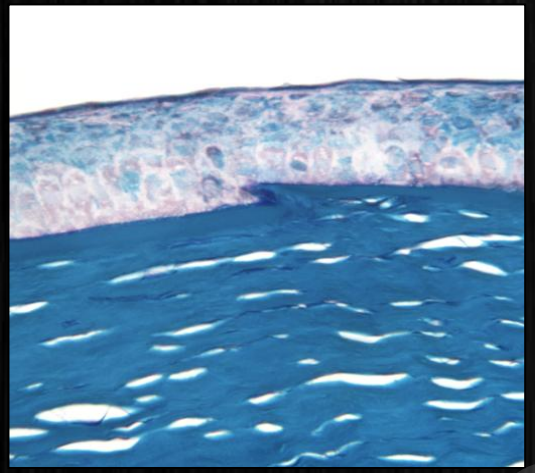
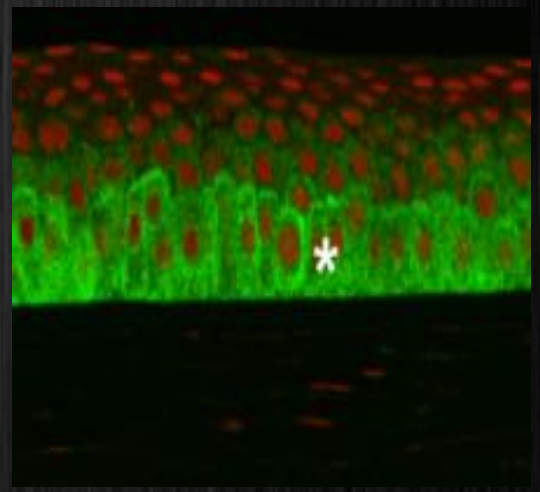
What role does the epithelium play in the Pathogenesis of Keratoconus?





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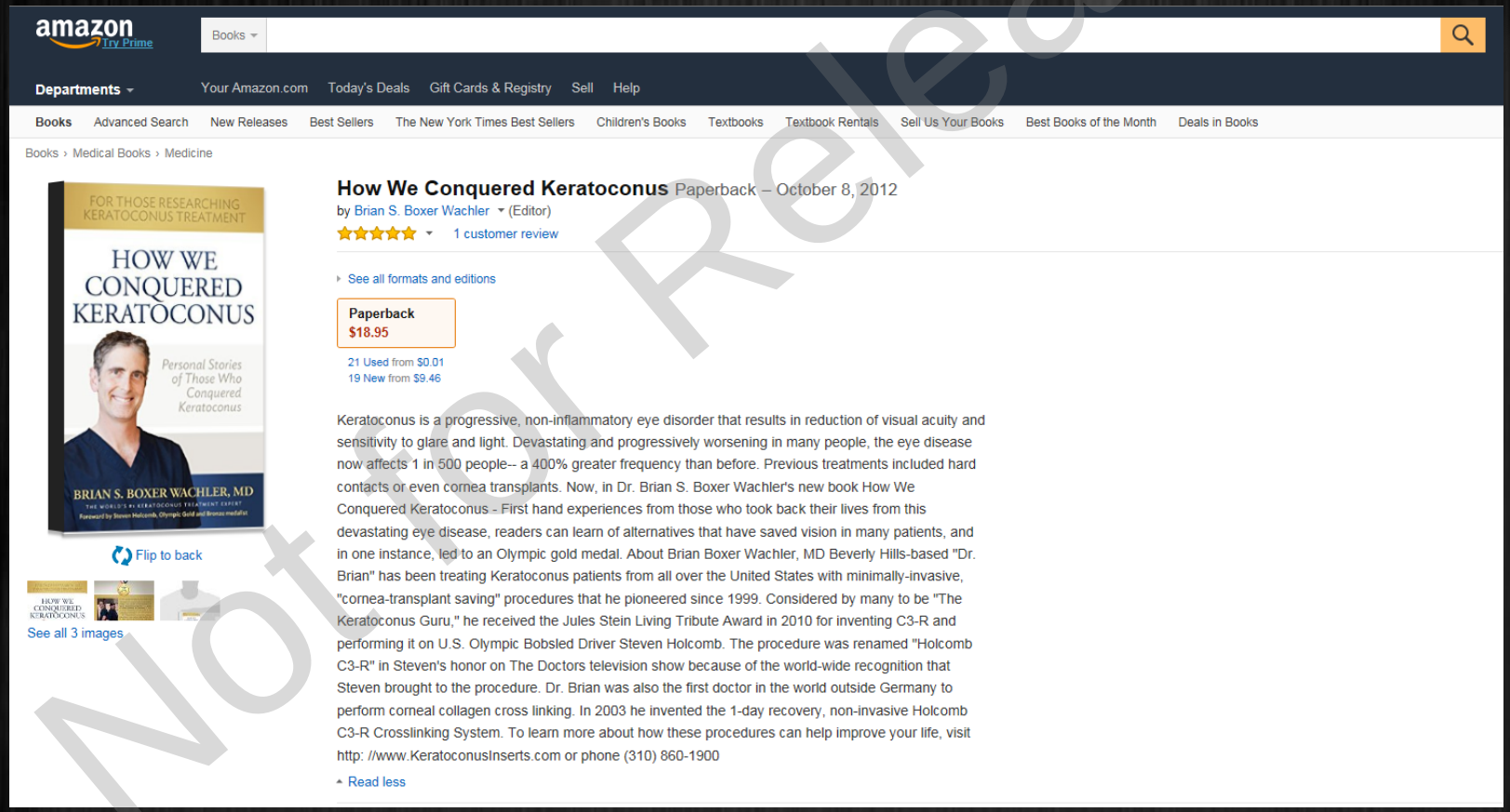
December 2016

How we conquered keratoconus?



Delphi Panel Collaboration: Definition/Diagnosis/Treatment of Keratoconus (April 2015)

SPECIAL ARTICLE

Global Consensus on Keratoconus and Ectatic Diseases

José A. P. Gomes, MD, PhD, Donald Tan, MD, PhD,† Christopher J. Rapuano, MD,‡ Michael W. Belin, MD,§ Renato Ambrósio, Jr, MD, PhD,¶ José L. Guell, MD,‖ François Malecaze, MD, PhD,** Kohji Nishida, MD,†† and Virender S. Sangwan, MD‡‡, the Group of Panelists for the Global Delphi Panel of Keratoconus and Ectatic Diseases*

Background: Despite extensive knowledge regarding the diagnosis and management of keratoconus and ectatic corneal diseases, many controversies still exist. For that reason, there is a need for current guidelines for the diagnosis and management of these conditions.

Purpose: This project aimed to reach consensus of ophthalmology experts from around the world regarding keratoconus and ectatic diseases, focusing on their definition, concepts, clinical management, and surgical treatments.

Methods: The Delphi method was followed with 3 questionnaire rounds and was complemented with a face-to-face meeting. Thirty-six panelists were involved and allocated to 1 of 3 panels: definition/diagnosis, nonsurgical management, or surgical treatment. The level of agreement considered for consensus was two thirds.

Results: Numerous agreements were generated in definitions, methods of diagnosing, and management of keratoconus and other ectatic diseases. Nonsurgical and surgical treatments for these conditions, including the use of corneal cross-linking and corneal transplantations, were presented in a stepwise approach. A flowchart describing a logical management sequence for keratoconus was created.

Conclusions: This project resulted in definitions, statements, and recommendations for the diagnosis and management of keratoconus and other ectatic diseases. It also provides an insight into the current worldwide treatment of these conditions.

Key Words: keratoconus, corneal ectasia, consensus, corneal cross-linking, corneal transplantation

(*Cornea* 2015;0:1–11)

Keratoconus and ectatic corneal diseases have been recognized for more than 150 years.[1,2] Over the last 2 decades, there has been a revolution in the knowledge related to the diagnosis and management of these conditions. In terms of diagnosis, the advent of corneal topography, and more recently corneal tomography, has increased the ability of ophthalmologists to identify corneal ectasia at a much earlier stage than was previously possible.[3] As a result, the previously established prevalence of keratoconus of approximately 1/2000 among the general population[4] has been challenged with much higher prevalence rates found in many parts of the world.[5,6]

The surgical treatment for keratoconus reflects this evolution.[7] Alternative procedures, such as the use of intrastromal corneal ring segment(s) (ICRS),[8,9] corneal cross-linking (CXL),[10–12] therapeutic excimer laser treatments including phototherapeutic keratectomy[13] and photorefractive

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Supported by the Asia Cornea Society, The Cornea Society, EuCornea, and the Panamerican Cornea Society (PanCornea).
The authors have no conflicts of interest to disclose.
Study development and scientific support by Coordinators of the panels. Web portal development, conduction of statistical analyses, and medical writing support by Eurotrials, Scientific Consultants S.A. Coordinators who were involved in development of the round questionnaires, moderation of the panels, discussion of the round results, and writing of the manuscript (J.A.P.G., D.T., C.J.R., J.L.G., R.A., M.W.B., F.M., K.N., and V.S.). Panelists who were involved in the questionnaires responses and discussion at the face-to-face meeting in Chicago: Alan El Danasoury, Aldo Caporossi, Beatrice Cochener, Choun-Ki Joo, Christopher R. Crnadale, Daniel H. Scorsetti, Deborah Jacobs, Denise de Freitas, Enrique Graue-Hernandez, Enzo Samicola, Farhad Hafezi, Friedrich Kruse, Florence Malet, George D. Kymionis, Gerard Sutton, Harminder S. Dua, Irving Raber, Jodhbir Mehta, Juan C. Abad, Luis Izquierdo Jr, Luis A. Rodriguez, Marian Macsai, Mauro SQ Campos, Nasuyki Maeda, Penny A. Asbell, Prema Padmanabhan, Rajesh Fogla, Richard Davidson, Robert Feder, Roberto G. Albertazzi, Samar Basak, Sheraz Daya, Shigeto Shimmura, Stephen Kaufman, Victor L. Perez, and Wolf Wonneberger.
Asia Cornea Society, Cornea Society, EuCornea, and PanCornea also contributed in both logistical support and in funding the face-to-face meeting in Chicago.
This project was primarily fundad by an independent educational grant by the Asia Cornea Foundation. The funding body had no role in the design, implementation, or interpretation of the results of this project. This funding defrayed the cost of statistical analysis, 4 rounds of the Delphi Panels, printed materials, portfolio, supplements, questionnaires, and travel of coordinators of Eurotrials to the face-to-face meeting in Chicago. All the corneal societies contributed in both logistical support and also in funding the face-to-face meeting in Chicago and potential publication expenses.
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- Currently, there is no clinically adequate classification system for keratoconus

- The following findings are mandatory to diagnose keratoconus
  - Abnormal posterior elevation
  - Abnormal corneal thickness distribution
  - Clinical noninflammatory corneal thinning

- The pathophysiology of keratoconus is likely to include the following components
  - Genetic disorder
  - Biochemical disorder
  - Biomechanical disorder
  - Environmental disorder

Gomes JA, Tan D, Rapuano CJ, Belin MW, Ambrósio R Jr, Guell JL, Malecaze F, Nishida K, Sangwan VS; Group of Panelists for the Global Delphi Panel of Keratoconus and Ectatic Diseases. Global consensus on keratoconus and ectatic diseases. Cornea. 2015 Apr;34(4):359-69.

Published Rates of Eye Disease

- Variable across populations and studies
- Highest published rate of keratoconus: 2.34%

First Author	Year	Sample Size	Age Range	Location	Source	Prevalence Rate (per 100,000)
Hofstetter	1959	13395	1-79	USA	General	600
Tanabe	1985	2601	10-60	Japan	Hospital	9
Kennedy	1986	64 KC	12-77	USA	Hospital	54.5
Santiago	1995	670	18-22	France	Army	1190
Gorskova	1998	-	-	Russia	Hospital	0.2-0.4
Pearson	2000	382	10-44	UK	Hospital	57 (white) 229 (Asian)
Nielson	2007	772	-	Denmark	National Registry	86
Jonas	2009	4667	> 30	India	General	2300
Milodot	2011	981	18-54	Israel	Student	2340
Reeves	2009	-	> 65	USA	Medicare	15.7 18.3
Alalyeoba	2007	1144	4-24	Nigeria	Student	440
Ziaei	2012	536		Iran	General	24.9
Waked	2012	92	23.6 Mean	Lebanon	Student	3300
Owens	2007	198	16.8 Mean	Maori New Zealand	Student	1538 (KC) 26,900 (Suspect)

Incidence of Keratoconus in Australia[5]

- How many subclinical or “form fruste”?

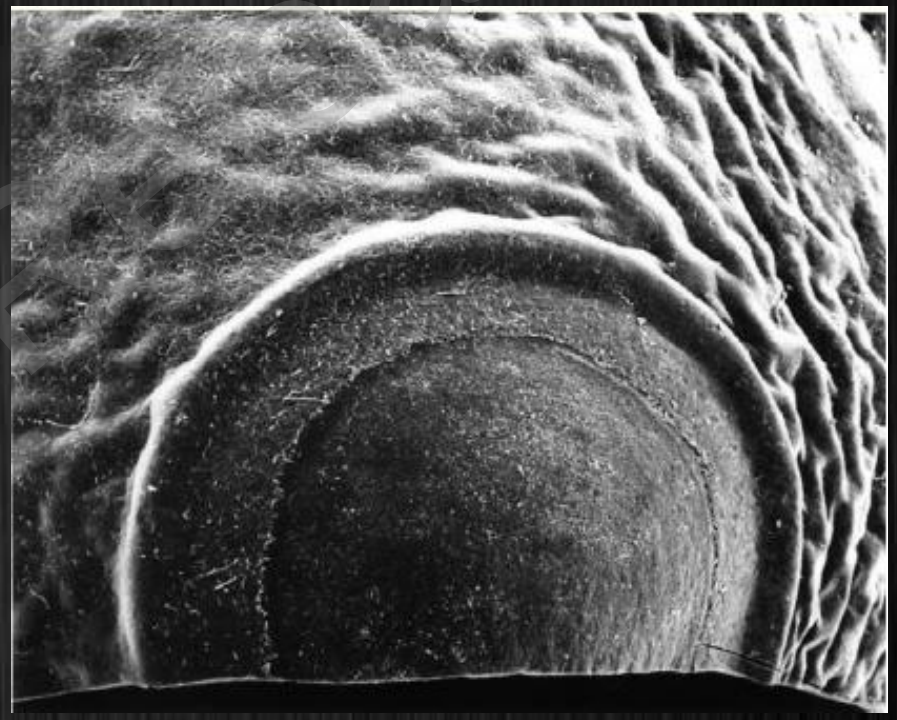
- **Investigation of keratoconus in an Australian refractive population.** Hodge C, Chan C, *Sutton G.* Clin Experiment Ophthalmol. 2014 Nov;42(8):796-8.

