Altered Sphingolipid metabolism in Vernal Keratoconjunctivitis: Newer Insights.

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To the Editor,

Vernal keratoconjunctivitis (VKC) is a chronic bilateral allergic inflammation of the ocular surface, seasonal or perennial affecting children in their first decade of life presenting with varying grades of severity. A small subset borders on refractory disease not amenable to interventions(1). Allergic response in terms of bioactive lipids is underexplored in ocular surface disorders and this study focused on sphingolipids. Ceramide and sphingosine are inter-convertible components of the "sphingolipid rheostat" that regulate immune response. The phosphorylated forms, ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P) regulated by the various kinases, phosphatases, hydrolases and lyases act as bioactives. They accumulate in cell membrane and compartmentalize intracellularly to play a major role in immune cell trafficking, inflammation, barrier cell integrity and cell survival(2–4).

A prospective observational study was conducted at a tertiary eye care hospital in South India in 87 cases of VKC (74 M/13 F; Mean age - 12.5 ± 5.3 years) and 60 age matched healthy controls (37M/23F; Mean age -13.7±5.2 years), as approved by the Institutional review board. The study was conducted in strict adherence to the tenets of Declaration of Helsinki after written informed consent from all study participants.

Based on Bonini's classification(1), VKC patients with grade 1 and 2 were considered non-refractory (NR-VKC) and those with grade 3 and 4 as refractory (R-VKC).

Gene expression of enzymes of sphingolipid metabolism evaluated in the conjunctival cells of VKC cases obtained by imprint cytology(5) showed a significant increase compared to the control, namely in Alkaline ceramidase (ACER) ($p<0.05$), Sphingosine kinase (SPHK1) ($p<0.001$, 5 fold increase); Sphingosine-1-phosphate lyase (SGPL) ($p<0.001$, 20 fold increase); UDP- Glucose ceramide glucosyltransferase (UGCG)($p<0.05$), while acid sphingomyelinase (ASMA) was significantly reduced ($p<0.01$), indicating altered sphingolipid metabolism in VKC with potential accumulation of S1P over that of C1P (**Fig 1A**). Concomitantly, S1P receptor, S1PR3 in the conjunctival imprint cells was significantly upregulated indicating cellular accumulation of S1P. While, Bcl2/Bax ratio was down-regulated, vimentin was significantly upregulated in VKC cases compared to control indicating a profibrotic and reduced apoptotic milieu **(Fig 1 B, C).** An altered sphingolipid metabolism was observed also at systemic level in the VKC cases, wherein there was significant lowering of C1P and an increase in S1P along with raised IgE in serum as per ELISA **(Fig 1D).**

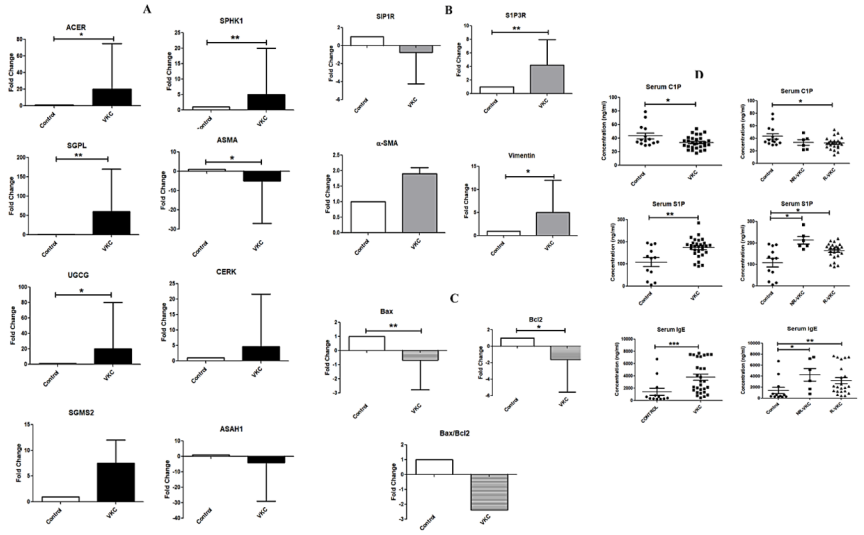

FIGURE 1

Figure 1: mRNA expression in conjunctival cellsof VKC.

