Altered Sphingolipid metabolism in Vernal Keratoconjunctivitis: Newer Insights

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January 30, 2024

Abstract

Background: The etiopathogenesis of vernal keratoconjunctivitis (VKC), is incompletely understood. Bioactive lipids play a key role in the allergic disorders and there are no studies exploring it in the ocular surface allergic disorder such as VKC. This study focused on the sphingolipid metabolism in VKC and explored the association of ocular surface sphingolipids with the refractory nature of the disease. Methods: Active VKC cases and age-matched healthy controls were recruited as part of a one-year prospective study at our tertiary eye care centre in South India. Imprint cytology was used to assess gene expression of enzymes of sphingolipids metabolism in conjunctival cells. Sphingolipids levels were estimated in the tears by LC-MS/MS analysis. Systemic levels of Sphingosine-1-phosphate (S1P) and Ceramide-1-Phosphate and IgE were estimated by ELISA. IgE induced regulation of S1P receptor gene expression was assessed by western blot analysis of histone deacetylases in cultured mast cells. Results: Significantly altered gene expression of the sphingolipids enzymes was noted in VKC in conjunctival cells. Pooled tears showed significantly lowered levels of S1P (d17:1), S1P (d17:0) and S1P (d20:1) in VKC. Additionally, Cer (d18:/17:0) was significantly lowered in R-VKC compared to NR-VKC. Lowered C1P and raised IgE were observed at serum level in VKC. Increased S1P receptors and histone deacetylases expression indicate regulation by histone modification as evaluated in IgE induced mast cells. Conclusion: Altered sphingolipid metabolism is associated with VKC pathogenesis and is differential in refractory cases that showed lower ceramide and specific sphingosines compared to non-refractory VKC.

Introduction:

Vernal keratoconjunctivitis (VKC) is a chronic, bilateral allergic inflammation of the ocular surface, seasonal or perennial affecting children in the first decade of their life. It can present as limbal, palpebral or as a mixed disease with varying grades of severity, with a small subset bordering on refractory disease not amenable to currently available treatment modalities(1). Being a tropical country, the incidence is high in India and almost 36% children have the perennial form of VKC(2).

The exact etiopathogenesis of allergic disorders is still not completely understood. Pathways involving IgE antibodies or T-cells and inflammatory cytokines(3,4) are well known. However, the ones involving bioactive lipid mediators are still being explored. Of the four classes of immune modulating lipids; platelet activating factor, leukotrienes, prostanoids and sphingolipids, immense focus has been on the sphingolipid group in influencing the pathogenesis of allergic disease as seen in the last decade(5). Sphingolipids (SL) comprise a large family of bioactive lipids containing a sphingoid base (aliphatic amino alcohol) as backbone that is acylated with various fatty acids to form several SL species. Ceramide is the central molecule which is generated by *de novo* synthesis or formed by degradation from sphingomyelin at plasma membrane

level, while sphingosine is formed from ceramide by ceramidase(6). Cytosolic conversion to their respective phosphorylated forms, ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P) and further to other sphingolipids including glycosphingolipids constitute the bioactives that are regulated by the various kinases, phosphatases, hydrolases and lyases **(Figure 1)** . Activation of the pathway occurs in response to a variety of cellular stresses due to UV radiation, pathogen exposure, allergens and chemotherapeutic agents, leading to the production of the bioactive lipid ceramide(7). Ceramide and sphingosine are interconvertible components of the "sphingolipid rheostat" that regulates immune function(8). These immune modulating sphingolipids accumulate in cell membrane and compartmentalize intracellularly to play a major role in immune cell trafficking, inflammation, barrier cell integrity and cell survival(6,7,9).

In light of the above recent understanding from reports of systemic allergic disorders, we decided to explore such bioactive lipids in the ocular allergic disorder, specifically VKC. The etiopathogenesis of VKC is still being explored. In addition, with the available reports in literature, we are presently unable to explain the etiopathology of refractoriness of this condition, barring reports of higher levels of inflammatory cytokines in these severely affected eyes compared to the less severe ones(10). We therefore specifically studied the sphingolipid metabolism in patients with vernal keratoconjunctivitis.

Method:

As part of a two-year observational prospective study in the year 2017, VKC cases and controls were recruited for one year in our tertiary eye care center, 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.

VKC was diagnosed and graded based on the classification by Bonini*et al* (11). 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.

VKC cases were further graded based on their severity as non-refractory (NR-VKC) and refractory (R-VKC). On basis of Bonini's classification, patients with grade 1 and 2 (mild to moderate form of seasonal VKC) were considered as non-refractory and those with grade 3 and 4 (severe to very severe form of perennial VKC) were grouped as refractory. 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. A 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).