Diagnosis	Number of cases	Mean age ± SD (range)	Male/Female Ratio
FFKC	98 (1.53%)	37.5 ± 11.6 years (20 - 67)	53.1% / 46.9%
KC	63 (0.99%)	37.7 ± 11.5 years (17 - 64)	55.6% / 44.4%
Corneal Warpage	19 (0.30%)	38.3 ± 8.7 years (24 - 61)	10.5% / 89.5%

2 “Hit” Hypothesis[14]

- Genetically predisposed Individual[15]

- 2nd Hit
  - Eye rubbing [16]
  - LASIK[17]
  - Second Genetic Defect[18]
  - CL wear [3]



Courtesy Prof. J.Marshall PhD

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The Keratoconus Mystery

- “Keratoconus” may be the end stage of multiple disease processes with varied pathogeneses, all of which share final anatomical and clinical similarities

- “The mechanical stress on the cornea imposed by eye rubbing may not be as much as a second hit evoking the structural changes of a predisposed cornea … but rather the necessary trigger and sine qua non of the keratoconus process.”

Role of inflammation in keratoconus

Comea

Elevated Expression of Matrix Metalloproteinase-9 and Inflammatory Cytokines in Keratoconus Patients Is Inhibited by Cyclosporine A

Rohit Shetty,[1] Anuprita Ghosh,[2] Rayne R. Lim,[3] Murali Subramani,[2] Krina Mihir,[2] Reshma A. R.,[2] Ashwini Ranganath,[1] Sriharsha Nagaraj,[1] Rudy M. M. A. Nuijts,[4] Roger Beuerman,[5] Reshma Shetty,[2] Debashish Das,[2] Shyam S. Chaurasia,[3,5,6] Abhijit Sinha-Roy,[2] and Arkasubhra Ghosh[2,3]

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RS and AG contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Purpose. The present study was designed to understand the role of inflammatory cytokines secreted by corneal epithelial cells in keratoconus (KC) and the response to treatment with cyclosporine A (CyA).

Methods. The study involved 129 Indian KC patients clinically graded according to Amsler-Krumeich classification and 20 healthy, nonectatic subjects as controls. Tear levels of matrix metalloproteinase-9 (MMP9), interleukin-6 (IL6), and tumor necrosis factor-α (TNFα) were measured using ELISA kits. Gene expression was measured by qPCR in corneal epithelial cells obtained by debridement from subjects undergoing ocular surface surgeries. In addition, epithelial cells were stimulated with TNFα and treated with CyA to study its role on MMP9 expression. Finally, 20 KC patients (27 eyes) with inflammatory symptoms were treated with topical CyA application.

Results. We observed that MMP9, TNFα, and IL6 levels were strongly upregulated at the mRNA level in KC patient epithelia. Similarly, tears collected from KC patients exhibited high levels of MMP9 and IL6 protein. Cyclosporine A treatment significantly reduced the mRNA expression levels of IL6 and TNFα in both short- and long-term treatments; however, it reduced MMP9 levels only in long-term treatment in cultured corneal epithelial cells. Subsequent treatment of KC patients with CyA for approximately 6 months reduced tear MMP9 levels and led to local reduction in corneal curvatures as determined by corneal topography maps.

Conclusions. The data indicate that corneal epithelium contributes to elevated MMP9 and inflammatory cytokine expression in tears of KC patients. Cyclosporine A treatment reduced MMP9 and inflammatory cytokine levels in an in vitro inflammation model system. In KC patients, CyA treatment reduced MMP9 levels measured in tears with concomitant arrest of disease progression. Therefore, CyA might be a novel treatment strategy in KC patients but requires additional evaluation in larger cohorts. (ClinicalTrials.gov number, NCT01746823.)

Keywords: keratoconus, cyclosporine A, MMP9, TNFα, IL6, ectasia, cornea, epithelial cells

Keratoconus (KC) is a common dystrophy of the cornea causing stromal thinning and astigmatism resulting in loss of visual acuity.[1,2] The etiology of KC is poorly understood, and factors driving ectasia still remain unclear. However, atopy, eye rubbing, inflammatory factors, and hard contact lenses have been associated with KC.[3–5] Previous reports suggest that progression of KC could be related to allergy[6,7] or oxidative stress.[8,9] Recent clinical reports on KC have suggested that its pathogenesis involves inflammatory mediators and matrix degrading proteins.[4,6,10–12] However, currently, it is not clear whether these proteins are the major factors driving ectasia. Furthermore, there are no drugs available to halt the progression of the disease; thereby, this necessitates surgical intervention such as corneal crosslinking and keratoplasty.[13,14]

Several studies investigating biochemical and pathologic changes[15–17] in structural and cellular levels of cornea have been reported,[18] but specific molecular mechanisms involved in KC pathogenesis are not fully elucidated. Although the structural integrity of the cornea is disrupted in KC,[17] there are few differences in the type or content of collagen architec-

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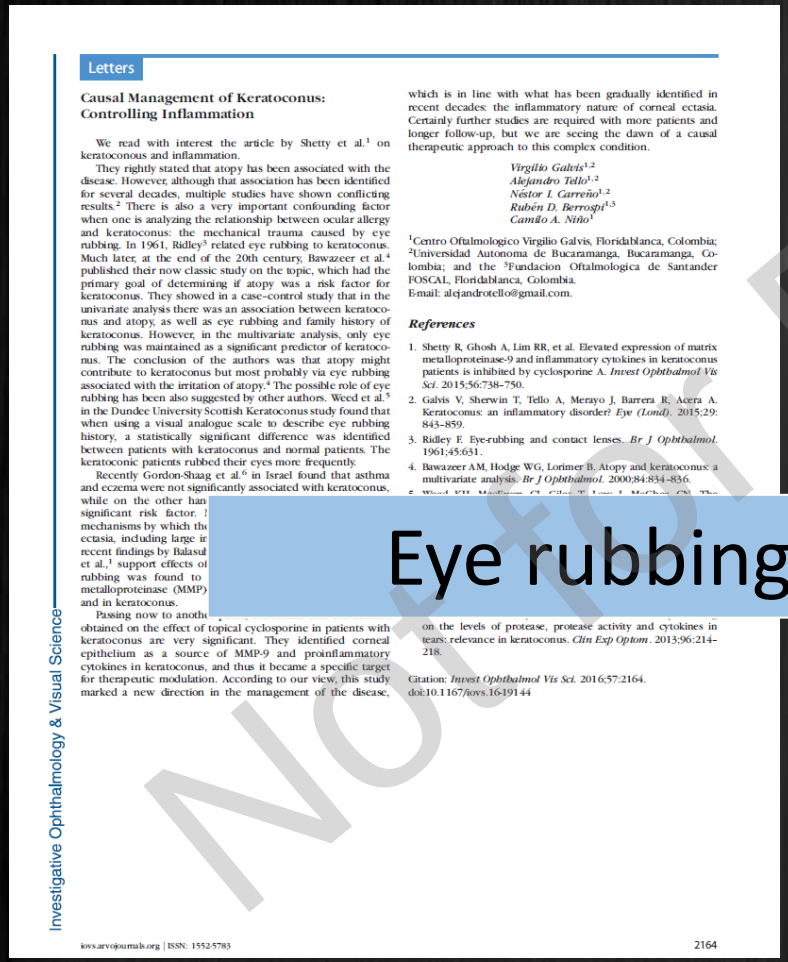
Investigative Ophthalmology & Visual Science

Role of inflammation in keratoconus



Letters

Causal Management of Keratoconus: Controlling Inflammation

We read with interest the article by Shetty et al.[1] on keratoconous and inflammation.

They rightly stated that atopy has been associated with the disease. However, although that association has been identified for several decades, multiple studies have shown conflicting results.[2] There is also a very important confounding factor when one is analyzing the relationship between ocular allergy and keratoconus: the mechanical trauma caused by eye rubbing. In 1961, Ridley[3] related eye rubbing to keratoconus. Much later, at the end of the 20th century, Bawazeer et al.[4] published their now classic study on the topic, which had the primary goal of determining if atopy was a risk factor for keratoconus. They showed in a case-control study that in the univariate analysis there was an association between keratoconus and atopy, as well as eye rubbing and family history of keratoconus. However, in the multivariate analysis, only eye rubbing was maintained as a significant predictor of keratoconus. The conclusion of the authors was that atopy might contribute to keratoconus but most probably via eye rubbing associated with the irritation of atopy.[4] The possible role of eye rubbing has been also suggested by other authors. Weed et al.[5] in the Dundee University Scottish Keratoconus study found that when using a visual analogue scale to describe eye rubbing history, a statistically significant difference was identified between patients with keratoconus and normal patients. The keratoconic patients rubbed their eyes more frequently.

Recently Gordon-Shaag et al.[6] in Israel found that asthma and eczema were not significantly associated with keratoconus, while on the other han
significant risk factor.
mechanisms by which th
ectasia, including large ir
recent findings by Balasul
et al.,[7] support effects o
rubbing was found to
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Passing now to anoth
obtained on the effect of topical cyclosporine in patients with keratoconus are very significant. They identified corneal epithelium as a source of MMP-9 and proinflammatory cytokines in keratoconus, and thus it became a specific target for therapeutic modulation. According to our view, this study marked a new direction in the management of the disease, which is in line with what has been gradually identified in recent decades: the inflammatory nature of corneal ectasia. Certainly further studies are required with more patients and longer follow-up, but we are seeing the dawn of a causal therapeutic approach to this complex condition.

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Citation: *Invest Ophthalmol Vis Sci*. 2016;57:2164.
doi:10.1167/iovs.16-19144

Letters

Author Response: Causal Management of Keratoconus: Controlling Inflammation

We are glad to receive comments from Galvis et al.[1] on our study published in *IOVS*.[2] We agree with their suggestions and appreciate the fact that inflammation is now considered one of the major components of keratoconus (KC) pathophysiology. As they rightly point out, our study in fact comes at the heels of an increasing number of reports indicating that the molecular drivers of KC may be inflammation-dependent factors causing matrix degradation.[3–7] Furthermore, the inflammation observed in KC is somewhat skewed, resembling chronic inflammation associated with systemic diseases rather than acute inflammation.[8] This is evidenced by the fact that acute inflammation proteins such as TNF-α are not overly expressed in KC, but chronic inflammation-associated target genes such as matrix metalloproteinase (MMP) 9 are significantly high. In fact, MMP9 is known to reduce barrier functions,[9] providing a chronic low-grade stimulation for the sustained inflammatory milieu. The KC disease pathology shows a similar phenomenon in not being associated with red eyes but evidently correlated with atopy, eye rubbing, and mechanical damage, which may be due to low-level irritation caused by an underlying subclinical, chronic inflammation. This is supported by the fact that ectasia progresses at variable rates in keratoconic patients. Rehany and Rumelt[10] have reported an association of vernal keratoconjunctivitis (VKC) and early occurrence of corneal hydrops in KC, suggesting a role of inflammation in disease pathophysiology. In our previous clinical study, we noticed a higher incidence (55.5%) of progressive KC in patients with VKC. In the same study, we also observed a higher failure rate (17.6%) after corneal collagen cross-linking in

factor to be considered in KC.[13] It is known that inflammation can actually lead to a transcriptional deregulation of the collagen genes[14] essential for maintaining tissue architecture. This might be an important contributor to the structural weakness observed in KC patient corneas.[15] However, one wonders whether patients with inflammatory-driven dry eye disease or ocular allergies are predisposed to KC. In addition, can we restore corneal structural strength by controlling inflammation? These questions remain to be solved and thereby open new avenues to investigate mechanisms involved in KC. Most importantly, we feel that management of inflammation can be an important first step in the management and progression of KC.

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doi:10.1167/iovs.16-19532

Eye rubbing vs. Inflammation

Eye rubbing as an integral factor in keratoconus

IJKECD

10.5005/jp-journals-10025-1114

REVIEW ARTICLE

Eye Rubbing, a *Sine Qua Non* for Keratoconus?

Damien Gatinel

ABSTRACT

Keratoconus, a dystrophy of unknown origin, remains an ophthalmic enigma. The contrast between the presence of marked structural changes and deformation of the corneal wall and the relative absence of specific genetic and biomolecular findings continues to intrigue ophthalmologists. In Marfan syndrome, where genetic and molecular abnormalities are well identified, and similar changes in collagen observed, the cornea tends not to be steeper, irregular or ectatic, but are globally flatter. This suggests that an external mechanical force may be necessary to induce the apparition and progression of the ectatic process in keratoconus. Eye rubbing has long been acknowledged as a risk factor for keratoconus and its progression, but could it in fact be the root cause? Many clinical observations and reports support the hypothesis of eye rubbing as a first and necessary hit for inducing progressive ectatic deformation of the corneal wall. Validating or refuting this hypothesis on the basis of patient admission may be impossible. It is difficult to document the frequency, duration and intensity of eye rubbing in patients with keratoconus, and virtually impossible to prove that every patient who denies the habit truly does not rub his eyes. Both the increase in incidence of atopy and the time spent in front of the computer screen in the general population may account for an increased tendency for eye rubbing, and lead to the perceived increased prevalence of keratoconus in both urban and non-urban areas. This paper explores the possibility that the mechanical stress imposed on the cornea by rubbing may not be as much a second hit evoking the structural changes of a predisposed cornea exhibiting unknown collagen progressive alteration, but rather the necessary trigger and sine qua non of the keratoconic process. Even if this provocative hypothesis is impossible to prove, it is equally difficult to refute, and acknowledging eye rubbing as a possible root cause increases awareness within the general population and if true, could dramatically reduce the incidence of keratoconus, and halt its progression in eyes already affected.

