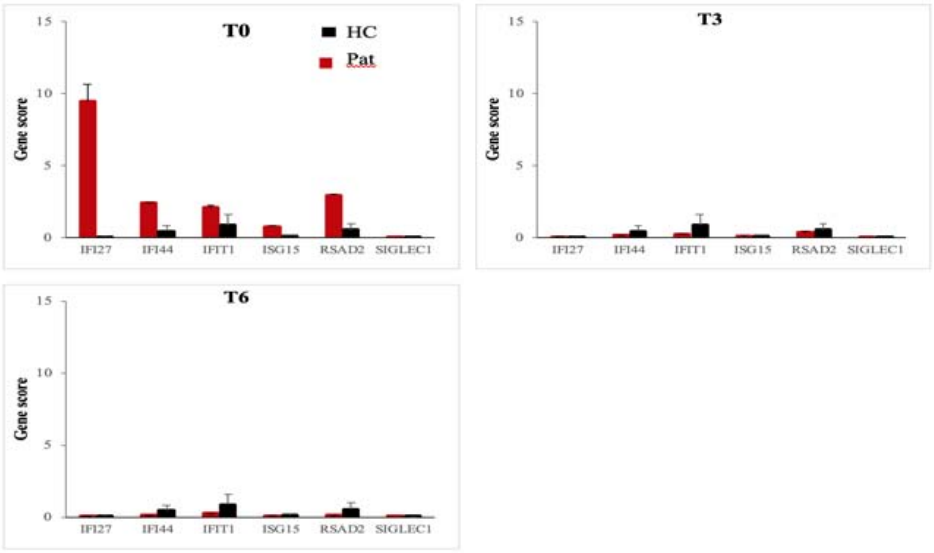

Figure 2: Peripheral blood type I interferon signature assessment shows increased expression of the indicated interferon stimulated genes in the Pat compared to HCs (n=30) before therapy (T0), and their complete normalization after 3 (T3) and 6 (T6) months of therapy. Pat: Patient; HCs: Healthy Controls.

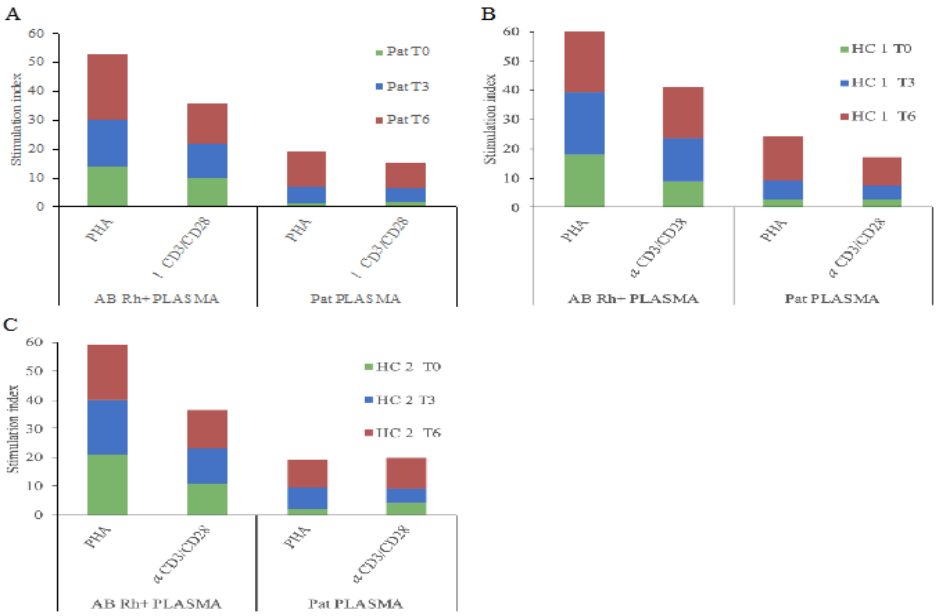

Figure 3: Comparison of SI in response to PHA and αCD3/CD28 proliferative stimuli measured by CFSE intensity performed on flow cytometry Fortessa LSR, Becton & Dickinson (BD) using the following BD antibodies: αCD3 (BV 420), αCD4 (APC) αCD8 (PE-Cy7). (A) Proliferative response of the STSL Pat lymphocytes stimulated in medium respectively enriched with 10% of control AB Rh+ plasma and Pat plasma, evaluated at T0, T3 and T6. It is noticeable the progressive SI normalization of cells incubated with Pat autologous plasma during therapy. (B and C) SI of T-helper lymphocytes of two HCs selected by age and blood group homologous to the Pat, cultured in medium alternately enriched with control plasma or Pat plasma. As well, there is a progressive SI normalization of lymphocytes incubated with Pat plasma during therapy, suggesting an interference in the proliferation due to plasmatic phytosterols concentration. SI: Stimulation Index; PHA: Phytohaemagglutinin; CFSE: Carboxyfluorescein Succinimidyl Ester; Pat: Patient; STSL: Sitosterolemia; T0: Time at Diagnosis; T3 and T6 respectively time at three and six months of treatment; HCs: Healthy Controls.

following therapy at T3 and T6, confirming the reduction of the background inflammatory state (Figure 2).