Fig 1A: Enzymes of sphingolipid metabolism

UGCG: UDP-Glucose Ceramide-Glucosyltransferase; CERK: Ceramide Kinase; SPHK1: Sphingosine Kinase-; ACER: Alkaline Ceramidase; SGMS2: Sphingomyelin Synthase; ASMA: Acid Sphingomyelinase; ASAH1: Acid Ceramidase; SGPL: Sphingosine-1-phosphate lyase; Control: n=60; VKC: n=87

Fig 1B: S1P1R and S1P3R receptor

S1P1R: Control& VKC n=13

S1P3R: Control n=13; VKC n=9

Fig 1C: α-SMA, Vimentin, BCl_2 and Bax

Control, n=22; VKC, n=19

Figure 1D: Serum C1P, S1P and IgE in VKC by ELISA

Control: n=14; VKC: 29; NR-VKC: n= 6; R-VKC: n=23

(S1P, IgE: Control: n=12)

*p<0.05; **p<0.01;***p<0.01: control versus VKC

Altered levels of sphingolipids in the ocular surface was evaluated by estimation in the pooled sets of Schirmer collected tears(6) by LC-MS/MS analysis that showed significant alterations in the VKC cases when classified as Refractory (R-VKC, n= 22 sets) and Non-Refractory (NR-VKC, n=6 sets). Accordingly, 4 of the specific sphingolipids namely,S1P (d17:0); S1P (d20:1); S1P (d17:1) and Cer (d18:/17:0) were significantly lowered in R-VKC. While Cer (d18:1/17:0) was significantly increased (by 30%) and C1P (d18:1/8:0) (by 65%) in NR-VKC than control, this response was not observed in R-VKC **(Fig 2A, B).** A lower level of ceramide in the conjunctival epithelium probably alters barrier permeability and reduced apoptosis may further contribute to the refractory behavior in a select group of VKC patients. Looking at the cumulative levels of tear sphingolipids a relatively lower level of total sphingolipids, S1P, C1P and ceramide (d18:/17:0) were seen in R-VKC compared to NR-VKC, possibly indicating the defense in NR-VKC which R-VKC fails to elicit. Of

the refractory cases classified based on intervention, those who were on topical immunomodulators > 8 weeks and still active, showed maximal lowering of tear ceramide and raised S1P/ceramide ratio thus correlating with the more severe/refractory grade of VKC **(Fig 2C).**

