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Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki

Rozprawa doktorska przygotowana w Instytucie Genetyki Człowieka
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Promotor: prof. dr hab. n. med. Marzena Gajęcka

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Prof. dr hab. n. med. Marzenie Gajęckiej
za przekazaną wiedzę, wszelką pomoc i wsparcie.

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Publikacje stanowiące podstawę rozprawy doktorskiej:

Jaskiewicz K and Gajecka M.
The Genetics of Keratoconus and Related Diseases.
W Traboulsi E, Utz V, and Kha A. Genetic Diseases of the Eye 3rd Edition.
Oxford University Press
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1. **Publikacje** (nie obejmujące prac stanowiących podstawę rozprawy doktorskiej):

• Gajecka M, Gutaj P, **Jaskiewicz K**, Rydzanicz M, Szczapa T, Kaminska D, Kosewski G, Przyslawski J, Ploski R, Wender-Ozegowska E.
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2. **Prezentacje plakatowe:**

• **Jaskiewicz K**, Maleszka-Kurpiel M, Kabza M, Ploski R, Rydzanicz M, Gajecka M. Transcriptomic profiles of corneal epithelium reflect irregularities in epithelial thickness maps of post-refractive ectasia patients. 75th ASHG Annual Meeting, Washington D.C. USA, 1-5.11.2023 *(zaakceptowana prezentacja plakatowa)*

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• Gajecka M, Gutaj P, **Jaskiewicz K**, Rydzanicz M, Szczapa T, Kaminska D, Kosewski G, Przyslawski J, Ploski R, Wender-Ozegowska E. The effect of a maternal type 1 diabetes on the neonatal microbiome. 55th Annual Diabetic Pregnancy Study Group Meeting, Poznan, Poland, September 7-9, 2023.

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• **Jaskiewicz K**, Maleszka-Kurpiel M, Rydzanicz M, Karolak JA, Płoski R, Gajecka M. Estrogen and androgen related genes in the corneal epithelium in Keratoconus. European Society of Human Genetics Conference 2022, Vienna, Austria, 11-14.06.2022.

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• **Jaskiewicz K**, Maleszka-Kurpiel M, Rydzanicz M, Matuszewska E, Matysiak J, Ploski R, Gajecka M. Characteristics of keratoconus corneal epithelium in multi-omic approach. ASHG 2021, Virtual Meeting, October 18-22 2021.

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diabetes. 51st Annual Meeting 2019 Diabetic Pregnancy Study Group of the EASD. Graz, Austria, September 5-7, 2019.

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3. **Kontynuacja prac badawczych podjętych w doktoracie:**

NARODOWE CENTRUM NAUKI

Preludium 2022/45/N/NZ5/02989: *Metylom warstw i regionów topograficznych rogówki w stożku rogówki, badany z wykorzystaniem sekwencjonowania całego genomu* (kierownik: mgr Katarzyna Jaśkiewicz, okres realizacji: 2.02.2023- 1.02.2026).

Spis treści

Wykaz skrótów:

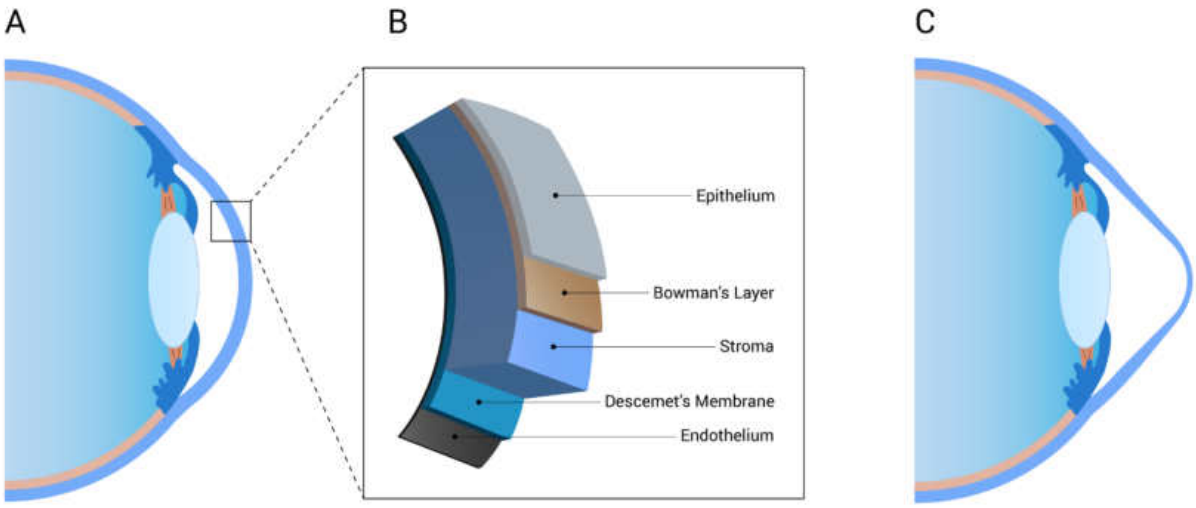
AS-OCT	tomografia przedniego odcinka oka (ang. *anterior segment optical coherence tomography*)
CE	nabłonek rogówki (ang. *corneal epithelium*)
CXL	zabieg sieciowania włókien kolagenowych (ang. c*orneal collagen crosslinking)*
DALK	keratoplastyka warstwowa przednia głęboka (ang. *deep anterior lamellar keratoplasty*)
ES	sekwencjonowanie eksomu (ang. *exome sequencing*)
Kmax	maksymalna keratometria (ang. *maximal corneal curvature*)
KTCN	stożek rogówki (ang. *keratoconus*)
K1	keratometria płaska (ang. *flat keratometry*)
K2	keratometria stroma (ang. *steep keratometry*)
LSCs	komórki macierzyste rąbka rogówki (ang. *limbal stem cells*)
PRK	fotokeratektomia refrakcyjna (ang. *photorefractive keratectomy*)
RNA-Seq	sekwencjonowanie RNA (ang. *RNA sequencing*)
TCT	najmniejsza wartość grubości rogówki (ang. *thinnest corneal thickness*)
WGS	sekwencjonowanie całego genomu (ang. *whole genome sequencing*)

Wykaz nazw genów

BCL2L1	*Bcl-2-like protein 1*
CAPZB	*Capping Actin Protein Of Muscle Z-Line Subunit Beta*
CLTB	*Clathrin Light Chain B*
CNOT3	*CCR4-NOT Transcription Complex Subunit 3*
CTDSP2	*CTD (Carboxy-Terminal Domain, RNA Polymerase II, Polypeptide A) Small Phosphatase 2*
DOCK9	*Dedicator Of Cytokinesis 9*
ENO1	*Enolase 1*
IPO5	*Importin 5*
JUNB	*JunB Proto-Oncogene, AP-1 Transcription Factor Subunit*
KRT12	*Keratin 12*
LTBP3	*Latent Transforming Growth Factor Beta Binding Protein 3*
NFYA	*Nuclear Transcription Factor Y Subunit Alpha*
PDPK1	*3-Phosphoinositide Dependent Protein Kinase 1*
PTPN1	*Protein Tyrosine Phosphatase Non-Receptor Type 1*
SPATA13	*Spermatogenesis Associated 13*
SPRA	*Sepiapterin Reductase*
STK24	*Serine/Threonine Kinase 24*
TCHP	*Trichoplein Keratin Filament Binding*
TFDP1	*Transcription Factor Dp-1*
TGFB2	*Transforming Growth Factor Beta 2*
TGFBI	*Transforming Growth Factor Beta Induced*
TLN1	*Talin 1*
TP53	*Tumor Protein P53*
TRIM8	*Tripartite Motif Containing 8*
VSX1	*Visual System Homeobox 1*
WNK1	*WNK Lysine Deficient Protein Kinase 1*

Wprowadzenie

Stożek rogówki (ang. *keratoconus*, KTCN) jest postępującym schorzeniem degeneracyjnym rogówki oka [1]. Choroba charakteryzuje się pogłębiającym się ścieńczeniem rogówki, w wyniku którego przyjmuje ona stożkowaty kształt. Uproszczony schemat budowy rogówki oraz zmian w stożku rogówki przedstawiono na Rycinie 1. Ze względu na proces ścieńczenia, KTCN należy do szerokiej grupy ektazji rogówki [2]. Szczyt stożka, miejsce największego uwypuklenia i ścieńczenia, jest zazwyczaj umiejscowiony w części dolno-skroniowej rogówki [3]. Powstające w strukturze rogówki nieprawidłowości prowadzą do aberracji optycznych, co w efekcie przyczynia się do znacznego upośledzenia wzroku i pogorszenia jakości życia pacjentów [4]. KTCN jest opisywany jako choroba obojga oczu, jednak często objawia się jako zaburzenie asymetryczne [2]. Pierwsze symptomy choroby mogą pojawić się już w okresie dojrzewania, by kolejno ulegać progresji przez trzecią/czwartą dekadę życia, a pacjenci z rozpoznanym KTCN w młodym wieku cechują się szybszą progresją [5].



Rycina 1. Schemat budowy oka ludzkiego. A. Prawidłowa rogówka z normalną grubością. B. Warstwy rogówki. Rogówka jest złożoną tkanką składającą się z pięciu warstw (od zewnętrznej): nabłonka, błony Bowmana, istoty właściwej, błony Descemeta i śródbłonka. C Rogówka ze stożkiem rogówki, z charakterystycznym ścieńczeniem i stożkowatym uwypukleniem. Źródło: *Karolak JA, Gajecka M. Mol Genet Genomics, 2017.*

KTCN jest najczęstszym typem ektazji rogówki i dotyka światową populację z częstością 1 na 2000 osób [1]. Choroba dotyczy wszystkich grup etnicznych i obu płci, jednak w badaniach epidemiologicznych wskazywane jest częstsze występowanie KTCN wśród mężczyzn, jak również u mieszkańców Bliskiego Wschodu i Azji [2, 6]. Brak jest aktualnych analiz dotyczących rozpowszechnienia KTCN w Polsce, a szacowana częstość występowania, zgodnie z Polską Kartą Problemu Zdrowotnego, wynosi 200-250 na 100 000 mieszkańców [7]. W jednoośrodkowym (szpitalnym) badaniu epidemiologicznym, opublikowanym w 1989 roku, nie podano częstości występowania KTCN w Polsce, wykazano jednak, że również w polskiej populacji KTCN w większym stopniu dotyczy mężczyzn (64% pacjentów z KTCN stanowili mężczyźni) [8].

Zaawansowaną postać KTCN można rozpoznać bez użycia sprzętu okulistycznego obserwując tzw. objaw Munsona, będący uwypukleniem powieki dolnej w kształcie litery V przy spojrzeniu w dół [9]. Z kolei w lampie szczelinowej można zaobserwować obecność złogów żelaza w głębokich warstwach nabłonka (pierścień Fleischera), linie napięcia błony Descemeta na szczycie stożka (linie Vogta) oraz blizny obecne w istocie właściwej rogówki [10]. Co ważne, u pacjentów z wczesnym i średniozaawansowanym KTCN, rogówka w lampie szczelinowej może wyglądać prawidłowo. W diagnostyce początkowych etapów rozwoju choroby wykorzystuje się topografię rogówki (krążki Placido), tomografię rogówki (kamera Scheimpfluga) oraz tomografię przedniego odcinka oka (ang. *anterior segment optical coherence tomography*, AS-OCT) [11–13]. Tomografia, która pozwala ocenić grubość rogówki, uniesienie powierzchni przedniej oraz tylnej jest obecnie głównym narzędziem wykorzystywanym do oceny parametrów rogówki u pacjentów z KTCN [2]. W ostatnich latach zwraca się uwagę na fakt, że pierwsze objawy KTCN można zaobserwować w nabłonku rogówki (ang. *corneal epithelium*, CE) obrazowanym za pomocą AS-OCT [14, 15]. Często początkowe stadia choroby są niemożliwe do rozpoznania w rutynowym badaniu okulistycznym, a zgłaszane przez pacjentów objawy podmiotowe (takie jak pogorszenie ostrości wzroku, dwojenie jednooczne, nadwrażliwość na światło, swędzenie i zaczerwienienie oczu) nie są patognomoniczne [5]. W celu rzetelnej oceny stanu pacjenta, u każdej osoby, u której podejrzewa się KTCN należy wykonać kompleksowe badanie okulistyczne obejmujące ocenę nieskorygowanej i skorygowanej ostrości wzroku, badanie odcinka przedniego w lampie szczelinowej oraz mapowanie rogówki (tomografia rogówki) i CE (AS-OCT) [16]. Konieczność wykonania badań z użyciem zaawansowanej aparatury (niedostępnej w każdym gabinecie okulistycznym) sprawia, że pacjenci trafiają do specjalistycznych gabinetów dopiero z zaawansowanymi stadiami choroby. Zatem rzeczywista częstość występowania KTCN w populacji wydaje się być znacznie niedoszacowana. Mimo trudności diagnostycznych, rozpoznanie KTCN w najwcześniejszym stadium zaawansowania (m.in. w postaci subklinicznej) pozostaje szczególnie ważne w kontekście efektywniejszego leczenia pacjentów i długoterminowego rokowania.

Leczenie KTCN ma na celu zatrzymanie postępu ektazji i poprawę widzenia. Wybór najlepszej metody leczenia zależy od stopnia zaawansowania choroby, ostrości wzroku oraz wieku pacjenta [2]. Korekcja ostrości wzroku za pomocą okularów jest zwykle możliwa tylko u pacjentów z łagodnym KTCN [17]. Ze względu na nieregularny astygmatyzm stosuje się tzw. „miękkie” soczewki (hydrożelowe, silikonowo-hydrożelowe, miękkie toryczne), jednak w przypadku dużej wady rozważa się tzw. soczewki „twarde” (sztywne soczewki gazoprzepuszczalne, twardówkowe lub hybrydowe) [18]. Niekiedy stosuje się implantację pierścieni śródrogówkowych lub kombinacje wyżej wymienionych metod [19]. Metodą, którą należy rozważyć u pacjentów z postępującym KTCN oraz KTCN o wysokim ryzyku progresji (w przypadkach rozpoznania KTCN u dzieci i młodzieży) jest zabieg sieciowania włókien kolagenowych (ang. c*orneal collagen crosslinking*, CXL) [20]. W trakcie zabiegu CXL na rogówkę podaje się ryboflawinę, która w warunkach tlenowych pod wpływem promieniowania UVA powoduje powstanie tlenu singletowego, który wchodzi w reakcję, z tworzącymi warstwę istoty właściwej, włóknami kolagenowymi, powodując tworzenie dodatkowego usieciowania tych włókien wiązaniami krzyżowymi [21]. Zabieg CXL wykonywany jest w celu zwiększenia wytrzymałości mechanicznej tkanki poddanej zabiegowi, co ma prowadzić do spowolnienia

progresji stożka rogówki, dodatkowo w niewielkim stopniu poprawia jakość widzenia pacjentów [22]. W przypadku pacjentów z najbardziej zaawansowanym KTCN przeprowadza się keratoplastykę. KTCN jest drugą przyczyną przeszczepów rogówki na świecie, przy czym tradycyjna keratoplastyka drążąca obejmująca wszystkie warstwy rogówki, często zastępowana jest keratoplastyką warstwową przednią głęboką (ang. *deep anterior lamellar keratoplasty*, DALK) [23]. Natomiast potencjalne terapie komórkowa i genowa wciąż pozostają w sferze badań naukowych i klinicznych [24, 25].

Nieprawidłowości histopatologiczne rogówki pacjentów z rozpoznanym KTCN obejmują wszystkie warstwy rogówki [26, 27]. Średnica rogówki u dorosłego człowieka wynosi około 11.5 mm [28], z kolei jej średnia grubość w części centralnej u zdrowych osób mieści się najczęściej w zakresie od 520 µm do 600 µm [29]. Rogówka zbudowana jest z pięciu warstw: nabłonek rogówki (CE), błony Bowmana (blaszka graniczna przednia), istoty właściwej (zrąb), błony Descemeta (blaszka graniczna tylna) oraz śródbłonka [10]. CE składa się z 5-6 warstw nabłonka wielowarstwowego płaskiego nierogowaciejącego, o łącznej grubości ok. 50 µm [30]. Komórki nabłonka rogówki podlegają nieustannemu ścieraniu (m.in. w wyniku mrugania), a za ich ciągłą regenerację i przebudowę odpowiadają komórki macierzyste rąbka rogówki (ang. *limbal stem cells*, LSCs), zlokalizowane na granicy rogówki i spojówki [31]. U pacjentów z KTCN mapy nabłonka rogówki odzwierciedlają charakterystyczną strukturę obwarzanka (ang. *doughnat pattern*), wynika to z faktu, że nabłonek, próbując ‘ukryć’ powstające uwypuklenie istoty właściwej staje się najcieńszy na szczycie stożka [32, 33]. W fizjologicznie bezkomórkowej błonie Bowmana pojawiają się komórki apoptotyczne oraz pęknięcia [26]. Z kolei najbardziej charakterystyczną zmianą histologiczną jest ścieńczenie istoty właściwej rogówki będące wynikiem zmniejszonej ilości, zmienionej orientacji i nieregularnego rozmieszczenia włókienek kolagenowych. W konsekwencji tych zmian dobrze zorganizowana architektura istoty właściwej zapewniająca przezroczystość rogówki zostaje zaburzona. Dodatkową cechą obserwowaną w przebiegu KTCN jest zmniejszona gęstość keratocytów w tej warstwie [10, 26, 27]. Z kolei z błonie Descemeta mogą pojawić się pęknięcia, a komórki śródbłonka ulegają wydłużeniu i uszkodzeniu [10].

KTCN jest uznawany za chorobę wieloczynnikową, a przyczyny jego powstawania nie są w pełni poznane. Wśród czynników środowiskowych zwiększających ryzyko rozwoju KTCN wymienia się częste i intensywne pocieranie oczu, katar sienny czy astmę [2]. Z kolei wśród czynników genetycznych zwiększających ryzyko rozwoju KTCN wskazuje się rozpoznanie u pacjenta zespołu Downa, wrodzonej ślepoty Lebera, zespołu Ehlersa-Danlosa czy też zespołu Noonan [5]. Oszacowano, że ryzyko rozwoju KTCN u krewnego osoby obarczonej chorobą jest od 15 do 67 razy większe niż u osoby, u której w rodzinie nie rozpoznano KTCN [34].

Podłoże genetyczne KTCN obejmuje liczne zidentyfikowane *loci* chromosomowe, w tym 13q32 [35], 5q31.1-q35.3 [36], 2q13-q14.3, 20p13-p12.2 [37] oraz warianty sekwencji w obrębie genów kandydujących, do których należą m.in. *VSX1, DOCK9, STK24, IPO5 i TGFBI* [36, 38, 39]. Dotychczas raportowano replikację tylko jednego *locus* KTCN (5q21.2)[36], a szczegółowe badania kluczowych genów kandydatów nie przyniosły jednoznacznych wyników. Często odkryte warianty uznawane są za istotne jedynie w przypadku konkretnej grupy etnicznej pacjentów czy wyłącznie rodziny, co wskazuje na dużą heterogeniczność

choroby. W większości przypadków KTCN występującego rodzinnie wskazuje się na dziedziczenie tej cechy w modelu autosomalnym dominującym z niepełną penetracją [40]. Najprawdopodobniej genetyczna predyspozycja (rozumiana jako nagromadzenie wielu sprawczych wariantów genetycznych) wraz z czynnikami środowiskowymi i behawioralnymi powodują rozwój KTCN [41]. Wskazane elementy podłoża genetycznego opisane zostały w pracy przeglądowej – w rozdziale „The Genetics of Keratoconus and Related Disease" w książce „Genetic Diseases of the Eye 3rd Edition".

Poza badaniami wykonywanymi w celu poznania dziedzicznych wariantów genetycznych w KTCN, prowadzone są rozległe badania molekularne obejmujące analizy cząsteczek RNA i białek [42]. Zidentyfikowano zaburzenie syntezy i szlaków dojrzewania kolagenu, a także obniżony poziom ekspresji elementów szlaków sygnałowych TGF-β, Hippo i Wnt, wpływających na organizację rogówki [43, 44].

Historycznie KTCN był uznany za chorobę niezapalną, co obecnie jest podważane przez klinicystów i badaczy [45, 46]. Wielokrotnie w badaniach naukowych odnotowano powiązanie KTCN ze znaczącymi zmianami w zakresie mediatorów stanu zapalnego, m.in. w analizach cytokin i proteaz płynu łzowego pacjentów, i tym samym wskazywano na utrzymujący się stan zapalny [47–50]. Dodatkowo przypuszcza się, iż nagromadzenie w rogówce pacjenta z KTCN reaktywnych form tlenu wywołuje kaskadę zdarzeń (poprzez apoptozę komórek aż do włóknienia tkanki), co pośrednio przyczynia się do wspomnianego zapalenia [51–53]. Równocześnie nie wykazano genetycznych przyczyn/podstaw nawracających/przetrwałych reakcji zapalnych układu odpornościowego u pacjentów z KTCN.

Podobnie jak w procesie poszukiwania czynników sprawczych w etiologii innych chorób wieloczynnikowych, aby zrozumieć procesy biologiczne leżące u podstaw KTCN, konieczna jest równoczesna analiza różnych aspektów biologicznych i danych pochodzących z wysokoprzepustowych analiz omicznych. Ponadto podejmowane w przyszłości badania powinny koncentrować się na poznaniu podstaw molekularnych etapów kształtowania się stożka w KTCN z uwzględnieniem interpretacji zmian patologicznych w rogówce w osiach wertykalnej (podział na warstwy rogówki) oraz horyzontalnej (podział na regiony topograficzne w obrębie każdej warstwy rogówki). Dlatego też, zespół którego jestem członkiem, po raz pierwszy w historii badań nad KTCN przeprowadził sekwencjonowanie całego genomu (ang. *whole genome sequencing,* WGS) oraz zdefiniował trzy regiony topograficzne rogówki.

Cel rozprawy doktorskiej

W niniejszej rozprawie doktorskiej postawiono hipotezę, że przebudowa nabłonka rogówki w przebiegu KTCN stanowi istotny element obrazu klinicznego choroby. Mimo obserwowanych zmian okulistycznych, w dotychczasowych badaniach naukowych nie poświęcono dostatecznej uwagi tej warstwie rogówki, a charakterystyczne zmiany grubości nabłonka, rozpoznawane jako struktura obwarzanka, nie zostały uwzględnione podczas przeprowadzonych badań molekularnych. Podjęte dotychczas próby biologicznej interpretacji zintegrowanych wyników badań klinicznych i molekularnych były niedostateczne by zrozumieć procesy leżące u podstaw KTCN.

Celem badań przedstawionych w rozprawie było poszerzenie wiedzy na temat zmian molekularnych w poszczególnych regionach topograficznych nabłonka rogówki, w tym w aspektach transkryptomicznych, proteomicznych oraz dotyczących wariantów sekwencji w kodujących i niekodujących częściach genomu. Zaplanowano ponadto identyfikację czynników środowiskowych warunkujących fenotyp KTCN.

Cel badań został osiągnięty poprzez realizację następujących zadań badawczych:

1. Przegląd dostępnych danych literaturowych dotyczących klinicznych, środowiskowych i genetycznych aspektów KTCN.

2. Charakterystyka regionów topograficznych w nabłonku rogówki u pacjentów z KTCN:
   A. Zdefiniowanie trzech regionów topograficznych rogówki i ich charakterystyka morfologiczna,
   B. Analiza ekspresji genów i białek w nabłonku rogówki w trzech regionach topograficznych,
   C. Identyfikacja korelacji cech klinicznych pacjentów z danymi transkryptomicznymi,
   D. Wskazanie procesów kształtujących przebudowę nabłonka rogówki u pacjentów oraz propozycja mechanizmu warunkującego tę przebudowę.

3. Analiza wpływu czynników środowiskowych, behawioralnych i socjoekonomicznych na ryzyko wystąpienia stożka rogówki u pacjentów polskich:
   A. Charakterystyka środowiskowych, behawioralnych i socjoekonomicznych czynników ryzyka,
   B. Identyfikacja korelacji czynników ryzyka z danymi transkryptomicznymi.

4. Ocena zróżnicowania wariantów sekwencji DNA w regionach topograficznych rogówki:
   A. Charakterystyka wariantów sekwencji kodujących genomu,
   B. Charakterystyka wariantów sekwencji niekodujących genomu.

Omówienie prac włączonych do cyklu publikacji stanowiących rozprawę doktorską

Artykuł I:
Rozdział "The Genetics of Keratoconus and Related Diseases"
w książce "Genetic Diseases of the Eye, 3rd Edition". Praca przeglądowa.
Jaskiewicz K and Gajecka M.
Oxford University Press, praca zaakceptowana do publikacji (13-09-2023).

Rozwój wysokoprzepustowych metod w biologii molekularnej w ostatnich latach znacząco przyczynił się do poszerzenia wiedzy o czynnikach warunkujących choroby oczu, w tym KTCN. W konsekwencji niezbędne było uaktualnienie i uszeregowanie doniesień naukowych w książce „Genetic Diseases of the Eye", powstającej po ponad dziesięciu latach od poprzedniej edycji. W rozdziale będącym pracą przeglądową przedstawiono aktualny stan wiedzy o aspektach genetycznych w etiologii KTCN oraz powiązanych chorób, łącząc je z wybranymi elementami klinicznymi i środowiskowymi.

Dokonano przeglądu 282 pozycji literaturowych, co pozwoliło na sformułowanie treści siedmiu podrozdziałów. Pierwszy rozdział zawiera krótką charakterystykę KTCN. W drugim podrozdziale omówiono etiologię, częstość występowania oraz historię naturalną choroby. W kontekście niniejszej pracy doktorskiej ważnym elementem dyskutowanym w tym rozdziale jest tło zapalne w przebiegu KTCN. W kolejnym podrozdziale omówione zostały aspekty kliniczne KTCN obejmujące diagnostykę w różnych stanach zaawansowania choroby i zależne od tych stanów dostępne opcje terapeutyczne. Dodatkowo w tym miejscu pracy uwzględniono potencjalne możliwości terapii komórkowej i genowej. W podrozdziale czwartym przedstawiono środowiskowe i behawioralne czynniki ryzyka, podkreślając, że czynniki takie jak przewlekłe tarcie oczu, alergia, atopia, astma, ekspozycja na promieniowanie UV, czy też noszenie soczewek kontaktowych mogą wpłynąć na ujawnienie i rozwój KTCN u osób predysponowanych genetycznie. W najobszerniejszym z podrozdziałów, podrozdziale piątym, omówiono wyniki badań molekularnych umożliwiających zrozumienie natury KTCN. Podsumowano wyniki badań genomowych, transkryptomicznych, proteomicznych, metabolomicznych oraz mikrobiologicznych wskazując na doniesienia naukowe (m.in. dotyczące genów kandydatów) weryfikowane przy użyciu różnych technik molekularnych, jak również różnych materiałów biologicznych. W przedostatnim rozdziale wskazano na choroby współwystępujące/powiązane z KTCN, w tym zespół Downa, zespół Ehlersa-Danlosa i dystrofię Fuscha. Z kolei w ostatnim podrozdziale podsumowano złożoność aspektów molekularnych w KTCN i wskazano przyszłe kierunki badań opierające się na analizach omicznych.

Przedstawiona praca przeglądowa obejmuje najważniejsze zagadnienia związane z KTCN i może być przydatna zarówno klinicystom, jak i naukowcom. Wnioski dotyczące złożoności genetycznych aspektów choroby wysunięto na podstawie badań własnych jak i danych pochodzących z literatury światowej. Z punktu widzenia przedkładanej rozprawy doktorskiej kluczowym elementem tego rozdziału w książce są tabele, w których zestawiono najważniejsze sprawcze geny, ze wskazaniem na specyficzne dla KTCN warianty sekwencji czy różnice w ekspresji na poziomie RNA i białek. Zarówno mnogość doniesień, sprzeczności

pomiędzy nimi, jak również mała powtarzalność i spójność wyników pomiędzy różnymi populacjami wskazują na potrzebę prowadzenia wielopoziomowych analiz omicznych.

Wkład Katarzyny Jaśkiewicz w publikację: wybór opisanych danych literaturowych; przygotowanie figury i tabel; pisanie manuskryptu.

Third Edition of "Genetic Diseases of the Eye"

16. The Genetics of Keratoconus and Related Diseases

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1. INTRODUCTION

Keratoconus (KTCN) is a degenerative corneal disease characterized by progressive stromal thinning, which results in the conical shape of the cornea (Figure 16.1). These structural changes in the corneal layers induce optical aberrations, leading to a loss of visual acuity due to distorted blurred vision, which is caused by irregular astigmatism and high myopia[1]. KTCN belongs to the broad group of corneal ectasias, which are characterized by bilateral thinning of the central, paracentral, or peripheral cornea[2]. Although KTCN is sometimes referred to as

corneal dystrophy, it is not included in the IC3D Classification of Corneal Dystrophies[3], and should be distinguished from this group of corneal diseases. Reported co-occurrence of KTCN with many types of corneal dystrophies, including Avellino and Fuchs dystrophies[4–6], may indicate that common molecular mechanisms in the pathogenesis of these disorders are involved; however, further studies are needed[7]. Also other genetic syndromes have been associated with KTCN, including Down syndrome[8], Marfan syndrome[9], osteogenesis imperfecta[10], GAPO syndrome[11], and Ehlers–Danlos syndrome[12,13].

2. KTCN NATURE

Etiology

Constant eye rubbing[14], contact lens wear[15], atopy[16], and UV light[17] have been described as the major environmental and behavioral risk factors contributing to KTCN pathogenesis. Different lines of evidence, including familial inheritance[18], a concordance between monozygotic twins in contrast to dizygotic twins[19,20], and the occurrence of syndromic KTCN[21] support a genetic basis for the disease. Still, whether KTCN is inherited according to a Mendelian or non-Mendelian pattern remains unknown. In most cases of familial KTCN, the disease shows an autosomal dominant pattern but with incomplete penetrance. Therefore, a genetic predisposition to KTCN, along with environmental, behavioral, and other (still unknown) factors, most likely causes the development of KTCN. These results are in line with our hypothesis, introduced in 2011, that KTCN is a complex, multi-factorial trait[22].

Prevalence

Among the general population, the estimated frequency of KTCN is 1.38 per 1000 individuals[23]. However, the prevalence of KTCN may vary according to patient ethnic origin, applied diagnostic tests, and the groups of individuals selected for particular study types[24–26].

The disease affects both genders, but the differences in occurrence of KTCN between males and females remain unclear. A study in patients in the United States showed that KTCN occurs more frequently in men[27], and a higher incidence of KTCN in males has also been observed in Poland. Between January 2007 and March 2015, in Department of Ophthalmology at the Medical University of Warsaw, KTCN was diagnosed in a total of 1004 Caucasian patients, 806 of which were male (Prof. Jacek P. Szaflik, M.D. Ph.D, 2015, personal correspondence).

We still lack knowledge regarding the influence of sex hormones on the course of KTCN. Since sex-dependency and pregnancy-caused progression were observed in KTCN, and hormones could influence cell survival, proliferation, and differentiation, various studies have been conducted, and the effects of hormone status, gender, and gestational period on KTCN severity and progression were demonstrated[28–30]. Later these findings were explained as the action effects of estrogen, progesterone, and androgen receptors expressed by corneal tissue[31]. Still, despite reported upregulated expression of these receptors (on a transcript level) in KTCN patients[32], most of these receptors (on a protein level in IHC staining) have not been clearly validated. Also, based on altered expression of genes responsible for the transduction of hormonal signals to the nucleus (*RAF1, GRB2, JAK1, CREB1*), genes related to sex hormones' synthesis (*HSD17B2*), and the strongly overrepresented '*The Activated PKN1 stimulates transcription of AR regulated genes KLK2 and KLK3 pathway*' in pathway analysis of the corneal epithelium transcriptome in patients with KTCN, we concluded that secondary messengers could influence the observed hormonal dependencies[33]. However, the mechanism through which hormones regulate corneal homeostasis and influence changes in KTCN remains unclear.

Natural history

The first symptoms of KTCN usually appear in puberty, and the disease progresses to varying degrees over one to two decades. The progression of KTCN is understood as a consistent change in at least two of the following tomographic parameters: steepening of the anterior surface, steepening of the posterior corneal surface, and thinning and/or an increase in the rate of corneal thickness change between the corneal periphery and the thinnest point on the cone[34]. Reduced best spectacle-corrected visual acuity is often associated with an alteration in tomographic parameters, but it is not necessary to identify the advancement of ectasia[34]. In general, KTCN progression stops in the middle of the fourth decade, presumably due to physiological age-related cross-linking. Mild KTCN tends to progress in approximately 25% of patients, and this progression continues for 3.5 years on average[35]. KTCN may advance more rapidly in pregnancy and during childhood[34]. In 2.6–2.8% of patients, a corneal hydrops occurs. In this complication, aqueous from the anterior chamber enters the corneal stroma and epithelium as a result of a rupture of Descemet membrane and endothelial damage[36].

Inflammation in KTCN

KTCN is described as a non-inflammatory ectatic disease. There is increasing evidence of the influence of inflammatory factors in KTCN, but additional studies are needed. The inflammatory processes in KTCN eyes were first mentioned in 1991. Fabre and co-workers showed that corneal fibroblasts presented fourfold more IL-1 binding sites than normal/unaffected fibroblasts, suggesting that these corneal fibroblasts could have increased sensitivity to IL-1 compared with normal corneal fibroblasts[37]. IL-1 is critical for the inflammatory process and could participate in triggering apoptosis of the corneal cells, which might lead to the development of KTCN[38]. Subsequent analyses of the tear cytokine and protease

profiles of KTCN-affected patients showed a reduced anti-inflammatory capacity in KTCN, providing evidence of the role of inflammation in the thinning and weakening of the corneas[39,40].

In 2013, we identified numerous variants in genes encoding inflammatory molecules, *IL1A* and *IL1B,* in a KTCN family, but we found a random distribution of these variants between individuals with and without KTCN. In all patients with KTCN, we found a c.214+242C>T variant in the *IL1RN* gene, which encodes a protein that modulates the effect of IL-1. We were not able to confirm a role of interleukins in triggering KTCN, but the association of IL-1-related single-nucleotide variants (SNVs) with KTCN suggests that some inflammatory events may be responsible for KTCN[41]. Besides published transcriptomic and proteomic findings, the genetic aspects regarding immune system functioning have not been demonstrated in KTCN research. As discussed in the following subsections, the negative effect of eye rubbing and allergies combined with inflammatory aspects have been broadly reported in KTCN.

3. KTCN DIAGNOSIS, MANAGEMENT AND TREATMENT

Advanced KTCN

In KTCN patients, slit lamp biomicroscopy reveals thinning (usually located inferiorly), apical decentration and conical protrusion of the cornea with iron deposits in the deep layers of the epithelium at the base of the cone (known as a Fleisher ring, fine lines (Vogt's striae), and occasionally scarring at the apex of the cone. The other accompanying signs observed in KTCN eyes are epithelial bullae, anterior stromal scars, enlarged corneal nerves, increased intensity of the corneal endothelial reflex, and subepithelial fibrillary lines[1].

The typical external signs of the disease include Munson sign, a V-shaped molding of the lower lid produced by the ectatic cornea in downgaze (Figure 16.1), and Rizzuti sign, a sharply focused beam of light occurring at the nasal limbus when the cornea is illuminated laterally. The

appearance of a "scissor-like" reflex motion on retinoscopy suggests the development of an irregular astigmatism.

Early-stage of KTCN

While the signs mentioned above are evident in moderate and advanced stages of the disease, in early KTCN the cornea may appear normal without clear features by slit lamp biomicroscopy. Therefore, other diagnostic methods should be applied to detect early-stage corneal ectasia. Retinoscopy appears to be a sensitive and reliable test for detecting KTCN including early-stage disease[42]. Placido corneal topography allows for the detection of early forms of the disease in the absence of clinical signs by analyzing the anterior surface of the cornea. Advanced computer-assisted topographic analysis can reliably measure the minimal topographic criteria necessary to determine the presence or absence of the early stage of corneal ectasia. Useful algorithms that topographically quantify the phenotypic features of KTCN are based on four indices: the K-value (an expression of central corneal steepening), the I-S value (an expression of inferior-superior dioptric asymmetry), the AST index (which quantifies the degree of irregular astigmatism SimK1-SimK2), and the skewed radial axis SRAX index (an expression of an irregular astigmatism occurring in ectasia)[43,44].

Because videokeratography examines only the anterior surface of the cornea, this technology is insufficient for the detection of early stage KTCN. More technologically advanced imaging systems, such as Orbscan IIz (Bausch & Lomb), Pentacam (Oculus), and Galilei (Ziemer Ophthalmic System AG), use scanning slit or a rotating Scheimpflug camera and provide data to evaluate and analyze both the anterior and posterior corneal surface elevation, corneal thickness, pupil size, and anterior chamber (size, volume, and angle), which are diagnostically helpful in cases of early or subclinical ectasia.

Management and treatment

The management of KTCN is intended to stop the progression of the ectasia in the earliest phase and improve vision. The selection of the best treatment method depends on the stage of the disease, the visual acuity, and the patient's age and his/her visual acuity requirements.

As already mentioned, eye rubbing, also associated with allergies or eye irritation, is a known risk factor for KTCN progression. Therefore, patients should be informed not to rub their eyes, and in cases of allergies, topical anti-allergic medication should be prescribed[34]. The use of topical lubricants to decrease ocular irritation is also recommended.

Spectacles and contact lenses

Correction of visual acuity with spectacles is usually possible only in mild KTCN. Due to a high irregular astigmatism, spectacle glasses are not helpful in more advanced cases. The type of contact lens that can be used depends on the severity of the disease. In the early stage of KTCN, soft hydrogel, silicone hydrogel, soft toric, or custom soft toric contact lenses can be used to correct a mild irregular astigmatism. In more advanced stages and in patients with a high irregular astigmatism, rigid gas permeable, scleral, or hybrid lenses may be required to improve vision.

Corneal cross-linking

Corneal cross-linking (CXL) is a method that enables the formation of covalent bonds between collagen fibers to provide additional concentration and strength to their structure. CXL stabilizes the progression of the corneal ectasia, and, to a small degree, improves patients' quality of vision[45,46].

The standard Dresden Protocol of CXL procedure embraces removing of corneal epithelium using a blunt spatula, after 20 seconds exposure to 20% ethyl alcohol, under local anesthesia and topical administration of 0.1% iso-osmolar riboflavin solution - every two minutes during 30 minutes period. Next, after pachymetric measurements (a corneal thickness of

more than 400 μm), the cornea receives a UV-A irradiation for 30 minutes with an intensity of 3 mW/cm2[47,48]. This original protocol is sometimes replaced by either epi-on (with intact corneal epithelium) or accelerated (with irradiation in shorter time with higher intensity, e.g., 10 mW/cm 2 for 9 min) techniques. However, long-term studies have not shown an advantage of one protocol over the other[49,50].

Keratoplasty

For patients with advanced disease, keratoplasty is the only feasible therapeutic approach. In a large study, based on the Clinformatics Data Mart Database (OptumInsight, Eden Prairie, MN), which contains de-identified medical claims of all beneficiaries from a national insurance provider in the United States, 9% of 21,588 individuals with diagnosed KTCN underwent penetrating (PK) or lamellar keratoplasty[51]. KTCN is one of the most common indications for corneal transplantation in developed countries, but the traditional full-thickness PK has increasingly been replaced by deep anterior lamellar keratoplasty (DALK), in which the corneal endothelium is preserved in a patient[52,53].

Healthy recipient endothelium is the main criterion for qualification to proceed to anterior lamellar keratoplasty (ALK) or PK. However, there are reports of the use of modified dissection techniques (careful manual dissection down to near Descemet membrane) in cases of DALK following hydrops[54,55]. Anterior lamellar keratoplasty has several advantages over PK: it preserves the recipient's endothelium and causes less damage to the endothelium and fewer immune reactions, resulting in a lower rate of postoperative endothelial cell loss or endothelial graft rejection.

Modern surgical techniques focus on baring Descemet membrane. These methods include the big bubble (used in more than 51% of cases), and others: viscodissection, hydrodissection, manual layer-by-layer, air-assisted manual dissection, and femtosecond laser-assisted DALK

(performed in less the 25% of the patients[34]. The complete baring of Descemet membrane (descemetic DALK, dDALK) is important for good visual outcomes.

The development of modern keratoplasty techniques has improved visual outcomes and the number of anterior lamellar grafts has significantly increased. The number of patients undergoing PK decreased with an increase for CXL and DALK[20,51,52].

Cellular and gene therapy

The cornea as an immune privileged tissue/organ is an ideal candidate for gene therapy. This feature possibly enables the delivery of gene vectors without the resulting immunogenicity, and thus permits a longer expression of gene in tissue[56]. Unfortunately, the genetic component of multifactorial KTCN is still unclear. Most of the genetic findings in worldwide KTCN studies have been found to be specific to the studied populations / ethnic groups or even the families being assessed. Thus, the prospects of gene therapy for KTCN are limited.

For now, more promising in this field are regenerative therapies based on stem cells. Human adult adipose tissue is a good source of autologous extraocular stem cells and importantly constitutes an easily accessible source of stem cells. Autologous adipose-derived adult stem cells (ADASC) are able to differentiate into multiple cell types (keratocytes, osteoblasts, chondroblasts, myoblasts, hepatocytes, neurons, etc.) and the direction of this differentiation depends on very specific stimulating factors[57]. The safety and efficacy of ADASC implantation within the corneal stroma of five patients with advanced KTCN were assessed[58]. During this clinical study no intraoperative or postoperative complications were recorded, supporting the safety of the procedure. Interestingly, the improved visual function of 1 line of unaided and spectacle-corrected distance vision and 2 lines of rigid contact lens distance vision, as well as the improvement of 16.5µm in the mean central corneal thickness, and synthesis of

new collagen were reported[58]. Additionally, other clinical studies demonstrated the combined effect of ADASC implantation with decellularized laminas of corneal stroma as giving better results than other carrier laminas from biological or synthetic materials or ADASC alone[59,60]. However, further studies with representative groups of patients are required to verify these preliminary results.

Also, the bone marrow mesenchymal stem cells[61,62], umbilical cord mesenchymal stem cells[63], and corneal stromal stem cells[64] were studied with in *in vivo* protocols, while the embryonic stem cells[65] and induced pluripotent stem cells[66] were evaluated *in vitro*. Unfortunately, these studies based on the stem cells showed limitations such as the high laboratory costs and unrepeatable therapeutic efficacy in different patients with KTCN[57].

4. ENVIRONMENTAL AND BEHAVIORAL RISK FACTORS

Since the etiology of KTCN is multifactorial, the behavioral, environmental, and socioeconomic aspects should be carefully inspected in undertaken research due to possible effects on gene expression patterns and phenotype[67,68]. Clinical studies have shown that KTCN is associated with contact lens wear, chronic eye rubbing, allergy, atopy, asthma, and UV exposure[69]. Supposedly some of these factors constitute trigger(-s) in genetically predisposed individuals.

Multiple reports confirmed the association between eye rubbing and KTCN, finding this behavior as the most significant predictor of KTCN in multivariate analysis with an odds ratio of 6.31[16]. Studies of cases of asymmetric KTCN showed that the most vigorously rubbed eye was affected the most[70]. That vigorously rubbing in majority of cases is observed as fist or knuckle type eye rubbing, in which applied force is greater than in a fingertip type[14,71]. These findings

support the concept that chronic mechanical trauma to the cornea plays a role in the pathogenesis of KTCN. Proposed mechanism of the rubbing-related KTCN includes microtrauma caused by rubbing the eye, which damage the corneal epithelium, leading to the release of cytokines, elevated levels of matrix metalloproteinases MMP-1 and MMP-13, apoptosis of keratocytes, myofibroblast differentiation, a change in biomechanical forces, and thinning of corneal tissue[72–74].