Tear sample collection

Tear was collected using sterile Schirmer strip by placing it at the lower cul-de-sac for 5 min time to obtain tear fluid as reported in our previous study(12); transferred using forceps to a vial and stored at -80^{o} C until analysis.

Conjunctival impression cytology

Conjunctival imprints using sterile Whatman paper were obtained after local anaesthesia with proparacaine HCL (Ophthalmic solution of 0.5%) from superior, inferior, temporal, nasal regions of the conjunctiva as published earlier(13)[(13)]. The samples were stored at -80^{o} C until analysis.

Blood Collection:

Blood sample (plain) was collected in a sub-set of controls and cases who gave consent, the serum was separated and stored at -80 degree C until analysis of IgE, S1P and C1P by ELISA.

Estimation of IgE, Ceramide 1 phosphate (C1P) and Sphingosine 1 phosphate (S1P) by ELISA

The reagents, working standards and samples were prepared according to manufacture protocol for the estimation of serum IgE, (Human IgE ELISA Kit (ab195216); MA, USA), serum C1P (Human Ceramide-1-phosphate -MBS2601367 ELISA kit, MyBio Source, CA, USA) and serum S1P (Human S1P-MB5S2516132 ELISA kit, MyBio Source, CA, USA). Detection was based on TMB substrate and the absorbance at 450nm was measured (Spinco spectramax-M2e multimode reader, Molecular Devices, CA, USA).

Total RNA isolation from conjunctival imprints

The imprint samples stored were extracted for RNA using 300-400µl of trizol as published by us earlier(13). The concentration and purity of the RNA were estimated using Nano drop method and then converted to cDNA using Bio-Rad iScript cDNA conversion kit.

Sphingolipid Enzyme gene expression by qPCR

The gene expression studies were carried out using the 50 ng/µL concentration of cDNA for assessing the sphingolipid metabolism enzymes mentioned in **Supplementary Table 1.0.** One µL (50 ng total RNA equivalent) in 10 µL reaction mixture was used for 50 cycles reaction in the qPCR and the fold change were analysed by ΔCq method according to Thomas(14).

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 the 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.

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 specimens of tear in cases and controls **(Supplementary Table 2)** . 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 OpenLAB control panel software (Agilent Technologies, 1260 Infinity, Santa Clara, CA, USA), respectively.

Mast cell culture and sphingolipid receptor expression on IgE exposure:

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); Pen Strep (Gibco #15140, MA, USA); L-glutamine-200 mM (Gibco # 25030-081). At the end of 16 hours, cells were centrifuged at 3000g, washed in 1X-PBS. RNA was isolated from these cells using Trizol (FavorPrep Tri-RNA Reagent, Merck, St.Louis, USA) followed by cDNA conversion (Biorad-iScript 1708841, CA, USA). The gene expressions were analysed by RT-PCR (Biorad-Touch real time PCR, CA, USA) for the enzymes.

Mast cell expression and HDAC expression on IgE exposure:

Mast cell were grown to confluence in six well cell culture plates exposed to 10ng/ml & 20 ng/ml concentration of IgE for 16 hours in serum free medium. Cells were then collected and centrifuged at 3000g, washed in 1X-PBS, 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/µl) 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.

Statistical analysis

Statistical analysis was performed in all patient samples using Analysis of variance one-way ANOVA student-t test with Microsoft Excel and GrapPad Prism was used for graphical representation. A p-value <0.05 was considered statistically significant.

Results:

The study included 87 cases of vernal keratoconjunctivitis (NR-VKC-28, R-VKC-59), with a gender distribution of 74 male; 13 female and a mean age of 12.5 ± 5.3 years. Controls (n=60) were age matched healthy individuals with a mean age of 13.7 ± 5.2 years and the gender distribution of male and female as 37 and 23 respectively.

3. 1 Conjunctival gene Expression of Enzymes of Sphingolipids Metabolism:

Gene expression of enzymes of the sphingolipid metabolism was evaluated in the conjunctival cells of VKC cases compared to age matched control. A significant increase in the expression of the enzymes involved in the removal of ceramide to form sphingosine namely, Alkaline ceramidase (ACER) ($p<0.05$); Sphingosine kinase (SPHK1) that forms S1P from sphingosine ($p<0.001$); Sphingosine 1- phosphate lyase (SGPL) that acts on S1P to breakdown to phosphoethanolamine ($p<0.001$) were seen. CERK which converts ceramide to C1P was raised though not statistically significant. But UDP- Glucose ceramide glucosyl transferase (UGCG) which forms glycosphingolipid from ceramide was increased significantly ($p<0.05$) in VKC patients**(Figure 2A)** . However, the enzyme, acid sphingomyelinase (ASMA) involved in ceramide synthesis from sphingomyelin was significantly reduced ($p<0.01$). No significant difference between R-VKC and NR-VKC were observed in the gene expressions studied (data not shown).