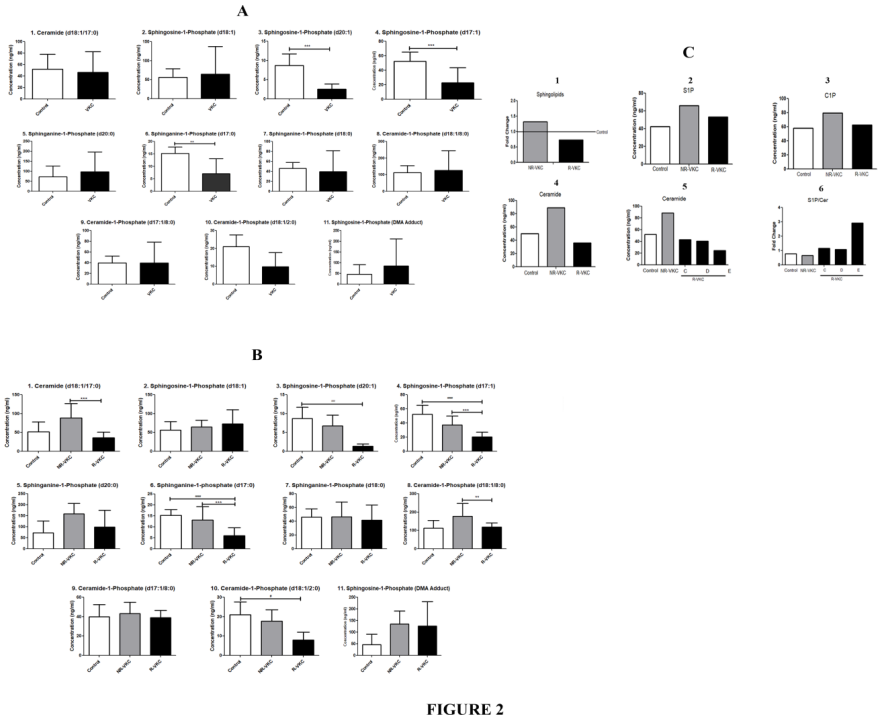

FIGURE 2

Fig 2A : Tear Sphingolipids in VKC by LC-MS/MS analysis.

Fig 2B: Tear sphingolipids in R-VKC and NR-VKC.

X-axis: Mean concentration (ng/ml) in pooled tear sets

Control: 7 pooled tear sets; VKC: 22 tear sets (NR-VKC: 6; and R-VKC: 16).

* $p<0.05$; ** $P<0.01$:***$p<0.001$: control vs VKC

Fig 3B: # $p<0.05$; ##$P<0.01$:### $p<0.001$: control vs R-VKC

Fig 2C: Cumulative tear Sphingolipids

1. Total sphingolipids in NR-VKC and R-VKC compared to control
2. Total S1P
3. Total C1P
4. Ceramide
5. Ceramide in R-VKC
6. S1P/ Ceramide classified based on intervention

C: steroid/immunomodulator <8 weeks D: without steroid/immunomodulator, E: steroid/immunomodulator > 8 weeks

To summarize, this study brings forth an association between altered sphingolipid metabolism and VKC pathogenesis observed both at ocular and systemic levels. Further, it unveils a possible explanation for the severe or refractory cases of VKC in terms of relatively lowered tear sphingolipids including ceramide, C1Pand S1P, thus opening fresh avenues for intervention.

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Author Contributions:

Menta Vignesh: Experimentation and data analysis, First draft of manuscript

Gurumurthy Srividya: Experimentation, analysis, manuscript drafting and discussion

Narayanasamy Angayarkanni: Concept and study design, data analysis, discussion, drafting, and funding

Agarwal Shweta: Clinical specimen and data, study design, discussion and drafting paper

Amrutha Anandan, Srinivasan Bhaskar, Iyer Geetha: Clinical data and discussion

Das S Ujjwalkumar, Lakshmi Moksha, Thirumurthy Velpandian: Analysis (LC-MS/MS) and data analysis

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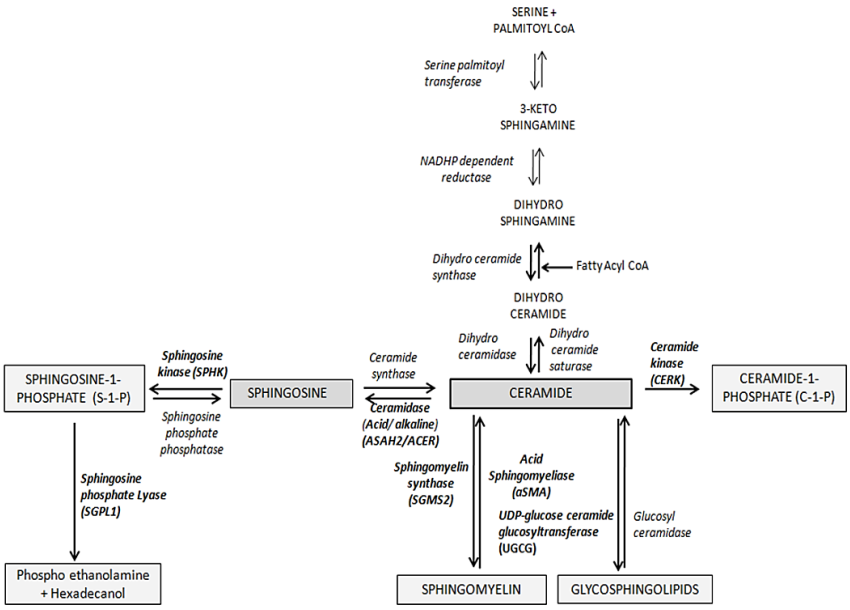
Supplementary Data



SUPPLEMENTARY FIGURE 1

Supplementary Figure 1: Sphingolipid Metabolism.