Some reports claimed that eye rubbing itself cause KTCN[75]; however not every individual who rubs his or her eyes chronically develops pathological changes, indicating other factors, including genetic susceptibility. Although some papers presented eye rubbing and/or eye compression at night as the only factors causing 'unilateral' KTCN[75,76], currently the global consensus on KTCN indicates that truly unilateral KTCN does not exist. Secondary or induced ectasia, caused by an exclusively mechanical process in a predisposed cornea, is the only mechanism by which KTCN can occur unilaterally[34]. The challenge that clinicians face is early detection of *forme fruste* KTCN. Cases with *forme fruste* KTCN may exhibit subtle topographic characteristics suggestive of early subclinical KTCN, but these are not sufficiently pronounced to reach the threshold of suspicion with automated classification systems[77]. Nevertheless, KTCN is an asymmetric progressive disease, and even if it is unilateral at the beginning, in the vast majority of patients both eyes will be affected over time.

Allergies contribute to intensified eye rubbing and also generate an imbalance of the immune system. In a case of recurrent KTCN after penetrating keratoplasty, allergic conjunctivitis seems to be the main trigger for eye rubbing[78]. Previously, Harrison et al. concluded that eye rubbing was stimulated by ocular itching or discomfort resulting from atopic disease [79]. Other studies also revealed higher prevalence of atopic conditions in KTCN, such as

allergic rash, eczema, asthma, and bronchial hyperresponsiveness[16,23,79–82]. A study in Dundee has shown an associated skin disorder in 34% of patients, hay fever in 36%, and asthma in 21%[83]. In addition, ethnic origin influenced the incidence of atopy in KTCN, with the presence of atopic disease significantly higher in White compared to Asian keratoconic patients[24]. Also, it has been reported that atopy was less common in patients with KTCN identified in one eye only[79].

KTCN has been reported in association with a history of contact lens wear [15]. Long-term contact lens wear appears to decrease the entire corneal thickness and increase the corneal curvature and surface irregularity[84]. In a retrospective KTCN study, an association between contact lens wear and the development of KTCN was found in patients diagnosed after a mean of 12.2 years of contact lens wear[85].

In accordance with available research data, excessive exposure to sunlight leads to oxidative damage in KTCN corneas[86]. Together with a reduced amount of antioxidative enzymes, such as aldehyde dehydrogenase class 3 (ALDH3) and superoxide dismutase, the effectiveness of the removal of reactive oxygen species (ROS) is insufficient[86–89]. The experimental finding showed degeneration of stromal collagen, stromal thinning, and loss of keratocytes in mice exposed to UV light[90]. As studies have shown that there is a higher incidence of KTCN in countries with warmer climates compared to Europe and North America, it is believed that high sun exposure is responsible for the high prevalence[69]. Consanguineous marriages may also be a factor, as they are more common in certain countries with warmer climates.

5. INTEGRATING OMICS-BASED RESEARCH TO UNDERSTAND NATURE OF KERATOCONUS

A multi-OMIC approach - embracing genomics, transcriptomics, proteomics, metabolomics, epigenomics, and microbiomics - allows for comprehensive analyses of DNA mutations and other sequence variants, distinct isoforms, protein and metabolite profiles, methylation patterns, and previously unrecognized microbiota in the studied samples.

To date, various approaches, including both simple molecular techniques and high-throughput technologies with computational and statistical methods, have been used for KTCN research. However, the vast majority of KTCN studies are focused on a single biological element only, such as a genome or transcriptome. As the molecular system responsible for the biological functioning of the human organism is complex, changes identified on one level may be reflected in other components of molecular networks. For example, disruption on the genomic or epigenomic level may lead to changes in the transcriptome, proteome, or metabolome. Thus, an analysis of a single part of whole biological network makes it difficult to systematically understand the molecular basis of the disease. This is why the holistic integration of different OMICS approaches allows for more optimal discovery of factors influencing the KTCN phenotype. This combination can not only identify genetic factors exclusively characteristic of KTCN but also factors associated with other eye diseases, as shown in Figure 16.2. The mentioned genetic, transcriptomic, proteomic and metabolomic aspects as well as characteristics of ocular surface and corneal microbiota in KTCN are characterized below.

GENOMIC APPROACHES IN KTCN

Numerous loci in keratoconus

One of the genomic approaches used to identify genes influencing the KTCN phenotype is linkage analysis, which has been performed in KTCN families with both affected and unaffected

individuals available for testing and has allowed for the identification of numerous chromosomal regions linked to KTCN (see Table 16.1). The KTCN loci have been mainly determined in families of Caucasian, Hispanic, or Italian ethnic origin, with the newer loci recognized in other populations, including Ecuadorian, Arabic, Caribbean African, Finnish, and Northwest Tasmanian. The listed loci have been identified for both isolated KTCN (e.g. 3p14), and KTCN associated with other genetic diseases such as Leber congenital amaurosis (e.g., 17p13)[91,92]. Among the reported chromosomal regions, only one locus (5q21.2) has been replicated[93,94]. The remaining KTCN susceptibility loci were representative for specific populations, small numbers of families, or most frequently, for single families only (Table 16.1). Finding the candidate gene is additionally complicated by the fact that chromosomal intervals mapped by linkage analyses are usually large in size (up to several megabases) and contain numerous candidate genes to be further evaluated[95]. Mutation screening of all putative genes from these regions requires substantial additional effort; thus, post-linkage analysis is often narrowed to the sequencing of a few functional genes localized in the genetic interval between markers with the maximum LOD score[95]. However, functional and positional candidate genes often do not contain mutations related to the disease phenotype. For example, molecular screening of *COL8A1* and *COL4A1* - *COL4A2* at 3p14-q13 and 13q34 KTCN loci, respectively, has not revealed any potentially pathogenic variants[91,96]. Also, the promising candidate genes may be localized in close proximity to the highest linkage peak and not exactly in the peak. For example, in an Ecuadorian family, we identified sequence variants that fully segregated with the KTCN phenotype and localized in the *IPO5*, *STK24*, and *DOCK9* genes (Table 16.1), which are mapped in distance (0.12 Mbp, 0.43 kbp, and 0.34 Mbp, respectively) to the *FARP1* gene, for which the maximum LOD score (4.1 in multipoint parametric linkage and 3.2 for multipoint nonparametric linkage) has been

obtained[97], but molecular screening of *FARP1* revealed no pathogenic variants in the affected members of the assessed family with KTCN[97,98]. In contrast, candidate genes might be mapped in genomic regions with low linkage signals [95]. For example, in two suggestive loci for KTCN—2q13-q14.3 and 20p13-p12.2, with LOD scores of 2.395 and 2.409, respectively, screening of candidate genes has revealed variants in *IL1RN* and *SLC4A11* genes, observed significantly more frequently in family members with KTCN[41].

In linkage studies it is important to ascertain a number of multigenerational families with many affected and unaffected individuals[95]. Although some of the KTCN loci have been identified using linkage analysis of small KTCN families, no causative mutations have been found in genes from those loci[99]. As indicated above, some patients may have a *forme fruste* or early form of the disease that could be not recognized. Thus, some affected individuals might be classified as unaffected in the family pedigree, causing difficulties or errors in phenotype-genotype correlations[95]. Furthermore, in a complex disease such as KTCN, in which both genetic and environmental factors influence the phenotype, identification of linked genes is more complicated than in the simple model of a monogenic disorder[20].

Association studies in KTCN

In KTCN genomic studies, the potential relationship between an allelic variant of a chosen genetic marker and disease phenotype has been frequently explored[22]. For example, Kim and colleagues performed a study to investigate the association between c.-31T>C and c.-511 C>T polymorphisms in the *IL1B* promoter and KTCN, and these two variants were found to be associated with a significantly increased risk of KTCN in Korean patients (Table 16.1)[100].

A genome-wide association study (GWAS) was first applied in KTCN research in 2011[101], where analyses based on cohorts from the USA, Australia, and Northern Ireland led to the

identification of a positive association of KTCN with rs1014091 and rs3735520 SNPs in the *HGF* gene (Table 16.1). Another GWAS revealed that rs4954218 SNP mapped upstream of the *RAB3GAP1* gene and was associated with KTCN in a Caucasian cohort from the USA[102] (Table 16.1). These findings were replicated in Australian Caucasian cases with KTCN[103]. Evidence of a genetic association was also found between KTCN and rs10519694 and rs2956540, located in the *LOX* gene in two independent case-control panels and in KTCN Caucasian and Hispanic families[104]. Also, six loci associated with central corneal thickness and KTCN, containing sequence variation in the *FNDC3B*, *MPDZ-NF1B*, *RXRA-COL5A1*, *COL5A1*, *FOXO1*, and *BANP-ZNF469* genes were identified[105]. The newest large scale genome-wide association study in KTCN, involving 4,669 cases and 116,547 controls, showed significant association with 36 loci, including the 15q22.33 locus with SNPs in *AAGAB, IQCH, SMAD3* genes and 20p13 locus with *STK35* gene[106]. A detailed list of loci/sequence variants reported to be associated with KTCN is presented in Table 16.1.

The interpretation of GWAS data is still a great challenge, especially if the associated sequence variant is located in a non-coding region or in a distance from known genes. Both the inter- and intra-genic regions of human genomes may contain regulatory elements, but little is known regarding how particular variants affect their functions and how this influences the phenotype[95]. Therefore, the statistically significant association of a particular SNP with a disease does not prove that this variant is actually causative for the disorder and further studies are required to assess its role in disease development[95]. For complex traits such as KTCN, meaningful results can be obtained with several hundred or thousand individuals having a well-defined phenotype. Poor phenotypic classification or inconsistent phenotype exams (using different inclusion criteria in various ascertainment places) in patients may lead to false positive

associations[95]. As GWAS recognizes only common risk alleles, the locus associated with GWAS signals may be far larger than assumed. Therefore, a good solution for revealing rare variants would be to extend the sequencing region to at least a few additional megabases around the GWAS signal[107]. Rare alleles may also be discovered through ES or WGS analyses, discussed later in this chapter.

Mutational screening by Sanger sequencing

Automated Sanger sequencing is routinely used in KTCN studies based on screening of candidate genes in patients with KTCN compared with unaffected persons, and numerous promising genes implicated in KTCN etiology have been revealed as indicated in Table 16.1. Among them are *VSX1* and *SOD1*, the first two genes widely investigated worldwide in KTCN research. Although potentially disease-causing pathogenic variants were initially identified in both genes[108,109], only a few subsequent studies have confirmed these findings[110,111] and many studies have failed to identify the causative sequence variants in these genes[112–115]. Since then, many other genes have been assessed as causative for KTCN (Table 16.1). However, no pathogenic variants have been identified that were reproducible between populations and studies in patients with KTCN, indicating that other genetic factors, including the not investigated intronic variants and other regulatory elements, could be causative in KTCN pathogenesis.

Capillary sequencing was utilized in the genetic screening of eight positional candidate genes at the 13q32 KTCN locus (Table 16.1), in which a possible pathogenic variant in *DOCK9,* c.2262A>C, was identified in Ecuadorian patients with a familial form of KTCN[98]. This affected splicing, as confirmed *in vitro*, revealing exon skipping and indicating the functional relevance of *DOCK9* substitution in KTCN[116]. Also, the analysis revealed that other sequence variants in intronic fragments of *DOCK9* (c.720+43A>G), *IPO5* (c.2377-132A>C), and *STK24*

(c.1053+29G>C) also segregated with the disease phenotype in the examined KTCN family. As mentioned above, the non-coding regions of genes contain many regulatory elements and intronic alterations, including single-nucleotide changes, which may trigger a deleterious effect on pre-messenger RNA splicing[117]. The identification of the three additional sequence variants in the 13q32 KTCN linked region could be meaningful rather than coincidence.

The screening of candidate genes conducted by Lechner and co-workers identified a significant enrichment of potentially pathogenic heterozygous alleles in *ZNF469*, which was found in 12.5% of European patients with KTCN[118]. To determine whether genetic variants in *ZNF469* contribute to the disease, further studies have been carried out in other cohorts but with contradictory results[119–121]. The high prevalence of *ZNF469* variants observed in patients with KTCN is typical for a common genetic variation observed in the general population. Moreover, the high phenotypic heterogeneity demonstrated by *ZNF469* gene mutations may indicate that similar processes are involved in diseases characterized by corneal thinning in general rather than only in KTCN[95,120].

In an analogous situation, mutations in *TGFBI* and *SLC4A11* were reported in several corneal diseases, including KTCN and corneal dystrophies[41,116,122–124]. Because *ZNF469*, *TGFBI* and *SLC4A11* are highly polymorphic genes, research carried out for larger groups of both patients and controls is required to further evaluate these candidates.

In addition to the numerous sequencing studies focused on genomic DNA, studies have also been conducted on the level of mitochondrial DNA. Assessment of the mitochondrial genome in 42 Polish individuals with KTCN revealed numerous sequence variants, including several novel sequence changes. However, the vast majority of these variants were observed in both affected individuals and in the control group, suggestive of polymorphic sequence

variations rather than causative variants[125]. An analysis of mitochondrial complex I genes (*ND1-ND6*) in 20 Indian KTCN patients revealed 84 variants, including two novel frameshift mutations[126]. A full mitochondrial genome investigation conducted in KTCN cases from Saudi Arabia revealed mitochondrial DNA mutations in 38.5% of KTCN patients. Among identified changes, one nonsynonymous variant was heteroplasmic, whereas the remaining nine were homoplasmic. There is insufficient data to determine whether heteroplasmy is involved in KTCN[127]. Because traditional PCR followed by direct Sanger sequencing is insufficient to detect heteroplasmy and also lacks the necessary sensitivity to identify low levels of mosaicism[128], techniques other than Sanger sequencing should be applied to assess heteroplasmy in KTCN.

Exome and whole genome sequencing as tools for high-throughput data generation

Screening of numerous candidate genes using Sanger sequencing is time-consuming and labor intensive, and is usually not successful in large-scale studies. These limitations can be reduced using next-generation sequencing (NGS), whole-genome sequencing (WGS) and targeted re-sequencing, including exome sequencing (ES)[129].

To date, WGS has not been applied in KTCN research. Since WGS is an effective method of detecting pathogenic variants in genetically heterogeneous diseases, including ophthalmic diseases[130], it can also be effectively used to decipher the observed locus and allele heterogeneity in the etiology of KTCN.

The literature data provide many examples of the efficacy of ES in identification of gene sequence variation in both rare and common complex disorders, including ocular diseases[131]. An ES approach involving American families of European ancestry revealed 23 potentially pathogenic variants in *HSPG2, EML6, CENPF, NBEAL2, LRP1B, PIK3CG, MRGPRD*, and *ITGAX* [132]. ES studies in Jordanian[133], Ecuadorian[134] and Chinese[135] families with KTCN showed

different variants in *MYOF, STX2, COL6A5, ZNF676, ZNF765*, and *SKP1, PROB1, IL17B, HKDC1*, and *TRANK1, ERMP1, SDK2, COL6A1, CNBD1, KRT82, NSUN5*, and *COL9A3* (Table 16.1). Again, the disclosed variants did not repeat between populations, although the majority of the listed genes were attributed to the same pathway, namely the pathway of cytoskeleton organization.

Targeted resequencing

In KTCN research a targeted resequencing of a subset of the human genome has been frequently utilized. For example, one study did a selective screen of all genes within the 5.5 Mb region of chromosome 15q22-q25, which was previously reported as linked with KTCN in a Northern Irish family. That study resulted in mutation detection in the seed region of the *miR-184* gene, indicating that this gene could be responsible for the familial form of KTCN[136,137]. Since then other targeted resequencing studies in patients with KTCN were carried out and sequence variation specific for KTCN, including *LOX, COL5A1, TIMP3, COL2A1, COL5A1, TNXB, and ZNF469*, and *COL12A1* was identified (Table 16.1)[116,138,139].

It should be emphasized that since ES studies are restricted to determining variants in the protein coding sequences, many potentially important variants localized in the remaining parts of the genome could be missed. Also, some difficulties in results interpretation may result from insufficient sequencing coverage or inappropriate algorithms used for variant calling and annotation, especially in case of variants from multi-allelic sites. Despite the expanded databases and sophisticated pipelines for data processing, both the narrowing of a large amount of initial data to a manageable number of variants and the filtering of candidate variants without a loss of possible causative ones are still challenging[95]. While trio analysis (patient and both parents are included) in search of *de novo* mutations is quite simple, looking for hereditable mutations in

large families with a complex disease such as KTCN could be problematic and requires additional Sanger-segregation analyses[95]. Despite those limitations, ES is still an effective tool in searching for causative sequence variants in multigenic diseases.

Transcriptome characteristics in KTCN

Over the last few years, various transcriptome studies have been performed to understand how the changes in gene expression influence the KTCN phenotype.

One technique used in KTCN transcriptome studies is microarray-based gene expression analysis, which allows profiling of a subset of relevant target genes and analysis of thousands of genes simultaneously. Microarray-based analysis of gene expression profiles in normal human donor corneas has revealed the expression of ~1200 genes, including six collagen genes that have not previously been reported as localized within the cornea tissue[140]. However, to expand the knowledge about corneal gene activity in KTCN, comparative gene expression studies are necessary. The first microarray-based comparison of the gene activity of KTCN and non-KTCN tissues was performed in human cultured keratocytes derived from five KTCN and five control corneas and included 164 apoptosis-related genes[141]. Three of nine genes identified as differentially expressed, *TNFAIP6*, *IGFBP5*, and *IGFBP3,* were indicated as involved in the mechanism underlying stromal thinning (Table 16.2)[141]. In another study, the abnormal expression of 56 genes in KTCN corneal epithelium samples compared to normal corneal epithelial cells was revealed[142]. Differential expression of five of these 56 previously indicated genes, *CLC*, *DSG3*, *EMP3*, *S100A2*, and *SLPI*, was confirmed in a subsequent study using different techniques[143]. In another study, stromal RNA samples derived from KTCN and non-KTCN corneas were investigated and led to the identification of several different up-regulated extracellular matrix components. In addition, down-regulation of collagen IV and versican encoding genes was observed[144]. Next, a genome-wide microarray transcriptome analysis was

conducted on 10 KTCN corneas and 10 control corneal tissues, in which 87 genes showed significantly differential expression, including the mucins, keratins, and genes involved in fibroblast proliferation[145]. A detailed description of array-based studies in KTCN research is presented in Table 16.2. Array-based studies have indicated a number of abnormally expressed genes in KTCN corneas. Since different types of corneal cells and various array probe sets were used in KTCN microarray studies, differences between the obtained results are not entirely unexpected.

Currently, the most commonly used approach in transcriptome profiling is RNA sequencing (RNA-seq), allowing for direct sequencing of both coding and non-coding RNA. This technology was firstly applied to identify expression markers specific for particular corneal cells, and three genes, *SLC4A11*, *COL8A2* and *CYYR1,* highly expressed in human corneal endothelial cells (HCECs) but not expressed in other studied corneal cells, were identified[146]. Another RNA-seq study discovered specific antigens overexpressed on HCECs surface that can be useful in the purification and detection of HCECs in cultures[147]. In 2017 the RNA-seq approach was used to study the transcriptome of full thickness corneas[148]. This revealed disruption of collagen and extracellular matrix (*BMP1, COL21A1, COL10A1, COL12A1, COL16A1, COL23A1, COL5A2, COL6A1, COL6A2, COL6A3, COL6A6, COL7A1*) and downregulation of the core elements of the TGF-β (*TGFB1, TGFB3, TGFBR1, TGFBR2, CTGF)*, Hippo (*FAT4, AMOT, DLG, PUMA, TEAD2, TEAD4*) and Wnt signaling (*WNT5A, FZD1, FZD7, APC*) pathways influencing corneal organization. Two other studies have reported transcriptomic analyses of corneas obtained during penetrating keratoplasty[149,150], and these highlight elements of TGF-β and WNT signaling pathways and extracellular matrix, as well as

the importance of cell migration/motility, cell adhesion, cytokine response, and inflammatory responses[149,150].

The tested biological material used in the above mentioned KTCN worldwide research has not been the most optimal study material as it consists of a heterogeneous mixture of different cells: epithelial, keratocytes, endothelial, inflammatory cells, and apoptotic cells[149]. Currently the greatest attention is focused on the studied separated corneal layers. For example, the corneal epithelium obtained during CXL procedures has been assessed using the RNA-seq approach[151–153]. In these studies, the strong effect of gender on gene expression in corneal epithelium was observable[153], and the Wnt signaling was again recognized as a KTCN-specific feature[151,153]. Moreover, cell-cell communication, cell junctions and cell signaling were highlighted as significant and consistent with morphological changes in corneal epithelium in KTCN[153].

Going further, the single-cell RNA-seq (scRNA-seq) was utilized to study the complex architecture of the cornea. The single cell atlas of human cornea presented by Colin et al.[154] provides a unique resource for better understanding and treatment of ocular surface disorders. In this study, 17 fetal corneas, five non-KTCN adult corneas and two KTCN corneas were evaluated using scRNA-seq and ATAC-seq. This investigation revealed activation of collagenase in the corneal stroma and a reduced pool of limbal suprabasal cells in KTCN[154]. Research embracing scRNA-seq technique and human samples obtained from three patients with KTCN undergoing the DALK procedure and two healthy donors confirmed dysregulation of collagen and extracellular matrix (ECM) in KTCN. Moreover, elevated cytokines in immune cells and dysregulation in subclasses of the epithelial cells (reduced basal cells and abnormally differentiated superficial cells) in KTCN corneas were found[155].

The implementation of high-throughput RNA sequencing methods improves the ability to examine genomic regions previously thought to be transcriptionally inactive, including the long intergenic noncoding RNAs (lincRNAs). To identify possible links between noncoding RNAs and KTCN genes, we looked for co-localization of lincRNAs with the KTCN loci found in our Ecuadorian families with KTCN to identify the possible relations between noncoding RNAs and KTCN genes. At the 13q32 chromosomal region, we identified TUCP (TCONS_00021879) localized ~1kb from 5' end of *STK24*, a gene in which a mutation that segregated with the disease phenotype has been previously reported. Interestingly, sequencing analysis revealed a g.99230266C>T (rs3809366) TUCP sequence alteration, showing segregation with the KTCN phenotype in a large, multigenerational Ecuadorian family[156]. Since some lincRNAs regulate the expression of nearby genes and may be implicated in a variety of biological processes[157], we suggest that this lincRNA, localized in close proximity to *STK24*, might play a role in the development and/or progression of KTCN in patients from Ecuador[156]. Our mutation screening was performed in material derived from blood. Compared to mRNA expression, lincRNA expression is typically more variable between tissues, and for this reason, analysis of corneal tissue is necessary to assess the detailed contribution of the identified sequence variant in familial KTCN etiology.

We created a platform (KTCNlncDB, http://rhesus.amu.edu.pl/KTCNlncDB/) to investigate lncRNAs expressed in human KTCN and non-KTCN corneas[158] and suggested that long non-coding RNAs (lncRNAs) might be involved in KTCN etiology. In addition, *in silico* functional studies based on predicted lncRNA:RNA base-pairings led us to recognition of a number of lncRNAs possibly regulating genes with known or plausible links to KTCN[158].

Proteomic studies in KTCN

The latest reports have demonstrated progress in proteomic investigations of the human eye in health and disease states. Corneal and tear proteomes in patients with KTCN have revealed a variety of proteins explaining the molecular basis of KTCN and indicating potential biomarkers of KTCN[159–161].

There are many methods for evaluating proteins in the human organism, and among them, the enzyme-linked immunosorbent assay (ELISA) has been widely used in KTCN research. It has been determined using ELISA that the tear film of affected individuals contained higher levels of collagen degradation products (telopeptides) comparing to control individuals[162] (Table 16.2). Collagen is a main corneal protein and plays a crucial role in the maintenance of corneal shape. Therefore, collagen degradation resulting in a decreased amount of total collagen may lead to a mechanical weakening of the cornea and loss of visual function in KTCN[163]. Evidence of corneal degradation was also provided by Lema and Durán, who performed ELISA to measure a level of matrix metalloproteinases and inflammatory molecules in tears in individuals with KTCN[164]. Matrix metalloproteinases are zinc-dependent endopeptidases capable of cleaving different proteins, including collagen. Since the main structural change in KTCN is thinning of the corneal stroma, a higher level of proteolytic activity observed in KTCN eyes (in the form of elevated levels of MMP-9) may strongly indicate degradation processes as a KTCN cause. In the same study increased levels of inflammatory molecules, IL-6 and TNF-alpha were found in KTCN tears, suggesting that both degradation and chronic inflammatory events may play a role in the disease pathogenesis[164] (Table 16.2). Analyses of the tear cytokine and protease profiles of KTCN-affected patients showed a reduced anti-inflammatory capacity in KTCN, providing further evidence of the role of inflammation in the thinning and weakening of the corneal tissue in KTCN[39,40,165]. The pro-inflammatory responses (as elevated level of IL1RN, IGKV1D-33,

IL18, C2, C4A, C9, CFD, CFH, TF, TOLIP) were confirmed in stromal samples of KTCN patients, with the greatest intensity for cone region[166]. In contrast, there were no statistically significant differences found in the level of inflammatory cytokines in serum between normal individuals and patients with KTCN[167].

A very useful approach for assessing protein profiles in different types of samples are two-dimensional electrophoresis (2-DE) followed by mass spectrometry-based techniques (MS). In KTCN, the 2-DE/MS approach was used in a proteomic comparison of corneal epithelium derived from KTCN and myopic patients and indicated abnormal levels of three proteins - gelsolin, alpha enolase, and S100A4[168]. Both up- and down-regulation of several proteins, including keratin 5, L-lactate dehydrogenase, annexin A8, and keratin 12, were identified in the epithelial and stromal layers of KTCN corneas compared to unaffected human corneas[169]. Moreover, the analysis of tears derived from 22 patients with bilateral KTCN and 22 control individuals revealed decreased levels of zinc-α2-glycoprotein, lactoferrin, and Ig-κ chain in KTCN tears[170]. In another tear film proteomic study, Ig-κ chain C and lipocalin-1 and Ig J chain proteins were found to have significantly increased levels in KTCN tears, while the members of the cystatin family, lipophilin-C, lipophilin-A, and phospholipase A2 proteins exhibited down-regulation in KTCN patients[171].

As the modern techniques are capable of detecting lower abundance and low mass proteins, the KTCN corneal proteome analysis using iTRAQ labeling and nano-electrospray ionization liquid chromatography tandem mass spectrometry has revealed as many as ~1000 proteins in human corneal epithelium and stroma. Among them, several proteins showed abnormal levels in KTCN corneas compared to reference corneal tissue. Up- and down-regulated proteins in human KTCN corneal epithelium, including keratins 6A and 16, vimentin, and iron

transporter lactotransferrin, suggest the involvement of degenerative changes and apoptosis in KTCN pathogenesis and progression, which is consistent with a previous report showing increased cell death and deteriorating processes[163]. These findings were verified by analysis of unstimulated tears from patients with KTCN and normal individuals using novel in-solution electrophoresis and linear ion trap quadrupole Fourier transform mass spectrometry. That study revealed increased proteases and decreased protease inhibitors levels in KTCN tears, suggesting the degenerative character of the disease[172]. Moreover, in KTCN corneas the members of ubiquitin proteasomal degradation pathway were reported to be elevated by implementing the liquid chromatography with tandem mass spectrometry (LC-MS-MS)[173]. Taken together, the results of proteomic studies partially correlated with transcriptomics data and demonstrated that structural remodeling and degradation processes might be involved in KTCN pathogenesis. However, there are few functional KTCN studies that could confirm the proteomic and transcriptomic findings, and it remains unclear whether cell degradation is a direct cause of the disease phenotype or a consequence of the pathophysiological process.

Metabolomic studies and oxidative stress in KTCN development

A metabolomic profile is the measurable consequence of the activity of genes and proteins within particular cells, tissues, organs, and body fluids, such as tears or blood. The corneal tissue/ and blood metabolome was assessed in a number of ophthalmic disorder investigations, including KTCN. However, the eye surface metabolome is still not well characterized.

Metabolomic profiles and their specific components can be analyzed with MS or nuclear magnetic resonance (NMR) spectroscopy. In KTCN research, the first study using NMR spectroscopy was performed by Greiner and colleagues in 1989 and different levels of phosphatic metabolites were found in KTCN-affected tissues compared to control tissues, indicating abnormal corneal metabolism in KTCN[174]. Using NMR spectroscopy and high-

performance liquid chromatography, the level of small soluble compounds in human corneas was investigated but no significant biochemical differences were revealed between the metabolic profiles of KTCN and unaffected corneas[175]. However, an increased number of free amino acids was observed in KTCN corneas as a consequence of the elevated activity of proteases in the disease-affected tissues[175,176]. Another study, performed in cultured normal human corneal keratocytes, fibroblasts, and KTCN cells using MS, revealed abnormal levels of lactate, glutathione, and arginine in KTCN cells indicating that KTCN corneal cells are under oxidative stress[177] (Table 16.2).

Oxidative stress is a consequence of the accumulation of reactive oxygen species (ROS), which cause a toxic effect on a number of cellular components, including proteins, nucleic acids, and membrane phospholipids. ROS may be induced by UV light, and since the cornea is constantly exposed to the UV radiation, it is particularly vulnerable to the oxidative stress and damage from ROS[178]. The healthy cornea has several mechanisms involved in the minimization of ROS damaging effects, including both enzymatic activities and non-enzymatic pathways, which are altered in KTCN[179]. It has been shown that level of antioxidants was decreased in KTCN corneas, while the level of oxidative stress markers was elevated, which suggests that mechanisms protecting against oxidative stress and cell degradation may be altered in this disease[17,180,181]. It is unclear whether oxidative cell degradation is a primary process in the disease development or if it is a secondary effect.

Also, unclear remains the role of sex and sex hormones in KTCN. Elevated levels of DHEA and PGF2α, PGA2, PGE2 and 5-HETE, contributing to steroidal hormone synthesis, were observed in the serum of patients with KTCN[182]. Since steroidal hormones could influence

gene expression and indirectly the extracellular matrix[183], these results could be significant for KTCN pathophysiology.

Are epigenetic factors involved in KTCN?

The epigenetic profile can vary among different cell types, and it is fundamental to human development. Epigenetic mechanisms include histone modification, nucleosome remodeling, DNA methylation, and regulation of gene expression by small and non-coding RNAs[184]. As epigenetic changes may be associated with the development and progression of several diseases, epigenomic studies need to be added to molecular-based investigations of human disorders[185,186].

Disruption of the balance between epigenetic factors could play a role in several ocular diseases, as reviewed by Yan and colleagues[187]. The first high-throughput epigenetic study in KTCN, based on the performed reduced representation bisulfite sequencing (RRBS) in human corneas, was published in 2019, in which genomic multiple KTCN-specific differentially methylated regions were detected and many of them overlapped previously identified KTCN linkage loci (3p14.3, 5q35.2, 13q32.3, 15q24.1, and 20p13) and chromosome arms that have been linked to KTCN (2q, 4q, 5p, 9p, 14q, and 17q)[188].

Previously, as already indicated above, a c.57 C>T mutation altering seed region of *miR-184* was found in a family with severe KTCN combined with early-onset anterior polar cataract. It was revealed that the mutant form of miR-184 failed to compete with miR-205 for overlapping target sites on the 3' UTRs of *INPPL1* and *ITGB4*[136]. The mutations in *miR-184* were also identified in three subsequent studies in two Chinese patients with isolated KTCN; in patients with a syndrome characterized by endothelial dystrophy, iris hypoplasia, congenital cataract, and stromal thinning (EDICT); and in members of family with congenital cataracts and corneal abnormalities including KTCN[189–191]. As the occurrence of *miR-184* mutations in KTCN

individuals was confirmed in independent studies, altered expression of *miR-184* target genes cannot be excluded as a possible factor in disease development.

Corneal microbiome and KTCN phenotype

The human microbiome consists of the genes and gene products (RNA, proteins, metabolites) produced by microbial communities[192]. Since the number of microbiota living in the human organism is enormous, it is postulated that some human metabolic features may be dependent on both microbial and human traits[193].

There is no evidence that microorganisms could be involved in etiology of KTCN. However, it is possible that through atopy and environmental factors, including eye rubbing and contact lens wear, microbiota populations in the KTCN ocular surface may differ from microbiome in the healthy ocular surface and may include an increased number of pathogens/opportunistic pathogens. KTCN patients, due to contact lens therapy and corneal hydrops that could co-occur with KTCN, have an elevated risk for corneal infection. It is known that contact lenses may increase the risk of pathogenic colonization of the ocular surface by the adherence of microorganisms to the lens and extended contact time of pathogenic bacteria to the corneal surface. Moreover, the loss of the epithelial-endothelial barrier of the cornea caused by ruptures of Descemet membrane associated with corneal hydrops may also significantly increase the risk for bacterial invasion. Thus, detailed characterization of the corneal microbiome could be helpful in future diagnostics and in the treatment of patients in whom KTCN co-occurs with corneal infection[194].

The ocular microbiome composition has only been partially characterized and the published investigations[195–197] focus on conjunctival rather than corneal samples. In one study, short RNA-Seq reads, obtained from a previous transcriptome study of 50 corneal tissues, including those derived from patients with KTCN, were mapped to the human reference genome

GRCh38 to remove sequences of human origin[198]. The assessment revealed significant variability of the corneal microbial communities, including fastidious bacteria and viruses [198] but no significant differences between unaffected and KTCN corneas were found. Still, further studies on ocular surface and corneal microbiota in KTCN are necessary.

6. KERATOCONUS COEXISTING WITH OTHER DISEASES

In the majority of patients KTCN manifests as an isolated sporadic disease. However, in some cases it may be a sign of a syndromic condition. Among those underlying disorders are Down syndrome[199], sleep apnea[200], asthma[201], Leber congenital amaurosis[21], and various connective tissue disorders[12,13]. Direct causal relationships between these disorders and KTCN have not been established.

A strong coexistence of Down syndrome and KTCN has been recognized, with a reported incidence ranging from 0.2% to 15%, depending on the type of the study and whether it was nation- or hospital-based[8,202–204]. The nationwide study in Norway showed an estimated prevalence of KTCN of 5.5% among persons with Down syndrome, which was 30 times higher than that in the general population[8,205]. Moreover, biometric measurements revealed thinner corneas and higher keratometry values in all adolescents with Down syndrome regardless of KTCN status[206]. Proposed explanations for this association are collagen-related abnormalities and a greater frequency of eye rubbing and atopy being diagnosed in patients with Down syndrome[204,206,207]. Scientists have also suggested a genetic link between KTCN and the disease locus at chromosome 21[207], however this speculation has not been confirmed and sequence variants related to phenotype were not found.

KTCN was observed in 29% of children with autosomal recessive Leber congenital amaurosis in Israel in 1994, pointing to the genetic association[21]. Familial segregation analyses revealed a nonsense variant c.834G>A (p.Trp278X) in the *AIPL1* gene[208]. Sequence variants in this gene as well as in the *CRB1* gene were suggested as causative in two unrelated families[209]. Moreover, various mutations in the *CEP290* gene were reported among patients with Leber congenital amaurosis and KTCN (Figure 16.3)[210,211].

Fuchs' corneal endothelial dystrophy is the most frequent corneal dystrophy diagnosed in patients with KTCN with the combined incidence approximately 1 per 100,000 [212]. In a 10 year long hospital study at Wills Eye Hospital in the USA, in the group of 51 patients with KTCN associated with another corneal dystrophy, 52.9% were diagnosed with Fuchs' corneal endothelial dystrophy diagnosed within time of 10 years[7]. First, a c.1920G>T (p.Gln640His) variant in the *ZEB1* gene was identified in a family affected with KTCN and Fuchs' endothelial corneal dystrophy[213]. Then, the same variant was discovered in a patient diagnosed with KTCN, epithelial basement membrane corneal dystrophy, and Fuchs' endothelial corneal dystrophy[214]. Besides the evidence of sequence variation, there is a hypothesis that combined incidence of KTCN and Fuchs' corneal dystrophy is an effect of some (unknown) disturbance in the differentiation of the neural crest cells as the corneal stroma and endothelium originate from the neural crest[5]. Although co-occurrence of these diseases is supported by multiple clinical data, the cause(-s) have not been identified.

Connective tissue disorders, such as subtypes of Ehlers-Danlos syndrome, osteogenesis imperfecta, mitral valve prolapse, and joint hypermobility, have been linked to an increased risk of KTCN due to weakening of the connective tissue within the cornea[13,138,215–217]. A group of 50 patients with classical Ehlers-Danlos syndrome had macro- and microstructural changes of the

cornea, including thinner and steeper corneas and lower keratocyte densities irrespective of KTCN diagnosis[218]. Genes found as involved in Ehlers-Danlos syndrome, *COL5A1, TNXB, ZNF469, COL12A1,* appear to play a role in KTCN, which was proven by the shared genetic variation between these two diseases[138].

Also, there is an evidence of sequence variants in *ZNF469* gene for both KTCN and brittle cornea syndrome[219]. Although, *ZNF469* pathogenic gene variants were reported in patients with brittle cornea syndrome[220] and KTCN[221], the association between these diseases have not been shown. Further studies have revealed this gene as a determinant affecting central corneal thickness, however central corneal thickness may be abnormal in a wide variety of corneal diseases[222].

The association of KTCN with an intellectual disability is a frequent one. One study has shown that among 212 institutionalized persons with intellectual disability, there were 16 patients with KTCN (7.5%)[223]. In a study by Kirby and coworkers, performed on siblings with severe mental retardation and bilateral KTCN, an autosomal recessive trait was discussed (one female and two males from siblings of seven from a consanguineous Pakistani family)[224]. In other study, among 94 patients with KTCN, 35 individuals (37.2%) had a psychiatric diagnosis, 13 (13.8%) moderate-severe depression, and 20 (21.2%) moderate-severe anxiety[225]. Additionally, the link between KTCN and autism has been suggested as eye rubbing is frequently observed in children with autism spectrum disorders, and this behavioral factor is reported as the most important[226].

Other reported risk factors for KTCN include mitral valve prolapse, collagen vascular disease, ocular trauma, pigmentary retinopathy, and Marfan syndrome[23]. As already mentioned, KTCN has been linked to asthma, eczema, atopic disorder and other immune-related disorders.

However, the nature of the relationship - whether it is the only effect of intensified eye rubbing or some genetic variants disturbing immune responses - remains to be elucidated.

In Figure 16.2 genes previously recognized for KTCN, ocular dystrophies and other ocular diseases are listed. Only genes specific to KTCN and those which are also important in other eye diseases are included, further pointing to the complex nature of the disease (Figure 16.2).

7. SUMMARY AND FUTURE DIRECTIONS

KTCN is a multifactorial eye disease, with limited treatment options and without reliable biomarkers and regenerative therapies available. KTCN research to date has mostly focused on selected molecular and/or clinical aspects. The studies conducted so far have led to the identification of numerous genetic features specific for KTCN, including KTCN loci, pathogenic variants, and other sequence variants in candidate genes, for some of which the significance has been verified in transcriptomic, proteomic and metabolomic assessments. Summarizing all the above-mentioned findings, the vast majority of the identified aspects are specific to individual populations or even individual families and cannot be implicated as causing KTCN in the general population. Apart from heterogeneity in KTCN, it is characteristic that the majority of the results obtained previously have not been replicated in the subsequent studies.

It has become clear that etiology of KTCN may involve more than one causative factor and that mentioned molecular techniques used separately in different KTCN studies may not be effective in identifying the causes of KTCN. The rapid advances of high-throughput technologies have led to the development of innovative OMICS-based approaches allowing relationship analyses between disease phenotype and variation in the human genome and the transcriptome, proteome, metabolome, epigenome, and microbiome. The OMICS-based results obtained so far in medical research have been promising. However, in the field of ophthalmic genetics, and

KTCN in particular, this type of approach has been rarely implemented. Future directions of KTCN research should be based on multidisciplinary, high-throughput and comprehensive screening strategies.

The major limitation for KTCN large-scale OMICS studies may be the relatively high cost of the experiments. High-throughput OMICS studies require sophisticated platforms. Moreover, despite the rapid development of computational methods, the analysis and interpretation of the huge amount of data generated in OMICS assessments is challenging. Another issue possibly limiting KTCN studies may be a problem with the collection of the appropriate material for analyses. To perform complex correlation analyses, several different material samples should be derived from the same individual. While blood samples are usually not difficult to collect from all studied individuals, the collection of whole or part of corneal tissues is limited to patients with corneal transplantation or other invasive surgical procedures. Though KTCN is the one of the major indications for keratoplasty, this procedure is not performed in all KTCN patients. Moreover, it is not possible to obtain "healthy" control cornea for comparative analyses. Therefore, the corneal tissues are often derived from non-KTCN individuals who were referred for corneal transplantation because of different corneal events, such as bullous keratopathy or corneal scarring, ulcers, and perforations. Corneas may be also obtained from the eye and tissue bank. However, for research purposes, it is possible to obtain only tissues that have been disqualified for transplantation, so tissue bank corneas cannot be treated as unaffected corneas.

An alternative material useful for the study of KTCN is tear film, which has direct contact with the corneal surface. However, while reflex tears after eye irritation caused by stimulation may be collected in quite a large amount, basal, unstimulated tears are difficult to obtain in patients with KTCN. In our practice, it is difficult to collect more than 6–8 µl of tear fluid

without induction in control individuals and even less in KTCN patients (unpublished results). A good model for KTCN analysis could be corneal cell lines. However, the establishment and cultivation of cell lines is difficult and often fails. Still, corneal cell lines as well as other cell lines derived from different cell types are not well characterized, have a limited life span and may not reflect the properties of *in vivo* cells.

Despite these limitations, the implementation of OMICS studies and collaboration between specialists in many scientific fields promise to identify new and unresolved biological aspects towards understanding the complex nature of KTCN.

TABLES/FIGURES CAPTIONS

Table 16.1. Representative genetic findings in keratoconus worldwide research. Keratoconus loci, population studied, number of families/ cases/ control individuals involved, inheritance patterns, locus/sequence variation identification techniques (including the LOD values for linkage studies), candidate genes, and the sequence variants identified in patients with keratoconus are listed in the chromosomal order.