3.2 Sphingolipid receptor expression in the conjunctival cells:

Alteration in the sphingolipid metabolism was further supported by the increased S1P receptor 3 expressions (S1P3R) in conjunctival cells of VKC cases compared to control. S1P1R did not show significant change**(Figure 2B)** showing that the cellular uptake of S1P is S1P3R mediated. The receptor expression however did not show significant variation between NR-VKC and R-VKC (data not shown).

3.3 Gene expression of fibrotic, apoptotic and inflammation marker in the conjunctival cells

The mRNA expression of IL6, vimentin and Bcl2/Bax were evaluated. The conjunctival IL6 expression was increased by 2-fold though not statistically significant indicating a pro-inflammatory status in VKC.

Bcl2/Bax ratio was down regulated, however; vimentin was found to be significantly upregulated in VKC cases compared to control**(Figure 2C)** .

3.4 Tear Sphingolipid concentration assessed by LC-MS/MS

Pooled tear specimen of the normal and VKC group were analysed by mass spectrometry to quantify the sphingolipids on the ocular surface.Based on standards available and sensitivity of detection, 11 different sphingolipids were estimated by LC-MS/MS analysis. Amongst these, S1P (d17:1), S1P (d17:0) (both $p<0.01$) and S1P (d20:1) ($p<0.001$) sphingolipids were significantly decreased in the tear of the total VKC patients when compared to the control. Further, S1P (d20: 0) and S1P (D17:1 DMA adduct) were raised more than two-fold in VKC though not statistically significant **(Figure 3A)** .

The difference in the levels of these sphingolipids was also noted within the VKC subgroups when classified as NR-VKC and R-VKC**(Figure 3B)** . Looking at characteristic changes in R-VKC, specific sphingolipids were found to be significantly low as compared to control namely, S1P (d17:1) by 4-fold, ($p<0.001$),S1P (d17:0) by 3 folds ($p<0.001$), S1P (d20:1) by nearly 2.5-fold ($p<0.01$) and C1P (d18:1/ 2:0) by nearly 2-fold ($p<0.05$). All these SL were also relatively lower in R-VKC in comparison to NR-VKC of which S1P (d17:0) and S1P (d20:1) were significantly lower ($p<0.001$). In addition, significant lowering of Cer(d18:1/17:0) and C1P(d18:1/18:0) ($p<0.001$ and $p<0.01$ respectively) was observed. Both Cer(d18:1/17:0) and C1P(d18:1/1/8:0) showed an increase by nearly 1.5 fold in NR-VKC compared to control but were not statistically significant.

3.5 Altered serum Sphingosine 1-phosphate (S1P); ceramide-1-phosphate (CIP) and serum IgE in VKC

In order to check, changes in the systemic level, the metabolites namely S1P and C1P were estimated by ELISA. The serum C1P level was lowered in the total VKC group compared to the control ($p<0.05$) and this was seen in both R-VKC and NR-VKC **(Figure 4A)** . Serum SIP was significantly higher in the non-refractory VKC compared to control ($p<0.05$), while R-VKC had levels similar to control**(Figure 4B)** .

IgE levels were significantly increased in VKC ($P=0.0007$) and this was observed both in NR-VKC ($p=0.013$) and in R-VKC ($p=0.004$) with no difference amongst the two **(Figure 4C)** . The quantitative estimation revealed a mean of 1433 ± 2061 ng/mL in control (median = 504 ng/mL), while VKC group revealed a mean of 3801 ± 3719 ng/mL (median = 3719 ng/mL) with > 95 % case showing IgE levels more than the median of the control.

3.6 IgE induced expression of the sphingolipid receptors in mast cells is mediated by HDAC: *In vitro studies*

In order to evaluate the altered sphingolipid metabolism in mast cells, the mast cells treated with IgE were evaluated for the S1P receptor expression. A significant increase in the expression of both receptors S1P1R and S1P3R ($p<0.05$) was observed in response to IgE treatment **(Figure 5A)** .

Histone deacetylation was evaluated by HDAC protein expression to see if IgE mediated sphingolipid metabolism is regulated through histone deacetylation. HDAC1 ($p<0.05$), HDAC2, HDAC4 and HDAC 6 ($p<0.05$) were found to be increased in response to IgE treatment in the mast cells cultured *in vitro* **(Figure 5B, C)** .

Discussion:

In the last few decades sphingolipids have emerged as vital structural and signaling mediators that regulate varied cellular activities like cell death, proliferation and inflammation(15,16). Ceramide, sphingosine, C1P, S1P are all bioactive lipids and are key modulators in various inflammatory cascades(16,17). Next to cholesteryl esters and waxes, sphingolipids are the major lipid components in the tear and along with phospholipids contribute to the amphiphilic lipids in tear as shown by mass spectrometry analysis which not only comes from the meibum but also from the cellular ocular surface(18). The first link between altered sphingolipids and eye originated from lysosomal storage disease(19). However, recently they have been implicated in dry

eye disease, corneal infections, diabetic cornea and age-related macular degeneration(20,21). But their role in conjunctival disorders including VKC needs to be explored.