Keywords: Computer vision syndrome, Corneal biomechanics, Crosslinking, Ectasia, Eye rubbing, Etiology of keratoconus, Keratoconus, Marfan syndrome.

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Conflict of interest: None

INTRODUCTION

"When the wise man points at the Moon, the idiot looks at the finger." This famous quote is attributed to Confucius and should encourage every clinician and researcher to look beyond the realm of existing evidence when facing the challenging mysteries of various diseases.

Keratoconus (KC) has long fascinated ophthalmologists, and its etiology has remained an enigma since this disease was first characterized in the mid-19th century. As an ophthalmologist whose practice is focused on KC detection and management, I have been continually intrigued by the pathophysiology of such a mysterious disease, with the exact mechanism of its hallmark characteristics of progressive thinning and deformation still not fully understood. Ironically, to better understand KC, it may be necessary to forsake the Confucius doctrine and focus our attention literally on the patient's fingers. In doing so, we may better understand the innate mechanism of induction and progression of this enigmatic disease. This shall be the focal point of my article.

"Keratoconus" refers to the abnormal conical shape of the corneal contour, which steepens centrally and flattens peripherally. Slit lamp findings of stromal thinning, scarring, and macroscopically detectable deformation of the corneal dome giving rise to Munson's sign are frequently encountered in patients with a long documented history of KC. A "cone" is a mathematical figure that is infinitely steep at its apex and completely flat on its envelope. Hence, "keratoconus" is merely a descriptive term, which fails to detail the underlying mechanism that results in this unique change to the corneal shape.

Through the many years of my clinical practice, I have encountered seemingly countless patients with KC, either newly diagnosed or with long-standing disease. In my practice, men seem slightly more affected than women, but the age at which the disease is discovered is quite variable, as is the stage of the disease at the time of diagnosis. It is not rare to observe large differences between the right and left eyes of the same patient.

Several years ago, as I was examining a patient with a severe form of the disease, where the corneas were so scarred and protruding that there was no need of a slit lamp exam to raise the suspicion of KC, a question inadvertently arose in my mind, as if I was looking for answers to the enigmatic origin of KC for the first time: I suddenly wondered how a quiescent organ such as the cornea could undergo such a dramatic morphological change, when there were so few histopathological findings and no real established biological cascade to explain it?

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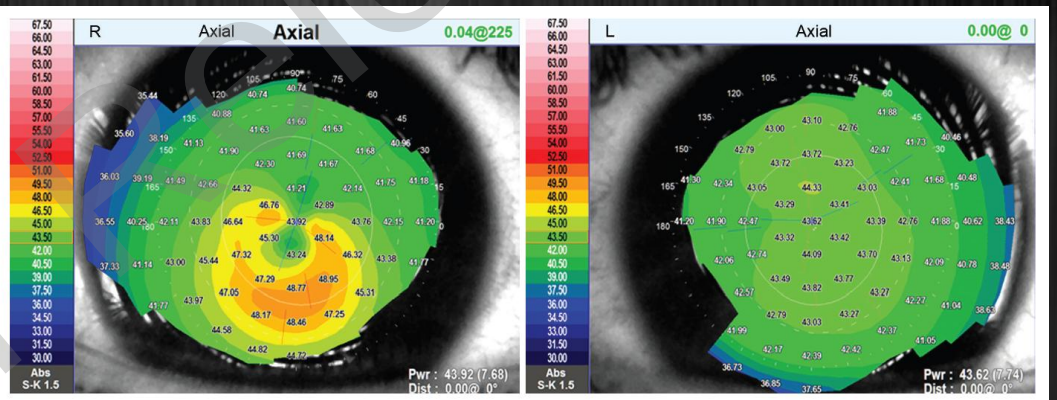


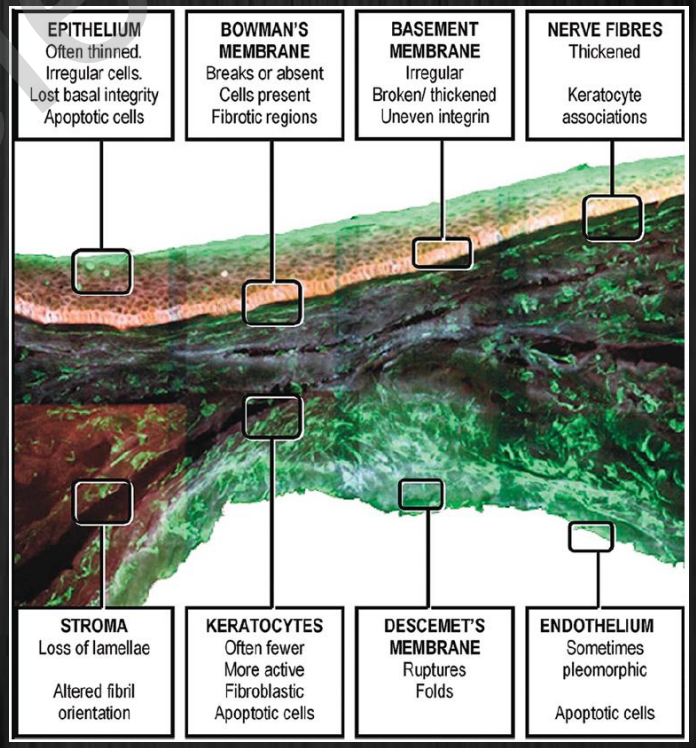
Fig. 1: Topographical aspect of a "unilateral" keratoconus in the right eye (axial map, OPDscan III) in a 34-year-old patient who denies rubbing his eyes vigorously. The corneal pachymetry was 550 µm in both eyes centrally. However, the patient admitted sleeping on the right side, with the pillow placed under his head and applied to the right side of his face by his right hand. During a subsequent visit, the patient recognized that he realized that he often rubbed his eyes, once having been warned of this eventuality. The combination of repeated daytime rubbing and nighttime compression of the right eye's cornea may explain the increased severity of its topographic deformation

Cornea Research Team
Save Sight Institute, Sydney University

- Dr Jing Jing You
- A/Professor Michele Madigan
- Dr Li Wen
- Dr Chris Hodge
- Dr Simon Cooper
- Dr Meidong Zhu
- Dr Con Petsoglou
- Dr John Males
- Dr Athena Roufas
- Professor Gerard Sutton

Histopathology[10]

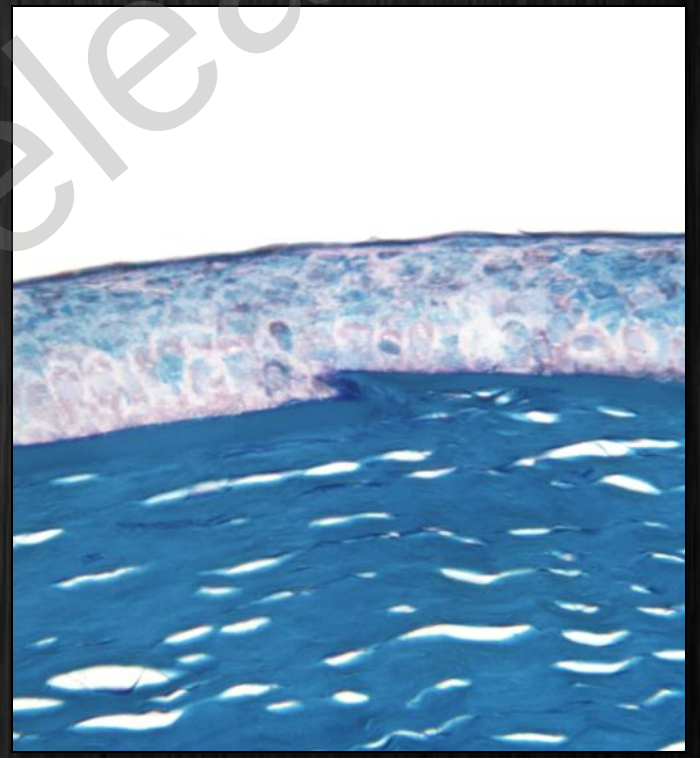
- Central, Paracentral thinning
- Breaks in Bowman's
  - (Descemet's membrane)
- Keratocyte density reduced
- Keratocyte apoptosis



10. Erie, J.C., et al. Am J Ophthalmol 2009;147(5):741-748.

Focus on KC epithelium

- Earliest changes (elongated epithelial cells)[11]
- Lack of basal epithelial integrity and oedema[12]
- Apoptosis in basal epithelium >90% cf 0% control[13]
- Removal of epithelium causes keratocyte apoptosis not vv



[11] Sherwin et al . Clin exp Ophthal 2004
[12] Somadi et al Ger J ophtal 1997
[13] Kaldawy et al Cornea 2002

Keratoconus: Cascade

Genetically predisposed individual

↓

Second hit

↓

Altered signaling to stromal keratocytes, neural abnormalities

↓

Apoptosis, enzymatic degradation

↓

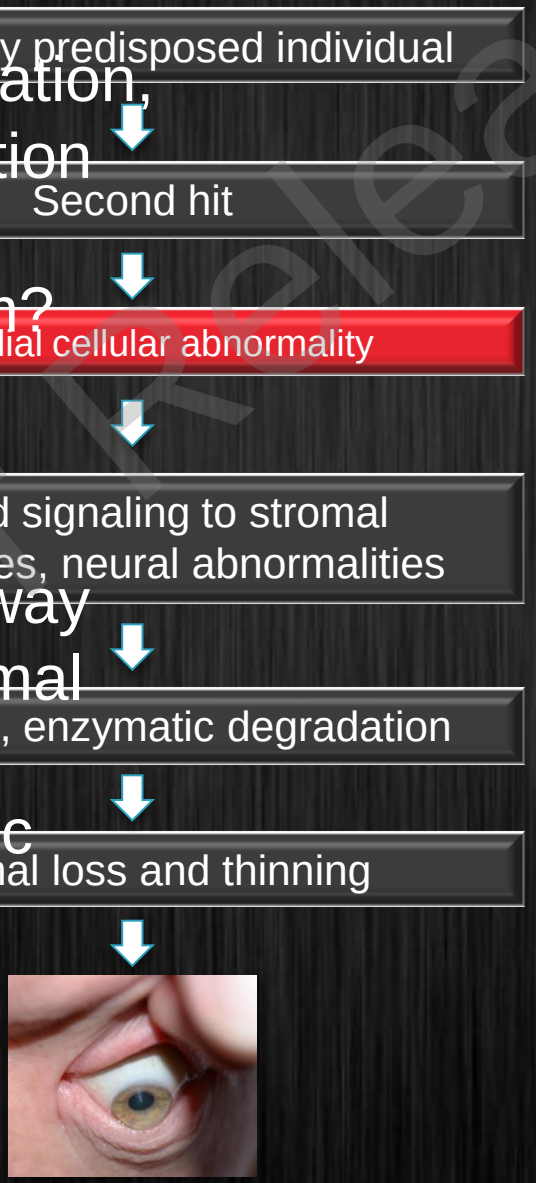
Stromal loss and thinning

↓

Keratoconus: Questions

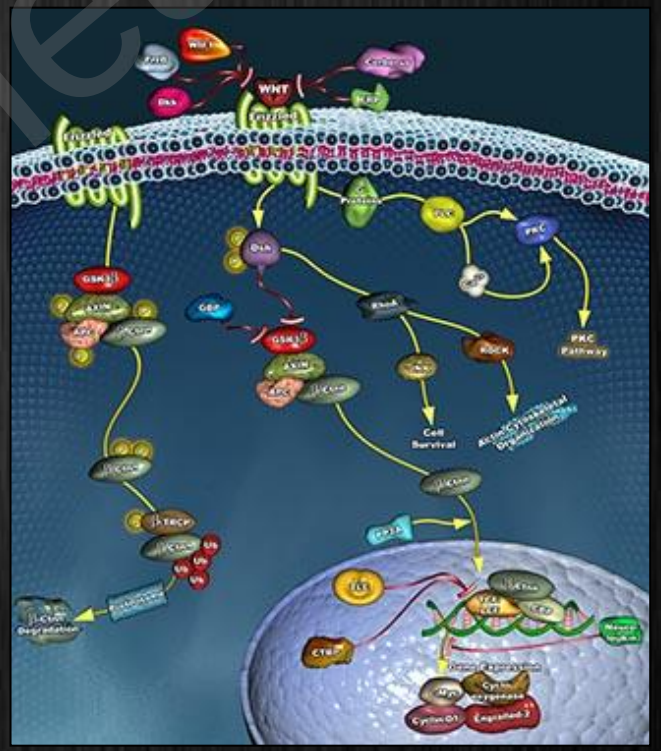
Is KC an epithelial migration, differentiation, proliferation and/or a stromal communication problem?

Is there a candidate cellular metabolic pathway or protein that is abnormal that we can target for diagnostic of therapeutic purposes?