KTCN locus/ MIM number/*Gene*	**Population/ number of families of familial cases, inheritance /sporadic cases, controls**	**Method (LOD for linkage analysis)**	**Detected sequence variation**	**References**
1p36.23-36.21 /-/-	Australian/ 1, AD/-	Linkage analysis (1.9)	-	Burdon et al. (2008)[227]
-/-/*FLG*	European/ -/ 89, -	PCR	multiple	Droitcourt et al. (2011)[228]
2p24/609271/-	European, Arab, and Caribbean African/ 28, AD	Linkage analysis (5.12)	-	Hutchings et al. (2005)[229]
2q/-/-	Hispanic/ 17, AD/-	Linkage analysis (2.3)	-	Li et al. (2006)[230]
-/-/*COL4A3*	Slovenian/ -/ 104, 157	SSCA, Sanger sequencing	multiple	Stabuc-Silih et al. (2009)[231]
-/-/*COL4A4*	Slovenian/ -/ 104, 157 Iranian/ -/ 140, 150	SSCA, Sanger sequencing Nested-PCR, ARMS-PCR, RFLP-PCR	multiple rs2228557, rs2228557	Stabuc-Silih et al. (2009)[231] Yari et al. (2020)[232]
-/-/*WNT10A*	Australian, Dutch/-/ 2709, 621	GWAS, genotyping/ PCR	rs121908120	Cuellar-Partida et al. (2015)[233]
2q13-14.3/-/*IL1RN*	Ecuadorian/ 1, AD/-	Linkage analysis (-)	rs2071459 (c.214+242C>T)	Nowak et al. (2013)[41]
-/-/*RAB3GAP1*	Caucasian/ -/ 222, 3324 Australian/ -/ 524, 2761 European/ -/ 165, 193	GWAS, microarray GWAS, microarray Allele-specific PCR	rs4954218 rs4954218 rs4954218	Li et al. (2012)[102] Bae et al. (2013)[103] Liskova et al. (2017)[234]
-/-/*IL1A*	Chinese/-/97, 101	Genotyping/ microarray	rs2071376	Wang et al. (2013)[111]
-/-/*IL1B*	Japanese/-/169, 390	Genotyping/ PCR	rs1143627, rs16944	Mikami et al. (2012)[235]
2q21.1/617928/ *TUBA3D*	Chinese/ 1, AR/-	ES	c.31 C > T, p.Gln11stop	Hao et al. (2017)[236]
3p14-q13/ 608586/-	Italian/ 1, AD/-	Linkage analysis (3.09)	-	Brancati et al. (2004)[237]
-/-/*GPX1*	Iranian/-/ 140, 150	RFLP-PCR	rs1050450	Yari et al. (2018)[238]

-/-/*TF*	Polish/ -/ 216, 228	RFLP-PCR	g.3296G>A	Wojcik et al. (2013)[239]
-/-/*FNDC3B*	Australian, Caucasian/ -/ 874, 6085	GWAS, microarray	rs4894535	Lu et al. (2013)[240]
4q/-/-	Caucasian, Hispanic/ 67, AD/-	Linkage analysis (2.2)	-	Li et al. (2006)[230]
5p/-/-	Hispanic/ 17, AD/-	Linkage analysis (2.5)	-	Li et al. (2006)[230]
5q14.1-21.3/614622/-	Caucasian/ 1, AD/-	Linkage analysis (3.48)	-	Tang et al. (2005)[241]
-/-/*CAST*	Caucasian/-/ 566, 518	TaqMan genotyping	rs4869307, rs27654	Li et al. (2013)[242]
5q15-21.1	Caucasian/ 1, AD/-	Linkage analysis (2.49)	-	Bykhovskaya et al. (2016)[243]
5q21.2/-/*LOX*	Italian/ 25, AD/-	Linkage analysis (0.49)	-	Bisceglia et al. (2009)[93]
-/-/*LOX*	Caucasians/ -/ 222, 3324	GWAS, microarray	rs10519694, rs2956540	Bykhovskaya et al. (2012)[104]
	Iranian/ -/ 112, 150	Allele-specific PCR	rs1800449	Hasanian-Langroudi et al. (2015)[244]
	European/ -/ 165, 193	Allele Specific PCR	rs2956540 (protective)	Dudakova et al. (2015)[245]
	Brazilian/-/ 140, 50	PCR, Sanger sequencing	c.1474T>A (Thr392Pro)	Gadelha et al. (2020)[110]
	Chinese/ -/ 53, 100	NGS, PCR, Sanger sequencing	c.95 G>A	Xu et al. (2020)[246]
5q31/-/-	Caucasian, Hispanic/ 67, AD/-	Linkage analysis (2.01)	-	Li et al. (2006)[230]
5q31/-/-	Ecuadorian/ 1, AD/-	Linkage analysis (2.32)	-	Rosenfeld et al. (2011)[247]
-/-/ *TGFBI*	Chinese/-/ 30, 30	PCR, Sanger sequencing	c. 535G>T	Guan et al. (2012)[248]
	Caucasian/-/42, 50	ES, PCR, Sanger sequencing	c.1598G>A (lack of significance)	Karolak et al. (2016)[116]
5q32-q33	Southern Italian/ 25, AD/-	Linkage analysis (2.45)	-	Bisceglia et al. (2009)[93]
-/-/*TNF*	Pakistani/ -/ 257, 253	RFLP-PCR	rs1800629	Arbab et al. (2017)[249]
-/-/*HGF*	Caucasian/ -/ 525, 518	GWAS, microarray	rs3735520, rs3735520, rs17501108, rs1014091	Burdon et al. (2011)[101]
	Australian/ -/ 157, 673	Tag SNP (PCR)	rs2286194	Sahebjada et al. (2014)[250]
	European/ -/ 165, 193	Allele Specific PCR	rs3735520	Dudakova et al. (2015)[245]
-/-/*IMMP2L*	Caucasian/ -/ 222, 3324	GWAS, microarray	rs214884, rs757219	Li et al. (2012)[102]
9p/-/-	Hispanic/ 17, AD/-	Linkage analysis (3.8)	-	Li et al. (2006)[230]
-/-/ *NFIB*	Australian, Caucasian/-/ 157, 673	Microarray	rs1324183	Sahebjada et al. (2013)[251]
	Australian, Caucasian/-/ 874, 6085	Microarray	rs1324183	Lu et al. (2013)[240]

-/-/ *MPDZ-NF1B*	European/ -/ 933, 5946	GWAS, microarray	rs66720556	Iglesias et al. (2018)[252]
9q34/614623/-	Caucasian, Hispanic/ 67, AD/-	Linkage analysis (4.5)	-	Li et al. (2006)[230]
-/-/ *COL5A1*	Australian, Caucasian/-/ 874, 6085	Microarray	rs7044529	Lu et al. (2013)[240]
	Caucasian/-/ 222, 3324	Microarray	rs7044529, rs1536482	Li et al. (2013)[242]
	Chinese/ -/ 53, 100	NGS, PCR, Sanger sequencing	c.1372C>T	Xu et al. (2020)[246]
-/-/*ZEB1*	Caucasian/ -/ 70, -	PCR, Sanger sequencing	c.1920G>T (p.Gln640His)	Lechner et al. (2013)[213]
	Italian/ -/ 1,-	Direct sequencing	c.1920G>T (p.Gln640His)	Mazotta et al. (2014)[214]
11p/-/-	Caucasian/ 40, AD/-	Linkage analysis (2.3)	-	Li et al. (2006)[230]
-/-/ *CAT*	Iranian/-/ 140, 150	RFLP-PCR	rs7943316 (protective)	Yari et al. (2018)[238]
12p	Caucasian, Hispanic/ 67, AD/-	Linkage analysis (2.5)	-	Li et al. (2006)[230]
-/-/*FOXO1*	Caucasian, Australian/ -/ 874, 6085	GWAS, microarray	rs2721051	Lu et al. (2013)[240]
	European/ -/ 165, 193	Allele-specific PCR	rs2721051	Liskova et al. (2017)[234]
	European/ -/ 933, 5946	GWAS, microarray	rs2755238	Iglesias et al. (2018)[252]
13q32/614629/-	Ecuadorian/ 18, AD/-	Linkage analysis (4.1)	rs9516572, rs3825523	Gajecka et al. (2009)[97]
-/-/ *DOCK9*	Ecuadorian/ 15, AD/-	PCR, Sanger sequencing	c.2262A>C; c.720+43A>G;	Czugala et al. (2012)[98]
	Polish/ -/ 42, 50	PCR, Sanger sequencing	lack of significance	Karolak et al. (2016)[116]
-/-/ *IPO5*	Ecuadorian/ 15, AD/-	PCR, Sanger sequencing	c.2377-132A>C	Czugala et al. (2012)[98]
	Polish/ -/ 42, 50	PCR, Sanger sequencing	lack of significance	Karolak et al. (2016)[116]
-/-/ *STK24*	Ecuadorian/ 15, AD/-	PCR, Sanger sequencing	c.1053+29G>C	Czugala et al. (2012)[98]
	Polish/ -/ 42, 50	PCR, Sanger sequencing	lack of significance	Karolak et al. (2016)[116]
13q34/-/	Ecuadorian/ 18, AD/-	Linkage analysis (2.86)	-	Gajecka et al. (2009)[97]
-/-/*COL4A1*	Ecuadorian/ 15, -/-	PCR, Sanger sequencing	no variants	Karolak et al. (2011)[96]
-/-/*COL4A2*	Ecuadorian/ 15, -/-	PCR, Sanger sequencing	no variants	Karolak et al. (2011)[96]
14q	Caucasian, Hispanic/ 67, AD/-	Linkage analysis (2.6)	-	Li et al. (2006)[230]
14q11.2	Southern Italian/ 25, AD/-	Linkage analysis (2.09)	-	Bisceglia et al. (2009)[93]
14q24.3/614628/-	White English, Iranian, Indian, Pakistani/ 6, AD/-	Linkage analysis (3.58)	-	Liskova et al. (2010)[253]
15q22.33-q24.2/-/-	Northern Irish/ 1, AD/-	Linkage analysis (8.13)	-	Hughes et al. (2003)[137]
-/-/ *mir184*	Northern Irish/ 1, AD/-	MPS	c.57C>T	Hughes et al. (2011)[254]
-/-/*POLG*	Polish/ -/ 284, 353	TaqMan Genotyping, RFLP-PCR	c.1370T>A	Wojcik et al. (2014)[255]

	Egyptian/ -/ 76, 77	RFLP-PCR	c.1370T>A	Awd-Allah et al. (2020)[256]
16q22.3-q23.1/608932/-	Northern Finnish/ 20, AD/-	Linkage analysis (4.11)	-	Tyynismaa et al. (2002)[257]
-/-/BANP-ZNF469	Caucasian, Australian/ -/ 874, 6085	GWAS, microarray	rs9938149	Lu et al. (2013)[240]
	Australian/ -/ 157, 673	Tag SNP (PCR)	rs9938149	Sahebjada et al. (2014)[250]
-/-/ZNF469	British, Middle Eastern/ 11, -/ -	PCR, Sanger sequencing	multiple	Davidson et al. (2015)[258]
	Polish/ -/ 42, 317	PCR, Sanger sequencing	no variants	Karolak et al. (2016)[120]
	Chinese/ -/ 30, 100	PCR, NGS	multiple	Yu et la. (2017)[221]
	Australian/ -/ 385, 576	ES	no variants	Lucas et al. (2017)[121]
17q	Hispanic/ 17, AD/-	Linkage analysis (3.9)	-	Li et al. (2006)[230]
-/-/XRCC1	Polish/ -/ 284, 353	TaqMan Genotyping, RFLP-PCR	c.1196A>G	Wojcik et al. (2014)[255]
	Egyptian/ -/ 76, 77	RFLP-PCR	c.1196A>G	Awd-Allah et al. (2020)[256]
20p11.21/148300/*VSX1*	Caucasian/- / 63, 277	PCR-SSCP	multiple SNVs	Héon et al. (2002)[108]
-/-/ VSX1	Italian/14, AD/66, -	PCR, Sanger sequencing	multiple SNVs	Bisceglia et al. (2005)[259]
	Caucasian/ 75, -/ 77,71	RFLP-PCR	no variants	Tang et al. (2008)[260]
	Korean/ -/ 249, 208	PCR-SSCP, Sanger sequencing	multiple	Mok et al. (2008)[261]
	European/ 27, -/ 39,-	PCR, Sanger sequencing	no variants	Dash et al. (2010)[262]
	Slovenian/ -/ 113, 110	PCR, , Sanger sequencing	no variants	Stabuc-Silih et al. (2010)[263]
	Italian/77,- / 225,-	PCR, Sanger Sequencing	c.715G>C (p.G239R)	De Bonis et al. (2011)[264]
	Indian/ -/ 117, 108	PCR, Sanger Sequencing	multiple	Verma et al. (2013)[265]
	Polish/ -/ 42, 50	PCR, Sanger Sequencing	lack of significance	Karolak et al. (2016)[116]
20p13-p12.2/-/*SLC4A11*	Ecuadorian/ 1, AD/-	-	c.2558+149_2558+203del54	Nowak et al. (2013)[41]
20q12/-/*MMP9*	Northwest Tasmanian/1, -/ 6,-	Linkage mapping, PCR, cSNP analysis	- no variants	Fullerton et al. (2002)[266]
-/-/SOD1	Muliethnic/ 15, -/ -	PCR, Sanger Sequencing	IVS2+50del7 mutation	Udar et al. (2006)[109]
	Greek/ -/ 33, 78	PCR, Sanger sequencing	c.169 + 50 delTAAACAG	Moschos et al. (2015)[267]
	Brazilian/-/ 140, 50	PCR, Sanger Sequencing	IVS2+50del7 mutation	Gadelha et al. (2020)[110]
-/-/TIMP3	Chinese/ -/ 53, 100	NGS, PCR, Sanger sequencing	c.476C>T	Xu et al. (2020)[246]
-/-/TIMP1	Iranian/ -/ 140, 150	Nested-PCR, ARMS-PCR, RFLP-PCR	rs4898	Yari et al. (2020)[232]

VSX1 chosen sequence variation is listed

Table 16.2. Selected transcriptomic, proteomic, and metabolomic abnormalities found in keratoconus world-wide research in the alphabetical order.

Protein/ transcript/ metabolite	Direction of change	Method	Material	Remarks	References
ADAM10	↓	SWATH-MS	Human corneal epithelum	Corneal degradation	Yam et al. (2019)[166]
ALDHA1	↓	TMT LC-MS	Whole human corneas	Corneal degradation	Shinde et al. (2019)[161]
COL12A1	↓	Immunofluorescence staining	Human corneal buttons	Alteration of cell-matrix interactions	Cheng et al. (2001)[268]
COL1A1	↓	Quantitative real-time PCR, immunohistochemistry	Human corneal epithelium; whole human corneas	Corneal degradation, inflammatory events	Shetty et al. (2015)[269]
	↓	TMT LC-MS	whole human corneas	Corneal degradation	Shinde et al. (2019)[161]
COL3A1	↓	TMT LC-MS	Whole human corneas	Corneal degradation	Shinde et al. (2019)[161]
COL4A1	↓	Microarray	Whole human cornea	Deregulation of the matrix arrangement	Stachs et al. (2004)[144]
	↓	Quantitative real-time PCR, immunohistochemistry	Human corneal epithelium; whole human corneas	Corneal degradation, inflammatory events	Shetty et al. (2015)[269]
COL4A3	↓	Microarray	Whole human cornea	Deregulation of the matrix arrangement	Stachs et al. (2004)[144]
COL6A1	↓	Nano-ESI-LC-MS	Human corneal epithelium	Loss of epithelial integrity	Chaerkady et al. (2013)[163]
COL6A2	↓	Nano-ESI-LC-MS (MS); 2D-DIGE	Human corneal epithelium and stroma	corneal remodeling	Joseph et al. (2011)[169]
ENO1	↓	2-DE, MS, MALDI-TOF, ES-MS/MS, Immunohistochemistry, Western Blot, RT-PCR	Human corneal epithelium	Cytoskeleton remodeling, Degradation processess	Nielsen et al. (2006)[168]; Srivastava et al. (2006)[270]
FBLN1	↓	TMT LC-MS	Whole human corneas	Corneal degradation	Shinde et al. (2019)[161]
IFN-γ,	↓	Multiplex immuno-bead assays, ELISA	Human tears	Chronic inflammatory events	Jun et al. (2011)[271]

IGFBP3	↓	cDNA microarray, RT-PCR, Real Time PCR	Human cultured keratocytes	Apoptosis, decrease of keratocyte density	Ha et al. (2004)[141]
IGFBP5	↓	cDNA microarray, RT-PCR, Real Time PCR	Human cultured keratocytes	Apoptosis, decrease of keratocyte density	Ha et al. (2004)[141]
IL17	↑	Multiplex immuno-bead assays, ELISA	Human tears	Chronic inflammatory events	Jun et al. (2011)[271]
IL6	↑	ELISA	Human tears	Chronic inflammatory events	Lema & Durán (2005)[164]
	↑	ELISA	Human tears	Chronic inflammatory events	Lema et al. (2009)[272]
	↑	Multiplex immuno-bead assays, ELISA	Human tears	Chronic inflammatory events	Jun et al. (2011)[271]
	↑	Quantitative real-time PCR, immunohistochemistry	Human corneal epithelium; whole human corneas	Chronic inflammatory events	Shetty et al. (2015)[269]
KRT12	↑	Nano-ESI-LC-MS (MS); 2D-DIGE	Human corneal epithelium and stroma	corneal remodeling	Joseph et al. (2011)[169]
KRT14	↑	Nano-ESI-LC-MS (MS); 2D-DIGE	Human corneal epithelium and stroma	corneal remodeling	Joseph et al. (2011)[169]
KRT16	↑	Nano-ESI-LC-MS	Human corneal epithelium	Loss of epithelial integrity	Chaerkady et al. (2013)[163]
KRT5	↑	Nano-ESI-LC-MS (MS); 2D-DIGE	Human corneal epithelium and stroma	corneal remodeling	Joseph et al. (2011)[169]
KRT6A	↑	Nano-ESI-LC-MS	Human corneal epithelium	Loss of epithelial integrity	Chaerkady et al. (2013)[163]
KRT78	↑	Microarray, RT-PCR, Real Time PCR	Whole human cornea	corneal remodeling	Macé et al. (2011)[145]
LOX	↓	Immunohistochemistry; fluorometric assay	Whole human corneas, cultured corneal fibroblasts	Inadequate collagen cross-linking	Dudakova et al. (2012)[273]
	↓	Quantitative real-time PCR, immunohistochemistry	Human corneal epithelium; whole human corneas Human corneal epithelium and stroma	Corneal degradation, inflammatory events	Shetty et al. (2015)[269]
	↓	SWATH-MS		Corneal degradation	Yam et al. (2019)[166]
LTF	↓	2-DE, MALDI-TOF MS, Western blot	Human tears	Pathogenesis involves an immune and inflammatory component	Lema et al. (2010)[170]
	↓	ELISA	Basal tears	Inflammatory component	Balasubramanian et al. (2012)[274]
	↓	Nano-ESI-LC-MS	Human corneal epithelium	Inflammatory component	Chaerkady et al. (2013)[163]
MMP1	↑	Immunohistochemistry	Whole human corneas	Corneal damage	Seppälä et al. (2006)[74]
	↑	Nano-LC-MS/MS	Tear samples	Inflammatory events,	Pannebaker et al.

				apoptosis	(2010)[275]
MMP9	↑	ELISA	Human tears	Chronic inflammatory events	Lema & Durán (2005)[164]
	↑	ELISA	Human tears	Chronic inflammatory events	Lema et al. (2009)[272]
	↑	Quantitative real-time PCR, immunohistochemistry	Human corneal epithelium; whole human corneas	Chronic inflammatory events	Shetty et al. (2015)[269]
	↑	SWATH-MS	Human corneal epithelium	Chronic inflammatory events	Yam et al. (2019)[166]
NOTCH1	↓	RNA-Seq	Human corneal epithelium	Corneal degradation	You et al. (2019)[276]
NRF2	↓	RNA-Seq	Whole human corneas	Corneal degradation	Shinde at el. (2020)[150]
S100A2	↓	Microarray, quantitative RT-PCR	Human corneal epithelum; whole human cornea	Corneal epithelium degeneration	Nielsen et al. (2003)[142]; Nielsen et al. (2005)[143]
S100A4	↑	2-DE, MS	Human corneal epithelium	Cytoskeleton remodeling	Nielsen et al. (2006)[168]
	↑	Nano-ESI-LC-MS (MS); 2D-DIGE	Human corneal epithelium and stroma	Corneal remodeling	Joseph et al. (2011)[169]
S100A6	↓	Microarray	Tear samples	Corneal remodeling	Recalde et al. (2019)[277]
SMAD7	↓	RNA-Seq	Whole human corneas	Corneal degradation	Kabza et al. (2017)[148]
	↓	RNA-Seq	Whole human corneas	Corneal degradation	Shinde et al. (2020)[150]
SOD1	↓	TMT LC-MS	Whole human corneas	Corneal degradation	Shinde et al. (2019)[161]
TGFB1	↓	RNA-Seq	Whole human corneas	Corneal degradation	Kabza et al. (2017)[148]
	↓	RNA-Seq	Whole human corneas	Corneal degradation	Shinde et al. (2020)[150]
TGFBI	↓	Immunohistochemistry	Human corneal buttons	Decrease mechanical stability of corneal stroma	Takács et al. (1999)[278]
	↓	In situ hybridization	Whole human corneas	Corneal degradation, loss of cell adhesion	Zhao et al. (2002)[279]
	↓	Nano-ESI-LC-MS (MS); 2D-DIGE	Human corneal epithelium and stroma	corneal degradation and remodeling	Joseph et al. (2011)[169]
TIMP1	↓	RT-PCR, Southern Blot, Immunohistochemistry, Western Blot	Human corneal buttons	Oxidative stress and tissue degradation	Kenney et al. (2005)[280]
	↓	Annealing control primer™-based GeneFishing™ PCR, RT-PCR, Real Time PCR	Human cultured keratocytes	Apoptosis	Lee et al. (2009)[281]

	↑	ELISA, Immunohistochemistry	Human cultured corneal stromal cells	Corneal stromal cell Apoptosis	Matthews et al. (2007)[282]
	↑	Nano-LC-MS/MS	Tear samples	Inflammatory events, apoptosis	Pannebaker et al. (2010)[275]
	↓	SWATH-MS	Human corneal epithelium	Inflammatory events, apoptosis	Yam et al. (2019)[166]
TIMP2	↓	TMT LC-MS	Whole human corneas	Corneal degradation	Shinde et al. (2019)[161]
TIMP3	↑	ELISA, Immunohistochemistry	Human cultured corneal stromal cells	Corneal stromal cell Apoptosis	Matthews et al. (2007)[282]
	↓	Annealing control primer™-based GeneFishing™ PCR, RT-PCR, Real Time PCR	Human cultured keratocytes	Apoptosis	Lee et al. (2009)[281]
TNFα	↑	ELISA	Human tears	Chronic inflammatory events	Lema & Durán (2005)[164]
	↑	ELISA	Human tears	Chronic inflammatory events	Lema et al. (2009)[272]
	↓	Multiplex immuno-bead assays, ELISA	Human tears	Chronic inflammatory events	Jun et al. (2011)[271]
	↑	Antibody array	Basal tear samples	Chronic inflammatory events	Balasubramanian et al. (2012)[39]
TNFβ	↑	Antibody array	Basal tear samples	Chronic inflammatory events	Balasubramanian et al. (2012)[39]
WNT10A	↓	TaqMan quantitative PCR, Immunohistochemistry	Human corneal epithelium	Corneal degradation	Foster et al. (2021)[283]

Figure 16.1 Keratoconus of left eye. Note bulging of inferior lid on downgaze (Munson sign).

Figure 16.2 Overlap of keratoconus-related genes including multi-level findings (genes, proteins and transcripts) and other ocular diseases-related genes. Presented based on research results included in Tables 16.1 and 16.22, and discussed in this chapter.

Figure 16.3 Keratoconus in an adult with *CEP290*-related Leber congenital amaurosis.

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Figure 16.1

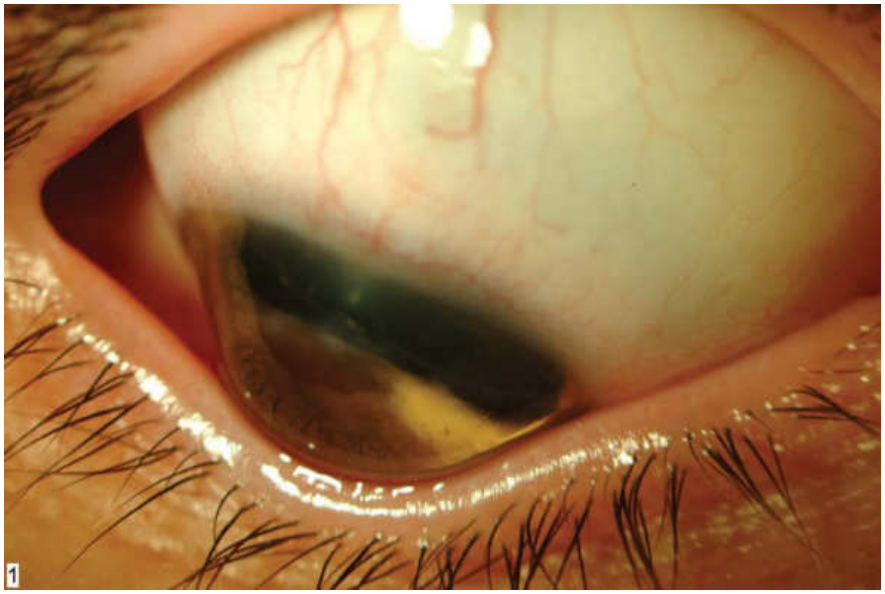
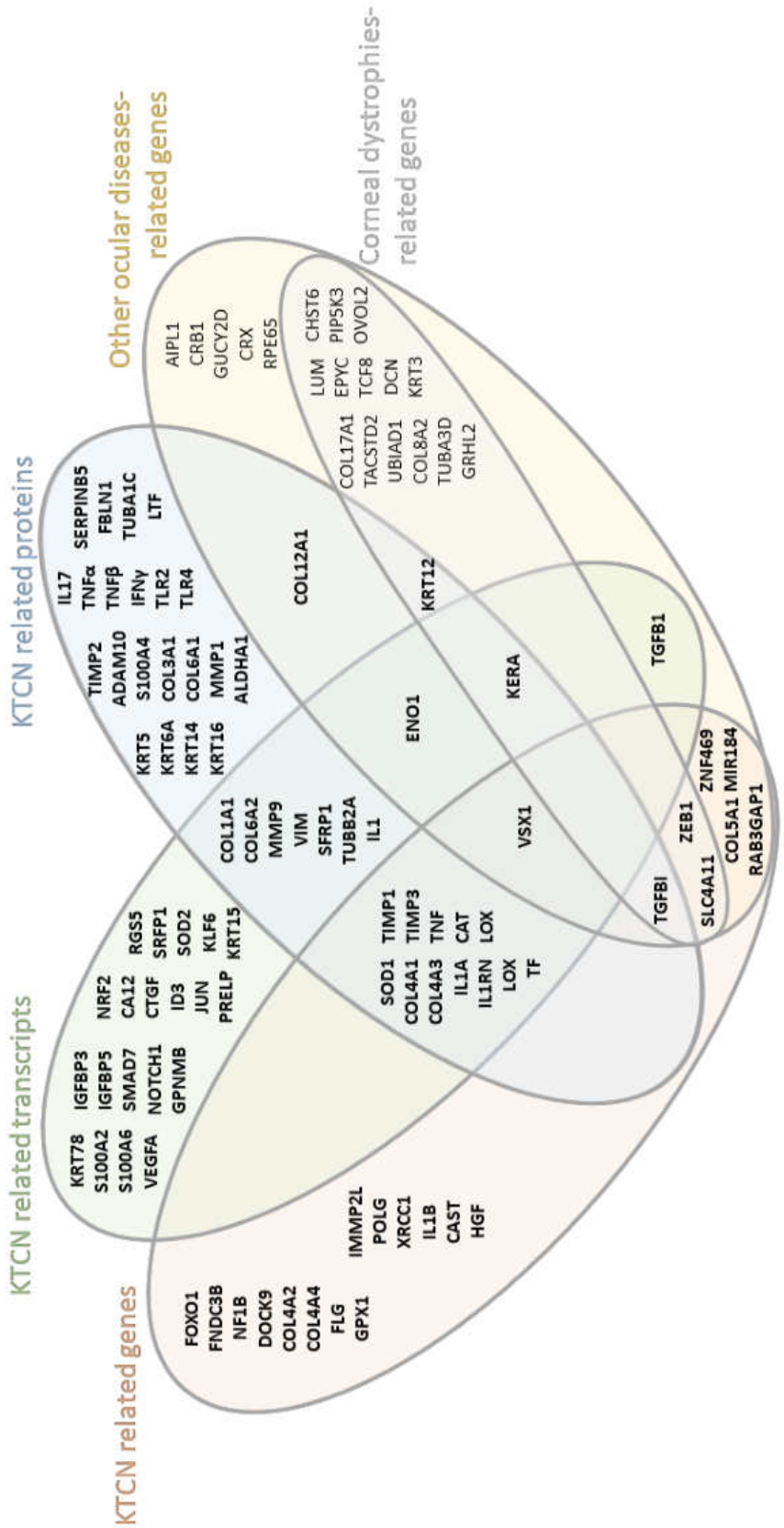


Figure 16.2


KTCN related genes
KTCN related transcripts
KTCN related proteins
Other ocular diseases-related genes
Corneal dystrophies-related genes
FOXO1
FNDC3B
NF1B
DOCK9
COL4A2
COL4A4
FLG
GPX1
IMMP2L
POLG
XRCC1
IL1B
CAST
HGF
KRT78
S100A2
S100A6
VEGFA
IGFBP3
IGFBP5
SMAD7
NOTCH1
GPNMB
NRF2
CA12
CTGF
ID3
JUN
PRELP
RGS5
SRFP1
SOD2
KLF6
KRT15
SOD1
COL4A1
COL4A3
TIMP1
TIMP3
TNF
IL1A
IL1RN
LOX
TF
COL1A1
COL6A2
MMP9
VIM
SFRP1
TUBB2A
IL1
VSX1
KRT5
KRT6A
KRT14
KRT16
TIMP2
ADAM10
S100A4
COL3A1
COL6A1
MMP1
ALDHA1
IL17
TNFα
TNFβ
IFNγ
TLR2
TLR4
SERPINB5
FBLN1
TUBA1C
LTF
COL12A1
ENO1
KRT12
KERA
TGFBI
TGFB1
ZEB1
SLC4A11
ZNF469
COL5A1
MIR184
RAB3GAP1
AIPL1
CRB1
GUCY2D
CRX
RPE65
COL17A1
TACSTD2
UBIAD1
COL8A2
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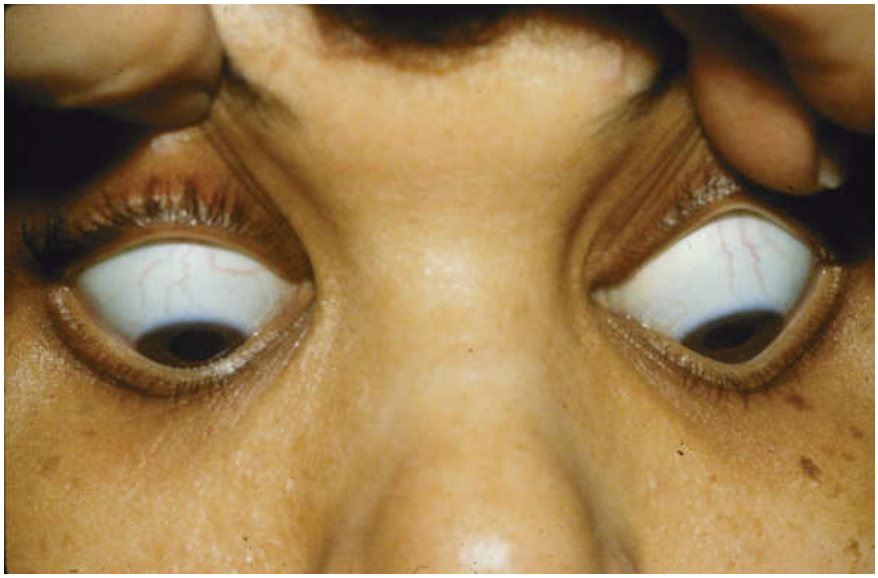
OVOL2

Figure 16.3



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September 13, 2023

Katarzyna Jaskiewicz and Marzena Gajecka
Institute of Human Genetics
Polish Academy of Sciences
Strzeszynska 32
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Poland

Dear Professor Gajecka and Ms. Jaskiewicz,

This letter serves as confirmation that the book *Genetic Diseases of the Eye 3rd Edition*, edited by Elias Traboulsi, Virginia Utz, and Arif Khan, is under contract for publication with Oxford University Press (OUP). Additionally, I confirm that Ms. Jaskiewicz is a co-author to Chapter 24, "The Genetics of Keratoconus and Related Diseases." This chapter is accepted for publication by OUP and the planned publication date is fall 2024.

A copy of the current version of the chapter is attached. Please note that, while it is accepted for publication, the manuscript is not in production yet as we are still awaiting the submission of a few chapters.

Please let me know if there are any questions. Thank you.

Best regards,

Marta Moldvai

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Artykuł II:

The Impaired Wound Healing Process Is a Major Factor in Remodeling of the Corneal Epithelium in Adult and Adolescent Patients with Keratoconus.

Jaskiewicz K, Maleszka-Kurpiel M, Matuszewska E, Kabza M, Rydzanicz M, Malinowski R, Ploski R, Matysiak J, Gajecka M.

Invest Ophthalmol Vis Sci. 2023 Feb 1;64(2):22.

Wstęp: Fenotyp stożka rogówki jest prawdopodobnie efektem wielopoziomowych zmian strukturalnych i czynnościowych w rogówce, w tym w nabłonku rogówki (CE). Mając na uwadze rozpoznane przez klinicystów charakterystyczne zmiany w grubości nabłonka w rogówce pacjentów z KTCN (strukturę obwarzanka), wskazano i wyodrębniono trzy regiony topograficzne CE. Postawiono hipotezę, że zmiany molekularne w CE pacjentów z KTCN są różne w poszczególnych regionach topograficznych i wpływają na przebudowę CE w przebiegu choroby. Ponieważ nieprawidłowości w zakresie krzywizn rogówki nasilają się wraz z postępem choroby (wzrost wartości), porównanie materiału biologicznego pozyskanego od pacjentów nastoletnich i dorosłych może ukazać istotę tej przebudowy.

Wyniki: Badania prowadzono wykorzystując próby CE od 17 dorosłych i sześciu nastoletnich pacjentów z KTCN, zebrane podczas zabiegów sieciowania włókien kolagenowych (ang. *corneal collagen crosslinking,* CXL). Materiałem kontrolnym były próby CE od pięciu osób poddanych laserowej korekcji wady wzroku metodą fotokeratektomii refrakcyjnej (ang. *photorefractive keratectomy*, PRK).

Stwierdzono statystycznie istotne różnice w wartościach parametrów klinicznych takich jak keratometria płaska (ang. *flat keratometry*, K1), keratometria stroma (ang. *steep keratometry*, K2), maksymalna keratometria (ang. *maximal corneal curvature*, Kmax), uniesienie przednie, uniesienie tylne oraz najmniejsza wartość grubości rogówki (ang. *thinnest corneal thickness*, TCT), porównując pacjentów z KTCN i osoby z grupy kontrolnej. Ponadto, parametrem różnicującym dorosłych i nastoletnich pacjentów z KTCN było uniesienie tylne rogówki.

Na podstawie badań klinicznych i morfologicznych scharakteryzowano trzy regiony topograficzne CE w rogówkach pacjentów z KTCN: centralny, pośredni i obwodowy. Regiony te odzwierciedlają charakterystyczne dla KTCN zróżnicowanie grubości nabłonka tworzące tzw. strukturę obwarzanka, ze ścieńczeniem w regionie centralnym i zgrubieniem w otaczającym go regionie pośrednim. Rozpoznano istotne różnice w grubości CE pomiędzy tymi regionami topograficznymi, jak również znamienne różnice w ilości i gęstości jąder komórkowych.

Przeprowadzono analizę danych z RNA-Seq, w tym zestawów genów hallmark (MSigDB Collections) i wskazano, że w poszczególnych regionach topograficznych CE w KTCN, w różnym stopniu nadreprezentowane są procesy apoptozy, naprawy DNA, przejścia epitelialno-mezenchymalnego oraz ścieżek interleukin i interferonów. Dokonano oceny korelacji cech klinicznych pacjentów z danymi transkryptomicznymi, w efekcie której rozpoznano korelacje pomiędzy poziomami ekspresji 8 genów (*TCHP, SPATA13, CNOT3, WNK1, TGFB2, KRT12, ENO1, CLTB*) a wartościami uniesienia tylnego rogówki lub keratometrii.

Z wykorzystaniem techniki spektormetrii mas (MALDI-TOF/TOF Tandem Mass Spectrometry) zidentyfikowano 11 białek sklasyfikowanych jako odróżniające centralny region CE u dorosłych pacjentów z KTCN w porównaniu z grupą kontrolną. W porównaniu pośredniego regionu CE u dorosłych pacjentów z KTCN wskazano pięć różnicujących białek. Z kolei dla regionu obwodowego rozpoznano 14 białek o zmienionym poziome ekspresji.

Zaproponowano mechanizm przebudowy nabłonka rogówki w KTCN, w którym zarówno pocieranie oczu, jak i rozregulowana odpowiedź immunologiczna nieprzerwanie uaktywniają procesy gojenia się ran (ang. *wound healing process*). W mechanizmie tym wskazano na indukcję apoptozy i różnicowania mezenchymalnego wynikające z nagromadzenia czynników wzrostu, cytokin i produktów degranulacji neutrofilów. Dodatkowo, zaburzony proces migracji komórek powoduje, że proces gojenia się ran z chwilowego staje się permanentny. Początkowo ochronny stan zapalny przyczynia się do postępującego osłabienia biomechanicznego, a region centralny CE odpowiadający szczytowi stożka stanowi strefę najbardziej podatną na dalsze uszkodzenia. Nasilona migracja komórek z peryferyjnego do pośredniego regionu topograficznego CE i jednocześnie zmniejszona migracja komórek z regionu pośredniego do centralnego, zmiana orientacji wrzeciona podziałowego z poziomej do pionowej, a także zmiany w cyklu komórkowym, procesach naprawy DNA i apoptozy powodują akumulację komórek i zwiększoną grubość nabłonka w środkowym regionie topograficznym CE. Jednocześnie czynniki te prowadzą do zmniejszenia liczby komórek i grubości nabłonka w regionie centralnym.

Wnioski: Po raz pierwszy w światowych badaniach nad KTCN zdefiniowano i oceniono morfologicznie oraz molekularnie trzy regiony topograficzne CE. Rozpoznane cechy molekularne, morfologiczne i kliniczne wskazują na wpływ rozregulowanego procesu gojenia się ran na przebudowę CE w KTCN, co pośrednio wpływa na powstawanie charakterystycznego fenotypu stożka.

Wkład Katarzyny Jaśkiewicz w publikację: koncepcja i plan badań, zbieranie i przygotowanie materiału biologicznego od pacjentów, przeprowadzenie wywiadów z pacjentami; przygotowanie bibliotek RNA-Seq; przygotowanie materiału do analizy MALDI-TOF/TOF Tandem Mass Spectrometry; przeprowadzenie oceny morfologicznej materiału; przeprowadzenie eksperymentów RT-qPCR; udział w analizach bioinformatycznych i statystycznych; analiza i interpretacja danych transkryptomicznych, proteomicznych, morfologicznych i klinicznych; przygotowywanie rycin i tabel; pisanie manuskryptu.

Załączniki i informacje dodatkowe związane z niniejszą publikacją (62 strony) są dostępne pod następującym adresem internetowym:
https://iovs.arvojournals.org/article.aspx?articleid=2785390.

Genetics

The Impaired Wound Healing Process Is a Major Factor in Remodeling of the Corneal Epithelium in Adult and Adolescent Patients With Keratoconus

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KJ and MMK contributed equally to this work.

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Purpose. Keratoconus (KTCN) is the most common corneal ectasia, characterized by pathological cone formation. Here, to provide an insight into the remodeling of the corneal epithelium (CE) during the course of the disease, we evaluated *topographic* regions of the CE of adult and adolescent patients with KTCN.

Methods. The CE samples from 17 adult and 6 adolescent patients with KTCN, and 5 control CE samples were obtained during the CXL and PRK procedures, respectively. Three *topographic* regions, *central*, *middle*, and *peripheral*, were separated toward RNA sequencing and MALDI-TOF/TOF Tandem Mass Spectrometry. Data from transcriptomic and proteomic investigations were consolidated with the morphological and clinical findings.

Results. The critical elements of the wound healing process, epithelial–mesenchymal transition, cell–cell communications, and cell–extracellular matrix interactions were altered in the particular corneal *topographic* regions. Abnormalities in pathways of neutrophils degranulation, extracellular matrix processing, apical junctions, IL, and IFN signaling were revealed to cooperatively disorganize the epithelial healing. Deregulation of the epithelial healing, G2M checkpoints, apoptosis, and DNA repair pathways in the *middle* CE *topographic* region in KTCN explains the presence of morphological changes in the corresponding *doughnut* pattern (a thin cone center surrounded by a thickened annulus). Despite similar morphological characteristics of CE samples in adolescents and adults with KTCN, their transcriptomic features were different. Values of the posterior corneal elevation differentiated adults with KTCN from adolescents with KTCN and correlated with the expression of *TCHP*, *SPATA13*, *CNOT3*, *WNK1*, *TGFB2*, and *KRT12* genes.

Conclusions. Identified molecular, morphological, and clinical features indicate the effect of impaired wound healing on corneal remodeling in KTCN CE.

Keywords: cornea, corneal epithelium, corneal wound healing, keratoconus, MALDI-TOF/TOF Tandem Mass Spectrometry, posterior corneal elevation, proteomics, transcriptomics

Keratoconus (KTCN) is the most common corneal ectasia, characterized by the conical shape of the cornea, leading to substantial vision impairment. KTCN is described as bilateral, but often manifests as a highly asymmetric disorder.[1] The disease usually occurs in adolescence and progresses through the third or fourth decades of life.[2]

KTCN affects the general population with the prevalence assessed at 1.38 per 1000 individuals, but strongly differs regarding gender and ethnicity.[3] There are no current data available concerning the KTCN prevalence in Poland. The hospital-based epidemiological study had not revealed KTCN frequency, however, showed that 64% of patients with KTCN were male.[4] According to the Polish Health Problem Card, the estimated prevalence of KTCN is 200 to 250 per 100,000 residents.[5]

KTCN is a multifactorial disease with a nebulous etiology. Environmental components including UV exposure and lifestyle habits (eye rubbing, contact lens wear) have been

thoroughly indicated.[6,7] Also, a complex genetic background comprising numerous identified KTCN loci, candidate genes, and pathways have been revealed.[8–11]

In KTCN diagnosis, various diagnostic methods based on the mapping of both epithelium and corneal thickness, as well as the anterior and posterior corneal surface topography assisted with advanced computer software, are being used.[12] Changes in the posterior surface of the cornea are often the earliest observed feature of KTCN.[13] In contrast, the topographically "normal" eye of a patient with KTCN may have features of KTCN detectable by the epithelial thickness mapping.[1] The automated computerized algorithm's data allow pointing to a clear distinction between unaffected and KTCN corneas based solely on epithelial and stromal thickness parameters.

KTCN is the second leading cause of corneal transplantation worldwide,[14] although the traditional penetrating keratoplasty performed in the advanced disease state is being frequently replaced by deep lamellar keratoplasty.[11] The management of mild to moderate KTCN is primarily aimed at improving visual acuity with glasses, contact lenses (rigid gas permeable), and intracorneal ring segments implantation, or a combination of these modalities.[15] In addition, the corneal collagen cross-linking (CXL) has been implemented successfully as a treatment option to stiffen the cornea and halt the progression of KTCN.[16–18]

Histopathological abnormalities in KTCN corneas comprise all corneal layers, of which the most characteristic or symptomatic are epithelial thinning, breaks in Bowman's layer,[19] the presence of apoptotic cells in physiologically acellular Bowman's membrane, and Descemet's membrane disruptions.[20] Irregular cell arrangement and cell loss in the corneal epithelium (CE) and stroma were also observed.[21]

The KTCN cone, observed as a presumable consequence of multilevel structural and functional changes in corneal tissue, including those in the epithelium (superficial, wing, basal),[22–25] speaks for a study design based on separately assessed, differently affected *topographic* regions of the CE. In this study, taking into account corneal thickness, especially the epithelial thickness, including the thinnest epithelium on the top of the KTCN cone, and the location of the apex or center of the cone in the cornea, three *topographic regions* of the CE, *central*, *middle*, and *peripheral*, were chosen to be investigated separately in patients with KTCN, and then compared with the adequate material derived from control individuals.

We hypothesize that molecular abnormalities in the CE of patients with KTCN are divergent in particular *topographic* regions, and, therefore, they shape the *doughnut* pattern in the morphologically disturbed CE in KTCN. Moreover, these molecular and morphological changes in particular regions of the CE may reflect an interplay between stroma and epithelium. Because corneal curvature abnormalities worsen as the disease progresses and to provide insight into the role of CE remodeling in KTCN, we examined numerous features comparing adolescent and adult patients. We undertook histological and transcriptomic profiling of the CE and proteomic assessments, pointing to discriminative clinical and molecular features in the assessed *topographic* regions of the CE in patients with KTCN.