Enzymes in bold were analysed for gene expression in the VKC and controls

Diagnosis of VKC and inclusion criteria for study recruitment: Patients with ocular itching, photophobia, presence of active tarsal/limbal papillae, bulbar congestion, Horner Tranta's dots or superficial punctate keratitis were diagnosed as active VKC. The quiescent stage was diagnosed based on inactive or flat-topped papillae, a non -inflamed ocular surface with previous history of chronic itching. All cases were included after a complete ophthalmic examination and ruling out any other ocular or systemic morbidity or history of ocular surgery. Tear specimen and conjunctival imprints were collected in cases and controls, while blood sample was collected in a sub-set based on consent of the study participant. All cases with active VKC and age $>$ 6 years were included in the study. Patients with history of previous ocular surgery, ocular co-morbidity other than VKC, any systemic disease other than allergic disorders; age $<$ 6 years and quiet eye at presentation were excluded from the study. On basis of Bonini's classification, patients with grade 1 and 2 (mild to moderate form of seasonal VKC) were considered as non-refractory (NR-VKC) and those with grade 3 and 4 (severe to very severe form of perennial VKC) as refractory (R-VKC). Patients with refractory VKC were further sub-grouped based on the duration and/ or use of topical steroid or immunomodulator, (C) those who were on topical steroid/immunomodulator $<$8 weeks; (D) those without steroid or immunomodulator, who presented as fresh case with a repeat episode; (E) those who were on

steroid/immunomodulator > 8 weeks. Duration of 8 weeks was considered so that the maximum effect of topical immunomodulator could be assessed. All cases had active VKC at recruitment and were advised to defer use of any topical on the day of collection. Non refractory and fresh refractory cases (group D) were only on lubricants at presentation whereas refractory (groups C and E) were on dual acting mast cell stabilizer (Olopatadine 0.2% w/v, Alcon Laboratories, India) in addition to lubricants and topical steroid / immunomodulator (Fluoromethalone 0.1%w/v, Allergan India Private Limited/Tacrolimus ointment 0.03% w/w, Aurolab, India).

LC-MS/MS analysis of sphingolipids in tear: The sphingolipid standards were procured from Avanti Polar Lipids (Alabama, USA). Based on calibration of the LC column and linearity check, the following standards were finalized for the assay in pooled specimen of tear in cases and controls LC-ESI-MS/MS experiments were performed using a triple quadrupole tandem mass spectrometer (4000 Q-Trap, AB Sciex, Foster City, CA, USA) coupled with high performance liquid chromatography system (HPLC, Agilent Technologies, 1260 Infinity, Santa Clara, CA, USA) that consisted of quaternary pump (G1311C), multi-sampler (G7167A), HIBAR (30 x 2.1mm, 2µm) column compartment (G1316A) with variable wavelength UV detector (G1314F) and online degasser. All the parameters of tandem mass spectrometer and HPLC were controlled by Analyst software, version 1.5.2 (AB Sciex, Foster City, CA, USA) and Open LAB control panel software (Agilent Technologies, 1260 Infinity, Santa Clara, CA, USA), respectively.

Supplementary Table 1: List of Sphingolipids optimized in LC-MS/MS.