Our present study revealed altered markers of sphingolipid metabolism in the ocular surface in VKC patients along with differences in the refractory cases. The conjunctival epithelial cells in VKC had significantly reduced levels of ASMA which facilitates ceramide synthesis. However, the ceramide that was estimated in tear namely Cer(d18:1/17:0) showed differential levels in NR and R-VKC. Increased expression of SPHK1 (5-fold) along with increase in SGPL (20-fold) as compared to 4 folds in CERK was noted indicating accumulation of S1P over that of C1P. These findings corroborated with the tear levels of sphingolipids too. Concomitantly, we also found S1PR3 receptor in the conjunctival imprints to be significantly upregulated indicating cellular accumulation of S1P. An altered sphingolipid metabolism was observed at systemic level too with raised S1P and lower C1P in VKC both in NR-VKC and in R-VKC. Both S1P and C1P promote chemotaxis, cell survival and inflammation. A critical balance between these interconvertible metabolites is crucial for normal cell function and any derangement can be detrimental to cells(22,23).

SIP plays an important role in the egress of lymphocytes, while C1P and S1P are involved in eicosanoid synthesis; both prostaglandins and leukotrienes. Besides, they also regulate the cellular arm of inflammatory cascade by promoting neutrophil migration, extravasation, macrophage survival and cytokine production. S1P receptors are present on dendritic cells, eosinophils, Natural Killer cells in addition to mast cells(24). Mast cells exposed to IgE in vitro showed increase in expression of S1PR1 in addition to SIPR3. Activated mast cells release S1P which acts in an autocrine fashion via S1P receptors to enhance mast cell function in addition to causing chemotaxis and degranulation(25).

Refractory VKC shows low levels of tear SL

Interestingly, tear sphingolipids showed differential levels based on the refractory nature of the VKC disease. Specific S1Ps and C1P (4 of them) were found to be characteristically lowered in R-VKC compared to control as well as NR-VKC. While Cer(d18:1/17:0) was significantly increased (by 30%) in NR- VKC than control, this response was not observed in R-VKC. Looking at the cumulative levels of tear sphingolipids a relatively lower level of the total sphingolipids, S1P, C1P and ceramide were seen in R-VKC compared to NR-VKC, while NR-VKC showed an increase compared to the control (**Supplementary Figure 1**). 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 (Supplementary Figure 1). Ceramide is an important component of the lipid raft in cell membranes and plays a pivotal role in signal transduction via FceRI receptors in mast cells. Among the multiple roles of ceramide, apoptosis is considered as the key function which it induces by both intrinsic and extrinsic pathways. The effect depends on the chain length of the fatty acids in the ceramide as short (C16; C18) or long (C24)(26,27). Apoptosis is necessary to resolve inflammation and delayed apoptosis has been observed in autoimmune diseases(28). A higher or raised ceramide probably induces apoptosis and the inflammation eventually subsides in the non-refractory group whereas reduced levels of ceramide in the refractory group with a raised S1P/ceramide ratio inhibits apoptosis and the inflammation persists contributing to the refractory status. Epithelial barrier permeability is influenced by ceramide and its deficiency reportedly contributes to the pathogenesis of atopic dermatitis(29). Similarly, a lower level of ceramide in the conjunctival epithelium probably alters the permeability making them more susceptible to allergens and reduced apoptosis further contributes to the refractory behavior in a select group of VKC patients. The reduced apoptosis in VKC and persistence of inflammation was further validated by the lower expression levels of Bcl2 and Bax and a 2-fold increase in the gene expression of IL6 expression in the conjunctival cells of VKC cases noted in our study(30). Moreover, there was an increase in the epithelial mesenchymal transition (EMT) marker namely vimentin, indicating pro-survival and EMT changes in VKC. EMT and sphingolipid metabolism has been explored in cancer and each inducing the changes in other is reported(31).

Since altered sphingolipid metabolism has been reported in allergic asthma, atopic dermatitis and many other autoimmune diseases, they have also been considered as therapeutic targets(23). Topical ceramide cream has

been found to improve the transepidermal barrier permeability in patients with atopic dermatitis(32). Topical liposomal C6-ceramide was found to inhibit corneal inflammation in murine models(33). SphK inhibitors like SK1-I and DMS have been found to reduce airway inflammation in mice(16,34). S1P receptor modulators like FTY 720 are being used in allergic rhinitis, asthma, allergic skin diseases such as contact hypersensitivity or atopic dermatitis(35). Owing to its lesser side effects than immunomodulators, topical FTY720 has been attempted to reduce rejection in corneal allograft as it was found to decrease infiltration of CD4 T cells in corneal grafts in animal experiments(36). A similar approach based can be explored in VKC.