Here we present the comprehensive evaluation of clinical, morphological, transcriptomic, and proteomic data concerning the three *topographic* regions of the CE in adult and adolescent patients with KTCN, evaluated together and separately in the studied subgroups.

Methods

Ophthalmic Examination and Patients' Inclusion and Exclusion Criteria

We included three subgroups of patients: adults with KTCN, adolescents with KTCN, and control individuals (the non-KTCN mild myopia patients). Each individual underwent a complete ophthalmological examination, including the assessments of both uncorrected and best corrected visual acuity, IOP, corneal tomography with rotating Scheimpflug camera WaveLight Oculyzer II (Alcon, Fort Worth, TX, USA), epithelial thickness mapping spectral-domain optical coherence tomography device (Zeiss Cirrus 5000, Carl Zeiss Meditec, Dublin, CA, USA), slit-lamp, and dilated funduscopic examination. The inclusion and exclusion criteria for adults and adolescents with KTCN, and control individuals are described in details in Supplementary Material 1.1.

A questionnaire comprising the behavioral, environmental, and socioeconomic aspects, including eye rubbing, use of contact lenses, atopy, UV exposure, smoking, reading habits, time spent in front of a screen (computer, tablet, smartphone, or electronic reader), hormone intake, education level, and place of living, was completed by each participant. JASP Software[26] was used in statistical analyses of clinical parameters, and the tests applied are described in Supplementary Material 1.2.

CXL and Photorefractive Keratectomy (PRK) Procedures

CXL in patients with KTCN was performed in accordance with the standard Dresden protocol,[27–29] and PRK was performed as a refractive error correction procedure[30] in control individuals, as described in Supplementary Material 1.3 and 1.4.

Material Collection and Sample Preparation

Stamps toward the nose and eyebrow were made on the CE before the excision in the CXL and PRK procedures to enable correct tissue orientation during cutting and separation of the *topographic* regions. The obtained tissues were submersed in an RNA stabilization solution (RNAlater; Qiagen, Hilden, Germany) immediately after excision and stored at −80°C until nucleic acids and proteins isolation. Further details of the sample preparation are included in Supplementary Material 1.5.

The procedure of designation of the particular *topographic* region is shown in Figures 1D, E, and Supplementary Figure S1. Before the CE cutting, the epithelium thickness values measured automatically using the OCT for the particular regions of CE were evaluated. Then, the specific *topographic* regions were assessed manually together with the determination of cone and apex (for KTCN and controls, respectively) location (central, superior or inferior, and nasal or temporal) at the same time by the operating surgeon and the researcher processing the material.

Assessment of Morphological Abnormalities in CE

Nuclei cell morphology and density in *topographic* regions of the assessed epithelia, in both patients with KTCN and

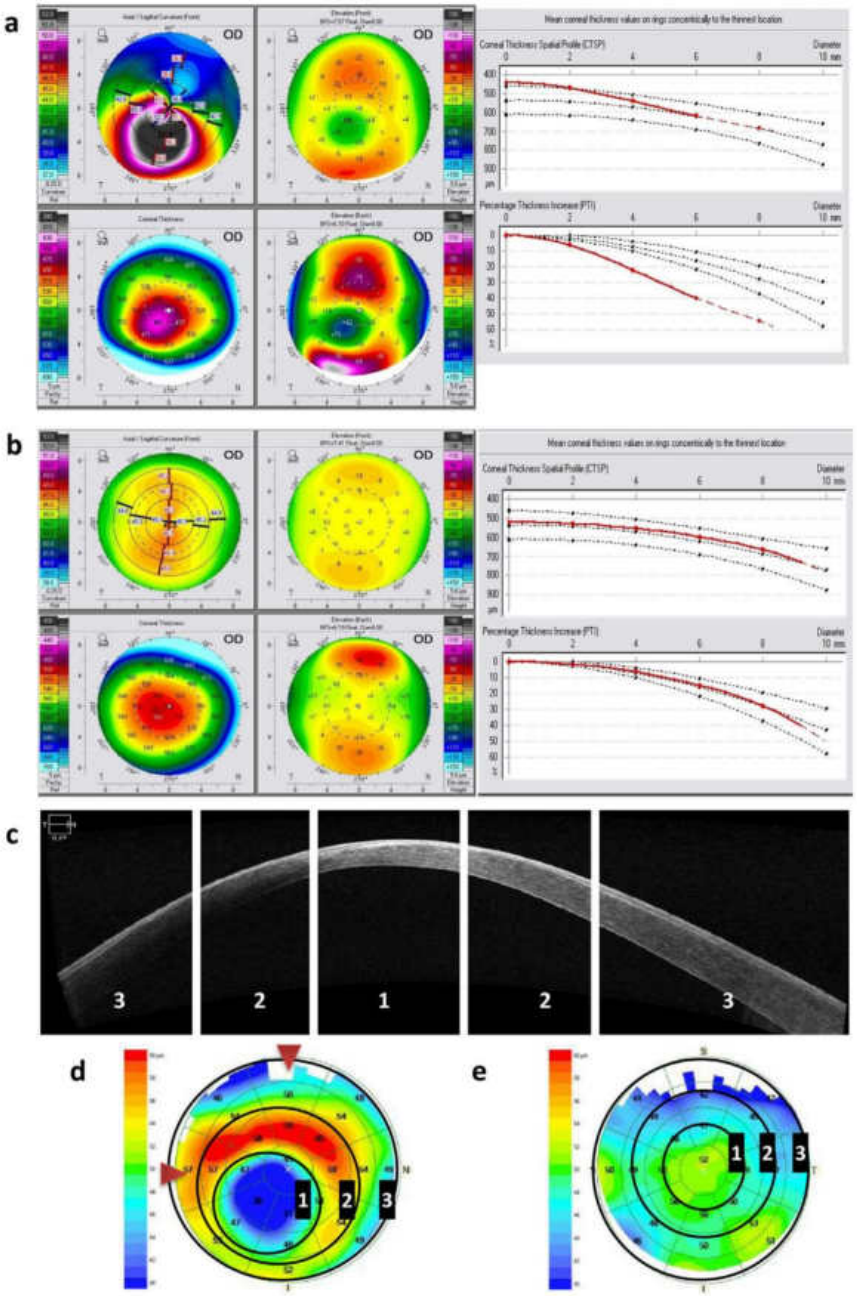


Figure 1. Results of ophthalmological examination of KTCN and control individuals, and the designation of corneal *topographic* regions. Differences between KTCN and control cornea in axial curvature, corneal thickness, anterior elevation, and posterior elevation levels are presented, based on the corneal tomography results obtained with rotating Scheimpflug camera WaveLight Oculyzer II for (**A**) the KTCN adult patient (4 OPT/KTCN/OD) and (**B**) control individual (1 OPT/M/OS). For the exact values of parameters evaluated, check the Supplementary Table S2. In the control cornea, the steepest part of the organ, called the apex, is most often located centrally on the visual axis, in the case of KTCN, the corneal apex is decentered off. (**C**) The cross-sectional image of the cornea and the segment of the anterior eye (spectral-domain optical coherence tomography [SD-OCT]) with indicated three distinct *topographic* regions (1, *central*; 2, *middle*; 3, *peripheral*) in KTCN patient 17 OPT/KTCN/OS. The decreased epithelial thickness and stromal thinning at the *central* region are shown. (**D, E**). The *topographic* regions—1, *central*; 2, *middle*; and 3, *peripheral*—are designated based on the steepness of epithelial thickness in KTCN (**D**, 4 OPT/KTCN), and control individual (**E**, 1 OPT/M), respectively. (**D**) The red arrows indicate the stamps for the correct tissue orientation, made before the CE removal during the CXL procedure. Then, the designated CE *topographic* regions were separated and processed further.

controls, were evaluated as described in Supplementary Material 1.6. At least 100 nuclei and the surface of 12,000 μm^2 of each *topographic* region were screened.

RNA and Protein Extraction

Separated CE samples were transferred from the microscope slides to the lysis solution (Norgen Biotek, Thorold, Ontario, Canada). Total RNA, DNA, and proteins were extracted and purified according to the instructions supplied with the RNA/DNA/Protein Purification Plus Micro Kit (Norgen Biotek). RNA extraction included DNase I treatment (RNase-Free DNase Set, Qiagen). The quality and quantity of the purified RNA and protein samples were assessed as described in Supplementary Material 1.7.

Total RNA Library Preparation and Sequencing and RNA Sequencing (RNA-Seq) Data Analyses

Total RNA libraries were prepared according to a previously established protocol,[31] using TruSeq Stranded Total RNA Library Prep Gold (Illumina, San Diego, CA, USA) in accordance with the manufacturer's protocol and details presented in Supplementary Material 1.8. A 100-bp paired-end sequencing run was performed on a NovaSeq 6000 platform (Illumina). The CE samples were sequenced with an average coverage of 100 million read pairs per sample. Bioinformatic analyses were executed according to a previously established protocol[32] with some modifications as presented in Supplementary Material 1.8.

Genes were considered to be differentially expressed based on the following cutoffs 0.01 false discovery rate threshold value and 1.5-fold change (FC). Reactome[33] pathway enrichment analysis was performed using the CAMERA method implemented in the limma package.[34] Pathways were considered to be differentially expressed based on the cutoff of the 0.01 false discovery rate. The enrichment analysis of hallmarks was executed using a published Hallmark Gene Set Collection being a part of the Molecular Signatures Database. In this approach, each hallmark consists of defined and concise sets of genes to most adequately summarize a specific biological state or process that, by decreasing variation and redundancy, helps in uncovering the most important but hidden molecular signatures.[35]

Bioinformatic analyses, including differential expression analyses and/or hallmarks enrichment, were executed in the following scheme: (1) *central* versus *middle* versus *peripheral* CE regions in each KTCN patient and control individual; (2) *central*, *middle*, or *peripheral* CE region in adult KTCN versus the *central*, *middle*, or *peripheral* region in control individuals; (3) the *central*, *middle*, or *peripheral* CE region in adolescent KTCN versus the *central*, *middle*, or *peripheral* region in control individual; and (4) the *central*, *middle*, or *peripheral* CE region in adolescent KTCN versus the *central*, *middle*, or *peripheral* region in adult KTCN.

RNA-Seq Data Verification Using RT-Quantitative PCR (RT-qPCR)

To verify the RNA-Seq data, the RNA samples used in the original RNA-Seq assessment were reverse transcribed to cDNA with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (Thermo Fisher Scientific, Vilnius, Lithuania), according to the manufacturer's procedure. Expression levels of six candidate KTCN genes—*KRT12*, *IFI27*, *TGFB2*, *B4GALT1*, *SPATA13*, *and WNK1*—and two housekeeping transcripts—*B2M* and *GAPDH*—were assessed using the FastStart Essential DNA Green Master (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol, using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 20 µL with 0.5 ng cDNA. Each reaction was performed in duplicate. The primer sequences and annealing temperatures are shown in Supplementary Table S1.

The relative quantification of gene expression was normalized to the level of the GAPDH and B2M transcripts with the comparative CT method. The log2 transformed FC values of gene expression levels between KTCN and control samples were calculated for the RT-qPCR and RNA-Seq data. Pearson correlations between log2 FC values for the evaluated genes were calculated using the JASP Software.[26]

Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF/TOF) Tandem Mass Spectrometry Protein–Peptide Profiling, Classification, and Identification of Discriminative Peaks

The methodology is described in detail in Supplementary Material 1.9. MALDI-TOF/TOF Tandem Mass Spectrometry proteomic analysis was performed after tryptic digestion, concentration, and purification of the samples. The research was conducted with an UltrafleXtreme (Bruker Daltonics, Billerca, MA, USA) mass spectrometer in the m/z (mass-to-charge ratio) range of 700 to 3500. The proteomic identification was based on the SwissProt protein sequence database.

Statistical analyses were performed in ClinProTools 3.0 (Bruker Daltonics) software with mathematical classification algorithms. Discriminative peaks for CE samples of adolescents with KTCN (compared with controls) were calculated in JASP Software[26] using statistical tests.

Pathways and Genes of Biological Importance

Consolidation of the obtained transcriptomic, proteomic, and clinical data was performed. Relationships between differentially expressed genes, gene pathways, and enriched hallmarks, and proteomic profile of KTCN specific *topographic* regions of the CE were identified using Venn diagrams and Pearson correlation tests, with an indication of association networks (STRING tool,[36] REACTOME database,[37] and gene ontology enrichment analysis[38]). To complement the results of these analyses, and indicate genes in differentially expressed pathways recognized using the CAMERA method and Hallmark Gene Set Collection, we checked the expression of genes with a mean expression level of at least 1 transcript per million from particular pathway gene sets, comparing the data between the subgroups of samples (the analysis was limited to males due to gender bias), and defined as significant based on the following cutoffs: P value ≤ 0.05 (t-test) and FC ≥ 1.5 or FC ≤ 0.5. The STRING tool,[36] Pathway Commons database,[39] SignaLink 3.0 database,[40] and SIGNOR tool[41] were used for the reconstruction of selected pathways' networks.

The clinical data, including flat keratometric readings (K1), steep keratometric readings (K2), maximum simulated keratometry, anterior elevation, posterior elevation,

difference in best fit sphere, thinnest corneal thickness, axial length, IOP, difference in best fit sphere, and thickness of the *central*, *middle*, and *peripheral* CE region values, were examined in detail using Pearson correlation tests, if correlated with transcriptomic data. Comparative analyses with the data obtained during the assessment of whole thickness corneas[31,32,42,43] are described in Supplementary Material 1.10.

At the same time, the genetic assessment of studied CE samples was performed. The CE samples from four unrelated adolescent patients with KTCN and two control individuals were analysed using the whole genome sequencing. The DNA samples were extracted according to the instructions of the RNA/DNA/Protein Purification Plus Micro Kit (Norgen Biotek), and their quantity was measured by Qubit dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific) and quality was assessed by 1% gel electrophoresis. Whole genome sequencing was performed with a TruSeq Nano DNA HT Library Prep Kit (Illumina) and the HiSeqX platform (Illumina) with mean coverage depth 30× at CloudHealth Genomics (Shanghai, China). The short reads were trimmed using the BBDuk2 program from the BBTools suite (http://jgi.doe.gov/data-and-tools/bbtools/) to remove Illumina adapters and poor-quality regions (mean Phred quality, <5). Then, reads were mapped to reference genome GRCh38 (source: Ensembl release 100) using BWA-MEM.[44] Duplicated reads were marked using Sam Bamba.[45] Single nucleotide polymorphisms and indel calling has been performed using Platypus[46] and the detected variants were annotated using Ensembl Variant Effect Predictor software.[47] The coding and noncoding sequence variation was assessed to show the link with here reported transcriptomic outcomes for the same CE samples.

Data Accessibility

Additional research data are presented in Supplementary Materials. Processed data of the RNA-Seq are shared in Mendeley Data Repository (https://data.mendeley.com/datasets/stpz7s5s5j/draft?a=94dfc03c-4459-40b6-888f-6d40b9a89249, DOI:10.17632/stpz7s5s5j.1). The other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Results

Clinical Characteristics of Patients

There were 17 adult patients with KTCN (2 females and 15 males), 6 adolescent patients with KTCN (2 females and 4 males), and 5 control individuals with mild myopia (2 females and 3 males) involved in this study. The clinical characteristics of the examined individuals and eyes subjected to the surgery are presented in Table 1, and details are compiled in Supplementary Table S2A; Supplementary Table S2B encompasses the information collected for both eyes in the studied individuals. Examples of results of corneal tomography with a rotating Scheimpflug camera and corneal thickness mapping of the examined individuals are presented in Figures 1A, B.

Different values of clinical parameters such as K1, K2, maximum simulated keratometry, anterior elevation, posterior elevation, difference in best fit sphere, TCT, and thickness of *central*, *middle*, and *peripheral* CE regions were found in the subgroups of adults and adolescents with KTCN in comparison with control individuals. The difference between the posterior elevation in adults (58.24 ± 13.89) and adolescents (35.00 ± 13.11) was found (P = 0.0018) (Supplementary Fig. S2). Differences in average values (with standard deviations) comparing the thickness of *central*, *middle*, and *peripheral* CE regions were found (Fig. 2). Other findings concerning both automatically and manually assessed CE thickness values are shown in Supplementary Table S3 and commented on in Supplementary Material 2.1.

The cone and apex locations in the eyes subjected to surgery for KTCN and controls, respectively, are reported in

Table 1. Clinical Characteristics of Examined Individuals and the Eyes Subjected to the Surgery

	Adults with KTCN		Adolescents with KTCN		Controls	
	Average ± SD	Median	Average ± SD	Median	Average ± SD	Median
Age at examination	27.94 ± 7.96	**25.0***	15.33 ± 1.97	**15.0***	28.00 ± 4.30	30.0
Age at diagnosis KTCN	24.56 ± 7.92	22.5	14.83 ± 2.14	15.0	*n/a*	*n/a*
K1, D	45.77 ± 3.71	45.8*	44.8 ± 2.46	44.2*	42.72 ± 2.29	43.3
K2, D	45.59 ± 11.11	47.2*	47.72 ± 4.00	46.3*	43.84 ± 2.11	44.2
K_{max}, D	57.14 ± 5.79	56.8*	54.15 ± 7.28	51.95*	44.14 ± 2.25	44.4
Anterior elevation, µm	26.88 ± 7.03	24.0*	19.67 ± 12.19	13.0*	1.60 ± 1.14	2.0
Posterior elevation, µm	**58.24 ± 13.89**	55.0*,†,‡	**35.00 ± 13.11**	30.5*,†,‡	**1.00 ± 2.74**	1.0
Diff BFS, µm	43.12 ± 19.35	41.0*	26.00 ± 20.47	20.5*	1.40 ± 1.52	1.0
TCT, µm	459.35 ± 40.06	461.0*	440.33 ± 34.99	443.5*	517.2 ± 24.12	515.0
AL, mm	24.59 ± 1.13	24.53	23.45 ± 0.82	23.64	24.23 ± 1.10	24.19
IOP, mm Hg	10.75 ± 1.61	11.0	12.80 ± 3.27	13.0	13.75 ± 2.22	14.0
Thinnest epithelial thickness (in range 0.0–7.0 mm), µm	39.65 ± 4.52	39.0*	38,50 ± 3.10	39.5*	44.00 ± 1.79	45.0
Average thickness of *central* region, µm	40.76 ± 4.13	40.0*	40.33 ± 3.08	41.0*	50.00 ± 2.35	49.0
Average thickness of *middle* region, µm	53.24 ± 3.11	52.0*	51.50 ± 4.09	50.0*	47.2 ± 1.92	47.0
Average thickness of *peripheral* region, µm	46.12 ± 1.69	46.0	45.33 ± 1.37	45.0	44.8 ± 3.19	45.0

K1, flat keratometric readings; K2, steep keratometric readings; K_{max}, maximum simulated keratometry; diffBFS, difference in best fit sphere; TCT, thinnest corneal thickness; AL, axial length; n/a, not applicable.

* Statistically significant differences between patients with KTCN and controls ($P \leq 0.05$).

† Statistically significant differences between patients adults/adolescents subgroups ($P \leq 0.05$).

‡ $P = 0.0018$.

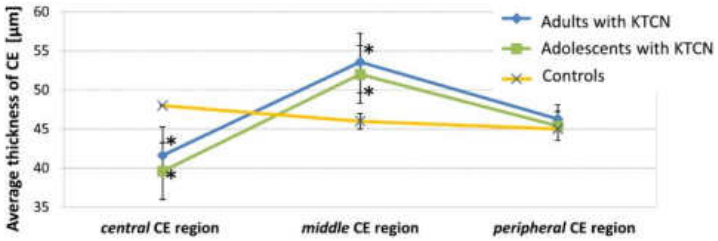
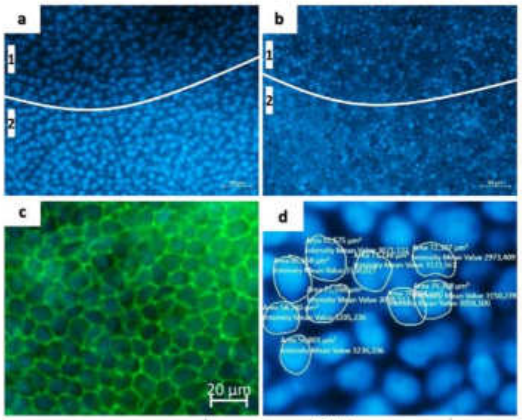


FIGURE 2. Differences in thickness of *central/middle/peripheral topographic* regions of CE in patients with KTCN and controls, and between patients with KTCN and controls. The average values with standard deviation are presented. *Statistically significant differences between patients' subgroups; for *central* CE region: adults with KTCN versus controls, $P = 0.001$, and adolescents with KTCN versus controls, $P = 0.004$; for *middle* CE region: adults with KTCN versus controls, $P = 0.0008$, and adolescents with KTCN versus controls, $P = 0.0336$.



e

	KTCN			CONTROLS		
	Central CE region- 1	*Middle CE region- 2*	*Peripheral CE region- 3*	*Central CE region- 1*	*Middle CE region- 2*	*Peripheral CE region- 3*
nuclei density (nuclei/mm²)	5 250	**7 417**	6 250	5 750	**5 750**	5 417
nucleus surface (µm²)	101	**88**	96	95	**106**	117

FIGURE 3. The morphology and density of the remodeled CE in KTCN in regions 1 (*central*) and 2 (*middle*) in KTCN (**A**) and control individuals (**B**); staining with DAPI (**A, B, D**) and wheat germ agglutinin (WGA) conjugate (**C**) (Zeiss Imager Z.2 with ApoTome.2). (**E**) Average nuclei density and single nucleus surface in the *central*, *middle*, and *peripheral topographic* regions of CE, calculated for two KTCN and two control individuals, based on the microscopic assessment of DAPI stained samples with a screening of 12,000 µm² of each region' surface, and counting of at least 100 nuclei per each CE region. Significant differences are bolded.

Supplementary Table S2A. Both, the central–temporal and inferior–temporal locations were found to be more common in patients with KTCN compared with controls ($P = 0.0048$). According to the project assumption, the analyzed subgroup of adolescent patients with KTCN was definitely different in age at examination (15.33 ± 1.97) compared with adults with KTCN (27.94 ± 7.96) and controls (28.00 ± 4.30). A total of 84 samples of CE (3 *topographic* regions × 28 ascertained individuals) were collected and proceeded in accordance with a study scheme (Supplementary Fig. S1).

The Morphologically Different *Middle* Region of KTCN CE

No morphological differences were found comparing the three assessed *topographic* regions of the CE in controls. The differences in average nuclei density and average single nucleus surface of the *middle* CE region comparing the patients with KTCN and control individuals were revealed (Fig. 3). In line with observations of irregularities visible at epithelial thickness maps, more nuclei (7417/mm² vs. 5750/mm²) and nuclei smaller in size (88 µm² vs. 106 µm²) in the *middle* CE region in patients with KTCN, were observed. We have not noticed morphological differences in the CE samples comparing the adult and adolescent patients with KTCN.

The Impact of Gender in Transcriptomic Profiling

We found that the gender of the studied patients influenced the sample clustering into male and female subgroups in the transcriptomic evaluation, as documented in Supplementary Figure S3A. Consequently, because the majority of our processed samples were derived from male individuals, further bioinformatics analyses were narrowed down to these samples. A UMAP plot after removing the data derived from samples of female patients is presented in Supplementary Figure S3B.

KTCN-Specific Gene Expression Manifested in the *Peripheral* CE Region of Adult Patients With KTCN

In control samples, no differentially expressed genes were identified in the opposing *central*, *middle*, and *peripheral topographic* CE regions. However, comparing the particular *topographic* CE regions of patients with KTCN with the corresponding CE regions of controls, the differentially expressed genes were found, with the majority of them observable in the *peripheral topographic* CE region of adults with KTCN (compared with the *peripheral* CE region in controls) (Supplementary Table S4), in which 258 genes were downregulated (130 protein coding), including *ADAMTS4*, *MEGF11*, *PROX1*, *RAX*, *ANK2*, *NLRP4*, *TRABD2B*, *MUC13*, *VSX1*, *LTF*, *ROS1*, and three upregulated, (*RN7SL2*, *STON2*, and *RN7SL4P*). Moreover, the genes *CDH13*, *EDNRB*, *PTN*, *NUPR1*, *BCL11B*, *SGPP*, *CDH2*, *DCN*, *PTN*, *TRIL*, *RTN4RL1*, and *LRRC3B* showed decreased expression in the *central topographic* region in adults with KTCN compared with the *peripheral* region of CE in these individuals. In contrast, *CYFIP2*, *LEFTY1*, *CLMP*, *DSCAM*, *RGL3*, and *CHST2* were upregulated in the *central* region of the CE.

In adults with KTCN, we found 62 genes with downregulated expression (25 protein coding) in the *peripheral* region of CE, including *KRTAP4-16*, *TBX5*, *ASB5*, *MT-TM*, *MT-TQ*, compared with adolescents with KTCN. No differentially expressed genes for KTCN CE *middle* region were recognized compared with *central* or *peripheral* CE regions in adults and adolescents with KTCN, and comparing the data with the corresponding CE *middle* region of the controls.

Over-represented Immune System Pathways and Under-represented Extracellular Matrix Organization Pathways in CE in Patients With KTCN

Of 984 the KTCN *topographic*-specific pathways, the 10 most differentiated in the comparison of particular CE regions between and within evaluated subgroups are presented in Supplementary Table S5A-O. The IFN alpha-beta signaling, IFN gamma signaling, and overall IFN signaling pathways were enriched within upregulated genes in the *central*, *middle*, and *peripheral topographic* regions of the CE of adults with KTCNcompared with adequate CE regions of controls, whereas, senescence pathways (cellular senescence and senescence-associated secretory phenotype) were mostly enriched in the *peripheral* CE region of adults with KTCN (Supplementary Table S5C). Moreover, the analysis revealed that the upregulated genes were associated with DNA methylation pathway in the *central* CE region of the adults with KTCN and adolescents with KTCN in comparison with controls (Supplementary Table S5D). The same trend of change was observable for the activated PKN1 stimulates transcription of androgen receptor regulated genes KLK2 and KLK3 pathway (Supplementary Table S5A, D). In adolescent patients with KTCN the *middle* CE region disclosed features of the selenoamino acid metabolism and translation-related pathways in analysis within genes with downregulated expression (Supplementary Table S5E).

We also compared the enriched pathways by juxtaposing the particular *topographic* regions with each other in the analysed subgroups of patients (excluding results from the control subgroup, since they may reflect physiologically various gene expressions, Supplementary Tables S5J–O), as described in greater detail in Supplementary Materials 2.3. The results of comparative analyses of CE data with data corresponding with the whole thickness corneas are described in Supplementary Materials 2.3.

Distinct Hallmarks of the *Middle* CE Region of Adult Patients With KTCN

In the hallmark gene set analysis, the apoptosis and G2M checkpoint gene sets were found to be upregulated in *central* and *peripheral* CE, and unchanged in the *middle* region in adults with KTCN, in comparison with the adequate *topographic* region in the CE samples derived from controls. The same trend was revealed for androgen response, DNA repair, MYC targets, peroxisome, protein secretion, unfolded protein response, oxidative phosphorylation, reactive oxygen species pathway, and Wnt beta-catenin signaling hallmarks. These findings do not apply to CE samples derived from adolescents with KTCN. The hallmarks of the IFN alpha response and IFN gamma response were upregulated in all the *topographic* regions of CE patients with KTCN, compared with controls (Table 2, Supplementary Table S6).

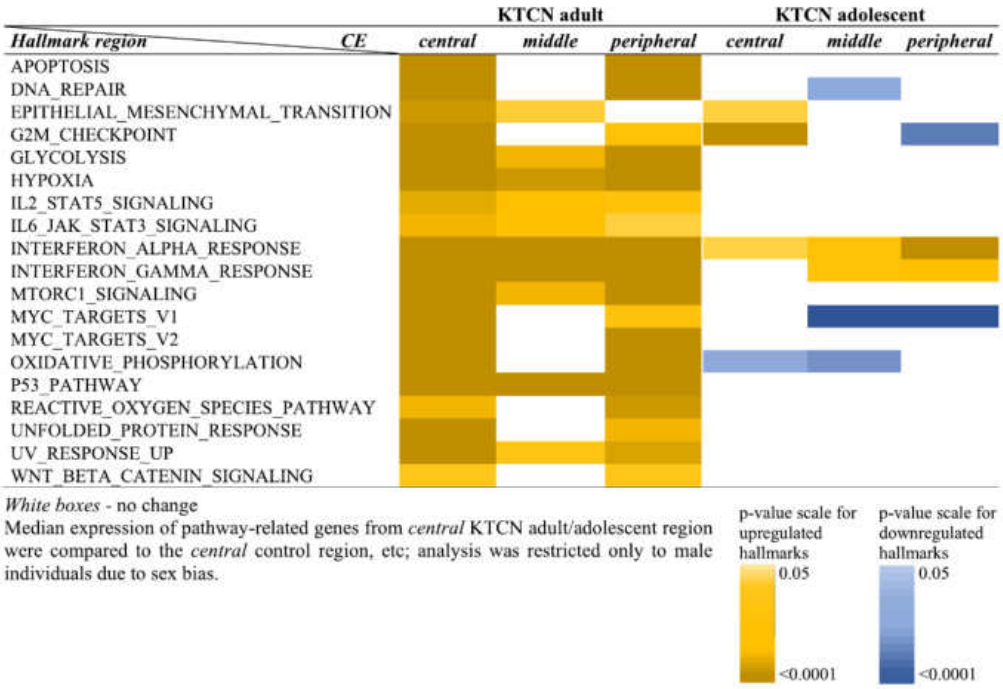
RNA-Seq Data Verification Using RT-qPCR

A high positive correlation between the RNA-Seq and RT-qPCR data was found, as presented in Supplementary Table S7. Shown is RT-qPCR data of *GAPDH*, *B2M*, *KRT12*, *IFI27*, *TGFB2*, *B4GALT1*, *SPATA13*, and *WNK1* gene expressions in each of the 84 originally collected CE samples used for FC calculations. Further results are presented in Supplementary Table S8 (log2FC values) and Supplementary Figure S4 ($R^2 = 0.7854$; Pearson correlation coefficient = 0.8857).

Discriminative Proteomic Features in the CE of Adult Patients With KTCN

The total average proteomic spectra of the CE samples of the adults with KTCN and controls are presented in Supplementary Figure S5. Because the two applied chemometric algorithms quick classifier and genetic algorithm are based on different mechanisms, the peaks classified as discriminative between these algorithms are divergent. We identified 11 unique proteins classified as discriminative between the *central* CE region of adults with KTCN versus controls. In a comparison of the *middle* CE region of adults with KTCN versus controls, we identified five proteins differentially expressed, and between *peripheral* CE region of adults with KTCN compared with controls, we identified 14 discriminative proteins. Lists of discriminative m/z ions for each algorithm together with values of chemometric parameters calculated for the algorithms are presented in Supplementary Table S9. A list of identified proteins classified as discriminative between particular CE regions of adults with KTCN and controls, together with the direction of change is presented in Table 3. The chemometric parameters (parameters of cross-validation and recognition capability) calculated for implemented algorithms in particular statistic schemes (e.g., the *central* region of the CE samples of the adults with KTCN vs. controls), assessing the accuracy of the obtained models are indicated in Table 3.

Table 2. Selected Hallmark Pathways Differentially Represented in Particular Regions of CE in Adults and Adolescents With KTCN, in Comparison With Corresponding CE Regions of Controls



White boxes - no change
Median expression of pathway-related genes from *central* KTCN adult/adolescent region were compared to the *central* control region, etc; analysis was restricted only to male individuals due to sex bias.

Table 3. Identified Proteins Classified as the Discriminative for Adults With KTCN Using Chemometric Algorithms

	***Central* CE Region**		***Middle* CE Region**		***Peripheral* CE Region**	
Direction algorithm	↑	↓	↑	↓	↑	↓
Genetic algorithm	ENO1 PGBD2	HEATR4 CSDE1 TTBK1 SDHA SELENOO TCF20	IGHV3-30	ZBTB4 HSPB1	TKT	IQCF2 WNK4 CLU CHERP
Quick classifier	KRT12 SLC8A1 F199X	HEATR4 SDHA	NDOR1	LENG9	SCN5A PTPRU TBC1D4 IL1RAP F5	RMI1 ABCA13 CLU ANKRD36C
Parameter	Cross-validation (%)	Recognition capability (%)	Cross-validation (%)	Recognition capability (%)	Cross-validation (%)	Recognition capability (%)
Genetic algorithm	95.8	100.0	78.2	100.0	86.5	95.8
Quick classifier	86.8	97.1	67.4	70.3	75.0	91.7

Arrows show the direction of change comparing the specific CE region of adults with KTCN to the corresponding CE region of controls. Values of chemometric parameters calculated for algorithms are indicated.

Molecular Indicators of Specific Clinical Features of KTCN

Based on the correlations of the transcriptomic findings with the clinical data we found the expression of *TCHP* (R = −0.47; $P = 0.029$), *SPATA13* (R = −0.75; P= 6.6e-05), *CNOT3* (R = −0.25; $P = 0.26$), *WNK1* (R = −0.34; $P = 0.12$), *TGFB2* (R = −0.59; $P = 0.0035$), and *KRT12* (R = −0.43; $P = 0.046$) genes to be correlated with the posterior elevation (Fig. 4A). In addition, considering exclusively clinical data of adults

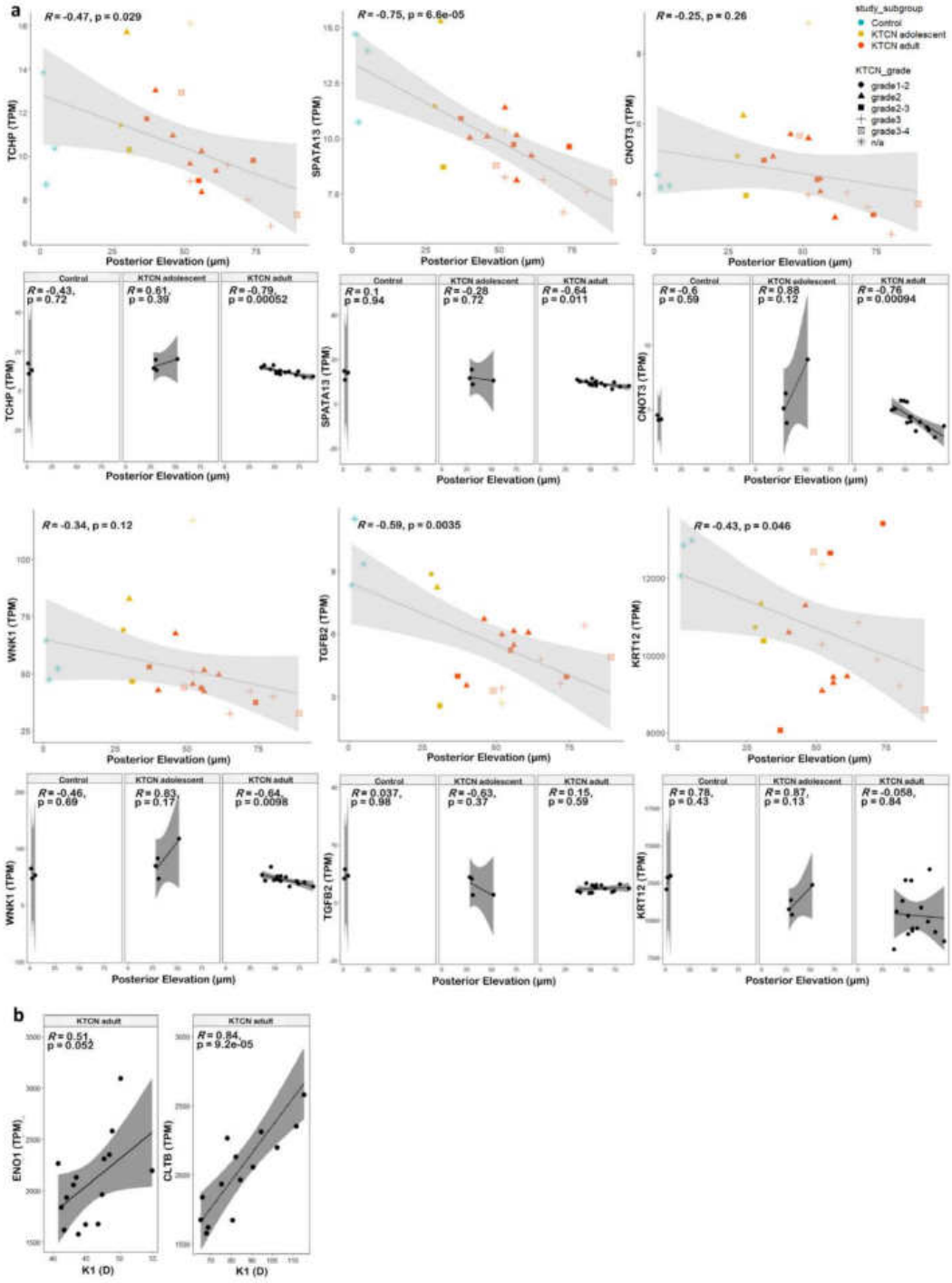


FIGURE 4. Pearson' correlation results between expression of selected genes, *TCHP*, *SPATA13*, *CNOT3*, *WNK1*, *TGFB2*, *KRT12*, *ENO1*, and *CLTB* (in TPMs), in the *central* CE region and particular clinical features: (**A**) posterior elevation (in micrometers), and (**B**) K1 (in **D**). (**A**) The color of dots and their shape indicate the studied subgroups and the grade of KTCN, respectively. (**B**) Plots show the regression analysis results in the subgroup of adults with KTCN only. Values of regression and statistical significance are presented in the plots.

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Table 4. Pathways and Genes of Biological Importance from Transcriptomic (RNA-Seq) and Proteomic (MALDI-TOF/TOF Tandem Mass Spectrometry) Assessments

		KTCN ADULT			*KTCN ADOLESCENT*		
Pathway Module, Pathway	**Study**	***Central* CE Region**	***Middle* CE Region**	***Peripheral* CE Region**	***Central* CE Region**	***Middle* CE Region**	***Peripheral* CE Region**
Cytokine signaling, IFN alpha/beta	RNA-Seq	**IRF9, OAS2, IRF1, IFI27**	**IRF9, OAS2, IFI27, ISG15, HLA-A, HLA-F, STING1**	**IFI27**, ROS1*, ANP32B	**IFI6, IRF9, IRF1**	**IRF9, HLA-H, MX2, MX1**	**IFI27, HLA-H**
	MALDI-MS	**KRT12**		KRT12			
Cytokine signaling, IFN gamma	RNA-Seq	**IRF9, OAS2, IRF1, GBP1,** CES1	**IRF9, OAS2, HLA-A, HLA-F,** FAM220A, CES1	VCAM1*, ROS1*, CES1	**B2M, IRF9, IRF1**	**IRF9, PML, HLA-H, HLA-DRA**	**HLA-H**
	MALDI-MS						
Innate immune system, neutrophil degradation	RNA-Seq	**B4GALT1**	**B4GALT1, STING1, HLA-A**	ADAMTS4*, CXCL1*, LTF*, MMP20*, S100P, MGST1	**PGM2, S100A9, ADAM10, B4GALT1, TICAM2, B2M, SERPINB6**	**S100A9, B4GALT1, HLA-H, STING1, SERPINB6, ALOX5**	**HLA-H, S100A9,** SRP14, EEF2, SNAP23
	MALDI-MS		ABCA13	ABCA13			
Adaptive immune system, class I MHC antigen processing and Presentation	RNA-Seq	**MYD88,** ZBTB16, PRKN	**HLA-A, HLA-F, CD74**	CCDC135*, ANK2*, ASB5*, ZBTB16	**B2M, S100A9,** ZBTB16, FBXO2	**HLA-H, S100A9**	**HLA-H, S100A9,** PAG1, ZBTB16, SNAP23
	MALDI-MS						
Signal transduction, signaling by receptor tyrosine kinases, (including signaling by EGFR)	RNA-Seq	**MT1A**	**ACTB**	PGR*, HGF*, PTN*, NTRK3*, ID4, FGF23, ERBB4, FUS, HNRNPM, PCK1	**CHEK1, ADAM10, STMN1, PPP2R1B, PCSK5,** PCK1	**ID3, PRKCA, NRG2, PDGFA, ID1, HBEGF, ACTB, DUSP7**	PAG1, CHEK1
	MALDI-MS		HSPB1	**PTPRU**			
Signal transduction, signaling by TGFB	RNA-Seq	TGFB2	**CDKN2A**	BMP10*, TGFB2, TCF4	**ITGB6, RBL1,** EMB		EMB
	MALDI-MS		**NDOR1**				
Signal transduction, signaling by WNT	RNA-Seq			AXIN2*, LGR6*, PRKCG*, TBX3*, TRABD2B*, ZEB2, MYEF2, PLCB1, TCF4, PCK1	**H2BC8, H2BC7, H4C13, H2AC17, PPP2R1B,** EMB, PCK1, H2AC18	**PRKCA, WNT9A, H4C12, H4C13**	**H4C12,** PLCB1, EMB
	MALDI-MS			**PTPRU**			
Cell–cell communications, cell–cell junction organization	RNA-Seq	**AJAP1, B4GALT1**	**AJAP1, B4GALT1, ZYX, ACTB**	MAGI2, CDH2*, CDH13*	**PRR4, B4GALT1**	**ZYX, ACTB, KRT7, B4GALT1**	
	MALDI-MS	**ENO1,** KRT7, SHROOM2	**SH3GLB2**	**PTPRU,** WNK4, ENO1	KRT7, SHROOM2	**SHROOM2**	**SHROOM2**
ECM organization, collagen formation	RNA-Seq			MMP20*, COL8A1, **TIMP3**			
	MALDI-MS						
ECM organization, ECM proteoglycans	RNA-Seq	TGFB2, ADARB1	MUSK*, DCN*, TGFB2	**ITGB6**			
	MALDI-MS						

Table 4. Continued

Pathway Module, Pathway	Study	KTCN ADULT *Central* CE Region	KTCN ADULT *Middle* CE Region	KTCN ADULT *Peripheral* CE Region	KTCN ADOLESCENT *Central* CE Region	KTCN ADOLESCENT *Middle* CE Region	KTCN ADOLESCENT *Peripheral* CE Region
ECM organization, degradation of ECM	RNA-Seq	ADARB1, KLKB1	ADAMTS4*, MMP20*, ELN*, DCN*	**ADAM10**			
	MALDI-MS			ADAM17	ADAM17		ADAM17
ECM organization, integrin cell surface interaction	RNA-Seq	ADARB1	**ZYX**	VCAM1*, ANP32B, COL8A1	**ITGB6**		
	MALDI-MS						
Programmed cell death, apoptosis	RNA-Seq		**DAPK2, TICAM1, CDKN2A**	VIM*	**H1-0, TICAM2, RIPK1**	**TICAM1, H1-0, DAPK2**	HMGB2
	MALDI-MS		HSPB1	**IL1RAP**			
DNA repair, base excision/double-strand breaks repair/mismatch repair	RNA-Seq	**MT1A**	**ISG15, ACTB,** GTF2H2	PLSCR4, CDC45	**H2BC8, H2BC7, H4C13, H2AC17, POLE2, POLQ, CDK1, CHEK1,** H2AC18	**ACTB, H4C12, H4C13**	**H4C12,** CHEK1
	MALDI-MS	TCF20		RMI1	SUMO2	RBBP8	RBBP8
Transcription, positive/negative regulation	RNA-Seq	ZBTB16	**ACTB†, TP53INP2**	ZBTB16*, BLK*, GPC5*, GAD2*, DLX6*, TAL1*, ZNF521*, ANP32B	**H2BC8†, H2BC7†, H4C13†, H2AC17†,** H2AC18†, ZBTB16	**H4C12†, H4C13†**	**H4C12†**
	MALDI-MS	CSDE1, TTBK1	ZBTB4*				
Cell cycle, mitosis/cell cycle checkpoints	RNA-Seq	MAP9	**CDKN2A**	MAP9, GABRG1*, IRGM*, NEFM*, CDK11A, CDC45, CENPM	**AURKA, BUB1B, CHEK1, CENPH, SGO2, BUB1, MAD2L1, SPC24, NUF2, PPP2R1B, CDK1, CENPE, CENPF, CENPA, SKA2, CCNB2, ZWINT, NEK2, CENPJ, MAD2L1, SPC24, CCNB2, RBL1, CENPE, CENPF, CENPA, NCAPG, BUB1B, TOP2A, SGO2, HMMR, ESCO2, POLE2,** EMB, CDKN1C	**PRKCA, KIF2C, CENPA,**	EMB, CHEK1, DMC1, CENPH, CDC45, SMC4, Q9NTJ3, SMC2
	MALDI-MS			RMI1		RBBP8	RBBP8
Developmental biology, Keratinization	RNA-Seq	**KLK13**		KRT26*,KRTAP4-16*, KLK5, KRT13	**KLK13, KRT24, KRT8**	**KLK13, KRT7, KRT24,** SPINK5, KRT6A	**KRT24,** SPINK5, KRT6A
	MALDI-MS	**KRT7, KRT12**	KRT3	KRT7, KRT12	**KRT78**	**KRT78**	

Presented are genes/proteins with upregulated (FC of ≥1.5) or downregulated (FC of ≤0.5) expression/protein levels in the particular CE regions of adults or adolescents with KTCN, attributed to biological pathways revealed as significant in pathway enrichment and hallmarks analyses. In RNA-Seq we performed the differential expression analysis using limma package. Additionally, we calculated the expression of genes [with a mean expression level of ≥1 transcript per million (TPM) from a particular pathway gene set, and defined it as significant based on the following cutoffs: $P \leq 0.05$ (t test) and FC of ≥1.5 or ≤0.5. For MALDI-TOF/TOF tandem mass spectrometry data analyses, we implemented mathematical algorithms (quick classifier [QC] and genetic algorithm [GA]) regarding CE samples of adults with KTCN and controls, whereas discriminative peaks for CE samples of adolescents with KTCN (compared with controls) were calculated in JASP Software. Extended list of pathways and genes of biological importance is presented in Supplementary Table S10.