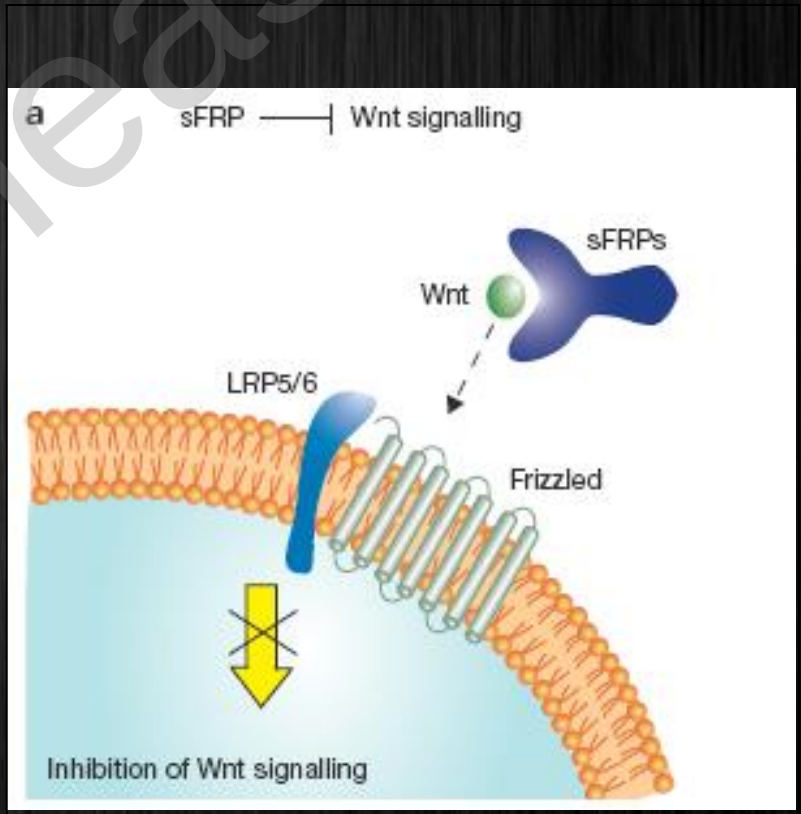
Wnt Signalling Pathway

- The Wnt signaling pathway describes a complex network of proteins
- Physiological processes including cell polarity, proliferation, migration, apoptosis
- Ubiquitous in mammals

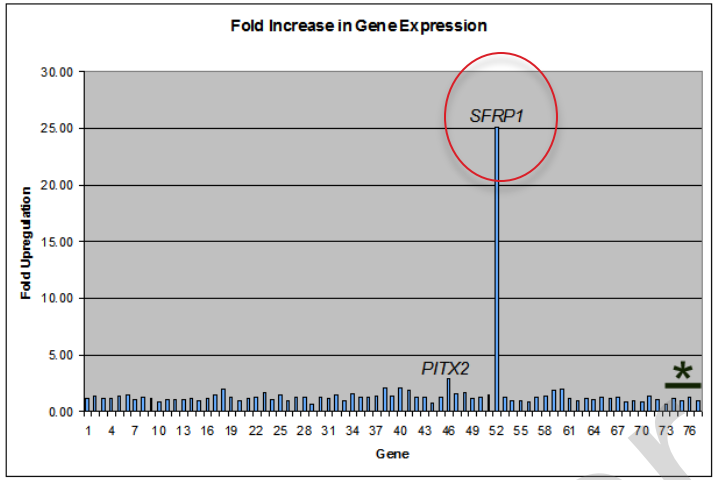


sFRP

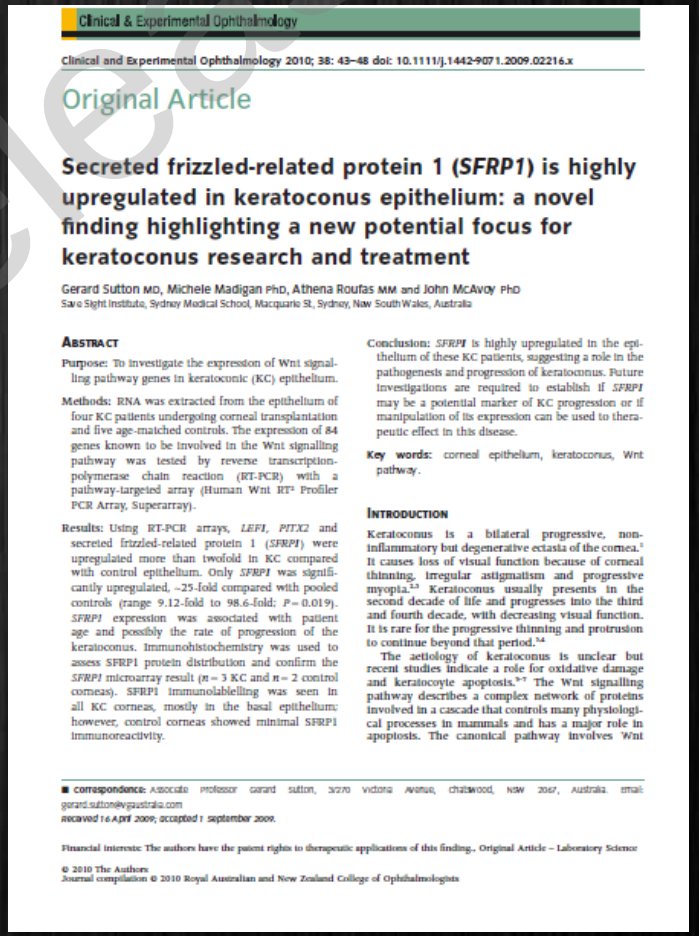
- Secreted Frizzled related Proteins
- 5 in humans
- Act as extracellular signalling ligands
- Apoptosis, cellular migration and proliferation



sFRP1: Initial Results



Gene	Fold Change (KC vs Control)	P-value
FOSL1	-1.5674	NS (P=0.073)
FZD7	1.77	NS (P=0.065)
JUN	1.5721	NS (0.515)
LEF1	**2.2082**	**P=0.013**
LRP5	**1.605**	**P=0.0106**
SFRP1	**10.865**	**P=0.0017**
WISP1	1.8256	NS (P=0.191)
WNT2B	1.589	NS (P=0.117)
WNT5A	-2.253	NS (P=0.16)
WNT16	-2.0258	NS (P=0.254)
Control Genes	**Fold Change (KC vs Control)**	**P-value**
B2M	1.1827	NS (P=0.49)
RPL13A	1.0324	NS (P=0.741)
ACTB	-1.272	NS (P=0.203)



Clinical & Experimental Ophthalmology

Clinical and Experimental Ophthalmology 2010; 38: 43–48 doi: 10.1111/j.1442-9071.2009.02216.x

Original Article

Secreted frizzled-related protein 1 (*SFRP1*) is highly upregulated in keratoconus epithelium: a novel finding highlighting a new potential focus for keratoconus research and treatment

Gerard Sutton MD, Michele Madigan PhD, Athena Roufas MM and John McAvoy PhD
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Abstract

Purpose: To investigate the expression of Wnt signalling pathway genes in keratoconic (KC) epithelium.

Methods: RNA was extracted from the epithelium of four KC patients undergoing corneal transplantation and five age-matched controls. The expression of 84 genes known to be involved in the Wnt signalling pathway was tested by reverse transcription-polymerase chain reaction (RT-PCR) with a pathway-targeted array (Human Wnt RT² Profiler PCR Array, Superarray).

Results: Using RT-PCR arrays, *LEF1*, *PITX2* and secreted frizzled-related protein 1 (*SFRP1*) were upregulated more than twofold in KC compared with control epithelium. Only *SFRP1* was significantly upregulated, ~25-fold compared with pooled controls (range 9.12-fold to 98.6-fold; $P = 0.019$). *SFRP1* expression was associated with patient age and possibly the rate of progression of the keratoconus. Immunohistochemistry was used to assess SFRP1 protein distribution and confirm the *SFRP1* microarray result ($n = 3$ KC and $n = 2$ control corneas). SFRP1 immunolabelling was seen in all KC corneas, mostly in the basal epithelium; however, control corneas showed minimal SFRP1 immunoreactivity.

Conclusion: *SFRP1* is highly upregulated in the epithelium of these KC patients, suggesting a role in the pathogenesis and progression of keratoconus. Future investigations are required to establish if *SFRP1* may be a potential marker of KC progression or if manipulation of its expression can be used to therapeutic effect in this disease.

Key words: corneal epithelium, keratoconus, Wnt pathway.

Introduction

Keratoconus is a bilateral progressive, non-inflammatory but degenerative ectasia of the cornea.[1] It causes loss of visual function because of corneal thinning, irregular astigmatism and progressive myopia.[2,3] Keratoconus usually presents in the second decade of life and progresses into the third and fourth decade, with decreasing visual function. It is rare for the progressive thinning and protrusion to continue beyond that period.[3,4]

The aetiology of keratoconus is unclear but recent studies indicate a role for oxidative damage and keratocyte apoptosis.[5–7] The Wnt signalling pathway describes a complex network of proteins involved in a cascade that controls many physiological processes in mammals and has a major role in apoptosis. The canonical pathway involves Wnt

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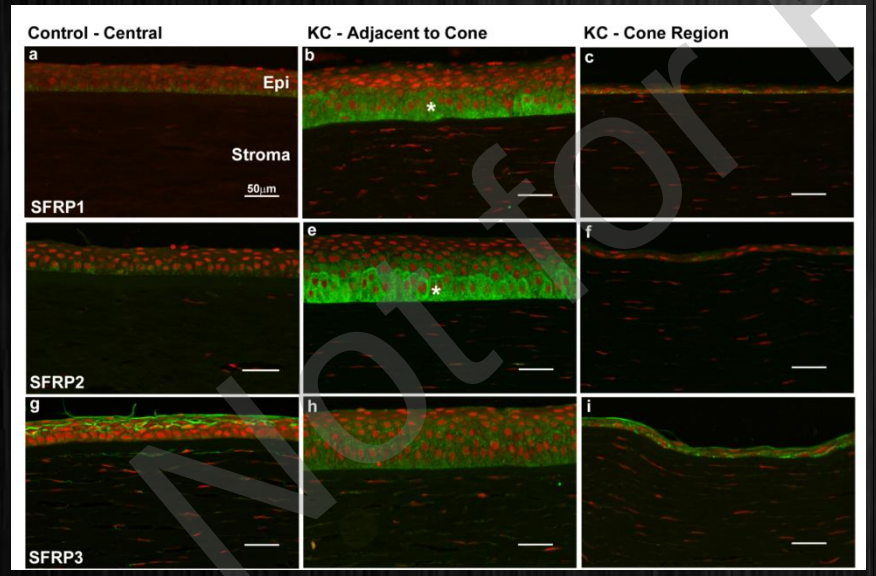
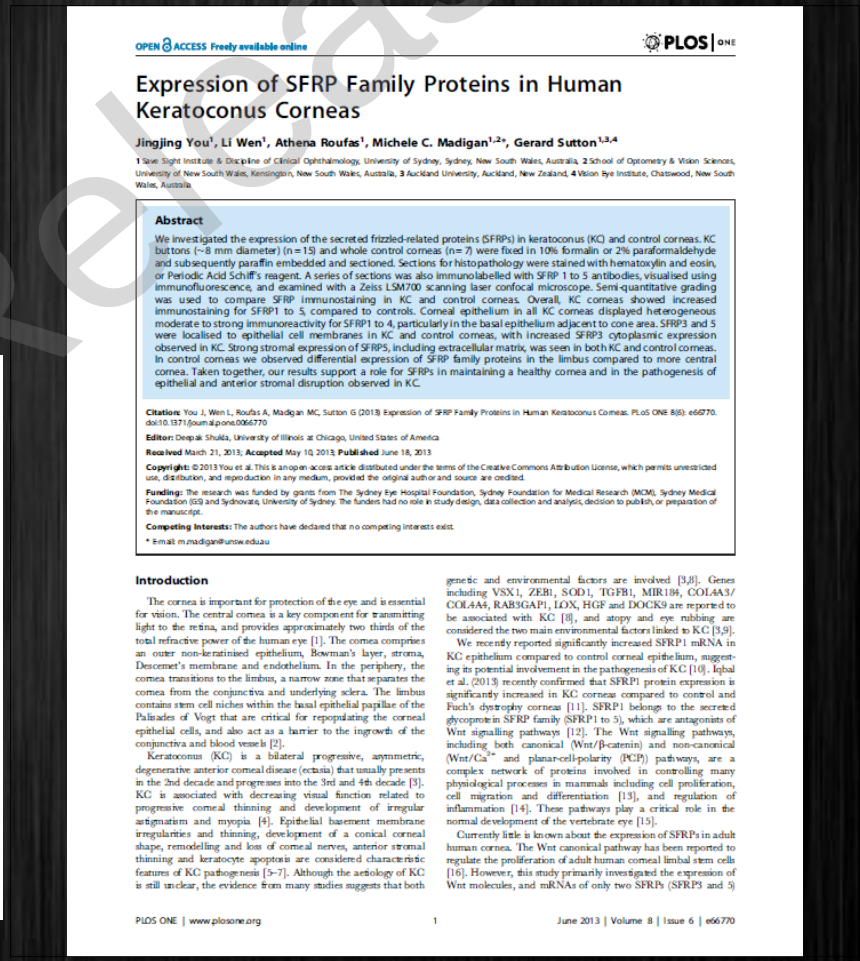
Financial interests: The authors have the patent rights to therapeutic applications of this finding., Original Article – Laboratory Science

Immunolabeling of SFRP family

- To examine the SFRPs (SFRP1-5) expression in KC and control corneas





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PLOS ONE

Expression of SFRP Family Proteins in Human Keratoconus Corneas

Jingjing You[1], Li Wen[1], Athena Roufas[1], Michele C. Madigan[1,2]*, Gerard Sutton[1,3,4]

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Abstract

We investigated the expression of the secreted frizzled-related proteins (SFRPs) in keratoconus (KC) and control corneas. KC buttons (~8 mm diameter) (n = 15) and whole control corneas (n = 7) were fixed in 10% formalin or 2% paraformaldehyde and subsequently paraffin embedded and sectioned. Sections for histopathology were stained with hematoxylin and eosin, or Periodic Acid Schiff's reagent. A series of sections was also immunolabelled with SFRP 1 to 5 antibodies, visualised using immunofluorescence, and examined with a Zeiss LSM700 scanning laser confocal microscope. Semi-quantitative grading was used to compare SFRP immunostaining in KC and control corneas. Overall, KC corneas showed increased immunostaining for SFRP1 to 5, compared to controls. Corneal epithelium in all KC corneas displayed heterogeneous moderate to strong immunoreactivity for SFRP1 to 4, particularly in the basal epithelium adjacent to cone area. SFRP3 and 5 were localised to epithelial cell membranes in KC and control corneas, with increased SFRP3 cytoplasmic expression observed in KC. Strong stromal expression of SFRP5, including extracellular matrix, was seen in both KC and control corneas. In control corneas we observed differential expression of SFRP family proteins in the limbus compared to more central cornea. Taken together, our results support a role for SFRPs in maintaining a healthy cornea and in the pathogenesis of epithelial and anterior stromal disruption observed in KC.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The cornea is important for protection of the eye and is essential for vision. The central cornea is a key component for transmitting light to the retina, and provides approximately two thirds of the total refractive power of the human eye [1]. The cornea comprises an outer non-keratinised epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium. In the periphery, the cornea transitions to the limbus, a narrow zone that separates the cornea from the conjunctiva and underlying sclera. The limbus contains stem cell niches within the basal epithelial papillae of the Palisades of Vogt that are critical for repopulating the corneal epithelial cells, and also act as a barrier to the ingrowth of the conjunctiva and blood vessels [2].