HDAC mediated regulation of the sphingolipids

In addition, recently role of epigenetic regulation in allergic disorders has gained interest. The most common mechanisms are DNA methylation, histone modification and noncoding RNA's. There are 4 subclasses of HDAC enzymes of which classI/II have been most widely studied.Evidence suggests contribution of HDAC enzymes in allergic disorders primarily in two areas; one is regulation of cells contributing to allergy (Tcells, macrophages) and the other is with various allergic phenotypes(37). Grausenburger et al showed in conditional HDAC1 knockout mice that HDAC was important for development of Th2 cytokine production and allergic airway inflammation(38). We found significantly raised levels of HDAC1 and 6 when mast cells were exposed to IgE along with increased levels of S1PR1and 3 receptors, similar to published reports(39). Increased S1PR1 and 3 expressions that internalize the S1P seem to target HDAC for transcriptional modulation in mast cells. However, further studies are required to study the role of HDAC enzymes in pathogenesis of VKC.

One of the drawbacks of our study was that all samples were analyzed only in the active stage and not compared with the quiet stage of the disease to further delineate the cause and effect of the sphingolipids. Besides this was the first time that role of sphingolipid metabolism was being evaluated in VKC and not all the species of the sphingolipids including various ceramides in the tear were estimated. However, altered enzymes expression, receptor levels and corresponding variation in the levels of specific sphingolipids confirmed an altered metabolism in VKC which is differential in R-VKC in terms of relatively lower levels of ceramide and specific S1P compared to NR-VKC.

To summarize, this study brings forth for the very first time an association between altered sphingolipid metabolism and pathogenesis of VKC which was observed both at ocular and systemic levels. In addition it unveils a possible explanation for the severe or refractory cases of VKC as compared to the seasonal ones and thus opens up fresh avenues in therapeutic intervention.

Acknowledgement : We acknowledge High Precision Bioanalytical Facility (HPBAF) with Mass spectrometry (LC-MS/MS) at Ocular Pharmacology and Pharmacy Division, RP Center, AIIMS, New Delhi, India.

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

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Figure Legends

Figure 1: Sphingolipid Metabolism

Figure 2A: mRNA expression of the key enzymes of sphingolipid metabolism in the conjunctival cells of VKC.

UGCG: UDP-Glucose Ceramide Glucosyltransferase; CERK: Ceramide Kinase; SPHK1: Sphingosine Kinase 1; ACER: Alkaline ceramidase; SGMS2: Sphingomyelin Synthase; ASMA: Acid sphingomyelinase; ASAH1: Acid ceramidase; SGPL: Sphingosine-1-phosphate lyase

*p<0.05; **p<0.01 is the significance based on comparison of control versus VKC for the corresponding enzyme.

Control: n=60 and VKC: n=87

Figure 2B: S1P1 and S1P3 receptor expressions in the conjunctival cells of VKC

*p<0.01 is a comparison of control vs VKC;

S1P1R: Control, n=13 and VKC, n= 13; S1P3R: Control, n=13 and VKC, n=9

Figure 2C: Expression of fibrosis marker α-Smooth Muscle Actin, Vimentin and apoptosis markers BCl_2 and Bax

p<0.05; ** P<0.01: are the comparison between control and VKC

Control, n=22 ; VKC, n= 19

Figure 3: Tear sphingolipids in VKC patients analyzed using LC-MS/MS.

Figure 3A : Tear Sphingolipids in control and Total VKC

Figure 3B: Tear sphingolipids in Refractory VKC (R-VKC) and Non-Refractory VKC (NR-VKC). Details of pooled sample sizes in each group is given in the methods section.

1: C17 Ceramide (d18:1/17:0); 2: Sphingosine-1-Phosphate (d18:1); 3: Sphingosine-1-Phosphate (d20:1); 4: Sphingosine-1-Phosphate (d17:1); 5: Sphinganine-1-Phosphate (d20:0); 6: sphinganine-1-phosphate (d17:0); 7: Sphinganine-1-Phosphate (d18:0); 8: C8 Ceramide-1-Phosphate (d18:1/8:0); 9: C8 Ceramide-1-Phosphate (d17:1/8:0); 10: C2 Ceramide-1-Phosphate (d18:1/2:0); 11: Sphingosine-1-Phosphate (d17:1) (DMA Adduct). X-axis represents the Mean concentration in (ng/ml) in the tear samples Y axis represents the different groups. * p<0.05; ** P<0.01:***p<0.001 is the comparison between control vs VKC in Fig 3a. and R-VKC vs NR-VKC in Fig 3b.# p<0.05; ## P<0.01:###p<0.001 is the comparison between control vs R-VKC

Tear specimen were analyzed after making pooled sets of each group.