MALDI-MS, MALDI-TOF/TOF Tandem Mass Spectrometry.

Bold text indicates the upregulated expression/level of gene/protein.

* Differentially expressed genes revealed using limma package.

† Genes involved in the regulation of gene expression by methylation.

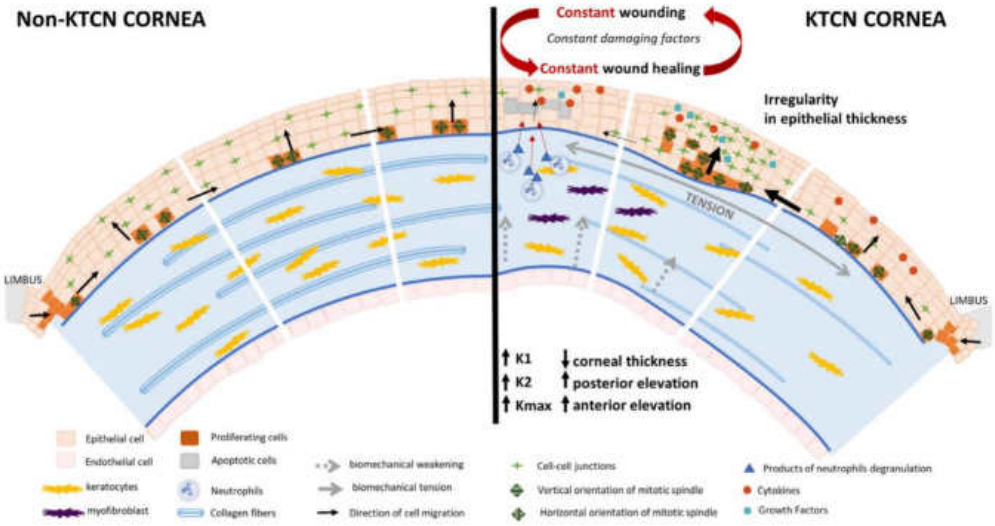

Figure 5. Hypothetical mechanism of CE remodeling in KTCN (white vertical lines indicate boundaries of the corneal *topographic* regions). In KTCN, the physiological conditions of cornea biomechanics, dynamic interactions of cells, and homeostasis of cytokines and growth factors are disturbed. The progressive biomechanical weakening of the cornea, being a consequence of changes in collagen fibers and other elements of extracellular matrix in the stroma, contributes to the additional tension of the cornea and results in increased posterior elevation, anterior elevation, and keratometry as well as the decreased corneal thickness. The CE remodeling is triggered by constant wounding stimulated by both eye rubbing and dysregulated immune responses. Simultaneously, the released growth factors, cytokines, and products of neutrophil degranulation induce apoptosis, mesenchymal differentiation, and wound healing. Additional dysregulation of the cell migration process causes a constant, instead of a transient, wound healing state. The initially protective inflammation contributes to the progressive corneal weakening manifested by cone formation, which constitutes the most susceptible zone for further corneal injuries. Intensified cell migration from the *peripheral* to the *middle topographic* region of CE and simultaneously reduced migration of cells from *middle* to *central topographic* region, modifications of mitotic spindle' orientation from horizontal to vertical, as well as changes in cell cycle checkpoints, DNA repair, and apoptosis cause the accumulation of cells and increased epithelial thickness in the *middle topographic* region of CE. Concurrently, these factors lead to decrease in the cell number and epithelial thickness in the *central topographic* region.

with KTCN, the relationships with clinical outcomes showed the following expression products correlating with K1: *ENO1* (K1; R = 0.51; $P = 0.52$) and *CLTB* (K1; R = 0.84; $P = 9.2e\text{-}05$) (Fig. 4B).

Pathways and Genes of Biological Importance: Abnormal Immune Responses, ECM Organization, and Cell Cycle, and Mechanisms of CE Remodeling

Results of the transcriptomic and proteomic data consolidation are presented as 17 pathways' branches in Table 4, and in the extended version of this table in Supplementary Table S10 (28 pathways' branches). Indicated pathways correspond with Supplementary Figure S6, as a visualization of networks for particular *topographic* regions of the CE. Deregulation of biological pathways such as IFN alpha/beta signaling, IFN gamma signaling, and neutrophil degranulation was revealed. Moreover, the decrease in the elements of the ECM (integrin cell surface interaction, collagen formation, and proteoglycans) was disclosed. Cell cycle and transcription, as well as DNA repair, were the processes substantially upregulated in the *central* and *middle* CE regions of adolescent patients compared with both adults with KTCN and controls. Combined data regarding the selected distinguishing pathways, together with the identified alternations are presented in Supplementary Figure S7, and the remaining results are compiled in Supplementary Figure S8. Additionally, the discovered high-impact and missense variants revealed in the four youngest patients with KTCN in our study group, assumed to contribute to the molecular picture of KTCN discussed here, in particular CE, are presented in Supplementary Table S11.

The hypothetical mechanism of formation of the CE *doughnut*, embracing elements of the corneal biomechanical weakening, dysregulation of immune responses, and altered cell migration, resulting in constant wounding and wound healing of CE and remodeling of CE, are presented and described in detail in Figure 5.

Discussion

Thus far, pathological changes in the cornea during KTCN development, leading to keratoconic cone formation, have been explained frequently by the incorrect arrangement of the stroma, and thus impairment of its physiological function.[48–50] To compliment previous, solely clinical findings, we delved into the corneal *topographic* regions of the KTCN cornea.

Discriminating Clinical Features, Epithelial *Doughnut*, and Corneal *Topographic* Regions

In line with previously reported epithelial thickness irregularity and asymmetry, as discriminating factors between keratoconic and healthy eyes,[51,52] we found a decrease in the

average epithelial thickness at the corneal apex in adults and adolescents with KTCN compared with unaffected eyes. Our finding of the most common cone locations in central temporal and inferior temporal, in both adults and adolescents with KTCN, corresponds with previously described keratoconic cones decentered to the inferior temporal location.[51,53]

On the maps of the epithelial thickness of our adult and adolescent patients with KTCN, the central cone, which is the thinnest region, was found to be surrounded by a thickened *middle* region of full- or open-ring shape, which has been reported and named as an epithelial *doughnut*.[21,51,53] Despite that well-known pattern, aspects of clinical, morphological, and molecular abnormalities in the CE of patients with KTCN and especially in the *topographic* regions of the CE have not been evaluated extensively. These days, first experiments are being performed based on the separation of corneal layers[54,55] or single-cell approaches[22] toward a more detailed assessment, but the design of these studies have not reflected the KTCN-specific *doughnut* pattern.

Moving toward unraveling the novel mechanistic aspects of KTCN development in the CE, we designated *central*, *middle*, and *peripheral topographic* regions of the CE based on the CE thickness irregularity observed in adults and adolescents with KTCN, and we compared the obtained data with the corresponding *topographic* regions of the CE in controls to determine biological diversity. To date, there was no KTCN study assessing three distinct *topographic* regions of the CE based on the CE thickness maps. While in two studies the separation of two *topographic* regions, the conical and nonconical zones has been implemented, the distinctive pattern of the epithelial *doughnut* in KTCN has not been evaluated.[54,56] Molecular analyses restricted to selected genes,[56] no details of the clinical examination of control individuals,[56] and lack of separation of control corneas into the two examined cone and non-cone zones[54] were additional limitations of these studies.

Morphological Diversity of the CE *Topographic* Regions

Morphological examination of the CE samples of controls showed a balance in the number of cells, confirmed by the nuclei number measurements, between the particular *topographic* regions. In contrast with controls, in the CE samples of patients with KTCN, an imbalance in the number and size of nuclei, with the greatest increase of number and decrease of size of nuclei in the *middle* CE region was observed. As predicted, a decrease in nuclei number in the *central* region of the CE was found. Morphological alterations were also visible in the *peripheral* CE region, somewhat reminiscent of the changes observed in the *middle* region, and in particular as an increase in the number and a decrease in the size of the cells. The identified features of the *peripheral* CE region of patients with KTCN were not detectable in the epithelial thickness mapping.

Deregulated Immune Responses in KTCN CE

Since 2017, we have been constantly confirming the hypothesis that the deregulated pathways, and not the disturbance of the expression of individual genes, constitute the molecular signature of KTCN.[31,42,43] Consequently, here, by consolidation of multi-omic data, we demonstrated the overall upregulation in IFNs alpha/beta and gamma signaling in both evaluated subgroups of patients with KTCN, which corresponds with previous reports on cytokines profile in the tear fluid.[57,58] However, in addition to the IFNs, we have found dysregulation of antigen processing and presentation pathway and allograft rejection hallmark. Additionally, the molecular changes found on genomic level indicate a more complex inflammatory background in KTCN as previously anticipated (Jaskiewicz et al. 2022, unpublished data). The presence of variants in genes involved in antigen processing and presentation (Jaskiewicz et al. 2022, unpublished data), together with the eye rubbing and allergy, could contribute to chronic corneal epithelial injury and trigger constant stimulation of wound healing in KTCN, as previously suggested.[59] In line with these results, here we presented the decreased level of clusterin (CLU) protein in the *peripheral* region of CE, which was previously demonstrated to be repressed during closure of wounds in tissue-engineered human cornea,[60] and the increased level of keratin 12 (KRT12) protein in the *central* region of CE, earlier reported as an effect of repairing response after injury.[61]

Impaired Wound Healing Process in KTCN CE

Because of constant wounding and stimulation of wound healing in KTCN,[59] and prolonged inflammation leading even to corneal scarring and vision impairment,[62] a properly functioning repair mechanism is crucial to provide homeostasis in CE.[63] Here, the expression of genes involved in response to wounding (*ITGB1*, *LOX*, *CD44*, *PECAM1*, *DST*, *DCN*, *B4GALT1*, *WNT5A*, and *TNFSRF12A*) was identified to be upregulated in the *central* and *middle* CE regions, but downregulated in the *peripheral* region of KTCN CE, suggesting that this process is differentially altered, depending on the region. Also, by combining the clinical and molecular data, we revealed the correlation between posterior corneal elevation and expression of the *WNK1* gene, which was previously found to contribute to the healing of corneal wounds in the tissue-engineered human cornea.[64] Additionally, in the *peripheral* region of CE of adults with KTCN, we found downregulated expression of the *HGF* gene, which could explain dysregulation of the migration of epithelial cells[65] and corneal epithelial wound healing.[66] Moreover, a previously not reported in the CE pathology aspect concerning the neutrophil degranulation pathway was found to be upregulated in the *central* and *middle* regions of the CE, but downregulated in the *peripheral* region of the CE of adults with KTCN. The role of neutrophils in stromal wound healing has been established previously.[59,67] At this point, we hypothesize that the observed dysregulation may be an effect of the interplay of stromal and epithelial wound healing rather than the infiltration of neutrophils into the CE from the stroma. Moreover, the revealed single high-impact sequence variants (*CEP290*, *MMRN1*, and *FN1*), and missense variants (*PECAM1*, *VWF*, *CD109*, *HRG*, *TLN1*, *HPSE*, *EPO*, *SIGLEC10*, *DOCK8*, *ERBB3*, and *AP3B1*) in CE samples of adolescents with KTCN (Jaskiewicz et al. 2022, unpublished data) confirm the importance of this supposition, as may have contributed to this molecular dysregulation of wound healing, although we have not determined their impact.

Because wound healing induces cell migration,[68] its efficacy could be also affected by the epithelial–mesenchymal transition and cell–cell communications, including the

cell–cell junctions and cell–ECM interactions.[63] In the *peripheral* region of CE of adults with KTCN, we found decreased expression of fibronectin (*FNDC1*), previously described as contributing to the scaffold of ECM and reported to be a mesenchymal marker.[69] Additionally, the hallmark of epithelial–mesenchymal transition was recognized to be elevated in the *central* and *middle* CE regions of adults with KTCN and in the *central* CE region of adolescents with KTCN. Regarding the aspect of the cell–cell communications, we revealed the upregulated pathways of tight and adherent junctions' organization in the *middle* CE region of adults with KTCN. Also, the claudin-related genes' expression was mostly increased in the *middle* CE region of adults with KTCN. Given both pro-proliferative and antiapoptotic functions of these genes,[70] this factor may explain the observable morphological pattern of CE in the *middle* CE region in patients with KTCN.

The cell–ECM interdependencies are another recognized, crucial element of cell migration.[71] The disruption of the organization of ECM was one of the features previously revealed in the transcriptome or pathways analysis in whole-thickness corneas,[31,43] cultured stromal cells from KTCN corneas,[72] and now in CE of patients with KTCN. Here, the downregulation of integrin cell surface interactions, collagen formation, ECM proteoglycans, and degradation of the ECM in the *peripheral* CE region of patients with KTCN was revealed as the most significant finding. Moreover, in the aspect of integrins-mediated cell adhesion to the ECM substantial overlap between transcriptomic and genomic findings (Jaskiewicz et al. 2022, unpublished data), which emphasize the importance of these results. Summarizing here, the revealed impaired ECM organization and cell–cell communication in the *central* and *middle* CE regions of adults with KTCN might cause the decreased epithelial cell motility in these regions through deregulation of cells migration, as previously found in rabbit normal corneas,[63] and corneal epithelial cell line.[73] Downregulated expression of cadherin *CDH13* taken together with the decreased levels of ENO1 protein (as elements of cell adherent junctions) and WNK4 protein (as an element of tight junctions) in the *peripheral* region, could intensify cell migration from the *peripheral* to *middle* CE region, leading to an accumulation of cells in the *middle* region of the CE and forming the aforementioned *doughnut* pattern. Also, based on the obtained results, we take into consideration that the *doughnut* pattern may result from an alteration in the cells' proliferation and cell cycle progression through the upregulation of apoptosis, G2M checkpoints, and DNA repair pathways in the *central* and *peripheral* CE regions. Additionally, in the aspects of cell proliferation, we found the upregulated hallmark of the mitotic spindle in all the *topographic* regions of adults with KTCN and in the *central* region of adolescents with KTCN. Previously, the vertical and horizontal orientation of the mitotic spindle has been described in normal CE as a feature possibly influencing both cell migration and cell division.[74] Delving into the genetic aspects we identified high impact variants in *INSC* gene in CE samples of adolescents with KTCN (Jaskiewicz et al. 2022, unpublished data), which is involved in spindle orientation during mitosis.[75] However, we have no evidence to verify the observable increase in the number of cells in the *middle* region compared with the *central* and *peripheral* region of CE, and to confirm whether it is a result of an increased proliferation of cells or their arrest at a given stage of the cell cycle.

Dysregulated Gender-related Pathways

We also focus on sex hormones, which were reported as the players in epithelial–mesenchymal transition,[76] cytoskeleton modulators,[77] and transcriptional activators.[78,79] The influence of gender on expression of more than 600 genes in CE of unaffected individuals[80] and patients with KTCN[55] was previously reported. Because such gender dependence embraces the genes responsible for cell growth, migration, and proliferation, we limited our further analyses to males instead of discriminating off all genes with gender-dependent expression. Regardless of the fact that female patients were excluded from molecular analyzes, we observed disturbances in hormone-related pathways, with the activated PKN1 stimulates transcription of androgen receptor regulated genes KLK2 and KLK3 pathway as the most upregulated in the CE of patients with KTCN.

Impact of the Stage of Progression in KTCN, Indicators, and Translational Relevance

Because the same morphological abnormalities of the CE were demonstrated in adults and adolescents with KTCN, to differentiate these patients' subgroups the transcriptomic and proteomic features together with clinical findings were evaluated, also in direction of identification of KTCN-specific indicators. In the comparison between adolescents and adults, the enrichment in transcription and mitosis pathways in adolescents were revealed, whereas other features were specific for adults with KTCN, including the ECM organization, epithelial–mesenchymal transition, inflammatory response, and TGF-β signaling. We noticed significantly higher values of posterior elevation in adults with KTCN in comparison with adolescents with KTCN.

Although the difference in the posterior elevation between adults with KTCN and adolescents with KTCN may be explained by the CXL procedure performed before the advanced stage of pediatric KTCN owing to the high risk of rapid progression,[81–83] the clinical–transcriptomic correlations found in this study allowed us to point to the KTCN indicators. Because posterior elevation has been reported as a strong discriminating factor of normal and KTCN eyes allowing the diagnosis before a patient becomes symptomatic,[84] and we pointed to the correlations of clinical and transcriptomic elements, the latter might be of translational relevance. For example, we can apply a noninvasive method, an impression cytology, to harvest CE cells. Here, we demonstrated a significant negative correlation between the expression of proapoptotic *TCHP*,[85] regulating cell migration *SPATA13*,[86] promitotic *CNOT3*,[87] wound healing–related *WNK1*,[64] cells' growth affecting *TGFB2*,[88] involved in CE organization *KRT12*,[89] and the value of posterior corneal elevation. We also found other correlations of clinical–transcriptome elements that, however, do not differentiate adults and adolescents with KTCN. The expression of *ENO1* involved in allergic responses[90] and *CLTB* involved in clathrin-dependent endocytosis[91] showed a strong positive correlation with the K1 value.

Mechanism of CE Remodeling in KTCN

Finally, summarizing all the findings, we propose the mechanism of cell and molecule migration in the three *topographic* regions leading to CE remodeling. Under physiological conditions, the proper dynamics of cells, cytokines,

growth factors, and lipids between the epithelium and the stroma promote epithelial integrity and protective inflammation, as well as limiting corneal fibrosis.[92,93] In KTCN, the physiological conditions of cornea biomechanics, dynamic interactions of cells, and homeostasis of cytokines and growth factors are disturbed. In KTCN, the progressive biomechanical weakening of the cornea, being a consequence of changes in collagen fibers and other elements of extracellular matrix in the stroma, contributes to the additional tension of the cornea and results in increased posterior elevation, anterior elevation, and keratometry, as well as decreased corneal thickness.[56,94] The contribution of CE to the overall biomechanical properties of the cornea has been confirmed in studies showing the association between corneal stiffness parameters A1 and corneal epithelial thickness[61] or even removal of epithelium.[95] Interestingly, in KTCN the CE is able rebuild in response to underlying stromal irregularities, allowing the creation of a symmetrical optical surface.[55,96] This remodeling is triggered by constant wounding stimulated by both eye rubbing and dysregulated immune responses. Disturbance of the immunological homeostasis may cause stimulation of the keratocyte into myofibroblast, due to a release of TGF-β2 from CE cells,[97] whereas loosing of TNF-α expression in stromal cells results in thinning of CE.[92] The released growth factors, cytokines, and products of neutrophil degranulation induce apoptosis, mesenchymal differentiation, and wound healing. Nevertheless, additional dysregulation of the cell migration process causes a constant, instead of a transient, wound healing state. Initially protective inflammation contributes to the progressive corneal weakening manifested by keratoconic cone formation, which is the most susceptible zone for further injuries. Intensified cell migration from the *peripheral* to the *middle topographic* region of CE and simultaneously decreased migration of cells from *middle* to *central topographic* region and modification of mitotic spindle orientation from horizontal to vertical, as well as changes in cell cycle checkpoints, DNA repair, and apoptosis, cumulatively cause the accumulation of cells and increased epithelial thickness in the *middle topographic* region of CE. Concurrently, these factors lead to a decrease in the cell number and epithelial thickness in the *central topographic* region.

Taking into account the multiple molecular and isolated clinical differences between adults and adolescents with KTCN, the disease duration and the likely sequence of changes in the layers of the cornea during disease development (changes in the posterior surface of the cornea are the earliest observed feature of KTCN[13]), we concluded that the recognized correlations of posterior elevation and transcriptome features might have translational relevance. In our study, the *doughnut* pattern of the CE was observed in both subgroups of patients with KTCN; therefore, we assume that morphological compensation of CE occurs quickly after the first lesions of the posterior surface of the cornea, which is in line with previous data.[1,13] Subsequently, further progression of KTCN in adults, as the increased steepening of posterior surface curvature leading to aggravating of cornea's properties, results in increased biomechanical and molecular interactions between the stroma and epithelium.

We are aware of the limitations of our study. It is not possible to obtain the CE from healthy individuals for research purposes; this is why patients with mild myopia undergoing the PRK surgery as non-KTCN controls were ascertained, as previously practiced by Pahuja et al.,[56] You et al.,[55] and Karolak et al.[42] Moreover, obtaining the CE samples from the control individuals toward transcriptomic assessment was especially challenging, because this type of material could be collected during a PRK procedure only, which nowadays is rarely performed, having been replaced with the newer techniques of vision correction in which the CE is not removed. The PRK procedure is performed only in adult individuals and this fact also results in the lack of age-matched controls in analyzes embracing adolescents with KTCN. We were able to collect only the samples of CE; therefore, our suggestions regarding the interaction between CE and subsequent corneal layers are based only on clinical data and need to be verified in further study involving both CE and stroma samples. More men than women were identified in the examined study subgroups, which is in line with the predominance of male patients among 110 individuals undergoing the CXL procedure in 2020 through 2022 in the Optegra Clinic (19 female and 91 male patients, unpublished data, Dr. Maleszka-Kurpiel, personal communication), and with other reports.[98–100]

To conclude, we found characteristic and distinguishing features for particular CE *topographic* regions in KTCN, pointing to their involvement in the stated mechanism of CE remodeling. The revealed changes in the CE molecular profiles are a consequence of the progression of KTCN rather than the cause of the disease. The presented molecular, morphological, and clinical evidence supports our hypotheses regarding the impaired wound healing in KTCN, the disturbed epithelial–stromal interactions, and biomechanical compensation of the posterior corneal surface. Importantly, the clinical–molecular indicators identified here are the measurable findings related to KC pathogenesis.

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Author Contributions: KJ participated in research design, sample collection, and patient surveys; performed sample preparations toward RNA-Seq, MALDI-TOF/TOF Tandem Mass Spectrometry, and morphological experiments; participated in RNA-Seq experiments; participated in bioinformatics analyses; figures and tables preparation; participated in the transcriptomic, proteomic, morphological and clinical data interpretation; performed biological interpretation of the results; wrote the manuscript. MMK performed patients' recruitment and clinical evaluation; facilitated sample collection; performed the clinical data interpretation; participated in the transcriptomic, proteomic, morphological and clinical data interpretation; performed biological interpretation of the results; wrote the manuscript. EM participated in the setup of MALDI-TOF/TOF Tandem Mass Spectrometry experiments and proteomic data interpretation; performed MALDI-TOF/TOF Tandem Mass Spectrometry experiments, MALDI-TOF/TOF Tandem Mass

Spectrometry data analysis, preparation of MALDI-TOF/TOF Tandem Mass Spectrometry figures, and tables. MK performed setup of bioinformatics pipeline, bioinformatics analyses of transcriptomic data; preparation of figures and tables. MR performed RNA-Seq experiments and transcriptomic data interpretation. RM: participated in the setup of morphological assessment and data interpretation. RP participated in the transcriptomic data interpretation. JM participated in the setup of MALDI-TOF/TOF Tandem Mass Spectrometry experiments and proteomic data interpretation. MG performed research design; secured research funding; participated in the transcriptomic, proteomic, morphological, and clinical data interpretation; performed biological interpretation of the results; and edited the manuscript.

All authors contributed to critical revisions and approved the final manuscript.

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Investigative Ophthalmology & Visual Science

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Investigative Ophthalmology & Visual Science

Artykuł III:

Non-allergic eye rubbing is a major behavioral risk factor for keratoconus.

Jaskiewicz K, Maleszka-Kurpiel M, Michalski A, Ploski R, Rydzanicz M, Gajecka M.
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Wstęp: Czynniki środowiskowe, behawioralne i społeczno-ekonomiczne pozostają słabo poznane w etiologii stożka rogówki. Celem pracy było scharakteryzowanie wybranych czynników jako potencjalnych czynników ryzyka oraz elementów wpływających na kształtowanie fenotypu KTCN, a zwłaszcza na nieprawidłowości w CE. W badaniu wzięło udział 118 pacjentów z KTCN i 73 osoby z grupy kontrolnej, które zostały zbadane klinicznie i odpowiedziały na pytania dotyczące środowiskowych, behawioralnych i społeczno-ekonomicznych czynników ryzyka KTCN. Zebrane dane zostały opracowane statystycznie, a wybrane wyniki, rozpoznane jako specyficzne dla KTCN, skorelowano z wynikami badań transkryptomicznych uzyskanych dla prób CE.

Wyniki: W analizie wieloczynnikowej badanych czynników ryzyka wykazano, że płeć męska, pocieranie oczu, czas spędzony przed komputerem w czasie wolnym od pracy/szkoły oraz obecność kurzu/zapylenia w środowisku pracy są najistotniejszymi czynnikami ryzyka z wartościami ilorazu szans odpowiednio 8.66 (95% CI: 3.16-23.79), 7.36 (95% CI: 2.02-26.89), 2.35 (95% CI: 1.42-3.88) i 5.25 (95% CI: 1.23-22.33). Analizując geny, których poziom ekspresji w centralnym regionie topograficznym nabłonka rogówki był skorelowany ze sposobem/intensywnością pocierania oka, wykazano nadreprezentację ścieżek molekularnych apoptozy (*TP53, BCL2L1*), białek opiekuńczych (*TLN1, CTDSP2, SRPRA*), odpowiedzi na niesfałdowane białka (*NFYA, TLN1, CTDSP2, SRPRA*), adhezji komórkowej (*TGFBI, PTPN1, PDPK1*) i stresu komórkowego (*TFDP1, SRPRA, CAPZB*). Ponadto rozpoznano korelacje poziomów ekspresji trzech genów (*TRIM8, JUNB, LTBP3*) z czasem spędzonym przed komputerem w czasie wolnym od pracy/szkoły. Stwierdzono, że geny, których ekspresję ekstrapolowano na stan alergii, nie są powiązane ze szlakami zapalnymi, w tym z IgE-zależnymi.

Wnioski: Pocieranie oczu powoduje uszkodzenie CE i wyzwala stres komórkowy, który poprzez wpływ na apoptozę, migrację i adhezję komórek kształtuje fenotyp KTCN. Tym samym zweryfikowano wcześniejsze przypuszczenia dotyczące przewlekłego urazu mechanicznego rogówki w KTCN.

Wkład Katarzyny Jaśkiewicz w publikację: koncepcja i plan badań, zbieranie i przygotowanie materiału biologicznego od pacjentów, przeprowadzenie wywiadów z pacjentami; opracowanie danych klinicznych i ich analiza statystyczna; przygotowanie bibliotek RNA-Seq; analiza i interpretacja korelacji danych transkryptomicznych i klinicznych; przygotowywanie rycin i tabel; pisanie manuskryptu.

Załączniki i informacje dodatkowe związane z niniejszą publikacją (24 strony) są dostępne pod następującym adresem internetowym:
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PLOS ONE

RESEARCH ARTICLE

Non-allergic eye rubbing is a major behavioral risk factor for keratoconus

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Abstract

Since the environmental, behavioral, and socioeconomic factors in the etiology of keratoconus (KTCN) remain poorly understood, we characterized them as features influencing KTCN phenotype, and especially affecting the corneal epithelium (CE). In this case-control study, 118 KTCN patients and 73 controls were clinically examined and the Questionnaire covering the aforementioned aspects was completed and then statistically elaborated. Selected KTCN-specific findings were correlated with the outcomes of the RNA-seq assessment of the CE samples. Male sex, eye rubbing, time of using a computer after work, and dust in the working environment, were the substantial KTCN risk factors identified in multivariate analysis, with ORs of 8.66, 7.36, 2.35, and 5.25, respectively. Analyses for genes whose expression in the CE was correlated with the eye rubbing manner showed the enrichment in apoptosis (*TP53*, *BCL2L1*), chaperon-related (*TLN1*, *CTDSP2*, *SRPRA)*, unfolded protein response (*NFYA*, *TLN1*, *CTDSP2*, *SRPRA*), cell adhesion (*TGFBI*, *PTPN1*, *PDPK1*), and cellular stress (*TFDP1*, *SRPRA*, *CAPZB*) pathways. Genes whose expression was extrapolated to the allergy status didn't contribute to IgE-related or other inflammatory pathways. Presented findings support the hypothesis of chronic mechanical corneal trauma in KTCN. Eye-rubbing causes CE damage and triggers cellular stress which through its influence on cell apoptosis, migration, and adhesion affects the KTCN phenotype.

Introduction

Keratoconus (KTCN) is a bilateral corneal disease characterized by progressive thinning and scarring of the cornea, iron deposition, and breaks in Bowman's layer [1]. The usage of state-of-the-art technologies extends this definition to include detected abnormalities in both posterior corneal elevation and corneal thickness distribution, as well as in the corneal epithelium thickness profile [2, 3]. Compound myopic astigmatism is the most prevalent refractive error in the manifest refraction in KTCN patients, followed by hyperopic compound, mixed and

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myopic astigmatism [4]. Visual function support is provided with eyeglasses, contact lenses, or intrastromal corneal ring segments (ICRS) [5]. Progression of KTCN can be modified–slowed down with corneal cross-linking procedure (CXL), which normalizes corneal parameters and improves visual acuity [6]. Penetrating keratoplasty, deep anterior lamellar keratoplasty, and Bowman layer transplantation are treatment options in the advanced stages of the disease [5].

KTCN affects all ethnic groups worldwide and the disease prevalence varies between the described populations. One of the lowest incidence rates (0.0068%) was reported in North Macedonia [7] and the highest (4.79%) in pediatric patients in Saudi Arabia [8]. Based on the performed meta-analysis, the frequency of 1.3/1000 in the general population was estimated [9].

The majority of patients are diagnosed with KTCN at an early age, usually in the second decade of life [10]. In the youngest patients, under 10 years of age, KTCN has been reported with frequent eye rubbing and eye allergies [11, 12].

According to the *Global consensus on keratoconus and ectatic diseases* [2], Down syndrome, connective tissue disorders (Marfan syndrome, Ehlers-Danlos syndrome), Leber congenital amaurosis, floppy eyelid syndrome, Asian and Arabian ethnicity, KTCN diagnosed in family members, ocular allergy, atopy, and the eye rubbing as an exclusive behavior factor, are KTCN risk factors [2]. In addition to the listed genetic syndromes, familial aggregation [13], discordance between twins [14], identified numerous KTCN *loci*, candidate genes and sequence variants [13], and higher KTCN frequency in offspring in communities with high consanguinity rates, confirm the genetic background in KTCN [9, 15]. Therefore, a genetic predisposition together with environmental factors could trigger a biochemical cascade influencing the disease onset [16].

As aspects of the influence of environmental factors on KTCN onset remain a controversial and contentious subject in KTCN research, the aim of this study was to characterize various environmental/behavioral and socioeconomic factors potentially influencing the KTCN phenotype, and especially the corneal surface. We then correlated the clinical/behavioral identified outcomes with the relevant data from analyses of transcriptomic features found in the same assessed patients to further characterize the revealed relationships.

Materials and methods

Ophthalmic examination and patients' inclusion and exclusion criteria

This single-center (Optegra Eye Health Care Clinic in Poznan, Poland), prospective, case-control study was conducted from October 2019 to May 2022. The study protocol was approved by the Institutional Review Board at Poznan University of Medical Sciences, Poznan, Poland (resolutions no. 200/22). Subject recruitment, sample collection, and other performed procedures/methods were conducted following the institutional guidelines and regulations, and in accordance with the Declaration of Helsinki. The written informed consent, for study participation and publication without identifying information, before subject recruitment from subjects and/or their legal guardian(s) was obtained. The individual whose photographs are presented in Fig 1 in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish the case details.

Each individual underwent a complete ophthalmological examination, including the assessments of both uncorrected (UDVA) best-corrected distance visual acuity (BDVA), intraocular pressure (IOP), corneal tomography with rotating Scheimpflug camera Pentacam (Oculus Optikgeraete GmbH, Wetzlar, Germany), epithelial thickness mapping MS-39 (CSO, Costruzione Strumenti Oftalmici, Florence, Italy), slit-lamp and dilated funduscopic examination. Additionally, towards screening of the dry eye syndrome symptoms the Ocular Surface Disease

Fig 1. The identification of the eye rubbing manner in the examined individuals. The photographs, numbered from 1 to 8, ranked ascending by their severity/potential harmfulness to the eyeball, included in the Questionnaire for the indication of the eye rubbing manner in the examined individuals. In the Questionnaire, the aspect of eye rubbing manner was verified using the multiple question method (Study Questionnaire, question no. 9: '*Please select the photo that most closely resembles the way you rub your eyes*').

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Index (OSDI) was evaluated and in suspected individuals, the Tear Breakup Time (TBUT) was checked.

The inclusion criteria for patients with KTCN were the diagnosis of KTCN, the following indices were analyzed: Belin-Ambrósio Enhanced Ectasia Display Total Deviation Value, Ambrósio relational thicknessvalues, pachymetric progression indices, the index of vertical asymmetry, anterior and posterior elevation [17], and corneal epithelial thickness profile [3].

The experienced ophthalmologists subspecialised in cornea and refractive surgery rated all corneal images.

The group of controls consisted of volunteers or regular clinic patients or KTCN family members, who agreed on a long detailed clinical examination followed by a detailed Questionnaire. The inclusion criteria for control individuals included no clinical evidence/symptoms of KTCN.

Exclusion criteria for both studied groups were co-occurring severe ophthalmic condition and genetic diseases/disorders. Additionally, the ongoing ocular surface inflammation was the exclusion criterion.

Collection of environmental/behavioral/socioeconomic data

A detailed Questionnaire, embracing questions on demographics, behavioral, environmental and socioeconomic aspects, was completed by each participant, in the presence of the researcher. The place of living up to the age of 15 (village or city up to 20,000 residents or city from 20,000 to 100,000 residents or city from 100,000 to 500,000 residents or city with over 500,000 residents); education level (primary or vocational education or high school or university); time of outdoor physical activity up to the age of 15; previous conjunctivitis, dry eye

syndrome, eye trauma, contact lenses use, ocular diseases, allergy (drug/food/pollens/dust/grass), asthma, atopic dermatitis, thyroid gland diseases, genetic diseases, smoking, past pregnancies were recorded.

Detailed questions regarding eye rubbing manner of study individuals embracing frequency of this behavior (occasionally or sometimes or often), dominant hand used, eye preference, preference of a part of hand used to rub, preference of a part of eye to rub, and details about the eye rubbing right after waking up were asked. Additionally, the 8 photographs showing different manners of eye rubbing and ranked ascending by their severity/potential harmfulness to the eyeball (ordinal scale) were presented to the examined person. The order of the photos was determined based on the expertise of ophthalmologists who performed the clinical examination and the researcher processing the surveys.

Also, aspects of professional occupation, presence of dust in working environment (defined as an exposition to filings of metals/building materials/construction materials or excessive dust), excessive UVA exposure, leisure activities (hobbies), reading habits, time spent in front of a screen (computer/tablet/smartphone/electronic reader/television set), hormones intake and sleeping habits (body position, head orientation and hand/arm position) were evaluated.

Statistical analyses of clinical parameters and environmental/ behavioral/ socioeconomic data

The JASP Software 5 [18] was used in statistical analyses. The normality of the continuous data was assessed by Kołomogorov-Smirnov and Shapiro-Wilk tests. The t-test was applied for continuous variables with a normal distribution. If the normality assumption was not satisfied, the Mann–Whitney test was applied. In the case of nominal data, the Chi-square test was executed.

The odds ratio on univariate logistic regression with 95% confidence intervals were calculated. The multivariate logistic regression was used to create the logistic model of KTCN risk factors, where odds ratios and 95% confidence intervals for variables were recalculated. The confusion matrix (performance diagnostic) of final model was computed, followed by performance metrics including accuracy, area under curve, sensitivity, specificity, and precision. The ROC curve was prepared.

For all performed statistical tests p-values ≤ 0.05 were considered as statistically significant.

Integrative analyses and biological interpretation

The integration of the obtained clinical and environmental/behavioral/socioeconomic data with the corresponding transcriptomic experimental data derived from the RNA-seq investigation of the cone (central) region of CE was performed. The transcriptomic assessment of the samples, embracing sample preparation, sequencing, and bioinformatic analyses were performed as previously [19]. Briefly, the CE samples were obtained during CXL [20] and photorefractive keratectomy (PRK) [21] procedures performed in KTCN patients and control individuals undergoing refractive error correction, respectively. The cone (central) region of each CE sample was assessed manually (based on the corneal tomography and epithelial thickness mapping), positioned in the intended orientation on the microscope slide with the use of light microscope and previously made stamps, and separated. Separated CE samples were transferred from the microscope slides to the lysis solution (Norgen Biotek, Thorold, ON, Canada) and total RNA, DNA, and proteins were extracted according to the instructions supplied with the RNA/DNA/Protein Purification Plus Micro Kit (Norgen Biotek). Total RNA libraries were prepared according to a previously established protocol [19], using TruSeq Stranded Total RNA Library Prep Gold (Illumina, San Diego, CA, USA) in accordance with

the manufacturer's protocol. A 100-bp paired-end sequencing run was performed on a NovaSeq 6000 platform (Illumina). The CE samples were sequenced with an average coverage of 100 million read pairs per sample. Bioinformatic analyses were executed according to a previously established protocol [19].

The relations between expression of particular genes (in TPM, transcript per million) and selected environmental/behavioral/socioeconomic data was executed using Pearson correlation or t-test. The set of dysregulated genes was analyzed using ShinyGO [22], and p-value ≤ 0.05 and/or FDR ≤ 0.05 were the cutoffs for pathway enrichment analyses. The Reactome database [23] was used as a pathway term source.

For visualization of the integrative analyses the ggplot2 package [24] in Rstudio (v. 4.1.3) [25] was used.

Results

Clinical characteristics of patients and controls

The 118 patients with KTCN (21 females and 97 males) and 73 control individuals (42 females and 31 males) were involved in this study. Among examined control individuals, no signs of KTCN have been disclosed. Clinical characteristics, including UDVA, BDVA, flat keratometric readings (K1), steep keratometric readings (K2), maximum simulated keratometry (Kmax), anterior and posterior elevation of the cornea, thinnest corneal thickness (TCT), thinnest epithelial thickness, axial length (AL), and IOP data of the examined study groups are compiled in Table 1. All presented clinical data differed significantly between the studied groups. No statistical differences in clinical parameters such as K1, K2, Kmax, anterior and posterior elevations, and TCT, have been found in comparison between male and female patients with KTCN, and between adult and adolescent patients with KTCN (S1 and S2 Tables).

The familial occurrence of KTCN concerned 11 patients with KTCN, including two pairs of siblings (brother-brother and sister-sister), two pairs of fathers and their sons, and one trio of an affected father with two daughters with KTCN.

Behavioral, environmental, and socioeconomic aspects in KTCN

The outcomes of analyzes of selected behavioral, environmental, and socioeconomic aspects evaluated in this study are presented in Tables 2 and 3, together with their statistical significance. All analysed data are compiled in S3 Table, while additional results of comparison of male and female patients with KTCN and adult and adolescent patients with KTCN in aspect of selected behavioral, environmental, and socioeconomic factors are presented in S4 and S5 Tables, respectively. Studied groups have differed in the aspects of age, sex, and place of living, with a predominance of younger males living in villages and minor cities in the KTCN group.

Substantial differences were found in Questionnaire answers concerning the manner of eye rubbing (Table 3). In the multiple questions, the KTCN group more often admitted to the eye rubbing and indicated the fist as part of the hand used for the eye rubbing. Moreover, the patients rubbed most of the eye surface, upper and/or lower eyelid in contrast to the inner and/or outer eye corner rubbed in controls. These behaviors were confirmed by the more frequent selection of the photographs no. 5–8 (as presented in Fig 1) from the remaining photographs included in question no. 9 in the Questionnaire. While we did not found differences in the manner of eye rubbing between adult and adolescent patients with KTCN, there was a significant difference considering sex of patients, that is male patients more frequently used knuckles, a base of hand, all fingers and/or a fist (as presented in photographs no. 5–8) (S4 and S5 Tables). Moreover, in the KTCN group the patients were more frequently left-handed.

Table 1. Clinical characteristics of the examined patients with KTCN and control individuals. The statistically significant differences, $p \leq 0.05$ or $p < 0.001$, are indicated.

	KTCN (n = 118)		Controls (n = 73)	
	x ± SD	Median	x ± SD	Median
UDVA OD	0.46 ± 0.37	0.30†	0.74 ± 0.46	0.85
BDVA OD	0.80 ± 0.35	0.90‡	1.12 ± 0.20	1.10
UDVA OS	0.44 ± 0.35	0.30‡	0.79 ± 0.46	0.95
BDVA OS	0.78 ± 0.34	0.90†	1.14 ± 0.14	1.10
K1 OD [D]	45.35 ± 5.14	43.95‡	42.93 ± 1.34	42.90
K2 OD [D]	48.07 ± 6.11	46.10‡	43.86 ± 1.47	43.90
Kmax OD [D]	53.93 ± 9.53	51.35‡	44.28 ± 1.53	44.30
anterior elevation OD [μm]	23.55 ± 18.42	18.00‡	2.81 ± 1.77	2.00
posterior elevation OD [μm]	50.78 ± 37.95	39.50‡	6.60 ± 4.83	6.00
TCT OD [μm]	465.34 ± 56.02	471.00‡	542.49 ± 35.51	544.00
thinnest epithelial thickness OD [μm]	43.16 ± 5.45	44.00‡	48.59 ± 3.81	48.00
K1 OS [D]	45.45 ± 4.71	43.75‡	43.01 ± 1.40	43.00
K2 OS [D]	48.32 ± 5.63	46.45‡	43.87 ± 1.59	44.10
Kmax OS [D]	54.54 ± 9.16	52.10‡	44.30 ± 1.58	44.30
anterior elevation OS [μm]	26.11 ± 18.40	22.50‡	2.82 ± 1.99	2.00
posterior elevation OS [μm]	52.72 ± 32.55	49.50‡	6.44 ± 4.15	5.50
TCT OS [μm]	470.64 ± 53.55	474.50‡	541.93 ± 35.21	542.00
thinnest epithelial thickness OS [μm]	43.52 ± 5.67	44.00‡	49.33 ± 4.08	49.00
AL OD [mm]	24.00 ± 0.89	23.95†	23.57 ± 0.98	23.40
AL OS [mm]	23.92 ± 0.88	23.85†	23.55 ± 0.92	23.40
IOP OD [mmHg]	13.17 ± 3.18	13.00‡	16.46 ± 2.62	16.00
IOP OS [mmHg]	13.13 ± 3.30	13.00‡	16.54 ± 2.39	16.00
KTCN in a family member	n = 18 (15.25%)†		n = 4 (5.48%)	
Any eye disease in a family member	n = 60 (50.85%)		n = 29 (39.73%)	

Abbreviations and symbols in the Table: x–average, SD–standard deviation, OD–oculus dexter, OS—oculus sinister, UDVA–uncorrected distance visual acuity, BDVA–best-corrected distance visual acuity, K1 –flat keratometric readings, K2 –steep keratometric readings, Kmax–maximum simulated keratometry, TCT–thinnest corneal thickness, AL—Axial length, IOP–intraocular pressure

† indicates statistically significant differences between KTCN patients and controls with p-value ≤0.05

‡ indicates statistically significant differences between KTCN patients and controls with p-value <0.001

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There were no differences in the frequency of reported conjunctivitis, dry eye syndrome, eye trauma, asthma, atopic dermatitis, and allergy to neither food nor pollens/grass/dust, between the studied groups (S3 Table).