Keratoconus (KC) is a bilateral progressive, asymmetric, degenerative anterior corneal disease (ectasia) that usually presents in the 2nd decade and progresses into the 3rd and 4th decade [3]. KC is associated with decreasing visual function related to progressive corneal thinning and development of irregular astigmatism and myopia [4]. Epithelial basement membrane irregularities and thinning, development of a conical corneal shape, remodelling and loss of corneal nerves, anterior stromal thinning and keratocyte apoptosis are considered characteristic features of KC pathogenesis [5–7]. Although the aetiology of KC is still unclear, the evidence from many studies suggests that both genetic and environmental factors are involved [3,8]. Genes including VSX1, ZEB1, SOD1, TGFB1, MIR184, COL4A3/COL4A4, RAB3GAP1, LOX, HGF and DOCK9 are reported to be associated with KC [8], and atopy and eye rubbing are considered the two main environmental factors linked to KC [3,9].

We recently reported significantly increased SFRP1 mRNA in KC epithelium compared to control corneal epithelium, suggesting its potential involvement in the pathogenesis of KC [10]. Iqbal et al. (2013) recently confirmed that SFRP1 protein expression is significantly increased in KC corneas compared to control and Fuch's dystrophy corneas [11]. SFRP1 belongs to the secreted glycoprotein SFRP family (SFRP1 to 5), which are antagonists of Wnt signalling pathways [12]. The Wnt signalling pathways, including both canonical (Wnt/β-catenin) and non-canonical (Wnt/Ca^{2+} and planar-cell-polarity (PCP)) pathways, are a complex network of proteins involved in controlling many physiological processes in mammals including cell proliferation, cell migration and differentiation [13], and regulation of inflammation [14]. These pathways play a critical role in the normal development of the vertebrate eye [15].

Currently little is known about the expression of SFRPs in adult human cornea. The Wnt canonical pathway has been reported to regulate the proliferation of adult human corneal limbal stem cells [16]. However, this study primarily investigated the expression of Wnt molecules, and mRNAs of only two SFRPs (SFRP3 and 5)

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Methods in eye research

Using soybean trypsin inhibitor as an external loading control for Western blot analysis of tear proteins: Application to corneal disease

Jingjing You [a,*], Chris Hodge [a,b], Li Wen [a], John W. McAvoy [a], Michele C. Madigan [a,c], Gerard Sutton [a,b]

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1. Introduction

The quest for novel proteins that can be measured and quantified in biological fluids and related to stages of various diseases is an exciting field for biomarker and proteomics research. The most commonly accepted method of quantification is enzyme-linked immunosorbent assay (ELISA). However, commercial ELISA kits are not available for all proteins that have been detected, and subsequent preparation is both time consuming and expensive to develop. As a result, a reliable method of relative quantification using Western Blotting (WB), that has high specificity and sensitivity, and is low cost, is highly desirable at the early stages of quantifying these potential biomarkers.

The current procedures for quantitative WB have significant limitations. The absolute quantification approach using a standard curve constructed from purified proteins to measure the protein detected lacks a quality control step to ensure consistent protein loading or transfer efficiency. It also does not truly reflect the concentration of the protein in samples, because there is no guarantee of the complete transfer of proteins from the gel to the membrane. The protein concentration detected by WB is also usually less than the corresponding ELISA results, as shown for example in Ida et al. (1996).

Tears are a unique body fluid predominantly composed of mucins, proteins, lipids and salts, and are critical for maintaining the integrity of the ocular surface, including the conjunctiva and cornea. Proteomic investigation of the human tear proteome has identified approximately 500 proteins (de Souza et al., 2006; Green-Church et al., 2008). With the advantage of easy accessibility, tears have become the focus of biomarker research for a variety of both ocular (Grus et al., 2005; Jacob and Hamr, 2008; Pannebaker et al., 2010), and systemic conditions (Grus et al., 2002; Molloy et al., 1999; You et al., 2010).

Although the main functions of tears relate to protection of the eye from bacteria and lubrication of the ocular surface, tears can also provide nutrients and remove metabolic products from the corneal epithelium and anterior corneal stroma (Tiffany, 2003). It remains possible, therefore that subtle changes within the cornea itself may be evident through analysis of the precorneal tear film. It has also been suggested that the biochemistry of tears is similar to blood serum (Sastri and Ghafoori, 2008). Methods designed to identify and quantify proteins in tears can thus potentially be applied to other biological fluids.

Keratoconus (KC) is a bilateral, progressive degenerative disease of the cornea (Rabinowitz, 1998). The pathogenesis of KC remains

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Tear levels of SFRP1 are significantly reduced in keratoconus patients

Jingjing You,[1] Chris Hodge,[1,2] Li Wen,[1] John W. McAvoy,[1] Michele C. Madigan,[1,3] Gerard Sutton[1,2,4]

[1]Save Sight Institute, Discipline of Clinical Ophthalmology, The University of Sydney, Australia; [2]Vision Eye Institute, Australia; [3]School of Optometry & Vision Sciences, UNSW, Australia; [4]Auckland University, New Zealand

Purpose: To measure secreted frizzled-related protein 1 (SFRP1) levels in human tears and to investigate tear SFRP1 as a potential biomarker for keratoconus (KC).

Methods: Tears were collected from control (n=33) and KC patients (n=33) using micropipette tubes. Total tear protein was measured using a FluoroProfile Protein Quantification kit. An in-house enzyme-linked immunosorbent assay (ELISA) was developed to measure SFRP1 in control and KC tears. Statistical analyses of age, gender, the association of SFRP1, and total tear protein with KC were conducted.

Results: Tear SFRP1 was significantly decreased in KC, compared to age-matched controls (3.41 ng/μl±3.12 versus 5.55 ng/μl±5.62, respectively; p=0.039). Conversely, total tear protein was significantly increased in KC, compared to age-matched controls (12.38 μg/μl±4.76 versus 9.40 μg/μl±3.88, respectively; p=0.038). The ratio of SFRP1/total tear protein was also found to be significantly decreased in the KC group (p=0.007). No significant association between tear SFRP1 and total tear protein was detected.

Conclusions: Tear SFRP1 was significantly decreased in age-matched KC versus control patients, and may be further reduced in moderate KC. Tear-SFRP1 levels alone do not provide an obvious biomarker for KC; however, our results provide further evidence that tear-protein profiles are altered in KC, and suggest the involvement of SFRPs in the pathogenesis of KC.

Keratoconus (KC) is the most common primary degenerative corneal disease, with a prevalence of around 1 in 2,000 worldwide [1]. The condition often presents bilaterally with asymmetric progression, leading to corneal thinning and the development of an irregular corneal shape. Although it does not cause blindness, KC has been shown to significantly reduce perceived quality of life [2].

The clinical symptoms of KC vary depending on the stage of progression. In the early stages, clinical findings may be limited to specialized diagnostic tests such as corneal topography. In more advanced cases, visual acuity may not be adequately corrected with optical aids. Various management strategies and treatments are available, including soft and rigid gas-permeable contact lenses for mild to moderate cases and surgical interventions such as collagen cross-linking, intracorneal ring segments, and corneal transplantation for moderate to severe cases [3]. The major challenge for clinicians is to determine which treatment is most appropriate for the individual patient. While clinical and surgical experience is integral to patient management, it is limited by our understanding of the etiology and pathogenesis of KC. Biomarkers have been widely used in other diseases, such as cancer and diabetes, and a reliable biomarker for detecting patients before clinical symptoms associated with KC are reported would be clinically useful in providing more effective prognostication and options for treatment and management.

We previously detected significantly increased mRNA and protein expression of secreted frizzled-related protein 1 (SFRP1) in KC corneal epithelium, compared to controls [4]. The SFRP family of glycoproteins inhibits Wnt signaling pathways by binding to Wnts or Frizzled (Fzd) proteins, preventing formation of the Wnt-Fzd complex, essential for the activation of Wnt pathways [5]. SFRPs may also function independently of Wnt signaling pathways [6]. Altered SFRP1 expression has been reported to be associated with cell apoptosis in various conditions, including cancer [7], periodontitis [8], and bone disease [9]. In KC, apoptosis of the anterior stromal keratocytes is associated with loss of stromal extracellular matrix and corneal thinning [10].

Tears are increasingly used as a source for discovering protein biomarkers for both ocular and systemic diseases. We previously developed an immunoblotting technique to relatively quantify proteins in biological fluids such as tears [11]. To validate this technique, we analyzed a small number of KC and control samples and found relatively less tear SFRP1 in KC, compared to controls [11]. This contrasted with our earlier findings in KC corneal epithelium [4], so a

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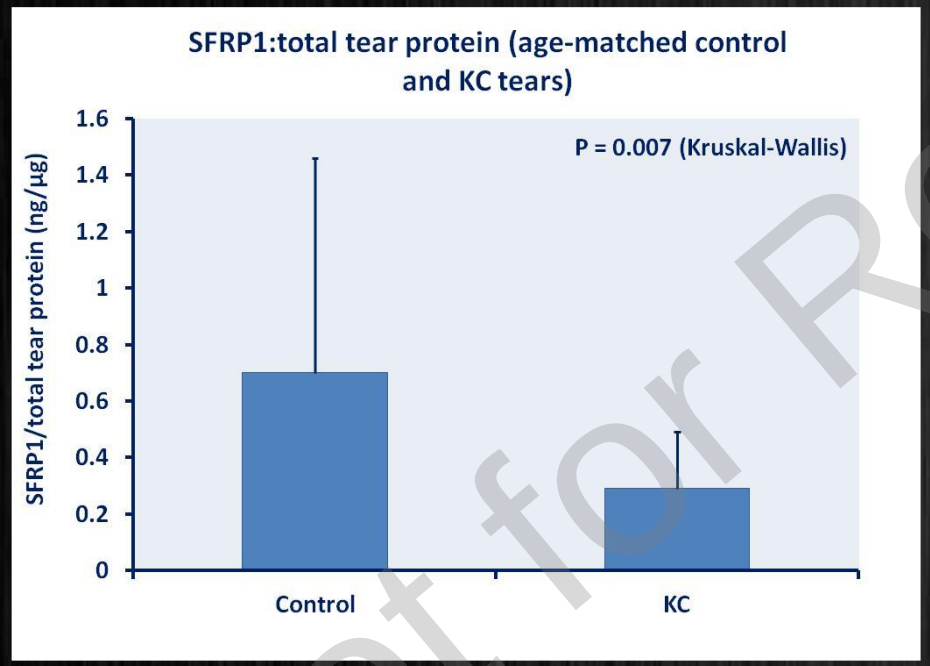
Tear Levels of SFRP1 in keratoconus



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[1]*Save Sight Institute, Discipline of Clinical Ophthalmology, The University of Sydney, Australia;* [2]*Vision Eye Institute, Australia;* [3]*School of Optometry & Vision Sciences, UNSW, Australia;* [4]*Auckland University, New Zealand*

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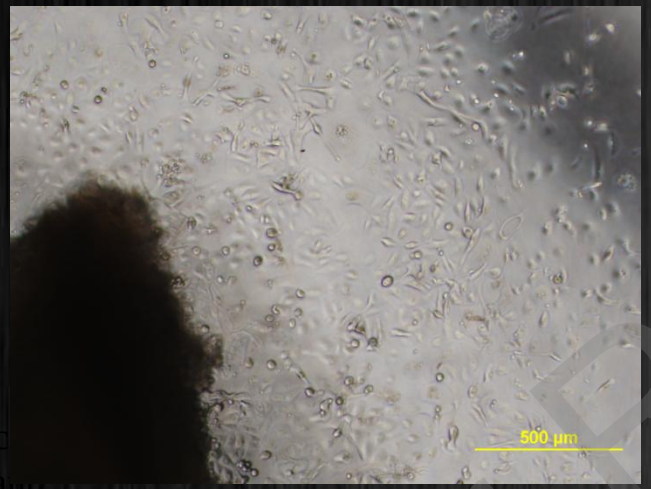
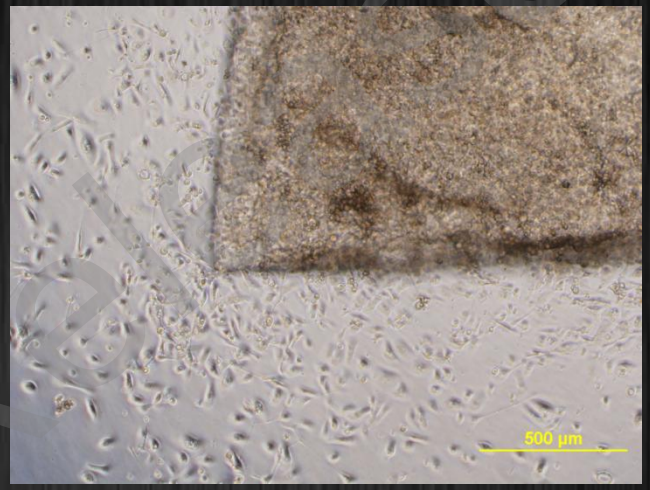
Control corneal epithelial cell outgrowth after 1 week