	Sample ID	Standard ID	Chemical Formula	Exact Mass	$[M+H]^+$	Name	Commercial Name
Ceramide -1- phosphate	**L- 10**	860594	$C_{34}H_{71}N_2O_5P$	601.49	602.49	N-palmitoyl-3-deoxy-ceramide-1-phosphate (ammonium salt)	16:0 3-deoxy-C1P
	L- 15	860533	$C_{34}H_{71}N_2O_6P$	617.48	618.5	N-palmitoyl-ceramide-1-phosphate (ammonium salt)	C16 Ceramide-1-Phosphate (d18:1/16:0)
	L- 05	860599	$C_{36}H_{73}N_2O_6P$	644.50	644.5	N-oleoyl-ceramide-1-phosphate (ammonium salt)	C18:1 Ceramide-1-Phosphate (d18:1/18:1(9Z

	Sample ID	Standard ID	Chemical Formula	Exact Mass	$[M+H]^+$	Name	Commercial Name
	L-01	860527	$C_{42}H_{87}N_2O_6P$	729.60	730.6	N-lignoceroyl-ceramide-1-phosphate (ammonium salt)	C24 Ceramide-1-Phosphate (d18:1/24:0)
	L-11	860530	$C_{20}H_{43}N_2O_6P$	421.52	422.5	N-acetoyl-ceramide-1-phosphate (ammonium salt)	C2 Ceramide-1-Phosphate (d18:1/2:0)
	L-13	860531	$C_{30}H_{63}N_2O_6P$	561.41	562.4	N-lauroyl-ceramide-1-phosphate (ammonium salt)	C12 Ceramide-1-Phosphate (d18:1/12:0)
	L-06	860532	$C_{26}H_{55}N_2O_6P$	505.36	506.4	N-octanoyl-ceramide-1-phosphate (ammonium salt)	C8 Ceramide-1-Phosphate (d18:1/8:0)
	L-03	860652	C25H53N2O6P	491.3	492.3	N-octanoyl-ceramide-1-phosphate (C17 base) (ammonium salt)	C8 Ceramide-1-Phosphate (d17:1/8:0)
Sphingosine -1- phosphate	**L-12**	860641	$C_{17}H_{36}NO_5P$	365.233	366.2	D-erythro-sphingosine-1-phosphate (C17 base)	Sphingosine-1-Phosphate (d17:1)
	L-14	860492	$C_{18}H_{38}NO_5P$	379.249	380.3	D-erythro-sphingosine-1-phosphate	Sphingosine-1-Phosphate (d18:1)

	Sample ID	Standard ID	Chemical Formula	Exact Mass	$[M+H]^+$	Name	Commercial Name
	L-16	860494	$C_{20}H_{45}N_2O_5P$	424.307	425.3	D-erythro-sphingosine-1-phosphate (DMA Adduct)	Sphingosine-1-Phosphate (DMA Adduct)
	L-09	860662	$C_{20}H_{42}NO_5P$	407.28	408.3	D-erythro-sphingosine-1-phosphate (C20 base)	Sphingosine-1-Phosphate (d20:1)
Sphinganine-1-phosphate	**L-08**	860536	$C_{18}H_{40}NO_5P$	381.264	382.3	D-erythro-sphinganine-1-phosphate	Sphinganine-1-Phosphate (d18:0)
	L-07	860655	$C_{17}H_{38}NO_5P$	367.249	368.3	D-erythro-sphinganine-1-phosphate (C17 base)	sphinganine-1-phosphate (d17:0)
	L-04	860675	$C_{20}H_{44}NO_5P$	409.296	410.3	D-erythro-sphinganine-1-phosphate (C20 base)	Sphinganine-1-Phosphate (d20:0)
Ceramide	**L-02**	860517	$C_{35}H_{69}NO_3$	551.28	552.3	N-heptadecananoyl-D-erythro-sphingosine	C17 Ceramide (d18:1/17:0)