Regarding the working environment, despite the lack of difference in the type of professional occupation between studied groups, we found that the presence of dust in the working environment was more frequently indicated by patients with KTCN (Table 2), and especially male patients with KTCN (S4 Table). Moreover, by assessing responses to questions regarding leisure activities, we found that KTCN patients spend more time using a computer after work and a smartphone in the dark (Table 2). There was no difference in the declared hobbies (S3 Table).

Finally, considering the sleeping position including head orientation and hand/arm position, no differences were found comparing the studied groups (S3 Table).

Additional statistics regarding presence of dust in the working environment and eye rubbing, most frequently rubbed eye and right / left-handedness, most frequently rubbed eye and more affected eye, eye rubbing and absolute differences in TCT between left and right eye, eye

Table 2. Qualitative and quantitative data/results of analyzes of selected behavioral, environmental, and socioeconomic aspects.

Variables		KTCN (n = 118)	Control (n = 73)	p-value
Age (years), mean±SD		27.3 ± 8.7	31.5 ± 10.7	0.004
Sex				< 0.001
	Female	21 (17.78%)	42 (57.53%)	
	Male	97 (82.20%)	31 (42.47%)	
Level of education				0.002
	Primary	14 (11.87%)	2 (2.74%)	
	Vocational education	11 (9.32%)	1 (1.37%)	
	High school	51 (43.22%)	27 (36. 99%)	
	University	42 (35.59%)	43 (58.90%)	
Place of living up to the age of 15				0.004
	Village	43 (36.44%)	32 (44.44%)	
	City up to 20000 residents	19 (16.10%)	17 (23.61%)	
	City from 20000 to 100000 residents	7 (5.93%)	11 (11.11%)	
	City from 100000 to 500000 residents	30 (25.43%)	3 (4.17%)	
	City with over 500000 residents	19 (16.10%)	12 (16.67%)	
Allergy				0.128
	Yes	45 (38.14%)	20 (27.40%)	
	No	73 (61.86%)	53 (72.60%)	
Food Allergy				0.223
	Yes	8 (6.78%)	2 (2.74%)	
	No	110 (93.22%)	71 (97.26%)	
Pollen/grass/dust Allergy				0.114
	Yes	42 (35.59%)	18 (24.66%)	
	No	76 (64.41%)	55 (75.34%)	
Professional occupation				0.128
	Student	27 (23.68%)	9 (12.50%)	
	Non-office worker	55 (48.25%)	36 (50.00%)	
	Office worker	32 (28.07%)	27 (37.50%)	
Dust in the working environment				0.016
	Yes	34 (28.81%)	10 (13.70%)	
	No	84 (71.19%)	63 (86.30%)	
Using a computer at work (hours per day)		5.7±2.8	6.1±2.3	0.617
Using a computer after work (hours per day)		2.4±1.2	1.6±0.9	< 0.001

Note: the respondents did not always answer all the questions, therefore the summation of the answers in individual questions in the table may be incomplete.

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rubbing and allergy status, and eye rubbing and time spent using a computer are presented in S6 Table.

Risk factors for KTCN in univariate analyses

The results of risk analysis of behavioral, environmental, and socioeconomic factors in KTCN, presented as the odds ratio, 95% confidence interval (CI), and p-value are compiled in Table 4. Statistical significance was found for 12 analyzed variables. The male sex (odds ratio 6.26), living in the median size city (100,000–500,000 residents) (OR 6.32), eye rubbing (OR 5.57), frequent rubbing of the left eye (OR 8.91), left handedness (OR 6.39), eye rubbing with fist (OR 4.75), rubbing of both eyes simultaneously with all fingers (photography no. 6; OR 5.73) or fists (photography no. 8; OR 5.52), rubbing of the most of the eye surface with the use of

Table 3. The data/results of analyzes of the eye rubbing behavior.

Variables		KTCN (n = 118)	Control (n = 73)	p-value
Eye rubbing				< 0.001
	Yes	109 (92.37%)	50 (68.49%)	
	No	9 (7.62%)	23 (31.51%)	
Frequent eye rubbing				0.011
	Yes	10 (8.48%)	0 (0.00%)	
	No	108 (91.52%)	73 (100.00%)	
Dominant hand				0.006
	Right	100 (84.75%)	71 (97.26%)	
	Left	18 (15.25%)	2 (2.74%)	
More frequently rubbed eye				0.020
	Both	79 (72.48%)	44 (1.67%)	
	Right	14 (12.84%)	3 (6.25%)	
	Left	16 (14.68%)	1 (2.08%)	
Part of the hand used for rubbing				0.052
	Fingertips	37 (41.11%)	23 (48.94%)	
	Base of hand	2 (2.22%)	3 (6.38%)	
	Knuckles	29 (32.22%)	18 (38.30%)	
	Fists	22 (24.45%)	3 (6.38%)	
Eye rubbing with a fist				0.009
	Yes	22 (24.44%)	3 (6.38%)	
	No	68 (75.56%)	44 (93.62%)	
The upper eyelid as the most frequently rubbed part				0.033
	Yes	45 (38.14%)	17 (23.29%)	
	No	73 (61.86%)	56 (76.71%)	
The lower eyelid as the most frequently rubbed part				0.004
	Yes	40 (33.90%)	11 (15.07%)	
	No	78 (66.10%)	62 (84.93%)	
Type of eye rubbing indicated in response to presented photographs				0.075
	Photography no. 1	11 (11.96%)	14 (28.00%)	
	Photography no. 2	14 (15.22%)	11 (22.00%)	
	Photography no. 3	9 (9.78%)	4 (8.00%)	
	Photography no. 4	7 (7.61%)	7 (14.00%)	
	Photography no. 5	10 (10.87%)	4 (8.00%)	
	Photography no. 6	9 (9.78%)	2 (4.00%)	
	Photography no. 7	6 (6.52%)	2 (4.00%)	
	Photography no. 8	26 (28.26%)	6 (12.00%)	
Photographs no. 1–4 or 5–8				0.002
	Photography 1 or 2 or 3 or 4	41 (44.56%)	36 (72.00%)	
	Photography 5 or 6 or 7 or 8	51 (55.44%)	14 (28.00%)	
Rubbing the eyes immediately after waking up				0.191
	Yes	50 (42.37%)	24 (32.88%)	
	No	68 (57.63%)	49 (67.12%)	

Note: the respondents did not always answer all the questions, therefore the summation of the answers in individual questions in the table may be incomplete.

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Table 4. Selected results of the risk analysis of behavioral, environmental and socioeconomic factors in KTCN. For each variable, the odds ratio, 95% confidence interval (CI), and p-value are presented.

Variable	Odds Ratio	95% CI	p-value
Sex (*male*)	**6.26**	3.23–12.13	**<0.001**
Level of education			
University (*vs* high school)	**0.52**	0.28–0.97	**0.041**
Place of living up to the age of 15			
City with 100,000–500,000 residents (*vs* city with more than 500,000 residents)	**6.32**	1.57–25.34	**0.009**
Allergy (*yes*)	1.63	0.87–3.08	0.130
Food allergy (*yes*)	2.58	0.53–12.51	0.239
Pollen/grass/dust allergy (*yes*)	1.69	0.88–3.24	0.115
Asthma (*yes*)	1.60	0.48–5.29	0.444
Smoking (*yes*)	0.86	0.421–1.77	0.686
Eye rubbing (*yes*)	**5.57**	2.41–12.91	**<0.001**
Dominant hand (*left*)	**6.39**	1.44–28.41	**0.015**
More frequently rubbed eye			
Left (*vs both*)	**8.91**	1.14–69.47	**0.037**
Eye rubbing with fists (*yes*)	**4.75**	1.34–16.80	**0.016**
Rubbing of the upper eyelid (*yes*)	1.39	0.70–2.79	0.349
Rubbing of the lower eyelid (*yes*)	2.08	0.96–4.50	0.064
Type of eye rubbing indicated in response to presented photographs			
Photography no.6 (*vs* No.1)	**5.73**	1.02–32.10	**0.047**
Photography no.8 (*vs* No.1)	**5.52**	1.68–18.10	**0.005**
Photographs no. 1–4 or 5–8 (*vs* No. 5–8)	**3.20**	1.52–6.72	**0.002**
Working environment with dust (*yes*)	**2.55**	1.17–5.55	**0.018**
Time of using a computer at work (*hours per day*)	0.95	0.82–1.09	0.438
Time of using a computer after work (*hours per day*)	**1.91**	1.31–2.77	**<0.001**
Using a smartphone (*yes*)	2.21	0.79–6.22	0.133
Time of using a smartphone during the day (*hours per day*)	1.20	0.93–1.54	0.154
Using a smartphone during the night (*yes*)	0.88	0.47–1.62	0.671
Time of using a smartphone during the night (*hours per day*)	1.51	0.85–2.68	0.157

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greater force (rubbing with knuckles/base of hand /all fingers /fists, photographs no. 5–8; OR 3.20), presence of dust in the working environment (OR 2.55), and longer time of using a computer after work (OR 1.91) were factors recognized as significantly increasing the KTCN risk. Higher education (university; OR 0.52) was the only variable reducing the risk of KTCN.

In addition, in Table 4 the non-significant but previously discussed factors (such as allergy or asthma) are presented.

Multivariate analysis and the resulting KTCN risk factor model

Male sex, eye rubbing, time of using a computer after work, and dust in the working environment, were the substantial KTCN risk factors identified in multivariate analysis, with OR

Table 5. Substantial KTCN risk factors, odds ratios, the confusion matrix, and performance metrics in a multivariate analysis.

A. Odds ratios			
Variable	**Odds Ratio**	**95% CI**	**p-value**
Sex (*male*)	8.66	3.16–23.79	<0.001
Eye rubbing (*yes*)	7.36	2.02–26.89	0.003
Time of using a computer after work (*hours per day*)	2.35	1.42–3.88	<0.001
Dust in the working environment (*yes*)	5.25	1.23–22.33	0.025
B. Confusion matrix			
	Predicted		
Observed	**Control**	**KTCN**	**% Correct**
Control	39	14	73.585
KTCN	11	67	85.897
Overall % Correct			80.916
C. Performance metrics			
	Value		
Accuracy	0.809		
AUC	0.882		
Sensitivity	0.859		
Specificity	0.736		
Precision	0.827		

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values of 8.66, 7.36, 2.35, and 5.25, respectively. The parameters of the recognized model, including the confusion matrix and performance metrics (accuracy, Area Under the Curve (AUC), sensitivity, specificity, and precision) are presented in Table 5A–5C, whereas the Receiver Operating Characteristic (ROC) curve for these variables in the logistic model, is shown in Fig 2.

Effect of eye rubbing on corneal surface transcriptome and biological interpretation

The results of the integration of transcriptomic data of the cone (central) region of the corneal epithelium (CE) of 23 KTCN patients and five controls and chosen environmental and behavioral factors are presented in Figs 3–5 and Table 6.

The pathway enrichment analysis for genes whose expression in the cone region of CE was found to be correlated with the eye rubbing manner (recognized by selecting one of the photographs presented in Fig 1 by study individuals) showed the enrichment in apoptosis, chaperon-related, and unfolded protein response pathways (Fig 3). Particularly, the expression of *TGFBI* (R = 0.79, p = 0.0039), *BCL2L1* (R = 0.65, p = 0.31), *CAPZB* (R = 0.73, p = 0.011), *CTDSP2* (R = 0.74, p = 0.0086), *PTPN1* (R = 0.77, p = 0.0057), *PDPK1* (R = 0.8, p = 0.0029), *RAPGEF3* (R = 0.79, p = 0.0041), *TFDP1* (R = 0.68, p = 0.022), *SRPRA* (R = 0.72, p = 0.013), *TLN1* (R = 0.62, p = 0.042), and *TP53* (R = 0.78, p = 0.0042) genes, was correlated with severity/potential harmfulness of the eye rubbing to the eyeball (Fig 4).

The expression of *TRIM8* (R = 0.73, p = 0.0006), *JUNB* (R = 0.64, p = 0.0043), and *LTBP3* (R = 0.66, p = 0.0028) genes were found to be correlated with time spent using a computer after work, as presented in Fig 5.

Without taking into account the environmental/behavioral aspects in the correlation analysis, the expression of all the genes mentioned above did not differentiate the studied groups. (S1 Fig).

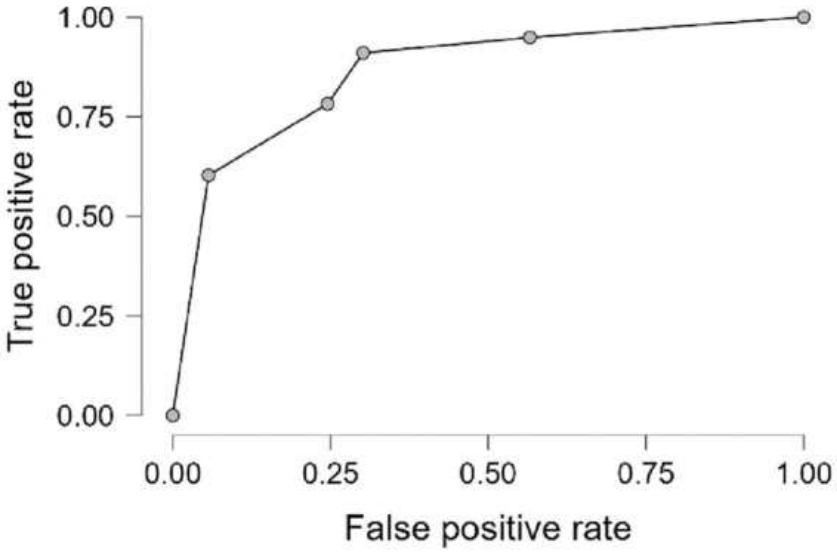

Fig 2. ROC curve of multivariate model of KTCN risk factors. ROC curve for the multivariate logistic regression analysis illustrating the performance of KTCN risk factors model. Model statistic values are presented in Table 5.

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Influence of allergy status on transcriptomic features and biological interpretation

Evaluating the allergic / non-allergic factors contributing to the phenotype we found that the genes whose expression was extrapolated to the allergy status didn't contribute to IgE-related pathways, such as 'allergen dependent IgE bound FCERI aggregation'. No allergy-related inflammation of any kind has occurred (e.g., 'interleukin-4 and interleukin-13 signaling'), as presented in S2 Fig, and S7 Table.

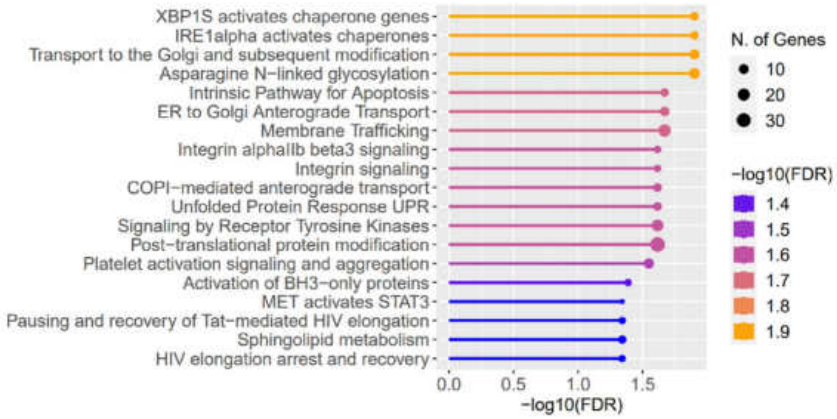

Fig 3. Results of pathway enrichment analysis. Results of pathway enrichment analysis for genes which expression in corneal epithelium was found to be correlated with the eye rubbing manner. The type of eye rubbing was indicated in response to presented photographs, included in Fig 1. Transcriptome data was obtained for males and females from both study groups. The Reactome database was chosen as the source of the pathways.

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Table 6. The results of the pathway enrichment analysis. The results of the pathway enrichment analysis for genes whose expression in the cone region of corneal epithelium was found to be correlated with the eye rubbing manner.

Pathway	Fold Enrichment	Enrichment FDR	nGenes	Pathway Genes	Genes
Asparagine N-linked glycosylation	3.5712	0.0126	14	305	*CTSA, CAPZB, ARFGAP1, NAPA, ST3GAL4, GORASP1, TBC1D20, TMEM115, PRKCSH, B4GALT3, DCTN5, LMAN2, MAN1B1, DYNLL2*
Transport to the Golgi and subsequent modification	4.6261	0.0126	11	185	*CAPZB, ARFGAP1, NAPA, ST3GAL4, GORASP1, TBC1D20, TMEM115, B4GALT3, DCTN5, LMAN2, DYNLL2*
XBP1S activates chaperone genes	9.7253	0.0126	6	48	*ARFGAP1, WFS1, KLHDC3, TLN1, CTDSP2, SRPRA*
IRE1alpha activates chaperones	8.8078	0.0126	6	53	*ARFGAP1, WFS1, KLHDC3, TLN1, CTDSP2, SRPRA*
Membrane Trafficking	2.4349	0.0214	21	671	*DVL2, AP2B1, CAPZB, PACSIN2, AP1B1, ARFGAP1, STX4, STX10, ANKRD27, NAPA, GORASP1, TBC1D20, TMEM115, KIF1C, VPS4A, DCTN5, YWHAB, LMAN2, ALS2CL, AP1G2, DYNLL2*
Intrinsic Pathway for Apoptosis	7.6527	0.0214	6	61	*TP53, YWHAB, STAT3, BCL2L1, TFDP1, DYNLL2*
ER to Golgi Anterograde Transport	4.5469	0.0214	9	154	*CAPZB, ARFGAP1, NAPA, GORASP1, TBC1D20, TMEM115, DCTN5, LMAN2, DYNLL2*
Post-translational protein modification	1.8475	0.0242	36	1516	*SCMH1, CTSA, OTUD5, CAPZB, PIGS, JOSD1, ARFGAP1, AXIN1, NAPA, TNKS2, DPH1, WFS1, ST3GAL4, GORASP1, TBC1D20, TMEM115, PRKCSH, DDA1, UBE2M, TRIM28, CKAP4, TP53, USP21, PEX10, B4GALT3, UBE2Z, DCTN5, LMAN2, MAN1B1, BTBD6, POMK, WDR5, H4-16, GPAA1, RAET1G, DYNLL2*
Unfolded Protein Response UPR	5.3394	0.0242	7	102	*NFYA, ARFGAP1, WFS1, KLHDC3, TLN1, CTDSP2, SRPRA*
COPI-mediated anterograde transport	5.3922	0.0242	7	101	*CAPZB, ARFGAP1, NAPA, GORASP1, TMEM115, DCTN5, DYNLL2*
Signaling by Receptor Tyrosine Kinases	2.5233	0.0242	18	555	*AP2B1, ATP6AP1, COL5A3, PXN, POLR2C, MET, PPP2R5D, YAP1, DUSP6, PDPK1, GRB7, POLR2D, MAPKAPK2, YWHAB, STAT3, NELFB, PTPN1, COL5A2*
Integrin alphaIIb beta3 signaling	11.5262	0.0242	4	27	*RAPGEF3, TLN1, PDPK1, PTPN1*
Integrin signaling	11.5262	0.0242	4	27	*RAPGEF3, TLN1, PDPK1, PTPN1*
Platelet activation signaling and aggregation	3.1648	0.0283	12	295	*STXBP2, GNB1, RAPGEF3, GNA11, STX4, GNAI2, TLN1, PDPK1, CHID1, PTPN1, PSAP, ACTN4*
Activation of BH3-only proteins	9.7253	0.0409	4	32	*TP53, YWHAB, TFDP1, DYNLL2*
Pausing and recovery of Tat-mediated HIV elongation	9.1532	0.0455	4	34	*POLR2C, CDK9, POLR2D, NELFB*
Tat-mediated HIV elongation arrest and recovery	9.1532	0.0455	4	34	*POLR2C, CDK9, POLR2D, NELFB*
Sphingolipid metabolism	5.2451	0.0455	6	89	*CTSA, KDSR, CSNK1G2, SMPD1, CERS6, PSAP*
MET activates STAT3	38.9010	0.0455	2	4	*MET, STAT3*
HIV elongation arrest and recovery	8.6447	0.0456	4	36	*POLR2C, CDK9, POLR2D, NELFB*
Pausing and recovery of HIV elongation	8.6447	0.0456	4	36	*POLR2C, CDK9, POLR2D, NELFB*
MHC class II antigen presentation	4.3569	0.0456	7	125	*AP2B1, CTSA, CAPZB, AP1B1, ACTR1B, DCTN5, DYNLL2*

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Discussion

The genetic predisposition to KTCN, comprising the presence of sequence variation in genes related to KTCN [26, 27] and KTCN-specific transcriptomic alterations [19, 28] along with environmental [9, 29], behavioral [9, 30, 31], and other unknown factors, most likely cause the onset of KTCN.

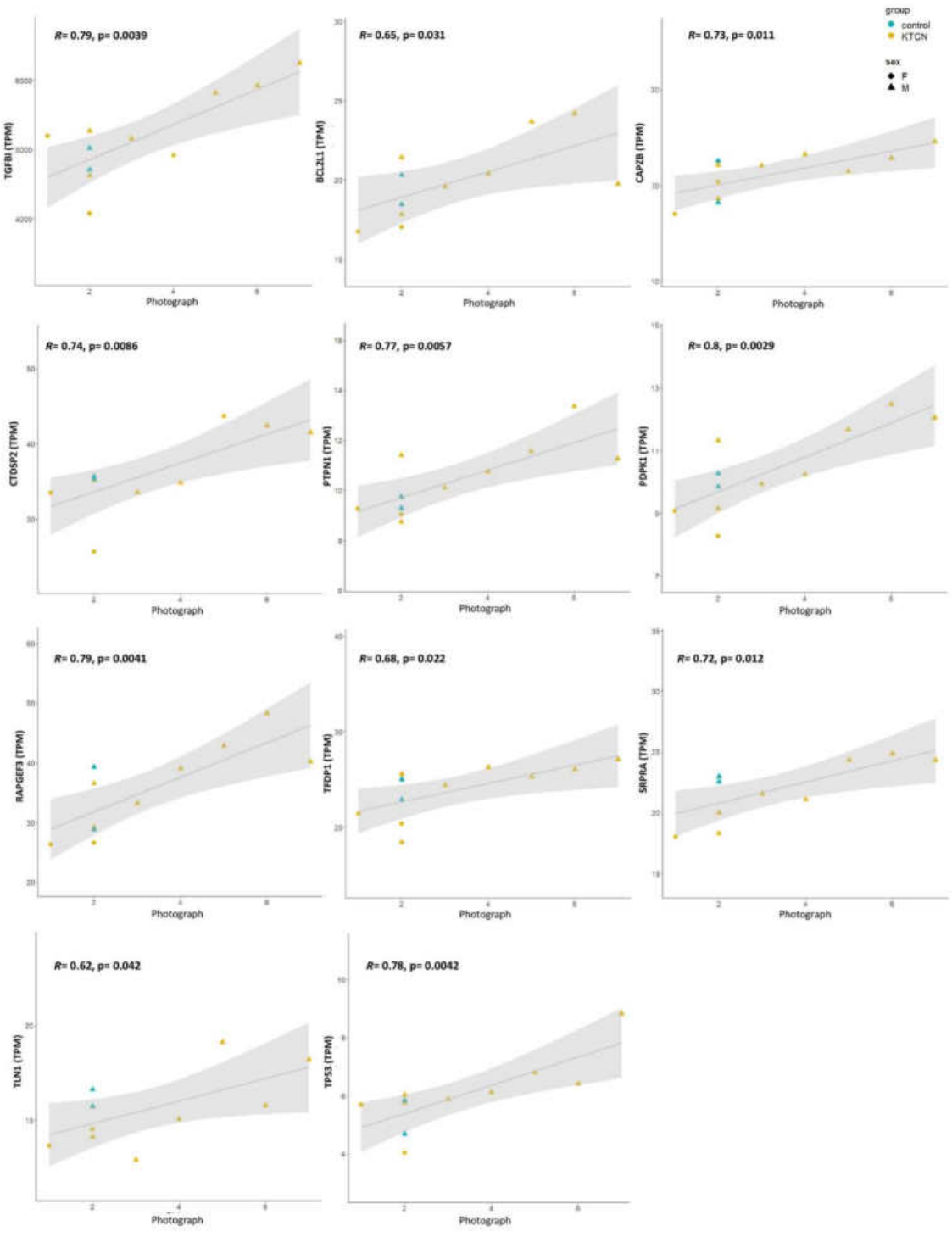
group
control
KTCN
sex
F
M
R= 0.79, p= 0.0039
TGFBI (TPM)
R= 0.65, p= 0.031
BCL2L1 (TPM)
R= 0.73, p= 0.011
CAPZB (TPM)
R= 0.74, p= 0.0086
CTDSP2 (TPM)
R= 0.77, p= 0.0057
PTPN1 (TPM)
R= 0.8, p= 0.0029
PDPK1 (TPM)
R= 0.79, p= 0.0041
RAPGEF3 (TPM)
R= 0.68, p= 0.022
TFDP1 (TPM)
R= 0.72, p= 0.012
SRPRA (TPM)
R= 0.62, p= 0.042
TLN1 (TPM)
R= 0.78, p= 0.0042
TP53 (TPM)
Photograph

Fig 4. Results of Pearson's correlation. Pearson's correlation results between the expression of selected genes, *TGFBI*, *BCL2L1*, *CAPZB*, *CTDSP2*, *PTPN1*, *PDPK1*, *RAPGEF3*, *TFDP1*, *SRPRA*, *TLN1*, and *TP53* (in TPM), in the cone (central) region of corneal epithelium and severity/potential harmfulness of the eye rubbing to the eyeball recognized by selecting one of the photographs presented in Fig 1 by study individuals. Values of regression and statistical significance are presented in plots.

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Previously the strong relation between eye rubbing and KTCN was reported in the case reports [32], case series [33], multivariate analyses [30], and meta-analysis [9]. Here we verified this aspect and further characterized the eye rubbing behavior practiced by patients with KTCN. Patients used their fists more frequently to rub the eye, and they also rubbed most of the eye surface, i.e., the upper and/or lower eyelid, as opposed to the inner and/or outer corner of the eye. Moreover, this disclosed manner involved the use of greater force when patients with KTCN rubbed their eyes. Previously, the mechanical forces exerted on the eyelids by KTCN patients by rubbing the eyes were reported to be different, depending on which part of the hand was used [34].

Comparing the corneal thickness and other clinical parameters between less and more frequently rubbed eyes, we didn't find the substantial differences. Although we've revealed relation between eye rubbing and absolute differences in TCT (TCT diff) between right and left eye, the specific manner of eye rubbing (e.g., eye rubbing with fist) or its severity did not show correlation with this variable. Since central corneal thickness is one of the most heritable human characteristics [35], we concluded that the effect of eye rubbing on the KTCN phenotype was rather due to the generation of cellular stress in the CE.

Previously, *in vitro* study showed the alteration of cytoskeleton and migratory behavior of corneal epithelial cells exposed to shear stress [36]. In line with these findings, here the increased expression of *TFDP1*, *SRPRA* and *CAPZB*, being elements of cellular responses to stress, and the increased expression of *TLN1*, *RABGEF3*, *PDPK1*, and *PTPN1*, being elements of extracellular matrix (ECM) affecting cell adhesion and migration were revealed, as the *in vivo* evidence of influence of severe eye rubbing on CE. Importantly, these results were consistent for the patients of both sexes, while previously reported transcriptomic data [37] and epidemiological [38] observations were sex-specific.

Moreover, we revealed the important eye rubbing-related changes in expression of genes contributing to apoptosis (*BCL2L1*, *TP53*, *BAX*), chaperone activation (*TLN1*, *CTDSP2*, *SRPRA*), and unfolded protein response (*NFYA*, *TLN1*, *CTDSP2*, *SRPRA*). Previously, among the listed genes, high levels of protein p53 were found in unaffected CE of various vertebrate

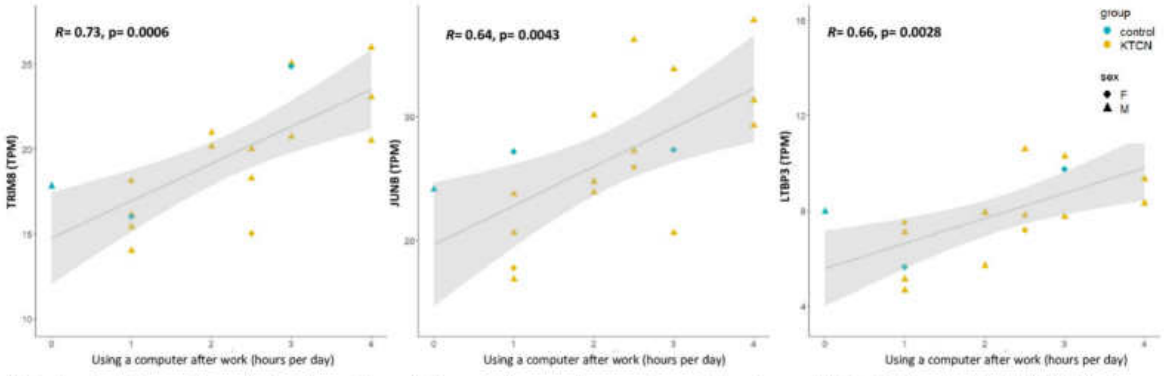

Fig 5. Results of Pearson's correlation. Pearson's correlation results between the expression of selected genes, *TRIM8*, *JUNB*, and *LTBP3* (in TPM), in the cone (central) region of corneal epithelium and time spent using a computer after work. Values of regression and statistical significance are presented in plots.

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species [39], and more importantly in the interpretation of our outcomes, the experimental study showed the increased cytoplasmic expression of p53 after the cell stress stimulation resulting from ultraviolet irradiation [40].

Notably, in a relation to severity of eye rubbing a dysregulated expression of elements of the TGFB signaling pathway was identified, previously reported as the KTCN-specific feature [19] and discussed as influencing the activation of corneal cells to produce various types of collagen and other ECM components [41]. Upregulation of *TGFBI* revealed here was found to be correlated with more severe eye rubbing, whereas increased expression of *JUNB* and *LTBP3* were strongly related to the hours spent in front of the computer after work. The longer time of using a computer after work was revealed as an independent risk factor for KTCN. However, patients with KTCN were younger and definitely more addictive to electronics and that should be considered in the data interpretation.

Moreover, the presence of dust in the working environment increased the risk of both the eye rubbing and KTCN itself. Interestingly, this factor may have similar influence on corneal surface as the living in median size cities, in which exposure to air pollution, particularly to particles with size less than 2.5 μm, was reported to be higher as an effect of urbanization degree and household heating systems [42, 43].

Here, the allergy has not been disclosed as a risk factor for KTCN. Regarding the aspect of allergy and atopy in patients with KTCN, it has been previously discussed as more prevalent in KTCN than in general population [30, 44, 45]. However, in some reports using multivariate analyses and meta-analyses, the atopy and allergic eye diseases were not significantly associated with KTCN [46, 47] and in many developing countries the constant increase in occurrence of allergic and atopic diseases has been reported [48, 49]. For example, the incidence of at least one allergic condition changed in years 2001–2005 from 18.9% to 24.2% in patients registered in England database (QRESEARCH 2,958,366 patients) [50]. In the analysis of transcriptome in the full-thickness corneas no significance for the allergy status was found, no differentially expressed genes comparing the KTCN patients with allergy vs. without allergy were indicated [51]. Moreover, no significant differences in the ocular surface immune cell subset proportions and tear fluid soluble factor levels were observed between KTCN eyes of different grades [52]. Also in that study, the influence of ocular allergy, history of systemic allergy and eye rubbing on the immune cell profile was investigated, and no differences were found [52]. In contrast, the significantly higher tear fluid levels of IL-1α, IL-9, IL-10, IL-13, TNFα, sVCAM, sTNFRII and IgE were observed in KTCN patients with history of systemic allergy and significantly higher levels of cytokines (IL-2, IL-12/23p40, IL-12p70, IL-13, IL-17A, IFNα), chemokines (MIG/CXCL9; ITAC/CXCL11), and growth factors (TGFβ1, EPO, VEGF) were revealed in KTCN patients with a history of eye rubbing [52]. In our KTCN patients the allergy was found to be not related to the severity of eye rubbing. Importantly, allergy status has not affected CE transcriptomic outcomes obtained for the same evaluated patients. Also, other inflammatory conditions, reported in the past in medical history, conjunctivitis, both bacterial and viral and autoimmune diseases, were not found to be more frequently observed in patients with KTCN. We conclude here that the eye rubbing behavior is not a consequence of the allergic condition.

We observed that patients with KTCN were more frequently left-handed comparing to control individuals being right-handed in general. Although we did not find a relation between the most frequently rubbed eye and right/left-handedness, the relation between the most frequently rubbed eye and the more affected eye, in particular between the left eye and the preference of left-eye rubbing, was revealed.

It has been reported that patients with KTCN more frequently than controls manifested dry eye syndrome [53]. Here the relation of KTCN with the dry eye syndrome, verified by TBUT and/or OSDI (data not shown), was not confirmed.

As outdoor activity is a verified factor influencing other multifactorial eye diseases, the myopia and high myopia phenotypes [54], and it has not been investigated in KTCN research, we also assessed this aspect in patients with KTCN as a risk or protective environmental factor and found it as not differentiating between the groups of patients and controls which we evaluated.

Also, patients with KTCN less frequently read paper books and used the electronic readers, probably due to the joint effect of a generational change as well as a problem with visual acuity that cannot be adjusted through glasses or contact lenses.

In this paper, we presented the level of higher education as the only variable reducing the risk of KTCN (OR = 0.52, univariate analysis), but this result should be treated with caution as it may be a consequence of selection bias.

Other, previously discussed factors as sleeping position and creating additional pressure on the eyeball [30] or smoking [31] weren't confirmed in our study. Moreover, the effect of pregnancy on KTCN progression could not be verified due to a small number of female patients in KTCN group. In this study more men than women were ascertained in KTCN group, which is in line with the predominance of male patients among 110 individuals undergoing the CXL procedure in 2020–2022 in Optegra Clinic (19 female and 91 male patients, unpublished data, Maleszka-Kurpiel, MD, PhD, personal communication) and with the previous studies [55, 56].

As this is a single center patient recruitment study, we interpreted our results with caution. Questions asked, based on the applied Questionnaire, were somehow biased, for example in recalling of habits/behaviors and quantifying their degree. Therefore, in the case of the behavioral aspects, we used the multiple question method to minimize such biases. In addition, the study groups were not matched in terms of age and sex, as the aim of the study was also to analyze the impact of these characteristics. We did not identify clinical features corelated with sex or age, however, the sex and age could potentially influence study results regarding the life habits. The absence of differences in clinical parameters between adult and adolescent patients indicates a rapid progression in the latter [57–59], pointing to the importance of increasing patient awareness [60] as well as early diagnosis due to substantial risk of progression. Generally, we focused on the regular patients, who best represent the overall patient population of the clinic. Hence in our control group, the KTCN family members occur, but differences in their life habits in comparison to KTCN-affected family members could be a valuable practical and clinical information. Importantly, the study groups do not differ in the aspect of contact lens wear, which could have influence the issue of frequency of the eye rubbing. Moreover, the absence of differences in clinical parameters between adult and adolescent patients, and male and female patients, in contrast to previous reports [38, 61], enabled a superior appreciation of both, behavioral and molecular findings. Regarding other limitations, obtaining the CE samples from the control individuals towards transcriptomic assessment was especially challenging, as this type of material could be collected during PRK procedure only, which nowadays is rarely performed and replaced with the newer techniques of vision correction, in which the CE is not removed.

So far, the impact of shear stress on function/mechanics of epithelial cells in single-layer model [36] and changes in mechanical forces exerted on the eye depending on eye-rubbing manner [34] have been reported. However, currently the effects of repetitive eye rubbing on corneal biomechanics have not been confirmed in a more complex model such as the ex-vivo porcine eyes [62]. Therefore, the influence of the eye rubbing, confirmed in the corneal epithelium cultured cells under flow conditions [36] should be further investigated in the studies on the biological mechanisms of this behavior on the physiology of the cornea, involving the 3D experimental models.

Overall, environmental / behavioral factors were shown to have a significant influence on the corresponding KTCN CE transcriptomic findings in the patients with KTCN. More importantly, the evaluated transcriptomic differences found between KTCN and control samples were only revealed after taking environmental / behavioral factors into account.

In conclusion, the male sex, eye rubbing, time of using a computer after work, and the dust in the working environment were found or verified as the most significant risk factors for KTCN and the effect of selected environmental/behavioral features on ocular surface in KTCN was revealed. Presented transcriptomic findings, integrated with environmental/ behavioral aspects, support the hypothesis of chronic mechanical corneal trauma affecting the pathogenesis of KTCN. This trauma in the form of eye rubbing-caused damage of the CE, leaving the allergy as a redundant condition, contributes to the cellular stress, which through influence on cell apoptosis, migration, and adhesion affects the KTCN phenotype.

Supporting information

S1 Fig. The boxplots of selected genes' expression. The boxplots presenting the expression of selected genes, *TGFBI*, *BCL2L1*, *CAPZB*, *CTDSP2*, *PTPN1*, *PDPK1*, *RAPGEF3*, *TFDP1*, *SRPRA*, *TLN1*, *TP53*, *TRIM8*, *JUNB*, and *LTBP3* (in TPM, Transcripts per Million), in the cone (central) region of corneal epithelium in the studied groups of patients with KTCN and controls.
(TIF)

S2 Fig. Results of pathway enrichment analysis. Results of pathway enrichment analysis for genes whose expression in corneal epithelium was found to be correlated with the allergy status. Transcriptome data was obtained for males and females from both study groups. The Reactome database was chosen as the source of the pathways.
(TIF)

S1 Table. Results of clinical comparison of male and female patients with KTCN.
(DOCX)

S2 Table. Results of clinical comparison of adult and adolescent patients with KTCN.
(DOCX)

S3 Table. Complete information on the qualitative and quantitative data/results of analyzes of behavioral, environmental, and socioeconomic aspects.
(DOCX)

S4 Table. Results of comparison of male and female patients with KTCN in aspect of selected behavioral, environmental, and socioeconomic factors.
(DOCX)

S5 Table. Results of comparison of adult and adolescent patients with KTCN in aspect of selected behavioral, environmental, and socioeconomic factors.
(DOCX)

S6 Table. Additional statistical analyses.
(DOCX)

S7 Table. Results of pathway enrichment analysis for genes whose expression in corneal epithelium was found to be related to the allergy status.
(DOCX)

S8 Table. STROBE statement—checklist.
(DOC)

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Popularyzacja wyników badań nad stożkiem rogówki

Wyniki prac badawczych dotyczących czynników środowiskowych, a w szczególności charakterystyki sposobów pocierania oczu przez pacjentów ze stożkiem rogówki (**Artykuł III: Non-allergic eye rubbing is a major behavioral risk factor for keratoconus.** Jaskiewicz K, Maleszka-Kurpiel M, Michalski A, Ploski R, Rydzanicz M, Gajecka M. PloS One. 2023 Apr 13;18(4):e0284454. doi: 10.1371/journal.pone.0284454), zyskały aprobatę amerykańskiej Narodowej Fundacji Stożka Rogówki w USA (ang. *The National Keratoconus Foundation,* NFKC, https://nkcf.org/) oraz zostały przedrukowane w NKCF May 2023 Update newsletter: *A Deeper Dive into Eye Rubbing* (https://nkcf.org/may-2023-update-newsletter/, w załączeniu).

MAY 2023 UPDATE

A Deeper Dive into Eye Rubbing

Doctors who diagnose and treat keratoconus spend part of each visit reminding patients that eye rubbing is to be avoided. Doctors have reported that some of their patients with keratoconus describe eye rubbing as a calming, almost enjoyable, experience. Others report some patients are not even aware they rub their eyes.

Doctors remind their patients the friction and the micro-trauma that accompanies eye rubbing can further weaken a cornea that is genetically predisposed to thinning.

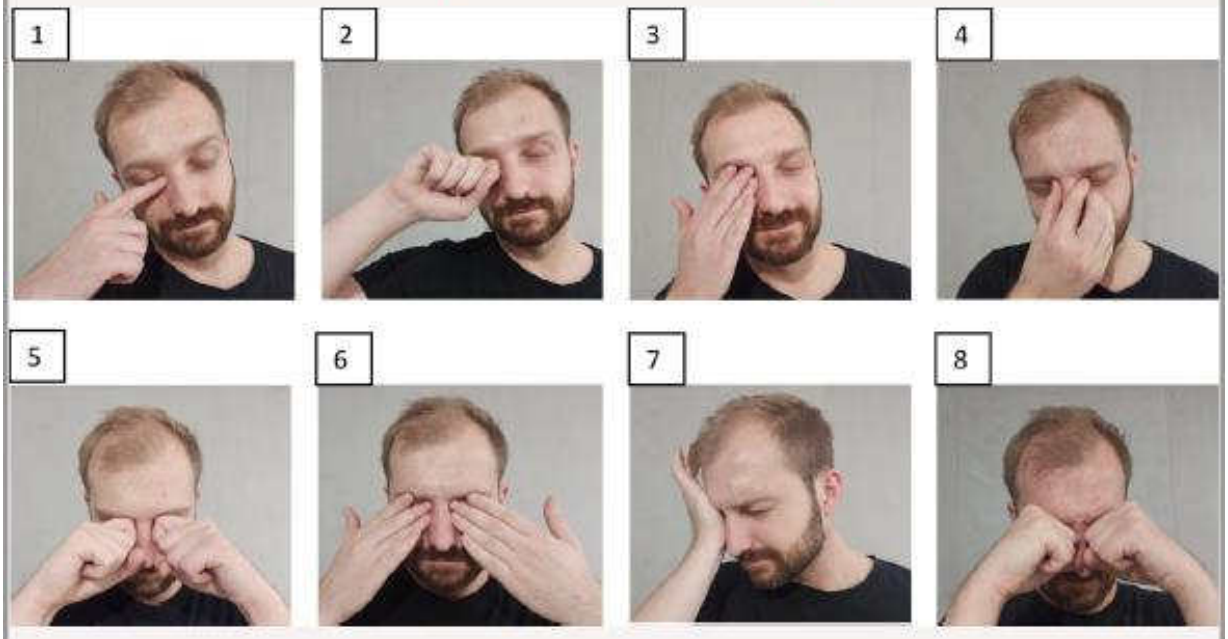
Researchers in Poland compared patients with keratoconus to controls in a study aimed at learning more about keratoconus. 118 individuals with keratoconus and 73 with no eye disease underwent complete eye exams and answered an extensive survey. The doctors found that individuals with keratoconus did not have allergies at a higher rate than the control subjects, although this result conflicts with many previous studies. They did discover that those who worked outdoors or in dusty environments (like construction) were more likely to be affected by KC.