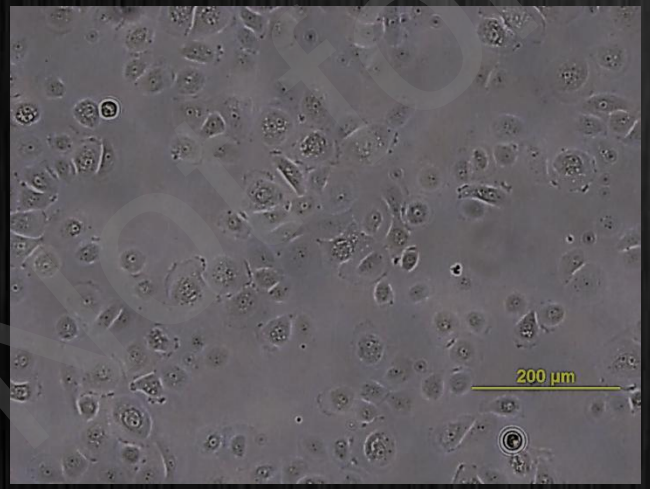


KC cornea epithelial cell outgrowth after 1 week

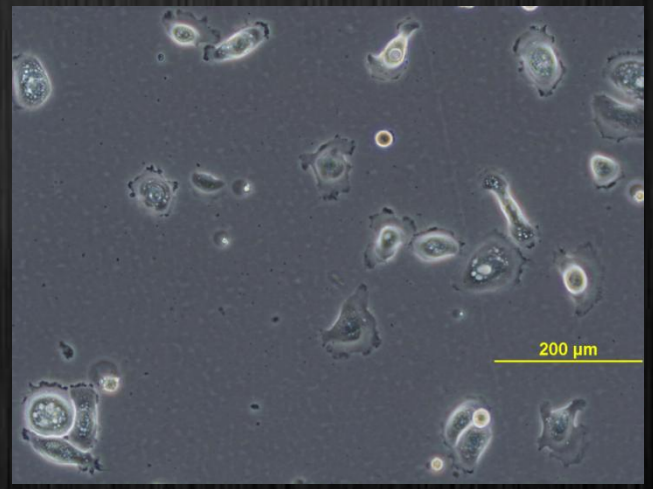


Control c
expla

Primary control corneal epithelial cells harvested from explant outgrowth

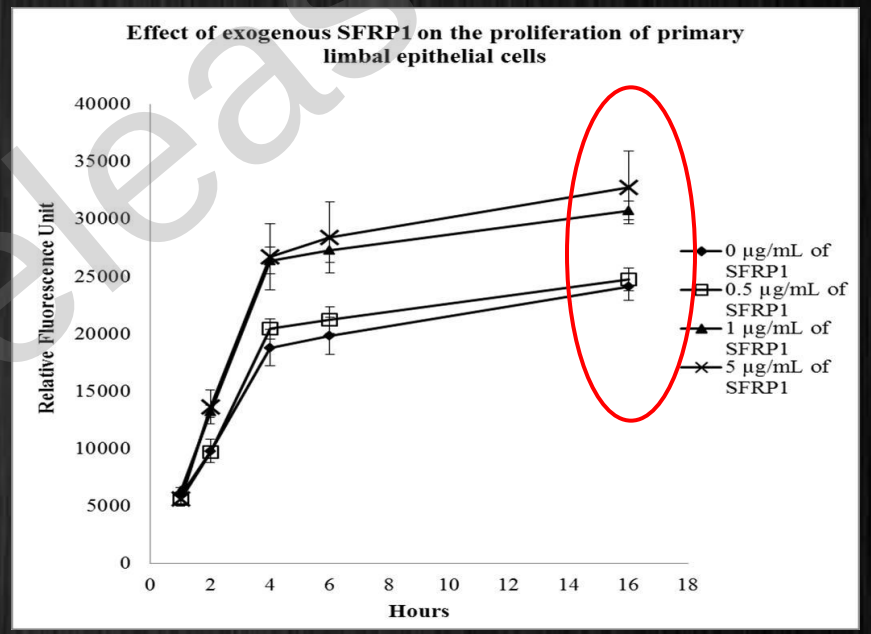


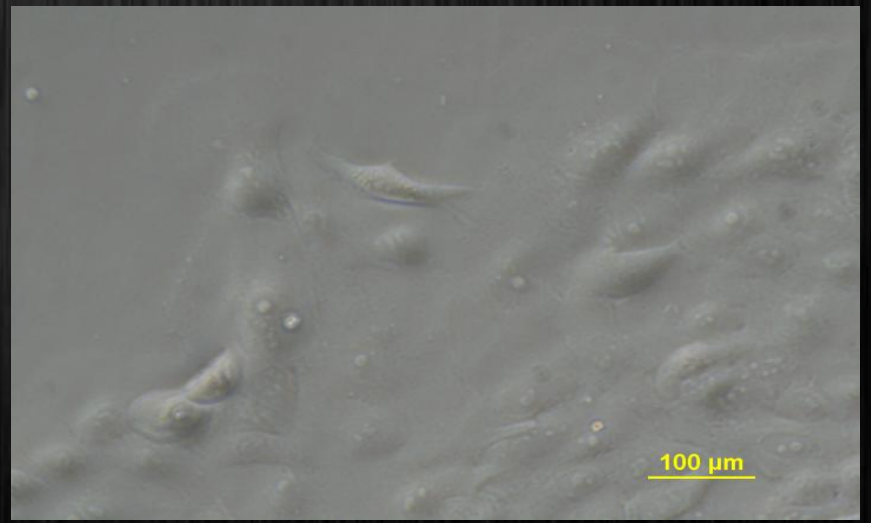
Primary KC corneal epithelial cells harvested from explant outgrowth



SFRP1 effect on human corneal epithelial cell proliferation

- SFRP1 induced a dose-dependent increase in cell proliferation, compared to untreated controls.
- (~1 to 1.3 fold difference).

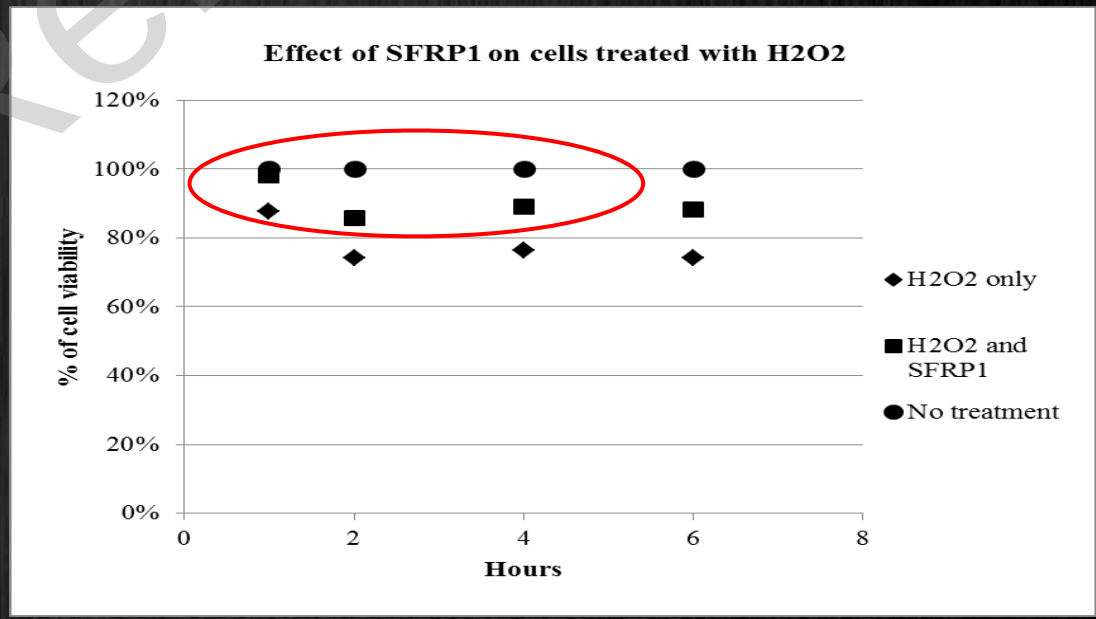




SFRP1 effect on human corneal epithelial cells – preliminary results

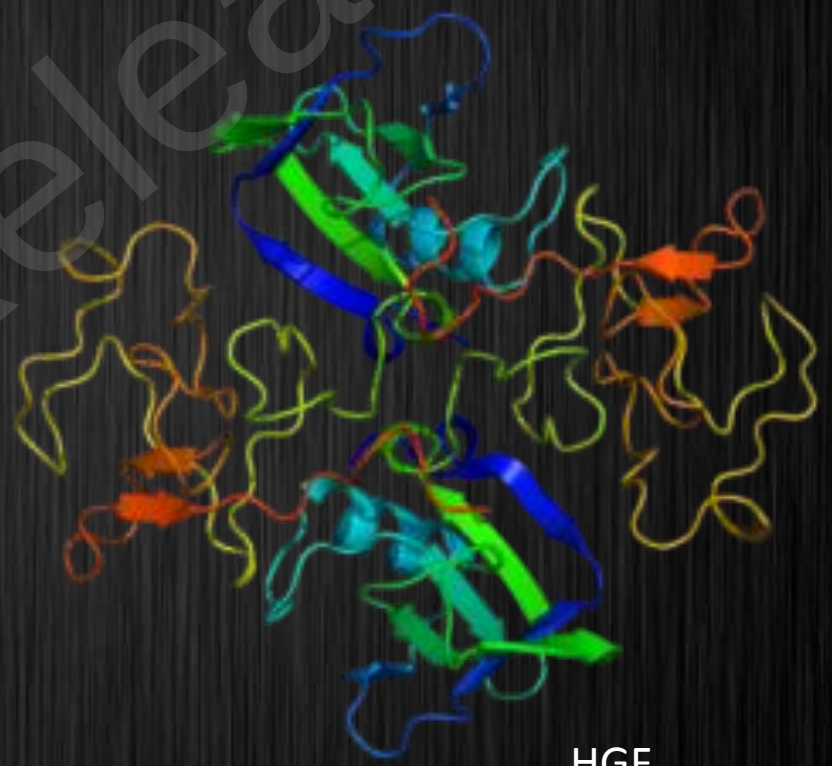
Anti-oxidative potential of SFRP1

- Viability was significantly increased for cells with H_2O_2 and SFRP1, compared to cells exposed to H_2O_2 alone (p=0.04)



Recent evidence: KC + HGF

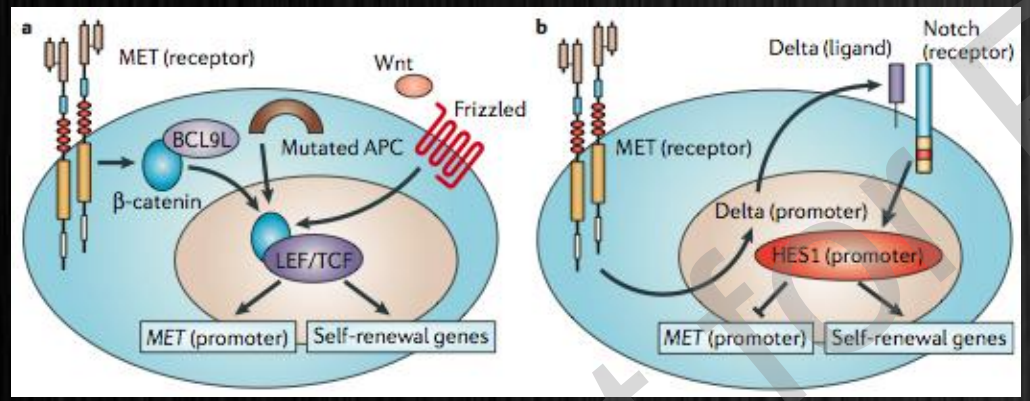
- Two parallel genome-wide association studies identifying potential SNPs associated with KC.*
- Reported a significant association between KC and the hepatocyte growth factor (*HGF*) gene.



HGF

* K. P. Burdon, S. Macgregor, Y. Bykhovskaya et al., *Investigative Ophthalmology & Visual Science*, vol. 52, no. 11, pp. 8514–8519, 2011.

Keratoconus HGF:cMET Corneal cellular regulation



- Initiates Corneal Epithelial Cell Migration*
- Corneal Proliferation & Layer Formation of Corneal Epithelial Cells**
- Implicated in Corneal Wound Healing***

*Daniels, et al *Investigative Ophthalmology and Visual Science* 2003
**Wilson S et al *Investigative Ophthalmology and Visual Science*, 1993.
***Carrington *Journal of Cataract and Refractive Surgery*, 2005.