Control: 7 pooled sets; VKC: 22 sets (NR-VKC: 6 sets; and R-VKC: 16). One set is a pool of 3 tear specimen in the group

Figure 4: Serum S1P, S1P and IgE in VKC estimated by ELISA

Figure 4A : Serum C1P in VKC, NR-VKC and R-VKC (Control: n= 14; VKC: 29; (NR-VKC: n= 6; R-VKC: n=23)

Figure 4B : Serum S1P in VKC, NR-VKC and R-VKC (Control: n= 9; VKC: 29; (NR-VKC: n= 6; R-VKC: n=23)

Figure 4C : Serum IgEin VKC, NR-VKC and R-VKC (Control: n= 12; VKC: 29; (NR-VKC: n= 6; R-VKC: n=23)

Figure 5: Effect of IgE on gene expression of the S1P receptors in cultured Mast cells

Figure 5A: S1P Receptors expression in Mast cells exposed to IgE

Figure 5B : Representative western blot showing protein expressions of the Histone Deacetylases, HDAC1, HDAC2, HDAC5 and HDAC6

Histone Deacetylase (HDAC) protein expression in mast cells exposed to IgE (10 and 20 ng/mL) for 16 hours compared to control *in vitro*as detailed in method section.

Lanes L1, 3, 5, 7- treated with 10ng/ml of IgE; L2,4,6,8- treated with 20ng/ml of IgE)

Figure 5C : Densitogram

Densitometry of the bands of the western blot was done using ImageJ software,

Y-axis: fold variation normalized to control

Student's–t test was performed to obtain the statistical significance between control and treated *p<0.05; ** P<0.01.