As part of the survey, pictures that depicted eye rubbing were presented in ascending order of rubbing severity (See below). Study subjects were asked to select the picture that best represented how they rubbed their eyes. 72% of those without KC selected among photographs #1-4, and only 28% selected a photograph from #5-8. 55% of individuals with KC selected #5-8 to indicate their eye rubbing style. Those without KC most often selected #1 (28%) and those with keratoconus most often selected #8 (28.2%).

The authors did not find that eye rubbing changed over time. Adolescents with KC in the study had similar eye rubbing habits as adults with the disease. This study confirms that individuals with keratoconus have a harsher, more severe way of eye rubbing than most members of the public.

Reference: Jaskiewicz K, Maleszka-Kurpiel M, et al, Non -allergic eye rubbing is a major behavioral risk factor for keratoconus. PLoS ONE 18:e0284454.

Which picture best describes you?



Artykuł IV:

Sequence variants contributing to dysregulated inflammatory responses across keratoconic cone surface in adolescent patients with keratoconus.

Jaskiewicz K, Maleszka-Kurpiel M, Kabza M, Karolak JA and Gajecka M.

Front. Immunol. 2023 Jul 6;14:1197054. doi: 10.3389/fimmu.2023.1197054

Wstęp: Sekwencjonowanie całego genomu (ang. *whole genome sequencing*, WGS) umożliwia globalną analizę pełnego zestawu wariantów genetycznych w DNA pacjenta. WGS w chorobach heterogenicznych, w tym w chorobach okulistycznych i w identyfikacji zmienności sekwencji DNA jest bardziej skuteczny niż sekwencjonowanie eksomu (ang. *exome sequencing*, ES). Technika ta do tej pory nie była wykorzystywana w badaniach nad KTCN.

Historycznie KTCN był uznany za chorobę niezapalną, jednak w ostatniej dekadzie aspekt ten jest dyskutowany i kwestionowany przez specjalistów w dziedzinie okulistyki. Aktualnie przyjęte przez większość klinicystów stanowisko dotyczące stożka rogówki z roku 2015 (ang. *Global Consensus on Keratoconus and Ectatic*), nie odnosi się do tej kwestii. Wcześniej opublikowane wyniki badań transkryptomicznych i proteomicznych dostarczyły informacji dotyczących możliwego wpływu czynników zapalnych na rozwój KTCN. Zasugerowano, że niektóre cytokiny zapalne (takie jak IL6, TNFα i MMP9), których podwyższony poziom wykryto w płynie łzowym i surowicy pacjentów, przyczyniają się do ścieńczenia i osłabienia rogówki w przebiegu KTCN. Jak dotąd nie wykazano jednak genetycznych aspektów zaburzeń w funkcjonowania układu odpornościowego w przebiegu KTCN.

Zróżnicowana morfologia, różna intensywność napięcia biomechanicznego, jak i zmienna intensywność pro-zapalnych bodźców środowiskowych (m.in tarcia oczu) w obrębie CE dodatkowo uzasadnia wybór tej warstwy rogówki do badania aspektów zmienności genomowej na przekroju powierzchni stożka rogówki u pacjentów z KTCN i interpretacji rozpoznanych wariantów genetycznych w kontekście funkcjonowania układu odpornościowego.

Wyniki: Nabłonek rogówki (CE) czterech niespokrewnionych nastoletnich pacjentów z KTCN i dwóch osób kontrolnych uzyskano podczas procedur CXL lub PRK. Trzy regiony topograficzne, centralny, pośredni i obwodowy, zostały rozdzielone w kierunku badania WGS, obejmującego łącznie 18 próbek eksperymentalnych. Dla każdego pacjenta z KTCN został zdefiniowany zestaw genów z rozpoznanymi wariantami genetycznymi, z którego usunięto warianty: I) zidentyfikowane u osób z grupy kontrolnej, II) dla których maksymalna częstość występowania allelu w populacji europejskiej (nie-fińskiej) jest ≥0.1 (na podstawie danych z baz 1000 Genomes Project phase three, ESP6500SI-V2 oraz gnomAD v2.1).

Zmienność sekwencji kodujących i niekodujących, w tym zmienność strukturalną, oceniono, a następnie zinterpretowano wraz z wcześniej opublikowanymi wynikami transkryptomicznymi dla tych samych próbek CE (PMID: 36811882) oraz wynikami poprzednich badań dla rogówek pełnej grubości (PMID: 28145428).

W analizie nadreprezentacji ścieżek molekularnych obejmującej geny z wariantami w sekwencji kodującej (specyficznymi dla KTCN i obecnymi u co najmniej dwóch pacjentów) wykazano nadreprezentację ścieżek związanych z układem immunologicznym, w tym ścieżek

przeszczep przeciwko gospodarzowi (ang. *graft versus host disease*) oraz procesowania i prezentacji antygenu (ang. *antigen processing and presentation*).

Zarówno kodujące, jak i niekodujące warianty sekwencji znaleziono w genach (lub w ich bliskim sąsiedztwie) powiązanych z wcześniej rozpoznanymi komponentami komórkowymi specyficznymi dla KTCN, w tym cytoszkieletem aktyny, macierzą zewnątrzkomórkową, macierzą zewnątrzkomórkową zawierającą kolagen, jak również z wcześniej identyfikowanymi procesami biologicznymi adhezji ogniskowej, ścieżki sygnałowej Hippo i ścieżki sygnałowej Wnt. Porównując oceniane regiony topograficzne, nie stwierdzono heterogeniczności genomu w CE rogówki. Rozpoznano trzydzieści pięć regionów chromosomalnych wzbogaconych zarówno w kodujące, jak i niekodujące warianty sekwencji specyficzne dla KTCN, z najbardziej reprezentatywnymi *loci* 5q, 9q i 16q, które już wcześniej uznano za istotne w etiologii KTCN.

Wnioski: Po raz pierwszy przeprowadzono sekwencjonowanie całogenomowe prób materiału pochodzącego od pacjentów z KTCN. Chociaż pojedyncze warianty mogą wpływać na fenotyp KTCN w niektórych rodzinach, uzyskane dane genomowe (warianty sekwencji zarówno kodujących, jak i niekodujących) pozwalają przypuszczać, że KTCN w dużej mierze może być efektem wzajemnego oddziaływania wielu wariantów, które zaburzają procesy fizjologiczne rogówki. Prezentowane wyniki analizy ontologii genów z wariantami sekwencji kodujących i niekodujących genomu, podobnie jak wyniki analizy nadreprezentacji ścieżek molekularnych, wskazują na spójność na poziomie procesów biologicznych. Założenie o jednoczesnym upośledzeniu funkcji wielu genów potwierdza również wzbogacenie sekwencji w warianty w 13 regionach chromosomowych w *locus* 5q.

Po raz pierwszy w badaniach nad KTCN znaleziono liczne warianty sekwencji kodujących i niekodujących w genach mogące mieć znaczenie w procesach wrodzonej i adaptacyjnej odpowiedzi układu odpornościowego, co wskazuje na genetyczne aspekty w podłożu zapalnym KTCN. Zaburzenia układu odpornościowego, w tym prezentacja antygenu i degranulacja neutrofilów, wpływają na hemostazę rogówki, co może skutkować ścieńczeniem i osłabieniem tkanki.

Wkład Katarzyny Jaśkiewicz w publikację: koncepcja i plan badań; zbieranie i przygotowanie materiału biologicznego od pacjentów; przeprowadzenie wywiadów z pacjentami; przygotowanie prób DNA do eksperymentów WGS; udział w analizach bioinformatycznych; analiza i interpretacja danych genomowych i klinicznych; przygotowywanie rycin i tabel; pisanie manuskryptu.

Załączniki i informacje dodatkowe związane z niniejszą publikacją (29 stron) są dostępne pod następującym adresem internetowym:
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Sequence variants contributing to dysregulated inflammatory responses across keratoconic cone surface in adolescent patients with keratoconus

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Background: Keratoconus (KTCN) is the most common corneal ectasia resulting in a conical shape of the cornea. Here, genomic variation in the corneal epithelium (CE) across the keratoconic cone surface in patients with KTCN and its relevance in the functioning of the immune system were assessed.

Methods: Samples from four unrelated adolescent patients with KTCN and two control individuals were obtained during the CXL and PRK procedures, respectively. Three *topographic* regions, *central*, *middle*, and *peripheral*, were separated towards the whole-genome sequencing (WGS) study embracing a total of 18 experimental samples. The coding and non-coding sequence variation, including structural variation, was assessed and then evaluated together with the previously reported transcriptomic outcomes for the same CE samples and full-thickness corneas.

Results: First, pathway enrichment analysis of genes with identified coding variants pointed to "Antigen presentation" and "Interferon alpha/beta signaling" as the most overrepresented pathways, indicating the involvement of inflammatory responses in KTCN. Both coding and non-coding sequence variants were found in genes (or in their close proximity) linked to the previously revealed KTCN-specific cellular components, namely, "Actin cytoskeleton", "Extracellular matrix", "Collagen-containing extracellular matrix", "Focal adhesion", "Hippo signaling pathway", and "Wnt signaling" pathways. No genomic heterogeneity across the corneal surface was found comparing the assessed *topographic* regions. Thirty-five chromosomal regions enriched in both coding and non-coding KTCN-specific sequence variants were revealed, with a most representative 5q locus previously recognized as involved in KTCN.

Conclusion: The identified genomic features indicate the involvement of innate and adaptive immune system responses in KTCN pathogenesis.

KEYWORDS

cornea, corneal epithelium, inflammation, non-coding sequence variation, whole genome, WGS, keratoconus

1 Introduction

Keratoconus (KTCN) is the most common corneal ectasia, characterized by progressive stromal thinning and the presence of the keratoconic cone (1). The structural changes, which often manifest asymmetrically, lead to a loss of visual acuity as an effect of irregular astigmatism (2). The first symptoms usually occur in adolescence, and the disease progresses through the third or fourth decade of life (1). Keratoconus affects both genders, but the reason of its higher occurrence in male patients remains unclear (3). The occurrence of KTCN may vary depending on the ethnicity of patients (1), with an estimated prevalence of 1.38 per 1,000 in a general population (4).

Advanced KTCN remains one of the most common indications for full-thickness (penetrating keratoplasty) and partial-thickness (deep anterior lamellar keratoplasty) corneal transplantation in developed countries (5). In the face of the organ shortage crisis in worldwide transplantation, there is a need for other therapies that inhibit the progression of KTCN to an advanced stage. A corneal collagen cross-linking (CXL) is an effective alternative used to stabilize the progression of KTCN and often improves the quality of patients' vision (6).

Histopathological abnormalities are present in all corneal layers of the KTCN cornea (7), with diverse manifestations in central and peripheral zones being an effect of different biomechanical tension across corneal curvature in KTCN (8). Changes in collagen fibers and other elements of the extracellular matrix (ECM) form stromal irregularities (8) contributing to the KTCN cone formation and also affect the corneal epithelium (CE). The resulting rebuilt epithelial structure is characterized by a central thinning surrounded by a thickened annulus, named *doughnut* pattern (9). In the morphological observations of the CE (including confocal and immunofluorescence microscopy), the decreased cell density and their elongated shape, disruptions in basal integrity, increased number of apoptotic cells, and depositions of iron particles have been previously described (7, 10, 11). Moreover, some molecular proteomic and transcriptomic findings showed disruption of elements of Wnt signaling (e.g., *WNT10A*) (12), cell–cell communications (e.g., CDH13) (13), pro-inflammatory cytokines (e.g., *IL6*) (14), matrix metalloproteinases (e.g., MMP9) (15), and innate immune system (e.g., TLR2 and TLR4) (16).

The available reports regarding molecular and environmental findings in KTCN studies (17–22) indicate the multifactorial nature of the disease. The behavioral, environmental, and socioeconomic factors supposedly induce disease manifestation/progression in genetically predisposed individuals. In clinical studies, KTCN was found to be related to chronic eye rubbing, allergy, atopy, asthma, and UV exposure (18).

Historically, KTCN has been described as a non-inflammatory disease (2), but in the last decade, this aspect has been widely studied and questioned by ophthalmologists. However, the currently valid Global Consensus on Keratoconus and Ectatic Diseases (23) has not addressed this issue.

Previously published transcriptomic and proteomic findings derived a hint for the influence of inflammatory factors in KTCN as the increased levels of inflammatory cytokines (as *IL6*, *TNFα*, and *MMP9*) were found in patients' tear fluid and serum samples and suggested to contribute to the thinning and weakening of the corneal tissue in KTCN (17, 24–26).

So far, the genetic aspects regarding the functioning of the immune system in the KTCN have not been demonstrated. Numerous genetic findings, including familial inheritance (27, 28), a concordance between monozygotic twins in contrast to dizygotic twins (29), and the occurrence of syndromic KTCN (30), imply the presence of a genetic component for the disease development. The previous results obtained by our research group confirmed the postulated genetic heterogeneity between patients and the involvement of numerous genetic factors in the pathogenesis of KTCN (19, 31–33). Besides the identification of the 13q32 (31), 5q31.1-q35.3 (32), 2q13-q14.3, and 20p13-p12.2 (33) KTCN loci, the numerous sequence variants in *VSX1, TGFBI, DOCK9, STK24, IPO5, SKP1* and *IL17B* candidate genes (19, 32) have been demonstrated. So far, whole-genome sequencing (WGS) has not been applied in KTCN research. As only WGS provides an overview of the entire human genome, and it is a suitable method for causative variant discovery in genetically heterogeneous diseases, this approach is a natural next step in investigating the pathogenesis of KTCN. We hypothesize that variants in non-coding regions of the genome complement the previously identified features specific to the KTCN exome.

The multilevel structural and functional changes in cornea, including the varied morphology and various intensities of inflammatory-causing internal biomechanical tension and external environmental stimuli (e.g., eye rubbing) acting on particular zones of CE, suggest the diverse molecular features across corneal curvature. These premises support the designation and separation of three *topographic* regions of the CE, namely, *central*, *middle*, and *peripheral*. Here, we assessed the genomic diversity across keratoconic cone surface implementing a unique study design embracing the three *topographic* regions of the CE of adolescent patients with KTCN.

Abbreviations: BCVA, Best-Corrected Visual Acuity; CE, Corneal Epithelium; CXL, Corneal Collagen Cross-linking; DEG, Differentially Expressed Gene; ECM, Extracellular Matrix; ES, Exome Sequencing; FDR, False Discovery Rate; IF, Intermediate Filaments; IGV, Integrative Genomics Viewer; IL, Interleukin; IOP, Intraocular Pressure; KIR, Killer cell Immunoglobulin-like Receptor; KTCN, Keratoconus; MAF, Maximum Allele Frequency; NK, Natural Killer cells; PRK, Photorefractive Keratectomy; RE, Regulatory Element; SD-OCT Spectral-Domain Optical Coherence Tomography; SNP, Single-Nucleotide Polymorphism; SNV, Single-Nucleotide Variant; TF, Transcription Factor; TFBS, Transcription Factor Binding Site; UCVA, Uncorrected Visual Acuity; WGS, Whole-Genome Sequencing.

2 Materials and methods

2.1 Ophthalmic examination and patients' inclusion and exclusion criteria

Unrelated adolescent patients with KTCN and the youngest available controls (the non-KTCN individuals with mild myopia)

were involved in this study. The study protocol was approved by the Bioethics Committee at Poznan University of Medical Sciences, Poznan, Poland. The possible consequences of the study were explained, and informed consent was obtained from all participants, according to the Declaration of Helsinki.

Each individual underwent a complete ophthalmological examination, including the assessments of both uncorrected (UCVA) and best-corrected visual acuity (BCVA), intraocular pressure (IOP), corneal tomography with rotating Scheimpflug camera WaveLight® Oculyzer™ II (Alcon, TX, USA), epithelial thickness mapping [Spectral-Domain Optical Coherence Tomography (SD-OCT) device, Zeiss Cirrus 5000, Carl Zeiss Meditec, Dublin, California, USA], and slit-lamp and dilated funduscopic examination. A questionnaire comprising the behavioral, environmental, and socioeconomic aspects, including eye rubbing, use of contact lenses, atopy, UV exposure, smoking, reading habits, time spent in front of a screen, hormone intake, education level, and place of living, was completed by each participant.

The inclusion and exclusion criteria for adolescents with KTCN, and control individuals are described in detail in Supplementary Methods 1.1.

2.2 CXL and PRK procedures

CXL in patients with KTCN was performed in accordance with the standard Dresden Protocol (34), while the PRK was performed as a refractive error correction procedure (35) in control individuals as described in Supplementary Methods 1.2, 1.3 respectively.

2.3 Material collection and sample preparation

Stamps towards the nose and eyebrow were made on the CE before the excision in the CXL/PRK procedures to enable correct tissue orientation during cutting and separation of the *topographic* regions. The obtained tissues were submersed in an RNA stabilization solution (RNAlater; Qiagen, Hilden, Germany) immediately after excision and stored at −80°C until further proceeding.

The procedure of designation of the particular *topographic* region is shown in Figures 1, S1. The details of the sample preparation are described in Supplementary Methods 1.4.

2.4 DNA extraction

Separated CE samples were transferred from the microscope slides to the lysis solution (Norgen Biotek, Thorold, ON, Canada). Total RNA, DNA, and proteins were extracted and purified according to the instructions of the RNA/DNA/Protein Purification Plus Micro Kit (Norgen Biotek). The quantity of DNA samples was measured by Qubit dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and quality was assessed by 1% gel electrophoresis.

2.5 Library preparation, sequencing, and WGS data analyses

WGS of the 18 CE samples was performed with a TruSeq Nano DNA HT Library Prep Kit (Illumina, San Diego, CA, USA) and the HiSeqX platform (Illumina) with mean coverage depth 30× at CloudHealth Genomics (Shanghai, China) as previously described (36). WGS data processing is summarized in the Supplementary Methods 1.5.

The following subset of sequence variants were analyzed: high impact variants (nonsense mutations/stop gain, stop lost, frameshifts, splice site mutations, etc.), missense variants (missense mutations considered deleterious by either SIFT or PolyPhen), and variants in regulatory regions [overlapping promoters, promoter flanking regions, enhancers, CCCTC-binding factor binding sites, transcription factor (TF) binding sites, or open chromatin regions, based on the Ensembl Regulatory Build for fibroblasts], classified as single-nucleotide variant (SNV)/deletion/insertion/indel/sequence alteration (substitution).

Motif analysis of variants located in regulatory elements (REs) was executed using the web-based FABIAN-variant application (37), and SNVs were additionally verified using MotifbreakR (38).

A set of impacted genes (high impact/missense/regulatory regions variants) was assessed for each patient after removing variants identified in control patients and variants with a MAF_AF (Maximum observed allele frequency in 1000 Genomes, ESP, and gnomAD) ≥0.1 (1000 Genomes Project phase three, ESP6500SI-V2, gnomAD v2.1). The non-redundant set of impacted genes in KTCN patients was analyzed using ConsensusPathDB (39), ShinyGO (40), STRING tool (41), and Reactome database (42). In pathway enrichment and gene ontology analyses, the p-value ≤ 0.05 and/or FDR ≤ 0.05 were the cutoffs.

The identification of genomic regions significantly enriched in genes with KTCN-specific sequence variants, compared to the density of genes in the background, was performed using ShinyGO (40). A "sliding window" is applied to scan the genome and multiple hypergeometric tests to determine whether the evaluated genes are significantly overrepresented within the analyzed window with a size of 6 Mbp and the FDR ≤ 0.05 as a cutoff.

The Integrative Genomics Viewer (IGV) (43) was used for visual exploration of genomic data.

To determine if there is any difference between the genomic characteristics of the particular CE *topographic* regions and full-thickness corneas, we compared the data obtained for the CE samples with our previously generated molecular data for the same population (Polish Caucasians) (19, 32, 44, 45) and with other published data on candidate genes and gene pathways (31, 46–51).

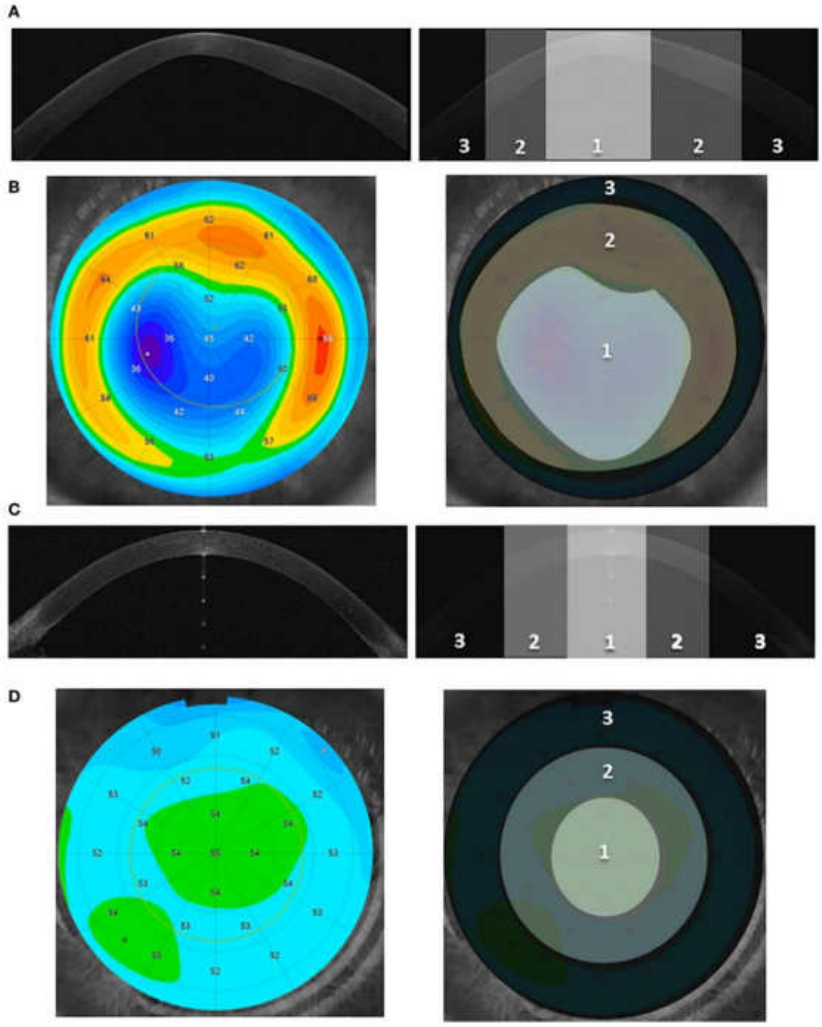

FIGURE 1
The designation of the three corneal *topographic* regions. **(A, C)** Examples of the cross-sectional image of the anterior segment of the eye (Anterior Segment Optical Coherence Tomography, AS-OCT) for the KTCN adolescent **(A)** and control individual **(C)**, respectively. Three distinct *topographic* regions (1—*central*, 2—*middle*, and 3—*peripheral*) are indicated. The KTCN-specific thinning at the 1—*central* region is visible. **(B, D)** Example of the epithelial thickness mapping for the KTCN adolescent **(B)** and control individual **(D)**, respectively. Representative images were obtained using MS-39 AS-OCT (CSO, Firenze, Italy). The *topographic* regions: 1—*central*, 2—*middle*, and 3—*peripheral*, were designated based on the steepness of epithelial thickness and corneal tomography with a rotating Scheimpflug camera (not shown). The designated CE *topographic* regions were separated and processed towards the DNA extraction.

All experimental samples were analyzed separately and at no step of the analysis (including the analysis of raw next generation sequencing reads) were data from the three regions of one individual combined. In the Results section, the variant(s) referred as recognized in the KTCN patient were present in all three *topographic* regions.

2.6 Data integration

Simultaneously, the transcriptomic and proteomic assessments as well as morphological evaluation in the same CE samples were performed, as described in detail elsewhere (13). All the data of the mentioned investigation were considered in final interpretation of the study outcomes.

3 Results

3.1 Characteristics of patients and DNA samples

Four unrelated adolescent patients with KTCN (one female/three male patients) and two control individuals (two male individuals) were involved in this study. There was no history of

KTCN and genetic diseases (including autoimmune diseases) in families of ascertained patients and controls. Clinical characteristics of the examined individuals and the eyes subjected to the surgery are presented in Table 1, while the information collected for both eyes in the studied individuals is compiled in S1 Table. Selected behavioral, environmental, and socioeconomic aspects evaluated in the questionnaire are presented in S2 Table.

A total of 18 experimental samples of CE (three *topographic* regions in each of the six ascertained individuals) were collected and used in the WGS experiments, in accordance with a study scheme (Figures 1, S1). The results of quantity and quality control of the 18 DNA samples and quality control of WGS reads are presented in S3 Table.

3.2 Genes shaping the inflammatory responses

Analyzing the coding sequence, a total of 646 variants were identified in KTCN patients, including high impact and missense variants (classified as SNVs, deletions, and insertions). In aspects of molecular function, biological process, and their cellular location, genes with identified variants contributed to ECM structural constituent, collagen-containing ECM, and cytoskeleton organization (S2 Figure and S4 Table). What is important, the pathway enrichment analysis comprising all genes with recognized sequence variation revealed the "Antigen presentation" and "Interferon alpha/beta signaling" as the most overrepresented Reactome pathways (FDR < 0.001, S5 Table). In STRING analysis, embracing the same set of genes with the 646 variants, the significant enrichment of 63 clusters, including network of interferon alpha/beta signaling, was recognized (S3 Figure and S6 Table).

Seventeen out of 646 variants were found as recurring in patients with KTCN, whereas the remaining 158, 159, 187, and 165 variants identified in patients 10 OPT/KTCN, 13 OPT/KTCN, 18 OPT/KTCN, and 30 OPT/KTCN, respectively, were unique for the evaluated individuals. The recurrent coding variants were as follows: rs687485 in *KIR2DL*; rs643861 and rs652641 in *KIR3DL1*; rs878913005, rs878949904, and rs1269243287 in *MUC5AC*; rs767573621 and rs752917826 in *TRAV19*; rs17229 in *TRBV12-5*; rs2257251 in *NANOGP8*; rs1227351091 in *NBPF19*; rs148235978 in *NFE2L3*; rs151080920 in *TSC22D2*; rs140771568 in *UCMA*; rs28575804 (A>G) in *UGT2B25P*; rs1563130264 in *AC011005.1*; and rs879192097 in *AL354822.1* (S7 Table).

Furthermore, analyzing the high-impact and missense variants identified in at least two patients with KTCN, the "Graft-versus-host disease" and "Antigen processing and presentation" were found to be the most enriched pathways (Figure 2A and S8 Table).

3.3 KCTN-specific non-coding sequence features

Excluding variants recognized in control individuals and variants with MAF>0.1, 15,213 sequence variants in REs in

TABLE 1 The clinical characteristics of the ascertained adolescent patients with KTCN and control individuals (non-KTCN mild myopia patients).

ID	Diagnosis	Study subgroup	Sex	Age at examination	Age at diagnosis	K1 [D]	K2 [D]	Kmax [D]	Anterior elevation [μm]	Posterior elevation [μm]	TCT [μm]	AL [mm]	Cone/apex[a] location	Thinnest epithelial thickness	Average thickness of corneal region 1 [μm]	Average thickness of middle region 2 [μm]	Average thickness of periphery region 3 [μm]	KTCN grade
Adolescent KTCN																		
10 OPT/KTCN	KTCN	Adolescent	M	14	14	49.5	54.8	66.2	+39	+52	391	24.2	Central	39	35	37	47	3
13 OPT/KTCN	KTCN	Adolescent	M	16	16	44.1	45.8	50.3	+11	+30	489	22.45	Inferior, temporal	39	42	49	45	2
18 OPT/KTCN	KTCN	Adolescent	M	14	13	42.8	44.5	49.6	+14	+28	413	23.71	Inferior, central	34	41	47	44	1-2
30 OPT/KTCN	KTCN	Adolescent	F	13	12	45.1	49.9	58.9	+31	+50	437	24.31	Central, temporal	35	39	56	47	2-3
Controls																		
5 OPT/M	Myopia	Control	M	24	nd	43.3	44.2	44.4	+2	+2	531	23.31	Central	41	32	50	49	n/a
6 OPT/M	Myopia	Control	M	30	nd	41.3	42.8	43.1	+1	+1	489	25.09	Central	46	49	46	45	n/a

M, male; F, female; K1, flat keratometric readings; K2, steep keratometric readings; Kmax, maximum simulated keratometry; OD, right eye; OS, left eye; nd, data not available; n/a, not applicable; TCT, thinnest corneal thickness; AL, Axial length; [x], applicable for control. Clinical data concerning only the eyes subjected to surgery are presented.

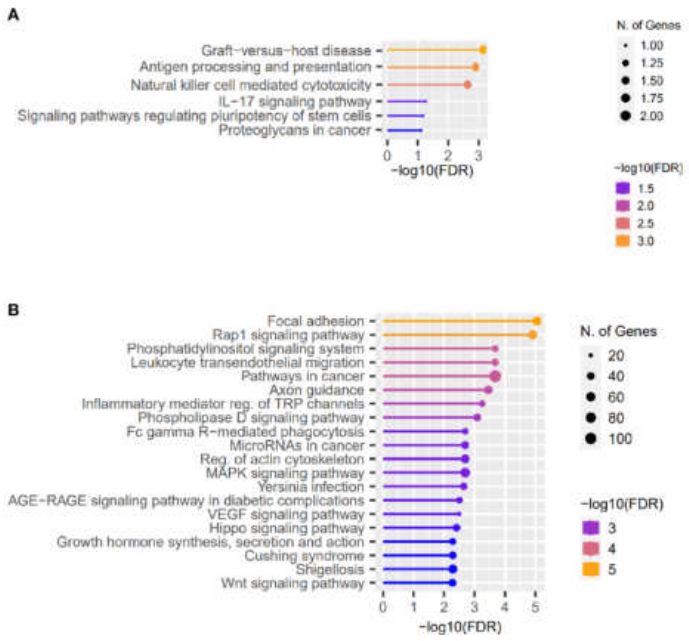

FIGURE 2
Representative results of the pathway enrichment analysis. Representative results of the pathway enrichment analysis of **(A)** genes with the same coding sequence variants identified in more than one KTCN patient, pointing to the involvement of innate and adaptive immune system responses in KTCN pathogenesis and **(B)** genes located close to the variants in REs. On *x* axes, −log(FDR) values of enriched pathways are presented, and on *y* axes, the KEGG pathways are presented. The size of the bubbles indicates the number of genes attributed to the particular pathway and present in our set.

KTCN patients, classified as SNVs, deletions, insertions, indels, and substitutions, were identified.

Next, using the FABIAN-variant application that predicts the effects of DNA variant located in RE on transcription factor binding sites (TFBSs), we narrowed down the number of variants and genes for further evaluation to 7,886 and 5,709, respectively. We assessed genes in which close proximity in at least one sequence variant in RE per KTCN patient was recognized as potentially forming or abolishing TFBS. Pathway enrichment analysis of these genes disclosed "Focal adhesion", "Rap1 signaling pathway", and also "Hippo signaling pathway", "Wnt signaling", and "TGF-beta signaling" pathways (Figure 2B and S9 Table).

3.4 Coexistence of coding and non-coding sequence variants in genes influencing the KTCN-specific pathways

Both coding and non-coding sequence variants of KTCN patients were found in the genes/genes' close proximity linked to the revealed KTCN-specific cellular components, namely, "Actin cytoskeleton", "Extracellular matrix", and "Collagen-containing extracellular matrix", and KTCN-specific pathways such as "Focal adhesion", "Hippo signaling pathway", and "Wnt signaling" (S4 Figure and S10 Table). Genes with coding and non-coding sequence variants, attributed to biological pathways from Reactome database, are listed in Table 2, and in detail in S11 Table.

To determine if the evaluated genome regions are significantly enriched, genes with both coding and non-coding sequence variants were analyzed and 35 enriched regions were revealed, as presented in Figure 3, with majority of these enriched regions at chromosomes 5q (13 regions), 9q (9 regions), and 16q (5 regions).

3.5 Genomic heterogeneity across corneal surface and structural variation

All identified coding-sequence variants were assessed for differences between *topographic* regions, separately for each adolescent patient with KTCN. No genomic heterogeneity across corneal surface was found. Although identified sequence variants in *KIR3DL1* (in two KTCN patients), *UMAD1* (in two KTCN patients), and *GOLGA6A2* (in one KTCN patient) genes have met quality criteria of reads in the applied bioinformatic algorithms and differentiated the *topographic* CE regions, the BAM files visualized with the IGV showed a variation in one of 26–43 reads only in these listed genes (the total number of reads for a particular genome site varied), indicating uncertain results. Expanding the same analysis for control samples (but applying the criterion of MAF ≤ 0.1), the additional coding sequence variants in 15 genes (*AC018682.2, AC092384.1, AC092490.1, AC117481.1, AC118281.1, ANKRD36BP2, CA15P2, CRACD, HLA-DRB5, KRTAP2-2, LINC00634, NBEAP1, OR2T35, SAMD1, UGT2B25P*, and *ZNF571*) were recognized as potentially differentiating the corneal surface, but none of these variants was confirmed by the BAM files.

TABLE 2 Chosen WGS results, list of genes with high impact, missense, and variants in regulatory elements attributed to the selected biological pathways.

Pathway module, Pathway	Genes with variants
Cytokine signaling, Interferon α/β	***HLA-A*, *MX2*, *XAF1***, *IRF1*[‡], *IFITM3*[‡], *IRF2*, *IFI35*, *ISG20*, *IFI27*, *TYK2*, *OAS1*
Cytokine signaling, Interferon γ	***HLA-A*, *ICAM1*, *GBP7***, *IRF1*[‡], *IFITM3*[‡], *GBP5*, *GBP1*, *MT2A*, *PTPN2*, *PML*, *OAS1*, *IRF2*, *TRIM31*, *SUMO1*, *CD44*, *NCAM1*, *IFNGR2*
Cytokine signaling, Interleukin-1	***PTPN14*, *SQSTM1*, *PSMD3***, *IL1R1*[‡], *PTPN12*[‡], *PELI2*, *TAB3*, *PTPN20*, *TAB1*, *IL1RAP*, *PTPN2*, *APP*, *SIGIRR*, *IL1RL1*, *UBE2N*, *PSMD1*, *UBE2V1*
Cytokine signaling, Interleukin-6	***CNTFR***, *OSM*, *TYK2*, *IL6ST*, *OSMR*, *STAT3*
Innate immune system, Neutrophil degranulation	***PECAM1*[†‡], *ATPSCKMT*, *HPSE*, *A1BG*, *HLA-A*, *TOM1*, *GALNS*, *PFKL*, *CEP290*, *GUSB*, *PIEZO1*, *EPO*, *PSMD3*, *LILRA6*, *ITGAD*, *EPX*, *IQGAP2*, *ATAD3A***, *ANO6*[§], *TMC6*[§], *GDI2*[‡], *HSPA6*[†], *S100Z*[§], *FCGR3B*[§], *RAB7B*[‡], *XRCC5*, *KCMF1*, *FCGR3B*, *GOLGA7*, *ATP8B4*, *PRC1*, *RAB18*, *IQGAP1*, *CPNE1*, *RNF38*, *RAP1B*, *PTPRC*, *RAP1A*, *PLD1*, *ADA2*, *CTSC*, *SH3GLB2*, *CEACAM6*, *RHOG*, *TMC6*, *IST1*, *CEACAM1*, *CEACAM3*, *PSMB1*, *RAB31*, *RHOA*, *PKP1*, *CYFIP1*, *CFD*, *STK10*, *CD44*, *DDX3X*, *DOCK2*, *RAB7B*, *SLC27A2*, *ITGAV*, *LTF*, *KCNAB2*, *GDI2*, *PSMA5*, *RAB44*
Adaptive Immune System, Class I MHC antigen processing and presentation	***HLA-A*, *UBE2Q2*, *TOM1*, *FBXO27*, *PSMD3*, *RNF213*, *KBTBD13*, *SH3RF1*, *CDRT1*, *ANAPC10***, *NEDD4L*[‡], *SMURF1*[‡], *S100Z*[§], *ZNRF1*[‡], *SPSB1*[‡], *ASB2*[‡], *PJA2*[‡], *ANAPC5*, *TRIM9*, *RNF126*, *TRIM41*, *FBXL7*, *ANAPC1*, *MIB2*, *PSMD1*, *FGG*, *FBXL20*, *UBE3B*, *UBA6*, *UBE3D*, *UBE3C*, *RNF19A*, *CDC27*, *HERC4*, *TRIM32*, *LNX1*, *TRIM36*, *UBE2N*, *PSMC6*, *PRKN*, *FBXO32*, *FBXO15*, *SMURF2*, *HECTD2*, *FBXO17*, *UBE2H*, *UBE2K*, *CDRT1*, *RNF220*, *KLHL3*, *PSMB1*, *LMO7*, *RNF217*, *PXK*, *ITGB5*, *ITGAV*, *UBE2V2*, *UBE2V1*, *FBXW9*, *FBXW7*, *ANAPC13*, *PSMA5*, *PATJ*
Signal transduction, Signaling by TGFB family members	***TGIF1*, *CCNT1***, *NEDD4L*[‡], *SERPINE1*[‡], *SMURF1*[‡], *FST*[#], *CDKN2B*, *EMB*, *SMOX*, *SMURF2*, *WWTR1*, *FKBP1B*, *STAG1*, *E2F5*, *ITGA8*, *TGFB3*, *FKBP1A*, *USP15*, *LTBP1*, *RHOA*, *TFAM*, *HDAC1*, *NCOR2*, *ITGB5*, *CCNT2*, *ITGAV*, *FBN1*, *SMAD4*, *SMAD1*, *CDK6*, *PRKCZ*, *SMAD6*, *CDK9*
Signal transduction, Signaling by WNT	***LGR5*, *ROR2*, *PRKCB*, *PSMD3*, *HECW1*, *CCDC88C***, *NFATC1*[†], *SMURF1*[‡], *PPP2R1A*[‡], *LRP5*[‡], *RAC2*, *CSNK1E*, *EMB*, *WNT3A*, *H2AC6*, *PRKCB*, *FZD1*, *VANGL2*, *WLS*, *PSMD1*, *HECW1*, *YWHAZ*, *TCF7L2*, *GNB5*, *ITPR1*, *ITPR2*, *LGR4*, *CUL3*, *TCF4*, *LGR5*, *APC*, *TNRC6C*, *KRAS*, *PRKG1*, *TNRC6B*, *H3-3B*, *GSK3B*, *H3-3A*, *GNG2*, *PPP3CA*, *BCL9*, *CREBBP*, *PSMC6*, *AXIN1*, *H3C2*, *AXIN2*, *SMURF2*, *H2BC6*, *PDE6A*, *WNT5B*, *DACT1*, *PSMB1*, *TLE4*, *RHOA*, *CTBP1*, *CTBP2*, *ROR1*, *LRTM1*, *HDAC1*, *PLCB1*, *PPP2R5C*, *RSPO2*, *NLK*, *DAAM1*, *PSMA5*
Extracellular matrix organization, Integrin cell surface interaction	***PECAM1*[†‡], *TNC*, *FN1*, *COL23A1*, *VWF*, *COL5A1*, *ITGAD*, *ICAM1***, *ITGA4*[‡], *COL5A2*[‡], *COL8A1*, *COL6A3*, *ITGAD*, *COL13A1*, *ITGA11*, *COL5A1*, *CD44*, *ITGB5*, *VTN*, *ITGB7*, *ITGAV*, *FBN1*, *JAM3*, *ITGA8*, *TNC*, *ITGA2B*, *HSPG2*, *FGG*
Extracellular matrix organization, Collagen formation	***LAMC2*, *P3H1*, *COL23A1*, *COL5A1*, *COL17A1*, *COLGALT1***, *COL27A1*[‡], *ADAMTS2*[‡], *P4HA1*[‡], *COL5A2*[‡], *COL21A1*, *COL8A1*, *COL6A3*, *COL25A1*, *COLGALT2*, *COL5A1*, *LOXL4*, *DST*, *PXDN*, *COL17A1*, *LOXL2*, *LAMC2*, *PLOD2*, *COL28A1*, *COL14A1*, *LOX*, *COL13A1*, *COL12A1*
Extracellular matrix organization, ECM proteoglycans	***TNC*, *FN1*, *LAMB1*, *COL5A1*, *LAMA5*, *ITGAD***, *TNR*[‡], *COL5A2*[‡], *SERPINE1*[‡], *COL6A3*, *MUSK*, *COL5A1*, *LRP10*, *ITGA8*, *TGFB3*, *ITGA2B*, *ACAN*, *DCN*, *LAMC1*, *LAMA1*, *ASPN*, *LAMA2*, *APP*, *LAMA5*, *PTPRS*, *ITGB5*, *ITGAV*, *MATN1*, *TNC*, *NCAM1*, *VTN*, *ITGAD*, *TNXB*, *HSPG2*
Extracellular matrix organization, Degradation of ECM	***FN1*, *LAMB1*, *LAMC2*, *COL23A1*, *COL5A1*, *COL17A1*, *LAMA5*, *CAPN9*, *CAPN12*, *NID1***, *COL5A2*[‡], *COL8A1*, *COL6A3*, *COL25A1*, *COL5A1*, *GPR37*, *CAST*, *MEGF11*, *MME*, *SUB1*, *ACAN*, *COL17A1*, *DCN*, *HTRA1*, *LAMC1*, *LAMC2*, *COL14A1*, *COL13A1*, *TLL2*, *COL12A1*, *MMP17*, *CD44*, *FBN2*, *LAMA5*, *ADAMTS9*, *ADAM17*, *FBN1*, *PLG*, *ELN*, *CAPN13*, *NID1*, *CAPN15*, *HSPG2*
Developmental biology, Keratinization	***EVPL*[†], *PCSK6*[†], *KRTAP10-7*, *KRT35*, *FLG*, *KRT33B***, *PKP3*, *KRTAP5-5*, *PKP4*, *PKP1*, *PI3*, *KRTAP4-11*, *KRTAP3-1*, *SPRR1B*, *KAZN*, *KRT39*
Cell–cell communication, Cell–cell junction organization	***LAMC2*[†‡], *COL17A1*[†‡], *SDK1*[†‡], *NECTIN2*, *SDK2***, *CLDN6*[†], *FLNC*, *CLDN8*, *LIMS1*, *LIMS2*, *PARVA*, *CLDN2*, *PARVB*, *MEGF11*, *DST*, *VASP*, *PARD6B*, *ACTB*, *CDH6*, *LAMC2*, *CLDN16*, *FERMT2*, *CTNNA1*, *NECTIN3*, *CLDN20*, *ACTN1*, *PATJ*
Chromatin modifying enzymes, Methylation	***WDR5B*, *KDM2D*, *SMYD3***, *PRDM16*[†], *KDM7A*[†], *H3C2*, *KDM4C*, *KDM4B*, *KDM2B*, *SMYD3*, *EHMT1*, *WDR5*, *SETD7*, *DOT1L*, *SETD3*, *EZH2*, *CCND1*, *H2AC6*, *SMARCA2*, *ARID1B*

Bold text indicates genes with coding sequence variants identified in more than one KTCN patient; † stands for genes with variants in both and non-coding genome sequence; ‡ stands for genes with the same variant identified in more than one KTCN patient.
Variants identified in control samples and variants with MAF≥0.1 in the gnomAD database were excluded (the completed list of genes with sequence variants is presented in S9 Table).

FIGURE 3
The display of genes, in which both coding and non-coding variants unique for adolescent patients with KTCN were identified, across chromosomes. Genes are represented by red dots. The violet lines indicate 35 regions statistically enriched. The genome was scanned with a sliding window in of size 6 Mbp. Within each window, the hypergeometric tests were used to determine if genes of both coding and non-coding variants were significantly overrepresented. The used FDR cutoff was ≤0.05.