Figure 3 reports patient's and HCs' lymphocytes response to proliferative stimuli (PHA and αCD3/CD28) assessed by SI. No significant differences were found in SI of patient's and HCs' PBMCs incubated with AB Rh+ plasma at different time points. Interestingly, patient's and HCs' PBMCs incubated in patient's plasma-enriched medium do not proliferate at T0, present a slight response at T3, and show substantial normalization of proliferation at T6.

Discussion

STSL is a rare disease and diagnosis can be challenging for clinicians [17]. Hematological presentation commonly described

includes stomatocytic anemia and macrothrombocytopenia [8]. Interestingly, our patient presented also leukopenia, particularly lymphocytopenia, and high inflammation indexes.

Assuming a correlation between phytosterol accumulation and these immunological abnormalities, we evaluated the response to treatment with EZE, since it is described in literature reduce the circulating plants sterols levels and increasing PLT and Hb [10]. Indeed, a progressive normalization of blood exams was stated during treatment, in particular we noted increase in WBCs and reduction in phytosterols, ESR and CRP. These data have been further investigated by the experiments performed. Negativization of IFN signature at T3 suggests high sensitivity of pro-inflammatory mechanisms to small variations in phytosterol levels. These results are in according to Berdiel et al. [18] that describe an increase of interleukin 2 and IFN-$_{\gamma}$ secretion in apolipoprotein E–deficient mice lymphocyte in presence of 2% dietary plant sterol supplementation.

The impact of altered serum lipid composition on inflammatory events is well described [19] Abnormal integration of lipids into the cytoplasmic membranes seems to warp lipid rafts and to alter surface receptors response such as TLRs activity [7,20]. Normally, TLRs trigger inflammatory cascade increasing IFN production, consequently to pathogen- and damage-associated molecular patterns mediated activation (PAMPs and DAMPs respectively) [21]. Our data support these hypotheses, suggesting that accumulation of circulating plant sterols due to ABCG5 or ABCG8 mutations and their incorporation in the lipid membranes could contribute to the state of basic inflammation.

Besides, integration of phytosterols in RBCs and PLTs membranes has been described and it is supposed cause their typical morphological anomalies [4]. Thus, plants sterols could be accumulated in the membranes of the whole organism including WBCs. Absence of response to proliferative stimuli demonstrated both in patient's and two HCs' PBMCs incubated with the patient's plasma (rich in phytosterols) at T0 and T3, suggests that phytosterols interfere with cells replication mechanisms. A SI comparable to the one obtained in PBMCs incubated in medium enriched with AB Rh^{+} plasma was found at T6, suggesting that, unlike the inflammatory response, lower levels of phytosterols are already sufficient to interfere with the homeostasis of the membranes.

Our study has several limitations. First, results should be validated on a larger number of patients. Nevertheless, the rarity of the pathology represents an intrinsic limitation to these types of studies. Second, we could not evaluate *in vitro* plant sterols' concentration that inhibit lymphocytes proliferation. Finally, we do not exclude that leukopenia could be secondary to production of soluble factors due to inflammatory state. However, we know that inflammation is closely related to the excess of plasmatic phytosterols, thus we can assume as indirectly mediated by plant sterols itself.

In conclusion, we suggest that leukopenia and inflammatory status are other STSL immunological manifestations that clinicians should consider. EZE has proven effective in reducing inflammation and promoting the increase of WBCs both clinically and *in vitro* experiments. Data support that these hematological abnormalities are consequent to high levels of circulating phytosterols. Nowadays, phytonutrients are often recommended, attributing to phytosterols anti-inflammatory properties [2,22], but we have to be aware that an excess can induce a paradoxical effect [18].

Declaration

Author Contributions: DRM and VS were the clinicians primarily responsible for the patient's care. LRA, IF and VS planned the study design. IF, PF e BP were involved in laboratories experimentation and data analysis. LRA prepared the draft of the article; DRM and VS reviewed it. All the authors approved the final submitted version. No previous similar article was found, and We declare not simultaneous publications.

Funding: Funding were obtained from the Italian Ministry of Health

Informed consent: Written consent to blood samples collection, for experimentation and publish were obtained from the tutor of the patient.

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