The data is expressed as mean ± SD of three independent experiments

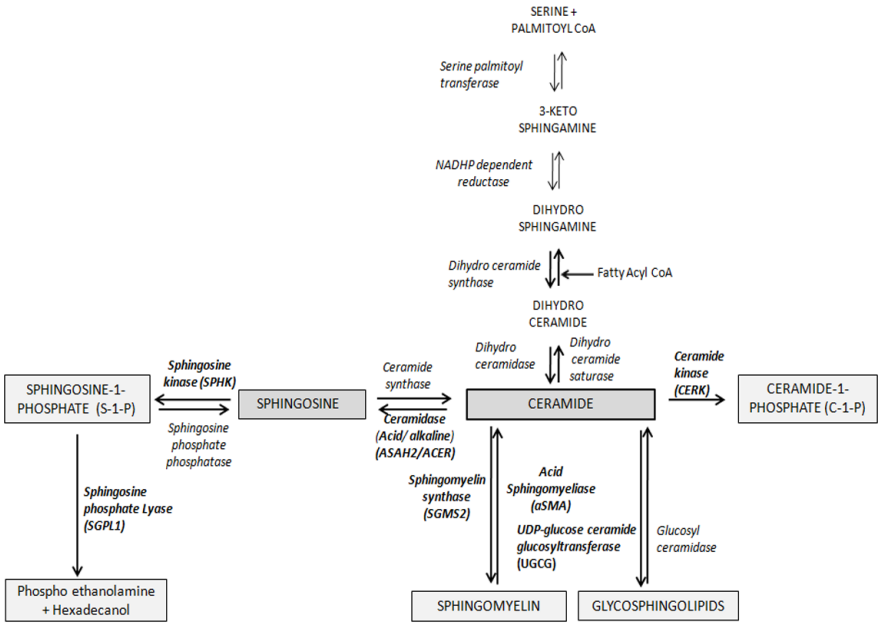


Figure 1

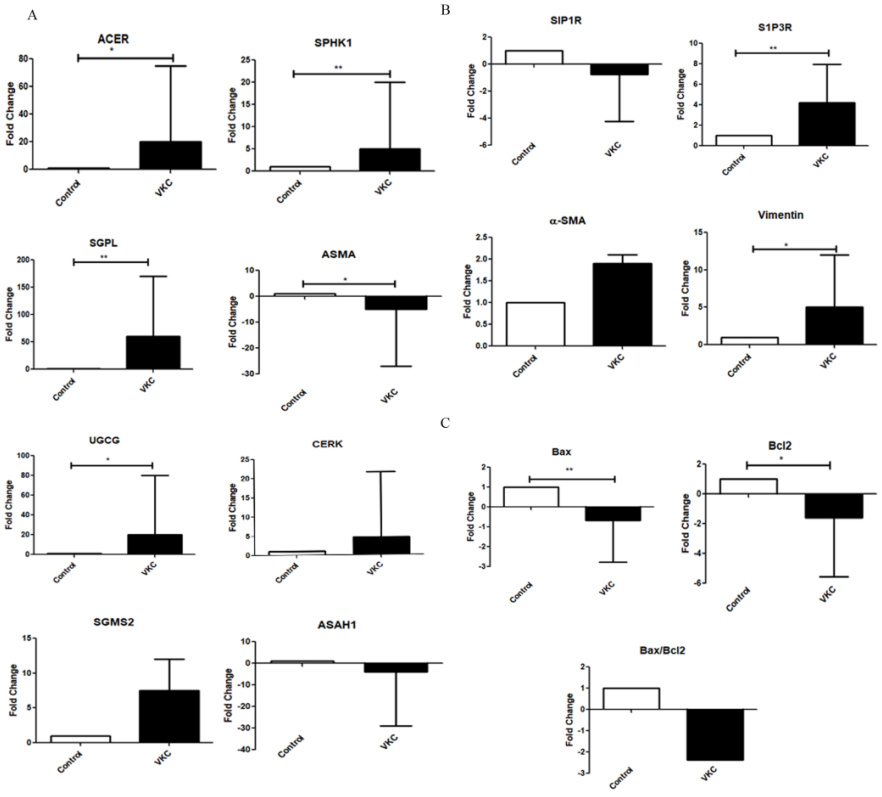
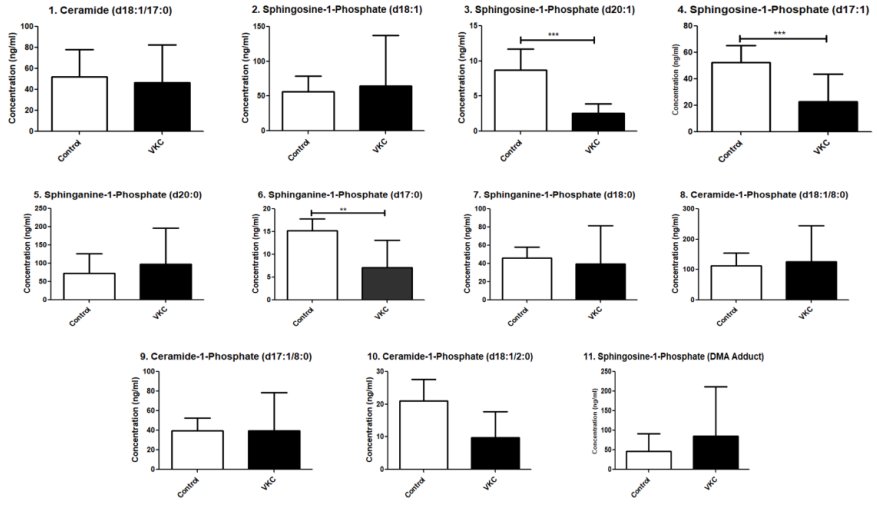


Figure 2

A



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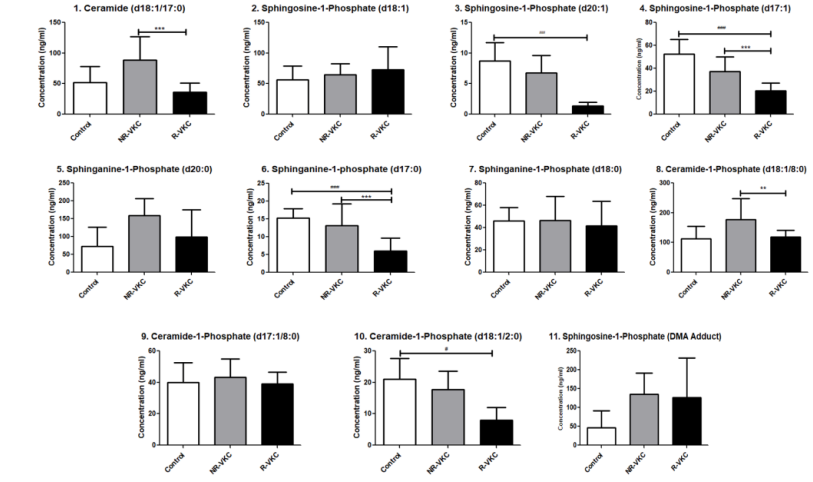