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Research Article

Expression of HGF and c-Met Proteins in Human Keratoconus Corneas

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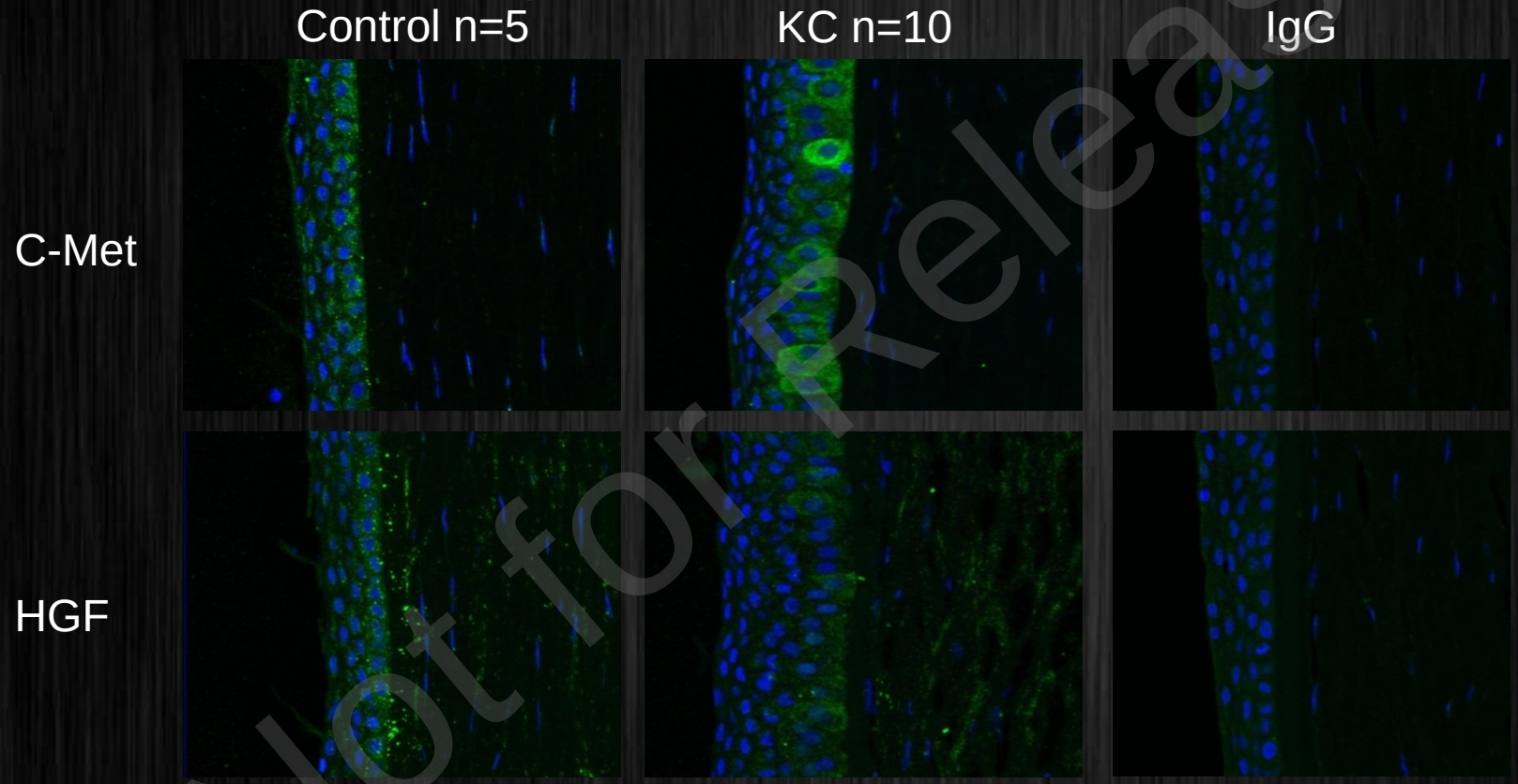
Keratoconus (KC) is a progressive degenerative inflammatory-related disease of the human cornea leading to decreased visual function. The pathogenesis of KC remains to be understood. Recent genetic studies indicate that gene variants of an inflammation-related molecule, hepatocyte growth factor (*HGF*), are associated with an increased susceptibility for developing KC. However HGF protein expression in KC has not been explored. In this initial study, we investigated late-stage KC and control corneas for the expression of HGF and its receptor mesenchymal-epithelial transition factor (c-Met/Met). KC buttons (~8 mm diameter) ($n = 10$) and whole control corneas ($n = 6$) were fixed in 10% formalin or 2% paraformaldehyde, paraffin embedded and sectioned. Sections were immunolabelled with HGF and c-Met antibodies, visualised using immunofluorescence, and examined with scanning laser confocal microscopy. Semiquantitative grading was used to compare HGF and c-Met immunostaining in KC and control corneas. Overall, KC corneas showed increased HGF and c-Met immunostaining compared to controls. KC corneal epithelium displayed heterogeneous moderate-to-strong immunoreactivity for HGF and c-Met, particularly in the basal epithelium adjacent to the cone area. Taken together with the recent genetic studies, our results further support a possible role for HGF/c-Met in the pathogenesis of KC.

1. Introduction

Keratoconus (KC) is the most common primary human degenerative corneal disease with a prevalence of around 1 in 2000 worldwide [1]. It is bilateral, asymmetric, and progressive, leading to corneal thinning and irregularity [2]. Onset primarily occurs in the 2nd decade of life and is associated with significant decreasing visual function [2] and morbidity [3]. KC is the main indication recorded for corneal grafts in Australia [4], and currently its progression can only be halted through surgical interventions including collagen cross-linking that stiffens the cornea using riboflavin and UVA [5]. More recently a surgical procedure was developed transplanting isolated Bowman's layer from donor corneas to KC eyes as a further late-stage intervention [6].

The histopathology of KC is well described and includes epithelial and stromal thinning within the apical cone region, breaks in the Bowman's layer, focal fibrosis, and anterior stromal keratocyte apoptosis [2, 7]. However the underlying pathogenesis of KC remains unclear. Recent evidence indicates a role for inflammation in the disease, with increased recruitment of inflammatory cells (e.g., macrophages, lymphocytes, and antigen presenting cells) [8] and inflammatory markers such as interleukin-1 (IL-1) and transforming growth factor-beta (TGF-β) [9] observed in KC corneal tissue sections. Increased expression of inflammatory markers such as interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), and matrix metalloproteinase 9 (MMP-9) has also been found in tears collected from KC patients compared to controls [10]. Furthermore, a recent review examining the biochemical

Immunostaining results



C-Met as a membrane bound ligand showed strong staining in the basal epithelial cells of KC compared to control. HGF also tends to express more in the basal layer of KC epithelium.

Keratoconus RNA sequencing

RNA sequencing:

- Use next generation sequencing technique to profile the transcriptome (RNA)

- 40,900 genes proflied in the corneal epithelial samples

- Identified 83 differentially expressed genes 83 using “Deseq” (a bioinformatics tool)

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Symbol	Gene_type	Chr	length
ENSG00000099864	201.9947217	-1.251746457	0.248436382	-5.03849897	4.69E-07	0.003853181	PALM	protein_coding	19	39390
ENSG00000004660	178.1381219	-0.982319019	0.196793679	-4.991618765	5.99E-07	0.003853181	CAMKK1	protein_coding	17	34576
ENSG00000078114	9815.232202	0.36187407	0.073166191	4.945919242	7.58E-07	0.003853181	NEBL	protein_coding	10	394214
ENSG00000114737	111.6750307	-0.905202716	0.196626303	-4.603670519	4.15E-06	0.015829145	CISH	protein_coding	3	5341
ENSG00000277196	64.91040756	-1.126549209	0.252127367	-4.468175045	7.89E-06	0.022989686		protein_coding	KI270734.1	23770
ENSG00000167656	897.4938334	-1.100095477	0.247832461	-4.438867574	9.04E-06	0.022989686	LY6D	protein_coding	8	1712
ENSG00000167325	2617.502386	0.180758144	0.041035747	4.404894665	1.06E-05	0.02306151	RRM1	protein_coding	11	44169
ENSG00000135373	11919.24305	0.287641106	0.065807056	4.370976658	1.24E-05	0.023583429	EHF	protein_coding	11	39964
ENSG00000007933	20.75697911	1.084945707	0.250433772	4.332265967	1.48E-05	0.025011955	FMO3	protein_coding	1	26942
ENSG00000196411	1144.13835	-0.532563903	0.123856009	-4.299863271	1.71E-05	0.026067914	EPHB4	protein_coding	7	24956
ENSG00000102934	650.1614936	-0.576352961	0.137770569	-4.183425871	2.87E-05	0.033083994	PLLP	protein_coding	16	36140
ENSG00000197020	740.8147248	0.57719732	0.138393312	4.170702427	3.04E-05	0.033083994	ZNF100	protein_coding	19	44862
ENSG00000135083	25.4007865	-1.050541027	0.251225704	-4.181662186	2.89E-05	0.033083994	CCNJL	protein_coding	5	93744
ENSG00000164850	108.0732756	-0.810405166	0.192905357	-4.201050614	2.66E-05	0.033083994	GPER1	protein_coding	7	11607
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ENSG00000155744	4212.210963	0.319646685	0.077782715	4.109482209	3.97E-05	0.039207143	FAM126B	protein_coding	2	97953
ENSG00000101224	263.7524923	-0.636676122	0.157242149	-4.049016918	5.14E-05	0.046147707	CDC25B	protein_coding	20	19349
ENSG00000168079	78.66468146	-0.968608426	0.240047811	-4.035064611	5.46E-05	0.04625665	SCARA5	protein_coding	8	122845
ENSG00000003137	136.2264839	-0.789718019	0.197983654	-3.98880415	6.64E-05	0.04861094	CYP26B1	protein_coding	2	18800
ENSG00000070950	527.4428342	0.266101005	0.066742903	3.986955796	6.69E-05	0.04861094	RAD18	protein_coding	3	188371
ENSG00000081026	3399.563627	0.489267037	0.122708677	3.987224455	6.69E-05	0.04861094	MAGI3	protein_coding	1	295174
ENSG00000279141	88.43235684	-0.808963178	0.205656661	-3.933561762	8.37E-05	0.058028181	LINC01451	lincRNA	9	5157
ENSG00000114480	1676.506477	0.290955183	0.074183993	3.922074968	8.78E-05	0.058219816	GBE1	protein_coding	3	272462
ENSG00000131669	437.6379309	-0.882589643	0.229199972	-3.850740623	0.000117761	0.060190465	NINJ1	protein_coding	9	12799
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ENSG00000102879	27.54455679	-0.887194853	0.228431669	-3.883852256	0.000102814	0.060190465	CORO1A	protein_coding	16	6249
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ENSG00000188483	75.97196887	-0.638976041	0.166095096	-3.847049412	0.000119549	0.060190465	IER5L	protein_coding	9	2710
ENSG00000135736	81.60556285	-0.742618389	0.192151257	-3.864759464	0.000111199	0.060190465	CCDC102A	protein_coding	16	24421
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ENSG00000100092	383.662174	-0.736583744	0.193116309	-3.814197504	0.000136626	0.063150273	SH3BP1	protein_coding	22	32278
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ENSG00000182103	96.91567289	-0.596336167	0.169871902	-3.510505036	0.000447256	0.092189201	FAM181B	protein_coding	11	3923
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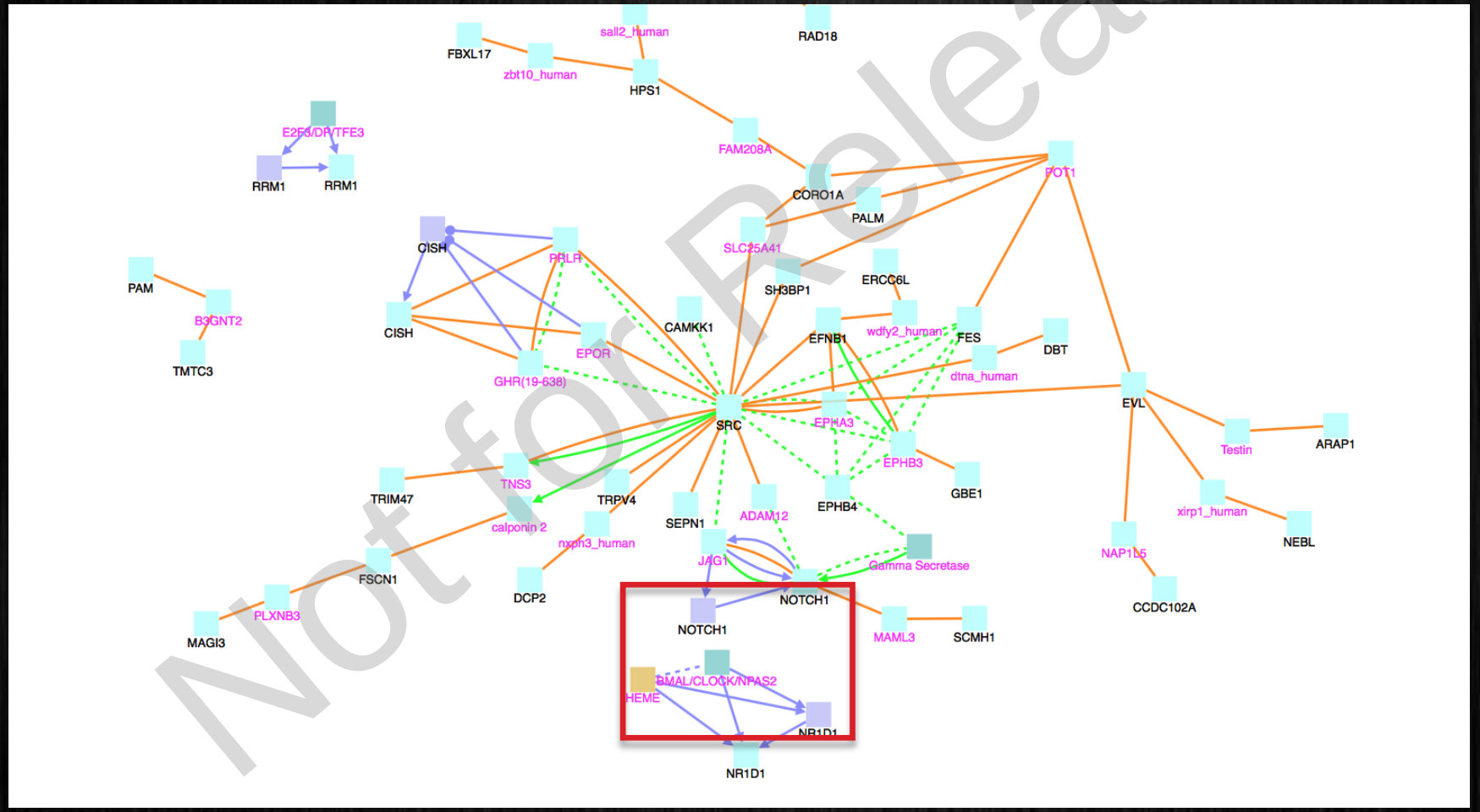
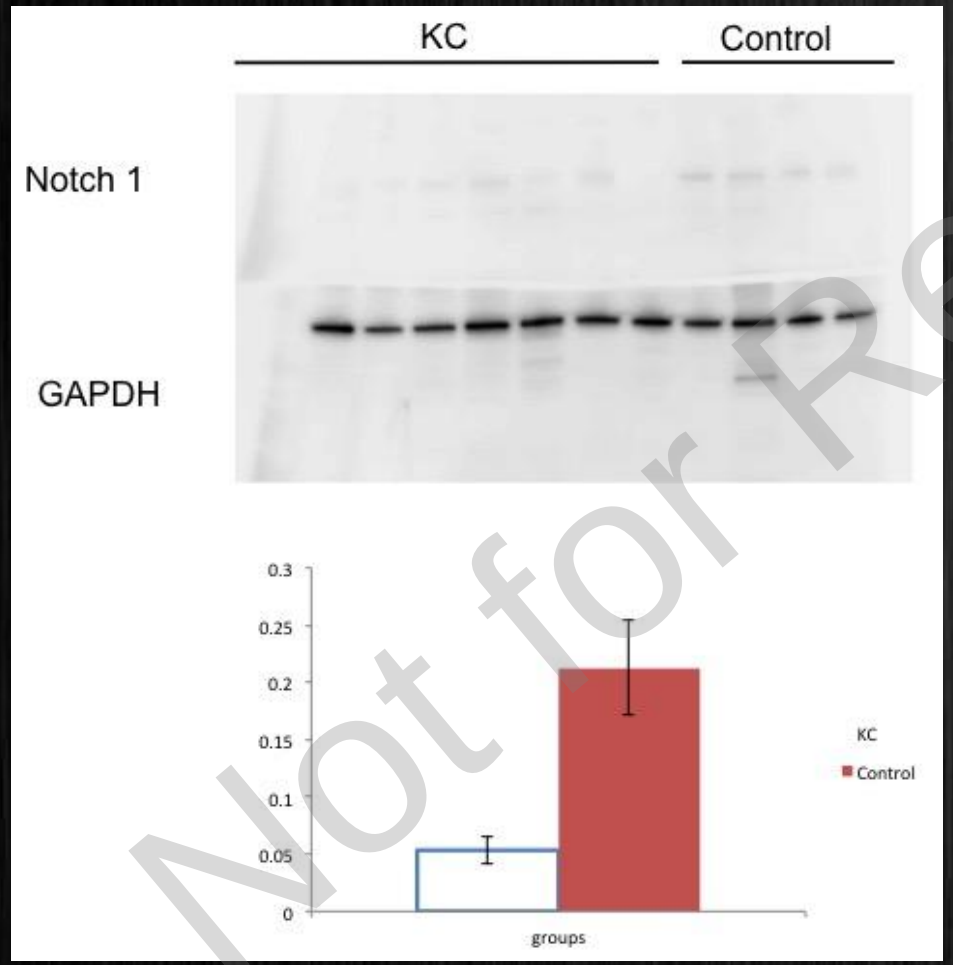
ENSG00000178498	82.27516945	-0.63811209	0.18431927	-3.461993365	0.00053619	0.098536286	DTX3	protein_coding	12	5182