Assessing structural variation, deletions longer than 50 bp were characterized in detail. The length of the deletions and their density in genome are presented in S5 Figure. Most of the deletions were localized in non-coding regions of the genome and in gene introns. We did not observe a difference in the number of deletions comparing patients with KTCN and control individuals, assessing the previously published KTCN-specific loci (S12 Table) (52). Nevertheless, upstream and downstream regions of genes involved in the innate immune system (*ITGAM, PRKCE, COL11A2, STK10, ADGRG3, IGHV2-70, IGHG4, MAP2K1, IGHG2, GSDMD, MLC1, MYO9B, IGHV1-69, DEFB131A, KIR2DS4, NKIRAS2, RNF38*, and *RPS6KA5*); the adaptive immune system (*TRAC, INPP5D, IGHV2-70, ASB3, PRKG1, TAP2, HMCN2, KIR3DL2, KIR3DL1, IGHV1-69, KIR2DL4, RASGRP3*), cytokine signaling (*ITGAM, HNRNPA2B1, NUP155, CAMK2A, INPP5D, IGHG4, MAP2K1, CRLF2, GSDMD, NKIRAS2, BOLA2*, and *RPS6KA5*); ECM including collagen biosynthesis, formation, and trimerization (*ITGA9, FBLN1, LOXL4, COL6A6, ITGAM, ADAMTS14, P4HA3, COL11A2, COL27A1*, and *COL26A1*); and others were found to contain deletions (51–5,098 bp in size) in patients with KTCN in contrast to control individuals (MAF > 0.1 was an exclusion criterion).

3.6 Influence of the presence of RE variants on gene expression

We evaluated the influence of the presence of identified RE variants on expression of genes localized in close proximity, based on data of RNA-seq performed in the same CE samples, and full-thickness corneas (45). No substantial features were recognized in the assessment of the CE. However, 722 of the previously recognized differentially expressed genes (DEGs) in full-thickness KTCN corneas were re-recognized as the gene containing variants in REs. Then, in the pathway enrichment analysis of the re-recognized genes and transcripts, the ECM pathway as the most overrepresented was revealed (Figure 4 and S13 Table).

4 Discussion

The complex background of KTCN, in addition to environmental factors, includes heterogeneous genetic characteristics. The multiple candidate genes and variants identified using different molecular techniques indicate that this heterogeneity requires the introduction of high-throughput investigation methods, which may be more effective in unraveling the KTCN-specific determinants.

Each individual genome differs from the reference human genome by an average of 3.5 million SNPs, but only a minority of those variations contributes to the phenotypic differences and disease predisposition (53). Only WGS can provide an overview of the entire human genome, allowing the global analysis and the full set of variants to be assessed (54–56). The majority of SNVs and indels is identifiable by both WGS and exome sequencing (ES), but still approximately 3% of coding variants is missed in the ES approach (54). Therefore, WGS is more efficient than ES for detecting coding sequence variants, besides the undoubted benefit of identification of non-coding sequence variation. To date, WGS

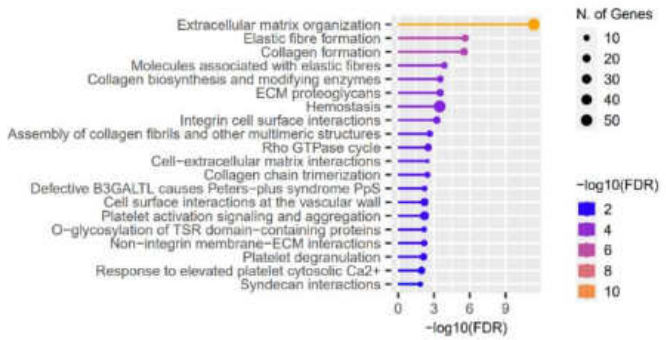

FIGURE 4
Representative results of the pathway enrichment analysis of 722 genes previously recognized as differentially expressed (DEGs) in full-thickness KTCN corneas and re-recognized as containing variants in the REs localized in close proximity. On the *x* axis, −log(FDR) values of enriched pathways are presented, and on the *y* axis, the KEGG pathways are presented. The size of bubbles indicated the number of genes attributed to the particular pathway and present in our set. The Reactome database was chosen for the definition of pathways.

has not been applied in KTCN research. Since WGS is an effective method of detecting mutations in genetically heterogeneous diseases, including ophthalmic diseases (57), and is more efficient than ES in the identification of sequence variation, this technique is crucial in deciphering the genetic aspects of KTCN.

Here, we found that the genetic features of patients with KTCN correspond with those previously reported regarding sequence variants in components of ECM (19, 50). Also, we identified other sequence variants in genes previously reported in KTCN research in the same population (19), including *COL17A1* [playing a role in the integrity of hemidesmosome and the attachment of basal keratinocytes (58), whose expression was decreased in corneal epithelial cells in single-cell RNA-seq analysis of KTCN corneas (59)]; *FREM2* [protein required for maintenance of the integrity of the skin epithelium (60) and eye morphogenesis (60)]; *SSX2IP* [which may connect the nectin–afadin and E-cadherin–catenin system through alpha-actinin and may be involved in the organization of the actin cytoskeleton (61)]; *MINK1* [mediating stimulation of the stress-activated protein kinase MAPK14/p38 MAPK downstream of the Raf/ERK pathway (62)]; *CAMSAP1* [Calmodulin-regulated spectrin-associated protein 1; key microtubule-organizing protein (63)]; and *RGS12* [Regulator of G-protein signaling 12 (64)]. Moreover, we identified variants previously reported in *EML6* (50), which is an element of cytoskeletal network and microtubules in cultured keratocytes (50, 65); *MAGI1* (50), which regulates cell–cell and cell–matrix adhesion (66); and *COL5A1* (48, 49), whose knockout in corneal stroma in mouse showed severe dysfunctional regulation of fibrillogenesis resulting in decreased fibril number and a disorganized lamellae structure with decreased stroma thickness (67).

Also, we have revealed variants in genes involved in keratin-related pathways (namely, the "keratinization" pathway from the Reactome database), such as *EVPL, PCSK6, KRTAP10-7, KRT35, FLG*, and *KRT33B*. As keratins participate in forming intermediate filaments (IF), which provide mechanical support, and form desmosomes between cells and hemi-desmosomes with basement membranes for epithelium integrity (68), this result may be of special interest regarding changes in biomechanics of keratoconic cornea.

Importantly, non-coding variants are now considered to be potentially pathogenic (69, 70). Sequence variation in the form of SNVs or indels in the regulatory regions can either generate or abolish TF binding sites, modulating the transcriptional activity of genes and regulating chromatin state (71). The disruption of the TF binding site and its effect on the disease phenotype have already been reported in many cancer types such as T-cell acute lymphoblastic leukemia (72) and esophageal and gastric cancers (73). However, while great advances in predicting the effects of coding variants have been made, the assessment of non-coding variants remains challenging. In particular, the immense number of variants revealed per patient sample impedes the analysis and biological interpretation. In our investigation, all variants located in REs, including promoters, promoter flanking regions, enhancers, CCCTC-binding factor binding sites, TFBS, or open chromatin regions of fibroblasts, were evaluated *in silico* towards functional effect (TFBS gain/lost) based on the motif analysis. The obtained list of RE variants showed unity on the pathway level with previously reported variants, namely, focal adhesion (19), Wnt signaling (19), and TGF-beta signaling (74). Moreover, the *LAMC2, COL17A1, SDK1, PECAM1, TJP2, MINK1, NEIL3, EVPL*, and *PCSK6* genes were revealed to have unique variants for patients with KTCN in both coding and non-coding genome sequence, which again were found as involved in the ECM, cell–cell communications, cytoskeleton organization, and keratinization pathways. To further evaluate the effect of variants located in REs, the functional approaches such as gene reporter assay are needed, but firstly transcriptome data from adequate biological material is necessary to be integrated with the genomic data. Therefore, we evaluated the data overlap between DEGs in full-thickness KTCN corneas (45) and variants recognized in the REs. We found the involvement of RE variants in numerous elements of ECM and that fact supports our assumption concerning the role of RE sequence variation in KTCN pathogenesis.

Although single variants could influence the KTCN phenotype in some families (75), our genomic data (both coding and non-coding sequence variation) suggest that KTCN largely occurs

through the interplay of multiple variants that interfere with major physiological processes in the cornea. The presented connection between coding and non-coding sequence variants in the gene ontology ("Actin cytoskeleton", "Extracellular matrix", and "Collagen-containing extracellular matrix") and in the pathway assessments ("Focal adhesion", "Hippo signaling pathway", and "Wnt signaling") corroborates this assumption, as well as the identified 13 regions enriched in variants in the 5q locus that had previously been found to be specific to KTCN in distinct populations (32, 46, 47).

It has been known that DNA structural variation modifies the interactions between REs and their target genes (71). Here, the majority of the deletions were found to be located in non-coding and intron regions. Overall, assessing the previously published KTCN-specific loci, we did not observe a difference in the number/density of deletions comparing patients with KTCN and control individuals. However, numerous genes involved in the innate immune system; adaptive immune system; ECM including collagen biosynthesis, formation, and trimerization; and others were found to contain deletions in size of 51–5,098 bp in patients with KTCN in contrast to control individuals. The extent to which this finding is important should be clarified in further research on this aspect. To date, deletions >50 bp in size themselves and their effect on the KTCN phenotype were only once evaluated, in an Ecuadorian family with KTCN (31), but that study did not reveal any copy number variation on evaluated 13q32 in the tested individuals.

We have anticipated that the various intensities of both internal biomechanical tension and external environmental stimuli (e.g., eye rubbing) acting on various *topographic* regions of CE could result in diverse molecular features across corneal curvature. Our study performed in the designated three *topographic* regions of the CE, *central*, *middle*, and *peripheral*, enabled the conclusion that there is no genomic heterogeneity across the corneal surface in KTCN. However, in a comprehensive morphological, transcriptomic, and proteomic evaluation performed in the same corneal samples, we identified the variability shaping the distinct features of CE zones (13). Continuing the topographic aspects of the CE, we have considered features of mosaicism to be involved in the process of remodeling of the KTCN CE. To date, based on the results of exome and/or Sanger sequencing performed in matched KTCN cornea–blood pairs, we found no evidence of inter-tissue variants in human KTCN corneas, since all variants possibly related to KTCN were identified in both corneal tissue and peripheral blood of patients (19). Again, here we did not identify features of mosaicism.

In our opinion, the most important findings of this study are those concerning the inflammation in KTCN. Nowadays, the inflammatory aspects in KTCN are widely discussed. The multiple analyses of the tear cytokine and protease profiles of patients with KTCN have shown a persistent inflammation (17, 76). Previously, we identified numerous, randomly distributed variants in genes that encoded inflammatory molecules (*IL1A* and *IL1B*) and one variant (c.214+242C>T in the *IL1RN*) presented in all KTCN individuals (33). Here, the enrichment in variants of genes involved in the innate (neutrophil degranulation) and adaptive (Class I MHC antigen processing and presentation) immune system was revealed. In our study, *TRAV19* and *TRVB12-5* genes were recognized with three recurring variants (one as a stop gain). Because these genes encode variable regions of T-cell receptors, it could indirectly influence the process of antigen processing (77). Importantly, in a single-cell study of full corneas, the upregulation of the T-cell receptor signaling pathway has been recognized (59). Identified recurring missense variants in *KIR2DL1* and *KIR3DL1* could influence the activity of the immune system, since they are members of the highly conserved killer cell immunoglobulin-like receptors (KIRs) family (78), which are transmembrane glycoproteins expressed by natural killer (NK) cells and subsets of T cells (79). It was previously reported that the variant in *KIR3DL1* has affected the NK cell function (80). NK cells activate neutrophils and induce both their infiltration to the inflamed sites and degranulation (81). Also, NK cells were reported to participate in acute immune response in dry eye syndrome and trigger Th-17 response, manifested by an increase of IL17 (82). Moreover, the involvement of NK cells in the corneal wound healing has been confirmed in a murine model (83). The evidence for migration of cells into corneal limbus and central stroma after a 2-mm epithelial abrasion of the mouse cornea and altered inflammatory reaction after antibody-induced depletion of NK cells were presented (83). Previously, as elements of innate immune responses, the expression of TLR2 and TLR4 has been studied by flow cytometry in corneal epithelial cells and blood samples of KTCN patients and their relatives (16, 84, 85), and significant upregulation has been shown, indicating a potential of the TLR2 and TLR4 proteins to be the KTCN biomarkers. However, no sequence variants in the TLRs were recognized here as well as in our current gene and protein expression assessment (13). Instead, we recognized sequence variants of other innate immune system elements (e.g., *MUC5AC*, as mucins consist the first-line defense). Moreover, the increased proportions of activated NK cells as well as neutrophils and γδT cells have been reported in KTCN studies (86). Also in many studies of tear fluid collected in KTCN patients, the higher levels of interleukins (IL1β, IL6, and IL17A), interferons (IFNα/β/γ), and other pro-inflammatory factors (TNFα, TGFβ1, LIF, granzyme-B, perforin, and MMP2) were recognized (14, 24, 86–88). Besides published transcriptomic and proteomic findings, the genomic aspects regarding disruption of immune responses have not been demonstrated in KTCN research. Also, the here-identified single high-impact sequence variants (*CEP290, MMRN1*, and *FN1*) and missense variants (*PECAM1, VWF, CD109, HRG, TLN1, HPSE, EPO, SIGLEC10, DOCK8, ERBB3*, and *AP3B1*) could contribute to molecular dysregulation of wound healing, followed by inflammation, although we have not experimentally determined their impact.

Therefore, we conclude here that the disturbance of the immune responses in KTCN affects various elements of corneal hemostasis, including the antigen presentation and neutrophil degranulation processes, and possibly results in thinning and weakening of the KTCN corneas.

Summarizing, applied WGS allowed for identification and confirmation of numerous genomic features in KTCN. In future studies, WGS data should be integrated with other genome-wide analyses data, including chromatin accessibility (Assay for Transposase Accessible Chromatin with sequencing, ATAC-Seq), transcriptomics, and proteomic data, in order to obtain the most

reliable research results. In this study, owing to a limited number of included patients, we intently did not focus on particular sequence variations but rather on the sets of impacted genes and the pathways in which those genes are involved. Additionally, we decided to evaluate adolescent patients with KTCN only, assuming the more pronounced genetic background of the disease. The recruitment of the control group was especially challenging, as nowadays the photorefractive keratectomy (PRK) procedure, during which the CE is removed, is rarely performed and is replaced with the newer techniques of vision correction without CE removal. The youngest possible patients with minimal vision impairment undergoing the PRK procedure were ascertained into the control group to minimize potential bias. The PRK procedure is performed in adults, and this fact also results in the difference in age between the compared groups of patients. Although only four patients and two controls were involved, 18 samples were tested in the WGS experiments and data obtained from those 18 samples were carefully curated. In addition, the sequence variant interpretation was performed using additional allele frequency data derived from the reference databases (1000 Genomes, ESP and gnomAD). Owing to the small size of the compared study groups, we were unable to subdivide these groups into subgroups of patients, taking into account the presence of an allergic disease or dry eye syndrome.

The multiple coding and non-coding sequence variants were found in genes (or in their close proximity) contributing to the previously discussed KTCN-specific cellular components, and newly presented aspects of innate and adaptive immune system responses, pointing to the involvement of inflammatory aspects in KTCN. Therefore, we conclude that variants in non-coding regions of the genome have complemented the previously identified features specific to the KTCN exome.

Additionally, the 35 chromosomal regions shown enriched in both coding and non-coding KTCN-specific sequence variants confirmed the heterogenous background of KTCN with a limited number of common elements.

We did not find the diversity in genetic features of the assessed *topographic* regions of CE. This lack of genomic heterogeneity across the corneal surface and no evidence of inter-tissue variants in human KTCN corneas suggest other molecular mechanisms of KTCN-specific changes in CE.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/clinvar/ under the accession number SUB13057607.

Ethics statement

The studies involving human participants were reviewed and approved by Bioethics Committee at Poznan University of Medical Sciences, Poznan, Poland. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

KJ: participated in research design, sample collection, and patient surveys; performed sample preparations towards WGS experiments; participated in bioinformatics analyses; figures and tables preparation; participated in the genomic and clinical data interpretation; and wrote the manuscript. MM-K: performed recruitment of the patients and clinical evaluation; facilitated sample collection; performed the clinical data interpretation; participated in the genomic data interpretation; and manuscript writing. MK: performed setup of bioinformatics pipeline; bioinformatics analyses of the genomic data; and preparation of figures and tables. JAK: participated in the genomic data interpretation. MG: research design; secured research funding; participated in the genomic data interpretation; and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1197054/full#supplementary-material

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Podsumowanie i wnioski

Stożek rogówki jest chorobą wieloczynnikową i aby zrozumieć procesy biologiczne leżące u jego podstaw, konieczna jest równoczesna analiza różnych aspektów biologicznych i danych pochodzących z wysokoprzepustowych analiz omicznych. W przeprowadzonych badaniach uwzględniono zróżnicowanie cech morfologicznych w rogówce, po raz pierwszy wyznaczając trzy regiony w obrębie nabłonka rogówki i wprowadzając termin regionu topograficznego. W ramach niniejszej pracy wykorzystano technikę sekwencjonowania całego genomu (ang. *whole genome sequencing*, WGS), która do tej pory nie była stosowana w badaniach nad KTCN.

1. W oparciu o dotychczasowe badania nad stożkiem rogówki obejmujące głównie wybrane aspekty molekularne i/lub kliniczne jak również wyniki badań własnych stwierdzono, że jedynie wielopoziomowe analizy/badania molekularne i łączenie aspektów różnych dziedzin nauki dają możliwość pełniejszego zrozumienia aspektów biologicznych w złożonej naturze KTCN.

2. Charakterystyka trzech regionów topograficznych rogówki, odzwierciedlających strukturę obwarzanka w nabłonku rogówki u pacjentów z KTCN, pozwoliła na wskazanie mechanizmu przebudowy nabłonka rogówki w KTCN, w którym kluczowymi elementami są rozregulowane ścieżki przejścia epitelialno-mezenchymalnego, komunikacji komórka-komórka i interakcji komórka-macierz zewnątrzkomórkowa, biorące udział w procesie gojenia się ran i pośrednio wpływające na powstanie charakterystycznego fenotypu stożka.

3. Pocieranie oczu powoduje uszkodzenie CE i wyzwala stres komórkowy, który poprzez swój wpływ na apoptozę, migrację i adhezję komórkową kształtuje fenotyp KTCN.

4. Wskazano 35 *loci* chromosomowych istotnie wzbogaconych w rozpoznane warianty sekwencji kodujących i niekodujących, specyficzne dla KTCN, w tym w regionach 5q, 9q i 16q, poprzednio rozpoznanych jako powiązane etiologicznie z KTCN.
W genach lub w ich bliskim sąsiedztwie znaleziono liczne warianty sekwencji kodujących i niekodujących, mogące mieć znaczenie w procesach wrodzonej i adaptacyjnej odpowiedzi układu odpornościowego, co wskazuje na bardziej złożone niż wcześniej przewidywano podłoże zapalne w KTCN.

Streszczenie

Stożek rogówki (ang. *keratoconus*, KTCN) jest postępującym schorzeniem degeneracyjnym rogówki oka występującym w populacji światowej z częstością 1 na 2000 osób. KTCN charakteryzuje się pogłębiającym się ścieńczeniem rogówki, w wyniku którego przyjmuje ona stożkowaty kształt, co prowadzi do znacznego upośledzenia wzroku. Pierwsze symptomy choroby mogą pojawić się już w okresie dojrzewania by kolejno ulegać progresji w trzeciej/czwartej dekadzie życia. Choroba częściej występuje u mężczyzn.

KTCN uznawany jest za chorobę wieloczynnikową o niejasnej etiologii, ze złożonym podłożem genetycznym obejmującym liczne zidentyfikowane *loci* chromosomowe, w tym 13q32, 5q31.1-q35.3, 2q13-q14.3, 20p13-p12.2, oraz warianty sekwencji w obrębie genów kandydujących, do których należą m.in. *VSX1*, *DOCK9*, *STK24*, *IPO5* i *TGFBI*. Również czynniki środowiskowe są wskazywane jako warunkujące KTCN. Historycznie KTCN uznany został za chorobę niezapalną, co obecnie jest podważane przez klinicystów i badaczy ze względu na podniesiony poziom ekspresji cytokin i proteaz w płynie łzowym i surowicy pacjentów.

U pacjentów z wczesnym i średniozaawanowanym KTCN, rogówka w lampie szczelinowej może wyglądać prawidłowo. W celu rozpoznania KTCN, u każdego pacjenta, u którego podejrzewa się chorobę należy wykonać kompleksowe badanie okulistyczne obejmujące ocenę nieskorygowanej i skorygowanej ostrości wzroku, badanie odcinka przedniego w lampie szczelinowej, tomografię rogówki (kamera Scheimpfluga) oraz tomografię przedniego odcinka oka (ang. *anterior segment optical coherence tomography*, AS-OCT).

Nieprawidłowości histopatologiczne rogówki pacjentów z rozpoznanym KTCN obejmują wszystkie warstwy rogówki. Najbardziej charakterystyczną zmianą histologiczną jest ścieńczenie istoty właściwej rogówki. Z kolei nabłonek rogówki (ang. *corneal epithelium*, CE), wobec powstającego uwypuklenia istoty właściwej staje się najcieńszy na szczycie stożka i przyjmuje charakterystyczną strukturę obwarzanka (ang. *doughnat pattern*).

W rozprawie doktorskiej postawiono hipotezę, że przebudowa nabłonka rogówki w przebiegu KTCN stanowi istotny element obrazu klinicznego choroby. Charakterystyczne zmiany grubości nabłonka, rozpoznawane jako struktura obwarzanka, nie były wcześniej uwzględnione w badaniach molekularnych. Podjęte dotychczas próby biologicznej interpretacji zintegrowanych wyników badań klinicznych i molekularnych były niedostateczne.

Rozprawa doktorska obejmuje rezultaty badań przedstawione w zbiorze czterech publikacji będących efektem eksperymentalnych badań transkryptomicznych, proteomicznych, kodujących i niekodujących elementów genomu w nabłonku rogówki, badań epidemiologicznych identyfikujących czynniki środowiskowe warunkujące fenotyp KTCN oraz przeglądu literaturowego.

W oparciu o dotychczasowe badania nad stożkiem rogówki, obejmujące głównie wybrane aspekty molekularne i/lub kliniczne, jak również wyniki badań własnych stwierdzono, że jedynie wielopoziomowe analizy molekularne i łączenie aspektów różnych dziedzin nauki dają możliwość pełniejszego zrozumienia aspektów biologicznych w złożonej naturze KTCN.

W przeprowadzonych w ramach pracy badaniach uwzględniono zróżnicowanie cech morfologicznych w rogówce, po raz pierwszy wyznaczając trzy regiony w obrębie nabłonka

rogówki i wprowadzając termin regionu topograficznego. Na podstawie rozpoznanych zmian w grubości CE u pacjentów z KTCN scharakteryzowano trzy regiony topograficzne (centralny, pośredni i obwodowy), w których zidentyfikowano specyficzne zmiany morfologiczne, transkryptomiczne oraz proteomiczne. W wyniku integracji zebranych danych wskazano mechanizm przebudowy nabłonka rogówki, w którym kluczowymi elementami są rozregulowane ścieżki przejścia epitelialno-mezenchymalnego, komunikacji komórka-komórka i interakcji komórka-macierz zewnątrzkomórkowa, odpowiadające za proces gojenia się ran (ang. *wound healing process*), który pośrednio wpływa na powstawanie charakterystycznego fenotypu stożka. Otrzymane wyniki pozwalają wnioskować, że na etapach projektowania i interpretacji biologicznej przeprowadzanych badań molekularnych powinno się uwzględniać występowanie trzech regionów topograficznych rogówki.

Zweryfikowano wcześniejsze przypuszczenia dotyczące przewlekłego mechanicznego urazu rogówki u pacjentów z KTCN. Pocieranie oczu, nieskorelowane z alergią, powoduje uszkodzenie CE i wyzwala stres komórkowy, który poprzez swój wpływ na apoptozę, migrację i adhezję komórkową również kształtuje fenotyp KTCN.

W ramach niniejszej pracy wykorzystano również technikę sekwencjonowania całego genomu (ang. *whole genome sequencing*, WGS), która do tej pory nie była stosowana w badaniach nad KTCN. W genach lub w ich bliskim sąsiedztwie znaleziono liczne warianty sekwencji kodujących i niekodujących, mogące mieć znaczenie w procesach wrodzonej i adaptacyjnej odpowiedzi układu odpornościowego, co wskazuje na bardziej złożone niż wcześniej przewidywano podłoże zapalne w KTCN. Ponadto wskazano 35 *loci* chromosomowych istotnie wzbogaconych w rozpoznane warianty sekwencji kodujących i niekodujących, specyficzne dla KTCN, w tym w 5q, 9q i 16q, poprzednio rozpoznanych jako powiązane etiologicznie z KTCN.

W efekcie przeprowadzonych prac badawczych potwierdzono złożoność podłoża genetycznego/molekularnego KTCN oraz znaczenie nawracającego/przetrwałego stanu zapalnego, mogącego mieć podłoże genetyczne, na obraz kliniczny stożka.

Summary

Keratoconus (KTCN) is a progressive degenerative disease of the cornea that affects the general population with a frequency of 1 in 2,000 people. KTCN is characterized by increasing thinning of the cornea, resulting in its conical shape, which leads to significant visual impairment. The first symptoms of the disease may appear in adolescence and then progress through the third/fourth decade of life. A higher occurrence of the KTCN in male patients has been reported.

KTCN is considered a multifactorial disease, in which a complex genetic background embraces numerous identified chromosomal *loci*, including 13q32, 5q31.1-q35.3, 2q13-q14.3, 20p13-p12.2, and sequence variants in candidate genes, such as *VSX1*, *DOCK9*, *STK24*, *IPO5,* and *TGFBI*. Some environmental factors have been previously reported as causative in KTCN etiology. Historically, KTCN was considered a non-inflammatory disease, however, this aspect is now questioned by clinicians and researchers due to the reports indicating the elevated expression of cytokines and proteases in patients' tear fluid and serum samples.

In patients with early and moderate KTCN, the cornea evaluated by slit lamp biomicroscopy may appear normal. In order to diagnose KTCN, a patient should undergo a comprehensive ophthalmological examination, including assessment of uncorrected and corrected visual acuity, slit lamp examination of the anterior segment of the eye, corneal tomography (Scheimpflug camera), and anterior segment tomography (AS-OCT).

Histopathological abnormalities of the cornea of patients diagnosed with KTCN comprise all corneal layers. The most characteristic histological change is the thinning of the corneal stroma. In turn, the corneal epithelium (CE), trying to 'mask' the emerging stromal irregularities, becomes the thinnest at the top of the cone and forms a characteristic *doughnut pattern*.

In this doctoral dissertation, it was hypothesized that the remodeling of the corneal epithelium in the course of KTCN is an important element of the clinical picture of the disease. Previously the characteristic alternations in the thickness of the corneal epithelium, called *doughnut pattern*, have not been considered in the molecular studies. Therefore, attempts to interpret the integrated clinical and molecular findings biologically have been insufficient.

This doctoral dissertation is a collection of four publications resulting from experimental studies of transcriptomic, proteomic, coding, and non-coding genome features of the corneal epithelium, epidemiological studies identifying environmental factors determining the KTCN phenotype, and a literature review.

Based on previous KTCN studies, covering mainly selected molecular and/or clinical aspects, as well as the results of own research, it was concluded that solely multi-level molecular approach and integration of different scientific fields provide the opportunity for a deeper understanding of the biological aspects in the complex nature of KTCN.

The conducted research took into account the diversity of morphological features in the cornea, defining for the first time three regions within the corneal epithelium and introducing a new definition of the topographic regions. Based on abnormalities in CE thickness profiles in patients with KTCN, the three topographic regions were characterized (central, middle, and peripheral) with specific morphological, transcriptomic, and proteomic alternations. As a result of the integration analyses, a mechanism of corneal epithelium remodeling was proposed,

highlighting the dysregulation of epithelial-mesenchymal transition, cell-cell communication, and cell-extracellular matrix interactions pathways, contributing to the wound healing process, which indirectly influences the formation of the keratoconic cone. These recognized alternations allow to conclude that the presence of three topographic corneal regions should be taken into account during the experimental study design and biological interpretation of the molecular findings.

The previous assumption regarding chronic mechanical corneal injury in KTCN was verified. The eye rubbing, not correlated with an allergy, causes CE damage and triggers cellular stress, which, through its effect on apoptosis, migration, and cell adhesion, also influences the KTCN phenotype.

During this study the whole genome sequencing (WGS) technique, which has not been used in the worldwide research on KTCN so far, was utilized. The multiple coding and non-coding sequence variants were found in genes or in their close proximity, contributing to innate and adaptive immune system responses, which indicates a more complex inflammatory background in KTCN than previously anticipated. Moreover, 35 chromosomal *loci* were identified as significantly enriched in recognized coding and non-coding KTCN-specific sequence variants, including 5q, 9q, and 16q *loci*, previously recognized as etiologically related to KTCN.

The conducted studies confirmed the complexity of the genetic/molecular background of KTCN and the importance of recurrent/persistent inflammation (which may have a genetic basis) on the clinical picture of the keratoconus.

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OŚWIADCZENIE

Niniejszym oświadczam, że miałam wiodący udział w powstaniu rozdziału książki, którego jestem współautorem pt.:

„*The Genetics of Keratoconus and Related Diseases*"

Jaskiewicz K and Gajecka M. w Traboulsi E., et al. *Genetic Diseases of the Eye (3rd Edition).* Oxford University Press.

Mój wkład w powstanie publikacji był następujący: wybór opisanych danych literaturowych; przygotowanie figury i tabel; pisanie manuskryptu.

Niniejsza publikacja jest częścią mojej rozprawy doktorskiej pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki", którą realizuję pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

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Niniejszym oświadczam, że miałam wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

"The Impaired Wound Healing Process Is a Major Factor in Remodeling of the Corneal Epithelium in Adult and Adolescent Patients with Keratoconus"

Jaskiewicz K, Maleszka-Kurpiel M, Matuszewska E, Kabza M, Rydzanicz M, Malinowski R, Ploski R, Matysiak J, Gajecka M.

Invest Ophthalmol Vis Sci. 2023 Feb 1;64(2):22.

Mój wkład w powstanie publikacji był następujący: koncepcja i plan badań, zbieranie i przygotowanie materiału biologicznego pozyskanego od pacjentów do prac eksperymentalnych; przeprowadzenie wywiadów z pacjentami; przygotowanie bibliotek RNA-Seq; przygotowanie materiału pod analizę MALDI-TOF/TOF Tandem Mass Spectrometry; przeprowadzenie oceny morfologicznej materiału; przeprowadzenie eksperymentów RT-qPCR; udział w analizach bioinformatycznych i statystycznych; analiza i interpretacja danych transkryptomicznych, proteomicznych, morfologicznych i klinicznych; przygotowywanie rycin i tabel; pisanie manuskryptu.

Niniejsza publikacja jest częścią mojej rozprawy doktorskiej pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki", którą realizuję pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

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Niniejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:
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Mój wkład w powstanie publikacji był następujący: rekrutacja pacjentów do projektu; badanie kliniczne pacjentów; przeprowadzenie wywiadów z pacjentami; zbieranie materiału biologicznego od pacjentów; opracowanie i interpretacja danych klinicznych, udział w interpretacji danych transkryptomicznych, proteomicznych i morfologicznych; pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

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OŚWIADCZENIE

Ninejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:
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Invest Ophthalmol Vis Sci. 2023 Feb 1;64(2):22.

Mój wkład w powstanie publikacji był następujący: zaplanowanie i wykonanie eksperymentów MALDI-TOF/TOF Tandem Mass Spectrometry; analiza wyników eksperymentów proteomicznych i ich opracowanie w postaci figur oraz tabel; edycja manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

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Ninejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

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Mój wkład w powstanie publikacji był następujący: analiza bioinformatyczna danych transkryptomicznych oraz edycja manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

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Jaskiewicz K, Maleszka-Kurpiel M, Matuszewska E, Kabza M, **Rydzanicz M**, Malinowski R, Ploski R, Matysiak J, Gajecka M. *Invest Ophthalmol Vis Sci*. 2023 Feb 1;64(2):22.

Mój wkład w powstanie publikacji był następujący: przygotowanie bibliotek RNA-Seq, przeprowadzenie sekwencjonowania NGS, interpretacja danych transkryptomicznych oraz edycja manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

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Mój wkład w powstanie publikacji był następujący: zaplanowanie i udział w ocenie morfologicznej preparatów materiału biologicznego.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

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OŚWIADCZENIE

Niniejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

"The Impaired Wound Healing Process Is a Major Factor in Remodeling of the Corneal Epithelium in Adult and Adolescent Patients with Keratoconus"

Jaskiewicz K, Maleszka-Kurpiel M, Matuszewska E, Kabza M, Rydzanicz M, Malinowski R, **Ploski R**, Matysiak J, Gajecka M.

Invest Ophthalmol Vis Sci. 2023 Feb 1;64(2):22.

Mój wkład w powstanie publikacji był następujący: udział w interpretacji danych transkryptomicznych.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Rafał Płoski

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UNIWERSYTET MEDYCZNY IM. KAROLA MARCINKOWSKIEGO W POZNANIU

KATEDRA I ZAKŁAD CHEMII NIEORGANICZNEJ I ANALITYCZNEJ

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Poznań, 10.09.2023

Prof. dr hab. Jan Matysiak

Kierownik Katedry i Zakładu Chemii Nieorganicznej i Analitycznej

OŚWIADCZENIE

Ninejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

"The Impaired Wound Healing Process Is a Major Factor in Remodeling of the Corneal Epithelium in Adult and Adolescent Patients with Keratoconus"

Jaskiewicz K, Maleszka-Kurpiel M, Matuszewska E, Kabza M, Rydzanicz M, Malinowski R, Ploski R, **Matysiak J**, Gajecka M.

Invest Ophthalmol Vis Sci. 2023 Feb 1;64(2):22.

Mój wkład w powstanie publikacji był następujący: udział w zaplanowaniu eksperymentów MALDI-TOF/TOF Tandem Mass Spectrometry oraz w interpretacji ich wyników.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

KIEROWNIK
Katedry i Zakładu Chemii
Nieorganicznej i Analitycznej
prof. dr hab. Jan Matysiak

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Poznań, 25.09.2023

prof. dr hab. n. med. Marzena Gajęcka
Zakład Genetyki Nowotworów

OŚWIADCZENIE

Ninejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

"The Impaired Wound Healing Process Is a Major Factor in Remodeling of the Corneal Epithelium in Adult and Adolescent Patients with Keratoconus"

Jaskiewicz K, Maleszka-Kurpiel M, Matuszewska E, Kabza M, Rydzanicz M, Malinowski R, Ploski R, Matysiak J, **Gajecka M**.

Invest Ophthalmol Vis Sci. 2023 Feb 1;64(2):22.

Mój wkład w powstanie publikacji był następujący: koncepcja i plan badań; zabezpieczenie finansowania badań; interpretacja danych transkryptomicznych, proteomicznych, morfologicznych i klinicznych; edycja manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod moim kierunkiem.

Z poważaniem,

Oświadczenia współautorów publikacji

Oświadczenia współautorów Artykułu III

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Poznań, 2.10.2023

mgr Katarzyna Jaśkiewicz
Zakład Genetyki Nowotworów

OŚWIADCZENIE

Ninejszym oświadczam, że miałam wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

„Non-allergic eye rubbing is a major behavioral risk factor for keratoconus"

Jaskiewicz K, Maleszka-Kurpiel M, Michalski A, Ploski R, Rydzanicz M, Gajecka M.

PLoS One. 2023 Apr 13;18(4):e0284454.

Mój wkład w powstanie publikacji był następujący: koncepcja i plan badań; zbieranie i przygotowanie materiału biologicznego pozyskanego od pacjentów do prac eksperymentalnych; przeprowadzenie wywiadów z pacjentami; opracowanie danych klinicznych i ich analiza statystyczna; przygotowanie bibliotek RNA-Seq; analiza i interpretacja korelacji danych transkryptomicznych i klinicznych; przygotowywanie rycin i tabel; pisanie manuskryptu.

Niniejsza publikacja jest częścią mojej rozprawy doktorskiej pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki", którą realizuję pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Katarzyna Jaśkiewicz

Poznań, 10.09.2023
dr n. med. Magdalena Maleszka-Kurpiel
Katedra Chorób Oczu i Optometrii
Uniwersytet Medyczny im. Karola Marcinkowskiego w Poznaniu

OŚWIADCZENIE

Niniejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:
„Non-allergic eye rubbing is a major behavioral risk factor for keratoconus"
Jaskiewicz K, **Maleszka-Kurpiel M**, Michalski A, Ploski R, Rydzanicz M, Gajecka M.
PLoS One. 2023 Apr 13;18(4):e0284454.

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Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Poznań, 10.09.2023

dr n. med. Andrzej Michalski
Katedra Chorób Oczu i Optometrii
Uniwersytet Medyczny im. Karola Marcinkowskiego w Poznaniu

OŚWIADCZENIE

Ninejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:
„Non-allergic eye rubbing is a major behavioral risk factor for keratoconus"
Jaskiewicz K, Maleszka-Kurpiel M, **Michalski A**, Ploski R, Rydzanicz M, Gajecka M.
PLoS One. 2023 Apr 13;18(4):e0284454.

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Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Andrzej Michalski

WARSZAWSKI UNIWERSYTET MEDYCZNY
MEDICAL UNIVERSITY OF WARSAW

Zakład Genetyki Medycznej
Centrum Biostruktury I Wydziału Lekarskiego

Warszawa, 11.09.2023

prof. dr hab. n. med. Rafał Płoski
Kierownik Zakładu Genetyki Medycznej
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

Niniejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

„Non-allergic eye rubbing is a major behavioral risk factor for keratoconus"

Jaskiewicz K, Maleszka-Kurpiel M, Michalski A, **Ploski R**, Rydzanicz M, Gajecka M. *PLoS One*. 2023 Apr 13;18(4):e0284454.

Mój wkład w powstanie publikacji był następujący: interpretacja danych transkryptomicznych.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Rafał Płoski

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Zakład Genetyki Medycznej
Centrum Biostruktury I Wydziału Lekarskiego

Warszawa, 11.09.2023

dr n. med. Małgorzata Rydzanicz
Zakład Genetyki Medycznej
Warszawski Uniwersytet Medyczny

OŚWIADCZENIE

Niniejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

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Jaskiewicz K, Maleszka-Kurpiel M, Michalski A, Ploski R, **Rydzanicz M**, Gajecka M. *PLoS One*. 2023 Apr 13;18(4):e0284454.

Mój wkład w powstanie publikacji był następujący: udział w przygotowaniu bibliotek RNA-Seq, przeprowadzenie sekwencjonowania NGS, interpretacja danych transkryptomicznych oraz edycja manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Małgorzata Rydzanicz

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Poznań, 25.09.2023

prof. dr hab. n. med. Marzena Gajęcka
Zakład Genetyki Nowotworów

OŚWIADCZENIE

Ninejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:
„Non-allergic eye rubbing is a major behavioral risk factor for keratoconus"
Jaskiewicz K, Maleszka-Kurpiel M, Michalski A, Ploski R, Rydzanicz M, **Gajecka M**.
PLoS One. 2023 Apr 13;18(4):e0284454.

Mój wkład w powstanie publikacji był następujący: koncepcja i plan badań, zabezpieczenie finansowania badań, interpretacja danych transkryptomicznych i klinicznych, edycja manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod moim kierunkiem.

Z poważaniem,

Oświadczenia współautorów publikacji

Oświadczenia współautorów Artykułu IV

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Poznań, 2.10.2023

mgr Katarzyna Jaśkiewicz
Zakład Genetyki Nowotworów

OŚWIADCZENIE

Ninejszym oświadczam, że miałam wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

„Sequence variants contributing to dysregulated inflammatory responses across keratoconic cone surface in adolescent patients with keratoconus"

Jaskiewicz K, Maleszka-Kurpiel M, Kabza M, Karolak JA and Gajecka M.

Front. Immunol. 2023 Jul 6;14:1197054.

Mój wkład w powstanie publikacji był następujący: koncepcja i plan badań; zbieranie i przygotowanie materiału biologicznego pozyskanego od pacjentów do prac eksperymentalnych; przeprowadzenie wywiadów z pacjentami; udział w analizach bioinformatycznych; analiza i interpretacja wyników badań; przygotowywanie rycin i tabel; pisanie manuskryptu.

Niniejsza publikacja jest częścią mojej rozprawy doktorskiej pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki", którą realizuję pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Katarzyna Jaśkiewicz

Poznań, 10.09.2023
dr n. med. Magdalena Maleszka-Kurpiel
Katedra Chorób Oczu i Optometrii
Uniwersytet Medyczny im. Karola Marcinkowskiego w Poznaniu

OŚWIADCZENIE

Niniejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:
„Sequence variants contributing to dysregulated inflammatory responses across keratoconic cone surface in adolescent patients with keratoconus"
Jaskiewicz K, **Maleszka-Kurpiel M**, Kabza M, Karolak JA and Gajecka M.
Front. Immunol. 2023 Jul 6;14:1197054.

Mój wkład w powstanie publikacji był następujący: rekrutacja pacjentów do projektu; badanie kliniczne pacjentów; zbieranie materiału biologicznego od pacjentów; opracowanie danych klinicznych; udział w interpretacji wyników badań; pisanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Poznań, 10.09.2023

dr n. biol. Michał Kabza

Michał Kabza Konsulting

OŚWIADCZENIE

Ninejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

„Sequence variants contributing to dysregulated inflammatory responses across keratoconic cone surface in adolescent patients with keratoconus"

Jaskiewicz K, Maleszka-Kurpiel M, **Kabza M**, Karolak JA and Gajecka M.

Front. Immunol. 2023 Jul 6;14:1197054.

Mój wkład w powstanie publikacji był następujący: analiza bioinformatyczna danych WGS; przygotowanie tabel i figur; edycja manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z poważaniem,

Michał Kabza

Poznań, 18.09.2023

dr hab. n. med. Justyna Anna Karolak
Katedra i Zakład Genetyki i Mikrobiologii Farmaceutycznej
Uniwersytet Medyczny im. Karola Marcinkowskiego w Poznaniu
ul. Rokietnicka 3, Pok. B1.0.06
60-806 Poznań
tel. (61) 641 83 14
e-mail: jkarolak@ump.edu.pl

OŚWIADCZENIE

Oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu:

„Sequence variants contributing to dysregulated inflammatory responses across keratoconic cone surface in adolescent patients with keratoconus" Jaskiewicz K, Maleszka-Kurpiel M, Kabza M, Karolak JA and Gajecka M. *Front. Immunol*. 2023 Jul 6;14:1197054.,

którego jestem współautorem.

Mój wkład w powstanie publikacji polegał na udziale w interpretacji wyników sekwencjonowania całogenomowego oraz edycji manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim mgr Katarzyny Jaśkiewicz pt.: „*Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki*", realizowanym pod kierunkiem prof. dr hab. n. med. Marzeny Gajęckiej.

Z wyrazami szacunku,
Justyna Karolak

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Poznań, 25.09.2023

prof. dr hab. n. med. Marzena Gajęcka
Zakład Genetyki Nowotworów

OŚWIADCZENIE

Ninejszym oświadczam, że mgr Katarzyna Jaśkiewicz miała wiodący udział w powstaniu manuskryptu, którego jestem współautorem pt.:

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Mój wkład w powstanie publikacji był następujący: koncepcja i plan badań; zabezpieczenie finansowania badań; interpretacja wyników; edycja manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie powyższej publikacji w przewodzie doktorskim pt. „Wielopoziomowa charakterystyka molekularna regionów topograficznych rogówki w stożku rogówki" realizowanym pod moim kierunkiem.

Z poważaniem,