Figure 3

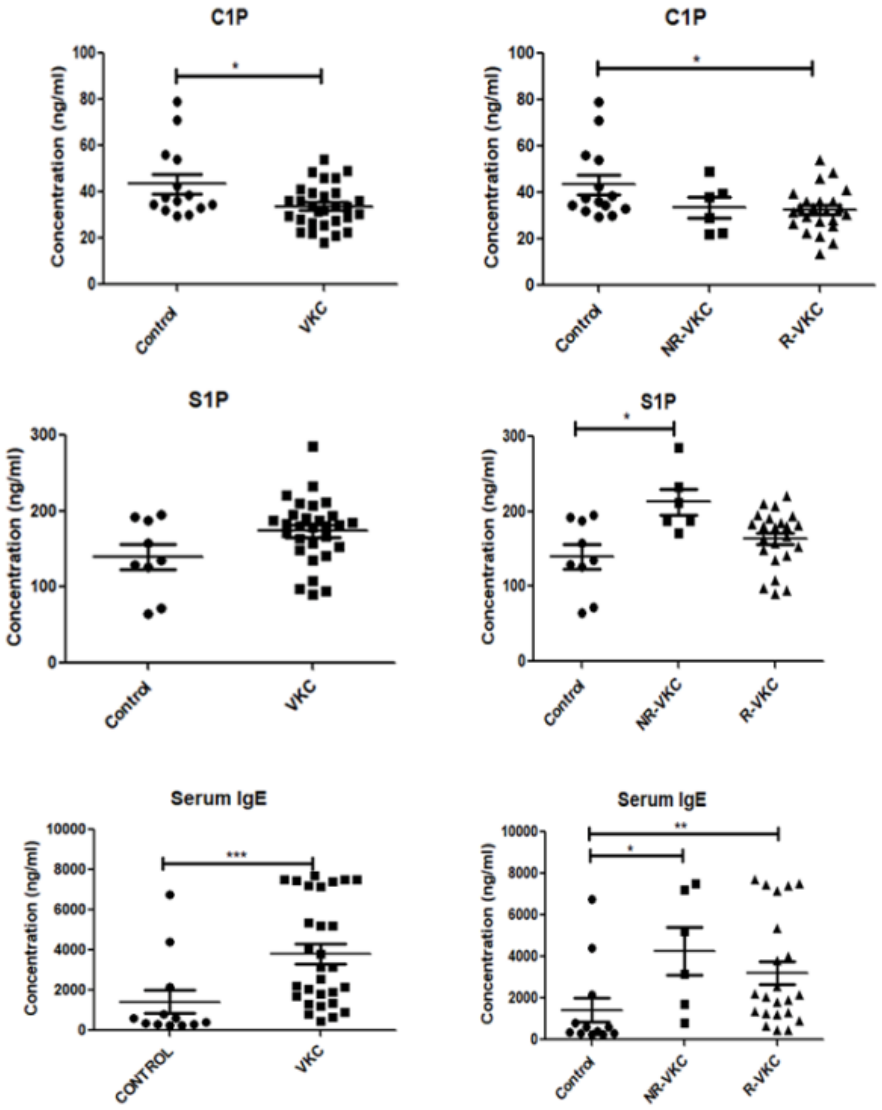

Figure 4

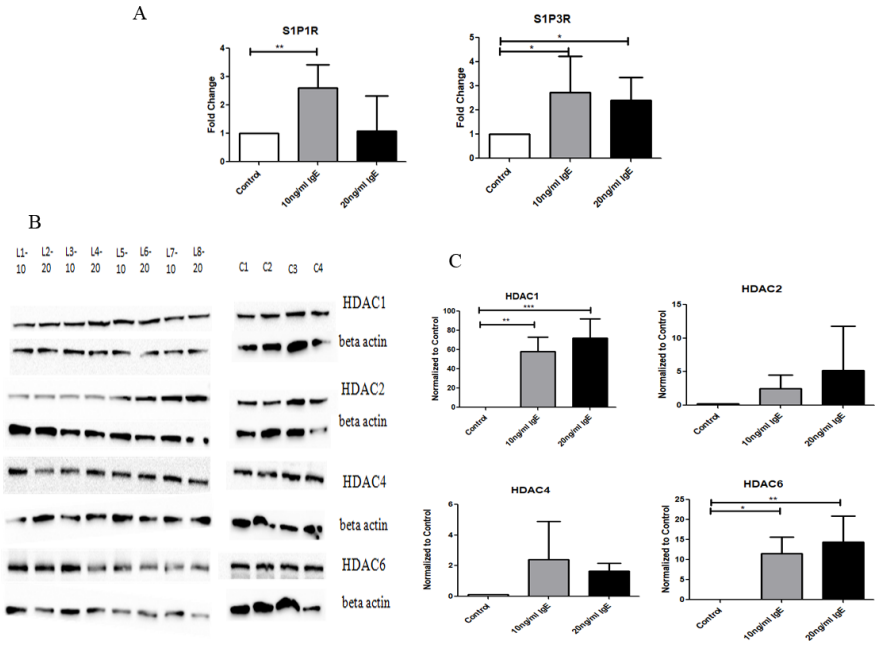

Figure 5