Network analysis using Consensus Path Database



Purple colour: gene; Light Blue: protein; Dark green: protein complex
Green line: protein activation; purple line: gene activation; orange line: protein interaction; Dash line: activation direction uncertain

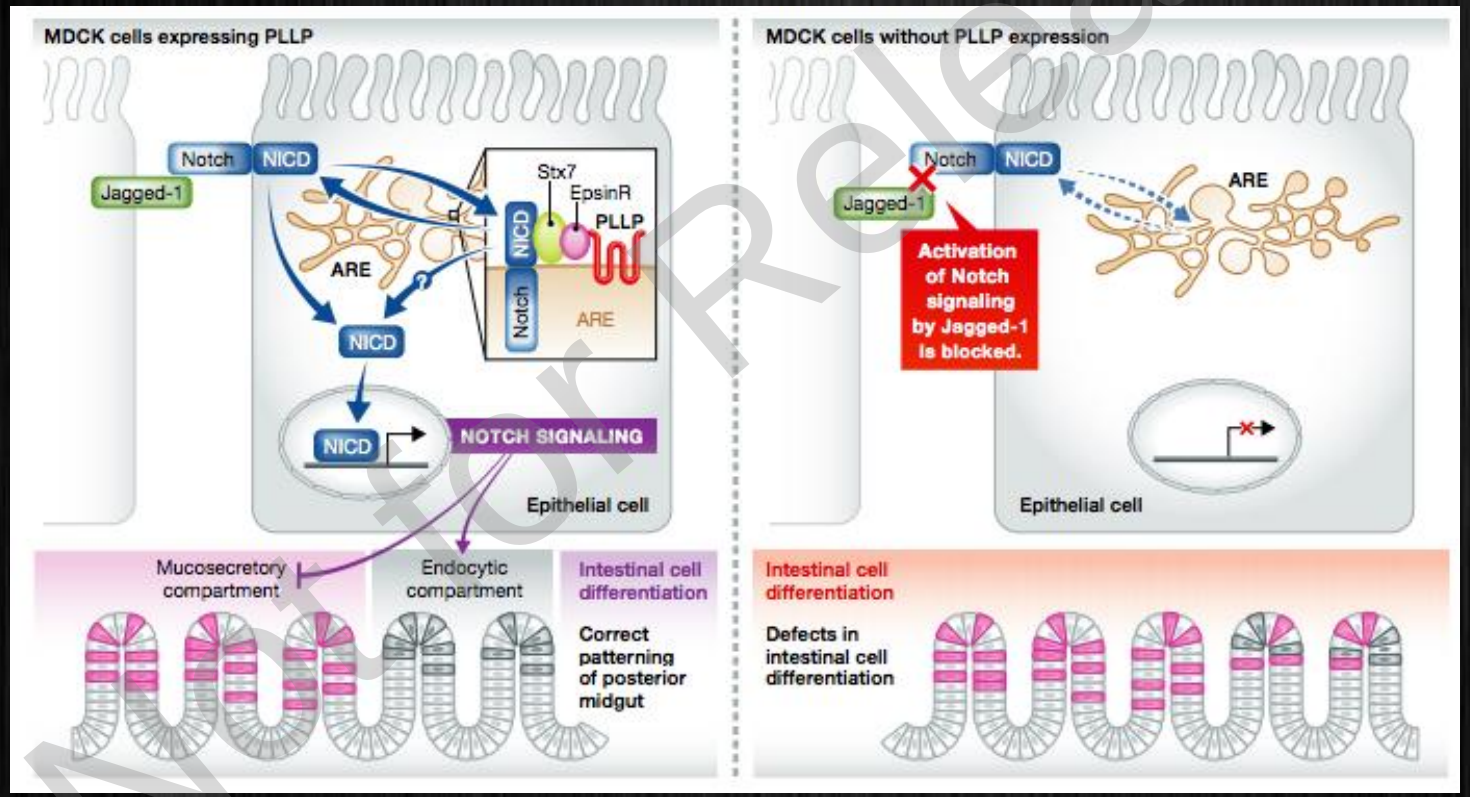
Reduction of Notch 1 protein expression



P=0.004
Notch 1 protein is significantly reduced in KC compare to control epithelial cells.
(GAPDH is internal control)

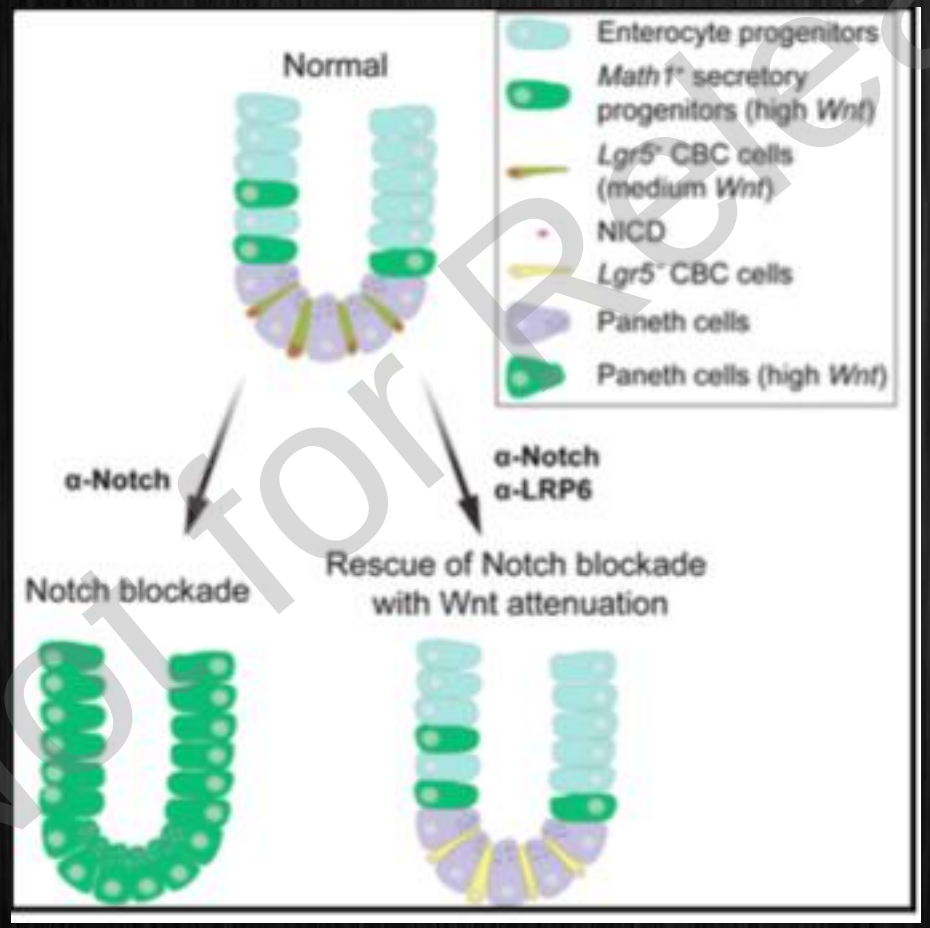
The reduction of Notch 1 in KC compared to control epithelium suggests involvement of abnormal epithelial cell differentiation in the pathogenesis of KC.

Notch Implicated in Intestinal Epithelial Cell Regulation



*VanDussen K2012 Feb 1; 139(3): 488–497

Wnt & Notch Pathways Work In Tandem*



* Stem Cells. 2013 Jun;31(6):1086-96.

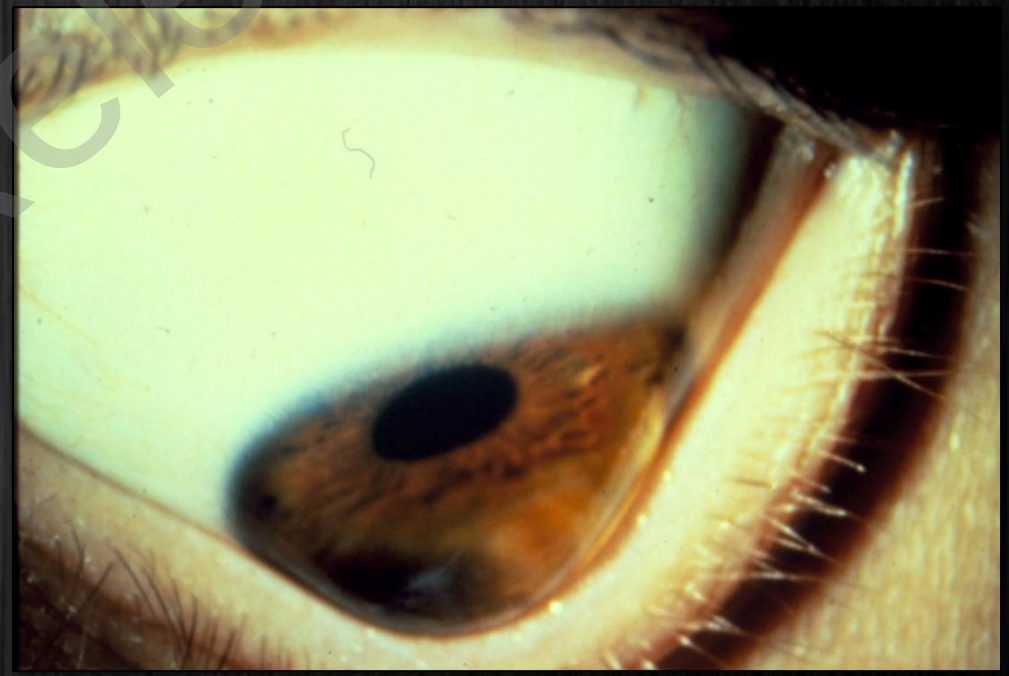
Ogaki S(1), Shiraki N, Kume K, Kume S.

Summary: KC Unit Sydney University

- Evidence mounting that corneal epithelial migration differantiation and proliferation are abnormal in Keratoconus

- We hypothesise that the abnormal epithelium leads to abnormal signalling to keratocytes and subsequent defective collagen production

- We have identified a number of target pathways with diagnostic and therapeutic potential.



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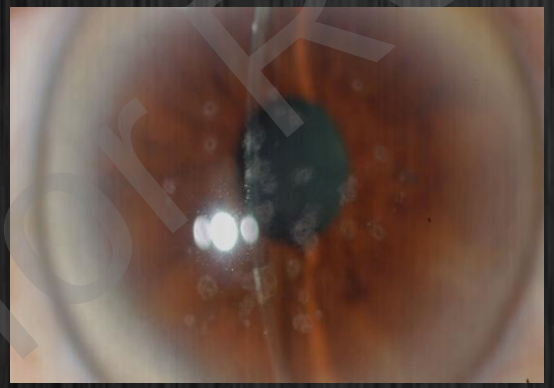

4 February 2017

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