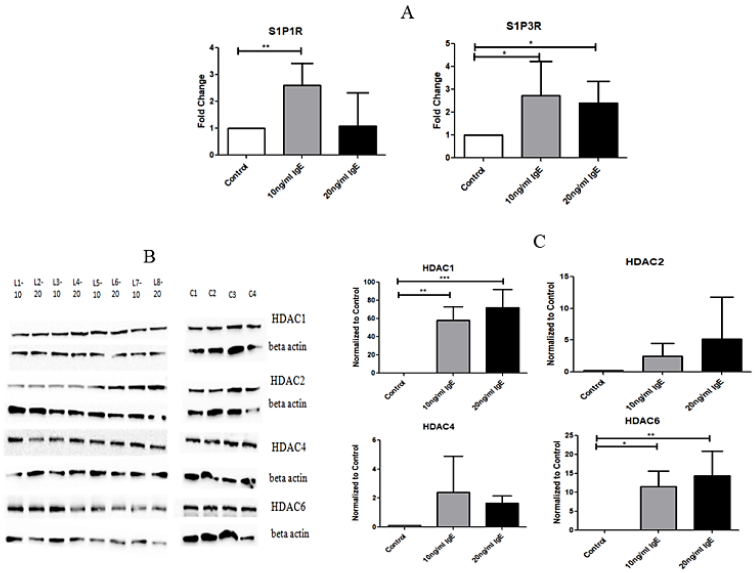
Tear Lipid extraction: For tear lipid analysis, one tear specimen each from 3 patients was pooled (tear collected using Schirmer's strip) in both control and VKC groups. The analysis was done in 7 pooled sets in controls and 22 in VKC (NR-VKC: 6 sets; and R-VKC: 16 sets). Briefly, the Schirmer strips were cut and measured volume of the extraction solvent, $CHCl_3$: Methanol Reagent (1:1) was added, vortexed and sonicated for 5 minutes. Samples were then transferred to a fresh vial and nitrogen purged. Extraction solvent was further added to the sample followed by vortexing for 1 minute. Sample was centrifuged for 5 min at 10000 rpm. Supernatant (20 µl) was loaded for analysis in LC-MS/MS.

In vitro **study in mast cells:**

Mast cell lines were purchased from (Kerafast-LUVA-Human Mast Cell Line, (EG1701-FP, MA, USA). Suspended cells were grown to confluence in six well culture plate and were exposed to 10ng/ml & 20ng/ml concentration of IgE (Abcam-Native human IgE protein (ab65866, MA, USA) for 16 hours in serum free medium (STEM PRO-34 nutrient supplement (Gibco #10641-025, MA, USA); At the end of 16 hours, cells were centrifuged at 3000g, washed in 1X-PBS. A) The gene expressions were analysed by RT-PCR (Biorad-Touch real time PCR, CA, USA) for the enzymes. B) Lysed using RIPA buffer. The lysate after protein estimation (Pierce BCA Protein Assay Kit Cat No: 23225, MA, USA) was used for western blot analysis (30 µg/ul) to assess endogenous Histone Deacetylase (HDAC) expression using Antibody Sampler Kit (#9928, Cell signalling Technology, MA,USA) using the corresponding antibodies namely, HDAC1, HDAC2, HDAC4,

HDAC6 and 7 (representing Zinc dependent class I, class IIa and IIb) as well as the Beta actin (β-Actin Antibody (AC-15) sc-69879, Santa Cruz Biotechnology, CA, USA) used as the loading control. Chemidoc-XRS+ (Biorad, CA, USA) was used for gel documentation and image lab tool (Biorad, CA, USA) was used for estimating the pixel density of the bands.

An *in vitro* study to evaluate the altered sphingolipid metabolism in mast cells that infiltrate conjunctival cells was done. Mast cells were treated with IgE that showed an increased expression of both S1P1R and S1P3R ($p<0.05$). Protein expression of HDAC (1and 6) were increased significantly ($p<0.05$). Increased S1P1R and 3R expressions that internalize S1P seem to target HDAC for epigenetic modulation in mast cells (Supp Fig 2).



SUPPLEMENTARY FIGURE 2

Supplementary Fig 2A: S1P Receptors expression in Mast cells exposed to IgE.

Supplementary Fig 2B : Representative western blot showing protein expressions of the Histone Deacetylases, HDAC1, HDAC2, HDAC5 and HDAC6

Mast cells exposed to IgE for 16 hours.Lanes L1, 3, 5, 7: treated with 10ng/ml of IgE; L2,4,6,8:treated with 20ng/ml of IgE; C1-C4: untreated

Supplementary Fig 2C : Densitogram of western blot by ImageJ software,

Y-axis: fold variation normalized to control. *$p<0.05$; ** $P<0.01$: control vs IgE treated.

Data expressed as mean ± SD of three independent experiments.